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The Effects of Facilitatory Drugs at the Skeletal Neuromuscular Junction Using Intracellular End-Plate Recordings

Daryl Dean Christ
Loyola University Chicago

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THE EFFECTS OF FACILITATORY DRUGS

AT THE SKELETAL NEUROMUSCULAR JUNCTION

USING INTRACELLULAR END-PLATE RECORDINGS

A thesis presented by

DARYL DEAN CHRIST

for the degree of

DOCTOR OF PHILOSOPHY

at the

DEPARTMENT OF PHARMACOLOGY

LOYOLA UNIVERSITY STRITCH SCHOOL OF MEDICINE

February, 1969

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ABSTRACT

The effects of facilitatory drugs (neostigmine, edrophonium, ambenonium and methoxyambenonium) at the skeletal neuromuscular junction have been studied using intracellular microelectrodes in the isolated tenuissimus muscle of the cat. These drugs produced small changes in miniature end-plate potential (m.e.p.p.) frequency. The m.e.p.p.s were prolonged by neostigmine, ambenonium and methoxyambenonium, but not by edrophonium. High concentrations of neostigmine and edrophonium depressed the m.e.p.p. amplitude concurrent with a decrease in the resting membrane potential. High concentrations of ambenonium and methoxyambenonium depressed the m.e.p.p. amplitude with no change in the resting membrane potential.

The amplitude of (+)-tubocurarine end-plate potentials (e.p.p.s) was increased without change in time-course by all four drugs at a concentration which had no effect on m.e.p.p.s. The half-decay of (+)-tubocurarine e.p.p.s was prolonged at the same concentration which prolonged the m.e.p.p.s. On the other hand the facilitatory drugs produced nearly equivalent changes in (+)-tubocurarine e.p.p.s and iontophoretic acetylcholine-potentials. The drugs did not increase the quantal release in
deficient calcium and high magnesium ion concentrations. Benzoquinonium e.p.p.s were not affected by the facilitatory drugs. These results are discussed in conjunction with the current theories of neuromuscular pharmacology.
LIFE

Daryl Dean Christ was born in Buffalo Center, Iowa on November 3, 1942. He graduated from Lakota Consolidated High School, Lakota, Iowa in 1960. He attended Wartburg College, Waverly, Iowa and graduated from the State University of Iowa with the Degree of Bachelor of Science and a major in zoology.

In September, 1964, the author became a graduate student in the Department of Pharmacology, Loyola University Stritch School of Medicine. In August, 1966 he was married to Bonnie Jean Grabow. During the graduate program he has been a graduate assistant and a trainee of the National Institute of Health.
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I wish to express my utmost appreciation to Dr. L. C. Blaber for his inspiration, guidance and extreme patience, thereby making this dissertation possible. I am also indebted to many others for their moral support.

Dr. John Goode
Robert Jacobs
Joel Gallagher
Bonnie Christ
Mr. and Mrs. George Christ
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>ACh</td>
<td>acetylcholine</td>
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<tr>
<td>amp</td>
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<td>Bz</td>
<td>benzoquinonium</td>
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<tr>
<td>°C</td>
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<td>ChAc</td>
<td>choline acetyl transferase</td>
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<tr>
<td>3-CH₃-PTMA</td>
<td>3-methylphenyltrimethylammonium</td>
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<td>cm</td>
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<tr>
<td>DFP</td>
<td>diisopropylphosphofluorodate</td>
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<td>e. p.</td>
<td>end-plate</td>
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<td>e. p. p.</td>
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<td>kg</td>
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<td>m</td>
<td>quantal content</td>
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<td>molar</td>
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<td>m. e. p. p.</td>
<td>miniature end-plate potential</td>
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<td>mg</td>
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<td>mV</td>
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<td>N</td>
<td>normal</td>
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<td>O. D.</td>
<td>outside diameter</td>
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<td>3-hydroxylphenyltriethylammonium</td>
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<td>3-OH-PTMA</td>
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<td>p. a. p.</td>
<td>post-activation potentiation</td>
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<td>p. t. p.</td>
<td>post-tetanic potentiation</td>
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<td>sec</td>
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<td>Tc</td>
<td>(+)-tubocurarine</td>
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<td>TEA</td>
<td>tetraethylammonium</td>
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<td>TEPP</td>
<td>tetraethylpyrophosphate</td>
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INTRODUCTION
Motor nerve axons originate in the ventral root of the spinal cord and extend to the periphery where they make synaptic contact with skeletal muscle fibers (Sherrington, 1925 and Tiegs, 1932). The motor nerve axon is a large diameter neuron surrounded by layers of myelin, produced by an associated Schwann cell (Geren, 1954 and Robertson, 1955). Because a single motor nerve branches in the nerve trunk (Adrian, 1925) and near the muscle fiber, it can innervate between 100 and 200 muscle fibers (Fulton, 1926 and Clark, 1931). The nerve, together with its muscle fibers, is called a 'motor unit' (Sherrington, 1925). The muscle fibers of a motor unit are arranged in a longitudinal or chain-like manner (Adrian, 1925 and Cooper, 1929).

Until the development of electronmicroscopy, there was much debate concerning the anatomy of the neuromuscular junction. The nerve loses its myelin sheath at the terminal. The nerve terminal, which has a diameter of 2 to 3 microns lies in a synaptic trough on the surface of the muscle fiber (Anderson-Cedergren, 1959 and DeHarven and Coers, 1959). A Schwann cell always encloses the surface of the nerve terminal away from the synapse (Birks, Huxley and Katz, 1960). The axoplasm does not make contact with the sarcoplasm (Reger, 1954) but is separated by a five-layered membrane, the synaptolemma. The synaptolemma is com-
posed of neurilemma and sarcolemma which combine in the area of the neuromuscular junction (Robertson, 1954; Couteaux, 1958 and Reger, 1958). The nerve terminal and muscle end-plate (e.p.) are separated by a primary synaptic cleft which is 200 Angstrom units wide (Palay, 1958). In the region of the nerve terminal the muscle membrane is thrown into a series of junctional folds--secondary synaptic clefts (Palade and Palay, 1954; Reger, 1954 and Robertson, 1954). They are approximately one micron long and one-tenth micron wide, being narrower toward the axonal surface (Robertson, 1956). The junctional folds probably are the "subneural apparatus" which is so prominent in Couteaux's (1955) light microscope sections.

The axoplasm which does not dip into the junctional folds is distinguished by the presence of mitochondria (Robertson, 1956). Another distinguishing feature of the axoplasm is the occurrence of "synaptic vesicles" (300 to 500 Angstrom units in diameter) which are concentrated near dense presynaptic membrane areas directly opposite a junctional fold (Birks, Huxley and Katz, 1960 and Hubbard and Kwanbunbumpen, 1968).

Birks (1966) made an unusual observation. By using acrolein as a fixing agent, rather than osmium tetroxide, a tubular system appeared in the nerve terminal axoplasm. Thus vesicles may be a fixation artifact.
Physiology of the neuromuscular junction

In a quiescent nerve-muscle preparation, small depolarizations of the membrane occur at the end-plate region of the muscle fiber. These miniature end-plate potentials (m.e.p.p.s)—initially called "end-plate noise" (Fatt and Katz, 1950)—have been recorded in several species, including frog (Fatt and Katz, 1950), rat (Liley, 1956a), guinea pig (Brooks, 1956b), cat (Boyd and Martin, 1956a) and human (Elmqvist, Johns and Thesleff, 1960). Each m.e.p.p. is due to the spontaneous release of a single quantum, which contains many molecules of transmitter (Fatt and Katz, 1952; Liley, 1956a and Boyd and Martin, 1956a). These quanta are released from the nerve terminal (Fatt and Katz, 1952), although they may originate from other structures (Birks, Katz and Miledi, 1960).

Upon activation of the motor nerve an action potential invades the motor nerve terminal, where it initiates the release of transmitter (Hubbard and Schmidt, 1962 and Katz and Miledi, 1965c). In the unmyelinated terminal the action potential has a slow conduction velocity and long after-potentials. These characteristics are typical of an unmyelinated C-fiber (Werner, 1960a; Hubbard and Schmidt, 1961; Blaber and Bowman, 1963b; Katz and Miledi, 1965c).

When transmitter release is measured in the presence of deficient calcium ions or excess magnesium ions, the end-plate depolarization (end-plate potential—e.p.p.) becomes quite small. If the calcium ion con-
centration is sufficiently low, the e.p.p. amplitude fluctuates considerably with many of the e.p.p.s having exactly the same amplitude and time course as m.e.p.p.s. The smallest e.p.p.s which occur are essentially m.e.p.p.s and the larger e.p.p.s are multiple m.e.p.p.s (Fatt and Katz, 1952). The e.p.p. amplitude in deficient calcium follows a Poisson distribution indicating an independent release of quanta from the activated terminal (del Castillo and Katz, 1954b; Martin, 1955; Boyd and Martin, 1956b; and Liley, 1956b). On the basis of these results Liley (1956c) and Katz (1962) concluded that a nerve stimulus merely increases the rate of spontaneous release of transmitter.

On first analysis there appeared to be a correlation between the amplitude of the nerve terminal depolarization and the release of transmitter. This relationship was arrived at by correlating the calculated depolarization induced by various extracellular potassium ion concentrations with the changes of m.e.p.p. frequency induced by the corresponding potassium ion concentration. Liley (1956c) stated that a 15 millivolt (mV) depolarization produces a ten-fold increase in m.e.p.p. frequency. According to his hypothesis, the nerve terminal action potential increases the release of transmitter by increasing the m.e.p.p. frequency in a logarithmic fashion. No other steps were considered necessary for release. On the basis of Liley's results it was possible to account for
all the quanta released by an action potential. Due to the logarithmic nature of the release, most of the release would occur at the peak of the action potential, when the m.e.p.p. frequency would be extremely high (Eccles and Liley, 1959). This theory also accounted for the observation that depolarization of the nerve terminal decreased the amplitude of the e.p.p. (Hubbard and Willis, 1962b and 1968 and Vladimirova, 1964) and hyperpolarization of the terminal increased the amplitude of the e.p.p. (del Castillo and Katz, 1954b: Hubbard and Willis, 1962a and 1962c and Vladimirova, 1964). On the basis of Lloyd's results (1950) in the spinal cord, Eccles and Liley (1959) concluded that depolarization decreased the amplitude of the terminal spike and thereby, decreased transmitter release; while hyperpolarization increased the amplitude of the terminal spike and increased transmitter release.

Although this simple theory was quite attractive, data began to accumulate indicating that transmitter release involves much more than just depolarization of the nerve terminal (Hubbard and Schmidt, 1963; Katz and Miledi, 1965d, 1965e and 1967a and Hubbard, Jones and Landau, 1967). It has been shown that calcium ions are essential for neuromuscular transmission (Fatt, 1936; Cowan, 1940; Kuffler, 1944; del Castillo and Stark, 1952; Fatt and Katz, 1952; del Castillo and Katz, 1954b; Boyd and Martin, 1956b and Liley, 1956c) and for spontaneous release of transmitter.
(Boyd and Martin, 1956a; Elmqvist and Feldman, 1965a; Hubbard, 1961 and Hubbard, Jones and Landau, 1968a). Lowering the calcium ion concentration decreases transmitter release with no effect on the nerve terminal action potential (Katz and Miledi, 1965d, 1965e and 1967a). Thus Liley's theory had to be modified to include a calcium step. The exact mechanism by which calcium ions affect release of transmitter is not known. Del Castillo and Katz (1954a and 1954c) hypothesized that calcium acts with an external component of the nerve terminal (X). The complex (CaX) moves intraterminally during the terminal action potential and causes the release of a quantum of transmitter (Gage, 1967; Hubbard, Jones and Landau, 1968a; Rahamimoff, 1968 and Katz and Miledi, 1968). Rahamimoff (1963) and Dodge and Rahamimoff (1967) have further hypothesized that four calcium ions are necessary to release one quantum of transmitter. This action of calcium is quite rapid and can be shown to occur even during the falling phase of the terminal action potential (Katz and Miledi, 1967a and 1967c). Calcium can be replaced in this action by strontium or barium ions (Elmqvist and Feldman, 1965a and Miledi, 1966).

All the actions of calcium ions which have been previously described at the neuromuscular junction are antagonized by magnesium ions. The magnesium ions decrease release of transmitter by competitively inhibiting the action of calcium ions (Del Castillo and Engback, 1954; Del Castillo and

Some workers also involve sodium ions, directly, in the release of transmitter, rather than through any effects on the sodium conductance of the terminal action potential. Changing the sodium ion concentration has no effect on release in a normal ionic solution (Del Castillo and Katz, 1955b and Colomo and Rahamimoff, 1968). When the muscle is immersed in tetrodotoxin, which blocks sodium conductance changes in nerve or muscle, release is normal (Elmqvist and Feldman, 1965c and Katz and Milcidi, 1967b). But in a deficient calcium ion concentration, a decrease in the sodium ion concentration will increase m.e.p.p. frequency, augment the e.p.p. and increase the quantal content of the e.p.p. (Gage and Quastel, 1965b and 1966; Kelly, 1965; Rahamimoff and Colomo, 1967 and Colomo and Rahamimoff, 1968). It has been hypothesized that sodium ions compete extraterminaly with the calcium ions for the receptor (X).

Digoxin and ouabain inhibit the sodium-potassium pump by inhibiting ATPase. They cause an increase in the release of transmitter which must
be due to a build up of sodium ions intraterminally (Birks, 1962 and 1963 and Elmqvist and Feldman, 1965b). Thus it has been further hypothesized that sodium ions compete with calcium ions intraterminally, as well as extraterminally (Birks and Cohen, 1965, 1968a and 1968b).

Gage and Quastel (1965a) and Parsons, Hofmann and Feigen (1965) have involved potassium ions in the release of transmitter; although it is quite difficult to distinguish between indirect effects on the nerve terminal membrane potential and direct effects on transmitter release.

Chloride ions are not involved in the release of transmitter (Elmqvist and Feldman, 1966).

Hypoxia, stretch, increased osmotic pressure and increased temperature all increase m.e.p.p. frequency and all, except hypoxia, increase the release of transmitter. Hypoxia decreases release, and probably does so by blocking the sodium-potassium pump, thereby depolarizing the terminal membrane (Hubbard and Loyning, 1966). Stretch, increased osmotic pressure and increased temperature act by different mechanisms to increase release (Fatt and Katz, 1952; Boyd and Martin, 1956a and 1956b; Furshspan, 1956; Hutter and Trautwein, 1956; Liley, 1956a; Takeuchi, 1958b and Hubbard, Jones and Landau, 1968b).
Since the work of Dale and his co-workers, it is generally accepted that acetylcholine (ACh) is the transmitter at the neuromuscular junction. ACh has been isolated from a neuromuscular preparation following indirect stimulation (Dale, Feldberg and Vogt, 1936) and this ACh arises from the nerve (Straughan, 1960 and Krnjevic and Mitchell, 1961). Injected ACh mimics the effects of indirect stimulation (Brown, Dale and Feldberg, 1936 and Brown, 1937). Also, the synthesizing and degradating enzymes for ACh are present (Marney and Nachmansohn, 1933 and Nachmansohn and Machado, 1943).

ACh is synthesized from choline and acetyl co-enzyme A in the presence of the enzyme, choline acetyl transferase-ChAc (Nachmansohn and Machado, 1943 and Korkes, Del Campillo, Korey, Stern, Nachmansohn and Ochoa, 1952). The site of synthesis is not established. It has been suggested that synthesis may take place in the terminal axoplasm (Whittaker, Michaelson and Kirkland, 1964 and Michaelson, 1967), mitochondria (Bodian, 1942 and Hebb and Smallman, 1956) or synaptic vesicles (Hebb and Whittaker, 1953; Whittaker, 1959 and DeRobertis, Arnaiz, Salganicoff, Iraldi and Zieher, 1963). The ACh is stored in a bound form (Elliot and Henderson, 1951 and Brodkin and Elliot, 1953). Many workers feel that the vesicles in the electronmicrograph represent the bound form of ACh (DeRobertis and Bennett, 1955; Del Castillo and
The transmitter which is present in the nerve terminal can be divided into two functionally, distinct stores--"the available store" which is readily releaseable, and the "unavailable store" which is not readily releaseable. An indirect stimulus depletes the available store. This store is rapidly refilled (Gage and Hubbard, 1965) from the unavailable store. The unavailable store is comparatively, slowly filled; as it depends on synthesis for its transmitter (Perry, 1953; Birks and MacIntosh, 1957 and 1961 and Thies, 1960). The available store may be represented by the vesicles which are located near the region of the presynaptic membrane, while the unavailable store may be the vesicles which are further from the membrane (Hubbard and Kwanbunbumpen, 1968). Elmqvist and Quastel (1965) and Elmqvist (1965) estimate the total releaseable store to be around 200,000 quanta.

The mechanism whereby the vesicle extrudes its contents from the nerve terminal is unknown, but probably involves contact of the vesicle with the presynaptic membrane. In this way the vesicle ejects the transmitter directly into the synaptic gap (Katz, 1962; Bass and Moore, 1966 and Hubbard, Jones and Landau, 1967). Hubbard and Kwanbunbumpen (1968) were able to observe a few vesicles which were fused with the
terminal membrane. Once the transmitter is released, it rapidly diffuses throughout the synaptic gap (Eccles and Jaeger, 1958) and comes into contact with at least two types of receptors -- acetylcholinesterase and the junctional receptor. The neuromuscular junction contains a high concentration of the degrading enzyme for ACh, acetylcholinesterase (Feng and Ting, 1938; Marney and Nachmansohn, 1938; Augustinsson and Nachmansohn, 1949; Koelle and Friedenwald, 1949 and Koelle, 1950 and 1951). Most of the acetylcholinesterase occurs in the post-synaptic regions; although it is present at four sites in the junction: 1) the plasma membrane of muscle covering the junctional folds, 2) the primary and secondary synaptic clefts, 3) the plasma membrane covering the terminal, and 4) the vesicles of the terminal (Barnett, 1962 and 1966 and Miledi, 1964). The acetylcholinesterase catalyzes the hydrolysis of ACh to choline and acetate, (Matthes, 1930 and Stedman, Stedman and Easson, 1932), thereby reducing the activity of the transmitter at the cholinergic receptor by a factor of 1000 (Bacq and Brown, 1937).

The ACh also acts at the nicotinic, cholinergic receptors (Langley, 1907 and Bacq and Brown, 1937) which are located in the e.p. region of the muscle fiber (Nastuk, 1953a; Del Castillo and Katz, 1955a and Katz and Miledi, 1961a). As the muscle membrane is impermeable to ACh
(Del Castillo and Katz, 1955a), the receptors must be located on the external surface of the muscle membrane. When ACh activating a receptor, the membrane resistance decreases (Katz, 1942 and Takeuchi and Takeuchi, 1960a), due to the simultaneous increase in permeability to both sodium and potassium ions, but not to chloride ions (Del Castillo and Katz, 1954d; Takeuchi and Takeuchi, 1960b and Takeuchi, 1963). ACh does not carry the current (Nastuk, 1953b and Del Castillo and Katz, 1955a). Due to this flow of sodium and potassium ions, the e.p. membrane potential approaches the equilibrium potential of the muscle fiber -- 10 to 20 millivolts, negative (Del Castillo and Katz, 1954d and Takeuchi and Takeuchi, 1959). When the membrane potential reaches a critical depolarization--threshold--sufficient current flow is established to activate the all or none processes of the muscle fiber (Eccles, Katz and Kuffler, 1941; Kuffler, 1942 and Fatt and Katz, 1951). The action of the transmitter is very short and impulsive (Eccles, Katz and Kuffler, 1941) and the level of depolarization is determined by the concentration of transmitter in the area of the cholinergic receptor (Eccles and Jaeger, 1958). When the transmitter release is complete, the concentration of transmitter falls rapidly, as it is inactivated by acetylcholinesterase (Marney and Nachman- sohn, 1938) or diffuses from the region of the e.p. (Ogston, 1955 and Eccles and Jaeger, 1958). Then the e.p. p. passively decays in a logarithmic
fashion, until it returns to the resting membrane potential (Eccles, Katz and Kuffler, 1941; Katz, 1942 and Fatt and Katz, 1951). Although the e.p. is chemically excitable, it is probably electrically inexcitable (Werman, 1960).

Normally, the e.p.p. is quite large and always initiates a muscle action potential (Kuffler, 1942). In order to record a pure e.p.p., it is necessary to block the initiation of the muscle action potential. This can be accomplished by stimulating the nerve during a refractory state or administering (+)-tubocurarine (Göpfert and Schaefer, 1938 and Eccles and O'Connor, 1939 and 1941). In this manner it is possible to study neuromuscular transmission by studying changes in the extracellular or intracellular e.p.p. (Fatt and Katz, 1951; Nastuk, 1953b and Del Castillo and Katz, 1956).

The e.p.p. follows the stimulus after a period of latency -- the synaptic delay. The synaptic delay is not due to diffusion of transmitter across the junction or to activation of the receptor substance. It must be due to a delay in the release of transmitter, probably involving the electro-secretory coupling process (Katz and Miledi, 1965a, 1965b, 1965d, 1967a, 1967c and 1967d).
Stimulation of the nerve sets up conditions in the motor nerve terminal such that after the stimulus the probability of release is greater than in the control state (Feng, 1940; Eccles, Katz and Kuffler, 1941 and 1942; Hutter, 1952; Liley and North, 1953; Lundberg and Quilisch, 1953a and 1953b; Brooks, 1956a and 1956b; Liley, 1956a and 1956b and Hubbard, 1959 and 1963). This is referred to as post-activation potentiation (p.a.p.) or post-tetanic potentiation (p.t.p.) and occurs independently of release from the conditioning impulse (Del Castillo and Katz, 1954c and Hubbard, 1963).

The quanta of transmitter (m) which a nerve impulse releases depends on the number of quanta available for release (n) and the probability that a single quanta will be released (p).

\[ m = np \] (Martin, 1965)

During the period of post-activation potentiation each quantum has a greater probability of being released (Mallart and Martin, 1967 and 1968) and under appropriate conditions will result in an increase in the quanta released and a larger e.p.p. (Lundberg and Quilisch, 1953a and 1953b; Del Castillo and Katz, 1954c and Liley, 1956b).

There are several mechanisms by which this change of m can occur. The nerve terminal action potential has a positive (or hyperpolarizing) after-potential (Hubbard and Schmidt, 1961 and 1963) due to a prolonged increase of potassium conductance (Gage and Hubbard, 1964 and 1966a).
According to Liley's theory, this hyperpolarization would increase the amplitude of the succeeding terminal spikes and increase the release of transmitter (Eccles and Liley, 1959). But there is not complete correlation between the time course of effects of hyperpolarization and the increased release (Hubbard, 1959 and 1963; Hubbard and Willis, 1962a and 1962b and Hubbard and Schmidt, 1962 and 1963). Hubbard (1963) observed two phases of facilitation, each due to increased release of transmitter; and hypothesized that the early phase was due to mobilization of transmitter to the release sites, while the secondary phase was the result of a larger presynaptic spike from hyperpolarization. Katz and Milèdi (1967b) observed that facilitation did not depend on a change in the terminal action potential. Gage and Hubbard (1966b) concluded that post-tetanic potentiation was not due to mobilization in the terminal. They also eliminated several other possibilities, including presynaptic swelling, increased intracellular sodium and decreased intracellular potassium.

Changing the extracellular calcium ion concentration directly affects the release of transmitter, as described previously. Due to this pronounced effect of calcium ion on release, it was suggested that residual calcium intraterminally may account for facilitation (Katz and Milèdi,
Katz and Miledi (1967a and 1967c) observed that the process of transmitter release continues to operate and even increases during the falling phase of the terminal action potential. Calcium utilization occurs during the depolarization of the nerve terminal (Katz and Miledi, 1967d) and facilitation is increased only when calcium ions are present during the conditioning stimulus (Katz and Miledi, 1968). Rahamimoff (1968) suggested that four calcium ions act at a receptor to release one quantum of transmitter. Facilitation would be expected to occur if one or more calcium ions remain attached to the receptor until the next impulse arrives.

Facilitation of the e.p.p. does not occur in all situations involving more than one indirect stimulus. When the muscle is blocked by (+)-tubocurarine, the second e.p.p. of mammalian preparations and subsequent e.p.p.s of amphibian preparations become depressed (Eccles, Katz and Kuffler, 1941 and 1942 and Lundberg and Quilisch, 1953a). This depression has been shown to be due to a decreased quantal content of the test e.p.p. (Hutter, 1952; Perry, 1953; Del Castillo and Katz, 1954c; Takeuchi, 1958a; Thies, 1960 and 1965; Brooks and Thies, 1962 and Elmqvist and Quastel, 1965). Thesleff has shown some evidence for a postsynaptic desensitizing action from accumulated ACh, as a result of
the tetanus (Axelsson and Thesleff, 1958 and Thesleff, 1959); but these results have been directly countered by others who show no evidence for a desensitizing action during a tetanus (Otsuka and Endo, 1960a; Lilleheil and Naess, 1961 and Otsuka, Endo and Nonomura, 1962). It is possible that a tetanic depression may be due to a propagation failure in the fine nerve terminals (Krnjevic and Miledi, 1958 and 1959); but this type of failure occurs only at high frequencies of stimulation and would be quite obvious with unicellular recording techniques, if it did occur.

Evidence indicates that the decreased quantal content is due to a depletion of the available stores of ACh (Perry, 1953 and Hubbard and Kwanbunbumpen, 1968), even though there is an increased probability of release during the depression (Elmqvist and Quastel, 1965 and Mallart and Martin, 1968). The results from the deficient calcium experiments are different as they do not involve a depletion of the available store. Thus they show only the increased probability of release (Mallart and Martin, 1968).

In the in vivo experiments the muscle contracts supramaximally when tetanically stimulated. The tetanic contraction reaches a plateau and begins to relax from the supramaximal contraction. Upon cessation of the
tetanus, the twitch again becomes supramaximal--p.t.p. of the twitch (Guttman, Horton and Wilber, 1937; Rosenblueth and Morison, 1937; Feng, Lee, Meng and Wang, 1938 and Von Euler and Swank, 1940). The unusual aspect of this observation is the fact that the muscle can contract greater than maximally. This has been shown to occur upon direct stimulation of denervated or curarized muscles with no change in the gross muscle action potential (Brown and Von Euler, 1938 and Von Euler and Swank, 1940). Thus the supramaximal contraction involves the active state of the muscle fibers and not the neuromuscular junction (Hill, 1949; Macpherson and Wilkie, 1954; Ritchie and Wilkie, 1955 and Close and Hoh, 1968). In the frog part of the p.t.p. of the twitch can be explained by recruitment of quiescent muscle fibers (Grumback and Wilber, 1940), but this is probably due to the low safety factor of transmission in some muscle fibers of the frog (Wakabayashi and Iwasaki, 1962). This would not be expected to occur in mammals which have a high safety factor of neuromuscular transmission (Paton and Waud, 1967).

The tetanic depression is due to a decreased release of ACh, as a result of depletion. Thus many of the e.p.p.s are below threshold and the gross muscle twitch becomes smaller (Dale, Feldbert and Vogt, 1936; Brown and Von Euler, 1938 and Li and Ting, 1941).
When the motor nerve to a skeletal muscle is cut, the muscle becomes inactive for a period of 12 to 15 days (Hines, Thomson and Lazere, 1942). The distal nerve degenerates and loses its ability to initiate muscle activity. At the same time there is a decrease in the ACh and ChAc activities (Feldberg, 1943; Bannister and Scrase, 1950 and Berry and Rossiter, 1958). The nerve completely degenerates leaving only the Schwann cells (Birks, Katz and Miledi, 1960). The m.e.p.p.s which had completely disappeared shortly after sectioning the nerve, reappear after several days and must originate from a site other than the nerve -- possibly the Schwann cell (Birks, Katz and Miledi, 1960). During degeneration the whole muscle fiber becomes sensitive to ACh (Luco and Eyzaguirre, 1955; Axelsson and Thesleff, 1959; Miledi, 1959 and 1960a; Frank, 1959; Jenkinson, 1960 and Katz and Miledi, 1961b) and there is a decrease in the acetylcholinesterase concentration around the original e.p. (Snell and McIntyre, 1955 and Frank, 1959).

The distal stump of the regenerating neuron contains high concentrations of acetylcholinesterase and choline acetyl transferase, indicating that these enzymes are produced by the nerve cell body (Sawyer, 1946; Hebb and Waites, 1956 and Hebb and Silver, 1961). The regenerating stump
approaches the muscle fiber and induces the formation of an e.p. Also, there is a sensitivity decrease to ACh at the non-e.p. regions of the muscle and an increase in the acetylcholinesterase concentration at the e.p. It has been concluded that this effect of the nerve on the muscle is due to a neural factor (probably not ACh) which is released and can induce profound changes in the muscle fiber (Buller, Eccles and Eccles, 1960a and 1960b and Miledi, 1960b and 1962).
Drug-induced facilitation of neuromuscular transmission

Facilitation is defined as an increase in the twitch tension of an indirectly stimulated skeletal muscle or antagonism of (+)-tubocurarine paralysis. Drugs which produce facilitation are termed 'facilitatory drugs'. The majority of these drugs have been shown to produce some inhibition of cholinesterase.

As early as 1900, it was shown that physostigmine is capable of antagonizing neuromuscular paralysis induced by curare and this observation has since been reported many times (Pal, 1900; Rothberger, 1901; Rosenblueth, Lindsley and Morison, 1936 and Koppanyi and Vivino, 1944). Also, physostigmine can facilitate the maximal indirect twitch of the cat gastrocnemius muscle. It has no effect on nerve conduction and is ineffective when the muscle is stimulated directly in a curarized or denervated preparation. The facilitation is associated with repetitive action potentials in the muscle fibers, which occur at intervals near the absolute refractory period of the muscle fiber. This repetition acts as a tetanus and induces a tetanic-like contraction of the muscle fiber, resulting in facilitation of the muscle contraction (Brown, Dale and Feldberg, 1936 and Brown, 1937). ACh, administered close-arterially, also elicits a brief, asynchronous tetanus. Thus the drug-induced repetitive firing is probably due to maintenance of transmitter
at the muscle e.p. As physostigmine is well known for its ability to prolong the action of ACh by inhibition of cholinesterase and as it also slightly increases the ACh contraction (Englehart and Loewi, 1930; Matthes, 1930 and Bacq and Brown, 1937), it seemed reasonable for the early workers to conclude that physostigmine maintains transmitter levels at the e.p. by inhibiting junctional cholinesterase.

Later studies using extracellular recording techniques in frog and cat muscle, have shown that tubocurarine diminishes the amplitude of the e.p.p. by competing with the transmitter at the cholinergic receptor (Jenkinson, 1960) but has no effect on the time-course of the e.p.p. When the e.p.p. is decreased below threshold by tubocurarine, it is possible to record a pure e.p.p. with no distortion from muscle action potentials. Administration of physostigmine or neostigmine increases the amplitude and duration of the curarized e.p.p. (Feng, 1940; Eccles, Katz and Kuffler, 1942 and Eccles and MacFarlane, 1949). Physostigmine also prolongs the active phase of the e.p.p. by a factor of two or three. Thus the transmitter is acting for a longer period of time at the e.p. when physostigmine is present (Katz, 1942).

In a non-curarized preparation (refractory) physostigmine increases
the amplitude and time-course of the e.p.p. When the e.p.p. is longer than the refractory period of the muscle fiber, repetitive discharges are initiated in the muscle fiber. Even in the presence of physostigmine the fast e.p.p. is quite rapid, indicating that a physostigmine-resistant mechanism of halting the action of the transmitter is occurring—probably diffusion.

These results on the fast e.p.p. are compatible, and indeed broaden, the theory of cholinesterase inhibition. Thus it was hypothesized that physostigmine and neostigmine by inhibiting cholinesterase prolong the e.p.p., such that repetitive activity is induced in the muscle. The repetitive activity gives rise to a tetanic muscle contraction.

Physostigmine also sets up a long secondary negative potential change. The secondary potential has a maximal amplitude of 40% of the spike height and it may persist for a second or more. This wave is readily abolished by subparalytic doses of curare which have little effect on the fast e.p.p. As these effects occur with no change in the muscle membrane electric time constant, and as tubocurarine has no effect on cholinesterase inhibition in vitro, it is difficult to explain this action of tubocurarine (Eccles, Katz and Kuffler, 1942).
Although most of the previous results were compatible with the theory that facilitatory drugs acted solely by inhibiting cholinesterase, none of the experiments provided conclusive evidence that prolongation of transmitter action was due to cholinesterase inhibition. Thus many workers searched for evidence which would establish that cholinesterase activity at the neuromuscular junction was decreased during the action of the facilitatory drugs. The most common study involved correlating inhibition of cholinesterase (such as, serum cholinesterase or electric eel cholinesterase) with the anticycurare potency of these drugs (Bacq and Brown, 1937; Eccles, Katz and Kuffler, 1942; Bulbring and Chou, 1947; Blascho, Bulbring and Chou, 1949; Randall and Lehmann, 1950; Hobbiger, 1952 and Karczmar and Howard, 1955) The results of these experiments were quite conflicting, and the probable reason for the discrepancies was brought out in a statement by Augustinsson (1959), "Plasma cholinesterases are regarded as a group of esterases with much divergent properties with regard to specificity against various choline and non-choline esters, sensitivity to inhibitors and activity-substrate relationships. It is not possible to apply results obtained with the plasma of one species to any other species." This statement was well illustrated with muscle cholinesterases by Blaber and Bowman (1962). They reported that ambenonium was 30 times as potent as neostigmine in inhibiting cat muscle
cholinesterase, but 1/200 as potent as neostigmine in inhibiting hen muscle cholinesterase. Thus conclusive experiments must involve a study of the acetylcholinesterase of the neuromuscular junction and facilitatory or anticurare action of a drug at a neuromuscular junction of the same species.

If cholinesterase is previously inhibited, any drug which acts by inhibiting cholinesterase should be ineffective. Hobbiger (1952) observed that previous inhibition of cholinesterase blocked the anticurare action of the facilitatory drugs. Neostigmine, applied iontophoretically, blocked potentiation of the ACh-potential by edrophonium (Katz and Thesleff, 1957a). Koelle (1957), using the observation that physostigmine pretreatment (Koster, 1946) or neostigmine pretreatment (Koelle, 1946) protected cholinesterase from irreversible inhibition by DFP, observed by histochemical methods that ambenonium and methoxyambenonium in concentrations, which were pharmacologically active, protected the junctional cholinesterase from DFP inhibition. These results indicated that ambenonium and methoxyambenonium were inhibiting junctional cholinesterase when they antagonized curare; but as stated by Koelle (1957) this does not show that cholinesterase inhibition was the important mechanism of action.
The facilitatory drugs cause not only potentiation of the neuromuscular twitch, but also depression at high doses (Bacq and Brown, 1937; Rosenblueth and Morison, 1937; Van Der Meer and Meeter, 1956b and Riker, Roberts, Standaert and Fujimori, 1957). In toxicity studies this depression appeared to be due to cholinesterase inhibition (Bodansky and Mazur, 1946 and Nachmansohn and Field, 1947). TEPP, tetraethylpyrophosphate, caused a depolarization of the e.p. at high doses, but was not capable of eliciting a twitch at any dose level. Thus Douglas and Paton (1954) concluded that TEPP induced depression of the e.p. by inhibiting junctional cholinesterase. Yet many workers have shown that total inhibition of cholinesterase did not block the indirect twitch (Berry and Evans, 1951; Barnes and Duff, 1953; Barstad, 1956b and Van Der Meer and Meeter, 1956a). Douglas and Matthews (1952) observed that TEPP blockade was readily reversible, even though TEPP was an irreversible cholinesterase inhibitor. On the basis of this type of data Barstad (1956a) concluded that DFP, in addition to its cholinesterase inhibiting properties, also exhibited a curare-like action at the e.p.

The theory of cholinesterase inhibition by facilitatory drugs was generally accepted until 1946. Then Riker and Wescoe (1946) observed that neostigmine produced a contraction of skeletal muscle, even though the acetylcholinesterase was previously inhibited by DFP--
diisopropylphosphofluoridate, a very potent, irreversible cholinesterase inhibitor (Mazur and Bodansky, 1946; Mackworth and Webb, 1948 and Michel and Krop, 1951). They concluded the primary effect of neostigmine was a direct, depolarizing action (like ACh) at the e.p. and did not involve cholinesterase inhibition. Several analogues of neostigmine, including 3-hydroxy phenyltrimethylammonium (3-OH-PTMA), which facilitated the neuromuscular twitch and antagonized tubocurarine blockade of the twitch, also stimulated denervated muscle. As 3-OH-PTMA lacked the carbamate grouping which was responsible for cholinesterase inhibition (Stedman, 1926), Riker concluded these compounds acted via a direct, stimulatory action and that other facilitatory drugs had the same mechanism of action (Randall, 1950; Randall and Lehmann, 1950 and Riker and Wescoe, 1950).

Edrophonium (3-hydroxy phenyldimethylethylammonium) was more specific than neostigmine in its anticurare action at the neuromuscular junction—it had fewer cholinergic side effects. Randall and Jampolsky (1955) hypothesized that neostigmine acted by cholinesterase inhibition, while edrophonium acted by a direct, stimulatory action on skeletal muscle.

Smith, Cohen, Pelikan and Unna (1952) observed that neostigmine, physostigmine and edrophonium exhibited direct stimulatory effects on the
frog rectus preparation which were 1/560, 1/75,000 and 1/216 as effective, respectively, as ACh. These drugs were at least one-thousand times as effective in potentiating an ACh contracture, as in producing a direct, stimulatory effect. Furthermore there was no correlation between the stimulatory effect and the potentiation of ACh contractures. They concluded that the effects of the facilitatory drugs on the frog rectus could not be due to a direct, stimulatory action.

Edrophonium did not depolarize the muscle e.p. except at concentrations which were much higher than those necessary to produce facilitation of the muscle twitch in the frog (Nastuk and Alexander, 1954).

Katz and Thesleff (1957a), using microelectrode iontophoretic techniques, applied ACh to a frog muscle e.p. If minimal doses of edrophonium were applied, the ACh-potential was augmented. This same dose of edrophonium, alone, produced no depolarization. Only at much higher doses was edrophonium capable of depolarizing the e.p. Also, edrophonium did not potentiate a depolarization due to carbachol (a stable choline ester), as would be expected if edrophonium was acting by a direct, depolarizing action. These results could only be explained by the theory that edrophonium inhibited cholinesterase. Although it was not possible to determine what concentration of edrophonium was necessary
to inhibit cholinesterase, these results were the first to show conclusively that acetylcholinesterase inhibition at the neuromuscular junction produced a physiological effect—prolongation of the action of ACh.

Randall (1950) and Riker (1953) suggested that displacement of tubocurarine from the e.p. receptor could explain the antagonistic action of edrophonium. Yet when tubocurarine was displaced from the receptor by rinsing the muscle in non-curarized Ringer, the amplitude of the e.p.p. was augmented with no change in time-course. Edrophonium increased both the amplitude and time course of the e.p.p. (Nastuk and Alexander, 1954).

Succinylcholine, like decamethonium, acted at the mammalian neuromuscular junction by depolarizing the muscle e.p. (Burns and Paton, 1951). Initially this depolarization increased the excitability of the muscle, such that facilitation of the muscle twitch occurred. After prolonged depolarization, there was a secondary depression of the muscle twitch (Zaimis, 1951). Methoxyambenonium was capable of antagonizing depolarization block by succinylcholine and decamethonium (Karczmar, 1957) and competitive block by tubocurarine (Lands, Karczmar, Howard and Arnold, 1955). These results seemed to contradict the classical theory of neuromuscular pharmacology—blocking drugs must act by depolarization or competition, and depolarizers and competitors
are mutually antagonistic. In addition pretreatment with methoxyambenonium increased the succinylcholine facilitation, but decreased the secondary blockade. Karczmar (1957 and 1961) stated that the underlying mechanism of neuromuscular facilitation was more complex than originally thought and "may depend on more sites of action."

ACh was known to have a desensitizing action in isolated amphibian preparations (Thesleff, 1955a, 1955b and 1958 and Axelsson and Thesleff, 1958) and methoxyambenonium readily restored the sensitivity of muscles which had been desensitized by ACh (Kim and Karczmar, 1967). Thus a sensitizing action which had been hypothesized for other facilitatory drugs (Zaimis, 1951; Cohen and Posthumus, 1955 and Van Der Meer and Meeter, 1956b) was hypothesized by Karczmar, Kim and Koketsu (1961) to be involved in the mechanism of action of methoxyambenonium.

This theory was countered by other evidence. Edrophonium was not capable of potentiating the depolarization from an iontophoretic application of carbachol (Katz and Thesleff, 1957a). Blaber (1960) observed that methoxyambenonium and ambenonium exhibited curarimimetic properties at doses higher than were necessary to antagonize tubocurarine. Thus it was concluded that antagonism to depolarizing block was due to this
curarimimetic activity and not due to sensitization. This was further supported by the observation that succinylcholine blockade was antagonized by appropriate mixtures of neostigmine and tubocurarine (Blaber and Karczmar, 1967a and 1967b).

Masland and Wigton (1940) investigated the origin of spontaneous twitching which occurred after administration of facilitatory drugs (Langley and Kato, 1915). Upon administration of neostigmine, spontaneous antidromic action potentials and antidromic potentials after an indirect stimulus were recorded in the ventral root of the cat spinal cord. Masland and Wigton concluded that the antidromic impulses originated in the region of the motor nerve endings. As ACh also produced the spontaneous activity in the nerve it seemed likely that neostigmine acted by causing an accumulation of ACh at the neuromuscular junction, thus allowing ACh to act at the nerve endings (Eccles, 1964 and Hubbard, 1965).

Physostigmine also produced antidromic activity in the cat. Feng and Li (1941) concluded that physostigmine acted directly on the presynaptic nerve endings in the mammalian preparation. Yet there was not complete correlation between repetitive firing of the nerve and repetitive firing of the muscle. Due to this inconsistency, it seemed likely that the nerve terminal was not the site of drug action, but was
merely stimulated by the prolonged e.p. current in the presence of physostigmine (Eccles, Katz and Kuffler, 1942). Antidromic activity was obvious in the nerve following direct stimulation of the muscle. After indirect stimulation the repetitive activity of the muscle appeared to occur earlier than the repetitive activity in the nerve. Also, antidromic activity occurred in nerves which were not stimulated and was decreased when the nerve stimulus was desynchronized. These results indicated that muscle action potentials were capable of depolarizing the nerve terminals, such that antidromic activity was initiated. This depolarization of the nerve terminal in turn induced repetitive activity in the muscle, resulting in a tetanic-like twitch (Lloyd, 1941 and 1942 and Brown and Matthews, 1960). According to these theories all the effects of physostigmine can be explained on the basis of inhibition of junctional cholinesterase.

Barstad (1962) observed that antidromic nerve discharges could be elicited even though the muscle fibers were cut and could not respond to a stimulus. Upon more careful examination, two types of antidromic nerve activity were observed by Werner (1960a, 1960b and 1961) in the presence of the facilitatory drugs. The early phase had a very short latency and was due to the ephaptic backfiring from the muscle action
potentials as described by Lloyd and Brown and Matthews. But there was also a secondary phase of antidromic activity which had a longer latency. This secondary phase could only be explained by the theory that a long generator potential occurred in the motor nerve terminal (Werner, 1960b). This generator potential probably was the negative afterpotential of the nerve terminal spike. Thus paired stimuli with a short interval increased the antidromic activity in the nerve and paired stimuli of a long interval depressed antidromic activity in the nerve (Werner, 1960a and Blaber and Bowman, 1963b). This theory was supported by the observation that antidromic activity which occurred in unstimulated nerve fibers had a short latency. The secondary activity, which had a longer latency, occurred only in nerve fibers which had been stimulated (Werner, 1961).

There was one discrepancy in these results. Werner (1960a) by using paired stimuli concluded that the negative afterpotential of the motor nerve terminal had a duration of only 5 msec. Yet the antidromic activity after an orthodromic stimulus in the presence of a facilitatory drug had a much longer duration; thus Blaber and Bowman (1963b) concluded that the negative afterpotential was also longer. The short duration observed by Werner (1960a) was probably due to extinction of orthodromic stimuli by antidromic stimuli (Blaber and Bowman, 1963a). Hubbard, Schmidt and Yokota (1965) also observed a longer duration for the negative
afterpotential of the motor nerve terminal.

Pentobarbital, procaine and cyclopropane blocked antidromic nerve activity induced by 3-OH-PTEA (3 hydroxyphenyltriethylammonium), but these anesthetics had no effects on the facilitation of neuromuscular twitch which was produced by 3-OH-PTEA (Riker, Werner, Roberts and Kuperman, 1959). Thus the nerve terminal generator was distinguished from the muscle generator of repetitive activity. Tubocurarine blocked the nerve terminal generator potential at lower doses than it blocked the ephaptic activity. On the basis of these results it was concluded that the safety factor for axonal excitation by the terminal generator was narrower than the safety factor for transmission to postsynaptic units (Werner, 1959 and 1961).

The facilitatory drugs produced facilitation of the mammalian neuromuscular twitch, rather easily. The amphibian muscle was much less responsive to the actions of these drugs (Raventos, 1937), although it was possible to produce facilitation of the amphibian muscle twitch with physostigmine or edrophonium, when a stimulus rate no greater than one per minute was used (Feng, 1937; Hodes and Steiman, 1939 and Nastuk and Alexander, 1954). This difficulty in obtaining facilitation
was paralleled by a lack of antidromic activity in the presence of the facilitatory drugs (Dun and Feng, 1940). These results may point to a fundamental difference in the pharmacological action of the facilitatory drugs at the mammalian as compared to the amphibian neuromuscular junction (Blaber and Karczmar, 1967b).

Riker and co-workers (1957) began an investigation of the nerve terminal activity of a series of phenolic compounds. These compounds were structurally similar to neostigmine, except they were considerably less potent than neostigmine in inhibiting acetylcholinesterase. 3-OH-PTMA facilitated the neuromuscular twitch at low doses and blocked the neuromuscular twitch at high doses. When the hydroxyl group was eliminated, as in PTMA (phenyltrimethylammonium) or PTEA (phenyltriethylammonium), the ability to facilitate the twitch was lost. As 3-CH₃-PTMA (3 methylphenyltrimethylammonium) was ineffective, the ability to facilitate the twitch seemed to require the presence of a hydroxyl or carbamyl grouping in the meta position. The meta grouping was also necessary to initiate antidromic activity in the motor nerve. On the basis of these results Riker, Roberts, Standaert and Fujimori (1957) concluded that 3-OH PTMA and its analogues which also facilitated the twitch produced their facilitatory effects by an action on the motor nerve terminal (Kuperman, Gill and Riker, 1961). As the ethyl analogues showed
no depression, they further concluded that the depressing activity was due to depolarization and was related to the number of methyl groups on the quaternary nitrogen (Randall, 1950 and Smith, Cohen, Pelikan and Unna, 1952).

Edrophonium, ambenonium and neostigmine facilitated the neuromuscular twitch and induced antidromic activity in the ventral root. Methoxyambenonium depressed the sensitivity of the e.p. and nerve terminal and probably, for this reason could not facilitate the muscle twitch or induce antidromic nerve activity (Blaber, 1960). Goode, Blaber and Karczmar (1965), using Koelle's (1957) technique, observed that it was possible to facilitate neuromuscular transmission with doses of edrophonium and neostigmine which did not protect cholinesterase from inhibition by DFP. This may indicate another mechanism of action for these drugs although such a conclusion, at least with edrophonium was not completely justified as kinetic studies have shown that inhibition of cholinesterase by edrophonium was very rapidly reversed, and therefore could not be expected to protect from DFP (Wilson, 1955 and Katz and Thesleff, 1957a).

Intravenous edrophonium and close-arterial edrophonium and neostigmine facilitated the neuromuscular twitch but did not increase the contracture from close-arterially injected ACh. Methoxyambenonium
(intravenously or close-arterially) antagonized tubocurarine but did not augment the ACh-contracture. If the facilitatory drugs acted by inhibiting junctional cholinesterase, the ACh-contracture and indirect muscle twitch should be increased at the same dose. Furthermore, there was no correlation between facilitation or ant curare action and the cholinesterase inhibiting activity of these drugs. On the basis of these results Blaber and Bowman (1959 and 1963a) and Blaber (1960 and 1963) concluded that the facilitatory drugs produced their effects by a direct action on the motor nerve terminal.

The development of unicellular recording techniques opened a new realm in which to study the effects of drugs at the neuromuscular junction. Fatt and Katz (1950 and 1952) in their early experiments in the frog observed that neostigmine increased the amplitude and half-decay time, but not the frequency of m.e.p.p.s. As hypothesized by Katz (1958 and 1962) experimental changes of m.e.p.p. time-course involve a postsynaptic mechanism while experimental changes of m.e.p.p. frequency involve a presynaptic mechanism. Liley (1956a) reported similar observations with neostigmine in the rat diaphragm, but Boyd and Martin (1956a) observed small increases in the frequency of m.e.p.p.s in the cat tenuissimus.
Neostigmine augmented the amplitude of a curarized e.p.p. (Fatt and Katz, 1951 and Boyd and Martin, 1956b) but produced relatively small changes in the time-course. Neostigmine greatly prolonged the time-course of an e.p.p. which was recorded in a ringer solution that had 20% of the control sodium ion concentration (Fatt and Katz, 1951). Increases of the e.p.p. were accompanied by proportional increases in e.p. current (Takeuchi and Takeuchi, 1959).

Several drugs have been shown to produce effects on the e.p.p. which are very similar to those reported for neostigmine, but these drugs do not act by inhibiting junctional cholinesterase. TEA (Stovner, 1958 and Koketsu, 1958), guanidine (Otsuka and Endo, 1960b) and phenol (Otsuka and Nonomura, 1963) have been shown to increase the release of transmitter. Thus the present investigation will be undertaken to try to determine the mechanism by which the facilitatory drugs produce their effects at the neuromuscular junction. The drugs which will be used are: 1) ambenonium, a very potent acetylcholinesterase inhibitor; 2) neostigmine, a classical facilitatory drug; 3) edrophonium, a potent facilitatory drug which has been reported to be a very weak acetylcholinesterase inhibitor and 4) methoxyambenonium, a potent antagonist of tubocurarine blockade that is not able to facilitate the maximal muscle twitch. It also has weak acetylcholinesterase inhibiting properties.
NEOSTIGMINE

EDROPHONIUM

AMBENONIUM

METHOXYAMBENONIUM
METHODS
I. Anatomy of the Tenuissimus Muscle of the Cat

All experiments were performed on the tenuissimus muscle of the cat. This muscle was chosen as it is very similar pharmacologically to the gastrocnemius and tibialis muscles which have been used in in vivo studies (MacLagen, 1962). The tenuissimus muscle originates from the transverse process of the second caudal vertebra. It lies between the biceps femoris and semimembranous muscles in the sciatic notch and inserts on fascia of the shank (Hyman, 1962). As the tenuissimus muscle is quite long (>5 cm.) and thin (1 mm) it makes a very good isolated tissue preparation. Ringer solution can reach most of the fibers in the muscle, which is not true of such large muscles as the tibialis anterior and gastrocnemius. The tenuissimus nerve arises from the sciatic nerve. Just before entering the tenuissimus muscle, the nerve trunk divides -- one branch passing anteriorly and the other branch passing posteriorly -- and enters the muscle. Each branch passes superficially down the middle of the muscle giving off nerve fibers to the muscle fibers.

II. Removal of Muscle

Cats were anesthetized with 70 mg/kg alpha-chloralose intraperitoneally, 5 mg/kg pentobarbital sodium facilitated induction and 1.5 mg/kg atropine aided in blocking mucous secretions. The sciatic notch was
opened to expose the tenuissimus muscle and nerve. The tenuissimus nerve was dissected free from the sciatic nerve so that a segment of nerve about 3 cm long could be obtained. The muscle was then dissected free. In general, the distal portion of the muscle (below the entry of the nerve) was used in the experimental procedure. The muscle was placed in a muscle bath. Removal of connective tissue from the muscle made it possible to see the superficial nerve fibers with the aid of a microscope. Pinning the muscle securely to the bottom of the bath eliminated extra-neous movements. The ends of the muscle were then cut distal to the pins. The nerve was placed over the bipolar platinum electrodes (5 mm apart) in a separate compartment of the bath (except in m.e.p.p. studies when the nerve was not removed from the animal).

III. Perfusion of Muscle

Krebs solution was used to superfuse the isolated tissue. All reagents except NaCl, D-glucose, and NaHCO₃ were kept in stock solutions. Thus addition of 2 ml of each of the stock solutions to a liter of distilled water gave the proper concentrations (Table 1) (Boyd and Martin, 1956a). The NaCl, D-glucose, and NaHCO₃ were measured as dry weight. This solution was aerated with 95% O₂ and 5% CO₂ through a sintered glass aerator in a 50 ml bath before passing into the isolated muscle bath.
Table 1

Composition of the Kreb's solution superfusing
the isolated tenuissimus muscle.

<table>
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<th>Value</th>
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<tr>
<td>NaCl</td>
<td>115 mM</td>
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<tr>
<td>KCl</td>
<td>4.60 mM</td>
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<tr>
<td>KH₂PO₄</td>
<td>1.15 mM</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>24.1 mM</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>2.46 mM</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>1.15 mM</td>
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<tr>
<td>Glucose</td>
<td>8.85 mM</td>
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</table>
(Another bath was used for drug-Krebs solution.) The 50 ml bath had a warm water jacket, controlled by a Haake constant temperature circulation pump, so that the temperature of the Krebs solution was raised to approximately 40°C. The Krebs solution then flowed into the muscle bath via a narrow diameter (PE 90 - I. D. 0.034", O. D. 0.050") polyethylene tubing of approximately 16 inches in length. The tubing also ran through a warm water jacket again to reach a constant temperature before entering the muscle bath. The polyethylene tubing was placed in the muscle bath and the Krebs solution was allowed to run freely into the muscle bath. The flow was constant for any experiment (200-250 ml/hr) and was determined by the length of polyethylene tubing and height of the 50 ml bath. The temperature was constant (it depended mainly on flow rate) but could be varied by a temperature control on the circulation pump such that the final temperature in the muscle bath was constant at approximately 37°C (36-38°C).

The muscle bath had the following dimensions -- 0.6 cm x 8.0 cm x 0.5 cm. The nerve compartment measured 1.0 cm x 1.0 cm x 0.5 cm. Thus the total volume of the bath was approximately 3 ml. This allowed for rapid turnover of solutions (2x volume in 1.5 min.) with a minimal amount of movement. The solution was removed by overflow into a drain.
The only adjustment necessary to change to new solution was to move a clamp from one tubing to another. The rate and final temperature were constant throughout the experiment. A muscle could be maintained for at least 10 hours without significant decay of membrane potential or neuromuscular transmission.

IV. The Making and Filling of Electrodes

Microelectrodes were made from pyrex capillary tubing (0.1 cm diameter - 7 cm length). The tubing was pulled to a narrow tip with a David Kopf #700 vertical pipette puller using nichrome heater coils. The electrodes were floated on distilled water and examined under a microscope (100x magnification factor) to obtain a rough estimate of the tip size (determined by distance water passes up electrode) and general contour of the tip and shank of the electrode. Only those electrodes with narrow tips (little water passing in) and narrow shanks were filled. All others were discarded. The electrodes were then boiled in filtered methyl or ethyl alcohol for 3 minutes using a Precision Scientific vacuum pump to fill the electrodes with alcohol. The alcohol was then replaced with distilled water by immersing the alcohol filled electrodes in filtered distilled water for 30 minutes. Finally, the electrodes were immersed in filtered 3M KCl until the day of use (at least 24 hours).
When electrodes were made for iontophoretic studies, they were immersed in ACh or carbachol (0.3 M to 3M), rather than KCl. By storing in a refrigerator, these electrodes were usable for periods up to several months.

V. Recording Apparatus

Recordings were made using the intracellular technique developed by Ling and Gerard (1949) and Nastuk and Hodgkin (1950). The electrode was supported in a glass tubing (I. D. 0.2 cm) which was filled with an agar - 3 M KCl column. The agar - 3 M KCl column made connection with a silver chloride coated-silver wire spiral to eliminate a junction potential. An indifferent electrode which made contact with Krebs solution consisted of an agar - Krebs column in contact with a silver-silver chloride spiral wire. (The silver wire spiral was coated with silver chloride by electrolysis in 0.1 N HCl. (2Cl^- + Ag -> AgCl_2 + 2 e^-.) The two inputs (one from each agar bridge) were connected to a Nikkon hi-input impedance amplifier. The output of this pre-amplifier led into a Tektronics Type 502 dual beam oscilloscope. This trace was used to monitor the membrane potential of the muscle cell. A second output from the preamplifier led into a Tektronics Type 122 low-level preamplifier. This output led into two Tektronics Type 502 dual beam oscilloscopes. The first oscilloscope which was monitoring
DIAGRAM OF PERFUSION AND RECORDING APPARATUS
the membrane potential (low gain) was also used as a monitoring scope for the end-plate phenomena (high gain). The second oscilloscope had only the end-plate phenomena and was used for recording purposes. Permanent records were made with an Analab oscilloscope camera. Thirty-five millimeter still or continuous motion pictures could be taken of the potentials depending on the particular experiment.

VI. Selecting an Electrode

Electrodes were selected on the basis of their resistance and ability to penetrate the muscle fiber without causing considerable damage. In general, a resistance of less than 20 megohms was necessary to make the noise level sufficiently small to record miniature potentials. If the resistance was less than 5 megohms, the electrode tip was usually too large and therefore damaged the muscle fiber. After an electrode of the correct resistance had been obtained, the electrode was placed in several muscle fibers. If the electrode gave a deflection equivalent or greater than 60 mV and there was no significant decay of the resting membrane potential, the electrode was considered usable. If there were no deflections of greater than 60 mV for resting membrane potentials, or the resting membrane potential decayed constantly, the electrode was discarded. An AC square wave signal was passed through the electrode
and by means of a capacity compensator in the Nikkon hi-input impedance amplifier, it was possible to keep the time-constant of the system below 70 µsec.

VII. Locating an End-Plate and Recording M. e. p. p. s

Connective tissue on the surface of the muscle was removed to expose the nervous structure of the muscle (small superficial branches come off a large central nerve). The superficial nerve branches were located with the aid of a binocular stereo microscope (magnification - 15x). The microelectrode and indifferent electrode were carried on a Narishige micromanipulator. The microelectrode was generally inserted in the area of a small superficial nerve. As a result, the probability of locating an e. p. was quite high. The presence of m. e. p. p. s indicated that the electrode was near the e. p. region of the muscle. The electrode was usually reinserted into the muscle until a point was found where the m. e. p. p. s were of maximal amplitude and shortest time-course. The resting membrane potential was also recorded to show that the muscle fiber was in good condition and not decaying over a long time period. Pictures of the m. e. p. p frequency were taken on a slow sweep (0.1 cm/sec) and time course were taken on a rapid sweep (2 msec/cm).
VIII. Locating an End-Plate and Recording (+)-Tubocurarine, High Magnesium and Benzoquinonium E.p.p.s

The nerve to the tenuissimus muscle was placed over bipolar platinum electrodes (0.5 cm apart) in a separate compartment of the bath. The nerve was stimulated at 2 x maximal voltage with a pulse of 50μsec at a rate of 1/2.5 to 1/10 stimulus per second. The muscle twitch was blocked by the lowest possible concentration of (+)-tubocurarine (2 to 5 μg/ml), benzoquinonium (1.5 to 3 μg/ml) or decreased calcium and increased magnesium. The blocking agent was added to the Ringer solution and infused through a second 50 ml bath system. An e.p.p. was localized in a manner similar to that for the m.e.p.p.s. Amplitude was the major criteria. An amplitude of greater than 3 mv was generally used (except in quantal content studies). The half-decay was usually below 2 msec (rise to one-half fall) and the rise-time was less than 1 msec. This e.p.p. was then used as control for drug-induced effects.

IX. Iontophoretic Application of Drugs

In this series of experiments the recording apparatus was the same as previously described, except only DC-amplification was used. The muscle twitch was always blocked with (+)-tubocurarine (4 μg/ml). The iontophoretic electrode (with ACh or carbachol) was supported in a second micromanipulator. Two stimulators were necessary, one to
eject the drug and another to retard diffusion of the drug from the tip of the electrode. Each stimulator was connected to an isolation unit. The two isolation units were connected in series, with one lead from the isolators passing through a 20 megohm resistance into the iontophoretic electrode. The other lead passed through 47 kohm resistance into the bath. The end of each lead was coated with silver chloride. By recording the voltage change across the 47 kohm resistance it was possible to determine the amount of current (as ACh or carbachol) which passed from the electrode. This current recording was used as a monitor to be certain that equal doses of drug were ejected with each pulse.

After an e.p.p. had been located, the iontophoretic electrode was moved into the region of the e.p. By careful maneuvering of this electrode, it was possible to produce a rapid depolarization of the e.p. from application of ACh or carbachol. These drug-induced potentials remained relatively constant in amplitude and time-course. Rapid iontophoretic potentials (half-decay less than 100 msec) required precise localization at the e.p. Any movement produced large changes in the iontophoretic potential. As these experiments required long infusion times, it seemed desirable to use slower potentials (100 to 500 msec half-decay). Then movements were not so critical. The pulse voltage and duration for
drug ejection were varied such that the amplitude of the iontophoretic potential was similar to the amplitude of the e.p.p. The braking current (opposite polarity) was adjusted, such that no decay of the membrane was obvious from diffusing drug.

X. Infusion of Facilitatory Drugs

The agents used were neostigmine bromide, edrophonium chloride, ambenonium chloride, and methoxyambenonium chloride. The facilitatory drug was added to a volume of the control Krebs-Ringer solution and infused through a second 50 ml bath. The initial concentration of the facilitatory drug was $10^{-8}$ M (except in the case of ambenonium where due to its high potency, the initial concentration was $10^{-10}$ M or $10^{-12}$ M) and the concentration was increased by 10 times every 15 - 20 minutes until a predominate postsynaptic action occurred - twitching, depolarizing (decreased amplitude and decreased resting membrane potential) or competitive blockade (decreased amplitude but no effect on the resting membrane potential). The muscle was then returned to a control Krebs-Ringer solution to determine if the drug was reversible or irreversible. Drugs were infused for a period of ten minutes before recordings were made.

XI. Measuring the End-Plate Phenomena

The film which had been exposed to the e.p. phenomena was projected on a Durst 606 Enlarger such that the potentials were magnified 2.5x in
order to make the necessary measurements.

M.e.p.p. frequency was determined from a series of traces which were filmed at a sweep speed of 0.1 sec/cm or 1 sec/sweep for a period of 25 seconds. Five such samples were taken at 2 minute intervals for each experimental situation. The standard error of the five samples was calculated as well as t-values for significant changes due to the drugs.

M.e.p.p. time-course was recorded on fast multiple sweeps (2 msec/cm) by holding the camera shutter open until a m.e.p.p. occurred on the monitor scope. This resulted in a thick base line. In each experimental situation, 10 m.e.p.p.s were measured for 1) amplitude, 2) rise-time, and 3) half-decay time and the average was calculated.

E.p.p.s were also recorded on a fast sweep (2 msec/cm) which could be triggered to give a single sweep. Again the 1) amplitude, 2) rise-time and 3) half-decay time were measured from ten samples in each experimental situation.

In iontophoretic studies e.p.p.s and iontophoretic potentials were recorded on single, slow sweeps (100 to 500 msec/cm). 1) E.p.p. amplitude and iontophoretic potential 2) amplitude 3) rise time and 4) half-decay time were measured from five records for each experimental
condition.

Standard deviation and t-values were calculated for each group of samples.

The effect of facilitatory drugs on quantal content was calculated from the number of e.p.p. failures during 200 nerve stimuli with decreased calcium (0.92nM) and increased magnesium (5-7 mM) ion concentrations or by comparing the amplitudes of 50 m.e.p.p.s and 50 e.p.p.s with increased magnesium ions (12-15 mM) (Del Castillo and Katz, 1954c).
RESULTS SECTION 1

EFFECTS OF FACILITATORY DRUGS ON M. E. P. P. S AND (+)-TUBOCURARINE E. P. P. S.
INTRODUCTION

Several microelectrode techniques are commonly used to distinguish presynaptic and postsynaptic mechanisms of drug action. The most direct technique involves the effects of drugs on m.e.p.p. frequency and time-course (Katz, 1958, and 1962). Also, a comparison of the amplitude and time-course of m.e.p.p.s and e.p.p.s, and observation of the e.p. membrane potential at various drug concentrations should enable a distinction between: 1) a direct, depolarizing action, 2) sensitization of the e.p. receptor, 3) cholinesterase inhibition and 4) a pre-junctional action.
RESULTS

Effects of neostigmine on m. e. p. p. s

The effect of neostigmine on m. e. p. p frequency was to produce a small but significant increase in three out of four experiments. The effects on frequency were not readily reversible and the frequency remained at the increased level after the muscle had been returned to Krebs solution for 90 minutes.

No effects on amplitude or rise-time were noted when the perfusion solution contained $10^{-8}$ M neostigmine. With $10^{-7}$ M neostigmine, the increase in both the amplitude and the time course became very significant ($P' = 0.001$) and the parameters were further increased by $10^{-6}$ M neostigmine. With $10^{-5}$ M the amplitude and time course of m. e. p. p. s. decreased as the membrane potential fell from 60 to 39 mV. At $10^{-6}$ M the amplitude increased by 67%, the rise-time by 86% and the greatest increase was the half-decay which increased by 188%. The effects on amplitude and time course, and the depolarization of the end-plate membrane were all reversed after returning the muscle to Krebs solution for 90 min. An example of the effects of the neostigmine on m. e. p. p. s. is given in Table 2A.
### TABLE 2A

**Effects of Neostigmine on M. e. p. p. s.**

<table>
<thead>
<tr>
<th>Concentration of neostigmine (M)</th>
<th>Frequency of m. e. p. p. s. (sec⁻¹)ᵃ</th>
<th>Amplitude (mV)ᵇ</th>
<th>Rise-time (msec)ᵇ</th>
<th>Half-decay (msec)ᵇ</th>
<th>Membrane potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.9±0.4</td>
<td>0.6±0.1</td>
<td>0.7±0.1</td>
<td>0.8±0.2</td>
<td>61</td>
</tr>
<tr>
<td>10⁻⁸</td>
<td>c</td>
<td>0.7±0.2</td>
<td>0.7±0.1</td>
<td>0.9±0.3</td>
<td>61</td>
</tr>
<tr>
<td>10⁻⁷</td>
<td>2.4±0.2*</td>
<td>0.9±0.2**</td>
<td>1.0±0.2**</td>
<td>1.6±0.4**</td>
<td>65</td>
</tr>
<tr>
<td>10⁻⁶</td>
<td>2.5±0.3**</td>
<td>1.0±0.2**</td>
<td>1.3±0.3**</td>
<td>2.3±0.7**</td>
<td>60</td>
</tr>
<tr>
<td>10⁻⁵</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>39</td>
</tr>
</tbody>
</table>

ᵃ Standard error of the mean.

ᵇ Standard deviation.

c Film recording poor with this observation for accurate measurement, but no change in frequency was apparent.

* Difference statistically significant at 1% level.

** Difference statistically significant at 0.1% level.

--- M. e. p. p. s too small to measure accurately.
<table>
<thead>
<tr>
<th>Concentration of neostigmine (M)</th>
<th>Amplitude (mV)(^a)</th>
<th>Rise-time (msec)(^b)</th>
<th>Half-decay (msec)(^b)</th>
<th>Membrane potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.4±0.2</td>
<td>0.6±0.1</td>
<td>0.9±0.1</td>
<td>68</td>
</tr>
<tr>
<td>10(^{-8})</td>
<td>6.9±0.3**</td>
<td>0.6±0.1</td>
<td>0.9±0.1</td>
<td>68</td>
</tr>
<tr>
<td>10(^{-7})</td>
<td>8.5±0.3**</td>
<td>0.8±0.1*</td>
<td>1.3±0.1**</td>
<td>68</td>
</tr>
<tr>
<td>10(^{-6})</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Muscle began to twitch</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^{a}\) Standard error of the mean.

\(^{b}\) Standard deviation.

\(^{c}\) Film recording poor with this observation for accurate measurement, but no change in frequency was apparent.

* Difference statistically significant at 1% level.

** Difference statistically significant at 0.1% level.

--- M. e. p. s too small to measure accurately.
Effects of neostigmine on (+)-tubocurarine e.p.p.s.

At $10^{-8}$M neostigmine, a very significant increase in amplitude was produced with no change in time course. The amplitude and time course both increased at $10^{-7}$M neostigmine; however, the increase in amplitude was greater (57%) than the increase in rise-time (33%) or half-decay (44%). At $10^{-6}$M neostigmine the muscle began to twitch and the recordings were lost. Table 2B illustrates the effects of neostigmine on single e.p.p.s.

Effects of edrophonium on m.e.p.p.s

In four experiments infusion of concentrations of edrophonium of $10^{-8}$M or greater produced a very significant increase in m.e.p.p. frequency (Figure 1). This effect was reversed after the muscle had been returned to Krebs solution for 60 min. (Table 3A).

There were no significant changes in amplitude or time course of the m.e.p.p.s. with edrophonium concentrations up to $10^{-5}$M. At $10^{-4}$M the e.p. membrane potential was depolarized to 41mV and the m.e.p.p.s. were no longer visible. The control readings were again obtained 60 min after the infusion of drug solution had been replaced by Krebs solution.
Figure 1. The effect of edrophonium on m.e.p.p. frequency. (a) Control (b) In the presence of edrophonium $10^{-8}$M. Voltage calibration, 1 mV; time calibration, 0.2 sec.
<table>
<thead>
<tr>
<th>Concentration of edrophonium (M)</th>
<th>Frequency of m.e.p.p.s (sec^-1)</th>
<th>Amplitude (mV)</th>
<th>Rise-time (msec)</th>
<th>Half-decay (msec)</th>
<th>Membrane potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.7+0.1</td>
<td>0.6+0.2</td>
<td>1.1+0.2</td>
<td>1.4+0.3</td>
<td>70</td>
</tr>
<tr>
<td>10^-8</td>
<td>2.7+0.4**</td>
<td>0.6+0.1</td>
<td>1.2+0.2</td>
<td>1.3+0.3</td>
<td>70</td>
</tr>
<tr>
<td>10^-7</td>
<td>2.8+0.5**</td>
<td>0.6+0.1</td>
<td>1.0+0.2</td>
<td>1.5+0.3</td>
<td>71</td>
</tr>
<tr>
<td>10^-6</td>
<td>2.5+0.2**</td>
<td>0.7+0.2</td>
<td>1.3+0.2</td>
<td>1.5+0.3</td>
<td>70</td>
</tr>
<tr>
<td>10^-5</td>
<td>2.5+0.2**</td>
<td>0.6+0.2</td>
<td>1.2+0.1</td>
<td>1.5+0.2</td>
<td>66</td>
</tr>
<tr>
<td>10^-4</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>48</td>
</tr>
</tbody>
</table>

For symbols see Table 2A.
### TABLE 3B

Effects of Edrophonium on E.p.p.s (4 μg/ml Tc)

<table>
<thead>
<tr>
<th>Concentration of edrophonium (M)</th>
<th>Amplitude (mV)(^b)</th>
<th>Rise-time (msec)(^b)</th>
<th>Half-decay (msec)(^b)</th>
<th>Membrane potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.5±0.2</td>
<td>0.7±0.1</td>
<td>0.9±0.1</td>
<td>65</td>
</tr>
<tr>
<td>(10^{-8})</td>
<td>3.6±0.1</td>
<td>0.7±0.1</td>
<td>0.9±0.1</td>
<td>65</td>
</tr>
<tr>
<td>(10^{-7})</td>
<td>4.0±0.2**</td>
<td>0.7±0.1</td>
<td>1.0±0.1</td>
<td>65</td>
</tr>
<tr>
<td>(10^{-6})</td>
<td>5.5±0.2**</td>
<td>0.8±0.1</td>
<td>1.2±0.1**</td>
<td>65</td>
</tr>
<tr>
<td>(10^{-5})</td>
<td>9.3±0.3**</td>
<td>1.2±0.1**</td>
<td>2.4±0.1**</td>
<td>65</td>
</tr>
</tbody>
</table>

Muscle began to twitch

For symbols see Table 2A.
Effects of edrophonium on (+)-tubocurarine e.p.p.s.

In three experiments $10^{-8}$ M edrophonium produced no significant changes in the amplitude or time course of the e.p.p.s; $10^{-7}$ M increased the amplitude with no change in time course. $10^{-6}$ M and $10^{-5}$ M increased both amplitude and time course. At $10^{-6}$ M the increases were: amplitude 57%, rise-time 14%, half-decay 33%, and at $10^{-5}$ M, amplitude 165%, rise-time 71% and half-decay 167%. Ten minutes after perfusion with $10^{-5}$ M edrophonium, the muscle began to twitch and the recording was lost (Table 3B).

Effects of ambenonium on m.e.p.p.s

In four experiments ambenonium did not produce any significant increase in m.e.p.p. frequency. In the experiment illustrated by Table 4A, the frequency decreased at $10^{-9}$ M and $10^{-8}$ M but there was no change at $10^{-7}$ M. The amplitude and time course were very significantly increased at concentrations of $10^{-6}$ M and $10^{-7}$ M. At $10^{-7}$ M the amplitude was increased by 71%, the rise-time 57%, and the half-decay 170%. The half-decay decreased at $10^{-6}$ M and the m.e.p.p.s disappeared in $10^{-5}$ M ambenonium. The m.e.p.p.s returned when the drug solution was replaced by Krebs solution at the increased amplitude and time course which did not return to control values after three hours perfusion.
Figure 2. The effect of ambenonium on e.p.p. and m.e.p.p. amplitude and time course. (a) E.p.p.s. recorded in the presence of 5 µg/ml tubocurarine; A--control; B--with $10^{-9}$ M ambenonium; C--with $10^{-8}$ M ambenonium. (b) M.e.p.p.s.: A--control; B--with $10^{-7}$ M ambenonium. The thick base line is due to the record being photographed from a recurrent sweep, the camera shutter being left open until a m.e.p.p. occurred. Voltage calibration, (a) 5 mV, (b) 0.5 mV; time calibration, (a) 1 msec, (b) 1 msec.
### TABLE 4A

**Effects of Ambenonium on M. e. p. p. s**

<table>
<thead>
<tr>
<th>Concentration of ambenonium (M)</th>
<th>Frequency of m. e. p. p. s (sec(^{-1}))(^a)</th>
<th>Amplitude (mV)(^b)</th>
<th>Rise-time (msec)(^b)</th>
<th>Half-decay (msec)(^b)</th>
<th>Membrane potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.5±0.1</td>
<td>0.7±0.1</td>
<td>0.7±0.1</td>
<td>1.0±0.3</td>
<td>66</td>
</tr>
<tr>
<td>(10^{-10})</td>
<td>1.3±0.3</td>
<td>0.7±0.1</td>
<td>0.7±0.1</td>
<td>1.1±0.2</td>
<td>65</td>
</tr>
<tr>
<td>(10^{-9})</td>
<td>1.1±0.3**</td>
<td>0.7±0.1</td>
<td>0.7±0.1</td>
<td>1.0±0.2</td>
<td>61</td>
</tr>
<tr>
<td>(10^{-8})</td>
<td>1.2±0.1**</td>
<td>0.8±0.2</td>
<td>0.8±0.1</td>
<td>1.3±0.3</td>
<td>58</td>
</tr>
<tr>
<td>(10^{-7})</td>
<td>1.5±0.2</td>
<td>1.2±0.1**</td>
<td>1.1±0.2**</td>
<td>2.7±0.4**</td>
<td>56</td>
</tr>
<tr>
<td>(10^{-6})</td>
<td>1.3±0.2*</td>
<td>1.2±0.1**</td>
<td>1.1±0.2**</td>
<td>2.3±0.6**</td>
<td>60</td>
</tr>
<tr>
<td>(10^{-5})</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>65</td>
</tr>
</tbody>
</table>

Note: Changes in m. e. p. p. frequency show a statistically significant decrease.

For symbols see Table 2A.
### TABLE 4B

**Effects of Ambenonium on E.p.p.s. (5 µg/ml Tc)**

<table>
<thead>
<tr>
<th>Concentration of ambenonium (M)</th>
<th>Amplitude (mV)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Rise-time (msec)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Half-decay (msec)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Membrane potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.8±0.3</td>
<td>0.7±0.1</td>
<td>1.0±0.1</td>
<td>---</td>
</tr>
<tr>
<td>10&lt;sup&gt;-10&lt;/sup&gt;</td>
<td>6.3±0.2**</td>
<td>0.7±0.1</td>
<td>1.0±0.1</td>
<td>78</td>
</tr>
<tr>
<td>10&lt;sup&gt;-9&lt;/sup&gt;</td>
<td>6.5±0.3**</td>
<td>0.7±0.1</td>
<td>1.0±0.1</td>
<td>78</td>
</tr>
<tr>
<td>10&lt;sup&gt;-8&lt;/sup&gt;</td>
<td>13.8±0.7**</td>
<td>1.1±0.1**</td>
<td>1.8±0.1**</td>
<td>80</td>
</tr>
<tr>
<td>10&lt;sup&gt;-7&lt;/sup&gt;</td>
<td>Muscle began to twitch</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

For symbols see Table 2A
When using ambenonium, it was difficult to maintain a stable end-plate membrane resting potential throughout a complete experiment due to the muscle twitching at concentrations between $10^{-9}$M and $10^{-7}$M. The shift in membrane potential in the experiment illustrated in Table 4A was not due to depolarization but due to movements of the electrode caused by the muscle twitching. In some experiments it was possible to observe a stable resting potential up to a concentration of $10^{-8}$M. In these experiments there was no change in amplitude or time course of the m.e.p.p.s with $10^{-10}$M or $10^{-9}$M. However, there were significant changes in amplitude, rise-time and half-decay at $10^{-8}$M and, therefore, the change in membrane potential shown in Table 4A did mask changes occurring with $10^{-8}$M ambenonium.

Effects of ambenonium on (+)-tubocurarine e.p.p.s

In three experiments $10^{-10}$M and $10^{-9}$M ambenonium produced a very significant increase in e.p.p. amplitude with no change in time course (Figure 2). At $10^{-8}$M the amplitude was increased by 138%, the rise-time by 57% and the half-decay by 80%. At $10^{-7}$M the muscle began to twitch and no further recordings were possible. (Table 4B).
Effects of methoxyambenonium on m.e.p.p.s

In three experiments methoxyambenonium produced a significant increase in m.e.p.p. frequency at $10^{-8}$M. No significant increase in amplitude or rise-time was observed. The half-decay increased at $10^{-7}$M and $10^{-6}$M. At $10^{-5}$M the m.e.p.p.s. disappeared with no change in end-plate membrane potential. The drug solution was replaced by Krebs solution and the m.e.p.p.s. returned at an increased amplitude and time course which did not completely return to control values after 150 min. In one experiment the m.e.p.p. frequency was greatly increased in the final rinse solution. (Table 5A).

Effects of methoxyambenonium on (+)-tubocurarine e.p.p.s

Methoxyambenonium, at a concentration of $10^{-8}$M, increased the amplitude of the e.p.p.s without change of time course. At $10^{-7}$M and $10^{-6}$M both amplitude and time course were increased; $10^{-6}$M increased the amplitude by 219%, the rise-time by 114% and the half-decay by 211%. After 15-min perfusion with $10^{-6}$M methoxyambenonium, the muscle began to twitch and the recording was lost. (Table 5B).

Effects of acetylcholine

In four experiments acetylcholine (ACh) produced no consistent


**TABLE 5A**

**Effects of Methoxyambenonum on M.e.p.p.s.**

<table>
<thead>
<tr>
<th>Concentration of methoxy-ambenonum (M)</th>
<th>Frequency of m.e.p.p.s (sec⁻¹)ᵃ</th>
<th>Amplitude (mV)ᵇ</th>
<th>Rise-time (msec)ᵇ</th>
<th>Half-decay (msec)ᵇ</th>
<th>Membrane potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.1±0.3</td>
<td>0.7±0.1</td>
<td>0.6±0.1</td>
<td>1.0±0.2</td>
<td>73</td>
</tr>
<tr>
<td>10⁻⁸</td>
<td>2.7±0.4*</td>
<td>0.6±0.1</td>
<td>0.5±0.1</td>
<td>1.0±0.2</td>
<td>73</td>
</tr>
<tr>
<td>10⁻⁷</td>
<td>2.1±0.4</td>
<td>0.8±0.1</td>
<td>0.7±0.1</td>
<td>1.5±0.2**</td>
<td>71</td>
</tr>
<tr>
<td>10⁻⁶</td>
<td>2.0±0.2</td>
<td>0.6±0.1</td>
<td>0.7±0.1</td>
<td>1.5±0.1**</td>
<td>73</td>
</tr>
<tr>
<td>10⁻⁵</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>71</td>
</tr>
</tbody>
</table>

For symbols see Table 2A.
### TABLE 5B

**Effects of Methoxyamenonium on E. p. p. s. (3µg/ml Td)**

<table>
<thead>
<tr>
<th>Concentration of methoxy-ambenonium (M)</th>
<th>Amplitude (mV)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Rise-time (msec)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Half-decay (msec)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Membrane potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.1 ± 0.2</td>
<td>0.7 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>66</td>
</tr>
<tr>
<td>$10^{-8}$</td>
<td>3.6 ± 0.2**</td>
<td>0.8 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>66</td>
</tr>
<tr>
<td>$10^{-7}$</td>
<td>6.6 ± 0.4**</td>
<td>1.0 ± 0.1**</td>
<td>1.3 ± 0.1**</td>
<td>68</td>
</tr>
<tr>
<td>$10^{-6}$</td>
<td>9.9 ± 0.6**</td>
<td>1.5 ± 0.1**</td>
<td>2.8 ± 0.1**</td>
<td>68</td>
</tr>
</tbody>
</table>

*For symbols see Table 2A*
changes in m.e.p.p. frequency, either as the concentration was increased during the same experiment or at the same concentration in different experiments. The frequency varied throughout the experiment and it is unlikely that any changes were drug-induced. ACh did not increase the amplitude or time course of the m.e.p.p.s, but at concentrations greater than $10^{-4} M$, the amplitude diminished as the end-plate membrane was depolarized. (Table 6).

Effects of tubocurarine

Experiments using tubocurarine alone showed no significant changes of amplitude or time course of e.p.p.s over a period of 2 hr. In experiments using 5-10 µg/ml tubocurarine, similar changes to those recorded above were observed but higher concentrations of the facilitatory drugs were required. Concentrations of tubocurarine of 10-15 µg/ml completely prevented any increases in amplitude and time course in the presence of the facilitatory drugs.

DISCUSSION

All the facilitatory drugs used produced statistically significant changes in m.e.p.p. frequency. However, the changes were small and assuming that a ten fold increase in frequency of the m.e.p.p.s repre-
TABLE 6

Effects of Acetylcholine on M.e.p.p.s

<table>
<thead>
<tr>
<th>Concentration of acetylcholine (M)</th>
<th>Frequency of m.e.p.p.s (sec⁻¹)ᵃ</th>
<th>Amplitude (mV)ᵇ</th>
<th>Rise-time (msec)ᵇ</th>
<th>Half-decay (msec)ᵇ</th>
<th>Membrane potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.2±0.2</td>
<td>0.8±0.2</td>
<td>0.6±0.1</td>
<td>1.0±0.2</td>
<td>70</td>
</tr>
<tr>
<td>10⁻⁸</td>
<td>1.1±0.1</td>
<td>0.9±0.2</td>
<td>0.7±0.1</td>
<td>1.0±0.2</td>
<td>71</td>
</tr>
<tr>
<td>10⁻⁷</td>
<td>1.3±0.4</td>
<td>0.9±0.2</td>
<td>0.7±0.1</td>
<td>0.9±0.2</td>
<td>70</td>
</tr>
<tr>
<td>10⁻⁶</td>
<td>1.5±0.3*</td>
<td>0.8±0.2</td>
<td>0.6±0.1</td>
<td>1.0±0.2</td>
<td>71</td>
</tr>
<tr>
<td>10⁻⁵</td>
<td>1.5±0.1**</td>
<td>0.8±0.1</td>
<td>0.6±0.2</td>
<td>1.1±0.1</td>
<td>71</td>
</tr>
<tr>
<td>10⁻⁴</td>
<td>1.5±0.2**</td>
<td>0.7±0.1</td>
<td>0.6±0.1</td>
<td>1.0±0.2</td>
<td>70</td>
</tr>
<tr>
<td>10⁻³</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>49</td>
</tr>
</tbody>
</table>

For symbols see Table 2A.
sented a 15 mV depolarization of the nerve terminal (Liley, 1956b), depolarization of the terminal could only have been in the order of 1 to 3 mV. Also, m.e.p.p. frequency varies over an extended period of time (Boyd and Martin, 1956a) thus some of the changes noted may have been spontaneous and not drug-induced.

Boyd and Martin (1956a) also noted a change in m.e.p.p. frequency using $3.3 \times 10^{-7}$ M neostigmine and a decrease at $3.3 \times 10^{-6}$ M, but as in the present results, the changes recorded were only small. The reduction of frequency at higher concentrations may simply reflect the difficulty in observing the m.e.p.p.s which were greatly decreased in amplitude when the end-plate was depolarized. No change in m.e.p.p. frequency has been observed in the frog sartorius using neostigmine (Fatt and Katz, 1952) or in the rat diaphragm using neostigmine (Liley, 1956a).

It is difficult to assess the importance of the small changes in nerve terminal membrane potential that these increases in m.e.p.p. frequency reflect. The change in spontaneous release of ACh indicated by the change in m.e.p.p. frequency is probably not physiologically significant. However, this small effect on the terminal may be indicative of a presynaptic action which does have significant effects on the release of ACh due to nerve action potentials.
The changes in time course of the m.e.p.p.s were much more easily observed and more consistent than the changes in amplitude. Since the changes in amplitude were small and only occurred in the presence of changes of time course, it can be concluded that the drugs produced no significant changes in the sensitivity of the end-plate. This conclusion confirms that of Blaber (1963), and the changes in end-plate sensitivity produced by methoxyambenonium in frog muscle (Karczmar et al., 1961) point to a species difference in the effect of these drugs at the neuromuscular junction. A logarithmic plot of amplitude during the decay of the e.p.p. was found to be linear with time, both in the control recordings and in the presence of facilitatory drugs; thus an increase of the time to half-decay indicates prolongation of time course. The degree of prolongation gives a rough estimate of the anticholinesterase activity of these compounds. Thus ambenonium is the most potent cholinesterase inhibitor, while neostigmine, methoxyambenonium and edrophonium exhibit decreasing potencies. Edrophonium produces no effects on m.e.p.p. amplitude or half-decay. This indicates that edrophonium does not inhibit cholinesterase up to a concentration of $10^{-6}$ M. Changes in amplitude and time course of m.e.p.p.s have been reported in other species previously, neostigmine ($3.3 \times 10^{-6}$M) in frog sartorius (Fatt and Katz, 1952), neo-
stigmine (3.3 X 10^{-6} M) in rat diaphragm (Liley, 1956a) and also in the cat
tenuissimus muscle, neostigmine (3.3 X 10^{-7} M) (Boyd and Martin, 1956a).

Edrophonium (10^{-4} M) and neostigmine (10^{-5} M) produced depolarization
of the end-plate, confirming the conclusions of Nastuk and Alexander
(1954), and Katz and Thesleff (1957). These concentrations are higher
than those necessary to facilitate transmission and would indicate that
depolarization of the end-plate is not a factor in the production of
facilitation of neuromuscular transmission by these drugs, confirming the
conclusion of Blaber and Bowman (1959).

Ambenonium (10^{-5} M) and methoxyambenonium (10^{-5} M) produced a
decrease in the amplitude and time course of the m.e.p.p.s without
any change in the resting potential of the e.p. membrane. These drugs
have been shown previously to produce neuromuscular paralysis
resembling that of (+)-tubocurarine (Blaber, 1960). This potent curare-
like action may account for the unusual observation that methoxyam-
benonium increased the half-decay time but not the amplitude of the m.e.
p.p.s.

The facilitatory drugs also increased and prolonged the (+)-tubocura-
rine e.p.p. but the effects on e.p.p.s occurred at concentrations which had
no effect on the m.e.p.p.s. This fact, together with an increase in amplitude and no change in time-course of the e.p.p. indicates a presynaptic action for these drugs.

Masland and Wigton (1940) and Eccles (1964) have suggested that ACh may be mediating in the action of the facilitatory drugs on the motor nerve terminal after inhibition of cholinesterase. However, the present results show that the time course of the e.p.p. was not prolonged at the time it was augmented, showing cholinesterase was not inhibited. Also, ACh was found not to produce any change in frequency of m.e.p.p.s, thus confirming the results of Hubbard, Schmidt and Yokota (1965). These results, although not eliminating ACh as a mediator, would suggest that nerve terminal effects are not produced by a previous inhibition of cholinesterase, though the drugs may sensitize the nerve terminal to an action of ACh. The action of ACh would not be to depolarize the terminal, since no change in m.e.p.p. frequency was produced.

Because the amplitude of the m.e.p.p.s was not increased in the absence of a prolonged time course, it can be assumed that the size of the quantum is not increased by facilitatory drugs. The increase in ACh release for each stimulus must, therefore, be due to an increase in the quantal content of the e.p.p.
Further work is required to support the present conclusions since they are based upon the assumption that m.e.p.p.s and e.p.p.s differ only in their mechanism of release. But m.e.p.p.s are well known for their small and highly variable amplitude (Fatt and Katz, 1952). E.p.p.s are comparatively large and very constant in amplitude. It is possible that the present results show no significant changes of the m.e.p.p.s only because of this high variability. This can be illustrated by the results with methoxyambenonium (Table 5). \(10^{-8}\text{M}\) methoxyambenonium increased the e.p.p. amplitude by 16%. The same percentage increase of the m.e.p.p. amplitude (0.7 to 0.8 mv) would not have been significant. Thus the initial conclusion that the facilitatory drugs act by a pre-junctional action is weakened and must be tested by further experiments.
RESULTS SECTION 2

EFFECTS OF FACILITATORY DRUGS ON (+)-TUBOCURARINE E. P. P. S AND IONTOPHORETIC ACETYLCHOLINE AND CARBACHOL POTENTIALS.
INTRODUCTION

From the previous experiments it was concluded that the e.p.p. amplitude was augmented at a concentration of facilitatory drug which was approximately one-tenth the concentration necessary to produce an effect on m.e.p.p. amplitude or time-course. Since m.e.p.p.s have a high variability and low amplitude, further experiments were performed to provide additional information regarding the mechanism of action of the facilitatory drugs. Therefore experiments were performed with the same drugs, using a microelectrode drug application technique (Nastuk, 1953a and Del Castillo and Katz, 1955a). This technique was used for the following reasons. First, the membrane potential change resulting from iontophoretic application of ACh is not as variable as m.e.p.p.s. Second, the ACh-potential can be adjusted to any desired amplitude, simply by increasing the current which passes through the electrode. Finally, it is possible to record both the e.p.p. and ACh-potential at the same muscle e.p., thereby eliminating any variability of drug response which may occur between different muscle fibers.

RESULTS

It proved to be quite difficult to maintain ACh-potentials at a
constant amplitude for long periods of time. Thus both ACh-potential amplitude and half-decay time were observed to determine the presence of drug-induced changes to the response of ACh at the e.p. membrane. (The half-decay seemed very constant for long periods of time, even though the amplitude decreased considerably.)

Effects of the facilitatory drugs on (+)-tubocurarine e.p.p.s and ACh-potentials.

Neostigmine.

Table 7 shows the results obtained from two experiments with neostigmine. In EXP #1 neostigmine produced no significant changes at $10^{-8}$M, although there were small effects (less than 10%) on the ACh-potential. At $10^{-7}$M there were large changes in both the e.p.p. and ACh-potential. In EXP #2 the e.p.p. was augmented by $10^{-8}$M neostigmine. The increase was 28% of control and was highly significant. Also, the ACh-potential was prolonged by 17%, although this increase was not as significant as the effect on the e.p.p.

Edrophonium.

It was quite difficult to produce the e.p.p. changes at $10^{-7}$M edrophonium which had been observed in the previous section. However,
TABLE 7

Effects of Neostigmine on E.p.p.s and ACh-potentials (4 µg/ml. Tc)

<table>
<thead>
<tr>
<th>Concentration of neostigmine (M)</th>
<th>E.P.P. amplitude (mV)</th>
<th>ACh-potential amplitude (mV)</th>
<th>ACh-potential half decay (msec)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EXP #1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.6±0.2</td>
<td>1.4±0.1</td>
<td>480±30</td>
</tr>
<tr>
<td>10⁻⁸ M</td>
<td>2.5±0.1 (0)</td>
<td>1.5±0.2 (7%)</td>
<td>520±30 (8%)</td>
</tr>
<tr>
<td>10⁻⁷ M</td>
<td>3.6±0.2** (38%)</td>
<td>3.3±0.2** (136%)</td>
<td>760±50** (58%)</td>
</tr>
<tr>
<td><strong>EXP #2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.8±0.1</td>
<td>1.3±0.1</td>
<td>120±10</td>
</tr>
<tr>
<td>10⁻⁸ M</td>
<td>2.3±0.1** (28%)</td>
<td>1.3±0.1 (0)</td>
<td>140±10* (17%)</td>
</tr>
</tbody>
</table>

* Difference statistically significant at the 1% level.

** Difference statistically significant at the 0.1% level.

b ± Standard deviation.

Values in parentheses indicate the percentage increase over control.
TABLE 8

Effects of Edrophonium on E. p. p. s. and ACh-potentials (4 µg/mlTc)

<table>
<thead>
<tr>
<th>Concentration of edrophonium (M)</th>
<th>E. P. P. amplitude (mV) (^b)</th>
<th>ACh-potential amplitude (mV) (^b)</th>
<th>ACh-potential half-decay (msec) (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EXP #1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.0±0.1</td>
<td>1.9±0.1</td>
<td>110±10</td>
</tr>
<tr>
<td>(10^{-7}) M</td>
<td>1.1±0.1</td>
<td>1.5±0.1</td>
<td>110±10</td>
</tr>
<tr>
<td></td>
<td>((10%))</td>
<td>((0))</td>
<td>((0))</td>
</tr>
<tr>
<td>EXP #2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.4±0.1</td>
<td>1.8±0.3</td>
<td>230±10</td>
</tr>
<tr>
<td>(10^{-6}) M</td>
<td>2.2±0.2**</td>
<td>2.1±0.2</td>
<td>350±20**</td>
</tr>
<tr>
<td></td>
<td>((57%))</td>
<td>((17%))</td>
<td>((25%))</td>
</tr>
<tr>
<td>EXP #3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.5±0.2</td>
<td>1.7±0.1</td>
<td>550±50</td>
</tr>
<tr>
<td>(10^{-8}) M</td>
<td>1.4±0.2</td>
<td>1.4±0.1</td>
<td>500±60</td>
</tr>
<tr>
<td></td>
<td>((0))</td>
<td>((0))</td>
<td>((0))</td>
</tr>
<tr>
<td>(10^{-7}) M</td>
<td>1.5±0.2</td>
<td>1.8±0.1</td>
<td>620±60</td>
</tr>
<tr>
<td></td>
<td>((0))</td>
<td>((6%))</td>
<td>((20%))</td>
</tr>
<tr>
<td>(10^{-6}) M</td>
<td>2.0±0.2**</td>
<td>2.7±0.2**</td>
<td>710±40**</td>
</tr>
<tr>
<td></td>
<td>((33%))</td>
<td>((59%))</td>
<td>((29%))</td>
</tr>
</tbody>
</table>

For symbols see Table 7
there were large changes in both the e.p.p. and ACh-potential at $10^{-6}$M edrophonium (Table 8).

**Ambenonium.**

The experiments with ambenonium resulted in almost identical increases in e.p.p. amplitude and ACh-potential half-decay (Table 9 and Figure 3). The only difference occurs in EXP #2 at $10^{-9}$M ambenonium when the e.p.p. was augmented, significantly, by 16% while the ACh-potential half-decay was increased by 8%, which was insignificant.

**Methoxyambenonium.**

EXP #1 shows large increases in e.p.p. amplitude and ACh-potential amplitude at $10^{-8}$M methoxyambenonium. $10^{-8}$M was much less effective in EXP #2, although the small increase of e.p.p. amplitude was significant (Table 10).

**Time-course of anti-Tc action.**

In a series of four experiments effects of the facilitatory drugs were observed continuously from the beginning of drug infusion. In this way it was possible to determine if effects on e.p.p. amplitude followed the same time-course as effects on ACh-potential amplitude or half-decay. The results of one experiment are illustrated in Figure 4. Records were
Figure 3. The effect of ambenonium on e.p.p.s and ACh-potentials recorded in the presence of 4 µg/ml (+)-tubocurarine: A--control e.p.p. and ACh-potential; B--with 10⁻⁸M ambenonium. Voltage calibration, 5mV; time calibration, 100 msec; current, 2.5 x 10⁻⁷ amps.
TABLE 9

Effects of Ambenonium on E. p. p. s and ACh-potentials (4 µg/ml Tc)

<table>
<thead>
<tr>
<th>Concentration of ambenonium (M)</th>
<th>E. P. P. amplitude (mV)</th>
<th>ACh-potential amplitude (mV)</th>
<th>ACh-potential half-decay (msec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EXP#1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.7±0.1</td>
<td>2.4±0.2</td>
<td>80±10</td>
</tr>
<tr>
<td>10^{-9} M</td>
<td>1.7±0.1</td>
<td>1.4±0.1</td>
<td>90±10 (13%)</td>
</tr>
<tr>
<td>10^{-8} M</td>
<td>3.0±0.2** (76%)</td>
<td>2.8±0.3</td>
<td>140±10** (75%)</td>
</tr>
<tr>
<td>EXP #2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.9±0.1</td>
<td>2.0±0.2</td>
<td>130±10</td>
</tr>
<tr>
<td>10^{-9} M</td>
<td>2.2±0.1** (16%)</td>
<td>1.9±0.1</td>
<td>140±10 (8%)</td>
</tr>
<tr>
<td>10^{-8} M</td>
<td>5.1±0.5** (168%)</td>
<td>2.2±0.1</td>
<td>350±10** (169%)</td>
</tr>
</tbody>
</table>

For symbols see Table 7
TABLE 10

Effects of Methoxyambenonium on E. p. p. s and ACh-potentials (4 µg/ml Tc)

<table>
<thead>
<tr>
<th>Concentration of methoxyambenonium (M)</th>
<th>E. P. P. amplitude (mV)</th>
<th>ACh-potential amplitude (mV)</th>
<th>ACh-potential half-decay (msec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.6±0.3</td>
<td>4.2±0.1</td>
<td>60±10</td>
</tr>
<tr>
<td>10⁻⁸ M</td>
<td>11.4±0.2**</td>
<td>6.6±0.8**</td>
<td>50±10</td>
</tr>
<tr>
<td></td>
<td>(73%)</td>
<td>(57%)</td>
<td>(0%)</td>
</tr>
<tr>
<td>EXP #2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.1±0.1</td>
<td>1.7±0.1</td>
<td>140±10</td>
</tr>
<tr>
<td>10⁻⁸ M</td>
<td>2.4±0.1*</td>
<td>1.5±0.1</td>
<td>150±10</td>
</tr>
<tr>
<td></td>
<td>(14%)</td>
<td>(0)</td>
<td>(7%)</td>
</tr>
<tr>
<td>10⁻⁷ M</td>
<td>3.8±0.3**</td>
<td>1.2±0.1</td>
<td>180±10**</td>
</tr>
<tr>
<td></td>
<td>(81%)</td>
<td>(0)</td>
<td>(29%)</td>
</tr>
</tbody>
</table>

For symbols see Table 7
Figure 4. The effect of ambenonium on e.p.p. (•) and ACh-potential (x) amplitude recorded in the presence of 4 µg/ml (+)-tubocurarine versus time: $A_1$--beginning of $10^{-8}$M ambenonium infusion; $A_2$--beginning of $10^{-7}$M ambenonium infusion.
made every thirty seconds throughout the experiment. The controls were relatively constant. $10^{-8}$M ambenonium produced parallel increases in both e.p.p. amplitude and ACh-potential amplitude. Two minutes after beginning to infuse $10^{-7}$M ambenonium, both potentials increased very rapidly in amplitude.

Effects of the facilitatory drugs on (+)-tubocurarine e.p.p.s and carbachol-potentials.

None of the drugs studied (neostigmine, edrophonium, ambenonium and methoxyambenonium) produced changes in the carbachol-potential at doses which caused considerable augmentation of e.p.p. amplitude. This is illustrated in Figure 5. After control records, $10^{-6}$M methoxy-ambenonium was administered. The e.p.p. and carbachol-potential were recorded every thirty seconds. After three minutes of methoxyambenonium infusion there was a rapid augmentation of the e.p.p. amplitude, but no change in the carbachol-potential. After four minutes, the e.p.p. was not recorded to avoid any subsequent muscle twitches. Even after ten minutes there was no change in the carbachol-potential.

DISCUSSION

In five of seven experiments there was a statistically significant
Figure 5. The effect of methoxyambenonium on e.p.p.s and carbachol-potentials recorded in the presence of 4 µg/ml (+)-tubocurarine: A--control e.p.p. and carbachol-potential; B--with $10^{-6}$M methoxyambenonium. Voltage calibration, 5 mV; time calibration, 500 msec; current, $3.2 \times 10^{-8}$amps.
increase in ACh-potential amplitude or half-decay at the same minimal concentration as the increase in e.p.p. amplitude. Two experiments in which there was no correlation (ambenonium EXP #2 and methoxyambenonium EXP #2) showed small, but significant, increases in the e.p.p. amplitude at concentrations which did not significantly affect the ACh-potential. Yet the changes of the Ach-potential are 50% as large as the changes in the e.p.p. at the low concentrations. In fact, in ambenonium EXP #2 $10^{-8}$ M ambenonium produced exactly the same percentage changes (168% and 169%) in e.p.p. amplitude and ACh-potential half-decay. On the basis of these results it seems likely that there is a good correlation between the effects of facilitatory drugs on the e.p.p. and ACh-potential in a curarized mammalian neuromuscular preparation.

This conclusion is substantiated even further by the observation that augmentation of the e.p.p. and ACh-potential amplitudes follow approximately the same time course, as shown in Figure 4. These conclusions are very similar to those of Goldsmith (1963) and Otsuka and Nonomura (1963). Both papers gave results showing the effects of edrophonium on e.p.p.s and ACh-potentials at the frog neuromuscular junction.

Correlation between effects of facilitatory drugs on e.p.p.s and ACh-potentials can be explained by several postsynaptic mechanisms of
action. The most likely mechanism involves the cholinesterase inhibiting properties of the facilitatory drugs. This theory receives strong support from the observation that the action of a stable choline ester (carbachol) is not affected by facilitatory drugs. This would eliminate the other possibilities, sensitizing and depolarizing actions, which have been hypothesized (Riker and Wescoe, 1946 and Karczmar, 1957). If sensitizing or depolarizing actions were occurring, an increase in the carbachol-potential should have been observed.
RESULTS SECTION 3

EFFECTS OF FACILITATORY DRUGS ON
QUANTAL CONTENT OF THE E.P.P.
INTRODUCTION

Calcium ions are essential for transmitter release at the neuromuscular junction. By decreasing the calcium ion concentration or increasing the magnesium ion concentration in the Ringer solution, release of transmitter can be decreased to any level. At appropriate concentrations of calcium and magnesium the average quanta in a single e.p.p. is near one (equivalent to a m.e.p.p.). At this level of blockade it is possible to quantify transmitter release per indirect stimulus (Del Castillo and Katz, 1954c).

The facilitatory drugs were tested in a deficient calcium preparation to determine if they were capable of increasing the quantal content of the e.p.p.

RESULTS

The effects of neostigmine, edrophonium, ambenonium and methoxy-ambenonium are illustrated in Tables 11 to 14, respectively. The quantal content was measured by two methods:

1) \[
\frac{\text{ave. e.p.p. amplitude of 50 samples}}{\text{ave. m.e.p.p. amplitude of 50 samples}}
\]

and

2) \[
\log_e \frac{\text{no. of e.p.p.s}}{\text{no. of failures}} \text{ in 200 trials (Figure 6).}
\]
Figure 6. Effect of edrophonium on quantal release. Indirect stimulation at the artifact. (a) control; (b) with $10^{-6}$ M edrophonium. Voltage calibration, 1 mV; time calibration, 5 msec.
TABLE II

Effect of Neostigmine on Quantal Content

<table>
<thead>
<tr>
<th>Concentration of neostigmine (M)</th>
<th>Mg (10-15mM)(^a)</th>
<th>Ca (0.92 mM)</th>
<th>Mg (5-7 mM)(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Muscle 1</td>
<td>Muscle 2</td>
<td>Muscle 3</td>
</tr>
<tr>
<td>Control</td>
<td>2.1</td>
<td>1.9</td>
<td>0.54</td>
</tr>
<tr>
<td>(10^{-8})</td>
<td>2.1</td>
<td>2.0</td>
<td>0.42</td>
</tr>
<tr>
<td>(10^{-7})</td>
<td>2.1</td>
<td>1.9</td>
<td>0.68</td>
</tr>
<tr>
<td>(10^{-6})</td>
<td>1.7</td>
<td></td>
<td>0.64</td>
</tr>
<tr>
<td>(10^{-5})</td>
<td>1.8</td>
<td></td>
<td>0.82*</td>
</tr>
</tbody>
</table>

Average amplitude of 50 e.p.p.s

\(a\) - Average amplitude of 50 m.e.p.p.s

\(b\) - Log number of stimuli

\(\text{number of failures}\)

* indicates a significant increase of 33% or a significant decrease of 25% (Edwards and Ikeda, 1962).
TABLE 12

Effect of Edrophonium on Quantal Content

<table>
<thead>
<tr>
<th>Concentration of edrophonium (M)</th>
<th>Mg (10-15 mM)\textsuperscript{a}</th>
<th>Ca (0.92 mM)</th>
<th>Mg (5-7 mM)\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle 1</td>
<td>Muscle 2</td>
<td>Muscle 3</td>
<td>Muscle 4</td>
</tr>
<tr>
<td>Control</td>
<td>1.2</td>
<td>2.4</td>
<td>0.36</td>
</tr>
<tr>
<td>(10^{-8})</td>
<td>1.2</td>
<td>2.5</td>
<td>0.45</td>
</tr>
<tr>
<td>(10^{-7})</td>
<td>1.2</td>
<td>2.9</td>
<td>0.34</td>
</tr>
<tr>
<td>(10^{-6})</td>
<td>1.6\textsuperscript{*}</td>
<td>2.6</td>
<td>0.44</td>
</tr>
<tr>
<td>(10^{-5})</td>
<td></td>
<td>2.4</td>
<td></td>
</tr>
</tbody>
</table>

For symbols see Table 11
TABLE 13

Effect of Ambenonium on Quantal Content

<table>
<thead>
<tr>
<th>Concentration of ambenonium (M)</th>
<th>Quantal Content</th>
<th>Mg (10⁻¹⁵ mM)ᵃ</th>
<th>Ca (0.92 mM)</th>
<th>Mg (5⁻⁷ mM)ᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Muscle 1</td>
<td>Muscle 2</td>
<td>Muscle 3</td>
<td>Muscle 4</td>
</tr>
<tr>
<td>Control</td>
<td>1.6</td>
<td>1.5</td>
<td>0.67</td>
<td>1.9</td>
</tr>
<tr>
<td>10⁻¹⁰</td>
<td>1.8</td>
<td>1.6</td>
<td>0.81</td>
<td>1.8</td>
</tr>
<tr>
<td>10⁻⁹</td>
<td>1.8</td>
<td>1.7</td>
<td>0.66</td>
<td>1.9</td>
</tr>
<tr>
<td>10⁻⁸</td>
<td>1.8</td>
<td>2.0*</td>
<td>0.53</td>
<td>1.7</td>
</tr>
<tr>
<td>10⁻⁷</td>
<td>2.0</td>
<td>2.2*</td>
<td>0.33*</td>
<td>2.0</td>
</tr>
<tr>
<td>10⁻⁶</td>
<td>1.8</td>
<td>1.4</td>
<td>0.28*</td>
<td>1.7</td>
</tr>
<tr>
<td>10⁻⁵</td>
<td></td>
<td></td>
<td>1.5</td>
<td>1.5</td>
</tr>
</tbody>
</table>

For symbols see Table 11.


TABLE 14
Effect of Methoxyambenonium on Quantal Content

<table>
<thead>
<tr>
<th>Concentration of methoxy-ambenonium (M)</th>
<th>Quantal Content</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mg (10-15 mM)\textsuperscript{a}</td>
<td>Ca (0.92 mM)</td>
<td>Mg (5-7 mM)\textsuperscript{b}</td>
</tr>
<tr>
<td></td>
<td>Muscle 1</td>
<td>Muscle 2</td>
<td>Muscle 3</td>
</tr>
<tr>
<td>Control</td>
<td>2.5</td>
<td>3.6</td>
<td>0.79</td>
</tr>
<tr>
<td>\textsuperscript{10}^{-8}</td>
<td>2.5</td>
<td>3.6</td>
<td>0.89</td>
</tr>
<tr>
<td>\textsuperscript{10}^{-7}</td>
<td>3.4*</td>
<td>3.9</td>
<td>0.59</td>
</tr>
<tr>
<td>\textsuperscript{10}^{-6}</td>
<td>3.2</td>
<td></td>
<td>0.92</td>
</tr>
</tbody>
</table>

For symbols see Table 11
With either of these techniques the facilitatory drugs did not produce consistent changes in the quantal content of the e.p.p. at any concentration.

DISCUSSION

The quantal content of the e.p.p. in deficient calcium or increased magnesium ions is not increased significantly by facilitatory drugs. This is similar to the conclusion of Hubbard, Schmidt and Yokota (1965), except they observed a small decrease in quantal content of the e.p.p. in the presence of neostigmine.

M.e.p.p.s and e.p.p.s in high magnesium or low calcium are augmented at the same concentrations. Also, both are depressed at high concentrations, indicating the e.p.p.s and m.e.p.p.s are affected by the same postsynaptic mechanisms.

Augmentation of the e.p.p.s and m.e.p.p.s is compatible with the theory of cholinesterase inhibition by facilitatory drugs. Depression at higher concentrations is probably due to direct competitive or depolarizing actions.

Thus these results can be explained completely on the basis of postsynaptic actions and they indicate that in a calcium deficient solution
facilitatory drugs do not increase release of transmitter from presynaptic nerve terminals.
RESULTS SECTION 4

EFFECTS OF BENZOQUINONIUM

AT THE NEUROMUSCULAR JUNCTION
INTRODUCTION

Benzoquinonium (Bz) produces competitive blockade of neuromuscular transmission, and in this respect acts much like (+)-tubocurarine. The distinguishing feature of benzoquinonium blockade is that it is not antagonized by facilitatory drugs (Hoppe, 1950; Hougs and Johansen, 1957; Bowman, 1958 and Blaber and Bowman, 1962). As Blaber and Bowman (1963a and 1963b) concluded facilitatory drugs act at the motor nerve terminal, Blaber and Bowman (1962 and 1963a) further concluded that benzoquinonium prevents the action of facilitatory drugs at the motor nerve terminal.

Alternatively, it is possible that the potent anticholinesterase activity of benzoquinonium (Hoppe, 1951 and Blaber and Bowman, 1962) prevents any subsequent inhibition of cholinesterase by facilitatory drugs.

The following experiments attempt to elucidate the sites of action of benzoquinonium and thereby obtain presumptive evidence for the mechanism of action of facilitatory drugs.
BENZOQUINONIUM
RESULTS

Benzoquinonium control

The e.p.p. recorded when the muscle was blocked by Bz progressively decreased in size as a function of time. This was especially evident in the amplitude, which decreased by approximately 50% after one hour of recording (Figure 7). There were much smaller decreases in rise-time and half-decay (Figure 8).

Effects of the facilitatory drugs on the benzoquinonium e.p.p.

Infusion of concentrations of neostigmine, edrophonium, ambenonium, or methoxyambenonium which increased the amplitude and/or time course of e.p.p.s recorded during To blockade, produced little change in the rise-time, half-decay, or amplitude of the Bz e.p.p. (Figure 9). There was a continuous decrease of the amplitude; but the rate of decline resembled closely that observed in the control e.p.p. with Bz alone. Since the drug concentrations were changed at 15 minute intervals, the slopes of Figure 7 and 9 can be compared. At high concentrations of the facilitatory drugs, effects on the e.p.p. were observed. Concentrations of $10^{-4}$ M edrophonium caused a facilitation of all parameters. $10^{-5}$ M neostigmine increased the time to half-decay. Ambenonium prolonged the half-decay at concentrations of $10^{-7}$ M and $10^{-6}$ M. Also, $10^{-5}$ M amben-
Figure 7. E.p.p. amplitude recorded in a muscle fiber blocked by Bz (2 µg/ml). Zero time represents the time at which an e.p.p. was located and the first record taken.
Figure 8. End-plate potentials recorded in a muscle fiber blocked by Bz (1.5 μg/ml). E.p.p.s recorded at 30 mins (upper trace), 60 mins (center trace, 90 mins (lower trace) after beginning of the Bz infusion have been superimposed. Voltage calibration 5 mV; time calibration 1 msec.
Figure 9. The effects of facilitatory drugs on the amplitude of e.p.p.s recorded in a muscle blocked by Bz. C represents the control reading with Bz alone. The facilitatory drugs were added 15 min after the control reading and the concentrations increased at 15 min intervals.
onium and methoxyambenonium depressed the amplitude and rise-time.

Comparison of the benzoquinonium e.p.p. with the (+)-tubocurarine e.p.p.

It was observed that the values for the rise-time and half-decay of the Bz e.p.p. were prolonged when compared to a Tc e.p.p. In three experiments a muscle was initially blocked with the lowest possible concentration of Tc. After a rapid e.p.p. was found (rise-time < 1 msec and half-decay < 1.5 msec), the Tc was removed by washing until the m.e.p.p.s became visible. Then the same end-plate was blocked with Bz. This gave the results in Figure 10. When e.p.p.s of the same amplitude were compared for any effect on time course, it was found that the rise-time and half-decay of the e.p.p.s in Bz were about twice the duration when compared to the rise-time and half-decay of the e.p.p.s in Tc. When the Bz was washed out and replaced by Tc, the time course returned to that of the Tc control level. Each change-over from Tc to Bz, or from Bz to Tc, took approximately one hour. If a facilitatory drug was added to Ringer solution of the final Tc e.p.p., the facilitating action of these agents was considerably less potent than in the control Tc e.p.p. This decreased potency is obvious in the increased latency of action for ambenonium. Normally, $10^{-7}$M ambenonium produced its anticurare action in about five minutes, but in the final Tc solution, the anticurare
Figure 10. Comparison of the time course of e.p.p.s recorded in a muscle fiber blocked by Tc (2 µg/ml) and Bz (1.5 µg/ml). Voltage calibration 5 mV; time calibration 1 msec.
action of ambenonium was delayed to about 15 minutes. In addition, the augmented amplitude of the e.p.p. occurred without any increase in the rate of rise (Figure 11). An action of the facilitatory drugs increasing the rate of rise of the e.p.p. during Tc blockade has been previously reported (Kuperman and Okamoto, 1964).

**Effect of benzoquinonium on m.e.p.p. frequency and time course**

Bz had little effect on m.e.p.p. frequency. There was only a slight increase which occurred at $10^{-8}$M. There was no significant increase at $10^{-7}$M.

Concentrations of Bz up to $10^{-7}$M produced no effect on the m.e.p.p. amplitude and time course. At $10^{-7}$M Bz, the amplitude of m.e.p.p.s was depressed without any change in the resting membrane potential, thus confirming that Bz does not produce any depolarization of the end-plate. At $10^{-6}$M the m.e.p.p.s were too small to measure accurately. These effects are illustrated in Table 15A.

**Effect of benzoquinonium on Tc e.p.p.s**

In three of four experiments Bz produced a facilitation of the Tc e.p.p. As shown in Table 15B, this effect occurred at $10^{-8}$M and $10^{-7}$M Bz. The response was very slow to develop and the level of facilitation
Figure 11. Comparison of the effects of facilitatory drugs in a muscle fiber blocked by Tc before Bz (a) and after Bz(b). In a, A is a control e.p.p. recorded in a muscle blocked by 3 µg/ml Tc; B is an e.p.p. recorded when a concentration of $10^{-6}$ M edrophonium was added to the perfusion solution. Between a and b the Tc was removed and the muscle blocked with Bz (1.5 µg/ml). The Bz was then removed and the muscle re-infused with Tc. In b, A is a control e.p.p. recorded in a muscle blocked by 3 µg/ml Tc; B is an e.p.p. recorded when a concentration of $10^{-6}$ M edrophonium was added to the perfusion solution, and C when $10^{-5}$ M edrophonium was added to the perfusion solution. Voltage calibration 5 mV; time calibration 1 msec.
## TABLE 15A

**Effects of Benzoquinonium on M. e. p. s**

<table>
<thead>
<tr>
<th>Concentration of benzoquinonium (M)</th>
<th>Frequency of m. e. p. s (sec⁻¹)ᵃ</th>
<th>Amplitude (mV)ᵇ</th>
<th>Rise-time (msec)ᵇ</th>
<th>Half-decay (msec)ᵇ</th>
<th>Membrane potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.0 ± 0.4</td>
<td>1.4 ± 0.3</td>
<td>0.7 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>62</td>
</tr>
<tr>
<td>10⁻¹⁰</td>
<td>3.0 ± 0.7</td>
<td>1.5 ± 0.1</td>
<td>0.7 ± 0.1</td>
<td>1.3 ± 0.2</td>
<td>60</td>
</tr>
<tr>
<td>10⁻⁹</td>
<td>3.1 ± 0.3</td>
<td>1.5 ± 0.3</td>
<td>0.8 ± 0.1</td>
<td>1.3 ± 0.2</td>
<td>60</td>
</tr>
<tr>
<td>10⁻⁸</td>
<td>3.7 ± 0.5*</td>
<td>1.4 ± 0.2</td>
<td>0.7 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>60</td>
</tr>
<tr>
<td>10⁻⁷</td>
<td>3.3 ± 0.6</td>
<td>0.9 ± 0.2**</td>
<td>0.7 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>60</td>
</tr>
<tr>
<td>10⁻⁶</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>60</td>
</tr>
</tbody>
</table>

* * Difference statistically significant at the 1% level.
** Difference statistically significant at the 0.1% level.
--- M. e. p. s too small to measure accurately.

ᵃ Standard error of the mean.
ᵇ Standard deviation.
TABLE 15B

Effects of Benzoquinonium on E.p.p.s

Recorded in the Presence of 4 µg/ml Tubocurarine

<table>
<thead>
<tr>
<th>Concentration of benzoquinonium (M)</th>
<th>Amplitude (mV)(^b)</th>
<th>Rise-time (msec)(^b)</th>
<th>Half-decay (msec)(^b)</th>
<th>Membrane potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.1±0.4</td>
<td>0.7±0.1</td>
<td>1.3±0.1</td>
<td>73</td>
</tr>
<tr>
<td>10(^{-10})</td>
<td>5.1±0.3</td>
<td>0.8±0.1</td>
<td>1.4±0.1</td>
<td>75</td>
</tr>
<tr>
<td>10(^{-9})</td>
<td>4.9±0.3</td>
<td>0.8±0.1</td>
<td>1.4±0.1</td>
<td>77</td>
</tr>
<tr>
<td>10(^{-8})</td>
<td>5.6±0.4*</td>
<td>0.8±0.1</td>
<td>1.3±0.1</td>
<td>75</td>
</tr>
<tr>
<td>10(^{-7})</td>
<td>7.0±0.3**</td>
<td>0.9±0.1**</td>
<td>1.4±0.1</td>
<td>75</td>
</tr>
<tr>
<td>10(^{-6})</td>
<td>Muscle began to twitch</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

For symbols see table 15A.
varies greatly between muscles. At concentrations above $10^{-7}$ M, the three muscles in which facilitation occurred began to twitch; in the other, the level of blockade was increased. Bz, therefore, appeared to have a weak facilitatory action which occurred at $10^{-8}$ M and $10^{-7}$ M. However, Bz significantly depressed the amplitude of the m.e.p.p.s at $10^{-7}$ M; therefore, the weakness of the facilitatory action may have been due to the presence of both facilitatory and curare-like actions at this concentration.

Effect of benzoquinonium on quantal release

Bz, at concentrations which did not decrease the amplitude of the m.e.p.p.s and e.p.p.s, produced no significant effect on the quantal release in the presence of a high magnesium ion concentration (12-15 mM) (50 m.e.p.p. amplitude/50 e.p.p. amplitude) or in low calcium (0.92 mM) and high magnesium (5-7 mM) concentrations, based on the number of failures in 200 stimuli (Table 16). At concentrations where the m.e.p.p.s and e.p.p.s were very small, due to the curare-like action of Bz, a just significant reduction in the quantal content was observed.
**TABLE 16**

Effect of Benzoquinonium on Quantal Content

<table>
<thead>
<tr>
<th>Concentration of benzoquinonium (M)</th>
<th>QUANTAL CONTENT</th>
<th>( \text{Mg (10-15 mM)}^a )</th>
<th>( \text{Ca (0.92 mM)}^b )</th>
<th>( \text{Mg (5-7 mM)}^b )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Muscle 1</td>
<td>Muscle 2</td>
<td>Muscle 3</td>
<td>Muscle 4</td>
</tr>
<tr>
<td>1.6</td>
<td>2.3</td>
<td>0.7</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>( 10^{-10} )</td>
<td></td>
<td>0.6</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>( 10^{-9} )</td>
<td></td>
<td>0.5*</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>( 10^{-8} )</td>
<td>1.4</td>
<td>2.5</td>
<td>0.4*</td>
<td>1.4</td>
</tr>
<tr>
<td>( 10^{-7} )</td>
<td>1.7</td>
<td>1.9</td>
<td></td>
<td>0.7*</td>
</tr>
</tbody>
</table>

\textbf{a} Average amplitude of 50 e.p.p.s

\textbf{b} Log\text{e} number of failures

\* Indicates a significant decrease (25\% or more; Edwards and Ikeda, 1962).
DISCUSSION

The potency of Bz in blocking neuromuscular transmission in the isolated tenuissimus of the cat is approximately twice that of Tc, agreeing with the potency reported by Bowman (1958) for the anterior tibialis muscle of the cat in vivo.

The e.p.p. observed during Bz paralysis had a longer time course than that observed during Tc paralysis; the rise-time and half-decay being approximately double. These effects can be explained on the basis of cholinesterase inhibition by Bz. At the concentration used to block the muscle, $2.4 - 4.8 \times 10^{-6}$ M, cholinesterase would be inhibited approximately 70-90% (Blaber and Bowman, 1962). The e.p.p. in the presence of Bz decreased in amplitude as a function of time. The reason for this failure to reach an equilibrium may have been due to a progressive decrease in quantal content, however, the reduction in quantal content was only observed at concentrations of Bz which reduced the amplitude of the e.p.p.s and, therefore, the accuracy with which the number of failures could be counted was also reduced. Quantal release at the concentrations of Bz used for blockade could not be measured by the techniques used.

Facilitatory drugs, at concentrations which increase the amplitude of e.p.p.s in the presence of Tc produced no effect on the e.p.p.s in the presence of Bz, thus confirming the lack of effect of facilitatory drugs.
in antagonizing Bz paralysis (Hoppe, 1950; Randall, 1951; Bowman, 1958; Blaber and Bowman, 1962).

Ambenonium (10⁻⁷ M) produced a prolongation of the time course of the Bz e.p.p. thus showing the possibility of the further inhibition of cholinesterase in the presence of Bz. Bowman (1958) showed that TEPP would produce a small amount of antagonism against Bz; ambenonium is also a powerful cholinesterase inhibitor (Blaber, 1960) comparable in potency to TEPP. 10⁻⁵ M ambenonium and methoxyambenonium produced depression of the e.p.p., and high concentrations of these two drugs have been shown to have a curare-like action (Blaber, 1960).

Neostigmine at 10⁻⁵ M increased the time to half-decay, and slowed the rate of decline in the amplitude of the Bz e.p.p.s. Edrophonium at a concentration of 10⁻⁴ M increased the amplitude and time course of the Bz e.p.p. At these concentrations edrophonium and neostigmine depolarize the end-plate and also antagonize the blocking action of Bz in vivo in the cat (Blaber and Bowman, 1959). The depolarizing action produced at high concentrations of edrophonium and neostigmine is not thought to contribute to the facilitatory action (Nastuk and Alexander, 1954; Katz and Thesleff, 1957a; Blaber, 1963). Thus it must be concluded that the facilitatory action of the drugs has been prevented by Bz.
Bz (10⁻⁸M) increased the amplitude of the Tc e.p.p. without any significant change in the time course, also there was no effect on the m.e.p.p.s at this concentration. No increase in quantal release has been shown for Bz by the techniques used in the present study. In this respect, it resembles the facilitatory drugs used in this study which produced similar effects. At $10^{-7}$M, Bz also prolongs the rise-time of the Tc e.p.p. Higher concentrations of Bz produced only depression of the Tc e.p.p. due to its curare-like effect. Bz has been previously shown to antagonize the paralysis due to Tc in vivo in soleus and tibialis anterior muscles (Hougs and Johansen, 1958).

Most of the effects of benzoquinonium can be explained on the basis of its ability to inhibit junctional cholinesterase. Thus benzoquinonium inhibits cholinesterase as it produces competitive blockade of the muscle. This cholinesterase inhibition accounts for the slight augmentation by benzoquinonium of the (+)-tubocurarine e.p.p. Also the facilitatory drugs are not able to augment the benzoquinonium e.p.p. except at concentrations which produce higher levels of cholinesterase inhibition (e.g. ambenonium) or produce direct, depolarizing actions (e.g. edrophonium and neostigmine).

Although most of the evidence supports the theory of cholinesterase inhibition, this theory is not supported by the experiment illustrated in
Figure 11. Edrophonium was not able to antagonize (+)-tubocurarine after previous administration of benzoquinonium. This is similar to the ability of (+)-tubocurarine to decrease a drug-induced prolongation of an e.p.p. (Karczmar, Koketsu and Soeda, 1968). This prolongation is generally considered to be due to cholinesterase inhibition. Yet it is decreased by tubocurarine, even though tubocurarine has no effect on cholinesterase inhibition, in vitro (Eccles, Katz and Kuffler, 1942 and McIntyre and King, 1943). This may be explained somewhat by the fact that (+)-tubocurarine is capable of slightly decreasing the half-decay of a normal e.p.p. (Beranek and Vyskocil, 1968).
GENERAL DISCUSSION
The experimental results indicate that facilitatory drugs have several actions at the mammalian neuromuscular junction. Some of these results implicate a presynaptic mechanism of drug action for facilitatory drugs. The (+)-tubocurarine e.p.p. amplitude is augmented at a concentration which has no statistically significant effect on m.e.p.p.s. This is illustrated in Table 17. E.p.p. amplitude is augmented at 1/10 the concentration necessary to affect m.e.p.p. amplitude or time-course. Furthermore drug-induced changes in m.e.p.p.s, when they occur, are much smaller than drug-induced changes in e.p.p.s. This differential effect of facilitatory drugs on m.e.p.p.s and e.p.p.s suggests that facilitatory drugs have a presynaptic mechanism of action. This conclusion is based on the assumption that m.e.p.p.s and e.p.p.s are identical with exception that m.e.p.p.s do not require a nerve stimulus for release. This is not completely valid, as there are several limitations in the technique.

1) M.e.p.p.s and e.p.p.s were recorded in different experiments.

2) M.e.p.p.s were recorded in normal Ringers solution but e.p.p.s were recorded in the presence of (+)-tubocurarine.

3) The amplitude of m.e.p.p.s averaged less than one mV while e.p.p.s averaged more than three mV.

4) M.e.p.p.s involved one quantum of transmitter while e.p.p.s
## TABLE 17

**Minimal Molar Concentration of Drugs Producing Significant Changes in M. e. p. p. s and E. p. p. s**

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amplitude</td>
<td>Half-decay</td>
</tr>
<tr>
<td>Neostigmine</td>
<td>$10^{-7}$</td>
<td>$10^{-7}$</td>
</tr>
<tr>
<td>Edrophonium</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Ambenonium</td>
<td>$10^{-8}$</td>
<td>$10^{-8}$</td>
</tr>
<tr>
<td>Methoxyambenonium</td>
<td>--</td>
<td>$10^{-7}$</td>
</tr>
</tbody>
</table>
involved more than one-hundred quanta.

5) M.e.p.p.s were more variable in amplitude and time-course than e.p.p.s.

These considerations, especially the greater variability of m.e.p.p.s, make it questionable whether the effects summarized in Table 17 are attributable to a presynaptic action of the facilitatory drugs. It is possible that these results are due to a general technical inability to detect small changes in m.e.p.p. amplitude or time-course.

These complications are overcome by the method of iontophoretic application of ACh to the e.p. However, this method also has limitations.

1) Iontophoretic applications of ACh are not physiological. They may not be acting at the same receptors and do not exhibit the same time-course of receptor activation as the normal release of ACh.

2) Prolonged application of ACh may affect neuromuscular transmission.

3) The ACh-potentials are not always stable for the duration of an experiment.

Facilitatory drugs produce much larger changes in ACh-potentials than in m.e.p.p.s; thus the ACh-potentials seem more responsive to the
effects of facilitatory drugs than the m.e.p.p.s. At high drug concentrations there are no significant differences between the magnitude of augmentation of (+)-tubocurarine e.p.p.s and the magnitude of augmentation of ACh-potentials. On the basis of this observation it would appear that most of the changes of the e.p.p. which are induced by facilitatory drugs can be explained on the basis of postsynaptic actions. In order to explain all the changes observed with facilitatory drugs on the basis of postsynaptic actions it is necessary to show augmentation of the ACh-potential and (+)-tubocurarine e.p.p. at the same minimal concentration of facilitatory drug. These results are summarized in Table 18. There is very good correlation between minimal concentrations of drug necessary to augment (+)-tubocurarine e.p.p.s and ACh-potentials. There is a discrepancy in the results of the iontophoretic studies. The minimal drug concentrations necessary to augment (+)-tubocurarine e.p.p.s in iontophoretic studies are consistently higher than minimal concentrations necessary to augment (+)-tubocurarine e.p.p.s in m.e.p.p.-e.p.p. studies. At these higher concentrations facilitatory drugs are also capable of prolonging m.e.p.p.s. The only difference in experimental technique is the presence of an ACh-electrode in the iontophoretic studies. Thus this electrode must be depressing the effects of facilitatory drugs.
TABLE 18

Minimal Molar Concentration of Drugs Producing Significant Changes in E.p.p.s and ACh-Potentials

<table>
<thead>
<tr>
<th>Drug</th>
<th>E.P.P.</th>
<th>ACH-POTENTIAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neostigmine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exp # 1</td>
<td>$10^{-7}$</td>
<td>$10^{-7}$</td>
</tr>
<tr>
<td>Exp # 2</td>
<td>$10^{-8}$</td>
<td>$10^{-8}$</td>
</tr>
<tr>
<td>Edrophonium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exp # 1</td>
<td>$10^{-6}$</td>
<td>$10^{-6}$</td>
</tr>
<tr>
<td>Exp # 2</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Ambenonium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exp # 1</td>
<td>$10^{-8}$</td>
<td>$10^{-8}$</td>
</tr>
<tr>
<td>Exp # 2</td>
<td>$10^{-9}$</td>
<td>$10^{-8}$</td>
</tr>
<tr>
<td>Methoxyambenonium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exp # 1</td>
<td>$10^{-8}$</td>
<td>$10^{-8}$</td>
</tr>
<tr>
<td>Exp # 2</td>
<td>$10^{-8}$</td>
<td>$10^{-7}$</td>
</tr>
</tbody>
</table>
There are several possible explanations for this depressant effect.

1) A depressant action of ACh at the neuromuscular junction.

ACh depolarizes the motor nerve terminal (Hubbard and Yokota, 1964 and Hubbard, Schmidt and Yokota, 1965), and this action may depress the presynaptic actions of facilitatory drugs in iontophoretic experiments.

2) Damage to the nerve terminal.

Placement of an ACh-electrode involves considerable manipulation. Such movement may damage the terminal such that it does not respond normally to pharmacological tests.

3) Use of low amplitude e.p.p.s.

Facilitatory drugs are relatively ineffective in augmenting low amplitude e.p.p.s in the presence of high concentrations of (+)-tubocurarine. This is generally explained by a second action of (+)-tubocurarine, but it is possible that the facilitatory drugs are just not as effective in augmenting low amplitude e.p.p.s as high amplitude e.p.p.s.

It is difficult to determine which of the above effects is responsible for a lack of drug action in the iontophoretic experiments. As the ACh-electrode had little effect on e.p.p. amplitude, it is unlikely that nerve terminal
damage can explain these results. Any damage which would decrease the pharmacological effectiveness of a drug should also decrease physiological release of transmitter. Any depolarization of a nerve terminal by ACh would also be expected to decrease physiological release; although ACh may depress actions of facilitatory drugs by another mechanism.

In spite of this discrepancy high concentrations of facilitatory drugs are very effective in augmenting or prolonging ACh-potentials. In fact the ability of facilitatory drugs to affect ACh-potentials is nearly equivalent to their ability to affect e.p.p.s. On the basis of this observation it is necessary to conclude that most of the anti-Tc action of facilitatory drugs on isolated curarized mammalian muscle preparations is due to postsynaptic actions. To determine conclusively that there is not a small presynaptic action by these drugs in the presence of (+)-tubocurarine, it would be necessary to augment e.p.p.s and ACh-potentials at lower concentrations in iontophoretic studies.

Low concentrations of a facilitatory drug produce an increase in the rate of rise of (+)-tubocurarine e.p.p.s without prolongation of time to half-decay. This effect can be explained by increased release of transmitter, sensitization of the e.p. to the transmitter or possibly, depolarization of the e.p. by facilitatory drugs (Otsuka and Endo, 1960b). It is
unlikely that facilitatory drugs have sensitizing or depolarizing actions, as these drugs are not capable of augmenting carbachol-potentials. This appears to leave only a presynaptic mechanism.

It is generally assumed that cholinesterase inhibition will augment and prolong the e.p.p. with no change in the rate of rise. Yet in several experiments facilitatory drugs did increase the rate of rise of ACh-potentials without prolongation. Although not reported as such, the records of Katz and Thesleff (1957a) especially figure 1 -- give similar results with edrophonium in frog muscle. This increase in ACh-potential amplitude with no prolongation indicates that cholinesterase affects only the quantity of ACh reaching the e.p. In other words cholinesterase inhibition, especially low levels of inhibition, produces no prolongation of ACh action. Diffusion must be the major mechanism of termination of the action of iontophoretic ACh. It is difficult to determine if the same mechanism holds true for the involvement of cholinesterase in the generation of the e.p.p. It has been hypothesized that diffusion is quite important in the decay of transmitter action (Ogston, 1955), but it is unknown how important cholinesterase is in determining the initial quantity of transmitter reaching the e.p. This observation on the effects of facilitatory drugs does not support the idea that cholinesterase inhibition can not increase the rate of
rise of (+)-tubocurarine e.p.p.s. This increase in the rate of rise is most prominent at concentrations of facilitatory drug, that do not prolong the e.p.p. At higher drug concentrations there is prolongation of the e.p.p., indicating that cholinesterase inhibition does lengthen the action of the neuromuscular transmitter.

The primary effect of facilitatory drugs on (+)-tubocurarine e.p.p.s in isolated mammalian muscle is cholinesterase inhibition. This conclusion is supported by studies on quantal content. None of the drugs tested produce increases in the quantal release of the e.p.p. at concentrations which produce large increases in (+)-tubocurarine e.p.p.s. Thus changes in amplitude of e.p.p.s in a high magnesium ion concentration parallel changes in amplitude of m.e.p.p.s. This result indicates a mechanism of action for the facilitatory drugs which is postsynaptic, and this action may well be cholinesterase inhibition.

Benzoquinonium blocks neuromuscular transmission much like (+)-tubocurarine, but blockade by benzoquinonium is not antagonized by facilitatory drugs (Bowman, 1958). As benzoquinonium e.p.p.s are prolonged (compared to (+)-tubocurarine e.p.p.s), it is likely that cholinesterase is at least partially inhibited by benzoquinonium. In vitro
results indicate that benzoquinonium is nearly as potent as neostigmine in inhibiting cholinesterase (Blaber and Bowman, 1962). This inhibition may explain the ability of benzoquinonium to augment the (+)-tubocurarine e.p.p. amplitude. Also, previous inhibition of cholinesterase by benzoquinonium limits the inhibition of cholinesterase produced by the facilitatory drugs.

The theory of cholinesterase inhibition by facilitatory drugs is supported by experiments on neuromuscular depression produced by paired stimuli to isolated curarized frog muscle preparations. Neuromuscular depression is not affected by edrophonium or physostigmine (Takeuchi, 1958a; Otsuka, Endo and Nonomura, 1962 and Otsuka and Nonomura, 1963). If the facilitatory drugs increase the release of transmitter, they should increase the depletion-induced depression resulting from paired stimuli. Although this work was performed in frogs it is probably applicable to mammalian preparations.

Although cholinesterase inhibition appears to be the primary action of facilitatory drugs on e.p.p.s, this action does not explain the spontaneous twitching observed in the presence of ambenonium. Originally, it was thought that m.e.p.p.s are augmented to such an extent by facilitatory
drugs that they give rise to propagated muscle action potentials. But Katz and Thesleff (1957b) concluded that even in small muscle fibers, cholinesterase inhibition could not augment m.e.p.p.s sufficiently to induce muscle action potentials. It is possible that spontaneous twitching is due to spontaneous giant potentials (Liley, 1957), except giant potentials have been observed only in the rat diaphragm. Furthermore if they can give rise to fibrillations, they can not cause fasciculations. Fasciculations must be induced by motor unit activity.

A possible origin of drug-induced fasciculations is suggested by the experiments of Hubbard, Schmidt and Yokota (1965). Neostigmine (3.3 x 10^{-6} M) reduces the stimulus threshold of a nerve terminal in the presence of (+)-tubocurarine or excess magnesium and decreased calcium Ringers. This apparent depolarization may initiate propagated activity in the motor nerve, which could spread throughout the motor unit giving rise to a fasciculation and antidromic activity (Masland and Wigton, 1940).

Neostigmine, edrophonium and ambenonium initiate spontaneous fasciculations and spontaneous antidromic nerve action potentials in vivo (Blaber and Goode, 1968). As neostigmine and edrophonium do not produce spontaneous twitching in isolated muscle, it is likely that in vitro preparations are not as responsive to facilitatory drugs as in vivo preparations.
Methoxyambenonium is as potent as neostigmine and edrophonium in augmenting the (+)-tubocurarine e.p.p., but is unable to facilitate an indirect, maximal twitch (Blaber, 1960). If the sole mechanism of action of facilitatory drugs is cholinesterase inhibition, methoxyambenonium should also produce facilitation of the maximal twitch. Blaber and Goode (1968) observed that methoxyambenonium is incapable of initiating spontaneous antidromic nerve activity. Initiation of spontaneous activity may involve the same mechanism of action as facilitation of transmission. If this is the case, methoxyambenonium would not be expected to facilitate the muscle twitch.

Repetitive muscle activity after an orthodromic stimulus lasts for a period of approximately 50 msec in the presence of a facilitatory drug (Blaber and Bowman, 1963a). The longest (+)-tubocurarine e.p.p. observed in the presence of high concentrations of a facilitatory drug, has a half fall of only 2.8 msec. This degree of prolongation would not give rise to prolonged repetitive muscle activity. It is necessary to assume that (+)-tubocurarine drastically decreases the prolongation induced by the facilitatory drugs (Eccles, Katz and Kuffler, 1942 and Karczmar, Koketsu and Soeda, 1968). Only in the presence of a deficient sodium ion concentration do facilitatory drugs prolong e.p.p.s to such an extent
(Fatt and Katz, 1951 and Kordas, 1968). This discrepancy between e.p.p. half-decay time and length of repetitive activity suggests that a mechanism other than cholinesterase inhibition is involved in facilitation.

The occurrence of spontaneous twitching makes it seem likely that a presynaptic mechanism is involved in the action of facilitatory drugs. The explanation, as to why it does not occur in the e.p.p. studies, may be involved in the depressant action of tubocurarine on the motor nerve terminal. Small doses of tubocurarine depress the supernormal period of the nerve terminal spike (Hubbard and Schmidt, 1961 and Hubbard, Schmidt and Yokota, 1965) and drug-induced antidromic nerve activity (Werner, 1961; Blaber and Bowman, 1963b and Standaert, 1964). As the concentration of (+)-tubocurarine used in these experiments was well over the concentration necessary to produce 100% block of the muscle twitch, it is quite possible that the nerve terminal action of facilitatory drugs was completely depressed in these experiments.

Deficient calcium ions and benzoquinonium are even more effective in blocking the terminal actions of facilitatory drugs. For this reason a terminal effect would not be expected to occur in their presence either (Hubbard, Schmidt and Yokota, 1965 and Blaber and Bowman, 1963a).
This depressant effect of blocking drugs may be amplified by the abnormal environment of the isolated muscle.

**THEORY OF THE MECHANISM OF DRUG-INDUCED FACILITATION AND ANTI-Tc ACTION AT THE SKELETAL NEUROMUSCULAR JUNCTION**

The mechanisms of drug-induced facilitation and anti-Tc action by facilitatory drugs are at present in hypothetical stages. All facilitatory drugs appear to act at at least three sites:

1) cholinesterase,
2) directly at the e.p., and
3) presynaptically.

The relative importance of each site of action depends on the particular drug being considered.

The presynaptic action of neostigmine, edrophonium, ambenonium and methoxyambenonium has not been clearly elucidated. It does not involve depolarization of the motor nerve terminal in the same manner as potassium ions. Yet neostigmine, edrophonium and ambenonium do depolarize the terminal at another site, possibly the first node, as hypothesized by Hubbard, Schmidt and Yokota (1965) and Blaber and Goode (1968). This
initiates spontaneous twitching. Methoxyambenonium blocks at this site, thus it is not capable of inducing spontaneous antidromic activity. This is probably a direct action (not mediated by ACh) (Hubbard, 1965) as ACh did not produce spontaneous twitching. Also edrophonium and neostigmine should have produced spontaneous twitching at some concentration if the effect was due to ACh accumulation.

Neostigmine increases the duration of the nerve terminal action potential and negative after potential (Hubbard and Schmidt, 1961 and Hubbard, Schmidt and Yokota, 1965). A longer duration of the terminal action potential should lead to increased release of transmitter. The prolonged negative afterpotential may also increase release somewhat; although according to Liley's theory (1956c), this release would be insignificant. To hypothesize that an augmented negative afterpotential increases the release of transmitter, it is necessary to hypothesize that an additional factor is involved (maybe calcium is accumulated, intraterminally).

The augmented negative afterpotential probably generates antidromic nerve activity. This antidromic activity passes into the ventral root where it is recorded. Also, when it reaches a nerve terminal branch, it may
pass orthodromically into another terminal. This reverberation could lead to a continuous release of transmitter.

In addition to a presynaptic action, facilitatory drugs inhibit junctional cholinesterase. It is difficult to assess the role of cholinesterase inhibition in facilitation. Yet this appears to be the primary mechanism involved in antagonism of high concentrations of (+)-tubocurarine.

The direct actions of facilitatory drugs have only a very minor role in facilitation and antagonism of (+)-tubocurarine but they may be responsible for blockade of neuromuscular transmission at high concentrations.

These same mechanisms probably are involved in the action of every drug at the neuromuscular junction. The importance of each action is determined by the chemical structure of the drug being studied. Depolarizing drugs act primarily by a direct, depolarizing action, but they also act at the nerve terminal and inhibit cholinesterase. Competitive blockers have a potent competitive blocking action, but may act at the terminal and inhibit cholinesterase (e.g. benzoquinonium). TEA and guanidine act primarily at the nerve terminal, but also exhibit direct actions. Thus the effect of a drug depends not on any one site of action but upon the sum of actions at many sites.
SUMMARY

1) The facilitatory drugs (neostigmine, edrophonium, ambenonium and methoxyambenonium) produced only small changes in m.e.p.p. frequency. As this effect was small compared to the effect of potassium ions, it was concluded that the facilitatory drugs did not significantly change the nerve terminal membrane potential.

2) In all experiments in which no blocking drugs were present ambenonium produced spontaneous twitching.

3) Each of the facilitatory drugs augmented but did not prolong the Tc e.p.p. Furthermore this augmentation of e.p.p. amplitude occurred at low concentrations of facilitatory drug which had no effect on m.e.p.p. amplitude or time-course. This lack of correlation implicated a presynaptic site of drug action.

4) When ACh-potentials and Tc e.p.p.s were recorded, simultaneously; both were augmented by the same minimal concentration of facilitatory drug. However, the concentration of drug necessary to augment the Tc e.p.p. was higher when ACh was applied to the e.p. by iontophoresis than in the experiments summarized in 3) above.

5) The facilitatory drugs did not augment the carbachol-potential at any concentration.
6) The facilitatory drugs did not increase the quantal content of the e.p.p. recorded in the presence of a low calcium ion and high magnesium ion concentrations.

7) Benzoquinonium administration, unlike Tc, caused a continuous decline in e.p.p. amplitude. The benzoquinonium e.p.p. had a longer half-decay time, which is indicative of its ability to inhibit junctional cholinesterase. The facilitatory drugs were ineffective in augmenting the benzoquinonium e.p.p.

8) The correlation between the minimal concentrations necessary to augment the Tc e.p.p. and ACh-potential indicates that most of the effects of the facilitatory drugs in antagonizing Tc are due to a postsynaptic action. This postsynaptic action is probably cholinesterase inhibition, as the facilitatory drugs had no effect on the carbachol potential. The lack of correlation between effects on m.e.p.p.s and Tc e.p.p.s indicates that these drugs might have a weak presynaptic action in the antagonism of Tc.

9) The conclusion that the primary effect of the facilitatory drugs is due to cholinesterase inhibition is supported by the lack of effect of these drugs on quantal release. Also most of the actions of benzoquinonium can be explained by it cholinesterase inhibiting properties.
10) Cholinesterase inhibition can not account for the production of spontaneous twitching by ambenonium. Thus a presynaptic effect must occur in the absence of Tc.

II) On the basis of these results it seems that a presynaptic action of the facilitatory drugs is minimal in the presence of high concentrations of Tc which are necessary for use of microelectrode recording techniques.
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The dissertation submitted by Daryl Dean Christ has been read and approved by five members of the faculty of the Graduate School.

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval with reference to content, form and mechanical accuracy.

The dissertation is, therefore, accepted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

26 April 1969

Signature of Advisor
L. C. Blaber, Ph. D.