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PURIFICATION BY AFFINITY CHROMATOGRAPHY OF ACETYLCHOLINESTERASE FROM ELECTRIC ORGAN TISSUE OF THE ELECTRIC EEL SUBSEQUENT TO TRYPTIC TREATMENT

YADIN DUDAI, ISRAEL SILMAN, NURIT KALDERON AND SHMARYAHU BLUMBERG
Department of Biophysics, The Weizmann Institute of Science, Rehovot (Israel)

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SUMMARY

1. A Sepharose–acetylcholinesterase inhibitor conjugate was prepared by synthesis of the inhibitor [*N*-(ε-aminocaproyl)-*p*-aminophenyl]trimethylammonium bromide hydrobromide and its covalent linkage to the CNBr-activated resin.

2. The Sepharose–inhibitor conjugate was employed for purification of acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) from electric organ tissue by affinity chromatography. Thus the enzyme was selectively adsorbed on the resin and specifically eluted with the soluble acetylcholinesterase inhibitor, decamethonium bromide.

3. The Sepharose–inhibitor conjugate adsorbed the different molecular species of acetylcholinesterase (differing in sedimentation coefficient) present in electric organ tissue. Since two of these species aggregate at low ionic strength, the partially purified enzyme also displayed this property.

4. Highly purified acetylcholinesterase was obtained if affinity chromatography was preceded by controlled tryptic digestion or prolonged autolysis causing conversion of the enzyme to an 11-S form which does not aggregate at low ionic strength.

5. Purified acetylcholinesterase was obtained in an overall yield of 40%. It was essentially homogeneous on acrylamide-gel electrophoresis, and its sedimentation coefficient (approx. 11 S), specific activity and amino acid composition resembled those previously reported for purified acetylcholinesterase.

6. Active site titration of the purified enzyme yielded an equivalent weight of 107 000 per active site. Acrylamide-gel electrophoresis in the presence of sodium dodecyl sulfate and mercaptoethanol revealed two major polypeptide components of molecular weights approx. 88 000 and 64 000.

7. The properties of the purified acetylcholinesterase are compared with those of the purified preparations previously reported, and its relationship to the molecular species present in intact electric organ tissue is discussed.

Abbreviations: PTA, phenyltrimethylammonium; ε-aminocaproyl-PTA, [*N*-(ε-aminocaproyl)-*p*-aminophenyl]trimethylammonium bromide hydrobromide; DFP, diisopropylfluorophosphate.

INTRODUCTION

Acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) is widely distributed in excitable membranes of nerve and muscle and its molecular properties are of interest because of its involvement in synaptic transmission[1]. The enzyme has, so far, only been purified extensively from the electric organ of the electric eel[2,3]. A general and convenient preparative method, allowing the isolation in high yields of acetylcholinesterase, is therefore desirable.

The technique of affinity chromatography permits convenient purification of an enzyme by its selective adsorption on a suitable resin to which a specific inhibitor of the enzyme has been covalently attached[4–7]. Resins in which inhibitors are covalently linked to CNBr-activated Sepharose have proved particularly suitable[7,8]. In a preliminary communication[9], we reported the preparation and application of an inhibitor–Sepharose conjugate suitable for the purification of acetylcholinesterase. The inhibitor synthesized for covalent linkage to Sepharose was [*N*-(ε-aminocaproyl)-*p*-aminophenyl]trimethylammonium bromide hydrobromide (ε-aminocaproyl-PTA, Fig. 1). This compound was chosen since phenyltrimethylammonium

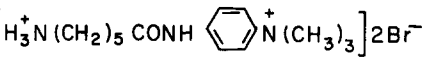

Fig. 1. Structure of [*N*-(ε-aminocaproyl)-*p*-aminophenyl]trimethylammonium bromide hydrobromide, the acetylcholinesterase inhibitor attached to Sepharose.

(PTA) is a good inhibitor of acetylcholinesterase[10], and the ε-aminocaproyl chain serves to covalently link the quaternary group to the activated resin. Berman and Young[11] have also recently described the preparation and utilization of acetylcholinesterase inhibitor–Sepharose conjugates.

Massoulié and co-workers[12,13] have reported that acetylcholinesterase occurs in extracts of electric organ tissue as three molecular species, with sedimentation coefficients of about 8, 14 and 18 S. These forms are all converted, on treatment with trypsin or other proteolytic enzymes, to a form with a sedimentation coefficient of about 11 S, similar to that reported for the preparations of purified enzyme isolated from toluene-treated eel tissue[14,15]. In the following we wish to describe simple procedures, utilizing affinity chromatography, for the purification in high yields of acetylcholinesterase from trypsin-treated electric organ tissue. Some properties of the purified enzyme will be presented and compared with those previously reported for purified preparations of acetylcholinesterase. The relation of the purified enzyme to the species present in the tissue extract will also be discussed.

MATERIALS AND METHODS

Materials

Sepharose 2B, Sephadex G-100 and Sephadex G-200 were obtained from Pharmacia (Uppsala, Sweden). Cyanogen bromide and 2-mercaptoethanol were obtained from Fluka, A.G. (Buchs, Switzerland), acetylcholine bromide, acetylthiocholine iodide, and 5,5′-dithiobis-(2-nitrobenzoic acid) from Calbiochem (Los Angeles, Calif.), and decamethonium bromide from K and K Laboratories (Plainview, N.Y.). Sodium dodecyl sulfate was the specially pure grade of B.D.H. Chemi-

cals (Poole, England). Crystalline trypsin and crystalline soybean trypsin inhibitor were obtained from Worthington Biochemical Corp. (Freehold, N.J.). *o*-Nitrophenyl *N,N*-dimethylcarbamate was synthesized according to Bender and co-workers[16,17]. Electric organ tissue from freshly slaughtered eels was stored frozen at −20° and thawed before use. Toluene-treated electric organ tissue was a gift from Prof. David Nachmansohn and was stored at 4° prior to use. Sucrose and other reagents were of analytical grade.

Buffers

Unless otherwise specified, the following two buffers were routinely employed: Buffer 1, 0.01 M phosphate–0.1 M NaCl (pH 7.0); Buffer 2, 0.01 M phosphate–1.0 M NaCl (pH 7.0).

Preparation of the acetylcholinesterase inhibitor [N-(ε-aminocaproyl)-p-aminophenyl] trimethylammonium bromide hydrobromide

Synthesis of N-(N-benzyloxycarbonyl-ε-aminopcaproyl)-N',N'-dimethyl-p-phenylenediamine. *N*-Benzyloxycarbonyl-ε-aminocaproic acid (60 mmoles, 15.9 g) was dissolved in 200 ml ethyl acetate, cooled well in a salt–ice bath and stirred vigorously. Triethylamine (60 mmoles, 6.1 g) was added and then isobutylchloroformate (60 mmoles, 8.2 g). After 20 min, *p*-dimethylaminoaniline (60 mmoles, 8.2 g) dissolved in 50 ml cold ethyl acetate was slowly added, with continued stirring, and the reaction mixture was left for 5 h at room temperature with stirring. A large part of the product precipitated directly; this material was filtered, washed with water, ethyl acetate and light petroleum (b.p. 40–60°), and dried by suction. Yield of crude material 14.5 g (63%).

Additional crude material could be obtained by extracting the ethyl acetate supernatant 2 times with equal volumes of water, drying the ethyl acetate solution with anhydrous Na_2SO_4, evaporating to dryness *in vacuo*, and triturating the product with light petroleum.

The crude material could be purified by treatment with activated charcoal in hot methanol and crystallization from the same solvent.

The total yield of recrystallized product was 13.1 g (57%), m.p. 110–111°. Analysis calculated for $C_{22}H_{29}N_3O_3$: N, 10.96. Found: N, 10.77.

Synthesis of [N-(N-benzyloxycarbonyl-ε-aminocaproyl)-p-aminophenyl]trimethylammonium iodide. *N*-(*N*-Benzyloxycarbonyl-ε-aminocaproyl)-*N',N'*-dimethyl-*p*-phenylenediamine (11.5 g, 30 mmoles) was suspended in 50 ml methanol. Methyl iodide (7.14 g, 150 mmoles) was added. The reaction mixture was refluxed for 3 h, then taken to dryness *in vacuo*, triturated with 250 ml absolute diethyl ether, and crystallized from 100 ml ethanol.

Yield 10.8 g (68%) m.p. 143–145°. Analysis calculated for $C_{23}H_{32}IN_3O_3$: N, 8.00; I, 24.15. Found: N, 7.85; I, 24.26.

Synthesis of [N-(ε-aminocaproyl)-p-aminophenyl]trimethylammonium bromide hydrobromide (ε-aminocaproyl-PTA). [*N*-(*N*-Benzyloxycarbonyl-ε-aminocaproyl)-*p*-aminophenyl]trimethylammonium iodide (5.25 g, 10 mmoles) was dissolved in 10 ml glacial acetic acid, and 20 ml of anhydrous HBr in glacial acetic acid was added. After 40 min at 25° the product was precipitated with 400 ml dry diethyl ether, triturated with successive batches of diethyl ether until it solidified, and left

overnight *in vacuo* over dry NaOH pellets. The product was crystallized from 200 ml ethanol, and then recrystallized from the same solvent. Yield 3.15 g (74%), m.p. 191–194°.

Analysis calculated for $C_{15}H_{27}Br_2N_3O$: N, 9.88, Br, 37.6; neutral equivalent, 425. Found: N, 9.48; Br, 36.3; neutral equivalent, 435.

ε-Aminocaproyl-PTA had an absorption maximum at 245 nm in 0.1 M sodium borate, pH 9.3, with $\varepsilon = 15\,500$; it was found to be a good inhibitor of acetylcholinesterase with a K_i of 6 μM when tested under standard assay conditions.

Preparation and characterization of ε-aminocaproyl-PTA–Sepharose conjugates

Sepharose was activated with cyanogen bromide and reacted with ε-aminocaproyl-PTA, according to the general procedure of Axén *et al.*[8]. The detailed experimental procedure was that described by Blumberg *et al.*[18], starting from 70 ml of Sepharose slurry, except that Sepharose 2B was utilized. Inhibitor solutions containing 15–250 μmoles of ε-aminocaproyl-PTA in 30 ml of 0.5 M $NaHCO_3$ were added to the activated Sepharose. The amount of inhibitor coupled to the resin was estimated by spectrophotometric determination of the unreacted inhibitor in the supernatants and washings. Resins containing 0.1–2.5 μmoles ε-aminocaproyl-PTA per ml resin were thus obtained.

Protein determinations

Protein was determined at different stages of the purification procedure by measuring the absorbance at 280 nm.

In the purified enzyme preparations, the nitrogen content was determined by a micro-Kjeldahl procedure[19], and the amount of protein was calculated assuming a nitrogen content of 16.0%.

Assay methods

Enzymatic activity was routinely determined by the pH-stat method using acetylcholine as substrate, and assay conditions similar to those of Kremzner and Wilson[14]. The assay was performed at pH 7.0 and 25°, and the reaction mixture contained 2.5 mM acetylcholine, 0.1 M NaCl, 0.02 M $MgCl_2$, and 0.01% gelatin. Fractions from sucrose gradients were usually assayed by the method of Ellman *et al.*[20], using acetylthiocholine as substrate.

Throughout the text the specific activity of enzyme solutions is expressed as units/$A_{280\ nm}$ where one unit of enzyme is the amount hydrolysing 1 μmole of acetylcholine per min under the standard assay conditions specified above, and $A_{280\ nm}$ is the absorbance of the enzyme solution at 280 nm.

Active-site titrations

Active-site titrations were performed essentially as described by Bender and co-workers[16,17]. A 1-ml cuvette with a 10-mm light path was filled with 0.8 ml of a solution of acetylcholinesterase in 0.06 M sodium phosphate–0.1 M NaCl (pH 7.6). The amount of protein in the solution was 200–800 μg. The extinction at 415 nm was determined in a Cary-15 spectrophotometer using the 0.1 slide wire. After determining a base line, 25 μl of a 20 mM solution of *o*-nitrophenyl *N*,*N*-dimethylcarbamate in 50% (v/v) acetonitrile–water was added. An immediate increase in

absorbance of about 0.010–0.040 absorbance units was recorded, followed by the slow increase in absorption reported by Bender and co-workers[16,17], which was extrapolated back to zero time. The molarity of active sites was then calculated as described by the same authors.

Acrylamide-gel electrophoresis

Disc-gel electrophoresis was performed on 7.5% acrylamide gels in Tris–glycine buffer (pH 8.3) according to the procedure of Davis[21].

Acrylamide-gel electrophoresis in the presence of sodium dodecyl sulfate was routinely performed according to the method of Shapiro *et al.*[22]. The gels contained 7.5% acrylamide, and both the gel buffer and reservoir buffers contained 0.1% sodium dodecyl sulfate–0.075 M sodium phosphate (pH 7.2). The reservoir buffer also contained 0.01 M β-mercaptoethanol, and the gels were pre-electrophoresed at 8 mA per tube for 2 h before applying the samples. Acrylamide-gel electrophoresis was also performed in gels containing 1% sodium dodecyl sulfate as described by Lenard[23].

When acetylcholinesterase samples and appropriate marker proteins were applied to the sodium dodecyl sulfate–acrylamide gels, they were denatured prior to application to the gel. Denaturation was performed by heating the protein for 3 min at 100° in a solution of 0.01 M sodium phosphate buffer (pH 7.2) containing 0.5% sodium dodecyl sulfate and 0.01 M β-mercaptoethanol. When the procedure of Lenard[23] was employed, the denaturation medium contained mercaptoethanol and 3% sodium dodecyl sulfate, and heating was again at 100° for 3 min.

Staining of gels was carried out either by staining for protein with Coomassie blue or Amido black, or for esterase activity with α-naphthyl acetate by a modified Gomori procedure[24]. Routinely, staining with Coomassie blue was performed for 2–4 h using a solution of 0.25% dye in methanol–acetic acid–water (5:1:5, by vol.). Destaining was accomplished by soaking the gels for 30 min in an aqueous solution containing 7.5% acetic acid and 5% methanol, followed by electrophoretic destaining in a solution of the same composition. When 1% sodium dodecyl sulfate gels were run according to Lenard[23], this author's staining procedure was employed.

Sucrose gradient centrifugation

The procedure used was essentially that of Martin and Ames[25]. A cushion of 0.6–0.7 ml of 50% sucrose in 0.01 M phosphate buffer (pH 7.0), containing either 0.1 M NaCl (Buffer 1) or 1.0 M NaCl (Buffer 2) was layered on the bottom of a 5-ml centrifuge tube. On the sucrose cushion was layered 4 ml of a linear gradient of 5–20% sucrose in the same buffer solution. The sample, together with a catalase marker, in a volume of 0.1–0.2 ml, was layered on top of the gradient. Centrifugation was carried out for 6–12 h at 36 000–39 000 rev./min at 4° in an SW-39 rotor using a Spinco Model L ultracentrifuge. Fractions of 0.15–0.2 ml were collected. The catalase marker was located by measuring the absorbance at 407 nm, and its sedimentation constant was assumed to be 11.4 S (ref. 25). Acetylcholinesterase was located by enzymic assay.

Amino acid analysis

Samples for amino acid analysis were hydrolysed for 22 h at 110° in 6 M HCl

in vacuo. Amino acid analysis was performed according to the method of Spackman *et al.*[26].

Procedures for purification of acetylcholinesterase

(A) Purification from trypsin-treated frozen electric organ tissue: 100 g of frozen tissue was thawed and homogenized with 250 ml Buffer 1 at 4° for 4 min using a Sorvall Omnimixer at full speed. The homogenate was treated with a solution of 35 mg trypsin in 5 ml water, and incubated at 25° for 16 h with stirring. Soybean trypsin inhibitor (35 mg in 5 ml water) was then added. After 30 min further incubation, the homogenate was centrifuged for 50 min at 105 000 × *g* and the pellet discarded. The supernatant was dialysed for 5 h against Buffer 1. After dialysis the supernatant was applied at a rate of 30–40 ml/h to an affinity column with a total bed volume of 12 ml (ε-aminocaproyl-PTA content, 1 μmole per ml resin), previously equilibrated with the same buffer. After application of the supernatant, the column was washed with 5 column vol. of Buffer 1. Acetylcholinesterase was then eluted with 4–5 column vol. of a solution of 10 mM decamethonium bromide in Buffer 1. The column was then washed with 4–5 column vol. of Buffer 2.

(B) Purification from a tryptic digest of a salt extract of toluene-treated electric organ tissue: 175 g of toluene-treated electric organ tissue[27] were homogenized with 360 ml of 5% (w/v) $(NH_4)_2SO_4$ at 4° for 3 min using a Sorvall Omnimixer at full speed. The homogenate was centrifuged at 4° for 40 min at 13 000 × *g*. The pellet was discarded and solid $(NH_4)_2SO_4$ was added to the supernatant to yield a concentration of 15%. After centrifugation at 4° for 40 min at 13 000 × *g*, the pellet was again discarded and solid $(NH_4)_2SO_4$ added to the supernatant to yield a final concn. of 36%. After centrifugation at 4° for 40 min at 13 000 × *g*, the supernatant was discarded, and the pellet was dissolved in 20 ml of Buffer 1. A solution of 17 mg trypsin in 1 ml water was added and the incubation mixture was stirred at 25° for 16 h. After centrifugation at 12 000 × *g* for 10 min the pellet was discarded and the supernatant was applied to a Sephadex G-100 column of dimensions 3.9 cm × 110 cm, previously equilibrated with Buffer 1. The enzyme was eluted with the same buffer, and the peak of enzyme activity was collected in a total volume of 98 ml. The enzyme was then applied to the affinity column and eluted as described for Procedure A.

(C) Purification from toluene-treated electric organ tissue without tryptic digestion: 175 g of toluene-treated electric organ tissue, which had been stored under toluene at 4° for more than 2 years, was homogenized and fractionated with $(NH_4)_2SO_4$ as described under Procedure B. The pellet which precipitated between 15 and 36% $(NH_4)_2SO_4$ was dissolved in 20 ml of Buffer 1, and dialyzed against the same buffer for 16 h at 4°. The solution was then applied to the affinity column and eluted as described under Procedure A.

RESULTS

Isozymic forms of acetylcholinesterase

Acetylcholinesterase extracted by salt solutions from homogenates of electric organ tissue appears in several isozymic forms as detected by sucrose gradient centrifugation at high ionic strength. The sedimentation coefficients of the main

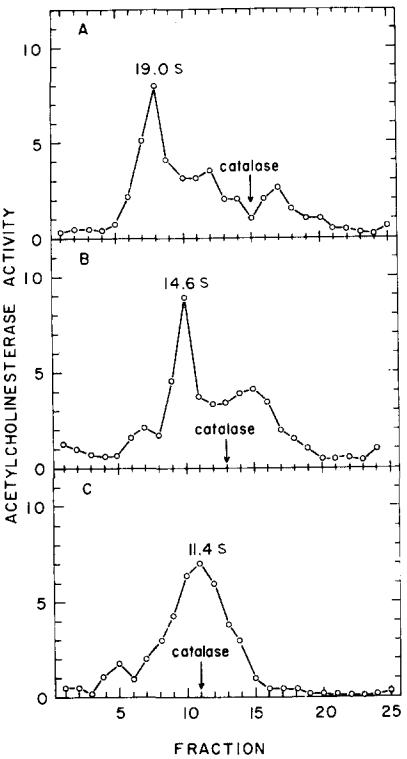

Fig. 2. Sucrose gradient centrifugation of extracts of electric organ tissue with Buffer 2. In all cases the tissue was homogenised in 2.5 vol. of Buffer 2 and the supernatant obtained after centrifugation at 10 000 × *g* for 15 min was examined. The sucrose gradients were also performed in Buffer 2 as described under MATERIALS AND METHODS. (A) Fresh electric organ tissue. (B) Electric organ tissue after storage under toluene at 4° for 2 months. (C) Electric organ tissue after storage under toluene for about 2 years.

components extracted from fresh electric organ tissue were found to be about 8, 14 and 18 S in agreement with the results of Massoulié and Rieger[12] (Fig. 2A). It was also confirmed that on sucrose gradient centrifugation at low ionic strength the two heavier species aggregate and only the lighter component does not aggregate. However, when salt extracts of toluene-treated electric organ tissue[27], which had been stored under toluene at 4° for several months, were similarly analysed, the proportions of the isozymic species were found to have changed drastically. Thus at high ionic strength the amount of the 14-S component relative to the 18-S component had increased, and a significant shoulder had appeared in the 11-S region (Fig. 2B). At low ionic strength most of the enzyme appeared as an aggregate, and only a peak at 11 S appeared. After toluene-treated tissue had been stored for about 2 years at 4°, the main peak of acetylcholinesterase activity on the sucrose gradient was located in the 11-S region (Fig. 2C), and little aggregation occurred at low ionic strength.

In our preliminary communication on purification of acetylcholinesterase by affinity chromatography[9], we reported that the product obtained was largely or wholly aggregated at low ionic strength, and most of it did not migrate on acryl-

amide-gel electrophoresis in 7.5% gels. When a purified preparation of the type described in the preliminary communication was analyzed on a sucrose gradient, at high ionic strength, it was found to yield a major 14-S species and a sizeable shoulder at about 11 S. At low ionic strength only the 11-S species remained unaggregated (Fig. 3).

It thus seemed plausible that the 11-S enzyme purified by previous workers from toluene-treated tissue[2,3] comprised only a part of the native enzyme which had been modified by toluene treatment. The observation of Massoulié and coworkers[12,13] that acetylcholinesterase from both fresh and toluene-treated tissue could be converted to a non-aggregating form of 11 S by treatment with trypsin,

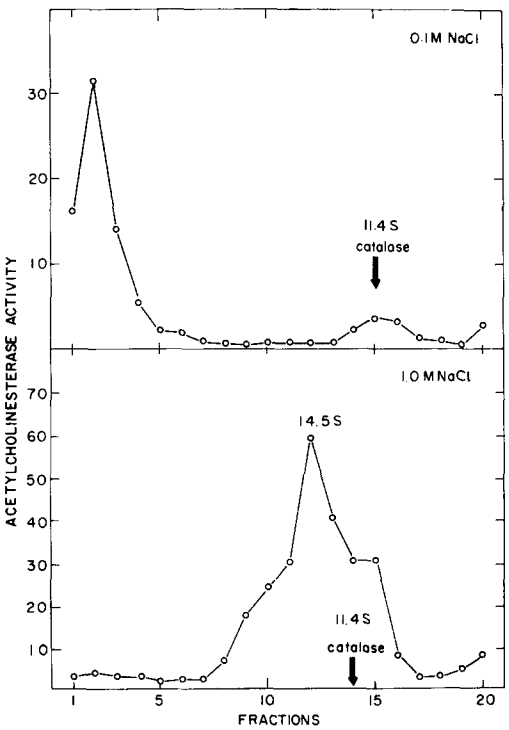

Fig. 3. Sucrose gradient centrifugation of acetylcholinesterase, purified as described by Kalderon *et al.*[9]. Centrifugation was performed at low ionic strength using Buffer 1 (upper gradient), or at high ionic strength utilising Buffer 2 (lower gradient).

prompted us to precede affinity chromatography by a step of tryptic digestion in order to obtain a high yield of enzyme as a single non-aggregating species. It was found that if acetylcholinesterase was extracted from electric organ tissue, fresh or toluene-treated, conditions could be found where tryptic digestion, without causing significant loss of enzymic activity, caused complete conversion to the 11-S non-aggregating form. Fig. 4 shows an experiment in which toluene-treated tissue was digested with trypsin for 6 or 16 h, applied to the affinity column as described in Procedure B under MATERIALS AND METHODS, eluted with Buffer 2, and applied to

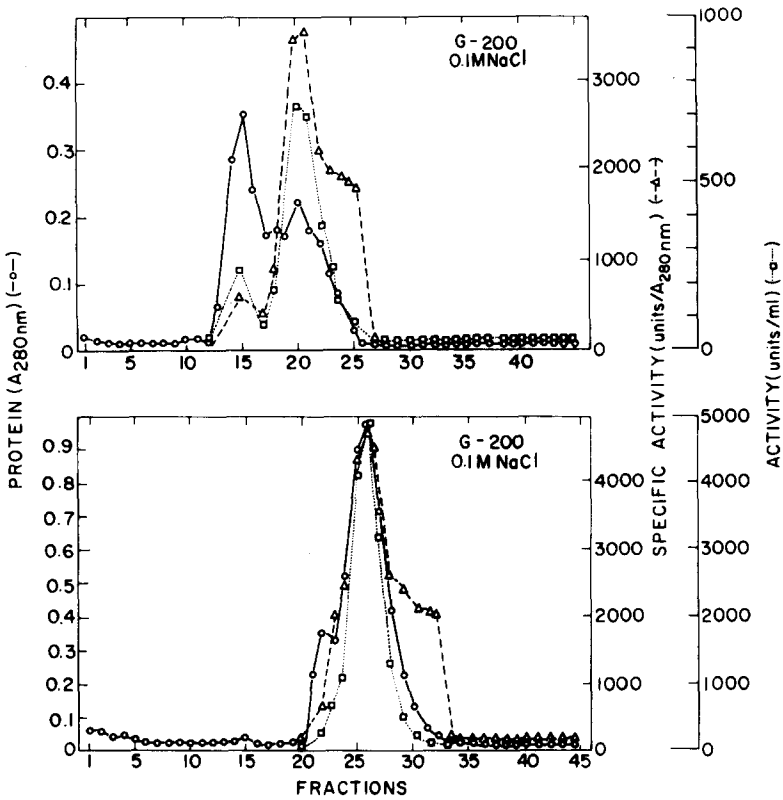

Fig. 4. Gel filtration on Sephadex G-200 of acetylcholinesterase extracted from toluene-treated tissue, treated with trypsin and partially purified by affinity chromatography. The 15–40% $(NH_4)_2SO_4$ precipitate of a homogenate of toluene-treated tissue was dissolved in Buffer 1 and treated with 2.5% trypsin (w/w protein) for 6 or 16 h at 25°. After tryptic digestion the reaction mixtures were immediately cooled to 4° and centrifuged at 15 000 × *g* for 10 min. Samples of the supernatants containing 7000 units of acetylcholinesterase (6-h digest) or 20 000 units (16-h digest) were immediately applied to an ε-aminocaproyl-PTA column with a bed volume of 5 ml and an inhibitor content of 2.5 μmoles per ml resin. The column was washed with 5 column vol. of Buffer 1 and the enzyme was eluted with Buffer 2, dialysed against Buffer 1 and applied to a Sephadex G-200 column (dimensions 1.5 cm × 130 cm) equilibrated and developed with Buffer 1. Upper figure, elution pattern obtained subsequent to 6-h tryptic digestion. Lower figure, pattern obtained subsequent to 16-h tryptic digestion.

a Sephadex G-200 column. If digestion was performed for 6 h the enzyme separated into two peaks (Fig. 4A). The first of these did not enter the gel on acrylamide-gel electrophoresis (Figs. 5A and 5B), whereas the second peak entered the gel as one main component (Figs. 5C and 5D) and also appeared as one component of 11 S on sucrose gradient centrifugation. After 16 h tryptic digestion, almost all the enzyme appeared as one peak on Sephadex G-200 (Fig. 4B), gave a pattern similar to those shown in Figs. 5C and 5D on acrylamide-gel electrophoresis and migrated as an 11-S species on sucrose gradients at both high and low ionic strength, yielding a pattern similar to that shown in Fig. 7.

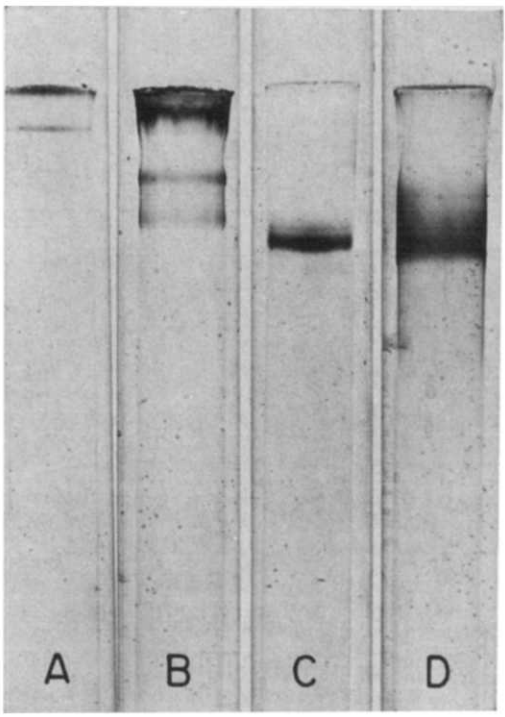

Fig. 5. Acrylamide-gel electrophoresis in Tris–glycine buffer (pH 8.3) of purified acetylcholinesterase fractions eluted from Sephadex G-200 columns in the experiment presented in Fig. 4 (upper diagram). Staining for protein and for esterase activity were performed as described under MATERIALS AND METHODS. (A) sample from Tube 15 stained for protein. (B) Same sample as in A, run in parallel and stained for esterase activity. (C) Sample from Tube 22, stained for protein. (D) Same sample as in C, run in parallel and stained for esterase activity.

Purification of the 11 S form of acetylcholinesterase by affinity chromatography

The basic approach involved was first to obtain an acetylcholinesterase extract in which essentially all the enzyme was in the 11 S form as judged by sucrose gradient centrifugation. This extract was then applied to a column of ε-aminocaproyl-PTA–Sepharose in a solution of low ionic strength, the dimensions of the column permitting adsorption of almost all the applied enzyme. After washing the column, the enzyme was specifically eluted with a solution of the powerful acetylcholinesterase inhibitor decamethonium[28,29]. When the column was further treated with a buffer solution of high ionic strength, both non-specifically adsorbed protein and a further amount of acetylcholinesterase were removed from the column, which could then be reutilized.

Three procedures were developed for purifying the enzyme by the general approach outlined above. In the first procedure (Procedure A under MATERIALS AND METHODS) the enzyme was converted to an 11-S form by tryptic digestion of frozen electric organ tissue and the digest applied to the affinity column. The second procedure (Procedure B) involved tryptic digestion of toluene-treated tissue and separation of the acetylcholinesterase from other proteins and the products of tryptic digestion by gel filtration on Sephadex G-100 prior to affinity chromatography. The

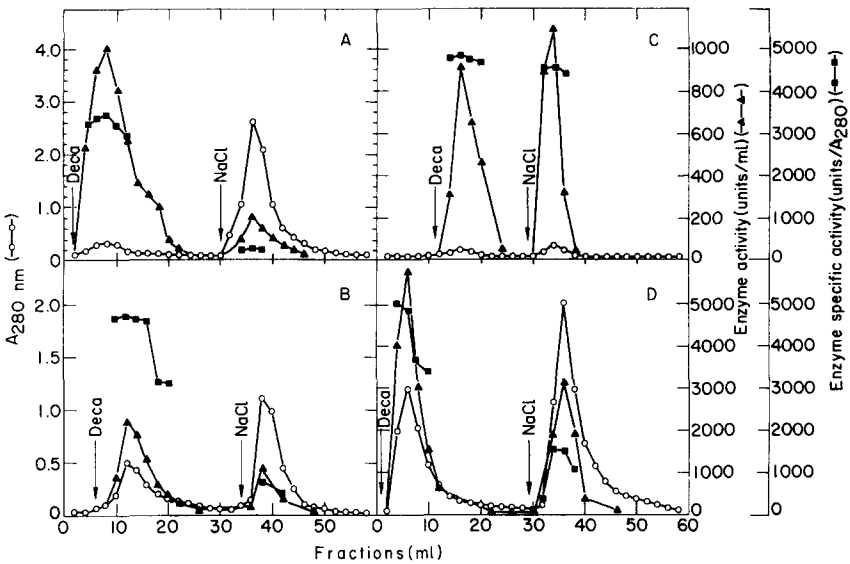

Fig. 6. Purification of acetylcholinesterase by affinity chromatography on ε-aminocaproyl-PTA columns. Affinity chromatography was performed on columns with a bed volume of 5 ml and an inhibitor content of 1 μmole per ml resin. Samples of enzyme obtained by use of the three procedures described under MATERIALS AND METHODS were applied to the column in Buffer 1, and the column was washed with 5 column volumes of the same buffer. Elution with 0.01 M decamethonium bromide in Buffer 1 was initiated at the arrows marked "Deca", and with Buffer 2 at the arrows marked "NaCl". (A) Purification by Procedure A. (B) Purification by Procedure B. (C) Rechromatography of the peak fractions, with specific activity of about 4900 units/$A_{280\ nm}$, eluted with decamethonium in B. (D) Purification by Procedure C.

third procedure (Procedure C) utilized the observation that prolonged storage of toluene-treated tissue yielded mainly 11-S acetylcholinesterase without tryptic digestion (see above Fig. 2C); thus, after $(NH_4)_2SO_4$ fractionation as in Procedure B, tryptic digestion and Sephadex G-100 chromatography were omitted.

Figs. 6A, 6B and 6D show the patterns obtained on affinity chromatography utilising Procedures A, B and C, respectively. In addition, Fig. 6C shows the pattern obtained on rechromatography of the enzyme peak eluted with decamethonium in Procedure B (Fig. 6B).

It can be seen that both Procedures B and C yield, on elution with decamethonium, an enzyme preparation with a specific activity of 4700–5100 units/$A_{280\ nm}$. However, using Procedure B the specific activity in different fractions of the peak was usually more constant than using Procedure C. In Procedure A, the specific activity was lower and much more variable (1500–3500 units/$A_{280\ nm}$). On rechromatography of the enzyme peak on the affinity column, all three preparations yielded a uniform peak with specific activity 4700–5100 units/$A_{280\ nm}$ on elution with decamethonium. On further elution at high ionic strength the enzyme produced by Procedure B (acetylcholinesterase B), yielded a second enzyme peak with specific activity as high as that of the fraction eluted with decamethonium (Fig. 6C).

In much of our work we routinely used Procedure B which was most convenient and reproducible in our hands, and allowed purification of the enzyme in high yields. Furthermore, the pattern obtained on rechromatography of the enzyme

TABLE I

PURIFICATION OF ACETYLCHOLINESTERASE BY PROCEDURE B

Step	*Total protein ($A_{280\ nm}$)*	*Total activity of acetyl cholinesterase (units)*	*Specific activity of acetylcholinesterase (units/$A_{280\ nm}$)*	*Recovery of acetylcholinesterase (%)*	*Purification*
15–36% $(NH_4)_2SO_4$ precipitate	825	140 000	170	100	(1)
Tryptic digest supernatant	588	151 000	257	108	1.5
Sephadex G-100 chromatography	95	144 000	1800	103	10.6
Affinity chromatography	12	56 500	4700	40*	27.6

* An additional 40% of the acetylcholinesterase applied to the affinity column could be recovered in fractions of lower specific activity on the trailing edge of the decamethonium-eluted peak and in the fraction eluted with 1.0 M NaCl.

indicates a high degree of homogeneity and will be referred to in the discussion. Table I shows the yields and specific activity obtained at the successive stages of a typical purification by Procedure B. It can be seen that tryptic digestion indeed did not cause apparent loss of enzymic activity, and that Sephadex G-100 gel filtration after proteolysis yielded more than tenfold purification. The affinity column was routinely capable of absorbing about 8000 units of enzyme activity per ml of resin containing 1 μmole of covalently bound ε-aminocaproyl-PTA per ml resin. The yield of purified acetylcholinesterase with specific activity of 4700 units/$A_{280\ nm}$ shown in Table I was 40% of the activity in the initial 15–36% $(NH_4)_2SO_4$ precipitate. Another 40% of the enzyme, with lower specific activity, could be recovered from the column.

Characterization of purified 11-S acetylcholinesterase preparations

Sucrose gradient centrifugation. When purified acetylcholinesterase preparations eluted with decamethonium by Methods A, B and C, or after rechromatography, were analyzed on sucrose gradients, single peaks were always obtained whether in 0.1 or 1 M NaCl. A typical sucrose gradient is given in Fig. 7, using the product of Procedure B (acetylcholinesterase B). It can be seen that a single peak is obtained, of sedimentation constant similar to that of the catalase marker.

Acrylamide-gel electrophoresis. When acetylcholinesterase B was electrophoresed on 7.5% acrylamide gels in Tris–glycine buffer, one major protein component was consistently observed (Fig. 8A), whether staining was performed with Amido black or Coomassie blue. There also sometimes appear to be two minor components, one migrating just behind the major component so that it is almost indistinguishable from it, and the other much more slowly. The major component and both minor components, when present, displayed enzymic activity when the gels were stained with α-naphthyl acetate.

Acrylamide-gel electrophoresis on 7.5% acrylamide gels in 0.1% sodium dodecyl sulfate–0.01 M mercaptoethanol, after prior denaturation of the protein

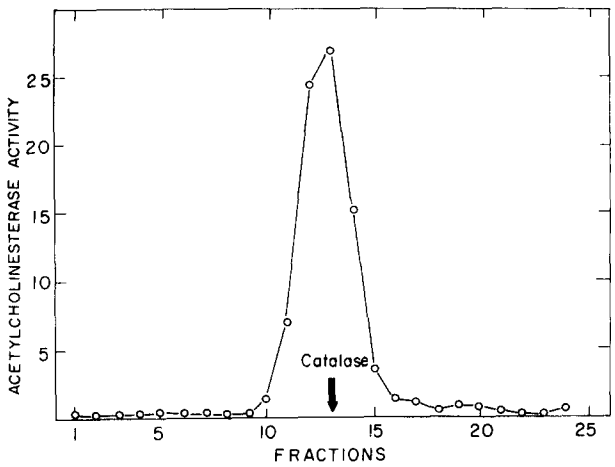

Fig. 7. Sucrose gradient centrifugation of acetylcholinesterase B (*i.e.* acetylcholinesterase purified by Procedure B in MATERIALS AND METHODS). Centrifugation was performed in Buffer 1.

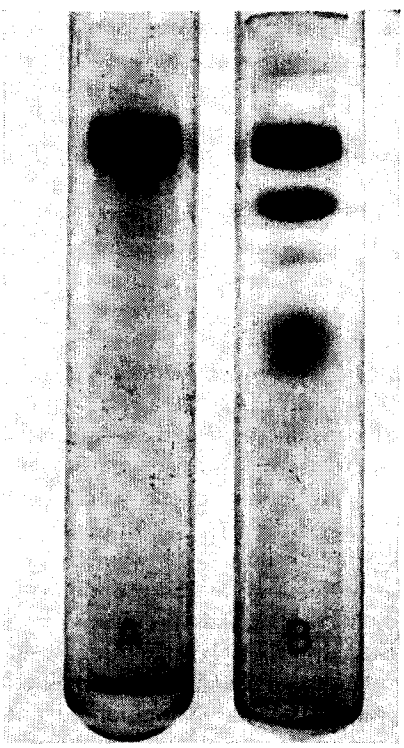

Fig. 8. Acrylamide-gel electrophoresis of a sample of acetylcholinesterase purified by Procedure B with specific activity 4900 units/$A_{280\ nm}$. (A) Electrophoresis in Tris–glycine buffer (pH 8.3). (B) Electrophoresis in 0.1% sodium dodecyl sulfate–0.01 M mercaptoethanol (pH 7.2). Staining for protein was with Coomassie blue.

with sodium dodecyl sulfate and mercaptoethanol, revealed two major components (Fig. 8B), with molecular weights of 88 000 ± 6 000 and 64 000 ± 5 000, using the procedure of Shapiro *et al.*[22] for molecular weight calibration, with bovine serum albumin, trypsin and lysozyme as calibrating proteins. The heavier component appeared to be present in larger amounts as judged by the intensity of staining. Traces of lower molecular weight species, of molecular weight 40 000 and lower, could also be discerned. The pattern observed and the ratio of the major components did not vary under different conditions of denaturation and electrophoresis. Thus, neither the extreme conditions utilized by Lenard[23], nor pretreatment of the enzyme with mercaptoethanol in 6 M guanidine hydrochloride followed by treatment with iodoacetamide, had any discernible effect. Electrophoresis of acetylcholinesterase A and acetylcholinesterase C in the presence of sodium dodecyl sulfate and mercaptoethanol also revealed the same two major components, and no major difference in the ratio of the two components could be detected. It should be noted that when acetylcholinesterase B was labelled with [^{3}H]diisopropylfluorophosphate (DFP) and electrophoresed in the presence of sodium dodecyl sulfate and mercaptoethanol, both major protein components were labelled[30].

Extinction coefficient. The extinction coefficient of acetylcholinesterase B at 280 nm was determined on the basis of micro-Kjeldahl analysis of two different samples of the enzyme, and assuming a nitrogen content of 16.0%. On this basis the value was found to be $\varepsilon^{1\%}_{280\ nm} = 17.6 \pm 1.0$.

TABLE II

AMINO ACID ANALYSIS OF PURIFIED PREPARATIONS OF ACETYLCHOLINESTERASE

Values are expressed as moles per 100 moles of total amino acids recovered. Acetylcholinesterase A, B and C were purified according to Procedures A, B and C as described under MATERIALS AND METHODS. The samples of acetylcholinesterase A and C were rechromatographed on the affinity column prior to amino acid analysis. All three preparations had specific activities of 4700–5100 units/$A_{280\ nm}$.

Amino acid	*Acetylcholinesterase A*	*Acetylcholinesterase B*	*Acetylcholinesterase C*	*L-I**	*L-II**
Lys	4.5	4.5	5.1	4.5	4.2
His	2.2	2.2	2.0	2.3	2.3
Arg	4.9	5.2	4.8	5.4	5.5
Asp	12.2	12.9	11.4	9.6	12.1
Thr	3.9	4.4	4.0	4.5	4.2
Ser	6.5	6.8	7.2	7.1	6.7
Glu	10.9	10.3	12.0	9.6	9.2
Pro	6.8	7.2	6.8	8.4	7.9
Gly	8.6	9.1	9.1	8.0	7.5
Ala	7.5	6.0	8.8	5.6	5.4
Cys	0.8	1.1	0.7	1.1	1.1
Val	7.0	6.7	7.1	7.2	6.8
Met	2.6	–	1.4	3.0	3.0
Ile	3.9	4.0	4.1	3.8	3.6
Leu	8.3	9.1	7.3	9.2	8.8
Tyr	2.9	3.6	2.3	3.9	3.8
Phe	5.2	5.4	4.8	5.3	5.4
Hexosamine	1.3	1.6	1.0	1.7	2.7

* Calculated from the data of Leuzinger and Baker[3].

Amino acid analysis. Table II shows the amino acid composition of samples of acetylcholinesterase purified by Procedures A, B and C. The preparations obtained by Procedures A and C were rechromatographed on the affinity column prior to amino acid analysis. The amino acid analyses of purified acetylcholinesterase given by Leuzinger and Baker[3] are included for purposes of comparison.

Active-site titrations. The number of active sites in acetylcholinesterase B was determined by the method of Bender and co-workers[16,17], utilizing the initial "burst" of release of *o*-nitrophenol from *o*-nitrophenyl *N,N*-dimethylcarbamate. Active-site titrations were performed on 3 different samples of acetylcholinesterase B, and the results obtained are summarised in Table III. On their basis an average molecular weight per site of 107 000 ± 11 000 can be calculated.

TABLE III

ACTIVE SITE TITRATION OF ACETYLCHOLINESTERASE B USING *o*-NITROPHENYL *N,N*-DIMETHYLCARBAMATE

Preparation	*Protein (μg)*	*o-Nitrophenol released (nmoles)*	*Equivalent weight per active site*
I	226	2.1	108 000
II	338	3.0	113 000
	338	2.8	121 000
III	613	6.4	96 000
	613	6.4	96 000

On the basis of the active site titrations and the measured specific activity for acetylcholinesterase B (4700–5100 units/$A_{280\ nm}$ under standard assay conditions), one can calculate a turnover number of $(9.2 \pm 1.4) \cdot 10^5$ substrate molecules per min per active site.

DISCUSSION

The inhibitor ε-aminocaproyl-PTA was selected for the preparation of a Sepharose conjugate suitable for the affinity chromatography of acetylcholinesterase, utilising the principles generally found desirable in designing a compound for this purpose[7]. Thus the phenyltrimethylammonium moiety is a good competitive inhibitor of acetylcholinesterase with a K_i of 53 μM (ref. 10), and ε-aminocaproyl-PTA is an even better inhibitor ($K_i = 6\ \mu M$) under standard assay conditions. The ε-amino group provides a means of attachment to the CNBr-activated Sepharose; furthermore, the insertion of an ε-aminocaproyl chain as a "spacer" between the inhibitor group interacting with the enzyme and the solid matrix has been shown in other cases to improve the efficiency of resins employed for affinity chromatography[7,31]. Indeed, Blumberg and Katchalski[32] have shown that close proximity of an inhibitor moiety to a polymeric backbone interferes with inhibitor–enzyme interaction.

Since the best inhibitors of acetylcholinesterase are quarternary ammonium compounds, resins prepared for affinity chromatography of this enzyme will in general bear a positive charge the size of which will depend on the amount of inhibitor

covalently bound. As a result they will tend to bind negatively charged proteins non-specifically as a result of electrostatic interaction. Indeed, in our preliminary communication[9] we mentioned that resins with a higher content of covalently bound ε-aminocaproyl-PTA adsorbed more enzyme but also more contaminating protein. This drawback could be overcome by specifically eluting the enzyme from the column with the acetylcholinesterase inhibitor decamethonium[28,29], a process which served to differentiate between specifically and non-specifically bound proteins. However, it can be seen from Fig. 6 that, even after elution with decamethonium, a portion of the acetylcholinesterase was retained on the resin, and was only removed, together with contaminating material, when the column was regenerated by washing with a solution of high ionic strength. It was considered possible that the acetylcholinesterase which was not eluted by decamethonium differed from the fraction eluted in having a different affinity for the resin. If, however, purified acetylcholinesterase B eluted with decamethonium, was rechromatographed (Fig. 6C), it was found that part of it was retained on the column on elution with decamethonium, and could only be removed at high ionic strength; however, the two fractions so eluted did not differ in specific activity or in other properties examined. It thus seems that the ε-aminocaproyl-PTA–Sepharose adsorbs acetylcholinesterase both by specific binding *via* the PTA ligand, and non-specifically by virtue of its polyelectrolyte nature. In principle the polyelectrolyte behavior of the resin could be masked by working at a higher ionic strength[33], which would also offer the advantage that forms of the enzyme which aggregate at low ionic strength would be dissociated. However, as shown by Changeux[29] the inhibition constants of acetylcholinesterase inhibitors decrease markedly with increasing ionic strength. Thus, he observed that the K_i for PTA was 2.55 μM in 1 mM veronal buffer (pH 7), changing to 115 μM in the presence of 0.2 M $MgCl_2$. We have similarly observed that the K_i of PTA changes from 12 μM in 0.1 M NaCl–0.01 M Tris buffer (pH 8) to 100 μM in 0.25 M $MgCl_2$–0.01 M Tris buffer (pH 8) and also that the K_i of ε-aminocaproyl-PTA changes from 6 μM in 0.1 M NaCl–0.02 M $MgCl_2$ (pH 7) to 60 μM in 1 M NaCl–0.01 M $MgCl_2$ (pH 7) (Y. Dudai and I. Silman, unpublished results). When enzyme was eluted from the affinity column with solutions of increasing ionic strength, instead of with decamethonium, acetylcholinesterase began to be eluted, together with contaminating proteins, in about 0.25 M NaCl. It is, however, possible that a resin containing a ligand with a higher inhibition constant could be employed at high ionic strength.

In our preliminary experiments[9], affinity chromatography yielded a product which aggregated at low ionic strength, whereas purified enzyme preparations previously described did not display this property. The observations of Massoulié and Rieger[12] led us to consider the possibility that previous workers had preferentially purified a part of the enzyme already present in toluene-treated tissue as a non-aggregating 11-S species, whereas our affinity chromatography procedure had yielded a mixture of the isozymic species described by Massoulié and Rieger[12]. Indeed we confirmed (Fig. 2B) that extracts of toluene-treated tissue contain the heavier species of acetylcholinesterase, which aggregate at low ionic strength, and that the amount of 11-S acetylcholinesterase in the extract increases with time, this being possibly a result of autolysis. It therefore seemed worthwhile obtaining a homogeneous non-aggregating preparation of the enzyme in high yield by controlled tryptic digestion of toluene-treated tissue, thereby converting all the enzyme to the 11-S species under

the conditions of Massoulié and Rieger[12]. As can be seen from Figs. 2 and 4 and Table I, tryptic digestion does not affect the overall acetylcholinesterase activity of a salt extract of toluene-treated tissue, while converting practically all the enzyme to a form which enters 7.5% acrylamide gels as a single species, has a sedimentation coefficient of about 11-S and does not aggregate at low ionic strength. As indicated by Fig. 2C, prolonged storage of toluene-treated tissue produces a similar conversion of a large part of the enzyme to an 11-S form, by a process of autolysis which may involve endogenous proteolytic enzymes but concerning which no direct evidence is available. Affinity chromatography by Procedures A, B and C, starting from trypsin-treated frozen electric organ tissue, toluene-treated tissue digested with trypsin, and toluene-treated tissue stored for 2 years, in all three cases yields highly purified acetylcholinesterase (Fig. 6). In all three cases the purified enzyme represented a high percentage of the activity in the initial extract. The degree of purification and homogeneity of the peak eluted with decamethonium differs for the three procedures, Procedure B being the best. On rechromatography of the products of all three procedures, the peak eluted with decamethonium is homogeneous, and has a specific activity of 4700–5100 units/$A_{280\ nm}$. No significant difference in specific activity was observed between the products of the three procedures. On rechromatography of the product of Procedure B both the decamethonium peak and the peak eluted with 1 M NaCl possessed the same specific activity (Fig. 6C, see above). This activity was similar to that obtained prior to rechromatography and suggests that the product of the first step of affinity chromatography is already fairly homogeneous when Procedure B is utilized.

The specific activity per absorbance unit at 280 nm (4700–5100 units/$A_{280\ nm}$ for all three preparations after rechromatography) is similar to that given for the previously described purified preparations of acetylcholinesterase. Thus, the maximal value reported by Kremzner and Wilson[14] of 660 mmoles/h per mg protein is derived using $\varepsilon^{1\%}_{280\ nm} = 2.29$, and if expressed as units/$A_{280\ nm}$ would be 4800 units/$A_{280\ nm}$. In a recent paper Leuzinger[34] reports an activity of 730 mmoles/mg per h using $\varepsilon^{1\%}_{280\ nm} = 1.90$, and assaying at pH 8.0. If this specific activity is normalised to pH 7 using the pH–activity curves of Bergmann *et al.*[35] or Silman and Karlin[36], a specific activity of 5300 units/$A_{280\ nm}$ is obtained. Thus discrepancies in reported specific activity of purified acetylcholinesterase may arise largely from differences in the method of determinating the extinction coefficient of the protein. As can be seen from Table II the amino acid analyses of acetylcholinesterase A, B and C are not very different from those reported for the acetylcholinesterase purified by Leuzinger and Baker[3], and the small difference may be partly a result of the different methods of converting the enzyme to an 11-S form.

The purified products obtained by Procedures A, B and C have similar specific activities, similar sedimentation coefficients on sucrose gradients, and display similar patterns on acrylamide-gel electrophoresis both in the presence and absence of sodium dodecyl sulfate and mercaptoethanol. Moreover, with respect to sedimentation constant, specific activity and amino acid analysis they closely resemble previous preparations of purified acetylcholinesterase. However, these criteria cannot be used to conclude that the preparations are completely identical with each other, or with previous preparations of purified acetylcholinesterase. In fact, Massoulié *et al.*[13] have detected a difference of 0.7 S between the sedimentation coefficient of a sample of

purified acetylcholinesterase prepared by Leuzinger and that of the species which they obtain by tryptic digestion of electric organ tissue. Thus it is likely that more careful inspection of the three preparations obtained by Procedures A, B and C will reveal both microheterogeneities within each preparation and differences between the preparations, these being the result of different degrees of proteolysis or autolysis. It should also be remembered that electric organ tissue contains, apart from the excitable membrane of the electroplax, other components, such as nerve endings and blood vessels, so that more than one type of acetylcholinesterase may be present. As already pointed out, acetylcholinesterase B sometimes displays two minor components on acrylamide-gel electrophoresis, both possessing esterase activity. Moreover, preliminary examination by acrylamide-gel electrophoresis of fractions of low specific activity on the sides of the peak eluted with decamethonium in Procedure A suggests the presence of other minor components with esterase activity (Y. Dudai and I. Silman, unpublished results).

Acrylamide-gel electrophoresis in the presence of sodium dodecyl sulfate and mercaptoethanol yields two major protein components (see Fig. 8B) present in similar ratios in acetylcholinesterase A, B and C. In a separate communication we have presented data showing that when acetylcholinesterase B is treated with radioactively labelled DFP, both these components are labelled, indicating that they both contain active sites[30]. Their molecular weights, as determined by standard methods for calibration of sodium dodecyl sulfaste–acrylamide gels, are about 88 000 and 64 000. The study of Leuzinger *et al.*[15] shows that in guanidine hydrochloride in the presence of mercaptoethanol the enzyme purified by Leuzinger and Baker[3] yields only subunits of molecular weight about 64 000. However, it should be noted that Leuzinger *et al.*[15] observed two components on acrylamide-gel electrophoresis of acetylcholinesterase in the presence of urea and mercaptoethanol. The recent study of Millar and Grafius[37], on a purified sample of acetylcholinesterase which they prepared, indicates subunits of even lower molecular weight, *i.e.* about 42 000. The results of active site titrations on different preparations of acetylcholinesterase also yield rather different results. Kremzner and Wilson[14] reported an equivalent weight of 54 000 per active site, a result which they considered would best fit a tetramer. However, Leuzinger[34] reports a value of 130 000 per active site, rather greater than the value of 107 000 that we obtain. The results of Leuzinger would indicate two active sites per molecule, and, taking into account his ultracentrifuge results, two catalytic subunits and two noncatalytic subunits. It should be remembered, however, that Kremzner and Wilson[14] used a different technique for titrating active sites than that used by Leuzinger and ourselves, and also that they assumed a rather different extinction coefficient for the protein.

It is difficult to suggest a simple model for the structure of our purified acetylcholinesterase, taking into consideration both the active site titration data and the results of sodium dodecyl sulfate–acrylamide-gel electrophoresis. On the one hand we appear to have only one active site per 107 000. On the other hand the enzyme appears to contain only polypeptide chains bearing active sites, the average molecular weight of which should be somewhere between 64 000 and 88 000. One obvious possibility is that acrylamide-gel electrophoresis is not yielding correct values for the molecular weights of the polypeptide chains, although it has been shown to yield reliable molecular weight values for the polypeptide chains of a large body of proteins

(see for example, refs. 22, 38 and 39). A second possibility is that tryptic treatment, or autolysis, or other stages of the purification procedure, inactivated some of the catalytic sites, so that they no longer react with *o*-nitrophenyl *N*,*N*-dimethylcarbamate. In this connection it should be remembered that our purified preparations of acetylcholinesterase have specific activities similar to other purified preparations (see above). A third possibility is that the tryptic treatment involved in the purification procedure hydrolyses peptide bonds in the polypeptide chain containing the catalytic site, without necessarily affecting catalytic activity, but that this treatment does not lead to the release of peptides or polypeptide fragments until the protein is denatured. This kind of behavior has been observed by Ullmann *et al.*[40] (see also ref. 41) in their studies on the structure of β-galactosidase. The catalytic polypeptide chain in native acetylcholinesterase might thus have a molecular weight of about 100 000, as indicated by our data on the labelling with [^{3}H]DFP of acetylcholinesterase in salt extracts of electric organ tissue[30]. This polypeptide chain might then be the main species present in our purified preparations, with some of its peptide bonds already cleaved, but with its quaternary structure maintained. On denaturation, small fragments would be released, leaving residual active site polypeptide chains of two sizes, 88 000 and 64 000. This would also be in line with the appearance of polypeptide chains of much lower molecular weight on acrylamide-gel electrophoresis in the presence of sodium dodecyl sulfate and mercaptoethanol (Fig. 8B). Obviously, accurate molecular weight determinations, by ultracentrifuge techniques, on the purified enzyme and on its subunits will be necessary in order to resolve this problem.

Our data seem to indicate that some proteolysis of polypeptide chains containing active sites occurs concomittantly with conversion of acetylcholinesterase from the 18-, 14- and 8-S species present in fresh extracts to the 11-S non-aggregating form of the purified enzyme. However, this does not necessarily mean that proteolysis of these polypeptide chains is the only step involved in the transformation or even that is involved at all. Massoulié *et al.*[42] have recently presented evidence that a similar type of transformation can be accomplished by sonication of salt extracts of electric organ tissue. Moreover, it is certainly possible that digestion of a separate polypeptide chain, such as the "aggregating factor" reported by Kremzner and Fei[43], is involved, or that phospholipids are partially responsible for aggregation, as suggested by Grafius and Millar[44]. In order to resolve this problem it will be necessary to purify the native forms of acetylcholinesterase and to compare their polypeptide components with those of the 11-S form.

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