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On the Question: Is Acetylcholinesterase an Allosteric Protein?

RICHARD J. KITZ,[1] LEON M. BRASWELL,[1] AND SARA GINSBURG

Departments of Anesthesiology and Neurology, College of Physicians and Surgeons, Columbia University, New York, New York 10032 and Anesthesia Laboratories, Massachusetts General Hospital, Boston, Massachusetts 02114

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SUMMARY

Classically, neuromuscular blocking agents are considered to function as competitive inhibitors of acetylcholinesterase, i.e., to interfere with acetylcholine hydrolysis by binding to the active surface of the enzyme. This and other evidence has led to the suggestion that acetylcholinesterase may also function as the "cholinergic receptor substance."

We have studied the influence of some neuromuscular blocking agents on the rate of inhibition of acetylcholinesterase by carbamate compounds (physostigmine, carbachol, neostigmine) and recovery of enzyme activity. It was found that these large, bulky drugs accelerate rather than inhibit carbamylation and decarbamylation of the active site. Under conditions in which there is no evidence of inhibition of substrate hydrolysis (active site binding), curare-like compounds accelerate the recovery of catalytic activity of neostigmine-inhibited acetylcholinesterase. The values of binding constants were measured, and the effects of varying concentrations of $MgCl_2$ and acetylcholine, different temperatures and pH, and other agents are reported.

The data are interpreted as evidence for an allosteric site mechanism capable of modulating activity at the catalytic surface, presumably through conformational changes in the enzyme.

INTRODUCTION

"Acid-transferring inhibition" is a term conceived by Wilson and applied to the mechanism of inhibition of acetylcholinesterase (EC 3.1.1.7) by certain organophosphate (tetraethyl pyrophosphate), carbamate (physostigmine), and methanesulfonate (methanesulfonyl fluoride) compounds (1, 2). These agents react with the enzyme in a manner analogous to acetylcholine hydrolysis, in which an intermediate derivative, acetylacetylcholinesterase, is formed and hydrolyzes in less than 0.1 msec (3). In the reaction with acid-transferring anticholinesterase agents, the acid moiety of the inhibitor is presumably transferred to the esteratic subsite of the active surface, forming diethylphosphorylacetylcholinesterase, monomethylcarbamylacetylcholinesterase, and methanesulfonylacetylcholinesterase, respectively, from tetraethyl pyrophosphate, physostigmine, and methanesulfonyl fluoride. Unlike acetylacetylcholinesterase, these intermediate enzyme compounds derived from the acid-transferring inhibitors are relatively stable and react with water only slowly (minutes, hours) or not at measurable rates. Thus the enzyme in the

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[1] Present address, Departments of Anesthesia, Harvard Medical School and Massachusetts General Hospital, Boston, Massachusetts 02114.

form of a stable covalent derivative is catalytically unreactive.

Most simple quaternary ammonium compounds (tetraethylammonium ion) at appropriate concentrations are inhibitors of acetylcholinesterase (4). The mechanism is distinct from acid-transferring inhibition and involves the formation of a readily dissociable complex with a negatively charged group on the enzyme, presumably the anionic subsite of the active surface. Covalent enzyme derivatives are not formed with these agents.

Classically, neuromuscular blocking agents interfere with cholinergic transmission by interfering with the action of acetylcholine on a postulated receptor substance (5). Two general mechanisms have been proposed to account for the action of these drugs. Curare and gallamine (Flaxedil) block depolarization of the membrane by acetylcholine and interfere with the access of acetylcholine to the receptor. Decamethonium and succinylcholine mimic the action of acetylcholine by producing a prolonged depolarization because they are not hydrolyzed by acetylcholinesterase, and produce a state refractory to further action by acetylcholine.

Most potent, clinically useful neuromuscular blocking agents are quaternary ammonium compounds. The mechanism of interaction of these drugs with acetylcholinesterase has traditionally been considered one of competitive inhibition, presumably by forming a complex with the anionic subsite of the enzyme. Because of these and other reactions with neuromuscular blocking drugs, it has been suggested that acetylcholinesterase may function as the "cholinergic receptor substance" (6, 7).

Changeux has recently shown (6) that certain neuromuscular blocking agents in low salt media are not strictly competitive inhibitors of acetylcholine-catalyzed hydrolysis, but produce only partial inhibition even at high concentrations. Gallamine and curare have also been shown to decrease the affinity of several reversible anionic site inhibitors for the enzyme but to enhance the binding of another. These data have been interpreted in terms of conformational changes of the enzyme. Kitz and Kremzner have shown (8) that highly purified acetylcholinesterase from *Electrophorus electricus* does undergo an alteration in conformation in response to the binding of substrates and inhibitors.

In this paper we report the effects of neuromuscular blocking drugs and other pharmacological agents on the reaction of acetylcholinesterase with some acid-transferring inhibitors. It was found that certain neuromuscular blocking compounds accelerate the rates of acid-transferring inhibition and spontaneous recovery of enzyme activity whereas others retard the rates. The results are interpreted as possible evidence for an allosteric site mechanism and for the occurrence of conformational changes of the enzyme.

MATERIALS AND METHODS

Enzyme. Acetylcholinesterase (EC 3.1.1.7) was prepared from the electric organ of *E. electricus* (9). At 25°, pH 7, and in a high salt medium consisting of 0.3 M $MgCl_2$, 0.005% gelatin, and 10^{-5} M EDTA, the enzyme had a specific activity of 50 mmoles of acetylcholine hydrolyzed per hour per milligram of protein, measured by automatic titration with a pH-stat. The protein concentration was 0.4 mg/ml, and the K_m value for acetylcholine was 9.1×10^{-5} M. Under the same conditions, but in a low salt medium of 0.003 M $MgCl_2$, 0.005% gelatin, and 10^{-5} M EDTA, the K_m value was 2.1×10^{-5} M. Several studies were also performed with one enzyme preparation having a specific activity of 8 mmoles/hr/mg and another of 500 mmoles/hr/mg.

Assay method. Most measurements were made with a pH-stat titrator modified for use with the dual-syringe technique. The instrument is designed to advance automatically the plungers of a matched pair of 0.5-ml syringes. One syringe contained standardized 0.01 N NaOH, and the other, 0.01 M acetylcholine adjusted to pH 7. The rate of addition of base to neutralize the acetate produced during the hydrolytic reaction is automatically recorded and is equal to the rate of substrate hydrolysis. The simultaneous addition of similar amounts of acetylcholine maintains the concentration of substrate. A pH-stat modified in this manner

allows activity measurements to be made at low but constant substrate concentrations.

In a typical inhibition experiment, sufficient enzyme was added to the reaction medium, containing 0.003 M $MgCl_2$ with 0.005% gelatin, 10^{-5} M EDTA, and 5×10^{-4} M acetylcholine, to obtain a satisfactory constant rate of substrate hydrolysis as measured with the pH-stat titrator. At zero time an appropriate concentration of the inhibitor, dimethylcarbamyl fluoride, was added to the reaction medium. The rate of inhibition was measured as a progressive loss of enzyme activity by automatically recording as a function of time the decreasing amount of base required to maintain the pH constant. The experiment was then repeated in the presence of an appropriate amount of the neuromuscular blocking drug or other agent in addition to the acid-transferring compound, and the progressive decrease in enzyme activity was again measured. The data were plotted semilogarithmically as a function of time and in accordance with the equations developed below (see Fig. 1).

In the usual recovery (of activity) experiments, a stock enzyme solution was incubated in 0.2 M NaCl at 25°, buffered with 0.001 M potassium phosphate, pH 7, with a concentration of neostigmine (usually 10^{-6} M) sufficient to produce 100% enzyme inhibition. An aliquot of the inhibited enzyme was diluted (250–500-fold) in the $MgCl_2$ reaction medium described above. The inhibitor was thus reduced below an effective concentration, and the rate at which the inhibited enzyme spontaneously recovered activity was followed by automatic titration. The experiment was repeated in the reaction medium containing an appropriate concentration of the myoneural blocking drugs or other agents to be tested. The data were plotted semilogarithmically as a function of time, and the half-time or rate constant values were measured.

Additional experiments were performed in which the $MgCl_2$ and substrate concentrations, pH, and temperature were varied to measure their influence on the phenomenon.

Compounds. All compounds are commercially available except for carbamyl fluoride, which was prepared according to the description of Schrader (10). Gallamine triethiodide (Flaxedil) was a gift of the Lederle Corporation, Pearl River, N. Y., and Pancuronium (2β, 16β-dipiperidino-5α-androstane-3α, 17β-diol diacetate dimethobromide) was generously supplied by N. V. Organon, Oss, The Netherlands.

RESULTS

When data for the reaction of acetylcholinesterase with dimethylcarbamyl fluoride are plotted semilogarithmically as a function of time, a state is reached in which the rate of inhibition is equal to the rate of spontaneous reactivation (Fig. 1). A simple steady-rate relationship is indicated (11).

$$E + I \xrightarrow{k_3} E' + P_1 \quad \text{and} \quad \frac{k_3 I}{k'_4} = \left(\frac{E'}{E}\right)_{ss} \tag{1}$$

$$E' + H_2O \xrightarrow{k'_4} E + P_2 \tag{2}$$

The symbols may be defined as follows: E = enzyme; I = inhibitior (dimethylcarbamyl fluoride); E' = dimethylcarbamyl-acetylcholinesterase (inhibited enzyme); P_1 = first product of the reaction, HF; P_2 = second product of the reaction, dimethylcarbamic acid; k_3 = second-order rate constant for the inhibition reaction; k'_4 = second-order rate constant for the recovery-of-activity reaction; and the subscript "ss" refers to the steady state. E'/E can be evaluated from the steady-state slope. k'_4 is measured separately (recovery experiments) by extensively diluting the inhibited enzyme (thus reducing the concentration of inhibitor) and following the rate of spontaneous recovery (Fig. 2). k_3 can be calculated from the known values of I, k'_4, and E'/E.

Metzger and Wilson have shown that tetraethylammonium ion accelerates the inhibition of acetylcholinesterase by dimethylcarbamyl fluoride (12). That observation is confirmed in this study (Fig. 1). In this case the semilogarithmic plot of fractional activity with respect to time is linear, indicating that spontaneous recovery of activity is not accelerated and may be impeded. For an accelerated reaction the following relationships have been described (12).

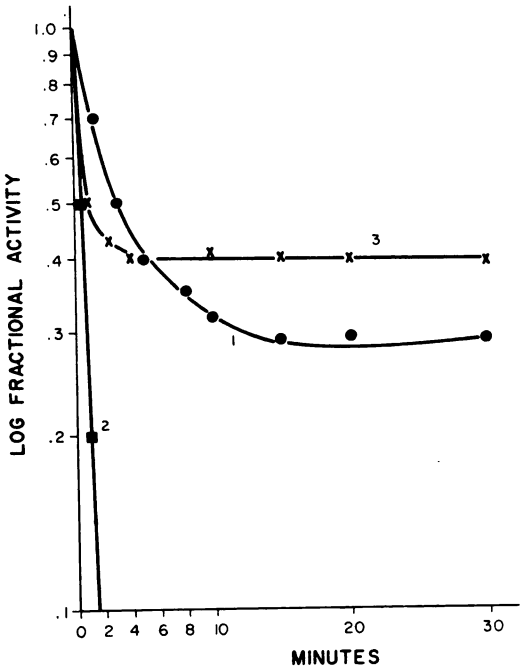


FIG. 1. *Effect of tetraethylammonium ion and gallamine on inhibition of acetylcholinesterase by dimethylcarbamyl fluoride*

Measurements were made in a pH-stat at pH 7 and 25° in a medium of 3×10^{-3} M $MgCl_2$, 10^{-5} M EDTA, and 5×10^{-4} M acetylcholine. Curve *1*, 1×10^{-4} M dimethylcarbamyl fluoride; *2*, 1×10^{-4} M dimethylcarbamyl fluoride and 2×10^{-3} M tetraethylammonium ion; *3*, 1×10^{-4} M dimethylcarbamyl fluoride and 5×10^{-5} M gallamine.

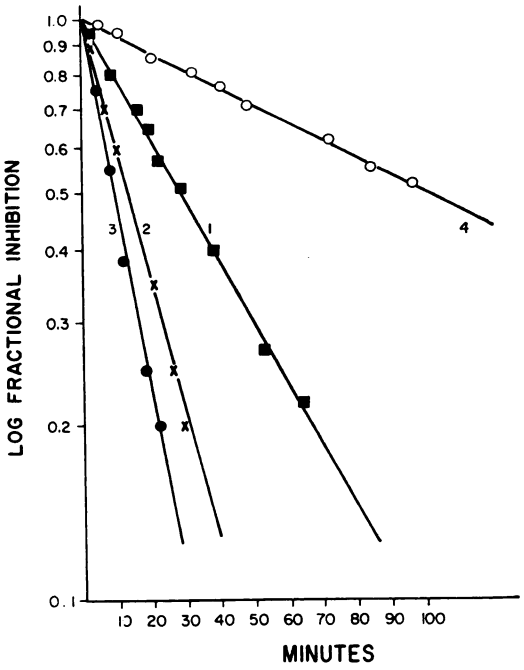


FIG. 2. *Recovery of activity of dimethylcarbamyl fluoride-inhibited acetylcholinesterase plotted as a function of time*

The assay technique of Hestrin was used (see the text for details). Curve *1*, normal recovery; *2*, recovery in the presence of 5×10^{-5} M gallamine; *3*, recovery in 5×10^{-4} M choline; *4*, 1×10^{-5} M succinyldicholine.

$$E + A \underset{}{\overset{K'_A}{\rightleftharpoons}} E \cdot A \qquad (3)$$

$$E \cdot A + I \xrightarrow{k'_3} E' + P_1 + A \qquad (4)$$

where A is the accelerator and k'_3 the rate constant for the accelerated reaction.

The data plotted in Fig. 1 indicate that the nondepolarizing neuromuscular blocking agent gallamine accelerates the rate of carbamylation of acetylcholinesterase. The slope, however, forms a plateau, indicating a steady-state relationship in which the rate of recovery must also be accelerated. This is different from the reaction in the presence of tetraethylammonium ion, in which only the rate of inhibition was accelerated; if the rate of hydrolysis of dimethylcarbamylacetylcholinesterase had also accelerated, the slope

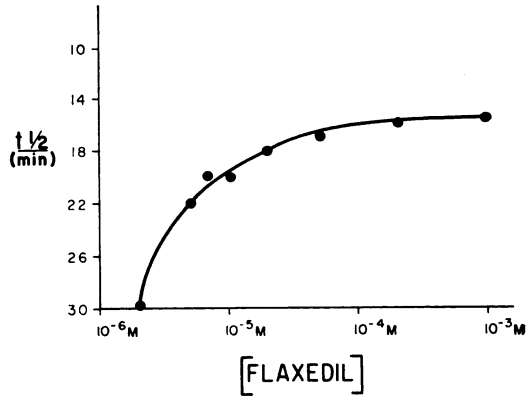


FIG. 3. *Half-time values for gallamine-accelerated recovery of activity of dimethylcarbamylacetylcholinesterase as a function of effector concentration*

The reactions were measured in a pH-stat at pH 7 and 25° in a medium of 3×10^{-3} M $MgCl_2$, 10^{-5} M EDTA, and 5×10^{-4} M acetylcholine.

would have formed a plateau, contrary to the observations (Fig. 1).

The decarbamylation component was evaluated separately by extensively diluting a sample of carbamylated enzyme and following the rate of recovery with and without the test compounds. The rate of recovery is a logarithmic function of time, indicating the relation (derived from Eq. 2)

$$\ln \frac{E'}{E} = -k'_4 t$$

where E is the initial concentration of enzyme. Figure 2 is a typical plot of the data. It is apparent that succinyldicholine slows the rate of spontaneous recovery of enzyme activity whereas choline and gallamine accelerate it.

When the half-time values (or first-order rate constants) for accelerated recovery are plotted against the concentrations of effector (accelerator), a curve is developed (Fig. 3), indicating a Michaelis-Menten type of relationship. The data in Fig. 3 are plotted as a function of the logarithm of concentration to illustrate better the wide range of concentrations employed. These data indicate relationships that can be expressed as

$$\begin{array}{l} E' + A \underset{}{\overset{K''_A}{\rightleftharpoons}} A \cdot E' \xrightarrow{k''_4} E + P_2 + A \\ \Big\downarrow k'_4 \qquad\qquad (5) \\ E + P_2 \end{array}$$

The following consequences can be derived from Eq. 5.

$$-\frac{dE}{dt} = \frac{k'_4 \varepsilon'}{1 + ([A]/K''_A)} + \frac{k''_4 ([A]/K''_A)\varepsilon'}{1 + ([A]/K''_A)} = k_{4(app)} \varepsilon'$$

where ε' is inhibited enzyme in all forms, and

$$k_{4(app)} = k'_4 \frac{1 + \alpha([A]/K''_A)}{1 + ([A]/K''_A)}$$

where $\alpha = k_4''/k'_4$ and

$$\left(\frac{k_{4(app)}}{k'_4} - 1\right)^{-1} = \frac{K''_A}{\alpha - 1} \cdot \frac{1}{A} + \frac{1}{\alpha - 1}$$

A plot of $[(k_{4(app)}/k'_4) - 1]^{-1}$ against $[A]^{-1}$ yields a linear relationship from which we can evaluate K''_A (Fig. 4).

To determine the simple inhibition of the enzyme-catalyzed hydrolysis of acetylcholine, the binding constant was evaluated by measuring the initial velocities of substrate hydrolysis at different concentrations of acetylcholine in the presence of the test compound. The data were plotted in a double-reciprocal manner as described previously (13).

A number of depolarizing and nondepolarizing neuromuscular blocking agents and other compounds were tested as accelerators of the decarbamylation reaction. The values of their binding constants to the enzyme when accelerating the decarbamyla-

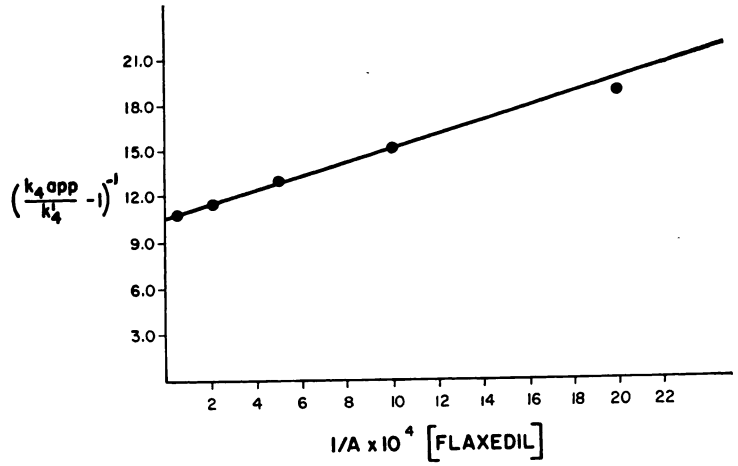


FIG. 4. *Reciprocal of the rate constant values for accelerated recovery of activity of dimethylcarbamylacetylcholinesterase plotted against the reciprocal values for concentration of effector (gallamine)*

The binding constant to the carbamyl-enzyme, K''_A, can be evaluated from the slope.

tion reaction are compared with the values obtained for the inhibition of acetylcholine hydrolysis in Table 1. Some of the drugs are accelerators, and others inhibit the reaction. All depress the rate of substrate hydrolysis. Except for choline, drugs which accelerate decarbamylation are also nondepolarizing neuromuscular blocking agents. Metzger reported (12) that simpler compounds (tetramethylammonium ion, tetraethylammonium ion) function as inhibitors of decarbamylation, but others (14) have indicated that tetraethylammonium accelerates the decarbamylation reaction.

The data recorded in Table 1 indicate a marked similarity when the values of the binding constants (K''_A) for the neuromuscular blocking compounds acting as accelerators are compared with the values (K_I) obtained for those acting as inhibitors of acetylcholine hydrolysis. This may be interpreted as evidence for binding (a) to the same site (active surface) and evoking two different mechanisms, acceleration and inhibition, or (b) to different functional sites

TABLE 1

Comparison of binding constants measured when the compounds act as accelerators and as inhibitors

K''_A is the binding constant of the compound when it is functioning as an accelerator of the decarbamylation of acetylcholinesterase (see Eq. 5). K_I is the constant measured for the inhibition of acetylcholinesterase-catalyzed hydrolysis of acetylcholine (see the text). See the text for details of measurement.

Compound	K''_A (as accelerator)	K_I (as inhibitor)
	M	*M*
Curare	3.6×10^{-6}	3.4×10^{-6}
Dimethylcurare	—[a]	3.5×10^{-6}
Pancuronium	5.7×10^{-5}	3.2×10^{-5}
Gallamine	6.4×10^{-6}	5.3×10^{-6}
"Flax. I"[b]	1.6×10^{-5}	1.4×10^{-5}
Ethocholine	5×10^{-3}	1.1×10^{-4}
Choline	4×10^{-2}	8.8×10^{-5}
Succinyldicholine	—[c]	6.5×10^{-6}
Decamethonium	—[d]	6.7×10^{-8}

[a] Dimethylcurare is an accelerator, but too weak for precise measurement.

[b] "Flax. I" is the ethyl iodide of the diethylaminoethyl ether of phenol.

[c] Succinyldicholine retards reactivation.

[d] Decamethonium has no effect on rehydrolysis.

TABLE 2

Influence of accelerator combinations on decarbamylation of acetylcholinesterase

All measurements were made at pH 6 and 25° in 3×10^{-3} M $MgCl_2$, 10^{-5} M EDTA, and 5×10^{-4} M acetylcholine in a pH-stat. Half-time values are averages of at least three experiments.

Effector	Choline	$t_{1/2}$	Inhibition of acetylcholine hydrolysis
	M	*min*	%
Normal recovery		230	
None	5×10^{-4}	60	15
5×10^{-5} M gallamine	None	89	0
5×10^{-5} M gallamine	5×10^{-4}	36	15
1×10^{-5} M curare	None	149	21
1×10^{-5} M curare	5×10^{-4}	50	32

having similar binding features. The values for the binding constants of choline and ethocholine to the carbamyl-enzyme are 50 times greater than for binding to the free enzyme, suggesting binding features that may differ from those of the blocking drugs. All nondepolarizing neuromuscular blocking agents tested accelerate decarbamylation. Three depolarizing drugs were tested: choline, a weak depolarizer, was an accelerator; succinylcholine, an inhibitor; and decamethonium had no effect at high concentrations. Because of its complementarity with the active surface, it is reasonable to assume that choline is bound to the anionic site in the acceleration reaction. Curare, gallamine, dimethylcurare, and Pancuronium are relatively much less complementary yet accelerate recovery. Do these neuromuscular blocking agents facilitate recovery by being bound to the same site (anionic subsite) as choline, or is an allosteric site involved? Information to help answer this important problem was developed by measuring the rates of recovery of dimethylcarbamylacetylcholinesterase at pH 6 in the presence of both choline and gallamine. If both accelerators are bound to the same site, then the reaction rate should fall between the values for the reactions measured separately, as one agent presumably would interfere with the binding of the other. If, however, the rate is greater when two compounds are used simultaneously than when either is

used alone, two sites are probably involved. The results in Table 2 support the latter hypothesis and indicate that the nondepolarizing neuromuscular blocking agents are presumably bound to separate sites. The values of K''_A (Table 1) may therefore reflect the binding of the neuromuscular blocking agents to this separate site.

Is the accelerated decarbamylation reaction dependent on the presence of substrate? This question was studied by extensively diluting the carbamyl-enzyme in a medium of 3×10^{-3} M $MgCl_2$, 1×10^{-3} M potassium phosphate buffer (pH 7), and 5×10^{-5} M gallamine (or other effector) at zero time. The recovery reactions were followed by withdrawing 0.1-ml aliquots of the diluted enzyme solution at appropriate time intervals and assaying the activity in the presence of substrate, using the technique described by Hestrin (15). The experiment was then repeated by following the recovery in a control medium that did not contain gallamine. Linear relations were observed when the increase in enzyme activity was plotted semilogarithmically as a function of time. A typical plot of the data (Fig. 2) indicates that gallamine accelerates decarbamylation in the absence of substrate. Apparently acetylcholine is not required for the accelerated reaction.

TABLE 3

Effects of acetylcholine concentration on gallamine-accelerated decarbamylation

The concentration of gallamine was 5×10^{-5} M in all experiments. The Hestrin technique was used in the experiments at zero substrate concentration, and a pH-stat in all other studies (pH 7, 25°, 3×10^{-3} M $MgCl_2$), as described in the text. Half-times are averages of three experiments. $t_{1/2}$ is related to $k_{4(app)}$ by $t_{1/2} \times k_{4(app)} = 0.693$.

Acetylcholine	$t_{1/2}$	$k_{4(app)}$	Inhibition of acetylcholine hydrolysis
M	*min*	min^{-1}	%
0	11	0.063	
5×10^{-5}	10	0.069	86
1×10^{-4}	12	0.058	74
2×10^{-4}	17	0.041	63
5×10^{-4}	17	0.041	45
1×10^{-3}	18	0.038	36
2×10^{-3}	19	0.037	16
5×10^{-3}	25	0.028	0
1×10^{-2}	36	0.019	0

TABLE 4

Effect of neuromuscular blocking agents on decarbamylation in presence of ethyl acetate as substrate

The concentration of ethyl acetate was 0.5 M in all experiments (pH 7, 25°, 3×10^{-3} M $MgCl_2$); a pH-stat was used as described in the text. Data are included for similar experiments in which 5×10^{-4} M acetylcholine was the substrate. Half-times are average values of at least three experiments.

Compound	Concentration	Inhibition of ethylacetate hydrolysis	$t_{1/2}$	Inhibition of acetylcholine hydrolysis	$t_{1/2}$
	M	%	*min*	%	*min*
Gallamine	2×10^{-4}	10	13	62	16
Choline	2×10^{-4}	11	<2	24	16
Succinyldicholine	5×10^{-5}	10	40	65	>130
Decamethonium	2×10^{-6}	9	26	55	30
Normal recovery			26		30

Do high concentrations of substrate affect the acceleration phenomenon? The reactivation experiments were repeated at various substrate concentrations with and without 5×10^{-5} M gallamine in 3×10^{-3} M $MgCl_2$, pH 7, at 25°, using the pH-stat technique. The difference in half-time values between the accelerated and control reactions was determined. The degree of inhibition by gallamine of substrate hydrolysis at the various concentrations of acetylcholine used was also evaluated. The data in Table 3 indicate that the concentration of substrate does influence the reactivation. At high substrate concentration accelerated recovery is prevented, and, as the concentration is reduced, acceleration progressively increases. Acetylcholine at high concentrations (0.01 M) may interfere with the binding of gallamine to an allosteric site. Or perhaps the substrate retards its own hydrolysis by being bound to the free anionic subsite of the acetyl-enzyme, as suggested by Wilson and Alexander (4).

Does the cationic head of acetylcholine

influence accelerated recovery? Ethyl acetate, a poor substrate, was substituted for acetylcholine. The effects of gallamine, decamethonium, succinyldicholine, and choline on decarbamylation in the presence of 0.5 M ethyl acetate were measured in a pH-stat at pH 7 and 25° in 3×10^{-3} M $MgCl_2$. Data are recorded in Table 4. In comparison with acetylcholine, ethyl acetate does not qualitatively influence the reaction of these agents with the dimethylcarbamyl-enzyme: gallamine and choline accelerate recovery, decamethonium has no effect, but succinyldicholine retards decarbamylation. Apparently the cationic head of acetylcholine does not influence the acceleration reaction.

What is the influence of pH on accelerated decarbamylation? This question was studied by measuring spontaneous recovery with and without 5×10^{-5} M gallamine at five pH values, from 6 to 8, at 25°, in 3×10^{-3} M $MgCl_2$ and 5×10^{-4} M acetylcholine in a pH-stat. Enzyme instability prevented accurate measurements at more extreme values of pH. Half-time values for the reaction are plotted against pH values in Fig. 5. As the pH of the reaction is reduced, both the normal and the accelerated recovery rates are reduced, the former to a greater degree. If the $MgCl_2$ concentration is increased from 0.003 to 0.3 M, the acceleration phenomenon is prevented.

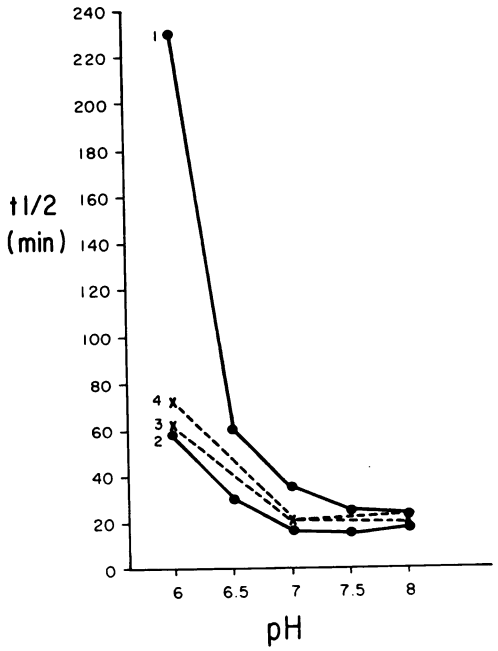


FIG. 5. *Influence of pH on decarbamylation*

All measurements were made in a pH-stat at 25° with 5×10^{-4} M acetylcholine. Solid lines indicate a medium of 3×10^{-3} M $MgCl_2$: curve *1*, normal recovery; *2*, 5×10^{-5} M gallamine added. Dashed lines indicate a medium of 0.3 M $MgCl_2$: curve *3*, normal recovery; *4*, 5×10^{-5} M gallamine added. The $t_{1/2}$ values are averages of at least three experiments.

TABLE 5

Influence of pH on gallamine-accelerated recovery

The concentration of gallamine was 5×10^{-5} M in all experiments. The measurements were made with a pH-stat in a medium of 3×10^{-3} M $MgCl_2$ at 25°, as described in the text.

pH	$t_{1/2}$		Inhibition of acetylcholine hydrolysis by gallamine
	Without gallamine	With gallamine	
	min		%
6.0	229	58	0
6.5	58	26	31
7.0	30	17	45
7.5	25	16	55
8.0	22	19	59

In Table 5 half-time values for gallamine-accelerated recovery and normal recovery are compared with the percentage inhibition of acetylcholine hydrolysis at various pH values. At pH 6, 5×10^{-5} M gallamine markedly accelerates decarbamylation but does not inhibit substrate hydrolysis. Under these conditions gallamine apparently is bound not to the active site on the free enzyme (no inhibition of substrate hydrolysis), but rather to an allosteric site on dimethylcarbamylacetylcholinesterase (accelerates decarbamylation). The data suggest that the anionic subsite and postulated allosteric site may have different pK_a values. This is currently being studied.

Is the accelerated reaction temperature-dependent? Rates of accelerated recovery in the presence of 5×10^{-5} M gallamine were measured under the usual low salt conditions at 10°, 20°, 25°, 30°, and 40°. Half-time values are plotted against temperature and compared with control values in Fig. 6. The rates of both the accelerated and the normal

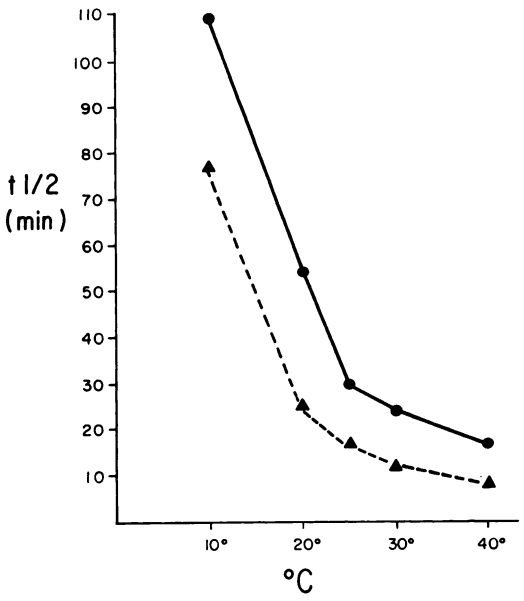


FIG. 6. *Influence of temperature on decarbamylation*

All measurements were made with a thermostated pH-stat in a medium of 3×10^{-3} M $MgCl_2$, 10^{-5} M EDTA, and 5×10^{-4} M acetylcholine. The solid line indicates normal recovery, and the dashed line, recovery in the presence of 5×10^{-5} M gallamine. All values are averages of at least three experiments.

reactions are reduced to the same extent. Temperature is apparently not critical to accelerated decarbamylation.

What is the influence of $MgCl_2$ concentration on accelerated decarbamylation of acetylcholinesterase? Table 6 contains data measured at four concentrations of $MgCl_2$. High concentrations (0.3 and 0.05 M) of $MgCl_2$ hasten normal recovery and retard acceleration by gallamine when compared with $t_{1/2}$ values measured at low salt concentrations. This phenomenon of accelerated recovery is dependent upon a low salt concentration, as was recognized by Changeux for other cholinesterase reactions (6).

Is accelerated decarbamylation $MgCl_2$-dependent? $CaCl_2$ and NaCl were substituted for $MgCl_2$ at the same ionic strength of 0.009, and the recovery experiments with and without gallamine were repeated. Decarbamylation was accelerated by gallamine in both $CaCl_2$ and NaCl solutions, indicating that the reaction is not ion-specific.

Does a potent, reversible active site inhibitor of acetylcholinesterase affect the acceleration phenomenon? Evidence indicates that 3-hydroxyphenyltrimethylammonium ion is simultaneously bound to both the anionic and esteratic subsites of the enzyme (16). The value of its binding constant to the carbamyl-enzyme under our conditions of measurement is 1.2×10^{-7} M, and to the free enzyme, 3.5×10^{-8} M. Data for the effect of this potent inhibitor on decarbamylation are recorded in Table 7. At approximately the I_{50} concentration of 3-hydroxyphenyltrimethylammonium the spontaneous hydrolysis of dimethylcarbamyl-acetylcholinesterase is retarded ($t_{1/2}$ = 40 min). Gallamine at its I_{50} value is an accelerator ($t_{1/2}$ = 17 min). When decarbamylation is measured in the presence of both 3-hydroxyphenyltrimethylammonium and gallamine at their I_{50} concentrations, the half-time value for the reaction is again 17 min. Inhibition of substrate hydrolysis is increased from 50% to 67%. These data provide evidence for the simultaneous binding of gallamine molecules at two sites: the traditional active site of the free enzyme (inhibition of substrate hydrolysis) and an allosteric site on the carbamyl-enzyme (acceleration of decarbamylation). In this case the allosteric site–gallamine interaction can apparently override the effects of a potent active site inhibitor and accelerate de-

TABLE 6

Influence of $MgCl_2$ concentration on gallamine-accelerated decarbamylation

The gallamine concentration was 5×10^{-5} M in all experiments. Conditions of measurements were 25°, pH 7, 5×10^{-4} M acetylcholine, using automatic pH-stat techniques as described in the text. All values are averages of at least three experiments.

$MgCl_2$ concentration	$t_{1/2}$		Inhibition of acetylcholine hydrolysis by gallamine
	Without gallamine	With gallamine	
M	*min*		%
0.003	30	17	46
0.01	25	18	26
0.05	20	20	1
0.3	20	21	0

TABLE 7

Influence of 3-hydroxyphenyltrimethylammonium ion on decarbamylation

All measurements were made at pH 7 and 25° in 3×10^{-3} M $MgCl_2$, using a pH-stat apparatus.

Effector	3-Hydroxyphenyltrimethylammonium ion	$t_{1/2}$	Inhibition of acetylcholine hydrolysis
	M	*min*	%
None	0	30	
None	1×10^{-6}	40	48
5×10^{-5} M gallamine	0	17	45
5×10^{-5} M gallamine	1×10^{-6}	17	67
5×10^{-4} M choline	0	10	38
5×10^{-4} M choline	1×10^{-6}	19	61
2.5×10^{-6} M succinyldicholine	0	60	13
2.5×10^{-6} M succinyldicholine	1×10^{-6}	74	50

carbamylation as though the inhibitor were not present.

Choline, a reactivator, was substituted for gallamine, and the effects on decarbamylation with and without 3-hydroxyphenyltrimethylammonium were measured under the usual conditions. The data in Table 7 indicate that the values for $t_{1/2}$ are: choline, 10 min; 3-hydroxyphenyltrimethylammonium, 40 min; and choline plus 3-hydroxyphenyltrimethylammonium, 19 min. These results may be interpreted as evidence that choline and 3-hydroxyphenyltrimethylammonium, which are both closely complementary to the active surface, are bound to the same site, since one interferes with the binding of the other and the resultant half-time lies between the values recorded separately.

Succinyldicholine may also have more than the usual degree of complementarity with the active site, as the configuration may be viewed as 2 molecules of acetylcholine covalently linked at the acetate functions. It is a potent inhibitor of decarbamylation and, when combined with 3-hydroxyphenyltrimethylammonium, further retards the rate of spontaneous decarbamylation. These data are consistent with the explanation that both drugs are bound to the active site. This does not rule out the possibility that one or both drugs may be bound to an allosteric site which inhibits activity at the active site.

Does the degree of purity of the enzyme preparation affect accelerated decarbamylation? Samples of eel enzyme were prepared with specific activities of 8, 50, and 500 mmoles of acetylcholine hydrolyzed per hour per milligram of protein (9). These samples were inhibited; accelerated decarbamylation was studied in the standard manner described under MATERIALS AND METHODS. No differences in reaction rates were found. Presumably the accelerated decarbamylation phenomenon is not a function of enzyme purity.

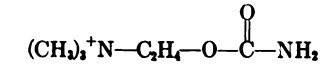
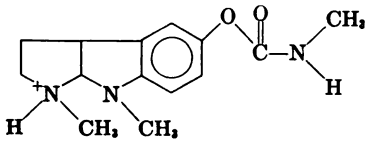
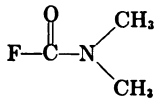
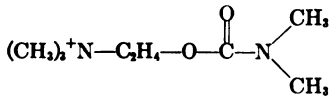
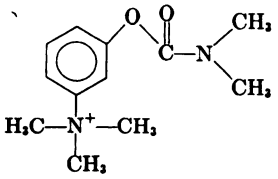
Does gallamine accelerate the recovery of enzyme activity after inhibition by other carbamates? Samples of the enzyme were inhibited by carbamylcholine (carbachol), physostigmine (eserine, a monomethyl carbamate), and three dimethyl carbamate agents. Normal recovery and gallamine-accelerated decarbamylation were studied in the usual manner. The results are recorded in Table 8. Gallamine accelerated recovery in all cases, and to the same degree, for the enzyme inhibited by the three dimethyl carbamates. The acyl-enzyme derived from carbachol (carbamylacetylcholinesterase) recovered activity too rapidly ($t_{1/2} < 2$ min) for accurate measurement. Spontaneous and gallamine-accelerated recovery in this case were slowed to a convenient rate by the addition of the simple quaternary ammonium agent tetraethylammonium ion; the concentration of the ion was the same in both cases. Normal recovery was slowed to a $t_{1/2}$ value of 15 min, and gallamine accelerated recovery to a half-life of 5 min. Apparently this phenomenon is not dependent upon the group covalently bound to the esteratic site. We have also accelerated the recovery of acetylcholinesterase after it had been inhibited by some organophosphate compounds; these data will be reported separately.

Are specific structural features of the gallamine molecule required for it to function as an accelerator? Some derivatives of gallamine were prepared and their effects on decarbamylation were studied. The results

TABLE 8

Influence of carbamyl group on accelerated recovery

The half-time values were measured under the usual conditions; they represent the averages of at least three experiments. Because the half-time of spontaneous reactivation for the inhibited enzyme derived from carbachol was less than 2 min, 2×10^{-3} M tetramethylammonium ion was added to the recovery medium in the control (no gallamine) and accelerated (with gallamine) experiments. In both cases, reactivation was slowed to more convenient rates.

Effector	$t_{1/2}$	
	Without gallamine	With gallamine
	min	
 Carbachol	15 ± 2.1	5 ± 2.0
 Physostigmine	38 ± 3.2	26 ± 1.4
 Dimethylcarbamylfluoride	30 ± 2.4	17 ± 0.83
 Dimethylcarbamylcholine	30 ± 2.0	17
 Neostigmine	30 ± 2.6	17 ± 0.8

are recorded in Table 9. As ethocholine groups are successively removed from the benzene ring, the accelerating qualities of the compounds are reduced but do not disappear. However, if the benzene ring is replaced by a methyl group (triethyl-β-methoxyethylammonium bromide), the resulting compound becomes an inhibitor rather than an accelerator. Perhaps the benzene ring is important in allosteric site interactions. Ethocholine and choline are reactivators but, because of their complementarity, are presumed to act at the active site.

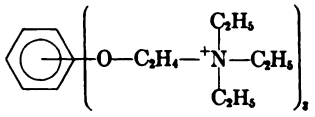
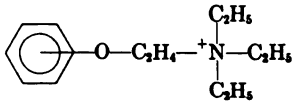
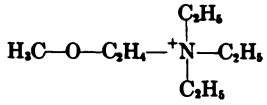
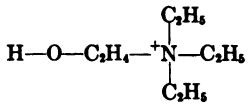
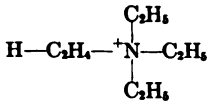
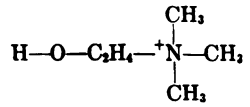
DISCUSSION

Spectropolarographic measurements indicate that acetylcholinesterase does undergo changes in conformation when interacting with certain substrates and inhibitors (8).

TABLE 9

Activity-structure relationships

The influence of the effectors on the spontaneous reactivation of dimethylcarbamylacetylcholinesterase was measured in a pH-stat under the usual conditions. The concentrations selected inhibit acetylcholinerase hydrolysis approximately 50%. Compounds with benzene rings and hydroxyl groups are accelerators.

Effector	Concentration	$t_{1/2}$	Inhibition of acetylcholine hydrolysis
	M	*min*	%
Normal recovery		30	
 Gallamine	1×10^{-4}	16	50
	5×10^{-4}	17	51
	1×10^{-3}	67	46
	1×10^{-3}	25	51
	1×10^{-3}	73	46
	1×10^{-3}	7	53

Some simple quaternary ammonium ions have been shown to accelerate the rate of inhibition of this enzyme by methanesulfonyl and carbamyl inhibitors. This has been interpreted as kinetic evidence for induced conformational changes (17, 18).

It has been suggested that acetylcholinesterase may also function as the "cholinergic receptor substance" (7). Neuromuscular blocking agents play important roles in these studies. Župančič measured the value of the equilibrium constant between tubocurarine and the anionic centers of horse plasma cholinesterase; it was found to be independent of whether the esteratic site was active or inhibited by physostigmine or heat (19). Changeux found (6) that some neuromuscular blocking agents are well bound to acetylcholinesterase but act only as partially competitive inhibitors of acetylcholine hydrolysis, even at high concentrations. It was also demonstrated that gallamine can reduce the amount of inhibition produced by some simple, reversible quaternary ammonium agents and enhance the inhibitory properties of others. These data

suggest the existence of a binding site distinct from the traditional active surface.

Much of the information reported in this paper supports the presence of an allosteric site topographically distinct from the active surface, which is able to modulate the properties of the catalytic center. "Bulky" agents, particularly those containing aromatic groups and producing nondepolarizing types of myoneural blockade in physiological preparations, are found under our conditions of measurement to accelerate inhibition and recovery of enzyme activity following interaction with some acid-transferring inhibitors. Their lack of complementarity with the active surface and their ability to accelerate rather than retard esteratic site activity suggests an alternative binding site. The most significant data are the following. (a) At pH 6 gallamine accelerates decarbamylation (and therefore must be bound to the inhibited enzyme) but does not inhibit substrate hydrolysis (and therefore is apparently not bound to the active site of the free enzyme). (b) When choline, a reactivator of dimethylcarbamylacetylcholinesterase (and, because of its complementarity, presumed to bind at the active surface), is combined with gallamine or curare, the rate of decarbamylation is faster than the rates measured separately. These measurements indicate separate binding sites. (c) The *similarity* of the values of the binding constants of the blocking agents to the carbamyl-enzyme and free enzyme may also indicate an allosteric site. Metzger and Wilson (18) reported markedly *different* values for the binding of tetraethylammonium and tetra-*n*-propylammonium to the carbamyl-enzyme and free enzyme. They interpreted their data as evidence of binding to the same site, presumably the anionic subsite. (d) 3-Hydroxyphenyltrimethylammonium ion, a potent, specific active site inhibitor, retards decarbamylation at a concentration equal to its I_{50} value. Gallamine at its I_{50} level completely reverses the inhibitory properties of 3-hydroxyphenyltrimethylammonium and substitutes acceleration at a rate equal to that measured in the absence of the ion. Changeux reported that gallamine enhances the binding of 3-hydroxyphenyltrimethylammonium but antagonizes the binding of its diquaternary derivative (6). (e) The specific active site inhibitor, 3-hydroxyphenyltrimethylammonium, was combined with the reactivator, choline, at concentrations equal to their I_{50} values and their effect on decarbamylation was studied. The half-time for recovery fell between the values measured for each separately. These data are consistent with both agents acting at the same site; when gallamine is substituted for choline [see (c) above], different sites may account for the action.

The evidence indicates that gallamine and choline accelerate decarbamylation of acetylcholinesterase by interaction with the enzyme at different sites. Are the mechanisms of acceleration the same? Choline reactivates the enzyme inhibited by some organophosphate and carbamate agents (14, 20). Although choline does not have appreciable intrinsic nucleophilic properties, its ability to reactivate the enzyme can be explained if we assume that its activity is promoted through interaction at the anionic site (19, 20). This mechanism, postulated by Wilson, would involve a nucleophilic attack by the oxygen of choline on the phosphorus atom (alkyl phosphate-inhibited enzyme) or carbamyl carbon atom (alkyl carbamate-inhibited enzyme) similar to the reactivation scheme proposed for pyridine-2-aldoxime methiodide (14, 21, 22). Although it is highly plausible, proof for this mechanism by isolation of the products, alkylphosphorylcholine and alkylcarbamylcholine, has not yet been accomplished.

Gallamine, curare, Pancuronium, and other nondepolarizing agents are not nucleophiles. Their ability to accelerate recovery of enzyme activity must be explained by a different mechanism. The data discussed in this paper are most consistent with the existence of a site topographically distinct from the active surface of acetylcholinesterase and capable of binding curare-like compounds. In addition, the site is able to modulate catalytic center activity, presumably through the mechanism of conformational changes in the enzyme. The allosteric site may preferentially bind aromatic compounds. The physiological significance of these observations is not yet clear.

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