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Major Component of Acetylcholinesterase in *Torpedo* Electroplax Is Not Basal Lamina Associated†

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ABSTRACT: Electroplax tissue from *Torpedo californica* contains two major structural forms of the enzyme acetylcholinesterase. One form, composed of tetrameric protomers which are further aggregated by interactions among associated collagenous "tail fibers", has been well characterized previously. This form is associated in situ with the basal lamina. The other form is described and characterized herein. This latter form accounts for at least 50% of the acetylcholinesterase activity of the tissue. This enzyme associates with the tissue phospholipids. It aggregates in aqueous solution but readily dissociates to dimers in 1% sodium cholate solution, a solvent in which it is both soluble and catalytically fully active. The same dimer is obtained in sodium dodecyl sulfate solution where the enzyme is denatured. Denaturation in the presence of the reductant dithiothreitol results in the formation of a single 80 000-dalton subunit. The purified enzyme contains no collagenous component. It is not derivable from the collagenous "tailed-enzyme" form in the tissue homogenate. However, the two enzymes have similar molecular weight catalytic subunits and the same substrate-dependent turnover numbers (per active site) for a variety of choline esters which are generally utilized to distinguish specific esterase function. In the tissue homogenate each form of the enzyme is associated with a characteristic structural component (phospholipid or collagen). By implication, acetylcholinesterase function is localized in situ in the phospholipid membrane as well as at the basal lamina.

Both the structure and the catalytic activity of acetylcholinesterase, derived from a variety of sources, have received considerable attention. A major impetus for structural studies derives from physiological interest in the spatial relationships among three components of the synapse, the acetylcholine receptor, acetylcholine, and acetylcholinesterase. Most structural and catalytic studies have focused on two enzyme- and receptor-rich organs, the electroplax from the species *Electrophorus* and *Torpedo*. Since quantitative enzymological assays are usually carried out in homogeneous solution (an environment different from that of the structured synaptic cleft), most studies of catalysis and its modulation have been performed under environmental conditions designed for homogenization of the enzyme solution. Homogeneity can be achieved by a variety of different procedures. Soluble acetylcholinesterase protein can be isolated and purified by treatment of electroplax homogenates with trypsin, a treatment which is successful both for *Electrophorus electricus* (Rosenberry, 1975) and *Torpedo californica* (Taylor et al., 1974). This trypsin-solubilized enzyme has been utilized in most studies of enzyme activity and of the effects of ligand on the binding of substrates and substrate analogues to the active site (Rosenberry, 1975; Taylor & Lappi, 1975; Taylor et al., 1975). A solubilized form of the enzyme can also be produced by treatment of the electroplax homogenates with collagenase (Dudai & Silman, 1974; Lwebuga-Mukasa et al., 1976), a

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procedure which argues for the involvement of collagen-like polypeptide association with the enzyme protein. In high salt concentration, enzymatic activity can be solubilized; it corresponds to a mixture of molecular forms of different but discrete molecular weights (Massoulié & Rieger, 1969; Lwebuga-Mukasa et al., 1976; Rieger et al., 1976a). In contrast to the trypsin-solubilized protein, these forms of the enzyme contain substantial quantities of hydroxyproline and hydroxylysine (Lwebuga-Mukasa et al., 1976), amino acids characteristic of collagen polypeptides. The high salt extracted protein has been used in a variety of studies, all of which probe the nature of the interaction between two protein components, the collagenous polypeptides ("tails") and the catalytic subunits (Rieger et al., 1973a; Rosenberry & Richardson, 1977; Anglister & Silman, 1978). The catalytic subunits appear to form a discrete aggregate of *four* polypeptides. The studies indicate that up to three of these tetrameric aggregates can be held in a tight macromolecular unit via interaction among the associated collagenous tails. The occurrence of collagenous material in the tailed enzyme suggests an interaction with the basal lamina. Consequently, the relationship between the tailed enzyme and basal lamina fractions of the electroplax homogenate has been investigated by Lwebuga-Mukasa et al. (1976). These authors also report the occurrence of a second acetylcholinesterase fraction of substantially lower density in sucrose density gradient sedimentation. Rieger et al. (1973b) reported on interactions between phospholipids and the tailed enzyme, whereas Watkins et al. (1977) subsequently reported an interaction between the tailed enzyme and sphingomyelin micelles.

Ultimately, studies of solubilized enzyme in homogeneous solution must be correlated with the behavior of the enzyme in the highly localized heterogeneous milieu of the synapse. Analogous "in situ" studies have usually been carried out with electroplax tissue homogenates of either *Electrophorus* (Robaire & Kato, 1974, 1975) or *Torpedo* (Foidart & Gridelet, 1974). Some differences in the catalytic and regulatory properties of the in situ vs. the solubilized enzyme have been reported (Robaire & Kato, 1974, 1975), whereas others have found no difference in function (Pattison & Bernhard, 1978). Such comparative studies are difficult to interpret due both to the problem of acquiring quantitative isothermal data in heterogeneous solution and to the possible existence of a variety of specific ligand binding sites, other than those of the enzyme, in the heterogeneous mixture. The results of the present study were obtained in the process of searching for an acetylcholinesterase-enriched membrane fraction, so as to apply the quantitative analytical techniques to a physiologically more relevant system. As will be shown, another physical form of the enzyme exists, in *Torpedo* electroplax, which is distinct from the "tailed" enzyme and its trypsin-solubilized derivative. In situ, this form interacts exclusively with phospholipid. The usual methods for the isolation of either the trypsin-solubilized or the tailed enzyme preclude the isolation of this enzyme protein, although it represents a major fraction of the total acetylcholinesterase activity of the electroplax homogenate. The isolation, purification to homogeneity, and characterization of this form and its differentiation from previously reported and well-characterized forms of the enzyme from electroplax are the major subjects of this report.

Experimental Procedures

Materials

Reagents and Chemicals. Four buffered solvents were used in this study. Buffer I contained 0.1 M NaCl, 40 mM $MgCl_2$, and 10 mM $NaHCO_3$. Buffer II contained 0.1 M NaCl, 40 mM $MgCl_2$, and 10 mM Tris.[1] Buffer III contained 0.5 M NaCl, 40 mM $MgCl_2$, and 10 mM Tris. Buffer IV contained 2 mM Tris. All solvents were adjusted to pH 8.0 at 25 °C.

All organic chemicals utilized in this study were reagent grade or the best grade available. Ellman's reagent [dithiobis(dinitrobenzoate)], acetylthiocholine chloride, decamethonium bromide, and *N*-carbobenzyloxy-ε-caproic acid were obtained from Sigma Chemical Co. and were utilized without further purification. Cholic acid (Aldrich Chemical Co.) was recrystallized from hot ethanol prior to use.

Carboxymethyl-Sephadex and Sepharose samples were obtained from Pharmacia Co. β-(2-Furyl)acryloyl phosphate was synthesized according to the method of Malhotra & Bernhard (1968).

Enzyme standards for sedimentation studies were obtained as follows: β-galactosidase (16 S) was from Boeringher and liver alcohol dehydrogenase (5 S) was a gift from D. Anderson. Trypsin-solubilized acetylcholinesterase was also used as a reference with a sedimentation coefficient of 11 S (Taylor et al., 1974).

Biological Materials. (*1*) *Bungarus multicinctus* venom was purchased from Sigma Chemical Co.

(*2*) *Torpedo californica* were supplied frozen by the Marine Biological Station of the University of Oregon at Charleston, OR. The frozen electric organs were dissected at 4 °C and kept frozen at −70 °C until needed. A variety of enzyme-rich fragments were derived from this tissue as are defined and described below.

(*3*) *Tissue homogenate* was prepared by homogenization of 50 g of electric organ with 150 mL of buffer I for 30 s with a Virtis 23 homogenizer at medium speed at 4 °C. The suspension was then centrifuged for 1 h at 28000*g* at 4 °C. The resultant pellet was homogenized in buffer I.

(*4*) *Protein suspension* was prepared according to the procedure described for the tissue homogenate, except that the initial homogenate was filtered through eight layers of cheesecloth to remove nonsuspendable fibrous material prior to centrifugation.

(*5*) *Acetylcholinesterase-enriched suspension* ("low-density fraction") was obtained by the following flotation method. Eight milliliters of protein suspension in buffer IV was prepared according to Pattison & Bernhard (1978). The suspension was brought to >40% w/v sucrose by the addition of 80% w/v sucrose. The homogenate was placed at the bottom of a centrifuge tube. Fifteen milliliters of 35% w/v sucrose solution and 2 mL of 15% w/v sucrose solution (both in buffer IV) were successively layered above the homogenate. The tubes were centrifuged for 5 h at 78000*g*, in a Beckman rotor 30 at 4 °C. After centrifugation, the upper part of the gradient (~5 mL) was collected. This fraction had light-scattering material which had moved up through the 35% sucrose layer. This upper part of the gradient was diluted fivefold with buffer IV, and the membrane fragments were pelleted by centrifugation at 78000*g* for 1 h at 4 °C. The pellets were then resuspended in the appropriate buffer.

Methods

All procedures, with the exception of enzyme assays, were carried out at 4 °C.

Solubilization of Enzyme Forms. Three different procedures have been used to solubilize acetylcholinesterase activity. These procedures are defined and described below.

[1] Abbreviations used: Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; CM, carboxymethyl.

(*1*) *2 M $MgCl_2$ Extraction.* A fraction of the acetylcholinesterase activity could be solubilized upon extraction of the tissue homogenate and protein suspension with 2 M $MgCl_2$ (in 10 mM Tris buffer, pH 7.5). After incubation for 10 min, the $MgCl_2$ extract was diluted by a factor of 3–4 with distilled water and centrifuged for 1 h at 78000*g*. This dilution of $MgCl_2$ is necessary in order to allow sedimentation of an otherwise floating band of phospholipid and protein. As is shown below, this low-density sedimentable material corresponds to the low-density fraction described above. There is no observable exchange of enzyme activity between the high-salt solution and the pellet.

(*2*) *Trypsin Solubilization.* Some solubilization of acetylcholinesterase from the enzyme fractions described above was achieved by trypsin treatment according to the method described by Taylor et al. (1974).

(*3*) *Cholate Extraction.* A fraction of the acetylcholinesterase activity from the enzyme fractions described above could be solubilized by the addition of sodium cholate to a final concentration 2% in buffer I, II, or III. The cholate-solubilized material was centrifuged for 1 h at 78000*g* in order to remove associated scattering material.

Purification of Solubilized Enzyme Forms. The solubilized enzyme forms described above could be further purified by affinity chromatography. The affinity column was prepared according to the method of Rosenberry et al. (1972) but with (*m*-aminophenyl)trimethylammonium as the affinity ligand and 6-aminocaproyl-6′-aminocaproyl as the spacer arm. The concentration of the affinity ligand in the wet gel was 1.6 μmol/mL of wet gel. A maximum of 15 μmol of enzyme sites/mmol of affinity ligand was applied to the column in the purification procedure. The same affinity column was used for the purification of the three enzyme forms, as is described in the following section. The affinity column was recycled by washing with 2 M NaCl in buffer I, followed by preequilibrating with the appropriate buffer.

(*1*) *$MgCl_2$-Extracted ("Tailed") Enzyme.* The diluted $MgCl_2$ extract from tissue homogenate (described above) was placed on the affinity column preequilibrated with buffer III. All of the enzyme activity was retained on the affinity column. The column was then washed with a large excess of buffer II. There was no trace of enzyme activity in the wash. The enzyme was then eluted with 1 mM decamethonium bromide in buffer III.

(*2*) *Trypsin-Solubilized Enzyme.* Tryptic digestion of the tissue homogenate was carried out according to the procedure of Taylor et al. (1974). Following the digestion, the extract was centrifuged for 1 h at 78000*g* at 5 °C. The clear supernatant was placed on the affinity column previously equilibrated with buffer I. Virtually all of the enzyme activity was retained on the column. The column was washed with an excess of buffer I without the elution of any enzyme activity. The enzyme was eluted with 1 mM decamethonium bromide in buffer I. The pooled enzyme fractions were freed of most of the decamethonium inhibitor by passage through a CM 25-Sephadex column (0.9 × 20 cm) preequilibrated with buffer I. The active fractions were then dialyzed against 25 volumes of buffer I, and the dialysis procedure was repeated a second time with fresh buffer.

(*3*) *Cholate-Extracted Enzyme.* This enzyme form was purified by affinity chromatography following one of two alternate procedures, as described below.

(a) The acetylcholinesterase-enriched suspension prepared by the floatation technique described above was homogenized in a 2% cholate solution in buffer III. The total protein concentration was ~1 mg/mL. The homogenate was then centrifuged at 78000*g* for 1 h. All the activity was found in the supernatant fraction. Twenty milliliters of the supernatant was then passed through a Bio-Gel P-10 column (350 mL) preequilibrated with a 1% sodium cholate solution in buffer III. The enzyme fractions were pooled and dialyzed against 20 volumes of buffer III. The dialysis was repeated 4 times. This procedure, which removed virtually all of the cholate, retained 80% of the original activity in the dialysate. After centrifugation of the final dialysate at 78000*g* for 1 h, there was no observable pellet. The solution was placed on the affinity column preequilibrated with buffer III. Approximately 20% of the enzyme activity passed directly through the column or was rapidly eluted during the wash with buffer III. The remaining activity was eluted with 1 mM decamethonium bromide in buffer III.

(b) The protein suspension in buffer I was brought to 2% sodium cholate by the addition of 10% sodium cholate. The turbid suspension was then centrifuged for 1 h at 78000*g*. A substantial pellet containing some enzyme activity was observed (see Table I). The supernatant was diluted 1:1 with buffer I and placed on the affinity column, preequilibrated with 1% sodium cholate in buffer I, and washed with a large excess of the buffered cholate solution. The enzyme was eluted with 1 mM decamethonium bromide in buffer I containing 1% sodium cholate.

Activity Assays. (*1*) *Acetylcholinesterase Activity.* Enzyme activity was determined by the method of Ellman et al. (1961) using 0.59 mM acetylthiocholine chloride and 0.08 mM 5,5′-dithiobis(2-nitrobenzoic acid) in buffer I at 25 °C. Alternatively, pseudocholinesterase activity was assayed by utilizing butyrylthiocholine (0.9 and 0.09 mM) instead of acetylthiocholine. Active site titrations were performed in 2 mM Tris buffer at pH 8.0 and 25 °C according to the method of Rosenberry & Bernhard (1971).

(*2*) *Acetylcholine Receptor Activity.* Receptor activity was assayed by a filter technique adapted from Schmidt & Raftery (1973), utilizing a diethylaminoethylcellulose (DEAE-cellulose) filter. The receptor activity was determined from the amount of radioactive [^{3}H]acetyl-α-bungarotoxin (beyond the control) adsorbed to the filter by interaction with the acetylcholine receptor. α-Bungarotoxin was prepared as described previously (Clark et al., 1972). Acetylation with tritiated acetic anhydride was carried out by following the procedure of Ostrowski et al. (1970). The monoacetyl derivative was separated from acetic acid by absorption of the reaction mixture on a CM-cellulose gel and from nonacetylated toxin by elution with a 0.2–0.4 M ammonium acetate (pH 5.5) linear gradient.

(*3*) *ATPase Activity.* Na^+,K^+-ATPase activity was assayed by means of the chromophoric substrate analogue, β-(2-furyl)acryloyl phosphate (Odom et al., 1980). The sodium–potassium-mediated activity is determinable by measuring the difference in hydrolysis rate in the absence vs. the presence of an excess of the potent specific Na^+,K^+-ATPase inhibitor, strophantidin (Odom et al., 1980). Ninety percent of the hydrolytic activity against the substrate is inhibitable by strophantidin.

Chemical and Physical Analyses. (*1*) *Protein Concentration.* Protein concentration was routinely determined with the Bio-Rad protein assay reagent. For more accurate and sensitive determinations, the method of Lowry et al. (1951) was preferred. γ-Globulin was used as a protein standard.

(*2*) *Phosphorus Content.* This was determined according to the method of Chen et al. (1956).

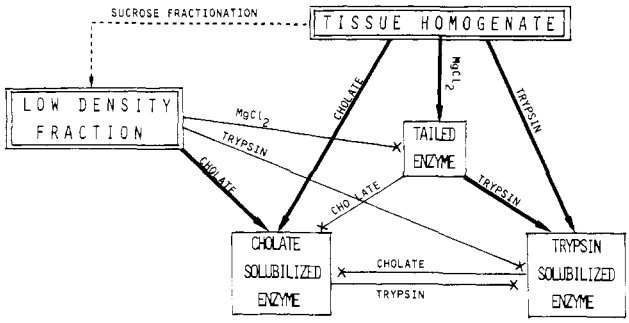

FIGURE 1: Relationships among the different acetylcholinesterases derived from *Torpedo* electric organ. $MgCl_2$, trypsin, and cholate refer to treatment with 2 M $MgCl_2$, trypsin, and 1% sodium cholate, respectively (as described under Experimental Procedures). ➞ represents observable transformations from one species to another. —× represents an unobservable transformation.

(*3*) *Hydroxyproline.* Hydroxyproline was determined after a 24-h digestion of protein samples in 6 M HCl at 110 °C in sealed tubes under vacuum. The hydrolyzed samples were analyzed with a Durrum autoanalyzer as described by Moore & Stein (1963).

(*4*) *Sedimentation Coefficients.* These were estimated by utilizing the sedimentation properties of proteins in a linear sucrose gradient (5–20% in buffer III in the presence of 1% sodium cholate). One milliliter of sample was layered over 30 mL of the sucrose gradient, and the tubes were centrifuged at 25000 rpm for 36 h at 5 °C by using a Beckman SW 27 rotor. Fractions of 1.5 mL were collected and assayed for activity. The sedimentation standards utilized were β-galactosidase (16 S), liver alcohol dehydrogenase (5 S), and trypsin-solubilized acetylcholinesterase (11 S).

(*5*) *Gel Electrophoresis.* Gel electrophoresis was carried out on a 4% acrylamide gel by using Fairbanks conditions (Fairbanks et al., 1971). Either 1% sodium dodecyl sulfate ($NaDodSO_4$) or 1% sodium cholate was used as a protein solvent.

For protein detection, gels were stained as described in Fairbanks et al. (1971) and scanned at 560 nm in a Gilford gel scanner. Carbohydrate was estimated qualitatively by using the "PAS" color test according to Fairbanks et al. (1971).

Results

Three structurally distinct species of acetylcholinesterase can be derived from *Torpedo* electroplax. These are as follows: (1) a "tailed" enzyme,[2] (2) a "trypsin-solubilized" enzyme, and (3) a "cholate-solubilized" enzyme. The derivation of these forms and their relationship to one another are summarized in the flow sheet of Figure 1. Three different treatments of electroplax tissue homogenate, $MgCl_2$ solubilization, trypsinolysis, and cholate solubilization, each lead to a single and distinctive form of the enzyme. These forms are identifiable by their different sedimentation properties in a linear sucrose gradient, as is shown in Figure 2. The $MgCl_2$ solubilization yields two components with sedimentation coefficients of 18 S and 14 S. These components are generally referred to as tailed enzyme (Rieger et al., 1976a). Trypsinolysis of the tissue homogenate yields an active form with a sedimentation coefficient of 11 S. Cholate-solubilized enzyme has a sedimentation coefficient of 8 S. Neither the $MgCl_2$-extracted and purified 18S plus 14S mixture nor its trypsin-solubilized 11S derivative is convertible to an 8S form by the presence of cholate. On the other hand, the tailed enzyme is convertible to the 11S form (Lwebuga-Mukasa et al., 1976). We have been unable to convert the 8S form to the 11S form by trypsin treatment. Therefore the 8S form is a species distinct from the tailed enzyme or its derivative, the 11S enzyme. These two different forms (the tailed and the cholate-solubilized) are both present in the tissue homogenate.

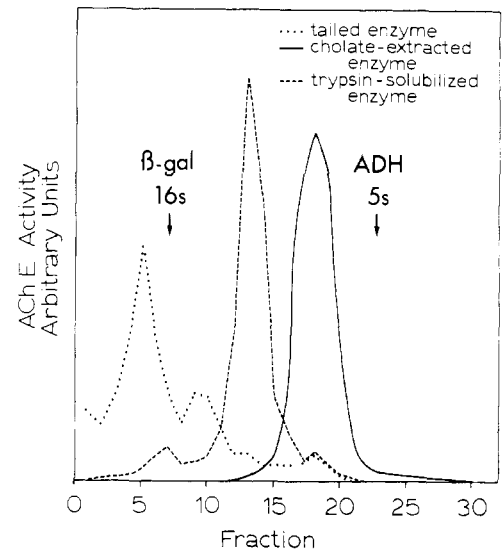

FIGURE 2: Sedimentation coefficients of the various species of acetylcholinesterase. Sedimentations were run on a 5–20% sucrose gradient in 1% sodium cholate, as described under Experimental Procedures. Purified tailed and 11S enzymes (see Exerimental Procedures) were brought to 1% sodium cholate before layering on the gradient. The same sedimentation pattern was obtained with solubilized but unpurified enzyme. Cholate-solubilized enzyme was obtained by cholate treatment of the low-density fraction without further enzyme purification.

On the basis of the following arguments, we conclude that the two enzymes do not interconvert during or after the tissue homogenization. (1) One gram of electroplax tissue contains ~200 units of activity (Table I). Solubilization either by $MgCl_2$ extraction (or trypsinolysis) or by cholate extraction yields ~100 units of activity/g of electroplax. (2) After solubilization by trypsin, there is no additional solubilized activity upon treatment of the residue with 2 M $MgCl_2$. (3) Cholate extraction of either the entire trypsinized tissue homogenate or the residual insoluble pellet after removal of the trypsin-solubilized enzyme yields an additional activity of 100 units/g of electroplax (Table I). The fact that $MgCl_2$ treatment does not extract any activity after trypsinolysis is as expected from the known relationship between the tailed enzyme and the 11S form. The conditions used for the extraction of solubilized enzyme activity from the tissue homogenate are, however, different in $MgCl_2$ extraction (4 °C, 10 min) vs. trypsinolysis (37 °C, 15 min). Therefore, if there were a time-dependent tailed enzyme–8S enzyme interconversion, there should be a difference in the activity ratio for tailed enzyme vs. trypsinized enzyme relative to incubation of cholate-extractable enzyme. Incubation of the tissue homogenate in 2 M $MgCl_2$ for 5 min vs. 1 h followed by dilution to 0.5 M $MgCl_2$ and centrifugation does not affect the yield of solubilized enzyme activity. Moreover, the amount of enzyme solubilized by cholate is independent of whether or not the homogenate has been pretreated with trypsin, a strong argu-

[2] The "tailed" enzyme structure is in reality a mixture of aggregates of tetrameric enzyme molecules held together by collagenous tails and consisting mainly of trimers and dimers of enzyme tetramers (with a sedimentation coefficient of 18 S and 14 S, respectively) (Lwebuga-Mukasa et al., 1976).

Table I: Solubilization of Acetylcholinesterase Activity

aqueous phase	activity (units/g of orginal tissue) total assayable	solubilized
(A) From Tissue Homogenate		
buffer[a]	124	2
buffer plus 2% cholate	120	106
buffer plus 2 M $MgCl_2$	203	102
buffer plus trypsin	176	92
buffer plus trypsin plus 2% cholate[b]	235	205
buffer plus trypsin plus 2 M $MgCl_2$[b]	195	102
(B) From Residual Pellet Obtained after Trypsin Treatment[c]		
buffer	100	5
buffer plus 2% cholate	125	120
buffer plus 2 M $MgCl_2$	94	6
(C) From Protein Suspension		
buffer	109	1.5
buffer plus 2% cholate	114	106
buffer plus 2 M $MgCl_2$	101	17
buffer plus trypsin	102	22
buffer plus trypsin plus 2% cholate[b]	115	121
buffer plus trypsin plus 2 M $MgCl_2$[b]	104	24

[a] The same results are obtained with buffer I or buffer II. For 2 M $MgCl_2$ treatment, buffer II was preferred. [b] After trypsin treatment, soybean trypsin inhibitor was added prior to further extraction procedure. [c] Tissue homogenate in buffer I was treated with trypsin as described under Experimental Procedures and centrifuged for 1 h at 28000*g*. The supernatant was discarded, and the pellets were homogenized in the various solvents.

ment against the interconvertability of these two enzyme forms in the tissue homogenate.

The tailed and the cholate-solubilized enzymes are associated with different components of the electroplax homogenate. Three different fractions have been prepared from electroplax (see Experimental Procedures). The *tissue homogenate* contains both the tailed and the 8S enzyme in equal quantity (by activity assay). The *protein suspension* (the tissue homogenate from which the nonsuspendable fibrous material has been removed by filtration through cheesecloth) has an activity ratio of only ~20% of tailed enzyme relative to 8S enzyme (Table I). In the *low-density fraction* (which contains up to 50% of the enzyme activity of the *protein suspension*), <2% of the activity can be solubilized by either $MgCl_2$ treatment or trypsin. All of the *low density fraction* activity is solubilized with cholate. By filtration through cheesecloth, most of the tailed enzyme is removed along with the fibrous nonsuspendable material. This localization of the tailed enzyme to the fibrous material is consistent with prior conclusions that the tailed-enzyme is associated with the basal lamina (Lwebuga-Mukasa et al., 1976). As long as the tailed enzyme is associated with this fibrous material, its activity is nonsuspendable and therefore not assayable by the classical spectrophotometric assay of Ellman (1961). Upon solubilization, with either $MgCl_2$ or trypsin treatment, an increase of the total assayable activity is observed, as described previously by Taylor et al. (1974). The cholate-extractable enzyme, in the absence of detergents, is a phospholipid-associated enzyme, suspendable and filtrable through cheesecloth. The addition of cholate to the tissue homogenate, unlike the addition of trypsin or $MgCl_2$, does not affect the total assayable activity.

Further studies were designed in order to differentiate between the tailed enzyme (18 S and 14 S) and the 8S form.

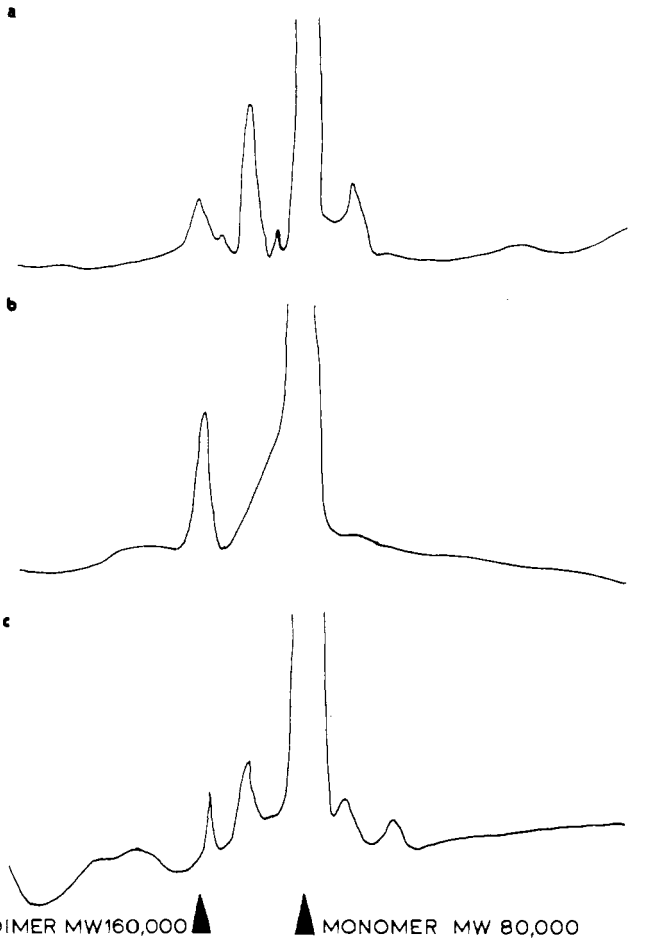

FIGURE 3: Electrophoretic behavior of tailed enzyme and cholate-solubilized enzyme on $NaDodSO_4$–polyacrylamide gel, in the presence of reducing agent. Gel electrophoresis was performed in 4% acrylamide gels, as described under Experimental Procedures. Enzymes were purified as described under Materials and Methods by using the protein suspension as the cholate-solubilized enzyme source. The samples were (a) tailed enzyme, (b) cholate-extracted enzyme, and (c) a mixture of the two enzymes. Sample layering was on the left, and the dye migration corresponds to the right part of the scan. The position of the monomeric (M_r 80 000) polypeptide characterized in the denaturation and reduction of the 11S trypsin-solubilized enzyme is indicated by the lower marker.

A characteristic of the tailed enzyme is its hydroxyproline content (a characteristic of collagenous polypeptides). We have found a hydroxyproline content of 89 nmol/mg of protein (~1% of the amino acid content) in purified tailed enzyme. The 8S form of the enzyme (from the low-density fraction), after purification by affinity chromatography, contains no hydroxyproline; the hydroxyproline content of tailed enzyme is unchanged when purified tailed enzyme is incubated with 1% sodium cholate and then purified by affinity chromatography, following the procedure described for the 8S enzyme.

The properties of the two purified enzymes have been examined by $NaDodSO_4$ gel electrophoresis, both in the presence and in the absence of reducing agent. In the presence of dithiothreitol, tailed enzyme migrates primarily as a single band (Figure 3). This band has been described as the enzyme subunit (Lwebuga-Mukasa et al., 1976). Two other faint bands, similar to those described by these authors, are observed. The slower band migrates as does the unreduced dimer. The other band (whose migration rate indicates a molecular weight of 10^5 daltons) could not be uniquely identified (as a tail vs. a catalytic component). The fact that we observe the same band by using a totally different purification

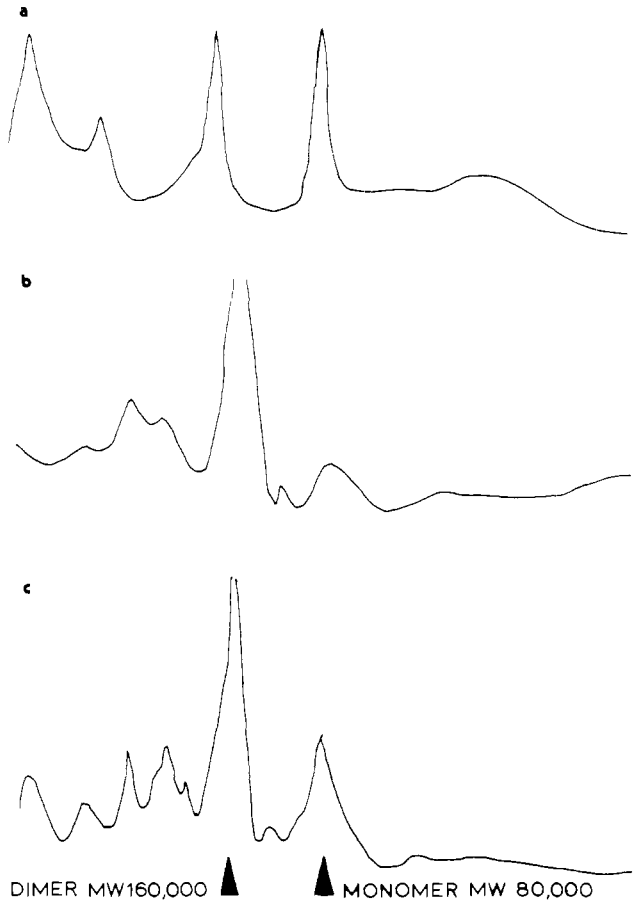

FIGURE 4: Electrophoretic behavior of tailed enzyme and cholate-solubilized enzyme on $NaDodSO_4$–polyacrylamide gel in the *absence* of reducing agent. Conditions were as described in Figure 3, with the omission of dithiothreitol in the samples. The position of the dimeric (160 000-dalton) polypeptide characteristic of the denatured, but not reduced, 11S trypsin-solubilized enzyme is indicated by the lower marker.

procedure makes the assumption that it is the tail component more likely. A comparable electrophoretic pattern is observed in the presence of dithiothreitol with the 8S enzyme except that the 120 000 band is not observed. When the two reduced enzymes are electrophoresed together in $NaDodSO_4$, there is no evidence for two main bands. In the absence of reducing agent, a variety of bands are observed upon $NaDodSO_4$ gel electrophoresis of the tailed enzymes, as is shown in Figure 4. Similar migration patterns have been described previously (Lwebuga-Mukasa et al., 1976); the various electrophoretic bands have been reported to arise from a variety of oligomers of the catalytic subunits and their further aggregation via interactions among their disulfide-linked tails. The same fastest migrating "monomer" and "dimer" bands are observable when the $NaDodSO_4$ electrophoresis is carried out with the trypsin-solubilized enzyme. Under these same non-reducing electrophoretic conditions, 8S enzyme migrates primarily as the dimer; no high molecular weight components are evident.

Trypsin-solubilized and cholate-solubilized enzyme each exhibit one principal band upon gel electrophoresis in the presence of 1% cholate. When the two enzymes are run together, cholate-solubilized enzyme migrates nearly twice as rapidly.

The absence of hydroxyproline in the cholate-solubilized 8S enzyme and its behavior on $NaDodSO_4$ electrophoresis indicate that the basic unit of the cholate-solubilized enzyme is a noncollagenous dimer in which the two polypeptides are linked

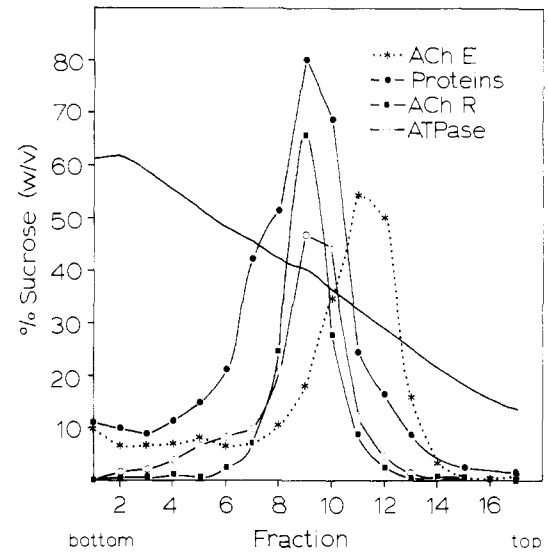

FIGURE 5: Fractionation of electric organ protein suspension in a linear sucrose gradient. Protein suspension prepared in buffer IV was homogenized in a 62% (w/v) sucrose solution. 5 mL of homogenate was placed at the bottom of a sucrose gradient. 35 mL of a linear sucrose gradient, between 60% and 10% (w/v), in buffer IV was layered above. Centrifugation was carried out at 81500*g* for 12 h in a Beckman SW 27 rotor; fractions of 2.5 mL were collected from the bottom to the top. The assays for acetylcholinesterase, acetylcholine receptor, ATPase, and proteins are described under Experimental Procedures.

together via disulfide bridges. The sedimentation coefficient (8 S) compared to that of the trypsin-solubilized enzyme (11 S) suggests that the cholate-solubilized enzyme does not aggregate so as to form tetramers. It might perhaps be argued that an asymmetrically shaped higher aggregate of protein could also have a low sedimentation coefficient. This possibility is unlikely since during electrophoresis in the presence of cholate, cholate-solubilized enzyme migrates faster than trypsin-solubilized enzyme. The discrete 8S band of cholate-solubilized enzyme is hence dimeric.

Since both the tailed enzyme and its derived trypsin-solubilized form have been well characterized structurally and enzymatically, we shall turn our attention to the 8S dimeric form.

Protein suspension, in buffer IV, has been fractionated in a linear sucrose gradient [from 60% to 10% sucrose (w/v)]. The sample was introduced in 62% sucrose (w/v) at the bottom of the gradient. Only material which floated is considered as membrane-bound material. When density equilibrium is attained, most of the protein is found in a broad peak at a sucrose density of 1.15 (Figure 5). This band contains both the acetylcholine receptor and the Na^+,K^+-ATPase. However, more than 50% of the acetylcholinesterase activity of the membrane suspension is found in a lower density fraction. The existence of such an acetylcholinesterase-rich low-density fraction has previously been observed (Cohen et al., 1972; Duguid & Raftery, 1973; Lwebuga-Mukasa et al., 1976). The routine preparation of this low-density acetylcholinesterase fraction involved the use of a *discontinuous* sucrose gradient, as described under Experimental Procedures. About 40% of the total suspendable enzyme activity is recovered in the low-density (<35% sucrose) fractions. This latter method provides a total separation of the low-density enzyme from the higher density proteins (there is no acetylcholine receptor in the low-density fraction). The specific activity of the low-density enzyme fraction (200 units/mg) is fivefold greater than that of the membrane suspension. Note that there is no de-

tectable hydroxyproline in this entire (impure) low-density fraction as well as in its affinity-chromatographed acetylcholinesterase component. Hence there is no evidence for the presence of basal lamina collagen components in the low-density fraction. On the contrary, this fraction contains 80 μg of phosphorus/mg of protein which corresponds to a ratio of 2 mg of phospholipid/mg of protein. This preparation is therefore the material of choice for studies of the physical properties of the phospholipid-associated 8S dimer.

The specific activity in the low-density fraction is unaffected by the presence of cholate, indicating that the active site of the phospholipid-associated dimer is accessible to the substrate. The acetylthiocholine turnover numbers determined from active site titration with M7C (Rosenberry & Bernhard, 1971) are the same for the phospholipid-associated dimer, the detergent-solubilized dimer (3.0×10^5 min^{-1}, reported herein), and the trypsin-solubilized tetramer reported previously (Pattison & Bernhard, 1978). The specific activity of the purified enzymes is 4000 units/mg for the dimer, 4300 units/mg for the trypsin-solubilized enzyme, and 3400 units/mg for the tailed enzyme. Therefore, there is hardly any difference in the activity toward acetylthiocholine among the various forms of the enzyme.

The possibility that the dimeric form of the enzyme is a "pseudocholinesterase", with little selectivity among aliphatic acylcholines, has been considered. The hydrolytic activity toward 0.9 mM butyrylthiocholine was determined with purified dimer and with the trypsin-solubilized enzyme. The hydrolysis is 6000 times slower than that of acetylthiocholine in both cases. With both enzymes, this rate is decreased by a factor of 2 when the butyrylthiocholine concentration is reduced 10-fold.

Discussion

We have not found any difference in the catalytic activity of the various forms of the enzyme. The occurrence of structurally different forms would therefore appear to bear on the localization of enzyme in different parts of the synapse rather than on the catalytic function per se. The trypsin-solubilized enzyme has been reported to be a glycoprotein (Taylor et al., 1974). The absence of carbohydrate in the 8S dimer might result in the loss of water solubility. A qualitative test for carbohydrate (see Methods), however, showed that both the trypsin-solubilized 11S tetramer and the purified 8S dimer stained positively with comparable intensity. This result does not distinguish possible differences in the nature or the number of glycosidic units present per protomer.

The change from water solubility to phospholipid association can arise from differences in the primary sequence of the enzymes. The identity of catalytic properties and the common subunit size suggest that there is no gross change in amino acid sequence and that there is active-site sequence homology. A detailed comparative analysis of amino acid composition and of peptide maps are complicated with these large polypeptide subunits.

The importance of (hydrophobic) interaction between the cholate-solubilized dimer and its environment is evident from sedimentation studies: After removal of cholate, the 8S peak is no longer present (Figure 6). Instead, higher molecular weight aggregates are evident. These aggregates are partially, but not completely, reversibly dissociated to ~8S material when they are once again treated with 1% cholate. The finding that cholate extraction of the tissue homogenate yields exclusively 8S material in reproducible quantities suggests that in *Torpedo* tissue either the phospholipid–enzyme complex never dissociates and hence never aggregates in aqueous solution or the phospholipid, unlike cholate, completely reverses polypeptide aggregation in aqueous solution. The nature of the interactions among dimers in these aqueous aggregates is different from the interactions between dimers within the trypsin-solubilized tetramer. These latter tetramers are not dissociated in the presence of 1% cholate. When the tetramers are mixed with the low-density phospholipid fraction (which contains the cholate-extractable enzyme) no additional enzyme is incorporated into this low-density fraction.

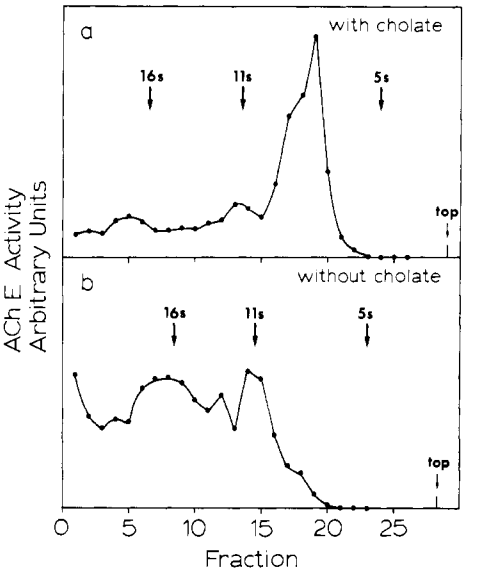

FIGURE 6: Sucrose gradient centrifugation of the cholate-extracted enzyme. Purified enzyme (by affinity chromatography) from the low-density fraction was (a) brought to 1% sodium cholate and layered on a sucrose gradient in the presence of 1% sodium cholate or (b) layered on a sucrose gradient without sodium cholate.

It might be imagined that the dissociation of a precursor to dimers (e.g., the catalytic tetramers of the tailed enzyme), followed by transfer of the dimers to phospholipid, might occur artifactually during homogenization of the electric organ. This hypothesis appears to be unlikely since the homogenization of electroplax tissue directly in 1% cholate solution yields the same amount of cholate-solubilized enzyme activity as when tissue homogenized in the absence of cholate is subsequently cholate extracted. Moreover, the yield of extractable enzyme, is not sensitive to the method of homogenization (Vertis vs. Brinkmann homogenizers) or to the time of homogenization (3–10 min).

The interaction between acetylcholinesterase and phospholipids, described herein for the first time for the enzyme from *Torpedo* electroplax, is a known feature of the acetylcholinesterase isolated from other sources. The erythrocyte enzymes from bovine (Beauregard & Roufogalis, 1977) and human (Ott & Brodbeck, 1978) sources are solubilized only by detergent. Acetylcholinesterase from sheep erythrocyte membranes can be released in a vesicular membrane fragment (Lutz et al., 1976). These fragments have a higher specific activity for acetylcholinesterase than does the entire erythrocyte membrane. Human erythrocyte acetylcholinesterase can be selectively extracted from the erythrocyte membrane by homogeneous phospholipid vesicles (Bouma et al., 1977). Under denaturing and reducing conditions, this enzyme has a molecular weight of 80 000–91 000 (Bouma et al., 1977). This is comparable to that of *Torpedo* acetylcholinesterase (Lwebuga-Mukasa et al., 1976). The sedimentation coefficient of the Triton-extracted enzyme from human erythrocytes is 7 S in the absence of reducing agents (Ott & Brodbeck, 1978). Hence it seems likely that for erythrocyte acetylcholinesterase,

as well as for the phospholipid membrane associated enzyme from electroplax, the functional form is a dimer. The similarities reported herein justify further comparative studies of the enzyme from different sources.

The presence of both the tailed enzyme and the phospholipid-associated dimer in *Torpedo* electroplax tissue has not been previously reported. This finding poses a number of questions. Is the dimeric form an intermediate in either the formation or the breakdown of the tetrameric tailed enzyme? Or do the two enzymes originate from two totally different biosynthetic pathways? It has been shown that both myoblasts and neuroblastoma cells can synthetize acetylcholinesterase in cell culture. However, myoblasts are able to synthesize the heavy form of the enzyme (corresponding to the tailed enzyme) along with the light forms (Sugiyama, 1977; Koenig & Vigny, 1978), whereas neuroblastoma cells synthesize only lower molecular weight enzyme (4 S–9.6 S, Chang & Blume, 1976; 4.6 S–10.3 S, Rieger et al., 1976b).

Are these two species localized in different regions of the electroplax? The tailed enzyme from innervated muscle (16 S) was first proposed to be the end-plate enzyme (Hall, 1973; Vigny et al., 1976; Weinberg & Hall, 1979) and, in the neuromuscular junction, is associated with the basal lamina (McMahan et al., 1978). However, the presence of the 16S enzyme has now been demonstrated in noninnervated muslce sections (Carson et al., 1979) and in sciatic nerve (Di Giamberardino & Couraud, 1978), probably in its motor neurons (Fernandez et al., 1979). These non-end-plate enzymes could be anchored in extrajunctional basal lamina (Sketelj & Brzin, 1979). The lighter forms of the enzyme are not necessarily characteristic of the end-plate region (Hall, 1973; Sketelj et al., 1978). They are present in both sensory and motor neurons (Fernandez et al., 1979). Little is known about their cellular localization. Sketelj et al. (1978) suggest that the 7S and 11S forms of the chicken muscle enzyme are present in the sarcoplasm.

Does the phospholipid environment of our 8S enzyme allow it to be more uniformly distributed than the tailed enzyme which is anchored to the basal lamina? The mobility of the phospholipids might serve to redistribute the associated enzyme over wide areas of the membrane. The effect of magnesium ion concentration in the density fractionation of the electric organ homogenate is relevant to this point. In the presence of sufficient Mg^{2+} or Ca^{2+} (>10 mM), all membrane-bound proteins are found in a peak of uniform density on a sucrose density gradient. At lower Mg^{2+} concentrations there is a structural transition resulting in a low-density acetylcholinesterase-associated phospholipid fraction. The effect of ionic environment on the appearance of this low-density enzyme fraction has been noted previously (Cohen et al., 1972). The formation of the low-density fraction is dependent on the concentration of ions *during* the fractionation procedure but not on the ionic concentration during the tissue homogenization. After homogenization in the absence of Mg^{2+}, addition of Mg^{2+} to the homogenate prior to sucrose density fraction yields no low-density phospholipid fraction containing acetylcholinesterase (Figure 7). Our results suggest that at high Mg^{2+} concentrations all phospholipid membrane components are tightly interacted and hence all of their associated protein migrate together. Likewise, in sheep erythrocyte membranes, acetylcholinesterase-enriched vesicles cannot be isolated in the presence of Ca^{2+} although vesicle formation occurs (Lutz et al., 1976). These in vitro results suggest that the fluidity of the phospholipid and the consequent mobility of its associated proteins are dependent on the ionic environment.

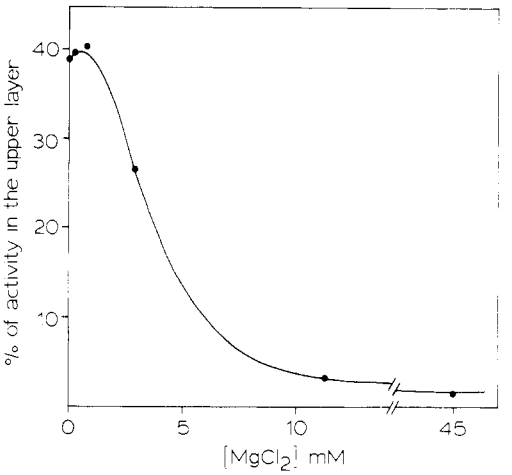

FIGURE 7: Effect of Mg^{2+} on the presence of acetylcholinesterase in a low-density fraction. Protein suspension was homogenized in buffer IV plus concentrated sucrose. 2 mL of homogenate plus 0.2 mL of $MgCl_2$ solution (from 2 mM to 0.5 M) was placed at the bottom of a centrifuge tube. After overnight incubation, 15 mL of sucrose (35% w/v) and 2 mL of sucrose (15% w/v) both in buffer IV were successively layered, and the tubes were centrifuged for 5 h at 78000*g*. The upper layers of the gradients were collected and analyzed for acetylcholinesterase content. The activity below was also checked; there was no [Mg^{2+}]-dependent inactivation of the enzyme. Final concentrations of Mg^{2+} in the overnight incubation mixture are given in the figure.

It is reasonable to assume that the various interactions of the disulfide-linked dimers with the environment (phospholipids, water and collagenous polypeptides) produce a different localization of each of the enzymes.

The outer structure of an enzyme is of primary importance for its immunological properties. It has already been pointed out that, in the case of acetylcholinesterase, the antibodies recognize the surface of the protein rather than the active-site region (Michaeli et al., 1969; Gurari et al., 1974; Rieger et al., 1976a). It has been shown that there are only minor differences in the antigenic determinants of tailed and trypsin-solubilized enzymes from eel (Gurari et al., 1974; Greenberg et al., 1977; Trevor & Greenberg, 1978). Anglister et al. (1979) have recently demonstrated antigenic determinants in the tailed form of acetylcholinesterase which are cross-reactive with rat tail tendon collagen. These determinants are absent from the trypsin-solubilized tetrameric enzyme. There are common antigenic sites between tailed enzymes from eel or *Torpedo* (Rieger et al., 1976a). However, no cross-reactivity is evident between acetylcholinesterases from eel enzyme and either bovine erythrocyte (Williams, 1969) or bovine caudate nucleus (Greenberg et al., 1977; Trevor & Greenberg, 1978).

Such an absence of cross-reactivity is as expected from enzymes which difffer in their interaction with their environment. It would be more meaningful and of considerable interest to study the cross-reactivity of enzymes which are normally phospholipid associated, for example, the 8S enzyme described here and erythrocyte acetylcholinesterase. It has already been shown that antibodies against bovine caudate nucleus acetylcholinesterase react with bovine erythrocyte enzyme and incompletely with rat brain enzyme (Trevor & Greenberg, 1978). The use of such more specific antibodies could allow a more precise study of the cellular localization of the acetylcholinesterase.

Conclusions

(1) At least 50% of the total acetylcholinesterase extractable from the electroplax tissue of *T. californica* is phospholipid

associated. It has a unique sedimentation coefficient of 8 S in cholate solution, and it contains no hydroxyproline, properties inconsistent with the basal lamina- or collagen-associated tailed enzyme previously isolated and characterized.

(2) Neither form of the enzyme (tailed or cholate-extracted) arises from the other artifactually, as a consequence of the isolation procedure.

(3) The two forms of acetylcholinesterase isolated from *Torpedo* electroplax have virtually identical catalytic properties (both are true *acetyl*cholinesterases) but differ markedly in some of their physical properties, particularly in regard to their interactions with components of the environment (phospholipids, water, and collagenous polypeptides). It is evident that the enzymes must differ in their "outer" structure presumably due to differences in amino acid composition or to differences in the composition of the covalently linked carbohydrate of these glycoproteins. The common size of the polypeptide subunit ($M_r \simeq 80\,000$) and the common catalytic properties of the two enzymes argue in favor of a common "inner" polypeptide folding and, presumably, to homologous or identical sequences for interior residues.

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