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Ligand Exclusion on Acetylcholinesterase†

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ABSTRACT: This paper examines covalent reactivity of AchE with respect to *cationic* and *uncharged* methylphosphonates and substrates in the absence and presence of cationic ligands selective for the active center and the peripheral anionic site. The organophosphorus inhibitors are enantiomeric alkyl methylphosphonothioates (**1–5**) containing cycloheptyl and isopropyl phosphono ester groups and *S*-methyl, *S*-*n*-pentyl, and *S*-[β-(trimethylammonio)ethyl] leaving groups; these agents differ in their configuration about phosphorus and their steric, hydrophobic, and electrostatic characteristics. The synthetic substrates examined are acetylthiocholine, *p*-nitrophenyl acetate, and 7-acetoxy-4-methylcoumarin (7AMC). Antagonism of the methylphosphonothioate reaction by cationic ligands is strongly dependent on the nature of both the cation and the methylphosphonate but independent of the configuration about phosphorus. While all cations cause *linear* mixed inhibition of acetylthiocholine hydrolysis, there are observed a variety of inhibition patterns of 7AMC and *p*-nitrophenyl acetate hydrolysis that are distinctly *nonlinear*, as well as patterns in which the reciprocal plots intersect in the upper right quadrant. Strong antagonism of cationic (methylphosphonyl)thiocholines correlates very well with linear inhibition of acetylthiocholine. Ligands that cause only negligible antagonism of the uncharged methylphosphonates display nonlinear inhibition of uncharged substrates. These relationships, since they are most pronounced for peripheral site ligands and are strongly dependent on the charge carried by the reactant, suggest that the peripheral anionic site alters enzyme reactivity through an electrostatic interaction with the net negative active center. Such behavior indicates a potential role for the peripheral anionic site in conserving AchE catalytic efficiency within a narrow range of values.

Acetylcholinesterase (AchE)[1] plays a central role in neuromuscular transmission by hydrolyzing acetylcholine released following depolarization of the presynaptic nerve terminal. The enzyme exists in nerve and muscle as a polymorphic family of molecular forms associated with the basal lamina and plasma membranes (Rotundo, 1987; Toutant & Massoulie, 1988). Since inhibition of AchE leads initially to a prolongation of end-plate currents and eventually to blockade of neuromuscular transmission (Katz & Miledi, 1973; Hartzell et al., 1975), removal of Ach^+ is essential to efficient neuromuscular transmission.

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[1] Abbreviations: AchE, acetylcholinesterase; CPM, *N*-[4-[7-(diethylamino)-4-methylcoumarin-3-yl]phenyl]maleimide; NMA, *N*-methylacridinium; decyl-TMA, decyltrimethylammonium; hexyl-TMA, hexyltrimethylammonium; PTMA, phenyltrimethylammonium; 7AMC, 7-acetoxy-4-methylcoumarin; 7HMC, 7-hydroxy-4-methylcoumarin; M7C, *N*-methyl-7-[(dimethylcarbamoyl)oxy]quinolinium iodide; $AcSch^+$, acetylthiocholine; Ach^+, acetylcholine.

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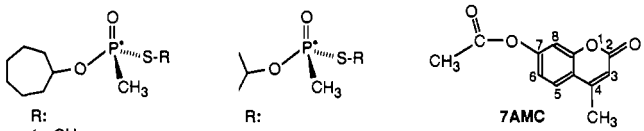
Hydrolysis of Ach^+ by AchE occurs with bimolecular rates of $k_{cat}/K_M \approx 10^9$ M^{-1} min^{-1}, the highest of any enzyme yet studied (Rosenberry, 1975; Quinn, 1987). This high catalytic efficiency is attributable in part to the bipartite structure of the active center, containing elements that subserve substrate recognition separate from those involved in covalent catalysis. One such element is the anionic subsite, a net negative locus at which a variety of cationic ligands are known to bind. This anionic site exists in proximity with an esteratic region containing a nucleophilic residue, serine-200 (Schumacher et al., 1986), that undergoes covalent reaction with esters of acetic acid, carbamic acid, sulfonic acid, and phosphoric and phosphonic acids. Recent studies employing resolved enantiomeric methylphosphonothioates have revealed a more complex arrangement in that the active center contains, in addition to anionic and esteratic regions, a predominantly hydrophobic alkyl-binding region situated within 5 Å of the nucleophilic serine (Berman & Leonard, 1989; Berman & Decker, 1989). Association of uncharged methylphosphonates within this region promotes irreversible inhibition of the enzyme and may provide an alternative orientation for hydrolysis of uncharged substrates. In line with this finding are observations that AchE displays a capacity to hydrolyze uncharged synthetic substrates (Hasan et al., 1980, 1981) and to associate with uncharged ligands (Hasan et al., 1981; Cohen et al., 1982, 1989; Coleman et al., 1987).

This paper examines cation antagonism of covalent reactions within the AchE active center. The covalent reactions examined are inhibition by cationic and uncharged methylphosphonates and hydrolysis of cationic and uncharged substrates. The organophosphorus inhibitors are enantiomeric cycloheptyl methylphosphonothioates containing *S*-methyl, *S*-*n*-pentyl, and *S*-[β-(trimethylammonio)ethyl] leaving groups (**1**–**3**) and isopropyl methylphosphonothioates containing *S*-*n*-pentyl and *S*-[β-(trimethylammonio)ethyl] leaving groups (**4** and **5**). These enantiomeric probes contain cycloheptyl and isopropyl phosphono ester moieties and thioic leaving groups (*SR'*) that differ in their steric, hydrophobic, and electrostatic characteristics. The *S*-methyl and *S*-*n*-pentyl leaving groups engender different degrees of steric interaction with the active center and differ from the thiocholine leaving group in their lack of a positive charge. Similarly, the steric and hydrophobic characteristics of the cycloheptyl group differ from those of the isopropyl group. The synthetic substrates examined are acetylthiocholine ($AcSch^+$), *p*-nitrophenyl acetate, and 7-acetoxy-4-methylcoumarin (7AMC). Hydrolysis of 7AMC produces 7-hydroxy-4-methylcoumarin (7HMC), which is highly fluorescent and therefore readily measurable.



Covalent reaction of these substrates and irreversible inhibitors is monitored in the presence and absence of cationic ligands selective for the active center and peripheral anionic sites. Edrophonium, phenyltrimethylammonium (PTMA), and *N*-methylacridinium (NMA) are aromatic cations representative of active-center-selective monoquaternary ligands. Propidium and *d*-tubocurarine are bisquaternary cations that associate exclusively at the peripheral anionic site, a topographically distinct site more than 20 Å removed from the active center that exerts an allosteric influence on enzyme activity (Taylor & Lappi, 1975; Berman et al., 1980). Decamethonium and decyltrimethylammonium (decyl-TMA) are typical of extended polymethonium bis- and monoquaternary ligands that associate at the active center. Decamethonium is of added interest because association of bisquaternary cations is mutually exclusive with ligand association at both the active center and peripheral anionic sites.

Materials and Methods

Materials. AchE from *Torpedo californica* was isolated by affinity chromatography as described by Taylor et al. (1974). In all experiments reported the enzyme specific activity was in the range 5–7 μmol of Ach^+ hydrolyzed min^{-1} (μg of protein)$^{-1}$. *N*-[4-[7-(Diethylamino)-4-methylcoumarin-3-yl]phenyl]maleimide, *N*-methylacridinium iodide, propidium diiodide, and 7-hydroxy-4-methylcoumarin were obtained from Molecular Probes (Eugene, OR). Edrophonium chloride was a gift from W. E. Scott of Hoffmann-La Roche; hexyltrimethylammonium iodide and decyltrimethylammonium bromide were gifts from David J. Triggle. Hexamethonium bromide was obtained from K & K Chemicals/ICN (Plainview, NY). All other reagents were from Sigma Chemical Co. (St. Louis, MO). Phenyltrimethylammonium and decamethonium were present as bromide salts; *d*-tubocurarine was present as the chloride salt. All kinetic determinations were carried out at 23 °C in a 0.01 M sodium phosphate buffer, pH 7.0, containing 0.2 M NaCl, except where noted.

7-Acetoxy-4-methylcoumarin, now available as β-methylumbelliferyl acetate (Molecular Probes, Eugene, OR), was synthesized according to Tsuchiya et al. (1982) and recrystallized from ethanol as fine, white needles (mp 143–144 °C). Purity and structure were confirmed by elemental analysis and proton NMR.

All fluorescence measurements were made on a SPEX 212 spectrofluorometer. Changes in absorbance were recorded on a Perkin-Elmer Lambda 3B spectrophotometer connected through a Data Translation 2805 analog-to-digital converter resident in an IBM PC computer; data acquisition was controlled with LabTech Notebook (Laboratory Technologies, Cambridge, MA).

Determination of Inhibition Rate Constants in the Absence and Presence of Cationic Ligands. Covalent inhibition of AchE by organophosphonates was assayed by measuring residual activity through an adaptation of the method of Parvari et al. (1983). Methylphosphonothioate inhibition of AchE was initiated in the absence or presence of cationic ligand in a 0.01 M sodium phosphate buffer, pH 7.0, containing 0.2 M NaCl. Aliquots (5 μL) of the inhibition mixture were removed at different times following initiation of the reaction, added to 1.5 mL of a 0.1 M sodium phosphate buffer, pH 7.0, containing acetylthiocholine (2×10^{-4} M), and allowed to incubate for 30 min. The 300-fold dilution served to cause dissociation of the cationic ligand and to stop inhibition by the methylphosphonate, while the indicated incubation period was found to be optimal for amplifying the presence of active enzyme. An aliquot (100 μL) of the amplification mixture was added with thorough mixing to an equal volume of isopropyl alcohol containing *N*-[4-[7-(diethylamino)-4-methylcoumarin-3-yl]-phenyl]maleimide (CPM; 1×10^{-4} M) and diluted with 3.0 mL of H_2O. This step served to denature AchE, quenching the reaction between enzyme and $AcSch^+$, and to generate the fluorescence signal. Fluorescence at 480 nm upon excitation at 390 nm was read within 15 min of quenching. Baseline fluorescence in the absence of enzyme activity was estimated by measuring the fluorescence intensity in a separate cuvette

containing all materials except enzyme; this served also to correct for any spontaneous hydrolysis of CPM that occurred after quenching of the reaction but before measurement of fluorescence. Fluorescence values were plotted against the time at which the aliquot was removed from the original inhibition mixture.

Enzyme was present at an active-site concentration of (0.8–3) × 10^{-9} M. The methylphosphonothioates were present at concentrations of $K_D/4$, where K_D denotes the dissociation constant derived from kinetic assay (Berman & Leonard, 1989). All ligands were present at concentrations 10–20-fold their dissociation constants. Inhibition by uncharged inhibitors was determined at 23 °C; the phosphonylthiocholines were determined at 4 °C to slow the inhibition to measurable rates. The observed rate constants (k_{obs}, min^{-1}) were related to the apparent bimolecular inhibition constant by the relationship k_i (M^{-1} min^{-1}) = k_{obs}/[methylphosphonate].

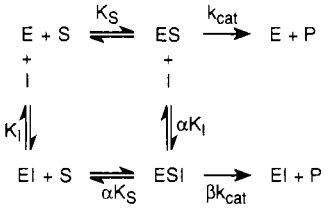
Kinetics of Reversible Inhibition. Enzymatic hydrolysis of $AcSch^+$ in the presence of DTNB (3.3 × 10^{-4} M) and the presence or absence of cationic ligands was measured by monitoring absorbance at 412 nm as a function of time (Ellman et al., 1961). For each determination spontaneous breakdown of substrate was accounted for in a reference cuvette containing all materials except enzyme. Reaction velocities were calculated as the change in absorbance per unit time. Liberation of thiocholine was quantitated by employing an extinction coefficient for the thionitrobenzoate dianion of 14 150 M^{-1} cm^{-1} (Riddles et al., 1979). Substrate concentration was varied over the range (0.2–3.2) × 10^{-4} M, where the K_S value was determined to be (5.6 ± 0.4) × 10^{-5} M. Hydrolysis of a single concentration of *p*-nitrophenyl acetate (1 mM) by AchE was carried out by monitoring absorbance of the *p*-nitrophenylate anion at 405 nm.

Enzymatic hydrolysis of 7AMC was measured by monitoring fluorescence of the product 7HMC at 450 nm upon excitation at 360 nm. This excitation wavelength was chosen because measurement of hydrolysis upon excitation at 325 nm, the absorption maximum for 7HMC, engendered substantial inner filter effects due to absorption by 7AMC. These inner filter effects were evident as leftward shifts of the double-reciprocal plots to lower apparent values of K_S and V_{MAX}. Rates of spontaneous hydrolysis were subtracted from rates obtained in the presence of enzyme and amounted to less than 1% of the enzymatic rate. In all cases the evolution of fluorescence was linear, and the reaction velocities were calculated as the change in fluorescence per unit time. Substrate concentration was varied over the range (0.75–6.0) × 10^{-4} M, the upper limit being governed by solubility; K_S was determined to be (5.6 ± 0.2) × 10^{-4} M. The reaction medium was identical with that described above but contained a small amount of acetonitrile (<0.5%), which caused no discernible loss of enzyme activity.

Emission of NMA (λ^{em}_{MAX} = 487 nm) when measuring inhibition of 7AMC was eliminated by monitoring 7HMC at 430 nm. In the determinations of inhibition by propidium, activity of 7AMC was corrected for quenching due to absorbance by propidium (λ^{abs}_{MAX} = 490 nm). The correction factor, the ratio of the fluorescence intensity of a fixed concentration of 7HMC (0.2 μM) in the presence and absence of the concentrations of propidium employed in the kinetic assays, was in the range 1.0–1.12.

Noncovalent Inhibition of AchE. A general form for equilibrium reversible inhibition is described by Scheme I, where E, S, and ES denote free enzyme, free substrate, and enzyme–substrate complex, respectively. ES undergoes covalent reaction to regenerate free enzyme and product, P. In the presence of inhibitor, I, two noncovalent complexes can form, the enzyme–inhibitor complex, EI, and a ternary complex, ESI, containing enzyme, substrate, and inhibitor. K_S and K_I refer, respectively, to the substrate and inhibitor dissociation constants. This scheme ignores formation of any covalent intermediates that occur after formation of ES and ESI but before production of P.

Scheme I



The reciprocal form of the velocity equation for enzyme inhibition that satisfies the above scheme is given in eq 1, where $[E_0]$ refers to the initial enzyme concentration and V_{MAX} = $k_{cat}[E_0]$.

$$1/V = (K_S/V_{MAX})\{(1 + ([I]/K_I))/(1 + (\beta[I]/\alpha K_I))\} \times (1/[S]) + 1/V_{MAX}\{(1 + ([I]/\alpha K_I))/(1 + (\beta[I]/\alpha K_I))\} \quad (1)$$

When $\beta = 0$, ESI is nonproductive and *linear inhibition* is obtained; in this case, the reaction velocity can be driven to zero with increasing concentrations of I. When $\alpha \gg 1$, formation of the ternary complex ESI is negligible, and the above scheme reduces to competitive inhibition. In all cases for which $\alpha \geq 1$, the slope versus [I] replots are linear and intersect the [I]-axis at a value equal to $-K_I$. In pure noncompetitive ($\alpha = 1$) and mixed inhibition ($\alpha > 1$), the *y*-intercept versus [I] replots also are linear and intersect the [I]-axis at a value equal to $-\alpha K_I$.

When $\beta > 0$, both ES and ESI are productive and *nonlinear inhibition* is obtained; in this case, increasing concentrations of inhibitor do not drive the reaction velocity to zero. When $\alpha > 1$ and $\beta = 1$, ES and ESI yield product with equal facility and the reciprocal form of the velocity equation reduces to competitive inhibition, in that increasing concentrations of inhibitor lead to increases in K_S without alteration in V_{MAX}. When $\alpha = 1$ and $\beta < 1$, ES yields product more readily than ESI and the reciprocal form of the velocity equation reduces to noncompetitive inhibition, in that increasing concentrations of inhibitor lead to reductions in V_{MAX} without alteration in K_S. In both cases slopes of the reciprocal plots approach a finite limiting value with increasing concentration of I as indicated by curvature of the slope versus [I] replots. These patterns are termed *nonlinear* (or *hyperbolic*) *competitive* and *nonlinear* (or *hyperbolic*) *noncompetitive* inhibition. Inhibition constants for nonlinear inhibition can be obtained from plots of $1/\Delta$ slope versus $1/[I]$ and $1/\Delta y$-intercept versus $1/[I]$, where Δ slope and Δy-intercept refer to the change in slope and *y*-intercept, as described by Segel (1975).

Results

Cation Antagonism of Irreversible Inhibition of AchE by Enantiomeric Methylphosphonothioates. Inhibition of AchE by five structurally related enantiomeric methylphosphonothioates, **1–5**, was examined in the presence and absence of cationic ligands selective for the active center and peripheral anionic site. Residual enzyme activity remaining after initiation of inhibition was assessed through measurement of fluorescence of CPM, a thiol-selective reagent that reacts

Table I: Inhibition Rate Constants for Reaction of Enantiomeric Methylphosphonothioates with Acetylcholinesterase in the Presence and Absence of Reversible Cationic Ligands[a]

	Cycloheptyl Methylphosphonothioates					
	CHMP-SMe		CHMP-S*n*Pe		CHMP-thiocholine	
ligand	S_P	R_P	S_P	R_P	S_P	R_P
none						
k_{obs}	2.6 ± 0.1	0.25 ± 0.02	7.4 ± 0.2	0.055 ± 0.003	1.9 ± 0.05	0.73 ± 0.03
k_i	1.3×10^5	640	2.1×10^5	190	2.2 ± 10^7	7.7×10^4
decamethonium						
k_{obs}	2.3 ± 0.1	0.16 ± 0.002	0.79 ± 0.04	0.017 ± 0.001	0.076 ± 0.004	0.037 ± 0.0005
C/L	1.1	1.5	9.4	3.2	25	20
decyltrimethylammonium						
k_{obs}	7.1 ± 0.4	0.60 ± 0.03	6.2 ± 0.2	0.085 ± 0.004	0.24 ± 0.01	0.11 ± 0.002
C/L	0.37	0.42	1.2	0.64	7.8	7.0
edrophonium						
k_{obs}	0.14 ± 0.007	0.015 ± 0.0004	0.52 ± 0.01	0.010 ± 0.002	0.048 ± 0.003	0.021 ± 0.0007
C/L	19	17	14	5.5	39	35
phenyltrimethylammonium						
k_{obs}	0.27 ± 0.02	0.057 ± 0.004	0.92 ± 0.02	0.017 ± 0.0002	0.12 ± 0.002	0.053 ± 0.001
C/L	9.6	4.4	8.0	3.2	15	14
N-methylacridinium						
k_{obs}	0.19 ± 0.004	0.04 ± 0.006				
C/L	13	6.1				
propidium						
k_{obs}	1.6 ± 0.07	0.11 ± 0.02	3.6 ± 0.2	0.045 ± 0.006	0.055 ± 0.004	0.034 ● 0.001
C/L	1.6	2.3	2.1	1.2	34	21
d-tubocurarine						
k_{obs}	1.5 ± 0.1	0.17 ± 0.005	9.0 ± 0.3	0.098 ± 0.004	0.21 ± 0.01	0.10 ± 0.001
C/L	1.7	1.5	0.82	0.6	8.8	7.1

	Isopropyl Methylphosphonothioates			
	iPrMP-S*n*Pe		iPrMP-thiocholine	
ligand	S_P	R_P	S_P	R_P
none				
k_{obs}	0.45 ± 0.02	0.68 ± 0.08	2.5 ± 0.12	0.16 ± 0.005
k_i	1.0×10^3	1.3×10^3	1.1×10^6	5.9×10^3
decamethonium				
k_{obs}	0.051 ± 0.009	0.082 ± 0.002	0.096 ± 0.004	0.0098 ± 0.0002
C/L	8.8	8.3	26	16
edrophonium				
k_{obs}	0.046 ± 0.002	0.072 ± 0.0006	0.071 ± 0.006	0.0079 ± 0.0001
C/L	9.8	9.5	35	20
propidium				
k_{obs}	0.27 ± 0.03	0.14 ± 0.009	0.16 ± 0.006	0.024 ± 0.0004
C/L	1.7	4.9	15	6.8

[a] The reaction medium was a 0.01 M sodium phosphate buffer, pH 7.0, containing 0.2 M NaCl. The observed inhibition rate constant, k_{obs} (min^{-1}), was obtained with the methylphosphonothioate present in at least a 40-fold excess over the enzyme subunit normality and at concentrations 0.25 times the dissociation constant ($K_D/4$):(S_P)-CHMP-SMe (2.0×10^{-5} M), (R_P)-CHMP-SMe [($3.7–4.0$) $\times 10^{-4}$ M], (S_P)-CHMP-S*n*Pe (3.6×10^{-5} M), (S_P)-CHMP-thiocholine (8.6×10^{-8} M), (R_P)-CHMP-thiocholine (9.5×10^{-6} M), (S_P)-iPrMP-S*n*Pe (4.4×10^{-4} M), (R_P)-iPrMP-S*n*Pe (5.1×10^{-4} M), (S_P)-iPrMP-thiocholine (2.3×10^{-6} M), (R_P)-iPrMP-thiocholine (2.7×10^{-5} M). (R_P)-CHMP-S*n*Pe, for which no K_D value is available, was present at a concentration of 2.9×10^{-4} M; this value was approximately 0.25 times the K_D determined from a limited examination of the concentration dependence of inhibition and was limited by solubility. Propidium and *d*-tubocurarine were present at concentrations 10 times their dissociation or inhibition constants. All other cationic ligands were present at approximately 20 times their dissociation constants. The dissociation constants for decamethonium (1.2×10^{-6} M), decyl-TMA (1.7×10^{-5} M), edrophonium (2×10^{-7} M), PTMA (1.6×10^{-5} M), NMA (1.2×10^{-7} M), propidium (3×10^{-6} M), and *d*-tubocurarine (5.0×10^{-5} M) come from Berman and Decker (1986b), Taylor and Lappi (1975), and Berman et al. (1981). The value of k_{obs} is the average ± SEM for 2–16 independent determinations. The apparent bimolecular inhibition constant, k_i (M^{-1} min^{-1}), was derived as k_{obs}/[methylphosphonothioate]. C/L denotes the ratio of k_{obs} in the absence (C, control) and presence (L, ligand) of noncovalent cationic ligands.

quantitatively with thiocholine liberated upon hydrolysis of $AcSch^+$ by AchE (Parvari et al., 1983). Inhibition constants for inactivation of AchE by (R_P)- and (S_P)-CHMP-SMe (**1**), CHMP-S*n*Pe (**2**), and CHMP-thiocholine (**3**) in the absence and presence of different cationic ligands are presented in Table I, along with results for enantiomeric isopropyl methylphosphonothioates (**4** and **5**). In all cases, inhibition displayed exponential behavior and could be monitored from as early as 3 s out to several hours following initiation of reaction. In the absence of cationic ligand, bimolecular inhibition constants for the uncharged methylphosphonothioates (**1**, **2**, and **4**) determined at a single concentration ($K_D/4$) were in excellent agreement with values derived from detailed examination of their concentration dependences (Berman & Leonard, 1989). Bimolecular inhibition constants for the [(cycloheptyloxy)- (**3**) and [(isopropyloxy)methylphosphonyl]thiocholines (**5**) determined at 4 °C were approximately 10-fold slower than the rates determined at 23 °C and were within 2-3-fold of those predicted for a 20-deg reduction in temperature. In all cases the respective chiral preferences of 220, 1000, 220, 0.67, and 190 for **1**–**5** were in excellent agreement with values determined previously (Berman & Leonard, 1989).

When the different organophosphonates were present at equal fractions of their dissociation constants ($K_D/4$), leading to equal degrees of occupation, the observed rate constant (k_{obs}) reflected the unimolecular phosphonylation constant (k_p). For the above agents, the chiral preferences expressed as the ratio of k_{obs} for the S_P and R_P enantiomers (**1** 12; **3**, 2.6; **4**, 0.6; **5**, 16.5) were in excellent agreement with those determined from

Table II: Reversible Inhibition by Cationic Ligands of Acetylcholinesterase Hydrolysis of $AcSch^+$ and 7AMC[a]

ligand	$AcSch^+$		7AMC	
	pattern of inhib (α)	10^6K_I (M)	pattern of inhib	10^6K_I (M)
edrophonium	linear mixed (7.1)	0.15	linear competitive	0.14
phenyltrimethylammonium	linear mixed (5.9)	20.0	linear competitive	17
N-methylacridinium	linear mixed (1.9)	0.046	linear competitive	0.11
decamethonium	linear mixed (3.2)	0.56	*b*	0.87
hexamethonium	linear mixed (4.2)	13	*b*	30
decyltrimethylammonium	linear mixed (8.4)	34	*b*	33
hexyltrimethylammonium	linear mixed (3.3)	142	*b*	200
propidium	linear mixed (1.6)	1.1	nonlinear competitive	1.2
d-tubocurarine	linear mixed (3.4)	40	nonlinear noncompetitive	85

[a] Inhibition was determined in a 0.01 M sodium phosphate buffer, pH 7.0, containing 0.2 M NaCl. $AcSch^+$ and 7AMC hydrolysis products were monitored as described under Materials and Methods. [b] The intersection point occurred in the upper right quadrant. For decamethonium, hexamethonium, and hexyl-TMA slope replots were linear; the values reported for K_I represent the [I]-intercept of these plots. For decyl-TMA the slope replots were nonlinear; the value for K_I was obtained from analysis of $1/\Delta$ slope versus $1/[I]$.

analysis of k_p (Berman & Leonard, 1989). Considered in light of the multiple steps in this procedure, comprising separate stages of inhibition, amplification, and signal development (see Materials and Methods), the agreement with previously reported rate constants, some of which approached the diffusion limit and required monitoring with stopped-flow techniques, was remarkable and indicated the suitability of this procedure for evaluating cation antagonism of irreversible inhibition by organophosphonates.

Inhibition of AchE by uncharged cycloheptyl methylphosphonothioates (**1** and **2**) was slowed in the presence of cationic ligands. The noteworthy finding was that the extent of slowing varied with both the individual cationic ligand and the nature of the methylphosphonothioate. Edrophonium and PTMA, active-center-selective aromatic cations, slowed irreversible inhibition of AchE by **1** and **2** by 10–20-fold. *N*-Methylacridinium showed similar behavior with respect to reaction of **1**. The presence of decamethonium had no significant effect on irreversible inhibition by **1** but slowed inhibition by the *n*-pentyl homologue **2** approximately 9-fold. Decyl-TMA caused slight acceleration of irreversible inhibition by **1** and showed no significant slowing of inhibition by **2**. Reaction of **1** and **2** was essentially unaffected by the peripheral anionic site ligands propidium and *d*-tubocurarine. Overall, aromatic active-center-selective cations slowed reaction of all uncharged methylphosphonothioates; with the exception of slowing of **2** by decamethonium, all other ligands did not appreciably antagonize inhibition by the uncharged agents.

All noncovalent cationic ligands examined slowed reaction of the [(cycloheptyloxy)methylphosphonyl]thiocholines (**3**). The reaction rates were generally at least 15-fold slower than the rates observed in the absence of ligand. The magnitude of this antagonism was far greater than that observed for the uncharged agents **1** and **2**. For all enantiomeric cycloheptyl methylphosphonothioates, cation antagonism of irreversible reaction displayed no marked chiral preference; antagonism of the R_P enantiomers was within 2–3-fold that of the S_P enantiomers (Table I).

These results were not remarkably different for the isopropyl methylphosphonothioates (**4** and **5**). Decamethonium and edrophonium exerted nearly equal effects on irreversible inhibition of AchE by (R_P)- and (S_P)-*i*PrMP-S*n*Pe (**4**), slowing it 9–10-fold. While propidium caused no significant reduction in reaction rate of the S_P enantiomer, the reduction in reaction rate of the R_P enantiomer was slightly greater. This slight reversal of chiral preference for enantiomeric isopropyl *S*-*n*-pentyl methylphosphonothioates in the presence of cationic ligand was notable since it was observed also in the absence of ligand and was similar to that observed previously in a more detailed examination of the concentration dependence of inhibition (Berman & Leonard, 1989). Edrophonium, decamethonium, and propidium antagonized irreversible inhibition by [(isopropyloxy)methylphosphonyl]thiocholine (**5**); these cations generally slowed inhibition by at least 15-fold. As seen for the cycloheptyl methylphosphonothioates, antagonism of irreversible inhibition by enantiomeric *i*PrMP-thiocholines exceeded that of the uncharged agents and displayed no significant chiral preference.

Reversible Noncovalent Inhibition of AchE Hydrolysis of Acetylthiocholine. Noncovalent inhibition was examined by measuring the capacity of cationic ligands to inhibit AchE hydrolysis of acetylthiocholine (Table II). All ligands displayed *linear mixed* patterns of inhibition; this was indicated by double-reciprocal plots that intersected in the upper left quadrant and slope versus [I] replots that were linear. Inhibition constants, K_I, derived from analysis of slope versus [I] replots, were in close agreement with the respective dissociation constants. *y*-Intercept replots afforded estimates of αK_I, where α measures the difference in the inhibitor affinity for free enzyme and enzyme–substrate complex. On the basis of Scheme I, the higher values of α seen for edrophonium, PTMA, and decyl-TMA indicated that these ligands display a higher affinity for free enzyme than for the Michaelis complex; the lower values of α seen for NMA, decamethonium, hexamethonium, hexyl-TMA, propidium, and *d*-tubocurarine indicated that these ligands show comparable affinity for both species.

Reversible Noncovalent Inhibition of AchE Hydrolysis of Uncharged Substrates. Reciprocal plots for inhibition of AchE hydrolysis of 7AMC by PTMA, propidium, and decamethonium displayed clear differences from those observed for $AcSch^+$ as substrate. PTMA was a *linear competitive* inhibitor of 7AMC hydrolysis (Figure 1A). A similar pattern was observed for edrophonium and NMA (Table II). Inhibition by propidium was competitive (Figure 1B), in strong contrast to the nearly noncompetitive behavior against $AcSch^+$, but the slope versus [I] replot was hyperbolic rather than linear. The value for K_I from plots of $1/\Delta$ slope versus $1/[I]$ was calculated to be 1.2×10^{-6} M ($\alpha = 6.0$; $\beta = 1$), a value in reasonable agreement with the known dissociation constant (Taylor & Lappi, 1975) and the inhibition constant determined against $AcSch^+$. *d*-Tubocurarine also displayed nonlinear behavior, but in this case a nonlinear noncompetitive pattern of inhibition was obtained. The calculated value of K_I (Table II) was in close agreement with its published value determined against Ach^+ and $AcSch^+$ (Mooser & Sigman, 1974), with the dissociation constant, K_D (Berman et al., 1981), and with the value determined against $AcSch^+$. While propidium and *d*-tubocurarine showed nonlinear behavior over a concentration

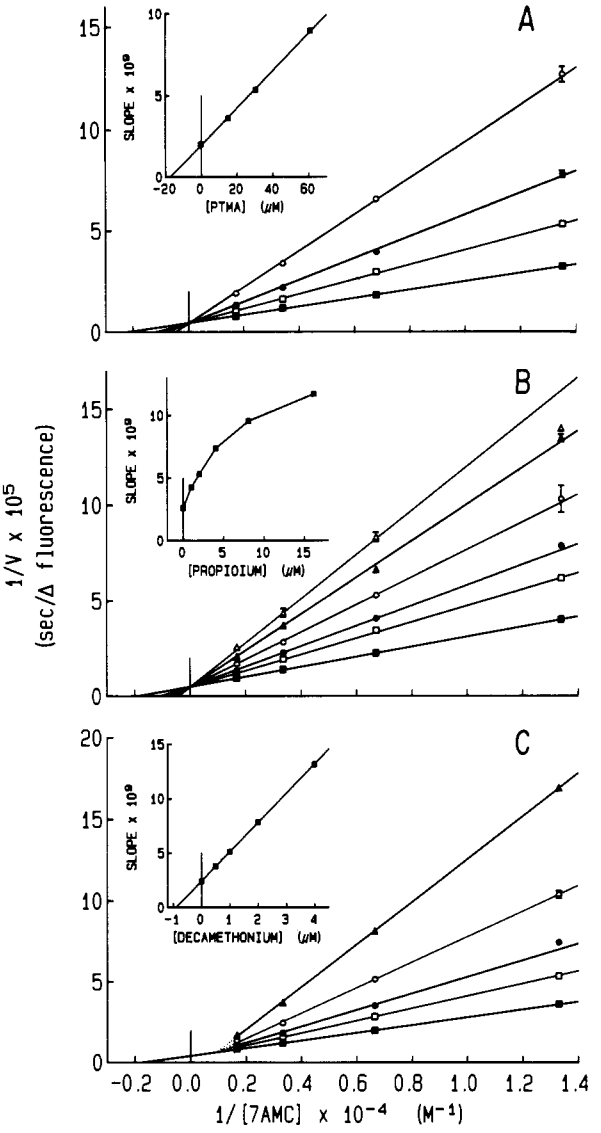


FIGURE 1: Double-reciprocal plots of reaction velocity versus 7-acetoxy-4-methylcoumarin concentration in the presence and absence of PTMA (A), propidium (B), and decamethonium (C). Panel A: (■) no PTMA; (□) 15.2 μM; (●) 30.5 μM; (○) 60.9 μM. Panel B: (■) no propidium; (□) 1.01 μM; (●) 2.02 μM; (○) 4.05 μM; (▲) 8.09 μM; (△) 16.2 μM. Panel C: (■) no decamethonium; (□) 0.50 μM; (●) 1.00 μM; (○) 1.99 μM; (▲) 3.99 μM. The value for k_{cat} was calculated to be $(1.5 \pm 0.1) \times 10^4$ min^{-1} ($n = 3$). For this calculation, the number of equivalents of 7HMC liberated upon hydrolysis was obtained by comparison of the fluorescence value from the reciprocal of the y-intercept with the intensities of known concentrations of 7HMC. The value for K_S was $(5.6 \pm 0.2) \times 10^{-4}$ M ($n = 17$). The insets present replots of the reciprocal plot slopes versus inhibitor concentration. For PTMA, the x-intercept of the replot affords an inhibition constant of 17 μM. For propidium, analysis of a $1/\Delta$ slope versus $1/[I]$ plot (not shown) affords an inhibition constant of 1.2 μM. For decamethonium, replot of the slopes is linear, suggestive of linear inhibition; the x-intercept affords an inhibition constant of 0.87 μM. Enzyme active center normality was in the range (1–2) $\times 10^{-9}$ N.

range spanning 6-fold their known dissociation constants, the reaction velocity in both cases was reduced no more than 2–4-fold.

The intersection point for decamethonium occurred in the upper right quadrant (Figure 1C). Similar behavior was seen for all other n-alkyl mono- and bisquaternary cations examined (Table II). This pattern was not attributable to accelerated spontaneous hydrolysis of 7AMC, since hydrolysis was unaltered in the presence of these ligands. Fluorescence emission spectra of the hydrolysis product 7HMC were unchanged by

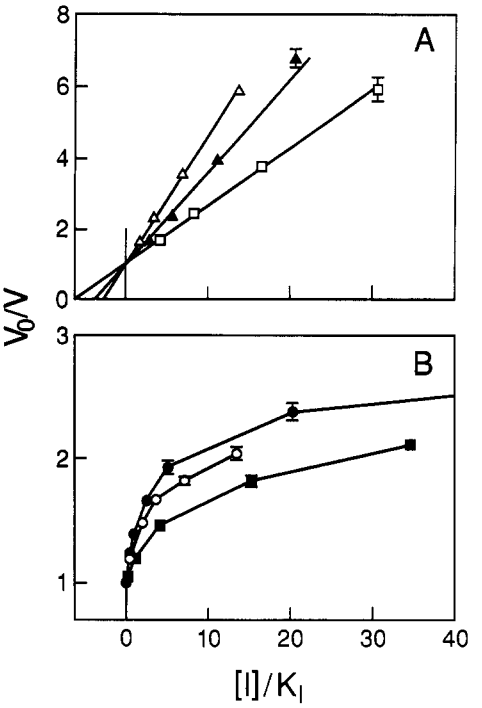
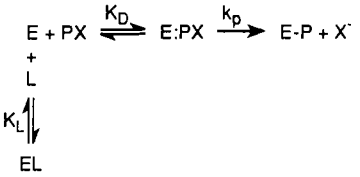


FIGURE 2: Inhibition of p-nitrophenyl acetate hydrolysis by cationic ligands. Velocities of AchE-catalyzed hydrolysis of p-nitrophenyl acetate (1 mM) were assayed by monitoring generation of the p-nitrophenylate ion at 405 nm. The y-axis represents the ratio of the reaction velocity in the absence (V_0) and presence (V) of inhibitor. The x-axis represents the ratio of the inhibitor concentration and the inhibition constant (K_I) versus AcSch$^+$ (from Table II). Panel A: (△) PTMA; (▲) edrophonium; (□) decamethonium. Panel B: (●) propidium; (○) d-tubocurarine; (■) decyl-TMA. Enzyme active center normality was 2×10^{-9} N.

these ligands, indicating that these long-chain alkyl ligands caused no sequestration of substrate or hydrolysis product in a clathrate cavity of the ligand. As shown in Figure 1C, the slope replot for decamethonium was linear, affording an apparent value for K_I of 0.87×10^{-6} M. This value was in close agreement with the value determined from decamethonium inhibition of AcSch$^+$ hydrolysis (Table II) and also with the equilibrium dissociation constant (Berman & Decker, 1986b). The upper range of substrate concentrations employed was limited by solubility, and reaction velocities at concentrations appropriate for approaching the intersection point could not be examined. However, reciprocal plots for inhibition by n-alkyl mono- and bisquaternary ligands appeared to converge at a common line representative of that in the absence of inhibitor rather than at a common point on one of the axes. Linear inhibition was observed for hexamethonium and hexyl-TMA, while nonlinear inhibition was observed for decyl-TMA. For all mono- and bisquaternary ammonium ligands the apparent inhibition constants determined against 7AMC were compatible with those seen with AcSch$^+$ as substrate (Table II).

To assess the general nature of these results, linear and nonlinear inhibition was examined by employing p-nitrophenyl acetate as another uncharged substrate. Inhibition of hydrolysis of p-nitrophenyl acetate, present at a single concentration (1×10^{-3} M), was monitored in the presence of different concentrations of ligand (Figure 2). Linear inhibition was observed for PTMA, edrophonium, and decamethonium (Figure 2A), and the x-intercepts were within 3–6-fold of the K_I with either AcSch$^+$ or 7AMC as substrate (Table II). Inhibition by propidium, d-tubocurarine, and decyl-TMA was markedly nonlinear (Figure 2B). These inhibitors, at the

Scheme II



highest concentrations examined, reduced the reaction velocity only 2–3-fold with respect to the activity in the absence of inhibitor (Figure 2B). This behavior was similar to that seen with 7AMC as substrate.

Discussion

Cation Antagonism of Organophosphonate Reactions. Irreversible inhibition of acetylcholinesterase by the enantiomeric methylphosphonothioates can be analyzed with reference to the mechanism in Scheme II in which collision between inhibitor (PX) and enzyme (E) results in formation of a reversible complex (E:PX) prior to covalent reaction and subsequent formation of a phosphonylated enzyme (E-P) concomitant with dissociation of the leaving group (X^-) (Berman & Leonard, 1989).

The equilibrium dissociation constant (K_D, M) and the unimolecular phosphonylation constant (k_p, min^{-1}) are related to each other and to the bimolecular inhibition constant (k_i, M^{-1} min^{-1}) by the relationship $k_i = k_p/K_D$. Binding of the cation, L, is described in terms of its dissociation constant, K_L (M). For mutually exclusive binding at a single class of homogeneous sites, all ligands present at concentrations that afford equal degrees of occupation are predicted to cause equal degrees of antagonism. If mutually exclusive binding is not the case, then different degrees of antagonism for the different ligands are anticipated.

As seen from examination of Table I, the site-selective cationic ligands do not cause comparable degrees of antagonism of the methylphosphonate reaction. This is seen with respect to aromatic active-center-selective cations (edrophonium, PTMA, and NMA), which slow reaction of **1** to a greater degree than do decamethonium and the peripheral site ligands propidium and *d*-tubocurarine. With respect to slowing reaction of **2** and **4** the aromatic active-center-selective cations and decamethonium display nearly equal efficacy. With respect to slowing reaction of the (methylphosphonyl)thiocholines (**3** and **5**), antagonism by all cations is substantial and falls within a 5-fold range of each other.

Similarly, a single ligand does not cause equal antagonism of all methylphosphonates. Decamethonium slows reaction of long-chain (**2** and **4**) but not the short-chain agents (**1**). Decyl-TMA, propidium, and *d*-tubocurarine show only small effects on reactions of **1** and **2** but cause a marked slowing of the (methylphosphonyl)thiocholines (**3** and **5**). Indeed, while all ligands effectively antagonize reaction of the (methylphosphonyl)thiocholines (**3** and **5**), the distinctions among the different ligands are most evident with respect to the kinetic behavior of the uncharged methylphosphonothioates (**1**, **2**, and **4**).

Decamethonium antagonism of **2** more than of **1** indicates a clear steric effect related to the dimensions of the thiol leaving group (SR′). Since edrophonium antagonizes inhibition by methylphosphonothioates containing short- and long-chain leaving groups, the locus for edrophonium association is concluded to exist in proximity of the nucleophilic residue, serine-200, within the esteratic region of the active center (Figure 3). The locus for decamethonium association is concluded to be spatially removed from serine-200 and distal

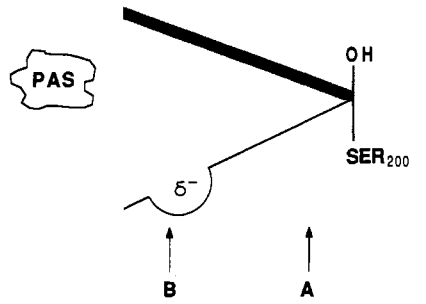


FIGURE 3: Model of ligand binding loci on the acetylcholinesterase subunit. The subunit of AchE contains multiple loci for binding of cationic ligands. The active center, comprising the anionic subsite (δ^-) and the alkyl-binding region (shading), is shown to be topographically distinct from the peripheral anionic site (PAS). Edrophonium and other active-center-selective aromatic cations bind in close proximity to the reactive serine, residue 200, as indicated by A. Decamethonium and other mono- and bisquaternary polymethonium cations associate within the active center but farther from serine-200, as indicated by B. Propidium and *d*-tubocurarine bind at the peripheral anionic site.

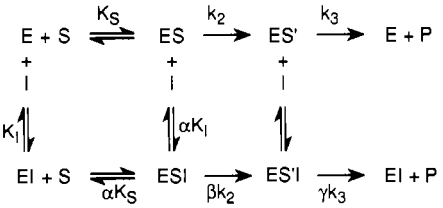
to the edrophonium binding locus. These findings are not compatible with competitive antagonism at a single class of sites and require that *aromatic* ligands associate at a locus separate from that for *n*-alkyl mono- and bisquaternary ligands. These findings therefore provide a kinetic index establishing the presence in the active center of a heterogenous population of cation binding sites and serve to independently substantiate earlier conclusions derived from equilibrium measurements (Berman & Decker, 1986a).

The behavior of decyl-TMA stands out in that this ligand *accelerates* inhibition of **1** 3-fold, exerts virtually no effect on inhibition by **2**, and causes a relatively low degree of antagonism of reaction with **3**. The acceleration is comparable in magnitude with that seen for selected aromatic and *n*-alkyl monoquaternary ligands that are known to accelerate covalent reaction of AchE with acetyl (Metzger and Wilson, 1967) methanesulfonyl (Kitz & Wilson, 1963; Krupka, 1974; Belleau et al., 1970; Belleau & DiTullio, 1970), and dimethylcarbamyl fluorides (Metzger & Wilson, 1963) and uncharged esters of acetic acid (Barnett & Rosenberry, 1977). These results have been explained with reference to formation of a ternary complex of enzyme, cation, and reactant. The present studies extend these earlier studies by separating the steric and electrostatic characteristics of the reactants. A steric component is indicated since only short-chain reactants undergo acceleration; an electrostatic component is indicated since only uncharged reactants are accelerated.

Overall, antagonism of covalent reaction at the active center of AchE is not related only to cation site selectivity but shows a dramatic dependence on the electrostatic nature of the agent undergoing covalent reaction within the active center. This behavior is most pronounced for the peripheral site ligands since they show little capacity to antagonize inhibition by the uncharged methylphosphonates (**1**, **2**, and **4**) but inhibit reaction by the (methylphosphonyl)thiocholines (**3** and **5**) with the same efficacy as seen for the aromatic active-center-selective cations.

In all cases cation antagonism of irreversible inhibition by enantiomeric methylphosphonates shows no remarkable dependence on the initial configuration about phosphorus. This is true also with respect to decyl-TMA acceleration of the reaction, which is observed for both the R_P and S_P enantiomers. These results complement previous findings that ligand association with enantiomeric methylphosphonyl conjugates and oxime reactivation of those conjugates show no dependence

Scheme III



on the initial configuration about phosphorus even though the (R_P)- and (S_P)-methylphosphonyl adducts with AchE display kinetically distinct behavior (Berman & Decker, 1989).

Ligand Exclusion Kinetics. Inhibition of AchE can be analyzed also with reference to an accepted alternative mechanism for AchE catalysis (Scheme III) where the enzyme–substrate complex (ES) undergoes covalent reaction to form a transient acyl intermediate (ES′), which reacts with water to form acetate and free enzyme. In such cases, $k_{cat} = k_2k_3/(k_2 + k_3)$ and depends on the rates of acylation, k_2, and deacylation, k_3. As shown by Krupka and Laidler (1961), apparent noncompetitive inhibition can arise when binding of I with a reaction intermediate (e.g., ES′) alters the velocity of the rate-determining step. For Ach$^+$, AcSch$^+$, and phenyl acetate $k_3 \leq k_2$, and the rate-limiting step is deacylation [Froede & Wilson, 1984; reviewed by Rosenberry (1975) and Quinn (1987)]. An estimated value for k_2/k_3 of 6 is known for turnover of Ach$^+$ by AchE from *Electrophorus* (Wilson & Cabib, 1956; Rosenberry, 1975). For AcSch$^+$, a k_{cat} of (1.9 ± 0.1) × 10^5 min^{-1} leads to a calculated value for k_3 of 2.2 × 10^5 min^{-1}. In this case, noncompetitive inhibition reflects binding of I to the acyl-enzyme ES′, even though formation of EI may be mutally exclusive with formation of ES. This mechanism gains support from observations that noncompetitive and mixed inhibition are not seen for those substrates for which $k_2 < k_3$, that is, when acylation is the rate-limiting step in hydrolysis (Krupka & Laidler, 1961; Wilson & Cabib, 1956). 7AMC falls in this latter category. For 7AMC, the k_{cat} of (1.5 ± 0.1) × 10^4 min^{-1} and the above value of k_3 lead to a k_2 of 1.6 × 10^4 min^{-1}, significantly less than k_3. These estimates are conditional since they are based on turnover of Ach$^+$ with crude enzyme from *Electrophorus* (Wilson & Cabib, 1956); however, the difference in relative magnitude of k_2 and k_3 for AcSch$^+$ compared with 7AMC far exceeds the much smaller differences (<20%) in turnover efficiency between AcSch$^+$ and Ach$^+$ [cf., Rosenberry (1975)].

All ligands examined cause linear mixed inhibition of AcSch$^+$ hydrolysis. This behavior can be attributed to inhibitor association with the acetyl-enzyme, ES′. In contrast, no inhibitor displays mixed inhibition of 7AMC hydrolysis. Indeed, other than the noncompetitive inhibition observed for *d*-tubocurarine, all aromatic inhibitors display competitive inhibition of 7AMC hydrolysis. Since the acetyl-enzymes formed from AcSch$^+$ and 7AMC are identical, the different patterns of inhibition must reflect substrate-specific differences in the relative rates of k_2 and k_3. The absence of mixed inhibition of 7AMC hydrolysis is consistent with $k_2 < k_3$, with the consequence that inhibitor association with the acetyl-enzyme is kinetically silent. Competitive inhibition of 7AMC hydrolysis must therefore reflect inhibitor interactions that occur during the initial substrate binding step. With respect to hydrolysis of AcSch$^+$, for which deacylation is rate determining, mixed inhibition by propidium and *d*-tubocurarine would reflect a noncompetitive reduction in the deacylation rate, k_3. This study therefore provides evidence that occupation of the peripheral anionic site alters hydrolysis of AcSch$^+$ by slowing the rate of deacylation.

Linear inhibition of AcSch$^+$ by peripheral site ligands signifies that the reaction velocity can be driven to zero by sufficiently high inhibitor concentrations. This finding, typical of the inhibition seen for Ach$^+$ (Mooser & Sigman, 1974), indicates that the ternary complex (ESI) formed with a cationic substrate is inactive. *Nonlinear* inhibition of 7AMC and *p*-nitrophenyl acetate by propidium and *d*-tubocurarine signifies that high concentrations of inhibitor do not drive the reaction velocity to zero. Such nonlinear inhibition arises because ES and ESI remain productive and continue to undergo covalent reaction. For propidium, the nonlinear competitive inhibition of 7AMC hydrolysis reflects an increase in K_S ($\alpha > 1$) without effect on V_{MAX}, since product arises from both ES and ESI with equal facility ($\beta = 1$). For *d*-tubocurarine, the nonlinear noncompetitive inhibition reflects a reduction in V_{MAX}, since product arises from ES and ESI but at different rates ($\beta < 1$), without effect on K_S ($\alpha = 1$). These findings demonstrate a distinguishing feature of the effect of peripheral site occupation on AchE reactivity, in that peripheral site occupation blocks acylation by *cationic* but not *uncharged* acetyl ester substrates.

Double-reciprocal plots for hexa- and decamethonium and hexyl- and decyl-TMA inhibition of 7AMC intersect in the upper right quadrant. This behavior is interesting since it is not evident with aromatic cations or with peripheral site ligands and, of the ligands examined, appears only with two structurally related classes of *n*-alkyl mono- and bisquaternary ligands. High substrate concentrations produce a reaction velocity in the presence of inhibitor that approximates the reaction velocity seen in the absence of inhibitor. Similar behavior has been reported for reaction of the cationic carbamate M7C with AchE from *Electrophorus* in buffers of low ionic strength in the presence of peripheral site ligands (Tomlinson et al., 1978, 1980). This behavior is inconsistent with formation of an enzyme–substrate complex containing only a single molecule of substrate and suggests that binding of a second molecule of substrate precludes association of inhibitor. Indeed, coumarins, coumarin-containing organophosphates (Aldridge & Reiner, 1969; Radic et al., 1984), and other aromatic organophosphates (Fibroulet et al., 1990) have been reported to bind at a peripheral site on AchE and to alter reactivity at the active center. While such an explanation may find support for 7AMC, a coumarin-based molecule, it is not entirely satisfactory since it requires that association of 7AMC at the second site occur with an affinity comparable to that for the first site. The presence of a second substrate site for 7AMC is not apparent by kinetic criteria. Caution is necessary in interpreting this pattern, however, since examination of 7AMC hydrolysis at concentrations exceeding K_S is precluded by its limited solubility.

Electrostatic Regulation of Covalently Reactivity. The behavior of peripheral site ligands stands out with respect to the antagonism of cationic and uncharged acetyl esters and methylphosphonates. Linear inhibition of AcSch$^+$ hydrolysis parallels the linear inhibition of Ach$^+$ (Mooser & Sigman, 1974) and the substantial antagonism of (methylphosphonyl)thiocholines (Table I). Nonlinear inhibition of 7AMC hydrolysis is similar to the nonlinear inhibition of *p*-nitrophenyl acetate hydrolysis and parallels the negligible antagonism of uncharged methylphosphonates. These distinctions are significant since they indicate that peripheral site occupation blocks acylation of AchE by *cationic* but not uncharged acetate esters. Since the primary characteristic distinguishing these acetyl esters is the presence or absence

Table III: Influence of Ionic Strength on AchE Hydrolysis of Acetylthiocholine and Irreversible Inhibition by (S_P)-CHMP-thiocholine[a]

Hydrolysis of Acetylthiocholine[b]			
[NaCl]	K_S (μM)	$10^{-5}k_{cat}$ (min^{-1})	$10^{-9}k_{cat}/K_S$ (M^{-1} min^{-1})
0.50	121	3.5	2.9
0.20	56	1.9	3.5
0.10	55	2.3	4.0
0.05	36	1.8	4.9
0.01	24	1.7	7.0
0.0	23	1.2	5.9
Irreversible Inhibition by (S_P)-CHMP-thiocholine[c]			
[NaCl]	K_D (μM)	k_p (min^{-1})	$10^{-8}k_i$ (M^{-1} min^{-1})
0.50	1.1	150	1.4
0.20	0.88	170	2.1
0.10	0.42	140	3.3
0.05	0.49	190	3.9
0.01	0.29	160	5.6
0.0	0.35	140	4.0

[a] Data are taken from Berman and Leonard (1989). [b] The catalytic parameters K_S, k_{cat}, and k_{cat}/K_S refer to the equation

$$E + S \overset{K_S}{\rightleftharpoons} ES \xrightarrow{k_{cat}} E + P$$

[c] The kinetic parameters K_D, k_p, and k_i refer to the equation

$$E + PX \overset{K_D}{\rightleftharpoons} E{:}PX \xrightarrow{k_P} E\text{-}P + X^-$$

of a cationic moiety, it is reasonable to conclude that peripheral site occupation alters covalent reactivity through an electrostatic interaction with the net negative active center.

Noncovalent ligand association at the active center and peripheral anionic site is exquisitely sensitive to changes in ionic strength of the medium, showing increases of 30–100-fold in affinity with modest reductions in ionic strength (Nolte et al., 1980; Taylor & Lappi, 1975; Berman & Decker, 1986b). Cationic substrates, such as Ach$^+$ and AcSch$^+$, in contrast, show relatively modest 2–5-fold changes in K_S and 2–3-fold changes in k_{cat} and k_{cat}/K_S over a wider range of ionic strength as shown in Table III [see also Mooser and Sigman (1974) and Nolte et al. (1980)]. A shallow ionic strength dependence is seen also for irreversible inhibition by (methylphosphonyl)thiocholines (Table III; Berman & Leonard, 1989). These results signify that covalent reactivity of AchE is conserved within a relatively narrow range and is essentially independent of ionic composition of the surrounding medium.

While the presence of a peripheral anionic site on the AchE subunit has been known for nearly a quarter of a century (Changeux, 1966), the function of this site has remained elusive. It is noteworthy that occupation of this site by propidium fails to exclude cationic noncovalent ligands such as NMA or edrophonium from the active center (Taylor & Lappi, 1975; Berman et al., 1981). Taken with the near-constant reactivity of AchE with changes in ionic composition, these selective actions of peripheral site ligands on *cationic, covalent reactants* indicate a potential role for the peripheral anionic site in conserving enzyme reactivity in an inconstant ionic environment. Such a function would be of considerable importance in neuromuscular transmission since an acute dependence of AchE catalysis on ionic composition of the synapse, resulting in large excursions in catalytic efficiency, would be incompatible with the requirement for rapid removal of Ach$^+$ from the synaptic cleft (Kuffler et al., 1984).

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Intrinsic Fluorescence of Binding-Site Fragments of the Nicotinic Acetylcholine Receptor: Perturbations Produced upon Binding α-Bungarotoxin†

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ABSTRACT: Synthetic peptides corresponding to sequences contained within residues 173–204 of the α-subunit in the nicotinic acetylcholine receptor (nAChR) of *Torpedo californica* bind the competitive antagonist α-bungarotoxin (BGTX) with relative high affinity. Since the synthetic peptide fragments of the receptor and BGTX each contain a small number of aromatic residues, intrinsic fluorescence studies were used to investigate their interaction. We examined a number of receptor-derived peptide fragments of increasing length (4–32 amino acids). Changes in the λ_{max} and quantum yield with increasing polypeptide chain length suggest an increase in the hydrophobicity of the tryptophan environment. When selective excitation and subtraction were used to reveal the tyrosine fluorescence of the peptides, a significant red shift in emission was observed and was found to be due to an excited-state tyrosinate. The binding of BGTX to the receptor-derived peptide fragments resulted in a large increase in fluorescence. In addition, at equilibrium, the λ_{max} of tryptophan fluorescence was shifted to shorter wavelengths. The fluorescence enhancement, which was saturable with either peptide or BGTX, was used to determine the dissociation constants for the complexes. At pH 7.4, the apparent K_d for a dodecameric peptide (α185–196), consisting of residues 185–196 in the α-subunit of the nAChR from *Torpedo californica*, was 1.4 μM. The K_d for an 18-mer (α181–198), consisting of residues 181–198 of the *Torpedo* α-subunit, was 0.3 μM. No binding or enhanced fluorescence was observed with an irrelevant synthetic peptide of comparable composition. The enhanced fluorescence upon binding was attributable to a change in the environment of the aromatic residues, formation of an excited-state tyrosinate, resonance energy-transfer mechanisms, and a possible reduction in the intrinsic quenching of the tryptophan fluorescence in native BGTX. Trp–Trp energy-transfer calculations suggest that the minimum distance between the tryptophan side chain of the dodecamer (α185–196) and the tryptophan in BGTX is ~12 Å. Distance constraints in this range serve as a useful complement to NOE-derived distance constraints (≤4 Å) obtained from 2D NMR experiments.

The nicotinic acetylcholine receptor (nAChR)[1] mediates signal transduction at the neuromuscular junction by using the binding of acetylcholine to trigger the opening of a cation channel within the receptor. The nAChR is a pentameric array, $\alpha_2\beta\gamma\delta$, of four subunits (Karlin, 1980), where the α-subunit contains the binding site for agonists such as acetylcholine, for α-neurotoxins, and for other competitive antagonists. The binding of competitive antagonists such as the curaremimetic snake toxins (e.g., BGTX) leads to functional blockade of neuromuscular transmission (Changeux et al., 1984). It has been suggested that both agonists and antagonists produce conformational changes in the nAChR upon binding [e.g., see McCarthy & Stroud (1989)]. Many groups have used fluorescence measurements, sensitive to conformational alterations, to study ligand and neurotoxin binding to the nAChR (Weber et al., 1971; Cohen & Changeux, 1975; Eldefrawi & Eldefrawi, 1977; Heidemann & Changeux, 1978). Agonist binding to the nAChR from *Torpedo marmorata* (Bonner et al., 1976; Barrantes, 1978) or from *Narke*

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[1] Abbreviations: nAChR, nicotinic acetylcholine receptor; BGTX, α-bungarotoxin; *A*, absorbance, *e*, efficiency; R_0, critical transfer distance; *F*, fluorescence; λ_{max}, emission maxima, Φ, quantum yield; λ_{ex}, excitation wavelength; λ_{em}, emission wavelength; Trp, tryptophan; Tyr, tyrosine.

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