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Site Selectivity of Fluorescent Bisquaternary Phenanthridinium Ligands for Acetylcholinesterase

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SUMMARY

The synthesis of decidium and hexidium diiodides, their spectroscopic properties, and association with acetylcholinesterase from *Torpedo californica* are described and compared with those for propidium. Decidium, hexidium, and propidium, bisquaternary analogs of the fluorescent phenanthridinium ligand ethidium, contain 10, 6, and 3 methylene carbons, respectively, interposed between the exocyclic and endocyclic quaternary nitrogens. The three ligands exhibit linear competitive inhibition of enzyme carbamylation by *N*-methyl-7-dimethylcarbamoxyquinolinium. Dissociation constants for decidium, hexidium, and propidium are found by direct fluorescence titration to be $2.1 \pm 0.2 \times 10^{-8}$, $5.8 \pm 1.4 \times 10^{-7}$, and $3.7 \pm 0.4 \times 10^{-6}$ M, values in close accord with the inhibition constants obtained from kinetic analyses. Association of the three ligands is characterized by a stoichiometry of one fluorescent ligand per 80-kDa molecular weight subunit and occurs with respective 6.5-, 4.5-, and 3-fold increases in both quantum yield and fluorescence lifetime. Decidium and hexidium, in marked contrast with propidium, are dissociated by ligands selective for the active center and undergo pronounced reduction in affinity upon modification of the active center with pyrenebutyl methylphosphonofluoridate. Whereas the kinetics reveal no clear distinctions in inhibitory action of the three ligands, the fluorescence studies indicate that the alkyltrimethylammonium moiety of decidium and hexidium occludes the active center; propidium, in contrast, associates solely with the peripheral anionic site and does not occlude the active center. The temperature dependence of binding indicates that decidium association engenders a substantial increase (+55 eu) in entropy. The data indicate that the active center and peripheral anionic sites are separated by a crevice which can accommodate the hydrocarbon portion of extended *n*-alkyl cationic ligands, thereby affording entropic stabilization of complex formation. This stabilization is realized, however, only when the anionic subsite of the active center is not occluded, enabling electrostatic interaction between cationic ligand and the anionic active center.

AchE exists in several molecular forms which differ in solubility but exhibit little difference in catalytic behavior. The catalytic subunits are of similar molecular weight (70,000–80,000), and each contains at least two topographically distinct anionic sites (1, 2). The active center contains an anionic subsite and binds simple cations typified by the substrate acetylcholine and an inhibitor, edrophonium. The other locus, the peripheral anionic site, selectively binds structurally diverse ligands such as gallamine, *d*-tubocurarine, and propidium, and appears to regulate enzyme activity. Bisquaternary ligands, typified by decamethonium, associate with the enzyme in a manner that is competitive with ligand association at both the active center and the peripheral site. Since the binding of these bisquaternary ligands shows 1:1 stoichiometry with each subunit, this behavior would suggest that the distance separating these sites cannot exceed the distance separating the cationic termini of the decamethonium molecule, i.e., 14.5 Å. Fluorescence energy transfer measurements indicate, however, that the distance between propidium bound at the peripheral site and fluorescent methylphosphonates covalently linked at the active center is greater than 20 Å (1). The disparity between distances based on the structure of a ligand which can span the sites and those derived from spectroscopic methods can be explained with the proposition that either the peripheral site is not a distinct anionic locus but a matrix of anionic loci, or that association of bisquaternary ligands such as decamethonium induces a change in protein conformation such that the two anionic sites come into closer proximity (1).

A ligand combining the structural characteristics of propidium and decamethonium would be of utility in assessing the topographic basis for ligand exclusion on AchE. This paper presents the synthesis and characterization of decidium diio-

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ABBREVIATIONS: AchE, acetylcholinesterase; PBMP-AchE, pyrenebutyl methylphosphono-acetylcholinesterase.

dide,[1] a bisquaternary 10-carbon analog of ethidium possessing the 14-Å distance between the cationic termini characteristic of the decamethonium molecule, and hexidium diiodide, a bisquaternary 6-carbon congener. We compare their behavior with that of propidium, which has been previously studied (1, 3). By virtue of their fluorescence enhancement upon complexation with AchE, association of decidium and hexidium with the enzyme is readily monitored by employing steady state and nanosecond fluorescence spectroscopy. Fluorescence characteristics of the complexed ligands afford an additional index of binding site environment on the enzyme.

Materials and Methods

The 11 S form of AchE was isolated from electric organ membranes of *Torpedo californica* by light trypsin digestion and purified to homogeneity by affinity chromatography (4). Enzyme normality was ascertained from the protein absorption spectrum employing the extinction coefficient, $\epsilon^{1\%}_{280} = 17.5$.

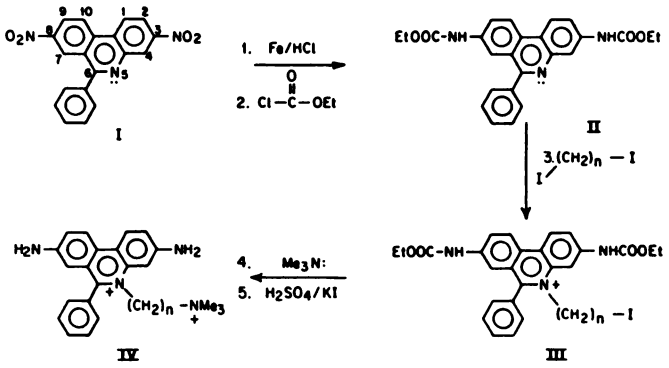
N-Methyl-7-dimethylcarbamoxyquinolinium iodide (Eastman Kodak; Molecular Probes), propidium diiodide (Calbiochem), decamethonium (K and K Chemicals), hexamethonium, acetylthiocholine iodide (Sigma Chemical Co.), and edrophonium chloride (Hofmann-LaRoche) were used without further purification. 3,8-Dinitro-6-phenylphenanthridine was a gift from Professor T. I. Watkins or was purchased from Calbiochem. Except where noted, the medium was a 0.01 N Tris-Cl buffer, pH 8.0, containing 0.1 N NaCl and 0.04 M $MgCl_2$.

Absorption spectra were obtained at 23° on a Perkin-Elmer Lambda 3B spectrophotometer. Absorption difference spectra using tandem quartz cuvettes (0.46 cm path length) were obtained on a Cary 219 spectrophotometer. Fluorescence titrations and corrected excitation and emission fluorescence spectra were recorded on a SPEX 212 fluorescence spectrometer equipped with cooled photon counting detection and a Hamamatsu R928P photomultipler tube. Nanosecond lifetime studies were conducted on a time-correlated fluorescence lifetime spectrometer from Photochemical Research Associates (London, Ontario, Canada). Emission decay rates were deconvolved employing the least squares procedure of Grinvald and Steinberg (5). Experimental conditions and data analysis have already been described (6).

Kinetic studies. Reaction of enzyme with *N*-methyl-7-dimethylcarbamoxyquinolinium iodide was carried out in a 0.01 N Tris-Cl buffer, pH 8.0, containing 0.1 N NaCl and 0.04 M $MgCl_2$, as described previously (3, 7). Reaction of AchE with acetylthiocholine iodide in the presence of 5,5′-dithiobis-(2-nitrobenzoic) acid was carried out in a 0.01 N sodium phosphate buffer, pH 7.0, containing 0.1 N NaCl and 0.02 N $MgCl_2$ (8). Data were recorded from a Perkin-Elmer Lambda 3B spectrophotometer or an Aminco Ratio II fluorescence spectrometer through a Data Translation 2805 analog-to-digital converter resident in an IBM PC computer. Data acquisition was controlled using the *Labtech Notebook* (Laboratory Technologies, Cambridge, MA). Data were analyzed by employing a linear regression program, Graph-Pad (Version 2.0), kindly provided by Dr. H. J. Motulsky, or a nonlinear regression procedure provided with the *Labtech Notebook*.

Analysis of fluorescence titration data. All fluorescence values are corrected for dilution resulting from added titrant, lamp fluctuations, incident scatter, and inner filter effects. Decidium, hexidium, and propidium fluorescence at 610 nm was monitored upon excitation at 535 nm. For competitive titrations decidium or hexidium was present at concentrations 20–40 times its dissociation constant and at least 2 times the concentration of enzyme sites. Other aspects of the data treatment have already been described (3, 6, 9, 10).

Synthesis of decidium and hexidium diiodides. Decidium and hexidium were synthesized as shown in Scheme 1. Reduction of 3,8-dinitro-6-phenylphenanthridine with iron powder in ethanolic HCl to give 3,8-diamino-6-phenylphenanthridine (step 1) and inactivation of the exocyclic amino groups with ethyl chloroformate (step 2) were carried out as described by Watkins (11). The methanol-insoluble fraction was then boiled in water for 2 hr to convert it to the high melting compound (II) (m.p. 211–213°) as described by Watkins (11) and Walls (12). Anal. calculated for $C_{25}H_{23}N_3O_4 \cdot H_2O$: C, 67.1, H, 5.6, N, 9.4, O, 17.9. Found: C, 67.1; H, 5.63; N, 9.39; O, 17.88.



Scheme 1

3,8-Carbethoxyamino-5,10′-iododecyl-6-phenylphenanthridinium iodide (IIIa, $n = 10$). A mixture of 3,8-carbethoxyamino-6-phenylphenanthridine (1.0 g; 2.3×10^{-3} mol) and diiododecane (15 g; 3.8×10^{-2} mol) was allowed to react at 130–140° for 48 hr. After allowing the reaction to cool, addition of ethyl ether (200 ml) caused formation of a dark yellow precipitate (1.4 g; 75%), which was used without further purification.

3,8-Diamino-5,10′-(trimethylammonium)decyl-6-phenylphenanthridinium diiodide (IVa, $n = 10$). Compound IIIa (0.5 g; 6.1×10^{-4} mol) and trimethylamine (25 ml, 25% in methanol) were heated at reflux for 10 hr; trimethylamine was added to keep the volume constant. The reaction was allowed to cool to room temperature, and addition of ethyl ether (100 ml) caused formation of an orange precipitate (0.28 g; 52%). This material (0.28 g; 3.2×10^{-4} mol) was dissolved in a mixture of methanol (25 ml) and 9 M H_2SO_4 (25 ml) and allowed to react at reflux for 24 hr. After cooling to room temperature, the solution was made neutral with NH_4OH and then poured into a solution of KI (0.5 M; 500 ml). Upon further cooling the product was obtained as purple crystals (0.10 g; 43% m.p. 166–177°). Anal. calculated for $C_{32}H_{44}N_4I_2$: C, 52.04; H, 6.01; N, 7.59; I, 34.37. Found: C, 52.25; H, 6.21; N, 7.40; I, 34.25.

3,8-Diamino-5,10′-(trimethylammonium)hexyl-6-phenylphenanthridinium diiodide (IVb, $n = 6$). This material was prepared as described above, except that diiodohexane was employed. The final product was isolated as purple crystals, m.p. 198–201°, dec. Anal. calculated for $C_{28}H_{36}N_4I_2 \cdot 2H_2O$: C, 46.81; H, 5.61; N, 7.80; I, 35.33. Found: C, 46.43; H, 5.45; N, 7.73; I, 35.04.

Results

Decidium, hexidium and propidium inhibition of AchE. Decidium, hexidium, and propidium inhibition of AchE catalysis was examined in a high ionic strength buffer ($\mu = 0.22$), by measuring the influence of ligand association on carbamylation by *N*-methyl-7-dimethylcarbamoxyquinolinium iodide (7). Reaction of this synthetic substrate with AchE proceeds with an initial liberation of the fluorescent alcohol *N*-methyl-7-hydroxyquinolinol in amounts proportional to the

[1] There exists no convenient yet consistent nomenclature for phenanthridinium derivatives. The name *eth*idium emphasizes the presence of the ethyl moiety while conveying no information that the ligand is a monoquaternary derivative. *Prop*idium derives its name from the presence of the propyl moiety irrespective of the fact that the ligand is a bisquaternary derivative. Since the new ligands reported here contain decamethylene and hexamethylene hydrocarbon chains conjoining their cationic termini, we propose the names *dec*idium and *hex*idium, with the tacit understanding that these are bisquaternary molecules.

normality of the enzyme sites. Hydrolysis of the carbamyl-ester proceeds more slowly than the carbamylation reaction, obviating consideration of the kinetic parameters associated with the decarbamylation step.

As shown in Fig. 1, reciprocal plot slopes increased with increasing concentrations of decidium, hexidium, and propidium and the curves converged at a common *y* intercept. Slope replots (Fig. 1, *insets*) revealed decidium, hexidium, and propidium to be linear competitive inhibitors of carbamylation, characterized by inhibition constants of 1.9×10^{-8}, 1.0×10^{-6}, and 6×10^{-6} M, respectively. The three ligands exhibited a mixed-type inhibition with respect to hydrolysis of acetylthiocholine, a fast turnover substrate (data not shown). Mixed inhibition might be anticipated if the inhibitor affected kinetics of deacetylation (13). Apart from marked differences in their respective K_I values, no clear difference could be discerned in the modes of decidium, hexidium, and propidium inhibition of AchE with respect either to hydrolysis of acetylthiocholine or carbamylation by *N*-methyl-7-dimethylcarbamoxyquinolinium.

Spectroscopic characterization of the free and complexed probes. The absorption spectrum of decidium was shifted from 480 nm, its position in buffer, to 505 nm without alteration in the extinction coefficient after complexation with AchE (Fig. 2). The corresponding fluorescence excitation spectra of decidium appeared at 480 and 505 nm, respectively, consistent with the absorption spectra. Under equivalent conditions the excitation maximum for hexidium was shifted from 484 nm, its position in buffer, to 512 nm after complexation with AchE. Previous studies with propidium showed that, upon complexation with AchE, its excitation wavelength shifted from 490 to 535 nm.

The corrected fluorescence emission spectrum for decidium was shifted from 645 nm, its position in buffer, to 610 nm after complexation with AchE, with a 6.5-fold increase in quantum yield estimated from integration of the emission wavenumber spectrum (Fig. 3A). Complexation of hexidium with AchE resulted in shift of the emission spectrum from 650 nm, its position in buffer, to 620 nm, with a 4.5-fold increase in quantum yield (Fig. 3B). Complexation of propidium with AchE resulted in shift of the emission spectrum from 662 nm, its position in buffer, to 630 nm, with a 3-fold increase in quantum yield.

The fluorescence lifetimes of decidium, hexidium, and propidium were found to be approximately 1.7, 1.4, and 1.1 nsec, respectively. The quantum yields and fluorescence lifetimes increased 3-fold upon transfer of the probes from buffer to D_2O, consistent with operation of a quenching mechanism governed by excited state proton transfer (14). Decidium, hexidium, and propidium complexed with AchE exhibited respective lifetimes of 11, 7, and 3.3 nsec, values that were 6.5-, 5-, and 3-fold greater than those found for the probes free in buffer. These increases in lifetime closely parallel the corresponding changes in steady state quantum yield. Therefore, although the three ligands displayed qualitatively similar behavior upon complexation with AchE, the fluorescence quantum yields and corresponding lifetimes of the bound ligands differed.

Association of decidium, hexidium, and propidium with AchE. Association of decidium, hexidium, and propidium with AchE was followed by the enhancement of their fluores-

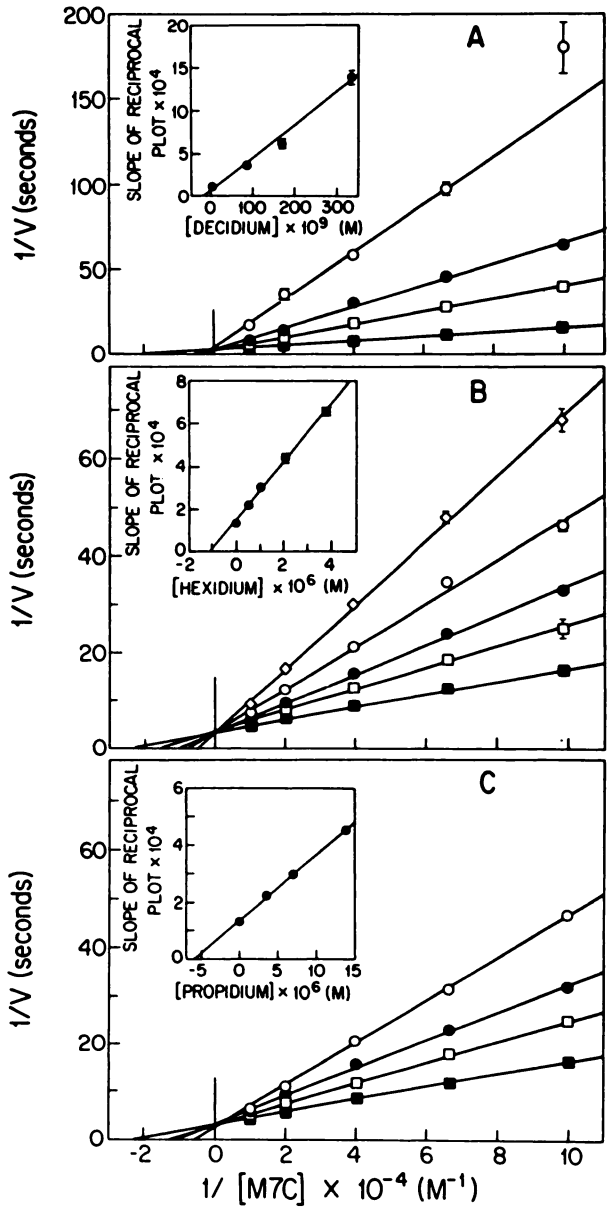


Fig. 1. Reciprocal plots of reaction velocity versus *N*-methyl-7-dimethylcarbamoxyquinolinium iodide concentration for AchE in the presence and absence of decidium (A), hexidium (B), and propidium (C). The reaction medium was a 0.01 N Tris-Cl buffer, pH 8.0, containing 0.1 N NaCl and 0.04 M $MgCl_2$. *N*-Methyl-7-dimethylcarbamoxyquinolinium is only weakly fluorescent but upon hydrolysis releases the fluorescent alcohol *N*-methyl-7-hydroxyquinolinol which is observed at 500 nm upon excitation at 400 nm. In all kinetic experiments, the inhibitor was present in at least 4-fold equivalents of enzyme active sites. A. ■, no decidium; □, 8.37×10^{-8} M; ●, 16.7×10^{-8} M; ○, 33.3×10^{-8} M. B. ■, no hexidium; □, 0.51×10^{-6} M; ●, 1.0×10^{-6} M; ○, 2.0×10^{-6} M; ◇, 3.8×10^{-6} M. C. ■, no propidium; □, 3.5×10^{-6} M; ●, 7.0×10^{-6} M; ○, 14.0×10^{-6} M. For the scheme

$$\text{AchE} + CQ \underset{}{\overset{K_C}{\rightleftharpoons}} \text{AchE}{:}CQ \xrightarrow{k_2} \text{AchE-C} + Q$$

where *CQ*, *Q*, and AchE-C represent *N*-methyl-7-dimethylcarbamoxyquinolinium, *N*-methyl-7-hydroxyquinolinium, and dimethylcarbamyl-acetylcholinesterase, respectively, k_2 and K_C may be calculated from the *y* intercept and slope·k_2, respectively of the reciprocal plot. Values of k_2 and K_C, averaged over four determination, were 0.32 ± 0.02 sec^{-1} and $4.2 \pm 0.3 \times 10^{-5}$ M, respectively. The *insets* present replots of the reciprocal plot slopes against inhibitor concentration, and are indicative of linear competitive inhibition. The competitive inhibition constants for decidium, hexidium, and propidium were calculated to be 1.9×10^{-8}, 1.0×10^{-6}, and 6.0×10^{-6}M, respectively.

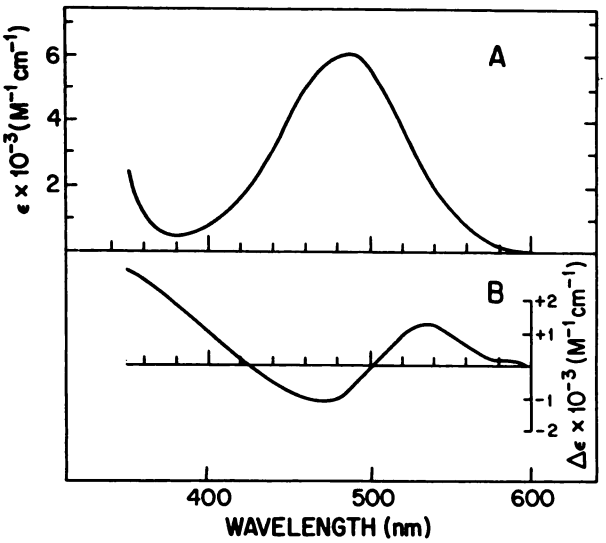


Fig. 2. Absorption spectra of decidium. A. Absorption spectrum of decidium in buffer. The *ordinate* values represent the molar extinction coefficient. B. Difference spectra generated between decidium (2.5×10^{-6} M) and AchE (2.8×10^{-6} M) in a 0.01 N Tris-Cl buffer, pH 8.0, containing 0.1 N NaCl and 0.04 M $MgCl_2$.

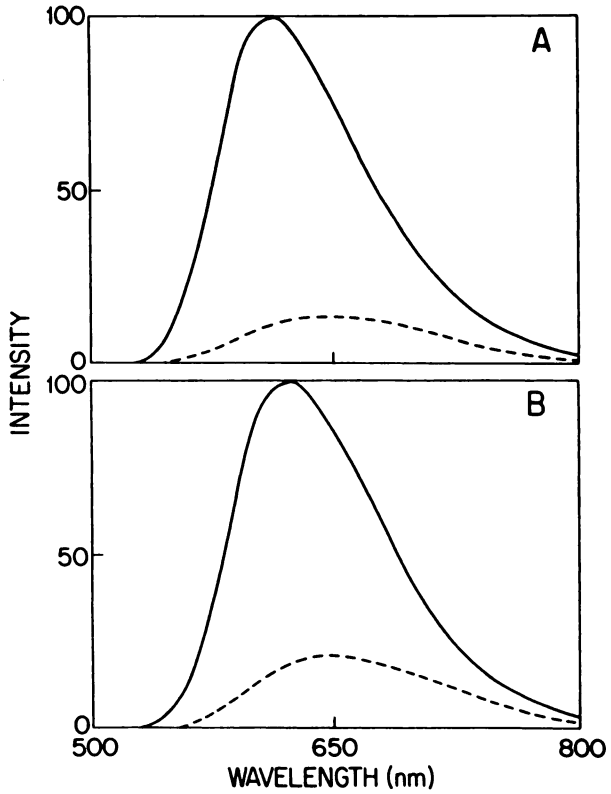


Fig. 3. Corrected fluorescence emission spectra for complexes of decidium and hexidium with AchE. A presents the spectra for decidium in buffer (– – –) and complexed with AchE (—). Enzyme was present at concentrations of $1.0–1.3 \times 10^{-5}$ M in subunit sites in the presence of $5–6 \times 10^{-6}$ M ligand; under these conditions more than 98% of the probe was bound as calculated from the dissociation constant of 2.1×10^{-8} M. The path length was 0.3 cm, obviating the need to consider distortion through inner filter effects (26). The excitation wavelength was set at the absorption maxima of 505 nm for study of the enzyme complex and 480 nm for study of the free ligand. After integration over the corrected wavenumber spectra, the quantum yield of the decidium complex with AchE was calculated to be 6.5-fold greater than that of the ligand free in buffer. B. Fluorescence spectra of hexidium in buffer (– – –) and complexed with AchE (—). Enzyme was present at concentrations of 2.4×10^{-5} M in subunit sites in the presence of 2.3×10^{-5} M ligand; under these conditions more than 98% of the probe was bound as calculated from the dissociation constant of 5.8×10^{-7} M. Fluorescence was monitored upon excitation at the absorption maxima of 512 nm for study of the enzyme complex and 484 nm for study of the free probe in buffer. After integration over the corrected wavenumber spectra, the quantum yield of the hexidium complex with AchE was calculated to be 4.5-fold greater than that for the ligand free in buffer.

cence at 610 nm upon excitation at 535 nm. The equilibrium titration profiles exhibited saturable binding and were compatible with a stoichiometry of one ligand per 80,000 molecular weight subunit. Scatchard plots afforded dissociation constants of $2.1 \pm 0.2 \times 10^{-8}$ M for decidium and $5.8 \pm 1.4 \times 10^{-7}$ M for hexidium (Fig. 4A). A value of $3.7 \pm 0.4 \times 10^{-6}$ M was obtained for propidium (Fig. 4B), in agreement with previous studies (3). The dissociation constants were compatible with inhibition constants determined with respect to carbamylation by *N*-methyl-7-dimethylcarbamoxyquinolinium. Dissociation constants for decidium measured at 15° ($1.0 \pm 0.2 \times 10^{-8}$ M), 19.5° ($2.0 \pm 0.2 \times 10^{-8}$ M), 23° ($2.1 \pm 0.2 \times 10^{-8}$ M), 30° ($2.0 \pm 0.3 \times 10^{-8}$ M), and 35° ($2.4 \pm 0.2 \times 10^{-8}$ M) were nearly invariant with temperature. An Arrhenius plot of these data revealed that decidium association engendered increases of 5.8 kcal/mol in enthalpy and +55 eu in entropy.

Dissociation of bound decidium and hexidium by nonfluorescent ligands. The dissociation constants for several cationic inhibitors of AchE were determined from their capacity to dissociate decidium and hexidium. In all cases dissociation determined over the concentration range of competing ligand was greater than 90%.

Dissociation constants of edrophonium, decamethonium, and hexamethonium determined through competitive displacement of decidium were $2.0 \pm 0.1 \times 10^{-7}$, $1.2 \pm 0.1 \times 10^{-6}$, and $1.3 \pm 0.1 \times 10^{-5}$ M, respectively (6). When measured through competitive dissociation of hexidium, the respective dissociation constants were $7 \pm 3 \times 10^{-8}$, $5 \pm 3 \times 10^{-7}$, and $4 \pm 2 \times 10^{-6}$ M. The greater uncertainty in these values arose from the smaller quantum yield for hexidium, which leads to a smaller excursion in fluorescence signal upon ligand dissociation. The dissociation constants of the nonfluorescent ligands determined by competition were consistent with their capacities to inhibit hydrolysis of cationic substrates (15). It is noteworthy that the dissociation constants determined for edrophonium, decamethonium, and hexamethonium in competition against hexidium were within 2 to 3-fold of the values determined against decidium; when considered with the competitive nature of the dissociation, these data indicate that the fluorescent and nonfluorescent ligands share a common locus.

Displacement of fluorescent bisquaternary ligands by edrophonium differs in the case of propidium. The competitive relationship between the active center-selective ligand edrophonium and propidium was assessed by direct titration of AchE with propidium in the absence and presence of edrophonium. For a simple competitive relationship, the presence of edrophonium would be expected to increase the propidium dissociation constant by $1 + [I]/K_C$), where I and K_C represent the edrophonium concentration and dissociation constant, respectively. In this case the presence of edrophonium at concentrations in the range of $1–4 \times 10^{-5}$ M would cause a 51 to 205-fold increase in the dissociation constant for propidium. As shown in Fig. 4B, the propidium K_D in the presence of edrophonium was within 2-fold of the value in the absence edro-

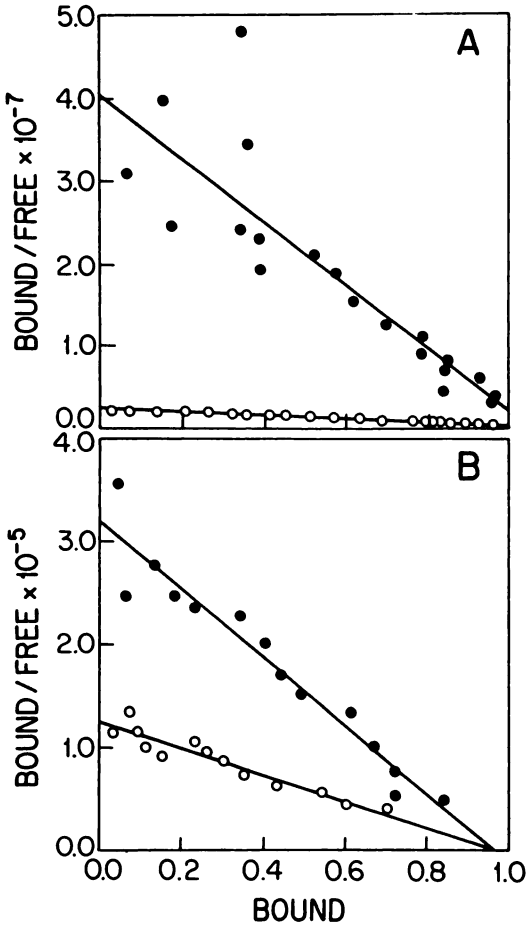


Fig. 4. Scatchard plots of decidium, hexidium, and propidium association with AchE. A. Decidium (●) and hexidium (○) association with AchE. From the equilibrium profile obtained for association of decidium with AchE, a dissociation constant of 2.3×10^{-8} M is calculated. When averaged over five determinations, the dissociation constant was calculated to be $2.1 \pm 0.2 \times 10^{-8}$ M. For hexidium a dissociation constant with AchE is calculated to be 6.6×10^{-7} M. When averaged over 13 determinations the dissociation constant was calculated to be $5.8 \pm 1.4 \times 10^{-7}$ M. B. Scatchard plots of propidium association with AchE in the presence (○) and absence (●) of edrophonium. Edrophonium was present at concentrations in the range 50 to 200-fold its dissociation constant of 2.1×10^{-7} M (6). From the negative reciprocal slope, the K_D for propidium is calculated to be 3.0×10^{-6} M in the absence and 7.8×10^{-6} M in the presence of edrophonium. Averaged over four determinations, the dissociation constant for propidium in the absence of edrophonium is $3.7 \pm 0.4 \times 10^{-6}$ M, in agreement with previous studies (3). Averaged over four determinations the propidium dissociation constant in the presence of edrophonium is determined to be $7.0 \pm 0.8 \times 10^{-6}$ M.

phonium. In contrast to decidium and hexidium, therefore, propidium and edrophonium do not share a common locus.

Decidium and hexidium association with PBMP-AchE. Equilibrium association of decidium, hexidium, and propidium with PBMP-AchE results in quenching of pyrenebutyl fluorescence (16) as a consequence of overlap of the phenanthridinium absorption spectrum with the pyrenebutyl emission spectrum (17). As a result, decidium and hexidium binding with PBMP-AchE can readily be followed by measuring the extent of quenching of the pyrenebutyl fluorescence at 400 nm upon excitation at 348 nm. Scatchard plots of the equilibrium profiles were indicative of ligand association at a single class of independent sites characterized by dissociation constants of $2.1 \pm 0.7 \times 10^{-6}$ M for decidium and $2.8 \pm 0.4 \times 10^{-6}$ M for hexidium (Fig. 5). These values were 100- and 6-fold higher than the respective values obtained for native enzyme. Conjugation of the pyrenebutyl methylphosphonyl group at the active center does not alter propidium binding (1, 16).

Discussion

Decidium and hexidium site specificity. AchE contains at least two topographically distinct anionic sites, one of which is localized within the active center. Another, the peripheral anionic site, is physically remote from the active center but can regulate enzyme activity. Whereas ligand association at the active center can be expected to produce competitive inhibition of substrate hydrolysis, association at the peripheral anionic site is less predictable, producing differing degrees of competitive, uncompetitive, and noncompetitive inhibition upon occupation by a variety of structurally unrelated cationic ligands (2, 3, 18, 19).

The analyses of enzyme inhibition kinetics of decidium, hexidium, and propidium in high ionic strength buffers revealed no clear distinctions in their binding. The three ligands exhibit competitive inhibition with respect to enzyme carbamylation by *N*-methyl-7-dimethylcarbamoxyquinolinium (Fig. 1). Inhibition of enzyme carbamylation focuses only on the initial step in the turnover of substrate. The competitive or near-competitive inhibition reveals that inhibitor binding, irrespective of its locus of binding, is mutually exclusive with carbamylation.

Despite the similarities in their inhibitory behavior, direct analysis of decidium, hexidium, and propidium association with AchE reveals clear distinctions in their binding modes. Equilibrium binding profiles derived from fluorescence measurements indicate that a primary binding locus for decidium and hexidium resides within the active center. Decidium and hexidium are dissociated by active center-selective ligands such as edro-

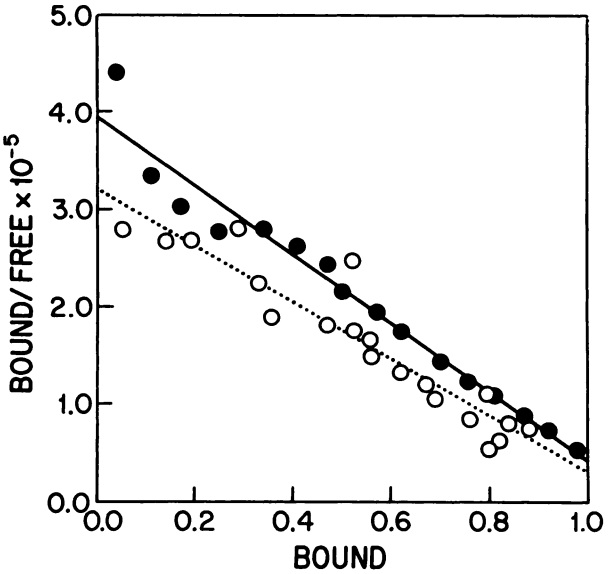


Fig. 5. Scatchard plots of decidium and hexidium association with PBMP-AchE. ●, decidium; ○, hexidium. PBMP-AchE was obtained by reaction of pyrenebutyl methylphosphonofluoridate with AchE, passage through a gel filtration column, and extensive dialysis against buffer (16). Association of decidium and hexidium was monitored by following the quenching of pyrenebutyl fluorescence at 400 nm upon excitation at 348 nm. The dissociation constant for decidium and PBMP-AchE, averaged over eight determinations, was $2.1 \pm 0.7 \times 10^{-6}$ M, while the value for hexidium, averaged over four determinations, was $2.8 \pm 0.4 \times 10^{-6}$ M.

phonium, and undergo respective 100- and 6-fold reductions in affinity upon modification of the active center with pyrenebutyl methylphosphonofluoridate. The influence of active center modification on decidium and hexidium association may also be compared with the 30-fold reduction in decamethonium affinity for PBMP-AchE (9).

These characteristics contrast with those found for propidium; this ligand associates at a peripheral anionic site and is dissociated by peripheral site-selective (gallamine, *d*-tubocurarine) but not active center-selective (edrophonium) ligands at concentrations consistent with their dissociation constants (Fig. 4B) (3). Propidium binding is unaffected by active center modification with pyrenebutyl methylphosphonofluoridate (1, 9). As indicated in resonance energy transfer studies of AchE, the propidium site is separated from the active center by greater than 20 Å, verifying the spatial separation of these sites (1).

In contrast to decamethonium, which contains two trimethylammonium groups, decidium and hexidium contain both exocyclic and endocyclic quaternary moieties. Bisquaternary and *n*-alkyl monoquaternary trimethylammonium ligands, typified by decamethonium and *n*-decyltrimethylammonium, respectively, are competitive inhibitors of methanesulfonylation of the active center and appear to associate with AchE at a locus within the active center (20, 21). Propidium, a 3-carbon congener containing a phenanthridinium moiety chemically equivalent with that in decidium and hexidium, appears not to occlude the active center of AchE and associates exclusively with the peripheral anionic site (1, 3). It seems likely, therefore, that the trimethylammonium portion of decidium and hexidium binds within the active center, whereas the phenanthridinium moiety is directed to an anionic locus remote from the active center.

Consequently, the phenanthridinium moieties of the bound decidium and hexidium may not associate with identical regions of the enzyme subunit, as reflected in the differing spectral shifts, quantum yields, and lifetimes of the bound species. The quantum yields and corresponding fluorescence lifetimes of phenanthridinium ligands reflect the accessibility of the fluorophore to the bulk aqueous medium (14). As evidenced by the changes in their fluorescence lifetimes and quantum yields upon complexation with AchE, the bound propidium, hexidium, and decidium show a progressive diminution in contact with the bulk aqueous medium.

Topographic requirements for ligand association with AchE. The enhanced affinity conferred by either the *n*-alkyl chain or the increased separation between quaternary groups is evident from the near-linear dependence of the binding free energy on the ligand chain length (Fig. 6). The value of 0.42 kcal/mol per methylene group falls in reasonable agreement with the value of 0.36 kcal/mol per methylene group found for association of *n*-alkyl bisquaternary ligands with AchE from *Torpedo* (6) and similar values derived for *n*-alkyl mono- and bisquaternary ligands with bovine erythrocyte enzyme (20–22). Since the relationship between affinity and chain length for the phenanthridinium ligands is similar to that for the *n*-alkyl polymethonium ligands, and therefore independent of the nature of the cationic termini, the increase in affinity through the series of mono- and bisquaternary ligands is concluded to originate from a common structural element, the *n*-alkyl chain.

Previous studies concerned decidium association with AchE prior to and after dealkylation ("aging") of branched alkyl

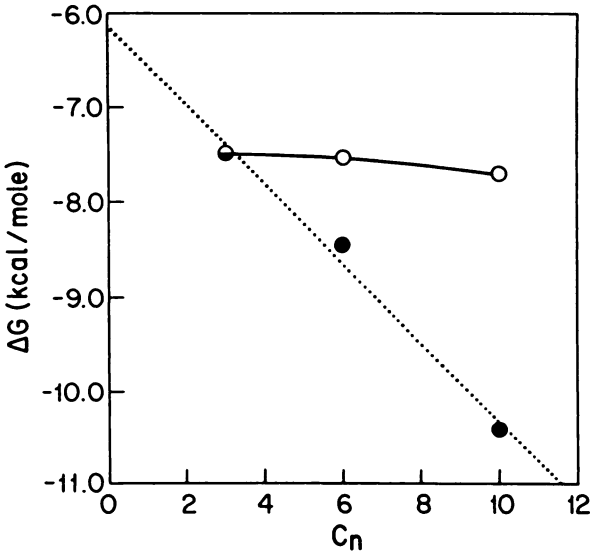


Fig. 6. Relationship between the binding free energy and chain length of bisquaternary phenanthridinium ligands. *Ordinate* values were calculated for 25° from the equation $\Delta G = RT \ln K_D$, where ΔG denotes the free energy (kcal/mol), R is the gas constant (1.98 cal/mol-°K), T is temperature (°K), and K_D is the dissociation constant (M). The slope affords an estimate of 0.42 kcal/mol per methylene group for ligand association with AchE (●). This value falls in reasonable agreement with the value of 0.36 kcal/mol per methylene group obtained from data presented by Berman and Decker (6) for association of *n*-alkyl bisquaternary ligands with AchE from *Torpedo*. The chain length dependence is abolished after modification of the active center with pyrenebutyl methylphosphonofluoridate (○).

methylphosphonyl conjugates of the enzyme, a reaction which increases by one unit the net negative charge within the active center (6). If Coulombic attraction within the active center were the sole determinant governing ligand association, then the mono- and bisquaternary ligands, since they contain identical trimethylammonium termini, would be expected to display equivalent enhancements in affinity after dealkylation of the methylphosphonyl group. Instead, longer chain bisquaternary ligands undergo greater enhancements in affinity than do their shorter chain counterparts following aging of the enzyme; shorter chain monoquaternary ligands, in contrast, experience greater increases in affinity than do their longer chain homologs. Taken together, these data signify that the entropic contribution conferred by the hydrocarbon chain of extended *n*-alkyl mono- and bisquaternary ligands represents a predominant component underlying ligand affinity. Indeed, the large entropy (+55 eu) gained upon decidium binding suggests that association with the protein results in removal of the hydrocarbon chain from contact with the aqueous environment and displacement of bound water from the protein surface. The presence of a crevice of limited steric dimensions separating in the active center and the peripheral anionic site affords a molecular explanation for the different binding affinities of the mono- and bisquaternary polymethonium ligands.

Active center modification increases the dissociation constants for decidium and hexidium to values ($2–3 \times 10^{-6}$ M) that are comparable with that for propidium and invariant with methylene chain length (Fig. 6). Decidium affinity for 2-(trimethylammonium)ethyl methylphosphono-acetylcholinesterase, a cationic conjugate, falls within the same range of values as that seen for the uncharged pyrenebutyl-containing conjugate (10). That the dissociation constants converge to a com-

mon value, one that is invariant with ligand methylene chain length, indicates that a free active center is required to achieve the additional stabilization energy conferred by the longer hydrocarbon chain. The indicated crevice for *n*-alkyl chains, therefore, appears not to be involved when association of the trimethylammonium moiety with the active center is precluded.

The reduction in binding energy accompanying occlusion of the anionic subsite of the active center indicates that realization of the additional entropy conferred upon association of the hydrocarbon chain with the protein surface requires an appropriate orientation of the cationic ligand by the net negative charge within the active center. Coulombic attraction between cationic ligand and the enzyme active center satisfactorily accounts for the diffusion-controlled bimolecular rates of association of simple cations (e.g., *N*-methylacridinium) with AchE (23, 24). Loss of the electrostatic component of binding energy after active center modification reveals that affinity and site selectivity of extended *n*-alkyl bisquaternary ligands depend not only on the net negative charge within the active center but also on the presence of a crevice contiguous with the active center. This dual dependence of extended *n*-alkyl ligand association on enzyme topography and surface charge is distinct from the less restrictive determinants underlying diffusion-limited association of cationic ligands exclusively at the peripheral anionic site or active center (23–25).

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References

1. Berman, H. A., J. Yguerabide, and P. Taylor. Fluorescence energy transfer on acetylcholinesterase: spatial relationship between peripheral site and active center. *Biochemistry* **19**:2226–2235 (1980).
2. Rosenberry, T. L. Acetylcholinesterase. *Adv. Enzymol.* **43**:103–218 (1975).
3. Taylor, P., and S. Lappi. Interaction of fluorescence probes with acetylcholinesterase. The site and specificity of propidium binding. *Biochemistry* **14**:1989–1997 (1975).
4. Taylor, P., J. W. Jones, and N. M. Jacobs. Acetylcholinesterase from *Torpedo:* Characterization of an enzyme species isolated by lytic procedures. *Mol. Pharmacol.* **10**:78–92 (1974).
5. Grinvald, A., and I. Z. Steinberg. On the analysis of fluorescence decay kinetics by the method of least squares. *Anal. Biochem.* **59**:583–598 (1974).
6. Berman, H. A., and M. M. Decker. Kinetic, equilibrium, and spectroscopic studies on dealkylation ("aging") of alkylphosphonyl-acetylcholinesterase: electrostatic control of enzyme topography. *J. Biol. Chem.* **261**:10646–10652 (1986).
7. Rosenberry, T. L., and S. Bernhard. Studies of catalysis by acetylcholinesterase. I. Fluorescent titration with a carbamoylating agent. *Biochemistry* **10**:4114–4120 (1971).
8. Ellman, G. L., K. D. Courtney, V. Andres, Jr., and R. M. Featherstone. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* **7**:88–95 (1961).
9. Berman, H. A., W. Becktel, and P. Taylor. Spectroscopic studies on acetylcholinesterase: influence of peripheral-site occupation on active center conformation. *Biochemistry* **20**:4803–4810 (1981).
10. Berman, H. A., and M. M. Decker. Kinetic, equilibrium, and spectroscopic studies on cation association at the active center of acetylcholinesterase: topographic distinction between trimethyl and trimethylammonium sites. *Biochim. Biophys. Acta* **872**:125–133 (1986).
11. Watkins, T. I. Trypanocides of the phenanthridine series, Part I. The effect of changing the quaternary grouping in dimidium bromide. *J. Chem. Soc.* 3059–3064 (1952).
12. Walls, L. P. Researches in the phenanthridine series, Part VIII. Further investigation of trypanocidal types. *J. Chem. Soc.* 67–74 (1947).
13. Krupka, R. M., and K. J. Laidler. Molecular mechanisms for hydrolytic enzyme action. I. Apparent non-competitive inhibition, with special reference to acetylcholinesterase. *J Am. Chem. Soc.* **83**:1445–1447 (1961).
14. Olmstead, J., III, and D. R. Kearns. Mechanism of ethidium bromide fluorescence enhancement in binding nucleic acids. *Biochemistry* **16**:3647–3654 (1977).
15. Mooser, G., and D. S. Sigman. Ligand binding properties of acetylcholinesterase determined with fluorescent probes. *Biochemistry* **13**:2299–2307 (1974).
16. Berman, H. A., and P. Taylor. Fluorescent phosphonate label for serine hydrolases, pyrenebutyl methylphosphonofluoridate. *Biochemistry* **17**:1704–1713 (1978).
17. Forster, Th. Transfer mechanisms of electronic excitation. *Discuss. Faraday Soc.* **27**:7–17 (1959).
18. Changeux, J.-P. Responses of acetylcholinesterase from *Torpedo mamorata* to salts and curarizing drugs. *Mol. Pharmacol.* **2**:369–392 (1966).
19. Roufogalis, B. D., and E. E. Quist. Relative binding sites of pharmacologically active ligands on bovine erythrocyte acetylcholinesterase. *Mol. Pharmacol.* **8**:41–49 (1972).
20. Belleau, B., H. Tani, and F. Lie. A correlation between biological activity of alkytrimethylammonium ions and their mode of interaction with acetylcholinesterase. *J. Am. Chem. Soc.* **87**:2283–2285 (1965).
21. Belleau, B., and V. DiTullio. Kinetic effects of alkyl quaternary ammonium salts on the methanesulfonylation of the acetylcholinesterase active center. Significance of substituent volumes and binding enthalpies. *J. Am. Chem. Soc.* **92**:6320–6325 (1970).
22. Bergmann, F., and R. Segal. The relationship of quaternary ammonium salts to the anionic sites of true and pseudo cholinesterase. *Biochem. J.* **58**:692–698 (1954).
23. Rosenberry, T. L., and E. Neumann. Interaction of ligands with acetylcholinesterase. Use of temperature-jump relaxation kinetics in the binding of specific fluorescent ligands. *Biochemistry* **16**:3870–3878 (1977).
24. Nolte, H.-J., T. L. Rosenberry, and E. Neumann. Effective charge on acetylcholinesterase active sites determined from the ionic strength dependence of association rate constants with cationic ligands. *Biochemistry* **19**:3705–3711 (1980).
25. Bolger, M. B., and P. Taylor. Kinetics of association between bisquaternary ammonium ligands and acetylcholinesterase: evidence for two conformational states of the enzyme from stopped-flow measurements of fluorescence. *Biochemistry* **18**:3622–3629 (1979).
26. Chignell, C. Fluorescence spectroscopy: drug interactions with biological systems. *Methods Pharmacol.* **2**:33–61 (1972).

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