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Modulation of Acetylcholinesterase Activity by Peripheral Site Ligands

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SUMMARY

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The steady-state hydrolysis of acetylthiocholine by soluble acetylcholinesterase from *Electrophorus electricus* (EC 3.1.1.7) under conditions of low ionic strength is inhibited by "peripheral" ligands such as gallamine, propidium, and *d*-tubocurarine. Curvature in the Lineweaver–Burk plots obtained when either gallamine or propidium is introduced to the enzyme at the same time as substrate is eliminated by 10-min preincubation of the enzyme with ligand and the resulting (inhibited) rates exhibit a moderate enhancement. No preincubation effects are detectable with *d*-tubocurarine; however the uncompetitive component of inhibition is more pronounced than that observed with gallamine or propidium. Metal ions such as Ca^{2+} and Mg^{2+} are activators at high substrate concentrations; in contrast, Zn^{2+} is a powerful inhibitor. The pre-steady-state carbamoylation of acetylcholinesterase by 7-(dimethylcarbamoyloxy)-*N*-methylquinolinium iodide (M7C) is subject to substrate activation at M7C concentrations above 5 μM. Gallamine and propidium inhibit this reaction at lower substrate concentrations but have little influence at higher substrate concentrations. The decarbamoylation of the dimethylcarbamoyl enzyme is accelerated by both ligands, as well as by Ca^{2+}, Mg^{2+}, and M7C itself. *d*-Tubocurarine similarly has little effect upon the carbamoylation *rate* at high M7C concentrations; instead the *amplitude* of the reaction is decreased. This effect approaches a maximum at around 4 mM *d*-tubocurarine and corresponds to approximately 50% of the original amplitude. Zn^{2+} also decreases the carbamoylation amplitude; in this case the ligand is capable of abolishing *all* of the carbamoylation amplitude and at an order of magnitude lower concentration than is required for maximal *d*-tubocurarine effects. The effect of Zn^{2+} is reversed by addition of EDTA. These results are discussed in terms of peripheral ligand-induced conformational changes in acetylcholinesterase.

INTRODUCTION

The ability of AChE[1] to undergo ligand-induced conformational changes was first suggested by Changeux (1) who in 1966 observed that gallamine inhibition of *Torpedo marmorata* AChE was not strictly competitive in nature. The finding that gallamine could relieve inhibition by decamethonium and other bisquaternary inhibitors led to the proposal that AChE possesses "peripheral" anionic sites, distinct from the catalytic site, whereby binding of cationic ligands could influence the catalytic properties of the enzyme. Subsequent studies, e.g., by Kitz *et al.* (2), Roufogalis and Quist (3), Rosenberry and Bernhard (4), Mooser and Sigman (5), and Taylor and Lappi (6), have lent additional support to this hypothesis, which can now be considered to be well established although the functional significance, if any, is not well defined.

The recent availability of site-specific fluorescent probes such as *N*-methylacridinium (7) and propidium (3,8-diamino-5,3′-diethylmethylamino-*n*-propyl-6-phenylphenanthridinium) (6, 8) as well as the fluorogenic pseudosubstrate 7-(dimethylcarbamoyloxy)-*N*-methylquinolinium (9) has greatly aided in the delineation of peripheral effects, not only under equilibrium conditions but also during catalysis. We have recently pointed out some differences between AChE from *Electrophorus electricus* and that from *Torpedo californica*, with respect to binding of propidium (10). Preliminary kinetic studies also reported at this time suggested that peripheral ligands such as gallamine and propidium might stabilize a form of the enzyme having enhanced catalytic ability.

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[1] Abbreviations used: AChE, acetylcholinesterase; ASCh, acetylthiocholine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); M7C, 7-(dimethylcarbamoyloxy)-*N*-methylquinolinium; M7H, 7-hydroxy-*N*-methylquinolinium.

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In the present study we examine in more detail the effects of peripheral ligands on catalysis by AChE from *E. electricus*. Our results further indicate that there are differences between the eel and torpedo enzymes in their responses to peripheral ligands, and provide additional evidence that AChE is "activated" by certain of these ligands (Ca^{2+}, Mg^{2+}, propidium, and gallamine) but "inactivated" by others (Zn^{2+}, *d*-tubocurarine). In general, our results support the conclusions drawn from recent studies with *T. californica* enzyme by Pattison and Bernhard (11), although the two-state model proposed therein may require extension in order to fully explain our results.

METHODS

Materials. ASCh and DTNB were purchased from Sigma, M7C from Eastman, propidium iodide and *d*-tubocurarine chloride from Calbiochem, and gallamine triethiodide from ICN. Metal salts were of analytical grade and purchased from Fisher Scientific. AChE was purchased from Sigma (type VI-S) and was further purified by affinity chromatography as described previously (10, 12). The low-ionic-strength buffer used in all of the experiments was 1.0 mM Tris, pH 8.0.

Kinetic studies. The reaction of AChE with both ASCh and M7C may be described by the scheme

$$E + RCOX \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E.RCOX \xrightarrow{k_2} E-COR \xrightarrow{k_3} E + P_2 \qquad [1]$$

$$(K_S) \qquad + \; P_1$$

where K_s is the dissociation constant for the enzyme–substrate complex (k_{-1}/k_1), k_2 is the acetylation (carbamoylation) rate constant, and k_3 is the deacetylation (decarbamoylation) rate constant. Routine assays of enzyme activity were carried out by the method of Ellman *et al.* (13), using ASCh as substrate. The production of the yellow thionitrobenzoate anion, generated by the reaction of DTNB with the hydrolysis product thiocholine, was monitored at 412 nm on a Beckman Model 25 spectrophotometer. Detailed studies of the steady-state kinetics of this reaction at low ionic strength were carried out in a modified Aminco–Bowman stopped-flow apparatus. The signal derived from the photomultiplier was collected on a Biomation 805 waveform recorder interfaced with a Heathkit H-11 computer and simultaneously displayed on an oscilloscope. The sensitivity of this instrument permits the use of ASCh concentrations as low as 1.0 μM. The apparatus was operated under the control of a Basic program developed in our laboratory. Up to 2048 data points from each kinetic run are transferred from the waveform recorder to the computer, where they are converted to absorbance values. Initial rates are determined by linear least-squares regression on points visually selected from the oscilloscope trace. All reactions were carried out in 1.0 mM Tris (pH 8.0) containing 31.4 mM DTNB and ASCh concentrations of 1.0 to 100.0 μM. Use of the stopped-flow apparatus enabled study of the rate of ASCh hydrolysis for periods of several milliseconds to several seconds after mixing of the reactants. In no case was the total change in ASCh concentration during the reaction greater than 7.5 μM, which was determined in separate experiments to produce no detectable change in the pH of the reaction mixture. In the absence of added ligands, data were analyzed according to the Michaelis–Menten equation,

$$v = k_{cat}[E_0][S] \;\; (K_m + [S]), \qquad [2]$$

where $k_{cat} = k_2k_3/(k_2 + k_3)$ and $K_m = k_3(k_{-1} + k_2)/k_1 \cdot (k_2 + k_3)$ in terms of Eq. [1].

The effect of varying concentrations of gallamine, propidium, *d*-tubocurarine, and metal salts (specified below) upon the rate of ASCh hydrolysis was also studied in this manner.

The reaction of AChE with the carbamoylating agent M7C is sufficiently slow and the two rate constants k_2 and k_3 are sufficiently well separated that both the transient phase and the steady-state portion of the reaction can be easily examined using conventional mixing techniques (7, 9). The production of the fluorescent product M7H with time t may be described by

$$[P_1] = At + B(1 - e^{-bt}). \qquad [3]$$

In this case, where $k_2 \gg k_3$ and $[S_0] \gg K_m$, A may be taken to be the steady-state velocity, which in turn is given by

$$A = k_3[E_0]. \qquad [4]$$

Under the same conditions, B is the amplitude of the transient phase and approximates very closely the enzyme normality, $[E_0]$. The constant b is the observed pseudo-first-order rate constant of carbamoylation (k_{obs}) and its dependence upon substrate concentration is given by the reciprocal relationship

$$1/k_{obs} = 1/k_2 + K_s/k_2 \cdot 1/[S_0]. \qquad [5]$$

The rate of M7H production in the absence and presence of the effectors described above was measured on a Perkin–Elmer MPF-44 spectrophotofluorometer operated in the energy mode, using excitation and emission wavelengths of 405 and 505 nm, respectively. M7C concentrations were in the range 0.5–40 μM and the enzyme normality was typically 1–4 × 10^{-8} N. All kinetic measurements were carried out at 25°C.

RESULTS

Steady-State Kinetics of Acetylthiocholine Hydrolysis

In the absence of added inhibitors, AChE exhibits Michaelis–Menten behavior as judged from the linearity of Lineweaver–Burk plots of initial rate data obtained from measurements of acetylthiocholine hydrolysis at low ionic strength. The steady-state parameters of the Michaelis–Menten equation (Eq. [2]) are k_{cat} = 3.5(±0.5) × 10^5 min^{-1} and K_m = 4.3(±0.2) × 10^{-6} M (10).

In the presence of gallamine or propidium, the plots are curved when inhibitor is introduced to the enzyme at the same time as the substrate. If the enzyme is preincubated for 10 min with the inhibitor prior to the introduction of substrate, the curvature is less pronounced and a moderate rate enhancement (relative to the nonpreincubated case) is observed over most of the substrate concentration range (Fig. 1).

The activation process may be directly observed in a stopped-flow spectrophotometer provided the enzyme

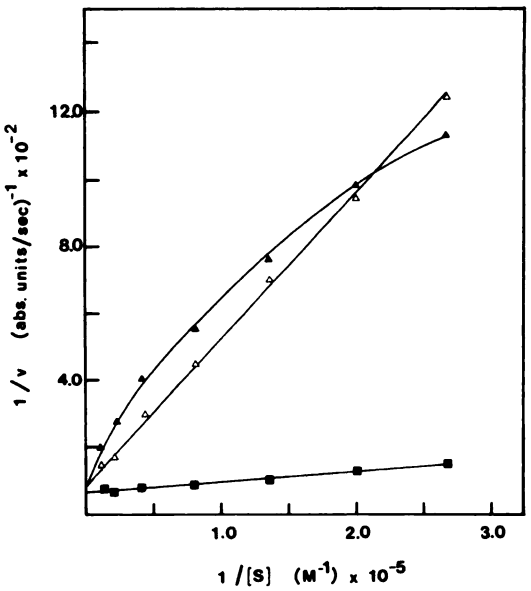

FIG. 1. *Effect of preincubation of AChE with 5 × 10^{-7} M propidium on the rate of acetylthiocholine hydrolysis (1.0 × 10^{-10} N AChE)*
(■) No propidium; (▲) + propidium, no preincubation; (△) + propidium, 10-min preincubation.

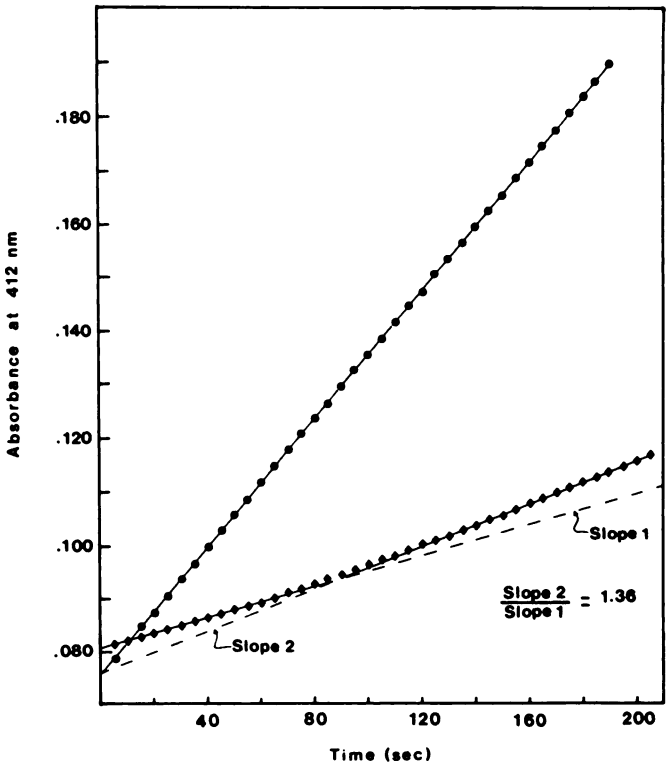

FIG. 2. *Activation of AChE by 5 × 10^{-7} M propidium*
Enzyme solution was mixed in the stopped-flow apparatus with an equal volume of a solution containing either substrate + DTNB or substrate + DTNB + propidium. The increase in absorbance of 412 nm was monitored immediately thereafter (final concentration: 2.5 × 10^{-5} M ASCh; 3.14 × 10^{-2} M DTNB; 9.3 × 10^{-12} N AChE). (●) No propidium; (◆) + propidium.

concentration is sufficiently low that only a small fraction (<20%) of the substrate is hydrolyzed during the time course of the activation (Fig. 2). Under these conditions there is a gradual increase in the rate of thionitrobenzoate production which reaches a constant value of about 1.36 times the rate observed in the first few seconds following mixing of all of the reactants.

Preincubation with either propidium or gallamine produces inhibition patterns which are of a predominantly competitive nature. No detectable preincubation effects are obtained with *d*-tubocurarine over the inhibitor concentration range studied. However, the uncompetitive component of inhibition appears to be more pronounced than that observed in the presence of propidium or gallamine (Fig. 3).

Alkaline earth cations such as Ca^{2+} and Mg^{2+} enhance

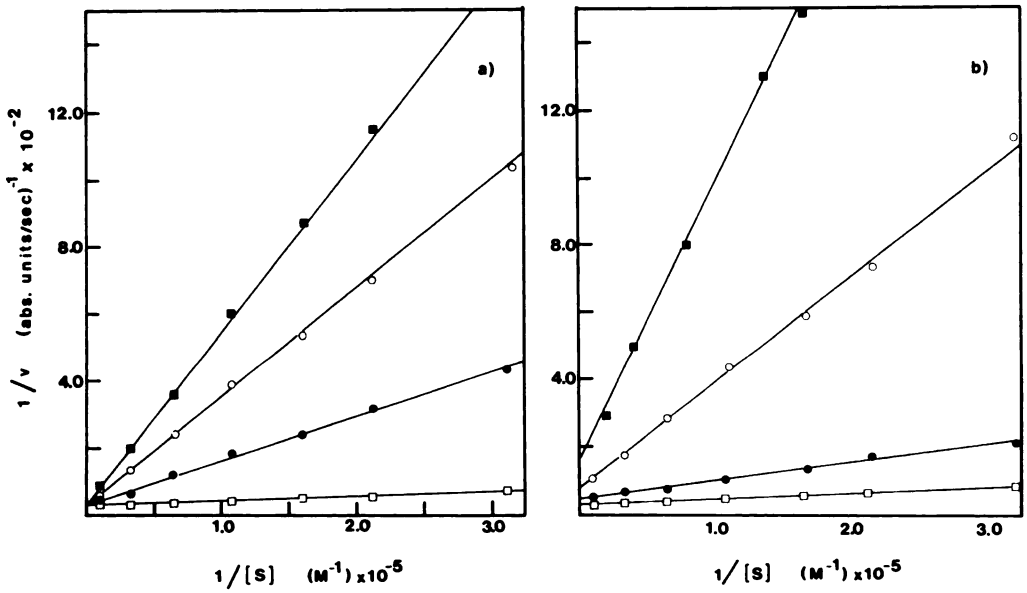

FIG. 3. *Inhibition of acetylthiocholine hydrolysis by gallamine and d-tubocurarine following 10-min preincubation with effector*
(a) Gallamine: (□) none; (●) 3.9 × 10^{-7} M; (○) 9.7 × 10^{-7} M; (■) 1.9 × 10^{-8} M. (b) *d*-Tubocurarine: (□) none; (●) 2.0 × 10^{-6} M; (○) 8.0 × 10^{-6} M; (■) 2.0 × 10^{-5} M.

the rate of ASCh hydrolysis at high substrate concentration (Fig. 4a). In contrast, other divalent metal ions such as Zn^{2+} and Cu^{2+} are inhibitors of the reaction (Fig. 4b). In both cases, the observed phenomenon appears to reflect hyperbolic binding of the particular metal ion.

Kinetics of Carbamoylation and Decarbamoylation

(a) In the absence of effectors. The carbamoylation of AChE by 7-(dimethylcarbamoyloxy)-*N*-methyl quinolinium iodide (M7C) obeys first-order kinetics over all of the observable reaction and over a wide range of substrate concentration (0.5–40 μM). The pseudo-first-order rate constant of carbamoylation appears to increase in a hyperbolic manner with M7C concentration up to about 1.0×10^{-5} M, which permits estimates of the enzyme–substrate dissociation constant ($K_s = 1.3 \times 10^{-6}$ M) and the carbamoylation rate constant ($k_2 = 0.08\ s^{-1}$) to be made.

Above 10 μM M7C, however, substrate activation is observed and pseudo-first-order rate constants as high as $0.23\ s^{-1}$ are obtained (Fig. 5a).

The increased rate is not accompanied by any change in the *amplitude* of the carbamoylation reaction. The rate of decarbamoylation of the dimethylcarbamoyl enzyme is also substrate concentration dependent, with k_3 increasing from $3.3 \times 10^{-4}\ s^{-1}$ at 1.0 μM M7C to $6.5 \times 10^{-4}\ s^{-1}$ at 40.0 μM M7C (Fig. 5b).

(b) In the presence of effectors. Gallamine, propidium, and *d*-tubocurarine each inhibit the carbamoylation of the enzyme when the M7C concentration is subsaturating (i.e., about 5×10^{-6} M and lower). At high M7C concentrations, these ligands have little influence on the carbamoylation rates; carbamoylation rate constants at least as high and in some cases higher than those obtained in the absence of added ligands are found, despite the wide range of inhibitor concentrations used (Fig. 6). Again, no change in the carbamoylation amplitude is observed at all substrate and effector concentrations except for relatively high concentrations of *d*-tubocurarine (see below).

Ca^{2+} and Mg^{2+} likewise have little effect on either the carbamoylation rate or amplitude. The effects of the above ligands upon the decarbamoylation reaction are summarized in Table 1. *d*-Tubocurarine is not included owing to its more complex behavior. All of the ligands increase the rate of decarbamoylation in the presence of low concentrations of M7C. At high M7C concentration, gallamine and propidium do not enhance the decarbamoylation rate beyond that observed with M7C alone, whereas Ca^{2+} and Mg^{2+} are capable of accelerating de-

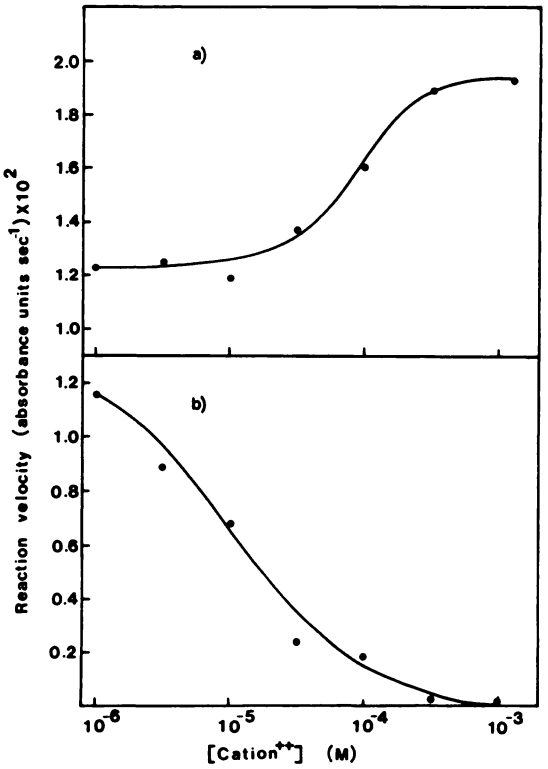

FIG. 4. *Effect of divalent metal ions on the rate of acetylthiocholine hydrolysis ([ASCh] = 50.0 μM)*

(a) Ca^{2+} (as $CaCl_2$); $MgSO_4$ shows similar behavior. (b) Zn^{2+} (as $ZnSO_4$); $CuSO_4$ shows similar behavior.

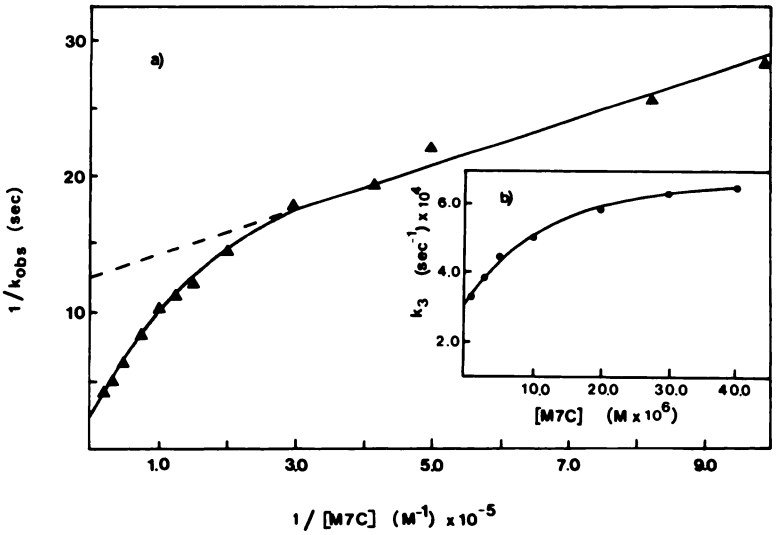

FIG. 5. *Kinetics of carbamoylation of AChE by M7C and subsequent decarbamoylation of the dimethylcarbamoyl enzyme*

(a) Double-reciprocal plot of the pseudo-first-order rate constant of carbamoylation as a function of M7C concentration. (b) Variation in k_3 with M7C concentration.

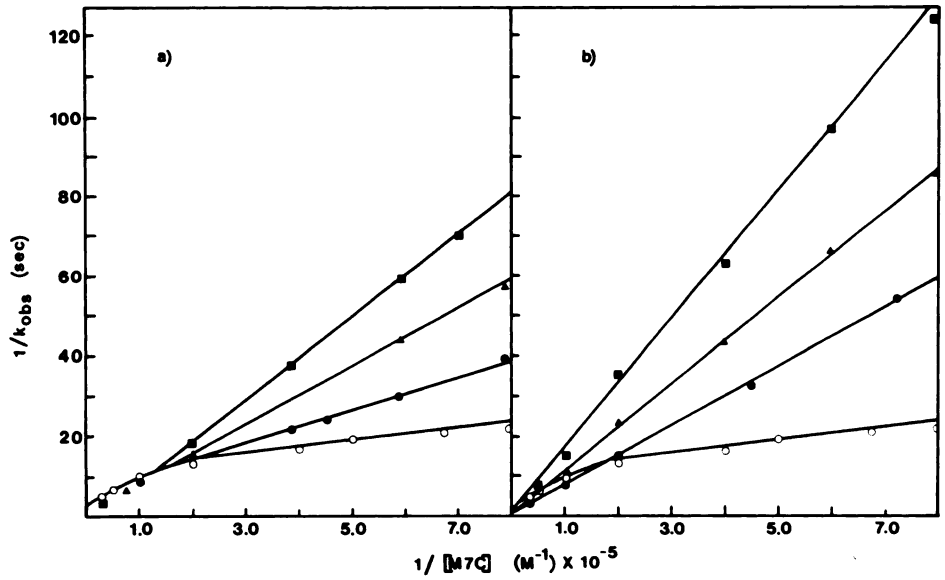

FIG. 6. *Inhibition of the carbamoylation of AChE with M7C by propidium and d-tubocurarine*
(a) Propidium: (○) none; (●) 1.3×10^{-8} M; (▲) 1.3×10^{-7} M; (■) 1.3×10^{-6} M. (b) *d*-Tubocurarine; (○) none; (●) 4.0×10^{-5} M; (▲) 2.0×10^{-4} M; (■) 4.0×10^{-4} M.

carbamoylation at all M7C concentrations studied. Thus, while the organic cations can influence both phases of the reaction, the effect of the two metal ions is confined to the decarbamoylation step.

(c) Amplitude effects. *d*-Tubocurarine has an additional effect so far not observed with gallamine or propidium. The *amplitude* of the carbamoylation reaction as well as the rate is progressively decreased by increasing the *d*-tubocurarine concentration. This phenomenon is most readily observed if the enzyme is preincubated with the effector for about 10 min, and is apparent even at high M7C concentrations, where *d*-tubocurarine as high as 1 mM has little effect on the *rate* of carbamoylation. It thus appears that the tubocurarine-induced amplitude decrease is a "peripheral" effect of the ligand. The amplitude decreases in an approximately hyperbolic fashion as the *d*-tubocurarine concentration is increased, and approaches a final value which corresponds to about 50% of the initial amplitude in the absence of *d*-tubocurarine).

The decrease in the carbamoylation amplitude can also be brought about by incubation of the enzyme with Zn^{2+} ions. In contrast to *d*-tubocurarine, Zn^{2+} is capable of abolishing *all* of the carbamoylation amplitude and is effective at a considerably lower concentration (Fig. 7). Cu^{2+} behaves similarly and the effects of this group of metal ions are antagonized by addition of EDTA (Table 2).

TABLE 1
Influence of effectors on the decarbamoylation rate constant, k_3, following reaction of AChE with M7C

Effector	[M7C] (μM)	k_3 ($s^{-1} \times 10^4$)
None	1.2	3.3
None	24.0	6.2
None	38.3	6.5
$CaCl_2$ (1 mM)	1.2	5.8
$CaCl_2$ (1 mM)	24.0	9.9
$MgSO_4$ (1 mM)	1.2	5.3
$MgSO_4$ (1 mM)	24.0	10.5
Gallamine (5 μM)	1.2	6.6
Gallamine (5 μM)	24.0	6.8
Propidium (5 μM)	1.2	4.4
Propidium (5 μM)	38.3	5.9

DISCUSSION

The results reported in this paper support the idea of peripheral ligand-mediated effects upon the catalytic properties of AChE. In the case of organic ligands such as gallamine and propidium, these effects are generally superimposed upon those due to competitive interactions with the catalytic site, owing to the cationic and hydrophobic nature of these compounds. Nevertheless, "peripheral" effects can be identified in a number of ways. Activation of the enzyme is observed during the steady-state hydrolysis of ASCh, in the pre-steady-state carbamoylation of the enzyme by M7C, and during the decarbamoylation of the dimethylcarbamoyl enzyme. The activation observed at high M7C concentrations in the absence of added ligands may also be a peripheral effect of M7C itself, especially since gallamine and propidium have little effect on the carbamoylation rates at high M7C concentrations. This result would be expected if gallamine, propidium, and M7C belong to a class of peripheral site activators which bind to the same or overlapping sites on the enzyme molecule. Substrate activation is not observed during the steady-state hydrolysis of acetylthiocholine; in fact at high ASCh concentrations substrate inhibition becomes apparent. While this may be a peripheral effect, an alternative explanation involving binding of ASCh to the acetyl enzyme is also plausible (14).

Activation by a group of metal ions exemplified by Ca^{2+} and Mg^{2+} may well be a better model for the kinetic demonstration of activation through interactions with peripheral sites. The work of Roufogalis and Wickson (15, 16), who demonstrated that the sensitivity of erythrocyte AChE toward Ca^{2+} activation is eliminated with-

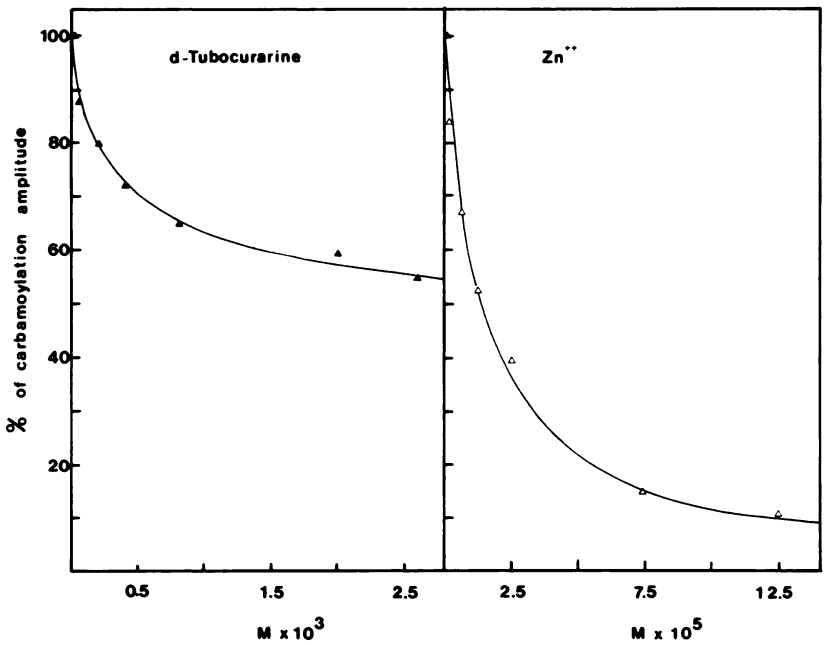

FIG. 7. *Effect of d-tubocurarine and Zn^{2+} on the amplitude of the carbamoylation reaction of AChE with M7C ([M7C] = 25.0 μM)*

out impairment of catalytic function following treatment with a water-soluble carbodiimide, strongly suggests that activation by this cation is indeed a "peripheral" effect. The available data thus support the proposal that AChE may exist in at least two catalytic states (1–3, 17). This suggestion in no way rules out additional explanations for such phenomena as noncompetitive inhibition arising out of ligand interactions with both the free enzyme and the acyl enzyme intermediate (14, 18) or for the acceleration of the rates of reaction of AChE with certain carbamates and methanesulfonates as a result of a conformational change induced by the binding of cations to the anionic subsite of the catalytic site (19). All of these effects may be operative to a greater or lesser extent, depending on the particular combination of enzyme, substrate, and effector. We suggest, however, that the ability of the enzyme to exist in at least two distinct catalytic states dependent on the occupancy of peripheral sites is a fundamental property of the enzyme itself. On the basis of a lack of antagonism between Ca^{2+} or simple monoquaternary ions such as tetraethylammonium, and the tridentate ligand gallamine, Roufogalis and Quist (3) proposed a multiplicity of peripheral binding sites (designated P_1 through P_4 in the terminology of Rosenberry (20)). Our observations that the principal effects of Ca^{2+} and Mg^{2+} are on the decarbamoylation reaction (acceleration), whereas gallamine and propidium increase the rates of both carbamoylation and decarbamoylation are consistent with this model. Evidently ligand binding to the various peripheral sites can induce different effects on the microscopic conformation of the catalytic sites.

A third state is required in order to explain the additional effects of *d*-tubocurarine and the group of divalent metal ions of which Zn^{2+} is representative. This class of effectors appears to cause a slow conversion of the enzyme to an "unreactive" form as judged by the ligand-dependent decrease in the amplitude of the "burst" or pre-steady-state phase of the reaction of AChE with M7C. The inability of *d*-tubocurarine to lower the amplitude below approximately 50% of the uninhibited amplitude may be related to the inability of *Electrophorus* AchE to bind more than two "bulky" peripheral ligands simultaneously. We have previously shown that this tetrameric enzyme binds more than two molecules of propidium only with great difficulty (10). In contrast, the enzyme from *Torpedo californica* binds four molecules of propidium per tetramer, with equal affinity (6). That the two enzymes are functionally very similar, however, is shown by the total abolition of the carbamoylation amplitude by Zn^{2+} with both the eel enzyme (this study) and the torpedo enzyme (11). The nature of the Zn^{2+} binding site(s) and the relationship between Zn^{2+}- and *d*-tubocurarine-induced effects on the enzyme remain to be elucidated.

It has been customary to draw analogies between AchE and the acetylcholine receptor, principally on the basis of certain parallelisms in the interactions of cholinergic agonists and antagonists with each of these proteins. While it is now generally recognized that the two proteins are indeed separate entities, the demonstration of an "unreactive" form of AchE is nonetheless reminiscent of the "desensitized" state of the receptor which may be induced by prolonged exposure to many drugs (21).

TABLE 2

Effect of peripheral ligands on the amplitude of the pre-steady-state carbamoylation of AChE by M7C ([M7C] = 25.0 μM)

Effector	Percentage of uninhibited amplitude
None	100
d-Tubocurarine (1.5 mM)	55
Zn^{2+} (73 μM)	15
Zn^{2+} (73 μM) + EDTA (122 μM)[a]	72
Cu^{2+} (48.4 μM) + EDTA (242 μM)[a]	70

[a] EDTA added after 10-min preincubation with metal ions.

In summary, we propose that AChE may exist in two catalytically active states; the more active state is stabilized by peripheral ligands such as gallamine and propidium, as well as by alkaline early cations such as Ca^{2+} and Mg^{2+}. A second, slow transition converts the enzyme to a third "unreactive" form which is stabilized by *d*-tubocurarine and transition metal ions such as Zn^{2+}.

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