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Acetylcholinesterase from *Torpedo*: Characterization of an Enzyme Species Isolated by Lytic Procedures

PALMER TAYLOR, JONATHAN W. JONES, AND NORMAN M. JACOBS

Division of Pharmacology, Department of Medicine, University of California, San Diego, La Jolla, California 92037

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SUMMARY

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An acetylcholinesterase has been purified from *Torpedo californica* by mild proteolysis of electroplax membranes with trypsin (5 μg/ml for 5 min) to solubilize the enzyme, followed by affinity chromatography of the soluble enzyme. The procedure yields an apparently homogeneous enzyme whose molecular weight (approximately 335,000), frictional coefficient (1.65), and amino acid composition all distinguished the *Torpedo* acetylcholinesterase from that which has been prepared by lytic procedures from *Electrophorus*. The enzyme is composed of four similar, if not identical, subunits, each of which possesses a catalytic and inhibitor binding site. *Torpedo* acetylcholinesterase contains 7.9% carbohydrate present as hexoses, hexosamines, and sialic acid, and at least some of these residues are exposed on the outer surface of the molecule. Concanavalin A, a plant lectin with specificity toward mannose residues at nonreducing positions, forms a sedimentable complex with the purified acetylcholinesterase which can be partially reversed by α-methyl-D-mannoside. Addition of concanavalin A to electroplax membranes markedly inhibits trypsin-induced acetylcholinesterase release from the membrane surface.

INTRODUCTION

Despite a plethora of kinetic investigations on various acetylcholinesterases (acetylcholine hydrolase, EC 3.1.1.7), detailed studies on the physical properties of the enzyme have been restricted largely to that obtained from the gymnote *Electrophorus electricus*. Early investigations revealed that solubilization of this membrane-bound enzyme to a nonaggregating form with a sedimentation coefficient of 11 S could be accomplished by prior immersion of the tissue in toluene for periods of months to years (1, 2). However, the more recent findings of Massoulié and his colleagues have shown that three species of acetylcholinesterase with sedimentation coefficients of 18, 14, and 8 S are obtained when fresh tissue is extracted with high ionic strength solutions (3–5). Each species exhibits sedimentation and other hydrodynamic behavior which differs from that of the 11 S purified enzyme obtained from toluene-immersed tissue. Treatment of the three species extracted at high ionic strength with trypsin results in their conversion to an 11 S species which, like the purified enzyme,

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does not aggregate at low ionic strength (4, 6). These findings have provided a strong indication that autolysis was essential for the purification of the 11 S species.

The concern might be raised that any purification procedure employing lysis yields a protein lacking a fragment which existed while the native enzyme was associated with the membrane. Also the lytic process may contribute to polydispersity in the isolated macromolecule. Nevertheless acetylcholinesterase preparations obtained by lytic procedures show a reasonable degree of homogeneity and a specificity toward substrates and other ligands that very closely resembles native membrane enzyme preparations (4). Moreover, as has become evident with other membrane-associated proteins such as cytochrome b_5 (7), a comparison of properties of an enzyme isolated by lytic and nonlytic methods can yield valuable information regarding molecular organization of the protein with respect to the membrane surface.

This communication describes the properties of an enzyme isolated by mild and controlled tryptic treatment of electroplax membranes obtained from *Torpedo californica*. A thorough characterization of the protein is essential for detailed studies on ligand interactions with acetylcholinesterase; the initial portion of this work is described in the companion article (8). Distinct differences not only in physical properties but also in susceptibility toward release from the membrane surface are apparent when the *Torpedo* and *Electrophorus* enzymes are compared.

METHODS

Materials. Yeast β-galactosidase, bovine serum albumin, and yeast alcohol dehydrogenase were purchased from Sigma. Catalase, trypsin, and trypsin inhibitor (soybean) were obtained from Worthington. Concanavalin A was a product of Pharmacia. Rabbit muscle phosphorylase *b* and dogfish muscle lactate dehydrogenase were gifts from Dr. S. R. Gross and Dr. S. S. Taylor, respectively, at this institution. *N*-Methyl-(7-dimethylcarbamoxy)quinolinium iodide was synthesized according to a published procedure (9). Edrophonium was kindly provided by Dr. W. E. Scott, Hoffmann–La Roche, Nutley, N. J.

Enzyme purification. Torpedo californica weighing between 4 and 28 kg were obtained primarily through the Scripps Institute of Oceanography. The electric organs, which constituted 15–20% of the body weight, were dissected and frozen at −40°.

For individual preparations of enzyme, 0.5–1.0 kg of tissue was homogenized with 2 volumes of 0.01 M Tris-Cl buffer, pH 8.0, containing 0.1 M NaCl and 0.04 M $MgCl_2$ (Tris-Na-Mg buffer) in a Sorvall OmniMixer at 4°. The homogenate was centrifuged for 2 hr at 27,000 × *g*. The resulting supernatant fraction, which contained 10–15% of the total acetylcholinesterase activity, was discarded, and the particulate fraction was rehomogenized with 0.5 volume of the original tissue weight of a buffer containing 0.01 M $NaHCO_3$, 0.04 M $MgCl_2$, and 0.1 M NaCl, pH 7.8 (HCO_3-Na-Mg buffer). The resuspended membranes were placed in a 37° water bath. When the stirred membranes reached 25°, trypsin was added to give a final concentration of 5 μg/ml. After 5 min the reaction was stopped by the addition of soybean trypsin inhibitor (10 μg/ml) and cooling the suspension in an ice bath. Following centrifugation at 27,000 × *g* for 2 hr, the supernatant fraction was run over a 0.9 × 15 cm affinity column at a rate of 20–40 ml/hr. The affinity resin was made by attaching *m*-aminophenyltrimethylammonium through an extended arm to Sepharose 4B as described by Berman and Young (10). By coupling a ^3H-labeled derivative of the ligand to the matrix, the level of substitution was found to be 1.2 μmoles/ml of packed resin. After washing the adsorbed protein with 5–10 column volumes of the HCO_3-Na-Mg buffer, elution was achieved by addition of 10 mM edrophonium to the buffer and reducing the flow rate to about 5 ml/hr. Acetylcholinesterase appeared within the first 6 ml of the elution front in concentrations up to 2.5 mg/ml in peak tubes. Edrophonium was removed by dialysis against 100 volumes of Tris-Na-Mg buffer (four changes), and the samples were stored at −20° in polypropylene tubes. Chromatography and dialyses were conducted at 4°.

Enzyme activity and protein determinations. Enzyme activity was monitored at 25° by a pH-stat method with a Radiometer titrator. The assay medium consisted of 0.1 M NaCl, 0.04 M $MgCl_2$, and 1.25 mM acetylcholine chloride, and the pH was adjusted to 8.0. Protein concentrations were determined by the method of Lowry *et al.* (11), using bovine serum albumin as a standard.

Sedimentation studies. The sedimentation coefficient for the purified enzyme was measured in a Beckman-Spinco model E ultracentrifuge with schlieren optics. The enzyme concentration was 1.84 mg/ml, and the Tris-Na-Mg buffer medium was used. Molecular weight was determined by sedimentation equilibrium measurements in the model E instrument, using the high-speed meniscus depletion method of Yphantis (12). Runs were made in the same buffer with protein concentrations between 0.3 and 0.4 mg/ml. Apparent weight-average molecular weights, $\bar{M}_{app}$, were calculated with the following equation:

$$\bar{M}_{app} = \frac{2RT}{(1 - \bar{\nu}\rho)\omega^2} \frac{d \ln c}{dr^2}$$

where ω is the angular velocity and the other symbols are standard.

Sedimentation velocity measurements in 5–20% sucrose gradients were carried out as described by Martin and Ames (13). The buffered medium differed from the standard buffer in that the total NaCl concentration was 1.0 M. One hundred microliters of sample in this buffer were layered over a 5.0-ml gradient. After centrifugation at 148,000 × *g* for 8 hr in a Spinco SW-50L rotor, 120-μl samples were collected by puncturing the bottom of the tube.

Amino acid and carbohydrate composition. Hydrolyses were performed with 6 N HCl in evacuated, sealed glass tubes at 110°, and the hydrolysates were run on a Beckman amino acid analyzer according to the procedures of Moore and Stein (14). For tryptophan, hydrolyses were performed with *p*-toluenesulfonic acid in the presence of tryptamine as described by Liu (15). Samples of lactate dehydrogenase and carboxypeptidase were also run to ensure that tryptophan recovery was quantitative.

The thiobarbituric acid method (16) was used to determine neuraminic acid content. Samples were initially hydrolyzed in 0.1 N H_2SO_4 for 6 hr. For neutral sugar determinations the samples were first hydrolyzed with 1 N H_2SO_4 for 8 hr at 100° in evacuated ampoules. A neutral sugar fraction was obtained by passing the hydrolysate over a tandem resin column containing Dowex 50 (H^+) and Dowex 1 (formate) as detailed by Spiro (17). Neutral sugars in the resulting effluent were assayed with the anthrone reagent (17). Individual sugars were identified by thin-layer chromatography on silica gel G, using a 4:1 propanol–water solvent. Naphthylresorcinol reagent was employed to detect reducing sugars.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was conducted on 5.6% acrylamide gels as described by Fairbanks *et al.* (18).

Electrofocusing measurements. Electrofocusing experiments with the purified acetylcholinesterase were conducted on 110-ml LKB 8101 columns, using the pH 5–10 LKB ampholyte solution. Focusing in the electric field was carried out for 36 hr with a 400-V potential difference. The temperature was maintained at 4°.

Gel filtration. An upward flow column 2.5 × 60 cm containing Sephadex G-200 and the Tris-Na Mg-buffer was used in the gel filtration studies. The columns were maintained at 4°, and the flow rate was regulated between 10 and 16 ml/hr. Columns were calibrated using blue dextran, yeast β-galactosidase, urease, catalase, lactate dehydrogenase, and human carbonic anhydrase C. The acetylcholinesterase diffusion coefficient was estimated according to the formulae described by Porath (19).

Equivalence titrations. Active site serines were titrated with the carbamoylating agent *N*-methyl-(7-dimethylcarbamoxy)-quinolinium iodide (9). Kinetics of carbamoylation was measured with a Farrand spectrophotofluorometer coupled to a recorder. At high substrate concentration it was necessary to use a Durrum stopped-flow spectrophotometer equipped with fluorescence detection. Normality of inhibitor binding sites was determined with the fluorescent inhibitor bis(3-aminopyridinium)-

1,10-decane (20) or by quenching of enzyme tryptophan fluorescence using 2,5-bis(3-diethyl-*o*-chlorobenzylammonium-*n*-propylamino)benzoquinone chloride (8).

RESULTS

Release of Acetylcholinesterase from Its Membrane-Associated Form

Figure 1A shows the time course of acetylcholinesterase solubilization from a crude particulate fraction from *Torpedo* electroplax. Trypsin at 5 μg/ml causes a rapid release of enzyme from the membranes, and after 5–10 min no further solubilization is evident. In the absence of trypsin solubilization proceeds at a far slower rate. Although incubation with trypsin appears to solubilize about 90% of the acetylcholinesterase originally detectable in the resuspended membranes, treatment with the proteolytic enzyme also increases the total amount of assayable enzyme. Thus, by the latter criterion, only 60–70% of the total acetylcholinesterase is solubilized.

Concanavalin A, a lectin from jack bean which possesses agglutinating activity, binds with high specificity to α-D-mannosyl and α-D-glucosyl residues at nonreducing termini in polysaccharides and glycoproteins (21–23). Addition of 0.5 mg/ml of concanavalin A appears to prevent acetylcholinesterase solubilization by trypsin (Fig. 1B). Two additional observations demonstrate that the probable action of concanavalin A is to prevent the initial solubilization process rather than to aggregate solubilized acetylcholinesterase. First, addition of 10 mM α-methyl-D-mannoside, which acts as a haptenic determinant for concanavalin A (21, 23), does not solubilize or dissociate acetylcholinesterase from the membrane preparation which was incubated in the presence of concanavalin A and trypsin. Second, incubation with trypsin followed by a 10-min exposure to concanavalin A results in the major portion of the enzyme remaining solubilized.

Properties of Enzyme in Crude Extract

When acetylcholinesterase is extracted from the crude membrane preparation by high ionic strength buffer, multiple peaks are observed on density gradient centrifugation,

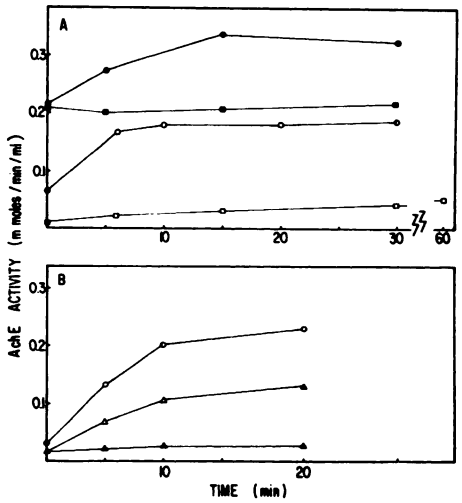

FIG. 1. *Solubilization of acetylcholinesterase (AchE) from Torpedo electroplax membranes*

A. A particulate fraction from electroplax was prepared and resuspended as described in METHODS. Two-milliliter fractions of the suspension were added to tubes in a 37° water bath. When the temperature reached 25° (about 40 sec), trypsin was added to give a concentration of 5 μg/ml and incubation was allowed to proceed with gentle mixing for the designated period of time. The reaction was terminated by addition of soybean trypsin inhibitor (10 μg/ml) and cooling of the samples to 4°. Samples were centrifuged at 45,000 × *g* for 30 min. □——□, incubation without trypsin, 45,000 × *g* supernatant fraction; ○——○, incubation with trypsin, 45,000 × *g* supernatant fraction; ■——■, incubation without trypsin, total suspension; ●——●, incubation with trypsin, total suspension.

B. The incubation procedure was the same except for the use of concanavalin A. ○——○, 5 μg/ml of trypsin without concanavalin A; ▲——▲, 5 μg/ml of trypsin incubated in the presence of concanavalin A (0.5 mg/ml), both incubations terminated by addition of 10 μg/ml of trypsin inhibitor and 10 mM α-methyl-D-mannoside; △——△, incubation with 5 μg/ml of trypsin, terminated by trypsin inhibitor followed by 10 min of incubation with concanavalin A and subsequent addition of 10 mM α-methyl-D-mannoside. Acetylcholinesterase activity was measured on 45,000 × *g* supernatant fractions.

with the major portion of the enzyme sedimenting as a 7.8 S species (Fig. 2A). In contrast, a 5-min tryptic digestion of the membrane preparation yields a soluble enzyme having a discrete sedimentation profile at 11 S (Fig. 2B). Conversion of the acetyl-

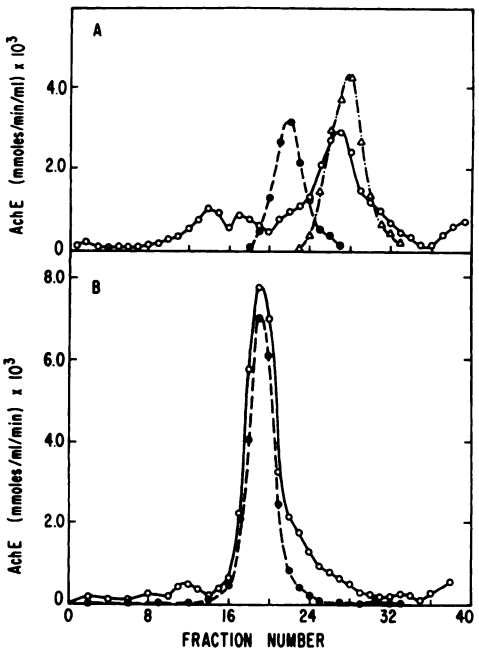

FIG. 2. *Sedimentation behavior of acetylcholinesterase (AchE) solubilized from electroplax membranes*

A. High ionic strength extract from fresh tissue. Freshly dissected electroplax was homogenized in 2 volumes of pH 8.0 buffer containing 1.0 M NaCl, 0.04 M $MgCl_2$, and 0.01 M Tris-Cl. Then 0.1 ml of a 27,000 × *g*, 60-min supernatant fraction was layered over 5 ml of a 5–20% sucrose gradient in the above buffer with yeast alcohol dehydrogenase, 7.4 S, and catalase, 11.2 S, added as marker proteins. The samples were centrifuged at 148,000 × *g* for 8 hr. ●- - -●, catalase; △-·-·△, alcohol dehydrogenase; O——O, acetylcholinesterase.

B. Trypsin-solubilized acetylcholinesterase. The enzyme was solubilized by incubation of electroplax membranes with 5 μg/ml of trypsin for 5 min as described in METHODS, and 0.1 ml of the supernatant fraction was layered on the gradient described above. ●- - -●, catalase; O——O, acetylcholinesterase.

cholinesterase by trypsin to a soluble 11 S species, which is found only in minor amounts upon high salt extraction of membranes, confirms the general observations of others on lytic conversion of acetylcholinesterase species (3, 6). However, two differences should be noted. First, upon lysis the major portion of the *Torpedo californica* enzyme exhibits an increase in sedimentation coefficient (7.8 S to 11 S). In contrast, high ionic strength extraction of electroplax from *Electrophorus* and *Torpedo marmorata* yielded three species (approximately 8, 14, and 18 S) with only 10% of the enzyme having a sedimentation coefficient in the vicinity of 8 S (3, 4, 6).

Second, an apparent difference from *Electrophorus* exists in the sensitivity toward tryptic digestion. Extensive solubilization and complete conversion of the solubilized *Torpedo* acetylcholinesterase occur after 5–10 min of digestion with 5 μg/ml of trypsin. For *Electrophorus* a 16-hr incubation of the total homogenate with 140 μg/ml of trypsin was necessary to achieve complete conversion to an 11 S species (6). Thus, while proteolytic enzymes influence the sedimentation properties of acetylcholinesterase from the various species of electric fish, differences in the distribution of the enzyme forms and in the susceptibility to lytic conversion are evident.

Purification of Acetylcholinesterase Obtained by Lytic Procedures

Exposing larger volumes of the crude membrane fraction to 5 μg/ml of trypsin for 5 min was the initial step employed in the purification of acetylcholinesterase. The solubilized enzyme could then be purified in high yield by affinity chromatography. The results of a typical purification scheme are described in Table 1. Between 70 and 85% of the solubilized enzyme was retained by the columns. Most of this was specifically removed in a 4–6-ml volume by 10 mM edrophonium. Subsequent elution with 1 M NaCl removed another 5–15% of the total enzyme, but of lower specific activity. A second cycle of the complete procedure usually yielded about 25% of the acetylcholinesterase obtained initially.

Properties of the Purified Enzyme

Electrophoretic behavior. Upon electrofocusing at 4° the purified enzyme behaves essentially as a single species, exhibiting an isoelectric point of 5.36 (Fig. 3). It has not been possible to compare this isoelectric point with that of acetylcholinesterase species obtained from salt-extracted membranes, owing to aggregation of a large amount of protein in the latter case.

TABLE 1

Summary of solubilization and affinity chromatography purification procedure for Torpedo acetylcholinesterase

The initial pellet fraction was resuspended in the HCO_3-Na-Mg buffer and incubated for 5 min with 5 μg/ml of trypsin at 25°. After centrifugation at 27,000 × *g* for 1 hr, the supernatant from the trypsin-treated fraction was placed over the affinity column as described in METHODS.

Procedure	Acetylcholinesterase activity	Protein	Specific activity
	mmoles/g tissue hr^{-1}	*mg/g tissue*	*mmoles/mg protein* hr^{-1}
1. 1:2 homogenate of electroplax	11.6	11.3	1.03
Supernatant fraction (27,000 × *g*, 2 hr)	1.23	7.4	0.17
Pellet (27,000 × *g*, 2 hr)	10.8		
2. Supernatant fraction (II) following incubation of resuspended pellet with trypsin	6.48	1.14	5.7
3. Edrophonium eluate from affinity chromatographic column	5.36	0.0147	363
4. 1 N NaCl eluate from affinity chromatographic column	0.42	0.030	14.0
5. Supernatant fraction (III) following second incubation of pellet with trypsin	3.48	0.51	6.8

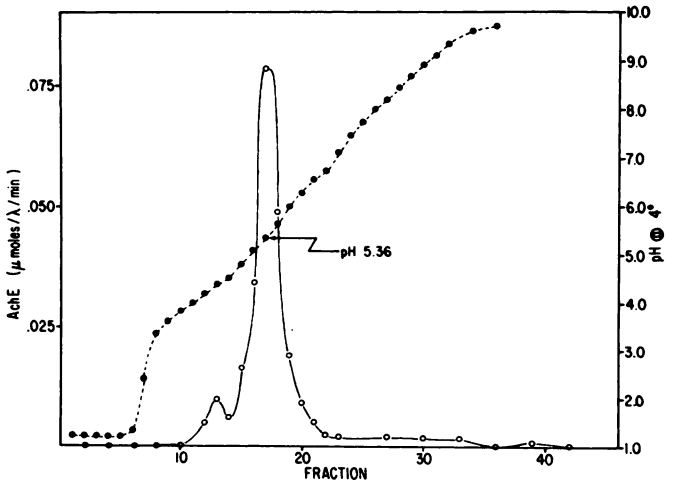

FIG. 3. *Isoelectric focusing of purified acetylcholinesterase*

One-half milligram of acetylcholinesterase purified as described in METHODS was subjected to electrofocusing for 36 hr at 4° in a 110-ml column. The potential difference was 400 V. The pH was measured at 4°, with the electrodes standardized at the same temperature.

As shown in Fig. 4, polyacrylamide gel electrophoresis in the presence of SDS[1] reveals the existence of a single band, which by comparative staining density can be judged to be approximately 95% pure. The band carries essentially all of the [^{3}H]-diisopropoxyphosphoryl counts following labeling of the protein with diisopropyl 1-[^{3}H]fluorophosphate. Higher molecular weight bands are observed when electrophoresis is carried out in the absence of dithiothreitol. Calibration of the rate of migration of the acetylcholinesterase band shows that it resides between phosphorylase

[1] The abbreviation used is: SDS, sodium dodecyl sulfate.

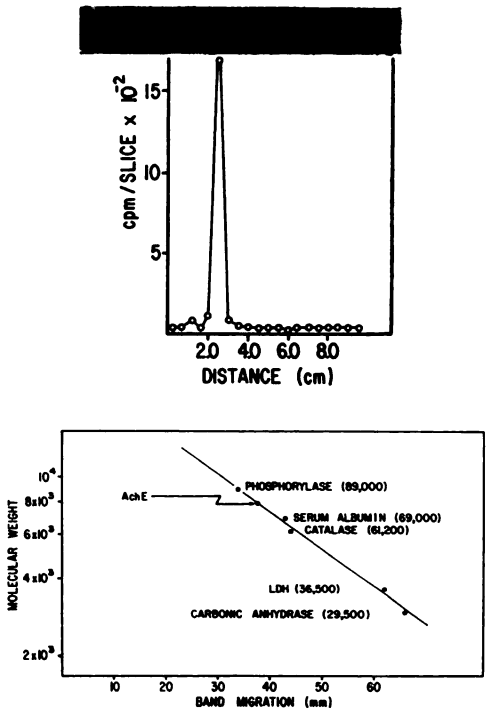

FIG. 4. *Polyacrylamide gel electrophoresis in the presence of SDS and dithiothreitol*

Upper panel: Shown below the gel is the distribution of ^3H after inhibition of acetylcholinesterase by diisopropyl 1-[^{3}H]fluorophosphate. Prior to preparation for gel electrophoresis, the enzyme was allowed to react with 50 μM diisopropyl 1-[^{3}H]fluorophosphate until the activity had decreased by 97%.

Lower panel: Calibration of the migration of acetylcholinesterase (AchE) relative to other protein subunits of known molecular weight. Distances of migration for the standard proteins were determined on parallel gels within the same run. LDH, lactate dehydrogenase.

b and bovine serum albumin (Fig. 4). This has yielded molecular weight estimates of 79,000–82,000 for the SDS-dissociated subunit.

Sedimentation and hydrodynamic behavior. Sedimentation velocity measurements on the lytic purified acetylcholinesterase, using schlieren optics, also show the presence of a single species and yield an $s_{20,w}$ value of 10.8 S at a protein concentration of 1.84 mg/ml in the standard Tris-Na-Mg buffer. This absolute measurement agrees well with the estimate of 11.0 S obtained from density gradients using far lower protein concentrations.

In sedimentation equilibrium, plots of ln Δy (fringe displacement) vs. r^2 are linear (Fig. 5). Thus the purified acetylcholinesterase exhibits behavior characteristic of a single macromolecule which does not undergo dissociation or aggregation under conditions of the experiment. The partial molar volume, $\bar{v}$, has been calculated from the amino acid (24) and carbohydrate composition (25) to be 0.716. Using this value, the molecular weight is estimated to be 338,000 (Table 2).

Acetylcholinesterase is eluted just slightly ahead of β-galactosidase but behind the void volume marker, blue dextran, upon gel filtration on upward flow Sephadex G-200 columns (Fig. 6). Elution behavior in molecular exclusion chromatography provides a more accurate reflection of the effective Stokes radius of the molecule than molecular weight (26, 27). Using the empirical relationship between the diffusion coefficient and

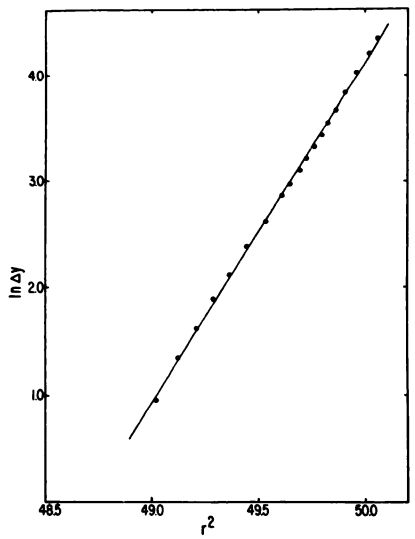

FIG. 5. *Sedimentation equilibrium analysis of purified acetylcholinesterase*

Acetylcholinesterase (0.33 mg/ml) in 0.1 M NaCl, 0.04 M $MgCl_2$, and 0.01 M Tris-Cl, pH 8.0, was centrifuged in the Spinco model E ultracentrifuge at 12,590 rpm at 20°. Photographs were taken of Rayleigh fringes after 16 and 22 hr. Δy, vertical fringe displacement; r, distance from the center of rotation.

TABLE 2

Physical properties of Torpedo californica acetylcholinesterase

Details of individual procedures are described in METHODS.

$s_{20,w}$	10.8 S
Molecular weight (sedimentation equilibrium)	338,000
Molecular weight (gel filtration and sedimentation velocity)	333,000
f/f_0	1.65
Stokes radius (gel filtration)	76 A
Subunit molecular weight (SDS)	78,000–82,000
Equivalent weight (serine carbamoylation)[a]	77,500 ± 7,200
Equivalent weight [titration with bis(3-aminopyridinium)-1,10-decane]	87,000
Equivalent weight [titration with 2,5-bis(3-diethyl-*o*-chlorobenzyl-ammonium-*n*-propylamino)benzoquinone]	84,000
$E^{1\%}_{280}$	17.5
Specific activity[b]	345 ± 44
Turnover No.[c]	4.81×10^5

[a] Mean value of six determinations ± standard deviation. The determinations were made on the same batch of enzyme.

[b] Mean ± standard deviation for six separate preparations of enzyme, in millimoles per hour per milligram of enzyme. The extinction coefficient given above or the Lowry protein assay, with the correction described in the text, was employed to ascertain enzyme concentration.

[c] Millimoles per minute per milliequivalent of enzyme, assuming four active sites per 335,000 molecular weight.

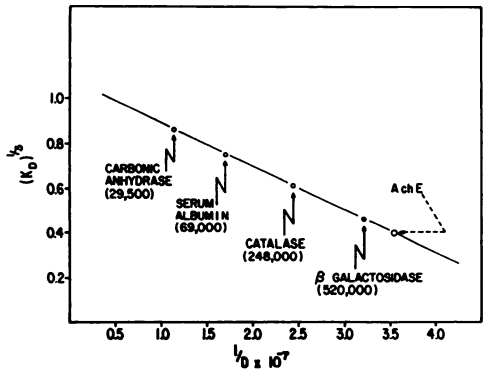

FIG. 6. *Upward flow gel filtration of acetylcholinesterase (AchE)*

Elution volumes were measured on upward-flow, 2.5 × 60 cm Sephadex G-200 columns equilibrated with 0.1 M NaCl, 0.04 M $MgCl_2$, and 0.01 M Tris-Cl, pH 8.0, at 4°. K_D, the distribution coefficient, was obtained from the elution volumes as described by Porath (19) and related to the diffusion coefficient as shown.

elution volume described by Porath (19) (Fig. 6), the Stokes radius of acetylcholinesterase is estimated to be 76 A. The diffusion coefficient, D, obtained from gel filtration and the $s_{20,w}$ value of 10.8 from the sedimentation velocity measurements have been used to calculate a molecular weight of 333,000 from the formula

$$M = \frac{RT}{(1 - \bar{v}\rho)} \frac{S}{D}$$

In turn, a frictional coefficient, f/f_0, of 1.65 may be calculated from the formula

$$\frac{f}{f_0} = \frac{a}{(3\bar{v}M/4\pi N)^{1/3}} \quad (26)$$

In the above formulae, a denotes the Stokes radius, R the gas constant, and N Avogadro's number. The high frictional coefficient is reflected in the observation that, while acetylcholinesterase possesses a sedimentation coefficient similar to catalase, which has a molecular weight of 248,000, on exclusion chromatography it is eluted just ahead of β-galactosidase, with a molecular weight of 520,000.

Amino acid and carbohydrate composition. The amino acid composition obtained from sequential hydrolyses of the purified enzyme is given in Table 3. These values may be compared with those reported for the *Electro-*

TABLE 3

Amino acid and carbohydrate composition

Residue	*Torpedo californica*[a]				*Electrophorus electricus*[c]	Difference[d]
	24 hr	48 hr	72 hr	Mean or selected integral value[b]		
						%
½Cys	9.8	9.8	10.2	10	10.6	−7.8
Asp	72.4	75.5	73.9	74	84.7	−15.7
Met	19.3	19.6	20.9	20	17.5	+9.8
Thr	27.9	26.9	28.5	28	29.2	−4.6
Ser	52.9	50.3	47.5	55	44.1	+17.9
Glu	79.7	75.3	73.3	75	66.9	+17.7
Pro	35.8	34.3	35.7	35	38.4	−7.0
Gly	53.9	54.8	53.8	54	56.2	−4.2
Ala	29.3	29.4	29.3	29	40.3	−31.6
Val	37.6	42.8	44.5	44	45.6	−19.2
Ile	23.2	26.0	27.9	28	24.3	−4.6
Leu	58.0	60.0	61.6	61	55.5	+4.4
Tyr	18.7	18.6	18.1	18	23.5	−32.2
Phe	39.8	37.2	38.1	38	34.2	+15.1
His	21.3	20.4	20.1	20	14.8	+36.1
Lys	34.6	34.0	32.9	34	29.6	+15.6
Arg	31.0	31.5	29.8	31	33.4	−7.5
Trp[e]				19	12.9	+39
Hexosamine[e]				19		
Sialic acid[f]				2.9		
Hexose[g]				11.1		

[a] Calculations based on a 82,500 subunit.

[b] The value for serine was taken from an extrapolation to zero time; the 72-hr hydrolysis time was used for valine, leucine, and isoleucine

[c] From Rosenberry *et al.* (28), 24-hr hydrolysis. Because carbohydrate residues were not assayed, data are based on a 76,000 unit.

[d] Difference in amino acid content is taken as the difference between species (*T. californica* − *E. electricus*) divided by the average. Comparison is made from 24-hr hydrolysis values.

[e] Determined following *p*-toluenesulfonic acid hydrolysis (15) at 24, 54, and 64 hr and extrapolation to zero time value.

[f] Thiobarbituric acid method (16).

[g] Anthrone method; calculated as mannose residues (17).

phorous enzyme (28), and substantial differences ranging between 30 and 40% are apparent for particular residues (e.g., alanine, histidine, tyrosine, and tryptophan).

An analysis of sugar content indicates that the total carbohydrate in acetylcholinesterase is substantial (7.9% by weight) and must be taken into account in estimating the equivalent weight of the protein. Glucosamine has been identified as the hexosamine by the position of the peak on the basic column. As expected for glycosidically linked neuraminic acid, color development in the thiobarbituric acid assay only occurs with prior acid hydrolysis. Treatment of the purified enzyme with *Clostridium perfringens* neuraminidase released 2.75 residues of the 2.90 neuraminic acid residues/82,500 subunit that are released by hydrolysis with 0.1 N H_2SO_4. Thin-layer chromatography of the neutral sugar fraction reveals the presence of mannose and galactose and a trace of glucose. Serial dilutions would indicate that mannose comprises 70–80% of the total neutral sugar content.

Specific activity. A protein analysis which is dependent on peptide bond or nitrogen content will yield a low estimate of the amount of acetylcholinesterase. Since we have routinely used the Lowry assay for protein, we have corrected that value by 7.9% to allow for the carbohydrate contribution in estimating the amount of acetylcholinesterase. With this correction, we find an extinction coefficient, $\epsilon_{280}^{1\%}$, of 17.5, where $\epsilon_{280}/\epsilon_{254} = 2.05$. Using the above criteria to determine enzyme concentration, the specific activity of the purified acetylchoinesterase in the various preparations averaged 345 ± 44 (SD) mmoles/mg of enzyme per hour. Assuming a molecular weight of 335,000 and four catalytic sites, the turnover number would be 4.81×10^5 min^{-1}.

Equivalent weight titrations. Two high-affinity bisquaternary inhibitors, bis(3-aminopyridinium)-1,10-decane iodide and 2,5-bis(3-diethyl-*o*-chlorobenzylammonium-*n*-propylamino)benzoquinone chloride, were employed in ascertaining the equivalence of binding sites. The former is fluorescent and shows a decrease in quantum yield when bound to acetylcholinesterase (20). Progressive addition of enzyme results in a decrease in ligand fluorescence, and the equivalence point can be estimated from the titration (Fig. 7A). The latter inhibitor quenches the tryptophan fluorescence of acetylcholinesterase upon binding (8); thus titration of a known weight of enzyme will also yield an

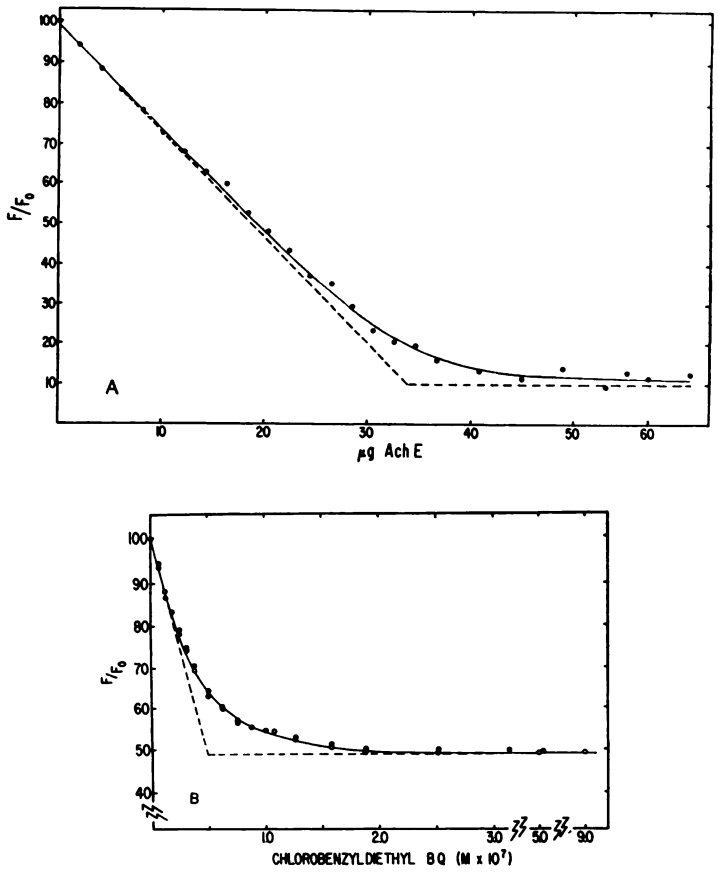

FIG. 7. *Determination of binding site equivalence using bisquaternary inhibitors*

A. Titration of 2.0 μM bis(3-aminopyridinium)-1,10-decane with purified acetylcholinesterase (AchE) in 0.1 M NaCl–0.04 M $MgCl_2$–0.01 M Tris-Cl (pH 8.0) buffer. Excitation wavelength, 322 nm; emission, 405 nm. The initial volume was 0.2 ml, so that 4×10^{-10} mole of inhibitor was present.

B. Titration of 0.039 mg/ml of acetylcholinesterase with 2,5 bis(3-diethyl—*o*-chlorobenzylammonium-*n*-propylamino)benzoquinone (BQ) chloride in the same buffer as above. Excitation wavelength, 290 nm; emission, 335 nm.

estimate of the stoichiometry of binding sites (Fig. 7B). Titrations in which quantum yield differences in the ligand are measured give an equivalent weight estimate near 87,000. Monitoring of the protein fluorescence upon titration with the higher-affinity benzoquinone ligand yields a value of 84,000.

A second means of estimating stoichiometry involves the use of acid-transferring substrates which react with the enzyme serines at a rate greatly exceeding the subsequent hydrolysis of the serine ester bond (9). The nonfluorescent substrate *N*-methyl-(7-dimethylcarbamoxy)quinolinium iodide (CQ) releases the fluorescent product *N*-methyl-7-hydroxyquinolinium (Q) concomitant with carbamoylation with the serine residues as shown below.

$$E + CQ \underset{}{\overset{K_c}{\rightleftharpoons}} E\cdots CQ \xrightarrow{k_2}$$

$$E\text{—}C + Q \xrightarrow{k_3} E + C$$

As described by Rosenberry and Bernhard (9), upon addition of substrate the fluorescence increase is characterized by an initial burst followed by slower enhancement. The excursion of the initial burst stoichiometrically reflects the carbamoylation of the active site serines. From six separate reactions we

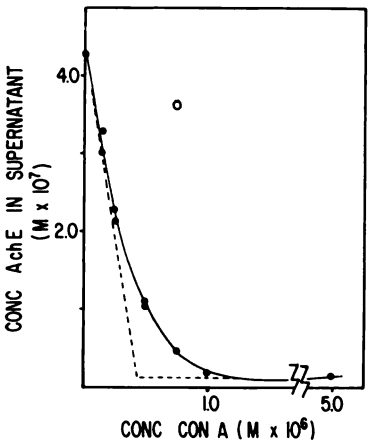

FIG. 8. *Association between acetylcholinesterase (AchE) and concanavalin A (con A)*

Purified acetylcholinesterase was mixed with increasing concentrations of concanavalin A at 4° in 0.05 M sodium acetate buffer, pH 5.5, containing 1 mM $MnCl_2$ and 1 mM $CaCl_2$. After 5 min, samples were centrifuged at 27,000 × *g* for 30 min and the supernatant fractions were assayed for acetylcholinesterase activity. O, extent of aggregation when 10 mM α-methyl-D-mannoside was added to acetylcholinesterase prior to concanavalin A.

find a mean equivalent weight of 78,000 for active site serines[2] (Table 1).

Reaction with concanavalin A. The high mannose content in acetylcholinesterase indicates that it will specifically interact with concanavalin A provided that the carbohydrate residues are exposed on the outer surface of the acetylcholinesterase molecule. Titration of the enzyme with increasing quantities of concanavalin A results in the formation of a sedimentable complex (Fig. 8). Prior to sedimentation, acetylcholinesterase activity in the saturated complex is diminished by 70%, indicating that the enzyme–concanavalin A aggregate shows a reduced catalytic efficiency. Concanavalin A possesses four identical subunits, and each 25,200 subunit species contains a single carbohydrate binding site (23). Apparent 1:1 stoichiometry between the 82,000 acetylcholinesterase and the 25,000 concanavalin A subunits is observed. This macroscopic stoichiometry cannot be taken to indicate that at saturation each acetylcholinesterase subunit contains an associated concanavalin A subunit. Formation of a multidimensional complex of this nature may well be precluded by steric constraints. Nevertheless, the observation that the complex is sedimentable by centrifugation at 27,000 × *g* for 30 min would be indicative of formation of a polyaggregated species.

The point scatter precludes an accurate determination of the dissociation constant of the aggregate; however, it can be estimated to be between 10^{-8} and 10^{-7} M. The interaction between the two proteins shows a specific involvement of the carbohydrate residues on acetylcholinesterase, since it is largely inhibited by α-methyl-D-mannoside, a sugar which serves as a haptenic determinant for concanavalin A.

DISCUSSION

By employing mild and controlled tryptic digestion of electroplax membranes from *Torpedo californica*, an acetylcholinesterase has been purified to apparent homogeneity and characterized. Although acetylcholinesterase purified by the above procedure from *Torpedo* has a sedimentation coefficient similar to that of an *Electrophorus* enzyme whose preparation also involves lysis (10.8 vs. 11.0 S), discrete differences in physical properties between the two enzymes are evident. Molecular weight estimates for the purified *Electrophorus* enzyme vary between 240,000 and 265,000 (2, 29, 30). These values clearly fall outside the range of 338,000 and 333,000 estimated from our sedimentation equilibrium and gel filtration data (Table 2). Moreover, for *Electrophorus* acetylcholinesterase, gel filtration measurements in which molecular weight was directly related to elution volume gave an estimate of 245,000 (28). A direct correlation between elution volume and molecular weight can only be achieved with globular proteins; thus the gel filtration molecular weight estimate would be consistent with a previously reported frictional coefficient of 1.23 for the *Electrophorus* enzyme (29).

[2] We have observed that the maximal rate constant for carbamoylation, k_2, $\cong$45 min^{-1} and K_s $\cong$0.4 mM in the pH 8.0 standard buffer at 25°. These constants differ by an order of magnitude from those reported for the *Electrophorus* enzyme (9, 20) and apparently reflect intrinsic differences in substrate specificity of acetylcholinesterases from the two sources.

In contrast, the *Torpedo* enzyme migrates slightly ahead of β-galactosidase (520,000) upon gel filtration. When nonglobular or dimensionally asymmetrical macromolecules are considered, elution volumes more closely correlate with the hydrodynamic radius of the macromolecule than its molecular weight (26, 27). The Stokes radius and $s_{20,w}$ values were used to calculate a frictional coefficient of 1.65 for *Torpedo* acetylcholinesterase. The above hydrodynamic differences indicate that the *Torpedo* enzyme is not only a heavier but a dimensionally more asymmetrical molecule than *Electrophorus* acetylcholinesterase.

SDS–acrylamide gel electrophoresis of the *Torpedo* enzyme reveals a single band which calibrates as an 80,000 molecular weight species. This value is in accord with equivalence measurements on catalytic and inhibitor binding sites (78,000–87,000) and indicates the presence of four active subunits.

Dudai *et al.* (6) reported the presence of two major bands of 80,000 and 60,000 molecular weight for *Electrophorus*. With the same species Bock *et al.* (31) have observed an 80,000 band and three lower molecular weight bands. In their brief note these authors indicated that the more rapidly migrating bands may arise from excessive proteolysis during the toluene extraction. Dissociation of *Electrophorus* acetylcholinesterase by guanidine HCl has yielded subunit species of 64,000 (29), although recent studies involving low pH guanidine dissociation have yielded values of 42,200 and 21,500 (32). Estimates of stoichiometry for the *Electrophorus* enzyme have produced disconcordant results, and two, three, and four active sites have been proposed (20, 28, 33, 34).

The turnover number of 4.8×10^5 min^{-1} for *Torpedo* acetylcholinesterase is slightly lower than values obtained for the *Electrophorus* enzyme. Extensive measurements by Rosenberry *et al.* (28) have yielded a value of 8.0×10^5 min^{-1} for the latter species. These authors used a correction factor averaging 12% to account for inactive enzyme (28), which was not employed in our estimates of *Torpedo* acetylcholinesterase turnover numbers.

Some of the differences in physical properties of the *Electrophorus* enzyme that have been reported may reflect varied degrees of proteolysis which occur in the preparation of a purified 11 S species. Likewise, the dissimilarities between the *Torpedo* and *Electrophorus* acetylcholinesterases could arise from the procedures used to obtain a solubilized enzyme. However, this possibility would seem less likely, since Dudai *et al.* (6) have demonstrated similar properties and composition in acetylcholinesterases isolated by either toluene extraction or trypsin treatment. Thus we feel that the distinctions in physical properties between the gymnote and torpednidae acetylcholinesterases would also be evident if the native membrane-associated enzymes were compared. That such differences exist is indicated by the different composition of acetylcholinesterase species which are extractable at high ionic strength (Fig. 2A) and by the relative susceptibility toward proteolytic solubilization (Fig. 1). The requirement of incubation with only 5 μg/ml of trypsin for 5 min may prove to be a fortuitous advantage for *Torpedo* extracts in yielding more monodisperse subunit species.

The acetylcholinesterase isolated and characterized in this study possesses a substantial carbohydrate content and a high frictional coefficient. The ratio of frictional coefficients (f/f_0) relates the asymmetry of the macromolecule to that of a rigid sphere of equivalent mass. Both shape and hydration factors contribute to this value. Typical globular proteins have frictional coefficients of 1.14–1.31; thus higher ratios result from dimensional asymmetry or additional bound solvent (35). While the greater hydrogen bonding capacity of surface glycoproteins would tend to immobilize additional solvent, it would seem unlikely that a frictional coefficient of 1.65 would be a consequence of hydration factors alone. Thus the isolated acetylcholinesterase should exist as a dimensionally asymmetrical structure.

The presence of about 33 carbohydrate residues per subunit, of which 3 are sialic acid and are usually found at terminal positions, would indicate that multiple oligosaccharide chains are attached to each subunit core. Determination of the precise number of oligosaccharide chains must await isolation

of individual glycopeptides. Specific interaction between concanavalin A and acetylcholinesterase, which can be prevented by α-methyl-D-mannoside, demonstrates the accessibility of the sugar moieties. The attached oligosaccharides, being hydrophilic, may function to orient the enzyme relative to the membrane surface.

The protection by concanavalin A from trypsin-induced solubilization of membrane-associated acetylcholinesterase (Fig. 1B) could well arise from binding of concanavalin A to the same oligosaccharide chains shown to be present on the purified acetylcholinesterase molecule (Fig. 8). Concanavalin A predominates as a 100,000 molecular weight tetrameric species above pH 7 (28); thus, being of size comparable to an acetylcholinesterase subunit, it could prevent access of trypsin to peptide bonds in the enzyme susceptible to cleavage. Alternatively, protection could be afforded by concanavalin A binding to the presumed acetylcholinesterase fragment which is cleaved, or to a neighboring saccharide-containing macromolecule on the membrane surface. Brown (36) has observed recently that concanavalin A adsorption to L-cells grown in culture protects against trypsin-induced release of particular classes of glycopeptides from the cell surface. We have observed that concanavalin A also will inhibit tryptic conversion of the solubilized 7.8 S species to the 10.8 S species. However, it is difficult to compare quantitatively the amount of concanavalin A required for protection in the crude membrane and the high salt-solubilized acetylcholinesterase preparations.[3]

The relationship between the purified 10.8 S and the 7.8 S species which can be extracted from the membranes by high ionic strength solutions remains an unresolved but intriguing problem. The dependence of the conversion between species on trypsin and other hydrolyases (3, 6) and its inhibition by concanavalin A would suggest the involvement of direct peptide bond hydrolysis. However, the present findings on the crude membrane system cannot rule out the possibility that trypsin releases or activates another enzyme which catalyzes the conversion.

If proteolysis of acetylcholinesterase occurs upon addition of trypsin, the increase in sedimentation coefficient from 7.8 S to 10.8 S is indicative of a larger decrease in frictional coefficient than equivalent mass loss. This could arise if lysis were to remove a tail-like structure or were to result in effective contraction of the protein structure. An alternative, involving removal of a fragment of low specific gravity, seems less likely in view of the finding of Massoulié *et al.* (5) that the salt-extracted 8, 14, and 18 S species and the purified 11 S species in *Electrophorus* exhibited similar buoyant densities.

Recently Massoulié and his colleagues (37) have observed with electron microscopy tetrameric species associated with elongated fragments in fractions isolated by density gradient centrifugation from high salt extracts of *Electrophorus* electroplax. They have also suggested that the 8, 14, and 18 S enzymes characterized by sedimentation analysis show 1:1, 2:1, and 3:1 ratios of tetrameric to elongated species, respectively.

The tendency of the salt-extracted enzymes to aggregate in low ionic strength media is lost upon lytic conversion to the 11S enzyme (3, 6). The moieties responsible for the aggregation phenomena may be involved in the association of acetylcholinesterase with the membrane. Thus a portion of the native acetylcholinesterase which is cleaved or reoriented upon lytic treatment could well serve, in conjunction with the saccharide residues, to orient the enzyme molecule with respect to the membrane surface.

By using detergent extraction procedures, cytochrome b_5 (7) and cytochrome b_5 reductase (38) have been purified in higher molecular weight forms than were characterized originally from lipase and lysosomal enzyme treatment of microsomal membranes. The presence of the entire molecule is essential for reconstituting these proteins within the membrane (7, 38). The fragment which is lost

[3] An active fluorescent concanavalin A derivative has been prepared by fluorescamine modification of the lectin. Binding studies with the fluorescent derivative show that the binding capacity of the membrane greatly exceeds that which could be due to the acetylcholinesterase molecules alone (P. Taylor and N. M. Jacobs, unpublished observations).

by the lytic treatment contains a preponderance of hydrophobic residues, which appear to partition into the membrane, providing the proper orientation of the catalytic site (7). However, these proteins cannot be dissociated from the membrane by high salt (7), whereas acetylcholinesterase can be, indicating an involvement of different molecular forces in membrane association. Moreover, membrane association of a tetrameric molecule may be subject to additional symmetry constraints not required for the monomeric proteins studied in the hepatic microsomal membranes.

Recently Dudai *et al.* (39) have purified a fraction composed of native 14 S and 18 S acetylcholinesterase species from fresh *Electrophorus* tissue with an affinity column containing an acridinium ligand. Following purification these species retained the sedimentation coefficients observed in the crude extract and still aggregated when exposed to low ionic strength conditions. Upon gel electrophoresis in the presence of SDS no difference between the 14 and 18 S and the lytic purified 11 S species could be discerned. However, the authors stated that lower molecular weight fragments may go undetected in such a procedure.

We have purified the *Torpedo* 7.8 S species to about one-sixth of the specific activity of the lytic species by adsorption of a high ionic strength extract to a concanavalin A–Sepharose column and subsequent elution with α-methyl-D-mannoside.[4] Complete purification of this species should make it possible to determine the structural basis for the 7.8 S to 10.8 S conversion and to compare the physical properties of individual enzyme forms.

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