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Synaptic Effects Of Some Synthetic Mono-onium Compounds

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SYNAPTIC EFFECTS OF SOME SYNTHETIC MONO-ONIUM COMPOUNDS

by

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ABSTRACT

The pharmacological actions of three series of mono-onium compounds (phenoxyalkyltrimethylammonium, phenoxyalkyltriethylammonium and phenylalkyltrimethylammonium) have been studied with respect to their synaptic stimulant and/or blocking activity, particularly at the skeletal neuromuscular junction. The phenoxyalkyltrimethylammonium (alkyl chain 2-10 carbons), a series which is an extension of choline phenyl ether, were studied for synaptic stimulant activity on the frog rectus abdominis *in vitro*, chick skeletal muscles *in vivo*, and cat superior cervical ganglion (cat S.C.G.) *in vivo*, and for synaptic blocking activity upon the cat S.C.G. and cat tibialis anterior *in vivo*, and rat phrenic nerve-diaphragm *in vitro*. The analogous series of phenylalkyltrimethylammoniums (0 to 5 C's) in which the oxygen is replaced by a carbon and phenoxyalkyltriethylammoniums (2 to 6 C's) in which the trimethylammonium-cationic head is replaced by triethylammonium, were studied on some of these preparations.

Increasing the carbon chain length from 2 to 3 in the phenoxyalkyltrimethylammonium series abolished ganglion stimulant activity on the cat S.C.G. and reduced synaptic blocking activity at both ganglionic and neuromuscular sites. Ganglion blocking activity thereafter returned (being optimal with the 8-carbon homologue) ; neuromuscular blocking activity returned on both the cat and rat preparations (being maintained

to the 10-carbon compound on the cat and to the 9-carbon on the rat).

The long chain phenoxyalkyltrimethylammoniums were shown to be partial agonists on the "nicotinic" receptors of the frog rectus. The partial return of agonist activity and of neuromuscular blocking activity is discussed with reference to Courtauld Atomic Models and a hypothesis is proposed that for maximal acetylcholine-like activity both the criteria of planar conformation and an optimal oxygen-to-nitrogen atomic distance must be met.

Inter-series comparisons between the phenoxyalkyltrimethylammoniums, phenoxyalkyltriethylammoniums, and phenylalkyltrimethylammoniums indicate that the oxygen atom can be replaced by a carbon atom without loss of synaptic activity. (The marked nicotinic activity of phenoxyethyltrimethylammonium (choline phenyl ether) is thus not critically dependent upon the ether oxygen atom).

The triethylated mono-oniums (phenoxyalkyltriethylammoniums) were, as expected, devoid of synaptic stimulant activity; yet they proved, with the possible exception of the ethyl (2-carbon) analogue, to have an affinity similar to their trimethylated counterparts. The discrepancy here is thought to be due to the profound acetylcholine-like depolarizing ability of the phenoxyethyltrimethylammonium, a property not shared by the triethylated compound.

The neuromuscular blockade induced in cats and rats by all three series was not reversed by the anticholinesterase edrophonium, and was thus not curare-like.

A series of experiments was conducted in which the motor nerve was alternately stimulated at 12 and at 60 or 120 (cat and rat , respectively) times per minute during the development of blockade.

Maximal rate-dependent blockade was observed with the phenylalkyltrimethylammoniums (greater than with d-tubocurarine) and was minimal with the phenoxyalkyltriethylammoniums. The relevance of these tests to possible pre-synaptic, triethylcholine-like, activity is discussed.

During these rate-dependent studies it was of interest to note that the clinically-used drug, gallamine triethiodide, was markedly rate-dependent, suggesting that future investigations with this drug should be carried out.

Taken together the results clearly demonstrate that merely increasing alkyl-chain length and cationic head size do not lead to transformation from a "nicotinic" , depolarizing-type blockade to a "curare-like" , competitively reversible-type blockade.

I. INTRODUCTION

The mode of propagation of an impulse from a nerve fibre to the effector organ has long occupied the attentions of pharmacologists and physiologists.

Since 1850, when Pelouze and Bernard showed that transmission could be interrupted at the neuromuscular junction, a vast amount has been done to elucidate not only the nature of such synaptic junctions but also what factors were responsible for the transmission of the impulse across the synaptic gap.

It has now been confirmed that acetylcholine is the chemical substance responsible for transmission at some peripheral synapses, including the skeletal myoneural junction. It has also become apparent that many chemical substances can modify such transmission, those which mimic the actions of the normal transmitter being agonists, and those which interfere with the action of the normal transmitter being antagonists. Since the early experiments with natural alkaloids such as curare, the evolutionary sequence in this field has been to establish the chemical nature of such compounds acting at synaptic sites to determine how they act, what conditions are likely to modify their actions, and what changes in chemical structure might alter their activity.

The present thesis is concerned with three series of chemical substances kindly synthesized by R.B. Barlow in Edinburgh. These series are the phenoxyalkyltrimethylammonium, phenylalkyltrimethylammonium, and phenoxyalkyltriethylammonium salts. The main purpose has been to more closely investigate the structure-activity relationships of such compounds, primarily at the neuromuscular synapse. It was hoped that such a study might throw more light on the mechanism of action of such mono-onium compounds. Their activity has been compared and contrasted to that of the commonly used, clinical neuromuscular blocking drugs, and a number of other new synthetic compounds which are believed to have presynaptic neuromuscular inhibitory actions. The latter compounds, such as triethylcholine, produce symptoms similar to those of myasthenia gravis. In addition, such studies as these may be justified as there is still a need for an improved, clinically useful, neuromuscular relaxant which is readily reversible and short-acting. As there are numerous publications in this field of study, only the more relevant reviews could be mentioned in the historical section. This deals briefly with the evidence for chemical transmission of nervous impulses followed by a discussion of compounds which are known to affect transmission acting at a postsynaptic site, and by those which are thought to interfere with transmission at a presynaptic site. This section is concluded by a survey of the literature concerning structure-activity relationships at the neuromuscular junction with specific emphasis on compounds related to those studied herein.

II. HISTORICAL REVIEW

A. Chemical Transmission at Peripheral Synapses

Over one hundred years ago Pelouze and Bernard (1850) were impressed by the fact that in an animal killed by a lethal dose of a substance or by decapitation , one could still elicit a muscle contraction on stimulation of a motor nerve. If , however , the animal was killed by curare, no such muscle contraction occurred. By tying a ligature around one leg of a frog so as to occlude the circulation, but not damage the nerve, they demonstrated that on giving curare extract into the dorsal lymph sac only the leg with the circulation occluded contracted on immersing in acid, whereas the rest of the animal was paralysed. The observation that only the limb with the occluded blood supply contracted was taken as evidence that curare was acting at the neuromuscular junction.

Within a short time, Crum-Brown, and Fraser (1868 , 1869) realizing that curare extracts contained quaternary ammonium salts, examined many other such compounds and showed that the methiodides of atropine, morphine, strychnine, codeine and many other compounds reproduced the paralytic effects of curare extracts. These pharmacological studies of the neuromuscular junction and other synapses were helpful in the later elucidation of the physiology of such

synapses. It is really only within the last fifty years that the mechanism of the propagation of a nerve impulse and its effect on the muscle or the effector organ has been at least partly clarified. Early investigations were preoccupied with whether transmission at peripheral synapses was electrical or chemical. Lapique (1926) reviewed the electrical theory and in fact the argument was continued as late as the 1940's (Hodgkin, 1937; Rosenblueth, Lissak and Lanari 1939; Eccles, 1945; Kuffler, 1948). Rosenblueth in 1950 reviewed the two contending theories and concluded that the evidence favoured chemical transmission from the nerve ending to the receptive tissue on the effector organ.

A brief summary of the work which led to the identification of the transmitting chemicals is presented at this point. The study of chemoreceptor function at motor end-plates began in 1904 when Elliott (1905), being aware of the work of Lewandowsky (1899) and Langley (1901) on the action of adrenal extracts, noted the similarity between their results and the effects of sympathetic nerve stimulation. Elliott (1905) was the first to propose a chemical basis for transmission of a nerve impulse. Elliott (1905, 1907) also noted the "biochemical similarity" between ganglion cells and "nerve endings" and argued in favour of regarding striated muscle motor nerve endings as homologous with autonomic ganglion cells not only on morphological grounds but also because of their common pharmacological sensitivity to curare and nicotine.

Dixon (1906, 1907) proposed that parasympathetic nerve endings might also release active substances and in fact was able to show that extracts from dog hearts excised during vagal stimulation

were capable of inhibiting isolated frog heart preparations. These experiments of Dixon are strikingly similar to those done later by Loewi (1921).

Complementary to these observations was the pharmacological investigation of choline and its esters (Hunt, 1901). In 1906 Hunt and Taveau discovered an acetic ester of choline, acetylcholine, which displayed 100,000 times the depressor activity of choline. Dale (1914) noted the similarity between the actions of acetylcholine and normally occurring parasympathetic effects and also distinguished between the nicotine-like and muscarine-like activity of acetylcholine, a classification which is still used today.

Loewi (1921), in his famous frog heart experiment, showed that the perfusate from a vagally stimulated frog heart slowed a second frog heart,thus demonstrating conclusively that there was indeed a chemical transmitter which he called "Vagusstoff" . In 1922 Loewi compared "Vagusstoff" with acetylcholine, and found that the solution containing "Vagusstoff" contained choline, but that choline itself was not very active and therefore was not the "Vagusstoff" .

In a somewhat similar fashion to Loewi's experiments with the vagally stimulated frog heart, Hess (1923) perfused the voluntary muscles of frogs and noted that, when the nerve supply was stimulated, an acetylcholine-like substance appeared in the perfusate.

Loewi and Navratil (1926a) compared the rate of destruction of acetylcholine and "Vagusstoff" and later in the same year (1926b) showed that the alkaloid eserine inhibited the destruction of both.

This chapter was completed in 1929 when Dale and Dudley successfully isolated acetylcholine from ox spleens, establishing it

to be a naturally occurring substance. They concluded that the "Vagusstoff" described by Loewi was acetylcholine and that acetylcholine was indeed the parasympathetic chemical transmitter.

Englehart and Loewi (1930) and Matthes (1930) showed the action of eserine was to inhibit the enzyme destroying acetylcholine and that it was effective in concentrations as dilute as 10^{-5} to 10^{-6} Molar. Kibjakow (1933) artifically perfused the superior cervical ganglion of the cat and demonstrated that, on electrical preganglionic stimulation, a substance appeared in the venous effluent which, on re-injection, acted as a stimulus to the ganglion cells. Chang and Gaddum (1933) observed acetylcholine-like activity in extracts from sympathetic ganglia and suggested that this substance was acetylcholine. Feldberg and Gaddum later in 1933 using Kibjakow's perfusion technique added eserine to the Locke's solution and showed beyond doubt that the active substance appearing in the venous fluid during preganglionic nerve stimulation was acetylcholine. Further conclusive evidence of the role of acetylcholine at autonomic ganglia was provided by Feldberg and Gaddum (1934) and Feldberg and Vartiainen (1935).

The role of acetylcholine at the skeletal neuromuscular junction was studied by Dale (1934). With the appreciation of the action of eserine on acetylcholine destruction, Dale removed the superior cervical ganglion of the cat and allowed the autonomic nerves to the tongue to degenerate. When he then perfused the tongue with Locke's solution, containing eserine, and stimulated the motor nerves acetylcholine appeared in the perfusate. Brown and Feldberg (1936) pioneered the investigation of the role of acetylcholine metabolism

at synapses, while Dale, Feldberg,and Vogt (1936) cut the motor nerves to various skeletal muscles of the cat and found that denervated muscles did not release acetylcholine into the perfusion fluid on direct stimulation, whereas innervated muscles did. Hence, it was shown that acetylcholine found in the perfusate after nerve stimulation was released from the nerve endings and gave added support to the now accepted theory that transmission across these neuromuscular synapses was mediated by acetylcholine.

The kinetics of acetylcholine liberation with and without nerve stimulation were studied by numerous workers including MacIntosh (1938) and Lorente de No (1938). Perry (1953), in a further analysis of acetylcholine release under different experimental conditions noted that acetylcholine liberation decreased exponentially with stimulation rate and also that in an eserinized ganglion, the liberation of acetylcholine is decreased. Shelley (1956), using guinea pigs also studied rates of acetylcholine synthesis in brain and found inhibition of synthesis with eserine and neostigmine, this inhibition being overcome by high concentrations of choline. Such results, Shelley suggested,are compatible with the widely held hypothesis that the amount of acetylcholine liberated from a ganglion by a single preganglionic volley is a constant fraction of the available acetylcholine and that the rate of synthesis (4 mμg./min.) of available acetylcholine is a constant, independent of the rate of stimulation. Shelley (1956) further concluded that the presence of tertiary and quaternary methylated nitrogen groups enable the anticholinesterases to replace choline at the active centre of choline acetylase and that high concentrations of choline could overcome this receptor

replacement. Birks and MacIntosh (1961) and MacIntosh (1963) demonstrated in ganglion perfusion experiments that acetylcholine synthesis was decreased with increased concentrations of anti-cholinesterase (e.g. eserine). Conceivably anticholinesterases might simply decrease the availability of choline for re-uptake rather than act upon choline acetylase or even the more obvious choline transferase mechanisms (MacIntosh, Birks, and Sastry 1956).

B. Postsynaptic Mechanisms of Drug Action

Burns and Paton (1951) concluded that d-tubocurarine caused a neuromuscular block by competitively impeding the access of acetylcholine to the end-plate, i.e. by causing a net increase of the threshold of the end-plate to acetylcholine. Such a hypothesis is compatible with that of Paton and Zaimis (1949) who noted that d-tubocurarine-blockade was reversed by eserine and proposed that eserine results in an increase in acetylcholine concentration which competitively displaces d-tubocurarine from its sites of action, or at least enables acetylcholine to reach sites of action which are not "saturated" (occupied) by the antagonist. Hence it has become generally accepted that the block of transmission caused by d-tubocurarine at the neuromuscular site is by a "mechanical" barrier erected by the "bulky" d-tubocurarine molecule to the effective access of acetylcholine to the end-plate: Paton and Zaimis (1949), Bovet (1951) and Paton (1953).

In 1948 Barlow and Ing introduced a new compound, decamethonium, which was shown to be a neuromuscular blocking drug by Paton and Zaimis (1948, 1949). Burns and Paton (1951) and Hunter and Pascoe (1951) noted that decamethonium caused a neuromuscular block, not by acetylcholine-competition, but rather by acting as a cholinomimetic and causing persistent depolarization of the end-plate. To substantiate this hypothesis, Hutter and Pascoe (1951) also noted that decamethonium antagonized the effect of d-tubocurarine on the cat tibialis anterior. Burns and Paton (1951) noted that successive doses of decamethonium produced successively less depolarization in the cat gracilis muscle and in this respect Zaimis (1953) studied the effect

of decamethonium on several species (cat, dog, rabbit, bird, man) . She found that the response of the drug on some muscles produced the expected depolarization features, but was followed by another phase which showed characteristics of d-tubocurarine-induced neuromuscular blockade.

Appreciation that a "block" could exhibit at one time a depolarizing type of action to be followed later by a non-depolarizing action was first made by Straub as early as 1907. He showed , upon perfusing Aplysia heart with muscarine that an initial depolarization of muscle occurred, followed by repolarization, but that the muscle remained insensitive to further stimuli while muscarine was still present.

Brown, Dale, and Feldberg (1936) and Bacq and Brown (1937) demonstrated the ability of nicotine and acetylcholine in high concentrations to cause a neuromuscular block, and Buchthal, Lindhard (1937), and Kuffler (1943) showed these agents acted by depolarizing the end-plates of skeletal muscle.

Subsequent studies demonstrated that such substances, along with, for example, decamethonium were capable of causing a "dual-block" (Jenden, Kamijo and Taylor 1951, Zaimis 1953, and Churchill-Davidson and Wise 1963), the early depolarizing phase passing into a non-depolarizing, apparently tubocurarine-like,phase.

The situation has been somewhat clarified by work on single muscle fibres, involving microelectrode measurements of the events occurring at the muscle end-plates after exposure to such drugs , showing the temporal relationship between neuromuscular block and membrane depolarization. These techniques enabled Thesleff (1955) to

study this second phase of block in greater detail using acetylcholine, nicotine, decamethonium, and succinylcholine, and he found that neuromuscular block was accompanied initially by depolarization which subsided spontaneously without the block being alleviated. Indeed, maximal neuromuscular block did not occur during the depolarization phase, but when the membrane potential had returned to normal. At this time, too, even though repolarization could be shown to have occurred, the end-plates were insensitive to the depolarizing action of acetylcholine (i.e. the end-plates were, in fact, "desensitized" to the normal action of acetylcholine). This period of desensitization of the end-plate appears related to the duration of exposure of the end-plate to the "desensitizing" type drug (Katz and Thesleff, 1957, and Sabawala and Dillon, 1959).

Foldes, Wnuck, Hodges, and Thesleff (1957), and Axelsson and Thesleff (1958) proposed that the desensitization which occurs is due to a reversible change of the receptor molecule from an "effective" to a "refractory" state. This change was noted to occur earlier or later during exposure depending upon the species studied. Others believe it is not the duration of exposure but rather the concentration of drug at the membrane which is significant in the desensitization phenomenon (Katz, Wolf, and Papper, 1965). Furthermore, increasing the dose results in an apparent change in the character of the block from depolarizing to non-depolarizing (Katz, Wolf, and Papper, 1963).

The elucidation of this phenomenon of mixed block or "desensitizing" block has been the subject of much clinical discussion as well, with the same observations seen in man as with other animals (Payne, Holmdahl, 1959). Here too, the apparent change in character

of block from a depolarizing type to one with the features of a competitive block, reversible with neostigmine, was observed (Brennan, 1956).

Paton (1956) has stressed, in evaluating the mode of action of neuromuscular agents, that the tissue blood flow and ionic environment especially with respect to sodium and potassium at the membrane is significant. More recently, the ability to recognize the development of dual block or mixed block has received attention. Churchill-Davidson and Christie (1959) and Churchill-Davidson , Christie and Wise (1960) using electromyography studied the changes in the hand muscles of man to stimuli applied to the ulnar nerve following administration of a depolarizing drug (succinylcholine and decamethonium). They measured the ability to sustain both twitch and tetanic rates of stimulation, the effect of anticholinesterases on neuromuscular block, and the presence or absence of post-tetanic facilitation. They too demonstrated that the total dose is the most significant factor in producing change in the usual depolarizing block characteristics (twitch and tetanus sustained, no post-tetanic facilitation, potentiation of block by anticholinesterase) to non-depolarizing block characteristics (twitch and tetanus not sustained, post-tetanic facilitation, block antagonized by anticholinesterase). Convenient nerve stimulators have been developed and described by Churchill-Davidson (1963, 1965) for elucidation of the type of block present in the clinical situation.

C. Presynaptic Mechanisms of Drug Action

In the past the study of neuromuscular synapse blockade has been mainly concerned with activity of drugs at the end-plate receptors, for example, the postsynaptic site. More recently, however, studies have been concerned with possible synaptic blockade resulting from an action at the site of acetylcholine synthesis, storage and release, for example, the presynaptic site.

In 1955 Schueler described a series of bisquaternary compounds, the hemicholiniums, which are a group of synthetic choline derivatives whose most striking pharmacological characteristic is their ability to cause respiratory failure. This initial study of the hemicholiniums by Schueler provided a new stimulus for the continued investigation of synaptic transmission.

MacIntosh, Birks, and Sastry (1956), Reitzel and Long (1959), and Wilson and Long (1959) demonstrated that hemicholinium (analogue HC3) is capable of producing transmission failure at various peripheral sites of the nervous system where acetylcholine is the transmitter. Schueler (1955), Giovinco (1957) and Reitzel and Long (1959) noted that choline protected animals from fatal doses of hemicholinium. Reitzel and Long (1959) demonstrated that hemicholinium-induced neuromuscular transmission failure was reversed by choline. The specificity of the choline molecule as an antagonist to hemicholinium has been studied by Giovinco (1957), and Reitzel and Long (1959).

MacIntosh, Birks and Sastry (1956) studied the activity of compound number 3 of Schueler's compounds (HC3) and showed that it inhibited acetylcholine synthesis of minced mouse brain prepared by

the method of Mann, Tennenbaum and Quastel (1939). This action of HC3 was antagonized by choline. MacIntosh et al (1956) noted that the action of HC3 on the acetylcholine synthesis of an acetone powder from rat brain was "so weak as to seem unrelated to its pharmacological activity" . As an alternative theory to an action on choline acetylase they thus suggested that HC3 and other hemicholiniums may compete with choline for transport by a specific carrier system to intraneuronal sites of acetylation. These findings were corroborated by other biochemical studies [e.g. Hebb and Smallman (1956), Gardiner (1957, 1961)] which gave further support to the hypothesis that HC3 acts on systems transporting choline into the cell.

Another substance recently shown to produce neuromuscular blockade associated with a presynaptic site of action is the triethyl analogue of choline, triethylcholine (Bowman and Rand 1961a, 1961b , Bowman and Rand 1962, Bowman, Hemsworth and Rand 1962a, 1962b). Triethylcholine and hemicholinium possess properties in common; both are more effective during high rates of nerve stimulation and both are antagonized by choline, suggesting a similar mechanism of action to hemicholinium on the synthesizing mechanism and in particular the choline transport process (Bowman and Rand 1961b, Bowman, Hemsworth and Rand 1962a).

When compared under identical conditions on the cat tibialis preparation, hemicholinium was shown to be 20 times as active as triethylcholine on a weight basis (Bowman and Rand 1961b). However, the dose of choline required to reverse the effects was larger in the case of hemicholinium.

Triethylcholine has been shown (Roberts 1962) to enhance

acetylcholine release from frog motor terminals, an action which might account for the observed slight initial increase in twitch tension [Bowman, Hemsworth and Rand (1962a) and Bowman and Hemsworth (1965)]. These authors, however, do not believe that the rate-dependent blockade produced by triethylcholine is a result of a decreased quantal output of acetylcholine resulting from the initial excessive release of transmitter. Such a stimulation of acetylcholine release from the nerve endings has been reported by Koketsu (1958), Stovner (1958), and Collier and Exley (1963) for tetraethylammonium; but whereas this action is much more marked with tetraethylammonium than with triethylcholine the reverse is true for the development of rate-dependent blockade (Bowman, Hemsworth and Rand 1962a).

In addition to tetraethylammonium and triethylcholine, other agents and procedures are known to affect acetylcholine release. Calcium enhances acetylcholine release (Harvey and MacIntosh 1940; Castillo and Stark, 1952; Kuffler 1952, and Castillo and Engbaek 1954), an effect which is antagonized by magnesium, which by itself decreases acetylcholine release (Castillo and Engbaek, 1954). Calcium and magnesium, however, both depress the muscle membrane (Jenkinson 1957). Simple procedures such as muscle stretching are also known to enhance acetylcholine release (Ralston and Libet 1953, and Hutter and Trautwein 1956). There is a great deal of interest at present in the possible presynaptic effects of a large number of facilitatory or anticurare agents. It is not possible in this review to deal adequately with the controversy surrounding these studies and reference instead is made to the studies and reviews of the following : -- Riker (1953), Riker, Roberts, Standaert and Fujimori (1957), Riker, Werner, Roberts and

Kuperman (1959), Krnjevic and Mitchell (1961), Werner and Kuperman (1963), Standaert and Adams (1965), and Paton and Waud (1967).

One difference between HC3 and triethylcholine is that the latter molecule is small compared to that of the bisquaternary hemicholinium compound. With this in mind Bowman and Rand (1961b) suggested that it is possible that, in addition to preventing the combination of choline with the carrier mechanism, triethylcholine may itself be transported into intracellular sites in place of choline. In vitro studies with brain tissue had previously shown (Burgen, Burke, and Desbarats-Schonbaum 1956) that triethylcholine could be acetylated to give acetyltriethylcholine which if released as "a false transmitter" instead of acetylcholine would be 0.0002 times as active as acetylcholine as an agonist (Holton and Ing 1949), and would thus be ineffective in causing muscle contraction (Bowman and Rand 1961b).

Added to the evidence of an action on the peripheral presynaptic nerve terminals there is some evidence that hemicholinium can also exert a post synaptic depression of end-plate sensitivity (Martin and Orkand 1961, Thies and Brooks 1961). This is a feature of most quaternary ammonium compounds including high concentrations of tetraethylammonium (Koketsu 1958 and Stovner 1958). Triethylcholine, however, is devoid of any depolarizing action on the end-plate or significant anticholinesterase activity (which would contribute to the observed facilitation of synaptic transmission) and the depressant action on the motor end-plate according to Bowman et al (1962b) is weak.

Instead of the more sophisticated, though usually in vitro, electrophysiological methods of demonstrating that a compound possesses

presynaptic activity most workers have employed more indirect means. It had previously been recognized (Preston and van Maanen 1953 , Wislicki 1958)that with d-tubocurarine there was a greater degree of neuromuscular block at fast than at slow rates of nerve stimulation. Blackman (1963), assuming that this drug probably has little or no effect on acetylcholine release, suggested that compounds producing a frequency dependent blockade of a greater magnitude than does d-tubocurarine might be suspected of having a presynaptic inhibitory component contributing to blockade.

Borison (1961), noting that whereas the neuromuscular actions of hemicholinium are undoubtedly important, quotes experiments in which respiratory depression had been obtained after administration of hemicholinium to cats but the cough reflex, and response to phrenic nerve stimulation remained intact. In addition he reported a decreased sensitivity to carbon dioxide and a depression of central vasomotor excitability, all of which led him to postulate that hemicholinium appears to have a central as well as a peripheral site of action. Gardiner (1961), however, observed that it would be difficult to attribute the effect of hemicholinium to a central respiratory paralysis because it is debatable whether such a bisquaternary ammonium compound would penetrate the central nervous system sufficiently.

Coincident with the basic studies relating to the mode of synaptic transmission has been a renewed interest into aspects of clinical neuromuscular transmission disorders. Of particular interest in this context is the etiology and pathogenesis of myasthenia gravis - a clinical entity resulting in voluntary muscle weakness, whose symptoms may be ameliorated by anticholinesterase drugs (Walker 1935), which serves

both as the basis for diagnosis and management of the disease (Grob 1958). Additional light is being shed on myasthenia, which appears to be due to a neuromuscular junction abnormality, although there is evidence that it may be a manifestation of an autoimmune phenomenon (Simpson 1960; Strauss, Seegal, Hsu, Burkholder, Nastuk and Osserman (1960).

Dahlback, et al (1961) studied intercostal muscle obtained by biopsy and found the response to applied acetylcholine to be that of normal muscle, as was the resting membrane potential, and amplitude and time courses of the miniature end-plate potentials. However, the frequency of these potentials was lower than normal and was not increased by potassium, indicating a presynaptic abnormality. Others (e.g. Churchill-Davidson and Richardson 1953) have shown that post-synaptic defects may exist in myasthenia gravis. There are also hypotheses (Stricker, Tholen, Massigni and Staub 1960) that the defect is caused by a substance circulating in the blood and this has been investigated by many (Wilson, Obrist and Wilson, 1953; Wilson and Wilson 1955; Nastuk and Strauss 1961; Nowell and Wilson 1961). Bowman , Hemsworth and Rand (1962) have drawn attention to the fact that if a circulating agent does exist, it might well exhibit properties like triethylcholine and there is evidence that an ethonium compound is normally present in nervous tissue. Calvey, Nowell and Wilson (unpublished data, quoted from Bowman and Rand 1962) found by chromatographic studies a substance with an Rf value similar to the ethylcholines present in the thymus gland of myasthenic patients and foetal whales. Bowman, Hemsworth and Rand (1962b) also showed that in triethylcholine-induced neuromuscular transmission failure that although

an improvement in muscular strength following anticholinesterase (neostigmine and edrophonium) was obtained, that choline itself was more effective. It has therefore been postulated by Bowman, Hemsworth and Rand (1962b) that prolonged treatment of myasthenics with choline might be beneficial. It should be noted, however, that the clinical treatment of myasthenia with choline was not found to be as effective in ameliorating the symptoms as was anticholinesterase therapy (Bowman and Rand 1962).

From the clinical viewpoint again Bowman and Rand (1961a) suggest that drugs such as triethylcholine (TEC) whose action is at least partly to retard the synthesis of transmitter substance might be used effectively to treat the spasms of tetanus. The same authors, (Bowman and Rand 1962b) in an analysis of seventeen choline analogues to determine the structural requirements for presynaptic blocking activity at the neuromuscular junction, concluded that compounds with ethyl groups (C_2H_5) attached to the quaternary nitrogen possessed optimal presynaptic activity, and suggested that myasthenia gravis could at least in part be attributable to a substance with this type of action.

Finally, Beaulnes, Bois and Carle (1966), studied the effects of curarizing drugs, anticholinesterases, and musculotropic substances on the dystrophic mouse phrenic nerve-diaphragm preparation. They showed an increased resistance to the neuromuscular blocking activity of d-tubocurarine and of gallamine and a corresponding increased sensitivity to the initial stimulant (depolarizing) effect of succinyldicholine. Such responses are opposite to those encountered in myasthenia gravis (e.g. Grob 1958). Beaulnes *et al* proposed two

hypotheses to explain these changes in sensitivity in dystrophic muscle. One, an increased production of an acetylcholine-like material (i.e. presynaptic factors), and two, an increase in the number of acetylcholine-sensitive sites on the cell membrane (postsynaptic factors) , which was initially postulated by Baker, Wilson, Oldendorf, and Bland (1960). Axelsson and Thesleff (1959) had previously noted that, following denervation of skeletal muscle, the entire surface of such a muscle cell assumed the same acetylcholine sensitivity as the normal muscle end-plate region. Such an increase in the acetylcholine sensitive area would be compatible with the increased sensitivity of dystrophic muscle to acetylcholine, and with the increased resistance shown by such muscle to curare-like agents. Beaulnes et al (1966) emphasize, however, that denervated muscle exhibits a lesser responsiveness to anticholinesterases, since cholinesterase activity is reduced, whereas in dystrophic muscle there is an enhanced effect to anticholinesterases , indicating a possible increased production of acetylcholine or acetylcholine-like material.

D. Structure-Activity Relationships

1. General Chemical Relationships

(a) Esters of Choline

The revelation that acetylcholine is the chemical transmitter at peripheral synapses has stimulated investigation into not only what chemical features were responsible for such activity but also the nature and location of the receptor sites which are acted upon by the transmitter or mimetic molecules (New York Acad. Sc. -- Symposium-Ehrenpreis 1967; Koelle, Douglas, and Carlsson, 1965; Triggle, 1965).

The relative activities of esters of choline to the acetic ester, acetylcholine, have been examined by several groups of investigators (Chang and Gaddum,1933; Willey,1955; Keyl, Michaelson, and Whittaker, 1957; Erspamer and Glasser, 1958; Holmstedt and Whittaker, 1958; Wurzel, 1959, 1960). In general, (Barlow, 1964, page 103), many of the aliphatic esters of choline are more active than choline itself, but the more bulky aromatic esters are generally less active than choline in producing stimulation.

Holmstedt and Whittaker (1958) noted that the agonist activity of such esters of choline (ability to cause a contracture of skeletal muscle) was not necessarily indicative of a parallel degree of antagonist activity, and suggested that this might be in part due to different susceptibilities to hydrolysis by cholinesterases. In particular, the duration of the neuromuscular blocking effect depends on the stability of the ester at the site of action and upon their stability when exposed to cholinesterases in plasma (Barlow, 1964 , page 106). Holmstedt and Whittaker (1958) found that the activity of

their compounds was potentiated by eserine.

(b) Alteration of the Choline Moiety

If the choline part of acetylcholine is altered it has been generally found that this results in a lessened degree of activity. For example, Simonart (1932) found that (±) acetyl-α-methylcholine was one half as active as acetylcholine and (±) acetyl-β-methylcholine was a hundredth to a hundred and eightieth as active (Simonart, 1932; Wurzel, 1959). An increase or decrease in the number of methylene groups in the acetylcholine molecule diminishes activity on all preparations (Holland, Klein, and Briggs, 1964).

(c) Alteration of the Carbon Chain Length

The effects of altering the carbon chain length of other simple onium compounds has also been of interest. The simple onium salts were investigated by Boehm (1908) and Kulz (1923) who studied the qualitative effects of alkyltrimethylammonium salts. In such a series the agonist (acetylcholine-like) activity is greatest in the butyl and pentyl (four and five carbon chain) homologues and it has subsequently been shown (Ariens, 1954; Stephenson, 1956) that such compounds with longer carbon chains demonstrate a transition from agonist to partial agonist to antagonist activity. Raventos (1937) made the initial quantitative comparisons of these alkyltrimethylammoniums on the frog rectus abdominis and dorsal muscle of the leech. When compared with acetylcholine on an equipotent molar basis, the experiments of Clark and Raventos (1937) showed that methyltrimethylammonium and tetramethylammonium were only a hundredth as active as acetylcholine and indeed the four and five

carbon chain compounds were a twenty-fifth to a hundredth as active on these preparations (Raventos, 1937; Ariens, Simonis, and de Groot, 1954). Philipott and Schlag (1956) have further confirmed that maximal activity in this series is found with the pentyltrimethylammonium. In these experiments they measured the effects of the drugs on the injury potential of the cat gracilis muscle as an indication of the ability to depolarize the neuromuscular junction.

Although the effects of altering the chain length in the choline part of acetylcholine have been studied at ganglia (Chang and Gaddum, 1933; Hey, 1952; Willey, 1955; Erspamer and Glasser, 1958; Holmstedt and Whittaker, 1958; Wurzel, 1959; Sekul and Holland, 1961) and post-ganglionic parasympathetic synapses (Hunt and Taveau, 1911; Hunt and Renshaw, 1925, 1934) there has been to date, little information about how such changes affect activity at the neuromuscular junction (Barlow, 1964).

Ing, Kordik, and Tudor Williams (1952) and Willey (1955) noted the effect of chain-lengthening on the ability of bis-onium compounds to cause a contracture of frog rectus. Maximal activity was obtained with the 12 carbon chain separating the two onium groups but even here the maximal activity was a fifth of that of acetylcholine , and the ten carbon compound, decamethonium, was 0.135 times as active as acetylcholine (Barlow, Blaschko, Himms, and Trendelenburg, 1955 ; Barlow and Zoller, 1962, 1964).

Other workers, for example Jewell and Zaimis (1954), have studied the effect of chain-lengthening on the type of blockade produced by these bis-onium compounds. They demonstrated, on increasing the carbon chain beyond ten, a decrease in acetylcholine-like blocking

activity and an increase in curare-like activity. Such a change in type of apparent blocking activity has given rise to the term "mixed block" (Jewell and Zaimis, 1954).

Barlow (1964) points out that the tridecamethylene homologue of decamethonium is less potent as a blocking agent than decamethonium but more active in causing a contracture. He suggests that lengthening the chain increases ability to block by a mechanism other than desensitization. In the analogous bistriethylammonium salts considerable blocking activity was found with the really long chain hexa-, and hepta-decamethylene bistriethylammonium (Barlow and Zoller, 1964).

In addition to these studies in which a simple carbon chain has been lengthened more complex homologous series have been investigated. Schueler and Hanna (1952), experimenting with substitutions within the decamethonium chain showed that replacement of one pair of methyl groups in the decamethonium chain by an amino group led to a decline in depolarizing type activity. The question of the effect of replacement of part of the polymethylene chain by a saturated ring has been investigated by Randall (1952). He found that such substitution results in conservation of activity better than by unsaturated ring substitution. Wien and Mason (1953) substituted a benzene ring in the chain and found synaptic blocking activity to be maximal with the six carbon chain compound, p-phenylhexamethylene bistrimethylammonium. This compound was about as active as d-tubocurarine on the cat gastrocnemius preparation, and was found to be capable of causing a contracture of the frog rectus. The blocking action was unaffected by eserine. The analogous ethyl-substituted ammonium compound (i.e. p-phenylhexamethylenebistriethylammonium) also revealed neuromuscular

blocking activity (Wien and Mason, 1953) but did not cause contracture of the frog rectus, and was reversed by eserine.

Levis, Preat and Dauby (1953) in studying the effects of substitution of an ether oxygen in place of a methylene group in decamethonium found that the duration of block was not only shorter-lasting but that there was diminution in blocking activity the greater the number of ether oxygen substitutions in the chain. The depolarizing activity (contracture of the frog rectus), however, was retained.

Substitution of an ether oxygen linkage for a sulphur atom or two sulphur linkages resulted in a similar activity to that of decamethonium (Andrews, Bergel and Morrison, 1953; Hunter 1953) , although compounds with more than one ether linkage were less active (Levis, Preat and Dauby 1953). The substitution of a sulphur for the ether oxygen atom in acetylcholine yields a substance, acetylthiocholine which is less active than acetylcholine (Wurzel 1959).

Replacement of part of a decamethylene chain by an ester group did not result in diminished activity (Bovet, Bovet-Nitti, Guarino, Longo and Fusco 1951). Indeed, the introduction into the chain of more than one ester group imparted greater blocking activity. An example of this type of compound is the widely used clinical neuromuscular blocking drug, succinylcholine (Bovet, Bovet-Nitti, Guarino, Longo and Marotta 1949; Phillips 1949; and Walker 1950), which was first described by Hunt and Taveau (1906), although its neuromuscular blocking activity was unrecognized at the time. Clinically, as in the frog, succinylcholine is more active than decamethonium. It is , however, less active than the eleven and twelve carbon chain bistrimethylammonium compounds whose activity on the frog rectus is greater

than decamethonium itself (Bovet et al, 1949).

Esters of choline with polymethylene biscarbamic acids have also been studied by Cheymol, Delaby, Chabrier, Najer, and Bourillet (1954) and Klupp, Kraupp, Stormann, and Stumpf (1953). They concluded that in this series acetylcholine-like activity was associated with a much longer chain length between the onium groups and that neuromuscular blocking activity reaches a maximum in heptamethylene, while acetylcholine-like activity (on frog rectus) reaches a maximum in the decamethylene biscarbamyl ester.

(d) Alteration of the Onium Group

i. General Considerations

Reviews of the importance of the trimethylammonium cationic head in determining synaptic activity have recently been presented by Barlow (1964) and Rubinstein (1966). The replacement of the methyl groups in acetylcholine, either by hydrogen or by ethyl groups greatly decreases the stimulant activity on the frog rectus. In fact, acetyltriethylcholine was 0.0002 times as active as acetylcholine. In addition, the corresponding sulphonium, phosphonium, and arsonium analogues are less active than acetylcholine.

The finding that substitution of ethyl for methyl groups on the quaternary nitrogen results in a decrease in depolarizing activity and an increase in curare-like blocking activity has been emphasized by Ariens, Simonis and de Groot (1954) and Thesleff (1955).

Ginzel, Klupp, Kraupp, and Werner (1953) found that phosphonium and sulphonium analogues of decamethonium have less depolarizing activity and were capable of antagonizing acetylcholine.

The replacement of a quaternary ammonium group in decamethonium by a primary amino group likewise decreased synaptic activity (Barlow, Blaschko, Himms, and Trendelenburg, 1955). From such a survey, Barlow (1964) points out that it appears that an increase in the size of the onium group, as in the phosphonium or sulphonium analogues, destroys acetylcholine-like activity, whereas replacement of one quaternary ammonium by a primary amino group merely reduces it to something comparable with a mono-onium salt. Thus, with such long chain bis-onium compounds as decamethonium, it is difficult to alter the chemical structure without significantly reducing its acetylcholine-like properties. Substitution in the onium groups in the analogous di-ester series, of which succinylcholine is a member, results in similar effects. The replacement of one methyl group by an ethyl radical results in slightly decreased agonist activity while substitution of more than one ethyl group results in a marked decrease in activity (Ariens and van Rossum, 1957).

The effect of the serial replacement of the methyl groups in the onium head by ethyl groups has also been studied by Barlow, Scott, and Stephenson (1963). These authors, realizing that the acetylcholine-like activity of a chemical compound depends not only upon the ability of a compound to become attached to the acetylcholine receptor but also upon its ability to activate them thereafter, attempted to study the effects of chemical structure on the affinity and the efficacy of compounds related to acetylcholine, as defined by Stephenson (1956). They studied two series of compounds in which the methyl groups in the onium head were serially replaced by ethyl groups. One series of such compounds contained an acetylcholine-like chain terminating in a

methyl group. The former compounds were all antagonists of acetylcholine, whereas most of the latter compounds mimicked acetylcholine (agonists). They determined the affinity constants of the antagonists and found that the variation in the affinity constants with the constitution of the onium group was sufficiently consistent from one series to another to suggest that corresponding changes in affinity with constitution of the onium group would occur in the agonist compounds. With the knowledge of the relative activity of the agonists and the extrapolation from the relative affinity of the antagonists, it was possible to assess the effects of structure on efficacy, at least on the acetylcholine receptors of the guinea-pig ileum. They concluded that substitution of one methyl in the onium group by an ethyl group in such compounds, increased affinity but decreased efficacy. The replacement of a second methyl by a second ethyl group, had little effedt on affinity but decreased efficacy still further. The replacement of the last methyl group by an ethyl group, however, often resulted in a moderate to marked decrease in affinity conceivably due, in some instances, to the steric interference between the third ethyl group and some constituent in the acetylcholine-like chain such as the close proximity of a carbonyl group.

ii. Specific Effects of Triethylation

Bowman and Rand (1962) studied 17 choline analogues in order to determine the structural requirements for presynaptic blocking activity at the neuromuscular junction. They concluded that compounds with ethyl groups attached to the quaternary nitrogen possessed optimal presynaptic activity. Moreover, these authors concluded from this

study that compounds with small groups on the quaternary nitrogen produced a depolarizing post synaptic effect and those with large groups a curare-like effect. Radicals intermediate in size were found to produce a presynaptic effect, the triethyl analogue being optimal in this respect.

2. Structure-Activity Relationships Particularly Relevant to This Study

There is a great deal of speculation in this field of study. The possible relationship between the structure of such synaptically-active compounds is discussed in almost all of the references cited above and a number of recent reviews have been concerned with this literature (Stenlake, 1963; Barlow, 1964; and Triggle, 1965).

Of particular relevance to this work are ideas concerning the effects of chain lengthening, the role of the ether oxygen atom, and the importance of the onium group in phenoxyalkyl- and phenylalkyl-mono-onium salts. The effect of chain lengthening on the activity of phenoxyethyltrimethylammonium (choline phenyl ether) has been studied by Hunt and Renshaw (1929). They found that phenoxypropyltrimethyl-ammonium (homocholine phenyl ether) was less active than the former as a ganglion stimulant. Hamilton (1963a) reported that the propyl compound was inactive as a stimulant on the cat superior cervical ganglion as it caused a ganglion blockade unaccompanied by a contracture of the nictitating membrane. Ginsborg and Hamilton (1963 unpublished results) subsequently found on the isolated frog sympathetic ganglion , using intracellular recording techniques, that phenoxypropyltrimethyl-ammonium, unlike nicotine, blocked the response to preganglionic

stimulation without any evidence of ganglion-depolarization.

In the same series of phenoxyalkyltrimethylammonium salts the effect of chain lengthening on in vitro anticholinesterase activity has been determined (Hamilton, McCurrach, and Hersey, 1964 - unpublished results - see Appendix II). These workers have demonstrated that the anti-acetylcholinesterase activity was markedly decreased as the chain length was increased from ethyl (2 carbon) to propyl and butyl (3 and 4 carbon). Further chain lengthening, however, was found to increase this activity, the decyl (10 carbon) compound being 500 times as active as the two carbon compound.

Thomas and Marlow (1963) and Thomas and Staniforth (1964) have studied the anticholinesterase and anti-acetylcholinesterase activity of aliphatic and aromatic quaternary ammonium compounds. In the phenylalkyltrimethylammonium series, they observed in general an increase in the anticholinesterase activity as the carbon chain length was increased from zero to five. The anti-acetylcholinesterase activity of these compounds decreased as the carbon chain length was increased from zero to three, although it increased somewhat again with the four and five carbon chain compounds. Thomas and Staniforth (1964) discussed these results with reference to the expected forces of attraction between the drug molecules and the enzyme receptor. They suggested that since an increase in the number of methylene groups in the aralkyl group should cause a regular increase in Van der Wäal's forces of attraction with both enzymes, as has been shown to be so with the alkyltrimethylammonium series (Bergmann, 1955) then the differences between the activity on the two esterases must be ascribed to the "coulombic component of the total absorption force" . The most reasonable

assumption they suggest is that the difference is a reflection of variations in the anionic sites in the two enzymes.

The role of the ether oxygen atom in such compounds as the very active nicotine-like substance choline phenyl ether has been the subject of study by a number of authors. A review of the literature led Hey (1949) to put forward a tentative hypothesis that maximum nicotine-like stimulant activity would be found, in ions of the type $ROCH_2CH_2N^+(CH_3)_3$, when the character of the group R is such that maximum mesomeric deviation toward the structure $R^-O^+CH_2CH_2N^+(CH_3)_3$ would be expected. This means that increased nicotine-like activity might be associated with a reduction in the electron density of the ether oxygen atom in such choline ethers and esters. Hey in 1952 provided further support for this hypothesis by a quantitative pharmacological study of twenty-one ring substituted compounds related to choline phenyl ether. This hypothesis has been extended to nicotine itself by Barlow and Hamilton (1962a) who studied a number of analogues of nicotine and provided some evidence that a partial positive charge (electron poor site) at a suitable distance from the cationic head may be necessary for nicotine-like blocking activity. The study of Barlow, Scott, and Stephenson (1963) previously referred to with reference to the effect of serially replacing methyl groups on the cationic head by ethyl groups, at the same time led these authors to conclude that, at least on the postganglionic parasympathetic receptors in the guinea-pig ileum, the acetylcholine-like activity depended upon the 3-ether oxygen atom and the trimethylammonium group for efficacy, and upon the 4-carbonyl group and presumably the onium group for affinity.

III. METHODS

A. Rat Phrenic Nerve-Diaphragm Preparation In Vitro

Healthy rats of either sex weighing between 150 - 250 G. were killed by a blow on the head and exsanguinated immediately by slitting the throat. The thorax was opened along the left sternal margin, exposing the left half of the diaphragm and the phrenic nerve. The left inferior costal margin was then incised to expose the left inferior aspect of the diaphragm. A fan-shaped strip of diaphragm about one to two cm. wide at the costal margin and a two to three cm. portion of the phrenic nerve was then excised. This was placed in a Petri dish containing Krebs' bicarbonate solution, containing KCL 1.76 G., $CaCl_2$ 1.41 G., KH_2PO_4 0.81 G., $MgSO_4$ 0.714 G., NaCl 34.5 G., $NaHCO_3$ 10.3 G., glucose 10 G. in 5 litres distilled-deionized water. The muscle and nerve were also moistened with Krebs' solution during the dissection. A fine silk thread was carefully tied around the tendinous portion of the muscle, which was then carefully mounted on a double hook pushed through the costal margin and suspended in a 50 ml. organ bath containing Krebs' solution at 37^{o}C. gassed with 5% carbon dioxide and 95% oxygen. The free end of the phrenic nerve was then carefully sucked into a fluid bipolar platinum electrode as originally

described by Furshpan and Potter (1959). The thread from the muscle was then carefully attached to a torsion lever for recording on a kymograph drum (Palmer Electric 12).

The nerve was stimulated supramaximally with a Grass S 5 stimulator with shocks of 0.6 - 0.7 msec. duration. The sequence used in all cases for the general pharmacological assay of each agent was: a one minute control tracing, following which the drug was introduced by a pipette into the organ bath and allowed to remain in the bath for three minutes. Only during the final one minute of this period was the recording drum switched on. Following this three minute exposure to the compound (test substance), the bath was thoroughly rinsed several times with gassed Krebs' solution and allowed to re-establish a base-line over the next three minutes, following which the cycle was resumed. The rate of stimulation was 0.2/sec. (12 per minute).

In the first series of experiments, antagonist activity was measured by estimating the molar concentrations of each compound which produced comparable degrees of block. The degree of block was measured as the percentage reduction in the height of contraction of the muscle, measured from its base-line. This activity was expressed as the equipotent molar ratio (EPMR) with reference to the standard reference compound. In each series the reference compound chosen was the one which most closely resembled choline in structure (i.e. phenoxyethyltrimethylammonium, phenoxyethyltrimethylammonium and phenylpropyltrimethylammonium). This indicates the number of molecules of the drug which produced the same percentage blockade of muscle contraction as one molecule of the reference drug. Two dose levels of each drug (reference S_1 and S_2 and unknown U_1 and U_2) were used where the dose

ratio S_2 / S_1 equalled U_2 / U_1 . Wherever possible a four-point assay, using a Latin square design, to reduce experimental bias , was employed and the potency ratio calculated as described in the British Pharmacopoeia, page 904, 1958 for penicillin. The reciprocal of this potency ratio gives the EPMR.

In a second series of experiments on the rat phrenic nerve-diaphragm preparation a method of quantifying possible rate dependence was evolved. The Differential Blocking $Index_{10}$ (DBI_{10}) was thus derived. This index is the percentage of the maximal twitch evoked by stimulation at a rate of 0.2 shocks per second which remains when the twitch due to stimulation at 2.0 shocks per second is 100 percent blocked. Depending on the amount of compound available at this stage of the study, this index was determined in a number of preparations and the mean and standard error of the mean calculated. In this and in all experiments, levels of significance between means (P values) were determined by means of a Student's t-test.

B. Experiments on the Cat Tibialis Anterior Nerve-Muscle Preparation In Vivo

Healthy cats, weighing between 1.6 and 3.0 kg. were anaesthetized with chloralose, 80 mg./kg. intravenously, after having first induced anaesthesia with ether-saturated absorbent cotton placed in an enclosed box. As soon as the animal was rendered unconscious and flaccid, it was removed from the box. The neck was suitably shaved and a plastic catheter placed in one external jugular vein , through which the chloralose solution was administered. While this was proceeding , anaesthesia was maintained by open drop ether placed on a nose cone. A tracheotomy was then performed and the animal artificially ventilated by means of a Harvard respiration pump.

The preparation of the tibialis anterior muscle was carried out basically as described by Brown (1938). The leg was shaved, and the popliteal space opened to identify the sciatic nerve and its bifurcation into anterior and posterior tibial nerves. The sciatic nerve was ligated and sectioned high in the popliteal space to sever its central connections. The anterior tibial nerve was then identified and a bipolar platinum electrode (C.F. Palmer, London) applied for subsequent nerve stimulation. The tibialis anterior tendon was identified and freed from its attachment over the antero-medial aspect of the ankle. The skin incision was then extended over the tibialis to expose part of the belly of the muscle and the transverse ligament of the ankle, which was cut, in order to free the muscle and expose the underlying anterior tibial artery. Large branches to the underlying extensor digitorum longus muscle were ligated. The inferior portion of

the anterior tibial artery having been suitably freed from the muscle, a miniature "bulldog" clip was applied to the proximal portion of this artery and a fine polyethylene cannula inserted and firmly tied in place. This was attached subsequently to a hypodermic needle for injection of the experimental drugs. Great care had to be taken during the dissection and cannulation of this artery in order to avoid trauma to the branches supplying the muscle.

The leg was then attached to a Brown-Schuster myographic stand (C.F. Palmer, London) by the lower end of the tibia and the femoral condyles. The tibialis anterior tendon was then attached to a semi-isometric myograph lever for recording on a smoked kymograph drum, by means of a strong silk thread sutured through the tendon.

The entire preparation was kept warm by means of a 60 watt electric bulb near the suspended leg, and by keeping the animal warm on an electrically heated table. Warmed paraffin oil was periodically dropped ontc the exposed tendon to prevent dessication. The skin was drawn over the popliteal space and ankle area leaving only the electrode and tendon protruding. Injections were made by attaching a tuberculin syringe filled with 0.3 cc. solution. Occlusion of the proximal end of the anterior tibial artery, as originally described by Brown, prior to injection in order to achieve adequate muscle response, was found to be unnecessary in these experiments.

All drugs were administered in exactly 0.3 ml. saline and indirect (nerve) stimulation was by means of a Grass S 5 stimulator. In one series of experiments the standard rate of 12 supramaximal shocks per minute was adhered to throughout the experiment. In a second series of experiments the rate of nerve-stimulation was either manually or

automatically changed at 30 second intervals from 12 to 60 shocks per minute in order to determine any possible presynaptic activity as described above (e.g. Blackman, 1963). It was determined in preliminary experiments that rates of nerve stimulation faster than 60 per minute resulted in premature fatigue of the preparation, unrelated to drug administration. The duration of stimulation was usually 0.7 msecs. in these experiments. In order to obtain an accurate estimation of the DBI_5 (see below) a gradually-developed 100 percent inhibition of the response due to rapid stimulation was obtained by means of a slow injection apparatus (C.F. Palmer, London) in series with the venous cannula. By this method it was possible to obtain a relative steady-state blockade at any desired level.

In a third series of experiments the muscle was alternately stimulated indirectly (nerve) and directly (muscle) for thirty second periods each minute. The electrodes for direct stimulation were the drills fixing the leg to the myograph stand and a hypodermic needle inserted deeply into the muscle. The stimulation parameters used were: supramaximal voltage, 12 shocks per minute and up to 2 milliseconds duration.

In the first series of experiments on the cat tibialis preparation the three homologous series of compounds were tested for their relative ability to block the muscle response to supramaximal nerve stimulation. In each series the compound which most closely resembled choline in structure was used as the standard for comparison. (phenoxyethyltrimethylammonium in the phenoxyalkyltrimethylammonium series, phenoxyethyltriethylammonium in the phenoxyalkyltriethylammonium series, an phenylpropyltrimethylammonium in the phenylalkyl-

trimethylammonium series). The equipotent molar ratio (EPMR) for each drug was determined as described for the rat diaphragm above.

In the second series of experiments on the cat tibialis a method of quantifying possible rate dependence was evolved. The Differential Blocking Index 5 (DBI_5) was thus derived. This Index is the percentage of the maximal twitch evoked by stimulation at a rate of 0.2 shocks per second which remains when the twitch due to stimulation at 1.0 shocks per second has been 100 percent blocked. Depending on the amount of compound available at this stage of the study, this Index was determined in a number of cats and the mean and standard error of the mean calculated.

A third series of experiments was designed to determine the reversibility of block with edrophonium chloride. In these a second slow injection apparatus in series with the contralateral femoral vein was used to infuse a 2-4 mg./cc. solution of edrophonium while maintaining a steady state block at the fast rate of stimulation due to simultaneous infusion of the relaxant drug. The rate of infusion of the blocking drug was very carefully titrated to exactly maintain the 100% block at the fast stimulation rate in order to avoid excess administration of drug.

These experiments involving edrophonium administration were often performed at the end of a DBI_5 determination and the percentage recoveries of the responses to slow (0.2/sec) and fast (1.0/sec) rates of stimulation were measured using the corresponding pre-drug responses as controls (i.e. as 100%).

C. Cat Superior Cervical Ganglion Preparation *In Vivo*

Healthy cats weighing between 2.5 - 4.0 Kg. were anaesthetized with ether followed by intravenous chloralose as described above for the tibialis anterior preparation. Following insertion of a tracheal cannula for controlled respiration, if necessary, the method was essentially similar to that used by Paton and Perry (1953).

A fine silk thread was sewn into the nictitating membrane , attached to an isotonic frontal writing lever (C.F. Palmer, London) with suitable magnification for kymographic recording, and adjusted so that the initial tension was one gram.

The neck tissues overlying the right common carotid artery, the lingual artery and the external carotid artery were dissected and retracted. All branches of the common carotid artery, except those supplying the superior cervical ganglion and all accessible side branches of the external carotid artery were cut between two ligatures, leaving the lingual and external carotid arteries intact. A thread was then passed beneath the latter and light tension was applied to this,making further dissection possible. The carotid artery was then sectioned between two ligatures as distal as possible leaving a stump suitable for insertion of a saline-filled needle cannula. During this cannulation the cut stump of this vessel was occluded proximally by means of a bulldog clamp operated remotely by means of a modified camera shutter-release. Having successfully completed this canmulation, the lingual artery was then ligatured. Alternately, if the external carotid cannulation was unsuccessful, the lingual artery was used as the injection site.

The right vago-sympathetic chain was then exposed below the level of the superior cervical ganglion and the sympathetic nerve was carefully isolated from the vagus. The sympathetic nerve was then crushed centrally and platinum bipolar electrodes applied for preganglionic stimulation with a Grass S 5 stimulator. In most experiments supramaximal stimulation with about four volts at 10 shocks per second, of 0.7 milliseconds duration was applied in order to produce a maximal sustained contracture of the nictitating membrane.

Drugs dissolved in saline were injected by this intra-arterial route in a constant 0.1 ml. volume, and were followed by 0.2 ml. injections of saline given between drug injections to wash out the cannula. These were repeated until no further nictitating membrane-response was observed.

Ganglion blocking activity was estimated by measuring the percentage relaxation of the nictitating membrane during continuous electrical stimulation.

On each preparation one compound was compared with a standard reference compound by means of a four-point assay design, doses being allocated randomly by means of a Latin square. Usually twelve to sixteen doses were used in each experiment and the equipotent molar ratios (EPMR) were determined graphically or by calculation as described for the cat tibialis anterior preparation.

D. Frog Rectus Abdominis Preparation In Vitro

(a) Determination of Equipotent Molar Ratios

Healthy frogs were pithed and the rectus abdominis muscle dissected. The muscle was sectioned longitudinally in the mid-line and one half mounted in a 5 ml. organ bath containing aerated Clark's modified Ringer solution (NaCl 32.5 G., $NaHCO_3$ 1.0 G., glucose 10.0 G., 10% KCl 7.0 ml., 10% $CaCl_2$ 6.0 ml., 5% NaH_2PO_4 1.0 ml. in 5 litres of de-ionized-distilled water). These experiments were conducted at room temperature, as described by Burn (1952). Contractions of the muscle strips were recorded with a Gimbal lever (C.F. Palmer, London) on a smoked kymograph drum. A four minute cycle was employed. The drugs were added by first emptying the bath and then filling it with 5 ml. of a solution of the test substance. The test solution was left in contact with the muscle for 90 seconds and then washed out with the Clark's modified Ringer solution. The muscle was then stretched for 90 seconds with a 2 gram weight suspended from the opposite side of the Gimbal lever fulcrum. Following this the muscle was left to re-establish a base line for one minute prior to the addition of the next dose.

The test substances were all appropriately diluted with Clark's modified Ringer solution from a 0.01 molar stock solution (M/100).

Equipotent molar ratios (EPMR) were determined only for the stimulant activity of the phenoxyalkyltrimethylammonium series, using the compound whose carbon chain length was two as the reference standard (i.e. taking phenoxyethyltrimethylammonium as having an EPMR of 1) .

Two dose levels of the reference standard and of the test compound were administered according to a Latin square design, using a 16 dose assay. The contractions were measured relative to the base line and the EPMR calculated as described for the rat phrenic nerve-diaphragm.

(b) Determination of Intrinsic Activities

The method used to determine the intrinsic activity of the compounds in the phenoxyalkyltrimethylammonium series was similar to that of Ariens (1954). For this group of experiments the rectus abdominis muscle was prepared as above except that a 30 ml. organ bath was used.

On each day both halves of the rectus were used, one to which acetylcholine was administered first and the test compound second, and the other to which the order of administration was reversed, the test drug being given prior to acetylcholine.

After dissection and mounting the muscle was stretched for 30 minutes by application of a 2 gm. weight. At the end of this period the weight was removed and a base line was recorded for 10 minutes.

Starting with a dose of 0.1 ml. of a 0.01 molar solution either acetylcholine or test compound dissolved in Clark's modified Ringer was added by pipette at 5 minute intervals by stepwise addition, each dose being double the previous one. When a maximal isotonic response had been obtained, the bath was then emptied and well washed out with fresh Clark's solution and the muscle was stretched for over an hour until it had relaxed to its previous base line. The procedure was then repeated for the second drug.

The Intrinsic Activity of a test compound was calculated by

determining the mean height of contraction of the two responses due to the test compound and dividing it by the mean height of contraction elicited by acetylcholine on the same frog. Depending upon supply of compound available, each compound was tested upon between one and eight frogs and the mean values of intrinsic activities (α) determined along with their standard error of the mean where applicable.

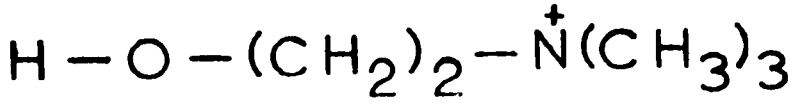
E. Unanaesthetized Chickens

Acetylcholine-like or curare-like neuromuscular activity was determined by the qualitative method described by Buttle and Zaimis (1949). Each compound of the three test series was injected intraperitoneally into a different chick in an amount suitable for the production of paralysis. When the response was at a maximum a photograph was made showing either the curare-like flaccid paralysis or the depolarizing, agonist type of acetylcholine-like response described by Buttle and Zaimis (1949) as "a spastic paralysis in which the legs are rigidly extended and the head thrust back" . Representative photographs were taken of control chickens similarly treated with d-tubocurarine and succinyldicholine.

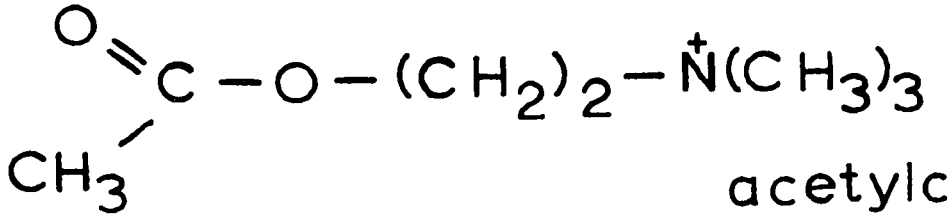
This series of experiments was purely qualitative in nature as, in order to conserve the supplies of test compounds, no attempt was made to compare their threshold doses or potencies.

FIGURE 1

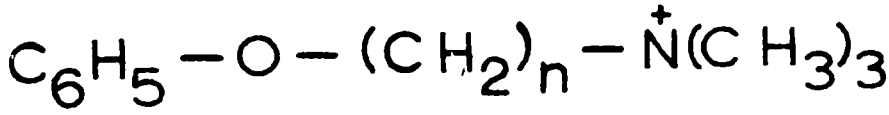
Compounds Studied



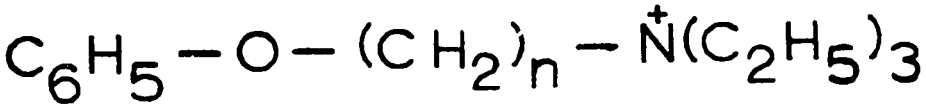
choline



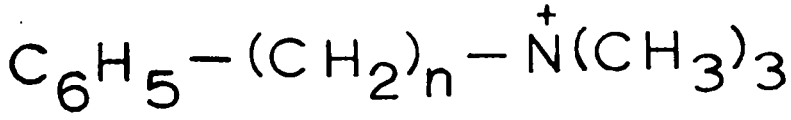
acetylcholine



phenoxyalkyltrimethylammonium
(n= 2-10)



phenoxyalkyltriethylammonium
(n=2-6)



phenylalkyltrimethylammonium
(n=0-5)

IV. MATERIALS

A. Compounds Specially Synthesized

The compounds comprising the three test series (Fig. 1) were all synthesized by R.B. Barlow at Edinburgh University, Department of Pharmacology, whose description follows: "The compounds were prepared from the appropriate phenoxyalkyl or phenylalkyl bromide by reaction with an excess of the tertiary base, dissolved in ethanol. They were crystallized from ethanol and/or ethylmethyl ketone. Sometimes it was necessary to induce crystallization by adding ether. All the compounds were dried *in vacuo* over $CaCl_2$ at a temperature not exceeding $50^{o}C$. The melting points of the phenylalkyltrimethylammonium bromides are in reasonable agreement with those of Thomas and Marlow (1963)."

The analytical results are shown in the accompanying tables in Appendix I.

B. Other Test Compounds Employed

1. Edrophonium chloride injection U.S.P., (Tensilon) Hoffmann-LaRoche, Montreal. Molecular weight 246.15 .
2. Acetylcholine iodide, British Drug Houses, Poole, England. Molecular weight 273.

3. Succinyldicholine dichloride, Burroughs Wellcome, Tuckahoe, New York. Molecular weight 397.34 Lot 4083.

4. Succinylmonocholine iodide, Burroughs Wellcome, Tuckahoe, New York. Molecular weight 331 Lot 32698.

5. Hemicholinium-3, Aldrich Chemical Co. Inc., Milwaukee, Wisc., U.S.A. Molecular weight 574.36 Lot 043051.

6. d-Tubocurarine chloride, Burroughs Wellcome, Tuckahoe, New York. Molecular weight 695.67 Lot 41495.

7. Gallamine triethiodide, (Flaxedil) Hoffmann-LaRoche, Montreal. Molecular weight 891.56 Lot M-5212.

8. Decamethonium bromide, Burroughs Wellcome, Tuckahoe, New York. Molecular weight 418.36 Lot 41091.

C. Other Pharmacological Substances Employed

Diethyl ether (for anaesthesia) , Mallinckrodt Chemical Works, Montreal, Canada. Chloralose, British Drug Houses, Poole, England. Heparin sodium U.S.P., Connaught Medical Research Laboratories, Toronto, Canada.

All test drugs used were dissolved in 0.9% sodium chloride and stored as concentrated stock solutions at 4°C. unless otherwise stated. Appropriate dilutions were made as required with 0.9% sodium chloride.

Chloralose was dissolved in distilled water before each experiment and administered as a 1% solution (10 mg./cc.) at 37°C.

V. RESULTS

The activity of the three series of test compounds relative to the reference standard used in each series has been calculated as the equipotent molar ratio (EPMR). This ratio indicates the number of molecules of one compound which produce the same effect as one molecule of the reference standard. The results expressed as the logarithm (base 10) are plotted on the accompanying graphs.

The quantitative results with each preparation are presented first, and are followed by results of experiments of a more qualitative nature. These included studies of reversibility of blockade by anticholinesterase, the type of paralysis in the chick, and the possible development of a frequency-dependent blockade of transmission, all of which were hoped to indicate the type and/or site of synaptic activity. Comparisons also have been made in these respects with some of the clinically used neuromuscular blocking agents.

A. Rat Phrenic Nerve-Diaphragm Preparation In Vitro

(a) Phenoxyalkyltrimethylammonium Series (Phenoxyalkyl TMA)

The EPMR's of the phenoxyalkyl TMA compounds relative to phenoxyethyl TMA (n=2) are given in Table 1 and Fig. 2 . On this preparation it can be seen that the effect of increasing the carbon-

TABLE 1

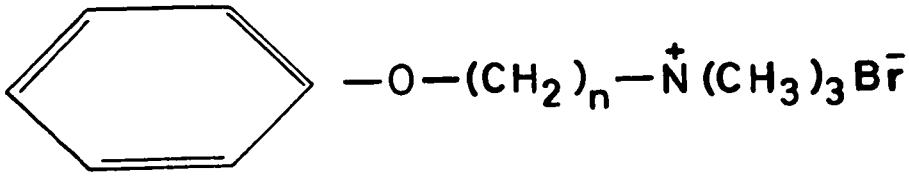
Effect of chain-lengthening on neuromuscular blockade

- Phenoxyalkyltrimethylammoniums
- Cat tibialis and rat diaphragm

TABLE 1

EFFECT OF INCREASING CHAIN LENGTH ON SYNAPTIC BLOCKADE -- NEUROMUSCULAR JUNCTION

PHENOXYALKYLTRIMETHYLAMMONIUM SERIES



EQUIPOTENT MOLAR RATIO/$_{N=2}$

CHAIN LENGTH N =	CAT TIBIALIS ANTERIOR	RAT PHRENIC NERVE DIAPHRAGM
2	1	1
3	18.28 ± 0.14 (4)	5.33 ± 0.203 (3)
4	12.90 ± 4.35 (2)	2.46 ± 0.196 (3)
5	3.19 ± 0.214 (3)	0.65 ± 0.015 (3)
6	9.12 ± 5.82 (2)	0.62 ± 0.054 (3)
7	9.13 ± 0.026 (3)	0.45 ± 0.020 (4)
8	4.22 ± 1.70 (3)	0.55 ± 0.036 (3)
9	3.48 ± 1.19 (3)	0.52 ± 0.007 (3)
10	3.48 ± 0.54 (3)	1.75 ± 0.104 (4)

( ) NUMERALS REPRESENT NUMBER OF ASSAYS

FIGURE 2

Effect of chain-lengthening on neuromuscular blockade*

- Phenoxyalkyltrimethylammoniums

(a) Cat Tibialis

(b) Rat Diaphragm

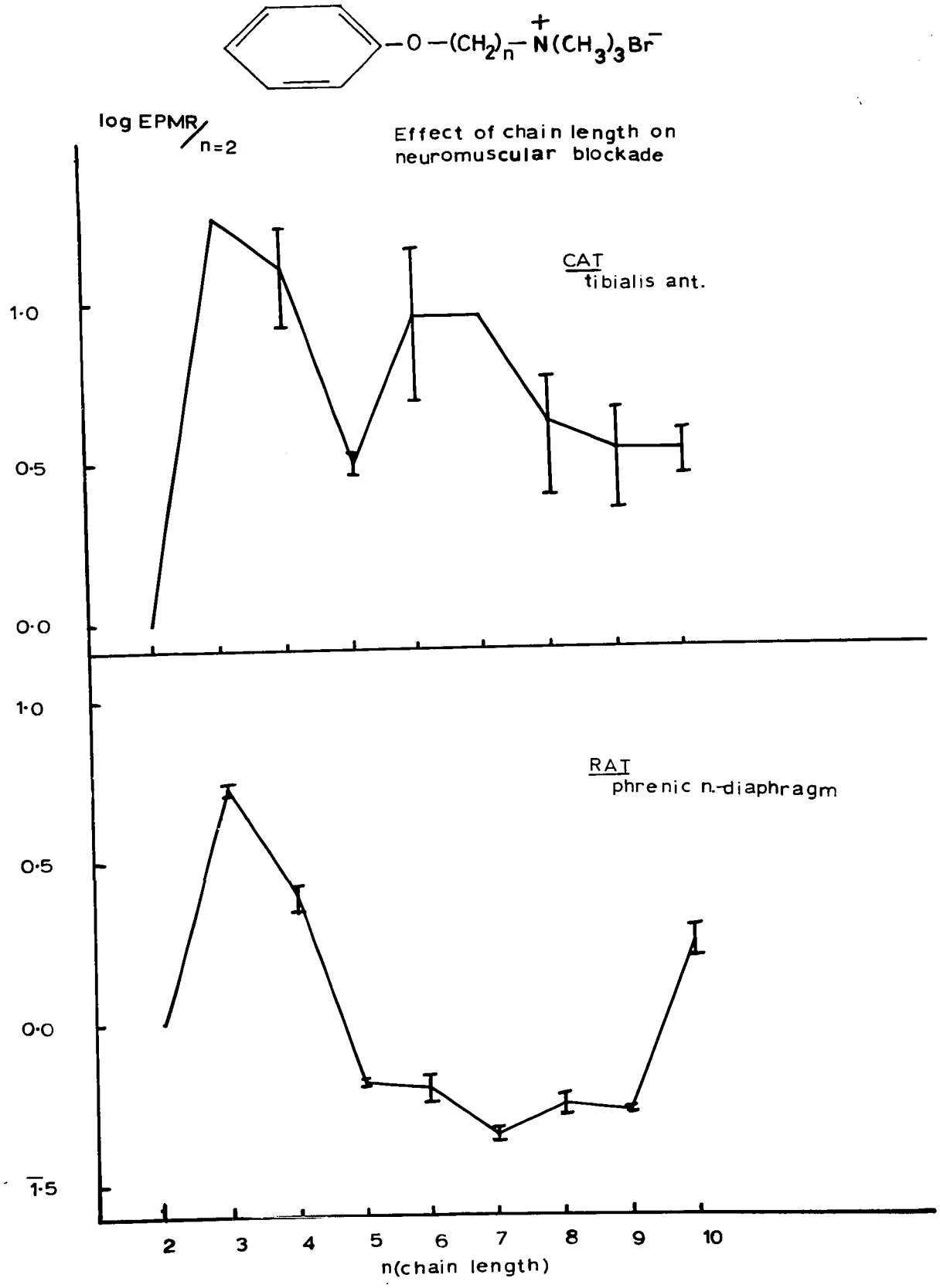
Note: (a) In cat tibialis the dose of $PhO(CH_2)_2$ TMA causing between 27 and 49% blockade was 30 to 120 nanomoles in 0.3 ml. saline intra-arterial (23 assays).

(b) In rat diaphragm assays the concentration of $PhO(CH_2)_2$ TMA causing between 24 and 61% blockade was $1.2 - 4.0 \times 10^{-5}$ Molar (26 assays).

* Activity is expressed as the log of the equipotent molar ratio (EPMR). The EPMR is the number of molecules of the "test" compound which produced the same effect (ganglion blockade) as one molecule of the "standard" reference compound phenoxyethyltrimethylammonium.

Note: A point above 0.0 indicates more molecules were required and thus a decreased relative activity.

All subsequent figures show EPMR $\pm$ S.E.M. unless otherwise indicated.


$-O-(CH_2)_n-\overset{+}{N}(CH_3)_3Br^-$
log EPMR/n=2
Effect of chain length on neuromuscular blockade
CAT
tibialis ant.
1·0
0·5
0·0
1·0
RAT
phrenic n.-diaphragm
0·5
0·0
$\overline{1}$·5
2
3
4
5
6
7
8
9
10
n(chain length)

chain length from ethyl (n=2) to propyl (n=3) is to significantly decrease the inhibitory action on the diaphragm stimulated maximally and indirectly via the phrenic nerve. 5.33 molecules of phenoxypropyl TMA were found to be equivalent in inhibitory activity to one molecule of the 2-carbon-chain phenoxyethyl TMA (P value 0.01 - 0.001). Further increasing the chain length from propyl (n=3) to butyl (n=4) and pentyl (n=5) leads to a progressive return of inhibitory activity; phenoxybutyl TMA being 0.41 times as active as phenoxyethyl TMA (P value 0.02 - 0.01) and phenoxypentyl TMA being 1.54 times as active as the parent phenoxyethyl TMA compound (P value 0.01 - 0.001). In addition, on this preparation the inhibitory activity of the phenoxyhexyl TMA (n=6), phenoxyheptyl TMA (n=7) phenoxyoctyl TMA (n=8) and phenoxynonyl TMA (n=9) may be seen to be significantly greater than that of the 2-carbon phenoxyethyl TMA. (n=6/n=2, 1.61 times; n=7/n=2, 2.22 times; n=8/n=2, 1.82 times; and n=9/n=2, 1.93 times respectively). Further increasing the chain length to give the longest phenoxyalkyl TMA compound studied, phenoxydecyl TMA (n=10), resulted once more in a decrease in blocking activity; the 10-carbon compound being 0.57 times as active as the 2-carbon compound (P value 0.01 - 0.001).

(b) Phenylalkyltrimethylammonium Series (Phenylalkyl TMA)

As above the EPMR's are given with reference to the standard phenylpropyl TMA compound where n=3 in Table 2 and Fig. 3. In this case, however, in an attempt to conserve the supply of the reference standard a number of bioassays were performed indirectly and the EPMR with reference to the phenylpropyl TMA was arrived at by calculation. In Table 2 and Fig. 3 where the comparisons with the standard propyl

TABLE 2

Effect of chain-lengthening on neuromuscular blockade

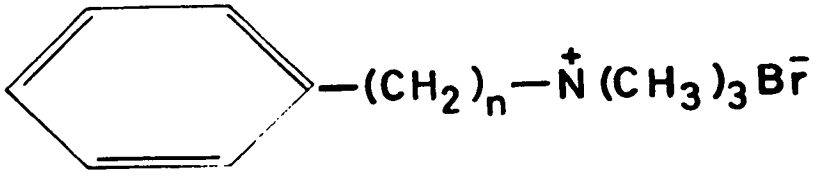
- Phenylalkyltrimethylammoniums

- Cat tibialis and rat diaphragm

TABLE

EFFECT OF INCREASING CHAIN LENGTH ON SYNAPTIC BLOCKADE -- NEUROMUSCULAR JUNCTION

PHENYLALKYLTRIMETHYLAMMONIUM SERIES



Equipotent Molar Ratio/$_{N=2}$

Chain Length N =	Cat Tibialis Anterior		Rat Phrenic Nerve Diaphragm	
0	2.71 ± 1.27	(3)	3.40 ± 0.145	(3)
1	1.08 ± 0.182	(3)	2.765- 3.23- 3.728‡	
2	0.77 ± 0.038	(3)	1.341- 1.67- 2.013*	
3	1.0		1.0	
4	---		2.98	(1)
5	8.54 ± 0.567	(5)	1.73 ± 0.187	(4)

‡ Indirect assay from 4 expts. $\frac{N=1}{N=0}$ and 3 expts. $\frac{N=0}{N=3}$.

Range calculated using ± one S.E.M.

* Indirect assay from 3 expts. $\frac{N=2}{N=0}$ and 3 expts. $\frac{N=0}{N=3}$.

Range calculated using ± one S.E.M.

( ) numerals represent number of assays

FIGURE 3

Effect of chain-lengthening on neuro-muscular blockade

- Phenylalkyltrimethylammoniums

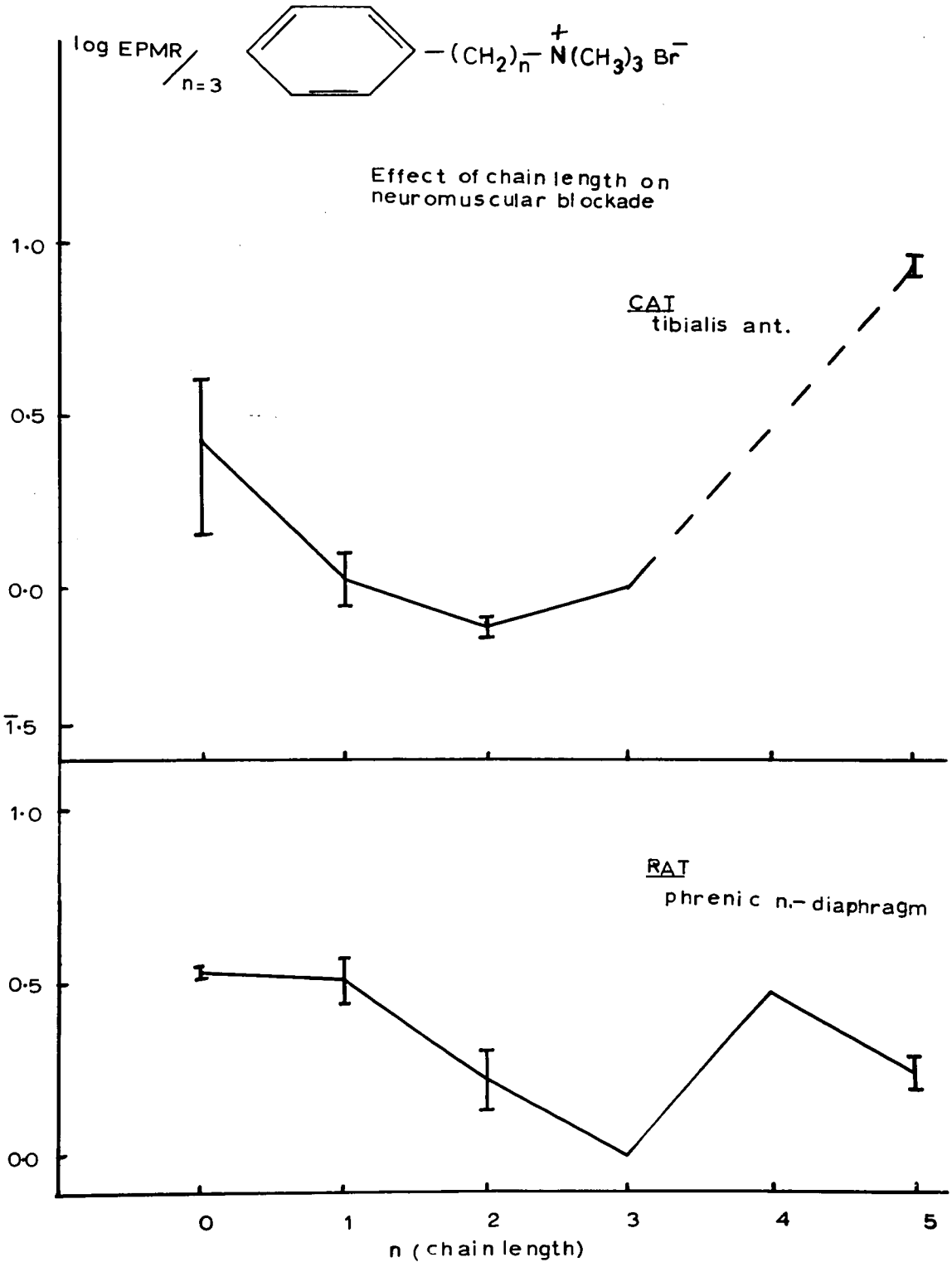
(a) Cat Tibialis

(b) Rat Diaphragm

Note: (a) In cat tibialis the dose of $Ph(CH_2)_3$ TMA causing between 28 and 54% blockade was 30 to 90 nanomoles in 0.3 ml. saline intra-arterial (14 assays).

(b) In rat diaphragm assays the concentration of $Ph(CH_2)_3$ TMA causing between 23 and 68% blockade was 1.0 - 4.0 X 10^{-5} Molar (8 assays).

S.E.M. is indicated.


log EPMR / n=3
$-(CH_2)_n-\overset{+}{N}(CH_3)_3\ Br^-$
Effect of chain length on neuromuscular blockade
1·0
0·5
0·0
$\bar{1}$·5
CAT
tibialis ant.
1·0
0·5
0·0
RAT
phrenic n.– diaphragm
0
1
2
3
4
5
n (chain length)

(n=3) compound were obtained by indirect methods an estimate of the error is given as a range: (e.g. the mean of 4 experiments comparing n=1/n=0 minus one S.E.M. multiplied by the mean of 3 experiments comparing n=0/n=3 minus one S.E.M. gave the lower range for the estimate of EPMR of n=1/n=3).

From the results available with this series on the rat diaphragm the phenylpropyl TMA compound was found to be the most potent inhibitor of the response to indirect stimulation (12 shocks / min). There was a progressive increase in inhibitory activity as the chain length was increased from n=0 (phenyl TMA) to n=3 phenylpropyl TMA); n=0 being 0.29 times as active as n=3; and n=2 being apparently 0.60 times as active as n=3 as determined indirectly. Although only one assay was performed comparing phenylbutyl TMA (n=4) and phenylpropyl TMA it appeared that the 4-carbon compound was again less active than the 3-carbon homologue; 2.98 molecules of n=4 being equivalent, in one assay, to one molecule of n=3. Phenylpentyl TMA likewise was 0.58 times as active as phenylpropyl TMA (P value 0.05 - 0.02).

(c) Phenoxyalkyltriethylammonium Series (Phenoxyalkyl TEA)

The results of experiments comparing the phenoxyalkyl TEA compounds with their phenoxyethyl TEA homologue on the rat diaphragm preparation are presented in Table 3 and Fig. 4. Lengthening the chain from n=2 (phenoxyethyl TEA) to n=3 (phenoxypropyl TEA) did not significantly alter inhibitory activity. Phenoxybutyl TEA (n=4), phenoxypentyl TEA (n=5) and phenoxyhexyl TEA (n=6) on the other hand were found to be more active than the standard reference compound where n=2; being 1.21 (P value 0.02 - 0.01), 1.70 (P value 0.02 - 0.01), and

TABLE 3

Effect of chain-lengthening on neuromuscular blocking activity

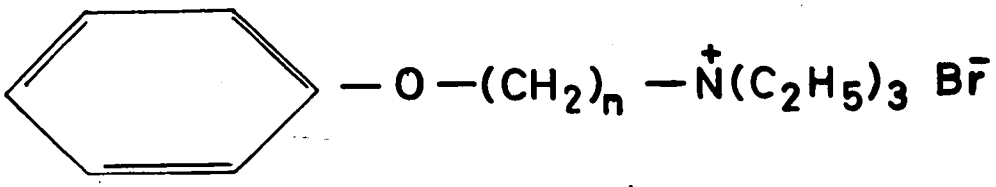
- Phenoxyalkyltriethylammoniums
- Cat tibialis and rat diaphragm

S.E.M. is indicated.

TABLE

EFFECT OF INCREASING CHAIN LENGTH ON SYNAPTIC BLOCKADE -- NEUROMUSCULAR JUNCTION

PHENOXYALKYLTRIETHYLAMMONIUM SERIES



EQUIPOTENT MOLAR RATIO/$_{N=2}$

CHAIN LENGTH N =	CAT TIBIALIS ANTERIOR	RAT PHRENIC NERVE DIAPHRAGM
2	1	1
3	1.40 ± 0.060 (2)	0.98 ± 0.143 (3)
4	1.22 ± 0.473 (3)	0.83 ± 0.018 (3)
5	0.55 ± 0.045 (4)	0.59 ± 0.048 (3)
6	0.40 ± 0.064 (3)	0.50 ± 0.026 (4)

( ) NUMERALS REPRESENT NUMBER OF ASSAYS

FIGURE 4

Effect of chain-lengthening on neuro-muscular blockade

- Phenoxyalkyltriethylammoniums

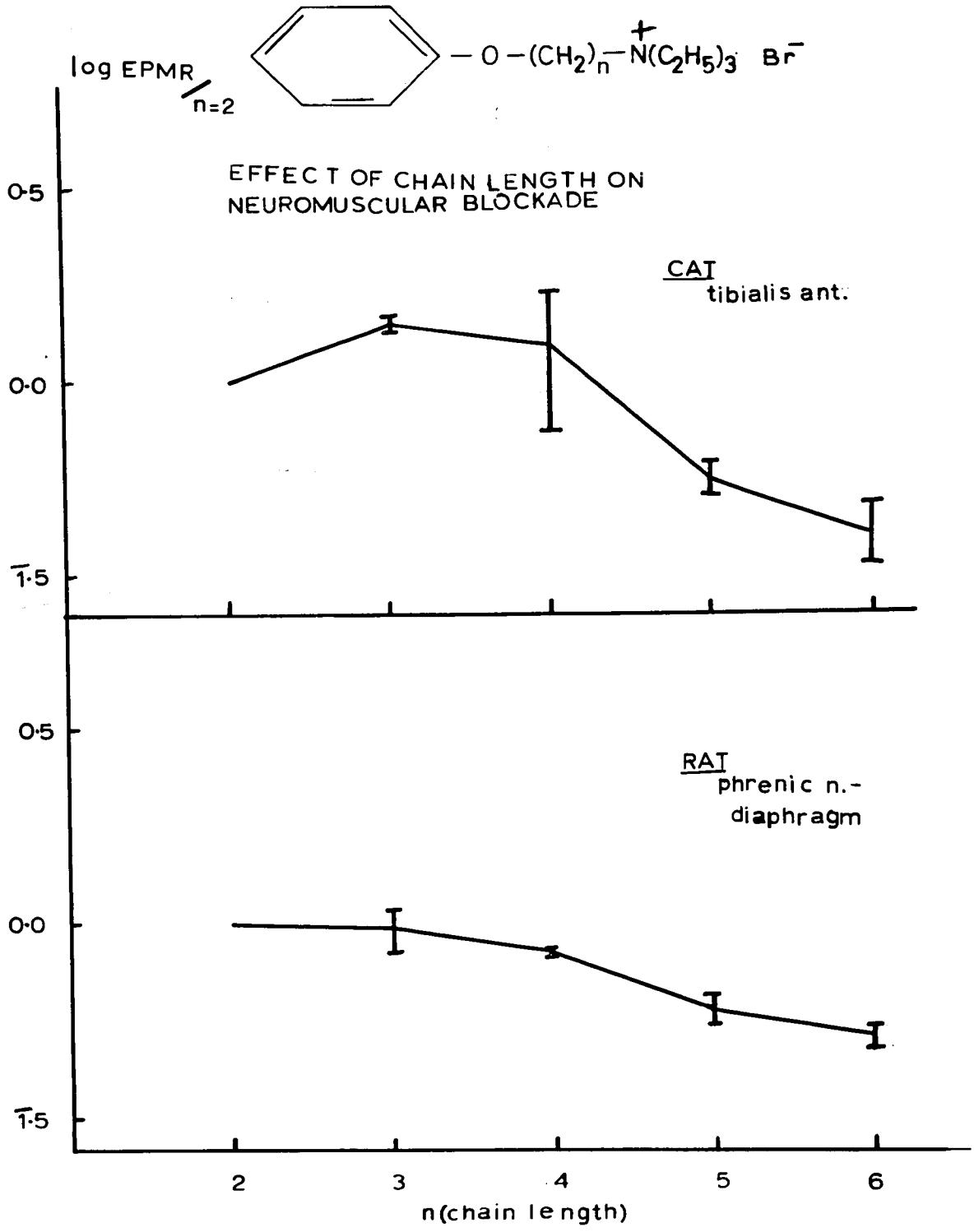
(a) Cat Tibialis

(b) Rat Diaphragm

Note: (a) In cat tibialis the dose of $PhO(CH_2)_2$ TEA causing between 16 and 60% blockade was 450 to 1800 nanomoles in 0.3 ml. saline intra-arterial (12 assays).

(b) In rat diaphragm assays the concentration of $PhO(CH_2)_2$ TEA causing between 13 and 46% blockade was $4.0 - 8.0 \times 10^{-5}$ Molar (13 assays).

S.E.M. is indicated.


log EPMR / n=2
$-O-(CH_2)_n-\overset{+}{N}(C_2H_5)_3\ Br^-$
EFFECT OF CHAIN LENGTH ON NEUROMUSCULAR BLOCKADE
CAT
tibialis ant.
0·5
0·0
1·5
RAT
phrenic n.-diaphragm
0·5
0·0
1·5
2
3
4
5
6
n(chain length)

2.0 (P value 0.001) times as active respectively as phenoxyethyl TEA.

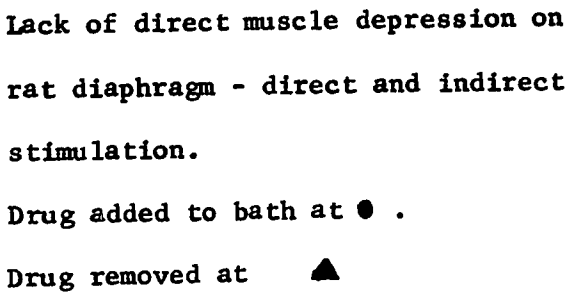
(d) Interseries Comparisons

The standard reference compounds were compared for inhibitory activity on the rat phrenic nerve-diaphragm preparation. Phenoxyethyl TMA was found to be slightly less active (though not significantly so -- P value 0.1 to 0.05) than the phenylpropyl TMA analogue: EPMR of 1.48 $\pm$ S.E.M. in 4 assays. Likewise, no significant difference was found between the phenoxyethyl TMA and phenoxyethyl TEA analogues: EPMR of TMA/TEA 0.78 $\pm$ S.E.M. of 0.302 in 3 assays (P value 0.6 to 0.5).

(e) Experiments Involving Direct Muscle Stimulation

Experiments involving alternate indirect (nerve) and direct (muscle) stimulation are illustrated for the phenoxyalkyl trimethylammonium series in Fig. 5. It may be seen at a time when the response to indirect stimulation had been abolished the response to direct stimulation remained. Identical results were obtained in experiments using the analogous phenoxyalkyltriethylammonium and phenylalkyltrimethylammonium series.

FIGURE 5



Lack of direct muscle depression on rat diaphragm - direct and indirect stimulation.

Drug added to bath at ● .

Drug removed at ▲

Fig 5

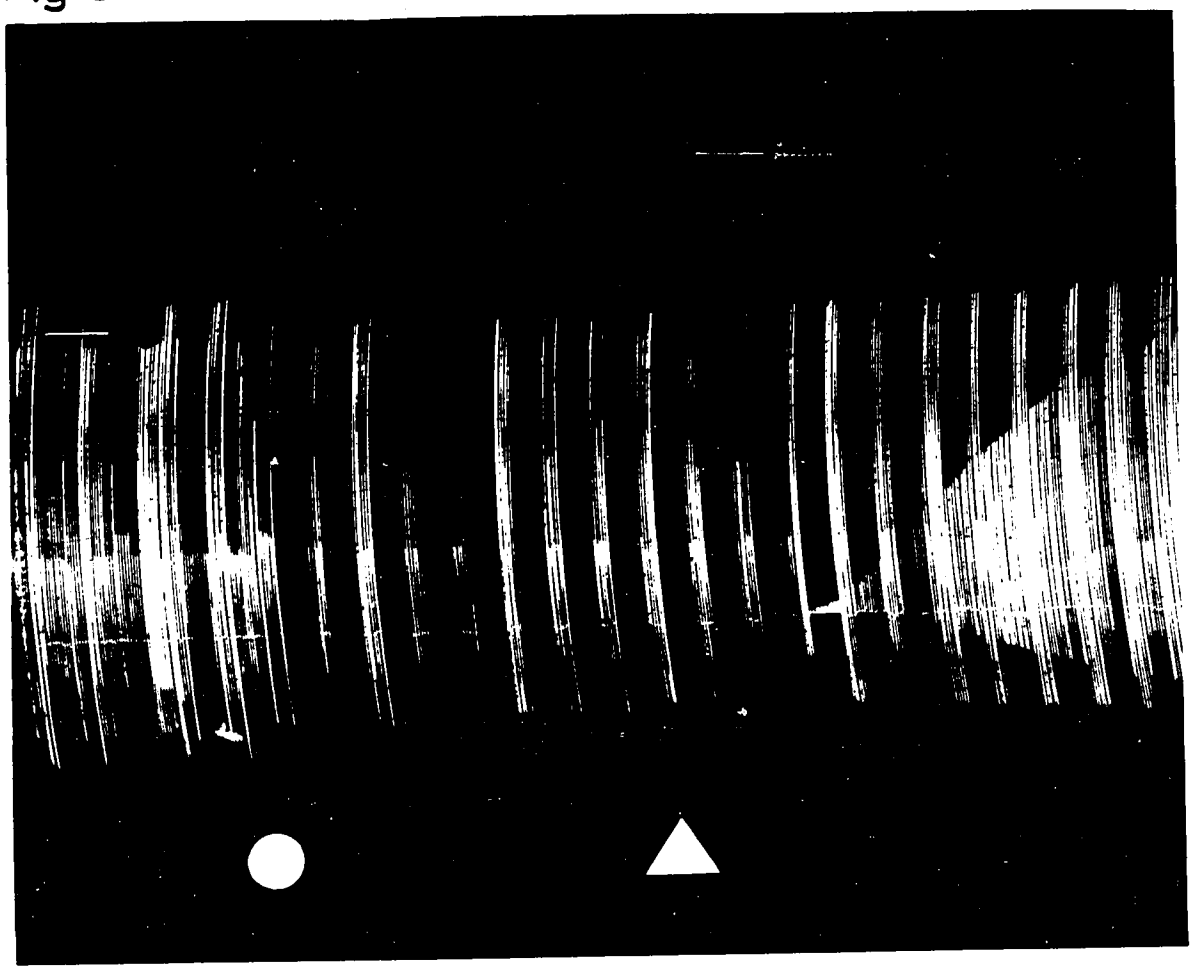
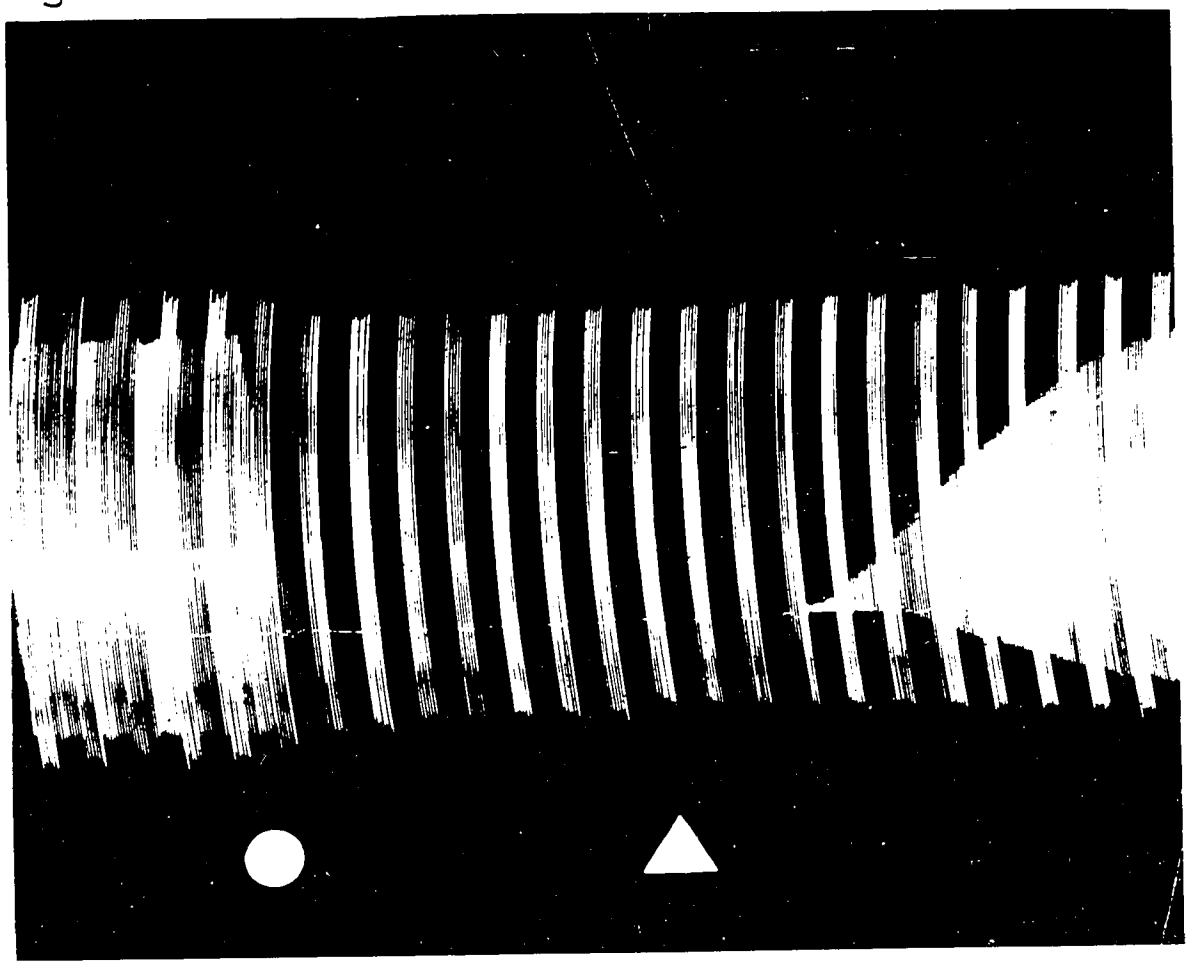


Fig 5



B. Cat Tibialis Anterior Preparation In Vivo

(a) Phenoxyalkyltrimethylammonium Series (Phenoxyalkyl TMA)

The mean EPMR $\pm$ SEM of the phenoxyalkyl TMA compounds relative to the homologue where the chain contains two carbon atoms (the most acetylcholine-like structure) are tabulated in Table 1. These results are presented graphically in Fig. 2 in which the logarithm (base 10) of the EPMR $\pm$ SEM are plotted against carbon chain length. On this preparation it can be seen that the phenoxyethyl TMA compound is the most effective inhibitor of the contractions of the tibialis elicited by anterior tibial nerve stimulation. Transition from the ethyl (n=2) to the propyl (n=3) homologue leads to a marked, 18-fold, decrease in blocking-activity. Further increasing the carbon chain length to five results in a partial return of activity, this compound being about a third as active as the 2-carbon compound. Lengthening the carbon chain to 6 suggested, from the two experiments performed that the activity was again decreased, and indeed a significant decrease occurred on further increasing the carbon chain to seven (n=5/n=7, P value $<$ 0.001). Thereafter the 8, 9 and 10-carbon chain compounds can be seen to have an activity resembling that of the five carbon homologue, and greater than that of the seven carbon, phenoxyheptyl TMA (e.g. phenoxyoctyl TMA was significantly (P value 0.05 - 0.02) more active than phenoxyheptyl TMA).

(b) Phenylalkyltrimethylammonium Series (Phenylalkyl TMA)

The inhibitory action of the members of this series of non-oxygen containing compounds on the tibialis preparation are presented

in Table 2 and Fig. 3 . In this series EPMR's were determined using phenylpropyl TMA (n=3) as the reference standard. This compound most closely resembles phenoxyethyl TMA structurally as the ether oxygen atom (O) has merely been replaced by a third methylene link (CH_2) .

From the number of bioassays performed no significant difference in activity was noted between phenyl TMA (n=0) and phenylmethyl TMA (n=1), (P value 0.4 - 0.3) . However, a significant decrease in inhibitory activity was noted on further increasing the alkyl chain length to n=5 , (phenylpentyl TMA) ; 8.5 molecules of phenylpentyl TMA being equivalent in blocking activity to one molecule of phenylpropyl TMA (n=3). Unfortunately there was insufficient supply of the 4-carbon, phenylbutyl TMA for its evaluation on this preparation.

(c) Phenoxyalkyltriethylammonium Series (Phenoxyalkyl TEA)

Fig. 4 and Table 3 show the effect of increasing chain length on the inhibitory activity on the tibialis preparation of the phenoxyalkyl TEA series from phenoxyethyl TEA (n=2) to phenoxyhexyl TEA (n=6). As with the corresponding phenoxyalkyl TMA series the standard chosen for comparison was the most acetylcholine-like structure, the phenoxyethyl homologue (n=2).

Increasing the chain length from 2 to 3 carbons did not change the inhibitory activity; the EPMR of the propyl to the ethyl homologue being 0.72 with a P value of 0.1 to .05 . Further increasing the chain length to n=4, n=5 and n=6 led to a progressive increase in activity , the phenoxypentyl TEA being 1.82 times as active (P value 0.01 - 0.001) as the phenoxyethyl TEA, and the phenoxyhexyl TEA being 2.5 times as active (P value 0.02 - 0.01) as phenoxyethyl TEA .

(d) Interseries Comparisons

Phenoxyethyl TMA and Phenylpropyl TMA, the two substances with a 3-atom chain separating the phenyl ring from the cationic head, were compared in one assay which indicated that they were similar in inhibitory activity on the tibialis preparation. This assay gave an estimate that 0.82 molecules of the phenylalkyl compound were equivalent in activity to one molecule of the corresponding-length phenoxyalkyl compound.

Likewise, interseries comparisons were made between corresponding members of the phenoxyalkyl TMA series and the phenoxyalkyl TEA series. Phenoxypropyl TMA/ phenoxypropyl TEA gave an EPMR of 1.03 in one assay and phenoxybutyl TMA/ phenoxybutyl TEA gave an EPMR of 0.99 as the mean of two assays.

(e) Experiments Involving Direct Muscle Stimulation

In a number of experiments the muscle was stimulated periodically by means of an electrode in the tibialis muscle. In all instances when a 100% reduction of the response to indirect muscle stimulation (nerve) had been obtained there was no reduction in the response to direct muscle stimulation. A typical tracing is shown in Fig. 6 a.

(f) Edrophonium Reversibility

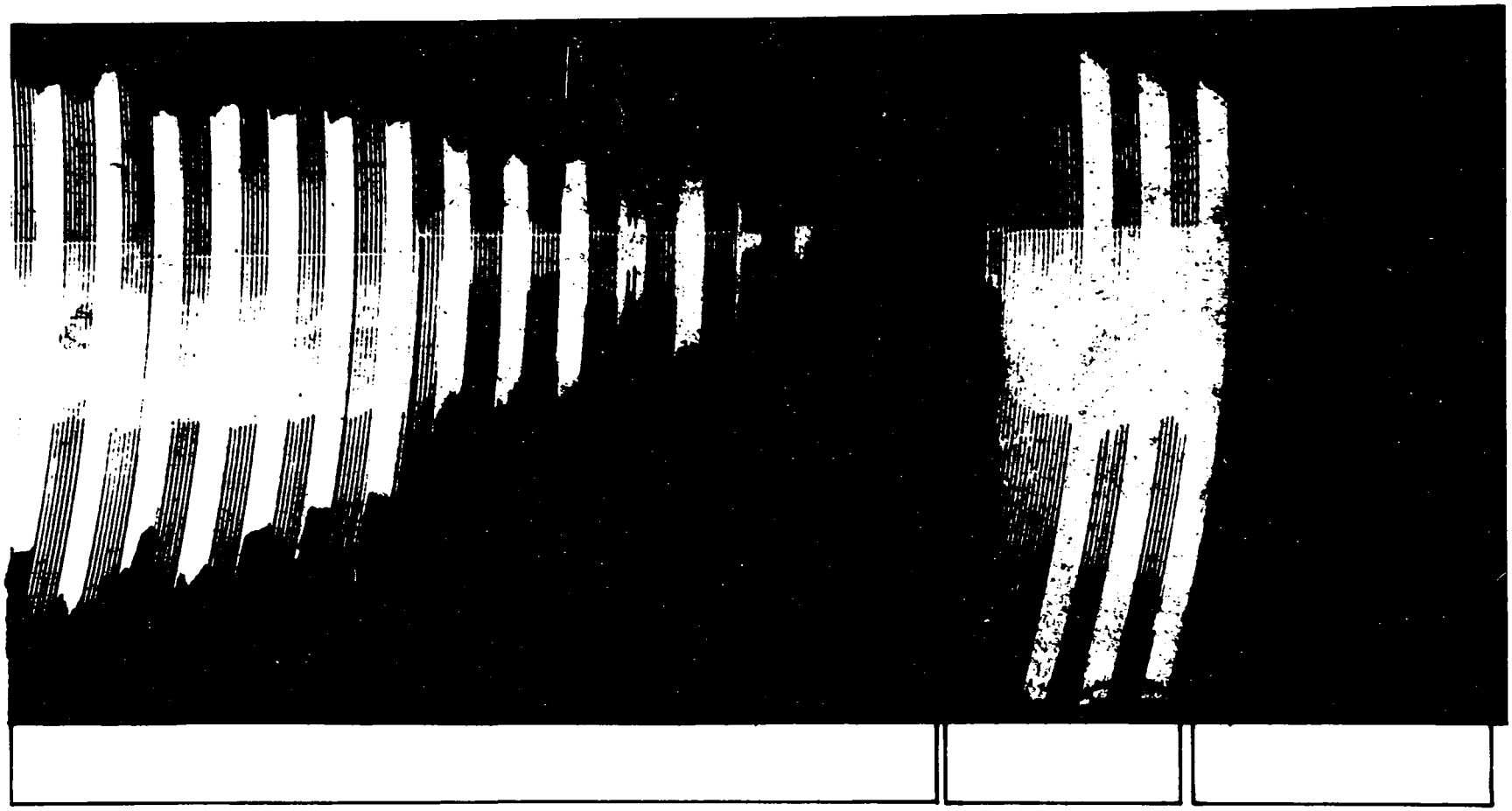
The maximal recovery (percent) of the response of the tibialis muscle to slow (0.2/sec) and to fast (1.0/sec.) nerve stimulation was determined after intravenous infusion of edrophonium (2-4 mg./ml.) .

FIGURE 6a

Lack of direct muscle depression on cat tibialis - direct and indirect stimulation. Drug, phenoxybutyl-trimethylammonium (10 micromoles/ml infusion) started at ● .

Note: Artefact, due to mechanical interference with recording needle and Kymograph drum. Normally, fast rate of stimulation decreases more rapidly than slow rate (see fig. 6b).

FIG. 6a



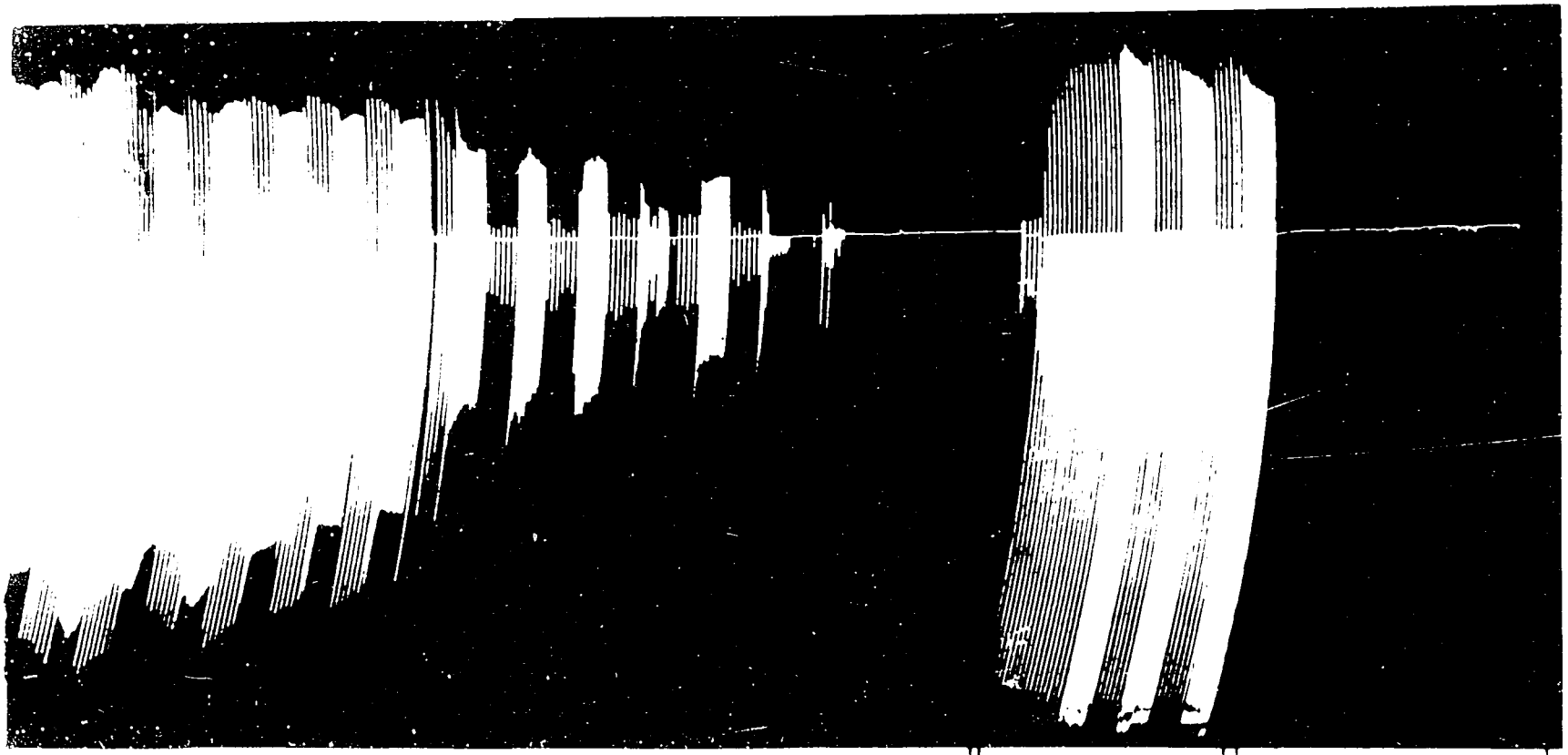


FIGURE 6b

Antagonism of neuromuscular blockade by edrophonium. Cat tibialis anterior. Stimulation rate alternately at 12 and 60 per minute.

Edrophonium infusion (2 - 4 mg./c.c.) begun at ● during blockade by:

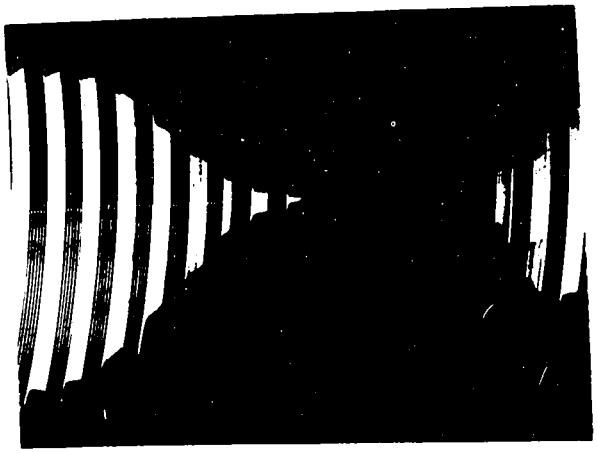
1. d-Tubocurarine) Note antagonism
2. Hemicholinium)
3. Succinyldicholine) Note non-antagonism
4. Phenoxyoctyltrimethylammonium)

at X in 3 and 4 infusion of relaxant and edrophonium discontinued.

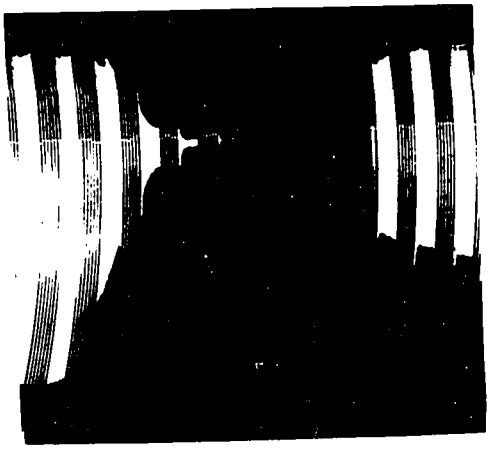
FIG. 6b

1

3

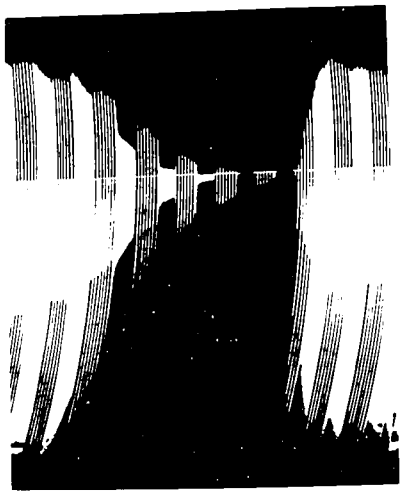


2



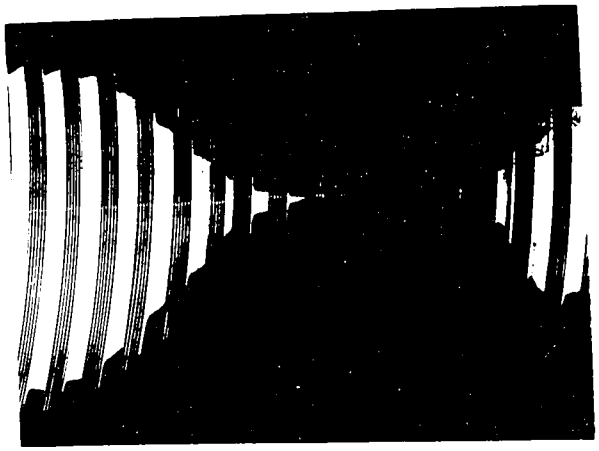
4

FIG. 6b

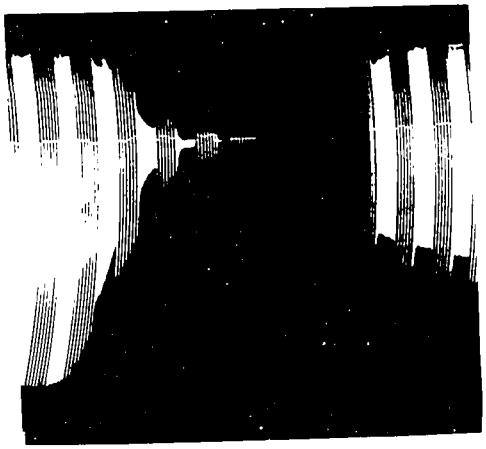


1

3



2



4

TABLE 4

Effect of edrophonium on neuromuscular blockade

- Interseries comparison with clinically used neuromuscular relaxants
- Cat tibialis

TABLE 4

MEAN RECOVERY AFTER EDROPHONIUM (%) ON CAT TIBIALIS ANTERIOR

Drug	I First Exposure		II Second Exposure	
	1.0/sec.+	0.2/sec.	1.0/sec.	0.2/sec.
d-tubocurarine	81 (6)	92 (6)	78 (5)	16 (5)
gallamine	101 (2)	101 (2)	15 (2)	13 (2)
succinyldicholine	0	0	0	0
Ph-O-$(CH_2)_n$-NEt_3 * n = 2,3,4,6	0	0	0	0
Ph-O-$(CH_2)_n$-NMe_3 * n = 4,5,6,8	0	0	0	0
Ph-$(CH_2)_n$-NMe_3 * n = 2	0	0	0	0
hemicholinium	58 (2)	72 (2)	33 (1)	46 (1)

+ Rate of nerve stimulation.

( ) Represents number of experiments.

* One or more tests with each compound of carbon-chain length = n

The edrophonium infusion was started at a time when the "rapid" response was totally inhibited and the "slow" response had reached equilibrium as indicated by a steady state block. These experiments, which involved the infusion of large quantities of relaxant drug were performed late in the study with those drugs which remained in sufficient supply. The percentage recoveries determined are tabulated in Table 4. A typical experiment is shown in Fig. 6 b.

Table 4 column (1) shows the ability of edrophonium to reverse the blockade produced by d-tubocurarine, gallamine, succinyldicholine, hemicholinium, and a number of the test compounds when administered to a fresh preparation for the first time without prior exposure to blocking drugs.

Table 4 column (II) presents similar results obtained when the preparation had previously been exposed to the relaxant compound and to attempted edrophonium reversal.

It can be seen that it was possible to reverse the blockade due to gallamine, d-tubocurarine, and hemicholinium by administration of edrophonium; but in no instance was a reversal obtained after succinyldicholine or any of the representatives of the phenoxyalkyltrimethylammoniums or phenoxyalkyl triethylammoniums studied.

The clinically used muscle relaxants, gallamine and d-tubocurarine exhibited a high degree of reversibility by edrophonium, at least on a fresh preparation; the mean percentage recoveries at the slow rate of nerve stimulation being 101% and 92% respectively. It can be seen, however, (Table 4 column II) that edrophonium may not be as effective in antagonizing the blockade due to either gallamine or d-tubocurarine after prior exposure to the same relaxant and to edrophonium.

This was particularly suggested in the case of gallamine in which there was only a 13% and 15% recovery at slow and fast rates respectively. Although the mean values (% recovery) obtained during second exposure to d-tubocurarine and edrophonium were lower than those obtained for first exposure the ranges of results obtained overlapped.

In the case of hemicholinium (HC_3), a drug (see above) which is commonly believed to have predominant effects on the presynaptic nerve terminals, it can be seen that a 58% to 72% recovery was obtainable on a fresh preparation and a 33% to 46% recovery (one experiment) was obtainable after previous HC_3 administration. Insufficient experiments were performed, as yet, to compare these statistically with the apparently greater antagonism of edrophonium to gallamine and d-tubocurarine. Again there may be a decreased effectiveness of edrophonium after previous exposure to the relaxant and to edrophonium .

TABLE 5

Effect of chain-lengthening on ganglion-blocking activity

- Phenoxyalkyltrimethylammoniums
- Cat superior cervical ganglion

Column 1: EPMR's relative to phenoxypropyltrimethylammonium.

Column 2: EPMR's calculated relative to phenoxyethyltrimethylammonium.

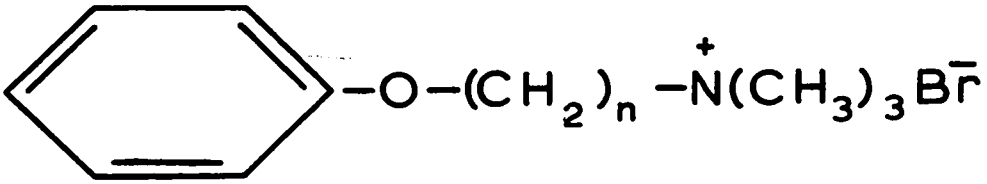
* Indicates mean values by indirect assay with reference to EPMR of n=3/n=2 in every case. In these cases a "range" is given rather than S.E.M. The "range" was estimated as follows:

EPMR of n=x/n=3 - S.E.M. X EPMR of n=3/n=2 + S.E.M.
and
EPMR of n=x/n=3 + S.E.M. X EPMR of n=3/n=2 - S.E.M.
where
x , 2 , 3 , etc. indicate carbon chain length.

TABLE 5

EFFECT OF INCREASING CHAIN LENGTH ON SYNAPTIC BLOCKADE -- SUPERIOR CERVICAL GANGLION

PHENOXYALKYLTRIMETHYLAMMONIUM SERIES



Chain Length	Cat Superior Cervical Ganglion			
n=	Equipotent Molar Ratio/$_{n=3}$ ± S.E.M.		Equipotent Molar Ratio/$_{n=2}$ ± S.E.M. or Range	
2	0.25 ± 0.099	(6)	1	
3	1		4.09 ± 0.679	(6)
4	0.97 ± 0.106	(4)	2.95 - 3.97 - 5.13*	
5	0.85 ± 0.061	(4)	2.69 - 3.48 - 4.35*	
6	0.76 ± 0.065	(4)	2.37 - 3.11 - 3.93*	
7	0.24 ± 0.061	(4)	0.61 - 0.98 - 1.43*	
8	0.10 ± 0.016	(4)	0.29 - 0.41 - 0.55*	
9	0.35 ± 0.007	(3)	1.17 - 1.43 - 1.68*	
10	2.06 ± 0.372	(4)	5.06 - 8.43 -11.59*	

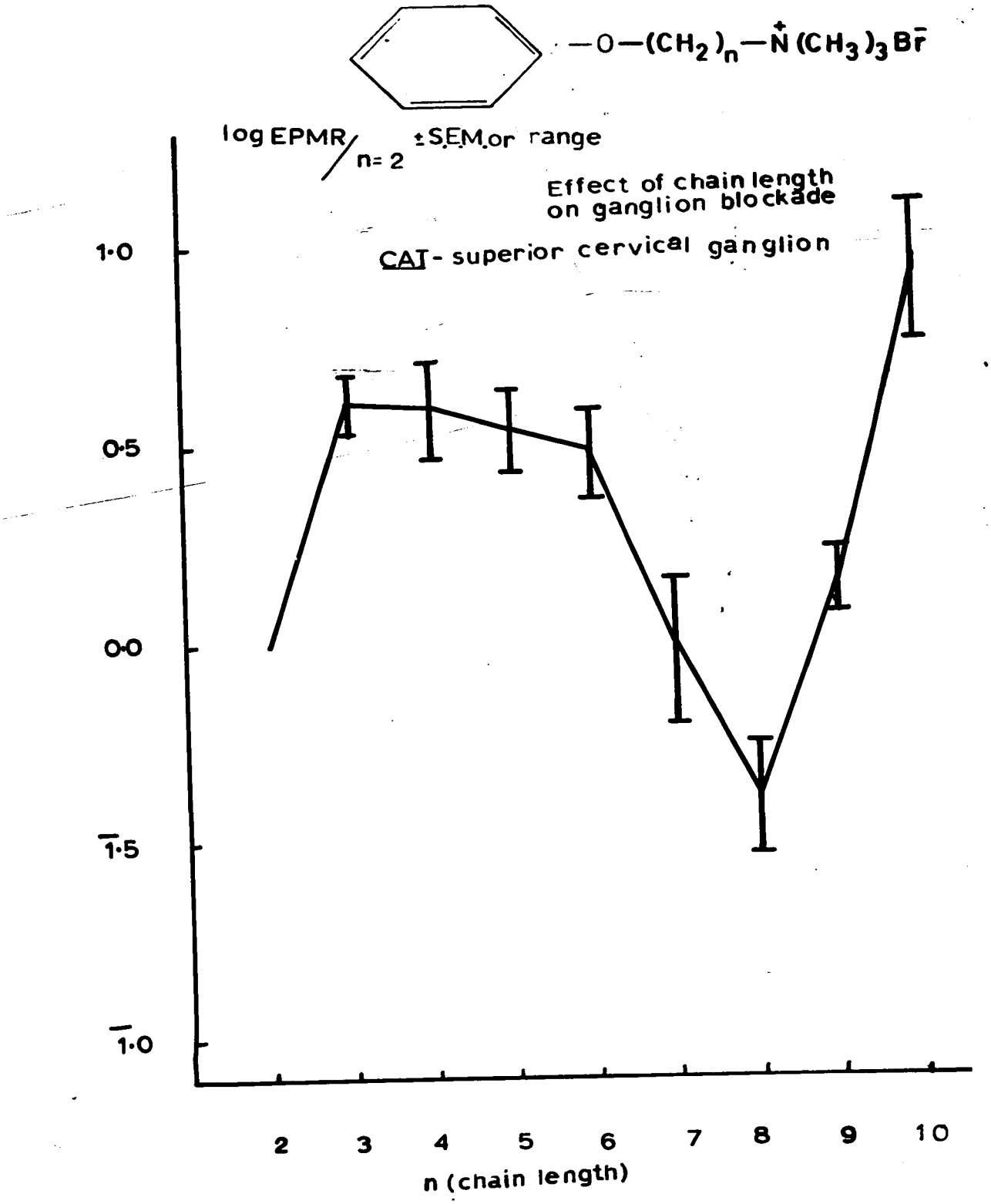
FIGURE 7

Effect of chain-lengthening on ganglion-blocking activity

- Phenoxyalkyltrimethylammoniums
- Cat superior cervical ganglion preparation

The doses of $PhO(CH_2)_3$ TMA (the standard used for comparison in most cases in the cat due to the added stimulant activity of $PhO(CH_2)_2$ TMA on this preparation) causing about 50% inhibition of the nictitating membrane during maximal preganglionic stimulation was between 50 and 200 nanomoles in 0.1 ml. saline intra-arterial (33 assays).

Note: This figure shows Ranges rather than S.E.M. (see table 5).
EPMR $\pm$ "Range" as defined on caption to table 5 is plotted.


—O—(CH2)n—N+(CH3)3Br−
log EPMR / n=2 ±S.E.M. or range
Effect of chain length on ganglion blockade
CAT - superior cervical ganglion
1·0
0·5
0·0
1·5
1·0
2
3
4
5
6
7
8
9
10
n (chain length)

C. Cat Superior Cervical Ganglion Preparation In Vivo

The activity of the phenoxyalkyl TMA salts in producing a ganglion blockade, as evidenced by the relaxation of the nictitating membrane during continuous 10/second stimulation of the preganglionic cervical sympathetic nerve, is presented in Table 5 and Fig. 7 . On this preparation it was noted that a marked qualitative difference existed between the usual reference compound, phenoxyethyl TMA (n=2) , and the rest of the series: this compound first produced a stimulation of the ganglion followed by a blockade (a contracture followed by a relaxation of the nictitating membrane) whereas all the longer-chain compounds (n=3 to n=10) merely produced a blockade without any initial stimulation. As it was felt to be undesirable to use such a compound as the standard reference against which all other compounds were to be bioassayed six assays comparing phenoxyethyl TMA (n=2) to phenoxypropyl TMA (n=3) were performed and thereafter each compound was bioassayed directly against phenoxypropyl TMA as the standard. The mean $\pm$ S.E.M. of the assays comparing ganglion blocking activity of the homologues to phenoxypropyl TMA are presented in the left-hand column of Table 5. For comparative purposes the EPMR's relative to the usual standard phenoxyethyl TMA (n=2) have been indirectly calculated and are presented in the right-hand column of Table 5 along with the "range" of error where the S.E.M. was not directly available. In each case the lower value given is the EPMR of (n=x / n=3) - S.E.M. multiplied by EPMR of (n=3 / n=2) - S.E.M. and the higher estimate is EPMR of (n=x / n=3) + S.E.M. multiplied by EPMR of (n=3 / n=2) + S.E.M. . The EPMR $\pm$ S.E.M. or the "range" with reference to phenoxyethyl TMA (n=2) is plotted log-

arithmically against chain length in Fig. 7 . Increasing the carbon-chain length from n=2 to n=3 led tc a fourfold decrease in inhibitory activity (EPMR n=3/n=2 of 4.09 $\pm$ S.E.M. of 0.679). Phenoxybutyl TMA (n=4) and phenoxypentyl TMA (n=5) were not significantly different from phenoxypropyl TMA (n=3), (P values 0.8 and 0.1 to 0.05 respectively). Phenoxyheptyl TMA (n=6) was somewhat more active than phenoxypropyl TMA (P value 0.05 to 0.02) though still less active than phenoxyethyl TMA (P value 0.01 to 0.001). Increasing the chain length to n=7 yielded a homologue with the same activity as phenoxyethyl TMA n=2 and further increasing carbon-chain length to n=8 (phenoxyoctyl TMA) gave a compound estimated to be 2.5 times as active as phenoxyethyl TMA although this was not a significant difference (P value 0.2 to 0.1). Thereafter further increasing chain length led again to a progressive decrease in ganglionic blocking activity phenoxynonyl TMA (n=9) being 0.29 times as active as phenoxyoctyl TMA (n=8) (P value $<$ 0.001) and phenoxydecyl TMA (n=10), the least active compound, being 0.12 times as active as phenoxyethyl TMA (n=2) (P value of 0.01 to 0.001).

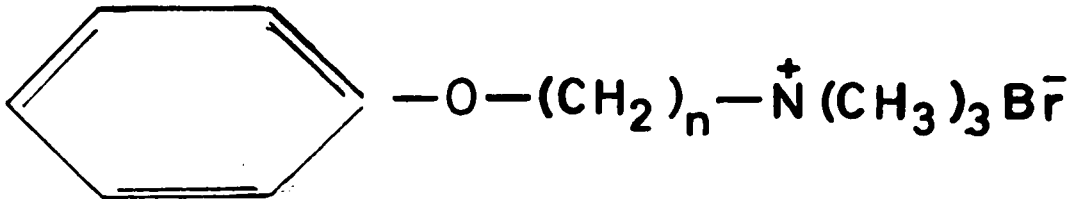
TABLE 6

Effect of chain-lengthening on agonist activity

- Phenoxyalkyltrimethylammoniums
- Frog rectus abdominis

TABLE

EFFECT OF INCREASING CHAIN LENGTH ON ACETYLCHOLINE-LIKE CONTRACTURE



PHENOXYALKYLTRIMETHYLAMMONIUM SERIES

Chain Length N =	Equipotent Molar Ratio/$_{N=2}$ Frog Rectus Abdominis	
2	1	
3	13.62 ± 1.29	(4)
4	12.20 ± 0.76	(4)
5	6.22 ± 0.44	(3)
6	30.40 ± 6.50	(3)
7	> 400	(2)
8	> 400	(2)
9	> 400	(3)

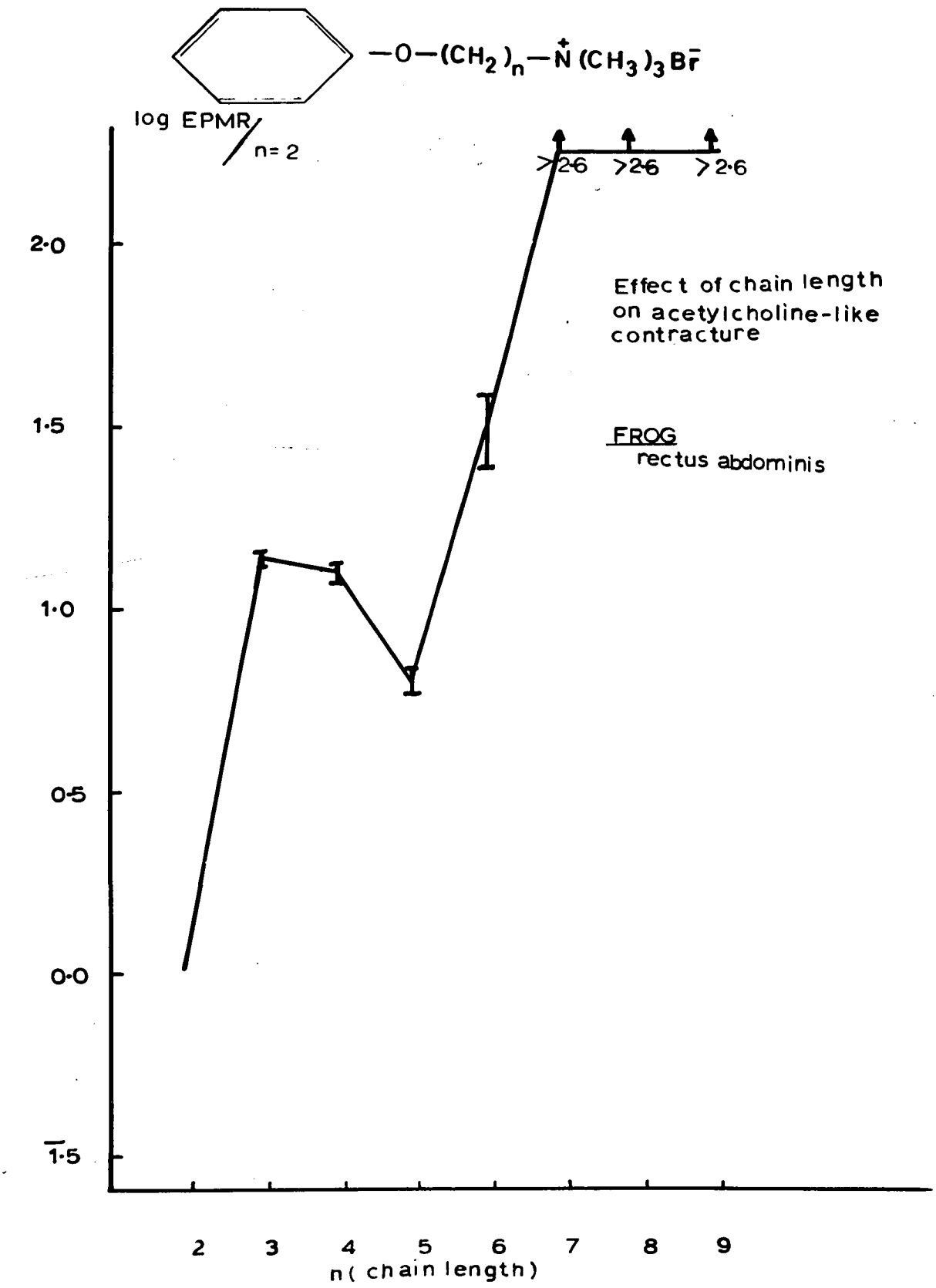
( ) NUMERALS REPRESENT NUMBER OF ASSAYS

FIGURE 8

Effect of chain-lengthening on agonist activity

- Phenoxyalkyltrimethylammonium
- Frog rectus abdominis preparation

Note: The concentration of $PhO(CH_2)_2$ TMA causing between 46 and 76% contracture of the rectus muscle was 2.5 to 5.0 X 10^{-6} Molar (21 assays).


—O—(CH2)n—N+(CH3)3Br−
log EPMR
n= 2
>2·6
>2·6
>2·6
2·0
1·5
1·0
0·5
0·0
1·5
Effect of chain length on acetylcholine-like contracture
FROG
rectus abdominis
2
3
4
5
6
7
8
9
n(chain length)

D. Frog Rectus Abdominis Preparation In Vitro

(a) Equipotent Molar Ratios (Phenoxyalkyl TMA Series)

The acetylcholine-like (agonist) activity of the phenoxyalkyl TMA series was determined using phenoxyethyl TMA (n=2) as the standard. The results of these experiments are shown in Table 6 and Fig. 8. On this preparation increasing the chain length from phenoxyethyl TMA (n=2) to phenoxypropyl TMA (n=3) led to a marked (14-fold) decrease in stimulant ability. Further increasing the chain length to four and then five carbon atoms led to a partial return of stimulant activity; the phenoxypentyl TMA (n=5) being 2.19 times as active as the phenoxypropyl TMA (n=3) homologue (P value 0.01 - 0.001). Thereafter further increasing the carbon-chain length led to a marked decline in stimulant activity, the phenoxyhexyl TMA (n=6) being 0.033 times as active as the reference (n=2) compound. The longer chain (n=7, n=8, n=9, and n=10) compounds were particularly inactive requiring more than 400 molecules to produce a contracture which even then was not as large as that produced by one molecule of phenoxyethyl TMA. The results for phenoxydecyl TMA (n=10) are not included in Fig. 8 and Table 6 but are included in the table of intrinsic activities below which indicates even less agonist activity than the phenoxynonyl TMA (n=9) homologue.

(b) Interseries Comparisons

In four assays the agonist activity of phenoxyethyl TMA was found to be identical to that of phenylpropyl TMA (EPMR 1.16 $\pm$ 0.102).

TABLE 7

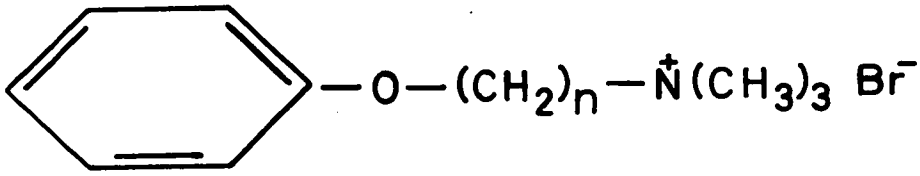
Effect of chain-lengthening on intrinsic activity

- Phenoxyalkyltrimethylammoniums
- Frog rectus abdominis

TABLE

EFFECT OF INCREASING CHAIN LENGTH ON INTRINSIC ACTIVITY
FROG RECTUS ABDOMINIS PREPARATION
ACETYLCHOLINE-LIKE CONTRACTURE

PHENOXYALKYLTRIMETHYLAMMONIUM SERIES



Chain Length N=	Intrinsic Activity ± S.E. of Mean	Number of Estimates
2	0.98 ± 0.020	6
3	0.81 ± 0.035	8
4	0.78 ± 0.059	7
5	0.64 ± 0.115	6
6	0.43 ± 0.051	8
7	0.22 ± 0.053	7
8	0.20	1
9	0.16 ± 0.050	2
10	0.07	1
Acetylcholine	1.00	

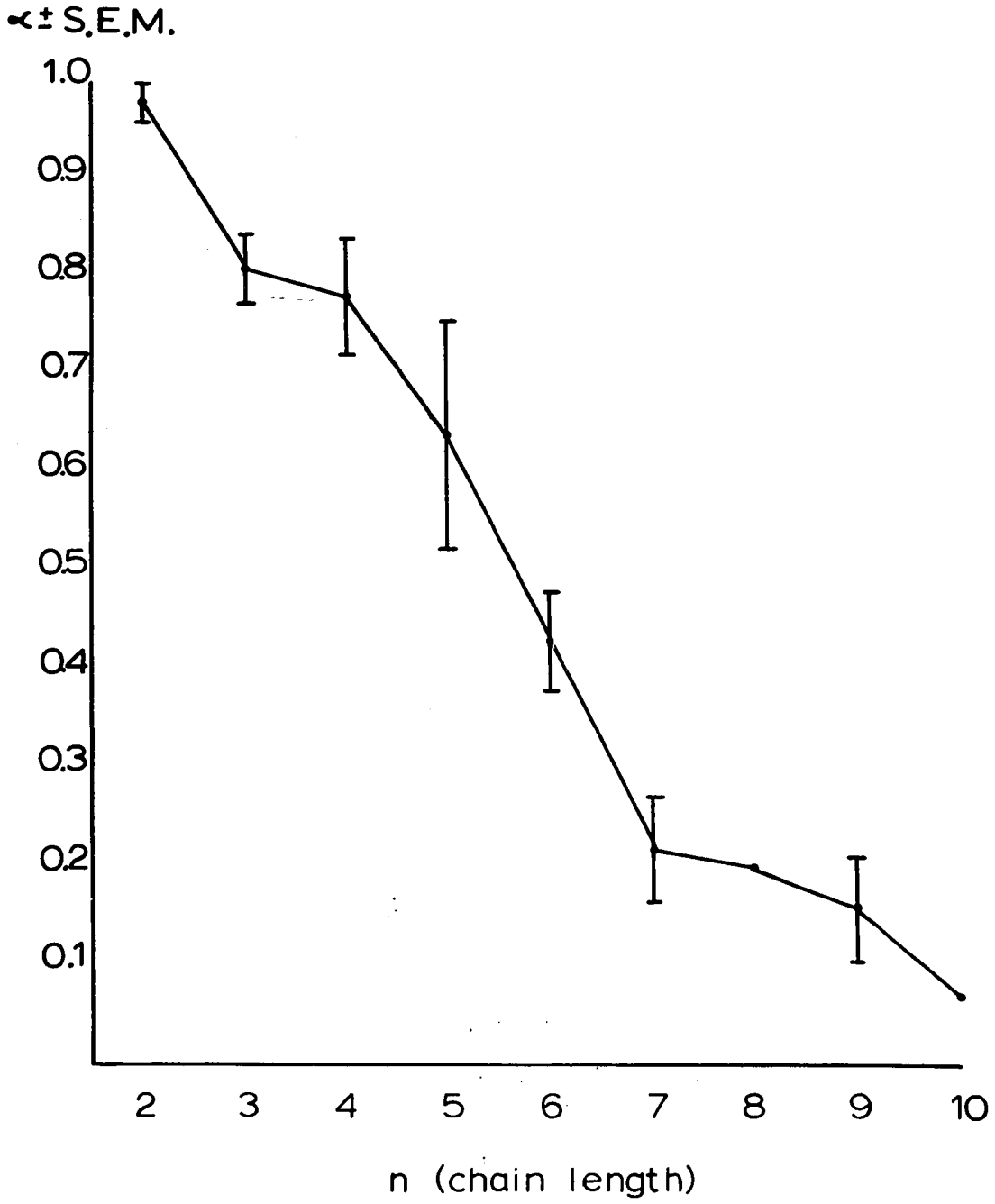
FIGURE 9

Effect of chain-lengthening on intrinsic activity

- Phenoxyalkyltrimethylammoniums
- Frog rectus abdominis preparation

FIG. 9

Effect of increasing chain length on Intrinsic Activity (α) Frog Rectus - $PhO(CH_2)_n$ TMA



(c) Intrinsic Activities

The intrinsic activities of the phenoxyalkyl TMA series are presented in Table 7 and Fig. 9 . The reference standard phenoxyethyl TMA (n=2) was not significantly different from the natural physiological transmitter acetylcholine having an intrinsic activity of 0.98 $\pm$ S.E.M. of 0.02 (in six experiments). Increasing the chain length from n=2 to n=3 led to a significant decrease (P value 0.001) in intrinsic activity. Thereafter further increasing the chain length to the phenoxydecyl TMA (n=10) homologue resulted in a progressive, almost linear, decline in intrinsic activity, the 10 carbon compound producing only 7% of the maximal contracture caused by acetylcholine.

TABLE 8

Effect of chain-lengthening on differential blocking index on rat diaphragm (DBI_{10}) and cat tibialis (DBI_5)

- Interseries comparison with clinically used neuromuscular relaxants

	DIFFERENTIAL BLOCKING INDEX ± S.E.M.					
	DBI_{10} Rat Phrenic Nerve Diaphragm Preparation			DBI_{5} Cat Tibialis Anterior Preparation		
Total Oxygen-Carbon Chain Length( )	$Ph-(CH_2)_n$TMA	$Ph-O-(CH_2)_n$TMA	$Ph-O-(CH_2)_n$TEA	$Ph-(CH_2)_n$TMA	$Ph-O-(CH_2)_n$TMA	$Ph-O-(CH_2)_n$TEA
Ph (0) N	50.0 ± 2.02 (6)					
Ph (1) N	34.0 ± 5.74 (4)					
Ph (2) N	45.3 ± 5.31 (4)					3.0 (1)
Ph (3) N	36.1 ± 4.86 (7)	18.3 ± 5.63 (8)	7.3 ± 1.32 (4)	<1.6 ± 1.27 (2)		< 1.2 (1)
Ph (4) N	30.0 (1)	24.0 ± 2.12 (4)	11.0 ± 1.00 (3)		18.0 (1)	32.0 (1)
Ph (5) N	38.5 ± 4.85 (4)	31.6 ± 1.85 (6)	8.3 ± 1.76 (4)		15.6 ± 3.93 (3)	
Ph (6) N		19.3 ± 1.47 (3)	12.0 ± 2.64 (9)		10.0 ± 1.00 (2)	12.0 (1)
Ph (7) N		25.6 ± 3.82 (12)	8.2 ± 2.55 (5)			
Ph (8) N		23.8 ± 6.03 (6)			8.5 ± 2.93 (4)	
Ph (9) N		13.6 ± 3.62 (5)				
Ph (10) N		13.8 ± 3.71 (7)				
Ph (11) N		3.6 ± 1.87 (3)				
d-Tubocurarine		29.3 ± 2.10 (19)			14.5 ± 1.77 (3)	
gallamine triethiodide		56.7 ± 12.93 (12)			17.5 ± 1.97 (3)	
decamethonium		25.0 ± 3.03 (6)				
succinyldicholine		18.8 ± 2.24 (4)			2.6 ± 0.61 (3)	
succinylmonocholine		29.0 ± 1.73 (3)				
HC_3		39.8			16.6 ± 2.97 (3)	
Triethylcholine		60.3				
edrophonium		69.0				

E. Effect of Stimulation Frequency on Neuromuscular Blockade

Differential Blocking Index (DBI)

1. Rat Phrenic Nerve-Diaphragm Preparation (DBI_{10})

The DBI_{10}'s for the three homologous series of compounds on the rat are listed in Table 8 column 1. The values $\pm$ S.E.M. are arranged so as to compare from left to right the compounds with the same total number of atoms separating the benzene ring from the quaternary nitrogen atom. The values obtained for the clinically used muscle relaxants studied are also presented in Table 8.

(a) Clinically Used Agents

The muscle relaxant activity of the clinically used compound, gallamine, was associated with a marked degree of rate dependence, giving a DBI_{10} of 56.7 ± 12.93 which was significantly greater than that of d-tubocurarine (P value 0.05 - 0.02) and decamethonium (P value 0.05 - 0.02). d-Tubocurarine itself was found to have a DBI_{10} which was not significantly different from the clinically used depolarizing compounds decamethonium and succinyldicholine (P value > 0.4).

(b) Phenylalkyltrimethylammonium Series (Phenylalkyl TMA)

The DBI_{10}'s for the phenylalkyl TMA compounds from n=0 to n=5 ranged from 30 to 50, (i.e. 30 or 50 percent of the maximal contraction due to stimulation at 0.2 shocks per second remained when the contractions due to stimulation at 2 shocks per second were abolished). These results are presented graphically as bar-graphs (indicating the S.E.M.'s) in Fig. 10. The highest mean DBI_{10}, 50.0 ± 2.02 , was

FIGURE 10

Effect of chain-lengthening on
differential blocking index (DBI_{10})
on rat diaphragm
- Phenylalkyltrimethylammoniums

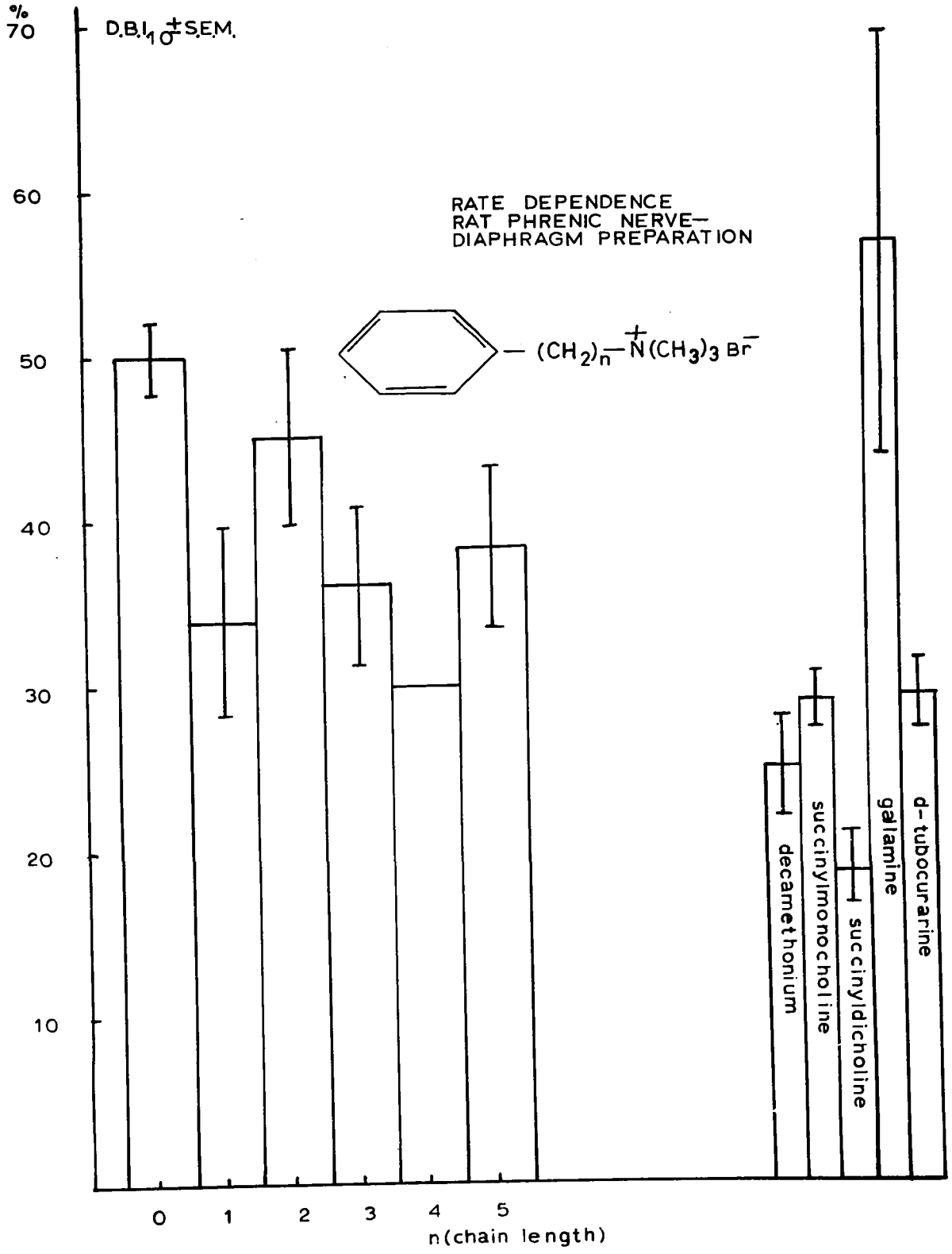
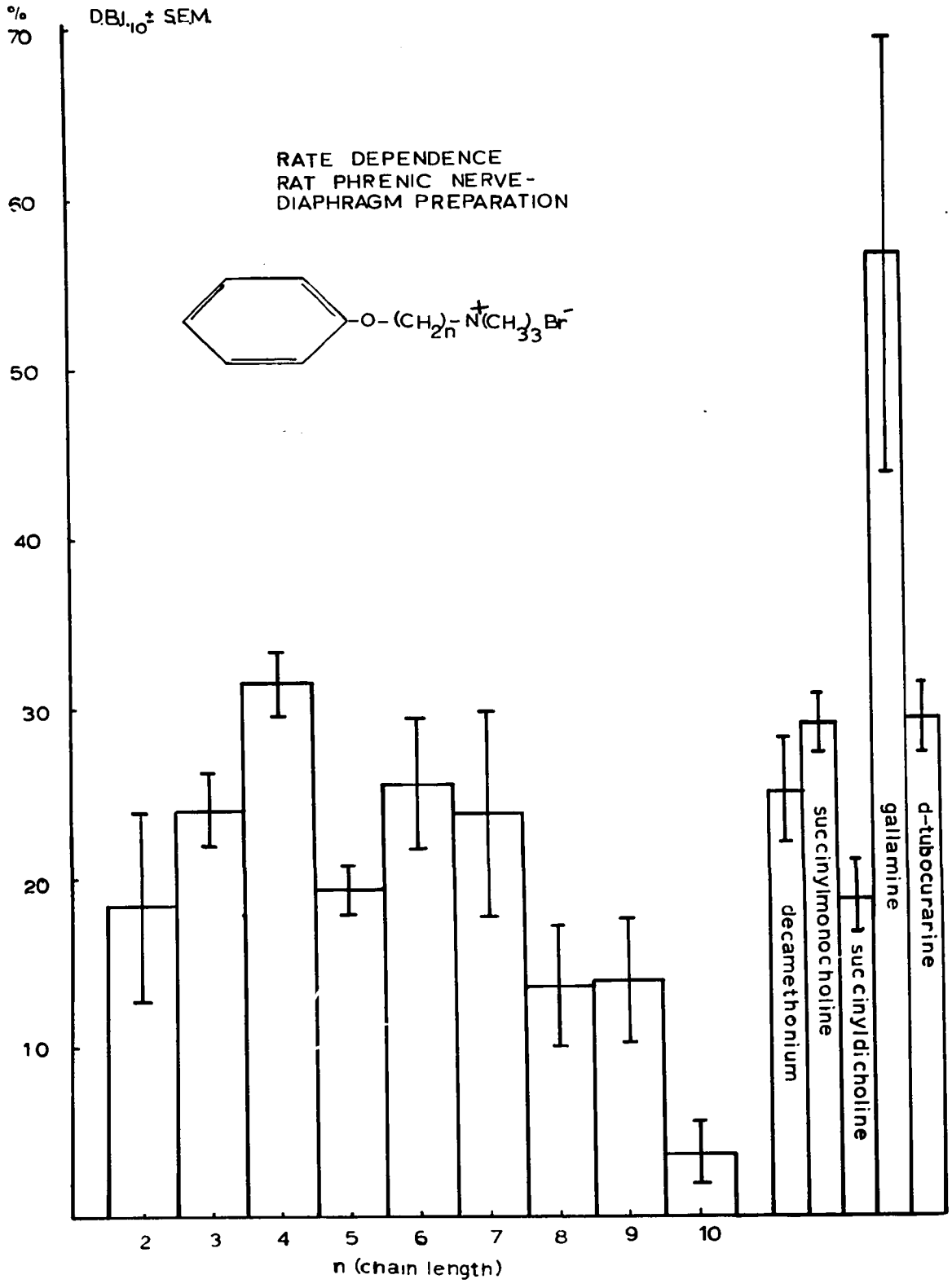

%
70
D.B.I$_{10}$ ± S.E.M.
60
50
40
30
20
10
RATE DEPENDENCE
RAT PHRENIC NERVE–
DIAPHRAGM PREPARATION
$-(CH_2)_n-\overset{+}{N}(CH_3)_3\ Br^-$
0
1
2
3
4
5
n(chain length)
decamethonium
succinylmonocholine
succinyldicholine
gallamine
d-tubocurarine

FIGURE 11

Effect of chain-lengthening on
differential blocking index (DBI_{10})
on rat diaphragm
- Phenoxyalkyltrimethylammoniums


%
DBJ10 ± SEM
70
60
50
40
30
20
10
RATE DEPENDENCE
RAT PHRENIC NERVE-
DIAPHRAGM PREPARATION
-O-(CH2)n-N+(CH3)3 Br-
2
3
4
5
6
7
8
9
10
n (chain length)
decamethonium
succinylmonocholine
succinyldicholine
gallamine
d-tubocurarine

obtained for phenyl TMA (n=0), being significantly greater (P value 0.05 - 0.02) than the values obtained with phenyl methyl TMA, phenylpropyl TMA and phenylpentyl TMA. The phenyl TMA homologue had a DBI_{10} similar to gallamine (P value 0.6) and significantly greater than those obtained for d-tubocurarine and decamethonium (P value < 0.001).

(c) Phenoxyalkyltrimethylammonium Series (Phenoxyalkyl TMA)

The DBI_{10}'s for the phenoxyalkyl TMA series are tabulated in the second column of Table 8 and are presented graphically in Fig. 11 . The values obtained range from 3.6 for phenoxydecyl TMA to 31.6 for phenoxybutyl TMA. Phenoxybutyl TMA showed a significantly (P value 0.05 - 0.02) greater degree of rate dependence than the first in the series, phenoxyethyl TMA. The highest DBI_{10} values were obtained with compounds from two to seven carbons (phenoxyethyl- , to phenoxyheptyl- , TMA) and least rate dependence was observed with the longer chain compounds: phenoxyoctyl TMA, phenoxynonyl TMA, and phenoxydecyl TMA. The latter homologue gave a DBI_{10} significantly (P value 0.05 - 0.02) less than phenoxyethyl TMA.

The most rate-dependent compound in this series, phenoxybutyl TMA, was similar to the most rate dependent clinical compound, gallamine (P value 0.1 - 0.05), and the least rate dependent, phenoxydecyl TMA , was significantly less rate dependent than the least rate dependent clinical compound, succinyldicholine (P value 0.001).

(d) Phenoxyalkyltriethylammonium Series (Phenoxyalkyl TEA)

The DBI_{10}'s for the phenoxyalkyl TEA series of compounds are tabulated in the third column on Table 8 and are presented graphically

FIGURE 12

Effect of chain-lengthening on differential blocking index (DBI_{10}) on rat diaphragm

- Phenoxyalkyltriethylammoniums

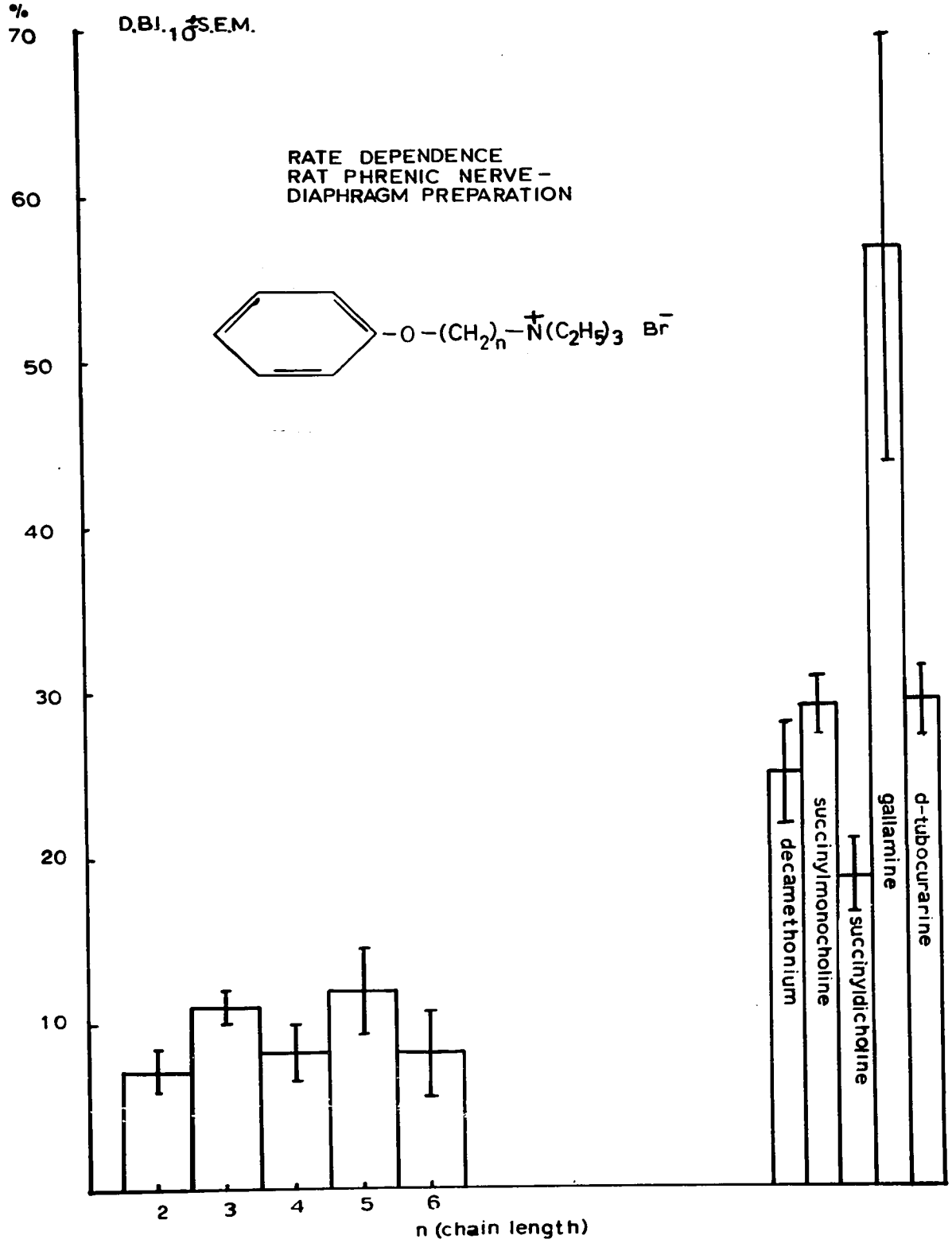
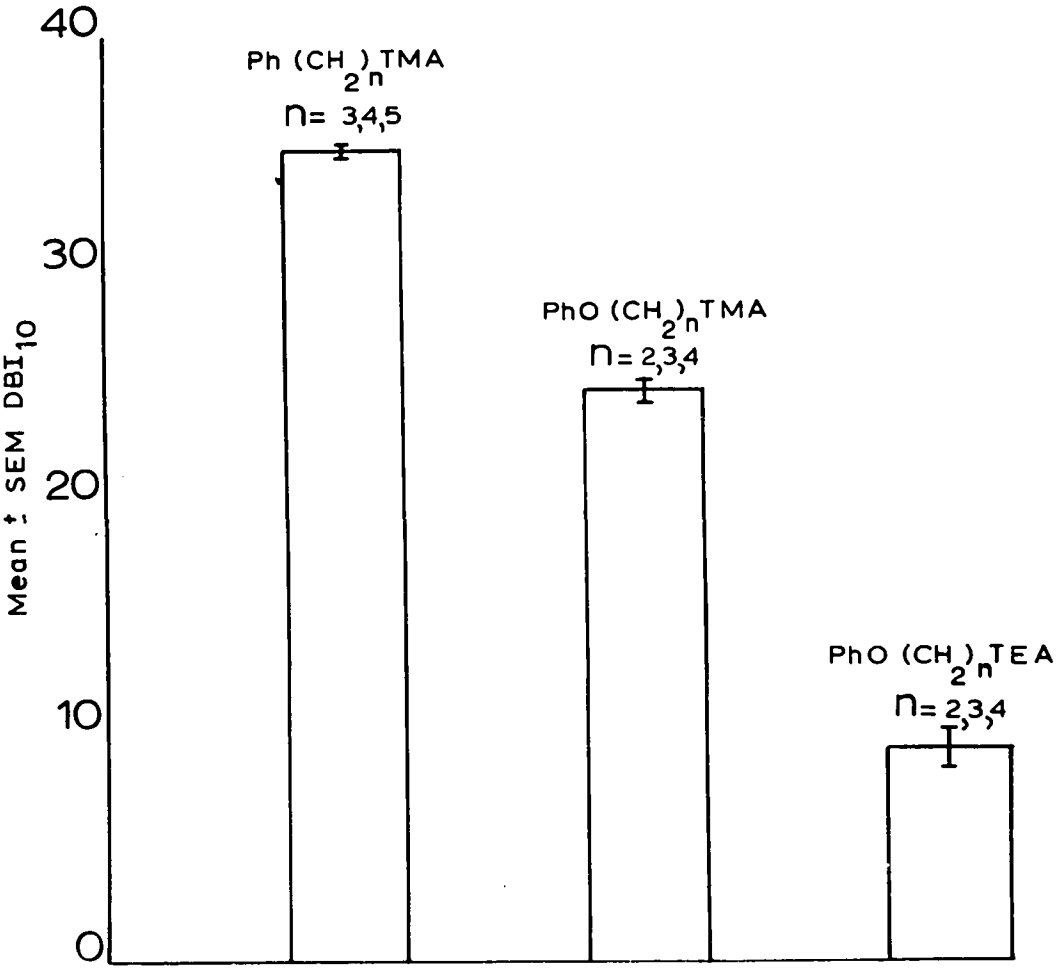

%
70
60
50
40
30
20
10
D.BI. ± S.E.M.
RATE DEPENDENCE
RAT PHRENIC NERVE -
DIAPHRAGM PREPARATION
$-O-(CH_2)_n-\overset{+}{N}(C_2H_5)_3\ Br^-$
2
3
4
5
6
n (chain length)
decamethonium
succinylmonocholine
succinyldicholine
gallamine
d-tubocurarine

FIGURE 13

Interseries comparison of DBI_{10} in compounds having 3 to 5 atoms separating the benzene ring from the quaternary nitrogen - rat diaphragm .

FIG. 13

Interseries Comparison of DBI_{10}'s in Compounds having 3 to 5 atoms separating the benzene ring (Ph) from the quaternary nitrogen ($\overset{+}{N}$) -Rat Phrenic Nerve - Diaphragm



in Fig. 12. It can be seen that this series of compounds showed little rate dependence with DBI_{10} values between 7.3 and 12.0; the least active phenoxyethyl TEA being not significantly different in this respect from the most active phenoxypentyl TEA (P value $>$ 0.1). The latter compound gave a DBI_{10} which was not significantly different (P value 0.1 - 0.5) from the least rate dependent clinical compound, succinyldicholine.

(e) Interseries Comparisons

It is possible from Table 8 and the composite results presented in Fig. 10, 11, and 12 to compare the rate-dependency of the analogues with the same number of atoms (counting both oxygen and carbon atoms) between the benzene ring and the quaternary nitrogen atom. Compounds with 3, 4 and 5 such atoms exist in all three homologous series. The mean DBI_{10}'s for the compounds with these atomic-lengths in the phenylalkyl TMA, phenoxyalkyl TMA, and phenoxyalkyl TEA series was 34.9 $\pm$ 0.26, 24.6 $\pm$ 0.39 and 8.9 $\pm$ 1.11 respectively (Fig. 13). This indicates the low rate dependence of the triethylated phenoxyalkyl TEA homologous compared to the corresponding trimethylated phenoxyalkyl TMA and phenylalkyl TMA salts. Phenylpropyl TMA, phenoxyethyl TMA, and phenoxyethyl TEA, the compounds chosen as reference standards in evaluating EPMR's above, show decreasing rate dependence in that order.

2. Cat Tibialis Anterior Preparation In Vivo (DBI_5)

Rate dependence experiments on the cat tibialis anterior preparation were carried out to the extent allowed by a very limited supply of drugs. The results are shown in Table 8.

(a) Clinically Used Agents

The muscle relaxant activity of the clinically used competitive compounds, gallamine (DBI_5 17.5 $\pm$ 1.95) and d-tubocurarine (DBI_5 14.5 $\pm$ 1.77) was associated with a significantly greater degree of rate dependence than was the depolarizing agent, succinyldicholine (DBI_5 2.6 $\pm$ 0.61) (P value 0.01) .

(b) Phenylalkyltrimethylammonium Series (Phenylalkyl TMA)

Phenylpropyl TMA used as the reference standard in other experiments with this series of compounds was the only representative tested on the cat tibialis preparation. As seen in Table 8 it exhibited no rate dependence in the two assays performed (DBI_5 1.6 $\pm$ 1.27) .

(c) Phenoxyalkyltrimethylammonium Series (Phenoxyalkyl TMA)

Assays for rate dependence of phenoxyalkyl TMA salts were carried out with phenoxybutyl TMA, phenoxypentyl TMA, phenoxyhexyl TMA and phenoxyoctyl TMA. There appeared to be a progressive decrease in rate dependence from a DBI_5 of 18.0 for phenoxybutyl TMA (one assay) to 8.5 $\pm$ 2.93 for phenoxyoctyl TMA (four assays).

(d) Phenoxyalkyltriethylammonium Series (Phenoxyalkyl TEA)

In the phenoxyalkyl TEA series single assays were performed with phenoxyethyl TEA, phenoxypropyl TEA, phenoxybutyl TEA and phenoxyhexyl TEA. The only compound likely to show any appreciable rate dependence is phenoxybutyl TEA which gave a DBI_5 of 32, about twice that of gallamine on the cat.

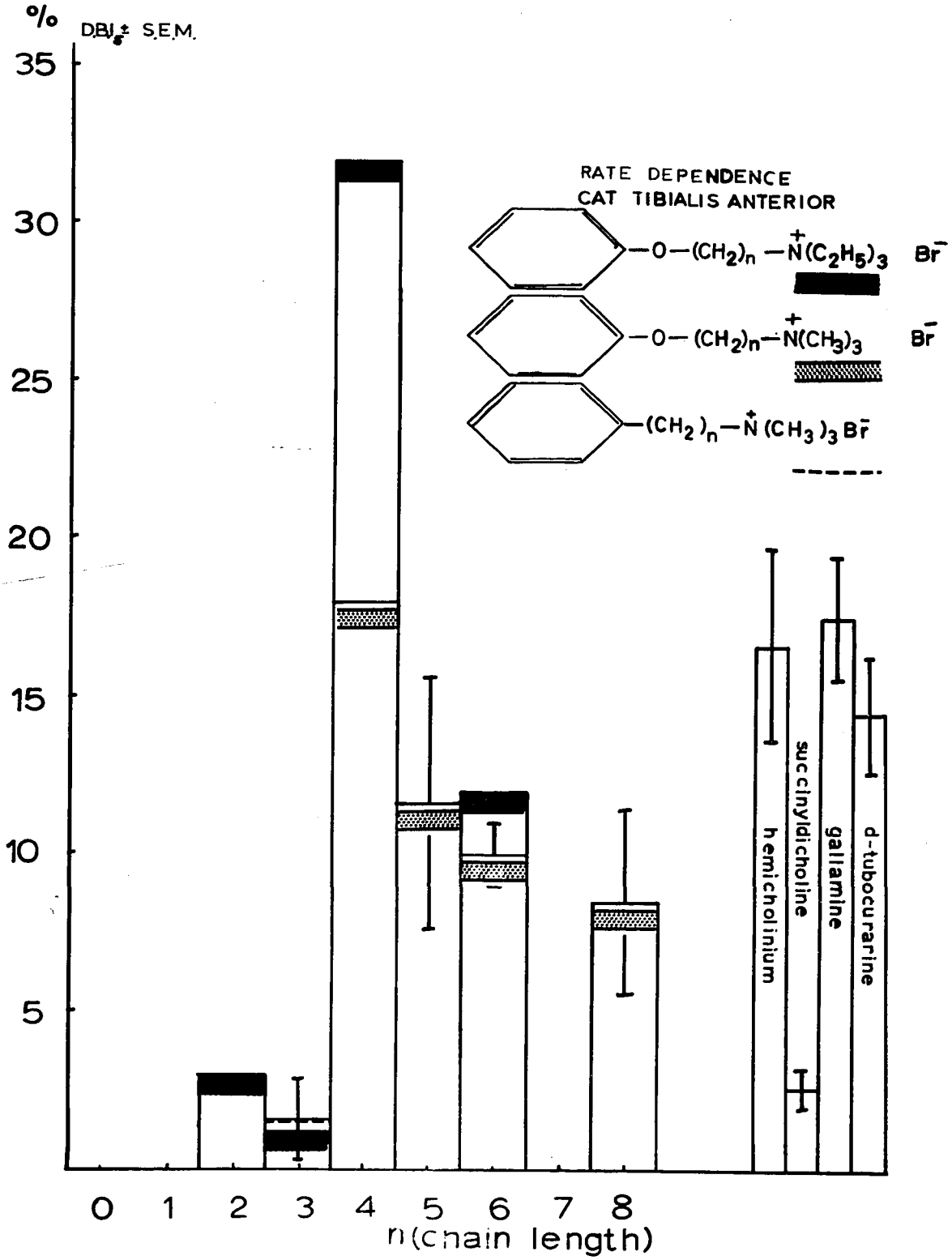
FIGURE 14

Effect of chain-lengthening on

differential blocking index (DBI_5)

- Interseries Comparison

- Cat Tibialis


%
DBI ± S.E.M.
35
30
25
20
15
10
5
RATE DEPENDENCE
CAT TIBIALIS ANTERIOR
—O—(CH2)n—N+(C2H5)3 Br−
—O—(CH2)n—N+(CH3)3 Br−
—(CH2)n—N+(CH3)3 Br−
hemicholinium
succinyldicholine
gallamine
d-tubocurarine
0 1 2 3 4 5 6 7 8
n (chain length)

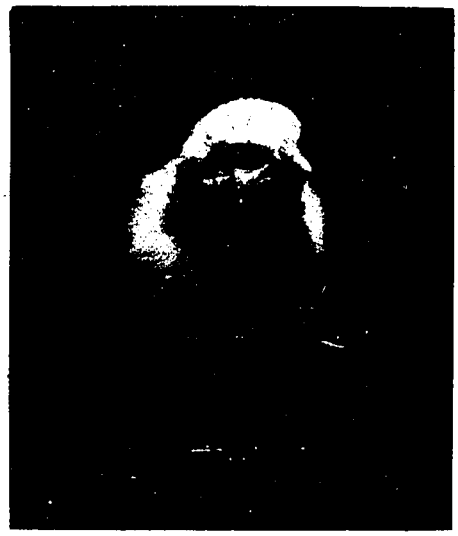
(e) Interseries Comparisons

Assays were performed on the cat which allowed a comparison of the rate dependency exhibited by compounds with a total of 3, 5, and 7 atoms separating the benzene ring from the quaternary nitrogen atom (Table 8, and composite Fig. 14). The phenylpropyl TMA analogue gave a DBI_5 similar to phenoxyethyl TEA, the corresponding analogue with 3 atoms in the chain, indicating no rate dependence in either case. The highest degree of rate dependency on the cat was recorded in the single assays with phenoxybutyl TMA (DBI_5 of 18) and phenoxybutyl TEA (DBI_5 of 32). The compounds with 7 atoms in the chain, phenoxyhexyl TMA and phenoxyhexyl TEA, gave DBI's of 10 and 12 respectively.

FIGURE 15

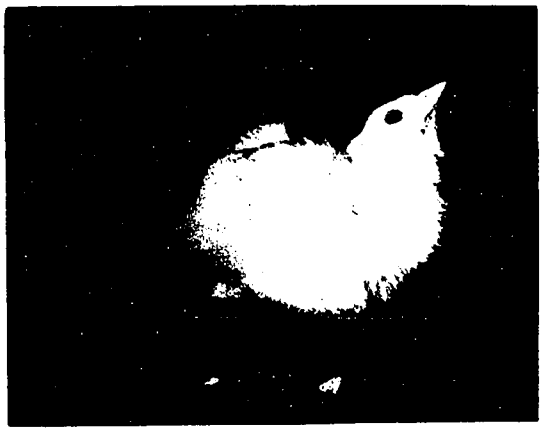
Effect of saline and clinically used neuromuscular relaxants on unanaesthetized chickens.

UNANAESTHETIZED CHICKENS



saline

10μM
d-tubocurarine

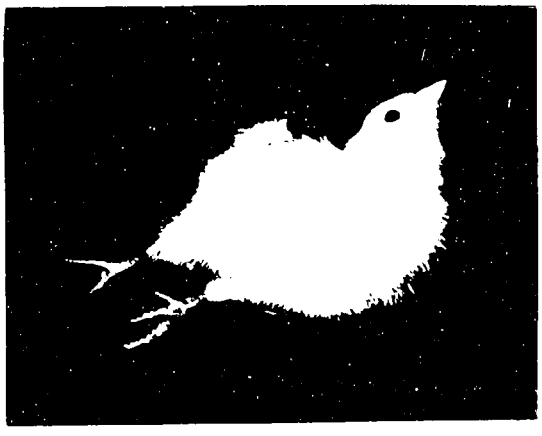


10μM
succinyldicholine

UNANAESTHETIZED CHICKENS

saline

10 μM
d-tubocurarine

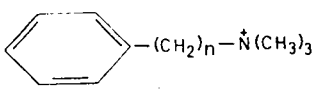


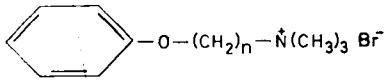
10 μM
succinyldicholine

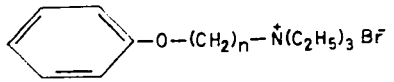
FIGURE 16

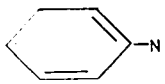
Type of paralysis caused by test compounds (intraperitoneal injections) to unanaesthetized chickens.

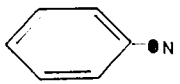
UNANAESTHETIZED CHICKENS - TYPE OF PARALYSIS

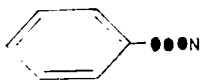




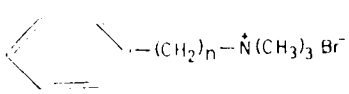


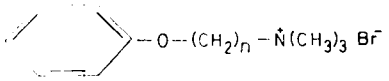


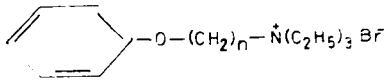


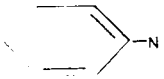


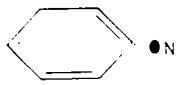
UNANAESTHETIZED CHICKENS - TYPE OF PARALYSIS

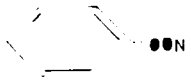


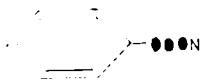


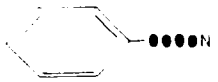


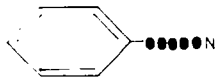


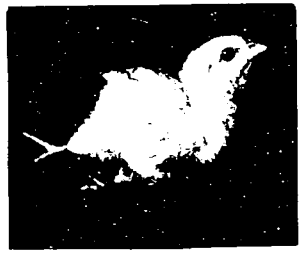


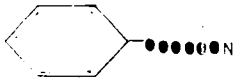


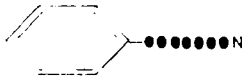


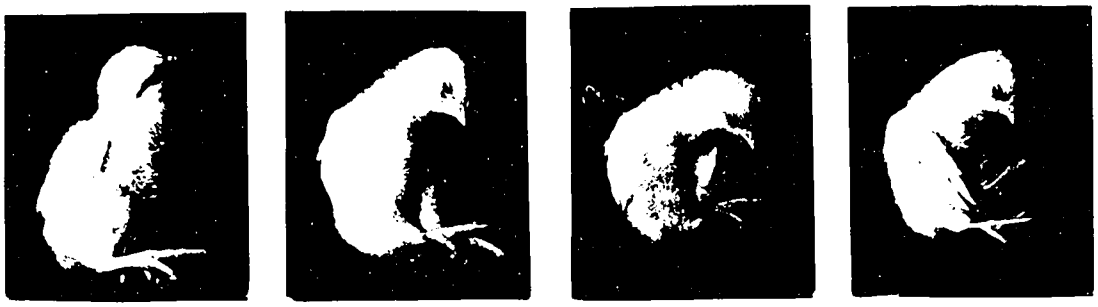


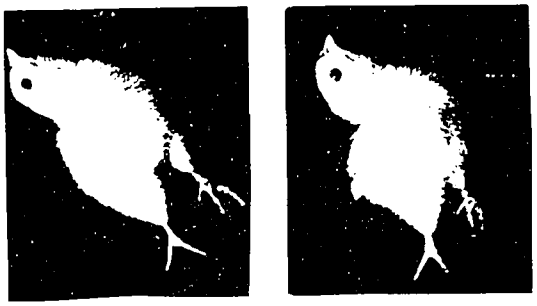












F. Unanaesthetized Chickens

The photographs in Fig. 15 and Fig. 16 show the type of paralysis produced in 6-day old chicks by intraperitoneal injections of adequate (10 - 40μM) doses of the clinically-used and test drugs respectively. Fig. 15 clearly demonstrates the flaccid-type of paralysis produced by d-tubocurarine as opposed to the spastic type of paralysis following succinyldicholine administration, and the lack of effect of a similar volume of saline. Fig. 16 shows the type of paralysis caused by the phenylalkyl TMA, phenoxyalkyl TMA, and phenoxyalkyl TEA salts. In this figure the representative photographs are arranged so as to compare horizontally the type of paralysis produced by analogous compounds (same number of atoms separating the benzene from the nitrogen) in each of the three series.

The phenylalkyl TMA salts available (phenyl TMA to phenylpentyl TMA) all produced succinyldicholine-like spastic paralysis. Similar spasticity was produced by all members of the phenoxyalkyl TMA series (phenoxyethyl TMA to phenoxydecyl TMA). The higher members of this series (heptyl to decyl) although exhibiting an initial spasticity often passed to a flaccid-type of blockade within 20 to 30 seconds (not illustrated).

The complete series of phenoxyalkyl TEA compounds available (phenoxyethyl to phenoxyhexyl) on the other hand failed to produce any evidence of spasticity, all of them like d-tubocurarine produced a flaccid paralysis.

The photographs presented in Fig. 16 are typical representatives of between 2 and 5 chicks tested on each drug. The amount of drug

administered in these non-quantitative experiments caused death,which was secondary to respiratory paralysis, in about 80% of the chicks.

VI. DISCUSSION

(a) Introduction

The investigation of the interference of chemical compounds with synaptic transmission has long been of interest and of great importance. Information about the interaction of some of the more specific synaptic blockers with the appropriate receptor molecules, however, is still obtainable only by indirect methods. Attempts to isolate the receptor and study more definitively the drug-receptor interactions are still fraught with difficulty and uncertainty.

Essentially the only available means of studying possible drug-receptor interactions is by extrapolating from drug-response relationships, the relationship between the former and the latter in the case of the neuromuscular synapse again being uncertain.

As drugs may mimic, potentiate, or inhibit synaptic transmission by various means, a study of activity at cholinergic synapses must involve both qualitative and quantitative studies. These should strive to elucidate the site and type of activity as well as the degree of effectiveness. In such a study as the present when some of the compounds are being studied for the first time it is of importance to study relative activity in both *in vivo* as well as *in vitro* preparations. By this means, using multicellular preparations, possible clinical usefulness can be predicted and also suggestions made as to which, if any,

of the new compounds deserve further, more definitive, studies on single muscle-fibres by means of intracellular electrophysiological recording techniques.

The present study has been an attempt to study the effect of altering the structure of choline phenyl ether (phenoxyethyl TMA) upon the observed synaptic stimulant (agonist) and blocking (antagonist) activity. By studying the effect of chain lengthening, (phenoxyalkyl TMA series), of replacement of the ether oxygen by a carbon (phenylalkyl TMA series) and of substituting ethyl radicals for the methyl radicals in the cationic head (phenoxyalkyl TEA) it was hoped that more information would be obtained as to the structural requirements necessary for agonist and antagonist activity of such compounds. In addition it was hoped that by means of some more qualitative screening programs some evidence as to the exact site or sites of action within the synapse and the type of inhibitory effect produced would be obtained.

(b) Agonist Activity

Agonist or cholinomimetic activity at neuromuscular synapses can be determined by intra- or extra-cellular recording techniques using micropipettes, but in a routine screening process this is costly, time-consuming and moreover the production of a depolarization of the cell membrane may or may not indicate the ability of the compound to produce a maximal contracture of the whole muscle mass. Many compounds produce a degree of depolarization associated with the blocking phase of activity (Burns and Paton, 1951). For these reasons valuable preliminary information can be obtained from studies upon the "slow" muscle fibres of the frog and chick. According to Ginsborg (1960) the contracture

produced by slow fibres of the chick biventer, similar to the frog rectus (Kuffler and Vaughan Williams 1953) is due to the depolarization caused by the drug acting on numerous end-plates scattered on the entire surface of the muscle fibre.

Information about acetylcholine-like activity at the neuromuscular junction is quite readily obtainable by testing compounds on slow-contracting muscle fibres: where contracture is indicated by recording base line changes, and block by alterations in contracture amplitude due to application of acetylcholine. Several muscle preparations have been described: Thesleff and Unna (1954) described the chick gastrocnemius, Ginsborg and Warriner (1960) the chick biventer cervicis, and Child and Zaimis (1960) the chick semispinalis. The chick biventer muscles contain both fast and slow-contracting fibres, so that drug effects on both types of fibre can be observed. Langley (1907) described the frog rectus abdominis preparation and Minz (1932) described the dorsal muscle of the leech, to demonstrate contracture. The former, because of its simplicity and basic accuracy in providing the necessary information relative to acetylcholine-like contracture was used in these experiments.

Recent publications by Eakins and Katy (1965, 1966a , b) suggest that an ideal preparation in which to study both the response of slow , multi- end-plate muscles, and fast twitch muscles in the mammal might be the anaesthetized cat in which the tension of the extraocular muscles (e.g. medial rectus) and tibialis muscles respectively are measured. In future studies, particularly of possibly clinically useful drugs, the former may prove to be a valuable addition to the battery of pharmacological tests.

Compounds of the phenoxyalkyl TMA series, available first in greatest amounts, were subjected to more investigation than the other two series. They were tested for acetylcholine-like, stimulant activity on the frog rectus abdominis *in vitro* and for both stimulant and blocking activity on the cat superior cervical ganglion *in vivo*.

Agonist activity was maximal with the original phenoxyethyl TMA compound (n=2) on the frog rectus, and, indeed, on the cat ganglion increasing the carbon chain length to n=3 (propyl) and longer abolished stimulant activity as evidenced by the failure to cause a contracture of the nictitating membrane. This latter observation is in agreement with previous intracellular electrophysiological studies with phenoxypropyl TMA on the isolated frog sympathetic ganglion preparation (Nishi and Koketsu 1960) and by Ginsborg and Hamilton (1963). These workers, using the apparatus described by Blackman, Ginsborg, and Ray (1963), showed that phenoxypropyl TMA caused a complete blockade of all orthodromic responses to preganglionic nerve stimulation without any measurable depolarization or effect upon the antidromic responses elicited by stimulation of the postganglionic nerve. This was in contrast to the effect of nicotine and phenoxyethyl TMA which depolarized the ganglion and abolished both orthodromic responses.

Hunt and Renshaw (1929) had previously found phenoxypropyl TMA to be much less active than phenoxyethyl TMA as a ganglion stimulant as indicated by the blood pressure (rise) response in a pithed, atropine-treated, cat. They found that the maximum rise obtained was 37 mm. Hg. with 20 mg. of propyl compound (I.V.) (compared to 95 mm. with 0.05 mg. of the ethyl compound) and that this was followed by a "comparatively brief" ..... "paralysing nicotine action." In the present experiments

in which injections of small amounts were made "close-arterially" to the ganglion only the phenoxyethyl compound caused a significant change in blood pressure in a few instances. The latter observation as well as the stimulant effect of the ethyl homologue on the ganglion(contracture of the nictitating membrane) led to the use of the phenoxypropyl TMA as the standard for comparison, in this series, rather than the phenoxyethyl TMA which was obviously qualitatively different in activity.

The stimulant activity of the phenoxyalkyl TMA compounds on the frog rectus was acetylcholine-like as it was inhibited by pretreatment of the preparation with 2×10^{-5} M d-tubocurarine. Of interest was the partial increase of agonist activity with the 5 carbon chain phenoxypentyl TMA after the 13 fold decrease in activity on increasing the chain length from 2 to 3 and 4 carbons. The pentyl homologue was significantly (2.19 times P value < 0.01) more active than the butyl and hexyl homologues (Table 6). That this is a "real" increase in activity is corroborated by the finding of Hamilton (unpublished results) that there is a similar and indeed parallel increase in agonist activity with the pentyl compound when this series was tested on the "slow" muscles of the chick biventer in vitro. As there is no reason to suspect the purity of any of these compounds (see analysis Appendix I Tables 1, 2 and 3), it may be that the 5-carbon chain molecule has the ability to closely approximate the position of the active sites in the acetylcholine (nicotinic) receptor postulated for the attachment of the phenoxyethyl TMA compound by Hey 1952. The 3 and 4 carbon compounds may be too short and the compounds with from 6 to 10 carbons may be too long to assume the appropriate position for receptor fit necessary for stimulation. (e.g. optimum distance between partial positive charge on ether oxygen and

cationic head (Hey 1952) .

It is of interest, however, as discussed later that the inability of these longer chain compounds to stimulate does not mean they have a low affinity for the receptors as they can still cause synaptic blockade (see cat ganglion and tibialis and rat diaphragm results).

In an attempt to describe further the change in the agonist activity of the phenoxyalkyl TMA's as the chain is lengthened the intrinsic activities have been determined by the method of Ariens (1954). By this means frog rectus muscles were exposed to step wise addition of drug until a maximal response was attained, and a linear relationship between increase in chain length and decrease in intrinsic activity was found,the longer members being partial agonists. The exact meaning of this estimate of activity, however, is in some doubt as if these values are expected to give a real indication of relative stimulant or agonist activity, one has to assume that to obtain the maximal response (100% contracture as obtained with acetylcholine), one hundred percent of the receptors have to be occupied. Stephenson (1956) suggests that this need not be the case and that active agonists may produce a maximal response by occupying only a small percentage of the available receptors leaving "spare receptors" unoccupied. Moreover, it is very likely that in the present experiments the exposure of the muscle to increasing concentrations of the longer chain compounds led to the production of a nicotine-like blockade or desensitization of the muscles. Such an ensuing blockade may or may not have been responsible for the inhibition of the contracture before a greater or even maximum response (intrinsic activity of 1.0) could be produced, the exact relationship between depolarization

and inhibition at the neuromuscular synapse not being clear (Katz and Thesleff 1957).

Despite this theoretical uncertainty, however, one may predict from these observations on the frog that any neuromuscular blockade of clinical usefulness would be associated with less initial stimulation (muscle fasciculations) when the longer chain phenoxyalkyl TMA compounds are used compared to the shorter more acetylcholine-like, phenoxyethyl TMA.

It is interesting to compare the effect of chain lengthening of these phenoxyalkyl TMA compounds with similar studies of the analogous simple alkyl TMA's studied for instance by Raventos (1937) also on the frog rectus. The most active homologue (agonist) on the frog rectus studied by Raventos was butyltrimethylammonium which was four times as active as tetramethylammonium. Further increasing the chain length to amyl (five-carbon polymethylene chain), hexyl, and heptyl resulted in EPMR's of 4, 8, and 40, respectively relative to one molecule of the butyl compound. The octyl trimethylammonium and longer homologues were reported to be inactive as agonists and were noted to be antagonists of the lower members of the series.

From the experimental tracings of the frog rectus records in Raventos' paper it is possible to conclude that a contracture of the rectus could be obtained with about 2.5×10^{-6} molar butyl TMA. This is similar to the concentration of phenoxyethyl TMA which produced about 50% maximal contracture in the present experiments. Heptyl TMA, however, which might be considered to be similar in overall length to the phenoxyethyl TMA and phenylpropyl TMA molecules, was calculated, from Raventos' results, to be only one fortieth as active as the latter benzene ring-

containing compounds. These observations suggest that the presence of a benzene ring in such alkylmono-onium compounds, instead of an equivalent polyethylene chain length, results in compounds which retain agonist as opposed to antagonist or partial agonist activity.

It is also apparent that, at least on the frog rectus preparation, nicotine stimulant activity (agonist activity) is not affected by substitution of a methylene (CH_2) for the ether oxygen (compare phenoxyethyl TMA and phenylpropyl TMA).

The present studies of the agonist activity of the phenylalkyl TMA series on the frog rectus indicated that activity increases as the number of carbon atoms separating the benzene ring from the cationic head are increased from zero to three. It will be of much interest to study the effect of further increasing chain length in this series to see if there is a decrease in activity from phenylpropyl to phenylbutyl TMA corresponding to the phenoxyethyl to phenoxypropyl TMA decrease.

It is likely that the newly acquired phenylalkyl TEA compounds will be antagonists rather than agonists or even partial agonists and will thus allow a determination of their affinity for the acetylcholine receptors (Affinity Constants). Eventually by the comparison of analogous blocking and stimulating compounds as did Barlow, Scott and Stephenson (1963) it should be possible to arrive at some conclusions regarding the relative importance of efficacy (or intrinsic activity) upon, and of affinity for the receptor in determining the stimulant (agonist) activity of such cholinomimetic compounds.

(c) Antagonist Activity - Cat Sympathetic Ganglion In Vivo

The results obtained with the phenoxyalkyl TMA series on the cat ganglion are interesting in that although stimulant activity is apparently abolished in compounds with an alkyl chain longer than 2 carbon atoms the synaptic blocking activity, initially decreased, again increased markedly up to the heptyl and octyl homologues. It would be tempting to suggest that phenoxyethyl TMA may be added to the list of drugs such as acetylcholine, nicotine and tetramethylammonium which Paton and Perry (1953) described as depolarizing ganglionic blockers and the compounds from phenoxypropyl TMA to phenoxydecyl TMA to the list of compounds which block transmission by "competition with acetylcholine" . This classification of ganglionic blocking drugs has been discussed at length by Hamilton (1961) and it is suggested that a better term to use for the latter category would be non-depolarizing rather than competitive. The term , competitive, suggests a situation similar to that recognized at the neuromuscular synapse on the administration of e.g. d-tubocurarine, (VanMaanen 1950 and Jenkinson 1960), i.e. a form of reversible competition obeying the mass-action laws. Until the blocking action of the long chain phenoxyalkyl TMA's has been shown to be reversible by increasing the concentration of agonist, as has been shown for the relationship between lobeline and some ganglion blocking drugs on afferent nerve endings in the cat (Suwandi and Bevan 1966), they should not be assumed to be competitive or surmountable in the commonly accepted meaning of the word.

The commonly used ganglion blocking drug, hexamethonium, is also known (Paton and Perry 1953) to block ganglionic transmission without any evidence of depolarization although it has been reported that in ganglia perfused with a solution deficient in potassium, in denervated

ganglia , or in anoxic situations , hexamethonium can cause a depolarization (Perry 1956, Perry and Reinert 1954, 1955). Such changes , although unphysiological, remind one of the complexity of such studies and of the possible effect of environmental ionic factors not only upon the degree of blockade but upon the type of blockade. It is perhaps in the use of ganglionic blocking drugs during anaesthesia and in particular in patients with hypokalemia resulting from excessive diuretic therapy that such unphysiological conditions might prevail and the effectiveness of the ganglion blocker might be affected. Indeed, such situations have been described with the use of d-tubocurarine at the neuromuscular synapse, where patients suffering from bowel obstruction with associated profuse vomiting, hypokalemia, hypochloremia and hyponatremia exhibit an abnormally prolonged blockade not relieved by an anticholinesterase, neostigmine (Hunter 1952). It is likely that the observations of Perry (1956) with hexamethonium on the potassium-deficient ganglion could be repeated with phenoxynonyl TMA as Hamilton (unpublished results) has shown that both hexamethonium and phenoxynonyl TMA, in concentrations which did not cause a contracture of the isolated chick biventer muscle, promptly caused a contracture when the potassium concentration of the Kreb's solution was lowered to a tenth of its normal level.

The ganglionic blocking activity of the most active phenoxyoctyl TMA homologue has been shown by Hamilton using a similar technique to be an eighth as active as the potent clinically used agent trimetaphan camphorsulphonate (Arfonad, Hoffmann LaRoche), (unpublished results). By inspection of the doses used it also appears likely that phenoxyoctyl TMA is at least as active as hexamethonium on the cat superior cervical

ganglion. This raised the question as to whether the phenoxyethyl moiety ($C_6H_5OCH_2CH_2$-) is as effective in non-specific binding as the second trimethylammonium group is considered to be by Gill (1959). This second point of attachment is considered by Gill to result in a stabilization of the membrane, preventing the re-orientation necessary for depolarization and resulting in a nondepolarizing type of inhibition.

The marked similarity between the graph of ganglionic blockade against chain length and those obtained by Hamilton, Hersey and McCurrach (see AppendixII) for anticholinesterase activity might suggest that anticholinesterase action was contributory to the observed ganglionic blockade. It is not easy with such a mode of intra-arterial injection to determine whether or not cholinesterase inhibitory concentrations are present at the ganglion-receptor sites, even if one was to assume that extrapolation was possible from ox red blood corpuscle acetylcholinesterase in vivo. However, it is unlikely that the blockade caused by the longer homologues, which did not appear by this technique to have any depolarizing component, was due to an anticholinesterase action. The phenoxyethyl TMA which did have a ganglionic stimulant action has also been shown to be a powerful direct nicotine-like agonist in its own right (e.g. frog rectus, frog ganglion, etc.) and thus an indirect ganglion stimulation due to acetylcholine build-up resulting from an anticholinesterase action is unlikely to contribute much to the observed effect particularly as the pI_{50} for phenoxyethyl TMA was only 2.7 ± 0.02 .

It is noteworthy that whereas ganglion-blocking activity in this series rose to a maximum at the phenoxyoctyl TMA (n=8), and decreased on further chain lengthening, the anticholinesterase activity

continued to rise with chain lengthening, phenoxydecyl TMA (n=10) having a pI_{50} of 5.4 ± 0.07 . This suggests that although there are similar receptors on the enzyme and ganglion as indicated by the initial parallelism on chain lengthening, that the decline in activity in the in vivo cat preparation after the octyl analogue might be due to a diffusion barrier or some impedance to the accumulation of adequate ganglionic concentrations. In the in vitro studies on the enzyme there is obviously a readily available receptor and here distribution of the drug to the site of action is not a complicating factor.

Studies on the ganglion with the other two series (phenylalkyl TMA and phenoxyalkyl TEA) and with a new series of phenylalkyl TEA analogues are now being performed in collaboration with others in this laboratory. The possible presynaptic inhibitory action of these new compounds on the cat superior cervical ganglion has not been investigated to date. Conceivably a study similar to that performed on the neuromuscular synapses used in this study would indicate, by evidence of frequency-dependent blockade, any likelihood of a significant presynaptic component.

(d) Antagonist Activity - Neuromuscular Junction

1. Introduction

It was of interest to study the effect of chain-lengthening upon the neuromuscular blocking activity of the phenoxyalkyl and phenylalkyl compounds. Relative neuromuscular blocking activity has been determined upon the cat tibialis preparation (Brown 1938) *in vivo* and the rat phrenic-nerve diaphragm preparation (Bulbring 1946) *in vitro*. These preparations were chosen in an attempt to obtain the maximum amount of information, keeping in mind the species and indeed muscle variation in sensitivity to the various classes of blocking drugs.

Species sensitivity to different drugs varies considerably. Generally, the variation in sensitivity to non-depolarizing type agents is much less than that shown by depolarizing agents. Paton and Zaimis (1949) have shown that sensitivity to d-tubocurarine decreases in the order rat, mouse, rabbit, cat; $\frac{\text{activity in cat}}{\text{activity in rat}} = 0.5$ i.e., the sensitivity in the cat is half that in the rat to d-tubocurarine, whereas decamethonium sensitivity decreased in the order cat, man, rabbit, with $\frac{\text{activity in cat}}{\text{activity in rat}} = 200$ i.e., sensitivity in cat is 200 times that in the rat. Estimates of decamethonium potency relative to d-tubocurarine may vary by as much as 100, from about 1/10 as active on the rat to about 10 times as active on the cat. Similarly, different muscles from the same animal may show marked differences in sensitivity: "white" muscles are less sensitive than "red" muscles to acetylcholine-like, desensitizing, drugs and become even more insensitive with repeated dosage when tachyphyllaxis occurs (Paton and Zaimis 1949). Such evidence serves to emphasize that conclusions about neuromuscular activity based

on but a single test are likely to be most misleading.

Experiments on the rat phrenic nerve-diaphragm preparation were thus carried out not only because it is an *in vitro*, mammalian, preparation which allows a steady state or equilibrium blockade to be established without relying upon constancy of blood flow and of metabolism or excretion, but also as it is a representative of the so-called "red" muscles (Jewell and Zaimis 1954). The cat tibialis nerve-muscle preparation is representative of "white" muscle and despite the problem of not really knowing the concentrations of drug at the receptor level might serve as an indicator of possible clinical usefulness. Pertinent to this discussion are the observations of Paton and Waud on the cat tibialis and sartorius muscles. These authors (1962, 1967) investigated the general relationship between the action of blocking agents at the end-plate and their effect on neuromuscular transmission. In this they determined an approximate index of the safety factor for transmission (i.e. "the extent of interference with the synaptic mechanism that can exist without failure of transmission"). They warn that the existence of a substantial margin of safety, (4.1 and 12 for the most and least sensitive groups of fibres) influences considerably the interpretation of the time course of action of blocking drugs and of comparisons between responses to nervous excitation and drug injection. In the present experiments all drugs were compared only in effectiveness in blocking the response to nerve stimulation and as the margin of safety is a property of the muscle independent of the drugs used (Paton and Waud 1967) it is likely that the results here obtained are still meaningful.

2. Type and Site of Blockade at Neuromuscular Junction

(a) Postsynaptic Activity

It is apparent from the experiments employing direct (muscle) stimulation compared to indirect (nerve) stimulation that the test compounds produce a synaptic blockade of the rat and cat muscles without any effect upon the responsiveness of the muscle fibres. The experiments discussed above, describing the marked stimulant activity of some on the acetylcholine-receptors of the frog rectus, suggest a post-synaptic receptor site of activity. The experiments on the frog in conjunction with those using unanaesthetized chicks suggest that the phenoxyalkyl TMA and phenylalkyl TMA series likely cause a succinyldicholine-like or indeed acetylcholine-like depolarizing blockade. It was regularly observed that, at least early in the experiment, the administration of these TMA compounds to the tibialis by the intra-arterial route caused the typical succinyldicholine-like potentiation of the maximal single muscle twitch elicited by nerve stimulation. Later in the experiments there was not a regular initial potentiating effect preceeding blockade of the tibialis, suggesting that the type of blockade had perhaps changed from the depolarizing type to a "dual-block" . This was also indicated by the increased time required for recovery of the maximal twitch height after a standard 0.3 ml. arterial injection.

In all cases in the chick when a TMA compound was used there was a clearly demonstrable initial period of spastic paralysis (i.e. initial stimulation evidenced by the extended neck and outstretched legs and feet). With the longer chain phenoxyalkyl TMA compounds, (heptyl to decyl) this period of stimulation was greatly shortened and within twenty seconds passed, before death, to one of flaccid paralysis. The phenyl-

alkyl TMA compounds tested all showed only the depolarizing component of paralysis and the phenoxyalkyl TEA analogues failed in any dose to cause spasticity and resulted always in a flaccid, apparently curare-like, paralysis.

It is important before comparing and contrasting the relative neuromuscular blocking activity of these homologues and analogues to have some idea of whether or not they are acting in the same way. To this end a study was made on the cat of the reversibility of the blockade on administration of edrophonium, (Tensilon, Hoffmann-LaRoche) an anti-cholinesterase. It was soon apparent that the degree of reversibility obtained by edrophonium during blockade by a curare-like drug bore a consistent relationship to the number of previous injections of the agent. The result was that following an initial injection of d-tubo-curarine or gallamine a considerably larger degree of reversal by edrophonium was observed than when similar, subsequent, tests were made on the same preparation. As it was feared that any tendency to be reversed by edrophonium would be masked by super-saturation of the preparation beyond concentrations causing 100% receptor occupancy a steady state, less than maximal, blockade was attained. Using an adjustable slow-infusion apparatus the blocking drug was injected intra-arterially to the muscle in a concentration and rate which caused a constant incompletely inhibited response at 12 shocks per minute. Once this was obtained an edrophonium infusion was given into the contra-lateral femoral vein and the effect on the height of the twitch was observed. In every case d-tubocurarine and gallamine were somewhat reversed but in no case was the neuromuscular blockade caused by succinyldicholine or any of the phenylalkyl TMA or phenoxyalkyl TMA or

TEA compounds reversed by edrophonium administration. In these experiments the dramatic reversal of the curare-like agents and lack of reversal of the synthetic compounds makes it extremely likely that the latter are non-curare-like, non-competitive in nature. It may be argued that rather than being non-competitive these compounds in fact exhibit the quasi-equilibrium state discussed by Paton and Waud 1967. They found that when agonists and antagonists were tested on cat nerve-muscle preparations over a wide range of dosage that they did not conform with the conditions of full competitive equilibrium. They concluded that this arose not because of some interfering non-competitive process, but because, during the relatively brief exposure to their agonists, the equilibrium between the antagonist and the receptors is not significantly disturbed. In the present experiments, however, there is no reason to suspect that the acetylcholine build-up in the presence of edrophonium was either temporally or quantitively inadequate to reverse a true competitively-acting drug particularly with the parallel d-tubocurarine and gallamine experiments as controls. Thus one might conclude that none of the phenyl or phenoxy analogues are competitive curare-like drugs. The phenyl and phenoxyalkyl TMA compounds are most likely depolarizing-desensitizing dual blockers like succinyldicholine and the phenoxyalkyl TEA analogues non-competitive desensitizing drugs as the latter showed no agonist activity on any preparation. This is contrary to what was expected and, in fact, hoped. However, further evidence of the non-competitive interaction of the long chain, phenoxy-nonyl TMA, and phenoxyalkyl TEA series has recently been provided by Hamilton (unpublished work). Hamilton studied the antagonism of these compounds to the acetylcholine contracture response of the frog rectus,

and found that whereas a 500-fold change in the concentration of d-tubocurarine resulted in only a five-fold change in the affinity constant (K) (Gaddum 1943) that a four to twenty-fold increase in the concentration of the phenoxyalkyl TEA compounds resulted in a 20 to a 140-fold change in the affinity "constant" . Similarly an eight-fold increase in the concentration of phenoxynonyl TMA resulted in a change in K from 71 x 10^4 to 49200 x 10^4 , clearly indicating that binding of the antagonist to the receptor is independent of the agonist concentration (acetylcholine) and that the blockade is indeed non-competitive.

Now that the phenylalkyl TEA series has been made available to us by R.B. Barlow similar studies will be performed again to search for characteristics resulting in a competitive type of synaptic blockade, although by analogy with the present study we may not expect these compounds to be competitive.

On the assumption that the phenoxy and phenylalkyl compounds studied to date act primarily upon the postsynaptic receptor resulting in either a depolarizing and/or a desensitizing type of blockade to the effect of acetylcholine released by nerve stimulation it would be appropriate now to discuss the structure-activity relationships suggested by this study. To have established the drug effect to be at the synapse as described would have sufficed in the so-called classical era of motor end-plate studies, (1836-1947). Couteaux demonstrated in 1947 the sub-neural apparatus, thus opening up a whole new area of investigation by histochemical methods and shortly thereafter this approach was extended to the electron microscope. It is thus necessary to try to evaluate at what area or areas of the synapse any given agent produces its effect. To this end, the limited anticholinesterase studies and rate dependence

studies were performed.

(b) Anticholinesterase Activity

As in the ganglion discussion it might be concluded that anticholinesterase activity, maximal in the long chain phenoxyalkyl TMA compounds, likely contributes little to the observed neuromuscular blockade caused by these compounds. The most active inhibitors of acetylcholinesterase were the least effective depolarizing compounds as evidenced by: initial muscle fasciculation and potentiated twitch in cat, contracture of frog and chick muscles and intrinsic activity measurements on the frog rectus. Anticholinesterase activity of the phenylalkyl TMA's has not been determined and the phenoxyalkyl TEA series, which failed on any test to show agonist effect, is extremely unlikely to possess such properties. Antagonism rather than potentiation of acetylcholine was observed on the frog rectus.

(c) Presynaptic Activity

As at least some of the blocking activity of these compounds might be due to an action upon the nerve terminal (presynaptic) site, experiments were performed on both the rat diaphragm and the cat tibialis to explore this possibility. It has been observed repeatedly that neuromuscular blockade by d-tubocurarine and a number of other compounds is greater at faster than at slower rates of nerve stimulation (e.g. Preston and VanMaanen 1953). Blackman (1963) suggested that the measurement of frequency dependency of block at a neuromuscular junction may provide a useful method of studying the effect of drugs on acetylcholine release (a presynaptic effect). It has been shown by Straughan

(1960) and Krnjevic and Mitchell (1961) that acetylcholine release declines relative to the frequency of stimulation, at fast rates. The latter authors have shown that on the rat diaphragm the mean release of acetylcholine was 0.12 p mole per impulse at 2 to 5 impulses per second and 0.025 p mole per impulse at faster rates (10 - 20 per second), and that output per impulse was not significantly affected by temperatures between 10 and 37°C. Thus, if an agent which has no effect on the presynaptic mechanisms is used then muscle fibres which are only just activated at a slow rate of stimulation will fail to be activated at a fast rate when the acetylcholine output is less. This may be simply the unmasking of the normal distribution of receptor sensitivities to acetylcholine, and a rate-dependent blockade would be expected primarily if the postsynaptic blocker was truly reversibly competitive in nature (Blackman and Ray 1964). Once a hundred percent receptor saturation has been attained, only competitively blocking drugs would show responses at the slower rates of nerve stimulation when acetylcholine concentrations adequate to disrupt the antagonist-receptor bond might be released. A desensitizing (non-competitive) or a depolarizing drug might not be expected to show a differential rate-dependent blockade by a similar argument. The desensitizing agent, obviously, will not be displaced by acetylcholine, the blockade being independent of agonist concentration. In the case of the depolarizing drug in the less than maximal blockade situation the larger amounts of acetylcholine released at slow rates of nerve stimulation, rather than causing a larger response than that at fast rates, might well act synergistically with the depolarizing-blocking compound resulting in a potentiation of the blockade. For this reason the low rate dependency observed in Blackman's (1963) study with the

depolarizing agent decamethonium and in this study with succinyldicholine and decamethonium would not be unexpected.

By the adaptation of Blackman's techniques it was hoped that the discovery of a degree of rate dependency in excess of that found with d-tubocurarine would indicate possible presynaptic activity. A compound with no postsynaptic blocking effect or one which competitively blocked the postsynaptic, acetylcholine, receptors might show a more profound rate dependency if it also in some way interfered with the formation of acetylcholine or its release. The method used to date has involved alternate stimulation of the motor nerve with maximal shocks at 12 and 120 per minute in the rat diaphragm and 12 and 60 per minute in the cat tibialis experiments. The smaller range in the case of the cat was dictated by finding that the response waned readily at rates faster than 60 per minute even in the absence of a blocking agent. The index of rate dependency for which, in this laboratory, we have coined the name Differential Blocking Index DBI_{10} (rat) and DBI_5 (cat) was determined by slowly arriving at a concentration in the organ bath (rat) and a rate of intravenous infusion (cat) which caused a steady-state , just maximal, blockade of the response at the fast rate. The residual response at the slow rate was then expressed as a percentage of its unblocked twitch height and used as a DBI_{10} or DBI_5 value.

The rate dependency studies on the rat are more complete and perhaps more meaningful. The wider range of 10 rather than 5-fold increase in the rate allowed a more vigorous test. In addition fewer compounds were tested upon the cat preparation due to the poor supply of compound which remained at this stage in the study and the necessity of conserving some for the edrophonium reversal studies.

On the rat, estimates of DBI_{10}'s for the phenoxyalkyl TMA compounds were all less than 32, the longer chain homologues being significantly less rate dependent (lower DBI_{10}'s) than the shorter ones. Values of DBI_{10} for the phenylalkyl TMA series were all higher and ranged from 30 (butyl) up to 50 for the non-carbon chain, parent phenyl TMA. Unexpectedly the phenoxyalkyl TEA series from the ethyl to the heptyl showed least rate dependency with DBI_{10}'s of between 7 and 10. On the rat a cross-comparison is possible between compounds in the three series with 3, 4 and 5 atoms separating the benzene ring from the nitrogen. The mean (and SEM) DBI_{10}'s for the compounds with these atomic lengths in the phenylalkyl TMA, phenoxyalkyl TMA and phenoxyalkyl TEA series were 34.9 ± 0.26 , 24.6 ± 0.39 and 8.9 ± 1.11 respectively (Fig. 13). The DBI_{10} found with d-tubocurarine was 29.3 ± 2.10 (19 Tests) and if only compounds showing significantly more rate dependence than this should be considered for further presynaptic studies obviously only the phenylalkyl TMA's qualify. If they do prove by further more critical tests (involving estimation of quantal size at rapid nerve stimulation and miniature end-plate potential frequencies (Gage and Quastel 1965) to be triethylcholine or hemicholinium-like then it may be that in molecules of this type the ether oxygen of the phenoxyalkyl salts is a hindrance to such activity perhaps encouraging preferential binding at other sites or interfering with attachment at the appropriate nerve terminal site. It will thus be of great interest to study our new series of phenylalkyl TEA salts for presynaptic activity as they also lack the ether oxygen but have a triethylated ammonium moiety.

The value for decamethonium of 25.0 ± 3.03 and for succinyldicholine and succinylmonocholine of 18.8 ± 2.24 and 29.0 ± 1.73

respectively seem rather high for depolarizing drugs at least by the above argument. It may be that at the stage in the experiment that these were randomly assessed a predominantly desensitizing rather than depolarizing blockade had ensued. Blackman (1963) also observed that later in the experiment the depolarizing compounds showed a higher rate dependency. It would be of interest to establish whether or not a second-phase of "dual block" had ensued at this stage (Jenden, Kamijo and Taylor 1951). The difference between succinyl di-, and succinyl mono-choline is interesting in that it might indicate the more depolarizing nature of the former (18.8 ± 2.24 and 29.0 ± 1.73). The high values of 39.8 and 60.3 obtained with the known presynaptic inhibitors hemicholinium (Schueler 1955, MacIntosh et al 1956,etc.) and triethylcholine (Bowman and Rand 1961 a and b, etc.) respectively, suggest that some meaning may be applied to this test. Moreover, the consistently high, though more variable, estimate of 56.7 ± 12.93 (12 tests) for gallamine triethiodide suggests that in addition to its postsynaptic d-tubocurarine-like activity it may well have a presynaptic effect.

The compound phenoxyethyl TEA studied in the present work, an analogue of gallamine triethiodide with one rather than three , $-O(CH_2)_2$ TEA,side chains on the benzene, however showed no rate dependency. It would be of interest to study in more detail the gallamine compounds earlier investigated by Bulbring and Depierre (1949).

More recent work in this laboratory (Hirst, unpublished results) has involved an improvement of this method of predicting presynaptic activity. In these experiments two hemidiaphragms are set up in the same organ bath, one indirectly stimulated (i.e. phrenic nerve) at 12 and the other at 120 shocks per minute. In addition, isometric

(Grass FT_{10}) strain gauges are used in conjunction with Grass P7 polygraphs instead of kymographical recording. The possible error in reading the "end-point" due to the mechanical interference of friction on the kymograph paper is thus obviated and a more rigorous test is allowed by keeping the rates constant without alternating. Perhaps a better method even than this would be an index involving a reading at 75 percent blockade of the fast response, at which the response can clearly be shown to be in an equilibrium steady-state, rather than at the 100 percent block stage.

The results on the cat tibialis, alas, are too few to allow any meaningful predictions. Likewise in these experiments, d-tubocurarine and gallamine triethiodide showed more rate dependency than the depolarizing drug succinyldicholine. In the cat experiments, however, hemicholinium showed remarkably low rate dependence and it is in retrospect that we now conclude that this should have been tested much earlier had it been obtainable. Only recently did we obtain an adequate supply and the address of a source of hemicholinium. It may simply be that enough patience was not exercised and the blockade produced within 20 minutes was caused by concentrations which acted post synaptically. The presynaptic inhibitory effects are usually slower in onset and apparently can be obtained with concentrations less than those producing significant postsynaptic blockade (Martin and Orkand 1961, Thies and Brooks 1961). A similar criticism may be levelled at the work of Ferry and Norris (communication presented at the Nottingham meeting 1967 of the British Pharmacological Society as personally cited by Hamilton) who claimed that bretylium produced only postsynaptic inhibition of the rat diaphragm. Bowman at the same meeting criticized them for not using smaller doses

and stimulating for a longer time.

The DBI_5 results on the cat with the test compounds showed no consistent pattern. Only one of the phenylalkyl TMA salts (the propyl) was tested and this caused no differential blockade in two cats. The four phenoxyalkyl TMA compounds studied ranged from 8.5 for the octyl to 18 for the butyl. On the cat the highest DBI_5 value was actually obtained with a triethylated compound, phenoxybutyl TEA but as this was only studied in one cat due to lack of compound further studies are necessary. This might suggest a species difference which would not be entirely unexpected considering the variable type of blockade known to occur in different species, muscles, and even ages of animals (Zaimis 1953, Jewell and Zaimis 1954, Churchill-Davidson and Wise 1963). Once more of these compounds are available a more critical test on the cat would involve perhaps a two-leg preparation in which both tibialis muscles are attached to a double myograph stand : one being stimulated at 12 and the other continuously at 60 per minute. In such a preparation minimal prolonged infusions into a jugular vein might be a suitable procedure to uncover pre-, rather than post-, synaptic blockade.

Thus, from the present pharmacological experiments designed to study the type of neuromuscular blockade produced there is no real evidence, except in the case of the phenylalkyl TMA series on the rat diaphragm, to suspect any significant presynaptic activity. Even in this case the concentrations used in the quantitative studies when blockade of 12 per minute responses was studied probably means that postsynaptic activity predominantly was studied. The edrophonium reversal studies along with the observations of others on the frog rectus

of non-reversibility on increasing agonist concentrations, strongly point to a non-d-tubocurarine-like blockade in all cases. The phenylalkyl TMA and phenoxyalkyl TMA compounds probably have depolarizing-desensitizing dual block effects on the postsynaptic end-plate receptors and can be compared with respect to the relative importance of chain lengthening and the presence of the ether oxygen atom. The phenoxyalkyl TEA compounds appear to be non-competitive postsynaptic inhibitors of acetylcholine lacking a depolarizing action, thus differing from the two TMA series, and should only with caution be compared and contrasted to the other series.

3. Structure-Activity Relationships - Neuromuscular Junction

The effect of lengthening the alkyl chain in the phenoxyalkyl TMA series has been studied on both the rat diaphragm and cat tibialis nerve-muscle preparations. The most profound change in activity in each case of adding one more carbon atom to the chain was between the ethyl to propyl increment (5 and 18-fold decrease in neuromuscular blocking activity in the rat and cat muscles respectively). As there was no such decrease between the 2-carbon (ethyl) and the 3-carbon (propyl) homologues in the analogous phenoxyalkyl TEA series which are devoid of stimulating or depolarizing activity it might be that the greater activity of the ethyl than the propyl in the phenoxyalkyl TMA series is associated with the more profound depolarizing activity of ethyl compound in this instance. Indeed phenoxyethyl TMA was found to be most potent in contracting the frog and chick slow fibres, which indicates depolarization as discussed above. Moreover, the marked acetylcholine-like activity of phenoxyethyl

TMA (choline-phenyl ether) is illustrated on the cat ganglion as it was the only phenoxyalkyl TMA compound shown to cause stimulation. The red muscle of the rat diaphragm is known to be more sensitive than the white muscle of the cat tibialis to curare-like agents than to depolarizing agents and vice versa (Jewell and Zaimis 1954). More importance may thus be attached to the above hypothesis when one notes that the greater (18-fold) difference between phenoxyethyl and phenoxypropyl TMA was observed on the cat tibialis. On the rat diaphragm , which is relatively less sensitive to depolarizing agents, the added benefit of greater depolarizing activity resulted in only a 5-fold advantage. In this laboratory we (Hersey and Hamilton , unpublished results) have shown that d-tubocurarine was only 8 times as active as phenoxyethyl TMA on the cat tibialis but as suspected was relatively more active (100 times) on the rat diaphragm.

The peculiar partial return of blocking activity on the cat tibialis at the phenoxypentyl TMA analogue (Fig. 2) may thus not be so surprising when one considers that a similar return of stimulant activity was observed on the frog rectus (Fig. 8) with this compound. This further suggests that compounds with predominantly depolarizing effects are more effective on the cat tibialis. As previously discussed this 5-carbon compound may be of optimum length to once again approximate the structural configuration necessary for acetylcholine receptor activation. The charges involved in the phenoxypentyl TMA molecule are as follows: the cationic nitrogen is, of course, positively charged, the phenoxy moiety is negatively charged. It seems plausible to assume that there can be attraction therefore between the two ends of the molecule. The resulting molecular configuration would therefore result

in an apparent shortening of the polymethylene chain, thereby displaying a strong spatial charge resemblance to the shorter phenoxyethyl TMA (See Fig. 17 and Table 9).

- Electronic and Spatial Considerations -

It would be enlightening to consider in more detail the electronic distribution in the phenoxy group. Certainly it is a rich pool of electrons, there being six electrons on the benzene ring, with the oxygen atom carrying two pairs of electrons. These two groupings do interact, resulting in a large degree of overlap and a consequent enrichment of electrons at the ortho and para positions of the aromatic ring and a corresponding reduction in electron availability at the oxygen atom. The oxygen atom does not, however, gain a positive charge and still retains a modecum of electropositive character.

The charge distribution in phenoxypentyl TMA may therefore be elaborated and presented in greater detail. If we assume there to be attraction between the cationic head and the electron sink of the phenoxy group, is this attraction between the ortho position and the positive nitrogen or is the oxygen participating in some subtle manner which is dependent on the electron levels at this atom ? Consequently it would be most interesting to compare the activity of phenoxypentyl TMA and phenylhexyl TMA. This latter compound although possessing the gross size of the phenoxypentyl TMA does not

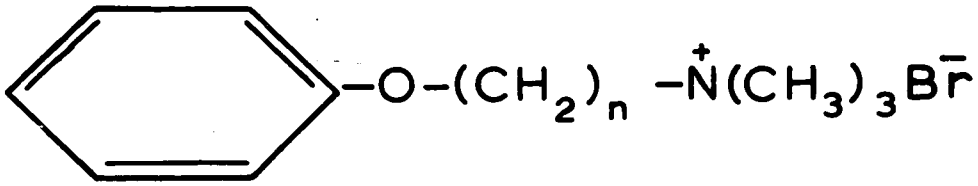
TABLE 9

Interatomic distances (Å) between the ether oxygen and the quaternary nitrogen in acetylcholine and phenoxyalkyltrimethylammoniums

TABLE 9

INTERATOMIC DISTANCES (Å) BETWEEN THE ETHER OXYGEN AND THE QUATERNARY NITROGEN IN ACETYLCHOLINE AND PHENOXYALKYL TMA

PHENOXYALKYLTRIMETHYLAMMONIUM SERIES



Compound	Minimum Distance	Maximum Distance	Closest Approximately Planar Distance
Acetylcholine	3.6	4.1	3.9
n = 2	3.4	4.0	3.5
n = 3	3.8	5.2	4.6
n = 4	4.2	5.3	4.5
n = 5	3.9	6.4	4.0
n = 6	4.6	8.2	5.0
n = 7	4.8	9.2	7.0

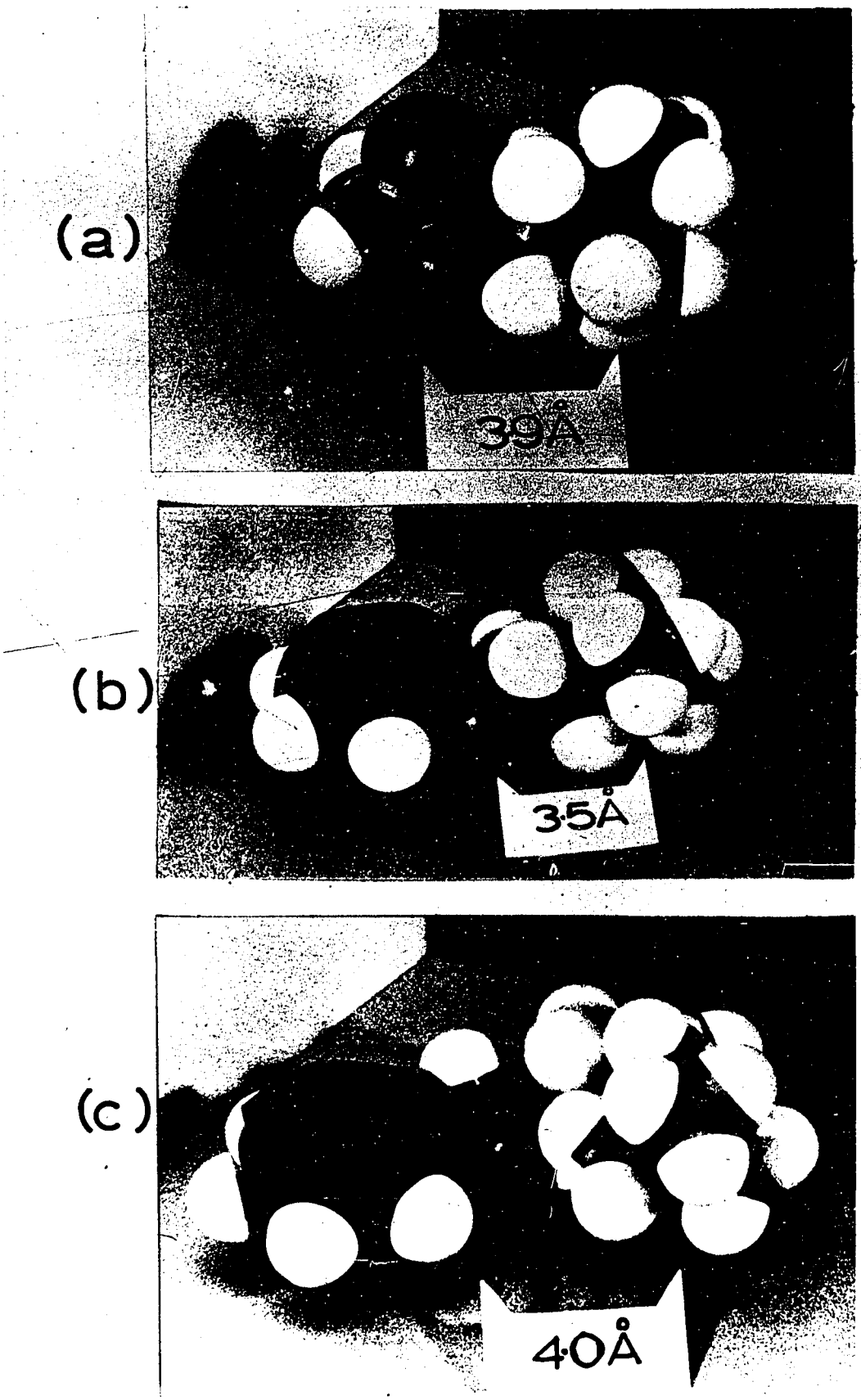
FIGURE 17

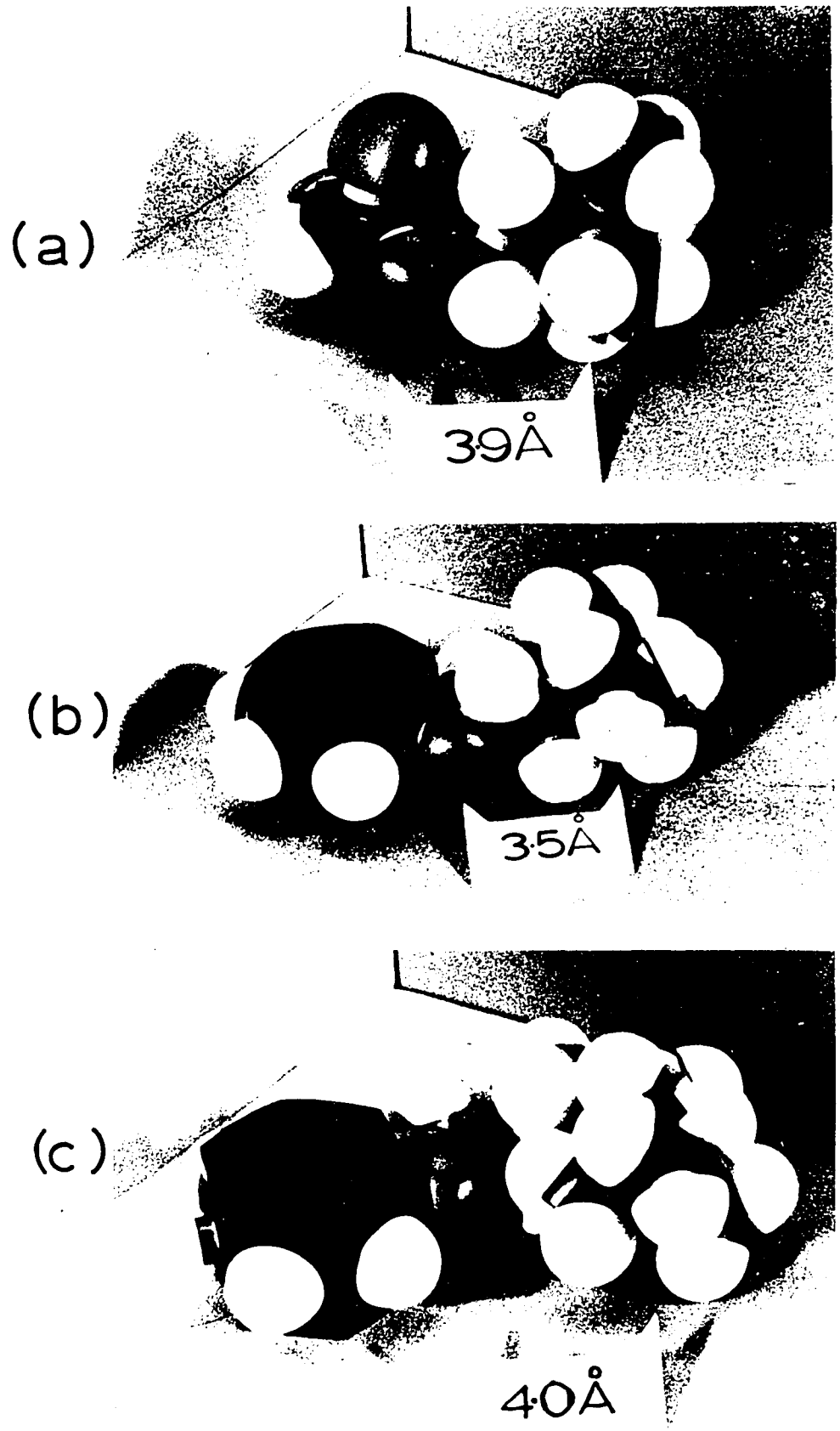
Interatomic distances (Å) between the ether oxygen and quaternary nitrogen atoms (Courtauld Atomic Models).

(a) Acetylcholine $CH_3\overset{O}{\overset{\|}{C}}\text{-}O\text{-}CH_2\overset{+}{N}(CH_3)_3$

(b) Phenoxyethyltrimethylammonium $Ph\text{-}O\text{-}CH_2CH_2\overset{+}{N}(CH_3)_3$

(c) Phenoxypentyltrimethylammonium $Ph\text{-})\text{-}CH_2CH_2CH_2CH_2CH_2\overset{+}{N}(CH_3)_3$


(a)
39Å
(b)
35Å
(c)
40Å


(a)
3.9Å
(b)
3.5Å
(c)
4.0Å

have the potentially influential oxygen atom. So although there might be some very slight enhancement of the aromatic electron pool from the alkyl substituent this would be much less than in the phenylalkyl ether. The cationic head should therefore not be as strongly attracted to the benzyl moiety and the spatial distribution of charges should be relatively uninfluenced. The above-mentioned hypothesis of the phenoxypentyl TMA assuming a quasi-phenoxyethyl TMA conformation may therefore be subjected to some trial. If the theory is a reasonable one the phenylhexyl TMA should not possess the peculiar agonistic activity of its ether analogue.

This discussion of molecular structure in the phenoxyalkyl TMA series may be further illustrated by reference to the interatomic distances measured from Courtauld molecular models (Table 9), some of which have been photographed in Fig. 17. In an attempt to test the hypothesis that the phenoxypentyl,and phenoxyethyltrimethylammoniums were the only compounds in this series capable of assuming an acetylcholine-like oxygen-nitrogen distance, the minimum and maximum oxygen-nitrogen separation (Å) was measured for the series ethyl to heptyl. From these values there was little to substantiate the above hypothesis as there were many compounds with possible distances overlapping that of the acetylcholine and phenoxyethyl trimethylammonium molecules (3.6 to 4.1 Å and 3.4 to 4.0 Å respectively). However, when the interatomic (O - N) distances were measured with the molecules in their most planar or flat form a special case

can be made for the phenoxyethyl and phenoxypentyl-trimethylammoniums being acetylcholine-like. The O - N distance in acetylcholine in its closest approximately planar configuration was 3.9 Å and in the phenoxyethyl- and phenoxypentyl-trimethylammoniums were 3.5 and 4.0 Å respectively. All other compounds in the phenoxyalkyltrimethylammonium series failed to allow manipulation into similar spatial configurations. The shorter chain phenoxypropyl and butyl compounds were planar at O - N separations of 4.6 and 4.5 Å and the longer chain phenoxyhexyl and heptyl compounds at 5.0 and 7.0 Å respectively.

It is particularly relevant that others have emphasized the importance of planarity for such receptor interactions as studied here. Barlow, Scott and Stephenson (1967) studied the acetylcholine-like activity of a number of mono-onium compounds and concluded that pyrrolidinium and pyridinium compounds with flat rings have a higher affinity than might be expected from their ionic weight alone. Moreover, Clark, Dawes and Williams (1968) concluded that the compound, here studied, choline phenylether (phenoxyethyltrimethylammonium) adopted a virtually "planar" configuration when interacting with and stimulating the ganglionic nicotinic receptor.

The overall effect of increasing carbon chain length in this series of phenoxyalkyl TMA's then, ignoring the parent phenoxyethyl TMA on the cat and rat and the phenoxypentyl TMA on the cat, is to

gradually increase blocking activity. On the cat tibialis (Fig. 2) the activity of the long chain, 8- , 9- and 10-carbon, compounds is about 5 times that of the propyl (3-carbon) analogue. On the rat diaphragm compounds with from 5- to 9-carbons are about 10 times as active as the propyl analogue. If, as the qualitative studies suggest, the blocking activity of these compounds is not curare-like but desensitizing, non-competitive, in nature then it would appear that the ability to desensitize increases with chain lengthening. Also it would suggest that desensitizing effects as well as curare-like effects are more readily measured on the rat diaphragm than on the cat tibialis (by comparing the propyl to the longer chain blocking activity). It is unfortunate, however, that there is no adequate method of assessing the concentration of the drugs at the receptor level in the in vivo cat preparation so that this hypothesis could be further tested by comparison with the bath concentration used in the rat diaphragm.

The decrease in activity with the 10-carbon phenoxydecyl TMA on the rat diaphragm is interesting in that no such decrease was observed in the cat tibialis muscle. It was observed that the long chain compounds did in fact become increasingly more difficult to get into solution at room temperature likely due to their increasingly paraffinic nature resulting in decreased water solubility and increased fat solubility. It is tempting to suggest that it is better to inject such solutions intra-arterially into the blood stream and allow the plasma to act as a solvent which carries the drug directly to the receptor site than merely to bathe such a "lean" muscle as the diaphragm in them. This argument, however, may be inconsistent with the previous

suggestion that anticholinesterase activity (in vitro) is increased whereas ganglion blocking activity (in vivo) is decreased with chains longer than 8-carbon atoms due to distribution and solubility factors.

The effect of lengthening the alkyl chain in the phenylalkyl TMA series has been partially studied on the cat tibialis and rat diaphragm preparations. On both of these preparations there was an increase in the neuromuscular blocking activity as the alkyl chain length was increased from zero to two carbon atoms. The three carbon, propyl, compound the analogue of phenoxyethyl TMA with the ether oxygen replaced by a methylene (CH_2) was not significantly different in activity from phenylethyl TMA. Insufficient phenylbutyl TMA was available for adequate evaluation but the phenylpentyl TMA was significantly less active than phenylpropyl TMA on both the rat diaphragm (EPMR 1.73 ± 0.187) (P value 0.05 - 0.02) and the cat tibialis (EPMR 8.54 ± 0.567 from 5 assays). It will be extremely interesting to study the phenylhexyl TMA compound, which has now been synthesized by Dr. Barlow, for the reasons discussed in detail above. The depolarizing activity of phenylpropyl TMA was found to be identical to that of phenoxyethyl TMA (EPMR 1.16 ± 0.102 from 4 tests), as indicated by measuring agonist activity on the frog rectus. This suggests that the oxygen atom surprisingly, is not as important as was originally thought. Moreover, interseries comparisons on the rat diaphragm showed that phenoxyethyl TMA was not significantly different in blocking activity from phenylpropyl TMA (P value 0.1 to 0.05 in 4 tests). One assay on the cat tibialis again gave an EPMR , for phenylpropyl TMA / phenoxyethyl TMA of 0.82 . These observations all indicate the lack of importance of the ether oxygen atom in determining stimulant or blocking activity on the acetylcholine receptors of the neuromuscular synapse. The problem which still

has to be resolved, however, is exactly how phenylpropyl TMA produces neuromuscular blockade. From the one experiment on the cat the lack of differential blockade at fast rates suggested there was no presynaptic depression of acetylcholine synthesis, but some rate dependence was observed on the more critical tests on the rat (DBI_{10} of 36.1 $\pm$ 4.86). However, the EPMR studies were performed using stimulus rates of 12 shocks per second so it is doubtful if such presynaptic depression could play a major role in the observed blockade.

Thus this study allows the conclusion that the oxygen atom is not significantly more influential than a methylene (CH_2) group in influencing either affinity for, or efficacy upon, the postsynaptic acetylcholine receptor. In longer chain compounds, however, the oxygen may have some intramolecular importance in determining the absolute configuration of the molecule facilitating at least in the case of the phenoxypentyl TMA a "depolarizing" conformation.

The neuromuscular blocking activity of the phenoxyalkyl TEA series was studied upon the rat phrenic nerve-diaphragm and the cat tibialis preparations. EPMR's were obtained in this series with all compounds from the pyridylethyl to pyridylhexyl TEA. Contrary to the findings with the analogous phenoxyalkyl TMA compounds there was no decrease in neuromuscular blocking activity when the carbon chain was lengthened from 2- (ethyl) to 3- (propyl) carbon atoms. The replacement of methyl groups in the cationic head by ethyl groups abolished the agonist activity on amphibian and bird slow-muscle fibres indicating, as expected, the inability of the TEA compounds to act as depolarizing compounds. Thus the marked activity of the phenoxyethyl TMA/ phenoxyethyl TEA on the cat tibialis EPMR estimated to be 0.079 from comparisons

of: (phenoxypropyl TEA/ phenoxyethyl TEA) x (phenoxypropyl TMA/ phenoxypropyl TEA) x (phenoxyethyl TMA/ phenoxypropyl TMA) i.e., $1.4 \times 1.03 \times \frac{1}{18.28} = 0.079$ is most likely due to the depolarizing ability of the TMA analogue as previously discussed. There was no difference in the neuromuscular blocking activity of the phenoxyethyl TMA and TEA analogues on the rat diaphragm (EPMR 0.78 ± 0.302), a preparation relatively insensitive to depolarizing drugs. Comparisons of phenoxypropyl TEA/ phenoxypropyl TMA and phenoxybutyl TEA/ phenoxybutyl TMA on the tibialis gave EPMR's of 1.03 (one assay) and 0.99 (mean of two assays) respectively. These studies on the acetylcholine receptors of the postsynaptic membrane indicate that when agonistic activity is low or absent the TMA and TEA moieties have similar blocking effects and presumably affinities. These studies can be compared with those of Barlow, Scott and Stephenson (1963) on replacement of methyls by ethyls on the nitrogen of their acetylcholine-like compounds as tested upon the guinea pig ileum. Barlow et al (1963) found that substitution of one methyl by an ethyl increased affinity and decreased efficacy, replacement of a second had little effect on affinity but decreased efficacy still further, and replacement of the last methyl group by an ethyl group often resulted in a marked decrease in affinity. This suggests that a change from phenoxyalkyl TMA to phenoxyalkyl TEA might well result in a compound with similar affinity. For this reason the intermediate phenoxyalkyl diethyl methyl ammonium analogues might well be worthwhile studying as they might have a greater affinity than either the TMA or TEA analogues resulting in a more effective neuromuscular blocking series of compounds.

The effect of increasing the alkyl chain length of the

phenoxyalkyl TEA series on neuromuscular blocking activity was similar on the rat diaphragm and cat tibialis nerve-muscle preparations. Increasing the chain length from two to three to four carbon atoms had no significant effect upon neuromuscular blocking activity whereas the five and six carbon chain (pentyl and hexyl) compounds were about twice as active as phenoxyethyl TEA. It would be of interest to extend this series even further to discover if affinity continues to increase and if in fact there is an optimum size.

Simply lengthening the alkyl chain does not appear to impart curare-like competitive blocking activity to these phenoxyalkyl monoonium compounds, although replacement of the methyls by ethyls in the cationic head abolished, at least, the depolarizing ability. Further studies might involve branched chains and indeed larger as well as heterogeneous substitution on the nitrogen.

Apart from basic interest in the structural requirements for competitive as opposed to non-competitive attachment to the various acetylcholine receptors there is still a place in clinical practice for other compounds with d-tubocurarine-like activity. d-Tubocurarine chloride is an invaluable drug when voluntary muscle relaxation is demanded as an aid to surgery. It is a predictable drug which can if necessary be reversed simply by the administration of an anticholinesterase such as prostigmine or edrophonium. However, for many surgical procedures its effects are unnecessarily long (40 minutes). This means that for most short procedures such as intubation and electroconvulsive therapy, succinyldicholine is the drug of choice. This depolarizing neuromuscular-blocking drug has a number of drawbacks being often less predictable. Abnormal, genetically determined, pseudocholinesterases

FIGURE 18

Comparison of duration of neuromuscular blockade due to d-tubocurarine and test compound.

▬▬▬ Duration of drug administration.

a. Phenoxybutyltriethylammonium.

b. d-Tubocurarine .

Alternate nerve stimulation at 60 and 12 per minute.

FIG 18

(a)

(b)

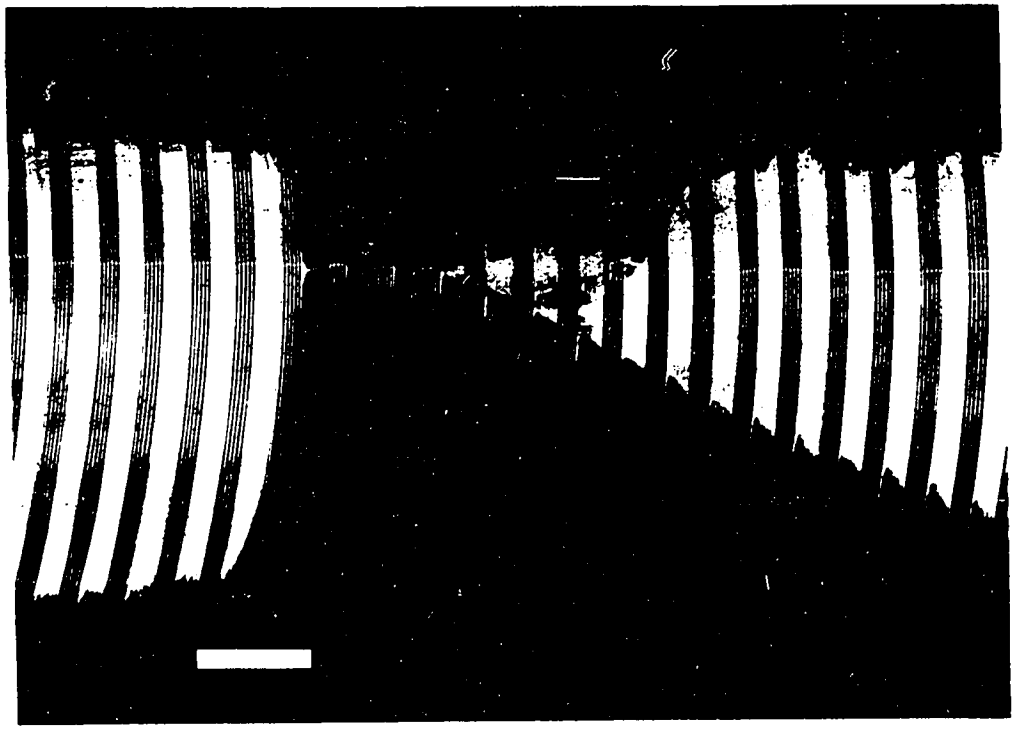
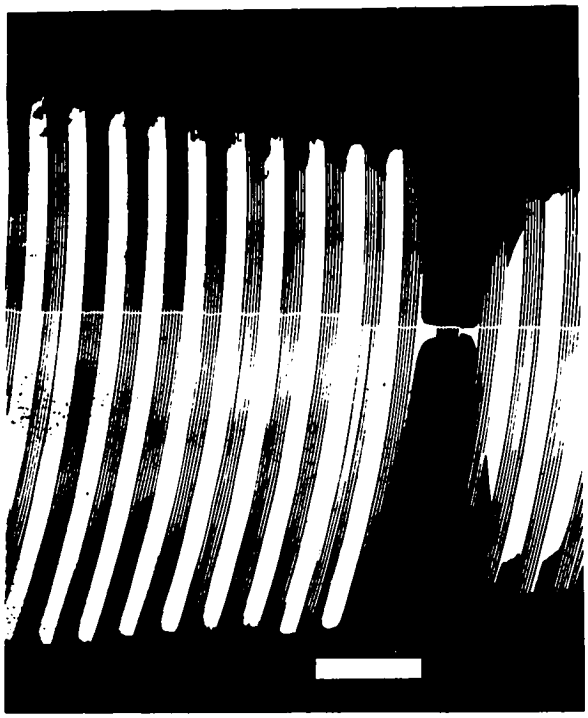
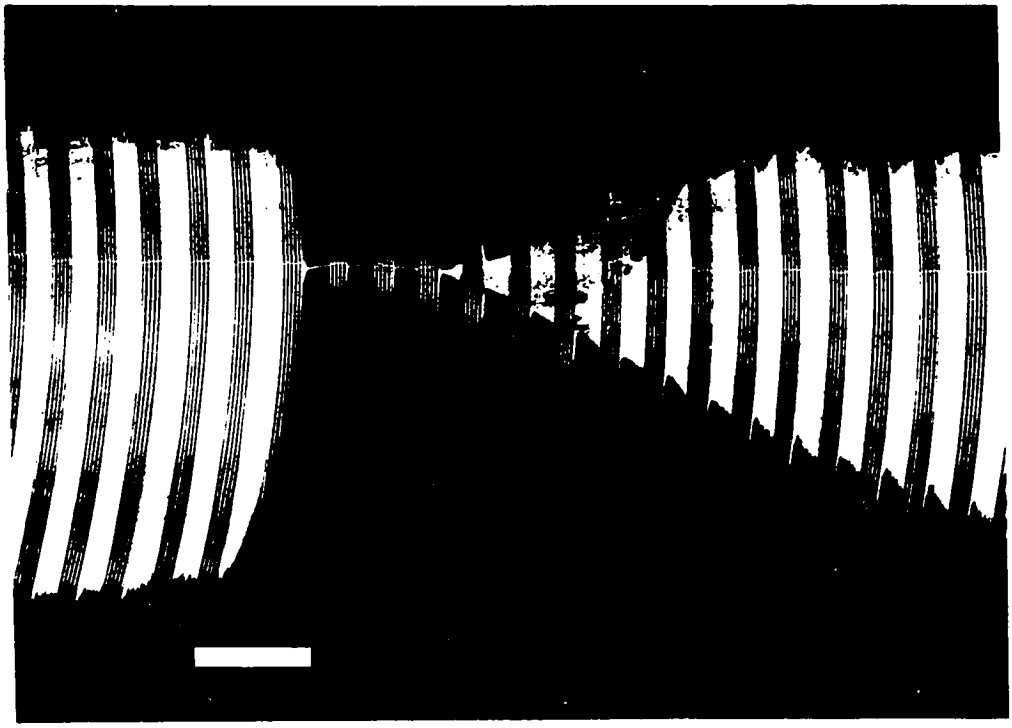


FIG 18

(a)



(b)



(Kalow 1962) cannot destroy this compound and can lead to very prolonged apnoea (Kalow 1956). The cholinergic nature of the compound suggests that premedication should include parasympatholytic drugs to protect the heart. Moreover, the transition from a depolarizing to a "phase-two" , nondepolarizing block is irregular, yet a constant possibility, resulting in a blockade of unknown duration. There can be much uncertainty as to whether it is safe to attempt reversal by the use of anticholinesterases. For these reasons the brief effects observed (Fig. 18) with the compounds studied might well fit them to replace d-tubocurarine if the structure could be altered to produce competitive blockade of the postsynaptic receptor. Future studies will take more cognizance of the relative ganglionic/neuromuscular blocking activity of these phenylalkyl TMA and phenoxyalkyl TEA series, as importance can only be attached to compounds with minimal ganglion blocking effects or at least not more than that of d-tubocurarine (Chou and de Elió 1948).

The structural requirements for presynaptic depression of acetylcholine formation have been discussed in some detail. Compounds with presynaptic depressant effects on acetylcholine formation and release may well offer a new approach to the management of diseased states. Any condition in which there is an abnormally high transmission across a cholinergic synapse would be selectively depressed by amounts which did not affect normal transmission. Certain hypertensive conditions and spastic neuromuscular disorders may well be suitable candidates for presynaptic blockers of sympathetic ganglion and neuromuscular synapses respectively. The problems to be overcome however, in such a search or molecular-manipulation study are many. The production of compounds selectively affecting abnormally rapid transmission without having marked

respiratory depressant effects might be the main stumbling block. The lack of specificity and low potency of hemicholinium and triethylcholine has thwarted their clinical application and suggests that new approaches with other chemical series such as the above might be profitable. Moreover, the close similarity between the effects of such compounds and the clinical symptoms of myasthenia gravis is challenge enough in itself to continue such research. If myasthenia is in part due to an interference with choline transferase activity then one approach might be the development of compounds which stimulate rather than inhibit such activity. Until much more is known about this whole field the naievity of this hypothesis cannot really be tested. However, with some of the structural changes suggested by this study future studies using analogous compounds should evaluate at cholinergic synapses: postsynaptic and presynaptic blocking activity, ganglionic and/or neuromuscular selectivity, and also effects on choline transferase and acetylcholinesterase activity. Such a study involving a number of different disciplines would be greatly improved by the enlistment of a research team including pharmacologists, biochemists, organic chemists, and clinicians.

VII. SUMMARY AND CONCLUSIONS

1. The acetylcholine-like stimulant activity of the mono-onium phenoxyethyl - to phenoxydecyl-trimethylammonium series has been demonstrated on the frog rectus abdominis muscle *in vitro* and the striated muscles of the chick *in vivo*.
2. The long-chain phenoxyalkyltrimethylammoniums were partial agonists on the frog rectus.
3. A difference between the nicotinic receptors of such striated muscles and those of the cat superior cervical ganglion must exist because only phenoxyethyltrimethylammonium in this series caused ganglion stimulation.
4. With the homologous series of phenoxyalkyltrimethylammonium compounds it was found in cats that increasing the carbon chain from 2 to 3-carbons caused a four-fold reduction in ganglion blocking activity. Optimal blocking activity was associated with the 8-carbon compound (3 times as active as the 2-carbon compound).
5. Similarly, it was shown that increasing the chain length from 2 to 3-carbons also decreased the neuromuscular blocking activity of the phenoxyalkyltrimethylammoniums (18-fold on the cat tibialis and 5-fold on the rat diaphragm). Neuromuscular blocking activity returned when the carbon chain was lengthened further: in the cat

preparation the 8-, 9-, and 10-carbon compounds were the most active (about 0.3 times that of the 2-carbon) and in the rat , greatest activity was associated with the 7-, 8-, and 9-carbon compounds (about twice that of the 2-carbon).

6. The partial return of agonist activity on the frog rectus and of blocking activity on the cat tibialis with phenoxypentyl-trimethylammonium suggests that this compound may pharmacologically resemble acetylcholine. An hypothesis to explain both how this pentyl derivative has agonist activity resembling that of acetylcholine and why among all these compounds greatest acetylcholine-like activity is associated with the ethyl and pentyl compounds, is suggested from spatial studies using Courtauld molecular models. Measurements of the distances between the oxygen and nitrogen atoms (O and N) suggests that although several of them can assume conformations such that this distance is similar to that in the acetylcholine molecule, when the molecules are in a planar conformation only the ethyl- and pentyl- derivatives have O - N distances resembling that of acetylcholine. It is therefore suggested that for maximal acetylcholine-like activity both the criteria of planar conformation and a correct O - N atom distance must be met.

7. Preliminary studies with the phenylalkyltrimethylammonium series indicate that both neuromuscular blocking and stimulant activity increase as 1, 2 and 3-carbons are placed between a benzene ring and a trimethylammonium moiety. Compounds with 4 and 5-carbons like the analogous phenoxypropyl- and phenoxybutyl- trimethyl ammoniums were less effective neuromuscular blockers.

8. Interseries comparisons between the phenoxyalkyl- and phenylalkyl- trimethylammoniums indicate that the oxygen atom can be replaced by a carbon atom without loss of synaptic activity. The marked nicotinic activity of phenoxyethyltrimethylammonium (choline phenyl ether) is thus not critically dependent upon the ether oxygen atom.

9. The effect of replacing the trimethylammonium moiety with a triethylammonium group has been studied. The triethylated monooniums were, as expected, devoid of any acetylcholine-like activity yet proved to have similar affinities for the acetylcholine receptor because a number of them were as effective neuromuscular blocking agents as their tri-methylated analogues.

10. No decrease in neuromuscular blocking activity of the tri-ethyl compounds was observed when the carbon chain was increased from 2 to 3. Interseries comparisons indicated that although the phenoxypropyltriethyl- and trimethylammonium compounds were equieffective, the phenoxyethyltrimethylammonium was much more active than the phenoxyethyltriethylammonium, particularly on the cat tibialis. This enhanced neuromuscular blocking activity of the phenoxyethyltrimethylammonium compound is associated with marked depolarizing (acetylcholine-like) depolarizing activity resulting in a succinyldicholine-like blockade.

11. The neuromuscular blockade induced in cats and rats by phenoxy- and phenylalkyltrimethylammonium and by phenoxyalkyltriethylammonium compounds was not reversed by edrophonium and was not curare-like. Thus merely increasing the carbon chain length and/or changing a trimethylammonium to a triethylammonium does not

change the neuromuscular blocking activity from succinyldicholine-like to curare-like.

12. Studies on blockade of the twitch responses of the rat diaphragm preparation revealed that most stimulus rate-dependency was obtained with the phenylalkyltrimethylammonium series. This rate dependence was greater than that with d-tubocurarine. Least rate dependent was phenoxyalkyltriethylammonium.

13. Future studies on the presynaptic activity of the phenylalkyl-trimethylammoniums will indicate whether or not this rate-dependent blockade is related to a triethylcholine-like decrease in acetylcholine activity.

14. The results also show that gallamine triethiodide is markedly rate dependent and may produce synaptic effects differing from those of d-tubocurarine. This new finding with such a much-studied and widely-used muscle relaxant as gallamine suggests that further study may reveal presynaptic affinity in addition to its classical postsynaptic site of action.

APPENDIX I

Chemical analysis of synthetic compounds supplied by Dr. R.B. Barlow, Department of Pharmacology , University of Edinburgh, Scotland.

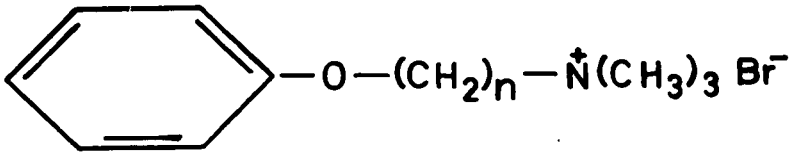
APPENDIX I

TABLE I

Chemical analysis of phenoxyalkyltrimethyl-ammonium bromides.

TABLE

*CHEMICAL ANALYSIS OF



CHAIN LENGTH N =	MOL. WT.	MELTING POINT °C	FOUND Br⁻	FOUND C	FOUND H	THEORY Br⁻	THEORY C	THEORY H
2	260	169	30.7	----	----	30.8	----	----
3	274	153	29.0	52.5	7.27	29.2	52.5	7.37
4	288	177	27.8	54.4	8.06	27.8	54.2	7.71
5	302	186 - 187	26.8 26.7	55.7	8.14	26.5	55.6	8.03
6	316	199 - 200	25.3 25.2	56.7	8.06	25.3	57.0	8.31
7	330	192 - 193	24.5 24.6	58.1	8.33	24.2	58.2	8.48
8	344	165 - 166	23.3 23.3	59.2	8.50	23.2	59.3	8.81
9	358	184 - 185	22.0 22.0	60.4	8.81	22.3	60.4	9.03
10	372	174 - 175	21.7 21.9	----	----	21.5	----	----

*BARLOW, R.B. (1965) UNPUBLISHED DATA

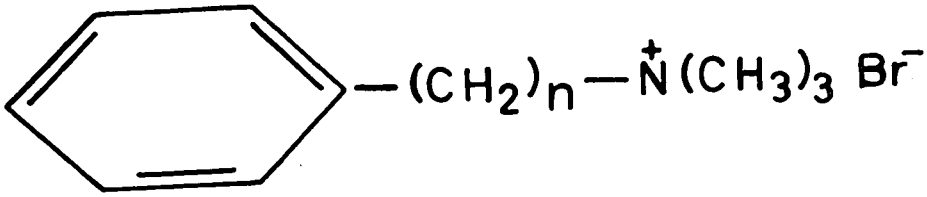
APPENDIX I

TABLE 2

Chemical analysis of phenylalkyltrimethyl-ammonium bromides.

TABLE

*CHEMICAL ANALYSIS OF



CHAIN LENGTH N =	MOL. WT.	MELTING POINT °C	FOUND Br^-	THEORY Br^-
0	216	214 - 215	36.9 36.9	37.0
+1	185	238 - 239	19.2	19.1
2	244	242 - 243	32.5 32.5	32.8
3	258	151 - 152	30.9 31.0	30.9
4	272	184 - 185	22.1 29.2	29.3
5	286	168 - 169	27.8 27.9	27.9

* BARLOW, R.B. (1965) UNPUBLISHED DATA

+ CHLORIDE

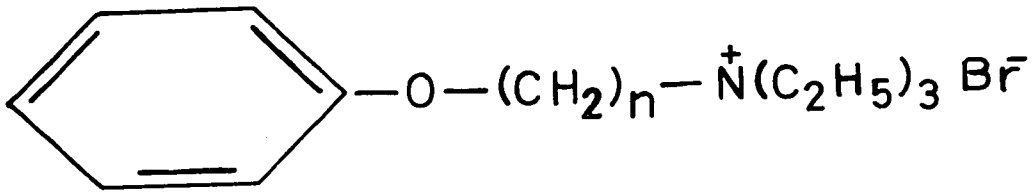
APPENDIX I

TABLE 3

Chemical analysis of phenoxyalkyltriethyl-ammonium bromides.

TABLE

* CHEMICAL ANALYSIS OF



CHAIN LENGTH N =	MOL. WT.	MELTING POINT °C	FOUND Br^-	THEORY Br^-
2	302	134 - 135	26.3 26.3	26.4
3	316	90 - 92	25.3 25.0	25.3
4	330	150 - 151	24.3 24.3	24.2
5	344	91 - 92	22.8 23.0	23.2
6	358	113 - 114	22.2 22.7	22.3

* BARLOW, R.B. (1965) UNPUBLISHED DATA.

APPENDIX II

Anti-acetylcholinesterase activity of phenoxy-alkyltrimethylammonium salts: J.T. Hamilton , P. McCurrach and L.W. Hersey. Unpublished Results, 1964.

ANTICHOLINESTERASE ACTIVITY OF PHENOXYALKYL-TRIMETHYLAMMONIUM SALTS

$(Ph\text{-}o\text{-}(CH_2)_n \overset{+}{N} (CH_3)_3 \; Br^-$

The anticholinesterase activity of the phenoxyalkyl-trimethylammonium salts was determined manometrically using a Gilson differential respirometer. (Gilson 1963, Umbriet, Burris and Stauffer, 1964). The rate of hydrolysis of acetylcholine (Acetylcholine Chloride-Hoffmann-LaRoche) by an acetylcholinesterase preparation (from ox red blood corpuscles, Nutritional Biochemical Corp.) was followed at 37°C at constant pressure by observing the evolution of carbon dioxide from Krebs' bicarbonate buffer due to the formation of acetic acid. The reaction was performed in an atmosphere of 5% carbon dioxide and 95% nitrogen and the volume of carbon dioxide evolved was read directly in microlitres from the digital volumometers every three minutes for 30 minutes. The initial rates of evolution were corrected by subtracting the appropriate enzyme blank (containing no acetylcholine) and adjusted to the volume of dry gas at 760 mm. mercury pressure. The volumes used in the calculations were the initial rates over the first ten minutes of reaction.

Preliminary Experiments:

Theoretically (Beck 1951) one unit of enzyme liberates one

microliter of carbon dioxide per minute. However, 16 units of this preparation (final concentration of four units/ml.) were necessary to give an adequate evolution of carbon dioxide.

A plot of the initial rate of evolution of carbon dioxide against the logarithm of the substrate concentration gave the classical bell-shaped curve (Table 1, Fig. 1) from which it was decided that 1.5×10^{-3} and 7×10^{-4} molar acetylcholine were suitable concentrations for the inhibitory experiments.

Determination of Anticholinesterase Activity:

In such experiments it is usual to express the anticholinesterase activity in terms of the negative logarithm of the molar concentration of the drug which produces 50% inhibition, the pI_{50}. However, this value varies with the substrate concentration and assuming competitive inhibition :

$$1 + s/Ks = I/Ki \qquad \text{(Barlow and Hamilton 1962)}$$

(where S is the concentration of substrate

Ks the Michaelis - Menten constant

I the concentration of inhibitor

and Ki the Inhibitor constant)

$$\text{i.e. } pKi = -\log Ki = pI_{50} + \log (1 + s/Ks)$$

So that a value of both pKi and pI_{50} could be obtained for each inhibitor, Ks was determined from a Lineweaver Burk plot. The results in Table 2 were plotted , 1/s against 1/v, in Fig. 2 and the intercept -1/Ks was -1700. This gives a Ks value of 5.88×10^{-4} for this preparation of acetylcholinesterase from ox rbc's. Augustinsson (1948)

gives a value of 2.9×10^{-4} for dog brain and Barlow and Hamilton (1962) found a value of 3.45×10^{-4} for an acetone preparation from dog caudate nucleus.

Thus $\log (1 + s/Ks) = 0.55$ where $s = 1.5 \times 10^{-3}$ M $= S_1$

and $\log (1 + s/ks) = 0.34$ where $s = 7.0 \times 10^{-4}$ M $= S_2$

Therefore with equal numbers of flasks containing S_1 and S_2

$$\log (1 + s/Ks) = 0.45$$

and $$pKi = pI_{50} + 0.45$$

The pI_{50} for each compound was determined as in Fig. 3 by plotting the percent inhibition against the logarithm of the inhibitor concentration (I). Equal numbers of flasks were incubated with 1.5×10^{-3} M and 7×10^{-4} M acetylcholine and usually two inhibitory concentrations of the drug were used in each experiment. The inhibitor was added to the enzyme in the flask 40 minutes before the start of the reaction.

Table 3 gives the estimated pI_{50}'s , the calculated pKi's , and the equipotent molar ratio (EPMR) of each compound relative to the compound with two carbons in the chain (n = 2, choline phenyl ether). This is the number of molecules of each homologue which produced the same inhibition as one molecule of the 2-carbon compound. i.e. molar concentration of homologue which produced 50% inhibition/ molar concentration of n=2 which produced 50% inhibition, or antilog.

(pI_{50} for n = 2) minus (pI_{50} for homologue) or antilog .

(pKi for n = 2) minus (pKi for homologue) .

The effect of increasing the chain length on the anti-cholinesterase activity of this series of compounds is shown graphically

in Fig. 4 .

TABLE I

Relationship between rate of hydrolysis (µl. of carbon dioxide/10 mins.) and substrate concentration (S), using 16 units of acetylcholinesterase per flask at 37°C.

Molar Concentration of Acetylcholine (S)	l. of Carbon Dioxide µl/10 mins.*
1×10^{-2} M	82
5×10^{-3} M	102
1×10^{-3} M	95
5×10^{-4} M	48

* mean of two experiments

TABLE 2

Results for estimation of Ks (Michaelis-Menten constant) for the enzyme - substrate complex.

(S) Molar Concentration of Acetylcholine	1/S	(V) l. of Carbon Dioxide /min. ± S.E.M.	1/V
1.5×10^{-3} M	667	2.99* ± 0.095	0.334 ± 0.011
7.0×10^{-4} M	1430	2.26* ± 0.081	0.442 ± 0.017

* mean of nine experiments

TABLE 3

Effect of chain lengthening on the anticholinesterase activity of the phenoxyalkyltrimethylammonium salts.

$C_6H_5 - O - (CH_2)_n - \overset{+}{N}(CH_3)_3\ Br^-$

n Chain Length	Number of Experiments	pI_{50} ± S.E.M.	pKi≠	Equipotent* Molar Ratio / n=2
2	4	2.66 ± 0.02	3.11	1.00
3	2	2.6	3.05	1.15
4	2	≒ 0	≒ 0	1.66×10^3
5	1	2.27 ± 0.02	2.72	2.40
6	2	3.18 ± 0.12	3.63	0.30
7	2	3.43 ± 0.02	3.88	0.17
8	2	3.61 ± 0.05	4.06	0.11
9	2	4.08 ± 0.02	4.53	0.04
10	1	5.42 ± 0.07	5.87	0.002

≠ pKi derived from equation $1 + S/Ks = I/Ki$ (Barlow and Hamilton 1962).

i.e. $pKi = -\log Ki = \log (1 + S/Ks) + pI_{50} = 0.45 + pI_{50}$

* Equipotent molar ratio = molar concentration of analog producing 50% inhibition molar concentration of the compound where n=2 which produces 50% inhibition.

= antilog (pI_{50} for n = 2) - (pI_{50} for homologue)

or = antilog (pKi for n = 2) - (pKi for homologue)

APPENDIX II

FIGURE I

Relationship between rate of hydrolysis (1. of carbon dioxide /10 mins.) and substrate concentration (S) using 16 units of acetylcholinesterase per flask at $37^{o}C$.

APPENDIX II

FIGURE 2

Lineweaver - Burk plot for determination of Michaelis-Menten Constant (Ks).

FIG. 1

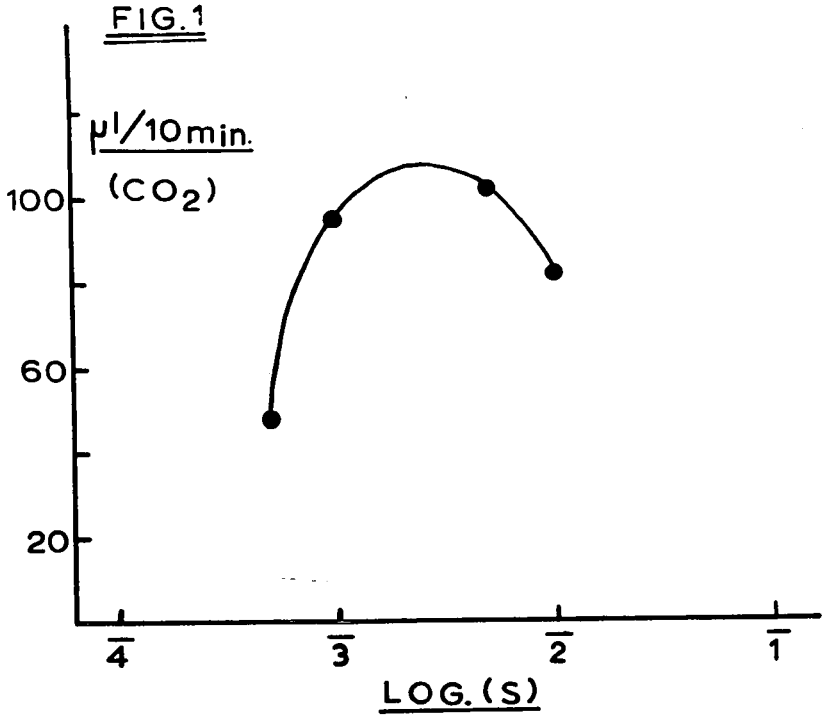
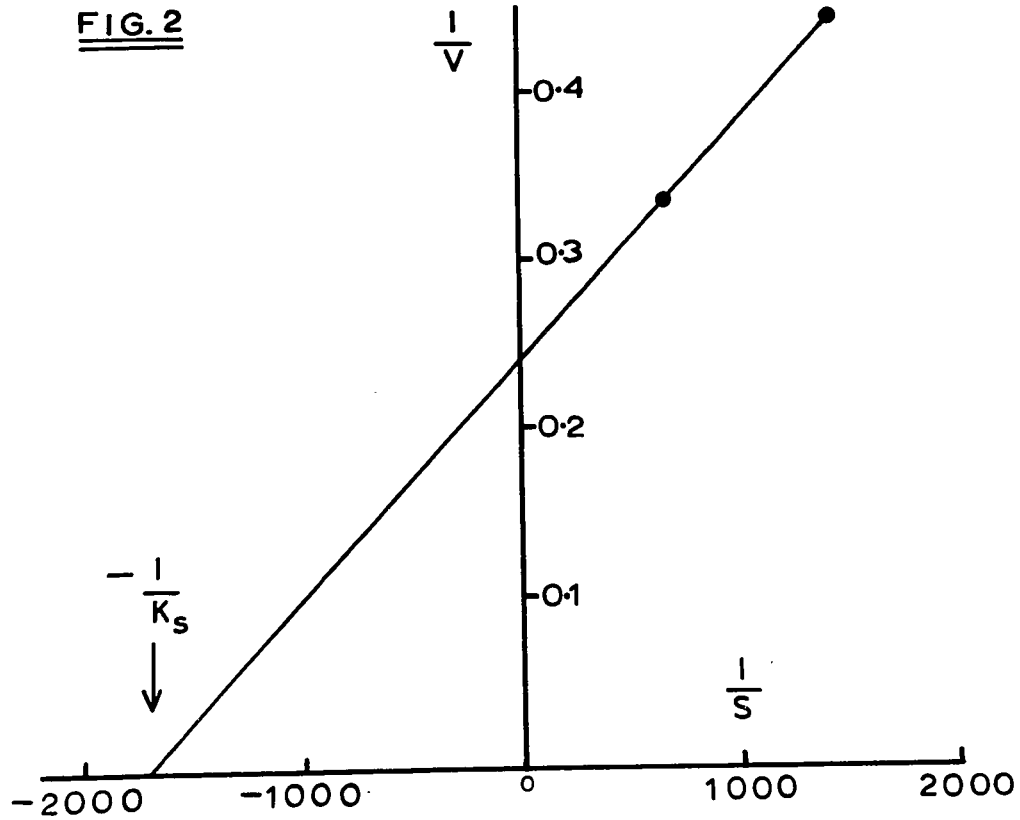

µl/10min.
(CO2)
100
60
20
4
3
2
1
LOG. (S)

FIG. 2

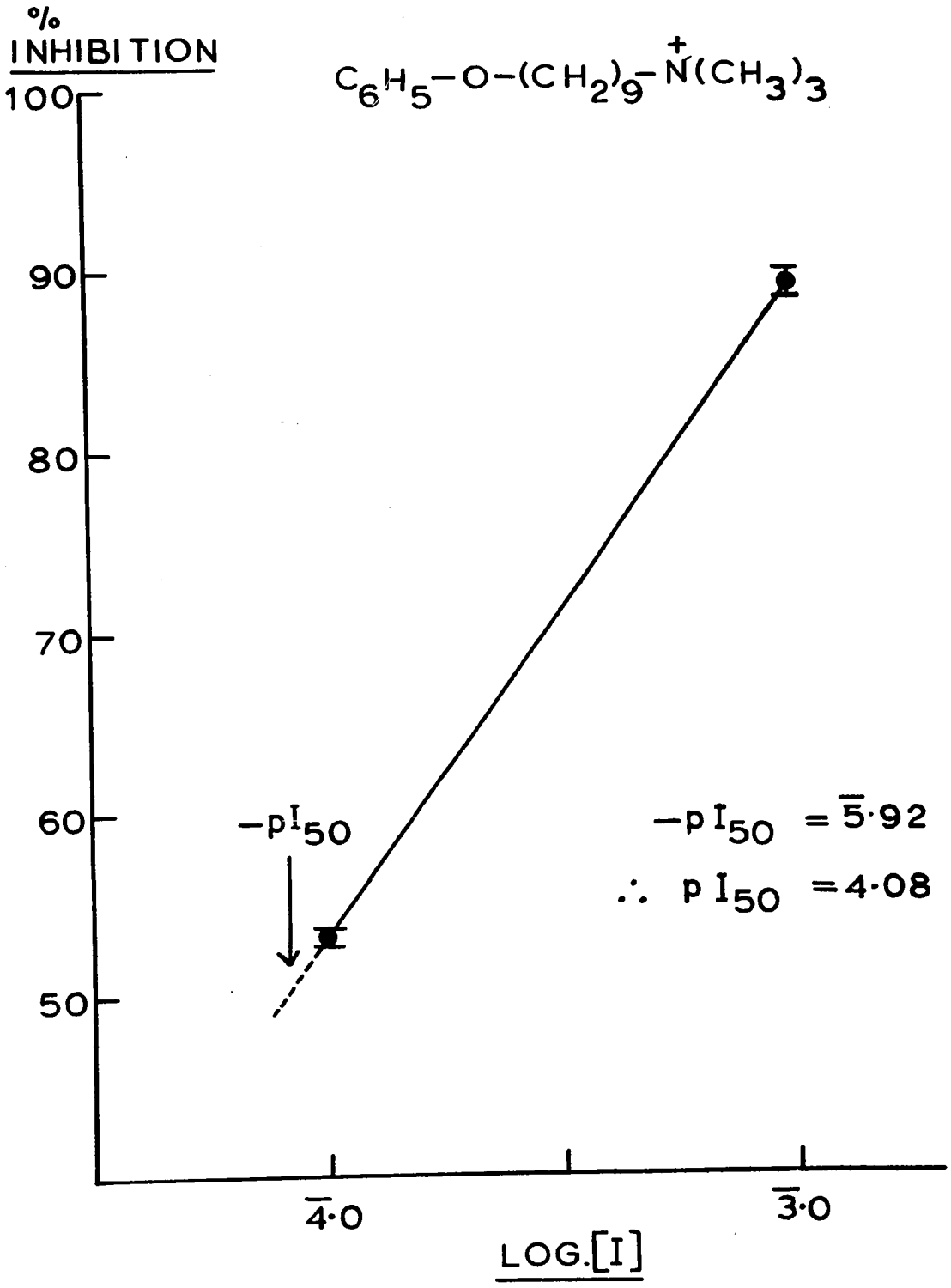

1/V
0·4
0·3
0·2
0·1
-1/Ks
1/S
-2000
-1000
0
1000
2000

APPENDIX II

FIGURE 3

Example of pI_{50} determination using phenoxynonyltrimethylammonium .

FIG. 3 DETERMINATION of pI_{50}



APPENDIX II

FIGURE 4

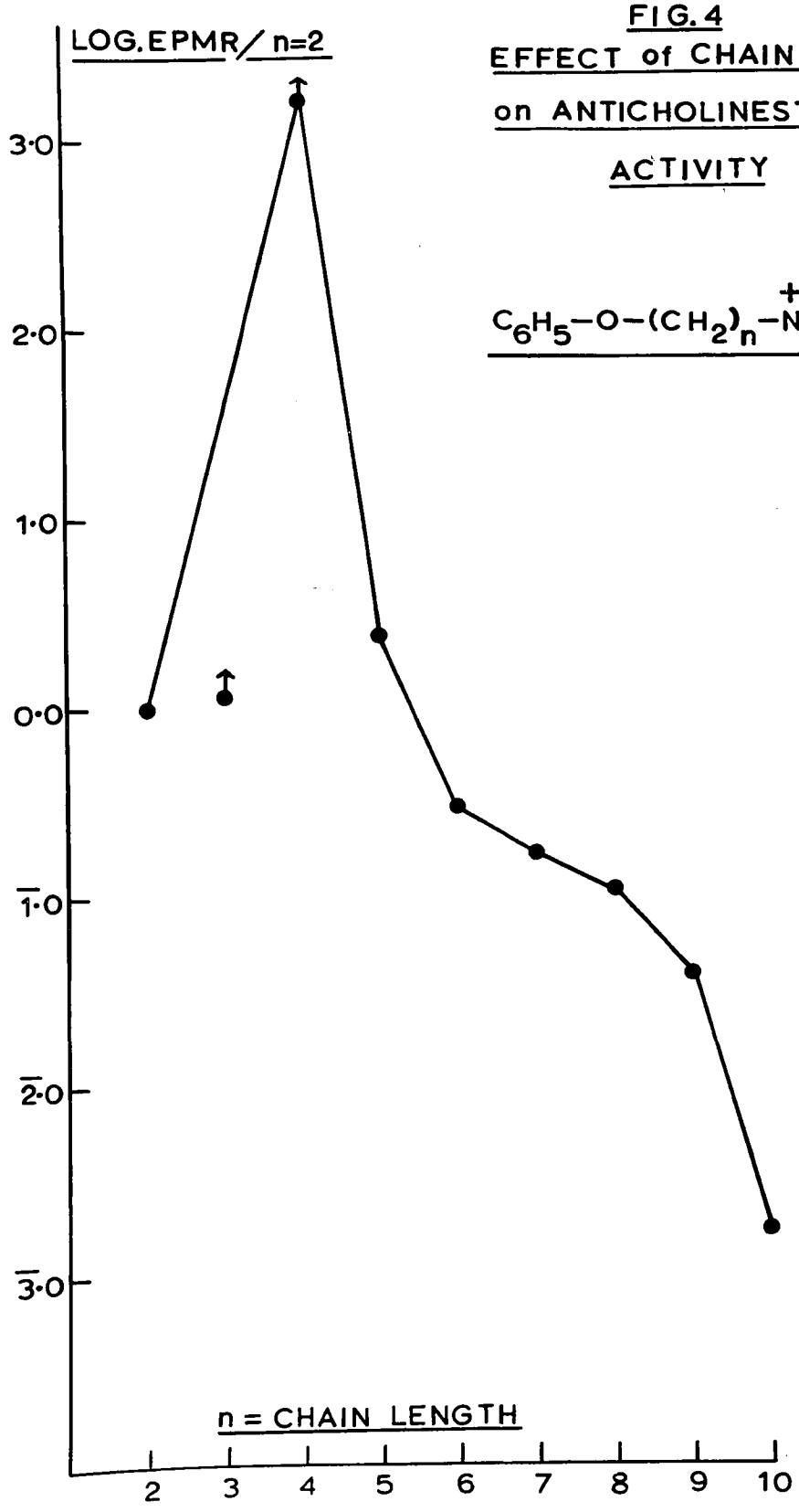
Effect of chain length on the anticholinesterase activity of the phenoxyalkyltrimethylammonium series.

(Log of equipotent molar ratio - carbon chain length) .

FIG. 4

EFFECT of CHAIN LENGTH on ANTICHOLINESTERASE ACTIVITY

$C_6H_5-O-(CH_2)_n-\overset{+}{N}(CH_3)_3\ Br^-$



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