Heinegard, D. (1977) *J. Biol. Chem. 252*, 1980-1989.
Heinegard, D., & Axelsson, I. L. (1977) *J. Biol. Chem. 252*, 1971-1979.
Heinrikson, R. L., & Meridith, S. A. (1984) *Anal. Biochem. 36*, 65-74.
Hoffmann, H. P., Schwartz, N. B., Rodên, L., & Prockop, D. J. (1984) *Connect. Tissue Res. 12*, 151-184.
Keiser, H. D., & Hatcher, V. B. (1979) *Connect. Tissue Res. 6*, 229-233.
Kimata, K., Hascall, V. C., & Kimura, J. H. (1982) *J. Biol. Chem. 257*, 3827-3832.
Kimura, J. H., Thonar, E. J. M., Hascall, V. C., Reiner, A., & Poole, A. R. (1981) *J. Biol. Chem. 256*, 7890-7897.
Krueger, R. C., & Schwartz, N. B. (1987) *Anal. Biochem. 167*, 295-300.
Laemmli, U. K. (1980) *Nature (London) 227*, 680-685.
Oike, Y., Kimata, K., Shinomura, T., Nakazawa, K., & Suzuki, S. (1980) *Biochem. J. 191*, 193-207.
Oike, Y., Kimata, K., Shinomura, T., Suzuki, S., Takahashi, N., & Tanabe, K. (1982) *J. Biol. Chem. 257*, 9751-9758.
Olson, C. A., Krueger, R., & Schwartz, N. B. (1985) *Anal. Biochem. 146*, 232-237.
Pèrin, J. P., Bonnet, F., & Jollès, P. (1984) *FEBS Lett. 176*, 37-42.
Rosenberg, L., Wolfenstein-Todel, C., Margolis, R., Pal, S., & Strider, W. (1976) *J. Biol. Chem. 251*, 6439-6444.
Schwartz, N. B., & Rodèn, L. (1974) *Carbohydr. Res. 37*, 167-180.
Schwartz, N. B., Habib, G. M., Campbell, S. C., D'Elvlyn, D., Gartner, M., Krueger, R., Olson, C., & Philipson, L. (1985), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 44*, 369-372.
Stevens, J. W., & Hascall, V. (1986) *J. Biol. Chem. 261*, 15442-15449.
Thonar, E. J. M., Lohmander, L. S., Kimura, J. H., Fellini, S. A., Yanagishita, M., & Hascall, V. C. (1983) *J. Biol. Chem. 258*, 11564-11570.
Thyberg, J., Lohmander, S., & Heinegard, D. (1975) *Biochem. J. 151*, 157-166.
Vertel, B. M., Upholt, W. B., & Dorfman, A. (1984) *Biochem. J. 217*, 259-263.
Wessel, D., & Flugge, U. I. (1984) *Anal. Biochem. 138*, 141-143.
Zanetta, J. P., Breckenridge, W. C., & Vincendon, G. (1972) *J. Chromatogr. 16*, 291-304.

Interaction of an Organophosphate with a Peripheral Site on Acetylcholinesterase†

Alain Friboulet,*,‡ François Rieger,§ Danièle Goudou,§ Gabriel Amitai,∥,⊥ and Palmer Taylor∥

Laboratoire de Technologie Enzymatique, URA 523 du CNRS-UTC-BP649, 60206 Compiègne, France, Unitê de Biologie et Pathologie Neuromusculaires, INSERM U 153-17, rue du Fer-à-Moulin, 75005 Paris, France, and Department of Pharmacology, M-036, University of California, San Diego, La Jolla, California 92093

Received April 5, 1989; Revised Manuscript Received September 8, 1989

ABSTRACT: *O*-Ethyl *S*-[2-(diisopropylamino)ethyl] methylphosphonothioate (MPT) is an active site directed inhibitor of acetylcholinesterase (AChE). Inhibition of the *Electrophorus electricus* (G4) enzyme follows classical second-order kinetics. However, inhibition of total mouse skeletal muscle AChE and inhibition of the individual molecular forms from muscle, including the monomeric species, do not proceed as simple irreversible bimolecular reactions. Similarly, complex inhibition kinetics are observed for the purified enzyme from *Torpedo californica*. AChE can be cross-linked with glutaraldehyde into a semisolid matrix. Under these conditions the abnormal concentration dependence for MPT inhibition is accentuated, and a range of MPT concentrations can be found where inhibition of polymerized AChE is far less than that observed at lower concentrations. Inhibition in certain concentration ranges is partially reversible after removal of all unbound ligand. Thus, there are two different modes of organophosphorus inhibition by MPT: the classical irreversible phosphorylation of the active site and a reversible interaction at a site peripheral to the active center. Propidium, a well-studed peripheral site ligand, can prevent the later interaction. Hence, the second site of MPT interaction with AChE may overlap or be linked to the peripheral anionic site of AChE characterized by the binding of propidium and other peripheral site inhibitors.

Acetylcholinesterase (AChE;[1] acetylcholine hydrolase; EC 3.1.1.7) is a widely distributed enzyme found in neural and nonneural tissues (Hall, 1973; Rieger & Vigny, 1976; Ott & Brodbeck, 1978; Massoulié & Bon, 1982). The enzyme exists in several molecular forms. In skeletal muscle, AChE is present as soluble and membrane-bound globular forms and as dimensionally asymmetric forms. The asymmetric forms are found not only in the motor end plate (Hall, 1973; Vigny et al., 1976), where they are primarily localized in the synaptic basal lamina, but also in intracellular loci (Dreyfus et al., 1983). In the electric organ of *Torpedo* both classes of molecular species have been found, whereas in *Electrophorus* only the asymmetric species have been identified. The catalytic properties of all the molecular forms of AChE appear identical in kinetic parameters or susceptibility to inhibition. The AChE active center is known to include a nucleophilic subsite that

†Supported in part by USPHS Grant GM 18360, DAMD Contract 17-87C7109, and DRET Grant 84/054.
‡URA 523 du CNRS-UTC-BP649.
§INSERM U 153-7.
∥University of California, San Diego.
⊥Present address: Israel Institute for Biological Research, P.O. Box 19, Ness Ziona, Israel 70450.

[1] Abbreviations: AChE, acetylcholinesterase; MPT, *O*-ethyl *S*-[2-(diisopropylamino)ethyl] methyl phosphonothioate; BuChE, butyrylcholinesterase; DFP, diisopropyl fluorophosphate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

0006-2960/90/0429-914$02.50/0

can form covalent bonds with acyl, carbamoyl, sulfonyl, or phosphoryl groups and an anionic subsite. The latter is selective for positively charged groups that are nonhydrated by virtue of carrying alkyl substituents (Kitz & Wilson, 1963; Krupka, 1966).

Consistent with an early suggestion of Changeux (1966) for *Torpedo* AChE, it has been shown that AChE possesses additional peripheral site(s) that can bind various quaternary ligands (Wombacher & Wolf, 1971; Crone, 1973; Rosenberry, 1975; Roufogalis & Wickson, 1975). Propidium is of particular value in defining this site since its fluorescence properties permit a direct measure of binding (Taylor & Lappi, 1975). Peripheral site actions of organophosphates have not been demonstrated directly, and inhibition by these compounds is generally reported to follow simple second-order kinetics (Aldridge & Reiner, 1972; Hart & O'Brien, 1973). In a few cases, however, the irreversible inhibition of AChE by organophosphorus compounds was not completely explained by classical second-order kinetics (Aldridge & Reiner, 1969; Gentinetta & Brodbeck, 1976; Radic et al., 1984). The results with unequal rates of DFP inhibition were interpreted to be indicative of inequivalence in reactivity of the subunits in the oligomer (Gentinetta & Brodbeck, 1976), although others find equivalent rates of inhibition (Barnett & Rosenberry, 1975). With other organophosphates with a more complex structure, reaction at more than a single site has been inferred (Radic et al., 1984).

In the present study we have examined the inhibition of skeletal muscle and *Torpedo* electric organ AChE by a stable and specific organophosphate, *O*-ethyl *S*-[2-(diisopropylamino)ethyl] methylphosphonothioate (MPT). Crude muscle AChE and the individual molecular forms prior to and following cross-linking of the enzyme to form polymeric species all share unusual inhibition behavior. Similar inhibition profiles are seen with the purified *Torpedo* enzyme. The inhibition kinetics are consistent with an additional site of action of the organophosphate, distinct from the active site. Moreover, the unusual features of MPT inhibition are decreased or suppressed in the presence of propidium and an irreversibly acting azido analogue of propidium. The competition experiments further define a second site of association of the alkyl phosphonate.

Materials and Methods

Acetylcholinesterase. White mice (129 Re J strain, from our own breeding colony) were sacrificed under ether; their sternocleidomastoid muscles were dissected. Muscles were homogenized directly after excision or after separation of the motor end plate rich and motor end plate free regions using a cytochemical stain as previously described (Koenig & Rieger, 1981). Homogenization was performed by hand in a conical glass-to-glass ice-cooled homogenizer in 10 volumes of buffer containing 10 mM Tris-HCl (pH 7.0), 1 M NaCl, 1 mM EGTA, and 1% Triton X-100. The resulting homogenate was centrifuged at 27000*g* for 15 min at 4 °C. Supernatants were used for inhibition studies or further fractionation. The individual forms of *Torpedo* AChE were isolated and purified as previously described (Taylor & Jacobs, 1974; Lee et al., 1982).

Separation of the Molecular Forms of AChE. Continuous gradients of 5–20% sucrose in the above buffer were prepared in 13-mL tubes, and 75-μL aliquots of the 27000*g* supernatant were layered on the gradient. Centrifugations were performed in an SW-41 Beckman rotor at 38 000 rpm and 4 °C for 15 h, or 43 h in the case of the monomeric form of AChE. Fractions were collected, and aliquots were assayed for esterase activity (Ellman et al., 1961). A computational Gaussian deconvolution of the gradient sedimentation patterns, using a laboratory-made program (Dreyfus et al., 1984), was used for a more quantitative analysis of the different AChE molecular forms.

Cross-Linking Procedure. The artificial AChE films were prepared following cross-linking with bovine serum albumin as a support (Thomas & Broun, 1976). An aliquot of 0.25 mL of muscle homogenate or gradient fraction and 0.25 mL of a solution containing 40 mg·mL^{-1} glutaraldehyde and 20 mM phosphate buffer (pH 7.0) were mixed and gently spread on a planar glass surface. After 3 h, complete polymerization occurred and a 10-cm^2, 50 μm thick, protein film was obtained. The film was rinsed with the buffer solution and then immersed into a 10 mg·mL^{-1} glycine solution in order to scavenge free reactive groups. Discs of 1-cm^2 area were stamped out of the film and used in the study of enzyme inhibition.

Inhibition Studies. Concentrated aqueous solutions of the inhibitor (5–20 μL) were combined with 100 mM sodium phosphate buffer, pH 7.0, containing 1.5 mM DTNB and the free (5–10 μL of extracted enzyme supernatant) or insolubilized enzyme in 1-mL total volume. The inhibitor was in substantial stoichiometric excess over AChE subunits. Assuming a turnover number similar to the values proposed for the *Electrophorus electricus* enzyme (Vigny et al., 1978a), we generally used enzyme concentrations not exceeding 2×10^{-13} M. The enzyme was incubated initially with various MPT concentrations for 45 min at 20 °C. After the substrate acetylthiocholine (1.5 mM final concentration) has been added, inhibition was followed by measuring substrate hydrolysis, usually for 45-min periods. After extensive dialysis of the inhibitor, spontaneous reactivation of the soluble enzyme was not observed (Vigny et al., 1978a). To assess whether components of muscle homogenate may interfere with the inhibition kinetics, inhibition experiments were performed on active *Electrophorus* enzyme mixed with the 27000*g* supernatant of crude muscle homogenate previously inhibited by diisopropyl fluorophosphate (DFP). An excess of DFP, 0.57 mM, and 10 min of reaction were used to inhibit the enzyme. The muscle extract was then dialyzed for 12 h against two to three changes of buffer to remove all unbound DFP. AChE from *Electrophorus* was then added to the solution, cross-linked with albumin, and tested for inhibition with MPT. In another set of experiments, propidium was added simultaneously with MPT at concentrations of 10–30 μM and inhibition monitored in the usual manner.

On the basis of the kinetics of irreversible inhibition of soluble AChE from *Torpedo* (Amitai & Taylor, 1983), monoazidopropidium (3 μM) was photolyzed with the insolubilized muscle AChE. After 45-min incubation with the inhibitor in the dark, photolysis was achieved with a 5-min exposure to a 150-W xenon lamp in a 1 mM phosphate buffer, pH 7.8. A cut-off filter removed most of the energy below 330 nm, and the sample was placed approximately 10 cm from the light source. The films were in a suspended, stirred solution to achieve equivalent exposure on both surfaces. The reaction was stopped by immersing the films in a solution of 10 mM Tris-HCl buffer, pH 7.0, containing 1 M NaCl. The films were washed twice for 1 h in the above buffer and then for 1 h in 10 mM phosphate buffer, pH 7.0, before exposure to MPT.

Chemicals. *O*-Ethyl *S*-[2-(diisopropylamino)ethyl] methylphosphonothioate (MPT) was a gift from Dr. Leterrier (Hŏpital Percy, Clamart, France). Purity of MPT was checked by gas chromatographic procedures, and aqueous

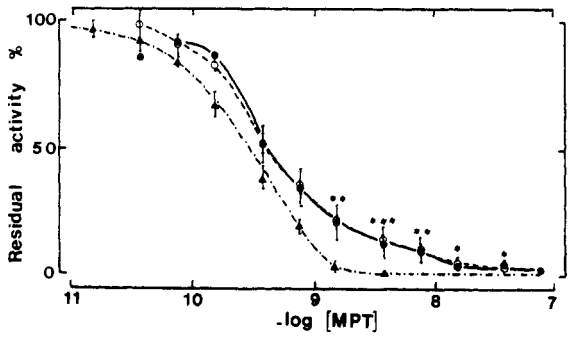


FIGURE 1: Inhibition of muscle AChE by MPT. The enzyme was incubated with MPT at various concentrations for 45 min, and the substrate was then added to initiate the enzymatic reaction. *E. electricus* AChE (▲) experimental data are fitted by the theoretical curve (-·-), with residual activity $[([E_0] - [EP])/[E_0]]$ calculated according to eq 2 using $K_d = 1.3 \times 10^{-7}$ M, $k_2 = 6.9$ min^{-1}, and $t = 45$ min. Crude muscle AChE (●) and AChE from motor end plate rich regions (○) extracted in the presence of high salt and 1% Triton X-100 and centrifuged as described under Materials and Methods. Means of four independent determinations are shown (± standard error of the mean). The statistical significance of the deviations of the experimental results from second-order kinetics calculated from data obtained with crude muscle AChE at low MPT concentrations, according to eq 2 ($K_d = 1.3 \times 10^{-7}$ M, $k_2 = 4.3$ min^{-1}, and $t = 45$ min), is indicated: (***) $P < 0.01$; (**) $P < 0.02$; (*) $P < 0.05$.

stock solutions were found to be stable for months. Dilute solutions in buffer show no degradation after 2 h as detected by chromatography. Acetylthiocholine iodide, 5,5′-dithiobis(dinitrobenzoic acid) (DTNB), bovine serum albumin (fraction V), and *E. electricus* AChE (fraction V.S) were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were of the highest available purity.

Monoazidopropidium was synthesized from propidium and $NaNO_2$ by methods described by Graves et al. (1977). The reaction products were separated by chromatographic means and on the basis of spectra of the reaction products (Graves et al., 1977; Yielding et al., 1984); the monoazido analogue was the fractionated entity as identified by mass spectrometry, TLC, and infrared spectroscopy (Amitai et al., unpublished results). This analogue was used in the photoaffinity labeling experiments.

RESULTS

MPT Inhibition of Skeletal Muscle AChE. The inhibition of AChE from *Electrophorus* by MPT is shown in Figure 1 and follows a classical pattern for irreversible inhibition (Aldridge & Reiner, 1969):

$$E + PX \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E{\cdot}PX \xrightarrow{k_2} EP + X \qquad (1)$$

where E and EP denote the free and phosphorylated enzyme, respectively, and E·PX is the complex between enzyme and inhibitor (PX). The integrated differential equation for reaction 1 is

$$\ln \frac{[E_0]}{[E_0] - [EP]} = \frac{k_2[PX]t}{K_d + [PX]} \qquad (2)$$

where $[E_0]$ is the total enzyme concentration, t is the time, and $K_d = k_{-1}/k_{+1}$.

The experimental data (Figure 1) are fitted by eq 2. The values of the constants K_d and k_2 of the theoretical curve were calculated by a weighted least-squares nonlinear regression: $K_d = 1.3 \times 10^{-7}$ M and $k_2 = 6.9$ min^{-1} ($t = 45$ min). Inhibition as a function of inhibitor concentration for the crude muscle homogenate AChE shows unexpected features since a shoulder exists at inhibitor concentrations between 10^{-8} and 10^{-9} M,

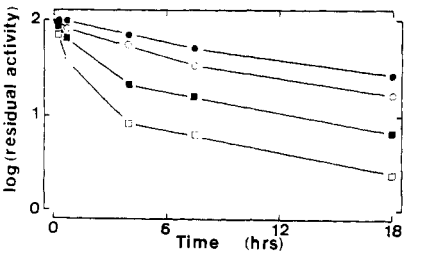


FIGURE 2: Kinetics of inhibition by MPT of muscle AChE. The enzyme was incubated for various periods with MPT before the residual activity was measured over a 45-min interval. MPT concentrations were 7.5×10^{-11} (●), 1.5×10^{-10} (○), 3.75×10^{-10} (■), and 7.5×10^{-10} M (□).

and complete inhibition could not be achieved until concentrations exceeded 10^{-8} M (Figure 1). These curves could only be fit by eq 2 for low MPT concentrations. The kinetic parameters $K_d = 1.3 \times 10^{-7}$ M and $k_2 = 4.3$ min^{-1} were obtained by assuming that for MPT concentrations less than 3.75×10^{-10} M inhibition proceeds by purely bimolecular second-order kinetics. Significant deviation of the experimental curves from the theoretical curve was revealed for MPT concentrations greater than 7.5×10^{-10} M (Students t test, Figure 1). Similar findings were obtained with acetylcholine as a substrate and pH-stat monitoring of hydrolysis with higher enzyme concentrations (data not shown). These results suggest that the anomalous inhibition kinetics are not generated by products of the Ellman reaction. The staining procedure used to dissect motor end plate rich regions, rich in the different forms of AChE, does not modify the inhibition pattern of muscle AChE shown in Figure 1.

We also found that the irreversible inhibition of crude muscle AChE presents nonlinear kinetics when the time of inhibition is the variable. Figure 2 shows the inhibition curves of the enzyme at various MPT concentrations after 0–18 h of exposure to the inhibitor.

Inhibition of the Main Molecular Forms of Muscle AChE. AChE activity from mammalian muscle was separated by sucrose density gradient centrifugation in the presence of Triton X-100 into several discrete molecular forms, which may bind the inhibitor differently. AChE present in the sternocleidomastoid motor end plate rich regions contains a high proportion of tail-containing, dimensionally asymmetric 16S form. The sedimentation profiles can be deconvoluted into as many as eight discrete molecular forms by computer analysis to separate Gaussian curves (Dreyfus et al., 1984). In the present work, the fractions corresponding to the three main peaks, with sedimentation coefficients of approximately 16, 10, and 4 S, and the monomeric species derived from the 4S peak were isolated and further studied. Inhibition profiles for MPT of the three individual AChE peaks (16, 10, and 4 S) also exhibit clear deviations from classical irreversible inhibition (Figure 3). The inhibition curves obtained for each of the three AChE peaks are similar and at least equally pronounced as that seen with total extractable muscle AChE. When the monomeric form of AChE was isolated in the presence of Triton X-100, the deviations appeared to be smaller but nevertheless were significantly different from that seen with the *Electrophorus* enzyme.

Inhibition of the Molecular Forms of AChE after Incorporation into a Matrix. The incorporation of AChE into a polymeric matrix not only stabilized enzyme activity but also allowed for convenient study of reversibility of inhibition following removal of the inhibitor. Evidence for apparent homogeneity of the separation of the active sites in the film has been obtained by transmission electron microscopy

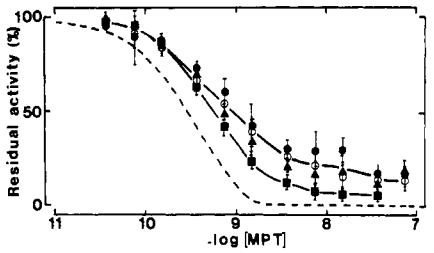


FIGURE 3: Inhibition by MPT of the isolated primary molecular forms of AChE from motor end plate rich regions of mouse muscle. Residual AChE activity as a function of MPT concentration after 45-min incubation with MPT. 16S (▲), 10S (●), 4S (○), and monomeric (■) AChE were separated in the presence of Triton X-100 as described in Dreyfus et al. (1984). The bars indicate standard error of the mean for three independent determinations. The best-fit curve obtained for the inhibition for the *E. electricus* enzyme shown in Figure 1 is included as a dotted line for reference.

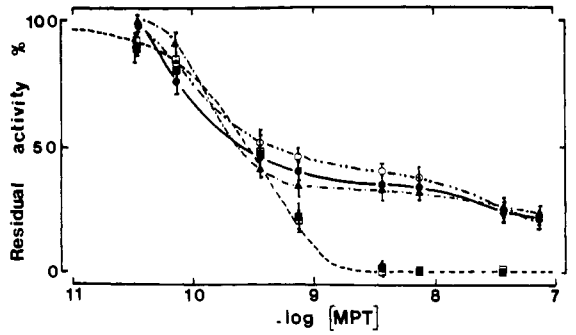


FIGURE 4: Inhibition by MPT of the major molecular forms of AChE from motor end plate rich regions after immobilization of the enzyme. The insolubilized major molecular forms of AChE were incubated with various MPT concentrations for 45 min. The substrate was then added directly in the incubation medium to measure the residual activity. (▲) 16S, (●) 10S, and (○) 4S molecular forms. The inhibition data of insolubilized commercial *E. electricus* AChE (□) in the absence or (■) in the presence of previously inhibited muscle homogenate can be fitted by the curve (– –) calculated for the soluble eel enzyme according to eq 2 with $K_d = 1.3 \times 10^{-7}$ M, $k_2 = 6.9$ min^{-1}, and $t = 45$ min. Bars show standard error of the mean for three independent determinations.

(Barbotin et al., 1984). Inhibition of the enzyme in polymerized discs can be studied with the same experimental procedure as for the soluble enzyme. With an active site concentration of insolubilized *E. electricus* enzyme as low as 10^{-13} M, we expect that diffusional limitations of protons released by catalysis and retained in the buffer-equilibrated matrix can be neglected. The experimental data (Figure 4) can be fitted by eq 2 with the same kinetic parameters as for the free enzyme. The inhibition curves obtained with the three main molecular forms of mouse muscle are not qualitatively different from that obtained with the corresponding free enzyme (Figure 4), although the intermediary plateau is more pronounced for inhibitor concentrations in the 10^{-8}–10^{-9} M range.

To assess whether nonclassical kinetics may have arisen from other components in muscle homogenates, we performed inhibition experiments on active matrix incorporated *E. electricus* enzyme added to a crude muscle homogenate whose AChE had been rendered inactive by previous treatment with 0.57 mM DFP. The data do not differ from those obtained in the absence of muscle homogenate (Figure 4). In another set of experiments, in order to test the possibility of a decrease in MPT concentration or the appearance of a degradation product during the period of 45 min of incubation, we used the muscle enzyme–MPT mixtures after incubation for 45 min to estimate their efficiency in inhibiting the soluble eel enzyme. The curves obtained are virtually the same as those when fresh

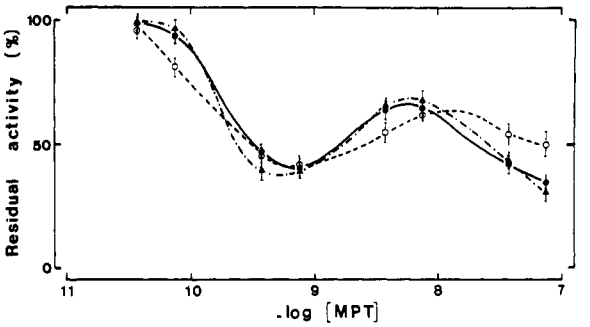


FIGURE 5: Biphasic curves of residual AChE activity of the immobilized major molecular forms after MPT inhibition and washing of excess free inhibitor. Residual activity as a function of MPT concentration during the inhibition period. After inhibition, the free inhibitor was eliminated from the artificial films by washing three times, 20 min each, before the substrate was added. Symbols as in Figure 4.

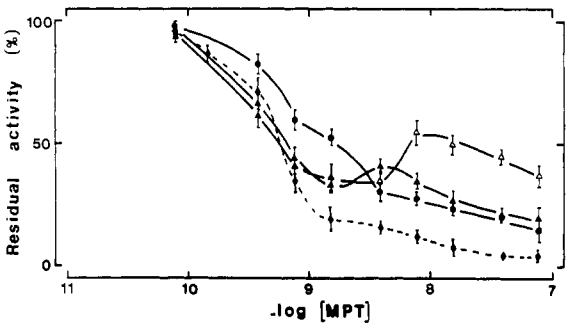


FIGURE 6: Inhibition by MPT of the purified molecular forms of AChE from *T. californica* electric organ. Residual activity of the soluble *T. californica* 11S AChE form as a function of MPT concentration after 45-min incubation (♦). With the immobilized purified forms of AChE from *Torpedo*, 5.6S (▲), 11S (●), and 17+13S (△), the residual activity was measured as a function of MPT concentration during the inhibition period. After the artificial films were washed to remove unreacted MPT and reaction products, activity is measured as in Figure 5.

MPT solutions are used (Figure 1), showing that there is no change in concentration of MPT and no evidence for the appearance of degradation products during incubation.

Complex Inhibition Patterns and the Absence of Spontaneous Reactivation of MPT-Inhibited Acetylcholinesterase. The immobilization of AChE in an artifical matrix allows for exposure to inhibitors and rinsing without dilution and denaturation of the enzyme. When the artificial films were exposed to the inhibitor as above and then washed (three times, 20 min each, with a solution containing phosphate buffer and DTNB), before addition of the substrate, the curves corresponding to 16S, 10S, and 4S AChE all exhibit an exaggerated dome shape (Figure 5), similar to those already reported for total AChE (Friboulet et al., 1986), with significantly increased activity at MPT concentrations of about 10^{-8} M. Thus, the inhibition appears to be much less efficient at higher (10^{-8} M) than at lower MPT concentrations (i.e., 10^{-9} M). In all cases, the curves obtained with the different molecular forms of AChE were nearly identical. Complex inhibition patterns are also found for the purified hydrophobic (5.6 S) and asymmetric (17 + 13 S) forms of *Torpedo* AChE (Figure 6). The AChE activities after inhibition and rinsing do not change for 48 h after inhibition, indicating the absence of spontaneous reactivtion of the insolubilized inactive enzyme.

Modification of the MPT Inhibition Curves of AChE by Propidium and Monoazidopropidium. The influence of propidium, a reversible peripheral site inhibitor, on the inhibition of AChE by MPT was tested by adding 10^{-5} or 3 ×

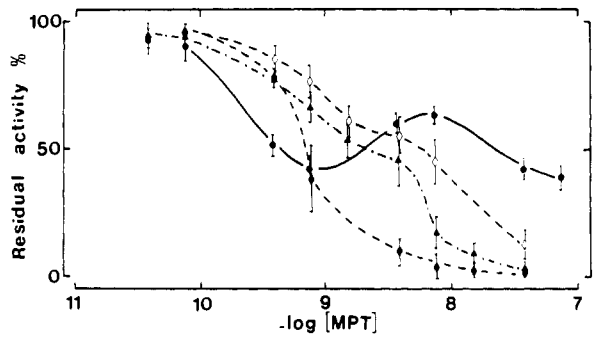


FIGURE 7: Influence of propidium and monoazidopropidium on the irreversible inhibition of immobilized mouse muscle AChE by MPT. The residual activity was measured after washing of excess free and reversibly bound ligands and reaction products. Inhibition of AChE activity by MPT alone (●) or in the presence of 1×10^{-5} M (◊) or 3×10^{-5} M (♦) propidium. Inhibition by propidium alone was nearly complete but is reversible. Upon washing the recovery of the enzymatic activity was complete. Inhibition of insolubilized mouse muscle AChE previously modified by 3×10^{-6} M (▲) monoazidopropidium: 40% of the original activity is retained after incubation and washing. Bars are standard errors of the mean for three independent determinations.

10^{-5} M propidium into the reaction mixture prior to MPT addition. Residual activities after MPT inhibition show a substantial decrease of the dome-shaped behavior (Figure 7) in the MPT concentration range of 10^{-9} to 5×10^{-8} M after protection with the reversible inhibitor of the peripheral site. Irreversible inhibition by MPT at low MPT concentrations is diminished by propidium while at higher MPT concentrations propidium induces greater inhibition. Both aspects of this behavior can be explained by propidium's inhibtion at a peripheral anionic site. At low MPT concentrations MPT may only be interacting with the active center of AChE. Propidium occupation of the peripheral site also inhibits catalysis of acetyl and carbamoyl esters in an uncompetitive fashion (Taylor & Lappi, 1975; Epstein et al., 1979). In this case phosphorylation at the active center is also inhibited. At higher concentrations of MPT, it also occupies the peripheral anionic site. In so doing, competition between propidium and MPT becomes evident, and MPT's secondary effect of preventing its own irreversible inhibtion becomes blunted. Thus, we observe with propidium a diminution of MPT action at the peripheral site influencing its activity at the active center. Consistent with this behavior of propidium, we found that several other peripheral site inhibitors, *d*-tubocurarine (5×10^{-4} and 1×10^{-3} M) and gallamine (5×10^{-4} and 1×10^{-3} M), also induce a convergence to a more sigmoid shape of the MPT inhibition curves (data not shown). This is to be expected since these agents interact with the same site as propidium at low ionic strength (Changeux, 1966; Taylor & Lappi, 1975).

The effect of the irreversible inhibitor monoazidopropidium on the inhibition profile is even more striking (Figure 7). Monoazidopropidium after incubation with the insolubilized enzyme was photoactivated, thereby reacting with the enzyme. The unconjugated reactants were removed by extensive washing, and the inhibition profile of various MPT concentrations was determined. The reaction with azidopropidium reduced the catalytic activity of the insolubilized enzyme to 40% of its original value. The azidopropidium-conjugated enzyme shows diminished sensitivity to MPT inhibition at low MPT concentrations. At higher concentrations, the dome-shaped curve that is manifest in the absence of monoazidopropidium is virtually removed. Similar results were obtained when monoazidopropidium was reacted with soluble enzyme and the enzyme–azidopropidium conjugate was incorporated into the insolubilized enzyme films (data not shown). Thus, the irreversible modification of the enzyme eliminates the minimum in the concentration-dependence curve, and the logarithmic dependence on the MPT concentration approaches a more sigmoid shape.

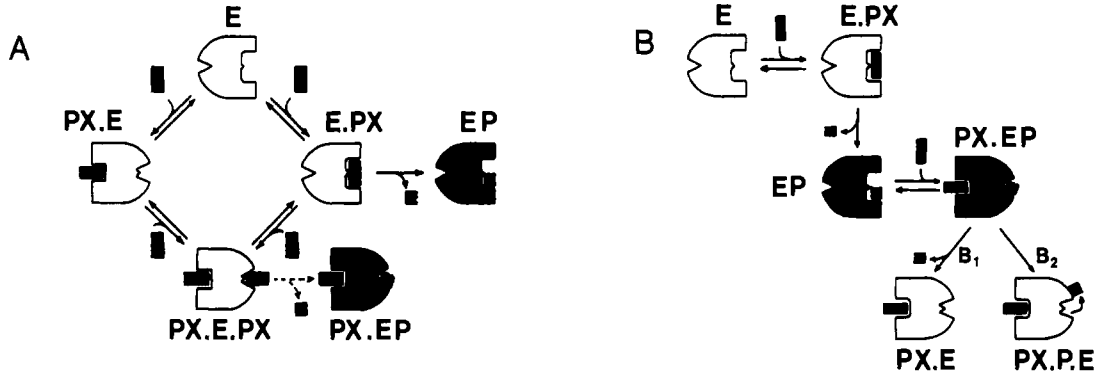
Discussion

O-Ethyl *S*-[2-(diisopropylamino)ethyl] methylphosphonothioate is an irreversible inhibitor of the active site of AChE (Bajgar & Patocka, 1977). The reaction has been shown to follow second-order irreversible inhibition kinetics at low inhibitor concentrations (Vigny et al., 1978a). In contrast to inhibition of the enzyme from *E. electricus*, we found inhibition of crude muscle AChE to deviate from second-order kinetics. By using specific inhibitors of butyrylcholinesterase (BuChE; EC 3.1.1.8) and AChE, we previously presented evidence that such a behavior could not be explained by sequential inhibition of AChE followed by BuChE (Goudou et al., 1983), as previously hypothesized by Vigny et al. (1978b) to explain the biphasic inhibition of rat superior cervical ganglion cholinesterases by diisopropyl fluorophosphate (DFP). AChE has been shown to exist under several molecular forms in mammalian muscle. A possible hypothesis to explain the unusual inhibition curves obtained with the crude muscle homogenate AChE is that the different molecular forms differ in their reactivity toward MPT. The three main peaks of AChE activity with approximate sedimentation coefficients of 4, 10, and 16 S were separated by sucrose gradient centrifugation. The inhibition curves obtained with the three distinct molecular forms are similar, and in fact, their deviations from a classical irreversible curve appear even more pronounced than that seen for crude muscle homogenate AChE (Figure 3). These results show that (i) all the molecular forms of muscle AChE have similar susceptibility to the inhibitor when deviations from a simple bimolecular reaction are evident; (ii) BuChE, which is negligible in the 16S AChE preparation (Goudou et al., 1983), is clearly not responsible for the complex inhibition curves; and (iii) it is highly improbable that contaminating proteins are responsible for the unusual inhibition kinetics, because they should exist at different concentrations in AChE-containing fractions of the continuous sucrose gradient. Moreover, homogeneous *Torpedo californica* AChE forms show also the unusual inhibition kinetics (Figure 6).

In order to ascertain whether subunit interactions could be responsible for the deviations in inhibition behavior, we isolated the monomeric forms of AChE and studied their inhibition by MPT. The inhibition curves obtained with the monomeric form of the enzyme isolated in the presence of detergent, although different, also deviate from second-order kinetics. The deviations from this sigmoid shape of the logarithmic concentration dependence are even more marked for monomer isolated in the absence of detergent (data not shown) and may relate to the amount of Triton associated with the enzyme.

When insolubilized into an artificial proteic film, the different molecular forms continue to exhibit unusual inhibition behavior by MPT (Figure 4). Conjugation of AChE in the film permits an extensive washing of excess free ligand, and we observed under these conditions a much less pronounced inhibitory action of MPT for high MPT concentrations. The resulting dome-shaped inhibition curve cannot be explained by the existence of more than one class of reactive catalytic subunits of AChE. Moreover, we did not find spontaneous reactivation of the inhibited enzyme. It thus seems that there is a partially reversible inhibition phase, for a defined range of MPT concentration values. Unusual irreversible inhibition of cholinesterases by organophosphorus compounds has been described by Main (1969) and by Aldridge and Reiner (1969)

Scheme I: Alternative Mechanisms of Interaction of MPT with the Peripheral Organophosphate Site[a]



[a] The site on the left is the peripheral site; the site on the right is the active site, composed of the esteratic and anionic subsites. The solid rod is the MPT molecule (PX). The open symbols correspond to the active forms of the enzyme, before and/or after washing of reversibly bound MPT. The black symbols correspond to the irreversibly inactivated forms of the enzyme. In (B), B_1 indicates the dephosphorylation of the active site while B_2 represents an intramolecular migration of phosphorus leaving the active site free.

and Radic et al. (1984) for the interaction of Tetram [*O,O*-diethyl *S*-[2-(diethylamino)ethyl]phosphorothioate] with BuChE and of Haloxon [bis(2-chloroethyl) 3-chloro-4-methylcoumarin-7-yl phosphate] with erythrocyte AChE, respectively. The inhibition curve in Figure 5 for MPT concentrations from 10^{-11} to 5×10^{-10} M can be explained on the basis of MPT binding to the active site of AChE, leading to irreversible inhibition. To explain the portion of the inhibition curve at higher concentrations (5×10^{-10} to 5×10^{-8} M MPT), we are led to propose the existence of a peripheral site of action of the organophosphorus compound. Two different mechanisms may be considered (see Scheme I): (A) A reversible binding of an additional MPT molecule to a peripheral site inhibits the irreversible step of the reaction at the active center. Such a scheme seemed plausible to explain the inhibition behavior of horse serum BuChE by Tetram (Main, 1974). (B) Alternatively, a reversible binding of a second MPT molecule to a peripheral site induces either dephosphorylation (B_1) or an intramolecular migration of phosphorus leaving the active site free (B_2). Either phenomenon would require a conformational change in enzyme structure.

While Scheme IA can explain the plateau level observed in the inhibition curve in the presence of MPT (Figure 4), a simple peripheral site binding mechanism cannot account for the biphasic inhibition behavior observed in Figure 5. A sequential model, as shown in Scheme IB, seems a more probable explanation of the inhibition curves of Figures 4 and 5. By stimulating catalysis of the organophosphate when the substrate is added, the substrate competes with MPT for the free active sites. Thus, a smaller fraction of the enzyme is irreversibly inhibited.

The curves obtained with the monomeric form of AChE strongly suggest an interaction between MPT and the peripheral site even on the monomer of AChE. Therefore, a cooperative phenomenon involving adjacent subunits (Monod et al., 1965) can be excluded. The interaction of MPT with this site probably induces an allosteric change of the monomer of AChE. Specific ligands for the peripheral site have better defined its specificity and physicochemical characteristics. The dramatically altered inhibition profiles with the peripheral site directed propidium derivatives (Figure 7) show an at least partial competitive behavior for propidium and MPT occupation at the periphral site. Propidium which is directed against a peripheral anionic site on AChE almost totally suppresses the abnormal features of the MPT inhibition curves. Studies with fluorescent alkylphosphonates show that peripheral site inhibitors alter active site conformation when the alkylphosphonate is conjugated to the active site serine (Epstein et al., 1979). From this study it also appears that organophosphate binding to the peripheral site can affect the turnover of the same organophosphate at the active center.

It cannot be ascertained whether the propidium site and the peripheral locus of organophosphate association are identical without a direct measure of peripheral site binding of the organophosphate. However, it is clear that propidium occupation greatly diminishes the abnormal MPT inactivation kinetics, which must be induced by binding at a site distinct from the active center. These considerations are underscored by the observation that prior modification of the enzyme by azidopropidium causes MPT inhibition to approach the simple kinetics described by eq 1. Thus the peripheral site, previously demonstrated with propidium and ligands that competitively dissociate the fluorescent inhibitor, shares common features with a secondary site of organophosphte association. The complete characterization of this site and its precise relationship with the propidium binding site have important potential implications for the elucidation of the mode of action of certain organophosphorus compounds.

Acknowledgments

We are grateful to Pr. D. Thomas for critical reading of the manuscript and to Dr. T. L. Rosenberry for suggestions regarding the study.

References

Aldridge, W. N., & Reiner, E. (1969) *Biochem. J. 115*, 147–162.

Aldridge, W. N., & Reiner, E. (1972) *Enzyme Inhibitors as Substrates* (Neuberger, A., & Tatum, E. L., Eds.) North-Holland, Amsterdam.

Amitai, G., & Taylor, P. (1983) *Fed. Prod., Fed. Am. Soc. Exp. Biol.*, 2034.

Bajgar, J., & Patocka, J. (1977) *Czech. Chem. Commun. 42*, 770–772.

Barbotin, J. N., Friboulet, A., Malpiece, Y., & Thomas, D. (1984) *Colloids Surf. 10*, 359–369.

Barnett, P., & Rosenberry, T. L. (1975) *Biochim. Biophys. Acta 517*, 154–159.

Changeux, J. P. (1966) *Mol. Pharmacol. 2*, 369–392.

Crone, H. D. (1973) *J. Neurochem. 20*, 225–236.

Dreyfus, P., Rieger, F., & Pinçon-Raymond, M. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6698–6702.

Dreyfus, P., Friboulet, A., Tran, L. H., & Rieger, F. (1984) *Biol. Cell 51*, 35–42.

Ellman, G. L., Courtney, K. D., Andres, V., & Featherstone, R. M. (1961) *Biochem. Pharmacol. 7*, 88–95.
Epstein, D. J., Berman, H. A., & Taylor, P. (1979) *Biochemistry 18*, 4749–4754.
Friboulet, A., Goudou, D., & Rieger, F. (1986) *Neurochem. Int. 9*, 323–328.
Gentinetta, R., & Brodbeck, U. (1976) *Biochim. Biophys. Acta 438*, 437–448.
Goudou, D., Friboulet, A., Dreyfus, P., & Rieger, F. (1983) *C. R. Acad. Sci. Paris 296*, 169–172.
Graves, D. E., Yielding, L. W., & Watkins, K. L. (1977) *Biochim. Biophys. Acta 479*, 98–104.
Hall, Z. W. (1973) *J. Neurobiol. 4*, 343–362.
Hart, G. J., & O'Brien, R. D. (1973) *Biochemistry 12*, 2940–2945.
Kitz, R., & Wilson, I. B. (1963) *J. Biol. Chem. 238*, 745–748.
Koenig, J., & Rieger, F. (1981) *Dev. Neurosci. 4*, 249–257.
Krupka, R. M. (1966) *Biochemistry 5*, 1988–1998.
Lee, S. L., Heinemann, S., & Taylor, P. (1982) *J. Biol. Chem. 257*, 12283–12291.
Main, A. R. (1969) *J. Biol. Chem. 244*, 829–840.
Main, A. R. (1974) in *Analysis in Toxicology* (Haynes, W. J., Ed.) Vol. 4, pp 59–105, Academic Press, New York.
Massoulié, J., & Bon, S. (1982) *Annu. Rev. Neurosci. 5*, 57–106.
Monod, J., Wyman, J., & Changeux, J. P. (1965) *J. Mol. Biol. 12*, 88–118.
Ott, P., & Brodbeck, U. (1978) *Eur. J. Biochem. 88*, 119–125.
Radic, Z., Reiner, E. & Simeon, V. (1984) *Biochem. Pharmacol. 33*, 671–678.
Rieger, F., & Vigny, M. (1976) *J. Neurochem. 27*, 121–129.
Rosenberry, T. L. (1975) *Adv. Enzymol. 36*, 103–218.
Roufogalis, B. D., & Wickson, V. N. (1975) *Mol. Pharmacol. 11*, 352–360.
Taylor, P., & Jacobs, N. M. (1974) *Mol. Pharmacol. 10*, 93–107.
Taylor, P., & Lappi, S. (1975) *Biochemistry 14*, 1989–1997.
Thomas, D., & Broun, G. (1976) *Methods Enzymol. 44*, 901–929.
Vigny, M., Koenig, J., & Rieger, F. (1976) *J. Neurochem. 27*, 1347–1353.
Vigny, M., Bon, S., Massoulié, J., & Leterrier, F. (1978a) *Eur. J. Biochem. 85*, 317–323.
Vigny, M., Gisiger, V., & Massoulié, J. (1978b) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2588–2592.
Wombacher, H., & Wolf, H. U. (1971) *Mol. Pharmacol. 7*, 554–566.
Yielding, L. W., Yielding, K. L., & Donoghue, J. E. (1984) *Biopolymers 23*, 83–110.