The Delayed Neurotoxic Effect of some Organophosphorus Compounds

IDENTIFICATION OF THE PHOSPHORYLATION SITE AS AN ESTERASE

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1. Organophosphorus compounds that produce a delayed neurotoxic effect in hens phosphorylate a specific site in the brain soon after administration. 2. Phosphorylation of the specific site by di-isopropyl [^{32}P]phosphorofluoridate *in vitro* is blocked by the prior addition of phenyl phenylacetate. 3. A small proportion of the total activity of hen brain hydrolysing phenyl phenylacetate *in vitro* was shown to be due to an enzyme different from two others previously described. 4. This enzyme is only slightly inhibited *in vitro* by concentrations of tetraethyl pyrophosphate and paraoxon (diethyl 4-nitrophenyl phosphate) up to 64 μM and is completely inhibited by 6 μM-di-isopropyl phosphorofluoridate and 128 μM-mipafox. 5. It is also inhibited *in vivo* by effective doses of neurotoxic organophosphorus compounds but not by high doses of non-neurotoxic analogues. 6. It is deduced that the active site of this enzyme is the phosphorylation site associated with the genesis of delayed neurotoxicity.

The delayed neurotoxic effect produced by some organophosphorus compounds appears 8–14 days after dosing, but an early event in hens has been shown to be phosphorylation of a site in nervous tissue within a few hours of dosing. The site was identified *in vitro* by selective labelling with [^{32}P]DFP* (Johnson, 1969). In this method two identical tissue samples were preincubated in media containing TEPP (16 μM), one of which also contained mipafox (128 μM); many possible [^{32}P]DFP-labelling sites were removed by phosphorylation with TEPP, and mipafox also phosphorylated the neurotoxic site. After preincubation the samples were incubated under identical conditions with [^{32}P]DFP and the difference between labelling of macromolecules in the two samples was a measure of the site.

Studies reported in this paper are based on the proposal that if a compound capable of forming a complex with the site were added to the medium just before [^{32}P]DFP then phosphorylation and labelling of the site would be prevented. I chose to investigate esters in this role since the phosphorylation site is probably a protein (Johnson, 1969) and it has often been suggested that inhibition of an esterase is involved with the genesis of delayed neurotoxicity. Bloch & Hottinger (1943) suggested that acetylcholinesterase was involved, whereas

Earl & Thompson (1952) pointed to brain pseudo-cholinesterase. Both these enzymes were excluded by Davison (1953), but Aldridge (1954) pointed out that all organophosphorus compounds producing ataxia are esters with a general capacity to inhibit esterases *in vivo*. Further attempts to associate delayed neurotoxicity with inhibition of particular esterases have failed (Aldridge & Barnes, 1961, 1966), but Aldridge's (1954) statement above remains true for all the large number of compounds that have since been tested. In the present work it was shown that few esters are able to protect the site against labelling with [^{32}P]DFP. PPA is effective, however, and the site was shown to be an esterase capable of hydrolysing this substrate. A preliminary report of these findings has been given (Johnson, 1968).

Of the inhibitors mentioned frequently in this paper, DFP and mipafox produced delayed neurotoxic effects in hens and TEPP and paraoxon (diethyl 4-nitrophenyl phosphate) did not.

MATERIALS AND METHODS

Chemicals. The sources of most chemicals used were listed previously (Johnson, 1969). In addition, phenyl acetate, phenyl propionate, phenyl butyrate, phenyl benzoate, phenylacetyl chloride, aniline, ethyl acetate, benzyl benzoate, benzyl salicylate, tri-*n*-butyrin and Brij 35 detergent (polyethylene glycol lauryl ether) were from BDH (Chemicals) Ltd., Poole, Dorset; ethyl phenylacetate was from Kodak Ltd., Kirkby, Liverpool; ethyl mandelate,

* Abbreviations: DFP, di-isopropyl phosphorofluoridate; TEPP, tetraethyl pyrophosphate; PPA, phenyl phenylacetate.

n-butyl mandelate and n-butyl phenylacetate were from K & K Laboratories Inc., Plainview, N.Y., U.S.A.; L-leucine amide hydrochloride, L-phenylalanine hydrochloride benzyl ester, L-phenylalanine hydrochloride ethyl ester, N-acetyl-L-phenylalanine ethyl ester, N-benzoyl-L-tyrosine ethyl ester, N-benzoyl-L-arginine hydrochloride ethyl ester and Folch brain fraction type VI were from Sigma (London) Chemical Co. Ltd., London S.W.6; Folch brain fractions I, III and V were from Koch–Light Laboratories Ltd., Colnbrook, Bucks.; Tween 80 was from Honeywell–Stein Ltd., Carshalton, Surrey; Triton X-100 was from Lennig Chemicals Ltd., London W.C.1. Butyrylcholine perchlorate was supplied by Dr W. N. Aldridge. PPA, phenyl 3-phenyl-propionate and phenyl 4-phenylbutyrate were synthe-sized as described by Poulsen & Aldridge (1964). Phenyl-acetanilide was synthesized by reaction of phenylacetyl chloride with excess of aniline in 1,2-dichloroethane and recrystallized from benzene: the m.p. (uncorr.) was 119–121° (lit. m.p. 118°). Inhibitors were supplied as follows: phenyl saligenin phosphate, di-isopropyl 4-nitrophenyl phosphate and bisdimethyl phosphorodiamidic fluoride from Albright and Wilson Ltd., Oldbury, Lancs.; 2,2-dichlorovinyl dimethyl phosphate from Shell Research Ltd., Sittingbourne, Kent; 4-bromo-3,6-dichlorophenyl dimethyl phosphorothionate (OMS 658) from the World Health Organisation, Geneva, Switzerland. Ethyl 4-nitro-phenyl ethylphosphonate and ethyl 4-nitrophenyl n-butyl-phosphonate were gifts from Dr E. L. Becker, Walter Reed Army Institute of Research, Washington, D.C., U.S.A.

Birds, dosing and neurotoxicity testing. All details were as described before (Johnson, 1969), except that where prophylactic treatment with oximes against the acute anticholinesterase effects of some compounds was known to be ineffective eserine (0·1 mg./kg. body wt.) was ad-ministered subcutaneously with atropine (20 mg./kg.) in 0·9% NaCl 20 min. before the organophosphorus compound and no oxime reactivator was given. This variation did not affect the time of onset or severity of delayed neurotoxic symptoms produced by DFP.

Tissue and buffer. Homogenates (10%, w/v) of brain were prepared in buffer by using the rotating (1100 rev./min.) smooth Perspex pestle as described by Aldridge, Emery & Street (1960) with 0·005 in. difference in diameter of the tube and pestle. The buffer used for homogenization and inhibition experiments was 50 mM-tris–HCl containing EDTA (0·2 mM); the pH was 8·0 determined at 20°.

Assay of PPA hydrolysis in vitro. The method depends on the colorimetric determination of the liberated phenol by the method of Gottlieb & March (1946). Homogenate (1 ml.) was incubated in centrifuge tubes for 30 min. at 25° with 0·5 ml. of buffer alone or buffer containing inhibitor(s). After incubation with inhibitors 2 ml. of suspension of PPA in unbuffered 0·03% Triton X-100 at 20° was added; incubation was continued at 25° for 14 min. and the reaction was stopped by addition of 2 ml. of 0·3 M-HClO₄. The tubes were cooled in ice and then centrifuged at 1500 g for 20 min. Then 4 ml. of clear supernatant was transferred to a tube containing 2 ml. of 4-aminoantipyrine (0·025% in 0·5 M-tris–HCl, pH 9·0); the contents were mixed and 1 ml. of aq. 0·4% K₃Fe(CN)₆ was added. The developed colour was stable for at least 1 hr. and was measured at 510 nm. The PPA suspension was prepared by dissolving about 20 mg. of the ester in 2 ml. of dimethylformamide and then adding Triton X-100 solution rapidly with shaking to give a final

concentration of 0·33 mg. of PPA/ml. Under the conditions described liberation of phenol was linear with respect to tissue concentration and time for both inhibited and uninhibited samples.

Labelling experiments in vitro. The procedure has been described in detail previously (Johnson, 1969); all reactions were at 25°.

Standard test for determination of the neurotoxic site in vitro *by radiochemical or colorimetric assay.* The standard test for the determination of DFP-sensitive phosphoryla-tion sites and activity of PPA-hydrolysing enzymes relevant to delayed neurotoxicity is summarized below.

(i) Paired samples of brain homogenate were first pre-incubated with either (a) non-neurotoxic inhibitors (e.g. TEPP or paraoxon) or (b) mipafox plus inhibitors as in (a). (ii) Both samples were then incubated with either [³²P]-DFP to measure the quantity of remaining phosphorylation sites or with PPA to measure activity of remaining esterases as described above. (iii) The difference between results obtained after preincubations (a) and (b) was calculated and is referred to as (a) − (b). Johnson (1969) showed that for labelling experiments (a) − (b) represents a quantity of phosphorylation sites probably associated with delayed neurotoxicity. The results of the present paper show that the difference in esterase activity measured after preincuba-tions (a) and (b) represents an enzyme associated with delayed neurotoxicity.

For determination of the phosphorylation site the inhibi-tor concentrations were 16 μM for TEPP and 128 μM for mipafox. For determination of the esterase TEPP and mipafox were used in concentrations as for the phosphoryla-tion site and paraoxon (64 μM) was included in the media for both preincubations (a) and (b).

RESULTS

Protection of the neurotoxic site against [³²P]*DFP labelling* in vitro. Paired samples of normal hen brain homogenate were preincubated under con-ditions (a) and (b) as described in the Materials and Methods section. After 30 min. 1 ml. of a suspension (2–10 mg./ml.) of the chosen ester in buffered 0·03% Brij 35 was added to each sample followed im-mediately by [³²P]DFP (final concentration 32 μM). Reaction was allowed to continue for 6 min. and the value of (a) − (b) was determined. PPA decreased the labelling of the site to 20–50% of the control and phenyl phenylpropionate decreased it to 70%. A wide variety of esters that showed no significant affinity for the site are listed below: (i) phenyl acetate, phenyl propionate, phenyl butyrate, phenyl benzoate and phenyl phenylbutyrate; (ii) L-phenyl-alanine benzyl ester, benzyl benzoate and benzyl salicylate; (iii) ethyl acetate, ethyl phenylacetate, ethyl mandelate, L-phenylalanine ethyl ester, N-acetyl-L-phenylalanine ethyl ester, N-benzoyl-L-tyrosine ethyl ester and N-benzoyl-L-arginine ethyl ester; (iv) butyl phenylacetate and butyl mandelate; (v) tributyrin, triolein and Tween 80; (vi) Butyryl-choline, leucine amide and phenylacetanilide; (vii) Folch brain fractions I, III, V and VI.

Inhibitor studies with brain enzymes hydrolysing PPA in vitro. Hen nervous tissues have been shown to hydrolyse PPA very rapidly *in vitro* and two esterases accounting for most of the activity have been characterized (Poulsen & Aldridge, 1964; Aldridge 1964). These workers showed that both enzymes were inhibited *in vitro* by low concentrations of the non-neurotoxic agents TEPP and paraoxon and were less susceptible to DFP and mipafox, which are neurotoxic. By a direct test *in vivo* Aldridge & Barnes (1966) showed that there was no correlation of the neurotoxic effects of a range of organophosphorus compounds with their inhibitory effect on either of these enzymes. The present finding that PPA protects the neurotoxic site *in vitro* has prompted a search for other PPA esterases in hen brain. Samples of brain homogenate were preincubated with various inhibitors for 30 min. and the residual PPA-hydrolysing activity was then determined. Fig. 1 shows that activity progressively decreased as paraoxon concentration was increased to 64 μM; 50% inhibition was found with about 0·6 μM-paraoxon at 25° and pH 8·0 and this is in reasonable accord with the value of 0·16 μM at 37° and pH 7·8 found by Poulsen & Aldridge (1964). Fig. 1 also shows that a residue of about 10% of the total activity was barely affected by paraoxon concentrations above 64 μM, and this suggests the presence of an enzyme(s) different from those previously characterized. When TEPP (2 μM) was included in the preincubation medium the paraoxon-sensitive activity was substantially inhibited and the paraoxon-insensitive activity was not (Fig. 1). TEPP did not inhibit the same proportion

of the paraoxon-sensitive activity at all concentrations of paraoxon. This implies that the paraoxon-sensitive activity is due to two or more enzymes, each with different I_{50} for TEPP; these are probably the two enzymes described by Poulsen & Aldridge (1964). Fig. 2 shows the results of a similar experiment in which the effect of TEPP was studied. Activity was steadily decreased as TEPP concentrations increased to about 128 μM, leaving a residual 8% only slightly affected by higher concentrations; 50% inhibition was found with 2 μM-TEPP (cf. 1 μM under the conditions of Poulsen & Aldridge, 1964); Fig. 2 also shows that the activity sensitive to lower concentrations of TEPP (up to 64 μM) was inhibited by paraoxon (0·5 μM) and the TEPP-insensitive activity was not.

Effect of neurotoxic inhibitors on the PPA-hydrolysing activity insensitive to TEPP and paraoxon. Pairs of homogenate samples were preincubated with paraoxon (64 μM); one tube of each pair also contained mipafox at concentrations up to 256 μM. After preincubation samples were assayed for PPA-hydrolysing activity. It was found that about 20% of the paraoxon-insensitive activity was sensitive to mipafox (Fig. 3) and that there was insignificant increase in inhibition at mipafox concentrations above 128 μM. Fig. 4 shows that, when the mipafox-sensitive activity was considered alone, there was a linear relationship of log(percentage activity remaining) to inhibitor concentration with the line extrapolating through log value 2·0 (± 0·01 s.d.) at zero inhibitor concentration. This shows that the mipafox-sensitive activity behaved as a single enzyme towards this inhibitor. Since the activity of
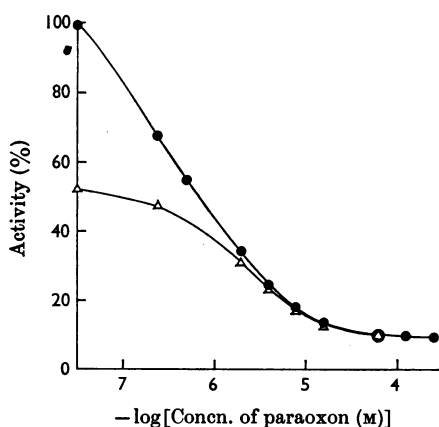
Fig. 1. Effects of paraoxon (●) and of paraoxon + 2 μM-TEPP (△) on the hydrolysis of PPA by hen brain homogenate. Experimental conditions were as described in the Materials and Methods section.

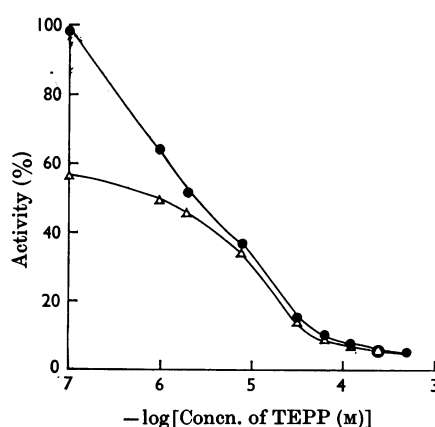Fig. 2. Effects of TEPP (●) and of TEPP + 0·5 μM-paraoxon (△) on the hydrolysis of PPA by hen brain homogenate. Experimental conditions were as described in the Materials and Methods section.
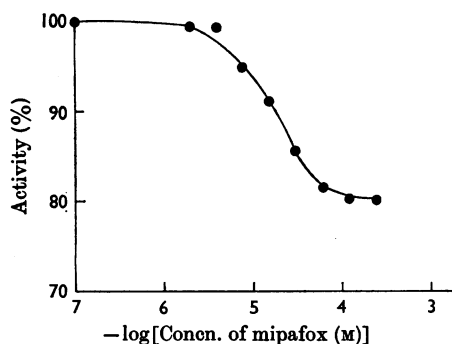
Fig. 3. Effect of mipafox on the Paraoxon-insensitive hydrolysis of PPA by hen brain homogenate. Experimental conditions were as described in the Materials and Methods section. All samples included 64μM-paraoxon in the medium; 100% activity is that observed in the absence of mipafox.
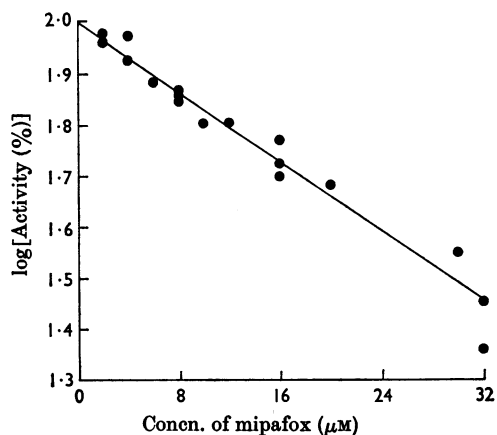

Fig. 4. First-order plot for the PPA esterase activity sensitive to mipafox but insensitive to paraoxon. Results of three experiments similar to that shown in Fig. 3 have been combined.

this mipafox-sensitive enzyme could be determined by comparing the PPA-hydrolysing activity of pairs of samples preincubated with paraoxon with and without mipafox, it was then possible to study its sensitivity to any other inhibitor by including such an inhibitor in the preincubation medium of both samples. By use of this method it was shown that the mipafox-sensitive enzyme was completely inhibited by 30min. incubation with 6μM-DFP at 25° and pH8·0. The concentrations of mipafox and DFP needed to inhibit the mipafox-sensitive enzyme are very similar to those needed to prevent phosphorylation of the neurotoxic site by [32P]DFP (Johnson, 1969); the resistance to high concentrations of TEPP and paraoxon is also common to the enzyme and the neurotoxic site. With the supporting evidence that PPA blocks phosphorylation of the site, it was reasonable to postulate that the neurotoxic site was identical with the active site of the enzyme.

A standard test was used to measure this enzyme *in vitro*. It is analogous in principle to that used to measure the phosphorylation site associated with delayed neurotoxicity (see the Materials and Methods section), but the nature and concentration of non-neurotoxic inhibitor necessary to eliminate much irrelevant activity differs in the two tests. In order that the results of the two tests might be compared (see below) TEPP (16μM) was included in the preincubation media (a) and (b) for esterase assay, although in practice this concentration of TEPP was found not to increase the inhibition above that caused by paraoxon (64μM). The converse is not true: inclusion of paraoxon (64μM) with the usual concentration of TEPP (16μM) used in determining the DFP-sensitive phosphorylation site decreases

the quantity of site determined and this must be allowed for (see below). The inhibition of the enzyme *in vivo* caused by dosing hens with a range of neurotoxic esterase inhibitors and with non-neurotoxic analogues was determined by the standard test to see whether a correlation with delayed neurotoxicity could be established.

Response of the paraoxon-insensitive mipafox-sensitive esterase to administration of organophosphorus compounds in vivo. Table 1 shows the activity of PPA esterase and quantity of the neurotoxic phosphorylation site remaining in hen brain 4–24hr. after birds had been dosed with the listed compounds. In some but not all cases both determinations were carried out on the same specimen of brain. Some measurements of the phosphorylation site are quoted from Johnson (1969). After dosage with neurotoxic compounds having a wide range of structures and physical properties both enzyme activity and quantity of phosphorylation site were markedly decreased, but they were both much less affected by non-neurotoxic analogues. This evidence, together with the ability of PPA to prevent labelling of the site by [32P]DFP *in vitro*, strongly supports the view that the neurotoxic phosphorylation site previously characterized is responsible for the PPA esterase activity measured in this test.

Catalytic-centre activity of the esterase. All the conditions except paraoxon concentration were identical in the determination of esterase activity and of quantity of phosphorylation site (see above). In two separate experiments it was found that inclusion of paraoxon (64μM) with TEPP (16μM) in the

Table 1. ^{32}P (from [^{32}P]DFP) bound to or PPA hydrolysed by brain of normal and dosed hens in vitro under the conditions of the standard test

Hens (1·8–2·8kg., paired for weight) were dosed as indicated in pairs, one with a neurotoxic compound and one with a structurally analogous non-neurotoxic compound, and killed after 4hr. unless otherwise noted. Control dosed birds of similar weight were observed for at least 3 weeks for signs of neurotoxic effect. Labelling by [^{32}P]DFP and hydrolysis of PPA were measured by the standard tests described in the Materials and Methods section. In each case results shown are the difference between values obtained after preincubations (a) and (b). Values were means of duplicates agreeing within 3%. Route of administration: p.o., oral; s.c., subcutaneous; i.p., intraperitoneal.

Compound	Dose (mg./kg.) and route of administration	Difference of results after preincubations (a) and (b)	
		^{32}P bound to brain (pmoles/g. wet wt.)	PPA hydrolysed (nmoles/min./g. wet wt.)
None [mean ± S.D. (no. of hens)]		37 ± 8 (8)	72 ± 9 (7)
Non-neurotoxic			
Paraoxon	15 s.c.	23, 34	63
TEPP	10 s.c.	29	54
2,2-Dichlorovinyl dimethyl phosphate	30 s.c.	20	46
Bisdimethyl phosphorodiamidic fluoride	5 s.c.	32	71
4-Bromo-3,6-dichlorophenyl dimethyl phosphorothionate	400 p.o.	36	72
Ethyl 4-nitrophenyl n-butylphosphonate	4 s.c.	23	54
Di-isopropyl 4-nitrophenyl phosphate	15 s.c.	—	71
Neurotoxic			
Di-2-chloroethyl 4-nitrophenyl phosphate	100 p.o.	10	6
Mipafox	25, 30 s.c.	4, 3	Nil
DFP	1·7 s.c.	3, 3	5, 7
2-Methylphenyl diphenyl phosphate*	120 s.c.	5, 2	5
Phenyl saligenin phosphate	2 s.c.	2–9†	Nil
Ethyl 4-nitrophenyl ethylphosphonate	8 s.c.	13	10

* Test performed 24hr. after dosing.
† Duplicate values differed by 8%.

preincubation medium for a standard-test determination of phosphorylation site decreased the quantity measured to 65% (58–72%) of that measured with TEPP alone. The mean value of 37pmoles of phosphorylation site/g. of normal brain (Table 1) must therefore be corrected to 24 pmoles/g. under the conditions of the esterase assay. Table 1 shows that this quantity catalyses formation of 72nmoles of phenol/min./g., giving a catalytic-centre activity of about 3000 molecules/min. at pH8·0 and 25°.

Investigation of the 'neurotoxic' esterase in hen spinal cord. When the standard test was applied to spinal cord taken from a normal hen, PPA-hydrolysing activity was found to be considerably lower than in whole brain (14 compared with 72nmoles/min./g. wet wt.). This difference is less when calculated on a protein basis: 0·33 compared with 0·9nmole/min./mg. of protein. The cord activity was completely inhibited in vivo 2hr. after administration of DFP (1·7mg./kg.).

DISCUSSION

The evidence presented in this paper confirms the long-standing hypothesis that inhibition of an esterase of the nervous system is an early event in the process of delayed neurotoxicity caused by organophosphorus compounds. Most of the activity of this enzyme in homogenates of whole brain was found to be inhibited after dosage with neurotoxic compounds. The enzyme in spinal cord responded similarly when tested with DFP, and it is not clear why the pathological effect is seen only in long axons. The products of the reaction normally catalysed by the 'neurotoxic' esterase may be essential nutrients, and failure of supply may manifest itself first in regions furthest removed from the neuron cell-body. Another possible function of the esterase is as a scavenger removing potentially toxic materials that initiate irreversible damage if allowed to accumulate. More understanding of the neurotoxic process may be gained by investigating
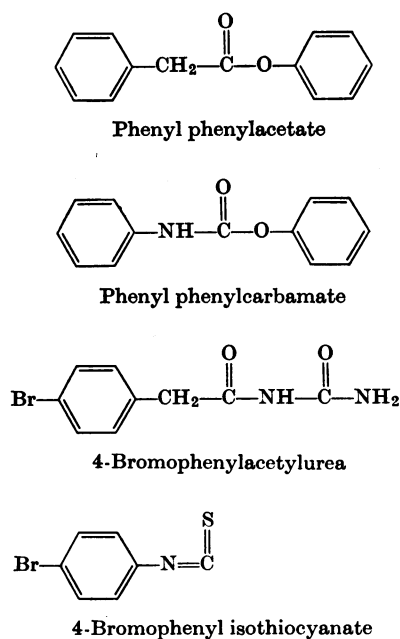
Fig. 5. Structures of PPA and related compounds.

metabolic reactions involving precursors or products of the esterase step. Recent studies with carbamates inhibiting the esterase (Johnson & Lauwerys, 1969) suggest that comparatively few variations of the chemical structure of the PPA molecule give an acceptable 'fit' at the active site. It is the more interesting therefore that two other compounds, which can be considered formally as derivatives of phenylacetic acid (see Fig. 5), produce delayed neurotoxic symptoms in some species and that the histological lesions are very similar to those produced by organophosphorus compounds in hens. 4-Bromophenyl isothiocyanate produces delayed ataxia in sheep (Lessel & Towlerton, 1967) and it would be possible for this compound to react directly (or via an intermediate thiocarbamate) with the active site of an esterase to give an inactive thiocarbamoylated enzyme. With 4-bromophenylacetylurea, which produces effects in rats (Cavanagh, Chen, Kyu & Ridley, 1968), reaction with an esterase seems unlikely on chemical grounds. However, competiton at some part of an essential metabolic sequence, which could include the esterase, seems possible. In considering whether the mechanisms of delayed neurotoxicity produced by these compounds on the one hand and organophosphorus compounds on the other may be related, it is a considerable problem that no functional effects are seen when rats are given single doses of organophosphorus

compounds (Barnes & Denz, 1953; Majno & Karnovsky, 1961) or when hens receive an oral dose of 4-bromophenylacetylurea (Cavanagh et al. 1968).

More than 80% of the PPA-hydrolysing activity of hen brain has previously been shown to be due to two distinct enzymes, both very sensitive to paraoxon (Poulsen & Aldridge, 1964; Aldridge, 1964). The present work shows that the remaining paraoxon-insensitive activity can be subdivided into two components: about one-fifth of the activity is due to a single mipafox-sensitive enzyme associated with delayed neurotoxicity, and four-fifths is insensitive to mipafox but can be at least partially inhibited by DFP (M. K. Johnson, unpublished work). These results demonstrate the value of irreversible inhibitors in distinguishing between enzymes having similar catalytic ability. Future investigations of the mechanisms of neurotoxicity would be greatly facilitated if a more specific substrate than PPA could be found.

Since ester substrates and carbamate inhibitors react with esterases by analogous mechanisms (Wilson, Hatch & Ginsburg, 1960; Reiner & Aldridge, 1967), information can be gained about the specificity of the esterase by studying the effect of a variety of carbamates in the standard assay. This is more convenient than studying a variety of substrates requiring new assay procedures. Thus far no normal cell constituent has been found to be a substrate, but recent work has suggested some variations of the PPA molecular structure that may lead to increased specificity.

Preliminary attempts have been made to obtain purified preparations of the 'neurotoxic' esterase, and it may eventually be possible to carry out studies of the phosphorylation process occurring at inhibition and to devise reagents to hydrolyse the protein–phosphate bond, thus regenerating active enzyme. Such compounds might be of therapeutic value. Various oximes are known that carry out a regenerative process with inhibited acetylcholinesterase (see Heath, 1961), but none is known to affect delayed neurotoxicity.

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