*Biochimica et Biophysica Acta*, 657 (1981) 243—256


BBA 69182

# PURIFICATION AND PROPERTIES OF THE MEMBRANE-BOUND ACETYLCHOLINESTERASE FROM ADULT RAT BRAIN

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(Received July 3rd, 1980)

*Key words: Acetylcholinesterase; Affinity chromatography; (Rat brain)*

## Summary

The membrane-bound acetylcholinesterase (acetylcholine acetylhydrolase, EC 3.1.1.7) from adult rat brain has been purified to homogeneity using sequential affinity chromatography on Con A-Sepharose and on dimethylaminoethylbenzoic acid-Sepharose 4B followed by DEAE-cellulose chromatography. The yield of the purified enzyme (specific activity: 3068 U/mg protein) is higher than 50%. Polyacrylamide gel electrophoresis in the presence of Triton X-100 gives only one band with acetylcholinesterase activity. With the exception of electrofocusing and pore gradient electrophoresis, where a multiple band pattern was detected (which seems to be artefactual), the enzyme appears to be homogeneous. Gel filtration and sucrose density gradient centrifugation in the presence of Triton X-100 give only one symmetrical peak, with a calculated molecular weight of 328 000. Since polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) and mercaptoethanol gives only one band with a molecular weight of 74 500, a tetrameric structure can be postulated for the membrane-bound acetylcholinesterase from rat brain.

## Introduction

Some confusion is present in the literature concerning the molecular species of acetylcholinesterase (acetylcholine acetylhydrolase, EC 3.1.1.7) present in mammalian brain. As reviewed by Rieger and Vigny [1] and Trevor et al. [2] molecular weights ranging from 60 000 to 650 000 have been described. A complex heterogeneity has been shown by the use of analytical electrofocusing [3—

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5], electrophoresis [4,6—11], gel filtration [1,4,5,7,11], density gradient centrifugation [1] or affinity chromatography on immobilized lectins [3]. However, this heterogeneity was also shown to be dependent on the method used to solubilize the enzyme [8]. In the case of lectin interactions, concanavalin A precipitates all the acetylcholinesterase activity of the enzyme in rat brain [1], whereas columns of the *Lens culinaris* lectin (which has the same sugar specificity) can adsorb only a part of it [3].

Gel filtration methods seem to give different molecular weights depending on the method of enzyme extraction or chromatographic conditions. If divalent cations are sometimes used to avoid aggregation, some of the solubilization methods use EDTA [3—5]. In a great number of studies, it is not clear if the enzyme is true acetylcholinesterase or acetylcholinesterase and cholinesterase [1,3—8].

Recent studies [1,12—19] have demonstrated that molecular species with different sedimentation coefficients are detected in proportions which are characteristic of the tissue or of its degree of maturation. In the adult rat brain, only two forms are detectable (4 S and 10 S), the latter being quite characteristic of the membrane-bound form (i.e. solubilized only with detergents) [1]. However nothing was known about the structure of the purified enzyme in contrast with the case for electric fish [20—22].

Since it has been suggested that acetylcholinesterases have a great affinity for concanavalin A [1] (a lectin specific for terminal glucose and mannose residues) and since the rat brain enzyme has a strong affinity for dimethylaminoethylbenzoic acid coupled to Sepharose through a spacer arm [23], the purification of this enzyme seemed to be quite easy. Moreover, since in rat brain a high proportion of acetylcholinesterase has the properties of a membrane-bound molecule [1] we focused our work on this part of the brain enzyme. The acetylcholinesterase which is soluble in the absence of detergent is thus eliminated prior to purification.

We present here the results of the purification to homogeneity of the membrane-bound enzyme solubilized in the presence of Triton X-100 and its molecular properties.

## Materials and Methods

### *Chemicals*

All reagents were of analytical grade. Special chemicals were obtained from the following sources: acetylthiocholine iodide, butyrylthiocholine iodide, α-methyl-D-mannoside, cytochrome *c*, ovalbumin, aldolase (EC 4.1.2.13), catalase (EC 1.11.1.6), β-galactosidase (EC 3.2.1.23), thyroglobulin, acetylcholinesterase inhibitor: 1,5-bis-4-allyldimethylammonium-phenyl/pentan 3-one dibromide and cholinesterase inhibitor tetraisopropylpyrophosphoramide from Sigma Chemicals Co. (St. Louis, U.S.A.), fluorescent sheep anti-rabbit immunoglobulins from Pasteur Production (Paris, France); 5,5'-dithiobis(dinitrobenzoic acid) from Merck (Darmstadt, F.R.G.); Triton X-100 from Serva (Heidelberg, F.R.G.); tetraethylammonium bromide from Fluka AG (Buchs, Switzerland), diethylaminoethyl (DEAE)-cellulose from Whatman (Springfield, U.K.) carrier ampholyte and Ultrodex from LKB Products (Stockholm, Sweden); Con

A-Sepharose, Sepharose 4B, Blue Dextran 2000, polyacrylamide gradient gel PAA 4/30, high molecular weight calibration kit from Pharmacia Fine Chemicals (Uppsala, Sweden); Ultrogel AcA (2/2) and AcA 3/4 from Industrie Biologique Française S.A. (Clichy, France) and Coomassie brillant blue R250 from Gurr (Chadwell Heath, Essex, U.K.).

*Preparation of rat brain acetylcholinesterase*

All operations were carried out at 4°C. 60 adult rat brains were homogenized in 600 ml of saline buffer (12.5 mM sodium phosphate buffer, pH 7.2/0.4 M NaCl) in a glass homogenizer with Teflon pestle and the suspension was centrifuged at 53 000 × *g* for 2 h. The supernatant (S1) was discarded and the pellet (P1) was homogenized in 600 ml of distilled water. The suspension was added slowly to a solution containing 5.4 l of saline buffer (13.75 mM phosphate buffer, pH 7.2/0.44 M NaCl) containing 0.55% Triton X-100 and stirred for 30 min. The suspension was then centrifuged as above. The pellet (P2) was discarded and the supernatant (S2) was used as starting material for acetylcholinesterase purification.

*(a) Adsorption of acetylcholinesterase on the Con A-Sepharose columns.* Con A-Sepharose was washed before and after use as previously described [24, 25]. The supernatant (S2) fraction was applied to a previously equilibrated (saline buffer containing 0.5% Triton X-100) 40 × 2 cm Con A-Sepharose column. The flow rate through the column was kept constant with a peristaltic pump (70 ml/h). The column was then washed with 10 column vol. of buffer. The enzyme was eluted with 6—8 column vol. of the same buffer containing 0.5 M α-methyl-D-mannoside.

*(b) Affinity chromatography on m-dimethylaminoethyl benzoic acid-Sepharose 4B column.* Dimethylaminoethylbenzoic acid-Sepharose 4B was prepared by the method of Massoulie and Bon [23]. The α-methyl-D-mannoside eluent from the Con A-Sepharose column was applied to a dimethylaminoethylbenzoic acid-Sepharose 4B column (40 × 2 cm) previously equilibrated in saline/Triton X-100 buffer and washed with 10 column vol. of the same buffer. Bound acetylcholinesterase was eluted with 1 column vol. of the washing buffer containing 0.2 M tetraethylammonium bromide. A flow rate of 70 ml/h was kept constant during chromatography. This fraction was dialyzed against large volumes of 12.5 mM phosphate buffer, pH 7.2, containing 0.5% Triton X-100, for 24 h, with two changes to remove NaCl, α-methyl-D-mannoside and tetraethylammonium bromide.

*(c) DEAE-cellulose chromatography.* The dialysed tetraethylammonium bromide eluate was applied to a 15 × 1 cm DEAE-cellulose column previously equilibrated with 12.5 mM phosphate buffer containing 0.5% Triton X-100. The elution was performed with a 300 ml linear gradient of NaCl (0—0.4 M). The flow rate (15 ml/h) was kept constant during chromatography.

*(d) Second affinity chromatography on dimethylaminoethylbenzoic acid-Sepharose 4B column.* The most active fractions were pooled and applied to a small (10 × 1 cm) column equilibrated as described above. The column was then washed with 10 column vol. of saline buffer containing 0.05% Triton X-100 and eluted with the smallest volume of the same buffer containing 0.2 M tetraethylammonium bromide. Fractions (0.5 ml) were collected and assayed

for acetylcholinesterase activity. A flow rate of 15 ml/h was kept constant during chromatography. The purified enzyme was dialysed against 0.05% Triton X-100 and stored at −20°C.

*Enzyme and protein determination*

The activity of acetylcholinesterase was determined at 37°C, at pH 7.2, by the spectrophotometric method of Ellman et al. [26], using acetylthiocholine as substrate, by continuous recording of the difference of absorbance obtained in the presence and absence of the acetylcholinesterase inhibitor 1,5-bis-4-allyl-dimethylammonium-phenyl/pentan 3-one dibromide ($1 \cdot 10^{-5}$ M). 1 unit (U) of enzyme activity is defined as the amount of enzyme that hydrolyses 1 μmol acetylthiocholine/min at 37°C. Cholinesterase was determined in similar conditions using butyrylthiocholine as substrate and tetraisopropylpyrophosphoramide ($1 \cdot 10^{-5}$ M) as inhibitor.

*Gel filtration*

Gel filtration was performed at 4°C either on Ultrogel AcA 2/2, Ultrogel AcA 3/4 or Sephadex G-200 columns (90 × 1 cm) in saline buffer containing 0.05 or 0.5% Triton X-100. The flow rate was 4 ml/h. Enzyme samples (1.5 ml) containing the molecular weight markers (each 1 mg/ml) were always used. Fractions of 0.5 ml were recovered.

The following compounds were used as standards of molecular weight ($M_r$) and Stokes' radius ($R_e$): cytochrome *c* ($M_r$, 12 400; $R_e$, 1.74 nm), ovalbumin ($M_r$, 45 000; $R_e$, 3.05 nm), aldolase ($M_r$, 158 000; $R_e$, 4.81 nm), fluorescent IgG ($M_r$, 160 000; $R_e$, 5.22 nm), catalase ($M_r$, 250 000; $R_e$, 5.22 nm), β-galactosidase ($M_r$, 540 000; $R_e$, 8.2 nm), thyroglobulin ($M_r$, 750 000; $R_e$, 8.5 nm) and Blue Dextran 2000. The positions of the markers were determined by the absorbance (610 nm for Blue Dextran, 527 nm for cytochrome *c*, 280 nm for proteins) or fluorescence (fluorescent IgG) or enzyme activity for β-galactosidase [28] and catalase [29].

*Sucrose density gradient centrifugation*

The sedimentation coefficient of the membrane-bound acetylcholinesterase was estimated in 5—20% (w/v) sucrose gradient in saline buffer containing 0.5% (w/v) Triton X-100 using β-galactosidase (16 S), catalase (11.3 S) and fluorescent IgG (6.9 S), as sedimentation markers. Centrifugation was performed at 4°C in a Spinco L2-65 ultracentrifuge with a SW 65 rotor (8—9 h at 230 000 × *g*). 35 fractions of 200 μl were recovered from the gradient. The size of the sample was 200 μl.

*Polyacrylamide gel electrophoresis*

Disc electrophoresis in 5% polyacrylamide cylindrical gels was performed according to Davis [30], except that 0.1% Triton X-100 was added to the gels and buffers (Tris-glycine, pH 8.9, for upper reservoir buffer and Tris-HCl, pH 8.1, for lower reservoir buffer).

Pore gradient electrophoresis [31] was performed in the absence of detergent on gradient polyacrylamide gels (PAA 4/30 from Pharmacia). Gel slabs were pre-run for 20 min at 70 V in a 0.09 M Tris/0.8 M borate buffer (pH 8.4)

containing 2.5 mM EDTA. Electrophoresis was then carried out for 16 h at 150 V.

For gradient polyacrylamide gel electrophoresis in the presence of SDS, gel slabs were pre-run for 60 min at 70 V in 0.04 M Tris/0.02 M sodium acetate (pH 7.4) buffer containing 2 mM EDTA and 0.2% SDS. 12% acrylamide gel slabs were also run in identical conditions.

*Electrofocusing*

Analytical electrofocusing was carried out at 4°C on 5% polyacrylamide gel slabs (125 × 125 × 0.5 mm) containing 0.05% Triton X-100. Gels containing ampholytes (pH range 3—10) were polymerized by ammonium persulfate. Following polymerization, gels were pre-electrofocused at 200 V for 60 min. Samples were applied in the basic region of the pH gradient (around pH 8.5) and then submitted to electrofocusing for 3 h at 300 V with cooling at 4°C.

The preparative flat-bed electrofocusing system was also used on Ultrodex plates (55 × 110 × 5 mm) in the presence of 0.05% Triton X-100. Plates were pre-run and electrofocusing was performed as described above.

*Staining after polyacrylamide gel electrophoresis and electrofocusing*

Proteins were stained overnight with Coomassie brilliant blue R250 (0.1%, w/v, in methanol/acetic acid/water: 4 : 1 : 5, v/v) and destained in the same mixture devoid of dye. Acetylcholinesterase activity was detected on the gels either with the $\alpha$-naphthylacetate method [32] or the copper-thiocholine method [33]. Blanks were performed by preincubation (30 min) and incubation in the presence of $1 \cdot 10^{-5}$ M 1,5-bis-4-allyldimethylammonium-phenyl/pentan 3-one dibromide.

## Results

*Purification of acetylcholinesterase*

When a 10% (w/v) homogenate of rat brain in phosphate saline buffer was centrifuged at 53 000 × *g* for 2 h, only 8% of the acetylcholinesterase activity was obtained in the soluble fraction S1 (Table I). By treatment of the pellet

TABLE I

FLOW SHEET OF THE PROCEDURE OF THE EXTRACTION OF RAT BRAIN ACETYLCHOLINESTERASE

Fractions are defined in Materials and Methods

| Fraction | Total protein (mg) | Volume (ml) | Activity (U/ml) | Total activity (U) | Acetylcholinesterase solubilized (% of total) |
|---|---|---|---|---|---|
| Homogenate * | 1179 | 134 | 1.54 | 206.8 | |
| $S_1$ (soluble in saline) | 286 | 120 | 0.119 | 14.3 | 8 |
| $S_2$ (soluble in saline containing Triton X-100) | 559 | 1155 | 0.138 | 159.0 | 90 |
| $P_2$ (Insoluble residue) | 119 | 210 | 0.018 | 3.9 | 2 |

* Starting from 12 rat brains.

TABLE II

PURIFICATION OF MEMBRANE BOUND RAT BRAIN ACETYLCHOLINESTERASE

This control preparation was obtained from 12 rat brains. The solubilization and chromatographic procedures are given in the text. Preparative experiments were performed starting from 100—120 rat brains. The overall yield of the preparations was always greater than 50%. n.d. = not determined (due to the very dilute protein solution in 0.5% Triton X-100).

| Fraction | Volume (ml) | Total protein (mg) | Total activity (U) | Spec. act. (U/mg protein) | Purification (X-fold) | Yield (% of $S_2$ activity) |
|---|---|---|---|---|---|---|
| Homogenate | 134 | 1179 | 206.8 | 0.18 | 1.00 | — |
| $S_2$ (solubilized only by Triton X-100) | 1155 | 559 | 159.0 | 0.29 | 1.64 | 100 |
| Con A-Sepharose 4B chromatography | 425 | 25.9 | 130.4 | 50.3 | 28.8 | 82 |
| I. Affinity chromatography (dimethylaminoethyl benzoic acid-Sepharose 4B) | 10.7 | 0.096 | 120.8 | 1254.6 | 7176.6 | 76 |
| DEAE-cellulose chromatography | 48.0 | n.d. | 84.3 | n.d. | n.d. | 53 |
| II. Affinity chromatography (dimethylaminoethyl benzoic acid-Sepharose 4B) | 1.8 | 0.025 | 81.2 | 3068.0 | 17 551 | 51 |

with Triton X-100 (0.5% in phosphate saline buffer) and centrifugation, approx. 98% of the total acetylcholinesterase activity from the pellet (P1) remained in the supernatant fraction (S2) (Table I).

A summary of a typical purification of Triton-solubilized enzyme is given in Table II. As can be seen, acetylcholinesterase was quantitatively adsorbed by a Con A-Sepharose 4B column and more than 80% of the enzyme can be eluted from the column with α-methyl-D-mannoside. It should be noted that desorption of acetylcholinesterase from Con A-Sepharose columns is quite difficult and the enzyme is eluted with a tailing peak. Increasing the volume of the α-methyl-D-mannoside eluant would give a 100% recovery of the enzyme. The second affinity column is very efficient, since during this purification step, the enrichment is about 250-fold (Table II). Such enrichment is however much lower (about 40-times) when S2 was directly passed through the same column, probably due to increasing nonspecific adsorption. The DEAE-cellulose step was introduced to eliminate the material which could have been adsorbed non-

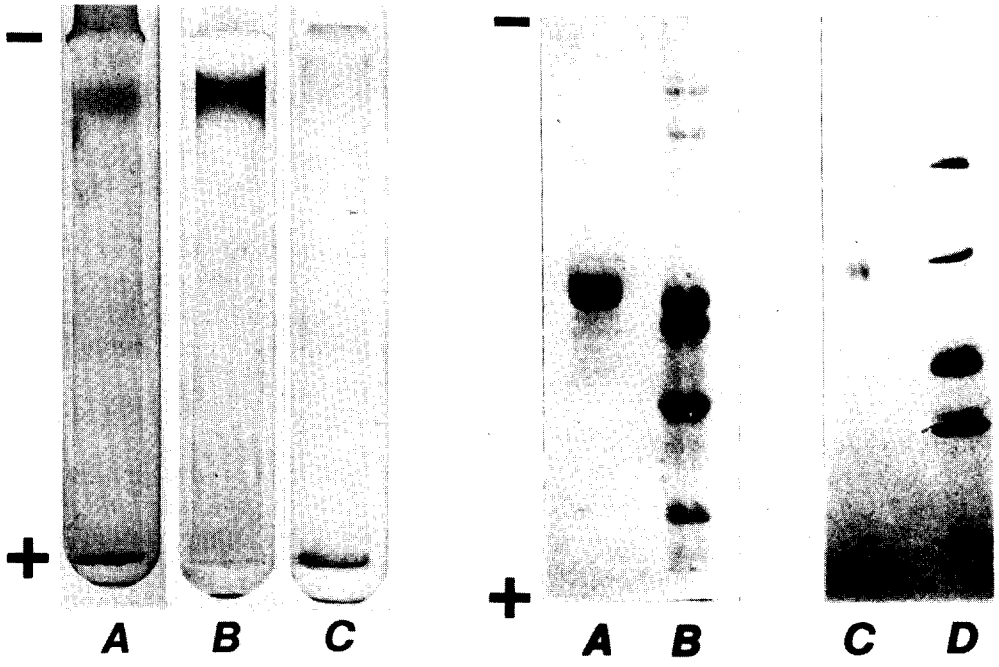



Fig. 1. Polyacrylamide gel electrophoresis of the purified acetylcholinesterase (10 μg) in 5% acrylamide gels containing Triton X-100. A: protein stain with Coomassie brillant blue R250. B: acetylcholinesterase stain with the copper thiocholine method [33]. C: same as B except that 1,5-bis-4-allyldimethylammonium-phenyl/pentan 3-one dibromide ($1 \cdot 10^{-5}$ M) was added to the incubation mixture.

Fig. 2. (A) SDS-polyacrylamide gel electrophoresis of the purified acetylcholinesterase (10 μg) in the presence of 2-mercaptoethanol on a polyacrylamide gradient. Electrophoretic conditions are given in Material and Methods. $M_r$ was determined by using the following proteins as standards (B): ferritin (18 500); lactate dehydrogenase (36 000); catalase (60 000); albumin (67 000); ferritin (220 000) and thyroglobulin (330 000). (C) Pore gradient electrophoresis without detergent of the purified membrane-bound acetylcholinesterase (5 μg). Only a small proportion of enzyme penetrate the gel. Note the tailings of the major bands. $M_r$ values were determined using the following proteins as standard: (D) albumin (67 000); lactate dehydrogenase (140 000); catalase (232 000); ferritin (440 000); thyroglobulin (669 000).

TABLE III

MOLECULAR PROPERTIES OF THE PURIFIED MEMBRANE-BOUND ACETYLCHOLINESTERASE FROM RAT BRAIN

| | Values ± S.D. | Number of experiments |
|---|---|---|
| Molecular weight of the monomer (SDS-polyacrylamide gel electrophoresis) | 74 500 ± 3000 | (4) |
| Stokes' radius (gel filtration) | 7.15 ± 0.35 nm * | (15) |
| | 8.05 ± 0.35 nm ** | (15) |
| Sedimentation coefficient | 9.75 ± 0.2 S | (4) |
| Calculated molecular weight | 294 000 * ± 20 400 | |
| | 328 000 ** ± 20 100 | |
| Isoelectric point (major band) | 5.21 ± 0.02 | (6) |

* Calculated according to Siegel and Monty [34].
** Calculated according to Ackers [35].

specifically on the two affinity columns. Elution by a linear NaCl gradient produced a symmetrical peak of activity eluted between 0.07 and 0.15 M sodium chloride. The last step of affinity chromatography was introduced to concentrate the purified enzyme and to reduce the concentration of Triton X-100.

The specific activity of the purified enzyme was found to be 3068 U/mg protein which represents a 17 550-fold purification. The overall yield of the preparation (about 50%) should be increased if control aliquots at various stages are taken into account.

It should be noted that the acetylcholinesterase activity decreases during storage at 4°C. During the 5 days of the preparation, the enzymatic activity in S2 decreases by 25%. With the purified enzyme, virtually all the activity is lost if it is stored for 1 month at 4°C. The activity is however very stable if the enzyme is stored frozen at −20°C.

*Purity of the Triton-soluble acetylcholinesterase*

Polyacrylamide gel electrophoresis in 5% acrylamide, in the presence of 0.05% Triton X-100, of the purified acetylcholinesterase gave only one slowly migrating protein band (Fig. 1) with enzyme activity. No other protein bands were detected. Polyacrylamide gel electrophoresis in the presence of 5 mM mercaptoethanol and 0.2% SDS in a gradient of acrylamide (Fig. 2) gave only a single band with a molecular weight around 74 500 ± 3000 (Table III). When the gels are overloaded and stained with Coomassie brilliant blue R250, two other bands of lower $M_r$ could be detected as traces (2—5% of the total area of the densitometric tracing).

*Gel filtration and sucrose gradient centrifugation of acetylcholinesterase*

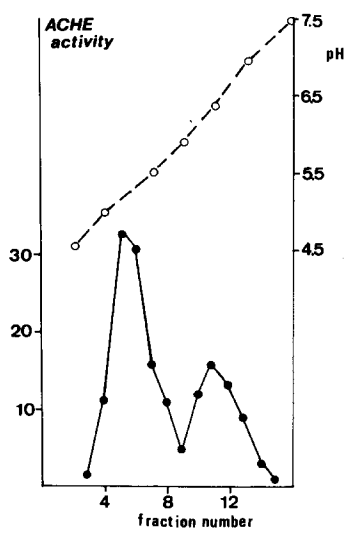
Gel filtration of the purified acetylcholinesterase in the presence of 0.05% Triton X-100 (w/v) allowed the determination of its apparent molecular weight. A mean value of 487 000 ± 21 000 was found for the purified enzyme. The enzyme migrates as a symmetrical peak and shoulders or multiple peak patterns were never detected. The Stokes' radius was calculated from the gel filtration parameters by two different equations [34,35]. With the method

of Siegel and Monty [34] a radius of 7.15 ± 0.35 nm was found which differs substantially from that found using the equation of Ackers [35], 8.05 ± 0.35 nm (Table III). Since in our conditions $(-\log K_d)^{1/2}$ [34] was not found to be proportional to the Stokes' radii of globular marker proteins, and since we found consistent results with the method of Ackers [35] (for Ultrogel AcA 3/4 the effective pore radius was 23.3 ± 1.0 nm) we believe that the value $R_e$, 8.05 ± 0.35 nm is more probable.

A symmetrical peak with sedimentation constant $s$ = 9.75 ± 0.2 S (Table III) was also observed both for S2 enzyme and the purified acetylcholinesterase by centrifugation in sucrose density gradients in the presence of detergent.

*Electrofocusing of acetylcholinesterase*

Electrofocusing of purified acetylcholinesterase gave different patterns depending on the system used. Preparative electrofocusing on Ultrodex plates gave only one peak of activity at pH 5.2. In the presence of 2-mercaptoethanol, two peaks of activity (Fig. 3) at pH 5.2—5.3 and 6.4, respectively, were detected. The picture obtained with analytical electrofocusing is more complex either in the presence or absence of reducing agents (Fig. 4). In both cases, a major protein band with enzymatic activity is detected at pH 5.21 and minor bands at pH 5.12 and 4.95. In the absence of 2-mercaptoethanol, two other weakly stained bands are detected (pH 4.85 and 5.38, respectively). In the presence of



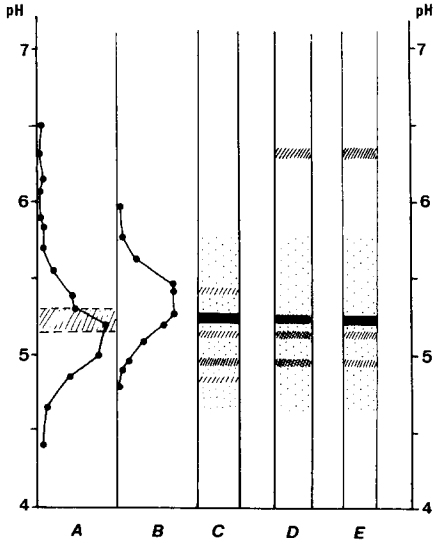




Fig. 3. Isoelectric focusing of the purified acetylcholinesterase (ACHE) on Ultrodex plates in the presence of mercaptoethanol. Fractions were assayed for enzyme activity by the method of Ellman et al. [26]: ●———●, enzyme activity (arbitrary units): ○- - - - -○, pH.

Fig. 4. Drawing of the pattern obtained by electrofocusing of purified acetylcholinesterase on 5% acrylamide gels. (A) 5 mm fractions of the gel were cut and acetylcholinesterase activity was measured by the method of Ellman et al. [26]. (B) The enzyme found in the dashed zone in A was subjected to re-electrofocusing under the same conditions. (C) The gel was run in the absence of mercaptoethanol and stained for proteins (Coomassie brillant blue R250). (D) The gel was run in the presence of mercaptoethanol and stained for acetylcholinesterase activity [32]. (E) The gel was run in the presence of mercaptoethanol and stained for proteins (Coomassie brillant blue R250).

2-mercaptoethanol, these two bands disappeared, whereas a protein band with acetylcholinesterase activity is detected at pH 6.35. It should be emphasized that diffuse protein and enzyme stain is always present between pH 4.65 and 5.75.

We have tested the possibility that the multiple band pattern could be due to artefacts. The major band (pH 5.21) was isolated after analytical electrofocusing (Fig. 4a) and submitted to the same procedure in identical conditions. As shown in Fig. 4b, this band gives rise to a diffuse pattern, suggesting artefacts due to electrofocusing.

*Pore gradient electrophoresis on polyacrylamide gradient in the absence of detergent*

The picture obtained was seen in Fig. 2 with a preparation of purified acetylcholinesterase stored at −20°C. Only one band with a molecular weight of 420 000 can be detected within the gel. When the gels are overloaded (10—20 μg) two minor bands with molecular weight of 520 000 and 720 000, respectively, are also present. However, most of the protein was precipitated at the start of the gel. With preparations stored at 4°C for 1 month (which lost their activity), a multiple band pattern was detected with the purified acetylcholinesterase. Analysis of the electrophoretic mobility of the various bands (data not shown) indicated that all of them could be multiples of the faster-migrating component ($M_r$, 74 700): 150 000 (×2); 220 000 (×3); 370 000 (×5); 440 000 (×6); 560 000 (×8); 730 000 (×10) and 1 000 000 (×15).

## Discussion

*Method of purification of the membrane-bound acetylcholinesterase*

As suggested in the Introduction of this paper, contradictory views exist in the literature concerning the solubilization of rat brain acetylcholinesterase. Chan et al. [4] and Wenthold et al. [5] claimed that a sucrose/EDTA mixture is able to solubilize 'most' of the enzyme from rat brain. It is clear however that only 20% of the enzyme can be solubilized by this method including part of the 10% which is soluble in water or other aqueous buffers of high or low ionic strength (Ref. 1 and our own data). The other 10% are extracted after stirring overnight at 4°C. Since it has been demonstrated that, in the head of house-fly or in mouse brain, incubation in sucrose/EDTA mixtures at room temperature or 37°C is more efficient [36,37 or 11], it is possible that this solubilization is due to mild proteolysis.

Preliminary experiments have shown that extraction by water or buffered saline (0.4 M NaCl) yields only about 10% of the total acetylcholinesterase found in adult rat brain. Repeated extractions with the same media do not solubilize more enzyme. We considered thus that the remaining enzyme has the characteristics of a membrane-bound molecule.

*Purity of the enzyme*

Using the procedure described in Materials and Methods, the membrane-bound acetylcholinesterase can be purified within a week in very high yield.

The specific activity of our purified enzyme (3067 U/mg protein) represents

more than 17 500-fold purification and is very similar to activities of brain acetylcholinesterase reported in the literature: 4500 U/mg protein [5] for the sucrose/EDTA soluble enzyme and 4250 U/mg for the total bovine caudate nucleus enzyme [10]. The differences observed for these purified enzymes are possibly due to difficulties in the determination of very small amount of protein, mainly in the presence of Triton X-100, as observed by Ruess et al. [10], and also to the different conditions used for enzymatic determinations [5,10].

No further comparison of the purity of the enzyme can be achieved since electrophoretic photographs are not given by others [4,5,10]. Assuming that there are no contaminants with the same charge and molecular weight for the protein and the same molecular weight for the subunits, we can estimate from the scan of the SDS-polyacrylamide gels stained with Coomassie brilliant blue R250 at least a 95—98% purity for our enzyme. Our purified preparations are completely devoid of nonspecific esterase activities on the basis of the action of inhibitors. In fact, during the preparation, the low quantity of cholinesterase present in the S2 fraction is adsorbed on the Con A-Sepharose column but not adsorbed on the dimethylaminoethyl benzoic acid-Sepharose 4B column. Since this column is used twice during the preparation and since cholinesterase is not detectable in the purified preparation, we have good reason to believe that it is not a contaminant.

*Molecular properties of the membrane-bound acetylcholinesterase*

*(a) Molecular weight and oligomeric structure.* As mentioned above, gel filtration gave only one peak with an apparent molecular weight of 487 000 ± 21 000 which is in agreement with the results of Rieger and Vigny [1] but in contradiction with results showing a profile with multiple peaks [4,5,7,11]. In our working conditions, the recoveries of enzymatic activities from the gel filtration columns are quantitative and, thus, it cannot be postulated that some molecular forms are lost on the column. The possibility that they are lost during the preparation of the enzyme (50% yield) can be excluded since only one symmetrical peak is obtained from the S2 fraction which contained all the membrane-bound acetylcholinesterase.

The presence of a unique peak could be an artefact due to the interactions of Triton X-100 with the enzymes as suggested [38,39] for the acetylcholinesterase of erythrocyte membranes. Our attempts to eliminate Triton X-100 by prolonged dialysis or chromatography on Biobeads SX2 [40] were unsuccessful due to enzyme precipitation. Our observations confirm the results of Rieger and Vigny [1], who observed multiple and high molecular weight aggregates when the Triton-soluble enzyme from rat brain was submitted to gel filtration (or sucrose gradient centrifugation) in the absence of Triton X-100. It is thus more likely that artefacts are produced by the elimination of Triton X-100.

An artificial increase of Stokes' radius due to interactions of Triton X-100 with the enzyme, suggested by others [1], is possible, but we have not as yet an experimental proof that such a phenomenon occurs, since the enzyme precipitates as soon as the detergent was eliminated (see above). An increase of Stokes' radius has been observed [1] for a soluble 10 S form of acetylcholinesterase when this enzyme is chromatographed in the presence of Triton X-100 (from 7.4 to 8.5 nm). It is not clear however if this soluble 10 S form is identi-

cal to the 10 S membrane-bound form and further studies are necessary to clarify this point. However, the value determined here (8.05 ± 0.35 nm) is in the same range as the previous one.

The *s* value of the purified membrane-bound acetylcholinesterase determined here (9.75 ± 0.2 S) agrees well with the data given in Ref. 1 either for the soluble 'hard to solubilize' form either in the presence or in the absence of detergent or for the membrane-bound 'hard to solubilize' form analysed in the presence of detergent (about 10 S).

The calculation of the molecular weight of the purified acetylcholinesterase is dubious since uncertainty exists for the determinations of Stokes' radii, sedimentation coefficients and partial specific volumes in the presence of detergents. However if we assume (i) that Stokes' radii and sedimentation coefficients determined in the presence or in the absence of detergents are not dramatically different [1,14] and (ii) that the partial specific volume ($\bar{v}$) is similar to those of the marker proteins and thus to those of the various forms of acetylcholinesterase found in various tissues and species [14] and to the 'hard to solubilize' 10 S form of the rat brain acetylcholinesterase ($\bar{v} = 0.76$) [1], calculations of the molecular weight from the Stokes' radius and sedimentation coefficient give 294 000 and 328 000, respectively, according to Siegel and Monty [34] or to Ackers [35]. These results as well as Stokes' radius and sedimentation coefficient are very close to those found for the globular G4 form from bovine superior cervical ganglia [14]. From our calculated values a tetrameric structure of a monomer with $M_r$ about 75 000 has thus to be expected.

*(b) Glycoprotein nature of the membrane-bound acetylcholinesterase.* As shown before, the membrane-bound acetylcholinesterase binds completely to Con A-Sepharose columns. The specificity of this lectin for terminal gluco- and manno-pyrannoside [41], also demonstrated by the specific elution of the enzyme by these sugars, indicates that acetylcholinesterase is a glycoprotein and that the membrane-bound enzyme appears to be homogeneous with regard to the affinity for the lectin. These results are also in agreement with the almost complete coprecipitation of concanavalin A and rat brain acetylcholinesterase found by others [1] but differ substantially from the heterogeneity observed by Gurd [3] with the membrane-bound enzyme prepared by the sucrose/EDTA extraction method.

*(c) Heterogeneity of the membrane-bound acetylcholinesterase: the role of artefacts.* As mentioned in the Introduction of this paper, a very complex heterogeneity of acetylcholinesterase has been shown using electrophoresis or electrofocusing together with density gradient centrifugation. It has not been possible to correlate these various kinds of heterogeneity [18]. Only two of the methods used by us to test the molecular properties of the pure membrane-bound acetylcholinesterase, i.e., pore gradient electrophoresis and isoelectric focusing give evidence of heterogeneity.

The method of pore gradient electrophoresis was introduced in an attempt to measure the apparent molecular weight of the purified membrane-bound acetylcholinesterase in the absence of detergent [31], since Triton X-100 (an uncharged detergent) does not penetrate the gel. The main result of this technique was that with fresh enzyme preparations, precipitation occurs as soon as the enzyme penetrates the polyacrylamide gel. Only a small proportion of the

enzyme migrated with tailing, giving a major band with an apparent $M_r$ of 420 000 and two minor bands. These results are very similar to those of Ruess et al. [10] for the bovine caudate nucleus enzyme and of Grossman and Lieflander [42] for the calf erythrocyte acetylcholinesterase, when these enzymes were submitted to polyacrylamide gel electrophoresis in the absence of detergent and have to be interpreted as artefacts due to this inappropriate technique.

However, the results obtained with old enzyme preparations are particularly interesting, since no precipitation occurs at the start of the gel and a definite multiple band pattern is observed with $M_r$ ranging from 75 000 to several millions. This heterogeneity is clearly not due to proteolysis since these old preparations, in SDS-polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol, give only one band identical to that obtained with fresh preparations. Identical results were obtained for the erythrocyte membrane acetylcholinesterase by Niday et al. [43], and our interpretations are very similar: during storage for a long period, denaturation processes occur, involving redistribution of the oligomeric structures of the membrane-bound enzyme. As observed by others [39], changes in sedimentation patterns are also detected in similar conditions with acetylcholinesterase from erythrocytes membranes.

The results of electrofocusing of the purified acetylcholinesterase could indicate a complex heterogeneity of the membrane-bound enzyme. However, in contrast with what has been observed by others for the sucrose/EDTA enzyme [5], rerunning of the major band (pH 5.21) gives in our results a diffuse and broad peak of activity. This means that the multiple band pattern obtained is an artefact due to the electrofocusing step itself, possibly by interactions between the enzyme and ampholytes [44]. The demonstration of others [39,45] that acetylcholinesterase isolated from human erythrocyte membranes with Triton X-100 is associated with lipids (mainly phosphatidylserine) has also to be taken into account in the interpretation of the heterogeneity obtained with electrofocusing. The effect of mercaptoethanol and the appearance of a protein band with acetylcholinesterase activity at pH 6.3 might indicate that an actual heterogeneity of the enzyme exists. It appears however (in preparation) that this band corresponds to an incomplete modification of the oligomeric structure of the membrane-bound enzyme due to the partial reduction of disulfide bridges.

Immunochemical studies are now in progress to obtain more precisions on the structure and properties of the rat brain acetylcholinesterase.

## Acknowledgements

We thank Drs. J. Massoulie, M. Vigny and S. Bon for useful discussion and Dr. O.K. Langley for reviewing this manuscript. J.-P.Z. is a Chargé de Recherche au CNRS.

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