Gel-electrophoretic identification of hen brain neurotoxic esterase, labelled with tritiated di-isopropyl phosphorofluoridate

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The particulate fraction from hen brain was labelled with [³H]di-isopropyl phosphorofluoridate (DiPF) and separated by polyacrylamide-gel electrophoresis. Four radioactive protein bands (1–4) of molecular weights 155 000, 92 000, 60 000 and 30 000 were resolved. Most of the labelling of bands 2, 3 and 4 was inhibited by preincubation with Paraoxon. The residue in band 4 was sensitive to pH 5.2. Successive treatments with Paraoxon and pH 5.2 resulted in the abolition of bands 3 and 4. Bands 1 and 2 contained one and two polypeptides respectively, whose labelling was sensitive to Mipafox, but one, in band 2, was sensitive to higher concentrations of Paraoxon. The concentrations of the other two polypeptides were 6.7 and 1.95 pmol of DiPF bound/g of brain in bands 1 and 2 respectively. Both were as sensitive to Mipafox as neurotoxic esterase and were also sensitive to phenyl benzylcarbamate. 4-Nitrophenyl di-n-pentylphosphinate given *in vivo* inhibited neurotoxic esterase and the labelling of the band-1 polypeptide by 82% and 84% respectively, but inhibited the labelling of the band 2 polypeptide by 51%. The phosphinate *in vitro* produced 98% inhibition of the labelling of the band-1 polypeptide, with only 26% inhibition of the band-2 polypeptide, under conditions sufficient to inhibit neurotoxic esterase totally. Both neurotoxic esterase and the band-1 polypeptide were found in the forebrain at 1.74-fold their concentration in the rest of the brain, whereas the band-2 polypeptide was uniformly distributed. The evidence indicates that the Mipafox-sensitive polypeptide in band 1 is the [³H]-DiPF-labelled active-site subunit of neurotoxic esterase. The catalytic-centre activity of the enzyme for phenyl valerate hydrolysis was found to be $2.6 \times 10^5 \text{ min}^{-1}$.

Neurotoxic esterase is an enzyme present in the nervous tissue of many animals, including man and the hen (Aldridge *et al.*, 1969). There is much evidence that the reaction of some organophosphorus esters with this enzyme *in vivo* is the initial event in a process that culminates in the delayed ataxic response (delayed neuropathy) (Johnson, 1975c; Davis & Richardson, 1980).

Neurotoxic esterase has only one known catalytic activity, namely ester hydrolysis. No endogenous substrates have yet been found for the enzyme, and simple inhibition of the enzyme for long periods does not produce any obvious effects *in vivo* (Johnson, 1970, 1974). This hydrolytic activity, then, is apparently not directly related to delayed

neuropathy. Delayed neuropathy is induced, however, as the inhibited enzyme ages, i.e. transfers a chemical group from the phosphorus atom (Johnson, 1975b). The processes disturbed by the aging event are unknown. It is important therefore to study the properties of neurotoxic esterase and the site receiving the aged chemical group, to identify these unknown processes involved in the development of delayed neuropathy.

Homogenates of hen brain can be covalently labelled by incubation with radioactive DiPF, a compound that produces delayed neuropathy (Johnson, 1969a). The neurotoxic phosphorylation site was defined as those DiPF-binding sites that were resistant to Paraoxon (not neurotoxic) but were blocked by Mipafox (neurotoxic) (Johnson, 1969a,b). The development of delayed neuropathy induced by an array of different compounds correlates with the initial modification of a large propor-

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

tion of the neurotoxic phosphorylation site. The degree of modification of this site also correlates with the degree of inhibition of neurotoxic esterase (Johnson, 1970). Since the reaction of the neurotoxic phosphorylation site with DiPF was partially prevented by the presence of the substrate for neurotoxic esterase, it was concluded that the neurotoxic phosphorylation site represents the active site of neurotoxic esterase. Although neurotoxic esterase is only operationally defined by a differential inhibition assay (Johnson, 1977) in the same way as the neurotoxic phosphorylation site, work with many different inhibitory compounds indicates that over 90% of this enzyme activity behaves homogeneously (Johnson, 1975a).

Until now, studies of neurotoxic esterase *in vitro* have concentrated on the chemical specificities of substrates and inhibitors of the enzyme (Johnson, 1975a), on the subcellular (Richardson *et al.*, 1979) and brain-regional distribution of the enzyme (Lotti & Johnson, 1980), and on the chemistry of the aging process that follows inhibition (Clothier & Johnson, 1980). Further study of the function of neurotoxic esterase in the nervous system would be facilitated by the availability of purified enzyme. The present paper describes the development of a procedure that, on an analytical scale, segregates radiolabelled organophosphorylated neurotoxic esterase from a large number of similarly labelled polypeptides. Since neurotoxic esterase is exclusively located in the particulate fraction of hen brain homogenate (Richardson *et al.*, 1979), the [^3H]DiPF-labelling of this fraction was investigated. Polypeptides in the particulate fraction were fractionated by polyacrylamide-gel electrophoresis in the presence of SDS. The segregation procedure also depends on the selective inhibitory properties of several previously studied irreversible inhibitors. This technique provides an analytical means of monitoring the purification of inhibited neurotoxic esterase. The technique does not require a differential measurement, unlike the esterase assay method. It is therefore possible to use the technique to monitor inhibited esterase through complex purification steps without the need to perform identical operations on Paraoxon-treated and Paraoxon-plus-Mipafox-treated homogenates, which would be necessary to follow the esterase activity. Although devoid of catalytic activity, the inhibited enzyme would be suitable for chemical and immunological investigations. The procedure is also applicable to comparisons of different regions of nervous tissue, and of different animal species. In addition, it may also be used to obtain information on the rate of turnover of inhibited enzyme *in vivo* in small animals. A direct measure of the minimum molecular size of neurotoxic esterase and an estimate of its molar concentration in brain were made.

Materials and methods

(1) Chemicals

The sources of inhibitors and of phenyl valerate were as given previously (Johnson, 1969a, 1970, 1974, 1975a). Instagel and Soluene-350 were from Packard, Caversham, Berks., U.K. [^3H]DiPF (6.5 Ci/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K. To remove small amounts of labelled impurities capable of reacting with proteins, the [^3H]DiPF was incubated at 37°C for 20 h after addition of Tris/citrate/EDTA buffer, pH 6.0 (0.85 ml) to the solution of DiPF in propylene glycol (0.2 ml) (Clothier & Johnson, 1979). The solution was then stored at -20°C.

(2) Buffers

(a) *Tris/EDTA buffer, pH 8.0 and pH 9.5.* Tris (50 mM)/EDTA (0.2 mM) was adjusted to pH 8.0 or 9.5 with HCl (12 M) at 20°C.

(b) *Citrate/EDTA buffer, pH 5.0, 4.5, 4.0, 3.5, 3.0 and 2.5.* Citric acid (50 mM)/EDTA (0.2 mM) and sodium citrate (50 mM)/EDTA (0.2 mM) were mixed together in suitable proportions to give the required pH at 20°C.

(c) *Tris/citrate/EDTA buffer pH 6.0.* Tris (50 mM)/EDTA (0.2 mM) and citric acid (50 mM)/EDTA (0.2 mM) were mixed together to give pH 6.0 at 20°C.

(d) *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.

(3) Animals and dosing

Hens were as described by Johnson (1969a). 4-Nitrophenyl di-n-pentylphosphinate (1.5 mg/kg body wt.) dissolved in glycerol/formal (1.2 ml) was given subcutaneously. Dosed birds were either killed after 18 h, for [^3H]DiPF-labelling, or challenged with a neurotoxic dose of DiPF after 18½ h. Before administration of DiPF, birds were given eserine (0.15 mg/kg) and atropine (20 mg/kg) subcutaneously to minimize the anticholinergic effects of DiPF. Then 10 min later the birds were given DiPF (1.7 mg/kg), dissolved in 0.3 ml of 60% (v/v) ethanol, subcutaneously.

(4) Preparation of subcellular particulate fraction from hen brain

One hen brain (weight 3–3.5 g) was homogenized as previously described (Johnson, 1969b) in ice-cold Tris/EDTA buffer, pH 8.0 (20 ml). Then 1 vol. of ice-cold sucrose (2 M) dissolved in Tris/EDTA buffer, pH 8.0, was added, with mixing. The homogenate was centrifuged at 19 000 $g_{av.}$ ($r_{av.} = 7.5$ cm) for 10 min at 4°C. The floating skin of myelin was

removed and discarded. The homogenate was re-centrifuged and the remaining myelin was discarded. The suspension was decanted, leaving a pink pellet, which was discarded. The suspension was diluted with ice-cold Tris/EDTA buffer, pH 8.0 (105 ml), and centrifuged in an MSE 8 × 35 ml titanium rotor for an integrated field time of $4.65 \times 10^6 \text{ g} \cdot \text{min}$ ($r_{\text{av.}} = 6.5 \text{ cm}$) at 4°C. The clear supernatant was discarded, and the whole pellet was dispersed into ice-cold Tris/EDTA buffer, pH 8.0 (34 ml), with a closely fitting rotating pestle.

(5) Treatment of particulate suspension with esterase inhibitors

Neurotoxic esterase is inhibited by Mipaflox, DiPF, phenyl benzylcarbamate and 4-nitrophenyl di-n-pentylphosphinate, but not by Paraoxon, under defined conditions of concentration, time of incubation, temperature and pH. These inhibitors have been used in the present work with minor modifications of these conditions, such as the use of lower concentrations of DiPF for extended time.

Particulate suspension (4 ml) derived from 350–410 mg of brain was warmed to 37°C and added to a solution containing Paraoxon and, in some cases, Mipaflox, in Tris/EDTA buffer, pH 8.0 (0.4 ml). Additionally, in some cases 26 μl of acetone or solutions of phenyl benzylcarbamate or 4-nitrophenyl di-n-pentylphosphinate in acetone was added 15 s after the first addition. Incubations were performed for 20 min at 37°C and were terminated by dilution with ice-cold Tris/EDTA buffer, pH 8.0 (31 ml). The suspension was centrifuged in an 8 × 35 ml titanium angle rotor for an integrated field time of $4.65 \times 10^6 \text{ g} \cdot \text{min}$ ($r_{\text{av.}} = 6.5 \text{ cm}$) at 4°C. The clear supernatant was discarded. Normally, the pellet was resuspended in citrate/EDTA buffer pH 5.0 (4 ml) (final measured pH 5.2 owing to the presence of the tissue), but in some cases buffers of different pH were used.

(6) Labelling of the particulate suspension by [^3H]DiPF

The pre-inhibited particulate suspension from step (5) was incubated at 37°C for 10 min before the addition of diluted [^3H]DiPF (50 μl) (final concn. 1.75 μM). Labelling was continued for 40 min and terminated by dilution with ice-cold Tris/EDTA buffer, pH 8.0 (31 ml). The I_{50} of neurotoxic esterase for DiPF is 0.7 μM for 20 min at 37°C (Johnson, 1975a). These conditions are thus sufficient to label 97% of the active sites of the enzyme with [^3H]DiPF.

The suspension was then centrifuged as described in section (5). This step was sufficient to remove 98% of the unbound tritium.

(7) SDS/polyacrylamide-gel electrophoresis

Each pellet from step (6), derived from 0.35–0.41 g of brain, was suspended in SDS solubilization buffer, pH 8.0 (2.0 ml), and boiled for 5 min to give a clear solution.

The electrophoresis system used is based on that described by Fairbanks *et al.* (1971), and uses the same electrophoresis buffer with the exception of using 0.1% (w/v) SDS instead of 1.0% (w/v) SDS. The polyacrylamide gel was formed by adding ammonium persulphate (80 mg) and *NNN'*-tetramethylethylenediamine (100 μl) to a solution of acrylamide monomer (4%, w/v) and methylene-bisacrylamide (0.106%, w/v) in electrophoresis buffer (100 ml). The gel was cast as a slab 16.0 cm long, 16.5 cm wide and 3 mm thick. Vertical electrophoresis was performed at room temperature with a voltage gradient of 2.4 V/cm for 16½ h.

The apparent molecular weights of protein were determined from their electrophoretic mobilities relative to a set of standard proteins: thyroglobulin (mol.wt. 330 000), β -galactosidase (mol.wt. 116 000), phosphorylase *b* (mol.wt. 94 000), bovine serum albumin (mol.wt. 67 000), catalase (mol.wt. 60 000), ovalbumin (mol.wt. 43 000), lactate dehydrogenase (mol.wt. 36 000) and carbonic anhydrase (mol.wt. 30 000) (Pharmacia, Uppsala, Sweden, and Sigma Chemical Co., Poole, Dorset, U.K.).

Proteins in the gel were fixed by shaking in 550 ml of methanol (45.5%, v/v)/acetic acid (9%, v/v) in water containing Kenacid Blue (BDH Chemicals, Poole, Dorset, U.K.) (0.002%) for 24 h at room temperature. The gel was destained by three washes in 1 litre of methanol (5%, v/v)/acetic acid (7.5%, v/v) in water for 2 h each.

(8) Measurement of tritium radioactivity

(a) *Total tritiated proteins in SDS.* Tritiated proteins in solution in SDS (0.2 ml) were precipitated and washed twice in 5 ml of ether (50%, v/v)/ethanol (35%, v/v) in water. This method gave the same results as the organic solvent system described by Johnson (1969a). The precipitate was solubilized in Soluene-350 (1 ml) and mixed with Instagel (10 ml) plus acetic acid (0.1 ml) before its radioactivity was counted in a Searle scintillation counter with external-standard ratio for quench correction. The efficiency of counting was 41%.

(b) *Tritiated proteins in polyacrylamide-gel slices.* The polyacrylamide gel after destaining was cut into 1.2 cm-wide tracks. Each track was cut into 33 0.5 cm slices. Each slice was incubated with H_2O_2 (30%, w/v; 1 ml) at 60°C in a capped scintillation vial for 48 h, to produce a homogeneous solution. Instagel (10 ml) was added and the radioactivity in the vial was counted after at least 24 h at 4°C in the dark. The average recovery of tritiated

proteins from the gels was $101.2 \pm 8.7\%$ (41 determinations). The efficiency of counting was 34%.

(9) Measurement of neurotoxic esterase activity

Neurotoxic esterase activity was assayed by a differential assay, measuring the phenol released from phenyl valerate, as previously described (Johnson, 1977).

Results

The 'neurotoxic phosphorylation site' was first studied by labelling whole hen brain homogenate with [^{32}P]DiPF at pH 8.0 (Johnson, 1969*a,b*). In the present study, the [^3H]DiPF-labelling of polypeptides in the particulate fraction of hen brain was investigated. The identification of neurotoxic esterase proceeded in two stages: firstly the selective removal of the labelling of most non-neurotoxic sites, and secondly the selective inhibition of neurotoxic esterase from the residual labelling. In the first section below, the experiments to characterize the conditions required to remove the labelling of most of the non-neurotoxic sites are described. The optimal conditions found were an incubation with Paraoxon ($160\mu\text{M}$) at 37°C and pH 8.0 for 20 min

before [^3H]DiPF treatment at 37°C at pH 5.2 for 40 min.

Effects produced by acid pH and Paraoxon

A washed particulate suspension derived from whole hen brain was treated with [^3H]DiPF in Tris/EDTA buffer at pH 8.0, or citrate/EDTA buffer at pH 5.2, without prior Paraoxon treatment. The particles were then washed, solubilized in buffer containing SDS and mercaptoethanol, and separated by electrophoresis in SDS on a 4% (w/v) polyacrylamide gel (see the Materials and methods section). Fig. 1 shows the tritium radioactivity in each 0.5 cm slice of gel. Four peaks of radioactivity were resolved (bands 1–4 in order of increasing mobility in the gel). The apparent molecular weights of these bands were 155 000, 92 000, 59 000 and 30 000 respectively. Throughout the present work, the positions of bands 1–4 on all gels is taken to be the position of bands 1–4 in an uninhibited sample labelled with DiPF at pH 8.0. No inhibitor treatment suggested the presence of other bands with different apparent molecular weight. However, the absolute positions of the bands from one gel to the next did vary slightly, presumably owing to differences in ambient temperature during electrophoresis. Less radioactivity was present in bands 2, 3 and 4 from the particle suspension that had been

Fig. 1. Polyacrylamide-gel electrophoretograms of membranes labelled at pH 8 or pH 5.2

Washed particles were suspended in either Tris/EDTA buffer, pH 8.0, or citrate/EDTA buffer, pH 5.0, and incubated with [^3H]DiPF. After a washing, the labelled particles were solubilized and separated by polyacrylamide-gel electrophoresis. The tritium radioactivity in each slice from particles labelled at pH 8.0 (▨) and at pH 5.2 (▩) is shown. For experimental details see the Materials and methods section. The origin is at the left. The arrowed numbers indicate the apparent molecular weight relative to a set of standard proteins.

Fig. 2. Effect of Paraoxon pretreatment on the $[^3\text{H}]\text{DiPF}$ -labelling of bands 1-4

Particles were treated with Paraoxon (0–160 μM) for 20 min at pH 8.0 and 37°C. The particles were subsequently labelled with $[^3\text{H}]\text{DiPF}$ at pH 5.2, solubilized and separated by SDS/polyacrylamide-gel electrophoresis. For experimental details see the Materials and methods section. The graph shows the mean tritium radioactivity in each of the four gel bands from two separate experiments as a function of the Paraoxon concentration (●, band 1; ▲, band 2; ■, band 3; ○, band 4).

labelled with $[^3\text{H}]\text{DiPF}$ at pH 5.2 than in the same bands labelled at pH 8.0. The labelling of band 4, in particular, was markedly diminished at pH 5.2.

In order to diminish the labelling of some of the irrelevant sites, the effects of Paraoxon on the DiPF-labelling of each of the bands was measured. The DiPF-labelling was again performed at pH 5.2, since this pH prevented the labelling of one of the irrelevant DiPF-binding sites that was resistant to Paraoxon (see below).

Particles were incubated with various concentrations of Paraoxon at pH 8.0 for 20 min at 37°C before $[^3\text{H}]\text{DiPF}$ labelling at pH 5.2. Fig. 2 shows that the radioactivity in all bands was decreased by Paraoxon treatment. Most of the Paraoxon inhibition of labelling was achieved with 10 μM -Paraoxon, but some Paraoxon-sensitive sites were still present in bands 2 and 3 after treatment with 40 μM -Paraoxon and were removed in particles pretreated with either 80 μM - or 160 μM -Paraoxon. The inhibition of labelling of band 1 by 160 μM -Paraoxon was small (17.8, 14.7 and 20.6% in three separate experiments).

To check whether the $[^3\text{H}]\text{DiPF}$ labelling of the neurotoxic phosphorylation site was inhibited by

lowering the pH to 5.2, the labelling sensitive to 50 μM -Mipafox was measured at several pH values. This concentration of Mipafox was used to inhibit neurotoxic esterase in the differential assay of the enzyme activity described by Johnson (1977). Particles were treated with 160 μM -Paraoxon with or without 50 μM -Mipafox, resuspended in either Tris/EDTA buffer at pH 8.6 or 8.0, or citrate/EDTA buffer, pH 5.2, and incubated at 37°C for 10 min. $[^3\text{H}]\text{DiPF}$ was added and radiolabelling was performed at the chosen pH. Reaction was stopped by the addition of 7 vol. of cold Tris/EDTA buffer, pH 8.0. Bound tritium was measured as described in the Materials and methods section. The quantity of Mipafox-sensitive sites was greatest at pH 8.0, but at pH 5.2 or 8.6 it had decreased by only 9% and 14% respectively (average of two experiments). The labelled components of particles treated with DiPF at pH 8.0 and 5.2 were separated by polyacrylamide-gel electrophoresis in the presence of SDS. Fig. 3 shows the radioactivity in each gel slice. Most of the Mipafox-sensitive labelling occurred in bands 1 and 2, with only very small amounts in bands 3 and 4. The labelling of bands 1 and 2 was only slightly decreased at pH 5.2 (Fig. 3b), consistent with the measurements of the total Mipafox-sensitive sites already described above. The labelling of band 4 was insensitive to Mipafox pretreatment and thus is not part of the neurotoxic phosphorylation site. However, the labelling of band 4 was practically eliminated if the treatment with $[^3\text{H}]\text{DiPF}$ was at pH 5.2. Thus part of the irrelevant, Paraoxon-resistant, labelling (band 4) can be conveniently removed by performing the DiPF-labelling at pH 5.2.

When DiPF-labelling was performed at pH values lower than 5.2 in citrate/EDTA buffers, it was found that there was a sharp inhibition of labelling of band 1, which contains most of the Mipafox-sensitive sites (77% inhibition at pH 4.3; 94% inhibition at pH 3.8). pH 5.2 was selected as the optimal pH for DiPF-labelling of the Mipafox-sensitive sites. This sharp inhibition of band-1 labelling at very low pH compared well with the inhibition of neurotoxic esterase at low pH described by Dudek (1979) and by Johnson (1982).

Attempts to discover the labelled polypeptide with the characteristics of neurotoxic esterase

In the following sections, the conditions of pre-inhibition with Paraoxon (160 μM) and DiPF-labelling at pH 5.2, described above, were used to prevent the DiPF-labelling of most of the irrelevant phosphorylation sites, without substantially inhibiting the DiPF-labelling of the neurotoxic phosphorylation sites. The second stage of the investigation, described below, is the attempted identification of labelled neurotoxic esterase by using

Fig. 3. Polyacrylamide-gel electrophoretogram of DiPF-labelled proteins labelled with and without Mipafox pretreatment at pH 8.0 (a) or pH 5.2 (b)

Washed particles were treated with Paraoxon (160 μ M) plus (▨) or minus (▧) Mipafox (50 μ M) at pH 8.0 at 37°C. The particles were washed and labelled with [3 H]DiPF in either Tris/EDTA buffer, pH 8.0, or citrate/EDTA buffer, pH 5.0. The particles were washed and then dissolved in SDS solubilization buffer, and the tritiated proteins were separated by electrophoresis in a polyacrylamide gel. For experimental details see the Materials and methods section. The origin is at the left.

pre-inhibition with specific inhibitors of the enzyme to prevent labelling by DiPF.

Neurotoxic esterase has an I_{50} of 7 μ M for Mipafox at 37°C for 20 min (Johnson, 1975a). The I_{50} for phenyl benzylcarbamate has been reported to be 10 μ M at 37°C for 20 min (Johnson 1975a), but is dependent on the acetone concentration (M. K. Johnson, unpublished work), and, under the con-

ditions used in the present study, the I_{50} was 2.5 μ M at 37°C for 20 min. These two compounds were selected as being chemically quite different, but nevertheless rather specific inhibitors of neurotoxic esterase in Paraoxon-treated tissue (Johnson, 1975a).

A particulate suspension was treated with Paraoxon and, in addition, either phenyl benzyl-

carbamate (12.5 μM) or Mipafox (50 μM) at pH 8.0. The particles were then labelled with [^3H]DiPF at pH 5.2. Table 1 shows that the labelling of bands 1 and 2 was significantly inhibited by phenyl benzylcarbamate or Mipafox. The Mipafox-sensitive labelling of band 2 was diminished (from 5.68 to 1.95 pmol/g) in particles treated with 160 μM -Paraoxon rather than 40 μM -Paraoxon. However, the phenyl benzylcarbamate-sensitive labelling of band 2 was not significantly different in the two situations (1.98 and 1.55 pmol/g respectively). Thus the higher concentration of Paraoxon inhibits the labelling of a site in band 2 that is sensitive to Mipafox but not to phenyl benzylcarbamate. Treatment of particles with phenyl benzylcarbamate (12.5 μM) in addition to Mipafox (50 μM) and Paraoxon (160 μM) caused only 2% and 0.6% increase in inhibition of gross labelling in two separate experiments. Thus phenyl benzylcarbamate and Mipafox are inhibiting the labelling of the same components under these conditions. These results indicate that most of band 1 (about 7 pmol/g) and only a small component of band 2 (about 2 pmol/g) are resistant to Paraoxon and sensitive both to Mipafox and to phenyl benzylcarbamate inhibition. These sites are therefore both candidates for the inhibited form of neurotoxic esterase. It was possible that these two sites were unrelated polypeptides or were related by being aggregates, subunits or the result of proteolytic cleavage of a common catalytic unit. The following

experiments were therefore performed in order to resolve these alternatives.

Titration of bands 1 and 2 with Mipafox. A washed particle suspension was treated with Paraoxon (160 μM) and a range of Mipafox concentrations in the pre-inhibition stage before [^3H]DiPF-labelling. Fig. 4 shows that the Mipafox-sensitive sites in band 1 and band 2 titrate over the same range of Mipafox concentrations and that both sets of data are scattered around the line drawn with an I_{50} of 10 μM . Thus the two sites cannot be distinguished by a difference in Mipafox-sensitivity, which is close to that determined for neurotoxic esterase ($I_{50} = 7 \mu\text{M}$; Johnson, 1975a).

Regional distribution of bands 1 and 2 in brain. It has been shown (Lotti & Johnson, 1980) that the highest concentration, on a wet-weight basis, of neurotoxic esterase is in the frontal lobes of the human brain. The distributions of Mipafox-sensitive bands 1 and 2 and of neurotoxic esterase were therefore measured in the forebrain and in the mid- and hind-brain of the hen.

Table 2 shows the Paraoxon-resistant Mipafox-sensitive labelling of bands 1 and 2 in both parts of the brain, together with the neurotoxic esterase activity in an identical particle preparation. Neurotoxic esterase activity and Mipafox-sensitive labelling in band 1 were present in the same ratio in both halves of the brain, whereas the Mipafox-sensitive labelling in band 2 showed no correlation. The

Table 1. *DiPF-binding sites in each labelled band after 40 μM - or 160 μM -Paraoxon treatment and the protection against labelling provided by Mipafox in phenyl benzylcarbamate*

Brain particles were treated with 40 μM - or 160 μM -Paraoxon at pH 8, and in addition with Mipafox (50 μM) or phenyl benzylcarbamate (12.5 μM). The particles were washed, treated with [^3H]DiPF at pH 5.2, solubilized and separated by gel electrophoresis (for details see the Materials and methods section). The radioactivity in each band was measured. Results are presented as means $\pm$ s.d. for the numbers of observations given in parentheses in the final column.

Quantity measured	Inhibitory conditions	[^3H]DiPF-binding sites (pmol/g of brain)			
		Band 1	Band 2	Band 3	Band 4
Quantity of sites	Paraoxon (40 μM)	8.96 $\pm$ 0.54	8.17 $\pm$ 1.04	1.87 $\pm$ 0.62	1.27 $\pm$ 0.31 (3)
Decrease in quantity of sites due to Mipafox pre-inhibition	Paraoxon (40 μM) + Mipafox (50 μM)	7.84 $\pm$ 0.64	5.68 $\pm$ 0.56	0.83 $\pm$ 0.38	0.59 $\pm$ 0.17 (3)
Decrease in quantity of sites due to phenyl benzylcarbamate pre-inhibition	Paraoxon (40 μM) + phenyl benzylcarbamate (12.5 μM)	6.47 $\pm$ 0.44	1.98 $\pm$ 0.42	0.66 $\pm$ 0.48	0.52 $\pm$ 0.27 (3)
Quantity of sites	Paraoxon (160 μM)	8.27 $\pm$ 1.21	4.61 $\pm$ 0.90	1.18 $\pm$ 0.46	0.71 $\pm$ 0.24 (14)
Decrease in quantity of sites due to Mipafox pre-inhibition	Paraoxon (160 μM) + Mipafox (50 μM)	6.68 $\pm$ 1.16	1.95 $\pm$ 0.23	0.26 $\pm$ 0.30	0.12 $\pm$ 0.08 (3)
Decrease in quantity of sites due to phenyl benzylcarbamate pre-inhibition	Paraoxon (160 μM) + phenyl benzylcarbamate (12.5 μM)	5.54 $\pm$ 0.53	1.55 $\pm$ 0.54	0.49 $\pm$ 0.11	0.05 $\pm$ 0.06 (4)

Fig. 4. Effect of Mipafox on the $[^3\text{H}]\text{DiPF}$ -labelling of the Paraoxon-resistant components of bands 1 and 2. Particles were treated with $160\mu\text{M}$ -Paraoxon and, in addition, $0\mu\text{M}$ -, $2\mu\text{M}$ -, $5\mu\text{M}$ -, $8\mu\text{M}$ -, $11.4\mu\text{M}$ -, $16\mu\text{M}$ -, $21.3\mu\text{M}$ - and $32\mu\text{M}$ -Mipafox for 20min at pH 8.0 and 37°C . The particles were subsequently labelled with $[^3\text{H}]\text{DiPF}$ at pH 5.2, solubilized and separated by SDS/polyacrylamide-gel electrophoresis. For experimental details see the Materials and methods section. The graphs show a semi-logarithmic plot of the percentage labelling of the Mipafox-sensitive component of the labelling of band 1 (●) and band 2 (▲) versus the Mipafox concentration. The plotted line shows the inhibition corresponding to an I_{50} of $10\mu\text{M}$.

concentrations of esterase and Mipafox-sensitive band 1 were increased 1.7-fold in the forebrain relative to the rest of the brain. Mipafox-sensitive sites in band 2 were present at slightly lower concentrations in the forebrain than in the rest of the brain. This evidence suggests that band-2 sites are not neurotoxic esterase.

Sensitivity of bands 1 and 2 to 4-nitrophenyl di-n-pentylphosphinate

In a further attempt to inhibit the DiPF-labelling of band 1 selectively, the effects of another inhibitor of neurotoxic esterase, 4-nitrophenyl di-n-pentylphosphinate, were investigated. This compound has been shown to react with the neurotoxic site *in vivo* (Johnson, 1974) and to have an I_{50} of 20nm for neurotoxic esterase (Johnson, 1975a).

A washed particle preparation was treated with Paraoxon ($160\mu\text{M}$) and either Mipafox ($50\mu\text{M}$) or 4-nitrophenyl di-n-pentylphosphinate (110nm) or both, before $[^3\text{H}]\text{DiPF}$ -labelling at pH 5.2. Table 3 shows the labelling of bands 1–4 and the inhibition of labelling produced by the two esterase inhibitors. Since there was virtually no additional blockade of labelling sites by 4-nitrophenyl di-n-pentylphosphinate in the presence of Mipafox, it appears that the Paraoxon-resistant sites sensitive to 4-nitrophenyl di-n-pentylphosphinate are also sensitive to Mipafox. On its own, 4-nitrophenyl di-n-pentylphosphinate (110nm) prevented the labelling of all the Mipafox-sensitive band-1 sites, but only 26% of

Table 2. Comparison of particulate neurotoxic esterase and Paraoxon-resistant Mipafox-sensitive $[^3\text{H}]\text{DiPF}$ -labelling in the forebrain and mid- + hind-brain

Results are presented as means $\pm$ s.d. for the numbers of observations given in parentheses. Particles were prepared from the two brain regions in exactly the same way as described for whole brain (for details see the Materials and methods section). Half of the particles were preincubated at pH 8 with Paraoxon ($160\mu\text{M}$), with or without Mipafox ($50\mu\text{M}$), and subsequently labelled with $[^3\text{H}]\text{DiPF}$ at pH 5.2. The other half was treated identically as regards centrifugation and buffers, but no inhibitors or $[^3\text{H}]\text{DiPF}$ were added. These particles were suspended in Tris/EDTA buffer, pH 8.0, and the neurotoxic esterase activity was measured. The tritiated particles were dissolved in SDS and separated by polyacrylamide-gel electrophoresis. The radioactivity in each band was measured and the DiPF binding was calculated.

	Forebrain	Mid- + hind-brain
Wet wt. (g)	1.83 ± 0.05 (3)	1.54 ± 0.17 (3)
Neurotoxic esterase in particles (nmol/min per g of brain) [E]	2466 ± 148 (5)	1424 ± 119 (5)
Esterase activity in particles (% of activity in original tissue homogenate)	69.4 ± 3.6 (3)	60.1 ± 1.1 (3)
Mipafox-sensitive $[^3\text{H}]\text{DiPF}$ -labelling of particles (pmol/g of brain) in:		
Band 1 [A]	9.51 ± 1.34 (3)	5.46 ± 0.28 (3)
Band 2 [B]	2.44 ± 0.44 (3)	2.64 ± 0.39 (3)
Ratio of enzyme activity to Mipafox-sensitive $[^3\text{H}]\text{DiPF}$ -labelling in band 1 (min^{-1}) [E/A]	2.59×10^5	2.61×10^5
Ratio of enzyme activity to Mipafox-sensitive $[^3\text{H}]\text{DiPF}$ -labelling in band 2 (min^{-1}) [E/B]	10.11×10^5	5.39×10^5

Table 3. *DiPF-binding sites in each labelled band after Paraoxon (160 μ M) treatment and the protection against labelling provided by Mipafox, 4-nitrophenyl di-n-pentylphosphinate or a combination of the two*

Brain particles were treated at pH 8.0 with Paraoxon (160 μ M) and, in addition, Mipafox (50 μ M) or 4-nitrophenyl dipentylphosphinate (110 nM) or both. The washed inhibited particles were treated with [3 H]DiPF at pH 5.2, then separated by polyacrylamide-gel electrophoresis, and the radioactivity was measured in each band on the gel. The results are expressed as means $\pm$ S.D. for three separate experiments.

Quantity measured	Inhibitory conditions	[3 H]DiPF-binding sites (pmol/g of brain)			
		Band 1	Band 2	Band 3	Band 4
Quantity of sites	Paraoxon (160 μ M)	8.57 $\pm$ 0.10	4.67 $\pm$ 1.13	1.03 $\pm$ 0.46	0.87 $\pm$ 0.12
Decrease in quantity of sites due to Mipafox pre-inhibition	Paraoxon (160 μ M) + Mipafox (50 μ M)	7.23 $\pm$ 0.25	2.81 $\pm$ 0.72	0.32 $\pm$ 0.22	0.18 $\pm$ 0.08
Decrease in quantity of sites due to phosphinate pre-inhibition	Paraoxon (160 μ M) + 4-nitrophenyl dipentylphosphinate (110 nM)	7.11 $\pm$ 0.21	0.72 $\pm$ 0.54	0.26 $\pm$ 0.30	0.05 $\pm$ 0.11
Decrease in quantity of sites due to Mipafox plus phosphinate pre-inhibition	Paraoxon (160 μ M) + Mipafox (50 μ M) + 4-nitrophenyl dipentylphosphinate (110 nM)	7.83 $\pm$ 0.20	2.90 $\pm$ 0.87	0.52 $\pm$ 0.52	0.36 $\pm$ 0.32

the Mipafox-sensitive band-2 sites. The virtually complete inhibition of labelling of the Mipafox-sensitive sites in band 1 by 110 nM-4-nitrophenyl di-n-pentylphosphinate is consistent with band 1 being the inhibited form of neurotoxic esterase (I₅₀ 20 nM; Johnson, 1975a). The low degree of blockade of the Mipafox-sensitive band-2 sites suggests that band 2 is not associated with neurotoxic esterase. To provide a more direct correlation between the delayed neurotoxic effect and band 1, the effects of 4-nitrophenyl di-n-pentylphosphinate dosing *in vivo* on the DiPF-labelling sites were investigated.

Hens were dosed *in vivo* with an amount of 4-nitrophenyl di-n-pentylphosphinate (1.5 mg/kg) that was sufficient to give protection from the delayed neurotoxic effects of a subsequent challenge dose of DiPF (Johnson, 1974). The animals were killed after 16 h. The brain was homogenized and the total neurotoxic esterase activity was measured. Particles were prepared, and treated as usual with Paraoxon (160 μ M), with or without Mipafox (50 μ M), before [3 H]DiPF-labelling. Table 4 shows that the 4-nitrophenyl di-n-pentylphosphinate-dosed hens contained only 16% of the control Paraoxon-resistant Mipafox-sensitive sites in band 1, whereas they contained 49% of the control Paraoxon-resistant Mipafox-sensitive sites in band 2.

The protective effect of the dose of 4-nitrophenyl di-n-pentylphosphinate *in vivo* against the delayed neurotoxicity of DiPF can be compared with the high degree of inhibition of both band 1 and of neurotoxic esterase produced by the dose. In contrast, a lower degree of inhibition of the Mipafox-sensitive band-2 sites is less indicative of a role for these band-2 sites in delayed neuropathy.

The activity of neurotoxic esterase remaining after the dose of 4-nitrophenyl di-n-pentylphosphinate was 18% (mean of three experiments). This correlated with the amount of Mipafox-sensitive band-1 sites available for DiPF-labelling (16%) rather than with the Mipafox-sensitive band-2 sites (49%).

Thus, here and in the section on the regional distribution of bands 1 and 2 in the brain, above, there is a good correlation between the activity of neurotoxic esterase and the DiPF-labelling of the Paraoxon-resistant Mipafox-sensitive sites in band 1. In neither case is there a similar correlation with band 2.

Catalytic-centre activity and molecular concentration of neurotoxic esterase

Table 2 shows that the catalytic-centre activity for the hydrolysis of phenyl valerate by neurotoxic esterase (the ratio of esterase activity to Mipafox-sensitive [3 H]DiPF-labelling of band 1) is $2.6 \times 10^5 \text{ min}^{-1}$. The average recovery of neurotoxic esterase in the particulate fraction of whole brain is 66%. Since Mipafox-sensitive band 1 is present at a concentration of 6.7 pmol/g of brain in the particles (Table 1), its concentration in whole brain should be 10.2 pmol/g of brain. The apparent molecular weight of band 1 is 155 000 (Fig. 1). Assuming this molecular weight to be correct, there should be 1.6 μ g of band 1/g of brain.

Discussion

Neurotoxic esterase is the phenyl valerate-hydrolytic activity, in homogenates of nervous tissue, that is resistant to Paraoxon (40 μ M) but inhibited by

Table 4. *DiPF-binding sites in each labelled band after Paraoxon (160 μ M) treatment, and the protection against labelling provided by Mipafox treatment of particles from normal and 4-nitrophenyl di-n-pentylphosphinate-dosed hens* Hens were dosed with 4-nitrophenyl dipentylphosphinate (1.5 mg/kg, intraperitoneally) as described in the Materials and methods section. After 18 h, particles were treated with Paraoxon (160 μ M), with or without Mipafox (50 μ M), at pH 8.0. After a washing, the particles were treated with [3 H]DiPF at pH 5.2, washed, solubilized and separated by polyacrylamide-gel electrophoresis. The radioactivity in each band was measured. The activity of neurotoxic esterase in brain homogenates from the dosed birds was $18.3 \pm 7.4\%$ of the activity in control homogenates. Results are expressed as means $\pm$ s.d. for three separate experiments.

Quantity measured	Inhibitory conditions	[3 H]DiPF-binding sites (pmol/g of brain)			
		Band 1	Band 2	Band 3	Band 4
Quantity of sites in control particles	Paraoxon (160 μ M)	7.50 ± 0.60	4.34 ± 0.69	0.93 ± 0.15	0.70 ± 0.31
Decrease in quantity of sites in particles from control hens due to Mipafox pre-inhibition	Paraoxon (160 μ M) + Mipafox (50 μ M) (particles from control hens)	6.35 ± 0.73	2.84 ± 0.75	0.35 ± 0.12	0.09 ± 0.30
Decrease in quantity of sites in particles from dosed hens due to Mipafox pre-inhibition	Paraoxon (160 μ M) + Mipafox (50 μ M) (particles from phosphinate-dosed hens)	1.03 ± 0.36	1.39 ± 0.26	0.13 ± 0.08	0.04 ± 0.06

Mipafox (50 μ M) under standard conditions of temperature, pH and time of incubation (Johnson, 1977). This hydrolytic activity behaves at least 90% homogeneously towards all inhibitors tested. As mentioned in the introduction, this activity also correlates with the availability of the neurotoxic phosphorylation site for DiPF-labelling.

The initial expectation was to find one DiPF-labelled protein, on gel electrophoresis, that was resistant to Paraoxon but sensitive to Mipafox. However, the Mipafox-sensitive labelling under inhibitory conditions similar to, although not identical with, those described by Johnson (1969*a,b*) was found to occupy three different polypeptide sites. Thus 58% appeared in band 1, 15% appeared in band 2 with similar properties with regard to Paraoxon, Mipafox and phenyl benzylcarbamate sensitivity, and a further 27% in band 2, although Mipafox-sensitive, was insensitive to the carbamate, and was also sensitive to higher concentrations of Paraoxon (Table 1).

Further experiments showed a correlation between the regional distribution of neurotoxic esterase and the Mipafox-sensitive sites in band 1 that was not paralleled by the Mipafox-sensitive sites in band 2. Moreover, a further correlation between the esterase and the band-1 sites was observed after inhibition by 4-nitrophenyl di-n-pentylphosphinate *in vivo*. The phosphinate was found to be even more selective in inhibiting band 1 *in vitro* at concentrations (110 nM) sufficient to inhibit neurotoxic esterase ($I_{50} = 20$ nM; Johnson, 1975*a*). The DiPF-labelling of the Mipafox-sensitive components of bands 1 and 2 has similar properties with regard to

Paraoxon, Mipafox and phenyl benzylcarbamate. In two different circumstances a correlation has now been observed between neurotoxic esterase activity and the DiPF-labelling of Mipafox-sensitive band 1. No correlation was observed with the Mipafox-sensitive component of band 2. This suggests that this band-2 polypeptide has little or no capacity to hydrolyse phenyl valerate. This hypothesis would be consistent with previous work demonstrating the correlation between neurotoxic esterase activity and the DiPF-labelling of the neurotoxic phosphorylation site (which includes the labelling of components in both bands 1 and 2) (Johnson, 1970). The Mipafox-sensitive band-1 sites represent only 58% of the neurotoxic phosphorylation site under the conditions of 40 μ M-Paraoxon pre-inhibition. However, there is much evidence from the study of a vast number of different chemicals for the direct relationship between inhibition plus aging of neurotoxic esterase and the induction of delayed neuropathy (Johnson, 1969*a*, 1975*b*). Thus there is no reason to doubt that band 1 is not only the inhibited form of neurotoxic esterase but is also the polypeptide whose modification is responsible for triggering delayed neuropathy.

The covalent reaction of neurotoxic esterase with DiPF has previously been shown to be followed by a rapid stoichiometric transfer of one side chain from DiPF to a different protein site under similar conditions to those used here (Clothier & Johnson, 1979). Only one tritium-labelled band has been found that can be identified as inhibited neurotoxic esterase. It therefore appears that the transferred tritiated side chain becomes attached to the same

polypeptide that contains the DiPF phosphoryl group. Alternatively, it is possible that the transferred group is attached to a different polypeptide that has the same apparent molecular weight as the initial DiPF-receptor polypeptide.

The identification of the bulk of band 1 as being the active-site polypeptide of neurotoxic esterase gives rise to several important possibilities. (1) The purification of labelled inhibited enzyme can be followed by using a simple electrophoretic assay for band 1 that, unlike the esterase activity, will not be affected by the use of detergents commonly used to solubilize neurotoxic esterase, but will give an indication of molecular integrity, which is not necessarily true with an enzyme assay. (2) Since band 1 apparently carries both the phosphorus atom and the aged side chain of DiPF at different positions on the polypeptide chain, further information on the aging process could be obtained by isolating radiolabelled band 1 by gel electrophoresis, to characterize chemically the phosphorylating site and the site accepting the aged group. (3) Most of band 1 is the neurotoxic esterase polypeptide. It is feasible to inject labelled DiPF *in vivo*, and identify labelled band 1 subsequently. Thus one could monitor the rate of turnover of inhibited enzyme *in vivo*. (4) The identification of the neurotoxic esterase polypeptide allows a comparison of the concentrations and physical properties of the polypeptide in different tissues and in different species.

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