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ISOLATION AND PROPERTIES OF SOME MOLECULAR SPECIES OF HUMAN BRAIN ESTERASES

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SUMMARY

Preparations of the carboxylic ester hydrolases of human brain were obtained by $(NH_4)_2SO_4$ fractionation and DEAE-Sephadex chromatography procedures. Organophosphate-sensitive esterases (B-type) were precipitated by 40% $(NH_4)_2SO_4$ saturation. Esterases resistant to organophosphate derivatives (A-type) and activated by *p*-chloromercuribenzoate (PCMB) (C-type) were precipitated from the remaining supernatant by 65% $(NH_4)_2SO_4$ saturation. The A- and C-type esterases were further fractionated by column chromatography into cathodic- and slow and fast anodic-migrating enzymes.

1. The major anodic enzymes showed hydrolytic activity toward aryl esters of acetic, propionic and butyric acids and were devoid of peptidase, amidase or cholinesterase activity.

2. The faster-migrating anodic enzymes were shown by starch-gel electrophoresis to be inactivated by air oxidation and could be reactivated by reducing agents. Reducing agents also reverse the inactivation of the enzyme by 8 M urea. The C-type esterase remained active only in the presence of sulfhydryl-reacting compounds or reducing agents capable of forming sulfhydryl derivatives. The esterases of intermediate mobility are inactivated by high concentration of reducing agents or sulfhydryl-reacting compounds and irreversibly inactivated by 8 M urea.

3. It was concluded that the fast-moving esterases require either free SH groups or SH groups reacted with small reducing molecules, such as iodoacetamide or mercaptol derivatives, in order to maintain the conformation required for activity. In contrast, the intermediary anodic esterases appear to require both free SH and S–S groups for activity, since they are inhibited by PCMB and inactivated by high concentrations of reducing agents. It appears that the 3 esterase groups described are SH–enzymes, but differ from each other in the role of the SH group in the determination of enzyme activity.

Abbreviation: PCMB, *p*-chloromercuribenzoate.

INTRODUCTION

This laboratory has previously reported on the separation of carboxylic ester hydrolases of brain by starch-gel electrophoresis and the variety of enzymatic molecular species demonstrable[1,2]. With α-naphthyl acetate as a substrate, these procedures reveal that as many as 14–16 isozyme bands can be extracted in aqueous medium, and though they vary in their sensitivity to inhibitors and activators, are generally resistant to diethyl-*p*-nitrophenyl phosphate and classified as A-esterases. A C-esterase which is activated by some sulphydryl reagents can also be detected[3]. A bound esterase, localized in the endoplasmic reticulum, extracted by 1% Triton X-100 and sensitive to organophosphate inhibitors (B-esterase) has also been reported[4]. Purification of non-specific esterases from plasma or kidney[5] and intestine[6] has been reported and RAMACHANDRAN AND ÅGREN[7] have investigated the DFP-bound esterases in liver cell fractions.

This work presents a method for the separation and isolation of some of the carboxylic acid esterases from human brain by fractionation with $(NH_4)_2SO_4$, their purification on DEAE-Sephadex columns, and an examination of their differential stability and behavior toward SH-reagents. The isolation of the various purified fractions may lead to an understanding of their specific properties and the role these enzymes may play in brain metabolism.

METHODS AND MATERIAL

Human brain without any apparent neurological involvement was obtained at autopsy and placed in a deep freeze at $-60°$. The elapsed time between death and freezing varied between 4 and 24 h but experiments on the effect of *post-mortem* autolysis showed no loss of esterase activity during this interval. When ready for use, the brain was allowed to thaw at room temperature for 2 h and the required brain areas were dissected. Homogenates (1:3) of whole brain in 0.005 M phosphate buffer in 1% Triton X-100 (pH 7.5), were prepared in a Waring blender for 2 min at room temperature. After centrifugation at 105 000 $\times$ *g* for 2 h (Spinco L40, No. 30 rotor) the clear supernatant was decanted. A fluffy precipitate that formed in this procedure and was difficult to centrifuge could be sedimented by suspending in phosphate buffer before recentrifugation. The well-packed sediment was washed twice in buffer and the washings combined with the first supernatant. This solution was saturated to 40% $(NH_4)_2SO_4$ (ref. 8) and after removal of the precipitate (Fraction I), the solution was adjusted to 65% $(NH_4)_2SO_4$ saturation by addition of the solid crystals. After standing for 0.5 h, the suspension was centrifuged (Fraction II). The sediments were dissolved in phosphate buffer, dialyzed and concentrated. To concentrate the solutions two methods were used: (1) immersion of the dialysis tube containing the solution to be concentrated in powder aquacide II (Calbiochem, Los Angeles, Calif.); (2) using the Amicon diaflo cell membrane filtration apparatus (Amicon Corp., Lexington, Mass.). The fractions were kept frozen at $-20°$ if not used immediately.

Column chromatography

A jacketed chromatographic column (2 cm $\times$ 40 cm), (Pharmacia Fine Chemicals, Piscataway, N.J.) was prepared by packing with DEAE-Sephadex A-50 beads

equilibrated with 0.1 M Tris–HCl, 0.001 M EDTA, and 0.001 M mercaptoethanol buffer (pH 8.6). Solutions of Fraction II prepared as described above were dialyzed against 8 l of Tris–HCl buffer (pH 8.6) for 14 h at 4°. The clear supernatant was used for chromatographic fractionation. The temperature of the column was kept constant at +8° with the aid of a Lauda circulating-water bath (Brinkman Instruments, Inc., Westbury, N.Y.). The column was washed with 120 ml of buffer after addition of the sample. A Varigrad chamber[9] (Buchler Instruments, Fort Lee, N.J.) was used to produce a LiCl linear gradient from 0 to 0.5 M. The proteins were eluted from the column with 800 ml of buffer at a rate of 0.2 ml/min. 5-ml fractions were collected in a fraction collector (Gilson Medical Electronics, Middleton, Wisc.) for 48 h. To avoid bacterial growth, butanol was added to the buffer to a final concentration of 1% (ref. 10). Proteins were determined by 280 mμ absorption for each tube, and the fractions were then assayed for esterase activity using α-naphthyl acetate and α-naphthyl butyrate as substrates. Since enzymatic activity towards both substrates showed a marked decrease with time (3–4 days) and no α-naphthyl butyrate hydrolysis could be detected after 2 weeks, the assays were done as soon as possible after collection. Aliquots from tubes that showed esterase activity were pooled and the enzyme was concentrated by precipitation with solid $(NH_4)_2SO_4$ to 65% saturation. After centrifugation, the sediment was suspended in 0.05 M phosphate buffer (pH 7.0) and freed of excess salts by passing the sample through a Sephadex G-50 resin equilibrated with the phosphate buffer. The fractions containing esterase activity were concentrated by ultrafiltration using the Diaflo Membrane U.M.2 with a molecular exclusion weight of 1000. The concentrated purified sample was frozen between use.

Enzymatic assays

Fractions obtained by $(NH_4)_2SO_4$ precipitation were assayed using *o*-nitrophenyl acetate as substrate and the *o*-nitrophenol liberated was measured at 410 mμ. Assays were performed in a Beckman DB Spectrophotometer at 25° (pH 7.2) and the initial velocity determined for 5 min during which period zero-order kinetics were observed. The cell compartment temperature was kept constant by a Haake Thermostatic bath (Polyscience Corp., Evanston, Ill.) and absorption changes were followed in a linear-log recorder (E. H. Sargent and Co., Chicago, Ill.) attached to the spectrophotometer. Corrections for non-enzymatic hydrolysis were made by reading absorption of the substrate against water and after complete hydrolysis with 0.1 M NaOH (ref. 11). Extinction coefficients of $4.18 \cdot 10^3$ in 0.1 M NaOH and $1.97 \cdot 10^3$ at pH 7.2 were used to calculate *o*-nitrophenol concentration[12]. The substrate (0.001 M) was prepared by dissolving appropriate amounts of each substrate in 10 ml of absolute methanol and adding to 0.005 M phosphate buffer (pH 7.2) at 4° while stirring. The low temperature is required to minimize hydrolysis of the substrate.

Esterase activity of the samples eluted from the DEAE-Sephadex column was determined by a modification of the method of EMERSON[13] as utilized by ALDRICH[14]. The α-naphthol substrates ($1.38 \cdot 10^{-3}$ M) were prepared by dissolving appropriate amounts of each compound in methanol (final concentration, 4%) and adding this to a solution of 40% propylene glycol in 0.1 M Tris–HCl buffer (pH 8.0). The enzymatic reaction was followed by determination of the amount of α-naphthol produced after 30 min incubation of 1.5 ml of the substrate at 37°. The α-naphthol produced was measured by adding 0.1 ml of 0.4% 4-amino-antipyrine and 0.1 ml of 2% $K_3Fe(CN)_6$,

and absorption at 510 mμ was determined. A calibration curve of α-naphthol was used to measure hydrolysis. Enzymic units are expressed as the amount required to liberate 1 μM of *o*-nitrophenol or α-naphthol/min and specific activity is the number of enzyme units per mg protein as determined by the method of LOWRY *et al.*[15]. The unit designations follow the recommendation of the Commission on Enzymes.

To study the substrate specificity, emulsions of α-naphthyl myristate and α-naphthyl palmitate were made in the same buffer as the other esters, but 1% Triton X-100 buffer was used to emulsify the substrate. Cholinesterase was determined by the method of ELLMAN *et al.*[16]. Peptidase activity was determined using l-leucyl-β-naphthylamide hydrochloride or benzoyl l-arginyl-β-naphthylamide by the method of GOLDBARG AND RUTENBURG[17]. Partial reduction of disulfide bonds was accomplished by incubation of the enzyme with mercaptoethanol or dithiothreitol in buffer solution[18].

Electrophoresis

For the determination of the enzymic species population, the enzymes were separated on starch gel according to the method of SMITHIES[19]. Esterase activity was determined by hydrolysis of α-naphthyl acetate previously described[3]. Proteins were stained with Amido Black 10-B and destained by electrophoresis with carbon electrodes (Otto Hiller, Madison, Wisc.). To study the effect of urea, enzyme preparations were adjusted with buffered urea to a final concentration of 8 M. After incubation of the sample at 37° for 0.5 h with urea, mercaptoethanol or buffer, each fraction was applied to the gel which was free of urea or mercaptoethanol. Preparation of the gels, separation of the enzymes and inhibitor studies with diethyl-*p*-nitrophenyl phosphate, eserine sulfate and *p*-chloromercuribenzoate (PCMB) were carried out as previously described[3]. Controls where the inhibitor solutions were replaced by buffer were assayed at the same time. The effect of diethyl-*p*-nitrophenyl phosphate and eserine were also studied *in vitro*. The enzyme inhibitor solutions were preincubated for 0.5 h at 37° and then added to the α-naphthyl ester substrate solution. Enzymatic activity was determined as described above.

Reagents

o-Nitrophenol acetate, *o*-nitrophenol butyrate, α-naphthyl acetate, as well as the longer chain α-naphthyl ester, octanol and 2-mercaptoethanol and iodoacetamide were purchased from Sigma Chemical Co., St. Louis, Mo., and used without purification. 4-Amino-antipyrine was obtained from Eastman Organic Co., Rochester, N.Y. Tris-201 base was used to prepare Tris–HCl buffers. DEAE-A-50 Sephadex beads and Sephadex G-50 were supplied by Pharmacia Fine Chemicals, Inc., Piscataway, N.J. Diethyl-*p*-nitrophenyl phosphate was purchased from K and K Laboratories, Plainview, N.Y. Eserine sulfate and PCMB were received from Mann Research Lab., New York, N.Y. Dithiothreitol was furnished by P. L. Biochemicals, Inc., Pabst Laboratory, Milwaukee, Wisc., leucyl-β-naphthylamide hydrochloride, iodoacetamide and *N*-benzoyl-l-arginyl-β-naphthylamide hydrochloride from Mann Research Laboratories, Inc.

TABLE I

$(NH_4)_2SO_4$ FRACTIONATION OF HUMAN BRAIN ESTERASES

Enzyme units: number of ml of enzyme solution that hydrolyze 1 μmole of *o*-nitrophenyl acetate per min in a total of 1.6 ml, using a Beckman cuvette of 0.5-cm path, at 25°. Details as described in METHODS. $(NH_4)_2SO_4$ concentrations calculated with the nomogram of DIXON[8].

Steps	*Description*	*Volume (ml)*	*Enzyme activity (units)*	*Proteins (mg/ml)*	*Specific activity (μM/mg)*	*Recovery (%)*
1	Total homogenate	270	440.1	25.2	0.06	
2	Supernatant	273	354.9	9	0.14	80.6
3	0–40% $(NH_4)_2SO_4$ saturation (Fraction I)	100	235	10.6	0.22	66.1*
4	Fraction I after dialysis, concentration, centrifugation	30	126	30.0	0.14	35.5*
5	40–65% $(NH_4)_2SO_4$ saturation (Fraction II)	32	40	22.4	0.06	11.2*
6	Fraction II, after dialysis, concentration and centrifugation	9	41.6	53.0	0.09	11.7*

* Recovery from supernatant.

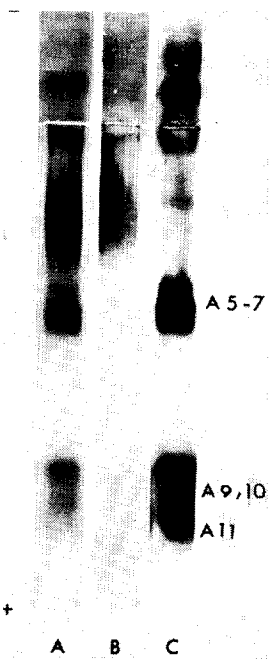


Fig. 1. Electrophoretic separation of human brain esterases with α-naphthyl acetate as substrate. A, Esterases extracted with 1% Triton X-100 in 0.005 M phosphate buffer (pH 7.5), 12.5 mg protein/ml. B, Fraction I precipitated by 40% saturation with $(NH_4)_2SO_4$, solubilized in 0.005 M phosphate buffer, dialyzed and concentrated to 30 mg protein/ml as described in text. C, Fraction II precipitated by 40%–65% saturation with $(NH_4)_2SO_4$, solubilized in 0.005 M phosphate buffer then dialyzed and concentrated to 53 mg protein/ml. The A5–7 bands are designated.

RESULTS

The results obtained by precipitation of the enzymes from the 40%- and 65%-saturated $(NH_4)_2SO_4$ are shown in Table I. With *o*-nitrophenyl acetate as substrate, appreciable increases in specific activity, which customarily demonstrate the degree of purification that occurs are not evident, but separation of the different molecular species can be achieved by this procedure (Fig. 1). The starch-gel electrophoresis patterns demonstrate that when compared to the total homogenate, the enzyme precipitated by 40% $(NH_4)_2SO_4$ saturation (Fig. 1B) consists of the diffuse Triton X-100-soluble enzyme which has been previously described as being localized in the endoplasmic reticulum, is organophosphate sensitive and classified as a B-esterase[20]. Recovery of this enzyme represents 66.1% of that of the total homogenate or approx. 82% of the activity that is extracted from the tissue. It can be seen from Table I that this enzyme is unstable in solution and it loses about half of its activity during the dialysis and concentration procedures employed. The instability of brain esterases during storage has been pointed out previously[21]. The 65%-saturated $(NH_4)_2SO_4$ precipitate (Fig. 1C) consists of all the isozymes that have been shown previously to be present in the cell sap, are diethyl-*p*-nitrophenyl phosphate resistant, and are classified as A-esterases. They represent 11.2% of the original enzymatic activity and about 14% of the aqueous-soluble enzyme. In contrast to the B-esterase, they are stable during the dialysis and concentration procedures employed at this stage. In preliminary experiments, further attempts to separate specific esterase bands from

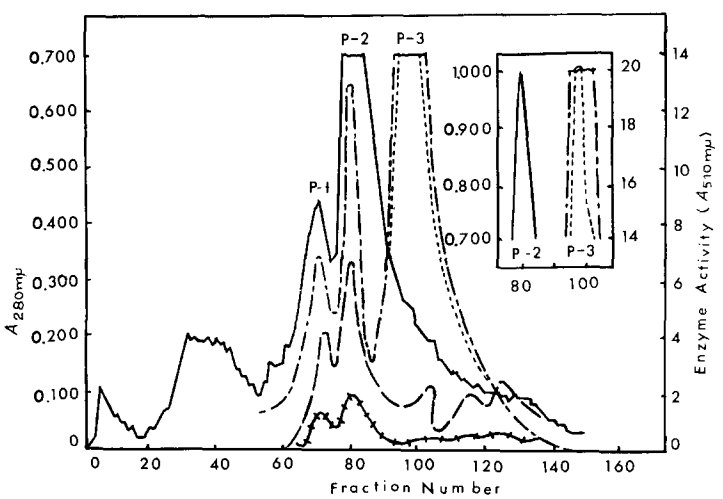


Fig. 2. Separation of human brain esterases on DEAE-Sephadex of a 40%–65% $(NH_4)_2SO_4$ saturation fraction. The fraction (420 mg protein) was applied on the column in 12.5 ml of 0.1 M Tris–HCl (pH 8.5), with 0.02 M mercaptoethanol. A linear LiCl gradient (0–0.5 M), volume 800 ml, was used for elution in the buffer containing 0.001 M EDTA and 0.001 M mercaptoethanol. 5-ml fractions were collected at a flow rate of 0.22 ml/min. $A_{280\ m\mu}$ refers to absorption at 280 mμ in a cell of 0.5 ml light path and $A_{510\ m\mu}$ to the α-naphthyl released in 30 min per ml enzyme solution. The 3 major peaks are designated P-1, P-2 and P-3. ———, protein; — · — · —, α-naphthyl acetate hydrolysis; — — —, α-naphthyl butyrate hydrolysis; /—/—/—/, α-naphthyl butyrate hydrolysis after 4 days storage; - - - - -, α-naphthyl acetate hydrolysis after 4 days storage. Samples were stored at 4° in the gradient buffer.

Fraction II were difficult due to apparent loss of enzymatic activity related to the precipitation of proteins during storage. In order to minimize this loss, either mercaptoethanol or dithiothreitrol was added to the buffers used to dissolve the proteins and was also added to the buffers used to elute the enzyme from the chromatagraphy columns. The results of a typical column separation are shown in Fig. 2. The major peaks are designated P-1, P-2 and P-3. When the enzyme activity of some fractions was determined several days later, a considerable loss in activity was observed for the hydrolysis of α-naphthyl butyrate, as can be seen in Fig. 2. Since turbidity was observed in those fractions, the loss of butyrate activity appeared to be related to the denaturation of proteins. The recovery of α-naphthyl acetate activity was 95%, and it was 45% for α-naphthyl butyrate activity when the assays were done immediately. Other chromatographic separations where mercaptoethanol was not added either to the sample or to the elution buffer gave only 30–45% recovery for α-naphthyl acetate activity and a total loss of α-naphthyl butyrate hydrolyzing activity. Higher concentrations of mercaptoethanol (0.1 M) interfered with 280 mμ absorbance readings.

Electrophoresis patterns of the three fractions corresponding to maximal esterase activity (tubes No. 70, 80 and 100) can be seen in Fig. 3. With α-naphthyl acetate as substrate, tube No. 70 of the P-1 fraction contains the cathodic esterase isozymes principally, with the anodally migrating esterase band proximal to the origin also present. Tube No. 80 from the P-2 fraction consists of the slow-moving anodic esterases principally, and tube No. 100 from the P-3 fraction, the bands previously

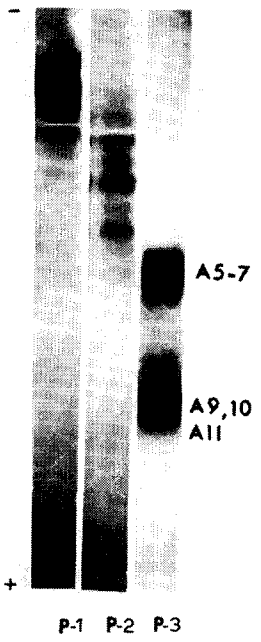
TABLE II

SUBSTRATE SPECIFICITY OF FRACTION P-3

Solutions containing 1 μM/ml of each substrate were prepared in 0.1 M Tris–HCl buffer as indicated in the text. The samples were incubated with the substrate during 30 min. The specific activity is expressed as μM of substrate hydrolyzed/min per mg protein.

Substrate	*Specific activity*
α-Naphthyl acetate	3.27
α-Naphthyl propionate	0.63
α-Naphthyl butyrate	0.07
α-Naphthyl valerate, -laurate, -myristate, -palmitate	0
Acetyl thiocholine	0
N-Benzoyl-L-arginyl-β-naphthylamide hydrochloride	0
L-Leucyl β-naphthylamide	0

identified from the cell sap as number A5–7 and A9–11 (ref. 3). No activity toward α-naphthyl butyrate was demonstrable. The purified desalted enzyme of Fraction P-3 obtained after chromatography on Sephadex G-50 and concentrated as described earlier (Fraction P-3b) when assayed with α-naphthyl esters of acetate, butyrate, propionate, caprylate, myristate, laurate and palmitate, and with acetylthiocholine, showed esterolytic activity only toward the first three substrates with no activity apparent toward the longer chain esters and no cholinesterase activity (Table II). Hydrolase activity measured using l-leucyl β-naphthylamide and *N*-benzoyl-l-arginyl-β-naphthylamide hydrochloride indicated that Fraction P-3b does not have either peptidase or amidase activity. No apparent inhibition of this fraction was



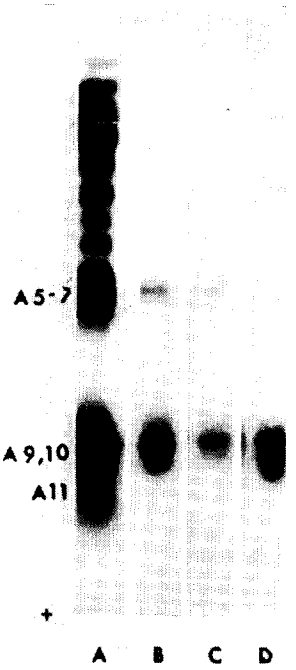


Fig. 3. Electrophoretic separation of esterases of tubes No. 70 (P-1), No. 80 (P-2) and No. 100 (P-3) collected from the DEAE-Sephadex column as described in Fig. 2, with α-naphthyl acetate as substrate. Separation of the cathodal migrating enzymes (P-1), the slow-moving anodal enzymes (P-2) and the major A5–7 and A9, 10 bands can be seen. The A11 band is missing due to air oxidation.

Fig. 4. A, Electrophoresis of Fraction II (36.5 mg protein/ml) before chromatography. B, Fraction P-3 (0.7 mg protein/ml). C, Fraction P-3b (0.7 mg protein/ml) after desalting on Sephadex G-50. D, Same as C but with the addition of mercaptoethanol at a final concentration of 0.4 M. Note the preservation of the A11 band and the inhibition of the A5–7 bands in the presence of the reducing agent. Esterase activity was determined with α-naphthyl acetate as substrate.

observed with diethyl-*p*-nitrophenyl phosphate ($5 \cdot 10^{-6}$ M), eserine (10^{-5} M), iodoacetamide (10^{-3} M), *N*-ethyl maleimide ($5 \cdot 10^{-5}$ M) and PCMB ($5 \cdot 10^{-5}$ M). A number of divalent cations, Fe^{2+}, Ca^{2+}, Mn^{2+}, Mg^{2+} and Zn^{2+} were tested at 10^{-3} M, but no significant inhibition or activation was found. When diethyl-*p*-nitrophenyl phosphate and eserine were used at higher concentrations of 10^{-4} M and 10^{-2} M, respectively, *in vitro*, 52.4% of the control activity was shown with the former and 36.6% with the latter inhibitor. The inhibition *in vitro* is applicable to the A5–7 and A9–11 enzymes *in toto*, and no differential inhibition of Fraction P-3b was determined.

The effect of mercaptoethanol on enzymatic activity is shown in Fig. 4. The zymogram pattern of the 40%–65% $(NH_4)_2SO_4$ fraction is shown in A, and the variety of esterase species previously characterized as A-esterases can be seen. The pooled P-3 fraction obtained by elution with the buffer system described and which contained mercaptoethanol (0.001 M) is shown in Fig. 4B. The differences in the density of the enzyme bands are due to the marked difference in protein concentration of the two preparations, Fraction II containing 36.5 mg protein/ml and the P-3 fraction 0.66 mg protein/ml. If the P-3 fraction is passed through a Sephadex G-50 column to remove salts and excess mercaptoethanol there is a marked decrease in

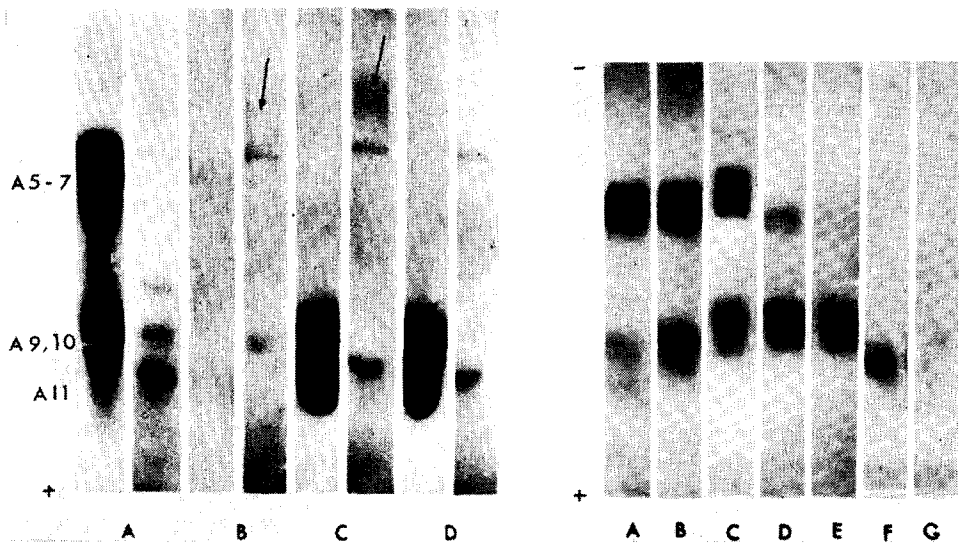


Fig. 5. Proteins and esterase activity of Fraction P-3b obtained from Fraction III by desalting on Sephadex G-50 and concentrated in a Diaflo cell membrane system. For each pair, esterase activity is on the left with its protein electrophorogram adjacent. A, Fraction P-3b (0.62 mg protein); B, same as (A), but in 8 M urea; C, same as (A), but in 8 M urea and 0.3 M mercaptoethanol; D, same as (A), but in 0.3 M mercaptoethanol. Note the appearance of a broad protein smear proximal to the origin with urea, as well as the protective effect of mercaptoethanol on esterase activity. Esterase activity determined with α-naphthyl acetate as substrate. Arrows designate reaggregated proteins due to dilution of urea concentration during electrophoresis as described in text.

Fig. 6. Effect of dithiothreitol on the stability of esterase activity of Fraction P-3b (0.26 mg protein). A, No dithiothreitol; B, 0.02 M dithiothreitol; C, 0.2 M; D, 0.5 M; E, 0.6 M; F, 1.5 M dithiothreitol; G, 2.0 M dithiothreitol. The electrophoretic separations were done on different starch gels, which accounts for the non-alignment of the bands.

the band densities in both the proximal (A5–7) and distal bands (A9, 10). This is seen in Fig. 4C. If mercaptoethanol is added to the P-3b fraction (0.67 mg protein/ml) to a final concentration of 0.4 M, the A5–7 bands are completely inhibited, while the distal bands seen in Fraction P-3 recover their original activity, and show an additional band (A11) which can be seen and which has been designated previously as a C-esterase, activated by sulfhydryl reagents[3]. Thus, mercaptoethanol appears to inhibit the A5–7 bands, reactivate band A11 and prevent loss of activity in A9, 10 which occurs in the absence of a SH- protecting reagent.

Fraction P-3b was reduced with mercaptoethanol in the presence or absence of 8 M urea at 4° for 24 h (Fig. 5). Compared to the control (Fig. 5A), mercaptoethanol (0.3 M) inhibited the A5–7 bands and reactivated the A11 band as described previously (Fig. 5D). Hydrolysis of the substrate was inhibited completely by previous incubation with 8 M urea (Fig. 5B), but the loss of esterase activity in the A9, 10 regions is prevented if mercaptoethanol is also present in the pretreatment mixture (Fig. 5C). Examination of the proteins of Fraction P-3b in the same picture show that 5 molecular species are present before the addition of urea. Correlations between enzyme and protein bands cannot be made because decoloration of background staining of the gels causes distortions which vitiate such comparisons. With 8 M urea, the fast-moving protein bands have been altered and replaced by another species in

the same region and a broad diffuse area of protein activity nearer the origin. This area presumably represents protein that has been disaggregated by treatment with 8 M urea and then has reaggregated during the electrophoresis procedure due to the dilution of the urea by the buffer.

The effect of sulfhydryl reagents is demonstrated further in Fig. 6. The P-3 fraction was desalted on a Sephadex G-50 column, and concentrated in a Diaflo cell membrane filtration system. Aliquots of the sample were incubated at 37° for 30 min in varying concentrations of dithiothreitol. In the absence of dithiothreitol there is a loss of activity in the A9, 10 bands while the A5–7 bands appear to be stable. The addition of dithiothreitol (0.02 M) reactivates the A9 band without inhibiting the A5–7 bands. At 0.2 M dithiothreitol, some slight inhibition of the proximal bands appear and at 0.5 M dithiothreitol almost completely inhibits these bands while fully activating the A9, 10 bands. Complete inactivation of the A5–7 zones is achieved with 0.6 M dithiothreitol, with complete inhibition achieved of all esteratic activity at 2.0 M dithiothreitol. Some differential effect of dithiothreitol on the activation of the A9 and 10 bands seems to occur. The A9 band appears to be reactivated prior to the A10 band. At higher concentrations of dithiothreitol the A9 band seems to be more sensitive than the A10 band.

DISCUSSION

Because of the variety and complexity of human brain esterases, the isolation of individual enzyme species would appear to be a requisite for studies of their specific activity and properties. The differential behavior of the various molecular species toward a number of inhibitors and activators[2], differences in solubilities and subcellular localization[20] and their varied affinities for α-naphthyl esters[22] indicate that the esterases do not constitute a single isozymic series. That groups of isozymes exist is not excluded. However the enzymes have in common an ability to hydrolyze certain carboxylic acid esters. Whether this hydrolase activity is the major biochemical function of all esterases demonstrated here is unclear at this time, though the major bands do not appear to possess peptidase, amidase or acetylcholinesterase activity.

A crude separation of esterases using DEAE-Sephadex A-50 chromatography was accomplished because inspection of the starch-gel electrophorogram showed it was possible to divide the esterases into cathodic, slow-moving anodic, intermediary and fast-moving anodic bands. The positively charged cathodic bands were eluted by washing the weakly anionic exchange resin with the initial buffer. The fast-moving esterases were most strongly retained, and were eluted with the higher molarity of the buffer used. The slow-moving esterases were more weakly adsorbed and consequently separated with a more dilute buffer.

The problem of stability and the nature of any instability of some of the esterase species of human brain was the concern of this study. It appeared that reducing conditions had to be maintained during purification procedures to preserve the activity of the A9–11 bands. Because of the alkaline pH required in some of the procedures, a series of undesirable side reactions could be initiated[23] and had to be considered. Electrophoretic separation of the esterases enabled us to demonstrate that the A5–7 bands were more stable to atmospheric O_2 than the A9–11 bands. The

data with 8 M urea treatment prior to electrophoresis and subsequent dilution by the buffer during electrophoresis indicated inactivation of all the esterases present in the isolated fraction and suggest the formation of depolymerized material some of which reaggregates during electrophoresis. Under the most favorable conditions, reaggregation to the original active protein may occur after dilution of the protein from a 8 M urea buffer to lower concentrations of urea. If conditions are not favorable, a new conformational aggregate or denaturated aggregates could be formed. A transition form combining denaturation and partial reformation to the original protein is also possible. Aggregates of protein bands were observed closer to the point of application of the sample in the gel. Since the A5–7 bands were inactivated by 8 M urea with or without a reducing agent, the intact tertiary structure appeared to be required for enzymatic activity, and under the experimental conditions employed here, a new conformational aggregate devoid of enzymatic activity was formed. The faster-migrating A9–11 esterases were also denaturated by urea, but the presence of thiol reagents protected the enzyme against the inactivation by urea. Thus these esterases remain active if oxidation is prevented, and any depolymerization that may have occurred does not destroy the catalytic nature of the protein.

The importance of sulfhydryl groups for the stability of proteins has been reviewed by CECIL AND MACPHEE[24]. SUND AND WEBER[25] in a more recent article on the quaternary structure of proteins enumerate several enzymes in which association or dissociation of polypeptide chains may or may not affect the enzyme activity. WHITE AND ANFINSEN[26] reported that ribonuclease could be inactivated by reduction with mercaptoethanol, cysteine or thioglycollic acid. ANFINSEN AND HABER[27] removed the excess of reducing agent by column chromatography and reactivated the reduced enzyme by standing at room temperature in alkaline pH. It appears that the presence of easily reactive free sulfhydryl groups on proteins will tend to form disulfide bonds unless the conditions are carefully controlled. This process of oxidation with or without polymerization appears to be favored when the enzyme in consideration is isolated in a purified state and in relatively dilute solution.

While the effects of organic mercurials, aklylating agents and other SH-reacting compounds on esterases has been demonstrated[2], the diverse behavior of the group precludes any generalizations. The A5–7 enzymes are stable toward atmospheric O_2 but are inactivated by high concentrations of mercaptoethanol and dithiothreitol, as well as by iodoacetate and PCMB (ref. 3). One can conclude that these enzymes are SH- group active enzymes because of their sensitivity to SH–reacting compounds. In addition, the inactivation by high concentrations of mercaptoethanol and dithiothreitol could result from a reduction of S–S linkages in the molecule with the resultant alteration in conformation of the protein. This interpretation is supported by the data that demonstrated an inability to reactivate the enzyme with reducing agents after cleavage with 8 M urea. Thus the A5–7 enzymes appear to require free SH groups as well as S–S linkages to exhibit catalytic activity*. The difficulties in interpretation of the data obtained with the A5–7 bands do not appear to pertain to the data on the A9, 10 enzymes. These enzymes are sensitive to atmospheric O_2, are inactivated by PCMB or iodoacetate, and reducing agents prevent loss in activity even after treatment with 8 M urea. The data are compatible with the

* That both SH and S–S groups may be present in the same enzyme has been demonstrated[28].

explanation that one or more free SH groups are required for the protein to exhibit enzymatic activity. In contrast to the A5–7 and A9, 10 enzymes, the A11 band is PCMB-activated. Such activity could be a result of an alteration of the conformation of the protein by the addition of the organomercurial to SH groups which either make available hidden active centers for substrate binding or alter steric hindrance factors to enable facilitation of enzyme–substrate interactions.

The complexity of the non-specific esterase activities of tissue has been commented on previously[29,30] and the characterization of the various enzyme forms so that some understanding of their role can be gained appears to be desirable. The ubiquity of these enzymes in all neural tissue thus far examined and their appearance early in evolutionary development[31] would point to a fundamental function of these enzymes in metabolism. The molecular forms of the three groups described are all SH- group active enzymes but differ from each other in other behavioral characteristics toward reducing compounds and SH reagents. Further studies are underway to determine the nature of these differences.

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