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# The Aryl Acylamidases and Their Relationship to Cholinesterases in Human Serum, Erythrocyte and Liver

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Human serum aryl acylamidase associated with serum cholinesterase was purified to homogeneity. Evidence for the identity of the two enzymes was based on co-elution profiles, co-purification in the different steps including affinity chromatography with constant ratios of specific activity and percentage recoveries, co-migration on gel electrophoresis, parallel inhibition by typical cholinesterase inhibitors and co-precipitation by antibody raised against the purified enzyme. Human liver aryl acylamidase was partially purified. Based on the elution profiles, purification data, inhibitory characteristics and gel electrophoresis it was concluded that aryl acrylamidase of liver was not associated with liver cholinesterase. More conclusive evidence for the non-association of the liver aryl acylamidase and cholinesterase came from their clear-cut separation on procainamide-Sepharose affinity chromatography. Both the serum and liver aryl acylamidase were compared with the purified erythrocyte aryl acylamidase associated with acetylcholinesterase. While the erythrocyte and serum aryl acylamidases showed some similarities in their sensitivities to amines like serotonin or tryptamine and choline derivatives, the liver enzyme was unaffected by any of these compounds. A notable observation was the activation by tyramine of the serum aryl acylamidase but not the erythrocyte and liver aryl acylamidases. The liver aryl acylamidase also differed from the other two in its relative insensitivity to inhibition by eserine, neostygmine and other cholinesterase inhibitors. Immunodiffusion and immunoprecipitation studies showed that the aryl acylamidases from the liver and erythrocytes were immunologically non-identical with the serum enzyme.

The existence of acetylcholinesterases (true cholinesterases) and pseudocholinesterases (non-specific cholinesterases or cholinesterases) has been known for several years. Their distinction has been mainly based on their substrate specificities and inhibitor sensitivities [1–4]. Acetylcholine and acetyl-$\beta$-methylcholine are preferred substrates of acetylcholinesterase [1] and the compound bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide (BW284C51) is a selective inhibitor of acetylcholinesterase [5]. Similarly butyrylcholine and benzoylcholine are considered to be preferred substrates of pseudocholinesterase and the compounds tetraisopropylpyrophosphoramide and ethopropazine are selective inhibitors of pseudocholinesterase [1, 5, 6]. These two classes of cholinesterase also differ in their occurrence. Caudate nucleus of brain, erythrocyte membrane and electric eel are known sources of acetylcholinesterase and serum is a well known source of pseudocholinesterase [7–9]. The active site of both acetylcholinesterase and pseudocholinesterase is known to possess an esteratic and an anionic site [10, 11] but acetylcholinesterase may possess additional anionic centers [11]. Koelle et al. [12–14] suggested the possibility of pseudocholinesterase acting as a post-translational precursor of acetylcholinesterase in the cat superior cervical ganglion. However in the rat superior cervical ganglion this could not be confirmed [5, 15].

Evidence from this laboratory and from other workers [16–19] suggest that an aryl acylamidase activity specifically sensitive to serotonin is associated with acetylcholinesterase of different sources such as electric eel, brain and erythrocytes. An aryl acylamidase of liver which is insensitive to serotonin is also known [20, 21]. It was therefore of interest to know whether aryl acylamidases in general are associated with cholinesterases of different sources and whether any differences in the amine sensitivities of the aryl acylamidases exist. Such a study may lead to methods of differentiating the cholinesterases on the basis of the amine sensitivities of the aryl acylamidase with which they are associated. In the present work we have purified the aryl acylamidase of human serum and shown that it is associated with the serum cholinesterase. We have also compared the properties and amine sensitivity of the human serum aryl acylamidase with the human erythrocyte aryl acylamidase associated with acetylcholinesterase and with the human liver aryl acylamidase.

*Enzymes.* Aryl acylamidase or aryl-acylamide amidohydrolase (EC 3.5.1.13); acetylcholinesterase or acetylcholine acetylhydrolase or true cholinesterase (EC 3.1.1.7); cholinesterase or acylcholine acylhydrolase or pseudocholinesterase (EC 3.1.1.8).

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

## MATERIALS AND METHODS

### *Materials*

Procainamide was obtained from Sarabhai M. Chemicals (India), and anti-(rabbit IgG) was from Miles Laboratories, USA. All other chemicals were from Sigma Chemicals Co., USA or from sources mentioned earlier [18, 19].

Human erythrocytes and plasma were obtained from the blood bank of the hospital.

### *Purification of Serum Aryl Acylamidase*

*Step. 1. Preparation of Serum.* 200 ml of citrated human plasma irrespective of blood groups stored at 4 °C for 5–7 days

was used as the starting material. The plasma was clotted by the addition of $CaCl_2$ to a final concentration of 2 mM. After keeping for 5 h at 4 °C the fibrin clot was removed by allowing the serum to filter through a nylon cloth.

*Step 2. DEAE-cellulose Chromatography.* DEAE-cellulose (Whatman DE 32) was packed in a column (3.5 cm × 40 cm) and equilibrated with 20 mM sodium acetate buffer pH 4.1 containing 1 mM EDTA and 1 mM 2-mercaptoethanol. The serum after dialysis against 12 l of equilibrating buffer was centrifuged to remove the precipitated proteins. The clear serum was applied on the DEAE-cellulose column and washed with 6 l of the equilibrating buffer. The enzyme was eluted by applying a 0 – 0.2 M NaCl gradient (total vol. 1.5 l) in the equilibrating buffer. The flow rate was maintained at 40 ml/h and fractions of 25 ml were collected. The active fractions (fractions 26 – 36) were pooled.

*Step 3. Concanavalin-A – Sepharose Chromatography.* Concanavalin A was coupled to Sepharose 4B according to the method of Cuatrecasas [22, 23]. The gel (containing 7 mg concanavalin A/ml) was packed in a column (1.7 cm × 6.5 cm) and equilibrated with 20 mM sodium acetate buffer pH 5.0. The pooled fractions from DEAE-cellulose were dialysed against 12 l of the same buffer and applied on the concanavalin-A – Sepharose column. After washing with 0.5 M NaCl in 20 mM acetate buffer pH 5.0, the enzyme was eluted at 25 °C with 0.05 M methyl α-glucoside in the wash buffer. The flow rate was maintained at 10 ml/h and fractions of 10 ml were collected. The active fractions (fractions 1 – 7) were pooled.

*Step 4. Procainamide-Sepharose Affinity Chromatography.* The method was essentially the same as that used by Lockridge and La Du [24] for the purification of serum cholinesterase. However the length of the spacer arm was increased from six to ten carbon atoms to enhance the efficiency of binding of the enzyme to the ligand. The preparation of the affinity media was done according to the method of Cuatrecasas [22, 23]. The spacer arm was synthesised by the successive addition of hexanediamine and succinic anhydride to cyanogen-bromide-activated. Sepharose 4B. Finally 10 μmol procainamide/ml gel was coupled to the carboxyl end of the spacer arm using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride [19]. The affinity gel was packed in a column (1.4 cm × 10 cm) and equilibrated with 20 mM potassium phosphate buffer pH 7.0. The pooled concanavalin-A – Sepharose eluate was dialysed against 2 l of the equilibration buffer and applied on the column. The column was washed with 125 ml of 20 mM potassium phosphate buffer pH 7.0 containing 0.1 M NaCl. The enzyme was eluted by applying a linear gradient of 0.1 – 0.6 M NaCl (total volume of 100 ml) in the equilibration buffer at a flow rate of 15 ml/h. Fractions of 5 ml were collected. Active fractions were pooled and dialysed against 20 mM potassium phosphate buffer pH 7.0.

### *Purification of Human Erythrocyte Aryl Acylamidase*

The purification of human erythrocyte aryl acylamidase was done as described earlier [19] using *m*-aminophenyl-trimethylammonium chloride-hydrochloride – Sepharose 4B affinity chromatography.

### *Purification of Human Liver Aryl Acylamidase*

*Step 1. Extraction of the Enzyme.* 10 g of frozen human liver of a 55-year-old male was minced and homogenized in 80 ml of 20 mM potassium phosphate buffer pH 7.0 containing 0.5% (v/v) Triton X-100 in a Waring blendor at maximum speed. The homogenate was centrifuged at 55000 × *g* for 1 h and the supernatant collected.

*Step 2. Ammonium Sulfate Precipitation.* The supernatant was subjected to a 33 – 60% $(NH_4)_2SO_4$ fractionation and the precipitated protein centrifuged down at 12000 × *g* for 30 min. The pellet was dissolved in 3 ml of 20 mM potassium phosphate buffer pH 7.0 and dialysed against 1.5 l of the same buffer.

*Step 3. Concanavalin-A – Sepharose Chromatography.* Concanavalin- – Sepharose was packed in a column (2.2 cm × 17.5 cm) and equilibrated with 20 mM potassium phosphate buffer pH 7.0. The dialysed $(NH_4)_2SO_4$ suspension was passed through the column and the column washed with 300 ml of equilibration buffer containing 0.5 M NaCl. The enzyme was eluted at 25 °C with the equilibration buffer containing 0.5 M methyl α-glucoside and 0.5 M NaCl. The active fractions (fraction numbers 1 – 9) of 5 ml each were pooled and dialysed against 3 l of equilibration buffer.

*Step 4. Sepharose 6B Gel Filtration.* The dialysed concanavalin-A – Sepharose eluate was concentrated by lyophilization, dialysed against 20 mM potassium phosphate buffer pH 7.0 containing 0.15 M NaCl and applied on a Sepharose 6B column (2.2 cm × 47 cm) previously equilibrated with the same buffer. The enzyme was eluted by the same buffer at a flow rate of 15 ml/h and fractions of 5 ml were collected. Active fractions (fractions 14 – 20) were pooled.

The purified liver enzyme was free of any detectable monoamine oxidase activity.

### *Inhibition or Activation Studies*

The purified enzymes were dialysed against 20 mM potassium phosphate buffer pH 7.0 and used for the above studies. The inhibitors or activators were added to the assay blanks to avoid any interference by them. Cholinesterase inhibition studies were performed by preincubating the enzymes with the inhibitor for 10 min at 37 °C before assay.

### *Gel Electrophoresis*

Polyacrylamide gel electrophoresis was done according to the method of Davis [25]. The samples concentrated by aquacide about tenfold and dialysed against 0.05 M Tris/HCl buffer pH 7.0 were subjected to electrophoresis at 4 °C on 7% gels at 3 mA per tube in 0.05 M Tris/glycine buffer pH 8.0 till the tracking dye bromophenol blue emerged. The gels were fixed in 12.5% trichloroacetic acid and stained with 0.05% Coomassie brilliant blue. For estimating the enzyme activities in the gel, the gels were sliced to 1.5-mm thickness, the enzyme extracted into 0.5 ml 20 mM potassium phosphate buffer pH 7.0 containing 0.5% Triton X-100 for 48 h and the activity in the extract was estimated.

### *Sodium Dodecyl Sulfate Gel Electrophoresis*

The method of Laemmli [26] in a Tris/glycine system was employed for sodium dodecyl sulfate gel electrophoresis. The gels were cast in 0.1% sodium dodecyl sulfate. Samples were boiled for 2 min in 2% sodium dodecyl sulfate containing 5% 2-mercaptoethanol and 20 μg of sample protein was applied on gel. Electrophoresis was carried out at a constant current of 3 mA per tube at room temperature. Proteins were stained as described by Laemmli [26].

*Preparation of Antibody*

*Step 1.* Antibody against serum aryl acylamidase was raised by injecting intradermally into a young rabbit 300 μg of purified aryl acylamidase in 1 ml water mixed with 1 ml of Freund's complete adjuvant. Booster doses of 200 μg and 150 μg of the purified enzyme were injected on the 22nd and 25th day respectively. A week later the rabbit was bled through the marginal vein of the ear.

*Step 2. $(NH_4)_2SO_4$ Precipitation.* The clotted blood was centrifuged and the serum collected. The serum was saturated with $(NH_4)_2SO_4$ to 52% and the precipitated proteins centrifuged down at 12000 × *g* for 30 min. The pellet was suspended in 20 mM potassium phosphate buffer pH 6.8 and dialysed against 3 l of the same buffer.

*Step 3. DEAE-cellulose Chromatography.* Precycled DEAE-cellulose (DE32) was packed in a column (1.8 cm × 18.5 cm) and equilibrated with 20 mM potassium phosphate buffer pH 6.8 and the $(NH_4)_2SO_4$ suspension was applied to the column. The column was washed with the same buffer and the washings were collected and dialysed against water and lyophilized. 5 mg lyophilized antibody was dissolved in 1 ml of phosphate-buffered saline pH 7.0. The purified antibody was free of any detectable aryl acylamidase or cholinesterase activities.

*Immunodiffusion Studies*

Double immunodiffusion experiments were done on 1% agarose gel made in phosphate-buffered saline pH 7.0 containing 0.02% sodium azide as described by Ouchterlony [27].

*Immunoprecipitation Studies*

To 0.15 ml of the purified serum, erythrocyte or liver enzyme preparations, 0.15 ml of the reconstituted antibody was added and kept at 4 °C for 48 h and centrifuged at 3500 × *g* for 30 min. The supernatant was separated and estimated for both aryl acylamidase and cholinesterase activities. Controls consisted of 0.15 ml of each enzyme and 0.15 ml of phosphate-buffered saline pH 7.0.

*Enzyme Assays*

Aryl acylamidase was assayed as described earlier [19]. Serum aryl acylamidase was assayed at pH 8.0, erythrocyte at pH 7.0 and liver at pH 6.5 based on their pH optima profile. Cholinesterase was assayed according to Ellman et al. [28] as described earlier [18]. Cholinesterase activities of all the three sources were estimated at pH 8.0. Serum cholinesterase was assayed using butyrylthiocholine as substrate, erythrocyte cholinesterase using acetylthiocholine and liver cholinesterase using both acetyl thiocholine and butyrylthiocholine as substrates. 1 unit of aryl acylamidase corresponds to 1 μmole *o*-nitroacetanilide hydrolysed/h and 1 unit of cholinesterase to a change in absorbance of 1/min at 412 nm under standard assay conditions. Monoamine oxidase was assayed according to the method of Green and Haughton [29] with tyramine hydrochloride as substrate.

*Protein Estimations*

Protein concentration were determined according to the method of Lowry et al. [30] using crystalline bovine serum albumin as standard. Protein in column fractions was monitored by measuring absorbance at 280 nm.

All purification procedure were carried out at 4 °C unless otherwise mentioned.

## RESULTS

*Purification of Human Serum Aryl Acylamidase*

Fresh serum aryl acylamidase and cholinesterase were completely bound to the DEAE-cellulose, but with aged serum samples a small amount (about 12%) of both aryl acylamidase and cholinesterase activities did not bind to the DEAE-cellulose. The binding of the enzyme to concanavalin-A–Sepharose was minimal at pH 7.0 and complete at pH 5.0. A ten-carbon spacer arm was found superior to a six-carbon spacer arm for binding the enzyme to the procainamide-Sepharose column. Table 1 summarises the scheme of a typical purification of serum aryl acylamidase and cholinesterase. A final purification of 13850-fold was achieved for aryl acylamidase and 13620-fold for cholinesterase with about 44% recovery for both the enzymes.

In all the steps of purification, both aryl acylamidase and cholinesterase activities co-purified with the same ratio of activities and percentage recoveries (Table 1). The elution profiles in all the column chromatographic procedures were also identical for the two enzymes (Fig. 1).

Table 1. *Purification of human serum aryl acylamidase and cholinesterase*
Enzyme units are defined in Materials and Methods. AAA = aryl acylamidase; ChE = serum cholinesterase

| Purification step | Volume | Total activity | | Protein | Specific activity | | Ratio of activities | Purification | | Recovery | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | AAA | ChE | | AAA | ChE | ChE/AAA | AAA | ChE | AAA | ChE |
| | ml | units | | mg | units/mg protein | | | -fold | | % | |
| Plasma | 200 | 100 | 15200 | 15800 | 0.00632 | 0.962 | 152 | – | – | 100 | 100 |
| Serum pH 4.0 | 214 | 83.5 | 12550 | 7810 | 0.0071 | 1.607 | 150.4 | 1.17 | 1.11 | 83.4 | 82.6 |
| DEAE-cellulose | 275 | 64.3 | 9590 | 15.3 | 4.196 | 627 | 149 | 664 | 652 | 64.3 | 63.1 |
| Concanavalin-A–Sepharose | 20 | 52.8 | 7790 | 3.5 | 15.12 | 2223 | 147.3 | 2400 | 2312 | 52.7 | 51.2 |
| Procainamide affinity chromatography | 52 | 43.9 | 6760 | 0.52 | 84.4 | 13000 | 154 | 13850 | 13620 | 43.9 | 44.4 |

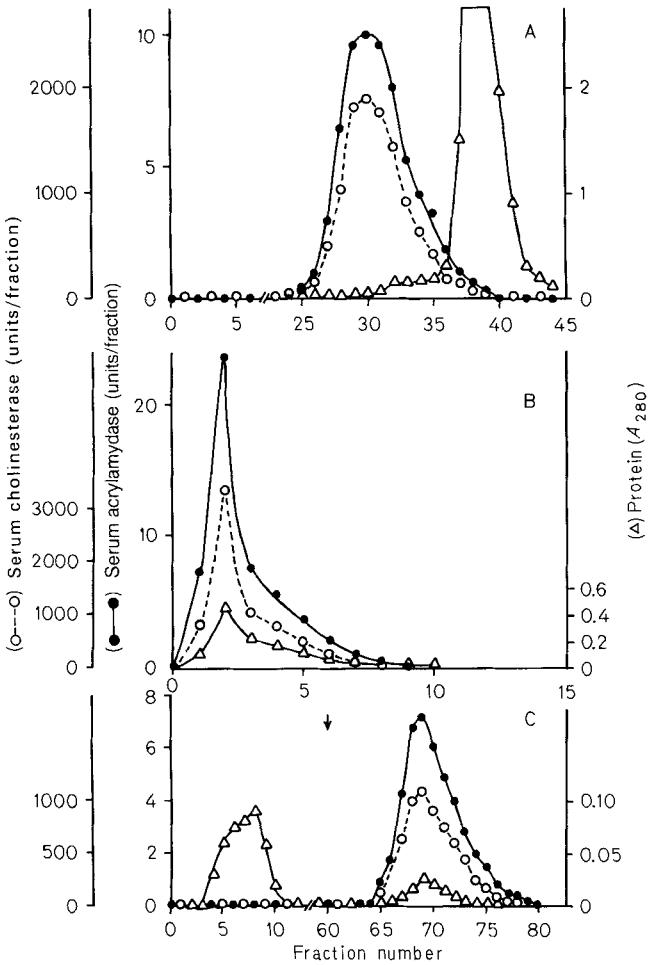


Fig. 1. *Elution profiles of serum aryl acylamidase and cholinesterase.* Chromatographic procedures are described under Materials and Methods. (A) DEAE-cellulose chromatography, (B) concanavalin-A – Sepharose chromatography and (C) procainamide-Sepharose affinity chromatography. The arrow indicates commencement of elution with NaCl gradient. (●—●) Aryl acylamidase; (○ – –○) cholinesterase; (Δ—Δ) absorbance at 280 nm

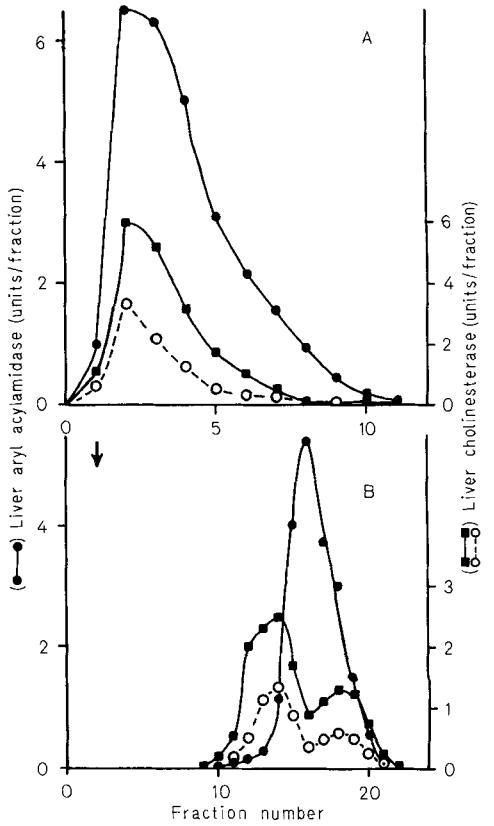


Fig. 2. *Elution profiles of liver aryl acylamidase and cholinesterase.* Experimental details are given under Materials and Methods. (A) Concanavalin-A – Sepharose chromatography; (B) Sepharose 6B gel filtration. The arrow indicates the void volume. (●—●) Aryl acylamidase; (■—■) cholinesterase activity with butyrylthiocholine as substrate; (○ – –○) cholinesterase activity with acetylthiocholine as substrate

### *Purification of Erythrocyte Aryl Acylamidase*

Details of the purification are given elsewhere [19]. Several lines of evidence indicating the identity of the erythrocyte membrane aryl acylamidase with acetylcholinesterase has been reported by us earlier [19].

### *Purification of Human Liver Aryl Acylamidase*

Table 2 summarises the purification of human liver aryl acylamidase and cholinesterase. The latter activity was measured using both acetylthiocholine and butyrylthiocholine as substrates. Although in the crude homogenate the cholinesterase activity measured using acetylthiocholine was slightly higher than that with butyrylthiocholine, in the final preparation the activity with butyrylthiocholine was double that obtained with acetylthiocholine. This may be due to the progressive removal of the acetylcholinesterase component of the liver [31] during the initial steps of purification. In the final step of purification the aryl acylamidase was purified about 43-fold with 35% recovery while the cholinesterase activity was purified only about 5 – 11-fold with a recovery of 4 – 9% (Table 2). The ratio of cholinesterase to aryl acylamidase varied from 1.77 – 1.56 in the initial step to 0.2 – 0.39 in the final step. Although both cholinesterase and aryl acylamidase activities of liver showed a similar pattern of elution in the concanavalin-A – Sepharose chromatography (Fig. 2A) their elution pattern on Sepharose 6B showed a clear difference (Fig. 2B).

### *Alternative Chromatographic Methods Tried for Liver and Serum Enzymes*

Attempts were made to purify the liver aryl acylamidase by methods similar to those used for the serum enzyme. DEAE-cellulose chromatography was not suitable because although the liver aryl acylamidase from the 55000 × *g* supernatant bound to the column at pH 7.0, no significant activity could be eluted with NaCl alone in the buffer. When 0.1% (v/v) Triton X-100 was included in the eluting buffer, aryl acylamidase activity was eluted but it was distributed in a large number of fractions.

Procainamide-Sepharose affinity chromatography was tried for the liver aryl acylamidase using either the fraction obtained after concanavalin-A – Sepharose chromatography or the 55000 × *g* supernatant. With the former it was found that about 99% of the aryl acylamidase along with the major protein content came out without binding to the column and almost all the cholinesterase activity (using both acetylthiocholine and butyrylthiocholine as substrate) in the fractions eluted from the affinity column. Although the

Table 2. *Purification of aryl acylamidase from human liver*
Enzyme units are defined in Materials and Methods. AAA = aryl acylamidase; AChE = Cholinesterase activity using acetylthiocholine as substrate; BuChE = cholinesterase using butyryl thiocholine as substrate

| Purification step | Volume | Total activity | | | Protein | Specific activity | | | Ratio of activities | | | Purification | | Recovery | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | AAA | AChE | BuChE | | AAA | AChE | BuChE | AChE/AAA | BuChE/AAA | AAA | AChE | BuChE | AAA | AChE | BuChE |
| | ml | units | | | mg | units/mg protein | | | | | | -fold | | % | | |
| Homogenate | 84 | 58.2 | 102.8 | 90.8 | 907 | 0.064 | 0.113 | 0.1 | 1.77 | 1.56 | – | – | – | 100 | 100 | 100 |
| 55000 × *g* Supernatant | 78 | 39 | 58.7 | 57.2 | 749 | 0.052 | 0.078 | 0.076 | 1.51 | 1.47 | – | – | – | 67 | 63 | 57 |
| $(NH_4)_2SO_4$ precipitation | 41 | 30.7 | 17.6 | 23.1 | 318 | 0.097 | 0.055 | 0.072 | 0.57 | 0.75 | 1.5 | 0.49 | 0.72 | 53 | 17 | 25 |
| Concanavalin-A – Sepharose | 44 | 24 | 8.9 | 15.7 | 21 | 1.13 | 0.42 | 0.74 | 0.37 | 0.65 | 17.7 | 3.7 | 7.4 | 45 | 8.6 | 17 |
| Gel filtration | 30 | 20.6 | 4.16 | 8.0 | 7.4 | 2.78 | 0.56 | 1.08 | 0.2 | 0.39 | 43.4 | 5.0 | 10.8 | 35 | 4 | 8.8 |

procainamide-Sepharose chromatography could not be used for purifying the liver aryl acylamidase, it gave a clear-cut separation of the liver aryl acylamidase from liver cholinesterase indicating the non-identity of the two enzymes. When the 55000 × *g* supernatant was used, a similar profile was obtained except about 15% of cholinesterase activity (using acetylthiocholine but not butyrylthiocholine as substrate) was detectable in the fractions not bound to the column. This may represent a small amount of acetylcholinesterase present in the crude 55000 × *g* supernatant of the liver [31].

Crude serum after subjecting to a procainamide-Sepharose chromatography (where almost 99% of both aryl acylamidase and cholinesterase activity was bound to the column and recovered in elution) was applied on a Sepharose 6B column of dimensions Similar to that used for the purification of liver aryl acylamidase (see Fig. 2B). It was found that both aryl acylamidase and cholinesterase activities co-eluted and the activity was distributed over fractions 10 – 15 with a single sharp peak of activity in fraction 12 (see Fig. 2B). This indicated a higher molecular weight for the serum aryl acylamidase compared to the liver aryl acylamidase.

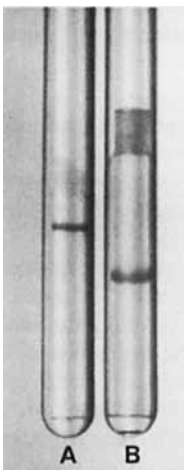


Fig. 3. *(A) Polyacrylamide gel electrophoresis of the purified serum aryl acylamidase; (B) sodium dodecyl sulfate gel electrophoresis of the purified serum aryl acylamidase.* Migration was from top to bottom towards anode. Details are given under Materials and Methods

*Polyacrylamide Gel Electrophoresis*

Purified serum aryl acylamidase showed a single protein band on polyacrylamide gel electrophoresis (Fig. 3A). The same enzyme on sodium dodecyl sulfate gel electrophoresis showed a single protein band (Fig. 3B) and its molecular weight as calculated from marker proteins (immunoglobulin G, bovine serum albumin, ovalbumin and trypsin inhibitor) was 68000.

Both the aryl acylamidase and cholinesterase activities of serum co-migrated on polyacrylamide gel electrophoresis under non-denaturing conditions and corresponded to the single protein band (Fig. 4A). The purified liver enzyme upon gel electrophoresis showed differences in the migration pattern of aryl acylamidase and cholinesterase activities (Fig. 4B). While all the cholinesterase and a part of the aryl acylamidase activities were located in the first two gel slices (stacking gel), a major portion of the aryl acylamidase activity was found in gel slices 14 – 20 without any cholinesterase activity. When electrophoresis of the purified liver enzyme was done on a 4% polyacrylamide gel (figure not given) instead of a 7% gel, a similar profile was seen. However a sample of the same enzyme without concentration by aquacide when subjected to electrophoresis on a 4% gel did not show any detectable aryl acylamidase activity in the first two gel slices but showed enhanced activity in gel slices 13 – 16 (see Fig. 4B). It is possible that on concentration a part of the purified liver aryl acylamidase migh have aggregated into a form that could not enter the separating gel. It should be noted that all the cholinesterase

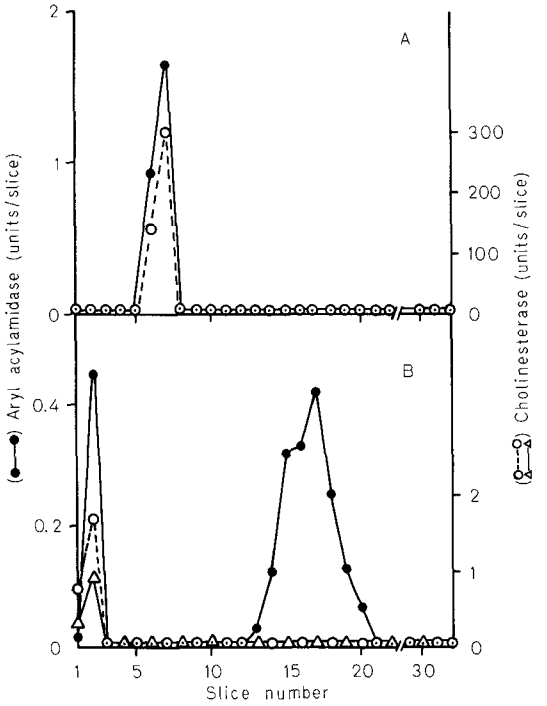


Fig. 4. *Profiles of enzyme activity in the gel slices after polyacrylamide gel electrophoresis.* (A) Purified serum aryl acylamidase (●—●) and cholinesterase (○--○); (B) purified liver aryl acylamidase (●—●) and cholinesterase with butyrylthiocholine as substrate (○--○) and acetylthiocholine as substrate (△—△). Gel slices 1 and 2 include the stacking gel

activity of both concentrated and dilute samples of the liver enzyme remained in the first two gel slices. Polyacrylamide gel electrophoresis of the erythrocyte enzyme showed co-migration of both aryl acylamidase and acetylcholinesterase activities as reported earlier [19].

The crude serum and liver enzymes were also subjected to electrophoresis on 4% polyacrylamide gels. Crude serum after being subjected to a procainamide-Sepharose chromatography (with 99% recovery of both aryl acylamidase and cholinesterase activities) showed on electrophoresis co-migration of both the activities. The activity profile was similar to that observed with the purified enzyme (see Fig. 4A) except that it was detectable in about six gel slices, slices 3–8, with a single sharp peak on slice 7. A 55000 × *g* supernatant of liver on electrophoresis also gave a profile similar to that obtained with the purified liver enzyme. Almost 98% of aryl acylamidase was located as a peak in the separating gel and about 2% was present in the stacking gel. All the liver cholinesterase activity was located in the stacking gel.

### *Effect of Amines and Choline Derivatives on the Aryl Acylamidases*

We had reported earlier [19] that acetylcholinesterase from brain, erythrocytes and electric eel have an aryl acylamidase activity which is inhibited specifically by the amine serotonin as well as by acetylcholine, its analogues and homologues. Tables 3 and 4 show the effect of different amines, their derivatives and choline derivatives on the purified aryl acylamidases from serum, erythrocytes and liver. Among the amines, serotonin followed by tryptamine was the strongest inhibitor of erythrocyte aryl acylamidase. The serum aryl acylamidase was also inhibited by serotonin to a lesser extent

Table 3. *Inhibition of purified aryl acylamidases of serum, erythrocytes and liver by amines and their derivatives*
n.s. = not significant (either 0% or <1% inhibition)

| Inhibitor | Concn | Inhibition of aryl acylamidase from | | |
|---|---|---|---|---|
| | | serum | erythrocyte | liver |
| | mM | % | | |
| Serotonin | 10 | 60 | 96 | n.s. |
| | 5 | 55 | 93 | n.s. |
| | 1 | 40 | 86 | n.s. |
| | 0.1 | 16 | 54 | n.s. |
| Tryptamine | 10 | 82 | 74 | n.s. |
| | 5 | 78 | 65 | n.s. |
| | 1 | 67 | 36 | n.s. |
| | 0.1 | 25 | 6 | n.s. |
| Dopamine | 10 | 29 | 18 | n.s. |
| | 1 | 18 | 8 | n.s. |
| | 0.1 | 5 | n.s. | n.s. |
| 5-Methoxy tryptamine | 10 | 47 | 8 | n.s. |
| | 1 | 18 | 4 | n.s. |
| | 0.1 | 2 | 2 | n.s. |
| Benzylamine | 10 | 9 | 28 | n.s. |
| | 1 | n.s. | 10 | n.s. |
| | 0.1 | n.s. | n.s. | n.s. |
| Kynuramine | 1 | 23 | 55 | n.s. |
| | 0.1 | n.s. | 10 | n.s. |
| Indole-3-propionic acid | 2 | 31 | 3 | 48 |
| | 1 | 18 | 2 | 41 |
| | 0.1 | 2 | n.s. | 7 |
| Indole-3-acetic acid | 0.5 | 13 | 5 | 14 |
| | 0.1 | 5 | 3 | 8 |
| 5-Hydroxyindole acetic acid | 1 | n.s. | n.s. | 5 |

Table 4. *Inhibition of purified serum, erythrocyte and liver aryl acylamidase by choline derivatives*
n.s. = not significant (either 0% or less than 1% inhibition)

| Inhibitor | Concn | Inhibition of aryl acylamidase from | | |
|---|---|---|---|---|
| | | serum | erythrocyte | liver |
| | mM | % | | |
| Acetylcholine | 10 | 73 | 87 | n.s. |
| | 1 | 44 | 28 | n.s |
| Propionylcholine | 10 | 67 | 84 | 5 |
| | 1 | 25 | 25 | n.s. |
| Butyrylcholine | 10 | 56 | 91 | n.s. |
| | 1 | 12 | 60 | n.s. |
| Succinylcholine | 10 | 97 | 73 | 3 |
| | 1 | 78 | 40 | n.s. |
| Benzoylcholine | 10 | 70 | 90 | n.s. |
| | 1 | 29 | 56 | n.s. |
| Acetyl-β- methylcholine | 10 | 66 | 70 | n.s. |
| | 1 | 32 | 21 | n.s. |
| Choline | 10 | 67 | 71 | n.s. |
| | 1 | 19 | 24 | n.s. |

and by tryptamine to a greater extent. The only other amine which gave a significant inhibition of both erythrocyte and serum aryl acylamidase was kyneuramine. 5-Methoxy-tryptamine, dopamine and benzylamine were much less inhibitory (Table 3). Other amines like norepinephrine, histamine and tyramine were poor inhibitors of the erythrocyte and serum aryl acylamidase. Tyramine was an activator of serum aryl acylamidase (see below). Amino acids like tryptophan, 5-methoxy-tryptophan, tyrosine and 4-aminobutyric acid had no effect up to 5 mM on the serum or erythrocyte aryl acylamidase. The liver aryl acylamidase was unaffected by all the above-mentioned compounds. However indole-3-propionic acid and indole-3-acetic acid inhibited the liver aryl acylamidase and to a lesser extent the serum aryl acylamidase with no significant effect on the erythrocyte enzyme (Table 3). These findings on liver aryl acylamidase were similar to our earlier observations [21]. Acetylcholine and other choline derivatives were inhibitory to both the erythrocyte and serum aryl acylamidase (Table 4). Succinylcholine and butyrylcholine were the most efficient inhibitors of serum and erythrocyte aryl acylamidase respectively. None of the choline derivatives had any significant effect on the liver aryl acylamidase.

Serotonin was a non-competitive inhibitor of aryl acylamidase of serum and erythrocyte and the $K_i$ values from Dixon plots were 4.5 mM and 0.125 mM respectively.

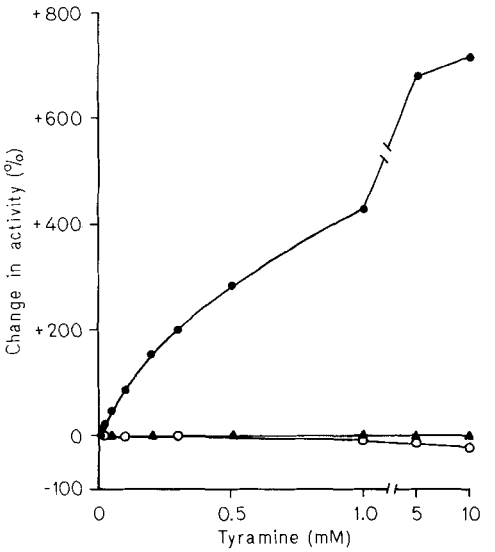


Fig. 5. *Effect of tyramine at various concentrations on the purified aryl acylamidase from serum (●—●), erythrocyte (○—○) and liver (▲—▲)*

### *Activation of Serum Aryl Acylamidase by Tyramine*

An interesting observation made was the activation of serum aryl acylamidase by tyramine (Fig. 5). There was a 4-fold activation at 1 mM and 6.5-fold activation at 5 mM tyramine. Neither the erythrocyte aryl acylamidase nor liver aryl acylamidase was significantly affected by tyramine up to 10 mM (Fig. 5). The activation of the serum aryl acylamidase was shown specifically by tyramine (4-hydroxyphenethylamine) and not by its structural analogues (3,4-dihydroxyphenyl)alanine(dopa) and 3,4-dihydroxyphenethylamine (dopamine). Tyramine (up to 5 mM) did not show any activation of the serum or liver cholinesterase. The ability of tyramine to activate the serum aryl acylamidase was also tested at different pH between pH 6 and 9 using 0.5 mM tyramine. About 3–4-fold activation was found at all pH between 6 and 8 and about 7-fold activation at pH 9. The erythrocyte and liver aryl acylamidases were not activated at any of these pH values by tyramine.

### *Inhibition by Eserine, Neostigmine and Selective Cholinesterase Inhibitors*

Eserine and neostigmine, potent inhibitors of cholinesterase, inhibited the aryl acylamidase as well as cholinesterase activities of both serum and erythrocytes. At 1 μM eserine and

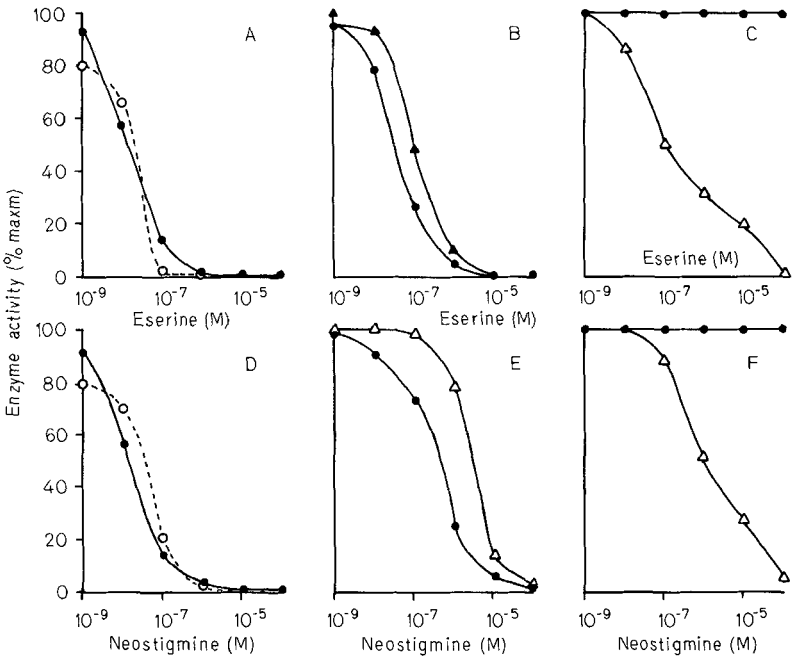


Fig. 6. *Inhibition by eserine and neostigmine of purified aryl acylamidase and cholinesterase from erythrocyte, serum and liver.* (A, B, C) Inhibition patterns of the erythrocyte, serum and liver enzymes respectively by eserine; (D, E, F) inhibition patterns of the respective enzymes by neostigmine. (●—●) Aryl acylamidase; (△—△) cholinesterase with butyrylthiocholine as substrate; (○--○) cholinesterase with acetylthiocholine as substrate

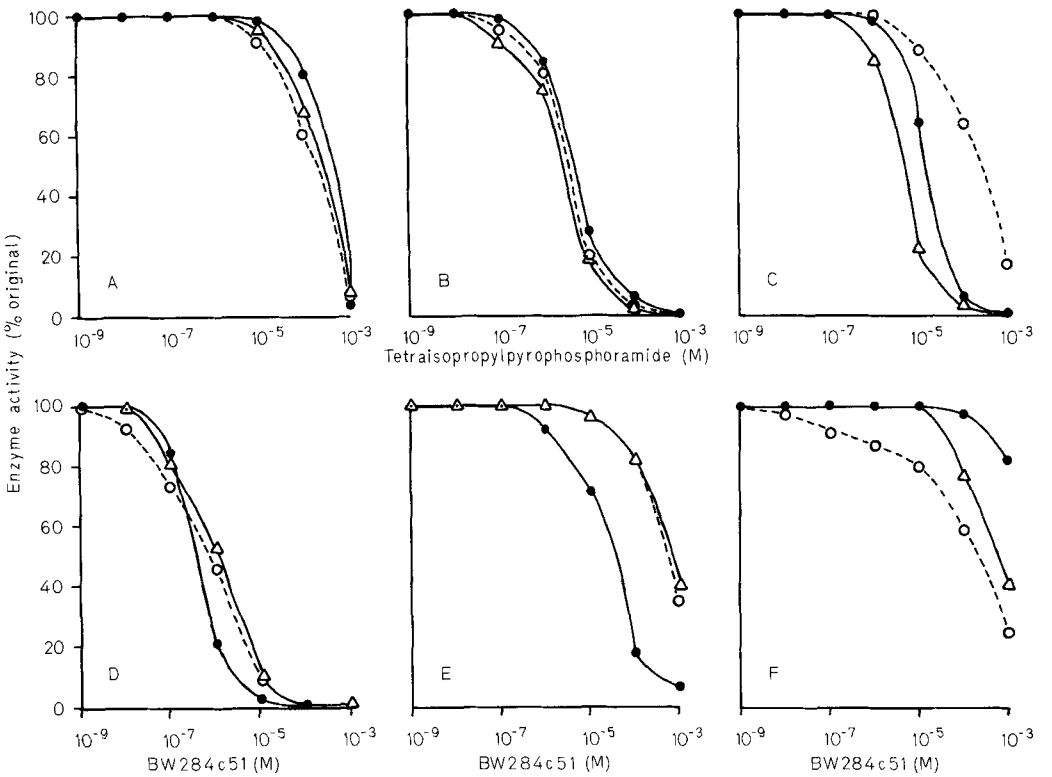


Fig. 7. *Inhibition of purified erythrocyte, serum and liver aryl acylamidase and cholinesterase by tetraisopropylpyrophosphoramide and BW284C51.* (A, B, C) Inhibition patterns of erythrocyte, serum and liver enzymes by tetraisopropylpyrophosphoramide; (D, E, F) inhibition patterns of the respective enzymes by BW284C51. (●—●) Aryl acylamidase; (△—△) cholinesterase using butyrylthiocholine as substrate; (○--○) cholinesterase using acetylthiocholine as substrate

10 μM neostigmine inhibition was nearly 100% of both the enzyme activities from both the sources (Fig. 6). However the liver aryl acylamidase was unaffected by either eserine or neostigmine up to 100 μM while the liver cholinesterase was completely inhibited at 100 μM (Fig. 6).

The ratio of cholinesterase activities (butyrylthiocholine to acetylthiocholine as substrate at equimolar concentrations) was 0.037, 1.96 and 2.14 for the purified erythrocyte, serum and liver enzymes. In order to classify the cholinesterases from the different sources a selective inhibitor of acetylcholinesterase, viz BW284C51 [5], and a selective inhibitor of pseudocholinesterase, viz. tetraisopropylpyrophosphoramide [6], were employed for inhibition studies of the arylacylamidases and cholinesterases from erythrocytes, serum and liver (Fig. 7). BW284C51 inhibited erythrocyte aryl acylamidase and acetylcholinesterase more than 90% at 10 μM concentration while tetraisopropylpyrophosphoramide inhibited both the enzymes only less than 10% at the same concentration. Conversely aryl acylamidase and cholinesterase of serum were inhibited more than 80% at 10 μM tetraisopropylpyrophosphoramide and only less than 30% by BW284C51 at the same concentration. Liver aryl acylamidase and cholinesterase were not significantly inhibited by BW284C51 at 10 μM while tetraisopropylpyrophosphoramide at 10 μM inhibited the enzymes to varying extent. At high concentrations of the inhibitors the extent of inhibition of the aryl acylamidase, acetylcholinesterase and butyrylcholinesterase activities of the liver enzyme varied widely. The liver cholinesterase exhibited more inhibition by tetraisopropylpyrophosphoramide when butyrylthiocholine was the substrate and by BW284C51 when acetylthiocholine was the substrate.

*Immunological Studies*

Purified antibody raised against serum aryl acylamidase showed cross-reactivity with the serum aryl acylamidase but not erythrocyte aryl acylamidase on double immunodiffusion in 1% agarose gel (Fig. 8). It gave precipitin lines with purified liver aryl acylamidase. However the serum precipitin lines and the liver precipitin lines did not fuse but crossed at the ends to reveal their immunological non-identity. In order to find out whether the precipitin lines formed in the double immunodiffusion were due to the enzyme proteins themselves, the precipitin lines were cut out from the gel after thorough washing of the gel and tested for the enzyme activities. Whereas the precipitin line formed between the antibody and the serum enzyme showed remarkable aryl acylamidase and cholinesterase activities, the precipitin line formed by the liver enzyme did not show any activity at all. This led us to conclude that the precipitin line formed by the liver enzyme must have been due to a non-specific protein other than the aryl acylamidase or cholinesterase. This conclusion is also in accord with our inability to immunoprecipitate the liver enzyme activities by the antibody as described below.

Immunoprecipitation of the enzyme activities by the antibody could be achieved only with the serum enzyme. Both aryl acylamidase and cholinesterase activities of serum could be precipitated and sedimented by centrifugation (Fig. 9). The ratio of aryl acylamidase to cholinesterase activity precipitated at different dilutions of the antibody was constant. Liver and erythrocyte enzymes could not be sedimented even with high concentrations of antibody. An attempt made to precipitate any complex formed between the antibody and the liver or

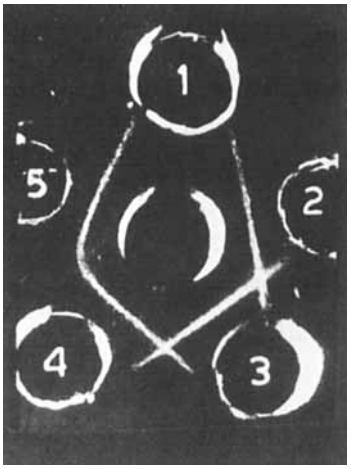


Fig. 8. *Double immunodiffusion pattern of the antibody raised against purified serum aryl acylamidase and the purified serum, liver and erythrocyte enzymes.* Center well contained 15 µl of reconstituted antibody (see Materials and Methods). Wells 2, 4 and 5 contained 15 µl of purified serum enzyme and wells 1 and 3 contained 15 µl of purified erythrocyte and liver enzymes respectively. The precipitin line formed by the serum enzyme showed remarkable aryl acylamidase and cholinesterase activities, but the precipitin line formed by the liver enzyme had no activity

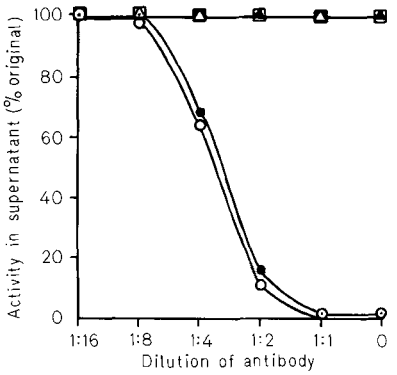


Fig. 9. *Immunoprecipitation of the purified serum, liver and erythrocyte enzymes by antibody raised against purified serum enzyme.* 0.15 ml of the reconstituted antibody (see Materials and Methods) at different dilutions was mixed with 0.15 ml of each of the enzymes and incubated at 4 °C for 24 h, centrifuged and the supernatant assayed for enzyme activities. The results are expressed as a percentage of original activity in the absence of antibody in the supernatant after centrifugation. (●—●) Serum aryl acylamidase; (○—○) serum cholinesterase; (■—■) erythrocyte aryl acylamidase; (□—□) erythrocyte cholinesterase; (▲—▲) liver aryl acylamidase; (△—△) liver cholinesterase assayed using butyrylthiocholine and acetylthiocholine as substrate

Table 5. *The comparative characteristics of purified aryl acylamidases from human erythrocytes, serum and liver*

| Property | Source of aryl acylamidase | | |
|---|---|---|---|
| | erythrocyte | serum | liver |
| 1. Relationship to cholinesterase | associated with erythrocyte acetylcholinesterase | associated with serum pseudocholinesterase | not associated with liver cholinesterase activity |
| 2. Amine sensitivity | inhibited by serotonin and tryptamine; not affected by tyramine | inhibited by tryptamine and serotonin; tyramine significantly activates | unaffected by serotonin, tryptamine and tyramine |
| 3. Sensitivity to choline derivatives | inhibited by butyrylcholine and other choline derivatives | inhibited by succinylcholine and other choline derivatives | unaffected by any of the choline derivatives tested |
| 4. Sensitivity to cholinesterase inhibitors | inhibited by eserine, neostigmine and selectively by BW284C51 | inhibited by eserine, neostigmine and selectively by tetraisopropyl-pyrophosphoramide | not inhibited by eserine and neostigmine; tetraisopropylpyrophosphoramide inhibits at relatively high concentration |
| 5. Immunological characteristics | immunologically different from serum enzyme | immunologically different from erythrocyte and liver enzymes | immunologically different from serum enzyme |

erythrocyte enzymes by forming a tertiary complex with anti-(rabbit IgG) also proved futile.

## DISCUSSION

In our earlier studies [19] we have provided several lines of evidence for the identity of the serotonin-sensitive aryl acylamidase with acetylcholinesterase from three different sources, viz. sheep basal ganglia, human erythrocytes and electric eel. In the present work we show the identity of the human serum aryl acylamidase (purified to homogeneity) with the serum cholinesterase on the basis of the following evidence: (a) their co-elution in all the column chromatographic procedures including affinity chromatography, (b) their co-purification in all the steps with constant ratios of activity and percentage recoveries, (c) their co-migration in gel electrophoresis, (d) their parallel inhibition by typical cholinesterase inhibitors and (e) their co-precipitation by antibody raised against the purified enzyme. The inhibition characteristics, particularly with tetraisopropyl-pyrophosphoramide, a selective inhibitor of pseudocholinesterase, and with BW284C51, a selective inhibitor of acetylcholinesterase, clearly differentiated between the erythrocyte and serum aryl acylamidase on the one hand and also confirmed

their identity with the respective cholinesterases on the other. The amine sensitivity studies indicate that the erythrocyte aryl acylamidase shows a higher sensitivity to serotonin inhibition as compared to the serum aryl acylamidase. Both enzymes were inhibited to varying extent by choline derivatives. A more significant observation was the activation by tyramine of serum aryl acylamidase but not erythrocyte aryl acylamidase.

The human liver aryl acylamidase appears to be an entirely different type of enzyme as compared to the erythrocyte and serum aryl acylamidases. The liver enzyme was not sensitive to inhibition by any of the amines or choline derivatives, it was not activated by tyramine and it was not inhibited by the typical cholinesterase inhibitors like eserine and neostigmine. Furthermore the liver aryl acylamidase did not appear to be associated with the liver cholinesterase as evidenced by the purification data (Table 2), their clear-cut separation on procainamide-Sepharose, elution profiles on Sepharose 6B (Fig. 2B), inhibition characteristics by eserine and neostigmine (Fig. 6) and the electrophoretic migration on polyacrylamide gel (Fig. 4B). Immunodiffusion and immunoprecipitation studies showed that aryl acylamidase from serum is different from those present in erythrocyte and liver. The comparative characteristics of the aryl acylamidases from the three sources are summarised in Table 5.

Although the evidence for the association of aryl acylamidase with cholinesterase has been mostly sought in the purified enzyme preparations of serum and liver, some experiments were also done on the crude enzymes. For example procainamide-Sepharose chromatography of crude liver enzyme (55000 × *g* supernatant) gave a clear-cut separation of the aryl acylamidase and cholinesterase activities indicating their non-association. Gel electrophoresis of the crude liver enzyme also indicated a clear separation of almost 98% of the aryl acylamidase activity from cholinesterase. Crude serum on procainamide-Sepharose chromatography showed co-elution of both aryl acylamidase and cholinesterase activity in 99% yield. This preparation on gel electrophoresis also showed co-migration of both the activities as found with the purified serum enzyme. Moreover Sepharose 6B gel filtration of this preparation resulted in the co-elution of both enzyme activities. These studies make it highly unlikely that any major form of aryl acylamidase not associated with cholinesterase activity in the serum or associated with cholinesterase activity in the liver might have been lost or gone undetected during the purification procedures. At least the separation techniques employed in the present studies indicate that such a possibility is remote.

The present studies indicate similarities between the serum and liver cholinesterases in their affinity towards procainamide-Sepharose, their inhibition characteristics by typical cholinesterase inhibitors and their specificity towards butyrylthiocholine as substrate. The apparent differences between the cholinesterases of the two sources are that the liver cholinesterase unlike the serum enzyme is not associated with aryl acylamidase, is immunologically non-identical with the serum cholinesterase and also differs from the serum cholinesterase in its electrophoretic mobility on polyacrylamide gel and elution profile on Sepharose 6B gel filtration.

It is likely that only those aryl acylamidase which are associated with cholinesterases (like those found in erythrocytes, serum, basal ganglia or electric eel) show sensitivity towards the amines serotonin or tryptamine and choline derivatives. Moreover tyramine activation appears to be a characteristic property of the aryl acylamidase associated with the serum type of cholinesterase. These characteristics of aryl acylamidase and those summarised in Table 5 may prove to be useful indexes of the type of cholinesterases present in the tissue or body fluids. These studies also indicate that the aryl acylamidases and cholinesterases from the three sources have major differences in their properties suggesting that they may be products of different genes.

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