IS THE ACETYLCHOLINE RECEPTOR AT THE NEUROMUSCULAR JUNCTION A CHOLINESTERASE?

by

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ABBREVIATIONS

ACh	acetylcholine
AChE	acetylcholinesterase
ChE	cholinesterase
DFP	diisopropyl fluorophosphate
I_{50}	molar concentration producing 50% inhibition of the enzyme in vitro
TEPP	tetraethylpyrophosphate
VOM	volt-ohmeter

GENERAL INTRODUCTION

The identification of acetylcholine as the chemical transmitter at the myoneural junction (of the cat) occurred approximately thirty years ago (1), and as early as 1907 Langley (2) had postulated the presence of a "neuro-receptive" area on skeletal muscle. In the literature of today we now encounter the term "receptor", which was coined at the beginning of this century by Ehrlich and which is defined by Furchgott (3) as "the postulated specific molecular site or structure in (or on) an effector cell with which molecules of a specific agonist must react in order to elicit the characteristic response of the cell to the agonist". At the myoneural junction the physiological agonist is the transmitter, acetylcholine, and the characteristic response is depolarization of the end-plate region. Facts concerning this cholinergic receptor are few -- neither its chemical identity nor its precise location have been established, and therefore the questions of its mechanism of action are far from being resolved.

What is the chemical nature of the receptor?

Several attempts have been made to identify the receptor by isolating the curare-binding substance from the electric tissue of eel, a structure held to be

homologous to the myoneural junction. On the basis of such studies Chagas (4) presented evidence that acidic mucopolysaccharides were sites of curare adsorption and postulated that the receptor itself might be a mucopolysaccharide. Ehrenpreis (5) used a fractionation procedure on the eel tissue extract to eliminate the chondroitin sulfuric acid, which he suspected was a curare "acceptor" but not the receptor due to the salt sensitivity of Chagas' receptor-curare complex. In 1960 Ehrenpreis concluded that the protein component which he extracted from electric tissue was the cellular acetylcholine receptor on the basis of a "striking parallelism between the binding strength of the various compounds tested in solution and their effectiveness in affecting the electrical activity of the isolated single electroplax prepared from electric tissue". However, in 1965 Beychok (6) demonstrated that the protein fraction extracted by the method of Ehrenpreis was readily resolved by chromatography into numerous sub-fractions, no one of which represented a large portion of the total protein in the fraction. When Beychok compared the curare-binding ability of these sub-fractions with other proteins obtained from the tissue, he found no differences among then and hence concluded that the acetylcholine receptor had not been isolated. The hazard of using

curare to tag receptor sites is related to the number of non-specific "acceptors" present in the tissue; possibly these sites of loss are far more numerous than the specific receptor sites. Therefore, curare-binding power has not been a successful criterion for the identification of the receptor.

Using the new technique of polarization microscopy, Csillik (7) presented evidence that the postsynaptic membrane of the myoneural junction consists of a regularly arranged, lead-reactive lipoprotein material. This lead-reactive lipoprotein substance he has identified as the acetylcholine receptor because of the structural rearrangement in the lipoprotein network which results from supramaximal stimulation of the nerve or treatment of the muscle with acetylcholine-eserine. His results support the theoretical suggestion of Watkins (8) that the physiological action of acetylcholine results from the dissocation of the membrane lipid-protein complexes. Liu and Nastuk (9) have collected data indicating that the post-junctional membrane receptors are phosphate-containing macromolecules; this conclusion is partially based on blockade of the neuromuscular junction to carbachol depolarization by uranyl ions ($UO2^{+2}$), which have a high affinity for phospholipids. Indeed it seems possible that changes in the lipoprotein

structure of the membrane could be associated with depolarization. Still these changes might result from conformational changes of a protein receptor activated by its agonist.

It would be presumptuous to draw a conclusion regarding the chemical nature of the receptor on the basis of any of the above-mentioned evidence.

Where is the receptor?

Due to the unique anatomical organization of the sub-neural apparatus (10) and the identification of acetylcholine-rich vesicles in presynaptic nerve endings (11, 12) coupled with denervated muscle data (13) it has seemed logical to locate the receptor on the postsynaptic side of the synaptic cleft. Certainly the proximity of receptors to nerve endings -- or their concentration in the motor end-plate region was established by Fatt and Katz (14) in 1951. According to the findings of Miledi (15) the receptors in frog neuromuscular junctions are not strictly junctional but extend about the periphery of the motor end-plate. Since efforts to depolarize the end-plate by micropipetting acetylcholine into the interior of a frog muscle fiber were unsuccessful (16), it would seem that the receptive substance is located on the surface of the postsynaptic

membrane; though an intramembranous site of action is not excluded.

Jenden et al. (17) envisage receptors being disposed throughout a three-dimensional structure rather than on a surface. This view is founded upon work using decamethonium, a bis-quaternary depolarizing ammonium salt. Waser (18), using a radioaudiographic technique, agrees that this depolarizing substance distributes itself more diffusely in the end-plate region than does curare, a bulky molecule and the classical non-depolarizing blocking agent of the myoneural junction.

Acetylcholine sensitivity of the muscle-tendon junction was studied by Katz and Miledi (19), who evaluated sensitivity by dividing the amplitude, in millivolts, of the transient membrane depolarization by the coulomb ($\times 10^{-9}$) quantity passed through the acetylcholine-containing pipette. Peak sensitivities at the end-plate ranged up to 670; highest sensitivity observed at the muscle-tendon junction was 10; at 2-5 mm from the tendon, 5×10^{-1}. The acetylcholine sensitivity of the muscle-tendon junction is particularly interesting because there is a local zone in this region of certain frog muscles where acetylcholinesterase is found in relatively high concentration.

Axelsson and Thesleff (20) demonstrated that

acetylcholine receptors in a denervated muscle respond to drugs in a manner qualitatively similar to those of the innervated end-plate, and they attributed the supersensitivity of denervated muscle to an increase in the size of the receptor area. One must admit the possibility, however, that the amount of molecular substance with which acetylcholine combines, i.e. receptors, might be unchanged, and instead the permeability characteristics of the depolarizing agent-receptor would be more effective than usual. Denervated muscle is much less specific in its response to chemical agents than normal muscle preparations, and for this reason it is permissible to question the degree to which denervated tissue represents normal muscle tissue.

There is evidence for a presynaptic action of acetylcholine both at the sympathetic postganglionic nerve endings (21) and at autonomic ganglia (22). Koelle and Volle have shown that threshold doses of both acetylcholine and carbachol are higher in DFP-treated denervated than in DFP-treated normal ganglia. This has led Koelle to emphasize the apparently greater sensitivity of the presynaptic terminals than of the postsynaptic terminals to carbachol and, after DFP, to acetylcholine. In 1940 Masland and Wigton (23) discovered antidromic motor nerve

impulses when mammalian nerve muscle preparations were exposed to acetylcholine and certain anticholinesterases; the generation of these nerve impulses they attributed to nerve terminal depolarization by the drugs. Later other workers (24, 25) argued that the cause of the antidromic firing was not a direct presynaptic effect of these drugs but rather could be explained by the ephaptic action of muscle membrane responses. More recently, however, Werner, Barstad, and Hubbard (26) have experimental indication of direct acetylcholine reaction with cholinoceptive sites on, or adjacent to, nerve terminals. Because ACh does not alter the frequency of the miniature end-plate potentials (16, 27, 28), it is difficult to assign a physiological function to the presynaptic acetylcholine receptors.

Csillik (7) postulates a postsynaptic amplification effect of acetylcholine -- that is, the acetylcholine combines with receptors on the postsynaptic surface to release more acetylcholine. He cites the fact that Fe-labeled botulinum, which has been shown to interfere with the release (29) but not the synthesis (30) of acetylcholine, is localized in the synaptic clefts (31). This is rather weak evidence since this localization of botulinum might not be associated with its toxicity. Reports

of acetylcholine release from denervated muscle are not new to the literature. The 1945 observation by Abdon and Bjarke (32) that curare, in concentrations which blocked neuromuscular transmission, also abolished the formation of free acetylcholine in the muscle after nerve stimulation but not after direct muscle stimulation should be repeated. These workers believed that free acetylcholine appeared in the muscle only as a consequence of the breakdown of a labile acetylcholine complex. A recent paper by Hayes and Riker (33) also indicts muscle as the principal tissue of acetylcholine origin. They found that the amounts of acetylcholine liberated by innervated and chronically denervated paired hemidiaphragms did not differ whether the muscles were at rest or driven by electrical stimuli. Since these results could not be duplicated by others (34, 35), Dale's evidence (1) for the neural origin of acetylcholine has not been disproved; his 1936 paper remains a classic. Moreover, Hebb (36) has recently calculated that the .5% of normal choline acetylase activity remaining in the distal intramuscular portion of degenerating nerves is sufficient to account for the ACh released spontaneously in denervated voluntary muscle.

There are a number of questions needing answers. At present it still appears most likely that the myoneural

receptive substance is primarily located on the post-synaptic surface, though additional presynaptic cholinoceptive sites may exist.

How does the receptor work?

The response of the skeletal muscle cell to the activation of the receptor by the agonist is depolarization, which represents a change in ionic permeability. But what do we know of the relationship between the receptor-agonist complex and depolarization?

The "occupation theory" derives from Paul Ehrlich and postulates that depolarization is the consequence of the receptor-agonist combination and continues as long as the receptor is occupied by the agonist. Most workers have confined their thinking within the framework of this theory, although certain modifications have been proposed. The action of a drug at a receptor was viewed in 1937 by Clark (37) as all-or-none -- an agonist causes an effect and an antagonist causes no effect; thus the activity of a drug of either kind was simply a measure of its affinity for the receptors. However, Stephenson (38) contributed the idea that different drugs might have varying capacities to initiate a response and consequently occupy different proportions of the receptors when producing equal responses.

This property he referred to as the efficacy of the drug. The activity of agonists would thus be the product of their affinity and efficacy. Some experimental evidence for this theory was obtained from an examination of the actions and interactions of the homologous series of alkyl trimethylammonium ions and atropine.

There are, though, obstacles to using a structure-activity relationship (SAR) approach at the myoneural junction. Cavallito (39) has stated that coulombic bonding characteristics, steric influences, and molecular lipophilic-hydrophilic balance are all essential factors determining the activity of neuromuscular blocking agents. Changing the structure of a drug most often alters more than one of these parameters, and yet this fact is often not considered when interpreting results.

Nevertheless, the concept of "spare receptors", which arises from Stephenson's hypothesis that a "maximum effect can be produced by an agonist when occupying only a small proportion of the receptors", is difficult to ignore. The theoreticians have been much intrigued by this notion. Van Rossum and Ariëns (40) in a recent paper discussed the possibility of a nonlinear relationship between receptor occupation and effect (depolarization or contraction) and the existence of receptor reserve in two different

theoretical situations. Because of the possible presence of an excess of receptors, MacKay (41) has devised a general method for the analysis of drug-receptor interactions which eliminated all assumptions about the relationship between stimulus and response by application of a null method.

More experimental data to document receptor reserve is needed. Using the ileum, Nickerson (42) has shown that occupancy of only about 1% of the histamine receptors is adequate to produce a maximal response. As the total number of free receptors is progressively decreased by irreversible combination with a blocking agent, larger concentrations of agonist are required to provide the receptor occupancy necessary for any given response. The appreciable shift in these dose-response curves before any decrease in maximal response can be detected provides for Furchgott (3) "decisive evidence against the concept that effect is proportional to the concentration of the receptor-agonist complex and for the concept of spare receptors."

Because an irreversible inhibitor for the neuromuscular junction is not available, similar information has not been obtained about these receptors. The concept of spare cholinergic receptors does emerge from Waser's calculation (43) that 8×10^{-6} molecules of curare comprise

the minimal paralytic dose at one mouse diaphragm end-plate. From this he calculates the total number of cholinergic receptors in one end-plate to be about 10^{-7}. Other workers (44) have estimated that a minimum of 10^{-6} molecules is released at the end-plate by one impulse. These estimates thus would suggest an approximate 10-fold reserve of cholinergic receptors.

The occupation theory has recently been challenged by Paton (45), who suggests that stimulant action is proportional to the rate of association between drug molecule and receptor. The obvious corollary would be that receptor occupation interferes with stimulant action by diminishing the pool of free receptors. This theory is a convenient way to explain the differences between depolarizing and blocking compounds by stating that the former dissociate rapidly from receptors, the latter very slowly.

To give weight to his ideas Paton compares the end-plate depolarizations evoked by a simple series of alkyl-trimethylammonium compounds and notes that depolarization declines as chain length increases -- an observation which he relates to the increased binding force of the molecule at the receptor. He has also calculated the rate constants for onset and offset of response under simple occupation

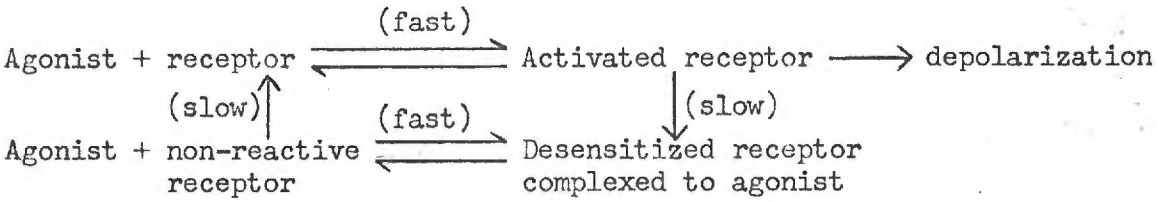
and simple rate theory. In rate theory, onset would be characterized by a maximal response at zero time followed by the exponential "fade" of the response to equilibrium. With alkyltrimethylammonium compounds he demonstrates the phenomenon of fade on the guinea pig ileum. It should be pointed out, however, that his results might also be attributed to an unspecific noncompetitive antagonism by these ions which would increase with increasing chain length. The difficulties inherent in SAR studies at the neuromuscular junction have already received comment.

Unfortunately at the present time there is insufficient evidence to decide between the rate and occupation theories of drug activity. One must then admit the difficulty of interpreting the degree of depolarization as a criterion of receptor activity due to our ignorance concerning the relationship between the two.

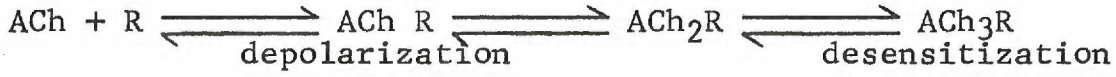
There is still another problem connected with depolarization at the myoneural junction which is as puzzling as the others. This problem relates to the phenomenon of "desensitization". Desensitization is a condition in which the end-plate has become refractory to depolarizing agents and from which it recovers only slowly after complete withdrawal of the agent. It has been suggested that this change arises from gradual

transformation of the agonist-receptor compound into an inactive form. The net result is that the desensitized receptor does not react characteristically -- by depolarization -- to the agonist.

First, desensitization was studied by Thesleff (46) with bulk application of acetylcholine to the isolated frog sartorius muscle and later by Katz and Thesleff (47) using iontophoretic application through micropipettes to the end-plate region. The scheme proposed by these workers to explain the results they observed is as follows:



Nastuk and Gissen (48) have proposed a second scheme in which the receptor is considered to be a polyvalent anion capable of binding acetylcholine:



Manthey and Nastuk (49) made the interesting finding that increasing the calcium concentration from 0 to 10 mM caused a 7-fold increase in the rate of desensitization of frog neuromuscular junction.

Thesleff (50) has speculated that the acetylcholine receptor combination only causes depolarization when a substance formed inside the fiber is utilized in the reaction, that desensitization results from a depletion of this substance, and that recovery occurs when the membrane is replenished from the inside by diffusion or synthesis of this substance. Paton (45) suggests that the substance is potassium ion. Shanes (51) has pointed out that multivalent ions and a variety of drugs which he classifies as "stabilizers" reduce the electrical effectiveness of sodium, potassium, and other ions in changing the resting potential; "labilizers" accentuate the ionic effects on membrane potential. Using his system of classification, he describes the desensitizing activity of the transmitter as a relatively non-specific action, like that of stabilizers, in preventing depolarization. It is clear that this phenomenon has not yet been explained, but one must be aware of it when evaluating data involving depolarization measurements.

Finally, it should be remembered that depolarization

is only the final effect in what may be a series of steps initiated by interaction of the receptor with its agonist (52).

INTRODUCTION TO THE PROBLEM

The hypothesis to be examined in this work states that the acetylcholine receptor is a cholinesterase. If this hypothesis could be proved, we would gain knowledge not only about the chemical nature of the acetylcholine receptor but also about its location and the manner in which it works, since we already have certain information about the enzyme. The experiments to be described later in this thesis involve a comparison of the effects of acetylcholine (ACh) and carbachol (Carb) (Fig. 1) on depolarization at the end-plate region of frog skeletal muscle. A comparison of these two depolarizing agents is relevant to the hypothesis because of their differential rate of hydrolysis by the enzyme.

Much of the information which we have about acetylcholinesterase comes from the laboratory of Wilson (53, 54, 55), who has analyzed the active sites of the enzyme and proposed the model illustrated by Fig. 2a to describe them. Evidence for the presence of the anionic site is given by the change in inhibitory capacity of eserine with pH (the structure of eserine is dependent on pH), in contrast to the behavior of prostigmine[1], which has a structure independent of pH and inhibits equally well at all pH's over the

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[1] It has been called to my attention that neostigmine is the official USP name of the drug referred to throughout this thesis as prostigmine.

Fig. 1. Indicates the similarity between the chemical structures of (a) acetylcholine and (b) carbachol.

Fig. 2. Representation of (a) the active sites of acetylcholinesterase (55), (b) the acylated enzyme, or ES' when the substrate is acetylcholine, and (c) the carbamylated enzyme, or ES' when the substrate is carbachol.

Fig. 3. Inhibition of acetylcholinesterase by prostigmine (·) and eserine (+) as a function of pH (53).

Fig. 4. Inhibition of acetylcholinesterase by TEPP as a function of pH. The concentrated enzyme was exposed to TEPP at various pH values, then rapidly diluted, and the activity tested at pH 7. The symbols represent two different sets of data (53).

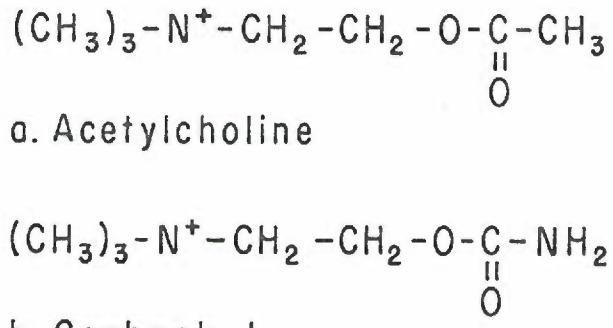


fig. 1

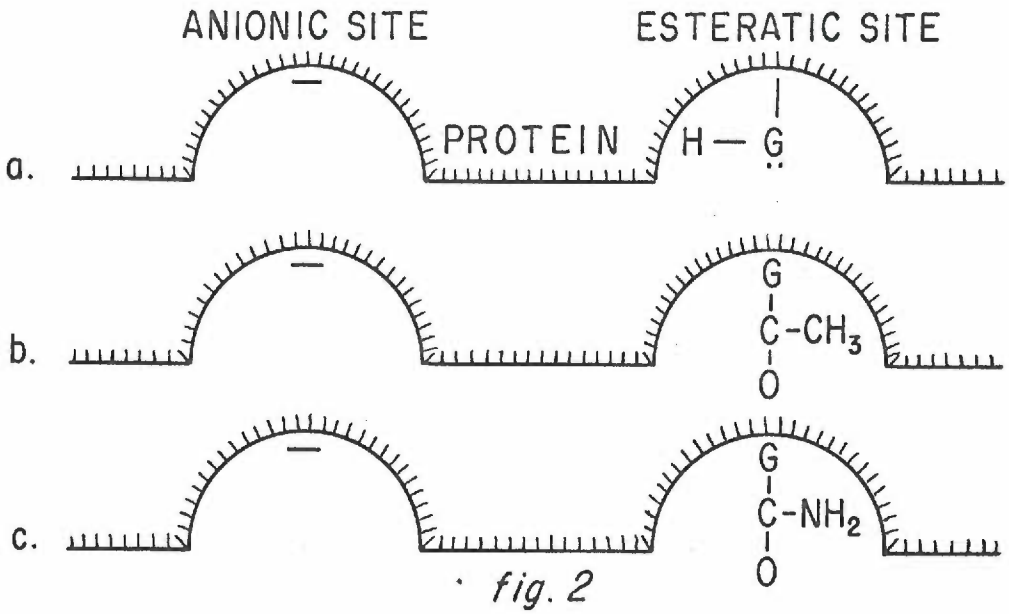


fig. 2

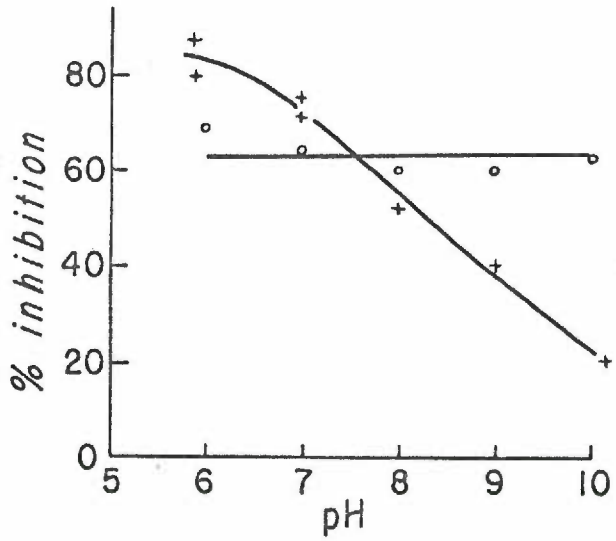


fig. 3

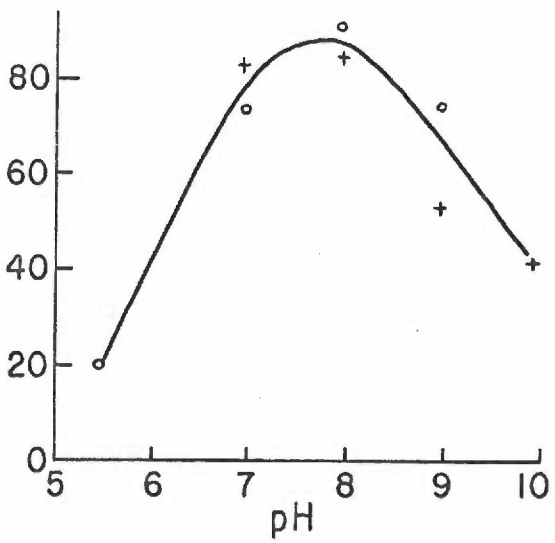


fig. 4

Fujita (57) have demonstrated that the hydrolysis of acetylcholine by the enzyme is inhibited competitively when ACh and Carb are added simultaneously to the enzyme but noncompetitively when the enzyme is pre-incubated with Carb (Fig. 5).

The stability of the carbamylated enzyme is best explained by the fact that the unshared nitrogen electrons attenuate the positive charge induced on the carbon atom as a result of the polarization of the C - O bond. Therefore, the hydroxyl ions of water are not as strongly attracted to carbon. The rate of hydrolysis is necessarily slowed.

Before reviewing the literature which directly concerns itself with the hypothesis, some definitions and general information might be helpful. Cholinesterases (ChE) are enzymes which catalyse the hydrolysis of choline esters. Acetylcholinesterase (AChE), or true cholinesterase, is the cholinesterase which hydrolyzes acetylcholine at the highest rate, while pseudocholinesterase hydrolyzes butyrylcholine or propionylcholine at a higher velocity than ACh. Histochemical methods have shown that the myoneural junction of striated muscle is rich in acetylcholinesterase but also contains some butyrylcholinesterase (58, 59). Electron

Fig. 5. Lineweaver-Burk plot of bovine erythrocyte acetylcholinesterase inhibition with carbachol. A, no inhibitor added; B, inhibitor pre-incubated 30 min. with enzyme; C, inhibitor added simultaneously with substrate (57).

Fig. 6. Anticholinesterase, decurarizing, and paralyzing activities in the diiodomethylate series of bis-(p-dimethylaminophenoxy)-α -ω alcanes as functions of the number of carbons in the alkyl bridge (77).

Fig. 7. The relationship between the specific inhibitory activity of a drug against true or pseudocholinesterase and its pharmacological action. The B/M ratio is I_{50} brain cholinesterase/I_{50} mucosa cholinesterase. A B/M ratio >1 denotes that the pseudocholinesterase is preferentially inhibited, a ratio <1 that true cholinesterase is preferentially inhibited (78).

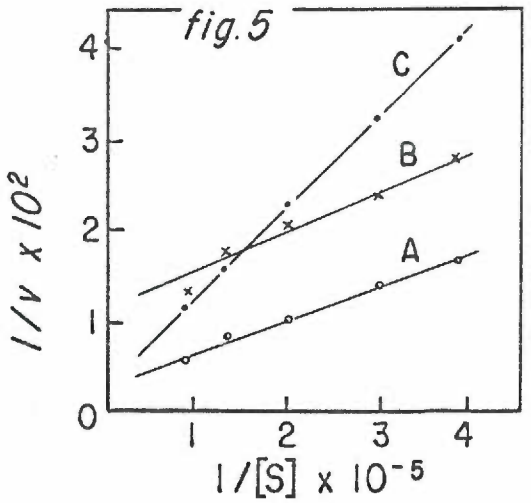
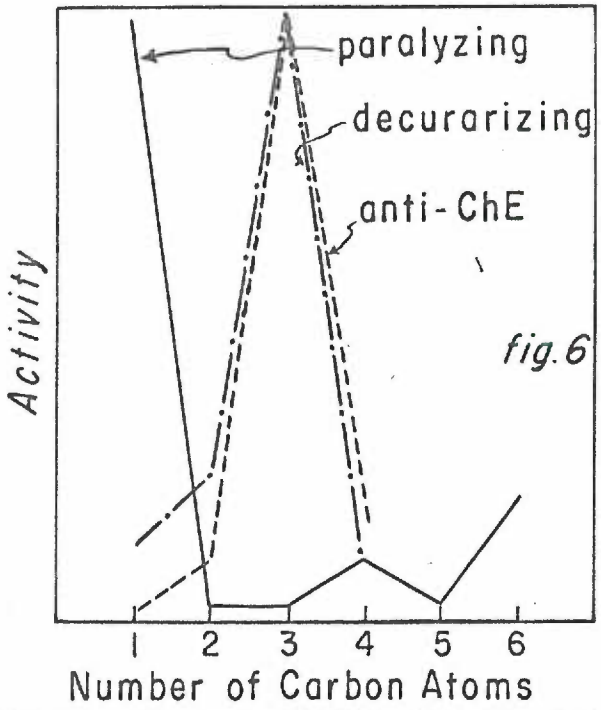


fig. 5
1/v x 10²
1/[S] x 10⁻⁵
A
B
C


paralyzing
decurarizing
anti-ChE
Activity
Number of Carbon Atoms
fig. 6

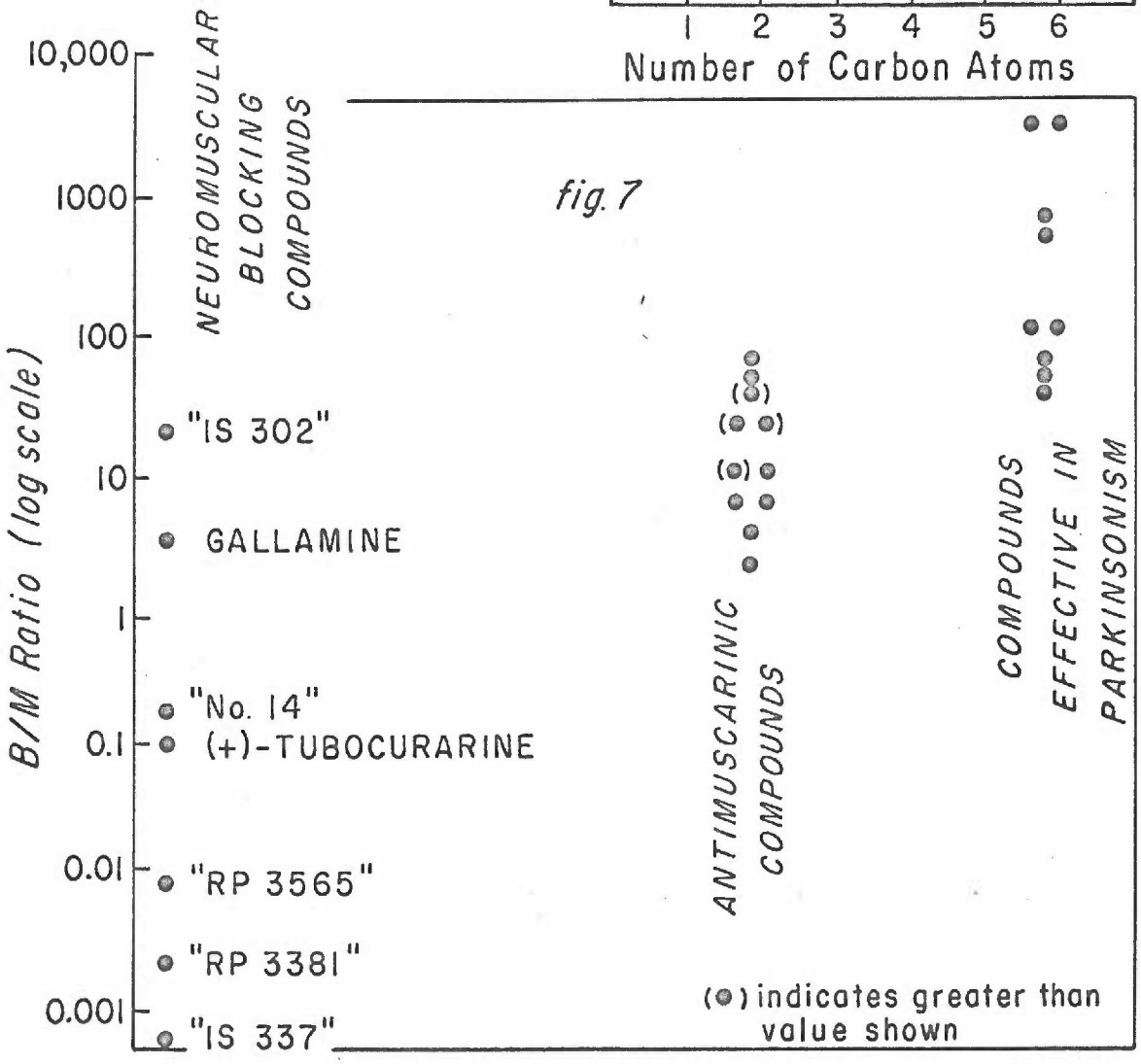


fig. 7
B/M Ratio (log scale)
NEUROMUSCULAR BLOCKING COMPOUNDS
"IS 302"
GALLAMINE
"No. 14"
(+)-TUBOCURARINE
"RP 3565"
"RP 3381"
"IS 337"
ANTIMUSCARINIC COMPOUNDS
COMPOUNDS EFFECTIVE IN PARKINSONISM
(●) indicates greater than value shown

microscopists have been unable to agree about the precise localization of this myoneural AChE; some believe the enzyme is situated extracellularly within the spaces of the primary and secondary clefts, while others assign a postsynaptic membrane site to it (60, 61). Karlin (62) has subfractionated the cells of eel electric tissue, a rich source of AChE, and correlated the presence of AChE with the presence of large fragments of the cell membrane. The erythrocytes and brain grey matter are other sources of AChE. Glial cells and blood serum are sources of pseudocholinesterase.

Roepke (63) first made the suggestion that the ACh receptor and ChE might be similar in a 1937 paper reporting the results of his study of the affinities of various choline esters for horse serum ChE. Because he found little difference in affinities, he speculated that the difference in pharmacological activity of the choline esters might be attributed to variations in the activity of the complexes formed when these substances united with the receptor and not to variations in the amount of drug fixed by the cells.

The concept that ChE and cellular receptor sites were essentially similar was again utilized in 1951 by Wescoe and Riker (64), who were interested in the direct

action of prostigmine, an anticholinesterase, upon the receptor. They were impressed by the fact that in both normally innervated and chronically denervated mammalian muscle, perfused with a huge intra-arterial dose of DFP, prostigmine simulated the action of ACh. Further evidence for a direct prostigmine action on the receptor was based on the immediate contractile response that occurs following close intra-arterial injection of this substance. At that time there was no consideration of the possibility of pre-synaptic activity for the drug.

Two years later Župančič (65) published his argument that the active groups of the ACh receptor were not only similar to the active groups of ChE -- but were identical to them.[1] This suggestion simplifies the picture of events at the neuromuscular junction. Acetylcholine would not be required to combine with the receptive protein in preference to ChE, later disassociate from it, and finally combine with ChE for removal by hydrolysis. Instead the combination of ACh with the ChE-receptor would initiate depolarization, and in the next fraction of time hydrolysis

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[1] This idea can be traced back to Karassik, who was probably the first to advocate the identity of cholinoreceptors with a negative group of the cholinesterase which differs from the hydrolyzing site of the enzyme.

would occur freeing the receptor for combination with another molecule of ACh, since, according to Župančič, choline has a markedly higher dissociation constant for the reaction than ACh. Župančič does not confine all ChE to the postsynaptic membrane, i.e. the receptor site, but also acknowledges the presence of barrier ChE in the interstitial fluid which interferes with the ester before it reaches its site of action -- the ChE-receptor. Thus he explains the potentiating influences of anticholinesterase by inhibition of barrier ChE. To support his basic contention this investigator cited the well-known phenomenon of ACh stimulation at low concentrations and inhibition (block) at high concentration; as explanation he offered the fact that AChE is inhibited by high concentrations of ACh.

Župančič believed and still believes that the active site of the ChE molecule is the anionic site, pointing to the fact that stable choline esters stimulate but are not hydrolyzed. Thus a ChE preparation with inhibited esteratic centers but with functioning anionic sites should be identical with the receptor protein.

His experimental approach (66, 67, 68) to the subject has centered upon the determination of equilibrium

constants for reactions between anionic centers of ChE and various cholinomimetic or cholinolytic substances. Butyryl-cholinesterase from horse blood and eel AChE were purified until a known concentration of esteratic centers per liter was achieved; this was calculated from the enzymatic activity and the turnover number for the ChE. After incubation with DFP, the esteratic centers of ChE were irreversibly inhibited, while its anionic centers remained accessible. Assuming equal numbers of both types of centers, the total concentration of anionic centers (R_t) in the preparation was known. After adding a given quantity of ACh, the total concentration of the ester (A_t) was known. An undiluted sample of the mixture was biologically assayed for the concentration of the free acetylcholine (A_f). Applying the mass-action law and assuming a bimolecular reaction of the type -- R + A = RA, one can write:

$$\frac{R_t - (A_t - A_f) \times A_f}{A_t - A_f} = K$$

Using this method or modifications of it, he has calculated dissociation constants for ACh or tubocurarine and ChE anionic sites which lie around 10^{-7}. He feels that the cholinesterases therefore display affinities for ACh and d-tubocurarine as required for cholinoreceptors. There is

disagreement in the literature concerning these values, however (69, 70).

Because of the relative simplicity of the ACh molecule, Wilson, Nachmansohn, and Altamirano (71, 72) believed that AChE and the receptor could not significantly differ, thus allowing the former to serve as a model for the latter. It was of interest to them that an essential characteristic of curare and curare-like compounds was the presence of two cationic quaternary nitrogens at a distance of 12 - 15 A^{o}, indicating the presence of two negative charges in the receptor at a similar distance. Bisquaternary nitrogen derivatives in which the two cations are separated by a distance of 12 to 15 A^{o} are more strongly bound to the enzyme protein than the corresponding monoquaternary compounds; their K_I's are two orders of magnitude less than the K_I's of monoquaternary compounds, which means they are stronger inhibitors by a factor of about 100, and this fact draws a parallel between structural aspects of enzyme and receptor.

In order to test the hypothesis that the receptor is an AChE, Altamirano et al. (71) monitored the electrical activity of eel electric tissue with microelectrodes and compared this index of receptor activity with the AChE

activity of intact cells assayed manometrically using ethyl chloroacetate as a substrate. They demonstrated that certain compounds such as ACh, Carb, procaine, and d-tubocurarine chloride block conduction without affecting the esterase, and hence concluded that blocking action could not be attributed to the effect on the esterase but must be due to that upon a different but similar cell constituent -- the long postulated receptor.

This summarizing statement conveys scant idea of the difficulties encountered in evaluating the esterase activity in the intact cell. The advantage of attempting an intact cell assay as opposed to tissue homogenization is illustrated by the fact that only a small fraction of various inhibitors may penetrate to the active site; homogenization without washing brings the enzyme into contact with a large excess of the inhibitor and makes the determination meaningless. Ethyl chloroacetate was used as the substrate in the eel experiment because ACh can penetrate to only 15% of the AChE of intact electroplax in sufficient concentration to permit assay, whereas ethyl choloracetate penetrates to 60% of the AChE. Aside from the problem that 40% of the AChE did not contribute to the assay, another difficulty was the discovery of a

second enzyme hydrolyzing this substrate. Furthermore, they reported that even high concentrations of Carb did not inhibit eel ChE -- a result which disagrees with Fellman and Fujita (57), who did find inhibition of red cell ChE by Carb.

One must read the work from Nachmansohn's group with care, for the electrical phenomenon which they are measuring is not a synaptic potential but an action potential propagated along the cell, similar to the spikes of nerve and striated muscle, which can be elicited either by direct or nerve stimulation. The ACh receptor for which Nachmansohn (73) searches functions in conduction; transmission at the synapse he considers an electrical affair.

In 1964 Webb (74), also a member of Nachmansohn's group at Columbia, determined the dissociation constants between certain benzoquinonium and ambenonium analogs and the ACh receptor of the isolated electroplax and compared them with those obtained from the electric organ of Electrophorus electricus. These compounds are strong competitive and reversible inhibitors of both the receptor and the esterase, but the effects of structural modification on reaction with the receptor differ markedly from those in which the inhibitory action on the AChE is tested.

Webb thus offers this as new evidence that the active sites of the ACh receptor and of AChE are different in chemical structure.

However, in his discussion Webb recognizes that there are differences between dissociation constants determined with an enzyme in solution and those tested on intact membranes. For example, in the organized structure of the membrane it is difficult to ascertain whether the concentrations of activator and inhibitor are the same in the immediate vicinity of the receptor sites as they are in the externally applied solution. Barriers may be present which impede the penetration of Carb and particularly large molecules such as the benzoquinonium or ambenonium analogs. Another factor which must be considered is the effect of an organized structure on the interation between micro- and macromolecules. Studies with insoluble enzymes (75, 76) have shown the strong effects which positive or negative charges surrounding the protein may have on the reaction; compounds with opposite charge may be attracted while those with the same charge may be repelled. Thereby the local concentration may be greatly increased or reduced. A 20 - 50 fold increase in the K_m has been observed with the insoluble enzyme compared

to the same enzyme in solution.

Other workers who have wrestled with the task of comparing receptor activity with anticholinesterase activity include Jacob and Tazieff-Depierre (77), who summarized evidence which points to a divergence between anti-ChE activity and muscle depressant potency within certain homologous series of agents (Fig. 6).

Todrick (78), who recently has concerned himself with studies using specific inhibitors for true or pseudo-cholinesterase, examined 46 substances for inhibitory action on true (typified by rat brain homogenate) and pseudo- (typified by rat intestinal mucosa) ChE. He classified these 46 substances into six groups -- neuromuscular blocking agents, antimuscarinic agents, drugs effective in parkinsonism, antihistaminic agents, anticholinesterases, and miscellaneous substances. Next he ranked the compounds according to their I_{50} brain ChE/I_{50} mucosal ChE values. A compound having a B/M ratio less than one would be more specific for inhibiting the true cholinesterase.

His results indicated in Fig. 7 led Todrick to suggest that there might be a correlation between the pharmacological action of the compounds examined and their specificity as ChE inhibitors. The author felt his data

supported the idea that the active center of true cholinesterase resembles in structure the ACh receptor at the neuromuscular junction. And he suggested that the correlation of anti-muscarinic activity with specificity for the pseudocholinesterase reflects a structural resemblance between the ACh receptors in the parasympathetic nervous system and the active center of the pseudocholinesterase.

Koelle (79), however, states that the I_{50} (molar concentration producing 50% inhibition of the enzyme in vitro) should be in the range of 10^{-7} or lower to demonstrate high affinity between enzyme and inhibitor. All of the I_{50} values reported by Todrick are considerably above this concentration, and thus Koelle would probably attach dubious significance to the results reported by Todrick.

Sensitization by diisopropyl fluorophosphate (DFP) of the rectus abdominus muscle of the frog toward ACh, butyrylcholine, succinylcholine, and non-ester compounds -- choline and decamethonium was demonstrated by Cohen and Posthumus (80) in 1955. They concluded that inhibition of the esterase could not be solely responsible for the sensitization afforded by anticholinesterases and explained their data in the following way. The receptor of the

involved muscle consists of two sites, one of which is an esterase grouping which they designated the B group. When direct binding of DFP occurs at this site the second component of the receptor -- the A group, which is the normal anionic site of action of effectors, would be sensitized. This same data could be used to support the hypothesis that choline and decamethonium (depolarizing substances which lack the carbonyl group) act indirectly by displacing bound ACh at the junction; the sensitization to ACh and butyrylcholine can easily be explained by the anticholinesterase activity of DFP.

In a paper a year later Cohen and a group of associates (81), studied the relationship between the pharmacological action of neuromuscular drugs and their capacity to inhibit esterases. The authors concluded that all drugs which are capable of acting on the end-plates of striated muscles strongly inhbited both cholinesterases. Furthermore, the end-plate depolarizing drugs were far stronger inhibitors of true cholinesterase than curare-like end-plate blocking agents. Included in this paper was evidence that liver-esterase is weakly inhibited by any of the drugs used. They also made the suggestion that the pattern of anionic sites on the end-plate receptor is

reflected in the structure of ChE.

More recently two new techniques have been used in an attempt to examine the hypothesis concerning the structure of cholinergic receptors and their relationship to AChE.

The first of these is autoradiography, which is being used by Waser (18). He injected a minimal lethal dose of C^{14}-curarine into the tail veins of mice, which died of asphyxia within two minutes. The diaphragms were isolated, stretched and dried on steel rings, and finally placed on x-ray films. Two to six months later the contact films were developed and at the same time the end-plates in the diaphragms were separately stained for ChE by Koelle's method.

Simultaneous intravenous injection of a minimal lethal dose of radiocurarine and varying doses of prostigmine resulted in autoradiographs without recognizable interference at the receptor sites. Although nervous transmission through the end-plate was restored within a short time saving the mice from asphyxiation, radioactivity in the end-plates was not noticeably reduced by low doses of prostigmine. Even with a lethal dose of prostigmine, 8 - 10 times higher, only a small change in the radioactivity of the end-plates was detected. Waser believes

that this indicates that the receptor blocked by curarine and the ChE blocked by prostigmine are located in different units.

When mouse diaphragm was denervated by cutting the phrenic nerve, ChE activity and binding of radioactive curare first increased and then decreased in a parallel fashion during the 4 to 66 day period following denervation; both ChE activity and binding of radioactive curare were completely lost after 60 days. In another group the phrenic nerve was coagulated with dry ice leaving the nerve intact for regeneration; when ChE activity reappeared after 30 days, the binding of curare once again could also be demonstrated. These denervation experiments would suggest a structural link between the two.

The second new technique to be applied to this problem is that of polarization microscopy. Csillik (7), studying the postsynaptic membrane after fixation in lead nitrate solutions, has noted a strong birefringence of that region which cannot be demonstrated after freezing or formalin fixation of the tissue. As lipoproteins are also sensitive to these procedures, it is possible that the lead affinity is exerted by the organization of membrane lipoprotein. Cholinesterase activity, which seems closely associated with the lead-reactive material, does not

disappear after freezing or formalin fixation.

Imbibition experiments[1] on the lead-fixed myoneural junctions reveal that their birefringence is a result both of micellar (form birefringence) and molecular (intrinsic birefringence) organization. Frog muscles which had been stimulated by tetanizing impulses, fixed in lead nitrate, and subjected to imbibition experiments showed no difference from control muscles until after acetone extraction. Then the imbibition curve of the stimulated muscle dropped to zero indicating "serious disintegration in the arrangement of non-lipid particles". The same decrease in intrinsic birefringence accompanied ACh-eserine treatment of the muscle, and curare prevented the alterations in intrinsic birefringence which usually followed supramaximal stimulation.

Csillik then equates the lead-reactive birefringent structure to the ACh receptor and formulates the question --

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[1] The imbibition experiment introduced into the polarization optical analysis of biological objects by Schmidt and Bennett utilized the fact that when using a fluid of an appropriate refractive index, the differences between the refractions of the micellar units and of their surrounding media can be abolished. Thus when the birefractive index equals the index of the micellar unit, the optical effect due to the micellae themselves is discontinued. Then if birefringence remains, it means that their molecular organization is regular.

"What is the relation of the enzyme to the receptor?" The temperature-sensitivity and imbibition experiments point to the lipoprotein character of the so-called "ACh receptor", whereas there is no information establishing the presence of lipids in purified AChE preparations. Csillik therefore believes that enzyme and receptor are two closely-related, but distinct entities. Yet the interesting birefringence changes which Csillik observes could represent membrane permeability changes resulting from ACh-receptor activation; in other words, there is no proof that the lead-reactive sites are also ACh receptors.

Finally, the work of Kimura (81) should be mentioned. In an effort to compare the ACh receptor with ChE he has attempted to measure the pK_a values of the two anionic sites and of the two esteratic sites using the frog rectus abdominus for receptor experiments. Because pK values of 2.08 - 3.9 were obtained for the association constant between organophosphate and the esteratic site of the ACh receptor as compared with a pK_a of 7.2 for ChE, he concludes that the ACh receptor is different from the ChE molecule. Nevertheless his work implies that there is an esteratic site in the ACh receptor; thus it could hydrolyze ACh and would by definition be a ChE.

Can these divergent results be amalgamated into any conclusion whatsoever? Perhaps the soundest summary statement that can be made is the following: The allegation that the acetylcholine receptor is a ChE has neither been proved nor disproved. It remains a tenable hypothesis and as such should serve to stimulate further experimentation.

Consequently, another examination of that hypothesis has been undertaken in the experiments to be described. As mentioned earlier, there is evidence that ACh and Carb react quite differently with the enzyme AChE. The former is rapidly hydrolyzed, whereas the latter forms such a stable intermediate that its rate of hydrolysis is quite slow. ACh and Carb both react with the receptor, for both are depolarizing agents. Hence a comparison of the depolarizing characteristics of the two substances could be expected to show a difference if the receptor were a ChE. The stable carbamyl intermediate (carbachol intermediate) might either prolong the duration of depolarization, or more probably the carbamylated receptor would be removed from the active receptor population. On the basis of the enzyme studies, then, one can predict that Carb-induced depolarization would decrease with subsequent doses while ACh-induced depolarization would remain constant -- if the

hypothesis that enzyme and receptor are identical is true. If the receptor is not a ChE, the ACh and Carb molecules must first disassociate from the receptor before being split. In this case one would not expect to find a difference between ACh and Carb-induced depolarization, for the electrostatic nature of the two molecules (Fig. 1) would not warrant such a prediction.

For these depolarization comparison studies the neuromuscular junction of a frog twitch-type muscle seemed an ideal preparation. This amphibian zone of ACh receptors has been used successfully in many neurophysiological end-plate investigations (83, 84). Fatt (85) recorded electrical events from both the sartorius and the extensor longus digiti quarti (Rana temporaria) in his study of the effect of low sodium on ACh-produced depolarization. In 1960 Jenkinson (69) used the same two muscles in his quantitative study of the antagonism between ACh and tubocurare at the receptor; in this paper he reported that two different methods of collecting data -- micro-electrode measurement of individual end-plate activity and extracellular recording from the end-plate region of the whole muscle -- gave him similar values for affinity constants. Studies by Castillo and Katz (86) established that the frog sartorius end-plate receptors which respond to iontophoretically applied drugs are identical, or at least representative of

the normal neuro-receptors. The rates of effect of bath-applied agents on frog sartorius end-plate potentials and iontophoretic ACh potentials was measured by Goldsmith (87). He, too, found the effects of drugs on end-plate potentials and ACh potentials produced near the end-plate to be very similar, thus supporting the view that the neuro-receptors were reached by diffusion from the bath.

The extensor longus digiti IV muscle (m. ext. long. dig. IV) of Rana pipiens was chosen after some initial experiments with the sartorius muscle because (1) the former muscle is much thinner so that equilibrium between the diffusing agonist and the receptor is more rapidly reached and (2) the larger sartorius permits a greater loss of electrical charge into the tissue (external shunting) so that the potential differences which follow ACh application in the bathing fluid are less. A slight disadvantage to the use of m. ext. long. dig. IV is the fact that 6-20% of its fibers of diameter greater than 10 micra have the 'Felderstrucktur' taken to be characteristic of slow fibers having a distributed innervation (88). This would not complicate the present experiments, however, because the end-plates of the twitch fibers form zones of maximal depolarization which are quite discretely localized.

MATERIAL AND METHODS

Preparation:

Unrefrigerated summer and winter frogs (Rana pipiens) were used. Although an increase in sensitivity to depolarizing substances in summer frogs was suspected, statistical treatment of the data did not prove the suspicion true. Refrigeration seemed to reduce the sensitivity of the preparation. Best results were obtained by using frogs within a day of mail delivery from the biological suppy outlet. A wire-covered 12" x 14" plastic tub partitioned with a wood strip to separate moss from tap water was used to house the frogs overnight at room temperature when necessary. "Red leg" was a continual problem during the winter; only active frogs were selected for experimentation. If the frogs were encouraged to jump just prior to dissection, muscular blood flow was enhanced -- a fact that aided in the dissection by providing greater contrast under the microscope. Double pithing preceded dissection.

For dissection of the m. ext. long. dig. IV (89) the foot of the intact frog was fastened in a petri dish, the bottom of which had been filled with black silicone potting compound (G. E. RTV-6164 and RTV-616B). The toe

muscle was dissected under Ringer's solution. In order to provide transverse illumination, the upper edge of the petri dish was supported in a circular opening in a flat platform. Beneath the platform, surrounding the petri dish, was a 22 watt circular fluorescent tube. Thus, light entered the petri dish only through the vertical sides and afforded the equivalent of dark field illumination which greatly facilitated the visualization of the muscle. Dissection was carried out at a magnification of 16X.

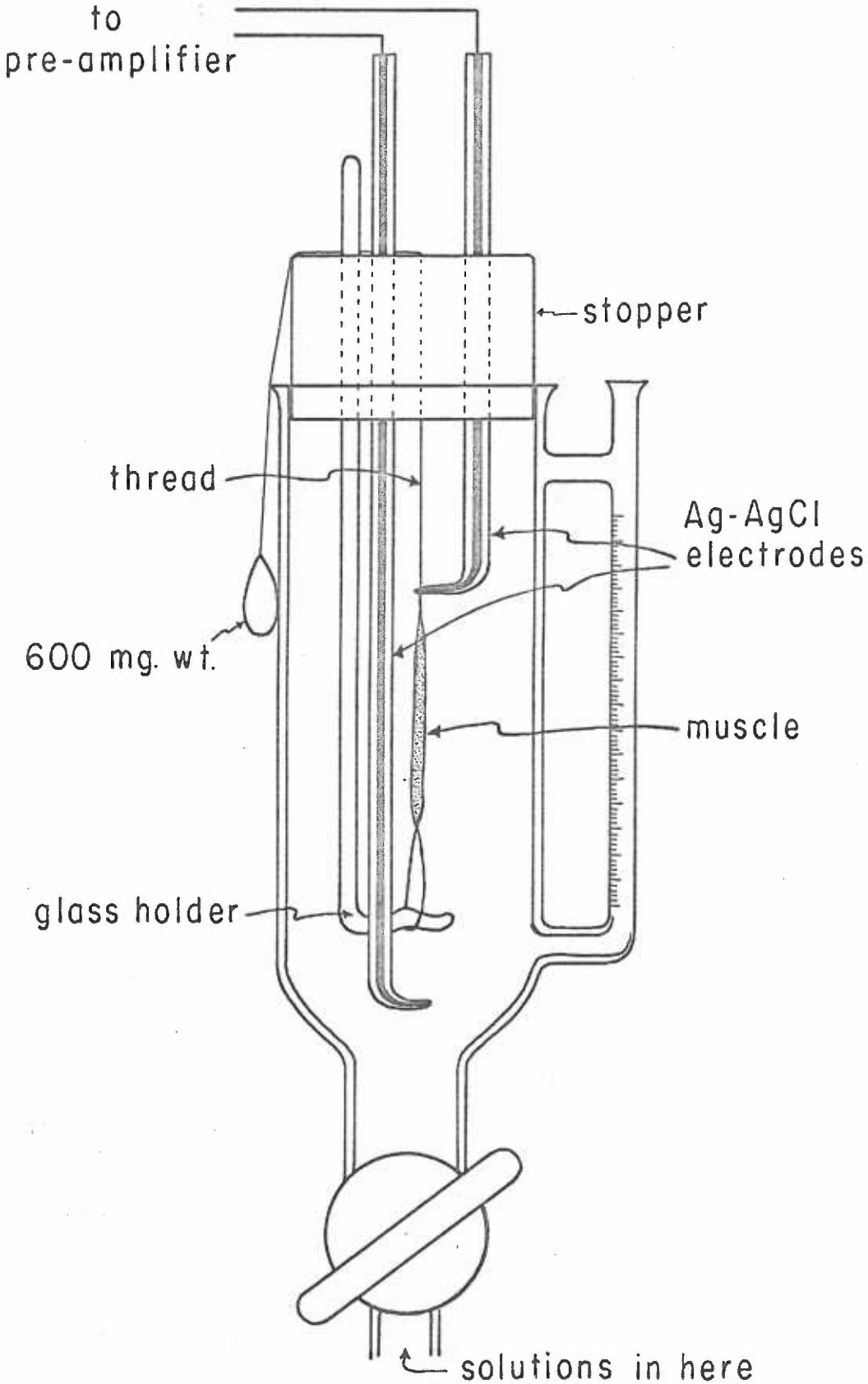
Extreme care had to be used in excising the muscle; a pair of Vanna dissecting scissors was very helpful in this operation. To avoid injury it was best to begin by cutting the connective tissue at about 1 mm. from each muscle edge. Sometimes the muscle branches at the distal end making injury difficult to avoid; in these cases the branching is generally bilateral. The distal tendon was tied with 7-0 silk. After cutting the foot from the leg, the proximal tendon was dissected free and tied with 4-0 silk. Using this 4-0 silk tie, one can gently pull and cut the muscle away from the surrounding tissue; the nerve was cut at its junction with a larger trunk. Muscles which registered more than 4 mv. injury potential at the beginning of an experiment were discarded; in most cases the injury potential was 1 - 2 mv. Many times an injury potential

of 5 - 8 mv. would subside to within useful range after an hour in Ringer's solution.

Apparàtus:

The interface electrode method of Fatt (85) was employed. The muscle was mounted vertically in an ambered glass chamber (Fig. 8) so constructed that bathing fluids could be changed through the bottom of the vessel and the chamber fluid level could be monitored by the calibrated side-arm. One Ag-AgCl electrode electrode was placed on the proximal tendon, while the other remained near the bottom of the chamber in contact with the solution. With part of the muscle immersed in Ringer's the potential difference recorded was that between the upper end and a point on the muscle at the level of the bathing solution. Solutions entered and left the chamber at a constant rate determined by a two-syringe Harvard infusion-withdrawal pump (114 cc/min; Model 600-910/920); one syringe held depolarizing fluid, the other Ringer's solution. With the rising and falling level of the fluid in the bath serving as a uniformly moving contact on the muscle, the pen recorder gave a continuous tracing of the potential differences along the muscle surface. The syringe plunger which changed the depolarizing fluid level also operated a linear potentiometer

Fig. 8. Glass chamber described in text. The stopper, electrodes, and glass muscle holder were removed as a unit for storage. Approximately 30 ml. of solution filled the chamber to the upper electrode.


to
pre-amplifier
stopper
thread
Ag-AgCl
electrodes
600 mg. wt.
muscle
glass holder
solutions in here

so that movement was transduced to potential difference. Thus, the recording system yielded a graph relating potential difference to distance along the muscle. This procedure will be referred to hereafter as "tracking".

Silver wires for electrodes were first ground to a tip at one end on pumice, cleaned in concentrated HCl diluted 1:1, rinsed and then inserted into the lumen of braided Ethicon 0 silk ligature to protect the AgCl (to be subsequently deposited) against light. The covered wires were next placed in glass tubing with a silk wick extending through the glass tip, soldered at the tops to miniature phone jacks, and plated from physiological saline according to the method of Bureš et al. (90), who recommend that electrolytic chlorination be carried out with low current density (0.1 to 10 A/m^2) overnight and in the dark. Both electrodes were connected in parallel as the anode, a piece of silver plate serving as the cathode. Two percent agar solution was then prepared, and the electrodes were filled from the bottoms. Any pair of electrodes registering a bias potential greater than 1 mv. after aging was not acceptable. When not in use, electrodes were shorted together in physiological saline and kept in brown bottles away from light.

Potential differences were fed into a Grass Model

5P1 d.c. pre-amplifier and driver amplifier. A short coaxial cable (the shielding of which was grounded to prevent stray signal pick-up) connected the miniature phone jacks to the input cable of the Grass so that negativity detected by the lower electrode caused an upward pen deflection. Input impedance of the pre-amplifier was 1 meg. The equipment was calibrated prior to each experiment by use of an internal standard voltage. This internal voltage source consisted of a battery and two resistors; these components were checked at the completion of the experimental series with a visual voltmeter and a VOM and were thus shown to be functioning accurately.[1] One hundred 2 mv. calibration signals into each of the two channels tested consistently registered that amount. Reading resolution was 4%.

Experimental Procedure:

The muscle was first tracked with Ringer's solution to ascertain the magnitude of the injury potential. If the control reading indicated an acceptable preparation (injury potential not greater than 4 mv.), the muscle was then

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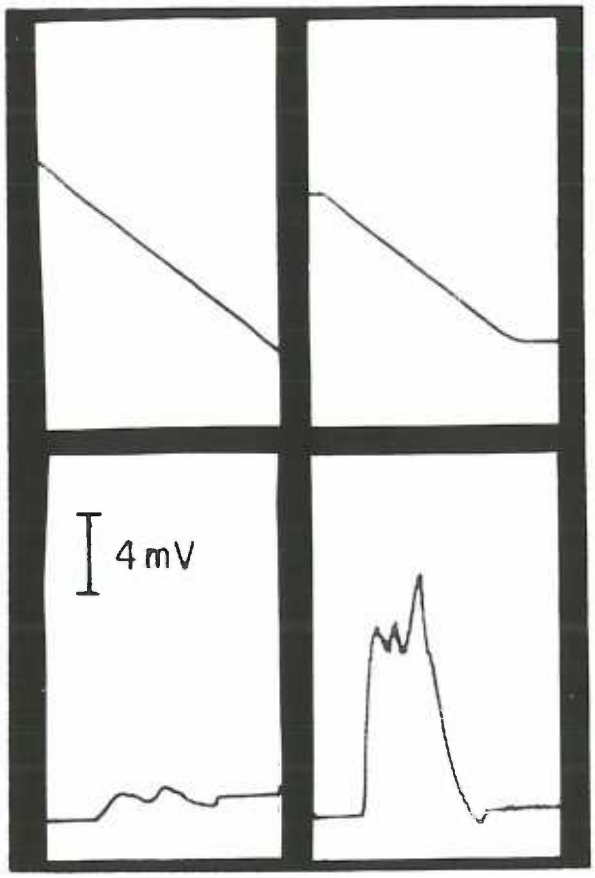
[1] The 1.35 volt batteries registered 1.346 volts in one channel and 1.348 volts in the other channel. The five-percent resistors were within 5% of their coded values.

tracked with 2.7 x 10^{-4}M ACh to locate the regions of greatest end-plate density. (Sample tracking record Fig. 9). There is ample evidence that the zones of maximal depolarization represent the nerve endings (91). The pump was then stopped at the peak of one of these depolarized regions so that the fluid level indicated on the sidearm could be noted. An additional exposure was made before the depolarizing fluid was removed from the chamber in order to confirm the accuracy of the measurement. After three two-minute washes in Ringer's solution, the muscle was allowed to rest 15 - 20 min. in Ringer's to eliminate the effects of this initial exposure to ACh.

The first "desensitization exposure" to a depolarizing agent consisted of a ten-minute exposure of the marked end-plate area to a very high concentration of depolarizing solution and a simultaneous recording of potential changes developed at this level. The depolarizing solution was withdrawn; five washes in Ringer's solution followed, and after an hour a second "desensitization exposure" reading was done. The same procedure was repeated until four records had been obtained. Note that one muscle only "sees" one test solution (excluding tracking dose), or, in other words, the same depolarizing


4 mV

solution is used throughout all four desensitization exposures. In several experiments a tracking dose was re-administered an hour after four desensitization exposures. Experiments were performed at room temperature (20 - 27° C).

Solutions:

The Ringer's solution had the following composition (mM): Na, 117; K, 2.1; Ca, 1.8; Cl, 121.0; phosphates, 2. It was brought to a pH of 7 with 10N NaOH. A week's supply of six liters was made up at one time. The Na and K content of an initial batch was checked by flame photometry and found to be correct.

Acetylcholine chloride (Merck) was used for locating end-plates if the experiment subsequently employed only Carb. One hundred mg. were diluted to 100 ml. with Ringer's solution to produce 5.5 x 10^{-3}M ACh; five ml. of this solution were diluted to 200 ml. with Ringer's to give the tracking concentration of 2.75 x 10^{-4}M. Acetylcholine iodide (K & K Laboratories) was used in preparing "desensitization doses", and these solutions were made up fresh each experiment -- 754 mg., weighed out on a Mettler analytical balance, were brought up to 50 ml. with Ringer's solution in a graduated cylinder (5.5x10^{-2}M ACh).

Carbamylcholine chloride (K & K Laboratories)

5.5×10^{-2}M solutions were prepared by bringing 500 mg. of the chemical to 50 ml. with Ringer's solution. Carb solutions were sometimes refrigerated for several days but were at room temperature when used.

One hundred mg. of prostigmine (Neostigmine methylsulfate; K & K Laboratories) were diluted to 100 ml. with Ringer's solution. Five ml. of this stock solution (1 mg/ml) were diluted to 500 ml. to give a concentration of 10^{-5} g/ml, or 3×10^{-5}M. The stock solution was refrigerated but never kept more than one week.

All bulk chemicals used were guaranteed by the manufacturer to be 95 - 99% pure.

RESULTS

Preliminary experiments:

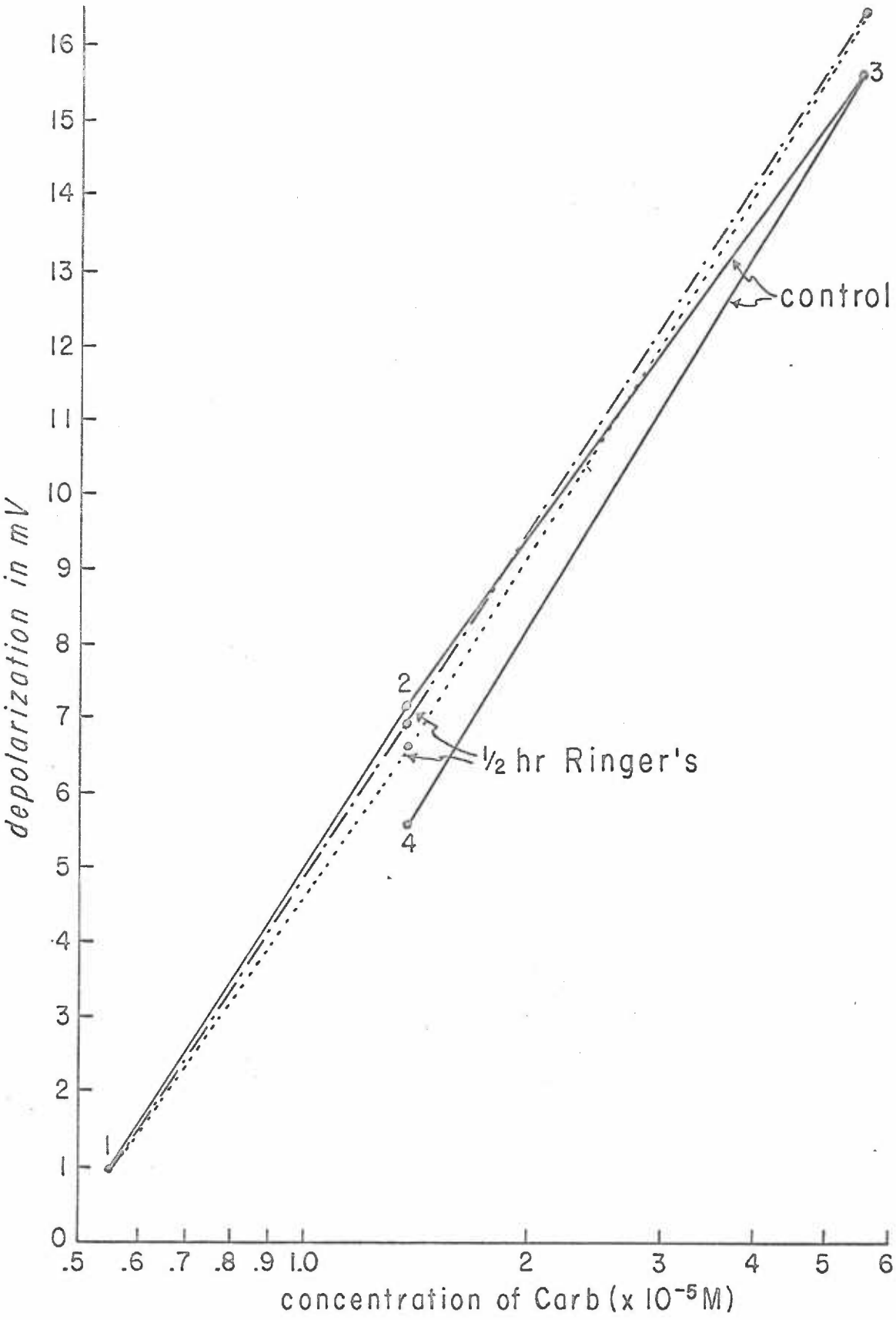
Initial work with the muscle involved lower concentrations of depolarizing agent than those later employed in testing the hypothesis. Since there is no evidence that a maximal response represents 100% receptor participation, concentrations 200 times those giving a maximal response were used in the final acquisition of data. Nevertheless the results of early experiments utilizing lower agonist concentrations contributed information influencing the final experimental design.

Evidence that a reproducible quantitative response could be elicited from this preparation is contained in Fig. 10, an illustration of three Carb dose-response curves obtained on the same muscle allowing one-half hour intervals between curves (disregard point 4, to be discussed in the next paragraph). Each point represents the maximal depolarization obtained by tracking the muscle four times with a given concentration of Carb. Clearly the responses of this m. ext. long. dig. IV are related to the strength of the depolarizing solution, and the capacity of the muscle to exhibit this responsiveness is undiminished in successive trials.

Fig. 10. Three Carb dose-response curves obtained on the same muscle separated by intervals of one-half hour. The first, or control, curve is represented by the solid line. After one-half hour in Ringer's the muscle responded to three different doses of Carb in the manner indicated by (-·-·-). The muscle was again placed in Ringer's Solution for one-half hour before the values for the third curve (·····) were determined.

Each plotted point represents the maximum depolarization produced by tracking the muscle four times with a given dose of Carb, a procedure requiring less than a minute. Following this the muscle was washed twice with 30 ml. of Ringer's Solution; this washing period totaled six minutes. Therefore, it required approximately one-half hour to gather the data represented by the solid line. After the collection of this data, the muscle was left in Ringer's Solution for one-half hour before another curve was attempted.

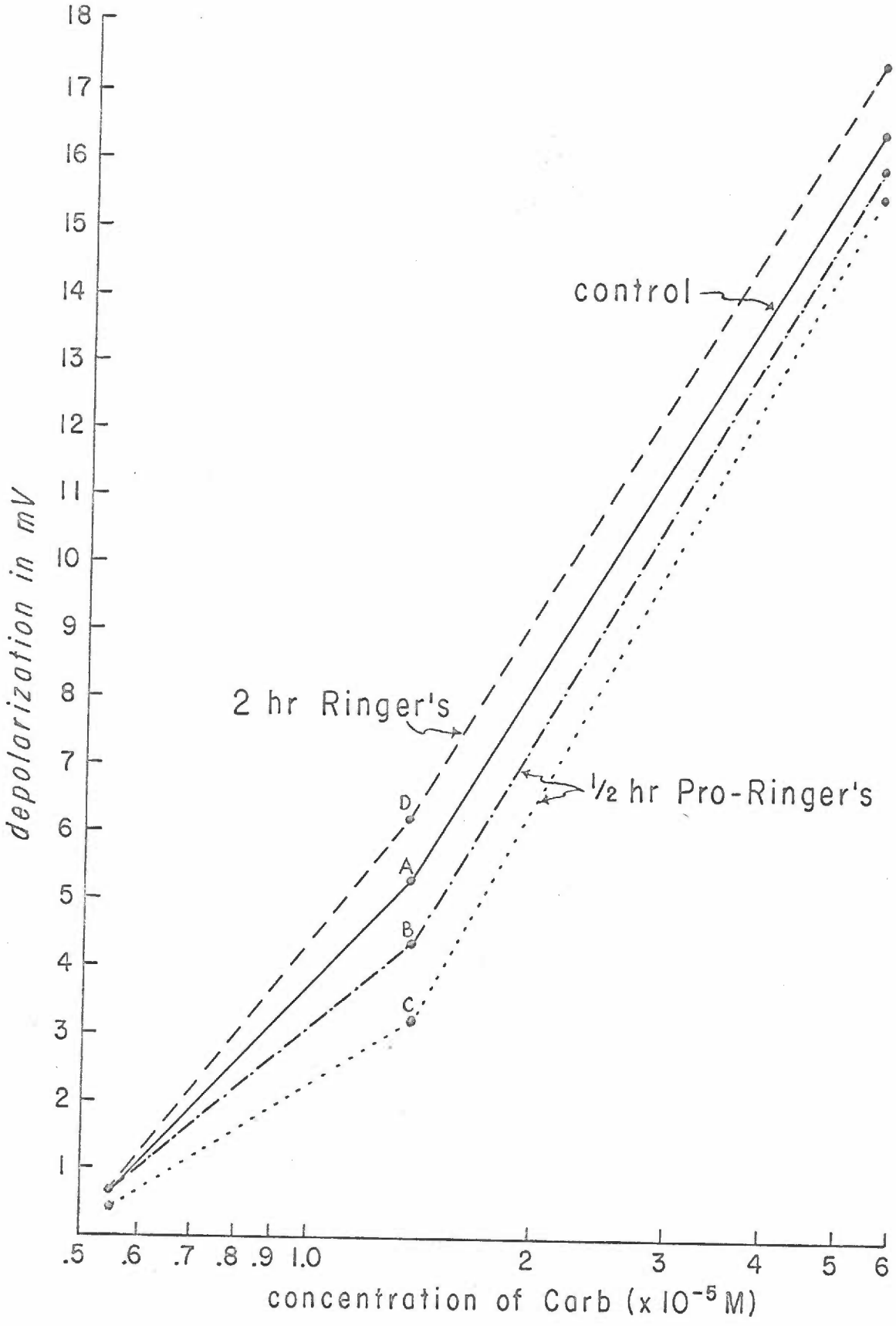
It should be noted that the curve indicated by the solid line involved two exposures (points numbered 2 and 4) to 1.37×10^{-5}M Carb, and the data yielding point 4 was taken from a muscle depolarized only 7 minutes previously by 5.5×10^{-5}M Carb. After sufficient time had elapsed (at least one-half hour) the muscle was able to reproduce the depolarization to 1.37×10^{-2}M Carb noted by point 2.


depolarization in mV
concentration of Carb (x 10⁻⁵M)
control
½ hr Ringer's
1
2
3
4
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
.5
.6
.7
.8
.9
1.0
2
3
4
5
6

That the problem of desensitization existed but could be circumvented by allowing time intervals of at least one-half hour to elapse between exposures is indicated in the same figure. Although the muscle was washed with Ringer's twice after each reading was taken, the second response to 1.37×10^{-5}M Carb (point 4) indicates only 5.6 mv. of depolarization, a decrease of 1.6 mv. from the depolarization evoked by the same dose earlier in the experiment. It is known that the amount of desensitization varies with the concentration of the depolarizing agent. Therefore Jenkinson (69) chose to use extremely small doses of Carb in his studies of curare-carbachol competition at the motor end-plate. An alternative solution to the problem is to allow a sufficient time interval between exposures for the muscle to recover from desensitization. This is in agreement with the results of other workers (47, 85).

In the presence of prostigmine, however, the responsiveness of the muscle was not stable -- even though the recommended time interval was allowed for recovery. Data plotted on curves B and C of Fig. 11 were obtained with Carb on muscle which had been exposed to prostigmine for one-half hour prior to bioassay, or "prostigminized"

Fig. 11. The effect of prostigmine (10^{-5} g/ml) on Carb-dose-response relations. Curves A, B, C, D obtained in sequence from a single muscle. For each curve, the plotted points indicate the maximum value of initial depolarization obtained from 4 rapid exposures over the course of about one minute. The preparation was washed for 6 min. with the appropriate bathing solution before each new concentration. Between curves A and B the preparation was at rest in Pro-Ringer's for one-half hour. Between curves B and C the preparation was at rest again for one-half hour in Pro-Ringer's. Between curves C and D the preparation was at rest in several changes of normal Ringer's for 2 hours.


depolarization in mV
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
control
2 hr Ringer's
½ hr Pro-Ringer's
D
A
B
C
.5
.6
.7
.8
.9
1.0
2
3
4
5
6
concentration of Carb (x 10⁻⁵ M)

muscle. Observe that, in the presence of prostigmine, the curve shifted to the right, an effect which was reversed by washing the muscle in Ringer's solution for two hours (curve D). Four dose-response curves were obtained from another muscle (Fig. 12) in the following time sequence: Carb, ACh, prostigmine + ACh, Carb. The final Carb data indicated that some type of desensitization had occurred. When the prostigmine + ACh exposure was omitted (Fig. 13), the final Carb depolarization values were equal to or greater than the initial values. There is insufficient data to draw a firm conclusion regarding this effect of the anticholinesterase. Nevertheless the persistent drop in the responsiveness of a prostigminized muscle, as well as other reasons listed in the discussion, led to the abandonment of the anticholinesterase as a tool in the main task of evaluating the hypothesis.

A comparison of the depolarizing potencies of ACh and Carb at lower concentrations was helpful in resolving the problem of finding equivalent doses of the two agents. Note that in the dose comparisons (Fig. 12 and 13) ACh ($\times 10^{-3}$) was compared with Carb ($\times 10^{-5}$) -- that is, a molar ratio of 100ACh:1Carb was employed. This ratio was suggested by Nastuk and Gissen's results (92), but it would

Fig. 12. Dose-response curves. Numbers indicate the order in which the data was acquired. (———) initial response to Carb; (····) response to ACh after an hour in Ringer's solution; (-···-) response to prostigmine-ACh after one-half hour in Ringer's and one-half hour in prostigmine; (----) response to Carb after forty-five minutes in Ringer's solution.

Points were obtained as indicated in the legends for Figs. 10 and 11.

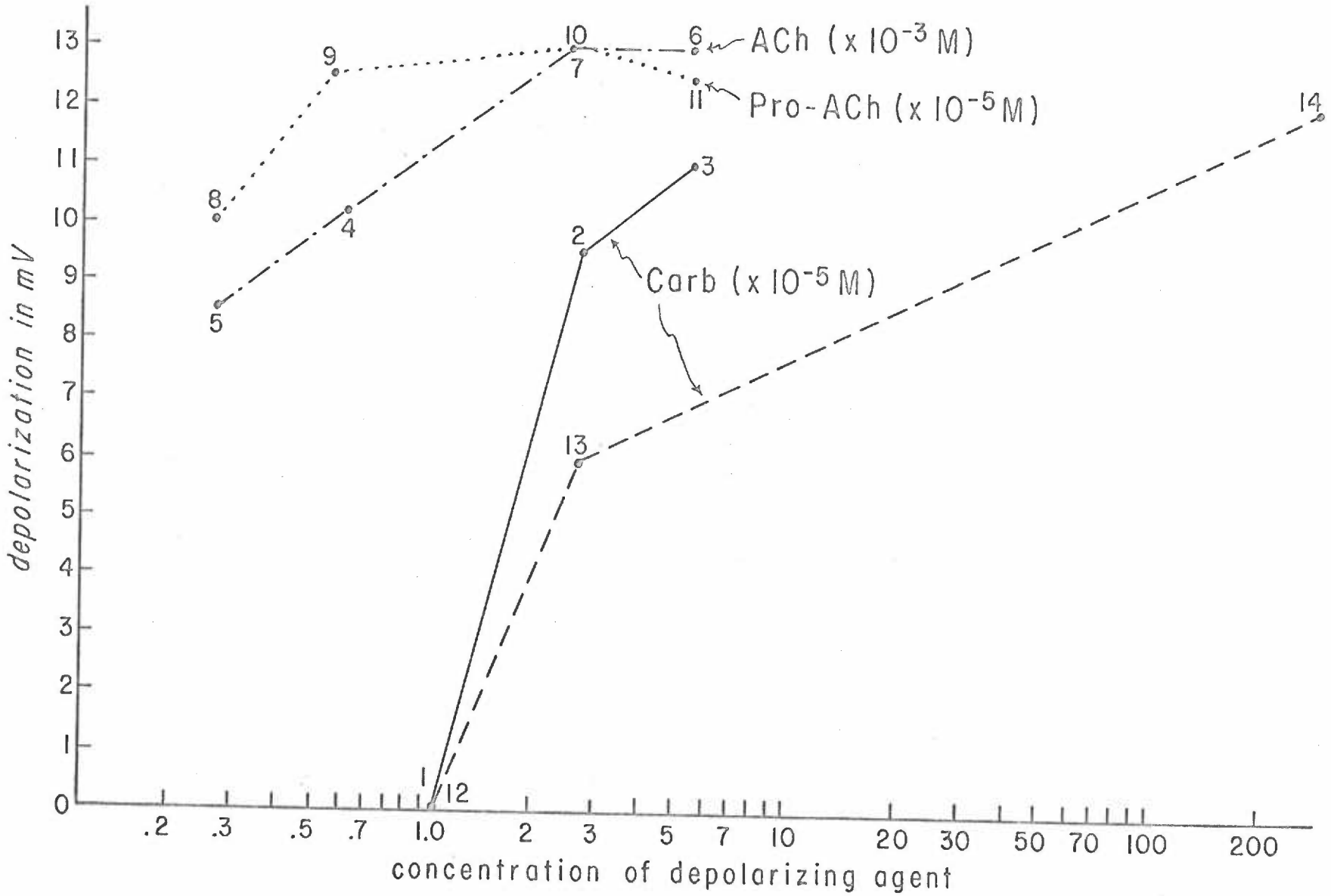
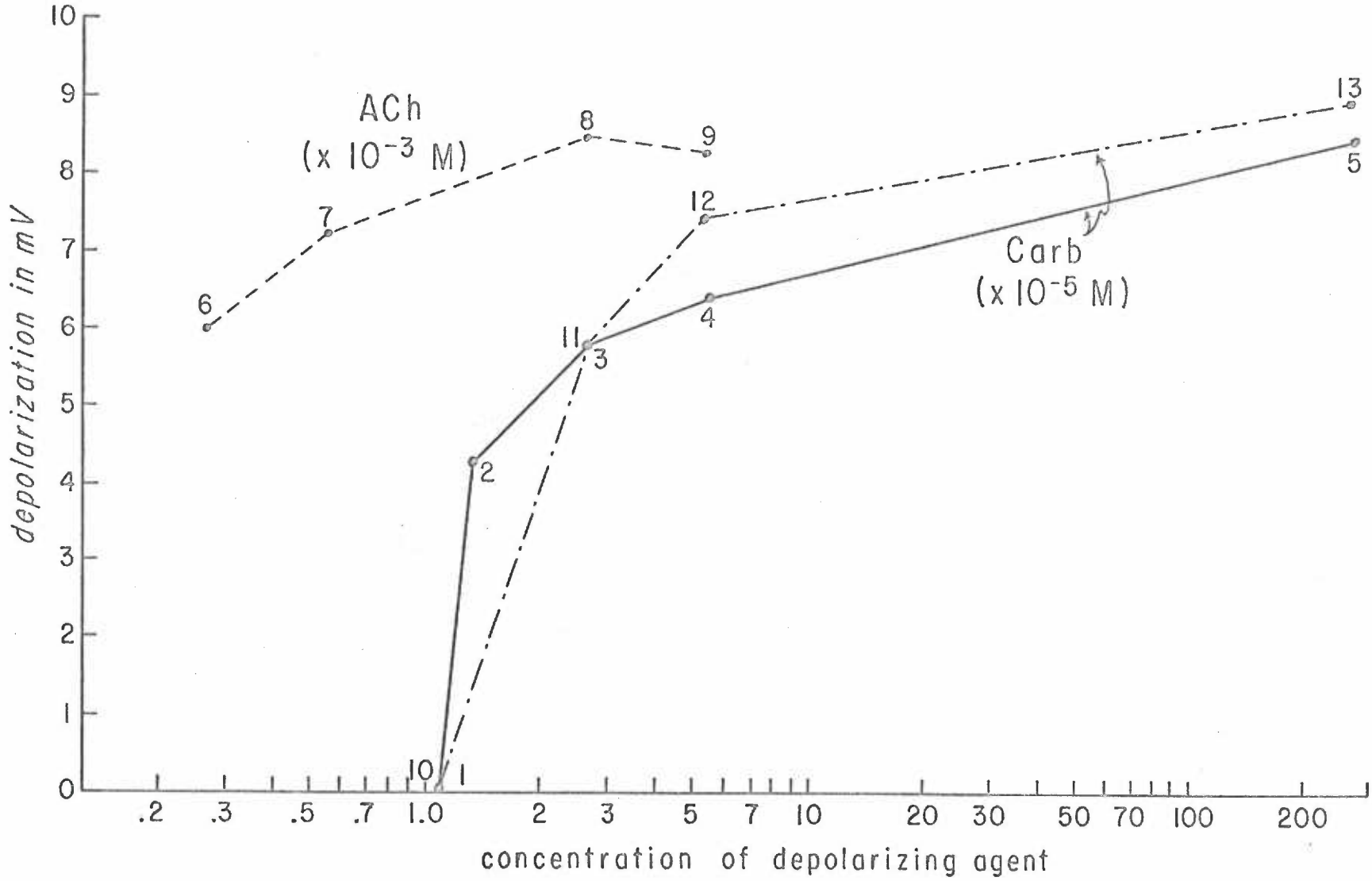

depolarization in mV
0
1
2
3
4
5
6
7
8
9
10
11
12
13
.2
.3
.5
.7
1.0
2
3
5
7
10
20
30
50
70
100
200
concentration of depolarizing agent
ACh (x 10⁻³ M)
Pro-ACh (x 10⁻⁵ M)
Carb (x 10⁻⁵ M)

Fig. 13. Dose-response curves. Numbers indicate the order in which the data was acquired. (——) initial response to Carb; (----) response to ACh after an hour in Ringer's solution; (-·-·-) response to Carb after forty-five minutes in Ringer's solution.

Points were obtained as indicated in the legends for Figs. 10 and 11.


depolarization in mV
0
1
2
3
4
5
6
7
8
9
10
ACh
(x 10⁻³ M)
Carb
(x 10⁻⁵ M)
.2
.3
.5
.7
1.0
2
3
5
7
10
20
30
50
70
100
200
concentration of depolarizing agent

appear from these dose-response curves that a higher concentration of Carb is necessary to achieve equivalence of depolarizing strength. Other ratios were then tried in single bioassay experiments and a 1:1 molar ratio of the two agents was finally selected. More will be said about this in the discussion. The use of lower concentrations in these preliminary experiments permitted the following conclusions: 1) that the preparation would give reproducible quantitative responses to a depolarizing agent; 2) that the effects of desensitization did not survive a half-hour time interval; 3) that the comparison of ACh and Carb should be made in the absence of prostigmine; and 4) that a molar dose ratio of 100ACh:1Carb did not yield equivalent responses.

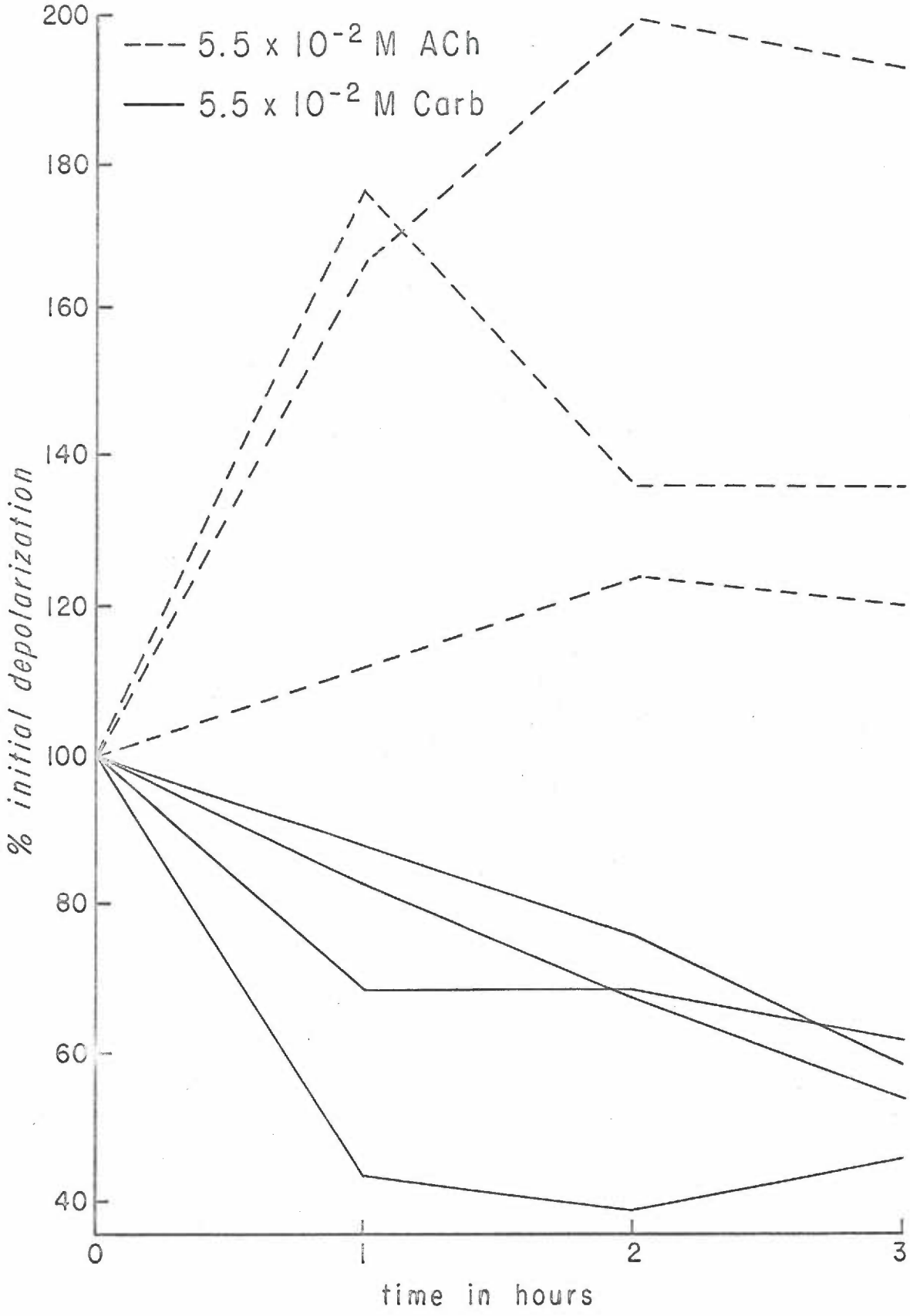
Comparisons of actions of ACh and Carb:

On the basis of the hypothesis that the ACh receptor is a cholinesterase, it is predicted that Carb-induced depolarization will decrease with subsequent doses, while ACh-induced depolarization remains constant. The criteria used to evaluate depolarization were 1) the initial amplitude and 2) the duration of depolarization.

Forty experiments were carried out at an agonist concentration of 5.5×10^{-2}M. In seven early experiments

Fig. 14. Comparison of amplitudes of depolarization obtained with 5.5 x 10^{-2}M ACh and 5.5 x 10^{-2}M Carb when exposure time equalled desensitization time. (-----) ACh; (———) Carb. Data from seven muscles -- three treated with ACh and four treated with Carb -- are presented in this figure.

Each muscle was exposed four times (0 hr., 1 hr., 2 hr., 3 hr.) to a depolarizing solution, the depolarization was recorded, and each time the drug remained in contact with the muscle until it was completely desensitized. The preparation was washed five times with 30 ml. of Ringer's (total wash solution = 150 ml.) after each exposure, and an interval of one hour separated exposures.


5.5 x 10⁻² M ACh
5.5 x 10⁻² M Carb
200
180
160
140
120
100
80
60
40
% initial depolarization
0
1
2
3
time in hours

(Fig. 14) the depolarizing agents were left in contact with the end-plate region until the tissue was completely desensitized, i.e. until depolarization had completely subsided and the pen had returned to the control baseline. Because this design involved a longer time period in the case of Carb than ACh, time of exposure was controlled in subsequent experiments. In all cases except the first Carb exposure, a ten-minute period was generally ample to allow for complete desensitization. Three experiments in which an hour drug exposure was attempted were dropped because the preparation did not return to control levels during washings. One 10-minute exposure experiment was excluded for the same reason, and two series of observations only extended through 2-hour periods. Seven other experiments were not included on the grounds that the 0-hour responses were less than 6 mv.

The results of the remaining twenty experiments are reported here. Typical records showing the responses of the m. ext. long. dig. IV to four exposures to 5.5×10^{-2}M ACh and to four exposures to 5.5×10^{-2}M Carb are reproduced in Fig. 15 and 16. The amplitude measurements were complicated by the fact that the Carb responses sometimes exhibited a biphasic character (Fig. 17). After the

Fig. 15. Exposure to 5.5 x 10^{-2}M ACh at 1-hour intervals. Records were obtained by bringing the depolarizing fluid to the endplate region and keeping the solution there for ten minutes. The initial downward deflection of the record indicates a slight positivity along this muscle as the endplate region was approached by depolarizing fluid. The initial portion of records from one muscle varies according to the control potential distribution for the muscle.

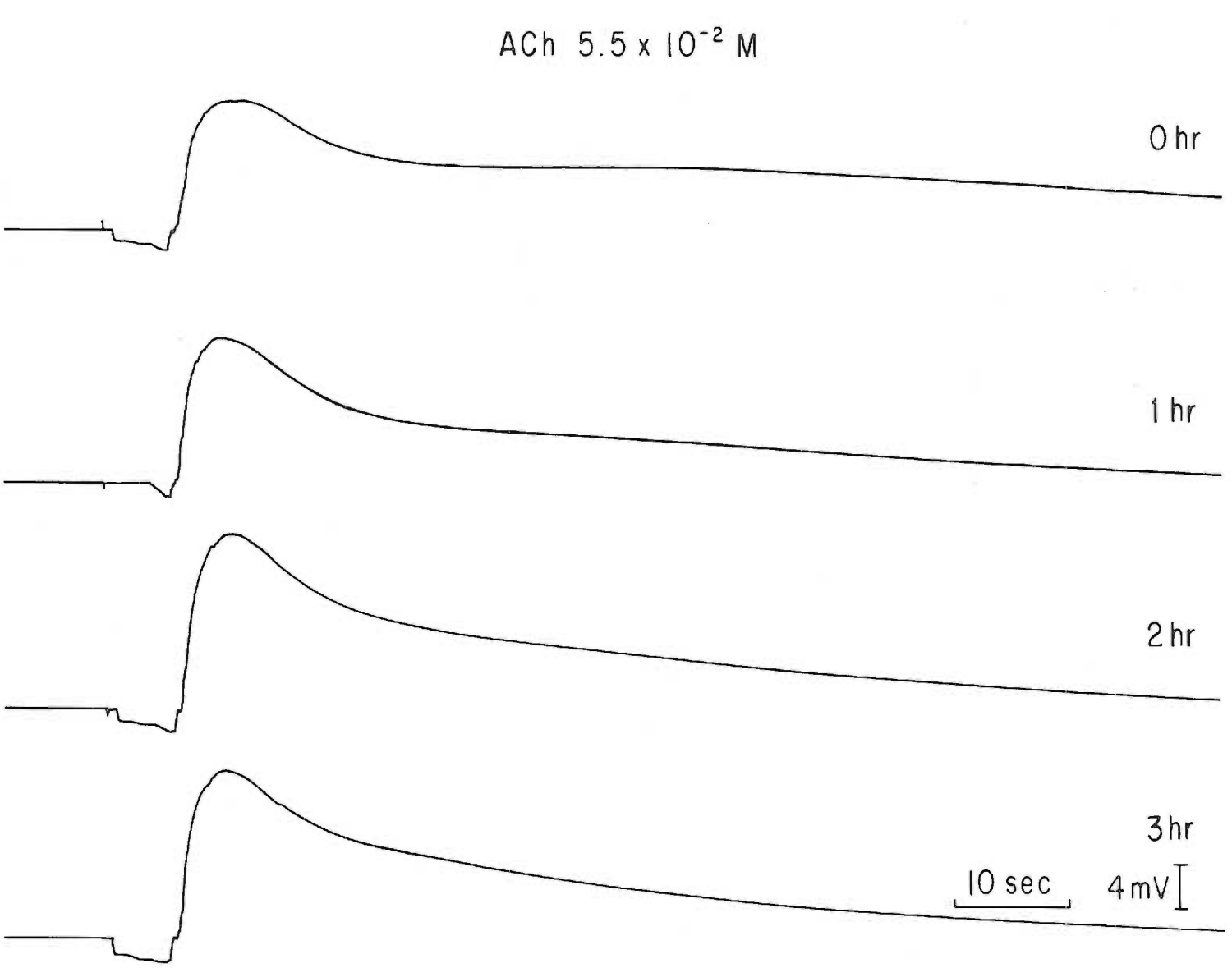

ACh 5.5 x 10⁻² M
0 hr
1 hr
2 hr
3 hr
10 sec
4 mV

Fig. 16. Exposure to 5.5 x 10^{-2}M Carb at 1-hour intervals.

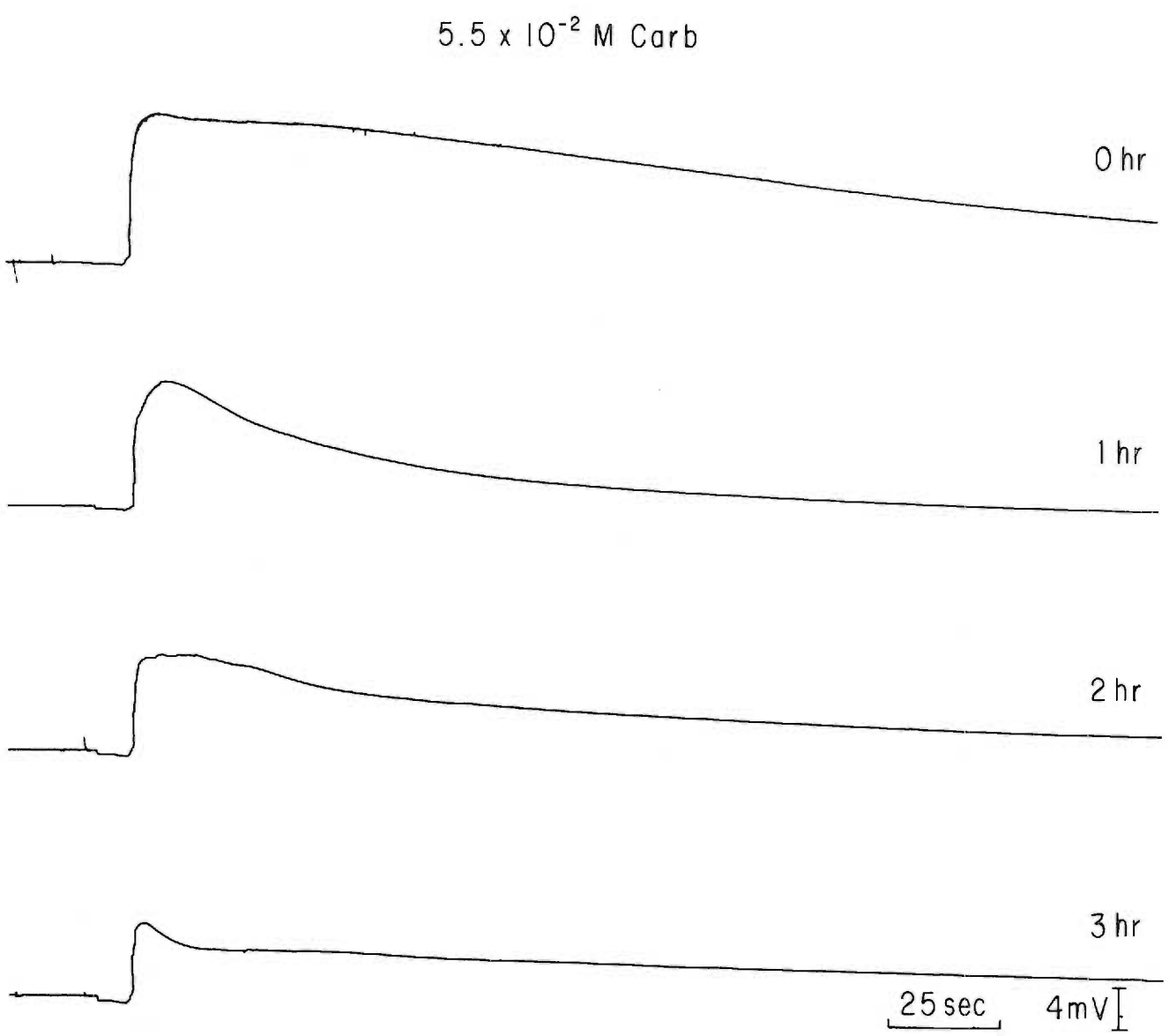
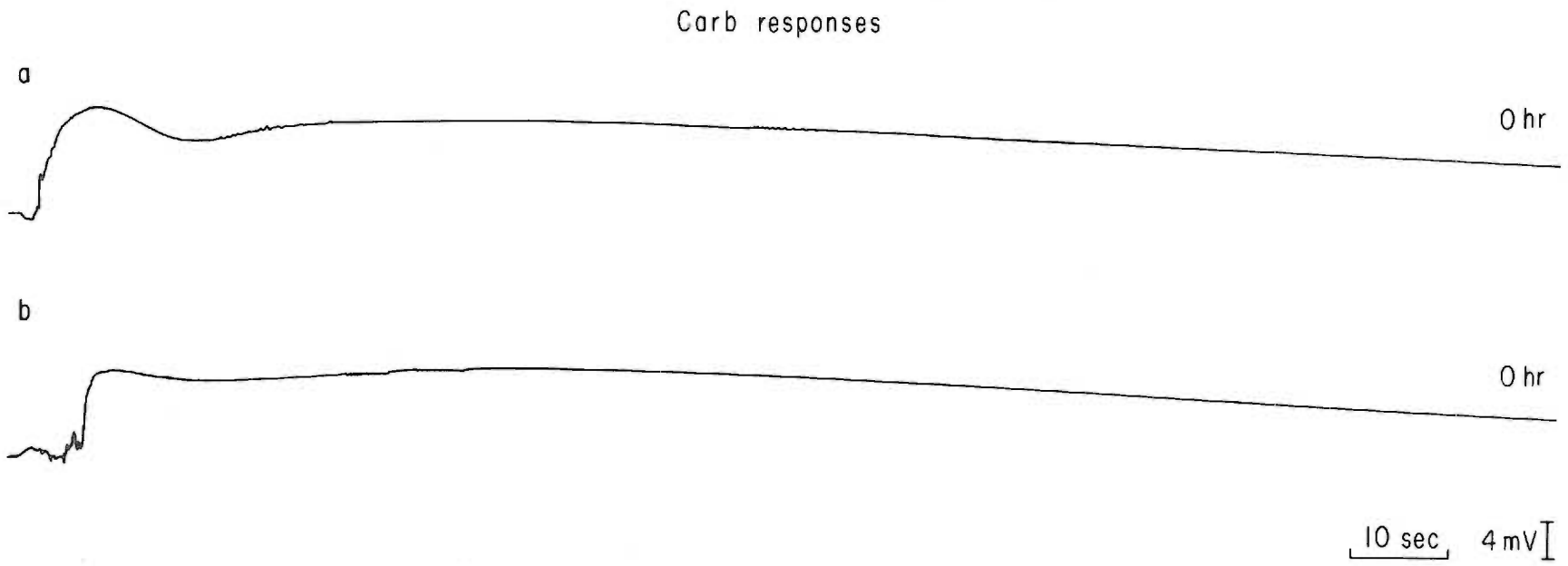

5.5 x 10⁻² M Carb
0 hr
1 hr
2 hr
3 hr
25 sec
4 mV

Fig. 17. Carb responses which illustrate a "secondary depolarization".
a) Initial exposure to 2.7×10^{-2}M Carb;
b) Initial exposure to 5.5×10^{-2}M Carb.


Carb responses
a
0 hr
b
0 hr
10 sec
4 mV

initial depolarization had begun to subside, a secondary increase in depolarization occurred that was sometimes greater in amplitude than the first response. Nevertheless, it was the amplitude of the initial depolarization that was measured. A 352 mM Ringer's solution, i.e. isosmotic with 5.5×10^{-2}M depolarizing solution, was made by adding excess NaCl and had no depolarizing effect on the frog muscle.

The results of these twenty experiments in which the amplitude of depolarization produced by 5.5×10^{-2}M ACh was compared with the depolarization induced by 5.5×10^{-2}M Carb in the manner outlined in the methods section are plotted in Fig. 18. The same data was normalized for plotting in Fig. 19. Means of the two populations were calculated and compared statistically using the two-tailed Student t test (Table I). The statistical significance of differences is indicated in the final row or column of all tables; percentages refer to the probability levels that the difference would occur by chance. If the null hypothesis had to be accepted at $\alpha = .05$, the difference was not considered to be significant and was so labeled.

Fig. 18. Comparison of 5.5×10^{-2}M ACh and 5.5×10^{-2}M Carb amplitudes of depolarization (raw data).

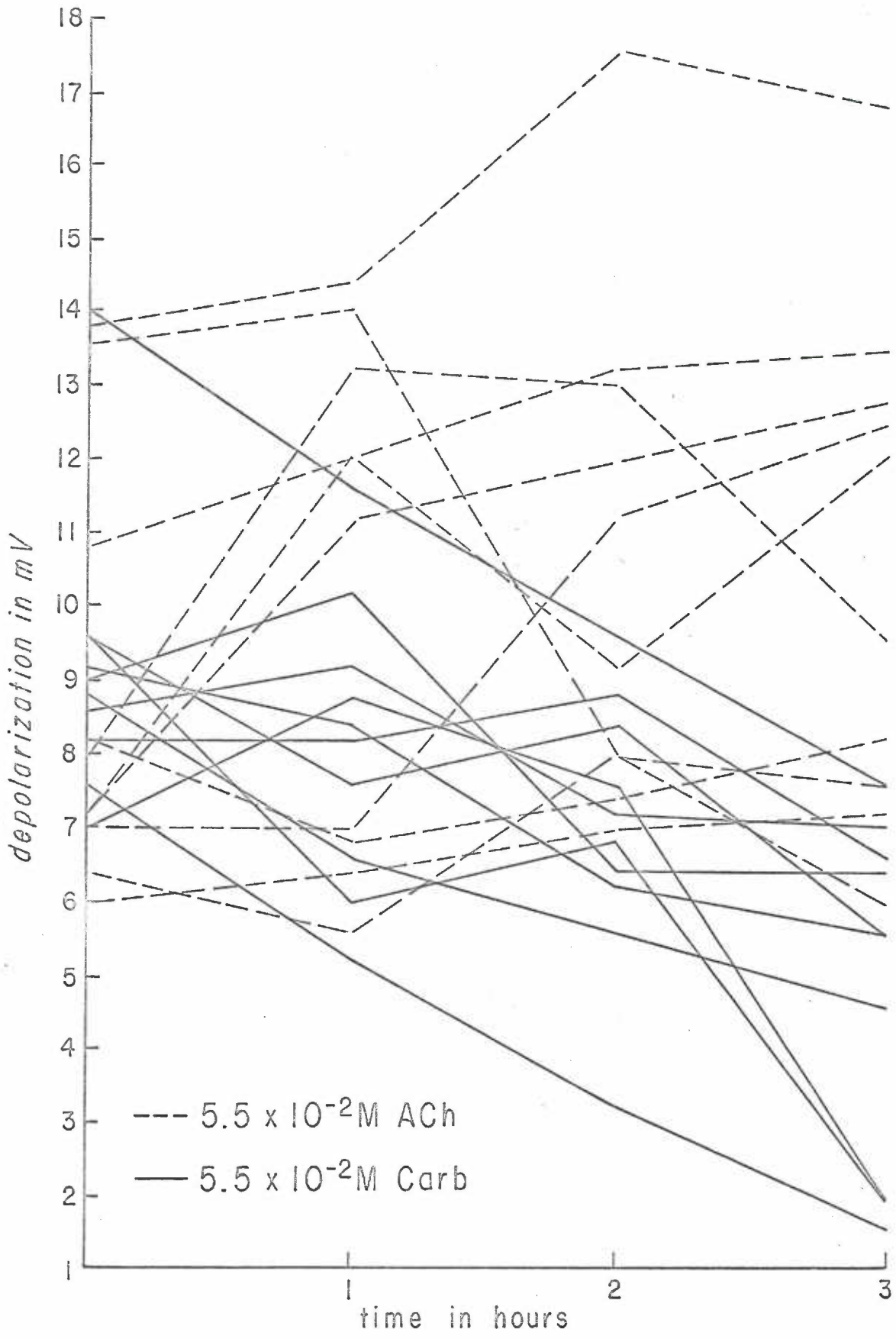

depolarization in mV
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
1
2
3
time in hours
--- 5.5 x 10-2M ACh
— 5.5 x 10-2M Carb

Fig. 19. Comparison of 5.5×10^{-2}M ACh and 5.5×10^{-2}M Carb amplitudes of depolarization (normalized data).

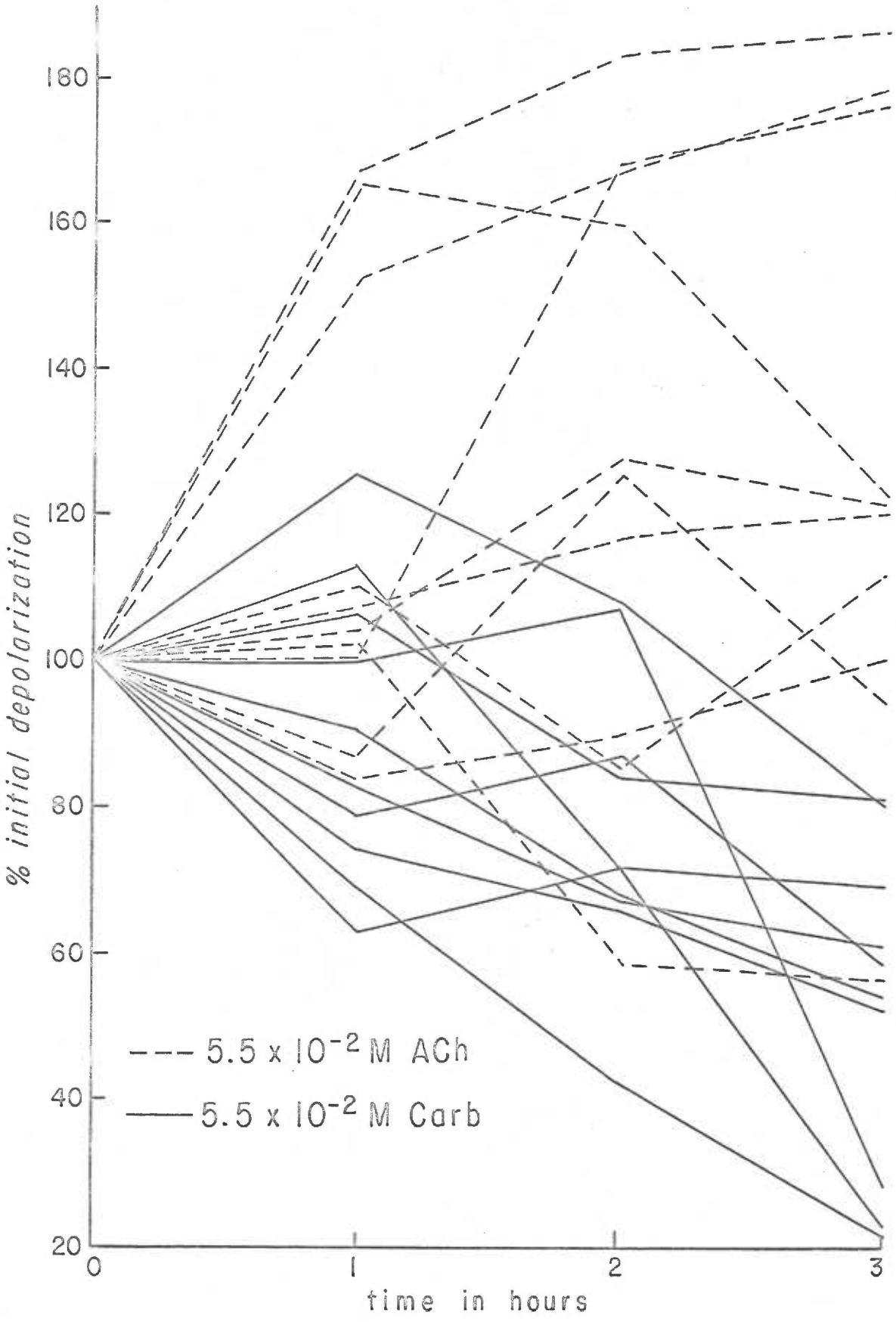

160
160
140
120
100
80
60
40
20
% initial depolarization
0
1
2
3
time in hours
--- 5.5 x 10⁻² M ACh
—— 5.5 x 10⁻² M Carb

Table I

Mean depolarization in mv. ± SD (N = 20)

	Initial	1st Hr.	2nd Hr.	3rd Hr.
ACh 5.5×10^{-2}M	8.82±2.74	10.24±3.24	10.64±3.19	10.80±3.07
Carb 5.5×10^{-2}M	9.16±1.80	8.18±1.84	6.98±1.72	4.90±2.12
	Not sig.	Not sig.	1%	1%

The 3rd hr. ACh value (10.80) was not statistically different from the initial ACh reading (8.82).

In three of these experiments the muscle was retracked with 2.75×10^{-4}M ACh, the dose originally used to locate the end-plates, an hour after the last desensitizing exposure. A muscle that had been subjected to four 10-minute exposures to 5.5×10^{-2}M Carb could not duplicate its initial responsiveness; the response to 2.75×10^{-4}M ACh was undiminished in the muscle which had been desensitized to 5.5×10^{-2}M ACh four times (Table II).

Table II

	Initial response to 2.7×10^{-4}M ACh	Final response to 2.7×10^{-4}M ACh
Carb-treated muscle	9.6 mv.	3.2 mv.
(four ten-minute exposures)	8.0 mv.	1.6 mv.
ACh-treated muscle	4.8 mv.	5.6 mv.
(four ten-minute exposures)		

Time to half-maximal values were also taken from the depolarization records in the twenty experiments previously discussed. A secondary depolarization (Fig. 17), when present, made this term somewhat meaningless, as in these instances the first eventappeared to have been superseded by a second one. Yet the time to half-maximal value did serve to emphasize the difference in the duration of depolarization brought about by the two depolarizing agents. Note the time scale difference in Figs. 15 and 16.

Half-maximal decay times from these twenty experiments are charted for comparison on Fig. 20. The results of a statistical analysis of this data are indicated in Table III.

Fig. 20. Comparison of 5.5×10^{-2}M ACh and 5.5×10^{-2}M Carb half-maximal decay times.

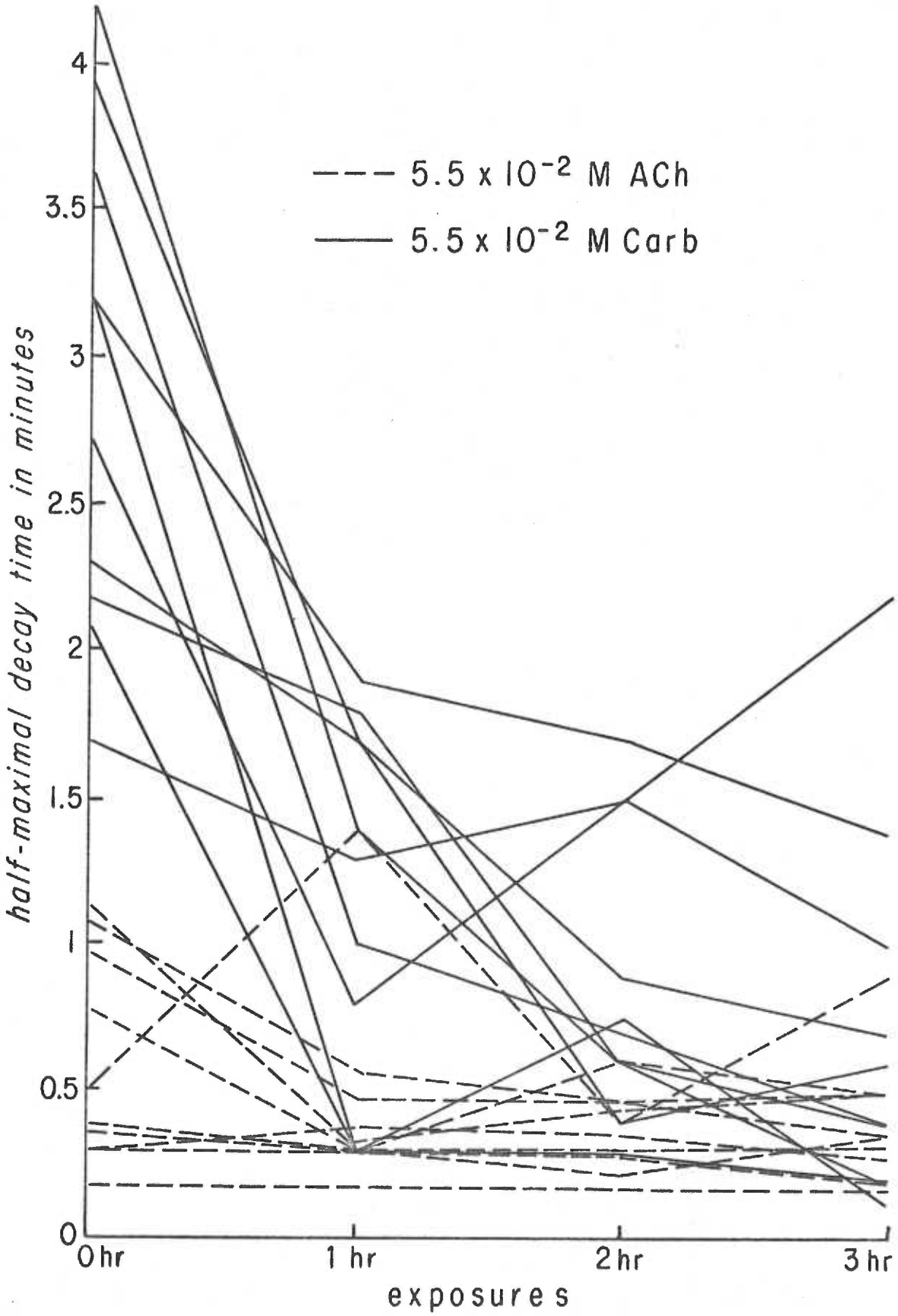

--- 5.5 x 10⁻² M ACh
—— 5.5 x 10⁻² M Carb
half-maximal decay time in minutes
4
3.5
3
2.5
2
1.5
1
0.5
0
0 hr
1 hr
2 hr
3 hr
exposures

Table III

Mean half-maximal decay time ± SD in minutes (N = 20)

	Initial	1st Hr.	2nd Hr.	3rd Hr.
ACh 5.5×10^{-2}M	.61± .34	.46± .33	.38± .12	.42± .19
Carb 5.5×10^{-2}M	2.91± .80	1.22± .57	.90± .47	.72± .62
	1%	1%	1%	Not sig.

The last figure in the ACh row is not statistically different from the first; the last figure in the Carb row is statistically different from the first at the 1% level of significance.

It is evident that the initial decay characteristic of Carb-induced depolarization is much greater than that of ACh-induced depolarization. However, the difference disappears with successive Carb desensitization doses, as the time course of the Carb response shortens significantly over the 3-hr. period.

Comparison of 2.75×10^{-2}M Carb + 2.75×10^{-2}M ACh and 2.75×10^{-2}M Carb:

These twenty experiments were performed to determine whether the presence of ACh would interfere with the Carb

amplitude effect. They were carried out in the same manner as the preceding experiments. Rather than combining 5.5×10^{-2}M ACh and 5.5×10^{-2}M Carb[1], the total molarity of the Carb-ACh solution was 5.5×10^{-2}M. Consequently, 2.7×10^{-2}M Carb experiments had to be run as a control. It was predicted that the ACh would exert a "protective" effect, or slow down the rate of receptor carbamylation. Unfortunately the results are equivocal (Tables IV and V). Although the data hints at such a possibility -- mean depolarization decreased 2.4% in the mixed group and 25.1% in the 2.7×10^{-2}M Carb group -- the variability of the results was great and a statistically significant difference could not be demonstrated between the two groups. Raw data and normalized data are plotted in Figs. 21 and 22. In Table IV the statistical comparison of the population means is indicated.

---

[1] The objection to this approach was on osmotic grounds; however the author may have been unduly cautious.

Fig. 21. Comparison of 2.7×10^{-2}M Carb and 2.7×10^{-2}M Carb + 2.7×10^{-2}M ACh amplitudes of depolarization (raw data).

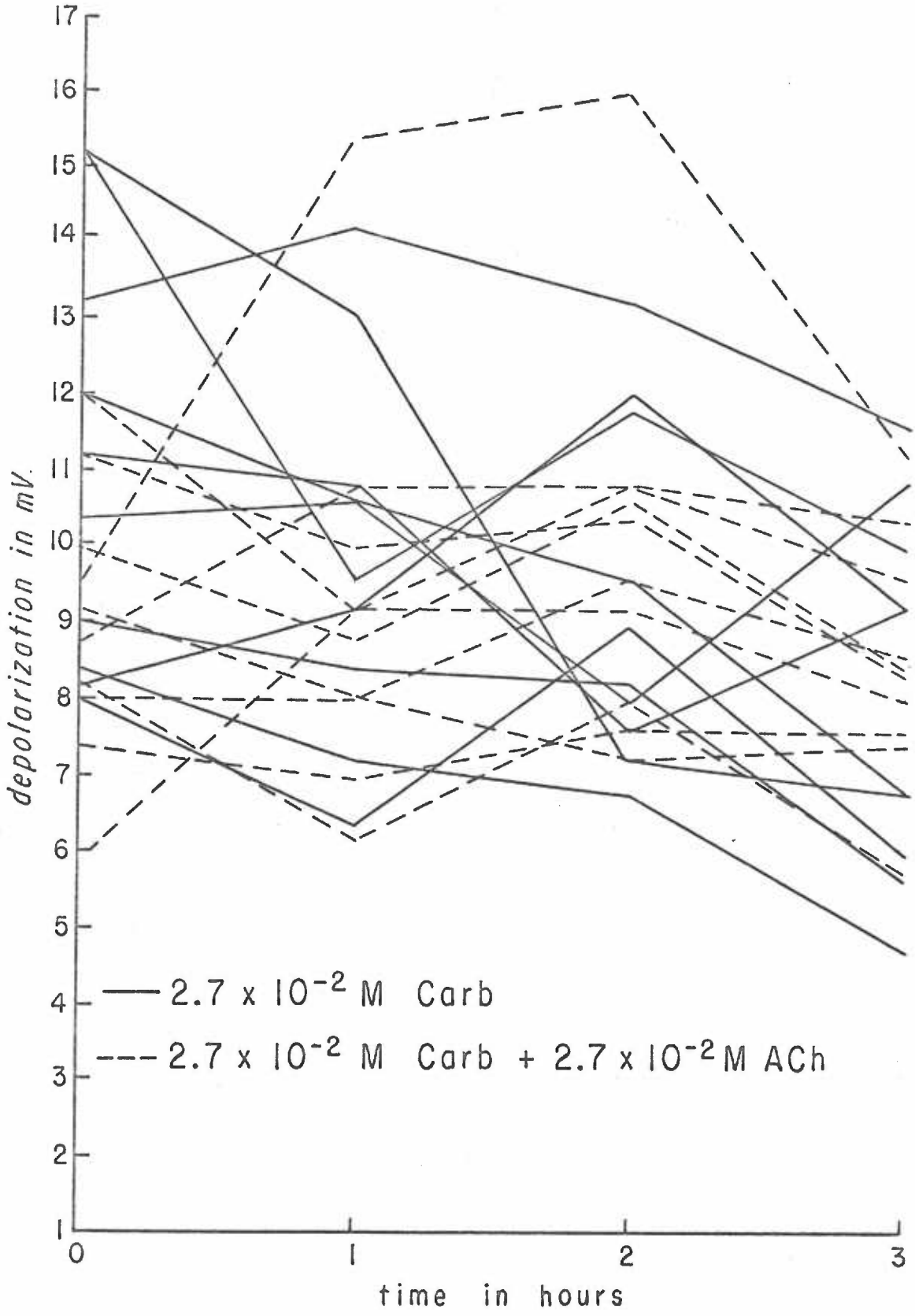

depolarization in mV.
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
1
2
3
time in hours
2.7 x 10⁻² M Carb
2.7 x 10⁻² M Carb + 2.7 x 10⁻² M ACh

Fig. 22. Comparison of 2.7×10^{-2}M Carb and 2.7×10^{-2}M Carb + 2.7×10^{-2}M ACh amplitudes of depolarization (normalized data).

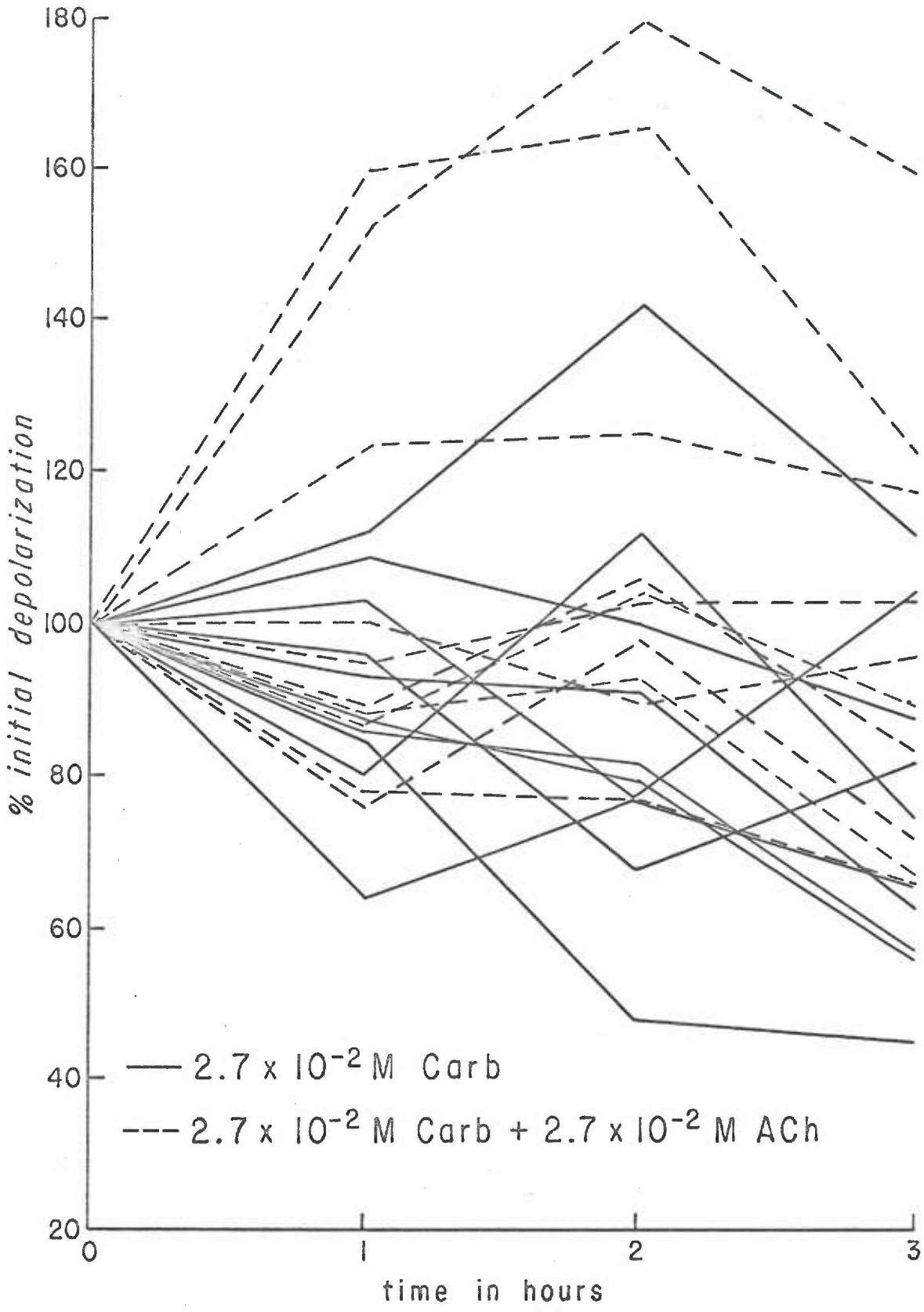

180
160
140
120
100
80
60
40
20
% initial depolarization
0
1
2
3
time in hours
2.7 x 10⁻² M Carb
2.7 x 10⁻² M Carb + 2.7 x 10⁻² M ACh

Table IV

Mean depolarization ± SD in mv.

N = 20

	Initial	3rd Hr.	
2.7×10^{-2}M Carb	11.08±2.63	8.09±2.23	5%
2.7×10^{-2}M Carb + 2.7×10^{-2}M ACh	9.04±1.69	8.54±1.47	5%
	Not sig.	Not sig.	

Because the initial mean depolarization in the 2.75×10^{-2}M Carb group was higher than that in the mixed group (in fact, at the 10% level significantly higher than the mean figured on all initial depolarizations excluding the 2.75×10^{-2}M Carb data) a statistical treatment of the normalized data is also included (Table V).

Table V

Mean % initial depolarization ± SD

N = 20

	Initial	3rd Hr.	
2.7×10^{-2}M Carb	100	74.9±20.5	1%
2.7×10^{-2}M Carb + 2.7×10^{-2}M ACh	100	97.6±28.08	Not sig.
		Not sig. (However it would be if one-tailed test were used.)	

Fig. 23. Comparison of 2.7×10^{-2}M Carb and 2.7×10^{-2}M Carb + 2.7×10^{-2}M ACh half-maximal decay times.

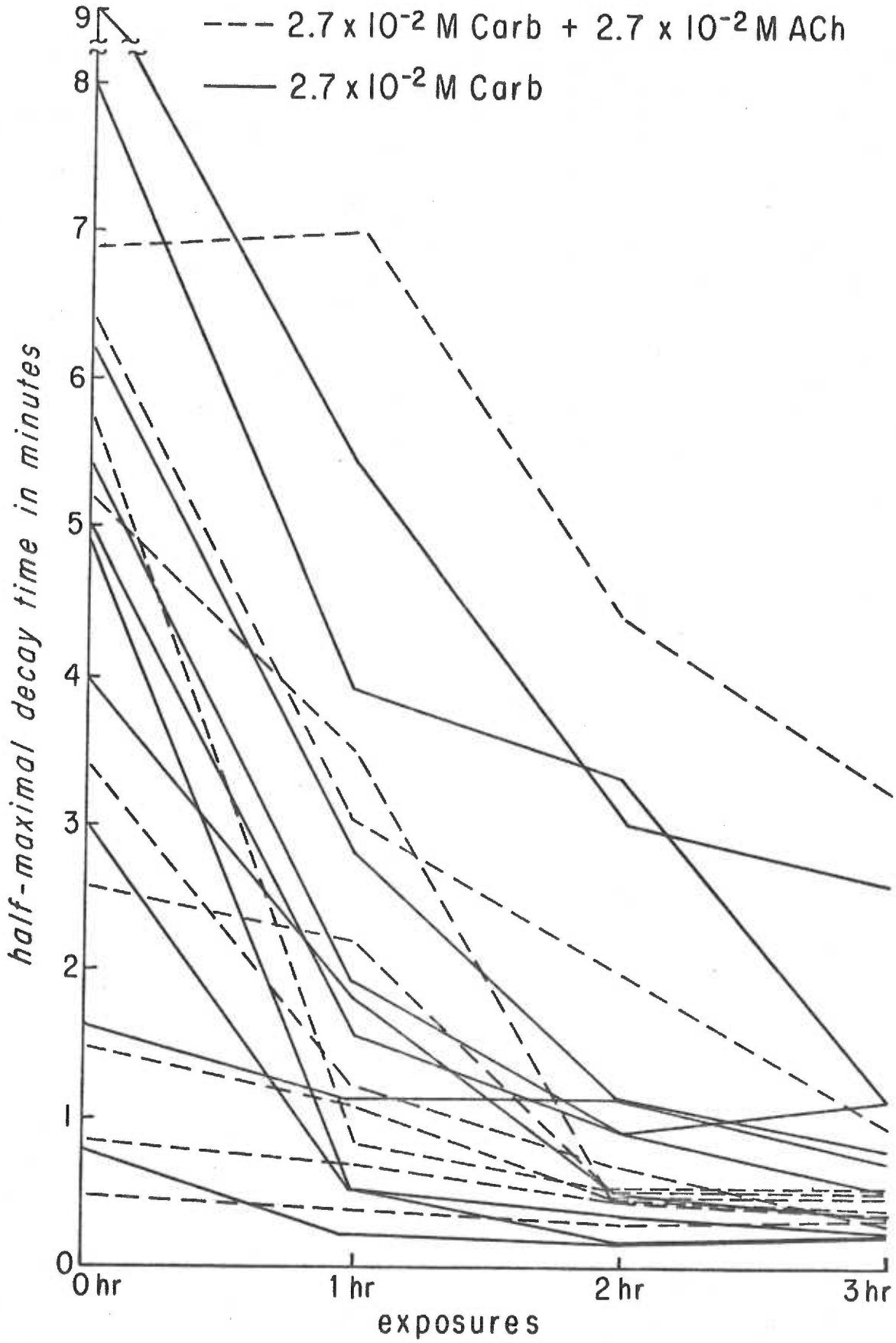

--- 2.7 x 10⁻² M Carb + 2.7 x 10⁻² M ACh
—— 2.7 x 10⁻² M Carb
half-maximal decay time in minutes
9
8
7
6
5
4
3
2
1
0
0 hr
1 hr
2 hr
3 hr
exposures

Fig. 23 presents the changes in half-maximal decay time which occurred in both groups. There was no difference between the two groups, and a statistically significant difference (1% level) exists between the first run and the last run in both groups (Table VI). These results indicate that the effect of Carb on time course was unaltered by the presence of ACh.

Table VI

Mean half-maximal decay time ± SD in minutes

N = 19

	Initial	3rd Hr.	
2.7×10^{-2}M Carb	4.74±2.41	.77± .71	1%
2.7×10^{-2}M Carb + 2.7×10^{-2}M ACh	3.44±2.03	.66± .89	1%
	Not sig.	Not sig.	

Other statistical comparisons:

Certain other comparisons, though not germane to the main body of the thesis seemed of interest. Tables VII and VIII compare the means of the two Carb groups.

Table VII

Mean depolarization ± SD in mv.

N = 20

	Initial	3rd Hr.	
2.7 x 10^{-2}M Carb	11.08±2.63	8.09±2.23	5%
5.5 x 10^{-2}M Carb	9.16±1.80	4.90±2.12	1%
	Not sig.	Not sig.	

From these results one can see that a concentration of 5.5 x 10^{-2}M Carb was more effective in reducing the amplitude of depolarization than was 2.7 x 10^{-2}M solution. As a single experiment indicated that four exposures to 2.7 x 10^{-3}M solution Carb did not produce an amplitude decrease, one can surmise that high concentrations are essential to demonstrate the reduction in amplitude of depolarization.

Table VIII

Mean half-maximal decay time ± SD in minutes

N = 19

	Initial	3rd Hr.	
2.7 x 10^{-2}M Carb	4.74±2.41	.77± .71	1% (previously noted)
5.5 x 10^{-2}M Carb	2.91± .80	.72± .62	1%
	1%		

The weaker Carb solution exhibits a longer half-maximal decay time. As rate of desensitization is contributing to the shape of this decay curve, one might predict the results here shown -- the higher the concentration of depolarizer, the faster desensitization will occur.

Finally all data from three solutions of 5.5×10^{-2} molarity is tabulated for comparison in Tables IX and X; here "mixed" refers to 2.7×10^{-2}M Carb + 2.7×10^{-2}M ACh.

Table IX

Mean depolarization ± SD in mv.

	ACh 5.5×10^{-2}M	Mixed 5.5×10^{-2}M	Carb 5.5×10^{-2}M
Initial	8.82±2.74	9.04±1.69	9.16±1.80
	No statistical differences between initial values		
3rd Hr.	10.80±3.07	8.54±1.47	4.90±2.12
	Not sig.	1%	

The initial depolarizing power of the three solutions is similar. After three hours only the Carb solution had produced any change in mean amplitude of depolarization. A protective effect cannot be claimed for the ACh in the mixed solution, though, for that same solution contains only 2.7×10^{-2}M Carb, a concentration which is shown by

Table VII to be less effective than 5.5 x 10^{-2}M Carb.

The last table (Table X) reemphasizes that factors other than concentration are influencing the decay curves and gives us data which allow us to reason concerning these other factors.

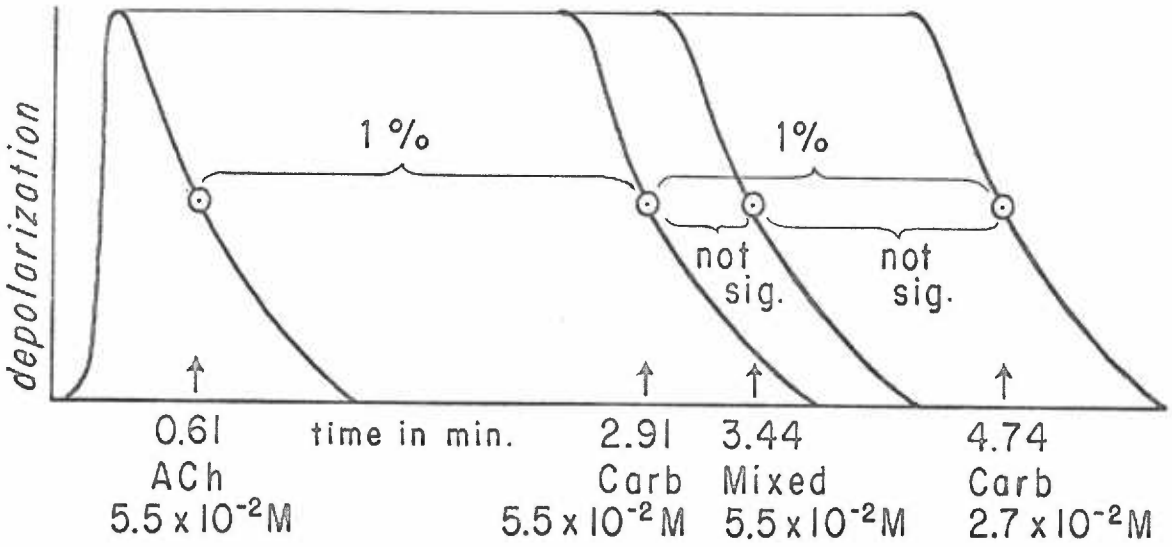
Table X

Mean half-maximal decay time ± SD in minutes

	ACh 5.5x10^{-2}M	Mixed 5.5x10^{-2}M	Carb 5.5x10^{-2}M
Initial	.61± .34	3.44±2.03	2.91± .80
	1%	Not sig.	

Fig. 24 is a compilation of all mean half-maximal decay values. Considering the facts that (1) the addition of 2.7 x 10^{-2}M Carb to 2.7 x 10^{-2}M Carb (5.5 x 10^{-2}M Carb) reduces the decay time course significantly -- i.e. 2.91 is significantly shorter than 4.74 and(2) the addition of 2.7 x 10^{-2}M ACh to 2.7 x 10^{-2}M Carb does not reduce the decay curve significantly -- i.e. 3.44 is not significantly smaller than 4.74, it is difficult to believe that the ACh molecule is more potent than Carb in producing some non-specific response -- desensitization.

Fig. 24. Graphical summary of mean half-maximal decay times. Percentages emphasize that differences indicated by brackets are statistically significant at the 1% level.


depolarization
1 %
1%
not sig.
not sig.
0.61
ACh
5.5 x 10⁻²M
time in min.
2.91
Carb
5.5 x 10⁻²M
3.44
Mixed
5.5 x 10⁻²M
4.74
Carb
2.7 x 10⁻²M

DISCUSSION

The data have yielded two primary differences between the responses to ACh and Carb. (1) There is no significant change in the amplitude of ACh-induced depolarization over four sequential exposures; there is a significant decrease in the amplitude of Carb-induced depolarization (Table I). (2) There is no significant change in the time course of ACh-induced depolarization coincident with repeated exposures; there is a significant decrease in the time course of Carb-induced depolarization (Table III).

The muscle end-plate apparently fully recovers its ability to respond after being depolarized and desensitized by a high ACh concentration. Since depolarization is a function of receptor activation, it would appear that the receptors are not "permanently" disabled by the exposures to high concentrations of ACh. Further evidence for this comes from the observation (Table II) that the response of a muscle to 2.75×10^{-4}M ACh is actually slightly greater, not less than the response to the same concentration registered before the four ACh desensitizing exposures. If the reaction of the receptor with ACh is represented by the classical enzyme-substrate equation (page 19), an

acetylated receptor would be formed as an intermediate (ES'). The forecast that ACh-induced depolarization would remain constant was based on the fact that this ACh intermediate is rapidly hydrolyzed. The results of the ACh studies are at least consistent with the hypothesis that the receptor has a dual function and the data serve as a control against which to compare the effects of Carb.

In contrast, the capability of the end-plate regions to be depolarized, as monitored by both amplitude and duration of depolarization, has decreased as a result of exposures to high concentrations of Carb. That this effect on amplitude is not immediately apparent (at 1-hr. exposure) could be due to the presence of spare receptors. Evidence that the functional ability of the ACh receptors is reduced after Carb treatment is indicated in Table II, where pre- and post-Carb comparisons of ACh depolarization amplitude is tabulated. It is possible that the receptor population available for combination has been diminished by the formation of an appreciable number of carbamylated receptor sites (Fig. 2c). Carbamylation of the enzyme AChE is known to occur (93) and is measured by the inhibition of ACh hydrolysis. Should the receptor and enzyme be identical, Carb would form a stable intermediate with the receptor, thus limiting the ability of the receptor to

respond, or to induce depolarization. The decrease in Carb-induced depolarization does occur as predicted by the hypothesis. That ACh and Carb have specifically different long-time effects on the receptor appears to have been demonstrated. These results then are consistent with the hypothesis that the ACh receptor is a ChE.

The interpretation of amplitude changes:

The most reliable measurement of ACh-receptor population that we can derive from this kind of data is the amplitude of depolarization. This statement is based on the two facts that curare is thought to compete with ACh for the receptor (69) and that it primarily effects the amplitude of end-plate potentials (83).

Nevertheless, changes in amplitude could mean either (1) an alteration in number of involved receptors, (2) some change in the receptors' specific ability to render the membrane permeable to ions, or (3) a change in ionic ratios not capable of being overcome by the membrane "pumps". Depolarization is but the electrical expression of ionic shifts which occur at the motor end-plate as a result of changes in muscle membrane permeabilities brought about by depolarizing agents such as acetylcholine. Originally it was concluded that permeability to sodium, potassium, and

chloride was increased by the transmitter. Recently, however, Takeuchi and Takeuchi (94) assessed the fraction of the total conductance due to each of these ion species by observing the effects that changes in concentration of each have on the equilibrium potential for the end-plate current. These studies have indicated that the transmitter produces a simultaneous indiscriminate rise of both Na and K permeability, but that Cl and anions in general seem to play no important part in this process. To reiterate -- amplitude of depolarization is only an indirect measurement of receptor population.

Although a specific differential effect of ACh and Carb on the receptor -- with the above reservations appended -- appears the most likely explanation for the amplitude changes seen, other possibilities have been considered. It is conceivable that the 1:1 molar dose ratio gives Carb a depolarizing advantage. The molar dose ratio used in these experiments was arrived at as the result of numerous trials based on the observations of previous investigators. The ratio of 100ACh:1Carb, found by Nastuk and Gissen (92), using isometric tension as the index of activity, proved in the present situation to be completely unusable. By a series of trial runs with 2.7×10^{-3}M ACh and varying concentrations of Carb, it was

eventually found that equal concentrations of ACh and Carb had equivalent depolarizing actions on the m. ext. long. dig. IV. Castillo and Katz (95) have discovered in iontophoretic studies that the coulomb dose which was required to produce a depolarization of given amplitude was on the average about three to four times greater for Carb than for ACh. They found large variations and cautioned that their method did not allow an accurate comparison of drug potencies to be made. Welsh and Taub (96) employed a very different preparation, the isolated heart of Venus mercenaria, and reported a molar ratio of 1ACh:80Carb. Burgen (97) found ACh to be "only slightly more active than Carb" in his experiments on the muscarinic receptor of the guinea-pig ileum. When bioassay material is treated with anticholinesterase, the ACh:Carb ratio shifts to indicate the greater potency of ACh under these conditions. Because the concentrations of depolarizing agents used in the experiments here reported are so great, tissue ChE would be largely inhibited; a 1:1 molar dose ratio would tend to favor ACh rather than Carb. In view of the almost identical molecular weights of ACh and Carb, a 1:1 ratio ought also to further insure equal diffusion rates of the two substances to the active sites.

Another argument against attributing the reduction

in the amplitude of Carb-induced depolarization to the notion that 5.5 x 10^{-2}M Carb is a stronger depolarizing agent than 5.5 x 10^{-2}M ACh can be invoked from the decay time data. If the decay time courses recorded represented mainly a differential time course of desensitization, then one could conclude that desensitization to 5.5 x 10^{-2}M ACh proceeded more rapidly than desensitization to 5.5 x 10^{-2}M Carb. Since desensitization has been shown to be related to agonist concentration (85), one could reason that an ACh concentration which was a more effective desensitizing solution than the comparable Carb concentration would be more likely to have the greater depolarizing potency.

Another interpretation of the amplitude data would be that Carb had a non-specific effect on membrane permeability (aside from thatgoverned by receptor activation), leading to a reduction in membrane resting potential. This would reduce the ionic concentration gradients and thereby the flow of charge which results from the action of a depolarizer. The fact that the reference electrode and upper portion of the muscle were never subjected to the high concentration of agonist plus the return of the muscle membrane potential to its control value in Ringer's solution would argue against the above idea. Katz and Miledi

(98) showed that 5.5×10^{-2} ACh (1% ACh) applied to the sartorius muscle membrane electrophoretically produced no effect. A 10% ACh solution (5.5×10^{-1}M) did produce a slow local depolarization and contraction but also probably caused osmotic damage. In experiments with m. ext. long. dig. IV depolarizations of 2 mv. or less were produced by both depolarizing solutions (5.5×10^{-2}M ACh and 5.5×10^{-2}M Carb) in regions away from the end-plate. This is most probably due to the depolarization of the slow fiber end-plates, though Nastuk and Gissen (92) did see depolarization of the sartorius muscle at regions away from the end-plate which recovered even after a two-hour bath exposure to $.027 \times 10^{-3}$M Carb.

It is possible that Carb, but not ACh, could exert a non-specific stabilizing effect on membrane permeability; however, there is no evidence for this and no a priori reason to argue for such an effect.

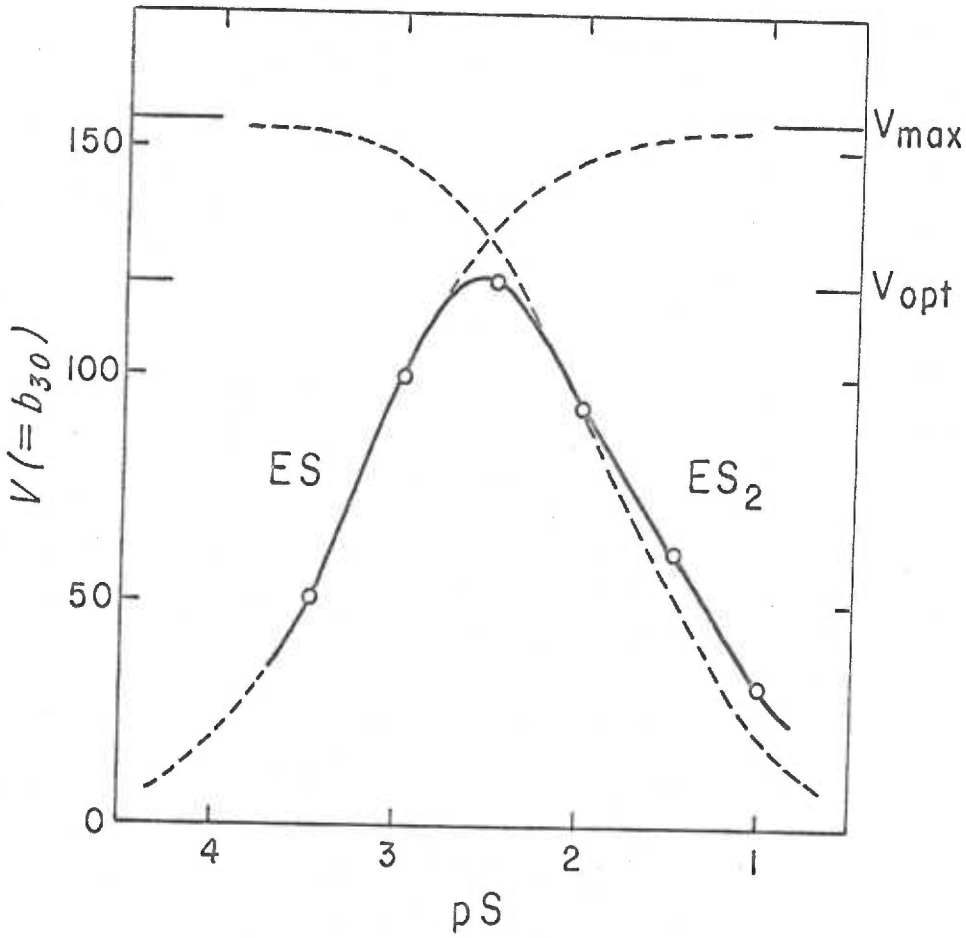
The question of the completeness of esterase inhibition should be raised in essaying alternative interpretations. Since any AChE located in the cleft would remove ACh from the receptor but ignore receptor-based Carb, an action of the entire Carb molecule rather than that of the carbamylated intermediate could be the

underlying explanation for the decrease of the amplitude data. For several reasons this appears unlikely. First, the substrate concentration is so high that no appreciable decrease in ACh could occur; second, most AChE should be inhibited in both cases (Fig. 25). At low concentrations of ACh, the inhibition of the esterase by prostigmine 10^{-5}g/ml produces a noticeable lengthening in the duration of the potential recorded at the end-plate. However, limited data from a prostigminized muscle would indicate that the duration of 5.5×10^{-2}M ACh-induced depolarization is not lengthened. A series of these experiments should be done to further document that the anticholinesterase is superfluous when such high depolarizing concentrations are maintained.

The large volume of the Ringer's wash solutions and the number of changes should also effectively reduce any concentration effect that would be responsible for an electrostatic bonding of the whole molecule to the membrane. Such a "wash-out" would not be as effective in removing a carbamyl group which is covalently linked to the membrane.

Due to the fact that irreversible complex with the esterase is claimed for Carb, but not for ACh, one might interpret the amplitude data on the basis of an endogenous

Fig. 25. Activity-pS curves for the enzymic hydrolysis of ACh of cow erythrocytes (99). ES_2 is an inactive form of the enzyme and represents the combination of two substrate molecules with the two active sites of the enzyme.



Erythrocytes

ACh blockade. That is, minute amounts of ACh could be released from the nerve and, in the case of Carb, accumulate to desensitize the postsynaptic membrane. It is known that all AChE inhibitors in sufficient concentration have a "curarizing" action on neuromuscular transmission. Some indication as to whether the block is attributable to excess ACh (as in the mechanism postulated above) or to depression of receptors has been obtained by studies of the effect of tubocurarine. The antagonism between the cholinesterase inhibitors and tubocurarine is rather limited. Only under certain conditions, when the dose of the inhibitor is relatively low and its application has been of short duration, can antagonism be shown. More generally, the blocking effect of ChE inhibitors adds significantly to that of tubocurarine (100). In addition, it seems unreasonable to think that the small amount of ACh released from the nerve would not diffuse out into the large volume of Ringer's solution surrounding the muscle.

The interpretation of changes in duration of depolarization:

In a number of earlier studies attention has been focussed on the factors which govern or modify the time course of disappearance of the depolarization induced by ACh and Carb. Eccles, Katz and Kuffler (83) have presented

the most careful descriptions of the recovery from depolarization induced physiologically by nerve impulses. This normal end-plate potential (e.p.p.) has a total duration of approximately 25 msec. The decay phase is exponential and appears to be governed solely by the resistive and capacitive properties of the membrane. On the basis of this assumption, they have calculated that transmitter released from the motor nerve ending is active for only 1 - 5 msec. According to this point of view, the duration of the normal e.p.p. is thus governed largely by the passive electrical properties of the membrane. When steps were taken to prolong the duration of transmitter action through the use of anticholinesterase (101), the duration of the e.p.p. was increased. Thus, the depolarizing action of the ACh-receptor complex is not apparently limited in time by the responsiveness of the membrane within the time limits covered by these studies. In order to avoid the uncertainties relative to time and amount of transmitter released from nerve terminals, Castillo and Katz (95) compared the time courses of ACh and Carb potentials using the elegant method of iontophoretic drug application. Though there was considerable variability in their data, the mean Carb-ACh ratio of half-decay times was 1.8, which was reduced after prostigmine to .9. They ruefully

decided that the time course of these drug potentials depended upon the kinetics of local diffusion and hydrolysis, rather than on the kinetics of the drug/receptor interaction. In other words, they used the rate of drug removal to explain the half-decay times -- measured in msec. -- which they observed. Though the depolarizing events here reported could only be obtained at the end-plate region of the muscle, they occurred in response to high constant concentrations of bath-applied agonists and lasted for many seconds or minutes. These decay curves represent a function of some quality of the agonist-receptor complex or of responses to this complex and are unrelated to rate of drug removal. Therefore these decaying responses are a different phenomenon than others have dealt with.

Because of the many variables to contend with in analyzing this decay time data, a stringent assessment of it is impossible. These decay curves may simultaneously be yielding information regarding duration of transmitter action at the receptor site, information relating to the resistive and capacitive properties of the membrane, and information relating to the somewhat baffling phenomenon of desensitization; also incorporated in them are delayed amplitude components.

A further difficulty in assessing the time-course data is associated with the occasional biphasic appearance of the Carb record (Fig. 17). If this break in the curve should be due to a slight though unnoticed movement artefact, then the Carb time course could more conveniently be viewed as a simpler event. Because of the size of the stretching weight, it seems likely that only isometric contractions could occur. To completely eliminate the possibility of muscle movement, the Carb experiments could be repeated in the altered osmotic or ionic environments known to eliminate muscle twitching (102, 69). Should the biphasic Carb records be inexplicable on the basis of movement, two sources of depolarization separated in time need to be found in the case of this depolarizing agent.

For example, Carb could be diffusing to a neighboring receptor population -- a feat impossible for ACh because of the ChE activity remaining in the upper portion of the muscle. The number and distance between end-plates are variable in the muscles. However, no correlation between biphasic Carb responses and adjacent upper end-plates in the tracking records was evident. Application of neither 5.5×10^{-2}M ACh nor 5.5×10^{-2}M Carb in a non-junctional region occasioned a slow "diffusion" response. The suggested time-course study of the initial responses of prostigminized

muscle to 5.5 x 10^{-2}M ACh should either strengthen or eliminate this explanation.

A second hypothesis would be that Carb at these concentrations releases ACh from the nerve as well as combining with the post-junctional membrane. There is good evidence for this mechanism at the sympathetic ganglion (22, 103). Thus one could attribute the primary rise in depolarization to neural ACh and the secondary event to the slower combination of Carb directly with the postsynaptic receptors. Since this latter effect diminishes the number of available receptors, a second Carb dose would not be as successful in directly depolarizing the membrane although a sufficient number of receptors would still be available for the initial neural ACh response. Or it might also be postulated that Carb requires a larger percentage of receptors to exert a maximal effect; therefore the time-course data, which is related to the slower-acting Carb, changes more rapidly than the amplitude data, which is related to the initial effect of ACh. Unfortunately there is no evidence for the release of ACh by Carb at the neuromuscular junction, nor have any experiments been undertaken to test this hypothesis. Such experiments would be desirable and such evidence would be essential before the above explanation could be

considered to be any more than speculation.

The problem of AChE inhibition:

Throughout the design and conduct phases of this study, it has been apparent that one possibly important uncontrolled variable was the amount of ChE available in the preparation. Serious consideration was given to the possibility of eliminating this variable by attempting to inhibit the ChE action completely by large concentrations of an anticholinesterase such as prostigmine. This approach was discarded for the reason that the use of prostigmine, for example, would raise more problems than it would solve. Prostigmine itself contains a carbamyl group, and there is evidence that eserine carbamylates the enzyme (104). There is also good evidence for the presynaptic activity of prostigmine (105, 106, 107). Furthermore, no proof could be found in the literature that prostigmine 10^{-5}g/ml (the concentration generally employed) did indeed inhibit 100% of the ChE. Quite probably this is because manometric assay methods are not sufficiently sensitive to permit such a firm conclusion. An attempt to demonstrate the esterase activity in a 3 mg. m. ext. long. dig. IV which had not been pre-treated with anticholinesterase was unsuccessful. Finally, if the hypothesis being tested is true, complete

ChE inhibition would probably gravely alter the activity of the receptor.

SUMMARY AND CONCLUSIONS

The significant decrease of carbachol-induced depolarizations compared with the lack of change in the acetylcholine-induced depolarizations lend credance to the postulate that a carbamyl group interferes with the activity of the end-plate receptors. Since acetylcholinesterase hydrolyzes acetylcholine rapidly but rids itself very slowly of the carbamyl group from carbachol, the enzyme and acetylcholine receptor demonstrate a common property.

The results of this work support the hypothesis that the acetylcholine receptor at the neuromuscular junction of twitch-type muscles is a cholinesterase.

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