EXCITATORY AND ANTI-CURARE PROPERTIES OF ACETYLCHOLINE AND RELATED QUATERNARY AMMONIUM COMPOUNDS AT THE NEUROMUSCULAR JUNCTION*

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I. AMPHIBIAN MUSCLE

A. Nature of contractile responses induced by acetylcholine

The most obvious characteristic of striated muscle is its contractile property. Undoubtedly this function served initially to attract the attention of experimental biologists to this tissue as a test object. The advantage of being able to visualize the effect of various agencies on function endowed the physiology of muscle with a unique fascination not only for the physiologist but also for the pharmacologist who was enabled to ascertain readily the excitatory and inhibitory potentials of various chemical substances. With continued refinements in our knowledge of muscle and the functional interrelationships of nerve and muscle, an appreciation has been acquired of the more subtle manifestations of neuromuscular processes. Nonetheless, the simple expedient of observing whether or not a substance can elicit or prevent a contractile response of the muscle still provides a valuable point of departure for the analysis of the mechanism by which a chemical substance modifies function. A large segment of research has been based on just such observation and for this reason initial remarks will be concerned with the actions of quaternary ammonium compounds on this most ostensible feature of muscle function.

From an historical standpoint, descriptions of drug actions on the contractile behavior of muscle were derived for many years almost exclusively from observations made on the frog striated muscle preparation. This was due in large part to the seeming pharmacologic insensitivity of mammalian striated muscle. The use of frog muscle, however, presented special problems that have only lately begun to be resolved. In this species the occurrence of a sustained shortening of the muscle, distinct from a tetanus, was noted to result from several agents. To this state the term contracture was applied, and the extensive attempts to analyze this form of muscle response may be appreciated from a perusal of Gasser's review on contractures of skeletal muscle, which summarizes the literature up to 1930 (107). Gasser's concluding remarks are still apropos to the problem and bear repetition, "... the known contractile mechanism in muscle can be set into states of activity differing in their properties from those resulting from the *normal* motor innervation ... an understanding of their properties (contractures) ... prevents the confusion of qualities, properly belonging to a contracture, with those possessed by a twitch."

The observations on the contractures occurring in frog striated muscles have been explained largely by the work of Kuffler *et al.* (160, 161). These workers demonstrated in the frog that the group of small diameter ventral root fibres averaging about 5μ possessed a function distinct from that of the large diameter ventral root fibres. Repetitive excitation of these small diameter nerve fibres

led to the appearance of considerable muscle shortening despite the fact that the response was localized to a restricted region of the muscle fibre. The electrical accompaniments of stimulation of these nerves confirmed the local nature of the response, since there arose in the junctional region a non-propagated potential that underwent spatial decay (160). Recently, Hunt and Kuffler (136) have called attention to the fact that the presence of muscle fibres innervated by the small nerve system is variable in extent in frog striated muscles and that twitch responses or contractures may be seen depending on the muscle under observation. Accordingly, the sartorius evidences quick responses and is innervated almost solely with large diameter motor nerve fibres; the rectus abdominis exhibits a characteristic contracture and receives an abundant supply of small diameter nerve fibres.

Unfortunately, little is known about the pharmacologic behavior of the small nerve innervated muscle fibre of the amphibian. For these reasons generalizations about the actions of drugs on neuromuscular function, drawn solely from experiments with amphibian muscle, should not be extended unconditionally to mammals without additional study in this class. The pharmacologist must consider the type of innervation and the nature of the contractile response in terms of ultimate aims when selecting a test object for purposes of pharmacologic study. Thus the frog rectus muscle, unlike the sartorius, is not comparable to the quick twitch system characteristic of the mammalian neuromuscular apparatus. On the other hand, the properties of the frog rectus abdominis are well suited for the assay of acetylcholine (ACh).

With the scarcity of experiments specifically designed to test the pharmacologic reactivity of small nerve innervated muscle fibres, it is possible only to anticipate results likely to be obtained from such direct experimentation. This information may be attained from a re-survey of reports especially concerned with the action of quaternary ammonium compounds on those frog striated muscles that evidence the contractural response. In 1921 Riesser and Neuschlosz (209), using the frog and toad gastrocnemius muscles, first demonstrated the contracture that resulted from exposure to Ringer's solution containing ACh. They were impressed by the duration of the effect which they noted, on occasion, to continue for several hours. However, easy reversibility to normal followed washing with fresh Ringer's solution and the direct excitability of the muscle was not altered. This contrasted with the irreversible and injurious effects of most substances then known to produce contracture. In addition, the important observation was made that this contractile response depended for its occurrence on exposure of the neural region to ACh; application of ACh to either the muscle or nerve alone was without effect. These authors also described the prominent contracture of the frog's rectus abdominis muscle in response to ACh and the antagonism of this effect by either atropine or curare. Several of the following citations furnish repeated confirmation of the antagonism of atropine to ACh and related amines acting on amphibian muscle. This effect is like that at parasympathetic neuro-effector junctions and contrasts sharply with the well-known inefficacy of atropine to modify the twitch response of mammalian striated mus-

cle. If an involvement of the small nerve system is assumed in this antagonism, a qualitative pharmacologic difference between the two motor systems must be recognized.

The pharmacologic behavior of frog striated muscle to ACh was extended by Sommerkamp (238) who classified the musculature on the basis of the recorded contractile responses to ACh applied *in vitro*. He recognized three types of response: (1) the quick twitch, exemplified by the sartorius, the semi-membranosus and the gracilis minor; (2) the prolonged contracture in which all of the fibres participate, characteristic for the rectus abdominis and the muscles of the shoulder girdle; (3) a prolonged contracture in which only part of the fibres are involved, exhibited by the gastrocnemius, ileofibularis and semitendinosus. The muscles of the lower extremities were found to belong to either the first or third type. Of special interest was the ileo-fibularis, in which he noted a distinct group of short fibres that ran between the proximal and distal tendons; these fibres reacted to the application of ACh with a contracture such that the remaining uncontracted fibres were thrown into folds as a consequence of the shortening. To this fibre group he applied the term, "tonus bundle." On isolation of this muscle bundle by dissection, he was able to show that the contracture produced by ACh resulted from an action specifically characteristic for these fibres and that the twitch response, seen with the whole muscle, resulted from the effect of ACh on the "non-tonic" fibres. In support of Sommerkamp's findings, the tonic contraction pattern elicited by ACh in the muscles of the frog's thorax, shoulder girdle and forelimbs was described in detail by Wachholder and Lebeduhr (252). Some few years later, the great sensitivity of the contracture mechanism of the frog rectus abdominis muscle to ACh suggested to Chang and Gaddum the use of this muscle for the bioassay of ACh (57).

The similarity between the responses to ACh and to nerve stimulation is evident in the data of Bremer (25). With appropriate indirect stimulation of the frog's gastrocnemius, a complex contraction was elicited in which relaxation of the quick response revealed the persistent contracture. The addition of either atropine, scopolamine or small amounts of curare largely diminished the contracture without visibly affecting the quick component. It appears from the data of Bremer that the contractural response is more sensitive to curare than is the twitch mechanism, but the more quantitative results of Raventos (206) as described below, support the opposite view.

Hess and v. Neergaard (125) re-examined, under more natural conditions, the behavior of frog muscle toward ACh. This was accomplished by perfusion *in situ* with oxygenated Ringer's solution delivered to the gastrocnemius by means of an intra-aortic cannula. ACh, introduced into the perfusion medium, produced a shortening equal to that occurring from maximal single shocks and, like the *in-vitro* response, it could also be inhibited by atropine. In a later study, the *in-vivo* activity of ACh on frog's striated muscle was determined by Brown (33) who employed the technic of intra-arterial injection. In these experiments with natural circulation, contractures having tensions in excess of the maximal twitch response to indirect stimulation could be developed by the close intra-arterial

injection of ACh. Large intra-arterial doses of atropine depressed the response to nerve stimulation as well as to ACh; the slow phase of the contraction was the more sensitive to the action of atropine. Curare also inhibited both the quick and the slow portions of the contractile response to intra-arterial ACh, but a clear-cut preferential action on one or the other component could not be ascertained. However, Raventos (206) defined the relationship between the dose of ACh and the nature of the contractile response in the frog's gastrocnemius, a muscle innervated by small and large diameter ventral root fibres. The technic of close arterial injection was adopted and it was determined that small doses of ACh (0.05–0.25γ) produced only contracture while larger doses (0.5–10γ) resulted in a twitch succeeded by a contracture. Atropine (0.5–10γ), administered intra-arterially, abolished the contracture but was without influence on the twitch. Small doses of curare (0.05–0.5γ) decreased or annulled the twitch but did not affect the contracture. Larger doses, as might be expected, abolished the contracture. These results provide a good comparison of the pharmacologic reactivity of the two motor systems present in frog muscle. They signify a greater sensitivity of the contracture mechanism to ACh and accordingly a somewhat higher resistance to curare block.

B. Reactivity to other quaternary ammonium compounds

The recognition of the reactivity pattern of amphibian striated muscle to ACh provided a reference standard for evaluating the effects of other quaternary ammonium compounds. It is a fact that the reactivity of frog striated muscle to quaternary ammonium compounds was known for many years before this prominent action was noted for ACh. Not only is ACh the naturial onium prototype, but with the realization of its functional role in synaptic transmission, studies of the structure-activity relationship among this class of compounds have assumed special significance. These pharmacologic studies have defined and related certain chemical characteristics to the initiation of excitation in a postsynaptic structure and as a consequence possible mechanisms of transmitter action have become evident. With regard to the neuromuscular junction (n.m.j.), it is a simple matter to proffer certain exceptions as an objection to the correlation of chemical structure with biologic activity at this site. While these exceptions may provide unanswerable and interesting problems, they bear no necessary connection to the fact that a remarkable relationship exists between certain structures of the quaternary ammonium class of compounds and specific biologic activity at a synapse like the n.m.j. The significance of this correlation is all the more enhanced by the recognition that the naturally occurring transmitter is a member of this group of compounds.

The response of amphibian striated muscle to simple alkyl onium salts was observed as early as 1908 when Boehm (21) saw generalized fasciculations in the frog after the injection of a solution of either tetramethylammonium or valeryltrimethylammonium salts into the ventral lymph sac. In this regard these bases were indistinguishable from nicotine. In an *in-vitro* experiment, using the isolated gastrocnemius or semimembranosus muscle, he applied separately mus-

carine as well as the methyl, the ethyl, the valeryl, the ethanol (choline) and the vinyl (neurine) derivatives of the trimethylammonium structure (—$\dot{N}Me_3$) and, in each instance, observed contracture.

Kulz (162), using the isolated frog gastrocnemius, studied the series: $\dot{N}Me_4$, $\dot{N}Me_3Et$, $\dot{N}Me_3$ propyl, $\dot{N}Me_3$ butyl, $\dot{N}Me_3$ amyl, $\dot{N}Me_3$ heptyl, and $\dot{N}Me_3$ octyl. With the exception of the heptyl and octyl derivatives, all produced contracture of the muscle. Quantitative comparison on a molar basis revealed that maximal excitatory action occurred with the propyl compound and that a progressive decrease in activity took place when the chain length was either increased or decreased, although the butyl derivative proved only slightly less potent than the propyl. For this same series Ing and Wright (143) found that equivalent isometric tensions were developed in the frog rectus abdominis by suitable equimolar concentrations of either $\dot{N}Me_4$, $\dot{N}Me_3$ Et, $\dot{N}Me_3$ propyl, $\dot{N}Me_3$ butyl or $\dot{N}Me_3$ amyl. The $\dot{N}Me_3$ hexyl was less effective and the octyl derivative gave a minimum response. Raventos (207) also examined this series with respect to its contracture-producing action on the frog's rectus abdominis. He found maximal activity with $\dot{N}Me_3$ butyl. The $\dot{N}Me_4$ and $\dot{N}Me_3$ amyl were next in potency and the heptyl compound fell sharply in potency when compared with the $\dot{N}Me_3$ hexyl; the octyl derivative was inactive. These results concur well with those of Kulz, when one considers that different muscles were employed in the respective investigations. Alles and Knoefel (8) also determined the activity of this series on the frog rectus muscle and reported the $\dot{N}Me_3$ butyl compound to be the most active and the $\dot{N}Me_3$ amyl to be only slightly less so. The hexyl and heptyl derivatives were close to the $\dot{N}Me_4$ salt in potency and activity fell sharply in the octyl compound. The nonyl compound was without excitatory effect. In general it may be concluded that chain lengths in excess of five carbon atoms sharply diminish the excitatory action until it nears extinction in the octyl compound.

In confirmation of earlier work, Chang and Gaddum (57) found that esterification of choline increased the excitatory potential of this base on striated muscle. Thus choline was only 0.14 per cent as potent as ACh for the initiation of contracture in the frog's fectus abdominis muscle. The propionyl ester proved to be 5.5 times as active as ACh, and butyrylcholine was only slightly less effective than the acetyl ester. Other esters of choline studied were the valeryl, glycollyl, pyruvyl, and carbamyl; all were active but to a lesser degree than the simple aliphatic esters. It is significant (see below) that the glycollyl ester (hydroxyacetylcholine) was the least active, having approximately one per cent of the effectiveness of ACh. In contrast, the others exhibited activity ranging upwards from 13 per cent of that of ACh. It is clear that the intensification of choline activity by esterification does not result simply from an increase in the length of the side chain or from the presence of the ester group itself. This is well illustrated by the experiments of Strack and Fosterling (243) and Burgen and Hobbigger (47) who prepared γ-carbomethoxyallyl trimethylammonium chloride, a compound in which the position of the ketonic and ether oxygens of ACh are

interchanged: $Me_3\dot{N}—CH_2—CH=CH=C(=O)—O—CH_3$. It also differs from ACh in

that unsaturation is present in the alkyl chain. This compound was found to produce contracture of the frog's rectus muscle similar to ACh except that the latency was longer and the rate of development of the contracture slower. The potency of this ester was about one third that of ACh and like ACh its action was opposed by d-tubocurarine (dTC) (47). Unlike ACh the sensitivity of the rectus muscle to this substance was not increased by physostigmine or TEPP and accordingly the ester was found refractory to esterasic hydrolysis. As in the case of choline, the nature of the esterifying group of $Me_3\dot{N}—CH_2—CH{=}CHCOOH$ is an additional determinant of potency. Consequently the larger ethyl (242) and benzyl (47) esters were found to be somewhat less active than the methyl ester. Of primary importance is the fact that the unesterified acid had only 1/100,000 the activity of ACh and only 1/2000 the activity of choline (243). These results emphasize the contributory role of esterification *per se*, as well as the ester link components, in the excitatory actions of these esterified derivatives and analogues of choline. It will be seen from later discussion that esterification primarily serves to enhance stimulatory action by providing a suitable chemical masking of the polar OH grouping.

Ethers of choline are also active stimulatory agents on the frog's rectus muscle. Thus the methyl ether of choline is more active than choline and the methyl ether of homocholine is considerably more effective than the choline ether (231).

A few aryl derivatives of $—\dot{N}Me_3$ studied also proved effective in the production of contracture in frog muscle. Ing and Wright (143) stated that the phenyl, benzyl and γ-phenylethyl derivatives of $—\dot{N}Me_3$ produced contracture of the rectus abdominis equivalent to that elicited by $\dot{N}Me_4$. Neostigmine, a more complex aryl quaternary ammonium compound, contains $—\dot{N}Me_3$ as its cationic head. In 1946 it was reported by Aeschlimann and Stempel (7), Lehmann (171), and Miquel (189) that this substance produced contracture of the frog's rectus muscle. As in the case of several other quaternary ammonium salts, the action of neostigmine differed quantitatively from that of ACh and like choline was found to be only one thousandth as potent as ACh. The base resulting from the hydrolysis of neostigmine, 3-OH phenyl $\dot{N}Me_3$ also causes contracture of the frog rectus (237) but it is more potent in this regard than neostigmine. This suggests that the carbamic acid ester link acts to diminish the direct excitatory capacity of the cationic head.

The researches of Hunt and Taveau (137) have suggested that a general increase in the nicotine-like activities of choline derivatives might be expected from the introduction of an aromatic group into the molecule. The results of Simonart (230, 231) provide a seeming exception in that a comparison of the activities of the methyl ether of homocholine and the γ-phenyl derivative of this compound on the frog rectus muscle show the former to be at least twice as active as the phenyl compound. However, many later studies have revealed the fact that several aryl quaternary ammonium compounds possess potent excitatory actions on striated muscle and it has become apparent that the benzene ring must be strategically located so that optimal spacing between two active molecular centers can be achieved.

The recent interest in the paralytic property of bis-trimethylammonium com-

pounds has furnished an opportunity to observe the effect of duplication of the onium center on the excitatory potential of derivatives of —$\dot{N}Me_3$. In general, the activity of such compounds is like the simple monoquaternary ammonium analogue and the influence of such factors as overall molecular size and the presence of certain groupings sharply modifies their action.

Paton and Zaimis (198) determined that contracture of the frog's rectus muscle could be produced by bis-trimethylammonium radicles connected by 6, 7, 8, 9, 10, 11, 12 and 18 methylene groups, respectively. The C12 compound was most effective, being one fifth as potent as ACh. Activity fell off sharply with decreasing chain length but the C10 and C4 compounds most closely approached the activity of C12. The C18 compound, like the C7 and C6 derivatives, possessed only weak actions. The contractures produced developed more slowly than those elicited by ACh, but like ACh the stimulatory action was antagonized by dTC and atropine. The action of ACh was enhanced slightly by C10. The shorter-chain compounds, C5 and C6, antagonized the contractures produced by the longer-chain compounds as well as those evoked by ACh. Paton and Zaimis concluded that the increase in excitatory action that occurs with the lengthening of the chain between the onium groups is a consequence of a steady approximation to an analogous monoquaternary salt. In accord with this, an excessive increase in the methylene chain, as in C18, would result in decreased activity equivalent to the effect of a nonamethylene moiety attached to —$\dot{N}Me_3$. However, the extremely weak activity of the short-chain compounds is not explained by such an analogy.

Bovet *et al.* (22) in their investigations of synthetic "curarizing" drugs, studied the group of compounds formed by the bis-esterification of the dicarboxylic acid series with choline and its ethyl analogues. The excitatory properties of these substances were evaluated on the rectus abdominis of the frog. In comparison with ACh, the succinyl derivative was approximately sixteen times more effective; the glutaryl derivative about twice as active; the adipic, the pimelic and sebacic derivatives ranged from about one half to one third the contractural activity of ACh.

These data reveal that ACh and other trimethylated quaternary onium cations have, as a pharmacologic characteristic, a prominent excitatory action on that amphibian muscle which responds to stimulation with a non-propagated, slow tonic contraction. The principal effect of various derivatives of —$\dot{N}Me_3$ is to modify the stimulatory action in a quantitative manner only. The three most important factors changing the excitatory potency of cations derived from —$\dot{N}Me_3$ may be summarized as: (1) the length of the substituent chain; (2) the presence of an ester linkage; and (3) the presence of an hydroxyl group. The first two factors have received considerable attention as determinants of any one of the several pharmacologic activities of quaternary ammonium cations. Hitherto, only brief mention has appeared in the literature concerning a possible attenuation of pharmacologic activity by an hydroxyl group in a substituent aliphatic chain attached to the —$\dot{N}Me_3$ moiety. Lee *et al.* (170) have described a pronounced decrease in the pressor potency of β-phenylethyl $\dot{N}Me_3$ iodide when

an hydroxy group replaced a hydrogen on the β-carbon; similarly the stimulant action of the 4-OH phenylethyl $\dot{N}Me_3$ was reduced by the introduction of the hydroxyl group onto the β-carbon. More recently Lands (165) noted that choline exerted a weaker negative inotropic action on the turtle auricle than did the $\dot{N}Me_3$ Et ion; he suggested that the alcoholic hydroxyl may interfere with this cholinergic action.

The results of the experiments cited above indicate that quaternary ammonium salts of the type $\dot{N}Me_2A(OH)$, where A(OH) is an hydroxy substituted aliphatic chain, are among the weakest contracture-inducing substances in this general class of compounds. Thus $\dot{N}Me_3EtOH$ (choline) is relatively impotent in its action on the frog rectus (57) and from the data it can be estimated that choline is approximately one fifth as active as $\dot{N}Me_3Et$; the difference is merely the elimination of the hydroxyl group. The potentiating effect of esterification has been cited; yet esterification of choline with hydroxyacetic acid offsets this general effect and, accordingly, the excitatory action of hydroxyacetylcholine is remarkably low in comparison with that of ACh (57). In this respect, it should be noted that contracture of amphibian muscle can be evoked by muscarine, a compound in which an hydroxyl group is present in a branched five-carbon chain attached to $-\dot{N}Me_3$. However, it is significant that from a teleologic view the drug lacks excitatory activity on more highly developed quick-twitch systems. The interpretation of this effect of an hydroxyl group is considered in the last section of this review.

The importance of the $-\dot{N}Me_3$ moiety itself in the action of quaternary ammonium compounds on frog striated muscle may be appreciated from studies involving replacement of the methyl groups with other alkyl groups. Boehm (21) first demonstrated the inactivity of the tetraethylammonium ion on the gastrocnemius. This lack of effect of $\dot{N}Et_4$ has since been confirmed by others using the frog rectus muscle (57, 143, 180, 207). In addition to $\dot{N}Et_4$, other symmetrical tetra alkylammonium salts are similarly unable to evoke contractural responses from the frog rectus muscle (143, 207).

As early as 1916, it was learned that when two of the methyl groups in $\dot{N}Me_4$ were replaced by two ethyl groups, the resulting dimethyl cation was without excitatory action on the frog's rectus abdominis (180). Consequently, the salts of $Me\dot{N}Et_3$ and $\dot{N}Et_2Me_2$ were also ineffective in this regard. These findings were confirmed for this simple alkyl ammonium series by Raventos (207). In an early review, Ing (140) concluded that, for the onium compounds to manifest an excitatory action on frog muscle, the attachment of at least three methyl groups to the central onium atom was a requisite. However, Holton and Ing (133) directly approached the problem as it applied to ACh. In this study, the specificity of $-\dot{N}Me_3$ in ACh was ascertained. The replacement of a single methyl by an ethyl group reduced the contracture action on the frog's rectus to only one fifth that of the parent compound. Obviously two methyl groups attached to the central nitrogen will suffice for activity and it appears that, in a cation such as dimethyl diethyl ammonium, the ethyl groups actively exert a detrimental effect on the excitatory potency (cf. p. 69). Thus in other recent

experiments designed to determine the neuromuscular action of the 3-hydroxy phenylethyl dimethylammonium ion (Tensilon), the frog rectus abdominis was employed as a test object and thereby further evidence has been furnished for the contractural action of a dimethyl onium cation (237). On the other hand, the replacement of two methyl groups seriously damages the stimulatory action, regardless of the nature of other attached groups. This suggests, for this n.m.j., that like the several diverse mammalian effectors responding to quaternary ammonium salts, two rather than three methyl groups on the central onium atom are the critical number. It must be appreciated, however, that maximal activity is associated with trimethylation of the nitrogen atom.

C. The effect of cholinesterase inactivation

The inherent cholinergic properties of amphibian nerve-skeletal muscle junctions can be verified to a large extent by the use of substances that inactivate cholinesterase (ChE). That appropriate inhibition of ChE by any one of the anti-esterase agents allows for the accumulation of ACh is a fact well attested by the voluminous evidence presented in the review prepared by Koelle and Gilman on anti-ChE drugs (154). The mere inactivation of ChE, though, is not a sufficient condition for subsequent pharmacologic response; this primary action must be followed by either the appearance of ACh or a related substance released from an endogenous store, or derived from an exogenous source. Thus in 1936 Feng (96) observed that the toad sartorius treated with physostigmine developed contracture when subjected to a brief indirect tetanic stimulation and particular interest was aroused in this contracture because of its intimate dependence on nerve activity. It was necessary to expose the tibial two-thirds (neural portion) of the muscle to the alkaloid if the contracture was to be elicited, and after nerve section and degeneration physostigmine was without action. Continued analyses of this contracture led Feng and Li (101) to note striking differences between the reactivities of the gastrocnemius and sartorius muscles in the manifestation of this response. The gastrocnemius removed from a toad previously injected with physostigmine exhibited pronounced contracture in response to repetitive nerve volleys whereas the sartorius excised from the same animal showed, with similar stimulation, considerably less tendency to develop contracture. That these disparities in sensitivity to physostigmine could be correlated with differing ACh sensitivities was clearly demonstrated by these workers; the gastrocnemius proved much more responsive to ACh than the sartorius. In all these experiments it is probable that the more striking contracture response of the gastrocnemius may be attributed to the prominence of the small nerve system in this muscle.

In the case of the isolated frog's rectus abdominis muscle such substances as di-isopropylfluorophosphate (DFP) and tetraethyl pyrophosphate (TEPP) do not by themselves cause shortening despite extreme inactivation of ChE (126, 202). This condition then could not result in the liberation of a significant amount of ACh. Miquel (189) and Quilliam and Strong (202) did note that toxic amounts of DFP caused contraction of the muscle but this effect cannot be considered to be a specific one. Finerty (103) has shown that the acidic character of DFP con-

tributes to its sensitizing action on the frog rectus and this has been considered the basis for its contractural action (202). In suitably high concentration, physostigmine will induce a contracture of the frog's rectus muscle (126, 189) but it possesses only about seven per cent of the direct excitatory capacity of neostigmine, a quaternary ammonium salt.

The relationship between the direct stimulating actions of neostigmine and physostigmine and their ability to sensitize to ACh has been well worked out for the frog rectus muscle (126). It is clear from this work that the optimal sensitizing action of neostigmine, and particularly of physostigmine, occurs with concentrations considerably below those required to stimulate directly. In the same study, sensitization to ACh by TEPP, physostigmine, neostigmine and two other dimethyl carbamate esters (Nu 683, Nu 5130) were compared with reference to their respective anti-ChE activities. Physostigmine and Nu 683 produced about 60 and 20 per cent, respectively, of the sensitization that could be established by TEPP; neostigmine equaled this action of TEPP. Surprisingly, at the peak sensitizing concentrations of any one of these agents, there were no significant differences in the extent to which muscle ChE had been inactivated, although the degrees of sensitization achieved varied widely. Inactivation of the ChE of the rectus muscle and increased susceptibility to ACh are inextricably associated, but the fact remains that a difference as great as 80 per cent exists between the sensitizing activity of two compounds whose anti-ChE activities are closely related. These data imply that a factor or factors as yet undescribed are operative in the production of sensitization of this junctional region to ACh.

Along similar lines, Hobbiger (126A) and Smith *et al.* (237, 237A) demonstrated a general correlation between the anti-ChE potency of certain aryl quaternary ammonium compounds and their ability to potentiate the action of ACh on the frog rectus muscle. In the series, 3-OH phenyl $\dot{N}Me_3$, 3-OH phenyl $\dot{N}Me_2Et$, 3-OH phenyl $\dot{N}MeEt_2$, 3-OH phenyl $\dot{N}Et_3$ and neostigmine, the potentiating effect of neostigmine was greatest and the potency of the 3-OH phenyl $\dot{N}MeEt_2$ derivative was somewhat less than one half that of neostigmine; 3-OH phenyl $\dot{N}Me_3$ and 3-OH phenyl $\dot{N}Me_2$ Et were approximately one tenth as effective as neostigmine; the 3-OH phenyl $\dot{N}Et_3$ was considerably weaker than any of the other derivatives (237A). The direct stimulant action of the members of this series could not be correlated with their separate abilities to potentiate ACh. Although the anti-ChE potencies of these agents as determined on eel, bovine erythrocytes and frog rectus ChE varied considerably according to the particular enzyme source studied, a general correlation existed between ChE inhibition and potentiation of the ACh response in the frog rectus preparation. It was concluded that potentiation of the characteristic ACh contracture of frog rectus muscle serves to indicate sensitization through ChE inactivation. There remains some question that sensitization may be so simply considered but the possible role of ChE inhibition in the production of the pharmacologic actions of 3-OH phenyl $\dot{N}Me_3$ and related compounds must be entertained. However, further study is indicated, particularly in the mammal where a rigid correlation between ChE inactivation and response is to be expected if the mode by which the response

is produced is uncomplicated. That this is in fact the case has been established by Jones *et al.* (147) who demonstrated for a series of organic phosphates a linear relationship between the negative log of the potency and response. Further it has been shown that a continuous relationship exists between the degree of ChE inhibition and the potentiation of ACh response in a mammalian neuro-effector system (212). This demonstration was achieved by the use of a single agent, DFP, in which possible multiple actions are obviously excluded. Finally, the interpretation of results obtained from a system such as the *in-vitro* frog rectus preparation must necessarily be limited, for in this system factors for the achievement of steady state conditions are ideal. This circumstance is inapplicable to the triggered response of the mammalian neuro-muscular apparatus.

It must be recognized that the pharmacologic action of the quaternary ammonium compounds is complex with regard to membrane action and ChE inhibition. It is not improbable that an overt excitatory action evolves as a visible manifestation of an effect on the post-junctional membrane whereby susceptibility to the action of ACh is increased. The complexities inherent in these combined drug actions are emphasized by the experiments of Zaimis (271) who demonstrated sensitization of the frog's rectus muscle by TEPP to the contractural action of C10, the latter a compound unaffected by ChE. As a corollary it was concluded that the excitatory action of C10 on the rectus could not be attributed to an anti-ChE action. Without doubt, the part played by ChE inactivation in enhancing the action of ACh is firmly established, and sufficient data now exist to encourage inquiries for additional mechanisms contributory to sensitization of response.

II. MAMMALIAN MUSCLE

A. Nature of contractile responses induced by acetylcholine

By 1930 there already existed a voluminous literature on the physiology and pharmacology of autonomic neuro-effectors but there was no specific report on the excitatory action of ACh on mammalian striated muscle. This circumstance can be attributed not to lack of interest but rather to the lack of a suitable method for the study of drugs acting on this structure. In fact, it was only through the hypersensitivity of denervated mammalian skeletal muscle and the peculiar hypersensitivity of mammalian extra-ocular muscles that the reactivity to ACh was eventually recognized. In 1931 Duke-Elder and Duke-Elder (81) recorded the *in-vivo* and *in-vitro* responses of the external rectus muscle of the dog and cat to ACh and in 1933 Feldberg (93) described the contractile response of the normal tongue musculature of the dog when ACh was introduced into the perfused lingual artery.

Although it had been generally accepted that the mammalian striated musculature was indifferent to ACh, Boehm (20) as early as 1885 noted the muscular contractions produced in the cat and in the rabbit by the subcutaneous injection of the nitrous ester of choline or of choline itself. In 1914 Dale (69) confirmed this action for the nitrous ester in the cat and also noted that the nitric ester possessed this property in somewhat lesser degree, but it was not until 1932 that Feldberg

and Mintz (95) noted the generalized fasciculatory responses that attended the intravenous injection of relatively large doses of ACh in the cat and in the dog. This effect was found to occur much more intensively when injection was made by an intra-arterial route. With this technique, it was realized that contraction of striated muscle could be produced regularly by small amounts of ACh and these authors made the important point that normally innervated mammalian striated muscle responded to ACh with sharp contractions. It was also pointed out that this action was uninfluenced by either acute nerve section or atropine. In 1934 Simonart and Simonart (235) recorded the isotonic contractions of the cat gastrocnemius following the intravenous injection of ACh and in the next year the technique was improved (233); injections were made into the hypogastric artery and the contractile responses of the muscle were recorded isometrically. From these studies emerged the now classic report of Brown *et al.* (36) on the reactions of the normal cat muscle to ACh. These investigators exploited the technique of close intra-arterial injection in a preparation in which the circulation was severely restricted to the muscle under study. With temporary interruption of the single arterial channel it was possible to apply ACh rapidly and to achieve contact with all of the muscle fibres. The results obtained stood in sharp contrast to the irregular results of the earlier workers. With this experimental method it was possible to demonstrate that twitch-like responses followed the close intra-arterial injection of as little as two micrograms of ACh. Larger doses produced contractions several times the maximal twitch tension. The importance of making the injection rapidly was recognized, and it was pointed out that the effect of a given dose would be much reduced if some slight delay were made in its delivery. The transient nature of ACh action was described, and the important observation was made that as soon as the contraction caused by ACh had terminated the response to indirect stimulation was entirely normal.

The nature of the response to ACh was suspected by Brown *et al.* to be in the nature of a short asynchronous tetanus and this impression was confirmed in a subsequent communication (32) in which restricted electrical recording from the cat's gastrocnemius was made. On the basis of this work, it was concluded that the contractile responses evoked by ACh were all or none in character and were initiated at the motor endplate.

Simonart and Simonart (233, 235) ascertained that curare abolished the response of the cat gastrocnemius to injected ACh but that relatively large doses of atropine were without effect on the contraction. This was confirmed by Brown *et al.* (36) who in addition showed that the response evoked by the close intra-arterial injection of ACh was prevented more readily by curare than was the response induced by nerve stimulation. This difference was conceived to result from the more effective application of ACh released by nerve stimulation as a consequence of the intimate relationship between the terminations of the motor nerve and the endplate. The insensitivity of the ACh-induced contraction of mammalian striated muscle to inhibition by atropine further delineates the unique position of the contracture mechanism in certain frog muscle. The quick-twitch muscle of the frog, like the mammalian striated muscle, is relatively un-

affected by atropine. Although Brown (33) was able to inhibit with atropine the effects of both ACh and nerve stimulation on the frog sartorius muscle, comparatively large amounts of the alkaloid were necessary to accomplish this. Luco and Altimirano (174) were able to abolish with atropine the ACh-evoked contractile response of the superior rectus muscle of the cat, but for this purpose intra-arterial doses of atropine ranging between one and eleven mgm. were necessary to block threshold doses of ACh.

Despite the extreme difference in susceptibility of the mammalian n.m.j. to the alkaloids of the curare and atropine series, certain workers (1, 174), on the basis of limited experiments, have implied qualitatively similar actions for curare and atropine irrespective of the synapse involved. Such an extreme view is not yet warranted if one considers the preponderant evidence for the widely dissimilar reactions to atropine and curare manifested by mammalian parasympathetic effectors, neuronal synapses, ganglionic synapses and the n.m.j. of striated and other types of muscle from various species. That atropine or curare may act as blockers at more than one synaptic site is not surprising; obviously certain chemical denominators are common to synaptic function in general; the transmitter agent itself is a prime example. The fact remains that the mammalian synapses of the autonomic, peripheral and central nervous systems exhibit extreme variation in susceptibility to the blocking actions of atropine and curare. It is most likely that these discrepancies are largely attributable to differences in the nature of the cellular receptors. Although ACh may be viewed as a generally effective excitant of post-synaptic structures, the many specific excitatory agents now known strongly support the concept of individual properties peculiar to each of the several synapses.

The importance of properly applying ACh to elicit a contractile response from mammalian striated muscle is again underscored when it is realized that initial attempts to observe such an action in man met with failure because injection was made into the portal of a large vascular area supplying a huge muscle mass (54, 105). Since then, however, the reactivity of human striated muscle to ACh has been clearly estabilshed. Harvey *et al.* (119) found that the rapid injection of ACh into the brachial artery produced a sensation of flexion which was quickly superseded by a temporary motor paralysis. The doses employed were excessive and perhaps quickly instituted an inexcitability of the endplate regions thereby aborting further initiation of contractile responses. Lanari (163) earlier had failed to note muscle contraction in normal man following the injection of similarly large doses of ACh into the brachial artery. Oddly, these workers (119, 163) did observe muscle contraction produced by the identical administration of ACh in human subjects afflicted with myasthenia gravis, myotonia and certain disorders of the nervous system. However, Acheson (2) could not confirm a difference in reactivity of normal and myasthenic muscle to ACh. The extension of this study by several groups of investigators provided a sufficiently wide experience and clearly established the fact that normal human muscle is no less responsive to ACh (4, 41, 121, 268). Acheson *et al.* (4) and Buchthal and Engbaek (41) revealed the wide variability of the threshold doses of ACh when injection is made into a large vascular bed such as that supplied by the brachial artery. Acheson

et al. decreased this variation somewhat by injection into the smaller area circulated by the radial artery. In any case, considerable variation of responses occurred and this likely reflects the inability, in the human experiments, to restrict ACh to the artery supplying a particular muscle. This is an important requirement if activating concentrations are to be presented to the several junctional regions in the total muscle with sufficient temporal uniformity.

B. Reactivity to other quaternary ammonium compounds

At the time that Brown *et al.* (36) introduced a technique appropriate for the pharmacologic study of mammalian skeletal muscle, almost nothing was known concerning the action of other quaternary ammonium compounds on this structure. Nearly all the then extant pharmacologic information regarding drug action on striated muscle had been derived from experiments on frog muscle, particularly the rectus abdominis, a type of muscle not strictly comparable to the mammalian form. In fact even present day information in this regard is still meager and much of the knowledge of structure-activity relationship obtained from studies on frog muscle still awaits re-evaluation on the mammalian neuromuscular apparatus. From the historical view, the early reports of Boehm (20) and Dale (69) described the effects of the nitrous and nitric esters of choline. Simonart (232) recorded the appearance of generalized post-mortem fasciculations in cats given lethal doses of the ethyl ether of choline. Actually, the Simonarts were engaged in a search for pure muscarinic substances and were therefore concerned with the appearance of any nicotine-like actions. In this way, they noted post-mortem fasciculations in cats that had received either the ethyl, vinyl or butyl ether of choline. It was remarked that the fasciculations depended intimately on a co-existing asphyxia. The mechanism of this effect is not clear and in none of these instances can it be certain that a direct stimulant action was involved. Further study is needed. However, it is noteworthy that post-mortem fasciculations did not occur in the animal poisoned with the ethyl ether of β-methylcholine (232). Utilizing the newly developed technique of the close intra-arterial injection in the mammalian neuromuscular preparation, Bacq and Brown (12) showed that other esters of choline, namely, the propionyl, the valeryl and the butyryl esters, produced contractile responses. These substances were effective in a range of doses approximating that for ACh but all were somewhat less potent than the acetyl ester. The acetyl ester of β-methylcholine was ineffective in intra-arterial doses as high as one mgm. and this result paralleled the known inability of this compound to excite the cells of a sympathetic ganglion. The carbamic acid ester of choline, carbaminoylcholine, produced a minimal contraction in a dose of 50 micrograms but this effect was followed by a long-lasting block of neuromuscular transmission. Choline itself produced a contractile response but it had only about 1/1000 the activity of ACh and this effect was succeeded by a depression of the response to indirect stimulation. The simple basic $\dot{N}Me_4$ ion was similar in action to choline but was effective in approximately one half the dose. Here again, as with the amphibian muscle, is an instance of a weakening of excitatory action by an hydroxyl group.

The recognition of the anti-ChE action of physostigmine by Engelhart and

Loewi (88) and the subsequent implication of the carbamate grouping as the responsible chemical moiety (239) fostered the preparation of many synthetic carbamic acid derivatives. The results of one of these programs led to the development of neostigmine (6). Because of the dramatic therapeutic effect that this intended physostigmine substitute exerted in myasthenia gravis and the simultaneous advances in the physiology and pharmacology of neuromuscular transmission, an especial interest centered around this compound. From the pharmacologic standpoint, neostigmine is a distinctive compound, for it exerts a strong inhibitory action on ChE activity and manifests, on skeletal muscle, an extremely rapid onset of action. This is of particular interest, since certain compounds react equally well with specific ChE but do not give rise to as prompt an excitatory effect at the n.m.j. The accumulated evidence presented thus far provides adequate illustration of the unique affinity of the substituted charged cationic grouping, —$\dot{N}Me_3$, with some post synaptic structure of the n.m.j. It was a consideration of this chemical structure in the neostigmine molecule that called attention to the possibility that this compound exerted a dual influence at the junctional region in striated muscle (211). Accordingly, it was demonstrated that the close intra-arterial injection of neostigmine into the cat gastrocnemius muscle evoked a contractile response as promptly as that initiated by the comparable administration of ACh. It was also shown that this action of neostigmine probably occurred independently from ChE inhibition since contraction could be evoked by neostigmine after extreme depletion of ChE by huge DFP dosage. Further analysis of the neostigmine molecule was directed towards ascertaining the functional contribution of the cationic head in the action on the n.m.j. A dissection of the molecule was begun by first replacing the dimethyl carbamate moiety with the simpler acetyl esterification; this was intended to eliminate the anti-ChE activity and to increase the resemblance to ACh (262). The resulting compound fell somewhat short of expectations since there remained some residual anti-ChE activity, but it was of a strictly competitive nature (261). When this substance, 3-acetoxy phenyl $\dot{N}Me_3$ bromide, was injected by the close intra-arterial route into the neuromuscular preparation of the cat, there resulted a contractile response which, like that produced by ACh, proved to be an asynchronous tetanus (216). The removal of the acetyl group revealed an identical action for the unesterified phenolic base, 3-OH phenyl $\dot{N}Me_3$ bromide (214). The basic ion $\dot{N}Me_3$ phenyl exhibited a similar initial action but differed in that the post-contractile fasciculations subsided rapidly and a transient depression of neuromuscular transmission readily occurred (259). This indicates a functional role for the hydroxyl grouping in the 3-OH phenyl $\dot{N}Me_3$ ion. This function will be discussed in a later section. However, it is evident from these data that the immediate contraction in skeletal muscle induced by any one of these agents is attributable to the strongly basic $\dot{N}Me_3$ phenyl ion and most probably derives primarily from the —$\dot{N}Me_3$ structure.

The effect of twinning the cationic grouping, —$\dot{N}Me_3$, with respect to excitatory action on the mammalian n.m.j., is illustrated in the work of Paton and Zaimis (198). C10 given by close intra-arterial injection into the cat produced a

rapid contraction when the dose was reduced to 4 micrograms. Some degree of neuromuscular block ensued but pronounced blocking action appeared only after larger doses. This action of C10 is qualitatively like that of $\dot{N}Me_4$ ion but of greater potency. Apparently the intensity of combination with receptor is fortified by the presence of two such reactive centers. Ginzel *et al.* (111) studied the pharmacology of a series of bis-choline esters and reported that the bis-choline ester of adipic acid evoked a strong contraction of the cat tibialis muscle when given by close intra-arterial injection in a dose as small as ten micrograms. As with the simple straight-chain bis-onium compounds, the initial excitatory effect was followed by neuromuscular depression. In the case of the ester compounds, however, the blocking action disappeared quickly but their effect could be prolonged by pre-treatment with physostigmine, as had been shown previously for the blocking action of succinyl choline (56). Most suggestive was the observation (111) that the contractile response was little affected by previous eserinization.

From these observations on the contractile effects produced in mammalian striated muscle by ACh and some few other quaternary ammonium compounds, it is immediately apparent that there is a woeful lack of any systematized study correlating the manifestation of this simple property with purposeful variations in the chemical constitution of quaternary ammonium derivatives. The few observations cited form no parallel for the well explored structure-activity relationship in the frog rectus muscle. The contrasting all or none character of the mammalian striated muscle fiber demands a separate evaluation. Such investigation could be ideally carried out on the single nerve-muscle fiber preparation.

C. The effect of cholinesterase inactivation

It is pertinent to inquire to what extent any anti-ChE property of these compounds will contribute to the initiation of contraction. In the absence of nerve stimulation, it is unlikely that an anti-ChE action is concerned in the contraction that follows the intra-arterial injection of an effective quaternary ammonium salt. The rapid intra-arterial injection of a near-fatal dose of DFP does not elicit an immediate contraction (211). It might be argued that this can be attributed to the comparatively slow interaction of DFP and ChE (191). However, the tertiary amine, physostigmine, like neostigmine, is water-soluble and reacts equally rapidly with ChE (191). Even if one assumes an instantaneous and extreme inactivation of ChE, it is difficult to imagine the simultaneous and uniform appearance of effective concentrations of ACh at the several motor endplates. The fasciculatory responses characteristically developing after inhibition of junctional ChE must reflect the small steady release of ACh, as suggested by Feldberg (94), and the asynchrony of its cumulation. This view receives substantial support from the recent work of Fatt and Katz (91) which describes the spontaneous, random and subthreshold electrical activity at the endplates of resting muscle fibers. The recorded miniature potentials are increased in size by neostigmine and reduced by curare. The conclusion was reached that the observed activity reflects the release of ACh by occasional activity in motor nerve terminals. The extra-ocular muscles of the cat, in response to the intra-arterial

injection of physostigmine, often manifest sufficient synchronization of fasciculatory response to produce tensions equal to or in excess of those evoked by maximal nerve shocks (38). This unusual situation finds explanation in the unique sensitivity of these muscles to ACh and their extremely low innervation ratio (1:3–1:10). In view of this, the threshold to ACh will be less and temporal dispersion of response slight. It must be concluded that the organized contraction of a skeletal muscle does not depend, for its initiation, on the inactivation of ChE.

D. Reactivity of chronically denervated muscle

1. *Acetylcholine.* The reactivity of chronically denervated muscle to ACh was first pointed out in 1922 (104). This initial observation was greatly extended by the studies of Dale and Gasser (73, 108) who made an elemental contribution to the pharmacology of ACh when they reported the unusual sensitivity of chronically denervated mammalian muscle to this substance. They concluded that the sensitivity was associated with the type of action that this substance shared with nicotine and that it was unrelated to the muscarine-like action of ACh. It was found in the cat that the distant intra-arterial injection of an amount of ACh as small as 0.2 microgram produced a perceptible response; after the i.v. administration of physostigmine, the threshold was reduced nearly one hundred fold. When the dose of ACh was optimal, the tension produced closely approached that obtainable by electrical stimulation (73). With the technique of close intra-arterial injection, Brown *et al.* (36) determined that the chronically denervated gastrocnemius muscle of the cat responded to as little as 0.001 microgram of ACh. Using larger doses these authors called attention, for the first time, to the compound nature of the response which consisted of an initial quick contraction succeeded by a secondary slow contracture. In contrast, the chronically denervated gastrocnemius of the frog manifested only a decrease in threshold to intra-arterially applied ACh (33).

The action of ACh on chronically denervated muscle was localized by Kuffler (158) to that region where the endplate would normally exist. These experiments were performed on chronically denervated frog sartorius muscle which after a period of seven weeks responded to concentrations of applied ACh less than 1:1,000,000,000. It was concluded that the principal change following denervation was a greatly increased excitability at the endplate region. As a consequence of the instability at this site, it was suggested that the spontaneous fibrillation occurring in denervated muscle might also originate from this point.

Brown (33) studied the electrical accompaniments of an ACh-induced response in the chronically denervated mammalian muscle. It was learned that the initial quick component is accompanied by an outburst of propagated action potentials like those that occur in normal muscle. In contrast to the normal situation the duration of the electrical discharge is considerably longer. With the onset of the sustained contraction there is an abrupt cessation of all electrical activity; this silence may persist as long as ten minutes after a single injection. The complete suppression of electrical activity by ACh also signifies a concomitant inhibition

of the usual fibrillatory potentials of chronically denervated muscle. These findings have been confirmed (221).

Gasser and Dale (108) concerned themselves with the effects of substances that might be expected to antagonize the action of ACh on denervated mammalian muscle. They reported that the action of curare was unimpressive but they did not explore dose ranges and were under the disadvantage of having to work with an impure preparation of alkaloid. Atropine, in doses several times those necessary to abolish muscarinic actions, was without influence on the response of the denervated muscle to ACh. The inefficacy of atropine to inhibit this contractural response, as opposed to the ready prevention of contracture in frog muscle by atropine, proved disturbing to Dale and Gaddum (72). To circumvent this seeming discrepancy they produced a chronic denervation of the cat diaphragm and after a suitable period sacrificed the animal and employed a strip of the muscle for *in-vitro* recording. This preparation proved very effective in exhibiting the characteristic response to ACh and under these conditions a concentration of 1:3000 atropine sulfate effectively obliterated the response. The implication was that it is necessary to maintain a sufficient local concentration of the drug if the effect is to be obtained. Although this provides another instance in which atropine can be shown to exert an effect like that produced by curare, it is distinctly artificial and actually serves to emphasize the singular affinity of the curare molecule for the n.m.j.

The apparent resistance of denervated muscle to the blockade of ACh action by curare also impressed Simonart (233) and was again remarked on by Brown *et al.* (36) and Brown (33). However, these latter workers did show that a sufficient dose of curare produced some inhibition of the response to ACh and that both the quick and the slow component of the response were equally depressed. On the other hand, Rosenblueth and Luco (221) inferred from their results a more pronounced antagonistic action of curare. Finally, McIntyre and co-workers (186) were able to demonstrate that the injection of a pure solution of dTC easily prevented the response of denervated dog muscle to ACh. McIntyre concluded that a curare-ACh antagonism exists independent of innervation (184). It is probable that the seeming refractoriness of denervated muscle to curare, as found by the earlier workers, can be traced to the lack of a purified curare alkaloid.

Gasser and Dale (108) also made the intriguing observation that epinephrine, injected immediately prior to ACh administration, annulled the characteristic effect. When epinephrine was injected with or instantly after ACh it did not influence the response. The inhibitory action of epinephrine could be reversed by ergotamine. Considerable indirect evidence was produced by Dale and Gaddum (72) to show that the inhibitory effect of epinephrine did not result from a true drug antagonism. They were able to demonstrate that pitressin had a similar action and that the epinephrine effect could be overcome by the simultaneous administration of histamine. These findings strongly suggested that the inhibitory action of epinephrine on the ACh response resulted from a reduction of capillary permeability. The absence of true drug antagonism between epineph-

rine and ACh also was well demonstrated on the isolated strip of denervated cat diaphragm. In this instance epinephrine did not modify the reactions to ACh.

2. *Effect of cholinesterase inactivation.* Brown (33) analyzed the effect of physostigmine on ACh action in denervated muscle and showed, with regard to magnitude and duration, that the enhancement of the effect was confined to the slow phase of the response. As in normal muscle, the parameters of the quick phase of the ACh contraction of denervated muscle were little affected by physostigmine. An identical effect of DFP on the response to ACh was later demonstrated (35). These findings parallel other already cited observations relating to the initiation of contraction in normal muscle; they argue strongly for the fact that ACh and related quaternary ammonium compounds detonate the response in a way quite different from that resulting from any inactivation of ChE that they may effect.

In the early part of this century. Langley and Kato (169) observed that physostigmine was without effect on chronically denervated muscle. This important observation has since been substantiated many times and is readily explained by the accepted fact that physostigmine produces its pharmacologic effects chiefly by inactivation of ChE. With the advent in 1943 of DFP, a potent irreversible inactivator of ChE, the veracity of this view was ascertained. It was revealed that, in the chronically denervated muscle of the cat, DFP was without effect (211) despite the fact that a comparatively huge dose was administered to the muscle by close intra-arterial injection. This finding was confirmed by Brown *et al.* (35). Similarly, the potent alkyl phosphate, HETP, was found to be without effect on the cat denervated muscle (59). The absence of an effect of anti-ChE compounds on denervated muscle directly relates to the degeneration of the motor nerve and the consequent absence of ACh, as shown by Dale *et al.* (71).

In light of these considerations, it must be concluded that the excitatory action of neostigmine at the n.m.j. is complex. This fact emerges from the demonstration that neostigmine produces a characteristic ACh-like contracture of chronically denervated cat skeletal muscle (211). The effect occurs even after large intra-arterial doses of DFP. It is obvious that this particular excitatory action is not mediated through the inhibition of esterase activity. The excitatory action of neostigmine has since been confirmed in the chronically denervated muscle of the dog (205).

Rosenblueth and Luco (221) noted contraction of chronically denervated cat muscle in response to either physostigmine or neostigmine; such an effect for physostigmine has not been noted by others (73, 169, 195, 260). It is likely that confusion may have arisen as a result of the assumption by these authors of identity between these drugs (cf. 223). In this regard it is significant to note that Rosenblueth and Luco (221) reported that the spontaneous electrical activity of the denervated muscle was temporarily increased after the administration of either physostigmine or neostigmine. Assuming only an anti-ChE effect, this result implies the improbable event of ACh liberation in a chronically denervated muscle. Brown and Harvey (38) using only physostigmine were unable to confirm this effect in the chronically denervated inferior oblique muscle of the

cat, a muscle exquisitely sensitive to cholinergic stimulation. It is likely that the report of Rosenblueth and Luco reflects the direct cholinergic effect of neostigmine at the endplate, in that neostigmine was probably employed in these experiments and inaccurately referred to as "eserine," thus providing a basis for misinterpretation.

3. *Other quaternary ammonium compounds.* The continued disruption of the innervation of skeletal muscle results in an increase in its sensitivity not only to ACh but also to other quaternary ammonium bases. Dale and Gasser (73) again pioneered in this respect when they showed that the simple $\dot{N}Me_4$ ion produced contracture of the denervated muscle but that bases such as natural muscarine and $\dot{N}Et_4$ were without such action. The Simonarts took advantage of the high sensitivity of chronically denervated mammalian muscle to nicotine-like drugs and employed it as a means of uncovering this property among the quaternary bases which they were testing for restricted muscarinic activity. Choline itself exerted only weak activity but when the propionyl and butyryl esters were tested on the denervated muscle of the cat, it was found that the former was approximately equal to ACh in activity and that the latter possessed about one half the potency of the naturally occurring ester prototype (232). It should be recalled that propionyl choline was also found more active than butyryl choline on the frog rectus muscle (cf. 57). These results signal the influence of esterification and of chain length on the excitatory potency of ACh analogues on mammalian muscle.

The simple methyl, ethyl, vinyl and butyl ethers of choline were noted by Simonart (232) to produce contracture of the denervated muscle but were very much weaker than the aforementioned esters. In each instance, their effects on the muscle were more prolonged than those of the esters, and this may reflect the participation of ChE in the termination of the choline ester action. It might be interjected, parenthetically, that the butyl and vinyl ethers of choline manifested strong pressor activities in the cat and yet were notably inactive on the denervated muscle. This serves to re-emphasize the individuality of synapses and points to the pitfalls inherent in a screening program in which one particular pharmacologic test is used to evaluate more general properties.

The addition of a methyl group to either the α or β carbon of choline neither augmented nor decreased the weak activity exhibited by choline itself on the denervated cat muscle (232). The striking increase in excitatory activity that is generally associated with esterification of choline was again evident with the acetyl ester of α-methyl choline. This compound possessed about one half the activity of ACh on the denervated muscle. It is noteworthy that it also exerts actions on the circulatory system of the cat like those of ACh and is only slightly less potent. However, when the acetyl ester of β-methyl choline was tested, the remarkable finding was made that this substance exerted a minimal action on the chronically denervated muscle; it was, in fact, no more active than its unesterified base. The profound muscarinic activities of this compound were described and are now widely recognized. The propionyl ester of β-methyl choline proved to be equally impotent on denervated muscle; its muscarinic activity

was only slightly less than the acetyl ester. Although the ethyl ether of choline was not exceptionally active on denervated muscle, the ethyl ether of β-methyl choline exerted a negligible action. The influence of a β-methyl substitution was also ascertained for the carbamino ester of choline. This drug was also inactive despite the fact that carbamyl choline is moderately effective in eliciting contracture from denervated muscle (236). From these data it has become apparent that the β carbon of choline analogues must be important in the determination of "receptor fit." The influence of other substituents on this carbon remains to be determined, but it may be that the production of asymmetry at this site hinders receptor union at the n.m.j. Simonart (234) tested the dextro and levo forms of acetyl-β-methyl choline on denervated muscle and found no significant difference in activity.

The contracture effect of neostigmine on chronically denervated muscle has been noted above. When the dimethylcarbamate grouping of neostigmine is replaced by an acetyl ester linkage, the resulting compound is definitely more active than neostigmine in this regard (205, 216). However, acetyl esterification by itself does not significantly increase the activity of the base, 3-OH phenyl $\dot{N}Me_3$. Conversely, esterification of this base with dimethyl carbamic acid, to form neostigmine, results in a decrease in the direct stimulatory capacity, as tested on denervated muscle. The same relationship has already been denoted to exist between these two compounds with respect to their excitatory actions on the frog rectus muscle. It must be concluded that the direct stimulatory potential inherent in the cationic portion of neostigmine is counteracted, to some extent, by the carbamate moiety. More generally, the comparison of the action of 3-OH phenyl $\dot{N}Me_3$ and neostigmine provides exceptions to the concept that esterification results in an increase of potency. Both 3-OH phenyl $\dot{N}Me_3$ and 3-acetoxy phenyl $\dot{N}Me_3$ exhibit approximately equal activity on the chronically denervated muscle (205, 214). However, the acetyl ester of 3-OH phenyl $\dot{N}Me_3$ is quite susceptible to esterasic hydrolysis (261) and the possibility exists that it may actually exert its effect as the hydroxy compound. On the other hand, this is not the case for neostigmine. Furthermore, several aromatic acid esters of 3-OH phenyl $\dot{N}Me_3$ have all been found slightly less active than the unesterified base (205). These were: the benzoyl, the benzyl, the *meta* chlorbenzyl, the *para* aminobenzoyl, the nitrobenzoyl, the *meta* toluyl and the *para* toluyl esters. Although the stability of these compounds is unknown, the enhancement of excitatory activity associated with esterification appears to apply only to aliphatic onium derivatives.

With regard to excitatory effects at the n.m.j., the contribution of an aromatic ring attached to the central onium atom cannot be minimized. This is evidenced by the finding that $\dot{N}Me_3$ phenyl is considerably more potent on the mammalian denervated muscle than is $\dot{N}Me_4$ (205). It is worth reiterating here that the activity of $\dot{N}Me_2Et_2$ on the sensitive frog rectus muscle was restored by the replacement of one of the ethyl groups with a phenyl ring. In this relatively simple aromatic onium series, the modifying influence of the hydroxyl group again is manifest. A comparison of the action of $\dot{N}Me_3$ phenyl and 3-OH phenyl $\dot{N}Me_3$

on denervated muscle discloses the phenolic derivative to have only one fourth the activity of the phenyl compound. However, when this is compared with the data for aliphatic hydroxyl onium compounds it is clear that the attenuating influence of the hydroxyl group is greater when it is attached to an aliphatic chain than when it is affixed to an aromatic ring.

In a series of investigations on the properties of several neostigmine analogues, Randall and Lehmann (205) and Randall (203) ascertained the importance of the position at which radicals were attached to the aromatic nucleus. The results noted with isomers of the 3-OH phenyl $\dot{N}Me_3$ ion are most illuminating. The shift of the OH grouping to a *para* position reduced the effectiveness fivefold but the placement of the OH group in the *ortho* position rendered the compound nearly inactive on the denervated structure. When the distance between the OH group and the onium center was re-established, as in the *ortho* hydroxyl benzyl derivative of $—\dot{N}Me_3$, it was found that activity returned closely toward that of the 3-OH phenyl $\dot{N}Me_3$ ion. These relationships are illustrated in figure 1.

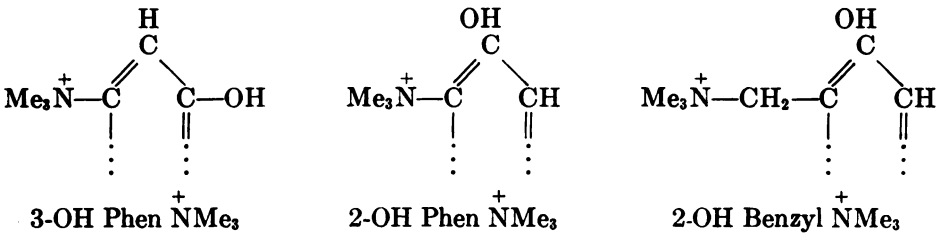

FIG. 1

These observations indicate that the proper spatial separation of the OH group from the central onium atom by the aromatic nucleus is essential for reaction of the amine with the receptor. This reaffirms the conclusion (214) that the 3-OH phenyl $\dot{N}Me_3$ ion represents a basic structural configuration that endows it and certain of its derivatives with a high affinity for the receptors of the n.m.j. An interpretation of the influence of the hydroxyl group on excitatory activity will be presented in the section on general considerations.

The importance of $—\dot{N}Me_3$ for excitatory activity is again evident from pharmacologic experiment with chronically denervated muscle. The report of Dale and Gasser (73), in which they noted the inactivity of $\dot{N}Et_4$, has been mentioned. In the 3-OH phenyl $\dot{N}R_3$ series, the earlier conclusions based principally on experiments with frog muscle have been substantiated. Accordingly, the replacement of a single methyl by an ethyl group in the compound 3-OH phenyl $\dot{N}Me_3$ reduces the activity on denervated muscle to about one fourth that of the trimethyl compound, but when two of the methyl groups are exchanged for ethyl groups the resulting compound retains only slight stimulatory action. Finally, the replacement of all the methyl groups to form the 3-OH phenyl $\dot{N}Et_3$ ion abolishes excitatory action (203). In recent unpublished work in this laboratory it has been shown that the close intra-arterial injection of 3-OH phenyl $\dot{N}Me_3$ into the chronically denervated muscle of the cat results in the immediate appearance of a propagated electrical response which is succeeded promptly by

the quick contractile component. The total effect is identical to that elicited by ACh. Following the close injection of 3-OH phenyl $\dot{N}MeEt_2$ an excitatory response did not appear even when the amount of this substance was increased to fifty times that of the methyl compound. In this way, the chronically denervated mammalian muscle provides another instance in which it may be shown that the initiation of excitation by quaternary ammonium ions depends largely on appropriate methylation of the onium atom and, more important, that the excitatory action is indistinguishable from that of ACh.

The recognition of the strong depolarizing action of C10 (39, 52) has substantiated the original suggestion of Paton and Zaimis (198) that C10 produces neuromuscular blockade by initiating some active response in the endplate or muscle fiber. On this basis, it might be expected that this compound would activate a contractural response in chronically denervated muscle. Such an effect was demonstrated by Zaimis (271) in the denervated muscle of the cat. The close intra-arterial injection of 2 micrograms of C10 produced a contracture manifesting the typical rapid and slow components; the duration of the latter phase was quite prolonged. As is the case with ACh, the quick portion of the response was accompanied by an outburst of propagated potentials and the slow phase was associated with an electrical silence. The injection of ACh, during the silent period, produced a slow contracture with no electrical activity. This is identical to the effect of two ACh injections (33) administered in a similar manner. Zaimis concluded that the activity of C10 on denervated muscle presented strong evidence for the view that this compound exhibits all of the characteristic actions of ACh at the n.m.j. From a consideration thus far of the excitatory actions of both C10 and 3-OH phenyl $\dot{N}Me_3$ on the mammalian n.m.j., it would be devious to seek to implicate ChE inactivation as an explanation for the primary action of these two compounds. Qualitatively the actions of these compounds at the n.m.j. are the same and they differ only in the intensity with which they produce each of several characteristic actions.

In an initial report, Jarcho *et al.* (145) described the obliteration by C10 of the fibrillatory potentials of denervated rat muscle. In subsequent experiments (146), a very brief initial increase in the fibrillatory potentials of denervated rat muscle was detected to follow the injection of C10. The increased electrical activity was striking but was followed by a complete electrical silence. With recognition of the hypersensitivity of the denervated muscle to C10, the dose was made suitably small and the fibrillatory potentials could then be enhanced without a succeeding depression. This result again expresses the typical ACh-like action of C10 at the n.m.j., and stresses the continuity of excitation and depression characterizing this class of depolarizing substances.

One of the most interesting findings concerned with the action of quaternary ammonium compounds on chronically denervated mammalian muscle has been made by McIntyre *et al.* (185, 186). These workers found that the close arterial injection of dTC produced a contracture of the denervated gastrocnemius muscle of the dog. As found for the other quaternary ammonium compounds, the contractile response consisted of a fast and a slow component. As in the case of ACh

and C10, the initial effect was accompanied by an increase in the electrical activity and the succeeding contracture was associated with an electrical silence. The amounts of dTC necessary to elicit this effect were, on a molar basis, approximately one hundred times greater than the minimal amount of ACh required. These results were seen to indicate a parallelism between the effects on denervated muscle of two quaternary ammonium bases whose pharmacologic actions are considered as mutually antagonistic. Perhaps this effect of dTC should not be unexpected. Rather, the surprising result might be considered to be its lack of any apparent excitatory effect on the normally innervated muscle. The direct excitatory action of dTC on denervated muscle was confirmed by Bean and Elwell (15) who also showed that dTC augmented the contractile response of the directly stimulated denervated muscle. In the denervated rat gracilis muscle, Jarcho *et al.* (146) recorded the same general sequence of events after the i.v. injection of dTC as had occurred after C10 administration. Usually the brief stimulatory effect after dTC was less intense and the succeeding depression of fibrillatory activity less complete than after C10. In this circumstance, it seems that dTC exerts an initial transient depolarization. A broad pharmacologic view of these substances, then, might recognize a basic unitary action for the quaternary ammonium compounds, but it would be an action qualified as one that undergoes full scale gradations in accord with the nature of the appended molecular structure.

There remain, however, some apparent exceptions that make any generalization tenuous. Thus Flaxedil, like $\dot{N}Et_4$, does not exert a contractile effect on the chronically denervated muscle of the cat; but in unusually high dosage such an effect has been elicited with Flaxedil (45, 215). At first, it was suspected that the absence of an evident excitatory effect with Flaxedil might be attributable to the ethonium cation. This idea has been dispelled by the fact that the nonamethyl analogue of Flaxedil is similarly inactive on the denervated structure (218). Notwithstanding, Flaxedil and certain of its analogues do exhibit some action in denervated muscle. Bülbring and Depierre (45) have shown that large intra-arterial doses of Flaxedil sensitize the denervated cat muscle to ACh.

III. AVIAN MUSCLE

In avian muscle, the characteristic effect of ACh, administered by close intra-arterial injection, is the quick development of tension from which recovery is slow (37). As in denervated mammalian muscle, the onset of the response is accompanied by oscillatory action potentials and the maintained contraction is associated with electrical silence. Unlike mammalian muscle, chronic denervation does not qualitatively change the pattern of response but, like in the mammal, the sensitivity of the muscle increases. Physostigmine only slightly augments the tension response to ACh but greatly prolongs the slow phase of the contraction.

The ACh-like properties of C10 were also manifest in the response of avian muscle to this substance; small amounts of C10 produced a sharp rise in tension which was maintained over a prolonged course (53, 112). In similar fashion,

the ACh-like effects of the bis-choline esters of dicarboxylic acids were evident in the neuromuscular preparation of the pigeon (112). The bis-choline ester of adipic acid equalled the intensity of C10 action but was much briefer in duration; it was in fact not significantly different from the action of ACh.

Like the amphibian and mammalian musculature, excitation of avian muscle by quaternary ammonium compounds is dependent on the degree of methylation of the central nitrogen atom. Although the *mono* ethyl analogue of the bis-choline ester of adipic acid was equiactive with the parent compound, the diethyl analogue possessed only a minimal excitatory action. A total elimination of excitatory action resulted when the methyl groups were replaced entirely by ethyl groups.

The influence of chain length on excitatory properties was also apparent in the study of the bis-choline esters on the pigeon preparation (112). It was found that activity decreased with an increase in the methylene chain length of the dicarboxylic acid. This was exemplified by the fact that the bis-choline ester of succinic acid was more active than the adipic homologue which in turn was more active than the sebacic member of the series.

In general the little information available for this tissue is consistent with the pattern of quaternary ammonium activity that has been outlined for amphibian and mammalian muscle.

IV. NEUROMUSCULAR TRANSMISSION

A. The effect of cholinesterase inactivation

Historically, pharmacologic studies on neuromuscular transmission had their origin in the development of a suitable technique (36) for the study of mammalian neuromuscular function. In these early experiments performed on the cat, it was noted that the contractile force of the muscle, responding to single maximal motor nerve shocks, could be augmented by the injection of physostigmine. Brown (32) investigated this effect of physostigmine in greater detail. After an intravenous dose of physostigmine, the maximum potentiation of the response to indirect stimulation was reached between five and ten minutes. This action of physostigmine was lasting, for it could be detected as long as 140 minutes after the administration of the drug. By the use of electrical recording, Brown revealed that the augmented "twitches" were, in fact, brief incomplete tetani. When the recording was restricted to a small number of muscle fibers, it was possible to show that physostigmine converted the response from a single nerve shock to a propagated repetitive discharge approaching, momentarily, a frequency as high as 600/sec. This was observed when the mechanical response had achieved an optimum. The discharge was at first synchronous but this phase usually did not continue for more than thirty to forty msec. and following this an extreme asynchrony developed. As the effect of physostigmine wore off, the discharge frequency developed by each nerve impulse fell progressively. It was concluded that the effect of physostigmine was mediated through its anti-ChE action, allowing for ACh to persist at the endplate in a supraliminal concentration which in turn initiated the repetitive discharges from this region. The stimulating effects of

physostigmine on neuromuscular transmission were eventually confirmed by Rosenblueth and co-workers (222, 223).

Bacq and Brown (12) explored the dose range over which physostigmine elicited potentiation of response. The maximum effect in the cat was achieved with a total dose of 25 microgram; higher doses produced a potentiation that was, after a short period, aborted by a secondary depression. The failure of potentiation after the large doses of physostigmine was laid principally to the excessive accumulation of ACh, but most significant was their finding that relates to the time course of the development of the characteristic physostigmine effect. Thus, the close intra-arterial injection of 20 microgram of physostigmine resulted in a gradual increase of the muscle response that reached its maximum in slightly more than two minutes. Clearly, the progressive development of the full action is associated with the accumulation of optimal concentrations of ACh at the several motor endplates.

Existing studies on amphibian muscle indicated that physostigmine did not enhance the response to indirect stimulation as it did in mammalian muscle. This seeming contrast was dispelled by the work of Feng (97) who demonstrated that the critical determinant for such an effect in amphibian muscle was the interval between stimuli. When the rate of indirect stimulation was as slow as one shock every minute or two, the addition of 1:10,000 physostigmine to the Ringer's solution bathing the toad's sartorius muscle resulted in the slow development of an increased response. The application of a direct shock to the aneural portion of the muscle elicited only the normal response. This result intimately associates the effect of physostigmine with the release of ACh through neural activity and further implies a low cholinergic capacity of amphibian as contrasted to mammalian tissue.

The results of Feng were confirmed by Cowan (68). In addition, Hodes and Steinman (128) were able to demonstrate in the nerve-sartorius preparation of the intact frog a typical augmentation of muscle response by physostigmine. For some unknown reason, they were unable to obtain this effect if the drug were administered by the close intra-arterial route.

The effect of an anti-ChE on the responses of mammalian muscle to maximal indirect shocks is well illustrated by experiments with DFP. Hunt (135) found that the close intra-arterial injection of 0.1 mgm./kgm. DFP potentiated the response of the cat's gastrocnemius in a manner similar to that previously described for physostigmine. This dose of DFP proved to be optimal for the production of this effect and potentiation developed progressively to reach a peak in approximately two minutes. A visible effect lasted for thirty minutes or more. Larger doses depressed the muscle response. Brown *et al.* (35) confirmed this observation but reported that an optimal DFP dose was followed by a latent period of about one minute after which the tension gradually rose and reached a peak in approximately four minutes. The duration of this effect accorded with the similar result of Hunt. When the dose of DFP was very large (1 mgm.), the maximum potentiation could be attained in about one minute but was followed by a profound depression of the response. Despite the rapidity of action of this

large DFP dose, the peak effect was developed through a graded response. Thus, it must be concluded that in the intact mammalian preparation the sufficiently close injection of a suitable dose of DFP provides a combination of circumstances leading to rapid inactivation of junctional ChE.

A recent communication describes (132) the pharmacology of the powerful dimethylamido-ethoxyphosphoryl cyanide (Tabun), another irreversible inactivator of ChE. The close intra-arterial injection of as little as 10 microgram potentiated the response of the cat's tibial muscle to maximal nerve shocks. In contrast to DFP, a very small range existed between the stimulating and paralyzing doses. As is the case for DFP and physostigmine, the attainment of the full effect required the passage of some few nerve impulses.

The high potency and affinity of the alkyl phosphates for ChE should furnish a decisive answer to the question of whether the rate of development of an effect on neuromuscular transmission is dependent solely on the rate of ChE inactivation. One group of investigators has described the pharmacology of HETP and TEPP in detail (48, 49, 59). The potentiation of the indirect response of cat muscle by these materials was shown; additionally, a simultaneous repetitive discharge of the muscle was recorded electrically (48, 59). Although the peak effect was reached in stepwise fashion, the experiment could not be considered crucial since all doses were administered intravenously. A closer approach to a solution of the speed with which TEPP exerts a maximal effect was made in an *in-vitro* experiment in which TEPP was added to the fluid bathing a rat phrenic nerve-diaphragm preparation (49). A considerable potentiation of the indirect response resulted but again the maximum was achieved gradually. In a recent unpublished experiment (218), the critical demonstration was made that TEPP injected by the close intra-arterial route augmented the indirectly stimulated cat gastrocnemius muscle and although the peak effect was achieved with unusual rapidity, it was gained through a progressively increasing response.

B. The effect of acetylcholine

Since the characteristic action of these several substances reflects the action of endogenous ACh, it is reasonable to anticipate a similar demonstration for this compound. This was immediately apparent to Brown *et al.* (36) who determined the effect of the close intra-arterial injection of ACh on the response of the cat gastrocnemius muscle to indirect stimulation. The results showed potentiation of the subsequent adjacent response and possibly a perceptible increase in the succeeding twitch. Although Simonart (233) in the previous year had reported that the intra-arterial administration of ACh potentiated the response of the cat's gastrocnemius to brief faradic shocks applied through the nerve, the effect of ACh, as observed by Brown *et al.*, was certainly not impressive. Briscoe (27) showed that this effect of ACh could be noted more readily when the stimuli were submaximal in strength. It is obvious that the difficulty met with in demonstrating potentiation by ACh expresses the factors making for the unique rapidity through which the effects of this compound are normally dissipated. Of these, there is an almost unanimous recognition of the catalytic activity of

ChE. However, the existence of some undetermined adaptive mechanism of the endplate membrane may be equally important in this regard (158).

The influence of ChE was investigated by Hunt (135) who found that small doses of DFP, ineffective by themselves, permitted injected ACh to persist sufficiently long to enhance the twitch tension to several succeeding impulses. Under these circumstances, it is pertinent to indicate that the effect of ACh was immediately maximal.

C. The effect of other quaternary ammonium compounds

In addition to the anti-ChE drugs, several quaternary ammonium compounds other than ACh have now been noted to produce the characteristic augmentation of the indirect responses of muscle. Neostigmine will be considered in this category, although it might have been equally well included with the anti-ChE agents. From the historical standpoint, the action of neostigmine on neuromuscular transmission was reported first by Wilson and Wright (269). Independently from the work of Brown *et al.*, it was observed that the administration of neostigmine enhanced the response of the cat gastrocnemius responding to an indirect stimulus. Rosenblueth *et al.* (222) also recorded this action of neostigmine. Bülbring (43) succeeded in demonstrating the potentiating effect of neostigmine (as well as physostigmine) on an *in-vitro* mammalian nerve-muscle preparation. Feng (97) and Cowan (68) both found the effect of neostigmine on amphibian muscle to resemble that of physostigmine. In all of these studies, the implication was that neostigmine, like physostigmine, achieved this effect solely through inhibition of ChE. With the realization of the direct component in neostigmine action, the effect of neostigmine on mammalian neuromuscular transmission was re-examined (260). In the cat gastrocnemius preparation, the rapid intra-arterial injection of an optimal dose induced a peak response by at least the second shock following the injection. The speed of development of this action provides strong presumptive support for an initial, direct excitatory action.

In the studies designed to investigate the action of the cationic head of neostigmine on neuromuscular function, attention was directed to the modification of the response to indirect stimulation by certain related cations (203, 205, 214, 216, 259). It became apparent that the close intra-arterial injection of either the $\dot{N}Me_3$ phenyl ion, or its 3-OH or 3-acetoxy congeners, resulted in a potentiation of the indirect response (214, 216, 259). In contrast with the action of the anti-ChE agents, the effects of these substances were, in each instance, immediately maximal. Unlike the usual anti-ChE action, the effects of these ions were comparatively short-lived and the control twitch tension was reached within two to three minutes. The high potency of these compounds in the production of this effect was evident from the effective dose range. A dose of 1 microgm./kgm. was threshold and affected only the next impulse; doses from 5–10 microgm./kgm. were optimum. The potentiating action of the unsubstituted $\dot{N}Me_3$ phenyl ion deserves special comment. It differed from its derivatives in that it produced only an enhancement of two or three twitches and the brief effect was usually

followed by a transient depression. Although this ion manifested an intense musculotropic action, it was difficult to find a dose that was purely stimulatory.

The data obtained by Randall (203) for the effects of many neostigmine analogues on neuromuscular transmission are of considerable value in the definition of structure-activity relationship. In a manner paralleling the action of 3-OH phenyl $\dot{N}Me_3$ and its congeners on the chronically denervated muscle, the replacement of methyl with ethyl groups resulted in a progressive diminution in the capacity to potentiate the indirect response of the dog tibialis preparation. The mono- and diethyl analogues were effective in the dose range of 3-OH phenyl $\dot{N}Me_3$. However, the fact that they were administered intravenously prevented further potency discrimination and precluded assessment of the speed of development of action. Surprisingly, the 3-OH phenyl $\dot{N}Et_3$ ion showed some activity but to produce the effect a dose one hundred times that of 3-OH phenyl $\dot{N}Me_3$ was necessary. However, the result does reveal for a triethyl onium cation a stimulatory action that was not in evidence on the chronically denervated muscle. This same communication also emphasizes the critical effect of the OH position in the three isomeric forms of OH phenyl $\dot{N}Me_3$. Although the enhancement of the response of the indirectly stimulated dog muscle was achieved readily with small doses of 3-OH phenyl $\dot{N}Me_3$ ion, the removal of the OH to the 2 or 4 positions abolished the excitatory effect. This corresponds with the effects observed for these isomers on denervated muscle. However, among the isomeric forms of OH phenyl $\dot{N}Me_3$, Depierre and Funke (78) found that paralysis of neuromuscular transmission was most readily induced by the 4-OH phenyl $\dot{N}Me_3$. The *ortho* isomer was only weakly active. If it is assumed that the paralyzing action of these compounds is in essence an extension of the excitatory property, this latter finding for the *para* form is contradictory to the report of Randall (203), although it must be borne in mind that different species were involved.

The effect produced by the phenyl $\dot{N}Me_3$ ion, and particularly its 3-OH and 3-acetoxy derivatives, on the electrical response of the motor unit stimulated through its nerve was shown to be characteristic of that resulting from the accumulation of ACh (214, 216). The duration of the entire effect rarely exceeded three minutes and subsequent electromyograms showed the prompt return of the simple propagated action current. The evanescence of action is to be contrasted with the prolonged repetitive activity observed after the administration of either physostigmine (32), neostigmine (119) or DFP (35, 118) in both cat and man. It must be concluded that the ambivalent position of neostigmine reflects a combined effect of the differing actions of the component phenyl $\dot{N}Me_3$ ion and the dimethyl carbamic acid.

A comparison of the effects of choline with its aromatic analogue, 3-OH phenyl $\dot{N}Me_3$, on neuromuscular transmission is illuminating. Like the phenyl compound, choline in optimal dose produced an immediately maximal potentiation of the indirectly excited cat tibialis (138). As for other related quaternary ammonium compounds, the potentiated response was associated with repetitive firing of the muscle. The elicitation of this effect required a dose of choline approximately ten fold the effective dose of 3-OH phenyl $\dot{N}Me_3$. The duration of

the twitch enhancement induced by choline was less than that which occurs after the phenyl analogue. This was due in part to the transcendence of a neuromuscular depression that can be attributed to the large doses of choline that were necessary to elicit an effect. The great quantitative differences between the excitatory actions of these analogues implies once again the functional contribution of the ring structure to the excitant properties of aryl quaternary amines. Depierre and Funke (78) arrived at a similar conclusion with regard to the phenyl ring of 3-OH phenyl $\dot{N}Me_3$.

Several references have been made to the gradation of action manifested by diverse quaternary ammonium substances, ranging in general character from predominantly excitatory to profound blocking agents. Thus it has been possible to illustrate an excitatory effect of dTC on denervated mammalian muscle (146, 185). In addition to this, perusal of an experiment depicted by Rosenblueth *et al.* (223) has revealed the remarkable finding of a transient potentiation of the indirect responses of the cat tibialis muscle by curare. Accompanying electrograms evidenced a concomitant repetitive discharge. Unfortunately, the authors make no mention of this recorded observation and from the technique employed it is possible that the indirect stimuli were submaximal. Nonetheless, it reveals an inherent excitatory action of curare, albeit minimal and fleeting.

In an analysis of the mode of action of polymethylene bis-trimethylammonium salts, notably C10, Paton and Zaimis (198) recorded the prominent increase in twitch tension produced in the cat by small intravenous doses of C10. It is significant that only stimulatory effects appeared with small doses. The usual acknowledgement of a cause and effect relationship between potentiating effect and inhibition of ChE led these workers to determine the anti-ChE activity of C10. They found that the "true" ChE from erythrocytes was inactivated by C10 in moderate concentration ($pK_i = 4.5 \times 10^{-5}$). They maintained the opinion, however, that the prominent actions of the drug were not mediated through the inhibition of ChE.

Zaimis (271) attacked this problem directly and revealed that the effects of C10 on the mammalian n.m.j. could not be correlated with an anti-ChE action. The results of this work are particularly revealing with regard to the complexity of action of quaternary ammonium compounds at the n.m.j. Having established the highly potent excitatory action of C10, exemplified by the conversion of the single twitch response into an incomplete tetanus, it was shown that this action could be augmented by the prior administration of physostigmine. This occurred with doses of physostigmine too small to be effective alone. A similar effect was demonstrable with small doses of neostigmine but the interaction of neostigmine and C10 at the n.m.j. was even more complex. This was evidenced by a mutual potentiation between the two compounds. Thus ordinarily ineffective doses of neostigmine, following two stimulant doses of C10, converted the single twitch into a pronounced tetanus and when this effect had passed off the repetition of the dose of C10 reproduced a similar tetanic spasm. It was found that the action of C12 was also potentiated by a previous dose of neostigmine. Compared with C10, C12 proved to have a weaker stimulant action and a correspond-

ingly weaker blocking action (198). On this basis, any correlation of either excitatory or blocking properties with anti-ChE activity was destroyed by the finding that C12 possessed a ten-fold more potent action against "true" ChE than did C10 (198). Obviously, the synergistic sensitizing action of any one of these compounds cannot be readily interpreted by the single assumption of ChE inhibition. Zaimis next demonstrated that the $\dot{N}Me_4$ ion increased the twitch tension of the indirectly excited cat muscle, and it was shown that neostigmine, in small doses, greatly sensitized the muscle to the stimulant action of this ion. Finally, the significant point was made that $\dot{N}Me_4$ is devoid of an anti-ChE action (14). Divergence from an action mediated primarily through ChE inhibition is increased by the fact that the phenyl $\dot{N}Me_3$ ion is also a very weak inhibitor of ChE (260). Despite this, its capacity to enhance the indirect twitch response is outstanding. In a comparable experiment, the effects of choline on transmission were not viewed as resulting from ChE inhibition (138). In this regard, it was demonstrated that small ineffective doses of physostigmine greatly increased the potentiation due to choline, as was found for C10. It was concluded that physostigmine non-specifically increases the excitability of the muscle.

Like the polymethylene bis-trimethyl ammonium salts, the bis-choline esters of succinyl and adipic acids injected into the proximate artery augmented the responses of cat skeletal muscle to indirect single maximal shocks (109, 110). No mention has yet been made of these compounds as anti-ChE agents. Evidently they may be considered so, since their ester linkages are susceptible to esterasic hydrolysis (56).

The interesting findings of Zaimis (271) and Hutter (138) accord with an early discovery of Kensler (149). It was shown that the action of $\dot{N}Et_4$ ion on the response of the indirectly stimulated cat muscle was nearly negligible but, if its administration was preceded by a small dose of neostigmine, striking potentiation of the response occurred (149). A consideration of this result in the light of the experiments of Zaimis suggests that the action of $\dot{N}Et_4$ at the n.m.j. is not anomalous. Thus the negative evidence afforded from experiments with $\dot{N}Et_4$ on frog rectus muscle and chronically denervated mammalian muscle does not necessarily exclude an excitatory action at the n.m.j. for this compound. Indeed, Ing and Wright (142) have shown that $\dot{N}Et_4$ increases the response of the frog sartorius to maximal single nerve shocks. Finally, the $\dot{N}Et_4$ potentiation of the response of mammalian muscle to indirect stimulation has been demonstrated on the kitten diaphragm *in vitro* (244). It is perplexing that this demonstration has not been achieved in the intact adult cat. In view of the positive evidence for an excitatory action of $\dot{N}Et_4$, it must be concluded that, like other onium ions, $\dot{N}Et_4$ combines at the receptor site but that this combination does not usually produce excitation. However, against a background of membrane instability the presence of $\dot{N}Et_4$ apparently leads to or supports active depolarization (cf. 127). This action should be readily demonstrable with electrical recordings.

In this same vein, it becomes apparent from the report of Barlow and Ing (14) that the exhibition of an excitatory action at the n.m.j. is not absolutely dependent on the presence of methyl groups. In this work, it was observed that in a

series of polymethylene bis-triethylammonium salts, whose methylene bridges numbered 2, 3, 4, 5, 7, 8, 9 and 10, all produced a variable augmentation of the twitch response of the isolated rat diaphragm stimulated through its nerve. The effect was most regularly seen with the C2 compound and was never observed when the methylene chain was increased to 13. Again, at this point, attention should be called to the fact that the 3-OH phenyl $\dot{N}Et_3$ ion augments the indirect response of dog muscle (203). Reference also has been made to the sensitizing action of Flaxedil on denervated muscle. For this compound with three ethonium groupings, a minimal but definite excitatory capacity has been revealed by the finding that small intravenous doses may augment slightly the indirect response of the cat tibialis (215). All these results signify a slight but definite excitatory capacity of ethonium compounds at the n.m.j.

D. The action of anticholinesterase drugs on the endplate potential

The recognition (82, 86, 99, 114) that the primary muscle response evoked by nerve stimulation is the development of a local non-propagated potential at the endplate served to establish the basis for understanding the manner in which the transmitter and other related agents act on the n.m.j. In a series of outstanding studies, Eccles and his co-workers employed this local potential to define the parameters of transmitter action. In an initial work, Eccles and O'Connor (86) found that physostigmine prolonged the endplate potential (e.p.p.) in cat muscle. This action of physostigmine was confirmed by Feng (99) in the toad nerve-sartorius preparation. Eccles, Katz and Kuffler (83) analyzed the effect of physostigmine on neuromuscular transmission in the cat and frog and observed in both species that the origin of repetitive muscle spikes was a consequence of the prolonged endplate negativity. It was concluded from this study that physostigmine lengthens the depolarizing action of ACh. It was also pointed out that the presence of repetitive activity alone does not furnish a reliable index of the persistence of transmitter action. The occurrence of repetitive muscle spikes after a drug may depend too on other factors, such as the excitability of the muscle fiber itself and its recovery or adaptation to prolonged depolarization. For these reasons, it would be desirable to ascertain the effect on the e.p.p. in any analysis of the action of quaternary ammonium compounds on neuromuscular transmission.

A few years later, Eccles and MacFarlane (85) extended their earlier observations to a determination of the actions of other anti-ChE agents on the e.p.p. of curarized frog muscle. In this study, they concerned themselves with physostigmine, neostigmine, three neostigmine analogues, a tertiary amine and DFP. Suffice it to point out that the neostigmine analogues were quaternary ammonium compounds containing the well known di-substituted carbamic acid grouping. Neostigmine produced the characteristic changes in the e.p.p. that had been described for physostigmine; but in contrast to physostigmine, neostigmine was active at much lower concentrations and the changes effected were of greater magnitude. Similar changes in the course of the e.p.p. of the curarized muscle were produced by each of the other anti- ChE drugs studied. However, in terms

of effective concentrations there were wide discrepancies. Most closely approaching the activity of neostigmine was the synthetic tertiary amine; the remaining compounds, including physostigmine and DFP, were considerably removed from the range of action of neostigmine. Considered together, none of these remaining five substances exerted an effect on the rise time of the e.p.p. or on the time to half decay of the e.p.p. of more than eight per cent of the activity manifested by neostigmine. Despite this, the effect of all these agents was interpreted in terms of their ability to inhibit ChE. In the absence of specific data on the anti-ChE activity of all these compounds, such correlation between changes in the e.p.p. and anti-ChE potency is not justified. Certainly the recent data of Augustinsson and Nachmansohn (11) would indicate a much closer relationship between the effective concentrations of neostigmine and physostigmine if ChE inhibition were the sole basis of action. From a pharmacologic consideration, the results of Eccles and MacFarlane indicate a highly specific action of neostigmine on the course of the e.p.p. in frog muscle. It is conceivable that the action of an agent lengthening the effect of either transmitter action or ACh develops through some alteration of membrane properties in addition to ChE inhibition (cf. page 55). It would be unsound to ignore the possibility that other compounds having a cationic head similar to ACh and neostigmine might also alter the characteristics of this membrane to subsequent transmitter action.

Most recently, Fatt and Katz (90) reported a remarkable analysis of the e.p.p. of frog muscle in which they employed an intracellular electrode to record actual changes in membrane potential. In confirmation of Eccles and MacFarlane, the e.p.p. recorded from curarized muscle was increased in amplitude and duration when neostigmine was added to the Ringer bath. However, the effect of neostigmine in the absence of curare was striking. This was elucidated by reducing the sodium content of the Ringer solution sufficiently to disrupt neuromuscular transmission. In this instance, neostigmine enormously lengthened the e.p.p. which, instead of rising to a peak, reached a plateau that was maintained for some 30 to 40 msec. after which it declined to one-half amplitude in 0.1 sec. (cf. 85). This finding supports the earlier report of Feng (100), in which the junctional negativity after physostigmine was noted to be more prolonged in the absence of curare.

From a determination of membrane capacity, Fatt and Katz estimated the discharge from the endplate of curarized muscle caused by the action of a single nerve impulse. In this circumstance, it was calculated that a quantity of 8×10^{-10} coulombs was transferred. This was noted to correspond with a net transport of 8×10^{-15} M. of univalent cation inward or of anions outward. In the absence of curare, this amount was figured to increase to $2\text{–}4 \times 10^{-14}$ M. and in the presence of neostigmine, the quantity of ions traversing the membrane was estimated to be about fifty times larger than that in the curarized muscle. The implication of this is that the quantity of ACh liberated by a single impulse must provide not only the electric charge for the ordinary e.p.p. but also the much greater amount which is needed to maintain the e.p.p. of neostigmine-treated muscle. The effect of neostigmine on the e.p.p. may be interpreted as preserving ACh and allowing it to maintain the e.p.p. in the face of simultaneous potential spread

along, and leakage of charge across, the membrane; but it seems more plausible to assume that the persistence of neostigmine at the endplate region serves directly to delay the recovery process of the depolarized membrane. Relevant to the latter point is the consideration by Fatt and Katz of the possibility of direct depolarization of the endplate by ACh ions. They concluded that this would be impossible since the amounts of ACh required would be absurdly large. As a corollary, the amounts of ACh needed to maintain the total action represented by the time course of the e.p.p. in the neostigmine-treated muscle (assuming a passive role for neostigmine) would be equally incongruous. Further insight into the mechanism of ACh and neostigmine action in the generation and maintenance of e.p.p. may be gained from similar experiments with compounds of closely related structure. Such studies are unfortunately lacking.

V. THE ELECTROGENIC ACTION OF ACETYLCHOLINE AND OTHER QUATERNARY AMMONIUM COMPOUNDS

A most direct approach to the determination of excitatory capacity of a substance at the n.m.j. is to ascertain its ability to depolarize the endplate region. In a sense this represents a more recent adaptation of Langley's earlier demonstration (168) of the specific reactivity of the nerve entry site in muscle to nicotine. Actually, the concept of a parallelism between the excitability of muscle and the modification of its resting potential by a substance finds its origin in the work of Höber and Waldenberg in 1909 (127). Using the frog sartorius they were able to demonstrate that the following quaternary ammonium substances depolarized the preparation: $\dot{N}Me_4$, $\dot{N}Et_4$, $\dot{N}$ propyl$_4$ and neurine. $\dot{N}Et_4$ and $\dot{N}$ propyl$_4$ ions were the weakest of this group. From the technique that was employed in these experiments, it is not possible to ascertain the site of action but in view of later work it is probable that the effects were not on the muscle membrane. This recorded depolarizing action for $\dot{N}Et_4$ lends strong support to the aforementioned suggestion that this ion exerts its weak excitatory effects in a manner similar to that of other quaternary ammonium compounds. Previous to this work, Henze (124) described a similar action of choline and muscarine on the curarized frog sartorius muscle. In both of these investigations the concentrations of the substances studied were excessively large. It can only be suspected that in concentrations approximating those of other active depolarizers, $\dot{N}Et_4$, $\dot{N}$ propyl$_4$, neurine, choline and muscarine produce a subcritical depolarization and possibly enhance the susceptibility of the endplate membrane to more active depolarizing agencies.

The earliest attempt to determine whether a relationship existed between the contractile responses induced by either nerve stimulation or ACh was made by Riesser and Steinhauser in 1923 (210). They found that exposure of the gastrocnemius muscle of the toad to ACh resulted in the development of a prolonged monophasic action current accompanying the mechanical contraction. This was confirmed by Schäffer and Licht (228) and Kruta and Paulian (156). That the initiation of the contractile response by ACh probably derives from the action of this quaternary ammonium compound on the motor endplate was demonstrated

in 1937 by Cowan (67). In this experiment, ACh was shown to produce a considerable increase in the injury potential of the isolated frog sartorius muscle. The experiment suffered somewhat from the necessity of having to expose the muscle continuously to a very dilute concentration of neostigmine to achieve the ACh effect. Despite the continued exposure to ACh and neostigmine the potential difference disappeared exponentially during the subsequent forty minutes. The adaptive mechanism of the endplate membrane to ACh was emphasized by the finding that an equivalent depolarization produced by KCl was maintained. The action of ACh could be largely prevented by exposure of the muscle to curare which was, of course, without effect on the injury potential. Cowan concluded that the liberation of ACh at nerve endings initiated the propagated response in muscle by establishing a local depolarization of sufficient magnitude.

The specific action of ACh was shown more exactly by Buchthal and Lindhard (42) who, by means of a micro-manipulator, applied a charge of ACh directly to the motor endplate of a fibre of the internal thoracic muscle of the lizard. The effect produced was likened to a brief and rapid tetanus and the liminal amount of ACh required was found to be as low as 5×10^{-6} microgram. Application of ACh to other sites on the fibre was without effect in concentrations tenfold those at the endplate; higher doses applied to the fibre produced tonic contracture. These findings were confirmed and extended to the frog sartorius muscle by Kuffler (158). Exposure of the endplate region to ACh in this preparation set up fibrillatory responses that lasted for several seconds. In this same study, a single nerve-muscle fibre preparation from the adductor longus muscle of the frog was prepared; commendable arrangements were made both for the application of a drug solution having a volume less than 0.3 microliter and for the recording of the electrical responses from a restricted area. In this manner, ACh applied directly to the endplate in appropriate concentration initiated a series of propagated potentials. As much as one thousand times the threshold concentration, applied other than at the endplate, produced at most a small local contracture. Subthreshold amounts at the endplate gave rise to a local negative potential which increased with larger concentrations until, at a critical value, a propagated spike potential was triggered. This depolarizing action of ACh was likened to the critical depolarization of the muscle membrane produced either by the normal e.p.p. or by an applied catelectrotonus. Curare sharply curtailed the depolarization by ACh.

The elegant experiments of Burns and Paton (52) have provided a quantitative picture of the depolarization of the mammalian motor endplate by both ACh and C10. In the cat, the close intra-arterial injection of 25 micrograms of ACh produced a considerable and immediate depolarization of the endplate region of the gracilis muscle. There was some slight spread of this effect from the endplate region but this tendency was obviated by the transient nature of the response. However, the prior administration of physostigmine caused the subsequent response to ACh to persist for several minutes and, as a consequence, spread of depolarization from the endplate region was evident. Larger doses of ACh injected after physostigmine rendered the endplate region electrically inexcitable, although stimulation elsewhere on the fibre was unaffected. In this

latter circumstance, the directly excited muscle impulse was unable to propagate past the endplate region. The strong depolarization of the endplate region produced by ACh could be reversed by the passage of anodal current.

The experiments recorded by Burns and Paton were designed principally to define the actions of C10 at the motor endplate. From these experiments it was found that the depolarization instituted by this compound was confined to the muscle membrane within 3 to 4 millimeters of the endplate. Using a technique described for the cat's gracilis muscle (34) and designed especially for the study of the e.p.p. in the mammal, they determined the magnitude of the depolarization with reference to the demarcation potential. Maximum depolarization obtained by C10 was about 95 per cent of the injury potential; this was achieved by intra-arterial doses of approximately 20 micrograms. The interesting observation was made that repeated doses of C10 produced a progressively diminishing depolarization. This provides a further analysis of the observation that repeated large doses of neostigmine, or 3-acetoxy phenyl $\dot{N}Me_3$, become increasingly less effective in establishing neuromuscular paralysis (260). Why the endplate membrane should become less sensitive to depolarizing compounds, despite an apparent recovery of the membrane potential, remains unanswered. The immediate effect of C10 administration gave rise to an intense depolarization in the center of the endplate with some slight spread beyond this locus. When the depolarization persisted, the spatial distribution of this effect increased and several millimeters of adjacent muscle membrane became involved. The effect of C10 was entirely analogous to the action of an applied cathodal current. With reference to earlier discussions, it is noteworthy that the endplate region depolarized by C10 proved to be initially more excitable, although diminished excitability rapidly transpired. Furthermore, the endplate depolarized by C10 could be restored to its initial state of polarization by the administration of dTC. These experiments precisely establish the ACh-like function of C10 at the motor endplate and indicate clearly that only qualitative differences exist between their actions.

A preponderance of evidence has been accumulated to indicate that the primary pharmacologic property of the 3-OH phenyl $\dot{N}Me_3$ ion and/or the phenyl $\dot{N}Me_3$ ion is ACh-like (9, 203, 205, 214, 216, 261, 262). The data derived from neuromuscular experiments (214, 216) led to the conclusion that the salts of these cations, like C10 and choline (89, 138), initiated excitation by a primary action on the motor endplate. This has been borne out by a recent experiment (259) in which the depolarizing action of 3-OH phenyl $\dot{N}Me_3$ on the endplate region of the frog's sartorius muscle was detected. The ion was without effect on the nonneural region of the muscle. The intensity of this action would be better determined in a mammalian preparation such as that described by Burns and Paton. The depolarizing action of neostigmine on the frog sartorius muscle has also been ascertained (194) but the concentration required was relatively large (approx. 10^{-4}M). It is likely, however, that the frog muscle is less sensitive to neostigmine than is mammalian muscle, since it was also observed (194) that the chronically denervated frog sartorius muscle was insensitive to neostigmine; this is strikingly opposite to the situation in mammalian muscle.

Although it is not the intent of this review to consider the blocking action of

quaternary ammonium compounds, it is apparent that the disruption of neuromuscular transmission by these simple derivatives of —$\dot{N}Me_3$, including ACh and excluding dTC, represents a more extreme expression of an initial excitatory process, *i.e.*, depolarization of the motor endplate. This view has already been advanced for the action of C10 (52), contradicting the earlier idea of Ing and Wright (143) that a continuity does not exist between the paralyzing and stimulatory properties of several onium salts. Ing and Wright determined blocking actions on the nerve-muscle preparation of the frog sartorius and stimulatory actions were measured by the contracture produced in the frog rectus muscle. This again emphasizes that caution must be exercised in applying conclusions obtained from one type of neuromuscular apparatus to another.

VI. ANTI-CURARE ACTION

A. Historical development

There appeared in 1871 the first evidence that the effects of a curare alkaloid could be counteracted by another pharmacologic agent. In that year, Traube (249) reported that the injection of nicotine into the fully curarized dog produced a resumption of spontaneous respiration. The observation of the curare-nicotine antagonism was confirmed in rats by Winterburg (270) in 1900. It is somewhat surprising that this pharmacologic antagonism did not receive more attention, in view of the general interest at that time in the subject of drug antagonism. The antagonism between atropine and pilocarpine was the only clear-cut example then known. Yet the anti-curare action of nicotine remained in relative obscurity until Langely (167) in 1905 focussed attention on the application of this antagonism to a general interpretation of drug action. However, the report by Pal (196) in 1900 that physostigmine could terminate the classical curare paralysis attracted the first real interest in anti-curare action. At the request of Pal, the pharmacologic analysis of the physostigmine-curare antagonism was extended by Rothberger (224).

The publications of Pal and Rothberger mark the beginning of what may be considered modern research on this topic. Rothberger's analysis of the physostigmine-curare antagonism is notable for its contribution to the mechanism involved. He succeeded in localizing the anti-curare action of physostigmine to the periphery, thereby dissociating the prominent central excitatory component of the drug action. Although previous experience had made recognizable the fact that strong central action, such as induced by strychnine or anoxia, did provide stimuli of a strength sufficient to break through a weak curarization, it was apparent that the effectiveness of physostigmine was not similarly limited by the depth of curarization. Not only did Rothberger localize the anti-curare action of physostigmine to the periphery but he further concluded that the site of action of physostigmine must be at the same point at which curare acts; this locus he specified as the intramuscular motor nerve terminations, the motor endplates.

The researches of Loewi (173) and Dale (70) introduced and established the concept of the humoral transmission of the nerve impulse at several synaptic sites.

The realization of the biologic lability of ACh led Loewi to the appreciation of ChE activity and finally to the recognition of a mechanism of physostigmine action on the vagal junctions of the heart (88). With special reference to the anti-curare action of physostigmine, the early results of Rothberger were confirmed on more refined neuromuscular preparations (44, 68, 83, 223) and, in each instance, the anti-curare action was associated with the inhibition of ChE.

A most notable advance in the localization and the mechanism of the physostigmine-curare antagonism was made by Eccles and his collaborators. Reference has already been made to their studies describing the e.p.p. and its modification by physostigmine and other anti-ChE drugs. At the same time, these investigations further clarified the mode of action of curare and as a consequence revealed the anti-curare action at a more fundamental level. In brief, curare was seen to lower the amplitude of the e.p.p. below a certain critical value and as a result the origin and the propagation of the spike potential did not occur. The effect of physostigmine on the e.p.p. proved opposite to that of curare and in the curarized muscle it increased the amplitude and the duration of the local potential. The restitution of spike propagation depended merely on the magnitude of this action.

Additonal developments in other areas also served to concentrate attention on the anti-curare properties of the ChE inhibitors. The recognition by Stedman (239) of the anti-ChE activity resident in compounds containing a substituted carbamic acid grouping led to the synthesis and testing of many such substances. Chief among these urethane compounds prepared by Stedman and his collaborators (240, 241) was the methyl carbamic ester of 3-hydroxy phenylethyl dimethylamine, referred to as miotine. This compound, a tertiary amine, had actions identical to physostigmine, including an anti-curare action in the cat (263). During the subsequent war years, the discovery of the irreversible inhibition of ChE by alkyl fluorophosphates provided another source of anti-curare agents. As an outgrowth of this, the inactivation of ChE by many alkyl polyphosphates was recognized, and a consequent anti-curare action has since been described for some of them.

The investigation of synthetic carbamates was extended by Aeschlimann and Reinert (6) whose series of 39 compounds included 21 quaternary ammonium salts. Several of them have since received prominent consideration in the numerous researches concerned with the structural considerations of drugs affecting neuromuscular function. The anti-curare action of neostigmine was readily demonstrated by Aeschlimann and Reinert on a simple nerve-muscle preparation of the cat. The same activity was later shown for the closely related substance 36 (29, 171). With the availability of a relatively pure curare alkaloid, Koppanyi and Vivino (155), in 1944, confirmed the anti-curare actions of neostigmine and physostigmine in rabbits paralyzed with a preparation of dTC.

B. Neostigmine, physostigmine and other chloinesterase inhibitors

Immediately following the introduction of neostigmine and the description of its efficacy in myasthenia gravis by Walker (253) and Pritchard (201), Bris-

coe (28) studied the antagonism between neostigmine and curare. In this work, a preparation of the cat's quadriceps muscle was employed and it was readily shown that, after large doses of curare had been given, the injection of neostigmine decreased the recovery time of the muscle to tetanic stimulation. After small doses of curare, the injection of neostigmine resulted in a prompt restoration of the response. In a simultaneous publication, Rosenblueth *et al.* (223) recorded their experiments on the decurarizing actions of neostigmine and physostigmine; Wilson and Wright (269) also described this action of neostigmine in the cat. In addition, the latter workers showed that the anti-curare action of potassium was increased by the prior administration of small doses of neostigmine, an effect manifested chiefly by a more sustained anti-curare action. This result might be considered as support for the aforementioned idea that neostigmine and related quaternary ammonium compounds may act directly to prolong recovery of a depolarized endplate membrane.

Jacobsohn and Kahlson (144) were the first to point out the difference in anti-curare activity between physostigmine and neostigmine. In their experiments on the frog gastrocnemius perfused through an aortic cannula, physostigmine was ineffective in restoring a response in the completely curarized muscle but neostigmine in doses between eight and twenty micrograms effected recovery of transmission. Burke *et al.* (50) redirected attention to the disparity between the anti-curare actions of physostigmine and neostigmine. In anesthetized dogs, experiments with nerve-muscle preparations revealed a more prompt recovery following i.v. neostigmine than after physostigmine similarly administered. In unanesthetized animals, the course of paralysis was determined for various doses of curare. For each curare dose, the average duration of drug action was established. Subsequent experiments tested the separate effects of a fixed dose of neostigmine or physostigmine. At each level of curarization, neostigmine proved superior to physostigmine in diminishing the time to recovery. The difference in efficacy between the drugs in each trial was highly significant. Most recently the discrepancy between the anti-curare action of physostigmine and neostigmine has been shown in the gastrocnemius of the cat (259). The drug comparison, in this case, was enhanced by the technique employed. Close intra-arterial injection was made with circulation restricted to the muscle under observation; the muscle was activated by a single maximal shock applied to the sciatic nerve. Under these conditions, recovery from a complete curare blockade following physostigmine was accomplished gradually over a period of several minutes. In sharp contrast, the restoration of the response after neostigmine was prompt, and complete recovery was usually achieved within thirty seconds.

Assuming the anti-curare effect of physostigmine to be mediated chiefly through an anti-ChE action, as is the case with regard to its potentiating action on the contractile response, there will be required, for the achievement of curare reversal, the liberation and accumulation of ACh. This is consistent with the progressive development of the anti-curare effect of physostigmine. A similar action has been demonstrated for DFP (135) and it is not possible to accelerate the full achievement of this effect by raising the dose or changing the mode of

administration (260). In fact, Tabun, one of the most potent irreversible inactivators of ChE known, when given to the cat by close intra-arterial injection, antagonized a complete curare blockade in a very gradual fashion; recovery progressed over a span of more than ten minutes (132). Corresponding with the observation of Jacobsohn and Kahlson (144) for physostigmine, Kensler (148) has described the relatively weak anti-curare activity of DFP in the intact frog. In this species, neostigmine exerts a sharp anti-curare action. These and other observations already cited imply the relatively slow formation and/or release of ACh by amphibian tissue. The qualitatively different anti-curare effect of neostigmine suggests that its action is mediated through some mechanism in addition to the inhibition of ChE.

If one considers the anti-curare actions of neostigmine and physostigmine to be exerted solely through ChE inactivation, it is logical to attempt to account for the divergence in their actions by seeking a difference in their reactivities with ChE. In this regard, the exhaustive reviews of Nachmansohn and Wilson (192), Augustinsson (10) and Koelle and Gilman (154) do not indicate any striking difference in the *in-vitro* reactivity of either neostigmine or physostigmine with the specific type of ChE isolated from any one of several sources. For ChE derived from the electric organ of the eel, physostigmine is a slightly more potent inhibitor than is neostigmine (191). For the mammal, there is no biochemical evidence to substantiate the existence of a significant difference between the susceptibility of the specific ChE to either of these substances. For example, the ChE activity of rat brain *in vitro* was found to be retarded nearly equally by physostigmine and neostigmine (79); the ChE from the caudate nucleus of the dog exhibited nearly the same sensitivity to inhibition by neostigmine and physostigmine (18), and no notable differences have been described for the inhibitory actions of these substances on erythrocyte ChE (226).

Another consideration that may account for differences among the *in-vivo* actions of anti-ChE agents would include an assessment of the ease with which any one of these substances enters an aqueous or a lipoidal phase. Nachmansohn (190) has indicated that those agents having a high water solubility will be most effective at a synapse like the n.m.j., while those having a predilection for lipoids will more readily affect neural tissue. Neostigmine belongs to the former class and DFP to the latter. Grob and Harvey (115) attempted to correlate, for three ChE inhibitors, the ratio of their oil-water solubility with their action on neuromuscular function in man. On extraction of aqueous solutions of TEPP, DFP and neostigmine with peanut oil, the anti-ChE activity of an aqueous solution of DFP was diminished approximately ninety per cent; the activity of neostigmine was reduced about fifteen per cent, while that of TEPP was scarcely affected. Although this fact undoubtedly accounts in part for the greater musculotropic actions of neostigmine and TEPP as compared with DFP, pharmacologic experiments clearly indicate the superior excitatory effects of neostigmine at the n.m.j. In addition, the water solubility of physostigmine is not inconsiderable and yet this alkaloid does not manifest an action equivalent to that of neostigmine on the n.m.j.

C. The phenyl trimethylammonium group

The recognition of a dual action of neostigmine at the n.m.j. engendered an interest in the pharmacologic properties of the basic residue of this compound. These studies were initiated by Wescoe *et al.* (262) who compared the general pharmacologic effects of 3-Ac phenyl $\dot{N}Me_3$ with neostigmine. This neostigmine analogue was first selected as an object of study because it provided a compound in which the carbamate moiety of neostigmine was replaced by an indifferent ester as regards ChE inhibition. The severe curtailment of anti-ChE action accompanying this change has been well established (19, 261) and it has been pointed out that the 3-Ac and 3-OH phenyl $\dot{N}Me_3$ ions, like choline and many of its analogues, weakly inhibit ChE in a competitive fashion (261).

The pharmacologic investigation of 3-Ac phenyl $\dot{N}Me_3$ established that the muscarinic effects, so prominent in the action of neostigmine, were largely lacking in the actions of the 3-acetoxy analogue. However, in the intact cat given an i.v. dose of the compound, intense generalized fasciculations of skeletal muscle occurred. These were at least equal in intensity to those that follow the administration of neostigmine. It also became evident that the lethal action of this compound resulted from a nicotine-like paralysis produced by relatively large i.v. doses, a course of events similar to that produced by lethal doses of such compounds as neostigmine and C10. The peripheral paralysis could be circumvented, and a fatal outcome avoided, by a brief period of positive pressure respiration. These gross observations revealed the expected musculotropic actions of this cation.

The most prominent action of 3-Ac phenyl $\dot{N}Me_3$ was its ability to antagonize a total curare paralysis in the intact animal (262). In these experiments a uniform dose of dTC was administered by i.v. infusion, and if apnea occurred the animal was intubated and respired artificially. With this method individual sensitivities to curare were ignored but from animal to animal the drug concentration was constant. Each cat served as its own control; the time and the course of recovery were recorded on one day, and in the same cat the influence of drug was noted on the succeeding day. At the conclusion of curare administration the cats exhibited, without exception, complete flaccidity and absence of postural tone; complete cessation of respiration occurred in one half of the animals. At this point, the i.v. administration of a single dose of 0.5 mgm./kgm. of 3-Ac phenyl $\dot{N}Me_3$ shortened the time for complete recovery to about one half of the control value. When apnea existed, spontaneous respiration was restored immediately and permanently by the neostigmine analogue. Following the initial decurarizing effect, recovery to normal in the treated cat proceeded at the same rate as in the untreated cat. The improvement produced by 3-Ac phenyl $\dot{N}Me_3$ was followed by a slight relapse only in the most severely curarized animals.

The anti-curare action of this compound was not accompanied by any side action worthy of note. In contrast to this, when neostigmine was administered under the same conditions and in the same dose, the animals showed profuse salivation, urination, defecation, nausea, vomiting and depression. Like neostigmine, however, the anti-curare action of 3-Ac phenyl $\dot{N}Me_3$ was evident almost

immediately after injection. From these observations, the possible application of the compound in clinical practice as a curare antagonist was suggested.

The antagonism between 3-Ac phenyl $\dot{N}Me_3$ and curare was confirmed in the neuromuscular preparation of the cat (216) and the dog (205). In this circumstance, the outstanding feature of the anti-curare action was the immediate manifestation of a maximal effect, comparable to the immediate anti-curare action of ACh (27, 269) and neostigmine (171). It was concluded that this effect was mediated by a direct action at the n.m.j. and was contrary to the more gradual restoration of response attained with relatively pure anti-ChE compounds.

Funke and Depierre (106A) attempted to evaluate the functional contribution of the carbamate group in compounds like neostigmine. For this purpose a series of iodo salts of bis-(p-trimethylaminophenoxy) alkanes was prepared and tested. These compounds served to eliminate the carbamate function and twin the phenoxy quaternary ammonium residues through either an ethane, propane, butane or pentane chain. All these substances exhibited antagonism to d-tubocurare or Flaxedil in the cat but maximum activity was achieved with the propane derivative. This compound was about as active in this regard as neostigmine but its lethal action was considerably less. Additionally, other cholinergic manifestations were found to be less in evidence than after neostigmine administration. Despite this, the propane derivative exhibited an intense inhibitory action *in vitro* against ChE activity, derived from dog red blood cells. A later report (106B) recorded this anti-esterase activity as greater than that of neostigmine. Because of these results, the carbamate grouping was introduced into the twinned phenoxyammonium function in one and in both of the *meta* positions (106B). The result of these alterations was, in general, to increase the anti-curare and the anti-ChE activities even further (106B). Most remarkable were the reported molarities required to produce near fifty per cent inhibition of the red blood cell esterase of the dog, namely, 10^{-14} and 10^{-16}M for the mono- and bis-carbamate derivatives, respectively, of the bis-(p-trimethylaminophenoxy)-1,3 propane ion. As would be expected, generalized cholinergic effects were intense and death was noted to ensue from an intravenous dose as small as five microgrm./kgm. These substances demand further investigation and should prove of unusual value as inhibitors of ChE in laboratory investigations.

The results of the several studies on the pharmacology of 3-Ac phenyl $\dot{N}Me_3$ and related agents (77, 78, 205, 216, 262) indicated the phenyl $\dot{N}Me_3$ ion as an agent possessing a high affinity and specificity for the n.m.j. Accordingly, the unesterified base, 3-OH phenyl $\dot{N}Me_3$, was examined with particular concern for its effects on neuromuscular function (213, 216). These and other experiments revealed that the potent anti-curare activity manifested by the 3-Ac phenyl $\dot{N}Me_3$ ion was no less evident in 3-OH phenyl $\dot{N}Me_3$ (77, 78, 205, 213, 214). The anti-curare action of 3-OH phenyl $\dot{N}Me_3$ was compared with that of neostigmine in the intact and unanesthetized cat (214). The experiments were, in essence, a cross-over assay performed on a group of six cats, in the manner described above for 3-Ac phenyl $\dot{N}Me_3$. At the end of the curare administration, a standard dose of 0.5 mgm./kgm. of anti-curare agent was injected i.v. An immediate and

dramatic anti-curare effect was observed after the administration of either drug. The greatest difference between the untreated and treated animals was the rapidity with which muscle tone returned, although arbitrary phases of recovery were also accelerated. In each case the total recovery time of the treated animals was one third that of the controls. By this method there was no significant difference in potency between neostigmine and 3-OH phenyl $\dot{N}Me_3$. However, the anti-curare action of neostigmine was accompanied by the several side actions noted above. These signs of toxicity were absent in animals treated with 3-OH phenyl $\dot{N}Me_3$. The total effect of neostigmine indicates the ubiquitousness of its actions and emphasizes the indiscriminate nature of its anti-ChE action in contrast with the more specific action of its cationic head. In the neuromuscular preparation of the cat (216) and the dog (205), 3-OH phenyl $\dot{N}Me_3$ produced an immediate restoration of the contractile response that had been suppressed by dTC. Electrical records from a motor unit of the cat disclosed the rapidity with which propagation was re-established (216).

The actual period of excitatory action of 3-OH phenyl $\dot{N}Me_3$ at the n.m.j. is brief; it lasts from two to three minutes as revealed by the duration of repetitive discharges set off following its administration. Despite this demonstrated brevity of action of 3-OH and 3-Ac phenyl $\dot{N}Me_3$ ions at the junction, the anti-curare effect after adequate doses is permanent. The permanent nature of this anti-curare effect presents a seeming paradox when viewed in the light of the apparently transient duration of action of these ions. However, the paradox may perhaps be explained by the assumption that these anti-curare agents effect a release of curare from the junctional region and that as a consequence, during the brief period of anti-curare action, a considerable fraction of the displaced curare is "eliminated"; after the brief period of action at the n.m.j. is ended, any "available" curare may recombine with the junctional receptor. This latter possibility may account for the relapse seen after anti-curare therapy in animals severely curarized. On the other hand it is possible that an antagonistic action toward curare continues to be exerted by 3-OH phenyl $\dot{N}Me_3$ after all excitatory effects have subsided. More recent evidence, to be discussed below, provides some substance for this view. In line with the former hypothesis is the observation that fixed doses of dTC can be repeated at brief intervals with the intensity and duration of the curare effect being reproducible on each occasion, but only if the effects of each curare dose are dissipated by a succeeding injection of 3-OH phenyl $\dot{N}Me_3$. This was shown in the neuromuscular preparation (260) wherein a fixed dose of dTC was given and the muscle response was reduced to 21 per cent of control. After recovery of the contractile response was complete, the dose of dTC was repeated and the response was abolished, this greater effect clearly reflecting curare accumulation. Subsequent to the recovery of the contractile response, a "clearing" dose of 3-OH phenyl $\dot{N}Me_3$ was administered i.v. A period of ten minutes was allowed to elapse and the dose of dTC again repeated. The recorded response revealed a depression of the muscular response to 14 per cent of the control, in good agreement with the initial effect of this dose of curare. This result demonstrates that the residual curare displaced

from the junctional region by the quaternary ammonium must be largely eliminated during a brief period of anti-curare action. This observation bears further implications regarding the pharmacology of curare, since it suggests that dTC is held in the organism largely by its affinity for the n.m.j. and that when this site is occupied by a suitable antagonist the free curare is eliminated with great rapidity. It should be understood that the elimination of curare may mean simply, as outlined by Marsh (179), a redistribution of the alkaloid into the extracellular fluid prior to its excretion via the kidney.

The short duration of action was also evidenced in man by the repeated effectiveness of multiple doses given four to twenty minutes apart (9, 175). In one of these investigations (9), the experiments were performed on patients undergoing abdominal surgery and anesthetized with cyclopropane. The measurement of respiratory exchange was chosen as the simplest and most accurate index of muscular function and quantitative determination of curarization was made, therefore, by recording the respiratory minute volume (RMV). For this purpose, a single fixed dose of dTC was given to each of six anesthetized patients and the RMV observed until it returned to the control level. Immediately subsequent to the recovery of the control RMV, a total i.v. dose of 5 mgm. of 3-OH phenyl $\dot{N}Me_3$ was given. In an average of four minutes (range 2–8) after this dose, the dose of dTC was repeated and the course of recovery recorded. In each case the speed of onset and intensity of the second curare dose did not differ from the first. Thus the action of curare was not impeded by a dose of 3-OH phenyl $\dot{N}Me_3$ given as closely as 2 minutes prior to the curare injection. Further, this result attests to the brief sojourn of the anti-curare agent at the n.m.j. That the treatment reduced the concentration of curare at the junctional region is reflected by the fact that significant accumulation was prevented.

The short duration of action of 3-OH and 3-Ac phenyl $\dot{N}Me_3$ ions at first seemed to present a serious drawback to the practical clinical usefulness of their salts. However, the unique anti-curare action described above for these substances made it apparent that the brevity of action provided a singular advantage in that repetition of doses was possible without fear of accumulation. Although the phenyl $\dot{N}Me_3$ and its derivatives are quaternary amines with a characteristic paralytic potential, this undesirable property becomes of negligible clinical significance when their short duration of action is considered. The prolonged neuromuscular depressant action of neostigmine, by contrast, makes for its ready accumulation and caution must be exercised in its use as an antagonist to curare.

The anti-curare action of the 3-OH phenyl $\dot{N}Me_3$ ion was confirmed by other observations in man (9, 92, 175). It was found that the extent of the anti-curare action was dependent upon the degree of curarization and the dose of the antagonist employed. When the dose of antagonist was sufficient, the dissolution of curarization occurred rapidly and return to the pre-curarization level was achieved in all instances within three minutes. The doses required to combat various levels of curarization were determined. In patients whose RMV was reduced to between 40 and 60 per cent of their control value, a dose of 5 mgm. 3-OH phenyl $\dot{N}Me_3$ was sufficient to restore approximately the control level.

The increase of curarization to an extent such that the RMV was 20–40 per cent of the control required for its dissipation a dose of 10 mgm. When the RMV was lowered to between 0 and 20 per cent of the control, the administration of 15 mgm. of 3-OH phenyl $\dot{N}Me_3$ was not quite sufficient to effect a complete restoration of function. In this group of individuals, following the peak anti-curare action, the reappearance of some degree of curarization was noteworthy. When ether was the anesthetic agent employed the extent of the anti-curare action at each level of curarization was somewhat less, due to the additive effect of ether and curare.

In the studies of MacFarlane *et al.* (175) on unanesthetized man, grip strength was employed as a criterion. Following a standard dose of dTC the i.v. injection of 10 mgm. 3-OH phenyl $\dot{N}Me_3$ produced objectively and subjectively a more immediate and dramatic relief than did neostigmine under the same circumstances. In contrast to neostigmine the duration of action was short (2–3 minutes) and some relapse to curarization occurred. Following this regression, the rate of recovery proceeded like that in the untreated subject. The anti-curare action of Tensilon in man has been recently demonstrated in most convincing fashion by Faulconer *et al.* (92). The action potential of the abductor digiti quinti muscle was recorded and a dose of dTC was given sufficient to depress the potential severely. At the peak of the curare action the i.v. administration of 5 mgm. of Tensilon resulted in a rapid but incomplete recovery of the electromyogram. A substantial return of the muscle action potential was evident in less than 90 seconds. Measurement of the respiratory ventilation rate in curarized surgical patients revealed a similar rapid anti-curare action of Tensilon in confirmation of the results of Artusio *et al.* (9). When the dose of Tensilon was raised to approximately 15 mgm., an almost immediate and nearly complete recovery from paralysis occurred. In the same circumstances the i.v. administration of 0.5 mgm. of neostigmine accelerated very slightly the recovery from curare. Higher doses of neostigmine would be undesirable from the standpoint of inducing unwanted side actions. In this clinical study (92), approximately 2000 administrations of Tensilon were made to counteract curare in patients undergoing surgical procedures or electroshock therapy. In this series minor reactions followed the administration of doses as large as 20 mgm. but in no instance was there an alarming side action.

D. Effect of aromatic OH group

The modifying influence of the OH group on the excitatory properties of several quaternary ammonium compounds has been discussed. It has been seen that the excitatory capacity of an aromatic onium compound varies strikingly with the position of the OH group on the aryl ring. Existing evidence (78, 203, 205) points to a similar attenuating effect of the OH group on the anti-curare potencies of certain aryl onium compounds, although there is the suggestion in some of these data that the anti-curare activity is less seriously altered by identical changes. Thus 2-OH phenyl $\dot{N}Me_3$ possessed about one fifth the anti-curare potency of the *meta* isomer when tested on the dog tibialis preparation

(203), but exhibited only a very minimal action when evaluated on the cat nerve-muscle preparation (78). In dog experiments (203), the anti-curare potency of 4-OH phenyl $\dot{N}Me_3$ proved equivalent to that of the *meta* isomer, while in the cat the anti-curare effectiveness of 3-OH phenyl $\dot{N}Me_3$ was reduced fifty per cent by removal of the OH group to the *para* position (78). The greatest anti-curare action was achieved in all cases with the OH group in the *meta* position. The restricting influence of the OH grouping on both excitatory and anti-curare activities reflects the importance of receptor affinity for either or both of these actions. The absence of an OH group, as in phenyl $\dot{N}Me_3$, is associated with an anti-curare action somewhat less than that of 3-OH phenyl $\dot{N}Me_3$ (77, 78, 205, 259). This may be attributable to the excessive affinity of this ion for the neuromuscular receptor analogous to the relatively limited anti-curare action of C10 (139).

E. Choline

The antagonism of curare by choline, another simple quaternary ammonium compound, has only recently been investigated (138). The results of this work reveal that a mutual antagonism exists between choline and dTC. The anti-curare potency of choline in the cat was such that a 50 per cent blockade of neuromuscular transmission by curare could be overcome temporarily by an i.v. dose of 20 mgm. of choline. Further increases in the dose lengthened the decurarizing effect, but with very large doses neuromuscular blockade due to choline itself supervened. It is interesting to contrast the action of choline and its aromatic analogue, 3-OH phenyl $\dot{N}Me_3$. The i.v. dose of choline requisite to achieve relief from a nearly complete curare paralysis was approximately ten to twenty times that of the phenol analogue. Furthermore, the anti-curare action of 3-OH phenyl $\dot{N}Me_3$ is more lasting. The brevity of the anti-curare action of choline in optimal doses was also evident when the compound was administered by the intra-arterial route in the nerve-muscle preparation of the cat. The difference between choline and its aromatic counterpart again signals a functional role for the aryl nucleus in counteracting the influence of the polar OH. Further explanation for these differences will be submitted in a subsequent section on general considerations.

F. Reciprocal antagonism

Briscoe (28) was most impressed by the fact that the antagonistic action of neostigmine occurred after the administration of a dose that would, by itself, cause a profound depression of the muscle response and, in this way, became aware of a mutual antagonism between neostigmine and curare. The demonstration of the reverse aspect of this antagonism was accomplished through the prior establishment of a profound neuromuscular depression in the cat quadriceps preparation by large doses of neostigmine. In this circumstance the administration of curare, in several instances, restored the myographic pattern towards a normal state. The speed of onset of this action of curare occurred in less than one minute and the maintenance of the effect depended primarily on the balance

between the doses of the antagonists. Cowan (68) also demonstrated the mutual antagonism that existed between neostigmine and curare in the nerve-sartorius preparation from the frog. In addition, Briscoe (29, 30) showed that the neuromuscular depression produced by the quaternary ammonium compounds, substance 36 and hordenine methyl sulfate, could be reversed by curare. To explain this action of curare, two possibilities existed: first, that curare counteracted the depressant effect of neostigmine by hastening the destruction of ACh; second, that curare acted to raise the threshold of the endplate to transmitter action. There is still no reason to doubt the general validity of Briscoe's conclusion that curare affects the receptor threshold in such a way that a normally depressant concentration of ACh now acts to cause excitation.

The mutual nature of these antagonisms strongly suggests a similar bilateral antagonism between ACh and curare. This is to be particualrly expected from the anti-curare action of ChE inhibition. In this regard, Briscoe (27) early recognized the necessity of testing the pharmacologic interaction of ACh and curare. Her experiments in the cat revealed that previous partial curarization abolished the depressant effect of a large intra-arterial dose of ACh. Additionally, a perceptible anti-curare action of ACh was noted, an effect probably facilitated by the circumstances of light curarization and submaximal nerve stimulation. Earlier, Rosenblueth *et al.* (223) had described an increased response of the partially curarized cat gastrocnemius muscle to indirect stimulation following i.v. ACh, but this was done in the eserinized animal. In a recent publication (138), the decurarizing action of ACh on the neuromuscular preparation of the cat has been described. An initial, intense and fleeting anti-curare action was superseded by a level of antagonism no more effective than a chemically equivalent amount of choline. The relatively feeble anti-curare effect of ACh must be attributed to the unique brevity of its action at the endplate membrane. Other researches have illustrated the opposing actions of ACh and curare at the level of the endplate (40, 67, 158).

An interest in the mutual antagonism between curare and a quaternary ammonium compound was revived by the observation that small amounts of dTC sufficed to protect the cat from fatal paralytic doses of 3-Ac phenyl $\dot{N}Me_3$ (262). In subsequent experiments (214, 216), it was possible to demonstrate for the first time an actual reversal, by curare, of a neuromuscular depression developing as a consequence of a large dose of either the 3-Ac or 3-OH phenyl $\dot{N}Me_3$ ions. In one of these experiments (214), the electrical recording of response from a motor unit revealed the prompt and complete restoration of propagation of the muscle action current by the injection of dTC. The success of these demonstrations derives from the propitious pharmacologic properties of the quaternary ammonium ions, 3-Ac and 3-OH phenyl $\dot{N}Me_3$, that is, their actions at the n.m.j. are neither sufficiently intense nor prolonged to result readily in neuromuscular block, yet their duration of action is longer than that of the fleeting ACh effect.

The reciprocal antagonism that exists between dTC and 3-OH phenyl $\dot{N}Me_3$ stresses that the primary action of such an ion, like that of ACh, is exerted at

the motor endplate. Additionally, the restoration of transmission by curare suggests that the paralyzing activity of these ions, like that of ACh, stems from a depolarization of the endplate, and that an appropriate amount of curare restores excitability by allowing repolarization to occur. These concepts should prove useful to the pharmacologist in arriving at a conclusion concerning either the mechanism of a neuromuscular blockade or the mechanism of action of an excitatory agent. In this way, Unna *et al.* (250) revealed the curare-like activity of the tertiary amines, erythroidine and dihydro-β-erythroidine, since the paralytic effects of these alkaloids in frogs, mice, rabbits, cats and dogs could be annulled by either neostigmine or physostigmine. Recent confirmation of this was obtained by showing that Tensilon abolishes the blockade established by dihydro-β-erythroidine as effectively as it counteracts the paralysis from curare (204). In opposite fashion, Paton and Zaimis (198) were led to realize the depolarizing action of C10, in that its effect was impeded by the presence of dTC. That the antagonism between C10 and dTC is mutual has since been shown (139, 197, 259). With the introduction of several synthetic curariform agents like Flaxedil it has been important, in some cases, to ascertain the nature of the resulting blockade. Thus, Bovet *et al.* (23) recognized immediately the resemblance between curare and Flaxedil from the simple fact that the latter was easily antagonized by neostigmine. This observation has since been well confirmed by the demonstration of a sharp and thorough dissipation of Flaxedil blockade in animals and man by anti-curare agents such as 3-OH phenyl $\dot{N}Me_3$ and Tensilon (177, 204, 259). Conversely, the lack of antagonism between C10 and neostigmine provided Paton and Zaimis (198) with another important clue in the revelation of the mechanism by which C10 and closely related bis-quaternary ammonium salts block neuromuscular transmission. Not only was the blockade produced by C10 not counteracted by neostigmine but it was, in fact, reinforced by this anti-curare agent.

The strong depolarizing action of C10 connotes its affinity for the receptors of the junctional region and this ACh-like property indicates its potential as an anti-curare agent. In the cat neuromuscular preparation, the anti-curare effect exerted by a given dose of C10 was immediately maximal (139, 259), suggestive of a direct interaction of C10 with the endplate receptors. The mutual antagonism between curare and a strong depolarizing agent such as the bis-choline ester of adipic acid has been demonstrated in the cat nerve-tibialis preparation (112). The characteristic effect of this latter compound, after its close intra-arterial administration, was evidenced by the suppression of the indirect response of the muscle. In addition there was a considerable diminution in the contraction evoked by direct stimulation. In light of the revealing experiments of Burns and Paton (52), it is most probable that the latter effect was a consequence of depolarization spread along the muscle fibre. Actually, the experiments of Ginzel *et al.* (112) do uncover the action of dTC as impedimental to depolarization spread, for they show that an amount of dTC insufficient to alter the indirect response of the muscle prevents a dose of the bis-choline ester, sufficient to abolish the indirect response, from depressing the direct response.

The development of contracture and simultaneous blockade of indirect stimulation in the pigeon nerve-muscle preparation by compounds like C10 (53) and the bis-choline esters affords an excellent indication of the nature of the blockade, *i.e.*, prevention of depolarization or excessive depolarization. With this preparation a striking demonstration has been made of the transition from one type of action to the other with relatively simple structural changes in the bis-ester series (112). Thus the contracture produced by the bis-choline ester of adipic acid is accompanied by suppression of the indirect response and some depression of the direct reaction, signaling an extreme depolarization. The diethyl analogue of this compound has a much weaker contracture-producing action and is similarly less effective as a blocker of transmission. When the blocking action of the diethyl analogue is fully developed there is no depression of the direct response of the muscle. Although it is not possible to ascertain from these experiments, it is probable that the action of this compound results from a restricted depolarization wherein multiple and opposing effects may arise from the one compound. This idea is strengthened by the appearance of a typical curare-like action in the triethyl analogue, in that it prevents the response of the muscle to indirect stimulation and it immediately annuls the contracture established by strong depolarizing agents such as C10 and the bis-choline ester of adipic acid. The importance of complete replacement of methyl by ethyl groups for the full development of this action may be appreciated by the comparatively weak contracture-inhibiting effect of the diethyl analogue. Most important is the recognition, from these and other studies, that mixed types of neuromuscular blockade may result from synthetic paralyzing compounds. A recent example of this is Mytolon, a bis-benzoquinone derivative. A definite antagonism of the action of this compound by neostigmine could not be established (134, 204), but Mytolon was shown to inhibit the development of C10 action in the dog (134). Correspondingly, Mytolon blockade was not antagonized by Tensilon and in fact was slightly enhanced by the anti-curare agent (204). On the other hand, the discrepant action of Mytolon was further revealed by the finding of a clear-cut antagonism between it and ACh on the frog rectus muscle (134). This observation also serves as an admonition against generalization when the information is obtained from a single species. It is obvious that the clarification of the mechanism of Mytolon action will require more precise methods of analysis. Randall (204) has described the paralytic action of a series of bis-quaternary polymethylene bipiperidines. The blocking effect of these compounds was not antagonized by Tensilon or neostigmine.

G. Stimulatory action and anti-curare activity

Previous reference has been made to the reports of Randall and Lehman (205) and Randall (203) in which the pharmacologic properties of several neostigmine analogues have been described. Re-examination of these data from the standpoint of excitatory activity discloses significant facts about the alteration of this function by certain structural modifications. The ability of several of these compounds to antagonize an established curarization now provides an opportunity to correlate the influence of structure on anti-curare activity and

to relate this to other effects on neuromuscular function. From this evaluation, a most important fact appears, namely, that good anti-curare activity is not necessarily associated with any significant, direct excitatory properties. Thus the homologues, 3-Ac or 3-OH phenyl $\dot{N}$Me Et_2, which were virtually without action on chronically denervated mammalian muscle, were equivalent in potency to 3-Ac or 3-OH phenyl $\dot{N}Me_3$ as anti-curare agents. Most important was the finding that 3-OH phenyl $\dot{N}Et_3$ retained one third of the anti-curare activity of the tri-methyl homologue, while losing nearly all excitatory properties. In this connection, attention must be redirected to the findings of Rothberger (225) and Kensler (149) on the efficient anti-curare action of the weakly excitatory $\dot{N}Et_4$ ion. On this basis, antagonism toward curare may be viewed as a consequence of a displacement of the alkaloid from the specific receptors of the end-plate membrane, without a concomitant depolarization. From the mass of evidence, it becomes clear that, although excitatory actions depend for significant activity on the presence of at least two methyl groups attached to the central onium atom, the anti-curare action of alkyl onium compounds cannot be similarly categorized. The question then arises,—does the anti-curare action of a quaternary ammonium compound require any prominent excitatory component in the action of the compound? The data indicate that such an action need not be associated with any direct or indirect stimulatory action, although the two properties may and usually do co-exist in one compound. The concept of competitive displacement is thus implicated as the prominent mechanism in the anti-curare action of quaternary ammonium compounds.

H. Competitive displacement

The competitive interaction of drugs and their antagonists on the effector cell was first explored, from a quantitative as well as a qualitative aspect, by A. J. Clark (61). For the most part, his researches were based on the reactions between ACh and its well-known antagonists, as examples of a type of drug action. In one of these studies on the interaction of ACh with the cell, Clark (60) utilized the antagonistic action of atropine and found that the presence of this drug in varying concentrations in the rectus abdominis raised the threshold to ACh but did not alter the shape of the response curve. He concluded that atropine and ACh probably affixed themselves to different receptors and that their antagonism was one of effects. Several years later he studied the influence of antagonists, such as atropine, curare and certain quaternary ammonium salts, to both ACh and $\dot{N}Me_4$ (62). It was found that all of these antagonists counteracted both substances and it was concluded that ACh and $\dot{N}Me_4$ combine with the same receptor. The log molar concentration of antagonist needed to increase by tenfold the concentration of the excitant drug was determined and it was found that this concentration for any one of the several antagonists was the same whether ACh or $\dot{N}Me_4$ was used. Contrary to his earlier thoughts, he then concluded that the antagonistic drugs competed for a common receptor.

It is important to recognize the fact that the antagonism between certain of the quaternary ammonium ions studied and curare is limited. This was early

recognized for the antagonism between physostigmine and curare, and more recently Van Maanen (251A) has pointed out the limited range of the anti-curare actions of Tensilon and neostigmine in the rat. For Tensilon, the three-fold range in anti-curare dose was relatively narrow when compared with the tenfold spread of the neostigmine dosage. It was implied that the limited *in-vivo* ranges of anti-curare dosage revealed an underlying inhibition of ChE as responsible for curare antagonism. This extrapolation is not warranted for there is no reason to assume that a direct competitive antagonism of curare *in vivo* will be unlimited. In fact, even under the more ideal *in-vitro* conditions, it was shown on the rat-diaphragm preparation that, when the amount of curare that could be antagonized by neostigmine had been exceeded, $\dot{N}Et_4$ produced a further action (149). The reverse of this was also true, thereby revealing a limited anti-curare action for $\dot{N}Et_4$. It is apparent from these complexities that the recognition of a limited antagonism does not necessarily furnish an insight into mechanisms involved.

More recently Van Maanen (251) determined the nature of the antagonism of each of the following to the ACh-induced contracture of the frog's rectus muscle: dTC, c-curarine-I, c-toxiferine-II and beta-erythroidine. Each of the four alkaloids shifted the dose-response curves only along the dose scale and, although the muscles were less sensitive to ACh, the slopes of the curves were not changed significantly. It was concluded that the antagonism between ACh and any one of these curare substances was a competitive one. This conclusion has now been confirmed by Kirschner and Stone (151), with regard to the actions of β-erythroidine, dTC and the dimethylether of dTC on the frog rectus muscle. The assumption was made that the development of muscle contraction, in response to ACh, is a function of drug concentration and that this function represents the reaction between drug and receptor. As long as the reaction between drug and receptor is not a limiting one, the velocity of subsequent reactions will prove proportional to the concentration of the drug-receptor complex and maximal effect will be attained when receptor groups are saturated. The formulations developed by Lineweaver and Burke (172) for the analysis of competitive and non-competitive inhibitory effects on an enzyme system, in which maximal reaction velocity is reached when the enzyme is substrate saturated, were therefore applied to determine the type of inhibition established by atropine and certain curariform agents on the development of ACh-induced contracture in the frog rectus muscle. By plotting the reciprocals of ACh concentration and muscle response in the presence and absence of varying inhibitor concentrations, it was found that an increase in concentration of any one of the curares progressively increased the value of the slope but that the slope intercept remained common to each curve. This result signified the competitive interplay between ACh and the curares. A similar finding was made for the blockade of the ACh contracture by physostigmine. It is significant that this action of physostigmine was demonstrated after ChE had been inactivated by continued exposure to TEPP and the authors concluded that physostigmine probably acted at more than one site. Finally, it was found that the action of atropine was complex, vindicating Clark's

idea, for reciprocal plot analysis revealed that both the slope and the intercept increased as the inhibitor concentration rose. Inspection of these data discloses an evident but small change in the intercept; however, more prominent is the general confluence of the curves suggestive of a predominantly competitive antagonism between atropine and ACh.

Reflecting on the work by himself and others, Clark (61) concluded that the type of drug antagonism exhibited by ACh and curare depended on some relatively simple physico-chemical process, because it was possible to express such an antagonism with simple formulae over enormous ranges of concentration. He likened the process to the displacement of one gas, coordinated to a hemoglobin molecule, by some other gas. Along these lines, the hypotheses of the pharmacologist have been borrowed from the physical chemistry of catalysis and, in analagous fashion, suppose a competition for common receptors in the active groups of proteins. The unusual fruitfulness of structure-activity pharmacology provides considerable support for this concept. In more detail, the probable mechanisms of drug-receptor union have been illuminated by the modern biochemical researches which have probed the mode of the interaction of small molecules with macro molecules such as the proteins. This type of approach should furnish essential clues for the process as it occurs on the cell surface *in vivo*. For example, a reaction at one site on a large molecule may make it harder or easier for a second molecule to react at another site. Thus a molecule of oxygen can react 6000 times as easily with one of the hemoglobin iron atoms if the reaction has already occurred with the three other metallic sites than if it has not taken place (227). Klotz and Curme (152) measured the influence of hydrogen ion on the binding of cupric ion by bovine serum albumin and found an effect not compatible with the simple displacement of a proton for each cupric ion added, *i.e.*, not quite every cupric ion displaced a proton. The important lesson of this experiment is that such a relationship does not require the cupric ion to react at the site from which the hydrogen ion is released, but that the liberation of the proton depends only on the net charge of the whole molecule. The application of these principles, then, to the antagonism between a quaternary ammonium ion and curare, suggests that ionic interaction with specific groups of the receptor protein may severely decrease the binding force between a similar receptor group and the larger curare molecule at another locus. Thus actual physical displacement need not be envisioned, an idea which is not commensurate with a constant ratio of antagonism, with very small concentrations of opposing drugs in the presence of a relatively high concentration of reactive receptor groupings. Fundamentally, the antagonism of curare at the endplate membrane by a quaternary ammonium compound may be conceived as requiring the close approach and electrostatic interaction of the cation with some oppositely charged group of the receptor protein. The end result of this reaction may so alter some membrane property that the curare molecule can no longer be held with adequate force; additionally, the effect of this ionic binding may result in an alteration of membrane potential sufficiently intense to give rise to excitation.

I. The interaction at the endplate

Electrophysiologic investigations on neuromuscular transmission provided most notable advances in the mode of action of curare and in the mechanism of its antagonism. The approach to the action of curare and anti-curare substances at a more fundamental level was begun independently and almost simultaneously by Gopfert and Schaeffer (114), Eccles and O'Connor (86) and Feng (98). All these studies precisely defined and demonstrated the e.p.p. in amphibian and mammalian muscle. Of most immediate importance was the discovery that this local potential was depressed but not abolished by curare (86, 114). Eccles and Kuffler (84) explored the details of this action in further experiments. It was found that increasing curare concentrations progressively lengthened the time at which the muscle spike rose from the e.p.p. Concurrently, the e.p.p. underwent a graded depression in amplitude until a level was reached at which spike propagation disappeared. By this technique it was found that the e.p.p. could be reduced to about one third of its original height before extinction of the conducted response occurred. Clearly, the origin of the propagated response was a function of the intensity and time-course of the local potential. In the light of this information, a reconsideration of the effects of physostigmine on the e.p.p. reveals an action directly opposite to that of curare. The early observations of Eccles and O'Connor (86) had indicated that physostigmine prolonged the e.p.p. in mammalian muscle. Feng (100), working with toad muscle, reported that the e.p.p. of a muscle soaked in a mixture of physostigmine and curare was always larger than that of a comparable muscle immersed only in solution of equivalent curare concentration. However, the recorded experiments of Eccles, Katz and Kuffler (83) provide a classic contribution to the analysis of physostigmine-curare interaction at the neuromuscular junction. By the use of appropriately timed double nerve volleys it was possible to show that physostigmine lengthened the e.p.p. by as much as ten times its original value. A small, subparalytic dose of curare, applied in the presence of physostigmine, greatly inhibited the prolonged endplate negativity. As the curare concentration was increased, the physostigmine effect was progressively diminished until a "normal" e.p.p. was attained. Conversely, the application of physostigmine to completely curarized frog muscle regularly produced an increase in the size and duration of the e.p.p. The prolonged e.p.p. from physostigmine was accounted for not only by the length of the decay process, but by an increase in the time of the rising phase so that it might require up to 3 times as long to reach its peak. Since the earlier work had shown that the transmitter action was reflected by the rising phase of the e.p.p., the prolongation of this phase would indicate that physostigmine has lengthened the duration of the transmitter action. The curare antagonism to physostigmine action was interpreted in terms of the diminution of a heightened e.p.p. by curare. This effect, in turn, was viewed as an action of curare opposing the depolarization of the endplate region by ACh.

The experiments of Kuffler (157) nicely delineated the local potential changes occurring at the n.m.j. Using a single muscle-nerve fibre preparation from the frog, he showed that the e.p.p. could be diminished by curare to approximately

40 per cent of the potential height before the spike was abolished. As the degree of curarization was increased, spike latency was prolonged and a longer time was required for the e.p.p. to reach the critical level. The antagonism between ACh and curare was demonstrated in an identical preparation (158). The concentration of curare producing a block of neuromuscular transmission served to lower, from 10 to 100 times, the sensitivity of the endplate to ACh. In this instance high concentrations of ACh set up only a localized contraction in the region of the endplate. The prevention of ACh depolarization by curare confirms an earlier findings by Cowan for the intact frog muscle (67).

The data of Eccles *et al.* (83) and Fatt and Katz (90) reveal that one effect of curare on the e.p.p. is to diminish its duration. This must be interpreted as an acceleration of forces normally acting to restore the polarized state of the membrane. To suggest that curare accomplishes this effect by accelerating the breakdown of ACh would be somewhat tenuous. Notwithstanding, there seems to be no hesitation to attribute the opposite action of physostigmine on the e.p.p. to a prolongation of ACh action by preventing its decomposition. While this latter may be true in part, it is not unreasonable that the presence of physostigmine *per se* obstructs forces acting to restore the membrane potential. Such an effect need not be associated with any intrinsic or direct depolarizing action. Thus, the attachment of either physostigmine, certain related tertiary amines, quaternary amines or phosphonium ions to membrane receptor sites may alter recovery processes by modifying permeability to potassium and/or sodium directly. This possibility is not without some foundation, since Holland and Grieg (130) have shown that physostigmine is highly effective in changing the permeability of the erythrocyte of the dog, cat and rabbit to these ions. They chose to attribute this action of physostigmine to its anti-ChE properties but neglected to consider the fact that the cat erythrocyte is devoid of ChE. Thus, their demonstration furnishes an excellent example of an action of physostigmine, independent of ChE, to alter the permeability of a cellular membrane to sodium and potassium. Recent data of Taylor *et al.* (246A) also reveal the important fact that physostigmine increases the net leakage of intracellular K^+ from the human erythrocyte. This was attributed primarily to an impedance of K^+ entry into the cell. With high concentrations of physostigmine the rate of K^+ leakage was increased and the overall effect was an acceleration of intracellular K^+ loss. A similar result was obtained with DFP, with the exception that this material affected only the entrance of K^+ into the red blood cell. In neither case could the effects be related to ChE inhibition since the drug concentrations necessary to affect threshold changes in permeability were in excess of those exerting complete inhibition of the erythrocyte ChE. Accordingly, it was decided that ChE is not essential for the maintenance of normal K^+ distribution in the erythrocyte. Secondly, the likely bearing of these effects of physostigmine and DFP on nerve function was recognized. Thus, the results of both these groups (130, 246A) proffer another possible mode of action for these and related substances on the endplate membrane.

Initial investigations of the junctional effects underlying the action of curare

antagonists were done in the presence of curare and necessarily observed as a resultant of opposing actions. In a recent remarkable study, Fatt and Katz (90) demonstrated that neostigmine, in the absence of curare, caused a profound prolongation of endplate negativity, several times that previously recorded. It will be important to determine whether other quaternary ammonium compounds having a prominent anti-curare action will institute similar electrical changes in the receptor membrane. The efficient anticurare effect of quaternary ammonium compounds, whose depolarizing action at the endplate must be negligible and whose anti-ChE potency is not remarkable, demands a reconsideration of the known changes produced by anti-curare substances in the electrical events at the n.m.j. In this respect, it will be of value to compare the effect of such ions as 3-OH phenyl $\dot{N}Me_3$ and 3-OH phenyl $\dot{N}Et_3$ on the e.p.p. developed in response to a nerve impulse. Thus far, this effect has been determined chiefly for those compounds whose predominant action is traditionally associated with the inhibition of ChE. A recent departure has been made by the precise study of Burns and Paton (52) wherein the effect of C10 on the endplate region of mammalian muscle is described. The intent of this work was to verify directly the locus, intensity and duration of the depolarization produced by C10, by way of explaining its blocking properties. In this it succeeded admirably and, in addition, it established the fact that a compound, considered to act other than through ChE inactivation, could directly prolong endplate negativity. The potent anticurare action of C10 has also been described by Hutter and Pascoe (139) who claimed that C10 decurarizes without changing the peak voltage or time course of the e.p.p. These results are difficult to interpret, for the height of the local potential can not be ascertained accurately in the presence of the superimposed muscle spike and in the presence of a rapidly changing curare concentration. In contrast, one of these authors (138) was able to demonstrate, in a similar preparation, an action of choline to increase the height of the curarized e.p.p. In this case, as with C10, no change in the time course of the e.p.p. could be detected. If, as Eccles and MacFarlane (85) have stated, the prolongation of the time to peak and to half-decay of the e.p.p. is associated with the inhibition of ChE, it appears that neither C10 nor choline decurarizes by inactivating the esterase, although both produce inhibition *in vitro*. It now remains to ascertain the effect on the e.p.p. of quaternary ammonium compounds whose direct depolarizing effects are weak or minimal and whose anti-ChE potency is negligible. If the e.p.p. induced by a nerve impulse is prolonged by these specified quaternary ammonium compounds, the experiment would reveal an effect of these substances on the restorative process of the depolarized membrane.

J. The significance of cholinesterase inactivation

A final consideration in the mechanism of anti-curare action relates to the part played by ChE inhibition. The profound effect of anti-ChE agents on neuromuscular function has been well reviewed (154). The effects, including anti-curare action, were seen to be consistent with the accepted ideas on chemical transmission. On this foundation, it follows that the anti-curare potency of

substances that act solely through ChE inhibition will be a direct function of their ChE inactivating power. For other compounds, the extent of participation of this component of action will vary with the compound in question. In the case of the homologous series of cations (3-OH phenyl $\dot{N}\equiv$) related to neostigmine, some answer to the relative roles played may be obtained. The several studies designed to correlate the anti-ChE and anti-curare activities of a heterogenous group of compounds have been cited and it has been pointed out that a general correlation can be shown. When the great discrepancy in the excitatory actions of 3-OH phenyl $\dot{N}Me_3$ and 3-OH phenyl $\dot{N}Et_3$ is considered conjointly with their relatively close anti-curare activities, and when both these effects are viewed in light of their similar anti-ChE potencies on frog muscle and eel ChE (64, 237A), an interpretation relating anti-curare action to the inhibition of ChE perforce renounces the association of their stimulatory actions with ChE inactivation as well as with curare antagonism. In these experiments (237A) the design was such that the anti-curare activities of these several compounds were evaluated in terms of their abilities to augment the anti-curare action of ACh. This action of these compounds quite reasonalby paralleled their respective capacities to increase the contractural action of ACh in the absence of dTC. In turn, the potentiation of ACh action by these compounds corresponded generally with their respective anti-ChE potencies. From this, the authors concluded that the anti-curare effect of phenolic quaternary ammonium compounds on the frog rectus results from the inhibition of muscle ChE and that the similar effect in higher species is consistent with this hypothesis. While there is no doubt that, in the *in-vitro* system described, the anti-curare effect of the phenolic quaternary ammonium compounds arose in part from ChE inhibition, it cannot be concluded generally that this is the sole basis for such an effect. Reference has been made to the fact that the non-propagated maintained response of the frog rectus muscle provides an ideal *in-vitro* preparation for the achievement of steady state responses. Under these circumstances, the sensitization of the muscle to ACh may be expected to maintain a continued and augmented antagonism of curare by ACh. It would be unjustified to extend conclusions from this circumstance to a system so different as the quick twitch propagated response of mammalian skeletal muscle *in vivo*. The fact remains that under this latter circumstance the injection of a weak anti-ChE substance, like 3-OH phenyl $\dot{N}Me_3$, is attended by an anti-curare effect which is immediately maximal. The intravenous dose of 3-OH phenyl $\dot{N}Me_3$, effective in relieving a curare blockade in man, would produce a maximal concentration of about 1.3×10^{-6} M in the body. This concentration was without significant inhibitory effect on erythrocyte ChE (261). The data of Smith *et al.* (237A) also reveal that considerably higher concentrations of this substance are needed to produce 50 per cent inhibition of ChE activity derived from either the electric eel, the bovine erythrocyte or the frog rectus muscle. Finally it must be emphasized again that, among the pharmacologic principles implicit in the definition of drug action, cognizance is demanded of the speed of onset of action. Experiments carried out on the frog rectus abdominis muscle cannot shed any light on this aspect as it pertains to the mammalian neuro-

muscular system. Their data and conclusions do, however, make the significant point that anti-curare action may be independent of stimulatory action, an expression of the similar fact inherent in the data of Randall (203) pertaining to the mammal.

Hobbiger (126A) found for Tensilon and 3 related analogs, in the cat tibialis preparation, that anti-curare activity reached a peak within one to two minutes after intravenous drug injection, but that a maximum effect was not sustained. In contrast neostigmine required approximately six to ten minutes for the full development of anti-curare action and the effect was maintained. When 2 to 5 mgm. per kgm. of intravenous TEPP preceded the injection of either neostigmine, Tensilon or its analogs, an anti-curare action was not in evidence, and in fact the administration of these substances resulted in a further depression of twitch response. Strangely, in this circumstance, C10, choline and ACh continued to exert an anti-curare effect. On the basis of this experiment, the activity of neostigmine, Tensilon and related analogs was attributed to ChE inactivation. Such a conclusion is an oversimplification of a complex situation. For example, the depression of the twitch response by the aromatic onium compounds, following curare and TEPP, is an expression of drug action. To what mechanism is this effect attributable? If it is thought of as indicating further ChE inhibition, the efficacy of ACh is paradoxical. The same dilemma confronts either a direct or indirect mechanistic interpretation. It is more likely that quantitative considerations are involved here, in that the anti-curare potency of ACh, choline or C10 after TEPP is perhaps altered; dose-effect relationships were not reported and it may be that smaller doses of Tensilon would have produced the desired result. The problem of sensitization is also re-emphasized when it is recalled that ChE inactivation will enhance the excitatory actions of both choline (138) and C10 (271) as well as that of ACh. Finally, there is no chemical basis to admit of a sharp qualitative dichotomy in the mechanisms of two closely related analogs: $Me_3\overset{+}{N}C_2H_5OH$ and $Me_3\overset{+}{N}C_6H_5OH$.

Using the rat diaphragm preparation, Hobbiger (126A) attempted to evaluate for Tensilon and its analogs the components of anti-curare action in terms of ChE inactivation and direct action. ACh and choline were chosen as prototypes of direct-acting agents and it was found that these substances were without anti-curare action on this preparation. C10 gave only a very brief improvement which was always considerably less than that seen with neostigmine analogs. It was assumed that these results demonstrated that anti-curare action arose from the inhibition of ChE. This conclusion is inconsistent with the failure to demonstrate an anti-curare action for ACh since it is obvious that an anti-curare action developed from the inhibition of ChE is in fact an expression of an anti-curare action of ACh. The more likely and more limited conclusion indicated by these data is that choline and ACh (in the presence of ChE), as indicated elsewhere in this review, do not possess a sufficient affinity for the motor endplate; this would curtail the displacement of curare. In this same report (126A) it is stated further that certain Tensilon analogs lose their anti-curare action on the frog rectus, as in the cat, after the muscle has been exposed to TEPP. Oddly,

an accompanying figure contradicts this statement for it exhibits a good anti-curare action for these analogs under just such a circumstance.

The efficient anti-curare action of DFP has been described in the cat (135) and in the rabbit (58), but a comparison of compounds like DFP with substances like neostigmine is rendered difficult by the marked differences in the physical properties of neostigmine and DFP. The striking influence of the lipoid solubility of DFP on its anti-ChE action has been mentioned. Another factor modifying the pharmacologic effects of DFP is its relatively greater affinity for pseudo-ChE (123, 182). Finally, the interaction of DFP and ChE is somewhat slower than is the interaction of ChE and quaternary ammonium compounds (191). The anti-curare action of the related alkyl phosphoryl cyanide (Tabun) is greater than is that of DFP and this undoubtedly relates to its greater anti-ChE potency, although its physical properties are similar to other phosphonium derivatives, including DFP (132). However, with the introduction of the alkyl phosphates it has been possible to obtain primarily water soluble compounds of extreme anti-ChE potency and whose reactivity with "true" ChE (80, 176, 191) is very rapid. This undoubtedly will prove to increase the efficiency of curare antagonism through ChE inhibition. In the neuromuscular preparation of the cat in which transmission was almost completely depressed by curare, an i.v. dose of about 0.5 mgm./kgm. of HETP produced quick recovery (48, 59). In the phrenic nerve-diaphragm preparation of the rat, the addition of TEPP to the bath produced a rapid but graded return from curare blockade (49).

In a recent communication, Berry and Evans (17) have dealt a serious blow to those attempts directed at establishing a causal relationship between ChE inhibition, as determined *in vitro*, and effects on the n.m.j., as determined *in vivo*. These experiments were performed on the phrenic nerve-diaphragm preparation of the rat. Amounts of either physostigmine, TEPP or DFP were added sufficient to produce a Wedensky inhibition on appropriate nerve stimulation. It was possible, in most of these instances, to re-establish neuromuscular transmission simply by washing the preparation, although direct determination revealed that a restoration of ChE activity by washing occurred only in the physostigmine-treated preparation. The inference was that either the ChE activity is not related to junctional transmission, or that the amounts necessary were so small that they fell within the limits of experimental error.

In the group of compounds that are both methylated quaternary amines and derivatives of substituted carbamates, such as neostigmine, a more complex situation is present in that the onium group has, as its natural prototype, the cationic moiety of ACh, and the carbamate grouping has, as its prototype, the functional grouping of physostigmine. In view of this fact and foregoing remarks, the complexity of the anti-curare activity of a drug such as neostigmine becomes apparent, for it may include a direct competitive displacement of the curare alkaloid, and indirect competitive and excitatory activity through ChE inactivation and a direct excitatory action *per se*. It has been only recently that attempts have been made to correlate anti-curare action with one of these factors, namely, the potency of ChE inactivation. In 1938, Cowan (68) sought to learn

whether a consistent relationship existed between anti-ChE activity and the ability to counteract a curare blockade. For this purpose he studied the anti-curare effects of several compounds. These compounds were: neostigmine, physostigmine, substance 36, substance 37 (dimethyl carbaminoyl quinoline HCl), a tertiary base; substance 38 (dimethyl carbaminoyl methyl quinoline methyl sulphate), a quaternary amine; preparation 3393 (dimethyl carbaminoyl phenyl methyl diethyl ammonium iodide) and preparation 1210 (3-OH phenyl $\dot{N}Me_3$), the latter two being quaternary ammonium compounds. All five quaternary ammonium compounds exhibited a similar anti-curare action on the frog nerve-sartorius preparation. Despite this, their anti-ChE activity ranged from one extreme to the other; preparation 1210, which had no carbamic acid grouping, had a negligible anti-ChE activity in comparison with the other compounds. The two tertiary amines, substance 37 and physostigmine, did exhibit a parallelism between anti-curare action and the inactivation of ChE. Substance 37 exhibited both these properties weakly; physostigmine manifested strong activity in both regards. Clearly, there was no regular consistency in the relationship between the inhibition of ChE and the antagonism of curare. In addition, Cowan was most impressed by the fact that the basic residue of neostigmine, 3-OH phenyl $\dot{N}Me_3$, exerted an action on neuromuscular transmission approximately 10,000 times that which could be expected from a consideration of its weak anti-ChE activity. He opined that this compound may affect neuromuscular transmission in some way other than ChE inhibition. The correctness of this view has since been substantiated (203, 213, 214).

Bülbring and Chou (44) made a comparison of neostigmine, physostigmine and certain neostigmine analogs. For this purpose they employed the rat phrenic nerve-diaphragm preparation and a nerve-muscle preparation in the intact cat. All substances were assayed with reference to neostigmine. In the *in-vitro* preparation, the ratio of the doses of dTC that caused 20 per cent inhibition in the presence of equimolar concentrations of antagonists and neostigmine, respectively, was determined. In this way physostigmine was slightly less effective than neostigmine; the significance of the recorded difference is questionable. Miotine was perhaps significantly more active than neostigmine. The ethyldimethylammonium and diethylmethylammonium homologues of neostigmine were significantly more active anti-curare agents. Concordantly, the anti-ChE activity of the diethyl homologue was approximately ten times more potent than neostigmine; the monoethyl homologue was intermediate in activity. To this extent, in the preparation employed, the anti-curare and anti-ChE potencies of these particular compounds ran parallel. Miotine, however, proved an exception in that its anti-curare activity was equivalent but it was found to be definitely weaker as a ChE inhibitor. In a further study, Blaschko *et al.* (18) compared the ethyl homologues of neostigmine, the amine oxide of neostigmine, the chlorphenylmethyl carbamate homologue of neostigmine and the tolylmethyl carbamate homologue of neostigmine. The series also included three other carbamate derivatives of quaternary ammonium compounds. In general, the results showed a statistically significant correlation between the inhibition of "true" ChE and

anti-curare activity. Specifically, exceptions to this generalization were evident. Thus, physostigmine and substance 38, having equal anti-ChE potencies, were significantly different in anti-curare activity; the tri-ethyl homologue of neostigmine and miotine, having equal inhibitory activity on "true" ChE, were also significantly different in anti-curare activity. The aforementioned carbamate homologues of neostigmine were approximately equal as anti-curare agents, but significantly different as inhibitors of "true" ChE. Most important in the data of these authors was the finding that the amine oxide homologue of neostigmine exhibited a weak anti-curare and anti-ChE potency. This compound was studied because it is a neostigmine derivative in which the basicity of the quaternary ammonium group is lost. It was concluded that strong basicity is indispensable for ChE inhibition. This clearly implies that receptor affinity among the quaternary amines is dependent on a highly charged cation, which results from the exclusion of further covalent combination by the nitrogen atom.

Casier and Verbeke (55) tested in the dog the decurarizing action of one of the neostigmine analogs studied by Blaschko *et al.* (18). With this compound, dimethyl carbamyl ether 3-methylpyridine, a striking anti-curare activity was observed with amounts which exerted only slightly inhibitory effects on erythrocyte ChE *in vitro*. In treated animals, no significant inhibition of erythrocyte or muscle ChE could be detected but the serum ChE was severely inhibited.

The studies on the derivatives of the phenyl $\dot{N}Me_3$ ion made it apparent that a potent anti-curare activity could exist in compounds whose anti-ChE properties were generally weak. It was Bloch (19) who first showed that 3-Ac phenyl $\dot{N}Me_3$ was a weak inhibitor of the ChE present in horse and human serum. Wescoe *et al.* (261) extended this work to the specific ChE's derived from human erythrocyte and rat brain cortex. It was found that the 3-Ac and 3-OH phenyl $\dot{N}Me_3$ inhibited the erythrocyte ChE activity by 50 per cent in a concentration of 2.5×10^{-5} M. These figures are to be compared with those for neostigmine and physostigmine whose pI_{50} values on ChE of dog caudate nucleus were 7.4 and 7.1 respectively (18). It is obvious that the equivalent anti-curare potencies of neostigmine and its cationic head cannot be associated invariably with the capacity to inactivate ChE.

The anti-curare activity of $\dot{N}Et_4$ is prominent and, although it requires the administration of relatively large amounts, the inhibitory effect of this compound on ChE is minimal (150). The discrepancy between anti-curare activity and ChE inhibition is also evidenced by the recognition of the highly potent anti-curare activity of C10. This compound, whose anti-ChE activity is approximately equal to that of 3-OH phenyl $\dot{N}Me_3$ acts *in vivo* in a concentration considerably less than the pI_{50} determined *in vitro*. An astute demonstration that the anti-curare action of C10 is independent of ChE inhibition was made in the cat neuromuscular preparation (139). The animal was heavily curarized by repeated treatments with alternate doses of physostigmine and dTC, until physostigmine no longer exerted an anti-curare effect and complete neuromuscular block remained. At this point adequate doses of C10 decurarized effectively. It was concluded that C10 and physostigmine could not act in the same manner.

Castillo and deBeer (56), in a report on the pharmacology of succinyl choline, showed that the blocking action of this compound was lengthened by the prior administration of physostigmine. This was presumed to be due to the prevention of the esterasic hydrolysis of the succinyl choline. More recently, deBeer *et al.* (76) have shown that the bis-succinyl amide of choline itself, which has no effect on neuromuscular function, will, like physostigmine, also prolong the paralyzing action of succinyl choline. The most striking revelation from this fact was the finding that the amide possessed no anti-ChE activity. Finally, despite the lack of ChE inhibition and any significant excitatory activity, the amide antagonized the action of dTC. Rightly, deBeer *et al.* wondered about the mechanism by which physostigmine prolongs the paralytic action of succinyl choline. These observations coincide with and support other demonstrations of the independence of excitatory and anti-curare properties and serve to sever a necessary relationship between anti-curare activity and ChE inhibition.

The study by Wescoe *et al.* (261) showed that compounds such as 3-OH phenyl $\dot{N}Me_3$ inhibit ChE in a competitive fashion and it was pointed out that even ACh itself inhibits its own enzymatic hydrolysis. It was also stated that, for quaternary ammonium compounds related to ACh, any strict classification of direct-acting agents and ChE inhibitors must be a quite arbitrary one. It was concluded that an affinity for the receptor simultaneously connotes an affinity for the ChE, and that the predominance of one affinity or another, *in vivo*, will depend upon specific molecular characteristics. The fact that inhibition occurs *in vitro* need not indicate that this effect is the primary mode of action *in vivo*.

The ultimate determination of the mechanisms involved in anti-curare action await combined application of pharmacologic and neurophysiologic technique. In this way preliminary assay by the pharmacologist should provide the physiologist with relatively simple quaternary ammonium ions for the determination of (1) their capacity to depolarize the endplate membrane, (2) their capacity to prolong an e.p.p. set off by nerve stimulation, and (3) their capacity to augment and/or prolong the e.p.p. set off by nerve stimulation in the presence of curare.

VII. MYASTHENIA GRAVIS

A. Pharmacologic aspects

The close resemblance of myasthenia gravis to curarization affords another opportunity to assess the differences between neostigmine and anti-ChE compounds of diverse nature, as manifested by their respective anti-myasthenic effects. Historically this chapter in therapeutics unfolds with the observation by Walker (253) that the subcutaneous injection of 1 mgm. of physostigmine in a patient afflicted with myasthenia gravis produced an extraordinary improvement. In this individual, the onset of the effect occurred in approximately 30 minutes. In a second communication (255), the effect of the then newly available synthetic, neostigmine, was reported to be generally more effective than physostigmine.

Understanding of the patho-physiology of myasthenia gravis was accelerated greatly by the quantitative study of neuromuscular function in man by Harvey

and his collaborators, who adapted the electrical stimulating and recording techniques employed so fruitfully in animal experiments. With their methods, the pharmacologic properties of the important drugs modifying neuromuscular function could be evaluated in the human. In addition, more detailed comparison could be made between the condition established by curarization and that pertaining as a result of the disease process in myasthenia gravis. Harvey and Masland (122) demonstrated that the abnormalities in the electrical responses of myasthenic and curarized muscle were identical. In such a patient, maximal stimulation of the ulnar nerve was followed by a depression in transmission for as long as two seconds, so that a second nerve volley excited a smaller number of muscle fibers. When the nerve was stimulated repetitively at frequencies up to 50/second, the recorded muscle action potentials showed a progressive decline in voltage. In a subsequent study, Harvey *et al.* (120) were able to demonstrate in one case neuromuscular facilitation similar to that described by Bremer and Homes (26) for the partially curarized animal. The decurarizing action of neostigmine was paralleled in these instances to the extent that this drug abolished the myasthenic abnormalities in neuromuscular transmission, as detected by the electromyogram (122). Finally, quinine intensified the electrical disturbances in myasthenia gravis, as would be expected from its synergistic action with curare (122). These and other more obvious features of the disease led Harvey and Lilienthal (117) to conclude that the resemblance of myasthenia gravis to partial curarization is not superficial.

B. Effects of cholinesterase inactivation

With the development and introduction of DFP, Harvey *et al.* (118) re-examined the actions of neostigmine in myasthenia gravis and compared them with the effects of DFP in this disease. The technique employed consisted of the delivery of a train of four maximal motor nerve shocks before and at intervals after the intra-arterial injection of either neostigmine or DFP. The results of this experiment revealed the remarkable rapidity with which neostigmine repairs neuromuscular transmission and, in contrast, disclosed the comparatively slow action of DFP. When the amplitude of the successive potentials in the stimulus train were expressed as per cent of the initial response, the decline was such that the last response might be as little as 10 per cent of the first. Within 15 minutes of the intra-arterial injection of neostigmine, all of the responses in the series were 100 per cent of the initial potential amplitude. After the intra-arterial administration of DFP, the second, third and fourth responses achieved a maximal increase in four hours and were 95, 89 and 80 per cent of the height of the first potential. Another striking difference between the action of neostigmine and DFP injected intra-arterially was manifested following the release of arterial occlusion. In the case of neostigmine, the release of the cuff was followed by an almost immediate systemic improvement; subsequent to DFP, on the other hand, the improvement remained sharply localized to the injected area. This suggests the local binding of DFP by protein and emphasizes the efficacy of small amounts of neostigmine. The differences between DFP and neostigmine may be in part

explainable by the low water solubility of the fluorophosphate. This possibility receives considerable support from the observations of Grob and Harvey (115) on the effects of TEPP in myasthenia gravis. The characteristic progressive depression in the response to a train of stimuli was entirely abolished within 15 minutes by the intra-arterial injection of TEPP. This suggests that a sufficient and rapid inactivation of ChE will permit the restoration of potential amplitude, although for purposes of comparing the speed of onset of action, continuous recording from the time of injection would be required. However, a comparison of this and other data (118) obtained by this group of workers suggests a slightly faster attainment of a maximal effect after the intra-arterial injection of neostigmine. Another difference between TEPP and neostigmine was evidenced by the fact that a relatively large initial dose of TEPP was necessary to produce an observable effect, suggesting the need for a critical diminution of ChE activity. The severity of the ChE depression was apparent from the narrow margin existing between effective and toxic doses. Since a similar therapeutic index does not exist for neostigmine, it would be illogical to attribute the action of neostigmine solely to ChE inhibition.

C. Effects of acetylcholine

The problem presented by myasthenia gravis revolves about a synaptic defect. This may arise from a deficiency of transmitter substance, presumably ACh, or from an increased threshold of the post-synaptic membrane to this substance. In either case, the administration of a sufficient quantity of ACh should briefly correct the defect. Lanari (164) tested this idea in 1937 in two patients seriously ill with myasthenia gravis. He found that injection into the brachial artery caused an intense contraction of the muscles of the hand and concluded that myasthenic muscle was excessively sensitive to ACh. Harvey and Lilienthal (117) confirmed this action of ACh in patients with myasthenia gravis and also concluded that these individuals manifested an increased sensitivity to ACh. This possibility presented a puzzling situation since myasthenia gravis in all other respects closely resembled partial curarization. Harvey and Lilienthal (117) suggested that an increased sensitivity of myasthenic muscle to ACh might arise from a deficiency in transmitter agent which would in turn be equivalent to a functional denervation. On the other hand, Buchthal and Engbaek (41) compared the effects of intra-arterial ACh in normals and in 7 patients with myasthenia gravis and found the threshold dose to be higher in the patients with myasthenia. These apparent differences between the reactions of normal and myasthenic muscle to ACh have been largely resolved by the experiments of Acheson and his collaborators (4). Using small groups of normal subjects and myasthenic patients, these workers were able to show that a considerable variation of the muscular response occurred when the same dose was repeated within one minute in a subject. Significant differences in threshold ranges or in the character of the response could not be detected between the two groups. In fact, a large variation in the threshold dose was manifest in all cases. This occurred regardless of whether a distant (brachial) or close (radial) intra-arterial injection

site was utilized. These observations have since been confirmed by Harvey *et al.* (121). It is now reasonably certain that there is no difference between the susceptibility of normal and myasthenic muscle to ACh. Such a difference, if it were to exist, would require for elucidation the technique of close intra-arterial injection, so that extraneous factors affecting drug distribution could be controlled as rigidly as is possible in an animal experiment.

D. Other quaternary ammonium compounds

The action of other esters and analogues of choline in myasthenia gravis has received little attention. Fraser *et al.* (105) observed the effects of intra-arterially injected ACh, acetyl-β-methylcholine, carbaminoyl choline and neostigmine in 2 patients afflicted with this disease. The injections were made into the femoral artery. ACh effected no evident change in muscle power, but the dose used was small in view of the large muscle mass involved. The β-methyl derivative of ACh, as might be expected, exerted no visible effect. The injection of carbaminoyl choline resulted in a rapid increase in the motor power of the injected limb; shortly after the release of venous compression there occurred a generalized improvement. The spread of drug action was like that which occurs after the local administration of neostigmine, as described by Harvey and Lilienthal (117). It is difficult to view this action of carbaminoyl choline as developing through ChE inactivation since its inhibitory potency compared with that of neostigmine is exceedingly small (219). Recently Tether (248) described an immediate but transient beneficial effect of 3-OH phenyl $\dot{N}Me_2Et$ (Tensilon) in myasthenia gravis. In each of 19 cases the improvement following i.v. injection ranged from "slight to marked" after doses of 10 to 20 mgm. The brief duration of action precludes any practical use of this drug in this disease, but it is noteworthy that in these unanesthetized patients the specific neuromuscular action of this substance was evidenced by its therapeutic effect and the absence of any other visible side effects. This observation militates against its action being evolved through an inhibition of ChE, for if this were a prominent pharmacologic action other cholinergic manifestations would be expected. Attempts at oral administration with Tensilon were unsuccessful, since this drug, like other quaternary ammonium compounds, is poorly absorbed from the gastrointestinal tract.

E. Pharmacological clues

The similarity between the neuromuscular blockades produced by curare and the disease process of myasthenia gravis is clearly established from the standpoint of physiologic considerations. However, the question of whether an actual curare-like metabolite is present at the neuromuscular synapse of the individual with myasthenia or whether some intrinsic change in the character of the postsynaptic membrane has occurred remains unresolved. With regard to the former postulation, the literature presents conflicting reports. An experiment of Walker (254) describes the release of venous occlusion in a myasthenic limb following exercise of that part; this procedure was attended by the development of weakness in distant muscles. This experience has been denied by some (113). It has

also been claimed that the serum taken from patients with myasthenia gravis interferes with neuromuscular transmission in the isolated nerve-muscle preparation of the frog (267). Others have not been able to confirm this observation (87, 113, 183). Still other experiments relate the defect to a deficiency in ACh release or synthesis (63, 245). A pharmacologic approach may go far in the achievement of a solution to the problem of the defect in myasthenia gravis. In this regard, the preceding sections have defined the characteristics of those quaternary ammonium compounds having pronounced excitatory actions on the motor endplate and it has also been pointed out that certain quaternary ammonium compounds, which have a negligible stimulatory action, do possess an efficient anti-curare action, *e.g.*, 3-OH phenyl $\dot{N}Et_3$. With the use of these specific and contrasting quaternary ammonium compounds in experiments such as described by Harvey *et al.* (117, 122), it should be possible to ascertain whether an excitatory action on the motor endplate is essential to the circumvention of the defect. If only agents having strong direct or indirect stimulatory actions were effective, it would suggest that the disease process involved either an alteration in the characteristics of the post-synaptic membrane or a deficiency in transmitter substance. On the other hand, if compounds having little or no direct or indirect excitatory properties were effective, strong presumptive evidence would be afforded for the existence of a curare-like substance at the neuromuscular synapse in myasthenia gravis. At present, guanidine is the only compound that has been studied sufficiently to afford a clue as to the reactivity of myasthenic muscle to an agent that has (1) a recognized anti-curare action (225), (2) a negligible inhibitory action on ChE (10), and (3) a questionable direct excitatory action (106). It is not, of course, a quaternary amine. Minot *et al.* (188) have described some efficacy for guanidine in myasthenia gravis but its action can in no way be likened to the incisive anti-myasthenic action of neostigmine or Tensilon (122, 248). From this, the assumption would be that a substance having a direct stimulatory action is a desideratum for therapy in myasthenia gravis. Obviously, experiments on this point are needed.

VIII. GENERAL CONSIDERATIONS

A. Ionic interaction of quaternary cation and receptor

The chemical approach to the problem of drug-receptor combination has furnished considerable insight into the mechanism by which quaternary ammonium compounds, including ACh, interact with the receptor protein of the endplate. The effect of this reaction undoubtedly gives rise to the initiation and development of the local excitatory response. Perhaps the most elementary observation that can be made regarding the interaction of this class of compounds at the motor endplate is the ionic nature of the reaction. As early as 1925, it was stated that pharmacologically active compounds of the onium group have as a distinguishing characteristic the ability to ionize (208). In this regard, it has been pointed out that members of the choline group are insoluble in lipids and have no influence on surface tension (142). In view of this and their strong electrolyte character an adsorptive attachment to the cell, as a primary bonding force, is

unlikely. The importance of the ionic charge can be appreciated from the fact that betaine, which at physiological pH exists largely as an electrically neutral inner salt, is inactive at the n.m.j. (260). The possibility that the trialkyl ammonium grouping of quaternary ammonium compounds may function like the ions of the alkali-metals, particularly K^+, was entertained by Ing and Wright (142) and Alles and Knoefel (8). The latter workers compared the alkyl trimethylammonium series of ions with the alkali-metal ions and showed similarities of action with some consistency in correlation between the respective members of each series. However, the action of K^+ on the neuromuscular apparatus is indiscriminate (158) and emphasizes the singular specificity of onium compounds at the n.m.j.

In 1932, Ing and Wright (142) first proposed the idea that the bond between simple monoquaternary ammonium salts and the neuromuscular effector was ionic in type. It was suggested that the reaction might be similar to that of an ion exchange, in which the quaternary ammonium ions replace inorganic cation on the receptor structure. The capacity of the cationic head of choline to displace calcium from calcium zeolite has been demonstrated (220) but it is unlikely that the physiological event of neuromuscular transmission liberates sufficient ACh molecules to displace the amount of inorganic cation necessary to produce the electromotive force needed for the generation of the excitatory wave (90).

From the preponderance of the foregoing data, the conclusion cannot be avoided that the excitatory and anti-curare activities of quaternary ammonium compounds are associated with their ionic reactivity. A fresh approach to determinants of the ionic interaction of the cationic head with the receptor was made by Holmes *et al.* (131). In this work the paralytic potencies of several symmetrical alkyl ammonium ions on the isolated rat phrenic nerve-diaphragm preparation were correlated with their positive charge densities. The approach was based on a consideration of the resonance that occurs between ionic and covalent bonds in an ion like $\dot{N}R_4$. The ionic-covalent bond resonance for $\dot{N}H_4$ has been described by Pauling (199) and recently discussed by Taylor (246) in a review on synthetic blocking drugs. Briefly, if all of the bonds in $\dot{N}R_4$ remain covalent, then the screening effect of the orbital electrons will be diminished and the effective nuclear charge will be increased. If, however, the ionic character of the bond is increased, the screening effect of valence electrons will be increased and the nuclear charge, in effect, will be diminished. The pharmacologic significance of charge density on the onium atom has been considered further by Taylor. On the basis of existing chemical studies, it was pointed out that for cations of a given charge the affinity between them and an exchanger increases as the distance of closest approach of the ion and the receptor decreases. It was decided that if the affinity of a quaternary ammonium ion for its receptor is related to the closeness of approach which it can make to the receptor, it would seem reasonable that those ions in which the positive charge is least diffuse would have the highest affinities. Presumably pharmacologic activity would depend on the achievement of a critical approach of the ionic and receptor charges.

The above view receives vigorous support from a consideration of the re-

searches describing the ionic interaction between ACh and ChE. The details of this interaction have been reported recently and are summarized in the reviews of Whittaker (264) and Nachmansohn and Wilson (192). Adams and Whittaker (5) measured the binding of choline by erythrocyte and plasma ChE, and estimated from the ratio of the affinities for the two enzymes the distance between the positive charge in the "substrate" and the anionic group in the active center of the enzyme. The value obtained was 5.3 Å. This corresponded well with a calculated distance of closest approach (4.9 Å) between a positive charge located in the center of $—\dot{N}Me_3$ and a carbonyl oxygen. The existence of an anionic site on ChE was demonstrated by Wilson and Bergmann (265). They showed that the inhibitory action of physostigmine was greater when it existed as a positively charged cation in acid solution than when it existed as an uncharged free base in alkaline solution. Similarly, the velocity of the ChE catalysed hydrolysis of the tertiary amine, dimethylamino-ethylacetate, proved to be three times as rapid in an acid medium. However, the hydrolysis of some dimethylamino-ethylacetate in strongly alkaline solution and the inhibitory effect of uncharged physostigmine molecules did suggest the existence of additional binding forces, although the primary union between a substance like ACh and the enzyme receptor is obviously ionic in nature.

The importance of the magnitude of the ionic charge in the reactivity of quaternary ammonium compounds has been revealed in another way. Very recently Schueler (229) has calculated the polarities of several moieties commonly occurring in cholinergic compounds. The result expresses the polarizing force (P.F.) in dynes of the grouping and represents a commendable attempt to relate cholinergic activities quantitatively with forces affecting molecular charge. Thus the grouping $(—\dot{N}(CH_3)_3)$ has a P.F. = +3.21, the grouping $(—\dot{N}(CH_3)_2H)$ a P.F. = +3.02, the grouping $(—\dot{N}CH_3H_2)$ a P.F. = +2.88, and $(—\dot{N}H_3)$ the P.F. = +2.64. A decrease in muscarinic activity was correlated with the corresponding loss in polarity, and represents another expression of the fact that primary, secondary and tertiary alkyl derivatives exist as covalent ionogens which in effect will diminish the influence of the formal charge.

The uniquely specific character of the $—\dot{N}Me_3$ grouping became apparent in the early part of the century, through the researches of Hunt and Taveau (137) and Burn and Dale (51). The later work of Ing *et al.* (142, 143) made clear that the ionic reactivity with the n.m.j. of compounds of the type $\dot{N}R_4$ would vary according to the nature of the substituent group but the peculiar inactivity of $\dot{N}Et_4$ proved a special enigma. Alles and Knoefel (8) attempted to explain the specificity of $—\dot{N}Me_3$ on the basis of its molecular size. In terms of optimal receptor fit, the butyl, amyl or hexyl members of the series would, on the basis of experiment, have the most suitable dimensions and the longer chain congeners would antagonize the lower by obstructing the attachment of the latter. If molecular size were to be a critical factor in determining the specificity of receptor fit, the inactivity of $\dot{N}Et_4$ had to be accounted for in view of the fact that amyl $\dot{N}Me_3$ exhibited optimal activity. Since the volume of $\dot{N}Me_4$ corresponded to a sphere with a radius of about 3.2 $Å^3$ it was assumed that the latter figure

exceeded an upper limit for symmetrical molecules to exhibit a stimulatory action. On this basis amyl $\dot{N}Me_3$, having a maximal length of 11.4 Å, could be visualized as oriented to the receptor site *via* the —$\dot{N}Me_3$ head of the molecule. The authors questioned the validity of this, however, for if this were the case, the influence of chain length on activity should be less than it actually is. The remaining alternative assumed an unsymmetrical receptor mechanism, as proposed by Clark (61).

In compounds of the type $\dot{N}R_4$, the influence of the substituent groups on the charge density of the onium ion has been considered by Holmes *et al.* (131). These workers assessed the influence of the R group by two means. An indication of the order of relative electro-negativity of R was obtained from the dissociation constants of the corresponding series of acids, RCOOH. The charge dispersing effect of R was also determined by measuring the dissociation constant of the corresponding primary, secondary and tertiary amines. In the series methyl through butyl, the ethyl group proved anomalous in that the bases were strongest and the acids weakest in the series. It was assumed from this that the electrophilic nature of the ethyl group was comparatively low and that as a consequence the positive charge density on the nitrogen atom in $\dot{N}Et_4$ fell below some critical level necessary for pharmacologic activity.

It has been seen for compounds of the type $R\dot{N}Me_3$ that the length of the alkyl chain modifies the activity. For the n.m.j. the optimal alkyl length approximates the distance covered by four or five methylene groups attached to the nitrogen atom. This corresponds well with the optimal five atom chain length of parasympathomimetic drugs as summarized by Ing (141). Welsh and Taub (257) found that the maximal inhibitory action of alkyltrimethyl ammonium ions on the heart of *Venus mercenaria* occurred with the n-amyl derivative. It was concluded from this study that the shape and size of quaternary ammonium ions, rather than physical chemical differences, determined the closeness of their relationship to ACh. The concept of molecular size as a critical operator in the specificity of drug receptor union is not seriously damaged by comparisons based on the blocking actions of quaternary ammonium compounds. It must be recognized that this pharmacologic action is more complex than the excitatory action and accordingly reference has been made to the inactivity of $\dot{N}$ propyl$_4$ and $\dot{N}$ butyl$_4$ on the frog rectus muscle (143, 207); it remains to be determined whether these ions are equally unable to excite the mammalian n.m.j. The conclusion must be reached that molecular size is an important determinant of the receptor union requisite for stimulation.

B. Effect of chain length on binding

In the absence of specific chemical union between receptor and a side chain, the effect of chain length must be attributed to dispersion forces which would tend to reinforce the primary ionic bond. This, of course, will apply particularly to the non-polar alkyl chain. Chemical precedent for this has been established by researches revealing that the reaction of lower fatty acids with serum albumin is enhanced by an increase in the length of the alkyl chain (13, 24). The fact

that excessive chain length impedes the drug-receptor union may be attributed to the change in physical properties in a situation where hydrophilic characteristics are the *desiderata*. The bis-quaternary ammonium compounds exhibit an optimal alkyl chain approximately twice that for the monoquaternary ammonium compounds. The two-ended attachment of the C10 or C12 molecule probably accounts for the effectiveness of a compound, with an alkyl chain, of a length that would normally exert a deleterious effect on the function of a single cationic head. Apparently the anionic receptor sites occur at some regularly spaced intervals.

C. The effect of the OH grouping

1. *Alcoholic OH.* Frequent mention has been made of the attenuating effect on excitatory activity of an OH group attached to an alkyl chain. Indeed, from the standpoint of excitatory action at the n.m.j. these derivatives of onium ions have a negligible action. It is obvious therefore, that the OH group counteracts in some way the affinity of the cationic head for the anionic receptor site. Certain possibilities present themselves in explanation of this effect. Firstly, however, it is unlikely that the alcoholic OH reacts with the receptor since this would tend to increase the binding of the compound. Further, from a chemical standpoint, this consideration is unlikely since the acidic dissociation of an aliphatic alcohol is relatively low. Electronic considerations suggest that some intramolecular inductive effect will be exerted by the polarity of the OH group. For example, the inductive effect of the OH grouping in choline may be appreciated from its P.F. of -1.46 (229). On the other hand, in a saturated aliphatic compound, like choline, it may be that the electronegativity of the OH group will be exerted through a direct field effect. Finally, a most important effect of an alcoholic OH grouping is its ability to associate, through bonding of its hydrogen, with water molecules of the solvent. Clearly, any one or all of these actions would tend to diminish the approach of the cationic head to the oppositely charged receptor locus. The detrimental effect of an OH group on the cholinergic action of aliphatic quaternary ammonium compounds seems, at present, most applicable to the n.m.j. Welsh and Taub (258), using the isolated ventricle of the clam as a test object, were impressed by the striking loss in cholinomimetic activity that occurred when an OH group was appended at any point on the alkyl chain of amyl $\dot{N}Me_3$. Although a weakening effect of an alcoholic OH group has also been noted for the muscarinic actions of other cholinomimetic compounds, the effect is variable and the OH group often appears unimportant in these particular actions (165, 166). Certainly, the effects of an OH containing compound, muscarine, on smooth muscle, exocrine glands and the heart serves as a prototype for drugs acting on these systems. With regard to the autonomic ganglia, a preliminary survey of the compounds studied by Hunt and Taveau (137) indicates that the OH group exerts an enfeebling effect on compounds normally active on the ganglionic cell.

From the realization of the effect of an OH grouping on the pharmacologic activity of an alkyl onium compound, it becomes apparent that the functional

effect achieved by acetylation of a terminal OH, in a compound like choline, is a masking of this group. That the acetyl group *per se* does not contribute a strong binding force between drug and receptor can be appreciated from the fact that the hydroxyacetic acid ester of choline is little more potent than choline at the n.m.j. (57). Another means of masking an OH group is accomplished through ether formation and attesting to this view is the ACh-like potency of choline ethers.

2. *Phenolic OH.* It is obvious from pharmacologic experiment (78, 203) that the attenuating effect of an OH group is least when it is a substituent on a benzene ring structure and oriented *meta* to the onium attachment. In fact, the mild antagonism to the interionic attraction of the cationic head and the negative receptor charge provided by the aromatic OH may well prevent a duration and degree of binding that would otherwise be conducive to the establishment of a depolarization blockade of neuromuscular transmission. It is probable that on this point rests the unusual excitatory and anti-curare properties of ions like 3-OH phenyl $\dot{N}Me_3$. Explanation for the peculiar effectiveness of these substances demands an inquiry into the chemical properties of such compounds.

Frequent allusions have been made to the contributory role of an aromatic ring in the pharmacologic activity of a compound. The most obvious participation of such a structure would be to enhance drug-receptor union through van der Waal's forces; to this end, the size and particularly the rigidity of the ring structure would furnish better conditions for the effectiveness of such attraction than would an alkyl chain. Under these circumstances, the phenolic OH in the *meta* position would be brought into a close relationship with the receptor. In this position, a reaction between the electronegative oxygen and some positively charged group of the receptor is not improbable. In this respect, it is noteworthy that the acid dissociation constant of phenol is much greater than that of the aliphatic alcohols and, in fact, the formation of a phenolate ion would be increased by the addition to the ring of the positively charged trimethylammonium grouping. The opportunity for reaction between the nucleophilic oxygen of the *meta* trimethylammonium phenolate ion and the receptor is further increased by the possibility that an electrophilic group of the receptor may be located at a complementary position. Pfeiffer (200) has postulated that the keto oxygen of ACh and related substances represents a reactive portion of the molecule in the union with the effector cell. This concept receives strong support from the *in-vitro* studies on the interaction of ACh and ChE (266), wherein the esteratic site of the enzyme is presumed to have a nucleophilic and an electrophilic group. The latter may unite with the negative carbonyl oxygen. Thus, the distance between the nitrogen and the oxygen atoms of 3-OH phenyl $\dot{N}Me_3$ approximates 5.1 Å, which is congruent with an estimated distance of 5.6 Å between the nitrogen and keto oxygen atoms of ACh. It is likely that all of these factors contribute to the fact that the aromatic derivative, 3-OH phenyl $\dot{N}Me_3$, is a more active excitatory agent at the n.m.j. than is the analogous aliphatic alcohol, choline.

Other pertinent information regarding drug-receptor interaction may be gleaned from a consideration of the structure-activity relations of other phenyl

trimethylammonium congeners. Description of the impotency of 2-OH phenyl $\dot{N}Me_3$ at the n.m.j. has been given. The failure of this isomer to activate finds explanation in the well-known *ortho* effect of organic reaction. This refers to the anomalous influence on a functional grouping of an *ortho* substituent in the phenyl ring. In particular, strong *ortho* effects are produced by the presence of an OH group situated in this relationship to the reactive grouping. Many chemical illustrations can be given, but an outstanding and not unrelated pharmacologic example of the influence of OH groupings on the behavior of a drug is illustrated by the streptomycin molecule which consists of a streptidine ring containing OH groupings at all ring positions adjacent to two charged guanidine groupings. The polarity of the OH groupings opposes the affinity of the cationic groups for protein (75). This is, in fact, an *ortho* effect. There are several possible interpretations of the *ortho* effect. Chelation has been advanced as a mechanism responsible for the *ortho* effect but in compounds of the type, $\dot{N}R_4$, this will not occur. It is also possible that the *ortho* OH introduces steric hindrance to the approach between drug and receptor; this point could be resolved by determining the excitatory activity of an isostere like 2-Me phenyl $\dot{N}Me_3$. However, the most outstanding factor is the direct inductive field effect arising from the proximity of the OH group to the charged onium center. Here, then, the overall result is to annul the effect of the charge on the nitrogen atom and simultaneously to abolish the receptor affinity of the compound. With regard to chemical reactivity it was long ago observed (187) that the *ortho* effect disappeared if the functional group were separated from the benzene nucleus by one or more carbon atoms. Similarly, reference has already been made (Fig. 1) to the fact that pharmacologic reactivity prominently reappears in the compound, *ortho* hydroxy benzyl trimethylammonium bromide.

The paucity of information on the activity of 4-OH phenyl $\dot{N}Me_3$ prevents assessment of the influence exerted by the OH group in the *para* position. It has been recognized (203) that the compound 4-OH phenyl $\dot{N}Me_3$ is somewhat less potent as an anti-curare agent than is the *meta* isomer. However, this observation does not reflect the relative excitatory capacity of the *para* isomer. Electronic consideration of the translation of an inductive effect through the benzene ring indicates that there may be a modification of the onium charge as a result of substitution in the *para* position. According to this theory the OH group acting as an electron repelling group would give rise to a tautomeric electron shift so that the *ortho* and *para* ring positions would be activated due to an increased electron density at these points. Thus the ion, phenyl $\dot{N}Me_3$, would be modified by the introduction of an OH group into the *para* position such that the naturally occurring electron sink, at the carbon atom to which the highly charged onium atom is attached, would be reinforced by the electron movement associated with the OH grouping. The increased electron density at the N substituted carbon atom would tend to diminish the positive charge concentration and would bespeak a lesser biologic reactivity. However, a recent study of the electrical effect of the $-\dot{N}Me_3$ grouping attached to a phenyl ring offers no support for the preferential increase of electron density at the *ortho* and *para* carbon

atoms via inductive effects transmitted through the ring (217). It was concluded that the inductive effect of a substituent group appears best regarded as falling off with distance, possibly in accord with Coulomb's law. On this basis the *para* isomer should prove the most active. There is some evidence to indicate that this may be the case. In a recent study Depierre and Funke (78) have shown that the 4-OH phenyl $\dot{N}Me_3$ ion is much more potent than the *meta* isomer in the production of neuromuscular paralysis in the cat. Presumably the blockade produced by this compound represents an extension of an initial stimulating action, as is the case for C10. Randall (203) reported only a paralysing action for the *para* isomer, but it is possible that sufficiently small dosage was not explored for the revelation of the potentiating effect.

In summary, it must be concluded that the effects of OH substitution on the biologic reactivity of phenyl $\dot{N}Me_3$ resolutely support the pharmacologic importance of onium charge concentration and, perforce, the significance of ionic interaction for the excitation of the motor endplate by quaternary ammonium compounds. It is tempting to speculate on the functional role of the —OH and $—OCH_3$ groupings in the dTC molecule. It might be assumed that these radicals counteract or disperse the onium charges of dTC sufficiently to prevent an excitatory result from drug-receptor union. As a corollary, the approach of a cation of dTC to the receptor surface might not be sufficiently close to achieve electrostatic union and the principal cohesive forces in this instance may develop from the van der Waal's attraction between the large dTC molecule and the receptor. This latter view accords essentially with that of Pfeiffer (200) for the blocking action of dTC.

D. Speculations on the cholinesterase of the neuromuscular junction

Some years ago, Roepke (141), while studying the inhibitory potencies and affinities of several compounds for ChE, noticed the parallelism between the affinities of the choline-like compounds and their known pharmacologic activity. He adroitly assumed that the ChE enzymes might serve as a model for the cell receptor. On this basis, the modern studies of Nachmansohn's group (16, 265, 266) afford considerable insight into the mechanism of the interaction of ACh and receptor. Their description of the ionic interaction between ACh and an anionic locus on the enzyme protein has been reviewed and is seen to complement the multitude of accumulated evidence revealing the primary ionic union between ACh and the cell receptor. On the other hand, the investigations of these workers on the reaction of the carbonyl grouping of ACh with an esteratic site on the enzyme furnishes a fresh impetus for a consideration of the counterpart of this reaction at the n.m.j. For this aspect of the reaction between ACh and ChE, they noted first the strong polarity of the carbonyl group of ACh and indicated that, in this circumstance, the carbonyl carbon atom possesses a definite electrophilic character. This was referred to the known mechanism of alkaline ester hydrolysis, in which this carbon atom is the point of attack by OH^- (116). Thus, in analogous fashion, it was assumed that a basic group of the enzyme reacts with the carbonyl carbon and that this union may be reinforced by reaction of the keto

oxygen. The esteratic site was visualized as containing a grouping, :G :H, of which the electron pair of the basic part first combines with the electrophilic carbon. The consequent weakening of the ether link would result in hydrolysis, with the protein group donating its proton to the alkyl fragment. The intermediate complex formed between the acyl fragment and the enzyme, G:C(O⁻)(CH₃), would be subject to attack by water and, in this way, the active site of the enzyme regenerated (16). As these workers have indicated, the formation of the complex would have to be the rate-controlling step in the reaction (192). It must be appreciated that these researches are initial efforts in the definition of this reaction and as such have been mildly criticized (264). It is indeed tempting to speculate that, in addition to the electrostatic attraction of the cationic head of ACh, the attachment of ACh to the endplate receptor is completed through reaction of its carbonyl carbon and/or oxygen atoms. If this plausible assumption were to be the case, it is obvious that activation of the ester link would follow and accordingly the receptor protein and the enzyme protein would be identical. This possibility seems peculiarly well suited to the synapse of the n.m.j. At this site, the ChE localized to the motor endplate is strategically placed. This localization was demonstrated originally in the frog by Marnay and Nachmansohn (178), later reaffirmed for the mammal (66) and more recently traced by histochemical method to the post-synaptic membrane (153). In this regard, it is worth reiterating that the highly reactive, chronically denervated muscle retains its ChE activity (31, 66). With the concept of the identity of enzyme and receptor, the evolution of the alkyl OH group by the esteratic site of the receptor would result in a simultaneous counteraction to the ionic interaction of the cationic head and the receptor. This concept might aid in providing some explanation for the inextricable and complex relationships between the *in-vivo* actions of quaternary ammonium compounds on the n.m.j. and their inhibitory actions on extracted enzyme protein *in vitro*. In this regard, the sensitizing actions of ChE inhibitors might be explained. Whittaker (264) has summarized the body of evidence establishing the fact that the phosphorous-containing anti-ChE compounds combine with an active center of the enzyme. It has been postulated that the mechanism of their action involves the splitting off of a phosphonium ion $[(RO)_2PO]^+$, which combines irreversibly with the site concerned with the activation of the ester link (46, 265). Assuming this reaction to be confirmed and taking cognizance of the large mass of data indicating that the initiation of excitation by a quaternary ammonium compound depends chiefly on the ionic reactivity of the cationic head, it becomes apparent that, if the ChE protein were the receptor, the phosphonium ion would not excite directly. The administration of ACh following anti-ChE substances having an electrophilic carbon or phosphorous atom, like either the carbamate or alkyl phosphate derivatives, would result only in receptor attachment through the cationic portion. The five-atom chain of

ACh would, in this circumstance, have no special virtue. In accord with this it is important to recall the prolonged pharmacologic action, relative to ACh, of γ-carbomethoxyallyl trimethyl ammonium chloride in which the position of the carbonyl carbon is on the "wrong" side of the ether link. In the case of neostigmine, union like that for ACh would occur, except that in neostigmine the ester link could not be activated and this receptor site would remain occupied. The modification of ACh action by blockade of an esterasic receptor site would not alter, but rather unify, existing theory of neurohumoral transmission, and therewith the complexities met in comparing anti-ChE action *in vitro* with effector response *in vivo* may be in large part artificial. The approaches of Nachmansohn's group to the mechanisms of ACh-ChE interaction may be profitably applied to suitable neuromuscular preparations for the determination of ACh-receptor union. Thus it would be important, with appropriate *in-vitro* methods, to determine the pH dependence of ACh action at the n.m.j., with the presumption that the reaction of the cationic head would continue independent of such change, whereas the fixation of the carbonyl grouping would be affected accordingly.

E. Reactivity of the carbonyl group

Pfeiffer (200) was duly impressed with the possibility that the keto and carbonyl oxygen atoms of the ester linkage might take part in the reactivity of such compounds with the cellular receptor. He cited several examples in which the intramolecular distances between these oxygen atoms and the onium center closely approximated interatomic distances of 5.2 and 7.0 Å. A recent commentary by Ing (141) serves to remind that several exceptions can be found to Pfeiffer's general formulations. The excitatory properties of the simple alkyl trimethylammonium salts make it evident that the ester linkage is not essential. However, the strong nucleophilic property of the carbonyl oxygen lends credence to the idea that ester structures will react through this site with the receptor. Recognizing the significance of the researches of Wilson and Bergmann (l. c.) for the theory of ACh-receptor interaction, Welsh and Taub (258) have recently made one of the first studies on the contributions of the carbonyl grouping in the reaction between drug and receptor. The sensitive heart of the clam, *Venus mercenaria*, was used, and it was learned that the cholinergic activity of amyl $\dot{N}Me_3$ was significantly enhanced by the inclusion of a carbonyl group at position 4 of the alkyl chain. The importance of the position of the C=O group was clearly demonstrated by recording the progressively steep loss in activity that occurred with the carbonyl grouping first in the 3 and then in the 2 position. It was also clear that the absence of an ether oxygen resulted in a comparatively smaller loss in activity. Most important was the fact that the ketone oxygen could not be reduced or replaced without engendering sharp losses in potency. It was concluded that the C=O grouping has a definitive role in the reaction of ACh and the cell receptor. As suggested by Wilson and Bergmann for the reaction between ACh and ChE, Welsh and Taub envisioned the possibility of a similar reaction between the C=O group and an appropriate site on the cell receptor. But they also recognized that such a site was not necessary for activity

since simple compounds such as amyl $\dot{N}Me_3$ were effective; the application of these carbonyl compounds in the definition of the receptor reactivity of the n.m.j. should provide especially useful information with regard to receptor specificity and excitation.

The importance of the ether oxygen atom in a number of ethers and esters of choline and certain related compounds has been considered very recently by Hey (125A). It was postulated that the electron density of this atom is an important determinant of pharmacologic activity, in that activity increases as the electron density of the atom decreases. To substantiate this, the greater nicotinic activity of the vinyl ether of choline, as opposed to the ethyl ether of choline, was cited and it was pointed out accordingly that the greater resonance of the vinyl group with the oxygen atom more effectively diminishes the electron density of the oxygen. Similarly, the strong nicotinic activity of choline phenyl ether was attributed to the high degree of resonance between the oxygen and the attached aromatic ring. For contrast it was pointed out that in the benzyl ether the resonance between the ether oxygen and the ring structure decreased and as a result the activity of this ether was less than that of the phenyl. For the ester structure it was proposed that activation of the ether oxygen atom depends on resonance with the carbonyl group and that any substituent attached to the carbonyl carbon influences activity depending on the resulting increase or decrease of resonance interaction of the carbonyl group with the ether oxygen atom. Hey determined nicotinic activity by measurement of the pressor effect; the compounds studied were nuclear substituted phenyl ethers of choline. It was found, in accord with hypothesis, that when the ring substituent impaired the resonance effect of the ring, activity was diminished. For example, *m*-tolyl ether of choline was considerably less active than the *m*-cholorophenyl and *m*-bromophenyl ethers of choline. Similar relationships were observed among the *para* derivatives and also between the 3,5-xylyl and 3,5-dibromophenyl ethers. It would be important to extend these observations to the neuromuscular junction with regard to anti-curare and excitatory activity.

F. Possible mechanisms of quaternary ammonium ion action

A final and most important question relates to the mechanism by which ACh, or some similar quaternary base, initiates the excitatory response at the motor endplate. The question may first be posed as to whether knowledge of certain steps in such a reaction, as derived from pharmacologic experiment, is applicable to the physiologic event of neuromuscular transmission. Regardless of the approach adopted, several unknown quantities exist. Thus, it is not possible to know precisely, or even approximately, the molar concentration of ACh or some similar drug attained at a single motor endplate following the close intra-arterial injection of a given effective dose of the substance. The micro-technique as employed by Nastuk (193) measured the depolarization produced at a single muscle endplate by ACh applied in a concentration of 110 mM/1. However, this concentration is excessive in light of the relationship between ACh concentration and local response as determined by Fatt (89). From the physiologic standpoint

there is still argument as to the site at which the transmitter substance is liberated. The predominant evidence at present still implicates the motor nerve terminals as the source. As Kuffler (159) has clearly stated, there is no definitive answer to the questions how liberated ACh reaches the underlying endplate, what concentrations of ACh are necessary to excite, and how close to the receptor is ACh liberated. The research of Dale *et al.* (71), demonstrating the liberation of ACh at the n.m.j. following motor nerve stimulation, is qualitatively significant but of dubious quantitative value when one considers the errors inherent in such an experimental preparation on which are superimposed the errors of bioassay. The question as to the validity of integrating evidence obtained from either a pharmacologic or physiologic approach may be resolved if the premise is accepted that ACh is the physiologic mediator. On this basis it will be seen that the action of ACh, whether liberated by a nerve impulse or introduced from without, will be the same despite the fact that wide variations in ultimate concentration may exist.

The results of intensive physiologic investigation have made quite plain the fact that excitation is attended by an extrusion of intracellular K^+. Certain important distinctions, however, have been made by Fatt and Katz (90). They point out that the characteristic response of the nerve or muscle membrane to an electric stimulus consists of a restorative electrochemical reaction in which Na^+ selectively enters the fiber; the process proceeds to an equilibrium reinforcing the initial electrical disturbance. On the other hand, the endplates do not respond to electric stimulation, but they do react to ACh. Therefore the latter reaction cannot be regenerative, and so the specificity of the endplate is further delineated. In this regard, Fatt (89) studied the electromotive action of ACh at the motor endplate to determine whether ACh rendered the membrane permeable to previously non-penetrating ions or whether ACh itself might penetrate the membrane at a rate sufficient to produce depolarization. Specifically, the ability of ACh to depolarize in the absence of Na^+ was tested and it was learned that ACh could accomplish this in the absence of Na^+, although the magnitude of this effect was reduced. It was suggested that Na^+, when present, may act to reinforce a depolarization initiated by ACh. This is in accord with the earlier suggestions of Hodgkin and Katz (129) with regard to the action of Na^+ on the excitatory process in nerve. Consideration was then given to the possiblity that ACh, by movement inward, might cause an outward flow of K^+. Mathematical considerations made it apparent that the extent of membrane depolarization could be related to the rate of the outward K^+ flux. However, the assumption that ACh will penetrate the membrane has no basis in fact, and existing evidence supports the probable surface action of ACh. In this respect, the important experiment of Cook (65) still prevails, in which it was demonstrated that the action of ACh on the frog heart could be inhibited by methylene blue, but that the process could be readily reversed by washing despite the penetration and fixation of the dye in the cell. The conclusion of a surface action of ACh is difficult to avoid. Further, the later experiments of Fatt and Katz (90) negate the hypothesis of an ACh action by direct penetration. It was estimated that if this

were to occur, ACh would have to be released in a quantity of about 1 to 2 × 10^{-12} mol. per junction per impulse. An anatomical consideration of the nerve endings revealed that the quantity of ACh necessary for only one or two impulses would be an absurdly large amount to be contained by these structures. As an alternative it was suggested that small quantities of ACh in some way alter the endplate surface to render it permeable to ions in general. In this connection, it was judiciously remarked that such a mechanism would satisfy the requirement for the very large amplification of ionic currents that occur at the point where the impulse is transferred from a minute nerve ending to the relatively enormous endplate surface. The magnitude of this disparity may be appreciated from the estimations by Clark with regard to the action of ACh on the cell of the frog heart (61). The results reveal that as huge a number of ACh molecules as 3 × 10^5 acting per cell would cover only 1/6000 of the cell's surface or, as Pfeiffer (200) has indicated, the size of the ACh molecule is infinitely small with respect to the cell surface.

The chemical factors concerned in the excitatory and anti-curare actions of ACh and other quaternary ammonium compounds furnish strong support for the surface action of ACh at the motor endplate. It has been seen that the precipitation of the excitatory response may be related to the ionic interaction of the cation, —$\dot{N}Me_3$. From this, one may raise the query as to why an equally strong cation like K^+ acts to depolarize either the muscle or endplate membrane indiscriminately, in contrast to the circumscribed depolarizing action of many quaternary ammonium ions. Again, the distinction between a penetrating and a surface action may be offered in explanation. If one takes into account the greater size of the cationic head of $\dot{N}Me_4$, the attraction of a surface for the side chain of a compound like $R\dot{N}Me_3$, the presence of hydrophilic groups in the side chain and ionic velocities, it becomes apparent that the smaller, more mobile alkali metal cation is much better suited for transport through the membrane. As a corollary, the action of K^+ at the endplate may be to depolarize directly by penetration and indirectly by a surface action. Finally, the fact that Mg^{++} does oppose the stimulatory action of K^+ (3) suggests that the transport mechanism for K^+ can be blocked by a related ion (102).

Danielli (74) has indicated the mechanism by which a low concentration of ACh in the pericellular milieu will be effectively increased by adsorptive concentration on the receptor surface. In the case of neuromyal transmission, the relatively few molecules of ACh involved must act by triggering a chain of reactions, as suggested by Welsh (256) in his speculations on the mode of action of ACh. What these are must remain speculation, but in agreement with Fatt and Katz (90) it seems most likely that ACh and related quaternary ammonium compounds render the endplate membrane generally permeable to intra- and extracellular ions. The occurrence of increased permeability at a specific locus or pore may initiate a rapid ionic propagation to adjacent membrane areas. Teorell (247) has discussed surface force gradients and comments on the little attention that they have received. In this way, it is possible that ionic surface flow will occur with considerable rapidity.

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REFERENCES

1. ABDON, N.-O.: On the influence of atropine on some nicotine-like actions of acetylcholine. Acta Physiol. Scandinav., 1: 153–170, 1940.
2. ACHESON, G. H.: Physiological and pharmacological aspects of neuromuscular diseases. J. Nerv. & Ment. Dis., 100: 616–620, 1944.
3. ACHESON, G. H.: Physiology of Neuromuscular Junctions: Chemical aspects. Federation Proc., 7: 447–457, 1948.
4. ACHESON, G. H., LANGOHR, J. L., AND STANBURY, J. B.: Sensitivity of skeletal muscle to intra-arterial acetylcholine in normal and myasthenic man. J. Clin. Investigation, 27: 439–445, 1948.
5. ADAMS, D. H., AND WHITTAKER, V. P.: The cholinesterases of human blood II. The forces acting between enzyme and substrate. Biochem. et Biophys. Acta, 4: 543–558, 1950.
6. ARSCHLIMANN, J. A., AND REINERT, M.: The pharmacological action of some analogues of physostigmine. J. Pharmacol. & Exper. Therap., 43: 413–44, 1931.
7. ARSCHLIMANN, J. A., AND STEMPEL, A.: Some analogs of prostigmine. Jubilee volume Emil Barell, Basle, 306–313, 1946.
8. ALLES, G. A., AND KNOEPEL, P. K.: Comparative physiological actions of alkyltrimethylammonium and alkalimetal salts. Univ. California Publ., Pharmacol., 1: 187–212, 1939.
9. ARTUSIO, J. F., JR., RIKER, W. F., JR., AND WESCOE, W. C.: Studies on the interrelationship of certain cholinergic compounds. IV. Anti-curare action in anesthetized man. J. Pharmacol. & Exper. Therap., 100: 227–237, 1950.
10. AUGUSTINSSON, K. B.: Cholinesterase—a study in comparative embryology. Acta Physiol. Scandinav., 15: (supp. 52) 1–181, 1948.
11. AUGUSTINSSON, K. B., AND NACHMANSOHN, D.: Studies on cholinesterase. VI. Kinetics of the inhibition of acetylcholine esterase. J. Biol. Chem., 179: 543–559, 1949.
12. BACQ, Z. M., AND BROWN, G. L.: Pharmacological experiments of mammalian voluntary muscle in relation to the theory of chemical transmission. J. Physiol., 89: 45–60, 1937.
13. BALLOU, G. A., BOYER, P. D., LUCK, J. M., AND LUM, F. G.: The heat coagulation of human serum albumin. J. Biol. Chem., 153: 589–605, 1944.
14. BARLOW, R. B. AND ING, H. R.: Curare-like action of polymethylene bis-quaternary ammonium salts. Brit. J. Pharmacol., 3: 298–304, 1948.
15. BEAN, J. W., AND ELWELL, L. H.: Influence of d-tubocurarine on contractile response of mammalian skeletal muscle *in situ*. Am. J. Physiol., 165: 716–726, 1951.
16. BERGMANN, F., WILSON, I. B., AND NACHMANSOHN, D.: Acetylcholinesterase. IX. Structural features determining the inhibition by amino acids and related compounds. J. Biol. Chem., 186: 693–703, 1950.
17. BERRY, W. K., AND EVANS, C. L.: Cholinesterase and neuromuscular block. J. Physiol., 115: 46P–47P, 1951.
18. BLASCHKO, H., BÜLBRING, E., AND CHOU, T. C.: Tubocurarine antagonism and inhibition of cholinesterase. Brit. J. Pharmacol., 4: 29–32, 1949.
19. BLOCH, H.: Darstellung und Pharmakologie des isobutyryl-3-oxyphenyltrimethyl-ammoniummethylsulfats und einiger verwandter Verbindungen. Arch. f. exper. Path. u. Pharmakol., 193: 292–304, 1939.
20. BOEHM, R.: Über das Vorkommen und die Wirkungen des Cholins und die Wirkungen des Kunstlichen Muscarine. Arch. f. exper. Path. u. Pharmakol., 19: 87–100, 1885.
21. BOEHM, R.: Über Wirkungen von Ammoniumbasen und Alkaloiden auf den Skelettmuskel. Arch. f. exper. Path. u. Pharmakol., 58: 265–270, 1908.
22. BOVET, D., BOVET-NITTI, F., GUARINO, S., LONGO, V. G., AND MAROTTA, M.: Proprietà farmacodinamide di alcuni derivati della succinilcolina dotati di azione curarica—Esteri di trialchiletanolamonio di acidi bicarbossilici alifatici. Rendiconti Instituto Superiore di Sanita, 12: 106–137, 1949.
23. BOVET, D., DEPIERRE, F., AND DE LESTRANGE, Y.: Propriétés curarisantes des éthers phénoliques à fonctions ammonium quaternaires. Compt. rend. Acad. Sc., 225: 74–76, 1947.
24. BOYER, P. D., LUM, F. G., BALLOU, G. H., LUCK, J. M., AND RICE, R. G.: The combination of fatty acids and related compounds with serum albumin I. Stabilization against heat denaturation. J. Biol. Chem., 162: 181–198, 1946.
25. BREMER, F.: Researches on the contracture of skeletal muscle. J. Physiol., 76: 65–94, 1932.
26. BREMER, F., AND HOMÈS, G.: Une Théorie de la sommation d'influx nerveus. Arch. internat. de Physiol., 35: 39–64, 1932.
27. BRISCOE, G.: The antagonism between curarine and acetylcholine. J. Physiol., 87: 425–428, 1936.
28. BRISCOE, G.: The antagonism between curarine and prostigmine and its relation to the myasthenia problem. Lancet, 1: 469–472, 1936.
29. BRISCOE, G.: The anti-curare action of substance 36. Lancet, 1: 621–623, 1937.
30. BRISCOE, G.: Changes in muscle contraction curves produced by drugs of the eserine and curarine groups. J. Physiol., 93: 194–205, 1938.
31. BROOKS, V. B., AND MYERS, D. K.: Cholinesterase content of normal and denervated skeletal muscle in the guinea pig. J. Physiol., 116: 158–167, 1952.
32. BROWN, G. L.: Action potentials of normal mammalian muscle. Effects of acetylcholine and eserine. J. Physiol., 89: 220–237, 1937.

33. Brown, G. L.: The actions of acetylcholine on denervated mammalian and frog's muscle. J. Physiol., 89: 438–461, 1937.
34. Brown, G. L., and Burns, B. D.: A convenient nerve-muscle preparation from the gracilis of the cat. J. Physiol. 106: 54P–55P, 1949.
35. Brown, G. L., Burns, B. D., and Feldberg, W.: The effect of di-isopropyl fluorophosphonate on neuro-muscular transmission in cats. J. Physiol., 107: 346–354, 1948.
36. Brown, G. L., Dale, H. H., and Feldberg, W.: Reactions of the normal mammalian muscle to acetylcholine and eserine. J. Physiol., 87: 394–424, 1936.
37. Brown, G. L., and Harvey, A. M.: Reactions of avian muscle to acetylcholine and eserine. J. Physiol., 94: 101–117, 1938.
38. Brown, G. L., and Harvey, A. M.: Neuro-muscular transmission in the extrinsic eye muscle. J. Physiol., 99: 379–399, 1941.
39. Brown, G. L., Paton, W. D. M., and Dias, M. V.: The depression of the demarcation potential of cat's tibialis by bis-trimethylammonium decane diiodide (C10). J. Physiol., 109: 15P, 1949.
40. Buchthal, F.: Interaction entre l'acetylcholine, l'adenosine triphosphate et les phénomènes électriques dans la transmission neuromusculaire. Arch. Sc. Physiol., 3: 603–606, 1949.
41. Buchthal, F., and Engbaek, L.: On the neuromuscular transmission in normal and myasthenic subjects. Acta psychiat. et neurol., 23: 3–11, 1948.
42. Buchthal, F., and Lindhard, J.: Direct application of acetylcholine to motor end plates of voluntary muscle fibers. J. Physiol., 90: 82P–83P, 1937.
43. Bülbring, E.: Observations on the isolated phrenic nerve diaphragm preparation of the rat. Brit. J. Pharmacol., 1: 38–61, 1946.
44. Bülbring, E., and Chou, T. C.: The relative activity of prostigmine homologues and other substances as antagonists to tubocurarine. Brit. J. Pharmacol., 2: 8–22, 1947.
45. Bülbring, E., and Depierre, F.: The action of synthetic curarizing compounds in skeletal muscle and sympathetic ganglia both normal and denervated. Brit. J. Pharmacol., 4: 22–28, 1949.
46. Burgen, A. S. V.: The mechanism of action of anticholinesterase drugs. Brit. J. Pharmacol., 4: 219–228, 1949.
47. Burgen, A. S. V., and Hobbiger, F.: The pharmacology of the methyl and benzyl esters of γ-crotonic betaine (γ-carboxyallyltrimethyl-ammonium chloride). Brit. J. Pharmacol., 4: 229–233, 1949.
48. Burgen, A. S. V., Keele, C. A., Chennells, M., del Castillo, J., Floyd, W. F., Slome, D., and Wright, S.: Physiological effects of alkyl polyphosphate. Nature, 160: 760–761, 1947.
49. Burgen, A. S. V., Keele, C. A., and Slome, D.: Pharmacological actions of tetraethylpyrophosphate and hexaethyltetraphosphate. J. Pharmacol. & Exper. Therap., 96: 396–409, 1949.
50. Burke, J. C., Linegar, C. R., Frank, M. W., and McIntyre, A. R.: Eserine and neostigmine antagonism to d-tubocurarine. Anesthesiology, 9: 251–257, 1948.
51. Burn, J. H., and Dale, H. H.: The action of certain quarternary ammonium bases. J. Pharmacol. & Exper. Therap., 6: 417–438, 1915.
52. Burns, B. D., and Paton, W. D. M.: Depolarization of the motor end-plate by decamethonium and acetylcholine. J. Physiol., 115: 41–73, 1951.
53. Buttle, G. A. H., and Zaimis, E. J.: The action of decamethonium iodide in birds. J. Pharm. & Pharmacol., 1: 991–992, 1949.
54. Carmichael, E. A., and Fraser, F. R.: The effects of acetylcholine in man. Heart, 16: 263–274, 1933.
55. Casier, H., and Verbeke, R.: Parasympathomimetic symptoms, sensitivity to acetylcholine and decurarization, in relation to inhibition of cholinesterases. Arch. internat. de pharmacodyn. et de thérap., 83: 453–463, 1950.
56. Castillo, J. C., and debeer, E. J.: The neuromuscular blocking action of succinylcholine (diacetylcholine). J. Pharmacol. & Exper. Therap., 99: 458–464, 1950.
57. Chang, H. C., and Gaddum, J. H.: Choline esters in tissue extracts. J. Physiol., 79: 255–285, 1933.
58. Chase, H. F., Schmidt, J. L., and Bhattacharya, B. K.: Antagonism of curare action by neostigmine, physostigmine, ephedrine and di-isopropyl fluorophosphate (DFP). J. Pharmacol. & Exper. Therap., 91: 236–245, 1947.
59. Chennells, M., Floyd, W. F., and Wright, S.: Action of condensed alkyl phosphates on the nerve-muscle preparation and the central nervous system of the cat. J. Physiol., 108: 375–397, 1949.
60. Clark, A. J.: The reaction between acetylcholine and muscle cells. J. Physiol., 61: 530–546, 1926.
61. Clark, A. J.: *Handbuch der Experimentellen Pharmakologie*. Berlin, Julius Springer, 1937.
62. Clark, A. J., and Raventos, J.: The antagonism of acetylcholine and of quaternary ammonium salts. Quart. J. Exper. Physiol., 26: 375–392, 1937.
63. Cohen, S. J., Kaufman, I. C., Spitz, E. B., and Stern, M. M.: Effect of blood of myasthenia gravis patients on acetylcholine sensitivity of prostigminized frog and leech muscle. Proc. Soc. Exper. Biol. & Med., 58: 181–183, 1945.
64. Cohen, H. L., and Unna, K. R.: Correlation between cholinesterase inhibition and acetylcholine potential of some phenolic quaternary ammonium compounds. J. Pharmacol. & Exper. Therap., 103: 340–341, 1951.
65. Cook, R. P.: The antagonism of acetylcholine by methylene blue. J. Physiol., 62: 160–165, 1926.
66. Couteaux, R., and Nachmansohn, D.: Changes of choline esterase at end plates of voluntary muscles following section of sciatic nerve. Proc. Soc. Exper. Biol. & Med., 43: 177–181, 1940.
67. Cowan, S. L.: The initiation of all-or-none responses in muscle by acetylcholine. J. Physiol., 88: 3P–5P, 1936.
68. Cowan, S. L.: The action of eserine-like and curare-like substances on the responses of frog's nerve-muscle preparations to repetitive stimulation. J. Physiol., 93: 215–262, 1938.

69. DALE, H. H.: The action of certain esters and ethers of choline and their relation to muscarine. J. Pharmacol. & Exper. Therap., 6: 147–190, 1914.
70. DALE, H.: Transmission of nervous effects by acetylcholine. Harvey Lect., 32: 229–245, 1937.
71. DALE, H. H., FELDBERG, W., AND VOGT, M.: Release of acetylcholine at voluntary motor nerve endings. J. Physiol., 86: 353–380, 1936.
72. DALE, H. H., AND GADDUM, J. H.: Reactions of denervated voluntary muscle, and their bearing on the mode of action of parasympathetic and related nerves. J. Physiol., 70: 109–144, 1930.
73. DALE, H. H., AND GASSER, H. S.: The pharmacology of denervated mammalian muscle Part 1. The nature of the substances producing contracture. J. Pharmacol. & Exper. Therap., 29: 53–67, 1926.
74. DANIELLI, J. F.: *Cell physiology and pharmacology*. Elsevier Publishing Co., Inc., New York, 1950.
75. DAVIS, B. D.: The binding of chemotherapeutic agents to proteins and its effect on their distribution and activity. New York Acad. Med. Symp. of Section on Microbiology, 3: 44–64, 1949.
76. DEBEER, E. J., CASTILLO, J. C., PHILLIPS, A. P., FANELLI, R. V., WNUCK, A. L., AND NORTON, S.: Synthetic drugs influencing neuromuscular activity. Ann. New York Acad. Sc., 54: 362–372, 1951.
77. DEPIERRE, F.: Propriétés décurarisantes de l'iodure de m-hydroxy-phényl-triméthyl-ammonium. Compt. rend. Acad. Sc., 231: 670–672, 1950.
78. DEPIERRE, F., AND FUNKE, A.: Action de l'iodure de tétraméthylammonium et des iodures d'hydroxyphényl-triméthylammonium sur la transmission neuromusculaire. Compt. rend. Acad. Sc., 230: 2242–2243, 1950.
79. DUBOIS, K. P., ERWAY, W. F., AND BYERRUM, R. U.: A comparison of cholinesterase inhibitors *in vitro* and *in vivo*. Federation Proc., 6: 326, 1947.
80. DUBOIS, K. P., AND MANGUN, G. H.: Effect of hexaethyl tetraphosphate on cholinesterase *in vitro* and *in vivo*. Proc. Soc. Exper. Biol. & Med., 64: 137–139, 1947.
81. DUKE-ELDER, W. S., AND DUKE-ELDER, P. M.: The contraction of the extrinsic muscles of the eye by choline and nicotine. Proc. Roy. Soc., 107B: 332–343, 1931.
82. ECCLES, J. C., KATZ, B., AND KUFFLER, S. W.: Nature of the "endplate potential" in curarized muscle. J. Neurophysiol., 4: 362–387, 1941.
83. ECCLES, J. C., KATZ, B., AND KUFFLER, S. W.: Effect of eserine on neuromuscular transmission. J. Neurophysiol., 5: 211–230, 1942.
84. ECCLES, J. C., AND KUFFLER, S. W.: Initiation of muscle impulses at neuromuscular junctions. J. Neurophysiol., 4: 402–417, 1941.
85. ECCLES, J. C., AND MAC FARLANE, W. V.: Actions of anti-cholinesterases on endplate potential of frog muscle. J. Neurophysiol., 12: 59–80, 1949.
86. ECCLES, J. C., AND O'CONNOR, W. J.: Responses which nerve impulses evoke in mammalian striated muscles. J. Physiol., 97: 44–102, 1939.
87. ENGBAEK, L. (Quoted from Buchthal, F.): Muscle, Ann. Rev. Physiol., 9: 119–148, 1947.
88. ENGELHART, E., AND LOEWI, O.: Fermentative Azetylcholinspaltung im Blut und ihre Hemmung durch Physostigmin. Arch. f. exper. Path. u. Pharmakol., 150: 1–13, 1930.
89. FATT, P.: The electromotive action of acetylcholine at the motor end-plate. J. Physiol., 111: 408–422, 1950.
90. FATT, P., AND KATZ, B.: An analysis of the end-plate potential recorded with an intracellular electrode. J. Physiol., 115: 320–370, 1951.
91. FATT, P., AND KATZ, B.: Spontaneous subthreshold activity at motor nerve endings. J. Physiol., 117: 109–128, 1952.
92. FAULCONER, A., LAMBERT, E. H., AND ROME, H. P.: A new curare antagonist in electroshock therapy. Neurology, 2: 226–232, 1952.
93. FELDBERG, W.: Die Empfindlichkeit der Zungenmuskulatur und dei Zungengefässe des Hundes auf Linguali-reisung und auf Acetylcholin. Arch. f. d. ges. Physiol., 232: 75–87, 1933.
94. FELDBERG, W.: The physiology of neuromuscular transmission and neuromuscular block. Brit. M. J., 1: 957–976, 1951.
95. FELDBERG, W., AND MINZ, B.: Die Wirkung von Azetylcholin auf die Nebennieren. Arch. f. exper. Path. u. Pharmakol., 163: 66–96, 1932.
96. FENG, T. P.: Studies on the neuromuscular junction II. The universal antagonism between calcium and curarising agencies. Chinese J. Physiol., 10: 513–528, 1936.
97. FENG, T. P.: Studies on the neuromuscular junction VI. Potentiation by eserine of responses to single indirect stimulus in amphibian nerve-muscle preparations. Chinese J. Physiol., 12: 51–58, 1937.
98. FENG, T. P.: Studies on the neuromuscular junction XIII. The localised electrical negativity of muscle around neuromuscular junction due to high-frequency nerve stimulation. Chinese J. Physiol., 14: 209–224, 1939.
99. FENG, T. P.: Studies on the neuromuscular junction XVIII. The local potentials around neuromuscular junctions induced by single and multiple volleys. Chinese J. Physiol., 15: 367–404, 1940.
100. FENG, T. P.: The local activity around the skeletal neuromuscular junctions produced by nerve impulses. Biol. Symp., 3: 121–190, 1941.
101. FENG, T. P., AND LI, T. H.: Studies on the neuromuscular junction XVI. Acetylcholine sensitivity of a muscle and its aptitude to give contracture of the eserine type. Chinese J. Physiol., 15: 197–212, 1940.
102. FENN, W. O., AND HAEGE, L. F.: The penetration of magnesium into frog muscle. J. Cell. & Comp. Physiol., 19: 37–46, 1942.
103. FINERTY, J. C.: Effects of di-isopropyl fluorophosphate (DFP) on acetylcholine stimulation of the frog rectus abdominis muscle. Am. J. Physiol., 151: 107–109, 1947.
104. FRANK, E., NOTHMANN, M., AND HIRSH-KAUFMAN, H.: Über die "tonische" Kontraktion des quergestreiften Saugetrymuskels nach Ausschaltung des motorischen Nerven. Arch. f. d. ges. Physiol., 197: 270–287, 1922.

105. Fraser, F. R., McGeorge, M., and Murphy, G. E.: The action of choline esters in myasthenia gravis. Clin. Sc., 3: 77–89, 1937.
106. Führer, H.: Curarestudien. 1. Die periphere Wirkung des Guanidins. Arch. f. exper. Path. u. Pharmakol., 58: 1–49, 1908.
106A. Funke, A. and Depierre, F.: Propriétés anticurarisantes de quelques sels d'ammonium quaternaire dérivés d'aminophénols. Compt. rend. Acad. Sc., 230: 245–247, 1950.
106B. Funke, A., Depierre, F., and Krucker, W.: Exaltation de l'activité anticholinestérasique des sels d'ammoniumquaternaires des phénoxyalcanes par l'introduction de groupements uréthanes. Compt. rend. Acad. Sc., 234: 762–764, 1952.
107. Gasser, H. S.: Contractures of skeletal muscle. Physiol. Rev., 10: 35–109, 1930.
108. Gasser, H. S., and Dale, H. H.: The pharmacology of denervated mammalian muscle II. Some phenomena of antagonism and the formation of lactic acid in chemical contracture. J. Pharmacol. & Exper. Therap., 28: 287–315, 1926.
109. Ginzel, K. H., Klupp, J., and Werner, G.: Zur Pharmakologie von α-ω-bis-quarternären Ammoniumverbindungen II. Vergleichende Untersuchungen über einigeraliphatischer Dicarbonsaureester. Arch. internat. de pharmacodyn. et de thérap., 87: 79–98, 1951.
110. Ginzel, K. H., Klupp, H., and Werner, G.: Zur Pharmakologie von α-ω-bis-quarternären Ammoniumverbindungen III. Die fermentative Spaltung einiger aliphatischer Dicarbonsäureester und die Stugerung ihrer Wirksamkeit durch Eserin. Arch. Internat. de pharmacodyn. et de thérap. 87: 351–365, 1951.
111. Ginzel, K. H., Klupp, H., and Werner, G.: Zur Pharmakologie von α-ω-bis-quarternären Ammoniumverbindungen I. Mitterlung: neuromusculare und ganglionare Wirkungen des Adipinsaure-bis-cholinesters. Arch. internat. de pharamcodyn. et de thérap. 86: 385–406, 1951.
112. Ginzel, K. H., Klupp, H., and Werner, G.: Die Wirkung einiger aliphatischer α-ω-bis-quarternären Ammoniumverbindungen auf die Skelettmuskulatur. Arch. f. exper. Path. u. Pharmakol., 213: 453–456, 1951.
113. Goni, A. R.: *Myasthenia Gravis*. Williams and Wilkins, Baltimore, 1946.
114. Gopfert, H., and Schaefer, H.: Über den direkt und indirekt erregten Aktionstrom und die Funktion der motorischen Endplatte. Arch. f. d. ges. Physiol., 239: 597–619, 1938.
115. Grob, D., and Harvey, A. M.: Observations on the effects of tetraethyl pyrophosphate (TEPP) in man, and on its use in the treatment of myasthenia gravis. Bull. Johns Hopkins Hosp., 84: 532–567, 1949.
116. Hammett, L. P.: *Physical Organic Chemistry*. McGraw-Hill Book Co., Inc. New York, 1940.
117. Harvey, A. M., and Lilienthal, J.: Observations on the nature of myasthenia gravis. The intra-arterial injection of acetylcholine, prostigmine, and adrenaline. Bull. Johns Hopkins Hosp., 69: 566–577, 1941.
118. Harvey, A. M., Lilienthal, J., Jr., Grob, D., Jones, B. K., and Talbot, S.: The administration of di-isopropyl fluorophosphate to man. IV. The effects on neuromuscular function in normal subjects and in myasthenia gravis. Bull. Johns Hopkins Hosp., 81: 267–292, 1947.
119. Harvey, A. M., Lilienthal, J , and Talbot, S.: On the effects of the intra-arterial injection of acetylcholine and prostigmine in normal man. Bull. Johns Hopkins Hosp., 69: 529–546, 1941.
120. Harvey, A. M., Lilienthal, J., Jr., and Talbot, S.: Observations on the nature of myasthenia gravis. The phenomena of facilitation and depression of neuromuscular transmission. Bull. Johns Hopkins Hosp., 69: 547–565, 1941.
121. Harvey, A. M., Lilienthal, J., Jr., and Talbot, S.: quoted from Acheson *et al.* (4).
122. Harvey, A. M., and Masland, R.: The electromyogram in myasthenia gravis. Bull. Johns Hopkins Hosp., 69: 1–13, 1941.
123. Hawkins, R. D., and Mendel, B.: Selective inhibition of pseudo-cholinesterase by di-isopropyl fluorophosphate. Brit. J. Pharmacol., 2: 173–180, 1947.
124. Henze, M.: Der chemische Demarcationsstrom in toxikologischer Beziehung. Arch. f. d. ges. Physiol., 92: 451–472, 1902.
125. Hess, W. R., and v. Neergard, K.: Die Beziehungen der Acetylcholinverkurzung des Skelettmuskels zur Einselzuckung und zum Tetanus. Arch. f. d. ges. Physiol., 205: 506–517, 1924.
125A. Hey, P.: On relationships between structure and nicotine-like stimulant activity in choline esters and ethers. Brit. J. Pharmacol. 7: 117–129, 1952.
126. Hobbiger, F.: The action of carbamic esters and tetraethylpyrophosphate in normal and curarized frog rectus muscle. Brit. J. Pharmacol., 5: 37–48, 1950.
126A. Hobbiger, F.: The mechanism of anticurare action of certain neostigmine analogues. Brit. J. Pharmacol. 7: 223–236, 1952.
127. Hober, R., and Waldenberg, H.: Über den Einfluss von Salsen starker organischer Basen auf den Ruhestrom und die Erregbarkeit von Froschmuskeln. Arch. f. d. ges. Physiol., 126: 331–350, 1909.
128. Hodes, R., and Steiman, S. E.: The effects of acetylcholine and eserine on frog muscle. Am. J. Physiol., 127: 470–479, 1939.
129. Hodgkin, A. L., and Katz, B.: The effect of sodium ions on the electrical activity of the giant axon of the squid. J. Physiol., 108: 37–77, 1949.
130. Holland, W. C., and Greig, M.: Studies on the permeability of erythrocytes. III. The effect of physostigmine and acetylcholine on the permeability of dog, cat and rabbit erythrocytes to sodium and potassium. Am. J. Physiol., 162: 610–615, 1950.
131. Holmes, P. E. B., Jenden, D. J., and Taylor, D. B.: Relationship between structure and action in drugs with curariform actions. Nature, 159: 86–88, 1947.
132. Holmstedt, B.: Synthesis and pharmacology of dimethylamido-ethoxy-phosphoryl cyanide (Tabun) together with a description of some allied anticholinesterase compounds containing the N-P bond. Acta physiol. Scandinav., 25 supp. 90: 7–120, 1951.

133. HOLTON, P., AND ING, H. R.: The specificity of the trimethylammonium group in acetylcholine. Brit. J. Pharmacol., 4: 190–196, 1949.
134. HOPPE, J. O.: A new series of synthetic curare-like compounds. Ann. New York Acad. Sc., 54: 395–406, 1951.
135. HUNT, C. C.: The effect of di-isopropyl fluorophosphate on neuromuscular transmission. J. Pharmacol. & Exper. Therap., 91: 77–83, 1947.
136. HUNT, C. C., AND KUFFLER, S. W.: Pharmacology of the neuromuscular junction. Pharmacol. Rev., 2: 96–120, 1950.
137. HUNT, R., AND TAVEAU, R. DEM.: The effects of a number of derivatives of choline and analogous compounds on the blood pressure. Hyg. Lab. Bull., No. 73, 1911.
138. HUTTER, O. F.: Effect of choline on neuromuscular transmission in the cat. J. Physiol., 117: 241–250, 1952.
139. HUTTER, O. F., AND PASCOE, J. E.: Decurarisation by decamethonium. Brit. J. Pharmacol., 6: 691–695, 1951.
140. ING, H. R.: The curariform action of onium salts. Physiol. Rev., 16: 527–544, 1936.
141. ING, H. R.: The structure-action relationships of the choline group. Science, 109: 264–266, 1949.
142. ING, H. R., AND WRIGHT, W. M.: The curariform action of quaternary ammonium salts. Proc. Roy. Soc., 109B: 337–353, 1931.
143. ING, H. R., AND WRIGHT, W. M.: Further studies on the pharmacological properties of onium salts. Proc. Roy. Soc., 114B: 48–63, 1933.
144. JACOBSOHN, D., AND KAHLSON, G.: Die Anticurarewirkung einiger Stoffe mit lahmender Wirkung auf die Acetylcholinesterase. Skandinav. Arch. f. Physiol., 79: 27–31, 1938.
145. JARCHO, L. W., ETZAGUIRRE, C., TALBOT, S. A., AND LILIENTHAL, J. L., JR.: Neuromuscular excitation: Response of normal and denervated mammalian muscle to bis-trimethylammonium decane (C10) and to d-tubocurarine. Am. J. Physiol., 162: 475–488, 1950.
146. JARCHO, L. W., BERMAN, B., ETZAGUIRRE, C., AND LILIENTHAL, J. L., JR.: Curarization of denervated muscle. Ann. New York Acad. Sc., 54: 337-346, 1951.
147. JONES, H. W., JR., MEYER, B. J., AND KAREL, L.: The relationship of cholinesterase inhibiting activity to the toxicity of some organic phosphorus compounds. J. Pharmacol. & Exper. Therap., 94: 215–220, 1948.
148. KENSLER, C. J.: The antagonism of curare by congo red and related compounds. J. Pharmacol. & Exper. Therap., 95: 28–44, 1949.
149. KENSLER, C. J.: Anti-curare action of TEA in the cat. Brit. J. Pharmacol., 5: 204–209, 1950.
150. KENSLER, C. J., AND ELSNER, R. W.: Tetraethylammonium and cholinesterase activity. J. Pharmacol. & Exper. Therap., 102: 196–199, 1951.
151. KIRSCHNER, L. B., AND STONE, W. E.: Action of inhibitors at the myoneural junction. J. Gen. Physiol., 34: 821–834, 1951.
152. KLOTZ, I. M., AND CURME, H. G.: The thermodynamics of metallo-protein combinations. Copper with bovine serum albumin. J. Am. Chem. Soc., 70: 939–943, 1948.
153. KOELLE, G. B., AND FRIEDENWALD, J. S.: A histochemical method for localizing cholinesterase activity. Proc. Soc. Exper. Biol. & Med., 70: 617–622, 1949.
154. KOELLE, G. B., AND GILMAN, A.: Anticholinesterase drugs. Pharmacol. Rev., 1: 166–216, 1949.
155. KOPPANYI, T., AND VIVINO, A. E.: Prevention and treatment of d-tubocurarine poisoning. Science, 100: 474–475, 1944.
156. KRUTA, V., AND PAULIAN, R.: Sur les variations de potentiel du muscle strié au cours de sa contracture acetylcholinique. Compt. rend. Soc. biol., 119: 1104–1105, 1935.
157. KUFFLER, S. W.: Electric potential changes at an isolated nerve-muscle junction. J. Neurophysiol., 5: 18–26, 1942.
158. KUFFLER, S. W.: Specific excitability of the endplate region in normal and denervated muscle. J. Neurophysiol. 6: 99–110, 1943.
159. KUFFLER, S. W.: *Modern Trends in Physiology and Biochemistry*. 277–289, Academic Press, New York, 1952.
160. KUFFLER, S. W., AND GERARD, R. W.: The small-nerve motor system to skeletal muscle. J. Neurophysiol., 10: 383–394, 1947.
161. KUFFLER, S. W., LAPORTE, Y., AND RANSMEIER, R. E.: The function of the frog's small-nerve motor system. J. Neurophysiol., 10: 395–408, 1947.
162. KULZ, F.: Quantitative Untersuchungen über die Wirksamkeit homologer quartärer Ammoniumbasen. Arch. f. d. ges. Physiol., 195: 623–625, 1922.
163. LANARI, A.: Contraction-provoking action of acetylcholine on the striated muscles in cases of myotonia. Rev. Soc. Argentina Biol., 12: 207–213, 1936.
164. LANARI, A.: Myasthenia gravis and chemical neuro-humoral transmission. Compt. rend. Soc. biol., 126: 827–828, 1937.
165. LANDS, A. M.: An investigation of the molecular configurations favorable for stimulation or blockade of the acetylcholine-sensitive receptors of visceral organs. J. Pharmacol. & Exper. Therap., 102: 219–236, 1951.
166. LANDS, A. M., NASH, V. L., AND HOOPER, K. Z.: The relationship between chemical structure and spasmolytic action of some new anticholinergic esters. J. Pharmacol. & Exper. Therap., 86: 129–137, 1946.
167. LANGLEY, J. N.: On the reaction of cells and of nerve-endings to certain poisons, chiefly as regards the reaction of striated muscle to nicotine and to curare. J. Physiol., 33: 374–413, 1905.
168. LANGLEY, J. N.: On the contraction of muscle, chiefly in relation to the presence of "receptive" substances. part I. J. Physiol., 36: 347–384, 1907.
169. LANGLEY, J. N., AND KATO, T.: The physiological action of physostigmine and its action on denervated skeletal muscle. J. Physiol., 49: 410–431, 1914–1915.
170. LEE, H. M., VAN ARENDONK, A. M., AND CHEN, K. K.: A study of twenty-three quaternary ammonium iodides. J. Pharmacol. & Exper. Therap., 56: 466–472, 1936.

171. LEHMANN, G.: The "curare-like" action of inhibitors of cholinesterase. Jubilee volume Emil Barell, 314–376, Basle, 1946.
172. LINEWEAVER, H., AND BURK, D.: The determination of enzyme dissociation constants. J. Am. Chem. Soc. 56: 658–666, 1934.
173. LOEWI, O.: Humoral transmission of nervous impulses. Harvey Lect. 28: 218–233, 1933.
174. LUCO, J. V., AND ALTAMIRANO, M.: A comparison of atropine and curare as antagonists of acetylcholine. Am. J. Physiol., 139: 520–524, 1943.
175. MACFARLANE, D. W., PELIKAN, E. W., AND UNNA, K. R.: Evaluation of curarizing drugs in man. V. Antagonism to curarizing effects of d-tubocurarine by neostigmine, m-hydroxyphenyltrimethylammonium and m-hydroxyphenylethyldimethylammonium. J. Pharmacol. & Exper. Therap., 100: 382–392, 1950.
176. MANGUN, G. H.: Toxicity and mechanism of action of TEPP. Federation Proc. 6: 353, 1947.
177. MARBURY, B. E., ARTUSIO, J. F., JR., WESCOE, W. C., AND RIKER, W. F., JR.: The effects of a synthetic curare-like compound, the tri-iodo salt of tris(triethylaminoethoxy) 1,2,3 benzene (Flaxedil), on the anesthetized surgical patient. J. Pharmacol. & Exper. Therap., 103: 280–287, 1951.
178. MARNAY, A., AND NACHMANSOHN, D.: Choline esterase in voluntary muscle. J. Physiol., 92: 37–47, 1938.
179. MARSH, D. F.: The distribution, metabolism, and excretion of d-tubocurarine chloride and related compounds in man and other animals. J. Pharmacol. & Exper. Therap., 105: 299–316, 1952.
180. MARSHALL, C. R.: Studies on the pharmacological action of tetra-alkylammonium compounds. II. The action of tetra-ethylammonium chloride. Trans. Roy. Soc. Edin., 50: 379–396, 1916.
181. MARSHALL, C. R.: Studies on the pharmacological action of tetra-alkylammonium compounds. III. The action of methyl ethylammonium chlorides. Trans. Roy. Soc. Edin., 50: 481–516, 1916.
182. MAZUR, A. AND BODANSKY, O.: Mechanism of *in vitro* and *in vivo* inhibition of cholinesterase activity of DFP. J. Biol. Chem., 163: 261–276, 1946.
183. MCEACHERN. D.: The thymus in relation to myasthenia gravis. Medicine, 22: 1–25, 1943.
184. MCINTYRE, A: *Curare, its History, Nature and Clinical Use*. University of Chicago Press, Chicago, 1947.
185. MCINTYRE, A. R., AND KING, R. E.: Contraction of denervated muscle produced by d-tubocurarine. Science, 97: 516, 1943.
186. MCINTYRE, A. R., KING, R. E., AND DUNN, A. L.: Electrical activity of denervated mammalian skeletal muscle as influenced by d-tubocurarine. J. Neurophysiol., 8: 297–307, 1945.
187. MEYER, V., AND SUDBOROUGH, J. J.: Das gesetz der Esterbildung aromatischer Säuren. Ber. d. Deut. Chem. Ges., 2: 1580–1592, 1894.
188. MINOT, A. S., DODD, K., AND RIVEN, S. S.: Use of guanidine hydrochloride in treatment of myasthenia gravis J. Am. M. A., 113: 553–559, 1939.
189. MIQUEL, O.: The action of physostigmine, di-isopropyl fluorophosphate and other parasympathomimetic drugs on the rectus muscle of the frog. J. Pharmacol. & Exper. Therap., 88: 67–71, 1946.
190. NACHMANSOHN, D.: Studies on permeability in relation to nerve function. I. Axonal conduction and synaptic transmission. Biochim. & Biophys. Acta, 4: 78–95, 1950.
191. NACHMANSOHN, D., ROTHENBERG, M. A., AND FELD, E. A.: Studies on cholinesterase. V. Kinetics of the enzyme inhibition. J. Biol. Chem., 174: 247–256, 1948.
192. NACHMANSOHN, D., AND WILSON, I. B.: Advances Enzymol. 12: 259–339, 1951.
193. NASTUK, W. L.: Membrane potential changes at a single muscle endplate produced by acetylcholine. Federation Proc., 10: 96, 1951.
194. NICHOLLS, J., AND KATZ, B. Personal communication, 1952.
195. NITSCHKE, A.: Quoted from Frank, E., Nothmann, M., and Guttmann, E., Arch. f. d. ges. Physiol., 201: 570–578, 1923.
196. PAL, J.: Physostigmin ein Gegengift des Curare. Centralbl. f. Physiol., 14: 255–258, 1900.
197. PATON, W. D. M.: The pharmacology of decamethonium. Ann. New York Acad. Sc., 54: 347–361, 1951.
198. PATON, W. D. M., AND ZAIMIS, E. J.: The pharmacological actions of polymethylene bistrimethylammonium salts. Brit. J. Pharmacol., 4: 381–400, 1949.
199. PAULING, L.: *Nature of the Chemical Bond*. Cornell University Press, New York, 1948.
200. PFEIFFER, C.: Nature and spatial relationship of the prosthetic chemical groups required for maximal muscarinic action. Science, 107: 94–96, 1948.
201. PRITCHARD, E. A. B.: The occurrence of Wedensky inhibition in myasthenia gravis. J. Physiol., 78: 3P–4P, 1933.
202. QUILLIAM, J. P., AND STRONG, F. G.: Some observations upon the pharmacological activity of di-isopropyl fluorophosphate. Brit. J. Pharmacol., 4: 168–176, 1949.
203. RANDALL, L. O.: Anticurare action of phenolic quaternary ammonium salts. J. Pharmacol. & Exper. Therap., 100: 83–93, 1950.
204. RANDALL, L. O.: Synthetic curare-like agents and their antagonists. Ann. New York Acad. Sc., 54: 460–479, 1951.
205. RANDALL, L. O., AND LEHMANN, G.: Pharmacological properties of some neostigmine analogs. J. Pharmacol. & Exper. Therap., 99: 16–32, 1950.
206. RAVENTOS, J.: The effects of arterial injections of drugs on the frog's gastrocnemius. J. Physiol., 90: 8P–9P, 1937.
207. RAVENTOS, J.: Pharmacological actions of quaternary ammonium salts. Quart. J. Exper. Physiol., 26: 361–374, 1937.
208. RENSHAW, R. R.: On the basis for the physiological activity of certain onium compounds. I. Introductory paper. Science, 62: 384–389, 1925.
209. RIESSER, O., AND NEUSCHLOSS, S. M.: Physiologische und Kolloidchemische Untersuchungen über den Mechanismus der durch Gifte bewirkten Kontraktur quergestreifter Muskeln. I. Über die durch Azetylcholin bewirkte Erregungskontraktur des Froschmuskels und ihre antagonistische Beeinflussung durch Atropin, Novokain, und Kurare. Arch. f. exper. Path. u. Pharmakol., 91: 342–365, 1921.

210. Riesser, O., and Steinhausen, W.: Über das elektrische Verhalten des Muskels bei Einwukung von Acetylcholin. Arch. f. d. ges. Physiol., 197: 288-299, 1922.
211. Riker, W. F., Jr., and Wescoe, W. C.: The direct action of prostigmine on skeletal muscle; its relationship to the choline esters. J. Pharmacol. & Exper. Therap., 88: 58-66, 1946.
212. Riker, W. F., and Wescoe, W. C.: The relationship between cholinesterase inhibition and function in a neuroeffector system. J. Pharmacol. & Exper. Therap., 95: 515-527, 1949.
213. Riker, W. F., and Wescoe, W. C.: Pharmacologic significance of m-hydroxyphenyltrimethyl-ammonium ion. Federation Proc., 9: 311, 1950.
214. Riker, W. F., Jr., and Wescoe, W. C.: Studies on the inter-relationship of certain cholinergic compounds V. The significance of the actions of the 3-hydroxyphenyltrimethyl ammonium ion on neuromuscular function. J. Pharmacol. & Exper. Therap., 100: 454-464, 1950.
215. Riker, W. F., and Wescoe, W. C.: The pharmacology of Flaxedil with obeervations on certain analogs. Ann. New York Acad. Sc., 54: 373-394, 1951.
216. Riker, W. F., Jr., Wescoe, W. C., and Brothers, M. J.: Studies on the inter-relationship of certain cholinergic compounds II. The effects of 3-acetoxy Phenyltrimethylammonium methylsulfate on neuromuscular function. J. Pharmacol. & Exper. Therap., 97: 208-221, 1949.
217. Roberts, J. D., Clement, R. A., and Drysdale, J. J.: The electrical effect of trimethylammonium group. J. Am. Chem. Soc., 73: 2181-2183, 1951.
218. Roberts, J., and Riker, W. F., Jr.: Unpublished obeervations.
219. Roepke, M. H.: A study of cholinesterase. J. Pharmacol. & Exper. Therap., 59: 264-276, 1937.
220. Roepke, M. H., and Welch, A. M. A comparative study of choline and certain of its analogues. II. Cationic exchange as a means of reaction of choline, acetylcholine and their analogues with cells. J. Pharmacol. & Exper. Therap., 56: 319-340, 1936.
221. Rosenblueth, A., and Luco, J. V.: A study of denervated mammalian skeletal muscle. Am. J. Physiol., 120: 781-797, 1937.
222. Rosenblueth, A., and Morison, R. S.: Curarization, fatigue and Wedensky inhibition. Am. J. Physiol., 119: 236-256, 1937.
223. Rosenblueth, A., Lindsley, D. B., and Morison, R. S.: A study of some decurarising substances. Am. J. Physiol., 115: 53-68, 1936.
224. Rothberger, J. C.: Über die gegenseitigen Beziehung zwischen Curare und Physostigmin. Arch. f. d. ges. Physiol., 87: 117-169, 1901.
225. Rothberger, J. C.: Weitere Mittheilungen über Antagonisten des Curarins. Arch. f. d. ges. Physiol., 92: 398-450, 1902.
226. Sanz, M.: Hemmung der Cholinesterase aus Erythrocyten und Serum durch Eserin, Prostigmin und homologe Verbindungen. Helvet. Physiol. & Pharmacol. Acta, 3: C14, 1945.
227. Scatchard, G.: Molecular interactions in protein solutions. Am. Scientist, 40: 61-83, 1952.
228. Schäffer, H., and Licht, H.: Beiträge zur Frage des Muskeltonus. II. Mitteilung: Die Azetylcholinkontraktur des Kaltblütermuskels und ihre elektrischen Erscheinungen. Arch. f. exper. Path. u. Pharmakol. 115: 196-204, 1926.
229. Schueler, F. W.: Polarizing forces of the muscarinic moiety. Science, 115: 548-549, 1952.
230. Simonart, A.: Étude physiologique de quelques dérivés d'homocholine. L'éther méthylique de la γ phénylhomocholine. Arch. internat. de pharmacodyn. et de thérap., 34: 15-32, 1928.
231. Simonart, A.: Étude physiologique de quelques dérivés d'homocholine. II. L'éther méthylé d'homocholine. III. Rapports avec les cholines. Arch. internat. de pharmacodyn. et de thérap., 34: 375-390, 1928.
232. Simonart, A.: On the action of certain derivatives of choline. J. Pharmacol. & Exper. Therap., 46: 157-193, 1932.
233. Simonart, A.: Étude de l'action de l'acétylcholine sur le muscle strié de mammifère. Arch. internat. de pharmacol. et de thérap., 51: 381-397, 1935.
234. Simonart, A.: Sur les isomeres de l'acétyl-β-methylcholine. Arch. internat. de pharmacol. et de thérap., 60: 209-212, 1938.
235. Simonart, A., and Simonart, E. F.: L'acétylcholine et le muscle strié normal de mammifère. Arch. internat. de pharmacol. et de thérap., 49: 302-328, 1934.
236. Simonart, A., and Simonart, E. F.: La Carbaminoyl-β-methyl choline. Arch. internat. de pharmacol. et de thérap., 51: 76-82, 1935.
237. Smith, C. M., Pelikan, E. W., and Unna, K. R.: Comparison between some phenolic quaternary ammonium compounds and cholinesterase inhibitors in their effects upon the rectus abdominus muscle of the frog. J. Pharmacol. & Exper. Therap., 103: 363, 1951.
237A. Smith, C. M., Cohen, H. L., Pelikan, E. W., and Unna, K. R.: Mode of action of antagonists to curare. J. Pharmacol. & Exper. Therap., 105: 391-399, 1952.
238. Sommerkamp, H.: Das Substrat der Dauerverkürzung am Froschmuskel. Arch. f. exper. Path. u. Pharmakol., 128: 99-115, 1928.
239. Stedman, E.: XCIV. Studies on the relationship between chemical constitution and physiological action. Part 1. Position isomerism in relation to the miotic activity of some synthetic urethanes. Biochem. J. 20: 719-734, 1926.
240. Stedman, E.: Studies on the relationship between chemical constitution and physiological action. Part 2. The miotic activity of urethanes derived from the isomeric hydroxy-benzyldimethylamines. Biochem. J., 23: 17-24, 1929.
241. Stedman, E., and Stedman, E.: The methylurethanes of the isomeric a-hydroxyphenylethyl dimethylamines and their miotic activity. J. Chem. Soc., 81: 609-617, 1929.

242. STRACK, E., AND FÖRSTERLING, K.: Über die biologische Wirkung der Ester des Crotonsäuerebetains. Zeit. f. Physiol. Chem., 257: 1-11, 1939.
243. STRACK, E., AND FÖRSTERLING, K.: Ein Verfahren zur biologischen Bestimmung von Betainen. Zeit. f. Physiol. Chem., 277: 74-86, 1943.
244. SULLIVAN, W. J., AND KENSLER, C. J.: Action of tetraethylammonium bromide and acetylcholine in the kitten phrenic nerve diaphragm preparation. Federation Proc. 9: 319, 1950.
245. TORDA, C., AND WOLFF, H. G.: Depression of acetylcholine synthesis by serum from working muscle. Healthy subjects and myasthenia gravis patients. Proc. Soc. Exper. Biol. & Med., 59: 13-16, 1945.
246. TAYLOR, D. B.: Some basic aspects of the pharmacology of synthetic curariform drugs. Pharmacol. Rev. 3: 412-444, 1951.
246A. TAYLOR, I. M., WELLER, J. M., AND HASTINGS, A. B.: Effect of cholinesterase and choline acetylase inhibitors on the potassium concentration gradient and potassium exchange of human erythrocytes. Am. J. Physiol., 168: 658-665, 1952.
247. TEORELL, T.: Permeability. Ann. Rev. Physiol., 11: 545-564, 1949.
248. TETHER, J. E.: Ann. New York Acad. Sc., 54: 475, 1951.
249. TRAUBE, L.: Versuche über den Einfluss des Nicotins auf die Hertsiätigkeit. Ges. Beitr. zur Path. u. Physiol. (Berlin: A. Hirschwald), 1: 302-309, 1871.
250. UNNA, K., KNIAZUK, M., AND GRESLIN, J. G.: Pharmacologic action of erythrina alkaloids 1. β Erythroidine and substances derived from it. J. Pharmacol. & Exper. Therap., 80: 39-52, 1944.
251. VAN MAANEN, E. F.: The antagonism between acetylcholine and the curare alkaloids, d-tubocurare, c-curarine-I, c-toxiferine-II and β-erythroidine on the rectus abdominis of the frog. J. Pharmacol. & Exper. Therap., 99: 255-264, 1950.
251A. VAN MAANEN, E. F.: Effectiveness of anticurarizing agents at different frequencies of stimulation. Federation Proc. 2: 398, 1952.
252. WACHHOLDER, K., AND V. LEDEBUR, J. R.: Acetylcholin der Muskeln normales erwashener Saügetiere; (Rote und Weisse Muskeln, Verhalten im Winterschlaf). Arch. f. d. ges. Physiol., 229: 657-671, 1932.
253. WALKER, M. B.: Treatment of myasthenia gravis with physostigmine. Lancet, 1: 1200-1201, 1934.
254. WALKER, M. B.: Myasthenia gravis; case in which fatigue of forearm muscles could induce paralysis of extraocular muscles. Proc. Roy. Soc. Med., 31: 722, 1938.
255. WALKER, M. B.: Case showing the effect of prostigmin in myasthenia gravis. Proc. Roy. Soc. Med., 28: 759-761, 1935.
256. WELSH, J. H.: Structure-activity relationship of acetylcholine and receptor substance. Am. Scientist. 38: 239-246, 1950.
257. WELSH, J. H., AND TAUB, R.: Structure-activity relationships of acetylcholine and quaternary ammonium ions. J. Pharmacol. & Exper. Therap., 99: 334-342, 1950.
258. WELSH, J. H. AND TAUB, R.: The significance of the carbonyl group and ether oxygen in the reaction of acetylcholine with receptor substance. J. Pharmacol. & Exper. Therap., 103: 62-73, 1951.
259. WESCOE, W. C., AND RIKER, W. F., JR.: The pharmacology of anti-curare agents. Ann. New York Acad. Sc., 54: 438-459, 1951.
260. WESCOE, W. C., AND RIKER, W. F., JR.: Unpublished observations on the action of physostigmine.
261. WESCOE, W. C., RIKER, W. F., JR., AND BEACH, V. L.: Studies on the inter-relationships of certain cholinergic compounds. III. The reactions between 3-acetoxyphenyltrimethylammonium methylsulfate, 3-hydroxyphenyltrimethylammonium bromide and cholinesterases. J. Pharmacol. & Exper. Therap., 99: 265-276, 1950.
262. WESCOE, W. C., RIKER, W. F., JR., AND BROTHERS, M. J.: Studies on the interrelationship of certain cholinergic compounds. 1. The pharmacology of 3-acetoxy phenyltrimethylammonium methylsulfate. J. Pharmacol. & Exper. Therap., 97: 190-207, 1949.
263. WHITE, A. C., AND STEDMAN, E.: On the physostigmine-like action of certain synthetic urethanes. J. Pharmacol. & Exper. Therap., 41: 259-288, 1931.
264. WHITTAKER, V. P.: Specificity, mode of action and distribution of cholinesterases. Physiol. Rev., 31: 312-343, 1951.
265. WILSON, I. B., AND BERGMANN, F.: Studies on cholinesterase. VII. The active surface of acetylcholine esterase derived from effects of pH on inhibitors. J. Biol. Chem., 185: 479-489, 1950.
266. WILSON, I. B., AND BERGMANN, F.: Acetylcholinesterase. VIII. Dissociation constants of the active groups. J. Biol. Chem., 186: 683-692, 1950.
267. WILSON, A., AND STONER, H. B.: Myasthenia gravis: a consideration of its causation in a study of fourteen cases. Quart. J. Med., 13(new series): 1-18, 1944.
268. WILSON, A., AND STONER, H. B.: The effect of the injection of acetylcholine into the brachial artery of normal subjects and patients with myasthenia gravis. Quart. J. Med., 16(new series): 237-243, 1947.
269. WILSON, A. T., AND WRIGHT, S.: Anti-curare action of potassium and other substances. Quart. J. Exper. Physiol., 26: 127-139, 1936.
270. WINTERBERG, H.: Ueber die Wirkung des Nicotins auf die Athmung nebst einem Anhange über die Wirkung des Nicotins auf den Kreislauf. Arch. f. exper. Path. u. Pharmakol., 43: 400-434, 1900.
271. ZAIMIS, E. J.: The action of decamethonium on normal and denervated mammalian muscle. J. Physiol., 112: 176-190, 1951.