Multiple Forms of Acetylcholinesterase from Pig Brain

By CHRISTOPHER H. S. MCINTOSH and DAVID T. PLUMMER

Department of Biochemistry, Chelsea College, University of London, London SW3 6LX, U.K.

(Received 11 January 1973)

1. A number of methods of solubilization of pig brain acetylcholinesterase (EC 3.1.1.7) were studied. The multiple enzymic forms of the resultant preparations were examined by polyacrylamide-gel electrophoresis. 2. Butanol extraction, Nagarase treatment and ultrasonication proved unsuitable as preparatory methods, but detergent treatment (Triton X-100, Triton X-100–KCl and lysolecithin) gave good yields. 3. Separation of soluble enzyme in three systems of polyacrylamide-gel electrophoresis were compared and the relative advantages are discussed. 4. By using a 6% (w/v) gel and continuous buffer system two forms of acetylcholinesterase were detected in Triton X-100-solubilized enzyme, but the incorporation of a sample and spacer gel and a discontinuous buffer system resolved this into four components. The forms of the soluble enzyme extracted by different methods differed in mobility. 5. With gradient polyacrylamide-gel electrophoresis between two and six forms were detected, depending on the method used for extraction. The average molecular weights of the five forms most frequently found were 60000, 130000, 198000, 266000 and 350000. 6. Treatment of the Triton X-100-extracted enzyme with 2.5M-urea altered the pattern and evidence of dissociation was observed. 7. The results are discussed in the light of present theories on the molecular structure of acetylcholinesterase.

A number of different methods have been used to solubilize acetylcholinesterase (EC 3.1.1.7) from brain. Among these are butanol extraction (Jackson & Aprison, 1966a), detergent treatment (Got & Polya, 1963; Jackson & Aprison, 1966b; Ho & Ellman, 1969; Crone, 1970, 1971), ultrasonication (Lawler, 1964), freezing and thawing (Bernsohn & Barron, 1964), and treatment with lysolecithin (Marples, *et al.*, 1959; McArdle, *et al.*, 1960) and bacterial protease (Ho & Ellman, 1969).

However, a comparative study on the resultant solubilized enzyme, to determine if the various physical and chemical treatments have any appreciable effect on the characteristics of the enzyme, has not been made.

Acetylcholinesterase exists in a number of multiple enzymic forms, the chemical nature of which is only partially understood. The number of forms reported varies widely and this variation appears to be dependent both upon species and method of extraction.

In human brain three enzymic forms are detected when either detergent or freezing and thawing are used (Barron & Bernsohn, 1968; Barron *et al.*, 1963; Bernsohn *et al.*, 1962, 1963). On the other hand, with rat brain the number varies, depending on the method of extraction, from three with a 105000g supernatant from frozen and thawed material (Bajgar & Žižkovsky, 1971) or bacterial protease treatment (Ho & Ellman, 1969) to a range of five to ten when detergent was used (Davis & Agranoff, 1968). The species variation is even more widely documented although comparisons cannot always be drawn owing

to the differences in extraction techniques. The number ranges from two in trout brain (Baldwin & Hochachka, 1970), three in chick brain (Maynard, 1966) and calf (Jackson & Aprison, 1966a), four in housefly (Eldefrawi *et al.*, 1970) and chicken (Iqbal & Talwar, 1971) to five in rabbit (Dolgo-Sakurov, 1969) and goldfish (Lim *et al.*, 1971).

A comparison of the multiple patterns is further complicated by the different electrophoretic techniques and conditions that have been used. Vertical and horizontal starch-gel agar-gel and polyacrylamide-gel electrophoresis have all been applied and the differences in sensitivity must be reflected in the results.

In the present study, disc electrophoresis (Allen & Gockerman, 1964; Ornstein, 1964; Davis, 1964) and vertical gradient electrophoresis (Margolis & Kenrick, 1967, 1968; Slater, 1969) on polyacrylamide gels were both utilized. The former method separates proteins primarily according to their net surface charge whereas the latter does so according to their molecular weights. Molecular-weight estimations of the various multiple enzymic forms have therefore been made.

Materials and Methods

Materials

Fresh pig brains were generously provided by Walls' Slaughterhouse, Acton, London W.4, U.K.

The enzyme substrates, acetylcholine, propionylcholine, butyrylcholine, acetylthiocholine and

butyrylthiocholine were obtained as the iodide salt from the Sigma (London) Chemical Co., London S.W.6, U.K.

Compounds used to 'solubilize' the enzyme were obtained from the following sources: Triton X-100, (Rohm and Haas Co., Philadelphia 5, Pa., U.S.A.) a non-ionic detergent, lysolecithin (Sigma), butan-1-ol (Fisons, Loughborough, Leics., U.K.) and bacterial protease (Nagarase) (Teikoku Chemical Industries Ltd., Osaka, Japan).

Reagents used in polyacrylamide-gel electrophoresis, riboflavin, acrylamide and *NN'*-methylene bisacrylamide were supplied by British Drug Houses Ltd., Poole, Dorset, U.K., and *NNN'*-tetramethylethylenediamine was supplied by Kodak Ltd., London W.C.2, U.K.

Compounds used to concentrate protein solutions were Lyphogel obtained from Hawksley and Sons Ltd., Lancing, Sussex, U.K., and Carbowax 20M from G. T. Gurr and Co. Ltd., London S.W.14, U.K. Sephadex G-200 was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden, and physostigmine sulphate from Burroughs Wellcome, London N.W.1, U.K.

Methods

Determination of acetylcholinesterase. Assays for acetylcholinesterase were carried out with a pH-stat (Radiometer, Copenhagen, Denmark). The acid produced during hydrolysis of acetylcholine at 30°C and constant pH (7.9) was measured in the following mixture: NaCl (0.15M), MgCl₂ (1.3mM), acetylcholine iodide (1mM) and tissue extract (0.3–1.0ml) in a total final volume of 8.0ml. After the rate of spontaneous H⁺ release had been determined for 5min, substrate was added (0.3ml of 26.67mM) and the enzymically catalysed rate of hydrolysis was determined for a further 5–10min. The rate was corrected by subtraction of spontaneous activity and non-enzymic hydrolysis of substrate. Enzyme activities were expressed in international units (μmol of substrate hydrolysed/min).

Assay for protein. This was determined by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin as standard. When (NH₄)₂SO₄ was present in the samples they were dialysed overnight against water before assay. If Triton X-100 was present a gelatinous precipitate was formed which was removed by centrifugation before determination. Standards with Triton X-100 present in them showed that this procedure had little effect on the determination.

For rapid protein measurement either a Biuret method (Plummer, 1971) was used or, with column fractions devoid of Triton X-100, the *E*₂₈₀ was measured.

Sephadex G-200 column chromatography. Sufficient

Sephadex G-200 for the column dimensions (33cm × 2.5cm) was stirred into 0.15M-NaCl and allowed to swell for 72h. After decantation of the fines the column was prepared.

Lyphogel was used to concentrate the samples and to give an enzyme activity of 6–10 μmol/min per ml; 1.0ml of the concentrate was then placed on the column and eluted with 0.15M-NaCl. Fractions (5ml) were collected and assayed for enzyme activity and protein concentration. Large volumes of solution containing a relatively low enzyme activity were concentrated by dialysis against Carbowax 20M. Molecular weights were determined by gel filtration (Andrews, 1965) by using Blue Dextran and the following proteins of known molecular weight as standards: cytochrome *c* (12000), ovalbumin (45000), bovine serum albumin (68000), lactate dehydrogenase (134000), γ-globulin (160000) and thyroglobulin (670000).

Polyacrylamide-gel electrophoresis. Three methods were used. (i) With a buffer system of Tris–glycine (10.04, pH 8.0) (Allen & Gockerman, 1964.) The gels were cast in glass cylinders (65mm × 5mm) and run on a Quickfit polyacrylamide-gel electrophoresis apparatus at a constant voltage of 240V for time-periods of 1–3h and at 4°C. The sample (30–50 μl) was mixed with concentrated sucrose solution and applied to the surface of the gel. Neither spacer gel nor sample gel were used (Gordon, 1969) and the concentration of polyacrylamide was varied from 6–10% (w/v). In some experiments Triton X-100 (0.1–1.0%, w/v) was incorporated into both gels and buffer as suggested by Singh & Wasserman (1970).

(ii) With the method of Ornstein (1964) and Davis (1964), the gels were prepared with tube dimensions given above and in the way described by Ornstein (1964). Tank buffer was 5mM-Tris–38mM-glycine (pH 8.3) and the small- and large-pore gels were 7% and 2.5% (w/v) polyacrylamide respectively. Electrophoresis was carried out at 2–3mA/tube for 1½–3h at 4°C.

(iii) For gradient gel electrophoresis, prepared Gradipore concave gradient polyacrylamide gels were obtained from Universal Scientific Ltd., London E.13, U.K. Gels were in the form of slabs of approximate dimensions 70mm × 70mm × 3mm and consisted of a polyacrylamide concentration gradient running from 4–26% (w/v). Samples were applied by using a plastic spacer after pre-running the gels for 1h at the voltage used for electrophoresis. Electrophoresis was carried out at 100V (constant voltage) for 24h with a buffer of 88mM-Tris–82mM-borate–2.5mM-EDTA (pH 8.3) and at room temperature. For molecular-weight estimates the gels were calibrated by running a number of standard proteins under identical conditions.

Staining of polyacrylamide gels. (i) For acetylcholinesterase and butyrylcholinesterase the thio-

choline method of Koelle (1951) as modified by Lewis & Shute (1966) was utilized. Incubation times of 30 min to 12 h were used for the deposition of reaction product. When subjecting the gels to the final ammonium sulphide treatment the gels were pre-washed with distilled water for 2–24 h.

When eserine was included in the incubation mixture the gels were initially soaked in a solution containing $10\text{ }\mu\text{M}$ -eserine for 1 h and then exposed to the incubation mixture containing $10\text{ }\mu\text{M}$ -eserine.

Esterase activity was also detected by the method of Paul & Fottrell (1960) in which the enzyme hydrolyses α -naphthyl acetate and the resulting α -naphthol is detected by coupling with *o*-dianisidine.

(ii) Staining for protein was carried out in two ways. (a) Gels were placed in 1% Amido Black in 7% (v/v) acetic acid for $\frac{1}{2}$ h and destained in 7% (v/v) acetic acid until the bands were visible. Electrophoretic destaining was not used because some bands disappeared if conditions were not very carefully controlled. (b) Gels were placed in 0.2% Coomassie Blue in methanol–acetic acid–water (5:1:5, by vol.) for 1 h and destained in the same solvent as described above.

Methods of solubilization. Pig brains, obtained a few hours after slaughter, were transported on ice to the laboratory. The external membranes were removed and the brains stored at -18°C until used. Extracts were prepared from slices of cerebral cortex, chopped and then homogenized in a Waring Blendor run at full speed for 3 min with intermittent cooling of the vessel in ice.

In the subsequent treatments assays were carried out at all stages of operation and, where necessary, controls of untreated sample were run in parallel.

Centrifugation at low *g* values was carried out in an MSE HS 18 centrifuge ($8\times 50\text{ ml}$ or $6\times 100\text{ ml}$ rotor) and for high *g* centrifugation either an MSE SS50 centrifuge ($10\times 10\text{ ml}$ or $8\times 50\text{ ml}$ rotor) or an MSE SS65 ($8\times 14\text{ ml}$ or $8\times 50\text{ ml}$ rotor) were used. The criterion of solubilization was taken to be the absence of sedimentable material after centrifugation for 1 h at $100\,000g$.

Butanol extraction was carried out by the method of Jackson & Aprison (1966a) up to and including the $(\text{NH}_4)_2\text{SO}_4$ -fractionation step.

Initial studies with Triton X-100 were done by the method of Ho & Ellman (1969). Later, the dilution to 8.0 mg of protein/ml was omitted and an $(\text{NH}_4)_2\text{SO}_4$ fractionation on the $100\,000g$ supernatant was incorporated. In a further set of experiments solubilization in a range of concentrations of Triton X-100 with and without the presence of various concentrations of KCl was used as described by Wright & Plummer (1970).

Lysolecithin treatment was carried out by incubating a 20% (w/v) homogenate for 30 min at 37°C with 12 mM-lysolecithin (Marples, *et al.*, 1959; McArdle *et al.*, 1960) after which the mixture was centrifuged

at $100\,000g$ for 1 h and the supernatant retained. Ultrasonication of a 20% (w/v) homogenate was maintained for 1 h at $22\,000\text{ Hz}$ and at $0\text{--}3^{\circ}\text{C}$ in an MSE ultrasonicator (Gordon & Rutland, 1967).

For bacterial protease treatment (Ho & Ellman, 1969) a 20% (w/v) homogenate was treated with Nagarase (0.16 mg/ml) for 20 h at $0\text{--}4^{\circ}\text{C}$ with occasional stirring. The mixture was then centrifuged at $100\,000g$ for 1 h and the supernatant retained.

Freezing and thawing of the homogenate was carried out by alternately placing it in a solid CO_2 –acetone mixture and a water bath at 30°C .

Results

Solubilization

When a 20% (w/v) homogenate of cerebral cortex was centrifuged at $100\,000g$ for 1 h 14% of the acetylcholinesterase activity was obtained in soluble form. Of the methods used to further solubilize acetylcholinesterase freezing and thawing, ultrasonication and Nagarase treatment all gave very low yields of enzyme. Although Nagarase inactivated the membrane-bound enzyme when a diluted homogenate was used (Ho & Ellman, 1969), treatment of undiluted homogenate resulted in 33% of the activity being solubilized and this was used for electrophoretic studies.

Butanol extraction resulted in a very low yield of active enzyme (18.84%) at the stage of 26–56% $(\text{NH}_4)_2\text{SO}_4$ fractionation and a purification factor of only 2.5.

Various detergent treatments gave much more successful solubilization but great variation in the amount of enzyme solubilized.

Treatment of pig cerebral cortex with Triton X-100 (1%) gave similar results to those of Ho & Ellman (1969) with rat brain with an overall purification of 1.67 but an appreciably lower yield. When the dilution step was omitted the majority of the acetylcholinesterase was precipitated from the $100\,000g$ supernatant by $(\text{NH}_4)_2\text{SO}_4$ at 20–40% saturation. This preparation with a purification of 5.9 and a 38% yield, was examined electrophoretically.

Incorporation of KCl into the detergent solution greatly enhanced the amount of solubilization (Table 1). The increase in measured enzyme activity appears to be due to an actual increase in the amount of enzyme solubilized and not just to activation by the reagents used. Thus addition of Triton X-100 to a brain homogenate caused little or no change in acetylcholinesterase activity, whereas KCl actually decreased the activity of the homogenate by about 20% at 2M-KCl. Despite the increase in solubilization indicated, this method was unsatisfactory since concentrations of salt above 0.6M salted out lipid material that was extremely difficult to remove. An aqueous solution of 1% Triton X-100 was therefore used in subsequent experiments.

Table 1. *Percentage of acetylcholinesterase activity in 100000g supernatant after treatment with Triton X-100-KCl mixtures*

A 20% (w/v) homogenate of cerebral cortex was treated with an equal volume of Triton X-100-KCl solution by shaking for 40 min at 22°C. The mixture was then centrifuged at 100000g for 1 h and the acetylcholinesterase activity in the supernatant was determined. The degree of solubilization was calculated by taking the uncentrifuged sample as 100%. The values given in the Table are the average of from four to eight experiments.

Triton X-100 (%)	Concn. of KCl (M) ...	Acetylcholinesterase activity (%)							
		0	0.1	0.3	0.6	1.0	1.5	2.0	
0		16	16	16	16	16	16	16	
0.1		20	22	24	20	20	29	36	
0.2		60	52	46	41	53	76	73	
0.5		68	66	65	63	93	93	107	
1.0		68	68	65	66	95	98	97	
2.0		81	81	70	75	74	99	101	

Lysolecithin was an extremely efficient solubilizing agent and yields of 85 and 83% solubilization were obtained in the two extractions carried out with only 2% loss in activity due to lysolecithin action.

Sephadex G-200 chromatography

The soluble enzyme and that obtained after detergent treatment were subjected to gel chromatography. Eluting with 0.15M- or 1.0M-NaCl gave the same elution profiles. However, with 0.05M-NaCl the acetylcholinesterase remained adsorbed on the column; 0.15M-NaCl was therefore used as the standard eluent.

The soluble enzyme when chromatographed gave one major peak of enzyme activity eluting close to the void volume and a shoulder to this peak was eluted from the column at a larger volume (Fig. 1a). The estimated molecular weight of the main peak was 437000 ± 14000 and that of the shoulder, which was eluted at 110 to 130 ml, encompassed the range 53000 to 127000.

Treatment of the homogenate with Triton X-100 produced a single peak of a higher-molecular-weight enzyme species since the bulk of the activity appeared in the void volume (Fig. 1b). This indicates either a very high-molecular-weight protein aggregate, a high-molecular-weight micellar structure or both. Ho & Ellman (1969) reported difficulties in eluting the activity from columns without the addition of Triton X-100 to their eluting medium. With the present system, 82% of the active enzyme was recovered from the column and the incorporation of 0.05, 0.5 or 1% Triton X-100 in the eluting medium did not significantly alter the elution volume of the main peak. However, there was a suggestion of a minor peak in the region of molecular weight 100000-150000 with the higher Triton X-100 concentrations.

Polyacrylamide-gel electrophoresis

(i) The electrophoresis method of Allen & Gockerman (1964) was applied to enzyme extracted by the methods previously described. Conditions of voltage (100-250 V), time (1-12 h) of run, and polyacrylamide concentration (5-10%, w/v) were all varied but the enzyme did not enter the gel. Staining for non-specific esterases with α -naphthyl acetate indicated that these species entered and gave a multiplicity of bands. A very complex protein banding (20-30 components) was also evident.

Incorporation of Triton X-100 (0.1-1.0%, w/v) into the gel and buffer system greatly facilitated the entry of a Triton extract of acetylcholinesterase. With a 1.0% Triton X-100 extract, 10% (w/v) polyacrylamide gel and 1% (w/v) Triton X-100 throughout the system the enzyme moved a short distance into the gel as two distinct bands (Fig. 2). On increasing the pore size (decreasing the gel concentration) the enzyme moved further into the gel as a single band or, with increased time of electrophoresis, as two distinct bands. Identical results were obtained with the sample fractionated with $(\text{NH}_4)_2\text{SO}_4$.

Since the technique of Triton X-100 incorporation was not satisfactory for studying the multiple enzymic forms of differently extracted enzyme, subsequent studies were made by using the system of Ornstein (1964) and Davis (1964) or gradient electrophoresis.

(ii) Far more satisfactory resolution was obtained by incorporating a sample gel and packing gel into the electrophoretic system (Ornstein, 1964; Davis, 1964). In all cases (Fig. 3) strong banding was observed with the thiocholine staining method of Lewis & Shute (1966).

With detergent solubilization (Triton X-100 and lysolecithin) three definite bands of activity were

Fig. 1. *Sephadex G-200 chromatography of (a) 100000g supernatant from untreated homogenate concentrated with Carbowax 20M and (b) 100000g supernatant from Triton X-100 (1%)-treated homogenate, concentrated with Carbowax 20M*

Elution was with 0.15M-NaCl. (a) $\circ$, Acetylcholinesterase activity (units/ml) and $\bullet$, protein (mg/ml). (b) Δ , Acetylcholinesterase activity (units/ml) and $\blacktriangle$, protein (mg/ml).

always obtained with dark staining at the origin and, in the case of lysolecithin, diffuse staining from the middle band to halfway down the gel. If the Triton X-100 extract was left overnight before electrophoresis the middle band dissociated into two bands of approximately equal intensity. The $(\text{NH}_4)_2\text{SO}_4$ fractionation made no difference to the multiple enzymic pattern. Upon incorporation of 2M-KCl into the extraction medium four bands were obtained with mobilities different from the pure Triton X-100 extract. This was observed when both extracts were run simultaneously (but on different gels) thus correcting for any difference incurred by voltage fluctuation, heating, etc., but not correcting for differences in gel composition. Both the untreated, concentrated supernatant and a sonicate gave four bands but once again mobility differences were apparent. Staining for protein showed a plethora of bands, some corresponding to the acetylcholinesterase activity.

(iii) Despite many obvious criticisms of gradient gel electrophoresis as a method of molecular-weight

determination, electrophoresis through gradient gels gives useful estimates of the molecular weights of multiple enzymic forms provided that the gel is first standardized. With standard proteins of the type used for Sephadex G-200 column calibration, the distance moved into the gel is approximately proportional to the molecular weight of the protein.

By using the standardized conditions given in the text, and with the calibration curve, molecular-weight determinations of the various forms separated on the gels were made, the distance from the origin to the centre of the band being taken as proportional to the average molecular weight. In all cases staining at the origin and between 5–10% (w/v) polyacrylamide gel indicates that a high percentage of the enzyme is of a very high molecular weight. The surface staining is due to a species of molecular weight greater than 2×10^6 and may be partly due to membrane fragments still present even after centrifugation at 100000g.

Fig. 4 and Table 2 give a comparison of the species

Fig. 2. Polyacrylamide-gel electrophoresis of acetylcholinesterase solubilized with 1% Triton X-100

Gels were prepared by the method of Allen & Gockerman (1964) and electrophoresis was at 250 V (constant) in Tris-glycine buffer (pH 8.0) with 1% (w/v) Triton X-100 incorporated throughout the system. The gels were stained by the acetylthiocholine method of Lewis & Shute (1966). Electrophoresis was on (a) 10% (w/v) polyacrylamide gel run for 1½ h, (b) 6% (w/v) polyacrylamide gel run for 1½ h, (c) 6% (w/v) polyacrylamide gel run for 3 h.

present. The nomenclature (bands I-IV) is purely for convenience of discussion and the grouping has been done on the basis of similar estimated molecular weights.

With 10 μ M-physostigmine sulphate (eserine) incorporated into the staining system no activity was detected. This indicates that the activity was due to a cholinesterase. The use of butyrylthiocholine iodide in place of acetylthiocholine iodide showed very slight staining in the region proximal to band I, indicating that the majority of the enzyme present was acetylcholinesterase and not butyrylcholinesterase.

Lowering the pH value of the reaction from 6.2 or over to 5.0 also showed very weak staining around band I. Finally, incorporation of 30 μ M-ethopropazine into the reaction mixture had no effect on the staining with acetylthiocholine iodide, but totally inhibited the band observed with butyrylthiocholine iodide showing conclusively that the enzyme detected was acetylcholinesterase and not cholinesterase.

(iv) Sonication of soluble acetylcholinesterase for more than 1 min caused total inactivation. Triton X-100 extract and concentrated supernatant multiple enzyme patterns were not altered by very short ultrasonic treatments.

Addition of Triton X-100 to the concentrated supernatant, butanol extract or sonicate did not alter the banding previously obtained. Butanol treatment of solubilized enzyme inactivates it very rapidly and no electrophoretic studies were therefore possible.

Fig. 3. Polyacrylamide-gel electrophoresis of soluble acetylcholinesterase

Gels were prepared by the method of Ornstein (1964) and Davis (1964). Electrophoresis was at 3 mA/tube for 1½ h for both large-pore gel (2.5%, w/v) and small-pore gel (7.0%, w/v) with a buffer system of Tris-glycine (pH 8.3). The gels were stained by the thiocholine method of Lewis & Shute (1966). The letters (A-H) under the gel profiles show the methods of extraction used and these are outlined in Table 2. The extra category B1, refers to the Triton X-100 preparation which was left overnight at 4°C before electrophoresis. Cross-hatching on diagrams indicates the depth of staining: ▨, weakest; ▩, medium; ■, strongest. For details see the text.

Fig. 4. Gradient polyacrylamide-gel electrophoresis of soluble acetylcholinesterase

Electrophoresis was carried out in 4–24% (w/v) concave gradients at a constant voltage of 100 V for 24 h at room temperature. The gels were stained by the thiocholine method of Lewis & Shute (1966). The methods of extraction used (A–H) are the same as those described in Table 2. Numbering of bands is for convenience in discussion and correspond to similar molecular weights. * Bands not always present. Shading indicates depth of staining: ■, least; ■, medium; ■, greatest.

Table 2. Estimated molecular weights of multiple forms of acetylcholinesterase separated by gradient polyacrylamide-gel electrophoresis

Acetylcholinesterase from cerebral cortex was solubilized by the methods shown in the Table and the molecular weights of the multiple forms were estimated by gradient gel electrophoresis. Full experimental details are given in the text.

Method of extraction	Band no.	...	Estimated molecular weight								
			I	Ia	II	IIa	IIb	III	IIIa	IIIb	IV
(A) Concentration of untreated supernatant				331 100	245 500			128 800			57 540
(B) 1% (w/v) Triton X-100		457 100*	363 100	269 200	190 500			131 800*			66 070
(C) Main peak of Sephadex G-200 chromatography of (B)					288 400	218 800					
(D) Butanol extraction					275 400	171 800	158 500	133 400			58 880
(E) Sonication			331 100	245 500				128 600	114 800	104 700	52 480
(F) Lysolecithin		402 700			263 000			128 000			57 540
(G) 2M-KCl-Triton X-100 (1%)		416 900			275 400				117 500		64 570
(H) Nagarase				371 500		208 900					

* Bands I and III were not always present.

Fig. 5. Inhibition of acetylcholinesterase by urea and guanidinium hydrochloride

The inactivation of acetylcholinesterase activity by ○, urea, and ●, guanidinium hydrochloride, is shown as the percentage of acetylcholinesterase activity remaining.

Dissociation of the high-molecular-weight acetylcholinesterase

In an attempt to dissociate the high-molecular-weight species of the enzyme under conditions not drastic enough to destroy enzyme activity the enzyme was treated with various concentrations of guanidine hydrochloride and urea. The concentration of guanidine which inactivates acetylcholinesterase was lower than for inactivation by urea (Fig. 5), which was therefore chosen as the disaggregating agent used to study effects on the multiple pattern.

The 100000g supernatant from a 1% (w/v) Triton X-100 extraction was pretreated with 1M- or 2.5M-urea and electrophoresis carried out in gels and buffer media containing the same concentration of urea.

With 1.0M-urea a very similar banding to untreated supernatant is apparent (Fig. 6), but an unexpected high-molecular-weight band appeared proximal to band I. Otherwise the differences in mobilities may be only due to the incorporation of urea into the electrophoretic system. With 2.5M-urea only two bands corresponding to groups II and IIa appeared. The loss of band IV may be explained since this is always of low activity but the loss of very-high-molecular-weight bands and the subsequent increase in band IIa (approx. mol. wt. 182000) may indicate dissociation of the high-molecular-weight species.

Discussion

The main purpose of this work was to determine the multiple character of the acetylcholinesterase solubilized by various methods and to compare the effectiveness of these methods as preliminary purification stages.

Of the various methods attempted the most effective were those based on detergent action. Lysolecithin

Fig. 6. Gradient polyacrylamide-gel electrophoresis of urea treated 100000g supernatant from 1% Triton X-100 extract

Electrophoresis was as previously described but 1.0M- or 2.5M-urea was incorporated into the electrophoretic system. Shading indicates depth of staining: ■, least; ■, medium; ■, greatest. Estimated molecular weights of species treated with 1.0M-urea (A) bands I, 524800; Ia, 182000; IV, 60260; with 2.5M-urea (B) bands II, 269200; IIa, 182000.

gave a soluble preparation rapidly and with a high yield but is expensive. Triton X-100 and Triton X-100-KCl both gave results similar to those already published (Ho & Ellman, 1969; Crone, 1970, 1971; Wright & Plummer, 1970), but the former would be the preferred method, despite 100% solubilization with 1% Triton-2M-KCl, because of the difficulty of removing lipids.

The multiple patterns of the extracted enzymes indicate that, apart from species differences, extraction procedures and electrophoretic techniques play important roles in the pattern produced (Figs. 3 and 4).

Ho & Ellman (1969) claimed that their Triton X-100 extract would not enter polyacrylamide gels

but did not give any details of electrophoretic conditions. In the present work by using the conditions of Allen & Gockerman (1964) similar results were obtained. However, incorporation of Triton X-100 into the electrophoretic system greatly facilitated enzyme entry and gave one to two bands depending on the conditions of electrophoresis (Fig. 2). It is possible that the Triton X-100 keeps the enzyme in a disaggregated form as suggested by Singh & Wasserman (1970).

The use of Ornstein (1964) and Davis's (1964) method gave far more distinct information about the multiple enzymic nature of acetylcholinesterase. Since the tank buffer was almost identical to that used in the preceding method it is concluded that enzyme concentration by means of the large-pore gels coupled with a differential buffer system greatly increases the resolution. With this electrophoretic system a constant four bands of activity were obtained, although with the Triton X-100 extract the middle band only split on leaving the preparation overnight before electrophoresis (Fig. 3). The differences observed in the mobilities of the species obtained by various extraction procedures may be due to differences in surface charge and/or molecular weight. Gradient gel electrophoresis was utilized to study this more thoroughly.

The application of gradient gels for the estimation of molecular weights has been criticized by Chrambach & Rodbard (1971) and by Rodbard *et al.* (1971). Although many of their criticisms of the claims of some workers (Margolis & Kenrick, 1968; Slater, 1969; Kopperschlaeger *et al.*, 1969) must be considered valid this method is useful as an estimate of molecular weights provided the gels are carefully standardized.

A complex series of banding is evident when this more sensitive method is used (Fig. 4). From two to six bands of activity were obtained depending on the extraction process. However there is strong evidence for at least three groups of extracted enzyme. First there is the soluble enzyme which gives bands Ia, II, III and IV, which may, in fact, be in equilibrium with membrane-bound enzyme as indicated by Shirachi *et al.* (1972). Sonication also produces these bands plus the extra bands IIIa and IIIb which may be due to release of further enzyme from membrane or, alternatively, they could be due to modification of the normal soluble enzyme by cleavage at points not essential for catalytic activity. The former is more likely since an extra 8% of enzyme is released by sonication.

In the second group may be included the enzyme extracted by detergent treatment: this is probably membrane acetylcholinesterase. In all cases bands I, II, III or IIIa and IV are generally present. The highest activity was always in the higher-molecular-weight bands. The effect of detergents on the apparent mole-

cular weights of these species is difficult to assess but the variation obtained in the higher-molecular-weight range could be due to differences in micelle formation.

The third group includes butanol and Nagarase treatment, both of which give unusual banding. The effect of butanol is to produce enzyme most of which lies in an intermediate-molecular-weight range, presumably owing to some type of modification of the enzyme structure. Nagarase has a disastrous effect on the enzyme, leaving only two bands probably because of complete inactivation of the low-molecular-weight species. The species obtained differ greatly in molecular weight from those obtained by Ho & Ellman (1969) and it is unlikely that any acetylcholinesterase solubilized by bacterial protease could be considered a normal form of that enzyme. The control experiments, in which soluble enzyme preparations were subjected to other treatments, tend to confirm the above classification.

A comparison of the estimated molecular weights with literature values (Table 3) shows close correlation in many cases if one allows for the different methods used for estimation. The soluble and released membrane enzyme purified by Shirachi *et al.* (1972) and Chan *et al.* (1972) consists of three species corresponding closely with three of the species in this work. With butanol extraction, although more species were detected in the present work, Jackson & Aprison (1966a) obtained three species in the range of molecular weights of the major species from pig brain. Finally, with the membrane-bound material extracted by detergent treatment, in agreement with Ho & Ellman (1969), most of the activity is of high-molecular-weight material, although two extra species of molecular weight approx. 60000 and 120000–130000 are present.

The results from Sephadex G-200 chromatography are anomalous. The Triton X-100 extract was eluted mainly in the void volume indicating a molecular weight far higher than the electrophoretic results would indicate. The presence of Triton X-100 or high concentrations of NaCl had very little effect on the elution profile of the major peak, but a small shoulder in the 100000–150000-molecular-weight region may be significant. Aggregation of the enzyme is the only explanation known by the authors which can account for the lack of correlation between the two methods. The Sephadex G-200 results for the concentrated supernatant are also different from the electrophoretic estimation, but measurement of molecular weights over 250000 is of doubtful accuracy and the shoulder in the profile encompasses the range of the other species separated by electrophoresis.

The majority of literature to date on acetylcholinesterase has been concerned with the *Electrophorus* enzyme since this has been obtained in the greatest purity. With early studies on enzyme of lower purity from *Electrophorus electricus* molecular weights

Table 3. Literature values for the molecular weight of acetylcholinesterase

Source	Method of extraction	Molecular weight	Reference
Calf caudate nucleus	Butanol	284000–360000 and 161400–204600	Jackson & Aprison (1966a)
Rat brain	Triton X-100	Over 200000	Ho & Ellman (1969)
	Bacterial protease	100000	Ho & Ellman (1969)
Bovine caudate nucleus	Repeated homogenization with EDTA	395000, 270000 and 130000	Shirachi <i>et al.</i> (1972)
<i>Electrophorus</i> electroplax	(Various)	260000	Millar & Grafius (1970)
		260000	Leuzinger <i>et al.</i> (1969)
		225000	Froede & Wilson (1970)
		Subunits	
		6×42200	Millar & Grafius (1970)
		4×64000	Leuzinger <i>et al.</i> (1969)
		4×49000	Froede & Wilson (1970)
Rabbit sciatic nerve	Triton X-100	170000 and 310000	Skangiel-Kramska & Niemierko (1971)

much higher than those shown were obtained and Grafius & Millar (1965) showed that aggregation occurs in low-ionic-strength media. However, present results indicate a molecular weight for the pure enzyme of 225000–260000 consisting of four to six subunits. In the present study all methods of extraction produced one species of molecular weight 59500 ± 5000 and a second of molecular weight 266100 ± 21200 . Although no previous information is available about the existence of the low-molecular-weight species having enzymic activity it is tempting to speculate that a polymerization sequence of type IV acetylcholinesterase is being demonstrated with the molecular weights of bands IV:III:IIa:II:Ia:I being in the approximate proportion of 1:2:3:4:6:7.

To investigate the possible dissociation of higher-molecular-weight polymers the effect of fairly high concentrations of urea was tried; 2.5 M-urea gave only two species, of molecular weight 269000 and 182000, the higher-molecular-weight species disappearing. Although these are only preliminary results it seems possible to dissociate the high-molecular-weight enzyme, but more conclusive results must await purification of the species studied.

The results presented here would tend to favour Leuzinger's (1971) second proposal for the subunit structure of acetylcholinesterase that ' α represents a catalytic subunit and β is bearing some other function not yet known', since if both α and β chains were necessary for activity a 60000-molecular-weight unit would not have activity.

We thank the Wellcome Trust for the provision of a pH-stat.

References

- Allen, J. M. & Gockerman, J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 617–633
- Andrews, P. (1965) *Biochem. J.* **96**, 595–606
- Bajgar, J. & Žižkovský, V. C. (1971) *J. Neurochem.* **18**, 1609–1614
- Baldwin, J. & Hochachka, P. W. (1970) *Biochem. J.* **116**, 883–887
- Barron, K. D. & Bernsohn, J. (1968) *J. Neurochem.* **15**, 273–284
- Barron, K. D., Bernsohn, J. & Hess, A. R. (1963) *J. Histochem. Cytochem.* **11**, 139–156
- Bernsohn, J. & Barron, K. D. (1964) *Int. Rev. Neurobiol.* **7**, 297–344
- Bernsohn, J., Barron, K. D. & Hess, A. R. (1962) *Nature (London)* **195**, 285–286
- Bernsohn, J., Barron, K. D. & Hedrick, M. T. (1963) *Biochem. Pharmacol.* **12**, 761–763
- Chan, S. L., Shirachi, D. Y. & Trevor, A. J. (1972) *J. Neurochem.* **19**, 437–447
- Chrambach, A. & Rodbard, D. C. (1971) *Science* **172**, 440–451
- Crone, H. D. (1970) *Proc. Aust. Biochem. Soc.* **3**, 52
- Crone, H. D. (1971) *J. Neurochem.* **18**, 489–497
- Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–427
- Davis, G. A. & Agranoff, B. A. (1968) *Nature (London)* **220**, 277–280
- Dolgo-Sakurov, V. B. (1969) *Ukr. Biokhim. Zh.* **42**, 671
- Eldefrawi, M. E., Tripathi, R. K. & O'Brien, R. D. (1970) *Biochim. Biophys. Acta* **212**, 308–314
- Froede, H. C. & Wilson, I. B. (1970) *Isr. J. Med. Sci.* **6**, 179–184
- Gordon, A. H. (1969) *Laboratory Techniques in Biochemistry and Molecular Biology* (Work, T. S. & Work, E., eds.) p. 50, North-Holland Publishing Co., Amsterdam
- Gordon, J. J. & Rutland, S. P. (1967) *Nature (London)* **214**, 850–851
- Got, K. & Polya, J. B. (1963) *Nature (London)* **198**, 884–885
- Grafius, M. A. & Millar, D. B. (1965) *Biochim. Biophys. Acta* **110**, 540–547
- Ho, I. K. & Ellman, G. C. (1969) *J. Neurochem.* **16**, 1505–1513
- Iqbal, Z. & Talwar, G. P. (1971) *J. Neurochem.* **18**, 1261–1267

- Jackson, R. L. & Aprison, M. H. (1966a) *J. Neurochem.* **13**, 1351–1365
- Jackson, R. L. & Aprison, M. H. (1966b) *J. Neurochem.* **13**, 1367–1371
- Koelle, G. B. (1951) *J. Pharmacol. Exp. Ther.* **103**, 153–171
- Kopperschlaeger, G., Diezel, W., Bierwagen, B. & Hofmann, E. (1969) *FEBS Lett.* **5**, 221–224
- Lawler, H. C. (1964) *Biochim. Biophys. Acta* **81**, 280–288
- Leuzinger, W. (1971) in *Cholinergic Ligand Interactions* (Triggle, D. J., Moran, J. F. & Barnard, E. A. eds), pp. 19–31, Academic Press, New York and London.
- Leuzinger, W., Goldberg, M. & Cauvin, E. (1969) *J. Mol. Biol.* **40**, 217–225
- Lewis, P. R. & Shute, C. C. D. (1966) *J. Cell Sci.* **1**, 381–390
- Lim, R., Davis, G. R. & Agranoff, B. W. (1971) *Brain Res.* **25**, 121–131
- Lowry, O. H., Rosebrough, A. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Margolis, J. & Kenrick, K. G. (1967) *Biochem. Biophys. Res. Commun.* **27**, 68–73
- Margolis, J. & Kenrick, K. G. (1968) *Anal. Biochem.*, **25** 347–362
- Marples, E. A., Thompson, R. H. S. & Webster, G. R. (1959) *J. Neurochem.* **4**, 62–70
- Maynard, E. A. (1966) *J. Exp. Zoo.* **161**, 319–33
- McArdle, B., Thompson, R. H. S. & Webster, G. R. (1960) *J. Neurochem.* **5**, 135–144
- Millar, D. B. & Grafius, M. A. (1970) *FEBS Lett.* **21**, 61–64
- Ornstein, L. (1964) *Ann. N.Y. Acad. Sci.* **121**, 321–349
- Paul, J. & Fottrell, P. (1960) *Biochem. J.* **78**, 418–424
- Plummer, D. T. (1971) *An Introduction to Practical Biochemistry* pp. 156–157, McGraw-Hill, London
- Rodbard, D., Kapadia, G. & Chrambach, A. (1971) *Anal. Biochem.* **40**, 135–157
- Shirachi, D. Y., Chan, S. L., Bhargava, H. N., Gardner, E. & Trevor, A. J. (1972) *Proc. West. Pharmacol. Soc.* **15**, 132–135
- Singh, J. & Wasserman, A. R. (1970) *Biochim. Biophys. Acta* **221**, 379–382
- Skangiel-Kramaska, J. & Niemierko, S. (1971) *Bull. Acad. Pol. Sci. Ser. Sci. Biol.* **19**, 389–393
- Slater, G. G. (1969) *Anal. Chem.* **41**, 1039–1041
- Wright, D. L. & Plummer, D. T. (1970) *Biochem. J.* **118**, 21P