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COMPARATIVE AFFINITY CHROMATOGRAPHY OF ACETYLCHOLINESTERASES FROM FIVE VERTEBRATE SPECIES

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SUMMARY

The efficacy of N-methylacridinium affinity chromatography in the purification of acetylcholinesterases from chicken, rat, calf and human brain and from the electric organ of the electric fish *Torpedo marmorata* has been investigated. Retention of the enzymes on the N-methylacridinium columns exceeded 90% in all instances except for the chicken enzyme, where 40–80% retention was observed depending on the acridinium concentration. Sucrose density gradient centrifugation profiles revealed no difference between the distribution of molecular forms in the crude extracts and in the partially purified fractions eluted from the columns by decamethonium iodide.

INTRODUCTION

Acetylcholinesterase (E.C. 3.1.1.7) is a polymorphic enzyme that occurs in globular forms (monomers, dimers, tetramers) and asymmetric forms (one, two or three tetramers linked to a collagen-like tail)[1–3]. The globular forms (G_1, G_2, G_4) are soluble at low ionic strength but the asymmetric forms (A_4, A_8, A_{12}) aggregate under these conditions[4–6].

Purification of the enzyme extracted in buffers of high ionic strength from the electric organs of electric fish was first achieved by a multi-step procedure involving ammonium sulphate fractionation[7]. The subsequent application of affinity chromatography greatly facilitated the purification, improved yields and rendered feasible the isolation of acetylcholinesterase from a variety of tissues[8–11].

The first affinity systems described were based on the cholinergic agonist phenyltrimethylammonium and were used to purify acetylcholinesterase from the electric fish, *Electrophorus electricus*[8,9,12,13]. This method suffered, however, from the limitation that the affinity of the ligand for acetylcholinesterase was reduced significantly in the high ionic strength conditions necessary to maintain the asymmetric forms of the enzyme in solution[10]. Dudai *et al.*[9] observed that the N-methylacridinium ion

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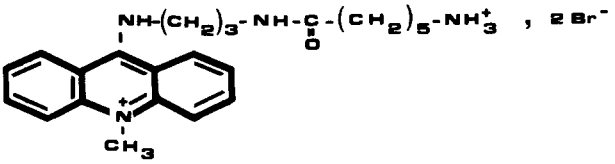
exhibits a high affinity for acetylcholinesterase even in solutions of high ionic strength and they therefore covalently linked a derivative of this ion to a Sepharose matrix, thus producing an affinity column capable of retaining acetylcholinesterase at high ionic strength. The widespread employment of this valuable ligand was nevertheless frustrated initially because of irreproducibility in its synthesis[14]. This problem was subsequently overcome by the publication of an alternative synthesis[11] and a modification of the original synthesis[15]. An additional obstacle to the general employment of this affinity system is posed by reports of differential behaviour of certain acetylcholinesterases and their different molecular forms on acridinium affinity columns[15,16]. Recently it has even been reported that with rat brain acetylcholinesterase, N-methylacridinium linked to agarose fails to retain more than 8% of the applied activity[17].

In this study an attempt has been made to establish conditions under which acridinium affinity column chromatography can be usefully applied to acetylcholinesterases from a variety of sources. A comparative study of the affinity chromatography of acetylcholinesterase from five vertebrate species has been performed. 10-Methyl 9-[3-(6-aminocaproyl)amino]propylaminoacridinium bromide, hydrobromide (MAc), synthesized following the methods of Rosenberry and Richardson[11] and Webb and Clark[15], was covalently bound to Sepharose 4B at concentrations of 1.7, 2.5 and 3.1 μmol per ml of resin. Acetylcholinesterase from rat, chicken, calf and human brains and from the electric organ of *Torpedo marmorata* were partially purified on columns prepared from each of the three N-MAc concentrations. Retention and recovery of acetylcholinesterase activity were monitored during affinity chromatography and the proportions of molecular forms present in the crude and partically purified extracts were compared.



10-Methyl 9-[3-(6-aminocaproyl)amino]propylaminoacridinium bromide hydrobromide (MAc).

EXPERIMENTAL

Preparation of crude acetylcholinesterase extracts

Acetylcholinesterase extracts were prepared by homogenization of the brain (Potter homogenizer) or electric organ tissue (Polytron homogenizer) in 10 volumes (w/v) of 0.01 *M* Tris (pH 7.5)–0.1 mg ml^{-1} bacitracin. The homogenates were centrifuged at 25,000 *g* for 30 min using a Sorvall SS 34 rotor. The supernatants were discarded and the pellets were resuspended and homogenized in 0.01 *M* Tris (pH 7.5)–0.1 mg ml^{-1} bacitracin–1% Triton X-100. These homogenates were centrifuged as above and the supernatants were retained as the crude acetylcholinesterase extracts.

Acetylcholinesterase activities

Activities were measured by the method of Ellman *et al.*[18], as described previously[19]. They are expressed as absorbance changes per minute, in 1 ml assay medium (1 ml path length) at 412 nm (optical density units, OD/min).

Measurement of protein concentrations

Protein concentrations were determined by the method of Lowry *et al.*[20]. Precipitates formed by the presence of Triton X-100 were removed by centrifugation and Triton X-100 was incorporated in the bovine serum albumin standards.

Synthesis of MAc

9-[3-(benzyloxycarbonyl)amino]propylaminoacridine was prepared according to Rosenberry and Richardson[11] by condensation of 1-(N-benzyloxycarbonyl)aminopropane hydrochloride[21] with 9-phenoxyacridine[22] and was then treated with 40% hydrogen bromide in acetic acid (we preferred this method to that of Webb and Clark[15] because in our experience the latter method yields a mixture of the desired 9-(3-aminopropylamino)acridine dihydrobromide and 1-(9-aminoacridinyl)propane). The solution of 9-[3-(aminopropyl)amino]acridine dihydrobromide produced by this condensation was rendered basic by the addition of excess of ammonia solution and was extracted with methylene chloride.

After drying on sodium sulphate the solvent was removed under vacuum and the resultant oil crystallized when ground in absolute ethanol.

10-Methyl-9-[3-(6-benzyloxycarbonylaminocaproyl)amino]propylaminoacridinium iodide was prepared according to Webb and Clark[15] by condensation of 6-(N-benzyloxycarbonylamino)caproic acid[13] with 9-(3-aminopropyl)aminoacridine dihydrobromide, followed by quaternization of the crude product with methyl iodide. The salt obtained was chromatographed on silica gel (Merck, Darmstadt, G.F.R.) and the pure aminoacridinium iodide salt was eluted with chloroform containing 3% methanol.

Acidolysis of this salt with a solution of 40% hydrogen bromide in acetic acid[15] yielded the desired MAc.

Product analysis

Melting points (*F*) were measured in capillary tubes in a Büchi–Tottoli apparatus, and were not corrected. Nuclear magnetic resonance spectra were recorded at 60 MHz in a Varian A60 spectrometer using 3-trimethylsilylpropionic acid (TMSP) as an internal reference. The element contents of the various compounds were measured by microanalysis and were within $\pm 0.3\%$ of the calculated values.

9-[3-(benzyloxycarboxyl)amino]propylaminoacridine. $F = 157°C$ (ethanol–diethyl ether). Yield: 80%.

9-(2-Aminopropyl)aminoacridine dihydrobromide. $F = 280°C$ (ethanol–water). Yield: quantitative.

9-(3-Aminopropyl)aminoacridine. $F = 130°C$. Yield: 30%. Analysis: $C_{16}H_{17}N_3$ (251); C,H,N. This base is rapidly carbonated on exposure to air.

10-Methyl-9-[3-(6-benzyloxycarbonylaminocaproyl)amino]propylaminoacridinium iodide. $F = 155°C$. Yield: 15%. Analysis: $C_{31}H_{37}N_4O_3I$ (640.5); C,H,N.

MAc. It was not possible to measure the fusion point and to perform the microanalysis of this product because of its extreme hygroscopicity, which makes storage under vacuum in a desiccator essential. UV spectrum (in ethanol): λ_{max} 393 (shoulder), 412 and 433 nm (see ref. 15). Yield: quantitative.

^1H NMR spectrum of MAc (in DMSO-d_6). 1.2–4 ppm (m, 19H, CH_3 and CH_2); 1.9 ppm (s, 3H, $\overset{+}{N}CH_3$); 6.8–8.4 ppm (m, 9H, aromatic and NHC–); 9.5 ppm (broad s, 3H, $\overset{+}{N}H_3$ exchangeable in D_2O).

Preparation of affinity resin

Activation of Sepharose 4B by cyanogen bromide. A 100-ml volume of Sepharose 4B (Pharmacia, Uppsala, Sweden) was resuspended in cold 1 *M* sodium carbonate solution and stirred magnetically during the addition of 8 g of cyanogen bromide dissolved in 20 ml of acetonitrile. The pH was maintained at 11 by the addition of 4 *M* sodium hydroxide solution and warming of the reaction mixture was prevented by the addition of crushed ice. After 15 min the Sepharose was rapidly washed on a 63-μm nylon sieve (Nytrel TI 63) under a water-suction vacuum with 2–3 l of cold water followed by 4 l of coupling buffer (8.4 g sodium hydrogen carbonate and 10.6 g of sodium carbonate per litre). The gel was then resuspended in fresh coupling buffer.

Attachment of ligand to activated Sepharose 4B. Depending on the desired concentration of covalently linked ligand, an appropriate weight of MAc (molecular weight 540.8) dissolved in coupling buffer was added to stirred, activated Sepharose 4B. The reaction was allowed to proceed at room temperature for 4 h and 4 ml of ethanolamine were then added to saturate unreacted groups. The mixture was then stirred gently for 12 h. At this point the absorbance at 410 nm (1 cm path length) of an aliquot of the liquid above the resin was measured. The product of this value and the volume of liquid yields the amount of unlinked MAc. This was compared with the amount of MAc initially added (volume and absorbance at 410 nm known) in order to calculate the concentration of MAc linked to the Sepharose 4B resin.

Finally, the gel was washed with 3 l of water followed by 0.01 *M* Tris (pH 7.5)–0.002% sodium azide, and was stored in this buffer at 4°C until used.

Affinity chromatography

All manipulations were performed at 4°C. For each acetylcholinesterase, three MAc affinity columns at MAc concentrations of 1.7, 2.5 and 3.1 μmol per ml of resin were used. The volume of each column was 5 ml, and 20 ml of crude extract were loaded on each at a flow-rate of 20 ml h^{-1}. After loading, the columns were washed with four column volumes of 0.01 *M* Tris (pH 7.5)–0.1 mg ml^{-1} bacitracin–0.1% Triton X-100 and the acetylcholinesterase was then eluted with decamethonium iodide (0.02 *M*). Fractions of 1 ml were collected and assayed for acetylcholinesterase activity.

The fractions containing the activity eluted by the decamethonium were pooled, as were those fractions containing activity not retained on the column. For those pools containing decamethonium the activity was determined in the following manner.

The activity was determined using a range of aliquot volumes taken from the pool (1–5 μl; at least three measurements for each volume), and by plotting the reciprocal of the measured activity as a function of the aliquot volume. Linear extrapolation to an aliquot volume of zero corresponds to the presence in the assay medium of zero decamethonium, thus giving the activity of the uninhibited enzyme.

Sucrose density gradient centrifugation

Crude and partially purified extracts were analysed on sucrose gradients (5–20%) in the presence of 1% of Triton X-100, 1 *M* sodium chloride, 0.05 *M* magnesium chloride, 0.01 *M* Tris (pH 7) and 0.1 mg ml^{-1} of bacitracin. Centrifugation was performed at 40,000 rpm for 24 h using a Beckman SW 41 rotor. Catalase and alkaline phosphatase were used as sedimentation coefficient markers. The relative proportions of the different molecular forms were established by integrating the areas under the centrifugation profiles.

Inhibition constants

Enzymatic activities of the crude extracts were measured in the presence of a range of acetylthiocholine concentrations (0–10^{-3} *M*) and in the presence of four different concentrations of MAc (0, $1 \cdot 10^{-6}$, $2.5 \cdot 10^{-6}$ and $5 \cdot 10^{-6}$). In the case of *Torpedo* acetylcholinesterase the MAc concentrations used were 0, $0.25 \cdot 10^{-8}$, $5.0 \cdot 10^{-8}$ and $2.5 \cdot 10^{-7}$ *M*. Assays were performed at 28°C, using a Varian Series 634 double-beam spectrophotometer coupled to a Servotrace chart recorder. The assay conditions have been described previously[19].

RESULTS

Inhibition constants

The inhibition constants for the interaction of each type of acetylcholinesterase

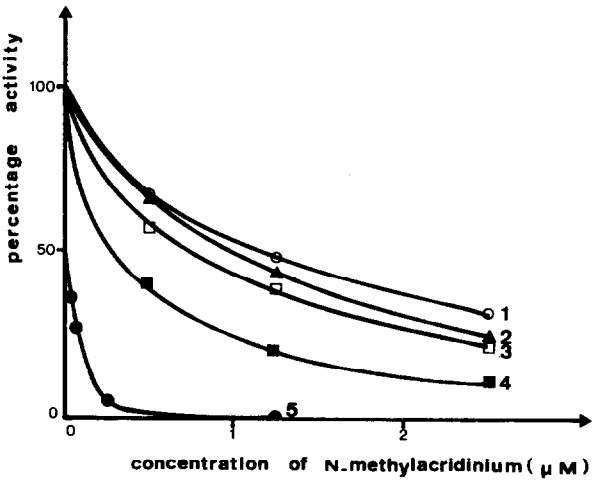


Fig. 1. Plot of the inhibition of acetylcholinesterase activity by N-methylacridinium. Percentage activity is plotted as a function of N-methylacridinium concentration. ○, Chicken; ▲, calf; □, rat; ■, human; ●, *Torpedo*. Approximate inhibition constants calculated by the method of Dixon and Webb[23] for acetylcholinesterase from chicken, calf, rat, human and *Torpedo* were 10^{-6}, 10^{-6}, 10^{-6}, 10^{-7} and 10^{-8} *M*, respectively. The substrate (acetylthiocholine) concentration was $8 \cdot 10^{-4}$ *M*.

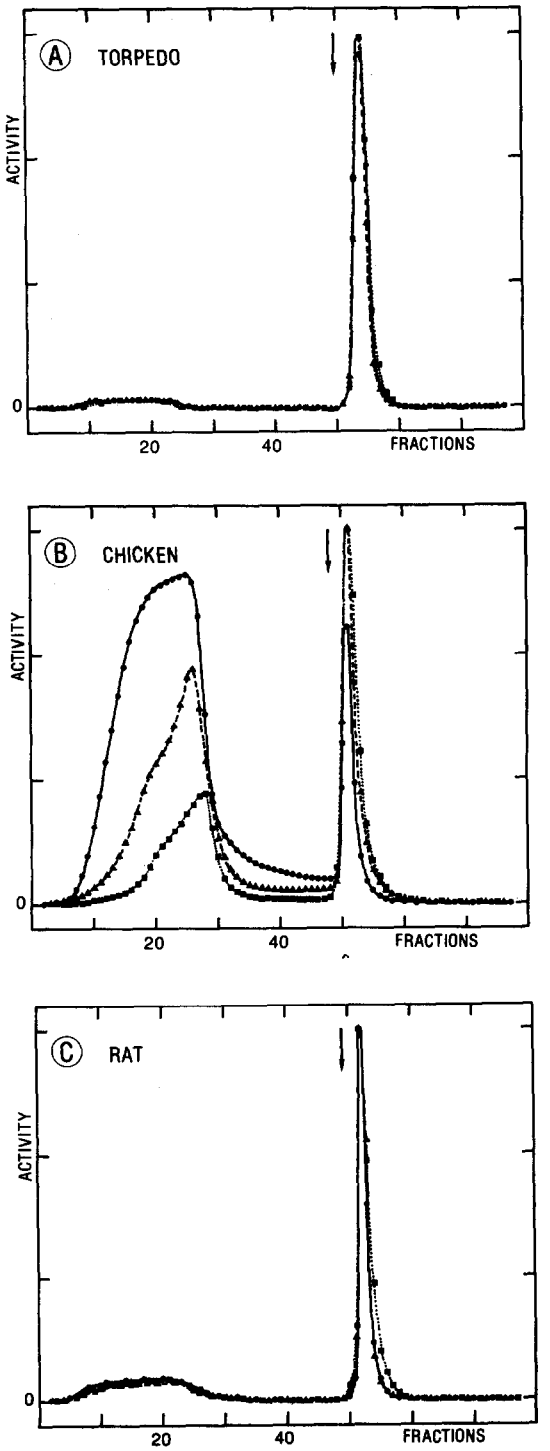

A
TORPEDO
ACTIVITY
0
20
40
FRACTIONS
B
CHICKEN
ACTIVITY
0
20
40
FRACTIONS
C
RAT
ACTIVITY
0
20
40
FRACTIONS

Fig. 2.

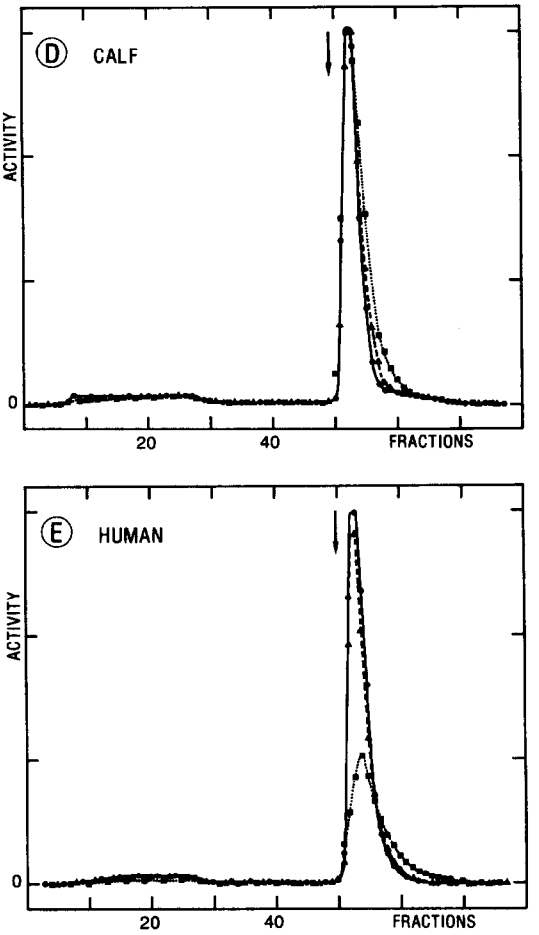


Fig. 2. Chromatography of acetylcholinesterase on MAc–Sepharose columns. Extracts of 20 ml from (A) *Torpedo* electric organ (3140 OD min^{-1}), (B) chicken brain (490 OD min^{-1}), (C) rat brain (150 OD min^{-1}), (D) calf caudate nucleus (620 OD min^{-1}) and (E) human caudate nucleus (30 OD min^{-1}), in 10 m*M* Tris-HCl (pH 7.5)–1% Triton X-100–0.1 mg ml^{-1} bacitracin, containing the acetylcholinesterase activities given in parentheses, were applied to 5-ml columns as indicated under Experimental. The unbound activity appears between fractions 5 and 30. The columns were washed with buffer [10 m*M* Tris–HCl (pH 7.5)–0.1% Triton X-100–0.1 mg ml^{-1} bacitracin] and acetylcholinesterase was eluted with 20 m*M* decamethonium iodide in the same buffer (indicated by arrow). The activities of the fractions are plotted on arbitrary but comparable scales for each species. The activities of the eluted fractions have not been corrected for inhibition by decamethonium. ●——● 1.7; ▲---▲ 2.5; ■···■ 3.1 μmol of MAc per ml of Sepharose.

with MAc were calculated using the Dixon and Webb plot[23] and are given in Fig. 1. Inhibition was mixed but predominantly competitive for each of the five acetylcholinesterases.

Acetylcholinesterases from chicken, rat and calf brain bind MAc with similar affinities whereas the human and *Torpedo* enzymes exhibit 10-fold and 100-fold higher affinities, respectively.

Affinity chromatography

The MAc affinity column profiles are shown in Fig. 2. Three profiles, corresponding to the three MAc concentrations linked to Sepharose 4B, are shown for each of the five acetylcholinesterases studied. Elution was performed in all instances with an excess of decamethonium (20 m*M*). The activities of the crude extracts varied considerably among the five enzymes and it was therefore not possible to load the same total activity on the columns in each instance. The total activities and the corresponding approximate weights of enzyme contained in the 20 ml of crude extract loaded in each instance were the following: chicken, 490 OD min^{-1}, 25 μg; rat, 150 OD min^{-1}, 4 μg; calf, 620 OD min^{-1}, 17 μg; human, 30 OD min^{-1}, 1 μg; and *Torpedo*, 3140 OD min^{-1}, 50 μg. The approximate weights were calculated by assuming the following values for the turnover numbers of each enzyme: *Torpedo* $1.64 \cdot 10^7$, chicken $1.05 \cdot 10^7$ and rat, calf and human $1.32 \cdot 10^7$ mol acetylthiocholine h^{-1} (ref. 24); with molecular weights of subunits: chicken 120,000 and *Torpedo*, rat, calf and human 70,000.

The columns retained more than 95% of the total activity for *Torpedo* electric organ and calf and human brain, about 90% for rat brain but only 40% (1.7 μmol ml^{-1} MAc) to 80% (3.1 μmol ml^{-1} MAc) for chicken brain. We found that with all three MAc concentrations, decamethonium eluted about 75% of the retained activity for calf brain, 50% for *Torpedo* electric organ and rat brain and 33% for chicken brain. The elution yield decreased from about 40% (1.7 μmol ml^{-1} MAc) to slightly less than 25% (3.1 μmol ml^{-1} MAc) for human brain. Thus the concentration of MAc influences markedly the retention with chicken acetylcholinesterase and the release with the human enzyme. It is remarkable that although the chicken enzyme is only partially retained, it is incompletely released by decamethonium.

The lower retention of the chicken enzyme is surprising in view of the fact that it possesses an affinity for MAc similar to that of the mammalian acetylcholinesterases (Fig. 1). To investigate this phenomenon further we examined the influence of tritiated diisopropyl fluorophosphate-inhibited chicken or calf brain extracts on the fixation of active calf brain acetylcholinesterase. We found that inhibited enzymes were not retained and that the corresponding extract did not affect the binding of active calf acetylcholinesterase. This indicates that the poor binding of the chicken enzyme is not due to any interference by non-enzymatic components.

Sucrose gradient centrifugation

Table I gives the relative proportions of the different molecular forms in the crude and partially purified extracts of each enzyme; 90% or more of the total enzymic activity is represented by the tetramer (10S) in the chicken, rat, calf and human enzymes and the *Torpedo* enzyme consists almost exclusively of the dimeric 6S form.

DISCUSSION

Affinity column chromatography, based on the affinity ligand N-methylacridinium, has been employed in the purification of acetylcholinesterase from the electric eel[9,11,15,16], bovine erythrocytes[16] and pig cerebral cortex[25]. In this study, we investigated the efficacy of the MAc ligand in the affinity chromatography purification of acetylcholinesterase from human, rat, calf and chick brain and from the electric organ of *Torpedo marmorata*.

TABLE I

RELATIVE PROPORTIONS OF THE DIFFERENT MOLECULAR FORMS PRESENT IN CRUDE AND AFFINITY-PURIFIED EXTRACTS

The values were obtained by integration of the areas under the peaks of sucrose gradient centrifugation profiles. The figures given for the partially purified extracts were obtained from the profiles of the pools eluted by decamethonium from the 2.5 μmol of MAc per ml of Sepharose columns.

Acetyl-cholinesterase		*Percentage of molecular forms present*			
Source	*State*	A_{12}	G_4	G_2	G_1
Chicken	Crude	0	90	10	0
	Purified	0	87	13	0
Rat	Crude	0	96	0	4
	Purified	0	91	4	5
Calf	Crude	0	97	0	3
	Purified	0	71	11	18
Human	Crude	0	95	0	5
	Purified	0	90	6	4
Torpedo	Crude	11	0	89	0
	Purified	6	0	94	0

Affinity columns containing three (and in some instances four) different concentrations of MAc covalently linked to Sepharose 4B were successful in retaining more than 90% of the applied acetylcholinesterase activity, except for the enzyme from chicken brain. Retention of this enzyme varied between 40 and 80% depending on the concentration of MAc. This poorer retention cannot be explained simply on the basis of relative affinities for MAc, as the inhibition constant for the chicken enzyme is very similar to those of the rat and calf enzymes (Fig. 1). The enzyme-binding capacity of affinity columns also depends on hydrophobic interactions of the enzyme with the spacer arm and ligand[26–28]. These hydrophobic interactions may be weaker with chicken acetylcholinesterase, thus accounting for the lower retention of this enzyme on the highly hydrophobic MAc columns.

It is of particular interest that while the phenylammonium ligand fails to bind the *Torpedo*[19] and chicken acetylcholinesterases (unpublished results), these enzymes may be at least partially retained, and therefore purified, on MAc columns.

In contrast, the phenylammonium ligands have been successfully employed in the purification of *Electrophorus*[19,28] and rat brain acetylcholinesterase[29]. This emphasizes how the same enzyme from different sources can display dramatically different ligand binding properties, presumably reflecting fine differences in the molecular architecture of active and peripheral anionic sites[28].

The efficient absorption of rat brain acetylcholinesterase by MAc obtained in our experimental conditions contrasts with recent work by Vidal *et al.*[17]. They observed very poor binding of rat acetylcholinesterase on columns prepared with MAc linked to agarose. The concentration of MAc employed in this conjugate is not specified, but reference is made to a previous report in which MAc at concentrations of 0.5–1.0 μmol ml^{-1} agarose had been applied successfully to the purification of acetylcholinesterase from pig cerebral cortex[25]. In this study, a concentration of 0.6 μmol of MAc per ml of gel efficiently retained rat brain acetylcholinesterase. The

reason for this discrepancy is unclear. MAc concentrations in this range have also been reported to be unsuccessful in retaining bovine eythrocyte acetylcholinesterase[16], even though the rat and bovine enzymes bind MAc with very similar affinities. It appears that this type of chromatography depends critically on the synthesis of the ligand and on its coupling with Sepharose, and underlines the necessity of establishing the optimal MAc concentration for each proposed purification.

The efficiency of elution of acetylcholinesterase by decamethonium was dependent both on the particular enzyme and on the concentration of MAc coupled to the Sepharose. Yields of acetylcholinesterase activity were generally in the region of 40–75 % of the activity retained by the column, although both the chicken and human enzymes were eluted from the columns in yields of less than 40 %. The poor yield of the human enzyme eluted from the 3.1 μmol of N-MAc per ml of Sepharose is in accord with the observation that the yield of electric eel acetylcholinesterase eluted from MAc columns decreases rapidly above a concentration of 1.0 μmol of MAc per ml of Sepharose[15].

Elution of *Torpedo* acetylcholinesterase was surprisingly quantitative at all MAc concentrations, in spite of its high affinity for the ligand. The Triton X-100 extracts of *Torpedo* electric tissue used contain almost exclusively the dimeric form of acetylcholinesterase, whereas in the other four extracts studied the tetrameric form is predominant. In order to test whether this difference could account for the facility of elution of the *Torpedo* enzyme, even at high MAc concentrations, the retention and elution of *Torpedo* acetylcholinesterase isolated from the spinal chord were studied, as this extract contains predominantly the tetrameric form of the enzyme[30,31]. No difference was observed in the percentage retention and in the elution between this extract and that containing the dimeric enzyme form. The facility of elution of *Torpedo* acetylcholinesterase at high MAc concentrations appears, therefore, to be independent of its quaternary association state in the case of the globular forms.

It should be emphasized that these observations do not necessarily extend to the asymmetric forms of *Torpedo* acetylcholinesterase. The high salt concentrations needed to solubilize these forms reduces their retention on the MAc affinity columns

TABLE II

SPECIFIC ACTIVITIES OF THE CRUDE EXTRACTS AND THE PARTIALLY PURIFIED EXTRACTS ELUTED FROM THE N-METHYLACRIDINIUM COLUMNS

All activities are expressed in terms of units of optical density per minute per milligram of protein. The figures in parentheses are the purification factors of the pools eluted from the columns as referred to the crude extracts.

Extract	*Source of acetylcholinesterase*				
	Chicken	*Rat*	*Calf*	*Human*	*Torpedo*
Crude extract	5.7	2.0	8.5	0.9	302
Material eluted from 1.7 μmol ml^{-1} MAc	48.4(8.5)	128(64)	222(26.1)	10.4(12)	1266(4.2)
Material eluted from 2.5 μmol ml^{-1} MAc	57.4(10)	116(58)	203(24)	8.7(9.8)	1374(4.6)
Material eluted from 3.1 μmol ml^{-1} MAc	31.6(5.5)	10.4(5.2)	21.6(2.5)	3.4(3.8)	624(2.1)

and, paradoxically, we have found that the enzyme is eluted in very poor yield by decamethonium (unpublished observations).

Sucrose gradient centrifugation showed that for each of the five acetylcholinesterases, the distribution of molecular forms in the partially purified extract is essentially the same as in the original crude extract (Table I). Calf acetylcholinesterase manifests the only significant difference between the crude and partially purified extracts, as a result of a small diminution in the proportion of tetramer and a concomitant increase in the proportions of dimer and monomer.

The specific activities of the crude and partially purified extracts, and the corresponding purification factors, are shown in Table II. The purification factors fall within the large range reported for a variety of acetylcholinesterases purified on various affinity columns[8,13,17,32]. Although outside the immediate scope of this study, we obtained some indications suggesting possible improvements of the purification method. A higher specific activity can be achieved by desorbing non-specifically bound contaminants from the column with an appropriate salt solution prior to elution of the enzyme by decamethonium. In addition, we found that the specific activities of all five enzymes eluted from the 3.1 μmol of MAc per ml of Sepharose columns are significantly lower than for the columns containing smaller concentrations of MAc. This effect is illustrative of the fact that affinity columns exhibit ion-exchange properties at high concentrations of affinity ligand, and bind proteins non-specifically[2,10,13].

In conclusion, the versatility of MAc affinity chromatography has been demonstrated by its successful application to the purification of acetylcholinesterases from five vertebrate sources, including *Torpedo* and chicken, for which other chromatographic methods had proved inadequate.

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