Intramolecular group transfer is a characteristic of neurotoxic esterase and is independent of the tissue source of the enzyme

A comparison of the aging behaviour of di-isopropyl phosphorofluoridate-labelled proteins in brain, spinal cord, liver, kidney and spleen from hen and in human placenta

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Neurotoxic esterase activity was measured in homogenates of human placenta and hen brain, spinal cord, liver, kidney and spleen. The activity in liver comprised less than 20% of the Paraoxon-resistant esterases, but in the other tissues neurotoxic esterase accounted for over 50%. The same tissues were labelled with [³H]di-isopropyl phosphorofluoridate, and any isopropyl group transferred on to protein during 'aging' of the labelled enzymes (alkali-volatilizable tritium) was measured. No Paraoxon-sensitive labelled sites were found to age in this way in any tissue. In brain, the Paraoxon-resistant alkali-volatilizable-tritium-labelled sites correlated with the number of neurotoxic esterase labelled sites, indicating that 'aging' and isopropyl group transfer were 100% efficient. The site receiving the transferred isopropyl group was characterized by analysing the distribution of radiolabelled proteins on gel-filtration chromatography in the presence of SDS. In particulate preparations from each tissue, the protein-bound alkali-volatilizable tritium (transferred isopropyl group) was attached to a polypeptide of M_r 178 000. This same polypeptide also bore the isopropyl-phosphoryl group of neurotoxic esterase, indicating that aging of neurotoxic esterase is an intramolecular group transfer. The apparent turnover number for the enzyme (average $1.6 \times 10^5 \text{ min}^{-1}$) was approximately the same in each hen tissue, confirming that closely similar enzymes were present in brain, spinal cord, liver and spleen. The apparent turnover for the human enzyme was 1.8-fold higher than that for the hen enzyme. The concentration of the neurotoxic esterase phosphorylated subunit in brain, spinal cord, spleen, placenta and liver was 14.6, 3.8, 7.4, 3.3 and 3.8 pmol/g of tissue. The evidence indicated that neurotoxic esterase is present in each tissue except kidney, and that isopropyl group transfer on 'aging' occurs on this enzyme only. This process is an intramolecular transfer of the group within the same polypeptide.

Delayed neuropathy is induced by some organophosphates in animals such as man and the hen. The common target for these neurotoxic organophosphates is an esterase, called neurotoxic esterase, present in nervous tissue. The primary event in the induction of the neuropathy is the covalent reaction of the organophosphate with neurotoxic esterase,

resulting in inhibition of the enzyme. The second event in the induction of the neuropathy is the aging of organophosphate-inhibited enzyme (Johnson, 1982). Aging is the irreversible cleavage of an alkyl or aryl group from the phosphorus atom on the inhibited enzyme. In the case of hen brain neurotoxic esterase inhibited by the neurotoxic organophosphate di-isopropyl phosphorofluoridate (DiPF), this liberated group becomes covalently attached to protein (Clothier & Johnson, 1979). In the work described below, this receptor protein was characterized. It was not known whether such transfer is common to other sites of DiPF-labelling. A search

Abbreviations used: DiPF, di-isopropyl phosphorofluoridate; SDS, sodium dodecyl sulphate.

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for such sites in six different tissues indicated that this transfer may be a distinctive characteristic of neurotoxic esterase.

An esterase similar to neurotoxic esterase has been detected in spleen and circulating lymphocytes (Richardson & Dudek, 1978) and human placenta (Gurba & Richardson, 1982). Inhibition of the enzyme in lymphocytes correlates with inhibition of the enzyme in brain (Dudek *et al.*, 1979). Since measurement of the degree of enzyme inhibition in lymphocytes could be of use in determining human exposure to neurotoxic compounds, it is necessary to show that the enzymes from brain and lymphocytes are identical. Were the human placenta to contain the same enzyme as nervous tissue, it would make an ideal starting material for purification of the human enzyme. In order to make tissue comparisons, the concentration, the apparent turnover number and subunit molecular weight were determined for the enzyme in spleen, liver and placenta and compared with those of the enzyme from brain and spinal cord. No significant amounts of neurotoxic esterase polypeptide were found in hen kidney. The extent of alkyl group transfer due to aging of the enzyme was determined in each tissue, and the protein receptor for the transferred alkyl group was characterized in each tissue by gel-filtration chromatography in the presence of sodium dodecyl sulphate.

Materials and methods

Animals

Hens were as described by Johnson (1969a).

Human tissue

Placentas were used fresh, from normal full-term births.

Chemicals

The sources of inhibitors and phenyl valerate were as described by Johnson (1969a, 1970, 1975). [^3H]DiPF (6.5 Ci/mmol) was from Amersham International, Amersham, Bucks., U.K. Before use it was treated to remove reactive impurities as previously described (Williams & Johnson, 1981). Soluene 350 and Instagel were from Packard, Caversham, Berks., U.K. All other chemicals were from BDH Chemicals, Poole, Dorset, U.K.

Solutions and buffers

(a) *Tris/EDTA buffer, pH 8.0.* Tris (50 mM)/EDTA (0.2 mM) was adjusted to pH 8 with HCl (12 M) at 20°C.

(b) *SDS solubilization buffer, pH 8.0.* Tris (20 mM)/SDS (5%, w/v)/glycerol (10%, v/v)/EDTA (2 mM) solution was adjusted to pH 8.0 with HCl

(12 M). To 19 vol. of this solution was added 1 vol. of 2-mercaptoethanol immediately before use.

(c) *Gel-filtration buffer, pH 8.0.* EDTA (0.1 M) was adjusted to pH 8.0 with 1 M-NaOH. Then 1 vol. of this solution was added to 99 vol. of SDS (1.01%, w/v, in deionized water).

(d) *Phenol reagent.* Phenol (50 g) was dissolved in a mixture of chloroform (50 ml) and 3-methylbutan-1-ol (1 ml).

Tissue homogenates

All tissues were homogenized in ice-cold Tris/EDTA buffer, pH 8.0, to produce a 20% (w/v) dispersion. Liver and kidney were initially homogenized in an Ultra-Turrax homogenizer (Janke und Kunkel) for 10 s. Placenta was dissected free of membranes and large blood vessels before homogenization for 60 s in the Ultra-Turrax homogenizer. Spleen and spinal cord were stripped of surface membrane before homogenization. Brain, spinal-cord, spleen, liver, kidney and placenta Ultra-Turrax homogenates were all homogenized in a glass tube with a closely fitting rotating Perspex pestle (Johnson, 1969b), with 12 strokes of the pestle.

Particulate preparation

Brain and spinal-cord homogenates (10 ml) were mixed with sucrose (10 ml; 2 M) in Tris/EDTA buffer, pH 8.0, and centrifuged at 19000 g ($r_{\text{av.}} = 7.5$ cm) for 5 min. The floating layer of lipid was removed and the centrifugation was repeated to remove the remaining lipid. The remaining homogenate was diluted with Tris/EDTA buffer, pH 8.0, to a volume of 70 ml and centrifuged in an MSE 8 $\times$ 35 ml titanium rotor for an integrated field time of 4.65×10^6 g-min ($r_{\text{av.}} = 6.5$ cm). Homogenates (10 ml) of other tissues were diluted directly with Tris/EDTA buffer, pH 8, to 70 ml and also centrifuged in an MSE 8 $\times$ 35 ml titanium rotor for an integrated field time of 4.65×10^6 g-min ($r_{\text{av.}} = 6.5$ cm). Each pellet was rehomogenized after the addition of Tris/EDTA buffer, pH 8.0 (5 ml).

Use of inhibitors and [^3H]DiPF

These were used essentially as previously described (Williams & Johnson, 1981). Homogenate or particle suspension (2 ml) was added to a solution (0.2 ml) containing Paraoxon (final concn. 160 μM) and, in some cases, Mipafox (final concn. 50 μM or 100 μM) in Tris/EDTA buffer, pH 8.0. After 20 min at 37°C, [^3H]DiPF (25 μl ; final concn. 1.646 μM) was added. Labelling was terminated after a further 40 min by denaturation of the enzymes.

Organic-solvent denaturation and washing

A 6 ml volume of ice-cold acetone/diethyl ether (2:1, v/v) was added to the labelled suspension and

the whole was mixed. The denatured protein was sedimented by centrifugation at 700 *g* ($r = 27.9$ cm) for 5 min. The pellet was washed five times by resuspension in 20 ml of diethyl ether/ethanol/water (10:7:3, by vol.).

SDS/phenol denaturation and washing

SDS (1.9 ml; 0.17 M) was added to the labelled suspension and the whole was mixed, NaCl (5.9 ml; 1.7 M) and phenol reagent (7.0 ml) were added and the whole was vortex-mixed. The mixture was centrifuged at 31 400 *g* ($r_{av.} = 8.7$ cm) for 10 min at 10°C.

The upper, aqueous, phase was carefully removed, and added to ice-cold ethanol (20 ml). The DNA precipitate was sedimented by centrifuging at 2250 *g* ($r = 22.3$ cm) for 5 min at 4°C. The pellet was washed five times by resuspension in 20 ml of ice-cold diethyl ether/ethanol/water (10:7:3, by vol.) and finally dissolved in Soluene 350 (1 ml), Instagel (10 ml) and acetic acid (0.1 ml) for scintillation counting.

Acetone (14.2 ml) was added to the lower, phenol, phase, which also contained the interface, and the mixture was vortex-mixed. The protein was sedimented by centrifugation at 9700 *g* ($r_{av.} = 8.7$ cm) for 5 min at 4°C. The pellet was washed five times by resuspension in 20 ml of ice-cold diethyl ether/ethanol/water (10:7:3, by vol.) followed by centrifugation at 2250 *g* ($r = 22.3$ cm) for 5 min at 4°C.

Buffer dilution and washing

In one experiment (subsection 3 of the Results section) labelling was terminated by dilution with ice-cold Tris/EDTA buffer, pH 8.0 (33 ml). The particles were sedimented by centrifugation for an integrated field time of 4.65×10^6 *g*-min ($r_{av.} = 6.5$ cm) in an MSE 8 × 35 titanium rotor. They were washed twice more by resuspension in Tris/EDTA buffer, pH 8.0 (35 ml), and centrifugation.

Solubilization of denatured protein in SDS

Organic solvents were removed from the final washed pellet by careful aspiration of the supernatant, and evaporation of the excess in a desiccator evacuated on a Venturi pump, for 2 min at room temperature, SDS solubilization buffer was added and the mixture was vortex-mixed. The suspension was incubated at 60°C for 30 min, and aggregated material was dissociated by gentle sonication in an ultrasonication water bath for 1 min. The undissolved residue was sedimented by centrifugation at 38 600 *g* ($r = 10.7$ cm) for 30 min at 30°C. The soluble extract was decanted, and the solid residue dissolved in Soluene 350 (1 ml), Instagel (10 ml) and acetic acid (0.1 ml) for scintillation counting.

Sephacryl-gel-filtration chromatography

The columns used were Sephacryl S300 (Pharmacia, Hounslow, Middx., U.K.) (1 cm² × 160 cm) and Sephacryl S400 (2 cm² × 83 cm). In both cases the flow rate was 4 ml/h, and 2 ml or 2.2 ml fractions were collected respectively. The applied sample consisted of 3 ml of protein solution plus 0.05 ml of Blue Dextran (Pharmacia) in SDS solubilization buffer. The sample was layered on the bed surface, and eluting buffer was overlaid on the sample as flow proceeded. Fractions were collected in polystyrene tubes (Sarstedt, Leicester, U.K.). The recovery of radioactivity in the column eluate compared with the original sample before chromatography was $92.7 \pm 3.2\%$ (mean $\pm$ S.D.; $n = 4$).

The column was standardized with Blue Dextran, ³H₂O and various proteins, applied in a volume of 3 ml. The standard proteins were thyroglobulin (M_r 330 000), myosin (M_r 212 000), β -galactosidase (M_r 116 000), phosphorylase *b* (M_r 94 000), bovine serum albumin (M_r 67 000), chicken ovalbumin (M_r 43 000), soya-bean trypsin inhibitor (M_r 20 100) and bovine insulin A-chain (M_r 2565).

Alkaline hydrolysis and radioactivity measurement

All radioactive ligands can be removed from [³H]DiPF-labelled brain proteins by alkaline hydrolysis (Clothier & Johnson, 1979). In addition, the isopropyl group, which was attached to protein during the aging of neurotoxic esterase, becomes volatile (Clothier & Johnson, 1979). The amount of this isopropyl group was measured in the present work as the loss of radioactivity from an alkaline hydrolysate of [³H]DiPF-labelled proteins when the hydrolysate was freeze-dried.

(a) Soluble extract in SDS solubilization buffer.

A 1 ml portion of the extract was mixed with NaOH (4.0 ml; 0.5 M) and incubated at 60°C for 2 h in stoppered tubes. After cooling, two samples (1 ml) were freeze-dried and each was redissolved in water (1 ml). These samples, plus a further two samples, were dissolved in Instagel (10 ml) and acetic acid (0.1 ml). To another sample (1 ml) was added aq. NH₃ (0.2 ml; 3.4 M), formic acid (0.1 ml; 6.7 M) and water (4 ml) followed by 10.5 ml of acetone/diethyl ether (2:1, v/v) and 0.1 ml of bovine γ -globulins (10 mg/ml) to precipitate the protein. After 18 h at 4°C, the protein was centrifuged at 300 *g*_{av.} for 5 min at 4°C. The pellet was resuspended in 20 ml of diethyl ether/ethanol/water (10:7:3, by vol.) and centrifuged at 1200 *g*_{av.} for 5 min at 4°C. The pellet was dissolved in Soluene 350 (1 ml), Instagel (10 ml) and acetic acid (0.1 ml).

For all tissues labelled after any of the pre-incubation conditions used, at least 91.8% of the protein-bound tritium was liberated by alkaline hydrolysis [average liberation of tritium: $96 \pm 1.9\%$ (mean $\pm$ S.D.; $n = 18$)].

(b) *Fractions from gel-filtration columns.* The fractions were made 0.18 M in NaOH by the addition of 2.2 M-NaOH (0.2 ml) to a 2.2 ml fraction. They were then capped tightly with a polythene stopper (Sarstedt) and incubated at 60°C for 2 h. From each fraction was taken two samples (1 ml). One sample was freeze-dried and redissolved in water (1 ml). Both samples were dissolved in Instagel (10 ml) and acetic acid (0.1 ml). Radioactivity was measured as previously described (Williams & Johnson, 1981). The yield of volatile radioactivity in the column eluate compared with that in the original sample before chromatography was $90.8 \pm 8.4\%$ (mean $\pm$ S.D.; $n = 4$).

Assay of neurotoxic esterase activity

Neurotoxic esterase activity was assayed by measuring the hydrolysis of phenyl valerate resistant to Paraoxon but sensitive to Mipafox. Because of the low activity of some of the homogenates, the method described by Johnson (1975) was used in which protein is precipitated before liberated phenol is measured.

Results

1. Neurotoxic esterase in six whole-tissue homogenates

Measurement and Mipafox-sensitivity. Neurotoxic esterase was measured in two nervous tissues, namely brain and spinal cord, two non-nervous tissues in which the enzyme has been demonstrated, namely spleen and placenta, and two tissues reported to be deficient in neurotoxic esterase, namely liver and kidney (Richardson & Dudek, 1978). The enzyme activity was measured as a differential inhibition of phenyl valerate hydrolysis (see the Materials and methods section). Table 1 shows the Mipafox-sensitive Paraoxon-resistant esterase (neurotoxic esterase-type) activities in homogenates of the six tissues. All tissues contained significant activity. This activity was a minor

component (less than 20%) of the Paraoxon-resistant esterases in liver and kidney (Fig. 1), but was the major component (more than 50%) in the other tissues. Titration of this Mipafox-sensitive component with Mipafox (Fig. 2) shows that this component had an I_{50} of approx. $10 \mu\text{M}$ in each tissue. This suggests that all six tissues contained neurotoxic esterase. Table 1 also shows that in all tissues except liver most of the neurotoxic esterase-type activity segregated in the particulate fractions of each homogenate, prepared as described in the Materials and methods section.

Fig. 1. Inhibition of Paraoxon-resistant esterases by Mipafox

Brain (●), spinal-cord (○), spleen (■), placenta (□), liver (▲) and kidney (△) homogenates were incubated with Paraoxon ($40 \mu\text{M}$) and Mipafox, at the concentrations shown on the graph, for 20 min at 37°C. Phenyl valerate was added and incubated for a further 15 min. Reaction was stopped and liberated phenol was measured as described by Johnson (1975). Esterase activity was calculated as a percentage of the activity in samples treated with only Paraoxon.

Table 1. Rate of Paraoxon-resistant Mipafox-sensitive hydrolysis of phenyl valerate in tissue homogenates and particles derived from them

The method used was described by Johnson (1975). Homogenates or particles prepared as described in the Materials and methods section were incubated with Paraoxon ($40 \mu\text{M}$) with or without Mipafox ($50 \mu\text{M}$) for 20 min at 37°C. Phenyl valerate suspension was added and the mixture was incubated a further 15 min. The reaction was terminated, the tissue was precipitated and removed, and the liberated phenol was then measured colorimetrically. Results are given as means $\pm$ S.D. for the numbers of determinations indicated in parentheses.

	Hen brain	Hen spinal cord	Hen liver	Hen kidney	Hen spleen	Human placenta
Rate of Paraoxon-resistant Mipafox-sensitive hydrolysis of phenyl valerate in whole homogenate (nmol/min per g of tissue)	2704 $\pm$ 270 (7)	536 $\pm$ 92 (6)	565.7 $\pm$ 230.0 (3)	358.5 $\pm$ 68.8 (3)	1078 $\pm$ 132 (7)	1130 $\pm$ 277 (6)
Rate of hydrolysis in particles (% of the rate in the original tissue homogenate)	68.0	62.5	44.7	113.9	73.9	66.5

Fig. 2. Titration of Paraoxon-resistant Mipafox-sensitive esterases with Mipafox
For key to symbols see Fig. 1. Mipafox-sensitive esterase activity was calculated as a percentage of the decrease in esterase activity caused by 50 μM-Mipafox in a Paraoxon-treated (40 μM) homogenate. The straight line indicates an I_{50} of 10 μM.

2. [³H]DiPF-labelling in six whole-tissue homogenates

Total and alkali-volatilizable radioactivity. Until now, only inhibited neurotoxic esterase has been shown to age in such a way that the group cleaved from the phosphorus atom becomes attached to protein. In order to determine whether this is a characteristic property of neurotoxic esterase, the protein-bound isopropyl group was measured in homogenates of the six tissues (described above) after reaction with [³H]DiPF.

Whole-tissue homogenates were treated with or without Paraoxon (160 μM) before incubation with [³H]DiPF at pH8.0, then precipitated, washed extensively with organic solvents and dissolved in SDS solubilization buffer (see the Materials and methods section). Table 2 shows that most of the bound radioactivity from each tissue was solubilized by the detergent. No more than 3.6% of the radioactivity in [³H]DiPF-labelled homogenates was rendered volatile by alkaline hydrolysis (Table 2). No volatilizable radioactivity could be detected in [³H]DiPF-labelled homogenates of liver, kidney and placenta that had not been pre-inhibited with Paraoxon. This suggests that only a very minor fraction of the sites reacting with DiPF can age in such a way as to produce volatile radioactivity on hydrolysis.

Paraoxon treatment of the homogenates before incubation with [³H]DiPF caused a large decrease (82–99%) in ³H incorporation into all tissues (Table 2). However, this Paraoxon pretreatment decreased

Table 2. [³H]DiPF bound to tissue homogenates with or without pretreatment by Paraoxon (160 μM): proportion of alkali-volatilizable radioactivity

Tissue	Fraction of ³ H solubilized in SDS (% of total ³ H bound)		³ H bound to solubilized proteins (pmol/g of tissue)		³ H lost on freeze-drying alkaline hydrolysate of solubilized proteins (% of total ³ H in hydrolysate)		(pmol of isopropyl group/g of tissue)	
	Untreated homogenate	Paraoxon-pretreated homogenate	Untreated homogenate	Paraoxon-pretreated homogenate	Untreated homogenate	Paraoxon-pretreated homogenate	Untreated homogenate	Paraoxon-pretreated homogenate
Hen brain	96.2 ± 1.3 (3)	97.0 ± 1.8 (6)	254.6 ± 39.7 (3)	37.3 ± 2.3 (4)	3.6 ± 0.2 (3)	23.0 ± 0.9 (4)	18.2 ± 3.7 (3)	17.1 ± 0.9 (4)
Hen spinal cord	96.8 ± 0.7 (3)	96.2 ± 1.9 (6)	156.3 ± 12.9 (3)	19.0 ± 0.9 (4)	2.8 ± 0.6 (3)	17.0 ± 0.6 (4)	8.5 ± 1.2 (3)	6.5 ± 0.1 (4)
Hen liver	98.2 ± 0.6 (3)	93.5 ± 4.7 (8)	386.4 ± 307 (3)	58.3 ± 6.9 (5)	0	7.2 ± 1.4 (4)	0	8.7 ± 2.8 (4)
Hen kidney	93.8 ± 3.0 (3)	96.6 ± 1.7 (8)	323.1 ± 809 (3)	45.3 ± 5.3 (5)	0.2 ± 0.3 (3)	3.6 ± 0.4 (4)	12.9 ± 10.9 (3)	3.2 ± 0.3 (4)
Hen spleen	95.2 ± 2.5 (3)	96.5 ± 2.1 (8)	249.1 ± 43.5 (3)	44.2 ± 4.8 (5)	2.0 ± 0.9 (3)	10.6 ± 1.2 (4)	9.2 ± 2.4 (3)	9.1 ± 0.5 (4)
Human placenta	92.7 ± 1.0 (3)	87.0 ± 1.8 (6)	364.4 ± 62.5 (3)	45.6 ± 6.0 (6)	0.7 ± 0.5 (3)	4.4 ± 0.8 (5)	5.0 ± 3.7 (3)	3.9 ± 0.4 (5)

the labelling of the sites giving rise to volatilizable tritium from 18.2 to 17.1 (6%), from 8.5 to 6.5 (24%) and from 9.2 to 9.1 (1%) in brain, spinal-cord and spleen homogenates respectively (Table 2). Table 2 indicates that less than 1% of the Para-oxon-sensitive sites of DiPF-labelling in brain, spinal cord and spleen give rise to volatile radioactivity on alkaline hydrolysis.

3. Radiochemical measurement of neurotoxic esterase in homogenates and particles derived from brain

Comparison with volatilizable radioactivity. In hen brain, it is established that most of the DiPF sites that are resistant to Paraoxon but sensitive to Mipafox are neurotoxic esterase (Johnson, 1969*a,b*, 1970; Williams & Johnson, 1981). A comparison was made between the quantity of this enzyme and the quantity of sites of volatilizable-tritium-labelling to ascertain whether these sites are limited only to those labelled during the aging of neurotoxic esterase.

In separate experiments, brain homogenate or particles, derived from homogenate as described in the Materials and methods section, were treated with Paraoxon with or without Mipafox and subsequently labelled with [³H]DiPF. Table 3 shows that virtually all of the labelled proteins were solubilized by SDS. It was found that 87% and 85% of the volatilizable radioactivity in the Paraoxon-treated homogenate or particles respectively was eliminated by pre-treatment with Mipafox. The ratio of the neurotoxic esterase labelled sites ('A' in Table 3) to the Paraoxon-resistant Mipafox-sensitive volatilizable-

tritium-labelled sites ('B' in Table 3) was 1.03 for both homogenate and particle preparations. This result indicates that at least 87% of the volatile radioactivity in hydrolysates of [³H]DiPF-labelled brain is derived from the protein site (Z) that accepts the isopropyl (iPr) group transferred during the aging of neurotoxic esterase (X):

This result also indicates that the aging and transfer reaction has gone to completion under the conditions used.

The work described below was done to identify the protein bearing site Z in brain, and to compare the Paraoxon-resistant Mipafox-sensitive DiPF-labelled protein in the other tissues with those in brain, to discover whether they correlate with the measured esterase activity, whether they resemble brain neurotoxic esterase in subunit molecular weight, whether they undergo group transfer during aging, and, if so, whether site Z is on the same protein in each tissue.

The DiPF-labelled subunit of neurotoxic esterase has been previously identified as a 155 000-*M_r* polypeptide by SDS/polyacrylamide-gel electrophoresis (Williams & Johnson, 1981). Gel-filtration chromatography was chosen in the present case to separate and characterize the tritiated proteins because, although it has a poorer resolving power than electrophoresis, there is a complete recovery of labelled proteins (elution from SDS/polyacrylamide gel gave only 60% recovery of labelled proteins), a larger volume of tissue extract can be analysed, and

Table 3. [³H]DiPF bound to brain homogenates and particles: correlation with volatilizable radioactivity

Brain homogenate and particle preparations were incubated with Paraoxon (160 μM) with or without Mipafox (50 μM) before being labelled with [³H]DiPF. The labelled tissue was precipitated and washed with organic solvents before solubilization in SDS and centrifugation. A portion of the supernatant was hydrolysed with NaOH (0.4 M), and the volatile tritium in the hydrolysate was measured by counting of radioactivity before and after freeze-drying (see the Materials and methods section). Results are given as means ± s.d. for the numbers of determinations indicated in parentheses.

	Brain homogenate	Particulate fraction from brain homogenate
Fraction of ³ H in [³ H]DiPF-labelled Paraoxon-treated preparation that was solubilized by SDS (%)	97.0 ± 1.8 (6)	99.0 ± 0.4 (3)
[³ H]DiPF bound to solubilized Paraoxon-treated preparation (pmol of DiPF/g of brain)	37.3 ± 2.3 (4)	22.0 ± 2.0 (3)
Decrease in [³ H]DiPF bound to solubilized Paraoxon-treated preparation due to Mipafox (50 μM) pretreatment (pmol of DiPF/g of brain) (A)	15.3 ± 1.2 (4)	11.5 ± 0.6 (3)
Volatile radioactivity in hydrolysates of solubilized labelled Paraoxon-treated homogenate (pmol of isopropyl group/g of brain)	17.1 ± 0.9 (4)	13.1 ± 1.0 (3)
Decrease in volatile radioactivity in hydrolysates of solubilized labelled Paraoxon-treated homogenate due to Mipafox (50 μM) pretreatment (pmol of isopropyl group/g of brain) (B)	14.8 ± 1.3 (4)	11.1 ± 0.6 (3)
Ratio A/B...	1.03	1.03

low-molecular-weight materials can be measured. Preliminary gel-filtration experiments on Sephacryl S300 showed that the concentration of Mipafox (50 μM) used was insufficient to completely abolish the ^3H -labelling of the neurotoxic esterase polypeptide. By doubling the Mipafox concentration to 100 μM , labelling of this component was eliminated. Also, the high viscosity of spleen particles solubilized in SDS caused a broadening of the void-volume peak on gel filtration of spleen particle extracts. This broadening was eliminated if DNA was removed from the particles by phenol extraction (see the Materials and methods section) before solubilization in SDS. Thus Blue Dextran applied in a 3 ml sample of solubilized spleen particles was eluted from a 160 ml Sephacryl S300 column in a peak occupying 22 ml. After extraction of the DNA, the Blue Dextran was eluted from the same column in a peak occupying only 8 ml.

The effects of this modification in the method of sample preparation on the measured tritium was investigated in a preliminary experiment. Brain particles were treated with Paraoxon with or without Mipafox and subsequently labelled with [^3H]DiPF. Duplicate samples of labelled particles were processed in three different ways: (A) dilution and washing with buffer (used by Williams & Johnson, 1981); (B) denaturation and washing with organic solvent; (C) denaturation with SDS/phenol and washing with organic solvent (see the Materials and methods section). Table 4 shows that methods (B) and (C) give essentially identical results. A substantial amount of labelling, resistant to both inhibitors, which is removed by methods (B) and (C) was not removed by method (A). The Paraoxon-resistant Mipafox-sensitive labelling determined by

method (A) was 24% greater than that measured by method (B) or method (C). This difference may be attributable to losses of bound tritium during the extensive organic-solvent-washing steps of methods (B) and (C).

4. Comparison of neurotoxic esterase with labelled proteins from [^3H]DiPF-labelled particles in six tissues

Neurotoxic esterase activity and total volatilizable radioactivity. Particulate fractions produced as described in the Materials and methods section were treated with Paraoxon, with or without Mipafox (100 μM), then labelled with [^3H]DiPF. The labelling was stopped by adding SDS, and the DNA was extracted by selective precipitation of the protein with phenol (see the Materials and methods section). The denatured labelled protein was then washed extensively with organic solvents and dissolved in SDS as described in subsection 3 above. Table 5 shows that most of the radiolabelled proteins were solubilized in SDS. The Paraoxon-resistant Mipafox-sensitive volatilizable-tritium-labelled sites (B) and DiPF-labelled sites (A) were present in equal amounts in both brain and spinal cord (Table 5). This indicates that aging of DiPF-labelled neurotoxic esterase and transfer of the isopropyl group had proceeded to completion in these tissues. No significant amounts of Paraoxon-resistant Mipafox-sensitive volatilizable-tritium-labelled sites were detected in kidney particles. The A/B ratio was greater than unity in the other tissues.

Since the E/B ratio (see Table 5) was very similar for brain, spinal cord, liver and spleen, it is likely that B represents the sites receiving the isopropyl group from neurotoxic esterase. The A/B ratio of greater

Table 4. Comparison of three different methods of preparation of [^3H]DiPF-labelled proteins from brain particles

A particulate preparation of brain was treated with Paraoxon (160 μM) with or without Mipafox (100 μM) before being labelled with [^3H]DiPF. The labelled particles were (A) washed with Tris/EDTA buffer, pH 8, or (B) precipitated and washed with organic solvents or (C) precipitated with SDS/phenol and washed with organic solvents. The washed particles were solubilized in SDS, and the soluble extract was hydrolysed with NaOH (0.4 M). Volatile tritium was measured by counting of radioactivity before and after freeze-drying (see the Materials and methods section). Results are given as means $\pm$ S.D. for the numbers of determinations indicated in parentheses.

	Method A (Tris buffer washing)	Method B (organic-solvent precipitation and washing)	Method C (SDS/phenol pre- cipitation and organic- solvent washing)
Paraoxon-resistant labelling by [^3H]DiPF (pmol of DiPF/g of brain)	43.0	20.7	19.7
Decrease in Paraoxon-resistant labelling due to Mipafox (100 μM) pretreatment (pmol of DiPF/g of brain)	17.6	13.5	13.2
Volatile radioactivity in alkaline hydrolysates of Paraoxon- treated labelled particles (pmol of isopropyl group/g of brain)	18.2	14.5	13.9
Decrease in volatile radioactivity in alkaline hydrolysates of Paraoxon-treated labelled particles due to Mipafox (100 μM) pretreatment (pmol of isopropyl group/g of brain)	16.0	13.0	12.8

Table 5. [^3H]DiPF bound to phenol-extracted particles derived from six tissues: correlation with volatilizable radioactivity and esterase activity

Particulate preparations derived from tissue homogenates were incubated with Paraoxon (160 μM) with or without Mipafox (100 μM) before being labelled with [^3H]DiPF. The labelled particles were precipitated with SDS/phenol and washed with organic solvents. The washed particles were solubilized in SDS, and the soluble extract was hydrolysed with NaOH (0.4 M). Volatile tritium was measured by counting the radioactivity of the hydrolysate before and after freeze-drying (see the Materials and methods section). The Paraoxon-resistant Mipafox-sensitive esterase activity of a portion of the untreated particulate preparations was measured as described in the Materials and methods section. The concentration of 178000- M_r polypeptide was calculated from B (column 6) by using the measured distribution of volatilizable tritium radioactivity of Paraoxon-resistant Mipafox-sensitive sites on chromatograms shown in Fig. 3(b). Results are given as means $\pm$ s.d. for the numbers of determinations indicated in parentheses.

	Fraction of ^3H in Paraoxon-treated particles solubilized by SDS (%)	[^3H]DiPF bound to solubilized proteins from Paraoxon-treated particles (pmol of DiPF/g of tissue)		^3H lost on freeze-drying alkaline hydrolysate of solubilized proteins from Paraoxon-treated particles (pmol of isopropyl group/g of tissue)		Ratio A/B	Paraoxon-resistant Mipafox-sensitive esterase activity (nmol/min per g of tissue) (E)	$10^5 \times$ Ratio E/B (min^{-1})	Concn. of 178000- M_r polypeptide (pmol/g of tissue) (C)	$10^5 \times$ Ratio E/C (min^{-1})
		Total	Decrease caused by Mipafox (100 μM) pretreatment (A)	Total	Decrease caused by Mipafox (100 μM) pretreatment (B)					
Brain	91.4 $\pm$ 2.8 (3)	20.8 $\pm$ 4.3 (3)	12.5 $\pm$ 2.3 (3)	13.2 $\pm$ 2.7 (3)	11.8 $\pm$ 2.7 (3)	1.06	1832 $\pm$ 232 (3)	1.55	9.9	1.85
Spinal cord	87.9 $\pm$ 3.8 (3)	7.3 $\pm$ 2.4 (3)	2.8 $\pm$ 0.3 (3)	3.6 $\pm$ 1.0 (3)	2.9 $\pm$ 0.5 (3)	0.97	341 $\pm$ 58 (3)	1.18	2.4	1.42
Liver	83.5 $\pm$ 8.7 (3)	29.3 $\pm$ 3.4 (3)	9.3 $\pm$ 3.4 (3)	3.4 $\pm$ 0.5 (3)	1.9 $\pm$ 0.5 (3)	4.89	253 $\pm$ 83 (3)	1.33	1.7	1.49
Kidney	89.1 $\pm$ 3.3 (3)	36.0 $\pm$ 4.9 (3)	7.7 $\pm$ 5.3 (3)	2.5 $\pm$ 0.6 (3)	1.0 $\pm$ 0.8 (3)	—	408 $\pm$ 128 (3)	—	—	—
Spleen	99.1 $\pm$ 0.1 (3)	34.4 $\pm$ 4.4 (3)	9.6 $\pm$ 0.8 (3)	8.2 $\pm$ 0.4 (3)	6.4 $\pm$ 0.4 (3)	1.50	834 $\pm$ 51 (3)	1.30	5.5	1.51
Placenta	93.5 $\pm$ 2.2 (4)	24.3 $\pm$ 4.0 (4)	8.7 $\pm$ 1.6 (4)	3.4 $\pm$ 0.5 (4)	2.5 $\pm$ 0.5 (4)	3.48	649 $\pm$ 110 (4)	2.60	2.2	2.95

than unity then suggests that liver, spleen and placenta contain additional Paraoxon-resistant Mipafox-sensitive sites of DiPF-labelling that do not undergo aging and group transfer. The *E/B* ratio for human placenta was different from that of the hen tissues. This may be due to a difference in the turnover number of neurotoxic esterase between the two species. The data in Table 5 indicate that neurotoxic esterase in brain, spinal cord, liver, spleen and placenta has aged and undergone isopropyl group transfer on to protein (site Z). To support this interpretation and to identify the polypeptide bearing site Z, the tritiated proteins in the same preparations of labelled particles were analysed by gel-filtration chromatography.

Characterization of [^3H]DiPF-labelled sites and sites of volatilizable-tritium-labelling. A sample of the SDS extract was separated by chromatography on Sephacryl S400 in the presence of SDS (see the Materials and methods section). The fractions from the Sephacryl column were hydrolysed with alkali. Two samples of each hydrolysate were taken for scintillation counting, one being freeze-dried beforehand in order to determine the amount of volatile radioactivity (see the Materials and methods section). Fig. 3(a) shows the radioactivity chromatograms of the SDS extracts from the five tissues treated with Paraoxon with or without Mipafox. Little radioactivity is present in the excluded volume (fractions 29–32), most of it being present in the partially included region of the column. In brain, spinal cord, spleen, liver and placenta, the volatile radioactivity (Fig. 3b) is distributed between two main peaks, which appear at the same position on each chromatogram. The larger peak (fractions 37–40) corresponds to a polypeptide of M_r 178 000 (Fig. 4), and the smaller peak (fractions 68–72) is very close to the totally included volume, corresponding to M_r <1000 (extrapolated value). Both peaks are eliminated by Mipafox (100 μM) treatment before labelling with [^3H]DiPF. The Mipafox-sensitivity of these peaks suggests that they may be derived from neurotoxic esterase.

The peak of DiPF-labelling (fractions 37–40; Fig. 3a) that is eliminated in Mipafox-pretreated particles derived from brain or spinal cord appears at the same position on the chromatogram as the main peak of volatile radioactivity (Fig. 3b). In the chromatogram of spleen particles (Fig. 3a), two peaks at fractions 39 and 44 are diminished by Mipafox pretreatment, but the main peak of volatile radioactivity, which is abolished by Mipafox pretreatment, co-migrates with the peak at fraction 39 (Fig. 3b). The chromatogram of placenta particles (Fig. 3a) also contains a peak of tritium-labelling at fraction 39, which is diminished in the Mipafox-treated particles. This peak coincides with the major peak of volatile radioactivity (Fig. 3b), which

is abolished in the Mipafox-treated particles. Unlike the brain, spinal cord and spleen, there is a substantial decrease in radioactivity in two other major peaks of labelling (fractions 43 and 51) in the Mipafox-pretreated particles. Neither of these peaks is co-eluted with volatile radioactivity. The chromatogram of the liver preparation shows a shoulder of Mipafox-sensitive DiPF-labelling at fractions 38–39 (Fig. 3a) co-migrating with a peak of Mipafox-sensitive volatilizable-tritium-labelling (Fig. 3b). The major part of the Mipafox-sensitive DiPF-labelling, however, is not associated with fractions 38–39.

To summarize, each tissue contains a polypeptide of M_r 178 000 whose labelling by DiPF is resistant to Paraoxon and sensitive to Mipafox. In addition, each tissue contains a polypeptide of the same molecular weight that has the same inhibition characteristics and that carries volatilizable tritium radioactivity. This evidence confirms previous work indicating that the 178 000- M_r polypeptide is part of neurotoxic esterase (Williams & Johnson, 1981), and indicates that aging results in an intramolecular transfer of the isopropyl group from the phosphorus atom in the active site on to an amino acid residue of the same polypeptide chain.

Degree of aging involving isopropyl group transfer in neurotoxic esterase from each tissue. If DiPF-inhibited neurotoxic esterase completely aged and transferred one of the two isopropyl groups on to itself, the number of Mipafox-sensitive sites of DiPF-labelling in the column fractions containing the 178 000- M_r polypeptide should equal the number of Mipafox-sensitive sites bearing a transferred isopropyl group, i.e. the Mipafox-sensitive volatile tritium radioactivity should be half the Mipafox-sensitive total radioactivity. In the examples shown in Fig. 3, the average ratio of Mipafox-sensitive volatile tritium radioactivity to Mipafox-sensitive labelling in fractions 37–40 inclusive was 0.505, 0.524, 0.424, 0.347 and 0.157 for brain, spinal cord, spleen, placenta and liver respectively, corresponding to a degree of aging involving isopropyl group transfer of $100.9 \pm 2.8\%$, $104.8 \pm 8.6\%$, $84.8 \pm 3.2\%$, $69.3 \pm 8.9\%$ and $31.3 \pm 3.0\%$ respectively (means $\pm$ S.D.; $n = 4$). This confirms the previous deduction from Table 5 that isopropyl group transfer is completely efficient in the brain and spinal-cord enzymes. The apparently incomplete aging and transfer in the other three tissues is probably due to the presence of other Mipafox-sensitive DiPF-labelled polypeptides contaminating the neurotoxic esterase peak. Fig. 3(a) shows that these three tissues do contain such Mipafox-sensitive DiPF-labelled polypeptides, which migrate close to the neurotoxic esterase peak and which do not contain volatilizable tritium. The results for brain and spinal cord indicate that the 178 000- M_r subunit

of neurotoxic esterase had completely aged and that all the aged group was transferred on to the same 178 000- M_r subunit.

The volatile Paraoxon-resistant Mipafox-sensitive label in the low-molecular-weight peak in the chromatograms (Fig. 3b) accounts for approx. 20% of the total Paraoxon-resistant Mipafox-sensitive label in the original soluble SDS extracts of the labelled particles. This component of labelling cannot be derived from the aging of DiPF-inhibited neurotoxic esterase, since all of the aged group from neurotoxic esterase has been found on the 178 000- M_r polypeptide. The distribution of volatile radioactivity on the chromatograms of brain particles is the same whether or not the labelled proteins were reduced with 2-mercaptoethanol before chromatography. In addition, 20% of the Paraoxon-resistant Mipafox-sensitive volatilizable-tritium-labelling was found to be lost on freeze-drying the initial SDS extracts of labelled brain particles, in the absence of both 2-mercaptoethanol and alkali. Thus it is probable that the low-molecular-weight peak of volatile-tritium-labelling resistant to Paraoxon but sensitive to Mipafox was not covalently bound to protein.

Radiochemical purity of chromatographically separated neurotoxic esterase. The radiochemical purity of the neurotoxic esterase peak (fractions 37–40) in the chromatograms of Paraoxon-treated particles shown in Fig. 3(a) can be assessed from the ratio of the number of moles of volatile isopropyl group (Fig. 3b) to the number of moles of DiPF-

Fig. 4. Calibration of Sephacryl S-400 chromatograms

A 2–10 mg portion of each standard protein was dissolved in SDS solubilization buffer (3 ml) (see the Materials and methods section) and applied to the Sephacryl S400 column described in Fig. 3. Blue Dextran and $^3\text{H}_2\text{O}$ were applied to the same column, also in a volume of 3 ml. Proteins and Blue Dextran were monitored at 280 ml. The positions of the peak maxima on the chromatograms are plotted against log (subunit M_r). The proteins are: 1, thyroglobulin; 2, myosin; 3, β -galactosidase; 4, phosphorylase b; 5, bovine serum albumin; 6, ovalbumin; 7, soya-bean trypsin inhibitor; 8, insulin A-chain. The excluded volume is at fraction 31, and the totally included volume is at fraction 71.

Fig. 3. Sephacryl S400 separation of [^3H]DiPF-labelled particles treated with Paraoxon with or without Mipafox: (a) total radioactivity, and (b) volatilizable radioactivity

Particulate preparations derived from tissue homogenates were treated with Paraoxon (160 μM) with or without Mipafox (100 μM) before [^3H]DiPF labelling. The labelled particles were precipitated with SDS/phenol and washed with organic solvents. The washed particles were solubilized in SDS and centrifuged. The supernatant (3 ml) was applied to a column of Sephacryl S400 equilibrated and eluted in 1% SDS/1 mM-EDTA, pH 8. Each column fraction was hydrolysed in NaOH (0.18 M) at 60°C for 2 h. The radioactivity of one sample of hydrolysate was counted directly, and a second sample was freeze-dried before counting of radioactivity. (a) shows the total radioactivity in each column fraction of particles treated with Paraoxon (●) or Paraoxon plus Mipafox (■). (b) shows the volatile tritium radioactivity in the same column fractions shown in (a). In brain, spinal cord, spleen, liver and placenta, 84%, 84%, 86%, 87% and 86% respectively of the Paraoxon-resistant Mipafox-sensitive volatilizable tritium radioactivity is in the peak centred at fraction 38.

labelled sites (Fig. 3a). If it is assumed that the number of moles of volatile isopropyl group in fractions 37–40 represents the number of moles of neurotoxic esterase in the peak comprising fractions 37–40 is 95.5%, 88.6%, 74.4%, 47.5% and 44.5% for brain, spinal cord, spleen, liver and placenta respectively. Thus this method is a useful technique for preparing radiochemically pure brain [^3H]DiPF-labelled neurotoxic esterase. Such a preparation would be of use in further chemical analysis of the sites modified by DiPF-phosphorylation and aging.

Number of moles of neurotoxic esterase in each tissue. The concentrations of the 178 000- M_r neurotoxic esterase polypeptide in the particulate fractions from brain, spinal cord, spleen, placenta and liver are 9.9, 2.4, 5.5, 2.2 and 1.7 pmol/g of tissue respectively (C in Table 5). Taking the recoveries of neurotoxic esterase activity in the particle preparations (Table 1) into account, the concentrations of the 178 000- M_r neurotoxic esterase polypeptide in the initial tissue homogenates would be 14.6, 3.8, 7.4, 3.3 and 3.8 pmol/g of tissue for brain, spinal cord, spleen, placenta and liver respectively.

Apparent turnover number for neurotoxic esterase in each tissue. Table 5 summarizes the Paraoxon-resistant Mipafox-sensitive esterase activity

(*E*) and concentration of the 178 000- M_r subunit of neurotoxic esterase (*C*) in each tissue. The ratio between these two quantities (*E/C*), i.e. the apparent turnover number, does not differ significantly among brain, spinal cord, liver and spleen, indicating that the same enzyme is present in all four tissues. The higher values for the apparent turnover number in placental neurotoxic esterase may be due to a difference in the catalytic rates of the hen and the human enzymes.

Conclusions

1. Tissue distribution of neurotoxic esterase

The results above confirm the presence of neurotoxic esterase in hen spinal cord, hen spleen and human placenta, and indicate that it is also present in hen liver but not kidney. The amino acid residue of hen brain neurotoxic esterase that can be phosphorylated by [^3H]DiPF is part of a polypeptide of M_r 178 000. The same polypeptide is also a part of the enzyme from spinal cord, spleen, liver and placenta. There is only a 4.4-fold range in concentration of the enzyme over the five tissues examined. Most of the enzyme segregates in the particulate fraction in homogenates of brain, spinal cord, spleen and placenta, and, apart from a small difference in the turnover rate between hen brain and human placenta, which may be due to species differences in the enzyme, there is nothing to distinguish the nervous-tissue enzyme from the non-nervous-tissue enzyme. The lack of tissue specificity may indicate a role for neurotoxic esterase in processes common to many types of cells. However, the fact that prolonged and substantial inhibition of the enzyme by compounds that do not age has no deleterious effect on the whole animal (Johnson, 1970, 1974) suggests that neurotoxic esterase may be only one of several enzymes that can perform this function.

2. Intramolecular group transfer of inhibited neurotoxic esterase

Serine esterases all react with DiPF, but they differ in their ability to age after the inhibition by DiPF. Di-isopropylphosphoryl-chymotrypsin does not age (Oosterbaan *et al.*, 1958), and di-isopropylphosphoryl-cholinesterase ages slowly with a t_4 of 4–6 h at 24°C (Berends *et al.*, 1959). Di-isopropylphosphoryl-neurotoxic esterase ages rapidly with a t_4 of about 3 min at 37°C (Clothier & Johnson, 1979). The results described in the present paper show that di-isopropylphosphoryl-neurotoxic esterase has a distinctive and characteristic mode of aging. No other proteins were found, in significant amounts, that contained a covalently attached transferred [^3H]isopropyl group that could be released as volatile tritium radioactivity on alkaline

hydrolysis. Furthermore, the transfer of the isopropyl group during the aging is an intramolecular event, which occurs with neurotoxic esterase from the non-nervous tissues, such as hen spleen and liver and human placenta, as well as from the nervous tissues brain and spinal cord. It is therefore a function of the active site(s) of neurotoxic esterase rather than being due, for instance, to the location of neurotoxic esterase in a unique environment in nervous tissue. At present, ester hydrolysis of phenyl valerate or phenyl phenylacetate and intramolecular group transfer in di-isopropylphosphorylated enzyme are the only reactions that neurotoxic esterase is known to perform. The high molecular weight of the phosphorylated polypeptide of neurotoxic esterase (178 000) is large enough to accommodate several different active centres. [For example, the eukaryotic fatty acid synthetase molecule is a single polypeptide chain with M_r 250 000, probably containing eight active centres (Block & Vance, 1977).] The aging reaction may possibly represent a co-operation between two active centres. The redundancy of the initial phosphorylated active centre is established. (Inhibition of neurotoxic esterase by compounds that do not take part in the aging process does not produce delayed neuropathy.) The essential requirement for inducing delayed neuropathy may be an inhibition of the functioning of the second active centre by the transferred alkyl group.

3. Purification of neurotoxic esterase

At present, brain has the highest known concentration of neurotoxic esterase per wet weight of tissue. However, one hen brain (3.5 g) contains only about 6 μg of enzyme (Williams & Johnson, 1981). A larger tissue containing more enzyme would be more suitable as a source. The results above indicate that spleen and placenta can be considered as alternatives to brain. Fresh human placenta is more available than human brain and thus appears to be an excellent source of human enzyme. Since the aim of the present work is the study of the biochemical mechanism of organophosphate-induced delayed neuropathy in animals in order to extrapolate the toxicity of such compounds in man, a direct study of the human enzyme would seem appropriate.

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