Biochem. J. (1973) **133**, 521–527
Printed in Great Britain

Multiple Forms of Acetylcholinesterase from Human Erythrocytes

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(*Received* 30 *November* 1972)

1. Acetylcholinesterase from human erythrocytes was solubilized with Triton X-100 in strong salt solution and partially purified by $(NH_4)_2SO_4$ fractionation. This preparation showed three main bands of enzyme activity after electrophoresis on polyacrylamide gel and incubation with either α-naphthyl acetate or acetylthiocholine as enzyme substrate. Two of the multiple forms were completely inhibited by 10μM-eserine and one only partially. Treatment with neuraminidase had no effect on the electrophoretic pattern; therefore sialic acid does not appear to determine or affect the ratios of the acetylcholinesterase multiple forms, unlike those of the serum cholinesterase. 2. Chromatography of the preparation on Sephadex G-200 revealed one major peak of enzyme activity and a suggestion of two minor zones of mol.wt. 546000, 184000 and 93000 (i.e. in the proportion 6:2:1). The main peak was almost completely separated from the Triton X-100 and the overall purification was about 600-fold. Further attempts to purify the enzyme by absorption on calcium phosphate gels were unsuccessful. 3. Electrophoresis of the enzyme preparation on a polyacrylamide gradient for 24h revealed three main bands that corresponded to the three values for molecular weights obtained by column chromatography. After 70h of electrophoresis a further three zones of activity developed making six molecular entities, the molecular weights of which were simple multiples of a monomer, thus resembling the cholinesterase found in serum.

The fact that cholinesterases appear in the tissues of almost all multicellular animals indicates the physiological importance of these enzymes. Mammalian plasma contains a soluble cholinesterase (EC 3.1.1.8) which comprises a family of isoenzymes deriving from molecular aggregation and deaggregation of the monomer (LaMotta *et al.*, 1968; LaMotta & Woronik, 1971; Juul, 1968). This polymerization appears to be controlled to some extent by neuraminic acid (Gaffney, 1970). The membrane-bound enzyme, acetylcholinesterase (EC 3.1.1.7), has also been the subject of considerable work and the molecular properties of the highly purified enzyme prepared from the electric organs of *Electrophorus electricus* have been extensively investigated. The native polymer has a molecular weight of between 225000 and 260000 and the active enzyme has been variously reported to consist of from 4 to 6 subunits (Leuzinger *et al.*, 1969; Millar & Grafius, 1970; Froede & Wilson, 1970). Acetylcholinesterase from brain has been studied by Bernsohn *et al.* (1962) and Maynard (1966), who detected at least three isoenzymes electrophoretically in this and other tissue.

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The erythrocyte membrane is also a rich source of acetylcholinesterase, and the molecular weight of the enzyme was estimated by labelling the active site followed by treatment with sodium dodecyl sulphate. Under these conditions, the enzyme was shown to exist as a dimer of mol.wt. 180000 that could be further cleaved with 2-mercaptoethanol to give units of mol.wt. 90000 (Bellhorn *et al.*, 1970).

The work discussed above clearly shows that there is a strong case for the systematic study of acetylcholinesterase of human erythrocytes to see whether multiple molecular forms of this enzyme exist and this is the subject of the present work.

Experimental

Materials

Acetylcholine iodide, acetylthiocholine iodide and Triton X-100 were purchased from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Eserine was obtained from Burroughs Wellcome Co., London N.W.1, U.K., and α-naphthyl acetate from British Drug Houses Ltd., Poole, Dorset, U.K. Sephadex and Sepharose were supplied by Pharmacia, London W.5, U.K., and Lyphogel by Hawksley and Sons Ltd., Lancing, Sussex, U.K. All other chemicals were of analytical reagent quality. Water doubly distilled in a glass still was used throughout.

Gradipore preformed polyacrylamide gradient gel slabs were obtained from Universal Scientific Ltd., London E.17, U.K.

Methods

Enzyme assay. Acetylcholinesterase activity was measured by means of the pH-stat (Jacobsen *et al.*, 1957). The reaction medium was an unbuffered solution of 0.15M-NaCl and 1.2mM-$MgCl_2$ with acetylcholine iodide added to a final concentration of 1mM to initiate the reaction. The acid produced during hydrolysis of acetylcholine at 30°C and a constant pH of 7.4 was determined automatically (Radiometer, Copenhagen, Denmark). Corrections were made to account for spontaneous hydrolysis of the substrate and any other non-enzymic release of H^+ ions, although this was usually very small. Units of enzyme activities were expressed in i.u. (μmol of substrate hydrolysed/min).

Protein determination. Samples of protein at concentrations above 0.5mg/ml were assayed by means of a biuret reaction (Plummer, 1971), and other samples were measured by the Folin–Lowry method (Lowry *et al.*, 1951). In the latter case it was necessary to centrifuge the final mixture before assaying if Triton X-100 was present, but otherwise the detergent did not interfere. Bovine serum albumin was used as the standard protein.

Determination of Triton X-100. The detergent concentration was measured by a modification of an absorptiometric method (Stevenson, 1954). The following solutions were added to 2ml portions of samples in centrifuge tubes with thorough mixing after each addition: 75μl of 2M-HCl, 50μl of 10% (w/v) $BaCl_2$ and 50μl of 10% (w/v) phosphomolybdic acid. The mixture was centrifuged at 3000rev./min for 10min and the supernatant was removed and discarded. The tube was then inverted and allowed to drain on to absorbent paper for a minute or so, then the precipitate was dissolved in 3ml of conc. H_2SO_4 with thorough mixing. Exactly 40min after adding the H_2SO_4 the E_{520} of the solution was read.

As a linear relationship between extinction and detergent concentration was obtainable only up to 0.05% (w/v) Triton X-100, it was necessary to dilute samples before assay.

Solubilization of the enzyme. A method of solubilizing the acetylcholinesterase of human erythrocytes based on the joint effect of the non-ionic detergent Triton X-100 and the neutral salt KCl was used (Wright & Plummer, 1972). Washed stromata from human erythrocytes were dissolved in a mixture of 1% (w/v) Triton X-100 and 1.2M-KCl to yield a solution of enzyme that could not be sedimented when centrifuged for 1h at 4°C and 100000*g*.

$(NH_4)_2SO_4$ fractionation. Solid $(NH_4)_2SO_4$ was added slowly, with stirring, to the standard solution of stromata, this was left for 30min at 4°C then the precipitate was removed by centrifugation for 20min at 4°C and 15000*g*. Protein fractions were collected at 0–20, 20–40, 40–60, 60–80 and 80–100% saturation with respect to $(NH_4)_2SO_4$ and assayed for enzyme activity. After the preliminary experiments a standard procedure was adopted of collecting the precipitate after adding $(NH_4)_2SO_4$ to 60% saturation, which precipitated about 75% of the enzyme.

Calcium phosphate gels. To purify further the acetylcholinesterase obtained as described above, the enzyme was treated with calcium phosphate gels at various pH values from 5.0–8.7 (Main *et al.*, 1959). The enzyme activity and protein concentration of the supernatant were assayed and the total activity and specific activity of each sample were compared with an untreated control sample.

Gel filtration chromatography. Three types of gel were initially employed for gel filtration: Sepharose 4B, Sepharose 6B and Sephadex G-200. The precipitate obtained after treatment with 60%-satd. $(NH_4)_2SO_4$ was dispersed in 0.15M-NaCl and centrifuged for 30min at 4°C and 35000*g*. The supernatant was concentrated about 5-fold with Lyphogel to give an enzyme activity of 6–14 units/ml, and 1ml of this solution was placed on a chromatography column (33cm×2.5cm) containing one of the three gels mentioned. The column was eluted with NaCl and 5ml fractions were collected automatically. Each fraction was assayed for acetylcholinesterase activity, protein and Triton X-100. With Sephadex G-200, NaCl concentrations of 0.05, 0.15, 0.50 and 1.0M were used in turn. Columns of Sepharose 4B and 6B were eluted with 0.15M-NaCl only. Molecular-weight estimations of peaks of enzyme activity were made by calibration of the column with markers [cytochrome *c* (12000), ovalbumin (45000), bovine serum albumin (68000), lactate dehydrogenase (134000), γ-globulin (160000), thyroglobulin (670000) and Blue Dextran (>2000000)] (Andrews, 1965).

Disc electrophoresis. The esterases were separated by electrophoresis on rods of polyacrylamide gel prepared by the method of Allen & Gockerman (1964). Spacer and sample gels were omitted (Gordon, 1969).

Before accepting a standard condition for electrophoresis, four buffers were investigated, namely KH_2PO_4–Na_2HPO_4, pH8.0; sodium barbitone–barbituric acid, pH8.0; Tris–glycine, pH8.0 and histidine–NaOH, pH7.5. Ionic strengths of 0.01, 0.02 and 0.05 were used with all these buffers to determine the effect of ionic strength on migration patterns. The sample (30–50μl) was mixed with sucrose and the solution was applied to the surface of the gel. Although 3 and 6% (w/v) polyacrylamide gels were used in initial experiments, 7.5% (w/v) gels were more effective and this concentration was used.

Gradient gel electrophoresis. Preformed Gradipore concave-gradient polyacrylamide gels, made up in the form of slabs of approx. 70mm×70mm×3mm were used (Universal Scientific Ltd., London E.17, U.K.). The slabs had a gradient that ran from about 4 to 26% (w/v) polyacrylamide. Samples (30μl in sucrose) were added and electrophoresis was done at 100V for 24h and at 50V for 70h. In the latter case the gels were initially subjected to electrophoresis for 2h at 30V (Margolis & Kenrick, 1968). In estimating molecular weights, the gradient function curve supplied with the gels was used to compare the migration distances of regions of activity with the positions of known protein markers.

Staining for acetylcholinesterase. Regions of acetylcholinesterase activity were made visible by using acetylthiocholine as substrate by the method of Lewis & Shute (1966). The only modification was the omission of the final ammonium sulphide treatment since the white bands of copper thiocholine could easily be observed. Alternatively, the zones of enzyme activity were determined by comparing the staining pattern obtained by using α-naphthyl acetate and tetrazotized *o*-dianisidine in the presence and absence of 10μM-eserine as described by Paul & Fottrell (1960).

Neuraminidase treatment. The enzyme solution (3ml), containing about 40mg of protein, was incubated with neuraminidase (6mg) in 3ml of buffer (0.15M-KCl–0.66mM-$CaCl_2$–0.1M-sodium cacodylate, pH5) for 1.5h at 30°C. The neuraminidase-treated enzyme and untreated enzyme were assayed for free sialic acid content (Warren, 1959) and both samples were subjected to electrophoresis on polyacrylamide-gel rods.

Results

Effect of aging

The acetylcholinesterase activity of human erythrocytes from 14 samples of freshly drawn blood was 12.16±1.24 (S.D.) units/ml of packed cells. The enzyme activity of 20 samples of time-expired transfusion blood was lower than this at 8.13±1.12 (S.D.) units/ml of packed cells, but apart from the amount of enzyme activity, the results obtained for the time-expired blood were essentially the same as those for fresh blood.

$(NH_4)_2SO_4$ precipitation

Treatment with $(NH_4)_2SO_4$ gave rise to considerable variation in the enzyme precipitated at 0–20, 20–40 and 40–60% of a saturated solution, but the total amount of activity precipitated between 0 and 60% was relatively constant. When 15 samples of stromata dissolved in 1% Triton X-100 containing 1.2M-KCl were treated with 60% satd. $(NH_4)_2SO_4$, an average of 87% of the original enzyme activity was precipitated and 13% remained in the supernatant.

While most of the enzyme separated out in a pinkish–brown leaflet (P_t), which was less dense than the $(NH_4)_2SO_4$ solution, a smaller percentage appeared in a denser white precipitate (P_b), which collected at the bottom of the centrifuge tube. Precipitate P_t comprised 64% and P_b 12% of the original activity.

The pH of the unbuffered solution was 4.5. The use of 0.1M-NH_3 to raise the pH to 5.0, 6.0 and 8.0 did not alter the yield of precipitable enzyme.

Similarly, alteration of the density of the solution with sucrose neither changed the yield of precipitable enzyme nor its distribution between forms P_t and P_b.

Absorption characteristics of calcium phosphate gels

Although calcium phosphate gels were prepared over a wide range of pH values, in no instance was any real increase observed in specific activity. These results led to the decision to use the precipitate P_t to investigate the isoenzymic character of the preparation.

Gel-filtration chromatography

Only precipitate P_t, dissolved in NaCl was used, because it was not possible to concentrate fraction P_b sufficiently for it to be assayed after dilution on the column. Of the three gels, only Sephadex G-200 gave results which encouraged further study, and therefore this was used for chromatography. Fig. 1 shows the characteristic elution pattern for acetylcholinesterase on Sephadex G-200 where the greater part of the enzyme activity has been separated from Triton X-100. It also shows that the greater part of the protein in the sample was separated from the fractions with enzyme activity. The results illustrated and other similar experiments all showed one main peak of enzyme activity with indications of two minor peaks representing lower-molecular-weight entities; resolution was not improved by using longer columns. For convenience, the major region of activity is referred to as 'peak I' and the two minor regions as 'peak II' and 'peak III'. Essentially the same results were obtained whether fresh or time-expired blood was used. The best purification on Sephadex G-200 was obtained by elution with 0.15M-NaCl (Table 1).

The molecular weights of the peaks measured by chromatography on Sephadex G-200 in 0.15M-NaCl were 588000, 199000, and 110000. Elution of a column with 0.5M-NaCl yielded molecular weights of 550000, 182000 and 77000 respectively, and a column eluted with 1.0M-NaCl gave the values 501000, 170000 and 91000.

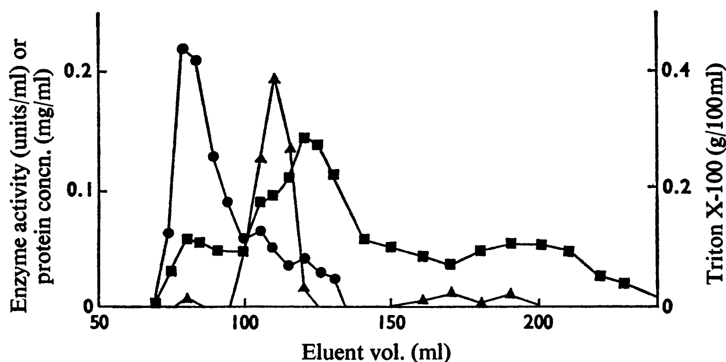

Fig. 1. *Elution of acetylcholinesterase activity (●), protein (■) and Triton X-100 (▲) from a column of Sephadex G-200 by* 0.15M-*NaCl*

Partially purified acetylcholinesterase containing from 6–14 units of enzyme activity was fractionated on Sephadex G-200 with 0.15M-NaCl as eluent. Experimental details are given in the text.

Table 1. *Specific activities of fractions obtained from Sephadex G-200 columns after elution with NaCl*

The average specific activity (μmol/min per mg of protein) of all fractions contributing to a peak on the Sephadex G-200 chromatography is listed for experiments at different ionic strengths. The percentage of total column activity is given in parentheses.

Concn. of eluent (M)	Acetylcholinesterase sp. activity (μmol/min per mg of protein)		
	Peak I	Peak II	Peak III
0.05	1.37 (77%)	0.56 (23%)	—
	1.04	—	—
0.15	4.06 (79%)	0.53 (15%)	0.20 (6%)
	8.30 (80%)	1.30 (14%)	0.40 (6%)
	9.40 (89%)	0.81 (7%)	0.26 (4%)
	4.20 (83%)	0.60 (10%)	0.18 (7%)
	6.51 (70%)	1.67 (24%)	0.46 (6%)
	3.60 (86%)	0.54 (9%)	0.31 (5%)
0.50	0.80 (70%)	0.51 (21%)	0.15 (9%)
	2.72 (70%)	1.05 (19%)	0.83 (11%)
	2.30 (70%)	0.53 (19%)	0.26 (11%)
1.0	0.85 (90%)	0.13 (10%)	—
	1.08 (43%)	0.45 (40%)	0.15 (17%)
	1.07 (51%)	0.18 (36%)	0.08 (13%)
	1.37 (86%)	0.30 (14%)	—
	1.86 (78%)	0.40 (22%)	—

Disc electrophoresis

Tris–glycine buffer was used for the polyacrylamide-gel electrophoresis of precipitate P_t, partly because of its advantages (ease of staining and reproducibility) and partly because of the disadvantages of the other buffers, namely, the obscuring effect of background staining and irreproducibility.

When acetylthiocholine iodide was used as staining substrate, electrophoresis of precipitate P_t gave rise to five bands of acetylcholinesterase activity, three (1, 2 and 3) of which developed within 30min to 1h. Incubation of the gels for a much longer time led to the development of two further bands (4 and 5) which were broader, but much less intense than the

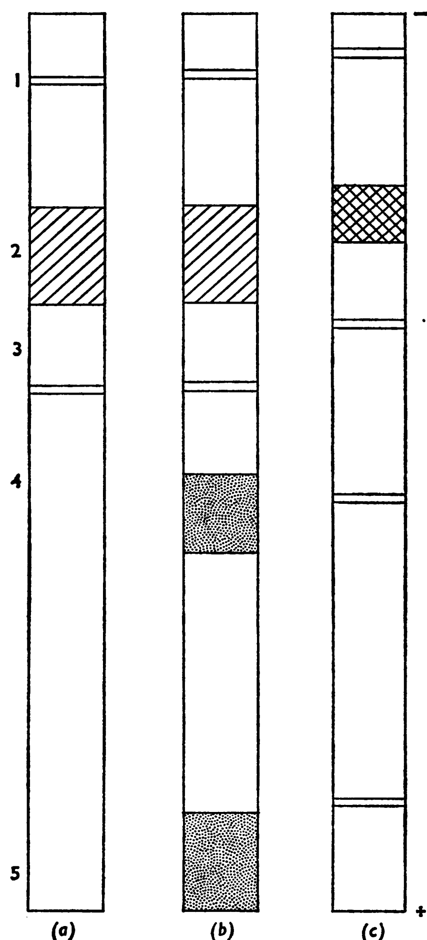

Fig. 2. *Electrophoresis of acetylcholinesterase from human erythrocytes on rods of polyacrylamide gel*

(*a*) Enzyme staining with α-naphthyl acetate as substrate; (*b*) enzyme staining with acetylthiocholine as substrate; (*c*) protein staining with Amido Black. ▩, Strong staining; ▨, moderate staining; □, weak staining; ▦, stain visible only after prolonged incubation. For further details of bands 1–5 see the text.

other bands (Fig. 2*b*). Staining with α-naphthyl acetate produced three bands identical in mobility and very similar in intensity to bands 1–3 revealed with acetylthiocholine iodide (Fig. 2*a*). In both instances all bands except number 3 were inhibited by 10μM-eserine. (There was only a partial inhibition of the third band.)

Table 2. *Molecular weights of acetylcholinesterase from chromatography on Sephadex G-200 and gradient electrophoresis*

In the case of the gradient electrophoresis, it was not possible to determine the peaks of activity within each band and therefore the range encompassed by each zone is given.

Molecular weights of acetylcholinesterase		
Column chromatography (average)	Gradient electrophoresis	
	24h	70h
93000	66000–83000	74000–91000
184000	158000–170000	186000–199000
		246000–251000
		354000–398000
		427000–501000
546000	427000–708000	562000

When the peaks obtained from chromatography of precipitate P_t were subjected to electrophoresis, three bands of activity were obtained in all cases, although bands 1 and 3 were not always visible if the activity was low.

Gradient-gel electrophoresis

Electrophoresis for 24h of the precipitate derived from KCl–Triton X-100 gave rise to three bands of enzyme activity, whereas six bands were observed after electrophoresis for 70h. The molecular weights of these bands and the average results from column chromatography are given in Table 2.

Neuraminidase treatment

The precipitate P_t was shown to contain sialic acid by hydrolysis of the sample with 0.05M-H_2SO_4 for 1h at 80°C and also by treatment with neuraminidase. The free sialic acid content of the control sample was 1.5nmol/mg of protein and this was increased to 42.2nmol/mg of protein on acid hydrolysis and 25.2nmol/mg of protein on enzyme treatment. The electrophoretic pattern of the acetylcholinesterase, however, was unaffected by incubation with neuraminidase.

Discussion

The best purification of acetylcholinesterase on Sephadex G-200 was achieved by elution with 0.15M-NaCl (Table 1). In two experiments, a purification

of approx. 800-fold was obtained for peak I, which included more than 80% of the total activity. The average purification for this peak, 567-fold, was more than three times that obtained with 0.5M-NaCl. Elution with 0.15M-NaCl gave as good a separation of isoenzymes as those obtained with 0.50M-NaCl and much better results than with 0.05M- or 1.0M-NaCl. Workers investigating acetylcholinesterase from *Electrophorus electricus* (Grafius & Millar, 1971) identified 'polydisperse components' of the enzyme by observing sedimentation coefficients and reported that 'three major but polydisperse components could be observed depending on the ionic strength of the medium'. We concluded that collection of the precipitate P_t in 0.15M- and 0.50M-NaCl (which consistently produced three peaks of acetylcholinesterase activity) provided an ionic environment most conducive to some dissociation of the higher-molecular-weight entity found in peak I.

The averaged molecular weights for the peaks are 546000 (peak I), 184000 (peak II) and 93000 (peak III). Bellhorn *et al.* (1970) used tritiated di-isopropyl fluorophosphate to show that erythrocyte acetylcholinesterase exists as a monomer (mol.wt. 90000) and a dimer (mol.wt. 180000). The molecular weights observed on chromatography are in the proportion 6:2:1 and the lowest molecular weight, 93000, is close to the reported value of the monomer and is also approximately twice the values reported for the monomer of the enzyme purified from *Electrophorus electricus* by Millar & Grafius (1970) (42000) and by Froede & Wilson (1970) (49000).

The observation that peak I, which contained from 70 to 90% of the total activity on the column, was usually free of detergent (Fig. 1) is important because it indicates that the association of the enzyme with Triton X-100 is reversible and that separation of acetylcholinesterase and Triton X-100 does not cause inactivation.

Electrophoresis of precipitate P_t revealed four, and possibly five, electrophoretically distinct forms of acetylcholinesterase (Fig. 2). The third band of activity may consist of cholinesterase and some non-choline esterases, since some of the activity was suppressed by eserine.

There were five bands of protein that approximately overlay the regions of enzyme activity, although four bands were extremely narrow. The presence of a broad zone of protein overlaying the major zone of acetylcholinesterase is indicative of the relative impurity of the enzyme at this stage.

None of the chromatography peaks may be said to differ electrophoretically from the crude preparation. The fourth and fifth bands of activity obtained from precipitate P_t by using acetylthiocholine were not observed in the column peaks. However, the dilution occurring during gel filtration could account for their absence during the pH-stat assay of the fractions and subsequent electrophoresis of the three observed peaks.

Gel filtration of precipitate P_t showed that there are probably three molecular forms of the enzyme. However, each of these forms (peaks I, II and III) behaved identically during electrophoresis, in which molecular seiving plays a major role. These results are explicable on the basis of a time-dependent equilibrium between the three molecular forms. Alternatively, the act of concentration could cause interconversion. In either case the three peaks would assume identical properties.

In both the 24 and 70h gradient-electrophoresis studies three main bands of activity were demonstrated, with molecular weights that corresponded fairly well with the three values obtained by column chromatography (Table 2). After 70h of electrophoresis a further three zones of activity developed, and these had molecular weights that were roughly multiples of the entity of lowest molecular weight, differing from it by factors of 3, 4 and 5 respectively. A complete series of multimolecular aggregates from monomer to hexamer was therefore indicated by the 70h electrophoretic run. The failure to observe the trimer, tetramer and pentamer during the 24h run and on Sephadex G-200 might be due to the slow formation of these aggregates. However, if these intermediate oligomers are formed by equilibration during the 70h electrophoretic run, they would be expected to be smeared out between the hexamer and the dimer positions and not to occupy the definite positions observed. These three new peaks could therefore be artifacts of the electrophoretic system. Whatever the final explanation, the existence of multiple forms of the partially purified acetylcholinesterase would appear to be clearly established.

Initial work showed a difference in the amount of acetylcholinesterase activity in fresh and time-expired erythrocytes, but electrophoresis and chromatography did not reveal any significant differences in enzyme heterogeneity suggesting that preferential loss of one or more forms does not occur during storage of the blood.

We are grateful to the Wellcome Trust for providing equipment and running expenses for this project.

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