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SEROTONIN-SENSITIVE ARYL ACYLAMIDASE ACTIVITY OF PLATELET ACETYLCHOLINESTERASE

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Abstract—Serotonin-sensitive aryl acylamidase (AAA, EC 3.5.1.13) was purified to apparent homogeneity from sheep platelets by affinity chromatography and it was shown to be associated with the platelet acetylcholinesterase (AChE, EC 3.1.1.7). The basis for the association of the two enzymes was the following. Both enzyme activities co-eluted from the affinity columns with constant ratios of specific activities and percentage recoveries. Both enzymes co-migrated on gel electrophoresis. Both enzymes co-eluted during Sepharose 6B gel filtration. Potent inhibitors of AChE such as bis(4-allyldimethyl ammoniumphenyl) pentan-3-one dibromide (BW 284C51), neostigmine and eserine also inhibited AAA potently. Both enzymes lost significant activity on treatment with deoxycholate or taurodeoxycholate and the loss could be partly restored by a mixture of phospholipids. The platelet AAA was specifically inhibited by serotonin and to a lesser extent by tryptamine but not by several other amines. It was also inhibited by acetylcholine and several of its analogues and homologues. It is suggested that in the platelets the two enzymes (AAA and AChE) are probably identical.

The concept of platelets being a model for monoaminergic neurons is based on the presence in the platelet of an amine system consisting mainly of serotonin and other amines [1]. Platelets possess an active transport system for serotonin and also have the capacity to store large amounts of this biogenic amine [2]. Almost all the serotonin circulating in the blood is carried by the platelets and it can be secreted from the platelets in response to a variety of stimulants [3]. Little is known, however, concerning the acetylcholine system of platelets. Choline acetyltransferase and AChE have been shown to be present in platelets [4]. But the AChE of platelets has not been purified or its characteristics studied in detail.

We have earlier shown the presence of a serotonin-sensitive AAA associated with AChE in such systems as the brain, basal ganglia, erythrocyte membrane and electric eel [5–7]. The serotonin sensitivity was exhibited by the AAA present in sources where it was found associated with AChE, but not in a tissue like liver where it was not associated with liver cholinesterase [8].

In the present paper we have provided evidence to indicate that the platelet AChE is associated with the serotonin sensitive AAA activity, thereby suggesting the similarity of platelets to other systems containing AChE.

MATERIALS AND METHODS

Sepharose 6B and 4B were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Bis(4-allyl dimethyl ammoniumphenyl) pentane-3-one dibromide (BW 284C51) was a gift from Wellcome Reagents Ltd. (Beckenham, U.K.). The various amines, phospholipids, deoxycholate, taurodeoxycholate, sodium cholate and marker proteins were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were either prepared or obtained as described earlier [5, 6].

Isolation of sheep platelets. All operations after collection of blood were carried out between 0 and 4°. Sheep blood was collected from the slaughter house at the time of killing, in siliconised glass beakers of one litre capacity containing 5 mM Na–EDTA, pH 7.2 (final concentration) as anticoagulant. Platelets were isolated according to Wolfe and Shulman [9] with slight modifications. Briefly, the whole blood was centrifuged at 350 *g* for 10 min. The supernatant was recentrifuged at 2400 *g* for 15 min and the sediment was suspended in the washing buffer described by Baenziger *et al.* [10]. Contaminating erythrocytes and leukocytes were removed by three further centrifugations at 120 *g* for 7 min. The supernatant was then centrifuged at 2400 *g* for 15 min. The sedimented platelets were resuspended in the wash buffer and recentrifuged at 3000 *g* for 15 min. The final sediment of platelets was suspended in 20 mM potassium phosphate buffer, pH 7.0 (protein 30 mg/ml). Microscopic examination revealed that there was only less than 0.1% contamination by erythrocytes in the final preparation.

Affinity chromatographic media. Two different affinity ligands, namely *m*-carboxyphenyl dimethyl ethyl ammonium chloride hydrochloride and *m*-aminophenyl trimethyl ammonium chloride hydrochloride, attached through a six carbon and a thirty carbon spacer arm, respectively, to Sepharose 4B, were used for enzyme purification. The structures of spacer arm and ligand are given in Fig. 1. The two affinity chromatography columns including the synthesis of the ligands and spacer arm were prepared as described earlier [5].

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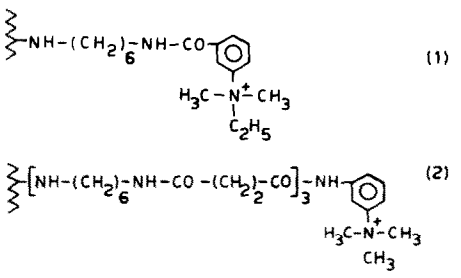

Fig. 1. Structure of the affinity ligands and spacer arms used in affinity chromatography. (1) *m*-Carboxyphenyl dimethyl ethyl ammonium chloride hydrochloride with a six-carbon spacer arm. (2) *m*-Amino phenyl trimethyl ammonium chloride hydrochloride with a thirty-carbon spacer arm.

Solubilization of platelet enzymes and affinity chromatography. Both AAA and AChE of platelets were membrane bound and only insignificant activity could be obtained in the 105,000 *g* supernatant, after homogenization in 20 mM potassium phosphate buffer pH 7.0 in a Potter–Elvehjem homogenizer. Two different solubilization procedures were adopted before affinity chromatography. (a) The platelet suspension in 20 mM phosphate buffer, pH 7.0 was sonicated in an MSE Mullard sonicator at maximum frequency for 10 min (with a 2 min interval after every 2 min of sonication). (b) The platelet suspension in buffer was first homogenized with 1% (v/v) Triton X-100 for 2 min and then sonicated as above. Both these preparations were centrifuged at 105,000 *g* for 1 hr and the supernatant was collected. The pellet was resuspended in the respective buffers and subjected to the sonication procedure, recentrifuged and the supernatant collected and combined with the first supernatant.

The affinity columns 1 and 2 (see Fig. 1) of 4 × 1 cm and 4.3 × 0.9 cm, respectively were equilibrated with 20 mM phosphate buffer/0.1% Triton X-100. The enzymes solubilized by either of the above two methods were loaded at a flow rate of 2–3 ml/hr till some of the enzymes emerged unadsorbed to the column. The columns were washed with the same buffer containing either 0.3 M NaCl (for column 1) or 1 M NaCl (for column 2) until there was no measurable protein in the effluent. Elution was carried out with 20 mM phosphate buffer, pH 7.0/0.1% Triton X-100/0.2 M tetraethyl ammonium bromide. Fractions of 2 ml were collected. Triton X-100 was essential in the elution buffer as without it neither AAA nor AChE was eluted. The fractions were dialysed extensively against 20 mM phosphate buffer, pH 7.0/0.1% Triton X-100 for 24 hr with three changes and assayed. Pooled active fractions were concentrated by aquacide II, redialysed against the same buffer and used for studying the properties or for electrophoresis. The purified enzyme had no detectable monoamine oxidase activity as measured by the method of Green and Haugton [11].

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was done according to Davis [12], using 7% gel in 0.05 M Tris/glycine pH 8.0 at 3 mA per gel for 2 hr. After electrophoresis the gels were either stained by Coomassie Blue for protein or assayed for activity. For enzyme activity, the gels were cut into slices of 1.5 mm thickness, extracted with 20 mM phosphate buffer, pH 7.0/0.5% (v/v) Triton X-100 and assays were carried out with 0.2 ml aliquots of extract. Incubation was done at 37° for 24 hr for AAA and 37° for 15 min for AChE.

Sodium dodecyl sulfate (SDS) gel electrophoresis. SDS gel electrophoresis was done according to Laemmli [13]. The purified enzyme prepared from affinity column 2 was used, either without removal of Triton X-100 or after removal of it. Removal of Triton was done by treating with 90% ethanol and collecting the precipitated protein in 0.1 ml 20 mM phosphate buffer [14]. Samples were treated with 4% SDS for 2 hr, boiled at 100° for 5 min in the presence of 6% 2-mercaptoethanol and subjected to electrophoresis in Tris/glycine buffer pH 8.3 for 2½ hr. Gels were stained with Coomassie Brilliant Blue R250.

Sepharose 6B gel filtration. Platelet suspension in phosphate buffer was sonicated in the absence of Triton X-100 centrifuged at 105,000 *g* for 1 hr and the supernatant collected as described above. The supernatant (6.5 ml) was concentrated by aquacide II to about 2.5 ml and dialysed against 20 mM phosphate buffer, pH 7.0/0.15 M NaCl. This was applied to a Sepharose 6B column (40 × 2.3 cm) pre-equilibrated with the same buffer at a flow rate of 10 ml/hr. Fractions of 2.5 ml were collected.

Enzyme assay and protein estimation. AAA was assayed as described before [7] except that the incubation period was 4 hr at 37°. Under the assay conditions the reaction rate was linear up to a period of 8 hr and up to 3 mg protein of the platelet suspension in the incubation mixture. AChE was assayed according to Ellman *et al.* [15] as described earlier [7]. One unit of AAA was defined as the amount of enzyme required to produce 1 μmole of *o*-nitroaniline in 4 hr and one unit of AChE as the amount which produces a change of 0.1 in absorbance at 412 nm in 1 min under the assay conditions.

Protein was estimated according to Lowry *et al.* [16] using crystalline bovine serum albumin as the standard. Appropriate blanks containing Triton X-100 were taken whenever the protein samples had Triton in them.

Bile salt treatment and effect of added phospholipid mixture. The crude platelet suspension (10 μl for AChE and 100 μl for AAA) was preincubated with sodium salts of either deoxycholate, taurodeoxycholate or cholate at 5, 10 and 20 mM concentrations at 37° for 10 min. Following this, they were assayed for AChE and AAA activities. A control which was pre-incubated without bile salt was run simultaneously. After the termination of the reactions, the reaction mixtures were centrifuged at 10,000 *g* for 5 min to remove any turbidity. To study the effect of phospholipids, a mixture of dipalmitoyl phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine in the ratio of 5:4:2 [17] in distilled water was sonicated for 5 min and 110 μg of this mixture was added to the platelet suspension prior to preincubation with bile salt detergents.

RESULTS

Solubilization and purification of the platelet AAA and AChE

Solubilization of the enzymes from the platelets into 105,000 *g* supernatant was possible by sonication and by the use of the detergent Triton X-100. Use of 1% Triton X-100 alone resulted in 25–30% solubilization of the enzymes, sonication resulted in 37–55% solubilization and a combination of 1% Triton X-100 and sonication gave 70–85% solubilization as evidenced from different experiments. The extent of solubilization was the same for both AAA and AChE in each of these methods.

Figure 2 shows the co-elution profiles of platelet AAA and AChE from the affinity columns and Table 1 gives the purification of AAA and AChE using the two affinity columns. In both chromatographic methods AAA and AChE were purified to similar extent with approximately constant ratios of specific activity and percentage recoveries.

Polyacrylamide gel electrophoresis of the purified enzymes

Figure 3 shows the gel electrophoretic patterns and the profiles of the enzyme activities on the gel slices. Gel electrophoresis of the purified enzyme

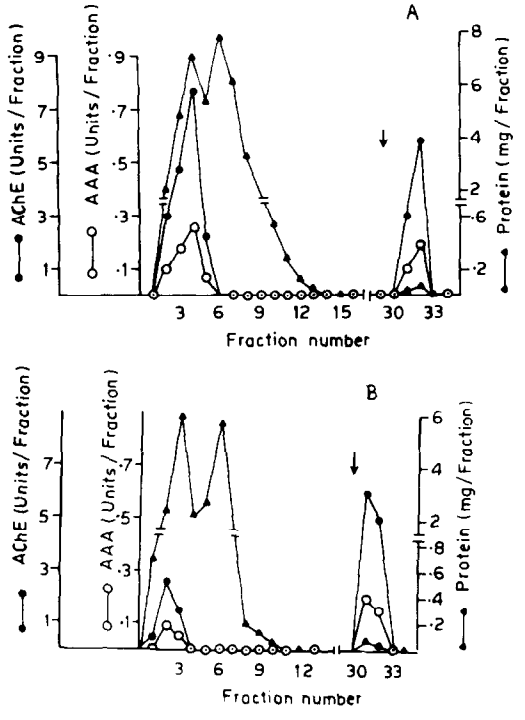

Fig. 2. Affinity chromatographic profiles of aryl acylamidase and acetylcholinesterase solubilised by sonication of platelets. Affinity columns used are given in Fig. 1. Details of chromatography are described under Methods. (A) Profile obtained on column 1. (B) Profile obtained on column 2. Washing buffer was added after the third fraction in A and after the second fraction in B. Arrow indicates commencement of elution with tetraethyl ammonium bromide. (○——○), aryl acylamidase; (●——●), acetylcholinesterase; (▲——▲), protein.

Table 1. Purification of aryl acylamidase (AAA) and acetylcholinesterase (AChE) from platelets by affinity chromatography

Condition of solubilization	Affinity chromatography method number	Specific Activity (units/mg) Before chromatography AAA	Before chromatography AChE	After chromatography AAA	After chromatography AChE	Purification fold AAA	Purification fold AChE	Ratio of activities AChE/AAA Before chromatography	Ratio of activities AChE/AAA After chromatography	Recovery* (%) AAA	Recovery* (%) AChE
Sonicated Without Triton X-100	1	0.021	0.67	2.5	75	118	112	31	30	90	85
	2	0.021	0.67	3.52	110	167	164	31	31	88	93
†Sonicated in presence of 1% (v/v) Triton X-100	1	0.06	1.57	6	145	100	93	26	24	72	75
	2	0.035	1.03	4.57	144	130	139	29	31	93	95

The methods of solubilization and affinity chromatography using chromatographic columns 1 and 2 are described under Materials and Methods. Enzyme units are also given under Materials and Methods.

* Recovery is taken as 100% for the enzyme activity bound to the affinity columns.

† Two different enzyme preparations have been used for affinity chromatography by methods 1 and 2.

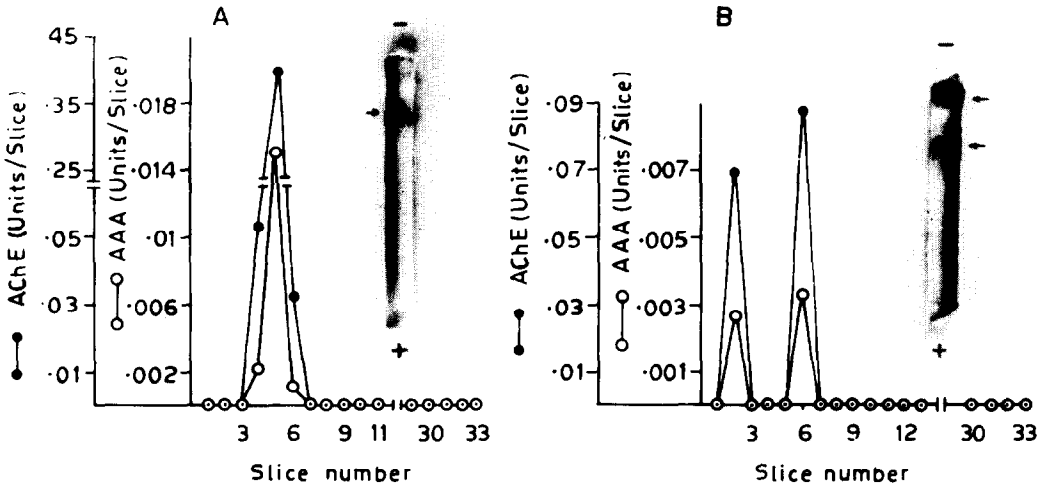

Fig. 3. Polyacrylamide gel electrophoresis of enzymes solubilized by sonication and purified by affinity chromatography and their activity profiles on the gel slices. Gel electrophoresis and assay of enzymes on slices are given under Materials and Methods. (A) Activity profile of purified protein (25 μg) from affinity column 2. Inset shows the protein band on the gel. (B) Activity profile of purified protein (12 μg) from affinity column 1. Inset shows the protein bands on the gel. (○——○), aryl acylamidase; (●——●), acetylcholinesterase; (→), protein band on the gel.

from affinity column 2 gave a single protein band which corresponded to both AAA and AChE activities (Fig. 3A). The enzyme obtained from column 1 gave two protein bands both corresponding to AAA and AChE activities (Fig. 3B). The appearance of two protein bands could be due to aggregation phenomenon which has been observed with AChE prepared from different sources under different conditions [18]. AAA activity always co-migrated with AChE activity during gel electrophoresis. Removal of Triton X-100 from the purified preparations prior to gel electrophoresis, either by using Bio-beads SM-2 [19] or hydroxyapatite chromatography [20] was not possible because in the former case both the enzymes and Triton bound to the Bio-beads SM-2 and in the latter case the enzymes could not be eluted from hydroxyapatite.

SDS gel electrophoresis

SDS gel electrophoresis of the purified enzyme from affinity column 2 showed one major protein band with a trace of a minor protein band just above it, irrespective of the presence or absence of Triton (Triton was removed by ethanol precipitation as described under Methods) in the enzyme (figure not shown). The molecular weight of the protein after removal of Triton was approximately 79,000 and without its removal about 50,000 as indicated by marker proteins. This difference in the molecular weight could be due to the interference of Triton in SDS gel electrophoresis [21]. The molecular weight of 79,000 is similar to the subunit molecular weight of AChE from bovine superior cervical ganglia [18], human erythrocytes [20] and adult rat brain [22].

Table 2. Inhibition of purified aryl acylamidase and acetylcholinesterase by acetylcholinesterase inhibitors

Inhibitors	Concentration (μM)	Inhibition (%) Aryl acylamidase	Acetyl-cholinesterase
Neostigmine	10.0	83	98
	0.1	67	87
	0.01	25	30
Eserine	10.0	90	97
	1.0	68	80
	0.01	23	25
BW 284C51	10.0	90	87
	1.0	72	68
	0.1	28	21
Tetra-isopropyl pyrophosphoramide	100.0	20	10
	10.0	3	0

Values are average of duplicates which did not differ from each other by more than 5%.

Table 3. Inhibition of purified platelet aryl acylamidase by amines and choline derivatives

Inhibitors	Concentration (mM)	Inhibition (%)
Amines		
Serotonin	5.0	92
	1.0	86
	0.5	60
Tryptamine	0.5	40
Choline derivatives		
Acetylcholine	5.0	61
	1.0	22
Succinylcholine	5.0	75
Butyrylcholine	5.0	81
Propionylcholine	5.0	69
Acetyl β-methylcholine	5.0	60
Choline chloride	5.0	59
Benzoylcholine	5.0	47

Values are average of duplicates which did not differ from each other by more than 5%.

Inhibition by AChE inhibitors

The purified AAA was potently inhibited by AChE inhibitors (Table 2). Eserine and neostigmine which are known to be potent inhibitors of AChE were also highly inhibitory to the platelet AAA. Neostigmine and eserine at 10 μM inhibited more than 95% AChE and 80% of the AAA activity. BW 284C51, a selective inhibitor of AChE gave about 90% inhibition of the enzyme activities at 10 μM, whereas tetra-isopropyl pyrophosphoramide, a selective inhibitor of pseudocholinesterase gave less than 5% inhibition of both AAA and AChE at a concentration of 10 μM.

Inhibition of AAA by various amines and choline derivatives

In Table 3 is shown the percentage inhibition of purified platelet AAA by different amines and choline derivatives. Among the amines, serotonin and tryptamine at 0.5 mM were found to inhibit to the extent of 60 and 40%, respectively. The inhibition by serotonin was found to be non-competitive in nature as observed from Lineweaver–Burk plots. Except for tyramine, which gave about 10% inhibition, all the following monoamines or polyamines such as dopamine, 5-methoxytryptamine, benzylamine, histamine, spermine and spermidine at 0.5 mM did not give any significant inhibition of AAA activity. Choline, acetylcholine and a number of its homologues and analogues were also inhibitory to the platelet AAA as shown in Table 3. Butyrylcholine gave the maximum inhibition of 81% at 5 mM. These inhibition characteristics are similar to those observed with AAA from brain and erythrocytes [5, 7].

Sepharose 6B gel filtration

Membrane bound AChE from different sources

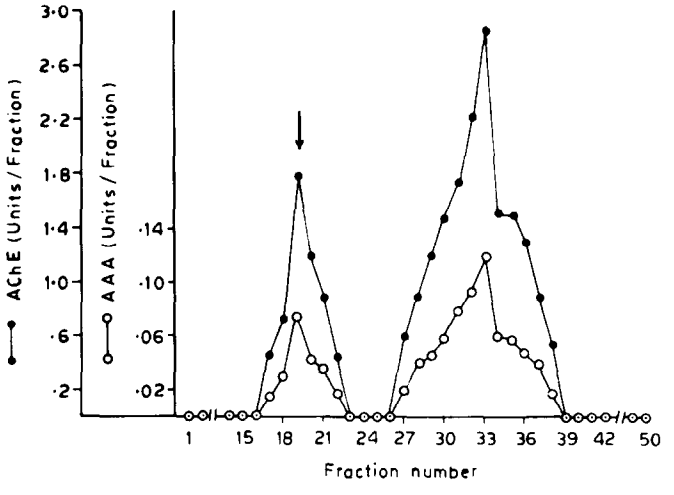

Fig. 4. Sepharose 6B gel filtration profile of aryl acylamidase (○——○) and acetylcholinesterase (●——●) solubilized from platelet suspension by sonication. Details of gel filtration are given under Methods. Arrow indicates the position of the first peak of blue dextran.

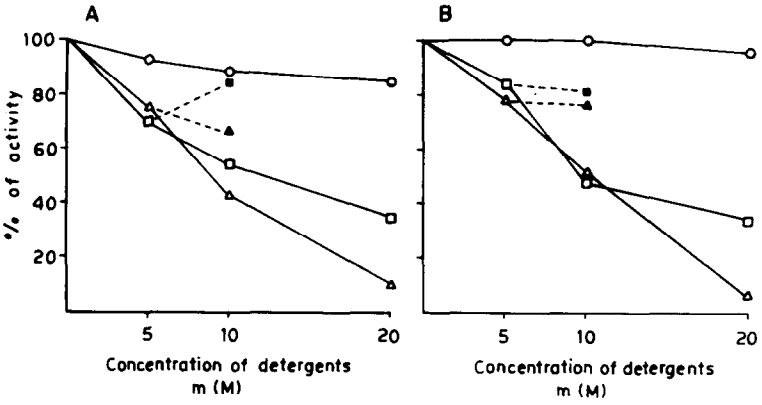

Fig. 5. Effect of bile salt detergents and phospholipid mixture on platelet aryl acylamidase (A) and acetylcholinesterase (B). Platelet suspension was treated with 5, 10 and 20 mM sodium salts of cholate (○——○), deoxycholate (△——△) and taurodeoxycholate (□——□) as described in Materials and Methods. Activity without treatment of bile salts is taken as 100%. Results are the average of duplicate experiments. The dotted lines indicate the enzyme activity when treated with deoxycholate (▲) or taurodeoxycholate (■) at 10 mM in the presence of a mixture of phospholipids (110 μg) as given under Materials and Methods.

are reported to exist in multiple molecular forms and this multiplicity might also depend on the methods of preparation [20, 23, 24]. The Sepharose 6B gel filtration profile of sonicated solubilized platelet enzymes is shown in Fig. 4. The elution profile shows two peaks of AAA and AChE activities, the proportion of peak II being higher than peak I. Both AAA and AChE were co-eluted from the column. The ratios of activities were maintained approximately constant and the recovery of both the enzymes from the column was about 70%. Both the peaks of AAA were inhibited by approximately 60 and 65% by 0.5 mM serotonin and 5 mM acetylcholine, respectively.

Effect of bile salts and phospholipid mixture on enzyme activities

The integrity of the lipid environment for the catalytic activity of AChE is known. The loss of AChE activity by treatment with deoxycholate and the restoration of activity by added phospholipid has been reported [25, 26]. We studied the effect of Na-salts of deoxycholate, taurodeoxycholate and cholate on the platelet AAA and AChE activities. Figure 5 shows that AChE activity was reduced to 53 and 48% and AAA activity was reduced to 44 and 56% in the presence of 10 mM deoxycholate and taurodeoxycholate, respectively, whereas cholate did not exert any effect on the enzymes. The addition of a mixture of phospholipids as given under Methods caused a reversal of the inhibition to approximately similar extent for both enzymes (Fig. 5). Addition of phosphatidylcholine alone at the same concentration did not show any significant restoration of activities of both the enzymes.

DISCUSSION

The present study shows that the platelets contain an AAA which is specifically sensitive to serotonin and which is associated with AChE. The evidences for the association and possible identity of the AAA with AChE are the following. (a) Both enzymes co-purify during two different affinity chromatographic procedures with approximately constant ratios of specific activity and percentage recoveries. (b) Both the enzymes show similar elution profiles during affinity chromatographic or gel filtration procedures. In the latter procedure two coincident peaks for both the enzyme activities were observed. (c) Both enzymes co-migrate on gel electrophoresis either as single or multiple species. (d) Both the enzyme activities are potently inhibited by typical AChE inhibitors such as eserine, neostigmine or BW 284C51. They are relatively unaffected by the selective pseudocholinesterase inhibitor tetra-isopropyl pyrophosphoramide. (e) Deoxycholate and taurodeoxycholate significantly reduce both the enzyme activities and the loss could be partly restored for both the enzyme activities by the addition of phospholipid mixture.

The platelet AAA and AChE are tightly membrane bound. The decrease in activities of the enzymes in the presence of deoxycholate may be due to the activation of phospholipases [17] which may act upon the phospholipids associated with the enzymes or which may change the conformation of the phospholipid part which modulates the catalytic site of the enzyme [27].

Platelet cholinesterase appears to be a true acetylcholinesterase for the following reasons. BW 284C51, a selective inhibitor of AChE is a potent inhibitor of the enzyme, while tetra-isopropyl pyrophosphoramide, a selective inhibitor of pseudocholinesterase has relatively no effect. In the purified enzymes, there was no detectable activity when butyrylthiocholine was used as substrate and in the crude platelet suspension butyrylthiocholine gave an activity 16 times lower than that with acetylthiocholine.

The collective results suggest that the platelet AAA is probably identical with the platelet AChE. The physiological substrate for the serotonin sensitive AAA and the significance of its association with AChE remain to be elucidated [28–30]. Since the platelets avidly take up serotonin and store it, it is possible that the platelet AAA may remain inactive under such conditions, but may be reactivated upon secretion or depletion of the serotonin from the platelets under the influence of a variety of stimulants. This could also serve as a control mechanism for the preservation or degradation of an *N*-acetyl compound susceptible to AAA action in platelets.

The demonstration in platelets of the presence of a serotonin sensitive AAA associated with AChE indicates the similarity of platelets to other AChE containing systems including the nervous tissue [5]. The present study also suggests that the association of the two enzymes may be a general phenomenon found in many sources containing AChE.

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