Active-Site-Directed Fluorescent Probes in the Kinetics and Spectroscopy of Purified Horse Serum Cholinesterase†

L. M. Chan, C. M. Himel,* and A. R. Main

ABSTRACT: 1-(5-Dimethylaminonaphthalene-1-sulfonamido)-3-*N*,*N*-dimethylaminopropane, an aminosulfonamide, and *N*-methylacridinium iodide, a heterocyclic aromatic quaternary salt, are active-site-directed, equilibrium fluorescent probes for the anionic subsite of purified horse serum cholinesterase. The organophosphate insecticide Maretin (*N*-hydroxynaphthalimide diethyl phosphate) is a fluorescent probe for the esteratic subsite. Spectral and kinetic data indicate the presence of one anionic subsite for each esteratic subsite. Diisopropyl phosphorofluoridate and Maretin give identical numbers of esteratic sites in the enzyme by titration. The dissociation constants of the enzyme complex with the three fluorescent probes range from 10^{-7} to 10^{-8} M, indicating binding at hydrophobic areas as well as the anionic or esteratic subsites. The dimethylaminopropane and Maretin compete for a common hydrophobic area, distinct from the hydrophobic area which binds *N*-methylacridinium ion. The dimethylaminopropane and *N*-methylacridinium ion compete for the anionic subsite in the enzyme. The phosphorylation constant for Maretin is 1.53 min^{-1} while its dissociation constant is 2.2×10^{-8} M. The rate of phosphorylation is decreased in the presence of *N*-methylacridinium ion in spite of the absence of competition for binding sites. The Förester distances for 50% energy transfer, measured with bound *N*-methylacridinium ion and with the dimethylaminopropane, are 19 and 17 Å, respectively.

Cholinesterases include acetylcholinestease (EC 3.1.1.7) usually obtained from erythrocytes or eel, and serum cholinesterase (EC 3.1.1.8), whose usual source is human serum or horse serum. Cholinesterases are involved in nerve transmission and the mechanism and mode of action of organophosphate and carbamate insecticides. As acylcholine hydrolases, they have anionic and esteratic subsites, and (like most proteins) hydrophobic areas. The anionic subsite determines specificity with respect to the choline moiety, while the actual catalytic process takes place at the esteratic subsite (Froede and Wilson, 1971). The presence of hydrophobic areas is well known (Mayer and Himel, 1972; Hovanec and Lieske, 1972; Bracha and O'Brien, 1970; Hillman and Mautner, 1970). However, the location(s) of hydrophobic areas relative to the anionic and esteratic subsites in cholinesterases is unknown.

The location and binding characteristics of hydrophobic areas are important. They may be a major factor in the determination of substrate differences between closely related enzymes. Their binding of inorganic ions, or organic molecules, can affect the conformation, kinetics, reactivity, and stability of enzymes.

The concept of active-site direction (Baker, 1967; Singer, 1967; Shaw, 1970) has been applied to equilibrium fluorescent probes (Himel *et al.*, 1970; Mayer and Himel, 1972; Mooser *et al.*, 1972). The nature and location of hydrophobic areas can have a major effect on the total free energy of binding of equilibrium inhibitors of enzyme systems (Himel and Chan, 1974).

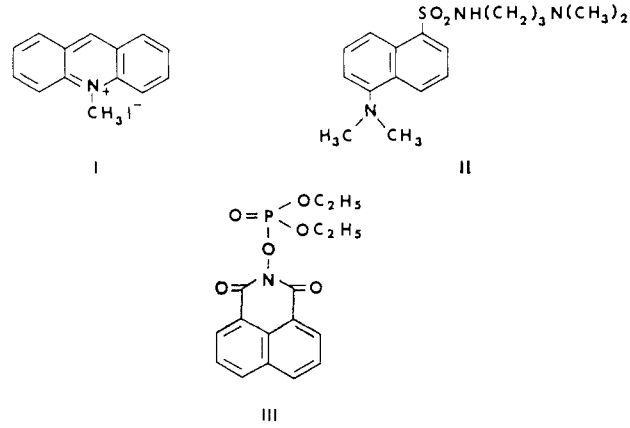
The availability of purified, stable horse serum cholinesterase (Main *et al.*, 1972) made possible this spectroscopic and kinetic study of the reaction of active-site-directed fluorescent probes with purified cholinesterase. Interrelationships between anionic and esteratic subsites and adjacent hydrophobic sites have been studied.

Experimental Procedure

Materials. Acetylcholine bromide and butyrylcholine iodide were obtained from Sigma, St. Louis, Mo. Quinine sulfate was obtained from Columbia Chemical Co., Columbia, S. C. Acridine was obtained from Aldrich, Milwaukee, Wis. They were recrystallized several times.

N-Methylacridinium iodide (I) was synthesized essentially as described by Mooser *et al.* (1972), except that the reaction was carried out at room temperature using *N*,*N*-dimethylformamide as solvent. Nearly pure crystals deposited during a 15-day period. The yield was 86% of a product which was readily filtered and easily purified. This procedure avoids coprecipitation of unreacted acridine. *Anal.* Calcd for $C_{14}H_{12}NI$: C, 52.36; H, 3.77; N, 4.36. Found: C, 52.64; H, 3.88; N, 4.06 (Midwest Microlab. Ltd.).

The synthesis and spectral properties of 1-(5-dimethylaminonaphthalene-1-sulfonamido)-3-*N*,*N*-dimethylaminopropane (II) have been described (Himel *et al.*, 1970; Mayer and Himel, 1972). Maretin (III) was obtained as an analytical



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grade from Chemagro, Kansas City, Mo. Diisopropyl phosphorofluoridate (iPr_2PF)[1] was obtained from Mann, Orangeburg, N. Y.

Cholinesterase was purified from horse serum by the method of Aull (1972). The preparation was judged to be about 70% cholinesterase by several criteria including its specific activity, disc gel electrophoresis, and analytical ultracentrifugation. The purification over serum was about 11,450-fold. The contaminant appeared to stabilize the enzyme and its presence would not have affected the results obtained in the present study. The purified enzyme hydrolyzes butyrylcholine 3.2 times faster than acetylcholine. It has a k_m value of 0.19 mM butyrylcholine, and a specific activity of 1.07 mmol of butyrylcholine/min per mg of enzyme at 25°, pH 7.5.

Methods. Absorption spectra were obtained with a Cary 15 spectrophotometer. All fluorescence measurements were made with a Turner 210 spectrofluorometer which gives corrected excitation and fluorescence spectra. The Turner was equipped with a time-drive unit for kinetic measurements. Quinine sulfate was used as the standard for quantum yield determinations. Its quantum yield was taken as 0.55 in 0.1 N H_2SO_4. Cholinesterase activity was measured with a Radiometer pH-Stat. The pH was maintained at 7.5 with 0.01 N NaOH. No salt was added. The protein concentration was determined spectrophotometrically. An extinction coefficient of 1.48 $(mg/ml \times cm)^{-1}$ was used. The enzyme normality was determined by iPr_2PF titration (A. R. Main, unpublished results).

Fluorescence Titration. Quartz cells (1 cm) with a total volume of 1.5 ml were used. The solvent used was sodium phosphate buffer, 0.05 M, pH 7.0. Hamilton microsyringes were used to add titrants. II has a long-wavelength absorption peak at 323 nm with an ϵ_{328} of 4.80×10^3 M^{-1} cm^{-1}. It fluoresces maximally at 580 nm with a quantum yield of 0.055. The complex of II with cholinesterase fluoresces maximally at 520 nm. *N*-Methylacridinium iodide has a major long-wavelength absorption peak at 358 nm with an ϵ_{358} of 2.14×10^4 M^{-1} cm^{-1}. It fluoresces maximally at 490 nm with a quantum yield of 0.75. The *N*-methylacridinium ion–cholinesterase complex is nonfluorescent (Mooser *et al.*, 1972). Maretin has a long-wavelength absorption peak at 342 nm with an ϵ_{342} of 1.46×10^4 M^{-1} cm^{-1}. It fluoresces maximally at 400 nm with a quantum yield of 0.33. The enzymatic hydrolysis product of Maretin is nonfluorescent. Figure 1 shows the absorption and fluorescence spectra of cholinesterase and the three probes. Despite the partial overlap of the spectra, competitive fluorescence titrations were made possible by selecting the proper combinations of excitation and fluorescence wavelengths.

Kinetics of Inhibition. The interaction of cholinesterase with Maretin can be represented as shown in eq 1, where E is the

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow[-P_1]{k_2} EI' \xrightarrow{k_3} E + P_2 \qquad (1)$$

enzyme, I is Maretin, EI is a reversible complex, EI′ is the phosphorylated enzyme, and P_1 and P_2 are products.

The method of Main (1964) was used. The data can be analyzed according to eq 2, where $K_I = k_{-1}/k_1$ is the dissociation constant of the Maretin–cholinesterase complex, v is the initial velocity, and i is the concentration of Maretin which is in excess and remains constant during the short reaction time t.

$$\frac{i\Delta t}{\Delta \log v} = \frac{2.303K_I}{k_2} + \frac{2.303i}{k_2} \qquad (2)$$

[1] Abbreviations used are: Dns, 5-dimethylaminonaphthalene-1-sulfonyl; iPr_2PF, diisopropyl phosphorofluoridate.

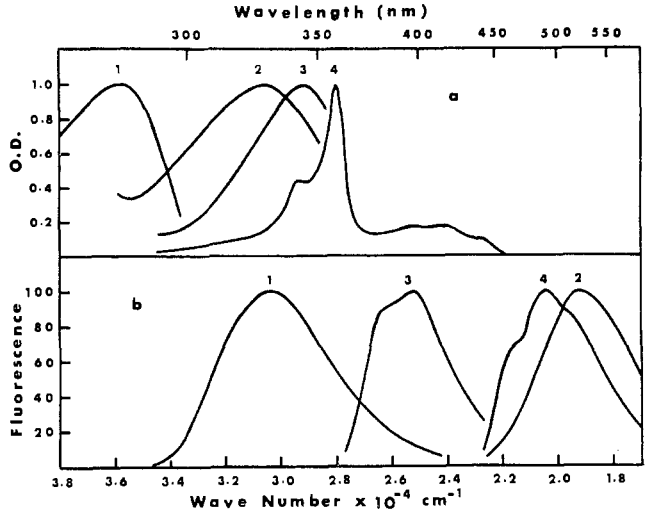

FIGURE 1: Absorption (a) and fluorescence (b) spectra: (1) cholinesterase; (2) II–cholinesterase complex; (3) Maretin; and (4) *N*-methylacridinium ion. For clarity, only part of the absorption spectra were shown.

Esteratic Site. Cholinesterase was allowed to interact with Maretin. The number of esteratic sites per equivalent of enzyme were determined by two methods: (1) measuring the residual enzymatic activity when excess enzyme was used; (2) measuring the residual fluorescence intensity of Maretin when excess Maretin was used.

Anionic Site. N-Methylacridinium iodide was used (Mooser *et al.*, 1972). The results were analyzed according to eq 3

$$r/C = (n/K_d) - (r/K_d) \qquad (3)$$

(Tanford, 1961), where, in our case, r is the number of moles of bound probe per equivalent of enzyme, C is the molar concentration of the free probe, n is the number of binding sites per equivalent of enzyme, and K_d is the intrinsic dissociation constant of the enzyme–probe complex.

The Förster critical transfer distance (R_0) is the distance between donor and acceptor at which transfer is 50% efficient. After substituting with the appropriate constants, the Förster equation becomes (Latt *et al.*, 1965)

$$R_0^6 = 8.8 \times 10^{-25}\kappa^2\phi J n^{-4} \qquad (4)$$

where n is the refractive index of the medium, κ is the orientation factor of the dipole pair, and ϕ is the donor quantum yield. $\phi = 0.055$, $n = 1.6$, and $\kappa = {}^2/_3$ were used in our calculations. The overlap integral J is given by

$$J = \int \frac{F_D(\bar{\nu})\epsilon_A(\bar{\nu})}{\bar{\nu}^4} d\bar{\nu} \qquad (5)$$

where $F_D(\bar{\nu})$ is the normalized fluorescence intensity of the donor at the wave number $\bar{\nu}$, and $\epsilon_A(\bar{\nu})$ is the molar extinction coefficient of the acceptor at the same wave number.

Results and Discussion

Maretin (III) inhibits cholinesterase with a pseudo-first-order phosphorylation constant (k_2) of 1.53 min^{-1} when

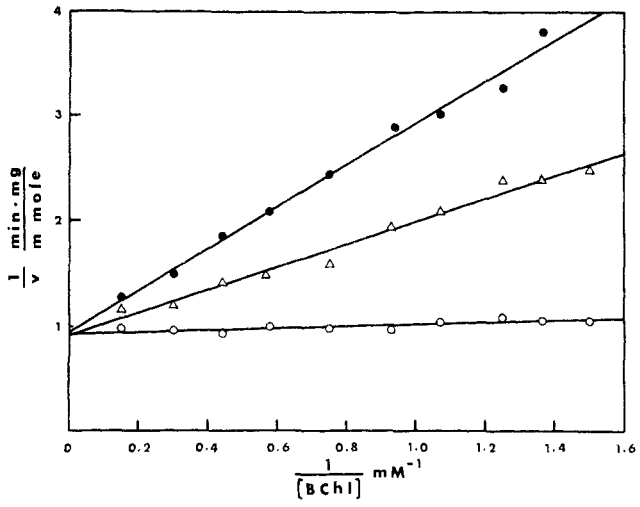

FIGURE 2: Kinetics of inhibition of cholinesterase (1.71×10^{-9} N) by II obtained with a pH-Stat. The concentrations of II were: zero (○); 5.0×10^{-6} M (△); and 1.0×10^{-5} M (●). The lines were fitted to the data by least-squares analysis.

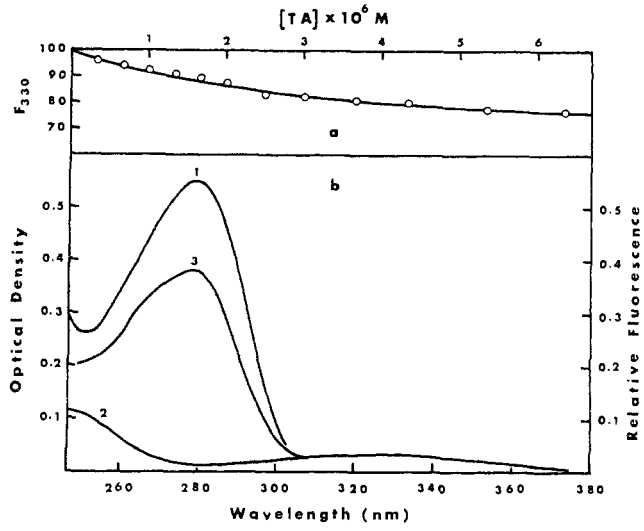

FIGURE 3: (a) Quenching of cholinesterase (2.07×10^{-6} N) fluorescence by II. Excitation and emission were set at 280 and 330 nm, respectively. (b) Curve 1, absorption spectrum of cholinesterase (2.77×10^{-6} N); curve 2, absorption spectrum of II (7.0×10^{-6} M); curve 3, excitation spectrum of a mixture of cholinesterase (2.77×10^{-6} N) and II (7.0×10^{-6} M). Emission was set at 520 nm. It was normalized at 330 nm to curve 2.

Maretin is in excess. The dissociation constant (K_I) of the Maretin–cholinesterase complex is 2.20×10^{-8} M (eq 2). Organophosphates can interact irreversibly by phosphorylation of the esteratic site of cholinesterase after initial complex formation (Froede and Wilson, 1971). The strong affinity of the complex makes Maretin a useful phosphorylation agent in spite of its modest phosphorylation constant (k_2). Furthermore, the intense fluorescence of Maretin at 400 nm allows spectroscopic detection of the phosphorylation reaction and an additional method of esteratic site titration. The results of these titrations are summarized in Table I.

The use of II as a fluorescence probe of cholinesterase has been described (Mayer and Himel, 1972). The enzyme used in that study was obtained from Sigma. iPr_2PF titrations show that the Sigma enzyme contains about 1% of cholinesterase. In this study, we have confirmed that II is a competitive inhibitor of purified cholinesterase with a K_I of 7.0×10^{-7} M (Figure 2). The fluorescence maximum of the II–cholinesterase complex is at 520 nm. By comparing this fluorescence spectrum with that of II in a series of organic solvents (Mayer and Himel, 1972), it can be concluded that the Dns moiety of II binds at a hydrophobic area. The quantum yield of the complex is low (about 0.1), considerably lower than the quantum yield of the II–cholinesterase complex using Sigma enzyme. In addition, with purified cholinesterase, the characteristic fluorescence spectrum of bound II can be observed only when it is excited at 280 nm, the region where cholinesterase absorbs. Further evidence for interaction of II with cholinesterase is shown in Figure 3. II quenches cholinesterase fluorescence (Figure 3a). The shape and intensity of the excitation spectrum of the II–cholinesterase complex (curve 3 of Figure 3b) indicate the transfer of electronic energy from the excited state of the tryptophanyl residues of cholinesterase to II (Stryer, 1968).

TABLE I: Relative Number of Active Sites of Cholinesterase.

Probe	Method	Subsite	Rel No. of Active Sites
iPr_2PF	Activity[a]	Esteratic	1.00[d]
Maretin	Activity[a]	Esteratic	0.96 ± 0.07
Maretin	Fluorescence[b]	Esteratic	1.08 ± 0.01
N-Methyl-acridinium ion	Fluorescence[c]	Anionic	0.95 ± 0.15

[a] Cholinesterase (2.74×10^{-7} N) was incubated with different concentrations of iPr_2PF or Maretin for 5 hr. The residual enzymatic activity was measured; [butyrylcholine iodide] = 6.64 mM. [b] Cholinesterase (2.19×10^{-7} N) was incubated with different concentrations of Maretin for 4 hr. The concentration of unreacted Maretin was determined by comparing the fluorescence intensity with a standard curve. Excitation and emission were set at 340 and 400 nm, respectively. [c] See the legend of Figure 5a for experimental details. [d] A cholinesterase solution with an optical density of 5.44×10^{-2} corresponds to $2.74 \pm 0.13 \times 10^{-7}$ N by iPr_2PF titration.

Unlike acetylcholinesterase (Mooser *et al.*, 1972), *N*-methylacridinium ion was a competitive inhibitor of cholinesterase with a dissociation constant (K_I) of 5.3×10^{-8} M under our assay conditions (Figure 4). In contrast to cholinesterase, acetylcholinesterase is subject to marked substrate inhibition (Froede and Wilson, 1971). Substrate inhibition and the uncompetitive component in the Lineweaver–Burk plot are a result of formation of a substrate (or inhibitor) acyl-enzyme complex in which deacylation is partially blocked (Krupka and Laidler, 1961a–c). Binding of substrate or inhibitor to the acyl-enzyme may or may not block deacylation (Krupka and Laidler, 1961c). The data show that *N*-methylacridinium ion binds to cholinesterase which is diethylphosphorylated (Figure 6). Since Maretin and butyrylcholine react with cholinesterase by a common mechanism (eq 1) (Froede and Wilson, 1971), *N*-methylacridinium ion also binds to the acyl-enzyme. The observed competitive inhibition (Figure 4) implies that the binding of *N*-methylacridinium ion to the acyl-enzyme does not block deacylation.

A typical Scatchard plot obtained from fluorescence titration for the interaction of *N*-methylacridinium ion with cholinesterase is shown in Figure 5a. The linear relationship suggests

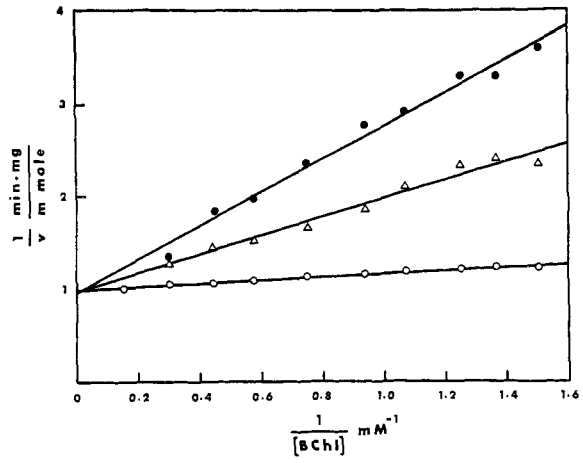

FIGURE 4: Kinetics of inhibition of cholinesterase (2.59×10^{-9} N) by *N*-methylacridinium iodide obtained with a pH-Stat. The concentrations of *N*-methylacridinium iodide were: zero (○); 2.5×10^{-7} M (△); and 5.0×10^{-7} M (●). The lines were fitted to the data by least-squares analysis.

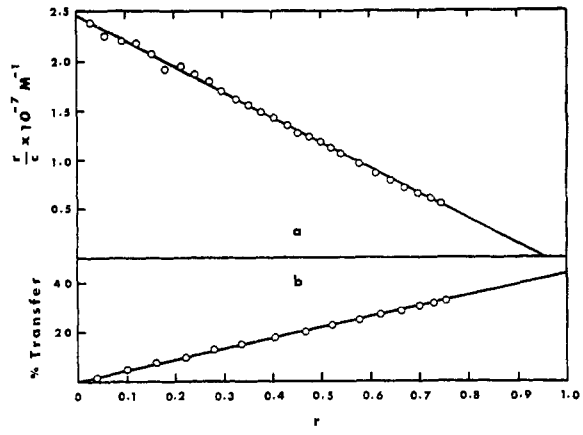

FIGURE 5: Interaction of cholinesterase (2.76×10^{-7} N) with *N*-methylacridinium iodide obtained by fluorescence titration. The concentration of *N*-methylacridinium iodide ranged from 1.0×10^{-8} to 3.4×10^{-7} M. The lines were fitted to the data by least-squares analysis: (a) the Scatchard plot; excitation and emission were set at 360 and 490 nm, respectively; (b) per cent energy transfer measured by the quenching of cholinesterase fluorescence by *N*-methylacridinium iodide; excitation and emission were set at 295 and 330 nm, respectively.

that there are 0.95 ± 0.15 identical, noninteracting *N*-methylacridinium binding sites for each iPr_2PF binding site of cholinesterase. The intrinsic dissociation constant (K_d) obtained from the Scatchard plots is $3.82 \pm 0.52 \times 10^{-8}$ M. The quaternary nitrogen of *N*-methylacridinium ion binds at the anionic subsite of cholinesterases (Mooser *et al.*, 1972; Froede and Wilson, 1971). In addition, the total free energy of binding includes coulombic forces and hydrophobic forces (Froede and Wilson, 1971). The low dissociation constant of the enzyme–*N*-methylacridinium ion complex indicates a substantial binding contribution from the acridinium ring system. Acridine, with a pK_a of 5.60 (Albert, 1966), also inhibits cholinesterase. As with its quaternary derivative, the fluorescence intensity of acridine (maximum at 450 nm, quantum yield = 0.37) is quenched when it is complexed with cholinesterase.

Active-site determinations by different methods are outlined in Table I. There is one anionic subsite associated with each esteratic subsite.

II binds to the enzyme at two sites. The amine group binds at the anionic site while the Dns moiety binds at an exo hydrophobic area (Mayer and Himel, 1972). The location of this exo hydrophobic area was identified as adjacent to the esteratic subsite by: (1) competition of II and Maretin for that hydrophobic area and (2) binding of II with the phosphorylated enzyme, indicating that II does not bind at the esteratic subsite.

Competitive reactions, monitored by fluorescence measurements, show that *N*-methylacridinium ion and II compete with each other for the anionic subsite of cholinesterase. II competes with Maretin only as long as the intact organophosphate occupies their common hydrophobic area exo to the esteratic site. When Maretin phosphorylates the esteratic subsite, the hydroxynaphthalimide leaving group no longer competes effectively for the exo hydrophobic area.

N-Methylacridinium ion binds as well to the phosphorylated enzyme (Figure 6) as to the active enzyme (Figure 5a). Maretin forms a ternary complex with the binary cholinesterase–*N*-methylacridinium complex. The hydrophobic area to which the acridinium moiety is bound is different from the hydrophobic area which binds II and the intact organophosphate.

Compounds which interact with acetylcholinesterase at its anionic site can inhibit or accelerate the reactions occurring at the esteratic site (Wilson, 1971; Kitz *et al.*, 1970; Rosenberry and Bernhard, 1971, 1972). *N*-Methylacridinium ion, Maretin, and cholinesterase form a ternary complex. In order to fit two such bulky inhibitors simultaneously into the active-site area a change in cholinesterase conformation may occur. Thus, binding of *N*-methylacridinium ion could affect the rate of phosphorylation of cholinesterase by Maretin. *N*-Methylacridinium ion at a concentration as low as 10^{-7} M decreases the rate of phosphorylation of cholinesterase by Maretin (Figure 7). II at a much higher concentration (10^{-5} M) also decreases the rate of phosphorylation of cholinesterase slightly.

Cholinesterase fluoresces maximally at 330 nm with a "protein" quantum yield of 0.064 (excited at 280 nm) and a "tryptophan" quantum yield of 0.055 (excited at 295 nm). The former is higher than the latter due to energy transfer from the tyrosine residues to the tryptophanyl residues of the enzyme

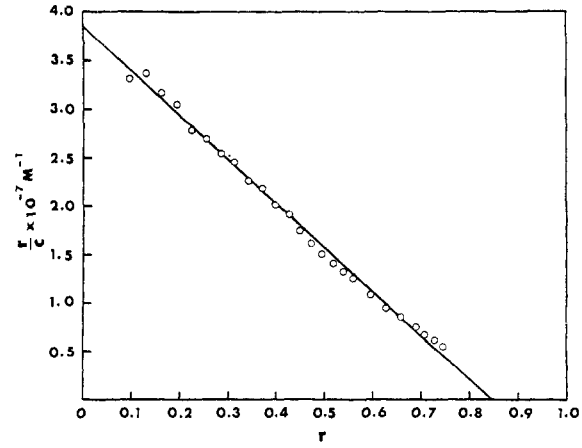

FIGURE 6: Interaction of phosphorylated cholinesterase (2.76×10^{-7} N) with *N*-methylacridinium iodide obtained by fluorescence titration. Cholinesterase was phosphorylated by incubating with Maretin (3.67×10^{-7} M) for 30 min. The pH-Stat showed no activity. Other conditions were the same as those stated in the legend of Figure 5a.

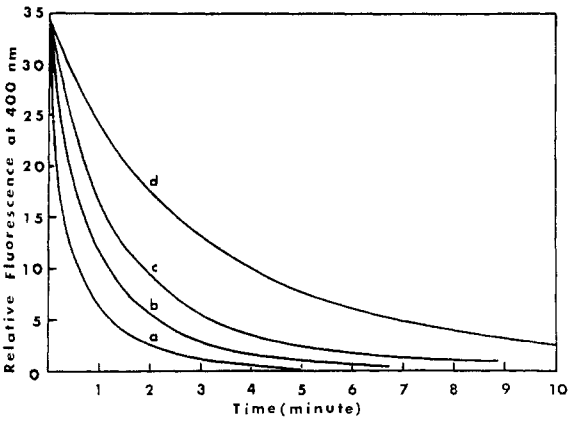

FIGURE 7: Effect of *N*-methylacridinium iodide on the kinetics of phosphorylation of cholinesterase (4.80×10^{-8} N) by Maretin (2.67×10^{-8} M). The concentrations of *N*-methylacridinium iodide were: (a) zero; (b) 1.0×10^{-7} M; (c) 2.0×10^{-7} M; and (d) 1.0×10^{-6} M. Excitation and emission were set at 340 and 400 nm respectively.

(Kronman and Holmes, 1971; Longworth, 1971). When cholinesterase is complexed with either II or *N*-methylacridinium ion, its fluorescence is quenched with no change in the peak position. This is the result of transfer of electronic energy from the tryptophanyl residues of cholinesterase to the bound probes. Figure 5b shows that the quenching of cholinesterase fluorescence is linearly proportional to the number of moles of bound *N*-methylacridinium ion. A necessary condition for excitation transfer by the Förster mechanism under very weak coupling is an appreciable overlap of the fluorescence spectrum of the donor and the absorption spectrum of the acceptor (Förster, 1965). Figure 1 shows that the fluorescence spectrum of cholinesterase overlaps the absorption spectra of all three probes. The overlap integrals calculated from eq 5 are 4.27×10^{-15} and 8.17×10^{-15} cm^6 mmol^{-1}, respectively, when the acceptor is II and *N*-methylacridinium ion. The corresponding Förster transfer distances for 50% transfer (R_0) are 17 and 19 Å, respectively (eq 4). Thus, resonance energy transfer from the tryptophanyl residues to the bound probes is quite probable. We have taken advantage of this energy transfer in the study of complex formation between II and cholinesterase.

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