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The Identity of the Serotonin-Sensitive Aryl Acylamidase with Acetylcholinesterase from Human Erythrocytes, Sheep Basal Ganglia and Electric Eel

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The identity of the serotonin-sensitive aryl acylamidase with acetylcholinesterase from three diverse sources, namely sheep basal ganglia, human erythrocyte membrane and electric eel, was examined. Both the enzymes co-purified with constant ratios of specific activity from all the three sources by different affinity chromatographic techniques. The ratio of acetylcholinesterase to aryl acylamidase activity was highest for basal ganglia, less for erythrocyte and lowest for eel enzymes. Both the purified enzymes co-migrated on polyacrylamide gel electrophoresis either as a single species or multiple species under different conditions. Gel density gradient electrophoresis indicated identical migration rates of both the enzymes. Extraction of the enzymes from the three sources by different techniques of membrane disruption and subsequent gel filtration on Sepharose 6B showed multiple peaks of enzyme activity. Both the enzymes had identical elution profiles on Sepharose 6B gel filtration. All the enzyme peaks from Sepharose 6B on gel electrophoresis showed co-migration of the enzyme activities. Apart from inhibition by serotonin and acetylcholine the purified aryl acylamidases from all the three sources were potently inhibited by neostygmine, eserine and BW284C51, all strong inhibitors of acetylcholinesterase. It is suggested that the serotonin-sensitive aryl acylamidase is identical with acetylcholinesterase.

The presence of an aryl acylamidase specifically sensitive to serotonin was first noticed in rat brain by Fujimoto [1]. This observation was confirmed by others using brain from different species [2–4]. We reported that brain aryl acylamidase was also sensitive to inhibition by acetylcholine, its analogues and homologues [3]. Subsequently Fujimoto [5] demonstrated that the serotonin-sensitive aryl acylamidase activity was a property of acetylcholinesterase. Several lines of evidence also indicated that in the monkey brain the serotonin-sensitive aryl acylamidase was associated with acetylcholinesterase [6].

In contrast to the serotonin-sensitive aryl acylamidase, a serotonin-insensitive aryl acylamidase has been noticed in the liver [1–3] as well as in several non-neural tissues of the rat [4]. Such an enzyme from monkey liver was also found to be insensitive to acetylcholine and its analogues and it was not associated with acetylcholinesterase activity [7]. It appeared, therefore, that the serotonin sensitivity may be an exclusive property of aryl acylamidase associated with acetylcholinesterase.

In our earlier work [6] a partially purified enzyme from monkey brain was used to demonstrate the association of the seronin-sensitive aryl acylamidase with acetylcholinesterase. Evidence for the association in the monkey brain included copurification of the two enzymes, potent inhibition of both enzymes by acetylcholinesterase inhibitors and similarity of both the enzymes in the subcellular and regional distribution. To obtain unequivocal evidence for the identity of the two enzymes and to find out whether acetylcholinesterases in general exhibit aryl acylamidase activity, we have purified the enzymes by diverse methods of affinity chromatography from three different sources of acetylcholinesterase: sheep basal ganglia, human erythrocyte membrane and electric eel. We show in the present paper that the serotonin-sensitive aryl acrylamidase was inseparable from acetylcholinesterase from all the three sources by affinity chromatographic procedures, electrophoretic techniques and gel filtration on Sepharose. Furthermore, the multiple

Enzymes. Arylacylamidase or aryl-acylamide amidohydrolase (EC 3.5.1.13); acetylcholinesterase (EC 3.1.1.7).

Trivial Name. BW284C51, bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide.

forms of both the enzymes behaved identically by different criteria.

MATERIALS AND METHODS

Materials

m-Dimethylaminobenzoic acid was obtained from Fluka, 3-aminopyridine from E. Merck, hexanediamine and succinic anhydride from Eastman, cyanogen bromide from Pierce, aquacide from Calbiochem, Sepharose 6B and 4B from Pharmacia fine chemicals and hydroxyapatite from BDH. Bis(4-allyldimethylammoniumphenyl)pentane-3-one dibromide (BW284C51) was a gift from Wellcome Reagents Ltd. All other fine chemicals were obtained from Sigma Chemical Company, USA or as described earlier [3, 6].

Heparinised human erythrocytes were obtained from the blood bank of the hospital. Basal ganglia were isolated from sheep brains obtained from the slaughterhouse. Acetylcholinesterase from electric eel (type V-S) was from Sigma Chemical Company, USA.

Synthesis of Ligands

Different ligands were used for the affinity chromatography of the enzymes. They were synthesized by published procedures with minor modifications. *N*-Methyl-3-aminopyridinium iodide was synthesized by methylating 3-aminopyridine with methyl iodide [8]. *m*-Carboxyphenyltrimethylammonium iodide was synthesized by methylating *m*-dimethylaminobenzoic acid with methyl iodide [9]. *m*-Carboxyphenyldimethylethylammonium iodide was synthesized by ethylating *m*-dimethylaminobenzoic acid with ethyl iodide in a similar manner. *m*-Aminophenyltrimethyl ammonium chloride hydrochloride was synthesized by acetylating *N*,*N*-dimethyl-*m*-aminophenylenediamine with acetic anhydride, extraction of the acetylated product into acetone, methylation with excess methyl iodide followed by deacetylation and conversion into the hydrochloride form by refluxing with hydrochloric acid and ethanol in equal proportions. Crystals of *m*-aminophenyltrimethylammonium chloride hydrochloride were formed on addition of acetone [10].

Preparation of the Affinity Media

The activation of Sepharose 4B was carried out according to the method of Cuatrecasas [11, 12]. In a typical method 5 ml washed Sepharose 4B was suspended in 5 ml 2 M Na_2CO_3 and 1.25 ml of a 1 g/ml solution of cyanogen bromide was added and stirred for 15 min at 4 °C. Immediately afterwards the gel was washed with 200 ml cold 0.1 M bicarbonate buffer, pH 8.5, followed by 50 ml 0.1 M cold borate buffer, pH 9.5. The washed gel was suspended in an equal volume of 0.1 M borate buffer, pH 9.5, and 1.16 g hexanediamine in 5 ml of the same buffer was added to the gel. The pH was adjusted to 9.5 with 5 M HCl and the gel stirred at 4 °C for 24 h. The excess amine was removed by washing the gel with 20 vol. of 1 M NaCl followed by water. The aminated gel was suspended in 5 ml water and 750 mg succinic anhydride and the pH adjusted to 8.0 with 5 M NaOH. The gel was stirred for 12 h at 4 °C and afterwards washed free of excess succinic anhydride and succinic acid and the succinylated gel was suspended in an equal volume of water. If the spacer arm has to be further extended, 2 mmol/ml gel of hexanediamine dissolved in water was added and the coupling done in presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (20 mg/ml gel). The gel was stirred for 24 h at 4 °C and the pH was maintained at 4.5. The spacer arm was further extended where necessary using succinic anhydride and hexanediamine. When the spacer arm is sufficiently long 0.5 mmol ligand/ml gel dissolved in water was added and the coupling done as before using carbodiimide. Finally 1 M acetic acid or ethanolamine (0.5 mmol/ml gel) was added and pH adjusted to 4.5 and stirred for 4 h to block any excess free amino or carboxylate groups respectively. After each stage of the synthesis of the spacer arm and the final coupling of the ligand, 2,4,6-trinitrobenzene sulfonate test [12] was done to determine the efficiency of the reaction.

Affinity Chromatography Methods of Enzyme Purification

The enzymes from basal ganglia, erythrocyte membrane and electric eel, used for affinity chromatography, were prepared as follows.

Basal ganglia, frozen at − 20 °C for at least 2 days, were homogenized in a Potter-Elvehjem homogenizer with 0.02 M potassium phosphate buffer, pH 7.0, containing 0.5% v/v Triton X-100 (8 ml/g tissue). Human erythrocyte membrane was prepared according to Marchesi [13] and washed with 15 mM Tris/HCl buffer pH 7.4. The membranes were suspended in 0.02 M potassium phosphate buffer pH 7.0 (protein 3.5 mg/ml) and kept frozen at − 20 °C for at least 2 days before use. The suspension was homogenized with 1% v/v Triton X-100 and kept for 1 h. Both basal ganglia and erythrocyte membrane homogenates were centrifuged at 105000 × *g* for 1 h in a Beckman model L ultracentrifuge and the supernatant collected for affinity chromatography. 75 µg electric eel acetylcholinesterase was dissolved in 5 ml 0.02 M potassium phosphate buffer, pH 7.0, and used.

The nine affinity methods tried for purification of the enzymes from the three sources are given in Table 1. The affinity ligands and spacer arms in each method

are also indicated in Table 1. In all, four different affinity ligands, *N*-methyl-3-aminopyridinium iodide, *m*-carboxyphenyltrimethylammonium iodide, *m*-carboxyphenyldimethylethylammonium iodide and *m*-aminophenyltrimethylammonium chloride hydrochloride were coupled to Sepharose 4B through a 6, 10, 26 or 30-carbon spacer arm.

The affinity columns (4 ml affinity gel) were pre-equilibrated with 0.02 M potassium phosphate buffer, pH 7.0, containing 0.5% v/v Triton X-100 (for methods 1–3, 7 and 9), 0.02 M potassium phosphate buffer pH 7.0 (for methods 4–6) or 0.02 M Tris/HCl buffer pH 7.4 containing 0.5% v/v Triton X-100 (for method 8). The enzymes (5 ml) were loaded and eluted at a flow rate of 5 ml/h and the washing of the column was done at 10 ml/h. Fractions of 2 ml were collected.

Removal of Triton X-100 from the Purified Enzymes of Basal Ganglia and Erythrocytes

The use of Bio-Bead SM-2 (Bio-Rad) for removal of Triton X-100 resulted in significant loss of activity of both aryl acylamidase and acetylcholinesterase. Therefore, hydroxyapatite was used for the removal of Triton X-100 [14]. A column (14 × 1 cm) of hydroxyapatite was equilibrated with 0.005 M potassium phosphate buffer pH 6.0. The purified enzyme, after dialysis against the same buffer, was loaded onto the column at a flow rate of 5 ml/h and washed with the same buffer until the effluent showed no absorption at 275 nm. The enzyme adsorbed onto the column was eluted with 0.2 M potassium phosphate buffer pH 7.4. The active fractions were dialysed against 0.05 M Tris/HCl buffer pH 7.0, concentrated by aquacide, redialysed against the same buffer and used for electrophoresis.

Preparation of Enzyme for Sepharose 6B Gel Filtration

Three different enzyme preparations were made from a human erythrocyte membrane suspension in 0.05 M potassium phosphate buffer pH 7.0 (protein 12 mg/ml). (a) The suspension was homogenized in a Potter-Elvehjem homogenizer for 2 min 0.5% v/v Triton X-100 and 1 M NaCl. (b) The suspension was homogenized with 1 M NaCl and sonicated for 10 min (with 2-min intervals after every minute of sonication) at maximum frequency in a Mullard MSE sonicator. Both these preparations were centrifuged at 105000 × *g* for 1 h and the supernatant was collected. The precipitates obtained at 55% saturation of $(NH_4)_2SO_4$ from the supernatant were collected by centrifugation at 55000 × *g* for 30 min. The precipitates were suspended in 0.05 M potassium phosphate buffer, pH 7.0, containing 1 M NaCl, dialysed against the same buffer for 8 h and were designated as 'Triton fraction' (protein 70 mg/ml) and 'sonicated fraction' (protein 60 mg/ml). (c) Solid sodium dodecyl sulphate was added (0.5% w/v) to the dialyzed Triton fraction, homogenized and allowed to stand for 1 h. The mixture was centrifuged at 12000 × *g* for 30 min and the supernatant, designated 'dodecyl sulphate fraction', was collected.

Basal ganglia from sheep brain were homogenized with 0.05 M potassium phosphate buffer, pH 7.0, containing 1 M NaCl (8 ml/g tissue). From the homogenate the Triton fraction, sonicated fraction and dodecyl sulphate fraction were made as described for the erythocyte membrane enzymes, except that the $(NH_4)_2SO_4$ fraction obtained at 60% saturation was used. Protein concentrations in the Triton and sonicated fractions were 40 mg and 55 mg/ml respectively.

100 µg electric eel acetylcholinesterase was dissolved in 2 ml potassium phosphate buffer pH 7.0, containing 1 M NaCl, and used.

Gel Filtration on Sepharose 6B

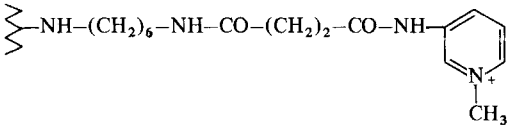
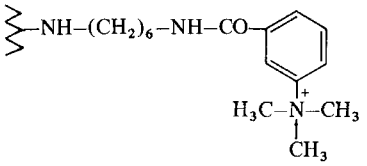
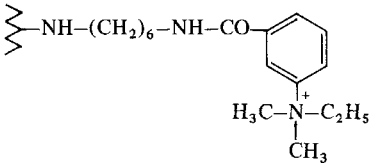
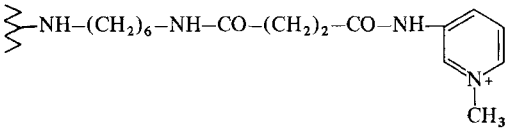
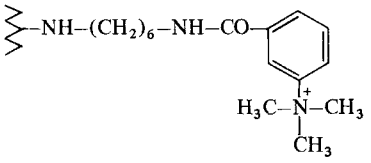
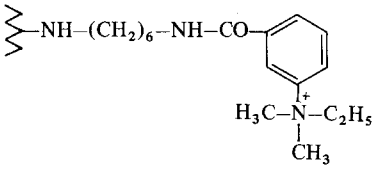
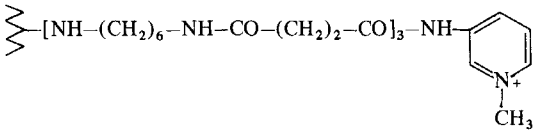
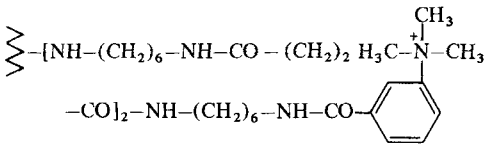
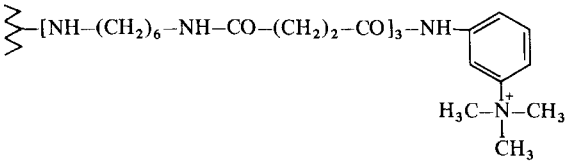
A Sepharose 6B column (2.3 × 42.5 cm), equilibrated with 0.05 M potassium phosphate buffer pH 7.0 containing 1 M NaCl, was used for gel filtration. 2 ml enzyme preparation was applied and fractions of 2 ml were collected. When dodecyl sulphate fraction was used sodium phosphate instead of potassium phosphate buffer was used for equilibration of the Sepharose column and for assays [15].

Gel Electrophoresis

Polyacrylamide gel electrophoresis was done on 7% gels in 0.05 M Tris/glycine buffer, pH 8.0, at 3.0 mA/tube according to Davis [16]. The current was passed until the tracking dye, bromophenol blue, emerged. This method was used for the gel electrophoresis of the enzyme peaks obtained by Sepharose 6B gel filtration as well as the purified electric eel enzyme. Electrophoresis of the purified enzymes from basal ganglia and erythrocytes was carried out in two different ways. In method 1 the Triton X-100 from enzymes was removed as described earlier and subjected to gel electrophoresis as above. In method 2 Triton X-100 was not removed from the purified enzymes and electrophoresis was done in gels prepared as above but containing 0.02% Triton X-100.

After electrophoresis, the gels were either stained for protein or assayed for enzyme activity. For protein staining, gels prepared according to method 1 were fixed with 12.5% trichloroacetic acid and stained with 0.05% Coomassie brilliant blue R dissolved in 10% trichloroacetic acid; and for gels prepared according to method 2, the protein bands were fixed and stained with 0.5% Coomassie brilliant blue R in 30% acetic acid. Both gels were destained in 5% acetic acid. For

Table 1. *Methods used for affinity chromatography of the serotonin-sensitive aryl acylamidase and acetylcholinesterase*
Other details of affinity chromatography of the enzymes are given under Materials and Methods. All the eluted enzymes were dialysed phate buffer, p H7.0, containing 0.5% v/v Triton X-100 and the eel enzyme against 0.02 M phosphate buffer, pH 7.0, containing 1 M NaCl

Source of enzyme	Method	Ligand used	Structure of spacer arm and ligand
Sheep basal ganglia	1	*N*-methyl-3-amino-pyridinium iodide	
	2	*m*-carboxyphenyltrimethyl-ammonium iodide	
	3	*m*-carboxyphenyldimethyl-ethylammonium iodide	
Electric eel	4	*N*-methyl-3-amino-pyridinium iodide	
	5	*m*-carboxyphenyltrimethyl-ammonium iodide	
	6	*m*-carboxyphenyldimethyl-ethylammonium iodide	
Human erythrocyte membrane	7	*N*-methyl-3-amino-pyridinium iodide	
	8	*m*-carboxyphenyl-trimethylammonium iodide	
	9	*m*-aminophenyltrimethyl-ammonium chloride hydrochloride	

for 24 h with six changes before assay as follows. The basal ganglia and erythrocyte enzymes were dialysed against 0.02 M potassium phos-

Wash buffer	Elution buffer	Comments
20 mM potassium phosphate pH 7.0 + 0.1 M NaCl + 0.5% v/v Triton X-100	linear gradient (0–0.1 M) tetraethylammonium bromide + 0.5% v/v Triton X-100 + 0.05 M NaCl + 20 mM potassium phosphate pH 7.0	–
20 mM potassium phosphate pH 7.0 + 0.3 M NaCl + 0.5% v/v Triton X-100	0.2 M tetraethylammonium bromide + 0.5% v/v Triton X-100 + 20 mM potassium phosphate pH 7.0	–
20 mM potassium phosphate pH 7.0 + 0.3 M NaCl + 0.5% v/v Triton X-100	0.2 M tetraethylammonium bromide + 0.5% v/v Triton X-100 + 20 mM potassium phosphate pH 7.0	–
20 mM potassium phosphate pH 7.0 + 0.5 M NaCl	0.5 M tetraethylammonium bromide + 0.5 M NaCl + 20 mM potassium phosphate pH 7.0	enzymes could not be eluted with even as high as 0.5 M tetraethylammonium bromide
20 mM potassium phosphate pH 7.0 + 0.5 M NaCl	0.5 M tetraethylammonium bromide + 0.5 M NaCl + 20 mM potassium phosphate pH 7.0	only 2% of the bound enzymes could be eluted
20 mM potassium phosphate pH 7.0 + 0.5 M NaCl	0.2 M tetraethylammonium bromide + 0.5 M NaCl + 20 mM potassium phosphate pH 7.0	–
20 mM potassium phosphate pH 7.0 + 0.1 M NaCl + 0.5% v/v Triton X-100		enzymes came out in the breakthough and washing
20 mM Tris/HCl pH 7.4 + 0.1 M NaCl + 0.5% Triton X-100	linear gradient (0–0.1 M) tetraethylammonium bromide + 0.5% Triton X-100 + 0.05 M NaCl + 20 mM Tris/HCl pH 7.4	–
20 mM potassium phosphate pH 7.0 + 1.0 M NaCl + 0.5% v/v Triton X-100	0.2 M tetraethylammonium bromide + 0.5% Triton X-100 + 20 mM potassium phosphate pH 7.0	–

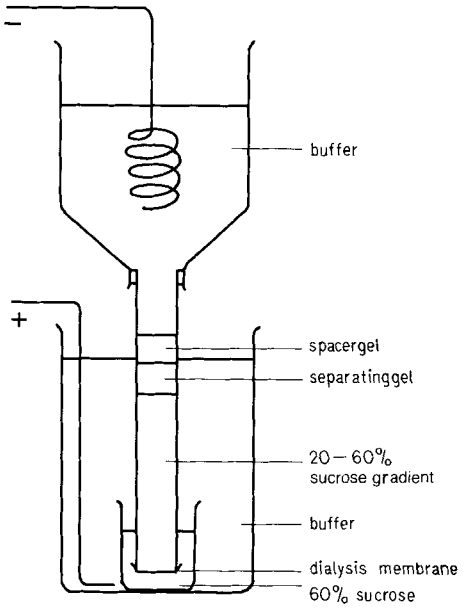


Fig. 1. *Design of the apparatus used for gel-density gradient electrophoresis*

assay of enzyme activity the gels were cut into slices of 1.5 mm or 3 mm thickness and extracted in 0.02 M potassium phosphate buffer, pH 7.0, containing either 0.5% v/v Triton X-100 (for erythrocyte and basal ganglia) or 0.5% Triton X-100 and 1 M NaCl (for electric eel enzyme) and assayed for aryl acylamidase and acetylcholinesterase.

Gel Density Gradient Electrophoresis

The method followed was essentially similar to that of Shuster and Schrier [17]. Fig. 1 shows the set-up used. A sucrose density gradient was made in a Pyrex glass tube (15 × 1.4 cm), the bottom of which was sealed by a dialysis membrane held in position by a rubber ring. A gradient of 20−60% sucrose in 0.375 M Tris/HCl, pH 8.3, was made by a three-veined peristaltic pump [18]. 2 ml 7% polyacrylamide, pH 8.3, prepared according to the method of Davis [16], was layered on top of the gradient and allowed to polymerise. 2 ml spacer gel (pH 6.5) was polymerised on top of the separating gel. The bottom of the tube was immersed in a glass beaker containing 20 ml 60% sucrose in 0.375 M Tris/glycine buffer, pH 8.0, to which 4.26 g NaCl was added to increase conductivity [19]. The whole set-up was immersed up to three-quarters the height in a 500-ml beaker containing 0.025 M Tris/glycine buffer, pH 8.0. This formed the anode vessel. The cathode vessel was a polythene reservoir fixed in position on top of the tube and filled with 300 ml 0.025 M Tris/glycine buffer, pH 8.0. After cooling at 4 °C, 1 ml of sample in 0.005% bromophenol blue and 5% glycerol was carefully layered on top of the gel and a current of 10 mA was passed for 4 h at 4 °C through platinum wire electrodes. After the run was over (till the dye reached the bottom), a double layer of Parafilm was fixed tightly over the dialysis membrane. A hypodermic needle was then introduced through the Parafilm and the membrane and 60% sucrose was pumped in slowly. The gradient which was displaced from the top was collected in fractions of 0.5 ml through a capillary tube inserted through the polyacrylamide gel. The fractions were dialysed against 0.02 M potassium phosphate, pH 7.0, and assayed.

Enzyme Assays and Analytical Methods

All operations of the enzyme preparation, gel filtration, gel electrophoresis and enzyme purification were done at 4 °C. Aryl acylamidase was assayed as described before [3], except that the concentration of *o*-nitroacetanilide in the reaction mixture was 10 mM. Acetylcholinesterase was assayed according to Ellman et al. [20], as described earlier [6]. 1 unit of aryl acylamidase corresponds to 1 μmol *o*-nitroacetanilide hydrolysed/h and 1 unit acetylcholinesterase to a change in absorbance of 1/min at 412 nm under standard assay conditions. Spectrophotometric measurements were made in a Zeiss PMQ II spectrophotometer. Protein was estimated according to Lowry et al. [21]. Appropriate blanks containing Triton X-100 were taken wherever the protein samples had Triton in them [22].

RESULTS

Comments on Affinity Chromatographic Methods

Table 1 gives the affinity chromatographic methods used for the purification of the aryl acylamidase and acetylcholinesterase from the sheep brain basal ganglia, human erythrocytes and electric eel. These methods were arrived at after trying different combinations of the spacer arm and ligands. Some of the methods are similar to those used by others for purification of acetylcholinesterase [8−10]. In some cases the enzyme could not be eluted from the column because of tight binding and in others different washing and elution buffers were used depending upon the tightness of binding of the enzymes to the affinity medium. Aryl acylamidase and acetylcholinesterase of both basal ganglia and erythrocytes failed to be eluted from the affinity column if Triton X-100 was absent in the elution buffer.

Methods 1−3 were useful in purifying the basal ganglia enzymes to varying degrees. Method 3 gave the highest purification. *m*-Carboxyphenyldimethylethyl ammonium iodide (method 3) appeared to have a higher affinity for the basal ganglia enzymes as

compared to the trimethyl derivative (method 2) because a linear gradient elution with tetraethylammonium bromide indicated that a higher concentration of this compound was needed to elute the enzymes in the former method as compared to the latter.

Methods 4 and 5 were not satisfactory for the eel enzymes because they were irreversibly bound to the column and could not be eluted to any significant extent with even as high as 0.5 M tetraethylammonium bromide. Method 6, however, was useful in purifying the eel enzyme.

A long spacer arm was necessary to prevent steric interaction for the affinity purification of the erythrocyte enzyme [10]. But even with a 30-carbon spacer arm *N*-methylaminopyridinium iodide (method 7) failed to retard the enzyme. Method 8 yielded purified enzymes but ionic strength in excess of 0.1 M NaCl in the wash buffer dissociated considerable amounts of the bound enzymes from the column. Method 9 was the best method for the purification of the erythrocyte membrane enzymes.

These results suggest that the affinity purification of the serotonin-sensitive aryl acylamidase and acetylcholinesterase from the diverse sources depends upon both the spacer arm as well as the ligand used. It may also be noted that a ligand useful for purification of the enzyme from one source need not necessarily be useful for another source [23].

Elution Profile of the Serotonin-Sensitive Aryl Acylamidase and Acetylcholinesterase from the Affinity Columns

Fig. 2 shows the elution profile of both enzymes from the affinity columns. The method best suited for purification of the enzymes from each source is given. It may be noted that both the enzymes had identical elution pattern from the column.

The elution profile of the enzymes by other methods given in Table 1 also showed the identity of the two enzyme activities (figures not given). Also in methods 4 and 5 both the enzyme activities were irreversibly bound to the column and in method 7 both the enzyme activities failed to bind to the column. Thus the various affinity chromatographic characteristics of the aryl acylamidase and acetylcholinesterase from all three sources were identical.

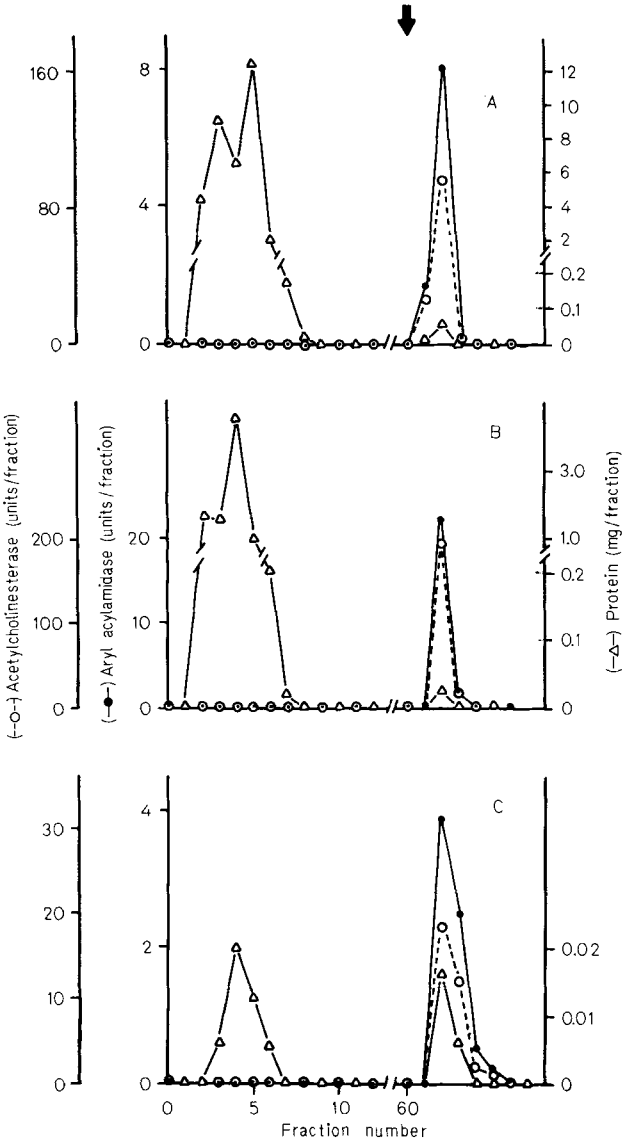


Fig. 2. *Affinity chromatography profiles of aryl acetylamidase and acetylcholinesterase.* (A) Basal ganglia enzyme on *m*-carboxyphenyldimethylethylammonium-iodide – Sepharose-4B (method 3). (B) Erythrocyte enzyme on *m*-aminophenyltrimethylammoniumchloride-hydrochloride – Sepharose-4B (method 9). (C) Electric eel enzyme on *m*-carboxyphenyldimethylethylammonium-iodide – Sepharose-4B (method 6). Methods of affinity chromatography are described in Table 1. Arrow (↑) indicates commencement of elution with tetraethylammonium bromide. (●——●) Aryl acylamidase; (o——o) acetylcholinesterase; (△——△) protein

Purification and Recovery of the Serotonin-Sensitive Aryl Acetylamidase and Acetylcholinesterase from the Affinity Columns

In Table 2 are given the purification data, the ratio of specific activities and recoveries of the serotonin-sensitive aryl acylamidase and acetylcholinesterase from the three sources, using the affinity chromatographic techniques. Both the enzymes were purified to similar extent by each method. The ratios of specific activities of the enzymes before and after each affinity chromatography were constant. Similarly the recovery of both the enzymes was the same in each method of purification.

It may be noted that the ratio of acetylcholinesterase activity to aryl acylamidase activity was highest for basal ganglia enzyme, much less for the erythrocyte enzyme and lowest for the eel enzyme.

Table 2. *Purification of aryl acylamidase and acetylcholinesterase from basal ganglia. Erythrocytes and electric eel*
The methods of affinity chromatography are described in Table 1. Specific activity is units of activity/mg protein. Enzyme units are as defined under Materials and Methods

Source	Affinity chromatography method number	Specific activity				Purification		Ratio of activities acetylcholinesterase/aryl acylamidase		Recovery	
		before affinity chromatography		after affinity chromatography		acetylcholinesterase	arylacylamidase	before affinity chromatography	after affinity chromatography	acetylcholinesterase	arylacylamidase
		acetylcholinesterase	arylacylamidase	acetylcholinesterase	arylacylamidase						
		units/mg				-fold				%	
Sheep basal ganglia	1	4.2	0.35	825	66	194	188	11.6	12.3	80	77
	2	4.4	0.36	658	52	150	145	12.2	12.5	77	74
	3	4.4	0.36	917	77	209	215	12.0	11.9	84	81
Electric eel	4	1001	205	–	–	–	–	4.87	–	0	0
	5	1001	205	–	–	–	–	4.87	–	2	2
	6	1001	205	1906	400	1.8	1.9	4.87	4.86	45	48
Human erythrocyte membrane	7	15.7	2.3	–	–	–	–	6.8	–	0	0
	8	15.7	2.3	5576	750	354	326	6.8	7.4	83	81
	9	15.7	2.3	6376	924	406	402	6.8	6.9	93	95

Gel Electrophoresis of the Purified Enzymes

Gel electrophoresis of the purified basal ganglia and erythrocyte enzymes in the presence of Triton X-100 gave single protein bands corresponding to enzyme activities. Removal of Triton-X-100 and gel electrophoresis invariably gave multiple protein bands each possessing enzyme activities. It is assumed that this multiplicity of bands result from the aggregated and non-aggregated forms of the enzyme, as reported for acetylcholinesterase by several workers [14, 26–28]. Fig. 3 shows the gel electrophoretic pattern of protein and the enzyme activities using the enzymes purified by the best affinity chromatographic method from each source. Both aryl acylamidase and acetylcholinesterase activities co-migrated and were associated with the major protein bands on the gel. Three peaks of enzyme activities were distinguished for the basal ganglia and erythrocyte enzymes after removal of Triton X-100. Only one peak of activity was seen for the eel enzyme. In the case of the eel enzyme a protein band was not visible due to the low amount of protein used.

Inhibition Characteristics of the Purified Aryl Acylamidase

The purified aryl acylamidases from all the three sources showed their characteristic inhibition by serotonin, acetylcholine and acetylcholinesterase inhibitors (Table 3). Neostigmine at 10 μM and eserine at 1 μM inhibited more than 95% of the activity. BW284C51, a selective inhibitor of acetylcholinesterase, gave more than 95% inhibition at 10 μM, whereas tetraisopropylpyrophosphoramide, an inhibitor of pseudocholinesterase, gave only less than 10% inhibition at the same concentration. The percentage inhibition of acetylcholinesterase by these inhibitors was approximately the same as that observed for aryl acylamidase (Table 3).

Serotonin was a non-competitive inhibitor of aryl acylamidase [6] and the K_i values for serotonin from Dixon plots were 0.145 mM, 0.125 mM and 0.1 mM respectively for the aryl acylamidase from basal ganglia, erythrocytes and eel (Fig. 4). Acetylcholine was also a non-competitive inhibitor [6] and Dixon Plots indicated K_i values of 2.15 mM, 1.65 mM and 1.05 mM respectively for the enzymes from the above three sources.

Gel Density Gradient Electrophoresis

Purified aryl acylamidase and acetylcholinesterase from all the three sources had similar rates of migration in the sucrose density gradient electrophoresis and appeared as single peaks of activity. In the case of the erythocyte enzyme the peak was preceded by a small shoulder of activity for both enzymes (Fig. 5).

Gel Filtration on Sepharose 6B

There is evidence that aryl acylamidase of brain and acetylcholinesterase from different sources are

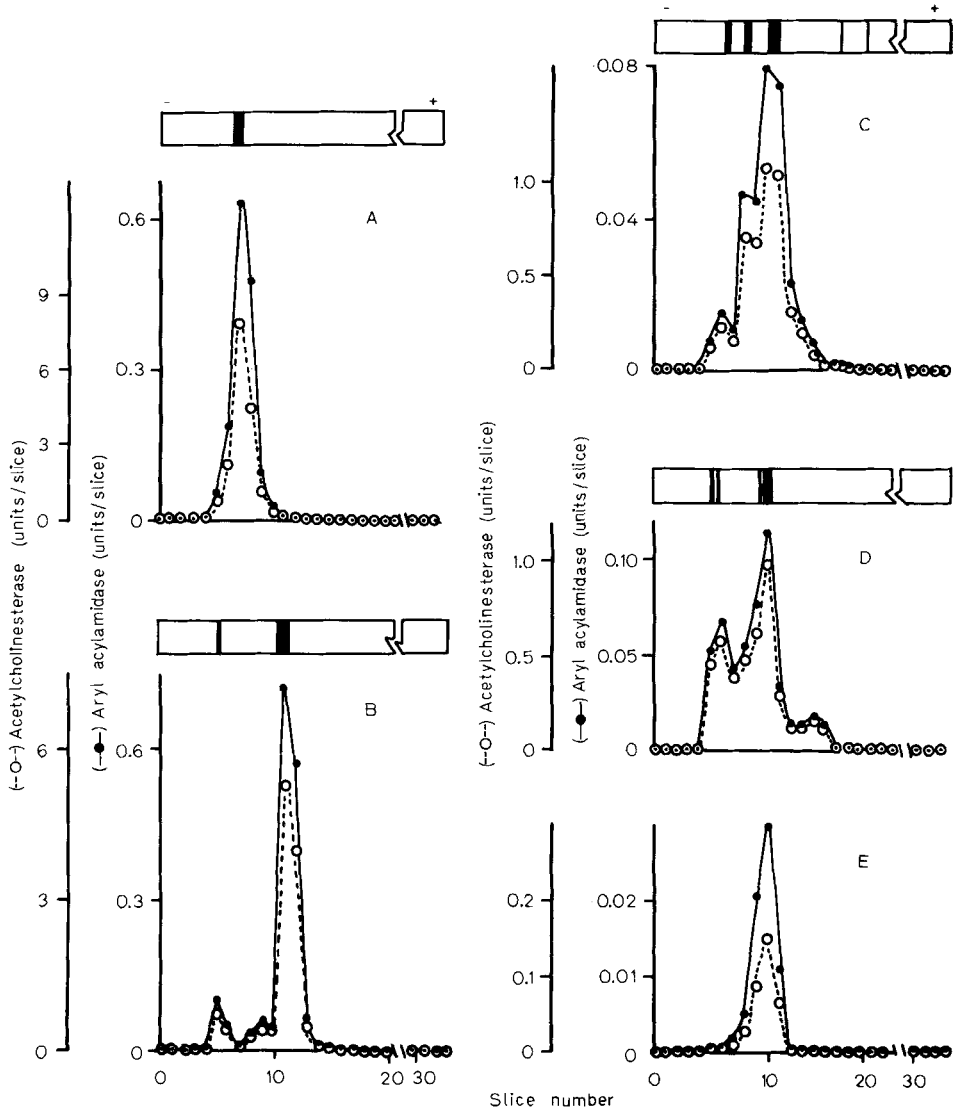


Fig. 3. *Gel electrophoresis of the purified enzymes.* The activity profiles of the enzymes in the gel slices and the protein bands on the gels are also shown. The enzymes were purified by affinity chromatography from basal ganglia, erythrocytes and electric eel by methods 3, 6 and 9 (described in Table 1). (A, B) Activity profiles of basal ganglia and erythrocyte enzymes respectively subjected to gel electrophoresis in the presence of Triton X-100; (C, D, E) the profiles of basal ganglia, erythrocytes and eel enzymes in the absence of Triton X-100. (For details of gel electrophoresis, see Materials and Methods.) Protein bands for the electric eel enzymes were not detectable because of low amount of protein used. Gel slice numbers 1 – 5 include the spacer gel. (●——●) Aryl acylamidase; (O---O) acetylcholinesterase

membrane-bound enzymes [3,24]. In particular acetylcholinesterase is known to exist in multiple molecular forms [14,25–27], the molecular size depending upon the method of preparation, ionic concentration, ageing or aggregation phenomena [14,24,28,29]. We have, therefore, utilized three general methods of preparation (given under Materials and Methods), which destablize membrane-bound or aggregated enzymes, and studied the gel filtration profile of the aryl acylamidase and acetylcholinesterase on a Sepharose 6B column.

Fig. 6 shows the gel filtration profile of both enzyme activities from the three sources. From the erythrocyte membrane, the Triton fraction gave a major peak I and a minor peak II (Fig. 6A) while the sonicated fraction gave only the peak I (Fig. 6B). The dodecyl sulphate fraction gave peaks I and II (Fig. 6C), the latter peak proportionately more as compared to the Triton fraction. The most noteworthy feature was that both aryl acylamidase and acetylcholinesterase showed the same profile of elution.

Fig. 6D – F show the elution profile of the enzyme from basal ganglia. Both the Triton fraction (Fig. 6D) and the dodecyl sulphate fraction (Fig. 6F) gave two peaks of activity, the proportion of peak II higher than peak I. With the sonicated fraction three distinct

Table 3. *Inhibition of purified arylacylamidase and acetylcholinesterase from erythrocytes, basal ganglia and electric eel*
The enzymes purified by affinity chromatographic methods 3, 9 and 6 (given in Table 1) respectively from basal ganglia, erythrocyte membrane and electric eel were used

Inhibitor	Inhibition					
	basal ganglia		erythrocytes		electric eel	
	arylacyl-amidase	acetylcholin-esterase	arylacyl-amidase	acetylcholin-esterase	arylacyl-amidase	acetylcholin-esterase
	%					
Serotonin						
5 mM	91	–	93	–	95	–
1 mM	85	–	86	–	89	–
0.2 mM	70	–	72	–	72	–
0.1 mM	48	–	54	–	55	–
Acetylcholine						
10 mM	86	–	86	–	92	–
4 mM	69	–	70	–	84	–
2 mM	46	–	42	–	58	–
Neostygmine						
10 μM	96	97	97	99	97	97
0.1 μM	79	75	80	79	77	60
0.01 μM	28	27	30	30	27	20
Eserine						
1 μM	94	94	95	98	98	95
0.1 μM	84	80	81	84	83	81
0.01 μM	33	29	36	38	34	37
BW284C51						
10 μM	98	98	97	92	98	100
1 μM	78	82	79	55	79	97
0.1 μM	14	13	16	13	14	53
Tetraisopropyl-pyrophosphoramide						
100 μM	8	8	10	54	12	11
10 μM	2	2	2	9	2	2

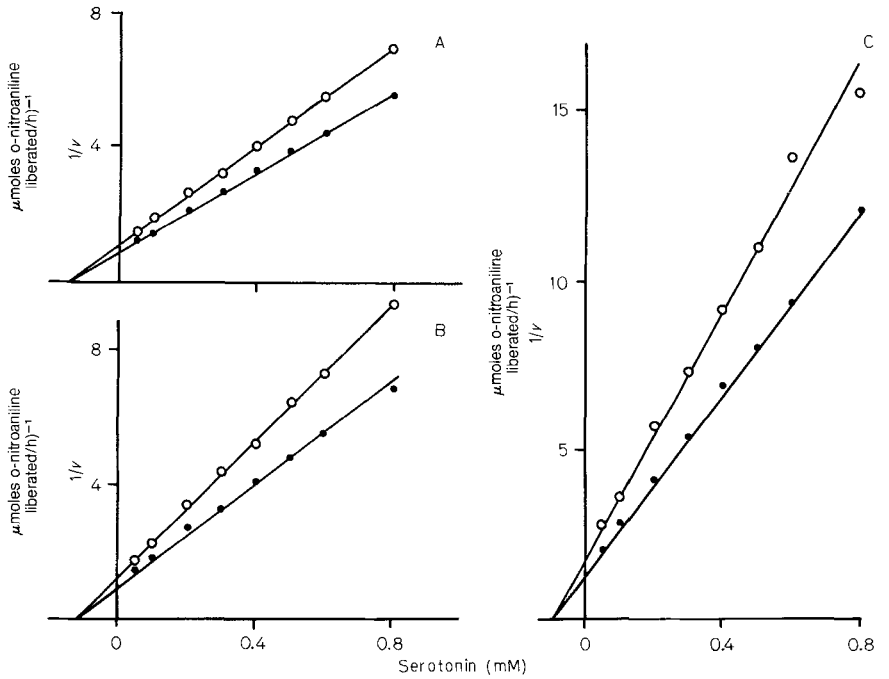


Fig. 4. *Dixon plots for serotonin inhibition of purified aryl acylamidases at 8.33 mM (○——○) and 11.66 mM (●——●) o-nitroacetanilide.* (A) Basal ganglia; (B) erythrocytes; (C) electric eel

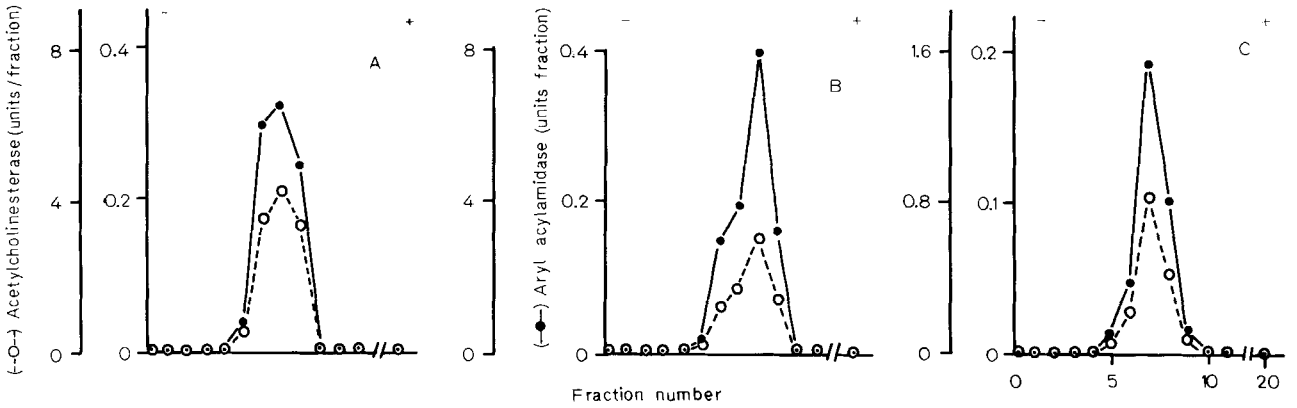


Fig. 5. *Gel density gradient electrophoretic pattern of purified aryl acylamidase (●—●) and acetylcholinesterase (O---O) from (A) basal ganglia, (B) erythrocytes and (C) electric eel*

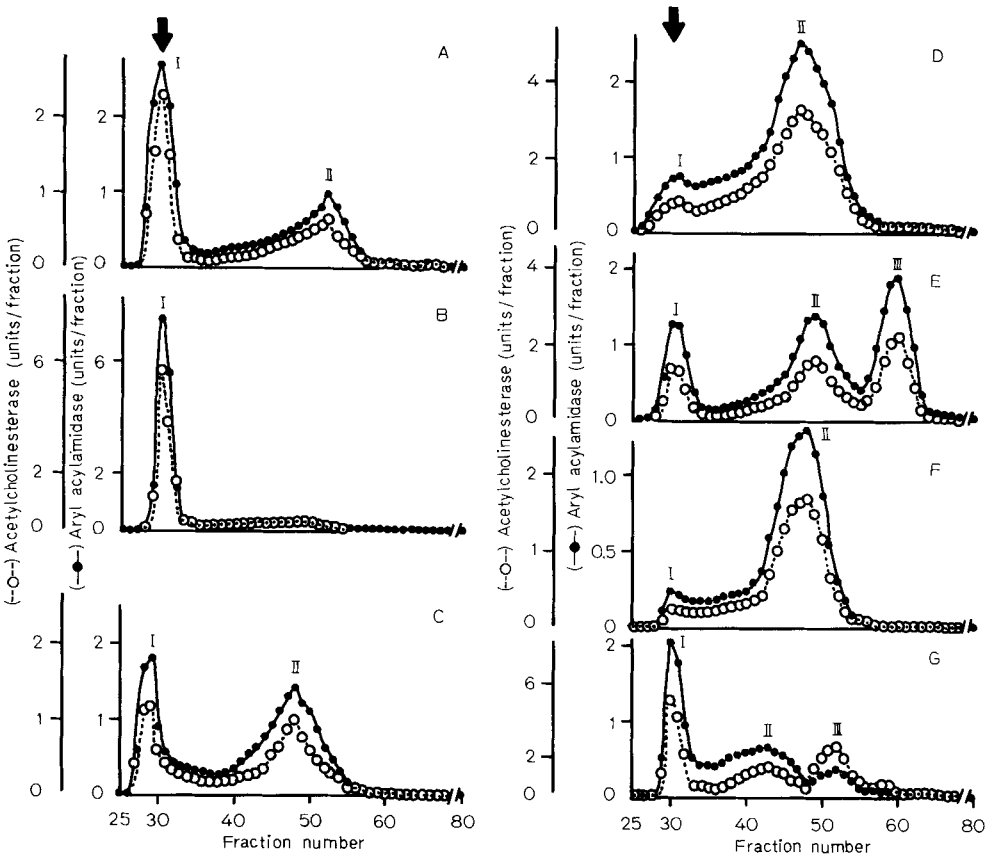


Fig. 6. *Sepharose 6B gel filtration profiles of aryl acylamidase (●——●) and acetylcholinesterase (O---O) from different sources.* Preparation of the enzymes for gel filtration on Sepharose 6B column were as described under Materials and Methods. Arrow (↓) indicates the position of the 1st peak of blue dextran. The recoveries of the enzymes from basal ganglia and erythrocyte membrane were 86–97% for aryl acylamidase and 83–90% for acetylcholinesterase. Recovery of electric eel enzymes were 51% for aryl acylamidase and 45% for acetylcholinesterase. (A) Erythrocyte 'Triton fraction'; (B) erythrocyte 'sonicated fraction'; (C) erythrocyte 'dodecyl sulphate fraction'; (D) basal ganglia 'Triton fraction'; (E) basal ganglia 'sonicated fraction'; (F) basal ganglia 'dodecyl sulphate fraction'; (G) electric eel enzyme

peaks of activity were obtained (Fig. 6E). Again both aryl acylamidase and acetylcholinesterase co-eluted from the column.

The elution of electric eel enzyme showed the presence of three peaks (Fig. 6G). Both aryl acylamidase and acetylcholinesterase showed a similar profile, except that the proportion of the third peak was somewhat higher for acetylcholinesterase as compared to aryl acylamidase. Preparation of the sonicated fraction or dodecyl sulphate fraction of electric eel

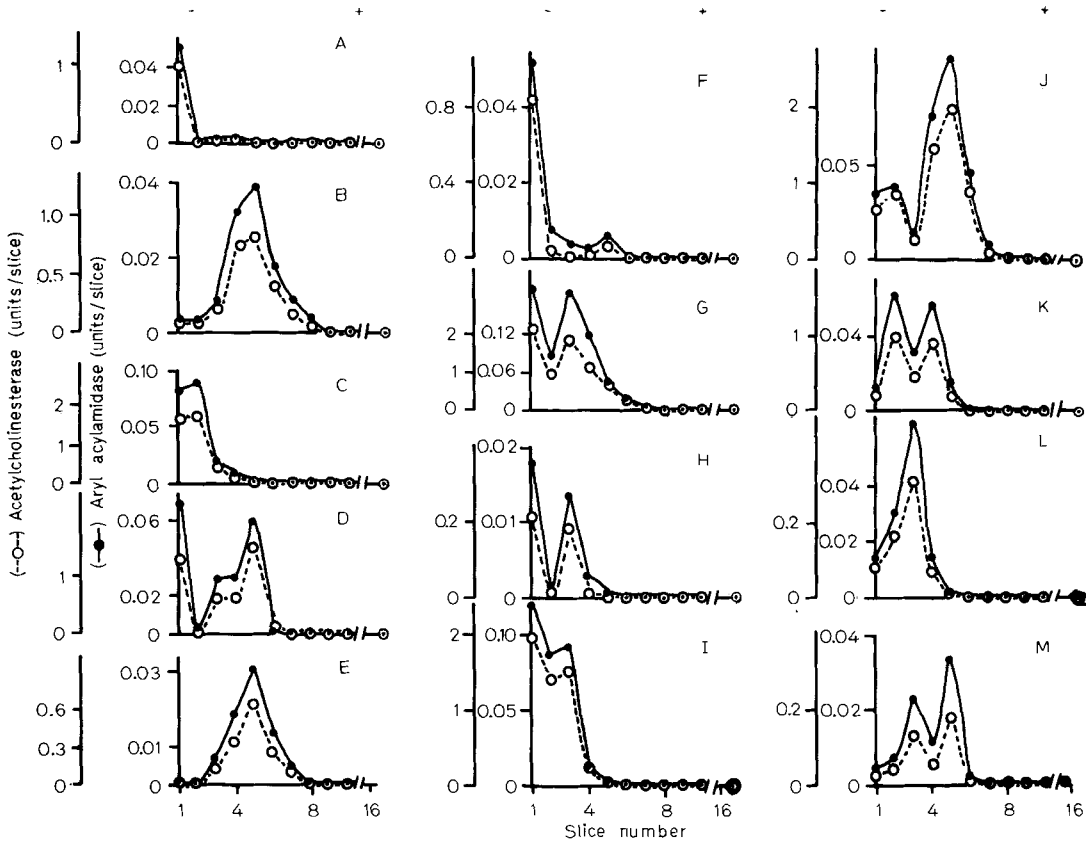


Fig. 7. *Polyacrylamide gel electrophoresis of enzyme peaks from Sepharose 6B filtration.* Electrophoresis and assay of aryl acylamidase (●——●) and acetylcholinesterase (O- --O) were done as described under Materials and Methods. Erythrocyte 'Triton fraction' peaks I (A) and II (B); erythrocyte 'sonicated fraction' peak I (C); erythrocyte 'dodecyl sulphate fraction' peaks I (D) and II (E); basal ganglia 'Triton fraction' peaks I (F) and II (G); basal ganglia 'sonicated fraction' peaks I (H), II (I) and III (J); basal ganglia 'dodecyl sulphate fraction': peak II (K); electric eel peaks I (L) and II (M). Peaks from Sepharose which were low in activity were not used for gel electrophoresis

enzyme resulted in approximately 90–100% loss of activity of both aryl acylamidase and acetylcholinesterase activities.

The ratios of activities (units of acetylcholinesterase/units of aryl acylamidase), although varying from source to source, were comparable among the various peaks of enzyme activities from the same source. Thus, for the erythrocyte enzyme the ratio was in the range of 6.4–8.4. For the basal ganglia the ratio ranged from 10.5–13.0. The electric eel enzyme peaks showed some variability in the ratio of activities. While both peaks I and II showed a ratio of 2.5, peak III had a ratio of 7.5. This was confirmed in repeated experiments. The electric eel enzyme, before Sepharose gel filtration, had a value of 4.8 for the ratio.

Polyacrylamide gel electrophoresis of all the different peaks of enzymes showed that both aryl acylamidase and acetylcholinesterase co-migrated (Fig. 7). The rate of migration of the enzymes appeared to be generally dependent on their molecular size, those with the highest molecular weight remaining near the origin and those with the lowest molecular weight moving the farthest. In many instances the enzymes did not show a single peak of activity, possibly because of changes in aggregation properties.

The effect of various inhibitors on the aryl acylamidase activity of the various enzyme peaks from the Sepharose column is shown in Table 4. Serotonin as well as acetylcholine were inhibitory to all peaks of enzymes. Neostygmine and eserine, potent inhibitors of acetylcholinesterase, also inhibited aryl acylamidase activity of all the enzyme peaks at 0.01 μM. Tetraisopropylpyrophosphoramide at 10 μM had only very little inhibitory effect on the enzymes. The percentage inhibition of all the peaks of enzymes from the three sources was about the same for the different inhibitors, except for a slightly enhanced inhibition of the electric eel aryl acylamidase by acetylcholine.

DISCUSSION

We have used three well-known sources of acetylcholinesterase [24,28] for proving the generality of

Table 4. *Effect of inhibitors on aryl acylamidase peaks eluted from Sepharose-6B*
Electric eel enzyme peak III was not used for inhibition studies because of insufficient activity of aryl acylamidase. See Fig. 6 for details of enzyme peaks from Sepharose-6B column

Inhibitor	Inhibition													
	erythrocyte membrane					basal ganglia							electric eel	
	Triton fraction		sonicated fraction	dodecyl-sulphate fraction		Triton fraction		sonicated fraction			dodecyl-sulphate fraction			
	peak I	peak II	peak I	peak I	peak II	peak I	peak II	peak I	peak II	peak III	peak I	peak II	peak I	peak II
	%													
Serotonin														
1 mM	80	77	82	80	76	80	77	80	78	84	81	77	85	87
0.2 mM	65	64	66	65	63	65	67	66	65	68	68	64	68	70
0.1 mM	47	49	50	50	46	46	45	47	45	45	47	45	53	54
Neostygmine														
0.1 μM	72	71	72	74	71	75	78	77	76	77	76	74	73	73
0.01 μM	26	23	27	26	23	26	24	26	25	28	27	23	20	22
Eserine														
0.1 μM	77	75	80	76	77	80	80	79	78	78	79	77	74	76
0.01 μM	35	34	36	32	34	33	31	34	31	33	34	30	30	31
Acetylcholine														
4 mM	64	66	65	64	62	64	62	61	64	64	65	60	77	78
2 mM	40	38	43	41	38	42	41	39	40	41	43	44	59	58
Tetraisopropyl-pyrophosphoramide														
0.1 mM	10	9	10	11	9	9	9	8	7	8	9	8	12	10
0.01 mM	2	2	2	3	2	2	2	3	2	2	2	1	3	2

the association of acetylcholinesterase with the serotonin-sensitive aryl acylamidase. The basis for the identity of the two enzymes is as follows. (a) Co-purification of both enzymes with approximately the same yield and constant ratio of specific activities from all three sources by different affinity chromatographic procedures. It is noteworthy that, although the ratios of activities differ from source to source, they remain constant during the purification procedures of the enzymes from all the three sources. (b) Gel electrophoresis of the purified fraction shows co-migration of both enzyme activities either as a single species or as multiple species from all the three sources. (c) Electrophoresis in a sucrose gradient also shows identical migration rates of both enzymes. (d) Multiple forms of the two enzymes, resulting from different methods of extraction, show identical profiles on Sepharose gel filtration as well as polycrylamide gel electrophoresis. (e) All the potent inhibitors of acetylcholinesterase: eserine, neostygmine and BW284C51 (a selective inhibitor of acetylcholinesterase), potently inhibit the aryl acylamidase in the purified or multiple forms from all the three sources. Tetraisopropylpyrophosphoramide, a selective inhibitor of pseudocholinesterase, does not inhibit the aryl acylamidase.

It is difficult to conceive that the identical behaviour of the two enzyme activities from all the three sources under the different experimental conditions is coincidental. The results rather seem to suggest that the acetylcholinesterase activity and the serotonin-sensitive aryl acylamidase activity are associated with the same protein or the same macromolecule. Previous studies [6] have indicated that the active sites of aryl acylamidase and acetylcholinesterase may be different. Large and complex proteins, like acetylcholinesterase, can have oligomeric structures in which several polypeptide chains are associated. Also in each subunit the polypeptide chain may be often folded up into several compact regions, called domains [30]. A search for such domains in the acetylcholinesterase protein may help further studies in localising the serotonin-sensitive aryl acylamidase associated with it.

The physiological role of the serotonin-sensitive aryl acylamidase, as distinguished from the serotonin-insensitive form, remains to be established. The brain aryl acylamidase does not seem to deacetylate melatonin or phenacetin [31, 32]. It remains to be seen whether it can *N*-deacetylate other naturally occurring acetylated compounds. Of particular interest is the recent finding of *N*-acetylendorphins with terminal

N-acetyltyrosine residues which are inactive physiologically and require *N*-deacetylation to be active [33]. The significance of the association of a serotonin-sensitive form of aryl acylamidase with acetylcholinesterase, which is well known for its involvement in neurotransmission, also remains to be elucidated.

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