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STUDIES ON THE CHARACTERIZATION OF HUMAN ERYTHROCYTE ACETYLCHOLINESTERASE AND ITS INTERACTION WITH ANTIBODIES

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Summary

Human erythrocyte membranes were solubilized in 5% Triton X-100 and the acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) was isolated by affinity chromatography utilizing a specific inhibitor, trimethyl-*p*-aminophenyl ammonium chloride, bound to Sepharose 4B. After a repeated chromatography acetylcholinesterase was found to be homogeneous by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Immunization of rabbits with acetylcholinesterase elicited the formation of an antiserum which gave single precipitin lines with the enzyme on immunodiffusion and rocket, crossed and immunoelectrophoreses. The purified enzyme had a specific activity of 418 units/mg protein. The K_m value of acetylcholinesterase with acetylthiocholine as substrate was 1.5×10^{-4} M. Isoelectric focusing of acetylcholinesterase in the presence of Triton X-100 and within the pH ranges of 3—10 and 3—6 exhibited a single peak of enzyme activity with a P*I* of 4.8. The results of amino acid and carbohydrate analyses showed that acetylcholinesterase is a glycoprotein with a carbohydrate/protein weight ratio of 0.16 and glucose, galactose, mannose, glucosamine, galactosamine and sialic acid as the sugar components. The N-terminal amino acid was blocked. Lipid, phosphorus and fatty acid analyses indicated phosphatidylserine and cholesterol as the major lipid components of acetylcholinesterase. The apparent subunit molecular weight estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis in the absence of 2-mercaptoethanol was 160 000 and in its presence, 80 000. The kinetic studies showed a competitive inhibition of acetylcholinesterase by its antibodies. Agglutination of human red cells by monospecific antiserum to acetylcholinesterase confirmed that the antigenic site(s) of the enzyme is localized on the outer surface of the erythrocyte membrane.

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Introduction

Human erythrocyte acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) is an enzyme strongly bound to the outer surface of the plasma membrane [1,2]. Although it seems to be the only one among the recognized membrane-bound enzymes to exhibit activity changes in some hematologic disorders [3], its phyiologic function in erythrocytes still remains unknown.

Several attempts to isolate human erythrocyte acetylcholinesterase by a variety of procedures including ammonium sulfate precipitation, gel permeation and ion-exchange chromatography resulted in only partially purified enzyme preparations [4—7]. Berman and Young [8] have established, however, that affinity chromatography with acetylcholinesterase inhibitors as ligands is the simplest and most efficient procedure for the isolation of electrophoretically homogeneous acetylcholinesterase preparations from eel electric tissue and bovine erythrocyte membranes. Recently, there were two separate reports [9, 10] describing successful application of this methodology to the isolation of human erythrocyte acetylcholinesterase. To study the molecular and kinetic properties of human erythrocyte acetylcholinesterase, we have also utilized affinity chromatography as the method of choice for the isolation of a highly purified enzyme preparation. This paper presents results on partial characterization of an electrophoretically and immunologically homogeneous acetylcholinesterase and its interaction with monospecific antiserum.

Materials and Methods

Preparation of the affinity column. The activation of Sepharose 4B and the preparation of Sepharose derivative were performed according to the method of Cuatrecasas [11]. Sepharose 4B (100 ml) was mixed with an equal volume of water and activated with CNBr (20 g). The pH was maintained at 11.0 with 8 M NaOH. The temperature was held at 20° C by adding ice to the mixture when necessary. The reaction was completed when the pH equilibrated at 11.0. The pH was then lowered to 10.0 by adding 6 M HCl to facilitate higher efficiency of coupling 6-aminohexanoic acid. Because of the instability of the activated Sepharose, the solution was packed in ice on a Buchner funnel, washed and reacted with 6-aminohexanoic acid within less than 90 s. The Sepharose was washed with the same buffer (1.5 l, 4° C) in which 6-aminohexanoic acid (2 mmols/ml, 100 ml) was dissolved. After reacting overnight at 4° C, the Sepharose was washed exhaustively with water and, upon addition of 100 ml of 3,3-diaminodipropylamine (2 mmols/ml), the pH was lowered to 4.7 with 1 M HCl. This pH was maintained while adding dropwise 500 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide dissolved in 3 ml water during a 10-min period. This suspension was stirred overnight at room temperature, then washed with water. An equal volume of succinic anhydride (1 mmol/ml) in water was added to the Sepharose derivative and the pH was raised to 6.0 with 20% NaOH. This pH was maintained until the solution equilibrated at pH 6.0. The suspension was stirred for 5 additional hours at 4° C. The activation, coupling and succinylation resulted in the formation of a Sepharose derivative with a free terminal car-

boxyl group as shown below:

Sepharose 4B-$NH(CH_2)_5$-CO-$NH(CH_2)_3NH(CH_2)_3$ NH-CO-$(CH_2)_2$-COOH.

The acetylcholinesterase inhibitor, trimethyl-*p*-aminophenyl ammonium chloride hydrochloride, was prepared form *N,N*-dimethyl-*p*-phenylene diamine dihydrochloride (Sigma, St. Louis, Mo.) according to the procedure described by Berman and Young [8] and was attached to the terminal carboxyl group of the Sepharose derivative (100 ml) by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide coupling.

Preparation of erythrocyte ghosts. Human erythrocyte membranes (ghosts) were prepared by the method of Dodge et al. [12]. 95 volumes of packed ghosts (protein concentration 3—4 mg/ml) were mixed with Triton X-100 (5 volumes) and the soluble fraction was obtained by centrifugation at 104 000 × *g* for 3 h in a Spinco Model L ultracentrifuge. The supernatant fraction was adjusted to pH 6.8 with 0.1 M HCl for application to the affinity column.

Isolation procedure. All the chromatogaphic procedures were performed at 4° C. After attachment of the ligand, the Separose derivative (100 ml) was mixed with Sephadex G-25 (100 ml) and packed onto a column (4 × 15 cm) using 10 mM Tris · HCl buffer containing 0.2% Triton X-100, pH 6.8, then with 0.5 M NaCl in the same buffer. The acetylcholinesterase was eluted with 10 mM Tris · HCl buffer containing 0.2% Triton X-100, 3 M NaCl and 0.3 M tetramethyl ammonium chloride, pH 6.8. The purified enzyme fraction was dialysed overnight against 10 mM Tris · HCl buffer containing 0.2% Triton X-100, pH 6.8, and rechromatographed in the same manner as above. Samples were concentrated by Amicon ultrafiltration with PM-10 filter (Amicon Corp., Lexington, Mass.).

Because of difficulty in removal of Triton X-100, the rechromatography step was also performed in the presence of sodium deoxycholate. The elution procedure was as described with the exception of final elution of acetylcholinesterase fraction. After elution with 0.5 M NaCl buffer, the column was washed with 10 mM Tris · HCl buffer containing 0.2% sodium deoxycholate, pH 7.4. The acetylcholinesterase was eluted from the column with 10 mM Tris · HCl containing 0.2% sodium deoxycholate and 1.0 M tetramethyl ammonium chloride, pH 7.4, 25° C. Maintenance of pH 7 or greater was necessary during isolation to avoid precipitation of sodium deoxycholate; the temperature was raised to 25° C to avoid gelling of the deoxycholate buffer. To remove tetramethyl ammonium chloride, this preparation was dialysed overnight at room temperature against 10 mM Tris · HCl buffer containing 0.2% sodium deoxycholate, pH 7.4, then concentrated by Amicon ultrafiltration. To remove the detergent, the sample was dialyzed exhaustively first against 50 mM Tris · HCl and then against double distilled H_2O.

Enzyme assay. Acetylcholinesterase activity was measured by the method of Ellman et al. [13]. 3 ml of phosphate buffer (0.1 M, pH 8, 37° C), 20 µl of substrate (acetylthiocholine iodide, 0.075 M), 100 µl of 5,5'-dithiobis-2-nitrobenzoate (0.01 M in 0.1 M phosphate buffer, pH 7, and 0.02 M $NaHCO_3$), and 50 µl of enzyme were mixed in the cuvette and immediately measured in a Beckman Model 25 spectrophotometer with a temperature control and recorder. A

unit of enzymic activity corresponds to 1 μmol of acetylthiocholine hydrolyzed per min.

Polyacrylamide gel electrophoresis. Analytical sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed as described previously [14]. This system is also applicable to molecular weight determination in which lysozyme (M_r 14 000), yeast alcohol dehydrogenase (M_r 37 000), ovalbumin (M_r 43 000) and bovine serum albumin (monomer M_r 68 000, dimer 136 000) were used as standards. Protein standards were purchased from Sigma C., St. Louis, Mo.

Isoelectric focusing. Isoelectric focusing was performed on a vertical column (LKB 8100, 110 ml) with LKB carrier ampholine at pH ranges 3—10 and 3—6. Light solution consisted of 48 ml of an acetylcholinesterase solution (15—20 μg/ml) in 1% Triton X-100 and 2 ml of carrier ampholine. Heavy solution consisted of 34 ml of 1% Triton X-100, 20 g sucrose and 2 ml of carrier ampholine. Heavy and light solutions were applied to the column by a gradient apparatus. The focusing was performed at 4°C. The voltage was gradually increased to maintain the power output at less than 1.5 W in order to prevent excess heat formation. After reaching 500 V the focusing was continued for an additional 2 days.

Preparation of antiserum. A monospecific antiserum to acetylcholinesterase was prepared by immunizing white New Zealand rabbits with the antigen (rechromatographed fraction III) at weekly intervals for 4 weeks. 1 ml of acetylcholinesterase (0.2 mg/ml) in Triton X-100 (0.5—1%) was mixed with an equal volume of complete Freund's adjuvant (Difco Lab, Detroit, Mich.) and injected intraperitoneally. The rabbits were bled by cardiac puncture. Precipitating antibodies to acetylcholinesterase were observed 3 weeks after the initial injection.

Agglutination analysis. One drop of freshly drawn whole blood was placed onto a microscope slide and mixed with one drop of antiserum prepared against purified human erythrocyte acetylcholinesterase. The slide was imspected after 2—3 min for agglutination of the red cells.

Preparation of immunoglobulins. The crude γ-globulin fraction was obtained by standard ammonium sulfate fractionation procedure [15]. 18 mg of crude γ-globulin fraction obtained from 6 ml of antiserum were redissolved in 6 ml of buffer containing 0.125 M NaCl and 0.025 M phosphate at pH 7.4.

Immunoinhibition titration. The inhibition of acetylcholinesterase activity by the immunoglobulins was studied by mixing 25 μl of a concentrated rechromatographed fraction III (8.4 μg acetylcholinesterase) with 1.5 ml of different concentrations of crude γ-globulin fraction and incubating at both room temperature and 4°C for 4, 12 and 48 h. The antigen-antibody suspension was assayed before and after centrifugation for 10 min at 10 000 rev./min in a Brinkmann 3200 centrifuge. The pseudocholinesterase activity present in the crude γ-globulin fraction was subtracted from the total activity.

Immunodiffusion and electroimmunoassays. Double immunodiffusion [16] and immunoelectrophoresis [17] were performed on glass slides (25 × 75 mm) coated with 1% agar (4—5 mm thick). Agar was dissolved in 0.05 M veronal buffer by heating in a boiling water bath for 20 min. After cooling to 55°C, Triton X-100 was added to a final concentration of 0.2%. Immunoelectrophoresis was carried out at 6.5 V/cm for 60 min. The plates were allowed to develop for 20—30 h.

Agarose plates for rocket electrophoresis were prepared as previously described [18]. Briefly, 8 ml of 2.5% agarose in 0.2% Triton X-100 was cooled to 55° C and mixed with antiserum to acetylcholinesterase, poured into a mold (100 × 100 × 1.5 mm) and allowed to congeal. Samples were diluted with electrophoresis buffer and applied to wells in the agarose plate with a microdispenser. The agarose plate was electrophoresed at 10 V/cm for 3.5 h. The plate was placed in a saline bath for 1—2 h, blotted with Whatman No. 1 filter paper and dried with hot air. Immunoprecipitates were stained with a 0.12% solution of Coomassie Brilliant Blue R (Coomassie Brilliant Blue R/water/95% ethanol/acetic acid (2 : 2 : 2 : 1, v/v) for 10 min, and destained to a clear background in a solution of acetic acid/95% ethanol/water (1 : 3 : 5, v/v).

Amino acid analyses. Protein samples were hydrolyzed with constant-boiling HCl in evacuated, sealed tubes at 110° C for 24, 48, and 72 h. The amino acid analyses and performic acid oxidation were done as previously described [19]. Tryptophan was determined according to the fluorimetric method of Sasaki et al. [20]. N-Terminal amino acids were determined by the dansylation procedure according to Weiner et al. [21].

Carbohydrate analyses. Neutral and amino sugars were determined by a previously described modification [22] of the gas-liquid chromatographic procedure of Griggs et al. [23]. Sialic acid was determined by the method of Warren [24].

Lipid, phosphorus and fatty acid analyses. Lipid analyses were performed according to the procedures described previously [25]. For the analyses, samples were extracted by chloroform/methanol (1 : 1, v/v). Phosphorus content was determined by the method of Gerlach and Deuticke [26]. Fatty acid composition of phospholipids were determined on samples extracted by chloroform/methanol. Phospholipids were transesterified as described by Mason and Waller [27]. Fatty acid esters were analyzed on a Packard Becker 420 gas chromatograp equipped with a dual flame ionization detector. The appropriate aliquots were injected into a glass column (16 ft × 2 mm) packed with 15% diethylene glycol succinate on 80/100 Chromosorb W AW at 185° C with a flow rate of 20 ml/min.

Protein analysis. The protein content of acetylcholinesterase preparations was determined by the method of Wang and Smith [28], a modification of Lowry's procedure [29], using bovine serum albumin as standard. The modification eliminated the interference of Triton X-100 by inclusion of sodium dodecyl sulfate in the assay system. To examine the discrepancy in specific activities between results obtained in this and other laboratories [9,10] the protein content of an acetylcholinesterase preparation was determined by the original method of Lowry et al. and by the modified procedure of Wang and Smith [28]. The data show that a constant specific activity was observed despite the variation of the sample aliquot. In contrast, protein determined by the Lowry procedure was not linear with respect to the sample aliquots resulting in varied activities ranging from 195.1 to 996.5 units/mg. These findings further substantiate that the presence of Triton X-100 strongly interferes with the protein determination by Lowry's procedure.

Samples containing tetramethyl ammonium chloride were dialyzed before analysis to prevent interference in the colorimetric determination.

Salt determination. NaCl concentration was determined using a conductivity meter (Radiometer, Copenhagen).

Results

Isolation of acetylcholinesterase

To establish the optimal conditions for the isolation of acetylcholinesterase the initial experiments on the elution of ghost proteins from each freshly prepared affinity column was carried out by a continuous salt gradient (Fig. 1). Results of such studies were utilized then for carrying out a stepwise elution of proteins and acetylcholinesterase (Fig. 2). The first fraction eluted with 10 mM Tris · HCl buffer contained lipids and a small amount of protein including hemoglobin that had not been completely removed during the preparation of ghosts. Approx. 65% of the total recovered protein was eluted with 0.5 M NaCl. The retarded elution of this protein fraction was attributed to non-specific ionic interactions. Although the retained acetylcholinesterase could be eluted with 3.0 M NaCl, a sharp symmetrical elution curve of enzymic activity was only obtained in the presence of 0.3 M tetramethyl ammonium chloride. To compensate for the inhibition of enzymic activity by tetramethyl ammonium chloride, the acetylcholinesterase assay used for monitoring the elution pattern was performed at high substrate concentration using 1.89 mM acetylthiocholine per test. Retention of acetylcholinesterase by the affinity column was greater at pH 6.8 than at pH 7.4. At pH 6.8, a decrease in the release of acetylcholinesterase into the first two fractions increased the amount of enzyme recovered in the third fraction. A summary of the purification procedure for acetylcholinesterase

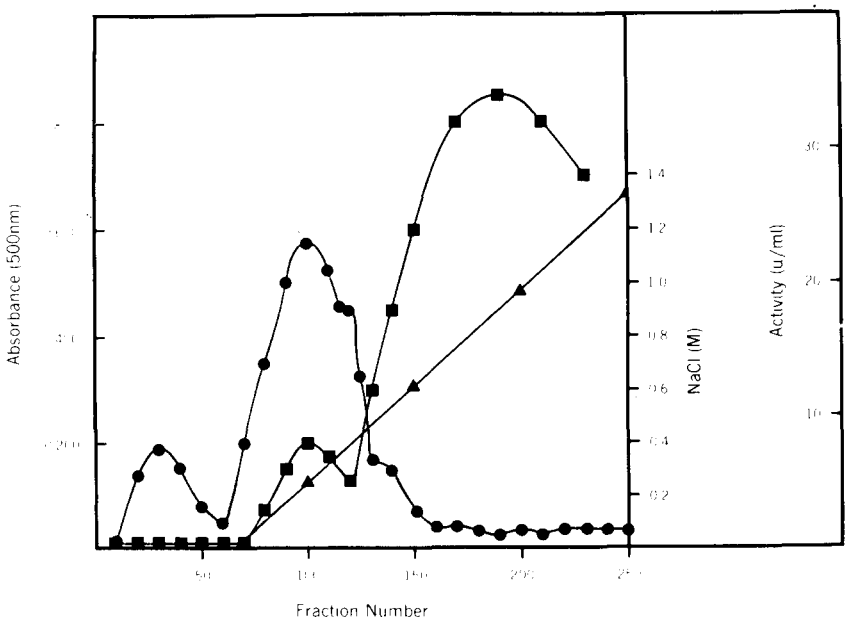


Fig. 1. Affinity chromatography of human erythrocyte acetylcholinesterase with a continuous linear NaCl gradient. ●, protein; ■, enzyme activity; ▲, NaCl concentration. Temperature, 4°C; solvent: 0.2% Triton X-100, 0.01 M Tris · HCl, pH 6.8.

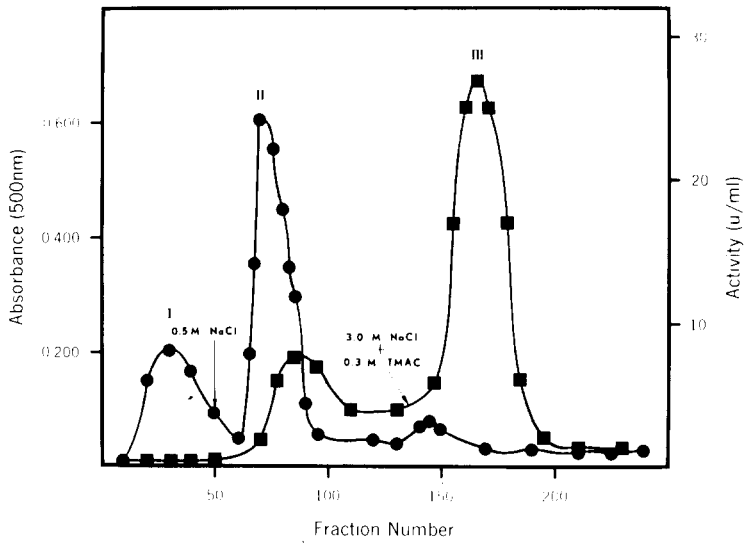


Fig. 2. Affinity chromatogaphy of human erythrocyte acetylcholinesterase with a stepwise gradient of 0.5 M NaCl, then 3.0 M NaCl plus 0.3 M tetramethyl ammonium chloride. See Fig. 1 for other experimental conditions.

is shown in Table I. The second chromatography of acetylcholinesterase resulted in a 332-fold purification of enzyme from erythrocyte ghosts with an overall yield of 28.4%.

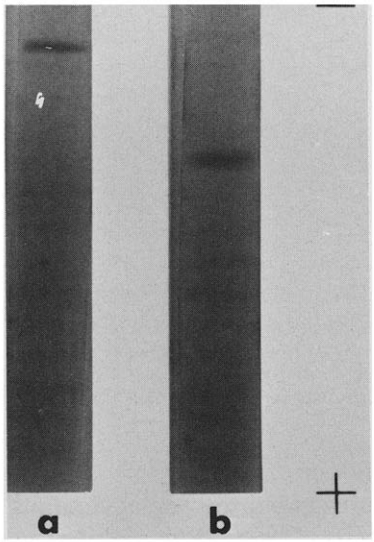
Characterization of acetylcholinesterase

After rechromatography, the acetylcholinesterase preparation exhibited single bands of different mobilities on sodium dodecyl sulfate polyacrylamide gel electrophoresis in the presence and absence of 2-mercaptoethanol (Fig. 3). Double diffusion analysis and rocket electrophoresis (Fig. 4) of Triton X-100-solubilized ghosts with antibodies to purified acetylcholinesterase showed single precipitin lines. On immunodiffusion (Fig. 5), immunoelectrophoresis, rocket electrophoresis and crossed immunoelectrophoresis the purified enzyme gave single precipitin lines with its antiserum. Human erythrocyte acetylcholinesterase antiserum gave no reaction with bovine erythrocyte enzyme or eel enzyme on immunodiffusion indicating the antigenic determinants of human

TABLE I

PURIFICATION OF ACETYLCHOLINESTERASE

	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Purification (X-fold)
Ghosts	2820.0	3550.4	1.3	100.0	1.0
Triton X-100-solubilized ghosts	1080.9	2984.3	2.8	84.1	2.2
Fraction III	71.3	1693.7	23.8	47.7	18.9
Second chromatography of fraction III	2.4	1006.6	418.6	28.4	332.4



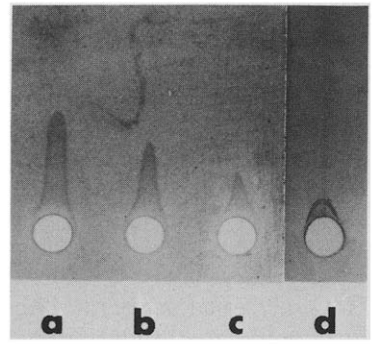


Fig. 3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of purified human erythocyte acetylcholinesterase. Acetylcholinesterase was dissolved in a solution of sodium dodecyl sulfate, sodium phosphate, urea and in the absence (a) and presence (b) of 2-mercaptoethanol as previously described [14]. The optimal protein concentration was 15—20 μg in a 25 μl aliquot. Protein was stained with Coomassie Brilliant Blue.

Fig. 4. Immunoprecipitation ("rockets") of human erythrocyte acetylcholinesterase. Dilutions of an acetylcholinesterase preparation (0.27 mg/ml), (a) 1 : 2, (b) 1 : 4, (c) 1 : 6, were placed in the wells and electrophoresed for 3.5 h against 0.5 ml of anti-acetylcholinesterase per 8 ml of agar in 0.2% Triton X-100; (d) undiluted soluble ghosts (1.87 mg/ml) were electrophoresed against 0.75 ml antiserum per 8 ml agar.

enzyme are different from those of bovine erythrocyte or eel electric tissue enzyme.

The K_m value of purified acetylcholinesterase with acetylthiocholine as substrate was $1.5 \cdot 10^{-4}$ M.

Isoelectric focusing of acetylcholinesterase in the presence of Triton X-100 and within the pH ranges of 3—10 and 3—6 showed a single peak of enzyme activity with a pI of 4.8 (Fig. 6).

The molecular weight determination of acetylcholinesterase on sodium dodecyl sulfate polyacrylamide gel electrophoresis in the absence of 2-mercaptoethanol gave an apparent subunit molecular weight of approx. 160 000. The molecular weight of the reduced subunit was 80 000.

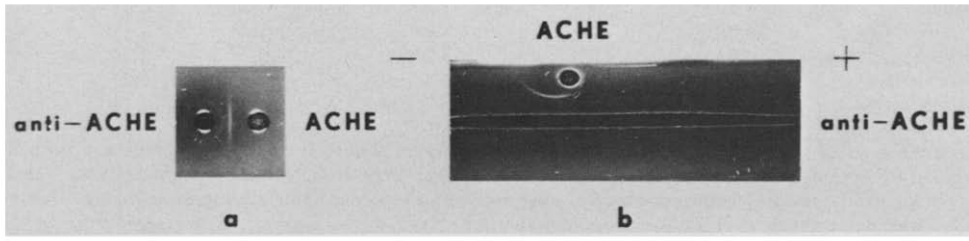


Fig. 5. Immunodiffusion (a) and immunoelectrophoretic (b) patterns of antiserum prepared against purified human erythrocyte acetylcholinesterase.

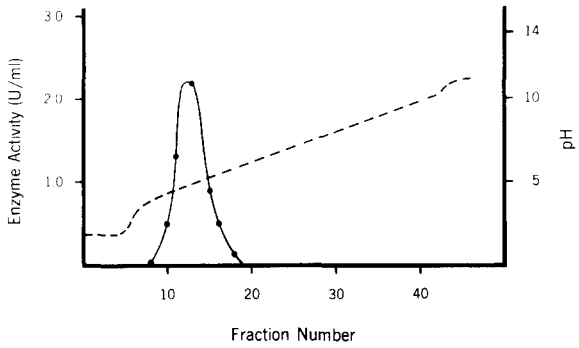


Fig. 6. Isoelectric focusing of human erythrocyte acetylcholinesterase. Protein (15—20 μg/ml) dissolved in 1% Triton X-100 was focused on a column (LKB 8100, 110 ml) with LKB carrier ampholine at pH range 3—10, 4°C. ●, enzyme activity; - - - - - -, pH.

Amino acid analyses of acetylcholinesterase demonstrated the presence of all common amino acids including half-cystine (Table II). Dansylation resulted in no detectable N-terminal amino acids indicating that acetylcholinesterase has a blocked N-terminal residue.

Carbohydrate analyses demonstrated that acetylcholinesterase is a glycopro-

TABLE II

AMINO ACID COMPOSITION OF ACETYLCHOLINESTERASE

Results are expressed in mol %.

	Human erythrocyte *	Bovine erythrocyte **	Eel electric tissue ***
Lysine	6.77	6.2	4.43
Histidine	3.08	1.6	2.28
Arginine	4.49	4.4	5.39
Aspartic acid	7.15	8.0	9.60
Threonine	4.90	5.4	4.45
Serine	9.14	10.9	7.04
Glutamic acid	10.15	11.2	9.51
Proline	4.93	4.7	8.33
Glycine	9.46	13.2	7.99
Alanine	10.78	10.2	5.61
Half-cystine	0.22	0.8	1.12
Valine	5.94	7.3	7.13
Methionine	1.35	1.5	2.97
Isoleucine	4.53	2.8	3.75
Leucine	10.88	7.5	9.11
Tyrosine	2.01	3.2	3.90
Phenylalanine	3.21	3.5	5.27
Tryptophan	1.02	—	2.11

* Analysis of three different acetylcholinesterase samples. Values for serine, threonine and tyrosine were obtained by linear extrapolation of average recoveries from 24- and 72-h hydrolyses. Values for valine and isoleucine represent average recoveries obtained after 72 h hydrolysis. Half-cystine was determined after performic acid oxidation. Tryptophan was determined according to the fluorimetric method of Sasaki et al. [20].

** Data of Berman [35].

*** Calculated from the data of Leuzinger and Baker [34].

tein containing mannose, galactose, glucose, glucosamine, galactosamine and sialic acid as the sugar components (Table III).

The thin-layer chromatography of chloroform/methanol extract of acetylcholinesterase indicated the presence of a small amount of phospholipids. Phosphatidyl serine seemed to be the only major phospholipid accounting for 90% of the total fatty acid content; lecithin, lysolecithin and sphingomyelin only occurred in trace amounts. The extract also contained cholesterol and a trace of cholesterol esters. Palmitic and stearic acids were identified as the major fatty acids (Table IV). Phosphorus analyses indicated that the total phospholipid content ranged from 2 to 5% of the dry weight of purified acetylcholinesterase. The ratio of total fatty acids to protein was 0.015—0.039 mg/mg protein.

Interaction of acetylcholinesterase with its antiserum

The enzymic activity of purified acetylcholinesterase was partially inhibited by its antiserum, reaching only 35% inhibition even in the presence of an excess amount of antibodies (Fig. 7). Equivalence was reached at approx. 75 μl of immunoglobulins. To further elucidate the antibody-induced change in the catalytic activity of acetylcholinesterase, the values for V were determined for both free and antigen-antibody complex of acetylcholinesterase (Fig. 8). Results of this kinetic study indicated competitive inhibition, since there was no difference between V of free enzyme and its antigen-antibody complex.

Topographic distribution of acetylcholinesterase

Agglutination of human red blood cells by monospecific antiserum to acetylcholinesterase confirmed the previous report [2] that acetylcholinesterase is localized on the outer surface of the erythrocyte membranes. We have also been able to reproduce the results obtained by Bender et al. [30] utilizing pronase as a probe for identifying proteins localized on the outer surface of the membrane. Upon treatment of the intact red cells, acetylcholinesterase was completely degraded along with the release of up to 98% of sialic acid, the

TABLE III

CARBOHYDRATE COMPOSITION OF HUMAN ERYTHROCYTE ACETYLCHOLINESTERASE

Analysis of two different acetylcholinesterase preparations. Values for neutral sugars were determined from hydrolysates (1 M HCl, 4 h) subjected to borohydride prior to acetylation; amino sugars from hydrolysates (4 M HCl, 6 h) subjected to the same treatment. Sialic acid was determined from samples hydrolyzed in 0.05 M H_2SO_4 for 1.5 h at 80°C by the method of Warren [24].

	Mol%
Glucose	21.83
Galactose	37.26
Mannose	11.00
Glucosamine	16.39
Galactosamine	11.26
Sialic acid	2.25

TABLE IV

FATTY ACID COMPOSITION OF HUMAN ERYTHROCYTE ACETYLCHOLINESTERASE

Values for fatty acids were determined from chloroform/methanol extracts of samples subjected to transesterification according to the method of Mason and Waller [27].

	Mol%
C_{14}	3.99
C_{16}	43.40
$C_{16:1}$	9.14
C_{18}	31.29
$C_{18:1}$	12.18

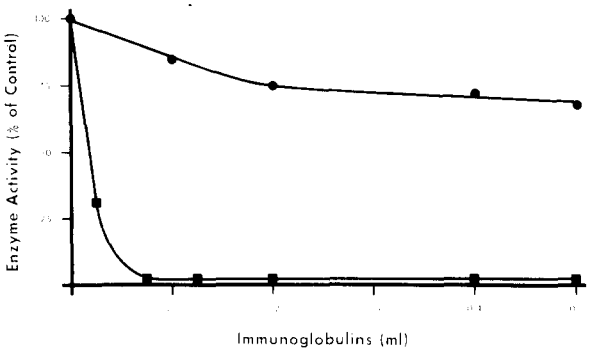


Fig. 7. Inhibition by antibodies of acetylcholinesterase activity. Acetylcholinesterase (8.4 μg) was incubated in the presence of immunoglobulins for 48 h at room temperature or at 4°C. Activity in antigen-antibody suspension before centrifugation (●); in supernatant after centrifugation (■).

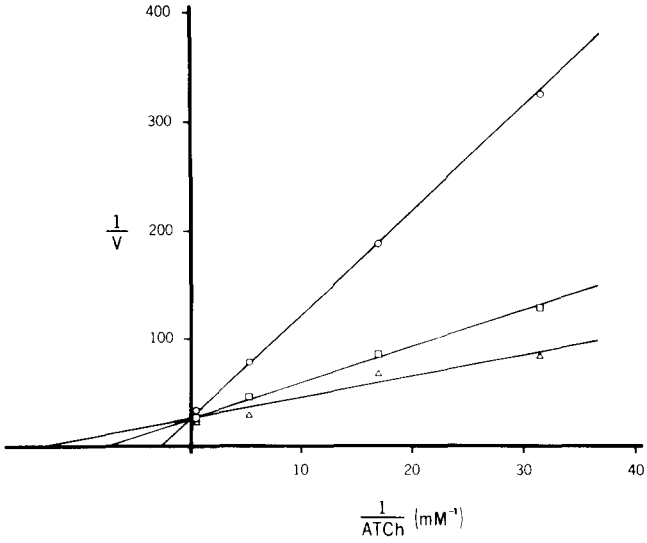


Fig. 8. Lineweaver-Burk plot of the inhibition of acetylcholinesterase activity. The antibodies were incubated with the enzyme (8.4 μg) in saline isotonic buffer (9 : 1, v/v) at room temperature for 48 h. 50-μl aliquots were assayed per test. △, no antibodies added; □, 0.4 mg antibodies/ml; ○, 1.6 mg antibodies/ml.

marker of the external surface. The cytoplasmic membrane marker NADH diaphorase [2], was unaltered by this treatment. Although our data indicate that the antigenic site(s) is on the surface, we cannot rule out the possibility that acetylcholinesterase may extend into or even span the membrane.

Discussion

Results of our study confirm previous reports [9,10] that the affinity chromatography with quaternary ammonium salts as ligands represents a very simple and efficient procedure for the isolation of acetylcholinesterase from human erythrocyte ghosts. An immunochemically and electrophoretically homogeneous enzyme preparation was obtained after two successive chromatographic runs as the only required steps. A 332-fold purification indicates that the enzyme represents only 0.3% of the membrane protein mass, which is consistent with an estimated value of 0.2% by Bellhorn et al. [31]. In contrast, others have reported a 10-fold greater purification value [9,10]. Recovery of the enzyme activities from the ghost preparations and specific activities of the ghost preparations are comparable among all three laboratories indicating that the discrepancy in specific activities of the final acetylcholinesterase preparations may be attributed to protein estimations by different methods. We suggest the discrepancy may be due to the underestimation of protein since Triton X-100 strongly interferes with the Lowry procedure [28,32,33]. The data obtained in our laboratory on the comparison of these methods indicate that the estimation of protein by the method of Lowry et al. in the presence of Triton X-100 but absence of sodium dodecyl sulfate is 5—10-fold less than the determination of protein in the presence of sodium dodecyl sulfate.

In our experience the elution procedure is highly dependent on the concentration of quaternary ammonium salt attached to the solid support and the size of the column used. Therefore, an efficient performance of affinity chromatography on any newly prepared affinity column requires a prior elution of proteins with a linear salt gradient to determine the optimum elution pattern.

Human erythrocyte acetylcholinesterase is characterized by the presence of all common amino acids including half-cystine. These results are comparable to those reported for eel electric organ and bovine erythrocyte acetylcholinesterase [34,35] as shown in Table II. No apparent difference can be seen among the three enzymes in the total hydrophobicity as defined by Tanford [36].

The dansylation study shows that the N-terminal amino acid of human acetylcholinesterase is blocked. This finding is similar to that of Leuzinger and Baker [34] who have reported that eel electric tissue acetylcholinesterase has a blocked N-terminal residue.

Results of quantitative carbohydrate determination provide the experimental evidence for the suggestion by several investigators [5,6,9,10] that acetylcholinesterase is a glycoprotein. It has been established that the weight ratio of carbohydrate/protein is 0.16. Because of insufficient amount of enzyme it has not been possible to determine whether glucose or sialic acid are integral components of the carbohydrate moiety or contaminations.

The detection of small amounts of phospholipid and cholesterol in purififed

acetylcholinesterase supports previous claims [1,37-39] that acetylcholinesterase may be lipoprotein. Sihotang [39] has shown that the phospholipid content of various acetylcholinesterase preparations depends on the concentration of deoxycholate used for their isolation and that the optimal enzyme activity is associated with a fixed phospholipid content. Identification of phosphatidylserine as the major phospholipid raises several questions regarding the significance of this finding. It still remains to be established, for example, whether its almost exclusive presence in purified enzyme is due to structural specificity or to a selective removal of other phospholipids by Triton X-100 and deoxycholate. Although Sihotang [39] used phosphatidylserine to demonstrate the activation of acetylcholinesterase, the phospholipid specificity of this reaction has not yet been established.

A molecular weight of 80 000 is in agreement with similar values reported recently for reduced acetylcholinesterase preparations isolated from human erythrocytes by affinity chromatography [9,10]. However, due to the anomalous behavior of glycoproteins on sodium dodecyl sulfate polyacrylamide gel electrophoresis [40], this value will have to be verified by independent methods.

Results of the present study suggest that the subunit of human erythrocyte acetylcholinesterase consists most probably of two covalently linked polypeptide chains of equal size. This conclusion is based on (a) the detection of a single peak of enzyme activity on isoelectric focusing, (b) the appearance of a single precipitin arc on immunoelectrophoresis, and (c) the presence of a single band of the non-reduced (M_r 160 000) and reduced (M_r 80 000) forms of acetylcholinesterase.

The occurrence of multiple molecular forms of human erythrocyte acetylcholinesterase has been interpreted as a result of either polymerization of the monomer [6,10] or presence of polymorphic forms [10]. The resolution of enzyme by isoelectric focusing into five molecular forms of slightly differing isoelectric points has been attributed to different contents of sialic acid [10]. In contrast, Wright and Plummer [6] observed no change in the electrophoresis pattern of acetylcholinesterase treated with neuraminidase. Our study shows that isoelectric focusing of acetylcholinesterase resulted in a single peak of enzyme activity with an isoelectric point of 4.8. The discrepancy between these observations may be due either to the differences in the purity of various acetylcholinesterase preparations or to the possible presence of proteolytic products [41] as another source of artifactual polymorphic forms. Since a highly purified acetylcholinesterase can be dissociated in the presence of sodium dodecyl sulfate into a monomeric form, we favor the aggregation process rather than polymorphism as a main reason for the appearance of multiple molecular forms of acetylcholinesterase. Whether human erythrocyte acetylcholinesterase occurs in the native state as a monomer or multimer cannot be answered by the results of this study.

The competitive inhibition of acetylcholinesterase by its monospecific antiserum suggests that the formation of antigen-antibody complex has little effect, if any, on the conformation of the enzyme. It seems that the antigenic determinant(s) is at or very close to the active site of the enzyme, which to our

knowledge represents the first demonstration of competitive inhibition of an enzyme by its monospecific immunoglobulins.

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