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CHARACTERIZATION OF HIGH-AFFINITY BINDING SITES FOR DIISOPROPYLFLUOROPHOSPHATE (DFP) FROM CHICKEN SPINAL CORD MEMBRANES*

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Abstract—The delayed neurotoxic organophosphate [^{3}H]diisopropylfluorophosphate ([^{3}H]DFP) binds with high affinity to membrane-bound proteins from the chicken spinal cord. The DFP binding proteins were solubilized from membrane preparations, using a detergent (CHAPS). The protein(s) sites that labeled with a low concentration of [^{3}H]DFP, e.g. 10^{-10}–10^{-9} M, were defined as the high-affinity binding sites. The density (or concentration) of the high-affinity binding sites in protein(s) was determined by the difference between total and non-specific binding. The high-affinity binding sites were saturable, and the maximal amount of binding sites was estimated at 400 fmol/mg protein. [^{3}H]-DFP binding to solubilized proteins was not completely reversible. Concentration-dependent curves suggested that the [^{3}H]DFP binding sites differ from the active sites of acetylcholinesterase, butyrylcholinesterase, and neuropathy target esterase, as well as from muscarinic acetylcholine receptors. The amount of DFP binding sites after a neurotoxic dose of tri-*o*-cresyl phosphate (TOCP) decreased markedly in membrane preparations from the chicken spinal cord. These results indicate that a TOCP metabolite(s) interacts with the DFP binding sites *in vivo*. Gel filtration chromatography of the solubilized membranes indicated at least two major high-affinity DFP binding proteins with apparent molecular weights of 300 and 110 kDa. The DFP binding sites corresponding to the 110 kDa protein were insensitive to eserine, a potent anti-cholinesterase agent.

Key words: diisopropylfluorophosphate; organophosphate; membrane-bound protein; delayed neurotoxicity; chicken

Some organophosphorus compounds such as TOCP‡, leptophos, triphenyl phosphite and DFP produce unique delayed neurotoxic effects in humans and other animals, including chickens [1, 2]. The neurotoxicity, which appears 1–2 weeks after acute exposure to neurotoxic organophosphorus compounds, is characterized by muscle weakness, ataxia and paralysis of the hind limbs. It has been proposed that inhibition of NTE is involved in the initiating stage of OPIDN [3]. Little is known, however, about the physiological role and the biochemical properties of NTE. Carrington [4] has pointed out that *in vitro* NTE assays may not be sensitive enough to identify the actual target, which could be present in much smaller amounts than that assayed as NTE.

Most investigations of DFP labeling proteins (or enzymes) have been performed at the micromolar level (around 10^{-6} M) *in vitro*, conditions under which DFP covalently binds to many proteins [5–7]. Modifications in the brain muscarinic receptors of animals chronically treated with DFP have been described [8–10]. However, little information is available about proteins labeled with low concentrations of [^{3}H]DFP, such as at the nanomolar range (10^{-10}–10^{-9} M). In preliminary experiments, we showed that [^{3}H]DFP binding sites (proteins), which are labeled at approximately 10^{-9} M, are present on membranes from the brain and spinal cord of chickens [11]. The presence of the "high-affinity" DFP binding site(s) was confirmed by means of a common radioligand binding assay.

We investigated the pharmacological and biochemical characteristics of the [^{3}H]DFP binding sites (proteins) in solubilized membranes. Additionally, the effect of TOCP *in vivo* on the number of high-affinity binding sites in membranes from chicken spinal cord was studied. The results indicated that the DFP binding sites interact with TOCP metabolite(s) *in vivo* and that they may be the target sites implicated in TOCP-induced delayed neuropathy.

MATERIALS AND METHODS

Chemicals. [1,3-^{3}H]Diisopropylfluorophosphate (sp. act. 111 or 148 GBq/mmol) was purchased from DuPont/NEN Research Products (U.S.A.).

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‡ Abbreviations: TOCP, tri-*o*-cresyl phosphate; DFP, diisopropylfluorophosphate; 2-PAM, pyridine-2-aldoxime methochloride; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; OPIDN, organophosphate-induced delayed neurotoxicity; AChE, acetylcholinesterase; BuChE, butyrylcholinesterase; and NTE, neuropathy target esterase.

Unlabeled DFP was obtained from Wako Pure Chemical Industries (Osaka, Japan). Paraoxon was obtained from the Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Tri-*o*-tolyl phosphate (TOTP or TOCP) was obtained from the Eastman Kodak Co. (Rochester, NY, U.S.A.). Eserine was obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). N, N'-Diisopropylphosphorodiamidic fluoride (mipafox) was synthesized by Dr. S. Sayama (Chemistry Department, Fukushima Medical College, Japan). All other chemicals and reagents used were of the highest purity commercially available.

Animals and treatment. Laying hens (*Gallus gallus domesticus*), aged 23 months and weighing 1.4 to 1.8 kg, were used. For intoxication studies, an i.v. solution of TOCP (20 mg/mL) was prepared by dissolution in a mixture of DMSO, Tween 80, and physiological saline (1:1:3). This formulation was injected into a wing vein at a dose of 2 mL/kg of body weight, while control chickens received the vehicle alone at 2 mL/kg.

Membrane preparation. Spinal cords from the chickens were disrupted in 10 vol. of ice-cold HEPES buffer (50 mM, pH 7.4) using a Teflon–glass homogenizer. The homogenates were then clarified by centrifugation at 1000 *g* for 10 min at 4°. The supernatants were combined and centrifuged again at 50,000 *g* for 10 min at 4°. The pellet was resuspended in HEPES buffer, and sedimented by centrifugation once again at 50,000 *g* for 10 min. The washed pellet was suspended in 10 vol. of HEPES buffer and dispersed with a Polytron homogenizer. This preparation (protein concentration of 4–6 mg/mL) was named intact membranes and stored at −80°.

Solubilization procedure. Solubilization proceeded essentially by the method of Pope and Padilla [7]. Briefly, the intact membranes (4–6 mg protein/mL) were diluted with 10 mM sodium phosphate buffer (pH 7.4), containing 1% CHAPS. The protein-detergent suspensions (2–3 mg protein/mL) were mixed gently by means of a magnetic stirrer at 0° for 60 min followed by centrifugation at 100,000 *g* for 60 min. Aliquots (400–800 μg protein/mL) of solubilized protein (100,000 *g* supernatant) were assayed for binding.

Radioligand binding. To measure the high-affinity binding of [^{3}H]DFP, 50 μL [^{3}H]DFP (final concentration of 0.2 to 15 nmol/L) and 100 μL of the appropriate unlabeled displacer or 10 mM phosphate buffer, pH 7.4, were incubated with 100 μL (50–300 μg protein/100 μL) of membrane preparations at 37°, usually for 60 min. The incubation was terminated by rapid vacuum filtration through a glass-fiber filter (Whatman GF/B) for intact membranes or a nitrocellulose filter (0.45 μm, Advantec, Japan) for solubilized membranes, followed by three 3-mL washes with ice-cold buffer (140 mM NaCl, 10 mM Tris–HCl, pH 7.4). The nitrocellulose filters were soaked in 0.1% polyethylenimine for 1–24 hr before use. Radioactivity trapped on the filters was measured in 5 mL of Aquasol-2 (DuPont) by liquid scintillation counting (Aloka, model 3500). High-affinity binding was defined as the amount of radioactivity bound after subtracting the non-specific binding assayed in the presence of unlabeled DFP (10 μM).

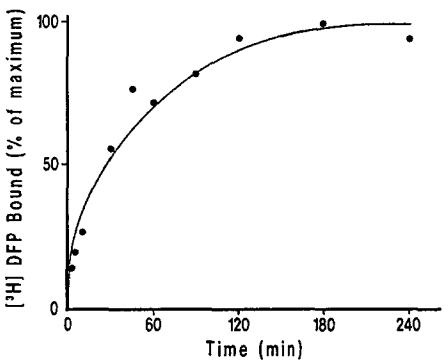

Fig. 1. Time-course of high-affinity [^{3}H]DFP binding to solubilized membranes. Binding was measured as described under Materials and Methods. High-affinity binding was defined as the difference between binding in the presence (non-specific) and absence (total) of 10 μM unlabeled DFP. Data points are the means of triplicates in a single experiment. Triplicate experiments gave similar results. The maximal value was 401 fmol/mg protein.

Gel filtration chromatography. For gel filtration studies, the solubilized fractions were concentrated to 1–4 mg protein/mL by ultrafiltration through a USY-1 membrane (molecular mass cutoff of 10 kDa, Advantec, Japan). Five hundred microliters of the concentrated solubilized fraction was applied to a column containing Superose 6HR 10/30 connected to an FPLC System, all obtained from Pharmacia. The column was equilibrated and calibrated with proteins of known molecular weight (blue dextran, 2000 kDa; apoferritin, 443 kDa; β-amylase, 200 kDa; alcohol dehydrogenase, 150 kDa; carbonic anhydrase, 29 kDa) using 10 mM sodium phosphate buffer, pH 7.4, containing 0.3% CHAPS. The sample was eluted with the same buffer at 30 mL/hr, and 1-mL fractions were collected. Each fraction was assayed for [^{3}H]DFP binding to solubilized proteins in duplicate as described above. The absorbance of each fraction at 280 nm was also determined.

Assay of AChE activity. AChE (EC 3.1.1.7) activity in the gel-filtered fractions was assayed by the method of Ellman *et al.* [12].

Protein assay. Protein concentrations were determined by the method of Lowry *et al.* [13], using bovine serum albumin, fraction V, as the standard.

Data analysis. Equilibrium binding data and inhibition curves were analyzed by non-linear least squares regression analysis, using the program LIGAND [14].

Statistics. For comparisons involving several groups, one-way analysis of variance followed by Duncan's multiple range test was performed.

RESULTS

Time-course of [^{3}H]*DFP binding to solubilized membranes.* The binding of [^{3}H]DFP to solubilized membranes was evaluated by incubating 5 nM [^{3}H]-

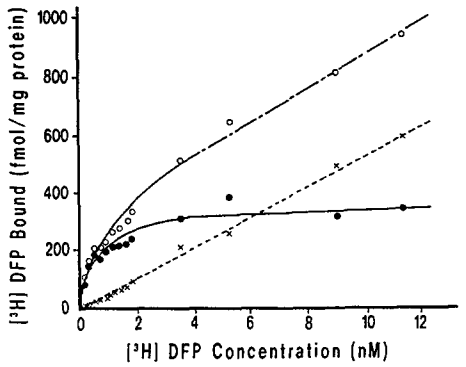

Fig. 2. Concentration-dependent curve for the binding of [³H]DFP (0.2 to 12 nM) to solubilized membranes from one typical experiment. Each point represents the mean value of triplicate assays. High-affinity binding (●-●) was obtained from the difference between total (○-○) and non-specific binding (x-x). Triplicate experiments gave similar results.

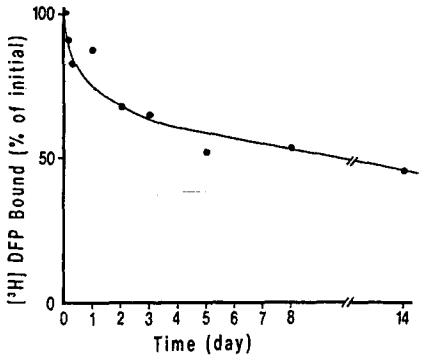

Fig. 3. Time-course of the dissociation of bound [³H]DFP from solubilized membranes. The solubilized membranes were labeled with [³H]DFP (5 nM) for 24 hr at 37°, and then 100 μM unlabeled DFP was added. At various times, aliquots were filtered, and the remaining high-affinity [³H]-DFP binding was determined. Each point represents the mean values of duplicate assays, and the data are representative of results obtained in three separate experiments. Initial values for [³H]DFP bound were 395 ± 36 fmol/mg protein (means ± SD, N = 4).

DFP with soluble preparations for various intervals (Fig. 1). At 37°, the binding reached a plateau at 90–120 min. Half-maximal binding was apparent between 10 and 30 min.

Concentration-dependence of [³H]DFP binding to solubilized membranes. [³H]DFP binding to solubilized membranes was examined over a wide range of concentrations (0.2 to 12 nM) after a 60-min incubation. The results of a typical experiment are shown in Fig. 2. Although total and non-specific binding increased in a concentration-dependent manner, high-affinity binding reached a plateau at concentrations of 5–10 nM. Half-maximal high-affinity binding was apparent at 1 nM, and the value was about 200 fmol/mg protein.

Dissociation of bound [³H]DFP from solubilized membranes. To examine the reversibility of the binding reaction, the solubilized membranes were incubated with 5 nM [³H]DFP for 24 hr at 37°, and then 100 μM DFP was added to prevent ligand reassociation. Although 35% of the [³H]DFP was dissociated from the membranes 3 hr after adding unlabeled DFP, the DFP-binding site complex did not completely dissociate within 13–14 days (Fig. 3).

Effects of various compounds on [³H]DFP binding to membrane preparations. To investigate the pharmacological profile of [³H]DFP binding to intact or solubilized membranes, inhibition studies were performed using various compounds including anti-cholinesterase (anti-ChE) agents. However, the inhibition of [³H]DFP binding did not conform to a simple, reversible bimolecular reaction that obeys the mass action law. Due to this difficulty, the IC_{50} values (Figs. 4 and 5) are the concentrations required to inhibit 50% of the high-affinity [³H]DFP binding. The results of the inhibition studies using intact membranes are shown in Fig. 4. The most potent agent examined was DFP itself with an IC_{50} value of about 10 nM. Among the organophosphorus compounds, paraoxon was a potent inhibitor, although the effect was about 10-fold lower than

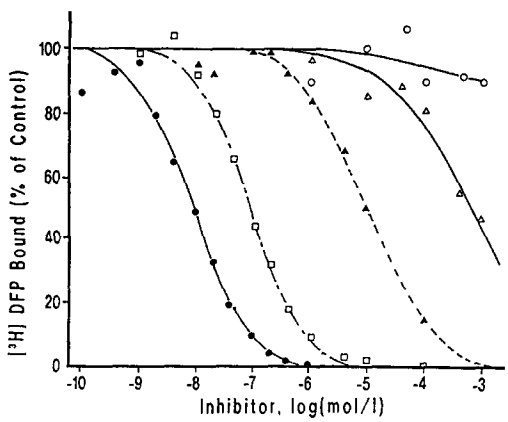

Fig. 4. Inhibition of 5 nM [³H]DFP binding to intact membranes by unlabeled DFP (●), paraoxon (□), mipafox (▲), carbachol (△) and atropine (○). Data are plotted as a percentage of high-affinity binding. Control values for [³H]DFP bound were 111 ± 10 fmol/mg protein (means ± SD, N = 4). Each point represents the mean value of duplicate assays, and the data are representative of results obtained in three separate experiments. The IC_{50} values were determined by non-linear least squares analysis. Approximate IC_{50} values: DFP, 10 nM; paraoxon, 100 nM; mipafox, 10 μM; carbachol, >100 μM; and atropine, >1 mM.

that of DFP. The delayed neurotoxic compound mipafox also inhibited [³H]DFP binding, but it was about 100 times less potent than paraoxon. In addition, carbachol and atropine exhibited very weak or no effects on [³H]DFP binding, thus

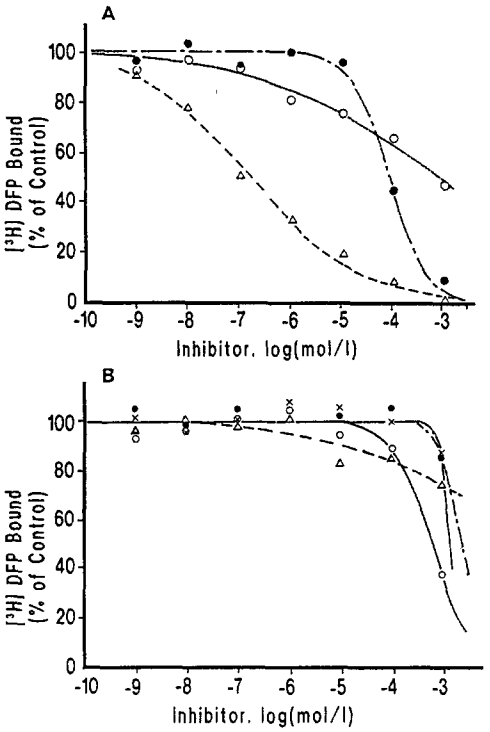

Fig. 5. Inhibition of 5 nM [^{3}H]DFP binding to solubilized membranes by (A) eserine (○), mipafox (●) and paraoxon (△) and (B) *p*-nitophenyl valerate (○), acetylthiocholine (●), 2-PAM (△), and butyrylcholine (×). Data are plotted as a percentage of the high-affinity binding. Control values for [^{3}H]DFP bound are 420 ± 33 fmol/mg protein (means ± SD, N = 4). Each point represents the mean value of duplicate assays, and the data are representative of results obtained in three separate experiments. Approximate IC_{50} values: paraoxon, 100 nM; mipafox, 100 μM; eserine, >100 μM; *p*-nitrophenyl valerate, >100 μM; acetylthiocholine, >1 mM; 2-PAM, >1 mM; and butyrylcholine, >1 mM.

excluding the possibility of [^{3}H]DFP binding sites to a class of muscarinic acetylcholine receptors.

The results of the inhibition studies using solubilized membranes are shown in Fig. 5. Of the three anti-ChE agents tested, paraoxon was the most potent inhibitor with an IC_{50} value of 100 nM. In contrast, mipafox was only 1% as potent as paraoxon, and eserine, a carbamate compound, displayed a very weak inhibitory effect upon these binding sites (Fig. 5A). As shown in Fig. 5B, acetylthiocholine, butyrylcholine and *p*-nitrophenyl valerate, which are substrates of AChE, BuChE and carboxylesterases (aliesterases), respectively, exerted little effect on the [^{3}H]DFP binding sites. The AChE reactivator 2-PAM was also a weak inhibitor.

Effect of TOCP in vivo *on the* [^{3}H]*DFP binding sites.* The number of the high-affinity [^{3}H]DFP binding sites on membranes from chicken spinal cord exposed to a neurotoxic dose of TOCP [15] *in vivo* for various periods before labeling with [^{3}H]-DFP *in vitro* is shown in Table 1. TOCP decreased [^{3}H]DFP binding to the spinal cord by 70–87% during the first day, but the binding recovered by 45–62% over 2–8 days. Though the [^{3}H]DFP binding under assay condition 2 (final [^{3}H]DFP concentration, 15 nM) was about three times greater than that under assay condition 1 (final [^{3}H]DFP concentration, 0.2 nM), an inhibitory effect of TOCP *in vivo* upon the density of [^{3}H]DFP binding sites was demonstrated under both assay conditions. As shown in Fig. 2 the high-affinity binding reached plateau values at concentrations of 5–10 nM DFP. Thus, we checked the binding profile under the two different assay conditions.

Gel filtration. To determine the approximate molecular weight of the [^{3}H]DFP binding protein(s) and the elution profile of AChE, solubilized membranes were layered onto a column containing Superose 6HR, followed by elution in the presence of 0.3% CHAPS. The fractions were assayed for AChE activity and [^{3}H]DFP binding activity. As shown in Fig. 6A, AChE activity eluted in fractions 9–14. [^{3}H]DFP binding activity eluted in two major peaks: one (fraction 12) had an estimated molecular weight of 300 kDa, whereas the other, larger peak (fractions 14 and 15) eluted at about 110 kDa. The high-affinity binding proteins were concentrated in the restricted fractions [Fig. 6B, eserine (−)]. When the binding was assayed in each fraction after an incubation with 10 μM eserine to inhibit AChE activity, the large peak (fractions 14 and 15) was resistant to eserine. In contrast, another small peak (fraction 12) was sensitive to eserine, although weak binding activity remained [Fig. 6B, eserine (+)].

DISCUSSION

Our preliminary study with membrane fractions from nerve tissues suggested the presence of high-affinity [^{3}H]DFP binding sites (or proteins) that are labeled with approximately 10^{-9} M DFP [11]. To examine the biochemical and pharmacological nature of these protein sites, the membrane fraction was solubilized using the detergent CHAPS. The solubilization of hen brain membrane-bound NTE with CHAPS has been described by Pope and Padilla [7]. The high-affinity DFP binding sites were also obtained from chicken spinal cord fractions following solubilization with CHAPS (Figs. 1 and 2). These results suggest that CHAPS is a suitable detergent for solubilizing the membrane-bound high-affinity DFP binding proteins. Equilibrium binding with 5 nM [^{3}H]DFP was established after 1–2 hr, and the high-affinity binding sites were saturable at ligand concentrations of up to 12 nM. In contrast, complete reversibility of the [^{3}H]DFP binding to solubilized membranes was not evident in the dissociation experiment (Fig. 3). This suggests that DFP-binding site interaction is not a simple bimolecular reaction. Thus, a Scatchard transformation [16], which is commonly used to quantify reversible ligand-receptor interactions, was not undertaken.

Carbachol, a muscarinic agonist, and atropine, a muscarinic antagonist, had very low or no affinity for [^{3}H]DFP binding sites (Fig. 4). There are several reports of modifications in the brain muscarinic receptors of animals chronically treated with DFP [8–10], which implies that the [^{3}H]DFP binding sites

Table 1. Effect of TOCP treatment *in vivo* on the high-affinity [^{3}H]DFP binding sites on spinal cord membranes from chickens

Time	No. of chickens	High-affinity binding sites (fmol/mg protein)	
		Assay condition 1†	Assay condition 2
Control*	4	34.6 ± 1.6^a (100)‡	129.7 ± 12.9^a (100)
6 hr	4	4.6 ± 2.3^b (13)	35.8 ± 8.1^b (28)
24 hr	4	10.5 ± 1.8^c (30)	36.9 ± 3.7^b (28)
4 days	4	20.4 ± 1.6^d (59)	58.8 ± 3.4^c (45)
8 days	4	21.6 ± 2.4^d (62)	67.1 ± 5.2^c (52)

* Control birds were treated with vehicle only and killed 24 hr later. Others were treated with a single i.v. injection of 40 mg/kg TOCP and killed from 6 hr to 8 days later.

† Intact membrane samples from each bird were incubated with 0.5 nM [^{3}H]-DFP (assay condition 1) or 15 nM [^{3}H]DFP (assay condition 2), at 37° for 60 min. The amount of the high-affinity binding sites was calculated as the total binding minus the binding in the presence of 10 μM unlabeled DFP.

‡ Values are means ± SD; the numbers in parentheses represent percent of control.

[a–d] Statistical comparisons between groups were determined by one-way analysis of variance followed by Duncan's multiple range test. Numbers in vertical columns not followed by the same superscript are significantly different ($P < 0.01$).

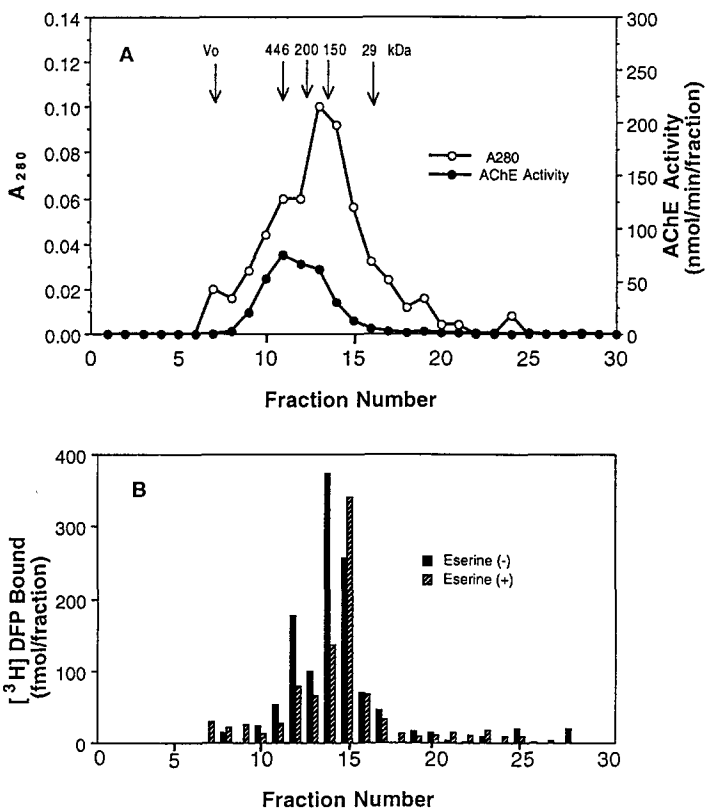

Fig. 6. Gel filtration chromatography of solubilized membranes from the spinal cord followed by assays for AChE activity and [^{3}H]DFP binding in each fraction. (A) Absorbance at 280 nm was monitored in individual fractions. The AChE activity in each fraction was assayed by the method of Ellman *et al.* [12]. Arrows indicate the positions of protein standards of known molecular weight. (B) Control, eserine(−): the fractions were labeled by incubation with 5 nM [^{3}H]DFP for 60 min at 37°. Eserine(+): the fractions were incubated with 10 μM eserine for 30 min at 37° prior to labeling with DFP. The other binding and filtration conditions were as described under Materials and Methods. The results of a single experiment, representative of two, are shown.

described here are not muscarinic cholinergic receptors.

Mipafox, which produces OPIDN, inhibited high-affinity [^{3}H]DFP binding in both intact (unsolubilized) and solubilized membrane fractions at concentrations in the micromolar range. The IC_{50} values of paraoxon, namely the concentrations that inhibited 50% of the AChE and [^{3}H]DFP binding activities in the chicken spinal cord *in vitro*, were 1.0 [11] and 100 nM (Figs. 4 and 5A), respectively. Therefore, it would be difficult to administer sufficient amounts of paraoxon (or parathion) to completely block the DFP binding sites *in vivo* because of its potent anti-ChE action. Although parathion has been considered the prototype of non-neurotoxic AChE inhibitors, de Jager *et al.* [17] have described a patient who suffered delayed polyneuropathy after a suicidal attempt through the ingestion of parathion. If animals are protected from acute cholinergic crisis, a high dose (such as 10–100 times the LD_{50}) of parathion (or paraoxon) might cause polyneuropathy. Thus, the high-affinity DFP binding sites, which are sensitive to paraoxon, cannot be excluded as initial targets of OPIDN.

DFP inhibits the activity of AChE [18], BuChE [18] and many carboxylesterases [19] *in vitro* and *in vivo*. However, the poor inhibitory effects of butyrylcholine and *p*-nitrophenyl valerate on DFP binding suggest that the binding sites are different from the active centers for BuChE and carboxylesterases. Additionally, even high concentrations of acetylthiocholine, 2-PAM and eserine have little or no effect upon the binding of [^{3}H]DFP. This indicated that the [^{3}H]DFP binding sites differ from the AChE active centers, although the binding sites were sensitive to paraoxon. NTE, which is a putative target site for OPIDN [3], is also inhibited by DFP [3] but is insensitive to 40–160 μM paraoxon *in vitro* [20, 21]. As shown in Figs. 4 and 5A, the DFP binding sites were totally inhibited by 10 or 100 μM paraoxon; thus, the DFP binding sites may differ from the NTE active sites.

Although there was no *in vitro* effect of TOCP on [^{3}H]DFP binding sites (data not shown), the DFP binding to membranes from spinal cords of chickens treated with TOCP *in vivo* was inhibited markedly, and full recovery did not occur during the experimental period of 8 days (Table 1). These results indicated that the TOCP metabolite(s) interacts with the DFP binding sites and that metabolite(s)-binding site complexes dissociate very slowly, or recover *de novo*. It is known that the metabolically activated form of TOCP is involved in OPIDN [22]. Therefore, the modification of unidentified DFP binding protein(s) described here may be implicated in the initiation of TOCP-induced delayed neuropathy.

Gel filtration chromatography was performed on CHAPS-solubilized proteins, and the fractions were assayed for [^{3}H]DFP binding. Two major peaks of [^{3}H]DFP binding were eluted in fractions 12 and 14–15 (Fig. 6B). These findings suggest that there are multiple high-affinity DFP binding proteins on the solubilized membranes. The large peak (fraction 15), in particular, was insensitive to eserine (Fig. 6B). This confirms that the binding site is different from AChE active sites, and it is in agreement with the inhibition studies shown in Figs. 4 and 5.

As shown in Fig. 2, the high-affinity binding sites will be fully occupied by 5–10 nM [^{3}H]DFP. The tissue (solubilized membranes) concentration of the binding sites is extremely low, since 1 mg of protein in the membranes bound about 400 fmol of [^{3}H]DFP at the most. It is possible, therefore, that the labeling experiments [5–7, 23, 24] reported previously failed to detect the high-affinity binding proteins described here.

In conclusion, we demonstrated that there are high-affinity binding sites for [^{3}H]DFP on not only intact but also detergent-solubilized membranes from the spinal cord. The inhibition studies *in vitro* suggested that the binding sites differ from the active sites of AChE, BuChE and NTE and from acetylcholine receptors. The results from gel filtration chromatography also suggested that these sites are distinguishable from AChE active sites. TOCP *in vivo* caused a significant decrease in the amount of the binding sites in membranes from the chicken spinal cord. The high-affinity DFP binding sites may be involved with the initial target sites of OPIDN. Further purification studies are required to identify the protein(s) containing the binding sites.

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