Reactivation and Aging of Neurotoxic Esterase Inhibited by a Variety of Organophosphorus Esters

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1. It was proposed [Johnson (1974) *J. Neurochem.* 23, 785–789] that both inhibition of neurotoxic esterase of nervous tissue and subsequent ‘aging’ of the inhibited esterase are necessary events in the pathogenesis of organophosphate-induced delayed neuropathy: aging has now been demonstrated with a number of neurotoxic compounds. 2. Reactivation by KF was observed for hen brain neurotoxic esterase inhibited by 14 organophosphates and phosphonates, and time-dependent loss of reactivability (aging) occurred in every case. 3. For five other compounds no reactivation occurred and aging could not therefore be established, but independent evidence for two compounds suggests that aging was rapid. 4. Half-lives of aging of neurotoxic esterase inhibited by phosphates ranged from less than 1 min to 10 min, and for phosphonates the range was 3–600 min. 5. The relationship of these findings to the mechanism of toxicity and to the prospects of therapy are considered. 6. Aging occurred rapidly with aryloxy and linear alkoxy groups attached to phosphorus and slowly with a highly branched alkoxy substituent: these effects seem incompatible with an S_N1 (dealkylation) mechanism.

Esterases that are known to be inhibited progressively by low concentrations of organophosphorus esters appear to react with inhibitor according to the steps shown in Scheme 1 (see Aldridge & Reiner, 1972). Reaction (3) (reactivation) may occur spontaneously and may also be catalysed by agents such as fluoride salts and some nucleophilic oximes. After inhibition, an alternative reaction is (4), which involves loss of the R group and generation of a charged substituent on the enzyme. For the cholinesterases, which have undergone most study, it is clear that once reaction (4) occurs reactivation in the manner of reaction (3) is no longer feasible. The enzyme is said to have aged (i.e. lost its responsiveness to reactivators) as a result of the group loss.

Many organophosphorus esters cause a delayed neuropathy in man and most experimental animals apart from small rodents (reviewed by Johnson, 1975a). This effect is quite separate from acute anticholinesterase effects, and the initial biochemical event is inhibition of a distinct esterase (neurotoxic esterase) in nervous tissue (Johnson, 1969). Structure–activity studies led to the proposition that in the genesis of neuropathy reaction

(4) is a necessary further step after inhibition of neurotoxic esterase (Johnson, 1974). With DiPF extremely rapid aging has recently been demonstrated both by the time-dependent loss of response to reactivation by KF and by a radiochemical method that showed conversion of di-isopropylphosphoryl-enzyme into the monoisopropylphosphoryl-enzyme (Clothier & Johnson, 1979). The rate and pH characteristics of the two phenomena that were monitored by these methods were indistinguishable, so that it then seemed valid to screen for the group-loss reaction that is predicted in the hypothesis for mechanism of neuropathy by using only the reactivation method, which does not require radiolabelled inhibitors. Studies reported in the present paper show that aging of neurotoxic esterase inhibited by a large variety of organophosphorus esters does occur, that the reaction is usually very rapid and that the relationship of structure of inhibitor to ease of aging is very different from that which is known for the cholinesterases. The implications of the last observation for mechanism of aging are considered.

Materials and Methods

Buffers

Tris (50 mM)/EDTA (0.2 mM) was neutralized to

Abbreviation used: DiPF, di-isopropyl phosphorofluoridate.

Scheme 1. Steps in the interaction of an esterase with an organophosphorus inhibitor
(1) Formation of Michaelis complex; (2) phosphorylation of enzyme; (3) reactivation; (4) aging.

pH 8.0 with HCl (12M) or else adjusted to desired pH by admixture with necessary amounts of citric acid (50mM)/EDTA (0.2mM).

Inhibitors

2,4-Dichlorophenyl methyl methylphosphonate (HOL 1957), ethyl 2,4,5-trichlorophenyl ethylphosphonate (HOL 1948) and 2-chloroethyl 2,2-dichlorovinyl ethylphosphonate (Bayer 48/86) were obtained from Bayer A.G. (Wuppertal, W. Germany). Pinacolyl methylphosphonofluoridate (Soman) and phenyl *OO*-saligenin phosphate were from the Chemical Defence Experimental Establishment (Porton Down, Salisbury, Wilts., U.K.). Bis-2-chloroethyl 2,2-dichlorovinyl phosphate was from Cooper Technical Bureau (Berkhamsted, Herts., U.K.). *NN'*-Di-isopropylphosphorodiamidic fluoride (Mipafos) was from Fisons Pest Control Co. (Saffron Walden, Essex, U.K.). Bis-(2-isopropylphenyl) phosphorochloridate was from FMC Corp. (Princeton, NJ, U.S.A.). From Shell Research (Sittingbourne, Kent, U.K.) came the following: 2,2-dichlorovinyl dimethyl phosphate (Dichlorvos), 2,2-dichlorovinyl diethyl phosphate, 2,2-dichlorovinyl di-*n*-propyl phosphate, di-*n*-butyl 2,2-dichlorovinyl phosphate, 2,2-dichlorovinyl di-*n*-pentyl phosphate, 2,2-dichlorovinyl diphenyl phosphate and 2,2-dichlorovinyl methyl *n*-pentyl phosphate. DiPF was from Boots Pure Drug Co. (Nottingham, U.K.). 4-Bromo-2,5-dichlorophenyl methyl phenylphosphonate (Leptophos oxon) was from Velsicol Co. (Chicago, IL, U.S.A.). Ethyl 4-nitrophenyl *n*-pentylphosphonate was from Professor E. L. Becker; 4-nitrophenyl *n*-pentyl *n*-pentylphosphonate came from Professor T. R. Fukuto; Dr. A. Ooms supplied the 4-nitrophenyl di-*n*-pentylphosphinate; and the benzenesulphonyl fluoride was prepared in this laboratory as described by Johnson (1975b).

Radiochemical study

The inhibitor 2,2-dichlorovinyl 2-di-*n*-[4,5(*n*)-³H]-pentyl phosphate was supplied (special synthesis) by The Radiochemical Centre (Amersham, Bucks., U.K.) as a solution (approx. 10mM) in propylene glycol. This was diluted 160-fold in acetone for use as a stock solution and kept at -25°C; at the time of use the specific radioactivity was about 600mCi/mmol. The labelling of neurotoxic esterase with this inhibitor and the assessment of aging were done as described for DiPF in subsections (3)(a)-(c) and (5)(a)-(c) of the Materials and Methods section of Clothier & Johnson (1979); the final concentration of inhibitor was 0.2-0.3μM and the inhibition was for 2 min at pH 5.2 and 37°C.

Inhibition, aging, reactivation and assay of neurotoxic esterase

The activity of the esterase is normally measured as the difference in activity between two samples that have been preincubated with selective progressive inhibitors. The procedure and reasons for the choice of inhibitors along with a standard protocol for study of aging have been fully described (Clothier & Johnson, 1979). Essentially the following steps are involved.

(1) Preparation of paired samples of hen brain particles differing only in the presence or absence of active neurotoxic esterase. These samples are referred to as 'P' and 'M' respectively.

(2) Inhibition of esterases in P and M with the compound under study by incubation for 2 min at 37°C in Tris/citrate/EDTA buffer, pH 5.2. The concentration of inhibitor was chosen to be 40-100 times the predetermined *I*₅₀ (20 min) for neurotoxic esterase.

(3) Dilution with 24 vol. of Tris/citrate/EDTA buffer of chosen pH (usually 5.2) and incubation at 37°C for a suitable aging period.

(4) Transfer of samples (10ml) of dilute inhibited tissue into reactivator (25ml) and incubation for 10min at 37°C. Unless otherwise noted the reactivator was KF (in Tris/citrate/EDTA buffer adjusted to pH 5.2) at a final concentration of 200mM; occasionally more concentrated solutions (up to 1M final) were used. It was necessary to perform the reactivation step in polypropylene centrifuge tubes: inhibitory material (presumably metal ions) was extracted from glass tubes by prolonged incubation in them of KF solutions, although no visible etching occurred.

(5) Cooling of the reactivated tissue, sedimentation of the particles and resuspension in Tris/HCl/EDTA buffer, pH 8.

(6) Assay in duplicate of the hydrolytic activity of the paired samples against phenyl valerate and calculation of neurotoxic esterase activity by difference. No inhibition was observed that might be due to KF (200mM) carried over from the centrifugation step. When higher concentrations of KF were used, step (5) was repeated, with no significant loss of neurotoxic esterase.

All glass and plasticware that came into contact with organophosphorus compounds was cleaned by detergent washing followed by an overnight soak in 1M-HNO₃ and extensive rinsing. In addition, cleaned polypropylene tubes were then filled with KF (0.2M in water) and incubated for several hours at 37°C and rinsed three times with de-ionized water: when this operation was omitted occasional and variable loss of enzyme activity was found in control samples that were put through the aging and reactivation procedure, and it was presumed that KF was interacting with traces of absorbed organophosphorus compounds of modest inhibitory power to form highly inhibitory phosphorofluoridates.

Results

Studies with DiPF showed that the rate of aging did not change detectably in the pH range 5.2–8.0, but that reactivation by KF was much faster at pH 5.2 (Clothier & Johnson, 1979). Therefore it was convenient for most of our experiments to screen with the pH at 5.2 throughout. Neurotoxic esterase is stable at this pH for several hours, but the reproducibility of the whole procedure declined when aging times beyond 30–60min were studied. In cases where aging times were prolonged we therefore used a 5-fold diluted pH 8.0 buffer for the 25-fold dilution step immediately after inhibition [see the Materials and Methods section step no. (3) and also Table 2 of Clothier & Johnson (1979)]: this enabled aging to proceed at pH 8.0 and reactivation to be carried out at 5.2 as usual; no significant loss of activity

occurred in experiments in which this procedure was used.

(1) Investigation of reactivation and aging under standard conditions

Table 1 shows the loss of response of inhibited neurotoxic esterase to KF reactivation during 5min aging periods after 2min inhibition by various organophosphorus esters. Comparison of columns (a) and (b) shows whether KF effected reactivation immediately after inhibition, and comparison of this effect with that seen 5min later [columns (c) and (d)] gives a guide to the extent of aging. Thus for the one compound having a structure that precludes group loss from the inhibited enzyme (the phosphinate, no. 20), the data show, as expected, that reactivation occurred without aging; the reproducibility of the recoveries at the two times in that experiment indicates the precision of the procedure used. Similar results have been obtained with the diethylphosphinyl-esterase under slightly different conditions (Clothier, 1979).

For the remaining 19 compounds several general comments should be made, as follows.

(i) The experiments were long and complex. Within each experiment all measurements were made in duplicate and were reproducible. However, performance of sufficient separate experiments for statistical evaluation of results with each compound was impracticable, and we have therefore assessed our results only semi-quantitatively. We have chosen to believe that changes in excess of 10% (almost exactly twice the S.D. of the mean control activities) signify real effects (scored +) and that changes of 5–10% may be significant (scored ±).

(ii) In cases where reactivation is not seen (scored –) these experiments do not show whether this is due to rapid aging or to resistance of the inhibited enzyme to KF.

(iii) The time for reactivation that was necessary for these experiments was not optimized for each inhibitor but was based on our experience with DiPF (Clothier & Johnson, 1979): this time was longer than the brief aging period. It follows that the extent of reactivation observed in these experiments is not simply relatable to the chemical structure of the acyl group: it has been influenced also by the rate of aging during the 2min inhibition period and the 40s transfer period as well as during the 5min observed aging period.

Bearing the above comments in mind, we have divided the compounds according to the observed response of the inhibited esterase to treatment with KF.

(a) *Inhibited neurotoxic esterase that was initially reactivated more than 10%.* Eleven compounds out of 19 were found in this group, which was then subdivided. Seven (compounds 2, 3, 4, 8,

Table 1. *Effect of delay on ability of KF to reactivate inhibited neurotoxic esterase*

As described in the Materials and Methods section, samples of preparations P and M were inhibited for 2 min at pH 5.2 by the compounds listed. The inhibitors are characterized by an acyl group that becomes attached to the enzyme and by a leaving group that is lost in the inhibitory reaction. Aging for 5 min was followed by reactivation with KF at pH 5.2 and assay of phenyl valerate hydrolase activity at pH 8. Neurotoxic esterase activity is derived from the difference in activity between samples of P and M put through the whole procedure and is calculated as a percentage of the mean activity of uninhibited samples [0.610 ± 0.019 A_{510} units (mean ± s.e.m.; 13 samples)]. The data represent single experiments with final samples assayed in duplicate: means of the closely agreeing values are given. In some, but not all, cases uninhibited controls were run through the whole procedure in parallel with inhibited samples: recovery of neurotoxic esterase was 83.0 ± 5.1% (mean ± s.d.; 26 samples) and was unaltered by treatment with KF. Times are measured from the commencement of inhibition, and there was a 30–40 s delay in the dilution and transfer steps before reactivation commenced. Extent of reactivation and of aging have been scored as follows: +, ≥10%; ±, ≥5%; –, <5%.

Activity of neurotoxic esterase after reactivation (%)										
Compound	Acyl group	Inhibitor leaving group	Concn. (μ M)	Started at 160 s		Started at 460 s		Scoring of		
				In buffer (a)	In KF (b)	In buffer (c)	In KF (d)	Reactivation	Aging	
Phosphates										
(1)	(MeO) ₂ PO–	Cl ₂ C=CH–O–	1000	18	26	14	13	±	±	±
(2)	(EtO) ₂ PO–		100	22, 23	41, 50	17, 17	22, 22	+	+	+
(3)	(PrO) ₂ PO–		2.5	18	44	13	18	+	+	+
(4)	(PrO) ₂ PO–		20	9, 6	50, 50	8, 5	17, 16	+	+	+
(5)	(BuO) ₂ PO–	Cl ₂ C=CH–O–	0.7	6, 4	46, 46	4, 5	14, 18	+	+	±
(6)	(C ₃ H ₁₁ O) ₂ PO–		0.15	3	13	1	2	+	±	–
(7)	(2-ClEtO) ₂ PO–		0.5	3	10	0	2	±	±	±
(8)	(PhO) ₂ PO–		0.35	15	38	21	33	+	+	+
(9)	(2-PrPhO) ₂ PO–	Cl	200	32	52	19	26	+	+	±
(10)	(C ₃ H ₁₁ O)(MeO) ₂ PO–	Cl ₂ C=CH–O–	2.5	31	37	31	33	±	±	–
(11)	PhO–P(O)–O $\overline{\text{O}}$ Ph–2-CH ₂		0.25	45	66	39	39	+	+	+
Phosphonates										
(12)	(MeO)(Me)PO–	2,4-Cl ₂ Ph–O–	100	5	4	4	2	–	–	–
(13)	[(Me) ₂ C–CH(Me)O]–(Me)PO–	F	10	45	62	50	59	+	+	±
(14)	(EtO)(Et)PO–	2,4,5-Cl ₃ Ph–O–	300	0	87	4	87	+	+	–
(15)	(2-ClEtO)(Et)PO–	Cl ₂ C=CH–O–	10	2	46	2	18	+	+	+
(16)	(EtO)(C ₃ H ₁₁)PO–	4-NO ₂ Ph–O–	50	16	85	18	86	+	+	–
(17)	(C ₃ H ₁₁ O)(C ₃ H ₁₁)PO–		40	12	0	9	0	–	–	–
(18)	(MeO)(Ph)PO–		3	39	19	35	14	±	±	–
(19)	Phosphoramidate (PrNH) ₂ PO–		100	6	15	3	11	±	±	+
(20)	Phosphinate (C ₃ H ₁₁) ₂ PO–	4-Br–2,5-Cl ₂ Ph–O–	8	3	71	3	54	+	+	–

10, 14 and 18) aged more than 10% in 5 min; the five phosphates appeared to have aged almost completely in that time but the phosphonates (14 and 18) showed slower aging.

Two phosphonates (compounds 13 and 15) did not age. We note that when the pinacolyl methylphosphonyl group (compound 13) is attached to acetylcholinesterase the aging rate is faster than is known for any other derivative of the enzyme (Michel *et al.*, 1967).

Compound (12) showed 8% loss of reactivability. For compound (7) the loss was just over 10%, but this value was achieved only because the 460s buffer value [column (c)] was greater than the 160s value [column (a)], which is clearly abnormal. We prefer therefore to score both these changes as $\pm$.

(b) *Inhibited neurotoxic esterase that was initially reactivated less than 5%.* Three compounds out of 19 (compounds 11, 16 and 19) produced inhibited esterase that was apparently not reactivated at all by KF; indeed with compound (16) activity was lower in the sample treated with KF; this odd result is discussed further below. It is impossible with these results to make any assessment of aging.

(c) *Inhibited neurotoxic esterase that was initially reactivated 5–10%.* Five compounds out of 19 (compounds 1, 5, 6, 9 and 17) fall in this group, in which, as noted above, the degree of reactivation may not be significant. Within this constraint the data suggest that aging may have been observed for compounds (1) and (5) (8.6 and 8.4% change respectively).

Summarizing the data in Table 1, we find that under the chosen conditions significant aging occurred within 5 min for neurotoxic esterase inhibited by seven out of 19 compounds and that aging may have occurred in four other cases. In two further cases no aging was observed, even though reactivation was high, but in the remaining cases no decision could be made because of the small reactivation observed.

(2) *Further investigation of aging with revised procedures*

For each compound where the evidence of Table 1 was uncertain or negative the test procedure was modified in one of several ways. The results in Table 2 are analysed below.

(a) *Reactivation was easy under standard conditions [subsection (1a) above].* The aging period was extended and aging was then observed in periods from 30 to 300 min for compounds (7), (12), (13) and (15). As noted above, more reproducible results were obtained during the longer periods of aging if pH of the aging solution was kept at 8 rather than 5.2. The aging observed for Soman (compound 13) was very similar at these different pH values.

(b) *Insufficient reactivation under standard con-*

ditions [subsections (1b) and (1c) above]. (i) Use of increased concentration of reactivator. When the concentration of KF was increased to 1 M, greater reactivation was not found in the three cases studied; indeed, in two cases (compounds 1 and 9) the KF-treated samples had less activity than had the buffer controls. The extra wash was adequate to remove KF from control samples, so we suggest that the high concentration of KF was converting some of the dilute inhibitor into more inhibitory phosphorofluoridates and causing significant further inhibition during the treatment with KF. This problem is intrinsic to the technique, but only significant in cases where the original inhibitor and the derived fluoridate have very different inhibitory powers, such as with the 4-nitrophenates [e.g. compound (16), which shows the effect even with 200 mM-KF (Table 1)]. We have shown (B. Clothier & M. K. Johnson, unpublished work) that even Paraoxon (a very poor inhibitor of neurotoxic esterase) is converted into a potent inhibitor on incubation with KF.

(ii) Use of inhibition times less than 2 min. Since aging was found to be rapid in many cases, it seemed reasonable that failure to obtain reactivation was sometimes due to aging having finished before the first of the two samples was placed in reactivator. Table 2 shows that, after inhibition with compounds (1) and (5) for only 1 min, reactivation could be obtained, and aging was complete 5 min later. No reactivation was seen with compounds (6) and (11), even when reactivation was attempted after only 12–15 s inhibition. The result with compound (9) was marginal, and limited by the fact that lack of solubility prevented use of a higher concentration of inhibitor to give more inhibition in the reference sample. In two experiments with ^3H -labelled inhibitor compound (6) we found that 30 s after a 2 min inhibition period 31–38% of the ^3H bound to the esterase was in a form that became volatile after alkaline hydrolysis; 10 min later the total bound radioactivity was unchanged, but the volatile tritiated derivative accounted for 45–53% of this radioactivity. The volatile material was not identified, but the situation was exactly analogous to that observed when DiPF-inhibited neurotoxic esterase aged, forming monoisopropylphosphoryl-(neurotoxic esterase) with simultaneous transfer of the other tritiated isopropyl group to a separate binding site (Clothier & Johnson, 1979); these experiments therefore support the inference of very rapid aging of di-n-pentylphosphoryl-(neurotoxic esterase).

Discussion

The present study was undertaken to answer questions in the realms of mechanisms of toxicity

Table 2. Further examination of compounds not shown to age in Table 1

The procedure and assessment of data was as described in the legend to Table 1 except that times for the inhibition step, the duration and the pH of aging or the concentration of KF were altered as noted. In cases where 1M-KF was used the reactivated P and M samples were washed once with Tris/HCl buffer, pH 8.0, and resedimented before the assay of phenyl valerate hydrolase activity.

Activity of neurotoxic esterase after reactivation (% of control)																					
Major change in procedure	Compound	Inhibition		Aging conditions			Concn. of KF (mM)	Before aging period						After aging period		Scoring of					
			Concn. (μ M)	Time (min)	Time (min)	pH		In buffer		In KF		In buffer (c)	In KF (d)	In buffer (c)	In KF (d)	Reactivation	Aging				
Longer aging period	(7)		0.8	2	30	8.0	200	8	70	4	18					+	+				
	(12)		100	4	30	5.2	200	18	54	3	13					+	+				
	(13)		1	20	300	5.2	200	0	79	3	61					+	+				
			1	20	300	8.0	200	2	74	3	55					+	+				
	(15)		10	2	30	5.2	200	14	81	22	71					+	+				
			1	20	300	8.0	200	17	72	11	37					+	+				
Increased concn. of KF	(1)		1000	2	5	5.2	1000	21	13	18	0					—	—				
	(6)		0.15	2	5	5.2	1000	2	5	0	0					—	—				
	(9)		1000	2	5	5.2	1000	15	3	13	2					—	—				
Briefer inhibition	(1)		2000	1	5	5.2	200	17	41	11	14					+	+				
	(5)		0.5	1	5	5.2	200	6	29	4	7					+	+				
	(6)		1.5	0.2	5	5.2	200	0	0	0	0					—	—				
	(9)		200	0.25	5	5.2	200	47	58	35	41					+	±				
	(11)		2.5	0.2	5	5.2	200	8	6	6	0					—	—				

and of enzymology, and the results are discussed under these headings.

Mechanism of toxicity

Group loss [aging, reaction (4) in Scheme 1] cannot occur with esterases inhibited by phosphinates, carbamates or sulphonyl fluorides. Pre-treatment of hens with esterase inhibitors of any of the above types prevents the development of neuropathy as a result of administration of neurotoxic phosphates or phosphonates (which in unprotected hens produce inhibited neurotoxic esterase that could age) (Johnson, 1974). It was therefore proposed that aging of the inhibited esterase is a necessary step in the genesis of delayed neuropathy produced by each and every neurotoxic organophosphorus ester. Group loss from the esterase inhibited by DiPF was shown both by reactivation studies and by radiochemical labelling (Clothier & Johnson, 1979). It is an assumption that an analogous group loss occurred in each case in our

present study where loss of reactivatability was demonstrated. We have presented evidence that this step occurs rapidly in many cases: Table 3 summarizes the results and shows that in 14 out of 19 possible cases aging was detected. In each of the remaining cases no reactivation was achieved, so that it was impossible to test for aging.

The case that neuropathy necessarily requires aging of inhibited neurotoxic esterase has been supported in every case where reactivation could be achieved within 1 min of inhibition, but our technique cannot be pushed further: limitations are imposed by the necessary differential techniques with mixtures of particle-bound enzymes. We offer below some comments on the five cases out of 19 where no reactivation was found.

(i) The radiochemical procedure appeared to show that group loss occurred from di-n-pentylphosphoryl-(neurotoxic esterase) so fast that it could not be detected by the reactivation method.

(ii) The cyclic phenyl saligenin phosphate (com-

Table 3. Approximate half-time for aging of neurotoxic esterase inhibited by various organophosphorus esters ($RR'P-O$ -esterase)

Approximate half-times were estimated from the data in Tables 1 and 2 and text assuming a first-order reaction in each case, as has been shown for DiPF (Clothier & Johnson, 1979). Times in the range 5–60 min are estimated to the nearest 5 min and times longer than 60 min are estimated to the nearest 60 min.

Compound	R	R'	Approx. t_f (min)
Phosphates			
(1)	MeO	Same as R	<2
(2)	EtO		<5
(3)	PrO		<5
(4)	Pr ⁱ O		3-4
(5)	BuO		<2
(6)	C ₅ H ₁₁ O		<1*
(7)	2-ClEtO		10
(8)	PhO		<5
(9)	2-Pr ⁱ PhO		<10
(10)	C ₅ H ₁₁ O	MeO	<2
(11)	PhO	2-HOPhCH ₂ O	Short*
Phosphonates			
(12)	Me	MeO	5
(13)	Me	(Me) ₃ C-CH(Me)O	600
(14)	Et	EtO	3
(15)	Et	2-ClEtO	300
(16)	C ₅ H ₁₁	EtO	†
(17)	C ₅ H ₁₁	C ₅ H ₁₁ O	†
(18)	Ph	MeO	10
Phosphoramidate			
(19)	Pr ⁱ NH	Same as R	†
Phosphinate			
(20)	C ₅ H ₁₁		Aging not possible

* Deduced indirectly; see the Discussion section.

† No aging measurable owing to failure to achieve reactivation.

pound 11) is known to form a benzylphosphorylated derivative of chymotrypsin that ages very rapidly with loss of the 2-hydroxybenzyl group (Ohkawa *et al.*, 1978; Toia & Casida, 1979). Most alkoxyphosphoryl-chymotrypsins are considered not to age, although some aryloxy derivatives do age (Lee & Turnbull, 1961; Bender & Wedler, 1972). It seems probable therefore that the tendency to age may be very great with inhibitory compound (11) no matter what enzyme is involved, and this may hold for neurotoxic esterase.

The failure to reactivate neurotoxic esterase after inhibition by Mipafos (compound 19) is believed to be due to the phosphoramidate structure of this compound, in which two C–N–P bond structures occur. To our knowledge no enzyme inhibited by this or other phosphorodiamidates has been reported to respond to reactivators. It is possible that nucleophilic reagents such as oximes and KF are unsuitable reagents for this reaction, or that the bond from phosphorus to enzyme is much more stable, or that one C–N–P bond is intrinsically unstable in the active-site environment, and that aging is virtually instantaneous after inhibition. However, resolution of these possibilities is beyond the scope of the present study.

For the two remaining compounds where no reactivation was found [the nitrophenyl phosphonate compounds (16) and (17)] there were problems caused by technical artifacts: it seemed that if any reactivation was occurring it was being overwhelmed by re-inhibition brought about by potent phosphonofluoridates generated from KF and the original inhibitor in spite of the large dilution factor. This problem is discussed in the Results section [subsection 2(b)(i)]: it might be soluble with the use of a different reactivator or inhibitors having unchanged R groups but a different leaving group. Some re-inhibition may have occurred in many of the cases we studied, but this did not usually obliterate the reactivation.

On the basis of the 14 proven cases, plus the arguments presented that aging did occur in two other cases and also the fact that in none of the doubtful cases was aging shown not to occur, we consider that the proposition can be accepted that aging of inhibited neurotoxic esterase is the second step in the biochemical mechanism of neuropathy. The speed of aging observed *in vitro* is such that it would be difficult in most cases to detect the time-dependent change *in vivo*: the low rate with Soman (compound 13) could not be observed, since, of all the compounds tested, it is the only one likely to be lethal at doses that cause negligible inhibition of the esterase (B. Clothier & M. K. Johnson, unpublished work). Aging *in vivo* might be detected with compound (15), and if it were sufficiently slow the possibility exists that the neurotoxicity could be

countered soon after poisoning by treatment with oximes capable of penetrating the nervous system.

The proposed mechanism (Johnson, 1974) regards the negative charge of the aged inhibited neurotoxic esterase as causing physiological upset. However, cross-linking on to another tissue site is also hypothetically possible, as is the possibility that it is the group that is lost that causes the damage. We showed (Clothier & Johnson, 1979) that after inhibition of the esterase by [³H]DiPF one isopropyl group was transferred stoichiometrically to another site on protein and that the transferred group was not trapped by glutathione or imidazole in the solution. A similar event appears to occur with di-n-pentoxyposphoryl-(neurotoxic esterase) (see the Results section), but we are doubtful whether alkylamino, aryloxy or arylamino groups would all transfer in the same way.

Enzymology

Aging of inhibited serine esterases has been most studied with the cholinesterases, and on the basis of studies with a few inhibitors the reaction was viewed as a dealkylation of the S_N1 type (Michel *et al.*, 1967). Aldridge (1975) has reviewed several lines of evidence that suggest that either S_N1 or S_N2 reactions may occur in aging, depending on both the compounds and the enzyme involved. The rapid aging of neurotoxic esterase inhibited by the simple n-alkyl phosphates (compounds 1, 2, 3, 5 and 6) and by the aryl phosphate (compound 8) and the very low rate after inhibition by the highly branched pinacolyl phosphonate (compound 13) certainly suggest that the mechanism is not S_N1 in these cases. The rates of aging of neurotoxic esterase inhibited by Soman (compound 13) were similar at pH 5.2 and 8.0. This is in accord with previous observations with DiPF (Clothier & Johnson, 1979), and again in marked contrast with the pH effect in the S_N1 mechanism (Michel *et al.*, 1967).

Rates of aging with the phosphates were so high that no ranking or relating of structure to activity was possible except to note that the bis-2-chloroethoxy derivative (compound 7) aged more slowly than the ethoxy derivative (compound 2). In general, phosphonates aged more slowly than did phosphates, but further comparisons are limited since all the phosphonates were racemic mixtures and it does not necessarily follow that the same optical configuration of groups was achieved in the phosphorylated active site in each case; indeed, it is not certain whether only one isomer was responsible for most of the inhibition produced in any particular case. However, it is worth noting that the chloroethoxy ester (compound 15) aged about 100-fold more slowly than did its unsubstituted analogue (compound 14).

Our studies of aging appear to be the first that

have used an enzyme that was not available in highly active semi-purified form. We have taken advantage of the insolubility of neurotoxic esterase to separate enzyme from reactivator, and have thereby avoided the massive dilution that is normally required if reactivator is not to interfere with the final assay. This procedure is applicable to any particle-bound esterase, although it cannot provide precise results if the rates are very rapid. Moreover, if aging rates are not very rapid (unlike the situation with neurotoxic esterase) and if inhibitors are not too lipophilic, then a centrifugation step may be inserted to separate inhibitor from enzyme. Experience in our laboratory has shown that DiPF and Paraoxon may be largely separated from particles by a single centrifugation, but the procedure is ineffective with the more lipophilic 2,2-dichlorovinyl di-n-pentyl phosphate. When the separation can be achieved the problems of interference by interaction of inhibitor and reactivator are eliminated.

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