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ELEVATED CHOLINE LEVELS IN BRAIN

A NON-CHOLINERGIC COMPONENT OF ORGANOPHOSPHATE TOXICITY

CHERYL J. FLYNN and LYNN WECKER*

Department of Pharmacology, Louisiana State University Medical Center, New Orleans, LA, U.S.A.

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Abstract—The role of cholinergic and non-cholinergic mechanisms in mediating organophosphate cholinesterase (ChE) inhibitor-induced elevations in choline levels in brain was investigated. The nerve agents soman and sarin, when administered to rats at doses greater than the IC_{50} for acetylChE inhibition, significantly increased the levels of choline and acetylcholine in both the striatum and hippocampus. The elevation in choline levels was evident 1 hr after injection with a maximal increase at 2 hr. Levels of choline returned to control by 4 hr. In contrast, the administration of diisopropyl phosphorofluoridate at doses greater than the IC_{50} for acetylChE inhibition increased the levels of acetylcholine, but did not alter the concentration of choline during the first 3 hr. Between 4 and 24 hr after injection, however, a significant decrease in choline levels was apparent. This effect persisted for 48 hr. When rats were pretreated with the anticonvulsant diazepam, the sarin- and soman-induced increases in choline levels were attenuated significantly. Results indicate that the organophosphates differentially alter the levels of choline in brain and suggest that the effect of soman and sarin to elevate choline levels is not a reflection of excessive cholinergic activity, but rather may be a consequence of the excitotoxic actions of these compounds.

The concentration of choline in brain is increased significantly within 1 hr following the administration of the highly toxic cholinesterase (ChE) inhibitor soman [1]. Although the biochemical and physiological significance of this effect is not understood, increased levels of choline may result from enhanced phospholipid hydrolysis through a predominantly non-cholinergic mechanism such as hypoxia [2, 3] or through direct muscarinic receptor stimulation mediated by elevated levels of acetylcholine (ACh) [4]. While the toxic effects of the organophosphates are generally ascribed to excessive cholinergic activity in both the peripheral and central nervous systems [5–7], not all effects produced by these compounds can be attributed to an enhanced activity of cholinergic neurons [8, 9]. Therefore, to determine the possible mechanisms involved in the soman-induced increase in choline levels, we investigated whether the choline increase was: (a) a characteristic effect of organophosphate ChE inhibitors; (b) dependent on an increase in the levels of ACh; (c) dependent on ChE inhibition; or (d) related to possible convulsant effects of the organophosphates. Results suggest that the increase in choline levels in brain following the administration of soman and sarin is a result of the convulsive properties of these compounds rather than a primary increase in cholinergic activity.

* Send correspondence to: Dr. Lynn Wecker, Department of Pharmacology, LSUMC, 1901 Perdido St., New Orleans, LA 70112.

MATERIALS AND METHODS

Male Sprague–Dawley rats (160–250 g, Harlan Industries, Indianapolis, IN) were used for all studies. Rats were group housed, maintained on a 12-hr light/dark cycle (6:00 a.m./6:00 p.m.), and had access to food (Purina Rat Chow 5001, Ralston Purina Co., Richmond, IN) and water *ad lib.*

Paraoxon (diethyl-*p*-nitrophenyl phosphate) was obtained from the Aldrich Chemical Co. (Milwaukee, WI) and was dissolved in saline to a stock concentration of 1.4 mg/ml. Solutions of soman (pinacolyl methylphosphonofluoridate) and sarin (isopropyl methylphosphonofluoridate) were obtained from the USAMRDC (Frederick, MD) and were diluted with saline immediately prior to use to a stock concentration of 0.35 and 0.57 mg/ml respectively. DFP (diisopropyl phosphorofluoridate), obtained from the Sigma Chemical Co. (St. Louis, MO), was dissolved in peanut oil to a stock concentration of 18 mg/ml. The organophosphate compounds were administered subcutaneously in microliter volumes (50 μl maximum). Diazepam (Hoffmann-LaRoche, Nutley, NJ) was dissolved in propylene glycol/ethanol/water (4/3/3, by vol.) containing one drop of 2 N HCl/10 ml to a final concentration of 4 mg/ml and was injected intraperitoneally (0.1 ml/100 g body wt). All other chemicals were of reagent grade and were used without further purification.

For the analysis of choline and ACh levels, rats were killed by head-focused microwave irradiation [10]. The brains were removed immediately and

chilled in ice-cold heptane, and striata and hippocampi were dissected. The brain regions were weighed, homogenized in acetonitrile containing propionylcholine as the internal standard, and prepared for the simultaneous determination of choline and ACh by pyrolysis gas chromatography [11, 12]. The concentrations of choline and ACh were calculated as nmoles/g tissue.

AcetylChE and ChE activities were measured spectrophotometrically using acetylthiocholine (3×10^{-3} M) as substrate [13]. Rats were killed by decapitation, and the brains were removed and chilled in ice-cold sodium phosphate buffer (0.1 M, pH 8.0). The striata and hippocampi were isolated and homogenized in phosphate buffer. For the determination of ChE activity, the reactions were initiated by the addition of substrate, and the rates of hydrolysis were monitored on a Beckman DU-8 spectrophotometer with a Kinetics II Module (Beckman Inst. Co., Houston, TX). For the determination of acetylChE activity, homogenates were incubated for 15 min with the specific BuChE inhibitor iso-OMPA (tetramonoisopropylpyrophosphortetramide, 10^{-5} M) prior to substrate addition. Enzyme activity was calculated as nmoles acetylthiocholine hydrolyzed · min^{-1} · (mg tissue)$^{-1}$.

All results are expressed as group mean values ± S.E.M. Data were analyzed on a DEC-10 by analysis of variance (ANOVA) and, depending on the experimental protocol, levels of significance were determined by either Newman-Keuls or Dunnett's test [14, 15]. P values of less than 0.05 were accepted as significant.

RESULTS

Initial studies determined the dose–response effects of soman, sarin, DFP, and paraoxon on acetylChE and ChE activities in rat brain. At 1 hr following the administration of these compounds, both acetylChE and ChE activities in striata and hippocampi were inhibited to a similar extent. Figure 1 shows the data obtained for acetylChE activity. To determine whether the increase in choline levels in brain observed following the administration of soman [1] was also evident after the administration of sarin, paraoxon, and DFP, the organophosphate compounds were administered at doses that inhibited acetylChE activity in brain by 90–100%. One hour after the injection of either soman (70 μg/kg) or sarin (100 μg/kg), a significant increase in the level of choline was evident in both brain regions (Fig. 2). The increase induced by sarin and soman were of similar magnitude, with the effect in the hippocampus (201–214% of control) larger than that in the striatum (133–152% of control). In contrast, no

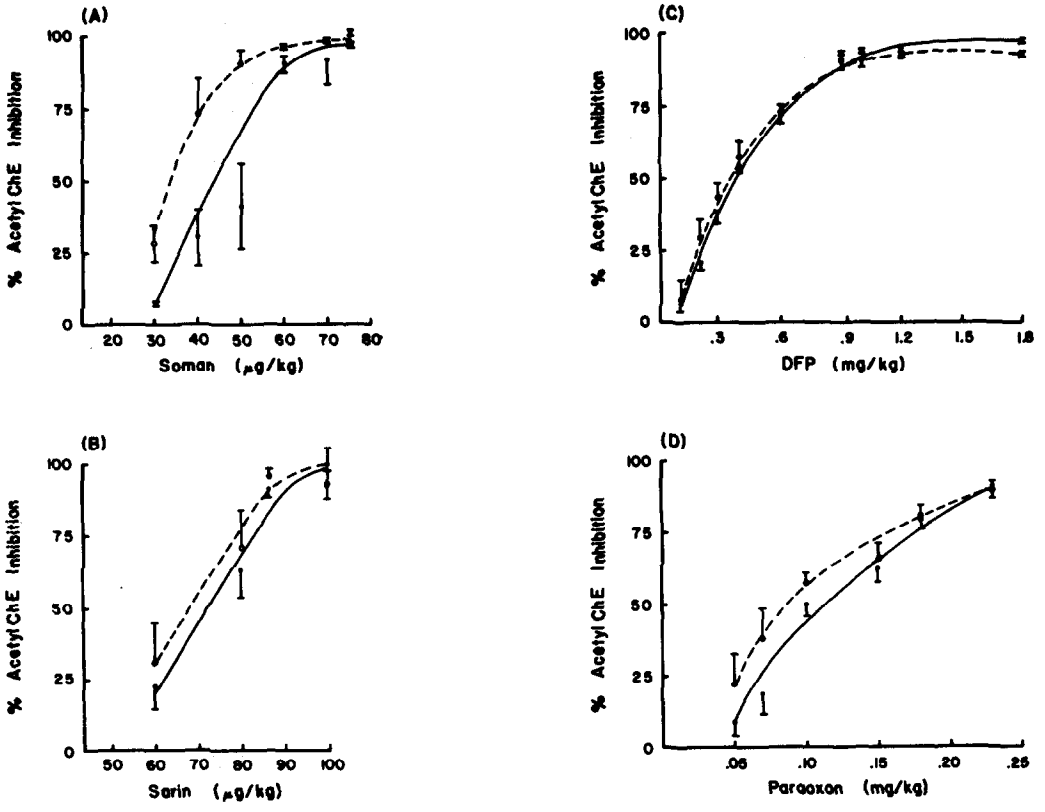

Fig. 1. Effects of soman, sarin, DFP and paraoxon on the activity of acetylChE in striatum and hippocampus of rat brain. Rats were injected subcutaneously with a range of doses of the agents or control vehicle and killed 1 hr after injection. A dotted line represents values obtained in the hippocampus and a solid line, those in the striatum. Each point represents the mean ± S.E.M. of determinations from two to eight rats per group. Control values (N = 22) for striatum and hippocampus were, respectively, 47.6 ± 1.88 and 6.63 ± 0.29 nmoles acetylthiocholine hydrolyzed · min^{-1} · (mg wet wt)$^{-1}$.

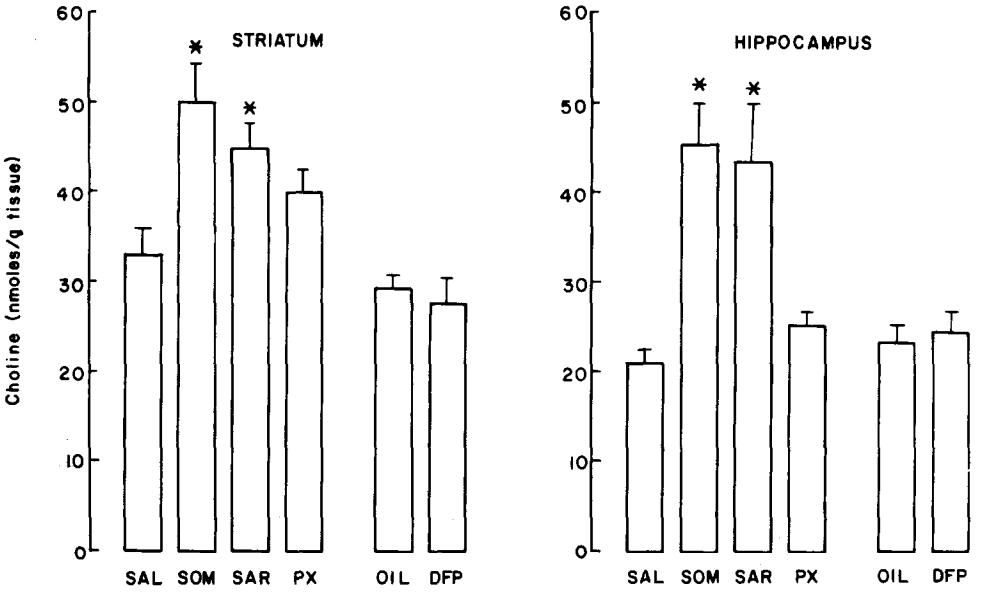

Fig. 2. Effects of organophosphates on levels of choline in rat brain. Rats received injections (s.c.) of soman (SOM, 70 μg/kg), sarin (SAR, 100 μg/kg), paraoxon (PX, 0.23 mg/kg), or DFP (1.8 mg/kg) and were killed 1 hr after injection by head-focused microwave irradiation. Choline levels were quantified by pyrolysis gas chromatography. Bars represent the mean ± S.E.M. of determinations from three to eighteen rats per group. Key: (*) significantly different from corresponding control values using Dunnett's test ($P < 0.05$).

changes were noted in either brain area from rats injected with paraoxon (0.23 mg/kg) or DFP (1.8 mg/kg).

Since studies by Shih [1] indicated that the effect of soman was time-dependent, it was possible that 1 hr of ChE or acetylChE inhibition following the administration of DFP and paraoxon was insufficient to produce an effect. Hence, the effects of the organophosphates on brain levels of choline were determined at intervals up to 96 hr following injection (Fig. 3). The effect of soman (70 μg/kg) was maximal at 2 hr with an 88 and 350% increase in choline

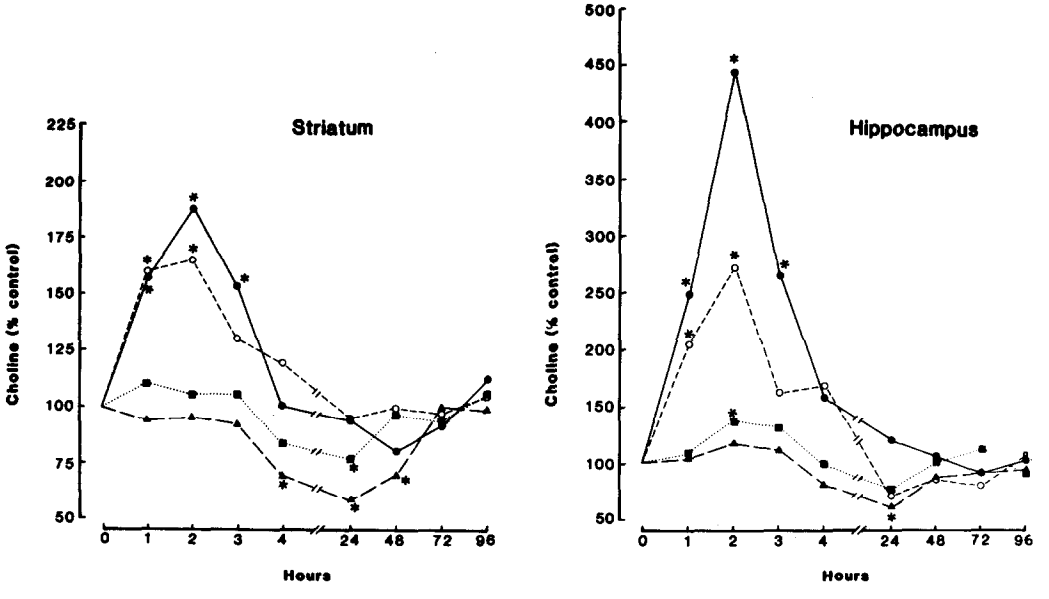

Fig. 3. Time course of effects of organophosphates on choline levels in rat brain. Rats received injections (s.c.) of soman (70 μg/kg, ●—●), sarin (100 μg/kg, ○---○), paraoxon (0.23 mg/kg, ■...■), or DFP (1.8 mg/kg, ▲--▲) and were killed at the times indicated by head-focused microwave irradiation. Each point represents the mean of 3–21 determinations. For clarity, error bars have been omitted. The error about each mean was approximately ±9%. Key: (*) significantly different from control using Dunnett's test ($P < 0.05$).

levels in striatum and hippocampus respectively. The concentration of choline remained elevated significantly for 3 hr following injection and returned to control by 4 hr. The effects of sarin (100 μg/kg) were less pronounced than those of soman (a 58 and 266% increase in striatum and hippocampus, respectively, at 2 hr), but the time course for changes was identical for both compounds. In contrast to the effects of sarin and soman, the administration of DFP (1.8 mg/kg) did not alter the levels of choline during the first 3 hr. However, a significant 30–45% decrease was evident in the striatum from 4 to 48 hr after administration. Similar, but less pronounced effects of DFP were noted in the hippocampus. The administration of paraoxon (0.23 mg/kg) did not alter choline levels in the striatum at early times after injection, but significantly decreased levels by 23% at 24 hr. In the hippocampus, paraoxon significantly increased choline levels by 38% at 2 hr, and this was followed by a decrease at 24 hr. Hence, results indicated that the effects of soman and sarin on choline levels in brain differed from those of DFP and paraoxon, suggesting that the choline increase was not a common characteristic of organophosphate ChE inhibitors.

Since studies have suggested that muscarinic receptor stimulation may increase the hydrolysis of phospholipids and enhance the release of choline from brain tissue [4, 16], we determined whether the increase in choline was related to or accompanied an increase in the levels of ACh. In the striatum, doses of DFP, paraoxon, and soman that inhibited acetylChE activity by 90–100% all produced the same relative increase in ACh levels at 1 hr following administration (Fig. 4). In the hippocampus, the increase in ACh levels following the administration of paraoxon and DFP was not different from that induced by sarin. Since neither DFP nor paraoxon increased choline levels in striatum, data suggested that muscarinic receptor stimulation was not mediating the choline increase. In addition, although the increase in ACh levels in the striatum induced by sarin was less than that induced by paraoxon and DFP, sarin did elevate choline levels significantly.

Since the doses of all the compounds studied inhibited acetylChE activity by 90–100%, results suggested that the choline increase was not merely a consequence of acetylChE inhibition. To substantiate this finding, a range of doses of sarin and soman were administered and choline levels were determined in striatum and hippocampus 1 hr after injection. Doses of sarin and soman below the IC_{50} for acetylChE inhibition did not alter choline levels, whereas doses above the IC_{50} all led to the same relative increase. Thus, there was a specific range of doses of soman (60–75 μg/kg) and sarin (80–100 μg/kg) that produced the effect. In addition, measurements of enzyme activity at various time intervals following the injection of sarin and soman indicated no recovery by 4 hr, even though choline levels had returned to control values. Hence, data indicated that the time-dependent alterations in choline levels did not parallel the inhibition or recovery of acetylChE activity.

During the course of these experiments, we observed that soman and sarin caused tremors that were much more severe than those observed following the administration of DFP and paraoxon. All animals showed the usual symptoms of organophosphate poisoning (muscle fasciculations, salivation, and diarrhea), while those injected with either sarin or soman also exhibited repetitive head bobbing, often lapsing into tonic extension with clenched jaws and Straub tail. Occasional overt clonic seizures were also observed. These symptoms persisted for several hours. Since it has been reported that soman and sarin, unlike paraoxon and DFP, can induce seizures that are abolished or prevented by

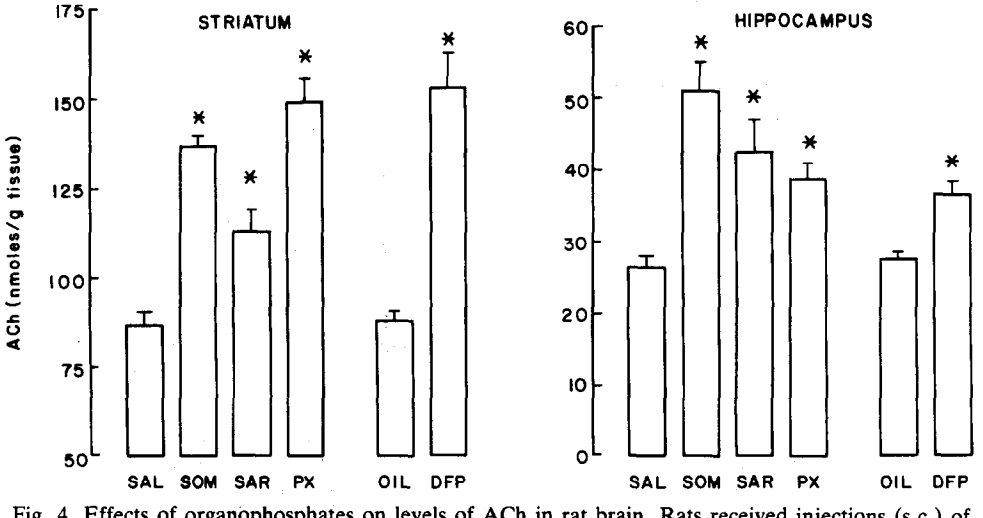

Fig. 4. Effects of organophosphates on levels of ACh in rat brain. Rats received injections (s.c.) of soman (SOM, 70 μg/kg), sarin (SAR, 100 μg/kg), paraoxon (PX, 0.23 mg/kg), or DFP (1.8 mg/kg) and were killed 1 hr after injection by head-focused microwave irradiation. ACh levels were quantified by pyrolysis gas chromatography. Bars represent the mean ± S.E.M. of determinations from three to nine rats per group. Key: (*) significantly different from corresponding control values using Dunnett's test ($P < 0.05$).

Table 1. Effects of diazepam on soman-induced increases in levels of choline in brain

Treatment	Choline (nmoles/g) Striatum	Hippocampus
Saline	31.6 ± 1.84 (8)	17.0 ± 1.40 (8)
Diazepam	32.1 ± 3.36 (7)	18.8 ± 1.02 (7)
Soman	54.2 ± 7.07* (7)	68.2 ± 15.1* (6)
Soman after diazepam pretreatment	33.0 ± 2.69† (7)	30.2 ± 5.39*† (6)

Rats received injections of saline or diazepam (4 mg/kg, i.p.) 30 min prior to the injection of either saline or soman (70 μg/kg, s.c.). Animals were killed by head-focused microwave irradiation 2 hr after the second injection, and choline levels were measured by pyrolysis gas chromatography. Each value is the mean ± S.E.M. The number of rats per group is in parentheses. Data were analyzed by ANOVA.

* Significantly different from corresponding control (saline or diazepam) values using the Newman–Keuls test ($P < 0.05$).

† Significantly different from soman-injected group using the Newman–Keuls test ($P < 0.05$).

diazepam [17–20], the effects of diazepam on the sarin- and soman-induced increase in choline levels were determined. Diazepam alone (4 mg/kg, i.p.) did not alter choline levels in either brain region (Table 1). However, when rats received an injection of diazepam 30 min prior to the administration of soman, the soman-induced increase in choline levels in the striatum was totally prevented (Table 1). In the hippocampus, diazepam attenuated the soman effect, with choline levels following the combined injection schedule at 178% of control, whereas soman alone increased choline levels to 401% of control. In addition to attenuating the choline increase, diazepam pretreatment also reduced the severity of the soman-induced tremors. When the effects of diazepam on sarin-induced alterations in brain choline levels were measured, a similar prophylactic effect was noted (Table 2). Measurements of acetylChE activity indicated that this dose of diazepam did not affect enzyme activity by itself nor did it protect acetylChE from inhibition by soman or sarin. Hence, results suggested that the choline increase may have been related to the convulsive properties of these compounds.

DISCUSSION

The steady-state concentration of choline in brain represents the summation of numerous metabolic processes. Choline is both released from and incorporated into phospholipids as well as ACh, the former representing the major (>95%) metabolic pathway for choline. Increased levels of choline in brain may result from either a decreased incor-

Table 2. Effects of diazepam on sarin-induced increases in levels of choline in brain

Treatment	Choline (nmoles/g) Striatum	Hippocampus
Saline	36.3 ± 1.57 (14)	21.1 ± 0.95 (13)
Diazepam	35.0 ± 1.33 (11)	22.0 ± 1.38 (10)
Sarin	50.9 ± 2.77* (13)	63.2 ± 5.70* (15)
Sarin after diazepam pretreatment	43.2 ± 1.85*† (11)	40.1 ± 2.94*† (11)

Rats received injections of saline or diazepam (4 mg/kg, i.p.) 30 min prior to the injection of either saline or sarin (100 μg/kg, s.c.). Animals were killed by head-focused microwave irradiation 2 hr after the second injection, and choline levels were measured by pyrolysis gas chromatography. Each value is the mean ± S.E.M. The number of rats per group is in parentheses. Data were analyzed by ANOVA.

* Significantly different from corresponding control (saline or diazepam) values using the Newman–Keuls test ($P < 0.05$).

† Significantly different from sarin-injected group using the Newman–Keuls test ($P < 0.05$).

poration of choline into phospholipids, or an increase in the hydrolysis of phospholipids. Studies have shown that choline levels in brain are increased following the administration of oxotremorine, physostigmine, and muscarine [16, 21], and it has been suggested that this choline increase reflects enhanced phosphatidylcholine hydrolysis mediated by muscarinic receptor stimulation [4, 16]. However, results from the present study do not support a muscarinic receptor-mediated event. Doses of soman and sarin that inhibited acetylChE activity by greater than 50% increased the concentrations of both choline and ACh in striatum and hippocampus 1–3 hr after administration. However, doses of DFP that similarly inhibited acetylChE activity and increased the levels of ACh in brain did not increase the levels of choline. Studies by Ladinsky *et al.* [16] also indicated that, although DFP increases ACh levels in mouse brain, it does not increase choline levels. They also reported that doses of physostigmine that increase choline levels in the cerebellum do not alter ACh levels in this brain area. Therefore, data suggest that another mechanism must be responsible for inducing phospholipid hydrolysis, with a consequent increase in choline levels.

The ability of soman and sarin to mobilize large amounts of choline may be related to their excitotoxic effects. Soman, when administered at doses comparable to those used in this study, produces convulsions and increases 2-deoxyglucose metabolism in brain [22]. It is well documented that seizures and hypoxia activate the hydrolysis of phosphatidylcholine, thereby releasing free fatty acids and, presumably, choline [23, 24]. In addition, prolonged seizure activity, especially when accompanied by respiratory impairment, leads to a cerebral energy deficiency [25] which could result in a decreased reutilization of choline for lipid synthesis. Since diazepam and other benzodiazepines abolish soman-induced seizure activity and convulsions [17–20], the present results, indicating that diazepam reduced the sarin- and soman-induced choline increase, suggest that choline mobilization may also be a consequence of seizure activity. It is likely that muscarinic receptor activation does not subserve this activity since atropine pretreatment is ineffective against soman-induced seizures [20]. Unfortunately, parallel experiments to determine whether atropine pretreatment prevents the induced choline increase are not feasible since atropine itself has been shown to increase choline levels in brain [26]. The failure of DFP to increase choline levels may be due to the absence of convulsive activity at the doses used in the present study. Although it is difficult to compare *in vivo* potency, studies using cortical slices have shown that soman is at least ten times more potent than DFP in inhibiting respiration in these slices [9].

In contrast to the effects of sarin and soman, the administration of paraoxon and DFP decreased choline levels 4–24 hr after administration. These findings are in agreement with those of Russell *et al.* [27] and Potter *et al.* [28]. In addition, Modak *et al.* [29] also observed a depression in choline levels in striatum and hippocampus between 3 and 24 hr after the administration of dichlorvos. It is likely that decreased choline levels result from inhibition of phospholipid hydrolysis *in vivo* by these organophosphates [30]. There is indirect evidence to indicate that DFP inhibits brain phospholipase A_2 [31], and several studies have shown that organophosphates phosphorylate other types of lipases [32, 33]. Studies in our laboratory have indicated that DFP and paraoxon inhibit the postmortem release of choline, whereas sarin and soman have no effect [34]. Since this release of choline is thought to reflect phospholipid hydrolysis [30, 35], results suggest that the DFP- and paraoxon-induced decrease in choline levels may be mediated by phospholipase inhibition. Preliminary studies in our laboratory demonstrating inhibition of phospholipase A_2 activity in brain following the administration of DFP support this hypothesis.

In conclusion, data indicate that both soman and sarin increase choline levels in brain, a characteristic not shared by all organophosphate ChE inhibitors. This choline increase appears to be unrelated to either acetylChE inhibition or elevated levels of ACh in brain and most likely reflects enhanced phospholipid hydrolysis secondary to alterations in brain metabolism. Hence, sarin- and soman-induced increases in brain levels of choline may represent a non-cholinergic component of organophosphate toxicity.

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