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RELATIONSHIP BETWEEN ORGANOPHOSPHATE TOXICITY AND CHOLINE METABOLISM

FINAL REPORT

LYNN WECKER

JUNE 6, 1986

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-83-C-3012

Louisiana State University Medical Center
1901 Perdido Street
New Orleans, Louisiana 70112

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) RELATIONSHIP BETWEEN ORGANOPHOSPHATE TOXICITY AND CHOLINE METABOLISM		5. TYPE OF REPORT & PERIOD COVERED FINAL REPORT NOV.15,1982 - DEC.15,1985 6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) LYNN WECKER		8. CONTRACT OR GRANT NUMBER(s) DAMD17-83-C-3012
9. PERFORMING ORGANIZATION NAME AND ADDRESS Louisiana State University Medical Center 1901 Perdido Street New Orleans, Louisiana 70112		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 6.27.34A 3M162734A875 AF.428
11. CONTROLLING OFFICE NAME AND ADDRESS USAMRDC - SGRD/RMI-S Fort Detrick Frederick, Maryland 21701-5012		12. REPORT DATE June 6, 1986 13. NUMBER OF PAGES 69
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)		15. SECURITY CLASS. (of this report) Unclassified 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

organophosphate cholinesterase inhibitors; brain; choline; acetylcholine; lipids; phospholipids; acetylchoolinesterase; convulsions; phospholipases, chemical warfare agents (JZ)

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Although research on the organophosphate cholinesterase (ChE) inhibitors has focused primarily on inhibition of acetylcholinesterase (AChE) and enhanced cholinergic transmission, these actions alone cannot explain the biochemical and physiological effects that persist long after enzyme activity has returned to normal. Although evidence indicates that some organophosphates alter the disposition and metabolism of choline and choline-containing compounds in the nervous system, the relationship of these changes to the toxicological manifestations of ChE inhibition has not been investigated. Therefore, one objective of this research was to investigate the relationship

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between the toxicity of the organophosphates and the metabolism of choline and choline-containing compounds in brain. Specifically, studies included: a) determination of the effects of choline supplementation on the biochemical and toxicological effects of the organophosphates; b) determination of the effects of the organophosphates on the disposition and metabolism of choline; and c) elucidation of the relationships among the disposition and metabolism of choline, the metabolism of acetylcholine (ACh), and the toxicological manifestations resulting from ChE inhibition.

Studies investigating the effects of choline supplementation on the actions of the organophosphates indicated that supplemental choline was ineffective in altering the biochemical or toxicological effects of these compounds in rats. Since previous results indicated that choline supplementation protected mice from the toxic effect of paraoxon, it appears that the mechanisms regulating the metabolism of choline, as well as the specific interactions of the organophosphates with biochemical systems, may differ between the two species.

The second major objective was to determine the effects of soman, sarin, diisopropylfluorophosphate (DFP), and paraoxon on choline/phospholipid metabolism and elucidate the relationship of these changes to inhibition of ChE. When rats received doses of sarin, soman, DFP, and paraoxon that totally inhibited ChE activity and caused similar increases in ACh levels, soman and sarin increased choline levels at early times after injection (one to two hours), whereas DFP and paraoxon had no effect at early times, but decreased choline levels at twenty-four to forty-eight hours after administration. Thus, alterations in choline levels were independent of both ChE inhibition and levels of ACh.

Since increased choline levels may result from phosphatidylcholine hydrolysis secondary to convulsions, the role of seizures and phosphatidylcholine catabolism in mediating the choline increase was investigated. Soman and sarin increased the levels of free fatty acids in brain and the increases in both choline and free fatty acids were prevented by pretreating animals with the anticonvulsant diazepam. In addition, bicuculline, a non-ChE-inhibiting convulsant also elevated choline levels. Thus, elevated levels of choline in brain induced by soman and sarin may be the result of excitotoxic effects and the associated catabolism of phosphatidylcholine.

To determine whether the organophosphates altered phosphatidylcholine catabolism, the activity of phospholipase A2 was investigated. Although soman, sarin, and DFP inhibited enzyme activity *in vitro* to the same extent, only DFP was active in inhibiting enzyme activity after *in vivo* administration. The physiological significance of enzyme inhibition was evaluated by investigating the postmortem accumulation of choline, a functional index of lipolytic activity. DFP and paraoxon inhibited the postmortem accumulation of choline, whereas sarin and soman had no effect.

Results indicate that sarin, soman, DFP, and paraoxon are similar in inhibiting ChE and elevating levels of ACh, but are dissimilar in their effects on choline levels, neuronal activity, and phospholipase A2 activity. These differential effects may explain the differences in the neurotoxicity of these compounds.

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SUMMARY

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Since increased choline levels may result from phosphatidylcholine hydrolysis secondary to convulsions, the role of seizures and phosphatidylcholine catabolism in mediating the choline increase was investigated. Soman and sarin increased the levels of free fatty acids in brain and the increases in both choline and free fatty acids were prevented by pretreating animals with the anticonvulsant diazepam. In addition, bicuculline, a non-ChE-inhibiting convulsant also elevated choline levels. Thus, elevated levels of choline in brain induced by soman and sarin may be the result of excitotoxic effects and the associated catabolism of phosphatidylcholine.

To determine whether the organophosphates altered phosphatidylcholine catabolism, the activity of phospholipase A2 was investigated. Although soman, sarin, and DFP inhibited enzyme activity *in vitro* to the same extent, only DFP was active in inhibiting enzyme activity after *in vivo* administration. The physiological significance of enzyme inhibition was evaluated by investigating the postmortem accumulation of choline, a functional index of lipolytic activity. DFP and paraoxon inhibited the postmortem accumulation of choline, whereas sarin and soman had no effect.

Results indicate that sarin, soman, DFP, and paraoxon are similar in inhibiting ChE and elevating levels of ACh, but are dissimilar in their effects on choline levels, neuronal activity, and phospholipase A2 activity. These differential effects may explain the differences in the neurotoxicity of these compounds.

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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I. EFFECTS OF CHOLINE AVAILABILITY ON ACTIONS OF THE ORGANOPHOSPHATES

Rationale

Choline is an ubiquitous molecule that plays a major role in both the synthesis of acetylcholine (ACh) and the metabolism of phospholipids. Although some controversy exists concerning the forms of choline available for the synthesis of ACh, it is generally believed that a significant portion of the choline used for the synthesis of neurotransmitter is generated from the hydrolysis of ACh (1-4). When this source of choline is limited under conditions of cholinesterase (ChE) inhibition, the steady-state concentration of choline may decrease (5,6). Dose-response studies utilizing paraoxon have shown a biphasic effect of this compound on free levels of choline in brain fifteen minutes following injection. Administration of 0.115 mg/kg paraoxon, which inhibits acetylcholinesterase (AChE) activity in brain by 40% and increases the levels of ACh by 20%, causes a significant 40% increase in the levels of free choline. However, administration of 0.23 mg/kg paraoxon, which inhibits brain AChE by >90% and increases the levels of ACh by 50%, causes a 25% decrease in the levels of free choline (6). Furthermore, the administration of choline chloride sixty minutes prior to paraoxon, which did not alter the levels of choline by itself, prevented the paraoxon-induced decrease in the levels of free choline. Results of recent studies have suggested that this effect of choline is mediated through alterations in choline-containing phospholipids (7). Hence, evidence suggests a relationship between AChE or ChE inhibition and the disposition and metabolism of choline and choline-containing compounds.

A. TOXICITY STUDIES

Objective:

a) to investigate the effects of dietary choline availability on the toxicity of the organophosphates in rats

Background

Studies have indicated the presence of a biochemical interaction between the neurotransmitter precursor choline and the organophosphate paraoxon. When choline was administered prior to paraoxon, choline potentiated the paraoxon-induced increase in the levels of ACh and prevented the paraoxon-induced depletion of choline in rat brain (6). In addition, the chronic administration of choline to mice protected animals from the toxic effects of paraoxon (8). Mice maintained on a choline-free diet were more susceptible to the toxic effects of paraoxon than mice maintained on a choline-supplemented (2.0% choline chloride) diet. Thus, the toxicity of paraoxon in mice is inversely proportional to the concentration of free choline in the diet (8). Since dietary choline deficiency decreases brain phosphorylcholine levels, whereas dietary choline supplementation increases the content of this phosphatidylcholine precursor (9), it is possible that the toxicity of paraoxon may be influenced by altering the dietary intake of choline. Therefore, it was determined whether choline supplementation could protect rats against the toxicity of paraoxon.

Methods

Male Sprague-Dawley rats (initial weight 140-160 g) were group housed and maintained on a twelve hour light/dark cycle with food and water available *ad libitum*. Rats were randomly assigned to one of three dietary groups and fed: choline-deficient chow (0% choline chloride), basal choline chow (0.2% choline chloride); or choline-supplemented chow (2.0% choline chloride) for twenty-eight to thirty-two days. Following maintenance on the dietary regimens, rats received various doses of paraoxon subcutaneously (sc) and the percentage of animals dying in each group was noted. Dose-response curves were constructed and the LD50 values (the dose necessary to kill 50% of the animals in each group) of paraoxon were calculated for each group.

Results

The LD50 values of paraoxon for rats maintained on the choline-deficient, basal choline, and choline-supplemented chows were 0.24, 0.21, and 0.21 mg/kg, respectively, suggesting no protective effect. In light of results from studies by Shih (personal communication) indicating that acute choline supplementation did not alter the toxicity of either sarin or soman, further toxicity studies were not pursued.

Discussion

On the basis of the results obtained, it appears that the chronic availability of choline in the diet does not have a profound influence on the toxicity of paraoxon in rats. It is interesting that while choline supplementation protects mice from the toxic effects of paraoxon, it has no effects in rats. This result implies that perhaps the mechanisms regulating choline and phospholipid metabolism differ between the two species. Since no significant effects of choline supplementation on the actions of organophosphates were detected, further experiments pursuing a possible interaction between organophosphate toxicity and choline supplementation were deemed futile.

B. CHOLINESTERASE STUDIES

Objectives:

a) to investigate the effects of dietary choline availability on the kinetics of AChE; and
b) to determine the effects of choline supplementation on the inhibition of AChE by paraoxon and fluoride

Background

Many studies have suggested that choline and phospholipids influence the activity of ChE and its susceptibility to inhibition by the organophosphates. A number of membrane-bound proteins, including AChE, are regulated by their phospholipid environment (10-12). Administration of a phospholipid-rich (20%) diet to rats has been shown to alter the content and composition of cellular phospholipids in both liver and brain (13). When the concentrations of non-choline-containing phospholipids in brain were significantly increased relative to choline-containing phospholipids, the activity of AChE was enhanced; both the maximal reaction velocity and affinity of the enzyme for substrate were significantly increased (13). Therefore, studies determined: a) the effects of dietary choline availability on the kinetics of AChE; and b) the effects of choline supplementation on the inhibition of AChE by paraoxon and fluoride.

Methods

Male Sprague-Dawley rats (initial weight 140-160 g) were group housed and maintained on a twelve hour light/dark cycle with food and water available *ad libitum*. Rats were randomly assigned to one of three dietary groups and fed: choline-deficient chow (0% choline chloride); basal choline chow (0.2% choline chloride); or choline-supplemented chow (2.0% choline chloride) for forty to fifty days. Following maintenance on the dietary regimens, rats were killed by decapitation, and the brains were removed and chilled in ice-cold 0.32 M sucrose. The striata and hippocampi were isolated, weighed, and homogenized (glass:teflon) in 5 ml 0.32 M sucrose. To obtain a particulate preparation from the crude mitochondrial fraction, samples were centrifuged at 1,000 x g for ten minutes at 2°C, followed by recentrifugation of the supernatant at 17,500 x g for twenty minutes at 2°C. The supernatants were discarded and the pellets (crude mitochondrial fractions) were resuspended in 5 mM phosphate buffer (pH=7.4) for a concentration of 0.5 ml buffer/100 mg original tissue wet weight. Samples were homogenized (glass:teflon), set on ice for twenty minutes, and centrifuged at 47,000 x g for twenty minutes at 2°C. The pellets thus obtained were washed with 0.5 ml phosphate buffer, rehomogenized, and recentrifuged. The crude mitochondrial membrane preparations obtained were resuspended in 0.1 M phosphate buffer (pH=8.0) for tissue concentrations of 15 mg original tissue wet weight per milliliter for striata and 30 mg original tissue wet weight per milliliter for hippocampi.

AChE activity was determined spectrophotometrically using acetylthiocholine as substrate (14). Tissue samples (0.1 ml) were preincubated for fifteen minutees at room temperature with tetramonoisopropylpyrophosphortetramide (iso-OMPA, 10 μM) to ensure inhibition of ChE. Substrate was added to initiate the reaction and the rate of the reaction was measured for a period of ten minutes using the Kinetics II module on a Beckman DU-8 Spectrophotometer. For kinetic determinations, five concentrations of acetylthiocholine were employed (0.01, 0.02, 0.1, 0.2, and 1.0 mM). For paraoxon inhibition studies, tissues were preincubated with paraoxon for fifteen minutes prior to the addition of substrate. The concentrations of paraoxon used for these studies were 0.005-50.0 μM. For allosteric inhibition studies, the samples were preincubated with sodium fluoride (NaF) for fifteen minutes. The concentrations of NaF employed were 0.1-4.0 mM.

Results

Dietary choline availability did not alter the kinetic parameters of particulate AChE from rat brain. The kinetic characterization of AChE from striata and hippocampi of rats fed the three dietary regimens is presented on page 8 of the 1982-83 Annual Report for this project.

Choline supplementation did not alter the inhibition of AChE activity by paraoxon or fluoride. Paraoxon inhibited AChE activity in tissue preparations from rats fed the basal chow in a concentration-dependent manner with maximal inhibition occurring at 10 μM; a concentration of 0.75 μM produced 50% inhibition. No differences were apparent for paraoxon-induced inhibition of AChE activity among tissues from the three dietary groups. Similarly, sodium fluoride, at concentrations of 0.5 to 4 mM produced a progressive concentration-dependent inhibition of enzyme activity. A maximal effect of 60% inhibition of enzyme activity in tissues from rats fed the basal chow was achieved with 4.0 mM fluoride and no differences were apparent for preparations from rats fed the supplemented or deficient chows. Total enzyme inhibition could not be achieved due to limited solubility.

Discussion

Results indicate that chronic alterations in the availability of choline in the diet do not affect the kinetic characteristics of AChE in a particulate preparation from a crude mitochondrial fraction of rat striatum and hippocampus. Furthermore, the susceptibility of the enzyme to inhibiton was also unaffected by modifications in the dietary intake of choline. Hence, while previous studies have indicated that alterations in dietary phospholipid composition modify enzyme activity (13), the present results suggest that modifications in the dietary intake of free choline do not produce similar effects.

C. PHOSPHOLIPID STUDIES

Objectives:

a) to determine the effects of choline supplementation on the release of choline from phospholipids; and
b) to determine the effects of choline supplementation on phospholipid profiles in rat brain

Background

It is well documented that whether administered intravenously, intraperitoneally, or directly into the brain, choline is metabolized first to water-soluble compounds followed by conversion to lipid-soluble esters (15-17). In light of the disappointing results obtained from the studies discussed above, it was necessary to determine whether dietary choline availability altered phospholipids in rat brain. Thus, both levels of phospholipids and the release of choline from phospholipids were determined in brain from rats maintained on the basal and choline-supplemented diets.

Methods

Male Sprague-Dawley rats (initial weight 140-160 g) were group housed and maintained on a twelve hour light/dark cycle with food and water available *ad libitum*. Rats were randomly

assigned to one of two dietary groups and fed: basal choline chow (0.2% choline chloride); or choline-supplemented chow (2.0% choline chloride) for twenty-eight to thirty-two days. For studies on the release of choline from phospholipids, half the animals in each group were killed by head-focused microwave irradiation and half were killed by decapitation. Brains were removed, chilled in ice-cold pentane, and the striata, hippocampi, and cortices dissected. Tissues were homogenized in acetonitrile containing propionylcholine as an internal standard, and prepared for the determination of the concentration of choline by pyrolysis gas chromatography (18,19). The rate of choline production was determined by subtracting the concentration of choline in brains from animals killed by microwave irradiation from the concentration of choline in brains from animals killed by decapitation.

For determination of the effects of dietary choline availability on membrane phospholipid profiles in rat striatum, animals were maintained on the basal or supplemented diets as described. Rats were killed by decapitation and the striata were removed, homogenized in 0.32 M sucrose, and synaptosomal membranes and microsomes were prepared (20). Phospholipids were isolated, separated by two dimensional thin layer chromatography, and analyzed for phosphorus by spectrophotometry (21,22).

Results

To provide a measure of the functional ability of the brain to convert choline esters to free choline, the rate of choline production postmortem was determined. The results of these studies are shown in Table 1. Supplementation with choline increased the rate of choline production in striatum, hippocampus, and cortex by 24%, 13%, and 53%, respectively. Hence, results suggested an effect of choline supplementation on the metabolism of bound sources of choline.

Since the production of choline postmortem is thought to be due to the release of choline from phosphatidylcholine, the concentration of this compound, as well as total lipid phosphorus, was measured in synaptosomal and microsomal fractions from striata. The results of these studies are shown in Table 2. It is evident that dietary choline supplementation did not alter the concentrations of either phosphatidylcholine or total lipid phosphorus in the synaptosomal membrane fraction. However, when lipids were quantitated in the microsomal fraction, a significant increase was apparent for the supplemented group.

Discussion

Results from these studies indicate that chronic supplementation with choline in the diet does not lead to alterations in the lipid composition of neuronal (synaptosomal) membranes. Although an increased intake of choline does significantly elevate circulating levels of choline and leads to an increased postmortem release of free choline, it does not increase the levels of either free choline or phosphatidylcholine in a neuronal membrane preparation. The functional significance of the increased phosphatidylcholine and total lipid phosphorus content of the microsomal fraction is unknown at this time.

II. EFFECTS OF THE ORGANOPHOSPHATES ON CHOLINE/PHOSPHOLIPID METABOLISM

Rationale

Administration of ChE inhibitors to animals causes the accumulation of ACh (23-25), which exerts a negative feedback effect on neurotransmitter synthesis (26-29). Since free choline, as well as choline-containing compounds, supplies precursor for ACh synthesis (30,31), it is reasonable to expect that organophosphate ChE inhibitors may disrupt the mechanisms regulating the synthesis of ACh.

Available data demonstrate that organophosphates have a complex spectrum of effects on choline metabolism via mechanisms that are not as well understood as those by which they affect ACh metabolism. Diisopropylfluorophosphate (DFP) is the agent most often studied, and data show that after acute or subchronic administration of this compound, choline levels in whole

Table 1

Effects of Dietary Choline Supplementation on the Postmortem Production of Choline

	Rate of Choline Production (nmol/g/min)	
	Dietary Regimen	
Brain Region	Basal	Supplemented
Striatum	30.2 ± 2.81	37.5 ± 2.18*
Hippocampus	24.0 ± 1.85	27.0 ± 2.25
Cortex	18.9 ± 2.39	28.9 ± 2.46*

Rats were maintained on either basal (0.2% choline chloride) or choline-supplemented (2.0% choline chloride) chow for twenty-eight to thirty-two days. Animals were killed by either decapitation or head-focused microwave irradiation. The rate of choline production is the difference between values obtained from animals killed by microwave irradiation and those obtained from rats killed by decapitation. Choline was analyzed by pyrolysis gas chromatography (18,19). Each value is the mean ± S.E.M. of determinations from seven to eight rats per group.
*Significantly greater than basal group values, $p < 0.05$.

Table 2

Effects of Dietary Choline Supplementation on Membrane Phospholipids in Rat Striatum

Subcellular Fraction	Dietary Regimen	Phosphatidylcholine (μmol/mg protein)	Total Lipid P
Synaptosomal Membranes	Basal	.3281 ± .0564	.8254 ± .1637
	Supplemented	.3313 ± .0362	.7398 ± .1059
Microsomes	Basal	.4895 ± .0151	.9874 ± .0552
	Supplemented	.5498 ± .0142*	1.208 ± .0694*

Rats were maintained on either basal (0.2% choline chloride) or choline-supplemented (2.0% choline chloride) chow for twenty-eight to thirty-five days. Animals were killed by decapitation and the striata isolated. Subcellular fractions were prepared (20) and phospholipids were isolated, separated by two dimensional thin layer chromatography, and analyzed for phosphorus by spectrophotometry (21,22). Each value is the mean ± S.E.M. of determinations from five groups, each group containing striata pooled from four rats.
*Significantly greater than basal group values, $p < 0.05$.

rodent brain are significantly depressed (5,32-34). In contrast, soman causes an acute elevation in choline levels (35), whereas dichlorvos causes an increase in choline immediately after administration and a decrease at later time points (36). There is no way of knowing whether these alterations reflect true differences in the mechanisms of action of different agents, or simply in pharmacokinetic variables. However, since the small free choline pool in brain (37) appears to be derived from several sources in addition to ACh hydrolysis, there are many points in the metabolic pathways of choline where organophosphates could exert an effect.

The few studies examining the mechanisms of organophosphate-induced changes in brain choline metabolism have suggested that phospholipid metabolism may also be affected (38,39). Most of the choline in brain is in the form of phosphatidylcholine (40). Thus, alterations in the metabolism of this compound are likely to result in quantitatively significant changes in brain choline levels. Free choline is incorporated into phosphatidylcholine via a series of enzymatically catalyzed intermediate steps described by Kennedy and Weiss (41). A study by Nelson and Barnum (38) demonstrated that DFP decreased the synthesis of phosphatidylcholine and the incorporation of radiolabelled phosphate into phospholipids in mouse brain one hour after administration, although a follow-up study (39) showed no effects of this agent on either choline phosphotransferase or choline-phosphate cytidylyltransferase.

In addition to inhibiting phospholipid synthesis, organophosphates may affect phospholipid degradation. Dross (32) showed that DFP decreased the postmortem release of choline and the concentrations of lysophosphatidylcholine and glycerophosphorylcholine in whole rat brain between thirty minutes and six hours after administration. The postmortem accumulation of choline is thought to be derived primarily from phosphatidylcholine (42) and may proceed via the actions of phospholipases A1 or A2, which cleave fatty acids from the one or two-position, respectively, followed by the actions of lysophospholipases, which remove the remaining fatty acid (43). The product, glycerophosphorylcholine (GPC), is readily hydrolyzed by GPC phosphodiesterase to glycerol phosphate and choline (31,44-46). Studies suggest the inhibition of phospholipase A as a possible mechanism for the depression of choline levels observed with DFP (32). Inhibition of phospholipases A by organophosphates has been demonstrated in some systems (47,48) although there appears to be only one study on nervous tissue that directly demonstrates inhibition of phospholipase A activity by organophosphates. Cooper and Webster (49) found that 200 μM DFP inhibits phospholipase A extracted from human brain. Phospholipase D and lysophospholipase D activities have also been identified in brain; they release free choline from phosphatidylcholine and lysophosphatidylcholine, respectively (31,50-52). The effects of organophosphates on these enzymes have not been investigated.

In general, available information suggests that altered choline levels in response to organophosphates are mediated by more than one mechanism. Some effects do not appear common to all agents, but what relationship, if any, these perturbations have to ChE inhibition is unclear. The variety of agents, doses, and experimental protocols used in previous studies makes valid comparisons impossible; thus, conclusions lending themselves to further investigations are difficult to draw. Nevertheless, there appears to be sufficient indication that organophosphate compounds could affect more than one site of choline metabolism. While it is unlikely that a simple relationship exists between organophosphate poisoning and choline metabolism, the implication of this relationship, viz., that structural or functional damage to neural membranes may be a toxic action of these compounds, is a significant one. To examine this relationship further, some of the effects of four organophosphates, viz., soman, sarin, DFP, and paraoxon, on choline levels in brain were characterized. Possible mechanisms linking the effects of the organophosphates on the steady-state levels of choline to ChE inhibition, ACh accumulation, and choline lipid metabolism were studied.

A. INITIAL CHARACTERIZATION OF THE EFFECTS OF THE ORGANOPHOSPHATES ON CHOLINERGIC PARAMETERS IN THE STRIATUM AND HIPPOCAMPUS OF RAT BRAIN

Objectives:

a) to establish dose-response relationships for AChE inhibition;
b) to determine whether any effects on choline levels were dose-dependent and whether the dose dependency was similar to that for AChE inhibition; and
c) to determine whether or how the concentrations of ACh were related to observed alterations in choline levels

Background

The organophosphate ChE inhibitors have been reported to share the ability to elevate ACh levels in brain, but their effects on the levels of free choline differ considerably. Several studies have shown that DFP causes a prolonged decrease in the steady-state levels of choline in brain between one and forty-eight hours after administration (5,32-34). In contrast, paraoxon causes a short-term increase in the concentration of free choline in striatum, but not in cortex or hippocampus (6). Similarly, increased levels of choline have been observed in several brain areas within one hour following the administration of soman (35). However, dichlorvos has a biphasic effect, increasing choline levels in striatum and cortex at fifteen minutes after administration and decreasing levels at three and twenty-four hours (36). Therefore, there is preliminary evidence to suggest that the organophosphate ChE inhibitors may differentially affect the metabolism of choline, but comparisons from the literature are difficult due to variations in routes of administration, dosages, and brain regions studied.

To ascertain whether the observed changes in choline levels are the result of ChE inhibition requires a comparative study of consistent design. Therefore, initial experiments: a) established dose-response relationships for AChE inhibition for sarin, soman, DFP, and paraoxon so that the effects of equipotent doses of each compound could be compared; b) determined whether any effects on choline levels were dose-dependent and whether the dose dependency was similar to that for AChE inhibition; and c) determined whether or how the concentrations of ACh were related to observed alterations in choline levels.

Methods

Male Sprague-Dawley rats (200-300 g) were group housed and maintained on a twelve hour light/dark cycle with food and water available *ad libitum*. Rats received injections of DFP (using peanut oil as the control), paraoxon, sarin, or soman (the latter three using saline as the control). Rats were killed one to two hours following the injection. For AChE activity measurements, rats were killed by decapitation; for ACh and choline determinations, rats were killed by head-focused microwave irradiation. The striata and hippocampi were dissected on ice and tissues were prepared for the spectrophotometric determination of AChE activity (14) or for the gas chromatographic determination of the levels of ACh and choline (18,19).

Results

The dose-response curves for inhibition of AChE activity in striatum and hippocampus by each of the compounds studied are shown in Figure 1. The IC50 values for both brain areas were similar and were for striatum and hippocampus, respectively: 44 and 33 µg/kg for soman; 73 and 68 µg/kg for sarin; 0.12 and 0.09 mg/kg for paraoxon; and 0.36 and 0.34 mg/kg for DFP.

The effects of the organophosphates on the levels of choline in brain are shown in Figure 2. When choline levels were measured one hour after the administration of soman (70 µg/kg) and sarin (100 µg/kg), doses that inhibited AChE activity by 90-100%, a significant increase in the levels of choline was evident in both brain regions. The increases 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 changes were noted in either brain area from rats injected with paraoxon (0.23 mg/kg) or DFP (1.8 mg/kg), doses that also inhibited AChE activity by 90-100%.

It was possible that one hour of AChE inhibition following the administration of DFP and paraoxon was insufficient to produce an effect because studies have shown that the effect of soman was time-dependent (35). Therefore, the effects of the organophosphates on brain levels of

Figure 1

Effects of Soman, Sarin, DFP, and Paraoxon on the Activity of AChE in the Striatum and Hippocampus

Rats received injections (sc) of a range of doses of the agents or control vehicle and were killed one hour after injection by decapitation. Dotted line represents values obtained in hippocampus and solid line represents those in striatum. Enzyme activity was determined spectrophotometrically (14). Each point is 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 nmol acetylthiocholine hydrolyzed per min per mg wet weight.

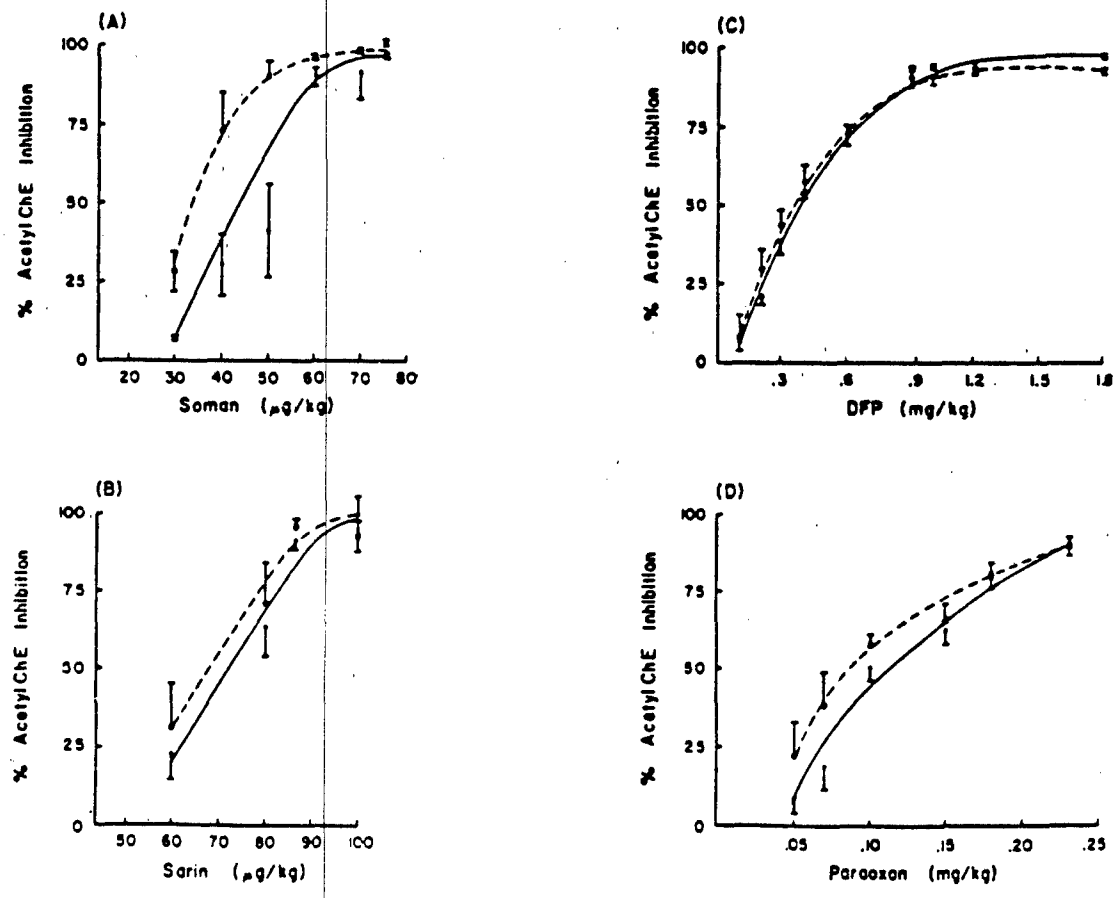
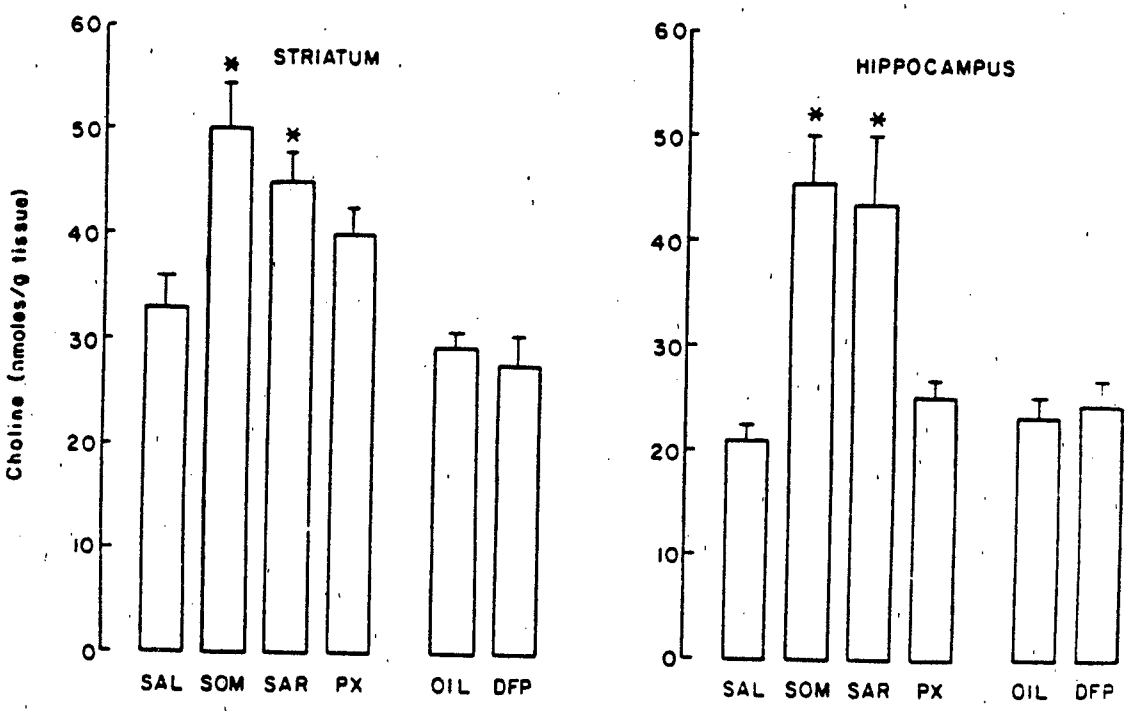


Figure 2

Effects of Soman, Sarin, DFP, and Paraoxon on the Levels of Choline in the Striatum and Hippocampus

Rats received injections (sc) of soman (SOM, 70 μg/kg), sarin (SAR, 100 μg/kg), paraoxon (PX, 0.23 mg/kg), DFP (1.8 mg/kg), or control vehicle (saline (SAL) or oil)), and were killed one hour after injection by head-focused microwave irradiation. Choline levels were quantified by pyrolysis gas chromatography (18,19). Bars are means ± S.E.M. of determinations from three to eighteen rats per group.
*Significantly different from corresponding control values, $p < 0.05$.



choline were determined at intervals up to ninety-six hours following injection (Figure 3). The effect of soman (70 μg/kg) was maximal at two hours, with increases to 188% and 450% of control in striatum and hippocampus, respectively. The concentration of choline remained significantly elevated for three hours following injection and returned to control by four hours. The effects of sarin (100 μg/kg) were less pronounced than those of soman (168% and 366% of control in striatum and hippocampus, respectively, at two hours), 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 three hours. However, a significant 30-45% decrease was evident in the striatum from four to forty-eight hours 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 them (by 23%) at twenty-four hours. In the hippocampus, paraoxon significantly increased choline levels by 38% at two hours and decreased levels at twenty-four hours. These results indicate that the effects of soman and sarin on choline levels in brain differ from those of DFP and paraoxon and suggest that the differential effects are due to intrinsic differences among the mechanisms of action of the organophosphates.

Since studies have suggested that muscarinic receptor stimulation may enhance the release of choline from brain and other tissue, it was determined whether the increase in choline was related to or dependent on an increase in the levels of ACh. In the striatum, doses of DFP, paraoxon, and soman that similarly inhibited AChE activity (by 90-100%) produced the same relative increase in ACh levels at one hour following administration, whereas a comparable dose of sarin produced less of an increase (Figure 4). In the hippocampus, the increase in ACh levels following the administration of paraoxon and DFP was less than that produced by soman and was not different from that induced by sarin. Since neither DFP nor paraoxon increased choline levels in striatum, data indicated that increased muscarinic receptor stimulation via increases in endogenous levels of ACh 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 significantly elevate choline levels.

Since the doses of the four compounds used for evaluating effects on choline levels inhibited AChE activity by 90-100%, it appeared that the choline increase elicited by soman and sarin was unrelated to AChE inhibition. To substantiate this finding, various doses of sarin (60-100 μg/kg) and soman (30-75 μg/kg) were administered and the choline levels in both striatum and hippocampus were determined one hour after injection (Tables 3 and 4). Doses below the IC50 for AChE inhibition did not alter choline levels, whereas doses above the IC50 all led to the same relative increase. Administration of paraoxon or DFP did not alter choline levels in these experiments, in agreement with the previous results. Therefore, while changes in choline levels did not reflect AChE inhibition, there was a specific range of doses of soman and sarin that produced the effect. Furthermore, measurements of AChE activity following the injection of sarin and soman indicated no recovery of enzyme activity for up to four hours (enzyme activity was inhibited to the same extent as determined one hour after injection as shown in Figure 1), even though data indicated that choline levels returned to control values by this time (Figure 3). Hence, the time-dependent alterations in choline levels did not parallel the inhibition or recovery of AChE. The independence of choline levels and AChE activity are futher apparent from time-course studies with paraoxon and DFP. These results are shown in Figure 5. Measurements of AChE activity between one and seventy-two hours after the administration of paraoxon (0.23 mg/kg) or DFP (1.6 mg/kg) indicated that enzyme activity remained substantially inhibited in both brain areas between one and four hours after the administration of these compounds and that the gradual recovery of enzyme activity after this time did not parallel the fluctuation in choline levels which were maximally depressed at twenty-four hours and recovered by seventy-two hours (Figure 3).

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

Figure 3

Time Course of the Effects of the Organophosphates on the Levels of Choline in the Striatum and Hippocampus

Rats received injections (sc) of soman (70 μg/kg, ●-----●), sarin (100 μg/kg, o-----o), paraoxon (0.23 mg/kg, ■-----■), DFP (1.8 mg/kg, ▲-------▲), or control vehicle (saline (SAL) or oil)), and were killed at the times indicated by head-focused microwave irradiation. Choline levels were quantified by pyrolysis gas chromatography (18,19). Each point is the mean of three to twenty-one determinations. For clarity, error bars have been omitted. The error about each mean was approximately ±9%.
*Significantly different from corresponding control values, $p < 0.05$.

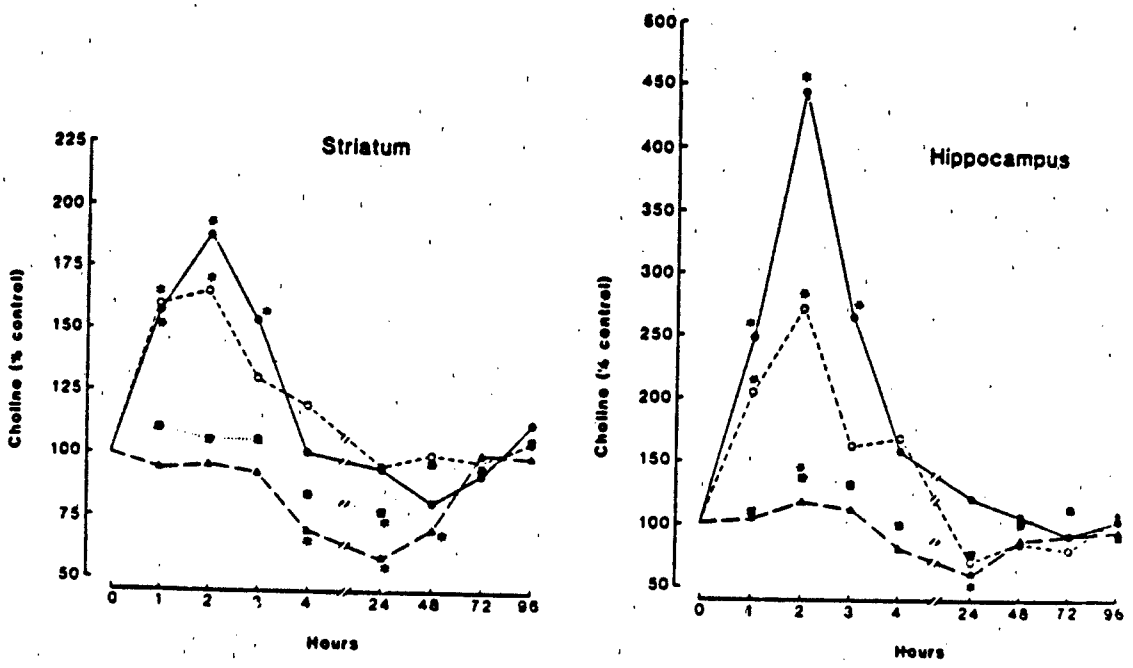


Figure 4

Effects of the Organophosphates on the Levels of ACh
in the Striatum and Hippocampus

Rats received injections (sc) of soman (SOM, 70 µg/kg), sarin (SAR, 100 µg/kg), paraoxon (PX, 0.23 mg/kg), DFP (1.8 mg/kg), or control vehicle (saline (SAL) or oil)), and were killed one hour after injection by head-focused microwave irradiation. ACh levels were quantified by pyrolysis gas chromatography. Bars are means ± S.E.M. of determinations from three to nine rats per group.
*Significantly different from corresponding control values, $p < 0.05$.

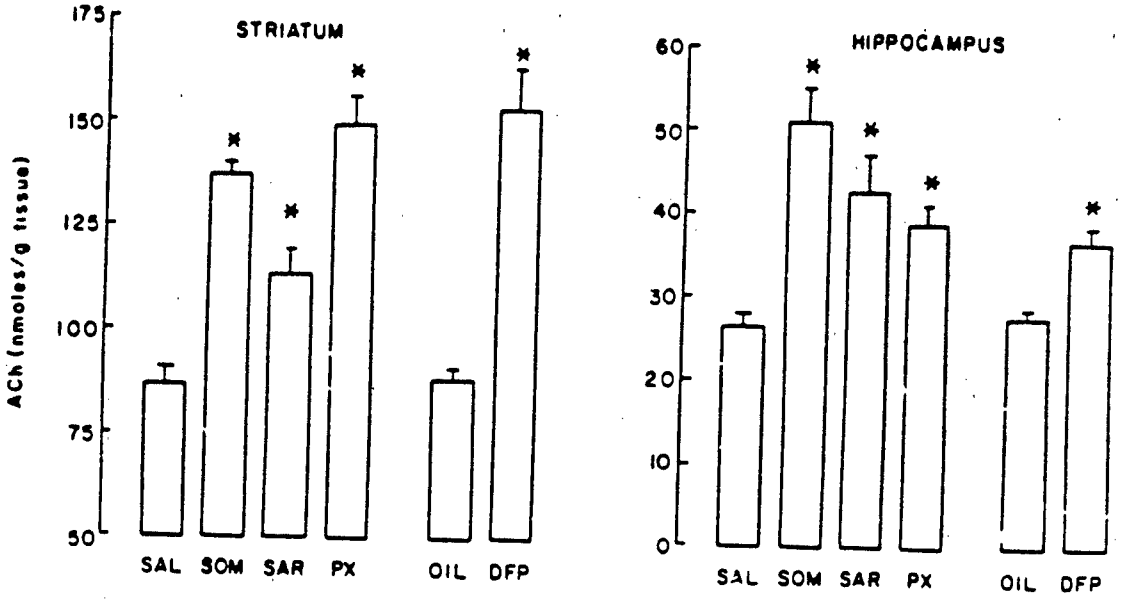


Table 3

Effects of the Organophosphates on the Levels of ACh and Choline in the Striatum

	Dose	ACh (nmol/g)	% Control	Choline (nmol/g)	% Control
Saline		82.1 ± 3.85 (6)		30.0 ± 1.95 (26)	
Soman	<IC50	92.0 ± 3.90 (10)	112	32.7 ± 2.17 (10)	108
	>IC50	113 ± 6.79* (12)	138	42.9 ± 4.30* (12)	142
Sarin	<IC50	81.7 ± 4.46 (3)	100	31.7 ± 2.14 (8)	105
	>IC50	112 ± 9.91* (8)	137	51.4 ± 5.33* (9)	170
Paraoxon	<IC50	78.3 ± 1.52 (6)	96	32.6 ± 3.21 (7)	108
	>IC50	120 ± 4.62* (25)	146	32.0 ± 2.14 (18)	106
Oil		85.3 ± 2.13 (13)		30.1 ± 1.76 (11)	
DFP	>IC50	113 ± 5.47* (17)	132	29.5 ± 1.79 (17)	98

Rats received injections (sc) of soman (30-75 µg/kg), sarin (60-100 µg/kg), paraoxon (0.05-0.25 mg/kg), DFP (0.1-1.8 mg/kg), or control vehicle (saline or oil) and were killed one hour after injection. ACh and choline levels were determined by pyrolysis gas chromatography (18,19). Analyses indicated that the effects of the organophosphates on ACh and choline levels did not differ among doses either below or above the IC50 for AChE inhibition for each compound. Thus, data were grouped for these values. The IC50 values were: 44 µg/kg for soman; 73 µg/kg for sarin; 0.12 mg/kg for paraoxon; and 0.36 mg/kg for DFP. The dose ranges below the IC50 were: 30-40 µg/kg for soman; 60-70 µg/kg for sarin; and 0.05-0.10 mg/kg for paraoxon. The dose ranges above the IC50 were: 50-75 µg/kg for soman; 80-100 µg/kg for sarin; 0.15-0.25 mg/kg for paraoxon; and 0.5-1.8 mg/kg for DFP. Each value represents the mean ± S.E.M. The number of rats per group is in parentheses.

*Significantly different from corresponding control group value, $p < 0.05$.

Table 4

Effects of the Organophosphates on the Levels of ACh and Choline in the Hippocampus

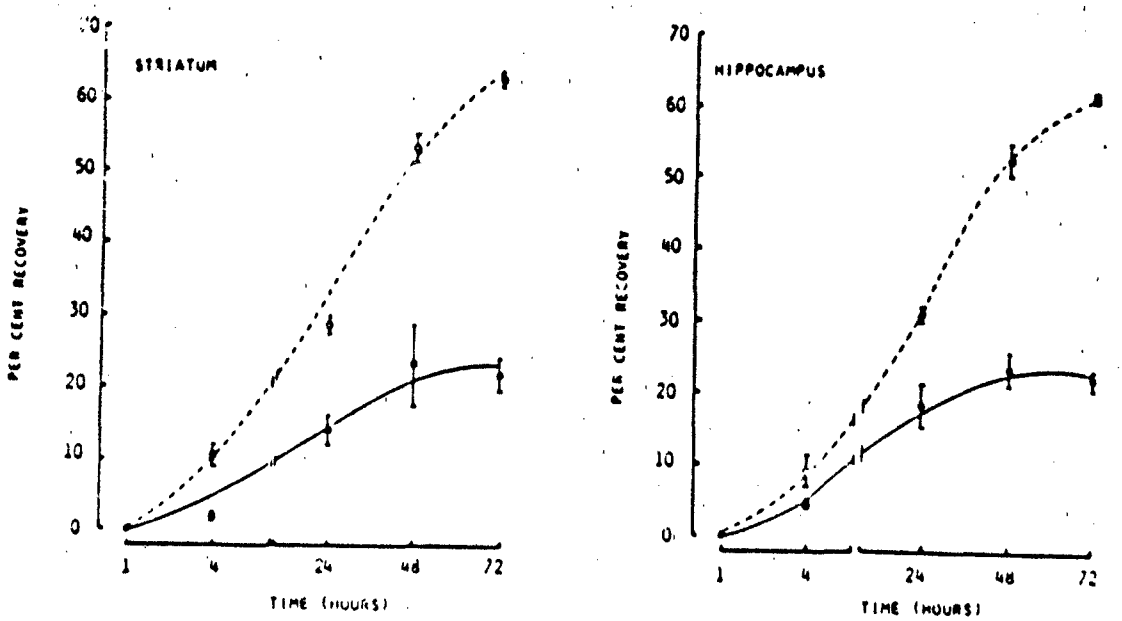
	Dose	ACh (nmol/g)	% Control	Choline (nmol/g)	% Control
Saline		24.5 ± 0.72 (7)		26.2 ± 2.10 (27)	
Soman	<IC50	25.8 ± 0.92 (8)	105	20.7 ± 3.01 (7)	79
	>IC50	39.9 ± 2.94* (14)	163	40.5 ± 6.66* (14)	155
Sarin	<IC50	26.3 ± 0.12 (3)	107	24.0 ± 4.86 (3)	92
	>IC50	37.5 ± 3.74* (9)	153	45.1 ± 7.32* (11)	172
Paraoxon	<IC50	23.5 ± 0.73 (8)	96	17.7 ± 0.82 (7)	68
	>IC50	30.7 ± 1.47* (23)	121	32.6 ± 3.87 (23)	124
Oil		24.8 ± 0.65 (12)		24.1 ± 2.68 (12)	
DFP	<IC50	23.3 ± 0.05 (2)	92		
	>IC50	29.9 ± 1.11* (18)	121	22.8 ± 1.74 (17)	95

Rats received injections (sc) of soman (30-75 µg/kg), sarin (60-100 µg/kg), paraoxon (0.05-0.25 mg/kg), DFP (0.1-1.8 mg/kg), or control vehicle (saline or oil) and were killed one hour after injection. ACh and choline levels were determined by pyrolysis gas chromatography (18,19). Analyses indicated that the effects of the organophosphates on ACh and choline levels did not differ among doses either below or above the IC50 for AChE inhibition for each compound. Thus, data were grouped for these values. The IC50 values were: 33 µg/kg for soman; 68 µg/kg for sarin; 0.09 mg/kg for paraoxon; and 0.34 mg/kg for DFP. The dose ranges below the IC50 were: 30-32 µg/kg for soman; 60-65 µg/kg for sarin; 0.05-0.07 mg/kg for paraoxon; and 0.10 -0.30 mg/kg for DFP. The dose ranges above the IC50 were: 40-75 µg/kg for soman; 75-100 µg/kg for sarin; 0.10-0.25 mg/kg for paraoxon; and 0.50-1.8 mg/kg for DFP. Each value represents the mean ± S.E.M. The number of rats per group is in parentheses.
*Significantly different from corresponding control group value, $p < 0.05$.

Figure 5

Recovery of AChE Activity in the Striatum and Hippocampus After Administration of DFP or Paraoxon

Rats received injections (sc) of 0.23 mg/kg paraoxon (o-----o) or 1.6 mg/kg DFP (●-------●) and were killed at the times indicated. Enzyme activity was determined spectrophotometrically (14). Control activity for striatum and hippocampus were, respectively, 48.3± 2.72 and 8.55 ± 0.92 nmol acetylthiocholine hydrolyzed per minute per milligram wet weight. Activity was inhibited by 90-96% at one hour. Values were calculated from the mean ± S.E.M. of three to seven determinations.



ACh, the former representing the major metabolic pathway (>95%) for choline. Increased levels of choline in brain may result from either decreased incorporation into or increased release from phospholipids. Studies have shown that choline levels in brain and choline efflux from brain and heart are increased following the administration of ACh, oxotremorine, physostigmine, and muscarine (53-56), indicating a possible role for ACh in elevating levels of free choline. The cholinergic agonist-induced changes were atropine-sensitive, indicating muscarinic receptor involvement. Nevertheless, results from the present study do not support an ACh-mediated event. Doses of soman and sarin that inhibit AChE activity by greater than 50% increase the concentrations of both choline and ACh in striatum and hippocampus one to three hours after administration. However, doses of DFP that similarly increase the levels of ACh in brain do not increase the levels of choline. Furthermore, studies by Ladinsky *et al.* (53) indicated that although DFP increased ACh levels in mouse brain, it did not increase choline levels. In addition, this group also reported that doses of physostigmine that increased choline levels in the cerebellum did not alter ACh levels in this brain area. Thus, both this and the present data suggest that factors other than increased levels of ACh must be involved in mediating the increased choline levels observed after soman and sarin intoxication.

One such factor is suggested by observations during these experiments that soman and sarin caused tremors that were much more severe than those observed following the administration of DFP and paraoxon. While all injected animals showed the usual symptoms of organophosphate poisoning (muscle fasciculations, salivation, and diarrhea), 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. It is possible that the accumulation of choline observed after soman and sarin poisoning was related to their excitotoxic effects. Soman, when administered at doses comparable to the higher doses used in this study, produces convulsions and increases 2-deoxyglucose phosphorylation (57-59), suggestive of generalized stimulation of metabolic activity. It is well documented that seizures, hypoxia, and cerebral energy deficit cause increased accumulation of free fatty acids in brain, thought to be derived from cell membrane phospholipids (60-62). This may be mediated by activation of phospholipases (63). Thus, the choline increase due to soman and sarin intoxication may be a consequence of seizure-related phosphatidylcholine hydrolysis.

In contrast to the effects of sarin and soman, the administration of paraoxon and DFP decreases choline levels four to twenty-four hours after injection. These findings are in agreement with those of Russell *et al.* (33) who reported that DFP decreased choline levels by 12-18% in whole rat brain between four and twenty-four hours after administration. No change was noted in the hippocampus at twenty-four hours, possibly because the dose of DFP administered was one half of that used in the present experiments. In addition, Modak *et al.* (36) also observed a depression in choline levels of striatum and hippocampus between three and twenty-four hours after the administration of dichlorvos. Since the present data indicated no temporal relationship between the recovery of AChE activity and the alterations in choline levels, it is possible that the reestablishment of normal choline levels more nearly parallels the recovery of an enzyme other than AChE, possibly a lipase. As mentioned, decreased choline levels may result from inhibition of phospholipid hydrolysis. Since no published data are available on the actions of soman and sarin on lipase activity, why they may be different from DFP in this regard is unknown.

In summary, results obtained in these initial studies indicate that both soman and sarin increase choline levels in brain, a characteristic not shared by all organophosphate ChE inhibitors. This choline increase appears causally unrelated to AChE inhibition or elevated levels of ACh and possibly reflects enhanced phospholipid hydrolysis secondary to alterations in brain metabolism. In contrast, DFP and paraoxon cause reductions of choline levels in brain that also appear unrelated to either AChE inhibition or elevated levels of ACh.

B. THE ROLE OF CONVULSIONS IN THE MOBILIZATION OF CHOLINE

Objectives:

a) to determine whether the sarin- and soman-induced increases in free choline levels in brain were unique effects of these agents or the nonspecific result of convulsive activity; and
b) to identify the likely source of the free choline

Background

The results obtained in the initial studies suggested that examination of the role of convulsions caused by the organophosphates was important for understanding the mechanisms responsible for the differential effects of these compounds on choline levels. It has been reported that during convulsions induced by convulsants or electroshock, the levels of free fatty acids in brain increase severalfold (60,64,65). Although one source of these free fatty acids is phosphatidylinositol (65,66), phosphatidylcholine has also been suggested as a source, based on the carbon chain length and degree of unsaturation of the accumulated fatty acids (67,68). However, no direct determinations have been made of whether or how much of the free bases are accumulated. Accumulation of free fatty acids following the administration of soman or sarin, in conjunction with elevated levels of choline, would provide more direct evidence that phosphatidylcholine is the source of the free choline accumulated in brain after sarin and soman poisoning. Therefore, experiments determined: a) whether bicuculline (a convulsant with no known activity on AChE) would increase levels of free choline in brain in a manner similar to that of sarin and soman; b) whether the anticonvulsant diazepam would prevent the sarin- and soman-induced increases in free choline; and c) whether soman would concomitantly increase the levels of free fatty acids.

Methods

Male Sprague-Dawley rats (160-300 g) were group housed, maintained on a twelve hour light/dark cycle, and had access to food and water *ad libitum*. Soman and sarin were diluted with saline immediately prior to use to a final concentration of 0.35 aand 0.57 mg/ml, respectively. Bicuculline was dissolved in acidified normal saline (3 drops 2 N HCl/5 ml saline) prior to use and was injected intraperitoneally (ip). Diazepam was dissolved in propylene glycol:ethanol:water (4:3:3, vol:vol:vol) containing one drop 2 N HCl/10 ml to a final concentration of 4 mg/ml and was injected ip.

To determine the effects of bicuculline on the levels of free choline in striatum and hippocampus, groups of animals received either bicuculline (6 mg/kg) or saline and were killed four minutes after injection by head-focused microwave irradiation. A four minute interval was maintained between injection and microwaving because this convulsant causes death at this dose in about five to seven minutes. To evaluate the effects of diazepam on the soman- and sarin-induced increase in choline levels in striatum and hippocampus, groups of animals received either vehicle or diazepam (4 mg/kg) thirty minutes prior to the injection of soman (70 µg/kg, sc) or sarin (100 µg/kg, sc). Animals were killed two hours after the second injection. The concentration of choline in brain for both series of experiments was determined by pyrolysis gas chromatography (18,19).

To determine the effects of soman on the levels of free fatty acids in brain, groups of rats received soman (65-70 µg/kg, sc) and were killed by head-focused microwave irradiation one hour after injection. To determine whether diazepam pretreatment altered the effects of soman on free fatty acids, animals received injections of diazepam (4 mg/kg, ip) thirty minutes prior to the administration of soman and were killed by head-focused microwave irradiation one hour after the second injection. (These studies were conducted on cerebral hemispheres rather than on striatum or hippocampus because larger tissue samples are required for the free fatty acid assay. This approach is justified in view of evidence that most regions in the brains of soman-poisoned rats had elevated levels of choline (35) and increases in free fatty acid levels occur in whole cerebrum of convulsing rats (61)). The cerebra were removed, chilled in saline, bisected sagitally, and prepared for the gas chromatographic separation and quantitation of free fatty acids (21,60,61). The specifics of this procedure are presented on page 12 of the 1984-85 Annual Report on this project.

Results

The administration of bicuculline to rats resulted in elevations of free choline in both striatum and hippocampus (Table 5). Even at this early time after the onset of convulsions, the choline levels in both areas were elevated by 82%. A control experiment verified that bicuculline did not inhibit AChE activity by itself. These results indicated that increased choline levels may be a consequence of seizures, regardless of cause. The results further supported previous evidence that AChE inhibition is not involved in the increases of free choline seen with soman and sarin intoxication.

When rats received an injection of diazepam (4 mg/kg, ip) thirty minutes prior to the administration of soman, the soman-induced increase in choline levels was totally prevented in the striatum and attenuated in the hippocampus (Table 6). When the effects of diazepam on sarin-induced alterations in brain choline levels were measured, a similar prophylactic effect was noted (Table 7). In addition to attenuating or preventing the increases of choline, diazepam pretreatment also reduced the severity of the sarin- and soman-induced tremors. Measurements of AChE activity indicated that this dose of diazepam did not affect enzyme activity by itself nor did it protect AChE from inhibition by soman or sarin (Table 8). Hence, these results suggested that the choline increase may be related to the convulsive properties of sarin and soman.

Consistent with previous studies showing that increased levels of free fatty acids in brain accompany convulsions (60,61), soman also caused increased free fatty acid levels (Table 9). There were marked increases in all molecular species measured, similar to the profile seen with other models of seizures (60.69,70), with the largest increase in the levels of oleic (18:1) and stearic (18:0) acids. The average increase for all fatty acids measured was 420% of control. Since diazepam prevented the soman- and sarin-induced increases in choline levels, suggesting that convulsions mediated this effect, it was useful to determine whether the free fatty acid increase was likewise suppressible with this agent. Therefore, the effects of diazepam pretreatment on soman-induced increases in free fatty acids were determined. When diazepam (4 mg/kg, ip) was administered thirty minutes prior to the administration of soman, the soman-induced increase of all species of free fatty acids was nearly totally prevented (Table 10).

Discussion

These studies suggest that the increases of free choline in brain by soman and sarin stem from their convulsant properties, and they further demonstrate that increased choline levels may occur after convulsions due to other agents. These data also establish that soman and sarin increase levels of free fatty acids in brain, an effect which has been observed using several models of seizures. The accumulation of free fatty acids is on the same order of magnitude as the accumulation of free choline. Therefore, it is possible that seizures result in the catabolism of phosphatidylcholine. Although the accumulation of free fatty acids and choline represents a relative minor loss of phosphatidylcholine in terms of the total pool, it may be functionally significant if it occurs in specific membranes, e.g., the synaptic plasma membrane. Phosphatidylcholine is a major phospholipid of cell membranes. It represents about 35-40% of the total lipid, as measured by phosphorus estimation, and is by far the largest source of choline in the brain. The fatty acid composition (expressed as percent of total fatty acids) of phosphatidylcholine is 51-57% 16:0; 12% 18:0; 22-29% 18:1; 4% 20:4; and minor amounts of other fatty acids (71,72). The results of the present studies indicate that there is significant accumulation of these fatty acids after soman poisoning. Excessive loss of these components from the cell membrane could result in increased cell permeability, functional impairment, and in later stages, degeneration of cell structure.

In addition, further cell damage may occur because free fatty acids and associated phospholipid catabolites, the lysophospholipids, have potent detergent effects on cell membranes (63,73-75). Lysophospholipids are rapidly degraded to glycerolphosphate and free fatty acid by the action of lysophospholipase. Polyunsaturated fatty acids are the primary target for lipid peroxidation (76) induced by free radicals generated from incomplete electron transport in mitochondria (73). Once lipid peroxidation has begun, it is chain propagating, thus exacerbating membrane damage and damage to other oxidizable components of cells, e.g., proteins and catecholamines (77).

Table 5

Effects of Bicuculline on the Concentrations of ACh and Choline in the Striatum and Hippocampus

Treatment	ACh (nmol/g)	Choline (nmol/g)
	Striatum	
Control	96.8 ± 3.65	36.6 ± 1.44
Bicuculline	64.3 ± 5.06*	66.7 ± 5.98*
	Hippocampus	
Control	30.1 ± 1.89	21.0 ± 1.19
Bicuculline	29.1 ± 2.33	38.2 ± 2.28*

Rats received injections (ip) of bicuculline (6 mg/kg) or saline and were killed four minutes after the injection by head-focused microwave irradiation. ACh and choline levels were determined by pyrolysis gas chromatography (18,19). Each value is the mean ± S.E.M. of determinations from six to nine animals per group.
*Significantly different from control values, $p < 0.05$.

Table 6

Effects of Diazepam on the Soman-induced Increase in Levels of Choline in the Striatum and Hippocampus

Treatment	Choline (nmol/g)	
	Striatum	Hippocampus
Control	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)
Diazepam + Soman	33.0 ± 2.69† (7)	30.2 ± 5.39† (6)

Rats received injections (ip) of control vehicle or diazepam (4 mg/kg) thirty minutes prior to the injection (sc) of saline or soman (70 μg/kg). Animals were killed two hours after the second injection by head-focused microwave irradiation. Levels of choline were determined by pyrolysis gas chromatography (18,19). Each value is the mean ± S.E.M. The number of rats per group is in parentheses.
*Significantly different from corresponding control (saline or diazepam) values, $p < 0.05$.
†Significantly different from soman-injected group values, $p < 0.05$.

Table 7

Effects of Diazepam on the Sarin-induced Increase in Levels of Choline in the Striatum and Hippocampus

Treatment	Choline (nmol/g)	
	Striatum	Hippocampus
Control	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)
Diazepam + Sarin	43.2 ± 1.85*† (11)	40.1 ± 2.94*† (11)

Rats received injections (ip) of control vehicle or diazepam (4 mg/kg) thirty minutes prior to the injection (sc) of saline or sarin (100 μg/kg). Animals were killed two hours after the second injection by head-focused microwave irradiation. Levels of choline were determined by pyrolysis gas chromatography (18,19). Each value is the mean ± S.E.M. The number of rats per group is in parentheses.
*Significantly different from corresponding control (saline or diazepam) values, $p < 0.05$.
†Significantly different from sarin-injected group values, $p < 0.05$.

Table 8

Effects of Diazepam on the Inhibition of ChE Activity by Soman and Sarin

Treatment	ChE Activity (nmol/min/mg tissue)	
	Striatum	Hippocampus
Control	57.2 ± 2.48 (7)	8.98 ± 0.22 (8)
Diazepam	53.5 ± 1.54 (5)	8.71 ± 0.25 (5)
Soman	3.90 ± 1.14* (5)	0.77 ± 0.34* (5)
Diazepam + Soman	5.04 ± 0.62* (4)	0.79 ± 0.15* (5)
Sarin	1.97 ± 0.57* (5)	0.40 ± 0.06* (5)
Diazepam + Sarin	2.72 ± 1.23* (4)	0.47 ± 0.04* (4)

Rats received injections (ip) of control vehicle or diazepam (4 mg/kg) thirty minutes prior to the injection (sc) of saline, soman (64-70 µg/kg), or sarin (100-110 µg/kg). Animals were killed two hours after the second injection by decapitation. ChE activity was determined spectrophotometrically (14). Each value is the mean ± S.E.M. The number of rats per group is in parentheses.
*Significantly different from corresponding control (saline or diazepam) values, $p < 0.05$.

Table 9

Effects of Soman on the Levels of Free Fatty Acids in Rat Cerebrum

	Saline (nmol/g tissue)	Soman
Total Free Fatty Acids	42.3 ± 4.38	177 ± 11.9*
16:0 (Palmitic)	16.8 ± 3.57	47.1 ± 1.62*
18:0 (Stearic)	8.72 ± 1.73	54.7 ± 4.24*
18:1 (Oleic)	4.91 ± 0.91	41.4 ± 3.58*
20:4 (Arachidonic)	4.45 ± 0.87	16.1 ± 2.23*
22:6 (Docosahexaenoic)	7.53 ± 1.23	17.3 ± 2.08*

Rats received injections (sc) of saline or soman (70 μg/kg). Animals were killed one hour after the injection by head-focused microwave irradiation. The levels of free fatty acids were determined by gas chromatography (60,61). Each value is the mean ± S.E.M. of determinations from four rats per group.
*Significantly different from corresponding control values, $p < 0.05$.

Table 10

Effects of Diazepam on the Soman-induced Increases in Free Fatty Acids in Rat Cerebrum

	Diazepam	Soman	Diazepam + Soman
		(% Control)	
Total Free Fatty Acid	93.3 ± 11.0	248 ± 26.6*	123 ± 14.7†
16:0 (Palmitic)	87.3 ± 22.2	175 ± 13.9*	120 ± 10.4†
18:0 (Stearic)	103 ± 14.5	354 ± 53.5*	146 ± 33.5†
18:1 (Oleic)	91.2 ± 8.91	198 ± 15.1*	97.2 ± 7.83†
20:4 (Arachidonic)	87.8 ± 6.26	561 ± 87.3*	143 ± 66.1†
22:6 (Docosahexaenoic)	46.2 ± 5.49	299 ± 46.6*	87.3 ± 23.1†

Rats received injections (ip) of control vehicle or diazepam (4 mg/kg) thirty minutes prior to the injection (sc) of saline or soman (65-70 μg/kg). Animals were killed one hour after the second injection by head-focused microwave irradiation. Levels of free fatty acids were determined by gas chromatography (60,61). Control values (± S.E.M.) for the free fatty acids were (nmol/g): 67.9 ± 9.90 (total); 27.7 ± 4.70 (16:0); 18.1 ± 3.98 (18:0); 9.79 ± 3.33 (18:1); 4.57 ± 0.52 (20:4); and 7.54 ± 0.58 (22:6). Each value is the mean ± S.E.M of determinations from three to eight rats per group.

*Significantly different from corresponding control (saline or diazepam) values, $p < 0.05$.

†Significantly different from soman values, $p < 0.05$.

Polyunsaturated fatty acids are also substrates for a family of monooxygenases such as prostaglandin synthase and lipoxygenase, resulting in the production of potent autacoids, prostaglandins, and leukotrienes (78). Collectively, these effects may result in the focal calcifications, glioses, and necroses associated with seizures, including those induced by soman and bicuculline (79-81).

The mechanism by which soman and sarin induce convulsions is not known. The possibility that soman causes convulsions by altering cerebral blood gases, sodium and potassium levels, or circulating insulin levels has been examined with negative results (82). However, the efficacy of the benzodiazepines in preventing or attenuating accumulations of free fatty acids in brain, as well as several other correlates of soman poisoning, including electroencephalogram (EEG) changes, convulsions, and increases in cGMP (83-85), points to a role for gamma-aminobutyric acid (GABA)-mediated neurotransmission. The GABA receptor antagonist properties of bicyclic organophosphates, which do not inhibit ChE, but are potent convulsants (86-88), suggest that soman and sarin may also act at the GABA receptor (82) in a manner similar to that of bicuculline (89). However, direct investigation of this has not been made.

Studies have shown that the levels of GABA in rat brain are unaffected by soman (90,91) and that soman and sarin increase glutamic acid decarboxylase activity only at lethal doses (92). GABA transaminase activity appears to be unaffected by soman (91,92). In contrast, DFP, which is not convulsant, does increase levels of GABA and glutamic acid and the activity of GABA transaminase in striatum (92). These differential effects suggest that there may be a mechanism unique among organophosphates by which soman and sarin generate seizures. Aminooxyacetic acid, which inhibits GABA transaminase and increases GABA levels, is effective against soman-induced seizures (91). This suggests that although soman and sarin may not directly alter levels of GABA and its metabolites, the actions of these organophosphates are attenuated by pharmacological enhancement of GABAergic function.

Increased cGMP may be a proximal factor in mediating the excitotoxic effects of soman and sarin. Soman elevates brain levels of cGMP (85) which has excitatory effects (87) and inhibits oxygen consumption in cortical brain slices, similar to soman (83,93). Diazepam not only prevents soman-induced increases in cGMP (85,87), but antagonizes the inhibition of oxygen consumption as well (83).

If soman-induced convulsions were due to increased levels of ACh, then atropine, a muscarinic antagonist that also blocks the increase of ACh induced by organophosphates (94), would be expected to be an effective antagonist. However, this has not proved to be the case (83,91). Atropine has little effect on soman-induced convulsions and does not block the *in vivo* elevation of cGMP levels induced by soman (85). Thus, evidence supports a role for GABA-related mechanisms rather than for ACh in mediating the convulsant effect of soman.

In conclusion, results from these experiments demonstrate that the accumulation of free fatty acids and choline in brain is a consequence of soman- and sarin-induced seizures. These effects of soman and sarin are antagonized by diazepam, a known anticonvulsant, supporting the hypothesis that soman and sarin caused a degradation of phosphatidylcholine, which may be a factor in the extensive neuropathology induced by these compounds.

C. THE EFFECTS OF THE ORGANOPHOSPHATES ON PHOSPHOLIPID METABOLISM

Objectives:

a) to determine whether the organophosphates alter the steady-state levels of phospholipids;
b) to determine whether the organophosphates alter the incorporation of choline into phospholipids;
c) to determine the direct (*in vitro*) effects of the organophosphates on phospholipase A2 activity in rat striatum;
d) to determine the effects of the organophosphates on phospholipase A2 activity after *in vivo* administration; and

e) to determine the effects of the organophosphates on the postmortem accumulation of free choline

Background

The data presented in the previous sections suggest that organophosphate-induced alterations in brain choline levels may be mediated by alterations in the metabolism of choline-containing phospholipids. Early studies investigating the delayed neurotoxicity induced by the demyelinating organophosphate compounds suggested a possible effect on phospholipid metabolism (95,96). In sciatic nerves from fowl poisoned with these agents, there was a decreased synthesis of phosphatidylcholine, phosphatidylethanolamine, and monophosphoinositide (95,96). Therefore, the first series of experiments involved determining whether the organophosphates altered the steady-state levels of phospholipids in brain.

In addition to determining steady-state concentrations, to fully assess the effects of the organophosphates on phosphatidylcholine metabolism, it is imperative to investigate dynamic processes, i.e., synthetic and degradative mechanisms. The enzyme choline kinase, which catalyzes the phosphorylation of choline to form phosphorylcholine, is the first step in the cytidine pathway originally described by Kennedy and Weiss (41) in which choline is incorporated into phosphatidylcholine. Although choline kinase activity in nerve terminals has been assumed to be localized exclusively to the cytosol (97,98), we have recently demonstrated significant enzyme activity in the membrane fraction of synaptosomes isolated from rat striata (99). To determine the relationship between membrane-associated and cytosolic enzyme activity and to provide insight as to a possible functional role of the membrane-associated enzyme, we investigated the kinetic mechanism of choline kinase located in both the cytosolic and membrane fractions of rat striatal synaptosomes and the effects of the organophosphates on enzyme activity.

In addition to synthetic processes, it is also possible that the organophosphates alter phospholipid hydrolysis which is mediated by the action of lipases. It is possible that inhibition of phospholipase A2 could account for the reduction in brain choline levels observed after DFP and paraoxon administration, while activation could account for increased levels of free fatty acids and choline following soman and sarin poisoning. Although studies indicate that organophosphates inhibit some types of lipases, no systematic pharmacological investigations of these agents on brain enzymes have been reported. Therefore, the effects of DFP, paraoxon, soman, and sarin on phospholipase A2 activity were determined.

Last, the effects of the agents on the postmortem accumulation of free choline were investigated. The source of the postmortem accumulation of choline is thought to be phosphatidylcholine (42) that is hydrolyzed by lipases activated by ischemia (60). Although the exact enzymes involved have not been identified, phospholipase A2 is thought to be the main enzyme mediating this effect. Thus, organophosphate-induced alterations in the postmortem accumulation of choline may serve as an index of the functional consequences of altered lipolytic activity.

Methods

Male Sprague-Dawley rats (225-300 g) were group-housed, kept on a twelve hour light/dark cycle, and had access to food and water *ad libitum*. To determine whether the organophosphates altered the steady-state levels of phospholipids, DFP (1.8 mg/kg) or peanut oil (control) was administered to rats, and the animals were killed two hours after the injection by decapitation. The striata and hippocampi were isolated, the microsomal fraction was prepared (20), and phospholipids were analyzed using thin layer chromatographic and spectrophotometric techniques (21,22).

To determine whether the organophosphates altered the incorporation of choline into phospholipids, choline kinase activity was assessed in striata from rats injected with sarin or soman. Animals received injections(sc) of saline, 70 µg/kg soman, or 100 µg/kg sarin and were killed by decapitation two hours later. The striata were dissected, and the synaptosomal fraction was isolated (20). The preparation was hypo-osmotically ruptured and soluble and particulate fractions were obtained. Choline kinase activity was determined radioisotopically (99).

The effects of the organophosphates on phospholipase A2 activity were determined both *in vivo*

and *in vitro*. For *in vivo* studies, rats received injections (sc) of saline, soman,(65 µg/kg), sarin (100 µg/kg), paraoxon (0.23 mg/kg), oil, or DFP (1.6 mg/kg) and were killed at various times after injection by decapitation. For *in vitro* studies, rats were killed by decapitation and a crude mitochondrial fraction from striatum was prepared (20). Phospholipase A2 activity was measured using a radioisotopic procedure (100). The specific methodology used for this assay is presented on pages 15 and 16 of the 1984-85 Annual Report for this project.

For determination of the effects of the organophosphates on the postmortem release of choline, rats received injections of the organophosphates at doses sufficient to cause 90-100% inhibition of AChE in brain. Animals were killed by either decapitation or head-focused microwave irradiation one to two hours after injection. The brains were removed and either processed immediately (for microwaved and decapitated zero times), or incubated for up to fifteen minutes in a moist chamber at 37°C. The levels of choline were quantitated by pyrolysis gas chromatography (18,19).

Results

The effects of DFP on microsomal phospholipids from rat striatum are shown in Table 11. At two hours following the administration of DFP, no changes were apparent in the steady-state concentrations of phospholipids in microsomes from striatum. A similar lack of effect, with the exception of an increase in phosphatidylethanolamine, was apparent in the hippocampus (Table 12). Similar results were obtained following the administration of paraoxon. Hence, results suggested that the organophosphates did not alter the steady-state concentrations of phospholipids.

To study the synthesis of phosphatidylcholine, it was first necessary to establish optimal conditions for measuring choline kinase activity. Since we had previously established that enzyme activity is associated with both the soluble and membrane fractions from rat brain (99), and that these fractions are indistinguishable on a biochemical basis, to provide insight as to possible functional differences, studies were initiated to determine whether we could differentiate these two forms on a kinetic basis (101). Therefore, the velocity of choline kinase was measured using various concentrations of MgATP at several concentrations of uncomplexed Mg^{2+} and a single concentration of choline. The experiment was repeated using different concentrations of choline. (Since the experimental procedures and mathematical analyses used for these experiments are rather lengthy, the reader is referred to reference number 101 for further details.) Analysis of these data indicated that MgATP binds in rapid equilibrium prior to Mg^{2+}, but the binding of MgATP and choline is random. Product inhibition by phosphorylcholine was noncompetitive versus both choline and MgATP. Hemicholinium-3, an analog of choline and competitive inhibitor of the sodium-dependent high affinity choline transport system, was noncompetitive versus choline and uncompetitive versus MgATP at high levels of Mg^{2+}. However, when the concentration of Mg^{2+} was decreased below the equilibrium constant for Mg^{2+}, hemicholinium-3 was noncompetitive versus MgATP. Thiocholine, another analog of choline, gave slope-linear intercept hyperbolic inhibition versus choline. Mg-5'-adenylyl imidodiphosphate, an analog of MgATP, was competitive versus MgATP and noncompetitive versus choline. Virtually identical results were obtained using either soluble or particulate forms of choline kinase from rat striata. Thus, the enzymes were kinetically indistinguishable, and, therefore, the soluble enzyme was used for studies investigating possible inhibition by the organophosphates.

When animals received injections of soman, sarin, DFP, or paraoxon that inhibited AChE activity by 90-100%, and choline kinase activity determined at various times after injections, no effects were noted by any of the compounds tested. Enzyme activity in the soluble fraction from a crude mitochondrial preparation from rat striatum was 4.63 ± 0.52 and was unaltered by soman, sarin, paraoxon, or DFP. It was thus concluded that the organophosphates did not alter the first step in the synthesis of phosphatidylcholine.

The dose-response curves for inhibition of phospholipase A2 activity by the organophosphates after preincubation *in vitro* are shown in Figure 6. Soman, sarin, and DFP all inhibited phospholipase activity with similar potency, with IC50 values of about 10 µM. Paraoxon was much less potent, causing only 50% inhibition at 1 mM. Since these assays were run at pH 8, the highly variable results obtained with paraoxon may have been due to base hydrolysis of this compound. Thus, the assays following preincubation with paraoxon were repeated at pH 7 to

Table 11

Effects of DFP on Microsomal Phospholipids in Striatum

Lipid	Control (Oil)	DFP
Total Lipid Phosphorus	40.2 ± 3.90	37.2 ± 1.99
Phosphatidylcholine	17.6 ± 1.53	16.8 ± 0.89
Phosphatidylethanolamine	13.9 ± 1.13	13.1 ± 0.61
Phosphatidylserine	5.83 ± 0.47	5.54 ± 0.23
Phosphatidylinositol	1.24 ± 0.16	1.01 ± 0.10
Phosphatidic Acid	0.24 ± 0.08	0.31 ± 0.07
Sphingomyelin	2.78 ± 0.25	2.34 ± 0.10
Lysophosphatidylcholine	0.52 ± 0.11	0.57 ± 0.12

Rats received injections (sc) of control vehicle (peanut oil) or DFP (1.8 mg/kg) and were killed two hours after the injection by decapitation. Each value is the mean ± S.E.M. of determinations from six rats per group. Phospholipids were isolated, separated by two dimensional thin layer chromatography, and analyzed for phosphorus by spectrophotometry (21,22). Values are expressed as micrograms of phosphorus per milligram of protein.

Table 12

Effects of DFP on Microsomal Phospholipids in Hippocampus

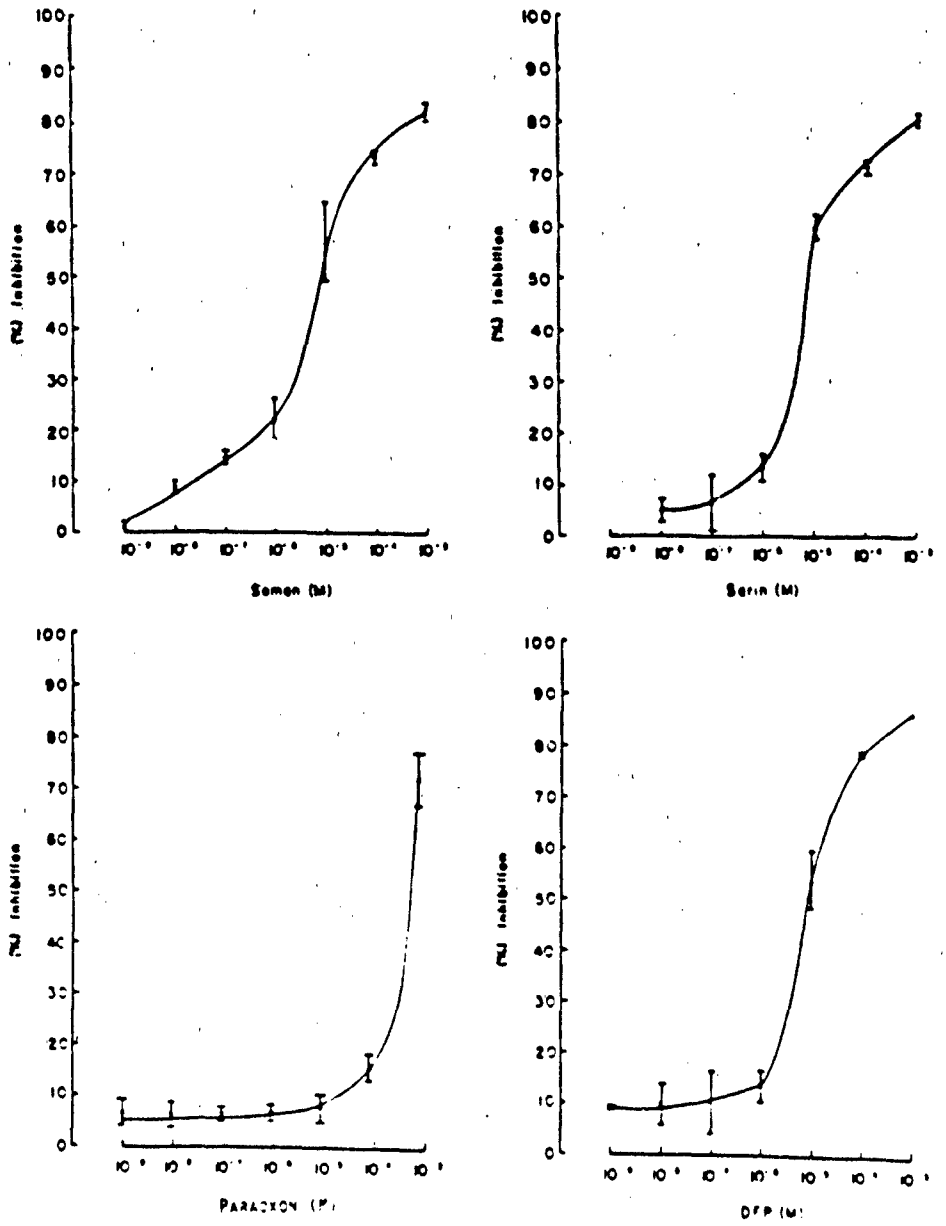
Lipid	Control (Oil)	DFP
Total Lipid Phosphorus	38.0 ± 1.29	41.4 ± 3.15
Phosphatidylcholine	16.3 ± 0.81	18.2 ± 1.10
Phosphatidylethanolamine	12.0 ± 0.43	14.1 ± 0.85*
Phosphatidylserine	4.87 ± 0.29	5.55 ± 0.32
Phosphatidylinositol	1.44 ± 0.06	1.38 ± 0.16
Phosphatidic Acid	0.29 ± 0.05	0.30 ± 0.09
Sphingomyelin	2.30 ± 0.09	2.45 ± 0.22
Lysophosphatidylcholine	0.51 ± 0.09	0.49 ± 0.16

Rats received injections (sc) of control vehicle (peanut oil) or DFP (1.8 mg/kg) and were killed two hours after the injection by decapitation. Each value is the mean ± S.E.M. of determinations from six rats per group. Phospholipids were isolated, separated by two dimensional thin layer chromatography, and analyzed for phosphorus by spectrophotometry (21,22). Values are expressed as micrograms of phosphorus per milligram of protein.
*Significantly different from control values, $p < 0.05$.

Figure 6

In Vitro Effects of the Organophosphates on the Activity of Phospholipase A2 in a Crude Mitochondrial Fraction from Rat Striatum

Naive rats were killed by decapitation and a crude mitochondrial fraction was prepared (20). The tissue preparations were incubated with the indicated concentrations of the organophosphates. Enzyme activity was determined radioisotopically (100). Each point is the mean ± S.E.M. of two to seven separate determinations run on different days. Phospholipase activity was calculated as picomoles of fatty acid released per hour per milligram of protein.



protect this agent from base hydrolysis. Control assays run at various pH values indicated that pH 7 resulted in only slightly lower control activity than those run at pH 8. Assays with paraoxon gave consistent results when run at a lower pH, suggesting that the agent was more stable under less alkaline conditions. Results from these assays did not differ significantly from those run at pH 8. However, all subsequent assays utilizing paraoxon were run at pH 7.

When phospholipase A2 activity was measured in crude mitochondrial fractions one hour after administration of soman and paraoxon, enzyme activity was not different from control (Table 13). Sarin caused a 10% inhibition at this time, while DFP inhibited activity by nearly 30%, as compared to its own controls (oil-injected), which were slightly higher than those obtained in saline-injected animals. (The difference in means between these control groups is probably due to the unequal sample size obtained by pooling values obtained from the saline-injected controls of experiments done on several different days.) To evaluate whether these results represented the maximal inhibition possible, enzyme activity was measured four and twenty-four hours after injection. At four hours both soman and sarin caused a 10% inhibition of phospholipase activity, while paraoxon still had no effect on enzyme activity four hours after administration. Twenty-four hours after a single injection of DFP, phospholipase activity was slightly recovered.

The last experiments in this series determined the effects of the organophosphates on the postmortem release of choline. The levels of free choline in both striatum and hippocampus increased with time postmortem from both control and DFP-poisoned animals, but at a much lower rate in the latter (Figure 7). The effect persisted for fifteen minutes after decapitation and the rate of choline accumulation in striata decreased to 68-76% of control and in hippocampus, from 70-66% of control. Paraoxon, which was relatively inactive against phospholipase activity *in vitro*, also depressed the choline accumulation in striatum and hippocampus over the fifteen-minute interval to 78-74% and 83-80% of control, respectively (Figure 8). In contrast, the postmortem accumulation of choline in striata from rats injected with soman and sarin (Figures 9 and 10) was the same as those from controls. These data indicated that although soman and sarin were as potent as DFP as phospholipase A2 inhibitors *in vitro*, they do not depress the postmortem release of choline *in vivo* in a manner similar to that of DFP. Furthermore, although soman- and sarin-induced convulsions may activate lipolysis, leading to increased choline levels in microwave-fixated brain, the activation is not evident postmortem.

Discussion

Results from these studies indicate that while the organophosphates do not appear to alter either the synthesis or steady-state concentrations of phospholipids, they do inhibit the degradation of phosphatidylcholine. DFP, soman, and sarin are equally potent inhibitors of phospholipase A2 *in vitro*. Since soman and sarin are more potent inhibitors of ChE activity than DFP, the data indicate that the potencies of organophosphates toward ChEs are not indices of relative potencies toward phospholipase A2. That DFP, soman, and sarin are structurally similar phosphorofluoridates may account for their similar inhibitory potency toward phospholipase A2 activity *in vitro*, although in general, structural similarity or lack of it cannot account for widely different potencies toward ChEs observed among organophosphorus agents (102,103). Comparative studies have demonstrated that stoichiometric anomalies may account for this. Ellin (103) showed that S-(2-diisopropylaminoethyl)ethylmethyl phosphonothioate (VX) titrates in a linear relationship with red cell ChE, while sarin demonstrates a linear logarithmic relationship and soman reacts with a mixed relationship. These data suggest that there are some differences in the molecular mechanisms governing ChE inhibition, even by agents that appear closely related structurally. The present results suggest that similar differences may not pertain in reactions of organophosphates with other enzymes.

Although brain tissue from rats poisoned with soman and sarin is rapidly hydrolyzing phospholipids (see previous sections), phospholipase A2 activity measured in crude mitochondrial fractions after administration of these agents was either unaffected or only slightly depressed. While it might be expected that phospholipase A2 activation is occurring during soman and sarin intoxication, the effect was not evident when measured *in vitro* following *in vivo* administration, possibly because the conditions under which phospholipases are activated *in vivo* are not preserved

Table 13

In Vivo Effects of the Organophosphates on the Activity of Phospholipase A2 in a Crude Mitochondrial Fraction from Striatum

Treatment	Time (hrs)	Phospholipase Activity (pmol/hr/mg protein)	% Inhibition
Saline		7.49 ± 0.11 (20)	
Soman	1	7.44 ± 0.30 (7)	0
	4	6.65 ± 0.31*† (10)	11
Sarin	1	6.72 ± 0.26* (6)	10
	4	6.78 ± 0.29* (7)	9.5
Paraoxon	1	7.72 ± 0.24 (7)	0
	4	7.79 ± 0.40 (8)	0
Oil		8.27 ± 0.14 (6)	
DFP	1	5.94 ± 0.42* (6)	28
	4	5.93 ± 0.20* (5)	28
	24	6.87 ± 0.46* (4)	17

Rats received injections (sc) of control vehicle (saline or peanut oil), 65 µg/kg soman, 100 µg/kg sarin, 0.23 mg/kg paraoxon, or 1.6 mg/kg DFP. Animals were killed at the times indicated by decapitation. Striata were isolated, crude mitochondrial fractions were prepared (20), and phospholipase activity was determined radioisotopically (100). Each value is the mean ± S.E.M. The number of animals per group is in parentheses.
*Significantly different from corresponding control values, $p < 0.05$.
†Significantly different from corresponding one hour values, $p < 0.05$.

Figure 7

Effects of DFP on the Postmortem Accumulation of Choline in the Striatum and Hippocampus

Rats received injections (sc) of 1.8 mg/kg DFP or peanut oil and were killed one or two hours after administration by head-focused microwave irradiation or decapitation. Values obtained at one and two hours did not differ and were therefore pooled. Following decapitation, brains were removed and either chilled immediately (0 time) or incubated for five minute intervals for up to fifteen minutes at 37°C in a moist chamber. Solid line represents control values and dotted line represents those from DFP-injected animals. Choline levels were determined by pyrolysis gas chromatography (18,19). Each point is the mean of determinations from five to seventeen animals ± S.E.M. All points except microwave (μ) are significantly different from control values, $p < 0.05$.

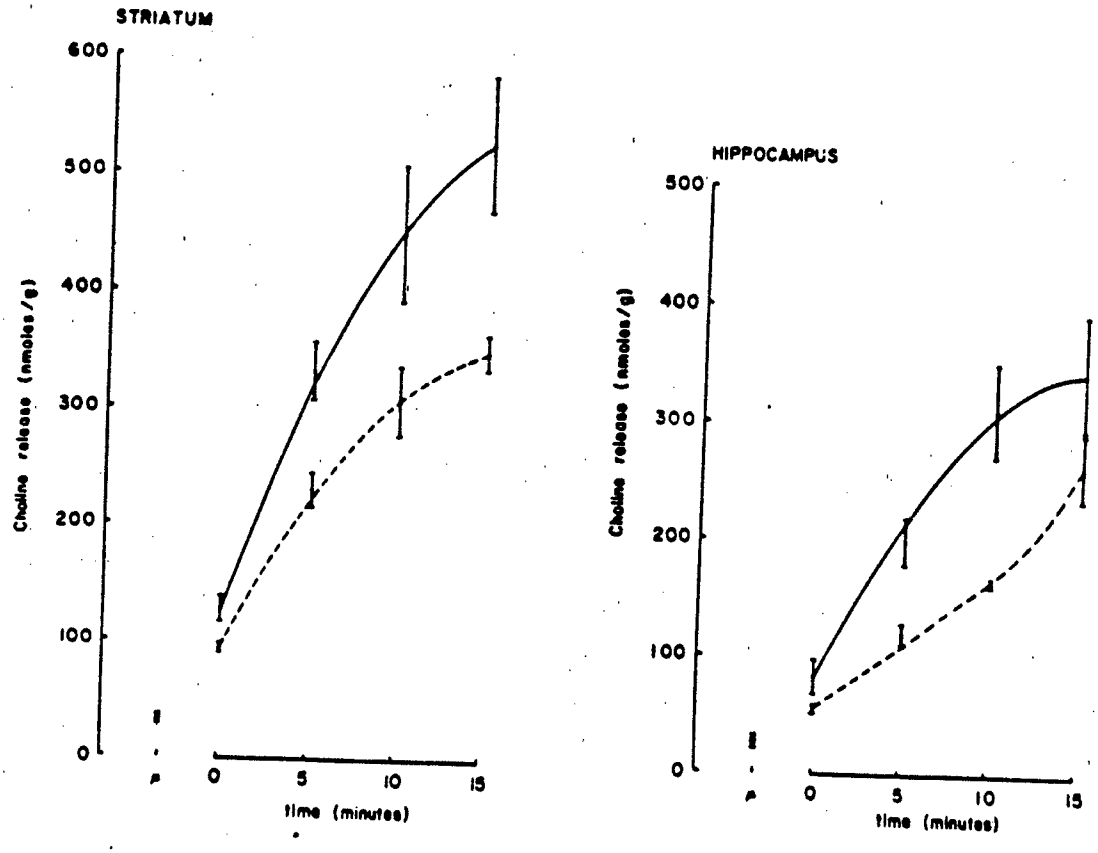


Figure 8

Effects of Paraoxon on the Postmortem Accumulation of Choline in the Striatum and Hippocampus

Rats received injections (sc) of 0.23 mg/kg paraoxon or saline and were killed one or two hours after administration by head-focused microwave irradiation or decapitation. Values obtained at one and two hours did not differ and were therefore pooled. Following decapitation, brains were removed and either chilled immediately (0 time) or incubated for five minute intervals for up to fifteen minutes at 37°C in a moist chamber. Solid line represents control values and dotted line represents those from paraoxon-injected animals. Choline levels were determined by pyrolysis gas chromatography (18,19). Each point is the mean of determinations from eight to twenty-six animals ± S.E.M. All points except microwave (μ) are significantly different from control values, $p < 0.05$.

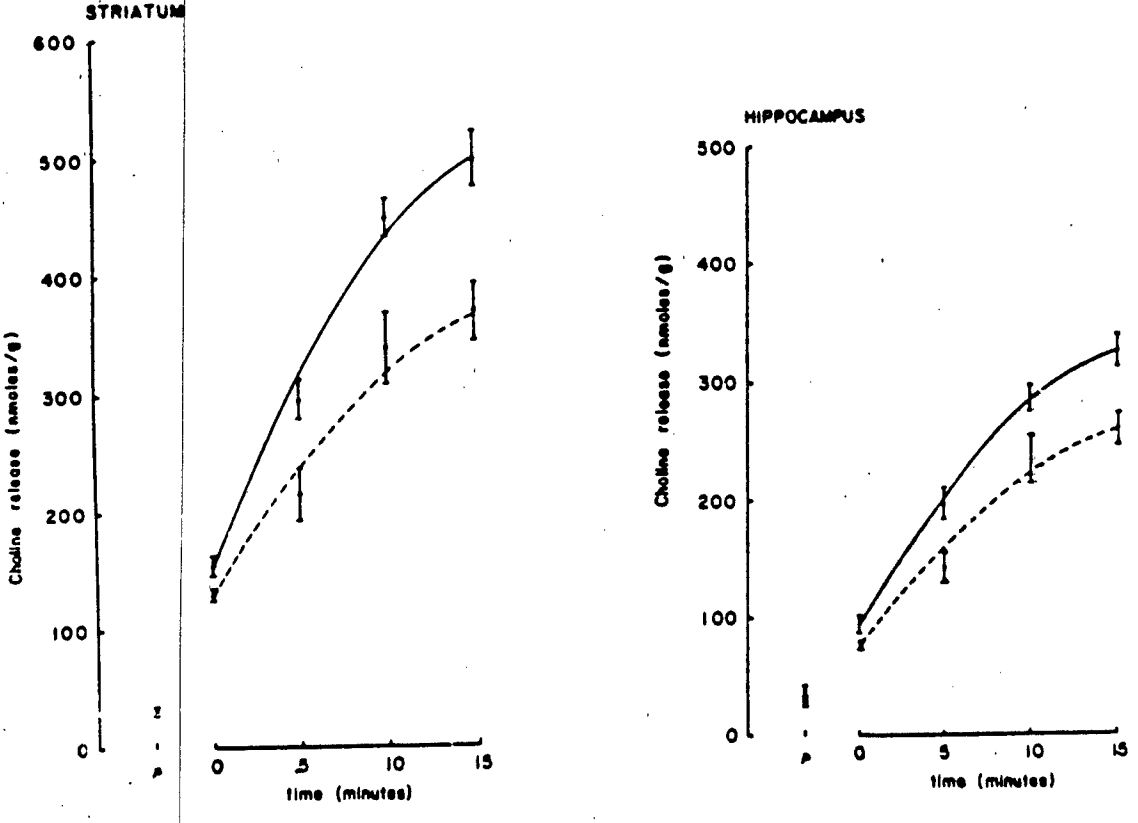


Figure 9

Effects of Soman on the Postmortem Accumulation of Choline in the Striatum and Hippocampus

Rats received injections (sc) of 70-80 µg/kg soman or saline and were killed one or two hours after administration by head-focused microwave irradiation or decapitation. Values obtained at one and two hours did not differ and were therefore pooled. Following decapitation, brains were removed and either chilled immediately (0 time) or incubated for five minute intervals for up to fifteen minutes at 37°C in a moist chamber. Solid line represents control values and dotted line represents those from soman-injected animals. Choline levels were determined by pyrolysis gas chromatography (18,19). Each point is the mean of determinations from six to twenty-four animals ± S.E.M.
*Significantly different from controls, $p < 0.05$.

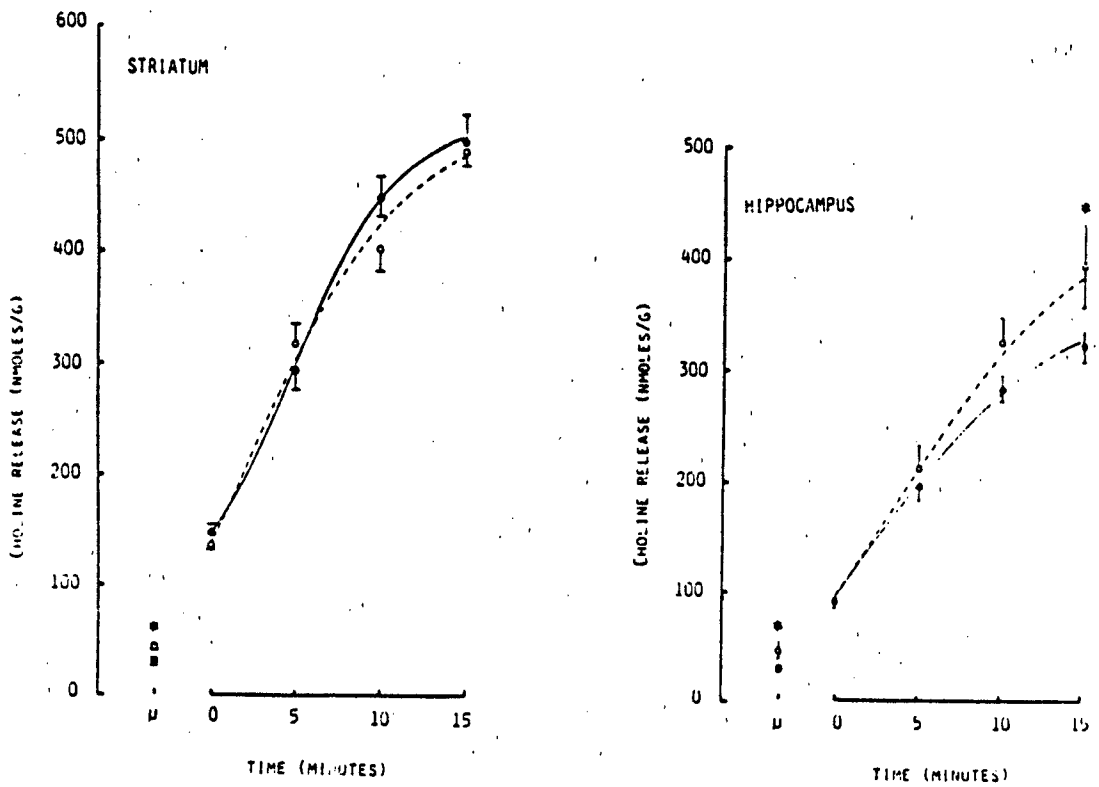
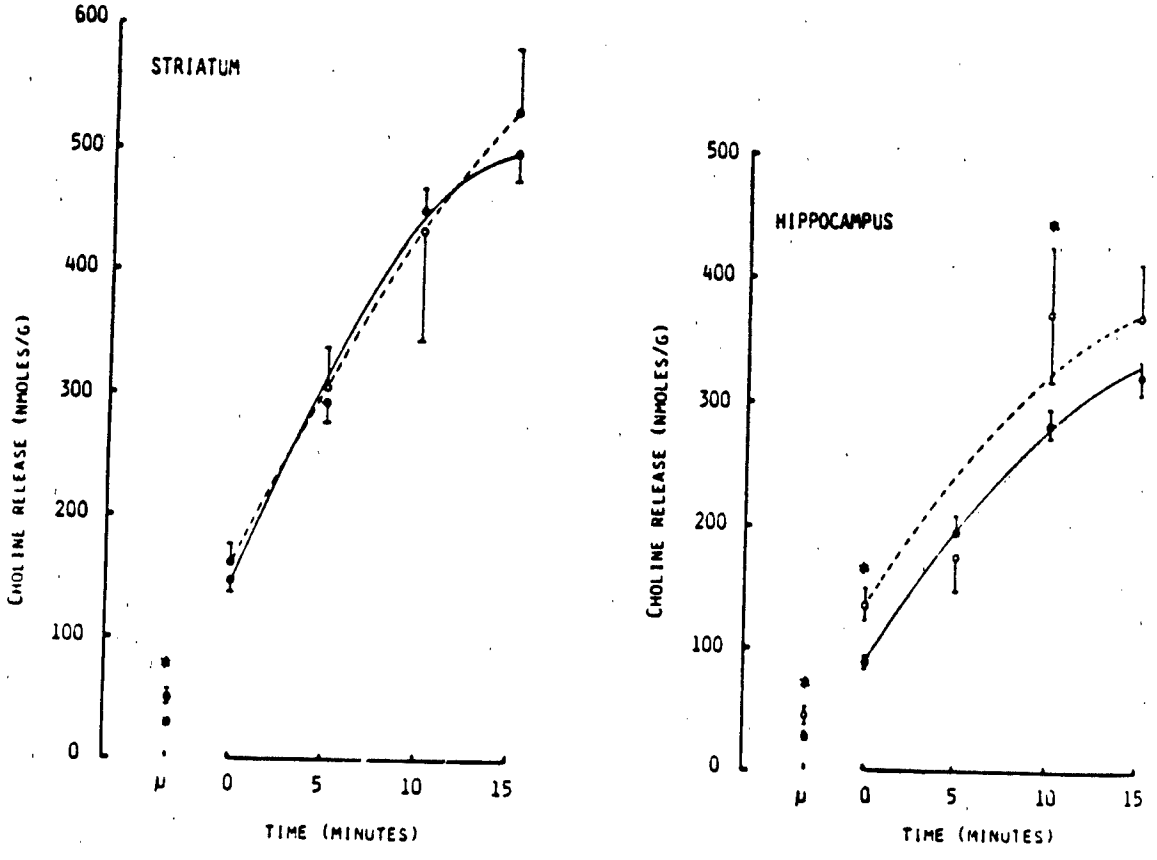


Figure 10

Effects of Sarin on the Postmortem Accumulation of Choline in the Striatum and Hippocampus

Rats received injections (sc) of 80-110 µg/kg sarin or saline and were killed one or two hours after administration by head-focused microwave irradiation or decapitation. Values obtained at one and two hours did not differ and were therefore pooled. Solid line represents control values and dotted line represents those from soman-injected animals. Following decapitation, brains were removed and either chilled immediately (0 time) or incubated for five minute intervals for up to fifteen minutes at 37°C in a moist chamber. Choline levels were determined by pyrolysis gas chromatography (18,19). Each point is the mean of determinations from seven to twenty-four animals ± S.E.M.
*Significantly different from controls, $p < 0.05$.



during subcellular fractionation. Covalent modification, usually via enzymatic phosphorylation, has been suggested as the mechanism of either activation or deactivation of many enzymes *in vivo*, but there is no evidence that phospholipase A2 activity is modulated by such a mechanism *in vivo* (104). Based on the effects of direct addition of the agents to crude mitochondrial fractions observed here, it appears that phosphorylation of this enzyme by soman and sarin results in inhibition. Although the assay used in the present study measured the activity of only one phospholipase A2 of the possibly several that lead to the release of choline in brain, it seems unlikely that other phospholipases A2 would activate in response to phosphorylation by organophosphates. While organophosphates do not inhibit all phospholipases A2 (105-107), they do not appear to activate them.

That soman and sarin affect phospholipase A2 activity only slightly as compared to DFP after *in vivo* administration reflects in part the molar differences in doses. Although the doses of DFP, soman, and sarin administered produced equivalent ChE inhibition, they corresponded to 8.7, 0.38, and 0.71 micromoles per kilogram, respectively. Therefore, at 100% ChE inhibition, the molar dose of DFP was more than 10 times greater than the corresponding doses of soman and sarin. The lack of response to paraoxon is understandable in view of its weak potency *in vitro*. Since inhibition of phospholipase A2 by soman was not evident until four hours after administration, the pharmacokinetic profile of phospholipase inhibition is likely to be different from that of ChE inhibition, which is complete within one hour after administration of all the agents. Furthermore, soman and sarin appear to be different from each other in this regard, since inhibition by soman was not apparent until four hours after administration.

That soman and sarin do not depress the accumulation of choline postmortem may also be a result of the relatively low doses administered. While significantly greater than those of normal animals, the choline levels evident in microwaved brains after soman and sarin poisoning represent only a small fraction of the total choline bound in phospholipids; this relatively small increase becomes undetectable as large amounts of choline accumulate in the brain postmortem. The 30% reduction in the postmortem accumulation of choline caused by DFP is in agreement with the *in vitro* results, which showed phospholipase A2 inhibition of about the same degree. The postmortem data are also in good agreement with the results of Dross (26), who additionally showed that DFP induced biochemical changes consistent with inhibition of phospholipase A2. Therefore, the similarity of paraoxon's effects on postmortem choline production to those of DFP is somewhat surprising, in view of its weak potency *in vivo*. Perhaps paraoxon is active against a lipase involved in the release of choline that is not the same as the one measured *in vivo*, while DFP is active against both. The calcium-independent phospholipase A2 activity measured in these assays is probably mitochondrial (43,108); it could be a different enzyme from the one mediating the accumulation of choline postmortem and may be more sensitive to DFP than to paraoxon. Furthermore, the *in vitro* assay of phospholipase A2 activity may have reversed paraoxon-induced inhibition by some unknown means. It is also possible that phospholipase A2 does not mediate the postmortem release of choline, a possibility that merits further evaluation in view of the central role of phospholipases in current hypothesis concerning ischemic and excitotoxic cell membrane injury in the central nervous system (73).

In conclusion, the results of these studies indicate that four organophosphates, DFP, soman, sarin, and paraoxon, are direct inhibitors of a calcium-independent phospholipase A2 activity in brain and that, *in vitro*, sarin and soman are as potent as DFP. However, the data also indicate that their potencies toward ChE are different from their relative potencies toward phospholipase A2, possibly limiting their effects on this enzyme *in vivo* at doses pharmacologically relevant to ChE inhibition. The role of this phospholipase A2 inhibition in mediating reduced choline accumulation seen postmortem is questionable, since paraoxon, a weak phospholipase inhibitor, is nearly as effective as DFP in producing this effect.

III. INDUCTION OF SKELETAL MUSCLE NECROSIS BY THE ORGANOPHOSPHATES: AN EVENT MEDIATED BY CHOLINESTERASE INHIBITION

Rationale

Results from studies on the actions of the organophosphates on the central nervous system have suggested that these compounds, through an action on choline/phospholipid metabolism, may produce biochemical changes that are deleterious to central neurons. As stated, phosphatidylcholine is a major component of cell membranes and perturbations in the metabolism of this phospholipid can lead to alterations in cell permeability, functional impairment, and possible degeneration of cell structure. Since the actions of the organophosphates may alter cell membranes in regions other than the central nervous system, it was of interest to determine whether peripheral tissue could also be affected.

Objectives:

a) to determine whether the chronic administration of low doses of paraoxon could alter the integrity of skeletal muscle fibers; and
b) to determine whether the effect of paraoxon on skeletal muscle could be related to inhibition of ChE activity in muscle, red blood cells, or plasma

Background

It is well documented that the administration of many organophosphate ChE inhibitors including paraoxon, soman, and phospholine, leads to necrosis of skeletal muscle fibers in the rat (109-113). This effect has been observed following the administration of doses of these compounds that cause excessive cholinergic activity, including prolonged muscle fasciculations, and it has been postulated that the development of the myopathy depends on a critical degree and duration of AChE inhibition, during which time biochemical and physiological alterations ensue (114). Studies on the effects of the organophosphates on the central nervous system indicated that some actions of these compounds may not be attributed to AChE inhibition, but rather, may involve alterations in choline/phospholipid metabolism. Thus, studies were designed to evaluate the hypothesis concerning the role of AChE inhibition and the development of a myopathy.

Methods

Male Sprague-Dawley rats (100-125 g) were housed two per cage and were maintained on a twelve hour light/dark cycle with food and water available *ad libitum*. Rats received daily injections (sc) of paraoxon (0.05 or 0.10 mg/kg) for up to sixty days. Control animals received daily injections of saline. At fifteen day intervals, animals were killed either with ether (for histological analyses) or by decapitation (for enzyme analyses). For histological analyses, the left hemidiaphragm was removed, cleaned, fixed in formalin, mounted, sectioned (10 µm), and stained with hematoxylin and eosin. Three sections from each muscle (100 fibers per section) were examined and the number of lesions per section was quantified (109).

ChE and AChE activities were determined spectrophotometrically (14). For determinations of enzyme activity in skeletal muscle, the left hemidiaphragm was removed, and a central strip (3 x 15 mm) containing nerve terminal branches was isolated for the endplate regions and two sections, above and below the junctional strip, were removed as representative of non-endplate regions. Muscle was weighed, minced and homogenized in phosphate buffer. For the isolation of plasma and red blood cells, blood was collected into heparinized tubes and centrifuged at 1500 x g for 15 minutes at 4°C.

Results

Administration of 0.05 mg/kg paraoxon to rats did not cause any symptoms of excessive cholinergic activity during the sixty days of injections. Similarly, rats injected with 0.10 mg/kg paraoxon did not exhibit parasympathomimetic effects during the first thirty days, but after this time, some rats exhibited mild salivation, diarrhea, and slight tremor which lasted for approximately one hour following injection. The symptoms exhibited by these rats were never as severe or as prolonged as those exhibited following a single large dose (0.23 mg/kg) of paraoxon (115).

Chronic low dose administration of paraoxon led to the development of a myopathy in diaphragm that was qualitatively similar to that following the administration of a single high dose of paraoxon (0.23 mg/kg). The lesion was characterized by the presence of central nuclei, fiber splitting, and breakdown of fiber architecture followed by phagocytosis and necrosis. The myopathy was significant after 30 days of injections of both 0.05 and 0.10 mg/kg (Figure 11). Although more muscle fibers were affected in the group injected with 0.10 mg/kg, as compared to the group injected with 0.05 mg/kg, there were no qualitative differences in lesion characteristics between the two groups. The severity of the myopathy observed at thirty days for both groups was not significantly enhanced by further injections of paraoxon (for up to sixty days).

To determine whether the development of the myopathy could be correlated with ChE inhibition in skeletal muscle, enzyme activity was measured in the endplate and non-endplate regions of diaphragm muscle (Figure 12). No significant enzyme inhibition was noted in either muscle section twenty-four hours after a single dose of 0.05 or 0.10 mg/kg paraoxon. With daily injections, however, there was a progressive inhibition of enzyme activity in the endplate region throughout the sixty-day period. In the non-endplate region, 0.05 mg/kg did not cause significant inhibition of enzyme activity until sixty days of injections, while with 0.10 mg/kg paraoxon, 32% enzyme inhibition was observed after fifteen days of injections and this level of inhibition remained constant throughout the remainder of the injection schedule.

To determine whether enzyme activity in blood could be correlated with the development of the myopathy, the effects of paraoxon on plasma ChE and red blood cell AChE activities were determined (Figure 13). Enzyme activity in plasma and red blood cells was not significantly different from control values when measured twenty-four hours following the administration of either 0.05 or 0.10 mg/kg paraoxon. However, chronic administration of paraoxon led to a significant progressive inhibition of enzyme activity during the first fifteen days. Further injections (up to sixty days) did not enhance enzyme inhibition, i.e., enzyme activity in both plasma and red blood cells remained at a constant reduced level despite further injections of paraoxon.

Discussion

Apart from the pathological alterations at the neuromuscular junction observed after acute toxic exposure to organophosphates, no consensus has been reached regarding the physiological and biochemical effects following prolonged exposure to sublethal levels of these compounds. Results from the present study indicate that chronic, low dose exposure to the organophosphate ChE inhibitor paraoxon has deleterious effects on skeletal muscle morphology that become apparent after prolonged (thirty-day) exposure. Furthermore, skeletal muscle fiber necrosis resulting from the administration of paraoxon occurs in the absence of other signs of organophosphate toxicity.

Studies with acute paraoxon administration have indicated that the organophosphate-induced myopathy is dose-dependent and depends on both a critical duration and degree of enzyme inhibition (114). It has been reported that 85% inhibition of muscle ChE activity is necessary to initiate severe fiber necrosis. The percentage of muscle fibers affected twenty-four hours following a single dose of paraoxon (0.23 mg/kg, sc) has been reported to be 3.2%, with a maximal effect (6.1% fibers affected) seen twenty-four hours after three consecutive days of injection (109). During this time, all animals exhibited signs of organophosphate toxicity immediately after each injection. Results from the present study indicate that with chronic exposure, although the level of enzyme inhibition in muscle never reaches the 85% level, necrosis is still manifest. Furthermore, the maximal number of lesions produced was 2.0%, not unlike the 3.2% observed following a single toxic dose. Hence, the duration of enzyme inhibition may play a more significant role than the actual degree of inhibition when one is considering repeated exposures. These results confirm previous observations, indicating a linear correlation between the time of enzyme inhibition and the severity of the myopathy (114). A further similarity between previous acute studies and the present chronic study includes the finding that there was no significant enzyme inhibition in the non-endplate region of diaphragm muscle with the lower dose of paraoxon (0.05 mg/kg), supporting the hypothesis of involvement of only the endplate enzyme (114).

There have been scattered case reports on neuromuscular function in agricultural workers

Figure 11

Effects of Chronic Paraoxon Administration on the Development of a Myopathy in Rat Diaphragm Muscle

Rats received injections (sc) of 0.05 (o——o) or 0.10 (o-----o) mg/kg paraoxon daily for up to sixty days. Muscle was analyzed for lesions using a histochemical technique (109). Each field represents 100 muscle fibers. Each point is the mean ± S.E.M. of determinations from four to five animals per group.
*Significantly different from saline-injected rats, $p < 0.05$.

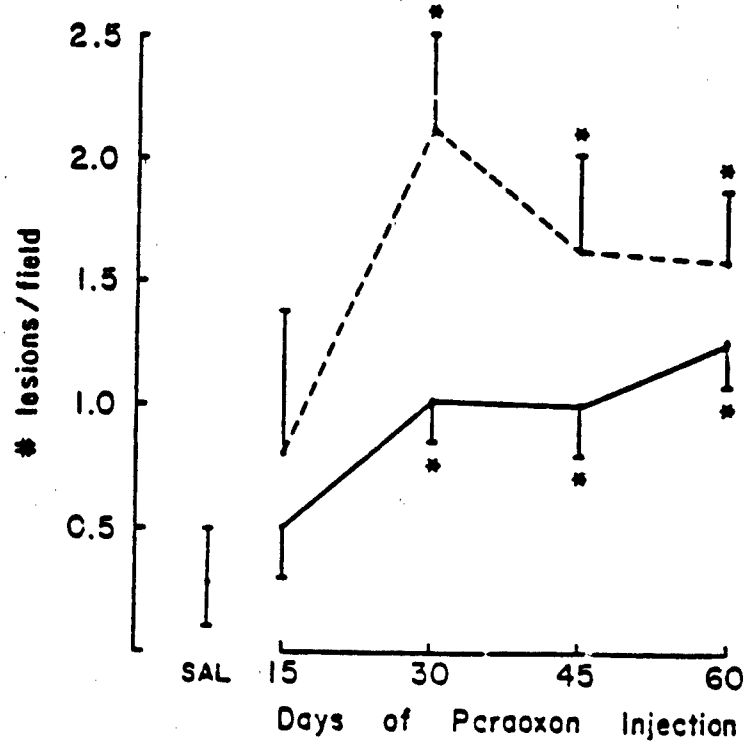
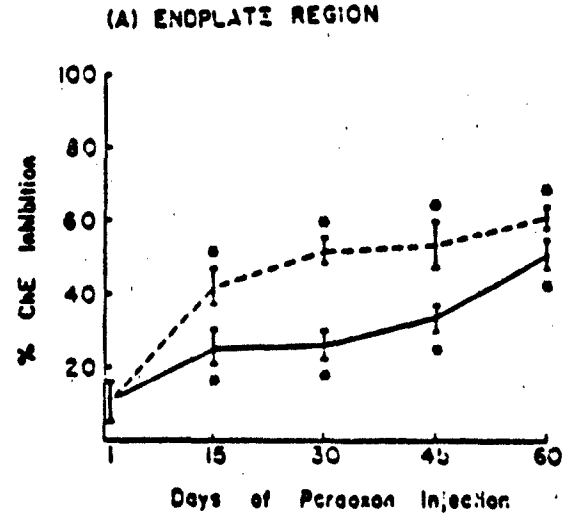


Figure 12

Effects of Chronic Paraoxon Administration on ChE Activity in the (A) Endplate and (B) Non-endplate Regions of Rat Diaphragm Muscle

Rats received injections (sc) of 0.05 (●——●) or 0.10 (●-----●) mg/kg paraoxon daily for up to sixty days. ChE activity was determined spectrophotometrically (14). Enzyme activity in the endplate and non-endplate regions from control rats was 3.39 and 1.50 nmol acetylthiocholine hydrolyzed per minute per milligram tissue, respectively. Each point is the mean ± S.E.M. of determinations from five to six animals per group.
*Significantly different from saline-injected rats, $p < 0.05$.



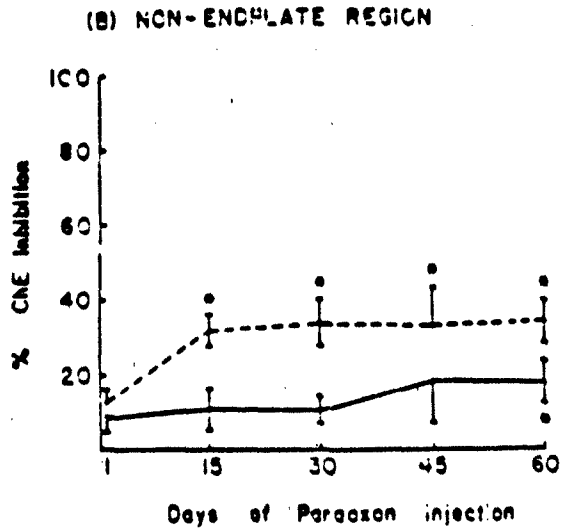
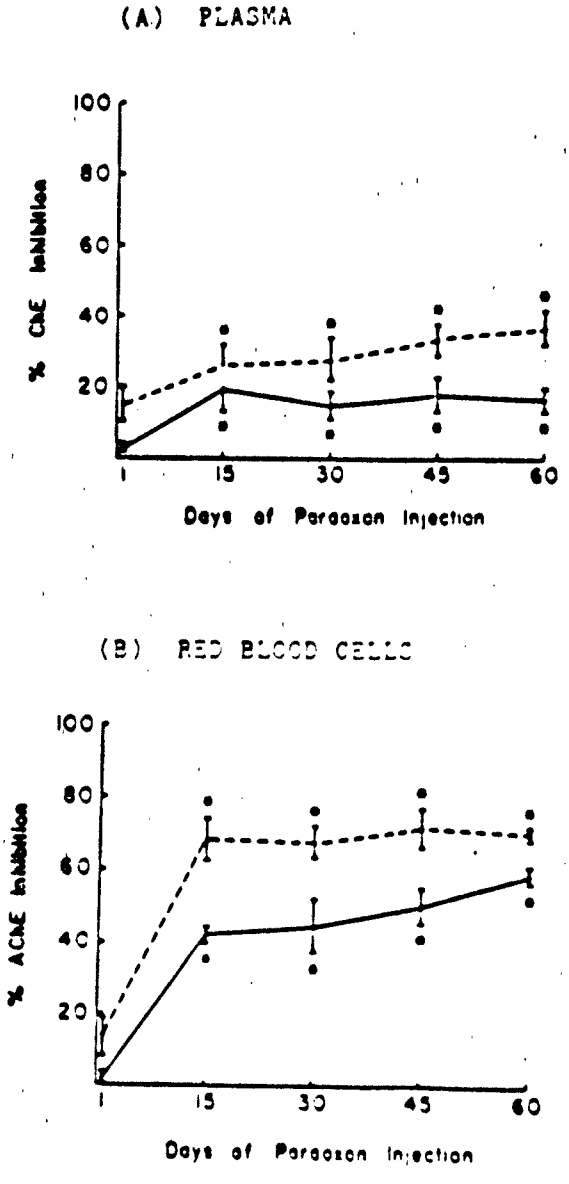


Figure 13

Effects of Chronic Paraoxon Administration on (A) Plasma ChE Activity and (B) Red Blood Cell AChE Activity

Rats received injections (sc) of 0.05 (●——●) or 0.10 (●-----●) mg/kg paraoxon daily for up to sixty days. ChE activity in plasma and AChE activity in red blood cells was determined spectrophotometrically (14). Enzyme activity in plasma and red blood cells from control rats was 0.67 and 1.40 µmol acetylthiocholine hydrolyzed per minute per milliliter, respectively. Each point is the mean ± S.E.M. of determinations from five to six animals per group.
*Significantly different from saline-injected rats, $p < 0.05$



exposed to organophosphate compounds. Although ChE activity was depressed, no definitive conclusions were reached concerning indications of abnormalities of neuromuscular function (116). Abnormal electromyographic recordings have been reported in 40% of a population of agricultural workers (117), but these findings could not be confirmed in a random group of occupationally exposed workers (118). Other possible evidences of alterations in neuromuscular function induced by chronic insecticide exposure include changes in the Achilles tendon reflex amplitude, low evoked muscle action potentials and low motor nerve conduction velocity (119,120), although the latter observations could not be reconfirmed in the same laboratory. It is possible that abnormalities in neuromuscular function observed in occupationally exposed workers may result from pathological alterations.

In summary, chronic exposure of rats to paraoxon, at doses that do not produce parasympathomimetic effects, leads to necrosis of skeletal muscle fibers. Hence, the organophosphate ChE inhibitors may cause deleterious effects in skeletal muscle in the absence of other signs of organophosphate toxicity.

REFERENCES

1. Perry, W.L.M. (1953) Acetylcholine release in the cat's superior cervical ganglion. J. Physiol. (Lond.) **119**: 439-454.

2. Collier, B. and MacIntosh, F.C. (1969) The source of choline for acetylcholine synthesis in sympathetic ganglion. Can. J. Physiol. Pharmacol. **47**: 127-135.

3. Potter, L.T. (1970) Synthesis, storage and release of (^{14}C) acetylcholine in isolated rat diaphragm muscles. J. Physiol. (Lond.) **206**: 145-166.

4. Hanin, I., Massarelli, R. and Costa, E. (1972) An approach to the study of the biochemical pharmacology of cholinergic functions. Adv. Biochem. Pharmacol. **6**: 181-203.

5. Dross, K. and Kewitz, H. (1968) Konzentration von acetylcholin, cholin and glycerylphosphorylcholin in gehirn wahrend der narkose and bei der DFP-vergiftung. Naunyn-Schmiedebergs Arch. Pharmak. Exp. Path. **260**: 107-108.

6. Wecker, L. and Dettbarn, W-D. (1979) Relationship between choline availability and acetylcholine synthesis in discrete regions of rat brain. J. Neurochem. **32**: 961-967.

7. Schmidt, D.E. and Wecker, L. (1981) CNS effects of choline administration: Evidence for temporal dependence. Neuropharmacology **20**: 535-539.

8. Wecker, L., Flynn, C.J., Stouse, M.R. and Tommer, B.A. (1982) Choline availability: Effects on the toxicity of centrally active drugs. Drug-Nutrient Interactions **1**: 125-130.

9. Millington, W.R. and Wurtman, R.J. (1982) Choline administration elevates brain phosphorylcholine concentrations. J. Neurochem. **38**: 1748-1752.

10. Coleman, R. (1973) Membrane-bound enzymes and membrane ultrastructure. Biochim. Biophys. Acta **300**: 1-30.

11. Beauregard, G. and Roufogalis, B.D. (1977) The role of tightly bound phospholipid in the activity of erythrocyte acetylcholinesterase. Biochem. Biophys. Res. Commun. **77**: 211-219.

12. Roufogalis, B.D. and Beauregard, G. (1979) Role of phospholipid (cardiolipin) in the modulation of substrate and inhibitor interactions with erythrocyte acetylcholinesterase. Mol. Pharmacol. **16**: 189-195.

13. Heger, H.W. (1979) Effect of dietary phospholipids on acetylcholinesterase activity in the rat brain and on phospholipid composition in liver and brain. Gen. Pharmacol. **10**: 427-432.

14. Ellman, G.L., Courtney, K.D., Andres, V., Jr. and Featherstone, R.M. (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. **7**: 88-95.

15. Ansell, G.B. and Spanner, S. (1968) The metabolism of (Me-^{14}C)choline in the brain of the rat *in vivo*. Biochem. J. **110**: 201-206.

16. Diamond, I. (1971) Choline metabolism in brain: The role of choline transport and the effects of phenobarbital. Arch. Neurol. **24**: 333-339.

17. Dowdall, M.J., Barker, L.A. and Whittaker, V.P. (1972) Choline metabolism in the cerebral cortex of guinea pigs. Biochem. J. **130**: 1081-1094.

18. Flynn, C.J. and Wecker, L. (1986) Elevated choline levels in brain. A non-cholinergic component of organophosphate toxicity. Biochem. Pharmacol. **35**: 3115-3121.

19. Flynn, C.J. and Wecker, L. (1987) Concomitant increases in the levels of choline and free fatty acids in rat brain: Evidence supporting the seizure-induced hydrolysis of phosphatidylcholine. J. Neurochem. **48**: 1178-1184.

20. Gray, E.G. and Whittaker, V.P. (1962) The isolation of nerve endings from brain: An electron microscopic study of cell fragments derived by homogenization and centrifugation. J. Anat. (Lond.) **96**: 79-88.

21. Folch, J., Lees, M. and Stanley, G.H.S. (1957) A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. **226**: 497-509.

22. Rouser, G., Fleischer, S. and Yamamoto, A. (1970) Two dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. Lipids **5**: 494-496.

23. Sethy, V.H. and van Woert, H. (1973) Antimuscarinic drugs-effect on brain acetylcholine and tremors in rats. Biochem. Pharmacol. **22**: 2685-2691.

24. Stavinoha, W.B., Modak, A.T. and Weintraub, S.T. (1976) Rate of accumulation of acetylcholine in discrete regions of the rat brain after dichlorvos treatment. J. Neurochem. **27**: 1375-1378.

25. Beani, L., Bianchi, C., Megazzini, P., Ballotti, P. and Bernardi, G. (1969) Drug induced changes in free, labile and stable acetylcholine of guinea-pig brain. Biochem. Pharmacol. **18**: 1315-1324.

26. Milosevic, M.P. (1969) Acetylcholine content in the brain of rats treated with paraoxon and pyridinium-2-aldoxime methylchloride. J. Pharm. Pharmacol. **21**: 469-470.

27. Szerb, J.C. and Somogyi, G.T. (1973) Depression of acetylcholine release from cerebral cortical slices by cholinesterase inhibition and by oxotremorine. Nature (Lond.) **241**: 121-122.

28. Szerb, J.C., Malik, H. and Hunter, E.G. (1970) Relationship between acetylcholine content and release in the cat's cerebral cortex. Can. J. Physiol. Pharmacol. **48**: 780-790.

29. Jenden, D.J., Jope, R.S. and Weiler, M.W. (1976) Regulation of acetylcholine synthesis: Does cytoplasmic acetylcholine control high affinity choline uptake? Science **194**: 635-637.

30. Ansell, G.B. and Spanner, S. (1977) The source of choline for acetylcholine synthesis. In: Advances in Behavioral Biology, Vol. 24 (Jenden, D.J., ed.), pp. 431-445. Plenum Press, New York.

31. Ansell, G.B. and Spanner, S. (1979) Sources of choline for acetylcholine synthesis in brain. In: Nutrition and the Brain, Vol. 5 (Barbeau, A., Growdon, J.H. and Wurtman, R.J., eds.), pp. 35-46. Raven Press, New York.

32. Dross, K. (1975) Effects of di-isopropylfluorophosphate on the metabolism of choline and phosphatidylcholine in rat brain. J. Neurochem. 24: 701-706.

33. Russell, R.W., Carson, V.G., Booth, R.A. and Jenden, D.J. (1981) Mechanisms of tolerance to the anticholinesterase, DFP: Acetylcholine levels and dynamics in the rat brain. Neuropharmacology 20: 1197-1201.

34. Potter, P.E., Hadjiconstantinou, M., Rubenstein, J.S. and Neff, N.H. (1984) Chronic treatment with diisopropylfluorophosphate increases dopamine turnover in the striatum of the rat. Eur. J. Pharmacol. 106: 607-611.

35. Shih, T.M. (1982) Time course effects of soman on acetylcholine and choline levels in six discrete areas of the rat brain. Psychopharmacology 78: 170-175.

36. Modak, A.T., Stavinoha, W.B. and Weintraub, S.T. (1975) Dichlorvos and the cholinergic system: Effects on cholinesterase and acetylcholine and choline contents of rat tissues. Arch. Int. Pharmacodyn. Ther. 217: 293-301.

37. Stavinoha, W.B. and Weintraub, S.T. (1974) Choline content of rat brain. Science 183: 964-965.

38. Nelson, W.L. and Barnum, C.P. (1960) The effect of diisopropylphosphorofluoridate (DFP) on mouse brain phosphorus metabolism. J. Neurochem. 6: 43-50.

39. Ansell, G.B. and Chojnacki, T. (1967) The effect of diisopropylfluorophosphonate on enzymes concerned in brain phospholipid synthesis. Progr. Biochem. Pharmacol. 3: 189-195.

40. Ansell, G.B. and Spanner, S. (1975) The origin and metabolism of brain choline. In: Cholinergic Mechanisms (Waser, P.G., ed.), pp. 117-129. Raven Press, New York.

41. Kennedy, E.P. and Weiss, S.B. (1956) The function of cytidine coenzymes in the biosynthesis of phospholipids. J. Biol. Chem. 222: 193-214.

42. Jope, R.S. and Jenden, D.J. (1979) Choline and phospholipid metabolism and the synthesis of acetylcholine in the rat brain. J. Neurosci. Res. 4: 69-82.

43. Woelk, H., Goracci, G., Gaiti, A. and Porcellati, G. (1973) Phospholipase A_1 and A_2 activities of neuronal and glial cells of the rabbit. Hoppe-Seyler's Z. Physiol. Chem. Phys. 354: 729-736.

44. Webster, G.R., Marples, E.A. and Thompson, R.H.S. (1957) Glycerophosphorylcholine diesterase activity of nervous tissue. Biochem. J. 65: 374-377.

45. Mann, S.P. (1975) Distribution of glycerophosphorylcholine diesterase in rat brain. Experientia 31: 1256-1258.

46. Tucek, S. (1984) Problems in the organization and control of acetylcholine synthesis in brain neurons. Progr. Biophys. Molec. Biol. **44**: 1-46.

47. Brown, J.H. and Bowles, M.E. (1966) Studies on the phospholipase A activity of *Crotalus atrox* venom. Toxicon **3**: 205-212.

48. Desnuelle, P., Sarda, L. and Ailhaud, G. (1960) Inhibition of pancreatic lipase by diethyl-p-nitrophenylphosphate in emulsion. Biochim. Biophys. Acta **37**: 570-571.

49. Cooper, M.F. and Webster, G.R. (1970) The differentiation of phospholipase A_1 and A_2 in rat and human nervous tissue. J. Neurochem. **17**: 1543-1544.

50. Wykle, R.L. and Schremmer, J.M. (1974) A lysophospholipase D pathway in the metabolism of ether-linked lipids in brain microsomes. J. Biol. Chem. **249**: 1742-1746.

51. DeVries, G.H., Chalifour, R.J. and Kanfer, J.N. (1983) The presence of phospholipase D in rat central nervous system axolemma. J. Neurochem. **40**: 1189-1191.

52. Taki, T. and Kanfer, J.N. (1979) Partial purification and properties of a rat brain phospholipase D. J. Biol. Chem. **254**: 9761-9765.

53. Ladinsky, H., Consolo, S. and Peri, G. (1974) Effect of oxotremorine and physostigmine on choline levels in mouse whole brain, spleen and cerebellum. Biochem. Pharmacol. **23**: 1187-1193.

54. Lundgren, G., Karlen, B. and Holmstedt, B. (1977) Acetylcholine and choline in mouse brain: Influence of peripherally acting cholinergic drugs. Biochem. Pharmacol. **26**: 1607-1612.

55. Dolezal, V. and Tucek, S. (1984) Activation of muscarinic receptors stimulates the release of choline from brain slices. Biochem. Biophys. Res. Commun. **120**: 1002-1007.

56. Corradetti, R., Lindmar, R. and Loffelholz, K. (1982) Physostigmine facilitates choline efflux from isolated heart and cortex *in vivo*. Eur. J. Pharmacol. **85**: 123-124.

57. McDonough, J.H., Jr., Hackley, B.E., Jr., Cross, R., Samson, F. and Nelson, S. (1983) Brain regional glucose use during soman-induced seizures. Neurotoxicology **4**: 203-210.

58. Pazdernik, T.L., Cross, R.S., Giesler, M., Samson, F.E. and Nelson, S.R. (1985) Changes in local cerebral glucose utilization induced by convulsants. Neuroscience **14**: 823-835.

59. Samson, F.E., Pazdernik, T.L., Cross, R.S., Geisler, M.P., Mewes, K., Nelson, S.R. and McDonough, J.H. (1984) Soman induced changes in brain regional glucose use. Fund. Appl. Toxicol. **4**: S173-S183.

60. Bazan, N.G. (1970) Effects of ischemia and of electroconvulsive shock on free fatty acid pool in the brain. Biochim. Biophys. Acta **118**: 1-10.

61. Bazan, N.G., Bazan, H.E.P. de, Kennedy, W.G. and Joel, C.D. (1971) Regional distribution and rate of production of free fatty acids in brain. J. Neurochem. **18**: 1387-1393.

62. Chapman, A.G., Meldrum, B.S. and Siesjo, B. (1977) Cerebral metabolic changes during prolonged epileptic seizures in rats. J. Neurochem. **28**: 1025-1035.

63. Bazan, N.G., Birkle, D.L., Tang, W. and Reddy, T.S. (1986) The accumulation of free arachidonic acid, diacylglycerols, prostaglandins, and lipoxygenase reaction products in the brain during experimental epilepsy. In: Advances in Neurology, Vol. 44 (Delgado-Escueta, A.V., Ward, A.A., Jr., Woodbury, D.M. and Porter, R.J., eds.), pp. 879-902. Raven Press, New York.

64. Bazan, N.G. (1971) Changes in free fatty acids of brain by drug-induced convulsions, electroshock and anaesthesia. J. Neurochem. **18**: 1379-1385.

65. Bazan, N.G. (1976) Free arachidonic acid and other lipids in the nervous system during early ischemia and after electroshock. In: Function and Metabolism of Phospholipids in the Central Nervous System (Porcellati, G., Amaducci, L. and Galli, L., eds.), pp. 317-356. Plenum Press, New York.

66. Bazan, N G. and Rodriguez de Turco, E.B. (1983) Seizures promote breakdown of membrane phospholipids in the brain. In: Neural Transmission, Learning and Memory (Caputto, R. and Marsan, C.A., eds.), pp. 187-194. Raven Press, New York.

67. Marion, J. and Wolfe, L.S. (1979) Origin of the arachidonic acid released post-mortem in rat forebrain. Biochim. Biophys. Acta **574**: 25-32.

68. Sun, G.Y. and Su, K. (1979) Metabolism of arachidonoyl phosphoglycerides in mouse brain subcellular fractions. J. Neurochem. **32**: 1053-1059.

69. Siesjo, B., Ingvar, M. and Westerberg, E. (1982) The influence of bicuculline-induced seizures on free fatty acid concentration in cerebral cortex, hippocampus and cerebellum. J. Neurochem. **39**: 796-802.

70. Rodriguez de Turco, E.B., Morelli de Liberti, S. and Bazan, N.G. (1983) Stimulation of free fatty acid and diacylglycerol accumulation in cerebrum and cerebellum during bicuculline-induced status epilepticus. Effect of pretreatment with α-methyl-p-tyrosine and p-chlorophenylalanine. J. Neurochem. **40**: 252-259.

71. Sun, G.Y. and Sun, A.Y. (1972) Phospholipids and acyl groups of synaptosomal and myelin membranes isolated from the cerebral cortex of squirrel monkey (*Saimiri sciureus*). Biochim. Biophys. Acta **280**: 306-315.

72. Breckenridge, W.C., Gombos, G. and Morgan, I.G. (1972) The lipid composition of adult rat brain synaptosomal plasma membranes. Biochim. Biophys. Acta **266**: 695-707.

73. Wieloch, T. and Siesjo, B. (1982) Ischemic brain injury: The importance of calcium, lipolytic activities, and free fatty acids. Pathol. Biol. **30**: 269-277.

74. Creutz, C.E. (1981) Cis-unsaturated fatty acids induce the fusion of chromaffin granules aggregated by synexin. J. Cell Biol. **91**: 247-256.

75. Poole, A.R., Howell, J.I. and Lucy, J.A. (1970) Lysolecithin and cell fusion. Nature **226**: 810-814.

76. Yoshida, S., Inoh, S., Asano, T., Sano, K., Kubota, M., Shimazaki, H. and Ueta, N. (1980) Effect of transient ischemia on free fatty acids and phospholipids in the gerbil brain: Lipid peroxidation as a possible cause of postischemic injury. J. Neurosurg. **53**: 323-331.

77. Demopoulos, H.B., Flamm, E.S., Pietronigro, D.D. and Seligman, M.L. (1980) The free radical pathology and the microcirculation in the major central nervous system disorders. Acta Physiol. Scand. Suppl. **492**: 91-119.

78. Wolfe, L. (1982) Eicosanoids: Prostaglandins, thromboxanes, leukotrienes, and other derivatives of carbon-20 unsaturated fatty acids. J. Neurochem. **38**: 1-13.

79. Dichter, M.A. (1983) The epilepsies and convulsive disorders. In: Harrison's Principles of Internal Medicine, Chapter 355, 10th edition (Petersdorf R.G., Adams, R.D., Braunwald, E., Isselbacher, K.J., Martin, J.B. and Wilson, J.D., eds.), p. 2018. McGraw-Hill, New York.

80. Martin, L.J., Doebler, J.A., Shih, T.M. and Anthony, A. (1985) Protective effect of diazepam pretreatment on soman-induced brain lesion formation. Brain Res. **325**: 287-289.

81. Griffiths, R., Evans, M.C. and Meldrum, B.S. (1983) Intracellular calcium accumulation in rat hippocampus during seizures induced by bicuculline or L-allylglycine. Neuroscience **10**: 385-395.

82. Clement, J.G. and Lee, M.J. (1980) Soman-induced convulsions: Significance of changes in levels of blood electrolytes, gases, glucose and insulin. Toxicol. Appl. Pharmacol. **55**: 203-204.

83. Boskovic, B. (1981) The treatment of soman poisoning and its perspectives. Fund. Appl. Toxicol. **1**: 203-213.

84. Lipp, J.A. (1973) Effect of benzodiazepine derivatives on soman-induced seizure activity and convulsions in the monkey. Arch. Int. Pharmacodyn. Ther. **202**: 244-251.

85. Lundy, P.M. and Magor, G.F. (1978) Cyclic GMP concentrations in cerebellum following organophosphate administration. J. Pharm. Pharmacol. **30**: 251-252.

86. Bellet, E.M. and Casida, J.E. (1973) Bicyclic phosphorus esters: High toxicity without cholinesterase inhibition. Science **182**: 1135-1136.

87. Coult, D., Howells, D. and Smith, P. (1979) Cyclic nucleotide concentrations in the brains of mice treated with the convulsant bicyclic organophosphate 4-isopropyl-2,6,7-trioxa-1-phosphabicyclo(2,2,2)octane. Biochem. Pharmacol. **28**: 193-196.

88. Bowery, N.G., Collins, J.F., Hill, R.G. and Pearson, S. (1976) GABA antagonism as a possible basis for the convulsant action of a series of bicyclic phosphorus esters. Proc. Brit. Pharmacol. Soc. **4**: 435-436.

89. Woodbury, D. (1980) Convulsants. In: Antiepileptic Drugs: Mechanisms of Action (Glaser, G.H., Penry, J.K. and Woodbury, D.M., eds.), pp. 249-302. Raven Press, New York.

90. Coudray-Lucas, C., Prioux-Guyonneau, M., Sentenac, H., Cohen, Y. and Wepeirre, J. (1984) Effects of physostigmine, paraoxon, and soman on brain GABA level and metabolism. Acta Pharmacol. Toxicol. 55: 153-157.

91. Lundy, P.M., Magor, G.F. and Shaw, R.K. (1978) Gamma aminobutyric acid metabolism in different areas of rat brain at the onset of soman-induced convulsions. Arch. Int. Pharmacodyn. Ther. 234: 64-73.

92. Ho, I.K., Fernando, J.C.R., Sivam, S.P. and Hoskins, B. (1984) Roles of dopamine and GABA in neurotoxicity of organophosphorus cholinesterase inhibitors. Proc. West. Pharmacol. Soc. 27: 177-180.

93. Jovic, R., Bachelard, H.S., Clark, A.G. and Nicholas, P.C. (1971) Effect of soman and DFP *in vivo* and *in vitro* on cerebral metabolism in the rat. Biochem. Pharmacol. 20: 519-527.

94. Wecker, L., Mobley, P.L. and Dettbarn, W.D. (1977) Effects of atropine on paraoxon-induced alterations in brain acetylcholine. Arch. Int. Pharmacodyn. Ther. 227: 69-75.

95. Austin, L. (1957) Lipid biosynthesis and chemically induced paralysis in the chicken. Brit. J. Pharmacol. 12: 356-360.

96. Porcellati, G. (1967) The effect of organo-phosphorus compounds on nerve phospholipid metabolism. Progr. Biochem. Pharmacol. 3: 49-58.

97. Spanner, S. and Ansell, G.B. (1978) Choline and ethanolamine kinase activity in the cytoplasm of nerve endings from rat forebrain. Adv. Exp. Med. Biol. 101: 237-245.

98. Spanner, S. and Ansell, G.B. (1979) Choline kinase and ethanolamine kinase activity in the cytosol of nerve endings from rat forebrain. Biochem. J. 178: 753-760.

99. Reinhardt, R.R. and Wecker, L. (1983) Evidence for membrane-associated choline kinase activity in rat striatum. J. Neurochem. 41: 623-629.

100. Lanni, C. and Franson, R.C. (1981) Localization and partial purification of a neutral-active phospholipase A_2 from BCG-induced rabbit alveolar macrophages. Biochim. Biophys. Acta 658: 54-63.

101. Reinhardt, R.R., Wecker, L. and Cook, P. (1984) Kinetic mechanism of choline kinase from rat striata. J. Biol. Chem. 259: 7446-7452.

102. Hayes, W.J. (1982) Pesticides Studied in Man. Williams and Wilkins, Baltimore, MD.

103. Ellin, R.I. (1982) Anomalies in theories and therapy of intoxication by potent organophosphorus compounds. Gen. Pharmacol. 13: 457-466.

104. van den Bosch, H. (1980) Intracellular phopholipases A. Biochim. Biophys. Acta 604: 191-246.

105. Webb, E.C. (1948) The action of alkyl fluorophosphates on esterases and other enzymes. Biochemistry 42: 96-98.

106. Salach, J.I., Seng, R., Tisdale, H. and Singer, T.P. (1971) Phospholipase A of snake venoms. J. Biol. Chem. 246: 340-347.

107. Roberts, M.F., Deems, R.A., Mincey, T. and Dennis, E.A. (1977) Chemical modification of the histidine residue in phospholipase A2 (*Naja naja naja*). J. Biol. Chem. 252: 2405-2411.

108. Woelk, H. and Porcellati, G. (1973) Subcellular distribution and kinetic properties of rat brain phospholipases A_1 and A_2. Hoppe-Seyler's Z. Physiol. Chem. Phys. 354: 90-100.

109. Wecker, L. and Dettbarn, W-D. (1976) Paraoxon-induced myopathy: Muscle specificity and acetylcholine involvement. Exp. Neurol. 51: 281-291.

110. Wecker, L. and Dettbarn, W-D. (1977) Effects of denervation on the production of an experimental myopathy. Exp. Neurol. 57: 94-101.

111. Laskowski, M.B., Olson, W.H. and Dettbarn, W-D. (1975) Ultrastructural changes at the motor end-plate produced by an irreversible cholinesterase inhibitor. Exp. Neurol. 47: 290-306.

112. Laskowski, M.B., Olson, W.H. and Dettbarn, W-D. (1977) An ultrastructural study of the initial myopathic changes produced by paraoxon. Exp. Neurol. 57: 13-33.

113. Dettbarn, W-D. (1984) Pesticide induced muscle necrosis: Mechanisms and prevention. Fund. Appl. Toxicol. 4: S18-S26.

114. Wecker, L., Kiauta, T. and Dettbarn, W-D. (1978) Relationship between acetylcholinesterase inhibition and the development of a myopathy. J. Pharmacol. Exp. Therap. 206: 97-104.

115. Wecker, L., Laskowski, M.B. and Dettbarn, W-D. (1978) Neuromuscular dysfunction induced by acetylcholinesterase inhibition. Fed. Proc. 37: 2818-2822.

116. Jager, K.W., Roberts, D.V. and Wilson, A. (1970) Neuromuscular function in pesticide workers. Brit. J. Ind. Med. 27: 273-278.

117. Drenth, H.J., Ensberg, I.F.G., Roberts, D.V. and Wilson, A. (1972) Neuromuscular function in agricultural workers using pesticides. Arch. Environ. Health 25: 395-398.

118. Jusic, A. (1974) Anticholinesterase pesticides of organophosphorus type: Electromyographic, neurological and psychological studies in occupationally exposed workers. In: Behavioral Toxicology (Xintaras, C., Johnson, B.L. and deGroot, I., eds.), pp. 182-190. United States Department of Health, Education, and Welfare, Washington, D.C.

119. Rayner, M.D., Popper, J.S., Carvalho, E.W. and Hurov, H. (1972) Hyporeflexia in workers chronically exposed to organophosphate insecticides. Res. Comm. Chem. Pathol. Pharmacol. 4: 595-606.

120. Roberts, D.V. (1976) E.M.G. Voltage and motor nerve conduction velocity in organophosphorus pesticide factory workers. Int. Arch. Occup. Environ. Health 36: 267-274.

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GRADUATE DEGREES RECEIVED

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"The Effects of Organophosphorus Cholinesterase Inhibitors on Choline Metabolism in Brain"

PUBLICATIONS

Flynn C.J. and Wecker L. (1983) Effects of Paraoxon on the Postmortem Release of Choline from Rat Brain. *Neurosci. Abst.* **9:** 962.

Wecker L., Flynn C.J., and Taylor S. (1984) Effects of Dietary Choline on Phospholipids in Rat Brain. *Trans. Amer. Soc. Neurochem.* **15:** 211.

Flynn C.J. and Wecker L. (1984) Effects of Cholinesterase Inhibitors on Choline Mobilization in Rat Brain. *The Pharmacologist* **26:** 225.

Reinhardt R.R., Wecker L., and Cook P. (1984) Kinetic Mechanism of Choline Kinase from Rat Striata. *J. Biol. Chem.* **259:** 7446-7452.

Thakkar J.K. and Wecker L. (1985) Phospholipase A_2 Activities in Subcellular Fractions from Rat Striatum. *Trans. Amer. Soc. Neurochem.* **16:** 309.

Flynn C.J. and Wecker L. (1985) Diazepam Prevents Organophosphate-Induced Increases in Brain Choline Levels. *Neurosci. Abst.* **11:** 1263.

Wecker L. and Stouse M. (1985) Effects of Chronic Paraoxon Administration on Skeletal Muscle Fiber Integrity. *Res. Comm. Chem. Pathol. Pharmacol.* **49:** 203-213.

Flynn C.J., Birkle D.L., and Wecker L. (1986) Diazepam Prevents Seizure-Induced Increases in Free Fatty Acids and Choline in Rat Cerebrum. *Neurosci. Abst.* **12:** 454.

Flynn C.J. and Wecker L. (1986) Elevated Choline Levels in BrainA Non-Cholinergic Component of Organophosphate Toxicity. *Biochem. Pharmacol.* **35:** 3115-3121.

Wecker L. (1986) The Utilization of Supplemental Choline by Brain. In: *Dynamics of Cholinergic Function* (I. Hanin, Ed.), Plenum Press, New York, pp. 851-858.

Flynn C.J. and Wecker L. (1987) Concomitant Increases in the Levels of Choline and Free Fatty Acids in Rat BrainEvidence Supporting the Seizure-Induced Hydrolysis of Phosphatidylcholine. *J. Neurochem.* **48:** 1178-1184.

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