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Organophosphorus and other Inhibitors of Brain 'Neurotoxic Esterase' and the Development of Delayed Neurotoxicity in Hens

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1. The delayed neurotoxic effects of some organophosphorus compounds are associated with phosphorylation of the active site of a nervous-tissue enzyme capable of hydrolysing phenyl phenylacetate. 2. Neurotoxic organophosphorus compounds and some carbamates and sulphonyl fluorides progressively inhibit the enzyme, attaching a substituent covalently at the active site. 3. Prolonged inhibition of the enzyme by phenyl *N*-benzyl-*N*-methylcarbamate or phenylmethanesulphonyl fluoride does not lead to neurotoxic effects. 4. Prior inhibition of the enzyme by carbamates or sulphonyl fluorides *in vivo* prevents the neurotoxic effects of several organophosphorus compounds. 5. After dosage of hens with protective compounds, protection lasts until about 70% of the enzyme site again becomes available for phosphorylation. 6. Reaction of all the inhibitors at the active site of the enzyme leads to the same inhibitory effect with respect to hydrolysis of phenyl phenylacetate but does not in all cases lead to delayed neurotoxicity. It is concluded that the nature of the group substituted at the enzyme active site determines the toxic response.

Numerous organophosphorus esters cause delayed neurotoxic effects in hens 10–14 days after administration (see review by Davies, 1963; Aldridge & Barnes, 1967). The effects are seen clinically as ataxia and histologically as a dying-back of some long axons of the nervous system. Effective compounds are all inhibitors of esterases or are converted into inhibitors *in vivo* (Aldridge, 1954), but not all inhibitors of esterases are neurotoxic. Of the inhibitors mentioned frequently in this paper DFP* and Mipafox cause ataxia whereas tetraethyl pyrophosphate and paraoxon do not.

Neurotoxic effects have been correlated with phosphorylation of a brain protein shortly after dosing: this protein can be selectively phosphorylated and assayed *in vitro* by using [^{32}P]DFP (Johnson, 1967, 1969*a*). The protein catalyses hydrolysis of phenyl phenylacetate *in vitro*, and a large variety of neurotoxic organophosphorus compounds inhibit this enzyme *in vivo* whereas non-neurotoxic analogues do not (Johnson, 1968, 1969*b*). Certain carbamates inhibit the esterase *in vitro*. After dosage of hens with such carbamates, esterase activity *in vivo* was decreased but returned to normal within 1–36h, depending on the structure of the carbamate used, and no neurotoxic effects were seen. These rates of return contrast with a much lower rate after inhibition by DFP *in vivo*. Delayed neurotoxic effects of DFP were prevented by predosing hens with phenyl benzylcarbamate shortly before DFP (Johnson & Lauwerys, 1969), and the protective effect persisted until the esterase activity had returned to about 70% of normal at the time when DFP was given. It was concluded that neurotoxic organophosphorus compounds, phenyl phenylacetate and phenyl benzylcarbamate all reacted at the active site of the 'neurotoxic esterase' and it was deduced that prolonged inhibition of this esterase caused the metabolic disturbance leading to dying-back of axons (Johnson, 1969*c*).

This paper reports further experiments confirming the identity of the 'neurotoxic phosphorylation site' and the 'neurotoxic esterase'. By studies with some inhibitors that do not contain phosphorus but do cause prolonged inhibition of the esterase it has been shown that the neurotoxic effect is a result of phosphorylation as such and not a consequence of prolonged deficiency of the esterase activity.

* Abbreviation: DFP, di-isopropyl phosphorofluoridate ('di-isopropyl fluorophosphate').

MATERIALS AND METHODS

Chemicals. Tri-*n*-butyl phosphorotrithiolate (DEF) was a specially purified preparation (purity >99%) from the Chemagro Corp., Kansas City, Mo., U.S.A.; 4-bromo-3,6-dichlorophenyl methyl ethylphosphonothionate (OMS

988) was from the World Health Organization, Geneva, Switzerland, and dimethyl 1-hydroxy-2,2,2-trichloroethylphosphonate (Dipterex) was from Baywood Chemicals Ltd., Bury St Edmunds, Suffolk, U.K. Tetraethyl pyrophosphate, *NN'*-di-isopropylphosphoryldiamidic fluoride (Mipafox) and [^{32}P]DFP were obtained from the sources stated previously (Johnson, 1969*a*).

Glycerol formal was supplied by Fluka A.-G. Chemische Fabrik, Buchs, Switzerland. Benzylmethylamine, benzenesulphonyl chloride and phenylmethanesulphonyl fluoride were from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. Phenylacetyl chloride, thiourea, phenyl chloroformate, benzylamine and *n*-butylamine were from BDH Chemicals Ltd., Poole, Dorset, U.K. The amines and phenyl chloroformate were redistilled before use. Phenyl phenylacetate was prepared by dropwise addition of phenylacetyl chloride (0.1 mol) to molten phenol (0.1 mol) at 140–150°C: the product was crystallized twice from light petroleum (b.p. 40–60°C), giving white crystals, m.p. 41–42°C. Carbamates were prepared by adding slowly a solution of phenyl chloroformate (0.10 mol) in benzene (50 ml) to a stirred solution of the appropriate amine (0.11 mol) and pyridine (0.12 mol) in benzene (100 ml). After 10 min the solution was warmed to dissolve any precipitate and poured into 300 ml of 2 M-HCl with stirring. The benzene layer was separated, washed once with 2 M-HCl (100 ml), twice with 2 M-NaOH (100 ml), twice with water and dried over Na_2SO_4. Benzene was evaporated in a stream of N_2. The solid phenyl benzylcarbamate was recrystallized twice from a mixture of 1,2-dichloroethane and light petroleum (b.p. 60–80°C), giving cream-coloured plates, m.p. 83–85°C. Each of the two liquid carbamates was washed twice with 4 vol. of cold light petroleum (b.p. 30–40°C) to remove unchanged reactants; phenyl *n*-butylcarbamate was obtained by fractional distillation under vacuum of the crude product (yield 40%, b.p. 143–146°C/2 mmHg). The washed phenyl *N*-benzyl-*N*-methylcarbamate contained an impurity showing an –NH– band absorption in the i.r. spectrum, so it was dissolved in diethyl ether and passed through a column (27 cm × 4 cm) of activated alumina (200–400 mesh; heated overnight at 135°C before use): the impurity was firmly bound while the pure product passed unretarded and was obtained free of ether by evaporating the effluent in a stream of warm N_2; it was not distilled. I.r. spectra of the monosubstituted carbamates had prominent bands characteristic both of carbonyl and –NH– groups, whereas the disubstituted carbamate gave the carbonyl band but not the –NH– band. *n*-Butanesulphonyl bromide was prepared from butan-1-ol, thiourea and conc. HBr by the general method (B) of Johnson & Sprague (1936): the pale-yellow product distilled over the range 72–98°C/2 mmHg (yield 24% based on thiourea); it had strong i.r. absorption at 1160 and 1350 cm^{-1} characteristic of the sulphonyl group (Bellamy, 1958) and liberated bromine on warming with conc. HNO_3. The crude *n*-butanesulphonyl bromide (32 mmol) was converted into the fluoride by stirring with finely powdered anhydrous NaF (160 mmol) in dimethylformamide for 6 h at 100°C (general method of Fahrney & Gold, 1963). The colourless purified product (yield 25%) distilled at 62–66°C/12 mmHg, had sulphonyl bands in the i.r. spectrum similar to those of the bromide, but did not yield bromine on treatment with conc. HNO_3. Benzenesulphonyl fluoride (b.p. 70°C/3 mmHg) was prepared similarly from benzenesulphonyl chloride.

Hens, dosing and neurotoxicity testing. Adult hens weighing 2.0–2.6 kg were used and all details were as described previously (Johnson, 1969*a*,*b*). Protective pretreatment against acute cholinergic effects of DFP and Mipafox was with eserine and atropine as described previously (Johnson, 1969*b*). No pretreatment was necessary for oral dosage with 2-methylphenyl diphenyl phosphate. Test compounds for intravenous dosing were dissolved in a suitable volume of glycerol formal (up to 0.8 ml/kg); control birds were unaffected by this volume of solvent.

Tissue and buffer. Homogenates of brain or spinal cord (each 10%, w/v) and sciatic nerve (four nerves ≡ 1.7 g/15 ml) were prepared in buffer by using the rotating (1900 rev./min) smooth Perspex pestle as described by Aldridge, Emery & Street (1960) with 0.005 in (1.25 mm) difference in diameter of the tube and pestle. The buffer was 50 mM-tris–HCl containing EDTA (0.2 mM); the pH was 8.0 at 20°C.

Standard assays of 'neurotoxic phosphorylation site' and of 'neurotoxic esterase'. These differential assays at 25°C were as described previously (Johnson, 1969*b*) except that for the determination of the 'neurotoxic esterase' tetraethyl pyrophosphate (16 μM) was no longer included in the preincubation medium since it was found to have no influence in the presence of the standard concentration of paraoxon (64 μM). The preincubation time with inhibitors before addition of [^{32}P]DFP or phenyl phenylacetate was 30 min. Compounds to be tested *in vitro* were dissolved in acetone and added to the medium immediately after the tissue, giving a final concentration of acetone of 1.7% (v/v); this concentration of acetone stimulated esterase activity slightly. All measurements were made in duplicate and were within 1% of the mean value. However, the activity of the esterase and quantity of phosphorylation site is calculated as a difference of two experimentally determined values, and the reported values are means lying within an extreme range of ±8%. Values determined for birds dosed with solvent only were not different from those for undosed birds. Means ±S.D. of all control values were 37±8 pmol of ^{32}P bound/g of brain (11 hens) for the phosphorylation site and 86±10 nmol of phenol produced/min per g of brain (26 hens) for the esterase activity.

RESULTS

Further evidence that a phosphorylation site and an esterase are involved in delayed neurotoxicity. Previous studies showed that both the 'neurotoxic phosphorylation site' and the activity of 'neurotoxic esterase' were substantially eliminated *in vivo* by doses of a number of neurotoxic organophosphorus compounds, but were only slightly affected by doses of non-neurotoxic analogues (Johnson, 1969*a*, *b*). The effects of three more compounds on either the site or the esterase are shown in Table 1 and support the previous conclusion. Thus after a non-neurotoxic dose of DEF the esterase activity was decreased only to 65% of normal but after a

Table 1. *Effect of administration of some organophosphorus compounds* in vivo *on the 'neurotoxic esterase' or 'neurotoxic phosphorylation site' of hen brain*

Hens (2.0–2.5kg, paired for weight) were dosed as indicated. One was observed for 3 weeks for signs of neurotoxic effects; the other was killed after 24h and the assays were performed on brain homogenate as described in the Materials and Methods section. Results for the esterase are calculated as percentages of the same parameter measured in the brain of a normal bird assayed at the same time. For the phosphorylation site results are calculated as percentages of the mean of all normal values quoted in the Materials and Methods section. Abbreviations: s.c., subcutaneous; i.p. intraperitoneal.

Compound	Dose (mg/kg) and route	Activity: Phosphorylation site available (% of control)	Activity: Esterase activity (% of control)
Non-neurotoxic dose			
Tri-*n*-butyl phosphorotrithiolate (DEF)	220s.c.		65
4-Bromo-3,6-dichlorophenyl methyl ethylphosphonothionate (OMS 988)	100i.p.	38, 73	
Dimethyl 1-hydroxy-2,2,2-trichloroethyl-phosphonate (Dipterex)	200s.c.		32
Neurotoxic dose			
Tri-*n*-butyl phosphorotrithiolate (DEF)	1100s.c.		23
4-Bromo-3,6-dichlorophenyl methyl ethylphosphonothionate (OMS 988)	100i.p. + 75i.p.*	11	14
Dimethyl 1-hydroxy-2,2,2-trichloroethyl-phosphonate (Dipterex)	200s.c. + 100s.c.*		32

* Two doses given 3 days apart.

neurotoxic dose it was down to 23% of normal. After a single dose of Dipterex 'neurotoxic esterase' activity was 32% of normal. From this result a slight neurotoxic effect in the observed control bird was expected but none was seen. However, after a second (smaller) dose activity was still only 32% of normal (Table 1), so that the deficiency of activity had been prolonged for a further 3 days and one out of the two observed control birds became ataxic. No ataxia was seen in birds given a single dose of OMS 988, but acute toxic effects and assay results for a group of eight birds dosed intraperitoneally with this compound were very variable, suggesting that absorption was not consistent in the group; this might account for the fact that only two out of four control birds receiving the double dose became ataxic although the esterase activity was highly inhibited in the individual bird that was assayed.

Effects of carbamate and sulphonyl fluoride inhibitors on the neurotoxic site and esterase. Table 2 shows that several carbamates and sulphonyl fluorides formally related to phenyl phenylacetate have similar effects on both the neurotoxic site and the esterase when tested *in vitro* and *in vivo*. This further confirms the identity of the site and the esterase. Inhibition of the esterase *in vitro* by both phenyl benzylcarbamate (50μM) and phenylmethanesulphonyl fluoride (50μM) was shown to be progressive with time, since no inhibition occurred if the compound was added to the homogenate at the same time as the substrate. Inhibition of the phosphorylation site was also progressive, but was not entirely stopped by addition of [^{32}P]DFP. It is presumed that the assay concentration of DFP (32μM) was insufficient to saturate the site.

Table 3 shows that whereas *in vivo* the activity of the esterase returns to normal quite rapidly after dosage with carbamates, the rate of return after a dose of phenylmethanesulphonyl fluoride cannot be distinguished from that after DFP.

Carbamate and sulphonyl fluoride inhibitors and delayed neurotoxicity. It has previously been shown that challenge doses of DFP given to hens during the comparatively brief period that the 'neurotoxic esterase' was inhibited by phenyl benzylcarbamate failed to cause ataxia (Johnson & Lauwerys, 1969). It was also shown that the carbamate did not prevent access of [^{32}P]DFP to the brain. Table 4 shows that sensitivity to DFP returned when about 70% of total esterase activity became available for inhibition by DFP. The Table also shows that an intravenous dose of phenyl *n*-butylcarbamate (510μmol/kg) protected against DFP given 24h later, when esterase activity was 39% of control, and gave partial protection after 48h, at which time the esterase activity was 78% of control. Phenyl *N*-benzyl-*N*-methylcarbamate also protected against DFP (Table 4), and in a further experiment with a single bird an intravenous dose of phenyl

Table 2. *Inhibition of 'neurotoxic phosphorylation site' and 'neurotoxic esterase' by some carbamates and sulphonyl fluorides*

Assay procedures and their analytical error were as described in the Materials and Methods section. For results obtained *in vitro* a stock solution of test compound in acetone was added to the preincubation medium immediately after the homogenate. The acetone concentration during preincubation was 1.7%. Preincubation was for 30min at 25°C before addition of [^{32}P]DFP or phenyl phenylacetate. Each result represents a single assay and was calculated as the percentage of the control value obtained when acetone was added to the homogenate. For tests *in vivo* hens were dosed as indicated with the compound dissolved in glycerol formal and were killed for assay at the indicated time after dosage: results were calculated as percentages of the same parameter measured at the same time in homogenate of brain from a control bird. Abbreviations: i.v., intravenous; s.c., subcutaneous. For each compound, site and esterase assays were performed at different times and with homogenates of brain from separate birds.

	In vitro			*In vivo*			
Compound	Concn. (μM)	Esterase activity (% of control)	Phosphorylation site available (% of control)	Dose (μmol/kg) and route	Time of assay after dosage (h)	Esterase activity (% of control)	Phosphorylation site available (% of control)
Phenyl benzylcarbamate	50	10	16	220 i.v.	1	17	14
Phenyl *N*-benzyl-*N*-methylcarbamate	50	37	48	166 i.v.	17	42	38
Phenyl *n*-butylcarbamate	100	53	50	305 i.v.	17	76	74
Phenylmethanesulphonyl fluoride	50	34	23	230 s.c.	24	7	12
n-Butanesulphonyl fluoride	50	76	82	29 i.v.	2	42	55
Benzenesulphonyl fluoride	100	92	92	200 s.c.	24	82	90

Table 3. *Rate of return of 'neurotoxic esterase' activity after inhibition* in vivo

Hens were dosed with test compounds dissolved in glycerol formal and the esterase was assayed in a homogenate of a single brain taken at the indicated time after dosage. Results are calculated as percentages of activity of brain from a control bird measured at the same time. Abbreviations: i.v., intravenous; s.c., subcutaneous.

		Esterase activity (% of control) at various times after dosage				
Compound	Dose (μmol/kg) and route	1–2 h	17–24 h	2 days	4 days	7 days
Phenyl benzylcarbamate	220 i.v.	20	90			
Phenyl *N*-benzyl-*N*-methylcarbamate	166 i.v.	18	42			
Phenyl *n*-butylcarbamate	510 i.v.	13	39	78		
Phenylmethanesulphonyl fluoride	230 s.c.		7	12	41	61
	20 i.v.	55	50			
n-Butanesulphonyl fluoride	2×36 i.v.*	15	29			
DFP	11 s.c.		7	16	35	58

* Divided dose given during 24h and assays timed from second dose.

benzylcarbamate (220μmol/kg) protected against the neurotoxic effect of Mipafox (137μmol/kg) given subcutaneously 1h afterwards. Since a neurotoxic dose of DFP caused prolonged inhibition of the esterase (Table 3), it seemed probable that ataxia would occur if inhibition due to carbamates could be prolonged by repeated dosing. Inhibition caused by phenyl *N*-benzyl-*N*-methylcarbamate

Table 4. *Effect of some inhibitors* in vivo *on 'neurotoxic esterase' and on delayed neurotoxicity caused by DFP*

Groups of hens were dosed with test compounds dissolved in glycerol formal; control birds received solvent only. Esterase activity was determined by using brain from one hen of the group killed at the indicated time after dosage. Results are calculated as percentages of the activity of homogenate from a control bird assayed at the same time. At the time of killing for assay, the remaining hens in the group received DFP (9.5 μmol/kg) subcutaneously after prophylactic treatment with eserine and atropine as described in the Materials and Methods section. Degree of protection was assessed clinically 3 weeks after dosage with DFP: ++, complete protection; +, decrease in severity; 0, no protection. Protection by each compound was confirmed by histological examination of tissues from at least one protected bird in that class 3 weeks after dosage with DFP. Control birds given only solvent before DFP became ataxic in every case. Abbreviations: i.v., intravenous; s.c., subcutaneous.

Test compound	Dose (μmol/kg) and route	Delay before assay of esterase or dosage with DFP (h)	Esterase activity (% of control)	Degree of protection and no. of birds ++	+	0
Phenyl benzylcarbamate*	220 i.v.	1	17	4/4		
		6	41	2/2		
		10	70		2/2	
		17	92		1/2	1/2
Phenyl *N*-benzyl-*N*-methylcarbamate	166 i.v.	17	42	1/1		
Phenyl *n*-butylcarbamate	510 i.v.	24	39	1/1		
		48	78		2/2	
Phenylmethanesulphonyl fluoride	87 s.c.	17	34	4/4		
	173 s.c.	48	15	1/1		
		72	37	2/2		
		96	45	1/1		
		120		1/1		
		168	67		1/1	
	29 i.v.	24	39	2/2		
	21 i.v.	24	50	2/2		
n-Butanesulphonyl fluoride	86 i.v.†	6	12	1/1		
	72 i.v.†	2	15	2/2		
		24	29	2/2		
	29 i.v.	2	42	2/2		
	13 i.v.	2	77		1/2	1/2

* Data taken from Johnson & Lauwerys (1969).
† Divided dose as Table 3.

was still substantial 17 h after a single dose (Table 3). This compound was therefore administered in divided doses to hens, so that a total of 5 mmol/kg was given in 14 oral doses during 17 days. Esterase activity was 25% and 32% of normal when measured 17 h after the first and last doses respectively. No ataxia developed in a single test hen during 18 days from the first dose. Esterase activity probably fluctuated considerably during the periods between doses, and it was thought possible that to cause ataxia there must be a severe continuous deficiency of activity for a few days, as occurs after a single dose of DFP.

Table 3 shows that a subcutaneous dose of phenylmethanesulphonyl fluoride (230 μmol/kg) caused inhibition *in vivo* of similar degree and duration to that caused by a highly neurotoxic subcutaneous dose of DFP (11 μmol/kg). It was therefore surprising that, when the hens dosed with phenylmethanesulphonyl fluoride were observed for up to 4 weeks, no signs of ataxia were seen and specimens of sciatic nerve and spinal cord were histologically normal (I am indebted to Dr G. Hard for the histological assessment). No neurotoxic effect was seen even when the dosing schedule was extended so as to keep enzyme activity at less than 20% of normal continuously throughout 2 weeks (an initial subcutaneous dose of 230 μmol/kg followed by six subcutaneous doses of 86 μmol/kg every other day). *n*-Butanesulphonyl fluoride caused brief convulsive reactions at intravenous doses above 25 μmol/kg and few experiments were

done with large doses. However, 85% inhibition of the 'neurotoxic esterase' was obtained after divided doses of this compound (Table 3) and no ataxia occurred in any of three birds that were observed for 3 weeks after receiving such a dose. The return of enzyme activity to normal was not followed in detail, but it was akin to the rate after phenylmethanesulphonyl fluoride.

It is evident that prolonged inhibition of the brain 'neurotoxic esterase' does not inevitably cause a metabolic disturbance leading to dying-back of long axons, and this is contrary to previous conclusions (Johnson, 1969*b*,*c*).

The histological lesions associated with delayed neurotoxicity are seen in spinal cord and peripheral nerve, rather than in brain. The biochemical correlations established so far have been based on studies with homogenates of whole brain. A measurable but low activity of 'neurotoxic esterase' has been found in sciatic-nerve homogenate (M. K. Johnson, unpublished work), but routine assay is not practicable. It has been shown that activity in spinal cord of both phosphorylation site and esterase is inhibited *in vivo* by both DFP (Johnson, 1969*b*) and 2-methylphenyl diphenyl phosphate (M. K. Johnson, unpublished work). At 3 days after inhibitory doses of DFP or phenylmethanesulphonyl fluoride activity of the esterase in spinal cord was respectively 33% and 41% of control, compared with 30–40% in brain. There is no reason therefore to believe that the present anomaly arises from studying brain instead of spinal cord or sciatic nerve.

One possible interpretation of the failure of phenylmethanesulphonyl fluoride to cause ataxia is that inhibition of the esterase is not connected with the lesion in spite of the correlations that have been accumulated. That this is not so is shown by the fact that, when birds dosed first with phenylmethanesulphonyl fluoride are given a challenge dose of DFP, no ataxia occurs (Table 4). The effect is exactly analogous to protection by carbamates, but the duration is greatly extended. Thus after a single subcutaneous dose of phenylmethanesulphonyl fluoride (173 μmol/kg) no ataxia was seen when DFP (9.5 μmol/kg) was given subcutaneously at any time up to 5 days after; partial protection was observed when DFP was given after 7 days, at which time 67% of normal esterase activity was available for inhibition by DFP (Table 4). Moreover, the same dose protected all of three birds against the neurotoxic effects of Mipafox (137 μmol/kg) given subcutaneously 1 day after, and protected two out of three against 2-methylphenyl diphenyl phosphate (350 μmol/kg) given orally and gave partial protection to the remaining bird. No protection occurred in any of three hens if the same dose of phenylmethanesulphonyl fluoride was given 1 h after DFP. It is reasonable to conclude that inhibition by phenylmethanesulphonyl fluoride prevents subsequent phosphorylation of the esterase by several different neurotoxic organophosphorus esters. The return of DFP-sensitivity of the birds can again be correlated with availability of about 70% of esterase activity (Table 4) and presumably with availability of 70% of the phosphorylation site. Table 4 shows that *n*-butanesulphonyl fluoride also protected against DFP. Further evidence that phenylmethanesulphonyl fluoride, phenyl benzylcarbamate and DFP all act at the same site is obtained from the results in Table 5: thus when phenylmethanesulphonyl fluoride (21 μmol/kg) was given intravenously to hens inhibition was typically prolonged and birds given a challenge dose of DFP 28 h afterwards did not become ataxic. However, Table 5 also shows that if phenyl benzylcarbamate (264 μmol/kg) is injected intravenously 1 h before the phenylmethanesulphonyl fluoride then the

Table 5. *Prevention of protective effect of phenylmethanesulphonyl fluoride by prior administration of phenyl benzylcarbamate*

Groups of four hens were dosed intravenously as indicated with test compounds dissolved in glycerol formal. 'Neurotoxic esterase' activity in homogenate of a single brain was determined 1.5 h and 28 h after the second dose and calculated as percentage activity of homogenate from an undosed bird assayed at the same time. At 28 h after dosage, two hens of each group received DFP (9.5 μmol/kg) subcutaneously after prophylactic treatment with eserine and atropine as described in the Materials and Methods section. Clinical assessment of ataxia was made after 2–3 weeks.

Compound (dose)	Time after second dose (h) ...	Activity of esterase (% of control) 1.5	28	Clinical effect of DFP given 28 h after second dose
Phenylmethanesulphonyl fluoride (21 μmol/kg) intravenously 1 h after solvent		45	50	No ataxia
Phenylmethanesulphonyl fluoride (21 μmol/kg) intravenously 1 h after intravenous phenyl benzylcarbamate (264 μmol/kg)		20	79	Ataxia
Phenyl benzylcarbamate (264 μmol/kg) intravenously 1 h after solvent		17	79	Ataxia

degree and duration of inhibition are identical with those after the carbamate alone, and similarly dosed birds became ataxic when given a challenge dose of DFP at 28h. This experiment has been repeated three times with slight variations of dose, giving qualitatively similar results. Also it was shown that 24h after a neurotoxic subcutaneous dose of DFP (9.5μmol/kg) the esterase activity was 11% of normal and less than 10% of the 'neurotoxic phosphorylation site' was available, but when the DFP was given 1h after an intravenous dose of phenyl benzylcarbamate (220μmol/kg) the respective values were 63 and 80% of normal.

DISCUSSION

The procedures used in the present work are complex and the conclusions to be drawn are surprising. I have therefore listed below a summary of the facts that are clearly established.

(1) Delayed neurotoxicity is associated both with phosphorylation of a protein site that can be identified by a specific assay and with inhibition of an esterase that can be specifically assayed. The esterase behaves as a homogeneous enzyme towards Mipafox *in vitro* (Johnson, 1969*b*). Complete correlation of delayed neurotoxicity with phosphorylation of the site has been established *in vivo* by using 16 organophosphorus compounds having a large variety of chemical structure (Johnson, 1969*a*,*b*; this paper, Table 1). Correlation of delayed neurotoxicity with inhibition of the esterase is also supported by studies with 16 organophosphorus compounds *in vivo* (Johnson, 1969*b*, and this paper).

(2) Phosphorylation of the 'neurotoxic site' and hydrolysis of phenyl phenylacetate by the 'neurotoxic esterase' are two properties of a single site associated with delayed neurotoxicity. There are four lines of evidence. (*a*) Both parameters were affected to a similar extent *in vivo* and *in vitro* by numerous compounds. Fig. 1 shows the close correlation of effect of organophosphorus compounds (fourteen *in vivo*), carbamates (three *in vitro* and *in vivo*) and sulphonyl fluorides (three *in vitro* and *in vivo*) on the site and esterase. The data are drawn from Tables 1 and 2 and from previously published work (Johnson, 1969*a*,*b*). It should be borne in mind that, apart from the analytical error due to the use of a differential assay (see the Materials and Methods section), some variation in the experiments *in vivo* is to be expected, since whenever possible the percentage inhibition was calculated with reference to separate control birds assayed at the same time. It was less desirable to use a mean of all the control values obtained for the phosphorylation site, since the specific radioactivity of some batches of [^{32}P]DFP supplied is now in doubt (personal communication from Mr R. Monks). (*b*) Labelling of the phosphorylation site *in vitro* was substantially diminished when phenyl phenylacetate was added to the incubation medium immediately before [^{32}P]DFP, but 27 other esters and amides tested were ineffective (Johnson, 1969*b*,*c*). A number of other esters have also been shown to be ineffective (M. K. Johnson, unpublished work). (*c*) Phenylbenzyl carbamate and phenylmethanesulphonyl fluoride are progressive inhibitors of both the 'neurotoxic esterase' and the process of phosphorylation of the 'neurotoxic site' and are also close structural analogues of phenyl phenylacetate. They would be expected to react at the same site as that responsible for the hydrolysis of phenyl phenylacetate, since a common site of action is consistent with all that is known of the mode of action of progressive inhibitors of esterases (Aldridge, 1969). Moreover, it has been shown that the active-site serine residue of at least one esterase (chymotrypsin) carries the

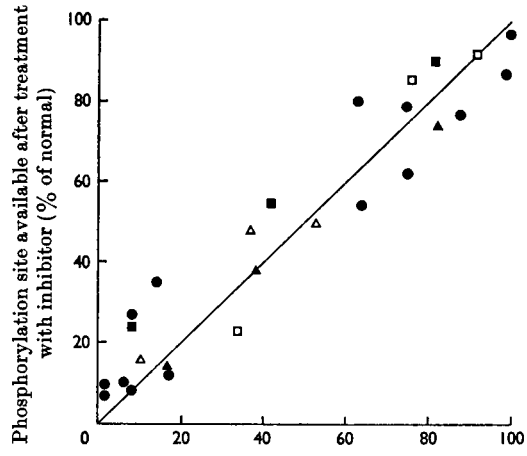

Fig. 1. Comparison of the effects of various compounds on the 'neurotoxic site' and the 'neurotoxic esterase' *in vivo* and *in vitro*. Data are taken from Tables 1 and 2 and text of this paper and from Johnson (1969*a*,*b*, Table 1). Each point represents results for a single compound for which the site and esterase assays were performed at different times with homogenates of brain from separate birds. Results from Tables 1 and 2 were calculated as percentages of the value for a normal bird assayed at the same time. Results from Johnson (1969*b*) were calculated as percentages of the mean of a number of values for normal birds that were not assayed at the same time as the dosed birds. The line is the theoretical line for complete correlation. Factors affecting the precision of the results are indicated in the Discussion, section 2(*a*). ●, Organophosphorus compounds *in vivo*; ■ and ▲, carbamates *in vivo* and *in vitro*; □ and △, sulphonyl fluorides *in vivo* and *in vitro*.

di-isopropylphosphoryl or the phenylmethanesulphonyl group after inhibition by DFP and phenylmethanesulphonyl fluoride respectively (Sanger, 1963; Gold, 1965; Cardinaud & Baker, 1970). (*d*) At 24h after a neurotoxic dose of DFP both parameters were profoundly affected and ataxia developed 8–14 days afterwards. When the DFP was preceded by a dose of phenyl benzylcarbamate both parameters were only slightly affected at 24h and no ataxia developed (see the Results section).

(3) Carbamate and sulphonyl fluoride inhibitors of the 'neurotoxic esterase' react at the same site as inhibitory organophosphorus compounds. This is shown by the following. (*a*) The evidence of 2(*c*) and 2(*d*) above. (*b*) The inhibitory carbamates and sulphonyl fluorides produce inhibition *in vivo* lasting from a few hours to several days, and this can be correlated with the duration of their capacity to prevent neurotoxic effects of DFP. Protection lasts until about 70% of the site is available to DFP (Table 4 and the Results section). This correlates with the fact that neurotoxic effects of organophosphorus compounds are not seen unless inhibition of the enzyme site shortly after the dose is at least 70% (Johnson, 1969*a*,*b*; and this paper, Table 1). (*c*) Phenylmethanesulphonyl fluoride protects against neurotoxic effects when given before DFP, but not when given after.

(4) After the enzyme site has been phosphorylated or phosphonylated *in vivo* ataxia follows. This has been established positively with 11 neurotoxic compounds and negatively with ten other compounds that are not neurotoxic although they are of related chemical structure to the neurotoxic ones and are all active esterase inhibitors *in vivo* (Johnson 1969*a*,*b*; and this paper, Table 1). Also, prior carbamoylation or sulphonation of the enzyme site *in vivo* prevents both the phosphorylative inhibition and the neurotoxic effects of a subsequent dose of several neurotoxic compounds (see the Results section and Table 4).

(5) After the enzyme site has been carbamoylated or sulphonated *in vivo* ataxia does not follow. This is difficult to establish incontrovertibly for carbamates, where repeated doses had to be given to achieve inhibition as long-lasting as that caused by a single dose of DFP, and it is probable that inhibition was not greater than 75% for any long continuous period. This objection does not apply to phenylmethanesulphonyl fluoride and probably not to *n*-butanesulphonyl fluoride. It is clear that phenylmethanesulphonyl fluoride is not even subclinically effective, for when the enzyme was only 40–50% inhibited by this compound it was not possible to produce ataxia by 'topping-up' the inhibition to near 100% with a dose of DFP, nor did extension of the period of profound inhibition (>80%) of the enzyme to more than two weeks by repeated doses of phenylmethanesulphonyl fluoride produce any neurotoxic effect.

Toxic effects that follow inhibition of an enzyme *in vivo* may be due to accumulation of an intermediate of metabolism rather than to lack of end product (e.g. convulsive effects due to accumulation of acetylcholine after poisoning by anticholinesterases). If delayed neurotoxicity caused by organophosphorus compounds were due to an accumulated intermediate, then it could be postulated that phenylmethanesulphonyl fluoride is not neurotoxic because it might inhibit formation of the toxic intermediate as well as inhibiting the esterase. However, this explanation is eliminated, since administration of phenylmethanesulphonyl fluoride shortly after DFP did not prevent neurotoxic effects.

(6) The inhibitory effects of DFP and phenylmethanesulphonyl fluoride *in vivo* are similar in brain and spinal cord.

(7) There is no essential difference between the protein bearing a phosphonyl, phosphoryl, carbamoyl or sulphonyl group at its active site, with respect to hydrolysis of phenyl phenylacetate *in vitro* or *in vivo*.

(8) There is an essential metabolic difference *in vivo* between the protein bearing a phosphonyl or phosphoryl group at its active site and the same protein bearing a carbamoyl or sulphonyl group at the same active site. There is no information on the nature of this difference that is directly associated with delayed neurotoxicity. All known neurotoxic esters are derived from phosphoric acid or phosphonic acid. It may be possible to synthesize phenyl phenylacetate esterase inhibitors derived from other acids and to explore further the relationship of neurotoxic effects to the nature of the substituent that is covalently bound to the active site.

It should be noted that, although prolonged inhibition of the esterase is not a sufficient indicator of a potentially neurotoxic ester, failure to inhibit the 'neurotoxic esterase' *in vivo* may still be taken as an indication that an organophosphorus compound is not neurotoxic at that dosage. The biochemical test has the advantage that the extent of inhibition can be determined and interpreted, whereas the clinical test is 'all-or-none'.

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