Molecular Forms of Acetylcholinesterase from *Torpedo californica:* Their Relationship to Synaptic Membranes†

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ABSTRACT: The 16S and 8S forms of acetylcholinesterase (AchE), which are composed of an elongated tail structure in addition to the more globular catalytic subunits, were extracted and purified from membranes from *Torpedo californica* electric organs. Their subunit compositions and quaternary structures were compared with 11S lytic enzyme which is derived from collagenase or trypsin treatment of the membranes and devoid of the tail unit. Upon sodium dodecyl sulfate–polyacrylamide gel electrophoresis in the absence of reducing agent, appreciable populations of monomeric through tetrameric species are observed for the 11S form. Under the same conditions, the 16S form yields only monomer and dimer in addition to a higher molecular weight species. If complete reduction is effected, only the 80 000 molecular weight monomer is dominant for both the 11S and 16S forms. Cross-linking of the 11S form by dimethyl suberimidate followed by reduction yields monomer through tetramer in descending frequency, while the 16S form again shows a high molecular weight species. A comparison of the composition of the 11S and 16S forms reveals that the latter has an increased glycine content, and 1.1 and 0.3 mol % hydroxyproline and hydroxylysine, respectively. Collagenases that have been purified to homogeneity and are devoid of amidase and caseinolytic activity, but active against native collagen, will convert 16S acetylcholinesterase to the 11S form. Thus, composition and substrate behavior of the 16S enzyme are indicative of the tail unit containing a collagen-like sequence. A membrane fraction enriched in acetylcholinesterase and components of basement membrane can be separated from the major portion of the membrane protein. The 16S but not the 11S form reassociates selectively with this membrane fraction. These findings reveal distinct similarities between the tail unit of acetylcholinesterase and basement membrane components and suggest a primary association of AchE with the basement membrane.

Acetylcholinesterase (AchE[1]) (EC 3.1.1.7) can be isolated from synaptic membranes of the electric fish in several molecular forms. Three species have been obtained by high salt extraction of the electric organ from *Electrophorus electricus* and are characterized by sedimentation constants of 8S, 14S, and 18S (Massoulié and Rieger, 1969). Electron micrographs show that the respective species contain an elongated tail unit (2–3 × 50 nm) attached to a variable number of globular subunits (Rieger et al., 1973; Dudai et al., 1973). Treatment with trypsin will convert these species to an 11S form which is devoid of the tail unit and is similar, if not identical, with the 11S species that has been extensively characterized from toluene-treated (autolyzed) electric tissue (Massoulié and Rieger, 1969; Dudai et al., 1972). Multiple forms of AchE in high salt extracts have also been observed in *Torpedo* (Massoulié and Reiger, 1969; Taylor et al., 1974) and the mammalian neuromuscular junction (Hall, 1972). Conversion to an 11S form by the lytic procedures occurs also with *Torpedo;* however, characterization of the 11S enzymes from the marine and fresh water fish reveals distinct differences in hydrodynamic properties and amino acid composition (Taylor et al., 1974).

Although histochemical studies show a preferential association of AchE with the postsynaptic membrane (Koelle and Friedenwald 1949; Bloom and Barrnett, 1966), these studies lack sufficient resolution to ascertain whether primary association of the enzyme is with the plasma membrane or an outer collagen-like matrix of basement membrane structure. Also, the role played by tail unit in the membrane association is unclear. Commercial collagenases will release AchE from the postsynaptic location leaving the receptor function intact (Hall and Kelly, 1971; Betz and Sakmann, 1973). Although the collagenases have specific sequence requirements for hydrolysis (Harper and Kang, 1970), the release experiments yield only minimal structural information since commercial collagenases are contaminated with a number of less specific proteases (Kono, 1968; Peterkofsky and Diegelmann, 1970). We have purified tailed AchE species from *Torpedo californica* and in this communication report on their structural properties and their susceptibility to purified collagenases. We also examine in more detail the nature of the interaction between the head and tail units and the role played by the tail in the reassociation of AchE with a membrane fraction.

Experimental Section

Materials

[^{3}H]DFP (1.13 mCi/mmol) was a product of New England Nuclear and [^{32}P]DFP (82.6 mCi/mmol) was ob-

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[1] Abbreviations used: AchE, acetylcholinesterase; AchR, acetylcholine receptor; DFP, diisopropyl fluorophosphate; DIP-AchE, diisopropylphosphorylacetylcholinesterase; IAA, iodoacetate; Ach, acetylcholine; BANA, benzoylarginylnaphthylamide; DMS, dimethyl suberimidate; CNBr, cyanogen bromide.

tained from Amersham/Searle. Unlabeled DFP was a product of Calbiochem. Trypsin (TRL grade) was purchased from Worthington. Soybean trypsin inhibitor and pyridine aldoxime methiodide were obtained from Sigma Chemical Co. 2-Aminoethyl *p*-nitrophenyl methylphosphonate was a generous gift of Dr. Irwin Wilson, University of Colorado, Boulder, Colo. Four collagenases were purified from freeze-dried extracellular extracts from *Clostridium histolyticum* (Worthington CLS-IV) and were identified on the basis of their isoelectric points (I, 5.50; II, 5.65; IIIa, 5.90; IIIb, 5.90). Collagenases IIIa and IIIb have different molecular weights and could be separated with DEAE-cellulose chromatography. Each of the collagenases appeared homogeneous on gel electrophoresis in the presence of sodium dodecyl sulfate and were active against native guinea pig skin collagen. They were also devoid of caseinolytic and amidase activity (<0.2% of an equal weight of trypsin) (Lwebuga-Mukasa, J., Harper, E., and Taylor, P., submitted for publication).

Methods

Electroplax tissue, which had been frozen at −70 °C since removal from *Torpedo californica,* was homogenized at 0 °C in 0.1 N NaCl, 0.04 M $MgCl_2$, 0.01 M Tris-HCl, pH 8.0, and filtered through four layers of cheese cloth. The pellet obtained after centrifugation at 40 000*g* for 1 h was resuspended in a solution of $MgCl_2$ or NaCl ($\Gamma/2$ = 1.0–6.0) buffered in 0.01 M Tris-HCl, pH 8, with a volume equal to one-half of the original tissue weight. The suspension was centrifuged at 40 000*g* for 1 h and the AchE activity in the supernatant was assayed titrimetrically using a Radiometer autoburet. Total AchE activity in the initial pellet was obtained by resuspending the pellet in 0.1% Triton X-100, 0.01 M Tris-HCl, pH 8.0, and assaying for AchE activity.

Purification of 8S AchE Species by Immunoadsorbent Chromatography. A crude extract of predominantly 8S AchE species was obtained by extracting the pellet obtained after the first centrifugation described above, with a volume equal to the original tissue weight of 0.01 M Tris-Cl, pH 8.0, containing 1.0 N NaCl and 0.04 M $MgCl_2$ (Taylor et al., 1974). After sedimenting a pellet at 23 000*g* for 2 h, the salt extraction procedure was repeated twice and the combined supernatants were applied to an immunoadsorbent column equilibrated with the same buffer. The immunoadsorbent column was prepared by coupling rabbit anti-AchE antibodies (prepared against the 11S enzyme) directly to CNBr-activated Sepharose 4B beads. From 50 to 80% of the added activity was retained by the column and 6–15% of the retained activity could be eluted with 2 M $MgCl_2$, 0.01 M Tris-Cl, pH 8.0. Although the procedure produced low and variable yields, greater than 80% of the purified AchE existed as 8S species. The purified enzyme had a specific activity of 230 mmol of Ach per hour per milligram of protein which may be compared with a mean value of 345 mmol of Ach per hour per milligram of protein for the 11S preparations (Taylor et al., 1974).

Purification of 16S AchE Species by Covalent Affinity Chromatography. An initial membrane pellet obtained as described above was resuspended in a volume equal to one-half the original tissue weight of 2 M $MgCl_2$, 0.01 M Tris-Cl, pH 7.83. The supernatant resulting from centrifugation at 40 000*g* for 2 h was dialyzed for 2–3 h against 1 N NaCl, 0.04 M MgCl, 0.01 M Tris, pH 8.0, using a Bio-fiber Beaker-80 (Bio-Rad) and put over a 0.9 × 15 cm covalent affinity column. All operations except for adsorption and elution from the column were conducted at 4 °C. For column adsorption the extract from the 4 °C reservoir was allowed to flow through a 22 °C bath immediately prior to adsorption on the column at the same temperature. The covalent resin consisted of 2-aminoethyl *p*-nitrophenyl methylphosphonate linked to Sepharose 4B beads through an extended arm composed of succinate and diaminopentane. It was prepared according to the procedures described by Voss et al. (1975). The bound phosphonate content was 0.16–0.3 μmol/ml of resin bed based on *p*-nitrophenol released from the column by 0.01 N NaOH. Elution of the enzyme was accomplished by 0.001 M pyridine aldoxime methiodide, 10% dimethyl sulfoxide in the 1 N NaCl containing buffer at 22 °C. Since elution of free enzyme was slow, the eluting buffer was allowed to remain in contact with the column for 12–16 h. About 60% of the extracted AchE was adsorbed to the column and of this 28% was eluted. The specific activity of 16S AchE eluted from the column varied between 150 and 180 mmol of Ach/h/mg of protein.

Conversion of 16S AchE to the 11S Form by Purified Collagenases. Tailed AchE (16S) obtained either by high ionic strength extraction or by extraction and subsequent purification by covalent affinity chromatography was incubated for 10 min at 37 °C with collagenase I or IIIa at a concentration of 3 μg/ml. One-tenth of a milliliter of the incubated sample was layered over a 5-ml 5–20% linear sucrose gradient and centrifuged at 40 000 rpm for 8 h in a Beckman SW-50L rotor. Twenty-five to forty fractions were collected and the conversion to the 11S species was ascertained from the activity profiles.

Purification of the Collagenase and Trypsin Released AchE. Trypsin-released AchE was purified according to Taylor et al. (1974). Collagenase-released AchE was prepared by the same procedure except that collagenase (3 μg/ml) was substituted for trypsin and the membranes were incubated at 37 °C for 20 min.

Characterization of the Enzyme Species. Formation of Diisopropylphosphoryl-AchE (DIP-AchE). Purified AchE species were labeled by reaction at 4 °C with 50 μM [^{3}H] or [^{32}P]DFP for 30 min. Unreacted ligand was removed by dialysis. Portions of [^{3}H]- and [^{32}P]DIP-AchE's were admixed and then subjected to various analytical procedures which have been described previously for the 11S enzyme (Taylor et al., 1974).

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. Purified AchE preparations were subjected to polyacrylamide gel electrophoresis in presence of 1% sodium dodecyl sulfate (Weber et al., 1972). Disulfide bonds were reduced by reaction with 1 mM dithiothreitol or 1% mercaptoethanol for 30 min. Alkylation with 3–5 mM IAA was employed to prevent subsequent reoxidation of sulfhydryl groups and to remove excess reducing agent if reduction was carried out on the undenatured enzyme. The gels were stained with Coomassie blue and staining densities ascertained on a Gilford gel scanner. Molecular weights of the species were estimated from comparative mobilities of protein standards of known molecular weight and cross-linked ovalbumin (Carpenter and Harrington, 1972).

Intramolecular Cross-Linking of AchE Using Dimethyl Suberimidate. Dimethyl suberimidate was synthesized from dicyanohexane and HCl in anhydrous methanol–ether (Davies and Stark, 1970; melting point 215–216 °C; lit. 216–217 °C). Cross-linking of various AchE's was done according to Davies and Stark (1970) by incubation with 0.5

Table I: AchE Solubilization in High Ionic Strength Solutions.

Salt	Γ/2	% of Total AchE Solubilized	Specific Activity (meq/(min mg) of protein)	Predominant Species
$MgCl_2$	1.5	56	0.098	16S
$MgCl_2$	3.4	68	0.105	16S
$MgCl_2$	6.0	81	0.101	16S
NaCl	1.0	20	0.025	8S
NaCl	1.5	18	0.018	8S
NaCl	2.0	19	0.017	8S

mM DMS at 4 °C for 12 h or at 22 °C for 3 h. The enzyme concentration ranged between 1.0 and 1.2 mg/ml for the 11S species and between 0.5 and 0.7 mg/ml for the 16S enzyme.

Assay Procedures. (a) Amino Acid Analysis. Amino acid analyses were carried out on a Beckman or Durrum automated amino acid analyzer as described by Moore and Stein (1967). Hydroxyproline was also measured colorimetrically by the method of Bergman and Loxley (1963), following 24-h hydrolysis in sealed evacuated tubes.

(b) Phospholipid Phosphate. Membrane lipids were initially extracted using the methanol–water–chloroform system as described by Bligh and Dyer (1959). An aliquot of the chloroform layer was transferred and evaporated to dryness and its inorganic phosphate content was then determined according to Chen et al. (1956).

(c) Na^+,K^+-Stimulated ATPase Activity. The Na^+,K^+-sensitive ATPase activity was monitored according to the method of Bonting et al. (1961) by measuring the difference in activity in the presence and absence of ouabain.

(d) Cobra Toxin Binding Activity (Acetylcholine Receptor). To determine the distribution of AchR, cobra α-toxin was labeled with ^{125}I to a specific activity of 10 Ci/mmol, and its binding to membranes was measured by the procedures of Duguid and Raftery (1973).

(e) Proteins. Total protein was measured by the method of Lowry et al. (1951).

Fractionation of Torpedo Membrane Fragments and Reassociation of Components with Membrane Fractions. Membrane fragments from fresh electric organ were prepared according to the differential and density gradient centrifugation methods of Duguid and Raftery (1973). Fractions were collected by reverse flow with an Isco density gradient fractionator. For the reassociation experiments [^3H]DIP-AchE was mixed at 4 °C with the resuspended membrane fraction prepared by differential centrifugation 30 min prior to layering the mixture on the density gradients. The membranes were resuspended in a buffer consisting of 0.1 N NaCl, 0.04 M $MgCl_2$, 0.01 M Tris-Cl, pH 7.8. If the labeled AchE preparations were to cause an appreciable change in ionic strength of the membranes to be layered on the gradient, they were passed over a 0.9 × 20 cm Sephadex G-25 column prior to layering on the gradient.

Results

Solubilization of AchE from the Membrane by High Salt Extraction. Purification and Sedimentation Properties of the Enzyme Forms. As shown in Table I, extraction of electric organ membranes with 1 N NaCl, 0.04 M $MgCl_2$, 0.01 M Tris-Cl, pH 8.0, solubilized 20% of the total AchE activity, which appeared largely as 8S species, although small amounts of 14S form could be detected (Taylor et al., 1974). Increasing the ionic strength neither increased the total AchE solubilized nor changed the observed S value of the predominant species. However, $MgCl_2$, at an ionic strength of 1.5, solubilized 56% of the total AchE activity which appeared primarily as 16S species. With $MgCl_2$ at a concentration of 2 M (Γ/2 = 6), 81% of the AchE could be released as 16S species with a small amount of 14S enzyme.

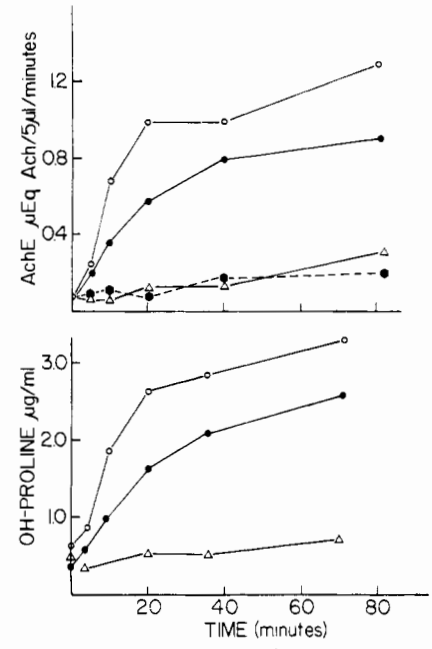

FIGURE 1: Release of membrane-associated AchE by collagenase IIIa with concomitant solubilization of hydroxyproline containing peptides. Membrane suspensions in 0.1 N NaCl, 0.04 M $MgCl_2$,.0.01 M Tris·HCl, pH 8.0, were prepared as described in the text and incubated at 37 °C with 5 mM $CaCl_2$ under the various conditions. Hydroxyproline was measured following hydrolysis in 6 N HCl for 20 h in vacuo: (○—○) 214 nM trypsin; (●—●) 21 nM collagenase IIIa (molecular weight 78 000); (●- - -●) 21 nM collagenase + 10^{-4} M *o*-phenanthrolene; (△—△) no added enzyme.

The 8S form purified by adsorption and elution from the immunoadsorbent column and the 16S species purified by covalent affinity chromatography retained the same sedimentation profiles observed following high salt extraction (cf. Figure 2). Thus, appreciable conversion between 8 and 16S was not evident nor was formation of 11S enzyme apparent during the purification procedures.

Release of Membrane-Associated AchE by Collagenases and Trypsin. The purified collagenases I, II, and IIIa at concentrations of 3–6 μg/ml at 37 °C promoted the release of AchE from electroplax membranes with a time course similar to that seen with trypsin incubation at 5 μg/ml (Figure 1). Collagenase I or IIIa was used in the majority of subsequent experiments. AchE release was rapid, being essentially complete in 20–30 min. It was not inhibited by soybean trypsin inhibitor or 1 mM phenylmethylsulfonyl fluoride. However, 10^{-4} M *o*-phenanthroline was an effective inhibitor of the collagenase-stimulated release which is consistent with the known inhibition by chelating agents of this putative metalloenzyme (Harper, 1974). AchE release by collagenases and trypsin was accompanied by release of hydroxyproline containing peptides that corresponded to the profile of release of AchE (Figure 1). On molar basis, collagenase was three- to tenfold more effective in releasing AchE from membranes than was trypsin.

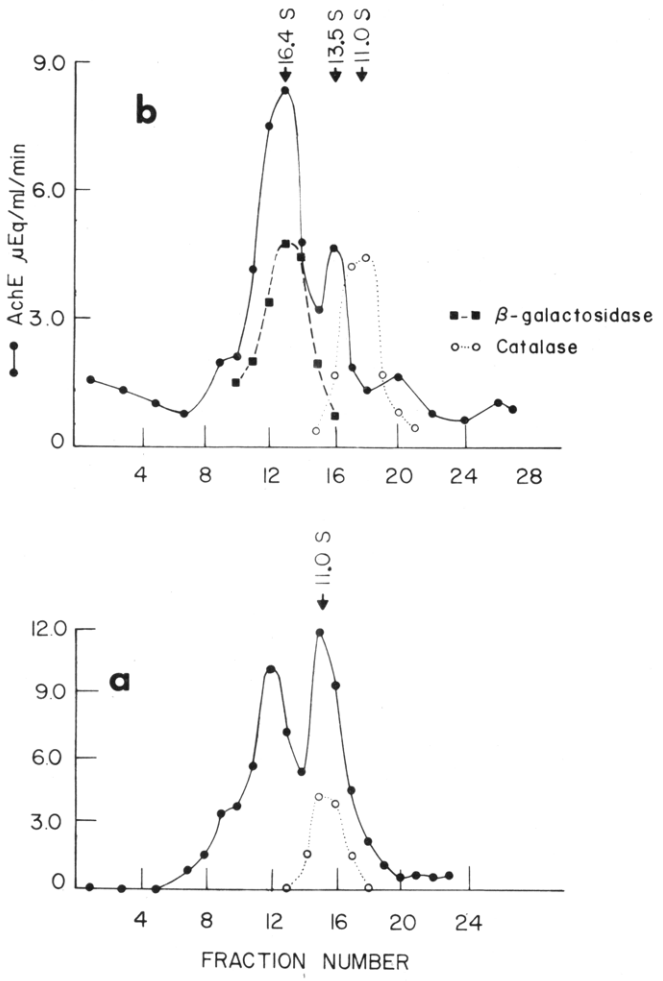

FIGURE 2: Conversion of 16S to 11S AchE by purified collagenase. AchE (16S; 0.3 mg/ml) extracted from the membrane by 2 M $MgCl_2$ and purified by covalent affinity chromatography was incubated in the absence and presence of collagenase IIIa (3 μg/ml) at 37 °C for 10 min. The medium was: 1.0 M NaCl, 0.04 M $MgCl_2$, 0.005 M $CaCl_2$, and 0.01 M Tris-Cl, pH 8.0. Aliquots of 0.1 ml were removed, layered on a 5–20% sucrose gradient containing 1.0 M NaCl, 0.01 M Tris·HCl, pH 8.0, and centrifuged in a SW 50L rotor at 40 000 rpm for 8 h: (a) incubation without collagenase IIIa; (b) incubation with collagenase IIIa.

Conversion of 16S to 11S AchE by Collagenases. Purified collagenases I and IIIa rapidly caused conversion of purified 16S AchE to 11S species (Figure 2). Control samples incubated at 37 °C without collagenase did not show such conversion (Figure 2). We have observed comparable rates of conversion for three AchE preparations obtained by separate techniques and at different levels of purity: the crude 2 M $MgCl_2$ extract, 16S AchE obtained after only sedimentation in a 5–20% sucrose gradient, and 16S AchE after elution from the covalent affinity column (shown here). To further exclude the possibility that collagenase released or activated a trypsin-like enzyme in the crude preparation which, in turn caused the conversion, we looked for collagenase dependent BANA-hydrolytic activity. When either the high salt extract or the homogenate was incubated with collagenase IIIa (3 μg/ml), no progressive increase in rate of BANA hydrolysis was evident. On prolonged incubation (4 h at 37 °C) of collagenase IIIa (20 μg/ml) with purified 11S AchE, there was no detectable change in AchE activity.

Electrophoretic Behavior of the Enzyme Forms. Upon reduction of disulfide bonds with 1 mM dithiothreitol in the presence of sodium dodecyl sulfate and after 30-min subsequent alkylation by 3 mM IAA, the 16S AchE purified by covalent affinity chromatography and the 11S trypsin derived enzyme exhibited similar patterns upon gel electrophoresis in the presence of sodium dodecyl sulfate. The major band in each case corresponded to a molecular weight of 75 000–80 000, and a very faint band could be observed at 150 000–160 000 (Figures 3 and 4 and Table II). These bands could be labeled with DFP in approximate proportion to their staining densities. A lightly staining band corresponding to a molecular weight of 120 000 was evident with the 16S preparation but the lower specific activity of the 16S enzyme would preclude a conclusion as to whether this band can be related to the tail unit. A high carbohydrate content of the tail unit or a collagen-like composition could be expected to yield low staining intensities and/or anomalous migration behavior (Furthmayr and Timpl, 1971).

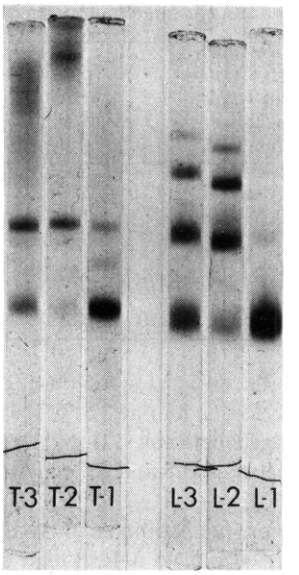

FIGURE 3: Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate for purified AchE. The 11S and 16S species were run on 3% polyacrylamide gels in the presence and absence of dithiothreitol. (L-1) 11S AchE reduced with 1 mM dithiothreitol and alkylated with 3 mM IAA in the presence of 1% sodium dodecyl sulfate; (L-2) 11S AchE that was alkylated by 3 mM IAA prior to addition of sodium dodecyl sulfate; (L-3) 11S AchE reduced with 1 mM dithiothreitol and alkylated with 3 mM IAA prior to addition of sodium dodecyl sulfate. The 16S tailed species are denoted by T-1 to T-3 where the numbers correspond to the reduction and alkylation conditions described for the 11S enzyme.

Electrophoresis of the trypsin derived enzyme following alkylation by IAA and subsequent denaturation in sodium dodecyl sulfate yielded four bands with molecular weights corresponding to the monomeric through the tetrameric species. Integration of the densitometer traces shows a ratio of staining densities for monomer through tetramer which would correspond to a *population* ratio of 41:35:18:6% for the respective species (Table II). For the 16S enzyme a totally different pattern emerged where only monomer and dimer were present in a *weight* ratio of 1:2, but the bulk of the enzyme subunits appeared in a rather diffuse band with a molecular weight greater than that for the tetrameric species ($\sim 10^6$ daltons).

If reduction and subsequent alkylation took place prior to sodium dodecyl sulfate denaturation, slightly more monomeric species was present in the 11S enzyme, and yet all

four species could still be detected. Thus, prior to denaturation, the intersubunit disulfide linkages should be at least partially occluded. The same procedure when applied to the 16S enzyme also yielded slightly more monomer, and the heavier diffuse peaks migrated farther into the gels which would be indicative of partial dissociation. Nevertheless, these species were of higher molecular weight than that expected for the tetramer of the 11S enzyme. If, as seems likely, the high molecular weight species detectable with the 16S form but not the 11S form, is a consequence of head-tail disulfide bonding, the interacting sulfhydryl species are also not directly accessible to solvent.

Cross-Linkin Studies. The population distribution of oligomeric species in the reduction-alkylation experiments prompted one to ascertain whether similar patterns might be achieved with chemical cross-linking agents. The profile for the 11S species following reaction with 1 mM dimethyl suberimidate at 4 °C for 12 h and subsequent reduction with dithiothreitol showed four major bands in diminishing proportions of 68:22:8:4%. Although some intermolecular cross-linking was evident, the molecular weights of the dominant peaks corresponded to those expected of monomer through tetramer (Figure 4 and Table II). Cross-linking at room temperature for 3 h gave a pattern similar to the one obtained after 12 h of cross-linking at 4 °C. If the cross-linked AchE was not reduced, there was an increase of trimer and tetramer with a concomitant decrease in monomer and dimer.

The tailed (16S) species, after cross-linking followed by complete reduction in the presence of sodium dodecyl sulfate, gave two protein bands with molecular weights corresponding to monomer and dimer in a weight ratio of 1:1; however, the bulk of protein existed as a diffuse band with a molecular weight range of $\sim 1.0 \times 10^6$. Thus, DMS can establish covalent cross-linkages between head and tail units. When the cross-linked tailed species was subjected to sodium dodecyl sulfate disc gel electrophoresis without reduction, three bands, all with molecular weights $>1.0 \times 10^6$, were discernible. No attempt has been made to calibrate the molecular weights of the large molecular weight species owing to their diffuse banding patterns.

Amino Acid Composition. Amino acid analyses of trypsin-derived AchE (Taylor et al., 1974) were compared with those of the 16S tailed species after 24-h hydrolysis in 6 N HCl (Table III). The most significant difference was the presence of small amounts of hydroxyproline and hydroxylysine and the enhanced glycine content in the 16S species.

Comparative Properties of Trypsin and Collagenase Released AchE. Although both collagenase and trypsin released AchE species that were soluble at low ionic strength, it seemed plausible that, since the two proteolytic enzymes exhibit different amino acid residue specificity, subtle differences exist which might be missed with separate analytical steps. To obviate this difficulty, the individual AchE's were labeled with different isotopes of DFP. The respective DIP-AchE's could then be admixed and subjected to identical analytical conditions. Isotopic ratios would be indicative of gross similarity of species.

Collagenase and trypsin derived AchE's had identical hydrodynamic properties when sedimented in 5–20% sucrose gradients (Figure 5b). Furthermore, upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis, their subunits showed identical mobilities (Figure 5c). These observations are consistent with near-complete removal of the tail unit by the two proteolytic enzymes.

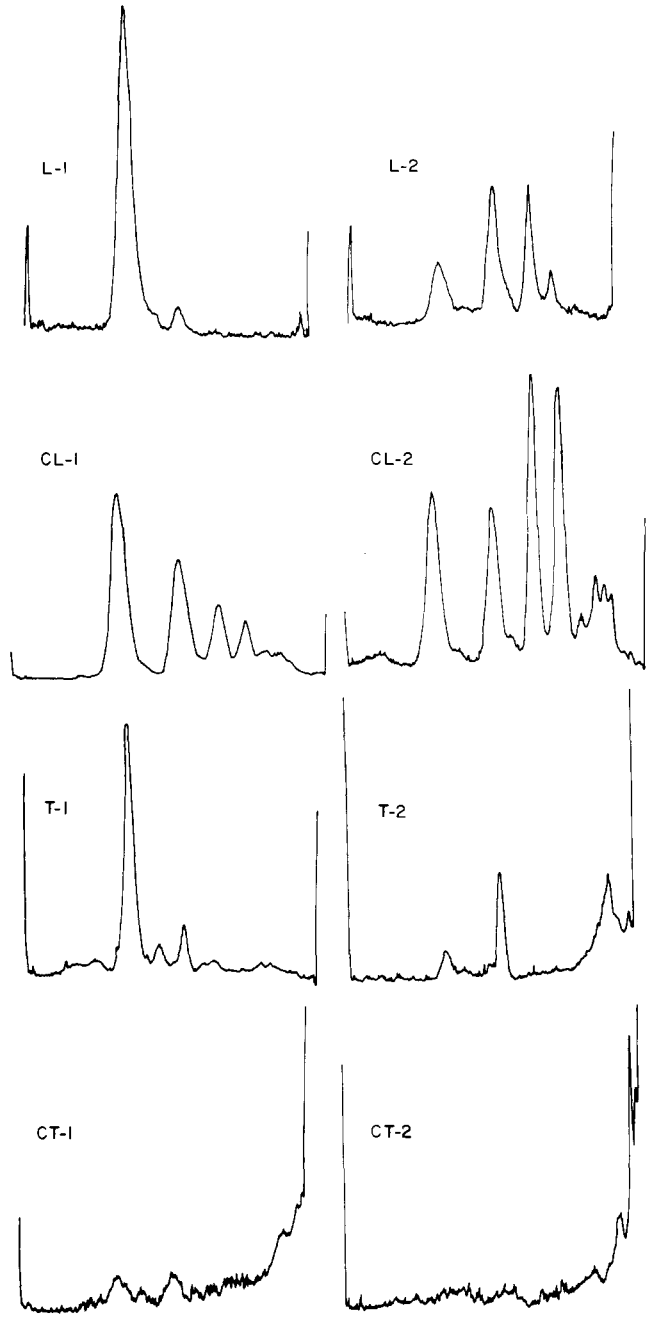

FIGURE 4: Densitometry traces of 3% polyacrylamide gels of the various forms of purified AchE. The 11S and 16S species and conditions of reduction have the same notation as in Figure 3. CL-1, CL-2, CT-1, CT-2 denote lytic (11S) and tailed (16S) enzyme that has been cross-linked by reaction with 0.5 mM DMS for 12 h at 4 °C. The numbers correspond to the reduction conditions of Figure 3. The bromophenol blue band was marked and is represented at the left margin with the top of the gel at the right margin.

Fractionation of Membrane Fragments by Sucrose Density Centrifugation. Upon density gradient centrifugation of the electric organ membranes, two distinct peaks of AchE activity were obtained: one centering at 48% sucrose and the other remaining near the top of the gradient (Figure 6). The slower moving peak was present in variable amounts in the individual experiments; but in most cases the bulk of AchE migrated with the fast sedimenting peak. AchE peaks were at least partially separable from the cobra α-toxin binding fraction, Na^+,K^+-stimulated ATPase activity, and the bulk of the other membrane proteins and phospholipids (Figure 6).

Table II: Oligomer Population of Lytic (11S) and Tailed (16S) AchE Species.

	% Monomer[c]	% Dimer[c]	% Trimer[c]	% Tetramer[c]	% High Mol Wt Species[c]
Lytic Enzyme (11S)[a]					
Complete disulfide reduction	97	3			
Disulfide reduction of the native enzyme	62	28	8	2	
Nonreduced	41	35	18	6	
Lytic (11S)[a] (crossed-linked with dimethyl suberimidate)					
Complete reduction	68	22	8	4	
Nonreduced	47	19	19	14	
Tailed enzyme (16S)[b]					
Complete disulfide reduction	89	11			
Disulfide reduction of the native enzyme	21	23			56
Nonreduced	8	19			73
Tailed (16S)[b] (cross-linked with dimethyl suberimidate)					
Complete reduction	11	12			78
Nonreduced	8	4			89

[a] Oligomer populations were calculated from the staining density divided by the number of 80 000 subunits in the oligomeric species. Separate preparations of 11S enzyme have been examined and the oligomer population varied by less than 5% between preparations. [b] In the case of the tailed enzyme, the precise molecular weight of the high molecular species has not been ascertained; thus oligomer populations cannot be calculated and only weight percentages of the various species are given. [c] The total percentages may not equal 100 because of rounding off to two significant figures.

Table III: Amino Acid Composition of 16S-Tailed and 11S-Lytic Acetylcholinesterase (24-Hour Hydrolysis).

Residue[a]	16S AchE (mol %)	11S AchE[b] (mol %)	Difference (mol %)
Asp	9.69	11.51	(−)1.82
Thr	4.72	4.09	(+)0.63
Ser	7.87	8.41	(−)0.54
Gln	11.82	12.66	(−)0.84
Pro	6.64	5.68	(+)0.96
Gly	11.42	8.65	(+)2.77
Ala	5.85	4.66	(+)1.19
Val	6.57	5.96	(+)0.51
Met	2.20	3.06	(−)0.86
Iso	4.64	3.69	(+)0.95
Leu	8.66	9.22	(−)0.56
Tyr	2.34	2.97	(−)0.63
Phe	5.08	5.69	(−)0.61
His	2.61	3.38	(−)0.67
Lys	5.17	5.50	(−)0.33
Arg	4.80	4.92	(+)0.08
Hyp	1.12		(+)1.12
Hyl	0.3		(+)0.3

[a] Since tryptophan, glucosamine, and cysteine have not been measured for the 16S species, mole per cent values are calculated on the basis of analyzed residues. [b] Taylor et al. (1974); 24-h hydrolysis.

The separation of the receptor and AchE containing membranes confirm earlier findings of Duguid and Raftery (1973). In addition, hydroxyproline containing proteins were concentrated in the fast-sedimenting AchE peak. Thus, the fast-sedimenting AchE peak appears enriched in components characteristic of the basement membrane and is separable from the bulk of membrane components.

Reassociation of AchE Species with Electric Organ Membrane Fragments. When purified 11S AchE was labeled with DFP and mixed with the membrane fraction that was layered on the gradient shown in Figure 6, it did not reassociate with the fast-sedimenting AchE peak upon centrifugation (Figure 7b). The tailed 16S form of AchE, however, whether a 2 M $MgCl_2$ extract or the fraction purified by covalent affinity chromatography, reassociated with the fast-sedimenting AchE peak (Figure 7c). A tailed 8S AchE species was also found to reassociate with the fast-sedimenting AchE peak. In the absence of the membrane fraction, the individual tailed enzyme species (16S) or the 11S form remained near the top of the gradient which reflects lack of formation of large aggregates (Figure 7a). The selective reassociation illustrates that the tail unit appears critical for in vitro AchE membrane association and a preferential association occurs with a fraction enriched in basement membrane components. It should be recognized that the membrane fraction with which the AchE associates is heterogenous and these reassociation experiments can not establish whether the microscopic disposition of the reassociated enzyme at all resembles that for the native membrane bound enzyme.

Discussion

Extraction of tailed species from membranes was achieved using either NaCl which produces 20% solubilization with an 8S species predominating or $MgCl_2$ which gave 16S species in much higher yields (80% solubilization). The S value of the released enzyme in some measure depends upon the conditions used to solubilize it and this finding suggests caution in considering a particular species native, because it predominated with a certain solubilization procedure. The AchE in *Torpedo* then differs from *Electrophorus* where 1 N NaCl was found to extract the bulk of AchE as 18S species and only smaller amounts of 14S and 8S enzyme were apparent (Massoulié and Reiger, 1969). The morphologic findings of Rieger et al. (1973) and Cartaud et al. (1975) indicate that the 18S, 14S, and 8S species in *Electrophorus* possess ratios of tetrameric head-to-tail units of 3, 2, and 1, respectively. In some cases the connecting filaments to each of the three sets of tetrameric head units in the larger 18S species have been observed (Cartaud et al., 1975).

Conventional separation methods have proved largely unsuccessful in the purification of tailed AchE species due to their tendency to aggregate at low ionic strength. Hence, we have restored to immunoadsorbent columns or the covalent affinity columns described by Voss et al. (1975) to effect a

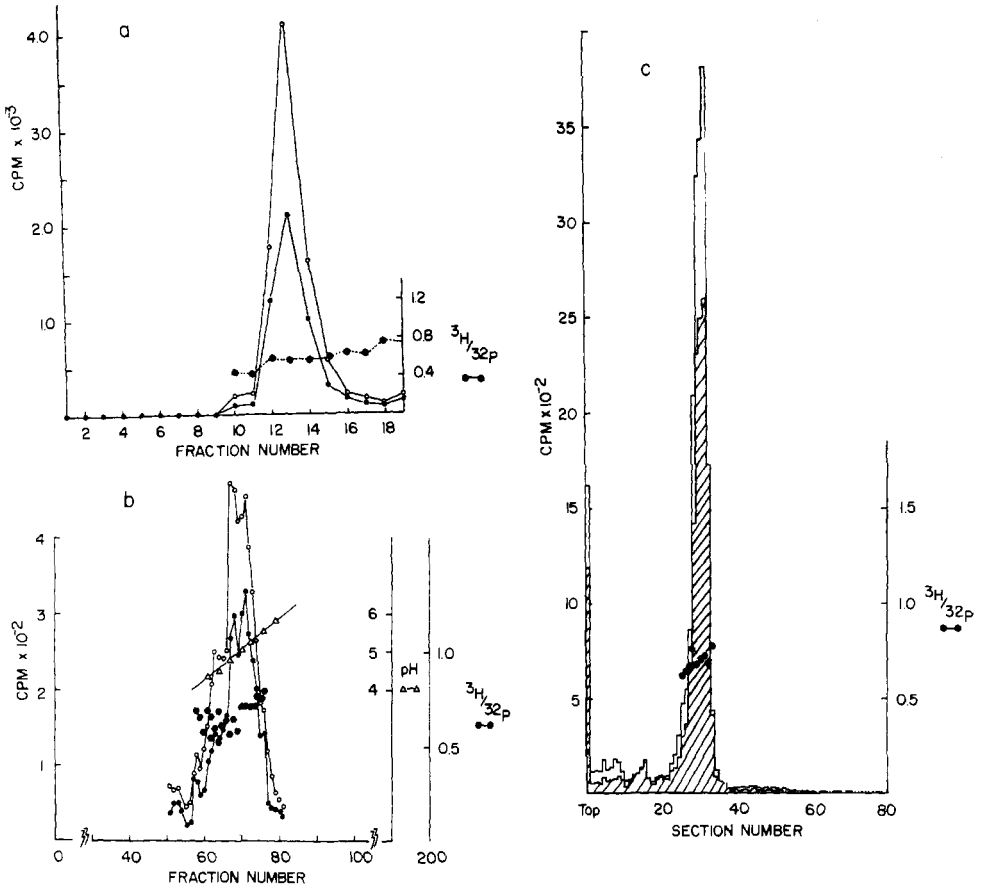

FIGURE 5: Comparative behavior of collagenase- and trypsin-derived AchE. The enzymes were purified by affinity chromatography following their dissolution from the membrane by the respective proteolytic enzyme. The labeled enzymes were labeled with the respective isotope of DFP, dialyzed, and then subjected to the various analytical procedures. The [^{32}P]DFP was diluted with unlabeled DFP so that the two isotopes had specific activities of the same magnitude prior to reaction. (O—O) Collagenase-derived [^{32}P]DIP-AchE; (●—●) trypsin-derived [^{3}H]DIP-AchE; (●- - -●) ratio ^{3}H/^{32}P. (a) Sedimentation in a 5–20% sucrose gradient in a SW 50L rotor at 40 000 rpm for 8 h, 4 °C. (b) Isoelectric focusing in a pH 3–10 ampholyte medium at 4 °C for 48 h. Approximately 0.5 mg of each enzyme was placed on the column and 200 fractions were collected from the 110-ml column. (c) Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, 7.5% acrylamide gels. The samples were reduced with 1 mM dithiothreitol prior to application to the gels. The clear and cross-hatched portions denote counts as ^{3}H and ^{32}P, respectively.

purification in 1.0 N NaCl. Although yields have been low, sufficient quantities of tailed AchE have been obtained to permit a partial characterization of the tailed species. Massoulié et al. (1971) have employed sequential gel filtration and density gradient sedimentation steps to purify tailed enzyme species from *Electrophorus,* while Dudai et al. (1973) have utilized affinity chromatography using *N*-methylacridinium as the reversibly associating ligand covalently attached to the gel matrix.

Upon gel electrophoresis in the presence of sodium dodecyl sulfate, the 16S enzyme purified from the covalent affinity column appeared to be ~90% pure if estimates are based on staining density of the 80 000 species; yet its specific activity is maximally 52% that of the 11S enzyme. The molecular weight of the tail unit in 8S *Electrophorus* AchE has been estimated on the basis of hydrodynamic measurements to be 60 000 (Massoulié et al., 1971). Assuming a ratio of four 80 000 catalytic subunits to a single tail unit, the contribution of the mass of the tail alone could not account for the lower specific activity of our tailed species. However, bleeding of the covalent affinity column is known to occur in which the enzyme serine remains phosphorylated, but becomes detached by cleavage of oligosaccharide residues in the agarose matrix (Voss et al., 1975) and this may account for the lower specific activity. The presence of an inactive phosphorylated species should not influence the structural data on the tailed enzyme since the oligosaccharide chain conjugated to the enzyme would be of low molecular weight.

Arrangement of Subunits (Quaternary Structure). Reduction by sulfhydryl reducing agents and reaction with the cross-linking agent, DMS, has enabled one to gain some insight into the arrangement of the head and tail units and of the individual subunits of AchE. First, the presence of monomeric through tetrameric species of AchE subunits in the absence of reduction supports the titrimetric and ligand binding stoichiometry data that the lytic enzyme consists of four identical 80 000 molecular weight subunits (Taylor et al., 1974). Secondly, the observation that reduction, alkylation, and removal of the excess reducing agent prior to denaturation still yields the oligomeric species suggests that the intersubunit disulfide bonds are not completely exposed to solvent.

The formation of a high molecular weight species (1×10^6 to 1.5×10^6) with the tailed (16S) enzyme only in the absence of reducing conditions demonstrates that the primary head–tail interaction involves disulfide bonds. This species appears diffuse on the gels which precludes a precise estimate of its molecular weight. If the tail contains a collagen-like sequence or is susceptible to partial proteolytic attack, anomalous or non-uniform migration in the gel might be anticipated (cf. Furthymayr and Timpl, 1971). As in the

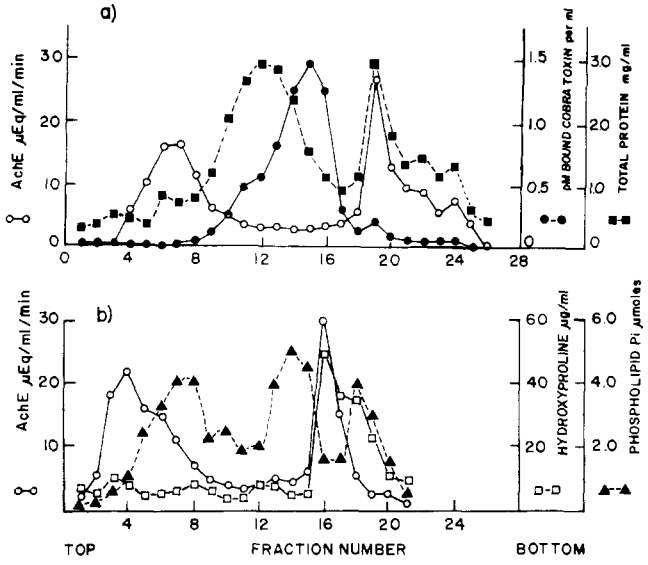

FIGURE 6: Fractionation of *Torpedo californica* electric organ membranes on a 17–60% sucrose gradient. Membranes were prepared by differential centrifugation and 5 ml of resuspended membrane fraction is layered over a 55-ml 17–60% sucrose gradient as described by Duguid and Raftery (1973) and centrifuged at 25 000*g* for 10 h in a Beckman SW 25.2 rotor. Gradient (a): (O—O) AchE (μeq/(min ml)); (●—●) cobra α-toxin bound (pM/ml); (■- - -■) total protein. Gradient (b): (O—O) AchE (μeq/(min ml)); (□—□) hydroxyproline in μg/ml; (▲- - -▲) phospholipid phosphorus (μeq/ml). Although not shown, the major peak of Na^+,K^+-sensitive ATPase centered between fractions 10 and 14 in gradient (a).

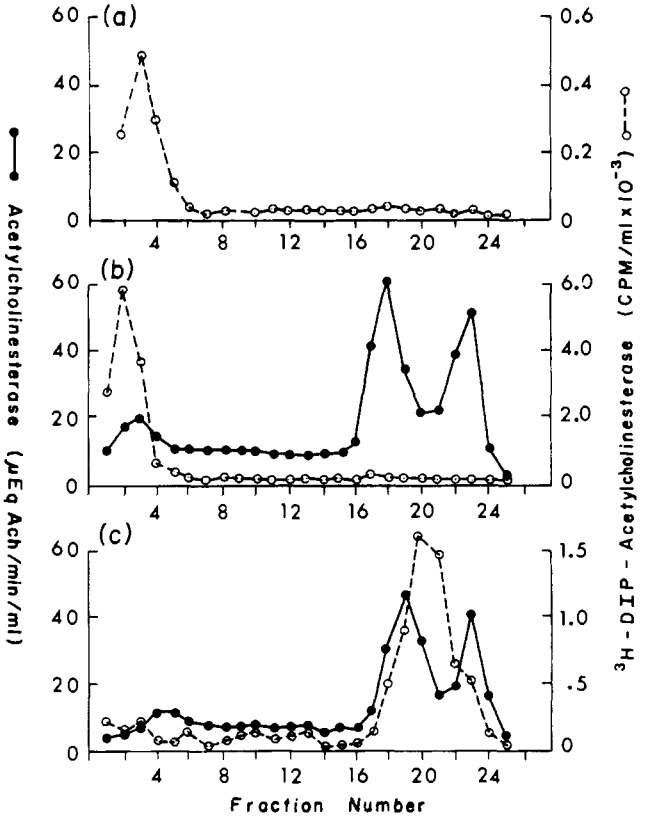

FIGURE 7: Reassociation of AchE species with a membrane fraction. The 16S and 11S AchE forms were purified following high salt or trypsin treatment of the membranes as described in Methods. The purified enzymes were labeled with DFP, dialyzed, and then mixed with the crude membrane preparation 30 min prior to centrifugation. The membrane preparation was identical with that put on to the gradient for the fractionation shown in Figure 6. The centrifugation conditions were the same as those shown in Figure 6. (●—●) Endogenous AchE; (O- - -O) [^{3}H]DIP-AchE: (a) 16S AchE in the absence of the membrane fraction; (b) 11S AchE with the membrane fraction; (c) 16S AchE with membrane fraction.

case of the interaction between subunits, the disulfide bonding between head and tail is at least partially sequestered from the solvent.

Arrangement of Subunits: 11S Enzyme. The oligomer distribution of unreduced *Torpedo* AchE contrasts with that of the *Electrophorus* AchE lytic species when run under similar conditions. The latter gave a population distribution of ~75% dimer with most of the remainder existing as monomer (Rosenberry et al., 1974) and was considered to be a dimer of dimers $(\alpha_2)_2$. The relatively uniform distribution in *Torpedo* where significant populations of trimer and tetramer are evident is indicative of more equivalent subunit interactions. Precise quantitation of intersubunit contacts in terms of heterologous and isologous subunit arrangements (Cornish-Bowden and Koshland, 1971) would be precluded by nonidentity of interacting species if residual tail fragments were present. A comparison of subunit distribution with *Electrophorus* AchE may be further complicated by a proteolytic cleavage of the 80 000 subunit in partially autolyzed *Electrophorus* preparations (Dudai and Silman, 1974; Rosenberry et al., 1974) which appears to be absent in the *Torpedo* enzyme.

If we assume that the rates of forming cross-links with a bifunctional reagent are solely dependent upon spatial arrangement of the subunits and their reactive groups, then analysis of the distribution of cross-linked species after intra-oligomeric cross-linking would reflect the quaternary structure of the enzyme. A descending ratio of monomer to tetramer would be observed in heterologous arrangements where the rates of intersubunit cross-linking were the same provided intra-oligomeric cross-linking does not approach completion. If the domains of subunit interfaces are not equivalent (e.g., an isologous arrangement), then dimer and tetramer could predominate over trimer. The latter situation has been observed after cross-linking α-isopropylmalate synthetase from *Salmonella typhimurium* using DMS (Kohlhaw and Boatman, 1971) and with the hexameric leucine aminopeptidase (Carpenter and Harrington, 1972).

In Torpedo AchE, cross-linking with DMS followed by denaturation and complete reduction gave profiles of integrated staining densities in decreasing order of monomer to tetramer (Figure 4). This observation has the following implications. (a) It is consistent with earlier evidence (Taylor et al., 1974) that lytic AchE is composed of four identical subunits with molecular weight of 80 000 daltons. The proposal of identical subunits differs from that of Powell et al. (1973) who postulated that AchE in *Electrophorus* consists of two pairs of nonidentical subunits; but is in accord with the findings of Rosenberry et al. (1974) who showed similarity of subunits on basis of cyanogen bromide fragmentation and gel electrophoresis. (b) The interfaces between one subunit and any two or more adjacent subunits exhibit similar reactivity.

Arrangement of Subunits: Tailed 16S Species. The spatial arrangement of the head subunits in the presence of the til is far more complex to analyze since dissimilar subunits must be considered. When the tailed 16S AchE is not reduced, no trimers of head subunits are detected despite substantial amounts of dimer (Table II). This contrasts with the lytic 11S species where trimer prevails and indicates that the tail remains affixed whenever three head subunits

are linked. With this situation the head–tail interaction should be asymmetric with respect to the four head subunits. The gel profile cannot be explained by partial formation of the 11S species since detectable quantities of monomer through tetramer then would be evident.

Structure of the Tailed 16S Species. The enhanced glycine content and presence of the collagen-containing amino acids, hydroxyproline and hydroxylysine in the 16S species, are indicative of the filamentous tail unit possessing a collagen-like sequence. The facile cross-linking of head to tail species by a bifunctional reagent and the observation that purified collagenases, demonstrated to be free of caseinolytic and amidase activity but active against native collagen, will promote the conversion of purified 16S AchE to an 11S form both support the contention that the collagen-like fragments are not simply associated with the salt extracted tailed preparations but form an integral part of the tailed structure. While we can not completely rule out the possibility that the collagenases activate or release another lytic enzyme that cleaves the tail, this possibility seems less likely since it would have to copurify with the 16S species. Nevertheless, we have been unable to achieve specific activities of the tailed enzyme consistent with only the molecular weight addition of the structural component.

Collagenases from *Clostridium* are known to have a high degree of substrate specificity, requiring the amino acid sequence Pro-X-Gly-Pro-Y, where X and Y can be any amino acid residue. The enzyme releases an N-terminal glycine (Harper and Kang, 1970). With this sequence requirement it seems unlikely that trypsin and collagenase would release 11S enzymes with very similar hydrodynamic and electrophoretic properties unless the above sequence existed near the point of head-to-tail attachment. The collagen characteristic amino acids, hydroxyproline and hydroxylysine, are found in sufficiently different substitution frequencies in other collagen-like sequences that their composition alone does not enable one to determine the extent of this sequence. However, in the collagen sequence, the nonunique amino acid, glycine, is present at every third residue and should constitute 33 mol % of the sequence. The glycine differences between the 16S and 11S species would account for a molecular weight of 40 000 attributable to a collagen-like sequence of a total molecular weight of 360 000. However, this value should be considered provisional since a difference in mole percent between two large numbers is subject to substantial error. Secondly, the presence of glycine in a noncollagenous portion of the tail would reduce its contribution to the collagen-like sequence.

Considerable sequence and structural detail is known about the subcomponent, Clq, of the human complement system. This 400 000 molecular weight plasma protein is fan-shaped where six terminal globular units are connected by rod-like connecting units (1.5 × 10–13 nm) to a central core (Shelton et al., 1972). Dissociation of the Clq with disulfide bond reduction yields three similar but not identical chains, A, B, and C, each of which contains hydroxyproline and hydroxylysine. Sequence analysis of the A chain shows 38% of the residues in collagenous peptides (N-terminal glycines are released with collagenase) and these are located in the center portion of the linear sequence (Reid et al., 1972; Reid, 1974). Thus, the Clq protein appears to differ from the 16S AchE in that AchE would contain the collagenous and non-collagenous regions in separate peptide chains which are interlinked only by disulfide bonds. An analysis of the C- and N-terminal residues of the 11S enzyme derived from collagenase and trypsin treatment of the 16S enzyme would provide more definitive information on this point. Also, procollagen (Bornstein et al., 1972), and perhaps basement membrane protein (Kefalides, 1973; Grant et al., 1973), contains collagenous and noncollagenous sequences within the same polypeptide chain. Owing to the presence of basement membrane material within the synaptic cleft (Pfenninger, 1973) and the nerve ectolemma, it might be considered that the tail unit is a component of the basement membrane.

Basement membranes are distinct extracellular matrices of wide distribution in nature (Kefalides, 1973). Although most structural investigations have been conducted on mammalian basement membranes, comparative studies have revealed similarities in basement layers from sources ranging from sea anemone to mammals (Norwack and Nordwig, 1974). Basement layer from the mammalian capillary basement membrane has been shown by electron microscopy to consist of a fine, partially oriented network of fibrils 3–4 nm in diameter, embedded in an amorphous matrix (Palade and Burns, 1964). The fibrils are composed of a collagen-like protein consisting of identical α_1 fibers in a triple helix arrangement. This component has glycine in a repetitive sequence and contains the collagen-characteristic amino acids hydroxylysine and hydroxyproline where the hydroxylysyl groups are glycosylated with the disaccharide galactosylglucose. Terminal regions of the α_1 chains have a noncollagen-like amino acid composition and fall outside the domains of the triple helix. Noncollagen-like components, along with the terminal regions of the α_1 chains, remain as a nondialyzable "core" when basement membrane is exhaustively digested with collagenase.

The possibility that the AchE tail unit is a component of the basement membrane and possesses a wider distribution than would a unique species associating with the catalytic units of AchE in discrete stoichiometric ratios will require further structural studies than have been reported here. However, our gross structural analysis shows distinct similarities between basement membrane constituents and the tail unit. It is also noteworthy that high ionic strength solutions will dissociate a tailed species while, at low ionic strength, the tailed species, but not the lytic species, will reassociate with a fraction enriched in basement membrane components. Whether the observed reassociation reflects simply structural similarity between the dominant components or bears a resemblance to the AchE within the synapse must also await further study. Nevertheless, these findings provide further evidence that the tail unit has a distinct structural role and that a primary association of the tail unit is with the basement rather than the plasma membrane.

Acknowledgments

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