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The Association of the Serotonin-Sensitive Aryl Acylamidase with Acetylcholinesterase in the Monkey Brain

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The serotonin-sensitive aryl acylamidase was partially purified from monkey brain. The aryl acylamidase activity was inhibited by serotonin (K_i = 0.425 mM) and tryptamine (K_i = 3.6 mM) but not by a number of other amines. It was also inhibited by acetylcholine (K_i = 2 mM) and its analogues and homologues. The relationship of aryl acylamidase to acetylcholinesterase was examined. The ratios of specific activities of aryl acylamidase and acetylcholinesterase in the different steps of purification were approximately constant and the percentage recoveries of both enzyme activities were comparable. Elution profiles of both enzyme activities from concanavalin-A–Sepharose, Sephadex G-200 and DEAE-Sephadex A-25 columns were similar. Both enzyme activities migrated in a similar fashion on gel electrophoresis in different percentage gels for different time intervals. Both enzymes showed similar distribution in the various anatomical regions and in the different subcellular fractions of monkey brain.

Eserine and neostigmine, potent competitive inhibitors of acetylcholinesterase also potently inhibited aryl acylamidase in a non-competitive manner. Inhibition of both enzymes was 100% at 10 μM of both the inhibitors. Tetraisopropylpyrophosphoramide, a selective inhibitor of pseudocholinesterase, did not inhibit either the brain acetylcholinesterase or aryl acylamidase at 10 μM. Serotonin inhibited acetylcholinesterase only at concentrations above 10 mM. Dixon plots of one inhibitor in the presence of additional inhibitors indicated that serotonin, butyrylcholine, acetylcholine, neostigmine and eserine (all non-competitive inhibitors of aryl acylamidase) acted at the same site as inhibitors of aryl acylamidase.

The serotonin-insensitive aryl acylamidase of monkey liver was also insensitive to acetylcholine and eserine and was not found associated with acetylcholinesterase [A. Oommen and A. S. Balasubramanian (1978) *Biochem. Pharmacol. 27*, 891–895]. Erythrocyte membrane known to contain true cholinesterase had aryl acylamidase activity sensitive to serotonin, acetylcholine and eserine. All these considerations suggest that the serotonin-sensitive aryl acylamidase activity is a property of true cholinesterase. Although some of the experimental results would suggest that in the monkey brain the two activities may be associated with the same protein with two different active sites further experiments are needed to confirm this.

The existence of a serotonin-sensitive aryl acylamidase in the rat brain was first reported by Fujimoto [1]. Since the enzyme was specifically sensitive to serotonin it was suggested that the brain aryl acylamidase could serve as a model for the study of the mechanism of action of serotonin in the central nervous system [1]. The uniqueness of the enzyme lies in the fact that it is sensitive to serotonin when prepared from the brain tissue but not from the liver [1,2]. We confirmed these observations of Fujimoto in the sheep and monkey brain and further observed that acetylcholine and several of its analogues and homologues also inhibit the brain enzyme but not the liver enzyme [3,4]. Fujimoto independently reported that purified acetylcholinesterase of *E. electricus* has aryl acylamidase activity which is serotonin-sensitive [5]. We have therefore considered the possibility of the association of the serotonin-sensitive aryl acylamidase with acetylcholinesterase in the brain. In an earlier communication [4] we reported that preparations of aryl acylamidase from monkey brain and liver differ from each other in their sensitivity to inhibitors, molecular weight and thermal stability. Furthermore, on Sepharose gel filtration acetylcholinesterase activity coeluted with aryl acylamidase activity from monkey

Enzymes. Aryl acylamidase or aryl acylamide amidohydrolase (EC 3.5.1.13); acetylcholinesterase (EC 3.1.1.7).

brain but not from monkey liver. These results and a study of the regional distribution of aryl acylamidase in monkey brain were considered as preliminary indications of a close relationship between brain aryl acylamidase and acetylcholinesterase. In the present communication we report several lines of evidence which suggest the association of the serotonin-sensitive aryl acylamidase with acetylcholinesterase in the monkey brain.

MATERIALS AND METHODS

The various amines, 5-methoxytryptamine, 5-hydroxyindole-3-acetic acid, tetraisopropylpyrophosphoramide, eserine sulphate, acetylcholine iodide and related choline derivatives, DL-α-phosphatidylcholine dipalmitoyl and trypsin inhibitor (soyabean) were from Sigma Chemical Co. (U.S.A.); pronase, B grade and aquacide II from Calbiochem (U.S.A.), trypsin (20000 Fuld-Gross units/g) from E. Merck (F.R.G.), α-chymotrypsin (thrice crystallized) from Worthington (U.S.A.) and neostigmine methylsulphate from Roche Products Ltd (India). Concanavalin A from *Canavalia gladiata* was prepared according to the method of Surolia et al. [6] and coupled to Sepharose 4B (activated at pH 8.5 in 0.1 M sodium bicarbonate buffer [7]) according to the method of Cuatrecasas and Parikh [8]. The final preparation had 12 mg concanavalin A per ml Sepharose 4B. All other chemicals were obtained as described earlier [3]. Monkey (*Macaca radiata*) brain was obtained immediately after sacrifice of the animal under Nembutal anaesthesia and stored frozen at −18 °C until required.

Purification of Monkey Brain Aryl Acylamidase and Acetylcholinesterase

All operations were carried out between 0−4 °C unless otherwise mentioned.

Extraction and Ammonium Sulphate Fractionation

Monkey brain (100 g) was homogenized with 200 ml of 0.05 M potassium phosphate buffer pH 7.0 containing 0.5% (v/v) Triton X-100 in a Potter-Elvehjem homogenizer. The homogenate was kept for 60 min and then centrifuged at 12000 × *g* for 30 min. The supernatant was subjected to ammonium sulphate fractionation using solid ammonium sulphate and the fraction precipitating between 30 to 65% saturation of ammonium sulphate was collected by centrifugation at 12000 × *g* for 30 min. It was dissolved using 5 ml of 0.05 M potassium phosphate buffer pH 7.0 and dialyzed against 100 volumes of the same buffer for eights hours with four changes.

Concanavalin-A − Sepharose Chromatography. The dialyzed ammonium sulphate fraction (20 ml) was kept in contact with 35 ml of Concanavalin-A − Sepharose for 48 h with frequent stirring. It was then poured into a column (13 × 1.8 cm) and washed with 10 bed volumes of 0.05 M potassium phosphate buffer pH 7.0 containing 0.5 M NaCl. Elution was carried out with 0.5 M α-methyl glucoside in the wash buffer. The column was kept in contact with 35 ml of the eluting buffer for 3 h before elution was started. Elution was performed at a flow rate of 10 ml/h and fractions of 5 ml were collected. The active fractions (4 − 11) were pooled and concentrated with Aquacide II to approximately 2 ml.

Sephadex G-200 Gel Filtration. The concentrated concanavalin A eluate (1.8 ml) was next subjected to gel filtration on a Sephadex G-200 column (26.5 × 2.2 cm) previously equilibrated with 0.05 M potassium phosphate buffer pH 7.0 containing 0.1 M NaCl at a flow rate of 8 ml/h. The first 24 ml of effluent was collected in bulk and thereafter 2-ml fractions were collected. The active fractions (3 − 9) were pooled and concentrated with Aquacide II to approximately 0.7 ml and then dialyzed against 70 volumes of 0.05 M potassium phosphate buffer pH 7.0 for 6 h with two changes.

Unless otherwise mentioned this concentrated and dialyzed Sephadex G-200 fraction was used as the source of enzyme.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was carried out according to the method of Davis [9] on 3.5%, 5% and 7% gels at pH 8.0 in 0.05 M Tris-glycine buffer and on 5% gels at pH 5.0 in 0.05 M β-alanine buffer at 3 mA/tube. The applied sample consisted of 0.15 ml enzyme together with 0.02 ml glycerol and 0.02 ml of tracking dye. Electrophoresis in the pH 8.0 buffer was run for different time intervals, viz. for 1 h and 20 min when the tracking dye (bromophenol blue) emerged from the gel tubes, and for 4 h. Similar electrophoretic runs were made at pH 5.0 using methylene green, as the tracking dye. At the end of the runs the gels were sliced into sections of 3-mm thickness and each section homogenized with 1 ml of 0.05 M potassium phosphate buffer pH 7.0 containing 0.5% (v/v) Triton X-100 and 0.1 M NaCl. The homogenate was kept at 4 °C for 24 h with frequent shaking and then centrifuged to settle the gel particles. Enzyme assays for both aryl acylamidase and acetylcholinesterase were carried out with 0.2 ml aliquots of the supernatants. Incubation was at 28 °C for 24 h for aryl acylamidase and at 37 °C for 10 min for acetylcholinesterase.

The gels were stained for protein with Coomassie brilliant blue. Sodium dodecyl/polyacrylamide gel

electrophoresis was carried out according to Weber and Osborn [10] with and without 2-mercaptoethanol treatment.

Subcellular Fractionation and Regional Distribution Studies

Subcellular fractionation of the monkey brain was carried out according to the method of Eichberg et al. [11], using one hemisphere of freshly obtained whole monkey brain.

When studying the distribution of aryl acylamidase and acetylcholinesterase in the different regions of the monkey brain, each region was homogenized in 2 volumes of 0.05 M potassium phosphate buffer pH 7.0 containing 0.5% (v/v) Triton X-100 and these homogenates were used for the assay of the enzymes.

Proteolytic Treatment of the Purified Enzyme

Trypsin Treatment. The purified enzyme (2 mg) was incubated with 0.2 mg of trypsin in a total volume of 0.7 ml in 0.05 M potassium phosphate buffer pH 7.0. After incubation for 1 h at 37 °C the reaction was stopped with 0.2 mg trypsin inhibitor and the mixture chilled in ice for 3 min.

α-Chymotrypsin Treatment. The purified enzyme (2 mg) was treated with 0.07 mg α-chymotrypsin in 0.05 M potassium phosphate buffer pH 7.0 in a total volume of 0.57 ml. After incubation for 1 h at 37 °C the mixture was chilled in ice for 3 min.

Pronase Treatment. The purified enzyme (3.25 mg) was incubated with 0.06 mg pronase in 0.05 M potassium phosphate buffer pH 7.0 in a total volume of 0.62 ml for 30 min at 37 °C and then chilled in ice for 3 min.

The protease-treated enzyme samples were subjected to gel filtration and polyacrylamide gel electrophoresis at the end of incubation periods as described above.

Aryl Acylamidase Assay

Aryl acylamidase was assayed according to a modified method of Hoagland and Graf [12] as described earlier [3]. The assay system consisted of 50 μmol potassium phosphate buffer pH 7.0, 4.5 μmol of *o*-nitroacetanilide and enzyme in a total volume of 0.5 ml. Incubation was carried out for 2 h at 37 °C and the absorbance of liberated *o*-nitroaniline read at 430 nm. Reaction rates in all the assays were linear with respect to time and protein concentration. One enzyme unit (U) is defined as the amount of enzyme required to produce 1 μmol of *o*-nitroaniline in 1 h under the standard assay conditions.

Acetylcholinesterase Assay

Acetylcholinesterase was assayed according to the method of Ellman et al. [13]. In a total volume of 0.5 ml, 50 μmol potassium phosphate buffer pH 8.0, 1.5 μmol acetylthiocholine iodide, 0.1 ml 5,5′-dithiobis(2-nitrobenzoic acid) reagent and enzyme (suitably diluted in 0.05 M potassium phosphate buffer pH 7.0) were incubated for 10 min at 37 °C. The reaction was stopped with 0.5 ml of 1 mM eserine sulphate and the absorbance of liberated product read at 412 nm. In all the studies, reaction rates were linear with respect to time and protein concentration. One enzyme unit (U) is defined as the amount of enzyme which produces a change of 0.001 in absorbance at 412 nm in 1 min under the standard assay conditions.

Protein Estimation

Protein was estimated according to the method of Lowry et al. [14] using crystalline bovine serum albumin as standard.

Specific activity is defined as enzyme units per mg protein. Spectrophotometric measurements were made in a Carl Zeiss spectrophotometer.

RESULTS

Purification of Aryl Acylamidase and Acetylcholinesterase

Using a combination of ammonium sulphate fractionation, concanavalin-A–Sepharose affinity chromatography [15], and Sephadex G-200 gel filtration a partial purification of the monkey brain aryl acylamidase and acetylcholinesterase was achieved (Table 1). Keeping the enzyme with concanavalin-A–Sepharose for 48 h was found to be necessary for maximal binding of aryl acylamidase to concanavalin A. The overall purification was approximately twenty-fold over the homogenate with the affinity technique providing about seven-fold purification. In both the concanavalin-A–Sepharose chromatography and Sephadex G-200 gel filtration steps aryl acylamidase and acetylcholinesterase co-eluted (Fig. 1). Furthermore as shown in Table 1 the ratios of the specific activities of aryl acylamidase and acetylcholinesterase were approximately constant in the different steps of purification and the percentage recoveries of the two enzymes at each step was about the same.

In an attempt to further purify the enzyme the Sephadex G-200 fraction was subjected to DEAE-Sephadex A-25 chromatography and linear gradient elution with NaCl. Although little purification and poor recoveries resulted, it was found that both the enzymes were eluted in the same fractions at this step also.

Table 1. *Purification of monkey brain aryl acylamidase and acetylcholinesterase*
Values are the average of 5 different sets of experiments. See Methods for details and definitions of enzyme units and specific activity

Fraction	Volume	Total protein	Total enzyme activity		Specific activity		Recovery		10^{-4} *a/b*
			aryl-acyl-amidase	acetyl-cholin-esterase	*a* aryl-acyl-amidase	*b* acetyl-cholin-esterase	aryl-acyl-amidase	acetyl-cholin-esterase	
	ml	mg	U		U/mg		%		
Homogenate	290	12227	544.43	5943250	0.045	486.1	100	100	0.925
12000 × *g* supernatant	109	2348	196.4	1508485	0.084	642.5	36	25.4	1.3
30–65% $(NH_4)_2SO_4$	20	607	78.5	732830	0.129	1207.3	14.4	12.3	1.06
Concentrated concanavalin A fraction	2.36	17.55	16.02	125745	0.913	7165	2.9	2.1	1.27
Sephadex G-200 fraction	0.78	5.61	5.22	49610	0.931	8843	0.96	0.84	1.05

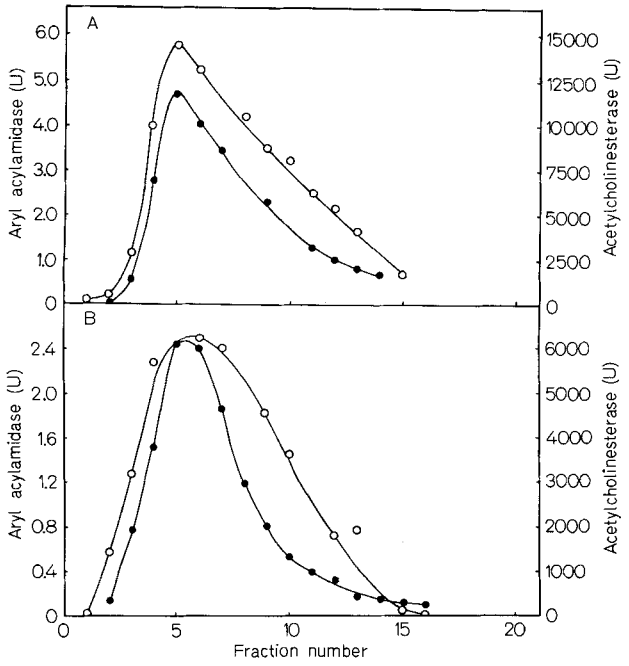

Fig. 1. *Elution profiles of aryl acylamidase and acetylcholinesterase.* (A) From the concanavalin-A–Sepharose column; (B) from the Sephadex G-200 column. See Methods for details. (●) Aryl acylamidase; (○) acetylcholinesterase

Although the ratios of specific activities of aryl acylamidase and acetylcholinesterase of the pooled concentrated fractions from the affinity column and Sephadex G-200 were approximately the same as in the other steps of purification (Table 1) a variation in the ratio of activities among the individual fractions after elution from concanavalin-A–Sepharose or Sephadex G-200 was noticed. In the affinity column step a 1.5–1.8-fold variation in the ratio of activities was observed as judged from three different experiments and in Sephadex G-200 gel filtration a 1.6–2.6-fold variation was seen in four different experiments.

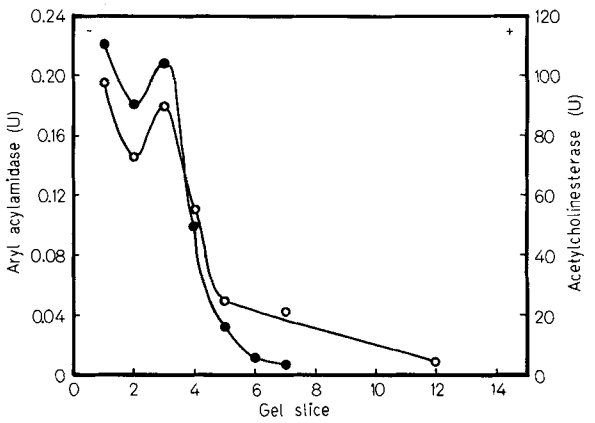

Fig. 2. *Polyacrylamide gel electrophoresis on 5% gels in 0.05 M Tris-glycine buffer pH 8.0.* Gels were cut into 3-mm thickness starting at the origin of the separating gel. Gel slice number 1 includes the origin. For details see text. Aryl acylamidase (●) and acetylcholinesterase (○) activity profiles after polyacrylamide gel electrophoresis for 1 h 20 min

Polyacrylamide Gel Electrophoresis of Aryl Acylamidase and Acetylcholinesterase

Both aryl acylamidase and acetylcholinesterase were observed to co-migrate when subjected to gel electrophoresis on polyacrylamide gels of different concentration (3.5%, 5% and 7%) as well as for different lengths of time. In Fig. 2 is shown the migration of the enzyme activities after gel electrophoresis for 1 h 20 min on 5% gels at pH 8.0. Both aryl acylamidase and acetylcholinesterase showed the same pattern of electrophoretic movement. During this period of electrophoresis a major peak of the enzyme activity remained at or near the origin with a second peak of activity on the separating gel (Fig. 2). During a 4 h electrophoresis the second peak of activity of both aryl acylamidase and acetylcholinesterase was found to further migrate into the separating gel as a diffused band of activity spread out in gel numbers 6 to 12 with a minor peak in gel number 10. The

Table 2. *Inhibition of brain aryl acylamidase by amines and choline derivatives*
Inhibitors were tested at 1 mM

Inhibitor	Inhibition
	%
Serotonin creatinine sulphate	83
Tryptamine hydrochloride	34
Succinylcholine chloride	58
Butyrylcholine iodide	60
Propionylcholine chloride	36
Acetyl-β-methylcholine bromide	33
Acetylcholine iodide	35
Choline chloride	38
Benzoylcholine chloride	44
Acetylthiocholine iodide	21

Table 3. *Inhibition of aryl acylamidase and acetylcholinesterase by acetylcholinesterase inhibitors*

Inhibitor	Concentration	Inhibition	
		aryl acylamidase	acetylcholinesterase
	μM	%	
Eserine	1	90	48
	10	100	100
Neostigmine	1	90	–
	7.5	–	93
	10	100	100
Tetraisopropylpyrophosphoramide	10	0	0
	100	19	0

major peak of activity due to both enzymes remained near the origin even after 4 h of electrophoresis. The same pattern of migration for the two enzymes was observed with 7.0% and 3.5% gels. The major enzyme activity near the origin may represent a higher molecular weight component or an aggregated form of the enzyme.

Staining for protein after gel electrophoresis (1 mg protein on 5% gel for 1 h 20 min) showed two closely spaced bands near the origin, two faint bands nearer the anode and a diffused band in between. The sodium dodecyl gel electrophoretic pattern with or without 2-mercaptoethanol treatment showed one protein band near the origin and about seven closely spaced faint bands in the middle of the gel.

When electrophoresis was carried out in pH 5.0 buffer no aryl acylamidase or acetylcholinesterase activity was detectable in the gels, possibly due to inactivation of the enzymes or non-entry of the enzymes into the gel.

Inhibition of Aryl Acylamidase by Various Amines and Choline Derivatives

It was shown in earlier studies that serotonin and some choline derivatives inhibit the sheep brain aryl acylamidase maximally around 1 mM. In Table 2 is shown the percentage inhibition of monkey brain aryl acylamidase by the amines and choline derivatives at 1 mM. Among the amines only serotonin and tryptamine were found to inhibit the enzyme with K_i values of 0.425 mM and 3.6 mM respectively as measured by Dixon plots. Tryptophan, 5-methoxytryptophan, 5-methoxytryptamine and 5-hydroxyindole-3-acetic acid were ineffective as inhibitors. The following monoamines and polyamines also did not have any significant inhibition on the enzyme at 1 mM: dopamine (3,4-dihydroxyphenethylamine), tyramine, histamine, benzylamine, norepinephrine, taurine, spermine and spermidine.

Choline, acetylcholine (K_i = 2 mM) and a number of their homologues and analogues were also inhibitory to the brain aryl acylamidase (Table 2). However the phospholipid dipalmitoyl phosphatidylcholine at 1 mM was not found to inhibit the enzyme.

Inhibition of Aryl Acylamidase by Acetylcholinesterase Inhibitors

Eserine and neostigmine which are known to be potent inhibitors of acetylcholinesterase were also highly inhibitory to the brain aryl acylamidase. Concentrations of 10 μM of either of these inhibitors caused a 100% inhibition of both enzyme activities. Tetra. isopropylpyrophosphoramide known to be a selective inhibitor of pseudocholinesterase at 10 μM [16] was observed to have no inhibitory effect on either aryl acylamidase or acetylcholinesterase at this concentration (Table 3).

Nature of Inhibition of Aryl Acylamidase and Acetylcholinesterase by the Various Inhibitors

Lineweaver-Burk plots showed that serotonin, tryptamine, acetylcholine, butyrylcholine, eserine and neostigmine were non-competitive inhibitors of brain aryl acylamidase (Fig. 3). The non-competitive nature of inhibition by serotonin and acetylcholine was shown earlier [1–3].

Eserine and neostigmine were found to be competitive inhibitors of the brain acetylcholinesterase (Fig. 4). Serotonin was found to inhibit acetylcholinesterase only at high concentrations, about 50% inhibition at 10 mM concentration of serotonin. Similar in nature was the inhibition of acetylcholinesterase by tryptamine. The 1/[S] against $1/v$ plots for the inhibi-

tion of acetylcholinesterase by serotonin showed a considerable scattering of the experimental points repeatedly and it was uncertain whether the inhibition was a competitive type or a mixed (competitive-non-competitive) type. The reasons for this finding are not obvious but it has been pointed out [17] that the amines spermine and spermidine can interact with and cause a non-enzymic hydrolysis of acetylthiocholine and their inhibition of acetylcholinesterase was more at higher substrate concentrations. Apparently more detailed investigations into the nature of inhibition of the monkey brain acetylcholinesterase by serotonin will be needed.

Semenza and Balthazar [18] have shown that the Dixon plot of one inhibitor is changed by the presence of an additional inhibitor at constant concentration and that when the two inhibitors compete with each other, the Dixon plots obtained in the presence and in the absence of the additional inhibitor are parallel. Semenza-Balthazar plots of aryl acylamidase when inhibited by serotonin together with butyrylcholine, neostigmine, eserine or acetylcholine gave approximately parallel plots (Fig. 5) suggesting that all the five non-competitive inhibitors of brain aryl acylamidase act at the same site on the enzyme.

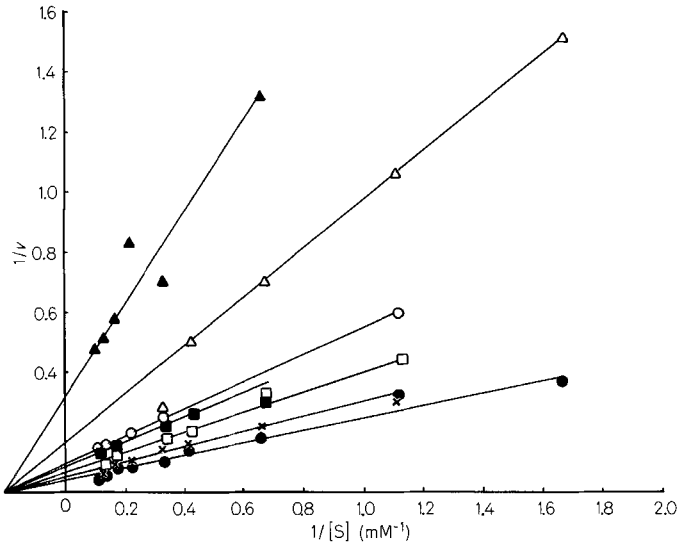

Fig. 3. *Lineweaver-Burk plots of the velocity of aryl acylamidase as a function of the concentration of* o-*nitroacetanilide and with various inhibitors.* In the absence of any inhibitor (●); in the presence of 4 mM acetylcholine iodide (×); 1 mM tryptamine HCl (□); 1 mM butyrylcholine chloride (■); 0.2 mM serotonin creatinine sulphate (○), 40 nM eserine sulphate (△) and 20 nM neostigmine methylsulphate (▲)

Regional Distribution of Aryl Acylamidase and Acetylcholinesterase in the Monkey Brain

A study of the regional distribution of both aryl acylamidase and acetylcholinesterase in the monkey brain indicated a parallel distribution profile of both enzymes in the different regions (Table 4). The region of highest specific activity for both aryl acylamidase and acetylcholinesterase was the basal ganglia followed by the pons.

Monkey Brain Subcellular Distribution of Aryl Acylamidase and Acetylcholinesterase

A study of the intracellular distribution of aryl acylamidase and acetylcholinesterase in the monkey brain showed that both enzymes had a similar distribution profile in the subcellular organelles (Table 5). The maximum specific activity of both enzymes was in the microsomal fraction followed by the small myelin and synaptosomal fractions.

Proteolytic Treatment of Aryl Acylamidase and Acetylcholinesterase

The purified monkey brain enzyme containing acetylcholinesterase and aryl acylamidase activities was subjected to proteolytic digestion with trypsin, α-chymotrypsin or pronase as given under methods. Trypsin and α-chymotrypsin caused a 40% decrease in activities of both enzymes after 60 min of incubation at 37 °C while pronase treatment for 30 min under the conditions described did not cause any loss in either enzyme activity.

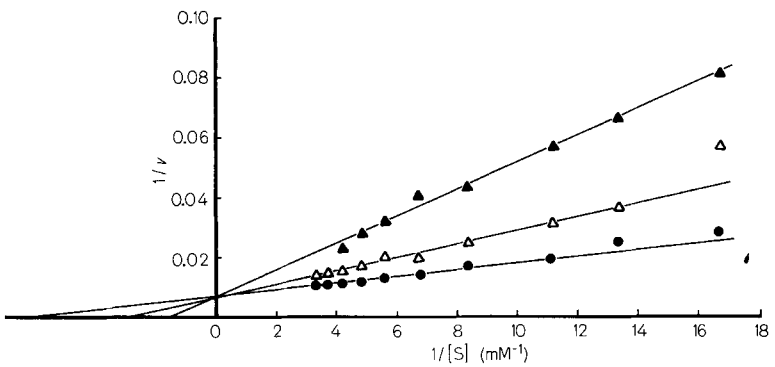

Fig. 4. *Lineweaver-Burk plots of the velocity of acetylcholinesterase as a function of the concentration of acetylthiocholine iodide in the presence and absence of inhibitor.* No inhibitor (●); in the presence of 40 nM eserine sulphate (△); in the presence of 0.3 μM neostigmine methylsulphate (▲)

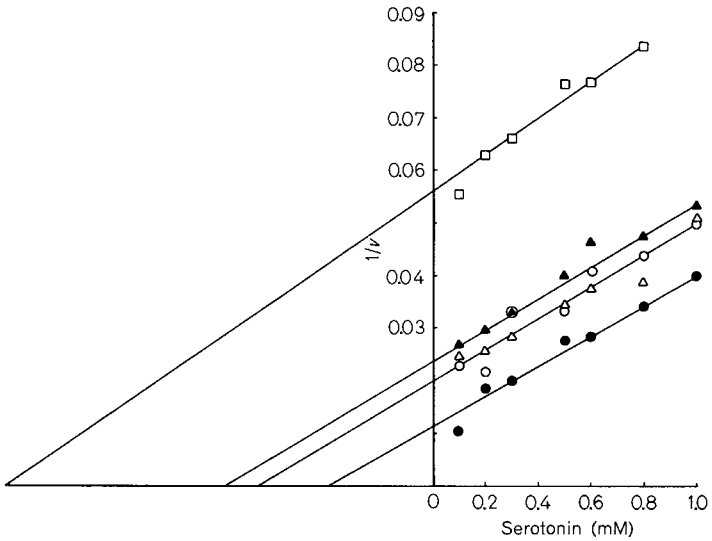

Fig. 5. *Dixon plots of monkey brain aryl acylamidase for inhibitors mutually competing.* Dixon plot of serotonin (●); serotonin with 30 nM neostigmine methylsulphate (△); serotonin with 1 mM butyrylcholine chloride (○); serotonin with 40 nM eserine sulphate (▲); and serotonin with 4 mM acetylcholine iodide (□)

Table 4. *Regional distribution of aryl acylamidase and acetylcholinesterase in the monkey brain*
Values are the mean of two sets of experiments. See Methods for details

Region	Specific activity	
	aryl acylamidase	acetylcholinesterase
	U/mg	
Basal ganglia	0.907	1548.5
Pons	0.128	623.2
Cerebellum	0.094	410.4
Mid-brain	0.084	514.4
Medulla oblangata	0.065	337.6
Temporal cortex	0.039	438.7
Parietal cortex	0.03	174.0
Frontal cortex	0.028	194.8
Corpus callosum	0.025	346.0
Occipital cortex	0.02	138.4

Table 5. *Subcellular distribution of aryl acylamidase and acetylcholinesterase in monkey brain*
See Methods for details

Fraction	Specific activity	
	aryl acylamidase	acetylcholinesterase
	U/mg	
P_1 (Crude nuclear)	0.048	220
P_2C (Mitochondria)	0.033	183
P_2B (Synaptosomes)	0.056	303
P_2A (Small myelin)	0.075	516
P_3 (Microsomes)	0.105	549
S_3 (Cytosol)	0.052	243

Proteolytic treatment and subsequent Sephadex G-200 gel filtration showed that such treatment did not significantly change the elution profile of both the enzyme activities. Similarly, gel electrophoresis after protease treatment did not significantly alter the migration patterns of both the enzyme activities.

DISCUSSION

The specific inhibition of the purified monkey brain aryl acylamidase by serotonin and tryptamine but not other amines confirms the earlier findings [3]. Furthermore the inability of tryptophan or 5-hydroxyindole acetic acid to inhibit indicates that the indole ethyl amine structure is the basic requirement for inhibition. The inability of 5-methoxytryptamine to inhibit suggests that a substitution in the 5-hydroxy group can cause a drastic change in the inhibitory capacity. The inhibition by several of the acetylcholine analogues and homologues also confirms the earlier findings [3]. The choline moiety appears to be essential for this inhibition though interestingly phosphatidylcholine does not inhibit the enzyme.

Our observations of the acetylcholine inhibition of aryl acylamidase coupled with the report of Fujimoto [5] that *Electrophorus electricus* acetylcholinesterase has aryl acylamidase activity prompted us to study the relationship between the two enzymes in the monkey brain. The following lines of evidence suggest a close association of the acetylcholinesterase and aryl acylamidase activities in the monkey brain. Firstly, the ratio of specific activities of both the enzymes at the various steps of purification of the enzyme

is approximately constant. Moreover the recovery of both the enzyme activities at the different steps of the purification procedure is also of the same order. Secondly, the elution profiles of both the enzyme activities from the concanavalin-A – Sepharose column, Sephadex G-200 column and DEAE-Sephadex columns were found to be the same. Thirdly, both enzyme activities co-migrate in gel electrophoresis when run in different percentage gels for different time intervals. Fourthly, potent inhibitors of acetylcholinesterase viz. eserine and neostigmine completely inhibit both aryl acylamidase and acetylcholinesterase of monkey brain at 10 μM. Eserine inhibition at a concentration as low as 10 μM is a distinguishing feature of cholinesterase from non-specific esterases [19]. Fifthly, brain acetylcholinesterase is considered to be a true cholinesterase and in the mouse brain it has been shown to be almost 99% true cholinesterase [20]. When tetraisopropylpyrophosphoramide which selectively inhibits pseudocholinesterase at 10 μM [16] was used there was no inhibition of either acetylcholinesterase activity or aryl acylamidase activity of the monkey brain enzyme. Sixthly, the distribution profile of both aryl acylamidase and acetylcholinesterase activities in the various anatomical regions of monkey brain closely resemble each other. The basal ganglia followed by the pons showed the highest activities. Finally, the subcellular distribution of both enzymes are identical with the highest specific activities in the microsomal fraction.

Apart from these observations the fact that monkey liver aryl acylamidase which is insensitive to serotonin and acetylcholine is also insensitive to eserine and is not found associated with acetylcholinesterase [4] lends further support to the postulation that the brain aryl acylamidase which is sensitive to serotonin is alone associated with acetylcholinesterase. Whether the absence of true cholinesterase in the liver is responsible for the observed differences between the brain and liver is not at present clear. We have observed that aryl acylamidase from several non-neural tissues of the rat such as the liver, spleen, kidney, intestine, lung and heart do not show the serotonin sensitivity but the enzyme from erythrocyte membranes which is known to contain almost 94% true cholinesterase activity [20] shows a significant sensitivity to serotonin, acetylcholine and eserine (55, 52 and 79% inhibition at 0.2 mM, 4 mM and 10 μM concentrations of the inhibitors respectively) and is insensitive to tetraisopropylpyrophosphoramide at concentrations studied for the brain enzyme (unpublished data).

Some of the experimental results indicate that the active sites for the brain aryl acylamidase and acetylcholinesterase reside at different loci in the same protein. Eserine and neostigmine which are competitive inhibitors of monkey brain acetylcholinesterase clearly show a non-competitive inhibition of aryl acylamidase. Added to this is the fact that acetylcholine which is a substrate for acetylcholinesterase acts as a non-competitive inhibitor of aryl acylamidase. Plots according to Semenza and Balthazar suggest that the non-competitive inhibitors of aryl acylamidase viz. butyrylcholine, acetylcholine, neostigmine, eserine and serotonin act at the same site. It is possible that this common site of inhibition could be the active site of acetylcholinesterase. Attempts at separation of the aryl acylamidase and acetylcholinesterase activities by proteolytic treatment and subsequent gel filtration or electrophoresis were unsuccessful because with the three proteases tried both enzymes lost considerable activity during incubation periods beyond 1 h.

Variations in the ratio of specific activities of aryl acylamidase and acetylcholinesterase have been observed among the eluted fractions of the enzyme from the concanavalin-A – Sepharose and Sephadex G-200 columns. Similar variations were also observed among the subcellular fractions and anatomical regions of the monkey brain. Acetylcholinesterase of brain is known to exist in different molecular forms [21 – 23] and it is not clear from the present experiments whether aryl acylamidase activity is associated with each form although the gel electrophoretic pattern would indicate that this may be so. It is also not known whether the same ratio of specific activities between the two enzymes is retained in all the different molecular forms. Moreover there is evidence for separate active sites for the two enzymes and inhibitors like serotonin or tryptamine are shown to affect the two activities to different extents. It is quite likely that endogenous factors present in the enzyme fractions could differentially affect the two activities and alter the ratio of specific activities. A final answer to these questions would depend upon purification of the brain enzyme to homogeneity.

Serotonin sensitive aryl acylamidase activitiy thus appears to be a property of true cholinesterase. The physiological substrate for the serotonin-sensitive brain aryl acylamidase is not known. It is however likely that analgesic drugs which have an acetamido group may serve as substrates for the enzyme. The interesting possibility exists that higher levels of serotonin in the brain may help in the longer survival of such drugs in the brain by inhibiting the aryl acylamidase.

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REFERENCES

1. Fujimoto, D. (1974) *Biochem. Biophys. Res. Commun. 61*, 72 – 74.
2. Paul, S. M., Halaris, A. E., Freedman, D. X. & Hsu, L. L. (1976) *J. Neurochem. 27*, 625 – 627.

3. Oommen, A. & Balasubramanian, A. S. (1977) *Biochem. Pharmacol. 26*, 2163–2167.
4. Oommen, A. & Balasubramanian, A. S. (1978) *Biochem. Pharmacol. 27*, 891–895.
5. Fujimoto, D. (1976) *FEBS Lett. 71*, 121–123.
6. Surolia, A., Prakash, N., Bishayee, S. & Bachhawat, B. K. (1973) *Ind. J. Biochem. Biophys. 10*, 145–148.
7. March, S. C., Parikh, I. & Cuatrecasas, P. (1974) *Anal. Biochem. 60*, 149–152.
8. Cuatrecasas, P. & Parikh, I. (1972) *Biochemistry, 11*, 2291–2299.
9. Davis, B. J. (1964) *Ann. N.Y. Acad. Sci. 121*, 404–427.
10. Weber, K. & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.
11. Eichberg, J., Whittaker, V. P. & Dawson, R. M. C. (1964) *Biochem. J. 92*, 91–100.
12. Hoagland, R. E. & Graf, G. (1971) *Enzymologia, 41*, 313–319.
13. Ellman, G. L., Courtney, K. D., Andres, V. & Featherstone, R. M. (1961) *Biochem. Pharmacol. 7*, 88–95.
14. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
15. Steele, R. W. & Smallman, B. N. (1976) *Biochim. Biophys. Acta, 445*, 147–157.
16. Evans, C. A. & Kerkut, G. A. (1977) *J. Neurochem. 28*, 829–834.
17. Anand, R., Gore, M. G. & Kerkut, G. A. (1976) *J. Neurochem. 27*, 381–385.
18. Semenza, G. & Balthazar, A. K. (1974) *Eur. J. Biochem. 41*, 149–162.
19. Augustinsson, K. B. (1960) in *Enzymes* (Boyer, P. D., Lardy, H. & Myrback, K., eds) vol. 4, p. 522, Academic Press, New York and London.
20. Adamson, E. D. (1977) *J. Neurochem. 28*, 605–615.
21. McIntosh, C. H. S. & Plummer, D. T. (1973) *Biochem. J. 133*, 655–665.
22. Rieger, F. & Vigny, M. (1976) *J. Neurochem. 27*, 121–129.
23. Adamson, E. D., Ayers, S. E., Deussen, Z. A. & Graham, C. F. (1975) *Biochem. J. 147*, 205–214.

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