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Characterization of [^{3}H]di-isopropyl phosphorofluoridate-binding proteins in hen brain

Rates of phosphorylation and sensitivity to neurotoxic and non-neurotoxic organophosphorus compounds

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The experiments described in this paper were designed to isolate [^{3}H]di-isopropyl phosphorofluoridate-binding proteins by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis for the purpose of characterizing and identifying potential initiation sites for organophosphorus-compound-induced delayed neurotoxicity. The major Paraoxon-insensitive Mipafox-sensitive binding protein (M_r 160000) was found to be identical with one previously identified as neurotoxic esterase, an enzyme that has been proposed to be the target site for organophosphorus-compound-induced delayed neurotoxicity. However, two other binding proteins with suitable binding characteristics were also found in smaller amounts, one of which has not been detected previously. Di-isopropyl phosphorofluoridate was found to phosphorylate all three of these proteins at rates similar to the rate at which neurotoxic esterase is inhibited by di-isopropyl phosphorofluoridate. Varying the concentration of di-isopropyl phosphorofluoridate or the time of incubation produced similar increases in binding to each of the labelled proteins. This suggests that the reaction rates of di-isopropyl phosphorofluoridate with proteins may be described by first-order kinetics, and the concentration of the Michael is complex formed during binding is minimal for all the phosphorylated proteins. The recovery of the binding activity in the 160000-M_r band was found to be similar to the recovery of neurotoxic esterase activity, lending further support to the contention that this band is identical with neurotoxic esterase.

A number of organophosphorus compounds are capable of causing a neuropathy in man and other animals at doses that are sublethal (Abou-Donia, 1981). Since the appearance of the signs and symptoms of the neuropathy (ataxia and paralysis) are delayed for several weeks, this phenomenon has been termed organophosphorus-compound-induced delayed neurotoxicity. Since the correlation between the acute toxicity and the delayed neurotoxicity of organophosphorus compounds is poor, the development of organophosphorus-compound-induced delayed neurotoxicity cannot be the result of the inhibition of acetylcholinesterase (Aldridge, 1954).

In order to identify the target site for organophosphorus-compound-induced delayed neurotoxicity, Johnson (1969*a*) identified a set of organophosphorus-compound-binding sites with specificity for Mipafox and other neurotoxic organophosphorus compounds by incubating hen brain proteins with *OO*-di-isopropyl [^{32}P]phosphorofluoridate (iPr_2P-F) after pre-inhibition of the sample with a non-neurotoxic organophosphorus compound to eliminate the irrelevant sites. Paraoxon is now thought to be the organophosphorus compound best suited for this purpose (Johnson, 1982). It has since been found that at least a portion of these binding sites have esterase activity (Johnson, 1969*b*; Williams & Johnson, 1981). Since this esterase has the correct specifity for organophosphorus-compound-induced delayed neurotoxicity it has been termed neurotoxic esterase (NTE) (Johnson, 1982).

With some differences, the experiments described in the present paper are similar to those of

Abbreviations used: NTE, neurotoxic esterase; iPr_2P-F, di-isopropyl phosphorofluoridate.

Williams & Johnson (1981), in which they conducted a study designed to identify the iPr_2P-F-binding polypeptide of NTE by separating [^{3}H]DFP-labelled proteins by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and ascertained that a 155000-M_r band is likely to be NTE. First, the resolution of the labelled proteins is increased by using either a greater number of slices, or by using fluorography. Secondly, the iPr_2P-F concentration range and the incubation period were varied in order to estimate the rates of phosphorylation of each band by iPr_2P-F. Thirdly, the rate of recovery of the binding activity following the administration of iPr_2P-F *in vivo* was monitored.

Methods

Animals

White Leghorn 14-month-old hens weighing approx. 1.5–2.0kg were obtained from Featherdown Farm (Raleigh, NC, U.S.A.).

Materials

[^{3}H]iPr_2P-F (*OO*-[1,3-^{3}H]di-isopropyl phosphorofluoridate) was obtained either from New England Nuclear Corp. (Boston, MA, U.S.A.) (5.2Ci/mmol) or from Amersham/Searle (Arlington Heights, IL, U.S.A.) (6.0Ci/mmol). Unlabelled iPr_2P-F was obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Physostigmine, phenylmethane sulphonyl fluoride and Paraoxon (*OO*-diethyl 4-nitrophenyl phosphate) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Atropine sulphate was obtained from Eli Lilly and Co. (Indianapolis, IN, U.S.A.). Mipafox (*NN*-di-isopropyl diamidophosphorofluoridate) was synthesized by the Midwest Research Institute (Kansas City, MO, U.S.A.). All materials used in the preparation of the polyacrylamide gels were obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.).

Dosing

In the recovery experiment, hens were dosed with 1.7mg of iPr_2P-F (17mg/ml in propylene glycol)/kg at 0, 3, 6, 9 or 12 days before they were killed. They were protected from the acute effects by the administration of 1.0mg of physostigmine/kg and 0.2mg of atropine/kg 15min before the organophosphorus compound. All compounds were administered subcutaneously.

Protein preparation and labelling with [^{3}H]DFP

The hens were killed by asphyxiation with CO_2. The brains were removed and homogenized in 50mM-sodium phosphate buffer, pH7.4, containing 0.32M-sucrose (10%, w/v) with a Polytron homogenizer for 15s. The homogenate was then spun at 1000*g* for 10min and the pellet was discarded. [^{3}H]iPr_2P-F in propylene glycol was then added to the supernatant (so that the final concentration was 1μM-iPr_2P-F in 1% propylene glycol) and the mixture was incubated for 1h at 37°C. The rate of the labelling was slowed to an insignificant value by the addition of excess unlabelled iPr_2P-F (1mM). Five experiments were conducted, each of which involved varying the incubation conditions in a different way. The first, third and fourth experiments were performed in quadruplicate, with brains from four different hens for each replication. Three replicates were used in the third experiment.

In the first experiment, the homogenate was preincubated with 100μM-Paraoxon, 100μM-Mipafox or 300μM-phenylmethanesulphonyl fluoride before the addition of the labelled iPr_2P-F. In the second experiment 50mM-Tris/HCl buffer, pH8.0, was used instead of phosphate buffer, and concentrations of Paraoxon and Mipafox were lowered to 40μM and 50μM respectively. The incubation period was shortened to 20min. These incubation conditions are identical with those used in the standard NTE assay method of Johnson (1977). In the third experiment, the concentration of iPr_2P-F was varied; the homogenate was incubated with 0.1μM-, 1.0μM-, 10μM or 100μM- iPr_2P-F. In the fourth experiment, the incubation time was varied; the homogenate was incubated with iPr_2P-F for 5min, 15min, 1h or 4h. In the fifth experiment the protein was preincubated with iPr_2P-F *in vivo* at various times before the hens were killed. The homogenates from these animals were incubated *in vitro* in 50mM-Tris buffer, pH8.0.

After incubation with [^{3}H]iPr_2P-F, the homogenate was then spun at 100000*g* for 1h. The pellet [membrane (P_2–P_3)] was resuspended in phosphate buffer. In order to concentrate the soluble proteins (supernatant), the proteins in the supernatant were precipitated with 10% (w/v) trichloroacetic acid and then resolubilized in phosphate buffer containing 1% (w/v) sodium-dodecyl sulphate. Protein concentrations of the samples were determined by using the method of Lowry *et al.* (1951) or that of Bradford (1976); bovine plasma protein was used as protein standard.

Polyacrylamide-gel electrophoresis

Proteins were separated by electrophoresis in polyacrylamide gels as described by Patton *et al.* (1983). Only membrane proteins were examined in the second experiment. For fluorography, samples containing 75μg of protein were run in 4mm lanes (20 per gel). For gel slicing, samples containing 100μg of protein were run in 8mm lanes (ten per

gel). Labelled bands were then detected by either slicing and scintillation counting (Expts. 1, 3 and 4) or by fluorography and microdensitiometry (Expts. 1 and 2).

Gel slicing and scintillation counting

After being destained, gels were stored in 10% (v/v) acetic acid for 4–96h. After removal of the stacking gel, the individual lanes were cut from the gel and sliced into 1.1mm slices with a Bio-Rad electric gel slicer (model 190). Two such slices were then inserted into 6mm plastic mini-vials to which 0.3ml of Protosol (New England Nuclear) was added to solubilize the ^3H from the gel. Scintillation fluid [Scintanalyzed Xylenes from Fisher Scientific Co. containing 0.3% 2,5-diphenyloxazole and 0.01% 1,4-bis-(5-phenyloxazol-2-yl)benzene from New England Nuclear] was added to the vial 24h after the Protosol. The radioactivity in each vial was counted with about 50% efficiency in a Packard Tri-Carb liquid-scintillation counter (model 3255).

Fluorography and microdensitometry

After being destained, gels were immersed in En^{3}Hance (150ml/gel; New England Nuclear) for 1h, and then soaked in water for 30min. Fluorographs were prepared and analysed as described by Patton *et al.* (1983).

NTE assays

NTE activity in the fifth experiment was assayed by the method of Johnson (1977).

Results

The binding profile of 1μM-iPr_2P-F to proteins from hen brain in 50mM-sodium phosphate buffer, pH7.4, as determined by gel slicing and scintillation counting, is given in Fig. 1. Fluorographs of gels run with the same samples are presented in Fig. 2. The amount of iPr_2P-F bound to eight membrane protein bands, as quantified by either scintillation counting or microdensitometry, is presented in Table 1. Table 2 contains similar data for seven bands from gels containing protein from the soluble fraction.

Both Tables 1 and 2 contain the percentage activity remaining after preincubation with 100μM-Paraoxon, 100μM-Mipafox or 300μM-phenylmethanesulphonyl fluoride for 1h. Although the results obtained by the two different detection methods are similar, the smaller bands are more prominent with scintillation counting than with autoradiography. A probable explanation for this is that the film may have a detection threshold that is proportionately greater for the less radioactive bands. Of the 15 bands, only one (M1) had inhibitor characteristics suitable for the target site for organophosphorus-compound-induced delayed neurotoxicity, i.e. it is more sensitive to Mipafox and phenylmethanesulphonyl fluoride than to Paraoxon. The fact that over 50% of the band was inhibited by Paraoxon may be either due to a non-homogeneous protein population in the band or because the concentration of Paraoxon was sufficiently high and the incubation period sufficiently long to permit inhibition of iPr_2P-F binding to the neurotoxic site by the non-neurotoxic organophosphorus compound. Another

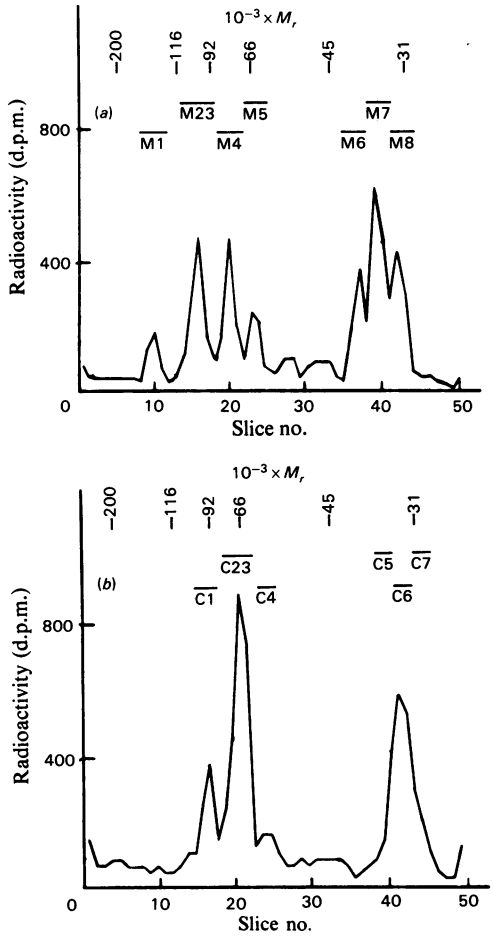

Fig. 1. *Quantification of the binding of iPr_2P-F to proteins from hen brain by gel slicing and scintillation counting*
The Figure shows the average radioactivities in slices of polyacrylamide-gel electrophoretograms of four protein samples from either a membrane (*a*) or a soluble (*b*) fraction from hen brain as determined by scintillation counting. The bands, which are the basis of comparison in Tables 1 and 2, are defined by the bars above the plots. The positions of the M_r standards are given at the top of each plot.

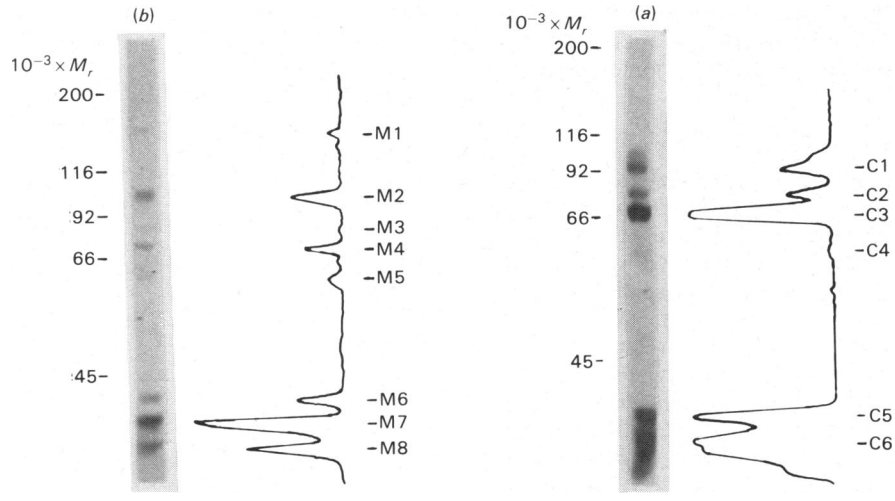

Fig. 2. *Quantification of the binding of iPr_2P-F to protein from hen brain by fluorography and microdensitometry*
The Figure shows fluorographs of polyacrylamide-gel electrophoretograms, containing samples of protein from either a membrane (*a*) or soluble (*b*) fraction from hen brain. Microdensitometric scans of the gels are illustrated to the right of each fluorograph. The positions of the M_r standards are given on the left.

Table 1. *Inhibition by Paraoxon, Mipafox and phenylmethanesulphonyl fluoride of iPr_2P-F binding by P_2–P_3 fraction from hen brain*

iPr_2P-F was bound to 100 μg samples of a P_2–P_3 fraction from hen brain with and without preinhibition by inhibitors. Values were obtained either by gel slicing and scintillation counting (radioactivity) or by microdensitometry of a fluorograph (area, in arbitrary units). The amount of bound ligand after preinhibition with 100 μM-Paraoxon, 100 μM-Mipafox or 300 μM-phenylmethanesulphonyl fluoride (PMSF) for 1 h at 37°C is expressed as a percentage of the uninhibited control (iPr_2P-F only). Statistical significance: *differs from iPr_2P-F only, $P<0.05$; **differs from iPr_2P-F only, $P<0.01$; ***differs from iPr_2P-F only, +Mipafox and +PMSF, $P<0.01$.

Band		iPr_2P-F only	+Paraoxon	+Mipafox	+PMSF
M1 (M_r 160000)	Radioactivity	382 d.p.m.	40%***	11%**	5%**
	Area	1.5	18%**	5%*	6%**
M2 (M_r 99000)	Radioactivity	1121 d.p.m.	14%*	15%**	43%**
	Area	8.8	0%*	6%*	60%
M3 (M_r 82000)	Radioactivity	363 d.p.m.	11%**	23%**	37%
	Area	0.9	0%**	18%**	201%
M4 (M_r 75000)	Radioactivity	624 d.p.m.	18%**	65%*	60%*
	Area	6.2	0%	16%**	90%
M5 (M_r 63000)	Radioactivity	569 d.p.m.	13%**	12%**	24%**
	Area	2.7	0%*	24%*	7%*
M6 (M_r 42000)	Radioactivity	813 d.p.m.	10%**	48%**	6%**
	Area	6.5	16%*	30%*	15%*
M7 (M_r 37000)	Radioactivity	1413 d.p.m.	10%**	68%*	18%**
	Area	45.4	1%**	64	5%*
M8 (M_r 32000)	Radioactivity	890 d.p.m.	30%**	22%**	7%**
	Area	27.0	8%**	34%**	6%**
Total radioactivity (mean ± S.E.M.)		7817 ± 712 d.p.m.	1539 ± 88 d.p.m.	2972 ± 254 d.p.m.	2228 ± 153 d.p.m.
iPr_2P-F bound		33.1 nmol/mg of protein	6.5 nmol/mg of protein	12.6 nmol/mg of protein	9.4 nmol/mg of protein

possibility is that, since Paraoxon was present during the iPr_2P-F incubation, Paraoxon may have been competing for the binding site in a reversible manner (Carrington & Abou-Donia, 1985). It is also possible that some of the other bands contain a minor component that is more sensitive to Mipafox and phenylmethanesulphonyl fluoride than to Paraoxon.

Table 2. *Inhibition by Paraoxon, Mipafox and phenylmethanesulphonyl fluoride of iPr_2P-F binding by soluble fraction from hen brain*

iPr_2P-F was bound to 100 μg samples of a soluble fraction from hen brain with and without preinhibition by inhibitors. Values were obtained either by gel slicing and scintillation counting (radioactivity) or by microdensitometry of a fluorograph (area, in arbitrary units). The amount of bound ligand after preinhibition with 100 μM-Paraoxon, 100 μM-Mipafox or 300 μM-phenylmethanesulphonyl fluoride (PMSF) for 1 h at 37°C is expressed as a percentage of the uninhibited control (iPr_2P-F only). N.D., the 28000-M_r and 32000-M_r bands were not distinguishable by using fluorography and microdensitometry. Statistical significance: *differs from iPr_2P-F only, $P<0.05$; **differs from iPr_2P-F only, $P<0.01$.

Band		iPr_2P-F only	+Paraoxon	+Mipafox	+PMSF
C1 (M_r89000)	Radioactivity	778d.p.m.	24%**	57%*	33%*
	Area	5.8	12%**	54%	20%**
C2 (M_r77000)	Radioactivity	948d.p.m.	10%**	59%*	92%
	Area	4.4	2%**	1%**	110%
C3 (M_r69000)	Radioactivity	1539d.p.m.	18%**	103%	79%
	Area	24.9	8%**	86%	25%
C4 (M_r58000)	Radioactivity	324d.p.m.	15%**	37%**	74%
	Area	3.5	6%**	0%**	25%*
C5 (M_r36000)	Radioactivity	775d.p.m.	7%**	19%**	7%**
	Area	18.5	4%**	46%	1%**
C6 (M_r32000)	Radioactivity	1226d.p.m.	15%**	51%*	60%
	Area	44.2	4%**	29%*	17%**
C7 (M_r28000)	Radioactivity	358d.p.m.	39%*	44%*	67%*
	Area	N.D.	—	—	—
Total radioactivity (mean ± S.E.M.)		8556 ± 1269d.p.m.	1706 ± 244d.p.m.	5134 ± 323d.p.m.	5450 ± 213d.p.m.
iPr_2P-F bound		36.2nmol/mg of protein	7.2nmol/mg of protein	21.7nmol/mg of protein	23.1nmol/mg of protein

Table 3. *Labelling of the P_2–P_3 fraction from hen brain by iPr_2P-F under the conditions of the NTE assay*

The labelling of seven membrane protein bands after incubation with 1 μM-iPr_2P-F for 1 h under the standard NTE assay conditions of Johnson (1977) was investigated. Labelling was quantified by fluorography and microdensitometry. The values for labelling in the absence of inhibitor represent the area under the curve for the peak; the units are arbitrary. The bands are equivalent to those described in Table 3, except that bands M7 and M8 are both contained in the band M7 above. The means ± S.E.M. for three determinations are given. Statistical comparisons were made by paired (comparisons of effects *in vitro*) or unpaired (for assessing the effects of iPr_2P-F *in vivo*) *t* tests: *differs from No inhibitor, $P<0.05$; **differs from No inhibitor, $P<0.01$; ***differs from No inhibitor and 40 μM-Paraoxon, $P<0.05$. The recovery rates were estimated from first-order (log) plots of the means of three determinations of the percentage recovery of the inhibited binding sites. NTE activity in the animals treated with iPr_2P-F was inhibited by 97 ± 3%, and 50% recovery was estimated to occur after 6.3 days.

Band	No inhibitor	40 μM-Paraoxon	50 μM-Mipafox	Paraoxon +Mipafox	1.7mg of iPr_2P-F/kg *in vivo*	Time for 50% recovery (days)
M1	350 ± 49	80 ± 18%	12 ± 4%*	3 ± 3%***	0 ± 0%**	7.7
M2	759 ± 83	13 ± 0%*	29 ± 3%*	30 ± 8%*	24 ± 3%*	4.3
M3	413 ± 45	66 ± 4%	47 ± 7%	30 ± 13%***	0 ± 0%**	8.1
M4	1956 ± 353	49 ± 10%*	86 ± 4%	49 ± 12%*	6 ± 4%**	7.3
M5	543 ± 120	7 ± 7%*	8 ± 8%*	2 ± 2%*	0 ± 0%**	3.4
M6	317 ± 25	0 ± 0%**	48 ± 25%	0 ± 0%**	61 ± 6%	3.5
M7	821 ± 23	23 ± 17%*	55 ± 4%**	35 ± 12%*	36 ± 12%*	5.7

Fluorographs of gels of hen brain membrane proteins incubated under the conditions of the standard NTE assay method of Johnson (1977) are presented in Fig. 3(*a*). Quantification of three sets of gels is presented in Table 3. With the shorter incubation period and lower Paraoxon concentration, only about 20% of band M1 was inhibited, although there was still nearly complete inhibition with Mipafox. There also appeared to be a Mipafox-sensitive Paraoxon insensitive component in band M3. In addition to the major bands labelled, there were a number of bands that were

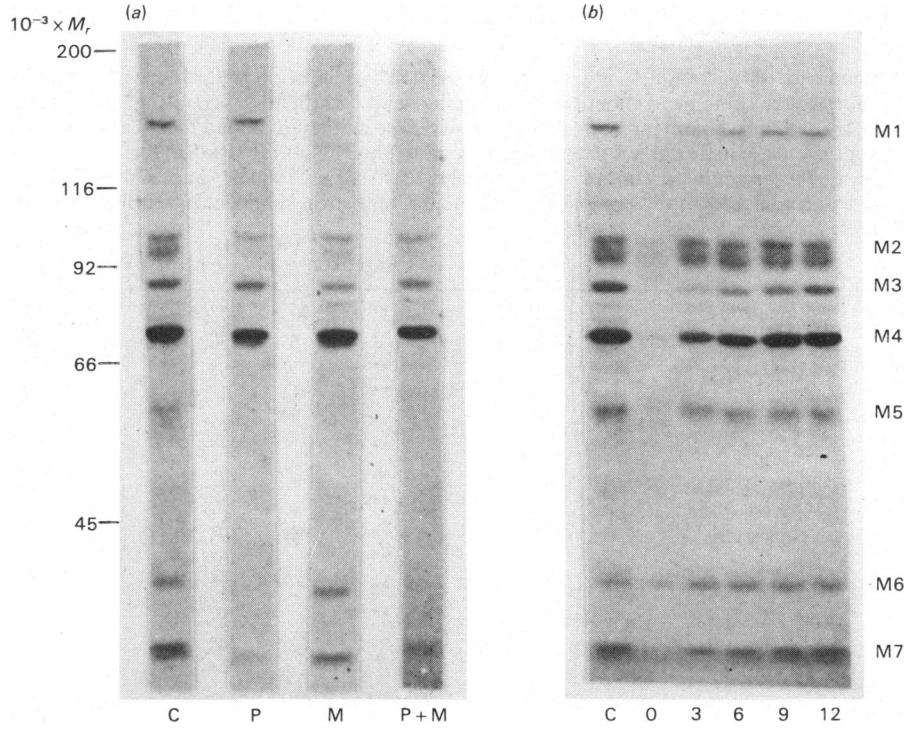

Fig. 3. *Electrophoresis and fluorography of membrane proteins from hen brain labelled by iPr_2P-F under the conditions of the NTE assay*

The Figure shows fluorographs of polyacrylamide gels containing membrane proteins from hen brain labelled by incubation with 1 μM-[^{3}H]iPr_2P-F for 1 h under the standard NTE assay conditions of Johnson (1977). The positions of M_r standards are given on the left. The bands quantified by microdensitometry (see Table 3) are identified on the right. (*a*) The protein was untreated (C) or pre-inhibited with either 40 μM-Paraoxon (P) or 50 μM-Mipafox (M) or both (P + M) for 20 min at 37°C before the labelling. (*b*) The proteins were labelled *in vitro* following the killing of birds 0, 3, 6, 9 or 12 days after administration of a neurotoxic dose of iPr_2P-F *in vivo*. The control lane (C) is from an animal that was not dosed with iPr_2P-F.

too faint to quantify by microdensitometry or to photograph. However, one of these, with an apparent M_r of about 115000, appeared to be completely sensitive to Mipafox but was unaffected by Paraoxon.

The rate of phosphorylation of each of the bands, as calculated by varying either the concentration of the ligand or the time of incubation, is gvien in Table 4. Although a number of other proteins were labelled with high concentrations of [^{3}H]DFP, analysis is restricted to the bands identified with 1 μM-[^{3}H]iPr_2P-F. In general, labelling of the low-M_r proteins was greatly increased by increasing the concentration of [^{3}H]iPr_2P-F whereas, the higher-M_r proteins were saturated with 10 μM- or 1.0 μM-[^{3}H]iPr_2P-F. One exception to this rule was a large increase in the labelling of band M1 when the concentration of [^{3}H]iPr_2P-F was increased to 100 μM; apparently there is a low-affinity protein with an M_r of about 160000.

Fig. 3(*b*) contains a photograph of a fluorograph of a gel of membrane proteins from hen brain exposed to iPr_2P-F *in vivo* at various times before the labelling with [^{3}H]iPr_2P-F *in vitro*. The amount of radioactivity in each band from three such gels is presented in Table 3. The labelling of three of the seven bands was completely blocked, but there was considerable residual radioactivity in bands M2 and M7 and a small amount of radioactivity remaining in band M4. The binding to band M6 *in vitro* was affected very little by the treatment *in vivo*. Some, or all, of these binding sites may have been protected by the physostigmine that was administered before the iPr_2P-F. The recovery rates for bands M1, M3 and M4 and the NTE activity returned at a lower rate than for the other four bands (Table 3).

Discussion

The characterizations of the iPr_2P-F-binding proteins described in the present paper are similar

Table 4. *Rates of phosphorylation by iPr_2P-F of proteins from hen brain*

Rate constants for the phosphorylation by iPr_2P-F of eight membrane and seven soluble protein bands from hen brain were calculated by varying either the time of incubation or the concentration of iPr_2P-F. The values for the amount of iPr_2P-F bound per mg of protein are for incubation with 1 μM-iPr_2P-F for 1 h and are the means for three experiments with four replications each. Bands C2 and C3 were difficult to distinguish with gel slicing and were combined for the latter calculation.

Band	Rate constants ($M^{-1} \cdot min^{-1}$)		iPr_2P-F bound (nmol/mg of protein)
	Variable time	Variable concentration	
M1	1.3×10^5	7.0×10^4	1.1 ± 0.1
M2	2.6×10^5	2.0×10^5	5.3 ± 0.5
M3	4.8×10^5	3.3×10^5	2.2 ± 0.2
M4	8.2×10^4	6.2×10^4	3.0 ± 0.1
M5	2.6×10^5	1.2×10^5	2.4 ± 0.1
M6	2.4×10^4	6.3×10^3	2.7 ± 0.3
M7	2.9×10^4	1.3×10^4	3.9 ± 0.6
M8	6.3×10^4	5.8×10^3	3.6 ± 0.5
C1	3.1×10^5	5.1×10^4	2.5 ± 0.3
C2	4.8×10^5	3.7×10^6	7.8 ± 0.8
C3	5.8×10^4	5.9×10^4	
C4	1.3×10^5	7.2×10^4	1.3 ± 0.2
C5	6.1×10^3	9.0×10^2	2.0 ± 0.4
C6	1.9×10^4	2.2×10^3	3.9 ± 0.5
C7	1.7×10^4	1.3×10^3	1.0 ± 0.5

to those reported by Williams & Johnson (1981). Band M1 appears to be identical with the 155000-M_r band that Williams & Johnson (1981) believed to be NTE, and the Mipafox-sensitive Paraoxon-insensitive component in band M3 may correspond to the NTE-like component in their 92000-M_r band. Although it is tempting to believe that these two bands are the NTE isoenzymes NTE_A and NTE_B (Chemnitius & Zech, 1983), the number of iPr_2P-F-binding sites does not necessarily correspond to phenyl valerate-hydrolysing activity. For instance, acetylcholinesterase is a major binding component, yet is only a small component of the Paraoxon-sensitive phenyl valerate-hydrolysing activity of hen brain. Consequently, the minor 115000-M_r band that we were barely able to detect could be the major component of the esterase activity measured by the NTE assay.

Another possibility is that, as some of the evidence of Williams & Johnson (1981) suggests, band M1 is in fact the major component detected by the NTE assay. The other NTE-like component of band M3 and the 115000-M_r band may have little or no esteratic activity. Nonetheless, the minor Paraoxon-insensitive Mipafox-sensitive binding proteins are still potential initiation sites for organophosphorus-compound-induced delayed neurotoxicity. The binding characteristics of these, or perhaps a putative band that went undetected, might be so similar to those of NTE that the assay is able to predict the delayed neurotoxicity of organophosphorus compounds in spite of the fact that NTE is not the initiation site.

The accuracy of the rate constants calculated for the bands is very poor for several reasons. (1) The assumption of saturation at 4 h incubation time or with 100 μM-iPr_2P-F may be unwarranted, particularly for the M_r bands. Underestimation of the total number of sites in a band would cause the rate constants to be overestimated. (2) There are multiple components in at least some of the bands, which presumably have different rates of phosphorylation. (3) The rates were calculated from concentrations that varied by factors of 10-fold or from incubation periods that varied by factors of 4-fold. The accuracy of the estimates are limited accordingly. (4) The range of concentrations or incubation periods employed probably varied over only a portion of the range of binding constants. Consequently, high reaction rates were underestimated, whereas low reaction rates were either undetected or overestimated. (5) iPr_2P-F is not stable in an aqueous solution, so that the effect of lengthening the incubation period may have been underestimated. However, the calculated rates do provide a rank order for the reactivity of the bands.

There are two pieces of evidence presented in this paper that band M1 is identical with the major component measured by the NTE assay. First, the rate constant estimated for band M1 is similar to the rate constant calculated for NTE_B ($1.6 \times 10^5 M^{-1} \cdot min^{-1}$) by Chemnitius & Zech (1983). Secondly, the rate of recovery of the binding activity of band M1 is similar to that of NTE (Johnson, 1974; Carrington & Abou-Donia, 1984). These findings confirm and extend the

reports that this protein has a tissue distribution and sensitivity to di-n-pentyl phosphinate (Williams & Johnson, 1981), and aging characteristics (Williams, 1983) that are similar to that of NTE.

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