Responses of Acetylcholinesterase from *Torpedo marmorata* to Salts and Curarizing Drugs

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SUMMARY

The structure and catalytic activity of acetylcholinesterase (Acetylcholine acetyl-hydrolase, EC 3.1.1.7) (AChE) from *Torpedo marmorata* depend upon the ionic strength, $\Gamma/2$, of the environment. The sedimentation coefficient of the enzyme is 14 S at $\Gamma/2 = 0.3$, but is polydisperse in the range of 10–80 S at $\Gamma/2 = 0.003$. The optimal velocity of the reaction catalyzed by AChE increases with ionic strength, while under the same conditions the affinity for the substrate and for several reversible competitive inhibitors decreases. The relative decrease of affinity as a consequence of increased ionic strength is higher for inhibitor molecules containing two quaternary ammonium ions than for compounds containing a single quaternary ammonium group. Among the monoquaternary inhibitors, this decrease is greater for phenyltrimethylammonium than for its 3-hydroxy analog.

In solutions of low salt concentration ($\Gamma/2 = 0.003$) significant affinity of the enzyme for two pachycurares, flaxedil and *d*-tubocurarine, can be demonstrated. Both compounds produce partial inhibition of AChE activity, antagonize its inhibition by reversible competitive inhibitors including some leptocurares, and enhance the inhibition by 3-hydroxyphenyltrimethylammonium. By measuring the degree of protection of AChE against thermal inactivation, the dissociation constant for the flaxedil–enzyme complex can be estimated to be about 3×10^{-7} M.

These observations and their possible physiological significance are interpreted in terms of conformational alterations of the AChE molecule in response to the binding of the pharmacologic agents.

INTRODUCTION

During the past few years a considerable amount of data has been accumulated and interpretive models have been proposed concerning the regulatory mechanism by which the activities of various enzymes are controlled by specific metabolites (1–6). In the majority of the known examples the circuits established between the macromolecular receptors and the diffusible regulatory signals operate within the limits of a single cell. Since in highly differentiated organisms chemical communication occurs between cells, it is of interest to determine whether some of the results and interpretations obtained for *intracellular* regulation can be extrapolated to *intercellular* systems.

An excellent example of an intercellular regulatory signal present in animals is acetylcholine (ACh), a chemical agent involved in the transmission of nerve impulses through a number of synapses (7–14). In a classical cholinergic synapse the following

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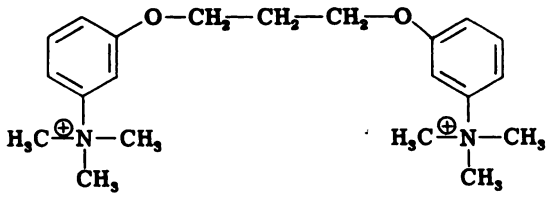
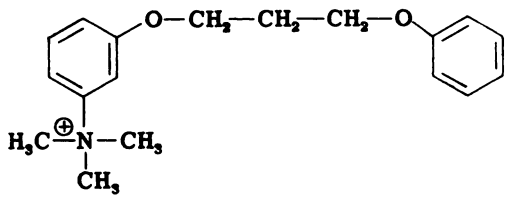
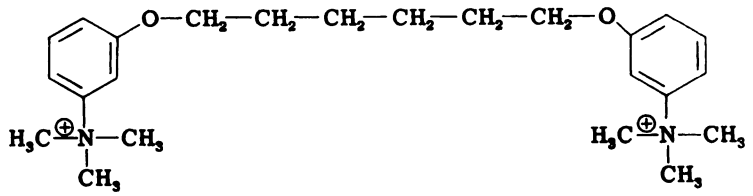
TABLE 1
Structure of some drugs to which AChE responds[a]



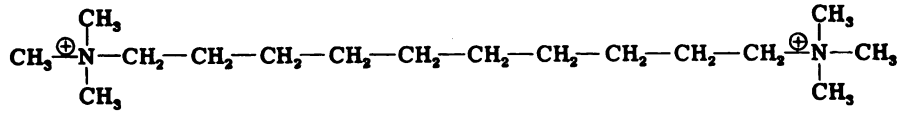
2842 CT



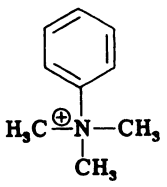
2983 CT



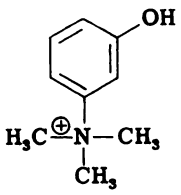
2817 CT



Decamethonium



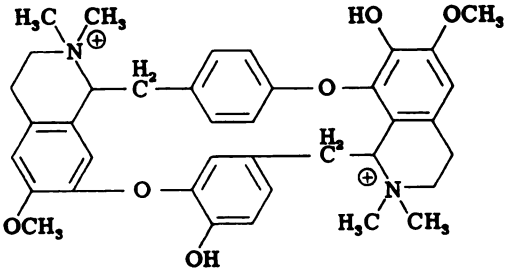
3MϕA



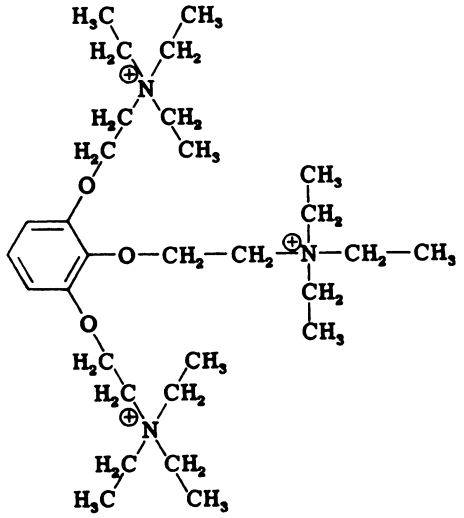
2561 CT

steps are thought to occur: (a) liberation and diffusion of ACh into the synaptic cleft as a consequence of a presynaptic impulse; (b) reception of ACh by a postulated stereospecific receptor located in the subsynaptic membrane accompanied by the triggering of permeability changes to ions in the postsynaptic cell; and (c) destruction of ACh by a specific enzyme, acetylcholinesterase (Acetylcholine acetyl-hydrolase, EC 3.1.1.7) (AChE) (11–14). Recognition of ACh by the subsynaptic membrane is a critical event in this biochemical sequence, and several investigations have been reported concerning the nature of the "physiological receptor of

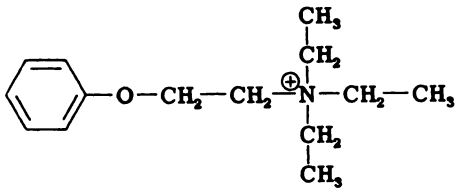
TABLE 1 (*Continued*)



d-Tubocurarine



Flaxedil



2512 CT

• Decamethonium is typical of the leptocurares or "depolarizing" agents; 2842CT and 2817CT are also classified as leptocurares; 2983CT has negligible leptocurare activity. Flaxedil and *d*-tubocurarine are typical pachycurares or "stabilizing" agents; 2512CT exhibits much smaller pachycurare activity.

ACh" and its attempted isolation (15–22). Although AChE necessarily contains in its catalytic center a stereospecific receptor of ACh for its participation in step 3, this enzyme has generally been distinguished from the "physiological receptor" involved in step 2, on the basis of its low affinity for drugs known to interfere *in vivo* with the postsynaptic action of ACh (13).

Among the drugs acting on the neuromuscular junction are curare and the curare-like agents (23–27). These com-

pounds have been assigned by Bovet (26) to two classes according to their structure and their *in vivo* mode of action.[2] The leptocurares, typified by decamethonium (Table 1), act like ACh insofar as they produce depolarization of the subsynaptic membrane. This depolarization is of greater duration than that induced by ACh, and subsequent repolarization at the motor end plate is delayed. The pachycurares, typified by *d*-tubocurarine and flaxedil (Table 1), block the ACh-induced depolarization, and are assumed to compete with ACh for its postsynaptic receptor.

The data presented in this paper concern the effect of several compounds including some lepto- and pachycurares on the AChE extracted from the electric tissues of an elasmobranch of the French Atlantic coasts, *Torpedo marmorata* Risso. First, it is demonstrated that the structure and the catalytic properties of isolated AChE vary dramatically with ionic strength. In solutions of low ionic strength the enzyme tends to form rapidly sedimenting aggregates and simultaneously exhibits high affinity for ACh and various inhibitors including some leptocurares. Under these conditions it is also demonstrated that some pachycurares, e.g., flaxedil and *d*-tubocurarine, known to be poor inhibitors of AChE (28, 29) and postulated to be bound with high specificity by the physiological receptor of ACh (12, 13, 30, 31) are actually bound to the AChE molecule.[3]

MATERIALS AND METHODS

Colorimetric Assay of AChE

The assay technique devised for these studies is based on the liberation of a proton upon hydrolysis of ACh in the presence of AChE. The extent of hydrolysis is followed spectrophotometrically by including a pH indicator, bromothymol blue (pK = 7.0), in the weakly buffered assay mixture. The assay medium regularly contains 10^{-3} M sodium Veronal, pH 7.4, and 7.5×10^{-5} M bromothymol blue. After the solution is prepared with distilled water,[4] dissolved CO_2 is eliminated by bubbling nitrogen for 30 min to 1 hr. The optical density (O.D.) at 620 mμ is then adjusted with NaOH or HCl to 1.6, corresponding to a pH close to pH 7. The assay is conducted in a total volume of 2.0 ml in cuvettes of 1-cm light path. After addition of substrate (generally 2.5×10^{-3} M ACh), the reaction is initiated by rapid mixing with the enzyme solution (generally 1 μl of a dialyzed preparation of AChE of specific activity: 4.4 moles of ACh split per hour per gram of protein and containing 3.6 mg of protein/ml). The decrease of O.D. at 620 mμ is followed in a recording spectrophotometer thermostatically maintained at approximately 23°. The graphs recorded within the range of O.D. 1.6 and 1.2 (corresponding to pH 7.0 ± 0.08) during the first 3 min after mixing are usually straight lines of which the slopes represent initial rates of ACh hydrolysis. In the few situations where recorded graphs are not linear, the slope at zero time may be taken as a measure of the initial rate. The calibration curve for relating the observed changes in O.D. at 620 mμ to the change of hydrogen ion concentration is established by measuring the optical density of the assay mixture to which known amounts of HCl have been added. For each modification of the assay medium a similar standard curve is prepared. The initial rates are directly proportional to the concentration of enzyme present in the assay medium. Activities estimated by the present colorimetric technique do not differ significantly from those measured in the same ionic environment with a pH meter. The specific activity is

[2] The leptocurares are also described as "depolarizing" or "decamethonium-like" drugs, and the pachycurares as "stabilizing" or "curare-like" drugs. Extensive discussions of these definitions and of the complex effects of these drugs are found in Bovet (26) and in Taylor and Nedergaard (27).

[3] A short communication of these results has been published: J.-P. Changeux, *Compt. Rend. Acad. Sci.* **262**, 937, 1966.

[4] Solubilization of crystals of bromothymol blue in water is greatly accelerated by first dissolving the crystals in the smallest volume of 95% ethanol and then adding water.

defined as the number of moles of ACh split per hour per gram of protein (32). Protein concentrations are measured by the method of Folin and Cioccalteau (33).

Preparation of AChE

Our method of extraction and purification of AChE from *T. marmorata* derives from those described by Kremzner and Wilson (32) and Lawler (34) for AChE of *Electrophorus electricus*. The extraction involves: (a) immersion of the electric organs under toluene for 3 weeks; (b) solubilization by dispersion of the electric tissue with a Waring blendor for 6 min in an aqueous solution containing 10^{-1} M $MgCl_2$ and 5×10^{-2} M sodium Veronal, pH 7.5; and (c) centrifugation to remove insoluble material. The purification steps are: (i) ammonium sulfate fractionation between 20 and 45% saturation; and (ii) chromatography on a DEAE-Sephadex column in Veronal buffer with a gradient of $MgCl_2$ concentration between 2.5×10^{-2} and 5×10^{-1} M. The last step gives a preparation of specific activity 4.4 moles ACh/hr per gram of protein. The specific activity can be increased to 74 moles ACh/hr per gram of protein by preparative ultracentrifugation in a sucrose gradient between 7 and 20% in the presence of 2×10^{-1} M $MgCl_2$.

The absence of esterasic activity other than AChE is indicated by the observation of the same optimal velocity whether ACh or acetyl-β-methylcholine is used as the substrate.

The commercial enzyme preparations used in a few experiments have included AChE from electric organs of *E. electricus* (Sigma), AChE from beef erythrocytes (Sigma), cholinesterase from human plasma (Sigma), and catalase (Sigma).

Chemicals

Several of the chemicals used have been prepared in the Service de Chimie thérapeutique of the Pasteur Institute (Paris) and are generous gifts of Dr. F. Tazieff-Depierre. They are designated in the list below by a four-digit number followed by the two letters CT. The others are commercially available.

acetycholine chloride (Sigma)
acetyl-β-methylcholine bromide (gift of Lematte et Boinot Ltd.)
phenyltrimethylammonium chloride: 3 MØA (Eastman Kodak)
(3-hydroxyphenyl)trimethylammonium iodide: 2561CT (= 2861CT)
[trimethylenebis(oxy-*m*-phenylene)] bis(trimethylammonium iodide): 2842CT
[trimethylene-bis(oxy-*m*-phenylene)] trimethylammonium iodide: 2983CT
[trimethylene-bis(oxy-*p*-phenylene)] bis(trimethylammonium iodide): 2785CT
[hexamethylene-bis(oxy-*m*-phenylene)] bis(trimethylammonium chloride): 2817CT
decamethylene bis(trimethylammonium bromide): decamethonium
1,2,3-tris(2-triethylammonium ethoxy) benzene triiodide: Flaxedil, gallamine triethiodide (Specia)
(2-triethylammonium ethoxy)benzene iodide: 2512CT (= 2512F)
d-tubocurarine (Sigma)
atropine sulfate (Pharmacie des Hôpitaux, Paris)

RESULTS

Effects of Salts on the Structure and Activity of AChE

Effect of Salts on the Sedimentation Coefficient of AChE

Conflicting reports have been published during the past few years concerning the sedimentation coefficient and the molecular weight of AChE from electric organs of fishes. Lawler (34) determined the infinite dilution value of the sedimentation coefficient of AChE from *E. electricus* to be 109 S, and the molecular weight from light scattering data to be 3.3×10^6. Kremzner and Wilson (35), on the other hand, found for a more highly purified preparation of the same enzyme a sedimentation coefficient of 10.8 S, and a molecular weight of 2.3×10^5. In agreement with the independent observations of Grafius and Millar (36) on the *E. electricus* enzyme, the sedimentation

analysis of AChE from *T. marmorata* in sucrose density gradients containing various salt concentrations shows that the slowly and rapidly sedimenting states of the enzyme can both be obtained from the same preparation. Figure 1 represents the

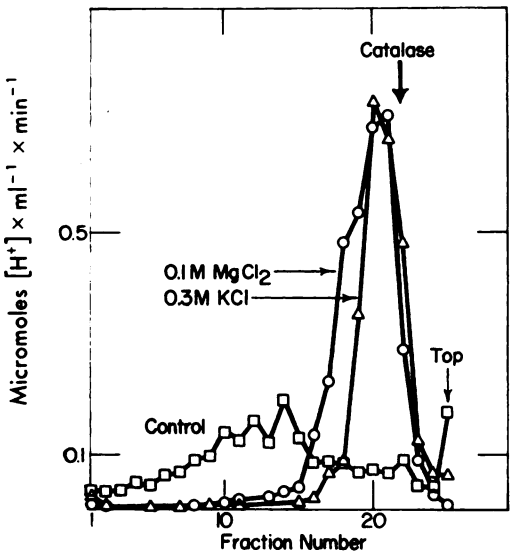


FIG. 1. *Effect of salts on the sedimentation of AChE in a sucrose density gradient*

Sucrose density gradients from 7 to 20% are established in 10^{-3} M potassium phosphate, pH 7.7, in the presence of 10^{-1} M $MgCl_2$ or 3×10^{-1} M KCl and in the absence of additional salts (control). Onto each 5 ml sucrose gradient is layered 0.15 ml of a partially purified preparation of AChE (specific activity 1.57 moles ACh/hr per gram of protein, 5.0 mg protein/ml), dialyzed overnight at 4° against 10^{-3} M potassium phosphate, pH 7.7. The samples to be centrifuged in the presence of high ionic strength are first mixed with the salts at their final concentration in the gradient and incubated several minutes at room temperature. Centrifugation is performed at 35,000 rpm during 3 hr 30 min in a SW 39 rotor, Spinco centrifuge, Model L. Fifty microliters of each collected fraction is mixed with 2.0 ml of the standard assay medium containing 2×10^{-1} M $MgCl_2$.

sedimentation profile in concentrated and dilute salt solutions of a preparation of AChE which had a specific activity of 1.5 mole ACh/hr per gram of protein and had been dialyzed overnight against 10^{-3} M potassium phosphate, pH 7.7. At low ionic strength polydispersity is observed, and the sedimentation coefficient, estimated by comparison with that of a catalase marker (11.3 S) varies from about 10 S to more than 80 S, with a peak at about 35 S. By contrast, the sedimentation profile of an aliquot of the same preparation of enzyme which had been incubated a few minutes at room temperature in the presence of 10^{-1} M $MgCl_2$ or 3×10^{-1} M KCl ($\Gamma/2 = 0.3$) and centrifuged in a sucrose gradient containing the same concentration of these salts displays a narrow peak of activity for which the sedimentation coefficient can be estimated by comparison with that of catalase as about 14 S.

An obvious interpretation of such dramatic changes in sedimentation behavior is that variation of the ionic strength of the medium induces a change in the state of aggregation of the enzyme. Although these findings are consistent with the interpretation that the slowly sedimenting state of the enzyme derives from the rapidly sedimenting species, they do not prove that the larger units result from the specific association of the smallest active unit with itself. Nonspecific association of the enzyme with other categories of molecules present in the incompletely purified preparation might also account for such a behavior. In order to establish the relationships among the various forms of the enzyme with more certainty, the following experiment was devised.

A preparative centrifugation of AChE was performed in a 25 ml sucrose gradient (7 to 20%), in the presence of 2×10^{-1} M $MgCl_2$, in order to collect appreciable amounts of the slowly sedimenting form of the enzyme. The fractions of enzyme sedimenting at approximately 14 S were pooled, dialyzed at 4° against buffer of low ionic strength (sodium Veronal 10^{-3} M, pH 7.5) and recentrifuged in the presence of the same buffer with and without 2×10^{-1} M $MgCl_2$. It is clear from the results in Fig. 2 that the rapidly sedimenting polydisperse form of the enzyme can be obtained from the slowly sedimenting one.

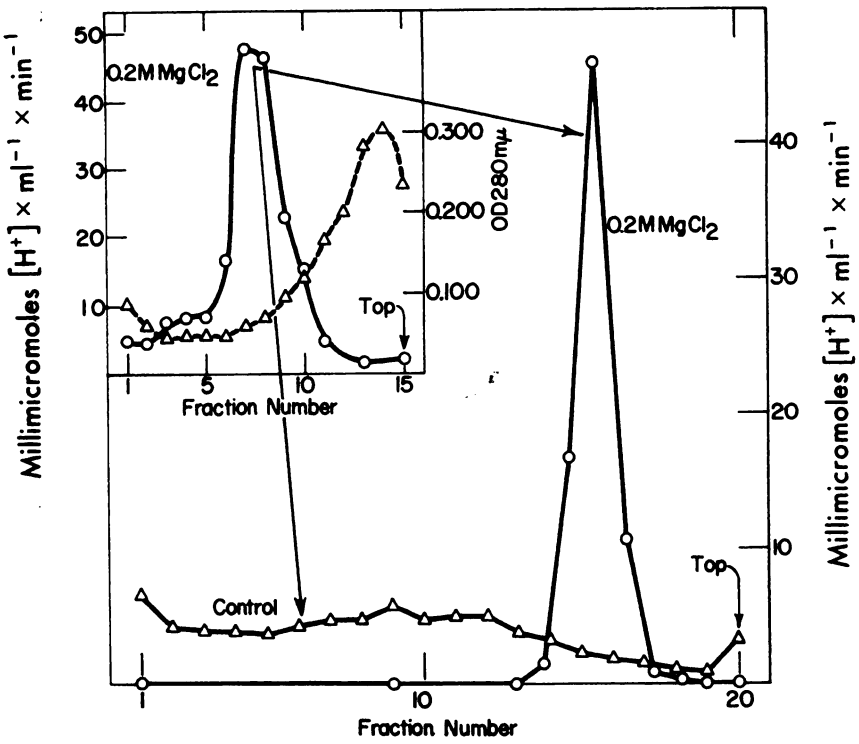


FIG. 2. *Reversibility of the association of AChE at low ionic strength*

Diagram in the left upper corner: A 25-ml sucrose density gradient is established in 2×10^{-2} M sodium Veronal, pH 7.5, supplemented with 2×10^{-1} M $MgCl_2$. On the top of the gradient are layered 1.5 ml of a partially purified preparation of AChE (specific activity 4.4 moles ACh/hr per gram of protein, 3.6 mg protein/ml) dialyzed overnight against 10^{-3} M sodium Veronal pH 7.5 and mixed with $MgCl_2$ at a final concentration of 2×10^{-1} M prior to centrifugation. AChE from this preparation exhibits the same sedimentation properties as those used in the experiment of Fig. 1. Fractions are collected immediately after centrifugation at 25,000 rpm for 19 hr in a SW 25 rotor, Spinco centrifuge Model L. The AChE activity (solid line) is assayed following the standard procedure: 20 μl of each fraction per 2.0 ml of assay mixture containing 2×10^{-1} M $MgCl_2$. Optical densities of each fraction are measured at 280 mμ (broken line).

Main diagram: Peak fractions from the former run are first pooled together then separated in two equal parts. The first one is dialyzed against 10^{-3} M sodium Veronal, pH 7.5, the second against 10^{-3} M sodium Veronal, pH 7.5, supplemented with 2×10^{-1} M $MgCl_2$. Of each dialyzed fraction, 0.2 ml is layered on 5 ml sucrose gradients, respectively, established in 10^{-3} M Veronal, pH 7.5, and 5×10^{-3} M Versene, (control) and in 10^{-3} M Veronal *pH* 7.5, 2×10^{-1} M $MgCl_2$ (0.2 M $MgCl_2$ on the figure). After centrifugation at 39,000 rpm for 2 hr and 45 min in a SW 39 rotor, 20 μl of each collected fraction is mixed with 2.0 ml of the standard assay medium containing 2×10^{-1} M $MgCl_2$. Recovery of activity: first preparative centrifugation, 40%; second centrifugation, control, 100%; 2×10^{-1} M $MgCl_2$, 85%.

Unless the enzyme preparation contains another molecule having the same sedimentation coefficient as AChE and with which the enzyme can associate, a reasonable explanation for these observations is that, at low salt concentrations, the smallest active unit of the enzyme associates reversibly with itself. Since neither the geometry of the association nor the nature of the bonds involved in this process has been investigated, it is possible that some nonprotein components, such as lipids, which could be strongly bound to the low molecular weight state of the enzyme, may contribute to the formation of the rapidly sedimenting aggregates.

Effects of Salts on the Activity of AChE and Its Inhibition by Various Reversible Competitive Inhibitors

General effects of salts. Although NaCl and $MgCl_2$ are classically known to be activators of AChE and assays of this enzyme are systematically performed in the presence of high concentrations of both these salts, the mechanism of their effect on the kinetics of catalysis by AChE is not clear (for references see 37). Our assay technique is suitable for an investigation of this subject since it allows measurement of AChE activity at ionic strengths in the range of less than 0.01 to greater than 0.6. The curves in Figs. 3 and 4 for the saturation of AChE by ACh in the presence and absence of KCl, NaCl, and $MgCl_2$ and the data of Table 2 show that an increase of ionic strength simultaneously increases the optimal velocity of the reaction and decreases the apparent affinity of the enzyme for ACh. Because of the high turnover number of AChE and its high affinity for the substrate, only rough estimates of the

TABLE 2

Effect of salts on the substrate affinity (apparent K_m) and optimal reaction velocity (V_{opt}) of AChE[a]

Parameter	Control	0.6 M KCl	0.6 M NaCl	0.2 M $MgCl_2$
Apparent $K_m \times 10^4$ M	0.95	2.1	2.5	5.0
V_{opt}/V_{opt} control	1	1.3	1.7	2.7

[a] Assay procedure the same as for Fig. 3.

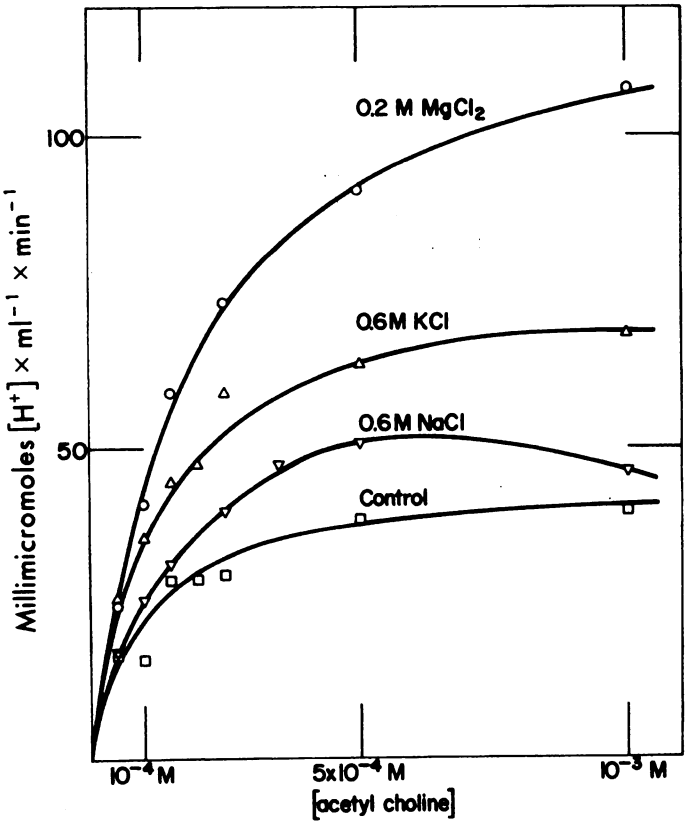


FIG. 3. *Effect of salts on the activity of AChE*

Initial rates of the AChE reaction are plotted as a function of ACh concentrations. Standard assay at low ionic strength (control) and in the presence of 6×10^{-1} M KCl and 2×10^{-1} M $MgCl_2$. Concentration of enzyme: 1 µl of a preparation of AChE (specific activity 4.4 moles ACh/hr per gram of protein, 3.6 mg protein/ml) per 2.0 ml of the assay mixture.

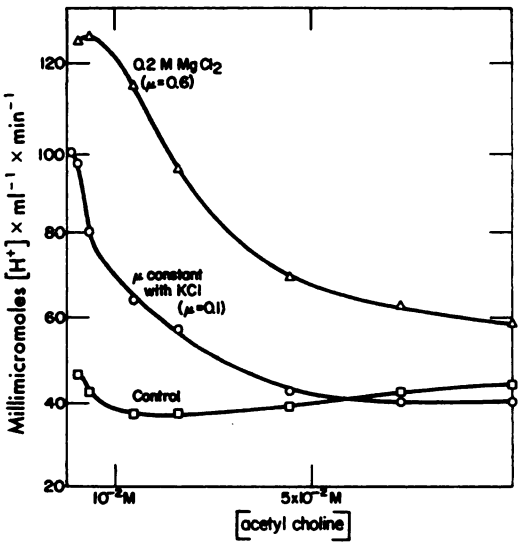


FIG. 4. *Effect of salts on the inhibition of AChE by excess substrate*

To the standard assay at low ionic strength (control) sufficient KCl to maintain $\Gamma/2 = 0.1$ or 2×10^{-1} M $MgCl_2$ ($\Gamma/2 = 0.6$) is added.

apparent K_m for ACh as a function of salt concentration have been made. It may be seen from the results in Table 2 and Figs. 3 and 4 that the concentration of ACh corresponding to the optimal velocity varies somewhat with the nature of the salt.

In experiments performed with extremely low salt concentrations the contribution of the substrate itself to the ionic strength must be taken in account. Figure 4 shows that when one does not take the precaution of maintaining the ionic strength of the assay medium at a constant value, the classical inhibition by excess substrate becomes negligible. The inhibition reappears when the ionic strength is maintained constant in all the experiments with KCl. The apparent absence of inhibition by excess substrate at low ionic strength results from two compensating effects; inhibition by excess ACh and activation by the increase of ionic strength produced by the addition of substrate.

The remarkable susceptibility of AChE to its ionic environment is also illustrated by the study of its inhibition by competitive inhibitors as a function of ionic strength, as shown in Figs. 5 and 6 and in Table 3. In agreement with the findings of Tazieff-Depierre *et al.* and others (29, 38–40), our results confirm that lowering the salt concentration of the assay medium increases the inhibition produced by each of these compounds. The lack of a significant difference between the inhibition curves obtained from assays performed in the presence of KCl or $MgCl_2$ at the same

TABLE 3

Effect of 0.2 M $MgCl_2$ on the inhibition of AChE by various reversible competitive inhibitors

Inhibitor	Control (I_{50}),[a] M	0.2 M $MgCl_2$ (I_{50} Mg), M	$I_{50}Mg/I_{50}$	Control K_i^+, M	0.2 M $MgCl_2$ $K_{i_{Mg}}$, M	$K_{i_{Mg}}/K_i$
Decamethonium	3.5×10^{-8}	9×10^{-6}	260	1.2×10^{-9}	1.5×10^{-6}	1250
2817CT	1.7×10^{-8}	4.5×10^{-7}	26.5	6×10^{-10}	7.5×10^{-8}	125
2785CT	7×10^{-8}	3×10^{-6}	43	2.45×10^{-9}	5×10^{-7}	205
2842CT	3×10^{-8}	3×10^{-6}	100	1.05×10^{-9}	5×10^{-7}	475
2983CT	1.1×10^{-5}	8×10^{-5}	7.3	3.85×10^{-7}	1.34×10^{-5}	35
3MϕA	7.3×10^{-5}	6.9×10^{-4}	9.4	2.55×10^{-6}	1.15×10^{-4}	45
2561CT	1.1×10^{-5}	1.50×10^{-5}	1.35	3.85×10^{-7}	2.5×10^{-6}	6.5

[a] I_{50} is the concentration of inhibitor in the presence of which the activity of the enzyme is half the activity measured in the absence of inhibitor. Standard method of assay at low ionic strength (I_{50}) and in the presence of 0.2 M $MgCl_2$ ($I_{50}Mg$); ^+Ki's are calculated from I_{50} using the following equation:

$$K_i = I_{50} \frac{K_m}{K_m + S}$$

where S is the substrate concentration. The following values of the Michaelis constant (K_m) for the substrate have been used: 9.5×10^{-5} M ACh at low salt concentrations, 5×10^{-4} M ACh in the presence of 0.2 M $MgCl_2$. The concentration of ACh is 2.5×10^{-2} M.

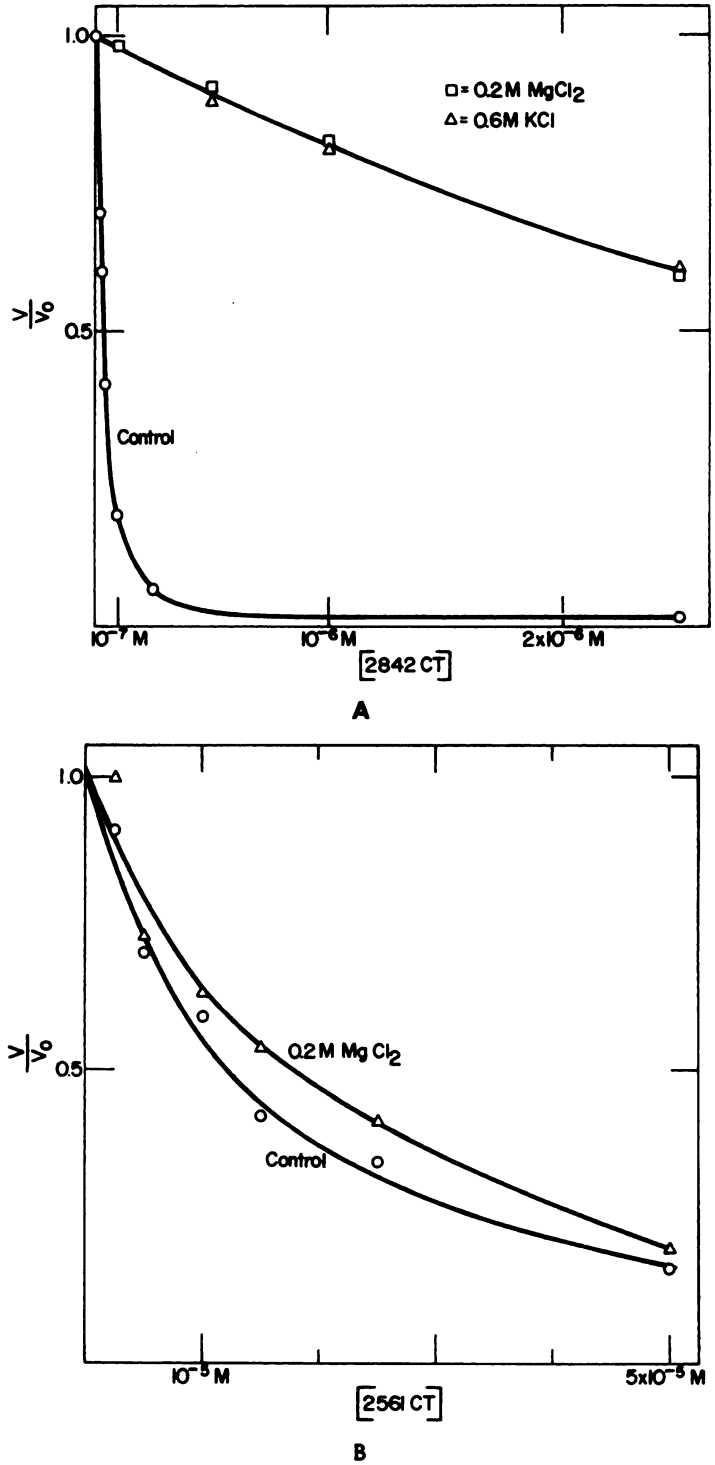


FIG. 5. *Effect of ionic strength on the inhibition of AChE by 2842CT and 2561CT*

Activities are plotted as a function of inhibitor concentration. Standard assay (2.5×10^{-2} M ACh) at low ionic strength for the control; in the presence of 2×10^{-1} M $MgCl_2$ and 6×10^{-1} M KCl as indicated.

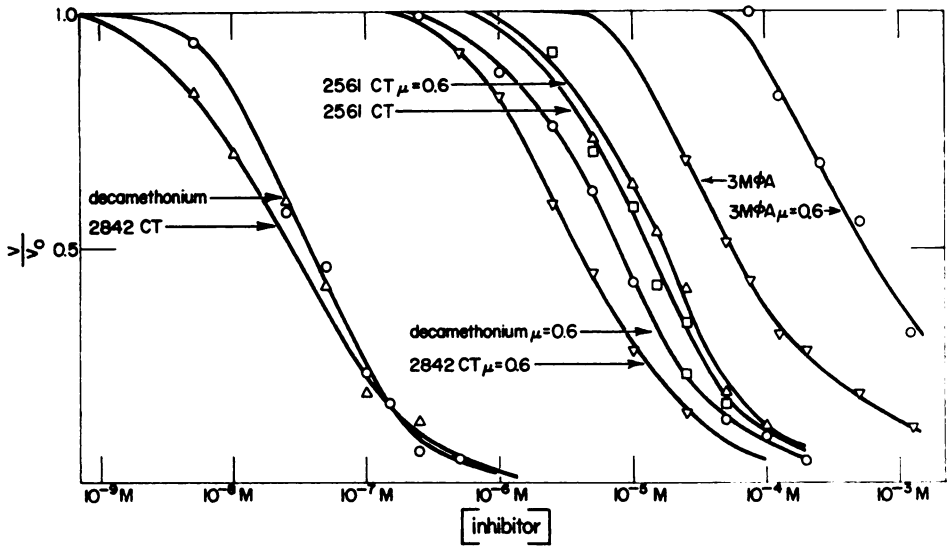


FIG. 6. *Effect of* 2×10^{-1} M *$MgCl_2$ on the inhibition of AChE by various reversible competitive inhibitors*

Activities are plotted as a function of the logarithm of the inhibitor concentration. Standard assay containing 2.5×10^{-3} M ACh in duplicate for each compound: at low ionic strength and in the presence of 2×10^{-1} M $MgCl_2$ (indicated by $\mu = 0.6$). Each curve is standardized to the same initial rate measured in the absence of inhibitor.

ionic strength, when the data are normalized to the same initial rate measured in the absence of inhibitor, again indicate that the salt effects observed are dependent mostly on the ionic strength, not on the nature of the salt.

Comparison of inhibition by mono- and bis-quaternary compounds. It has been well established by Tazieff-Depierre and coworkers (39, 41–44) and by others (for references, see 37) that, at a given ionic strength the affinity of AChE for competitive inhibitors with two quarternary ammonium ions exceeds its affinity for monoquaternary compounds. We have extended the comparison of these categories of inhibitors over a wide range of ionic strength. As shown in Fig. 6 and Table 3, variation of ionic strength from 0.6 to 0.003 decreases the inhibition constant K_i of phenyltrimethylammonium (3MØA) by a factor of 45, while the K_i of a bis-quaternary compound, 2842CT, is decreased by a factor of 475. The difference in the behavior of mono- and bis-quaternary inhibitors is even more striking when 2842CT and 2983CT are compared, since these compounds are identical except for the presence of a second quaternary ammonium in the former. The ratio of the K_i's measured at high and low ionic strength is 475 with 2842CT and 35 with 2983CT (Table 3). The data presented in Table 3 and Figs. 5 and 6 for a variety of other inhibitors support the general conclusion that decreasing the ionic strength increases preferentially the affinity of the enzyme for bis-quaternary inhibitors–among them that of a typical leptocurare, decamethonium.

Comparative inhibition by phenyltrimethylammonium (3MØA) and (3-hydroxyphenyl)trimethylammonium (2561CT). Several investigators have reported that AChE exhibits higher affinity for the hydroxyl-containing mono-quaternary inhibitor 2561CT than for its nonhydroxylated parent compound, 3MØA (43–45). For this reason we have studied the relative effects of ionic strength on the inhibition of AChE by these compounds. As shown in Table 3, decreasing the ionic strength increases the affinity of AChE for 3MØA by a factor of 45 while the affinity for 2561CT is increased by a factor of only 6.5.

Discussion of the Effects of Salts

The dramatic effect of added salts on the sedimentation behavior of AChE suggests that, in addition to their obvious effects on electrostatic interactions, their influence on the *kinetic* properties may be attributable in part to alterations of the enzyme structure. At present it is uncertain whether the changes that affect the reactivity of the enzyme toward its substrates and inhibitors can be identified with the conformational alterations responsible for the formation or dissociation of supermolecular aggregates. The putative ionic strength dependent structural changes in the catalytic unit may nevertheless be invoked for the interpretation of the selective enhancement of AChE inhibition by bis-quaternary versus mono-quaternary compounds or 3-hydroxyphenyl trimethylammonium versus its nonhydroxyl analog when the ionic strength is decreased. In an extensive study, the effect of ionic strength on the affinity of each charged group of the inhibitor for its complementary group on the enzyme, as well as the effect of ionic strength on the structure and the conformation of the inhibitor molecule should also be considered.

Effects of Pachycurares on the Kinetic Properties and Thermostability of AChE

Inhibition of AChE by Pachycurares

The foregoing results prompted an investigation under conditions of low ionic strength of the effects on AChE of certain neuromuscular blocking agents, the pachycurares, which had been classed as weak competitive inhibitors of the enzyme on the basis of conventional assay techniques (13, 28, 29, 37).

The primary evidence for the strong binding of pachycurares by the AChE molecule is their actual inhibition of its catalytic activity in assay media of low ionic strength. Figure 7 shows the kinetics of the inhibition of AChE by flaxedil. Insofar as the inhibition of AChE by flaxedil can be reversed by an excess of substrate, the inhibition by flaxedil is competitive (29). However, the graphs of enzymic activity measured at given substrate concentration as a function of increasing concentrations of flaxedil do not extrapolate to zero at infinite drug concentration as with competitive inhibitors, but lead to plateaus, The simple interpretation of this partial inhibition in terms of a heterogeneous molecular population does not account for the following observations.

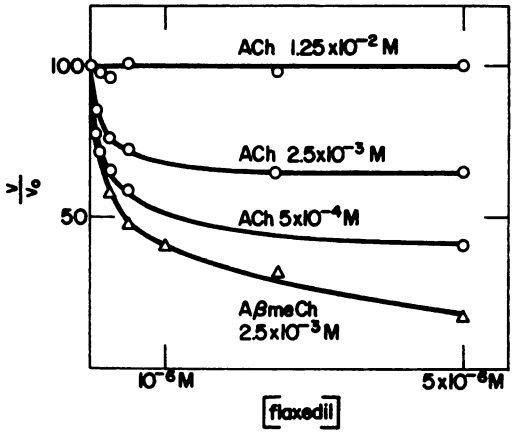
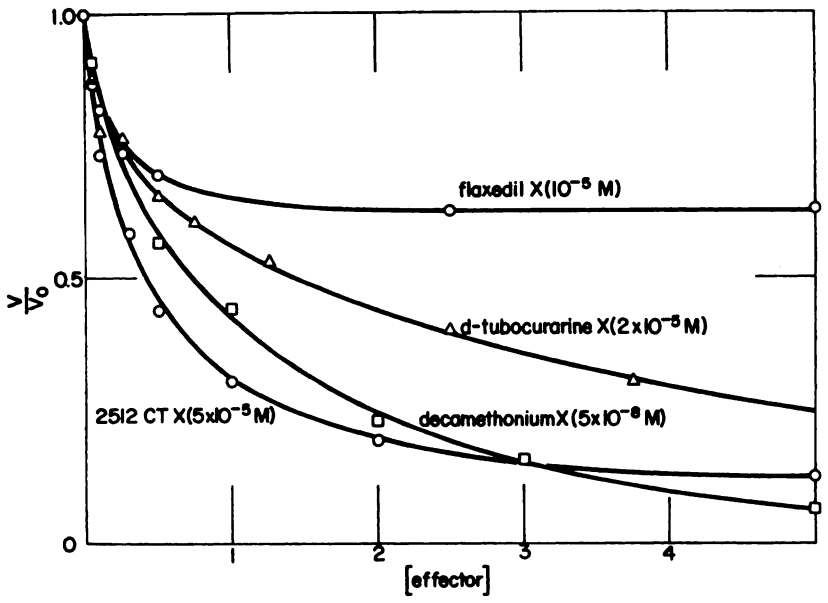


FIG. 7. *Inhibition of AChE by flaxedil at low ionic strength*

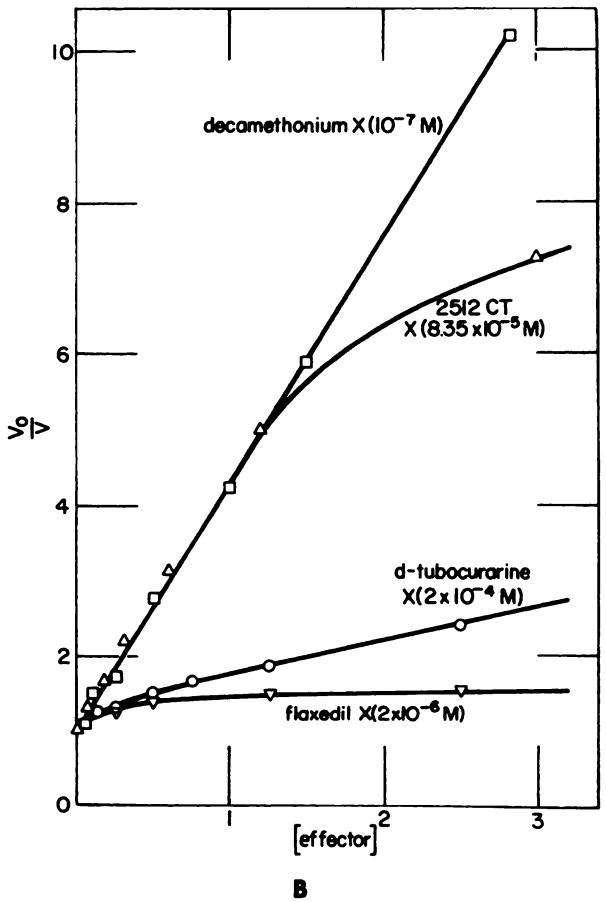
Activities are plotted as a function of flaxedil concentrations at three concentrations of ACh and one of acetyl-β-methylcholine (AβMeCh). Concentrations are indicated on the figure. All the curves are standardized to the same initial rate measured in the absence of flaxedil. Standard assay at low ionic strength.

1. The plateau values measured in the presence of high levels of flaxedil vary with the substrate concentration.

2. With acetyl-β-methylcholine as the substrate the plateau value observed at high levels of flaxedil is significantly different from that measured under the same conditions with ACh as the substrate. It is thus apparent that the inhibition of AChE by flaxedil does not follow the classical laws of simple competitive inhibition but resembles that observed for some regulatory enzymes and their natural feedback inhibitors (4, 46, 47). Following the nomenclature of Dixon and Webb (48), such inhibition is called "partially competitive inhibition"—which implies that the substrate and the inhibitor may be simultaneously bound by the enzyme.



A



B

FIG. 8. *Comparative inhibition of AChE by various curarizing drugs at low ionic strength*

(A and B) Activities or their reciprocal are plotted as a function of curarizing drug concentration. For each drug the scale of concentration is different as indicated on the figures. Standard assay at low ionic strength. 2.5×10^{-3} M ACh.

While similar but less pronounced nonclassical behavior is observed with *d*-tubocurarine, the kinetics of the inhibition by 2512CT (an analog of flaxedil) and decamethonium, which have limited or no pachycurare activity *in vivo* (25, 49, 50) follow classical competitive kinetics (Fig. 8). The partially competitive behavior of the drugs assayed can thus be correlated with their *in vivo* pachycurare activity (Table 1).

Antagonism between Bis-quaternary Inhibitors and Pachycurares

Further evidence for the binding of pachycurares to AChE is the observation

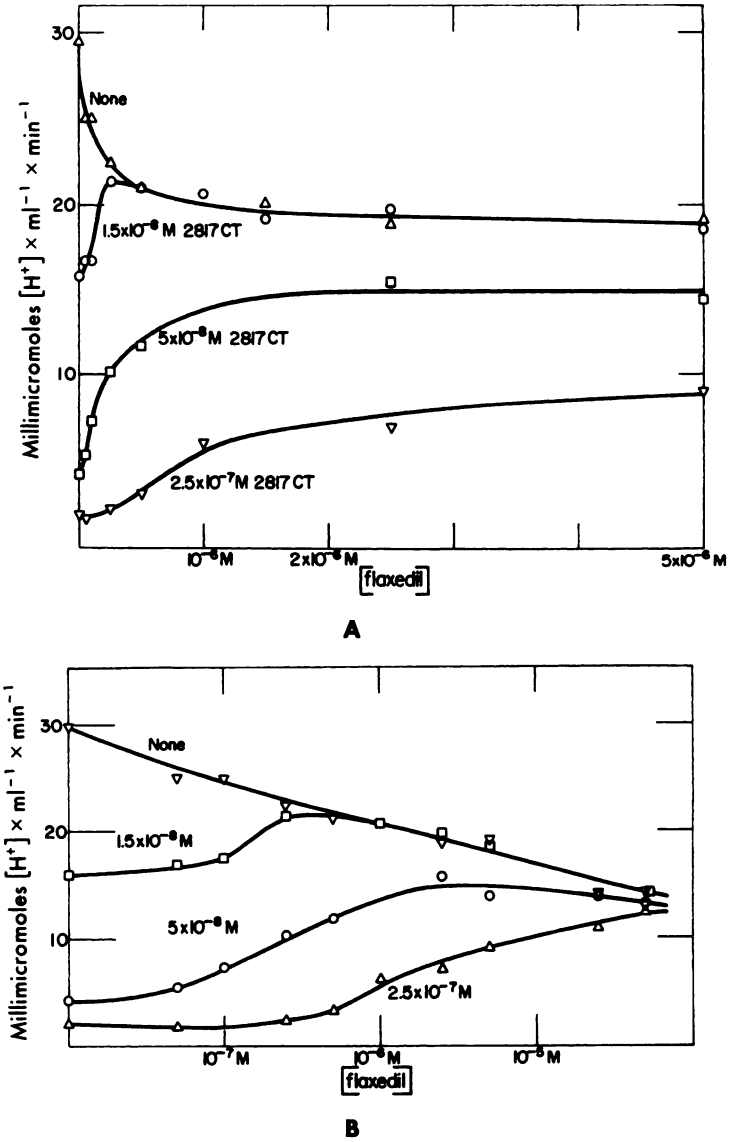


FIG. 9. *Effect of flaxedil on the inhibition of AChE by 2817CT*

(A) Activities are plotted as a function of increased flaxedil concentrations at three concentrations of 2817CT as indicated on the figure. (B) In abscissa, the logarithm of the flaxedil concentrations. Standard assay at low ionic strength, 2.5×10^{-5} M ACh.

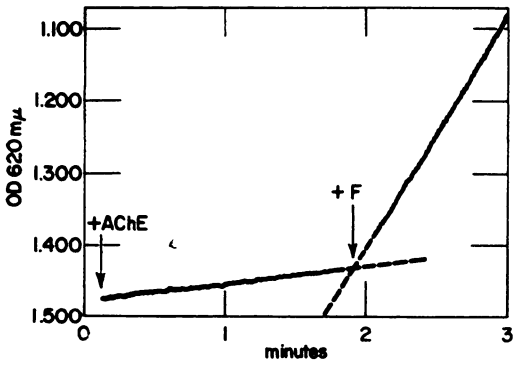


FIG. 10. *Kinetics of the reactivation by flaxedil of the AChE inhibited by 2817CT*

The arrow under *+AChE* indicates the time of addition of 5 μl of a dialyzed preparation of AChE (specific activity 4.4 moles ACh/hr, 3.6 mg protein/ml) to the standard assay medium of low ionic strength containing 2.5×10^{-3} M ACh and 2.5×10^{-1} M 2817CT. The arrow under *+F* indicates the time of addition of 1 μl of 10^{-1} M flaxedil (final concentration 5×10^{-5} M) to 2.0 ml of the reaction mixture.

that, at low ionic strength, in the presence of substrate, flaxedil and *d*-tubocurarine overcome the inhibition of the enzyme by bis-quaternary compounds. Figures 9-12 show that, at fixed concentrations of 2817CT and ACh, increased concentrations of flaxedil or *d*-tubocurarine reactivate the AChE in the range of concentration for which flaxedil or *d*-tubocurarine alone produce only partial inhibition. It should be emphasized at this point that this "reactivation" differs substantially from the phenomena described with irreversibly inhibited AChE.[5] With neither of the bis-quaternary inhibitors does the action of flaxedil exhibit a time dependence within the limits of accuracy of the technique used (Fig. 10), and the effects observed are immediately reversible.

[5] For example, reactivation by pyridine-2-aldoxime methiodide of diisopropylfluorophosphate-inhibited AChE.

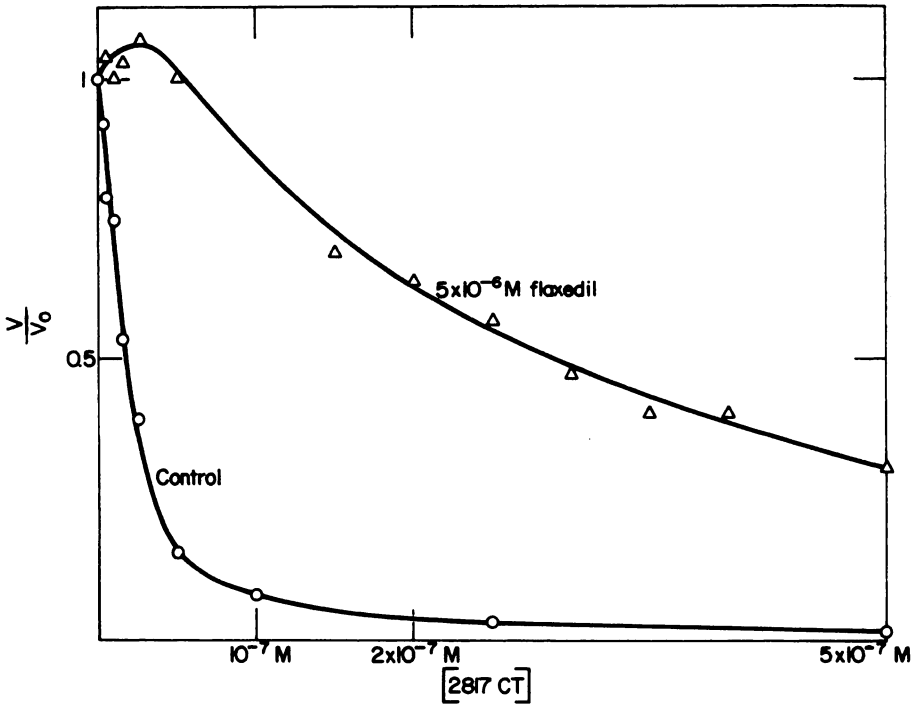


FIG. 11. *Effect of flaxedil on the inhibition of AChE by 2817CT*

Activities are plotted as a function of increased 2817CT concentrations. Standard assay at low ionic strength. 2.5×10^{-3} M ACh.

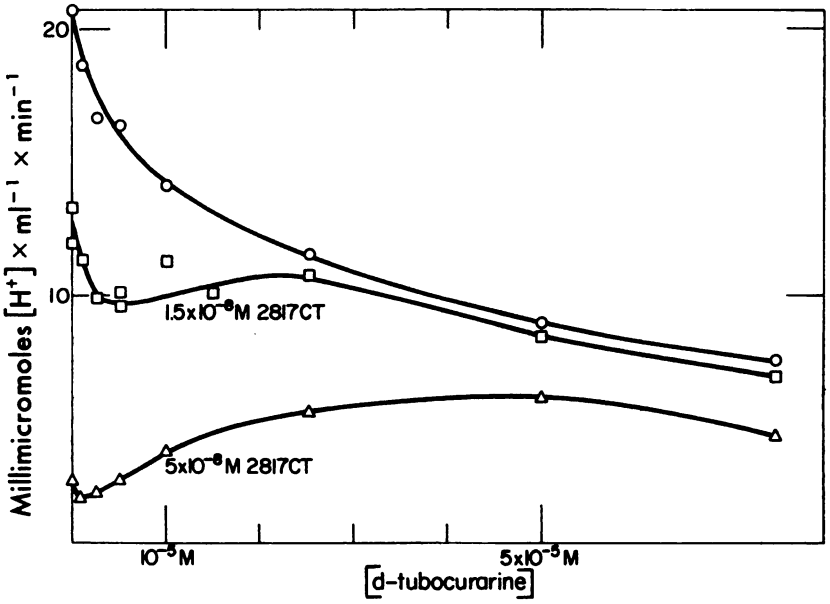


FIG. 12. *Effect of d-tubocurarine on the inhibition of AChE by 2817CT*
Same conditions as for Fig. 11.

The observation of the antagonism between pachycurares and bis-quaternary inhibitors depends on:

1. The ionic strength of the assay medium: In order to detect antagonism between bis-quaternary inhibitors and flaxedil or *d*-tubocurarine, the assay must be conducted in a medium of low ionic strength. In the presence of 2×10^{-1} M $MgCl_2$, the antagonism can be demonstrated only at

TABLE 4
Effects of flaxedil, 2817CT, and $MgCl_2$ on the activity of AChE from various sources

	Salt added	Control	2817CT, 2.5×10^{-7} M	Flaxedil, 5×10^{-5} M	2.5×10^{-7} M 2817CT, 5×10^{-5} M Flaxedil
Acetylcholinesterase	—	100[a]	7	48	42
Electric organs of *Torpedo marmorata*	0.2 M $MgCl_2$	210	143	—	—
Acetylcholinesterase	—	100	2.35	64	26.5
Electric organs of *Electrophorus electricus*	0.2 M $MgCl_2$	191	129	—	—
Acetylcholinesterase	—	100	8.2	109	32
Beef erythrocytes	0.2 M $MgCl_2$	172	150	—	—
Cholinesterase	—	100	35	15	3.65
Human plasma	0.2 M $MgCl_2$	34.5	29	—	—

[a] Numbers are percentages of specific activity relative to that of the control. Except for the *T. marmorata* enzyme, commercial preparations were used. Standard assay 2.5×10^{-1} M ACh.

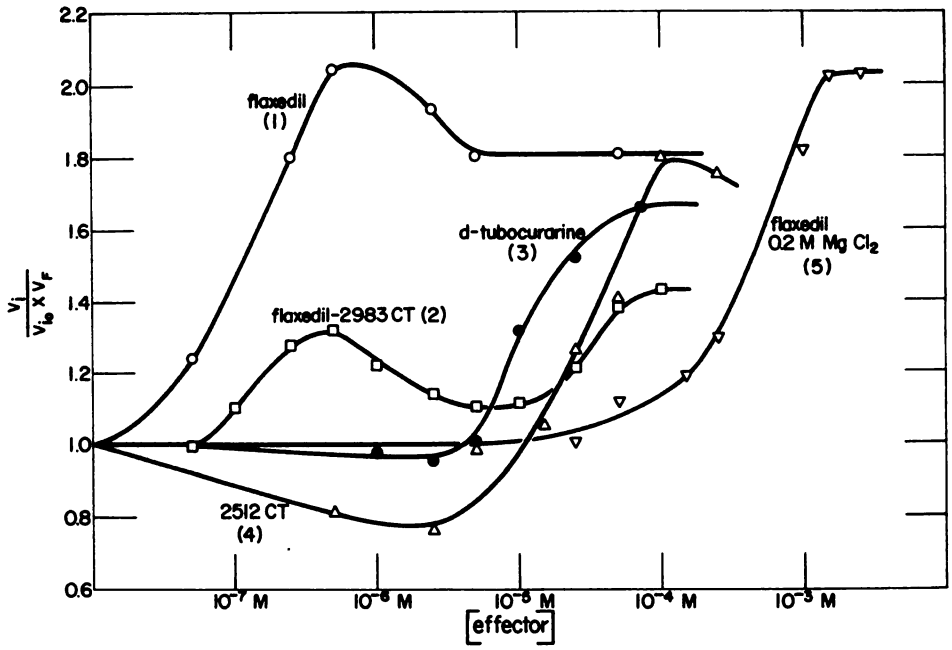


FIG. 13. *Antagonism between several ligands of AChE*

v_F is the activity in the presence of flaxedil and in the absence of inhibitor; v_{i_0}, the activity in the presence of inhibitor and in the absence of flaxedil; v_i, the activity in the presence of both flaxedil and inhibitor. The ratio $(v_i)/(v_{i_0} \times v_F)$ expresses the fractional inhibition of the enzyme by the inhibitor at various concentrations of flaxedil, standardized to the same fractional inhibition in the absence of flaxedil. All curves except (*5*) are based on the standard assay at low ionic strength; curve (*5*) is based on assays in the presence of 2×10^{-1} M $MgCl_2$. For curves (*1*), (*2*), and (*5*) the effector is flaxedil, for (*3*), *d*-tubocurarine, and for (*4*), 2512CT. The concentration of inhibitor used in each experiment corresponds to about 50% inhibition in the absence of antagonist. (*1*) 1.5×10^{-6} M 2817CT: 46% inhibition; (*2*) 5×10^{-5} M 2817CT: 47.5% inhibition; (*3*) 1.5×10^{-5} M 2817CT: 40% inhibition; (*4*) 1.5×10^{-5} M 2817CT: 47.5% inhibition; (*5*) 5×10^{-7} M 2817CT: 46% inhibition. In abscissa, the logarithm of effector concentrations. 2.5×10^{-3} M ACh.

concentrations of flaxedil about 1000 times greater than those used at low ionic strength (Fig. 13).

2. The source of the enzyme: Enzymes derived from the following sources in addition to *T. marmorata* have been tested for antagonism between 2817CT and flaxedil: AChE from electric organs of *E. electricus* and from red cells of beef and cholinesterase from human serum (Table 4). Antagonism between 2817CT and flaxedil can be demonstrated with the three AChE tested, and it is still demonstrable in the most highly purified preparation of AChE from *T. marmorata* (specific activity 74 moles ACh/hr per gram of protein). With the pseudocholinesterase from serum, flaxedil manifests no antagonism of 2817CT but produces additive inhibition (Table 4). The ability of flaxedil to restore the activity of AChE inhibited by bis-quaternary reversible inhibitors thus appears to be a characteristic property of the AChE molecule.

3. The structure of the inhibitor: Flaxedil antagonizes any of the several bis-quaternary compounds tested and none of the mono-quaternary drugs (Table 3). This absolute specificity for the bis-quaternary inhibitors is well illustrated by the relative effect of flaxedil on the inhibition by two compounds already compared, 2983CT and 2842CT, which possess exactly the same formula except for the number of quaternary ammonium ions (Fig. 13 and Table

5). Flaxedil does not overcome the inhibition by 2983CT, which has a single quaternary nitrogen atom, but it is a strong antagonist of 2842CT, which has two.

TABLE 5
Effects of flaxedil on the inhibition of AChE by various reversible competitive inhibitors

	Control (I_{50}),[a] M	Flaxedil 5×10^{-5} M (I_{50} *F*),[b] M	$I_{50}F/I_{50}$
Decamethonium	3.5×10^{-8}	2.3×10^{-6}	64
2817CT	1.7×10^{-8}	1.5×10^{-6}	88
2785CT	7.0×10^{-8}	6.9×10^{-6}	99
2842CT	3.0×10^{-8}	3.0×10^{-6}	100
2983CT	1.1×10^{-5}	1.8×10^{-5}	1.6
3MØA	7.3×10^{-5}	7.3×10^{-5}	1.0
2561CT	1.1×10^{-5}	1.7×10^{-6}	0.15
2817CT + $MgCl_2$ 0.2 M	4.5×10^{-7}	4.6×10^{-7}	1.0

[a] I_{50} is the concentration of inhibitor in the presence of which the activity of the enzyme is 50% inhibited.

[b] I_{50} F is the value obtained when 5.0×10^{-5} M flaxedil is present in the assay medium. Standard assay at low ionic strength 2.5×10^{-3} M ACh.

4. The structure of the antagonist: Among four drugs assayed as antagonists of 2817CT two are known to have a strong curare-like action on striated muscle, flaxedil (49, 50) which contains three quaternary ammonium ions, and *d*-tubocurarine, which has two of them. The other two drugs which have limited curare-like action on striated muscle are 2512CT, an analog of flaxedil (49,50) and atropine, both containing a single quaternary ammonium ion. It is clear from the results shown in Fig. 13 and Table 6 that the capacity to antagonize the inhibition by 2817CT is related to the number of quaternary ammonium ions in the antagonizing molecule. Furthermore, of the few drugs tested, the two best antagonists which possess several quaternary ammonium ions coincide with the best *in vivo* pachycurares (Table 6).

TABLE 6
Specificity of the antagonism between some pachycurares and 2817CT

Antagonist added at 5×10^{-5} M	I_{50} A[a] M	I_{50} A/I_{50}[b]
Control	1.7×10^{-8}	1
Atropine	1.5×10^{-8}	1.1
2512CT	4.0×10^{-8}	2.4
d-Tubocurarine	1.3×10^{-7}	7.4
Flaxedil	1.5×10^{-6}	87

[a] (I_{50} A) is the concentration of 2817CT required for 50% inhibition in the presence of the antagonist.

[b] I_{50} is the concentration of 2817CT required for 50% inhibition in the absence of antagonist.

The kinetics of the antagonism between bis-quaternary inhibitors manifest the following unusual characteristics:

1. The antagonistic action of the various pachycurares is superimposed on their intrinsic inhibitory effects.

2. Several of the curves in Figs. 9, 11, and 12 are not hyperbolic as they would be if simple Henri and Michaelis kinetics were followed.

3. The curves drawn from measurements at limited concentrations of inhibitor in the presence of increasing concentrations of the pachycurares do not extrapolate in a regular plot to the corresponding curve drawn from data obtained in the absence of inhibitor (Figs. 9 and 12).

Cooperative Inhibition of AChE by Flaxedil and 2561CT

As mentioned above, flaxedil does not overcome the inhibition of AChE by reversible inhibition with a single quaternary ammonium ion. In addition it may increase the inhibition by some of them. For example, the levels of inhibition produced by flaxedil and phenyltrimethylammonium (3MØA) are purely additive, while inhibition by the 3-hydroxy derivative of the latter (2561CT) is strongly enhanced by the addition of flaxedil (Fig. 14 and Table 5).

Protection of AChE by Flaxedil against Thermal Inactivation

The preceding kinetic data demonstrate clearly that such pachycurares as flaxedil and *d*-tubocurarine establish specific complexes with the AChE molecule. Observa-

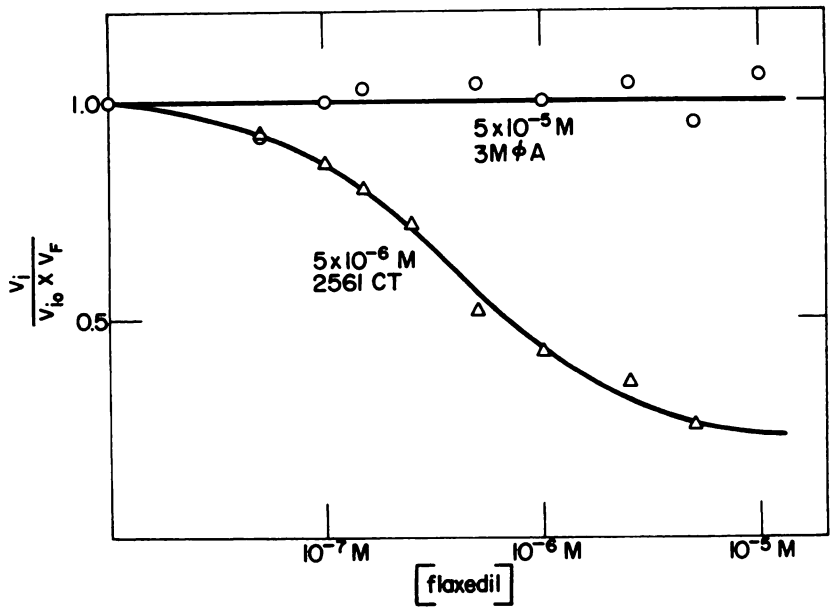


FIG. 14. *Comparative effects of flaxedil on the inhibition of AChE by phenytrimethylammonium (3MØA) and (3-hydroxyphenyl)trimethylammonium (2561CT)*

Data plotted as for Fig. 3. The abscissa gives the logarithm of effector concentrations. Standard assay at low ionic strength. 2.5×10^{-3} M ACh; 5×10^{-5} M 3MØA (55% inhibition in the absence of flaxedil); 5×10^{-6} M 2561CT (49% inhibition in the absence of flaxedil).

tions on the effect of flaxedil on the thermostability of the enzyme support this conclusion, and allow an indirect determination of the binding constant for flaxedil in the absence of substrate (51). As shown in Table 7 flaxedil effectively protects AChE against thermal inactivation at 35° at low salt concentrations. From the rates of inactivation measured in the presence of increasing amounts of flaxedil and plotted as shown on Fig. 15, a saturation curve of AChE by flaxedil may be drawn. The dissociation constant of the complex (AChE-flaxedil) is estimated to be at least 3×10^{-7} M at 35° under the conditions described in the legend of Fig. 15. Flaxedil thus binds to AChE with an affinity about 300 times greater than that of measured for ACh under the same conditions.[6]

TABLE 7
Protection of AChE by flaxedil against thermal inactivation[a]

Sample	0 minutes	5 minutes	10 minutes
Control	100[b]	55	13.5
Flaxedil 10^{-4} M	100	98	96

[a] Method is described in the legend of Fig. 15.
[b] Numbers are percentages of initial activity.

Conclusions on the Effects of Pachycurares

The enzymologic evidence presented in the preceding section indicates that both flaxedil and *d*-tubocurarine are strongly bound by the enzyme.

The singular partially competitive inhibition of AChE by the pachycurares, the reactivation by pachycurare of the enzyme inhibited by bis-quaternary compounds and

[6] This is a lower estimate; in Fig. 15, the fractional saturation of AChE by flaxedil is plotted as a function of the total and not of the free concentration of ligand. Because of the high affinity of AChE for flaxedil the free concentration of flaxedil could be significantly lower than the total concentration.

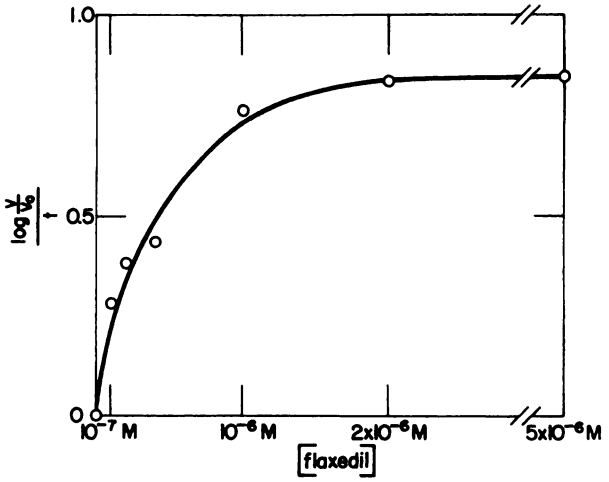


FIG. 15. *Protection of AChE against thermal inactivation as a function of flaxedil concentration*

A preparation of AChE of specific activity 4.4 moles ACh/hr per gram of protein containing 3.6 mg protein/ml dialyzed overnight against 10^{-3} M sodium Veronal pH 7.5 is diluted 1:10 with distilled water at 35°. Aliquots are sampled at given times and immediately frozen at −15°C. For each sample, the AChE activity is measured in the presence of 2×10^{-1} M $MgCl_2$ using the standard assay. The same experiment is performed at various concentrations of flaxedil in the inactivation medium. v_0 is the residual activity measured in the control after a given period of inactivation; v is the corresponding residual activity in the presence of the given concentration of flaxedil.

the stimulation by flaxedil of the inhibition of AChE in the presence of 2561CT, support the conclusion that the complex (enzyme-pachycurare) is active, i.e., that pachycurare and ACh may bind simultaneously to the enzyme. The binding locations of pachycurares could thus be, at least partly, topographically distinct from the active site of the enzyme.

DISCUSSION

Properties of the Receptors for the Lepto- and Pachycurares on AChE

The interference by the lepto- and pachycurares with the *in vitro* activity of AChE and the remarkable analogy of the observed antagonism between these two classes of "depolarizing" and "stabilizing" drugs at the level of AChE to their interaction at the neuromuscular junction (52, 53) tempt one to relate the effects on the isolated enzyme to the mechanism of ACh reception in the subsynaptic membrane. Although a role for AChE as the postsynaptic receptor of ACh *during* transmission, in addition to its function in destroying the chemical signal *after* passage of the impulse, was suggested by Roepke (5) about thirty years ago, the experimental data are still lacking for its definitive identification as the physiological receptor.

The requirements for such a "physiological receptor" include the ability to respond both to the chemical signal for synaptic transmission, i.e., ACh and to the neuromuscular blocking agents presumed, on the basis of *in vivo* experiments, to act at this level. The AChE molecule satisfies both requirements. The active site of AChE obviously constitutes a stereospecific binding area for ACh. The demonstrated affinity of AChE for the leptocurares and the pachycurares further indicates that the enzyme surface contains binding sites specific for these neuromuscular blocking agents.

The first receptors to be considered in detail are the leptocurare binding sites. To account for the high affinity of these bis-quaternary molecules for AChE, it is presumed that they interact multivalently with the enzyme. According to Nachman-

sohn and Wilson (55), part of each active site of AChE is differentiated into an anionic center. The binding of one of the quaternary ammonium ion of the leptocurares to this part of the active center would be consistent with a role of these drugs as competitive inhibitors of acetylcholinesterase activity. At least three postulates may be made concerning the origin of the additional anonic center(s) on the enzyme (37):

1. The two anionic centers involved in the binding of a bis-quaternary molecule are parts of active sites belonging to separate enzyme molecules held in proximity by the formation of a supermolecular aggregate.

2. The two anionic centers involved are parts of separate active sites which are located sufficiently close together on the surface of a single enzyme molecule.

3. Only one of the anionic centers to which the bis-quaternary inhibitor binds belongs to an active site, while the other is present in the neighborhood of this site (*peripheral anionic site*).

The first situation is eliminated by the observations that, under conditions of high ionic strength in which the slowly sedimenting form of the enzyme is present, AChE still exhibits higher affinity for bis- than for mono-quaternary inhibitors (Table 3) and the antagonism between bis-quaternary inhibitors and flaxedil can still be demonstrated (Fig. 13). In the absence of precise stoichiometric data, however, neither of the alternative arrangements of anionic sites may be ruled out.

The postulation of peripheral anionic centers, on the other hand, does not introduce serious geometrical restrictions, and offers an explanation for the existence of pachycurare receptors. Specifically, we infer from kinetic evidence, including the absence of competitive interactions between pachycurares and ACh, that the binding areas of the pachycurares are, at least partly, distinct from the active sites. The putative peripheral anionic centers would therefore provide the complementary structure for the binding of the pachycurares to the enzyme surface. A corollary to this model is that there are several peripheral anionic centers which are sufficiently close to bind simultaneously the several quaternary ammonium ions of a pachycurare molecule. These centers might also possess structures slightly different from those of the active sites, such that they can better accomodate the ethyl-substituted ammonium ions characteristic of the majority of the synthetic pachycurares (26) than the methyl-substituted quaternary ammonium ions found in the leptocurares. In addition, the peripheral anionic centers might serve as supplementary binding sites for ACh.

Extension of a Model for Allosteric Interactions to AChE

The conclusion that the regions of pachycurare binding are at least partly distinct from the catalytic sites of AChE suggests that the interactions between the compounds bound at these respective sites, such as flaxedil and ACh, might not be entirely direct (steric) interactions. It is tempting to postulate that, in addition, indirect (allosteric) interactions, mediated through conformational changes in the protein molecule, may intervene. To account for such effects observed in bacterial regulatory enzymes, a model has been proposed by Monod, Wyman, and Changeux (2). While it is premature to attempt a quantitative interpretation of the observations with AChE in terms of this mechanism, the adaptation of several pertinent statements of the model of Monod *et al.* (2) provides a convenient framework for discussion of the interactions among different ligands (heterotropic interactions) at the level of the enzyme:

1. The AChE molecule is postulated to exist spontaneously under at least two conformational states in reversible equilibrium, $P \rightleftharpoons D$.

2. These states differ with respect to their affinity toward ligands containing quaternary ammonium groups. The pachycurares, whose quaternary ammonium ions are substituted by ethyl or larger aliphatic chains, exhibit preferential affinity for one of the states designated as P. The leptocurares, whose quaternary ammonium ions

are substituted by methyl groups, exhibit preferential affinity for the other state, *D*. ACh binds preferentially, but not exclusively (56), to the same state *D* as the leptocurares (Fig. 16).

3. The maximal velocity of the AChE reaction, in particular the rate of hydrolysis of the acetyl enzyme, is greater for the *P* than for the *D* state.

4. No assumptions are made about the subunit (oligomeric) structure of the enzyme, which was an essential statement of the model of Monod *et al.*, because of the present lack of experimental evidence for allosteric interactions between identical ligands (homotropic interactions) in the case of AChE.

In the resting or polarized membrane AChE is predominantly in the *P* state, which binds ACh with low affinity. The binding of ACh therefore favors the transition of the *D* state, which corresponds to the depolarized membrane, and perturbs the membrane constraints in a certain domain. The fusion of such local perturbations might consequently shift the overall membrane structure into a depolarized state. Though such an amplification mechanism, the binding of ACh to AChE acting as a postsynaptic receptor might give rise to the triggering of a postsynaptic impulse.

The leptocurares, like the classical AChE inhibitors such as neostigmine and physo-

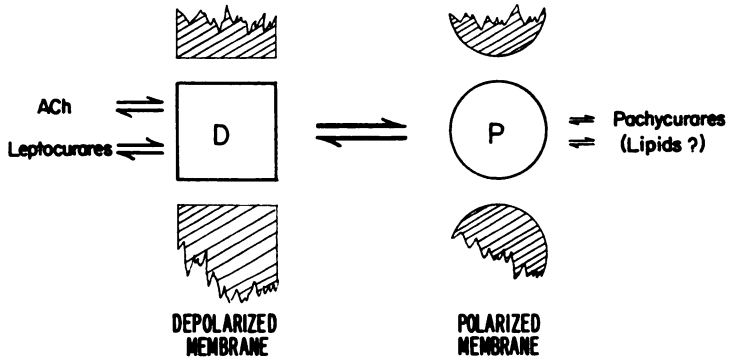


FIG. 16. *Extension of a model for allosteric interactions to AChE*
See text.

The *kinetic* properties of isolated AChE which may be interpreted in terms of this simple model include: the partially competitive inhibition by the pachycurares, the antagonism between lepto and pachycurares, and the inhibition by excess substrate (57).

To account for the postulated *physiological* function of AChE as a postsynaptic receptor of AChE, in addition to its cholinesterase function, one may further assume that: (1) Each state of the enzyme fits into a different structural state of the subsynaptic membrane to which it is integrated. (2) Reciprocal conformational constraints are established between the enzyme molecule and the neighboring protein molecules in the membrane (membrane constraints).

stigmine, could interfere with transmission by blocking the enzymic hydrolysis of ACh and thereby affecting its free concentration in the synaptic cleft. A more provocative interpretation is that the leptocurares, like ACh itself, stabilize the *D* state of AChE which corresponds to the depolarized membrane. The pachycurares, on the other hand, could prevent the triggering of postsynaptic events by binding preferentially to the *P* state of the molecule corresponding to the resting membrane (Fig. 16).

Finally, on the subject of the physiological implications of our results, one may wonder why the AChE molecule should contain on its surface differentiated areas, distinct from the active site, which are capable of binding such foreign drugs as

the pachycurares. Insofar as these peripheral receptors are specific for compounds possessing quaternary ammonium ions they could evidently serve as supplementary binding sites for ACh. A more intriguing explanation for their existence is based on the possible structural analogy between various curarizing agents and a class of naturally occurring compounds containing both quaternary ammonium ions and large hydrophobic residues, namely, the choline-containing lipids which are essential constituents of cell membranes (22). One may ask whether the existence of pachycurare binding areas on the several species of AChE tested is not directly related to the fact that all these AChE molecules (even that from erythrocytes) are membrane-bound enzymes and whether these areas may not coincide with the region of enzyme-membrane complex formation.

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