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Abstract

The activity and properties of cholinesterase (ChE) of the motor endplate and its fractions were studied in isolated human skeletal muscle. This preparation was used since the ChE activity of the membrane preparation was localized only in the motor endplate. The endplate ChE was stable in the isolated membrane for 4 weeks at 4 degrees C. The specific activity of the extracted ChE of human muscle membrane was 29.6% higher than that of the original membrane. Studies with specific substrates and ChE inhibitors indicated that most of the ChE of human muscle membrane and its fractions was acetylcholinesterase, and that the minor component was pseudocholinesterase. A Michaelis-Menten constant of 3.82 mM was estimated in the endplate ChE, and 0.88 mM in the extracted ChE of the endplate. The extracted human endplate ChE was separated into three fractions by Sephadex G-200 chromatography, and into two fractions by acrylamide gel electrophoresis.

KEYWORDS: acetycholinesterase, cholinesterase, moter endplates, cholinesterase inhibitors, isozymes

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MOTOR ENDPLATE CHOLINESTERASE IN HUMAN SKELETAL MUSCLE

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Abstract. The activity and properties of cholinesterase (ChE) of the motor endplate and its fractions were studied in isolated human skeletal muscle. This preparation was used since the ChE activity of the membrane preparation was localized only in the motor endplate. The endplate ChE was stable in the isolated membrane for 4 weeks at 4℃. The specific activity of the extracted ChE of human muscle membrane was 29.6 % higher than that of the original membrane. Studies with specific substrates and ChE inhibitors indicated that most of the ChE of human muscle membrane and its fractions was acetylcholinesterase, and that the minor component was pseudocholinesterase. A Michaelis-Menten constant of 3.82 mM was estimated in the endplate ChE, and 0.88 mM in the extracted ChE of the endplate. The extracted human endplate ChE was separated into three fractions by Sephadex G-200 chromatography, and into two fractions by acrylamide gel electrophoresis.

Key words : acetylcholinesterase, cholinesterase, motor endplates, cholinesterase inhibitors, isozymes.

Cellular and subcellular localization of cholinesterase (ChE) activity in mammalian skeletal muscle has been revealed by histochemical, biochemical and autoradiographical techniques (1-5). ChE activity is largely concentrated in the neuromuscular junction. The ChE of the neuromuscular junction plays an important role in the pathophysiology of diseases of neuromuscular transmission, and in the action of drugs which affect neuromuscular transmission. However, there was no quantitative study of the motor endplate ChE of human skeletal muscle. In a previous paper, we reported that only about 2 % of the total ChE of human skeletal muscle was estimated to be in the endplates compared with 20 % in rat skeletal muscle (3). The difference was due to the lower ChE of the motor endplate and the higher ChE of non-endplate components in human muscle than in rat muscle.

In the present study, the activity and properties of the motor endplate ChE of human skeletal muscle were studied in isolated muscle membrane preparation and its fractions. Intercostal muscle was chosen since this muscle was mainly used in microelectrophysiological studies of human motor endplates.

MATERIALS AND METHODS

Isolation of muscle membrane. Human intercostal muscle was obtained during postmortem study within several hours of death from patients with no history of neuromuscular diseases. The muscle membrane preparation was isolated by a modification of our previous method (2). The muscle was homogenized in a Polytron homogenizer with 50 mM $CaCl_2$ solution, and filtered through an 18 mesh plastic net. The filtrate was centrifuged at 1,000 g for 1 min, and the precipitate was washed three times with buffered KCl solution containing 45 mM KCl, 30 mM $KHCO_3$, 2.5 mM dl-histidine monohydrochloride, adjusted to pH 7.8 with 1 M tris hydroxymethylaminomethane solution. The precipitate was suspended in buffered KCl solution, incubated in a water bath at 37 ℃ for 30 min with gentle shaking, and left at 4 ℃ for 20 min. The suspension was centrifuged at 1,000 g for 1 min, and the precipitate was washed twice with 2.5×10^{-7} M NaOH solution, then left overnight suspended in the solution at 4 ℃. The suspension was washed twice with 25 mM glycine-NaOH buffer, pH 10.7. The muscle membrane preparation was obtained after washing the suspension twice with 2.5×10^{-7} M NaOH solution.

Extraction of motor endplate ChE. The membrane preparation was suspended in a solution containing 100 mM NaCl, 10 mM $MgCl_2$, 30 mM sodium phosphate buffer at pH 7.5 and 0.5 % (v/v) of Triton X-100, and incubated in a water bath at 37 ℃ for 30 min with shaking at 80 cycles/min. The suspension was then centrifuged at 1,000 g for 15 min, and the supernatant was recovered as the soluble fraction and the precipitate as the insoluble fraction.

ChE assay. ChE activity was measured by the hydroxamic acid method of Hestrin (6). Incubation was performed at 37 ℃ for 30 min in a shaking water bath at 80 cycles/min in a 2.0 ml medium containing the sample (0.4 mg protein), the substrate, 100 mM NaCl, 10 mM $MgCl_2$ and 30 mM sodium phosphate buffer at pH 7.5. The substrate used in this study was 4 mM acetylcholine (ACh) bromide, 8 mM acetyl-beta-methylcholine iodide or butyrylcholine iodide. The concentration of acetyl-beta-methylcholine was doubled since the compound was a dl form and ChE acts only on the l form. Diisopropylfluorophosphate (DFP) and 1,5-bis-(4-allyldimethyl-ammoniumphenyl)pentan-3-one dibromide (BW284C51) were used as inhibitors. The action of ChE inhibitors was determined by incubation of the sample in the medium with the inhibitor but without substrate for 15 min at room temperature prior to the addition of ACh as substrate. Protein was measured by the method of Lowry *et al.* (7) with bovine serum albumin as the standard following dissolving the sample in 1 M NaOH solution. The motor endplates were visualized in the membrane preparation by staining for ChE activity by the method of Karnovsky and Roots (8).

Separation of ChE isozymes. Sephadex G-200 column (30 × 2.0 cm) was eluted with 150 mM NaCl-20 mM sodium phosphate buffer, pH 8.0. The column was loaded with 1 ml samples containing 2.5 mg protein. Flow rate was 3 ml/h and the fraction size was 1 ml. The molecular weight (MW) of the ChE isozymes were estimated using aldolase, catalase, ferritin and thyroglobulin (MW: 158,000, 232,000, 440,000 and 669,000, respectively) to standardize the column, and blue dextran (MW: 2000,000) to determine the void volume. Acrylamide gel electrophoresis was performed by a modification of the method of Ornstein and Davis (9). The separation gel was a cast in two parts, a lower gel with small pore size and a upper gel with large pore size. The small pore gel contained 7 % (wt/v) acrylamide, 0.18 % (wt/v) N, N-methylene bisacrylamide, 0.03 % (v/v) N, N, N, N-tetramethylenediamide and 0.07 % (wt/v) ammonium persulfate in 380 mM tris hydroxymethylaminomethane, adjusted to pH 6.7 with 1 M HCl. The 0.2 ml samples containing 0.1 mg protein were applied

on top of the upper gel. The buffer in the electrode compartments was 5 mM tris hydroxymethylamino-methane-38 mM glycine, pH 8.3. A current of 2.5 mA per tube was applied for 2 to 2.5 h. Staining of the gel for ChE was performed by the method of Karnovsky and Roots. Protein in the gel was stained in a solution containing 0.1% (wt/v) Amino Black in 7% (v/v) acetic acid and destained in 7% acetic acid until the bands were visible.

RESULTS

Properties of motor endplates. Numerous motor endplates were visualized in the membrane by staining for ChE (Fig. 1). The fine structures of these endplates were similar to structures of endplates seen in muscle sections. However, the motor endplates of the isolated membranes were compressed, stretched and folded. There was no appreciable change in ChE activity when the membrane was stored at 4℃ in distilled water for 4 weeks. The activity in 6 samples was 1.25 ± 0.071 (μmol ACh hydrolyzed/30 min/mg protein) initially and 1.22 ± 0.070 at 4 weeks. The motor endplates were the only structure in the membrane demonstrated by staining for ChE.

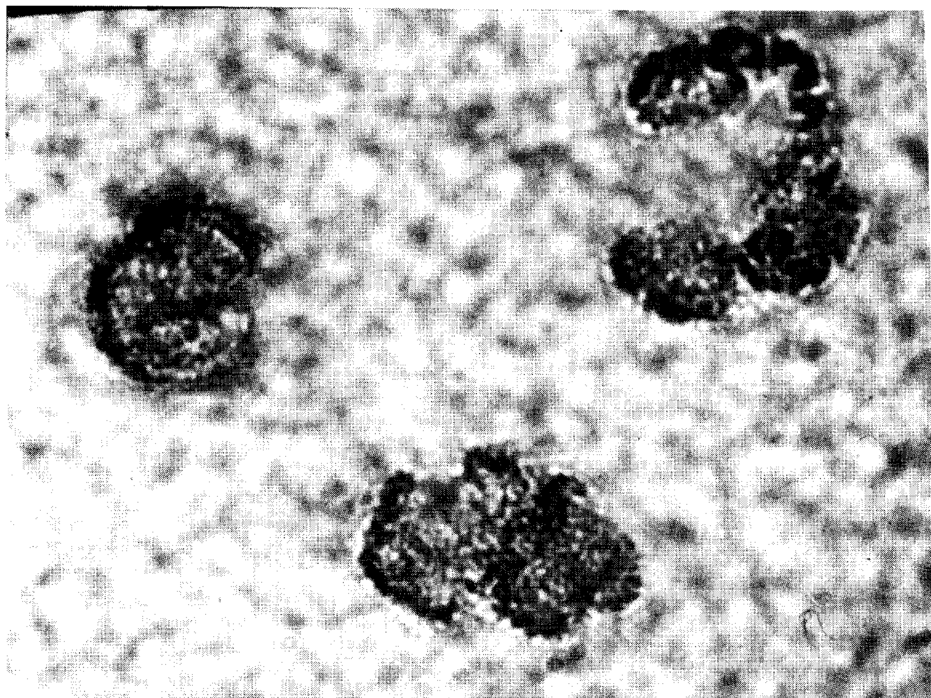

Fig. 1. Isolated human muscle membrane. Motor endplates demonstrated by cholinesterase staining, × 2198.

Extraction of endplate ChE. In the initial study there was no difference in

the extracted ChE activity of human muscle membrane by Triton X-100 in phosphate buffered saline solution following overnight incubation at 4 ℃, overnight incubation at room temperature or 30 min incubation at 37 ℃. Incubation at 37 ℃ longer than 1 h resulted in the growth of microorganisms. In the next step the membrane fraction was suspended in various concentrations of Triton X-100 from 0.1 to 5.0%, and the extracted ChE was measured (Table 1). The solubility did not seem to have any definite relation with these concentrations of Triton X-100. Using Triton X-100 in distilled water instead of phosphate buffered saline solution, the solubility was in the range of 20 to 25%. Therefore, 0.5% Triton X-100 in phosphate buffered saline solution was used exclusively.

In 15 muscle membranes 37.1 ± 0.46 (mean ± SEM)% of the total motor endplate ChE was extracted (Table 2). As calculated per mg of protein in each fraction, ChE activity was 1.25 ± 0.040 (μmol ACh hydrolyzed/30 min/mg protein) in 15 original membranes, 1.62 ± 0.033 in 15 soluble fractions and 0.81 ± 0.027 in 15 insoluble fractions. The mean specific activity of the soluble fraction was 29.6% higher than the original membrane.

TABLE 1. EXTRACTION OF CHOLINESTERASE OF HUMAN MUSCLE MEMBRANE BY VARIOUS CONCENTRATION OF TRITON X-100[a]

Triton X-100 concentration	% of extracted cholinesterase activity
0.1 %	35.3 ± 0.43
0.5 %	38.0 ± 0.61
1.0 %	37.1 ± 0.35
1.5 %	35.5 ± 1.03
2.0 %	35.7 ± 0.79
4.0 %	34.7 ± 0.64
5.0 %	34.9 ± 0.91

a Mean ± SEM in 10 samples.

TABLE 2. CHOLINESTERASE ACTIVITY OF HUMAN MUSCLE MEMBRANE AND ITS FRACTIONS USING ACETYLCHOLINE AS SUBSTRATE[a]

	Cholinesterase activity
Membrane	1.25 ± 0.040 (μmol ACh hydrolyzed/30 min/mg protein)
Soluble fraction	1.62 ± 0.033 (μmol ACh hydrolyzed/30 min/mg protein)
	37.1 ± 0.46 (% of total membrane cholinesterase)
Insoluble fraction	0.81 ± 0.027 (μmol ACh hydrolyzed/30 min/mg protein)

a Mean ± SEM in 15 samples.

Properties of endplate ChE. Using acetyl-beta-methylcholine as substrate, the ChE activity of the soluble fraction of human muscle membrane was 51.4% higher than the original membrane, and 22.9% lower in the insoluble fraction

(Table 3). Using butyrylcholine as substrate, the ChE activity of the soluble fraction was only 9.8% of the activity of the original membrane, and 36.3% in the insoluble fraction. The ratio of the ChE activity with acetyl-beta-methylcholine to the activity with butyrylcholine was 10.3 in the original membrane, 159.0 in the soluble fraction and 21.9 in the insoluble fraction.

DFP slightly inhibited the ChE activity, while BW284C51 greatly inhibited the activity (Table 4). DFP is an inhibitor of pseudocholinesterase (PChE), inhibiting less than 5% of acetylcholinesterase (AChE) at 10^{-7} M and 100% of PChE at 10^{-8} M or higher concentrations; BW284C51, an AChE inhibitor, inhibits more than 95% of AChE and less than 5% of PChE at concentrations between 10^{-6} and 10^{-5} M. These results indicate that human muscle membrane and its fractions contain two types of ChE, AChE accounting for most of the activity and PChE as a minor component.

TABLE 3. CHOLINESTERASE ACTIVITY OF HUMAN MUSCLE MEMBRANE AND ITS FRACTIONS USING ACETYL-BETA-METHYLCHOLINE AND BUTYRYLCHOLINE AS SUBSTRATES[a]

	Cholinesterase activity (μmol of substrate hydrolyzed/30 min/mg protein)	
	Acetyl-beta-methylcholine	Butyrylcholine
Membrane	1.05 ± 0.053	0.102 ± 0.0102
Soluble fraction	1.59 ± 0.054	0.010 ± 0.0023
Insoluble fraction	0.81 ± 0.038	0.037 ± 0.0097

a Mean ± SEM in 8 samples.

TABLE 4. EFFECT OF CHOLINESTERASE INHIBITORS ON CHOLINERASE ACTIVITY OF HUMAN MUSCLE MEMBRANE AND ITS FRACTIONS[a]

Inhibitor		Cholinesterase activity (%)		
		Membrane	Soluble fraction	Insoluble fraction
DFP	10^{-8} M	93.2 ± 0.71	98.4 ± 0.40	92.7 ± 0.95
	10^{-7} M	92.0 ± 1.14	95.4 ± 0.81	91.1 ± 1.89
	10^{-6} M	82.5 ± 1.50	88.0 ± 0.81	83.4 ± 2.09
BW284C51	10^{-6} M	10.7 ± 0.53	2.7 ± 0.29	10.0 ± 0.66
	10^{-5} M	9.5 ± 0.92	0	7.4 ± 0.73

a Mean ± SEM in 8 samples, expressed in % of the activity without inhibitor.

The soluble fraction of human muscle membrane had much greater ChE activity than the original membrane at all substrate concentrations studied (Fig. 2). Substrate inhibition of the enzyme was not observed up to 8 mM ACh concentration. Higher substrate concentrations were not recorded since the error in estimation became greater by the method used. When the data were

plotted by the method of Woolf (10), the Michaelis-Menten constant (Km) was 0.88 mM in the soluble fraction and 3.82 mM in the original membrane (Fig. 3).

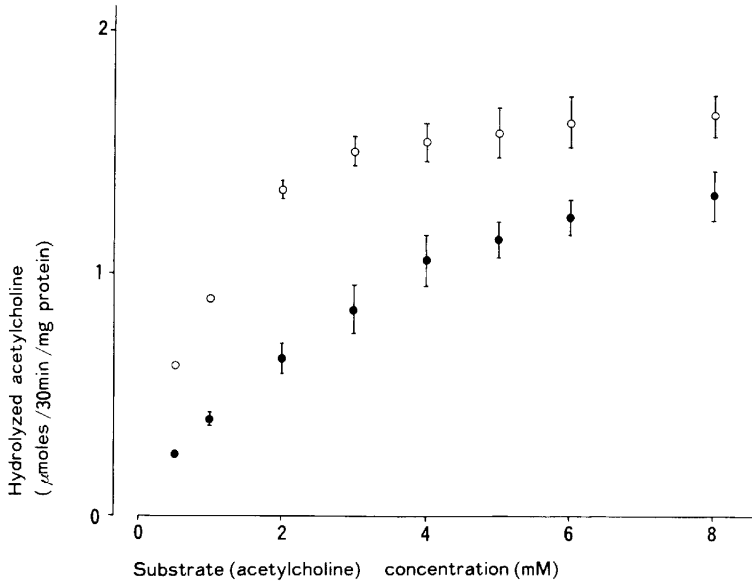

Fig. 2. Effect of substrate (acetyleholine) concentration on cholinesterase activity of the original membrane (●) and its soluble fraction (○) in human muscle. The circles indicate the mean, and the vertical lines the standard error of the mean, in 24 estimations.

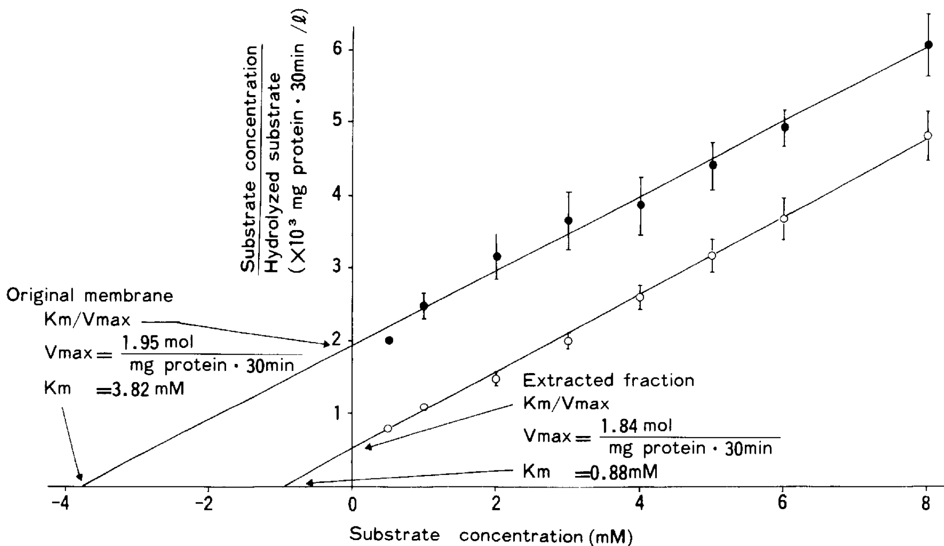

Fig. 3. The same data as in Fig. 2 plotted by the method of Woolf.

Endplate ChE isozymes. By Sephadex G-200 chromatography, the extract of human muscle homogenate was separated into five peaks of ChE and seven peaks of protein (Fig. 4). The specific activity and the molecular weight of ChE isozymes were estimated: isozyme 1- 2.50 (μmol ACh hydrolyzed/30 min/mg protein), 655,000, isozyme 2- 2.15, 535,000, isozyme 3- 2.00, 291,000, isozyme 4- 0.71, 117,000, isozyme 5- 0.70, 23,000, while the extract of human muscle membrane was separated into three peaks of ChE with three corresponding protein peaks. The specific activity and the molecular weight of ChE isozymes were estimated: isozyme 1- 3.48, 655,000, isozyme 2- 2.46, 291,000, isozyme 3- 1.847, 52,000. Recoveries of 90 to 95% were obtained for these enzymes.

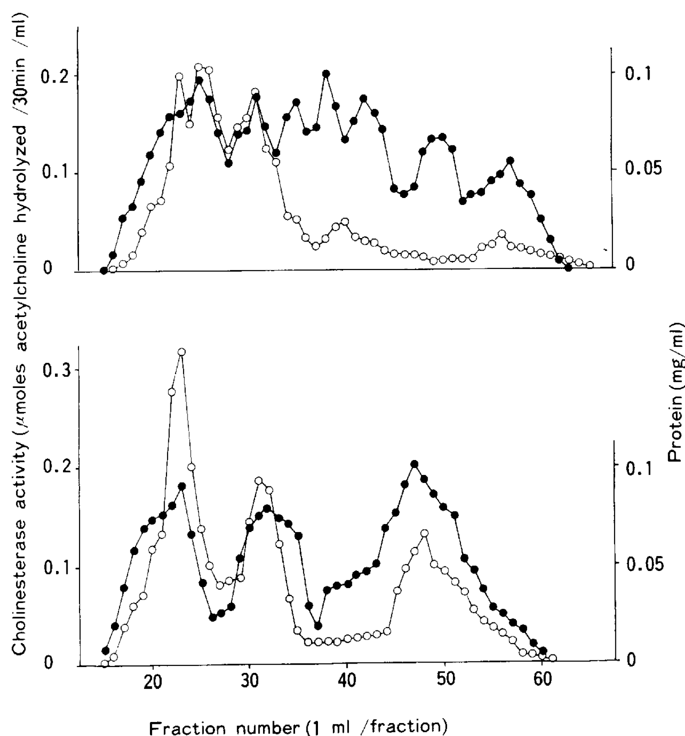

Fig. 4. Sephadex G-200 chromatography of extracted cholinesterase of human muscle homogenate (upper graph) and muscle membrane (lower graph). ○, cholinesterase activity; ●, protein concentration.

By acrylamide gel electrophoresis, the extract of human muscle homogenate was separated into three fractions of ChE, one weak band 1 and two strong bands 2 and 3, and eleven fractions of protein, while the extract of human muscle membrane was separated into two strong bands 2 and 3, and three fractions of protein (Fig. 5). The first peaked fraction of the membrane extract separated by Sephadex G-200 chromatography had a single strong band 2 of

ChE and a single corresponding band of protein. The recovery of the enzyme was 70 to 80% in these experiments.

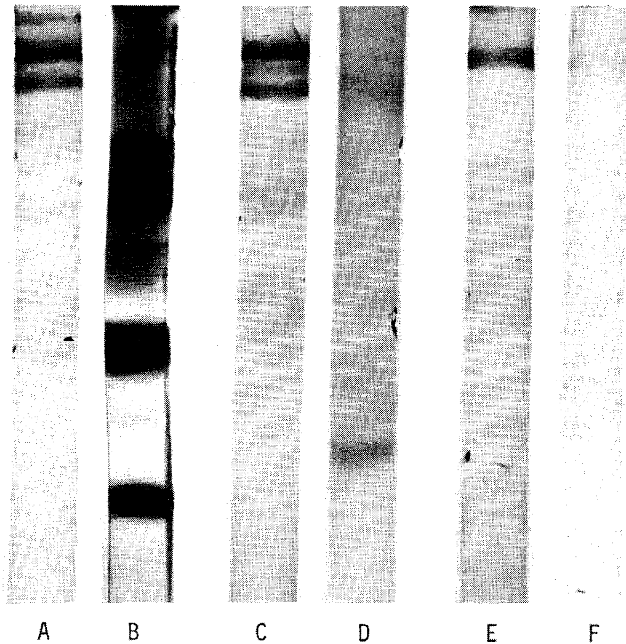

Fig. 5. Acrylamide gel electrophoresis of extracted cholinesterase of human muscle homogenate (A, B), muscle membrane (C, D), and first peak fraction of extracted muscle membrane by Sephadex G-200 chromatography (E, F). A, C, E, cholinesterase staining; B, D, F, protein staining.

DISCUSSION

Several studies have been reported on measurement of the ChE activity of the motor endplate. In indirect measurement, ChE activity was estimated from the difference of the activity between the muscle segments with motor endplates and the segments without motor endplates (2, 11). Direct estimation of endplate ChE was performed by chemical measurement of the activity of microdissected muscle segments which contained motor endplates (12, 13). However, this information has been limited by inaccuracy in isolating this enzyme activity from the muscle segment. The isolated muscle membrane is much more suitable for studies on the activity and properties of the motor endplate ChE.

The ChE of the muscle membrane seems to be localized entirely to the motor endplate since no other structure is stained for ChE activity, and since muscle membrane which does not contain motor endplates lacks ChE activity (3). Motor endplate ChE seems to be tightly bound to the membrane since storing the membrane does not change ChE activity as measured chemically and histochemically.

A number of different methods have been used to extract ChE from excitable tissues. Among these are butanol extraction (14), ultrasonication (15), detergent treatment (11, 16, 17, 18, 19, 20), bacterial protease treatment (17) and sucrose treatment (20, 21). Non-ionic detergent Triton X-100 has been most widely adopted for extraction study. With this detergent 86% of total ChE was extracted from guinea-pig brain synaptosome (16), 85% from rat brain homogenate (17), 97% from rat brain membrane (18), 47 to 94% from rat skeletal muscle homogenate (11, 20) and 80% from guinea-pig brain homogenate (22). These yield is extremely high compared with 37.1% solubility from human muscle membrane.

Many reports on the multiple molecular forms of ChE have appeared. The number of ChE isozymes described varies from two to five. Three isozymes were found in the skeletal muscles of human and rat by acrylamide gel electrophoresis of Triton X-100 extract (23). In electrophoretic purification of butanol extract of calf caudate ChE performed on Sephadex G-25, the enzyme was separated into three active components with corresponding protein peaks (14). With barbital buffer extract of rat brain three isozymes were separated by agar gel electrophoresis (24), while four forms were found with polyacrylamide in Triton X-100 extract of chick brain (25). Four isozymic forms from ChE extracted with EDTA from ox caudate nucleus were fractioned both by gel filtration on Sephadex G-200 and electrofocusing (26). In Triton X-100 extract of rat skeletal muscle four isozymes were characterized by Sephadex G-200 chromatography (27), and three isozymes with sedimentation coefficients of 4S, 10S, and 16S, which was specific for the motor endplate, were detected by sucrose gradient centrifugation (11, 19). The differences in number of ChE isozymes observed in these studies are probably related more to the techniques (separation methods, extraction media, purification shemes) than to the species employed. It is noteworthy that four isozymes were found in the brain of species as different as the chick (25), the ox (26) and the cockroach (28). A further reason for these differences is to be found in the tendency shown by the molecules of ChE to form aggregates of high molecular weight, which may not enter the gels (29, 30). Also, if interconversion of the isozymic forms can occur spontaneously, it would account for some of these separation patterns, by facilitating, under certain conditions, the transformation of two or three isozymic species into lower molecular weight forms, the ensuing combinations leading to a varying number of ChE isozymes (31, 32). The specific function of each isozyme of AChE is not yet clear. The existence of ChE isozymes showing differences in substrate and inhibitor specificities suggests the physiological significance of multiple isozymes of AChE.

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