Biochemical Pharmacology, Vol. 35, No. 7, pp. 1079–1085, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00

SYNTHESIS AND *IN VITRO* PROPERTIES OF A POWERFUL QUATERNARY METHYLPHOSPHONATE INHIBITOR OF ACETYLCHOLINESTERASE

A NEW MARKER IN BLOOD–BRAIN BARRIER RESEARCH

DRORA LEVY and YACOV ASHANI*
Israel Institute for Biological Research, Ness-Ziona 70450, Israel

(*Received* 18 *June* 1985; *accepted* 22 *August* 1985)

Abstract—To substantiate reported data and improve the properties of anticholinesterase drugs in blood–brain barrier (B-BB) research, 7-(methylethoxyphosphinyloxy)1-methyl-quinolinium iodide (MEPQ) was prepared and evaluated as an inhibitor of both acetyl- and butyrylcholinesterase (AChE and BuChE, respectively) from various sources. The second-order rate constants for the inhibition of cholinesterase from eel, mice brain and horse serum at 25° were found to be 5.3×10^8, 1.3×10^8 and $5.4 \times 10^7\ M^{-1}\ min^{-1}$ respectively. The inhibited enzyme could be reactivated by 1-methyl-2-hydroxy iminomethylpyridinium iodide (2-PAM). The two enantiomers of the racemic mixture MEPQ inhibited AChE at similar rates. Low concentrations of AChE could be determined by the residual enzyme activity and by fluorescence measurements of the leaving group, thus suggesting the application of MEPQ as a sensitive titrant of cholinesterase, as well as a potential tool in studying B-BB permeability changes.

Phospholine iodide, a quaternary anti-cholinesterase (anti-ChE) drug that cannot cross the blood–brain barrier (B-BB) of normal healthy rats, was demonstrated to be of potential value for studying permeability changes induced by various insults upon the B-BB of rats [1, 2]. Phospholine penetration through the B-BB was expressed in terms of irreversible inhibition of brain cholinesterase (AChE, EC 3.1.1.7; BuChE, EC 3.1.1.8). The inhibited enzyme in the brain homogenates was readily and completely reactivated *in vitro* by specific nucleophiles such as 1-methyl-2-hydroxyiminomethylpyridinium iodide (2-PAM). The following scheme depicts the mechanism underlying the above-mentioned approach.

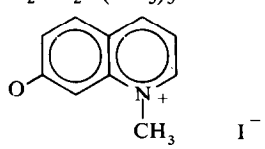
$$EOH + R'(RO)P(O)X \xrightarrow[in\ vivo]{inhibition} R'(RO)P(O)OE \xrightarrow[in\ vitro]{reactivation} EOH$$

where EOH is either AChE or BuChE and:

phospholine iodide: $R = C_2H_5$, $R' = OC_2H_5$ $\quad X = SCH_2CH_2\overset{+}{N}(CH_3)_3\ I^-$

DEPQ: $R = C_2H_5$, $R' = OC_2H_5$ $\quad X =$

MEPQ: $R = C_2H_5$, $R' = CH_3$ $\quad X =$



Scheme 1

To substantiate the data reported with regard to phospholine iodide [1, 2] and to further explore the potential application of quaternary anti-ChE drugs in B-BB research, we synthesized and evaluated the biological properties of a new compound 7-(methylethoxyphosphinyloxy)-1-methyl-quinolinium iodide (MEPQ). We chose to prepare and evaluate the methylphosphonate MEPQ for the following reasons:

(a) The diethylphosphoryl ester of 1-methyl-7-hydroxyquinolinium (DEPQ, Scheme 1) was found to display an exceptionally high rate of inhibition of AChE, *ca.* fifty times more rapid than phospholine iodide [3, 4]. Evidence in the literature indicates that the replacement of one alkoxy group on phosphorus in *O,O*-diethyl-phosphates $[(RO)_2P(O)X]$ by an alkyl group such as methyl $[CH_3P(O)(OR)X]$ enhanced nucleophilic substitution at tetrahedral phosphorus compounds by small nucleophiles (e.g. OH^-, H_2O) and macromolecules such as AChE [5–7]. Thus, it was anticipated that the methyl-

* Address all correspondence to: Yacov Ashani, Ph.D., Israel Institute for Biological Research, P.O. Box 19, Ness-Ziona 70450, Israel.

phosphonate, MEPQ, will be more potent than DEPQ in terms of enzyme inhibition. Moreover, by following the same rationale, MEPQ was predicted [6–8] to be less stable under physiological conditions than the analogous diethylphosphate ester and therefore blood contamination during brain tissue processing should be minimized. These properties are essential for developing efficient anti-ChE markers to be used in B-BB studies [1].

(b) The unique structure of MEPQ is particularly suited for the convenient and reliable radiolabeling of the methylphosphonyl (CH_3-P) conjugate of the inhibited enzyme for further applications of this compound as a histological marker in B-BB research.

In this report, we describe the synthesis, stability, and *in vitro* anti-ChE properties of MEPQ. A preliminary report describing the *in vivo* utilization of MEPQ as a marker for monitoring B-BB permeability changes in mice and rats was summarized recently [9].

EXPERIMENTAL

Materials

S - [2(*N, N, N* - Trimethylammonio)ethyl] - *O, O* - diethylphosphorothiolate iodide (phospholine iodide) was obtained from the Ayerst Laboratories (NY) as freeze-dried powder. 7-Hydroxyquinoline was purchased from Eastman Kodak (Rochester, NY) and used as obtained. 1-Methyl-2-hydroxyiminomethylpyridinium iodide (2-PAM) and *N*-tris(hydroxy-methyl)methyl-2-aminoethane sulfonic acid (TES) were obtained from Sigma (St. Louis, MO). 1-Methyl-7-hydroxyquinolinium iodide (7HQ) and 1-methyl-3-hydroxyiminomethylpyridinium iodide (3-PAM) were prepared according to the method of Rosenberry and Bernhard [10], and Ginsburg and Wilson [11] respectively. Diethyl methylphosphonate (DEMP) may be easily obtained from triethylphosphite and methyl iodide according to Ford-Moore and Williams [12]. Unless noted, all other chemicals were reagent quality.

AChE (11S from electric eel) and BuChE from horse-serum were obtained from either Worthington Biochemicals (Freehold, NJ) or the Sigma Co. A third source of AChE was brain homogenates of either mice or rats.

Synthesis

Nuclear magnetic resonance (NMR) spectra were recorded on Bruker 250 MHz and Varian XL-100 spectrometers. Mass spectrometry (MS) was performed on a Varian MAT 112 mass spectrometer. Melting points were determined on a Thomas Hoover melting point apparatus and are uncorrected. Microanalysis was performed by the microanalytical laboratories of the Hebrew University, Jerusalem. Thin-layer chromatography (TLC) was performed on aluminium plates, coated with neutral silica and visualization was carried out with either a UV lamp or iodine vapors.

MEPQ was prepared according to the synthetic route outlined in Scheme II.

Warning: All procedures should be carried out in a well-ventilated hood.

$$CH_3P(O)(OC_2H_5)_2 \rightarrow CH_3P(O)(OC_2H_5)Cl \rightarrow CH_3P(O)(OC_2H_5)OX$$

$$DEMP \rightarrow MPOC \rightarrow MEPQ$$

Scheme II

Methylethoxy phosphinyloxychloride (MPOC). Thirty grams (0.25 mole) oxalyl chloride (freshly distilled) was added dropwise at 4° while stirring to 30 g (0.2 mole) DEMP over *ca.* 15 min. An exothermic reaction and gas evolution were observed. The mixture was warmed for 90 min at 70°, and excess, unreacted oxalyl chloride was removed by distillation (760 mm Hg, 78°). The remaining crude oil was distilled through a Vigreux column under reduced pressure (20 mm Hg, 85°; lit. [13]: 21 mm Hg, 83°) to give 26 g (94% yield) of colorless MPOC. The purity and homogeneity of MPOC were confirmed by using ^1H and ^{31}P NMR spectroscopy.

7-(Methylethoxy phosphinyloxy) quinoline (tertiary ester). To a mixture of 7-hydroxyquinoline (1.9 g, 0.013 mole) and MPOC (2.0 g, 0.014 mole) in 20 ml of dry benzene was added dropwise under nitrogen at room temperature 1.7 g triethylamine (0.017 mole) in dry benzene. The mixture was warmed for 60 min at 60°; the 7-hydroxyquinoline had reacted completely (TLC). The precipitate was filtered off and the benzene solution extracted three times with 3% aqueous $NaHCO_3$ solution (4°) and finally washed with distilled water. After drying over $MgSO_4$ and filtering the solvent was removed under reduced pressure (20 mm Hg). Volatile impurities were removed from the remaining oil (3.2 g, 97% yield) under reduced pressure (0.1 mm Hg) at 50°. The purity of the tertiary ester was confirmed by:

(a) The ^1H decoupled ^{31}P NMR spectrum revealed one singlet at δ27.6 ppm (relative to H_3PO_4) and the ^1H NMR spectrum (in $CDCl_3$) gave the following (relative to TMS): δ(ppm): 1.33 (t, 3H, $OCH_2\underline{CH_3}$), 1.71 (d, 3H, P-$\underline{CH_3}$), 4.25 (m, 2H, $O\underline{CH_2}CH_3$), 8.09 (m, 6Ar).

(b) A peak of $m/z = 251$ was observed in the mass spectrum which equals the molecular peak of the tertiary ester.

(c) TLC with 5% methanol in $CHCl_3$ on silica showed a single spot.

7-(Methylethoxy phosphinyloxy)-1-methylquinolinium iodide (MEPQ). Methyliodide (1.8 g, 0.013 mole) and the foregoing tertiary ester (1.2 g, 0.0048 mole) were allowed to react overnight at room temperature in dry acetonitrile. The acetonitrile and unreacted methyliodide were removed under reduced pressure (20 mm Hg, 30°) and the remaining yellow oil crystallized from dry acetone-ether at −20° as yellow crystals (1.17 g; yield: 65%), m.p. 108–110°. Anal. Calcd. for $C_{13}H_{17}INO_3P$: C = 39.69; H = 4.32. Found: C = 39.90; H = 4.02. ^1H-NMR (d_6-acetone), δ(ppm, TMS): 1.32 (t, 3H, $OCH_2\underline{CH_3}$), 1.86 (d, 3H, P-$\underline{CH_3}$), 4.4 (m, 2H, $O\underline{CH_2}CH_3$), 4.83 (S, 3H, N-$\underline{CH_3}$), 8.33 (m, 6Ar). ^{31}P-NMR (d_6-acetone), δ29.07 ppm (H_3PO_4).

Enzyme activity determinations

AChE and BuChE activities and the kinetics of inhibition and reactivation were monitored spectrophotometrically by the method of Ellman *et al.* [14]. The assay mixture (3.1 ml) contained 0.5 mM acetylthiocholine iodide and 0.3 mM 5,5′-dithiobis-(2-nitrobenzoic acid) in 0.1 M phosphate buffer (pH 7.0). All measurements were performed at 25°.

Brain homogenates

Both mice and rat brain homogenates were prepared by homogenizing the whole brain in 0.1 M TES buffer containing 0.5% peroxide-free Triton X-100 [15], 0.1% bovine serum albumin (BSA) and 1 N NaCl at 4°. The homogenates were centrifuged for 60 min at 17,000 *g* (4°), and the supernatant fraction was collected and assayed for AChE. More than 96% of the enzyme activity was found in the supernatant fraction.

Kinetics of inhibition and reactivation

Standard stock solutions of MEPQ (1 mM) were prepared in acetone and kept at 4°. The concentration of the MEPQ stock solution was checked routinely by absorption spectroscopy (see below). At t = 0, inhibitor was diluted *ca.* 10^5 times in 0.5 ml enzyme solution (0.3 to 1 nM) in 0.1 M phosphate containing 0.1% BSA at pH 7.0. (Brain homogenates in TES buffer were diluted 1:10 in phosphate buffer.) At selected time intervals, 10–50 μl was diluted in 3.1 ml Ellman assay mixture for measurement of enzyme residual activity. Reactivation was initiated at t = 0 by diluting an inhibited enzyme 100-fold in 0.5 to 1 mM solution of the specific reactivator 2-PAM in 0.1 M phosphate buffer (0.1% BSA, pH 7.0). The reactivation medium (25–50 μl) was assayed for enzyme activity as described above. Controls contained 0.5 to 1 mM 3-PAM, a poor-reactivator analog of 2-PAM [16].

Stability measurements

Buffer and alkaline solutions. The alkaline hydrolysis of MEPQ was monitored at 406 nm and carried out directly in the spectrophotometer cell by diluting 100-fold, a 3 mM stock solution in acetone in 3 ml of 0.001 to 0.01 N KOH. The molar absorbance of 7HQ was found to be 1.01×10^4 (0.01 N KOH). Kinetic measurements of the hydrolysis in phosphate buffers (pH 6.0 to 8.0) were performed by incubating the inhibitor (5 mM) at 26° in the appropriate stock solutions. At selected time intervals, 30 μl were diluted in 3 ml 0.1 M phosphate buffer (pH 8.0). The amount of the released leaving group was measured at 406 nm and calculated from a calibration curve that was constructed by diluting known amounts of 7HQ in 0.1 M phosphate buffer at pH 8.0.

Whole blood. MEPQ or phospholine iodide were diluted into heparinized mixed blood that was collected from mice and incubated at 38°. Final concentrations of MEPQ and phospholine were in the range of 1 to 3 μM and 0.1 to 0.5 mM respectively. At selected time intervals, 10 μl was diluted 100–200 × into eel AChE solution, and the rate of inhibition of the eel enzyme was followed at 25°. From the rate of inhibition of the enzyme activity, the concentrations of MEPQ and phospholine could be determined. Enzyme activity was assayed in the Ellman's buffer medium, containing 0.1% Triton X-100.

Titration of AChE

The titration was performed in accordance with the methodology of Gordon *et al.* [4].

Residual activity method. Titration of enzyme solutions (0.3 to 1 nM) was carried out in 0.01 M phosphate buffer (0.1% BSA) at pH 7.0, by adding various amounts of inhibitor ranging approximately between 0.15 and 1.7 times the number of equivalents of enzyme present in the solution. The inhibition was allowed to proceed to completion (30 min) and the residual activity plotted versus the number of equivalents of MEPQ.

Fluorescence of the leaving group. MEPQ was added in excess to a buffer solution (0.01 M phosphate, pH 7.0) of AChE, containing 5–50 nM active-site concentration. The increase in fluorescence due to the release of the leaving group was monitored to completion at an excitation wavelength of 400 nm and an emission wavelength of 500 nm; the reaction was complete within 3 min. Calibration curves were constructed by diluting known amounts of 7HQ into the appropriate assay medium. Blanks were subtracted by repeating the same procedure with enzyme that was incubated previously for 30 min with 1 μM paraoxon (Aldrich), which is known to react specifically with the active-site serine of AChE [17]. This treatment should therefore prevent the release of the fluorescent chromophore 7HQ. The fluorescence observed in the blanks represents mainly the extent of hydrolysis of MEPQ. The concentration of the released leaving group was calculated from the corresponding calibration curve. The concentration of the leaving group is assumed to equal the normality of the enzyme on the basis of 1:1 stoichiometry.

Measurement of the affinity of 7HQ to AChE

Various amounts of 7HQ (0.05 to 1 μM) were added to Ellman's assay mixture (0.1 M phosphate, pH 7.0, 25°), where the concentration of acetylthiocholine ranged between 10 and 50 μM. The dissociation constant of the reversible complex between AChE and 7HQ was calculated by employing the Hunter–Downs equation [18], as described elsewhere [17].

RESULTS

Synthesis and purity of MEPQ

In contrast with a previous report [4], we found that methyliodide could be used as a methylating agent to produce MEPQ from the tertiary ester, provided that well-dried, reagent-grade solvents were used. After recrystallization, an overall yield of 60% MEPQ (based on 7-hydroxyquinoline) was obtained. NMR and absorption spectroscopy are in good agreement with the proposed structure and TLC confirmed the homogeneity of MEPQ.

Absorption spectroscopy (406 nm, phosphate buffer, pH 8.0) revealed that in several cases MEPQ

Table 1. Pseudo-first-order rate constants for the hydrolysis of MEPQ in phosphate-buffered solutions (26°)

pH	Molarity	$T_{1/2}$ (min)	k_{obs} (min^{-1})
6.0 ± 0.1	0.005	941	$7.4 \cdot 10^{-4}$
	0.01	641	$1.0 \cdot 10^{-3}$
	0.05	552	$1.3 \cdot 10^{-3}$
	0.10	498	$1.4 \cdot 10^{-3}$
7.0 ± 0.2	0.005	416	$1.7 \cdot 10^{-3}$
	0.01	248	$2.8 \cdot 10^{-3}$
	0.05	103	$6.7 \cdot 10^{-3}$
	0.10	54	$1.3 \cdot 10^{-2}$
8.0 ± 0.1	0.005	298	$2.3 \cdot 10^{-3}$
	0.01	136	$5.1 \cdot 10^{-3}$
	0.05	69	$1.0 \cdot 10^{-2}$
	0.10	39	$1.8 \cdot 10^{-2}$

was accompanied by small amounts of the free phenol (the leaving group) 7HQ. This contamination when present, did not exceed 5%. An analogous contamination was reported by Gordon *et al.* [4] with regard to DEPQ.

Hydrolysis of MEPQ and stability of stock solutions

MEPQ was found to be relatively stable in neutral aqueous solutions, but it decomposed rapidly in alkaline aqueous medium. The hydrolysis products $CH_3P(O)(OC_2H_5)OH$ + 7HQ were confirmed by absorption (406 nm) and NMR spectroscopy (not shown). Under the experimental conditions employed (excess of nucleophile), the rates of release of 7HQ were found to be first order and linearly related to the concentration of the attacking nucleophile. Table 1 summarizes the pseudo-first-order rate constants for the hydrolysis of MEPQ in phosphate-buffered solutions. A plot of the pseudo-first-order rate constants versus the buffer molarity at pH 7.0 provided a straight line (not shown), thus allowing the estimation of k_{H_2O} (k_{H_2O} = intercept). The second-order rate constants for the hydrolysis of MEPQ by H_2O ($k_{H_2O}/55$) and by OH^- at 26° were found to be $2.4 \times 10^{-5}\,M^{-1}\,min^{-1}$ and $2.0 \times 10^{2}\,M^{-1}\,min^{-1}$ respectively.

Crystalline MEPQ was found to be stable in storage over dessicant at −20° for at least 1 year. One-millimolar stock solution in dry acetone was kept at −20° for several weeks without a significant increase in the content of the free phenol, 7HQ. Also, 1-mM stock solutions in distilled water were found to be stable at 4° for several hours.

Stability of MEPQ and phospholine in mouse blood

The loss of anticholinesterase activity of both MEPQ and phospholine was measured *in vitro* in mouse whole blood incubated at 38°. As judged from the inhibition of eel AChE, >99% MEPQ was hydrolyzed within 10 min, whereas phospholine concentration under the same experimental conditions did not change significantly within the first 60 min of incubation. We note that the blood profile of phospholine in rats injected i.m. revealed a biological half-life of 2 min [1]; however, this low value is presumably due to rapid clearance rather than hydrolysis by blood constituents.

Inhibition of AChE and BuChE

The bimolecular rate constants for the inhibition of eel, mouse brain and horse-serum ChE by MEPQ in 0.1 M phosphate buffer at pH 7.0 (25°) were found to be 5.3 (±0.7) × $10^8\,M^{-1}\,min^{-1}$, 1.3 (±0.2) × $10^8\,M^{-1}\,min^{-1}$ and 5.4 (±0.7) × $10^7\,M^{-1}\,min^{-1}$ respectively.* At 35°, the biomolecular rate constant for the inhibition of eel AChE increased to 2.1 (±0.2) × $10^9\,M^{-1}\,min^{-1}$. Under the experimental conditions employed, the rates of inhibition of all enzymes studied here were found to be first order, at least for the first two half-life time periods (Fig. 1), and linearly related to the inhibitor concentration. The rates of inhibition of enzyme from either mouse or rat brain homogenate were found to be practically the same.

Reactivation of $CH_3P(O)(OC_2H_5)$-AChE conjugate

The kinetic profile for the induced reactivation of the methyl phosphonyl-AChE conjugate with 2-PAM is shown in Figs. 2 and 3. As is the case for many phosphonyl (or phosphoryl)-AChE conjugates, the reactivation in the presence of 0.5 to 1 mM 2-PAM went fairly rapidly, whereas 3-PAM did not affect significantly the stability of the enzyme–inhibitor conjugate. Similar rates were obtained for AChE inhibited by either MEPQ or $CH_3P(O)(OC_2H_5)Cl$ (Fig. 3). This observation strongly supports the assumption that the common

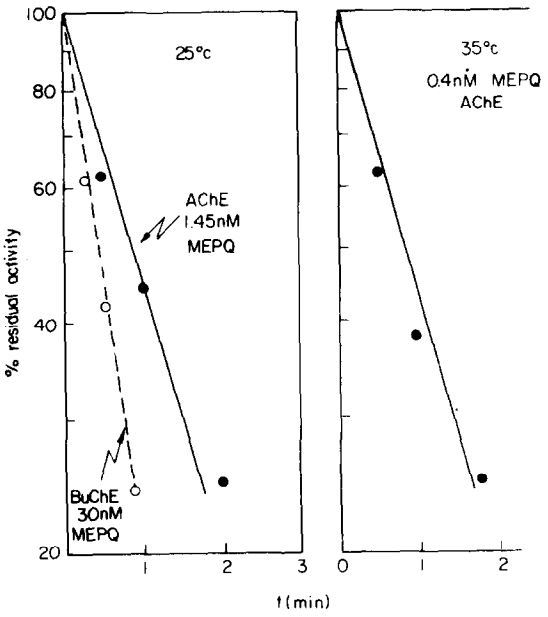


Fig. 1. Semi-logarithmic plots of percent residual activity versus time of incubation for eel AChE and horse-serum BuChE in the presence of MEPQ (phosphate 0.1 M, pH 7.0).

* The rate constants were calculated for the racemic mixture.

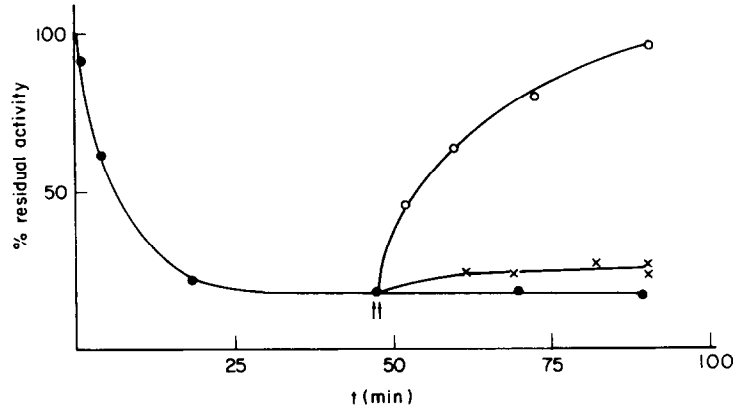


Fig. 2. Inhibition and reactivation profiles for ChE from mouse brain homogenates in the presence of 2.2 nM MEPQ in 0.1 M phosphate buffer (pH 7.0, 25°). Arrows indicate the addition of 0.5 mM 2-PAM (○—○) and 3-PAM (×—×).

inhibited intermediate is indeed $CH_3P(O)(OC_2H_5)$-AChE.

Dissociation constant (K_I) of [7HQ][AChE] conjugate

Since MEPQ was contaminated with small amounts of 7HQ and since quaternary ammonium compounds were reported in the literature to protect AChE from phosphorylation [4], it seemed worthwhile to measure K_I ($E + I \rightleftarrows EI$) and to estimate its protective efficacy in terms of preventing phosphonylation by MEPQ. K_I was calculated by using the Hunter–Downs equation [18].

$$E[I]/(E_o - E) = K_I + K_I[S]/K_m$$

where E and E_o denote enzyme activity with and without the inhibitor $[I]$, respectively, $[S]$ is the concentration of the substrate acetylthiocholine, and K_I and K_m are the dissociation constant of the inhibitor–enzyme reversible complex and the Michaelis constant of acetylthiocholine respectively. A plot of $E[I]/(E_o\text{-}E)$ vs $[S]$ provided a straight line (not shown), and K_I was found to be 1 (±0.1) μM. We note that, in experiments conducted in this study, the highest concentration of MEPQ was 0.1 μM and thereby the maximal expected concentration of 7HQ should not exceed 5 nM. The inhibition profiles of AChE by MEPQ with and without the presence of 10 nM 7HQ were found to be the same. The results indicate that under the experimental conditions described here, 7HQ did not protect the enzyme from phosphonylation by MEPQ.

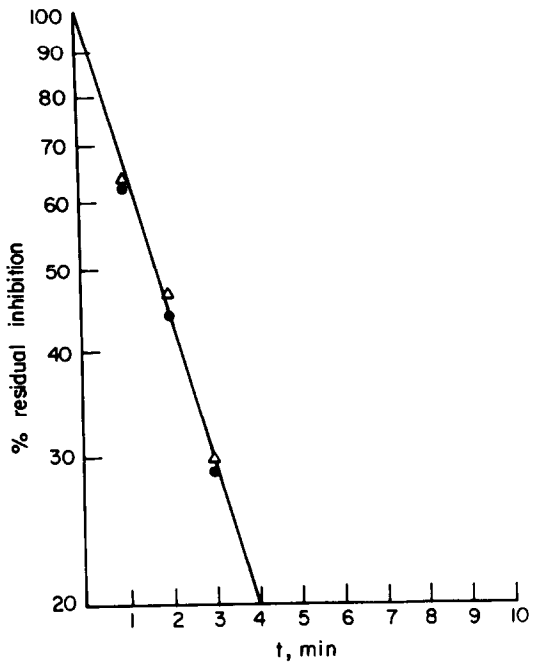


Fig. 3. Kinetics of 2-PAM-induced reactivation of eel AChE inhibited with either MEPQ (△) or MPOC (●). Eel AChE (0.25 nM) was partially inhibited with 0.4 nM MEPQ or 0.5 μM MPOC for 30 min (0.1 M phosphate buffer, pH 7.0, 25°). Reactivation was initiated by the addition of 1 mM 2-PAM.

Titration of AChE

In view of the high rate of inhibition of eel AChE by MEPQ, it was of interest to titrate low levels of enzyme concentration, as described in the past for DEPQ [4].

Stock solutions of AChE (10–100 nM) were titrated fluorimetrically by measuring the production of the leaving group 7HQ upon addition of 0.2 μM MEPQ. This enzyme solution was then diluted 10 to 100-fold in 0.01 M phosphate buffer solution (pH 7.0), and the AChE concentration was measured by the residual activity method. Figure 4 presents a plot of residual activity of AChE versus the number of equivalents of MEPQ added to a known concentration of fluorimetrically determined enzyme. If only one enantiomer of the racemic MEPQ (i.e. half of the MEPQ concentration) is assumed to be responsible for the inhibition of the enzyme, then the number of equivalents of the enzyme should equal the number of equivalents of MEPQ that produce 50% inhibition. However, the results shown in Fig. 4 indicate that the two isomers display similar rates of inhibition.

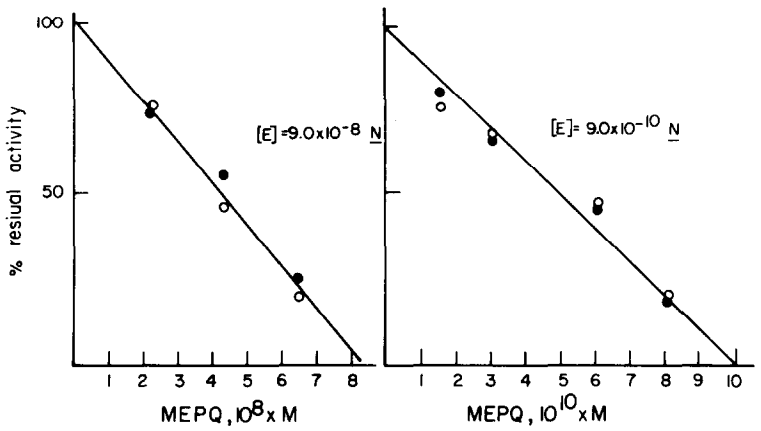


Fig. 4. Determination of eel AChE concentration by MEPQ in accordance with the residual activity technique: (a) Titration of AChE (9×10^{-8} M). The concentration of AChE was measured by the fluorescence method. (b) Titration of 9×10^{-10} M AChE. Concentration was determined by the fluorescence method as above, and the enzyme solution was diluted 100x. Enzyme activity was assayed after 10 min (○) and 30 min (●) of incubation with MEPQ.

DISCUSSION

The bimolecular rate constant for the inhibition of eel AChE was found to be 3 and 170 times the value reported for DEPQ [3, 4] and phospholine iodide [19] respectively. From the inhibition and reactivation data, we may assume, as is generally the case with organophosphate inhibitors of AChE, that the following scheme represents the mechanism underlying this reaction.

$$EOH + CH_3(C_2H_5O)P(O)X \rightarrow CH_3(C_2H_5O)P(O)OE + HX$$

Scheme III

A stoichiometry of 1:1 was established between the enzyme and MEPQ when a stoichiometric amount of the racemic mixture had reacted; both enantiomers thus displayed a similar rate of inhibition. This observation is in contrast with previous findings, where chiral phosphonates have been demonstrated to possess a remarkable stereospecificity towards AChE [20, 21].

Although it is difficult to compare results obtained under different experimental conditions, we note that MEPQ was found to be only three times as potent as DEPQ [3, 4], in terms of eel AChE inhibition, whereas by analogy with similar compounds we anticipated a somewhat higher increase (~10-fold) in the bimolecular rate constant for the inhibition of AChE [5–7]. This may be explained as follows. DEPQ is at least 100 times more powerful than would be anticipated [8] from the acidity of 7HQ (pK_a 4.7, Ref. 3). Consequently, one may argue that the unique properties of such a leaving group (e.g. binding and orientation at the active-site) control the rate-limiting step of the inhibition to such an extent that electronic differences between phosphates and phosphonates become less important.

As was reported in the past for analogous compounds [4, 10], the concentration of the leaving group 7HQ could be measured fluorimetrically at very low concentration and translated to AChE active-site concentration by assuming 1:1 stoichiometry (Scheme III). The method of residual activity has also been applied successfully to determine the normality of AChE. Due to the high bimolecular rate constant for the inhibition of AChE, concentrations as low as 0.3 nM AChE could be measured.

Our findings are in good agreement with the criteria for an improved anti-ChE marker in B-BB studies. The inhibition of both AChE and BuChE is very rapid ($1–5 \times 10^8\ M^{-1}\ min^{-1}$ and $5.4 \times 10^7\ M^{-1}\ min^{-1}$ respectively), and the inhibited enzyme is practically completely reactivated by 2-PAM. The extremely low stability of MEPQ under *in vitro* physiological conditions, as compared to phospholine iodide, suggests that the blood-level concentration should rapidly fall below the level that minimizes contamination from vascular breakage during processing of the brain tissue.

MEPQ is relatively stable in the solid state at −20° and displayed reasonable stability in acetone and distilled water stock solutions when kept in the cold.

The *in vivo* results [9] verify the usefulness and suitability of MEPQ as a new, promising and sensitive probe for the effective quantative (ChE activity in homogenates) and qualitative (histochemical staining) assessment of ChE in studies concerned with permeability changes in the B-BB. The preparation of radiolabeled MEPQ is currently under investigation in this laboratory.

REFERENCES

1. Y. Ashani, G. N. Catravas and W. A. Alter, *Biochem. Pharmac.* **30**, 2585 (1981).
2. Y. Ashani and G. N. Catravas, *Biochem. Pharmac.* **30**, 2593 (1981).
3. R. J. Kitz, S. Ginsburg and I. B. Wilson, *Molec. Pharmac.* **3**, 225 (1967).

4. M. A. Gordon, H. W. Carpenter, H. W. Barrett and I. B. Wilson, *Analyt. Biochem.* **85**, 519 (1978).
5. R. D. O'Brien, in *Drug Design* (Ed. E. J. Ariens), Vol. II, p. 162. Academic Press, New York (1971).
6. M. Eto, *Organophosphorus Pesticides, Organic and Biologic Chemistry*, p. 75. CRC Press, Cleveland (1974).
7. Y. Ashani and B. S. Green, in *Chemical Approaches to Understanding Enzyme Catalysis. Biomimetric Chemistry and Transition-State Analogs* (Eds. B. S. Green, Y. Ashani and D. M. Chipman), p. 169. Elsevier, Amsterdam (1981).
8. A. J. Kirby and S. G. Warren, *The Organic Chemistry of Phosphorus*. Elsevier, Amsterdam (1967).
9. Y. Ashani, D. Levy, E. Helman, Y. Grunfeld, T. Kadar and M. Spiegelstein, *Life Sci.* **36**, 493 (1985).
10. T. L. Rosenberry and S. A. Bernhard, *Biochemistry* **10**, 4114 (1971).
11. S. Ginsburg and I. B. Wilson, *J. Am. chem. Soc.* **79**, 481 (1957).
12. A. H. Ford-Moore and J. H. Williams, *J. chem. Soc.* 1465 (1947).
13. Z. Pelchowics, *J. chem. Soc.* **44**, 238 (1961).
14. G. L. Ellman, K. D. Courtney, V. Andres and R. M. Featherstone, *Biochem. Pharmac.* **7**, 83 (1961).
15. Y. Ashani and G. N. Catravas, *Analyt. Biochem.* **109**, 55 (1980).
16. I. B. Wilson and H. C. Froede, in *Drug Design* (Ed. E. J. Ariens), Vol. 2, p. 213. Academic Press, New York (1971).
17. W. N. Aldridge and E. Reiner, *Enzyme Inhibitors as Substrates*. North-Holland, Amsterdam (1975).
18. A. Hunter and C. E. Downs, *J. biol. Chem.* **157**, 427 (1945).
19. J. A. Maglothin and I. B. Wilson, *Biochemistry* **13**, 3520 (1974).
20. H. L. Boter and C. Van Duk, *Biochem. Pharmac.* **18**, 2403 (1969).
21. H. P. Benschop, C. A. G. Konigs, J. V. Genderen and L. P. De Jong, *Toxic. appl. Pharmac.* **72**, 61 (1984).