

## Spectroscopic Studies of Ligand Interactions with Acetylcholinesterase\*

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A series of ligands with suitable spectroscopic properties have been employed for investigating the sites and specificity of ligand association with an acetylcholinesterase purified to apparent homogeneity from *Torpedo californica*. Bisquaternary ammonium ligands in which the nitrogens are maximally separated by 14 Å, bind with 1:1 stoichiometry with each 82 000 molecular weight subunit on acetylcholinesterase. Placement of a benzoquinone moiety between the quaternary nitrogens provides a ligand which upon binding quenches the tryptophanyl fluorescence of the protein. The complex exhibits 49% of the fluorescence of the free protein and the quenching appears to take place by radiationless energy transfer between excited state dipoles. Since quenching appears uniform with binding on each subunit, it is unlikely that the ligand binding sites exist near a center or axis of symmetry between subunits. Modification of the active site serine forming the respective sulfonyl or phosphoryl esters enables one to study the orientation of the bound bisquaternary ligand with respect to the catalytic serine and the esterifying group. Propidium, a second ligand, binds to acetylcholinesterase with an enhancement in quantum yield and a shift in excitation and emission wavelengths. Propidium also exhibits binding which is stoichiometric with each subunit on the enzyme but is highly selective for a peripheral anionic site. Thus, agents such as edrophonium and *N*-methylacridinium which bind preferentially to the active center of the enzyme will not dissociate the bound propidium. Bisquaternary ligands such as decamethonium are mutually competitive with both the active center and the peripheral anionic site. The long excitation and emission wavelengths (488/632 nm) of propidium endow it with ideal acceptor properties for energy transfer studies with various donor ligands bound to the active center.

### INTRODUCTION

Most of the work to date on ligand binding to acetylcholinesterase has been based on the influence of the inhibitory or stimulatory ligand on steady state kinetic parameters in which substrates such as acyl or carbamyl esters are employed. By investigating spectroscopic changes associated with ligand binding, the ligand-enzyme interaction can be examined directly rather than rely on multiple kinetic parameters<sup>1-4</sup>. This approach carries a second inherent

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advantage for the spectroscopic measurements can yield additional information on the properties of the macromolecular complex. We describe here the use of absorption and fluorescence spectroscopy to study specificity and sites of ligand binding to an acetylcholinesterase purified to homogeneity from *Torpedo californica*<sup>5</sup>. In the fluorescence measurements, changes in both ligand and protein fluorescence offer suitable means for monitoring complex formation.

## METHODS

Acetylcholinesterase from the electric organ of *Torpedo californica* has been obtained in apparent homogeneous form by dissolution from the membrane with trypsin or collagenase and subsequent purification of the soluble enzyme by affinity chromatography<sup>6</sup>. Fluorescence titrations and spectral measurements were carried out in a Farrand spectrophotofluorometer in 1.0 cm<sup>2</sup> or 0.3 cm<sup>2</sup> cells at 25 °C. Absorption and difference spectra were measured in a Cary 16 spectrophotometer while a Durrum rapid kinetic spectrophotometer was employed for the stopped-flow work.

## RESULTS AND DISCUSSION

*Properties of the Lytic Enzyme*

Table I lists the major physical properties of the lytic enzyme derived from tryptic treatment of the isolated electric organ membrane. A collagenase from *Clostridium histolyticum* has been purified to homogeneity and found to be devoid of other hydrolytic activity. This collagenase in molar concentrations less than trypsin also promoted dissolution of acetylcholinesterase from its

TABLE I.

*Acetylcholinesterase — (Torpedo Californica) — Physical Properties*

Sedimentation Coefficient	10.8 S
Stokes Radius	76 Å
Frictional Coefficient, $f/f_0$	1.65
Molecular Wt. from Gel Filtration and Sedimentation Velocity	333 000
Molecular Wt. from Sedimentation Equilibrium	338 000
Subunit Molecular Weight SDS Dissociation	78 000 — 82 000
Functional Equivalent Weight <sup>a</sup> (Serine Carbamylation)	77 500 ± 7 200
Equivalent Weight (Inhibitor binding)	
a) bis-3-aminopyridinium 1,10 decane	87 000
b) 2,5 bis(3-diethyl- <i>o</i> -chlorobenzyl-ammonium- <i>n</i> -propylamino) benzoquinone	84 000
Isoelectric Point	5.36
Specific Activity <sup>b</sup>	345 ± 44

<sup>a</sup>Mean ± standard deviation for 6 determinations on the same preparation of enzyme.

<sup>b</sup>Mean ± standard deviation for 6 separate enzyme preparations in mmol h<sup>-1</sup> mg<sup>-1</sup>.

membrane associated form (Fig. 1). As expected for a Zn containing metalloenzyme<sup>6</sup>, *o*-phenanthroline was effective in preventing the collagenase activity. The enzymes prepared from trypsin and collagenase treatment of the membranes have identical sedimentation and electrophoretic properties when individually labelled by <sup>32</sup>P and <sup>3</sup>H-DFP, admixed and subjected to the respective separation procedure (Fig. 2). Both trypsin and collagenase have specific sequence requirements for peptide bond hydrolysis. The similarity of the corresponding lytic acetylcholinesterases suggests that random cleavages are not responsible for production of lytic enzyme. For the following spectroscopic studies we have employed the enzyme derived from tryptic dissolution of the membranes.

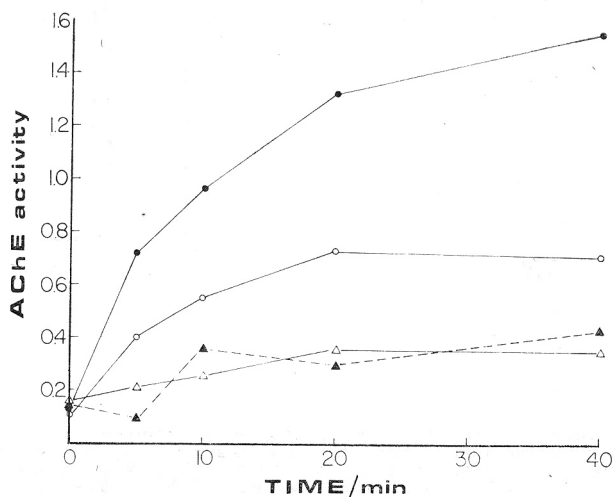
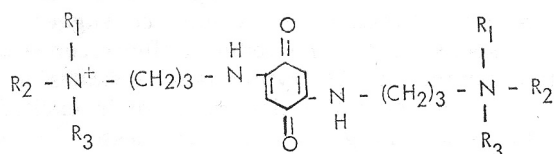


Fig. 1: Release of acetylcholinesterase (AChE) from *Torpedo californica* electroplax membranes by trypsin and collagenase at 37 °C: The collagenases were purified from *Clostridium histolyticum* to apparent homogeneity and found to be free of casein and benzoylarginyl-naphthylamide hydrolytic activities (<1 mole per cent of trypsin — Lwebuga-Mukasa, J. and Taylor, P.; to be published). The membranes were isolated by differential centrifugation<sup>5</sup>. The membranes were resuspended in 0.5 volume of the original tissue weight of 0.01 M Tris Cl buffer containing 0.04 M MgCl<sub>2</sub>, 0.1 M NaCl, pH = 7.8. For collagenase promoted hydrolysis 0.01 M CaCl<sub>2</sub> was also added. At the specified time aliquots of the stirred suspension were removed, cooled on ice, and immediately centrifuged at 45 000 g for 10 min. Acetylcholinesterase was assayed in the supernatant fractions and is expressed as  $\mu\text{Eq}/[\text{min} \cdot (5 \mu\text{l})]$  of cholinesterase. ●—●, incubation with trypsin,  $1.27 \times 10^{-7}$  M; ○—○, incubation with collagenase A,  $0.33 \times 10^{-7}$  M; ▲—▲, incubation with collagenase and  $10^{-4}$  M *o*-phenanthroline; △—△, incubation without added enzyme.

### Fluorescence quenching associated with bisquaternary benzoquinonium complex formation

The association of a series of bisquaternary benzoquinone ligands of the structure



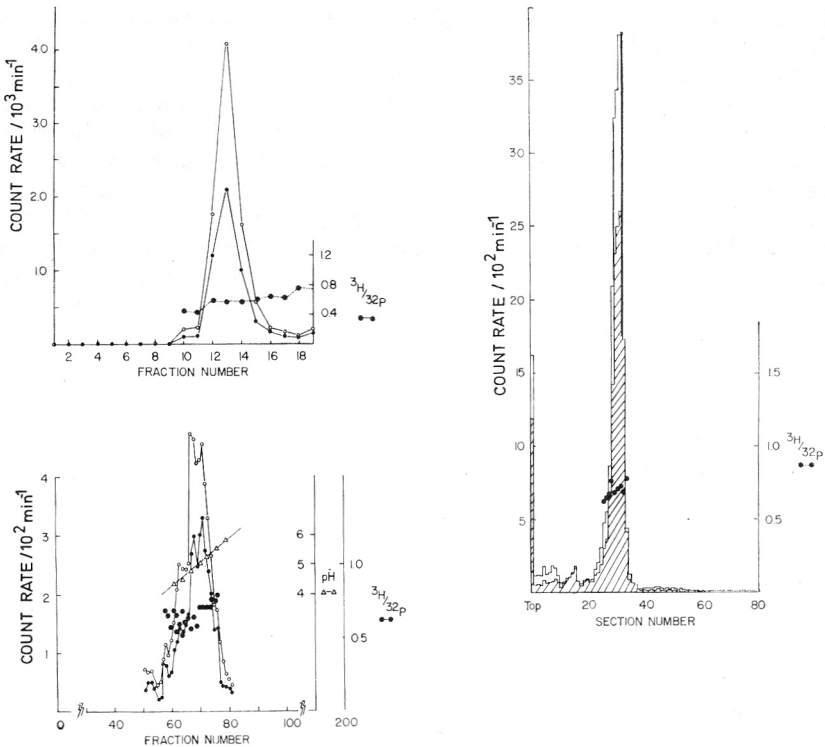


Fig. 2: Electrophoretic and sedimentation properties of acetylcholinesterase prepared by collagenase or trypsin dissolution of electroplax membranes: Acetylcholinesterase was purified from the membranes by lytic and affinity chromatographic procedures previously described<sup>5</sup> where 5  $\mu\text{g/ml}$  of trypsin or 2  $\mu\text{g/ml}$  of collagenase was employed. The purified enzymes were labelled by reaction with <sup>3</sup>H or <sup>32</sup>P diisopropylfluorophosphate by reaction with 50  $\mu\text{M}$  ligand for 20 min at 4 °C. Unreacted ligand was removed by dialysis. Portions of the <sup>3</sup>H and <sup>32</sup>P DFP enzymes were admixed and then subjected to sedimentation in a 5–20% sucrose gradient (upper left), isoelectric focusing (lower left) and polyacrylamide gel electrophoresis in the presence of SDS (right). ○—○, Collagenase derived acetylcholinesterase — labeled with <sup>32</sup>P—DFP; ●—●, trypsin derived acetylcholinesterase labeled with <sup>3</sup>H—DFP; hexagons: ratio of counts <sup>3</sup>H/<sup>32</sup>P. The analytical procedures were the same as those described previously.

with acetylcholinesterase yields the respective complexes that exhibit 48–51% of the fluorescence of the free enzyme (Table II). Back titration with the all aliphatic congener, decamethonium, results in competition between the two ligands and with excess decamethonium a return to the fluorescence value for the unbound enzyme is observed. Dissociation constants may be calculated for both ligands from the titrations. The benzoquinone moiety has an absorption maximum at 340 nm ( $\epsilon_{340} = 2.38 \times 10^4$ ) and its spectrum overlaps in large measure with that of the fluorescence emission of the tryptophanyl residues. The non-quenching decamethonium is devoid of absorption in this region. The large overlap integral with the quenching ligand and the low concentrations at which the fluorescence titrations are conducted suggest that radiationless energy transfer is responsible for the reduced fluorescence quantum yield of the complex. In this instance the 19 tryptophanyl residues on each acetylcholinesterase subunit serve as potential donors in their excited states and the benzoquinone residue is the energy acceptor. The lesser degree of fluorescence

TABLE II.  
Dissociation Constants,  $K_D$ , Determined from Ligand or Protein Fluorescence.  
Changes Associated with Complex Formation at 25 °C

Bisquaternary Ligand 2,5 bis (trialkylammonium- <i>n</i> -propylamino)benzoquinone	$K_D$ /M	% Quenching of Protein Fluorescence	$J^c$ cm <sup>6</sup> /mol × 10 <sup>15</sup>
Alkyl Groups			
Trimethylammonium	$1.2 \times 10^{-5}$	52	18.8
Diethylmethylammonium	$3.5 \times 10^{-6}$	51	
Triethylammonium	$3.1 \times 10^{-6}$	53	
Benzyl-diethylammonium	$1.9 \times 10^{-7}$	50	
<i>p</i> -Nitrobenzyl-diethyl- ammonium	$2.9 \times 10^{-6}$	56	
<i>o</i> -Chlorobenzyl-diethyl- ammonium	$2.8 \times 10^{-8}$	53	
bis-3-Aminopyridinium- -1,10-decane	$2.6 \times 10^{-7}$ <sup>a</sup>	35	4.21
Decamethonium	$5.9 \times 10^{-6}$ <sup>b</sup>	< 3	0

<sup>a</sup>Determined from ligand fluorescence

<sup>b</sup>Determined by back-titration of bis(3-aminopyridinium)-1,10-decane

<sup>c</sup>Overlap integral calculated from the corrected fluorescence emission spectrum of acetylcholinesterase and the absorption spectra of the respective ligands.

quenching that we observe with other bisquaternary inhibitors exhibiting smaller overlap integrals with the protein tryptophanyl fluorescence is also consonant with the above quenching mode<sup>4</sup>.

An equivalent degree of fluorescence quenching is observed upon titration of each of the four subunits on the acetylcholinesterase molecule (Fig. 3). Since

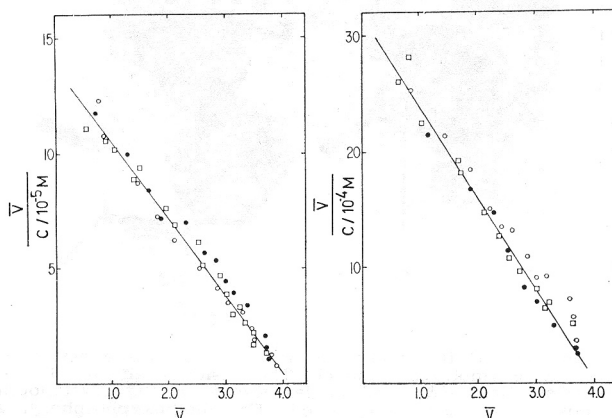


Fig. 3: Scatchard plots of fluorescence titrations of *Torpedo* acetylcholinesterase and various bisquaternary benzoquinone inhibitors: Conditions were the same as in Fig. 1.  $v$  is moles of ligand per bound mole of enzyme.  $c$  is the free ligand concentration: Left-hand panel, 2,5 bis (triethylammonium-*n*-propylamino)benzoquinone; right-hand panel, 2,5 bis (trimethylammonium-*n*-propylamino)benzoquinone.  $v$  was determined from the fractional approach to maximum quenching. At maximum quenching, which was determined from reciprocal plots, it is assumed that 4 ligand molecules are bound per mole of acetylcholinesterase. The different symbols denote separate titrations.

quenching is a linear function of the extinction coefficient of the acceptor ligand but dependent on the sixth power of the donor-acceptor distance and effective to distances<sup>4,7</sup> of 25 Å, equivalent quenching on each subunit can arise only if the distance between binding sites is large. These findings indicate that the subunit binding sites do not reside close to an intersubunit axis or center of symmetry on the macromolecule.

### *Chemically modified acetylcholinesterase*

An advantage inherent in the spectroscopic procedures for measuring ligand association is that chemically modified, but catalytically inactive, enzymes may be examined. We have made a series of alkanesulfonyl and alkoxyphosphoryl enzymes which are catalytically inactive but contain substituents of varying bulk esterified to the active site serine. For both the sulfonyl and phosphoryl enzymes the oxygens, alkoxy, and alkyl groups are tetrahedrally disposed around the sulfur and phosphorus. With tetrahedral geometry, it is to be expected that one of the sulfone oxygens and the phosphoryl oxygen will orient in the direction of the oxyanion hole. This structural feature has been characteristic in the serine hydrolases examined thus far crystallographically<sup>8</sup> and stabilization is conferred through hydrogen bonding

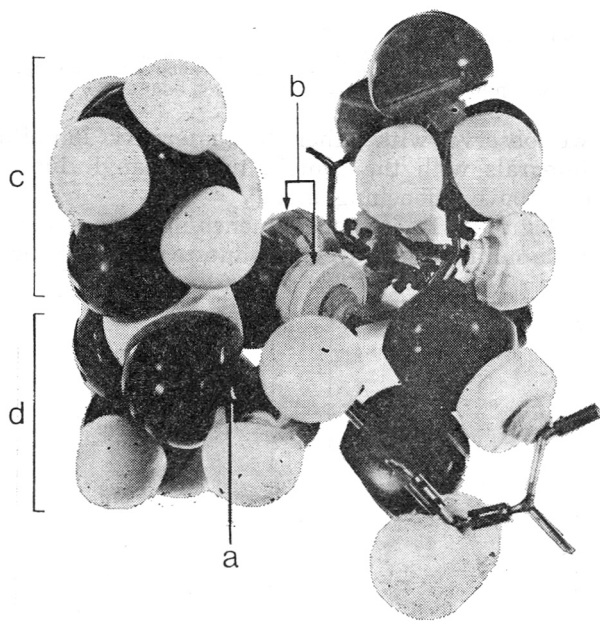


Fig. 4: Proposed orientation of the acid-transferring group in the active center of acetylcholinesterase, using the chymotrypsin model: The residues glycine-193, aspartate-194, and serine-195 have the orientation and bond angles shown in the crystallographic structure<sup>8</sup>. Space-filling hydrogens and oxygens are added. The diethoxyphosphoryl group is linked through the side chain oxygen of serine-195 (designated by a), and the phosphoryl oxygen is hydrogen bonded to the two donor hydrogens on the amide nitrogen backbone of glycine-193 and serine-195 (b). This area has been deemed the «oxyanion» hole<sup>8</sup>. Hydrogen bonding at this position would fix the orientation of the alkoxy groups with respect to the active site surface. One of the alkoxy groups (c) would be oriented toward the anionic subsite, while the other (d) would be directed into the acyl pocket. Accordingly (d) would have the same orientation as the acyl-CH<sub>3</sub> group of acetylcholine in its transition state configuration. The chymotrypsin model was constructed by Dr. J. J. Birktoft, Department of Chemistry, University of California, San Diego.

between the respective oxygen and amide backbone hydrogens in the protein (Fig. 4). With this orientation, the bulky alkoxy groups in the phosphoryl enzyme would be directed towards (1) the acyl pocket, and (2) the anionic subsite that associates with the quaternary choline group in the substrate. In the case of the alkane sulfonyl enzyme, where there exist two sulfone oxygens, the alkyl group could be directed either towards the acyl pocket or the anionic subsite.

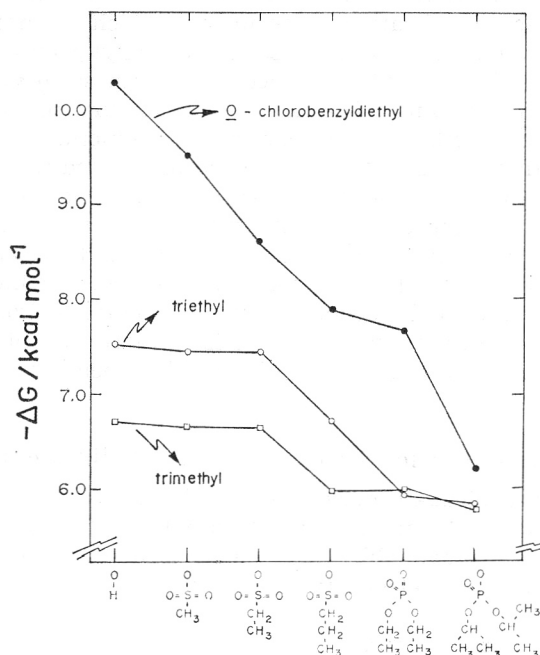


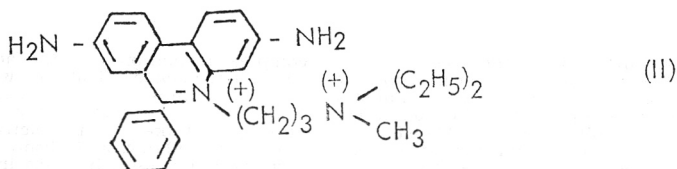
Fig. 5: Relationship between the free energy of complex formation and the acid transferring group for native and modified acetylcholinesterase: Fluorescence titrations were conducted using 2,5 bis (3-diethyl-*o*-chlorobenzylammonium-*n*-propylamino) benzoquinone, 2,5 bis (triethylammonium-*n*-propylamino) benzoquinone and 2,5 bis (triethylammonium-*n*-propylamino) benzoquinone. The following acid transferring groups were linked to the active site serine: methanesulfonyl, ethanesulfonyl, propanesulfonyl, diethoxyphosphoryl and diisopropoxyphosphoryl. Transfer to the enzyme was effected by the corresponding *N*-methyl-3-hydroxypyridinium ester, diethyl-*p*-nitrophenyl phosphate or diisopropylfluorophosphate. The reaction was carried to at least 96% of completion as measured by inhibition of catalysis and product was separated from reactants passing it through a Sephadex G-25 column<sup>5</sup>. Dissociation constants for the complexes were obtained from titrations which monitored tryptophanyl fluorescence quenching ( $\lambda_{\text{excitation}} = 290 \text{ nm}$ ,  $\lambda_{\text{emission}} = 340 \text{ nm}$ ).  $\Delta G$  was calculated from the dissociation constant.

Given these constraints on the orientation of the substituted phosphoryl and sulfonyl groups that result from the serine ester bond and hydrogen bonding at the oxyanion hole, we can better interpret differences in binding affinity for bisquaternary ligands with the enzyme. Binding constants have been obtained by direct fluorescence titration for the bis-*N*-trimethyl, triethyl and *o*-chlorobenzyl diethyl benzoquinonium derivatives with three substituted alkanesulfonyl enzymes and two phosphoryl acetylcholinesterases. In Fig. 5, we have plotted the binding free energy against the extent of substitution. For the *o*-chlorobenzyl derivative every substitution affects the binding energy and reduction in binding free energy roughly parallels the bulk of the este-

rifying substituent. In contrast, binding energy for complexes with the trimethyl and triethyl derivatives is reduced only by the propanesulfonyl and both phosphoryl substitutions. This structure-binding relationship is most consistent with the second quaternary nitrogen in the bisquaternary ligand directed away the active site serine and the chlorobenzyl group towards the serine. This interpretation is the same as that made by Wilson and Kitz<sup>9</sup> and later by Belleau<sup>10</sup> who, on the basis of lack of protection of the active site serine, suggested that the bisquaternary inhibitors bind »*exo*« to the active site serine. The direct fluorescence titration experiment, which is not dependent on differences in intrinsic reactivity of the serine hydroxyl group, also can provide refinement of the estimation of steric occlusion with serine modification and bisquaternary ligand binding. The marked reduction in the binding free energy occurring at the ethane to propanesulfonyl juncture indicates that only the methane and ethane substitution can be accommodated with the acyl pocket. For the propanesulfonyl enzyme, this orientation may be sterically precluded. Since either sulfone oxygen can associate with the oxyanion hole, the propane group would be directed towards the anionic subsite. Thus, interference with the optimal binding distance for the quaternary nitrogen would be anticipated. Steric occlusion in the acyl pocket would be predicted from a comparison of catalytic rates for various acyl substrates<sup>11</sup> where one observes a drastic fall in  $K_m$  at comparable acyl group lengths (*i. e.* between propionyl and butyryl choline).

#### *Propidium as a fluorescence probe for acetylcholinesterase*

Propidium (II), a bisquaternary ligand which differs from those described above in that only 5.5 Å maximally separate the quaternary nitrogens, has proven to be a useful reversible fluorescence probe for acetylcholinesterase because of its unique site selectivity.



Binding of propidium to acetylcholinesterase is characterized by an enhancement of fluorescence quantum yield, formation of a high affinity complex, and a wavelength shift in the fluorescence excitation and emission spectra (Fig. 6). By selecting optimal wavelengths, a large difference in fluorescence signal (10–12 fold) between free and bound ligand can be achieved in the fluorescence titrations (Fig. 7). These titrations also reveal one propidium binding site per 82 000 molecular weight subunit on the enzyme (Fig. 7) and complete dissociation of the bound propidium can be effected by stoichiometric addition of a high affinity bisquaternary inhibitor such as ambenonium<sup>12</sup>. A wide variety of ligands will compete at the propidium binding site and their effectiveness can be ranked as: bisquaternary ammonium > monoquaternary ammonium  $\approx$  divalent cation > monovalent cation (Fig. 8). Of particular interest, however, is the behavior of edrophonium and *N*-methylacridinium



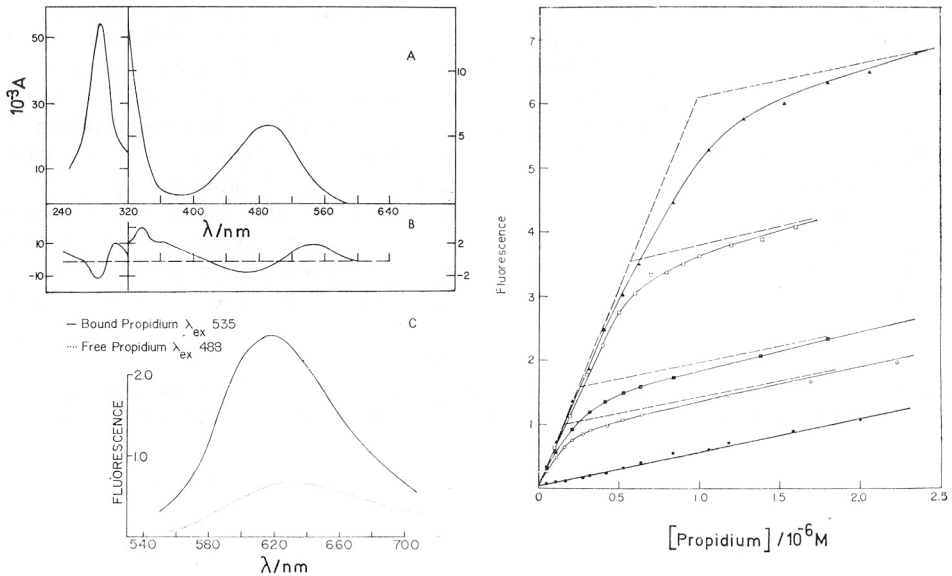


Fig. 6: Spectral properties of propidium: Panel A: Absorption spectrum of propidium diiodide in 0.001 M Tris Cl, pH = 8.0. The ordinate values represent molar extinction coefficients. Panel B: Difference spectrum generated between  $1.2 \times 10^{-5}$  M propidium and acetylcholinesterase ( $1.52 \times 10^{-5}$  equivalents of binding sites/liter) in 0.001 M Tris Cl, pH = 8.0, and equal concentrations of the unmixed ligand and enzyme in the reference path. Under these conditions about 95% of the propidium is bound to the enzyme. Samples were run using 0.46 cm tandem cells in the double beam Cary 16 spectrophotometer. Panel C: Fluorescence emission spectra of free propidium (---) and acetylcholinesterase bound propidium (—) in 0.001 M Tris Cl buffer, pH = 8.0. An excitation wavelength of 488 nm was used for free propidium and 535 nm for the bound ligand. A Hamamatsu R 818 photomultiplier tube was employed since its spectral response is relatively constant between 500 and 800 nm.

Figure 7: Fluorescence titrations of propidium—acetylcholinesterase complex formation in 1 mM Tris-Cl, pH = 8.0, 25 °C: ●—● blank titration in absence of enzyme; ○—○, 0.145 mg/ml of enzyme (0.18 μM); ■—■, 0.24 mg/ml (0.3 μM); □—□, 0.54 mg/ml (0.67 μM); ▲—▲, 0.88 (1.1 μM). Molarity has been designated as subunit molarity where 82 000 Daltons is the molecular weight<sup>4</sup>.

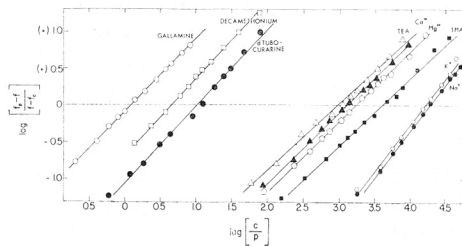


Fig. 8: Displacement of propidium from the propidium—acetylcholinesterase complex by various ligands: Incremental additions of the ligands were added to the propidium—acetylcholinesterase complex and the fluorescence was recorded. Data are plotted logarithmically where [C] and [P] are concentrations of the competing ligand and propidium, respectively.  $f_p$  denotes the initial fluorescence when all of the enzyme sites are saturated with [P],  $f_c$  denotes the fluorescence when propidium is completely displaced from the enzyme and  $f$  denotes the fluorescence reading during the titration. ○—○, gallamine; □—□, decamethonium; ●—●, d-tubocurarine; △—△, tetraethylammonium. ■—■, tetraethylammonium. ▲—▲,  $Ca^{2+}$ , pentagons,  $Mg^{2+}$ , open hexagons,  $K^+$ ; filled hexagons,  $Na^+$ . The cations were added as chlorides.

in the dissociation of propidium from the complex. Substantial evidence has accumulated over the years that edrophonium will bind specifically to the active site of acetylcholinesterase<sup>13,14</sup> and in 1:1 stoichiometry with each subunit<sup>14</sup>. We observe with the *Torpedo* enzyme a  $K_I$  of  $1.1 \times 10^{-7}$  M when inhibition of 7 dimethylcarbamoy-*N*-methylquinolinium decarbamylation is measured<sup>12</sup>. A similar  $K_D$  is obtained when edrophonium is used to back titrate the fluorescent species *N*-methylacridinium and bis-3 aminopyridinium 1, 10 decane (Table III). The first shows selectivity for the active center while with the second ligand one end of the molecule binds at the active center<sup>1</sup>. However, the competitive relationship between edrophonium and propidium yields an edrophonium dissociation constant that is 3.5 orders of magnitude

TABLE III.

*Dissociation Constants Determined for Edrophonium by Competition with Various Ligands or Inhibition of Catalysis, 0.001 M Tris Cl, pH = 8.0*

Ligand	$K_D/M$
Propidium <sup>a</sup>	$4.0 \times 10^{-4}$
<i>N</i> -methylacridinium <sup>b</sup>	$1.9 \times 10^{-7}$
bis-3-aminopyridinium 1,10-decane <sup>b</sup>	$3.3 \times 10^{-7}$
<i>N</i> -methyl 7-dimethylcarbamoyquinolinium <sup>c</sup>	$1.1 \times 10^{-7}$
Acetylcholine <sup>c</sup>	$1.3 \times 10^{-7}$

<sup>a</sup>Determined by back titration as described in the Experimental section

<sup>b</sup>Determined by back titration of *N*-methylacridinium and 3-aminopyridinium 1,10 decane fluorescence at 390/510 nm and 320/405 nm respectively.

<sup>c</sup>Inhibition constant calculated assuming competitive inhibition of *N*-methyl-7-dimethylcarbamoyquinolinium or acetylcholine hydrolysis.

greater than that obtained in the other back titrations. Thus, the binding of propidium is removed from the active center of acetylcholinesterase and occurs at a peripheral anionic site.

Although edrophonium and propidium are not competitive and bind to separate sites, we find that the bisquaternary inhibitors, where the nitrogens are separated by 10 methylene groups and provide a maximal span of 14 Å, are equally competitive with both ligands<sup>12</sup>. This finding can go no further than to demonstrate that binding of the 10 carbon bisquaternary inhibitor is mutually exclusive with two geometrically distinct binding sites, an anionic site in the active center and a peripheral anionic site. It can not establish that the bisquaternary inhibitor spans between these two anionic sites. However, fluorescence measurements could resolve this point since energy transfer will be highly efficient at distances of 14 Å or less. Thus, the long wavelength spectrum of propidium confers to it ideal acceptor properties and any ligand exhibiting substantial fluorescence emission between 400 to 500 nm should be a suitable donor. Nevertheless, site selectivity must be nearly absolute and we are currently examining the specificity of certain reversible ligands and a phosphoryl ester containing a dansyl group for their suitability as energy donors.

Fig. 9 summarizes the findings thus far obtained with the spectroscopic and other physical measurements. The *Torpedo* acetylcholinesterase consists of four similar, if not identical, 82 000 molecular weight subunits each containing a peripheral anionic site and an active center. The active center (A) contains the catalytic serine and is also the site of edrophonium and *N*-methylacridinium binding. Propidium binds at a peripheral anionic center (B). Ten carbon bisquaternary inhibitors are competitive with both sites and could span between them. Hydrodynamic measurements yield an effective Stokes radius of 76 Å and dimensional asymmetry is revealed in the macromolecule<sup>5</sup>. Fluorescence quenching also indicates that the ligand binding sites do not reside near an axis or center of symmetry.

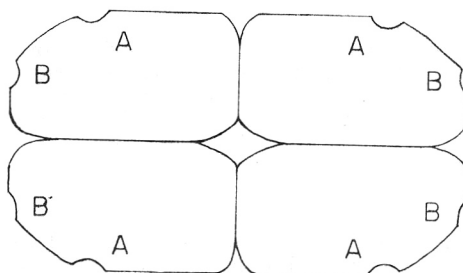


Fig. 9: Proposed scheme for ligand binding to acetylcholinesterase: The tetrameric enzyme is composed of 82 000 molecular weight subunits and shows dimensional asymmetry. Each subunit possesses a catalytic center, A, and a peripheral anionic site, B. Edrophonium and *N*-methylacridinium are relatively selective for the A site while propidium is specific for site B. Gallamine, *d*-tubocurarine, tetramethylammonium, tetraethylammonium and inorganic cations compete with propidium for site B. Bisquaternary ligands in which 14 Å separate the two quaternary nitrogens show binding which is competitive with both sites A and B and could span between them.

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## DISCUSSION

**I. Silman:**

Does residual enzyme remain bound to particulate material after your digestion procedure?

**P. W. Taylor:**

Yes, around 40%. A subsequent homogenization and digestion will remove additional material. This makes it difficult to ascertain whether there are multiple structural linkages or locations on the membrane.

**I. Silman:**

Have you tried displacing propidium with uncharged hydrophobic compounds, *e. g.* fatty acids aromatic molecules?

**P. W. Taylor:**

No.

**U. Brodbeck:**

(a) What are the effects of propidium on the catalytic properties of the enzyme? (b) Did you observe similar effects to the one described by Wermuth using acriflavine as structural probe. (c) In one of your first slides you showed that the enzyme is homogeneous upon SDS electrophoresis and rate sedimentation experiments, but heterogeneous upon isoelectric focusing. How do you account for that? What are the isoelectric points?

**P. W. Taylor:**

(a) Propidium at low ionic strength is uncompetitive with the substrate, N-methyl 7-dimethylcarbamoxiquinolinium (*cf.* ref. 12). (b) No, but we are not doing parallel experiments nor using the same substrate. (c) I doubt that there is substantial heterogeneity. The discontinuities in the profiles may have resulted from poor collection from the column. Note that the ratio of activities for the two enzymes is constant.

**R. D. O'Brien:**

Have you tried titrating the propidium binding with acetylcholine at concentrations at which excess substrate inhibition occurs? It is possible that excess substrate inhibition involves acetylcholine binding to that peripheral anionic site.

**P. W. Taylor:**

No, we have not, but it is a good suggestion. Propidium will bind to the methanesulfonylated enzyme so one could eliminate the complications of substrate hydrolysis during titration.

**W. N. Aldridge:**

Your results with propidium are extremely interesting. Would you please justify and clarify your nomenclature when you state that you consider that propidium is a specific reagent for the anionic site rather than the active site.

**P. W. Taylor:**

The existing data rather clearly illustrate that propidium binds to a site peripheral to the site of edrophonium binding and that a ternary complex involving the two ligands results. There is substantial evidence that edrophonium binds to the active center. I have considered the propidium site to be an anionic site since the analogue, ethidium, which does not contain the exocyclic quaternary ammonium binds with a 10-fold reduction in affinity. Also, a large number of cationic ligands compete with the binding of propidium. Nevertheless, it is also evident that hydrophobic binding forces are important in stabilizing the propidium-enzyme complex. Experimental evidence for this comes from the difference spectra where bathochromic shifts similar to those seen with the propidium-enzyme complex can be achieved when propidium is placed in certain solvents having a lower dielectric constant than water.

**G. Mooser:**

I am interested in your suggestion that decamethonium may bridge to a site other than what we have been calling the peripheral site. Could you elaborate on this?

**P. W. Taylor:**

That is a possibility. All that binding experiments can establish is whether binding of decamethonium is mutually exclusive with ligands that bind at both sites. Energy transfer experiments which enable one to measure intersite distances should shed some light on this issue. If the intersite distance exceeds 14 Å, obviously the decamethonium type ligands can not bind between both sites.

**SAŽETAK****Spektroskopska istraživanja interakcije liganada s acetilkolinesterazom**

*P. Taylor, J. Lwebuga-Mukasa, H. Berman i Shelley Lappi*

Istraživanja su načinjena s čišćenom acetilkolinesterazom vrste *Torpedo californica*, primjenom niza liganada prikladnih spektroskopskih svojstava. Biskvarterni amonijevi spojevi s najvećim razmakom između dušikovih atoma (14 Å), vezuju se sa svakom osnovnom jedinicom (M. t. 82 000) acetilkolinesteraze u omjeru 1:1. Uvrštenje benzokinonske skupine između kvarternih dušikovih atoma daje ligand koji pri vezivanju gasi triptofilnu fluorescenciju proteina. Kompleks pokazuje 49% fluorescencije proteina, a gašenje najvjerojatnije nastaje prijelazom energije bez radijacije između pobuđenih dipola. Kako je gašenje jednako pri vezivanju na svaku osnovnu jedinicu, malo je vjerojatno da se mjesta vezivanja liganada nalaze u blizini centra ili osi simetrije između osnovnih jedinica. Modifikacija aktivnog mjesta serina oblikovanjem odgovarajućih sulfonilnih ili fosforilnih estera, omogućava istraživanje smještaja biskvarternog liganada u odnosu na katalitičku serinsku i esterificirajuću skupinu. Drugi ligand, propidij, veže se na acetilkolinesterazu uz povećanje kvantnog iskorištenja i uz pomak valnih dužina ekscitacije i emisije. Propidij se također veže stehiometrijski sa svakom osnovnom jedinicom enzima, ali je izrazito selektivan za periferno anionsko mjesto. Prema tome, spojevi koji se vežu prvenstveno za aktivno mjesto enzima, kao edrofonij i *N*-metilakridinij, neće odcijepiti vezani propidij. Biskvarterni ligandi, kao što je dekametonij, uzajamno su kompetitivni i s aktivnim mjestom i perifernim anionskim mjestom. Velike valne duljine ekscitacije i emisije (488/632 nm) propidija, čine ga idealnim akceptorom u istraživanjima prijelaza energije s različitim ligandima-donorima koji se vezuju u aktivno mjesto.

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