Parker, C. A. (1967), *in* The Triplet State, Zahlan, A. B., Ed., Cambridge, The University Press, p 353.
Schmidt, O. (1958), *J. Phys. Chem. 39*, 59.
Silver, S. (1967), *J. Mol. Biol. 29*, 191.
Simon, M. I., and Van Vunakis, H. (1964), *Arch. Biochem. Biophys. 105*, 197.
Spikes, J. D., and MacKnight, M. L. (1970), *Ann. N. Y. Acad. Sci. 171*, 149.
Wacker, A., Dellweg, H., Träger, L., Kornhauser, A., Lodeman, E., Trück, G., Selzer, R., Chandra, P., and Ishimoto, M. (1964), *Photochem. Photobiol. 3*, 369.
Yamabe, S. (1969), *Arch. Biochem. Biophys. 130*, 148.

Fluorescent Probes of Acetylcholinesterase†

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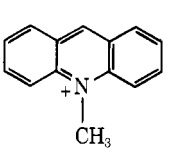
ABSTRACT: *N*-Methylacridinium ion and bis(3-aminopyridinium)-1,10-decane are two potent cationic inhibitors of acetylcholinesterase from *Electrophorus electricus* which should be useful as fluorescent probes of the various types of binding interactions in which the enzyme can participate. The fluorescence spectra of both inhibitors are strongly quenched upon binding to the enzyme, and one molecule of each inhibitor binds to the enzyme per catalytic site titratable by 7-dimethylcarbamyl-*N*-methylquinolinium iodide. The dissociation constant for each inhibitor determined by fluorometric titration agrees closely with the inhibition constants obtained from steady-state kinetic studies. For *N*-methylacridinium ion, the dissociation constant is $2.32 \pm 0.75 \times 10^{-7}$ M while for bis(3-aminopyridinium)-1,10-decane the dissociation constant is $5.16 \pm 1.41 \times 10^{-8}$ M. All the available data are consistent with both probes binding in a region which overlaps the acetylcholine specific site at the active site. In addition, one aminopyridinium moiety of bis(3-aminopyridinium)-1,10-decane appears to interact at a cationic specific binding site remote from the active site. By studying the competitive displacement of both probes from the enzyme, it should be possible to examine the binding specificity of both cationic binding sites toward various acetylcholine antagonists. The use of 7-dimethylcarbamyl-*N*-methylquinolinium as an active-site titrant has permitted the precise measurement of the turnover number per active site of a commercial enzyme preparation before and after purification by affinity chromatography. Using this value, the purified enzyme appears to have 4 catalytic subunits/260,000 molecular weight.

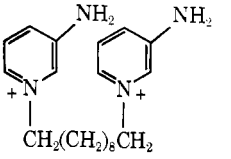
Recent studies on the ligand binding properties of acetylcholinesterase (AChE)[1] have indicated that the enzyme may possess binding sites for acetylcholine (ACh) and acetylcholine antagonists at loci remote from the active site (Changeux, 1966; Meunier and Changeux, 1969; Kato *et al.*, 1970; Kitz *et al.*, 1970). Kinetic studies have constituted the main experimental approach to suggest the existence of these peripheral sites although recently nuclear magnetic resonance (nmr) experiments have supported the existence of a specific binding site for atropine which is distinct from the binding site at the active site (Kato *et al.*, 1970).

An additional and potentially very useful approach for the study of the ligand binding properties of AChE would be to devise specific fluorescent probes of the different binding sites on AChE and use them as indicators of the types of interactions in which acetylcholinesterase can participate. Two properties of fluorescent probes make them well suited for the study of AChE. First, the sensitivity of fluorescence measurements permits the use of relatively low concentrations of the enzyme; and secondly, fluorescence spectra of ligands bound to proteins are frequently sensitive to the microenvironment in which they are located.

In the present communication, we wish to report that N-methylacridinium chloride (I) and bis(3-aminopyridinium)-1,10-decane diiodide (II), two highly fluorescent compounds



I



II

bind very tightly to AChE. They are suitable as fluorescent probes of the enzyme since the fluorescence spectra of both of them are strongly quenched upon binding to the enzyme. The fluorescence changes which occur as a consequence of the formation of the enzyme–inhibitor complex can be readily used to quantitate their interactions with the enzyme as well as those of the ligands which affect the binding of I and II. They can also be used to determine the binding site normality of a solution of electric eel AChE.

The enzyme normalities determined with I and II agree with those measured using 7-dimethylcarbamyl-*N*-methylquinolinium iodide (III) as an active-site titrant. The titrimetric method employing III is a single turnover assay which is based on the intense fluorescence of 7-hydroxy-*N*-methylquinolinium (IV) (Prince, 1966b) in its zwitterionic form (V) and the inhibition of AChE by III (Kitz *et al.*, 1967). This procedure has been reported by Rosenberry (1970) and by

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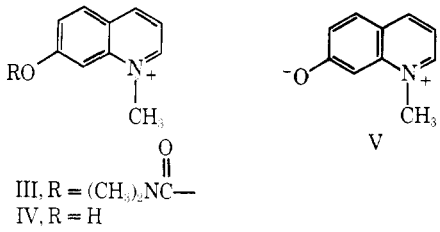
[1] Abbreviations used are: AChE, acetylchloinesterase; ACh, acetylcholine.



III, R = $(CH_3)_2NC$—
IV, R = H

V

Rosenberry and Bernhard (1971). Independently, we had investigated this method because a reliable method for measuring active-site normality was essential for the determination of the stoichiometry of binding of I and II. The various kinetic parameters obtained by us for the reaction of III with acetylcholinesterase are in close agreement with those that have been reported by Rosenberry and Bernhard (1971).

Experimental Section

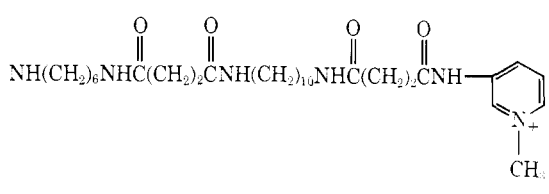
Materials

N-Methylacridinium iodide was synthesized by stirring 3.0 g of acridine (Aldrich) in 10 ml of iodomethane (Eastman) and 15 ml of acetone for 18 hr. The precipitate that formed was filtered and yielded red crystals on recrystallization from methanol–anhydrous ether. The chloride salt used in the experiments was made by passing the iodide salt through a Dowex 1-Cl^- column. A slight impurity could be detected in the chloride salt by descending paper chromatography using Whatman No. 3MM paper and eluting with 1-butanol–acetic acid–water (8:12:3, v/v). It was removed by passing the *N*-methylacridinium chloride twice through a 1 × 40 cm Sephadex G-10 column, eluting with water. Following lyophilization the yellow crystals were recrystallized from acetonitrile, 176–179° dec (lit. (Andrien, 1966) 183° dec).

Bis(3-aminopyridinium)-1,10-decane diiodide was synthesized by refluxing 4.0 g of 3-aminopyridine (Aldrich) and 2.1 g of 1,10-diiododecane (K & K) in 30 ml of acetone for 96 hr. The solution was evaporated to near dryness under reduced pressure and a precipitate formed. The solution was filtered and the precipitate was dissolved in methanol, treated with activated charcoal, and twice recrystallized from methanol–anhydrous ether. Light yellow crystals were recovered in a 51% yield, mp 180–181.5°. *Anal.* Calcd for $C_{20}H_{32}N_4$: C, 41.25; H, 5.54; N, 9.63. Found C, 41.28; H, 5.57; N, 9.46.

7-Hydroxy-N-methylquinolinium iodide was prepared according to Prince (1966a). 7-Dimethylcarbamyl-*N*-methylquinolinium iodide was prepared according to Kitz *et al.* (1967). Acetylcholine bromide (Eastman) was recrystallized from methanol–anhydrous ether. All other chemicals were reagent grade and used without further purification. Sepharose 2B was purchased from Pharmacia.

Acetylcholinesterase from *Electrophorus electricus* was obtained from Worthington Biochemical Corp. and used either directly or following purification with a Sepharose affinity column. The column was prepared according to Cuatrecasas (1970) and is similar to that reported by Berman and Young (1971) with the exception that the side chain and inhibitor attached to the Sepharose appeared as



In the preparation of the column, units successively attached were 1,6-diaminohexane, succinic anhydride, 1,10-diaminodecane, succinic anhydride, and *N*-methyl-3-aminopyridinium iodide.

The commercial AChE preparation (3 mg), in which the enzyme represents only 10% by weight of the total protein, was purified using 5 ml of the modified Sepharose in a 1-cm diameter column at 4°. After the enzyme was placed on the column, it was washed with 30 ml of a solution of 0.05 M NaCl, 0.02 M $MgCl_2$, and 0.001 M phosphate (pH 7.0) at a flow rate of 9 ml/hr. No activity could be detected in the eluate. The enzyme was eluted with the same buffer containing 0.01 M decamethonium bromide. The fractions containing the majority of activity (about 20 ml) were combined and concentrated to 4 ml on an Amicon ultrafiltration unit with a PM10 Diaflo membrane. The decamethonium was removed by dialyzing the concentrated solution three times for 18 hr against 4 l. of 0.1 M NaCl–0.02 M $MgCl_2$–0.001 M phosphate (pH 7.0).

Acetylcholinesterase purified in this manner shows a single band on acrylamide gel (7%) electrophoresis, pH 8.25, staining with Amido Black. Without purification, the commercial AChE preparation, upon acrylamide gel electrophoresis, contained three major protein components in addition to the enzyme. The enzyme was used within a few days after purification as it partially precipitated out of the buffered solution. The cloudy material was not used in any of the analyses, although its normality as measured with III and its activity as measured with ACh remained identical to that of the freshly prepared enzyme.

Methods

Enzyme Assay. AChE activity was measured with a Radiometer pH-Stat equipped with two syringes: one containing NaOH and the other containing an equimolar concentration of ACh (Jensen-Holm, 1961). With this apparatus the concentration of ACh in the reaction vessel remained constant during the reaction. Several rates were measured during a single assay by adding periodic aliquots of ACh. Standard assay conditions included a 20-ml volume of 0.1 M NaCl and 0.02 M $MgCl_2$ at 25°. The pH was maintained at 7.0 with 0.01 M NaOH. These standard assay conditions were used to determine the specific activity of AChE purified by affinity chromatography. The protein concentration used in the calculation of the specific activity was determined by quantitative amino acid analysis (Spackman *et al.*, 1958) following hydrolysis in 6 N HCl.

Fluorescent Ligand Titrations. All ligand binding fluorescence measurements were made on an Aminco-Bowman spectrophotofluorometer equipped with a 150-W xenon lamp, 1P21 photomultiplier tube, and a thermostatted cell compartment. Compound I fluoresces maximally at 490 nm when excited at 360 nm, and II fluoresces at 410 nm when excited at 330 nm at pH 7.0. The intense fluorescence of both chromophores is strongly quenched when bound to AChE.

Enzyme normality with respect to these ligands and the intrinsic dissociation constants of their complex with the enzyme may be determined by plotting the reciprocal of the bound ligand concentration against the reciprocal of the free ligand concentration according to eq 1 (Klotz, 1946),

$$\frac{1}{[F_b]} = \frac{1}{[E_0]} + \frac{K_{diss}}{[E_0][F_f]} \qquad (1)$$

where $[E_0]$ is the total enzyme normality for the given ligand,

K_{diss} is the dissociation constant of the enzyme–inhibitor complex, and $[F_b]$ and $[F_f]$ are the bound and free concentrations of fluorescent inhibitor, respectively.

The bound and free concentrations of the fluorescent inhibitor ($[F_b]$ and $[F_f]$, respectively) can be obtained if the intensity coefficient of the free ligand, γ (the proportionality factor between oberved fluorescence intensity and concentration) and the intensity coefficient of the bound ligand, γ', are known. The explicit expression for $[F_b]$ and $[F_f]$ are

$$[F_b] = \frac{I_t - I_{obsd}}{\gamma - \gamma'} \quad (2)$$

$$[F_f] = \frac{I_{obsd} - \gamma'[F_0]}{\gamma - \gamma'} \quad (3)$$

where I_t is the measured fluorescence intensity in the absence of enzyme, I_{obsd} is the fluorescence intensity in the presence of enzyme, and $[F_0]$ is the total concentration of ligand. The intensity coefficient of the free ligand (γ) is obtained in the absence of enzyme, and the intensity coefficient of the AChE-bound ligand (γ') is determined according to eq 4 which

$$\frac{[F_0]}{\gamma[F_0] - I_{obsd}} = \frac{K_{diss}}{[E_0](\gamma - \gamma')} + \frac{1}{\gamma - \gamma'} \quad (4)$$

is valid if $[E_0]$ is much greater than $[F_0]$ and where $[E_0]$ need only be known in relative units. For both I or II, γ' is insignificant relative to γ so that eq 2 and 3 reduce to eq 5 and 6.

$$[F_b] = \frac{I_t - I_{obsd}}{\gamma} \quad (5)$$

$$[F_f] = \frac{I_{obsd}}{\gamma} \quad (6)$$

Titrations with I and II following eq 1 were done in a 1-ml volume with 0.1 M NaCl–0.02 M $MgCl_2$–0.01 M phosphate (pH 7.0) at 25°. Enzyme normalities were in the range of 3×10^{-8} to 1.5×10^{-7} N. Fluorescence intensity was recorded after each of several 5-μl aliquots of fluorescent ligand was added to the enzyme solution (Figures 1 and 2).

Care is needed in pipetting II since concentrations of a stock solution near 5×10^{-6} M bind to the glass pipet. This problem can be alleviated by always using a clean, dry, "to contain" micropipet, drawing the solution into the pipet only once, and rinsing the contents several times into the cuvet. Binding to the cuvet walls was not a problem since the concentrations were well below the binding affinity for the glass. In the absence of enzyme, fluorescence intensity was linear with concentration of fluorescent ligand over the concentration range used in the experiments. Freshly prepared solutions of I were used in all fluorimetric measurements. Its fluorescence intensity was also linear over the concentration range used in the experiments.

Ligand binding stoichiometry was additionally evaluated for II and I binding to AChE by the method of continuous variations (Martell and Çharabek, 1959). In this procedure the ratio of ligand to enzyme is varied while the sum of their concentrations is kept constant. The enzyme–ligand complex, as measured by fluorescence quenching, reaches a maximum at their stoichiometric ratio.

Active-Site Titration. The titrimetric procedure using III is rapid and sensitive. The normality of enzyme solutions as low as 1×10^{-8} N could be accurately determined in 5–10 min using the Aminco-Bowman instrument. Its validity as a titrimetric method is a consequence of the kinetic scheme for AChE which can be represented as

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' \xrightarrow{k_3} E + P_2 \quad (7)$$
$$\qquad\qquad\qquad\qquad\quad + P_1$$

where E is the free enzyme, S is the substrate, ES′ is a covalent intermediate, P_1 is the first product released, and P_2 is the product of hydrolysis of ES′. The explicit equation for the production of P_1 as a function of time has been derived (Gutfreund and Sturtevant, 1956; Bender, 1971) for enzymes which proceed by the kinetic scheme indicated in eq 7. In the reaction of III with AChE where $k_2 \gg k_3$ and when the concentration of III is greater than $K_s k_3/k_2$ ($K_s = k_{-1}/k_1$), the initial burst of IV is equal to the enzyme concentration.

The values of k_2, k_3, and K_s have been determined by the procedure indicated below and are reported in Tables I and II. k_2 and K_s can be readily measured using eq 8 since k_{obsd}, the experimental pseudo-first-order rate constant describing production of dimethylcarbamyl-AChE from I is slow enough

$$\frac{1}{k_{obsd}} = \frac{1}{k_2} + \frac{K_s}{k_2}\frac{1}{[S_0]} \quad (8)$$

to be observed with conventional mixing-techniques. The rate of hydrolysis of the dimethylcarbamyl-enzyme, k_3, can be determined from the steady-state velocity (v_{ss}) of the reaction using eq 9.

$$v_{ss} = k_3[E_0] \quad (9)$$

The value of III as a titrant for AChE, as opposed to other dimethyl carbamate esters, such as *o*-nitrophenyldimethylcarbamate (Bender *et al.*, 1966) is that III is very weakly fluorescent while V fluoresces intensely with an emission maximum at 510 nm when excited at 410 nm. Since concentrations of V can be detected as low as 10^{-8} M, enzyme concentrations of AChE in this range can be routinely measured. Typical carbamylation experiments were performed by adding a 5-μl aliquot of III to a 1-ml solution of enzyme (about 0.1×10^{-7} to 1.0×10^{-7} M) to give a final III concentration

TABLE I: Effect of pH on the Reaction of 7-Dimethylcarbamyl-*N*-methylquinolinium with Acetylcholinesterase.[a]

pH	k_2 (min^{-1})	$k_3 \times 10^2$ (min^{-1})	K_s (μM)	Rel Enzyme Concn[b]
6.0	4.7	0.7	11.0	0.98 ± 0.08
7.0	4.9	2.6	8.6	1.00 ± 0.06
7.5	5.3	3.9	4.9	1.02 ± 0.06
8.0	5.2	4.9	4.5	1.01 ± 0.05

[a] Worthington enzyme (0.5×10^{-7}–1.2×10^{-7} M) used without further purification. 1-ml assay volume containing 0.1 M NaCl, 0.02 M $MgCl_2$, and 0.01 M phosphate, 25°. [b] Ratio of enzyme concentration measured at various pH's to the concentration measured at pH 7.0.

TABLE II: Effect of Ionic Environment on the Reaction of 7-Dimethylcarbamyl-*N*-methylquinolinium with Acetylcholinesterase.[a]

Condition	Ionic Strength	k_2 (min^{-1})	$k_3 \times 10^3$ (min^{-1})	K_S (μM)	Rel Enzyme Concn[b]
Control	0.17	4.9	2.6	8.6	1.00 ± 0.06
0.2 M $MgCl_2$	0.6	5.0	3.9	14	1.13 ± 0.07
0.6 M NaCl	0.6	8.0	2.3	23	1.07 ± 0.07
Low ionic strength	1.7×10^{-3}	4.0	1.7	2.5	0.98 ± 0.05

[a] Worthington enzyme ($0.5–1.2 \times 10^{-7}$ N) used without further purification. 1-ml assay volume, pH 7.0, 25°. Control contained 0.01 M NaCl, 0.02 M $MgCl_2$, and 0.01 M phosphate. Low ionic strength assay is a 1:100 dilution of the control. [b] Ratio of enzyme concentration measured under these conditions to that of the control.

of 6×10^{-6} to 2.5×10^{-5} M. To obtain an accurate measure of the concentration of V generated in the initial burst under the given experimental conditions, a 5-μl aliquot of V was added as an internal standard 5 min after the steady-state phase of the reaction had been achieved.

Results

Titration of AChE by III. The effects of pH, ionic strength, and ionic composition on $[E_0]$, k_2, k_3, and K_S are indicated in Tables I and II. $[E_0]$ and k_2 are unaffected by changes in the enzyme environment. On the other hand, k_3 and K_S are a function of pH and ionic conditions. Magnesium ion, in particular, promotes the hydrolysis of the dimethylcarbamyl-enzyme. In addition, k_3 increases 7-fold in going from pH 6.0 to 8.0 at a constant ionic strength. The values of k_2, k_3, and K_S determined at pH 7.0 are in close agreement with those reported by Rosenberry and Bernhard (1971) at pH 6.85 at comparable ionic strengths.

The titration of purified AChE with III was performed in the same way as the titrations previously described with the commercial enzyme preparation and yielded similar results. The turnover numbers of the crude and purified enzymes, based on the active-site normality determined by titration with III, are compared in Table III. The results in this table indicate that the turnover number based on the active-site normality is not a sensitive function of the purity of the enzyme preparation.

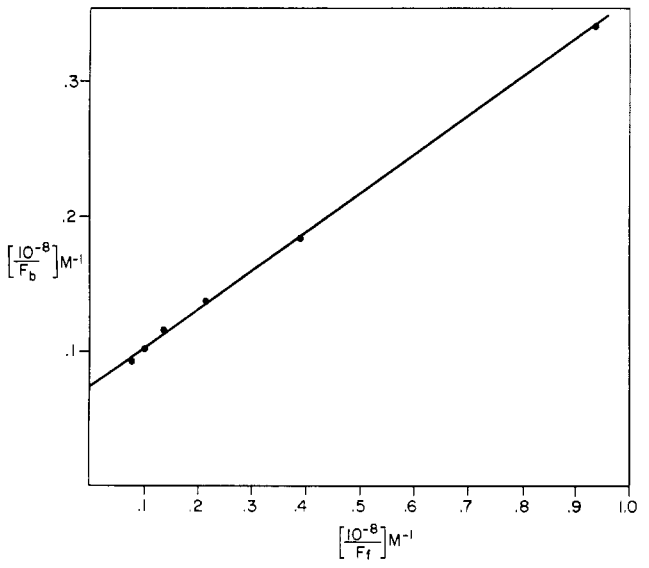


FIGURE 1: Titration of AChE with II according to eq 1. 1-ml volume containing 1.7×10^{-7} N AChE (Worthington enzyme without further purification), 0.1 M NaCl, 0.02 M $MgCl_2$, and 0.01 M phosphate, pH 7.0, 25°. Total II concentration from 4.0×10^{-8} to 24.0×10^{-8} M. The line is a linear least-squares fit of the points shown.

The specific activity of our purified preparation is 10.8 mmoles of ACh hydrolyzed per min per mg of protein which is comparable to the value of 12.5 mmoles of ACh hydrolyzed per min per mg of protein reported by Leuzinger and Baker (1967). The equivalent weight of the catalytic subunit using the turnover number reported here is therefore 67,500 mg/active site.

Stoichiometry of Ligand Binding. Titrations of AChE with bis(3-aminopyridinium)-1,10-decane (II) and *N*-methylacridinium ion (I) are illustrated in plots of $1/[F_b]$ *vs.* $1/[F_f]$ in Figures 1 and 2, respectively. The enzyme binding-site normality with respect to these ligands as determined from the ordinate intercept (eq 1) is compared to the normality measured by titration with III in Table IV. As indicated, both the I and II binding-site concentrations are equivalent to the catalytic site concentration. To further support these findings, the stoichiometry of I and II binding was investigated by the method of continuous variations. Figure 3 shows that the enzyme–ligand complex reaches a maximum when the ratio of AChE normality to the concentrations of I or II is 1. This confirms the observation that both of these fluorescent ligands have one binding site per catalytic site.

Loci of I and II Binding. The binding site of both fluo-

TABLE III: Turnover Number of Pure and Impure Acetylcholinesterase.[a]

	AChE (min^{-1})
Impure enzyme	6.92×10^5
Chromatographed enzyme	7.29×10^5

[a] Activity measured with acetylcholine on a pH-Stat in 0.1 M NaCl and 0.02 M $MgCl_2$ at pH 7.0, 25°. Enzyme normality used in the calculations is determined with III under the same conditions with the addition of 0.01 M phosphate (pH 7.0). Impure AChE is the Worthington enzyme without further purification. The chromatographed enzyme was purified on an affinity column described in Experimental Section.

TABLE IV: Fluorescent Ligand Titration of Acetylcholinesterase.[a]

Fluorescent Ligand	[Binding Site][b] / [Catalytic Site]	K_{diss}[c] (M)	K_I[d] (M)
Bis(3-aminopyridinium)-1,10-decane (II)	0.88 ± 0.15	$5.16 \pm 1.41 \times 10^{-8}$	$5.31 \pm 0.36 \times 10^{-8}$
N-Methylacridinium (I)	0.91 ± 0.22	$2.32 \pm 0.75 \times 10^{-7}$	$2.19 \pm 0.28 \times 10^{-7}$

[a] Worthington enyzme used without further purification. 1-ml assay volume containing 0.01 M NaCl, 0.02 M $MgCl_2$, and 0.01 M phosphate, pH 7.0, 25°. Enzyme concentration in II titrations are 0.9×10^{-7} to 1.7×10^{-7} M; enzyme concentration in I titration are 0.6×10^{-7} to 2.4×10^{-7} N. Data are averages with standard deviations of at least four determinations. [b] Binding-site normality determined by fluorescent ligand titration; catalytic site normality determined by III titration. [c] From fluorescent ligand titration. [d] From inhibition kinetics with ACh as the substrate.

rescent probes overlaps the catalytic site since there is good agreement between the dissociation constants of I and II using plots of eq 1 (Figure 1) and those measured as inhibition constants from the slopes of Lineweaver–Burk plots (Table IV). As indicated in Figure 4, the inhibition of ACh hydrolysis by I and II shows both a competitive and noncompetitive component in a Lineweaver–Burk plot that is characteristic of many AChE inhibitors. Krupka and Laidler (1961) have shown that the displacement of the intercept of a Lineweaver–Burk plot in AChE-catalyzed reactions rerults from the inhibitor binding to the acetyl-enzyme interimediate to form a complex in which the rate of hydrolysis is less than that of the free acetyl-enzyme. Compounds I and II therefore likely bind to the acetyl-enzyme intermediate as well as the free enzyme.

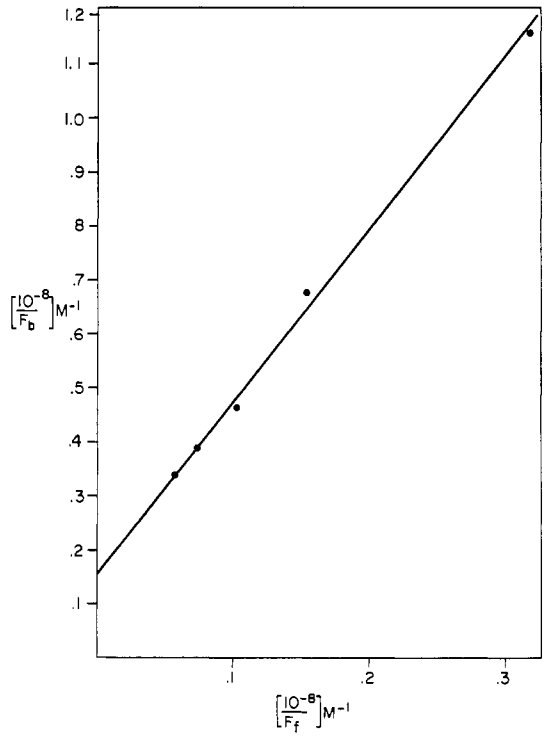


FIGURE 2: Titration of AChE with I according to eq 1. 1-ml volume containing 6.49×10^{-8} N AChE (Worthington enzyme without further purification), 0.1 M NaCl, 0.02 M $MgCl_2$, and 0.01 M phosphate, pH 7.0, 25°. Total II concentration from 4.0×10^{-8} to 20.0×10^{-8} M. The line is a linear least-squares fit of the points shown.

The inhibition of the presteady-state portion of III hydrolysis by I and II offers a means of separating the effects of the inhibitor on the reaction *prior* to formation of the intermediate (k_2) from the effects of the inhibitor on the reaction *subsequent* to the formation of the enzyme intermediate (k_3). If the noncompetitive component seen in the Lineweaver–Burk plot is due to binding of these ligands to the acetyl-enzyme intermediate, inhibition of the carbamylation step of the reaction of AChE with III should be strictly competitive. Figure 5 shows this is in fact the case. These

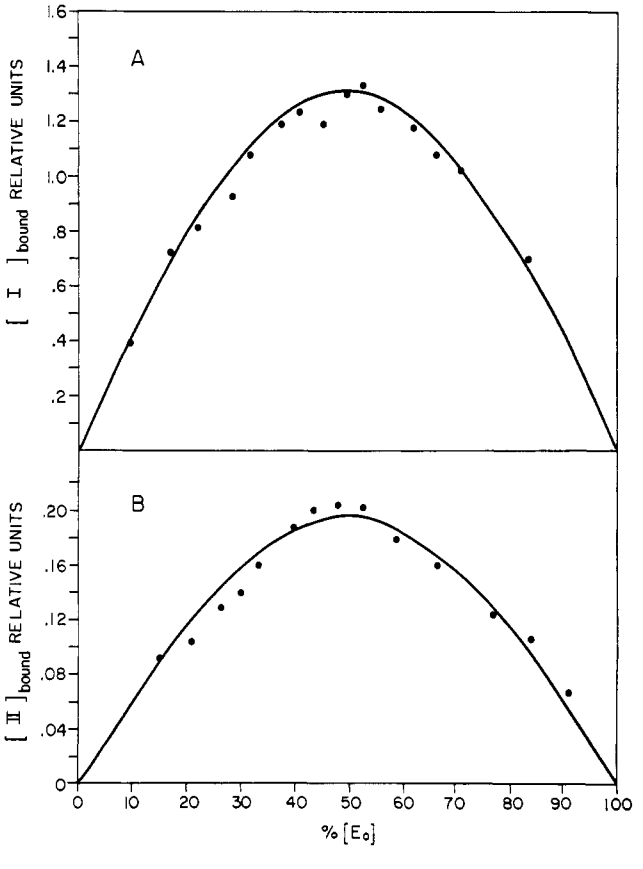


FIGURE 3: Analysis of stoichiometry of I and II binding to AChE by the method of continuous variation. Buffer media contains 0.1 M NaCl, 0.02 M $MgCl_2$, and 0.01 M phosphate, pH 7.0, 25°. (A) Total concentration of I plus AChE (Worthington enzyme without further purification) is 4.95×10^{-7} N. (B) Total concentration of II plus AChE is 2.02×10^{-7} N. The solid line represents the calculated theoretical curve.

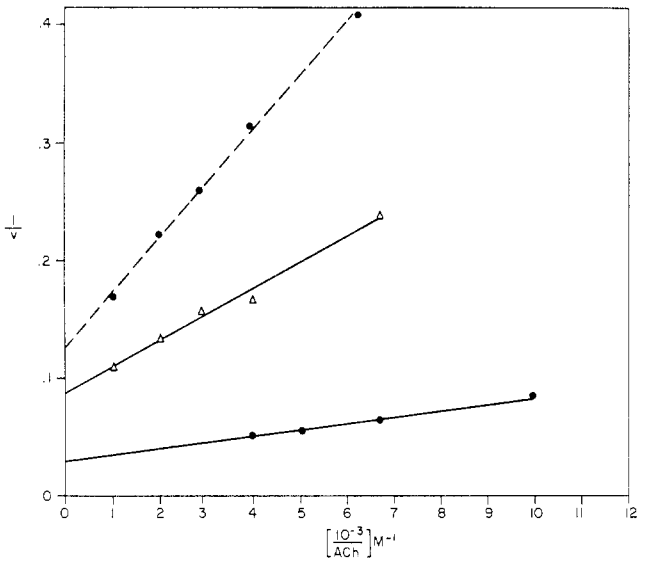


FIGURE 4: Lineweaver–Burk plot of II and I inhibition of AChE. Initial rates determined on a Radiometer pH-Stat with a 20-ml volume of 0.1 M NaCl and 0.02 M $MgCl_2$, pH 7.0, 25°. (●—●) No inhibitor; (△—△) 1.56×10^{-7} II; (●- - -●) 1.69×10^{-6} I. The lines represent linear least-squares fit of the points shown.

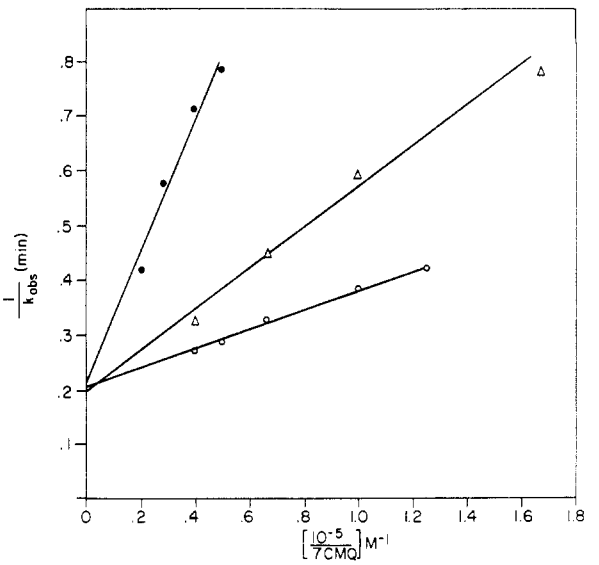


FIGURE 5: Inhibition of III carbamylation of AChE (Worthington enzyme without further purification). 1-ml assay volume containing 0.1 M NaCl, 0.02 M $MgCl_2$, and 0.01 M phosphate, pH 7.0, 25°. (○—○) No inhibitor, 3.23×10^{-8} N AChE; (●—●) 3.0×10^{-7} II, 3.23×10^{-8} AChE; (△—△) 5.0×10^{-7} I, 1.30×10^{-7} AChE. The lines represent linear least-squares fit of the points shown.

results together with the similarities of K_I's listed in Table IV strongly suggests that the sites for I and II binding are at least partially congruent with the substrate binding site.

Fluorescence Properties of I and II. The fluorescence spectra of both I and II are strongly quenched upon binding to the enzyme. The fluorescence intensity of either probe bound to the enzyme, as determined by eq 4, is less than 5% the intensity of the probe free in aqueous solution. The marked change in fluorescence intensity between the free and bound form of the probes is compatible with the observation that the fluorescence intensity of each probe is extremely sensitive to solvent environment in the absence of enzyme. For example, I-chloride in dioxane has a fluorescence intensity which is only 0.01% that measured in pH 7.0 phosphate buffer (excitation, 360 nm; emission, 490 nm). Similarly, II fluoresces only 3% as intensely in dioxane as it does in phosphate buffer (excitation 330 nm, emission, 410 nm). Although the precise mechanism by which the enzyme diminishes the fluorescence intensity of the probes is uncertain, the significant quenching observed when I and II bind to the enzyme surface is consistent with the apparent sensitivity of the fluorescence intensity of both probes to their enivronment.

Discussion

7-Dimethylcarbamyl-*N*-methylquinolinium iodide (III), *N*-methylacridinium chloride (I), and bis(3-aminopyridinium)-1,-10-decane diiodide (II) provide three different ways to study the ligand binding properties of acetylcholinesterase fluorometrically. Compound III can be used to measure the number of intact catalytic sites. Compound I binds reversibly to the active site and can be used as an indicator of the binding of other compounds in this region of the enzyme. Finally, compound II binds both to the active site and, by virtue of its bifunctional nature, to at least one specific binding site for cationic ligands remote from the active site and can be used to study binding phenomena of compounds at either of these two sites.

Although all of these compounds can in principle be used to determine the normality of acetylcholinesterase solutions, III is the most useful compound for this purpose. The titrimetric method with III can readily measure enzyme normalities as low as 1×10^{-8} N because of the intense fluorescence of the ionized form of 7-hydroxy-*N*-methylquinolinium at 410 nm when excited at 510 nm. Additional advantages of this method are (1) the dimethylcarbamyl ester does not fluoresce appreciably under these conditions; and (2) the 410-nm excitation maximum of V is removed from the spectral region where proteins absorb so there is virtually no background fluorescence due to AChE or any other proteins that might be present in the enzyme preparation. However, since the pK_a of the hydroxyl group of IV is 5.8 (Prince, 1966b), the sensitivity of the active-site titration is greatest at pH 7 and above where the concentration of the fluorescent ionized species, V, is greatest.

The titrimetric procedure using III also has distinct advantages relative to the several other procedures which have been used to measure the active site normality of AChE. For example, direct methods of titration have employed ^{32}P-labeled organophosphates which form stable phosphorylated derivatives at the catalytic site of AChE (Michel and Krop, 1951; Lawler, 1961; Kremzner and Wilson, 1964). These methods provide an accurate measure of normality, but the toxicity of the compounds and the time required for these determinations have prevented them from becoming routine. On the other hand, the method of Wilson and Harrison (1961), based on the ratio of carbamylated to native enzyme in the reaction of dimethylcarbamyl fluoride with AChE, requires a high concentration of enzyme (10^{-6} N) in order for the assay procedure to be performed in a reasonable time. The use of *o*-nitrophenyldimethylcarbamate as an active-site titrant (Bender *et al.*, 1966) is also relatively insensitive and is only useful for enzyme concentrations in the range where *o*-nitrophenol can be measured spectrophotometrically (*i.e.*, 2×10^{-6} M or greater). Nevertheless this latter method is identical in principle and can be performed as rapidly as the method reported here with III.

The accurate measurement of he active-site normality of AChE using III permits the precise determination of the turnover number per active site for the enzyme as well as the equivalent weight of the catalytic subunit. The turnover number for AChE before and after purification on the affinity column is presented in Table III. Using the turnover number of the purified enzyme reported in Table III and the specific activity of 10.8 mmoles of ACh hydrolyzed per min per mg of protein obtained here, the equivalent weight of the catalytic subunit is 67,500 mg/active site. With the specific activity of 12.5 mmoles of ACh hydrolyzed per min per mg of protein reported by Leuzinger and Baker (1967), the equivalent weight of the catalytic subunit is 58,500 mg/catalytic site using the turnover number reported in Table III. Since the reported molecular weight of the enzyme has ranged from 230,000 (Kremzner and Wilson, 1964) to 260,000 (Leuzinger *et al.*, 1969), the number of catalytic sites per molecule would be 3.41–3.86 with the 67,500 catalytic subunit equivalent weight or 3.9–4.5 with the 58,500 equivalent weight. These results are indicative of 4 catalytic sites per molecule which is in agreement with that found by Michel and Krop (1951), Kremzner and Wilson (1964), Froede and Wilson (1970), and Rosenberry and Bernhard (1971) but contrary to the two active sites per enzyme determined by Leuzinger (1971).

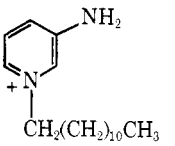
Both I and II interact very specifically with AChE. The enzyme possesses one binding site for each of these fluorescent probes for every catalytic site that is titratable by III. This stoichiometry is supported both by the enzyme normality determined from plots of eq 1 (Figures 1 and 2) and the method of continuous variations (Figure 3). The interaction of I and II with AChE is so specific that the fluorescent probes can be used with AChE preparations in which the enzyme is only 10% of the total protein present. For example, the dissociation constants for I, determined by fluorometric titration using both the crude and purified enzyme preparations, are the same. Therefore none of the protein impurities in the crude enzyme preparation bind I to a significant extent under the conditions where the interaction with AChE can be readily observed. Nonspecific interaction between protein impurities and I and II with an affinity roughly comparable to that of AChE are further excluded by the straight lines obtained in the double-reciprocal plots in Figures 1 and 2. Convex curves would be expected in these plots if any significant interactions took place between the impurities and the fluorescent probes (Klotz and Hunston, 1971).

All the data presently available indicate that I interacts strongly at the anionic binding site at the active site of AChE. The close agreement of the dissociation constant for I determined by direct fluorometric titration, and initial rate inhibition studies with acetylcholine indicate that the binding site of I overlaps that of the true substrate acetylcholine. The competitive inhibition by I of the carbamylation of AChE by III (Figure 5) is further evidence that I binds at the active site of the enzyme. Although the source of the strong fluorescence quenching of I observed upon binding to AChE cannot be determined precisely, it possibly reflects the region of low dielectric constant which might surround the anionic binding site. This conclusion is based on the weak fluorescence of I in solvents of low dielectric constant. Because of the marked difference in the fluorometric properties of I in aqueous solution and bound to the enzyme surface, the displacement of I from the enzyme can be readily monitored in competitive binding experiments with nonfluorescent ligands of AChE. Compound I, therefore, should be a useful probe to investigate the binding interactions of a wide variety of other compounds at the active site.

Compound II was chosen as a probe for AChE for two reasons. The first is that quaternary derivatives of 3-aminopyridine are strongly fluorescent. Second, II is similar in structure to one of the well-studied inhibitors of cholinergic systems, decamethonium. In a series of bis-quaternary ammonium compounds, the C_{10} analog, where the cationic nitrogens are separated by about 14 Å in the extended conformation, has been found to be the most effective alkyl chain length in blocking neuromuscular transmission (Barlow and Ing, 1948), inhibiting AChE (Bergmann, 1955), and accelerating the sulfonylation of AChE by methane sulfonyl fluoride (Belleau *et al.*, 1970).

Compound II must bind to the active site of the enzyme, since, as in the case of I, its dissociation constant is the same whether it is determined by direct fluorometric titration or by initial rate inhibition studies with acetylcholine. Since there is one binding site for II for each catalytic site that is titratable by III (Table IV), only one of the aminopyridinium moieties of II can bind to an active site. The possibility that II bridges two active sites with each of the two fluorescent aminopyridine groups at distinct active sites is excluded by the 1:1 stoichiometry revealed by fluorometric titration (Figure 1) and the technique of continuous variations (Figure 3).

The nature of the locus of interaction, if any, of the second aminopyridinium group of II, remains to be explored. Since II is over 20 times stronger as an inhibitor of AChE as the closely related monopyridinium compound (VI), the existence



VI

of a second site, distinct from the active site, for a cationic ligand such as a quaternary aminopyridine is strongly indicated. Further evidence for the binding of this second quaternary aminopyridine group to the enzyme is that the fluorescence of enzyme-bound II is completely quenched relative to II free in solution within the sensitivity of our measurements. Neither of the aminopyridinium groups of enzyme-bound II is therefore free in solution.

The binding of II to AChE both strongly suggests the existence of a binding site specific for cationic ligands remote from the active site and at the same time provides an experimental approach to investigate the binding specificity of this site. As noted above, several investigators have previously presented convincing evidence that different types of ligands bind at regions distinct from the catalytic site on AChE. Most notably, pachycurares (cholinergic antagonists) like gallamine have shown this characteristic. Kitz *et al.* (1970) found that gallamine will accelerate the decarbamylation of carbamyl-AChE under conditions of low ionic strength. Kato *et al.* (1970), using nmr have shown that eserine and atropine can concurrently bind to AChE. Changeux (1966) has presented evidence that suggests the main binding site of the pachycurares is not only separate from the catalytic site, but is the same site responsible for the enhanced inhibitory power of the C_{10} bis-quaternary compounds (*e.g.*, II) over their monoquaternary counterparts (*e.g.*, VI). By studying the displacement of enzyme-bound II by the pachycurares, it should be possible to determine under equilibrium condi-

tions, if their sites overlap either of the two binding sites of the aminopyridine moieties of II.

In summary, the three fluorescent compounds described here provide sensitive probes of different functional properties of acetylcholinesterase. Because of the intrinsic sensitivity of fluorescence, the value of these probes will not be restricted to the relatively readily available eel enzyme but will be also useful in studying acetylcholinesterase from mammalian sources.

References

Andrien, A. (1966), The Acridines, 2nd ed, New York, N. Y., St. Martin's Press, p 341.

Barlow, R. B., and Ing, H. R. (1948), *Brit. J. Pharmacol. 3*, 298.

Belleau, B., Di Tullio, V., and Tsai, Y.-H. (1970), *Mol. Pharmacol. 6*, 41.

Bender, M. L. (1971), Mechanisms of Homogeneous Catalysis from Protons to Proteins, New York, N. Y., Wiley-Interscience, p 441.

Bender, M. L., Beque-Canton, M. L., Blakeley, R. L., Feder, J., Brubacher, L. T., Gunter, C. R., Kézdy, F. J., Killheffer, J. V., Jr., Marshall, T. H., Miller, C. G., Roeske, R. W., and Stoops, J. K. (1966), *J. Amer. Chem. Soc. 88*, 5890.

Bergmann, F. (1955), *Discuss. Faraday Soc. 20*, 126.

Berman, J. D., and Young, M. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 395.

Cautrecasas, P. (1970), *J. Biol. Chem. 245*, 3059.

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

Froede, H. C., and Wilson, I. B. (1970), *Israel J. Med. Sci. 6*, 179.

Gutfreund, H., and Sturtevant, J. M. (1956), *Biochem. J. 63*, 656.

Jensen-Holm, J. (1961), *Acta Pharmacol. Toxicol. 18*, 379.

Kato, G., Yung, J., and Ihnat, M. (1970), *Biochem. Biophys. Res. Commun. 40*, 15.

Kitz, R. J., Braswell, L. M., and Ginsburg, S. (1970), *Mol. Pharmacol. 6*, 108.

Kitz, R. J., Ginsburg, S., and Wilson, I. B. (1967), *Biochem. Pharmacol. 16*, 2201.

Klotz, I. M. (1946), *Arch. Biochem. 9*, 109.

Klotz, I. M., and Hunston, D. L. (1971), *Biochemistry 10*, 3065.

Kremzner, L. T., and Wilson, I. B. (1964), *Biochemistry 3*, 1902.

Krupka, R. M., and Laidler, K. J. (1961), *J. Amer. Chem. Soc. 83*, 1445.

Lawler, H. (1961), *J. Biol. Chem. 236*, 2296.

Leuzinger, W. (1971), *Biochem. J. 123*, 139.

Leuzinger, W., and Baker, A. L. (1967), *Proc. Nat. Acad. Sci. U. S. 57*, 446.

Leuzinger, W., Goldberg, M., and Cauvin, E. (1969), *J. Mol. Biol. 40*, 217.

Martell, A., and Chabarek, B. (1959), Organic Sequestering Reagents, New York, N. Y., John Wiley, p 508.

Meunier, J. C., and Changeux, J.-P. (1969), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 2*, 224.

Michel, H. O., and Krop, S. (1951), *J. Biol. Chem. 190*, 119.

Prince, A. K. (1966a), *Arch. Biochem. Biophys. 113*, 195.

Prince, A. K. (1966b), *Biochem. Pharmacol. 15*, 411.

Rosenberry, T. L. (1970), *Diss. Abstr. 31*, 1063-B.

Rosenberry, T. L., and Bernhard, S. (1971), *Biochemistry 10*, 4114.

Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.

Wilson, I. B., and Harrison, M. A. (1961), *J. Biol. Chem. 236*, 2292.

Structure of the Light-Emitting Moiety of Aequorin†

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ABSTRACT: From 125 mg of disc electrophoretically pure aequorin extracted from *ca.* 2 tons of the jellyfish *Aequorea*, the light-emitting moiety in the bioluminescence reaction was separated as approximately 1 mg of a substance designated AF-350 (mol wt 277). The properties of AF-350, including ultraviolet and infrared absorption, nuclear magnetic resonance and mass spectra, pK_a values, and products of deuteration, acetylation, hydrogenation, and hydrolysis indicate that the structure of AF-350 is most likely 2-amino-3-benzyl-5-(*p*-hydroxyphenyl)pyrazine. This structure has further support through comparison with model compounds, namely, *Cypridina* etioluciferin and etioluciferamine. The AF-350 moiety is probably bound to the protein through the amidine part of the pyrazine ring, rather than through the phenolic hydroxyl.

The photoprotein aequorin, isolated from the bioluminescent jellyfish *Aequorea* (Shimomura *et al.*, 1962, 1963) emits visible light by an intramolecular reaction when Ca^{2+} is added. Aequorin contains a single chromophore, which is functional in the light-emitting reaction, and has been separated from the protein and designated AF-350 from its ultraviolet absorption maximum at 350 nm (Shimomura and

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