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CARBOXYLESTERASES OF HUMAN BRAIN EXTRACT

PURIFICATION AND PROPERTIES OF A BUTYRYLESTERASE

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Summary

1. A carboxylesterase (carboxylic-ester hydrolase, EC 3.1.1.1) from human brain extract was prepared to purity using DEAE-cellulose, Sephadex G-200, and fractionation with $(NH_4)_2SO_4$. The yield was about 20%.

2. Esters of butyric acid were split faster than esters of acetic, propionic and valeric acid, and the enzyme is tentatively designated as a butyrylesterase. Thiocholine esters were split at low rates.

3. The molecular weight was estimated as 340 000 using gel chromatography on Sephadex G-200. In isoelectric focussing the enzyme was resolved into several peaks (p*I* 4.0—4.7). The low isoelectric point does not seem to be due to terminal sialic acid residues.

4. The enzyme was irreversibly inhibited by diethyl-*p*-nitrophenyl phosphate (k_i = 206 $mol^{-1} \cdot l \cdot s^{-1}$) and by diisopropylfluorophosphate. The carboxylesterase inhibitor bis-*p*-nitrophenyl phosphate and eserine did not inhibit the enzyme.

5. The enzyme was progressively inhibited by *p*-hydroxy-mercuribenzoate, and reactivated by dithiothreitol and 2-mercaptoethanol. *N*-Ethylmaleimide inactivated the enzyme very slowly, whereas iodoacetate and iodoacetamide were without effect.

6. Ca^{2+}, Mg^{2+}, and Zn^{2+} or EDTA did not influence the enzyme activity.

Introduction

Human brain extract contains several eserine-resistant carboxylesterases (carboxylic-ester hydrolase, EC 3.1.1.1) which so far have been only partly separated by electrophoretic and chromatographic procedures [1—4]. More

Abbreviations: iPr_2P-F, diisopropylfluorophosphate; Nbs_2, 5,5′-dithio-bis-(2-nitrobenzoic acid).

than 30 bands of enzyme activity were separated by isoelectric focussing on polyacrylamide gel slabs (unpublished observations). Previous studies showed that these enzymes do not merely represent a series of isoenzymes with identical catalytic properties; at least four different patterns of substrate specificities could be discerned [4].

One of the enzymes catalyzed the hydrolysis of α-naphthylbutyrate at a higher rate than that of α-naphthylacetate. Its molecular weight was higher and its isoelectric point was lower than those of other brain carboxylesterases [4]. The present study concerns the purification and further characterization of this carboxylesterase which, until a better nomenclature is available, or until other functions of the enzyme are encountered, can be designated as a butyrylesterase.

Materials and Methods

Substrates. α-Naphthylacetate, α-naphthylbutyrate, α-naphthylpropionate, α-naphthylvalerate, *p*-nitrophenylacetate, *p*-nitrophenylbutyrate, *p*-nitrophenyl phosphate, acetylthiocholine iodide, butyrylthiocholine iodide and hypoxanthine were all from Sigma.

Marker substances. Alkaline phosphatase (type III from *Escherichia coli*) and xanthine oxidase (type II from bovine milk) were from Sigma. Alcohol dehydrogenase (from yeast) and apoferritin (from horse) were products of Calbiochem and Schwartz/Mann, respectively.

Inhibitors. Diethyl-*p*-nitrophenyl phosphate, bis-*p*-nitrophenyl phosphate, diisopropylfluorophosphate, and eserine salicylate were purchased from Sigma.

SH-reagents. p-Hydroxymercuribenzoate (sodium salt) and *N*-ethylmaleimide were from Sigma. Sodium iodoacetate (AnalaR) and iodoacetamide (AnalaR) were from B.D.H. 2-Chloromercuri-4-nitrophenol was obtained from Whatman, and 5,5′-dithio-bis-(2-nitrobenzoic acid) from Aldrich.

Sialidase (neuraminidase from *Vibrio cholerae*) was obtained from Behringwerke, dithiothreitol from Sigma, and sucrose (reinst) from Merck. Agarose was from Litex, Glostrup, Denmark, and potato starch from Nordisk Droge, Copenhagen; hydrolysis of the starch was performed according to the procedure given in ref. 5. Fast Blue RR was from Gurr and Co., amido-schwarz 10B from Merck.

Brain tissue. 16 human brains were obtained at autopsy 12—120 h after death. The causes of death were heart failure, traffic accidents, and suicide by hanging or shooting. The whole brain except for the cerebellum was used. Membranes and large vessels were removed and the tissue cut into small pieces, rinsed in ice-cold 0.9% NaCl, blotted on filter paper and weighed.

Extraction. Homogenization and extraction with 10 mM Tris · HCl buffer (pH 7.0) + 0.5 mM dithiothreitol were performed as described previously [4]. The slightly turbid extract was cleared by precipitating particulate matter as follows: pH was adjusted to 5.2 by addition of 0.1 M HCl to the extract at 10°C while stirring. After standing for 20 h at 5°C the precipitate was removed by centrifugation for 1 h at 14 000 × *g* (Sorvall RC2-B, rotor GS3). The pH of the supernatant was then adjusted to 7.0 by the addition of 0.1 M NaOH.

Precipitation with ammonium sulphate. This was performed by addition of

solid $(NH_4)_2SO_4$ to the solution at 22°C. After standing for 45 min the precipitate was collected by centrifugation for 15 min at 14 000 × *g*. Dialysis was carried out in cellophane tubing at 4°C.

Ion-exchange chromatography. DEAE-cellulose (DE 23, Whatman) equilibrated with 25 mM Tris · HCl buffer (pH 7.0) + 0.10 M NaCl + 0.25 mM dithiothreitol was packed in a column (20 cm × 19.6 cm^2) by means of a peristaltic pump as described previously [4]. The particle-free extract from 2—4 brains (3000—6000 ml, 10—20 g protein) was applied to the column. One bed volume of starting buffer was passed through the column, and elution carried out using a linear gradient from the starting buffer (1000 ml) to 25 mM Tris · HCl buffer (pH 7.0) + 0.40 M NaCl + 0.25 mM dithiothreitol (1000 ml). Sample application, rinsing and elution were carried out at a rate of 9 ml · cm^{-2} · h^{-1} using a peristaltic pump. The eluate was collected in 20-ml fractions. Conductivity of the eluate was measured at 25°C. All other operations were performed at 4°C.

Rechromatography on DEAE-cellulose was performed on DE-32 (Whatman) equilibrated with 10 mM sodium citrate buffer (pH 5.5) + 0.25 mM dithiothreitol packed in a column (15 cm × 4.9 cm^2) at a rate of 35 ml · cm^{-2} · h^{-1}. The sample (10 ml, 20—40 mg protein) was dialyzed against the starting buffer and applied to the column. One bed volume of starting buffer was passed through the column, and elution carried out using a linear gradient from the starting buffer (500 ml) to 25 mM sodium citrate buffer (pH 5.5) + 0.40 M NaCl + 0.25 mM dithiothreitol (500 ml) at a rate of 10 ml · cm^{-2} · h^{-1}. The eluate was collected in 20-ml fractions. The conductivity of the eluate was determined at 25°C.

Gel chromatography. Preparative gel chromatography on Sephadex G-200 (Pharmacia) was carried out at 4°C on a column (87 cm × 19.6 cm^2, Pharmacia K50/100) equilibrated with 25 mM Tris · HCl buffer (pH 7.0) + 1.0 M NaCl + 0.25 mM dithiothreitol. The sample was applied to the column and ascending elution carried out at a rate of 1.5 ml · cm^{-2} · h^{-1} using a peristaltic pump. The volume of the sample was at most 20 ml, and the eluate was collected in 20-ml fractions.

Estimation of molecular weight. This was performed on Sephadex G-200 at 4°C. The size of the column (Pharmacia K25/100 equipped with long adaptors) was 60 cm × 4.9 cm^2. The sample (at most 1.0 ml) including the marker substances was applied to the bottom of the column and ascending elution carried out at a rate of 1.5 ml · cm^{-2} · h^{-1}. The eluate was collected in 2.5-ml fractions. Two different buffer systems were used: 25 mM Tris · HCl (pH 7.0) + 1.0 M NaCl, and 25 mM Tris · HCl (pH 7.0) + 0.20 M KCl; both contained 0.25 mM dithiothreitol.

Marker substances. Molecular weights were taken from ref. 6.

Alkaline phosphatase (M_r = 80 000). 10 µl of the enzyme suspension was added to each sample. Assay: 0.2 ml of the eluate was added to 0.6 ml of substrate solution (3 mM *p*-nitrophenyl phosphate in 33 mM Tris · HCl buffer (pH 8.0)). The mixture was incubated for 15 min at 30°C and 0.8 ml of 0.2 M NaOH was added. A_{405nm} was measured against a blank and used as a measure of the enzyme activity.

Xanthine oxidase (M_r = 275 000). 150 µl of the enzyme suspension was centrifuged for 2 min at 17 000 × *g* (Microcentrifuge, Ole Dich, Copenhagen),

and the precipitate dissolved in the sample. Assay: 0.5 ml of the eluate was mixed with 0.5 ml of substrate solution (0.2 mM hypoxanthine + 0.10 mM EDTA in 50 mM Tris · HCl buffer (pH 7.0)) and incubated for 30 min at 30°C; A_{293nm} was determined against a blank and used as a measure of the enzyme activity.

Alcohol dehydrogenase (M_r = 150 000) and apoferritin (M_r = 485 000). 4 mg of alcohol dehydrogenase and 10 mg of apoferritin were used in each experiment. The concentrations in the eluate were estimated by measuring A_{230nm}. Apoferritin exhibited an additional peak in the exclusion volume.

Calculation of molecular weight [6]. The activities of butyrylesterase and of the marker enzymes or the concentration of the marker proteins were plotted against the elution volume, and the effluent volumes determined by extrapolating the slopes of the peaks to their apexes. The effluent volumes were plotted against log M_r.

Gel chromatography on Sephadex G-200 of inhibited enzyme. Samples of purified butyrylesterase were treated with 1.25×10^{-4} M *p*-hydroxymercuribenzoate until about 98% inhibition was achieved. Chromatography was performed on a column (58 cm × 4.9 cm^2) equilibrated and eluted with 25 mM Tris · HCl (pH 7.0) + 0.2 M KCl + $5 \cdot 10^{-7}$ M *p*-hydroxymercuribenzoate at a rate of 8.8 ml/h. Fractions of 2.5 ml were collected. In aliquots of the fractions the enzyme was reactivated by incubation for 1 h with 2-mercaptoethanol (10 mM) before determination of the enzyme activity.

Isoelectric focussing. This was carried out using a 110 ml column (8101, LKB, Stockholm) thermostatted at 4°C. 2% solutions of Ampholine pH 3–6 (LKB) were used as described previously [4]. The sample (2–4 mg of protein) was dialyzed against 1% glycine, and applied together with the sucrose gradient. A voltage of 500 V was applied for 40 h, and fractions of 2.0 ml collected. pH was determined at 25.0°C.

Treatment with sialidase. Treatment was performed as described previously [7]. For comparison, a sample of human plasma cholinesterase was treated in the same way.

Electrophoresis. This was performed on agarose and starch gel slabs in a horizontal apparatus at 10°C. 1% agarose slabs (110 × 90 × 1.5 mm) were cast on glass plates in the following three buffer systems: 10 mM sodium phosphate (pH 7.0), 10 mM sodium glycinate (pH 9.2), and 10 mM sodium acetate (pH 5.5). Slots (8.2 × 0.6 mm) for application of the sample were formed during the casting 10 mm from the cathodic end. Samples of 3 µl were applied, previously dialyzed against the gel buffer, and electrophoresis carried out for 90 min at 5 V/cm. The corresponding bridge buffers were 50, 100, and 100 mM, respectively. After the run the plate was broken longitudinally and one half stained for protein with amido-black 10B, and the other for butyrylesterase by immersing the gel in a solution of α-naphthylbutyrate (0.01%, w/v) and Fast Blue RR (0.02%, w/v) in a 0.2 M sodium phosphate buffer (pH 7.2) at 37°C.

Starch gel electrophoresis was carried out according to ref. 5, using the following buffers: gel buffer, 5 mM NaH_2PO_4 + 3 mM sodium citrate; bridge buffer, 0.25 M NaH_2PO_4 + 0.15 M sodium citrate (pH 5.9); gel buffer, 6 mM sodium phosphate; bridge buffer, 0.20 M sodium phosphate (pH 7.2); gel buffer, 7.5 mM Tris · HCl + 0.6 mM $MgCl_2$; bridge buffer, 0.25 M Tris · HCl + 3

mM $MgCl_2$ (pH 8.6). After the run the gel was sliced horizontally, one half was stained for esterase activity and the other for protein. The slabs were scanned by means of a densitometer (Kipp-Zonen, DD2).

Protein. Protein concentrations were determined by a modified biuret reaction [4]. The sample was dialyzed against 50 mM sodium phosphate buffer (pH 7.0) before analysis. The transmittance of the eluates from chromatographic experiments was continuously recorded at 254 nm (Uvicord, LKB, 0.3 cm light path) or A_{280nm} was measured.

Determination of dithiothreitol. This was performed in stored samples of enzyme as follows: 10 µl of the sample was added to 1.0 ml of 1 mM Nbs_2 in 50 mM sodium phosphate buffer (pH 8.0). A_{412nm} was measured against a blank, and the concentration calculated from the molar absorbtivity (13 600 $mol^{-1} \cdot l \cdot cm^{-1}$) [8].

Carboxylesterase activity. The activity of carboxylesterase was routinely determined at 30°C with α-naphthylacetate (0.47 mM, pH 7.5), α-naphthylbutyrate (0.19 mM, pH 7.5), *p*-nitrophenylacetate (0.47 mM, pH 7.0) and *p*-nitrophenylbutyrate (0.47 mM, pH 7.0) as substrates by continuous recording of the increase in absorbance at 322 nm (naphthyl esters) and 348 nm (*p*-nitrophenyl esters) [4]. Eserine salicylate ($5 \cdot 10^{-5}$ M) was present in the reaction mixture to inhibit cholinesterases, except for the inhibitor experiments performed on the purified enzyme. In comparative experiments the α-naphthyl esters of acetic, propionic, butyric, and valeric acid were used in a final concentration of 0.1 mM. The hydrolysis of acetylthiocholine and butyrylthiocholine was determined by the method of Ellman et al. [9].

Phosphatase activity. The activity of phosphatase was determined as described previously [5].

Inhibitor experiments. Solutions of *p*-hydroxymercuribenzoate, 2-chloromercuri-4-nitrophenol, sodium iodoacetate, *N*-ethylmaleimide, iodoacetamide, and Nbs_2 were prepared with 25 mM Tris · HCl buffer (pH 7.0 and 7.5). Solutions of diethyl-*p*-nitrophenyl phosphate (Paraoxon), bis-*p*-nitrophenyl phosphate, iPr_2P-F and eserine salicylate were prepared with water. The actual concentration of diethyl-*p*-nitrophenyl phosphate was determined by adding an equal volume of 1.0 M NaOH to the solution and measuring A_{405nm} after standing for 30 min at 80°C. The absorbance was determined against a blank prepared with unhydrolyzed inhibitor in water. The molar absorptivity (18 440 $mol^{-1} \cdot l \cdot cm^{-1}$) was taken from ref. 10.

Incubation of the inhibitors with the enzyme was performed at 30°C. At the end of the incubation period the reaction was stopped in an aliquot of the incubation mixture by its addition to the substrate solution.

Results

Purification

The crude extract was freed from particles by acidification and centrifugation. The particle-free extract was chromatographed on DEAE-cellulose at pH 7.0, and carboxylesterase activity determined with α-naphthylacetate and -butyrate as substrates (Fig. 1). The chromatogram exhibited a peak of α-naphthylbutyrate-splitting enzyme comprising about 50% of the total α-

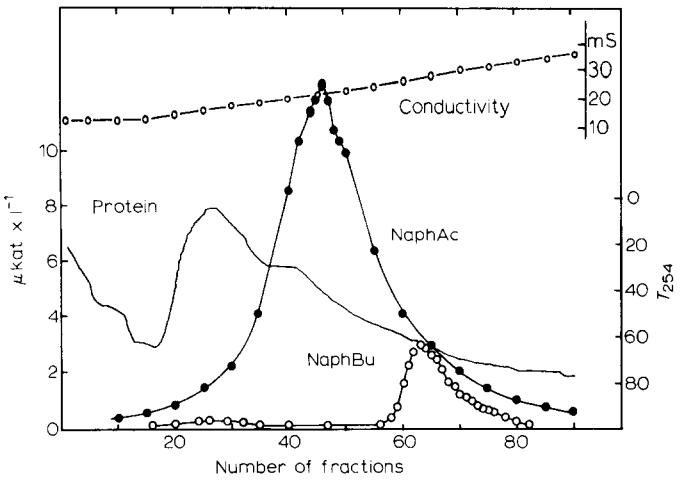

Fig. 1. DEAE-cellulose chromatography at pH 7.0 (4°C) of particle-free extract (3000 ml) from one brain. Abscissa: number of 20-ml fractions. Left ordinate: carboxylesterase activity as determined with α-naphthylacetate (●——●) and -butyrate (○——○) as substrates at pH 7.5 and 30°C. Right ordinate: protein concentration as estimated from continuous recording of transmittance at 254 nm; conductivity of the eluate as measured at 25°C.

naphthylbutyrate-splitting enzyme activity of the extract. About half of the α-naphthylbutyrate-splitting activity was associated with other carboxylesterases which were eluted by the starting buffer [4]. The peak was collected and the enzyme precipitated at 2.0 M $(NH_4)_2SO_4$. Most of the α-naphthylacetate-splitting enzymes remained in the supernatant. The precipitate was dissolved in 15 ml of 20 mM Tris · HCl buffer (pH 7.0) + 0.25 mM dithiothreitol, and subjected to gel chromatography on Sephadex G-200 (Fig. 2). The peak was collected and the enzyme precipitated at 1.5 M $(NH_4)_2SO_4$. This step resulted in a slight purification as well as a concentration of the sample. The precipitate was

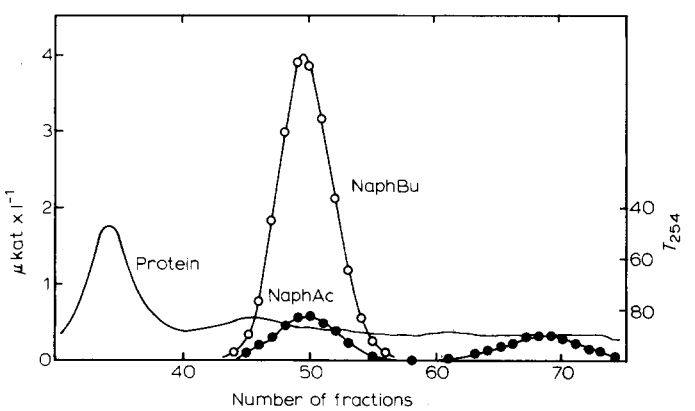

Fig. 2. Gel chromatography on Sephadex G-200 (4°C) (step III of the purification procedure). Bed volume: 1700 ml. Abscissa: number of fractions of 20 ml. Left ordinate: carboxylesterase activity as determined with α-naphthylacetate (●——●) and -butyrate (○——○) as substrates at pH 7.5 and 30°C. Right ordinate: protein concentration as estimated from continuous recording of transmittance at 254 nm.

dissolved in buffer (see above) and rechromatographed on DEAE-cellulose at pH 5.5 (Fig. 3). A final precipitation at 1.5 M $(NH_4)_2SO_4$ resulted in a further purification of the enzyme.

Three experiments, each based on 2—4 brains, resulted in a 650—800-fold increase in specific activity. The yield averaged 22% as calculated on the basis of the amount of enzyme obtained after step I. Calculation of the yield as related to the content of α-naphthylbutyrate-splitting enzyme activity in the extract is meaningless since about 46% (mean of three experiments) of this activity derived from other enzymes [4], see above. The results obtained in one of these experiments are given in Table I.

Higher yields (45%) were achieved in nine experiments, each based on one brain, but the purification was lower (280—350-fold) than that obtained in the three experiments referred to above.

Purity

The identical distribution of the enzyme activities as determined with different substrates in the fractions obtained by chromatography on DEAE-cellulose at pH 5.5 (Fig. 3), indicates that the ability of the preparation to split different esters represents an inherent property of the enzyme. The protein profile indicates only slight contamination with other proteins.

Electrophoresis on starch gel at pH 5.9, 7.2 and 8.6 and on agarose gel at pH 5.5, 7.0 and 9.2 showed only one single band of protein. Single bands of esterase activity with α-naphthylbutyrate as substrate were seen at the same locations, indicating a high degree of purity. The results from electrophoresis on agarose at pH 5.5 and 7.0 are shown in Fig. 4. The specific activity of the pure

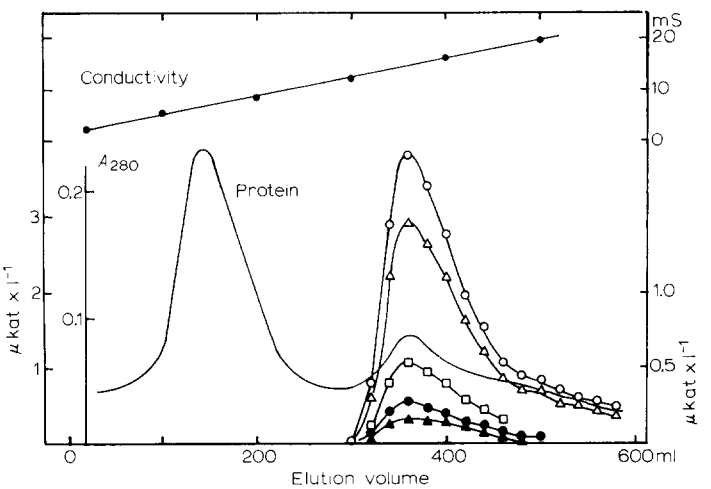

Fig. 3. Rechromatography on DEAE-cellulose at pH 5.5 (4°C) (step V of the purification procedure). Abscissa: number of 20-ml fractions. Left ordinates: carboxylesterase activities as determined with α-naphthylacetate (●———●), α-naphthylbutyrate (○———○), *p*-nitrophenylacetate (▲———▲), *p*-nitrophenylbutyrate (△———△). Protein concentrations as estimated from the absorbance at 280 nm. Right ordinates: enzyme activity with butyrylthiocholine as substrate (□———□). Conductivity of the eluate as measured at 25°C.

TABLE I

Purification of butyrylesterase from a particle-free extract (6900 ml) obtained from four brains. The enzyme activity was determined with α-naphthylbutyrate (0.2 mM) as substrate at pH 7.5 and 30°C. The concentration of protein was determined by a modified biuret method [4].

Purification step		Total enzyme (nkat) *	Recovery (%)	Protein concentration (mg/ml)	Enzyme activity (nkat/mg) **	Purification
Particle-free extract		4583		5.59	0.12	1
I	DEAE chromatography, pH 7.0	2066	100	3.78	1.17	9.9
II	Precipitation with $(NH_4)_2SO_4$ (2.0 M)	1816	88	34.3	2.58	21.9
III	Gel chromatography with Sephadex G-200	1252	61	0.82	10.53	89.2
IV	Precipitation with $(NH_4)_2SO_4$ (1.5 M)	1062	52	3.24	19.33	164
V	DEAE chromatography, pH 5.5	478	23	0.11	58.67	497
VI	Precipitation with $(NH_4)_2SO_4$ (1.5 M)	478	23	0.60	94.17	798

* nmol/s

** nmol/s per mg protein

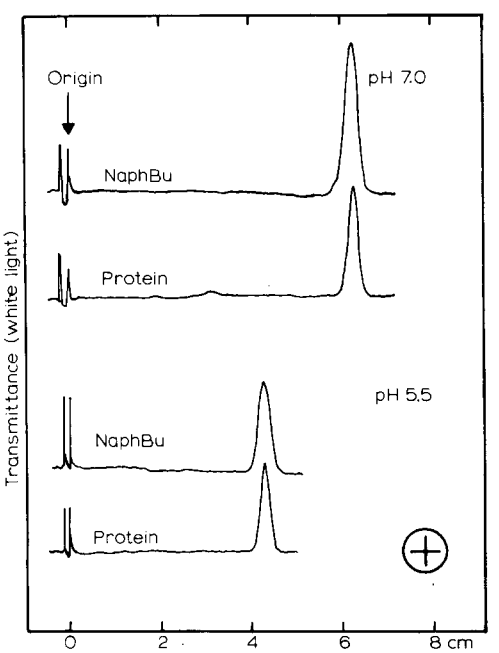

Fig. 4. Electrophoresis of the purified enzyme on agarose gel at pH 7.0 and 5.5 (10°C). After staining for enzyme activity with α-naphthylbutyrate (NaphBu) as substrate, and for protein with amido-black 10B, the slabs were scanned using a densitometer equipped with a tungsten light source without filter. The transmittance was recorded.

enzyme was about 0.1 kat/kg as based on protein determinations using the biuret method.

Stability

The activity of purified samples of the enzyme decreased rapidly in buffer solutions at pH 7 and 4°C, the half-life being less than 2 weeks. Inactivation was prevented by addition of 5 mM dithiothreitol + 1 mM EDTA. However, dithiothreitol was oxidized in the course of 3—4 weeks. Frequent determinations of the concentration of dithiothreitol were therefore performed, and when the concentration was less than 0.2 mM the sample was dialyzed against 5 mM dithiothreitol + 1 mM EDTA. Preparations of the enzyme treated in this way remained stable for several months.

Isoelectric focussing

The purified enzyme was resolved into several fractions by isoelectric focussing. In 11 experiments the enzyme was resolved into 3—6 peaks with isoelectric points at pH 3.9, 4.0, 4.1, 4.2, 4.5, and 4.7. In all experiments the recovery was about 75%. In each experiment there was a predominance of peaks with isoelectric points either about pH 4.0 (Fig. 5A) or about pH 4.7 (Fig. 5B). No explanation of this variation is available.

Molecular weight

The molecular weight of the enzyme was estimated as 340 000 (S.D. = 8000, $n = 4$) by gel chromatography on Sephadex G-200 in two different buffer systems, see Materials and Methods.

Effect of sialidase

The low isoelectric point of the enzyme might be due to terminal sialic acid

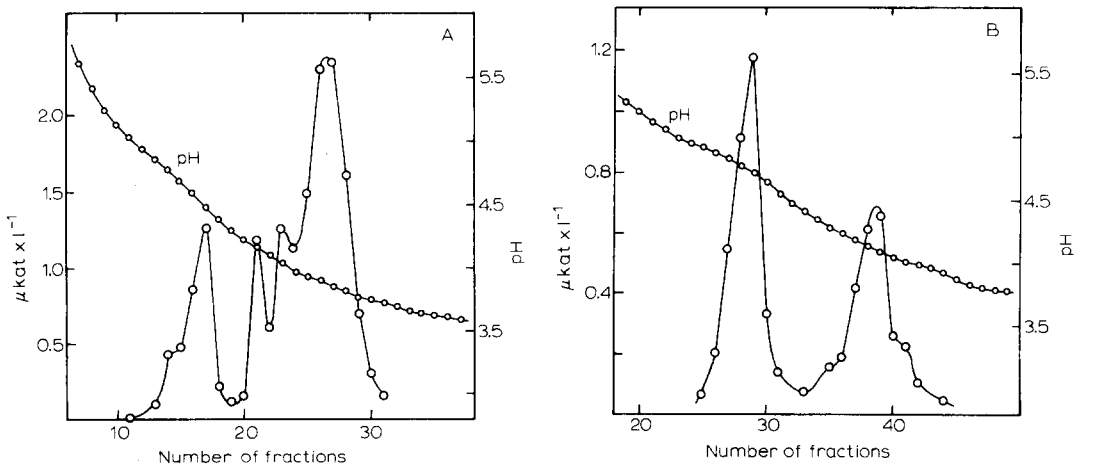

Fig. 5. Isoelectric focussing in Ampholine, pH 3—6 of the purified enzyme at 4°C. A and B represent two experiments on the same enzyme preparation (see text). Abscissa: number of 2-ml fractions. Ordinates: carboxylesterase activity as determined with α-naphthylbutyrate as substrate; pH of the eluate as measured at 25°C.

residues as shown for butyrylcholinesterases from human tissues [11]. The enzyme was therefore treated with sialidase and subjected to electrophoresis on starch gel at pH 7.2. A sample of human plasma cholinesterase was treated similarly for comparison. The electrophoretic migration did not change, indicating that sialic acid residues were not involved (Fig. 6).

Substrate specificity

The relative rates at which the enzyme preparation catalyzed the hydrolysis of α-naphthylacetate, α-naphthylbutyrate, *p*-nitrophenylacetate and *p*-nitrophenylbutyrate appear from Fig. 3, Tables II and III. The effect of the enzyme on different carboxylic acid esters was investigated by determining the enzyme activity with 0.1 mM solutions of α-naphthyl esters of acetic, propionic, butyric and valeric acid as substrates. The butyrate was split at the highest rate (Table II).

The purified enzyme catalyzed the hydrolysis of acetylthiocholine and butyrylthiocholine at low rates. To ascertain that this was not due to a contamination with cholinesterase, eserine was added in a concentration which abolished completely the activity of cholinesterase (Table III).

Lower activities of butyrylesterase were observed with phosphate buffers than with Tris buffers. It was therefore investigated whether the preparation exerted phosphatase activity. However, *p*-nitrophenyl phosphate was not split, either at pH 6.0 (sodium citrate buffer), or at pH 7.7 (Tris · HCl buffer).

Inhibitors

Diethyl p-nitrophenyl phosphate. The enzyme was irreversibly inhibited by diethyl *p*-nitrophenyl phosphate. The inhibition was followed as a function of time for 3—4 half-life periods with four different concentrations of the inhibitor ($0.53 \cdot 10^{-5}$—3.52×10^{-5} M) in 42 mM Tris · HCl buffer at pH 7.5 and 30°C.

The inactivation followed pseudo first-order kinetics, and the first-order rate constants were proportional to the concentration of the inhibitor. The second-order rate constant (k = 206 $mol^{-1} \cdot l \cdot s^{-1}$, S.D. = 13, n = 8) was calculated

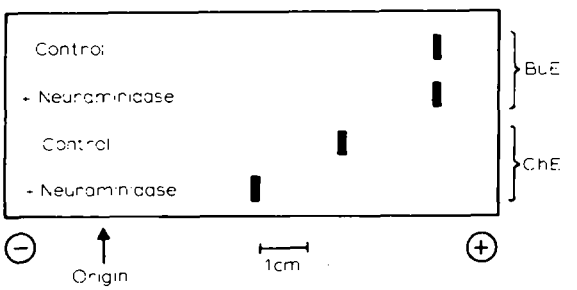

Fig. 6. Electrophoresis on starch gel of untreated and neuraminidase-treated butyrylesterase (BuE) and human plasma cholinesterase (ChE). Electrophoresis was performed for 4 h at 4°C at pH 7.2. Staining was performed with α-naphthylbutyrate as substrate.

TABLE II

Relative activity of butyrylesterase at pH 7.5 and 30°C with α-naphthyl esters of different carboxylic acids as substrates: acetic acid (NaphAc); propionic acid (NaphPro); butyric acid (NaphBu); valeric acid (NaphVal). The substrate concentrations were 0.1 mM. The enzyme activity with α-naphthylbutyrate as substrate was arbitrarily assigned the value of 1.00.

	Substrate			
	NaphAc	NaphPro	NaphBu	NaphVal
Number of carbon atoms in the carboxylic acid	2	3	4	5
Relative enzyme activity	0.11	0.42	1.00	0.35

from

$$k = \frac{\ln a - \ln x}{t \cdot b}$$

where a is the enzyme activity without inhibitor, x the enzyme activity after incubation with the inhibitor for t s, and b the molar concentration of the inhibitor.

Reactivation of the inhibited enzyme did not take place after removal of excess of inhibitor by dialysis, suggesting that the inhibitor binds strongly to the active site.

iPr_2P-F. The enzyme was rapidly inhibited by iPr_2P-F (Table IV). The inhibition was not reversed by dialysis.

Eserine salicylate ($8 \cdot 10^{-5}$ M). This compound did not inhibit the enzyme at all (Table IV). In control experiments, the splitting of α-naphthylbutyrate by human plasma cholinesterase was completely inhibited after incubation for 2 min with eserine ($5 \cdot 10^{-5}$ M).

Bis-p-nitrophenyl phosphate. This compound, which has been designated as a specific inhibitor of carboxylesterases [12], did not inhibit the enzyme significantly (Table IV).

TABLE III

The relative enzyme activity of butyrylesterase with α-naphthylbutyrate (NaphBu) (0.2 mM), α-naphthylacetate (NaphAc) (0.5 mM), acetylthiocholine, (AcThCh) (0.5 mM), and butyrylthiocholine (BuThCh) (0.5 mM) as substrates at pH 7.5, and with *p*-nitrophenylacetate (pNPAc) (0.5 mM) and *p*-nitrophenylbutyrate (pNPBu) (0.5 mM) as substrates at pH 7.0 and 30°C. The enzyme activity was determined in the absence and in the presence of eserine salicylate. The activity with α-naphthylbutyrate as substrate in the absence of eserine was arbitrarily assigned the value of 1.00.

Eserine (M)	Relative enzyme activity					
	NaphAc	NaphBu	AcThCh	BuThCh	pNPAc	pNPBu
0	0.14	1.00	0.004	0.14	0.10	0.83
5×10^{-5}	—	0.97	0.004	0.14	—	—

TABLE IV

Effect of inhibitors on butyrylesterase with α-naphthylbutyrate as substrate. The experiments were performed with 25 mM Tris · HCl buffers (pH 7.0 or 7.5) at 30°C.

Inhibitor	Concentration of inhibitor (M)	pH	Incubation time (min)	Inhibition (%)
Iodoacetate	5×10^{-3}	7.5	90	0
Iodoacetamide	5×10^{-3}	7.5	90	0
N-Ethylmaleimide	1×10^{-4}	7.5	60	13
	1×10^{-4}	7.5	150	32
	2×10^{-4}	7.5	60	18
	2×10^{-4}	7.5	150	44
Nbs_2	1×10^{-4}	7.5	12	16
	1×10^{-4}	7.5	33	19
	1×10^{-4}	7.5	150	30
iPr_2P-F	5×10^{-5}	7.5	3	100
	5×10^{-7}	7.5	2	71
Bis-*p*-nitrophenyl phosphate	1×10^{-5}	7.0	60	0
	1×10^{-4}	7.0	60	20
	5×10^{-4}	7.0	60	17
Eserine salicylate	8×10^{-5}	7.5	50	0

$CaCl_2$, $MgCl_2$, $ZnSO_4$ or EDTA. These, in concentrations of 1.0 mM, had no effect on the enzyme activity.

Inhibition by SH reagents.

p-Hydroxymercuribenzoate in low concentrations caused a slowly progressive inhibition of the enzyme. The inhibition was followed as a function of time at pH 7.5 (25 mM Tris · HCl) and 30°C with four different concentrations of the inhibitor (4×10^{-5}—1×10^{-4} M). The enzyme used in these experiments was meticulously dialyzed to remove any trace of dithiothreitol. The inactivation followed pseudo first-order kinetics for 4—5 half-life periods, i.e. the rate constant varied proportionally to the concentration of the inhibitor. The second-order rate constant was 40 $mol^{-1} \cdot l \cdot s^{-1}$ (S.D. = 2, $n = 4$). At pH 7.0 (25 mM Tris · HCl) the rate constant was about six times higher than at pH 7.5. Dialysis of a completely inhibited sample of the enzyme did not reactivate the enzyme to any extent. Addition of dithiothreitol (1 mM) or 2-mercaptoethanol (2 mM) reactivated the enzyme to 80% of the original activity in the course of 30 min.

The reaction with *p*-hydroxymercuribenzoate did not cause a dissociation of the enzyme into subunits. In gel chromatography on Sephadex G-200 native and inhibited enzyme behaved identically (four experiments).

2-Chloromercuri-4-nitrophenol also inhibited the enzyme. At pH 7.5 a concentration of $1 \cdot 10^{-5}$ M of the inhibitor caused a progressive inactivation of the enzyme with time. First-order kinetics were not followed, as the rate constant decreased with time. At a concentration of $1 \cdot 10^{-4}$ M complete inactivation was obtained within 2 min. The experiments were performed with the same concentration of enzyme and in the same buffer as described for *p*-hydroxy-

mercuribenzoate. Treatment for 24 h with 2-mercaptoethanol (1—10 mM) did not reactivate the enzyme.

Iodoacetate, *iodoacetamide*, *N-ethylmaleimide* and Nbs_2 exhibited no or only feeble inhibitory effect on the enzyme (Table IV).

Discussion

The occurrence in nearly all animal tissues of multiple forms of non-specific esterases has been widely reported. There are only few thorough investigations on the properties of separate enzymes although microsomal esterases from liver and kidney have been extensively studied [3—7].

Human brain contains an unusually high number of non-specific (or eserine-resistant) esterases, and it has been suggested that they may differ from those of other tissues [1—3]. Hitherto, no attempts have been made to investigate their properties in detail. Previous findings indicate that at least four different carboxylesterases might be isolated from human brain extracts [4]. This paper reports on the purification and properties of one of these enzymes.

The report concerns an enzyme which splits α-naphthylbutyrate at a higher rate than the acetate. The enzyme was purified about 800-fold, and the preparation appeared to be pure. Its molecular weight (340 000) was much higher than that of other brain carboxylesterases [4], and its isoelectric point was lower (pH 4.0—4.7). This did not seem to be due to terminal sialic acid residues. In isoelectric focussing several bands were formed.

The enzyme was rapidly inactivated in the absence of reducing agents such as dithiothreitol, which may indicate that the maintenance of SH groups is a prerequisite for the proper function of the enzyme.

Only artificial substrates were investigated; a search for biological substrates is in progress. α-Naphthylbutyrate and *p*-nitrophenylbutyrate were split at about the same rate; the corresponding acetates were split at much lower rates. Of the series α-naphthylacetate, -propionate, -butyrate, and -valerate, the butyrate was split at the highest rate. It is remarkable that *p*-nitrophenyl esters and α-naphthyl esters were split at about the same rate, since these substrates have been used to discriminate between aryl- and carboxylesterases [18]. The butyrylesterase also splits thiocholine esters at low rates both in the presence and absence of eserine, the butyrate was split at a higher rate than the acetate. The resistance to eserine shows that the activity is not due to contamination with cholinesterases. This finding suggests that the enzyme is identical to an esterase-fraction previously found in human brain extract (fraction D, Fig. 1 in ref. 7).

The irreversible inhibition of the enzyme by diethyl-*p*-nitrophenyl phosphate and iPr_2P-F indicates that the enzyme contains serine in the active site. The rate constant of the inhibition with diethyl-*p*-nitrophenyl phosphate ($206\ mol^{-1} \cdot l \cdot s^{-1}$) was low as compared to those found for liver carboxylesterase [19] ($1.3 \cdot 10^5\ mol^{-1} \cdot l \cdot s^{-1}$), acetylcholinesterase [20] ($0.8 \cdot 10^4\ mol^{-1} \cdot l \cdot s^{-1}$), and butyrylcholinesterase [20] ($2.5 \cdot 10^4\ mol^{-1} \cdot l \cdot s^{-1}$). This agrees with earlier suggestions that brain carboxylesterases are more resistant to diethyl-*p*-nitrophenyl phosphate than esterases from other tissues [21,22]. Bis-*p*-nitro-

phenyl phosphate, known as a specific carboxylesterase inhibitor [12], had no significant effect on the butyrylesterase.

The inactivation of the enzyme by *p*-hydroxymercuribenzoate is of particular interest because of its use to distinguish between two different types of esterases, namely aryl- or A-esterases (EC 3.1.1.2) with SH groups as components of their active sites, and carboxyl- or B-esterases (EC 3.1.1.1) with serine in the active site [23,24]. Indirect evidence has been presented previously that brain tissue contains esterases which are inhibited by organic mercurials as well as by organophosphorus compounds [1]. In the present study an enzyme was actually isolated which was inhibited by both types of inhibitors. The fact that the enzyme was irreversibly inhibited by diethyl-*p*-nitrophenyl phosphate and iPr_2P-F indicates that the active site comprises serine as an active group. On the other hand, the inactivation by *p*-hydroxymercuribenzoate does not by any means indicate that a reaction with SH groups in the active site does occur. Indeed, the low rate of the reaction, and the finding that other SH reagents did not inactivate the enzyme, or did so very slowly, indicates that groups of the active site are not involved [25]. This is also supported by the finding that the rate constant for the inactivation with *p*-hydroxymercuribenzoate was higher at pH 7.0 than at 7.5, i.e. the opposite effect to that expected for easily accessible SH groups.

A similar effect of pH was found for the slow reaction of *p*-hydroxymercuribenzoate with 'masked' SH groups in hemoglobin [26]. This reaction caused a dissociation of the molecule into subunits [27]. It is of interest to note that *N*-ethylmaleimide and iodoacetate did not react with 'masked' SH groups of the hemoglobin molecule [27].

The inactivation of butyrylesterase by *p*-hydroxymercuribenzoate however, was not caused by a disintegration into subunits since the molecular weight of the inactivated enzyme did not differ from that of the native enzyme. The inhibitory effect of *p*-hydroxymercuribenzoate may possibly be due to a reaction with 'masked' SH groups essential for the maintenance of the native conformation of the enzyme. It should be noted that the inactivation was reversible by treatment with dithiothreitol or 2-mercaptoethanol.

The findings reported in this study indicate that the butyrylesterase is a serine-enzyme, i.e. a carboxylesterase. It differs from other mammalian carboxylesterases investigated [13] in being inactivated by organic mercurials. However, in bacteria an esterase was found to be inhibited by organophosphates as well as by *p*-hydroxymercuribenzoate [28]. We have found recently that human red cells contain an enzyme with identical properties (to be published). Quantitative considerations completely exclude the possibility that its presence in the brain arises from contamination with blood. Possibly, the enzyme is identical with an esterase (B_1) found in several human tissues [29].

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