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A SIMPLE METHOD FOR PURIFICATION OF ACETYLCHOLINESTERASE FROM HUMAN ERYTHROCYTE MEMBRANES

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SUMMARY

A new and simple method for preparation of a highly purified and homogeneous acetylcholinesterase from human erythrocytes has been described This method consisted of (a) the preparation of hemoglobin-free ghosts (b) the solubilisation of the ghosts with deoxycholate (c) precipitation of the active protein with $(NH_4)_2SO_4$ and (d) its chromatography in Sephadex gel These steps led to a very high purification and specific activity, higher than any values reported in the literatures for human erythrocyte acetylcholinesterase that we are aware of The K_m of the purified enzyme using different substrates and the K_i using quinidine as inhibitor have been determined

INTRODUCTION

Acetylcholinesterase (acetylcholine hydrolase EC 3 1 1 7), a membrane-bound enzyme [1] has been the object of intense interest to the neurochemists because of its paramount importance in the control of electrical activity of excitable membranes [2] The enzyme has been successfully prepared in pure and homogeneous form from eel electric tissue [3, 4] Acetylcholinesterase from this source has been extensively studied and its molecular properties well characterised [5–10] On the other hand studies on the molecular properties of acetylcholinesterase prepared from human erythrocytes have lagged behind, hampered by a lack of the erythrocyte enzyme of comparable purity and homogeneity Despite numerous claims [11–14] no pure, homogeneous enzyme from human erythrocytes has yet been prepared The methods in use have a variety of drawbacks (1) The use of Tween 20 [11] involves complex steps which led to products of unpredictable properties (2) The use of Triton X-100 [12, 13] led to heterogeneous proteins (3) That of a hypertonic solution of NaCl and $CaCl_2$ [14] resulted in a considerable loss of enzyme activity An attempt to use sodium dodecylsulfate resulted in almost complete inactivation of the products [14] Apart from inherent drawbacks these methods can, at best, only lead to products which are still inhomogeneous and polydispersed and in our hands, not suitable for studies at the molecular level*

* Affinity chromatography is said to lead to high specific activity [4] but this method has so far only been found usefull for purification of the eel and the bovine enzyme [10], not for the human one

In this paper a method for preparation of a pure and homogeneous acetylcholinesterase from human erythrocytes is described using deoxycholate as the solubilising agent The method, while simple, resulted in the production of a highly purified enzyme suitable for use in studies at the molecular level It avoids all the pitfalls encountered in the methods available for the purification of the enzyme to date And above all the method requires little time, equipments and chemicals

MATERIALS

Acetylthiocholiniodide, dithiobisnitrobenzoic acid, glycine, sodium deoxycholate, quinidine sulfate, bovine erythrocyte acetylcholinesterase and serum albumin, were obtained from Sigma Chemical Co (St Louis, Mo), $MgCl_2$ and NaCl, sodium phosphates, phenol red and amido black from Merck (Darmstadt), $(NH_4)_2SO_4$, Tris and polyacrylamide gel from BDH Ltd (Poole), and Sephadex gels from Pharmacia AB (Uppsala)

Determination of enzyme activity

Small portions of the preparations resulting from the purification steps were desalted where possible by passing through Sephadex G-75 column (30 cm × 1 2 cm) equilibrated and eluted with 0 01 M Tris This step was followed by dialysis, for 2 periods of 6 h against distilled water then for 8 h against Tris buffer (1 mM, pH 8 6) prior to the determination of their activities

Enzyme activity was determined by the method of Ellman et al [15], when acetylthiocholine was used as the substrate, otherwise by either colorimetric method [16] or titrimetric method (using 0 001 M NaOH) when acetylcholine was used as the substrate For the Ellman's method the following modification was introduced to avoid the use of phosphate buffer 1 mM Tris made 0 10 M with respect to NaCl and $MgCl_2$ and adjusted to pH 8 0 with HCl was used as the buffer The concentration of acetylthiocholine was increased to 0 375 M A typical run contained 2 50 ml buffer, 0 20 ml enzyme solution (containing 1–10 μg protein), 0 02 ml substrate, 0 10 ml dithiobisnitrobenzoic acid and distilled water to make a volume of 3 14 ml For erythrocyte activity, 3 0 ml suspension (1 in 600 dilution in buffer) were used The activity was either followed continuously in a recorder or determined after a fixed period of incubation at room temperature using appropriate blank in an Acta III spectrophotometer (Beckman Instruments) The change in absorbance per min per μg enzyme was proportional to the activity This absorbance could be converted into μmoles/min per mg enzyme to arrive at units of activity [15]

Deoxycholate was determined by the method of Samuel et al [17], protein by the method of Lowrey t al [18], as modified by Miller [19], using bovine albumin as standard, with the exception that protein in chromatographic effluents was estimated by its absorbance at 280 nm

Purification of acetylcholinesterase

Preparation of ghosts Hemoglobin-free ghosts [20] were prepared in the cold room from human banked blood (acid–citrate–dextrose-preserved) by washing with phosphate buffer at pH 7 4 All subsequent preparative procedures were carried out at 4 °C unless otherwise stated

TABLE I

EFFECT OF DEOXYCHOLATE ON THE SOLUBILISATION OF ACETYLCHOLINESTERASE FROM ERYTHROCYTE MEMBRANES

Six ghost suspensions each with a volume of 6 ml and contained about 10 mg protein, were treated with deoxycholate of the concentrations indicated (another containing about 5 mg treated with 20 mM deoxycholate was included to check the effect of ghost concentration) They were stirred for 15 min and their absorbance at 600 nm was taken Then 5-ml portion of each was spun at 100 000 × *g* for 1 h The precipitate was resuspended in 5 ml water Protein content and activity of the precipitate and the supernatant are shown in the table The total activity is the activity multiplied by the total protein

	Protein			Total activity/h		
Deoxycholate (mM)	Precipitate (mg)	Supernatant (mg)	% in the supernatant	in precipitate	in supernatant	% in the supernatant
0	6 060	2 640	30	2332	276	10
10	5 340	3 340	39	1848	474	24
20	4 980	5 285	52	1248	1284	51
20	1 860	3 185	63	502	720	60
30	2 300	9 130	80	612	2096	78
40	0 840	10 550	93	160	1148	88
60	0 560	10 440	95	96	980	90

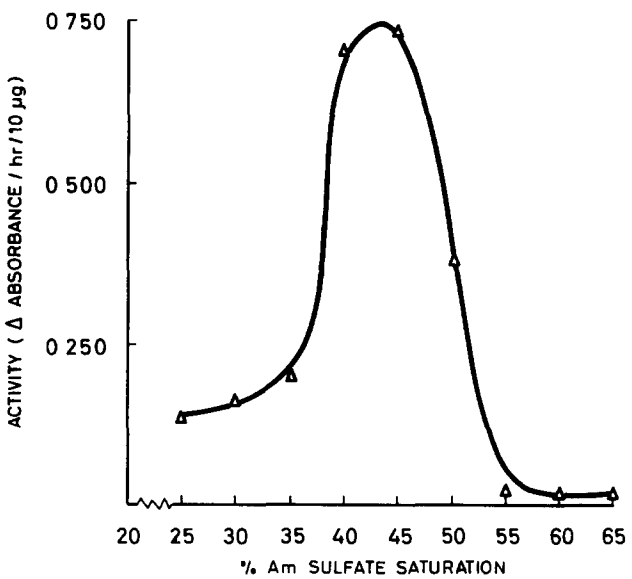

Fig 1 $(NH_4)_2SO_4$ precipitation of acetylcholinesterase 100 ml of deoxycholate-solubilised ghosts, adjusted to pH 9, was brought to 20% $(NH_4)_2SO_4$, stirred and then allowed to stand for 15 min before spinning ar 10 000 × *g* for 30 min The precipitate was set aside for analysis of protein and enzyme activity The supernatant was adjusted to pH 8 2, treated to the next higher sulfate concentration, stirred, allowed to stand and spun as before The precipitate was again set aside This procedure was repeated as necessary, each time adjusting the supernatant to pH 8 2 before treating it to the next higher concentration The activities of the precipitates measured as changes in absorbance/h per 10 µg are shown in the figure

Solubilisation of ghosts A preliminary study of ghost solubilisation for a fixed 15-min period showed that the extent of solubilisation depended on the relative amounts of deoxycholate and ghosts used Solubilisation was determined by the increase in the amount of protein in the supernatant following centrifugation of the deoxycholate-treated suspension at 100 000 × *g* for 1 h [21] in a Beckman preparative ultracentrifuge, using the SW-65L titanium rotor (Beckman Instruments, Calif) The results of such a preliminary study are presented in Table I, where it is shown that the enzymic activity of the supernatant was roughly proportional to the amount of protein in it for deoxycholate concentration of 10 to about 40 mM, but beyond which the activity decreased

Accordingly in a large scale preparation 70 ml of ghost suspension containing about 10 mg protein/ml was made to 30 mM with respect to deoxycholate concentration by adding 0 9 g sodium deoxycholate The pH was adjusted to 9 0 and the suspension was stirred for 15 min when the visible clumps of ghosts had disappeared

$(NH_4)_2SO_4$ fractionation and gel chromatography

$(NH_4)_2SO_4$ fractionation studies showed that 95% of the active protein was precipitated when the $(NH_4)_2SO_4$ saturation was 33–58% (Fig 1) Therefore the 70 ml of the ghost solution was brought to 33% satn with $(NH_4)_2SO_4$ (13 7 g) and stirred for 15 min After allowing to stand for 30 min it was centrifuged for 20 min at 10 000 × *g* in a Sorvall Superspeed SS-3 centrifuge The supernatant, adjusted to

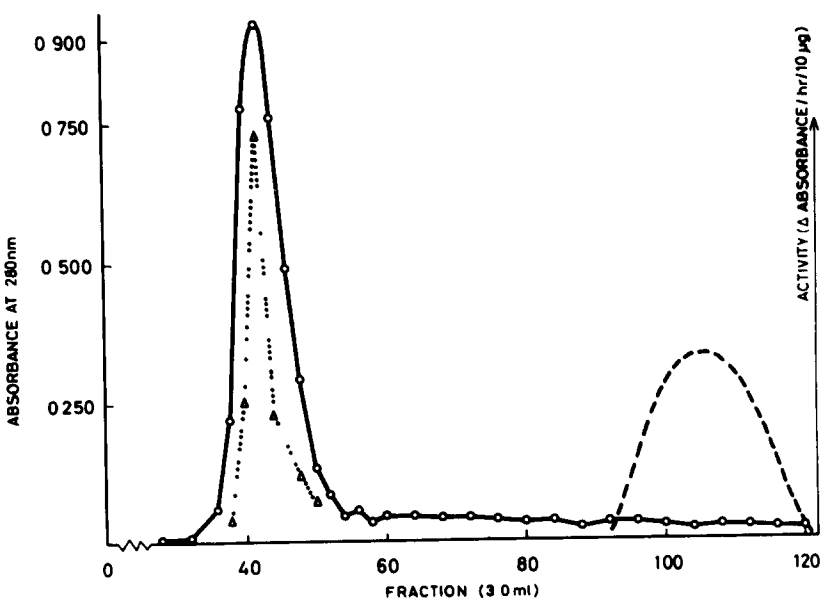

Fig 2 Purification acetylcholinesterase by gel filtration 3 ml of the enzyme solution (15 mg protein) obtained by $(NH_4)_2SO_4$ precipitation were run through a Sephadex G-200 column (2 5 cm × 70 cm) equilibrated with 0 05 M carbonate buffer (pH 9 2) containing 0 05 M NaCl The proteins were eluted with the same buffer 3-ml fractions were collected using Radi-Rack Fraction Collector (Type 3403B, LKB, Sweden) ○—○, absorbance of fractions at 280 nm, △—△, their corresponding activity (absorbance/h per μg) The broad peak (– – –) was that of hemoglobin commonly observed if the ghosts used were not hemoglobin-free

pH 8 2, was made to 58% satn with $(NH_4)_2SO_4$ (12 5 g) It was allowed to stand for 1 h before spinning for 45 min at 18 000 × *g* The precipitate, partly sticking to the sides of the centrifuge tubes was dissolved in 5 2 ml of 50 mM carbonate buffer (pH 9 2) containing 50 mM NaCl 3 ml of this solution (= 15 mg protein) were run through a Sephadex G-200 column, equilibrated with the same carbonate buffer Elution of the protein was also made with the same buffer Desired fractions were pooled and desalted by passing through Sephadex G-75 column, equilibrated with 0 01 M Tris buffer (30 cm × 1 2 cm) This preparation was then dialysed for 2 periods of 6 h against distilled water followed by 8 h against 0 001 M Tris buffer (pH 8 6) prior to activity determination, to use in electrophoresis or to lyophylisation for storage The protein concentration and the activity of each fraction are shown in Fig 2

Electrophoresis

A volume representing 4 μg of the dialysed enzyme preparation was electrophoresed in polyacrylamide gel (7 5 %) with Tris–glycine (pH 8 5) as buffer [22] A volume containing 50 μg of the protein precipitate between 26 and 60% (not between 3 and 58%) $(NH_4)_2SO_4$ satn was run concurrently for comparison (Fig 3) 0 8 μg of a marker protein run under identical conditions as a check for the sensitivity of the system was clearly detectable (not shown in the figure)

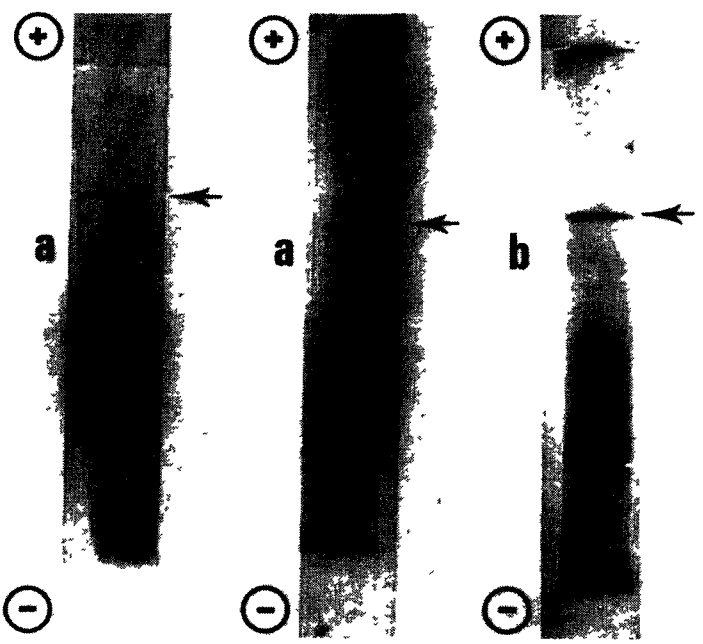

Fig 3 Electrophoresis of acetylcholinesterase preparation Disc electrophoresis of the enzyme preparation obtained at different stages of purification was carried out at room temperature in 7 5% polyacrylamide gel using Tris–glycine buffer (pH 8 5) at a current of 4 mA/tube for 30 min The gel was stained with amido black and destained by electrophoresis (a) (in duplicates) 0 05 ml containing 4 0 μg protein of the active peak from the Sephadex G-200 column (b) 0 10 ml containing 50 0 μg of the active protein precipitated on treatment with $(NH_4)_2SO_4$ between 26 and 60% satn The enzyme band is indicated by arrow

In Table II are shown each of the stages in the purification of the enzyme together with the corresponding protein and activity

TABLE II

PURIFICATION OF ACETYLCHOLINESTERASE FROM HUMAN ERYTHROCYTES

Successive stages in the purification The protein content and the corresponding activity at different stages are shown Activity is expressed in units (μmoles substrate cleaved/min) Total activity is the activity multiplied by the total amount of protein Values in brackets were obtained on using an optimum deoxycholate concentration of 60 mM and starting with a ghost concentration of 8 mg/ml Other values, on using suboptimum deoxycholate of 30 mM and ghost concentration of 10 mg/ml

Purification steps	Total protein (mg)	Total activity (units $\times$100)	Specific activity (units/mg $\times$100)	Yield (%)	Purification
Erythrocytes	51 700 0	113 300	2 (2 5)	100	1
Ghosts	750 0	87 375	102 (77)	77	51 (31)
Solubilised ghosts	679 0	78 200	98 (71)	69	49 (28)
Sulfate fraction 33–58% satn	36 2	16 845	466 (1860)	15	233 (744)
Sephadex G-200 gel	12 5	15 500	1243 (12 090)	14 (13)	622 (4836)

Determination of the optimum pH

The optimum pH was estimated by titration with 0 001 M NaOH at constant

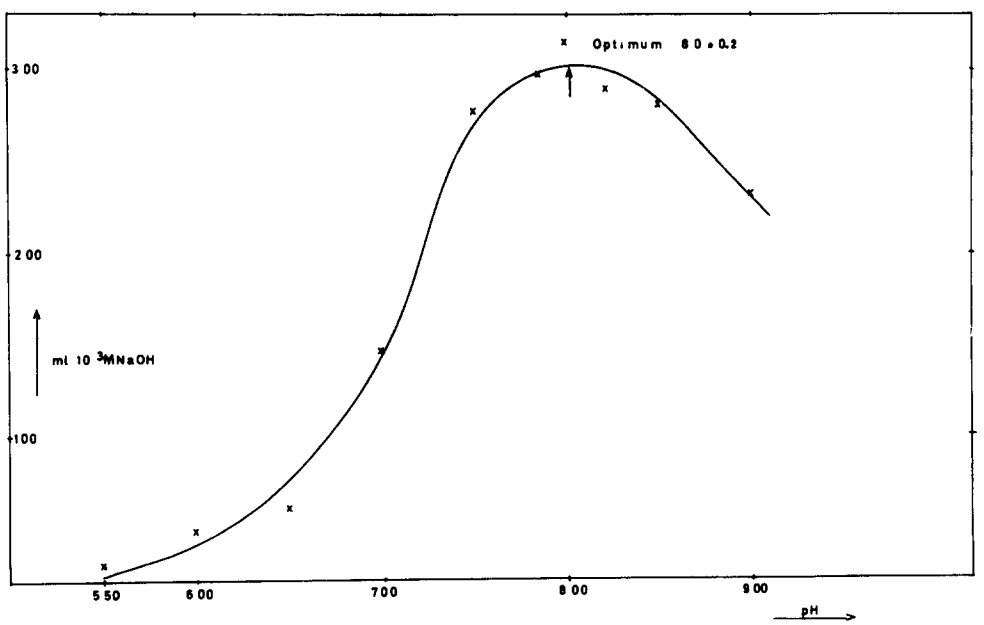

Fig 4 Hydrolysis of acetylcholine by acetylcholinesterase as a function of pH Reaction condition [acetylcholine] = 0 01 M, [NaCl] = 0 01 M, and [$MgCl_2$] = 0 01 M, temperature 26 °C and enzyme used 10 μg, Acetic acid produced was titrated with 0 001 M NaOH using Metrohm Combititrator pH-Stat (Switzerland)

pH of the acetic acid produced on incubating the enzyme with acetylcholine at different pHs, using an automatic titrator The results are shown in Fig 4, from which the optimum pH for the enzymatic reaction was estimated to be 8 2 $\pm$ 0 2 This value is in agreement with the values found by others, for acetylcholinesterase from bovine erythrocytes [23], eel electric tissue [24] and from brain tissue [25]

RESULTS AND DISCUSSION

After the first submission of this paper we have carried out additional investigations in the hope of further increasing the specific activity of the preparation We found that the optimum deoxycholate concentration, as far as specific activity and purification are concerned, is 60 mM Using this concentration and starting with a ghost concentration of 8 mg/ml we obtained an almost 10-fold increase in the specific activity and a purification of almost 5000-fold These values are presented here and in Table II in brackets following those obtained by using a suboptimal deoxycholate concentration of 30 mM

The method described led to a soluble acetylcholinesterase in good yields, 12–18% The specific activity ranged from 12 00–15 00 (121 00) units/mg from samples to samples and the purification of 600–700 (5000) fold using banked blood which was 6-weeks-old as the starting material Using a 13-week sample a value as low as 8 00 units/mg was once found A specific activity of 12 43 (121 00) units/mg recorded in Table II is considerably higher than any values reported in the literatures [11, 12, 26] for the human erythrocyte enzyme Although the method of affinity chromatography reportedly led to high specific activity for bovine and eel enzyme [4, 10], this method is yet to be tried for the purification of a human one And even if it turned out to be useful, the method is relatively expensive Besides it requires an already soluble acetylcholinesterase as the starting material

When this paper was submitted for publication an article by Paniker et al [29] appeared which described a preparation of acetylcholinesterase using Tween 20 or Triton X-100 These workers reported of a product with a purification and specific activity of 4176 and 118 units/mg, respectively, which are slightly lower than ours, 4836 and 121 00 units/mg*, respectively However, it would appear that the activity of their preparation was partly due to activation by Tween 20 or the Triton X-100 they used for the solubilisation of the ghosts These agents are known to bind tightly to membrane acetylcholinesterase and to strongly activate the enzyme [21, 14] They did not determine the amount of the detergent remaining in their final enzyme preparation as we did

The enzyme was colorless, very soluble in water, saline and Tris or phosphate buffer (pH 8 0) It was stable when kept frozen, losing only 20% of its original activity in a month However, at 4 °C, it lost 50% of its activity in 10 days, at room temperature, 50% in 10 h In the dry form the enzyme was found to be even more stable On electrophoresis in polyacrylamide gel the purified enzyme migrated as a single band towards the positive electrode (Fig 3a) In contrast a preparation obtained by $(NH_4)_2SO_4$ precipitation of 26–60% satn appeared to show nine bands (Fig 3b) Unfortunately it was not possible to demonstrate enzyme activity in the system used

* Their values were reported per h

Besides the amount of enzyme applied was too small for the purpose However, since there was only one band detectable (Fig 3a, marked with arrow), it must obviously be that of the enzyme 0 8 μg of a marker protein could be clearly seen when run under identical conditions

Time required for the preparation of the enzyme is about 20 h, starting from ghosts Solubilisation of ghosts, 15 min, first $(NH_4)_2SO_4$ precipitation, period of standing and centrifugation, 45 min, second $(NH_4)_2SO_4$ precipitation, standing and centrifugation, 90 min, and running in Sephadex G-200, 16 h

Determination of Michaelis constant, K_m, and inhibition constant, K_i, using a known competitive inhibitor (quinidine) [27] for the purified enzyme was carried out using two different substrates, acetylcholine and acetylthiocholine Values obtained are shown in Table III*

TABLE III

K_m AND K_i OF ACETYLCHOLINESTERASE

	K_m (mM)		K_i (quinidine) (mM)	
	Acetylcholine	Acetylthiocholine	Acetylcholine	Acetylthiocholine
Bovine acetylcholinesterase (Sigma)	0 66 ± 0 04	0 22 ± 0 02	0 40 ± 0 03	0 21 ± 0 02
Human acetylcholinesterase (our laboratory)	0 045 ± 0 004	0 28 ± 0 02	0 45 ± 0 03	0 13 ± 0 02

It is noteworthy that the K_m value, for the human enzyme, using acetylcholine as substrate, was significantly smaller than the corresponding value found for eel-enzyme [28] Using acetylthiocholine as substrate, the values were comparable The K_i values were comparable for the bovine and the human enzyme, in agreement with values reported in the literature [28] Unfortunately no comparable values have been reported by Paniker et al [29] for their preparation

The profile of the protein peak from the Sephadex G-200 column and that of the corresponding activity are symmetrical (Fig 2) Recovery was approx 85%, so possible interaction of the protein with the gel could be precluded The same protein migrated as a single band when electrophoresed (Fig 3a) These findings are complementary to each other and are suggestive of the purity and the homogeneity of the preparation The existence of multimolecular forms as suggested by the work of others [13, 26] was not confirmed

Deoxycholate determination showed that the enzyme was free from the detergent In any case, an investigation of the effect of deoxycholate up to a concentration of 5 mM (amount estimated in the preparation after $(NH_4)_2SO_4$ precipitation) on the activity of the enzyme showed no effect Nonetheless an initial drop in the total activity of the preparation on solubilisation with the detergent was observed It is noteworthy (Table I) that whereas the total protein in the supernatant was increased when the concentration of the deoxycholate was increased, the total activity reached a maximum and then decreased These were unexpected and remain to be clarified

* The detailed kinetics of this enzyme are under study

With the availibility of a simple method for the preparation of a pure and homogeneous enzyme, it is hoped that studies of this erythrocyte enzyme would be facilitated, studies largely hampered in the past for lack of a homogeneous preparation

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