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HUMAN ERYTHROCYTE ACETYLCHOLINESTERASE PURIFICATION, PROPERTIES AND KINETIC BEHAVIOR

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SUMMARY

1. Human erythrocyte acetylcholine hydrolase (EC 3.1.1.7) is purified 2537-fold, by isolating erythrocyte membrane fragments, by Triton X-100 extraction of the enzyme from the membranes and by DEAE-cellulose and calcium phosphate gel chromatographies.

2. The purified enzyme is a glycoprotein; it moves as a single band in acrylamide gel electrophoresis. Electron microscopy, gel filtration and density-gradient centrifugation indicate it to be a macromolecule, composed of small spherical units. The enzyme easily forms reversible aggregates and the presence of other proteins (*e.g.* bovine serum albumin) preserve the purified enzyme, as a catalytically active polymer.

3. The molecular characteristics, the substrate specificity, the influence of pH and temperature on hydrolysis, and the kinetic constants of the enzyme are different from other cholinesterases implicated in the acetylcholine receptor function.

4. The kinetic characteristics suggest that the enzyme possesses allosteric behavior. The polar substrate, acetylthiocholine, activates the enzyme; a specifically low binding constant for the substrate binding, outside the active center, is calculated. Other quarternary nitrogen carrying compounds (*e.g.* mytelase) are also found either to activate or inhibit the enzyme and compete with the substrate, depending upon the substrate concentration and hence upon the substrate activation of the enzyme. The kinetic behavior of the enzyme also depends upon the temperature, a highly ordered catalysis can only be observed above 32 °C; this result may also be explained by the allosteric behavior of the enzyme.

5. Specific anionic activators of the enzyme such as HCO_3^-, *cis*-oxaloacetate and fructose 1,6-diphosphate are established. Oxaloacetate binding to the enzyme is also found to be specific; this compound, similar to mytelase, is a better activator of the substrate activated enzyme.

Abbreviation: DTNB, 5,5'-dithio-bis-2-nitrobenzoic acid.

INTRODUCTION

The physiological function and the molecular properties of the erythrocyte acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) have not been adequately established. The membrane localization of this enzyme, similar to other membrane enzymes, has rendered its purification difficult[1]. The eel electroplax enzyme could be purified and crystallized[2], but no satisfactory high yield purification procedure has yet been established for the erythrocyte acetylcholinesterase[3]. Although its physiological function is unknown, its deficiency has been implicated in a human disease, paroxysmal nocturnal hemoglobinuria[4]. Previous work done in this field indicated its difference from the physiologically important eel electroplax and central nervous system acetylcholinesterases which are closely linked to the acetylcholine receptor function of the acetylcholine sensitive membranes[5,6]; and has presented the enzyme as an esterase rather than a specific cholinesterase, evidenced by substrate specificity and its inhibition pattern by various anticholinergic compounds and organophosphates[7,8]. The recent investigations on eel electroplax acetylcholinesterase have established the allosteric behavior of this enzyme[5,6]. However, no similar observations have been made for erythrocyte acetylcholinesterase.

Herein we report a purification method for human erythrocyte acetylcholinesterase by which a highly specific active protein can be obtained in high yield. This procedure does not resort to the use of organic solvents or hydrolases for the extraction of the enzyme from membranes. It has enabled us to investigate some of the chemical properties of the purified enzyme which is a macromolecular glycoprotein. The kinetic investigations using the purified protein indicated its allosteric behavior. Furthermore specific effectors are established; the enzyme is found to be activated by HCO_3^-, fructose 1,6-diphosphate and *cis*-oxaloacetate. The results are discussed in relation to other cholinesterases.

MATERIALS AND METHODS

Chemicals of C.P. or highest purity grade obtained from Sigma Chemical Co., U.S.A., and deionised water were used throughout. The potassium salts of organic acids were usually prepared for buffer solutions. The proteins, used as markers for molecular weight determinations, were either recrystallized or purified chromatographically. The bovine serum albumin was a 2× crystallized preparation obtained from Sigma Chemical Co. Latex particles with a standard 0.109 μm diameter of known concentration were obtained from Serva Chemical Co., Heidelberg, ambenonium chloride (mytelase) was a gift of Winthrop Arzneimittel GmbH, W. Germany.

Measurement of enzyme activity

The method of Ellman *et al.*[9] was used to measure the hydrolysis of thioesters. During the purification procedure the assay system consisted of 0.1 M phosphate buffer, 0.1 mM acetylthiocholine iodide, 0.07 mM 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) and the diluted enzyme at pH 8.0. The hydrolysis was measured at 412 nm at room temperature, in duplicate. One unit was equal to 1 μmole of thioester hydrolyzed per h. When the kinetic characteristics of the purified enzyme were studied, an autoanalytic system (Fig. 1) was used. The purified enzyme was diluted

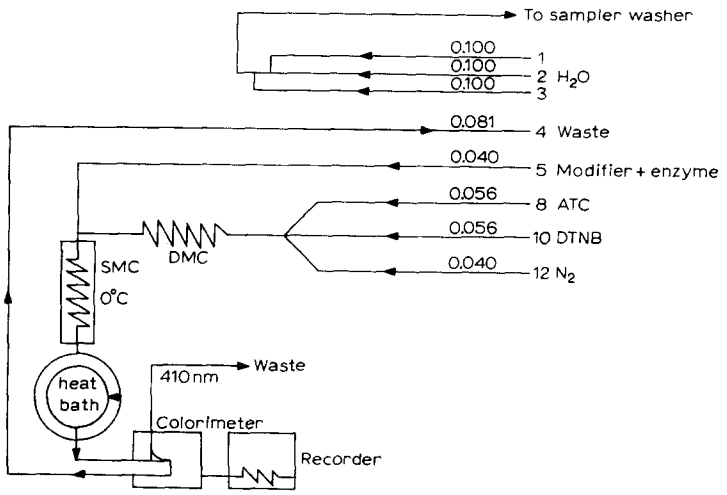


Fig. 1. The scheme of the autoanalyzer system (numbers above the lines indicate the internal diameter in inches. DMC and SMC refer to double mixing coil and small mixing coil, respectively). ATC = acetylthiocholine iodide.

with the desired concentration of modifier and albumin (2.5 mg/ml albumin in the enzyme preparation, 0.5 mg/ml albumin final concentration in the assay) 10 min before analysis during which time it was kept at 4 °C. The enzyme dilution was adjusted to give not more than 5% hydrolysis in either 15 or 8 min, the selected hydrolysis time for the particular experiment at 37 °C. The spacing was achieved by N_2, cleaned from O_2 (ref. 10). In between samples, the system was first washed twice with BRIJ SP-35 (0.08%, w/v), then twice with deionized water. The reproducibility of the autoanalytic assay was $\pm 0.5\%$ S.E. in 40 assays, and the stoichiometry was excellent within the hydrolysis limits selected.

The enzyme was activated by various anions; phosphate, various carboxylates, bicarbonate and Tris increased the activity. In order to minimize the bicarbonate contamination, a low working pH (pH 6.0) was selected to investigate the effect of various compounds. An inert buffer was required. The rates observed in the presence of adipate were low; in fact adipate had only a very slight activating effect between 1 mM and 100 mM but it didn't alter the percent and pattern of inhibition or activation by various modifiers. Similar to adipate, glutarate, gluconate and glucuronate also had little or no effect, whereas other shorter chain mono- and dicarboxylates activated the enzyme; hence adipate was selected as a suitable inert buffer.

Appropriate blanks were designed at each working temperature for each modifier tested. Triplicate controls with the enzyme in 2.5 mM adipate, pH 6.0, were run before and after each modifier assay; the assay was also run in triplicate, and 2.5 mM adipate were present in the medium when the effects of modifiers at pH 6.0 were studied. The control value given in the results, represents the average of all control experiments. The rates are expressed as μmoles of thioester hydrolyzed per h per mg protein at 37 °C.

The hydrolysis of choline esters was measured by the method of Hestrin[11]. Protein determinations were carried out according to the modified method of Lowry *et*

al.[12]. Sephadex G-200 and Sepharose 4B gel filtrations were carried out according to Ackers[13]; the density-gradient centrifugation and the electron microscopic examination were carried out according to methods of Martin and Ames[14] and Valentine *et al.*[15], respectively.

RESULTS

Purification of the enzyme

All procedures were carried out and preparations kept at 3 °C, unless indicated otherwise.

Red blood cells were obtained from healthy normal Blood Bank donors; the washed and packed fresh erythrocytes (100 ml) were added dropwise to 5 mM sodium citrate, pH 6.6 (900 ml), with continuous mixing. The mixture was left overnight. The membrane fragments were collected by centrifugation at 17 000 × *g* for 30 min, and washed at least 8 times successively with the same buffer until the supernatant was pale pink in color. The collected white flocculent fragments could be kept for 3 months. The yield was 50%, and the purification 18-fold (*cf.* Table I).

TABLE I

SUMMARY OF THE PURIFICATION PROCEDURE

The results are the average of various runs, the number of which is denoted by n.

Step	*Volume (ml)*	*Activity (units/ml)*	*Protein concentration (mg/ml)*	*Specific activity (units/mg)*	*Purification (-fold)*	*Yield (%)*
Hemolysate ($n=9$)	1000	27	38	0.70	—	100
Membrane fragments ($n=9$)	170	80	6.4	12.50	18	50
Triton X-100 extract ($n=5$)	100	127.7	1.84	70	100	47
DEAE-cellulose chromatography ($n=5$)	85	87.6	0.36	243	347	27
Calcium phosphate gel chromatography ($n=5$)	21	60.4	0.034	1776	2537	5

Triton X-100 was used to extract the enzyme from membrane fragments in the following manner; to an aliquot of membrane fragment suspension (100 ml), Triton X-100 was added to give a 0.5% (v/v) final concentration, and the mixture was then incubated in a metabolic shaker at 37 °C for 4 h. The supernatant was collected by centrifugation at 17 000 × *g* for 30 min; it was clear and pale yellow in color. The yield was 47%, and the purification 100-fold. The use of various proteolytic and lypolytic enzymes, phospholipases and neuraminidase as well as other detergents, ultrasonic treatment and various organic solvents such as butanol, chloroform *etc.* to extract the enzyme from the membrane, was not as satisfactory. Other Tritons such as CF-54, QS-15, DF-12 and N-101 could also solubilize the enzyme with high yield, however, the use of BRIJ SP-35 was not satisfactory.

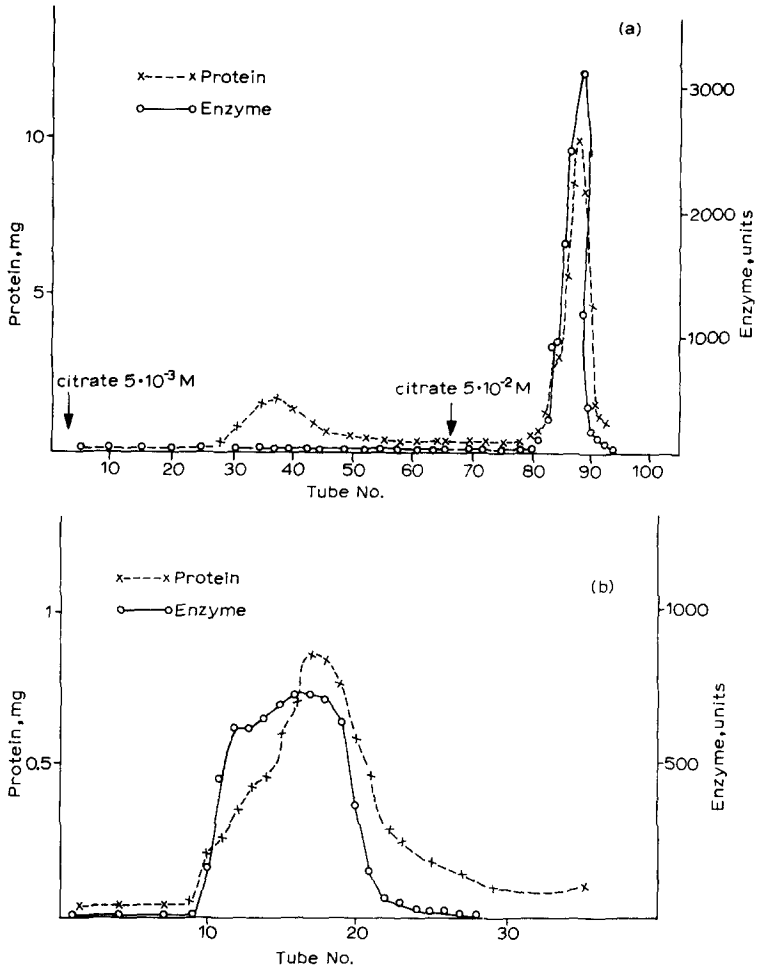


Fig. 2. Chromatography of the enzyme protein. (a) DEAE-cellulose chromatography. The results are the average of 5 runs. Total amount of protein and enzyme units are as indicated. (b) Calcium phosphate gel on cellulose chromatography.

The collected supernatant (95–98 ml) was immediately passed through a 2.5 cm × 62 cm DEAE cellulose column, previously equilibrated with 5 mM sodium citrate containing 0.5% Triton X-100 (v/v), pH 6.6. Flow rate, 2 ml/min; 15-ml fractions were collected. The column was then washed with the same buffer. The elution of the enzyme was carried out by 50 mM sodium citrate containing 0.5% Triton X-100 (v/v), pH 6.6 (Fig. 2a). The fractions with the highest specific activity were pooled (tubes 81–87). Hence, although 92% of the total applied activity could be recovered, the final yield was only 27% and the purification 347-fold. This preparation could be kept for 2 weeks.

The combined DEAE-cellulose purified enzyme (85 ml) was dialyzed against 5 mM phosphate containing 0.5% Triton X-100 (v/v), pH 6.6, for 18 h by repeated changes of the dialysis medium, and then passed through a 2.5 cm × 20 cm column

of calcium phosphate gel on cellulose at a rate of 10 ml per h at room temperature; 10-ml fractions were collected. The column was then washed using the same buffer. The fractions with highest specific activity were pooled (tubes 10–11). Hence, although 100% of total applied activity could be recovered, the final yield was 5%, and the purification 2537-fold (*cf.* Table I, Fig. 2b). The use of either Bentonite or Celite was not as satisfactory.

The pooled active fractions were immediately lyophilized and washed three times with toluene at 0 °C for 5 min in order to remove the Triton X-100 contamination, and the thin film of powder was collected by centrifugation; its toluene contamination was removed under vacuum.

This powder was immediately used as the "purified enzyme". No activity loss was observed during this last step.

The properties of the purified enzyme protein

The crude or purified enzyme hydrolyzes a non-polar substrate, α-naphtyl acetate, very rapidly. Thin-layer or vertical acrylamide-gel electrophoresis of Triton X-100 extracted enzyme, stained by α-naphtylacetate and Fast Blue RR, revealed that most of the present enzyme activity remained at the origin[16–18]. Hence a different method was sought in order to solubilize and resolve the enzyme protein. Among several methods used, the procedure described by Cotman and Mahler was found to be satisfactory (Fig. 3)[19–21]. This method involves the resolution of insoluble membrane

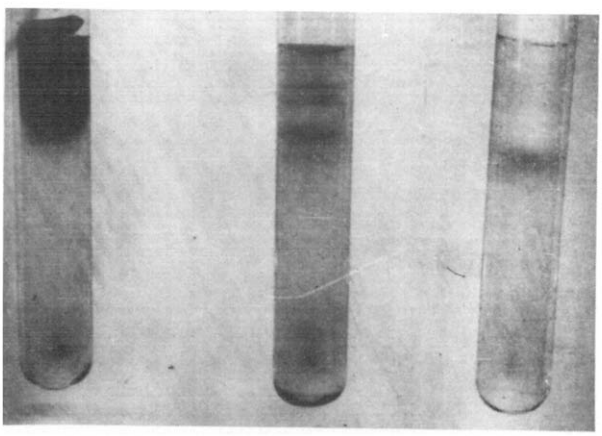


Fig. 3. Acrylamide gel electrophoretic patterns at various steps of purification: phenol–acetic acid–5 M urea was used. The protein was stained by Amido Black 10 B. From left to right: Triton X-100 extracted protein, DEAE-cellulose chromatography purified protein and final preparation (15 μg of protein was applied to each).

proteins by phenol, acetic acid and 5 M urea; whereas there were at least six bands in the Triton X-100 extracted enzyme and five in the DEAE-cellulose purified preparation; the final product was found to contain only a single band.

The periodic acid–Schiff stain showed that the purified enzyme was a glycoprotein[22] and could not be stained by Oil red O.

The purified enzyme gave a $A_{280\ nm}/A_{260\ nm}$ ratio of 0.8 and an absorbance maximum at 266 nm (Fig. 4).

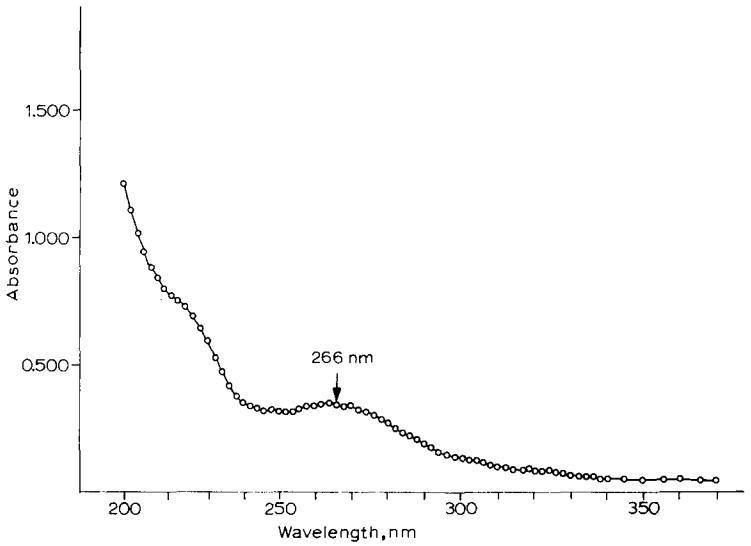


Fig. 4. The ultraviolet spectrum of the purified enzyme preparation: 22.5 μg/ml protein was present in 5 mM phosphate buffer at pH 6.6.

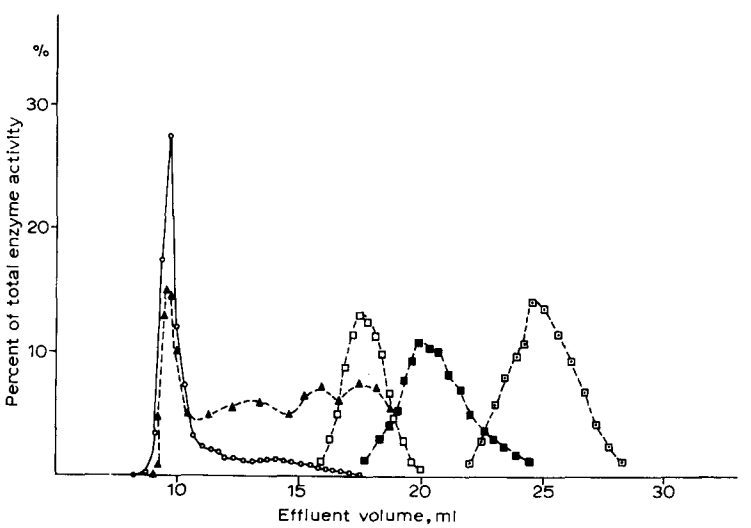


Fig. 5. Sepharose 4B filtration of acetylcholinesterase activity. A column of 1 cm × 46 cm Sepharose 4B previously equilibrated with 5 mM sodium citrate, pH 6.6, was used. The void volume was determined by Blue Dextran 2000 (▲---▲); 100 μg/0.1 ml of the purified enzyme (○—○); urease (mol. wt 510 000, □---□); glucose oxidase (mol. wt 152 000, ■---■) and peroxidase (mol. wt 40 000, ⊡---⊡) were applied. Acetylcholinesterase activity was measured in an autoanalytic system containing 20 mM oxaloacetic acid, 0.05 mM acetylthiocholine iodide, 0.05 mM DTNB, 0.5 mg/ml bovine serum albumin, pH 6.0, 37 °C. The results are the average of duplicate runs. The percentage of the total applied activity found in each fraction is plotted against the effluent volume.

The purified enzyme is a macromolecule because on filtration it was found in the void volume of the Sephadex G-200 column, and was collected at the bottom of the centrifuge tube after 24 h density-gradient centrifugation using a 10–40% (w/v) sucrose gradient in 5 mM citrate, pH 6.0. The enzymatic activity was also eluted in the void volume of the Sepharose 4B column (Fig. 5), indicating a minimum molecular weight of $2 \cdot 10^7$ for more than 90% of the molecules present. The addition of albumin does not effect the Sepharose 4B filtration pattern of the enzyme and no significant binding of albumin to the enzyme could be detected.

Electron microscopic examination confirmed the presence of a polymer, composed of small identical looking spherical units (Fig. 6). The addition of latex particles of 0.109 μm diameter, enabled the assessment of the diameter of the individual spheres to be in the order of 0.0106 μm.

The enzyme was found to hydrolyze the thioesters better than their respective choline ester analogues (Fig. 7); acetylthiocholine iodide was one of the better substrates.

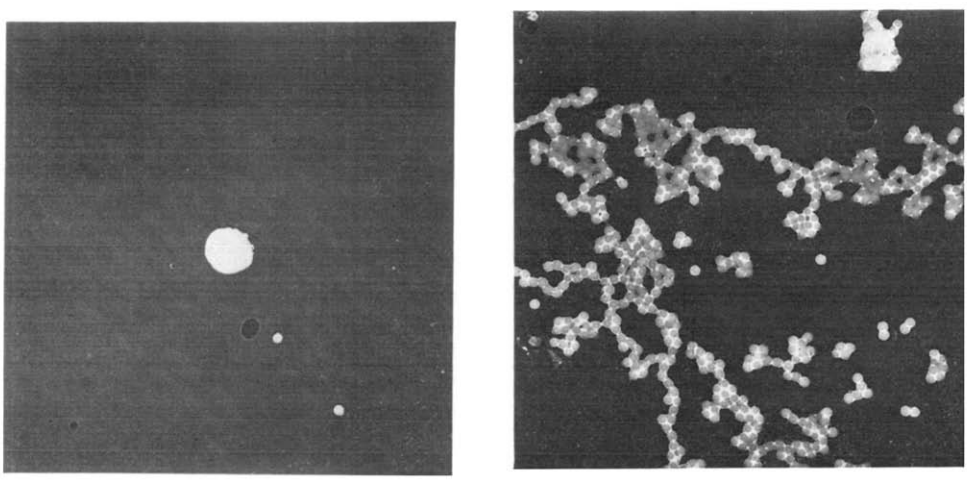


Fig. 6. Electron micrographs of negatively stained purified enzyme and latex particle. The purified enzyme was dissolved (9 μg/ml) in deionised water and a drop of this solution was dried on a collodion covered grid. A drop of 4% (w/v) sodium silicotungstate at pH 7.0 was applied over it and dried. To another sample latex particles of 0.109 $\pm$ 0.003 μm in diameter were added at $1.37 \cdot 10^{11}$ particles/ml[15]. The samples were investigated using an R.C.A. 3G electron microscope. Magnification 56 000 $\times$. Left, latex particle; right, purified enzyme.

The stability of the purified enzyme preparation

A rapid loss of activity (50% activity loss in 3 h) was observed when the purified enzyme was dissolved or dialyzed against deionised water which ended with the formation of a turbid, then a flocculent solution. The activity loss was not observed if the enzyme preparation was dissolved in deionised water containing albumin at a final concentration of 2 mg/ml (Fig. 8a). When albumin was added, at 2 mg/ml, to a two-weeks aged flocculent enzyme preparation, full activity could be restored. The effect of albumin concentration on enzyme activity is sown in Fig. 8b. Since a final concentration of 0.5 mg/ml albumin in the assay medium gave adequate protection this amount of albumin was always present in all the kinetic experiments. Ovalbumin,

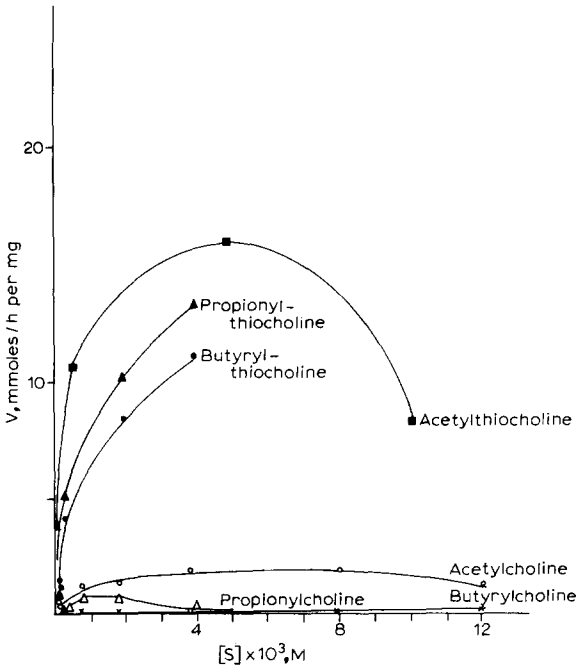


Fig. 7. Substrate specificity of the purified enzyme. The activity was measured in 20 mM oxaloacetic acid, 0.5 mg/ml bovine serum albumin, pH 6.0, 37 °C. The results are the average of two experiments. The substrate concentration is shown on the abscissa and the rate on the ordinate.

similar to bovine serum albumin, provided a protective effect. The addition of 20 mM oxaloacetic acid, at pH 6.0, in place of bovine serum albumin also protected the loss of enzyme activity and could reactivate the precipitated enzyme. Hence, when oxaloacetic acid was present the activation and protection by bovine serum albumin was not as great. The presence of bovine serum albumin did not prevent or change the inhibition or activation of several important modifiers used.

The kinetic characteristics of acetylthiocholine iodide hydrolysis

The Lineweaver–Burk plot of acetylthiocholine iodide hydrolysis, at pH 6.0 and 37 °C, was found to be linear in the presence of 20 mM oxaloacetic acid (Fig. 9a)[23]. At concentrations higher than 5 mM acetylthiocholine iodide, substrate inhibition was observed (*cf.* Fig. 7). The V was found to be 45 mmoles/h per mg enzyme, and the K_m for acetylthiocholine iodide 0.18 mM, using Michaelis–Menten kinetics[24]. In the presence of an inert buffer, 2.5 mM adipate, pH 6.0 and 37 °C, this plot was a hyperbole (Fig. 9b). The concavity of this curve was accentuated in the presence of a competetive inhibitor, 0.01 mM mytelase (Fig. 9c). Mytelase was preferred in place of eserine, because the inhibition of the latter compound gradually increased during incubation. These results were considered as an indication of the allosteric behavior of erythrocyte acetylcholinesterase, and assuming special binding sites, distinct from the catalytic center, some of the relevant microscopic dissociation constants for acetylthiocholine iodide and oxaloacetic acid were calculated[25]. The values obtained indicate low, hence

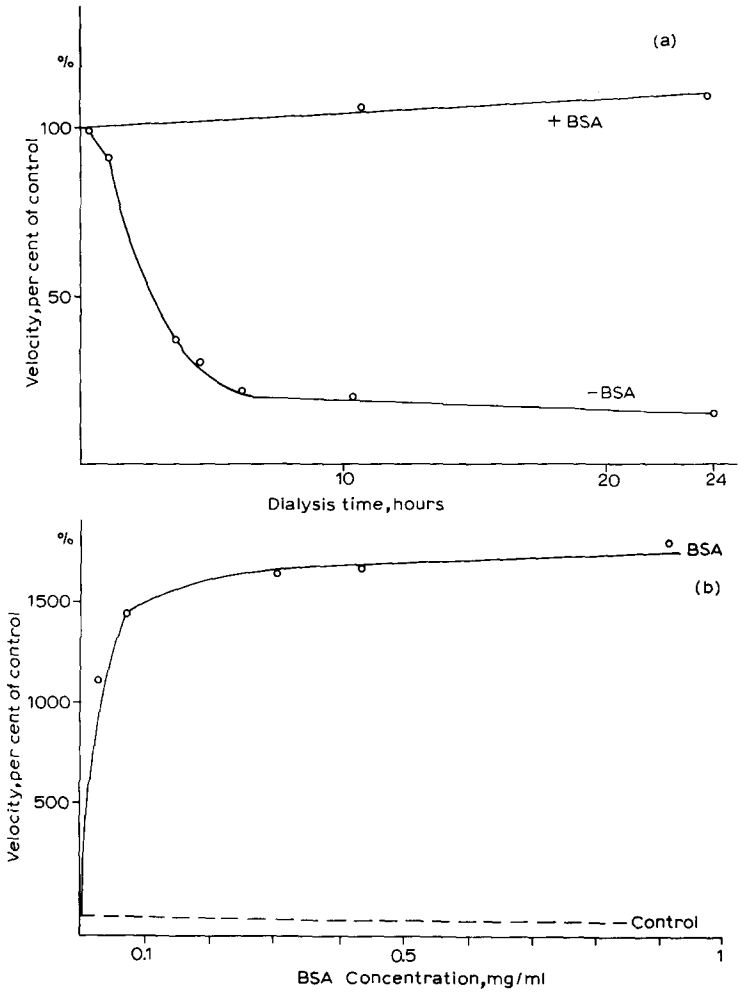


Fig. 8. The effect of bovine serum albumin on enzyme activity. (a) The protection of activity by the presence of bovine serum albumin during dialysis. The purified enzyme was diluted to 7.5 μg/ml and dialyzed against deionised water at 0 °C; when albumin was added it was present at 20 mg/ml concentration. The activity was measured using the autoanalytic system containing 20 mM oxaloacetic acid, 0.05 mM acetylthiocholine iodide, 0.05 mM DTNB, pH 6.0, 37 °C; the final albumin concentration was 0.67 mg/ml. On the abscissa is the dialysis period and on the ordinate the rates observed as percent of the zero time control. (b) The protection of enzyme activity by the presence of albumin in the assay medium. The enzyme was dialyzed against deionised water for 1 h at 0 °C, then the activity was measured in the autoanalytic system containing 2 mM citrate, 0.05 mM acetylthiocholine iodide, 0.05 mM DTNB and albumin concentration as indicated on the abscissa, pH 6.0, 37 °C. On the ordinate, the observed rates are expressed as percent of the control activity found in the absence of albumin at the end of dialysis. BSA = bovine serum albumin.

specific, regulator site binding constants for the erythrocyte acetylcholinesterase to acetylthiocholine iodide; in fact they were smaller than the active site dissociation constants (both K_2 and $K_3 < K_1$ and K_4). However, the maximal velocity of the substrate-activated enzyme was smaller ($k_5 e_0 > k_6 e_0$). The binding constants for oxalo-

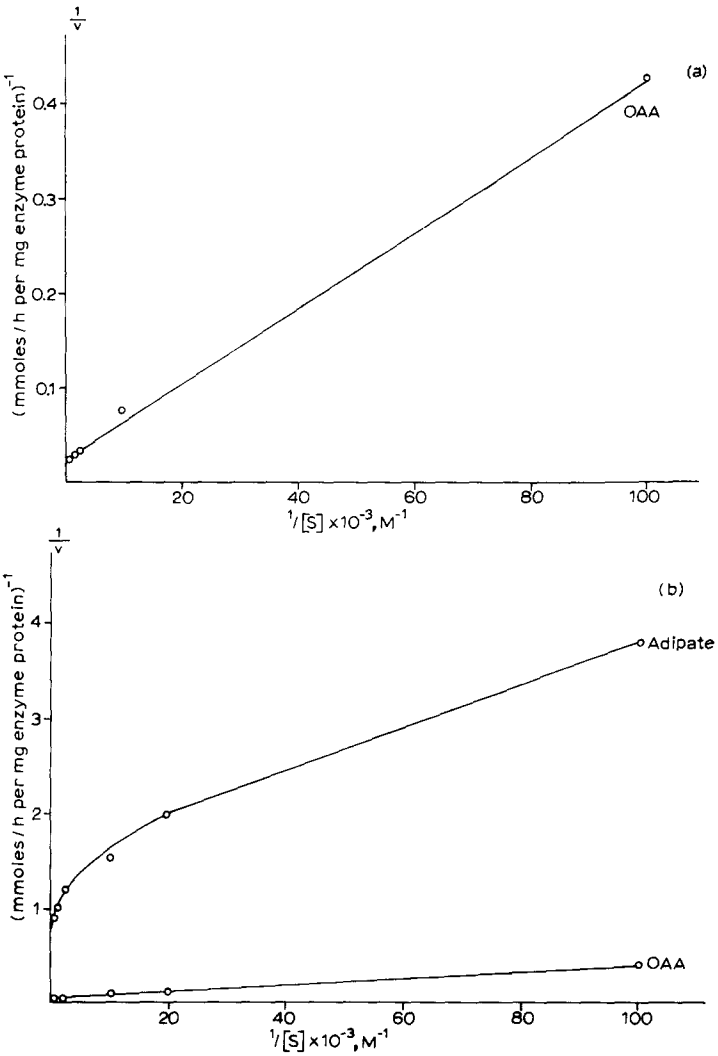
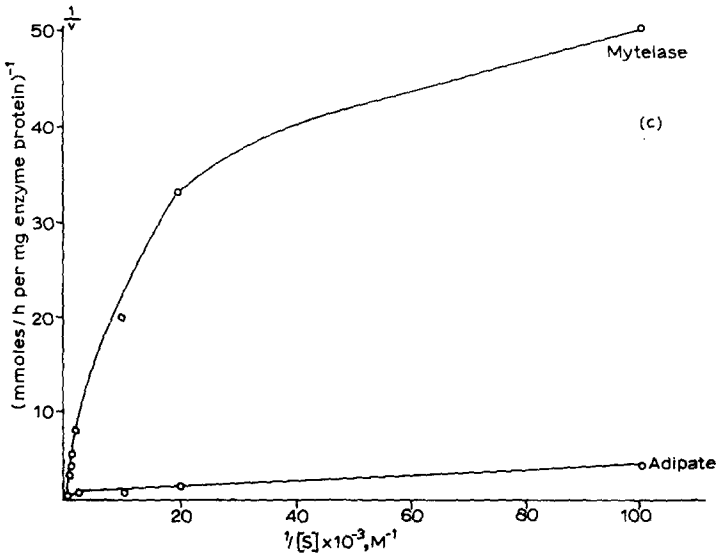


Fig. 9. Lineweaver–Burk plots of acetylthiocholine iodide hydrolysis. The autoanalytic assay system contained 0.05 mM DTNB, 0.5 mg/ml albumin, pH 6.0, 37 °C. (a) 20 mM oxaloacetic acid present. (b) 2.5 mM adipate was present. The control was 20 mM oxaloacetic acid. (c) 0.01 mM mytelase in 2.5 mM adipate was present. The control was 2.5 mM adipate. The reciprocal of the acetylthiocholine iodide concentration is shown on the abscissa, and the rate on the ordinate. The results are the average of six experiments. Assuming the model proposed by Frieden[25] which

is $E + S \overset{1}{\rightleftharpoons} ES$; $S + E \overset{2}{\rightleftharpoons} SE$; $ES + S \overset{3}{\rightleftharpoons} SES$; $SE + S \overset{4}{\rightleftharpoons} SES$; $ES \overset{5}{\rightarrow} E + P$; $SE_S \overset{6}{\rightarrow} E + P + S$; $E + M \overset{7}{\rightleftharpoons} ME$; $ES + M \overset{8}{\rightleftharpoons} MES$; $ME + S \overset{9}{\rightleftharpoons} MES$ and $MES \overset{10}{\rightarrow} M + E + P$; K_1, K_2 *etc.* indicating the microscopic dissociation constants, k_5, k_6 and k_{10} the rate constants and $b = (1 + \frac{K_1}{K_2})^{-1}$, the following values were calculated for acetylthiocholine iodide–enzyme



interaction: $K_1 = 8 \cdot 10^{-4}$ M; $K_2 = 4.4 \cdot 10^{-6}$ M; $K_3 = 2.2 \cdot 10^{-6}$ M; $K_4 = 4 \cdot 10^{-4}$ M; $b = 0.005$; $V_1 = k_6 e_0$ as 1.3 mmoles/h per mg; $V = k_5 e_0$ as 40 mmoles/h per mg. When oxaloacetic acid was present, the following values were obtained in addition: $K_7 = 4.8 \cdot 10^{-4}$ M; $K_8 = 4.2 \cdot 10^{-4}$ M; $K_9 = 2.3 \cdot 10^{-4}$ M and V (oxaloacetic acid) $= k_{10} e_0 = 45$ mmoles/h per mg. OAA = oxaloacetic acid.

acetic acid (K_7 to K_9) were in the range of $2.3 \cdot 10^{-4}$–$4.8 \cdot 10^{-4}$ M and the maximum velocity V (oxaloacetic acid) $= k_{10} e_0$ was 45 mmoles/h per mg enzyme, a value slightly higher than $V = k_5 e_0$ which was equal to 40 mmoles/h per mg enzyme (*cf.* Fig. 9).

The effect of temperature on kinetic parameters

The effect of temperature on acetylthiocholine iodide hydrolysis, determined in the presence of the optimum oxaloacetic acid concentration at pH 6.0, is shown in Fig. 10a; the rates remained linear during the 8 min incubation at both extremes of the experiment (0 and 50 °C). A sharp increase, both in V and K_m values, was found at temperatures higher than 32 °C. The Arrhenius plot (Fig. 10b) enabled the calculation of $\Delta S^{\neq}$ and ΔE_a[26]. A sharp increase in $\Delta S^{\neq}$ was found when the assay temperature was raised above 32 °C and was calculated to be +74.9 cal/degree per mole.[1] However the activation energy assumed a higher positive value.

The effect of pH on kinetic parameters

The effect of pH on acetylthiocholine iodide hydrolysis determined from pH 4.0 to 9.0 is shown in Fig. 11. A slight decrease in K_m and an increase in V was observed at pH 4.8; both values remained approximately the same between pH 4.8–8.5; the pK_{es1} and pK_{e1} values were found to be 4.8. As activity loss was observed below pH 4.0 and above pH 9.0, the other inflection, if there is one, could not be investigated.

The activator effect of various glycolytic and citric acid cycle intermediates

Among 75 glycolytic and citric acid cycle intermediates, their analogues,

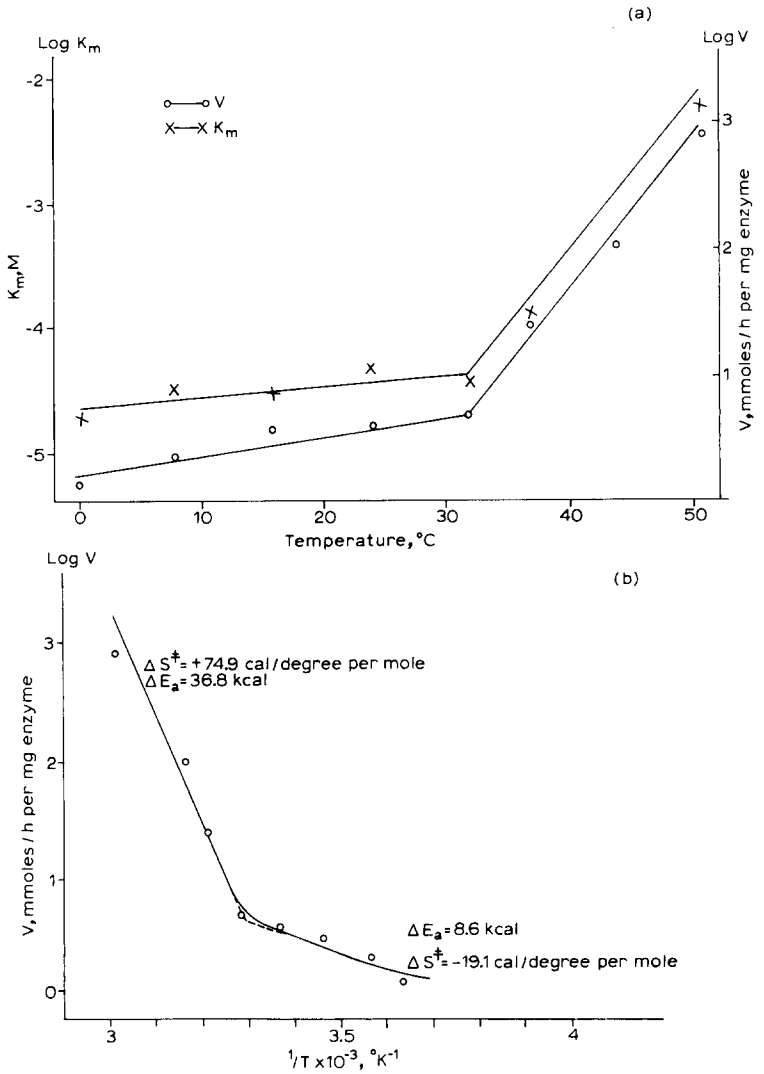


Fig. 10. Effect of temperature on acetylthiocholine iodide hydrolysis. (a) The hydrolysis was measured in 8 min from 0 to 50 °C in an autoanalytic system containing 20 mM oxaloacetic acid, 0.05 mM DTNB, 0.5 mg/ml albumin, pH 6.0, acetylthiocholine iodide concentration ranged from 10^{-5} to 10^{-3} M. The V and K_m values are calculated assuming classical Michaelis–Menten kinetics[24]. The results are the average of quadruplicate experiments; (b) the Arrhenius plot; $\Delta S^{\ddagger}$ and ΔE_a are calculated according to Dawes[26].

nucleotides and nucleotide sugars tested, the strongest activation was caused by HCO_3^-, oxaloacetic acid and fructose 1,6-diphosphate (Fig. 12). The activation by citrate was not as great; other citric acid cycle intermediates as well pyruvate, phosphoenolpyruvate, lactate and EDTA had only little activating effect (150% of control rate at 10 mM EDTA). Whereas glucose 6-phosphate, glucose 1-phosphate, fructose 1-phosphate, galactose 1-phosphate, galactose 6-phosphate and mannose 6-phosphate activated the enzyme much less than fructose 1,6-diphosphate. Fructose 6-phosphate

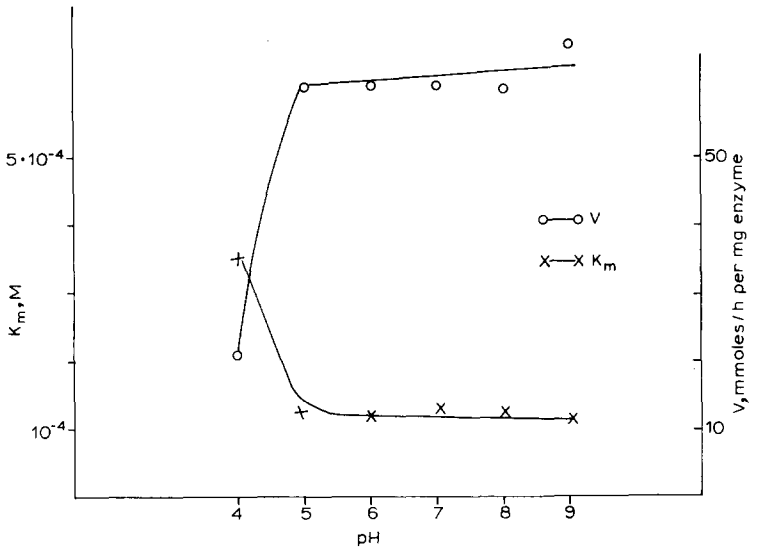


Fig. 11. The effect of pH on acetylthiocholine iodide hydrolysis. The autoanalytic assay contained 20 mM oxaloacetic acid, 2.5 mM Tris, 0.05 mM DTNB, 0.5 mg/ml albumin, 37 °C; acetylthiocholine iodide concentration ranged from 10^{-5} to 10^{-3} M, pH varied as shown on the abscissa. The V and K_m values are calculated assuming classical Michaelis–Menten kinetics[24]. The results are the average of quadruplicate experiments.

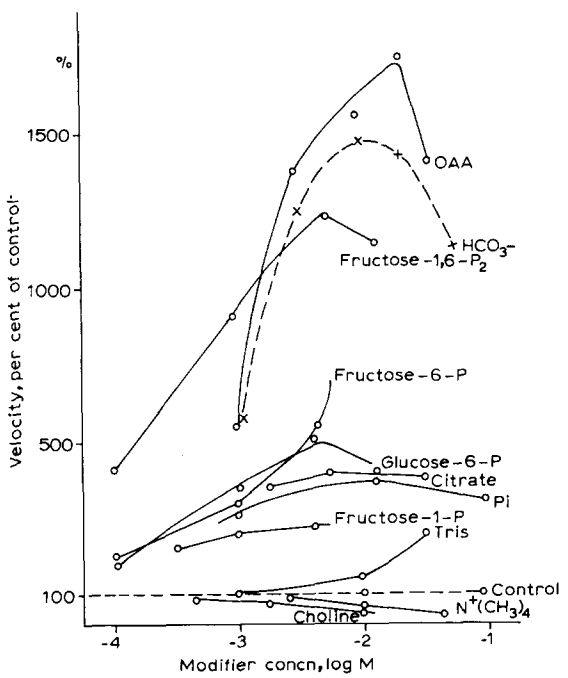


Fig. 12. The effect of anions on erythrocyte acetylcholinesterase activity. The autoanalytic assay system consisted of 2.5 mM adipate, 0.05 mM DTNB, 0.05 mM acetylthiocholine iodide, 0.5 mg/ml albumin, pH 6.0, 37 °C. The logarithm of the modifier concentration is shown on the abscissa. The results are the average of triplicate determinations and are expressed as the percent of averaged control rate ($n=120$) observed in the presence of 2.5 mM adipate on the ordinate. The effect of HCO_3^- (×—×) was investigated at pH 8.25. OAA = oxaloacetic acid.

was a good activator at high concentrations which was probably due to fructose 1,6-diphosphate contamination of the preparation used (approx. 7%). Triose mono- and diphosphates, pentose phosphates, amino or acetylamino sugars and their phosphate esters, several nucleotide sugars (UDP-glucose, UDP-glucuronic acid, UDP-galactose, UDP-*N*-acetylglucose and galactosamine), ATP, ADP and AMP tested at 1 to 10 mM concentrations and cyclic 3',5'-AMP at 0.01 mM had little or no activating effect.

Choline and tetramethyl ammonium ions inhibited the enzyme at high concentrations whereas their tertiary analogues were not effective; the ammonium ion also had no effect. Triton X-100 or BRIJ SP-35 failed to activate the enzyme.

Ouabain (0.1 mM) and stilbesterol (0.01 mM) had no effect on the rate. Whereas Cl^-, Br^- and I^- had no effect at 10 mM, 1 mM fluoride, inhibited the enzyme (approx. 90%). No significant activation or inhibition was observed in the presence of 50 mM Li^+, Na^+ or K^+.

The effect of oxaloacetic acid and mytelase at low substrate concentrations

In order to study the effect of a specific activator (oxaloacetic acid), and of a specific quarternary nitrogen compound inhibitor (mytelase) low substrate concentrations were chosen in view of the activating effect of acetylthiocholine iodide on the enzyme as has already been presented (*cf.* Fig. 9). The percent of oxaloacetic acid activation became considerably less when the substrate concentration was increased in the assay medium. This was also true for the inhibitory effect of mytelase. Furthermore, at 10^{-10} to $3 \cdot 10^{-9}$ M, mytelase was found to activate acetylcholinesterase; this effect was statistically significant (Fig. 13).

DISCUSSION

Erythrocyte acetylcholinesterase has not been purified to a great extent until recently[27]; hence we have tried to design a simple purification procedure that would also give a higher yield. Earlier efforts of purification consisted of *n*-butanol extraction of bovine erythrocyte membrane fragments followed by lyophilization. The enzyme could then be extracted by 0.1 M phosphate buffer at pH 8.0. This procedure yielded a 360-fold pure enzyme[28]. In another report, Tween 20 and toluene were used to extract the enzyme from the erythrocyte membrane fragments followed by $(NH_4)_2SO_4$ precipitation, calcium phosphate gel chromatography, acetone, ethanol and diethylether extraction. By this procedure the enzyme could be purified 240-fold[29]. Recently a method involving affinity chromatography for the purification of electric eel and bovine erythrocyte acetylcholinesterase has been described; the acrylamide gel electrophoresis of the 2500-fold (over crude commercial starting material) purified bovine erythrocyte acetylcholinesterase preparation yielded a single band[27].

Miller[30] has described the use of Triton X-100 as a suitable extraction agent for erythrocyte membrane (Na^+–K^+)-ATPase which could be virtually totally solubilized with little loss of activity; under the same conditions no activity loss for acetylcholinesterase was observed. Wright and Plummer[31] using ammonium sulfate precipitation, in addition to KCl–Triton X-100 solubilization, reported a 50-fold purification for human erythrocyte acetylcholinesterase; the polyacrylamide gel electrophoresis of their purified material at pH 8.1 revealed the presence of one to three bands.

The procedure described here extends these findings; by the use of DEAE-

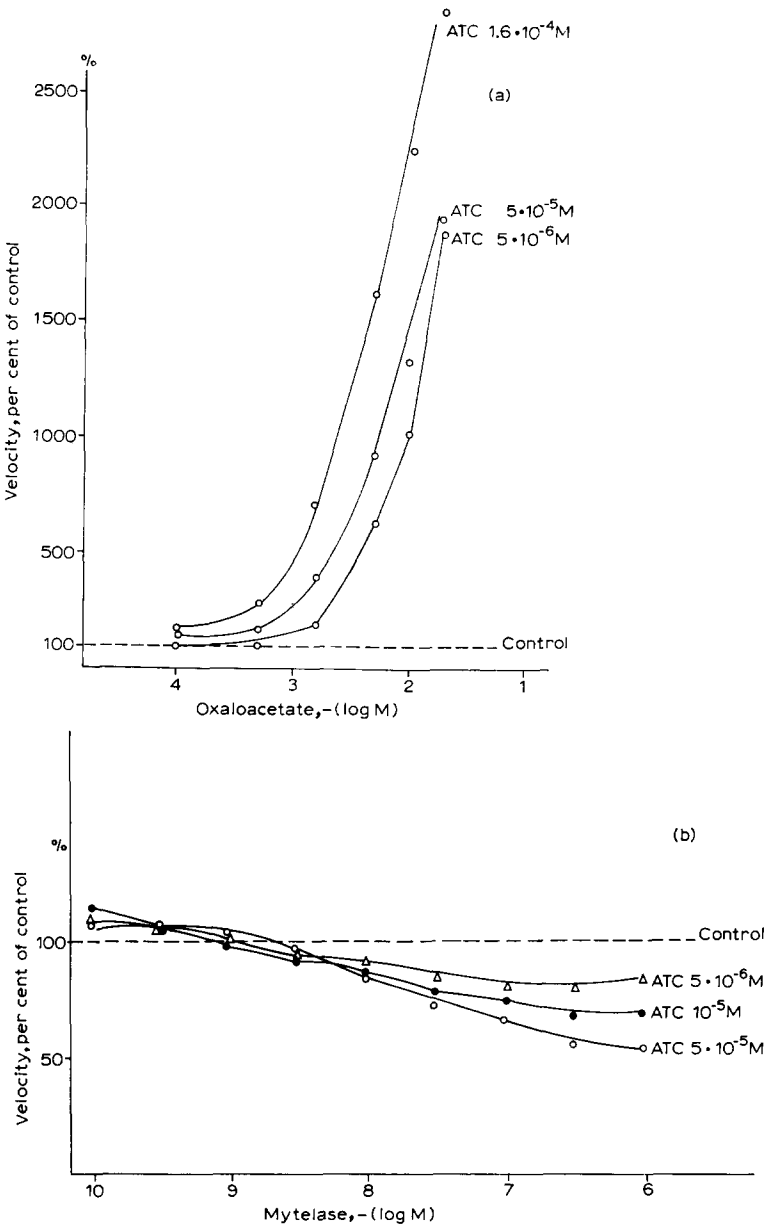


Fig. 13. The effect of oxaloacetic acid and mytelase at low substrate concentration. The results are the average of triplicate autoanalyzer assays containing 2.5 mM adipate, 0.05 mM DTNB, 0.5 mg/ml albumin, pH 6.0, 37 °C. The concentration of acetylthiocholine iodide is indicated on respective curves. The logarithm of modifier concentration is shown on the abscissa, and the rate observed as percent of the control on the ordinate. (a) Oxaloacetic acid, the control experiment $n = 72$; (b) mytelase, $n = 99$; the P values related to the activating effect of mytelase at 10^{-10} M and $3 \cdot 10^{-9}$ M concentrations are $0.01 > P > 0.001$ for all three acetylthiocholine iodide concentrations tested. ATC = acetylthiocholine iodide.

cellulose and calcium phosphate gel on cellulose chromatography, human erythrocyte acetylcholinesterase has been purified to a great extent, with little loss of enzyme activity. The yield here, is reported to be 5%; however care was taken to collect only the fractions with highest specific activity. Considering the large percent of discarded activity, only about 14% activity loss should be considered to occur during the two chromatography steps following the Triton X-100 extraction (*cf.* Table I). The equal effectiveness, and high solubilization yields of other Tritons as well, suggest a specific benefactorial solubilization effect of these detergents. Hence, it is considered that the enzyme is only damaged a little by the purification procedure reported here. This is to be expected, as the use of organic solvents which might have a deleterious effect[32], and also the use of hydrolytic, lipolytic or proteolytic enzymes were avoided[33,34].

The maximum velocity of human erythrocyte acetylcholinesterase, 45 mmoles of acetylthiocholine iodide hydrolysis per h per mg calculated in this report, is not high when compared to that reported for the eel enzyme, which is 750 mmoles acetylcholine hydrolysis per h per mg protein[2]. This lower value may be due to the possible presence of a protein contaminant in our preparation or it may be explained by the different chemical nature of our purified protein. In fact our method yields a protein, electrophoretically different from the purified bovine erythrocyte acetylcholinesterase. The purified human acetylcholinesterase requires pretreatment with a strong resolving solution for satisfactory polyacrylamide gel chromatography; whereas the latter can migrate electrophoretically by the conventional acrylamide disc electrophoresis technique[18,27].

The purified erythrocyte acetylcholinesterase is a protein as evidenced by the presence of a 215 nm peptide absorbance band in the ultraviolet spectrum, Amido Black 10 B staining and its reactability with the Folin–Ciocalteu reagent. The aromatic amino acid absorbance maximum at 266 nm and the ratio of the absorbances at 280 nm/260 nm < 1, are different from the eel acetylcholinesterase ,with an absorbance maximum at 280 nm (ref. 2).

The electrophoretic investigation which reveals the presence of a single protein band in purified human acetylcholinesterase is in accordance with the electrophoretic findings of the affinity chromatography of purified bovine erythrocyte acetylcholinesterase[27]. Erythrocyte membrane is shown to contain glycoproteins, and to be rich in sialic acid which can be solubilized by neuraminidase[35]. The enzyme protein was stained by periodic acid–Schiff stain reagent; and acrylamide gel chromatography of Triton X-100 extracted enzyme, stained both for enzyme activity and for carbohydrate presence indicated similar localization of the two stains. It is the only periodic acid–Schiff stained band (among various protein bands) detected in the Triton X-100 extracted or DEAE-cellulose purified protein fractions. Despite the strong resolving effect of phenol, acetic acid and 5 M urea, a considerable amount of periodic acid–Schiff stainable material stayed at the origin when intact erythrocyte membranes were used. Hence, the enzyme should be considered as the, or one of the, glycoprotein(s) of the erythrocyte membrane. This finding also supports its chemical difference from central nervous system acetylcholinesterase which does not stain with periodic acid–Schiff reagent[36].

Other kinetic characteristics of purified human acetylcholinesterase are also different from the eel enzyme, *e.g.* its K_m value for acetylthiocholine iodide is 0.18 mM compared to the K_m value of 0.06 mM of the eel enzyme[37], choline concentration

leading to 50% inhibition is different (*cf.* Fig. 12)[7,37] and it requires the presence of bovine serum albumin or another protein for optimum activity *etc.* The pH activity profile and the pK_{es1} and pK_{e1} values of 4.8 were also different from the pK_a of 6.5, reported for the eel enzyme[7]. This pK_{e1} value suggests the presence of a carboxyl group at the active center of erythrocyte acetylcholinesterase; whereas, an imidazole residue is implicated in the catalytic function of the latter[7].

These findings, together with the substrate specificity, *i.e.* the preferred hydrolysis of thiocholinesters as well as nonpolar substrates, compared to the hydrolysis of cholinesters support the view that this enzyme is substantially different from the eel enzyme and that it is an esterase rather than a specific cholinesterase[7,8].

Human erythrocyte acetylcholinesterase is a large polymer; its macromolecular composition is evidenced by Sephadex G-200, Sepharose 4B gel filtration and density-gradient centrifugation results. Electron microscopic examination reveals a large aggregate, composed of small, identical looking spheres. A similar appearance is also obtained for the eel enzyme under certain conditions[38]. The difficulties in resolving our protein into functional subunits has not yet permitted us to estimate the accurate molecular weight of each subunit. The electron microscopic appearance of the purified enzyme is reminiscent of another purified erythrocyte membrane protein, spectrin[39,40]; the subunit of which is much smaller than acetylcholinesterase. Spectrin is found to aggregate easily, unless either EDTA or β-mercaptoethanol is present; the presence of these compounds in the filtration medium does not alter the gel filtration pattern of purified acetylcholinesterase. The tendency of acetylcholinesterase to aggregate is further exemplified by the effect of bovine serum albumin. A turbid, then flocculent, appearance sets in, concomittant with activity loss unless bovine serum albumin is present; this process is reversible. The molecular aggregates thus formed are also all heavier than $2 \cdot 10^7$ daltons. The protecting and activating effects of bovine serum albumin, the lack of significant bovine serum albumin binding to enzyme in filtration experiments, ovalbumin as well as bovine serum albumin protection, and the failure of bovine serum albumin to change the modifier's activating and inhibiting action, all favor a nonspecific effect of bovine serum albumin. The role of some acidic proteins leading acetylcholinesterase to aggregate may be possible.

The physiological function of erythrocyte acetylcholinesterase is as yet unknown. Ouabain that inhibits erythrocyte (Na^+–K^+)-ATPase, and hence the ionic pump[41] and stilbesterol that inhibits the erythrocyte glucose transport system[42], both failed to inhibit the purified acetylcholinesterase. Although the purified erythrocyte acetylcholinesterase is markedly different from the eel enzyme, which is probably responsible for the acetylcholine receptor function, it also manifests an allosteric behavior[5,6].

The acetylcholinesterase of the electric organ of *Electrophorus electricus* has been crystallized and its physicochemical properties have been established in detail. It has been found to be a tetrameric protein with a molecular weight of $2.6 \cdot 10^5$ (refs 2, 43). Various experiments involving the calculation of the binding constants and thermodynamic interaction parameters of acetylcholine and its tertiary analogue, the affinity labelling of the enzyme as well as the inhibitory action of certain toxins (α-bungarotoxin), and antibiotics (tyrocidine), all led to the final conclusion that the eel enzyme manifests an allosteric behavior towards its substrates. Also that there are two topographically distinct, regulatory and allosteric sites besides the two active sites per tetramer[5,6,44].

The kinetic characteristics, the presence of specific modifiers and the effect of temperature on the purified erythrocyte enzyme presented here, indicate its possible allosteric behavior. The results may be explained in other ways; *e.g.* by the presence of isoenzymes, the presence of protein impurities bound to acetylcholinesterase, *etc.*[25]. However, its macromolecular structure, the single acrylamide electrophoretic band, and the relatively high specific activity, all furnish evidence for the existence of a relatively homogenous protein preparation and thus support the former conclusion.

The calculation of the acetylthiocholine iodide–enzyme microscopic dissociation constants, assuming the presence of a separate polar substrate binding site besides the active center, gives a low value; hence a specific binding for acetylthiocholine iodide can be assumed (*cf.* Fig. 9). The inhibitory action of other quarternary nitrogen compounds *e.g.* mytelase (*cf.* Fig. 9c), choline, and tetramethylammonium (*cf.* Fig. 12) may be explained both by competition between the quarternary nitrogen compounds and the substrate, and by the presence of other binding sites for these compounds[7]. The mytelase activating effect observed at low inhibitor concentrations suggests the existence of more than one binding site for this competitive inhibitor (*cf.* Fig. 13b). At low substrate concentration, within the range of K_2 and K_3, increasing the substrate concentration leads to a more effective inhibition by mytelase (*cf.* Fig. 13b); which is suggestive of a substrate-induced conformation leading to better inhibitory action by mytelase. At very high substrate concentrations the inhibitory action of mytelase disappears (*cf.* Fig. 9a); which in turn suggests a competitive interaction between the substrate and mytelase. Such competition should not be considered impossible, as both thiocholine and cholinesters inhibit the enzyme at high concentration, and this can be observed both in the presence and absence of an activator (*cf.* Fig. 7). This interpretation is further supported by the relative lack of effect of tertiary nitrogen-carrying analogues on the enzyme.

The activating effect of oxaloacetic acid, also, depends upon the substrate concentration. Again at low substrate concentration, within the range of K_2 and K_3 (*cf.* Fig. 13a) increasing the substrate concentration leads to a higher percent of activation by oxaloacetic acid, supporting the prerequisite of substrate induced conformational change, for a better oxaloacetic acid activating effect. At very high substrate concentrations, the activating effect of oxaloacetic acid decreases; the maximal velocities calculated for the oxaloacetic acid activated enzyme, and for enzyme in the absence of activator, in fact are close ($V_{\text{oxaloacetic acid}} = 45$, $V = k_5 e_0 = 40$ mmoles/h per mg protein (Fig. 9)).

The effect of temperature on catalysis may also be interpreted by the allosteric behavior of erythrocyte acetylcholinesterase. This effect has been investigated in the presence of optimum activating concentrations of oxaloacetic acid, in order to simplify the interpretation. A sharp increase, both in K_m and V, has been observed at temperatures higher than 32 °C. Hence, the Arrhenius plot shows two intersecting lines[26]. Similar Arrhenius plots have been observed for several other enzymes; a shift of the rate-limiting catalytic step, phase change in the catalytic system, two forms of the enzyme, and reversible inactivation or deactivation of the enzyme, have all been implicated[26]. When the substrate activation, and the existence of specific modifiers are considered, the results presented in Fig. 10 may be interpreted by a shift of the rate-limiting catalytic step, by a change of the aggregation state of the enzyme or by a reversible change of enzyme protein between two accessible states, *viz.* preferred for-

mation of a more reactive catalyzer at temperatures higher than 32 °C. The latter possibility has already been proposed for allosteric enzymes as a model to investigate the allosteric effects of inhibitor and activator modifiers[45,46]. When the complexity of the catalytic model proposed in the presence of substrate and modifier[25,45,46], and the effect of bovine serum albumin on the state of enzyme aggregation is considered, a valid interpretation of the Arrhenius plot (presented in Fig. 10) should depend upon the elucidation of the physical chemistry of erythrocyte acetylcholinesterase. The $\Delta S^{\neq}$ value calculated for temperatures below 32 °C is −19.1 cal/degree per mole, which is in the range reported for simple chemical catalysts. However this value is +74.9 cal/degree per mole at temperatures above 32 °C, indicating the presence of an ordered catalysis at the active center now; a similar high positive value for $\Delta S^{\neq}$ has been reported (+34; +53 cal/degree per mole) for eel acetylcholinesterase, by Wilson and Cabib[47], calculated with acetylcholine as the substrate. The ΔE_a value for temperatures below and above 32 °C are, however, more positive than the value reported for the eel enzyme by Wilson and Cabib[47]. This temperature behavior of the erythrocyte enzyme may also be responsible for the lack of documentation of its allosteric behavior, as most of the studies previously reported have used room temperature to investigate the hydrolysis.

The effect of anions is interesting; HCO_3^- and certain important cellular metabolic intermediates, such as fructose 1,6-diphosphate and oxaloacetic acid, have been found to activate the enzyme specifically. Assuming a special binding site for oxaloacetic acid, low and thus specific binding constants for the binding of this compound to the enzyme are calculated (*cf.* Fig. 9). The range and degree of activation of HCO_3^- and oxaloacetic acid, parallel those described for other allosteric systems by these compounds; *i.e.* acetyl-CoA carboxylase[48] and pyruvic carboxylase[49]. Purified erythrocyte acetylcholinesterase has been found to be strongly inhibited by divalent cations as Mg^{2+} and heavy metals, thus the activator effect of carboxylates might be attributed to their chelating effect; however, a specific chelator, EDTA, has not been an effective activator. The activating effect of fructose 1,6-diphosphate was interesting as the concentration of this compound in erythrocytes is reported to be high[50,51]. Its effect is specific, *i.e.* is not reproduced by P_i, fructose 6- or 1-phosphate, and other hexose monophosphates; also triose diphosphates (1,3- or 2,3-diphosphoglyceric acid) failed to activate acetylcholinesterase. The accumulation of 2,3-diphosphoglycerate within the erythrocyte, has been implicated for the action of thyroxine on erythrocyte because this compound effects the allosteric behavior of hemoglobin[52]. Other polyphosphates, nucleotide di- and triphosphates have also little or no activating effect on erythrocyte acetylcholinesterase.

It is hoped that these results, related to the behavior of erythrocyte acetylcholinesterase of which the physiological function is yet unknown, may shed light on the possible participation of the enzyme in erythrocyte physiology, and may explain the relation between its deficiency and paroxysmal nocturnal hemoglobinuria[4].

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