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Effects of Edrophonium, Eserine, Decamethonium, *d*-Tubocurarine, and Gallamine on the Kinetics of Membrane-Bound and Solubilized Eel Acetylcholinesterase

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SUMMARY

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The effects of edrophonium, eserine, decamethonium, *d*-tubocurarine, and gallamine on the kinetic parameters of membrane-bound and solubilized eel acetylcholinesterase were investigated. All assays were done using acetylcholine as substrate with a pH-stat in Ringer's solution. Edrophonium, an inhibitor of the anionic portion of the active site of acetylcholinesterase, was found to be a competitive inhibitor at a concentration of 0.1 μM and a mixed competitive-noncompetitive inhibitor at higher concentrations (1 and 10 μM) for both membrane-bound and solubilized acetylcholinesterase. Eserine (0.2–20 μM), a molecule which interacts with both the anionic and the esteratic portion of the active site of acetylcholinesterase, was a competitive inhibitor of the solubilized enzyme; inhibition by eserine of the membrane-bound enzyme was noncompetitive at 0.2 μM and mixed competitive-noncompetitive at higher concentrations of eserine (2 and 20 μM). The inhibition by decamethonium of both membrane-bound and solubilized acetylcholinesterase was noncompetitive at 3 and 100 μM decamethonium. *d*-Tubocurarine was found to affect the kinetics of both states of the enzyme in a mixed competitive-noncompetitive fashion. Gallamine, at concentrations as high as 100 μM, had no significant effect on either the K_m or V_{max} of membrane-bound or solubilized acetylcholinesterase. The effects of the membrane matrix on the interaction of these drugs with eel acetylcholinesterase are discussed.

INTRODUCTION

The properties of several membrane-bound enzymes are altered when they are extracted from their membrane environment (1–6). Such enzymes are referred to as allotopic enzymes (2). Acetylcholinesterase (acetylcholine acetyl-hydrolase, EC 3.1.1.7), a protein associated with cholinergic transmission, is one such enzyme. Silman and Karlin (7) have shown that in the absence of buffer, the pH vs. activity profiles of membrane-bound and solubilized eel electroplax acetylcholinesterase are different. Moreover, these two forms of the

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enzyme have been reported to differ in their K_m values, extent of inhibition by excess substrate, and their response to divalent cations (8, 9).

Various chemical agents have been reported to affect the kinetics of hydrolysis of acetylcholine by one or another of the forms of acetylcholinesterase (10–12), but there have been few studies comparing the effects of these agents on the kinetic parameters of membrane-bound and solubilized acetylcholinesterases from eel electroplax were thus investigated. The selection of these agents was guided by the following considerations. Edrophonium inhibits acetylcholinesterase by competing with the quaternary nitrogen moiety of the substrate acetylcholine for the anionic portions of the active site of the enzyme (13), thus providing a probe for this portion of the active site. Eserine was chosen because it interacts with both the anionic and esteratic portions of the active site of acetylcholinesterase (14); it thus provides a probe for the entire active site.

Decamethonium, *d*-tubocurarine, and gallamine are blockers of most cholinergic nicotinic synapses. In addition, each of these drugs has been shown to affect some of the kinetic parameters of acetylcholinesterase by binding to some peripheral site(s) on the enzyme (11, 12, 15–23). These sites have been proposed to be allosteric in nature (12, 15, 16). However, the previous studies were carried out using an enzyme obtained from such diverse sources as bovine erythrocytes, rat diaphragms, rat brain, and eel and *Torpedo* electroplax and the enzyme was assayed under widely varying conditions with respect to pH, ionic strength, temperature, and composition of assay medium. The properties of acetylcholinesterase have been found to depend both on its source and on the composition of the medium in which it is assayed (7, 9, 23–26). We thus felt that a comparative study of the effects of these drugs on membrane-bound and solubilized acetylcholinesterase obtained from the same source and assayed under identical conditions in a solution whose composition approximates that of eel extracellular fluid (27) would provide a clearer understanding of the importance of peripheral sites on the enzyme. The effects observed with these drugs are discussed in terms of possible differences in configuration between the active site of membrane-bound and solubilized acetylcholinesterase.

METHODS

Preparation of Membrane-Bound and Soluble Acetylcholinesterase

Membrane fragments from the electric organs of *Electrophorus electricus* were prepared according to the method of Karlin (28) as modified by Robaire and Kato (8). Sections of 2–5 cm were cut from the caudal end of the eel. The electric organ was isolated and cleaned of connective tissue, washed in a modified Ringer's solution (27) containing 180 mM NaCl, 5 mM KCl, 6 mM $CaCl_2$, and 1.5 mM $MgCl_2$, pH 7.20, blotted, and weighed.

The tissue was homogenized for 15 sec in the salt medium (4 ml/g of tissue) in a type OM Sorvall Omni-Mixer. The brei was then homogenized at 5700 rpm in a Potter-Elvehjem homogenizer by 10 up-and-down strokes of the pestle. The homogenate was filtered through Chrom-a-cord mesh (Travenol Laboratories) and then through a stainless steel sieve with openings of 96 μm (Newark Wire Company). The filtrate was rehomogenized in a Potter-Elvehjem homogenizer as above and then by three strokes of a tenBroeck tissue grinder; this suspension was centrifuged at 20,000 × *g* for 30 min in a Sorvall RC-2B refrigerated centrifuge. The pellet was washed three times with the above salt solution; the third wash had less than 1% of the acetylcholinesterase activity of the pellet. The pellet was resuspended in a volume of the salt solution equal to that of the original wet weight of the tissue; the suspension was stored at 4° for 48 hr and then centrifuged at 20,000 × *g* for 30 min. The pellet was resuspended in the Ringer's solution described above. This suspension is referred to as the membrane-bound enzyme preparation. Its supernatant was centrifuged at 100,000 × *g* for 2 hr, and the enzyme activity in the resulting nondialyzed supernatant is called solubilized acetylcholinester-

ase. The ratio of solubilized to solubilized plus membrane-bound acetylcholinesterase activity, or percentage solubilization, reached 50% after storage of the membrane preparation and remained constant for an additional 7 days (8).

Electron Microscopy

The 20,000 × *g* 30-min pellet obtained after the third wash of the membrane preparation was fixed for 3 hr at room temperature in 0.1 M phosphate buffer containing 3% glutaraldehyde, rinsed overnight with 0.1 M phosphate, and postfixed for 1 hr in 0.1 M phosphate buffer containing 2% OsO_4. It was then dehydrated with ethanol and washed with propylene oxide–Epon solutions containing increasing concentrations of Epon. After the last Epon wash (100%), the pellets were kept for 24 hr at 60°. Sections of 600–800 A were prepared using an MTI ultramicrotone (Porter-Blum), placed on copper grids, stained first for 2 min with 2% uranyl acetate, rinsed with water, stained for 10 min with lead nitrate, and then rinsed again with water. The sections were visualized and photographed on a Philips 300 electron microscope.

Examination of the electron micrographs of the membrane fragments revealed that the majority (90%) of these fragments were unsealed, although a small proportion (10%) of vesicular structures of 0.1-μm diameter was also observed (29). There was no evidence of presynaptic structures in the electron micrographs.

Sucrose Gradient Centrifugation

Sucrose gradients (8–17% sucrose, pH 7.2, 5.0 ml) were prepared with a Beckman density gradient former at room temperature and stored overnight at 4°. The concentrations of NaCl used in each gradient are specified in the legend to Fig. 1. A 50-μl cushion of 50% sucrose, pH 7.2, containing the appropriate NaCl concentration was layered at the bottom of each tube. The total volume of the sample applied to each gradient was 200 μl, of which 160 μl were concentrated solubilized acetylcholinesterase, 10 μl were catalase (10 mg/ml) from bovine liver (Sigma), and 30 μl were β-galactosidase suspension (grade IV, Sigma). The gradients were centrifuged for 7 hr at 290,000 × *g* (49,000 rpm) in a Beckman L2-65B ultracentrifuge using an SW 50.1 rotor. After centrifugation, the tubes were perforated at the bottom and fractions of 10 drops (approximately 100 μl) were collected.

β-Galactosidase was assayed in a medium containing 0.05 M Tris and 0.05 M NaCl, pH 7.6, using 1.5 mM *O*-nitrophenyl-β-D-galactopyranoside as substrate. β-Galactosidase activity was measured by determining the increase in optical density at 405 nm with time. Catalase was assayed in a medium containing 0.05 M Tris–0.05 M NaCl, pH 7.6, and enough H_2O_2 to give an optical density at 240 nm of 0.6 to 0.7 (approximately 5 μl of 30% H_2O_2 per milliliter of assay medium). Catalase activity was measured by determining the decrease in optical density at 240 nm with time.

Protein concentration was determined by the method of Lowry *et al.* (30), and acetylcholinesterase activity was measured according to Ellman *et al.* (31).

Assay of Acetylcholinesterase Activity

Acetylcholinesterase was assayed with a pH-stat assembly (Radiometer). The substrate used was acetylcholine iodide (Sigma). The assays were done at 30° under a nitrogen atmosphere in 1 ml of Ringer's solution containing 180 mM NaCl, 5 mM KCl, 6 mM $CaCl_2$, 1.5 mM $MgCl_2$, and 2.0 mM phosphate buffer, pH 7.20. The acetic acid formed was neutralized with 0.010 N NaOH. The electrodes were washed in 0.1 N HCl for 1 min between assays to prevent accumulation of acetylcholinesterase activity on the outer glass surface of the electrode.

Studies of the effects of drugs on acetylcholinesterase were done by adding the enzyme to a solution containing both substrate and drug (i.e., the enzyme was not previously incubated with the drug). Drugs were obtained from the following sources: gallamine triethiodide (Flaxedil), Poulenc, Ltd.; *d*-tubocurarine, Burroughs Wellcome and Company; decamethonium,

K & K laboratories, Inc.; edrophonium chloride, Hoffmann–La Roche; eserine sulfate, Nutritional Biochemicals Corporation.

Analysis of Kinetic Data

The apparent affinity of acetylcholine for acetylcholinesterase (K_m) and the maximal velocity of hydrolysis (V_{max}) were calculated from the initial velocities. Since estimates of the reliability of these values were desired and since all linear transformations of the Michaelis-Menten rate equation have certain drawbacks (32–35), it was decided to use a method first described by Wilkinson (36) to analyze the kinetic data. This method consists of fitting the data to a rectangular hyberbola using the computer analysis of Cleland (37). As suggested by Cleland (37), rates at concentrations of substrate higher than those at which the maximal rate is observed were not utilized.

RESULTS

Properties of Solubilized Acetylcholinesterase

Sucrose gradient centrifugation. When solubilized acetylcholinesterase is centrifuged on a sucrose gradient in the presence of 1.0 M NaCl (Fig. 1A) two peaks of acetylcholinesterase activity, having sedimentation coefficients of 11.4 S ± 0.2 and 7.4 S ± 0.1, are found. The two peaks contain approximately equal amounts of enzyme activity. A very similar pattern is observed when the NaCl concentrations are reduced to 0.1 M (Fig. 1B). When sucrose gradients are centrifuged in the absence of added NaCl, three peaks of activity are found, with values of 7.4, 11.0, and 12.8 S (Fig. 1C).

Kinetic properties of 11.4 S and 7.7 S acetylcholinesterase. The rate of hydrolysis of acetylcholine (50 μM–20 mM) was determined for the two forms of acetylcholinesterase by the pH-stat method. The K_m values for acetylcholine were obtained by fitting the results to a rectangular hyperbola, as described under METHODS. Percentage substrate inhibition has been previously defined as $(rate_2 - rate_{20})/rate_2$, where the subscripts 2 and 20 are the millimolar substrate concentrations (8). It is clear from Table 1 that, for the solubilized acetylcholinesterase and its two components, 11.4 S and 7.7 S, neither the K_m nor the extent of inhibition by excess substrate is significantly different.

Effects of Edrophonium

The effects of edrophonium on the hydrolysis of increasing concentrations of acetylcholine by both membrane-bound and solubilized acetylcholinesterase are shown in Fig. 2A and B, respectively. Increasing concentrations of edrophonium inhibit both states of the enzyme to apparently similar extents. This inhibition of acetylcholinesterase by edrophonium was quantitated by analyzing the results shown in Fig. 2 using the computer program of Cleland (see METHODS). The results of this analysis are presented in Table 2.

Three features of the inhibition of acetylcholinesterase are prominent. First, in the presence of 0.1 μM edrophonium, the V_{max} of neither the membrane-bound nor the solubilized enzyme is affected, while their K_m values are doubled; this indicates a competitive type of inhibition. Second, in the presence of high concentrations of edrophonium, there is a decrease in V_{max} in addition to the increase in K_m of both membrane-bound and solubilized acetylcholinesterase; this indicates that at the higher concentrations edrophonium acts as a mixed competitive-noncompetitive inhibitor. Third, there is no significant difference between the effects of edrophonium (at the concentrations studied) on membrane-bound and solubilized acetylcholinesterase.

Effects of Eserine

The rates of hydrolysis of acetylcholine by membrane-bound and solubilized acetylcholinesterase in the presence of increasing concentrations of eserine are shown in Fig. 3. There is no change in the V_{max} of the solubilized enzyme, while that of the membrane-bound enzyme decreases with increasing concentrations of eserine. This is demonstrated more clearly in Table 2, which summarizes the computer analysis

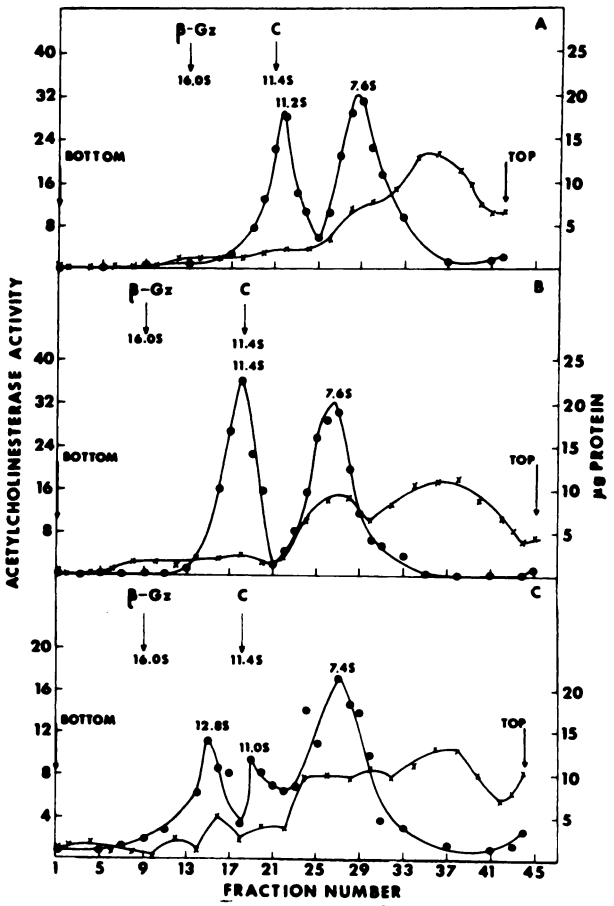

FIG. 1. *Sucrose gradient centrifugation profile of acetylcholinesterase solubilized from electroplax of Electrophorus electricus.*

All gradients were prepared as described in METHODS and were centrifuged in an SW 50.1 rotor at 49,000 rpm (290,000 × *g*). The NaCl concentrations used for each gradient were: A, 1.0 M NaCl; B, 0.10 M NaCl; C, no NaCl. β-Galactosidase(β-Gz) and catalase (C) were used as markers. Acetylcholinesterase activity (●——●) was assayed using the method of Ellman *et al.* (31), and protein concentration (x——x) was assayed according to Lowry *et al.* (30).

TABLE 1

K_m and percentage inhibition by excess substrate of solubilized acetylcholinesterase and its 11.4 S and 7.7 S components

K_m values and standard errors were obtained using Cleland's program (37) (see the text). Percentage inhibition by excess substrate is defined in the text.

Enzyme	K_m	Substrate inhibition
	μM	%
Mixture*	186 ± 24	32.2
11.4 S	166 ± 23	33.6
7.4 S	168 ± 18	32.4

* Mixture refers to the solubilized enzyme before separation on sucrose gradients.

of the data shown in Fig. 3. A low concentration of eserine, 0.2 μM (0.1 μM eserine sulfate), decreases the V_{max} of membrane-bound acetylcholinesterase by 28% and doubles the K_m of acetylcholine for the solubilized enzyme. Neither the V_{max} of solubilized acetylcholinesterase nor the K_m of acetylcholine for membrane-bound acetylcholinesterase is significantly altered at this concentration of eserine. Thus eserine at 0.2 μM is a competitive inhibitor of solubilized acetylcholinesterase and a noncompetitive inhibitor of the membrane-bound enzyme. Inhibition of the solubilized enzyme by higher concentrations of eserine (2 and 20 μM) remains competitive in na-

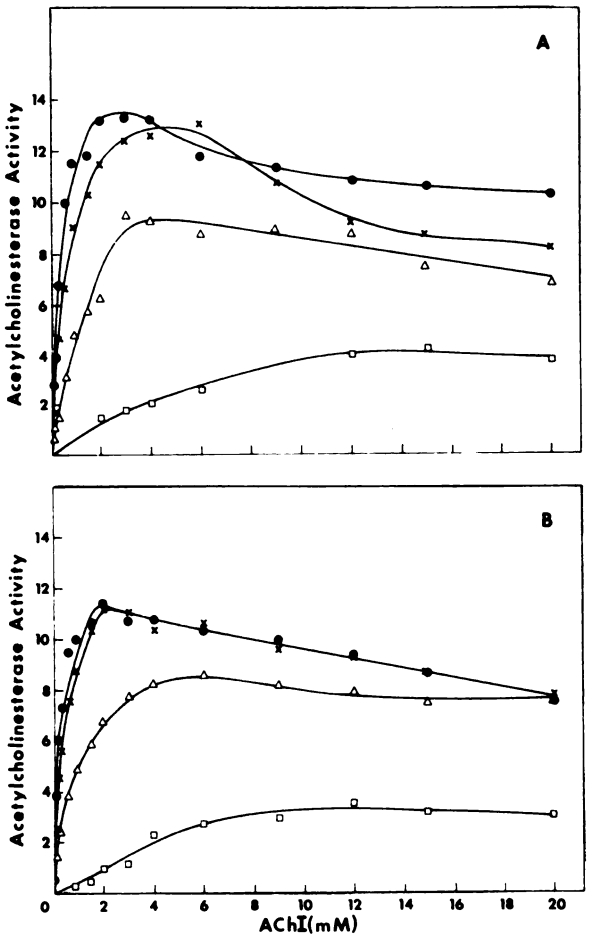

FIG. 2. *Effects of increasing concentrations of edrophonium on rate of hydrolysis of acetylcholine by soluble and membrane-bound acetylcholinesterase.*

Acetylcholinesterase activity is expressed in terms of micromoles of acid formed per minute per gram of tissue wet weight. The abscissa represents the concentration of substrate (acetylcholine iodide, AChI) used in each assay. All assays were done with a pH-stat in a solution containing 180 mM NaCl, 5 mM KCl, 6 mM $CaCl_2$, 1.5 mM $MgCl_2$, and 2 mM phosphate, pH 7.20. A. Rate of hydrolysis of acetylcholine by membrane-bound acetylcholinesterase. B. Rate of hydrolysis of acetylcholine by solubilized enzyme. Edrophonium concentrations: ●——●, none (control); X——X, 0.1 μM; △——△, 1 μM; □——□, 10 μM.

ture. At 20 μM eserine the V_{max} of solubilized acetylcholinesterase remains unaltered, while its K_m increases 8-fold. However, the inhibition of membrane-bound acetylcholinesterase by eserine at these higher concentrations is mixed competitive and noncompetitive; i.e., the V_{max} is decreased and the K_m of acetylcholine for the enzyme is increased.

Effects of Decamethonium

The inhibition of both membrane-bound and solubilized acetylcholinesterase by decamethonium is similar. From Fig. 4 it is apparent that the V_{max} of both states of the enzyme is decreased in the presence of 3 and 100 μM decamethonium. The computer analyses of these data (Table 2) reveal that decamethonium decreased the V_{max} of both membrane-bound and solubilized acetylcholinesterase while it does not significantly alter the K_m value for either state of the enzyme. These results indicate that decamethonium is a noncompetitive inhibitor of acetylcholinesterase.

TABLE 2

Effects of edrophonium, eserine, decamethonium, d-tubocurarine, and gallamine on V_{max} and K_m values of membrane-bound and solubilized acetylcholinesterase

Membrane-bound and solubilized acetylcholinesterase from *Electrophorus electricus* were prepared as described under METHODS. The assays were performed at 30°, under a nitrogen atmosphere, with a pH-stat in a medium containing 180 mM NaCl, 5 mM KCl, 6 mM $CaCl_2$, 1.5 mM $MgCl_2$, and 2 mM phosphate, pH 7.2, using acetylcholine iodide as substrate. V_{max} values are expressed as a percentage of the V_{max} in the absence of drug (control). Standard errors of V_{max} values and the mean K_m values and their standard errors were obtained by computer analysis according to Cleland (37).

Drug	V_{max}		K_m	
	Membrane-bound	Solubilized	Membrane-bound	Solubilized
	%	%	μM	μM
Control	100.0 ± 4.8	100.0 ± 2.1	444 ± 78	195 ± 20
Edrophonium				
0.1 μM	96.3 ± 4.8	106.9 ± 2.8	729 ± 122	406 ± 35
1 μM	78.6 ± 6.4	80.7 ± 2.1	1672 ± 208	832 ± 85
10 μM	34.2 ± 8.2	45.8 ± 9.0	5522 ± 878	8352 ± 1196
Eserine				
0.2 μM	71.7 ± 5.3	93.1 ± 6.5	585 ± 69	470 ± 45
2 μM	52.9 ± 2.1	109.9 ± 11.9	680 ± 88	758 ± 116
20 μM	40.1 ± 3.2	102.3 ± 11.1	2291 ± 376	1738 ± 169
Decamethonium				
0.1 μM	98.6 ± 4.9	103.4 ± 1.5	380 ± 59	174 ± 14
3 μM	76.1 ± 3.0	81.7 ± 6.2	351 ± 36	206 ± 56
100 μM	21.3 ± 2.3	23.3 ± 3.1	572 ± 166	249 ± 147
d-Tubocurarine				
0.1 μM	106.0 ± 4.5	93.6 ± 1.8	392 ± 52	156 ± 23
3 μM	107.0 ± 3.2	88.7 ± 1.3	351 ± 30	135 ± 12
100 μM	86.3 ± 3.4	71.0 ± 3.3	524 ± 57	359 ± 62
Gallamine				
0.1 μM	92.0 ± 3.3	96.1 ± 1.6	373 ± 26	204 ± 17
3 μM	91.7 ± 5.0	97.7 ± 3.0	313 ± 44	202 ± 29
100 μM	91.7 ± 5.3	98.2 ± 3.3	320 ± 68	278 ± 39

Effects of d-Tubocurarine

The effects of *d*-tubocurarine, over a concentration range of 0.1 to 100 μM, on the kinetics of hydrolysis of acetylcholine by membrane-bound and solubilized acetylcholinesterase are illustrated in Fig. 5. *d*-Tubocurarine appears to be a less potent inhibitor of the enzyme than is decamethonium. In the presence of 3 μM *d*-tubocurarine the only significant effect is a reduction by 10% of the V_{max} of solubilized acetylcholinesterase (Table 2). For both the membrane-bound and the solubilized enzyme in the presence of 100 μM *d*-tubocurarine there is a decrease in the V_{max} and an increase in the K_m of acetylcholine, sugesting a mixed competitive-noncompetitive type of inhibition at this concentration of *d*-tubocurarine.

Effects of Gallamine

The results of the studies in which the effects of gallamine on membrane-bound and solubilized acetylcholinesterase were investigated are shown in Fig. 6. The apparent lack of effect of this drug on the kinetics of acetylcholine hydrolysis by acetylcholinesterase was substantiated by the computer analyses of these data (Table 2). Table 2 clearly indicates that concentrations as high as 100 μM gallamine have no significant effects on the rate of hydrolysis of acetylcholine by either the membrane-bound or the solubilized enzyme.

DISCUSSION

A number of investigators (38–41) have established that the acetylcholinesterase activity found in the supernatant of the

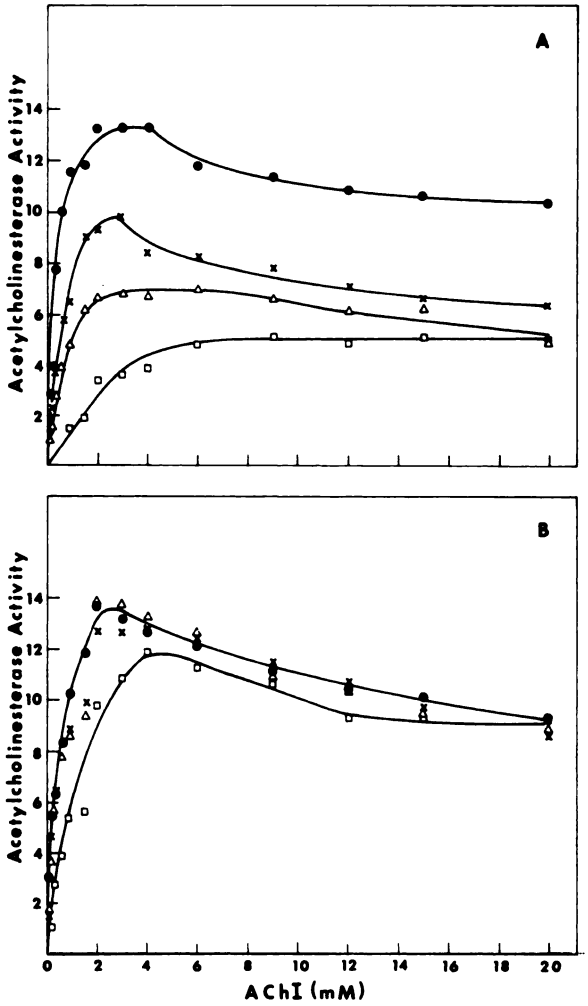

FIG. 3. *Effects of increasing concentrations of eserine on rate of hydrolysis of acetylcholine by soluble and membrane-bound acetylcholinesterase.*

The axes and the assay conditions were the same as those described in the legend to Fig. 2. A. Rate of hydrolysis of acetylcholine by membrane-bound acetylcholinesterase. B. Rate of hydrolysis of acetylcholine by solubilized enzyme. Eserine concentrations: ●——●, none (control); X——X, 0.2 μM; △——△, 2 μM; □—□, 20 μM.

homogenate of eel electroplax consists of three components having sedimentation coefficients of 18 S, 14 S, and 8 S. These three forms consist of clusters of three, two, and one tetramer, respectively. Electron micrographs illustrate that in each case the tetramer is attached to a rigid "tail" piece (42–44). The tetramer is an 11 S globular protein which can be dissociated into 7 S dimers by sonication (39–45).

The results presented above show that even though the solubilized enzyme was not sonicated, approximately 50% of the total acetylcholinesterase activity has a sedimentation coefficeint of 7.7 S. Furthermore, the ratio of the 7 S to the 11 S plus 7 S forms of the solubilized enzyme increases up to 80% after sonication for 90 sec.[3] These results indicate that acetylcholinesterase solubilized from electroplax membranes under gentle conditions sediments

[3] Unpublished observations.

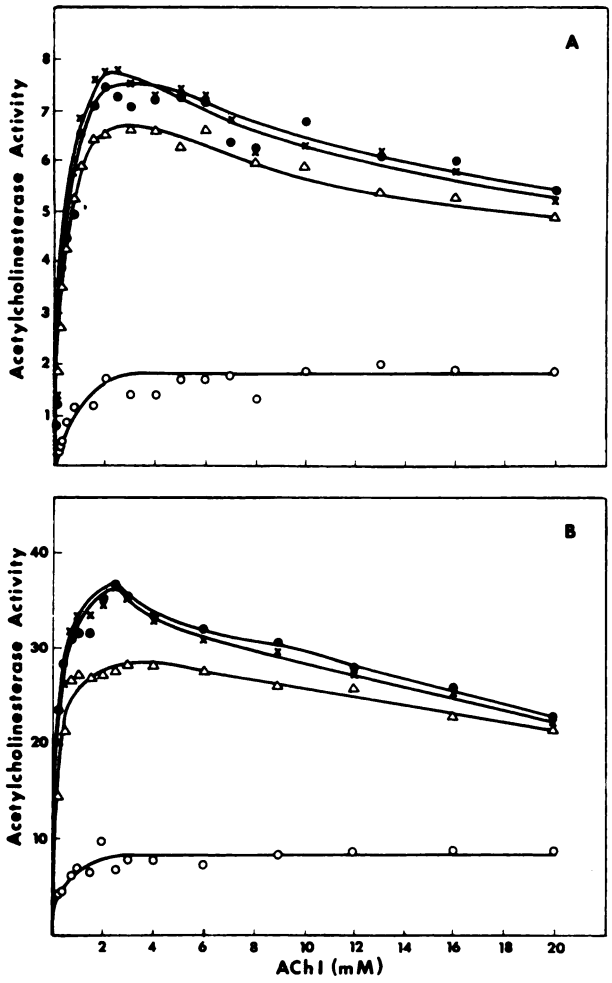

FIG. 4. *Effects of increasing concentrations of decamethonium on rate of hydrolysis of acetylcholine by soluble and membrane-bound acetylcholinesterase.*

The axes and the assay conditions were the same as those described in the legend to Fig. 2. A. Rate of hydrolysis of acetylcholine by membrane-bound acetylcholinesterase. B. Rate of hydrolysis of acetylcholine by solubilized enzyme. Decamethonium concentrations: ●——●, none (control); X——X, 0.1 μM; △——△, 3 μM; ○——○, 100 μM.

as though it did not contain the "tail" reported to be associated with the enzyme found in initial tissue homogenates (42, 43).

Millar *et al.* (45) reported that the dimeric form of acetylcholinesterase from eel electroplax was not inhibited by excess substrate and that its K_m (using acetylthiocholine as substrate) was twice that of the tetrameric form. However, the highest substrate concentration used was 1.1 mM acetylthiocholine, and only the K_m of the dimer was determined. Our results clearly indicate that the 11.4 S and 7.7 S forms of acetylcholinesterase have very similar K_m values are are inhibited to the same extent by excess substrate.

Millar has recently shown[4] that the differences between the dimeric and tetrameric forms of acetylcholinesterase were not observed when the substrate acetylcholine was used over a wider concentration range. Thus only the solubilized enzyme, a mixture of the 11.4 S and 7.7 S forms, was

[4] D. B. Millar, personal communication.

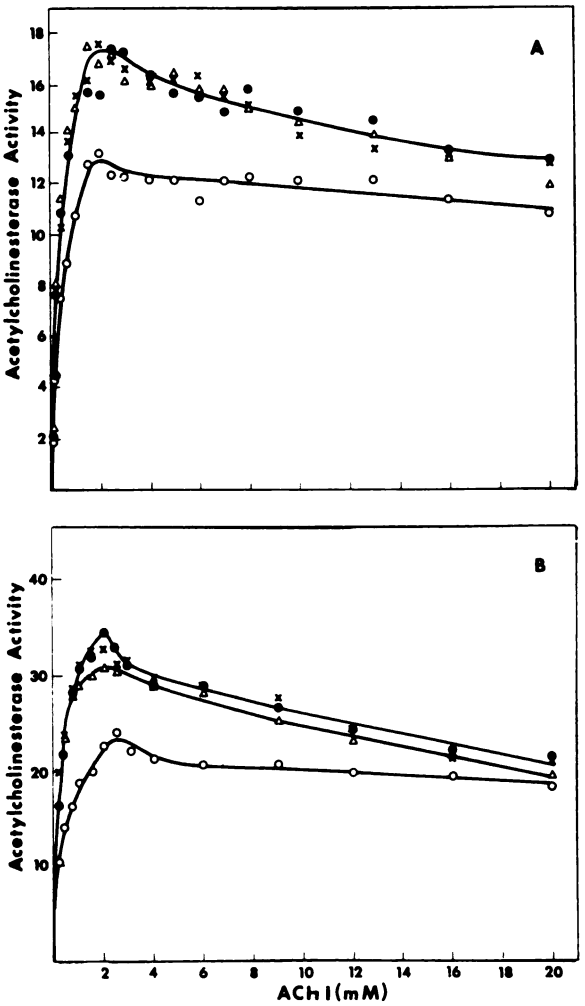

FIG. 5. *Effects of increasing concentrations of d-tubocurarine on rate of hydrolysis of acetylcholine by soluble and membrane-bound acetylcholinesterase.*

The axes and the assay conditions were the same as those described in the legend to Fig. 2. A. Rate of hydrolysis of acetylcholine by membrane-bound acetylcholinesterase. B. Rate of hydrolysis of acetylcholine by solubilized enzyme. *d*-Tubocurarine concentrations: ●——●, none (control); X——X, 0.1 μM; △——△, 3 μM; ○——○, 100 μM.

used to study the relative effects of drugs on membrane-bound and soluble acetylcholinesterase.

Edrophonium, a positively charged organic molecule, inhibits the hydrolysis of acetylcholine by acetylcholinesterase by competing with the substrate for the occupancy of the anionic portion of the active site of the enzyme (13). Our data on the effects of edrophonium on solubilized acetylcholinesterase are compatible with this proposal; furthermore, the finding that the effects of edrophonium on the membrane-bound and solubilized enzymes are similar suggests that the properties of the anionic portion of the active site of acetylcholinesterase, with respect to edrophonium binding, are unaffected by the membrane matrix.

It has been shown by Stein and Lewis (46) that the competitive inhibition of soluble acetylcholinesterase by eserine, first proposed by Krupka and Laidler (47), was observed only if the enzyme had not first

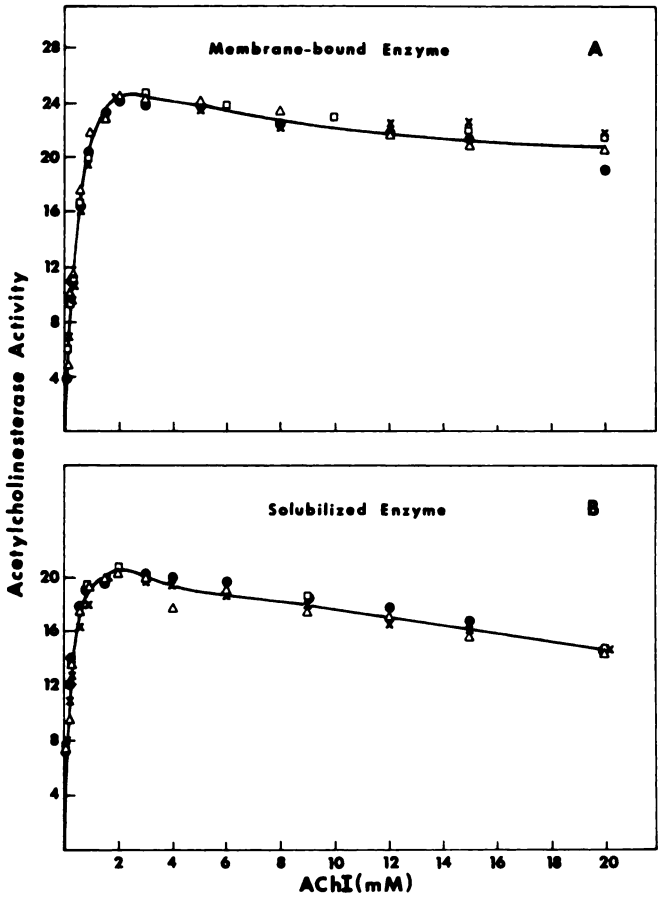

FIG. 6. *Effects of increasing concentrations of gallamine on rate of hydrolysis of acetylcholine by soluble and membrane-bound acetylcholinesterase.*

The axes and the assay conditions were the same as those described in the legend to Fig. 2. A. Rate of hydrolysis of acetylcholine by membrane-bound acetylcholinesterase. B. Rate of hydrolysis of acetylcholine by solubilized enzyme. Gallamine concentrations: ●——●, none (control); X——X, 0.1 μM; △——△, 3 μM; □——□, 100 μM.

been incubated with eserine; if, however, the enzyme was previously incubated with this drug for 6.8 or 18.9 min, the inhibition was gradually changed from the competitive to the noncompetitive type. The noncompetitive component of this inhibition is ascribed to the fact that eserine carbamylates the esteratic portion of the active site of acetylcholinesterase; the carbamylated form of the enzyme is unable to hydrolyze acetylcholine. Since under the conditions of our experiments acetylcholinesterase was not initially incubated with eserine, inhibition of the enzyme would be expected to be competitive. This was true for the solubilized enzyme; however, the inhibition of the membrane-bound enzyme was found to be noncompetitive. The simplest explanation of this apparent discrepancy is that the configuration of the active site of the membrane-bound enzyme is sufficiently different from that of the solubilized enzyme to allow, under our experimental conditions, the carbamylation of the enzyme to proceed fast enough so that when the initial rates of acetylcholine hydrolysis are measured the majority of the membrane-bound acetylcholinesterase is already carbamylated. The tertiary nature of eserine may also provide an explanation of the difference in its effects on membrane-bound and soluble acetylcholinester-

ase. Acetylcholine and the quaternary ammonium inhibitors gain access to the enzyme by diffusing through the layers separating the bulk aqueous medium from the active site. Eserine, however, might reach the sequestered enzyme more rapidly than acetylcholine by permeation through the membrane, a consequence of its partially unchanged nature. The effect would be the same as that of prior incubation with eserine.

The evidence that at least certain portions of the decamethonium and *d*-tubocurarine molecules bind to sites on acetylcholinesterase distinct from the active site of the enzyme is extensive (11, 12, 15–18, 21, 22, 48). The lack of major differences between the kinetic parameters of membrane-bound and solubilized acetylcholinesterase with respect to the inhibition observed with these two drugs indicates that the relationship existing between the peripheral site(s) and the catalytic site of this enzyme is not significantly affected by the membrane milieu.

Changeux (12) first demonstrated that gallamine is an allosteric effector of soluble *Torpedo* acetylcholinesterase. Kitz *et al.* (15) and Roufogalis and Quist (17) demonstrated similar effects of gallamine on eel and erythrocyte acetylcholinesterase, respectively. In our experiments, however, gallamine at concentrations as high as 100 μM was found to have no effect on the kinetics of acetylcholine hydrolysis by either membrane-bound or solubilized acetylcholinesterase. Crone (23) found that effects of gallamine on acetylcholinesterase similar to those described by Changeux (2), Kitz *et al.* (15), and Roufogalis and Quist (17) could be observed when the enzyme was assayed in low ionic strength medium. When the ionic strength of the medium was raised to 0.15 M, these effects of gallamine were no longer demonstrable (23). Thus it appears that the apparent discrepancy between our results and those of others (12, 15, 17) can be explained by the fact that our enzyme assays were done in Ringer's solution (approximately 200 mM) while the experiments of the authors mentioned above who demonstrated an effect of gallamine on acetylcholinesterase were done in low ionic strength medium (less than 4 mM).

In conclusion, it appears that the presence of acetylcholinesterase in a membrane milieu can induce subtle changes in the properties of the enzyme which can be detected by a detailed comparison of its membrane-bound and solubilized forms under carefully controlled conditions. However, the biological significance of these changes in the properties of acetylcholinesterase is not yet clear.

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