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AN IRREVERSIBLE ANTICHOLINESTERASE PROBE FOR STUDYING INCREASED PERMEABILITY OF THE RAT BLOOD–BRAIN BARRIER*

YACOV ASHANI,† GEORGE N. CATRAVAS‡ and WILLIAM A. ALTER III
Biochemistry Department, Armed Forces Radiobiology Research Institute, Bethesda, MD 20014, U.S.A.

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Abstract—Phospholine iodide, a potent anticholinesterase quarternary salt, was found to be a useful probe in studying changes in blood–brain barrier permeability in rats exposed to various insults. Based on brain acetylcholinesterase (AChE) activity measurements, it was concluded that phospholine iodide, injected i.m. at a dose of 65 μg/kg, inhibited brain AChE activity in three different regions, provided that the blood-brain barrier integrity was damaged by exposing rats to osmotic shock (glycerol), convulsions (metrazole), or γ-irradiation. Enzyme activity was not inhibited in sham-handled controls treated with phospholine iodide. Possible complication from blood contamination during brain processing was ruled out. It was suggested that phospholine iodide can be used as a semi-quantitative probe to detect an increase in blood–brain barrier permeability by the establishment of a unidirectional flow of the marker from brain capillaries into extravascular spaces and the phosphorylation of brain AChE. Based on the stability of the inhibited AChE and its 100 per cent reactivability in the presence of specific nucleophiles, this probe may be useful for the study of blood–brain barrier resistance in conscious, freely moving rats exposed to various stresses.

Changes in blood–brain barrier permeability as a result of chemophysical insults or certain diseases are of great interest from both the clinical and basic research points of view. Rapoport [1] and Bradbury [2] have reviewed and analyzed existing concepts and methodologies associated with various aspects of the resistance of the blood–brain barrier to insult. The basic methodologies that are currently in use were introduced by Crone [3] and Oldendorf [4]; they almost exclusively utilize radiolabeled tracers that are injected into the surgically exposed carotid artery of an anesthetized animal [3, 4] or into the femoral artery of a catheterized (and restrained) conscious animal [5]. By measuring the rate of extraction of radioactivity from brain-venous blood or uptake by brain tissue, a quantitative correlation can be established between permeability and various parameters such as drug-lipid solubility or cerebral blood flow [5–9]. Most of the techniques used to study the resistance of the blood–brain barrier to external insults are primarily dependent on radiotracers that are neither metabolized nor bound to brain tissue. These characteristics minimize the need for corrections usually required for apparent brain tissue and intravascular radioactivity measurements.

We recently began a search for a method of estimating the integrity of the blood–brain barrier which would permit injection of a probe intramuscularly into many conscious animals over a short period of time. This report describes the use of phospholine iodide, a powerful anticholinesterase drug known to rapidly inhibit acetylcholinesterase (AChE, EC 3.1.1.7) by forming a covalent stable phosphoryl–AChE conjugate. We found that, under normal conditions, this drug does not cross the blood–brain barrier of the healthy rat, as judged from AChE activity measurements in brain tissue homogenate. In contrast, animals that were exposed, before or after the administration of phospholine iodide, to experimental conditions known to increase the permeability of the blood–brain barrier did show significant reductions in enzyme activity. This activity could be restored by a specific reactivator.

We present here quantitative data indicating that phospholine iodide is a useful probe in rats for studying increased permeability of the blood–brain barrier.

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 with saline, in 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. The stability of the phospholine iodide solution was checked routinely by following the pseudo first-order

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† Recipient of a Senior NRC fellowship. Present address: Israel Institute for Biological Research, Ness-Ziona, Israel.

‡ Author to whom all correspondence should be addressed.

inhibition profile of acetylcholinesterase from rat brain homogenates, using a value of $2.7 \times 10^6\ M^{-1}\ min^{-1}$ and the experimental conditions described below for the inhibition reaction.

O-(1,Methyl-2,2-dimethylpropyl) methylfluorophosphonate (Soman) was prepared by a microscale technique that is described in the subsequent paper [10]. Soman is an extremely toxic compound; aqueous stock solutions of 0.1 to 0.4 mg/ml were safe to handle with adequate standard precautions. The concentration of Soman was determined by measuring the rate of inhibition of AChE, using experimental conditions described previously [11, 12].

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. (Milwaukee, WI). Stock solutions (50 mM) were made in distilled water and kept at −20° when not in use.

AChE either from the electric eel (1000 units/mg) or from bovine erythrocytes (2 units/mg) 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), weighing 250–320 g, were used throughout this study.

Brain homogenates

Rat brain homogenates were prepared by homogenizing the hypothalamus (20–30 mg), brain stem (130–180 mg), or corpus striatum (50–80 mg) in 1.5 ml of 0.1 M phosphate buffer containing 1% peroxide-free Triton X-100 [13] at 4°. The homogenates were centrifuged for 15 min at 15,000 *g* (4°), and the supernatant fraction was collected and assayed for AChE activity.

Assay of acetylcholinesterase

AChE from brain homogenates, electric eel, or bovine erythrocytes was assayed according to the method of Ellman *et al.* [14]. Ten to fifty microliters was diluted directly into the experimental cuvette containing Ellman's reaction mixture. Whenever brain homogenates were assayed, the appropriate amount of sample was also added to a blank cuvette containing the Ellman reagent without the substrate. Alternatively, 50 μl of brain homogenate was allowed to react in the cuvette for 3 min with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB, Ellman reagent) prior to the addition of acetylthiocholine. These alternative procedures were required because of the sulfhydryl content of the brain homogenate, which reacted quite rapidly with DTNB. Enzyme activities were recorded on a Cary-14 double-beam spectrophotometer, equipped with an expanded scale (0 to 0.1) and a thermostat.

Titration of brain AChE with Soman and DEPQF

Active site concentrations of AChE brain homogenates were determined according to general procedures reported previously [15–17]. Soman or DEPQF stock solution was added to the assayed homogenate to a final concentration of 0.4 to 1.6 nM, and AChE activity was measured until it reached a constant level.

Inhibition of AChE

Stock solution of inhibitor (phospholine iodide, Soman, or DEPQF) was added to the appropriate enzyme solution (0.3 to 1.0 nM) to a final concentration of 3–20 nM. The rate of inhibition was followed by diluting the incubated solution 60–100 times in the assay cuvette and monitoring the residual activity for periods of 1–2 min.

Reactivation of inhibited AChE

The stock solution of P2S was diluted to a final concentration of 0.5 to 0.7 mM in the reactivation medium. Regeneration of inhibited enzyme was measured as described above by diluting 50 μl in the assay cuvette. No correction was required for the oxime nucleophilicity at the final concentration obtained in the assay cuvette.

Octanol:phosphate-buffer partition coefficient

The octanol:phosphate-buffer partition coefficient was calculated by measuring the distribution of phospholine iodide between octanol and 0.1 M phosphate buffer (pH 7.0) at 30°. The concentration of phospholine in the organic layer was measured by shaking 20 μl of octanol with 1 ml of buffer solution (0.1 M phosphate) containing eel AChE. From the rate of inhibition of enzyme activity, the concentration of phospholine iodide could be determined in the organic phase.

Phospholine concentration in blood

The phospholine concentration in blood was determined by mixing 25–50 μl of plasma with 0.25 ml of eel AChE solution (0.3 nM) and following the rate of inhibition of enzyme activity at 30°. Heparinized, mixed blood was collected from rats that had been decapitated at specific time intervals after administration of phospholine iodide.

Induced opening of the blood–brain barrier

Glycerol (modified from Rapoport et al. [18]). Rats were anesthetized with sodium pentothal (75–100 mg/kg) before inserting a catheter into the left internal carotid artery. A 50% (v/v) glycerol/aqueous solution was injected (0.15 ml/100 g) within 2 sec and flushed in with 0.3 to 0.4 ml saline. Two minutes after administration of glycerol, the rats were injected i.m. with phospholine iodide solution (0.05 to 0.09 ml, 65 μg/kg). In rats that served as controls, glycerol solution was replaced by saline. Glycerol injection frequently resulted in respiratory arrest; therefore, artificial respiration was maintained, when required, for periods of 30 min, after which the rat was killed and the brain was perfused with 10–15 ml saline through the catheterized left internal carotid artery.

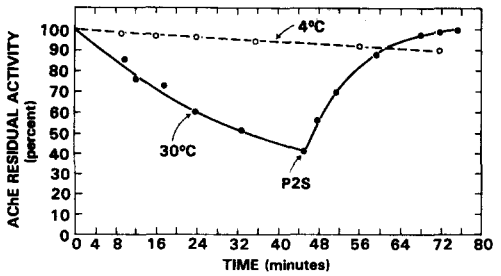


Fig. 1. Inhibition and reactivation profiles for brain AChE (corpus striatum) in the presence of 7.8 nM phospholine iodide. P2S (0.5 mM) was added 45 min after addition of phospholine.

Metrazole shock. This was induced by i.p. injection of 100 mg/kg metrazole (0.3 to 0.4 ml/rat) into rats at various times after administration of phospholine iodide i.m.

γ-Irradiation

Rats in lucite boxes were exposed, whole body, to 6 MeV effective bremsstrahlung photons using the Armed Forces Radiobiology Research Institute's (AFRRI) linear accelerator in a pulsed mode of 15 pulses/sec and an average dose of 0.23 rads/pulse. The rats were placed at a distance of 4 meters from the source to ensure a uniform whole-body dose. Dosimetry measurements were conducted in parallel with the irradiation experiments, and the absorbed dose was calibrated with thermoluminescence dosimeters.

RESULTS

Inhibition (and reactivation) of brain AChE in vitro *in the presence of phospholine iodide*

Figure 1 illustrates the rate profile for inhibition of AChE in homogenates of corpus striatum in the presence of 7.8 nM phospholine iodide both at 4° and 30°. A biomolecular rate constant of $2.7 \times 10^6\,M^{-1}min^{-1}$ was computed for inhibition of brain AChE by phospholine iodide at 30° in 0.1 M phosphate buffer at pH 7.0. This value is close to a rate constant previously reported when AChE from electric eel had been used [19]. No significant differences were observed between the various brain regions studied in this work (hypothalamus, brain stem, and corpus striatum) in terms of inhibition by phospholine iodide.

As shown in Fig. 1, the rate of inhibition was reduced significantly at 4°. From measurements of enzyme concentration in the homogenate and the maximum degree of inhibition obtained after *in vivo* administration of phospholine to rats exposed to insult (see below), it appeared that concentrations of phospholine higher than 0.1 nM in the hypothalamus or 0.5 nM in the brain stem and striatum homogenates were not likely to be present. The procedure applied in the present protocol, therefore, ensured that any significant quantity of inhibited enzyme (>3 per cent) measured in the homogenate (as calculated after reactivation) was presumably due to inhibition that took place *in vivo*.

Reactivation of inhibited brain AChE was practically complete within 30 min of incubation at 30° in the presence of 0.5 mM P2S (Fig. 1), provided that phospholine was present in concentrations of less than 8 nM. This was easy to determine by comparing the AChE activity after 30 min of incubation at 30° to the enzyme activity at 4°. The activity of brain AChE without the inhibitor did not change significantly after 30 min at 30°.

In addition to phospholine iodide, we also measured the bimolecular rate constant of the inhibition of AChE by Soman and obtained a value of $1.5 \times 10^8\,M^{-1}min^{-1}$ for brain AChE (30°, pH 7.0, 0.1 M phosphate buffer). Enzyme inhibited by Soman could not be reactivated by P2S, as shown in the past by many investigators [20].

Titration of brain-homogenate AChE with Soman and DEPQF

Table 1 summarizes the concentrations of AChE in three brain regions in terms of the active site concentration in 1 g wet brain tissue. For the lowest concentration obtained (hypothalamus), we calculated (see Eqn. 1 in Discussion) that 100 min would be required to complete 95 percent of the expected inhibition of enzyme activity when the AChE concentration in the homogenate was twice that of the active optical isomers of Soman and about 60 min when DEPQF was used as the titrant. Such long periods of incubation may introduce some error. This could explain the differences between relative activity and relative enzyme concentrations obtained

Table 1. Concentrations of AChE active sites in brain tissues as measured by titration with Soman or DEPQF*

Tissue	AChE				
	With Soman		With DEPQF		
	pmoles/g	Relative concn	pmoles/g	Relative concn	Relative activity*
Hypothalamus	18 ± 1.5†	0.27	15 ± 0.5	0.26	0.22
Brain stem	19 ± 4.0	0.28	18 ± 1.0	0.31	0.28
Corpus striatum	67 ± 9.0	1.0	57 ± 4.0	1.0	1.0

* Calculated from the rate of hydrolysis of acetylthiocholine (Ellman assay) and normalized per weight unit.

† Mean ± S.E.M. There were six rats in each group.

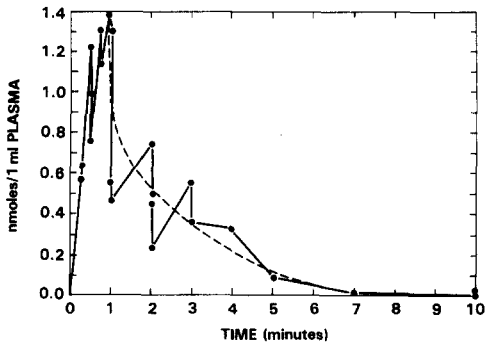


Fig. 2. Phospholine blood profile in rats injected i.m. with 65 μg/kg. The abscissa indicates minutes after injection. Each point represents an individual rat. The broken line is an assumed profile, constructed intuitively from experimental points.

for the hypothalamus. It may also explain the consistently higher results obtained with Soman as the titrant (see Discussion).

The average concentrations of the enzyme in the various brain homogenates were found to be 0.3, 2.0 and 2.4 nM, respectively, for the hypothalamus, brain stem and corpus striatum.

Octanol:phosphate-buffer partition coefficient

The coefficient for phospholine iodide was $2.2 \pm 0.2 \times 10^{-3}$ (N = 3).

Phospholine blood profiles

Blood levels of phospholine iodide assumed the profile shown in Fig. 2. The activities of both serum cholinesterase and RBC acetylcholinesterase were practically zero, even 0.5 to 1.0 min after i.m. administration of 65 μg/kg of phospholine iodide.

Residual activity of brain AChE after phospholine iodide administration

The standard dose of phospholine iodide chosen for this study was 65 μg/kg, i.m. (see also Ref. 10). This dose appeared to be optimal in terms of the blood concentrations needed to achieve brain AChE inhibition after damage to the blood–brain barrier by glycerol, metrazole, or γ-irradiation, while also inducing some peripheral symptoms indicating AChE inhibition in muscles (fasciculations). Table 2 clearly indicates that, without damage to the blood–brain barrier, brain AChE inhibition was negligible. If rats were killed within 0.5 to 1.0 min after phospholine administration, however, inhibition was observed in the brain stem during the incubation period at 30° ($P < 0.01$). No increase in inhibition was observed 2–5 min after phospholine administration.

Phospholine entry into the brain after experimentally induced blood–brain barrier damage

Table 3 summarizes the brain AChE inhibition by phospholine iodide that had penetrated the blood–brain barrier disrupted by various experimental methods. The concentrations of phospholine that were responsible for the apparent inhibition were computed from Tables 1 and 3.

In studying the glycerol-induced shock, we did not find a significant difference between rats that were saline-perfused or not perfused before brain dissection, and, therefore, we concluded that contamination from intravascular phospholine did not affect the results. Differences were noted, however, in AChE inhibition and phospholine entry between the left half hemisphere, ipsilateral to the exposed carotid, and the right side of the brain.

In metrazole-treated animals strong seizures began within 1–2 min after i.p. administration and persisted until the animals expired (5–7 min). The percentage of AChE inhibition by phospholine in metrazole-treated rats was time dependent. When metrazole was administered 2 min after phospholine iodide, less enzyme was inhibited compared to 0.5 min (Fig. 3). The activity of brain AChE in the presence of 0.003 to 0.02 M metrazole (*in vitro*) decreased from 78 to 50 percent of the original level with 0.5 mM acetylthiocholine as substrate; the enzyme activity was restored completely upon dilution. In addition, 7 mM metrazole reduced the inhibition of AChE in the presence of either phospholine or paraoxon (see Ref. 10) by 2.5-fold. No reactivation was observed under these conditions. Although we did not measure the uptake of metrazole by brain tissues, the

Table 2. Residual activity (%) of brain-homogenate AChE as measured after administration of phospholine iodide (65 μg/kg, i.m.)

Brain region	AChE* (%)			
		Time after phospholine injection (min)		
	Control†	0.5–1.0	2–5	6–10
Hypothalamus	99.5 ± 1‡	97 ± 3	100 ± 0.7	98.9 ± 3.0
Brain stem	99.5 ± 1	89 ± 4§	100 ± 1.8	99.3 ± 2.0
Corpus striatum	99.5 ± 1	97 ± 1	99 ± 0.5	99 ± 1.5

* One hundred percent activity was the enzyme activity at 4° immediately after collecting the homogenate supernatant fraction. Standard protocol is based on 30 min of incubation at 30° with or without 0.5 mM P2S.

† No phospholine.

‡ Mean ± S.E.M.; N ≥ 4 for each group.

§ Significance level: $P < 0.01$ (*t*-test).

Table 3. AChE inhibition and phospholine iodide entry into rat brain tissue after experimentally induced damage to blood–brain barrier

		Osmotic shock†				Convulsions§		γ-Irradiation‖	
		Left side of brain‡		Left plus right sides of brain					
Region	Control* (% inhibition)	% AChE (inhibition)	Phospholine (pmoles/g)	% AChE inhibition	Phospholine (pmoles/g)	% AChE inhibition	Phospholine (pmoles/g)	% AChE inhibition	Phospholine (pmoles/g)
Hypothalamus	1.0 ± 1.7 (8)¶	38.5 ± 7.0 (4) ($P < 0.005$)††	5.8**	21.3 ± 2.3 (4) ($P < 0.001$)	3.2	19.8 ± 2.6 (8) ($P < 0.001$)	3.0	11.9 ± 1.4 (10) ($P < 0.005$)	1.8
Brain stem	0.0 ± 1.2 (8)	25.3 ± 6.5 (4) ($P < 0.005$)	4.6	12.5 ± 2.5 (4) ($P < 0.005$)	2.3	15.7 ± 2.3 (8) ($P < 0.005$)	2.8	8.9 ± 3.5 (10) ($P < 0.01$)	1.6
Corpus striatum	1.3 ± 0.7 (8)	6.5 ± 2.3 (4) ($P < 0.01$)	3.7	6.5 ± 2.3 (4) ($P < 0.01$)	3.7	5.6 ± 1.8 (8) ($P < 0.01$)	3.2	2.0 ± 0.6 (10) NS	1.4

One hundred percent activity was the enzyme activity at 4° immediately after collecting the homogenate supernatant fraction. Standard protocol is based on 30 min of incubation at 30° with or without 0.5 mM P2S.

* Phospholine iodide (65 μg/kg) was injected into muscle.

† A 50% glycerol–water mixture (0.15 ml/100 g) was injected into the left internal carotid artery 2 min before phospholine injection i.m.

‡ Brain tissue ipsilateral to phospholine injection site.

§ Metrazole (100 mg/kg) was administered i.p. 0.5 to 2.0 min after phospholine.

‖ Ten thousand rads whole-body was delivered in 45 min. Phospholine was injected 48–72 hr post-irradiation. The sham-irradiated control did not show a significant decrease in brain AChE.

¶ Mean ± S.E.M. Numbers in parentheses indicate number of animals.

** Phospholine (pmoles/g brain tissue) calculated from % inhibition (the corresponding enzyme concentration is given in Table 1, under DEPQF).

†† Significance level for statistical differences between experimental group and group receiving only phospholine. NS denotes not significant (*t*-test).

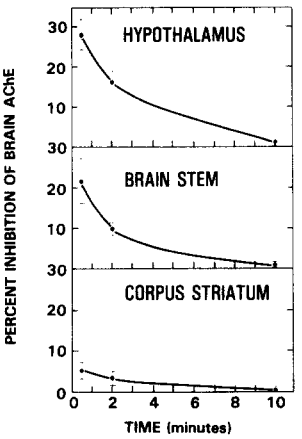


Fig. 3. Inhibition of brain AChE by phospholine (65 μg/kg, i.m.) after administration of 100 mg/kg of metrazole i.p. The abscissa indicates the time of metrazole injection after phospholine administration. Each point is the mean of four rats; bars denote S.E.M.

results obtained for inhibition of brain AChE after administration of metrazole and phospholine to rats are relevant to our discussion. In fact, the results reported in Table 3 may be even lower than the actual degree of inhibition of AChE.

Damage to the integrity of the blood–brain barrier in rats irradiated with 10,000 rads was detected only 48 hr post-irradiation, at which time 50 percent of the animals died 20–40 min after i.m. administration of phospholine (65 μg/kg), a nonlethal dose to normal rats. Sham-handled controls kept in the exposure room but shielded from the irradiation did not show significant inhibition of brain AChE. The enzyme activity of irradiated rats was not statistically different from that of non-irradiated controls.

DISCUSSION

In order to contribute significantly to current methodology and to extend the choice of markers to be used in blood–brain barrier studies, the following requirements were established. First, the probe should react very rapidly and specifically with widely distributed target compounds within the extravascular brain regions. Second, blood levels of the injected probe should be optimal after a single injection by the intramuscular or subcutaneous route, i.e. blood drug concentration should be high enough to allow reasonable penetration in the event of blood–brain barrier breakdown, and the blood–probe concentration should, within a reasonable time, fall below the level that minimizes contamination from vascular breakage during processing of the brain tissue. Third, the proposed marker should not cross the blood–brain barrier of normal animals (within the detection limits of the assay procedure).

An organophosphorus inhibitor of AChE seems to meet these three basic criteria. Of the various types of AChE inhibitors, phospholine iodide was the most promising due to the presence of a quaternary nitrogen that renders undetectable the penetration of such polar salts through the blood–brain barrier [21, 22]. Inhibition of AChE by organophosphorus esters is a bimolecular reaction (Scheme 1) that results in the corresponding 1 : 1 covalent and stable phosphoryl-enzyme conjugate [19, 20].

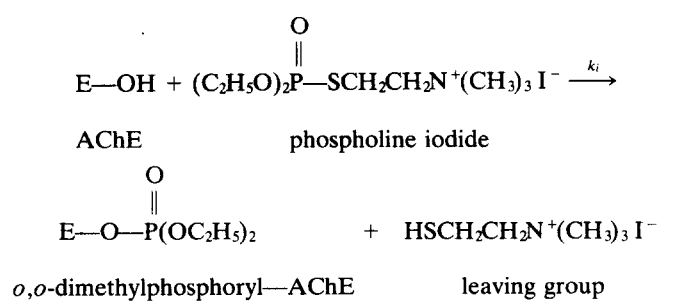
Furthermore, the *o,o*-diethylphosphoryl–AChE conjugate is readily reactivated by specific nucleophiles such as P2S, irrespective of the leaving group of the inhibitor [23].

Our findings are in good agreement with the above-mentioned criteria for a marker in blood–brain barrier studies. The inhibition *in vitro* of brain AChE obeyed pseudo first-order kinetics. The bimolecular rate constant for inhibition of brain AChE from rat brain homogenates was computed to be $2.7 \times 10^6\,M^{-1}min^{-1}$ at 30°. Maglothin and Wilson [19] reported a similar value for the inhibition of eel AChE at 25°.

The inhibited enzyme reactivated completely within 30 min of adding 0.5 mM P2S, even in the presence of a concentration of phospholine iodide as high as 7.8 nM (Fig. 1). Complete reactivation of inhibited enzyme in the brain homogenates in the presence of P2S enabled us to determine the original enzyme activity for each individual rat before the *in vivo* administration of phospholine iodide. Thus, each rat served as its own control.

To estimate the time required to complete 95 percent of the inhibition reaction *in vivo*, we assumed a local enzyme concentration of 10^{-5} N, as computed previously on the basis of biochemical and physiological data [24–26]. The equation used to estimate the time required to complete 95 percent of the total expected inhibition is a slight modification [17] of the standard bimolecular second-order equation, expressed as follows:

$$t = \frac{1}{k_i E_o[1 - (I_0/E_0)]}\left(\ln \frac{1.0\text{–}0.95\ (I_0/E_0)}{0.05}\right) \qquad (1)$$



(Scheme 1)

where k_i is the second-order rate constant for the inhibition, and E_0 and I_0 are initial concentrations of the enzyme and the inhibitor, respectively. According to Equation 1, for a ratio of $I_0/E_0 = 0.2$ and for $E_0 = 10^{-5}$ N, 95 percent of the phospholine iodide will be consumed by AChE within 8 sec. If the inhibition proceeds via a pseudo first-order reaction in terms of enzyme concentration, one would expect 95 percent completion of the reaction also within 8 sec, in accordance with Equation 2:

$$I_t = I_0 \, e^{-k't} \qquad (2)$$

where $k' = k_i \times E_0$.

Although we did not measure enzyme levels until 0.5 min after administration of phospholine, this observation supports the calculation that suggests very rapid inhibition of brain AChE in intact tissue by phospholine iodide. Similar conclusions have been reached by using the lipid-soluble paraoxon (see Ref. 10).

Based on the empirical relationship between cerebrovascular permeability and lipid solubility [5], we predicted a permeability value of 2×10^{-7} cm sec^{-1}, and, from the blood levels of phospholine and the permeability coefficient, we calculated (according to the technique of Rapoport *et al.* [5]) a possible accumulation of 10 pmoles phospholine iodide per gram of brain tissue within 5 min after an injection i.m. of 65 μg/kg of phospholine iodide. Table 2, however, indicates that the inhibition was negligible and that the phospholine iodide concentration was less than 0.5 pmole/g brain tissue. This deviation from the predicted value suggests a mechanism other than simple diffusion through lipid layers, interference with drug passage through the lipid membranes, or partial binding to plasma constituents.

Three different experimental procedures that have been shown previously to produce variable increases in permeability of the blood–brain barrier were used in this study to evaluate the utility of phospholine iodide as a probe (see Table 3), i.e., osmotic opening of the barrier as demonstrated by Rapoport [1] and Bradbury [2], convulsions or seizures induced by electrical shock or by drugs [27–32], and ionizing radiation which has been reported to induce small but significant changes in blood–brain barrier resistance [33, 34]. On the basis of brain AChE activity measurements, we have concluded that phospholine iodide reached the brain whenever the integrity of the blood–brain barrier was damaged.

Differences in the brain region responses to phospholine under the insults imposed (Table 3) are significant only in terms of percentage of inhibition of the enzyme. However, calculated values for the concentrations of phospholine in terms of pmoles/g brain tissue are approximately the same. It would not be possible, therefore, to indicate significant differences in the blood–brain barrier of the various regions.

Even though these results suggest a usefulness of this marker, several possible complications should be considered. Contamination from intravascular inhibitor, especially during the homogenizing process, may be ruled out for the following reasons. Peak concentrations of phospholine iodide were found to be 1.4 nmoles/ml plasma at about 1 min after administration i.m. of 65 μg/kg phospholine. Since the blood volume in various areas of the brain varies between 10 and 35 μl/g [5], and since the dilution factor during homogenization was in the range of 10–50, we may expect the maximum concentration of inhibitor stemming from intravascular origin to be in the range of 0.5 nM. The maximum expected contamination would be in the brain stem because of the large blood volume and the relatively small dilution factor in this area, compared to other brain regions. Based on Equation 1 we have concluded that, at 30°, 24 percent inhibition of the enzyme activity would require at least 80 min, assuming 0.5 nM phospholine iodide and 2 nM AChE (see Results). In practice, rats were killed when blood levels of phospholine were only one-fifth to one-tenth of the peak concentration (Fig. 2). We may rule out, therefore, intravascular contamination as an alternate explanation. This would be true also if blood volume were increased to two to three times the normal values. With regard to normal healthy rats, our observations indicate that in only one case, when rats were killed within 1 min after phospholine administration, did inhibition of AChE in brain stem homogenate occur after 30 min of incubation at 30° (Table 2). Since there was no significant inhibition of AChE in brain homogenates obtained from rats killed more than 2 min after injection, it can be concluded that phospholine from intravascular origin in the homogenate was minimal. In any event, we detected free inhibitor residues (>0.5 nM in homogenate) by comparing AChE activity after incubation at 4° and at 30°. In addition, the enzyme was considered to be inhibited in intact brain tissue only when the enzyme activity at 4° and 30° did not differ considerably, within the experimental error of the assay, and when enzyme activity increased significantly after addition of P2S.

We also considered the possibility that inhibited serum cholinesterase and RBC acetylcholinesterase would mix with brain tissue AChE during the homogenizing process and would contribute activity upon reactivation, which would be interpreted incorrectly. The AChE activity ratio, 1 g wet brain tissue/1 ml whole blood, however, was found to be 45. Since blood volume content in brain tissue is approximately 1.0 to 3.5% (v/w) [5], the final contribution to enzyme activity is practically negligible, even if brain tissue blood volume were to increase five times.

It is apparent from these results that phospholine iodide can be used as a convenient qualitative probe to detect increases in blood–brain barrier permeability in conscious rats exposed to various insults. There is also a good indication that this probe may be useful as a semi-quantitative tool to define permeability changes in terms of rate of transfer, but more experiments are required for further evaluation.

The main limitation of this method is the chemical nature of the inhibition of AChE by phospholine. Brain regions that contain high concentrations of AChE (e.g. corpus striatum) are expected to be less sensitive to the method in terms of reliable detection of inhibited enzyme. In this case, histological studies may overcome such a disadvantage. The application of an organophosphorus inhibitor of AChE as a probe to detect changes in blood–brain barrier integ-

rity may also be used in conjunction with various physiological states such as hypothermia [21, 35]. A marker such as phospholine might, therefore, be evaluated for blood–brain barrier studies on the basis of three different measurable criteria pertinent to its biological activity in the central nervous system (enzyme activity in brain homogenates, histology, and physiology).

On the basis of the present results, we have used phospholine iodide as a probe to study the effects of lipid-soluble anticholinesterase drugs on blood–brain barrier integrity in rats (see Ref. 10).

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