Journal of Chromatography, 157 (1978) 141–151

CHROM. 10,994

PURIFICATION OF ACETYLCHOLINESTERASE FROM PIG CEREBRAL CORTEX BY AFFINITY CHROMATOGRAPHY

CHARLES A. REAVILL* and DAVID T. PLUMMER

Department of Biochemistry, Chelsea College, University of London, Manresa Road, London SW3 6LX (Great Britain)

(Received March 6th, 1978)

SUMMARY

Acetylcholinesterase from pig cerebral cortex was solubilised with 1% (w/v) Triton X-100 and purified by affinity chromatography. Three different ligands were investigated and details are given for their preparation. The elution profile depended on the presence of Triton X-100, the ionic strength and the inhibitor used to remove the enzyme from the column as well as the nature of the affinity material.

The most efficient purification was obtained when the enzyme was eluted from a column containing the acetylcholinesterase inhibitor [1-methyl-9-(N$^{\beta}$-ε-amino-caproyl)-β-aminopropylamino] acridinium bromide hydrobromide covalently linked to Sepharose 4B. A recovery of 44% of the applied enzyme was eluted from the column with a specific activity of 148 μmoles min^{-1} mg^{-1} and a purification of 900-fold.

INTRODUCTION

Acetylcholinesterase (AChE; acetylcholine hydrolase, EC 3.1.1.7) is present in the central nervous system of all vertebrates[1] and reports from several groups of workers have shown that the brain enzyme exists in a number of molecular forms[2–4]. Work in our own laboratory has concentrated on pig cerebral cortex as the source of AChE and the effect of a number of methods of solubilisation has been studied together with the multiple enzymic forms of the resultant preparations[5]. This initial investigation was extended to a study of the naturally soluble and membrane forms of the enzyme[6] and the multiple molecular forms of isolated subcellular fractions[7]. In the above work, relatively crude preparations of the enzyme were used and in this paper we present the results obtained during attempts to purify the enzyme by affinity chromatography using several affinity materials.

* Present address: Department of Neurology, Institute of Psychiatry, Denmark Hill, London SE5 8AF, Great Britain.

MATERIALS AND METHODS

Materials

Fresh pig brains were kindly provided by Walls Slaughterhouse (Acton, Great Britain) and the Co-operative Society Slaughterhouse (Woolwich, London, Great Britain).

5,5'-Dithiobis-(2-nitrobenzoate) (DTNB) and acetylthiocholine iodide (ATChI) were obtained from Sigma (London, Great Britain) and Triton X-100 from BDH (Poole, Great Britain).

Materials for the three affinity columns were obtained from the following sources. Sepharose was bought from Pharmacia (London, Great Britain), N-benzyloxycarbonyl-ε-aminocaproic acid and 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride were purchased from Aldrich (Wembley, Great Britain). Triethylamine, *p*-dimethylaminoaniline and phenol (redistilled before use) were supplied by Fisons (Loughborough, Great Britain). Anhydrous HBr in glacial acetic acid, isobutylchloroformate, iodomethane, 9-chloracridine and aminopyridine were obtained from Eastman-Kodak (Rochester, N.Y., U.S.A.).

All other reagents used were analytical grade and all solutions were prepared in glass distilled water.

Preparation of the enzyme

Porcine brains were obtained from the slaughterhouse within 20 min of death, transported to the laboratory in crushed ice, then stored frozen until required. After thawing at 4°, the membranes and blood vessels were removed from the surface of the brains and the cortex excised. The AChE was then solubilised using the method of Ho and Ellman[2] with slight modifications. The brain cortex was finely chopped and a 20% (w/v) suspension prepared in sodium phosphate buffer (0.03 *M*, pH 7.0) by homogenising for 5 min at 4° in a Waring blender. The suspension was then centrifuged at 100,000 *g* for 1 h and the supernatant decanted. The AChE in this fraction was referred to as the "naturally soluble" enzyme. The pellet was resuspended in the buffer solution to give a protein concentration of 8 mg/ml and Triton X-100 added to a final concentration of 1% (w/v). The mixture was stirred for 10 min at room temperature and centrifuged at 100,000 *g* for 1 h. The supernatant was taken as the "Triton solubilised enzyme".

Assay of acetylcholinesterase

AChE activity was determined at 30° and pH 8.0 by a colorimetric method using acetylthiocholine as substrate[8]. For routine assay, 50 μl of enzyme was mixed with 3 ml of sodium phosphate buffer (0.1 *M*, pH 8) and incubated at 30° for 10 min. After this time 100 μl of DTNB (0.01 *M*) was added to the mixture followed by 20 μl of ATChI (158.5 m*M*) to give a final substrate concentration of 1 m*M*. The hydrolysis of acetylthiocholine produces thiocholine which reacts with DTNB to give 5-thio-2-nitrobenzoate, a yellow chromophore that absorbs at 412 nm. The enzyme activity was then measured by following the increase in absorbance at 412 nm in a Perkin-Elmer SP124 double beam spectrophotometer. The activity was determined in I.U. from the initial slope of the curve by calculating the extinction change (ΔE) with time (min) and allowing for the molar extinction coefficient of the chromo-

phore ($1.36 \cdot 10^4$ l mole^{-1} cm^{-1}), the volume of the enzyme (0.05 ml), the total volume of the reaction mixture (3.17 ml) and a factor of 1000 to obtain μmoles then:

$$\text{AChE activity} = (\Delta E \cdot 1000 \cdot 3.17)/(\text{min} \cdot 1.36 \cdot 10^4 \cdot 0.05)$$
$$= (\Delta E/\text{min}) \cdot 4.66 \ \mu\text{moles min}^{-1} \text{ ml}^{-1}$$

Estimation of protein

Protein was determined by the method of Lowry *et al.*[9] using crystalline bovine serum albumin as standard. However if Triton X-100 was present, a gelatinous precipitate was formed, but this interference was overcome by centrifuging the precipitate (1000 *g* for 5 min) and incorporating Triton X-100 in the reagent blank and standard[10,11].

Affinity column I (MAP-agarose)

Preparation of the ligand N-methyl-3-aminopyridinium iodide. Aminopyridine (53 mmoles; 5 g) was dissolved in 75 ml of acetone, added to iodomethane (292 mmoles; 15 ml) and stirred for 18 h. The precipitate was then filtered and washed with acetone.

N-methyl-3-aminopyridinium iodide (MAP) was prepared in 11.2 g (95%) yield; m.p. (found): 120–122°; m.p. (literature) 123°.

Preparation of MAP-agarose. The affinity material was built up stepwise by a method based on the procedures used by Berman and Young[12] and Goodkin and Howard[13]. Cyanogen bromide (30 g) was added with stirring to 100 ml of washed Sepharose 2B suspended in 100 ml of water. The temperature was kept below 20° with crushed ice and the pH at 11.0 with 4 *M* NaOH. After 12 min the suspension was filtered rapidly under suction with 1 l of sodium borate buffer (0.1 *M*, pH 9.5) at 4°. Diaminobutane (198 mmoles; 17.4 g) in 100 ml of sodium borate buffer (0.1 *M*, pH 9.5) was immediately added and the mixture shaken gently at 4° overnight. The excess amine was removed by filtration on a sintered glass funnel and the washed Sepharose dispersed in 100 ml of water. Succinic anhydride (100 mmoles; 10 g) dissolved in 105 ml water at 4° was added to the Sepharose and the pH raised to 6.0 with 4 *M* NaOH. The pH was carefully monitored and after it had stabilised the suspension was left for 5 h at 4°.

N-Methyl-3-aminopyridinium iodide (0.6 mmoles; 0.13 g) was added to 6 ml of the succinylated resin in the presence of 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (4.5 mmoles; 0.86 g), shaken gently overnight at 4° and thoroughly washed before use with elution buffer.

Affinity column II (ε-aminocaproyl-PTA-agarose)

Preparation of ligand [N-(ε-aminocaproyl)-p-aminophenyl] trimethylammonium bromide hydrobromide. The method was based on that of Dudai and Silman[14] with some modifications.

N-Benzyloxycarbonyl-ε-aminocaproic acid (60 mmoles; 15.9 g) was dissolved in 200 ml of ethyl acetate with vigorous stirring and cooled in an ice bath. Triethylamine (60 mmoles; 6.1 g) and isobutylchloroformate (60 mmoles; 8.2 g) were added with stirring and the mixture left to stand for 20 min. The solution was then vig-

orously stirred and 60 mmoles (8.2 g) of *p*-dimethylaminoaniline dissolved in 50 ml of ice cold ethyl acetate was added dropwise and the solution left at room temperature for a further 5 h. The resulting precipitate was filtered, washed successively with water, ethyl acetate and light petroleum (b.p. 40–60°) and dried by suction. This crude material was then treated with activated charcoal in hot methanol and recrystallised from hot methanol.

N-(N-Benzyloxycarbonyl-ε-aminocaproyl)-N,N'-dimethyl-*p*-phenylenediamine was prepared in 8.23 g (36%) yield; m.p. (found) 110–111°; m.p. (literature) 110–111°.

The above compound (22.1 mmoles; 8.23 g) was suspended in 40 ml of methanol with 110.5 mmoles (5.26 g) of iodomethane and refluxed for 3 h. The product was taken to dryness under reduced pressure, triturated with 200 ml of diethyl ether and recrystallised from 100 ml of absolute ethanol.

[N-(N-Benzyloxycarbonyl-ε-aminocaproyl)-*p*-aminophenyl] trimethyl ammonium iodide was prepared in 7.64 g (71%) yield; m.p. (found) 142.5–144°; m.p. (literature) 143–145°.

This compound (10 mmoles; 5.25 g) was dissolved in 10 ml of glacial acetic acid + HBr, left at 25° for 40 min, then precipitated with 400 ml of dry diethyl ether. The solidification was completed by trituration with five batches of dry diethyl ether. The solid was dried for 48 h in a vacuum desiccator over pellets of NaOH, then finally recrystallised from 200 ml of absolute ethanol.

[N-(ε-Aminocaproyl)-*p*-aminophenyl] trimethyl ammonium bromide hydrobromide was prepared in 2.95 g (69%) yield; m.p. (found) 190–192°; m.p. (literature) 191–194°.

For convenience this compound is referred to as ε-aminocaproyl-PTA ,using the convention of Dudai and Silman[14].

Preparation of ε-aminocaproyl-PTA-agarose. The material for the affinity column was prepared by a method based on that of Axen *et al.*[15] as modified by Blumberg *et al.*[16]. Cyanogen bromide (7 g) was stirred with 70 ml of water for 10 min during which time most of it had dissolved. A slurry of Sepharose 4B was washed with water and 70 ml of this was added to the stirred cyanogen bromide. The pH was immediately adjusted to 11.0 with 6 *M* NaOH and the mixture cooled below 20° with crushed ice for 8 min. The activated gel was then rapidly washed with 1 l of ice cold water on a Buchner funnel and the wet gel quickly added to a solution of ε-aminocaproyl-PTA (117 μmoles; 50 mg) in 36 ml of 0.5 *M* $NaHCO_3$. The mixture was shaken gently for 16 h in the cold room, filtered and washed thoroughly with 0.1 *M* $NaHCO_3$ and water. The amount of ligand coupled was found by estimating spectrophotometrically the quantity of ligand remaining in the washings. (At pH 9.8, ε-aminocaproyl-PTA, $\lambda_{max.} = 245$ nm, $E = 15{,}500$ l mole^{-1} cm^{-1})[14].

Approximately 1 μmole of ligand was coupled per ml of Sepharose. The affinity column was washed extensively with the elution buffer before use.

Affinity column III (MAC-agarose)

Preparation of ligand [1-methyl-9-(N$^{\beta}$-ε-aminocaproyl)-β-aminopropylamino] acridinium bromide hydrobromide. The method of preparation was based on that of Dudai and Silman[14] with some amendments advised by Silman (personal communication). Phenol (1.28 moles; 120 g) was heated at 70° and 93.6 mmoles (20 g) of 9-chloracridine added to the melt. After all the solid had dissolved, 100 ml of 1,2-

propylenediamine was added to the vigorously stirred mixture and the temperature raised to 120°. It was critical that this temperature was not exceeded. After 30 min, the 9-(β-aminopropylamino) acridine was precipitated by pouring the reaction mixture with rapid stirring into 1600 ml of 0.75 *M* NaOH. This was left overnight to complete the solidification, filtered and washed successively with 2 *M* NaOH and water. The crude product was dried in vacuo, refluxed in 1100 ml of benzene and filtered. The insoluble residue was discarded and the filtrate concentrated to 200 ml, mixed with 50 ml of petroleum and left in the cold room overnight. The crystalline product was filtered and washed with benzene.

9-(β-Aminopropylamino) acridine was prepared in 9.5 g (40%) yield; m.p. (found) 131–139°; m.p. (literature) 131–133°.

N-Benzyloxycarbonyl-ε-aminocaproic acid (30 mmoles; 7.9 g) was dissolved in 150 ml dry ethyl acetate in a salt ice bath at −10° and stirred vigorously. Triethylamine (30 mmoles; 3.05 g) followed by isobutylchloroformate (30 mmoles; 4.1 g) were added to the mixture which continued to be stirred for a further 20 min at −10°. The precipitated triethylamine hydrochloride was washed with 20 ml of dry ethyl acetate and the combined filtrate and washings returned to the salt ice bath when a solution of 9-(β-aminopropylamino) acridine (25 mmoles; 6.3 g) in 100 ml of dimethylformamide was added. The mixture was left for a further 10 min at −10° and then 12 h at room temperature. Thin-layer chromatography (TLC) on silica gel in glacial acetic acid indicated that the reaction had gone to completion. The mixture was evaporated to dryness under reduced pressure, the residue dissolved in 50 ml of absolute methanol and 8 ml of iodomethane were added to the solution which was then refluxed for 4 h and left overnight at room temperature. TLC on silica gel in ethyl acetate was used to check the reaction to be gone to completion. The solution was evaporated to dryness under reduced pressure, extracted twice with 100-ml aliquots of dry ethyl acetate and the product recrystallised from 80 ml of 2-propanol. The product was finally filtered and washed with ice cold 2-propanol followed by ice cold diethyl ether.

[N-(N-Benzyloxycarbonyl-ε-aminocaproyl)-β-aminopropylamino) acridinium iodide was prepared 7.2 g yield; m.p. (found) 155–157°; m.p. (literature) 156–157°.

The above quaternary compound (6.4 mmoles; 4.1 g) was dissolved in 40 ml of anhydrous glacial acetic acid and 80 ml of anhydrous HBr in glacial acetic acid were added with stirring. This solution was left to stand at room temperature for 30 min and the product precipitated with dry diethyl ether. The precipitate was triturated with five batches of diethyl ether, filtered, washed with diethyl ether and stored in a vacuum desiccator over dry NaOH pellets for 24 h. The product was finally recrystallized from absolute ethanol.

[1-Methyl-9-(N$^{\beta}$-ε-aminocaproyl)-β-aminopropyl amino] acridinium bromide hydrobromide was prepared in 2.5 g (72%) yield; m.p. (found) 236–240°; m.p. (literature) > 240° with decomposition. For convenience, this compound is referred to as MAC[14].

Preparation of MAC-agarose. The ligand was coupled to the Sepharose 4B in the same way as described for the preparation of the ε-aminocaproyl-PTA-agarose. The ligand MAC (0.09 mmoles; 50 mg) was mixed with 70 ml of the cyanogen bromide activated Sepharose 4B. The washings were assayed spectrophotometrically when 0.5–1.0 μmole of MAC was found to be coupled per ml of Sepharose. (At pH

8.3, $\varepsilon = 7{,}880\ \text{l mole}^{-1}\ \text{cm}^{-1}$ at 393 nm, $\varepsilon = 12{,}050\ \text{l mole}^{-1}\ \text{cm}^{-1}$ at 410 nm and $\varepsilon = 10{,}150\ \text{l mole}^{-1}\ \text{cm}^{-1}$ at 431 nm)[14].

Conditions for use of affinity columns

All operations were carried out at 4°.

Binding. The columns used had a bed volume of 12 ml and the enzyme was run through the affinity material at a rate of 30–40 ml/h.

Elution. The standard elution buffer was 0.03 *M* sodium phosphate, pH 7.0 containing 1% (w/v) Triton X-100 and all solutions used for elution were prepared in this medium. The column was first washed with 5–10 column volumes of buffer until the eluate gave a zero reading of protein. The elution was then continued with 5 column volumes of the standard buffer containing the competitive inhibitor edrophonium chloride (10 m*M*) or decamethonium bromide (10 m*M*) and 2–5-ml fractions were collected. Finally, the column was washed with five column volumes of buffer containing 1 *M* NaCl.

Washing. The column was prepared for further use by washing with 6 *M* guanidine hydrochloride and 50 column volumes of elution buffer.

Enzyme assay in fractions. The enzyme was assayed by the method previously described after dialysis for 72 h against three changes of 2 l of elution buffer.

RESULTS

Column I (MAP-agarose)

After equilibration of the column, 1 ml of the enzyme which contained approximately 25 mg of protein was applied to the column. The standard elution procedure was carried out with edrophonium chloride (10 m*M*) as inhibitor. The elution profile obtained (Fig. 1) showed two peaks of activity, one emerging from the column after edrophonium chloride and the other following elution with high salt concentration (1 *M* NaCl). The enzyme eluted in the edrophonium chloride peak gave only a 5-fold purification and contained 35% of the total AChE activity applied to the column. If Triton X-100 was excluded from the elution media, the AChE was quantitatively adsorbed onto the column and could not be removed by the inhibitor or high salt solution until the detergent was reintroduced into the buffer.

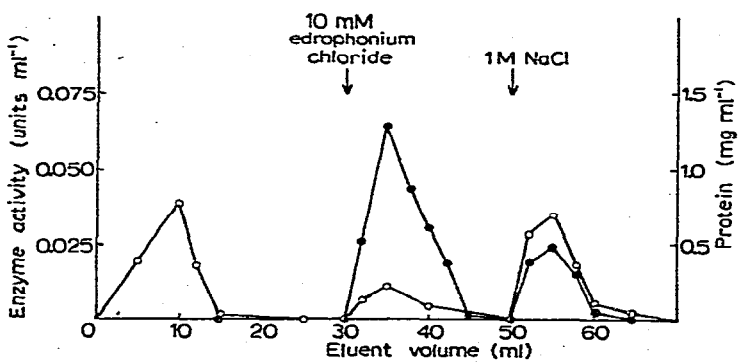

Fig. 1. Fig. 1. Elution of acetylcholinestetase activity (●) and protein (○) from an MAP-agarose column.

Column II (ε-aminocaproyl-PTA-agarose)

A much greater volume of the enzyme was used in this column (150–200 ml) which was washed with buffer until protein could no longer be detected in the eluate. The column was then eluted with buffer containing edrophonium chloride followed by high salt concentration as previously described. Rather surprisingly, only 0.4% of the AChE activity appeared in the inhibitor peak with no significant purification. Increasing the concentration of inhibitor 5-fold to 50 m*M* made no difference to the yield or degree of purification of the enzyme. In view of this, a different inhibitor, namely, decamethonium bromide was used to remove the AChE from the column and this proved to be more effective. The protocol was the same as that described above except that decamethonium bromide (10 m*M*) was substituted for the edrophonium chloride. With this modification, there was a significant improvement in the elution with 35% of the AChE activity appearing in the inhibitor peak and a 16-fold purification (Fig. 2). As with the previous column, exclusion of Triton X-100 from the elution buffer led to strong retention of the enzyme to the column with none appearing in the collected fractions.

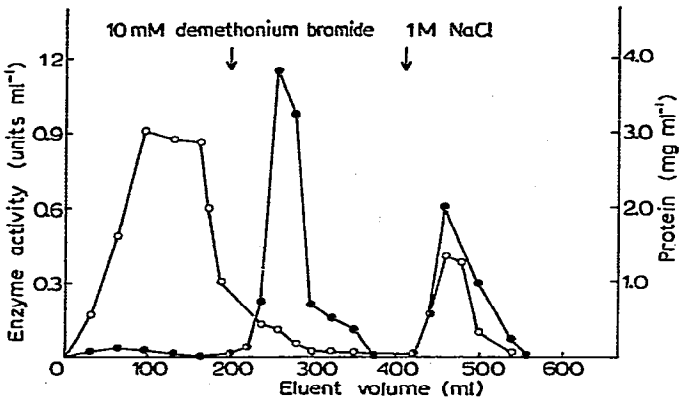

Fig. 2. Elution profile of acetylcholinesterase activity (●) and protein (○) from an ε-aminocaproyl-PTA affinity column.

Column III (MAC-agarose)

As with column II, large volumes of the enzyme could be applied without any loss in the collected fractions. The elution procedure was the same as for column II with a wash of successively decamethonium bromide and 1 *M* NaCl.

A total of 44% of the AChE activity was eluted in the decamethonium peak which had a specific activity of 148 μmoles min^{-1} mg^{-1}, a purification of 900-fold (Fig. 3). When this purified enzyme was rechromatographed on another MAC column and eluted with decamethonium bromide, the specific activity was at least doubled although the protein content could not be determined accurately due to its low level.

A summary of the results obtained for all three columns is shown in Table I.

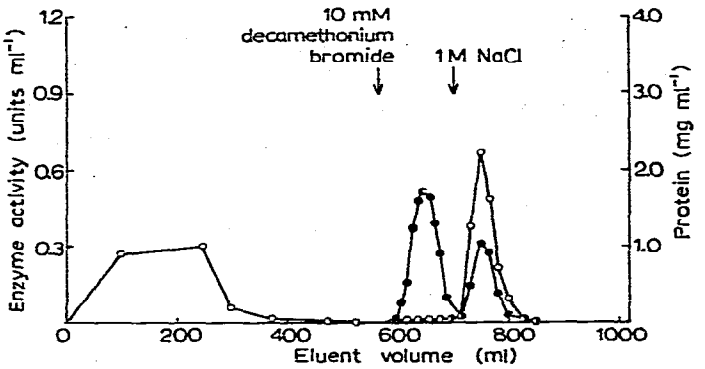

Fig. 3. Purification of acetylcholinesterase by affinity chromatography on MAC-agarose column. Acetylcholinesterase (●), protein (○).

TABLE I

AFFINITY CHROMATOGRAPHY OF ACETYLCHOLINESTERASE ON THREE DIFFERENT MATERIALS

*Affinity material**		*Protein (mg)*	*Enzyme activity (μmoles min⁻¹)*	*Specific activity (μmoles min⁻¹mg⁻¹)*	*Yield (%)*	*Purification (times)*
MAP-agarose	Total applied	25	1.671	0.067	—	—
	Recovered in peak	1.75	0.585	0.336	35	5
ε-Aminocaproyl-PTA-agarose	Total applied	955	150	0.157	—	—
	Recovered in peak	21	52	2.476	35	16
MAC-agarose	Total applied	713	117	0.164	—	—
	Recovered in peak	0.349	51.5	147.6	44	900

* See Fig. 4 for the formulae.

DISCUSSION

The first affinity column to be prepared was MAP-agarose as this had been used by Goodkin and Howard[13] to purify the AChE of rat brain synaptosomal membranes. The attraction of this method was that the rat brain enzyme had been solubilised with Triton X-100 and so a procedure was available which involved the presence of detergent in the elution medium. The results show that the column was effective in binding the AChE and other proteins and since detergent was present in the elution medium, it was unlikely that the enzyme was being retarded by hydrophobic interactions on the apolar side chain. Elution with edrophonium chloride removed 35% of the AChE activity from the column but with an overall purification of only 6-fold. This compared unfavourably with the results of Goodkin and Howard[13] in which they succeeded in purifying the rat brain enzyme by 100- to 150-fold. The reason for this difference possibly lies in the different properties of the rat brain and pig brain acetylcholinesterases. In view of the disappointing results obtained in the purification of AChE, another column was prepared. The phenyltrimethylammonium ligand was selected as this has been shown to be a good competitive inhibitor of AChE especially when bound to an ε-aminocaproyl side-arm. Dudai *et al.*[17] have shown this moiety to have an inhibitor constant (K_i) of 6 μM for AChE.

The ε-aminocaproyl-PTA-agarose was very efficient in binding the pig brain AChE as shown by the fact that large volumes of the enzyme could be applied without being eluted by the buffer. The largest volume used was 300 ml of extract containing about 150 units of AChE activity. Even with this load very little of the enzyme appeared in the eluate although contaminating protein was washed off the column. The only factor limiting the volume of extract applied to the column was getting sufficient starting material. However the initial experiments with this column were disappointing and only a trace of the enzyme activity (0.4%) was found in the crude edrophonium chloride wash. Increasing the inhibitor concentration to 50 m*M* made no difference. This may have been due to the fact that edrophonium chloride has only one positively charged nitrogen atom which would mean that if the enzyme was binding to the quaternary atom on the column by way of one of its peripheral anionic sites as well as by the active centre anionic site then the edrophonium chloride would not be very effective. For this reason, another competitive inhibitor, decamethonium bromide, was used in an attempt to elute the AChE. This molecule was chosen because it has two positive centres separated by a decamethylene bridge which has just the correct dimensions to span the distance between the active anionic and peripheral anionic sites. Furthermore, several other groups seem to have preferred to use this inhibitor in the affinity chromatography of AChE[17].

Decamethonium bromide was better than edrophonium chloride at removing the acetylcholinesterase from the column and a recovery of 35% was obtained. Again the purification was small, being only 16-fold, which compared unfavourably with the 700-fold, obtained by Chan *et al.*[18]. Another affinity column (MAC-agarose) was therefore prepared to try to improve purification.

The MAC-agarose was highly successful at binding the AChE and up to 250 ml of the crude extract could be run through the column without any of then enzyme being lost in the eluate. The yield in the decamethonium peak was the best obtained of the methods so far tried at 44% and the 900-fold purification with a specific activity of 148 μmoles min^{-1} mg^{-1} was also satisfactory. As with the previous two columns, a further 13% of enzyme activity was obtained with the salt wash so this compares favourably with the results of Dudai *et al.*[17], who eluted 50% of the electric eel AChE from the same column in the presence of 1 *M* NaCl. The preliminary experiments with this column did include 1 *M* NaCl in all the eluting media as carried out by Dudai *et al.*[17], but only very small amounts of the enzyme were retarded whereas the above group had great success in binding the eel AChE to the column at high ionic strength. The difference between these two results may have been due to the enzyme originating from separate species or to the fact that Dudai's group had no Triton X-100 in their preparation whereas this detergent was present at all stages of our preparation. However the column was very successful with the pig brain AChE where the enzyme was eluted with decamethonium bromide at low ionic strength. When this enzyme was rechromatographed on the column, the specific activity was at least doubled giving a usefully purified preparation. The area of uncertainty arose from the problem of measuring the protein content with any great accuracy because of its low level.

A second peak of activity following the inhibitor peak was obtained for all three columns when they were washed with 1 *M* NaCl. The ligands were chemically diverse (Fig. 4) and yet from 12–14% of the activity was consistently removed at

high ionic strength. This suggests that the enzyme, eluted with 1 *M* NaCl, may have been binding to the agarose matrix as this was the one feature common to all three columns. The binding of this minor peak of enzyme activity would appear to be electrostatic since a high salt concentration was required to remove it from the column. Electrophoresis of this peak on a gradient of polyacrylamide showed the same range of molecular forms as the purified enzyme obtained from the main peak and the crude enzyme extract[5]. This second peak does not therefore appear to be a different molecular form of pig brain AChE and as mentioned above probably represents a degree of non-specific binding of the enzyme to the agarose matrix. Little or no purification was obtained in this second peak which was therefore not used in subsequent work.

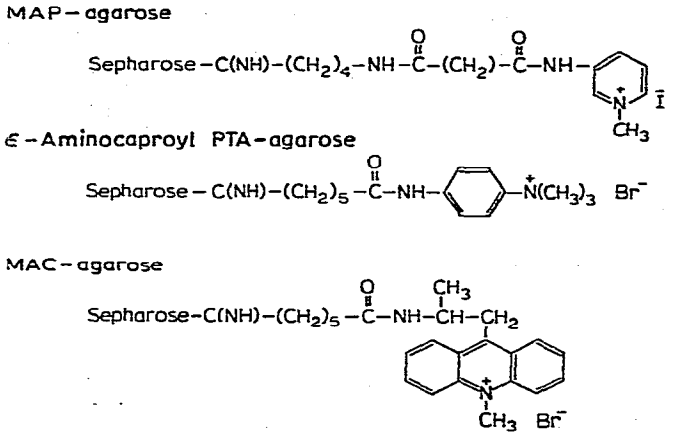

Fig. 4. The ligands used during the purification of acetylcholinesterase by affinity chromatography.

The other result common to all three columns was the fact that if Triton X-100 was excluded from the elution media then AChE was quantitatively bound to the column and could not be removed by changing the concentration of the inhibitor or salt. It seems likely that the detergent-depleted enzyme was binding hydrophobically to the apolar side chains linking the agarose and the ligand. However if only 0.1% (w/v) Triton X-100 was reintroduced into the decamethonium solution the enzyme could be removed and this was the method used for preparing a low-detergent sample of AChE.

The results with these columns clearly show that a published method for purifying an enzyme from one source by affinity chromatography cannot necessarily be applied to the same enzyme from another source. Some ligands that had been successfully used for AChE were found to be unsatisfactory for the pig brain enzyme. However MAC-agarose proved to be highly efficient with a satisfactory yield and a good degree of purification. This material has thus been used in further studies.

ACKNOWLEDGEMENT

The authors would like to thank Dr. I. Silman for his useful advice during this work.

REFERENCES

1 A. Nistri, A. M. de Bellis and E. Cammelli, *Neuropharmacol.*, 14 (1975) 427.
2 I. K. Ho and G. L. Ellman, *J. Neurochem.*, 16 (1969) 1505.
3 E. G. Hollunger and B. H. Niklasson, *J. Neurochem.*, 20 (1973) 821.
4 S. B. Vianna, S. L. Chan and A. J. Trevor, *Proc. West. Pharmacol. Soc.*, 17 (1974) 146.
5 C. H. S. McIntosh and D. T. Plummer, *Biochem. J.*, 133 (1973) 655.
6 D. T. Plummer, C. A. Reavill and C. H. S. McIntosh, *Croatica Chem. Acta*, 47 (1975) 163.
7 C. H. S. McIntosh and D. T. Plummer, *J. Neurochem.*, 27 (1976) 449.
8 G. L. Ellman, K. D. Courtney, V. Andres and R. M. Featherstone, *Biochem. Pharmacol.*, 7 (1961) 88.
9 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
10 E. F. Hartree, *Anal. Biochem.*, 48 (1972) 422.
11 J. Chandrarajan and L. Klein, *Anal. Biochem.*, 69 (1975) 632.
12 J. D. Berman and M. Young, *Proc. Nat. Acad. Sci.*, 68 (1971) 395.
13 P. Goodkin and B. D. Howard, *J. Neurochem.*, 22 (1974) 129.
14 Y. Dudai and I. Silman, *Methods Enzymol.*, 34 (1974) 571.
15 R. Axen, J. Porath and S. Ernback, *Nature (London)*, 214 (1967) 1302.
16 S. Blumberg, I. Schechter and A. Berger, *Eur. J. Biochem.*, 15 (1970) 97.
17 Y. Dudai, I. Silman, N. Kalderon and S. Blumberg, *Biochim. Biophys. Acta.*, 268 (1972) 138.
18 S. L. Chan, D. Y. Shirachi, H. N. Bhargava, E. Gardner and A. J. Trevor, *J. Neurochem.*, 19 (1972) 2747.