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Contribution to the Understanding of the Pharmacology of the Skeletal Neuromyal Junction, with Particular Reference to the Action of Depolarizers

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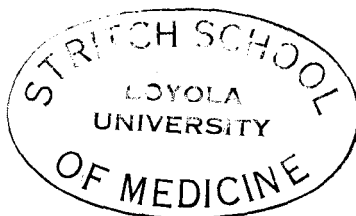
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CONTRIBUTION TO THE
UNDERSTANDING OF THE PHARMACOLOGY OF THE
SKELETAL NEUROMYAL JUNCTION, WITH PARTICULAR
REFERENCE TO THE ACTION OF DEPOLARIZERS

by
K. C. KIM, M.D.



A Thesis Submitted to the Faculty of the Graduate School
of Loyola University in Partial Fulfillment of
the Requirements for the Degree of
Doctor of Philosophy

June
1962

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I also wish to extend my thanks to Dr. K. Koketsu for his kind help and advice.

LIFE

Kil Chol Kim was born in Korea on April 22, 1919.

He was awarded the degree of Medical Doctor from TAE CU Medical School in Korea in 1944.

He had practiced until 1955.

He had his internship at Edgewater Hospital in Chicago in 1956.

He had anesthesia training at Billing Hospital, University of Chicago, at the County Hospital and at the Michel Reese Hospital in Chicago from 1957 to 1958.

He started to study pharmacology in the Department of Pharmacology at Loyola University School of Medicine in 1959. He was graduate assistant in 1961/1962. Part of his graduate training was carried out under Dr. K. Koketsu, Department of Neuropsychiatry, Illinois College of Medicine.

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A. Author's preface:

When I came to the U.S.A., I received training as an anesthesiologist and later was employed in this capacity. For a research-minded anesthesiologist, two avenues of investigation seemed natural; either research on physiology and pharmacology of respiration or research on the problem of neuromyal transmission.

Many anesthesiologists have done work on the nerve-muscle junction and contributed not only to applied pharmacology of muscle relaxants, but also to the understanding of the mechanisms of neuromyal cholinergic transmission. It should suffice to mention such anesthesiologists and authors of many research papers and important texts as F. Foldes, V. Collins, R. D. Dripps, H. C. Churchill-Davidson. My special interest in the neuromyal junction was aroused by the reading of their stimulating publications as well as by the baffling and protean behavior of the nerve-muscle transmission. No one who practices anesthesia could help but be challenged and mystified by the variable sensitivity and the qualitative change of the response of the endplate to neuromyal agents. Moreover, we deal here with a very basic physiological mechanism - that of cholinergic transmission. Altogether, the temptation to study the versatility of this transmission at the neuromyal site cannot be easily resisted. This explains why, along with anesthesiologists, many prominent neurophysiologists and neuropharmacologists either have made the neuromyal junction the subject of their main preoccupation, as did B. Katz, P. Fatt, G. L. Brown, S. W. Kuffler, S. Thesleff, E. Zaimis, or at least attacked this subject at one time or another, as exemplified by J. C. Eccles, D. Bovet, G. B.

Koelle, K. R. W. Unna, J. Hoppe, and others.

When, therefore, the chance was offered to me to be trained in the methodology of studying neuromyal transmission by Dr. K. Koketsu and to carry out research for a Ph.D. in this area under Dr. A. G. Karczmar, I could not but embrace this chance.

B. Introduction

I. Physiology of the neuromyal junction.

1. Historical developments leading to the modern concepts of neuromyal physiology.

Before the latter half of the nineteenth century it was assumed that a continuity exists between nerve and muscle (cf. Brazier, 1959). The muscle was supposed to be activated by a nervous fluid, possibly having properties of Newton's ether, which could fill the muscle and provide its tension and rigidity.

In 1862 Kuehne (cf. also Kuehne, 1888) noted histological differences between skeletal muscle and its innervation. He suggested that the action current of the nerve, discovered previously by Du Bois-Reymond (1848, 1849), invaded the muscle and caused it to contract. Du Bois-Reymond noticed a transmission delay at the neuromyal junction; ^{although} at first ^{he} considered that it may be due to chemical influences, ^{later} he believed that electrical fields of a nerve-muscle ^ucontinuum were responsible for contraction.

The importance of the junction was established by the researches of Claude Bernard (1857), which indicated that curare acts at a peripheral site on the motor nerve. Subsequently Langley (1905, 1907) showed that nicotine can act, upon denervation and motor nerve degeneration, at restricted sites of the muscle fiber.

At the turn of the century the concept of the chemical nature of neuromyal transmission was elaborated by Elliott (1905), Dale (cf. his Harvey Lecture, 1937), and Loewi (1921). Their work dealt principally with smooth and cardiac muscle. The thought that transmission at the parasympathetic nerve

endings is cholinergic in nature, originated from Dixon's (1907) suggestion that the transmitter may be muscarine, an alkaloid related to choline. Subsequently, acetylcholine (ACh) was implicated as a transmitter substance (Hunt and Taveau, 1906; Dale, 1914; Loewi, o.c.) at parasympathetic nerves. Finally, Dale and his associates (cf. Dale, Feldberg and Vogt, 1936) showed that motor nerve terminal releases upon stimulation a pharmacologically active substance, later identified as ACh.

This classical period of the studies of cholinergic transmission and of the neuromyal junction terminated with the studies of Sir Lindor Brown (1937, and with Dale, Feldberg, and Vogt, 1936) in which they showed that "close" (1) intra-arterial injection of ACh mimics, in both innervated and denervated muscle, the effects of nerve impulse; this was true with regard to mechanical and membrane responses of the muscle. It could be also shown that curare and physostigmine influence mechanical and membrane effects of injected ACh just as they do the responses of the stimulation of the motor nerve. Thus, the transmitter role of ACh at the motor nerve terminal was additionally substantiated by the similarity of the effects of drugs upon the muscle response to ACh, on one hand, and to motor nerve stimulation on the other.

Altogether, we can consider that by the 1940's not only the neuromyal transmission was proven to be cholinergic in nature, but also the importance of the endplate (e.p.), while

(1) "Close" intra-arterial injection is a pharmacological term. In this technique, the agent is injected into the artery as close as possible to its presumed site of action.

originating from the nineteenth century researches of Kuehne (o.c.), and Bernard (o.c.), was finally established as an ACh receptor. Both these achievements can be attributed mainly to the English school of investigators (Brown, Feldberg, Dale, and Eccles; cf. Eccles, Katz; and Kuffler, 1941).

2. Physiological events at the endplate.

a. Morphology of the endplate. The work of Cou-teaux (1947, 1955) established that "there are three sharply defined components of different cellular origin at the junction" (Fatt, 1959). The first of these is the terminal apparatus of the nerve; the second - the specialized region of muscle surface contacted by the nerve endings; the third - a layer of neuroglia, which at this site is referred to as teloglia, continuous with the Schwann cell envelope of the myelinated fiber. The teloglia contributes half the nuclei of the junctional region (the sole nuclei), the remainder being the fundamental nuclei in the muscle, and accompanies the nerve terminals as they sink into the grooves in the muscle fiber surface. Muscle fibers lining the groove form infoldings, as seen in the electron microscope (Robertson, 1956; Andersson-Cedergreen, 1959). While the picture of the junction as described above is generally characteristic for all species, there are differences between the mammalian and frog ("bush" endplate) junctions, the mammalian e.p. occupying a smaller site in relation to the muscle, while the nerve terminals of the frog endbush cover a wide area of the muscle fiber.

Most recently, an interesting contribution to the anatomy of the nerve terminal portion of the junction was offered by De Robertis (1958) and confirmed by Andersson-Cedergreen (1959) and other investigators. These investigators showed occurrence at the terminal nerve endings of small vesicular elements,

called synaptic vesicles. Indirect evidence (cf. Del Castillo and Katz, 1956a; and Eccles, 1958) suggests that synaptic vesicles contain ACh, presumably in the quantal form of "packages" (cf. also below).

b. Cholinesterases and the neuromyal junction.

It was shown in a series of researches by Nachmansohn and his associates (Marnay and Nachmansohn, 1938; Nachmansohn, 1940; cf. also Nachmansohn, 1946 and 1959) that cholinesterase (ChE) is an enzyme concentrated at the endplate. In terms of the kinetics of its action, ChE can account for the almost instantaneous hydrolysis of ACh, released at the nerve terminals. The rapidity of the action of ChE is a pre-requisite, as proposed by Nachmansohn, for functionability of the neuromyal junction. The importance of ChE's for the function of the neuromyal junction and of other synapses has been further extended by many investigators (for recent review, cf. Koelle, 1961a and b). While their specific function will be reviewed more in detail in subsequent sections of this introduction, it should be stated at present in general terms that chemical transmission may depend not only on the presence of a transmitter, but also upon the availability of a hydrolytic enzyme (Loewi, 1921, Giarman, 1957). In view of this, and particularly because of their role in the neuromyal function, the localization of ChE's in the junction is important.

The ChE of the endplate is predominately acetyl-cholinesterase (AChE), located mostly post-synaptically. Pseudocholinesterase (butyrylcholinesterase, BuChE) is present in smaller quantities (Denz, 1953; Holmstedt, 1957 and 1959). AChE is present also at the musculotendinous junctions. Some AChE or BuChE is present in the muscle fiber (Becket and Bourne, 1957).

It should be stated here that a physiologically and functionally important difference between AChE and BuChE is that the former is a faster and a more specific enzyme. As stated by Nachmansohn (o.c.), this enzyme, rather than BuChE, fulfills the criteria of an enzyme capable of terminating the actions of ACh. On the other hand, BuChE may be concerned with terminating the action of excesses of acetylcholine escaping into the blood stream (Koppanyi and Karczmar, 1953). In accordance with this difference between the two ChE's, AChE predominates in central and peripheral synapses, and BuChE in the blood and in certain barriers surrounding synapses (Volle and Koelle, 1961).

c. Electrical response at the e.p. and at the nerve terminal.

It was shown first by Eccles and O'Connor (1938) and Shaeffer and Haass (1939) that a non-propagated negative, monophasic potential arises in a curarized, non-contracting muscle. This potential, characterized by a brief rising phase and a slower return to the baseline, is referred to as the endplate potential (e.p.p.); it is focused at the nerve endings, and magnitude-wise correlated with their density (Eccles, Katz and Kuffler, 1941 and 1942). Upon reduction of the concentration of curare, action potentials of individual fibers appear as deflections superimposed on the e.p.p., till a full, synchronized action potential obscures the e.p.p. completely. To the contrary, when the concentration of curare is increased, the e.p.p. becomes reduced.

Fatt and Katz (1951) used the intracellular recording technique to study the e.p.p.'s at the individual muscle fibers as potentials obtained across the fiber membrane at the

position of insertion of the electrode. By means of this technique the frog muscle fiber was found "to have a resting membrane potential of above 90 mv (inside negative with respect to outside), which is the same in the junctional region as elsewhere along the fiber" (Fatt, 1959). In a frog nerve-muscle preparation, critically curarized to abolish contraction, muscle fibers display, in response to nerve stimulation, e.p.p.'s ranging from 10 to 40 mv. The e.p.p. is, in these conditions, a transient positive deflection, or reduction of membrane potential from its resting level (depolarization). The e.p.p. becomes attenuated and slower when measured progressively further away from the nerve terminal.

Threshold depolarization of 40 mv initiates the propagated, biphasic action potential. The spike portion of the action potential is the positive deflection of membrane potential, indicating depolarization, plus the overshoot or the reversal of the membrane potential. The spike at the e.p. differs in certain features from the spike evoked in the muscle fiber by direct stimulation. This may be due to the modification of the action potential by junctional activity. This activity consistently causes a deviation toward a level near zero membrane potential (Fatt, 1959). On the basis of further biophysical and experimental analysis, Fatt concluded that "the effect of junctional activity on the muscle fiber membrane can be represented as the addition of a conductance in a series with a fixed emf. This may further be interpreted as the creation of a new path for the diffusion of ions across the membrane" (o.c.).

While the above description of the e.p.p. is based on investigations of amphibian muscle fibers, it also applies,

except for certain numerical and pattern differences, to mammalian musculature. The differences are due to a higher conductance of the latter, with a consequent reduction in electric time and space constants.

An important aspect of ACh as the transmitter substance at the neuromyal junction is, as already stated, its behavior when applied to the junctional tissue. Whether applied to the whole muscle fiber, or only to the area invaded by the nerve endings, ACh depolarizes the junctional region alone (Cowan, 1936; Kuffler, 1943; Burns and Paton, 1951). This can be demonstrated by means of iontophoretic technique, in which ACh is applied by means of brief electric pulses from a micropipette. In this case, ACh was found to be ineffective along the whole muscle fiber (Nastuk, 1953; Del Castillo and Katz, 1955a). When amphibian muscle is exposed in the presence of ChE inhibitors to ACh, the concentration of $1\text{ }\mu\text{M}$ depolarizes the junctional region sufficiently to produce a spike. With lower concentrations, depolarization is proportional to the amount of ACh. With higher concentration, depolarization persists after a number of spikes, at the time when the neuromyal junction is not capable of further initiation of spikes. The viewpoint that ACh acts on the e.p. alone was recently attacked by Ochs et al. (1959), but even more recent findings of Katz (1959) seem to support the original contention that chemical sensitivity of the muscle is restricted. High sensitivity to ACh extends only over very limited regions in the neighborhood of the nerve terminal branches; in the frog, the sensitivity decreases or disappears already within 10 to 20 μ from the nerve terminal. Recently, Miledi (1960) thought that this region, while limited, extends somewhat further. Depolarization occurs only when ACh

is applied to the fiber surface; ACh is not effective when released within the muscle fiber. The time course of depolarization, while much longer than that of e.p.p., is consistent with the reasonable assumption that ACh must diffuse from its point of release to the receptor some microns away. Finally, depolarization elicited by ACh can be demonstrated (Del Castillo and Katz, 1955b) to be caused by the same conductance change which occurs during transmission.

The appearance, during quiescence of the nerve, of small (0.5 mv) transient changes of potential (miniature endplate potentials, m.e.p.p.), has an interesting bearing on neuromyal transmission. The m.e.p.p.'s share, in a small way, the characteristics of the e.p.p.'s; they have a similar time course, are attenuated away from the nerve terminals, and are affected like e.p.p.'s by certain drugs, such as curare and ChE inhibitors. The m.e.p.p.'s disappear upon denervation and subsequent degeneration of the nerve terminal. This and other considerations caused Fatt (1959) in particular to consider m.e.p.p.'s as being due to random collisions of the synaptic vesicles, already described as probably containing "packages" of ACh, with active sites in the nerve terminal membrane; these collisions would lead to release of "quanta" of ACh, and to depolarization at restricted receptor sites. The normal e.p.p., or the spike, depends on the other hand, on synchronized release of a very large number of "packages", leading to a constant spike amplitude. Not necessarily the same vesicles discharge per each spike, although their number, per each spike, is constant. On the other hand, an incomplete discharge of relatively few vesicles would lead to a reduced e.p.p., and finally to a m.e.p.p.

Some of the lines of indirect evidence for this viewpoint are as follows; first, withdrawal of calcium reduces the size of the e.p.p., till the e.p.p. becomes not much larger than the m.e.p.p.; it also fluctuates in size and can become intermittent (Boyd and Martin, 1956a and b; Del Castillo and Katz, 1954a; Liley, 1956). This is because (Fatt, 1959) the e.p.p. reveals itself under these conditions as being composed of a variable number of m.e.p.p.'s, the fluctuation being due to variation in number of units firing. Second, when trains of stimuli are used in the frog nerve muscle preparation, the second stimulus leads to a larger e.p.p. When the calcium concentration is reduced so that the e.p.p. has an amplitude corresponding to postulated discharge of a few synaptic vesicles as described before, the response to the second of the stimuli is again increased, and, what is most important, the fluctuation is reduced. This reduction of fluctuation can be due only to an increase in the number of units, or of synaptic vesicles, responding (Del Castillo and Katz, 1954b).

d. Mechanism of neuromyal transmission.

From various experiments described above, the amount of ACh released during a single nerve impulse at a single junction has been calculated as 10^{-18} moles (Emmelin and McIntosh, 1956). This figure, after certain corrections are made, corresponds well to the minimal quantity of ACh, applied iontophoretically, capable of evoking spike upon application to the endplate. ACh, released during the single impulse, is presumably liberated by concerted breakage of a constant (per each impulse) number of synaptic vesicles in the nerve terminals. It can be also calculated that ACh will exert its maximal effect before diffusing more than 1μ , a

distance which agrees with the morphological, electron microscope findings on the separation of the pre- and post-junctional surfaces. Finally, ACh will remain in effective concentration at the junction for less than 1 ms, and diffusion will terminate the first, high intensity transmitter action, expressed by the short duration of the rising phase of the e.p.p., or of the spike. On the other hand, descending phase of the e.p.p. will depend on factors other than the movement of ACh, for instance, on the activity of AChE (Fatt, 1959). Finally, upon combining with the receptor, ACh alters physical properties of the membrane and a new path appears for ionic diffusion; in electrical terms, an additional conductance short circuits existing potential differences. These concepts, when considered jointly with Grundfest's (1957; 1959) concept of electrical non-excitability of perhaps most synapses, lead to a picture of the skeletal neuromyal junction as one of the most explored, chemically operated synapses.

II. Pharmacology of the neuromyal junction.

1. General concepts.

Certain aspects of pharmacology of the neuromyal junction have been already presented, since they form an integral part of our concepts of the neuromyal transmission. Conversely, certain drug actions can be deduced from the knowledge of the physiology of the e.p. Thus, curare and its crystalline d-isomer, d-tubocurarine (d-Tbc), does not by itself affect electric properties of the membrane, but prevents ACh from combining with the receptor and from exerting the depolarizing action. ACh and d-Tbc interact competitively and the e.p.p. will be decreased by large concentrations of d-Tbc because under these circumstances depolarization will not suffice

to produce the spike and also it will embrace only relatively few receptor units (Bovet, 1951). A number of synthetic substances share the properties of d-Tbc (Bovet, 1951 and 1959; Hoppe, 1957). On the other hand, ChE inhibitors, while incapable of affecting the early phase of ACh action, may cause ACh accumulation at the e.p., ACh then becoming capable of displacing d-Tbc (Pelikan et al., 1956). By means of a similar mechanism, anti-ChE agents can increase the amplitude and possibly the frequency of m.e.p.p.'s, and prolong the e.p.p. (Del Castillo and Katz, 1954c); finally, in the presence of anti-ChE agents, single motor nerve stimulation will lead to repetitive firing rather than to single spike (Nastuk and Alexander, 1954; Karczmar and Fudema, 1957; Karczmar, 1961). Repetitive firing is accompanied by the macroscopically observable sustained contraction, indistinguishable from the tetanic twitch which is normally obtained upon repeated discharge.

Finally, certain substances which resemble ACh, can depolarize the e.p. similarly to the transmitter substance. These do not have to be choline esters, but must exhibit a cationic, usually quaternary head of certain electron density, the latter depending in part on the dimension of the quaternary or related radical (Riker, 1953, Riker et al., 1958). Among the most effective depolarizers are two bisquaternaries, decamethonium (C_{10}) and succinylcholine (SCh; Paton and Zaimis, 1949; Ginzl et al., 1951). Anti-ChE agents, presumably because of protection they afford ACh, can also depolarize (Douglas and Paton, 1954); this depolarization can occur only in the presence of large concentrations of the anti-ChE agents.

It is of interest that certain chemical resemblance exists between many anti-ChE agents, depolarizers and curaremimetics (Karczmar, 1961; Karczmar et al., 1961). In fact, many neuro-myally active agents cannot be easily classified from the pharmacological viewpoint; they may exhibit predominantly the actions characteristic for one type of drugs, and, somewhat less extensively, those characteristics for drugs of another type. Thus, certain curaremimetics as well as many cholinomimetics may have anti-ChE action (Hoppe, o.c.). This, in turn, may indicate certain similarities between cholinergic receptor and the ChE or particularly AChE moiety: (Karczmar, 1961; Karczmar et al., o.c.).

2. Neuromyal blocking agents and their antagonists.

a. Curare, ammonium ions and anti-ChE agents.

The mechanism of action of curare, d-Tbc, and of synthetic curaremimetics was discussed when basic properties of the e.p. were described (cf. above). As competitive antagonists of ACh, these agents can be antagonized by large doses or concentrations of ACh and of other depolarizing agents, principally quaternary ammonium compounds. Among the latter can be listed the phenyltrimethylammonium group investigated by Riker, Wescoe and their associates (cf. Wescoe et al., 1949, 1950; Riker et al., 1957, 1959a). Riker (1953) is of the opinion that these compounds act as anticurare agents not only by depolarization but also because they displace curare from the e.p., thus exposing curare to metabolic destruction. This hypothesis of displacement was originally proposed by A. J. Clark (1933). Part of Riker's evidence for this concept is as follows. The effective dose of curare or d-Tbc, used alone, may produce a 1/2 hr. blockade (cat data); depolarizing or excitatory action of

phenyltrimethylammonium compounds, as evidenced by repetitive discharges, is, on the other hand, brief. Yet, when d-Tbc is antagonized by a trimethylammonium compound, the blockade does not recur.

The effect of anti-ChE agents is, as already indicated, similar to that of large doses of ACh. In fact, Unna and his associates (Unna et al., 1944; Pelikan, Smith and Unna, 1956) could relate anticurare potency of a number of anti-ChE agents to the enzymic inhibitory potency of the latter. However, in the case of at least these anti-ChE agents, which possess a quaternary cationic head resembling that of trimethyl-ammonium compounds or of ACh itself, their potency to displace d-Tbc and also to produce the depolarization of the e.p. plays also a part in their anti-d-Tbc action. Prime examples are Edrophonium (Tensilon), and neostigmine (Prostigmine). Perhaps among the first investigators to point out the difference between the action of neostigmine and that of other anti-ChE agents were Jacobsohn and Kahlson (1938). For instance, neostigmine but not other anti-ChE agents, can produce muscle twitch in the cat upon close intra-arterial injection, and its anti-curare effect is more prompt than that of physostigmine or phosphonate anti-ChE agents (Riker, 1953). However, whether acting solely by AChE inhibition, or also directly, this group of curare antagonists provokes repetitive firing at the e.p., and causes potentiation of the mechanical response of the muscle to indirect stimulation.

b. Unstabilizers of motor nerve terminal and curare.

Masland and Wigton (1940) and Eccles et al. (1942) demonstrated that upon administration of neostigmine and physo-

stigmine short trains of repetitive antidromic discharges can be made to appear in motor nerve fibers stimulated with single or double shocks. More recently, Riker, Werner and their associates (Riker et al., 1957, 1959a and 1959b) studied a number of compounds, which are substituted trimethylammoniums characterized particularly by a hydroxy substitution, but also by substitutions of larger alkyl groups for the methyls of the parent compound. Riker and Werner stressed their presynaptic, motor nerve terminal site of action. It is Riker's opinion that these compounds are not anti-ChE agents, that they do not depolarize, and that they produce repetitive firing and twitch potentiation by unstabilization of the nerve terminal. Since anti-ChE agents, physostigmine and neostigmine, also produce antidromic firing, and since certain trimethylammonium compounds and their analogs (such as Edrophonium) exhibit anti-ChE potency (Nastuk et al., o.c.), it cannot be ruled out that hydroxyalkylammonium agents may unstabilize the nerve terminal because of their anti-ChE action. It would be difficult to prove this. Compounds of this structure would presumably inhibit reversibly cholinesterase action and this inhibition cannot be readily demonstrated in vivo (Karczmar, 1961). Tasaki (1958) used microelectrode methods on the e.p. of a cephalopod (these animals are particularly convenient for this type of study), and he measured directly the pre- and post-synaptic potentials. It is possible that his method should be used to test further the hypothesis of Riker and his associates.

It should be pointed out that Koelle and his associates (Koelle, 1961a and b; Volle and Koelle, 1961) postulated recently that ACh itself can exert a retrograde action at the

nerve terminal causing more release of ACh from its storage; while Koelle's postulate was derived from data dealing with a sympathetic ganglion, it may be extended to the neuromyal junction. Altogether, it may well be that some agents, whether cholinomimetic or anti-ChE in nature, or, finally, methylammonium derivatives, may produce excitatory and anti-curare effects at the e.p. by virtue of their nerve terminal site of action. This action may be facilitatory in some, at present, undefined way, or it may be due to release of ACh from presumably synaptic vesicles in the nerve terminal.

c. Action of tetraethylammonium (TEA) and Sodium Fluoride (NaF.).

Recently Stovner (1957a, b and c; 1958a, b, and c) and Koketsu (1958) have studied still another anti-curare substance, TEA. Stovner (o.c.), studied mostly the effects of TEA upon the blockade of neuromyal junction by curaremimetic substances. Koketsu (o.c.), pointed out that, paradoxically, TEA blocked ACh depolarization very much like a curaremimetic, and yet antagonized d-Tbc. They suggested that, somewhat like hydroxymethylammonium compounds, TEA acts presynaptically, and possibly releases ACh at that site. This interesting suggestion is based on indirect information and deserves further evaluation.

An interesting feature of the action of TEA is that its main effect is the augmentation of the e.p.p. While TEA causes some prolongation of the e.p.p., it does so to a much lesser extent than anti-ChE agents (Koketsu, 1958). A chemically unrelated compound, NaF, shares these characteristics with TEA (Koketsu and Gerard, 1956). These and other features of NaF action led these investigators to conclude that, without being an anti-ChE, it sensitizes the e.p. to ACh (o.c.).

d. Depolarizing substances.

Burns and Paton (Burns and Paton, 1951; Paton, 1952) compared depolarizing e.p. actions of ACh and of decamethonium (C_{10}) on the cat gracilis muscle. In both cases, depolarization was confined to the e.p., but it was more or less persistent only in the case of C_{10} . Depolarization by C_{10} resembled that by ACh, provided the muscle was pretreated with physostigmine. In both cases, the depolarization rendered the e.p. but not the muscle elsewhere electrically inexcitable. These experiments were extended by Zaimis and Paton (Zaimis, 1953 and 1959; Paton and Zaimis, 1949 and 1952). In 1952-1953 Zaimis was responsible for interesting conceptualization that depolarizing agents can be effective, provided they are long acting, as blocking agents. According to this hypothesis, all pharmacologic actions of depolarizers - muscle twitch upon i.a. administration as well as initial potentiation and subsequent blockade of neuromyal transmission - are interrelated and are the result of their capacity to depolarize the e.p. At that time then, two classes of blocking substances were distinguished, the competitively acting curaremimetics on one hand, and the depolarizing blocking agents, exemplified by succinylcholine (SCh) and by C_{10} on the other.

Several additional actions of depolarizers indeed are consistent with the conceptualization of the British investigators. For instance, anti-ChE agents which antagonize d-Tbc, are without effect upon SCh and C_{10} ; some of them actually enhance or prolong their blockade (Karczmar, 1957; Zaimis, 1959). As can be expected, competitive blockers can antagonize the depolarizers; pretreatment with a small, subliminal dose of d-Tbc prevents the action of SCh and C_{10}

(cf. for instance Karczmar and Fudema, 1957, and Karczmar, 1961). In fact, antagonism of SCh and C_{10} by any other than curaremimetic agents was, till recently (cf. below), never demonstrated. Finally, depolarizing agents produce characteristic spastic paralysis in the pigeon (Zaimis, 1952, 1953; Hoppe, 1955), as do sometimes ChE inhibitors (o.c.).

It appeared subsequently that the status of the so-called depolarization blockade cannot be quite so clear-cut. In a series of papers, Thesleff (1955a and b) showed that in the frog at least, C_{10} and SCh blocked the neuromyal transmission only after depolarization was over. Furthermore, the effects of methoxyambenonium (WIN 8078) in the cat and in the dog (Karczmar and Howard, 1955; Karczmar, 1957 and 1961; Lands, Karczmar, Howard and Arnold, 1955) upon C_{10} , SCh and d-Tbc blockade cannot be reconciled with the original hypothesis of Zaimis (cf. Karczmar, 1956, 1957 and 1961). Karczmar observed that methoxyambenonium antagonized the blockade by both curaremimetics and depolarizers; as can be easily perceived from the foregoing, this is a unique property for an agent active at the neuromyal junction.

Furthermore, the antagonism of SCh and C_{10} by methoxyambenonium takes a rather unusual form (Karczmar, 1956 and 1957). The usual effect of SCh and C_{10} upon mechanical response of the muscle to indirect stimulation consists of a brief twitch increase followed by blockade. This blockade is converted by methoxyambenonium into a continuous increase of the twitch response, which is not followed by a blockade (C_{10} and SCh "reversal"). Karczmar pointed out that if all the actions of SCh and C_{10} depended on their depolarizing effect, the increase and prolongation of the twitch increase due C_{10} and

SCh by methoxyambenonium, should be followed by increased and prolonged blockade. The action of methoxyambenonium, interesting because it bears upon original postulates of Zaimis with regard to SCh and C_{10} , has not been as yet explained; indeed, it is difficult to conceive how a compound which is a weak anti-ChE (effective at doses probably incapable to inhibit, in vivo, AChE; Karczmar, 1961) can antagonize d-Tbc; the coupling of this action of methoxyambenonium with its antagonistic actions upon SCh and C_{10} , is even more perplexing.

In view of these findings, Zaimis (1959) pointed out that SCh and C_{10} act as pure depolarizers only in the cat fast (white) muscles, such as tibialis or sartorius or in a "mixed" muscle such as the gastrocnemius. In the case of the slow muscle of the cat or in that of most muscles of certain species other than the cat, SCh and C_{10} act as curaremimetic blocking agents, i.e., as competitors of ACh. Also, both Zaimis (o.c.) and Foldes (1959) point out that, upon repeated administration, the action of SCh and C_{10} can shift, even in the case of fast muscles, from depolarizing to competitive. Finally, Blaber (1960) argued that anti- C_{10} effects of methoxyambenonium was obtained in the cat, at higher dose levels than its anti-d-Tbc actions, and this suggested that methoxyambenonium may antagonize C_{10} by preventing it from depolarizing. This, actually, may explain the antagonism between C_{10} and SCh on one hand and methoxyambenonium, but not the "reversal".

Altogether, it is fair to state that, at present, the mechanism of action of depolarizers, as well as that of methoxyambenonium, is controversial.

- e. Flexibility of the cholinergic receptor and conditioning of the neuromyal junction.

It appears from the foregoing that several mechanisms may exist at the neuromyal junction with regard to antagonizing blocking action of d-Tbc. Anti-ChE agents, TEA, NaF, methoxy-ambenonium, and hydromethylammonium compounds may antagonize d-Tbc in different ways. Moreover, these and still other compounds may modify the neuromyal transmission by means of several different mechanisms. It should be added that Perry (1953) speculated on synaptic facilitation, not based on ChE-inhibition, without specifying the mechanism involved.

While all this points to the great flexibility of the neuromyal junction, another line of evidence indicates still another type of control possible at that site. It was shown some time ago (Koppanyi and Karczmar, 1953; Karczmar, 1955) that whole organisms and certain synapses (Wills, unpublished data) may recover function relatively fast after large doses of ChE inhibitors, before significant regeneration or reactivation of ChE activity may take place (cf. also Barnes and Duff, 1953; McNamara et al., 1954; Robinson et al., 1954). Also at the neuromyal junction spontaneous recovery of function occurs relatively rapidly after large doses of certain anti-ChE agents (Wills, unpublished data). While even in the case of the so-called irreversible ChE inhibitors such as phosphonate anti-ChE agents and war gases the reversal of inhibition may occur in certain nerve structures faster than originally thought (Koenig and Koelle, 1960), yet the above data indicate that the whole organism or a specific synapse may adapt to low levels of ChE's and concomitant high levels of ACh.

Such an adaptation to ACh was observed directly in the case of autonomic ganglia (Krivoy and Wills, 1956). At the e.p., it was observed (Fatt, 1959) that ACh depolarization

decreases in continuous presence of ACh. Further evaluation of this problem at the neuromyal junction, and particularly data on the adaptation of mechanical response of indirectly stimulated skeletal muscle to ACh are still lacking.

III. Purpose of the present research.

Analysis of the available pharmacologic data pertinent to the skeletal neuromyal transmission suggest, first of all, that the mechanism of action of the so-called depolarizer blocking agents is not fully understood. Specifically, re-evaluation of the data of Thesleff (1955a and b) on the relation of depolarization to blockade of mechanical response of the muscle is indicated. Furthermore, the mechanism by means of which methoxyambenonium antagonizes d-Tbc and reverses C_{10} and SCh block, is not understood at present. Also, further study of newer, not fully understood, anti-d-Tbc agents such as TEA and NaF is desirable, particularly since TEA and NaF were not investigated as antagonists of C_{10} blockade. The proposed research deals with the effects of TEA, NaF and methoxyambenonium upon mechanical responses of the frog muscle to nerve stimulation, ^{and} with the effects of TEA and methoxyambenonium the e.p.p. and muscle membrane; moreover, the interaction of TEA and methoxyambenonium with C_{10} was studied in terms of the mechanical and e.p. parameters of transmission, and compared with the interaction of these compounds with d-Tbc. All this should throw additional light on the mechanisms of anti-d-Tbc, anti-SCh and anti- C_{10} action of TEA, methoxyambenonium and NaF, and thus on new sites of action within the neuromyal transmission pathway.

Since the results led to a concept of multiplicity of sites and mechanisms of the neuromyal transmission, i.e., to

the concept of the flexibility and multiple control system as being characteristic for this transmission, it was thought also important to study among the factors of this flexibility, also the adaptation and recovery processes of the e.p.

The exploration of these and still other possibilities may throw a light on the important general principle of the "modulating" of the cholinergic neuromyal transmission.

C. Methods.

1. Collection of materials and data.

Rana pipiens, the form used in this investigation, were obtained from a local supplier. Winter frogs were generally employed. Stock animals were kept in an appropriate aquarium, and the experiments were run at a temperature optimal for the frog nerve-muscle function, i.e. at 18-20°C; cooled organ bath was used for this purpose. The data consisted of photographic records (obtained by means of the Polaroid and of Dumont Oscilloscope Cameras) and of polygraph tracings of muscle twitch. Sufficient number of experiments were carried out to provide statistically dependable numerical values of the pertinent parameters.

2. Techniques.

Experiments were generally performed on nerve-sartorius muscle preparation of the winter frog. The preparation was bathed in Ringer solution, in muscle chambers of various types, the type depending on the experiment. Ringer solution consisted of 115.6 mM NaCl, 2 mM KCl, 1.8 mM CaCl_2 , and 0.2 mM NaHCO_3 ; the pH was 6.8. In experiments of long duration oxygen was bubbled into the solution.

a. Recording mechanical response of muscle.

Isometric mechanical responses to indirect and direct stimulation were recorded by means of a force transducer coupled with Offner Type R Dynograph or with Grass Model 5 Polygraph. The muscle was fixed at its pelvic end in the vertical muscle bath and connected by means of its tendon with the force transducer. When an indirect stimulus was employed, the nerve was separated from the muscle and suspended from platinum electrodes. The stimulator employed was from American Electronic Laboratories (Model 104A). Generally, the single stimulus had the strength (in the case of supramaximal shocks) of 1 to 5v, duration of 2 to 50 ms. Direct stimuli to the muscle were also employed at the stimulus strength of 1 to 8 v. Trains of five ^{to ten} stimuli, one stimulus every 1 second, were employed, the interval between each train being 5 to 10 minutes. There was a diminution of up to 20 per cent of twitch height in the controls within 2 to 3 hours. Accordingly, the twitch obtained after an experimental procedure was compared to that recorded simultaneously in the control preparation. This was particularly done to ascertain whether or not TEA or methoxyam-benonium, used alone or in combination with C₁₀, increased the muscle twitch above the control levels.

b. Measurements at the e.p. and at the muscle membrane.

Essentially, the following parameters were studied per se and with regard to the action of drugs and drug combination; 1) the resting potential of the muscle fiber and of the e.p., 2) the action potential of the muscle, 3) e.p. depolarization, 4) the e.p.p. Intracellular and extracellular measurements were employed.

In the former case capillary microelectrodes (Ling and

Gerard type) were drawn by means of a micropipette puller, (Industrial Science Associates Inc.) The micro-electrodes had a tip diameter of less than 0.5μ . They were filled with KCl by boiling in 3 M KCl solution, and showed a resistance of less than 20 megohms. In the measurement of the resting potential and of the e.p.p., the muscle was suspended in a vertical muscle bath, treated with a solution of d-Tbc or C_{10} sufficient to block transmission (generally 5×10^{-6} and $3 \times 10^{-6}M$, respectively), the solution discarded, and the e.p.p., induced by supra-maximal volleys to the nerve, was tested. The microelectrode was connected to a cathode-follower input stage, and its output connected to two amplifiers and to cathode-ray tubes (Techtronix Model 502). Resting potentials were observed by the cathode-ray tube connected to a d.c. amplifier. The same setup was employed to record the miniature endplate potentials (m.e.p.p.'s).

Extracellular electrodes were mainly used to measure the persistent depolarization of the e.p., shown by a difference in surface potential between the nerve-free pelvic end and of the sartorius and the e.p. region. The muscle-nerve preparation was mounted, in a vertical perspex chamber, in the Ringer bath. One Ag-AgCl electrode was placed on the pelvic end and the other made contact with the fluid below the tibial end. The distribution of the surface potential was measured by means of an arrangement allowing the movement of the muscle with respect to the surface of the fluid which acted as a movable electrode (Del Castillo et al., 1956a). All preparation with initial potential of over 0.5 mv were discarded. It should be pointed out at this time that the muscle has to be dissected and prepared with utmost care to prevent injury.

In these experiments the location of the e.p. had to be

ascertained. In the case of the microelectrode technique, the intracellular electrodes were inserted with the help of a binocular microscope at the site dictated by experience and verified by the presence of m.e.p.p.'s as well as by the presence of the e.p.p. of about 10 to 30 mv. As stated in the introduction, the e.p.p.'s decrease as the distance from the e.p. to the electrode became greater. In the case of the external electrode technique, the position was ascertained by the size of the depolarization by ACh, maximal in the center of the e.p. zone.

3. Solutions and chemicals.

N'-bis (2-diethylamino-ethyl)-oxamide bis-2-methoxybenzylchloride (methoxyambenonium, WIN 8078) and its bis-2-chlorobenzyl analog (ambenonium); chlorides of ACh, TEA and d-Tbc; C₁₀ bromide, physostigmine sulfate, sodium fluoride (NaF), prostigmine methylsulfate, and certain other agents were dissolved in Ringer solution. Most compounds employed were stable in solution; however, fresh solutions were prepared each day. The less stable eserine solutions were prepared fresh before each experiment. ACh, while stable at stronger concentrations, may be unstable in weak solutions, particularly in the case of 10⁻⁶M concentrations (Thesleff, personal communication). It was, however, ascertained by means of the leech muscle bioassay (MacIntosh and Perry, 1950; 9 experiments) that even dilute ACh solutions (10⁻⁶ to 10⁻⁷M) maintained their full strength for at least 10 minutes. As additional precaution, in the case of experiments dealing with prolonged exposure to ACh, solutions were prepared fresh every five minutes from a stable (1-2 per cent) stock solution.

Many experiments necessitated complex sequence of exposure of the muscle to various drugs. In the studies of effects of

TEA and methoxyambenonium, alone or in combination with C_{10} , on endplate sensitivity to ACh, each treatment was followed by several washes and by a test of ACh depolarization to ascertain that the neuromyal junction returned to control conditions prior to the next procedure. Somewhat different sequences of exposures are described in the legends to various figures and in the text.

D. Results.

I. Effects of pharmacologic agents on mechanical response of the muscle.

1. Effects of methoxyambenonium and of ChE inhibitors.

In the case of maximal or supramaximal stimulation of the nerve, methoxyambenonium did not increase the twitch amplitude over a wide range of concentrations (0.5×10^{-7} to 10^{-5} ; 15 experiments); stronger concentration ($10^{-4}M$ and higher; 14 experiments) caused slowly developing blockade which became complete within 30 to 50 minutes when the weaker concentration was used and faster with stronger concentrations (fig. 1). Also in the cat methoxyambenonium did not increase the twitch of indirectly stimulated muscle (Karczmar, 1957). It may have been expected that, as in the cat, so also in the frog, ineffectiveness of methoxyambenonium could be contrasted with the well-known effectiveness of ChE inhibitors in increasing the twitch height. This proved not to be the case, and the potent anti-ChE agents, such as eserine, ambenonium and neostigmine, proved incapable over a wide range concentration to potentiate the twitch. With higher concentrations blockade could be observed; weak blockade occurred, for instance, in the case of $5 \times 10^{-6}M$ concentration of ambenonium (4 experiments) and more complete blockade in that of $5 \times 10^{-5}M$ concentration

(3 experiments).

While generally not described in the literature, this lack of effect of anti-ChE agents upon the twitch of indirectly stimulated frog muscle is known (Koketsu, personal communication). It should be remembered that, in the mammal, it is difficult to potentiate by means of anti-ChE agents the response to preganglionic stimulation (McIsaachs and Koelle, 1959), unless the preganglionic nerve trunk is longitudinally split and a thin strand used for the stimulation, or unless submaximal stimulation is used.

It was accordingly attempted to test the effect of drugs upon the twitch amplitude in the frog after technically difficult splitting of the motor nerve, or by using submaximal stimuli. In these conditions, methoxyambenonium ($1-5 \times 10^{-6}M$) increased the twitch height in 12 out of 22 experiments from 20 to 40 percent.

The augmentation lasted for relatively short time periods (3 to 10 minutes); ambenonium and neostigmine proved more effective, increasing the twitch from 30 to 50 percent; also, the increase lasted longer (5 to 15 minutes; 5 experiments per compound).

Finally, TEA (0.3 to $1 \times 10^{-3}M$) did not augment muscle twitch whether supra- or sub-maximally stimulated (15 experiments); in higher concentrations (5 experiments) TEA produced initial increase of muscle twitch amplitude; this was rapidly followed by blockade. It should be pointed out that, in the past, TEA was found to increase the twitch of indirectly stimulated frog sartorius (Ing and Wright, 1933). This was also demonstrated in the kitten but not in the adult cat (Sullivan and Kensler, 1950).

It should be stated that neither TEA (5×10^{-5} to

$5 \times 10^{-3}M$) nor methoxyambenonium (10^{-7} to $10^{-4}M$) had any significant effect, within a wide range of concentrations, upon the twitch of the directly stimulated muscle (15 experiments per compound).

2. Interaction between C_{10} , methoxyambenonium and TEA.

C_{10} (3×10^{-6} to $1.5 \times 10^{-4}M$), produced, as is well known, blockade of indirectly, supra-maximally stimulated frog muscle (22 experiments). Interaction between C_{10} and TEA or methoxyambenonium was studied using supramaximal stimulation of either the divided or the intact nerve, or employing submaximal stimulation. With intact nerve and supramaximal stimulation, methoxyambenonium, used at the point of almost complete C_{10} blockade, produced partial restoration of transmission only rarely (fig. 2; 3 out of 15 experiments). However, more consistent partial antagonism between C_{10} and methoxyambenonium (3×10^{-6} to $1.5 \times 10^{-4}M$) occurred whenever divided nerve or submaximal stimulation was used (12 experiments, fig. 3). TEA (3×10^{-4} to $10^{-3}M$) was more effective against C_{10} than methoxyambenonium (10 cases); it produced regularly marked restoration of transmission which was blocked by C_{10} , whether sub-maximal or supra-maximal stimulation was used (10 experiments; figs. 4 and 5). Particularly in the cases of submaximal stimulation, the muscle twitch depressed by C_{10} was augmented by TEA to a height above that of controls obtained prior to C_{10} (6 experiments; fig. 5). The effect of TEA was especially pronounced in the case of the first few responses; the twitch amplitude subsequently decreased (fig. 4).

Several other agents were tried as possible C_{10} antagonists. NaF ($5 \times 10^{-3}M$ to $5 \times 10^{-5}M$), a "sensitizer" of the e.p. (cf.

Introduction, and Koketsu and Gerard, 1956), had negligible effect on C_{10} blockade when supramaximal stimulation was used (5 experiments); it exhibited antagonistic action when submaximal stimulation was used (7 experiments). The restoration of the twitch height amounts to about 30 percent. Anti-ChE agents, ambenonium (10^{-7} to $10^{-5}M$), neostigmine (10^{-6} to $5 \times 10^{-5}M$), and eserine (0.5 to $1.0 \times 10^{-5}M$) had no effect upon C_{10} blockade of maximally stimulated nerve-muscle preparation (15 experiments per compound); in fact, as sometimes in the mammal (Karczmar, 1957 and 1961), these compounds increased the blockade under these circumstances (fig. 6). When submaximal stimulation was used, partial (about 20 percent), temporary (3 to 5 minutes) restoration of transmission was sometimes observed. This was most noticeable in the case of neostigmine. An interesting result was noticed when neostigmine in relatively weak (10^{-7} to $10^{-6}M$) concentrations was employed simultaneously with C_{10} , (8 experiments; fig. 7). Under these circumstances, neostigmine delayed the onset of C_{10} blockade. This effect was not observed when eserine (9 experiments) was applied simultaneously with C_{10} . It is of interest that in a few experiments, a combination of eserine ($5 \times 10^{-6}M$) and of methoxyambenonium ($5 \times 10^{-6}M$) caused partial restoration of transmission blocked by C_{10} , even when supramaximal indirect stimulation was employed (in 7 out of 8 experiments; fig. 8). Other than eserine anti-ChE agents were not employed in combination with methoxyambenonium.

It appears from this section that TEA, methoxyambenonium and NaF, are, in this sequence, most effective antagonists of C_{10} blockade, anti-ChE agents being ineffective in this respect.

3. Interaction between d-Tbc, TEA and methoxyambenonium.

Generally, 3 to $5 \times 10^{-6}M$ concentration of d-Tbc was used to cause neuromyal blockade of the indirectly stimulated frog nerve-muscle preparation; used at this strength, d-Tbc generally caused a 70-90 percent reduction of the twitch. Methoxyambenonium (1.0 - $2.0 \times 10^{-6}M$; 12 cases) caused, within a few minutes, restoration of transmission lasting from 5 to 10 minutes (fig. 9); however, blockade returned subsequently. Faster deblocking as well as more rapid return or intensification of original d-Tbc blockade was noticed with stronger concentrations of methoxyambenonium.

TEA had similar, biphasic effect upon curarized muscle. Weaker concentrations (3 to $5 \times 10^{-4}M$; 15 experiments) produced restoration of transmission without subsequent blockade (fig. 10). As in the case of the effects of TEA upon C_{10} -blocked transmission, the action of TEA was particularly pronounced in the ^{case of the} first few responses. With higher concentration of TEA, the mechanical responses were initially augmented and gradually depressed. This confirms the earlier data of Koketsu (1958).

4. Effects of ACh in various experimental conditions, on the response of the indirectly stimulated muscle.

The most conspicuous action of effective concentration of ACh was its blocking effect upon transmission. This was noticed with a relatively wide range of ACh concentrations. Yet, it became apparent that twitch increase may be obtained with this agent, since frequently, after the depressant action of ACh was recorded and the preparation washed with Ringer, the twitch amplitude was increased by 20 to 40 percent,

presumably because traces of ACh remained on the receptor (12 experiments; fig. 11 and 12). It was not easy to find the concentration of ACh capable of increasing the twitch amplitude without recurring to washing; however, several experiments (18 out of 35) could be carried out in which ACh (5 to $7 \times 10^{-6}M$) caused increase of twitch amplitude (fig. 11). In this respect, ACh resembled C_{10} except that facilitatory effect of C_{10} could be more easily demonstrated than that of ACh (cf. below p.33).

Negligible recovery was generally noticed with the concentrations of ACh capable of producing marked neuromyal depression (10^{-5} to $10^{-4}M$). This was true even when experiments were carried out for 3 or 5 hours. It should be, however, pointed out that controls also exhibit reduction of the muscle twitch over comparable time periods (cf. Methods), and that therefore some recovery may have set in but was masked by deterioration of the preparation. This effect of strong concentrations of ACh resembled that of C_{10} , which was also found essentially irreversible (cf. below p.³³).

However, in the case of weaker concentration of ACh (5×10^{-6} to $10^{-5}M$), which produced relatively small, less than 50 percent depression of transmission, recovery could be observed repeatedly. It frequently occurred within 5 minutes of the onset of blockade (figs. 12 and 13). Similar results were recently reported by Smith (1960). It is of interest that in the presence of neostigmine spontaneous recovery would also occur (figs. 13 and 14; 9 experiments out of 15), when weak concentrations of ACh were employed.

It should be pointed out that, in the in vivo experiments in which ACh was administered i.a. or i.v. to cats or dogs to

produce neuromyal actions, the ACh could be disposed of by combined action of ChE's, tissue diffusion, and removal by the blood stream, but in the in vitro experiments ACh remains in touch with the muscle throughout the whole experiment. Thus, the recovery was spontaneous and occurred in the continuous presence of ACh. It could be stated that the recovery constituted a case of adaptation to depressant levels of ACh.

Another instance of adaptation to ACh was demonstrated in experiments conducted somewhat differently from the previous ones. In these experiments, the preparation was "conditioned" to increasingly higher and higher concentrations of ACh; it could be shown that, after the "conditioning", even a strong concentration of ACh ($5 \times 10^{-5}M$), capable of marked blockade of an "unconditioned" preparation, was ineffective (11 experiments). In other experiments of this type, concentrations of ACh, effective in control experiments, were found ineffective in "conditioned" muscles (12 experiments; figs. 15 and 16). This, then, is a case of ACh tachyphylaxis, which was so far described only by Smith (1960). It should be pointed out, however, that conditioning could occur only with concentrations of ACh capable of slight, 20 to 25 percent, blockade (fig. 16). In other words, ACh tachyphylaxis was never pronounced.

As already stated (cf. section 2 above), anti-ChE agents frequently synergized with C_{10} in the production of transmission blockade. Similarly, ACh generally synergized with neostigmine and eserine in the production of the blockade. In the presence of neostigmine (10^{-6} to $10^{-7}M$) or of eserine (10^{-5} to $10^{-4}M$), it sufficed to employ 10^{-7} to $5 \times 10^{-6}M$

concentration of ACh to produce 30 to 50 percent blockade of transmission; when used alone, ACh had to be used in concentrations of $5 \times 10^{-5}M$ or higher to produced similar degree of blockade (cf. supra). As described before, weaker concentrations of ACh and of neostigmine did not produce blockade (fig. 13).

Another agent which produced blockade synergistically with ACh was methoxyambenonium. As already described, used alone, methoxyambenonium never produced any blockade at concentration lower than $1.5 \times 10^{-5}M$ (cf. Section 1 above), while ACh was never markedly depressant at less than $10^{-6}M$. Used jointly, these agents were markedly blocking at concentrations of $10^{-8}M$ of ACh and $10^{-7}M$ of methoxyambenonium (6 experiments). The recovery from such blockade was slight within the usual experimental duration (2 hrs.).

5. Effects of C_{10} upon the response of the indirectly stimulated muscle.

C_{10} effect upon neuromyal junction differed in several respects from that of ACh. In the first place, it was relatively easy to obtain increase of the twitch response with concentrations ranging from 10^{-6} to $10^{-5}M$. In the case of weak concentrations of C_{10} , twitch increase lasted for the whole experimental period (up to two hours; 12 cases; fig. 17). This effect of C_{10} can be contrasted with that of ACh, in which case twitch increase could not be easily demonstrated, and when obtained, it was only temporary. In the case of stronger concentrations of C_{10} , the initial twitch increase subsided and progressive blockade took place, the rate of the occurrence of the blockade depending upon concentration. The blockade was complete in 30 minutes in the case of $5 \times 10^{-5}M$

solution of C_{10} . The following finding was also of interest. In the control preparation, as well as after the twitch magnitude was increased by a weak concentration of C_{10} , a typical "treppe" effect was observed in most of the cases (figs. 17 and 18).

As already stated, spontaneous recovery was not noticed in the case of either facilitating or blocking concentrations of C_{10} . Even in the case of concentrations of 5×10^{-6} to $6 \times 10^{-5}M$ of C_{10} , producing some facilitation first and relatively slight blockade within 30 or 40 minutes, no recovery but rather progressive blockade, terminating in complete block within 1 to 2 hrs., was noticed. This constitutes then another difference between C_{10} and ACh.

Finally, adaptation phenomena were never noticed with C_{10} . In several experiments, C_{10} solutions (10^{-6} to $5 \times 10^{-5}M$) causing some twitch increase at first and some blockade later, were replaced, when blockade ensued, by somewhat stronger solutions (6×10^{-5} to $10^{-5}M$); in all cases increased blockade was observed. When the preparation was washed in Ringer between the two applications of C_{10} , the block increased when the preparation was placed in the second C_{10} solution (fig. 19). Even when stronger solution was substituted for the weaker solution at the time when the latter produced twitch increase only, blockade occurred. This, non-adaptive behavior of C_{10} , again differed from the tachyphylaxis effect noticed in the case of ACh.

II. Effects of pharmacologic agents at the endplate.

1. Effects of methoxyambenonium, TEA and C_{10} on muscle membrane and at the endplate.

It was determined, first of all, that neither methoxyam-

benonium (1.5×10^{-7} to $1.5 \times 10^{-4}M$), nor C_{10} (3×10^{-6} to $1.5 \times 10^{-4}M$) nor finally TEA (10^{-3} to $10^{-5}M$) had any effect on both the resting and action potential of single fibers. TEA and methoxyambenonium resembled each other in that they did not have any direct effect upon the resting potential of the e.p. in concentrations of 1.5×10^{-7} to $1.5 \times 10^{-6}M$. Thus, they are not depolarizing agents; also, they presumably have no direct myogenic action, as already suggested by the lack of their action upon the directly stimulated frog muscle (cf. supra, Section I-1).

Methoxyambenonium showed one interesting activity at the e.p. in which it differed from TEA. At a relatively low concentration of $5 \times 10^{-6}M$, methoxyambenonium increased the amplitude (by 0.5 ± 0.1 mv), but not the frequency of m.e.p.p.'s (fig. 20; $250 \pm 18/30$ minutes; 7 experiments) while TEA did not seem to affect the m.e.p.p.'s. It appeared that, at optimal concentrations (10^{-6} to $5 \times 10^{-5}M$), eserine did not produce as much effect upon m.e.p.p.'s as methoxyambenonium (fig. 20). Control m.e.p.p.'s showed an average of 0.3 to 0.1 mv, while after eserine they averaged 0.4 ± 0.1 mv.

C_{10} had, as it is well known, a depolarizing action at the e.p., which was obtained with concentrations of 10^{-7} to $1.5 \times 10^{-4}M$. This action of C_{10} was almost entirely confined to the e.p.

2. Effects of TEA and methoxyambenonium on the e.p.p.

E.p.p. was obtained by blocking the transmission either by C_{10} (generally $5 \times 10^{-6}M$) or by d-Tbc (generally 3 to 5 $10^{-6}M$).

With regard to curarized preparations, the e.p.p. action of methoxyambenonium was, interestingly enough, very different

from that of TEA. In brief, methoxyambenonium markedly augmented the e.p.p. (fig. 21). Measured extracellularly, the e.p.p. varied between 2 and 4 mv; measured intracellularly, between 10 and 30 mv. The augmentation amounted to 1.2 ± 0.3 mv and 10 ± 0.4 mv, measured extra- and intra-cellularly, respectively. Yet, even when concentrations of methoxyambenonium reversed the action of d-Tbc ($1.5 \times 10^{-4}M$) and the e.p.p. was increased past the critical depolarization potential which produced a spike, repetitive firing was never noticed. The effect was prolonged and consistent. All e.p.p.'s of any particular train of stimuli were affected similarly. Still higher concentrations tended to lower the e.p.p.'s. Finally, methoxyambenonium did not change the pattern and shape of the e.p.p.; particularly, the falling phase of the e.p.p. was not prolonged (fig. 21). For instance, in the case of $1.5 \times 10^{-6}M$ methoxyambenonium solutions the time value of the decline of the e.p.p. to half its peak value was 1.8 ± 0.4 ms, and thus was quite similar to that of the control e.p.p. (1.7 ± 0.4 ms), although the latter was half the height of the former. Thus, methoxyambenonium caused augmentation but no prolongation of the e.p.p. of curarized preparation.

While TEA also increased the e.p.p. of the curarized preparation, this augmentation differed from that produced by methoxyambenonium. TEA, $2 \times 10^{-4}M$ to about $5 \times 10^{-4}M$ (threshold concentrations and higher), produced augmentation proportional to TEA concentration, combined with prolongation of the falling phase of the e.p.p. (duration of decline to half the peak value 2.1 ± 0.5 ms). In fact, the e.p.p. pattern was changed generally; the latency and the rising phase were also

prolonged, although the falling phase and the duration of the e.p.p. were most affected. It was also noticed that the effect of TEA was most conspicuous in the case of the first e.p.p.'s of each train of stimuli; repeated stimulation of the nerve brought about e.p.p.'s of control size and shape. In the case of these earlier e.p.p.'s, TEA ($5 \times 10^{-4}M$) produced augmentation amounting to 1.5 ± 0.3 mv and 14 ± 0.4 mv, measured extra- and intra-cellularly, respectively.

Higher (10^{-3} to $3 \times 10^{-3}M$) concentration of TEA caused manifold e.p.p. changes. Repetitive firing and considerable augmentation and prolongation of the first e.p.p.'s of each train of stimuli were noticed; depression of subsequent e.p.p.'s of each train of stimuli followed. $5 \times 10^{-3}M$ concentrations and higher led to immediate decrease of the e.p.p.'s in absence of repetitive firing. Some of these observations were already reported by Koketsu (1958).

It was of particular interest to record the changes produced by methoxyambenonium and TEA upon the e.p.p.'s obtained when the transmission was blocked by C_{10} . This e.p.p. was generally smaller than the e.p.p. obtained in curarized preparations (fig. 22 and 23). The rather surprising finding was that the effect of methoxyambenonium and of TEA was essentially similar or even identical with that produced by these agents in the case of the e.p.p.'s of curarized preparations. Again, TEA increased and augmented the e.p.p., when used in lower concentrations (3×10^{-4} to $5 \times 10^{-3}M$); used in these concentrations, TEA increased the e.p.p. by 1.4 ± 0.4 mv (extracellular recording). In higher concentrations ($10^{-4}M$) TEA actually produced repetitive firing

(fig. 22). As before, the restoration of the e.p.p. was particularly noticeable after the first nerve stimulation; thereafter, subsequent stimuli produced smaller e.p.p.'s. Higher concentration of TEA (5×10^{-4} to $10^{-3}M$) decreased the e.p.p. due to C_{10} , just as it decreased the e.p.p. of curarized preparations.

As in the case of e.p.p.'s of curarized preparations, the effects of methoxyambenonium upon e.p.p.'s obtained in the presence of C_{10} resembled those of TEA in one respect and differed in several others. Methoxyambenonium (5×10^{-5} to $5 \times 10^{-6}M$) augmented but did not prolong the e.p.p. (fig. 23). The effect was consistent, i.e., e.p.p.'s obtained by first stimuli of each train were not augmented more than those due to subsequent stimuli. However, neither repetitive firing nor prolongation of the e.p.p.'s was produced by methoxyambenonium within this range of concentrations.

It is well known that anti-ChE agents produce an increase of e.p.p.'s of curarized preparations, and, in the case of higher concentrations, spiking and repetitive firing (cf. for instance, Nastuk and Alexander, 1954); one of the most conspicuous e.p.p. effects of anti-ChE agents was the prolongation of the falling phase of the e.p.p. These data could be reproduced at present. In the case of eserine, the duration of the decline to half the peak value was 2.5 ± 0.5 ms, as against 1.7 ± 0.4 ms duration in the control experiments (fig. 23). On the other hand, anti-ChE agents (for instance eserine, 10^{-6} to $10^{-5}M$) did not increase the e.p.p. when the transmission was blocked by C_{10} ; the only effect noticed with these and higher concentrations of eserine was a decrease of the e.p.p. amplitude (fig. 23; 8 experiments).

3. Endplate action of C_{10} (3×10^{-6} to $1.5 \times 10^{-4}M$) was measured in these experiments simultaneously with the recording of the muscle twitch.

These measurements entirely confirmed the conclusions of Thesleff (1955a and b) that C_{10} depolarization in the frog is temporary, and that its time course is different from that of its transmission blockade.

When measured with the external electrodes, depending upon C_{10} concentrations, maximum depolarization varied between 2 and 7 mv, reached its peak within 1 to 15 minutes, decreased subsequently and completely subsided within 15 to 50 minutes (fig. 24). At the same time the blockade of mechanical muscle response to indirect stimulation was first noticed well after depolarization; as the muscle repolarized the maximal blockade of transmission occurred well after complete return of e.p. membrane to normal polarization (fig. 24).

4. Analysis of C_{10} blockade of neuromyal transmission, and of its antagonism by TEA and by methoxyam-benonium.

Since the above data indicate that C_{10} depolarization cannot account for transmission blockade, the experiments of Thesleff (o.c.) indicating that this blockade is due to ACh "desensitization" of the e.p. were repeated and expanded. C_{10} in all concentrations capable of initial depolarization and subsequent blockade (10^{-6} to $10^{-4}M$) decreased ACh depolarization at the e.p. The pattern of ACh depolarization was, essentially, not affected; in the presence of C_{10} , the time course of ACh depolarization was parallel to that due to ACh alone (15 experiments; figs. 25 and 26). Since this suggests competition between C_{10} and ACh, it may be predicted that TEA and methoxy-

ambenonium, antagonists of C_{10} transmission blockade, would sensitize the e.p. to ACh. Yet, this did not prove to be the case with TEA. Actually, the effect of TEA upon ACh depolarization was very similar to that of C_{10} itself (12 experiments; fig. 25); this confirms the data of Koketsu (1958).

On the other hand, methoxyambenonium (5×10^{-6} to $10^{-5}M$) markedly increased ACh depolarization by 2.1 ± 0.5 mv (external electrode measurement) in the case of stronger concentrations. An interesting feature of this sensitization was, that again, a family of parallel curves was obtained; ACh depolarization in presence of methoxyambenonium was increased over, and parallel to, that caused by ACh alone (11 experiments; fig. 27). In other words, methoxyambenonium increased but did not prolong ACh depolarization. In this, the methoxyambenonium effect differed markedly from the well-known action of anti-ChE agents, which as well as augmenting it prolongs ACh depolarization (13 experiments; fig. 28). Finally, at relatively high concentrations ($10^{-4}M$), methoxyambenonium depressed ACh depolarization (fig. 27), i.e., it desensitized the e.p. to ACh.

A reference should be made at this time to the blockade of neuromyal transmission recorded when a combination of very weak concentrations of methoxyambenonium ($10^{-7}M$) and of ACh ($10^{-8}M$; cf. p. 36) were tested. It could be shown at present that, employed in these concentrations, neither agent nor their combination produced measurable depolarization. On the other hand, when the block was produced by combining ACh with anti-ChE agents such as eserine and neostigmine (cf. p. 36, p. 32), the effective concentrations (10^{-5} to $10^{-6}M$) of ACh

could produce some depolarization, which was markedly intensified by the anti-ChE agents at concentrations employed (14 experiments; fig. 28).

It appears that while sensitization of the e.p. to ACh may be underlying the antagonism between methoxyambenonium and C_{10} , TEA must antagonize C_{10} by its e.p.p. action, since it actually desensitizes the depolarization of ACh. This could be substantiated by additional lines of evidence.

First, it could be shown that C_{10} synergizes with d-Tbc. When C_{10} (10^{-5} to $10^{-6}M$) was added to a curarized preparation, the e.p. was first augmented; then, since the resting potential of the e.p. was raised by the depolarizing action of C_{10} , the e.p.p. was decreased. Finally, when the C_{10} depolarization subsided, the base-line returned to normal and the e.p.p. was markedly reduced. With higher concentrations of C_{10} and d-Tbc (at concentrations of both agents of $10^{-5}M$), e.p.p.'s were completely blocked. TEA could increase the height and the duration of e.p.p.'s obtained by combined use of C_{10} and d-Tbc, producing repetitive firing with $10^{-4}M$ concentrations. As in the case of e.p.p.'s produced in the presence of C_{10} or d-Tbc alone, methoxyambenonium again resembled TEA in antagonizing e.p.p.'s obtained in the presence of C_{10} -d-Tbc combinations; again, it neither prolonged e.p.p.'s nor caused repetitive firing.

In another experimental series, ACh sensitivity was decreased by C_{10} , and the effects of TEA and of methoxyambenonium upon desensitized e.p. measured. TEA ($0.1 - 0.5 \times 10^{-4}M$) could never restore the e.p. sensitivity (fig. 29). Actually, at higher concentrations, TEA additionally reduced ACh depolarization after its partial reduction by C_{10} . This could be

expected on the basis of the blocking actions of TEA upon ACh depolarization (*vide supra*). To the contrary, methoxyambenonium in concentrations of 5×10^{-5} to 5×10^{-6} M could at least partially restore ACh depolarization reduced by C_{10} (15 experiments; fig. 26). The average increase was 1 ± 0.3 mv (extra-cellular recording). As in the case of the depolarization produced by ACh in the absence of C_{10} , methoxyambenonium augmented, but did not prolong ACh depolarization obtained in the presence of C_{10} . Finally, stronger concentrations of methoxyambenonium additionally decreased ACh depolarization, already reduced by C_{10} . In other words, methoxyambenonium exhibited in this, as in many other experiments, biphasic action.

5. ACh depolarization and neuromyal blockade.

The time course of ACh depolarization was compared in these experiments to that of ACh blockade of neuromyal transmission. At concentrations of 5×10^{-6} to 5×10^{-5} M of ACh, depolarization measured with external electrodes reached within 1 to 2 minutes a peak of from 3 to 8 mv.

Thereupon, in spite of the continued presence of ACh, or application of a fresh solution, the depolarization decreased and subsided in from 10 to 15 minutes, depending on ACh concentration (fig. 30 to 32). The response of the muscle to indirect stimulation, on the other hand, followed a different time course, since it was first noticed at a considerable time after the peak of depolarization was reached, and since it reached its maximum a considerable time after the depolarization was over (more than 100 experiments; fig. 30). Recovery from the blockade of transmission ensued later. In other words, the e.p. potential exhibits two functionally very different phases of ACh action; the first phase immediately after the reversal of ACh depolarization, when ACh blocked muscle response to

indirect stimulation; and the second phase when recovery from transmission blockade could be observed.

During both these phases the e.p. was resistant to depolarization action. In the continuous presence of ACh, eserine and neostigmine could not restore depolarization (15 experiments; fig. 29); methoxyambenonium was also ineffective under these conditions. However, increasing the ACh concentration produced some slight depolarization, even when the concentrations of ACh in question were capable, prior to first administration of ACh, of producing maximal (8 mv) depolarization (fig. 32). Even half-an-hour after the cessation of ACh depolarization, ACh sensitivity of the e.p. did not return to normal, i.e., strong concentrations of ACh could produce, at this time, only small depolarizations. Pretreatment with eserine prior to adding either high or low concentrations of ACh did not increase the effect of the latter; in fact, eserine seemed to decrease the effectiveness of ACh in depolarizing the desensitized e.p. On the other hand, methoxyambenonium at 10^{-6} to $10^{-7}M$ produced, combined with $10^{-6}M$ ACh, the highest depolarization of which the desensitized e.p. seemed to be capable, although even this depolarization (3-5 mv, 12 experiments; fig. 32) was significantly short of the maximal depolarization which ACh could produce in a fresh e.p. The effectiveness of methoxyambenonium was not increased by employing stronger concentrations of this compound with ACh in various concentrations (10^{-5} to $10^{-8}M$).

E. Discussion.

Two major problems will be considered in this discussion. One problem concerns the mechanism of C_{10} blockade, and that

of the interaction between C_{10} and various pharmacological agents, particularly as it reflects on the mechanism of the antagonism of C_{10} by TEA and methoxyammonium. The other problem is related to the effects of ACh at the e.p. and upon the neuromyal transmission, and it deals also with the comparison of the neuromyal effects of ACh and C_{10} .

1. Mechanism of the blockade by C_{10} of frog neuromyal transmission.

a. Blocking action of C_{10} at the neuromyal junction.

It seems likely that, as already proposed by Thesleff (1955a and b), C_{10} blockade of the neuromyal transmission in the frog is not due to depolarization. He cited several lines of evidence indicating just this; perhaps the strongest one being the difference between the time course of C_{10} neuromyal blockade and that of C_{10} e.p. depolarization. Also, simultaneously with producing the neuromyal blockade, C_{10} desensitized the e.p. to ACh, and the pertinent experiments suggested that these two compounds may compete for the receptor site. All these results could be confirmed at present. Moreover, in some of the experiments described herein, the e.p.p.'s could be decreased or even completely flattened by either excessive concentrations of d-Tbc and C_{10} , or by C_{10} -d-Tbc combinations; these experiments suggest that d-Tbc and C_{10} synergize with each other, and have a similar mechanism of action. Finally, the results obtained at present demonstrate the antagonistic action of methoxyammonium and TEA upon C_{10} blockade, and show that this antagonism is similar to that which methoxyammonium and TEA exert with regard to d-Tbc. It is difficult to explain

these antagonisms without assuming that C_{10} blockade is due to desensitization of the e.p. to ACh, although even then the full understanding of these phenomena is not easy, as will be shown below.

b. Anti- C_{10} effects of TEA, methoxyambenonium and NaF.

It should be stressed that these agents not only antagonized C_{10} , but also d-Tbc blockade. If C_{10} blocks by ACh desensitization or, possibly, by competing with ACh, and if this block is, therefore, similar to that produced by d-Tbc, antagonism of both C_{10} and d-Tbc by one and the same compound should be expected. While this concept would explain the "double" antagonism of d-Tbc and C_{10} , exhibited by TEA, methoxyambenonium and NaF, it offers also difficulties. One would, for instance, expect, that C_{10} , as a desensitizer of the e.p. or as a competitor of ACh, should be antagonized by anti-ChE agents; yet, as in the mammal (Zaimis, 1959; Karczmar, 1957 and 1961), so also in the frog C_{10} blockade is, if anything, deepened by ChE inhibitors such as ambenonium, eserine and neostigmine. Also, anti-ChE agents depress the e.p.p. obtained in the presence of C_{10} . It should be pointed out that the anti-ChE compounds employed are very potent inhibitors of AChE and thus should easily produce ACh accumulation capable of depolarizing even a desensitized e.p., which is one of the possible mechanisms underlying the antagonism of neuromyal blockade (Riker, 1953). In view of the ineffectiveness of anti-ChE agents, it may be suggested that the antagonism depends rather upon displacement of the blocker by the antagonist, as proposed by Riker (o.c.), and that the anti-ChE agents in question cannot displace C_{10} because they do not exhibit sufficient affinity to the neuromyal junction. However, it is not likely that lack of antagonism of C_{10} , particularly by an

benonium, is due to the lack of affinity to or activity of this compound at the e.p. Indeed, ambenonium produces direct muscle effect (Karczmar, 1957; Land et al., 1955) and actions upon neuromyal transmission at the levels of less than a microgram, and thus is a much more potent substance than C_{10} .

Even in the frog, where the desensitizing mechanism of C_{10} blockade, amply documented here as well as by Thesleff (1955a and b), may explain why certain agents can antagonize both C_{10} and d-Tbc, the behavior of the C_{10} blockade with regard to anti-ChE presents difficulties. The situation is even more obscure in the mammals (cats and dogs). In these species, the desensitizing character of C_{10} blockade is less well documented, and the evidence is mainly indirect. Foldes and his associates (cf. for instance, Foldes, 1959) and Zaimis (1959) presented data indicating that upon repeated C_{10} administration C_{10} blockade in slow and in fast muscles exhibits aspects of competitive blockade, since it is antagonized by anti-ChE agents. Actually, methoxyambenonium antagonizes and "reverses" C_{10} or SCh upon its very first administration to a fast muscle of the cat. This obviously suggests that also in the mammals, C_{10} and SCh action and block cannot be purely depolarizing in nature, even in the early phases of their action. If it were so, methoxyambenonium which potentiates the excitatory action of C_{10} and SCh, should subsequently produce increased blockade rather than cause "reversal", i.e. conversion of SCh and C_{10} blockade into a prolonged increase of the twitch response.

It should be stressed that recently Blaber (1960) argued that a similar conclusion (cf. Karczmar 1956, 1957 and 1961) is not warranted, and attempted to explain the anti- C_{10} effects of methoxyambenonium in terms of the original hypothesis

of Zaimis (1953). He pointed out that anti- C_{10} effects of methoxyambenonium were obtained upon i.a. administration in the cat at lower dose levels than its anti-d-Tbc actions. It was also suggested that higher doses of methoxyambenonium may antagonize C_{10} by desensitizing the endplate to depolarization. However, in the case of the i.v. route in the cat (Karczmar, 1957) as well as in that of the frog nerve-muscle preparation, doses of methoxyambenonium capable of antagonizing C_{10} and d-Tbc are comparable or identical. Furthermore, methoxyambenonium can antagonize C_{10} only at concentrations which are considerably below those producing ACh desensitization and concomitant blockade of neuromyal transmission (Karczmar, 1957). Also, if depolarization were indeed the mechanism underlying C_{10} blockade, facilitatory effects of methoxyambenonium confirmed by Blaber (o.c.) should lead to synergism between C_{10} and methoxyambenonium, which was not the case either in the cat or in the frog.

Even if we assume that action of methoxyambenonium upon C_{10} block, in the cat and frog, may depend on the desensitizing character of C_{10} and SCh block, two problems still remain: the explanation of the "reversal" of C_{10} and SCh action by methoxyambenonium, and of the lack of antagonism of SCh and C_{10} block by anti-ChE agents. These two problems will be analyzed further after a discussion of the action of the antagonists of C_{10} block, TEA, NaF, and methoxyambenonium. Indeed, it may well be that the answer to these problems lies in the nature of this action.

c. Mechanism of action of methoxyambenonium, NaF and TEA.

The difficulty in explaining the action of methoxyam-

benonium, NaF, and TEA, is that while depolarizers, curare-mimetics, anti-ChE agents or hydroxymethylammonium compounds show some of the characteristics of these three agents, yet the combination of properties exhibited by these three compounds is unusual (Table 1). TEA augments e.p.p. but is not an anti-ChE agent (Koketsu, 1958), or a depolarizer. In fact, TEA blocks ACh depolarization (Koketsu, 1958), and it increases ACh desensitization produced by C_{10} . Also, while TEA produces repetitive firing at the e.p., it does so when employed in high concentrations, not required for its anti-curare effects and for the augmentation of the e.p.p. Thus, TEA is not an anti-d-Tbc agent by virtue of the mechanism proposed by Riker et al. (o.c.). Similarly, methoxyambenonium augments e.p.p., increases the m.e.p.p.'s and sensitizes the e.p. to ACh; yet it is a weak anti-ChE (Lands et al., 1955; Karczmar 1957) and at concentrations (10^{-6} to $10^{-7}M$) capable of actions at the neuromyal junction it does not inhibit AChE (Arnold et al. 1954). ¹⁾ Also, methoxyambenonium produces neither depolarization nor repetitive firing. NaF also causes ACh sensitization, increases the e.p.p., and antagonizes d-Tbc (Koketsu and Gerard, 1956), without being a depolarizer, or an anti-ChE agent, or an unstabilizer of the nerve terminals. Finally, all these three compounds are, in the frog, not only d-Tbc but also C_{10} antagonists.

1) It is of interest that, in the case of the frog isolated nerve-muscle preparation, it is possible to compare the concentration found inhibitory to AChE in vitro, with the concentration effective pharmacologically. Usually, in vitro data on anti-ChE action have to be correlated with in vivo

data, based on i.v. administration, and expressed in mg./kg.; in view of the poor distribution of a bisquaternary compound such as methoxyambenonium, such two sets of data are not really comparable. Moreover, AChE inhibition, or its absence, upon i.v. administration cannot be easily measured, at least manometrically, in the case of a reversible inhibitor such as methoxyambenonium (cf. Koelle, 1961a, Karczmar, 1961), and, in such experiments, the deduction as to whether or not methoxyambenonium acted as an AChE inhibitor, is difficult to assess.

The explanation of these paradoxical effects of NaF, TEA and methoxyambenonium is difficult. It was already suggested (Koketsu, 1958) that TEA increases the release of bound ACh from the nerve terminals. This would then explain the phenomena observed by Koketsu (o.c.) as well as at present, such as the increase and slight prolongation of the e.p.p., compared to the marked e.p.p. prolongation obtained with anti-ChE agents; repetitive firing obtained with larger concentrations of TEA; anti-d-Tbc action; and the fact that TEA particularly effects the e.p.p. and the mechanical response obtained upon the first few nerve stimulations. Furthermore, by assuming this mechanism, the paradox of a substance which decreases ACh sensitivity and yet is an anti-d-Tbc and anti-C₁₀ agent is resolved; the amplitude of the e.p.p. and the deblocking action would be determined by two antagonistic actions of TEA: ACh release and ACh desensitization (Table 1). Finally, the present data on C₁₀ antagonism of TEA are consistent both with the view of d-Tbc-like action of C₁₀, and with the ACh-release mechanism of action of TEA.

It is more difficult to suggest the mechanism of action of methoxyambenonium and of NaF, besides pointing out certain

similarities and dissimilarities of their action. As shown by Koketsu (1956) with regard to NaF, and as demonstrated at present with regard to methoxyambenonium, neither agent produces augmentation of the mechanical muscle response to indirect stimulation; both sensitize the e.p. to ACh, and both increase the e.p.p.; they are not depolarizers or anti-ChE agents. While NaF does not prolong ACh depolarization and the e.p.p. as much as anti-ChE agents, yet it does so to a certain extent; it resembles then in this respect TEA, while methoxyambenonium remains an unique agent in that it produces "augmentation without prolongation" both with regard to the e.p.p. and to ACh depolarization. Yet, NaF resembles methoxyambenonium (and also TEA) in still another important respect, as shown at present; it is not only an anti-curare but also an anti-C₁₀ agent. Koketsu (o.c.) presented some indirect evidence that NaF does not increase the release of ACh at the nerve terminal; he proposed therefore that "NaF increases the affinity of ACh for the e.p. membrane and enhances the depolarizing action of ACh" (o.c.) by some specific, but hitherto unexplained, action. In other words, it acts at the postsynaptic site as a sensitizer or facilitator.

It should be stressed that these terms are used here in a sense more specific than that in which Perry (1956) used them. Perry (o.c.), speaking of facilitating compounds, had in mind simply compounds acting in a manner different from that of anti-ChE agents, but did not otherwise define their action. In the light of the present researches, methoxyambenonium is a facilitator agent, sensitizing the e.p. to the nerve impulse and to ACh; a facilitator can be defined therefore as a substance that augments, but does not prolong, the

e.p.p. and ACh depolarization. This is a rare type of action, hitherto not described.

In view of the similarities of action of NaF and methoxyambenonium, and since neither is an anti-ChE or ACh-like agent, these compounds may be considered as facilitators. It should be however remembered that, since methoxyambenonium does not affect the time course of ACh depolarization and of e.p.p. which is somewhat prolonged by NaF, the mechanism of action of methoxyambenonium and of NaF cannot be identical.

In any event, these views of action of methoxyambenonium, NaF and TEA, coupled with the conceptualization that C_{10} blockade depends, like that of d-Tbc, upon ACh desensitization or upon competition with ACh, may constitute a preliminary picture of the antagonism between these agents and C_{10} .

d. Concluding remarks on C_{10} -methoxyambenonium interplay.

The above considerations lead to certain conclusions and hypotheses which can be summarized as follows. In the frog as in the cat, C_{10} blockade must consist, to an extent at least, of desensitizing the e.p. to ACh; this, coupled with the many instances of ACh-sensitizing actions of methoxyambenonium at the e.p., shown at present, offers an explanation of its effect upon C_{10} . Otherwise, methoxyambenonium antagonism of both C_{10} and d-Tbc blockade in the cat, and in the frog cannot be readily understood.

As already stated, this does not explain methoxyambenonium "reversal" of C_{10} and SCh blockade in the cat (Karczmar, 1957 and 1961). While the final explanation of the "reversal" cannot be offered on the basis of these experiments, it may be suggested that methoxyambenonium may prevent the desensi-

tization or competition of C_{10} postsynaptic action (Table 1), and synergize with certain "facilitatory" actions of C_{10} . The possible site and mechanism of these "facilitatory" actions of C_{10} will be discussed subsequently (section 2a of this Discussion). Suffice it to say at present that these actions may be presynaptic and that methoxyambenonium may intensify them at that site (Table 1). Methoxyambenonium may have then two sites of action, postsynaptic and presynaptic. The facilitatory post-synaptic action of methoxyambenonium is amply documented at present, but its pre-synaptic effect can be only suggested. However, it may be speculated that both these effects are necessary for the prediction of the C_{10} "reversal" by methoxyambenonium.

It may be also suggested that the action of methoxyambenonium is kept within "physiological" limits, because its effects are not accompanied by a prolongation of the e.p.p. and of ACh depolarization, or by repetitive firing, and because methoxyambenonium exhibits depressant properties when employed at higher concentration, such as decremental action upon ACh depolarization and upon e.p.p. It should be stressed here that also TEA shows biphasic actions, and that this may contribute to its anti- C_{10} action in the frog.

This may have also a bearing on the other problem, pointed out above; namely the problem of the lack of antagonism - even in the frog - between C_{10} and anti-ChE agents. Possibly these agents lack a built-in safety factor. Also, lack of C_{10} antagonism by anti-ChE agents may be due, in some kind of a way, to the contributory action of C_{10} depolarization. The depressant effect of anti-ChE agents on the e.p.p. of C_{10} -treated neuromyal junctions should be also remembered in this context.

More specifically, C_{10} depolarization, while not playing a conspicuous role in the blockade by C_{10} , employed alone, may be important in the presence of anti-ChE agents. In fact, increased depolarization was noticed at present in the case of blocking combinations of C_{10} and eserine, or C_{10} and neostigmine; anti-ChE agents also produce depolarization, when used alone in high concentrations (Douglas and Paton, 1954). Thus, ChE inhibitors, by intensifying the depolarizing action of C_{10} , can cause a pure depolarization blockade, such as suggested by Zaimis (o.c.) for C_{10} alone; or, this component of combined action of anti-ChE agents and C_{10} can contribute to e.p. desensitizing action of C_{10} .

It appears that TEA, NaF and methoxyammonium antagonize in the frog both d-Tbc and C_{10} . This can be partially explained by the similarities in the d-Tbc and C_{10} blockade. This antagonism must be also based on a special characteristic of TEA, NaF and methoxyammonium, since so many other substances cannot antagonize both the C_{10} and d-Tbc block. This aspect of the action of these three agents may be their biphasic effect, allowing them to limit undue C_{10} depolarization, or it may depend on a "facilitatory" mechanism or mechanisms different from the mechanism of action of anti-ChE's.

Altogether, this investigation, similar to other recent analysis of the pharmacology of the neuromyal junction (Riker et al., 1959; Zaimis, 1959; Karczmar, 1961), indicate multiplicity of effects possible at the neuromyal junction. In fact, they may serve to emphasize the warning of Bovet (1951) that absolute distinction between agents active at the neuromyal junction is impossible. Indeed, causally committed terms

such as "depolarizers" and "competitors" should be perhaps avoided and each neuromyally active agent described in its own right and in terms of its characteristic relationships at the junction. Whether this state of matter exists because several sites of action are available within the neuromyal junction, or because receptor proteins may exhibit various types of responses depending upon the presence of chemical agents and on its physiological environment, cannot be answered at present.

2. Neuromyal actions of ACh and of C_{10} , and their mechanism.

One of the main aspects of the action of ACh emerging from this research and from that of other investigators, is the flexibility of this action.

Certain similar aspects of action of C_{10} were brought forward in the course of this study. In so far as ACh is concerned, it was found capable of at least three types of action at the neuromyal junction, which in turn can be modulated by the past history of the junction and by pharmacologic agents.

To start with, ACh exerted facilitatory actions, probably unrelated to its depolarizing effect, as well as blocking effects upon the twitch of the indirectly stimulated muscle. Secondly, adaptation to blocking concentrations of ACh seemed possible, and, the neuromyal junction could be rendered insensitive to blocking concentrations of ACh by means of pre-treatment with ACh. Finally - and this was brought up also in the earlier section of this discussion - neuromyal actions of ACh could be increased by agents other than anti-ChE's or releasers of ACh from nerve-end terminals, i.e. by facilitators.

a. Facilitatory and blocking actions of ACh as compared with those of C_{10} .

Weak concentrations of ACh increased the twitch response of the muscle to indirect stimulation. This lasted for 5 to 15 minutes, i.e. over several trains of stimuli. Additionally, similar twitch potentiation occurred after a muscle exposed to blocking concentrations of ACh was washed out in Ringer; it is conceivable that, even after several washes, traces of ACh remained at the junction and caused the potentiation.

It was shown at present that weak concentrations of C_{10} also produce facilitation, C_{10} resembling in this respect ACh. However, there seemed to exist some quantitative difference between C_{10} and ACh. C_{10} facilitation could be much more easily obtained than that by ACh. Also, in the case of suitable doses, C_{10} could produce facilitation lasting up to 2 hrs., while ACh facilitation was temporary.

The facilitatory actions of ACh and of C_{10} are not easy to explain. Twitch potentiation by anti-ChE agents is generally considered to be due to repetitive firing (cf. for instance, Nastuk and Alexander, 1954; Karczmar and Fudema, 1957, and Karczmar, 1961), i.e. to conversion of single twitches into short tetani; similarly, ACh and C_{10} can produce repetitive firing at the height of their depolarization. However, weak facilitatory concentrations of ACh and C_{10} certainly did not produce significant depolarization. In fact, the facilitatory actions of weak concentrations of ACh and of C_{10} continued, in the case of present experiments, past the period of depolarization (figs. 11 and 17); they also could be obtained with concentrations of ACh and of C_{10} incapable of significant depolarization, as for instance when facilitation was noticed

after ACh treatment, following several washes in saline. It cannot be ruled out, however, that C_{10} and ACh could produce, employed in concentrations in question, small, essentially not measurable, depolarization. Nevertheless, it cannot be easily explained why subthreshold depolarization should cause upon indirect stimulation increased twitch amplitude.

It may be speculated that C_{10} and ACh facilitation are analogous to post-tetanic facilitation, and to facilitation occurring in the case of trains of stimuli. Fatt (1959) and Dudel and Kuffler (1961) presented indirect evidence that these types of facilitation are due to increased release, upon stimulation, of ACh from the nerve terminals when the terminals are sensitized by earlier stimulation; this would be then a recruitment phenomenon, previous activity causing more nerve terminals to become active upon subsequent stimulation. Similarly, ACh and C_{10} could produce increased release of the endogenous transmitter from the nerve terminal. It should be added that Koelle recently (1961b) postulated on the basis of indirect evidence that, in the course of cholinergic synaptic transmission, the stimulus arriving at the synapse causes "direct" release of small quantities of ACh, which then acts upon the nerve terminals producing a more massive release of the transmitter. This suggestion is, in fact, related to the earlier hypothesis of Masland and Wigton (1940) and of Riker and his associates (1957, 1959), that certain ammonium ions facilitate and produce anti-d-Tbc action by a presynaptic facilitation at the nerve terminals. It was already suggested in an earlier part of this discussion (cf. p. 62) that this presynaptic action of C_{10} may underlie the C_{10} "reversal" by methoxyammonium.

Another action of ACh that has been studied at present is the blockade by this agent. This blockade resembled in some ways that by C_{10} but in other respects, differed from C_{10} block. As in the case of C_{10} the depolarization was temporary. Even in the case of strong concentrations of ACh, and in continued presence of ACh, the membrane returned spontaneously to its normal potential within 10 to 15 minutes; this recovery was faster in the case of weaker concentrations. Similar data were presented by Thesleff (1955a and 1955b) and Katz and Thesleff (1957). It was pointed out (Patt, 1959) that the neuromyal junction, after being depolarized by ACh, is not excitable; altogether, the data suggest that, as in the case of C_{10} , ACh blockade is not due to depolarization.

In the case of higher concentrations, ACh blockade was essentially irreversible, since it lasted from 2 to 4 hours, thus resembling C_{10} blockade. On the other hand, recovery from the blockade could be observed when weaker concentrations of ACh were employed. In this case ACh block differed from that by C_{10} , since even weaker concentrations of C_{10} , which produced early facilitation, caused subsequently irreversible, slowly progressing blockade.

It could be that stronger, irreversibly blocking concentration of ACh, as well as late phases of the progressive C_{10} blockade, may be due to changes in the muscle, as indicated by the increase in muscle fiber resistance (Kim et al., unpublished). On the other hand, while reversible blocking actions of weaker concentrations of ACh as well as early blocking actions of C_{10} do not depend on depolarization, they may differ in nature. As already stated, C_{10} blockade may be competitive in character; on the other hand, weak concen-

trations of ACh which cause blockade probably do not do so competitively. This is suggested by the fact that both neostigmine and methoxyambenonium synergized with ACh in the production of blockade. It may be thought that neostigmine, which augments and prolongs ACh depolarization, as shown at present and by other investigators (cf., for instance, Koketsu and Gerard, 1956), can in this way synergize with ACh blockade. Thus, ACh in combination with neostigmine may either produce pure depolarization blockade, or cause increased desensitization. The same mechanism may obtain in the case of synergism between C_{10} or SCh and anti-ChE agents, observed at present in the frog and previously in the cat or dog (Karczmar, 1957 and 1961; cf. also p. 32). However, methoxyambenonium seems even more synergistic with ACh than neostigmine. It synergized with ACh at concentrations lower than equi-effective concentrations of neostigmine; yet it is a much weaker anti-ChE than neostigmine (Lands et al., 1955), and does not produce prolongation of ACh depolarization. Moreover, concentrations of ACh, capable of blockade when combined with subliminal concentrations of methoxyambenonium, did not produce, whether used alone or with methoxyambenonium, any measurable depolarization. Thus, the synergism of methoxyambenonium with ACh in the production of ACh blockade cannot be due to its anti-ChE or depolarizing action.

Recently, Smith (1960) also agreed that ACh blockade cannot be due to depolarization; he speculated that ACh and/or its hydrolysis products accumulate at the e.p. and occupy it, preventing diffusion to the e.p. of added ACh. The blockade by subliminal concentrations of ACh combined with subliminal concentrations of methoxyambenonium seems to militate against

"occupancy" theory of Smith; this type of block actually suggests that ACh is capable of a desensitizing action; this action could be, speculatively, due to ACh-induced receptor change. It can be further speculated that, methoxyambenonium can intensify ACh blockade not by increasing ACh depolarization, but by intensifying the postulated desensitizing action of ACh at the receptor site.

On the other hand, this blocking action can be curare-mimetic in nature, dependent upon competition, and thus resemble, in the frog at least, C_{10} blockade. This may be borne out by the fact that after ACh depolarization is over and the blockade ensues, higher concentrations of ACh are still capable of producing depolarization. This is also consistent with the fact that anti-ChE agents do not depolarize after ACh conditioning, since in a quiescent muscle no free ACh would be available for the protective action of the anti-ChE compound. Yet, one would expect that "sensitizing" or "facilitatory" agents such as methoxyambenonium or NaF, should depolarize the refractory e.p. when used in continued presence of ACh, which was not the case. Altogether, it may be suggested that ACh blockade is not depolarizing in nature, and it may be speculated that it is not competitive in character, but dependent upon other hitherto unknown factors.

b. Additional components of action of C_{10} .

As already discussed, C_{10} blocks in the frog by desensitizing the e.p. to ACh or perhaps competing at the e.p. with ACh. As a competitor, C_{10} may be displaced by certain quaternary cations (Riker, 1953), including neostigmine. Competitive character of C_{10} blockade was also suggested by Thesleff (1955a and b) on the basis of his data. Some results

obtained here may be explained in a similar fashion. While neostigmine in concentrations of $10^{-5}M$ or higher intensified C_{10} blockade, in weak concentrations it delayed the block without decreasing its extent. This result cannot be due to anti-ChE action of neostigmine, since this effect would contribute to rather than delay the blockade; it may be due, however, to competition between C_{10} and neostigmine for the receptor site. If the C_{10} blockade is competitive in nature, it differs then from the ACh blockade if as speculated above, this blockade is not competitive. It must be remembered however that strong similarities - some suggested by the present data, some shown by other investigators - exist between C_{10} and ACh. Both exert depolarizing actions. C_{10} , like ACh, causes twitch potentiation when used at low concentrations, and both cause blockade which, in the frog at least, is independent of their depolarization. It is a matter of further experimentation to ascertain whether or not C_{10} blockade indeed differs from that produced by ACh.

c. Modulation of ACh action at the neuromyal junction.

The foregoing underlined the flexibility of the response of the neuromyal junction, exposed to ACh or to C_{10} , to pharmacologic agents. The action of ACh, C_{10} and SCh can be affected by competitively acting agents, anti-ChE compounds, stabilizers of the nerve terminal (Riker et al. 1958), releasers of ACh such as TEA, and by sensitizers. More than one type of substance may belong to this latter category, since NaF action differs in certain aspects from that of methoxyammonium.

Another dimension of flexibility of action of ACh and of C_{10} is added by the fact that they can act as facilitators and also as blocking agents. The depolarizing action of ACh, just

as that of C_{10} , seems not to be the basis of their blocking or facilitatory action.

Present experiments deal with changes occurring over long time periods. ACh blockade occurs, in continued presence of ACh, after 10 or 15 minutes-long depolarization. Somewhat similar data were presented by Foldes (1959) and Zaimis (1959) indicating that, employed over extended time periods or upon repeated administration, C_{10} ceases depolarizing and acts as a competitor of ACh, due presumably to a change in the receptor protein. Yet, these long duration experiments may also constitute a model of immeasurably faster physiological events occurring at the e.p. If this is the case, the termination of action of ACh upon each burst of stimuli may depend not only upon ChE's, or upon diffusion, as suggested by Koelle (1959) with regard to adrenergic transmitters, but upon a change in the receptor reactivity and accompanying change in the mode of action of ACh. Certain data by Del Castillo and Katz (1954c) obtained with iontophoretic techniques of ACh application may suggest the occurrence of similar phenomena.

The change in receptor protein under the impact of ACh seems to be the basis of the spontaneous recovery of the neuromyal junction from blocking actions of ACh when the preparation is exposed to continued presence of ACh. Similar changes may underlie the development of acute tolerance to ACh of the junction which can be demonstrated by "conditioning" of the e.p. to ACh. ACh was not previously considered as exhibiting tachyphylaxis, although recovery from and adaptation to high levels of ACh were reported before in the case of sympathetic ganglia (Krivoy and Wills, 1956). Adaptation to high doses of anti-ChE agents (Karczmar and Koppanyi, 1953;

Barnes and Duff, 1953) presumably constitute similar phenomena. Krivoy and Wills (o.c.) reported that ACh adaptation occurs irrespective of maintenance of electronegativity due to the presence of ACh at the ganglion. At the neuromyal junction ACh depolarization does not persist in the continued presence of ACh; and the adaptation to high levels of ACh may be due to the subsidence of depolarization and also to the fact that subsequent additions of high levels of ACh lead to a relatively small depolarization. In other words, the neuromyal junction adapts to a certain level of ACh and responds not in relation to absolute concentrations of ACh, but rather to increments or gradients of ACh.

The adaptation mechanism may act most importantly in physiological processes of transmission by modulating these processes, thus making it possible for the transmission to function in certain states of hyper-release of ACh and producing a buffer effect in these situations. Suddenly increased concentration of ACh at a synapse will still produce an effect even in continued presence of ACh derived from previous action; yet this effect will not be undue or excessive.

The difference between C_{10} and ACh with regard to recovery from and adaptation to their respective blockade is also of interest in this context. Indeed, it may be teleologically important that the natural transmitter, ACh, exhibits reversibility and tachyphylaxis while the exogenous substance, C_{10} , does not. While these considerations can be only speculative and the mechanism of pertinent phenomena not available, these differences between C_{10} and ACh bear stressing at this time.

3. General conclusions.

These investigations, both of actions of such pharma-

cologic agents as C_{10} , NaF, TEA and methoxyambenonium, as well as of the cholinergic transmitter ACh, leave us with new facts, mechanisms, and with certain speculations. It seems to be well substantiated that certain new mechanisms, independent of such better known mechanisms as anti-ChE and depolarizing action, contribute to the effect of substances capable of deblocking the neuromyal junction; among these new mechanisms a specific facilitator effect at the e.p. is described here for the first time. These mechanisms as well as the non-depolarizing aspects of C_{10} blockade provide the basis for the understanding of the relatively few substances capable of antagonizing both C_{10} and d-Tbc blockade. This antagonism has heretofore either been poorly or not at all explained.

All this points to a flexibility of processes and multiplicity of sites at the neuromyal junction available for drug action. Instead of thinking only in terms of depolarizing and competitive and anti-ChE actions, we can also think of releasers of ACh such as TEA, as well as of facilitatory agents such as NaF or methoxyambenonium. Finally, even the action of ACh at the neuromyal junction may be speculatively resolved into facilitatory and blocking components. Furthermore, some of the data here may suggest that neither of these actions depends on the usual transmitter action of ACh, i.e. upon its depolarizing property. This, plus the demonstrated recovery and adaptation processes occurring at the neuromyal junction with regard to the action of ACh, provides not only for the flexibility of cholinergic transmission, but may be considered as important in modulating cholinergic transmission and preserving its function in special physiological conditions.

F. Summary

I. Objectives and Methods

1. Tetraethylammonium (TEA), NaF, and methoxyambenonium were studied with regard to their actions at the neuromyal junction, as well as with regard to their interaction with C_{10} and d-tubocurarine (d-Tbc). Moreover, actions of C_{10} and of acetylcholine (ACh) at the neuromyal junction were compared.

2. The purpose of the study was: 1) to explain the antagonism of both d-Tbc and C_{10} by methoxyambenonium which is difficult to understand in the light of present theories of the blocking action of d-Tbc and of C_{10} ; 2) to study the effects of newer anti-d-Tbc agents, TEA and NaF, upon C_{10} blockade; 3) to compare these effects with those of anticholinesterase agents; and 4) to explore whether ACh and C_{10} exert other than depolarizing action at the neuromyal junction.

3. The study was carried out employing the mechanical twitch response to indirect stimulation of the frog sartorius muscle and measuring membrane phenomena such as endplate potential (e.p.p.), endplate and muscle membrane polarity, and miniature endplate potentials (m.e.p.p.'s).

4. Mechanical muscle response was studied by means of a force transducer and a polygraph. External and internal micro-electrodes and oscillographic recordings were employed for the study of the membrane phenomena.

II. Drug Effects on the Mechanical Response of the Muscle to Indirect Stimulation.

A. Effect of methoxyambenonium, ambenonium, and NaF on muscle twitch.

1. When supramaximal stimulation was used, methoxy-

ambenonium did not increase the twitch amplitude at concentration of 5×10^{-7} to $10^{-5}M$. Higher concentrations caused slowly developing blockade which was complete within 30 to 50 minutes.

2. When submaximal stimulation was employed, methoxyambenonium increased the twitch amplitude 20% to 40% above the control level.

3. Ambenonium produced some blockade at $5 \times 10^{-6}M$ concentration; higher concentrations caused a more complete blockade.

4. Employed at concentrations of 0.3 to $1 \times 10^{-3}M$, TEA produced increase of muscle twitch amplitude on the first stimulus of each train of stimuli both for supra- or sub-maximal stimulation.

B. Effects of methoxyambenonium, TEA and NaF upon C_{10} and d-Tbc blockade.

1. Methoxyambenonium produced partial restoration of transmission blocked by C_{10} in the case of supramaximal stimulation of intact nerve. More consistent antagonism between C_{10} and methoxyambenonium (3×10^{-6} to $1.5 \times 10^{-4}M$) occurred with divided nerve or submaximal stimulation.

2. TEA (3×10^{-4} to $10^{-3}M$) was more effective against C_{10} than methoxyambenonium.

3. For submaximal stimulation the muscle twitch depressed by C_{10} was augmented by TEA to an amplitude height above that of controls obtained prior to C_{10} treatment. The anti- C_{10} effect of TEA was especially pronounced in the case of the first few responses, and twitch amplitude subsequently decreased.

4. NaF exhibited antagonistic action to the C_{10}

blockade of submaximal stimulation, but not supramaximal stimulation.

5. Anti-ChE agents, ambenonium (10^{-7} to $10^{-5}M$), neostigmine (10^{-6} to $5 \times 10^{-5}M$) and eserine (0.5 to $1.0 \times 10^{-5}M$), used after the onset of C_{10} block, had no antagonistic effect upon C_{10} blockade.

6. When neostigmine was employed in weak concentrations simultaneously with C_{10} , the onset of C_{10} blockade was delayed; eserine did not exhibit this action.

7. A combination of eserine ($5 \times 10^{-6}M$) and of methoxyambenonium caused partial restoration of transmission blocked by C_{10} , even with supramaximal indirect stimulation.

8. Methoxyambenonium (1.0 to $2.0 \times 10^{-6}M$) produced within a few minutes restoration of transmission blocked by d-Tbc. Faster deblocking or intensification or original d-Tbc blockade was noticed with stronger concentrations.

9. TEA in weaker concentration (3 to $5 \times 10^{-4}M$) produced restoration of transmission. With higher concentration of TEA, the mechanical responses were initially augmented and then gradually depressed.

C. Effects of ACh and of C_{10} on Muscle Twitch.

1. Generally ACh had depressant actions upon neuromyal transmission. After washing out the ACh, the twitch amplitude was increased above the control level. Facilitation sometimes occurred also with weaker concentrations ($10^{-6}M$) of ACh following such a sequence.

2. C_{10} in weak concentrations (10^{-5} to $10^{-6}M$) exhibited pronounced and prolonged facilitatory effect on the transmission. "Treppe" was always noticed.

3. With weaker concentrations of ACh (5×10^{-6} to $10^{-5}M$) which produced relatively small depression of transmission, spontaneous recovery could be observed. This did not occur with higher concentrations of ACh, or with both low and high blocking concentrations of C_{10} ; in these instances, persistent blockade was noticed.

4. It was demonstrated that neuromuscular transmission can be conditioned to even higher concentrations of ACh, or to ACh with neostigmine.

5. Subliminal concentrations of methoxyambenonium ($10^{-7}M$) produced marked blockade when added to subliminal concentrations of ACh ($10^{-8}M$).

6. Adaptation or recovery phenomena were never noticed in the case of C_{10} blockade. Even with concentrations of C_{10} causing initial facilitation (5×10^{-6} to $6 \times 10^{-5}M$), subsequent blockade was progressive in nature.

III. Effects at the Endplate and on the Muscle Membrane.

A. Interaction of NaF, TEA and methoxyambenonium with d-Tbc and C_{10} .

1. It was determined that neither methoxyambenonium, NaF, nor TEA had any effect on the resting potential of the muscle fiber membrane or on the action potential of single fibers.

2. Methoxyambenonium increased the amplitude of m.e.p.p.'s but not their frequency. Eserine had no such action.

3. Methoxyambenonium, NaF and TEA all increased the e.p.p., whether the transmission was blocked by d-Tbc or by C_{10} .

4. Anticholinesterase agents markedly prolonged the e.p.p.. In the case of C_{10} -blocked transmission, they frequently decreased the e.p.p. amplitude.

5. TEA and NaF prolonged e.p.p. slightly. Methoxyambenonium produced e.p.p. "augmentation without prolongation". Methoxyambenonium is considered, therefore, a facilitator agent.

6. In high concentrations ($10^{-4}M$), TEA produced repetitive firing whether the transmission was blocked with C_{10} or with d-Tbc. Methoxyambenonium was devoid of this action.

7. ACh depolarization of the endplate was increased and prolonged by anti-cholinesterase agents. Methoxyambenonium increased ACh depolarization without prolonging it at low (10^{-6} to $10^{-5}M$) concentrations and decreased it at higher concentrations (10^{-5} to $10^{-4}M$).

8. TEA, in all effective concentrations (10^{-5} to $10^{-4}M$), decreased ACh depolarization.

B. Mechanism of the antagonism between C_{10} , and TEA and methoxyambenonium.

1. C_{10} depolarized the e.p., but the blockade of transmission began after the peak depolarization was reached; the block to indirect stimulation increased and persisted after the depolarization was over. This is in confirmation of earlier data of Thesleff (1955a and b).

2. C_{10} desensitized the endplate to ACh, which again confirms the data of Thesleff. This effect could be reversed by methoxyambenonium (10^{-6} to $10^{-5}M$), but ACh desensitization was intensified by all effective concentrations of TEA.

3. C_{10} and d-Tbc synergized in blocking the transmission and decreasing the e.p.p.

4. Both methoxyambenonium (5×10^{-5} to $10^{-6}M$) and TEA (0.1 to $0.5 \times 10^{-4}M$) increased the e.p.p. obtained in the presence of d-Tbc and C_{10} .

C. Effects of ACh.

1. ACh depolarization, similarly to that by C_{10} , runs a time course which is not parallel with ACh blockade of neuromyal transmission.

2. After ACh depolarization ceased, and before neuromyal transmission was restored, the endplate was depolarized to a small extent by higher concentrations of ACh. This confirms earlier data of Del Castillo and Katz (1954a).

3. The endplate, desensitized by ACh, could not be depolarized again by eserine or methoxyambenonium in continued presence of ACh.

4. The highest degree of depolarization of desensitized endplate was obtained by employment of ACh combined with weak ($10^{-6}M$) concentration of methoxyambenonium.

IV. Conclusions and hypotheses.

1. One of the reasons why NaF, TEA, and methoxyambenonium can antagonize both C_{10} and d-Tbc block of the frog nerve-muscle preparation must be that C_{10} , like d-Tbc, blocks mainly by desensitizing the endplate to ACh.

2. NaF and methoxyambenonium are facilitatory compounds, distinct from anticholinesterase agents. TEA antagonizes d-Tbc and C_{10} possibly by releasing ACh from motor nerve terminals.

3. Speculation as to why NaF, TEA, and methoxyambenonium (but not anticholinesterase agents) antagonize C_{10} , must relate to the fact that anticholinesterases do not increase e.p.p. of the endplate blocked by C_{10} . This may be due to

intensification of the depolarizing action of C_{10} by anticholinesterases.

4. C_{10} "reversal" by methoxyambenonium may be explained on the basis of two actions of the latter: A) the facilitating postsynaptic action, demonstrated here which antagonizes C_{10} desensitization of the endplate, and B) intensifying action of methoxyambenonium on the presynaptic component of C_{10} action, leading to the increase of twitch amplitude.

5. Flexibility of the neuromyal transmission is well expressed by diversified actions and mechanisms of action of agents studied here. Even C_{10} and ACh actions at the neuromyal junction differ.

6. Another dimension of flexibility of the neuromyal junction is expressed by the fact that neuromyal transmission can be conditioned to high concentrations of ACh, and may recover from ACh blockade.

7. Physiological significance of these findings is discussed.

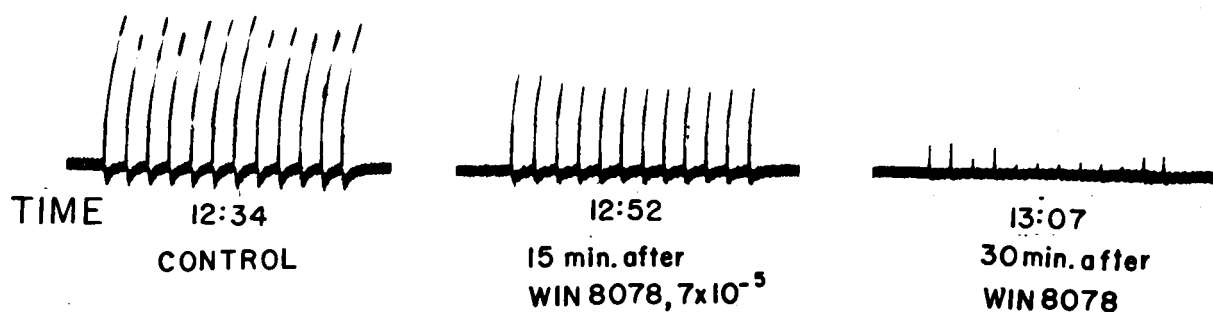


Fig. 1: Blocking effect of high concentrations of methoxyam-benonium (WIN 8078, $7 \times 10^{-5}M$) upon neuromyal transmission. Frog sartorius muscle stimulated indirectly. Cf. Methods for details of technique.

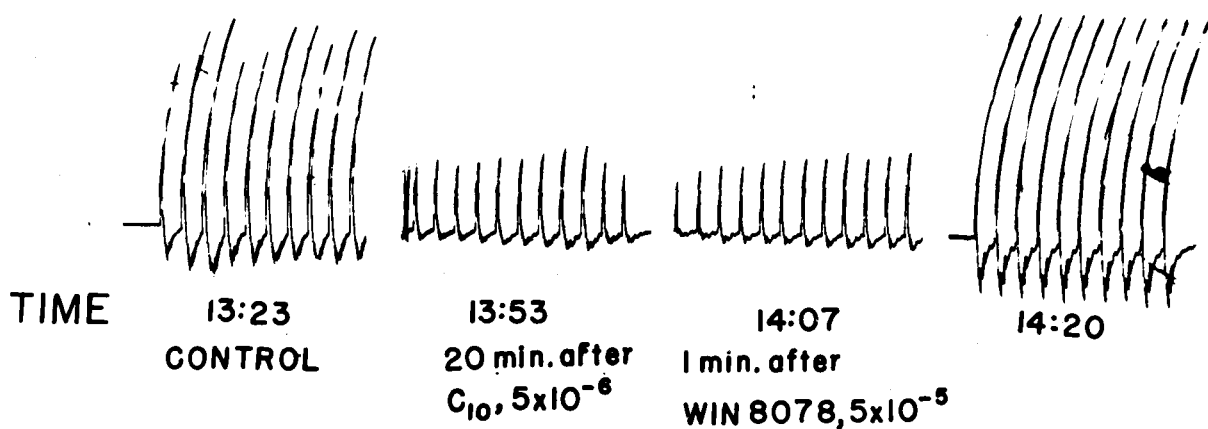


Fig. 2: Twitch response of frog sartorius to stimulation of undivided nerve. Rare example, in these conditions, of restoration by methoxyambenonium (WIN 8078, $5 \times 10^{-5}M$) of neuromyal transmission blocked by C_{10} ($5 \times 10^{-6}M$). Other indications as in fig. 1.

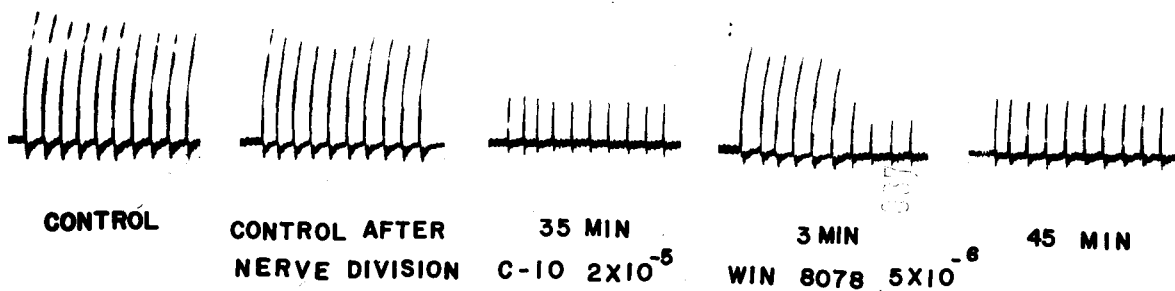


Fig. 3: Antagonism between C_{10} ($2 \times 10^{-5}M$) and methoxyambenonium (WIN 8078, $5 \times 10^{-6}M$). Stimulation of divided nerve. Other explanations as in fig. 1.

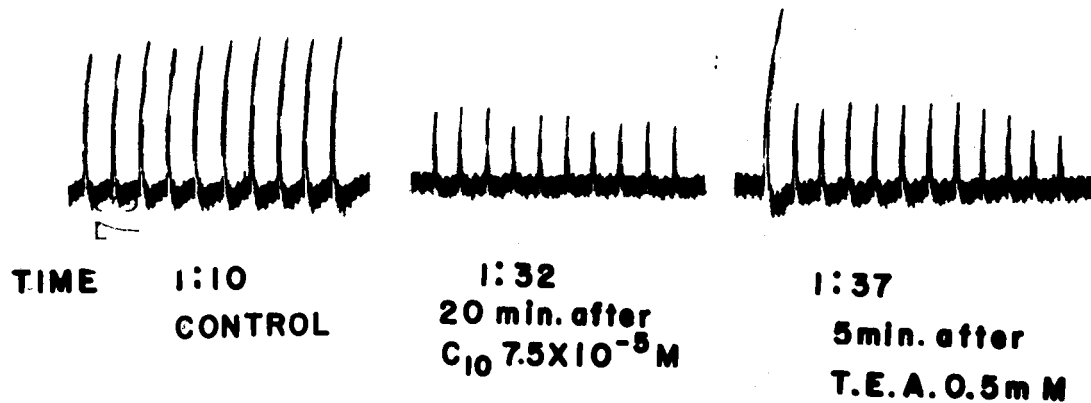


Fig. 4: Partial antagonism between TEA and C_{10} ($7.5 \times 10^{-5} M$). Responses to supramaximal nerve stimulation. Note particularly the increased amplitude of the first muscle response after TEA. Cf. also fig. 1.

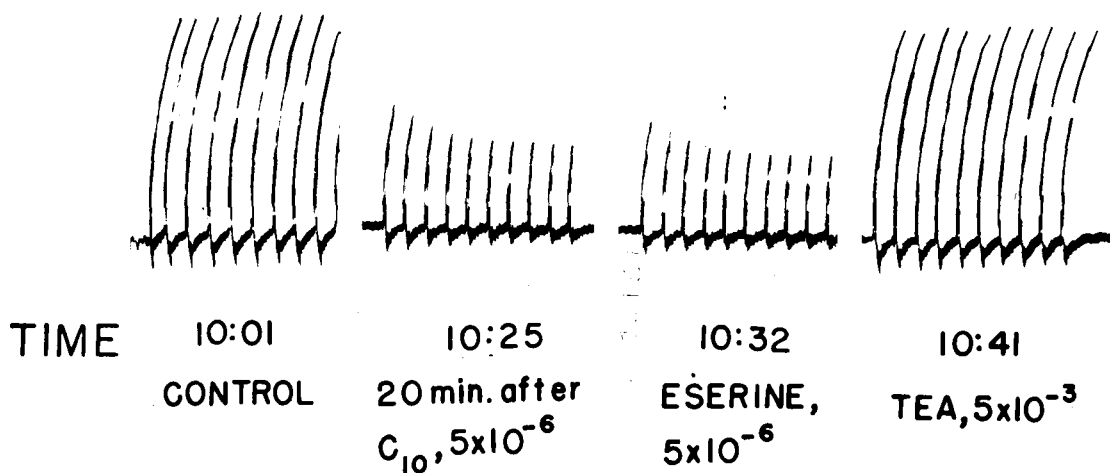


Fig. 5: Antagonism between TEA, $5 \times 10^{-3}M$ and C_{10} , $5 \times 10^{-6}M$. Submaximal stimulation of the nerve. Note ineffectiveness of eserine, $5 \times 10^{-6}M$, administered prior to TEA. Cf. also fig. 1.

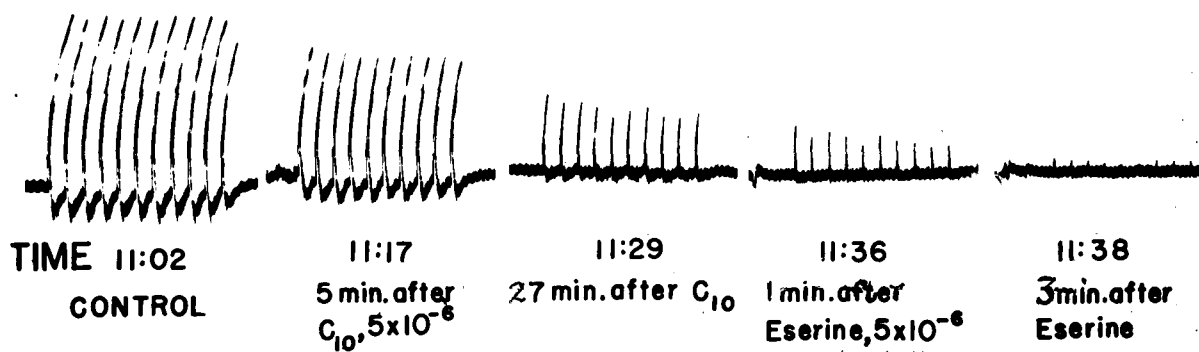


Fig. 6: Increase of C₁₀ ($5 \times 10^{-6}M$) blockade by eserine ($5 \times 10^{-6}M$). Cf. also fig. 1.

DELAY OF ONSET OF C-10 BLOCKADE BY PROSTIGMINE

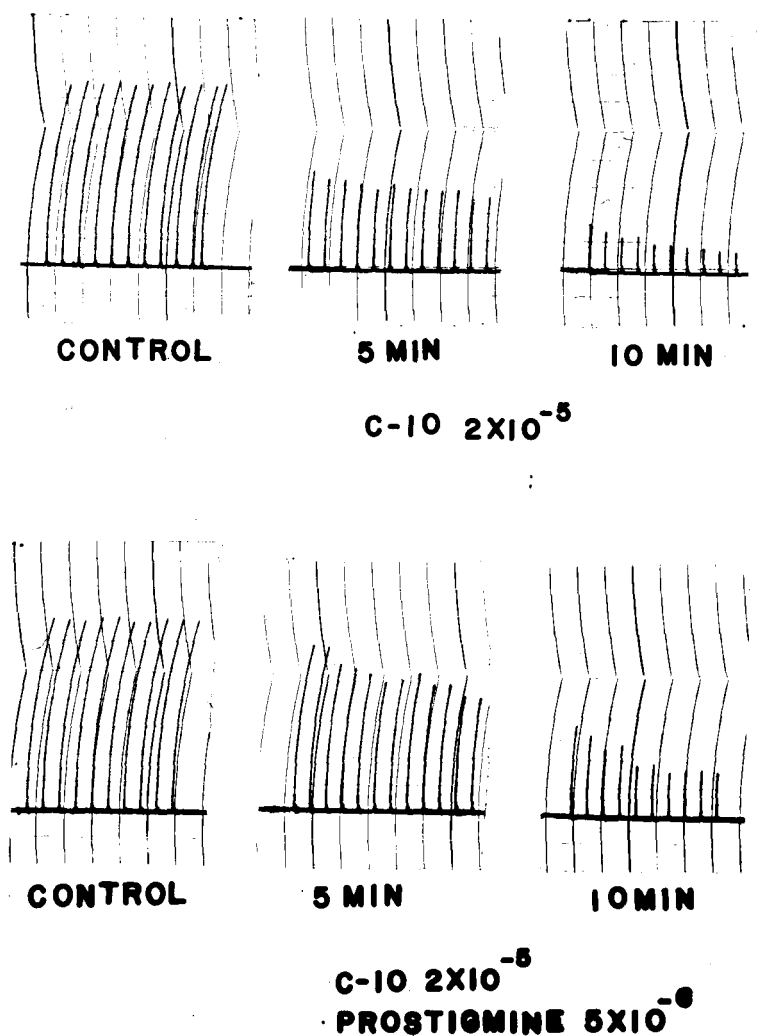


Fig. 7: Upper row: blockade of neuromuscular transmission by C_{10} , $2 \times 10^{-5}M$. After several washes in Ringer, the twitch returned to control levels (lower row, first record). Subsequently, C_{10} , $2 \times 10^{-5}M$, was added simultaneously with prostigmine, $5 \times 10^{-6}M$. Onset of blockade was delayed (compare top and bottom rows).

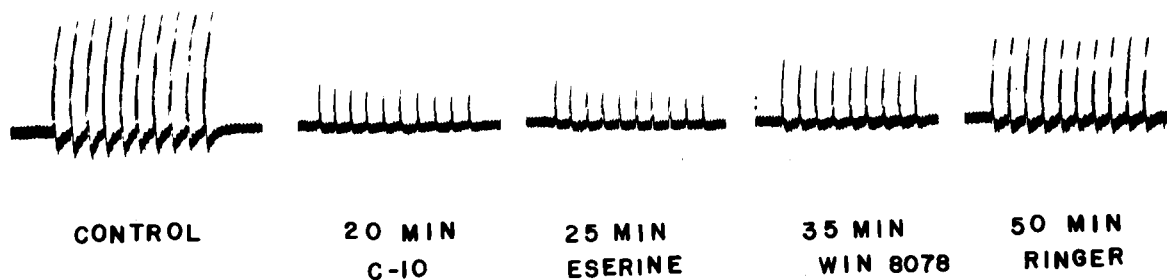


Fig. 8: Partial restoration of neuromyotonic transmission, blocked by C_{10} , $5 \times 10^{-5}M$, by methoxyammonium (WIN 8078), $5 \times 10^{-6}M$, preceded by eserine, $5 \times 10^{-6}M$. Supramaximal stimulation of undivided nerve (cf. also fig. 1).

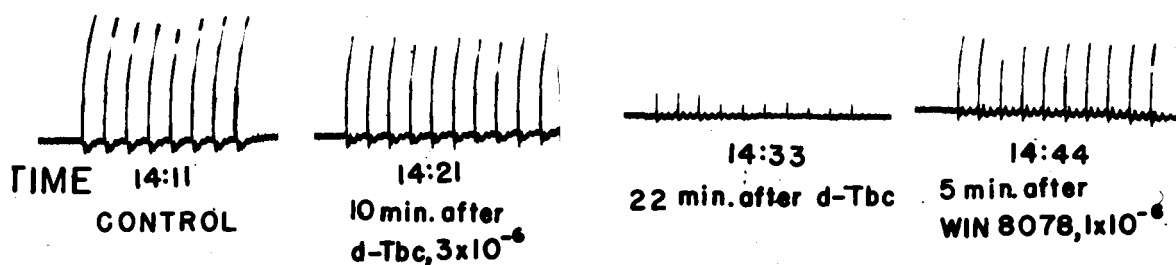


Fig. 9: Antagonism between d-tubocurarine (d-Tbc, 3×10^{-6} M) and methoxyambenonium (WIN 8078, 5×10^{-6} M). Supramaximal stimulation. Cf. also fig. 1.

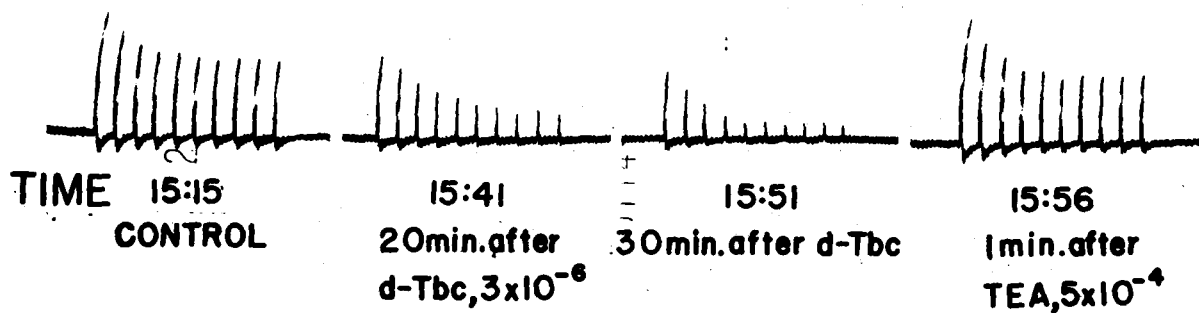


Fig. 10: Antagonism between d-tubocurarine (d-Tbc, 3×10^{-6} M) and TEA, 5×10^{-4} M. Other explanations as in fig. 9.

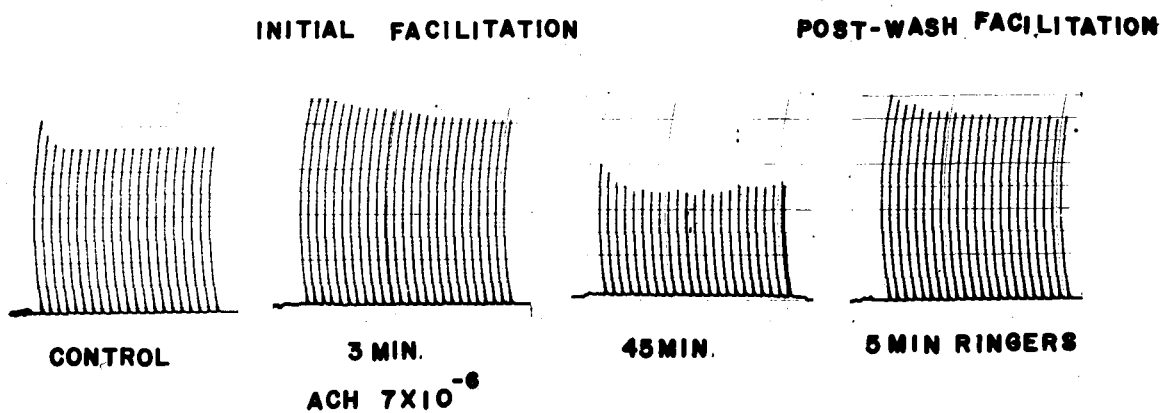


Fig. 11: Facilitation of neuromyal transmission by weak concentrations of acetylcholine (ACh, 7×10^{-6} M). Note also that, after facilitation ceased and blockade supervened (after 45 minutes of treatment), the wash in Ringer led to secondary facilitation. Supramaximal stimulation.

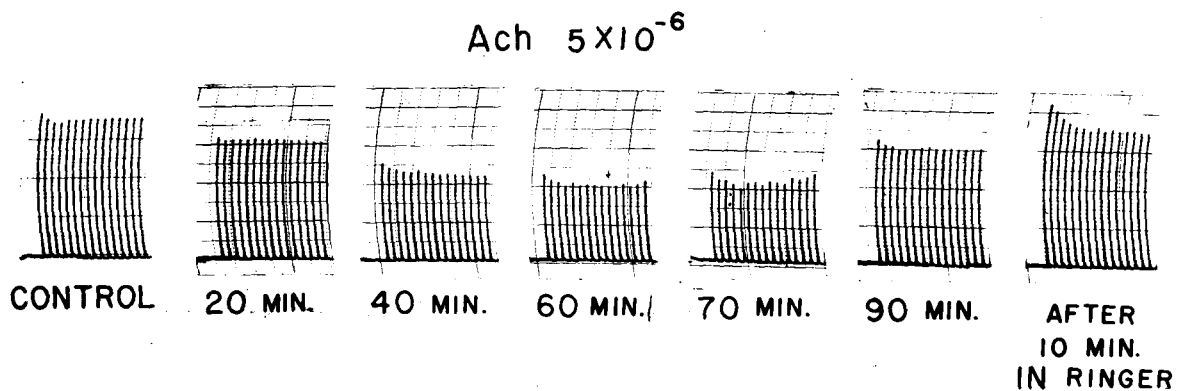


Fig. 12: Recovery of neuromuscular transmission after blockade by acetylcholine. Consecutive records, at time intervals indicated, of the twitch amplitude of indirectly stimulated sartorius muscle in acetylcholine ($\text{ACh}, 5 \times 10^{-6}\text{M}$). Fresh solution of acetylcholine employed every 5 minutes. Supramaximal stimulation.

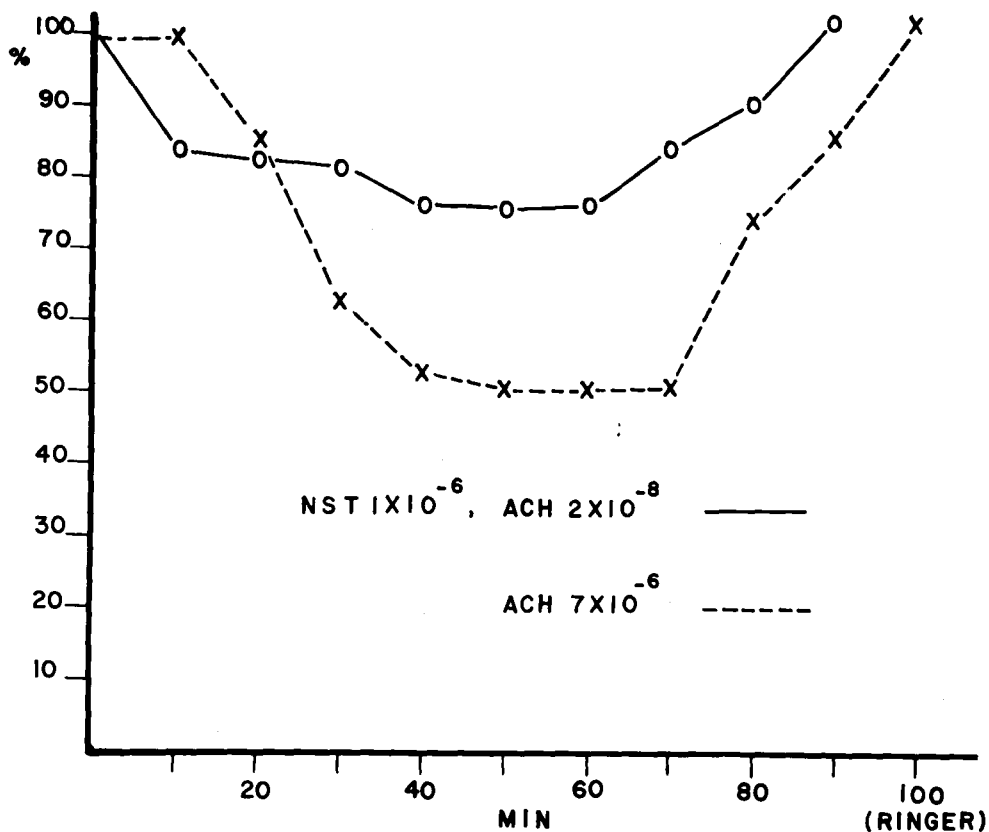


Fig. 13: Plot of spontaneous recovery of neuromyal transmission from partial blockade induced by acetylcholine (ACh, $7 \times 10^{-6}\text{M}$; crosses and dashed line), and by acetylcholine-neostigmine combination (ACh, $2 \times 10^{-8}\text{M}$ with NST, $1 \times 10^{-6}\text{M}$; circles and continuous line). Abscissae: time in minutes; ordinate: twitch amplitude in percent of the control twitch. The experiment dealing with recovery from ACh is different from the one illustrated in fig. 12.

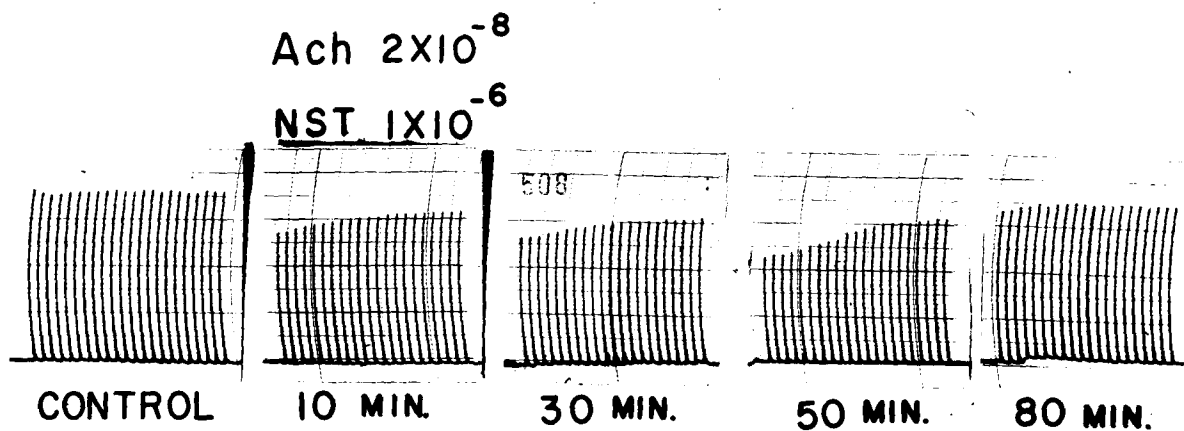


Fig. 14: Spontaneous recovery of neuromyral transmission recorded after partial blockade due to simultaneous employment of acetylcholine (ACh, $2 \times 10^{-8}M$) and neostigmine (NST, $10^{-6}M$). Indirect, supramaximal stimulation.

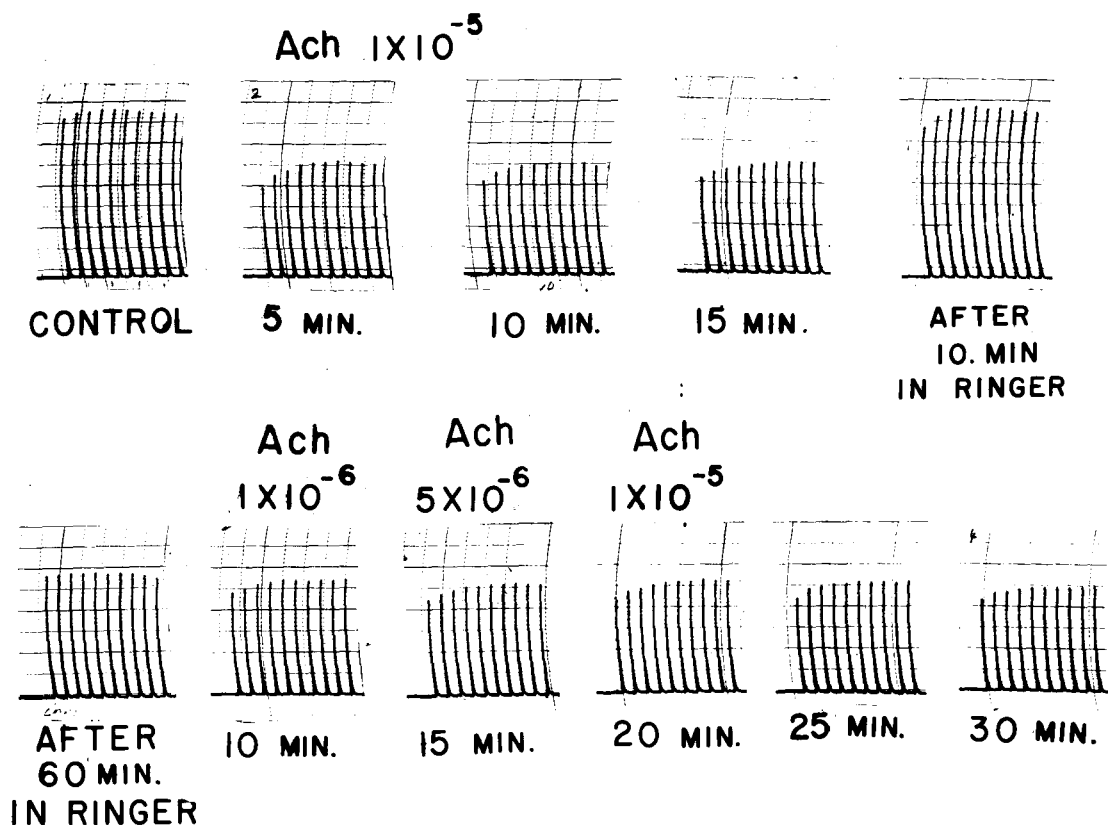


Fig. 15: "Adaptation" of neuromyal transmission to high, blocking concentrations of acetylcholine. Upper row: first treatment with acetylcholine (ACh, $1 \times 10^{-5} \text{M}$). After blockade was recorded, the preparation was washed. Subsequently, ACh (1×10^{-6} to $1 \times 10^{-5} \text{M}$) was found ineffective. Supramaximal stimulation.

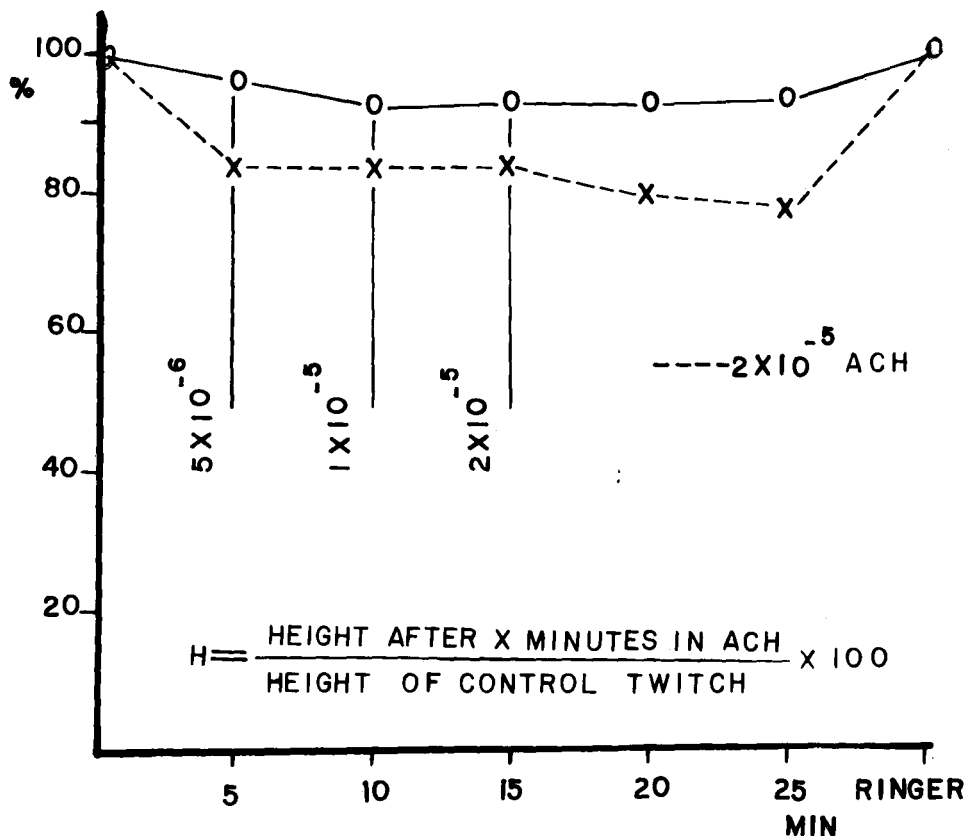


Fig. 16: Plot of the time course of an experiment leading to adaptation to ACh. Abscissa: time in minutes. Ordinate: twitch amplitude in percent of control twitch. The preparation was first exposed for 25 minutes to $2 \times 10^{-5}M$ ACh solution (crosses and dashed line); after wash in Ringer, the preparation was exposed, successively, to $5 \times 10^{-6}M$, $1 \times 10^{-5}M$, and $2 \times 10^{-5}M$ ACh solutions (circles and continuous line). This is an experiment different from the one illustrated in fig. 15.

FACILITATION BY C-10 4×10^{-6}

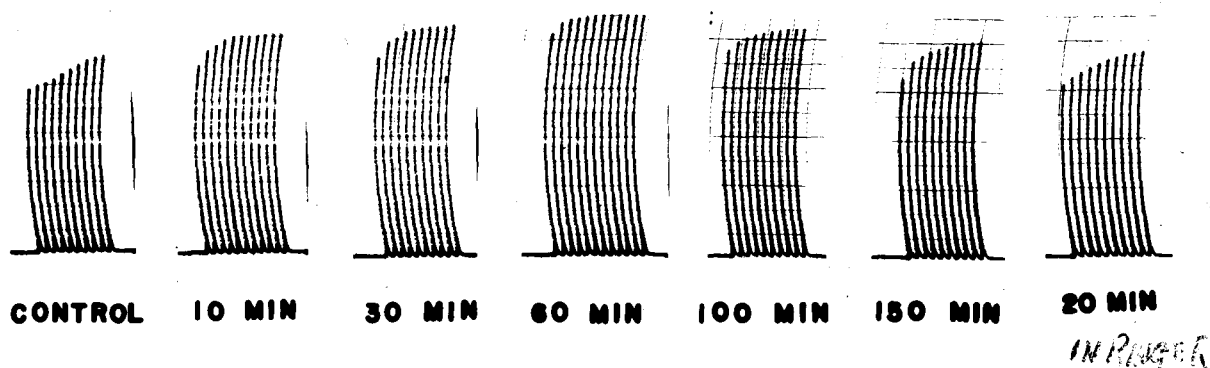


Fig. 17: Prolonged facilitation of neuromuscular transmission by C₁₀, 4×10^{-6} M. Notice repeated "treppe" phenomenon. Last set of responses indicates the effect of a 20 minutes saline wash.

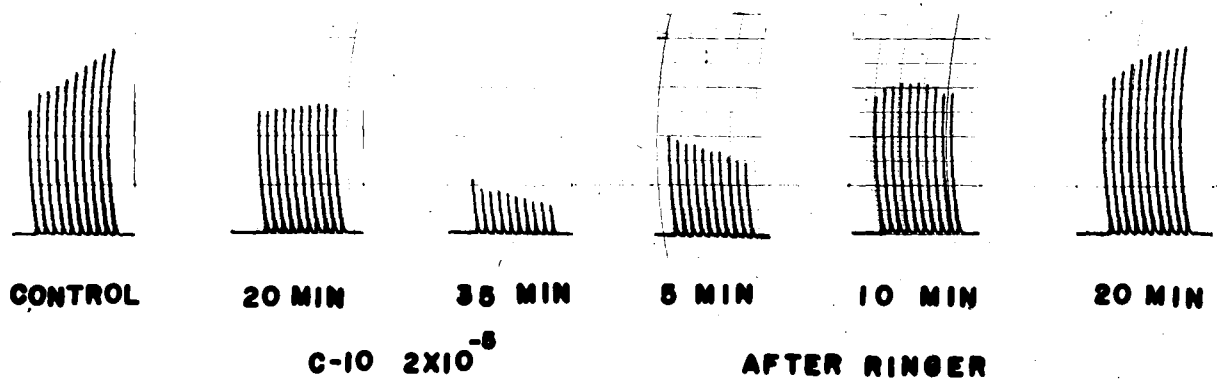


Fig. 18: Blockade of neuromyal transmission by C_{10} ($2 \times 10^{-5}M$), and the recovery after wash in Ringer. Note that the "treppe" occurred during the control period as well as following the wash, but disappeared when C_{10} blockade was present. Supra-maximal stimulation.

NO ADAPTATION TO $C_{10} \ 1 \times 10^{-5}$

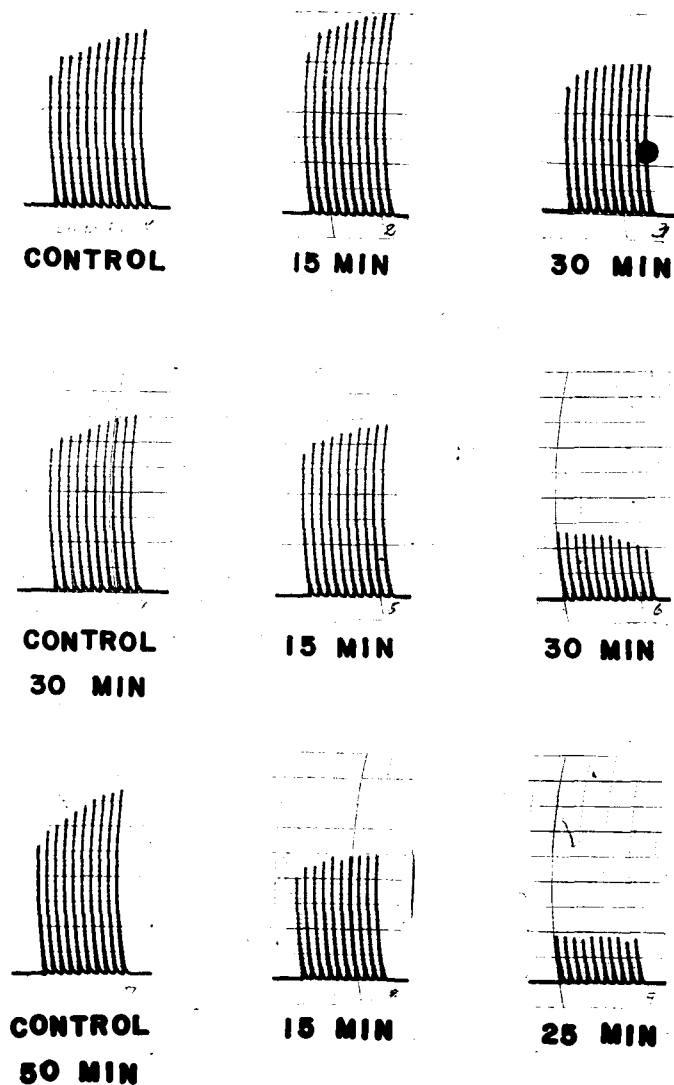


Fig. 19: The three rows of this figure illustrate three exposures of a frog nerve-muscle preparation to C_{10} , $10^{-5}M$. The exposures were separated by three wash periods. Notice that the blocking effect of C_{10} increased on each subsequent exposure. Supramaximal stimulation.

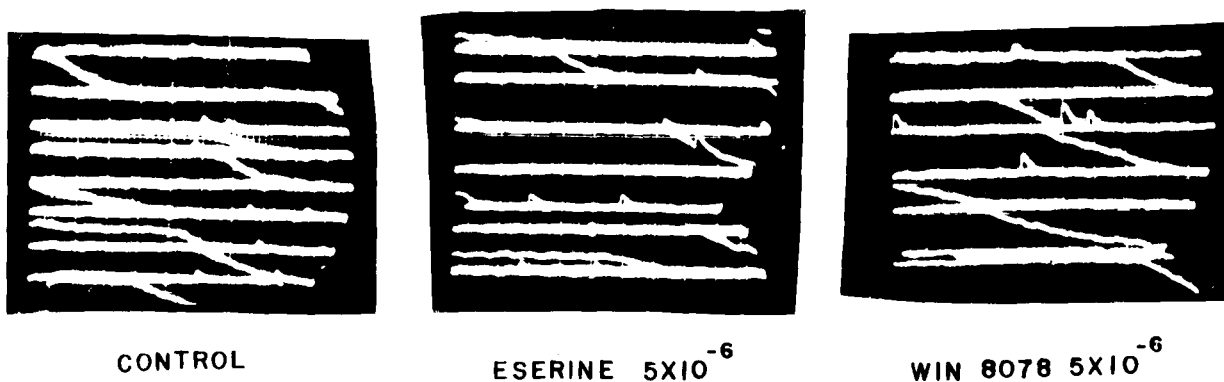
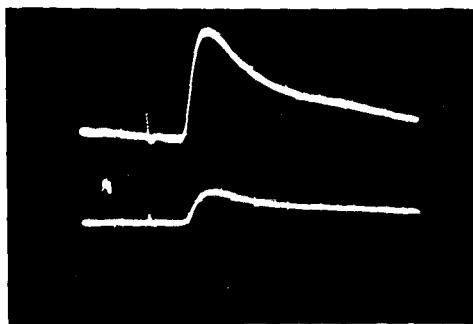
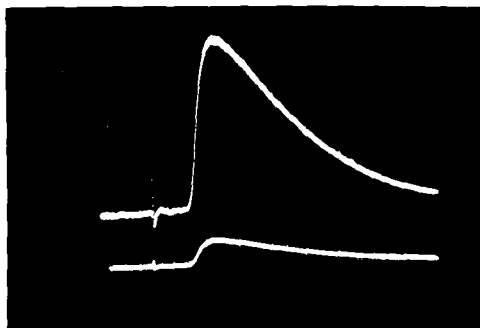


Fig. 20: Effect of methoxyambenonium (WIN 8078, $5 \times 10^{-6}M$) upon the amplitude of the miniature endplate potentials (m.e.p.p.'s). Notice that eserine had ^{slight} effect, and that the amplitude rather than frequency of m.e.p.p.'s was increased by methoxyambenonium. Each record shows from 6 to 8 sweeps (rate of the sweep 2ms/cm; approximate time interval between sweeps 0.1-0.2 sec.) on the oscilloscope screen. For further details on techniques, cf. Methods.



CONTROL E.P.P. reduced by
CURARE 1.5×10^{-6} M



2 min. after WIN 8078 1×10^{-6} M

Fig. 21: Augmentatory (facilitatory) effect of methoxyambonium (WIN 8078, 10^{-6} M) upon endplate potential (e.p.p.) of curarized frog nerve-muscle preparation. Lower and upper tracings, A.C. and D.C. recordings, respectively. Shock artefact visible in each tracing. Cf. also Methods.

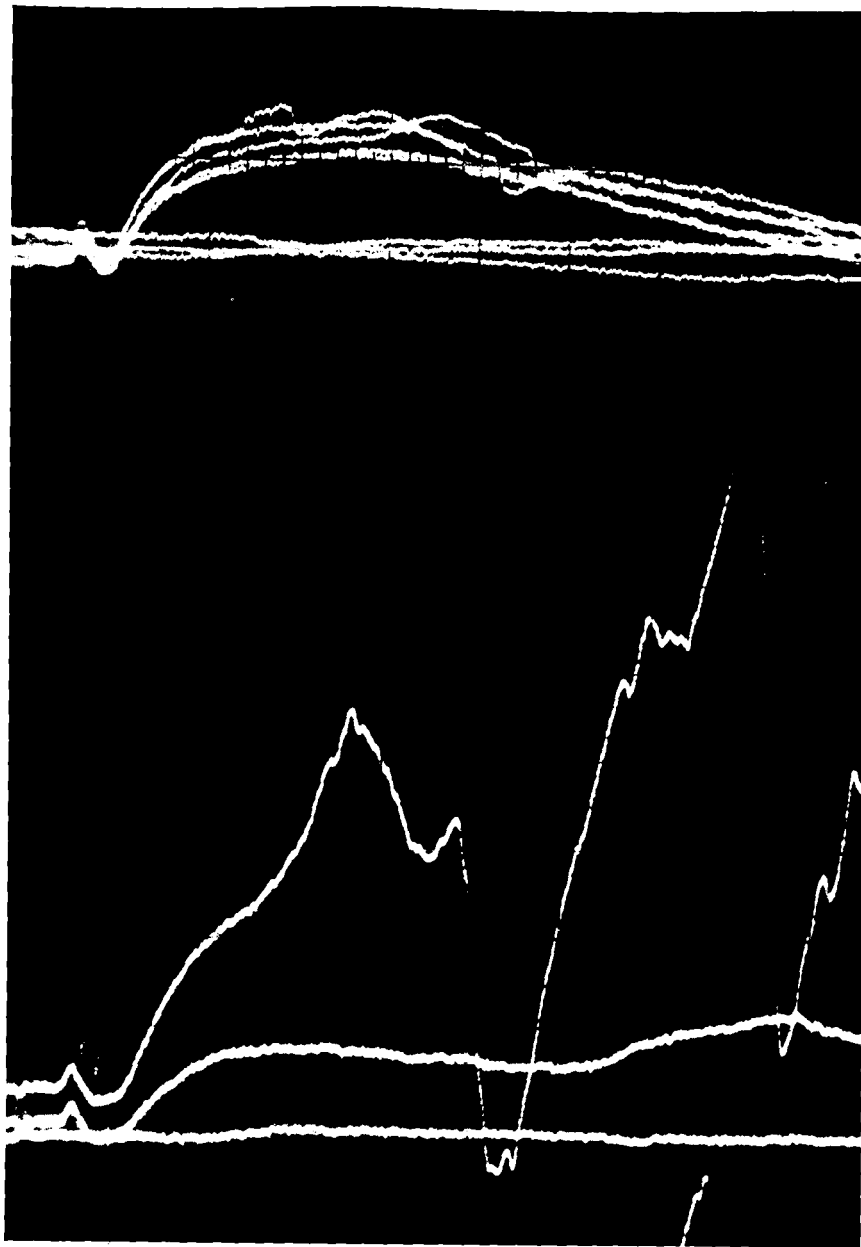
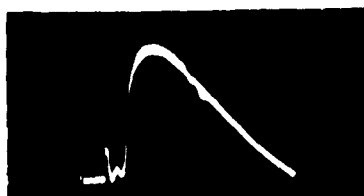


Fig. 22: Effect of TEA, $10^{-4}M$, upon endplate potential (e.p.p.) of nerve-muscle preparation. Transmission blocked by C_{10} , $5 \times 10^{-5}M$ (upper record). Four superimposable e.p.p.'s recorded. Lower record, two tracings 5 minutes after TEA. First tracing illustrates prolongation of e.p.p. by TEA. The very next sweep (after 0.1 sec.) shows spikes and repetitive firing. Cf. also Methods and Text.



CONTROL E.P.P.
C-10 5×10^{-7}



5 MIN AFTER
WIN 8078 5×10^{-6}



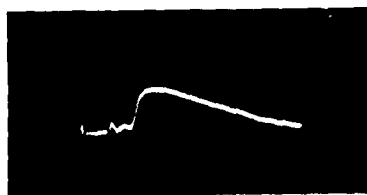
CONTROL E.P.P.
D-TBC 3×10^{-6}



15 MIN AFTER
ESERINE 3×10^{-6}



CONTROL E.P.P.
C-10 1×10^{-6}



20 MIN AFTER
ESERINE 3×10^{-6}

Fig. 23: The effects of various chemical agents upon the e.p.p. p. Molar concentrations throughout. Top-row: the effect of methoxyambenonium (WIN 8078) upon the e.p.p. after the blockade of transmission by C10. Observe that single spike seemed to have been induced by methoxyambenonium; note also that methoxyambenonium caused "augmentation without prolongation" of the e.p.p. Middle-row: the effect of physostigmine (eserine) upon the e.p.p. of the curarized preparation. Note the conspicuous prolongation of the e.p.p. by physostigmine. Bottom-row: the effect of physostigmine upon e.p.p. produced by C10. Note that the control e.p.p. is at a threshold level of depolarization (the beginning of a spike is observable), and that eserine produced e.p.p. reduction.

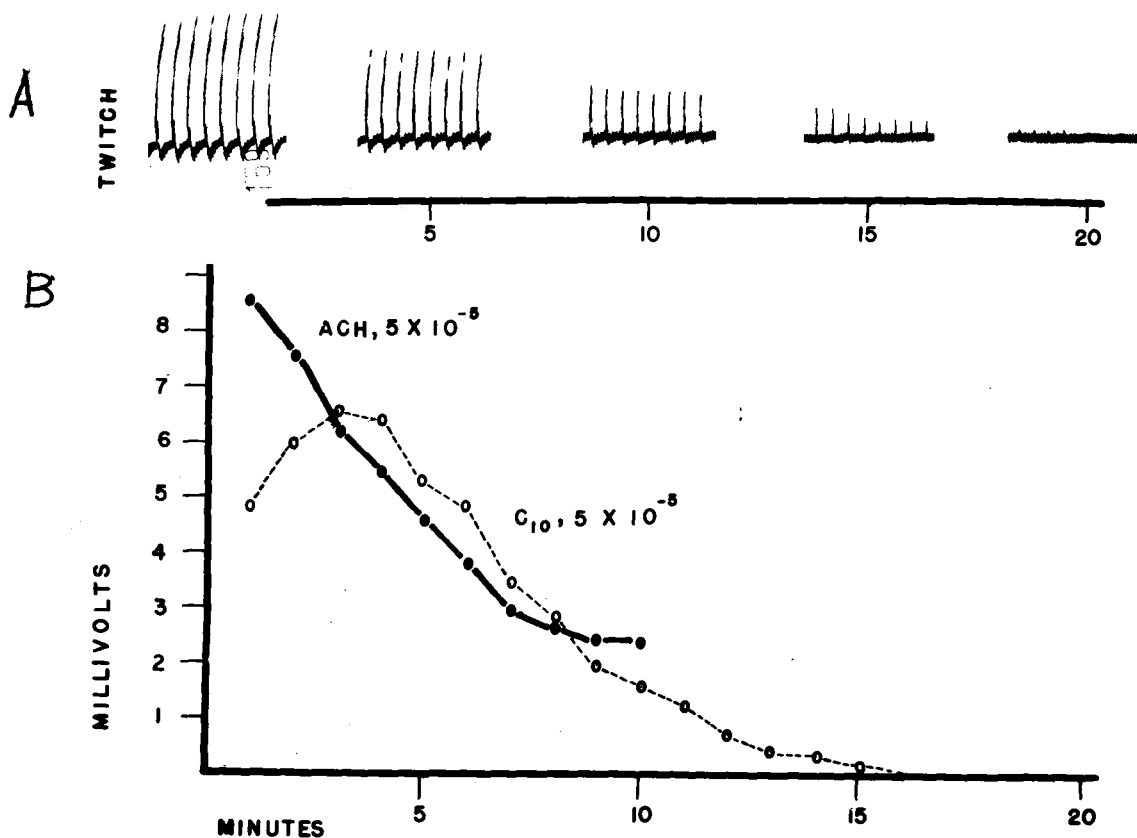


Fig. 24: Effect of C_{10} upon the endplate and neuromyal transmission. Upper row, twitch amplitude and time in minutes. Lower row: external electrodes recording at the e.p. (ordinate); abscissae: time in minutes. Time scale identical in both records. The preparation was first exposed to acetylcholine (ACh, 5×10^{-5} M) and depolarization recorded (full circles and continuous line). After wash, C_{10} (5×10^{-5} M) depolarization (lower row; empty circles and discontinuous line) and blockade of neuromyal transmission were recorded simultaneously.

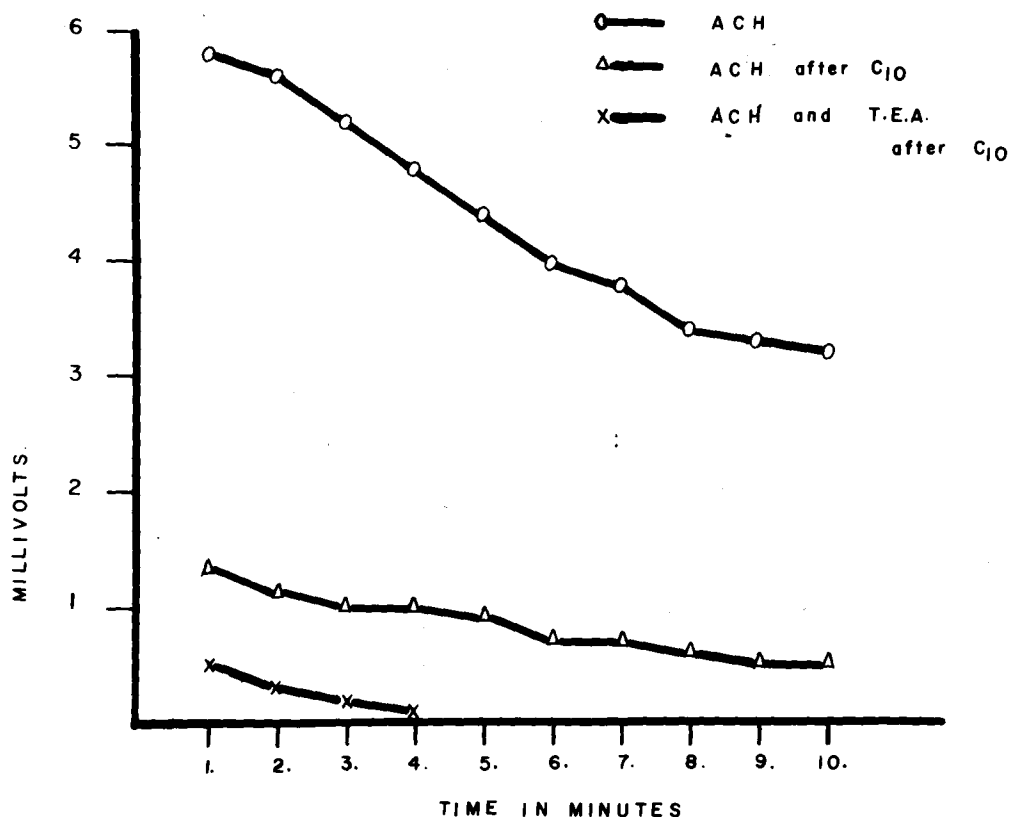


Fig. 25: Acetylcholine (ACh, $5 \times 10^{-5}M$) depolarization after and before C_{10} , $5 \times 10^{-5}M$. External electrodes recording. First, depolarization due to ACh alone was recorded (empty circles). After wash, and exposure of the preparation to C_{10} (5 minutes), ACh depolarization was diminished (triangles). After wash, sensitivity to ACh returned to normal (not shown) and the preparation was exposed first to C_{10} , and then to ACh and TEA, $10^{-4}M$. ACh depolarization was further decreased, See also text.

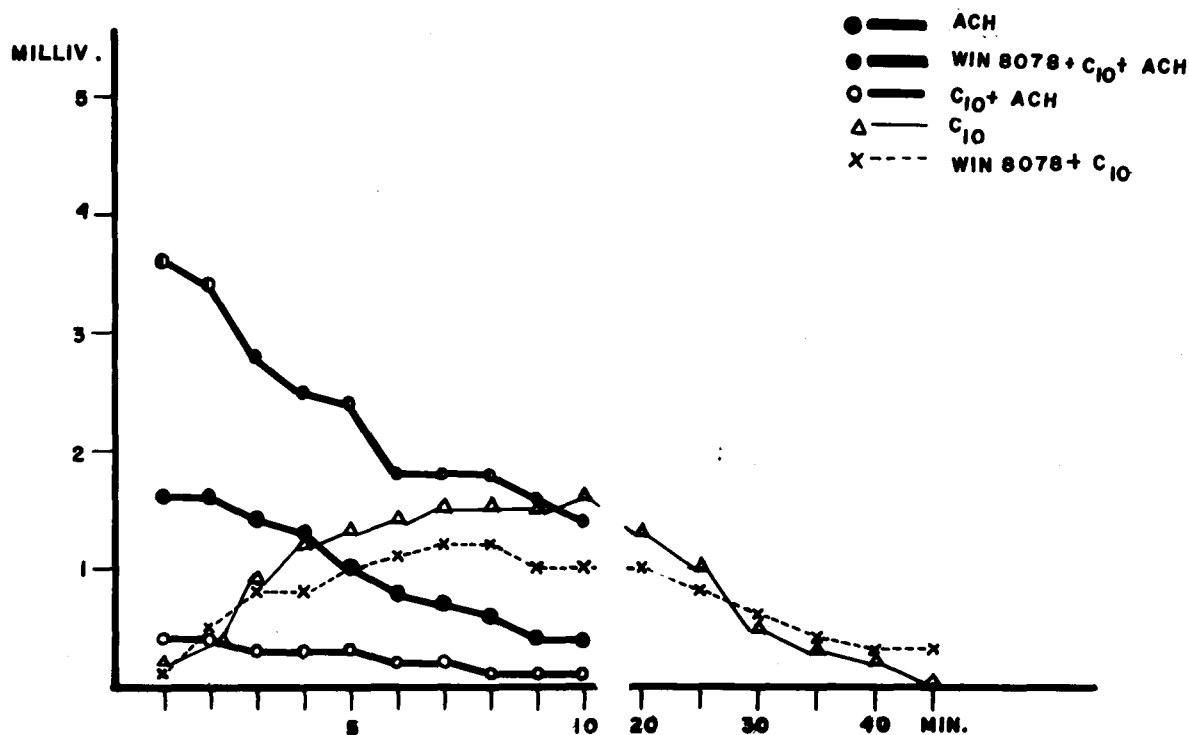


Fig. 26: The effect of methoxyammonium (WIN 8078) upon depolarization by acetylcholine (ACh), diminished by C₁₀. Firstly, C₁₀ ($5 \times 10^{-5}M$) depolarization was measured (triangles and continuous line). After wash in Ringer, essentially similar depolarization was obtained by C₁₀, $5 \times 10^{-5}M$, and WIN 8078, $10^{-5}M$, employed simultaneously (crosses and dashed line). After appropriate wash in Ringer, ACh ($5 \times 10^{-5}M$) depolarization was measured (semi-empty circles and continuous line); following wash in Ringer and treatment with C₁₀ ($5 \times 10^{-5}M$), ACh depolarization was diminished (empty circles and continuous line), finally, after one more wash, combined treatment with C₁₀, ACh and WIN 8078 ($5 \times 10^{-5}M$ in each case) caused increased depolarization (full circle and continuous line). External electrodes.

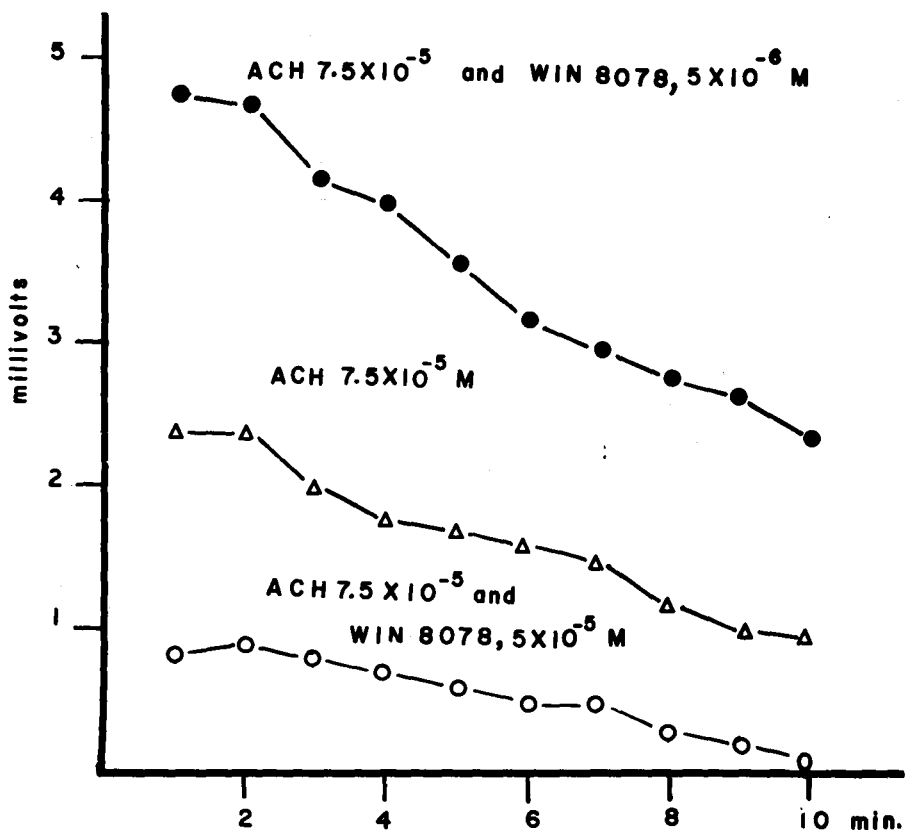


Fig. 27: Biphasic effect of methoxyambenonium (WIN 8078) upon acetylcholine (ACh) depolarization. The preparation was exposed, first, to ACh alone, then to ACh and weak concentration of WIN 8078, and, finally, to ACh and strong concentration of WIN 8078. The preparation was washed in Ringer between treatments. External electrodes. Other explanations in the figure and in the text.

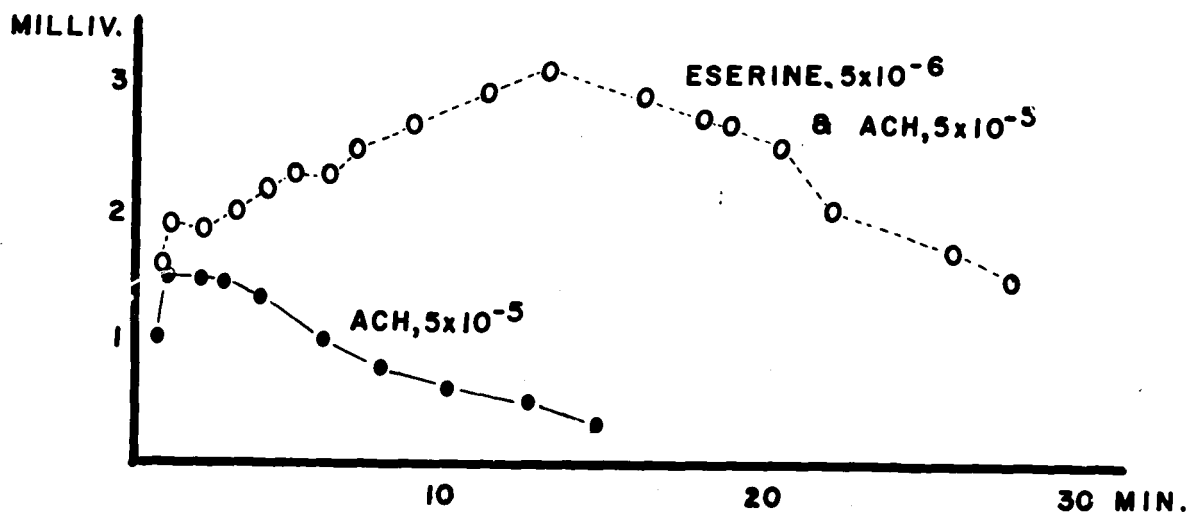


Fig. 28: Augmentation and prolongation of acetylcholine (ACh) depolarization by eserine. The neuromyal preparation was first exposed to ACh alone, and then, after appropriate wash in Ringer, to ACh and eserine. External electrodes recording. Other explanation in the figures and in the text.

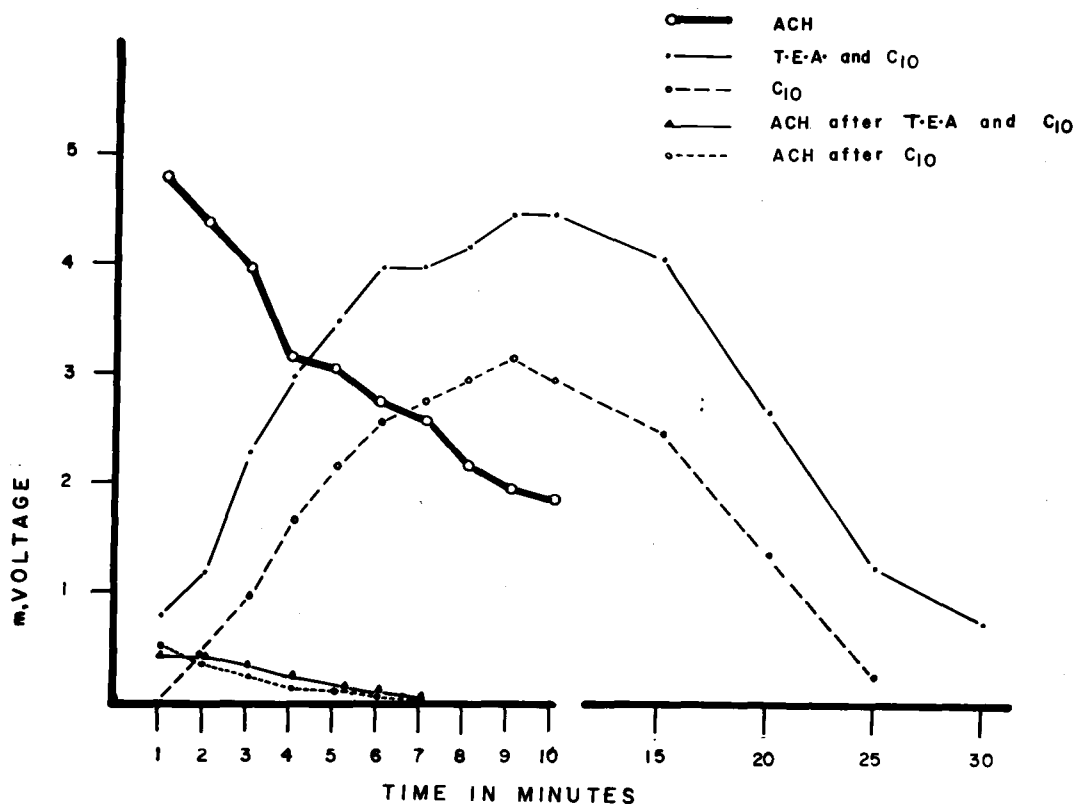


Fig. 29: Lack of effect of TEA upon acetylcholine (ACh) depolarization reduced by C₁₀ treatment. C₁₀ depolarization was first measured with (dots and thin line) and without (full circles and dashed line) TEA; TEA seemed to increase somewhat C₁₀ depolarization. Subsequently, ACh depolarization was measured with (full circles and continuous line, lower part of the illustration) and without (full circles and thick line) C₁₀. Addition of TEA did not increase ACh depolarization in presence of C₁₀ (full circles and thin line, lower part of the figure). Appropriate washes after each treatment. External electrodes recording.

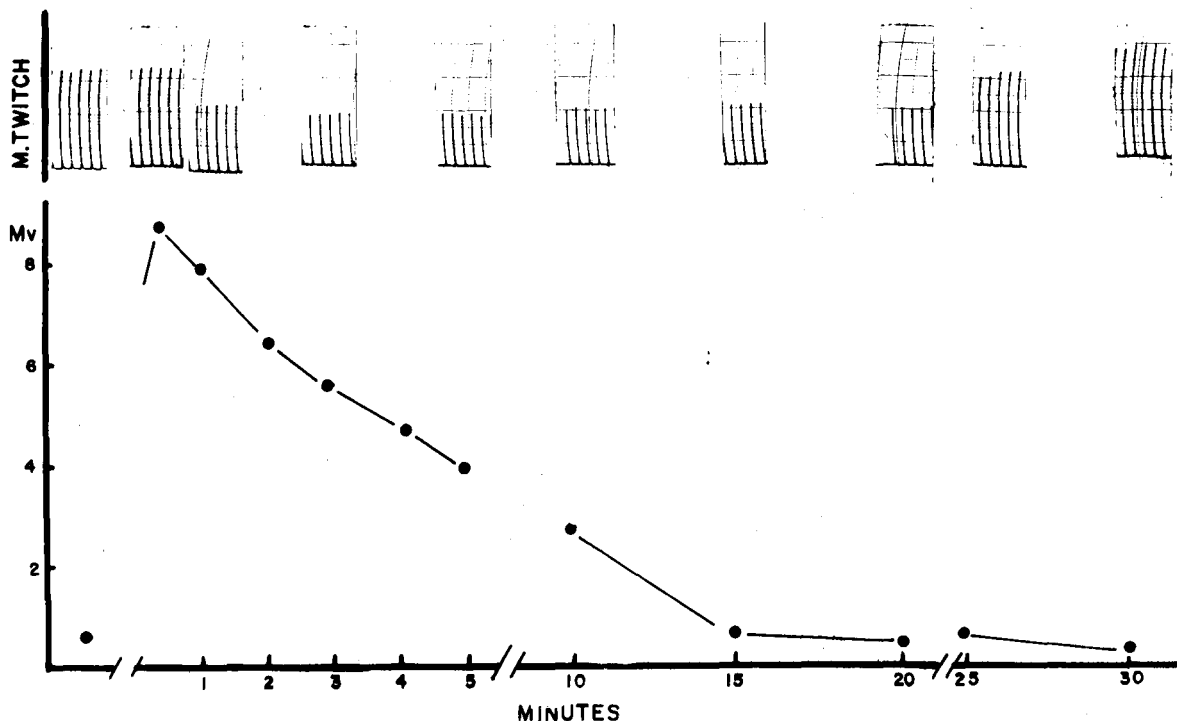


Fig. 30: Effect of acetylcholine (ACh) upon the endplate and neuromyal transmission. Upper record, recording of muscle twitch (indirect stimulation). Lower record, depolarization due to ACh ($5 \times 10^{-5}M$), measured with external electrodes. Identical scales (minutes) for the measurement of depolarization and of the muscle twitch. Note that partial ACh blockade developed after the peak depolarization, and persisted for some time after depolarization was over (15-20 minutes). Spontaneous recovery of transmission (25 - 30 minutes) without further change of the endplate membrane.

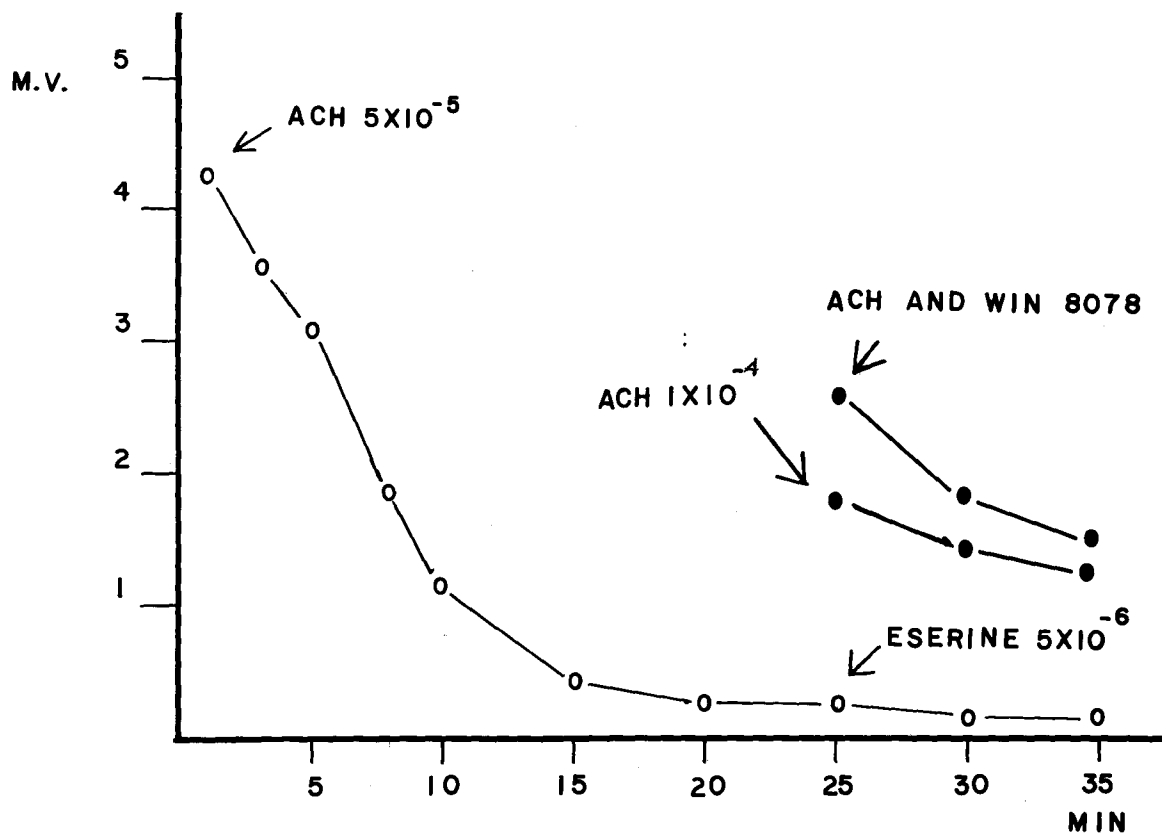


Fig. 31: Desensitization of the endplate by acetylcholine (ACh, 5×10^{-5} M). After ACh depolarization (circles and continuous line) was over, eserine (5×10^{-6} M) had no depolarizing action in continued presence of ACh, while strong concentration of ACh, and particularly, combined treatment with weak concentrations of ACh (5×10^{-6} M) and of methoxyammonium (WIN 8078, 5×10^{-6} M) were effective (full circles and continuous line). External electrodes recording. Other explanations in the figure.

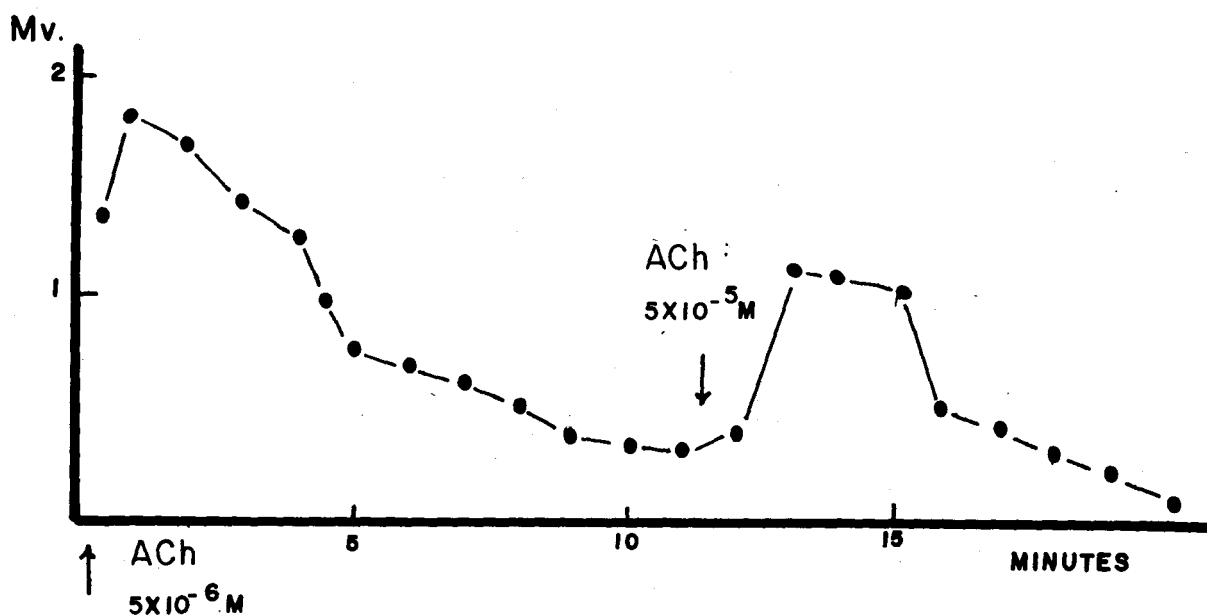


Fig. 32: Desensitization of the endplate by acetylcholine (ACh, $5 \times 10^{-6}M$). After ACh depolarization was over, addition of 10 times more concentrated ACh solution caused some depolarization. External electrodes. Other explanations in the text.

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TABLE I

	Interaction with Blocking Agents				Effect on		SITE OF EFFECT	TYPE OF EFFECT
	Effect on Mechanical Response		Effect on					
	d-TBC Block	C-10 Block	E.P.P. Produced by C-10	ACh Desensiti- zation by C-10				
d-TBC		Antagonism	Decrease	Increase	Decrease		Post-synaptic (ACh receptor)	Competition with ACh
C-10	Antagonism				Decrease	Decrease	Post-synaptic (ACh receptor)	ACh Desensitization
WIN 8078	Antagonism	Antagonism	Increase No Prolongation	Increase	Increase No Prolongation		Post-synaptic (ACh receptor)	Sensitization to ACh
TEA	Antagonism	Antagonism	Increase and Prolongation	Increase	Decrease	Increase and Pro- longation	Presynaptic	Increased Release of ACh
ESERINE	Antagonism	Synergism	Decrease	Increase	Increase Prolongation	Increase	Post-synaptic ChE	Anti-ChE
NaF	Antagonism	Antagonism			Increase Prolongation	Increase	Post-synaptic	Sensitization to ACh

APPROVAL SHEET

The dissertation submitted by K. C. Kim, M.D., 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.

May 22, 1962
Date

Alexander O. Kapur
Signature of Advisor