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MEMBRANE MARKER ENZYMES

CHARACTERIZATION OF AN ARYLESTERASE OF GUINEA PIG CEREBRAL CORTEX UTILIZING *p*-NITROPHENYL ACETATE AS SUBSTRATE

H. BRUCE BOSMANN

Department of Pharmacology and Toxicology, University of Rochester School of Medicine and Dentistry, Rochester, N.Y. 14642 (U.S.A.)

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SUMMARY

1. A guinea pig cerebral cortex esterase (EC 3.1.1.2) was purified 1372.5-fold with a recovery of 62.2% by centrifugation, $(NH_4)_2SO_4$ precipitation, and chromatography on Sephadex G-100 and Sephadex G-200.

2. The purified enzyme preparation was free of acetylcholinesterase activity (EC 3.1.1.7).

3. The reaction was dependent on $MnCl_2$, had an optimum pH of 7.6, was linear with added enzyme protein to 2.0 μg protein, and was linear with incubation time up to 80 min.

4. No ion would substitute for Mn^{2+} in the assay except for Cd^{2+}, which did so partially. Several divalent cations were very inhibitory to the reaction, the order of greatest inhibition being $Cu^{2+} = Hg^{2+} > Fe^{2+} > Ca^{2+} + Pb^{2+} > Co^{2+}$; Mg^{2+} and Cd^{2+} did not inhibit the arylesterase.

5. Enrichment of the enzyme was found in the synaptosomal fraction of the cerebral cortex and the highest activity of the arylesterase in the subsynaptosomal fraction was in the microsomal fraction.

6. The purified arylesterase fraction showed isoelectric points of pH 5.1 and 5.8; molecular weights of 78 000, 180 000; and a K_m apparent value of 0.43 μM and a V value of 1.42 μmoles/h per mg protein.

INTRODUCTION

Esters of many types and diverse forms occur in biologic material, and there is a wide variety of esterases which cleave the bonds of these diverse esters during catabolism. The major esterase of neural tissue, and in particular of cerebral cortex, is acetylcholinesterase (EC 3.1.1.7). The present report describes the isolation from cerebral cortex and the characterization of an arylesterase (aryl-ester hydrolase,

EC 3.1.1.2) that does not accept acetylcholine as a substrate. The general reaction catalyzed by an arylesterase is

$$\text{A phenyl acetate} + H_2O \xrightarrow{\text{Arylesterase}} \text{a phenol} + \text{acetate}^1$$

The specific reaction studied herein is:

$$p\text{-Nitrophenylacetate} + H_2O \xrightarrow[\text{cerebral cortex arylesterase}]{\text{Guinea pig}} p\text{-nitrophenol} + \text{acetate}$$

the yellow color formed at basic pH by the phenolate ion being the basis of the assay.

Esterases, and in particular arylesterases, have been identified in a variety of tissues, but much of the work has been at a histochemical or starch-gel electrophoresis staining level. Soluble esterases of human liver[2], kidney[3], brain[4], heart[5], smooth and skeletal muscle[6], and lung[7] have been identified on starch-gel electrophoresis. Rabbit serum arylesterases[8] with high esterase activity for several aromatic esters have been found by disc electrophoresis. In a series of papers Masters and co-workers[9–13] have described esterases from pig, sheep, horse, ox, oppossum, guinea pig, rat, and chicken, utilizing disc electrophoresis and specific staining methods in the presence of inhibitors. Non-specific esterase has been identified in the placenta and fetal membranes of the horse, sheep, cat, dog, ferret, rat, rabbit, guinea pig, and human[14]. Esterases have also been identified in tissue culture cells[15], houseflies[16], *Fusarium oxysporum* f.sp. *vasinfectum*[17], *Escherichia coli*[18], tomatoes[19], and sea urchin egg membranes[20]. In none of these studies was a serious attempt made to purify the esterases or to characterize the purified preparations. In the present study the biochemical characteristics of a guinea pig cerebral cortex arylesterase, purified 1372.5-fold, are given.

Esterase activity has been used as a rough endoplasmic reticulum marker enzyme[21–24] and Lansing *et al.*[25] have described the preferential enrichment of esterase in microsomes as opposed to plasma membranes in rat liver. Esterase has been identified in L5178Y mouse leukemic cells as a rough endoplasmic reticulum marker enzyme and has been demonstrated as peak enzyme in synchronous L5178Y cells[26]. Because of the interest in a non-acetylcholinesterase esterase in nervous tissue and because of the utility of this enzyme activity as a membrane marker, the present investigation was undertaken.

MATERIALS AND METHODS

Materials

Substrates were purchased from Sigma Chemical Co. or Pierce Chemical Co. "Enzyme grade" $(NH_4)_2SO_4$ and "density gradient grade" sucrose were purchased from Mann Chemicals Co. All solutions were made with deionized glass distilled water.

Enzyme assay

The arylesterase was assayed routinely in the following incubation mixture: 100 μl of enzyme preparation (0.1–5.0 μg protein), 200 μl of 0.1 M sodium phosphate buffer (pH 7.6), 500 μl of distilled water, 200 μl of *p*-nitrophenyl acetate (50 μmoles)

and 100 μl of 1.0 mM $MnCl_2$, at a final volume of 1.1 ml. The incubation mixture was incubated at 37 °C for 30 min in a Dubnoff metabolic shaker. The reaction mixtures were then immediately placed in an ice bath, centrifuged at 20 000 × *g* at 1 °C for 10 min and released *p*-nitrophenol was measured spectrophotometrically at 400 nm. From these data and a standard *p*-nitrophenol curve the amount (in nmoles) hydrolyzed per h was calculated. Parallel substrate and enzyme blanks were determined with each estimation.

Protein

Protein was determined according to the method of Lowry *et al.*[27]. Crystalline bovine serum albumin was used as a standard.

Purification of arylesterase from guinea pig cerebral cortex

All steps of purification were carried out at 4 °C unless otherwise specified. Four independent preparations of enzyme were made. Fractions from the columns were assayed for protein by the method of Lowry *et al.*[27]. All 0.1% Triton X-100 was made in 0.1 M Tris–HCl buffer (pH 7.6). At several steps (Steps 2, 4 and 5 below), solutions were concentrated by lyophilization.

Step 1. Extraction. 12 cerebral cortices were removed from guinea pigs (300 g) which had been starved for 16 h with water *ad libitum*. They were minced in 5 vol. of the 0.1% Trition X-100 solution and homogenized for 30 strokes in a Ten Broeck homogenizer.

Step 2. Centrifugation. This homogenate was then centrifuged at 40 000 × *g* for 10 min.

Step 3. $(NH_4)_2SO_4$ precipitation. To the supernatant was added sufficient $(NH_4)_2SO_4$, with stirring, to make the solution 20% in $(NH_4)_2SO_4$. This was stirred at 4 °C for 30 min and then allowed to sit for 30 min at 4 °C. The suspension was centrifuged at 20 000 × *g* for 20 min. Both the supernatant and the pellet were exhaustively dialyzed against the 0.1% Triton X-100 solution at 4 °C.

Step 4. Gel filtration on Sephadex G-100. A 10-ml sample (450 mg protein) from the 20% $(NH_4)_2SO_4$ dialyzed precipitate in the 0.1% Triton X-100 solution was applied to a Sephadex G-100 column (2.5 cm × 45 cm) packed in the same solution. The column was eluted with the same buffer and fractions of 2 ml were collected. The void volume of the column was about 120 ml, which corresponds to Tube 60 in Fig. 2.

Step 5. Gel filtration on Sephadex G-200. A 5-ml sample (110 mg protein) of the peak from the previous step was applied to the Sephadex G-200 column (45 cm × 2.5 cm) packed in the 0.1% Triton X-100 solution. The column was developed with the same buffer and 10-ml fractions were collected. The void volume of the column was 120 ml, which corresponds to Tube 12 in Fig. 3.

Enzyme characterization

All experiments were performed with the purified enzyme; purified enzyme from several purification runs were pooled for the experiments.

Synaptosome and subsynaptosomal fraction isolation

Synaptosomes (nerve ending particles, Fraction P_2) and subsynaptosomal

fractions were isolated exactly by the methods of Whittaker *et al.*[28–31] as described[32,33], at 0–4 °C from guinea pig cerebral cortex (brain stem transsected between superior and inferior colliculi). Male guinea pigs, 300 g, fasted for 16 h before death were the source of cerebral cortex. Twelve guinea pigs were used for each experiment, yielding a wet weight of cortex of approx. 30 g. The procedures used in these experiments are outlined in schematic form in Fig. 1. In each instance of fractionation, if the fraction was present as a pellet it was suspended in 0.1% Triton X-100, 0.1 M Tris buffer (pH 7.6). If the fraction was in sucrose it was diluted 1:3 with Tris buffer and centrifuged out of solution at 40 000 × *g* for 10 min before resuspension in 0.1% Triton X-100, 0.1 M Tris buffer (pH 7.6).

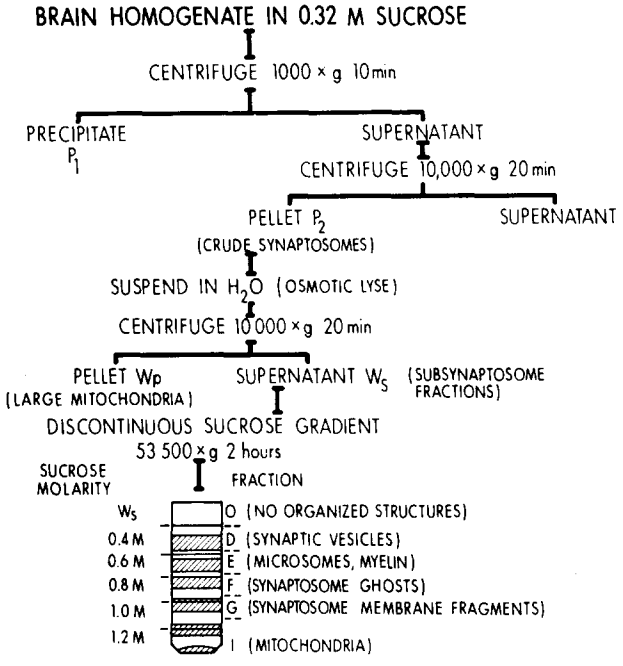

Fig. 1. Schematic diagram of procedures used to isolate synaptosomes and subsynaptosomal fractions. The methods of Whittaker and coworkers[28–21] were used. All procedures were carried out at 4 °C. All fractions were extracted with 0.1% Triton X-100 before assay, as described in Materials and Methods.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was carried out by the "mini-protein" method of Laico *et al.*[34] as described previously[35], omitting the dialysis step to prevent loss of polypeptides. Suitable standards were always run simultaneously. 50 μg (as protein) of each fraction were extracted at pH 7.1 in 0.1% sodium dodecyl sulfate and 0.1% 2-mercaptoethanol for 3 h at 37 °C. The samples were subjected to electrophoresis at 8 V/cm for 2 h in 125 mm 5% polyacrylamide gels which were 0.1 M phosphate and 0.1% sodium dodecyl sulfate. The electrophoresis buffer was 0.1 M phosphate buffer (pH 7.1), which was 1% sodium dodecyl sulfate. All gels were run toward the anode. Following electrophoresis the gels were stained for either protein or glycoprotein. For protein, the gels were fixed in 20% sulfosalicylic acid for 16 h,

stained with 0.25% Coomassie Blue for 3 h, and destained with several washes of 7% acetic acid. For glycoprotein, the gels were stained with a modified periodic acid–Schiff technique exactly as described by Zacharius *et al.*[36].

Acetylcholinesterase (EC 3.1.1.7)

Cholinesterase activity was estimated by the method of Hemsworth[37] by a null-point titration method using a Radiometer (Copenhagen) pH-Stat unit (Burette unit ABU12; Titration assembly TTA31; Titrator TTT11; pH Meter PHM28; and Recorder SBR2) with a 5-ml water-jacketed reaction chamber for automatic titration of 0.01 M NaOH from the 0.25-ml burette. The temperature was maintained throughout at 37 °C by a thermostated circulating water bath (Heto T6TB water bath).

pH measurements

pH measurements were performed with a Beckman Research Model pH meter. Corrections for solution temperature were made.

Isoelectric focusing

Isoelectric focusing was carried out utilizing the LKB 110 ml electrofocusing apparatus. Ampholytes were purchased from LKB. Runs were performed without added sample to determine ampholyte contribution to the absorbance at 280 nm; this contribution was subtracted from data presented herein. Runs were carried out for 24 or 48 h; in typical runs starting parameters would be 360 V, 4.3 mA or 335 V, 8 mA and finishing parameters would be 370 V, 1.3 mA or 365 V, 1.2 mA. All runs were performed at 2 °C.

RESULTS AND DISCUSSION

Purification of the guinea pig cerebral cortex arylesterase

The data presented in Table I indicate that the procedures outlined above resulted in a 1372.5-fold purification of the arylesterase with a 62.2% recovery of the enzyme. Chromatography on Sephadex G-100 (Fig. 2) and especially on Sephadex

TABLE I

PURIFICATION AND ISOLATION OF ARYLESTERASE ACTIVITY BY $(NH_4)_2SO_4$ PRECIPITATION AND COLUMN CHROMATOGRAPHY

Procedures were performed as given in Materials and Methods. All procedures were carried out at 0–4 °C. The experiments were repeated 4 times and were quantitatively and qualitatively reproducible. Starting material was 12 guinea pig cerebral cortices.

Fraction	*Total protein (mg)*	*Total activity (mmoles/h)*	*Recovery (%)*	*Specific activity (μmoles/h per mg protein)*	*Purification factor*
(1) 0.1% Triton X-100 extract	6600	45	100	6.8	1
(2) 40 000 × *g* supernatant	3900	38	84.4	9.7	1.4
(3) 20% $(NH_4)_2SO_4$ precipitate	450	34	75.5	75.5	11.1
(4) Sephadex G-100	110	30	66.6	272.7	40.1
(5) Sephadex G-200	35	28	62.2	9333.3	1372.5

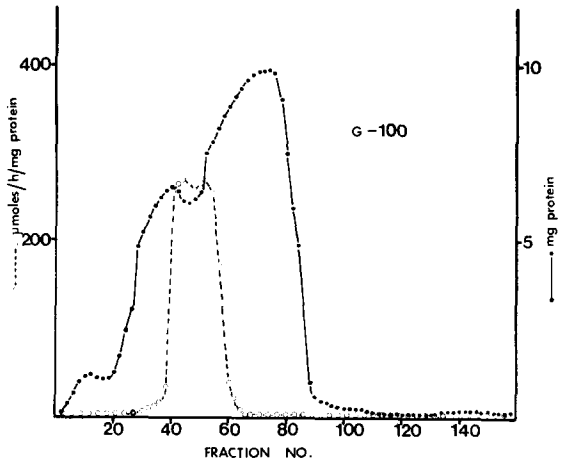

Fig. 2. Chromatography of the guinea pig cerebral cortex arylesterase activity on Sephadex G-100. Enzyme (34 mmoles/h; 450 mg protein) in 10 ml of 0.1% Triton X-100 in 0.1 M Tris–HCl buffer (pH 7.6) was applied to the column (45 cm × 2.5 cm). Elution was with the 0.1% Triton X-100 in 0.1 M Tris–HCl buffer (pH 7.6); 2-ml fractions were collected. All procedures were carried out in a jacketed column at 4 °C.

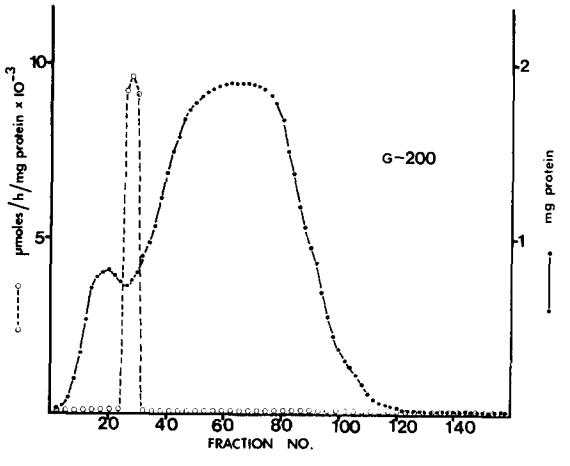

Fig. 3. Chromatography of the guinea pig cerebral cortex arylesterase activity on Sephadex G-200. Enzyme (28 mmoles/h; 110 mg protein) in 5 ml of 0.1% Triton X-100 in 0.1 M Tris–HCl buffer (pH 7.6) was applied to the column (45 cm × 2.5 cm). Elution was with the 0.1% Triton X-100 in 0.1 M Tris–HCl buffer (pH 7.6); 10-ml fractions were collected. All procedures were carried out in a jacketed column at 4 °C.

G-200 (Fig. 3) proved to be the most efficient steps of purification. The enzyme eluted essentially in the void volume of the Sephadex G-100 column indicating an approximate molecular weight of 100 000 or greater and was eluted after the void volume of the Sephadex G-200 column, again indicating an approximate molecular weight of over 100 000 but less than 200 000 (see below and Fig. 7). The degree of purification was extremely good, especially in the light of very substantial recovery (Table I) and probably indicates that the enzyme is very stable and certainly amena-

ble to gel chromatography. Pooled samples from Sephadex G-200 eluates of three purification runs were utilized to perform the experiments reported below.

Activity of the guinea pig cerebral cortex arylesterase as a function of enzyme protein concentration

Assays indicated that the reaction was essentially linear with the purified enzyme up to 2.0 μg of enzyme protein.

Acetylcholinesterase activity of the purified quinea pig cerebral cortex arylesterase

20 μg of the purified enzyme protein were entirely free of acetylcholinesterase (EC 3.1.1.7); comparison of this result with the data for *p*-nitrophenyl acetate as substrate indicates that this amount of purified enzyme protein is 10 times as much as the amount of enzyme protein that will catalyze the hydrolysis of 19 μmoles of *p*-nitrophenyl acetate per h.

Activity of the purified cerebral cortex arylesterase as a function of incubation time

The reaction of the arylesterase under the defined conditions of the assay utilizing 1 μg of purified enzyme protein was linear up to 70 min of incubation at 37 °C.

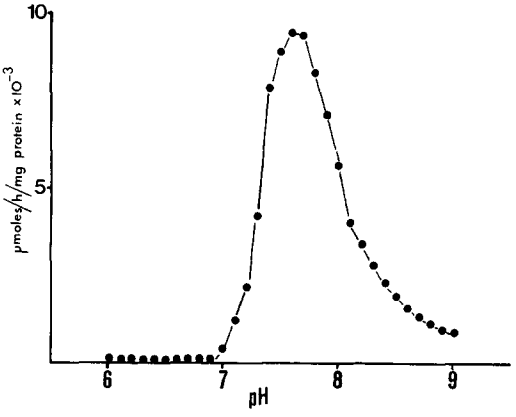

Fig. 4. Effect of incubation pH on the activity of the guinea pig cerebral cortex arylesterase. Experiments were performed as given in Materials and Methods.

Activity of the purified cerebral cortex arylesterase as a function of pH

The guinea pig cerebral cortex arylesterase showed a rather sharp pH–activity profile as shown in Fig. 4. Optimum pH of reaction was 7.6, and activity fell off rapidly on either side of this pH optimum. Below pH 7 there was absolutely no activity (although the final pH after incubation was raised to 10 for expression of the phenolate ion color) and above pH 9 there was essentially no activity.

Effects of various cations and boiling on the guinea pig cerebral cortex arylesterase

The data in Table II demonstrate that the $MnCl_2$ present in the assay was necessary for optimal activity although 48% of the activity was found when the $MnCl_2$ was omitted from the assay (Table II, Column II). Similarly, when EDTA

TABLE II

EFFECTS OF VARIOUS IONS AND BOILING ON THE CEREBRAL CORTEX ARYLESTERASE

All ions were tested as the chloride and were present at 0.1 mM final concn. EDTA (disodium salt) was present at 0.1 mM final concn. Data are percent control incubation (as given in Materials and Methods except that 0.1 mM $MnCl_2$ was omitted in Column II) ± S.D. That is, in Column I the normal $MnCl_2$ was present, so inhibition was being measured, while in Column II the normal $MnCl_2$ was omitted so activation was being measured.

Ion	*I* *(% control)*	*II* *(% control)*
None	100 ± 2	48 ± 2
EDTA	49 ± 2	51 ± 3
Mn^{2+}	96 ± 2	100 ± 2
Co^{2+}	42 ± 1	26 ± 1
Cu^{2+}	0	0
Fe^{2+}	11 ± 2	12 ± 1
Hg^{2+}	0	0
Mg^{2+}	102 ± 3	39 ± 3
Ca^{2+}	16 ± 4	16 ± 2
Cd^{2+}	104 ± 2	81 ± 2
Pb^{2+}	16 ± 3	8 ± 2
Boiled enzyme	0	2 ± 1

(0.1 mM final concn) was present in the normal defined assay (Table II, Column I), 49% of the control activity remained. When the $MnCl_2$ normally present in the assay was included (Table II, Column I), several of the ions tested were inhibitory to the arylesterase activity. Co^{2+} was moderately inhibitory while Fe^{2+}, Ca^{2+} and Pb^{2+} severely inhibited the arylesterase; Cu^{2+} and Hg^{2+} completely inhibited the enzyme, while Mg^{2+} and Cd^{2+} had no inhibitory effect. When the $MnCl_2$ normally present in the assay was omitted (Table II, Column II) none of the ions tested could effectively substitute for the Mn^{2+}. Cd^{2+} was able to substitute for the Mn^{2+} to 81% the control activity; none of the other ions tested resulted in activity greater than that found when no ion was present (Table II). Boiling of the purified arylesterase from guinea pig cerebral cortex completely abolished its activity.

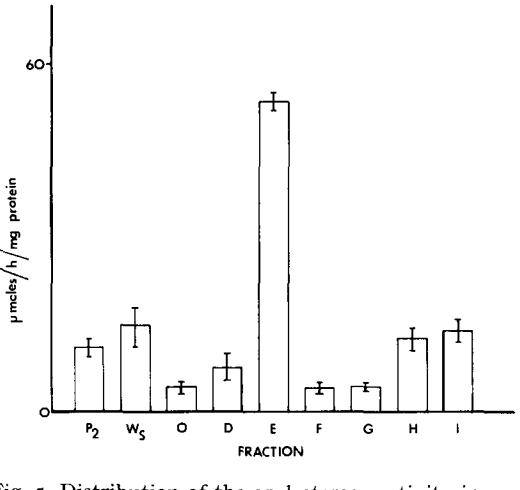

Fig. 5. Distribution of the arylesterase activity in synaptosomes and subsynaptosomal fractions. Experiments were performed as given in Materials and Methods (see also Fig. 1).

Synaptosomal and subsynaptosomal localization of the guinea pig cerebral cortex arylesterase

The fractionation of the cerebral cortex into a synaptosomal fraction, P_2 (see Fig. 1), resulted in an enrichment in arylesterase activity (Fig. 5). The crude homogenate of the guinea pig cerebral cortex contained 6.8 μmoles/h per mg protein of activity (Table I) while the P_2 fraction of synaptosomes contained 11.0 μmoles/h per mg protein of arylesterase activity. Fractionation of the synaptosomes into subsynaptosomal fractions (see Fig. 1) resulted in a very high arylesterase activity in Fraction E, the microsome–myelin fraction, of 53.4 μmoles/h per mg protein. The arylesterase activity was by far the highest in this fraction, indicating that even in brain tissue the arylesterase is a good membrane marker enzyme for microsomes or rough endoplasmic reticulum.

TABLE III

EFFECTS OF VARIOUS TREATMENTS OR INHIBITORS ON ACTIVITY OF THE GUINEA PIG ARYLESTERASE FROM CEREBRAL CORTEX

Experiments were performed as given in Materials and Methods and are means from 6 independent observations. All determinations were made with the purified enzyme.

Inhibitor or treatment	*Activity (% control)*
None	100
2 min at 60 °C (enzyme)	27
2 min in 10 M urea (enzyme)	21
Eserine, 10^{-3} M	99
Acetylcholine, 10^{-3} M	101
α-Naphthyl acetate, 10^{-3} M	34
β-Naphthyl acetate, 10^{-3} M	28
Diisopropylfluorophosphate, 10^{-4} M	94
p-Hydroxymercuribenzoate, 10^{-3} M	13

Effects of various treatments and potential inhibitors on the purified guinea pig cerebral cortex arylesterase

The data in Table III confirm the arylesterase character of the purified enzyme. Holmes and Masters[11] state that heat lability and urea instability are characteristics of arylesterases as opposed to cholinesterases, acetylesterases, or carboxylesterases: the data in Table III meet the criterion[11] for arylesterases with respect to stability at 60 °C and lability in 10 M urea. Eserine and acetylcholine would be expected to be potent inhibitors of cholinesterases while α- and β-naphthyl acetate would be expected to be competetive inhibitors of arylesterases: the data of Table III indicate that the enzyme presently being studied is an arylesterase by these inhibitor criterion. Finally, the substantial inhibition by *p*-hydroxymercuribenzoate and lack of inhibition by diisopropylfluorophosphate indicate that the enzyme being studied may be similar to the brain arylesterase described by Ecobichon[4].

Isoelectric focusing of the purified guinea pig cerebral cortex arylesterase

The isoelectric point of the purified Sephadex G-200 arylesterase fraction ranged from pH 4.9 to 5.9. Two peak isoelectric points were found as shown in Fig. 6 of pH 5.1 and 5.8.

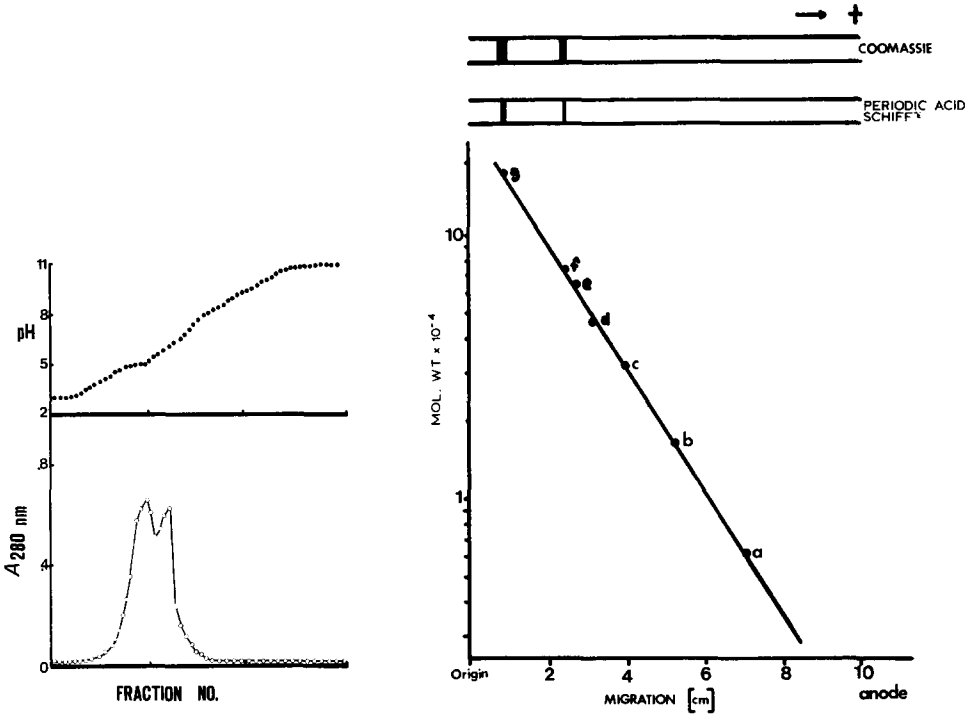

Fig. 6. Isoelectric focusing of the purified guinea pig cerebral cortex arylesterase fraction. Experiments were performed as given in Materials and Methods.

Fig. 7. Sodium dodecyl sulfate gel electrophoresis of the purified guinea pig cerebral cortex arylesterase fraction. The top portion of the figure gives the schematic representation of the gels. The bottom portion represents the migration *vs* molecular weight graph for the following samples and standards: (a) insulin (approx. mol. wt 6 000); (b) hemoglobin monomer (17 000); (c) hemoglobin dimer (34 000); (d) hemoglobin trimer (51 000); (e) hemoglobin tetramer (68 000); (f) arylesterase (76 000); and (g) arylesterase (180 000).

Polyacrylamide gel electrophoresis and molecular weight determination of the purified guinea pig cerebral cortex arylesterase

On polyacrylamide gel electrophoresis in the sodium lauryl sulfate system described in Materials and Methods, two bands were found for the purified arylesterase as shown in Fig. 7. Both bands stained for both proteins with the Coomassie Brilliant Blue reagent and glycoprotein with the periodic acid–Schiff technique. Using a molecular weight *vs* migration plot with appropriate standards (Fig. 7) according to the methods of Shapiro *et al.*[38] and Weber and Osborn[39], two approximate molecular weights were found of 78 000 and 180 000 for the cerebral cortex arylesterase: whether the former is a subunit of the latter is not known. The data of Fig. 7 correlate well with the Sephadex G-100 (Fig. 2) and Sephadex G-200 (Fig. 3) column chromatography data. Previous molecular weight estimates for arylesterases include 60 000 for green turtle arylesterase[40] and 48 000–72 000 for a variety of vertebrate arylesterases[13].

$K_{m(apparent)}$ and V values for the guinea pig cerebral cortex arylesterase

Linear double reciprocal plots, as shown in Fig. 8, gave a K_m of 0.43 μM and

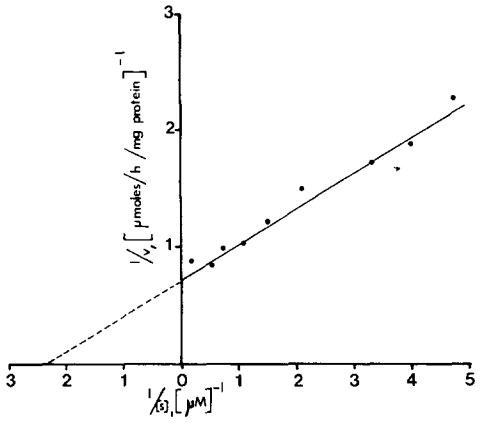

Fig. 8. Lineweaver–Burk plot of arylesterase activity of guinea pig cerebral cortex. Assays were carried out under the conditions described in Materials and Methods by varying substrate concentration. Incubation was for 5 min to insure initial rates of reaction and steady state conditions.

a V of 1.42 μmoles/h per mg protein for the guinea pig cerebral cortex arylesterase.

The data given here describe a highly purified arylesterase from guinea pig cerebral cortex which is free from cholinesterase activity. The data on the subcellular localization of the enzyme in the synaptosome microsomes and its ease of assay and purification indicate that the enzyme is excellent as a membrane marker enzyme.

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REFERENCES

1 M. Florkin and E. H. Stotz, *Comprehensive Biochemistry*, Vol. 13, *Enzyme Nomenclature*, Elsevier, Amsterdam, 1965, p. 126.
2 D. J. Ecobichon and W. Kalow, *Can. J. Biochem. Physiol.*, 39 (1961) 1329.
3 D. J. Ecobichon, *Can. J. Biochem.*, 42 (1964) 277.
4 D. J. Ecobichon, *Can. J. Physiol. Pharmacol.*, 44 (1966) 225.
5 D. J. Ecobichon, Ph. D. Thesis, Univ. of Toronto, Toronto, Canada (1964).
6 D. J. Ecobichon and W. Kalow, *Can. J. Biochem.*, 43 (1965) 73.
7 J. Brebner and W. Kalow, *Can. J. Biochem.*, 48 (1970) 970.
8 L. V. Albers, S. Dray and K. L. Knight, *Biochemistry*, 8 (1969) 4416.
9 R. S. Holmes and C. J. Masters, *Biochim. Biophys. Acta*, 132 (1967) 379.
10 R. S. Holmes and C. J. Masters, *Biochim. Biophys. Acta*, 146 (1967) 138.
11 R. S. Holmes and C. J. Masters, *Biochim. Biophys. Acta*, 151 (1968) 147.
12 R. S. Holmes and C. J. Masters, *Biochim. Biophys. Acta*, 159 (1968) 81.
13 N. Kingsbury and C. J. Masters, *Biochim. Biophys. Acta*, 200 (1970) 58.
14 G. A. Christie, *Histochemie*, 12 (1968) 189.
15 G. von Zeipel, *Acta Pathol. Microbiol. Scand.*, 78 (1970) 258.
16 H. H. Shatoury, *Experientia*, 26 (1970) 1295.

17 L. Miller and J. D. Macmillan, *Biochemistry*, 10 (1971) 570.
18 D. M. Barnes, A. C. Swindell and S. J. Wakil, *J. Biol. Chem.*, 245 (1970) 3122.
19 H. Delincée and B. J. Radola, *Biochim. Biophys. Acta*, 214 (1970) 178.
20 A. Grossman and A. G. Troll, *Biochim. Biophys. Acta*, 212 (1970) 192.
21 H. B. Bosmann, A. Hagopian and E. H. Eylar, *Arch. Biochem. Biophys.*, 128 (1968) 51.
22 H. B. Bosmann and S. S. Martin, *Science*, 164 (1969) 190.
23 H. B. Bosmann and B. A. Hemsworth, *J. Biol. Chem.*, 245 (1970) 363.
24 H. B. Bosmann, *J. Biol. Chem.*, 246 (1971) 3817.
25 A. I. Lansing, M. L. Belkhode, W. E. Lynch and I. Lieberman, *J. Biol. Chem.*, 242 (1967) 1772.
26 H. B. Bosmann, *Biochim. Biophys. Acta*, 203 (1970) 256.
27 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
28 E. G. Gray and V. P. Whittaker, *J. Anat.*, 96 (1962) 76.
29 V. P. Whittaker and M. N. Sheridan, *J. Neurochem.*, 12 (1965) 363.
30 V. P. Whittaker, I. A. Michaelson and R. J. A. Kirkland, *Biochem. J.*, 90 (1964) 293.
31 V. P. Whittaker, *Progr. Biophys. Mol. Biol.*, 15 (1965) 39.
32 H. B. Bosmann, *J. Neurochem.*, 19 (1972) 763.
33 H. B. Bosmann and B. A. Hemsworth, *Biochem. Pharmacol.*, 19 (1970) 133.
34 M. T. Laico, E. I. Ruoslahti, D. S. Papermaster and W. J. Dwyer, *Proc. Natl. Acad. Sci. U.S.*, 67 (1970) 120.
35 H. B. Bosmann, K. R. Case and M. B. Shea, *FEBS Lett.*, 11 (1970) 261.
36 R. M. Zacharius, T. E. Zell, J. H. Morrison and J. J. Woodlock, *Anal. Biochem.*, 30 (1969) 148.
37 B. A. Hemsworth, *Eur. J. Pharmacol.*, 15 (1971) 91.
38 A. L. Shapiro, E. Vinuela and J. V. Maizel, *Biochem. Biophys. Res. Commun.*, 28 (1967) 815.
39 K. Weber and M. Osborn, *J. Biol. Chem.*, 244 (1969) 4406.
40 N. Kingsbury and C. J. Masters, *Biochim. Biophys. Acta*, 227 (1971) 31.