Biochemical Pharmacology, Vol. 30, No. 18, pp. 2593–2601, 1981.
Printed in Great Britain.

0006-2952/81/182593-09 $02.00/0
Pergamon Press Ltd.

SEIZURE-INDUCED CHANGES IN THE PERMEABILITY OF THE BLOOD–BRAIN BARRIER FOLLOWING ADMINISTRATION OF ANTICHOLINESTERASE DRUGS TO RATS*

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(*Received* 25 *July* 1980; *accepted* 6 *January* 1981)

Abstract—Powerful inhibitors of acetylcholinesterase (phospholine iodide, paraoxon, and Soman) were used either separately or in combination with an anesthetizing drug (nembutal), an acetylcholine antagonist (atropine sulfate), or a convulsive drug (metrazole) to study the resistance of the blood–brain barrier to their effects. On the basis of measurements of acetylcholinesterase inhibition in rat brain stem and corpus striatum, it was concluded that these anticholinesterase drugs increased the permeability of the blood–brain barrier, provided that seizures were manifested shortly after administration of these drugs. Rats that were treated prophylactically with either nembutal or atropine sulfate did not convulse and, consequently, damage to the blood–brain barrier integrity was reduced significantly, despite a high degree of acetylcholinesterase and butyrylcholinesterase inhibition. It is suggested that anticholinesterase drugs enhance brain AChE inhibition by inducing strong convulsions and, thereby, increase their own penetration through the blood–brain barrier. It does not appear likely that acetyl- or butyrylcholinesterase located in the walls of the brain capillaries is involved in maintenance of the blood–brain barrier.

Inhibition of acetylcholinesterase (AChE, EC 3.1.1.7) following administration of organophosphorus esters can account for numerous physiological and clinical observations. Nevertheless, there are several indications in the literature that this class of compounds may exhibit mechanisms of biological activity different from just inhibiting AChE [1–9]. It is not clear, however, whether several non-AChE-related symptoms that have been reported in the literature originated from direct interaction between biochemical targets other than AChE and the inhibitors or, alternatively, were related to secondary physiological responses that were initiated by accumulated acetylcholine.

The presence of AChE in biological structures that are not associated with mediation by, or release of, acetylcholine raises the question of whether AChE has a function other than in cholinergic transmission. This question is especially pertinent to processes related to permeability and transport through membranes. Silver [10] reviewed several reports that suggest a possible role for AChE or butyrylcholinesterase (BuChE), in the walls of brain capillaries, in maintaining the integrity of the blood–brain barrier. The mechanism underlying the observed increase in capillary permeability as a result of administration of anticholinesterase drugs is not clear. For example, physiological responses as a possible explanation were not eliminated and, therefore, the conclusion is questionable that active AChE or BuChE in capillary walls is essential for maintaining blood–brain barrier integrity.

In the preceding paper [11], we described the use and unique properties of a peripheral anticholinesterase drug, phospholine iodide, in studying blood–brain barrier permeability changes. In those experiments, changes in blood–brain barrier integrity were induced by various experimental stresses such as osmotic shock, convulsions or γ-irradiation. In the present study we demonstrate the application of phospholine iodide to the study of the effects of paraoxon and Soman, centrally powerful anticholinesterase drugs, on the resistance of the blood–brain barrier in rats. Paraoxon and Soman are lipid soluble and exhibit strong CNS activity and thus pass through the blood–brain barrier, whereas phospholine iodide activity is restricted to the periphery, presumably due to its poor solubility in lipids and inability to traverse the blood–brain barrier [11].

Phospholine iodide, paraoxon, and Soman were administered to rat muscle, either separately or in various combinations. From measurements of brain AChE and plasma ChE, we were able to clarify several aspects of the resistance of the blood–brain barrier to insults in rats administered anticholinesterase drugs.

* This study was supported by the Armed Forces Radiobiology Research Institute, Defense Nuclear Agency, under Research Work Unit MJ 60202. The views presented in this paper are those of the authors. No endorsement by the Defense Nuclear Agency has been given or should be inferred.

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MATERIALS AND METHODS

S-[2(*N,N,N*-Trimethylammonio)ethyl]-*O,O*-diethylphosphorothiolate iodide (phospholine iodide) was obtained from the Ayerst Laboratories (New York, NY) as a freeze-dried powder. Stock solutions were made in saline at a concentration range of 0.15 to 0.3 mg/ml. (Although phospholine iodide is a powerful anticholinesterase drug, these solutions were safe to handle.) Stock solutions were kept at −20° and, then, at 4° when in use. Stability of the phospholine iodide solution was checked routinely by following the pseudo first-order inhibition profile of acetylcholinesterase from rat brain homogenates, using a value of $2.7 \times 10^6\ M^{-1}$ [11] and the experimental conditions described before [11] for the inhibition reaction.

o,o-Diethyl-*o*-(*p*-nitrophenyl) phosphate (paraoxon) was purchased from the Aldrich Chemical Co. (Milwaukee, WI). Stock solutions were made in saline at a concentration range of 1.0 to 1.5 mg/ml and kept at −20° when not in use. Paraoxon solutions were checked routinely after hydrolysis in 0.1 N NaOH by measuring the absorbance of the corresponding *p*-nitrophenol at 400 nm.

O-(1-Methyl-2,2-dimethylpropyl) methylfluorophosphonate (Soman) was prepared in our laboratory by the microscale technique. Soman is an extremely toxic compound, and to avoid hazards the reaction was carried out in a well-ventilated hood. The compound was prepared by adding, dropwise, a mixture of pinacoyl alcohol and triethylamine in ether, into a stirred mixture of methylphosphonodifluoridate and ether. Following completion of the reaction (15 min), the preparation was filtered through a filter paper (Whatman No. 1) and the filtrate was flash evaporated (20 mm Hg, 30°).

To prepare fresh aqueous Soman, 0.1 ml of the foregoing residue was stirred for 15 min with 5–10 ml of cold distilled water and filtered through filter paper (Whatman No. 1); the pH was adjusted to 7.0 at 25°. The concentration of Soman was determined, and checked routinely, by measuring the inhibition of eel acetylcholinesterase according to the method of Schoene [12]. The normality of the enzyme solution was determined by the method of Gordon *et al.* [13] by titration with DEPQF (see below) [11, 13]. Soman aqueous concentrations were in the range of 0.2 to 0.4 mg/ml and were confirmed by comparing the toxicity of the solution to rats (LD_{50} *ca.* 200 μg/kg, s.c.) with other reports [7, 14]. Decontamination of both the organic and aqueous solutions was done with 10% NaOH solution (1:1, water–methanol).

Pentylenetetrazole (metrazole) was purchased from K & K (Plainview, NY) and used as a 25% (w/v) solution in distilled water. 7-(Diethoxyphosphoryl)-*N*-methylquinolinium fluorosulfonate (DEPQF) was obtained in isopropanol solution as a gift from Professor I. B. Wilson, University of Colorado, Boulder, CO. 2-Pyridiniumaldoxime-1-methyl methansulfonate (P2S) was obtained from the Aldrich chemical Co. Stock solutions (50 mM) were made in distilled water and kept at −20° when not in use.

AChE (1000 units/mg) from electric eel was obtained from the Sigma Chemical Co. (St. Louis, MO). Enzyme stock solutions were made in 0.1 M phosphate buffer (pH 7.0) containing 0.05% bovine serum albumin.

Animals. Adult male rats [Tac:N(SD) fBR] (Taconic Farms, Germantown, NY) were used throughout this study.

Octanol:phosphate-buffer partition coefficient. The octanol:phosphate-buffer partition coefficient was calculated, as described for phospholine iodide [11]. The amount of paraoxon in either buffer or octanol was determined by measuring the rate of inhibition of AChE or the amount of *p*-nitrophenol released in 0.1 N NaOH, measured spectrophotometrically (400 nm).

The preparation of brain homogenates and the assay for acetylcholinesterase activity have been described in detail in the preceding paper [11]. Further information is included, when necessary, in Results, figure legends and table footnotes. Whenever death occurred, rats were processed immediately. In all cases, the first brain-AChE activity measurement was conducted within 15–20 min after dissection of the brain. The temperature of samples during this period was maintained at 4°.

RESULTS

Partition coefficients and kinetic parameters for inhibition of AChE by paraoxon and phospholine iodide in vitro. Table 1 summarizes the octanol:phosphate-buffer partition coefficients for paraoxon and phospholine iodide. Paraoxon was found to be *ca.* 10^5-fold more lipid soluble than phospholine iodide, as expected from the chemical structure of these inhibitors (see Scheme 1 in Discussion).

The rates of inhibition of both brain AChE and plasma ChE *in vitro*, in the presence of phospholine iodide, were found to be first order ([inhibitor] ≫ [AChE]) and linearly related to the concentration of the inhibitor. In contrast, inhibition in the presence of paraoxon displayed these characteristics only when brain AChE was used. The rate of inhibition of plasma ChE, although found to be first order (up to $2 \times T_{\frac{1}{2}}$ of the inhibition reaction) in the concentration range of 5.4 to 10.4 μM paraoxon, was not linearly related to the inhibitor concentration (Table 1). In addition, 2.7 μM paraoxon decreased plasma ChE activity by only 15 percent (data not shown), and incubation of this mixture with AChE from either rat brain homogenates or electric eel did not reveal freely available paraoxon. These results may indicate a loss of paraoxon either by hydrolysis, or by strong complex formation with plasma constituents, or by a combination of both pathways. A double-reciprocal plot did not change the line curvature.

Both inhibited brain AChE and plasma ChE reactivated completely within 30 min at 30° in the presence of 0.5 mM P2S, provided that the inhibitor concentration was below 8 nM.

Inhibition of plasma ChE and brain AChE in vivo *after sublethal administration of paraoxon.* Figure 1 illustrates the change with time of the inhibition of plasma ChE and brain AChE of rats by paraoxon administered i.m. (225 μg/kg). The inhibition curves

Table 1. Partition coefficients and kinetic parameters for the inhibition of rat brain AChE and plasma ChE by paraoxon and phospholine iodide (30°)

Inhibitor	Partition coefficient*	k_i (M^{-1} min^{-1})		k_i[Brain]
		Brain AChE†	Plasma ChE‡	k_i[Plasma]
Paraoxon	1.8 ± 0.2 ($\times 10^2$)	1.05 ± 0.15 ($\times 10^6$)	1.6–4.0 ($\times 10^5$)§	3.5
Phospholine iodide	2.2 ± 0.2 ($\times 10^{-3}$)‖	2.7 ± 0.3 ($\times 10^6$)‖	4.1 ± 0.4 ($\times 10^6$)	0.65

* Octanol:phosphate-buffer at 30°.
† In 0.1 M phosphate buffer, pH 7.0, containing 1% Triton X-100 (for details see Ref. 11).
‡ Inhibitors were added directly to undiluted plasma.
§ Inhibitor concentration range of 5.4 to 10.4 μM.
‖ From the preceding paper [11].

of brain AChE and plasma ChE followed similar time courses. The maximum inhibition was about 80–85 percent and was achieved within 10 min after injection of paraoxon. In no case could we demonstrate the presence of free inhibitor in rats after incubation of brain AChE or eel AChE with plasma samples obtained from rats administered paraoxon.

After the addition of 5 μM paraoxon to plasma or brain homogenates of rats that had been administered 225 μg/kg paraoxon approximately 30 min previously, enzyme inhibition increased from 80–85 percent to 95 percent within 5 min (data not shown). Further, the activities of AChE in brain homogenates and of ChE in plasma of rats administered 225 μg/kg paraoxon i.m. did not change after incubation at 30° for 30 min compared to enzyme activity at 4° (measured after killing the rats). We concluded, therefore, that the profiles in Fig. 1 represent the actual percentage of inhibited enzyme *in vivo* at the time of killing.

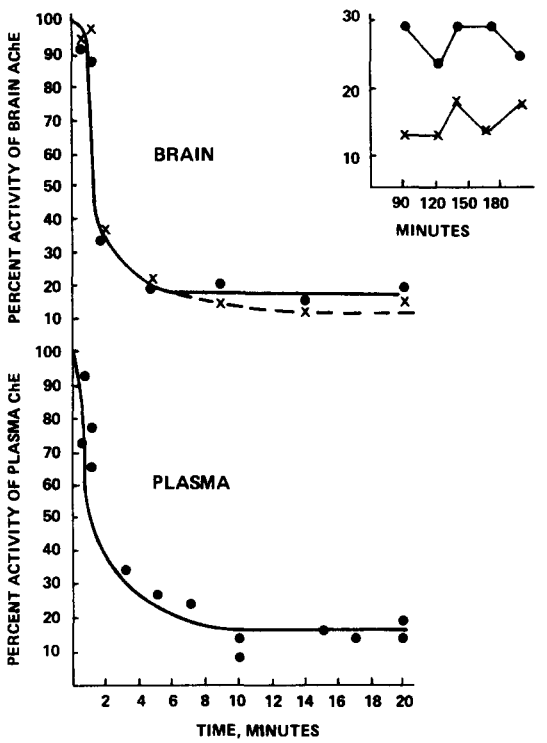

Fig. 1. Inhibition of brain AChE and plasma ChE after administration of 225 μg/kg paraoxon (i.m.) to rats. The inset represents brain AChE inhibition after injection of 200 μg/kg (i.m.). The mean body weight of the rats was 305 ± 15 g. Key: (●——●) brain stem, and (×——×) corpus striatum. Each point represents the average of three measurements of an individual rat. The abscissa indicates the time of killing after administration of paraoxon.

The inset to Fig. 1 indicates that the *o,o*-diethylphosphoryl-AChE conjugate was stable under *in vivo* conditions for at least 190 min after administration of paraoxon. Biosynthesis of acetylcholinesterase following the administration of anticholinesterase to rats has been shown to be negligible at least 3 hr after the poisoning [7].

Brain–enzyme–activity weight dependence. During these experiments we found a significant dependence of brain–enzyme inhibition on the body weights of the rats. Maintaining equal weight distribution in control and experimental groups for each set of experiments was, therefore, important.

AChE in the corpus striatum was consistently more accessible to paraoxon than was AChE in the brain stem. On the other hand the entry of phospholine iodide into brains of rats that had been exposed to experimental stresses, known to increase the permeability of the blood–brain barrier, was more prominent in the brain stem [11].

Combined administration of paraoxon and phospholine iodide. Intramuscular administration of phospholine iodide, 1 min after the injection of paraoxon, increased significantly the percentage of inhibited AChE both in brain stem and corpus striatum (Table 2 and Fig. 2 respectively), when compared to rats given only paraoxon. Phospholine iodide, when given separately, did not display any significant inhibition, up to a dose of 85 μg/kg (Table 2). A supralethal dose (150 μg/kg), however, caused a small but significant inhibition in brain stem AChE. Strong convulsions preceded the deaths of animals in this group. Administration of 65 μg/kg phospholine iodide 30 and 60 min after the injection of 200 μg/kg paraoxon increased significantly the inhibition of AChE in the brain stem but not in the corpus striatum (data not shown). After 90 min (not shown), intramuscular administration of 65 μg/kg

Table 2. Mortality ratio and brain stem AChE inhibition of rats administered paraoxon and phospholine iodide*

Paraoxon (μg/kg)	Concentration of phospholine iodide (μg/kg)											
	0		45		65		85		150			
	Mortality	AChE (% inhibited)	Mortality	AChE (% inhibited)	Mortality	AChE (% inhibited)	Mortality	AChE (% inhibited)	Mortality	AChE (% inhibited)		
0			0/6	1 ± 1 (NS)	0/6	1 ± 1 (NS)	5/6	2 ± 2 (NS)	4/4	7 ± 1 ($P < 0.001$)		
150	0/5	24 ± 7	0/5	31 ± 5 (NS)	4/4	60 ± 15 ($P < 0.005$)	4/4	66 ± 8 ($P < 0.001$)	NM†	NM		
200	0/6	67 ± 5	3/6	72 ± 8 (NS)	6/6	81 ± 4 ($P < 0.001$)	5/5	84 ± 5 ($P < 0.001$)	NM	NM		
250	0/5	75 ± 2	4/5	82 ± 3 ($P < 0.01$)	5/5	87 ± 3 ($P < 0.001$)	5/5	84 ± 2 ($P < 0.001$)	NM	NM		

* Mortality denotes the ratio of the number of rats that died, within 2 hr of the phospholine injection, to the total number of rats in the group. Weight distribution of rats: 270 ± 25 g. AChE inhibition (mean ± S.E.M.) is the percent reduction of AChE activity from the non-inhibited level of control AChE. Numbers in parentheses indicate the significance level of statistical differences between the experimental grotp and the group receiving only paraoxon. NS denotes not significant (*t*-test). Phospholine iodide was administered 1 min after paraoxon; both inhibitors were injected i.m.

† Not measured.

phospholine did not change significantly the concentration of AChE either in the brain stem or the corpus striatum, when compared to the control group, although the average value was found to be higher than that recorded for AChE activity of the group that received only paraoxon.

Combined administration of paraoxon and metrazole. Administration of 100 mg/kg metrazole i.p. was used to induce strong convulsions after rats were treated with paraoxon (Fig. 3). Metrazole increased significantly the percentage of enzyme inhibited, both in the brain stem and the corpus striatum, provided that the convulsive drug was administered within 10 min after paraoxon. We reported similar observations when phospholine iodide was used as the anticholinesterase drug [11].

On the basis of this observation and the data presented in Fig. 1, we have concluded that, after 10 min, paraoxon was essentially unavailable to inhibit further, when injected intramuscularly into rats. This, of course, is within the limits of the described procedure upon which the activity of brain AChE measurements was based.

To further demonstrate the penetration of phospholine iodide through the blood–brain barrier in rats pretreated with paraoxon, and to relate the increase in AChE inhibition to phospholine rather than to paraoxon, we conducted the following experiment. Metrazole and phospholine iodide were injected separately into two groups of rats 60 min after the administration of paraoxon. Table 3 clearly indicates a significantly greater increase in brain-AChE inhibition for the group given phospholine iodide, compared to rats that were treated with metrazole. Such differences were observed only when the paraoxon dose was above 200 μg/kg.

Although metrazole reversibly inhibited brain AChE *in vitro* at concentrations above 3 mM (see also Ref. 11), it is not likely that this finding was an artifact or led to misinterpretation of the reported results. Metrazole did increase enzyme inhibition when administered within 10 min after paraoxon (Fig. 3) but (as expected from Fig. 1) not after that time interval.

Combined administration of Soman and phospholine iodide. To produce various amounts of inhibited-aged (irreversibly inhibited) AChE before the administration of phospholine iodide, Soman was injected intramuscularly at a dose ranging from 40 to 135 μg/kg. We assumed that rapid aging of Soman-inhibited AChE would not interfere with the measurement of any phospholine iodide that entered the brains of these experimental rats and, thus, that any significant amount of reactivated enzyme would be accounted for by phospholine iodide.

Fifteen minutes after the injection of Soman i.m., rats received 65 μg/kg phospholine iodide i.m. Strong convulsions began 2–10 min after injection of phospholine iodide, depending on the dose of Soman. In acute cases death occurred 10–60 min after the second injection. In separate experiments, animals were protected partially from strong seizures by two injections of atropine sulfate (2 × 25 mg/kg, i.p., the first 5 min before Soman, and the second, 5 min after phospholine iodide injection).

The percentages of inhibited-aged enzyme and

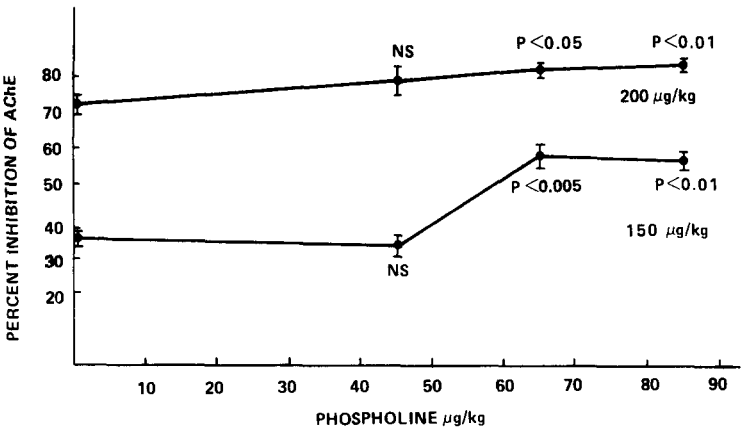

Fig. 2. Percent inhibition of corpus striatum AChE after combined administration of paraoxon and phospholine iodide (i.m.) to rats (wt = 275 ± 25 g). Phospholine iodide was administered 1 min after injection of either 150 or 200 μg/kg paraoxon. Measurements were made approximately 30 min after injections. Significance level refers to statistical differences between experimental group and control rats that received only paraoxon (see intersection at ordinate). Points are means ± S.E.M. ($N \geq 5$).

inhibited-non-aged AChE were calculated from the following equations (1 and 2 respectively):

% inhibited-aged

$$= 100 \times \left[1 - \frac{(\text{AChE})_{\text{P2S}}}{(\text{AChE})_{\text{control}}}\right] \quad (1)$$

% inhibited-non-aged

$$= \left[1 - \frac{(\text{AChE})_{\text{I}}}{(\text{AChE})_{\text{P2S}}}\right] [100 - \% \text{ aged}] \quad (2)$$

where $(\text{AChE})_{\text{P2S}}$ and $(\text{AChE})_{\text{I}}$ are the enzyme activities of brain homogenates after incubation with and without P2S, respectively, in the presence of an inhibitor. $(\text{AChE})_{\text{control}}$ represents average enzyme activity (of normal healthy rats) normalized per brain weight unit. To calculate the actual amount of phospholine iodide responsible for the inhibition of brain AChE in the presence of Soman we used the following equation (equation 3).

phospholine iodide (pmoles/g)

$$= [\% \text{ inhibited-non-aged}] \cdot [\text{AChE, sites/g}] \quad (3)$$

AChE active site concentration (pmoles/g) was taken from the preceding paper [11].

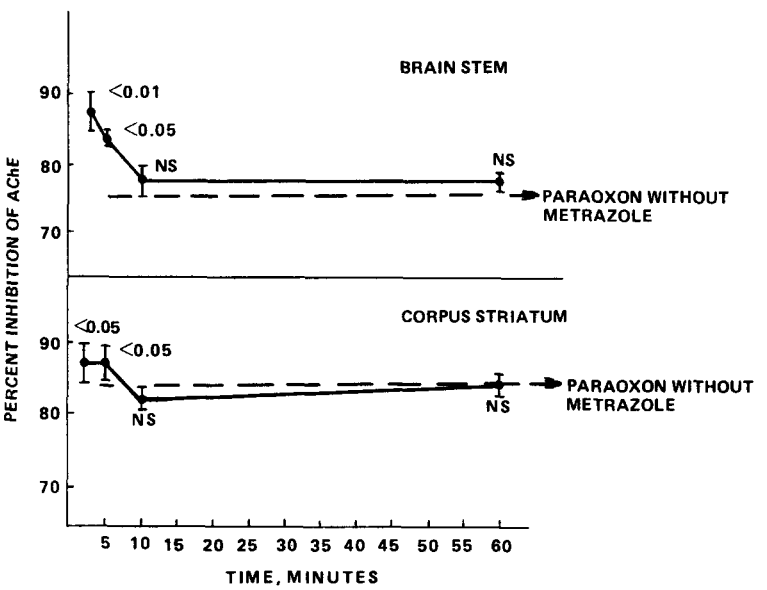

Fig. 3. Increase in brain AChE inhibition after administration of 100 mg/kg metrazole (i.p.) to rats (wt = 295 ± 17 g) at various time intervals after administration of 225 μg/kg paraoxon (i.m.) (normalized control line). Points and bars represent means and S.E.M. respectively. Significance level refers to statistical differences between the experimental group and the control that received only paraoxon (dashed line). NS = not significant.

Table 3. Percent inhibition of brain AChE after intramuscular injection of paraoxon i.m. followed 60 min later by phospholine iodide or metrazole*

Paraoxon dose (μg/kg)	Percent inhibition of brain AChE					
	Phospholine iodide (65 μg/kg, i.m.)		Metrazole (100 mg/kg, i.p.)		Significance (P)	
	Brain stem	Corpus striatum	Brain stem	Corpus striatum	Brain stem	Corpus striatum
170†	54 ± 4.0‡	55 ± 7.0‡	48.5 ± 2§	51 ± 5.0§	NS‖	NS
225¶	85 ± 0.5§	92 ± 0.5§	78.0 ± 1§	86 ± 1.5§	<0.001**	<0.01**

* Each value is a mean ± S.E.M.; N ≥ 4.
† Mean weight of the rats was 245 g ± 10 (S.D.).
‡ No death occurred within 2 hr. AChE was assayed after 120 min.
§ All rats convulsed strongly and died 10–30 min after the second injection. Brain AChE was assayed immediately after death.
‖ Not significant (*t*-test).
¶ Mean weight of the rats was 331 g ± 12 (S.D.).
** Significance level of statistical differences between the groups injected with metrazole or phospholine (*t*-test).

The results of the above-mentioned experiments are shown in Fig. 4. It appears that plotting the amount of phospholine iodide measured in the brain stem against the degree of aged enzyme induced by Soman before the administration of phospholine iodide tends to form a bell-shaped curve. The data with regard to the corpus striatum are less conclusive.

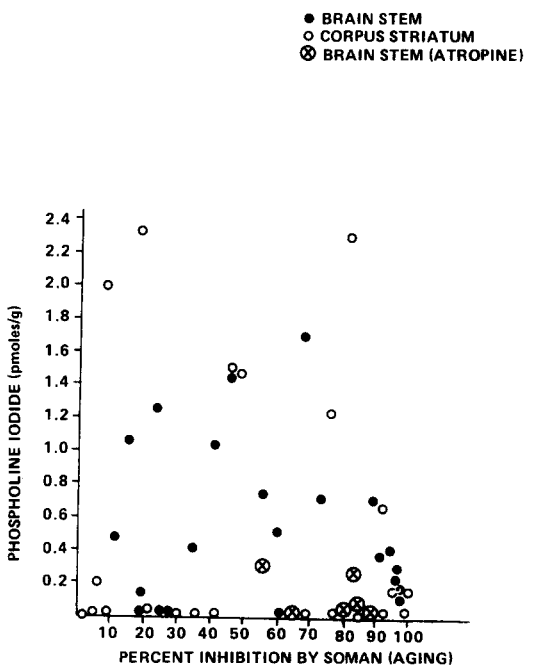

Fig. 4. Phospholine entry into brains of rats (275 ± 25 g) after i.m. administration of 65 μg/kg phospholine iodide to animals treated with 40–135 μg/kg Soman (i.m.), injected 15 min before phospholine. Each point represents the mean of three measurements of an individual rat. Atropine sulfate was administered 5 min before Soman and 5 min after phospholine iodide (2 × 25 mg/kg, i.p.). The abscissa indicates the percent inhibition of AChE.

Rats treated with atropine sulfate displayed significantly less phospholine in brain stem, compared to rats that exhibited the same percentage of aged enzyme but were not protected with atropine sulfate.

In a different set of experiments, the injection i.m. of 135 μg/kg Soman into rats 1 min after the injection of phospholine iodide (65 μg/kg, i.m.) resulted in AChE aging as high as 90–95 percent. In spite of the high degree of aging, we observed small but significant amounts of reactivable enzyme. One-third of the inhibited enzyme that was reactivated by P2S was formed *in vitro* (during the incubation at 30° without P2S), indicating that the inhibition by phospholine iodide was not completed *in vivo*. On the basis of our previous experiments [11], we have ruled out phospholine contamination from intravascular sources as an explanation for the above-mentioned observation. Table 4 was constructed to demonstrate the potential quantitative aspects of the present approach.

AChE levels in anesthetized rats given paraoxon, followed by either phospholine iodide or metrazole. To reduce the strong seizures that occurred after the administration of either phospholine iodide or metrazole to conscious rats pretreated with paraoxon, rats were anesthetized with nembutal (50 mg/kg, sodium nembutal) and the experiments were repeated. Results from these experiments are summarized in Table 5.

The residual activity of brain AChE, after the administration of either phospholine iodide or metrazole to anesthetized rats pretreated with paraoxon, did not differ from the control group which was given only paraoxon and anesthetized. There was an increase in brain AChE inhibition in anesthetized rats administered paraoxon (control, Table 5), compared to conscious rats treated with the same dose of paraoxon (Table 2). In this particular experiment, rats were killed 45 min after administration of either phospholine or metrazole. In contrast to similar experiments with conscious rats where death occurred within 10–30 min, all the rats of this experi-

Table 4. Comparative entry of anticholinesterase drugs into brains of rats injected i.m. with nonlethal doses*

Inhibitor	Dose (μg/kg)	N	Inhibitor entry† (pmoles/g)	
			Brain stem	Corpus striatum
Phospholine iodide	65	6	ND‡	ND
+100 mg/kg metrazole§	65	10	2.8 ± 0.3	3.2 ± 0.5
+200 μg/kg paraoxon‖	65	6	1.8 ± 0.3	ND
+70 μg/kg Soman¶	65	7	0.3 ± 0.1	0.7 ± 0.3
Paraoxon**	150	5	4.3 ± 1.4	21.7 ± 2.1
	250	6	13.6 ± 0.4	50.4 ± 0.6
Soman**	40	4	4.1 ± 0.5	4.5 ± 1.8
	70	7	15.0 ± 1.0	41.3 ± 5.7

* Calculated from % inhibition of AChE activity and active site concentration of AChE (see Results and Ref. 11).
† Inhibitor (pmoles) per g wet brain tissue; mean ± S.E.M.
‡ Not detectable.
§ One minute after phospholine; lethal combination.
‖ Sixty minutes before phospholine; lethal combination.
¶ Fifteen minutes before phospholine; lethal combination.
** Results refer to enzyme levels 45–120 min after administration of drug.

ment survived up to the time of killing. Seizures were almost undetectable in the group of anesthetized rats.

DISCUSSION

Phospholine iodide, paraoxon, and Soman were selected for this study on the basis of the following rationale (see Scheme 1).

First, AChE, inhibited by an *o,o*-diethylphosphoryl moiety, is readily reactivated *in vitro* in the presence of P2S, irrespective of the leaving group of the inhibitor [15–17]. Thus, a significant increase in brain-AChE inhibition after the administration of phospholine iodide, a peripheral, non-CNS, anticholinesterase, to rats pretreated with paraoxon, a powerful central AChE inhibitor, indicates entry of phospholine into brain tissues. This is true, of course, provided that an increase in AChE inhibition due to enhanced penetration of paraoxon has been ruled out.

Second, AChE activity restored after the addition of P2S to brain homogenates of rats treated with Soman (also a powerful central AChE inhibitor) and phospholine iodide should provide strong evidence for changes in blood–brain barrier permeability to phospholine iodide. This approach is based on the extremely rapid conversion of Soman-inhibited AChE to a nonreactivatable form [15–18].

(a) AChE $\xrightarrow{+(C_2H_5O)_2P(O)X}$ enzyme inhibited, non-aged $\xrightarrow{P2S}$ complete reactivation

X = *p*-nitrophenoxy (paraoxon)
X = $SCH_2CH_2N^+(CH_3)_3I^-$ (phospholine iodide)

(b) AChE + $CH_3C(CH_3)_2CH(CH_3)OP(O)F$ → enzyme inhibited, complete aging $\xrightarrow{P2S}$ no reactivation
Soman

(Scheme 1)

Third, phospholine iodide has been found to be a useful probe in studying changes in blood–brain

Table 5. Percent inhibition of brain AChE after administration of phospholine iodide or metrazole to anesthetized rats pretreated with 200 μg/kg paraoxon (i.m.)*

Drug	Brain stem		Corpus striatum	
	Experimental	Control	Experimental	Control
Phospholine iodide (292 ± 14)†	79.3 ± 2.0 (NS)‡	83.0 ± 1.8	84.0 ± 1.1 (NS)	87.3 ± 1.4
Metrazole (309 ± 8)†	86.5 ± 2.4 (NS)	86.3 ± 0.95	90.3 ± 1.3 (NS)	88.8 ± 2.1

* Each value is a mean ± S.E.M.; N ≥ 4. Rats were killed 45 min after the injection with either metrazole or phospholine iodide. Controls were anesthetized and given paraoxon. Phospholine iodide (65 μg/kg, i.m.) and metrazole (100 mg/kg, i.p.) were administered 1 min after paraoxon. Rats were anesthetized with 50 mg/kg nembutal sodium administered i.p. 10 min before paraoxon.
† Weight distribution of rats (average ± S.D., N = 8).
‡ Not significant when compared to control (*t*-test).

barrier permeability in conscious rats [11]. This anticholinesterase drug does not cross the blood–brain barrier of normal rats, and it displays certain advantages over radiolabeled tracers [11].

Administration of phospholine iodide immediately after paraoxon increased significantly the amount of inhibited brain AChE when compared to a control group that received only paraoxon (Table 2, Fig. 2). The magnitude of the increase was dependent on the doses of the two inhibitors and was associated strongly with severe convulsions and a high mortality rate. Since it has been reported by several investigators [19–23] that convulsions increase the permeability of the blood–brain barrier and since seizures are known to develop during acute poisoning from anticholinesterase drugs [1], it was of interest to substitute metrazole for phospholine iodide. Metrazole, a powerful CNS stimulant, has been used to induce strong seizures in experimental animals [11, 24], and it does not inhibit AChE irreversibly as phospholine iodide does [11]. Our experiments (Fig. 3) clearly indicate that convulsions increased the percentage of inhibited AChE in brains of rats administered sublethal doses of paraoxon. This increase was observed only within the first 10 min after paraoxon injection, and not later after injection. When metrazole or phospholine was administered to rats 60 min after the injection of paraoxon, increased inhibition of brain AChE was recorded only in the group that had received phospholine (Table 3). Since, as we demonstrated, freely available paraoxon was not likely to be present 10 min after administration i.m. (see Figs. 1 and 3), we suggest the following interpretation for the results of the experiments combining administration of paraoxon and phospholine iodide.

Physiological responses that are stimulated by either phospholine iodide or metrazole injection following paraoxon injection are most likely responsible for the enhanced penetration of the anticholinesterase drugs (see also Ref. 11). Although many insults alter autoregulation of cerebral blood flow [19], it seems that convulsion is the dominant event underlying the decreased blood–brain barrier that was observed in these experiments.

Physiological tolerance or perhaps adaptation was observed 60 min or more after paraoxon injection, even though the percentage of brain AChE inhibition was not changed (see also Fig. 1). This indicates that inhibition of AChE or BuChE was not enough to induce changes in the blood–brain barrier as far as the biological function of these enzymes in capillary walls is concerned.

To further evaluate the role of convulsions in the enhanced penetration of phospholine iodide, we repeated the experiments summarized in Table 2 (with conscious rats) with a group of anesthetized animals (Table 5). Neither metrazole nor phospholine iodide significantly increased the inhibition of brain AChE of the sedated, unconscious rats. We attribute these findings to the prevention of the strong seizures that were observed in conscious rats.

The experiments conducted with Soman and phospholine iodide (Fig. 4) are consistent with the conclusions reached thus far. The reactivated portion of the inhibited enzyme, although small, indicates clearly that the penetration of phospholine iodide depended on the physiological conditions induced by the combination of Soman and phospholine iodide. Whenever the percentage of inhibited-aged enzyme after the administration of Soman was high enough to produce seizures immediately after the administration of phospholine iodide, we detected reactivated enzyme in brain. Atropine sulfate reduced these seizures and decreased significantly the entry of phospholine despite the same degree of inhibited and aged enzyme as in non-atropinized rats. When brain AChE was aged (irreversibly inhibited) more than 90 percent, the sensitivity of detection of damage to the blood–brain barrier decreased. This observation resulted either from the immediacy of death of the animals or the phosphorylation of the majority of available brain AChE *in vivo* by Soman, or a combination of both.

Results of the present study suggest that the blood–brain barrier of rats treated with lipid-soluble inhibitors of AChE is damaged if the inhibition of AChE is enough to induce strong convulsions. Non-lipid-soluble anticholinesterases such as phospholine iodide have been shown previously [11], and in this work, to cross the blood–brain barrier whenever strong seizures occurred within 10 min following the injection of this drug. It is logical to assume, therefore, that the organophosphorus esters will display a self-enhancement of brain AChE inhibition in doses at which induced convulsions alter the blood–brain barrier.

The conclusions of this study can be extended to the role of capillary wall AChE and BuChE in maintaining the integrity of the blood–brain barrier [10]. Our findings indicate clearly that inhibition of AChE or BuChE is not sufficient to increase the permeability of the barrier. Presumably a physiological response stemming from the accumulation of acetylcholine is involved in the breakdown of the blood–brain barrier.

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