Correlation of neuropathy target esterase activity with specific tritiated di-isopropyl phosphorofluoridate-labelled proteins

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Neuropathy target esterase (NTE) is a membrane-bound carboxylesterase activity that has been proposed as the target site for initiation of organophosphate-induced delayed neuropathy. This activity is identified by its resistance to treatment with Paraoxon and sensitivity to co-incubation with Paraoxon and Mipafox. Sucrose-density-gradient centrifugation of membrane-associated proteins isolated from chick-embryo brains identified three proteins, M_r 161 000, 116 500 and 103 000, that were labelled with [^3H]di-isopropyl phosphorofluoridate in an NTE-like manner and that co-migrated with NTE. The 161 000- M_r and 116 500- M_r proteins were identified in both adult and embryo brain. One or both of these proteins may therefore contribute to the activity defined as NTE. In addition, a 61 000- M_r protein was identified that does not co-migrate with NTE, but that was labelled with [^3H]di-isopropyl phosphorofluoridate in a Paraoxon-resistant and Mipafox-sensitive manner. The effect of Mipafox on labelling, however, was reversibly blocked by co-incubation with Paraoxon. This protein, therefore, is not NTE, but has the necessary inhibitor-sensitivity to be the target site for organophosphate-induced delayed neuropathy.

INTRODUCTION

Neuropathy target esterase (NTE) is an integral membrane protein found in highest concentration in central and peripheral nerve tissue [1–3]. Inhibition of this enzyme by some organophosphate esters (OPs) and the subsequent dealkylation ('aging') of the enzyme-bound OP is correlated with the ability of these OPs to cause organophosphate-induced delayed neuropathy (OPIDN) [4–8]. This neuropathy, which appears 1–3 weeks after an acute exposure to neuropathic OPs, is characterized by muscle weakness, ataxia and paralysis of the limbs [9,10]. Each of these symptoms is accompanied either by distal degeneration of the long large-diameter peripheral nerves or by damage to the ascending and descending tracts of the spinal cord [9–11]. There is evidence that the target site at which OP binding initiates OPIDN is pharmacologically distinct from the active site of acetylcholinesterase [12]. The role of NTE in the proper function of the nerve axon and the mechanism by which OP modification of NTE possibly initiates the delayed degeneration of the peripheral nerves has not been determined.

NTE activity is detected on the basis of its inhibition by neuropathic OPs such as di-isopropyl phosphorofluoridate ($\text{iPr}_2\text{P-F}$) and Mipafox, and its resistance to inhibition by non-neuropathic OPs such as Paraoxon. Operationally, NTE is defined as that fraction of total phenyl valerate-hydrolysing carboxylesterase activity that is both Paraoxon-resistant and Mipafox-sensitive. To identify those Paraoxon-resistant sites that are Mipafox-sensitive, paired samples were treated with either Paraoxon, to block irrelevant $\text{iPr}_2\text{P-F}$ -binding sites, or with both Paraoxon and Mipafox. These samples

were then labelled with [^3H] $\text{iPr}_2\text{P-F}$ and analysed by SDS/PAGE [13,14]. This method identified several NTE-like proteins, which in decreasing order of abundance were reported to have apparent M_r values of 160 000, 82 000 and 115 000 [13,14]. Williams & Johnson examined the brain regional distributions of NTE and [^3H] $\text{iPr}_2\text{P-F}$ -labelled proteins and concluded that the 160 000- M_r protein was distributed similarly to NTE whereas the 82 000- M_r protein was not [13]. Carrington & Abou-Donia found that $\text{iPr}_2\text{P-F}$ phosphorylated each of these proteins at a rate similar to the rate at which $\text{iPr}_2\text{P-F}$ inhibits NTE [14]. These studies did not conclusively identify a specific protein as NTE, and the possibility remains that some of these proteins could be labelled in an NTE-like manner but not have any carboxylesterase activity. It is therefore important to use an analytical method that leaves the NTE activity intact, thereby allowing a direct comparison of the NTE activity and NTE-like labelled proteins.

The method described by Ishikawa *et al.* [15] for separating carboxylesterases on sucrose density gradients was chosen for this purpose. They reported that most of the carboxylesterase activity found in chicken brain migrated on sucrose gradients as a 4 S peak. This peak was substantially inhibited by Paraoxon, but only partially inhibited by Mipafox. A smaller peak with a 9 S sedimentation coefficient was found to have the Paraoxon-resistant Mipafox-sensitive properties of NTE. The 9 S activity had the same tissue distribution and relative activity as NTE, and it exhibited the same time course of inhibition and subsequent recovery as NTE [16].

In the present paper the sedimentation behaviour on sucrose density gradients of both NTE and NTE-like

Abbreviations used: $\text{iPr}_2\text{P-F}$, di-isopropyl phosphorofluoridate; NTE, neuropathy target esterase; OP, organophosphate ester; OPIDN, organophosphate-induced delayed neuropathy; PAGE, polyacrylamide-gel electrophoresis.

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labelled proteins from chicken brain extracts is examined. Samples containing either active carboxylesterases or [^3H]iPr₂P-F-labelled proteins are first fractionated on identical sucrose density gradients, and the fractions are then assayed for carboxylesterase activity or analysed by SDS/PAGE. By this method it is possible to identify the Paraoxon-resistant Mipafox-sensitive proteins that co-migrate with active NTE.

MATERIALS AND METHODS

Chemicals

[^3H]iPr₂P-F ([1,3- ^3H]di-isopropyl phosphorofluoridate; 3.5 Ci/mmol, Amersham, Arlington Heights, IL, U.S.A.) was prepared for use as described by Williams & Johnson [13], and stored at -25°C . Mipafox (NN'-di-isopropylphosphorodiamidic fluoride) and phenyl valerate were synthesized as previously described [15] in accordance with the methods of Johnson [17]. The purity of Mipafox was monitored by determination of its melting point (60.5°C , pure white crystals), and the purity of phenyl valerate was analysed by n.m.r. and i.r. spectroscopy. Paraoxon (phosphoric acid diethyl 4-nitrophenyl ester; Aldrich Chemical Co., Milwaukee, WI, U.S.A.) was analysed for interfering contaminants by t.l.c. and by determination of its I_{50} for NTE ($600\ \mu\text{M}$) as recommended by Johnson [18]. Stock solutions of Mipafox (0.1 M in 50 mM-Tris/citrate buffer, pH 6.0) and Paraoxon (0.01 M in acetone) were prepared and stored in a desiccator at -25°C . Scintillation-grade Triton X-100 was obtained from Research Products International Corp. (Elk Grove Village, IL, U.S.A.).

Animals

Fertilized White Leghorn chicken eggs were obtained from Donsing Hatcheries (Rio Linda, CA, U.S.A.) and incubated by the Department of Avian Sciences, University of California at Davis. White Leghorn laying hens were obtained from flocks maintained by the Department of Avian Sciences.

Assay for phenyl valerate hydrolysis

Phenyl valerate hydrolysis was assayed colorimetrically by the method of Johnson [17] with the following modifications. Sufficient phenyl valerate (12.5 mg/ml in dimethylformamide) was added to assay tubes to achieve an initial concentration of 1 mM [19]. Absorbance values were determined at 490 nm. This wavelength was determined to be the absorbance maximum with an absorption coefficient equal to $16812\ \text{M}^{-1}\cdot\text{cm}^{-1}$ (model 260 spectrophotometer; Gilford Instruments, Oberlin, OH, U.S.A.). Units of activity are reported as μmol of phenol produced/min.

Protein determinations

Protein was measured by using the method of Lowry *et al.* [20], with bovine albumin (fraction V; Sigma Chemical Co., St. Louis, MO, U.S.A.) as a standard. In order to avoid the interference caused by precipitation of Triton X-100, 0.1 ml of 24% (w/v) SDS was added to samples containing Triton X-100 (final assay volume 1.3 ml) [21,22].

Preparation of microsomal membranes from chick-embryo brains

Brains from day-20 chick-embryos were homogenized for 25 s in 10 ml of ice-cold buffer A (50 mM-Tris/HCl

buffer, pH 7.2 at 21°C , containing 2 mM-EDTA and 2 mM-EGTA)/g of tissue with a Polytron homogenizer on setting 7 (Brinkmann Instruments, Westbury, NY, U.S.A.). Homogenates were centrifuged in an SS34 rotor (Sorvall, Wilmington, DE, U.S.A.) at $1100\ g$ ($r_{\text{av.}}\ 8.26\ \text{cm}$) for 10 min at 4°C . The soluble fractions were centrifuged in a type 60 Ti rotor (Beckman, Palo Alto, CA, U.S.A.) at $100000\ g$ ($r_{\text{av.}}\ 6.15\ \text{cm}$) for 20 min at 4°C . Membrane pellets were resuspended in buffer A and stored in liquid N_2 for future use.

Inhibition and labelling of embryo brain microsomal membranes

Four identical membrane samples containing 19.1 mg of protein each (3.82 mg/ml in buffer A) were treated for 20 min at 37°C with buffer (C), Paraoxon (P), Mipafox (M) or Paraoxon + Mipafox (PM). The final concentrations of Paraoxon and Mipafox were $100\ \mu\text{M}$ and $50\ \mu\text{M}$ respectively. The reaction was stopped by diluting samples with 44 ml each of ice-cold buffer B (50 mM-Tris/HCl buffer, pH 8.0 at 21°C , containing 0.2 mM-EDTA). Samples were centrifuged in a type 60 Ti rotor at $100000\ g$ for 20 min at 4°C . The pellets from each sample were resuspended in 48 ml of buffer B and centrifuged as before. The membrane pellets were resuspended in 8 ml each of buffer B and labelled with $1.75\ \mu\text{M}$ -[^3H]iPr₂P-F at 37°C for 40 min [13]. Labelling was stopped by dilution of samples with 40 ml of ice-cold buffer B, followed by centrifugation as described above. Unbound [^3H]iPr₂P-F was removed by resuspending samples twice in 48 ml of ice-cold buffer B followed by re-centrifugation.

Extraction and concentration of [^3H]iPr₂P-F-labelled proteins

Each of the four [^3H]iPr₂P-F-labelled pellets was treated with 19.1 ml of ice-cold 1 mM-Tris/HCl buffer, pH 7.2 at 21°C , containing 0.2% (w/v) Triton X-100, 0.5 M-NaCl, 1 mM-EDTA and 1 mM-EGTA for 40 min. The concentrations of protein in these samples ranged from 0.58 to 0.66 mg/ml. At the same time 16.6 mg of uninhibited unlabelled microsomal membranes was treated with 19.1 ml of the above extraction solution. These samples were centrifuged in a type 60 Ti rotor at $100000\ g$ for 1 h at 4°C . The soluble fractions were recovered and concentrated overnight at 4°C against 5 litres of 1 mM-Tris/HCl buffer, pH 7.2 at 21°C , containing 0.025% Triton X-100, 1 mM-EDTA and 1 mM-EGTA in a Micro-ProDiCon apparatus with PA-30 dialysis membranes (30000- M_r cut-off; Bio-Molecular Dynamics, Beaverton, OR, U.S.A.). Fractions were concentrated 19–22-fold by this method.

Sucrose density gradients

Each concentrated fraction was run in quadruplicate in 12.6 ml sucrose density gradients of 5–20% (w/v) sucrose in 1 mM-Tris/HCl buffer, pH 7.2 at 21°C , containing 0.025% Triton X-100, 0.5 M-NaCl, 1 mM-EDTA and 1 mM-EGTA (1.1 mg of protein or approx. $225\ \mu\text{l}$ of sample/gradient). A single gradient containing $225\ \mu\text{l}$ of the uninhibited unlabelled sample was run simultaneously with each set of four gradients containing labelled samples. Yeast alcohol dehydrogenase (7.4 S), catalase (11.4 S) and β -galactosidase (16 S) were added to each gradient sample as marker enzymes. The gradients were centrifuged in an SW40 rotor (Beckman) at $154000\ g$

(r_{av} , 11.27 cm) for 20.5 h at 4 °C. Fractions (approx. 0.5 ml each) were collected from each gradient. The gradients with [^3H]iPr₂P-F-labelled samples were analysed by mixing 250 μl of each fraction with 750 μl of water and 10 ml of scintillation fluid containing PCS (Amersham)/xylene (2:1, v/v). The amount of [^3H]iPr₂P-F was then measured in a Beckman LS-3133P scintillation counter (counting efficiency 37.5%). The gradients with uninhibited unlabelled samples were assayed for phenyl valerate-hydrolysing activity in the presence and in the absence of inhibitors. The concentration of sucrose in each fraction was determined by its refractive index. The locations of marker enzymes in all gradients were determined by standard methods [23–25].

The individual fractions from each set of four gradients were combined into four larger fractions on the basis of their profiles of phenyl valerate-hydrolysing activity and the positions of marker enzymes: fraction 1, tubes 1–8 (P, M or PM) or tubes 1–7 (C); fraction 2, tubes 9–12 (P, M or PM) or tubes 8–11 (C); fractions 3, tubes 13–17 (P, M or PM) or tubes 12–16 (C); fraction 4, tubes 18–25 (P, M or PM) or tubes 17–25 (C). These samples were prepared for electrophoresis by dialysis at 4 °C against 7 litres of 1 mM-Tris/HCl buffer, pH 7.2 at 21 °C, containing 0.025 % Triton X-100, 1 mM-EDTA and 1 mM-EGTA. Samples were then concentrated 24–64 fold to a final volume of approx. 300 μl against 5 litres of the above buffer at 4 °C by using the Micro-ProDiCon system.

SDS/PAGE of concentrated sucrose-density-gradient fractions

Concentrated fractions 1, 2, 3 and 4 from each buffer- or inhibitor-treated sample were subjected to SDS/PAGE on 3 mm-thick 7.5 % (w/v) polyacrylamide gels according to the Laemmli method [26]. Electrophoresis samples were prepared by adding 100 μl of each concentrated fraction to an equal volume of 2-fold-concentrated sample buffer [0.125 M-Tris/HCl buffer, pH 6.8 at 21 °C, containing 4 % (w/v) SDS, 20 % (v/v) glycerol, 10 % (v/v) 2-mercaptoethanol and 0.002 % Bromophenol Blue] followed by heating in a boiling-water bath for 3 min. Myosin (M_r 200 000), β -galactosidase (M_r 116 000), bovine serum albumin (M_r 66 200) and fructose biphosphate aldolase (M_r 40 000) were used as M_r markers. Gels were stained with 0.025 % Coomassie Blue R250 in 25 % (v/v) propan-2-ol/10 % (v/v) acetic acid [27] and destained with 45 % (v/v) methanol/10 % (v/v) acetic acid. The locations and quantities of [^3H]iPr₂P-F-labelled proteins in the gels were determined both by scintillation counting of radioactivity of gel slices and by fluorography.

For scintillation counting of radioactivity, sample lanes were cut into 1 mm slices. Two adjacent slices were placed in each glass scintillation vial containing 1 ml of 90 % (v/v) Protosol (NEN Research Products, Boston, MA, U.S.A.) and gently shaken overnight. Then 10 ml of 27 mM-2,5-diphenyloxazole/0.22 mM-1,4-bis-(5-phenyloxazol-2-yl)benzene in toluene was added to each vial. The amount of [^3H]iPr₂P-F per vial was measured twice for 2 min (counting efficiency 45 %). In all cases more than 65 % of the radioactivity loaded was recovered. Reported values are the average of these two determinations.

For fluorography, samples were electrophoresed on 1.5 mm-thick gels, and stained with Coomassie Blue

R250 as indicated above. These gels were treated with En³Hance (NEN Research Products) according to the manufacturer's instructions and dried on to filter paper. Kodak X-Omat X-AR5 film (Rochester, NY, U.S.A.) was exposed to the gels at –70 °C for 10 weeks before development of the film.

RESULTS

For these experiments, microsomal membranes were prepared from chick-embryo brain membranes. The yield of NTE activity in the microsomal fraction was 47 % (2.8-fold purification) and the total recovery of NTE was 98 %. A portion of this fraction was then treated with buffer, Paraoxon, Mipaflox or Paraoxon + Mipaflox before labelling with [^3H]iPr₂P-F. A high-ionic-strength Triton X-100 solution was used to extract either phenyl valerate-hydrolysing esterases or [^3H]iPr₂P-F-labelled proteins from the microsomes. The yield of NTE activity in the soluble fraction was typically greater than 65 %. These extracts were then fractionated by sucrose-density-gradient centrifugation. The gradients were compared in order to identify those regions in which Paraoxon-resistant Paraoxon + Mipaflox-sensitive [^3H]iPr₂P-F label co-migrated with NTE activity. Fractions isolated from the sucrose gradients were then analysed by SDS/PAGE to identify specific NTE-like [^3H]iPr₂P-F-labelled proteins that had co-migrated with NTE.

Comparison of NTE activity and NTE-like [^3H]iPr₂P-F label on sucrose density gradients

Fig. 1(a) illustrates the profiles of total and inhibitor-resistant phenyl valerate-hydrolysing activities found on a gradient loaded with an uninhibited unlabelled extract. Most of the phenyl valerate-hydrolysing activity was located in a large peak with a low sedimentation coefficient (fractions 1–11) that did not contain any NTE activity. A smaller peak of phenyl valerate-hydrolysing activity was also present that extended into the high-density region of the gradient (fractions 12–23). NTE activity was spread fairly evenly through this region. This range of NTE activity was much broader than previously reported [15,16].

The sucrose density gradients containing [^3H]iPr₂P-F-labelled proteins were assayed to determine their distribution of [^3H]iPr₂P-F (Fig. 1b). The low-density peak (fractions 1–11) contained no Paraoxon-resistant Mipaflox-sensitive (NTE-like) label. The high-density region (fractions 12–25) contained a broad peak of NTE-like label with a distribution that matched that of the NTE activity (Fig. 1a). This supports the earlier work of Wilson *et al.* [16] in which the NTE activity and NTE-like label from whole brain extracts were found to co-migrate on sucrose density gradients. Fractions 13–15 contained a peak of Mipaflox-resistant but Paraoxon-sensitive label that had no corresponding peak of Mipaflox-resistant but Paraoxon-sensitive activity.

Identification of NTE-like [^3H]iPr₂P-F-labelled proteins isolated from sucrose density gradients

The next step was to determine by SDS/PAGE the relative mobilities of those NTE-like [^3H]iPr₂P-F-labelled proteins that matched the sucrose-density-gradient distribution of the NTE activity. Individual fractions from each set of gradients (buffer-, Paraoxon-, Mipaflox-

Fig. 1. Comparison of carboxylesterases and $[^3\text{H}]\text{iPr}_2\text{P-F}$ -labelled proteins from chick-embryo brain membranes by sucrose-density-gradient centrifugation

(a) Membrane-bound carboxylesterases were isolated from chick-embryo brain membranes by extraction with a high-ionic-strength Triton X-100 solution followed by centrifugation. Solubilized proteins were concentrated and then fractionated on a 5–20% (w/v) sucrose density gradient (----). A portion of each fraction was treated with buffer (○), Paraoxon (●), Mipafox (▲) or Paraoxon + Mipafox (△) and assayed for remaining phenyl valerate-hydrolysing activity (1 unit is defined as 1 μmol of phenol produced/ml). NTE is found in the region where Paraoxon-inhibited values are greater than Paraoxon + Mipafox-inhibited values (fractions 12–23). The positions of standard proteins of known sedimentation coefficient (S) are shown. (b) Four identical chick-embryo brain membrane fractions were treated with buffer (○), Paraoxon (●), Mipafox (▲) or Paraoxon + Mipafox (△) and labelled with $[^3\text{H}]\text{iPr}_2\text{P-F}$. Labelled proteins were extracted and isolated as above and fractionated on 5–20% (w/v) sucrose density gradients. The profiles of $[^3\text{H}]\text{iPr}_2\text{P-F}$ label from each of the four types of gradients have been plotted together. Individual fractions were combined into fractions 1, 2, 3 and 4 as indicated by the labelled bars, and subsequently analysed by SDS/PAGE. The concentrations of Paraoxon and Mipafox during labelling with 1.75 μM - $[^3\text{H}]\text{iPr}_2\text{P-F}$ were estimated to be less than 0.024 μM and 0.012 μM respectively. The values for the Mipafox-treated gradient have been divided by 0.65 in order to compensate for a lower amount of protein loaded on this gradient.

or Paraoxon + Mipafox-treated) were combined into four fractions as described in the Materials and methods section (see Fig. 1). These 16 combined fractions were concentrated and analysed by SDS/PAGE. The distribution of $[^3\text{H}]\text{iPr}_2\text{P-F}$ in each lane was determined both by scintillation counting of the radioactivity of gel slices and by fluorography. Fractions 3 and 4 are plotted in

Figs. 2 and 3. The values plotted in panel (a) of each Figure represent the total radioactivity (c.p.m.) detected per μg of sample loaded. Panel (b) in Figs. 2 and 3 is a plot of the distribution of NTE-like label in that fraction.

As shown in Fig. 1, fractions 3 and 4 both contained NTE-like labelled proteins. Analysis of fraction 3 by SDS/PAGE (Fig. 2) identified three bands of NTE-like

Fig. 2. SDS/PAGE of sucrose-density-gradient fraction 3 (see Fig. 1)

(a) Fraction 3 from the buffer-treated (○), Paraoxon-treated (●) and Paraoxon + Mipafox-treated (△) [³H]iPr₂P-F-labelled sucrose-density-gradient samples was analysed by SDS/PAGE. The distribution of [³H]iPr₂P-F label was quantified by gel slicing and scintillation counting of radioactivity. Slices containing the dye front have been positioned together to provide the best alignment of peaks. Amounts of protein loaded in each lane are: buffer-treated, 23 μg; Paraoxon-inhibited, 25 μg; Paraoxon + Mipafox-inhibited, 42 μg. Results for the Mipafox-inhibited fraction are not shown. The positions of *M_r* standards are shown ($10^{-3} \times M_r$). (b) The location of NTE-like labelling has been plotted by subtracting the Paraoxon + Mipafox-inhibited values in (a) from the Paraoxon-inhibited values. This difference has been plotted on the same scale in Fig. 3.

label at *M_r* 161 000 (slices 9–13), 108 000 (slices 15–18) and 78 000 (slices 20–24). In these three regions the Paraoxon and Paraoxon + Mipafox curves were statistically different from each other (Mann–Whitney test [28], $P \leq 0.005$, 0.05 and 0.005 respectively). Comparison of gel-slicing data with a fluorogram of these samples indicated that the 108 000-*M_r* band was a composite of 116 500-*M_r* and 103 000-*M_r* bands (results not shown). The 161 000-*M_r* and 78 000-*M_r* bands were resistant to Paraoxon inhibition and their labelling was completely eliminated by Paraoxon + Mipafox inhibition. The addition of Paraoxon + Mipafox to the proteins in the 108 000-*M_r* peak caused only a partial decrease in label.

Fraction 4 (Fig. 3) contained one major band of NTE-like label with an *M_r* of 161 000 (slices 9–13) and two

minor bands at *M_r* 116 500 (slices 14–16) and 103 000 (slices 16–19). The results of the Mann–Whitney test for these three regions were $P \leq 0.005$, 0.05 and 0.025 respectively. These bands were therefore found in both fractions 3 and 4 whereas the 78 000-*M_r* band was found only in fraction 3. The 161 000-*M_r* protein exhibited the strongest labelling in both fractions 3 and 4.

The SDS/PAGE profiles of fractions 1 and 2 confirmed that these fractions contained no NTE-like labelled proteins although they contained the majority of labelled proteins. A 61 000-*M_r* protein identified in fraction 1 deserves special attention. [³H]iPr₂P-F labelling of this band was only partially inhibited by Paraoxon pretreatment of membranes, and only a slight additional inhibition was seen with Paraoxon + Mipafox pretreat-

Fig. 3. SDS/PAGE of sucrose-density-gradient fraction 4 (see Fig. 1)

Details are similar to those described in Fig. 2 legend. Amounts of protein loaded in each lane are: buffer-treated, 15 μg ; Paraoxon-inhibited, 27 μg ; Paraoxon + Mipafox-inhibited, 14 μg .

ment (Table 1). However, pretreatment with Mipafox alone almost completely inhibited the labelling of this band. This band is therefore Paraoxon-resistant and Mipafox-sensitive but is not NTE-like (Paraoxon-resistant and Paraoxon + Mipafox-sensitive).

As shown in Fig. 1, NTE was found across a much broader region of the gradients than previously reported [15]. This was probably due to aggregation of NTE during the concentration step rather than to heterogeneity or to proteolysis of NTE. The concentration step, before sucrose-density-gradient centrifugation, resulted in a 20-fold decrease in volume and a 10-fold increase in the recoverable protein concentration, while the detergent concentration only increased 4-fold. Although these conditions can be expected to lead to aggregation, the recovery of NTE activity during concentration was much greater when the increase in detergent concentration was minimized. In addition, when NTE was extracted at higher protein concentrations, which eliminated the need for a concentration step, the NTE activity was located in

Table 1. SDS/PAGE analysis of the 61000- M_r band from sucrose-density-gradient fraction 1 (see Fig. 1)

Each fraction 1 from the buffer-, Paraoxon-, Mipafox- and Paraoxon + Mipafox-treated $[^3\text{H}]\text{iPr}_2\text{P-F}$ -labelled sucrose density gradients was analysed by SDS/PAGE. The distribution of $[^3\text{H}]\text{iPr}_2\text{P-F}$ was quantified by gel slicing and scintillation counting of radioactivity. The $[^3\text{H}]\text{iPr}_2\text{P-F}$ radioactivity is presented for a peak of label at M_r 61000.

Inhibitor treatment	$[^3\text{H}]\text{iPr}_2\text{P-F}$ (c.p.m.)	Protein loaded (μg)	Specific radio-activity (c.p.m./ μg)	Specific radio-activity (%)
Control	403	132	3.05	100
Paraoxon	322	199	1.62	53.1
Mipafox	87	206	0.422	13.8
Paraoxon + Mipafox	314	256	1.23	40.3

Fig. 4. Analysis of chick-embryo brain membrane carboxylesterases by sucrose-density-gradient centrifugation

Membrane-bound proteins were extracted from chick-embryo brain membranes (5 mg of protein/ml) in ice-cold 1 mM-Tris/HCl buffer, pH 7.2 at 21 °C, containing 0.3% Triton X-100, 0.5 M-NaCl, 1 mM-EDTA and 1 mM-EGTA for 30 min. Insoluble material was removed by centrifugation and soluble proteins were fractionated on a 5–20% (w/v) sucrose gradient (----) (150000 *g* for 20.5 h). Curves represent A_{280} (.....) and residual phenyl valerate-hydrolysing activity in each fraction after incubation with buffer (○), Paraoxon (●) or Paraoxon + Mipaflox (△). NTE is found in the region where Paraoxon-inhibited values are greater than Paraoxon + Mipaflox-inhibited values. The positions of proteins with known sedimentation coefficients (S) are shown.

a fairly distinct 9.6 S peak (Fig. 4). Proteolysis would be expected to result in either the loss of NTE activity or a decrease in the sedimentation coefficient of NTE rather than the increase seen in these experiments.

DISCUSSION

In these experiments, microsomal membranes were prepared from chick-embryo brains, treated with neurotoxic and/or non-neurotoxic organophosphates, and labelled with [^3H]iPr₂P-F. Labelled proteins were then extracted with a high-ionic-strength Triton X-100 solution and fractionated on sucrose density gradients. The identities of proteins in different regions of the gradients were then determined by SDS/PAGE. In this way the sedimentation behaviour of specific NTE-like [^3H]iPr₂P-F-labelled proteins was compared with the sedimentation behaviour of active NTE. Four bands of NTE-like [^3H]iPr₂P-F-labelled protein were identified with apparent M_r values of 161000, 116500, 103000 and 78000. With the exception of the 103000- M_r protein, these proteins have all been identified in microsomal membranes isolated from both hen and embryo brains (results not shown). Since the 103000- M_r protein appears to be embryo-specific it is not considered a suitable candidate for NTE or for the target site for initiation of OPIDN. Fluorographic analysis of hen brain microsomal membranes indicates that the 78000- M_r protein is actually a 79000- M_r and 77000- M_r doublet.

Only the 161000- M_r , 116500- M_r and 103000- M_r proteins were found to co-migrate with NTE in fractions 3 and 4 of the sucrose density gradients. The greatest amount of NTE-like label found in both fractions was associated with the 161000- M_r protein. This protein undoubtedly corresponds to the 155000- M_r region of Paraoxon-resistant label first identified by Williams & Johnson [13], and subsequently identified as a 160000-

M_r protein by Carrington & Abou-Donia [14]. The 116500- M_r protein appears to correspond to the 115000- M_r protein previously identified in low abundance [14]. The 78000- M_r protein, which is the second most abundant NTE-like [^3H]iPr₂P-F-labelled protein found in hen brain microsomal membranes ([14]; unpublished work performed in our laboratory), was found only in fraction 3. This result is consistent with the observation by Williams & Johnson that most of the label in a region described as Band 2 (M_r 61000–125000, designated as M_r 92000) does not share the same 4-nitrophenyl di-n-pentylphosphinate-sensitivity and forebrain and hind-brain distribution as NTE [13].

A 61000- M_r protein, which was resistant to Paraoxon inhibition and to Paraoxon + Mipaflox inhibition, was found in fraction 1. The existence of this protein has also been confirmed in microsomal membranes isolated from hen brains (results not shown). If this protein possesses carboxylesterase activity, it would be identified by the NTE assay as an 'inhibitor-resistant esterase activity (IRE)' and not as NTE [2]. However, the labelling of this protein is almost completely inhibited by treatment with Mipaflox alone. This protein therefore has the Paraoxon-resistant Mipaflox-sensitive characteristics expected of the target site for initiation of OPIDN despite the fact that it would not be classified as NTE by the NTE assay. Paraoxon appears to block reversibly the inhibitory effects of Mipaflox on this protein. This result is supported by evidence that Paraoxon reversibly inhibits the active sites of some carboxylesterases [29]. Since a direct causal link has not been established between OP modification of NTE and the onset of OPIDN, this protein should be considered a possible target site for the initiation of OPIDN.

In these experiments, brains were isolated from 19-day chick embryos rather than adult hens. Embryo brains were a more readily available tissue source, and several

criteria indicate they were a suitable tissue for this study. First, the specific activities of NTE in hen and 19-day-embryo brains were virtually identical. Secondly, high-ionic-strength Triton X-100 extracts of embryo brain microsomal membranes had the same profile of phenyl valerate-hydrolysing activity on sucrose density gradients as that seen previously with hen brain membranes [15]. Thirdly, with the exception of the 103 000- M_r protein, each of the NTE-like [^3H]iPr₂P-F-labelled proteins identified in this study have been identified in adult hen brains [14]. Finally, the method of analysis employed in these experiments is independent of the relative proportions of [^3H]iPr₂P-F-labelled proteins found in either adult or embryo brain. The absolute quantities of these proteins do not affect their sedimentation on sucrose density gradients or migration during SDS/PAGE.

A region of Mipafox-resistant and Paraoxon-sensitive labelling was found on the sucrose density gradients that did not correspond to a similar region of carboxylesterase activity (Fig. 3, fractions 13–15). This illustrates the point that Paraoxon- or Mipafox-sensitive labelling of a protein does not indicate that the protein has carboxylesterase activity. It is important, therefore, that the correlation between NTE activity and [^3H]iPr₂P-F-labelling also be investigated on the basis of characteristics, such as sedimentation velocity on sucrose density gradients, that are unrelated to differential inhibition by OPs. Failure to do so could lead to the inappropriate identification of a protein that does not possess carboxylesterase activity as NTE.

In summary, the results show that a 161 000- M_r protein and a 116 500- M_r protein are indistinguishable from NTE on the basis of their sedimentation on sucrose density gradients. This is the first time that the correlation between NTE and a set of [^3H]iPr₂P-F-labelled proteins has been made by use of a method that leaves the NTE activity intact and is not solely dependent on differential inhibition by OPs. In addition, a 61 000- M_r protein was identified that is distinct from NTE, but also has the necessary characteristics to be considered a possible target site for the initiation of OPIDN. Conclusive identification of the OPIDN target site will most likely not be possible until these proteins have been purified and characterized.

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