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A COMPARISON OF SARCOLEMMMA AND SARCOPLASMIC
RETICULUM.

The City University of New York, Ph.D., 1974
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PROPERTIES OF MEMBRANE BOUND CHOLINESTERASES,
A COMPARISON OF SARCOLEMA AND SARCOPLASMIC RETICULUM

by

ALICE Y-C LIU

A dissertation submitted to the Graduate
Faculty in Biomedical Sciences in partial
fulfillment of the requirements for the
degree of Doctor of Philosophy, The City
University of New York.

This manuscript has been read and accepted for the Graduate
Faculty in Biomedical Sciences in satisfaction of the
dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

PROPERTIES OF MEMBRANE BOUND CHOLINESTERASES,
A COMPARISON OF SARCOLEMMMA AND SARCOPLASMIC RETICULUM

by

Alice Y-C Liu

Adviser: Professor T.W. Mittag

The properties of membrane bound cholinesterases of both the sarcolemma (SL) and sarcoplasmic reticulum (SR) of rat diaphragm muscle were studied and compared. By the use of inorganic cations and d-tubocurare as test agents, it was found that the properties of membrane bound AChE of SL and SR are quite distinct from one another, and are also different from the soluble enzyme prepared from E. electricus. The results also show that junctional AChE has an allosteric anionic site in addition to the anionic site in the catalytic center. Binding of ligands, such as Ca^{++} or d-tubocurare at this allosteric site is capable of altering the activity of the catalytic center. SL-AChE outside the endplate region does not show the same behaviour to Ca^{++} and d-TC.

Attempts to solubilize the various types of membrane bound AChEs resulted in loss of their characteristic properties. These results suggest that perhaps incorporation of AChE into different membrane matrices is responsible for the difference in properties of various membrane bound AChEs. Collagenase treatment, which

digests collagen of the muscle, may modify the physicochemical properties of the membrane causing changes in the properties of membrane bound AChE. If many of the properties of AChE depend intimately on the immediate environment around the enzyme, it is to be expected that solubilization of membrane bound AChE will alter enzyme properties.

The effect of the nerve in regulating muscle cell membrane AChE activity is shown in studies done on denervated animals. Both SL- and SR-AChE activities decreased by about 50-60% after denervation (10 days). The difference in SL- and SR-AChE half-lives was reflected by the differential effect of denervation in decreasing SR-AChE activity at a rate faster than SL-AChE. Experiments on denervation together with cycloheximide suggest that the neurotrophic effect in regulating muscle cell membrane AChE activity is probably via induction of de novo enzyme synthesis. Furthermore, there is a specific trophic effect of the nerve in confining d-TC activated SL-AChE in the endplate region of a normal innervated muscle and that denervation resulted in the appearance of this specific AChE outside the endplate region.

When SL and SR membrane bound AChEs were solubilized and subjected to polyacrylamide gel electrophoresis, it was found that both types of soluble SL and SR enzymes have 4 electrophoretically separable bands of AChE activity. The relative migration rate of these four bands were closely similar and were indistinguishable from the AChE staining pattern of purified eel AChE. The four bands of AChE activity in acrylamide gel electrophoresis obtained from three enzyme preparations of different origin indicates that they are size-isomers of one enzyme, rather than different isoenzymes. The data from

these experiments emphasize conclusions from the experiments on membrane fractions, ie., properties of AChE depend intimately on the immediate environment in the membrane.

In summary, the present study shows that various types of membrane bound AChE do possess different properties, not only in terms of their cytological distribution, catalytic properties, biological half-lives, but also with respect to the influence that the motor nerve exerts on them. AChE in the membrane appears to interact with other components in the membrane matrix, which confers characteristic properties to the AChE molecule.

ACKNOWLEDGEMENTS

The author wishes to express sincere appreciation to Dr. Thomas W. Mittag for his support, guidance, and discussions throughout the course of this research.

The author also expresses her thanks to members of her supervisory committee, Professors J.P. Green, M. Orlowski, S. Wilk, D. Capra and A. Smith for making valuable suggestions during the preparation of this manuscript.

The author was the recipient of a fellowship from Mount Sinai Graduate School, City University of New York.

Dedicated with love to my parents for their emotional support and guidance throughout the years.

And to Dr. Jack P. Green for his dedication to teaching and research in the neurosciences.

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LIST OF ABBREVIATIONS

SL : sarcolemma of rat diaphragm muscle

SR : sarcoplasmic reticulum of rat diaphragm muscle

AChE : acetylcholinesterase

BuChE : butyrylcholinesterase

ATP : adenosine 5'-triphosphate

d-TC : d-tubocurarine

CHI : cycloheximide

+EP : endplate containing part of the rat hemidiaphragm muscle

-EP : non-endplate containing part of the rat hemidiaphragm muscle

AChR : acetylcholine-receptor

ChE : cholinesterase

I. INTRODUCTION

A. STRUCTURE OF THE NEUROMUSCULAR JUNCTION

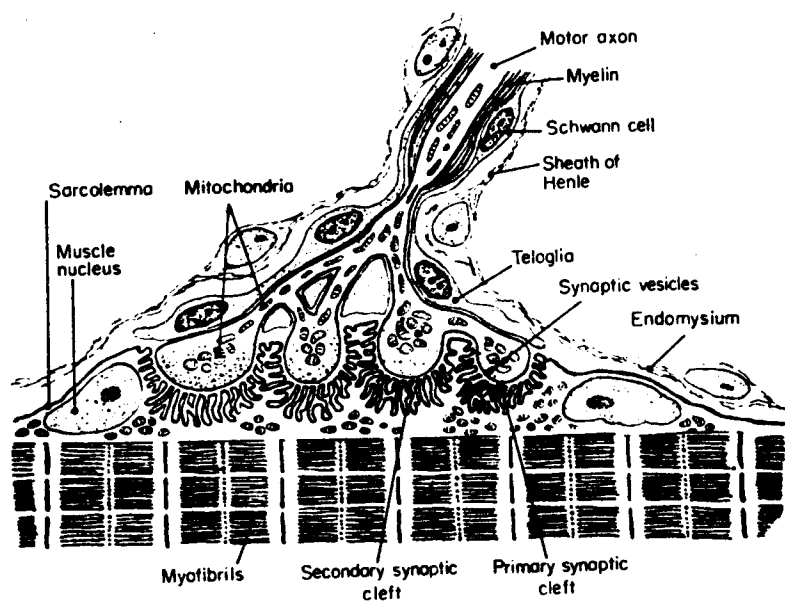
The modern era of knowledge of the morphology of the neuromuscular junction began with Couteaux' work. In 1947 he identified and described the subneural apparatus from pictures obtained by supravital staining with Janus Green B. He assumed that the pallisadelike structure, which outlined the motor arborization, represented the postsynaptic surface of the neuromuscular junction and was the site of the cholinesterase activity involved in neuromuscular transmission. This hypothesis was proved by the histochemical method for cholinesterase which clearly showed the selective localization of this enzyme in the subneural apparatus (Couteaux & Taxi, 1952; Coërs, 1953 a,b). The demonstration of a boundary between the axoplasm and the sarcoplasm was confirmed by electron microscopic observations (Palade, 1954; Robertson, 1956; Reger, 1958; Anderson-Cedergren, 1959; deHarven & Coërs, 1959; Birks et. al., 1960). This method together with histochemical techniques established beyond a doubt the discontinuity between nerve and muscle. In addition, electron microscopy showed that the striped appearance of the subneural apparatus was actually due to a profuse folding of the sarcoplasmic membrane.

A diagrammatic representation of a human neuromuscular junction is shown in Fig. 1. The motor axon loses its myelin sheath before branching to form the terminal arborization, and is instead covered by a thin cellular layer of terminal Schwann cells. The Schwann cells (teloglia) wrapping the terminal branches form a lid over the nerve terminals; their cytoplasm does not insert between the axoplasm and the sarcoplasm. At this level, the sarcoplasmic membrane facing the terminal arborization undergoes its post-synaptic

FIGURE .

Diagrammatic representation of a human neuromuscular junction.

(From International Review of Cytology 22, 239-267, 1967)



differentiation, the subneural apparatus.

The subneural apparatus is a pallisadelike differentiation of the sarcolemma. The subneural lamellae are inserted perpendicular to the hollow of the synaptic groove. Obviously, as a result of this arrangement the post-synaptic surface is made much larger than the presynaptic surface. Optical microscopic examination of histochemical preparations of the subneural apparatus shows that the cholinesterase activity is concentrated at the level of the primary and secondary synaptic clefts and not within the axonic endings (Couteaux & Taxi, 1952; Coërs, 1953b; Coërs & Woolf, 1959). This post-synaptic localization has been confirmed by denervation experiments. Both histochemical and biochemical studies have established that the cholinesterase activity of the neuromuscular junction persists after complete degeneration of the motor nerve (Coërs, 1953d; Schwarzacher, 1957; Savay & Csillik, 1956; Guth et.al., 1964). Results from electron microscopic studies also support the view that acetylcholinesterase of endplates (specific cholinesterase) is predominantly post-synaptic and located near the sarcoplasmic membrane (Lehrer & Ornstein, 1959).

These results fit in with the chemical transmission hypothesis. Acetylcholine, discharged at the nerve ending, diffuses into the synaptic clefts, reaches the receptors on the post-synaptic membrane, and modifies its ionic permeability, thus producing the endplate potential. Simultaneously, the acetylcholinesterase on the membrane hydrolyzes the acetylcholine and thereby terminates its action, in addition to diffusion. This restores the resting potential of the post-synaptic membrane and makes it responsive to a new discharge of acetylcholine.

B. PHYSIOLOGY AND PHARMACOLOGY OF NEUROMUSCULAR TRANSMISSION

1. Historical aspects

The brilliant researches of Otto Loewi, began in 1921, established the first real proof of the chemical mediation of nerve impulse by the peripheral release of specific chemical agents. He stimulated the vagus nerve of a perfused (donor) frog heart and allowed the perfusion fluid to come in contact with a second (recipient) frog heart used as a test object. A substance was liberated from the first organ that slowed the rate of the second. Loewi referred to this chemical substance as *vagusstoff* ("vagus-substance"); subsequently, Loewi and Navratil (1926) presented evidence for its identification as acetylcholine (ACh). Since then, numerous investigations have lead to the understanding that ACh, in addition to its role as the neuro-humoral transmitter of all post-ganglionic parasympathetic fibers and a few post-ganglionic sympathetic fibers, such as those to the sweat glands (Dale & Feldberg, 1934) and the sympathetic vasodilator fibers (Folkow & Uvnas, 1950; Uvnas, 1954) has been shown to have this transmitter function in three additional classes of nerves:

- a. Preganglionic fibers of both the sympathetic (Kibjakow, 1933; Feldberg & Gaddum, 1934) and the parasympathetic (Perry & Talesnik, 1953) systems.
- b. Motor nerves to skeletal muscle (Dale et. al., 1936; Brown et. al., 1936).
- c. Certain neurons within the central nervous system (Feldberg, 1945).

2. Steps involved in neuromuscular transmission

The arrival of an action potential (A-P) at the axonal terminals initiates a series of events (McLennan, 1963; Eccles, 1964; Nachmansohn, 1959):

a. Release of the transmitter:

It has been demonstrated that ACh is stored within synaptic vesicles. During the resting state, there is a continual slow release of isolated quanta of the transmitter, usually insufficient to cause initiation of a propagated impulse at the postjunctional site, but is recorded electrically as miniature endplate potentials (MEPP) in muscle. The A-P causes the synchronized release of several hundred quanta into the synaptic cleft.

b. Combination of the transmitter with postsynaptic receptors, and production of postsynaptic potential:

The transmitter diffuses across the synaptic or junctional cleft, a distance of 100 to 500 Å, and combines with specialized receptors on the postsynaptic membrane accompanied by the triggering of permeability changes to ions in the postsynaptic cell.

c. Initiation of postsynaptic activity.

d. Destruction of the transmitter:

When impulses can be transmitted across neuromuscular junctions in rapid succession (ranging from a few up to several hundred per second), it is obvious that there must be some effective means of terminating the action of ACh. This is indicated by the high turnover number of acetylcholinesterase (AChE). The turnover number of AChE gives a good index of the speed at which the active site reacts with ACh. It is defined as

the number of substrate (ACh) molecules reacting per minute per active site. AChE was calculated to have a turnover number of approximately 4×10^5 molecules of ACh per minute, one of the highest known for any enzyme.

Each of the above listed steps can either be blocked or enhanced by using a number of different pharmacological agents. Tetrodotoxin blocks A-P by preventing the selective increase in permeability to Na^+ associated with the rising phase of the spike of A-P. Hemicholinium blocks the uptake of choline by the nerve terminal thus preventing the synthesis of ACh. The process of release of ACh from the nerve terminal can be blocked by botulinum toxin, lack of Ca^{++} and excess of Mg^{++} . Agents such as d-tubocurarine cause transmission blockade by combining with the postsynaptic ACh-receptor (ACh-R). The depolarizing agents, succinylcholine and decamethonium, act at the same sites, but their blocking effect is preceded by activation. Anti-ChE agents (neostigmine, diisopropylfluorophosphate DFP) produce neuromuscular transmission blockade through the excessive accumulation of endogenous ACh. The predominant postsynaptic localization of AChE at the motor endplate has been suggested as indicating that its primary function here is to terminate transmitter action on the postsynaptic membrane.

C. MOLECULAR PHARMACOLOGY OF THE POST-SYNAPTIC MEMBRANE OF NEUROMUSCULAR JUNCTION

The molecular pharmacology of the postsynaptic membrane of the neuromuscular junction indicates that there are at least three related, but topographically distinct, sites located on the postsynaptic membrane:

- a/. Substrate site of cholinesterase
- b/. Cholinergic receptor
- c/. Ion-permeable sites.

These sites appear to be closely associated on the synaptic membrane at the level of the electron microscope. Precise knowledge of the number, type and location of these macromolecules at cholinergic junctions is obviously required for an understanding of the functional architecture of junctions and the mechanisms of transmission therein.

In the past, cholinesterase, by virtue of its essential feature, the enzymic reaction, has been more amenable to such analysis than other functional components there, eg. the receptor macromolecule. It is natural, therefore, that considerable attention has been paid to its localization at junctions. The majority of such studies have used methods based on the type introduced by Koelle and Friedenwald (1949) for cytochemical localization of ChE activity. Nevertheless, such studies have two major obstacles. Firstly, enzyme cytochemistry in general relies upon the deposition of the product of the enzyme reaction, by some secondary process such as reaction with a metal present. The problems of diffusion of the reaction product before deposition are well known (Danielli, 1953; Holt & Sullivan, 1958; Pearse, 1961) and may be particularly serious over the distance involved in electron microscope studies. Secondly, although the histochemical

methods are very valuable for qualitative surveys, the many poorly controlled or unknown variables that determine the amount of reaction product deposited, render the quantitation of enzyme by such methods virtually impossible. An alternative strategy, as employed by Ostrowski and Barnard (1961), is the application of agents which act irreversibly with the active center of the enzyme and quantitative autoradiography, which under rigorously controlled conditions can yield quantitative results of AChE distribution. These in situ cytochemistry studies (Zachs, 1961; Barrnett, 1959; Miledi, 1964; Lehrer, 1959; Brown, 1961; Barrnett, 1962; Couteaux, 1963; Ulbrecht, 1962) demonstrated that the enzyme AChE is concentrated over the junction folds. Very little is in axon or muscle regions. It is estimated that the number of AChE active sites per end-plate is of the order of 10^7 (Barnard & Rogers, 1964).

Although AChE necessarily contains in its catalytic center a stereospecific receptor or acceptor site for ACh in order to carry out the hydrolysis of this neurotransmitter, AChE has been clearly distinguished from the "ACh receptor" involved in mediation of the physiological response of acetylcholine. While the cytochemistry and biochemistry of AChE has been relatively well studied over the past fifty years, our knowledge of the ACh-receptor has been mostly fragmentary until the past few years.

Intracellular recording and micro-iontophoretic application of ACh have established that the ACh-sensitivity of normal muscle fibers is maximal in the region of neuromuscular contact and falls sharply therefrom (Miledi, 1960). Such experiments demonstrated the pattern of distribution of the ACh receptors but not their number. The irrefutable demonstration of the existence of ACh-R at the end-plate region

has recently been achieved by the use of certain snake toxins (Berg et.al., 1972; Miledi & Potter, 1971).

α -Bungarotoxin, a polypeptide of M.W. 8,000 obtained from the venom of the Formosan banded krait Bungarus multicinctus, and similar proteins from cobra and other elapid snake venoms have a curare-like action (Chang, 1960a,b) and bind with high specificity to acetylcholine receptors of striated muscle (Chang & Lee, 1963; Lee & Chang, 1966; Lee et.al., 1967; Miledi & Potter, 1971; Barnard et.al., 1971; Berg et.al., 1972; Fambrough & Hartzell, 1972), electroplax (Changeux et.al., 1970; Changeux et.al., 1971; Miledi, Molinoff & Potter, 1971) and brain (Bossman, 1972). In this way, neurotoxins such as α -bungarotoxin (α -BuTx) abolish neuromuscular transmission by blocking the action of ACh. Since the binding of α -BuTx to the AChR of muscle is not readily reversible in the systems examined, labeled α -BuTx can be used to assay AChR concentration as well as sites of synaptic connections (Lee et.al., 1967; Miledi & Potter, 1971; Barnard et.al., 1971; Berg et.al., 1972; Fambrough & Hartzell, 1972; Changeux et.al., 1970; Bossman, 1972).

Miledi and Potter using [^{125} I] labeled α -BuTx showed that most of the toxin is selectively bound at the endplate regions. There are about 4.7×10^7 α -BuTx binding sites per endplate of the rat hemidiaphragm (Barnard et.al., 1971). Furthermore, the amount of bound toxin per muscle increased after denervation, the time course and location of which coincides with the increase in extrajunctional ACh sensitivity.

The 1:1 ratio of AChE:AChR at the endplate region has recently been demonstrated by Barnard et. al. (1971). By using specific ligands and cellular autoradiography the estimated number of cholinergic

receptor sites is equal to the number of ChE active centers. The close spatial association of AChE and AChR on the postsynaptic membrane and the quantitative 1:1 relationship of the two macromolecules argue against any distribution pattern involving marked segregation of enzyme and receptor sites. The simplest model therefore involves a mosaic of receptor and ChE over the whole posynaptic membrane.

D. ACETYLCHOLINESTERASE

As early as 1914, Dale suggested that an enzyme is present in the blood which brought about the destruction of acetylcholine. Such an enzyme was shown to exist in nearly all animal tissues. Stedman et. al. (1932) prepared an enzyme from horse serum, called cholinesterase, which was considered to be a specific esterase for acetylcholine (ACh) and other choline esters. During the following years, a great number of papers appeared which dealt with the ACh-splitting activity of various animal tissues, vertebrates as well as invertebrates. These investigations indicated the existence of two types of choline ester-splitting enzymes. Acetylcholinesterase (acetylcholine acetyl-hydrolase, EC 3.1.1.7; AChE) occurs at the neuromuscular junctions, neurons, on the outer surface of mammalian erythrocytes and in certain other tissues and is usually membrane bound. In the cholinergic synapse, where AChE occurs in high concentration on the postsynaptic membrane, the enzyme is responsible for the hydrolysis of ACh released in the process of cholinergic transmission. Butyrylcholinesterase (BuChE, also known as pseudo-cholinesterase) is present in various types of glial or satellite cells of the central and peripheral nervous systems, in neurons, muscle, and in the plasma, liver and other organs; its' physiological function is unknown.

Although both types of enzymes can hydrolyze ACh and certain other aliphatic and aromatic esters and as a group are inhibited selectively by physostigmine, they can be distinguished by several criteria. AChE hydrolyzed ACh at a greater velocity than BuCh; it hydrolyzes acetyl- β -methylcholine (methacholine), but not benzoylcholine. BuChE, on the other hand exhibits a maximal velocity

of hydrolysis with butyrylcholine as substrate; it hydrolyzes benzoylcholine, but not methacholine, and it is more sensitive to inhibition by several organophosphorus agents, such as diisopropylfluorophosphate (DFP) and mipafox than is AChE (Augustinsson, 1963). Pharmacologically, the main reason for distinguishing between AChE and BuChE is that practically all the effects of the anti-cholinesterase agents are due to the inhibition of the AChE, with the consequent accumulation of endogenous ACh; inhibition of BuChE at most sites produces no apparent functional derangement (Holmstedt, 1959).

Leuzinger et. al. (1969) studied the properties of solubilized AChE from Electrophorus electricus. The molecular weight of this enzyme was determined by sedimentation equilibrium and found to be 260,000. The enzyme dissociates in the presence of guanidine and mercaptoethanol into four subunits each having one fourth of the molecular weight of the native enzyme. Examination of the C-terminal residues revealed that there are two types of polypeptide chains in the acetylcholinesterase. They suggested that acetylcholinesterase has a dimeric hybrid structure, with two α and two β chains. The α and β chains together make up an active site and there are two active sites per native enzyme molecule. A similar study on Triton X-100 solubilized human erythrocyte acetylcholinesterase (Shafai & Cortner, 1971) showed that the enzyme is a hybrid dimer composed of two unlike components (α and β) of equal size.

The molecular properties of the active center of AChE has long been an interesting problem. Nachmansohn and Wilson (1951) proposed a model of the active center of AChE. In this model there is an anionic site and esteratic site, which together constitute the active center of the enzyme molecule. Nevertheless, a number of recent

studies demonstrated the necessity of expanding such a basic model. Results showed that AChE from a variety of sources is an allosteric enzyme (Changeux, 1966; Wombacher & Wolf, 1971; Crone; 1973; Balleau & DiTullio, 1971; Mooser & Sugman, 1972; Kato et. al., 1972; Kato, 1972; Kitz et. al., 1969; Roufogalis & Quist, 1972; Rosenberry & Bernhard, 1971; Roufogalis & Wickson, 1973), which contains one or more peripheral anionic sites. Binding of ligands at these sites can modulate the activity of the catalytic center.

E. TROPHIC INFLUENCES OF NERVE ON MUSCLE

Broadly speaking, one can define the trophic influences of nerve on muscle as those functions of the nerve that affect or regulate the metabolism of the muscle cell. There are morphological, physiological and biochemical changes that occur when the muscle is denervated. Some of these changes are direct consequences of the loss of nerve innervation; others are perhaps secondary changes. Two aspects of neurotrophism will be discussed:

1. Neural regulation of ACh sensitivity

Studies using micro-iontophoretic application of ACh showed that in a normal innervated skeletal muscle ACh sensitivity is confined to the endplate region. After denervation, the neuromuscular junction retains its original degree of sensitivity, but the regions adjacent to it become gradually more sensitive to iontophoretic application of ACh (Axelsson, 1959). The chemosensitive area spreads centrifugally from the endplate region until by 1 or 2 weeks postoperatively the entire muscle fiber is as sensitive as the endplate region (which has retained its original sensitivity). Supersensitivity to ACh appears to result from increased synthesis of receptors (Fambrough, 1967). The increase in AChR after denervation can also be demonstrated by α -bungarotoxin binding, which binds specifically to AChR (Miledi & Potter, 1971).

The precise mechanism by which ACh sensitivity of the muscle fiber is controlled is unknown. There is evidence that spread of ACh sensitivity in denervated muscle results directly from the absence of ACh released from the nerve terminal, and there is also evidence that some other but as yet unspecified mechanism is involved.

2. Neural regulation of cholinesterase activity of muscle

Numerous attempts have been made to study the quantitative (Brzin, 1963; Lentz, 1971; Guth et.al., 1964; Stewart & Martin, 1966; Crone & Freeman, 1972) and qualitative (Wilson, 1971) changes of ChE after denervation. Certain discrepancies between the results obtained by different authors might be partially ascribed to the fact that different animals, different muscles, and different techniques have been employed. Histochemical methods for ChE seem to yield more uniform results concerning changes of this enzyme in the endplate of the denervated muscle. Most, if not all, workers agree that there is a remarkable decrease in ChE activity in the endplates after denervation (Beckett, 1957; Bergner, 1957; Coërs, 1953; Kupfer, 1951; Snell & McIntyre, 1955 & 1956). Biochemical measurements of ChE performed on whole homogenized or sliced denervated muscle do not offer a clear picture of the changes in ChE activity after denervation (Broods & Myers, 1952; Couteaux & Nachmansohn, 1950; Kovacs et.al., 1961). Considering the fact that muscle ChE consists of various pools of enzymes with different cytological distribution (eg. neuromuscular junction vs. myosin) and different specificity (eg. AChE vs. BuChE), it is not surprising that they would respond differently to the process of denervation and that such studies would yield rather unfruitful results.

The role of the nerve in regulating muscle cholinesterase activity was revealed quite clearly in a study in which young chickens were treated with DFP (an irreversible inhibitor of ChE), a treatment that abolished endplate ChE (as determined histochemically). Subsequently the ChE gradually reappeared at the endplate, but only in normally innervated muscle; reappearance of ChE did not occur if the muscle

had been denervated at the time of DFP treatment (Filogamo & Gabella, 1966). These observations indicate that the nerve is indispensable for the synthesis of new enzyme by the motor endplate. Experiments on the rat (Rose & Glow, 1967), using a quantitative rather than a histochemical technique for observing changes in ChE, further support this conclusion. After administration of DFP to rats the rate of presumed de novo synthesis of ChE (Denz, 1954; Koenig & Koelle, 1961) was determined. It was found that the rate of resynthesis was significantly and markedly reduced in denervated muscles, whereas normally innervated control muscles showed no such impairment of ChE resynthesis (Rose & Glow, 1967). Continuous infusion of ACh (5-10 ml/day of a 2% solution) was without effect on the resynthesis of ChE by the denervated muscles. This result, although negative, suggests that ACh availability may not be the principal factor determining ChE synthesis in muscle.

The effect of denervation on ChE activity changes is specific because denervation produces a more rapid decrease in ChE than in total protein, whereas tenotomy produces a loss of protein without a change in ChE activity of muscle (Guth et.al., 1964).

F. PROBLEMS ASSOCIATED WITH STUDIES ON ACETYLCHOLINESTERASES

Most of the information on AChE has been derived from studies on the solubilized enzyme from eel, Electrophorus electricus, and mammalian erythrocyte ghosts. In many instances the experiments on such solubilized enzyme preparations have been deficient in several important aspects concerning the physiological functions of this class of enzymes as indicated below:

- 1/. Acetylcholinesterases (AChE) are usually membrane bound and thus their reactivity with drugs is influenced by the local environment. The affinity constants (Webb & Johnson, 1969; Jenden, 1958), kinetics and even the mechanism of interaction (Changeux et. al., 1969; Changeux, 1969) of the reaction could be very different from that observed with the enzyme in solution.
- 2/. It is now known that all tissues contain a number of ChE types (most prominent being the true and pseudo varieties). All too often it is only the true enzyme in solution (particularly the solubilized eel AChE) which is used for determining ligand binding constants or reaction rates. For example, it was shown that pseudo-ChE is much more sensitive to inhibition by DFP than the true enzyme. The determination of inhibition rates for DFP with true ChE in solution might have little bearing on the membrane bound enzyme (Mittag et. al., 1971).
- 3/. Most of the enzymatic assays of AChE are generally done at the optimal substrate concentration, 10^{-3} M ACh, a concentration which the AChE molecule would never encounter under physiological conditions. Such high substrate concentrations could possibly affect the enzyme kinetics in the following manner:

a. Under low salt conditions, the contribution of ACh to ionic strength is substantial. It was shown that the conformation of solubilized AChE of Torpedo marmorata and Electrophorus electricus are remarkably susceptible to the ionic environment. Changeux (1966) studied AChE of Torpedo marmorata. He showed that depending on the ionic environment, both rapid and slow sedimenting species can be obtained from the same enzyme preparation. At low ionic strength, polydispersity is observed, which varied from about 10 S to more than 80 S, with a peak at about 35 S. By contrast, an aliquot of the same preparation of enzyme in the presence of 0.1 M MgCl_2 or 0.3 M KCl has a sedimentation constant of 14 S. It would not be surprising if the slow and rapid sedimenting species of AChE would display different enzyme kinetics. Acetylcholine at 10^{-3} M concentration could perhaps alter the physicochemical properties of the enzyme molecule, which in turn would influence the enzyme kinetics.

b. Silman and Karlin (1967) studied the pH-dependence of activity of membrane bound AChE of Electrophorus electricus. They observed an anomalous pH-dependence relative to soluble acetylcholinesterase when assayed in the absence of buffer. This is due to a drop in local pH (via accumulation of acetic acid produced from the enzyme reaction) in the vicinity of the membrane bound enzyme, consequent to the hydrolysis of substrates. This decrease in pH feeds back to inhibit the enzyme activity, thus producing the anomalous behavior. This local pH effect depends directly on the enzyme activity. Lowering the substrate concentration or using a poorer substrate or adding a competitive inhibitor would reduce such anomalous behavior.

4/. AChEs of *Electrophorus electricus* and mammalian erythrocytes are membrane bound enzymes in situ, and vigorous treatments are usually needed to solubilize these membrane bound enzymes. It is conceivable that important changes in the enzyme molecule could be induced during purification. In fact, recent findings of Massoulie and his colleague (Massoulie & Rieger, 1969, 1970, 1971) have shown that three species of AChE with sedimentation coefficients of 18, 14 and 8 S are obtained when fresh tissue from *Electrophorus electricus* is extracted with high ionic strength solutions. Each species exhibits sedimentation and other hydrodynamic properties which differ from that of the 11 S purified enzyme obtained from toluene-immersed tissue. Treatment of the three species extracted at high ionic strength with trypsin results in their conversion to an 11 S species. These findings have provided strong evidence that autolysis was essential for the purification of the 11 S enzyme. Other studies (Chan et. al., 1972) using brain AChE also offered a similar explanation (ie. proteolysis) to account for the different forms of AChE.

Studies on these solubilized and purified AChEs may, therefore, not be relevant to AChE in situ.

5/. Due to the presence of large amounts of AChE in the electric organ of *E. electricus* and *T. marmorata*, most of the earlier studies on AChE have been done on enzymes from these two sources. Certainly, there could be some very significant differences between an eel AChE and the AChE from the mammalian neuromuscular junction. One striking difference is illustrated by the effect of d-tubocurarine on AChE activity. Curare at 10^{-6} M concentration gives 70% inhibition of purified electroplax AChE which is of the

non-competitive type (Changeux, 1966). On the other hand, AChE of the intact rat diaphragm is activated by d-tubocurarine, and no inhibition is observed even at 10^{-5} M concentration (Mittag & Hehir, 1971 unpublished results).

Studies using whole tissue homogenates also have significant obstacles. For example, in skeletal muscle, AChE occurs in high concentration at the neuromuscular junction. There are, however, additional sites of AChE activity, such as the musculotendinous junction (Lubinska & Zelena, 1967), the sarcoplasmic reticulum (Ulbrecht & Kruckenberg, 1965), microsomes (Namba & Grob, 1967) and myosin (Varga et. al., 1955). It would be surprising indeed that these AChEs with different cytological distributions and functional significance represent a homogeneous class of enzymes. It is more likely that they represent specific isoenzymes which have unique distributions, catalytic properties and biological half-lives. Studies using tissue homogenates would therefore reveal little about the nature of the essential pool of junctional or synaptic enzyme under investigation, which may comprise only a small fraction of the total tissue activity. For example, endplate AChE comprises only about 20-30% of the total enzyme activity of muscle homogenates (Namba & Grob, 1968). Data obtained with a muscle homogenate might not be relevant to AChE at the endplate region at all.

Alternatively, experiments performed with intact tissues also have several inherent limitations, such as existence of diffusion barriers and enzyme compartmentation.

One way to circumvent these problems, at least in part, is to use membrane preparations with the enzymes still attached to the membrane.

II. SPECIFIC AIMS

The purpose of this study is to re-investigate some of the studies on muscle membrane AChE carried out by other investigators using both histochemical and biochemical approaches.

A radiometric assay method is used (Ehrenpreis, Mittag & Patrick, 1970; Mittag et. al., 1971). Because of the sensitivity of this method, which allows one to use a substrate concentration of 10^{-6} M, the problems associated with using high substrate concentrations are avoided. Furthermore, partially purified membrane preparations will be used; so as to circumvent the complications of having different pools of AChEs activity of different cytological distribution and possibly different properties as well. Such complications are inevitably the case when studying muscle homogenate AChE activity.

Histochemical methods are very valuable for quantitative surveys, and have yielded much valid information on the cytological distribution of AChE activity in the past. Nevertheless, because of the many poorly controlled or unknown variables that determine the amount of reaction product deposited, quantitation of enzyme activity by such methods is virtually impossible.

The results from the present study will be compared with such previous histochemical studies of others.

Specific aims of this study can be divided into three parts:

- A. To study the properties of muscle cell membrane bound AChEs. Two types of membranes were studied; one being the sarcolemma of rat diaphragm muscle together with the motor endplates that innervate them, the other is sarcoplasmic reticulum from the same muscle.

The presence of a high concentration of AChE at the endplate region of skeletal muscle has been proved by histochemical (Barnett &

Palade, 1959; Miledi, 1964; Barrnett, 1962), as well as biochemical methods (Gialobini & Holmstedt, 1960). AChE has also been shown to be present in the sarcoplasmic reticulum of skeletal muscle.

Ulbrecht and Kruckenbert (1965) using both histochemical and biochemical methods demonstrated the presence of AChE in the sarcoplasmic reticulum, which most probably is associated with the T apparatus.

The AChEs of these two types of membrane (ie. sarcolemma and sarcoplasmic reticulum) not only differ in terms of their cytological distribution, but they also have a different functional significance. The high concentration of AChE at the endplate region is required for rapid hydrolysis of the transmitter ACh. On the other hand, the functional significance of AChE on the SR membrane is not understood. It was suggested that AChE is important for propagation of electrical events of excitable membranes and that the AChE in the T-apparatus is involved in spreading excitation from the sarcolemma surface into the depth of the muscle cell.

The biochemical properties of AChE on these two types of membranes are studied in this thesis. Furthermore, for comparative purposes, AChE located on the outside of human erythrocytes as well as a solubilized preparation of eel AChE are investigated and compared. The biochemical results obtained in this study are compared with histochemical studies. The conclusions from both types of studies will be discussed and contrasted.

- B. The possibility that several isoenzymes comprise muscle AChE and that different forms of the enzyme are incorporated into different types of membrane will be examined.

Membrane bound AChEs have been solubilized and acrylamide gel electrophoretic patterns of these solubilized AChEs will be

compared with a solubilized eel enzyme.

- C. The neurotrophic regulation of both AChE and BuChE activity on both types of membrane (ie. sarcolemma and sarcoplasmic reticulum) will also be investigated by means of denervation.

Numerous studies have been done on denervation and cholinesterase activity changes. Both histochemical and biochemical studies have demonstrated the decrease in AChE activity in skeletal muscle after denervation. Nevertheless, there are certain limitations in both approaches. Histochemical studies usually only reveal high concentrations of enzyme activity, whereas biochemical assay of ChE activity of muscle homogenate or muscle slices does not provide a clear cut picture of the effect of denervation.

One of the aims of the present study will be to re-investigate the neurotrophic effect on the various types of membrane bound AChEs and BuChEs using purified membrane fractions. The possible existence of a specific trophic effect of the nerve on an AChE which is normally confined to the endplate region and which may be a specific iso-enzyme will also be investigated.

III. METHODS

A. MEMBRANE PREPARATION

All operations were carried out in polypropylene labware at 4°C unless otherwise stated.

Male Sprague-Dawley rats were killed by vertebral dislocation, diaphragm muscles were freshly excised and rinsed in isotonic saline. When required, the endplate region (+EP, middle part of the hemidiaphragm) was separated from the non-innervated portion (-EP) by cutting 25% of the muscle away from each edge at right angles to the muscle fibers. Diaphragms from 3-4 rats were first manually minced and then homogenized in 0.6 M KCl (20 ml) buffer in a polytron PT-10 homogenizer (Brinkman Instruments, Westbury, N.Y.) at setting 2 for 2 minutes. The muscle homogenate was centrifuged at 3,000 X g for 15 minutes. The pellet or supernatant was used for preparing sarcolemma (SL) or sarcoplasmic reticulum (SR) respectively.

1. Sarcolemma Membrane (SL). Sarcolemma was prepared by a simplification of the method described by Namba and Grob (1968), modified to obtain the SR from the same muscle. All centrifugations were 3,000 X g for 15 minutes at 4°C. The pellet obtained after homogenization described above was first washed in 20 ml of 0.6 M KCl and centrifuged (repeated 5 times) and then incubated in 20 ml of 0.6 M KCl at 37°C for 30 minutes. The pellet obtained by centrifuging the incubation mixture was washed with 20 ml aliquots of distilled water (repeated 6 times) and finally stored as a suspension in 2-3 ml of 0.32 M sucrose-5 mM histidine buffer, pH 7.6. The protein concentration was approximately 2 mg/ml with a yield of 0.7-0.8 mg protein per hemidiaphragm determined by the Lowry method (1951).

2. Sarcoplasmic Reticulum Membrane (SR). Supernatant from the homogenization step above was diluted 10 fold with distilled water and stored overnight (18 hours) at 4°C. The myosin was pelletized by centrifuging at 15,000 X g (15 minutes) and the pellet discarded. The final supernatant was centrifuged at 45,000 X g for 90 minutes and the resulting pellet stored as a suspension in 4-5 ml of 0.32 M sucrose-5 mM histidine buffer pH 7.6. The yield was 1.1 mg protein $\pm$ 0.09 per hemidiaphragm.

3. Erythrocyte Ghosts (RBC). Human erythrocyte ghosts were prepared according to the procedure of Steck et. al. (1970). Disodium ethylenediaminetetracetate (EDTA) was added to a concentration of 0.01 M to freshly drawn human blood. The red cells were washed three times with cold 0.15 M NaCl in 0.005 M sodium phosphate buffer, pH 8.0. Each milliliter of packed red cells was lysed by resuspension in 40 ml of cold 0.005 M phosphate buffer pH 8.0. The plasma membranes were centrifuged at 20,000 X g for 10 minutes to form pellets, which were washed twice more with the same buffer. The resulting pellets were white and were comprised of membrane ghosts.

B. CHOLINESTERASE ASSAYS

All assays were carried out at 37°C with a substrate concentration of 5 μ M unless otherwise stated.

Suspensions of the membrane preparations were added to a 37°C bath with a final volume of 10-20 ml buffer and a protein concentration of about 0.2 mg/ml. The final composition of the assay buffer was 0.1 mM Tris chloride, 1 mM histidine hydrochloride and 64 mM sucrose pH 7.6 which also contained CaCl₂, MgCl₂ or NaCl at appropriate concentration when required.

Cholinesterase activities were assayed by the radiometric

procedure described (Ehrenpreis, Mittag and Patrick, 1970; Mittag et. al. 1971). The specific substrated BL-acetyl-1-¹⁴C methylcholine iodide and butyryl-1-¹⁴C-choline iodide were used for assaying AChE and BuChE respectively. The enzyme assay was started by addition of substrates.

At predetermined time intervals (usually 10 minutes apart), a 1 ml aliquot was removed from the assay bath, the enzyme reaction stopped with 0.1 ml of 1 mg/ml neostigmine solution and added to a column (16 X 6 mm in diameter) of a cation-exchange resin (Bio Rad AG 50W, 200-400 mesh) equilibrated in 0.1 M sodium phosphate buffer (pH 7.2) and the effluent was collected in a scintillation vial. The column was then washed successively with 1 ml and 0.5 ml of phosphate buffer, and the washings were collected in the same vial. At the beginning and end of each assay a 1 ml aliquot was taken from the assay bath, the enzyme reaction stopped with 0.1 ml of the neostigmine solution and transferred directly to a scintillation vial. These two samples are taken as the total cpm/ml of bath. To each vial, 10 ml of Bray's solution was added and the sample vials, together with the vials for total radioactivity taken at the beginning and end of the assay were counted in a liquid scintillation counter (Beckman LS-250).

When radioactivity was plotted against time, a straight line was obtained, the slope of which represents membrane bound AChE activity together with spontaneous background hydrolysis which was found to be 0.03% per minute for acetyl-~~β~~-methylcholine under these conditions. Substitution of numerical values into the following equation gives a rate constant

$$k = \frac{R \times V_{to} \times 10^{-3}}{W (T - A_{to})}$$

for hydrolysis of substrates by the membrane preparation's AChE, the dimensions are liter min^{-1} mg^{-1} .

R = rate in cpm per ml of sample corrected for background
hydrolysis

V_{to} = bath volume in ml at zero time

W = mg. of membrane protein in the total assay bath

T = total cpm per ml of bath

A_{to} = cpm acetate per ml bath at zero time

k = rate constant liter/min/mg protein

Each assay comprised five points and was continued for an additional five points when additions were made to the incubation medium (eg. CaCl_2 or $3\text{ }\mu\text{M}$ d-tubocurarine).

The kinetics of substrate hydrolysis followed a pseudo first order rate equation at the substrate concentrations well below the K_m . The assay procedure gives a rate constant independent of variations in the substrate concentration below 0.1 mM.

Specific activities are calculated from the assay rate constant and are expressed in $\mu\text{moles per minute per gm Lowry protein for } [S] = 10^{-4}\text{M}$.

C. CHOLINESTERASE STAINING

A portion of the SL membrane suspension was recovered by suction on a 13 mm cellulose acetate filter (Millipore, $5.0\text{ }\mu\text{pore size}$) and fixed for 20 sec. in formalin vapor. Cholinesterase staining was done at room temperature by the method of Karnovsky and Roots (1964) in a medium containing 2 mM acetylthiocholine iodide, 65 mM sodium phosphate buffer pH 6.0, 5 mM sodium citrate, 3 mM cupric sulfate and 0.05 mM potassium ferricyanide. The filter disc was dehydrated in graded concentrations of ethanol, cleared in xylene and mounted in Canada

balsam.

D. ATPase ASSAY OF SR MEMBRANE

Mg⁺⁺-activated ATPase was assayed by the procedure of Martonosi and Feretos (1964 a,b) in a medium containing 100 mM KCl, 5 mM histidine buffer pH 7.4, 4 mM MgCl₂, 4 mM ATP and 0.05 mM CaCl₂. The total volume was 2 ml. The assay was started with the addition of known amounts of SR membrane preparation, after 30 minutes at 37°C the reaction was stopped with the addition of 0.5 ml of 10% trichloroacetic acid and 0.4 mM ammonium molybdate added. The phosphomolybdate complex was extracted into butyl acetate (4 ml) and read in a Beckman spectrophotometer at 340 mμ. ATPase activity was determined from a standard curve using KH₂PO₄ as standard.

E. UPTAKE OF Ca⁺⁺ BY SR MEMBRANE

The uptake of Ca⁺⁺ by SR membrane was measured by the procedure of Martonosi and Feretos (1964 a,b) in a medium containing 100 mM KCl, 5 mM histidine buffer pH 7.4, 5mM oxalate, 4 mM ATP, 4 mM MgCl₂ and 50 μM ⁴⁵CaCl₂. The total volume was 2 ml, the assay was started with the addition of known amounts of SR membrane preparation (final protein concentration in the assay medium was 0.04-0.2 mg/ml). After 30 minutes at 37°C the reaction was terminated by filtration through Millipore filters (type GS, with 0.45 to 0.3 μ average pore diameter). The filtration was complete within 2 seconds. The filters were dried and transferred to scintillation vials. 10 ml of Bray's solution was added to each vial and the samples were counted in a liquid scintillation counter (Beckman LS-250). The amount of bound Ca⁺⁺ was calculated from the difference between the radioactivity of the filtrates of SR-free incubation medium and of the SR-containing samples.

F. SURGICAL DENERVATION

Male Sprague-Dawley rats weighing 200-250 gm were used. Denervation of the left hemidiaphragm was carried out when the animal was under pentobarbital anesthesia (35 mg/Kg, intraperitoneally). The phrenic nerve was snared with a sterile hook inserted through a left thoracic incision, and was severed as close as possible to the diaphragm surface.

In some denervated rats, the effect of cycloheximide was tested. After full recovery from pentobarbital anesthesia a dose of 100 μ g of cycloheximide dissolved in isotonic saline was injected into the animal intraperitoneally. This was repeated daily for 10 consecutive days. This procedure, according to Kimura and Kimura (1973) is sufficient to suppress the increase in protein synthesis and ACh supersensitivity after denervation.

G. BINDING OF [^{125} I] α -BUNGAROTOXIN TO MUSCLE

The [^{125} I] α -bungarotoxin used in this series of experiments was kindly prepared by Dr. Mittag according to the methods of Berg et. al. (1972).

Rat diaphragms (either normal or denervated for 10 days) were removed with ribs attached and incubated with [^{125} I] α -bungarotoxin at room temperature in Krebs' solution at pH 7.3, containing 140 mM NaCl, 4 mM KCl, 2 mM CaCl_2 , 1 mM MgSO_4 , 1 mM KH_2PO_4 and 12 mM NaHCO_3 . Binding was carried out with 1.6 μ g α -bungarotoxin for two hours with constant shaking. In all cases, [^{125} I] α -bungarotoxin was used within 20 days of preparation. After incubation with labeled toxin, the muscles were washed overnight in several changes of a solution containing 0.15 M NaCl-0.4 mM EGTA-0.02 M Tris.HCl (pH 7.4). The hemidiaphragms were then trimmed of the rib attachments, excess fat, weighed and were manually cut into pieces of about 1 mm X 1 mm in size. The minced tissue from a hemidiaphragm

was then transferred to a scintillation vial. 1.5 ml of Protosol (NEN) was added. Alkaline digestion of the tissue was enhanced by overnight incubation at 54°C with constant shaking. After complete digestion of the hemidiaphragm pieces (which resulted in a clear yellowish viscous fluid) 10 ml of Aquasol (NEN) was added, the pH of the sample was then neutralized with glacial acetic acid. The amount of radioactivity was estimated in a Beckman LS-250 liquid scintillation counter. Internal standards were added to the samples to correct for quenching.

H. BINDING OF [^{125} I] α -BUNGAROTOXIN TO SL MEMBRANE PREPARATION

SL membrane preparations were prepared as described previously (p. 24). The final pellet was then resuspended in Krebs' solution and 1.6 μ g/ml of α -BuTx was added. Binding was done according to the procedure described for intact diaphragms, washing was done by centrifuging the membrane suspensions at 15,000 X g for 15 minutes, decanting off the supernatant and resuspending the pellet in fresh media. Samples were digested and counted as described above.

I. ACRYLAMIDE DISC GEL ELECTROPHORESIS

In order to obtain high resolution of the AChE isoenzymes, some modifications were applied to a standard disc electrophoretic technique (Davis, 1964). Glass tubes with an internal diameter of 5 mm were used. The separation gel with Tris buffer (pH 7.5), was cast in two parts, a lower gel with 8% acrylamide and an upper gel with 6%. The acrylamide concentration of the spacer and sample gel was 2.5%.

1 ml of 0.001% bromphenol blue in water was stirred into the upper buffer. Electrophoresis was conducted in the cold (5-15°C), and a current of 2.5 ma per gel tube was maintained until the tracking dye (bromophenol blue) had moved approximately 27 mm into the separation gel (about 1.5 hr).

Samples were concentrated by using a B-15 Minicon (Amicon Incorporation) macrosolute concentrator (15,000 MW cut off). Approximately 0.1-0.2 ml of sample solution with 150-200 μ g of protein was loaded onto each cylinder gel. Individual samples were run in pairs; one for protein staining and the other for AChE staining. Following electrophoresis, individual acrylamide gels were placed in test tubes containing one of the following reaction mixtures:

1. Protein Staining (Coomassie blue stain)

- a. preincubation for 30 minutes at room temperature in 10-40 fold of 12.5% TCA.
- b. incubation for 1-2 hours in a freshly prepared mixture of 1 part of 1% Coomassie blue (aqueous) solution plus 19 parts of 12.5% TCA.
- c. destaining for 1-8 hours in 12.5% TCA.
- d. storage in 10% TCA solution.

2. Acetylcholinesterase Staining (modified Koelle's thiocholine method)

- a. preincubation for one-half hour at 0°C in a freshly prepared solution containing $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, glycine, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ and maleate-NaOH buffer (pH 6.1) in the proportions used in Koelle's (1951) reaction mixture.
- b. incubation for 1-20 hours at room temperature (about 25°C) in a freshly prepared reaction mixture containing the above ingredients plus acetyl-thiocholine iodide, all in the proportions used in Koelle's mixture (1951).
- c. Following incubation, gels were washed in cold water and were examined against a black background. The staining appearing as white bands were recorded manually.

IV. RESULTS

A. STUDIES ON AChE AND BuChE ACTIVITIES OF SL, SR AND RBC MEMBRANE PREPARATIONS

These experiments were done to establish the integrity of the membrane preparations and to compare the AChE specific activities, the proportion of AChE and BuChE activity, and distribution of enzymes in these membrane preparations.

The SL membrane obtained by the method described is a fluffy white membranous suspension which aggregates on standing. The material can be trapped on a cellulose acetate filter and stained for cholinesterase by the method of Karnovsky and Roots (1964). Many unbroken endplates are readily identified, together with endplate fragments (Fig. 2).

The preparation of SR from the same muscle used for the SL preparation requires the use of 0.6 M KCl buffer. In view of the possibility that a modification in methods of preparation could result in a change in the properties of this subcellular fraction, some properties of SR obtained by this method are compared to SR prepared by a more conventional method (Martonosi and Feretos, 1964 a,b). The results in Table 1 show that there are no significant differences ($p > 0.05$) between the two preparations with respect to ATPase, AChE and BuChE activities. However, there is a significant difference with respect to Ca^{++} -uptake ($p < 0.01$). The reason for this discrepancy is not known.

Specific activities of both AChE and BuChE of sarcolemma, sarcoplasmic reticulum and erythrocyte ghosts were studied. Results are given in Table 2. Specific activity of AChE of SR is about four times that of the entire muscle SL membrane while they contain

FIGURE 2

1200 X magnification of a motor endplate of SL membrane preparation mounted on a cellulose acetate filter and stained for cholinesterase by the method of Karnovsky and Roots.

Whole endplate (large arrow) and fragments (small arrow) are present.



TABLE 1

Mean activities for ATP-ase, Ca^{++} uptake, AChE and BuChE at 30°C in sarcoplasmic reticulum prepared as under METHODS (A) and by the method of Martonosi and Feretos (B).

Activity	SR preparation A (n=2)	SR preparation B (n=2)	
ATP-ase*	11.5 $\pm$ 1.6	16.5 $\pm$ 2.5	(p>0.05)
Ca^{++} uptake*	6.25 $\pm$ 3.2	11.7 $\pm$ 2.6	(p<0.01)
AChE	2.4 $\pm$ 0.2	1.9 $\pm$ 0.9	(p>0.05)
BuChE	3.9 $\pm$ 0.4	3.0 $\pm$ 0.16	(p>0.05)

*Units of activity: ATP-ase - μ moles Pi/mg protein/30 minutes

Ca^{++} uptake - μ moles/g protein in 30 minutes

TABLE 2

Specific activities of AChE and BuChE in sarcolemma (SL), sarco-
plasmic reticulum (SR), and human erythrocyte (RBC) membrane
preparations. Results are expressed as means $\pm$ standard errors in
units of μ moles/min/g protein for acetyl- β -methylcholine 10^{-4} M
at 37 ° C.

Membrane	AChE	BuChE
SL	1.19 $\pm$ 0.3 (n=5)	3.5 $\pm$ 1.85 (n=3)
SR	4.6 $\pm$ 0.2 (n=5)	5.8 $\pm$ 0.52 (n=4)
RBC	180.0 $\pm$ 11.5 (n=11)	

comparable BuChE activities. However, both SL and SR have much lower AChE activity than the red cell ghosts.

Table 3 compares specific activities of SL membrane when the diaphragms were separated into an inner part containing most of the endplates (+EP) and an outer portion of non-endplate containing region. Sarcolemma membrane preparations were also prepared. The ratio of specific activities for the two regions shows the preferential location of AChE in the +EP region, presumably associated with endplates. The result also indicates that the entire muscle membrane contains a general background of AChE activity. BuChE activity is present in both the +EP as well as -EP region, however, the distribution of membrane-bound BuChE activity is opposite to that of AChE, ie, there is a preferential localization of BuChE activity outside the endplate-containing region.

From the data, one can calculate the approximate amount of SL AChE activity associated with endplates. It was estimated that 25% of the entire SL-AChE activity is endplate specific. The remaining 75% of AChE activity is probably not associated with endplates, rather, it is distributed along the entire muscle cell surface. This is termed the background AChE activity and is assumed to have a uniform distribution over the SL membrane. No difference in specific activity or enzyme type was found in SR membrane fractions prepared from +EP and -EP regions of the diaphragm muscle.

TABLE 3

Specific activities at 30°C of AChE and BuChE in sarcolemma from endplate containing (+EP) and non-innervated (-EP) regions of rat hemidiaphragms (n=5)

Protein yield mg/hemidiaphragm		AChE	BuChE	Ratio AChE:BuChE
+EP	0.31 $\pm$ 0.03	1.028 $\pm$ 0.06	0.80 $\pm$ 0.10	1.44
-EP	0.55 $\pm$ 0.05	0.58 $\pm$ 0.04	1.37 $\pm$ 0.13	0.42
Ratio				
+EP/-EP	0.71	1.84	0.61	-

B. SOLUBILIZATION OF MEMBRANE BOUND ACETYLCHOLINESTERASES

This series of experiments was designed to investigate the stability and mode of attachment of AChE to the various membrane types. Several solubilizing agents were used and compared with respect to their effectiveness in selectively solubilizing AChE from membranes.

The stability of attachment of AChE to the various types of membranes was investigated by storage of the membrane suspensions at 4°C for 18 hours, followed by incubation at 37°C for 30 minutes; except for erythrocyte ghosts which only went through the storage period. The results in Table 4 showed that under these conditions the membrane bound AChE on both the SR and RBC ghost preparation were gradually released into the incubation medium, whereas none was released from the SL, i.e., AChE activity of the SL preparation seems to be tightly bound to the membrane. These results suggested that the mode of attachment of AChE to the various membrane types might be different and therefore, different solubilizing agents would be required for the selective solubilization of the various types of membrane bound AChEs. The effect of various agents in solubilizing the different types of membrane bound AChEs were then investigated.

Collagenase (Hall & Kelly, 1971) EDTA (Heller & Hanahan, 1972; Chan et. al., 1972) and 0.5% Triton X-100 either alone (Ho & Ellman, 1969; Karazmar & Bernsohn, 1972) or in combination with 1 M NaCl (Hall, 1973) are known solubilizing agents for various types of membrane bound AChEs and were investigated on SL and SR membrane fractions.

All membrane preparations were freshly prepared. SL and SR pellets were washed and resedimented; ensuring that most if not all of the starting AChE activity is membrane bound. The membrane pellets were then resuspended in media containing the various solubilizing agents

TABLE 4

Stability of attachment of AChEs to the various types of membrane preparations.

	AChE specific activity		Distribution of AChE activity %	
	solubilized	membrane bound	solubilized	membrane bound
SR (n=5)	*7.88 $\pm$ 1.03	3.00 $\pm$ 3.5	52.3 $\pm$ 3.5	47.68 $\pm$ 3.5
SL (n=2)	**0.0	1.25 $\pm$ 0.15	0.0	100.0
RBC	130.0	210.0	13	87

* not sedimented at 100,000 X g (60 minutes)

** not sedimented at 15,000 X g (20 minutes)

Membrane preparations were prepared and stored in cold for 18 hours followed by a 30 minute incubation at 37°C, except for the RBC membrane which only went through the cold storage period. The membrane suspensions were re-pelletized, AChE activity in soluble (supernatant) and membrane bound form were determined.

AChE specific activities are expressed in units of μ moles/min/g protein for [S] = 10^{-4} M at 37°C.

and were incubated at 37°C for 30 minutes. The incubated SL and SR membrane suspensions were pelletized at 15,000 and 100,000 X g respectively. AChE activity of both the membrane bound and solubilized (supernatant) forms were determined.

Table 5 shows that collagenase (30 units/ml of incubation medium) was most effective in solubilizing SL membrane bound AChE, releasing 70% of the total AChE activity with a three fold increase in specific activity. Triton X-100 (0.5%) alone was ineffective while in 1 M NaCl solubilization was almost as effective as with collagenase (Hall, 1973). In the case of SR, both collagenase and EDTA treatment increased release of enzyme somewhat above control. However, only EDTA brings about a 6 fold increase in specific activity of the solubilized enzyme above the starting SR membrane preparation (Table 6).

The effect of consecutive EDTA extractions of SR membrane bound AChE was also investigated. Table 7 showed that successive EDTA treatments extract progressively less AChE with decreasing specific activities. About one-third of the SR membrane bound AChE activity was not extractable by EDTA.

These results suggest that the mode of association of AChE with the various membrane matrixes are quite different. AChE activity of SL membrane preparations appear to be tightly bound to the membrane, since storing the membrane fraction did not change SL-AChE activity. On the other hand, AChE on SR membrane gradually became solubilized during the storage and incubation period. The difference in mode of association of AChE with the different membrane types is also illustrated by the result on the effect of different solubilizing agents. Different solubilizing agents are required for the selective release of the various types of membrane bound AChEs.

TABLE 5

Degree of solubilization* of SL membrane-bound AChE after various treatments for 30 minutes at 37°C (n=2)

Treatment	AChE sp. activity	Distribution of AChE activity %
CONTROL		
soluble	0	0
membrane-bound	1.25	100
COLLAGENASE (30 U/ml)		
soluble	3.33	70
membrane-bound	0.56	30
EDTA (2.5 mM)		
soluble	0	0
membrane-bound	1.40	100
TRITON X-100 (0.5%)		
soluble	0	0
membrane-bound	1.1	100
TRITON X-100 (0.5%) IN 1 M NaCl		
soluble	2.06	67
membrane-bound	0.28	32

*not sedimented at 15,000 X g.

TABLE 6

Degree of solubilization* of SR membrane-bound AChE after various treatments for 30 minutes at 37°C (n=5)

Treatment	AChE sp. activity	Distribution of AChE activity, %
CONTROL		
soluble	7.88	52
membrane-bound	3.02	48
COLLAGENASE (30 U/ml)		
soluble	3.50	68
membrane	1.89	32
EDTA (2.5 mM)		
soluble	18.0	64
membrane-bound	2.48	36
TRITON X-100 (0.5%)		
soluble	0.85	18
membrane	2.70	82

*not sedimented at 100,000 X g.

TABLE 7

Consecutive extractions of SR-AChE by 2.5 mM EDTA

	Exp't I		Exp't II	
	Units	Sp. activity	Units	Sp. activity
S ₁	11.9	17	11.5	18.4
S ₂	3.5	5.2	3.5	6.5
S ₃	-	-	0.72	1.5
P	8.4	3.0	8.84	3.2
	Recovery 102%		Recovery 106 %	

S₁ - S₃ are sequential EDTA extractions of SR membrane preparation,
ie. 100,000 X g supernatant.

P - 100,000 X g pellet.

C. STUDIES ON THE INFLUENCE OF INORGANIC CATIONS ON MEMBRANE BOUND AChE ACTIVITY

It is well known that AChE activity is profoundly influenced by the presence of inorganic cations (Cohen & Oosterbaan, 1963). In fact, AChE assays are traditionally performed in the presence of added inorganic cations, particularly Na^+ and Mg^{++} . This series of experiments was done to re-investigate and compare the effect of Ca^{++} , Mg^{++} and Na^+ on membrane bound AChEs and on a partially purified soluble enzyme preparation from Electrophorus electricus.

Results in Table 8 showed that under the defined experimental conditions (ie., 37°C , $[\text{S}] = 5 \times 10^{-6}\text{M}$) all three cations decrease AChE activity of the membrane preparations in a concentration dependent manner. By contrast the soluble enzyme from Electrophorus was activated by all these ions in agreement with numerous previous observations (Meer, 1953; Myers, 1952; Nachmansohn, 1940). Since divalent cations were more effective in altering enzyme activity, the effect of Mg^{++} on AChE activity was studied as a function of the substrate concentration.

Typical double-reciprocal plots on the SL membrane-bound AChE are shown in Fig. 3 for substrate concentrations ranging from $5 \times 10^{-4}\text{M}$ to 2 mM. The plot is a straight line in the absence of added MgCl_2 , which extrapolates to $K_m = 2.6 \text{ mM} \pm 0.38$. In the presence of 10 mM MgCl_2 the plot is a curve which crosses the control line at $6.9 \times 10^{-4}\text{M} \pm 0.27$. Therefore at substrate concentrations below this value Mg^{++} inhibits AChE activity whereas above this activation of SL-AChE activity by the cation is observed.

Results of similar experiments on SR membranes are shown in Fig. 4. The apparent $K_m = 3.9 \text{ mM} \pm 0.81$ and the plot in the presence of 10 mM

TABLE 8

Effect of CaCl_2 , MgCl_2 and NaCl on AChE bound to SR and SL membrane and on soluble, partially purified AChE from *Electrophorus*. Enzyme activities are relative to control = 100 and were assayed with $5 \times 10^{-6}\text{M}$ acetyl- β -methylcholine.

Added ion, mM		SR (n=4)	SL (n=4)	Purified eel AChE
Ca^{++}	0	100.0	100.0	100
	0.2	84.2 $\pm$ 6.2	72.1 $\pm$ 8.5	150
	0.8	71.6 $\pm$ 8.6	52.7 $\pm$ 10.0	158
	2.0	59.5 $\pm$ 8.3	47.9 $\pm$ 6.4	160
Mg^{++}	0	100.0	100.0	100
	0.2	82.5 $\pm$ 2.0	79.2 $\pm$ 13.5	195
	0.8	74.5 $\pm$ 5.8	65.8 $\pm$ 4.1	262
	2.0	62.6 $\pm$ 10.5	56.2 $\pm$ 7.1	283
Na^+	0	100.0	100.0	100
	0.6	93 $\pm$ 4.3	89.5 $\pm$ 4.5	81
	6.0	74 $\pm$ 4.9	95.5 $\pm$ 5.5	83
	300.0	20 $\pm$ 1.0	45.5 $\pm$ 2.5	329

FIGURE 3

Plot of reciprocals of hydrolysis rate (V , micromoles per minute per gram of protein) against acetyl- β -methylcholine concentration (S , millimolar) for AChE bound to SL with ($\circ$) and without ($\bullet$) 10 mM $MgCl_2$ added to the buffer.

Points are the means $\pm$ standard errors (vertical bars) of three determinations.

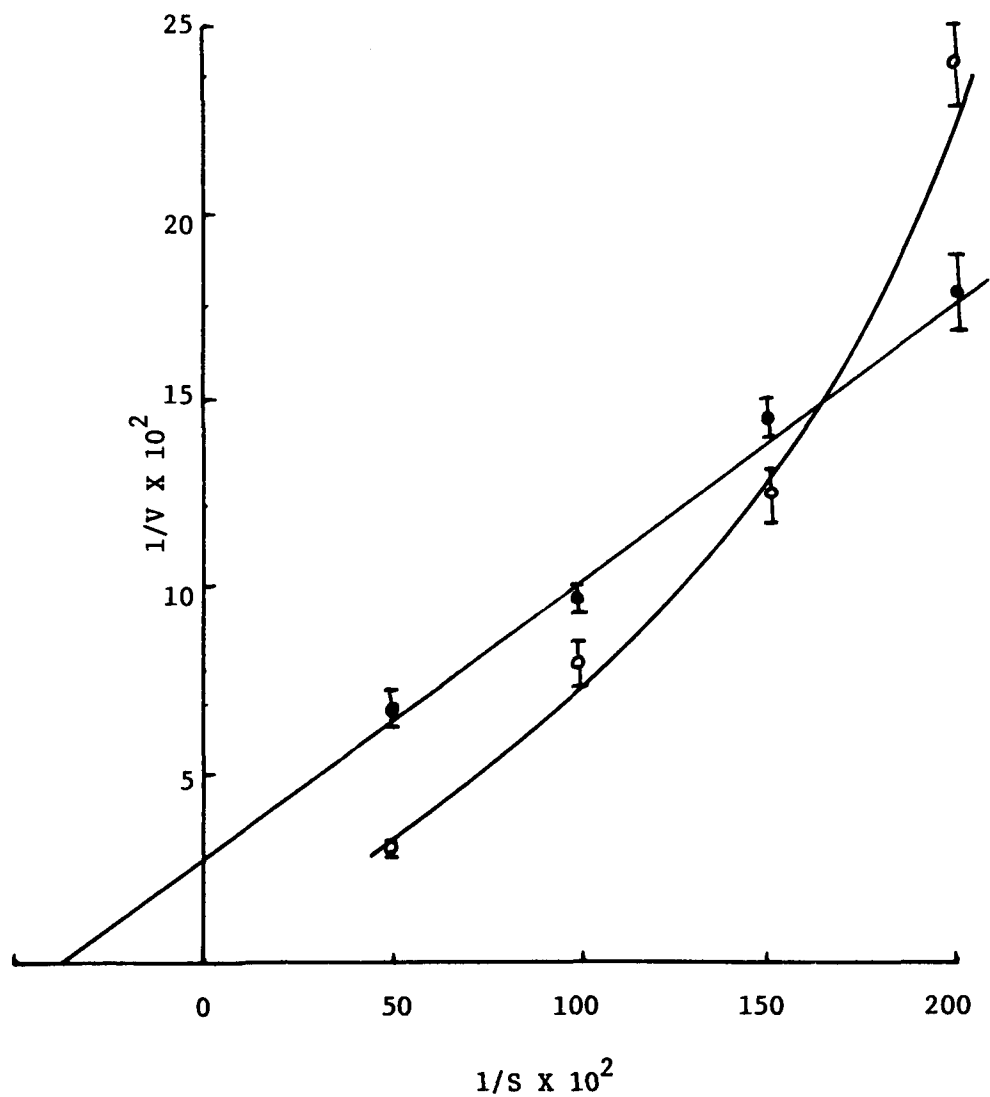
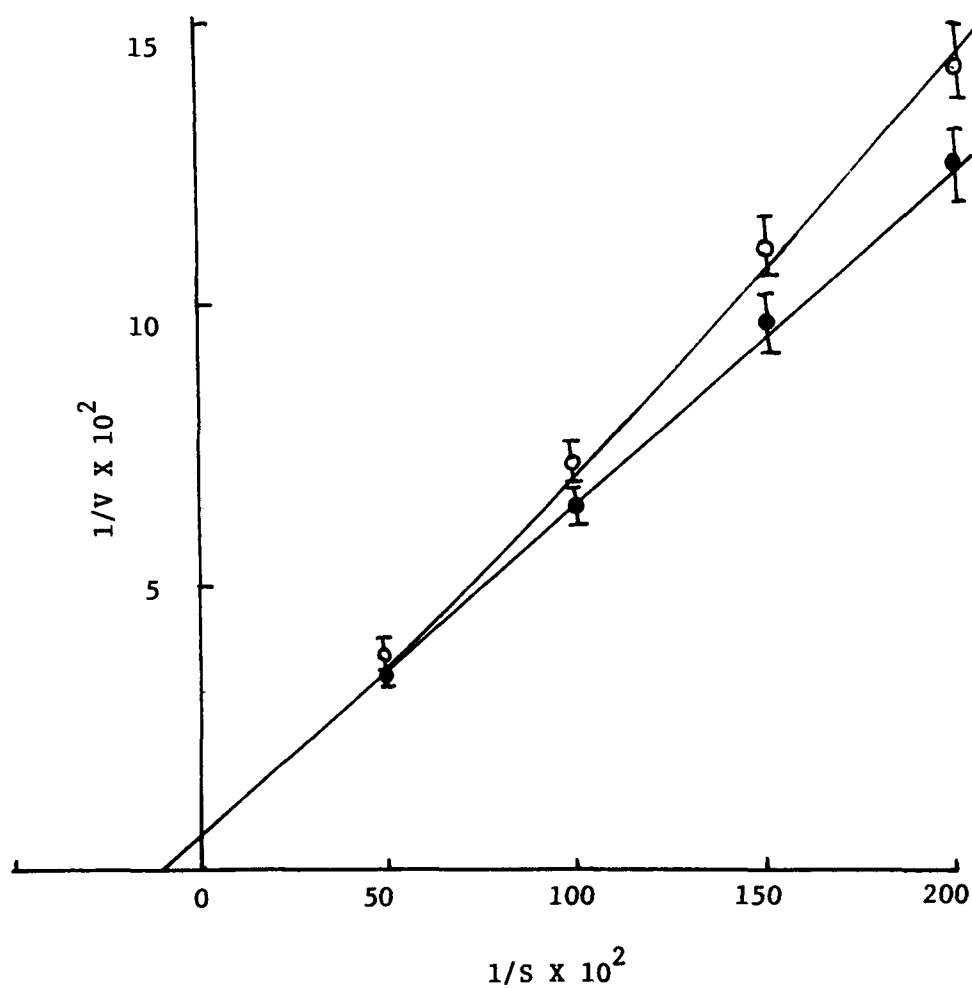


FIGURE 4

Plot of reciprocals of hydrolysis rate (V , micromoles per minute per gram of protein) against acetyl- β -methylcholine concentration (S , millimolar) for AChE bound to SR with ($\circ$) and without ($\bullet$) 10 mM MgCl_2 added to the buffer.

Points are the means $\pm$ standard errors (vertical bars) of three determinations.



Mg^{++} is also a curve which diverges from the control line at substrate concentrations below $5.6 \times 10^{-4} M$, and no activation is observed when the substrate concentration is above this value.

Therefore, by using cations as test agents, a clear difference between SL and SR membrane-bound AChE and solubilized eel AChE is revealed. While the membrane-bound enzyme activity is inhibited by the presence of divalent cations, the solubilized eel enzyme is activated. The activating effect of cations on the solubilized eel enzyme could possibly be due to disaggregating effect of salt and thereby an apparent increase in the number of enzyme active sites, resulting in an increase in activity.

A difference between the two types of membrane bound AChEs (ie., SL vs SR) is also observed. The effect of Mg^{++} on both types of membrane bound AChE varies as a function of substrate concentration. Depending on the substrate concentration, both activating and inhibitory effect of cations on SL-AChE can be obtained. On the other hand, SR-AChE activity is only inhibited by Mg^{++} at substrate concentrations below $5.6 \times 10^{-4} \pm 2.2$, with no activation at the highest substrate concentration studied (2 mM).

TABLE 9

K_m and crossover values of SL and SR membrane bound AChEs.

	K_m	Crossover value
SL (n=3)	$2.59 \pm 0.375 \text{ mM}$	$6.87 \times 10^{-4} \text{ M} \pm 0.27$
SR (n=3)	$3.88 \pm 0.809 \text{ mM}$	$5.58 \times 10^{-4} \text{ M} \pm 2.2$

K_m values were obtained by extrapolating the control slope on the double reciprocal plot to the Y axis.

The crossover point being the substrate concentration at which the slope of control and 10 mM MgCl_2 meet in a double reciprocal plot.

D. EFFECTS OF A NON-DEPOLARIZING NEUROMUSCULAR BLOCKING AGENT (d-Tubocurare) ON MEMBRANE-BOUND AND SOLUBILIZED AChE ACTIVITY

In earlier experiments by Hehir and Mittag (1971) by using the surface AChE activity of whole diaphragm muscles and the twitch response to phrenic nerve stimulation, the influence of d-tubocurare (d-TC) on the AChE and cholinergic receptor were studied simultaneously (Fig. 5). d-TC gave a dose-dependent activation of AChE, reaching a maximum of some 50% activation at a dose giving complete neuromuscular blockade, and it was postulated that there is an allosteric interaction between macromolecules bearing the substrate site of AChE and the binding site for the bis-quaternary neuromuscular drug, presumably AChR.

Because of inherent difficulties (ie., diffusion barriers, multiple isoenzymes etc.) involved in using an intact tissue preparation, such experiments were extended in the present study to both the SL and SR membrane preparations.

In the absence of added inorganic cations, d-TC at $3\mu\text{M}$ concentration has different effects on the two muscle membrane AChEs. The AChE of SR is inhibited by curare by about 60% whereas the SL-membrane bound AChE is unaffected. Under these experimental conditions, $3\mu\text{M}$ d-TC also inhibits the solubilized AChE from Electrophorus electricus by 70% (Table 10).

Results on the effects of $3\mu\text{M}$ d-TC on SR and SL membrane bound enzymes in the presence of added inorganic ions is shown in Table 11 and 12. A graded increase in ionic strength decreases and finally abolishes the inhibitory effect of d-TC on SR-AChE (Fig. 6). On the other hand, in the presence of 10 mM Ca^{++} , SL-AChE is activated by d-TC (Fig. 7). This activating (or dis-inhibiting) effect of d-TC on SL-AChE shows

FIGURE 5

The effect of d-tubocurarine added at molar concentrations indicated at the arrows on the rate of hydrolysis of C^{14} acetyl- β -methylcholine ($5 \times 10^{-6} M$) by the intact rat phrenic nerve-diaphragm preparation in vitro. The final curare addition ($7 \times 10^{-6} M$) produced complete block of the indirect twitch response.

(Data from Mittag & Hehir, unpublished results)

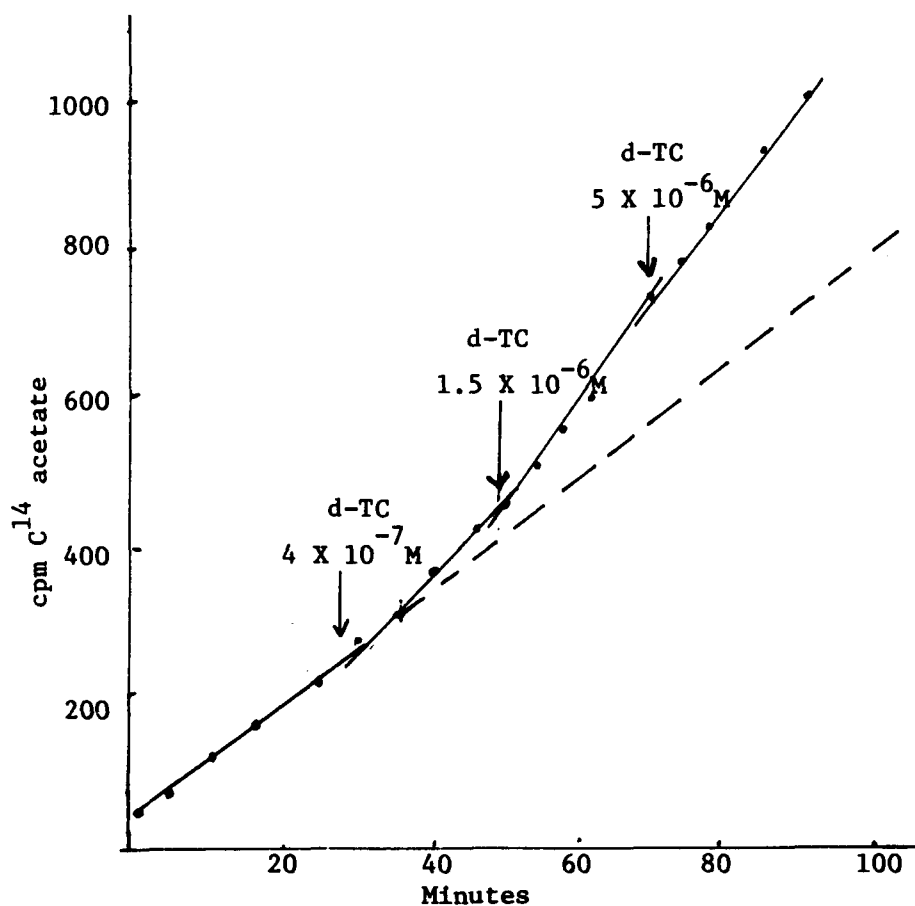


TABLE 10

Effect of 3 μ M d-TC on SL and SR membrane bound AChE and solubilized eel AChE in the absence of added inorganic cations.

	Sp. activity	Effect of 3 μ M d-TC
SL (n=3)	1.19 $\pm$ 0.3	N.E.*
SR (n=5)	4.6 $\pm$ 0.2	-58.5% $\pm$ 3.28
Eel (n=4)	N.D.**	-71.3% $\pm$ 6.5

* N.E. ie., no effect

**N.D. ie. not determined

Specific activities of enzymes are expressed in units of μ moles/min/g protein for [S] = 10^{-4} M at 37°C.

TABLE 11

Effect of 3 μ M d-TC on SR-AChE activity in the presence of different concentrations of divalent cations

Cation conc. (mM)	AChE activity	AChE activity in the presence of 3 μ M d-TC	% of ^{activation} inhibition of AChE due to d-TC
Ca⁺⁺			
0	100.0 $\pm$ 0.0	41.5 $\pm$ 3.28	-58.5 $\pm$ 3.28
0.2	84.2 $\pm$ 6.23	44.6 $\pm$ 2.45	-47 $\pm$ 2.45
0.8	71.6 $\pm$ 8.6	42.96 $\pm$ 0.25	-40.3 $\pm$ 0.25
2.0	59.5 $\pm$ 8.3	39.7 $\pm$ 1.75	-35.25 $\pm$ 1.75
10.0	31.89 $\pm$ 2.24	32.2 $\pm$ 0.04	+ 0.01 $\pm$ 0.04
Mg⁺⁺			
0	100.0 $\pm$ 0.0	41.5 $\pm$ 3.28	-58.5 $\pm$ 3.28
0.2	82.5 $\pm$ 2.0	42.07 $\pm$ 5.2	-49.28 $\pm$ 5.2
0.8	74.5 $\pm$ 5.8	43.21 $\pm$ 9.1	-42.46 $\pm$ 9.1
2.0	62.6 $\pm$ 10.5	45.0 $\pm$ 8.19	-27 $\pm$ 8.19
10.0	41.17 $\pm$ 2.01	39.11 $\pm$ 2.5	- 5 $\pm$ 2.5

Enzyme activities are expressed relative to control = 100 and were assayed with 5 X 10⁻⁶ M acetyl-~~S~~-methylcholine.

TABLE 12

Effect of 3 μ M d-TC on SL-AChE activity in the presence of different concentrations of divalent cations

Cation conc.	AChE activity	AChE activity in the presence of 3 μ M d-TC	% of activation of AChE due to d-TC
Ca^{++}			
0	100.0 $\pm$ 0.0	100.3 $\pm$ 4.9	+ 0.3
0.2	72.1 $\pm$ 8.5	76.42 $\pm$ 8	+ 6.0 $\pm$ 8
0.8	63.2 $\pm$ 5	67.3 $\pm$ 6	+ 6 $\pm$ 6
2.0	47.9 $\pm$ 6.4	60.83 $\pm$ 1.3	+27 $\pm$ 1.3
10.0	31.7 $\pm$ 5.36	44.69 $\pm$ 4.5	+41 $\pm$ 4.5
Mg^{++}			
0	100.0 $\pm$ 0.0	102 $\pm$ 9	+ 2
0.2	79.2 $\pm$ 13.5	90.2 $\pm$ 7	+14 $\pm$ 7
0.8	65.8 $\pm$ 4.1	61.2 $\pm$ 16	- 7
2.0	56.2 $\pm$ 7.1	57.32 $\pm$ 8	+ 2
10.0	37 $\pm$ 5.0	36.26 $\pm$ 4.8	- 2

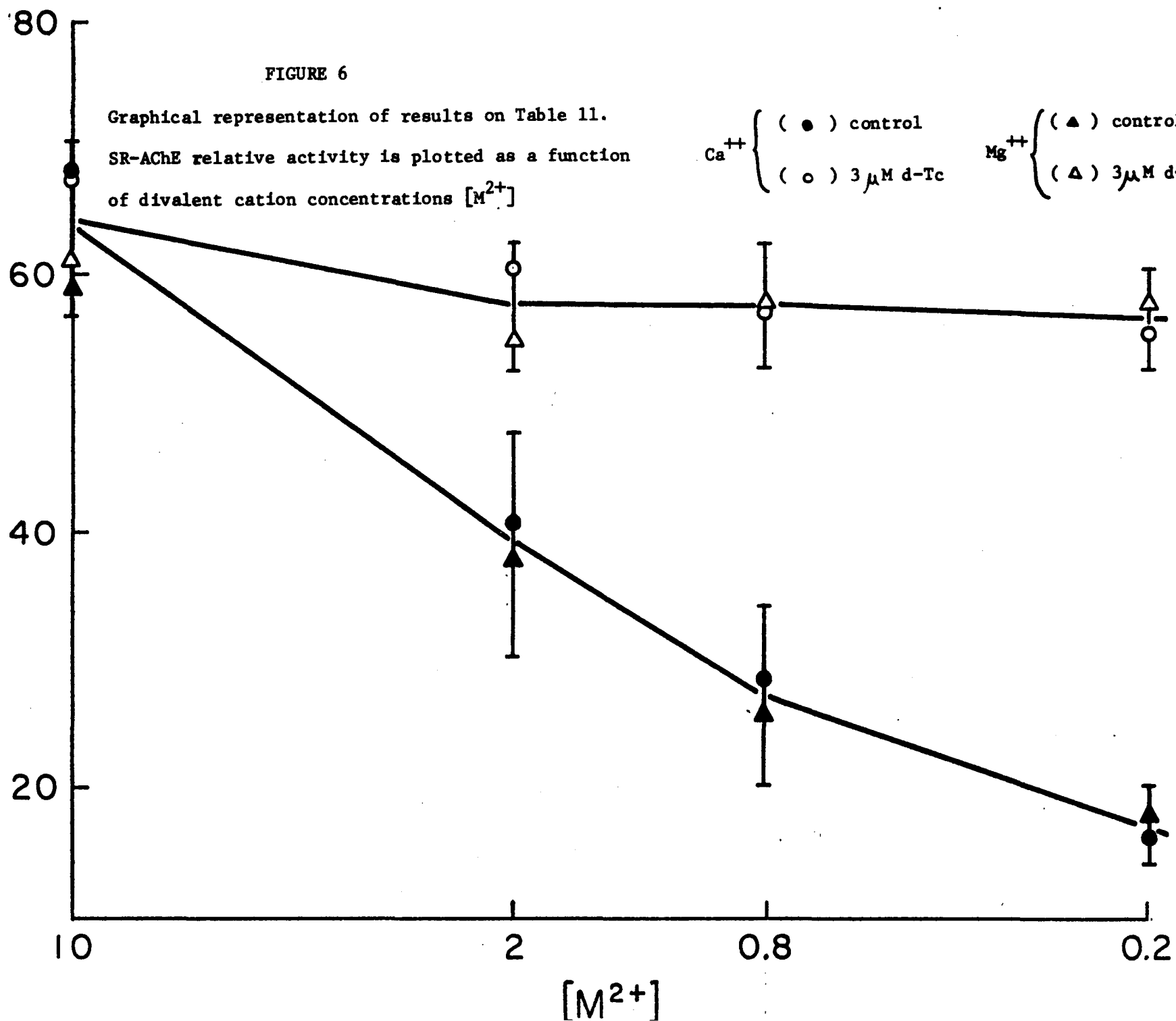
Enzyme activities are expressed relative to control = 100 and were assayed with 5 X 10⁻⁶ M acetyl- β -methylcholine.

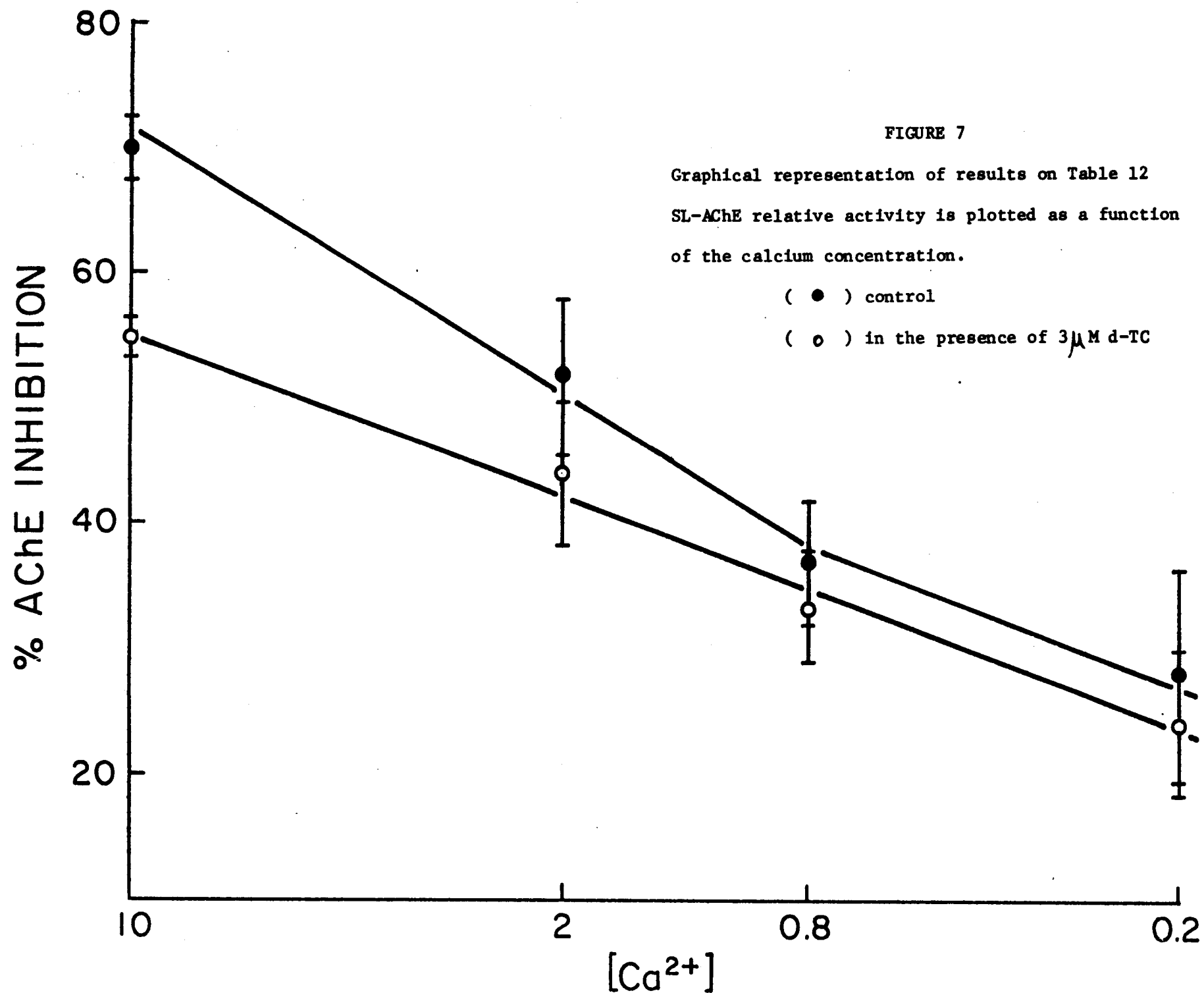
FIGURE 6

Graphical representation of results on Table 11.
SR-AChE relative activity is plotted as a function
of divalent cation concentrations $[M^{2+}]$

Ca^{++} { (●) control
(○) $3\mu M$ d-Tc }
 Mg^{++} { (▲) control
(△) $3\mu M$ d-Tc }

% AChE INHIBITION





cation specificity. It is Ca^{++} dependent, and d-TC activation of SL-AChE is not seen in the presence of Mg^{++} or Na^+ (Table 12 & Fig. 8). Furthermore, the Ca^{++} -dependent d-TC activation was found to be confined to SL derived from the inner or endplate containing (+EP) region of the diaphragm muscle. AChE attached to the SL membrane outside of the endplate area (-EP) is unaffected by $3\mu\text{M}$ d-TC either in the presence or absence of 10 mM Ca^{++} (Table 13).

Collagenase (30 units/ml) or $1\text{ M NaCl} - 0.5\%$ Triton X-100, which are both effective in solubilizing SL-AChE, were used to study the effect of $3\mu\text{M}$ d-TC on AChE in treated SL preparations and the enzyme released from the SL after such treatments.

$1\text{ M NaCl} - 0.5\%$ Triton X-100 solubilized about 60-70% of the total enzyme activity of both the +EP and -EP SL membrane preparations. AChE activity which remains membrane bound after such treatment behaves very much the same as untreated preparations. It is inhibited the same as untreated SL by 10 mM Ca^{++} . Similarly, the +EP, but not -EP, SL-AChE is activated by $3\mu\text{M}$ d-TC in the presence of Ca^{++} . However, the solubilized enzyme from both +EP and -EP SL preparations are now different, since both are inhibited by 10 mM Ca^{++} but are now unaffected by $3\mu\text{M}$ d-TC either in the presence or absence of added calcium ions (Table 14).

By comparison, collagenase treatment, renders all SL-AChE inhibitable by d-TC, irrespective of the physical state of the enzyme molecule (ie. solubilized vs. membrane attached) or the in situ localization of the enzyme (ie. +EP vs. -EP). This inhibitory effect of d-TC on collagenase treated preparations is not calcium dependent (Table 15). These results suggest that collagenase drastically alters the enzyme properties perhaps by enzymatic attack

FIGURE 8

SL-AChE activity in the absence (slope 1), in the presence of 10 mM Ca^{++} (slope 2) and in the presence of 3 μM d-TC plus 10 mM Ca^{++} (slope 3) as a function of time.

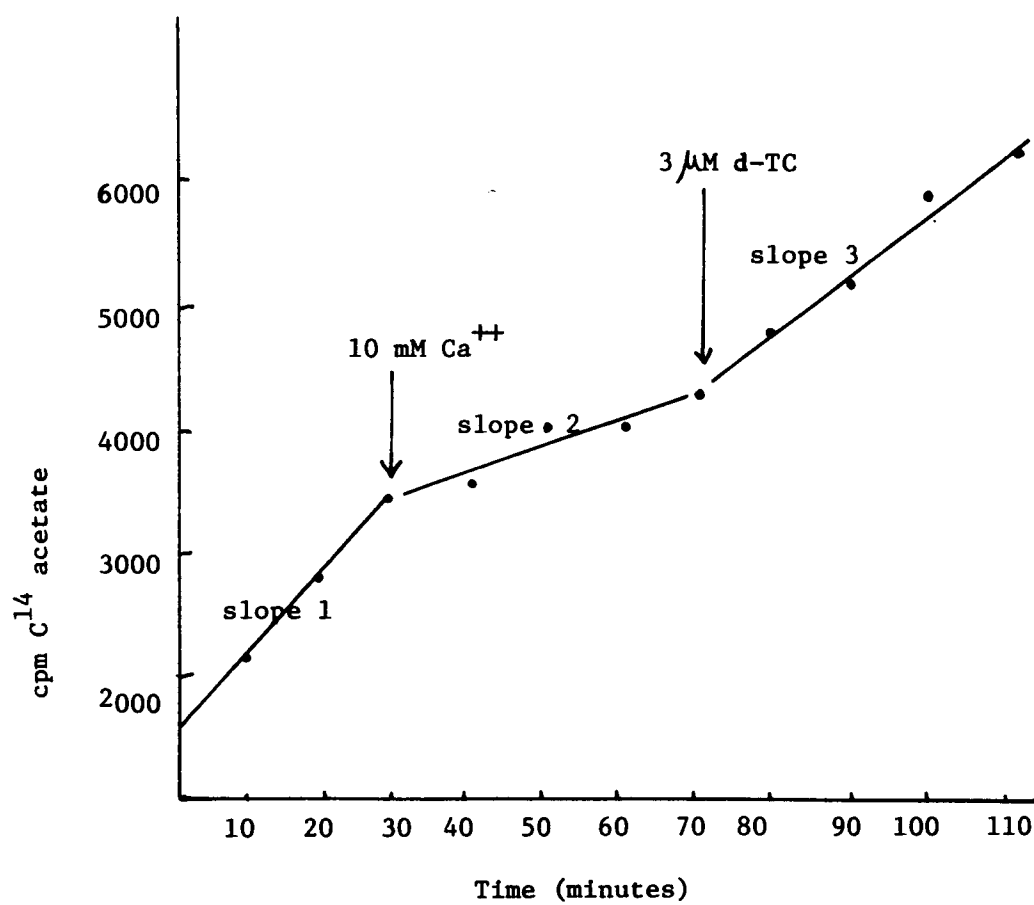


TABLE 13

Effect of $3\text{ }\mu\text{M}$ d-TC on +EP and -EP SL-AChE in the presence of 10 mM Ca^{++}

Sp. Activity	Effect due to 10 mM Ca^{++}	Effect of $3\text{ }\mu\text{M}$ d-TC in the presence of Ca^{++}	Protein yield mg/hemidiaphragm
+EP (n=8)			
1.405 ± 0.05	$-67.9 \pm 2.5\%$	$+43 \pm 6.8\%$	0.41 ± 0.03
 -EP (n=8)			
1.003 ± 0.9	$-70.2 \pm 1.9\%$	N.E.*	0.56 ± 0.06

* N.E. ie., no effect

AChE specific activities are expressed in units of $\mu\text{moles/min/g protein}$
for $[\text{S}] = 10^{-4}\text{ M}$ at 37°C .

TABLE 14

Effect of 10 mM Ca^{++} and 3 μM d-TC (in the presence and absence of Ca^{++}) on 1 M NaCl - 0.5% Triton X-100 treated SL-AChE (both solubilized and membrane bound form).

	AChE sp. activity	Effect due to 10 mM Ca^{++}	Effect due to 3 μM d-TC		Protein mg/hemidiaphragm
			- Ca^{++}	+ 10 mM Ca^{++}	
+EP	*Solubilized 21.5 $\pm$ 0.33	-27.5 $\pm$ 0.5%	+ 5 $\pm$ 2.5%	-2.5 $\pm$ 2.5%	0.068 $\pm$ 0/007
	**Membrane bound 3.0 $\pm$ 0.26	-25.0 $\pm$ 10%	+49 $\pm$ 12.5%	+7 $\pm$ 7 %	0.25 $\pm$ 0.004
-EP	*Solubilized 19.74 $\pm$ 1.7	-43.6 $\pm$ 4.2%	+ 4 $\pm$ 9 %	-2 $\pm$ 3.5%	0.071 $\pm$ 0.008
	**Membrane bound 2.62 $\pm$ 0.14	-31.3 $\pm$ 4.9%	+16 $\pm$ 10.2%	+8 $\pm$ 8.5%	0.235 $\pm$ 0.035

AChE specific activities are expressed in units of $\mu\text{mole}^{-1}/\text{min/g}$ protein for $[\text{S}] = 10^{-4}\text{M}$ at 37°C.

*Solubilized, ie., not sedimented at 15,000 X g for 20 minutes.

**Membrane bound, ie., 15,000 X g pellet.

TABLE 15

Effect of $3\text{ }\mu\text{M}$ d-TC on collagenase treated SL-AChE (both solubilized and membrane bound form) in the absence of inorganic cations.

	AChE sp. activity	Effect due to $3\text{ }\mu\text{M}$ d-TC	Protein yield mg/hemidiaphragm
+EP (n=4)	*Solubilized 33 ± 0.17	-38.0 $\pm 3.7\%$	0.105 ± 0.09
	**Membrane bound 8.0 ± 0.406	-46.7 $\pm 3.5\%$	0.235 ± 0.042
-EP (n=4)	*Solubilized 31.9 ± 1.93	-45 $\pm 2.5\%$	0.09 ± 0.03
	**Membrane bound 5.8 ± 1.04	-39 $\pm 2.38\%$	0.23 ± 0.026

*Solubilized, ie., not sedimented at 15,000 X g for 20 minutes.

**Membrane bound, ie., 15,000 X g pellet.

AChE specific activities are expressed in units of $\mu\text{mole}^{-1}/\text{min/g}$ protein for $[\text{S}] = 10^{-4}\text{ M}$ at 37°C .

which alters the enzyme molecule itself or alternatively changes the physicochemical properties of the membrane matrix where the enzyme is incorporated.

In summary, clear differences in properties of SL-AChE (both +EP and -EP) and SR-AChE are once again observed. This time using d-TC as a test agent. Furthermore, d-TC enables the identification of a specific AChE associated with the endplate part of the muscle sarcolemma. The molecular basis of the differential effect of d-TC on +EP SL-AChE, -EP SL-AChE and SR-AChE will be discussed in a subsequent section.

By using d-TC as a probe, it was also shown that properties of membrane bound AChE are quite distinct from AChE in solution. The possibility of changes in the physicochemical properties of the enzyme molecule after solubilization and/or changes in the immediate environment surrounding the AChE in the membrane that renders the enzyme molecule to behave differently will be discussed.

E. EFFECT OF TRITON X-100 TREATMENT ON PROPERTIES OF SL MEMBRANE BOUND AChE

Because of the close location of AChE and the ACh-receptor on the postsynaptic membrane (Barnard, Wieckowski & Chiu, 1971) it is quite possible that their association may even extend to the molecular level, and that d-TC activates AChE in the presence of Ca^{++} by binding to the ACh-receptor which, by allosteric interaction, can modulate AChE catalytic center activity.

In order to test this possibility, the effect of $3\text{ }\mu\text{M}$ d-TC on Triton X-100 treated SL membrane was therefore investigated. A number of studies (Miledi, Molinoff & Potter, 1971; Miledi & Potter, 1971; Berg et. al., 1972) have demonstrated that the AChR is a membrane-bound protein which is readily solubilized in an active state by Triton X-100 (judging by its ability to bind D-BuTx after solubilization).

Rat diaphragm muscle was homogenized in 1% Triton X-100 and Krebs' solution (Berg et. al., 1972). This procedure according to Berg et. al. solubilizes most if not all of the AChR. SL membrane preparation was prepared as usual. The effect of $3\text{ }\mu\text{M}$ d-TC on membrane bound AChE was then studied.

The results in Table 16 show that the properties of SL-AChE prepared from Krebs' muscle homogenate is identical to the standard SL-AChE (ie. prepared from KCl homogenate) with respect to two parameters studied, ie., inhibited by 10 mM Ca^{++} and that only the +EP SL-AChE is activated by d-TC in the presence of Ca^{++} . The specific activity of AChE of both +EP and -EP SL-membrane prepared in Krebs' solution are somewhat lower than the standard preparation. Addition of 1% Triton X-100 during homogenization further lowers the AChE specific activities of both +EP and -EP SL membrane preparations. Nevertheless, the characteristic effects of

TABLE 16

Specific activities of +EP and -EP SL-AChE and the effect of Ca^{++} and d-TC on the enzyme activity of SL membrane fraction prepared from Kreb's muscle homogenate

	AChE sp. activity	10 mM Ca^{++} effect	3 μM d-TC effect*	Protein mg/hemidiaphragm
+EP (n=3)	8.06 $\pm$ 0.115	-71 $\pm$ 7%	+67 $\pm$ 9.5%	0.49 $\pm$ 0.02
-EP (n=3)	6.69 $\pm$ 2.5	-66 $\pm$ 4 %	- 5 $\pm$ 5%	0.44 $\pm$ 0.09

+EP and -EP part of the diaphragm muscle were homogenized in Kreb's solution. SL-membrane fractions were prepared according to standard procedures. Enzyme specific activities are expressed in units of $\mu\text{mole}^{-1}/\text{min/g}$ of protein for $[\text{S}] = 10^{-4}\text{M}$ at 37°C .

*The effect of d-TC is expressed as a % of a baseline activity (ie. activity in the presence of 10 mM Ca^{++}).

both Ca^{++} and d-TC persist (Table 16, 17).

In a parallel series of experiments, SL membrane preparations of +EP and -EP regions of the diaphragm muscle were prepared as usual and the final membrane pellets were resuspended in standard buffer (0.32 M sucrose-5 mM histidine) with the addition of Triton X-100 to a final concentration of 1.5% (W/W). The membrane suspensions were then incubated for 30 minutes at 37°C, centrifuged at 15,000 X g for 15 minutes, the supernatants were separated from the membrane pellets, and the pellets resuspended. The effects of 3 μ M d-TC on such Triton X-100 extracted SL membrane, from which AChR presumably has been removed, were studied. Results are given in Table 18. No solubilized AChE was detected after treatment with Triton X-100 (see also Table 5). Specific activities of AChE of both +EP and -EP SL membrane preparations as well as their properties were similar to controls. The AChEs were inhibited by 10 mM Ca^{++} and the Ca^{++} -dependent d-TC activation is confined to AChE of the +EP region SL membrane (Table 18).

This series of experiments demonstrated that AChE and AChR are probably independent entities, adding to the evidence that the two macromolecules are indeed different and can be separated from one another (Bartels, 1968; Barnard, Wieckowski & Chiu, 1971). Triton X-100 which presumably solubilizes most if not all AChR does not solubilize AChE located on the SL membrane. Furthermore the specific property of +EP SL-AChE, ie. d-TC activation, is retained after such treatment, indicating that AChR is probably distinct from the bis-quaternary drug binding site and that the activating effect of d-TC is a property of the enzyme or some other associated macromolecule.

TABLE 17

Specific activities of +EP and -EP SL-AChE and effects of 10 mM Ca^{++} and d-TC on the enzyme activity of SL membrane fraction prepared from Krebs' Triton X-100 muscle homogenate.

	AChE sp. activity	10 mM Ca^{++}	3 μM d-TC effect*	Protein mg/hemidiaphragm
+EP (n=3)	6.77 $\pm$ 0.24	-68.6 $\pm$ 8.8%	+63 $\pm$ 7.4%	0.433 $\pm$ 0.04
-EP (n=3)	3.6 $\pm$ 0.61	-49.6 $\pm$ 6.7%	+ 3.2 $\pm$ 1.46%	0.425 $\pm$ 0.17

SL membrane fractions were prepared from Krebs'-1% Triton X-100 muscle homogenate, a procedure which solubilizes AChE (Berg et. al., 1972).

Specific activities of AChE and their properties, with respect to two parameters (Ca^{++} and d-TC) effect were examined.

Specific activities of enzyme are expressed in units of $\mu\text{mole}^{-1}/\text{min/g}$ of protein.

*The effect of d-TC is expressed as a % of baseline activity (ie. in the presence of 10 mM Ca^{++}).

TABLE 18

Effect of Ca^{++} and 3 μM d-TC on Triton X-100 extracted +EP and -EP SL-AChE

	AChE sp. activity	10 mM Ca^{++} effect	3 M d-TC effect*	Protein mg/hemidiaphragm
+EP	1.043 $\pm$ 0.21	-67 $\pm$ 3.5%	+49 $\pm$ 3.2%	0.45 $\pm$ 0.01
-EP	0.86 $\pm$ 0.12	-65 $\pm$ 2.7%	+ 3 $\pm$ 8%	0.43 $\pm$ 0.05

Standard +EP and -EP SL membrane fractions were prepared. Final membrane suspensions were extracted with 1.5% Triton X-100 at 37°C for 30 minutes. Membrane suspensions were repelletized and the effect of Ca^{++} and d-TC were examined.

Specific activities of AChE are expressed in units of $\mu\text{moles}^{-1}/\text{min}/\text{g}$ of protein for $[\text{S}] = 10^{-4}\text{M}$ at 37°C.

*The effect of d-TC is expressed as a % of baseline activity (ie., in the presence of 10 mM Ca^{++}).

F. STUDIES ON QUANTITATIVE AND QUALITATIVE CHANGES OF SL- AND SR-AChEs
OF THE DENERVATED DIAPHRAGM MUSCLE

1. Changes in Cholinesterase Activities of SL and SR Membrane of the
Denervated Diaphragm Muscle

It is well known that the nerve controls several functional and structural properties of muscle fibers (Guth, 1968; Gutmann, 1962). The trophic influence of nerve on muscle becomes evident after denervation. The rat diaphragm serves admirably for this purpose as it is possible to denervate one hemidiaphragm by unilateral phrenicotomy and to use the other side as a control. This technique was used in this study and the changes in SL-AChE and BuChE specific activities two, ten and twenty days after denervation are shown in Table 19. There is a 30-40% decrease in SL-AChE two days after denervation, reaching 60% by 10 days and remains at that level twenty days after denervation. On the other hand, the decrease in SL-BuChE activity is preceded by a lag period. No decrease in SL-BuChE activity is observed two days after denervation, by 10 days there is a 31% decrease which remains at this level up to twenty days after denervation (Table 20).

Since the fall in SL-AChE activity reaches a maximum at 10 days after denervation, this was chosen as the standard timing for further denervation experiments. The rate of change in +EP and -EP SL-AChE activities were investigated. Results are presented in Table 21. The rate of loss of +EP and -EP SL-AChE in denervated muscle was identical. This is indicated by the similar ratio of D/I AChE activity of the +EP and -EP membrane preparations. In all cases, the SL membrane protein yield per hemidiaphragm was not statistically different (Table 22).

TABLE 19

Changes in AChE specific activities of SL membrane preparations two, ten and twenty days after denervation.

AChE specific activities		D/I
Denervated	Innervated	
2 days (n=2) 9.4 $\pm$ 0.8	14 $\pm$ 0.2	0.67 (p < 0.05)
10 days (n=4) 4.4 $\pm$ 0.18	11.9 $\pm$ 0.17	0.32 (p < 0.01)
20 days (n=1) 5.58	13.16	0.42 (p < 0.01)

Enzyme specific activities are expressed as means $\pm$ standard errors in units of $\mu\text{mole}^{-1}/\text{min/g}$ protein for $[S] = 10^{-4}\text{M}$ at 37°C .

TABLE 20

Changes in BuChE activities of SL membrane preparation two, ten and twenty days after denervation.

BuChE specific activities			D/I
	Denervated	Innervated	
2 days	15.8	14.5	1.08
10 days	11.2	16.2	0.69
20 days	10.2	15.2	0.67

Enzyme specific activities are expressed as means $\pm$ standard errors in units of $\mu\text{mole}^{-1}/\text{min/g}$ protein for $[S] = 10^{-4}\text{M}$ at 37°C .

TABLE 21

Effect of denervation and cycloheximide on +EP and -EP SL-AChE
specific activities in diaphragm muscle denervated for 10 days.

+EP			-EP		
Denervated	Innervated	D/I	Denervated	Innervated	D/I
CONTROL (n=3) 5.00 $\pm$ 0.18	11.15 $\pm$ 1.4	0.45 (p<0.01)	4.5 $\pm$ 0.7	9.7 $\pm$ 1.5	0.46 (p<0.01)
CYCLOHEXIMIDE (n=3) 4.7 $\pm$ 0.36	8.96 $\pm$ 0.46	0.52 (p<0.01)	4.3 $\pm$ 0.41	6.3 $\pm$ 0.78	0.68 (p<0.05)
CHI/CONTROL (ratio) 0.94 (p>0.05)	0.78 (p<0.05)		0.95 (p>0.05)	0.65 (p<0.05)	

Enzyme specific activities are expressed in units of $\mu\text{mole}^{-1}/\text{min/g}$ protein
for $[S] = 10^{-4}\text{M}$ at 37°C .

TABLE 22

Effect of denervation and cycloheximide on mg of SL membrane protein
yield per hemidiaphragm.

	+EP		-EP	
	Denervated	Innervated	Denervated	Innervated
CONTROL (n=3)	0.6 $\pm$ 0.24	0.52 $\pm$ 0.06	0.75 $\pm$ 0.23	0.74 $\pm$ 0.10
CYCLOHEXIMIDE (n=3)	0.75 $\pm$ 0.03	0.57 $\pm$ 0.036	0.79 $\pm$ 0.09	0.72 $\pm$ 0.067

For the SR membrane preparations there was a 35% decrease in AChE specific activity 10 days after denervation (Table 23). There were no significant differences in SR membrane protein yield per hemidiaphragm under all conditions studied (Table 24).

Most membrane proteins turn over at a certain rate, and it is likely that AChEs are not permanent entities attached to the membrane. Turnover of AChE can be demonstrated by using DFP (an irreversible inhibitor of AChE) to abolish endplate ChE activity (as determined histochemically). The gradual reappearance of ChE is then presumably due to a de novo synthesis of new enzyme molecules (Denz, 1954; Koenig & Koelle, 1960, 1961). The role of the nerve in the regulation of muscle membrane AChE activity has been demonstrated in denervated muscle in the present study. Denervation could either reduce the rate of synthesis or increase the rate of degradation of the AChE molecule, both processes would result in a decrease in AChE specific activity (Table 19, 20, 21, 23).

To test these hypotheses, the effect of daily injections of cycloheximide, a protein synthesis inhibitor, on changes of AChE activity of denervated rats was studied. The rationale for using a protein synthesis inhibitor is as follows: the trophic effect of nerve on muscle cell membrane AChE can be either to stimulate the rate of de novo enzyme synthesis or to slow down the rate of degradation of membrane bound AChE. In both cases denervation would result in a decrease in AChE activity. If indeed, the trophic function of nerve was to decrease the degradation of membrane AChE, then the effect of denervation (removal of the inhibitory effect on the degradative process) together with cycloheximide (presumably inhibits de novo protein synthesis) would be synergistic in decreasing membrane bound AChE activity. On the other hand, if the neurotrophic effect is to induce de novo synthesis of AChE, which is blocked by

TABLE 23

Effect of denervation and cycloheximide on SR-AChE specific activities

	Denervated	Innervated	D/I
Control	17.74 $\pm$ 0.92	50.75 $\pm$ 2.2	0.35 (p<0.01)
Cycloheximide	14.4 $\pm$ 0.85	24.0 $\pm$ 1.9	0.6 (p<0.05)
CHI/Control	0.81 (p>0.05)	0.47 (p<0.01)	

Enzyme specific activities are expressed in units of $\mu\text{mole}^{-1}/\text{min/g}$ protein
for $[S] = 10^{-4}\text{M}$ at 37°C .

TABLE 24

Effect of denervation and cycloheximide on SR membrane protein yield per hemidiaphragm.

	Denervated	Innervated
Control	0.73 $\pm$ 0.23	0.81 $\pm$ 0.2 (D vs. I p>0.05)
Cycloheximide	0.64 $\pm$ 0.25	0.77 $\pm$ 0.21 (p>0.05)
CHI/Control	0.87 (p>0.05)	0.95 (p>0.05)

Enzyme specific activities are expressed in units of $\mu\text{mole}^{-1}/\text{min/g}$ protein for $[S] = 10^{-4}\text{M}$ at 37°C .

cycloheximide, then denervation and cycloheximide treatment would not act synergistically in decreasing membrane bound AChE activity.

The results in Table 21 show that ten consecutive days of intraperitoneal cycloheximide injections alone can reduce the +EP SL-AChE activity by 22% and -EP SL-AChE by 35%. The effect of cycloheximide in reducing SL-AChE activity, however, is not additive to the effect due to denervation, ie., SL-AChE activity of the denervated muscle is identical in the control and cycloheximide treated group.

For SR-AChE, the results are shown in Table 23. The effect of cycloheximide decreases membrane bound AChE activity by 53%, compared to a 22-35% decrease of SL-AChE activity. Again, the effect of cycloheximide is not additive to that of denervation in decreasing SR-AChE activity, ie., there is no significant difference in denervated SR-AChE specific activities between the control and cycloheximide treated groups.

2. Qualitative Changes of SL-AChE 10 days After Denervation

It was shown previously (Table 13) that the Ca^{++} -dependent d-TC activation of AChE is confined to SL derived from the +EP part of the hemidiaphragm. The enzyme attached to SL outside of the endplate area is unaffected. This however is not the case when the diaphragm is surgically denervated. Ten days after denervation, in addition to a general decrease of AChE specific activity over the entire muscle cell membrane, the Ca^{++} -dependent d-TC activated AChE appears in the entire muscle cell membrane including the area outside the endplate region (Table 25).

It was indicated earlier that at least part of the trophic effect of nerve in regulating AChE activity is to induce the de novo synthesis of the enzyme. To gain more insight into the problem of trophism, the effect of cycloheximide on the appearance after denervation of a specific SL-AChE (that which is activated by d-TC) outside the endplate region was investigated. There are two major questions:

- a/. Whether a trophic effect of nerve is the mechanism which confines the specific AChE isoenzyme in the endplate region and whether this mechanism depends on protein synthesis
- b/. Whether one can differentiate the effect of cycloheximide and denervation in terms of the specific activity and/or appearance of specific AChEs on the membrane

The results in Table 25 show that for the innervated muscle (both control and cycloheximide treated) Ca^{++} inhibits both +EP and -EP SL-AChE activity by about 60%. The Ca^{++} -dependent d-TC activated AChE is confined to the +EP region. This confinement process is probably less - dependent on protein synthesis, since in the cycloheximide treated animals the presence of this Ca^{++} -dependent d-TC activated AChE is still

TABLE 25

Effect of Ca^{++} and d-TC on SL-AChE of normal-denervated and cycloheximide-denervated diaphragm muscle.

Innervated			Denervated	
10 mM Ca ⁺⁺		3 μM d-TC	10 mM Ca ⁺⁺	3 μM d-TC
CONTROL				
+EP	-60 <u>±</u> 5.29%	+57 <u>±</u> 3.4%	-66 <u>±</u> 0.88%	+59.5 <u>±</u> 3.8%
-EP	-61 <u>±</u> 9.13%	N.E.	-67 <u>±</u> 6.08%	+56 <u>±</u> 8.9%
CYCLOHEXIMIDE				
+EP	-58 <u>±</u> 6.2%	+39.8 <u>±</u> 6.7%	-70 <u>±</u> 3.5%	+ 58 <u>±</u> 11%
-EP	-51.6 <u>±</u> 4.9%	N.E.	-61.8 <u>±</u> 4.8%	+64.7 <u>±</u> 7.9%

Effect of d-TC were examined in the presence of 10 mM Ca^{++} ; in the absence of Ca^{++} , d-TC has no effect on SL-AChE.

restricted to the +EP region even though the specific activity is decreased.

For denervated diaphragm muscles, Ca^{++} still exerts its inhibitory effect on SL-AChEs. The degree of inhibition of SL-AChE by 10 mM Ca^{++} is not statistically different in the various groups studied. This point is significant in view of the fact that d-TC activation of SL-AChE is Ca^{++} -dependent, and therefore it is necessary to have a stable baseline activity (ie. AChE activity in the presence of 10 mM Ca^{++}) to study the effect of d-TC on SL-AChE under the various conditions (eg. cycloheximide treatment, denervation). It is shown in Table 25 that denervation results in the appearance of Ca^{++} -dependent d-TC activated AChE in the -EP region, in addition to its presence in the +EP part of the muscle. Furthermore, this process (ie. appearance of a d-TC activated AChE outside the EP region) is unaffected by cycloheximide treatment at this dose level (ie. 100 $\mu\text{g/day/rat}$, intraperitoneally).

In summary, d-TC activated all denervated SL-AChEs, irrespective of their localization on the denervated muscle (ie. +EP vs. -EP) in the presence of Ca^{++} . Cycloheximide treatment either alone or combined with denervation had no influence on the location of d-TC activated AChE.

Similar experiments were conducted on SR-AChE. 10 mM Ca^{++} inhibits SR-AChE by 65-75%, irrespective of cycloheximide treatment or the denervation process (Table 26), ie., denervation does not result in change in SR-AChE with respect to Ca^{++} inhibition. Similarly, in the absence of inorganic cations, 3 μM d-TC inhibited SR-AChE activity in all cases by 55-65%. Addition of 10 mM Ca^{++} obliterates the inhibitory effect of the bis-quaternary neuromuscular blocking agent as observed in controls.

The overall result of this series of experiments therefore indicates that there is a specific trophic effect of the nerve in confining the Ca^{++} -dependent d-TC activated SL-AChE in the endplate region. Denervation

TABLE 26

Effect of Ca^{++} and d-TC on SR-AChE activity of normal-denervated and cycloheximide-denervated diaphragm muscle.

	10 mM Ca^{++}		d-TC	
	Denervated	Innervated	Denervated	Innervated
CONTROL	$-75 \pm 4.5\%$	$-68 \pm 3.8\%$	$-67 \pm 2.4\%$	$-65 \pm 7.5\%$
CYCLOHEXIMIDE	$-77 \pm 2.5\%$	$-76.6 \pm 5\%$	$-60.7 \pm 3.8\%$	$-54 \pm 3.5\%$

The effect of d-TC was examined in the absence of 10 mM Ca^{++} . The presence of divalent cations abolishes the effect of d-TC on SR-AChE.

results in the appearance of this specific isoenzyme over the entire muscle cell surface. This neurotrophic process is therefore probably independent of de novo protein synthesis. The implication is that while the nerve regulates the general level of muscle cell membrane AChE activity by a mechanism involving protein synthesis, there is a specific trophic effect in confining the specific AChE located in the neuromuscular junction, which is disrupted by the process of surgical denervation and appears either to be independent of protein synthesis, or alternatively is dependent on protein synthesis, but is not as sensitive to cycloheximide treatment as the general level of muscle cell membrane AChE activity.

G. BINDING OF [^{125} I] α -BUNGAROTOXIN TO MUSCLE

Acetylcholine (ACh) receptors are normally confined to the neuromuscular junction and nearby area, but after denervation the region sensitive to ACh increases, spreading from the endplate region to encompass the entire surface of the muscle fiber (Axelsson & Thesleff, 1959; Miledi, 1960). Studies on AChR have recently been greatly facilitated by the availability of α -bungarotoxin. α -Bungarotoxin (α -BuTx) is a component of the venom of a Formosan snake (Bungarus multicinctus). It blocks specifically and irreversibly the depolarizing action of acetylcholine at vertebrate neuromuscular junctions (Chang & Lee, 1963; Lee and Chang, 1966; Lee & Tseng, 1966). In addition, the toxin blocks the response to acetylcholine of denervated muscle fibers (Eaker, Harris & Thesleff, 1971).

Binding of [^{125} I] α -BuTx to muscle and SL membrane preparations was done for the following purpose:

- 1/. To check the process of denervation. Denervation results in an increase in ACh sensitive areas of muscle. This is due to an increased synthesis of AChR and is revealed by an increase in α -BuTx binding to denervated muscle.
- 2/. Kimura and Kimura (1973) reported that intraperitoneal cycloheximide treatment depressed the ACh supersensitivity of diaphragm 5 days after denervation. Their pharmacological results were re-investigated using α -BuTx binding.
- 3/. To gain further insight into the d-TC binding site of +EP SL-AChE. The curare-like effect of α -BuTx in neuromuscular transmission blockade is well documented. Furthermore, α -BuTx binding to AChR is at least partially blocked by d-TC (Miledi & Potter, 1971). If indeed d-TC binds to AChR and results in an increase in +EP SL-AChE activity, then α -BuTx should

TABLE 27

Binding of [^{125} I] α -bungarotoxin to muscle and SL membrane preparation.

	Denervated	Innervated	(D-I)
INTACT MUSCLE			
CONTROL	3.62 ± 0.416	2.29 ± 0.214	1.32 ± 0.22 ($p < 0.05$)
CYCLOHEXIMIDE	2.97 ± 0.16	2.01 ± 0.12	1.04 ± 0.19 ($p < 0.05$)
SL MEMBRANE	0.733 ± 0.15	0.937 ± 0.23	-0.20 ± 0.14 ($p > 0.05$)

Amount of toxin bound is expressed in units of p moles / hemidiaphragm

presumably have the same effect in activating or altering +EP SL-AChE activity.

The amount of [^{125} I] α -BuTx bound to whole normal (innervated) and denervated hemidiaphragms is shown in Table 27. There is an increase of 1.32 ± 0.22 p mole of toxin bound per hemidiaphragm 10 days after denervation. Under these conditions, cycloheximide decreases, but does not abolish the increase in toxin binding after denervation. The difference in amount of toxin bound between the innervated and denervated muscle is 1.04 ± 0.19 p mole per hemidiaphragm in cycloheximide treated animals.

Surprisingly, no difference in amount of toxin bound is observed in the SL membrane fractions prepared from innervated and denervated muscle (Table 27). It is therefore possible that during the isolation procedure AChR on the SL membrane is solubilized, or that the purification procedure of the membrane fraction (ie. high salt extraction) causes conformational changes in the extrajunctional AChR which then is incapable of binding α -BuTx.

In summary, the results show that there is an increase in extrajunctional AChR after denervation as revealed by the increase in α -BuTx binding by the intact muscle, confirming results of other workers. However, it is unlikely that the d-TC binding site (ie. site which binds d-TC and that results in an acceleration of +EP SL-AChE activity) is AChR. This is due to the fact that SL membrane preparations which lose their capability to bind α -BuTx by the procedure for preparing the membrane fraction from muscle is still capable of responding to d-TC in terms of activation of AChE activity.

H. STUDIES ON AChE ISOENZYMES USING ACRYLAMIDE GEL ELECTROPHORESIS

Solubilized AChEs of both SL and SR membrane of rat diaphragm muscle were investigated, using acrylamide disc gel electrophoresis. Results of these were compared with solubilized eel AChE. SL membrane bound AChE is solubilized by Triton X-100 and 1 M NaCl treatment, whereas SR-AChE is solubilized by EDTA extraction. These were subjected to acrylamide gel electrophoresis.

Fig. 9 shows that under these experimental conditions, both SL and SR solubilized enzymes have 4 electrophoretically separable bands of AChE activity in the 6% separation gel. Furthermore, the relative migration rate of these 4 bands are identical to that of the eel enzyme preparation. For all three types of enzymes investigated, the two bands closer to the origin are relatively sharp whereas the two faster migrating bands are broad and diffuse. The multiple forms of AChEs could represent AChEs with differences in charge, differences in size or a combination of both. Some AChE activity always remained in the spacer gel. This phenomenon was also observed by Wilson et. al. (1971), they also noted that the activity in the spacer gel was not removed by pretreating the enzyme with Triton X-100, sonication, extending the time of electrophoresis up to twice its usual length, varying the ionic strength of the buffer or using acrylamide concentrations as low as 5%.

Despite the similarity in the AChE staining pattern of the three different enzyme preparations, their protein pattern showed marked differences (Fig. 10). The purified eel AChE preparations, as expected, has the lowest number of protein bands. The solubilized SR-AChE preparation used for electrophoresis in the experiments consists of multiple protein bands. Triton X-100, which is used for solubilizing

FIGURE 9

Acrylamide gel AChE staining pattern of various solubilized enzyme preparations.

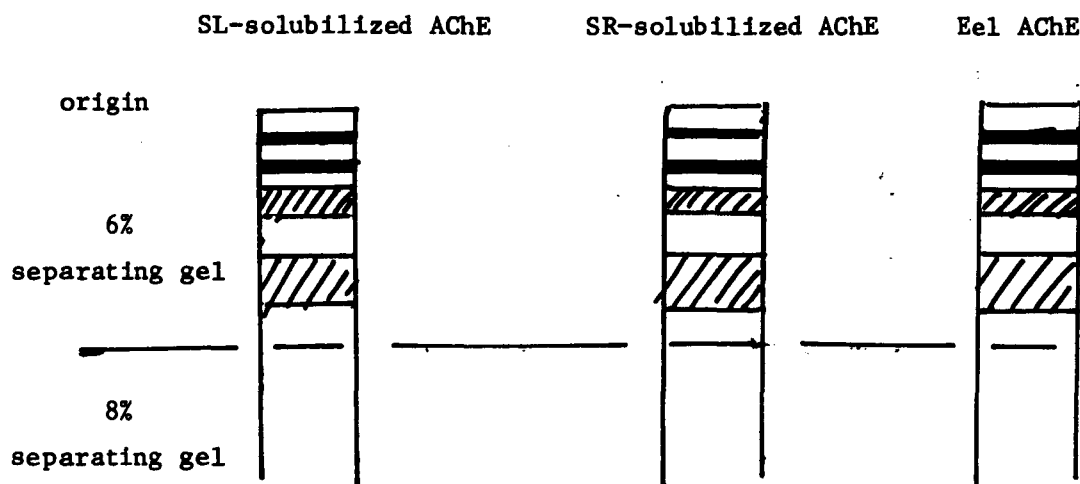
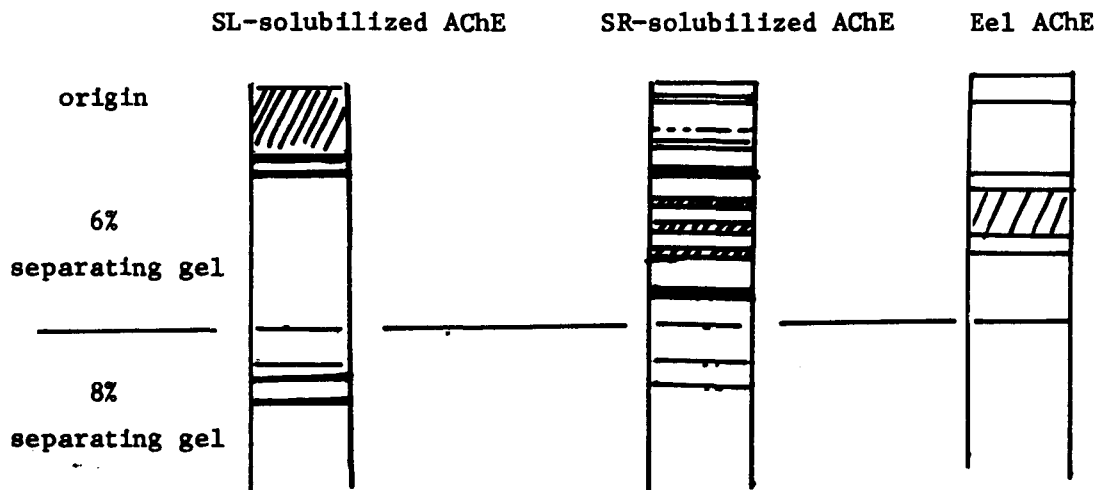


FIGURE 10

Acrylamide gel protein staining pattern of various solubilized enzyme preparations.



SL-AChE, interferes with the protein staining pattern. There is a large opaque bloud at the origin of the separation gel. This phenomenon is also observed when Triton X-100 alone is loaded onto a gel and subjected to electrophoresis. In this case, no stainable AChE activity is present.

In all cases, no attempt was made to correlate AChE staining patterns and protein patterns. The broad and diffuse bands obtained from histochemical staining of AChE (due to diffusion of enzyme hydrolysis product) and the multiple protein bands render such task extremely difficult to execute.

The results indicate that whatever differences in kinetics or ligand-binding properties of the various types of membrane bound AChE there is , these differences are not revealed in the electrophoretic mobility. The SL and SR enzymes behave identical to each other and are similar to the results observed when solubilized eel AChE is the source. This suggests that solubilized AChEs form molecular weight complexes of several sizes which do not depend on the source of the enzyme.

V. DISCUSSION

A. PROPERTIES OF MEMBRANE BOUND CHOLINESTERASES

The present results indicate that there is a general distribution of AChE and BuChE activity along the entire muscle cell surface. This also raises the question of why histochemical and quantitative methods yield different patterns of cholinesterase distribution in the muscle. The histochemical method demonstrated the presence of the enzyme only at the endplate region, whereas the present method demonstrated the presence of the enzyme activity throughout the entire muscle cell surface. This discrepancy, however, is more apparent than real. In as much as the endplate activity is very high, and the endplate occupies a very restricted region of the muscle fiber, it is only natural that the histochemical method would merely reveal such regions of high activity.

Differences between the solubilized eel enzyme and various forms of membrane bound AChEs are revealed by studying their behavior toward inorganic cations. Under the defined experimental conditions (ie 37°C, $[S] = 5 \times 10^{-6} M$) the eel enzyme is activated by the three types of inorganic cations added (Ca^{++} , Mg^{++} , Na^{+}). The membrane bound AChE activity on the other hand is inhibited. This inhibitory effect is also cation concentration dependent, and it also depends in part on the nature of the cation, divalent cations, such as Ca^{++} and Mg^{++} being more effective than monovalent cations on a molar basis.

The differential effect of d-TC on +EP vs. -EP SL-AChE and SR-AChE together with their differences in behavior toward Mg^{++} , as revealed in the double reciprocal plots, indicates that the various types of membrane bound AChE possess different catalytic and/or allosteric properties.

The effect of the nerve in regulating AChE activity is shown in the studies done on denervated animals. Both SL and SR-AChE activities decrease after denervation. Furthermore, a difference in SL and SR-AChE half-lives is reflected by the differential effect of denervation in decreasing SL and SR-AChE activity. SR-AChE activity decreases at a rate faster than SL-AChE. This point (ie. difference in half-lives) is again illustrated by the more dramatic effect of cycloheximide treatment on SR-AChE than on SL-AChE.

The specificity of the neurotrophic effect is shown by the confinement of a d-TC activated SL-AChE in the endplate region of a normal innervated muscle, and that denervation results in the appearance of this specific AChE outside the EP region.

The present study therefore shows that the various types of membrane bound AChEs present in the same tissue do possess different properties not only in terms of their cytological distributions, catalytic properties, biological half-lives, but also with respect to the influence that the nerve exerts on them.

The results also show that SL-AChE is relatively firmly attached to the membrane itself, contrary to the belief that AChE of the neuromuscular junction is anchored in a "cementing substance" (the ectolemmal sheath) in the synaptic cleft (Betz & Sakmann, 1971). It is, however, possible that the endplate AChE is situated in a relatively exposed position on the muscle cell surface, and is probably not an indispensable part of the structural component of the membrane. This is indicated by Hall's study (1971) which showed that collagenase treated rat diaphragm which lost most if not all of the endplate AChE, has the same mean resting membrane potential as the controls and were able to conduct action potentials in

response to nerve stimulation (Hall and Kelly, 1971).

The specificity of the various agents in selectively solubilizing the different types of membrane bound AChE indicates that AChEs are integrated into the various membrane matrices in a different manner. This phenomenon is further illustrated by the difference in stability of attachment of AChEs to the various membranes. While SL-AChE is firmly attached on the membrane, SR-AChE is gradually released into solution during storage and incubation.

Hall (1973) studied the properties of the cholinesterase activity in homogenates of whole rat diaphragm and of innervated (+EP) and non-innervated (-EP) regions. He observed that when a high speed supernatant fraction of muscle homogenized in 1 M NaCl, 0.5% Triton X-100 was subjected to velocity sedimentation in a sucrose gradient, three species of AChE activity with sedimentation constants of 4 S, 10 S and 16 S were present. Velocity sedimentation of extracts from +EP and -EP regions of muscle demonstrated that the 4 S and 10 S forms of the enzyme was found throughout the muscle while the 16 S form of the enzyme was found only in the +EP regions. The presence of a unique AChE isoenzyme associated with the neuromuscular region is also indicated in the present study. Only SL-AChE of the +EP part of the diaphragm muscle is activated by $3\mu\text{M}$ d-TC, this activation is dependent on the presence of Ca^{++} . The Ca^{++} -dependent d-TC activation is not observed in enzymes associated with non-endplate (-EP) containing sarcolemma, sarcoplasmic reticulum or solubilized eel enzyme. When +EP SL-AChEs are solubilized however, they no longer behave as enzymes in situ. Collagenase treated +EP SL-AChE, whether membrane bound or solubilized, is inhibited by $3\mu\text{M}$ d-TC, whereas enzymes solubilized by 1 M NaCl - 0.5% Triton X-100 are unaffected by

d-TC either in the presence or absence of 10 mM Ca^{++} . The anomalous behavior of SL-membrane bound AChE after collagenase treatment and of the solubilized SL-AChEs can be explained in the following way: AChE, rather than being an isolated macromolecule adsorbed on the membrane, is an enzyme which interacts with other membrane components in a specific manner. Incorporation of AChE into different membrane matrices is responsible for differences in properties of various types of membrane bound AChEs. Collagenase treatment, which digest collagen of the muscle, is likely to modify the physicochemical properties of the membrane and thereby results in changes of the properties of membrane bound AChE. If it is accepted that many of the properties of AChE depend intimately on their immediate environment, it is not at all surprising that solubilization of membrane bound AChE will result in a change of properties as documented by these studies.

B. IS AChE AN ALLOSTERIC ENZYME?

1. Effect of inorganic cations on membrane bound AChE activity.

A large number and variety of enzymes are known to be activated by inorganic ions, but the mechanism of activation generally is not well understood. It is well known that the presence of inorganic cations influences enzymic activity. Traditionally AChE activity is measured in media containing added inorganic ions, particularly Na^+ and Mg^{++} (Cohen & Oosterbaan, 1963), yet the influence of the inorganic ions on enzyme activity was rarely taken into account in kinetic studies until the recent few years.

In the present study, the effect of three types of cations were examined, Ca^{++} , Mg^{++} and Na^+ . The eel enzyme is activated by all three types of cations, Mg^{++} being the most potent activator. Studies by Changeux and Grafius et. al. (Changeux, 1966; Grafius & Miller, 1965) showed that the degree of aggregation of AChE from T. marmorata and E. electricus is inversely proportional to the ionic strength in the medium. High molecular weight aggregates are formed under low ionic strength conditions. The activation of AChE by the addition of salt could therefore be attributed in part to alterations of the enzyme structure. If the high M.W. forms persist during the assay (ie. assay in low ionic strength) it would be rather unlikely that these aggregated forms are so ordered that all or nearly all the active sites are arranged in contact with water and accessible to the substrate. While these factors of reversible aggregation and disaggregation are absent in membrane bound enzymes, interpretation of the effects of metal ions on membrane bound AChE is complicated by a number of factors. The anomalous behavior as shown in Fig. 3 and 4 could result from a mixture of more than one membrane

bound isoenzyme with different K_m 's and binding affinities for cations. A second complication may be cation binding by other structures in the membrane, particularly of divalent ions, which influences the enzyme indirectly. However, the results obtained using the membrane bound enzymes can be interpreted on the basis of cation binding to two anionic sites, one being the substrate anionic site, the other being peripheral anionic site(s), which are known to be present even in the purified AChE preparations (Changeux, 1966; Mooser & Sigman, 1972; Belleau et. al., 1970).

At low substrate concentrations (below 0.5 mM) Mg^{++} inhibits AChE of both muscle membrane preparations. This inhibitory phenomenon also occurs with soluble AChE from bovine erythrocytes (Smallman & Wolfe, 1954) but is not observed in soluble AChE from E. electricus. Cation inhibition of erythrocyte AChE is observed under conditions in which the rate limiting step is acetylation of the enzyme, eg., by the use of a specific substrate (Roufogalis & Thomas, 1968 a,b; Roufogalis & Thomas, 1969) dipropyl acetylcholine or at low concentrations of acetylcholine (Smallman & Wolfe, 1954). Therefore, the inhibitory effect of cations on membrane-bound AChE activity at a substrate concentration of $5 \times 10^{-6} M$ (standard condition) can be explained by a competitive binding of cations to the substrate anionic site, thereby inhibiting the rate limiting acetylation step.

The eel enzyme, on the other hand, shows only activation by Ca^{++} and Mg^{++} over the range of substrate concentrations from $1 \mu M$ to 1 mM. It is likely that cations do bind competitively to the substrate anionic site of this enzyme, in a way analogous to binding

to other AChEs, such as SR and SL-membrane bound AChE and solubilized erythrocyte AChE. Nevertheless, the results suggest that the enzyme disaggregating effect of cations, which causes an increase in enzyme activity, over-shadows the inhibitory effect of cations.

A recent study by Wins (Wins et. al. 1970) of membrane bound AChE prepared from *Electrophorus* showed activation by Mg^{++} and Ca^{++} in the presence of optimal substrate concentrations. The Mg^{++} activation phenomenon was found in the present study for the SL enzyme only and then only at substrate concentrations exceeding 0.7 mM. A number of studies have demonstrated that activation is maximal under conditions when deacetylation is rate limiting, eg. by the use of a specific substrate (phenylacetate) or at optimal acetylcholine concentration (Roufogalis & Thomas, 1968 a,b). The activation mechanism is thus acceleration of the rate of deacetylation by binding of cations to an allosteric anionic site of the acetylated enzyme intermediate.

For SL-AChE, the transition from inhibition to activation as the substrate concentration is raised provides evidence for the simultaneous binding of cations at two sites:

- a. the catalytic anionic site: which results in competitive inhibition in rate of acetylation.
- b. peripheral allosteric anionic site: which results in an increase in rate of deacetylation.

The overall effect of cations on SL-AChE activity under all conditions thus depends on the balance that these two opposing forces exert on the enzyme molecule.

Cation activation of AChE was not observed for the SR membrane preparations under all conditions studied, suggesting a weak or

absent binding of Mg^{++} to the allosteric anionic site of the enzyme in this membrane.

2. Effect of a bis-quaternary neuromuscular blocking agent (d-TC) on membrane bound AChE activity.

The effect of some bulky quaternary nitrogen cations (eg. gallamine, d-TC) on AChE activity (Roufogalis & Quist, 1972; Wombacher & Wolf, 1971; Changeux, 1966) and on reactions analogous to acetylation of the enzyme, namely carbamylation and sulfonylation (Belleau et.al., 1970; Kitz et. al., 1969; Kitz & Wilson, 1963; Metzger & Wilson, 1963) have been extensively studied. These, together with other studies using different approaches (Belleau & DiTullio, 1979; Mooser & Sigman, 1972; Kato, 1972; Kato et. al., 1972) such as NMR or active site labeling led to the concept that AChE is an allosteric enzyme, which in addition to the catalytic center, possesses anionic sites having a high binding affinity for bis-quaternary nitrogen compounds such as d-tubocurare and gallamine. In these studies, it was also shown that the regulatory effects of curare and gallamine on soluble (Changeux, 1966) as well as membrane bound AChE (Wins et. al., 1970) are antagonized by Mg^{++} and Ca^{++} , suggesting that the inorganic cation competes with the bulky bis-quaternary nitrogen compound for binding at the allosteric site(s).

The results with SR-AChE showed that while both Ca^{++} and d-TC alone are inhibitory, nevertheless, an increase in cation concentration gradually decreased and finally obliterated all effects of d-TC and thus both types of cations may act through the same mechanism.

Similar antagonism between Ca^{++} and d-TC is also observed in SL-AChE but in a different manner. The inhibitory effect of Ca^{++}

is partially relieved by the addition of d-TC. This activating effect is Ca^{++} -dependent, Mg^{++} or Na^+ is ineffective in this manner. Furthermore, it also depends on the concentration of added Ca^{++} . The activation is barely noticable at 2 mM Ca^{++} concentration and is quite distinct at 10 mM.

Based on available evidence the following two site hypothesis is offered for the behavior of SL-AChE in the endplate region. There exists in AChE two ligand-binding sites, ie, one regulatory site in addition to the familiar active center. The active center consists of an anionic site and an esteratic site, according to well-known models (Nachmansohn & Wilson, 1951). The regulatory site contains one or more negative charges and, in contrast to the active center possess no catalytic activity of itself. Bulky bis-quaternary nitrogen compounds bind preferentially to the negative charges of the regulatory site, whereas the relatively small inorganic cation is capable of binding to both the active center and the regulatory site. The regulatory site is the binding site for substrate as well, but its affinity for substrate molecules is considerably lower than that of the active center. The two sites exert a mutual influence on each other. Binding of ligands to the regulatory site can influence the catalytic center activity and vice versa. No attempt is made in this study to analyse allosterism kinetically. The presence of various other membrane macromolecules in these membrane preparations, which could interact with a number of ligands, as well as the possible existence of different AChE isoenzymes with different K_m 's and ligand binding affinities render such tasks not only difficult to execute but precludes unambiguous conclusions.

C. QUANTITATIVE AND QUALITATIVE CHANGES OF MUSCLE CELL MEMBRANE AChEs AFTER DENERVATION

Studies on changes in AChE activity of SL and SR membrane preparations and changes in AChE isozyme composition are of importance in relation to both the pharmacology of the neuromuscular synapse and the increased sensitivity to ACh observed in denervated muscle.

Denervation produces a selective decrease in both background and endplate SL-AChE activity reaching 45% of control within 10 days. The neural influence of SL-AChE activity is fairly specific inasmuch as denervation produces a decrease in cholinesterase activity without simultaneously decreasing membrane protein content.

The results of cycloheximide treated animals showed that the trophic function of nerve in regulating AChE activity is probably via induction of de novo protein synthesis. If the trophic function were to decrease the rate of AChE degradation, then the effect of cycloheximide treatment, which presumably blocks new synthesis of AChE, and the effect of denervation, which presumably eliminates the inhibitory effect of nerve on the degradative process of AChE, would be synergistic or at least additive. This was not found, indeed, the effect of cycloheximide treatment is not additive to that of denervation, suggesting that perhaps both are regulating AChE activity via a similar pathway----synthesis of new enzyme molecules.

The more dramatic effect of cycloheximide treatment on SR-AChE than on SL-AChE (53% vs. 28 %) implies that SR-AChE probably turns over at a faster rate than SL-AChE.

The trophic function of nerve is also indicated in changes in the SL-AChE isoenzyme distribution after denervation. Ca^{++} -dependent d-TC activated AChE, which is normally confined to +EP region, appears

in the entire muscle cell membrane after nerve sectioning. The appearance of this AChE enzyme, irrespective of a general decrease of SL-AChE activity, indicates that this is a rather specific effect of denervation. The appearance of this particular AChE enzyme outside the EP region after denervation could be due to spreading, unmasking of a pre-existing entity, or it could be due to de novo synthesis of new enzyme molecules. The latter possibility is made unlikely by the fact that the appearance of this enzyme outside the EP region after denervation is unaffected by cycloheximide treatment. The possibility of spreading or unmasking of AChE molecules after denervation is not distinguished in the present study.

The close spatial and temporal association between the ACh-receptor (AChR) and the specific d-TC-activated SL-AChE in both the innervated and denervated muscle prompts the possible identity of the AChR and the d-TC binding allosteric site of AChE. Such a possibility is however, ruled out by the following arguments:

1. Collagenase (30 units/ml) treatment solubilized 68% of SL membrane bound AChE, it also renders all forms (ie., either solubilized or membrane attached) of AChE inhibitable by d-TC. AChR, on the other hand, is unaffected by this process. Hall (Hall and Kelly, 1971) studied collagenase detachment of endplate AChE from muscle. Collagenase solubilized endplate AChE but the collagenase treated muscle has a normal resting membrane potential, and is able to conduct action potentials in response to ACh released by nerve stimulation.
2. Triton X-100 at a concentration of 1-1.5% is known to solubilize most if not all AChR (ie. specific α -BuTx binding macromolecules located on the postsynaptic membrane of a normal innervated muscle). However, it is demonstrated in the present study that the

Ca^{++} -dependent d-TC activation of +EP SL-AChE is unaffected by such treatment, furthermore, no SL-AChE activity is solubilized.

3. The phenomenon of d-TC activation of membrane bound AChE has been demonstrated not only with the intact hemidiaphragm, but also with isolated muscle cell membrane fractions, which bind little, if at all, [^{125}I] α -BuTx.
4. α -BuTx, a polypeptide of M.W. 8,000 which binds specifically to AChR, does not affect AChE catalytic activity over the range of concentration which it binds to AChR.

The d-TC binding site of the +EP SL-AChE, therefore, probably represents an integral part of the AChE macromolecule. Solubilization of the enzyme however, results in changes in the enzyme molecule, which is no longer capable of binding bis-quaternary nitrogen compounds at the allosteric anionic site.

D. BINDING OF [^{125} I] α -BUNGAROTOXIN TO MUSCLE

The phenomenon that denervation brings about a spread of ACh sensitivity outside of the endplate region is well known. This is due to an increase of AChR in the extrajunctional area. In the present study the background amount of toxin bound by the innervated muscle is very high in comparison with other studies, this also accounts for the relatively small % increase in amount of toxin bound after denervation.

A study done by Porter et. al. (1973) showed that there are considerable amounts of ACh-receptor-like molecules located extrajunctionally in the sarcoplasm of mouse diaphragm muscle. These molecules are capable of binding α -bungarotoxin irreversibly, but have a much slower rate of reaction. They summarized some of the properties of these molecules: "there is no evidence yet that these dispersed extrajunctional components are a form of ACh receptor, although they may confuse some isolations of the latter".

Non-specific binding of the toxin molecules by extrajunctional ACh-receptor-like molecules could therefore account for the high amount of toxin bound in the innervated muscle. The increase in α -BuTx binding after denervation is 1.32 ± 0.22 p mole per hemidiaphragm. This, if calculated according to Berg et. al.'s (1972) data, which show that normal innervated rat hemidiaphragm binds 0.1-0.2 p mole of toxin, would represent an 8 fold increase in AChR after denervation.

The result on lack of α -BuTx binding to the SL membrane preparation is rather surprising. Apparently during the isolation procedure of the SL membrane most if not all of the AChR are solubilized or alternatively, the high salt extraction procedure (0.6 M KCl buffer) renders the AChR molecule incapable of binding to α -BuTx. Thus the presence of AChR

induced by denervation in isolated SL membrane cannot be shown by α -BuTx while it can readily be shown in the whole hemidiaphragm.

In the past few years, the assumption that the AChR induced after denervation, and the innervated EP AChR are identical have been questioned. Qualitative changes in the cholinergic receptors of muscle after denervation were observed. Pharmacological studies have indicated that the ACh receptors which are induced after denervation are less sensitive to curare (Beranek & Vyskocil, 1967). Redfern & Thesleff (1971) showed that denervation produces a qualitative change in the action potential of muscle fibers. Following denervation, the mean maximal rate of rise of action potentials was reduced by about one third and the resting membrane potential was reduced from a mean of 82 mV in normal innervated muscle to a mean of 68 mV two days after denervation. These changes after denervation could be caused by an altered association outside the endplate between receptors and ion channels, or by a genuine difference in the receptors themselves. The presence of different classes of AChR was also suggested by Miledi and Potter (1971) in their study on the binding of α -BuTx to innervated muscles. These studies suggest that two different classes of ACh receptor may exist at the endplate itself, one of which has a low affinity for curare, and therefore, the binding of α -BuTx to these receptors is not blocked by the presence of excess amounts of curare.

The fact that there is no demonstrable increase in the amount of toxin bound to the denervated SL-membrane preparations after denervation suggests that perhaps there is a difference in sensitivity of the two types of receptors (ie. innervated vs. denervated-induced) to high salt extraction. It is possible that high salt extraction, a

procedure used for the preparation of the SL-membrane, solubilizes or inactivates the AChRs which appear after denervation and therefore the increase in AChR after denervation is not demonstrable.

For the intact diaphragm muscles, the increase in α -BuTx binding after denervation is unaffected by cycloheximide treatment. Kimura and Kimura (1973) showed that the dose-response curve of the ACh supersensitivity of diaphragms 5 days after denervation was depressed by cycloheximide at a concentration of 10 μ g/day/rat post-phrenicotomy. Such cycloheximide treatment, however, was not effective in blocking the ACh supersensitivity 2 weeks after denervation. The inability of cycloheximide to block the increase in α -BuTx binding 10 days after denervation perhaps reflects Kimura and Kimura's physiological observation at 2 weeks after denervation, i.e., the increase in ACh sensitivity at 2 weeks after denervation is not blocked by cycloheximide.

E. DO THE DIFFERENT MUSCLE CELL MEMBRANE AChEs REPRESENT THE SAME
OR DIFFERENT ISOENZYME(S)?

The differences in properties of muscle cell membrane bound AChEs could be due to :

1. Presence of a different isoenzymes in different membrane types
2. The same AChE isozyme incorporated into different membrane matrices,
which in turn confers different properties to the AChE molecule.

The present study revealed similar electrophoretic mobilities of various solubilized AChEs from different types of membranes (SL vs. SR) as well as from different phyla of animals (rats vs. Electrophorus). At present the significance of the multiple forms of AChE can only be speculated upon. It is difficult to say whether all the enzymatically active regions on the acrylamide gels represent distinct enzymes in vivo, different states of enzyme aggregation or whether some of them are subunits of larger enzymes that have been dissociated during their isolation.

The similarity of the four bands of AChE activity in acrylamide gel electrophoresis of three different enzyme preparations probably indicates that they are size-isomers of one enzyme, rather than different isoenzymes.

It is quite apparent that the properties of AChE depend intimately on the immediate environment. AChE in the membrane interacts with other components in the membrane matrix, which confers characteristic properties to the AChE molecule. Collagenase treatment of SL-membrane, which probably disrupts the physicochemical properties of the membrane also changes the properties of SL-membrane bound AChE. Solubilization of membrane bound AChE results in a loss of identity of various

types of membrane bound enzyme.

Due to changes in the enzyme properties after solubilization (eg. Ca^{++} -dependent d-TC activation of +EP SL-AChE), it is difficult as well as erroneous to extrapolate experimental data obtained with solubilized enzyme preparations to the enzyme in situ.

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