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Sex Differences in the Recovery of Brain Acetylcholinesterase Activity Following a Single Exposure to DFP

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SMOLEN, A., T. N. SMOLEN, P. C. HAN AND A. C. COLLINS. *Sex differences in the recovery of brain acetylcholinesterase activity following a single exposure to DFP.* PHARMACOL BIOCHEM BEHAV **26**(4) 813–820, 1987.—Male and female C57BL, DBA, and C3H mice were injected intraperitoneally with a single 6.33 mg/kg dose of diisopropylphosphofluoridate (DFP). The time course of recovery of acetylcholinesterase (AChE) activity as well as effects on choline acetyltransferase (ChAT) activity and brain muscarinic and nicotinic receptors were measured. DFP treatment did not affect ChAT activity or the muscarinic and nicotinic receptors. Near control levels of AChE activity were regained in female mice within the first 20 days. However, levels of whole brain AChE activity remained depressed for as long as 40 days following a single dose of DFP in male mice. An analysis of the recovery of AChE activity in several brain regions indicated that control activity was regained in striatum, hindbrain, and hippocampus, but not in cortex, midbrain, and hypothalamus. These data are discussed in terms of potential neurotoxicity induced by a single dose of DFP.

Acetylcholinesterase Diisopropylphosphofluoridate Choline acetyltransferase Sex differences
Muscarinic receptors Nicotinic receptors

ORGANOPHOSPHATES such as diisopropylphosphofluoridate (DFP) exert their pharmacological actions by inhibiting acetylcholinesterase (AChE) thereby allowing acetylcholine (ACh) concentrations to rise in cholinergic neurons. The enzyme is irreversibly inhibited and although there is some low level of spontaneous reactivation [19], for all practical purposes enzyme activity can return to normal levels only by synthesis of new enzyme.

Humans chronically exposed to organophosphates exhibit a variety of psychological modifications including fatigue, mood changes, excessive dreaming, mental confusion, and schizophrenic and depressive reactions [15,44]. In addition, changes in electroencephalogram activity are seen [11,23]. While the causes of these changes remain largely unknown, the biochemical adaptations to chronic exposure to organophosphates are better understood. Studies in animals have demonstrated that chronic exposure to organophosphates elicits a decrease in brain cholinergic receptors. Decreases in the number of brain muscarinic receptors have been reported after chronic treatment with DFP [7, 12, 37, 42, 49], tetram [14], paraoxon [43], disulfoton [9], and soman [6] as estimated by measuring the binding of ^{3}H-quinuclidinyl benzilate (QNB). Similarly, chronic treatment with organophosphates decreases brain nicotinic receptors as measured by ^{3}H-acetylcholine [39] or ^{3}H-nicotine binding [8]. The predominant hypothesis that has been presented to explain this decrease in brain cholinergic receptors is that the chronic increase in synaptic ACh content results in a down regulation of receptor number as accomplished by a decrease in synthesis or increase in destruction of the receptor molecules.

The biochemical and behavioral effects of a single exposure to sublethal doses of organophosphates are in some ways less well understood than are the chronic effects. The AChE inhibition following acute exposure to an organophosphate results in increased levels of ACh in brain areas rich in cholinergic neurons. It is generally believed that the increase in synaptic ACh is too transient to cause long-term effects since all available evidence suggests that the elevations last from only a few hours to 24 hours depending upon the brain area studied [36,40]. These studies did not provide information concerning the concentration of ACh in the synapse and the methods currently available do not allow such an estimate. There is evidence, however, that following

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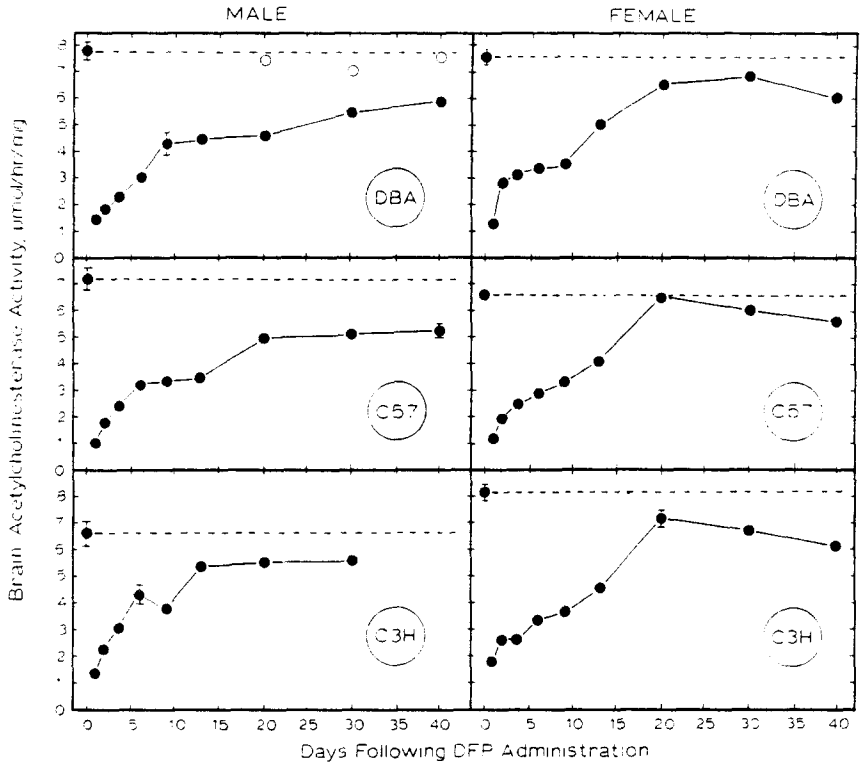

FIG. 1. Rate of recovery of whole brain AChE activity following a single dose of DFP in male and female DBA, C57BL and C3H mice. Mice were injected with DFP (6.33 mg/kg) and the brain AChE activity was measured on the days indicated. The open circles in the DBA male panel are saline-injected controls which were sampled along with the treated mice. The dotted line represents control AChE activity in each case. Each point is the mean±SEM of 6–12 mice per point.

acute exposure to various organophosphates, humans suffer a variety of intellectual and psychiatric disorders that are very long-term, and may even be permanent [23, 41, 44].

We have been investigating the genetic regulation of sensitivity to the acute effects of DFP in the mouse [46,47] and have found that the acute physiological effects of a single exposure to DFP are terminated within 3 to 4 hours. This finding is consistent with the observation that treatment with irreversible AChE inhibitors does not result in a prolonged (greater than 24 hours) increase in brain ACh content [36,40], but it is not consistent with the observation that humans exposed to large doses of organophosphates manifest long-term possibly irreversible toxic sequelae. Therefore, we carried out studies to ascertain the effects of a single, large dose of DFP on AChE, choline acetyltransferase (ChAT) and muscarinic and nicotinic cholinergic receptors in the brain. Changes in each of these could result in early termination of acute effects of DFP by altering the synthesis and degradation of ACh, or by receptor desensitization. It is possible that these could also be involved in the long-term effects of a single exposure to organophosphates. In view of our interests in the influence of genetic factors in regulating the responses of mice to cholinergic drugs, these studies were carried out using three inbred mouse strains.

METHOD

Animals

Male and female DBA/2Ibg, C57BL/6Ibg and C3H/2Ibg mice, 60–80 days of age were used in these studies. Mice were raised at the Institute for Behavioral Genetics, kept on a 12 hour light cycle and allowed free access to food (Wayne Lab Blox) and water.

Drug Treatment

Diisopropylphosphofluoridate (Sigma) was prepared in saline and injected intraperitoneally. DFP is often administered in an oil vehicle, yet it is stable for several hours in saline [47]. A saline solution is more quickly and easily administered and was better received by the animals because restraint time during injection is reduced. Solutions were used within one hour of preparation. Unless otherwise noted a dose of 6.33 mg/kg was used. This dose caused 30–40% lethality in the C57BL mice, 15% in the C3H mice and 1–2% of DBA mice. Animals generally died within 15 minutes of injection, and were immediately replaced with another animal, usually a littermate. For most of the following studies, the 6.33 mg/kg dose was chosen because it gives the maximal practical inhibition of AChE activity. Higer doses result in high mortality rates especially in the C57BL mice [45]. DFP dose response curves for AChE inhibition are similar in all three of the strains [45]. Cholinergic receptors and enzyme activities were measured at intervals over 40 days following a single exposure to DFP as indicated in the figure legends.

Preparation of Brain Tissue

Mice were killed by cervical dislocation, the brain was

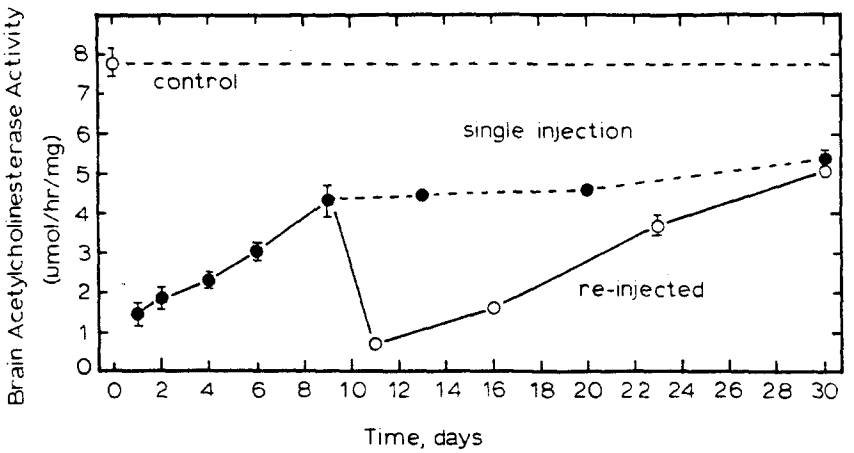

FIG. 2. Effect of a second injection of DFP on rate of return of brain AChE activity in male DBA mice. On day 9 after one injection of DFP (6.33 mg/kg), mice were re-injected with the same dose. Rate of recovery was measured as described in Fig. 1.

removed rapidly and dissected on ice into cortex, cerebellum, hippocampus, hypothalamus, striatum, midbrain (the tissue remaining after removal of the hippocampus, hypothalamus and striatum; primarily thalamus) and hindbrain (pons-medulla). The tissue was placed in 10 volumes of HEPES-buffered Ringer's solution (NaCl, 118 mM; KCl, 4.8 mM; $CaCl_2$, 2.5 mM; $MgSO_4$, 1.2 mM; HEPES, 20 mM; adjusted to pH 7.5 with NaOH) and frozen at −70°C. On the day of the assay the tissue was thawed and homogenized in a Potter-Elvehjem homogenizer. An aliquot of the homogenate was taken for the assay of AChE and ChAT activities. The particulate fraction of the remaining homogenate was prepared by centrifugation using the method described by Marks and Collins [28] for use in the binding assays.

For some studies only AChE activity was measured. For these experiments whole brain, or brain regions, were homogenized in 10 volumes of 50 mM potassium phosphate, pH 7.4, in all-glass (Dounce) homogenizer. These two buffer systems were equally suitable for the measurement of AChE activity.

Brain Acetylcholinesterase Activity

Brain AChE activity was measured using a modification of Ellman's method [13], as described previously [28]. Tissue homogenates were diluted (1:5 to 1:40) in 0.05% Triton X-100 in 50 mM potassium phosphate, pH 7.4. These diluted homogenates were also used for the assay of ChAT activity. For K_m determinations, 5 concentrations (31-500 μM) of the substrate, acetylthiocholine, were used. A saturating concentration (500 μM) was used for other determinations. Blanks contained the specific AChE inhibitor BW 254 C51 (10 μM) [28].

Choline Acetyltransferase Activity

Choline acetyltransferase activity was measured in Triton X-100 treated homogenates according to a modification of the method of Schrier and Schuster [38]. Each 200 μl incubation in 50 mM potassium phosphate pH 7.4, contained 5 mM choline Br, 400 mM acetyl CoA and 5 nCi of [^{14}C]-acetylCoA. Enzyme activity was measured as described previously [28] except that a 10 min incubation was used. ChAT activity was measured in cortex, striatum and hippocampus.

L-[^{3}H]-QNB binding. The binding of L-[^{3}H]-QNB (quinuclidinyl benzilate) (New England Nuclear) to muscarinic receptors was measured using a modification of the method of Yamamura and Snyder [50] as described previously [29]. A single concentration (150 pM) was used in the five smaller regions. In cortex, K_D and B_{max} were determined using five concentrations (10–150 pM) of ligand. Approximate protein concentrations in the assays were: cortex, striatum, hippocampus, and hypothalamus, 20 μg; midbrain and hindbrain, 100 μg. Blanks were obtained by omitting protein from the assays. A no-protein blank gave results identical to a 1 mM atropine-containing blank.

L-[^{3}H]nicotine binding. The binding of L-[^{3}H]nicotine (New England Nuclear) was measured as described previously [29]. A single concentration of radiolabelled nicotine (5 nM) was used to measure total binding. Samples contained 200–600 μg of protein. Blanks were determined by including 10 μM unlabelled L-nicotine in the assays.

α-[^{125}I]bungarotoxin binding. The binding of α-[^{125}I]bungarotoxin (BTX) (New England Nuclear) was measured as described previously [29]. A single concentration of labelled BTX (800 pM) was used for the five smaller regions. The binding parameters were determined in cortex using five concentrations (50–800 pM) of radiolabelled BTX. Samples contained 100–300 μg of protein. Blanks were obtained by including 1.0 mM unlabelled L-nicotine in the assays.

Liquid Scintillation Counting

All of the binding assays were done as filtration assays. The reactions were terminated by dilution and rapid filtration through glass fiber filters (Boehringer-Mannheim). After washing, the glass fiber filters were placed in 5 ml polypropylene scintillation vials (Sarstedt) and 2.5 ml of scintillation cocktail (1.35 L Toluene, 900 ml Triton X-100 and 10.5 g 2,5-diphenyloxazole) was added. The stoppered vials were shaken for 10 min and the radioactivity was determined in a Beckman 7000 liquid scintillation spectrometer. Tritium was counted at 40% efficiency, ^{14}C at 40% efficiency, and ^{125}I at 44% efficiency.

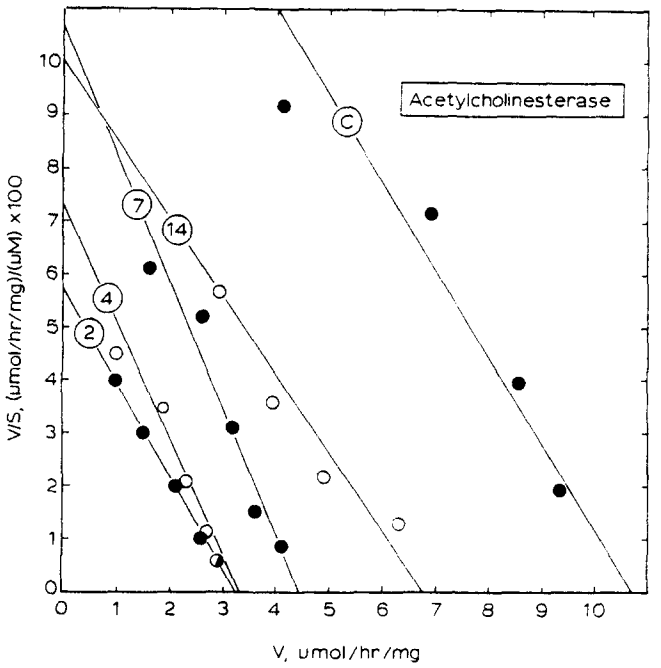

FIG. 3. Eadie-Hofstee plot of AChE activity from brain of male DBA mice. Mice were treated with one 6.33 mg/kg dose of DFP and 2, 4, 7 and 14 days later, the brain AChE activity was measured using five concentrations of substrate. Plotted values are means of 6 different K_m and V_{max} determinations.

Protein Assay

Proteins were measured by the method of Lowry *et al.* [26] with bovine serum albumin as the standard. An equal aliquot of HEPES-buffered Ringer's solution was included in the blank to correct for the slight contribution of the buffer to the protein assay.

Data Analysis

Data were analyzed using one or two way analysis of variance. Where appropriate, individual group differences were analyzed using Duncan's new multiple range test. For these experiments an α level of 0.05 was considered significant. Binding parameters were calculated using Scatchard plots.

RESULTS

Figure 1 shows the time course of recovery of brain AChE activity following the injection of a single, 6.33 mg/kg, dose of DFP in male and female DBA, C57BL, and C3H mice. One day after receiving DFP, whole brain AChE activity was markedly reduced. The activity increased gradually, and at a similar rate, over the next 8 days in both males and females. After that time, the brain AChE activity in the males plateaued at a value 75–80% of control and remained at that level as long as 40 days following a single exposure to DFP. Results were similar for all three strains, but the C3H mice tended to return more nearly to control levels than did the other two mouse strains. Saline-injected controls were tested along with the drug treated DBA males and their AChE activity did not change with time over the 40 day period. Time 0 controls had AChE activity of 7.7 μmoles/hr/mg protein, the values for controls 21, 30, and 40 days following saline were 7.40, 7.02, and 7.51 μmoles/hr/mg protein respectively. Thus, the observation that DFP-treated male mice do not return to control AChE activity is a result of DFP treatment, and not due to the fact that animals were tested at different ages. In the females of all three strains, brain AChE activity continued to rise such that by day 21 it was nearly at control level.

The long-term reduction of AChE activity could be the result of some direct neurotoxicity by DFP which was more pronounced in the males. If this were true, then a second injection of DFP might be expected to lower the level of recovery of AChE activity even further. Figure 2 shows the effect of a second injection of DFP on whole brain AChE activity in male DBA mice. Ten days after a single injection of 6.33 mg/kg DFP, male DBA mice were injected with a second 6.33 mg/kg dose. At 24 hours the AChE activity was reduced to a level lower than that of the singly-injected mice. Enzyme activity returned to a value similar to that of the singly-injected animals, and not a lower value. The rate of return of AChE activity was the same for singly and doubly injected mice. This indicates that any possible neurotoxic effect of DFP is confined to a small population of susceptible cells.

If the recovered AChE were kinetically different from the basal enzyme, the lowered AChE activity reported in Figs. 1 and 2 could result from the enzyme operating at a different percentage of V_{max}, and not be due to prolonged reduction of enzyme molecules. Since AChE activity was measured with a single concentration of substrate this was a potential problem. Figure 3 is an Eadie-Hofstee plot of brain AChE activity from control and DFP-treated male DBA mouse brain. The K_m of AChE remains constant; the only difference being a reduction of V_{max} in the DFP-treated mice. This indicates that the reduced level of measured activity is due to less enzyme being present, and not to altered substrate binding properties of the enzyme.

In all of the studies above, AChE activity was measured in whole brain. Table 1 shows that AChE activity in each of six brain regions follows the pattern of whole brain activity quite well. After 1 day, DFP causes a marked reduction of AChE activity in each region, although hindbrain and hypothalamus were somewhat spared compared to the other regions. By day 13, AChE activity was about 70% of control. Thirty days after DFP administration the AChE activity of the areas that account for the majority of total brain AChE activity, cortex and midbrain, was still significantly reduced below control. It appears that in hindbrain and striatum the AChE activity may be fully recovered. This indicates that there may be regional differences in the return of AChE activity, but that whole brain AChE activity is generally a satisfactory measure of AChE activity.

Because a single exposure to DFP caused a long-term reduction in AChE activity, other cholinergic systems were checked to see if they were similarly affected. Figure 4 compares ChAT activity with AChE activity in four brain regions following DFP treatment. Both assays were performed using tissue that had been frozen at −70°C. Freezing has no effect on ChAT activity, but it increases AChE activity slightly in cortex, midbrain and hippocampus, and markedly in striatum. This freeze-thaw treatment probably releases more enzyme from the membranes in a manner similar to detergent treatment [18]. Following DFP treatment, the long-term reduction of AChE was seen in all areas except striatum (see

TABLE 1

REGIONAL DISTRIBUTION OF ACETYLCHOLINESTERASE ACTIVITY IN BRAIN FOLLOWING A SINGLE INJECTION OF DIISOPROPYLFLUOROPHOSPHATE

	Acetylcholinesterase Activity (μmols/hr/mg protein)					
Day	C	M	H	P	S	T
Control	7.55 ± 0.29	8.7 ± 1.03	5.78 ± 0.29	4.53 ± 0.18	15.20 ± 1.03	5.25 ± 0.52
1	0.94 ± 0.07*	1.42 ± 0.16*	1.52 ± 0.23*	0.84 ± 0.07*	1.87 ± 0.29*	1.53 ± 0.15*
% Control	12.4	16.3	26.3	18.5	12.3	29.1
13	4.74 ± 0.23*	3.97 ± 0.14*	4.49 ± 0.40*	2.88 ± 0.09*	10.86 ± 1.24*	3.67 ± 0.26*
% Control	62.8	45.6	77.7	63.5	71.4	69.9
30	5.66 ± 0.47*	6.14 ± 0.57*	6.00 ± 0.97	4.06 ± 0.29*	14.38 ± 1.67	3.97 ± 0.23*
% Control	74.9	70.5	103.8	89.6	94.6	75.6

Male DBA mice were injected with DFP (6.33 mg/kg). At the times indicated AChE activity was measured in cortex (C), midbrain (M), hindbrain (H), hippocampus (P), striatum (S), and hypothalamus (T). The listed values represent the mean ± the standard error of the mean of 5–10 separate determinations. An * indicates significant differences from the saline-injected control group, $p<0.05$.

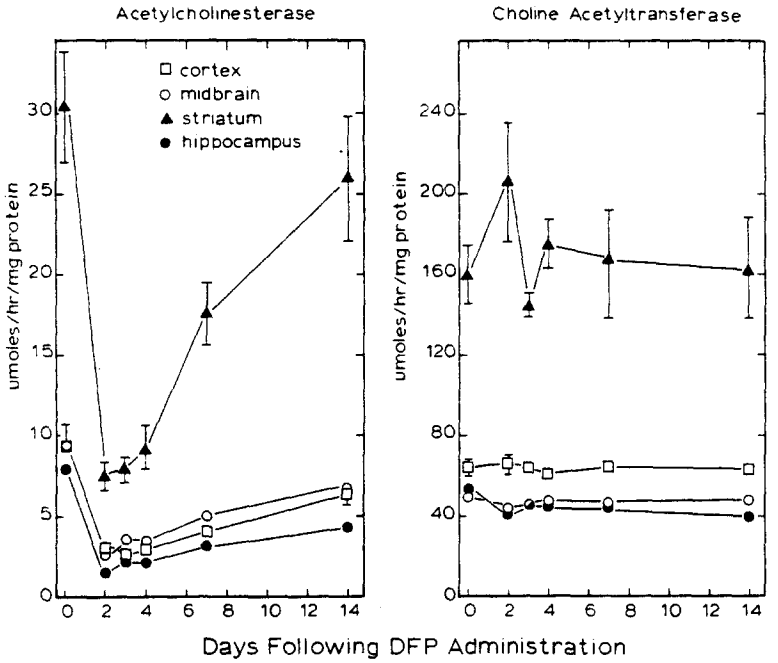

FIG. 4. Time course of AChE and ChAT activities in brain regions of male DBA mice following a single 6.33 mg/kg dose of DFP. In each panel the cortical activity is the V_{max} value calculated from Eadie-Hofstee plots. The activities of the other regions were determined using a single, saturating substrate concentration as detailed in the Method section. Each point is the mean±SEM of 6 individual mice.

also Table 1 which was a different experiment) but DFP had no effect on ChAT activity. There were no significant differences between saline- and DFP-treated animals in any region at any time.

Table 2 shows the effect of DFP on QNB binding in cortex 24 hours after exposure to DFP in three inbred mouse strains. There were no significant changes in either K_D or B_{max} for QNB binding in any of the strains. A complete time course of the effects of treatment with a single dose of DFP on QNB, BTX and nicotine binding was carried out in DBA mice. The data presented in Fig. 5 show that QNB, BTX or nicotine binding were not significantly changed by a single injection of DFP in any brain region for as long as 2 weeks post treatment. These results indicate that the long-term effects of DFP on AChE activity do not generalize to the entire cholinergic system.

DISCUSSION

The most important observation made in the present study is that brain AChE activity does not return to control levels in male mice following a single exposure to DFP,

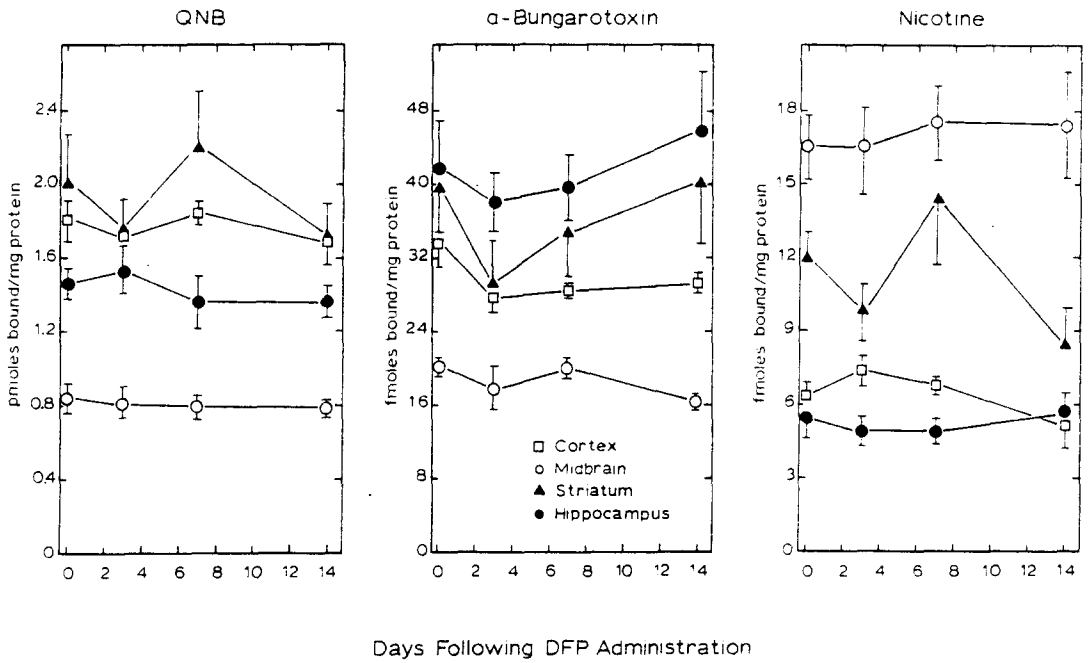

FIG. 5. Effect of a single exposure to DFP on ^{3}H-QNB, ^{125}I-α-BTX and ^{3}H-nicotine binding in brain regions as a function of time. Male DBA mice were injected with 6.33 mg/kg DFP and receptor binding assays were performed on the days indicated on the figure. In the QNB and α-BTX panels the cortical values are B_{max} values calculated for Scatchard plots. B_{max} was not determined for nicotine. All other values were determined at a single concentration of radiolabelled ligand as detailed in the Method section. Each point is the mean±SEM of 6 individual mice.

TABLE 2

^{3}H-QNB BINDING IN CORTEX 24 HOURS FOLLOWING A SINGLE INJECTION OF DIISOPROPYLPHOSPHOFLUORIDATE

Strain	Treatment	(pM)	B_{max} (pmoles bound/ mg protein)
DBA	Saline	27.2 ± 3.3	1.81 ± 0.04
	DFP	25.4 ± 4.7	1.80 ± 0.13
C57BL	Saline	24.3 ± 3.3	1.88 ± 0.09
	DFP	21.9 ± 2.7	1.62 ± 0.09
C3H	Saline	21.9 ± 2.8	1.90 ± 0.13
	DFP	24.2 ± 2.5	1.87 ± 0.20

Binding of ^{3}H-QNB was measured in cortical membranes. Five concentrations were used, and the binding constants were calculated from Scatchard plots of the data.

whereas control levels are regained in female mice. The extent of recovery of enzyme activity varied among brain regions in male mice. AChE activity was still significantly depressed in cortex, midbrain, and hypothalmus 30 days after DFP, but control, or near control, levels of activity had been regained in striatum, hypothalamus, and hippocampus. Because the return of AChE activity after inhibition by organophosphates is thought to be due to *de novo* synthesis of new enzyme molecules [10,17], it appears that the synthesis of new AChE molecules is impaired in some brain regions in male, but not female mice. We have previously demonstrated that full recovery of AChE activity is seen in reaggregate cultures obtained from fetal mouse brain [48]. Thus, sex and age influence the ability of neuronal cells to recover from DFP treatment.

Several other investigators have noted that AChE activity does not return to control levels following a single dose of an organophosphate. Kozar *et al.* [24] reported that 60 days following a single 1 mg/kg dose of DFP only 75–80% of control AChE activity was regained in selected regions (anterior preopitc hypothalamus, lateral hypothalamus, and caudate nucleus) of rat brain. Lemercier *et al.* [25] studied the return of AChE activity in several regions of rat brain following soman treatment and found that control levels were regained within 16 days in some brain regions (striatum, neocortex, cerebellum) but not in others (entorhinal cortex, medulla, mesencephalon). Similarly, Grubic *et al.* [16] have observed that AChE activity had not reached control activity in brain and the end plate region of the diaphragm 15 days after rats had been treated with soman, and Koelle *et al.* [22] noted that AChE activity in the stellate and superior cervical ganglia of cats treated with sarin was only 75% of control at equilibrium. Thus, it seems that a number of species fail to regenerate baseline AChE levels in a variety of tissues and selected brain regions following a single injection with various organophosphates. None of these investigators reported on sex differences.

A recent study of [^{3}H] DFP metabolism in the mouse [30] has demonstrated that radiolabelled DFP and its metabolites disappear from the blood and brain within minutes and only in liver and kidney were appreciable quantities of [^{3}H]-diisopropylphosphoric acid, the principal metabolite of DFP, found after 3 days. Thus DFP and its metabolites are no longer found in brain at a time when enzyme activity is still

less than 50% of control in our study. Therefore, the binding of DFP and its metabolites, in brain, do not correlate highly with AChE activity which is consistent with the suggestion [19] that irreversibly inhibited AChE is lost by protein degradation and that the recovery of enzyme activity represents the synthesis of new enzyme molecules.

Several recent studies have demonstrated that single doses of organophosphates can produce readily observable brain damage [3, 25, 27, 32, 33]. It has been argued [27.40] that these lesions may arise because of hypoxia that accompanies organophosphate-induced convulsions. We did not observe convulsions in our DFP-treated mice, but, at the doses we used, marked respiratory depression occurs [45]. This may have been sufficient to induce a mild hypoxia that affected a particularly sensitive subset of cells.

It is well known that some organophosphates, including DFP, can produce a characteristic neurotoxicity which is referred to as delayed peripheral neuropathy [1]. The cause of this peculiar neurotoxicity is not known, but there is an association between delayed peripheral neuropathy and an enzyme activity called neurotoxic esterase [20,21]. Organophosphates have the potential to interact with proteins other than AChE or neurotoxic esterase to cause a toxic reaction. For example, organophosphates irreversibly inhibit other serine-containing enzymes including peptidases [31], carboxylesterases [2, 4, 5], and cholinesterases [34]. In many cases the endogenous substrates for these enzymes is unknown. Therefore, it is conceivable that inhibition of one or more of these known enzymes (or other enzymes not yet described) could result in neurotoxicity by causing the buildup of a potentially toxic substrate, or the lack of a needed product. Differences between brain regions in the recovery of AChE activity may relate to regional differences in the buildup of these potential toxic substances. In addition, Reynolds *et al.* [33] have reported that the soman metabolite pinacolyl methylphosphonic acid (PMPA) binds covalently to proteins from a number of tissues in mice. This binding does not correlate well with the relative activities of AChE in these tissues. Detectable PMPA binding was seen as long as 7 days post treatment. This form of interaction is likely to be very non-specific, but an organophosphate or metabolite randomly binding to a critical protein could lead to neurotoxicity. If metabolites of organophosphates are neurotoxic the sex differences in return of AChE activity seen in mice might be due to sex differences in organophosphate metabolism. The fact that we observed full recovery of AChE activity in DFP-treated reaggregate brain cultures [48] is consistent with the suggestion that a metabolite of DFP alters regeneration of AChE activity.

In summary, a single dose of DFP results in inhibition of AChE which is not completely reversed in male mice. This failure to regain control activity may be a subtle indicator of central nervous system neurotoxicity. If a similar phenomenon occurs in humans, this may explain some of the long-term behavioral changes that are seen following organophosphate poisoning. Until more direct methods of assessing potential neurotoxicity of acute DFP exposure are developed, neurotoxic actions should be considered as a potential explanation for the failure to regain control AChE activity in DFP treated male mice.

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