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Interaction of Fluorescence Probes with Acetylcholinesterase. The Site and Specificity of Propidium Binding†

Palmer Taylor* and Shelley Lappi

ABSTRACT: A bis-quaternary fluorescence probe, propidium diiodide, has been found to exhibit a tenfold enhancement of fluorescence when bound to acetylcholinesterase from *Torpedo californica.* The complex is characterized by a high affinity, $K_D = 3.0 \times 10^{-7}$ *M,* and 1:1 stoichiometry with the 82,000 molecular weight subunit of acetylcholinesterase. A wide variety of other quaternary ammonium ligands such as decamethonium, gallamine, *d*-tubocurarine, tetraethylammonium, and tetramethylammonium will completely dissociate propidium from the enzyme as will monovalent and divalent inorganic cations. The competitive dissociation does not show cooperative behavior or a distinct requirement for occupation of multiple sites of different affinity to produce displacement. While a directly competitive relationship can be illustrated macroscopically, the various quaternary ligands show a different susceptibility toward inorganic cation displacement. The affinity of propidium relative to gallamine increases with ionic strength. This finding indicates that there is not complete equivalence in the negative subsites to which quaternary groups bind. Although edrophonium will also displace propidium from the enzyme, the dissociation constant obtained from this competitive relationship is 3.5 orders of magnitude greater than the constants obtained for inhibition of catalysis. By competitive displacement titrations it is shown that the primary binding site of edrophonium is distinct from that of propidium and a ternary complex with the two ligands can form on each subunit. In contrast to edrophonium, the binding of propidium is unaffected by methanesulfonylation of the active center serine and is uncompetitive with the carbamylating substrate, *N*-methyl-7-dimethylcarbamoxyquinolinium. Thus, it appears that propidium associates with a peripheral anionic center on the enzyme. Although propidium and edrophonium associate at separate sites on acetylcholinesterase, bis-quaternary ligands where the quaternary nitrogens are separated by 14 Å displace both ligands from the enzyme with equal effectiveness.

From steady-state kinetic studies using natural and synthetic substrates, it has become evident that various quaternary ammonium ligands and inorganic cations interact strongly with acetylcholinesterase at more than a single site (Changeux, 1966; Kitz et al., 1970; Wombacher and Wolf, 1971; Belleau et al., 1970). This evidence has been reinforced by nuclear magnetic resonance (Kato, 1972) and, more recently, fluorescence spectroscopic measurements of ligand association with acetylcholinesterase (Mooser et al., 1972; Mooser and Sigman, 1974). The latter approaches possess the inherent advantage that ligand association can be measured directly rather than relying upon the influence of the ligand on multiple catalytic parameters.

The monitoring of ligand–acetylcholinesterase complex formation by fluorescence, to date, has been based on ligands which exhibit greatly diminished quantum yields upon association with the enzyme (Mooser et al., 1972) or ligands whose absorption spectra are suitable to effect quenching of protein tryptophanyl fluorescence upon binding to the enzyme (Schnitzky et al., 1973; Taylor and Jacobs, 1974). In cases where the site of binding was analyzed, the above ligands were found to interact with the active center of acetylcholinesterase, although one end of the bis-quaternary ligands interact at a locus outside of the active center (Mooser et al., 1972; Taylor and Jacobs, 1974). We have recently observed that propidium (3,8-diamino-5,3′-diethylmethylamino-*n*-propyl-6-phenylphenanthridium) is a potent inhibitor of acetylcholinesterase catalysis and in vivo elicits competitive blockade at the neuromuscular junction

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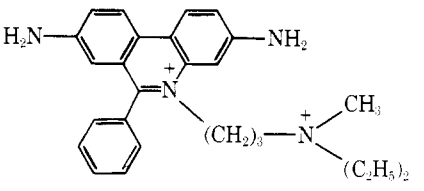
(Taylor et al., 1974b). When propidium binds to purified acetylcholinesterase it exhibits a tenfold enhancement of fluorescence, making it an ideal probe for the enzyme. A ligand of high affinity showing enhanced fluorescence in the bound state may be employed advantageously to examine rotational and segmental motion in the macromolecule (Yguerabide et al., 1970). Moreover, spectral properties of propidium should enable one to utilize energy transfer measurements to ascertain distances between the various sites on the acetylcholinesterase subunit (Stryer, 1968). For the above studies to be informative, the loci and stoichiometry of propidium–acetylcholinesterase complex formation should be rigorously established, and we report here on the specificity and site of interaction of propidium with the enzyme. The site of interaction of propidium differs from that of the other fluorescence probes which have been studied to date since it is shown that this ligand binds exclusively to a peripheral anionic site on the enzyme.

Experimental Procedure

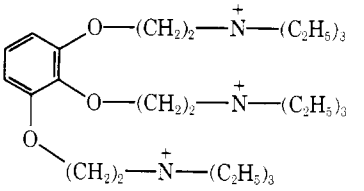
Enzyme Purification. Acetylcholinesterase from the electric organ of *Torpedo californica* was purified to apparent homogeneity by affinity chromatographic procedures as previously described (Taylor et al., 1974a). About 700 g of electroplax tissue was used in each preparative procedure and this yielded from 8 to 20 mg of purified enzyme. In some preparations, a minor nucleic acid contaminant came through the purification procedure which was not detected with gel electrophoresis. The presence of nucleic acid could be ascertained when an excess of gallamine or ambenonium did not completely eliminate the enhanced fluorescence of the bound propidium. In such cases, the enzyme preparation was incubated with 0.5 unit/ml of micrococcal nuclease (Sigma-200 unit/mg) at 25° for 30 min in the presence of 10 m*M* $CaCl_2$ and then was adsorbed and eluted a second time from the affinity column. Separation from nucleic acid could also be achieved by sedimentation in a 5–20% sucrose density gradient in 1.0 *M* NaCl, 0.04 *M* $MgCl_2$, and 0.01 *M* Tris-Cl (pH 8.0) and removing the acetylcholinesterase peak appearing at 11.0 S.

Acetylcholinesterase isolated by the above procedure has been designated as the "lytic" form since its dissociation from electroplax membranes is brought about by mild tryptic treatment of the membrane fractions. Its molecular weight has been estimated from sedimentation equilibrium and from sedimentation velocity–gel filtration measurements to be 330,000 and it is composed of four functionally and structurally similar, if not identical, subunits (Taylor et al., 1974a).

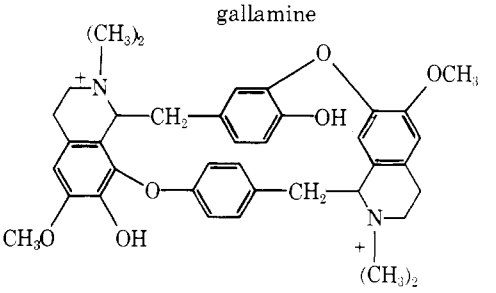
Materials. Propidium diiodide was obtained from Boots Chemical Co., Ltd., Nottingham, England, or Calbiochem and recrystallized from methanol. Edrophonium was a gift from Dr. W. E. Scott, Hoffmann-La Roche, Nutley, N.J. Ambenonium and 2,5-bis(triethylammonium-*n*-propylamino)benzoquinone were kindly provided by Dr. F. C. Nachod, Sterling-Winthrop Research Institute, Rensselaer, N.Y. *N*-Methylacridinium, bis(3-aminopyridinium)-1,10-decane, *N*-methyl-3-hydroxypyridinium iodide methanesulfonate, and *N*-methylacridinium were prepared and recrystallized according to the published procedures (Mooser et al., 1972; Rosenberry and Bernhard, 1972; Ginsburg, 1962). Decamethonium and *d*-tubocurarine were obtained from Sigma. Gallamine triethiodide was purchased from K & K Laboratories. Tetraethylammonium chloride and tetramethylammonium chloride were products from Eastman and were recrystallized from methanol. Structures of the ligands used in this study are shown below.



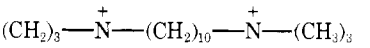
propidium



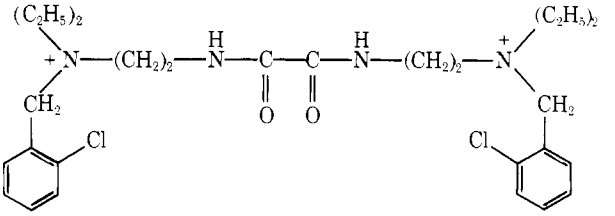
gallamine



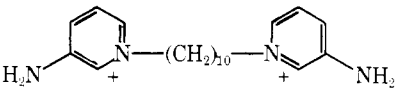
d-tubocurarine



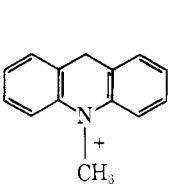
decamethonium



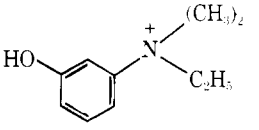
ambenonium



bis(3-aminopyridinium)-1,10-decane



N-methylacridinium



edrophonium

Sulfonylation of Acetylcholinesterase. The methanesulfonyl derivative of acetylcholinesterase was prepared by reacting *N*-methyl-3-hydroxypyridinium iodide methanesulfonate (Kitz and Wilson, 1962) with acetylcholinesterase as previously described (Taylor and Jacobs, 1974).

Absorption Spectroscopy. Spectral measurements were made using a Cary Model 16 recording spectrophotometer at 25°. For the difference spectra, tandem cells of 0.46-cm path length were employed. Initially the base-line signal with the unmixed enzyme and ligand in both light paths was

measured. The contents in the two compartments of the tandem cell in the sample beam were mixed and the difference spectrum was recorded. The cells in the two beams were interchanged and the inverted spectrum recorded. Then, the blank spectrum was recorded following the mixing of the contents in the reference cell.

Fluorescence Titrations. Details of the fluorescence titration procedure have been previously described (Taylor and Jacobs, 1974). For these studies, measurements were carried out in 1.0 cm^2 (2.0 ml total volume) or 0.3 cm^2 (0.2 ml total volume) cells. The fluorometer was equipped with a thermostated 4 cell turret so that a blank, a standard to correct for changes in excitation energy or inner filter effects, and two samples could be titrated simultaneously. Unless otherwise specified, titrations were carried out in 0.001 *M* Tris-Cl (pH 8.0) at 25°. The propidium absorption spectrum has maxima at 287 and 488 nm and shows a bathochromic shift in the presence of enzyme (Figure 3). Both the short and long wavelength transitions may be used for excitation and we have routinely employed excitation wavelengths of 290 and 535 nm to maximize the signal differences between free and bound species. Many of the competing ligands used in these studies have high extinction coefficients in the ultraviolet range and, in such cases, the longer wavelength band was employed. Emission was measured at 602 nm when a RCA IP 28 photomultiplier was used.

Analysis of Titration Data. The raw fluorescence data were corrected for volume dilution resulting from added titrant and light scatter changes incurred during the titration. For direct titrations Scatchard plots were employed and the fluorescence value for the ligand in the bound state was determined with excess acetylcholinesterase (2.2×10^{-5} equiv/l. in the 0.3-cm^2 cells). Stoichiometric equivalence is observed with the 82,000 molecular weight subunit (Taylor et al., 1974b).

For competitive displacement studies propidium was present at a concentration of 16–40 times its dissociation constant and usually in at least a twofold stoichiometric excess of acetylcholinesterase binding sites. Under these conditions the concentration of free enzyme can be considered negligible and the observed fluorescence, f, can be related to the concentrations of ligand–enzyme complexes by

$$f = f_c + \frac{(f_p - f_c)[E\text{–}P]}{[E\text{–}P] + [E\text{–}C]} \quad (1)$$

where f_p is the fluorescence of propidium when the enzyme sites are saturated by this ligand and f_c is the fluorescence of propidium when the enzyme is totally saturated with competing ligand. When propidium and the displacing ligand are competitive, f_c would also equal the fluorescence of free propidium. [E–P] and [E–C] are the concentrations of propidium–enzyme and competing ligand–enzyme complexes, respectively. By substituting for [E–P] and [E–C], terms from the following equilibrium relationships:

$$K_p = [E][P]/[E\text{–}P] \quad (2)$$

$$K_c = [E][C]/[E\text{–}C] \quad (3)$$

and rearranging one obtains

$$\frac{f_p - f}{f - f_c} = \frac{[C]}{[P]}\frac{K_p}{K_c} \quad (4)$$

[C] and [P] may be calculated from the equations:

$$[C] = [C_t] - \left(\frac{f_p - f}{f_p - f_c}\right)[E_0] \quad (5)$$

$$[P] = [P_t] - \left(\frac{f - f_c}{f_p - f_c}\right)[E_0] \quad (6)$$

where $[C_t]$ and $[P_t]$ represent the total concentrations of added competing ligand and propidium, respectively, and $[E_0]$ is the concentration of enzyme binding sites (equivalents per liter).

For eq 4 a logarithmic plot of $[(f_p - f)/(f - f_c)]$ vs. $([C]/[P])$ has a slope of unity and K_c may be calculated from the zero intercept on the abscissa and K_p. The logarithmic relationship of eq 4 is formally analogous to the Hill equation and a slope that differs from unity over the entire titration curve would reflect either inhomogeneity in binding sites or cooperative binding behavior (Flanagan and Ainsworth, 1968).

Calculations were done on a Tektronix Model 31 computer where corrected fluorescence values, corresponding volumes and concentrations of titrant, and the initial enzyme and propidium concentrations were supplied to the program. Data were calculated according to the logarithmic form of eq 4 and a slope was fitted to the data points by a weighted least-squares analysis. Details on the computer program may be obtained on request from the authors.

For the case where two ligands, C_1 and C_2, compete with propidium binding, eq 1 and 4 may be represented as

$$f = f_c + \frac{(f_p - f_c)[E\text{–}P]}{[E\text{–}P] + [E\text{–}C_1] + [E\text{–}C_2]} \quad (7)$$

and

$$\frac{f_p - f}{f - f_c} = \frac{([C_1]/K_{c_1}) + ([C_2]/K_{c_2})}{([P]/K_p)} \quad (8)$$

In the experiments where two ligands were employed, for example, a quaternary ammonium ligand and an inorganic cation, one ligand, C_1, was added at a fixed equivalence to propidium and then the mixture was titrated with the second ligand. Thus, if eq 8 is represented logarithmically where fluorescence is a function of free concentration of the second ligand, we obtain

$$\log\left[\frac{f_p - f}{f - f_c}\right] = \log\left[\frac{[C_1]}{K_{c_1}} + \frac{[C_2]}{K_{c_2}}\right] - \log\left[\frac{[P]}{K_p}\right] \quad (9)$$

Kinetic Studies. The reaction of the purified acetylcholinesterase with *N*-methyl-7-dimethylcarbamoxyquinolinium iodide was carried out as previously described (Rosenberry and Bernhard, 1971). Two buffer systems were employed: 0.001 *M* Tris-Cl (pH 8.0) and 0.1 *M* NaCl–0.04 *M* $MgCl_2$–0.01 *M* Tris-Cl (pH 8.0). At high substrate concentrations the initial carbamylation rates were too rapid to be monitored by the fluorometer and X–Y recorder so a Durrum stopped-flow spectrophotometer equipped for fluorescence detection was employed. In the latter instrument an excitation wavelength of 400 nm was used in combination with a Corning 3-72 cut off filter to exclude scattered light. Traces from the storage oscilloscope were photographed, measured, and analyzed by reciprocal plots.

Results

Propidium and Edrophonium Inhibition of the Transcarbamylation Reaction. Acetylcholinesterase hydrolysis of acyl-esters proceeds through the formation of an acyl-enzyme intermediate and inhibitors can affect steady-state kinetics by association with the transient acyl intermediate in

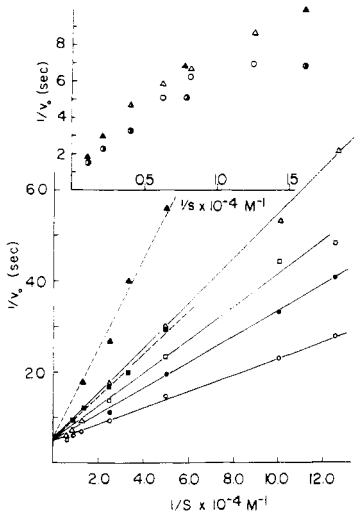


FIGURE 1: Reciprocal plots of the pseudo-first-order rate constants for serine carbamylation of acetylcholinesterase vs. substrate, *N*-methyl-7-dimethylcarbamoxyquinolinum, concentration. The buffer system was 0.01 *M* Tris-Cl (pH 8.0) containing 0.1 *M* NaCl and 0.04 *M* $MgCl_2$ at 25°. (O—O) No inhibitor; (●—●) 5.0×10^{-6} *M* propidium; (□—□) 1.0×10^{-5} *M* propidium; (△—△) 2×10^{-5} *M* propidium; (■- - -■) 4×10^{-7} *M* edrophonium; (▲- - -▲) 1×10^{-6} *M* edrophonium. The inset shows the data points at high substrate concentration measured without inhibitory ligand (◑—◑) and in the presence of 2×10^{-5} *M* propidium (▲—▲). Data shown by open circles were measured on a standard fluorometer with an X-Y recorder and the half-filled notations denote data obtained with a stopped-flow spectrophotometer using fluorescence detection. In all the kinetic experiments, the inhibitor was present in at least fourfold excess of the equivalents of enzyme. For the scheme:

$$\text{AchE} + \text{CQ} \overset{K_c}{\rightleftharpoons} \text{AchE}\cdots\text{CQ} \overset{k_2}{\longrightarrow} \text{AchE—C} + \text{QH},$$

where CQ, QH, and AchE-C represent *N*-methyl-7-dimethylcarbamoxyquinolinium, *N*-methyl-7-hydroxyquinolinium, and dimethylcarbamylacetylcholinesterase, respectively, k_2 and K_c may be calculated from the y intercept and (slope · k_2), respectively (Mooser *et al.*, 1972). If the deviations at high substrate concentration are ignored, $k_2 = 0.182\ \text{sec}^{-1}$ and $K_c = 32.2\ \mu M$.

addition to the free enzyme and enzyme–substrate complex (Krupka and Laidler, 1961). The use of a synthetic substrate where the longer lived carbamyl enzyme intermediate is formed enables one to examine the acyl transfer step alone and eliminate consideration of the kinetic parameters associated with deacylation of the complex (Rosenberry and Bernhard, 1971; Mooser et al., 1972). Since only the free enzyme species is titrated with the fluorescent ligand, propidium, equilibrium titrations can be more directly related to inhibition kinetics with the carbamylating substrate, *N*-methyl-7-dimethylcarbamoxyquinolinium, than to inhibition of acyl-ester hydrolysis.

In the higher ionic strength buffer (0.1 *M* NaCl–0.04 *M* $MgCl_2$–0.01 *M* Tris-Cl (pH 8.0)) both propidium and edrophonium give similar patterns of inhibition where both ligands appear to be nearly competitive with the substrate (Figure 1). However, a precise analysis of the mode of inhibition is complicated by deviations from Michaelis–Menton kinetics at high substrate concentrations. Complete saturation is not fully achieved as evidenced by the downward deviations in the reciprocal plots. Thus, at high substrate concentrations a second phase in the reaction kinetics is apparent. The second phase has not been reported for the *Electrophorus* enzyme (Rosenberry and Bernhard, 1971; Mooser et al., 1972) and we have yet to examine it under a variety of reaction conditions. The amplitude of the fluorescence change resulting from carbamylation is not enhanced at high substrate concentration which argues against the appearance of a second catalytic site at high concentrations of substrate. Negative deviations in reciprocal plots at high substrate concentration could be interpreted in terms of two orientational presentations of substrate resulting in catalysis, or the substrate at high concentrations may also bind to a modifier site which results in a stimulation of catalysis. Multiple sites for substrate association are consistent with observations that 1-naphthol and *N*-methylhydroxyquinolinium derivatives can inhibit the enzyme in an uncompetitive manner (Rosenberry and Bernhard, 1972).

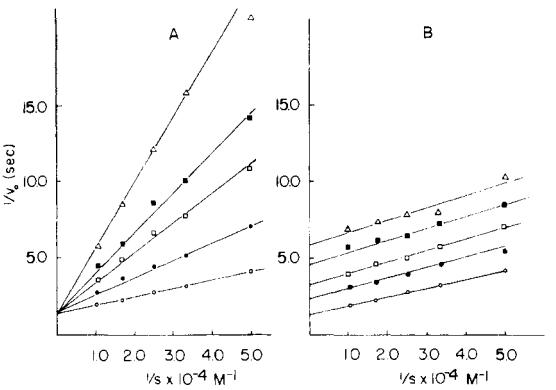


FIGURE 2: Reciprocal plots of the pseudo-first-order rate constant for serine carbamylation vs. *N*-methyl-7-dimethylcarbamoxyquinolinium concentration. The buffer was 0.001 *M* Tris (pH 8.0), 25°. (A) Edrophonium inhibition: (O—O) no inhibitor; (●—●) 1.25×10^{-7} *M* edrophonium; (□—□) 2.5×10^{-7} *M* edrophonium; (■—■) 3.75×10^{-7} *M* edrophonium; (△—△) 6.25×10^{-7} *M* edrophonium. (B) Propidium inhibition: (O—O) no inhibitor; (●—●) 1.5×10^{-7} *M* propidium; (□—□) 5.0×10^{-7} *M* propidium; (■—■) 12.5×10^{-7} *M* propidium; (△—△) 25.0×10^{-7} *M* propidium. For the scheme shown in Figure 1, $k_2 = 0.725\ \text{sec}^{-1}$ and $K_c = 39.4\ \mu M$.

At low ionic strength an enhancement of the rate of carbamylation is observed and the patterns of inhibition by edrophonium and propidium now differ substantially (Figure 2A and B). Inhibition by edrophonium remains competitive while for propidium uncompetitive behavior becomes dominant.[1] Uncompetitive inhibition can be interpreted in terms of formation of a ternary complex between the substrate, propidium, and the enzyme (Mahler and Cordes, 1971). Nearly parallel lines in reciprocal plots require in the simplest case that propidium exhibit a higher affinity for the enzyme–substrate complex than the free enzyme. Alternatively, the substrate could also bind at a noncatalytic site

[1] Using the representation of Rosenberry and Bernhard (1972)

$$\frac{1}{V_i} = \frac{1}{k_{cat}[E_0]}\left[1 + \frac{[I]}{K_{uncomp}} + \frac{K_m}{[S]}\left(1 + \frac{[I]}{K_{comp}}\right)\right]$$

where K_m and $k_{cat}[E_0]$ are the slope and intercept from a reciprocal plot of the Michaelis–Menton formulation and K_{comp} and K_{uncomp} are competitive and uncompetitive inhibition constants for the inhibitor. K_{comp} will influence the slope of the reciprocal plot and the ordinate intercept will be unaffected. K_{uncomp} will only affect the intercept. If $K_{uncomp} < K_{comp}$, in Lineweaver–Burk plots a series of parallel lines will result from successive increases in inhibitor concentration. This kind of inhibitory behavior has been called uncompetitive or coupled. It arises if the inhibitor has a higher affinity for the enzyme–substrate complex than the free enzyme and promotes formation of the transient complex (Mahler and Cordes, 1971; Webb, 1963). Parallel lines might also be apparent if the substrate binds to a modifier site as well as the catalytic site. If substrate binding at a second site affects V_0, additional terms are necessary to describe the rate profile.

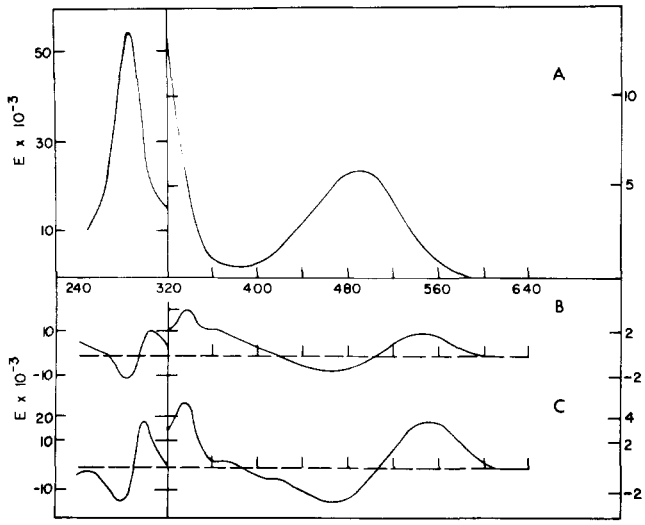


FIGURE 3: Spectral properties of propidium. (A) Absorption spectrum of propidium diiodide in 0.001 *M* Tris-Cl (pH 8.0). The ordinate values represent molar extinction coefficients. (B) Difference spectrum generated between 1.2×10^{-5} *M* propidium and acetylcholinesterase (1.52×10^{-5} equiv of binding sites/l.) 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 as described in the Experimental Section. (C) Difference spectrum between propidium in absolute methanol (sample cell) and in 0.001 *M* Tris-Cl (pH 8.0) (reference cell).

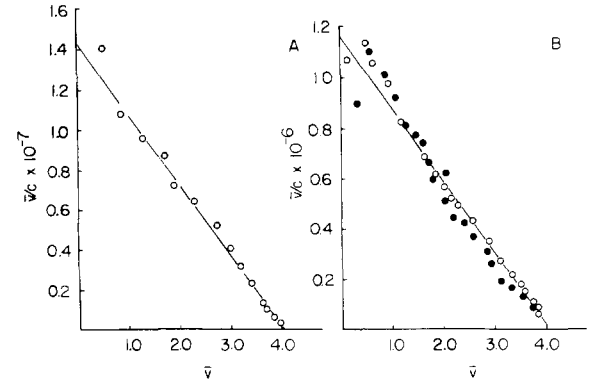


FIGURE 4: Scatchard plots of propidium binding to native acetylcholinesterase and the methanesulfonyl enzyme. Titrations were carried out in 1.0-cm² and 0.3-cm² cells at 25°. Calculations are based on a saturation value of 4.0 mol of ligand/mol of enzyme. $\bar{V}$ is the moles of ligand bound per mole of enzyme and c is the free ligand concentration. (A) 0.001 *M* Tris buffer (pH 8.0); (B) 0.01 *M* Tris buffer (pH 8.0) in 0.1 *M* NaCl and 0.04 *M* $MgCl_2$; (O—O) unreacted enzyme; (●—●) methanesulfonyl enzyme.

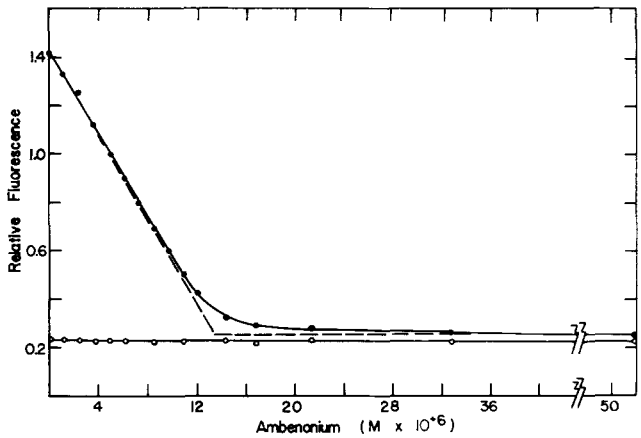


FIGURE 5: Back titration of propidium-acetylcholinesterase complex by ambenonium. Incremental additions of ambenonium were added to a solution containing 2.0×10^{-5} *M* propidium and acetylcholinesterase (1.52×10^{-5} equiv/l. based on an 82,000 molecular weight subunit). Titrations were carried out in a total volume of 0.2 ml in 0.001 *M* Tris (pH 8.0) 25°. (●—●) Propidium and enzyme; (O—O) propidium alone.

and the inhibitor competes with substrate at this site as well. While we have not attempted through replots to rigorously analyze the kinetics at low ionic strength in terms of primary inhibition constants, the influence of propidium on the carbamylation rate is instructive in that it shows a complex interaction involving substrate, inorganic cations, and the added ligand. Furthermore, the kinetics are suggestive of more than a single site for *N*-methyl-7-dimethylcarbamoxyquinolinium binding.

Difference Spectrum for the Enzyme Associated Propidium. The absorption spectrum of propidium in 0.001 *M* Tris-Cl buffer (pH 8.0) is shown in Figure 3A. The difference spectrum generated in the presence of acetylcholinesterase shows substantial shifts to longer wavelength for both the visible and major ultraviolet transition (panel B). It is of interest that we do not observe in these difference spectra superimposed peaks in the ultraviolet range that would be characteristic of protein tryptophan and tyrosine perturbations upon ligand complex formation (Donovan, 1969). In fact, a difference spectrum remarkably similar to the enzyme-associated propidium can be produced for propidium in methanol, a solvent of lower dielectric constant (panel C).

Fluorescence Titrations of Propidium Association with the Native and Sulfonylated Enzyme. The binding of propidium is stoichiometric with the 82,000 molecular weight subunit on the tetrameric acetylcholinesterase molecule (Taylor et al., 1974) and the approximate linearity of the Scatchard plots under two ionic strength conditions (Figure 4) indicates an absence of cooperative behavior for binding to this tetrameric enzyme. Certain sulfonyl esters will react with the active center serine to form a stable sulfonyl enzyme which is catalytically inactive (Wilson et al., 1962). This procedure has been shown to abolish edrophonium binding to the *Electrophorus* enzyme when measured by equilibrium dialysis (Suszkiw, 1973) and lowers the binding affinity of bis-quaternary ligands provided the groups surrounding the quaternary groups are bulky (Taylor and Jacobs, 1974). For propidium, the fluorescence binding profiles are very similar for the native and methanesulfonyl enzyme which indicates lack of overlap between the site of propidium occupation and the active center serine (Figure 4).

Dissociation of the Bound Propidium by Competing Ligands. Figure 5 shows a back titration of propidium fluorescence by the high affinity acetylcholinesterase inhibitor, ambenonium (Lands et al., 1958). The titration behavior which approximates a straight line can only yield a upper estimate of the dissociation constant but it does illustrate that propidium can be displaced completely through stoichiometric binding of this bis-quaternary ligand to each 82,000 molecular weight subunit. The other ligands we have employed show larger dissociation constants which may be readily calculated from the back titration data (Table I). Although their apparent dissociation constants differ markedly, a wide variety of cationic ligands can effect complete dissociation of the bound propidium (Figure 6). The ligands

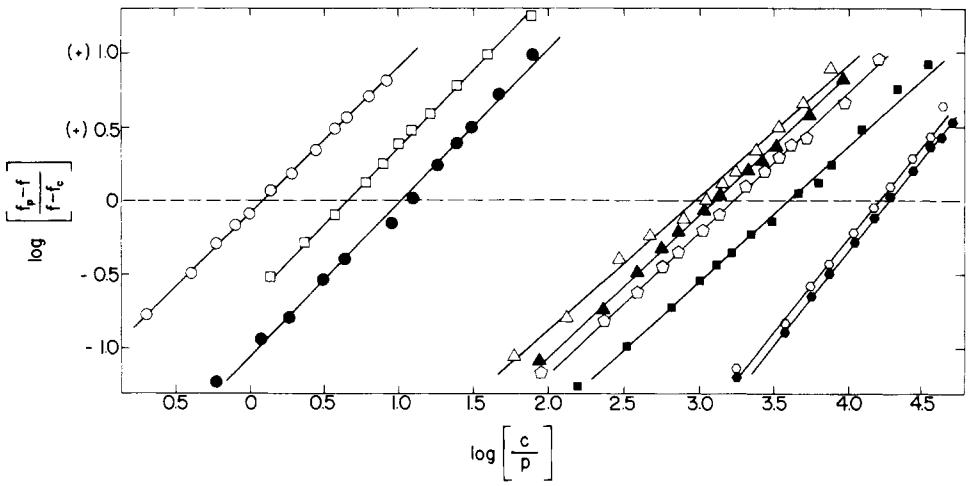


FIGURE 6: Displacement of propidium from the propidium-acetylcholinesterase complex by various ligands. Incremental additions of the ligands were added to the propidium-acetylcholinesterase complex and the fluorescence was recorded as described in the Experimental Section. Data are plotted logarithmically 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; (▲—▲) Ca^{2+}; (○—○) Mg^{2+}; (■—■) tetramethylammonium; (○—○) K^+; (●—●) Na^+. The inorganic cations were added as chlorides.

Table I: Dissociation Constants Determined for Various Ligands by Back Titration of Propidium Fluorescence.[a]

Ligand	$K_D \pm$ SEM	Slope[b]
Gallamine	$3.3 \pm 0.5 \times 10^{-7}$	0.91
d-Tubocurarine	$3.1 \pm 0.3 \times 10^{-6}$	1.03
Ambenonium	$<4 \times 10^{-9}$	
Bis(3-aminopyridinium)-1,10-decane	$\sim 6.0 \times 10^{-9}$	
Decamethonium	$1.5 \pm 0.1 \times 10^{-6}$	1.04
Tetraethylammonium	$2.9 \pm 0.1 \times 10^{-4}$	0.85
Tetramethylammonium	$1.1 \pm 0.2 \times 10^{-3}$	0.95
Edrophonium	$4.0 \pm 0.2 \times 10^{-4}$	1.02
Ca^{2+}	$4.3 \pm 0.4 \times 10^{-4}$	0.90
Mg^{2+}	$7.5 \pm 0.8 \times 10^{-4}$	0.89
Na^+	$5.9 \pm 0.7 \times 10^{-3}$	1.15
K^+	$3.6 \pm 0.5 \times 10^{-3}$	1.10

[a] Measurements were made in 0.001 *M* Tris-Cl (pH 8.0). The method of back titration and calculation of the dissociation constants are described in the Experimental Section. Values represent the mean ± the standard error for at least three titrations. [b] Slope measurements were determined from a least-squares analysis of the plots.

that are competitive for this site have apparent dissociation constants that extend over seven orders of magnitude and the respective affinities can be ordered as follows: bis-quaternary > monoquaternary ≃ divalent cations > monovalent cations.

The competitive titration curves for each ligand conform relatively well to the logarithmic form of eq 4 and the slopes do not deviate markedly from unity. Equation 4 assumes a competitive relationship between propidium and the dissociating ligand, and a unit slope would be indicative of: (1) a lack of heterogeneity in sites to which the competing ligands that dissociate propidium bind, and (2) the absence of cooperativity between subunits with respect to association of the competing ligand. For example, if binding at multiple nonequivalent sites is required for propidium displacement, a slope of unity would only be observed at the extreme ends of the graph. If cooperative behavior between subunits were evident slopes greater than unity would be anticipated. Divalent cations, tetramethylammonium and tetraethylammonium, yield overall slopes slightly less than one while for monovalent cations the slope is greater than one (Table I). The deviations from a unit slope, however, are small and, in view of the magnitude of the point scatter, it is difficult to attach a great deal of significance to slope differences (Figure 6). Thus, we are unable to detect substantial heterogeneity in binding sites or any cooperativity for the binding of these displacing ligands from the competitive displacement titrations.

Dissociation of Propidium in the Presence of Two Competing Ligands. Although propidium and other bis-quaternary ligands appear to be competitive for the same binding site, the stabilization energy conferred to the complexes for the bis-quaternary ligands results from Coulombic interactions at more than a single negative subsite on the macromolecule surface and these subsites may not necessarily be identical for each ligand. While inorganic cations would be expected to be competitive with any of the multidentate quaternary ligands, the cation's capacity for displacement of the quaternary ligands might be expected to differ and such behavior could be revealed in competition experiments. In such experiments, propidium was added in a concentration sufficient to achieve nearly complete saturation of its binding sites. The competing ligand, gallamine, was added, and the dimunition in fluorescence was recorded. Propidium fluorescence was then monitored at this fixed gallamine/propidium ratio in the presence of increasing concentrations of Mg^{2+}. If a simple competitive relation prevailed as suggested by displacement of bound propidium by either gallamine or Mg^{2+} (Figure 6), we would anticipate that the divalent cation would cause further displacement of propidium in the presence of a fixed ratio of gallamine and propidium. The ratio of bound gallamine and propidium would remain constant. For this situation plots of the logarithmic formulation of eq 8 are described by a curved line with limiting slopes of zero and unity at low and high Mg^{2+} concentrations.

Upon titration with Mg^{2+}, however, we observe an initial increase in fluorescence followed by a decrease at higher divalent cation concentrations (Figure 7a). In the logarithmic plots this behavior manifested by negative deviations from the theoretical lines (Figure 7b). It is most likely that the

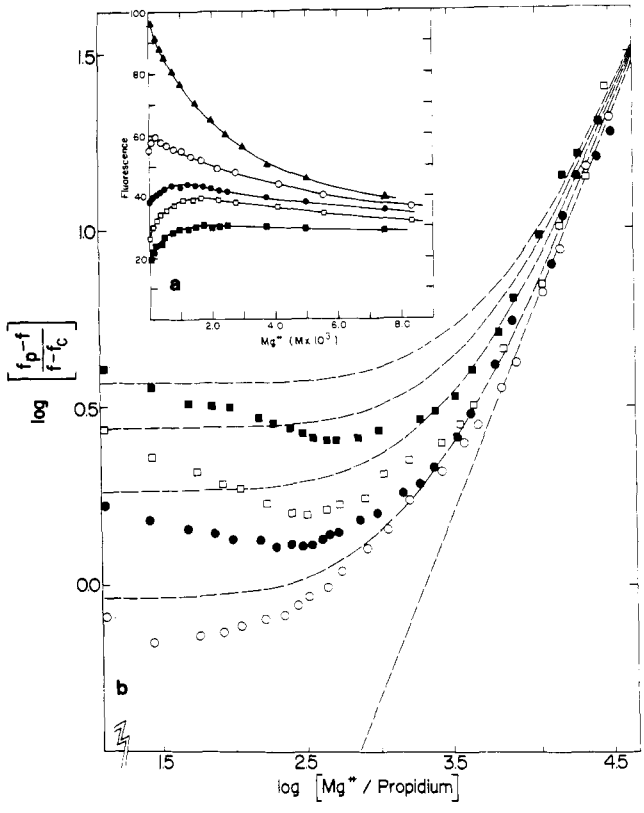


FIGURE 7: Back titration of propidium fluorescence by Mg^{2+} in the presence of fixed ratios of gallamine to propidium. The fluorescence of the propidium–enzyme complex was recorded before and after the addition of the competing ligand, gallamine, and then monitored following incremental additions of the divalent cation. In each titration shown here the propidium concentration was 5×10^{-6} M and acetylcholinesterase was present at 3.8×10^{-6} equiv/l. (○—○) 5.0×10^{-6} M gallamine; (●—●) 1.0×10^{-5} M gallamine; (□—□) 1.5×10^{-5} M gallamine; (■—■) 2.0×10^{-5} M gallamine. (a) Fluorescence changes during the initial phase of the Mg^{2+} titration. (b) Data from (a) plotted according to the logarithmic form of eq 8. The dotted lines represented the calculated fit to eq 8 where $K_p = 3.0 \times 10^{-7}$ M, $K_G = 3.3 \times 10^{-7}$ M, and $K_{Mg} = 6.5 \times 10^{-4}$ M and P, G, and Mg^{2+} denote propidium, gallamine, and magnesium, respectively.

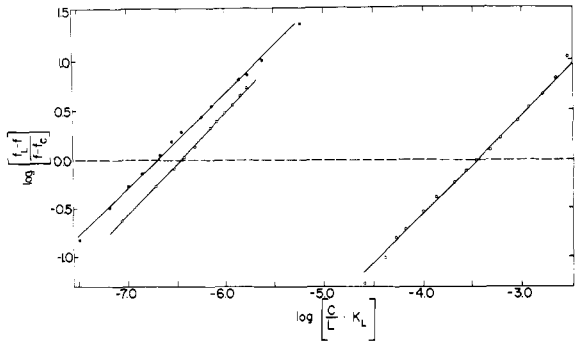


FIGURE 8: Back titration of propidium, N-methylacridinium and bis(3-aminopyridinium)-1,10-decane by edrophonium. The titration procedure is identical with that used in Figure 6 but the fluorescence of the dissociated ligand is measured at the following wavelengths: N-methylacridinium, λ_{ex} 390/λ_{em} 500 nm; bis(3-aminopyridinium)-1,10-decane, λ_{ex} 320/λ_{em} 402 nm. The graph differs from Figure 6 in that the abscissa is multiplied by K_L, the dissociation constant of the fluorescent ligand. K_L was measured independently by direct titration and the values used here are: K (propidium) = 3.0×10^{-7} M, K (N-methylacridinium) = 8.0×10^{-8} M, K (3-aminopyridinium-1,10-decane) = 6×10^{-9} M. (○—○) Propidium; (●—●) N-methylacridinium; (□—□) bis(3-aminopyridinium)-1,10-decane.

Table II: Dissociation Constants Determined for Edrophonium by Competition with Various Ligands.

Ligand	K_D
Propidium[a]	4.0×10^{-4}
N-Methylacridinium[b]	1.9×10^{-7}
Bis(3-aminopyridinium)-1,10-decane[b]	3.3×10^{-7}
N-Methyl-7-dimethylcarbamoxyquinolinium[c]	1.1×10^{-7}

[a] Determined by back titration as described in the Experimental Section. [b] Determined by back titration of N-methylacridinium and 3-aminopyridinium-1,10-decane fluorescence at 390/510 and 320/405 nm, respectively. [c] Inhibition constant calculated assuming competitive inhibition of N-methyl-7-dimethylcarbamoxyquinolinium hydrolysis.

observed fluorescence behavior reflects an initial increase in the fraction of propidium bound rather than an enhancement of quantum yield of the bound ligand since fluorescence increases were not observed when gallamine or Mg^{2+} were employed separately as displacing agents (Figure 6). Thus, Mg^{2+} dissociates gallamine more effectively than propidium which indicates that the individual negative subsites which confer stability to the quaternary ammonium groups of gallamine are not completely equivalent to those stabilizing the propidium–enzyme complex.

Ligands Selective for the Active Center of Acetylcholinesterase. A comparison of Figure 2 and Table I reveals that edrophonium inhibits serine carbamylation at concentrations far lower than are required for dissociation of propidium and indicates that occupation of different sites are involved in the two phenomena. Displacement of various fluorescent ligands by edrophonium is shown in Figure 8 and the apparent dissociation constants calculated from these studies are tabulated in Table II.

The dissociation constants for the fluorescent ligands were measured independently by direct fluorescence titration and these values were employed in the calculation of the edrophonium dissociation constants (cf. eq 4). Competitive dissociation of edrophonium by either N-methylacridinium or bis(3-aminopyridinium)-1,10-decane yields dissociation constants for edrophonium which are in good accord with its inhibition constant, K_I, calculated from competitive inhibition of enzyme serine carbamylation by N-methyl-7-dimethylcarbamoxyquinolinium. The locus of interaction involved here would be the active center. These observations are consistent with the earlier findings of Sigman and his colleagues where it was proposed on the basis of competitive inhibition with substrate that N-methylacridinium and bis(3-aminopyridinium)-1,10-decane associate with the active center of *Electrophorus* acetylcholinesterase (Mooser et al., 1972).

In contrast, competition for binding between propidium and edrophonium yields a dissociation constant for edrophonium that is 5000-fold greater than that obtained with the above ligands. Since competition between edrophonium and propidium can only be demonstrated at high edrophonium concentrations, the primary binding site of propidium differs from that of edrophonium. The differences in dissociation constants that we observe in Table II indicate that a ternary complex forms with propidium bound to one site and either edrophonium or N-methylacridinium on the second site. Scatchard plots for propidium binding to the free acetylcholinesterase and its edrophonium and N-methylacridinium complexes are similar (Figure 9) suggesting that

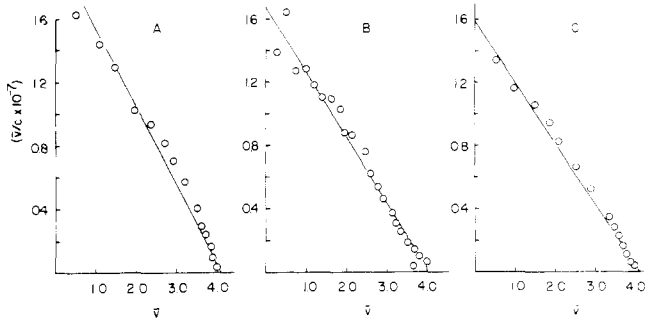


FIGURE 9: Scatchard plots of propidium binding to acetylcholinesterase and its *N*-methylacridinium and edrophonium complexes. Titrations were carried out in 1.0-cm^2 cell in 0.001 *M* Tris-Cl (pH 8.0) with 535 and 602 nm as the excitation and emission wavelengths. Calculations are based upon saturation of the binding sites resulting in 4.0 mol of ligand bound/mole of enzyme. (A) 7.5×10^{-7} equiv/l. of acetylcholinesterase; (B) 7.5×10^{-7} equiv/l. of acetylcholinesterase with 1×10^{-6} *M* *N*-methylacridinium; (C) 7.5×10^{-7} equiv/l. of acetylcholinesterase and 5×10^{-6} *M* edrophonium. Fluorescence signals of the ligands in the absence of enzyme were subtracted from the recorded values for the ligand–enzyme complexes. The fluorescence signals of B and C at saturation were 0.92 and 1.03 of A, respectively. $\bar{\nu}$ is the moles of propidium bound/mole of enzyme and *C* is the free propidium concentration.

the latter two ligands do not alter appreciably the affinity of the propidium–acetylcholinesterase interaction. Thus, it appears that propidium binds exclusively to a site peripheral to the active center of the enzyme.

Although edrophonium and propidium do not have a common primary binding site, both appear competitive with the bis-quaternary inhibitor, bis(3-aminopyridinium)-1,10-decane (Tables I and II). Thus, when the two quaternary groups are separated by 14 Å, binding of this ligand becomes mutually exclusive with ligands that associate at either the peripheral anionic site or the active center.

Discussion

Our findings on the site of propidium binding lend support to previous work indicating that two discrete sites for cationic ligands exist on acetylcholinesterase (Belleau et al., 1970; Kitz et al., 1970; Mooser and Sigman, 1974). Propidium clearly exhibits a different site specificity than do the bis-quaternary (Mooser et al., 1972; Taylor et al., 1974a) and acridinium (Mooser et al., 1974) ligands that have been employed previously for fluorescence titrations of acetylcholinesterase.

Edrophonium would appear to be a most satisfactory frame of reference for delineating the sites of ligand binding on acetylcholinesterase. Early investigations with trialkylphenylammonium analogs showed that a *m*-hydroxyl substitution greatly enhanced the inhibitory potency of these congeners, and it was suggested that hydrogen bonding with an active site serine might confer additional stabilization energy to the complex (Wilson and Quan, 1958). Inhibition by edrophonium is competitive with the carbamylating substrate, *N*-methyl-7-dimethylcarbamoxyquinolinium under conditions of low and high ionic strength (Figures 1 and 2). In addition, equilibrium dialysis studies of edrophonium binding to the *Electrophorus* enzyme have shown 1:1 stoichiometry with serine hydroxyl groups phosphorylated by diisopropyl fluorophosphate (Suszkiw, 1973). The high affinity binding of edrophonium ($K_D = 2.5 \times 10^{-7}$ *M*) is abolished by prior sulfonylation of the active site serine (Suszkiw, 1973).

Propidium shows 1:1 stoichiometry in binding to the 82,000 molecular weight subunit on acetylcholinesterase (Taylor et al., 1974b) and can be stoichiometrically displaced by the high affinity ligand, ambenonium (Figure 5). However, propidium, in addition to not being competitive with edrophonium binding at its primary site, shows inhibition that is uncompetitive with substrate but nearly complete at low ionic strength. Moreover, propidium binding does not appear to be influenced by methanesulfonylation of the active site serine. Using these comparative criteria, propidium appears to be highly selective for a single peripheral anionic site on each acetylcholinesterase subunit and exhibits no overlap with the active center. While propidium is a bis-quaternary compound, it differs from the other ligands studied here in that the two quaternary nitrogens are maximally separated by only 4.8 Å.

Although propidium and edrophonium do not competitively displace each other from their primary binding sites, bis-quaternary ligands where the quaternary nitrogens are maximally separated by 14 Å displace both edrophonium and propidium (Tables I and II). The dissociation constants estimated from the competitive back titrations agree closely with those obtained from direct binding measurements. This finding alone, however, can go no further than to demonstrate that binding of the bis-quaternary inhibitor is mutually exclusive with ligands that associate at either the active center or a peripheral anionic site. It cannot establish that the 14 Å bis-quaternary inhibitors span between both sites. More information on the latter possibility should be forthcoming since relatively specific spectroscopic probes for the two sites are now available. For example, energy transfer measurements for a suitably designed ternary complex should enable one to ascertain whether the intersite distance is compatable with a 14-Å span. Furthermore, some of the ligands may be useful in examining conformational changes associated with complex formation.

Our findings with propidium provide independent support for previous considerations of acetylcholinesterase ligand specificity using *N*-methylacridinium and bis(3-aminopyridinium)-1,10-decane (Mooser et al., 1974). From a comparison of edrophonium displacement of propidium and *N*-methylacridinium, it is evident that the latter ligand is relatively specific for the active center as proposed from kinetic studies by Mooser et al. (1972). Their investigations have also revealed that the 10-carbon bis-quaternary compounds, bis(3-aminopyridinium)-1,10-decane and decamethonium, are stabilized by interaction at two discrete sites, one of which is the active center. This possibility is also supported by fluorescence quenching studies of complex formation between bis-quaternary benzoquinonium derivatives and chemically modified acetylcholinesterase species (Taylor and Jacobs, 1974). The other quaternary group could well overlap with the propidium binding site provided there is no conformational change associated with binding of these ligands.

Mooser et al. (1974) observed that gallamine caused direct dissociation of *N*-methylacridinium and we have observed that gallamine will displace propidium. Thus, it is quite possible that the binding of gallamine, like the 10-carbon bis-quaternary ligands, is mutually exclusive with both sites even though the maximum distance which separates the quaternary nitrogens is 1.7 Å less than in the 10-carbon aliphatic derivatives. Various kinetic studies, however, would suggest that the mode of binding of gallamine differs from that of the 10-carbon bis-quaternary ligands. The lat-

ter ligands accelerate methanesulfonation of the active site serine while the binding of gallamine decreases the sulfonylation rate (Belleau et al., 1970). Also, gallamine will accelerate the decarbamylation of the dimethylcarbamyl enzyme while decamethonium has no effect on the rate of decarbamylation (Kitz et al., 1970).

The displacement of *N*-methylacridinium by *d*-tubocurarine indicates that the curare derivative binds at two sites having equivalent affinities but binding to only one of them will cause dissociation of *N*-methylacridinium (Mooser and Sigman, 1974). The binding of the first *d*-tubocurarine molecule destabilizes the complex for binding of the second *d*-tubocurarine molecule. If a similar alternative site situation prevailed for *d*-tubocurarine dissociation of propidium, rather large deviations from the logarithmic plots (Figure 6) would have been evident. Since single site occupation describes rather well *d*-tubocurarine dissociation of propidium, it is possible that *d*-tubocurarine binding at either of the two sites detected by Mooser and Sigman is sufficient to dissociate propidium from its peripheral anionic site. It should be recognized that Sigman and colleagues have used the fresh water species, *Electrophorus electricus*, as their enzyme source, while the *Torpedo* enzyme that we have used comes from a marine species. However, a comparison of findings from the two studies would seem justified since the ligand specificity and properties of the binding sites have remarkable qualitative similarity.

The wide variety of cationic ligands which are effective in dissociating propidium from its site on acetylcholinesterase emphasizes the essential role played by Coulombic forces in stabilizing the complexes. The high affinity observed for multidentate ligands makes it apparent that more than a single negative subsite is involved in stabilization of these complexes. Even though gallamine and propidium displace each other from the surface of acetylcholinesterase, these competing quaternary ligands show a different sensitivity towards their dissociation by divalent cations (Figure 7). With increasing concentrations of Mg^{2+} the apparent affinity of propidium relative to gallamine increases. Thus, a simple competitive relationship for occupation of a single site does not exist for the three ligands. The negative subsites which stabilize the quaternary groups in the complex, presumably two for propidium and three for gallamine, on a microscopic basis are not strictly equivalent. This situation might arise if the binding area on the macromolecule, which has been described as the peripheral anionic site, consists of an array of negative subsites that allow multiple orientations for the binding of multidentate ligands. Alternatively, acetylcholinesterase may exhibit an ionic strength dependent conformational change and the conformational states are reflected in different relative affinities for the two ligands. Different conformational states of acetylcholinesterase have been proposed previously as a possible explanation for synergistic inhibition shown by certain inhibitor combinations (Rosenberry and Bernhard, 1972) and for partially competitive inhibition of substrate catalysis observed for various ligands (Changeux, 1966). Equilibrium titrations do not enable one to resolve which of the two alternatives is correct but further work on the kinetics of ligand complex formation should prove helpful in this regard.

The availability of a fluorescence probe which shows enhanced fluorescence when bound affords the opportunity of examining conformation and topology of the acetylcholinesterase–ligand complexes. Such studies are dependent on delineating the stoichiometry and site specificity for the probe which fortunately, for propidium, has only a single site of interaction on each acetylcholinesterase subunit. Propidium also exhibits competitive blockade at the neuromuscular junction (Taylor et al., 1974b) and similar studies on the isolated cholinergic receptor may be equally informative in characterizing the properties and ligand specificity of that macromolecule.

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