Ligand-Induced Changes in Membrane-Bound Acetylcholine Receptor Observed by Ethidium Fluorescence. 2. Stopped-Flow Studies with Agonists and Antagonists†

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ABSTRACT: The kinetics of cholinergic ligand binding to membrane-bound acetylcholine receptor from Torpedo californica have been followed in a stopped-flow photometer, by using the fluorescent probe ethidium. The overall reaction amplitude, as a function of ligand concentration, can be fit to the law of mass action for both agonists and antagonists. All agonists show at least biphasic kinetics, and the concentration dependence of the kinetic parameters is fit by a common mechanism involving sequential binding of ligands with increasingly lower affinity. The receptor–ligand pre-complexes isomerize to different noninterconvertible final complexes depending on the number of ligands bound. In contrast, the kinetics observed with antagonists cannot be fit to a common model. These kinetics are always much slower than those observed with agonists, and the relaxation rates depend only weakly on antagonist concentration.

Various signals have been used to observe the interaction of cholinergic ligands with AcChR preparations in in vitro studies. The most direct method followed the changes in intrinsic fluorescence accompanying ligand binding (Bonner et al., 1976; Barrantes, 1976). The small overall change in fluorescence (±1%) superimposed on a large nonspecific fluorescence decrease renders these experiments extremely difficult. The inhibition of the rate of toxin binding to the receptor by cholinergic ligands provided an indirect signal by which slow ligand-induced changes in the affinity state of the AcChR were detected (Weber et al., 1975; Colquhoun & Rang, 1976; Weiland et al., 1976, 1977; Lee et al., 1977; Quast et al., 1978b). The interpretation of the data obtained in this way requires a careful study of the mechanism of toxin binding and the perturbation thereof by the ligand under study (see, e.g., Quast et al., 1978b). Recently, extrinsic fluorescent probes like quinacrine (Grünhagen & Changeux, 1976; Grünhagen et al., 1976, 1977) and ethidium (Schimerlik & Raftery, 1976; Quast et al., 1978a) have been used to monitor the kinetics of ligand binding to membrane-bound AcChR. Obviously, the interpretation of such data depends on a careful analysis of the interaction of the indicator dye with the membrane fragments.

In the preceding communication (Schimerlik et al., 1979a), it was shown that the fluorescence increase observed after mixing receptor–Eth complex with cholinergic ligands reflects specific ligand–receptor interactions since (1) no fluorescence increase is observed when receptor–toxin complex (which does not bind ligand in a specific manner; see, e.g., Weber & Changeux, 1974) was mixed with ligand and (2) the fluorescence increase observed after mixing receptor–Ethidium complex with varying ligand concentrations is described by the law of mass action, yielding equilibrium constants consistent with those observed by other methods. For the ligand Carb it was shown that the fluorescence increase was due to an increase in the quantum yield of bound Eth rather than to a dye uptake. Moreover, Eth does not seem to extensively alter the state of the membrane-bound receptor (see Schimerlik et al. (1979a)). In this paper we present the kinetics of the interaction of cholinergic agonists and antagonists with membrane-bound AcChR in the presence of Eth, as observed in a stopped-flow photometer, and we discuss fitting of the results to a variety of mechanisms.

Experimental Section

Torpedo californica membrane fragments, enriched in AcChR, were prepared as referenced in the preceding paper of this issue (Schimerlik et al., 1979a). The affinity state of membrane-bound AcChR was determined according to Quast et al. (1978b). The chemicals used are listed in the preceding paper.

Rapid mixing experiments were done in a Durrum stopped-flow photometer, Model D-110 (dead time ≈ 2.5 ms), set up in the fluorescence mode. The instrument was connected to a Tektronix 5103N storage oscilloscope and/or a Biomation Model 805 transient recorder and a Hewlett-Packard 7004 X-Y recorder. Excitation was at 493 ± 5 nm and emission was monitored by using a Corning C.S. 3–69 cutoff filter. Typical conditions were as follows. Membrane fragments (0.3–0.5 μM in [125I]α-BuTx sites) and Eth (2 μM) in one syringe were mixed with Eth (2 μM) and ligand in the other. All solutions were prepared in Torpedo Ringer's solution containing 0.02% sodium azide (at pH 7.4), and temperature was maintained at 25 °C.

Kinetic traces were analyzed by graphical methods (see, e.g., Figure 1). The fitting procedure of the kinetic parameters to mechanism 1 is described in the Appendix.

Results

1 Observations with Agonists. (1.1) Carbamylcholine. Qualitative Observations. Mixing of membrane-bound receptor with cholinergic ligands in the presence of Eth resulted in an increase in Eth fluorescence (Schimerlik & Raftery, 1979).
several days at
was observed at ligand concentrations higher than 500 nM
for Carb binding to the receptor with the exception that in
concentrations higher than 50 pM only the second phase
with buffer or mixing of receptor-toxin complex with cho-
many membrane preparations a third (very fast) exponential
value. The observed rate,
fluorescence following the fast phase
increase of bound Eth followed a single slow exponential with
concentrations, a faster phase arose (phase 2). Figure 1 shows
a half-time in the minute range (phase I). At higher ligand
was often observed
was mixed
with receptor originally in the low-affinity form (Weber et al.,
1976; Schimerlik et al., 1979a). Use of the stopped-flow
photometer technique provided the time resolution for an accurate
examination of the kinetics of that increase (Quast et al., 1978a).
In order to ensure that the observed effect was specific, the
following control experiments were undertaken: (a) rapid mixing of
membrane fragments, originally in the low-affinity form, with buffer in the stopped-flow photometer, did not
change the affinity state of the receptor as determined by the
assay described by Quast et al. (1978b); (b) mixing of
membrane fragments in the absence of Eth with buffer or
ligands did not result in any detectable changes in light
scattering; and (c) in the presence of Eth mixing of receptor with buffer or mixing of receptor-toxin complex with chol-
ergic ligands did not show any significant change in Eth
fluorescence.
When Carb at concentrations less than 1 μM was mixed with
receptor originally in the low-affinity form (Weber et al.,
1975; Lee et al., 1977; Quast et al., 1978b), the fluorescence
increase of bound Eth followed a single slow exponential with
a half-time in the minute range (phase 1). At higher ligand
concentrations, a faster phase arose (phase 2). Figure 1 shows
a kinetic trace measured at a final Carb concentration of 3
μM where the amplitudes of both phases were about equal.
With increasing ligand concentrations, the amplitude δA2 [see
Figure 2 (O)] and the relaxation rate τ−1 \(_2\) [Figure 2 (Δ)] of
phase 2 increased. Correspondingly, phase 1 decreased in both
amplitude and relaxation rate [see Figure 2 (A)]. At Carb concentrations higher than 50 μM only the second phase
remained with its amplitude, δA2, rapidly reaching a constant
value. The observed rate, τ−1 \(_2\), also began to level off although
a plateau value was not yet reached at a Carb concentration of
1 mM. The total amplitude, δA, [Figure 2 (Ω)], reached a
plateau at Carb concentrations higher than 10 μM.
The kinetic pattern described above was found consistently
for Carb binding to the receptor with the exception that in
many membrane preparations a third (very fast) exponential
was observed at ligand concentrations higher than 500 μM
(Quast et al., 1978a). Storage of membrane fragments for
several days at 4 °C, even in the absence of calcium (see also
Grünhagen et al., 1976) generally resulted in faster kinetics
for phase 2. In addition, a considerable slow decrease in
fluorescence following the fast phase δA2 was often observed
at high Carb concentrations. Also, the saturation value of δA2
was decreased. Similar aging phenomena were observed when
a fresh receptor preparation was exposed to calcium and Eth
at room temperature for more than 1 h. Often these effects
made a quantitative evaluation of the slow kinetics at low
ligand concentrations difficult.

**Reaction Mechanism.** Assuming that the fluorescence
increase of Eth (a) directly reflects ligand-induced changes in
the receptor and (b) that it is due to a change in quantum
yield of bound Eth (see preceding paper), we found that the
observed kinetics are consistent with the following mechanism
where bound Eth is omitted.

\[
R + 2L \rightarrow RL \rightarrow L + RL_2
\]

\[
K_i = \frac{[L][R]}{[RL]}
\]

\[
K_i' = \frac{[L][RL]}{[L][RL]}
\]

First, the receptor \(R\) rapidly combines with ligand \(L\), forming
the two precomplexes \(RL\) and \(RL_2\). These precomplexes then
isomerize slowly to the final complexes \(C_1\) and \(C_2\) with
\(RL_2 \rightarrow C_2\) faster than \(RL \rightarrow C_1\). \(C_1\) does not bind a further ligand
molecule so that \(C_2\) is exclusively formed from \(RL_2\).

The increase in quantum yield comes from the isomerization steps
\(RL \rightarrow C_1\) and \(RL_2 \rightarrow C_2\) and is assumed to be the same for
both reactions (see below, eq 6).

The concentration dependence of the two kinetic phases
expected from mechanism 1 is calculated in the Appendix. It
can be rationalized as follows: at low ligand concentrations,
\(L/K_r \gg L^2/(K_2K_3)\), the law of mass action predicts that
essentially no \(RL_2\) will be formed. Consequently, only \(C_2\) is
formed, resulting in a single kinetic phase (slow phase or phase
1). At higher concentrations, both \(RL\) and \(RL_2\) are formed
in fast equilibrium to the following isomerization steps.
Since formation of \(C_2\) is faster than that of \(C_1\), the pathway
\(R \rightarrow RL \rightarrow RL_2 \rightarrow C_2\) is first followed. This leads to a
transient increase in concentration of \(C_2\) above its equilibrium
value \(C_2 = R^2/(K_1K_2K_3)\) (fast phase = phase 2). Then
the slower reaction \(RL \rightarrow C_1\) occurs until the equilibrium
distribution of \(C_1/C_2\) is reached (slow phase = phase 1). With
increasing ligand concentration, the law of mass action drives the equilibrium toward $C_2$, since $C_1/C_2 \approx (K_2K'/K_1')$ (see eq A3). Therefore, the slow kinetic phase will disappear at high ligand concentrations.

As shown in the Appendix (see eq A8), the ligand-induced increase in fluorescence $F(t)$ contains two exponentials with the relaxation rates

$$\tau^{-1} \approx k_{-2} + k_{-1}'$$

(see A6) for the fast phase and

$$\tau^{-1} \approx k_{-1}' + k_{-1}$$

(3)

for the slow phase (see A7). The fast relaxation rate, $\tau^{-1}$, describes formation of $C_1$. It is decoupled from the slow reaction $RL \rightarrow C_1$ and reaches a limiting value of $k_{-2} + k_{-1}'$ complete saturation of $RL$. The slow relaxation rate, $\tau^{-1}$, depends on the kinetic parameters of $C_1$ formation but also contains the equilibrium parameters for $C_2$ formation since the reaction $RL_2 \rightarrow C_2$ constitutes a fast equilibrium with $RL \rightarrow C$. The overshoot phenomenon, although correctly derived, has been neglected in our preliminary report on Carb kinetics (Quast et al., 1978a). This approximation led to a low value for the equilibrium constant for the additional ligand which is given by (8) (see eq A3).

The parameter derived from a fit of the data shown in Figure 2 are listed in the first row of Table I. The sequence in which they are given reflects the extent to which they were directly obtained from the data (see Appendix). Table I also contains the parameters obtained for the reaction of Carb with receptor originally in the high-affinity state (see, e.g., Weber et al. (1975) and Quast et al. (1978b)).

The degree of variability of the kinetic parameters for the different membrane preparations mentioned above is comparable to that observed in other kinetic studies (see, e.g., Grünhagen et al. (1977)).

Ethidium Dependence. Figure 3 shows the Eth dependence of the Carb kinetics with the membrane preparation used in Figure 2, at a final Carb concentration of 2 $\mu$M. The total amplitude, $\delta A_2$, decreased considerably at ethidium concentrations greater than 1 $\mu$M (Figure 3), whereas the ratio $\delta A_2/\delta A_1$ increased (O). The relaxation rates depended only

\[ \delta A_2 = Q[\bar{C}_1/(\tau k_{-2}) + \bar{C}_2/(\tau k_{-1})] \]
The symbols denote dependence as that seen in Figure 3 and thiocholine, are listed in Table 4 together with the theoretical fits according to mechanism to toxin binding kinetics (Table I) and vary over a broad concentration range, according to the ligand examined. The very fast exponential decrease and increase to choline by AcChE. It is therefore expected that the receptor finally relaxes to the state which would have been induced by initially mixing receptor with choline (equilibrium level). Indeed, the total fluorescence amplitude at equilibrium as a function of AcCh concentration is described by the law of mass action with an apparent $K_d$ of 15 μM (data not shown). This value is close to that of choline (see Table I).

On the fast time scale, the second (fast) phase leveled off in both amplitude and rate at higher ligand concentrations as observed with all agonists (see Figures 2 and 4). At AcCh $> 50$ μM, $\delta A_2 \approx 90\%$ of $\delta A_4$ and $\tau_{2}^{-1} \approx 4 s^{-1}$. Above 50 μM, a faster phase (phase 3, $\tau_{2}^{-1} \approx 17 s^{-1}$) appeared at the expense of the second phase so that $\delta A_{2} + \delta A_{4} = \text{constant} = 90\% \delta A_{4}$. kinetic parameters, however, did not vary strongly for the different ligands.

(1.3) Acetylcholine. The kinetics of acetylcholine differed from the basic agonist pattern on the slow time scale due to the action of AcChE (in our receptor preparation enzyme sites equaled about 1% of the concentration of toxin sites). Figure 5 shows kinetics traces of the Eth fluorescence increase obtained at different AcCh concentrations. On the fast time scale, there was, at lower concentrations, only the fast phase (phase 2, $\tau_{2}^{-1} \approx 1-3 s^{-1}$). At high AcCh concentrations (Figure 5, trace C), an additional very fast phase appeared (phase 3, $\tau_{2}^{-1} \approx 18 s^{-1}$). In the slow domain there was first an intermediate fluorescence decrease (phase 4, $t_{1/2}(4) \approx 5-10 s$), followed by an increase (phase 5, $t_{1/2}(5) \approx 10 s$) which then slowly relaxed to the equilibrium level (phase 6, $t_{1/2}(6) \approx 100 s$). This last phase was missing at high ligand concentrations (see trace C). This kinetic pattern was reproducibly found with AcCh although the concentration at which the very fast phase appeared depended on the preparation as did the amplitude ratio between the intermediate fluorescence decrease and increase ($\delta A_{2}/\delta A_{4}$).

The complex reaction shown in Figure 5 reflects the fact that the receptor reacts with AcCh which is also hydrolyzed to choline by AcChE. The connecting bars for each of the parameters denote the deviation from the mean value, determined from four individual experiments. The connecting curves have no theoretical significance.

weakly on Eth concentration. The Eth dependence of the kinetics was also measured at a final Carb concentration of 200 μM where only the fast phase remained (see Figure 2). The total amplitude $\delta A_{4} (=\delta A_{2})$ showed the same concentration dependence as that seen in Figure 3 and $\tau_{2}^{-1}$ was independent of Eth concentration.

(1.2) Deca, Nicotine, Choline, and Thiocholine. All other agonists examined followed the kinetic pattern found for Carb except AcCh, which will be discussed in the next section. As examples, the data for Deca and nicotine are shown in Figure 4 together with the theoretical fits according to mechanism 1. The fitting parameters, together with those for choline and thiocholine, are listed in Table I. The very fast exponential at ligand concentrations higher than 500 μM, which was occasionnally observed with Carb and acetylcholine (see below), was also seen for thiocholine but not for any other agonist. As in the case of Carb, the equilibrium constants for the first ligand, $K_{1}$/$K_{1}'$, were much lower than the effective equilibrium constants for the second ligand, $K_{2n}$. The values of $K_{1}$/$K_{1}'$ were, in general, in agreement with the inhibition constants $K_{i}$ for toxin binding kinetics (Table I) and vary over a broad concentration range, according to the ligand examined.

FIGURE 3: Ethidium dependence of the kinetic parameters. The final concentrations were [AcChR]₀ = 0.05 μM in α-BuTx sites, originally in the low-affinity form and [Eth]₀ = 2 μM. The measurements were performed at a constant (low) photomultiplier voltage so that the total fluorescence (obtained after mixing of receptor plus ethidium with ethidium in the absence of Carb) increased linearly with ethidium concentration. The total amplitude (○) is given in arbitrary units; δA₄ (O) is normalized with respect to δA₄ (percent of δA₄ at the indicated [Eth]₀); δA₄ = δA₄ - δA₄ is omitted. (A) and (△) denote $\tau_{2}^{-1}$ and $\tau_{2}^{-1}$, respectively. The error bars for each of the parameters denote the deviation from the mean value, determined from four individual experiments. The connecting curves have no theoretical significance.

FIGURE 4: Kinetics of interaction of Deca and nicotine with the AcChR. The final concentrations were [AcChR]₀ = 0.2 μM in α-BuTx sites, originally in the low-affinity form and [Eth]₀ = 0.2 μM. The symbols denote (○), δA₄ normalized to 100% at saturation; (O) δA₄ (in percent of δA₄ at saturation); (A) $\tau_{2}^{-1}$; and (△) $\tau_{2}^{-1}$. The fitted curves were calculated as indicated in Figure 2 with the parameters from Table I.

FIGURE 5: Kinetic traces after rapid mixing of AcCh with AcCh. Concentrations after mixing were [AcChR]₀ = 0.5 μM in α-BuTx sites, originally in the low-affinity form; [Eth]₀ = 1.5 μM; and [AcCh]₀ = 0.5 (A), 5 (B), and 380 μM (C). Note the dual time base for the fast and the slow portion of the traces. Trace A is twofold expanded in vertical scale as compared to traces B and C. All amplitudes are given as percent of δA₄, at saturation. Trace A ([AcCh]₀ = 0.5 μM): a fast increase in fluorescence (δA₄ = 35%, $\tau_{1}^{-1} = 1 s^{-1}$) is followed by a short lag phase (δA₄ = 0, $t_{1/2}(4) = 4 s$), a fluorescence increase ($δA₄ = 16\%$, $t_{1/2}(5) = 11 s$), and a final fluorescence decrease ($δA₄ = -30\%$, $t_{1/2}(6) = 100 s$). Completion of the reaction is not shown but equilibrium level is indicated. The total amplitude is the difference between initial and final level of fluorescence, $\delta A_{4} = \sum \delta A_{4}$ is 18% of δA₄ at saturation. Trace B (10 μM): δA₄ = 88%, $\tau_{1}^{-1} = 3 s^{-1}$; δA₄ = -30%, $t_{1/2}(4) = 10 s$; δA₄ = 24%, $t_{1/2}(5) = 15 s$; δA₄ = -27%, $t_{1/2}(6) = 100 s$; δA₄ = 55%. Trace C (380 μM): δA₄ = 33%, $\tau_{1}^{-1} = 3.5 s^{-1}$; δA₄ = 5%, $\tau_{1}^{-1} = 18 s^{-1}$; δA₄ = -42%, $t_{1/2}(4) = 8 s$; δA₄ = 53%, $t_{1/2}(5) = 40 s$; δA₄ = 0; δA₄ = 95%.
Table II: Kinetics of AcChR with Antagonists

| Ligand | $K_d$ (µM)$^b$ | $K_l$ (µM)$^b$ | $\delta A_{10}\%$|$^c$ | Kinetics | $\delta A_{1}$ ($\%$)$^d$ | $\tau_1^{1}$ (s$^{-1}$)$^d$ | $\tau_2^{1}$ (s$^{-1}$)$^d$ |
|--------|-------------|-------------|----------------|----------|-----------------|----------------|----------------|
| d-Tc   | 0.05        | 0.2 ± 0.02  | 76 ± 5         | biphasic | $\approx 20$ | 0.02-0.04       | $\approx 0.05$ |
| Hexa   | 25.0        | 120 ± 14    | 87 ± 5         | biphasic | $\approx 20$ | 0.020          | $\approx 0.55$ |
| DAP    | 1.90        | 0.7 ± 0.3   | 80 ± 10        | monophasic | 100          | 0.025          |                |
| Gallamine | 4.0        | 11 ± 1      | 44 ± 1         | biphasic | concn dependent | 0.01          | 0.04-0.02       |

$^a$ Derived from concentration dependence of $\delta A_4$ (see the legend of Figure 6).
$^b$ Derived from inhibition of toxin binding kinetics (see preceding paper).
$^c$ $\delta A_{10}$ is $\delta A_4$ at saturation (in percent amplitude for Deca; see Table I).
$^d$ $\delta A_{1}$ given in percent of $\delta A_4$ at saturation for the ligand considered; subscripts 1 and 2 denote the slow and fast phase, respectively.

At still higher ligand concentrations, $\tau_1^{1}$ started to decrease. The appearance of an additional fast phase at high ligand concentrations, also occasionally observed in the case of Carb (Quast et al., 1978a) and thiocholine (see above), will be dealt with in the Discussion.

The half-times of the slow phases (Figure 5, phases 4, 5, and 6) depended either weakly or not at all on AcCh concentration ($t_{1/2}(4) \approx 10$ s, $t_{1/2}(5) \approx 10-40$ s, and $t_{1/2}(6) \approx 100$ s). In order to try to interpret the slow phases, the kinetics at [AcChR]$_0$ = 10 µM were followed in the presence of increasing concentrations either of solubilized AcChE from *Electrophorus* or of the esterase inhibitors DFP and physostigmine or of a phospholipase fraction from *Bungarus caeruleus* (Bon & Changeux, 1975; Moody & Raftery, 1978). Total blockage of AcChE led to kinetics as observed with Carb: a fast increase in fluorescence (phase 2), followed by a small, much slower increase (phase 1). Incubation with lipase led, with increasing incubation time, to a threefold decrease in the overall amplitude and to the appearance of the very fast phase (phase 3) at the expense of phase 2 in the fast time domain. Incubation with solubilized AcChE from *Electrophorus* affected only the slow phases but left $\delta A_4$ unchanged. At high concentrations of esterase, hydrolysis of 10 µM AcCh was complete in less than 1 s and yet the basic pattern of the AcCh kinetics was essentially conserved. This shows that a short contact of AcCh with the receptor was sufficient to condition the receptor for several minutes so that it reacted with choline in slow cycles of fluorescence decrease and increase (phases 4, 5, and 6). However, prolonged contact with AcCh (achieved by inhibition of the AcChE) resulted in the kinetic behavior found with the other agonists (see above).

(2) Kinetics with Antagonists. The kinetics observed after mixing receptor–Eth complex with various antagonists were very different from those measured with agonists. In general, the antagonist kinetics were biphasic with a poor separation in time of the two relaxation rates ($\tau_1^{1} \approx 4 \tau_1^{1}$) (d-Tc; gallamine and Hexa; see Figure 6 and Table II). Only DAP gave monophasic kinetics. The total amplitude as a function of ligand concentration is well described by the law of mass action [see Figure 6 (●)]. In the cases of DAP and d-Tc, the total amplitude decreased at higher ligand concentrations due to displacement of the dye by the ligand (see preceding paper) especially in the case of DAP. This may lead to a considerable error in the calculated equilibrium constant, which was obtained from a weighted double-reciprocal plot of $\delta A^{1}$ vs. $L$–1, by taking only the values at low ligand concentration into account. The concentration dependence of the two kinetic phases was in general weak, and the kinetics were slow. No common mechanism has been found to describe the kinetics. A survey of the empirically derived parameters is given in Table II.

Discussion

The fluorescence increase observed after mixing of receptor–Eth complex with a cholinergic ligand specifically reflects the interaction of that ligand with the membrane-bound AcChR, as shown in the preceding paper. Firstly, the receptor–toxin complex which does not bind ligands specifically (Weber & Changeux, 1974; Quast et al., 1978b) does not show any increase in ethidium fluorescence after addition of ligand. Secondly, the total fluorescence increase, as a function of added ligand concentration, is described by the law of mass action, and from this the equilibrium constant for that ligand can be calculated. The specificity of the Eth response is further confirmed by the kinetics of the ligand-induced fluorescence increase. As shown in this report, all agonist-induced kinetics consist of (at least) two phases. At low concentrations a slow phase was observed, and this was replaced at increasing ligand concentration by a fast phase with a time constant shorter than a second. In contrast, antagonist-induced kinetics did not follow a common pattern. In general, they show only a weak concentration dependence and were, in the ligand concentration range from 100 to 1000 µM, about 2 orders of magnitude slower than the kinetics with agonists. Note that this "pharmacological specificity" of the Eth response was also reflected in the kinetics measured with the agonist Deca and the antagonist Hexa [compare Grünhagen et al. (1977)].

The dependence of the kinetic parameters for Carb on Eth concentration (see Figure 3) is weak except for the total amplitude, $\delta A_4$. To a large degree this effect might reflect the nonlinear dependence of the quantum yield for Eth bound to the AcChR–Carb complex as was observed for Eth bound to the AcChR alone [see Schimerlik et al. (1979a) (preceding paper), Figure 2D). On the other hand the decrease of $\delta A_4$ with increasing Eth concentration might also reflect a weak perturbation of the membrane-bound receptor by the dye. This effect seems indicated by the weak dependence on Eth concentration of the rate of transition of AcChR from low to high affinity for Carb [see Schimerlik et al. (1979a) (preceding paper), Figure 8] or of the relaxation rate of HTX interacting with the AcChR [see Schimerlik et al. (1979b)] (following...
observed fluorescence changes to ligand-induced conformational changes in the receptor, we had to find a reaction mechanism which would allow for dramatic changes in agonist kinetics near saturation of the total amplitude: the first (slow)

phase decreased in both amplitude and relaxation rate as a faster phase arose with increasing ligand concentration. Some of the mechanisms examined are listed in Table III. First, mechanisms considering only two states of the receptor (R and R') and a single ligand molecule binding (Table III, 1-3) can be rejected on the basis of the calculated concentration dependence of the slow corresponding amplitude and relaxation rate. The fast relaxation rates which would reflect the ligand binding steps are not listed in Table III because they increase linearly with ligand concentration. (None of the experimental rates showed this property.) Mechanism 3 in Table III (the sum of (1) and (2)) is the two state model of receptor often used to describe desensitization of the receptor in vivo (Katz & Thesleff, 1957; Rang & Ritter, 1970) and in vitro [see, e.g., Weiland et al. (1977)]. Considering three state mechanisms of the receptor, a simple possibility is the sequential mechanism 4 in Table III, in terms of which Griinhagen et al. (1977) discussed their kinetics of agonist binding in the presence of the fluorescent local anaesthetic quinacrine. This mechanism predicts that both relaxation rates increase hyperbolically with ligand concentration (see Table III) and is therefore in disagreement with our observations. For the same reason, mechanisms involving a nonproductive binding state N were excluded, e.g., mechanism 5 in Table III.

Turning to mechanisms involving two or more ligands binding to the receptor, we can first exclude the concerted transition model (MWC) since it predicts only a single slow relaxation time [for a review, see, e.g., Hammes & Wu (1974)]. Induced-fit models involving sequential binding of ligands allow for many permutations of ligand binding and isomerization steps [see, e.g., Loudon & Koshland (1972)]. The most simple example of these models, a linear sequence (Table III, 6), can be ruled out because of the concentration dependence of \( r_1^{-1} \). Note that high-affinity binding of the first ligand requires \( k'_r \gg k_r \), so that \( r_1^{-1} \) in (6) will ultimately increase with increasing \( L \), in contrast to the experimental observations. As a more complex example of induced-fit models, it was considered that the same final complex was formed via two different kinetic pathways, depending on the number of ligands bound in the precomplexes, RL and RL-2 (see 7). This mechanism which is a simplified version of the model proposed by Bulger et al. (1977) for the interaction of \([^{125}I]-\alpha\)BuTx with membrane-bound AChR from Electrophorus predicts relaxation times in qualitative agreement with ours (see Figures 2 and 4). It requires, however, that the midpoints of \( \delta A_2 \) and \( \tau_2^{-1} \) be close together and thus cannot be brought to a quantitative fit with the data. Eq 1 shows the only mechanism involving two ligands that we found compatible with the data.

It would have been desirable to obtain independent evidence for the low-affinity binding of a second ligand. However, binding studies with \(^{3}H\)Carb are not feasible since, at AChR concentrations high enough to detect the weaker dissociation constant (20–50 \( \mu M \)), specific binding of the ligand is obscured by a large nonspecific binding component.

**Number of Ligands Bound.** There is substantial evidence that the number of high-affinity ligand sites equals half the number of toxin sites in both membrane-bound [Raftery et al., 1975; Quast et al., 1978a; Schimerlik et al., 1979a (preceding communication)] and solubilized receptor [Moody et al., 1973; Maelicke et al., 1977]. The number of toxin sites in the functional unit of the AChR, is, however, still uncertain.

There is increasing evidence that the receptor occurs predominantly as a dimer in membrane preparations [Suarez-Isla & Hucho, 1977; Chang & Bock, 1977; Hamilton et
Table III: Kinetics of Mechanisms Considered for AcChR-Agonist Interaction

<table>
<thead>
<tr>
<th>Mechanism</th>
<th>Conditions</th>
<th>(Slow) Kinetic Relaxation</th>
<th>Equilibrium Amplitude $\delta A_k$</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) $R + L \xrightarrow{k_1} RL \xrightarrow{k_2} R'L$</td>
<td>$z = R'L$</td>
<td>$\tau_1^{-1} = k_{-1} + k_1$; $\delta A_s = QR_0 \frac{L}{L + K_k K_{K_r}}$</td>
<td>$\delta A_1 = \delta A_s$</td>
</tr>
<tr>
<td>(2) $R \xrightarrow{k_3} R'$</td>
<td>$z = R' + R'L; K_{K_r} = \frac{R}{R'}$</td>
<td>$\tau_1^{-1} = k_{-1} + k_1$; $\delta A_s = QR_0 \frac{L}{L + K_k K_{K_r}}$</td>
<td>$\delta A_1 = \delta A_s$</td>
</tr>
<tr>
<td>(3) $L + R \xrightarrow{k_4} RL$</td>
<td>$z = R' + R'L; K_{K_r} = \frac{R}{R'}$</td>
<td>$\tau_1^{-1} = k_{-1} + k_1 + k_{1/2} L/K_k$; $\delta A_s = QR_0 \frac{L}{L + K_k K_{K_r}}$</td>
<td>$\delta A_1 = \delta A_s$</td>
</tr>
<tr>
<td>(4) $R + L \xrightarrow{k_5} RL \xrightarrow{k_6} R'L$</td>
<td>$z = Q, R'L + Q, R''L$ (step 1 faster than 2')</td>
<td>$\tau_1^{-1} = k_{-1} + k_1 + k_{1/2} L/(K_k K_{K_r})$; $\tau_2^{-1} = k_{-1} + k_1 + k_{1/2} L/(L + K_{K_r})$</td>
<td>$\delta A_4 = Q, R_0 \frac{L}{L + K_k (K_{K_r})}$</td>
</tr>
<tr>
<td>(5) $N \xrightarrow{k_7} R + L$</td>
<td>$z = N + R'L$ (step 1 rate limiting)</td>
<td>$\tau_1^{-1} = k_{-1} + k_1 + k_{1/2} L/(L + K_{K_r})$</td>
<td>$\delta A_4 = Q, R_0 \frac{L}{L + K_k (K_{K_r})}$</td>
</tr>
<tr>
<td>(6) $R \xrightarrow{k_8} RL \xrightarrow{k_9} R'L_2 \xrightarrow{k_{10}} R''L_2$</td>
<td>$z = R'L + R'L_2 + R''L_2$ (step 2' faster than 1')</td>
<td>$\tau_1^{-1} = k_{-1} + k_1 + k_{1/2} L/(L + K_k K_{K_r})$</td>
<td>$\delta A_4 = Q, R_0 \frac{L}{L + K_k (K_{K_r}) + L^2/(K_k K_{K_r})}$</td>
</tr>
<tr>
<td>$R + L \xrightarrow{k_{11}} RL \xrightarrow{k_2} R'L_2 \xrightarrow{k_{12}} R''L_2$</td>
<td>$z = R'L + R'L_2 + R''L_2 + C_2$ (step 2' faster than 1')</td>
<td>$\tau_1^{-1} = k_{-1} + k_1 + k_{1/2} L/(L + K_k)$</td>
<td>$\delta A_4 = Q, R_0 \frac{L}{L + K_k (K_{K_r}) + L^2/(K_k K_{K_r})}$</td>
</tr>
<tr>
<td>$R + L \xrightarrow{k_{13}} RL \xrightarrow{k_2} R'L_2 \xrightarrow{k_{14}} R''L_2$</td>
<td>$z = R'L + R'L_2 + C_2$</td>
<td>$\tau_1^{-1} = k_{-1} + k_1 + k_{1/2} L/(L + K_k)$</td>
<td>$\delta A_4 = Q, R_0 \frac{L}{L + K_k (K_{K_r}) + L^2/(K_k K_{K_r})}$</td>
</tr>
</tbody>
</table>

\(a\) Ligand L is buffered \((R \gg R_0)\); binding steps are considered fast as compared to the following isomerization reactions, and the corresponding relaxation rates are omitted. All isomerization rate constants are denoted with a prime. The isomerization equilibrium constants are defined as \(K'_k = k_{-1}/k_2\) and are assumed to be much smaller than unity \((K'_k << 1)\). \(z\) denotes the observed quantity; \(Q_l\) denotes different quantum yields. Kinetic phases are numbered starting with the slowest so that \(\tau_1^{-1} \ll \tau_2^{-1}\). The bars denote equilibrium concentrations. The conditions for evaluation of the different mechanisms were chosen such that the predictions corresponded as closely as possible to the observed data.
al., 1977; Witzemann & Raftery, 1978a). To date it is not known which form, monomeric (two toxin sites) or dimeric (four toxin sites), is the basic functional form in the membrane or at the synapse, and therefore we have discussed our model in terms of the simplest receptor unit, i.e., the monomeric form which binds 2 mol of \(\alpha\)-BuTx/mol of AcChR. This choice has the advantage of mathematical simplicity and the smaller number of fitting parameters required. The mechanism we propose for the monomeric receptor form (i.e., eq 1) cannot, however, accommodate the very fast phase (phase 3), observed occasionally at very high concentrations of the strongly binding ligands Carb, AcCh, and tiocoline. These results are fit instead by an expansion of eq 1 to the dimeric form of the receptor (see below). Phase 3 was not found with all membrane preparations and occurred more often with increasing age of the preparation. For these reasons we have not been able to collect sufficient reliable data for this phase. There is, however, substantial evidence that, at very high ligand concentrations, binding of a further ligand occurs and that, with an increasing shift of the membranes toward the high-affinity state, the equilibrium constant for the additional ligand decreases to experimentally attainable concentrations in the millimolar range. Formulation of a mechanism analogous to eq 1 for the dimeric receptor form includes binding of four ligands, two of them with high affinity. Binding of these two ligands leads first to the two precomplexes RL and RL; their respective isomerizations to C1 and C2 are reflected in the slow phase (phase 1). Formation of the triligand complex C3 from RL1 then would be responsible for the fast phase (phase 2) and, the step RL4 \(\rightarrow\) C4 would be responsible for the very fast phase (phase 4). A short mathematical analysis of this mechanism, considering only the first three ligands, has recently been published (Quast et al., 1978a). Since the four-ligand mechanism causes only an additional displacement of a complex of lower ligation \((C_1)\) to \(C_4\) on top of the earlier displacements, the mathematical treatment is placed in the Appendix.

**Interpretation of Data.** It has already been noted that the equilibrium constants for ligands obtained from the total amplitude are in good agreement with the value obtained by other methods, i.e., the inhibition of the rate of \([^{125}\text{I}]-\alpha\)-BuTx binding (see Tables I and II) and ultracentrifugation experiments with \([^1\text{H}]\)Carb (see Table I). The overall reaction amplitude, \(\delta A\), reflects essentially binding of the high-affinity ligand(s) (see text, eq 5), and the value of \(K_i/K_{i'}\) correlates well with the inhibition constant \(K_i\) found from the inhibition of the rate of \(\alpha\)-BuTx binding to the AcChR. The four- to fivefold tighter binding of \([^1\text{H}]\)Carb in the presence of 4 mM Ca\(^{2+}\) obtained in ultracentrifugation studies (Table I, column 12) was also reflected in a change in \(K_i/K_{i'}\) of two- to fourfold upon Ca\(^{2+}\) addition (Table I, column 2). This is in agreement with earlier work of Cohen et al. (1974) who found that Ca\(^{2+}\) increases the affinity of \([^1\text{H}]\)AcCh and Carb for AcChR by about twofold. The values of \(\delta A\), at saturation (see Tables I and II) were different when different ligands were tested on the same membrane preparation. Although the differences in \(\delta A\) were small for some ligands, they were statistically significant. These differences might be (partly) due to direct interactions between membrane-bound ligand and dye molecules; however, they may indicate either that the same final receptor conformation is induced by individual ligands to differing degrees [ligands as allosteric effectors; see, e.g., Janin (1973)] or that each ligand induces a unique conformation. Although the differing kinetics observed for agonists and antagonists favor the latter interpretation, more detailed spectroscopic studies are needed to decide between these possibilities.

Equation 1 with \(C_1\) and \(C_2\) both denoting the open channel form has recently been proposed as the mechanism describing channel opening at the frog neuromuscular junction (Stevens, 1975) and personal communication. The thermodynamic parameters are, however, very different (especially \(K_{i'} > 1\)), and the time scale is in the millisecond range (Stevens, personal communication). Despite these differences the complete formal analogy is intriguing.

In low-affinity membrane preparations, the isomerization from low to high affinity for Carb, measured by an increase in the inhibition of the rate of \([^{125}\text{I}]-\alpha\)-BuTx binding with incubation time, was observed in the presence of Eth [see Schimerlik et al. (1979a) (preceding paper)]. However, this did not seem to be reflected in the change in Eth fluorescence after mixing with Carb since the fast phase (phase 2) was about 10 times too fast and the slow phase had the wrong concentration dependence. A similar phenomenon has been observed by Briley & Changeux (1978) in a partially reconstituted membrane system where the slow agonist-induced affinity change was observed by the inhibition of \(\alpha\)-toxin kinetics while only fast quinacrine responses were observed. A correlation between the slow ligand-induced change in affinity and the conformational changes reflected by Eth fluorescence is rather difficult since similar kinetics were found with Eth, regardless of whether the AcChR was "initially" in the low- or high-affinity form (see Table I). Since the assay for the affinity form of the AcChR (Quast et al., 1978b) is slow, it was impossible to decide whether receptor characterized as initially in the high affinity form was actually in that state prior to addition of ligand or underwent the isomerization to the high-affinity form at a much faster rate \((t_{1/2} < 5\text{ s})\) than receptor in the low-affinity form.

In a recent publication, Grünhagen et al. (1977) have analyzed the kinetics of agonist binding to receptor in the presence of the fluorescent local anaesthetic quinacrine. They observed a fast fluorescence increase in the millisecond time range, followed by a decrease in the minute range. Fitting the kinetics to mechanism 4 in Table III, they correlated the fast phase with channel activation and the slow phase with desensitization (see also Grünhagen & Changeux, 1976). This interpretation is, however, in question for the following reasons.

(a) Electrophysiological experiments with various systems, using different methods, indicate that in vivo two agonist molecules are needed to open the ion channel, e.g., in *Electrophorus* electroplaque (Sheridan & Lester, 1977) or the frog neuromuscular junction [see, e.g., Dionne & Stevens (1975) and Adams (1975, 1977)]. In general, one would therefore expect a more complex ligand dependence of the rate constant corresponding to channel opening than that found by Grünhagen et al. (1977) (see also \(\gamma^2\) in mechanism 4 of Table III).

(b) Quinacrine at concentrations around 10 \(\mu\text{M}\) acts as a noncompetitive inhibitor of the Carb-induced steady-state depolarization of the *Electrophorus* electroplaque (Grünhagen & Changeux, 1976). Therefore, one would expect a strong quinacrine dependence of the observed kinetics in vitro in the concentration range explored by Grünhagen et al. (1976, 1977), but none was found. Voltage-jump relaxation experiments on the frog end plate (Adams & Feltz, 1977) suggest that the fast phase of the kinetics in vitro (Grünhagen et al., 1977) might reflect channel blocking by the local anaesthetic quinacrine rather than activation of the channel. (c) No correlation was made between the observed kinetics and the original affinity state of the receptor for Carb. Despite these
differences concerning the interpretation of the kinetics with quinacrine, the specificity of the quinacrine response is established beyond doubt (Gründhagen & Changeux, 1976) as it is in the case of ethidium. The very fact, however, that two specific but indirect probes of receptor mechanism show such different kinetics upon addition of agonist suggests that correlation of these kinetics with electrophysiological results has to remain on a tentative basis.

Nevertheless, the kinetic studies presented here have provided useful information on the interactions of cholinergic ligands with membrane-bound AcChR. Firstly, the overall fluorescence amplitude of the reaction, $\delta A$, was an accurate measurement of the saturation of the receptor sites that bind ligands with high affinity. Secondly, the pharmacological specificity of agonists and antagonists was reflected in their different kinetic mechanisms. Finally and most important, the kinetic results require that more than a single ligand binds to the AcChR in vitro, in agreement with the stoichiometry deduced from electrophysiological studies in vivo.

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Appendix

(1) Two-Ligand Binding Mechanism (eq 1 in text).

Equilibrium. By use of eq 6 in the text, $Q_1 = Q_2 = Q$. the total amplitude $\delta A_1$ of the ETh fluorescence increase after addition of agonist is equal to

$$\delta A_1 = Q(C_1 + C_2) \quad (A1)$$

where the bar denotes concentrations at equilibrium. Since $K_1'$ and $K_2'$ $\ll 1$ (see Table I), $C_1$ and $C_2$ can be approximated by

$$C_1 \approx R_0 L/(K_1 K_1') \quad C_2 \approx R_0 L^2/(K_1 K_2 K_2') \quad (A2)$$

Here, $R_0$ is the total concentration of receptor units comprised of two toxins sites and $L$ is the free ligand concentration. The ratio of $C_1$ to $C_2$ can be written as

$$C_1/C_2 = (K_2 K'_2)/(K_1 L) = K_{eff}/L \quad (A3)$$

defining an effective equilibrium constant $K_{eff}$ for the second ligand binding.

(1.2) Kinetics. The fluorescence signal $F$ is proportional to $C_1 + C_2$ formed at time $t$

$$F = Q(C_1 + C_2). \quad (A4)$$

From mechanism 1 (see text) one obtains the rate equations

$$C_1' = k_1 RL - k_{1'} C_1 \quad C_2' = k_2 RL_2 - k_2' C_2 \quad (A5)$$

Using the mass balance $R_0 = R + RL + RL_2 + C_1 + C_2$ and assuming that the binding steps in eq 1 of the text are fast ($RL = R L/K_1; RL_2 = R L^2/(K_1 K_2)$), the differential equation can be rewritten, using matrix notation, as

$$\begin{pmatrix} C_1 \\ C_2 \end{pmatrix}' = A \begin{pmatrix} C_1 \\ C_2 \end{pmatrix} + \frac{q_1}{q_2} R_0 \quad (A5)$$

where $q_1 = k_1 \phi L/K_1$, $q_2 = k_2 \phi L^2/(K_1 K_2)$, $\phi = [1 + L/K_1 + L^2/(K_1 K_2)]^{-1}$, and the elements of the reaction matrix A are given by $a_{11} = q_1 + k_{1'}$, $a_{12} = q_1$, $a_{21} = q_2$, and $a_{22} = q_2 + k_{2'}$. The calculation of the relaxation rates is simplified by the fact that formation of $C_2$ is fast compared to formation of $C_1$. The fast relaxation rate, $\tau_2^{-1}$,

$$\tau_2^{-1} \approx k_{2'} + k_{2''} L^2/(K_1 K_2) \quad (A6)$$

is then obtained from the trace of A, neglecting the terms containing $k_1'$ and $k_1''$. The slow relaxation rate, $\tau_1^{-1}$, is calculated from $\tau_1^{-1} = \text{det} A/\tau_2^{-1}$ to equal

$$\tau_1^{-1} \approx k_{1'} + k_{1''} L^2/(K_1 K_2) \quad (A7)$$

The fluorescence signal $F$ is obtained by linear superposition of the solutions for $C_1$ and $C_2$

$$F = Q(C_1 + C_2) = \delta A_1 - \delta A_1 e^{-\tau_1/\tau_2} - \delta A_2 e^{-\tau_1/\tau_2} \quad (A8)$$

with $\delta A_1$, $\tau_1^{-1}$, and $\tau_2^{-1}$ from eq A1, A2, A6, and A7. The amplitudes $\delta A_1$ and $\delta A_2$ are calculated from the initial conditions

$$F(0) = 0 = \delta A_1 - \delta A_1 - \delta A_2 \quad (A9)$$

Neglecting small terms of the order of $\tau_1^{-1}/\tau_2^{-1}$, one obtains

$$\delta A_1 = +Q[C_1[1 - (\tau_1 k_{1''})^{-1}] + C_2[1 - (\tau_1 k_{1''})^{-1}]] \quad (A9)$$

$$\delta A_2 = +Q[C_1/(\tau_1 k_{1'}) + C_2/(\tau_1 k_{1'})] \quad (A10)$$

(1.3) Fitting Procedure. The total amplitude, $\delta A_1$, was calculated according to eq A1 and A2 for a given concentration of the free ligand, $L$. The total ligand concentration, $L_0$, was then calculated from $L_0 = L + C_1 + 2C_2$ and a plot of $\delta A_1$ vs. $L_0$ was constructed. For the start of the fitting procedure, $K_1 K_2'$ was taken from the midpoint of the plot $\delta A_1$ vs. $L (L_{1/2} \approx K_1 K_2' + R_0/2)$ and the value of $K_1 K_2 K_2'$ from the concentration where $\tau_1^{-1}$ reaches its maximum value or starts to decline (see text, eq 4 and 5). In general, only small variations of the starting guesses were necessary to obtain a satisfactory fit for $\delta A_1$ vs. $L_0$. We calculated a second observable $\delta A_2$ according to eq A12 with the values of $K_1 K_2'$ and $K_1 K_2 K_2'$ determined from the fit for $\delta A_1$ and using the experimental values of $\tau_1^{-1}$, $k_{1'}$ and $k_{1''}$. The calculation of $\delta A_2$ was very sensitive to small variations of $\tau_1^{-1}$ and $k_{1'}$ and these values were (slightly) varied until a good fit was achieved. $\delta A_1$ was calculated from $\delta A_1 = \delta A_1 - \delta A_2$.

Next, $\tau_2^{-1}$ was fitted by using the experimental values of $k_{1'}$ and $k_{1''}$. [If $k_{1''}$ could not be obtained directly from the experimental data (see, e.g., Figure 2), it was varied with the constraint $k_{1''} > 10 k_{1'}$.] $K_1 K_2$ was calculated from $K_1 K_2 K_2'$ (see $\delta A_2$) and $K_1 K_2'$. $K_1$ was varied until a good fit was obtained. In the last step $\tau_1^{-1}$ was calculated with $k_{1'}$ as the only adjustable parameter with the constraint $k_{1'} \geq 10 k_{1'}$. In general, the five observed quantities $\delta A_1$, $\delta A_2$, $\tau_1^{-1}$, and $\tau_2^{-1}$ were well fit using a single consistent set of parameters (see Table I).

(2) Four-Ligand Binding Mechanism. In formal analogy to eq 1 (text) this mechanism is written

$$R + 4L \quad \rightarrow \quad R L + 3L \quad \rightarrow \quad R L_2 + 2L \quad \rightarrow \quad R L_2 + L + \quad \rightarrow \quad R L_4$$

where it is again assumed that (a) all isomerization steps $RL_d \quad \rightarrow \quad C_1$ are accompanied by the same change in quantum yield,
(b) all binding steps are fast, and (c) $K_i = k_i' / k_i'' \ll 1$. At equilibrium we have

$$
C_i = RL_i / K_i' \quad \text{with} \quad RL_i = RL / \prod_{j=1}^{m} K_j \quad i = 1, ..., 4
$$

(A12)

where all $K_j$ are phenomenological (macroscopic) equilibrium constants, containing statistical weight factors. As discussed in a previous paper (Quast et al., 1978a), the parameters must be adjusted so that (a) a Scatchard plot regarding the two high-affinity sites ($C_1$ and $C_2$) is linear and (b) formation of $C_1 + C_3$ is reflected in one single kinetic phase. The condition for linearity of the Scatchard plot is that the concentration of ligand bound to the two high-affinity sites, $L_0 = C_1 + 2C_2$, reduces to a rectangular hyperbola as a function of $L$. Using eq A12 one obtains the equation

$$
(2K_1 K_2')^2 = K_1 K_2 K_3' K_4'
$$

(A13)

which differs from the corresponding equation given earlier (Quast et al., 1978a) by a statistical factor of 2, due to differing definitions of $K_j$.

The rate equations for the final complexes are given by

$$
C_i = k_i' RL_i - k_i'' C_i \quad i = 1, ..., 4
$$

(A14)

For the sum $X = C_1 + C_2$ one obtains

$$
X = [k_i' L_i / K_i + k_i'' L_i / (K_i K_j)] R - k_i'' C_i - k_i' C_2
$$

(A15)

The concentration of free receptor sites can be expressed as

$$
R = (R_0 - X - C_1 - C_2) \phi \quad \text{with} \quad \phi = [1 + \sum_{i=1}^{m} L_i / \prod_{j=1}^{m} K_j]^{-1}
$$

(A16)

After insertion of eq A16 into eq A15 it becomes apparent that formation of $X$ will be described by a single kinetic phase only if one assumes

$$
k_i'' = k_i' = k_j'' = k_j'
$$

(A17)

The rate equations for $X, C_1$, and $C_4$ can now be calculated in a straightforward manner. In the matrix form, they read

$$
\begin{pmatrix}
\dot{X} \\
\dot{C}_3 \\
\dot{C}_4
\end{pmatrix}
+ \begin{pmatrix}
\gamma \phi + k_i' & \delta \phi & \gamma \phi + k_i'' \\
\gamma \phi & \delta & \gamma \phi + k_i''
\end{pmatrix}
\begin{pmatrix}
X \\
C_3 \\
C_4
\end{pmatrix}
= \begin{pmatrix}
\beta \\
\gamma \\
\delta
\end{pmatrix} R_0
$$

(A18)

where

$$
\beta = k_i' L / K + k_i'' L^2 / (K_i K_j)
$$

$$
\gamma = k_i' L^i / \prod_{j=1}^{m} K_j
$$

$$
\delta = k_i'' L^i / \prod_{j=1}^{m} K_j
$$

(A19)

The relaxation rates, $\tau_i$, of the reaction matrix are calculated by successive approximations, with $\tau_1 \ll \tau_2 \ll \tau_3$. They are

$$
\tau_1 \approx \delta \phi + k_i'' \quad \tau_2 \approx \gamma (\phi + \delta / k_i'')^{-1} + k_i'' 
$$

$$
\tau_3 \approx \delta (\phi + \delta / k_i'')^{-1} + \gamma / k_i'' + k_i''
$$

(A20)

and have the concentration dependence expected from mechanism A11 (see Discussion).

Calculation of the amplitudes pertaining to eq A18 from the initial conditions is tedious and leads to a complex mathematical expression. Since, however, at no concentration do more than two phases coexist, it is sufficient to consider eq A18 in the two concentration ranges where either (a) binding of the fourth ligand does not yet occur so that the fluorescence signal is given by $F_3(t) = Q(C_1 + C_2)$ or (b) the fourth ligand needs to be considered but $X$ can be neglected so that $F_4(t) = Q(C_3 + C_4)$. Then the formalism developed in eq A8–A10 holds, and one can write by direct analogy

$$
F_3(t) = Q(X + C_3) = \delta A_1 - \delta A_4 e^{-t/\tau_1} - \delta A_5 e^{-t/\tau_2}
$$

(A21)

with $\delta A_1 = X + C_3, \delta A_2 = Q(X/(\gamma_k' k'' + \gamma C_3/(\gamma_k' k'')), \text{and} \delta A_4 = \delta A_5 - \delta A_3$ (see eq A12, A20). Correspondingly in the high concentration range

$$
F_3(t) = Q(C_3 + C_4) = \delta R_0 - \delta A_4 e^{-t/\tau_1} - \delta A_5 e^{-t/\tau_2}
$$

(A22)

with $\delta A_4 \approx QC_1/(\gamma_k' k'' + \gamma C_3/(\gamma_k' k''), \text{and} \delta A_5 = QR_0 - \delta A_3$.

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