Interaction of Ligands with Acetylcholinesterase. Use of Temperature-Jump Relaxation Kinetics in the Binding of Specific Fluorescent Ligands†

Terrone L. Rosenberry* and Eberhard Neumann

ABSTRACT: The fluorescence of either N-methylacridinium (I) or 1-methyl-7-hydroxyquinolinium (II) is totally quenched on binding to the catalytic site of acetylcholinesterase. Equilibrium titrations of 11S acetylcholinesterase at 0.1 M ionic strength with I confirmed previous reports that binding shows high specificity for the catalytic site. Analogous titrations with II indicated that only the protonated, cationic form of II binds and that binding has a specificity and stoichiometry similar to that of I. Under most of the experimental conditions introduced here, the reaction of either I or II with acetylcholinesterase was characterized by a single relaxation time. The bimolecular association constants for the reaction were unusually high, at 23 °C and ~0.1 M ionic strength; for I, $k_{12} = 1.18 \pm 0.03 \times 10^9$ M$^{-1}$ s$^{-1}$; for II, $k_{12} = 2.18 \pm 0.15 \times 10^9$ M$^{-1}$ s$^{-1}$. These constants were obtained from observed relaxation times both by a conventional analysis of equilibrium reactant concentrations and by a new method introduced here in which only the total ligand concentration need be known. At relatively high concentrations of enzyme and I, a second relaxation was observed; analyses of relaxation amplitudes indicated that this relaxation reflected independent ligand binding at a second, peripheral site on the enzyme. It has recently been suggested by M. Eigen that certain specific ligands may have unusually high bimolecular association constants with their target macromolecules because they can bind initially to peripheral sites and proceed to the specific site by surface diffusion on the macromolecule. A test of this proposal for acetylcholinesterase and I was conducted by introducing 30 mM Ca$^{2+}$ to the solvent. No supporting evidence was obtained. Nevertheless, this proposal, applied to other sites with very low ligand affinities, may still partially account for the high bimolecular association rate constants.

The speed with which acetylcholinesterase catalyzes the hydrolysis of acetylcholine has long been appreciated. Studies by Michel and Krop (1951), Lawler (1961), Kremzner and Wilson (1964) and others are in quite close agreement on a maximum turnover number ($k_{cat}$) of $1.6 \times 10^6$ s$^{-1}$ at pH 8 and 25 °C (Rosenberry and Bernhard, 1971; Rosenberry, 1975a). Recently it has been emphasized (Rosenberry, 1975a) that this enzymatic hydrolysis is also extremely efficient at the perhaps more physiologically relevant acetylcholine concentrations below the apparent Michaelis constant $K_{app}$ (<0.1 mM). At these concentrations the appropriate rate parameter is the second-order rate constant $k_{cat}/K_{app}$. Values of $k_{cat}/K_{app}$ for acetylcholine and several other cationic substrates are around $2 \times 10^8$ M$^{-1}$ s$^{-1}$ at 25 °C and 0.1 M ionic strength (Rosenberry, 1975a) and thus approach a limit generally expected for diffusion-controlled enzyme reactions (Eigen and Hammes, 1963). In agreement with this suggestion, a low deuterium oxide isotope effect of 1.1 is associated with $k_{cat}/K_{app}$ for acetylcholine (Rosenberry, 1975b); this observation indicates that the rate-limiting step for acetylcholine hydrolysis at low substrate concentrations precedes general acid–base catalysis and is likely to involve either the bimolecular reaction step or a subsequent conformational change of the enzyme-substrate complex (Rosenberry, 1975b).

Because of the importance of the initial steps associated with the interaction of acetylcholinesterase with specific ligands, we have used temperature-jump relaxation kinetics to investigate the reactions of this enzyme with N-methylacridinium (I) and 1-methyl-7-hydroxyquinolinium (II). Both I and the zwitterionic form of II are highly fluorescent, and the fluorescence of both I and II is totally quenched when they bind with high affinity to acetylcholinesterase. Careful studies by Mooser et al. (1972), Mooser and Sigman (1974), and Taylor and Lappi (1975) have established that I binds with high specificity to each of the four independent catalytic sites in the 11S enzyme tetramer. The binding of II is less well defined, but kinetic data (Rosenberry and Bernhard, 1972) and our current study suggest that it is also highly specific for the catalytic sites.

In this study we show that these ligands bind to the enzyme with unusually high bimolecular rate constants. The possibility that peripheral anionic sites are contributing to these rate constants is considered.

Experimental Section

Materials. Acetylcholinesterase from electric organs of the eel Electrophorus electricus was purified as an 11S species free of detectable protein contaminants as described previously (Rosenberry et al., 1974). The preparation used in this study had been stored as a concentrated frozen solution for several months and had a specific activity of 5.7 mmol of acetylcholine hydrolyzed min$^{-1}$ (mg of protein)$^{-1}$ with 2.0 mM acetylcholine in 0.1 M NaCl at pH 7.4 and 25 °C. This corresponds to about 70% of the maximal specific activity observed for 11S acetylcholinesterase. Normalities of acetylcholinesterase solutions were measured according to either eq 1 or eq 11–13 below.

† From the Departments of Neurology and Biochemistry, College of Physicians and Surgeons, Columbia University, New York, New York 10032, and the Max-Planck-Institut fur Biochemie, D-8033 Martinsried bei Muenchen, West Germany. Received December 14, 1976. This investigation was supported, in part, by United States Public Health Service Grant NS-03304, National Science Foundation Grant PCM73-00744, and a NATO Senior Fellowship in Science to T.L.R.
The recrystallized perchlorate salt of N-methylacridinium was a gift from Dr. Meir Shinitzky. A λmax at 358 nm with εmax 21 300 M⁻¹ cm⁻¹ was observed for this compound in 0.1 M sodium phosphate buffer at pH 8.0. 1-Methyl-7-hydroxyquinolinium iodide (λmax 406 nm, εmax 9200 M⁻¹ cm⁻¹ in 0.1 M sodium phosphate, pH 7.0) was obtained by hydrolysis of 1-methyl-7-acetoxyquinolinium iodide (Rosenberry and Bernard, 1972). Sodium phosphate buffers labeled 0.1 M were prepared by mixing appropriate amounts of 0.1 M NaH₂PO₄ and 0.05 M Na₂HPO₄ to achieve the indicated pH.

Fluorescence Measurements. Most of the equilibrium and all of the kinetic data reported here were obtained in a fluorescence temperature-jump apparatus described previously (Rigler et al., 1974; Department of Biochemical Kinetics, Max-Planck-Institute for Biophysical Chemistry, Goettingen, West Germany). Excitation light from a mercury source was passed through a monochrometer adjusted to the adsorption maximum of the fluorescent ligand and focused on the sample compartment containing 0.8 mL of solution. Emission light was passed through cutoff filters, which removed scattered excitation light, and collected by two independent photomultiplier units placed at 90° angles to the excitation light. Equilibrium fluorescence values were read as photomultiplier voltages normalized by a reference photomultiplier voltage recorded directly from the light source.

Fluorescence relaxation spectra were induced by a 3.3°C temperature increase triggered by a 20-kV discharge from a 5 X 10⁻⁸ F capacitor through the sample compartment. The initial temperature was 20°C and the final temperature was 23.3°C. Transient photomultiplier signals were electrically damped to optimize signal to noise. In the presence of high equilibrium fluorescence, a time-delayed device allowed monitoring of only those fluorescence relaxations of interest. Relaxation spectra were displayed on a calibrated Tektronix storage oscilloscope and recorded on 35-mm film. Relaxation times and amplitudes were obtained by superposition of the film image on an oscilloscope screen whose voltage input was derived from a calibrated multiple exponential function generator developed by C. R. Rabl (Max-Planck-Institute for Biophysical Chemistry, Goettingen, Germany). Exposure of acetylcholinesterase to the described temperature-jump experimentation for 4 to 6 h gave no detectable loss of enzyme activity.

A few equilibrium fluorescence measurements were also made on a Perkin-Elmer MPF-3A spectrophotofluorometer.

Ligand-Interaction Schemes. Three alternative schemes were considered for the interaction of fluorescent ligands with acetylcholinesterase.

SCHEME I

E + L  $$\stackrel{k_{12}}{\rightleftharpoons}$$  EL

In Scheme I, ligands bind only to the catalytic site of the enzyme in a simple, one-step process given by a forward rate constant k₁₂ and a backward rate constant k₂₁. The ratio k₂₁/k₁₂ defines the equilibrium dissociation constant K₁. Schemes II and III extend Scheme I by including a second ligand-binding site. In general, Scheme II could apply to any enzyme catalytic subunit which has two ligand-binding sites; however, when these two sites are assumed to bind ligand independently, K₁ = K₄ and Scheme II becomes equivalent to Scheme III. In Scheme III, E and E' indicate independent binding-site species; interactions at the second site occur with rate constants k₁₁' and k₂₁' (equilibrium constant K₁₁'/K₁₂' = K₂).

SCHEME II

E + L  $$\stackrel{k_{12}}{\rightleftharpoons}$$  EL

E' + L  $$\stackrel{k_{12}'}{\rightleftharpoons}$$  EL'

SCHEME III

E + L  $$\stackrel{k_{1}}{\rightleftharpoons}$$  EL

E' + L  $$\stackrel{k_{1}'}{\rightleftharpoons}$$  EL'

E' + L  $$\stackrel{k_{1}'}{\rightleftharpoons}$$  EL'

SCHEME IV

E + L  $$\stackrel{k_{12}}{\rightleftharpoons}$$  EL

E' + L  $$\stackrel{k_{12}'}{\rightleftharpoons}$$  EL'

Scheme III assumes only a single ligand-binding site as in Scheme I. However, it extends Scheme I to include a second form of the enzyme–ligand complex EL* which is accessible via a conformational change of the initial complex EL. This conformational change has rate constants k₂₃ and k₁₂ and equilibrium constant K₃₂/K₃₃ = K₃.

Equilibrium Titrations. Our observations with N-methylacridinium are consistent with a previous report (Mooser et al., 1972) that the fluorescence of the ligand is totally quenched on binding to the enzyme (fluorescence of bound ligand ≤ 0.1% of fluorescence of free ligand). This is also the case for 1-methyl-7-hydroxyquinolinium. Consequently, the observed fluorescence F is directly proportional to the free ligand concentration cₐ such that F = fCₐ, where f is the fluorescence intensity coefficient obtained from observed linear F vs. cₐ plots in the absence of enzyme. For Scheme I, an equilibrium titration of enzyme with increasing amounts of ligand may be expressed by eq 1,

$$\frac{1}{fB'} = \frac{1}{fC_{E}^{init}} \left( 1 + \frac{K_{1}}{F} \right) \left( 1 + \frac{\Delta V}{V} \right)$$

where cₐ is the total ligand concentration; cₑₐ is the initial total enzyme concentration; ΔV/V is a dilution correction for incremental volume additions of ligand solution ΔV to initial total volume V; and B' is the concentration of bound ligand Bₐ multiplied by a dilution correction factor. F values were corrected for a slight background emission arising from solvent and enzyme alone. In a plot of (fB')⁻¹ vs. F⁻¹, cₑₐ is obtained from the (fB')⁻¹ axis intercept (fCₑₐ)⁻¹, and K₁ is obtained from the ratio (fK₁) of the slope to the (fB')⁻¹ axis intercept. An equation similar to eq 1 was used by Mooser et al. (1972) in their equilibrium titration of acetylcholinesterase with N-methylacridinium. Given the observed total lack of bound ligand fluorescence, eq 1 would also hold for Scheme III if K₃ is replaced by the overall K₁₁₁, where K₁₁₁ is given by eq 2,

$$K_{111} = \frac{c_{E_{L}}}{c_{E}^{init} + c_{E}^{*} + c_{E_{L}}^{*}} = \frac{K_{1} K_{3}}{1 + K_{3}}$$

with cₓ defined as the equilibrium concentration of species X.

For the more complex situation in Scheme II, the total bound ligand Bₐ is given by eq 3.

$$B_{L} = c_{L}^{init} - \bar{c}_{L} = \frac{c_{E_{L}}^{init}}{1 + \frac{\Delta V}{V}} \left( \frac{1}{1 + \frac{K_{1}}{\bar{c}_{L}}} + \frac{1}{1 + \frac{K_{2}}{\bar{c}_{L}}} \right)$$
Equation 3 may be rearranged in several ways to permit analysis by means of a reciprocal plot. One rearrangement, suggested by its analogy to eq 1, is given in eq 4

\[
\frac{\phi}{fB'} = \left[ 1 + \frac{\Delta V}{V} \right] \frac{\phi}{fE'_{\text{init}} - F} = \frac{1}{fE'_{\text{init}}} \left( 1 + \frac{\phi}{F \left[ \frac{1}{K_1} + \frac{1}{K_2} \right]} \right) \tag{4}
\]

where

\[
\phi = \frac{(K_1 + K_2 + 2z_L)(K_1 + K_2)}{K_1^2 + K_2^2 + z_L(K_1 + K_2)} \tag{5}
\]

Equation 4 is particularly useful when \( K_2 \gg K_1 \) and \( K_2 > z_L \), in which case \( \phi \approx \frac{(K_2 + 2z_L)(K_1 + K_2)}{K_1^2 + K_2^2 + z_L(K_1 + K_2)} \) and does not deviate greatly from 1. If preliminary estimates of \( K_1 \) and \( K_2 \) can be made, \( \phi \) can be calculated for each experimental point and \( \phi/fB' \) can be plotted vs. \( \phi/F \) to obtain precise values for \( c_{E'_{\text{init}}} \) and \( K_2 \).

Given values for \( c_{E'_{\text{init}}} \) and \( K_1 \) from the titration analysis, \( c_E \) in Scheme I and \( c_E \) in Scheme II can be calculated from eq 6.

\[
c_E = \frac{K_{1E'_{\text{init}}}}{(K_1 + z_L)(1 + \Delta V/V)} \tag{6}
\]

Equation 6 also holds for Scheme III if \( K_1 \) is replaced by \( K_{1I}' \).

**Relaxation Times.** Expressions relating the observed relaxation times \( \tau_1 \) and \( \tau_{1I} \) to the intrinsic rate constants in Schemes I–III have been given by Eigen and DeMaeyer (1963). For Scheme I, eq 7 obtains.

\[
\tau_1^{-1} = \tau_1^{-1} = k_{12}(c_E + z_L) + k_{21}
\tag{7}
\]

The observed \( \tau_1^{-1} \) is equal to the intrinsic variable \( \tau_1^{-1} \) defined by eq 7. Schemes II and III involve coupling through a species which participates in both reactions. To examine the effect of coupling in Scheme II we define \( \tau_{2^{-1}} = k_{12}c_E + k_{21} \), the reciprocal relaxation time for the second reaction in Scheme II' if it were completely uncoupled. One general condition which must obtain in Scheme II' is given by eq 8.

\[
\tau_{1^{-1}}\tau_{1I}^{-1} = \tau_{1^{-1}}\tau_{2^{-1}} - k_{12}c_E + k_{12}c_E
\tag{8}
\]

The analysis of coupled reactions is greatly facilitated if one relaxation time is much greater than the other. This is the case for all the relaxation measurements in this report; the faster relaxation is at least eight times the slower in every instance. If \( \tau_{1^{-1}} \gg \tau_{2^{-1}} \), then we ascribe \( \tau_{1I} \) to the second binding site in Scheme II' and assume \( \tau_{1^{-1}} \approx \tau_{2^{-1}}^{-1} \). Appropriate substitution into eq 8 gives eq 9

\[
\tau_1^{-1} \approx k_{12}(c_E + z_L) + k_{21}
\tag{9}
\]

where \( \theta^{-1} \equiv \frac{1}{1 + c_E/(K_2 + z_L)} \). The term \( \theta \) is a measure of the coupling between the two reactions; coupling becomes negligible as \( \theta \rightarrow 1 \).

The application of eq 7 and 9 in this study is referred to as method A and presumes that \( z_L, c_{E'_{\text{init}}}, K_1 \), and \( K_2 \) are known parameters and that \( c_E \) is obtained from eq 6. Plots of \( \tau_1^{-1} \) vs. \( (c_E + z_L) \) yield \( k_{12} \) as the slope and \( k_{21} \) as the \( \tau_1^{-1} \) axis intercept.

An alternative form of relaxation time analysis, based on a report by Eigen and Winkler-Oswatitsch (1977), is being introduced in this report. This analysis, called method B, avoids calculations of equilibrium concentrations and instead requires only that \( c_{E'_{\text{tot}}} \) be known and that \( c_{E'_{\text{tot}}} \), the total enzyme concentration, be constant. For Scheme I, the equilibrium concentrations in eq 7 can be expressed in terms of \( c_{E'_{\text{tot}}} \) and \( c_{E'_{\text{init}}} \) as shown in eq 10.

\[
\tau_1^{-1} = k_{12}(c_E + c_{E'_{\text{tot}}})^2 + 2k_{12}^2(c_E - c_{E'_{\text{tot}}})c_{E'_{\text{tot}}} + k_{12}^2(c_{E'_{\text{tot}}}^2) = A + Bc_{E'_{\text{tot}}} + C(c_{E'_{\text{tot}}}^2)
\tag{10}
\]

This second-order polynomial can be analyzed by least-squares regression analysis to give the best-fit values of \( A, B, \) and \( C \) which, in turn, can be converted to \( k_{12}, K_1 \), and \( c_{E'_{\text{tot}}} \). Unfortunately, eq 10 and Scheme I are insufficient for the analysis of some of our data. Equation 10 can be modified to apply to Scheme II' and to include a small dilution correction factor under certain conditions. In particular, if we define the constant \( \theta_0 \) as the limit of \( \theta \) (eq 9) as \( L \rightarrow 0 \) and assume that \( K_2 > z_L \), such that \( \theta \approx \theta_0 \) for the entire data set, eq 10 can be applied to Scheme II'. Furthermore, if \( \Delta V/V \) (eq 11) is both approximately proportional to \( c_{E'_{\text{tot}}} \) and small enough that \( (1 + \Delta V/V)^{-1} \approx 1 - \Delta V/V \), then coefficients \( A, B, \) and \( C \) in eq 10 applied to Scheme II' are given by eq 11–13.

\[
A \approx k_{12}^2(K_{1II} + c_{E'_{\text{init}}})\tag{11}
\]

\[
B \approx 2k_{12}^2(K_{1II} - c_{E'_{\text{init}}}[1 + \rho(c_{E'_{\text{init}}} + K_{1II})])\tag{12}
\]

\[
C \approx k_{12}^2(1 + \rho c_{E'_{\text{init}}}^2)\tag{13}
\]

where \( k_{12} = k_{12} \theta_0, K_{1II} \theta_0, \) and \( \rho \equiv \Delta V/c_{E'_{\text{tot}}} \). If \( \theta_0 \) and \( \rho \) can be estimated independently, \( K_{1II}, c_{E'_{\text{init}}} \), and \( k_{12} \) can be obtained from eq 11–13 by successive approximations.

**Competitive Inhibition.** In the presence of a competitive inhibitor I, Scheme I is extended to Scheme IV.

**SCHEME IV**

\[
E + L \xrightleftharpoons{k_{21}} EL \quad \text{and} \quad E + I \xrightleftharpoons{k_{w1}} EI
\]

The formal treatment of Scheme IV is identical to that of Scheme II', although the coupling occurs through \( E \) rather than \( L \). Thus, if \( \tau_{1^{-1}} \equiv [k_{12}(c_E + z_L) + k_{21}] \), the reciprocal relaxation time for the second reaction in Scheme II' if it were completely uncoupled, One general condition which must obtain in Scheme II' is given by eq 8.

\[
\tau_{1^{-1}}\tau_{1I}^{-1} = \tau_{1^{-1}}\tau_{2^{-1}} - k_{12}c_E + k_{12}c_E
\tag{8}
\]

The application of coupled reactions is greatly facilitated if one relaxation time is much greater than the other. This is the case for all the relaxation measurements in this report; the faster relaxation is at least eight times the slower in every instance. If \( \tau_{1^{-1}} \gg \tau_{2^{-1}} \), then we ascribe \( \tau_{1I} \) to the second binding site in Scheme II' and assume \( \tau_{1^{-1}} \approx \tau_{2^{-1}}^{-1} \). Appropriate substitution into eq 8 gives eq 9

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\tag{9}
\]

where \( \theta^{-1} \equiv \frac{1}{1 + c_E/(K_2 + z_L)} \). The term \( \theta \) is a measure of the coupling between the two reactions; coupling becomes negligible as \( \theta \rightarrow 1 \).

The application of eq 7 and 9 in this study is referred to as method A and presumes that \( z_L, c_{E'_{\text{init}}}, K_1 \), and \( K_2 \) are known parameters and that \( c_E \) is obtained from eq 6. Plots of \( \tau_1^{-1} \) vs. \( (c_E + z_L) \) yield \( k_{12} \) as the slope and \( k_{21} \) as the \( \tau_1^{-1} \) axis intercept.

An alternative form of relaxation time analysis, based on a report by Eigen and Winkler-Oswatitsch (1977), is being introduced in this report. This analysis, called method B, avoids
where $k_{on} = k_{12}b_0$, $\theta_{-1} = (1 + K_s/\tau_{II})$, $K_a = k_{ea}/k_{s}$, and $K_{obs} = K_{II}/b_0$. Treatment is similar to that following eq 14.

Relaxation Amplitudes. The total amplitude $\Delta F_{tot}$ of the one-step process in Scheme I has been derived by Eigen and DeMaeyer (1963) and is given by eq 16

$$\Delta F = \left[ \frac{-f_{cB} - f_{cE}}{c_{E} + c_{I} + K} \right] \left[ \frac{\Delta H \Delta T}{RT^2} \right]$$

where $\Delta F = \Delta F_{tot} = \Delta F_1 + \Delta F_2$. If it is assumed that only the free ligand fluoresces, then eq 16 obtains with $\Delta F = \Delta F_{tot}$, $K = K_{II}$ (eq 2), and $\Delta H = \Delta H_2$. In this case, the amplitude of the slower step $\Delta F_2$ is given by eq 17

$$\Delta F_2 = \left[ \frac{-f_{cB} - f_{cE}}{c_{E} + c_{I} + K} \right] \times \left[ \frac{\Delta H_2 + (1 - \theta) \Delta H_1}{RT^2} \right]$$

where $\theta$ is given below eq 9. The second and third terms on the right are virtually constant if $\theta$ is both approximately constant and close to 1, the case in the current data.

In Scheme III the two observed relaxation processes are most appropriately considered in terms of a single overall equilibrium with $\Delta F_{tot} = \Delta F_1 + \Delta F_2$. If it is assumed that only the free ligand fluoresces, then eq 16 obtains with $\Delta F = \Delta F_{tot}$, $K = K_{II}$ (eq 2), and $\Delta H = \Delta H_2$. In this case, the amplitude of the slower step $\Delta F_2$ is given by eq 17

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Results

Relaxation Spectra Associated with N-Methylacridinium and Acetylcholinesterase. Relaxation measurements were carried out in 0.1 M sodium phosphate for two reasons. (1) The fluorescence of N-methylacridinium was quenched by about a factor of 2 in 0.1 M NaCl relative to 0.1 M sodium phosphate. A Stern-Volmer analysis indicated that quenching arose from a complex between N-methylacridinium and Cl\(^{-}\). A rapid relaxation was observed when a mixture of N-methylacridinium and Cl\(^{-}\) was perturbed in temperature-jump experiments. Because this relaxation was coupled to that involving ligand association with the enzyme (a situation formally identical to Scheme II'), subsequent experiments avoided Cl\(^{-}\). (2) A complicated relaxation spectrum was observed for the interaction of either N-methylacridinium or 1-methyl-7-hydroxyquinolinium with acetylcholinesterase at pH 6.0 and 5.3. Because the spectrum was greatly simplified at higher pH values, the complicated spectrum at or below pH 6.0 suggests the involvement of both protonated and unprotonated enzyme forms in the binding of cationic ligands. This topic is beyond the scope of the present paper. However, it was decided to work at pH 8.0-8.5 where possible to avoid a contribution due to protonated enzyme forms. At this pH, 0.1 M sodium phosphate is required for sufficient buffering capacity.

Typical relaxation spectra obtained with N-methylacridinium and acetylcholinesterase at pH 8.0 are shown in Figure 1. At the relatively low ligand concentration in Figure 1a, the sudden temperature increase of about 3 OC gives rise to a relatively large increase in fluorescence; the overall fluorescence increase or total relaxation amplitude $\Delta F_{tot}$ corresponds to nearly 15% of the total fluorescence $F$ at these ligand concentrations. This fluorescence increase can be fit precisely to a single exponential curve and thus can be characterized by a single relaxation time. At higher ligand and high enzyme concentrations as in Figure 1b, $\Delta F_{tot}/F$ decreases and two distinct relaxation steps become apparent. The fast step is displayed with a decreased time scale in Figure 1c. The relaxation time of 33 $\mu$s calculated for this fast step can be clearly distinguished from the relaxation time or rise time associated with the temperature-jump forcing function itself. This rise time is shown in Figure 1d, where a temperature jump is ap-

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**Table 1: Thermodynamic and Kinetic Constants Observed for the Interaction of N-Methylacridinium and Acetylcholinesterase in 0.1 M Sodium Phosphate, pH 8.0 at 23 °C.**

<table>
<thead>
<tr>
<th>Method</th>
<th>$K_1$ (µM)</th>
<th>$k_{12}$ (nM⁻¹s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.149 ± 0.003</td>
<td>1.18 ± 0.03</td>
</tr>
<tr>
<td>B</td>
<td>0.103 ± 0.012</td>
<td>1.23 ± 0.05</td>
</tr>
</tbody>
</table>

* Method A involves separate determinations of $K_1$ and $k_{12}$ according to eq 4 and 9. In Method B, $K_1$ and $k_{12}$ are evaluated simultaneously according to eq 10 through 13 (see Figure 5). Data are from Figures 2, 4, and 5. Estimates utilize $\rho = 0.00225$ and $\theta = 1.140$. (Method B) and Figure 5 were made experimentally by analysis of ten random data sets generated by the equation $\tau_{IR} = \tau + (\tau\bar{\tau})^{1/2}(R)$ where $\tau$ is an observed mean relaxation time, $R$ is a random Gaussian number from a Gaussian distribution of standard deviation equal to 1, and $\tau_{IR}$ is the generated mean relaxation time.

All listed error estimates are standard deviations of the mean parameter estimate, defined as the square root of the variance of the mean parameter estimate.

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1 This application of eq 16 to Scheme III derives from the easily demonstrated equality

$$\Delta F_{tot} = \int \left[ \frac{1}{\tilde{c}_{E}} + \frac{1}{\tilde{c}_{L}} + \frac{1}{\tilde{c}_{EL}} \right] \times \left[ \Delta \ln K_1 + \Delta \ln K_2 + \Delta \ln K_3 \right] d\tau = \frac{-f_{cB} - f_{cE}}{c_{E} + c_{I} + K_{III}} \Delta \ln K_{III}$$

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applied to a ligand solution in the absence of enzyme. A rise time of 8 μs and a ΔF_{tot}/F = 0.0064 is observed for this control condition; this intrinsic change in ligand fluorescence was subtracted as the ΔF values for mixtures of ligand and enzyme were obtained.

Three or four temperature-jump measurements were made at each c_{1} value. At lower c_{1}, (e.g., the first six points in Figure 5), the standard deviation of the mean slow relaxation time was <5% of the mean. At higher c_{1}, the standard deviation was <10% of the mean slow relaxation time. An insufficient number of fast relaxation times were measured for adequate statistical analysis.

It should be noted that the fast relaxation step is observed only at high enzyme normalities (>1 μM). At lower enzyme normalities the amplitude of this step becomes vanishingly small.

**Equilibrium Titration of Acetylcholinesterase with N-Methylacridinium.** A quantitative evaluation of the relaxation data conventionally requires observation or calculation of both the equilibrium free ligand concentration c_{1} and the equilibrium free enzyme concentration c_{E}. An equilibrium titration of acetylcholinesterase with N-methylacridinium is displayed as a Scatchard plot in Figure 2. This titration was carried out simultaneously with relaxation measurements by sequential additions of ligand to the temperature-jump cell. The ligand fluorescence F was measured both prior to and after the relaxation measurements at a given total ligand concentration c_{tot}. At each c_{tot} the initial F was slightly greater than the final F. This progressive loss of fluorescence amounted to about 2% at each c_{tot}. The loss was less pronounced at lower pH and more pronounced at higher pH in the absence of Ca^{2+} (see below). We assumed that this fluorescence decrease represented a net loss of ligand from the system (see Discussion) and applied a cumulative correction to c_{tot} for the observed fluorescence loss in the temperature-jump cell.

The data in Figure 2 conform to a single-binding isotherm at low ligand concentrations, but a second, lower affinity binding site is also suggested at the highest ligand concentrations. The relaxation amplitude analysis described in the following section independently confirmed the presence of a second ligand-binding site, and final analysis of the data in Figure 2 was based on Scheme II'.

Inclusion of the correction for the second, low-affinity binding site was important to a quantitative characterization of the relaxation data at c_{tot} > 1.0 μM. For the initial estimate of K_{1}, in which Scheme I was assumed to apply to the first eight points in Figure 2, K_{1} was 5% less and c_{E}^{inv} 5% more than the final estimates assuming Scheme II'. Corresponding c_{E} estimates (eq 6) using these initial values were 6 to 10% less than the final estimates, and the experimental point scatter in Figures 3 and 4 below was significantly improved when the final c_{E} estimates replaced the initial estimates.

**Analysis of Relaxation Amplitudes.** The observation of two relaxation processes at high enzyme normality in Figure 1 implies the presence of at least two equilibria in the interaction of N-methylacridinium with acetylcholinesterase. Schemes II' and III represent two of the simplest systems which involve two equilibria. While the equilibrium titration data in the previous section suggest that Scheme II' obtains, we examined the relaxation data to see if it would independently confirm...
Scheme II' and reject Scheme III. The relatively small relaxation time $\tau_{II}$ associated with the fast step made its analysis in terms of $C_E$ and $C_L$ rather imprecise. The relaxation amplitude of this step was obtained with greater precision. An analysis of relaxation amplitudes offers the further advantage that, when $\tau_{II} > \tau_{I}$, a clear distinction between Schemes II' and III can be made without assumptions about the intrinsic rate constants for the two observed steps. Thus, for Scheme III, with the restriction that only the free ligand fluoresces, the total relaxation amplitude $\Delta F_{III}$ follows the simple concentration dependence given by eq 16 ff, while, for Scheme II', the relaxation amplitude $\Delta F_1$ (slow step) is given by eq 17. In Figure 3, amplitude analyses corresponding to these two alternatives are shown. A much better fit of the data is obtained when $\Delta F_1$, rather than $\Delta F_{tot}$, is the dependent variable. Thus, Scheme II', but not Scheme III, is consistent with the data at high enzyme normalities.\(^2\) Below $c_{E}^{init}$ values of 1.0 $\mu$M, $\theta$ values in Scheme II' are greater than 0.95 and Scheme I is an excellent approximation.

From the slope of the line in Figure 3, $\Delta H_1$ may be approximated (eq 17) with the assumption that $(1 - \theta) \approx 0$. (Assuming $K_2 = 20 \mu$M, $\theta$ varies between 0.88 and 0.91.) For the 3.3 °C temperature change, $\Delta H_1 \approx 34.3$ kJ/mol (8.2 kcal/mol).

\(^2\) Although equilibrium titration data indicate that the fluorescence of bound ligand is <0.1% of the free ligand fluorescence, it could be argued that within Scheme III a slightly fluorescent EL is present in low concentration relative to nonfluorescent EL*. If the fast relaxation observed here corresponded to the $K_2$ equilibrium in such a Scheme III, the amplitude of the slow step $\Delta F_1$ would be given by eq 18 (see Thusiou, 1972)

$$\Delta F_1 \approx \left(\frac{\tau_{II}^E}{c_{E}^* + 2 + K_{III}}\right) \left(\Delta \ln K_{III}\right) \left(\frac{f_L - f_{EL}}{f_{EL}(1 + K_3)^{-1}}\right)$$

where $K_{III}$ is defined in eq 2, $f_L$ is the fluorescence intensity coefficient for free ligand, and $f_{EL}$ is the fluorescence intensity coefficient for ligand bound in the EL species. The predictions of eq 17 for Scheme II' and of eq 18 for Scheme III' are very similar, and the amplitude data in Figure 3 cannot distinguish between them. However, Scheme III' can be rejected because it predicts that the relaxation times $\tau_{II}$ for the fast step should be independent of $C_L$ and $C_E$. In fact the relaxation times $\tau_{II}$ are observed to decrease about 30% as $C_L$ increases from 1.6 to 5.5 $\mu$M, a result consistent with Scheme II'.

Assuming Scheme II', analysis of $\Delta F_{II}$ permits an estimate of $K_2$. Trial values of $K_2$ were inserted in eq 16 to obtain the best correspondence to the $\Delta F_{II}$ data. The data were not highly precise, but $K_2 = 20 \mu$M could be estimated within a factor of two. This estimate was sufficiently accurate to allow calculation of $\phi$ values (eq 5) for use in the equilibrium titration data in Figure 2.

Analysis of Relaxation Times. The observed reciprocal relaxation times for the slow step ($\tau_{I}^{-1}$) were plotted against the equilibrium concentrations in accordance with eq 9, as shown in Figure 4. The calculated value for $k_{12}$ is given in Figure 4 and Table I (method A).

An independent analysis of the observed $\tau_{I}$ values was carried out assuming only $c_{E}^{init}$ as the independent variable. Data were analyzed with the use of eq 10, and $c_{E}^{init}$, $K_1$, and $k_{12}$ were obtained from eq 11–13 as outlined in the Experimental Section. This analysis is given in Figure 5 and Table I (method B), and a quite reasonable correspondence with the more conventional determination in Figure 4 (method A) was obtained despite the required slight approximation $\theta \approx \theta_0$ in method B. Methods A and B agree precisely on the value of $c_{E}^{init}$. This agreement is largely due to the condition $c_{E}^{init} \gg K_1$, thus allowing considerably greater accuracy in determining $c_{E}^{init}$ than $K_1$ in method B. Indeed, $K_1$ in method B is only about 70% of the more accurately determined $K_1$ value in method A and has a much higher relative standard deviation. Nevertheless, the difference between $K_1$ in methods A and B is larger than that predicted by the variance of the mean relaxation times. This suggests either that the approximations underlying the use of method B (see eq 10 ff) or, more likely, that some systematic inconstancy, e.g., slight progressive loss of E or L from the system, is influencing the estimates. Estimates of $k_{12}$ are also listed in Table I, and methods A and B agree within the limits predicted by the experimental variance.

The dissociation rate constant $k_{21}$ may be obtained directly from the $\tau_{I}^{-1}$ intercept in Figure 4 or as the product of $K_1$ and $k_{12}$ in Table I. Because $c_{E}^{init} \gg K_1$, the intercept in Figure 4 is too near the origin for an accurate estimate of $k_{21}$. However, combined estimates at lower $c_{E}^{init}$ indicate $k_{21} \approx 140$ s⁻¹, in...
reasonable agreement with a value of ~100 s⁻¹ estimated from dilution experiments involving N-methylacridinium and acetylcholinesterase (Mooser and Sigman, 1975).

Effect of Inhibitors on Relaxation Times. The effect of Ca²⁺ on the relaxation times observed with acetylcholinesterase and N-methylacridinium was observed in Tris buffer at pH 8.5. As noted above, a slow decrease in ligand fluorescence was particularly pronounced in the absence of Ca²⁺. Ligand fluorescence decreased about 6% over the course of the relaxation experiments at each CL' at the absence of Ca²⁺, while in the presence of 30 mM Ca²⁺ about 2% of the free ligand fluorescence was lost at each CL'.

At the 1.0 mM CL' conditions in these studies, Schemes I and IV were adequate approximations and relaxation times were analyzed according to eq 14 (method A). Data are given in Table II. Estimates of k on by method B were within experimental error of those listed in Table I. Both Ca²⁺ and Tris appear to act as competitive inhibitors. For Ca²⁺, k₁ ≈ 30 mM; for Tris at this pH, k₁ ≈ 50 mM.

Relaxation Measurements with Acetylcholinesterase and 1-Methyl-7-hydroxyquinolinium. Analysis of this interaction is slightly complicated by the fact that the ligand exists in two forms, the protonated cation and the unprotonated zwitterion. Only the zwitterion is fluorescent under the experimental conditions (Prince, 1966; Rosenberry and Bernhard, 1971), but our current study indicates that only the protonated form has significant affinity for the enzyme active site. The apparent ligand dissociation constants K'obsd at three pH values are shown in Table III. The high K'obsd value at pH 7.5 suggests that the protonated ligand form has higher enzyme affinity. To analyze this suggestion quantitatively, Schemes IV and V were combined. Protons are known to act as competitive inhibitors of cation binding to the catalytic site of the enzyme with a K₁ of 6.3 (see Rosenberry, 1973b), and the ligand has a pK₁ of 5.9 (Prince, 1966; Rosenberry and Bernhard, 1971). In this case, K'obsd = K₁(1 + κ₁/K₁)(1 + κ₁/κ₁). Calculated values of K₁ at each pH are also shown in Table III, and their agreement provides confirmation of this analysis.

Because the affinity of this ligand is greatly reduced at high pH, it was necessary to study relaxation phenomena at a pH lower than 8.0. Only a single relaxation time was observed at pH 7.0, and thus the study was carried out at this pH; at lower pHs, a complex relaxation spectrum was observed as noted above. At pH 7.0, τ'obsd followed eq 15 (method A). The observed value of k on was 0.162 ± 0.011 × 10⁶ M⁻¹ s⁻¹. This corresponds to a calculated k₁ of 2.18 ± 0.15 × 10⁶ M⁻¹ s⁻¹ (see eq 15). The observed value of k₁ was 444 ± 43 s⁻¹. In the calculation of k₁, k on was assumed to be a function of K₁ but not of K₁, in contrast to K'obsd. This assumption was based on two observations: the relaxation time for ligand protonation was too small to be observed, consistent with the assumption above eq 15; and inhibition due to enzyme protonation is more complicated than the simple competition proposed in Scheme IV and apparently occurs slowly enough to give multiple coupled relaxations. Thus, any effect of enzyme protonation on τ'obsd could not be analyzed by the given equations and was ignored. Such effects should be small at pH 7.0.

Discussion

Accuracy of Data. The equilibrium dissociation constant K₁ for the interaction of N-methylacridinium and acetylcholinesterase in Table I is in agreement with that observed by Mooser et al. (1972) when a slight difference in solvent is taken into account. Two independent methods of analysis in Table I agree quite well on the value of k₁ which characterizes this interaction. The k₁ value is very high and is discussed further below. Method B is introduced in this report and is an extension of a procedure introduced by Eigen and Winkler-Oswatitsch (1977); it is particularly elegant in that only one experimental variable, the total ligand concentration CL' (eq 1 in Mooser et al., 1972), need be known. From a single set of relaxation times at constant total enzyme concentration, this method allows simultaneous determinations of the bimolecular reaction rate constant, the equilibrium dissociation constant, and the total enzyme normality for the ligand-enzyme interaction. Furthermore, these determinations are accomplished by a rigorous least-squares solution of a second-order polynomial which can include appropriate variance analyses.

The greatest uncertainty in our observed values arises from the estimate of CL' and is due to the progressive loss in fluorescence with time at each CL'. Because this loss is increased at high pH and decreased at low pH and in the presence of Ca²⁺, it quite plausibly could arise from adsorption of the positively charged ligand on the quartz walls of the temperature-jump cell. An alternative explanation for the loss is suggested by a recent report on the photochemical dismutation of N-methylacridinium (Einarsen and Zeppenauer, 1975), a process catalyzed by either horse liver alcohol dehydrogenase or human serum albumin. A similar photochemical sensitivity is much less apparent in our experiments. Addition of acetylcholinesterase, if anything, decreases the rate of fluorescence loss, and the rate of loss is not greatly affected by whether the exposure to excitation light is intermittent or continuous.

The effect of uncertainty in CL' arising from this source on the kinetic rate constants is not large. If CL' is not corrected for fluorescence loss in method B in Figure 5, for example, k₁ is reduced by 11%, K₁ is increased by 19%, and CL' is increased by 8%. The uncertainty in CL' also affects the estimate of the fluorescence coefficient f; Figures 2-5 were plotted in the original units of photomultiplier voltage, but to convert to μM units in Figures 2-5 and Tables I-III a division by f was...
carried out. In this conversion $f$ was considered to have insignificant error, and thus the indicated standard errors of the thermodynamic and kinetic constants reflect the precision of the data. Because the uncertainty in $c_{\text{E}}^{\text{mol}}$ may have resulted in a standard error in $f$ of 5%, the absolute standard errors are somewhat larger than those indicated.

Estimates of $k_{\text{on}}$ and $k_{\text{off}}$ obtained by applying method B to a variety of relaxation data indicate a close correlation of $k_{\text{on}}$ and $k_{21} = k_{\text{on}}K_{\text{obsd}}$ with the corresponding values of $k_{\text{on}}$ and $k_{21}$ obtained from method A (eq 14). In other words, when the application of method A alone indicates a discrepancy between the equilibrium determination of $K_{\text{obsd}}$ (eq 1) and the kinetic determination of $K_{\text{obsd}} = k_{21}/k_{\text{on}}$ (eq 14) (e.g., the data on compound II above), the method B estimate of $K_{\text{obsd}}$ agrees with the kinetic estimate of method A. Such discrepancies probably indicate significant inconstancies in the data, as noted above. With the appropriate computer program, method B is very straightforward; because $r^2$ values are used, appropriate weighting is essential. The agreement of estimates of $c_{\text{E}}^{\text{mol}}$ and/or $K_{\text{obsd}}$ from this method with independent estimates from equilibrium data is an indication of the overall consistency of the data. The accuracy of method B appears greatest when $K_{\text{obsd}} \leq c_{\text{E}}^{\text{mol}} \leq 5K_{\text{obsd}}$ under which conditions the plot corresponding to Figure 5 has an inverted bell shape.

Mechanistic Schemes Examined. Over most of the concentration ranges examined, the interactions of both $N$-methylacridinium and 1-methyl-7-hydroxyquinolinium with acetylcholinesterase appear consistent with Scheme I. Only at high enzyme concentrations does $N$-methylacridinium begin to show a second, faster relaxation time. While the data indicate that this second relaxation is best explained by Scheme II', appropriate coupling of the two equilibria in Scheme III could have given qualitatively similar observations. A plausible candidate for the second ligand-binding site postulated by Scheme II' is the peripheral anionic site on acetylcholinesterase defined by the binding of propidium (Taylor and Lappi, 1975). This peripheral site is probably the anionic site approximately 14 Å away from the catalytic site between which bisquaternary ligands specifically bind to acetylcholinesterase (Mooser et al., 1972; Taylor and Lappi, 1975; Wee et al., 1976; see Rosenberry, 1975a). While no relaxation spectrum consistent with Scheme III was observed, it is likely that that binding of $N$-methylacridinium to the catalytic site of acetylcholinesterase does induce a conformational change of the enzyme–ligand complex. Aromatic cation binding has been suggested to involve conformational changes of acetylcholinesterase (Rosenberry and Bernhard, 1972; Rosenberry, 1975a), and $N$-methylacridinium is one of only a few aromatic cations which can accelerate the acetylcholinesterase-catalyzed hydrolyses of methyl or ethyl acetate (Barnett and Rosenberry, 1977). The acceleration phenomenon is highly suggestive of an "induced-fit" ligand–enzyme complex (Koshland, 1958; Rosenberry, 1975b). Induced fit can be formalized by Scheme III, but if this process occurs with $N$-methylacridinium and acetylcholinesterase the rate constants for the second step are sufficiently fast that this step rapidly equilibrates and, hence, is not observable.

Basis of High $k_{12}$ Values. The most important finding in our study is the high values of $k_{12}$ that characterize the binding of both $N$-methylacridinium and 1-methyl-7-hydroxyquinolinium to the catalytic site of acetylcholinesterase. These $k_{12}$ values are greater than $10^9$ M$^{-1}$ s$^{-1}$. Typical values of $k_{12}$ for the interaction of ligands with specific enzyme sites are in the range of $10^7$ to $10^8$ M$^{-1}$ s$^{-1}$, although $k_{12}$ values for NADH and certain dehydrogenases approach $10^9$ M$^{-1}$ s$^{-1}$ (see Hammes and Schimmel, 1971). An example of ligand binding closely analogous to those reported here is the interaction of the aromatic cation proflavin with the active site of chymotrypsin. A $k_{12}$ of only $1 \times 10^8$ M$^{-1}$ s$^{-1}$ at 12 °C and pH 9.2 was observed (Havsteen, 1967).

It is instructive to consider the theoretical encounter frequency $k_{12}^*$ of a ligand with an enzyme active site in aqueous solution. A first approximation of $k_{12}^*$ can be made on the basis of eq 18 (see, e.g., Eigen, 1974; Eigen and Hammes, 1963) for the association step of the cationic ligand (charge number $z_L = +1$) with the active site of the enzyme which carries at least one negative charge ($z_E = -1$).

$$k_{12}^* = \frac{2\pi N}{10^5} (D_E + D_L)(d_{\text{EL}})(\alpha)$$ (18)

In eq 18, $N$ is Avogadro's number, $2\pi$ is the physically plausible value for the solid angle of diffusional approach of the ligand to the active site, $D_E + D_L \approx D_L \approx 10^{-4}$ cm$^2$/s is the estimate for the sum of the diffusion coefficients of enzyme and ligand, respectively, and $d_{\text{EL}} \approx 5$ Å is the estimated "encounter distance" within which E and L react to form a complex. The term $\alpha$ accounts for the electrostatic contribution to the association. To a first approximation for an ionic strength of ~0.1 M and with $z_{\text{EL}} = -1$, we may use $\alpha \approx 1$. From eq 18 we then estimate $k_{12}^* \approx 2 \times 10^9$ M$^{-1}$ s$^{-1}$. Since the observed $k_{12}$ values in this study approach this $k_{12}^*$ estimate, the bimolecular reactions studied here approach diffusion control as defined by Eigen and Hammes (1963); i.e., any encounter between ligand and active site leads to a complex, independent of approaching ligand orientation. If the effective charge number $z_E$ of the active site is increased by neighboring fixed (negatively) charged groups, a larger electrostatic contribution results in a larger value of $\alpha$ and thus a larger value of $k_{12}^*$. Even in this event, the observed $k_{12}$ values are close to diffusion controlled.

It is of interest to consider whether any other mechanisms underlie the extremely high association rates with which acetylcholinesterase is able to react with cationic ligands like $N$-methylacridinium or 1-methyl-7-hydroxyquinolinium ions. Recently, Eigen (1974) discussed a way in which macromolecules can achieve $k_{12}$ values for specific ligand interactions that exceed the apparent theoretical maximum of $k_{12}^*$. If such macromolecules form extended surfaces in one (DNA) or two dimensions (membrane-bound proteins) and have numerous nonspecific binding sites, ligand encounters at nonspecific sites followed by rapid surface diffusion to the specific binding site can provide a considerable increase in the effective $d_{\text{EL}}$ in eq 18. Such an increase, amounting to two or three orders of magnitude, appears to obtain for the interaction of lac repressor with the operator site on DNA.

Acetylcholinesterase possesses several peripheral anionic sites for which, as yet, no physiological function has been found (see Rosenberry, 1975a). Since the $k_{12}$ values with aromatic cations here as well as the apparent bimolecular reaction rates obtained from steady-state studies with acetylcholine and other specific substrates (Rosenberry, 1975a; Rosenberry, 1975b) are somewhat higher than normally observed from enzyme–ligand interactions, it is pertinent to inquire whether surface diffusion enhances these rates. One indication of a surface-diffusion mechanism which is based on electrostatic interactions between ionic ligands and fixed surface charges is a high ionic strength dependence of $k_{12}$ (see Eigen, 1974). Increasing ionic strength tends to increase the dissociation rate constant from nonspecific sites and hence to reduce the surface diffusion contribution to $d_{\text{EL}}$. While in principle it would thus be relevant to investigate the ionic-strength dependence of ligand interactions with acetylcholinesterase, the enzyme undergoes rather
striking changes in ligand-binding properties as the ionic strength is raised from 0.001 to 0.1 M (Mooser and Sigman, 1974; Taylor and Lappi, 1975) which suggest multiple enzyme conformations. To avoid complications in analysis which could arise from ionic-strength-dependent enzyme conformational equilibria, we investigated the role of peripheral anionic sites by introducing 30 mM Ca(ClO4)2 to the control solvent, 0.1 M NaClO4, 50 mM Tris, pH 8.5. Ca2+ is known to bind with high affinity to peripheral anionic sites and much lower affinity to the catalytic site of acetylcholinesterase (Roufogalis and Quist, 1972; Taylor and Lappi, 1975). If peripheral anionic sites contribute to a surface diffusion component in the $k_{12}$ values observed here, this introduction of Ca2+ should significantly reduce these values. No such contribution was found; 30 mM Ca2+ reduced $k_{on}$ by less than a factor of 2 (Table II) or to about the extent expected for competition at the catalytic site (Scheme IV) with a $K_1$ for Ca2+ of about 30 mM. The apparent absence of a surface diffusion contribution from peripheral sites to $k_{12}$ at the roughly physiological ionic strength used here does not rule out the possibility of such a contribution from other surface sites with Ca2+ dissociation constants $>$30 mM.

Physiological Relevance of High $k_{12}$ Values. Acetylcholine in physiological systems is largely compartmentalized intracellularly such that it remains protected from hydrolysis by acetylcholinesterase located on the external cell surface (see Rosenberry, 1975a). During excitation, acetylcholine is released, and acetylcholinesterase and acetylcholine receptors, present in about equal amounts in excitable membranes (see Rosenberry, 1975a), compete for the released acetylcholine. The high value of $2 \times 10^8$ M$^{-1}$ s$^{-1}$ for $k_{cat}/K_{app}$ for acetylcholine noted in the Introduction defines a minimum value for the bimolecular rate constant for the reaction of acetylcholine with acetylcholinesterase. The even higher values of $k_{12}$ observed for the fluorescent ligands in this report suggest that this constant may even be higher. In any event, the destruction of acetylcholine by acetylcholinesterase is close to diffusion controlled. In contrast, a recent temperature-jump relaxation kinetic study found that acetylcholine interacts with solubilized and purified acetylcholine receptor with a $k_{12}$ of $2 \times 10^7$ M$^{-1}$ s$^{-1}$ (Neumann and Chang, 1976), an order of magnitude lower than the second-order enzyme-catalyzed hydrolysis rate. Thus, the competition of these two proteins in solution for acetylcholine would greatly favor acetylcholinesterase. Since the interaction of acetylcholine with receptor is vital to consequent membrane conductance changes, the in vivo structural organization inherent in the membrane localization of the two proteins may prevent them from competing for acetylcholine as they would in homogeneous solution.

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