PATHOPHYSIOLOGY OF ANTICHOLINESTERASE AGENTS

Annual Report

John E. Rash, Ph.D.
Julie K. Elmund, Ph.D.

September 21, 1987

Supported by

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Department of Anatomy and Neurobiology
Colorado State University
Fort Collins, Colorado 80523

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The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.
In this third Annual Report, we complete the description of the acute, delayed, and long-term effects on rat neuromuscular junction (NMJ) ultrastructure and physiology following single acute injections of very low to near lethal doses of physostigmine, a reversible anticholinesterase (anti-ChE) compound. We also complete the descriptions of the immediate and long term effects of subacute exposures at doses which produce sustained blood ChE inhibitions of 40% (±10%) and 80% (±10%) for up to 14 days and of the reversibility of effects during recovery for 3-28 days following termination of subacute exposure. In additional correlative experiments using guinea pigs as alternative models to rats, sub-lethal but acute high doses of physostigmine produced equally severe and apparently identical...
pathological alterations in endplate ultrastructure at similar blood ChE inhibition levels. We conclude that the rat and guinea pig are essentially equivalent as models for characterizing the ultrastructural alterations of neuromuscular junctions caused by acute high doses of physostigmine. Finally, we present additional physiological and ultrastructural data showing that the threshold for producing neuromuscular pathology is lowered (i.e., toxic alterations of the endplates are increased) by sustained neuromuscular activity. These data may be of value in evaluating and comparing anti-ChE medications and dosages used for protection against the irreversible anti-ChE agents.
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I. SUMMARY

In this third Annual Report, we complete the description of the acute, delayed, and long-term effects on rat neuromuscular junction (NMJ) ultrastructure and physiology following single acute injections of very low to near lethal doses of the anticholinesterase compound, physostigmine. We complete the descriptions of the immediate and long term effects of subacute exposures at doses which produce sustained serum ChE inhibitions of 40% (+10%) and 80% (+10%) for up to 14 days and of the reversibility of effects during recovery for 3-28 days following termination of subacute exposure. We also describe additional correlatative experiments using guinea pigs as alternative models to rats. In these experiments, the effects of sub-lethal but acute high doses of physostigmine are shown to produce similar alterations of endplate ultrastructure at similar dose levels as measured in mg/kg. Finally, we present additional physiological and ultrastructural data showing that sustained neuromuscular activity mimics many of the ultrastructural alterations produced by physostigmine, but does not result in the supercontraction of subjunctional sarcomeres that characterize high-dose physostigmine intoxication. Detailed composite descriptions and overall conclusions from all completed experiments will be provided in the Final Report (due approximately January 1, 1988).

In this report, we complete the ultrastructural comparisons of the low (0.01 LD50) to very low dose (0.001 LD50) acute exposure experiments with images from a much larger sample of "control" endplates from sham injected animals. We show that subtle "alterations" in endplate morphology as described in several previously published descriptions of low dose experiments are equally common in endplates from "control" (sham-injected) rats. Specifically, in endplates of control and treated rats, we observed a) similarly frequent examples of Schwann cell "fingers" interposed between nerve terminals and junctional folds; b) similarly frequent examples of junctional folds devoid of nerve terminals, and c) equally infrequent examples of small diameter nerve terminals near shallow junctional folds (previously identified by us and others as "collateral sprouts" characteristic of endplate remodeling). Thus, isolated examples of these atypical but normal variations are now recognized as probably not reflecting acute or delayed effects of low dose exposures to this (or any other) anti-cholinesterase agent. We conclude that acute exposure to physostigmine at low to very low doses (0.01-0.001 LD50 or <35% serum ChE inhibition) produced no discernible alterations of endplate ultrastructure, at least as can be discerned in thin section electron micrographs. Nor are ultrastructural alterations indicative of drug-induced pathology observed during the "recovery" phase 1-56 days following acute exposure.

In experiments using guinea pigs as an alternative model to rats, sub-lethal but high doses of physostigmine produced equally severe pathological alterations in endplate ultrastructure at similar exposure doses. At 1 hour post injection (PI) of 1.2 mg/kg physostigmine, virtually all neuromuscular junctions of the diaphragm, soleus, and EDL myofibers exhibited...
supercontraction of sarcomeres in the subjunctional sarcoplasm. Often, Z bands were missing, free thick and thin filaments were present in disorganized masses. In guinea pigs as in rats, similar concentric zones reflecting a continuum of severely to slightly damaged mitochondria were observed in the subjunctional sarcoplasm. Progressing from the junctional folds to undamaged perijunctional regions, the sequence of mitochondrial morphologies progressed from "exploded" to "swollen" to "frothy" to "blistered" mitochondria. The EDL muscles of guinea pigs were more severely affected than the EDL muscles of rats, the soleus muscles were less affected, and the diaphragm equally affected as in the rat. Overall, the NMJs of guinea pigs and rats appear to be equally susceptible to the pathological alterations caused by acute high doses of physostigmine.

In subacute exposure experiments in rats, we confirm and extend our previous observations that low to moderate doses (yielding sustained 40 ±10% inhibition) produce no (or very few) detectable alterations in endplate ultrastructure after 3, 7 or 14 days continuous exposure, nor were alterations detected during the recovery period 3-28 days following termination of "moderate" dose subacute exposure. Likewise, at relatively high dose subacute exposures (80% + 10% sustained serum ChE inhibition), most fibers exhibited no obvious changes in endplate fine structure. However, after 3 and 14 days continuous exposure, a few nerve terminal branches were depleted of synaptic vesicles and had increased numbers of coated vesicles. In addition, at 7 days post-implantation, a few soleus myofibers exhibited evidence for prior supercontraction and partial reorganization of subjunctional sarcomeres. A few fibers also appeared "edematous" and had swollen subsynaptic nuclei. We previously showed that when "pre-primed" pumps were employed (rather than unprimed pumps), much higher toxicity (and greater lethality) was observed. Thus, we conclude that a lower level of toxic symptoms occurs when a gradual decrease in serum ChE activity is induced by a gradual onset of exposure (even at high dose exposure levels). By introducing an even slower onset of pumping (by using unprimed pumps) or by inducing a slightly lowered ChE inhibition level, even these minor ultrastructural alterations might be avoided.

In parallel experiments assessing the effects of subacute physostigmine administration on muscle physiology, contractile properties were minimally affected during the subacute exposure regimens employed, and these quickly returned to normal during the recovery period after Alzet pump removal. These data demonstrate that recovery of function of nerve terminals and repair of muscle cytoplasm occur very rapidly in the rat following sublethal doses of physostigmine. However, the few severely affected fibers may have been much slower to recover, but because of their rarity (i.e., low percentage of fibers affected), the effects of damage were not discernible in the recordings of whole muscle contractile properties.

Muscle use is shown to be an important factor in the expression of drug toxicity at the motor endplate. Data obtained from stressed and physiologically stimulated rats (20-80 hz for 15 and 30 minutes) revealed that many of the ultrastructural changes
seen after an acute high dose of physostigmine are duplicated by continuous high frequency nerve stimulation in the absence of physostigmine. Of the major alterations associated with acute high-dose exposure, only supercontraction of subjunctional sarcomeres was not evoked by 30 minutes of 80 Hz stimulation. These data may help to explain why the constantly used diaphragm myofibers are most rapidly and severely affected in animals exposed to near lethal doses of physostigmine whereas unused voluntary muscles in the same animal show little or no damage. Thus, we conclude that the presence of supercontraction during exposure to high doses of anti-ChE agents reflected not only nearly continuous neuromuscular activity but also concurrent sustained endplate depolarizations that occur only during the simultaneous presence of anti-ChE drugs plus sustained nerve activity.

Based on the rapid repair of endplate ultrastructure and recovery of normal function during subacute exposure to physostigmine, and based on recent evidence for down regulation of transmitter release efficacy as a normal control mechanism for neuromodulation, we suggest that a combined physiological and ultrastructural (freeze-fracture) investigation be conducted to ascertain if the "protective" or compensatory alterations afforded by prior exposure to anticholinesterase agents reflect a decrease in transmitter release (i.e., decrease in quantal content), and if so, if alteration or partial disassembly of the "active zones" occurs in the period 0-12 hour following moderate to high dose exposures to anti-ChE agents. Such studies may prove of value in understanding the protective mechanisms to nerve agent exposure afforded by prior exposure to the reversible anti-ChE medications.
II. FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).
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VI. INTRODUCTION

The prototype anti-ChE agent, physostigmine (or eserine), is a toxic tertiary carbamate alkaloid isolated from the Calabar bean or esere nut of Physostigma venenosum (1). Systematic investigations of its chemical structure-activity relationships led to the introduction of neostigmine, which is now used in the symptomatic treatment of myasthenia gravis (1). The current major clinical application of physostigmine is in the treatment of glaucoma (1) and in the symptomatic treatment of atropine intoxication (1). Physostigmine and pyridostigmine have been suggested as possible chemotherapeutic agents for protection against exposure to the irreversible anticholinesterase "nerve gases" (2-5), with some authors suggesting that physostigmine provides substantially greater protection from soman poisoning than does pyridostigmine at an equitoxic dose (2).

Physostigmine, because of its tertiary amine structure, was also reported to have the additional advantage that it protected brain ChE, whereas pyridostigmine afforded little or no protection of brain ChE.

A. ANTICHOLINESTERASE AGENTS PRODUCE DEPOLARIZING NEUROMUSCULAR BLOCKADE AND SUPERCONTRACTION AT ENDPATES

It has been known since early in this century, that the anti-ChE agents neostigmine and physostigmine, and the depolarizing neuromuscular blocking agents nicotine and succinylcholine produce profound myopathies and neuropathies, as well as distinctive pathophysiologies of neuromuscular transmission (6-30). The myopathical and physiological alterations of neuromuscular transmission caused by anti-ChE agents are attributed primarily to severe and prolonged endplate depolarization. Some authors also suggest that Ca ++-activated proteases may be important in myofiber destruction and Z-bands dissolution (26,27). However, the rapidity of the repair process (this report - showing complete reassembly of subjunctional sarcomeres and return to normal contractile responses within 24 hours of subacute exposure - a period too brief for the requisite synthesis of large amounts of myofibrillar protein) makes attractive the suggesting for widespread proteolytic activity.

Koelle has suggested that "to exert a significant effect in vivo, an anti-ChE agent must generally inhibit from 50% to 90% of the functional AChE at a given site" (1). Hudson, et al., (28-30) refine this estimate by showing that with pyridostigmine (a quaternary anti-cholinesterase agent), severe morphological alterations (supercontraction of subjunctional sarcomeres) occur when blood ChE inhibition exceeds approximately 70%. On the other hand, in their studies of the effects of low to very low doses of pyridostigmine, Hudson and coworkers reported several subtle changes in nerve and muscle components, including an increase in the frequency of distended mitochondria in the nerve terminal as compared to nerve terminals of control (untreated) animals. They also described more frequent and greater separation of nerve terminal membranes from junctional fold.
membranes in the low-dose exposures than in the sham injected rats. However, they (28) explicitly stated their assumption as follows: "Assuming that sampling bias caused by microscopic examination of only a small portion (several thin sections) of each NMJ from a limited number of fibers is not the reason for the observed variability, the mechanisms underlying variable effects could be contributed to a number of factors," which they then proceeded to list. Nevertheless, we note that skeletal and cardiac muscle fixed by glutaraldehyde undergo substantial chemically induced hypoxia (31,32), and as a result, often exhibit characteristically swollen mitochondria as an artifact of glutaraldehyde fixation (33,34). Moreover, anti-ChE agents are also known to produce profound bradycardia, hypotension, bronchoconstriction, and release of epinephrine, resulting in substantial systemic tissue hypoxia (1). Thus, current fixation techniques, which also increase hypoxia (34), may be limited in their ability to allow unambiguous attribution of the similar changes in mitochondria to low-dose drug effects. Consequently, we have devised improved methods of fixation that usually eliminate artifactual alterations in mitochondria, thereby allowing at least some of the drug-induced alterations to be differentiated unambiguously from artifacts of specimen preparation. Moreover, we have developed additional criteria for distinguishing a variety of plane-of-section artifacts from these attributed to effects of anti-ChE.

B. ACUTE VS. DELAYED EFFECTS OF ANTICHOLINESTERASE TOXICITY

Numerous ultrastructural studies of acute anti-ChE toxicity report subtle to profound alterations of the neuromuscular junction at near-LD50 doses (11-30). These include initial supercontraction of subjunctional sarcomeres, with rapid progression to Z band disruption and myofibril disassembly; swelling and explosion of mitochondria in the nearby subjunctional sarcoplasm; and relatively severe mitochondrial swelling in the nerve terminals. With a single exposure, these initial changes appear to be partially ameliorated within 24 hours and virtually complete within a few days. However, additional delayed effects have been reported, including 1) at about 14-56 days, destruction and removal of some junctional folds and their replacement by vesicular debris similar to that seen in the autoimmune disease, myasthenia gravis (15,16,35-38); 2) at 7-28 days, the occasional disappearance of nerve terminal branches, yielding areas of junctional folds devoid of associated nerves (as found in partial endplate denervation) (39-41); and 3) at 14-56 days, the formation of small-diameter nerve terminal branches (collateral sprouts) similar to those seen in myasthenia gravis (15,39,40) and in human neurogenic neuromuscular diseases (41). More detailed examination of endplates from sham-injected ("control") rats (this third Annual Report) allows some of these "signs of delayed toxicity" to be reassigned to atypical but normal morphologies and allows more definitive discrimination of pathology from normal variability.
C. ANTI-CHOLINESTERASE AGENTS AS POSSIBLE PROPHYLACTIC AGENTS FOR SOMAN INTOXICATION

Following exposure to the irreversible anti-ChE agent soman, endplate acetylcholinesterase (AChE) is inactivated irreversibly. The resulting decrease in AChE activity lasts potentially until new AChE is synthesized. At high doses (near or above 1.0 LD₅₀), normal motor nerve activity results in prolonged endplate depolarizations. Severe pathological alterations in endplate physiology and ultrastructure ensue, potentially resulting in neuromuscular (including respiratory) failure. The quaternary carbamate anti-ChE pyridostigmine has been shown to be a moderately effective prophylactic agent against exposure to lethal doses of soman (5), while the tertiary carbamate physostigmine may prove even more effective. In both instances, the protective mechanism or mechanisms are entirely unknown. However, it has been suggested that the reversible anti-ChE agents competitively occupy the esterase sites during exposure to the irreversible anti-ChE agents. After termination of exposure to soman plus reversible anti-ChE agents, the protected AChE sites are thought to be unmasked by decarbamylation of the reversible agents, leading to sufficient reactivation of AChE to permit recovery of muscle function. However, this proposal leads to a dilemma: In the presence of two powerful anti-ChE agents, it is not clear how the more vital muscle activities (breathing, for example) are to continue during the prolonged period required to reactivate AChE. This reactivation period is estimated to be at least 30 minutes for neostigmine and substantially longer for physostigmine and pyridostigmine (1). Clearly, if the only protective mechanism were based on competition for and co-blockade of a single class of AChE molecules, death would still ensue during the prolonged neuromuscular/respiratory blockage produced by either/both agents. Since a substantial number of rats survive a dose of ?Soman of several LD₅₀'s, the protective mechanism(s) must be other than the one proposed.

To clarify the protective mechanisms afforded by reversible anti-ChE agents, detailed knowledge of their primary toxic effects must be obtained and compared to those produced by the toxic nerve agents. Moreover, compensatory changes in endplate physiology ("neuromodulation") produced by prophylactic doses of these anti-ChE agents must be identified and characterized. This study utilizes biochemical, ultrastructural, and physiological approaches to identify the acute and delayed effects on neuromuscular junctions following exposure to very low to near-lethal doses of physostigmine. In these experiments, the rat is used as a model system for analyzing chemoprophylactic agents for nerve agent exposure because, of all animals tested, the rat is among the least protected by prior or concurrent exposure to the reversible anti-ChE agents (3,5).

D. PURPOSE OF THIS STUDY

In our previous studies of neostigmine toxicity (24,25), we used conventional transmission electron microscopy and
intracellular recording techniques to show that neostigmine-treated endplates had severe pre- and post-synaptic alterations (as described above) and that these alterations persisted with "chronic" (sic) (i.e., multiple-injection) exposures. No long-term recovery or delayed effects after acute exposure were described in those reports. In this study, we have used combined ultrastructural, biochemical, and physiological techniques to analyze the neurotoxic and myotoxic effects of acute physostigmine exposure at doses spanning three orders of magnitude -- from 0.001 LD$_{50}$ to 1.1 LD$_{50}$. We have analyzed light and electron microscopic images from diaphragm, soleus, and EDL muscles of rats after acute (single subcutaneous injections) and subacute exposures (up to 14 days of exposure using Alzet osmotic minipumps) and from these same muscles during recovery (1-56 days of recovery after single acute drug exposure and up to 28 days following removal of Alzet pumps) an additional 5 guinea pigs were also examined following exposure to high acute doses of physostigmine (3 experimental, 2 control animals) and essentially identical alterations were observed in diaphragm, myofibers and EDL. In each rat, changes in nerve and muscle ultrastructure were correlated with temporal changes in serum cholinesterase (ChE) inhibition levels. Serum ChE levels were measured periodically as an independent measure of the effect and distribution of physostigmine. Following acute high dose exposure (0.8 LD$_{50}$ or >80% serum ChE inhibition), thin section transmission electron microscopy revealed a dose-dependent continuum of pre- and post-synaptic alterations of neuromuscular junctions, including supercontraction of subjunctional myofibrils; damage to membrane-bound organelles such as mitochondria, sarcoplasmic reticulum, and T-tubules; decreases in number of synaptic vesicles and increases in number of coated vesicles, and reversible swelling of nerve terminal mitochondria. In the first and second Annual Reports, we assessed the diaphragm, soleus, and extensor digitorum longus (EDL) muscles and determined that the constantly used "mixed" (fast, intermediate, and slow twitch) diaphragm muscle (used for breathing) provides the best model for assessing neuromuscular toxicity because it is most severely and consistently affected of the three muscle types examined.

Physiological studies of rats exposed to physostigmine included an assessment of twitch potentiation of EDL muscles following acute exposure to physostigmine, as well as an assessment of possible synergistic effects of prolonged high frequency stimulation in the presence and absence of physostigmine (0.1-0.3 LD$_{50}$). Rapid alterations in neuromuscular physiology were correlated with parallel changes in blood ChE inhibition levels. From the biochemical, ultrastructural, and physiological data obtained during this study, a better understanding of the pathophysiology of physostigmine is being gained. From these data, others may be able to make a more informed decision concerning its possible use as a prophylactic agent against the irreversible nerve agents.
E. Summary of First and Second Annual Reports

In the first Annual Report, we:
1) established the LD$_{50}$ of physostigmine as 0.75 mg/kg;
2) injected twenty four groups of 3 "experimental" and 2 "control" rats with 0.001 LD$_{50}$ to 1.1 LD$_{50}$ and obtained samples for electron microscopic analysis from diaphragm, soleus and EDL muscles 1 hr and 1, 7, 14, 21, and 56 days PI;
3) established regimen for obtaining continuous levels of blood ChE enzyme inhibitions of 30-50% ("low dose") and 70-90% ("high dose") using Alzet mini-osmotic pumps;
4) obtained diaphragm, soleus, and EDL muscles from these "subacute exposure" groups for ultrastructural analysis of possible changes at 3, 7, and 14 days post implantation and during recovery at 3, 7, 14 and 28 days after surgical removal of the pumps;
5) Obtained blood samples for ChE inhibition analysis before drug exposure and at 1 hr and 1, 3, 7, 14, 28 and 56 days PI (or until the animals were fixed for electron microscopy);
6) identified a synergistic effect of anesthesia and physostigmine on muscle physiology;
7) performed initial measurements of the effect of low to high doses (0.001 to 0.8 LD$_{50}$) of physostigmine on muscle twitch tension and resistance to fatigue;
8) performed ultrastructural analyses of acute high dose exposures;
9) obtained preliminary ultrastructural data from low to moderate dose exposure, and;
10) obtained preliminary data concerning "reversibility" of cytopathological alterations from 1 day to 56 days following a single acute injection.

In the second Annual Report, we:
1) completed measurements of the effect of low to high doses (0.001 to 0.8 LD$_{50}$) of physostigmine on muscle twitch tension and resistance to fatigue;
2) completed ultrastructural analyses of acute high dose exposures;
3) obtained additional ultrastructural data from low to moderate dose exposure, and;
4) obtained additional data concerning "reversibility" of cytopathological alterations from 1 day to 56 days following a single acute injection.
5) completed ultrastructural analyses of subacute low and high dose exposures;
6) began ultrastructural analyses of delayed effects of subacute high dose exposures;
7) began ultrastructural analysis of endplates following prolonged moderate frequency stimulation in the presence and absence of physostigmine;
8) established that the threshold of physostigmine toxicity is lowered substantially by prolonged neuromuscular stimulation.
In this third Annual Report, we complete all experiments involving acute and subacute exposure regimens, recovery from acute and subacute exposure, and comparison of "control" myofibers from sham injected and sham implanted rats. We also verify supercontraction of subjunctional myofibers in an alternative model, the guinea pig, following acute high dose exposures. We have established criteria that allow effects of drug exposure to be distinguished from most artifacts of specimen preparation. Consequently we are now able to show that recovery from the effects of high and low dose exposure is much more rapid and complete than previously suggested.
VII. EXPERIMENTAL METHODS

A. CARRIER SOLUTIONS FOR PHYSOSTIGMINE

Physostigmine sulfate in aqueous solution is labile above pH 6 (42). Thus, to facilitate comparison of acute and subacute exposure regimens, similar diluents consisting of rat Ringers solution made slightly acidic were used in all experiments. Physostigmine solutions and the diluents used in the sham-injection (control) experiments were made slightly acidic by the addition of glacial acetic acid diluted 1:2000 (i.e., 10 mM). The resulting solutions were pH 3 but were adjusted to pH 4 by dropwise addition of 2 M sodium acetate buffer. These dilute acetic acid solutions were well tolerated by the animals and did not produce evidence of tissue damage or of necrosis at the site of injection or implantation, nor did they produce any detectable alterations of endplate morphology in any of the muscles analyzed. (Pumps implanted into 1 series of 30 rats were not adjusted to pH 4 by sodium acetate but left at pH 3. No differences were noted between the two groups.)

B. ESTABLISHMENT OF LD₅₀ IN RATS FOR ACUTE EXPOSURE

As described in the first annual report, the LD₅₀ of physostigmine for unstressed ("acclimated") rats was determined to be 0.75 mg/kg (see first Annual Report). For correlation with other studies of anti-ChE agents, relative inhibitions of blood ChE enzyme levels were measured for all rats. As has been found for other anti-ChE agents, 0.8-1.0 LD₅₀ of physostigmine was found to depress blood ChE enzyme levels by 95% (see RESULTS).

C. PROCEDURE FOR ACUTE ADMINISTRATION OF PHYSOSTIGMINE

In the acute exposure experiments, high, moderate, low, and very low doses corresponding to 0.8, 0.1, 0.01, and 0.001 LD₅₀ (0.6, 0.75, 0.075, and 0.0075 mg/kg) were tested. For each experimental protocol or exposure interval, 3 treated and 2 control (sham-injected) rats were prepared for ultrastructural analysis. Seventy-three male Wistar rats weighing 180 to 250 g were given single S.C. injections of 0.001 to 1.1 LD₅₀ in the rostral mid-back region. Blood samples were obtained immediately before injection, at 30 minutes and 1, 7, 14, 28, and 56 days PI. Blood and tissue samples were coded to conceal dose and sampling time. Blood samples were analyzed for cholinesterase inhibition and the results correlated with ultrastructural analyses of the tissue samples. Fifteen sham-injected control rats were obtained for examination after the same exposure intervals (30 minutes to 56 days PI; age- and sex-matched controls).

As an alternative model system for assessing the effects of high but sublethal doses of physostigmine, 4 guinea pigs (581, 637, 677, and 678 g) were injected with 0.75-1.25 mg/kg of physostigmine and 2 guinea pigs were given sham injections. Blood samples for serum ChE analysis were obtained before injection and at 30 minutes PI blood ChE inhibitions were not obtained for the 3 treated animals due to equipment malfunction. Thus, ultrastructural alterations were correlated with dose in mg/kg.
TABLE 1. Experimental Protocols for Exposure of Guinea Pigs to 0.75-1.25 mg/kg of Physostigmine.

<table>
<thead>
<tr>
<th>Guinea Pig Weight (grams)</th>
<th>Physostigmine Dose (mg/kg)</th>
<th>Muscle Alteration (Diaphragm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>581</td>
<td>0.75</td>
<td>Frothy Mitochondria?</td>
</tr>
<tr>
<td>637</td>
<td>1.25</td>
<td>Supercontraction</td>
</tr>
<tr>
<td>677</td>
<td>1.25</td>
<td>Supercontraction</td>
</tr>
<tr>
<td>678</td>
<td>1.25</td>
<td>Supercontraction?</td>
</tr>
</tbody>
</table>
D. MUSCLE TYPES EXAMINED BY ELECTRON MICROSCOPY

The fast twitch EDL muscle, slow twitch soleus muscle, and mixed fast, intermediate, and slow twitch diaphragm muscle were selected for study. Muscles composed primarily of slow twitch fibers have been reported to be damaged much more severely by anti-ChE agents than those composed primarily of fast twitch fibers (16,17,24). Therefore, we have compared the effects of physostigmine on muscles with relatively pure populations of fast twitch (EDL) and of predominantly slow twitch (soleus) myofibers. However, it is possible that the relevant factor in expression of anti-ChE toxicity is not twitch contraction time (43-46) or pattern of firing of fast twitch vs. slow twitch fibers [i.e., phasic vs. tonic activation as suggested by Ward, et al. (17)], but that it may instead be related to the amount/duration of muscle activity. Thus, as a counter example to muscles with nearly pure fiber types, the diaphragm muscle was also selected because it is a mixed muscle containing fast, intermediate, and slow twitch fibers (43-45). The close proximity of three fiber types in the same muscle provides for an internal fiber-type control. Presumably, under respiratory insufficiency, all fiber types (fast, slow, and intermediate type) are activated continuously at near maximum levels. The mixed diaphragm muscle may then be contrasted with the fast twitch EDL muscle, in which the fibers are voluntarily rested during the initial period of neuromuscular distress following physostigmine injection. In rats in respiratory distress, even the fast twitch fibers in the diaphragm have relatively continuous firing of myofibers, presumably greater even than that seen in the postural muscles, such as the soleus (46). Consequently, both diaphragm and soleus muscles are relatively continuously activated until the animal collapses from exhaustion/muscle failure. Even then the diaphragm myofibers must continue to contract lest the animal die of respiratory failure. Thus, these three distinctive types of muscle (EDL, soleus, and diaphragm) provide a wide variety of patterns of muscle activity, thereby facilitating the evaluation of acute physostigmine toxicity in different muscle fiber types having a variety of firing patterns.

E. SUBACUTE ADMINISTRATION OF PHYSOSTIGMINE: Determination of Doses Required to Obtain Pre-Determined Enzyme Inhibition Levels

In our first Annual Report, we established that Alzet® Mini-osmotic pumps loaded with 0.25 mg/ml and 25 mg/ml and delivered at a rate of 2.5 μl/hr yielded sustained serum ChE inhibitions of 30-50% and 70-90%, respectively. As in the previous experiments, blood enzyme levels were measured before implantation of the pumps and after 24 hours, 2, 5, and 14 days of exposure and in the subacute recovery experiments at 7, 14, and 28 days after pump removal. Preliminary data from those experiments were described in the second Annual Report. The remaining data from those experiments are presented in this third Annual Report (see RESULTS).
F. FIXATION FOR ELECTRON MICROSCOPY

1. Perfusion Fixation

As described in greater detail in the first and second Annual Reports, virtually all drug-treated and sham injected control rats were fixed for electron microscopic examination by arterial perfusion via the left ventricle. (The exceptions were several immersion fixations to analyze artifacts of hypoxia during inadequate perfusion fixation. These data are not included in the analyses of drug effects.) The initial perfusate (30 seconds to 2 minutes) consisted of a 39°C rat Ringer's solution containing 10 units/ml of heparin. All solutions were pressurized with 95% O_2, 5% CO_2 at 150 mm Hg. After tissue clearing, a solution of 39°C oxygenated rat Ringer's buffer containing 2.5% glutaraldehyde (pH 7.2) was perfused for approximately 5 minutes.

Muscle samples were removed for electron microscopy only when 1) glutaraldehyde-induced rigor ensued within 30 seconds, 2) the liver and kidneys were uniformly blanched and yellowed within 2 minutes, and 3) exposed muscles were uniformly yellow (usually within 1-2 minutes). Whole EDL and soleus muscles and strips of diaphragm were immediately removed and placed in vials containing 2.5% glutaraldehyde in Ringer's buffer. The vials were then placed in an ice bath or in the refrigerator (0-4°C) for 1-24 hours or up to 3 days.

The quality of perfusion fixation of each muscle sample was graded from 1 (poor) to 10 (excellent) as it was removed from the rat. Ultrastructural analysis (see first Annual Report) revealed that small regions of inadequate perfusion (as confirmed by presence of blood cells in capillaries) are occasionally found in the highest-rated perfusions, and areas of adequate fixation are found even in the perfusions rated as poor. Since many of the changes induced by poor fixation at least superficially resemble toxic drug effects as reported by others (30), we identified several artifacts of inadequate perfusion technique by codifying those changes in additional (control) samples fixed by immersion only (next section).

2. Immersion Fixation; Identification of Artifacts

To identify the most common artifacts of inadequate perfusion fixation and, moreover, to identify the changes occurring with increasing depth of fixative penetration, whole EDL muscles from untreated rats were excised and placed intact into 37°C oxygenated 2.5% glutaraldehyde in rat Ringer's buffer (pH 7.2). After 3-5 minutes, the intact muscles were chilled and fixed for 16-20 hours in 4°C 2.5% glutaraldehyde. After primary fixation, 1.0 mm-thick wedges from surface to center were excised and prepared for electron microscopy according to the same procedures used for perfusion-fixed samples (see next section). Surface fibers were virtually identical to fibers in the highest rated perfusion fixation, but mitochondrial alterations characteristic of delayed fixative penetration/hypoxia (31) were found in fibers deeper than 0.1-0.25 m from the surface (32).
These data (see first and second Annual Reports) were instrumental in discerning artifacts of fixation from low-dose drug effects.

G. LOCATING ENDPLATES; EMBEDDING SAMPLES IN PLASTIC

After primary glutaraldehyde fixation, endplate regions were stained using a modified *in vitro* AChE stain (47,48). Control endplates were visible within 1-2 hours, whereas endplates from animals treated with high-dose anti-ChE required staining for 18 to 24 hours. (We attribute this delayed staining to the slow hydrolysis of the physostigmine originally bound to endplate ChE and the subsequent reactivation of AChE [1] to yield positive staining by the ChE reaction products. Rinsing for 1-3 days in drug-free fixative or in drug-free Ringers solution restored rapid stainability.) Small segments containing opalescent endplates were dissected free and post-fixed in OsO₄ for 1 hour. Samples were then stained in 0.5% aqueous unbuffered uranyl acetate for 16 to 20 hours, dehydrated in a graded ethanol series, transferred through a graded acetone-plastic series, and embedded in one of two plastic mixtures: 1) 10% Epon 812 (or Polybed 812), 20% Araldite 6005, 70% dodecenyl succinic anhydride with 1.5% DMP-30 (tri-dimethylamino methyl phenol) as catalyst or 2) Spurr's plastic (49), and polymerized at 70°C for 24 hours. (Plastic resins were purchased from Tousimis Research Corporation, Ted Pella, Inc., Earnest F. Fullam, Inc., and Polysciences, Inc.) Some of the results from this ancillary study of immersion vs. perfusion fixation are described in Lee, et al., (32).

H. MICROTOMY AND ELECTRON MICROSCOPY

Gray to pale gold sections were cut with EMS or Dupont diamond knives using Sorvall MT-2B or LKB Model IV ultramicrotomes, collected on 200 or 700 mesh copper grids, and post-stained with lead citrate (50,51). Specimens were examined at 80 kV on Philips EM 200 or Philips 400T transmission electron microscopes and photographed at initial magnifications of 1,000X to 40,000X using Electron Microscope Film (Kodak, Rochester, NY). Thick sections were stained with toluidine blue and photographed at 400-900X using a Zeiss Photoscope.

Initially, all samples for electron microscopic analysis were coded and examined without knowledge of prior treatment. At least 3 to as many as 12 endplates from each muscle obtained from each treatment regimen and control were photographed. This procedure was maintained during initial photography and preliminary evaluation, but was discontinued as distinctive features of drug cytotoxicity were identified and catalogued. However, to avoid secondary (discretionary) bias, especially in the evaluation of the low-dose samples, every endplate identified in every sample examined was photographed for detailed ultrastructural analysis. In excess of 3000 different endplates have now been photographed.
I. WHOLE BLOOD CHOLINESTERASE ENZYME ASSAY

We have used the radioisotope assay method of Siakotos, et al (52) to determine blood ChE enzyme activity levels. Using $^{14}$C-acetylcholine as the substrate, we have measured the relative activity of blood ChE after acute and subacute exposure to physostigmine. Samples were obtained before injection of physostigmine and at 30 minutes, 1, 7, 14, 28, and 56 days PI. Two 50 $\mu$l samples of whole blood were drawn into heparinized capillary tubes from a small incision in the tail vein. Samples were diluted 1:9 (50 $\mu$l:450 $\mu$l) with distilled water, mixed thoroughly, then frozen in 1 ml plastic vials by immersion in liquid N$_2$. For enzyme assays, the individual samples were thawed and within 3 minutes of thawing, injected into the enzyme reaction mixture.

According to the method of Siakotos et al. (52), blood ChE enzyme activity was determined by measuring the amount of $^{14}$C-acetate generated from $^{14}$C-acetylcholine after 5 minutes in the reaction mixture. Diluted whole blood (100 $\mu$l) was added to 100 $\mu$l of 0.1 M sodium phosphate (pH 7.38) and 100 $\mu$l of $^{14}$C-acetylcholine (containing 0.3 umoles of total acetylcholine and 0.10 $\mu$Ci of $^{14}$C-acetylcholine). This solution was mixed thoroughly and incubated at 25°C. The reaction was stopped by adding excess resin-dioxane mixture to reach a volume of 5 ml. The resin-dioxide slurry was prepared by adding 200 ml of dioxane to 50 g of AG 50W-X8 cation exchange resin (200-400 mesh from Biorad). The resin was previously converted to the Na$^+$ form, washed with acetone, and dried in vacuo. Five ml of dioxane was added to this mixture, mixed thoroughly, and then centrifuged at 1,500xg max, for 2 minutes. One ml of supernatant was added to 10 ml of scintillation cocktail and the radioactivity assayed on a Beckman liquid scintillation counter. For each assay, background radioactivity was determined by sham incubation with distilled H$_2$O replacing blood (to assess the condition of the radiosubstrate). The total amount of radioactive isotope used in each assay was also determined.

Normal control ChE activity was determined for each animal prior to drug exposure. This activity was designated as 100% activity for that animal. Relative ChE inhibition PI was plotted for each animal against its own control value.

We emphasize that enzyme inhibition levels were obtained at the prescribed intervals for all rats examined ultrastructurally and for most of the rats examined physiologically. Over 2,000 enzyme assays have been obtained. Thus enzyme histories are available for each rat, permitting direct correlation of altered ultrastructure with ChE enzyme inhibition.
In vivo twitch tensions were recorded from EDL muscles in rats following acute physostigmine administration, in non-injected rats receiving prolonged muscle stimulation at 20, 40, or 80 Hz stimulation, and in rats receiving both acute exposure to drug followed by prolonged muscle stimulation (Table 2). Spinal block anesthesia was used to avoid interaction between general anesthetic (chloral hydrate, ketamine/xylazine) and physostigmine. For the spinal block procedure, the rat initially was anesthetized with ether and a cannula inserted into the spinal canal at the L6 level. Sufficient 2% lidocaine was injected to maintain anesthesia in the caudal half of the body for about 25-30 minutes without producing generalized effects (decreased alertness, etc.). The rat was allowed to recover completely from the ether anesthesia prior to recording of twitch tensions.

**TABLE 2. Number of Animals in Each Experimental Group for the Study of Continuous Stimulation Without and With Physostigmine**

<table>
<thead>
<tr>
<th>Stimulus Duration</th>
<th>15 Min.</th>
<th>30 Min.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stimulus Frequency</strong></td>
<td>20 Hz</td>
<td>40 Hz</td>
</tr>
<tr>
<td>Stimulation only</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Physostigmine plus stimulation</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

After detaching the distal tendon of the overlying tibialis anterior muscle, the distal tendon of the EDL was dissected free, cut, and fastened to a Grass FT03C force displacement transducer. Resting tension was adjusted to 4 g. The peroneal nerve was isolated and cut for placement on a bipolar stimulating electrode. The rat's paw and femur were clamped in a stereotaxic apparatus, and the (alert) animal was immobilized in a restraining jacket. Exposed muscle and nerve were kept moist, and the rat's body temperature was maintained with a heating pad and radiant heat source.

Physostigmine was injected subcutaneously in the rostral midback region. Dose was 0.25 LD50 in total volume made up to 0.2 cc with 1:2000 glacial acetic acid and adjusted to pH 4.0 (as above). Blood samples for ChE inhibition analysis were taken prior to physostigmine administration and at appropriate intervals thereafter.
Supramaximal (5 times threshold or 5T) stimulus pulses of 0.1 ms duration were delivered at 0.1 Hz to elicit single twitches of the EDL muscle. "Percent sustained contraction" (PSC) values were used to assess overall performance of the contractile mechanism. In the first and second Annual Reports, we used the term "tension ratio". This term is now considered somewhat confusing because an increasing tension ratio indicates a decreased ability to sustain a tetanic contraction. Thus, we now use PSC, which represents tension developed by the EDL muscle at the end of a 20 Hz 10 sec train divided by the tension at the beginning of the train. Accordingly a decrease in PSC reflects a decreased ability to sustain a tetanic contraction.

For the control (non drug treated) group of rats, PSCs were measured and then continuous stimulation was applied at 20, 40, or 80 Hz for 15 or 30 minutes. PSCs were again determined at 6 to 30 minutes after termination of continuous stimulation. For physostigmine-treated rats, after measurement of initial PSCs, physostigmine was administered and single EDL twitches (0.1 Hz stimulus) were monitored on a Graphtec WR3101 chart recorder. An increase in single twitch tension signaled the onset of the whole muscle response to physostigmine. (For a representative chart recording, see Fig. 24, RESULTS, section B.6.) The stimulator was then switched to 20, 40, or 80 Hz for 15 or 30 minutes. Final PSCs were determined 8 to 30 minutes after stimulus termination (corresponding to 45-80 minutes post-injection of physostigmine). Table 2 presents the number of animals in each protocol of continuous stimulation with and without physostigmine administration.
VIII. RESULTS

A. Summary of RESULTS in First and Second Annual Reports

1. Effects of Acute physostigmine exposure
   a. Establishment of LD₅₀

In the first Annual Report, we established that the LD₅₀ for acute subcutaneous injection of physostigmine was 0.75 mg/kg. Within 3-10 minutes after injection of 0.8-1.1 LD₅₀ (or 0.6-0.825 mg/kg) physostigmine, all rats exhibited pronounced fasciculation and tremor of superficial muscles, frequent tail rigidity, heightened startle reflex, increased salivation, pronounced respiratory distress, and increased ocular secretions. After 30 minutes of exposure, surviving rats had greatly diminished or nearly normal startle reflexes, and by 6-24 hours, had resumed (near) normal activity. At 0.3 LD₅₀, rats showed moderate signs of respiratory distress, excess salivation, and moderate whole body fasciculations. At 0.1 LD₅₀, rats showed no signs of respiratory distress or excess salivation, and observable fasciculations were confined to the facial musculature. Rats exposed to 0.001-0.01 LD₅₀ exhibited no overt signs of drug intoxication. Rats were weighed periodically and immediately before perfusion fixation. Normal weight gain was noted in all experimental groups up to 56 days PI.

b. Blood ChE Inhibitions at defined doses

Blood ChE assays were completed on all rats before injections and at prescribed intervals (over 300 rats, more than 3000 assays). Blood ChE inhibitions 30 minutes PI were linearly proportional to the log of the physostigmine dose between 0.01-0.8 LD₅₀ (0.0075-0.6 mg/kg). However, blood ChE inhibitions were anomalously higher than predicted at the very low dose exposures (see below). These values for blood ChE inhibition are consistent with blood ChE inhibitions measured at similar doses of other anti-ChE agents (24-26).

Based on an approximate LD₅₀ of 0.75 mg/kg, experimental dose levels were defined and standardized and the average blood ChE inhibitions 30 minutes PI were determined as follows:

- high dose = 0.8 LD₅₀ (0.6 mg/kg) = 89% ±3% inhibition
- threshold dose = 0.3 LD₅₀ (0.225 mg/kg) = ≈80% inhibition
- moderate dose = 0.1 LD₅₀ (0.075 mg/kg) = 67% ±7% inhibition
- low dose = 0.01 LD₅₀ (0.0075 mg/kg) = 33% ±7% inhibition
- very low dose = 0.001 LD₅₀ (0.75 mcg/kg) = 28% ±15% inhibition

Interestingly, blood ChE activity of animals not exposed to physostigmine ("controls") fluctuated substantially from day to day, thereby accounting at least in part, for the variabilities calculated for blood ChE inhibition in drug-treated rats.

c. Physiology

Measurable potentiation of EDL twitch tension began 7 to 30 minutes after subcutaneous injection of a high dose of physostigmine, with time of onset related to time required for the ChE inhibition level to reach 75-80%. Duration of the response ranged from 10 to 46 minutes, the twitch tension returning to normal before the ChE inhibition level dropped below 80%. Maximum
twitch potentiation apparently was influenced by several factors including peak ChE inhibiton level, rise time to attain inhibition greater than 80%, duration of inhibition above 80%, resting tension placed on the EDL for recording, and stress response of individual animals.


d. Ultrastructure

At sub-lethal but high doses, a characteristic lesion of the neuromuscular junctions was produced within 30 minutes of acute exposure to this potent but reversible anti-ChE agent. Supercontraction of subjunctional sarcomeres and the formation of exploded, swollen, frothy, and blistered subjunctional mitochondria were confirmed as the most obvious markers for the characteristic toxic lesion of the neuromuscular junction produced by this anticholinesterase agent. Moreover, the dose that produced supercontraction in 50% of diaphragm myofibers was defined as the "toxic threshold dose" (or "threshold" dose.) Soleus and EDL myofibers in non-stressed rats showed no evidence for supercontraction in any myofibers at the defined "threshold" dose. No signs of supercontraction or myofiber damage were noted 30 minutes PI for doses of 0.001-0.1 LD₅₀.

2. Recovery from Acute Physostigmine Exposure

Blood ChE activities from all rats receiving acute exposures to sub-lethal doses of physostigmine recovered to the normal range within 24 hours, but showed widespread fluctuations over the next 56 days. (Similar fluctuations were also seen in controls, see above.) The physiological alterations (EDL twitch potentiation and sustained high frequency contraction) returned to normal within one hour after acute physostigmine administration. In all cases, this return to normal physiology occurred when blood ChE inhibition levels were still 80% or higher. The relative extent of damage done to EDL during this period was rapidly reversed in terms of whole muscle physiology, but endplate ultrastructure was maximally altered sometime between 1 hour and 24 hours PI.

For doses of 0.8 LD₅₀, the destructive effects observed in all myofibers of diaphragm and soleus fibers were partially reversed 24 hours PI, and blood ChE levels had returned to near normal. (See first Annual Report.) In contrast, at 24 hours PI, EDL fibers exhibited increased myofiber damage and increased "frothy" and partially distended mitochondria. Seven days PI of 0.8 LD₅₀, junctional folds in some diaphragm and soleus myofibers appeared to be devoid of attached nerve terminal branches, an alteration originally attributed to drug effect. However, numerous similar images from "control" endplates (see Section B, below) indicate that such images are not due to delayed drug effect, but instead represent normal endplate ultrastructure as revealed in unusual planes of section.

By 14 days PI, small diameter nerve processes were observed, some apparently not embedded within a primary synaptic cleft. Such images are found equally frequently in control endplates, and thus are presumed to represent normal endplate variations. However, in some endplate regions, junctional folds
appeared to be missing and were replaced by vesicular debris similar to that seen after exposure to other anticholinesterase agents and in myasthenia gravis. These more pronounced endplate alterations suggested that in the most severely affected fibers, a process of (partial) denervation and possibly of reinnervation via small collateral "sprouts" had occurred. We concluded that physostigmine may exert substantial influences on biological control mechanisms regulating endplate morphology.

3. Effects of Subacute Physostigmine Exposure: Physiology

Biochemical analyses of blood ChE enzyme inhibitions were monitored following subacute exposure to physostigmine from very low to very high doses. Blood ChE inhibitions of 40% ±10% and 80% ±10% were obtained and maintained for 3, 7, and 14 days. Rats were monitored physiologically at the end of the subacute exposure period (3, 7, 14 days exposure) and at the end of the recovery period (14 days exposure followed by 7, 14, 28, or 56 days of recovery) and compared to rats receiving sham-filled implants. Blood samples taken either immediately or within 15 minutes after the start of recording gave average enzyme inhibition levels of 79% for high dose, no recovery rats. Inhibition levels averaged 72% for high dose recovery animals prior to pump removal. All rats (both high and low dose) that were allowed to recover for 3 or more days showed 0% serum ChE inhibition. Average EDL twitch tensions for all subacute exposure regimens and durations were not significantly different from the normal population. Percent sustained contractions (PSCs) measured during the first 10-15 minutes of recording were not significantly different (P<.01) from controls for any of the rats with high dose Alzet pumps (recovery or non-recovery). However, after 14 days of implantation of high dose pumps, the EDL fatigued more easily than normal and lost some ability to sustain a contraction (i.e., had a reduced PSC, see Results, section 6.a.) During the recovery phase (i.e., 3-28 days after pump removal), there was a gradual increase in PSC values.

4. Recovery from Subacute Physostigmine Exposure

For rats in the subacute/recovery experiments, at 3 days recovery and thereafter, EDL twitch tension and high frequency contraction properties were normal. This would be expected because a) ChE inhibition levels had returned to 0% (i.e., normal) and b) because these physiological properties had been altered minimally in the period before recovery. Thus, subacute administration of moderate or high doses of physostigmine does not produce significant changes in maximum muscle twitch tension. However, resistance to fatigue was slightly lowered at high subacute doses. Our preliminary data (see second Annual Report) also showed that subacute exposure to physostigmine at low to moderate doses produced no detectable alterations in endplate ultrastructure. These studies are now complete and comprise a major portion of this report (See Results, Section 7.b., p107.).
B. Current Results.

1. Endplate Ultrastructure in Control Rats: Normal Variability vs. Artifacts of Fixation

In well-perfused muscles from sham-injected rats (diaphragm, soleus and EDL muscle, Fig. 1-7), the myofibers contained uniformly spaced sarcomeres, mitochondria with closely spaced cristae, and a fine network of tubular sarcoplasmic reticulum membranes and transverse T-tubules. Because the ribs were cut prior to perfusion fixation and the diaphragm was allowed to foreshorten naturally, sarcomeres in the diaphragm myofibers were usually foreshortened to 1.7 μm (Figs. 1a-6a). On the other hand, the hind limbs were usually extended during fixation. Thus, the myofibers in the EDL were usually slightly stretched, yielding sarcomeres of 2.4-2.8 μm (Figs. 1c-6c), while the myofibrils in the soleus were foreshortened to slightly less than rest length, or 2.1 μm (Figs. 1b-6b).

Nerve terminals of control muscles contained densely packed synaptic vesicles and normal or slightly distended mitochondria. Frequently, Schwann cell "fingers" were interposed between the nerve terminal plasma membranes and junctional folds (Figs. 5 and Fig. 8). In several endplates, junctional folds adjacent to some nerve branches were extremely foreshortened (Figs. 2c and 3b). In other areas, junctional folds appeared to be present without an adjacent nerve terminal profile or were associated with small diameter nerve processes (Fig. 1c), similar to the "collateral sprouts" seen during nerve re-growth and associated with endplate repair and remodeling. (Similar images were also seen in control endplates following implantation of sham-filled mini-osmotic pumps, see Section 7.b.1, Figs. 32b, 33a, and 34a). However, examination of subsequent consecutive and non-consecutive serial sections revealed that such images usually arise as plane-of-section artifact (See interpretive drawing, Fig. 5b). These observations are in contrast to those of Hudson, et al. (28-30), who reported a) Schwann cell fingers between nerve and muscle only in anti-ChE-treated tissue and b) junctional folds apparently devoid of nerve terminals only in pyridostigmine treated endplates but not in endplates from control rats. Our 10-fold larger sample size of control endplates likely allowed us to identify these morphologies as "normal but atypical" rather than as "alterations due to drug effect". We have also documented that artifacts of poor perfusion are present in limited areas even in the highest rated perfusions. These artifacts are often misinterpreted as drug effects. Such poor perfusions usually are characterized by the presence of erythrocytes in nearby capillaries (see our first Annual Report) and by the presence of characteristic swollen mitochondria in all areas of the myofibers [Figs. 3c, 5a. See also Fig. 33c in Section 7b.], in the nerve terminals [Figs. 1a,c; 2b,c; 3a,c; 4a; 5a; 6a,b], and in adjacent non-contractile cells [eg., Schwann cells and fibroblasts, Figs. 1b, 2b, 4b]. In severe cases of such fixation hypoxia, mitochondrial swelling and
swelling of sarcoplasmic reticulum superficially resemble the effects of acute physostigmine exposure. Thus, criteria were introduced to allow artifacts of poor fixation to be distinguished from drug effects. (See RESULTS section B.2, page 49.)
Figure 1. Comparison of Neuromuscular Junctions from Rat Diaphragm, Soleus, and EDL Muscles 30 Minutes after Sham Injection ("control"). The myofibers are characterized by the presence of uniformly spaced sarcomeres and normal (undistended) mitochondria, sarcoplasmic reticulum, and T-tubules. Due to the position of the hind limbs during perfusion fixation, the soleus and myofibers are usually preserved at these characteristic sarcomere lengths.) The nerve terminals are usually within a concave primary synaptic cleft, which is lined by relatively regularly spaced junctional folds. However, some junctional folds are seen without apparent opposition by nerve terminal profiles (Figs. 1a and 1c, arrows. Also see Figs. 32b, 8a, and 8c). Nerve terminals have numerous synaptic vesicles and either normal or slightly distended mitochondria. (About 30% of normal endplates exhibit swollen mitochondria, presumably reflecting artifacts of fixation resulting from normal variability in fibers stimulated during/by glutaraldehyde fixation.) The non-synaptic surface of the nerve terminal is covered by cytoplasmic processes of Schwann cell (*). Occasionally, Schwann cell processes are within the primary synaptic cleft, interposed between the nerve terminal and the junctional folds (arrowheads). In all micrographs, scale bars represent 1 μm, unless otherwise indicated.
Figure 2. Comparison of Neuromuscular Junctions from Diaphragm, Soleus, and EDL Muscles 1 Day after Sham Injection ("control"). Variations in depth of junctional folds reflect normal variability, while variations in mitochondrial preservation ("swolllen mitochondria") reflect typical artifacts of immersion fixation using oxygenated glutaraldehyde solutions, which unfortunately, carry inadequate oxygen for such metabolically active tissues as muscle.
Figure 3. Comparison of Neuromuscular Junctions from Diaphragm, Soleus, and EDL Muscles 7 Days after Sham Injection ("control"). Note variations in depth of junctional infolding, some of which arise from plane-of-section artifact (See Fig. 5b) and some from normal variability of endplate ultrastructure (Fig. 3b, arrowhead. Seen at higher magnification in Fig. 8d).
Figure 4. Comparison of Neuromuscular Junctions from Diaphragm, Soleus, and EDL Muscles 14 Days after Sham Injection ("control"). Misalignment of sarcomeres (Fig. 4a) and occasional swollen mitochondria, especially in Schwann cells reflect inadequacies of chemical fixation. A portion of Figure 4a (arrowhead) is shown at higher magnification in Figure 5a.
Figure 5. Higher Magnification Image from Figure 4a (Fig. 5a). The single lateral swellings of the presynaptic mitochondria are characteristic of hypoxia during fixation, while the multiple "blisters" seen following prolonged nerve stimulation (see Figs. 26-28) are diagnostic for and proportional to the amount of prolonged nerve activity. Similar changes in nerve terminal mitochondria following exposure to high doses of anticholinesterase agents (see Figs. 9-13) and following anti-ChE plus prolonged stimulation (Figs. 29-31) are consistent with this interpretation. Diagram Illustrating Images Arising from "Plane-of-Section Artifacts". Various planes-of-section may result in images of nerve terminals that appear to be widely separated from the junctional folds, as well as images of junctional folds that appear to be present without an associated nerve terminal profile. (See also Figs. 8a-d).
Diaphragm
Figure 6. Comparison of Neuromuscular Junctions from Diaphragm, Soleus, and EDL Muscles 28 Days after Sham Injection ("control"). Note minor variations in endplate preservation.
Figure 7. Comparison of Neuromuscular Junctions from Diaphragm, Soleus, and EDL Muscles 56 Days after Sham Injection ("control"). Note variability in the preservation of mitochondria.
Figure 8. Atypical Variations Seen in Nerve Terminals of Sham Injected Rats. Junctional folds are seen without (apparent) association with axon terminals (Figs. 8a, 8c). In some regions, the depth of junctional infoldings is less than one-half micrometer (Fig. 8d), while in other areas, the near tangential plane of section causes junctional folds appear to be several micrometers in depth (Fig. 8b).
2. Comparison of Rat and Guinea Pig Neuromuscular Junctions after High Dose (0.3-0.8 LD₅₀) Acute Exposure to Physostigmine

a. Rats

1) High Dose Exposures

After a single high-dose exposure to physostigmine (0.8 LD₅₀ or 0.6 mg/kg; perfusion fixation 30 minutes PI), all neuromuscular junctions of rat diaphragm myofibers (Fig. 9, including both fast twitch and slow twitch fibers) and all neuromuscular junctions of rat soleus myofibers exhibited supercontraction restricted to the subjunctional sarcoplasm. In contrast, only 1 of 6 EDL myofibers showed even limited areas of supercontraction (see first Annual Report).

In regions of maximum supercontraction in the diaphragm and soleus muscles, partial or complete destruction of the myofibrillar apparatus was observed, including the disappearance of Z bands and the dissolution of recognizable sarcomeres. Diaphragm fibers were rapidly, consistently, and grossly affected; soleus fibers were somewhat less affected; and EDL fibers were consistently the least affected 30 minutes PI. Thus, the diaphragm was proposed as the best model for analyzing ultrastructural alterations associated with one of the immediate life-threatening effects of physostigmine intoxication (i.e., blockage of respiration). Since the diaphragm is a "mixed" muscle (i.e., contains fast, intermediate and slow twitch fibers), and since all fiber types in the diaphragm were equally severely affected, we attributed these differences in expression of cytopathology to differing patterns of muscle use rather than to fiber-type differences. [By differing patterns of muscle use, we mean that under physostigmine-induced respiratory insufficiency, the diaphragm myofibers were activated maximally and virtually continuously; the soleus, a postural or support muscle, was used only sporadically (because the rat supported much of its weight on its abdomen during the period of maximum drug effect); and the EDL muscles, used to extend the digits during walking, were virtually unused because walking was minimal during the initial exposure period.]

In the supercontracted regions of diaphragm and soleus fibers, a continuum of severely to slightly damaged mitochondria was observed in the subjunctional sarcoplasm (Fig. 9). The most extreme cases seen in this study were "exploded" mitochondria, which had obvious discontinuities in their external membranes. The most severely affected mitochondria were in the diaphragm myofibers and were immediately adjacent to the junctional folds; those less affected ("frothy") mitochondria were at a greater distance; the least affected mitochondria (those that were "blistered") were at the margins of the supercontracted a-as (Figs. 9a and 9c). Thus, high dose exposures to physostigmine (i.e., 0.8 LD₅₀ or 0.6 mg/kg; >90% blood ChE inhibition) produced a characteristic lesion -- concentric hemispheres surrounding the endplate region, each successive zone nearer the endplate exhibiting increased damage to sarcomeres, mitochondria, and sarcoplasmic reticulum. This apparent continuum of mitochondrial changes associated with severe endplate depolarization included
the following sequence, starting with mitochondria in the unaffected perijunctional cytoplasm and terminating in the mitochondria adjacent to the junctional folds: normal mitochondria → mitochondria with single blisters → mitochondria with multiple blisters [frothy] → swollen mitochondria → exploded mitochondria.

This continuum of mitochondrial changes 30 minutes after exposure to a high dose of physostigmine was contrasted with the abrupt localized swellings and/or gross distentions of mitochondria that are seen under hypoxic conditions. In hypoxic myofibers (31), mitochondria had segments of normal diameter and with condensed matrix, but in addition, had distended localized regions, either containing electron-dense glycogen granules (or in samples stained with aqueous uranyl acetate, clear areas with strings of minute electron dense granules). These differences have been used to distinguish the effects of a) anti-ChE agents and b) excess or prolonged endplate activity vs. c) artifacts of hypoxia/glutaraldehyde fixation. (See Section B.6, for a description of alterations induced by prolonged stimulation in the absence of drug.)

At high doses of physostigmine, changes in nerve terminals were also detected. When compared to the normal variability in mitochondrial preservation in control endplates (Figs. 1-7), mitochondria in the nerve terminals of diaphragm myofibers were swollen and distorted (Fig. 9). (By comparison, however, the postsynaptic mitochondria were much more severely affected.) Occasionally, nerve terminal branches were virtually depleted of synaptic vesicles (SVs) and were greatly enriched in coated vesicles (CVs), thereby indicating substantial and prolonged SV exocytosis, as well as substantial ongoing endocytosis. Thus, we support in part the conclusion of Hudson (28-30) that nerve terminals are altered concommitant with the development and severity of supercontraction. However, during the ChE-induced depolarizing neuromuscular blockade (1), it is not yet clear whether increased presynaptic activity preceeds, coincides with, or follows the failure of neuromuscular transmission, nor is it clear when in this sequence that swelling of presynaptic mitochondria occurs.

2) Threshold for Development of Endplate Cytopathology

Titration of responses revealed an abrupt increase in ultrastructural alterations from 0.1 LD_{50} (67% ±7% inhibition) to 0.3 LD_{50} (75% ±5% inhibition) to 0.8 LD_{50} (89% ±3% inhibition). At 0.1 LD_{50}, no supercontraction or other evidence for endplate cytopathology was observed. At 0.3 LD_{50}, approximately 60% of diaphragm myofibers were supercontracted (Fig. 10), while soleus and EDL myofibers showed no evidence for supercontraction (Fig. 11). Consequently, using a) supercontraction and b) "frothy mitochondria" as the two morphological criteria for establishing the desired "morphological dose response curve" for physostigmine toxicity, it is apparent that there is a steep onset resembling a "threshold" for the development of cytopathology. In resting, non-stressed animals, threshold for cytopathology occurs at 0.3 LD_{50}.
or 75 ±5% enzyme inhibition.

b. Guinea Pigs

In myofibers obtained from a guinea pig 30 minutes after injection of 1.2 mg/kg ( >0.8 LD$_{50}$), virtually all diaphragm myofibers (as well as many myofibers in the EDL muscle) exhibited profound supercontraction of subjunctional sarcomeres, as well as the presence of swollen, frothy, and blistered mitochondria (Figs. 12a, 12c). Unexpectedly, soleus fibers (Fig. 12b) were less affected than EDL fibers. Few soleus myofibers had supercontracted sarcomeres and none exhibited exploded mitochondria. At 0.75 mg/kg, myofibers appeared nearly normal (Fig. 13). Sarcomeres were of normal length, but sub junctional mitochondria were enlarged and had occasional blisters between cristae. These alterations are consistent with those seen in the rats at 0.8 and 0.3 LD$_{50}$, respectively. However, at 30 minutes PI in guinea pigs (three animals tested), EDL fibers appear to be more sensitive to the toxic effects of physostigmine than soleus fibers. The small sample size does not permit conclusions concerning the sources of the differences between rats and guinea pigs, but the apparent reversal of fiber-type specificity for anti-ChE. effect may be due to differing muscle use patterns between rat and guinea pigs, species differences in susceptibility to anticholinesterase agents, or to a limited sample size.

In summary, supercontraction and the development of a similar characteristic series of mitochondrial alterations at similar LD$_{50}$ levels in guinea pigs and rats suggests that either animal model is appropriate for analyzing the alterations in endplate morphology at near lethal drug exposure levels. However, for long term experiments involving a large number of animals, rats provide an equally useful model for predicting pathophysiological alterations of skeletal muscle yet at a substantial cost savings.
Figure 9. Comparison of the Effects of Acute High Dose of Physostigmine (0.8-1.1 LD₅₀) on Neuromuscular Junctions of Rat Diaphragm, Soleus, and EDL Muscle 30 Minutes PI. Supercontraction with associated exploded, swollen, frothy and blistered mitochondria (Figs. 9a and 9b) provide unambiguous evidence for physostigmine toxicity at the neuromuscular function of diaphragm and soleus muscle. EDL myofibers are little affected at 30 minutes PI.
Figure 10. Comparison of the Effects of Acute Near Threshold Dose of Physostigmine (1.0 LD<sub>50</sub>) on Neuromuscular Junctions of Rat Diaphragm, Soleus, and EDL Muscles 30 minutes PI. Concentric hemipherical zones of decreasing destruction surround the neuromuscular junctions.
Figure 11. Comparison of Effects of Acute Near Threshold Dose of Physostigmine (0.3 LD$_{50}$) on Rat Diaphragm and Soleus Muscles 30 Minutes PI. No obvious ultrastructural alterations are evident even though these muscles are from the same rat as illustrated in Fig. 10.
Figure 12. Comparison of the Effects of Acute High Dose of Physostigmine (1.2 mg/kg) ChE on Neuromuscular Junctions of Guinea Pig Diaphragm, Soleus, and EDL Muscles 30 Minutes PI. Note the characteristic continuum of mitochondria changes associated with supercontraction (Fig. 12a and 12c) as well as the "vesicated" mitochondria in the nerve terminals (Fig. 12b).
Figure 13. Comparison of the Effects of Acute Threshold Dose of Physostigmine (0.75 mg/kg) inhibition on Neuromuscular Junctions of Guinea Pig Diaphragm, Soleus, and EDL Muscles 30 Minutes PI. Note the blistered, and frothy, and swollen mitochondria in Figure 13a and 13b. Consequently, this is considered a near but subthreshold dose in the guinea pig.
3. Rat Neuromuscular Junctions after Moderate to Very Low Doses (0.1 \text{LD}_{50} \text{ to } 0.001 \text{LD}_{50})

a. Moderate Dose (0.1 \text{LD}_{50})

After 30 minutes exposure to a moderate dose of physostigmine (0.075 mg/kg; ChE inhibition of 67\% \text{ + 7\%}), the nerve terminals of most endplates in diaphragm, soleus, and EDL (Fig. 14) appeared normal, containing numerous synaptic vesicles, a few coated vesicles, normal mitochondria (occasionally slightly swollen), and a few flattened cisternae. The increased number of coated vesicles and flattened cisternae constitutes evidence of increased presynaptic activity immediately prior to fixation (53-55), presumably as an effect of drug toxicity. In some cases (Fig. 14a), both pre- and post-synaptic mitochondria, as well as those in adjacent myofibers, were swollen and distorted. (Such mitochondrial changes are not attributed to drug effect, but instead, are attributed primarily to tissue hypoxia during aldehyde fixation. Although the hypoxia may arise from non-specific systemic drug effect, the mitochondrial alterations in the non-junctional sarcoplasm are consistent with increased muscle activity immediately before and during fixation combined with glutaraldehyde-augmented hypoxia.)

Post-synaptically, diaphragm myofibers revealed few gross changes in their myofibrils (Fig. 14a). None (0\%) of the myofibers in diaphragm, soleus, or EDL exhibited any evidence of supercontraction or for damage to mitochondria or to sarcoplasmic reticulum. However, a few blistered and frothy mitochondria were observed in the subjunctional sarcoplasm in diaphragm myofibers. Since all the other changes were within normal variations seen in the control rats, only those very distinctive changes in post-junctional mitochondria (blistered and frothy mitochondria) were identified as representing an initial stage in the sequence of degeneration as seen in the high dose acute exposures. (The blistered and frothy mitochondria seen at higher doses are attributed to a secondary consequence of the primary drug effect. The excess rate and/or duration of endplate depolarizations results in flooding of the endplate region with Na\textsuperscript{+} and Ca\textsuperscript{++} ions and associated water molecules. Mitochondria initially sequester Ca\textsuperscript{++} ions, but once "saturated" with calcium phosphate granules, apparently begin to develop numerous internal clear "blisters" and "frothy" areas of as yet undetermined origin. As ion and water influx continue, the frothy areas expand and the mitochondria continue to swell. At much higher dose levels, severe and prolonged endplate depolarizations ultimately result in rupture or "explosion" of the subjunctional mitochondria.) Thus, 0.1 \text{LD}_{50} (or 67\% enzyme inhibition) appears to represent a near- but sub-threshold level for induction of endplate cytopathology in unstressed animals. Whether threshold is static at 0.3 \text{LD}_{50} or is altered by muscle use (i.e., by indirect stimulation) is currently under investigation. (See
Section B.6, p. 88.)

b. Acute Low and Very low Doses (0.01 and 0.001 LD50)

After 30 minutes of exposure to very low doses of physostigmine (0.01 and 0.001 LD50, 33% (+7%) and 28% (+15%) inhibition of blood ChE), myofibers from diaphragm, soleus, and EDL revealed no detectable changes in nerve terminals or in the subjunctional cytoplasm (Figs. 15, 16). Sarcomeres were of normal length and the membrane-bound organelles (mitochondria, sarcoplasmic reticulum, triads and T-system) were normal. The nerve terminals were filled with synaptic vesicles and occasional flattened cisternae. In most nerve terminals, mitochondria were normal, with none of the distinctive lateral swellings attributed to hypoxia. However, in a few instances, just as in sham treated controls (Figs. 2b, 3c, 4b, 6b, and 25c, 27a, 28c, 29a), swollen mitochondria were also observed in nerve terminals (Fig. 16a, 16d) and in nearby fibroblasts and Schwann cells (Fig. 16a). In our first Annual Report, we showed that one class of swollen mitochondria contained numerous minute, electron-dense granules identified as the remnants of glycogen granules extracted by aqueous uranyl acetate (31). Thus, this type of mitochondrial swelling was characteristic of glutaraldehyde-fixed hypoxic tissue rather than of drug-induced swelling. Moreover, since other stages in the continuum of physostigmine-induced alteration (blisters, frothy and swollen mitochondria) were not present, these changes are attributed to artifacts of hypoxia and of glutaraldehyde fixation rather than to drug effect on neuromuscular junction ultrastructure. Thus, following low and very low acute doses of physostigmine (0.01 and 0.001 LD50), there were no detectable changes in nerve terminals or in the subjunctional cytoplasm.

4. Delayed Effects of Acute Exposure to Physostigmine

a. Acute High Dose

After a single high-dose injection (0.8-1.1 LD50), several unusual morphologies were noted during the period 1-56 days PI. (In many instances, it is not possible to differentiate between changes representing acute-delayed effects and changes representing recovery/repair. Therefore, additional details are described in Section B.5.) As described in the first and second Annual Reports and summarized above, destructive effects on sarcomeres and subjunctional mitochondria were consistently observed in the neuromuscular junctions of diaphragm and soleus muscles 30 minutes after a single high dose (0.8-1.1 LD50) injection of physostigmine. These effects were partially reversed in diaphragm and soleus muscle by 24 hours PI. In contrast, EDL myofibers were scarcely damaged at 30 minutes PI (0.8-1.1 LD50), but by 24 hours after a single exposure, extensive myofibrill disruption and damage to junctional folds was evident, including the presence of many frothy mitochondria in
the subjunctional cytoplasm. In addition, we noted (first Annual Report) that at 24 hours PI, clusters of closely packed nerve terminal branches were found in the neuromuscular junction of a few diaphragm muscles, but subsequently, similar images have been seen equally frequently in "control" endplates (Fig. 5a). In some fibers, areas of continuing myofibril dissolution were previously reported beneath junctional folds that apparently were devoid of attached nerve terminals. Those data originally were thought to support previous suggestions that partial denervation and reinnervation phenomena occur in some fibers during the acute recovery phase. In addition, several endplates from the diaphragm and soleus muscles 14, 28, and 56 days PI had vesicular debris in the synaptic clefts and areas of apparent junctional fold "simplification" similar to that found in the neuromuscular junctions of patients with myasthenia gravis (35-38,40,41,56,57) and after treatment with other anti-ChE agents (11-30). In other fibers, junctional folds appeared to be devoid of attached nerve terminals (Fig. 22, arrow). However, based on our subsequent analysis of a much larger number of sections from control endplates, we conclude that the very small diameter nerve terminals as well as those apparently not embedded within a primary synaptic cleft may not reflect denervation and the formation of new nerve terminals by collateral sprouting as previously proposed. Although many of these images show convincing evidence of repair of prior degenerative changes at the motor endplate, many images interpreted as evidence of residual damage are now thought to be indistinguishable from atypical but normal variations. However, the presence of a few necrotic fibers is evidence that at near lethal doses, a few muscle fibers had been damaged irreversibly. Nevertheless, we conclude that at recovery periods greater than 7 days following sublethal exposures to physostigmine, repair processes are both rapid and efficient and essentially complete in most myofibers and in their innervating nerves. As a consequence of these rapid repair processes, by 7-14 days PI, most fibers in all surviving high dose animals were indistinguishable from normal myofibers.

b. Acute Moderate to Very Low Doses of Physostigmine

At 24 hours PI (0.1 LD<sub>50</sub>-0.001 LD<sub>50</sub>), no damage to endplates was detected and no delayed effects were noted in any endplates. In contrast to data presented by Hudson and coworkers (24-26), no increase was noted in the normal frequency of separations of nerve terminal membranes from junctional folds, or in the frequency of areas with Schwann cell fingers interposed between nerve terminal and junctional folds. Moreover, from 1 to 56 days PI, no ultrastructural changes in neuromuscular junctions were detected in any of several hundred myofibers examined (Fig. 21 for 0.1 LD<sub>50</sub>; Fig. 22 for 0.01 LD<sub>50</sub>; and Fig. 23 for 0.001 LD<sub>50</sub>), nor were
any physiological deficits measured (See first and second Annual Reports.). In all well-fixed muscle samples, subjunctional sarcomeres exhibited the same length as extrajunctional sarcomeres; pre- and post-synaptic mitochondria and other membrane-bound organelles appeared normal; junctional fold depth, number, and distribution appeared normal; and Schwann cells had the same variability as in untreated controls. Moreover, delayed ultrastructural alterations were not observed at any stage from 1-56 days PI. Serial section reconstruction revealed that areas of junctional folds apparently devoid of axon terminals (in both control and treated endplates) resulted when the plane-of-section transected the crests of junctional folds but did not include the closely associated axon end-bulbs (see Fig. 5b). Thus, in the absence of serial reconstruction or detailed morphometric analysis of a very large number of sections, it must be concluded that, rather than representing degenerating endplates, these atypical images represent normal morphological variations as revealed in unusual planes-of-section.
5. REVERSIBILITY OF ULTRASTRUCTURAL ALTERATIONS

a. High-Dose

The acute effects of high dose (0.8-1.1 LD$_{50}$; 0.6-0.8 mg/kg) physostigmine seen at ½ hour and 24 hours PI were substantially reversed by 7 days and were virtually indetectable by 14 days and thereafter (see first Annual Report). Since at 0.8 LD$_{50}$, only a very small proportion of fibers sustained irreversible damage, the relatively small number of fibers examined (<300) and the very small number of endplates examined (<30) would permit an estimate sufficiently accurate only to conclude that the proportion of irreversibly damaged fibers is at most only a few percent. This conclusion was supported by numerous physiological measurements at greater than 7 days PI, which showed that in treated vs control rats, there were no detectable differences in muscle force generation, resistance to fatigue, or twitch potentiation.

b. Moderate and Low Doses

After moderate to very low doses (0.1 to 0.001 LD$_{50}$, 37% ± 7% to 28% ± 15% blood ChE enzyme inhibition), the junctional ultrastructure of diaphragm muscle at 7 days to 56 days PI (Figs. 17-23) was similar to that of normal or sham injected controls. None of the fibers in diaphragm, soleus, or EDL exhibited supercontraction or any other physiological or ultrastructural evidence of myofiber damage at 7-56 days. Moreover, we have found no evidence of delayed toxicity at the neuromuscular junction at 0.1-0.001 LD$_{50}$ at any period between 7 and 56 days PI.
Figure 14. Effects of an Acute Moderate (0.1 LD$_{50}$) Dose of Physostigmine on Rat Neuromuscular Junctions 30 Minutes PI. Diagram Illustrating Images Arising from "Plane-of-Section Artifacts".
Figure 15. Effects of an Acute Low Dose of Physostigmine (0.01 LD<sub>50</sub>) on Rat Neuromuscular Junctions 30 Minutes PI. No changes in endplate ultrastructure were noted.
Figure 16. Effects of an Acute Very Low Dose of Physostigmine (0.001 LD₅₀) on Neuromuscular Junctions 30 Minutes PI. Except for hypoxic mitochondria (Figs. 16a, 16c), the endplates appeared normal.
Figure 17. Recovery of Rat Diaphragm, Soleus, and EDL Neuromuscular Junctions 14 days after a Low Dose of Physostigmine (0.01 LD₅₀). Endplates have no recognizable alterations.
Figure 18. Recovery of Diaphragm, Soleus, and EDL Neuromuscular Junctions 28 days after a Low Dose of Physostigmine (0.01 LD50). Except for hypoxic mitochondria, endplates appear normal.
Figure 19. Recovery of Diaphragm, Soleus, and EDL Neuromuscular Junctions 56 days after a Low Dose of Physostigmine (0.01 LD$_{50}$). Except for hypoxic mitochondria, endplates appear normal.
Figure 20. Recovery of Rat Diaphragm, Soleus, and EDL Neuromuscular Junctions 7 days after a Very Low Dose of Physostigmine (0.001 LD$_{50}$). Endplates appear normal (no hypoxia.)
Figure 21. Recovery of Diaphragm, Soleus, and EDL Neuromuscular Junctions 14 days after a Very Low Dose of Physostigmine (0.001 LD$_{50}$). Endplates appear normal.
Figure 22. Recovery of Diaphragm, Soleus, and EDL Neuromuscular Junctions 28 days after a Very Low Dose of Physostigmine (0.001 LD$_{50}$). Endplates appear normal.
Figure 23. Recovery of Diaphragm, Soleus, and EDL Neuromuscular Junctions 56 days after a Very Low Dose of Physostigmine (0.001 LD$_{50}$). Endplates appear normal.
6. Effects of Stimulation on Muscle Physiology and Endplate Ultrastructure in the Presence and Absence of a Moderate Dose of Physostigmine

   a. Physiology

   The effects of muscle use on physostigmine-induced pathology have been assessed using low to high frequency stimulation of the common peroneal nerve, thereby simulating a continuous workload on the muscle. Stimulation patterns included 20, 40, and 80 Hz trains of 15 and 30 minutes duration. Rats were stimulated continuously in the absence of drug or following an acute subcutaneous dose of physostigmine at 0.25 LD$_{50}$. Blood cholinesterase inhibition levels averaging 76% were measured during stimulation (26 samples from 7 animals). Average twitch tension (single twitches elicited at 0.1 Hz prior to continuous stimulation and/or physostigmine administration) was 32 (+9) grams (n=27). Of the 27 rats included in this part of the study, 12 received physostigmine plus continuous stimulation, while 15 received continuous stimulation without drug. The number of rats in each experimental group is shown in Table I (see Materials and Methods).

   When 20, 40, or 80 Hz stimulation was applied, a large reduction in strength of contraction occurred over the 15 or 30 minutes of continuous stimulation. The nature of this response is illustrated in Figure 24. The decrease in strength of contraction averaged 82% during 20 Hz, 92% during 40 Hz, and 95% during 80 Hz stimulation. Physostigmine administration in combination with continuous stimulation produced no significant difference in final strength of contraction compared to continuous stimulation only.

   Percent sustained contractions (PSCs) were compared prior to and at selected times after continuous stimulation and/or physostigmine administration (Fig. 25). For control, non-drug treated rats (n=15), a two way analysis of variance (ANOVA) showed no significant change (P<.01) in PSCs due to variation in frequency (20, 40, or 80 Hz) or duration (15 or 30 minutes) of stimulation. An ANOVA comparing these controls to rats treated with continuous stimulation plus physostigmine demonstrated that treatment with drug plus stimulation produced a significant (P<.01) increase in PSC when compared to stimulation alone. For the physostigmine-treated rats, neither variation in stimulus frequency nor interaction between stimulus frequency and drug treatment was found to be significant.

   Final PSCs were measured at selected times after termination of continuous stimulation and/or physostigmine administration. Recovery times in control rats (stimulation only) ranged from 6 to 30 minutes after continuous stimulation was stopped, with some rats being tested at both 15 and 30 minutes. For physostigmine treated rats, recovery times ranged from 8 to 45 minutes after continuous stimulus termination, corresponding to 45 to 80 minutes after physostigmine administration. Within the time ranges measured, no significant differences in PSCs were measured for the recovery times either post-stimulation or post-injection.
Figure 24. Chart Record illustrating experimental protocol for effects of physostigmine intoxication plus high frequency stimulation on rat EDL muscle. Rats anesthetized by spinal block with 2% lidocaine and immobilized as described in Methods. The following steps describe experimental procedure:

A) normal twitch tension with stimulation of peroneal nerve at 0.1 hz (pulse duration 0.1 msecond; strength 5X threshold)

B) initial PSC obtained with 20 hz 10 s stimulus train

C) temporary twitch potentiation following sustained contraction elicited in B)

D) when twitch tension returns to normal, physostigmine administered (0.25 LD50 given subcutaneously)

E) approximately 10-25 minutes post-injection, twitch potentiation indicates beginning of whole muscle response to physostigmine

F) when potentiation in E) is observed, stimulation at 20, 40 or 80 hz is begun

G) strength of contraction gradually decreases during continuous high frequency (20, 40 or 80 hz) stimulation

H) high frequency stimulation terminated after 15 or 30 minutes and stimulation at 0.1 hz resumed

I) when twitch tension returns to normal final PSC determined with 20 hz 10 seconds stimulus train
FIGURE 25. Effect of physostigmine and continuous stimulation on Percent Sustained Contraction (PSC) in EDL.

Percent Sustained Contraction (PSC) is the tension (in grams) at the end of a 10 second 20 hz stimulus train divided by the tension at the beginning of the train. A lower PSC indicates a decreased ability of the muscle to sustain a tetanic contraction. Continuous stimulus trains at 20, 40, or 80 hz were applied for 15 or 30 minutes in the absence or presence of physostigmine intoxication. Physostigmine was injected subcutaneously at a dose of $0.25 \text{LD}_{50}$. 

Stimulation Only
no drug

Physostigmine
plus stimulation

- before experimental treatment
- residual tension

n=5 n=5 n=5 n=4 n=4 n=4
b. Ultrastructure

1) Stimulated only (no drug)

Neither supercontraction nor other evidence of endplate hyperactivity was seen in EDL from the unstimulated (contralateral) side. However, in stimulated myofibers, a continuum of changes was noted, directly proportional to duration and frequency of stimulation. After 15 minutes of 20 Hz stimulation, mitochondria in the nerve terminals of EDL myofibers exhibited a few "vesications". Post-synaptically, there were a few blistered and frothy subjunctional mitochondria (Fig. 26). After 30 minutes of 20 Hz stimulation, mitochondria in the nerve terminals were frequently "vesicated" but few alterations were noted in the subjunctional sarcoplasm. At 40 Hz and 80 Hz for 15 and 30 minutes, EDL myofibers exhibited substantial increases in vesication of pre-synaptic mitochondria (Figs. 27, 28). Most notable, however, was the appearance of vesicated, frothy, and swollen mitochondria in the immediate subjunctional sarcoplasm. Endoplasmic reticulum, golgi cisternae, and nuclear membranes were also swollen, but unlike endplates exposed to suprathreshold doses of physostigmine, there was no supercontraction of subjunctional sarcomeres. After 30 minutes of stimulation at a given frequency, endplates were altered approximately the same as endplates receiving stimulation at twice that frequency but for half that duration (i.e., 40 Hz at 30 minutes resembled 80 Hz at 15 minutes, while 20 Hz at 30 minutes produced changes equivalent to those seen after 40 Hz stimulation for 15 minutes). Thus, at these unusually prolonged and high rates of stimulation, the degree of mitochondrial damage (both pre- and post-synaptically) was proportional to the total number of nerve depolarizations, whether delivered in 15 or 30 minutes.

Since there was normally a 3-5 minute delay between the termination of stimulation and the onset of perfusion fixation, and since we were concerned that the ultrastructural alterations produced by prolonged and continuous stimulation might be reversed substantially during that delay, additional samples were prepared by a) immersion fixation while the stimulation was in progress ("Zero" delay; only the outermost surface fibers examined) and b) by perfusion fixation at 2, 5 and 15 minutes after termination of stimulation. Except for slightly greater hypoxic damage to mitochondria in the immersion fixed samples (especially in deeper fibers), essentially no differences were observed in mitochondrial morphology at 0-15 minutes of "recovery". Thus, we conclude that the observed changes in mitochondria following the prolonged stimulations accurately reflected the cumulative effects of the 18,000 to 144,000 endplate depolarizations elicited. Despite an enormous capacity for repolarization during normal rates and durations of stimulation, these levels of stimulation are 1 to 2 orders of magnitude greater than normally encountered during sustained neuromuscular activity. Apparently, it must be inferred that these ultrastructural changes reflect severe endplate fatigue. Under such circumstances, restoration of normal ionic and metabolite levels may require hours or even days (certainly much more than the 15 minutes following this experiment). If so, these normal repair mechanisms are qualitatively similar to those
occurring during endplate repair following acute high dose exposures to physostigmine. Conversely, recovery from an acute exposure to a sublethal dose of physostigmine is likely to be qualitatively and temporally similar to that following severe neuromuscular fatigue.

2) Stimulation Plus Subthreshold Dose (0.25 LD$_{50}$) of Physostigmine.

In rats exposed to 0.25 LD$_{50}$ physostigmine, myofibers from the normally stimulated diaphragm and from unstimulated soleus and EDL muscles had no detectable ultrastructural alterations. Likewise, in the contralateral muscles stimulated at 20 Hz for 15 or 30 minutes (Fig. 29), soleus and EDL myofibers were also essentially unchanged, except that pre-synaptic mitochondria exhibited a few "vesicated" intercristal spaces, especially in fibers stimulated at 20 Hz for 30 minutes. Endplates stimulated at 40 Hz for 15 or 30 minutes (Fig. 30), were essentially identical to those receiving similar stimulation in the absence of drug [i.e., sub-synaptic mitochondria were minimally altered after 15 minutes of stimulation plus drug (Fig. 30a), but following 30 minutes of stimulation at 40 Hz in the presence of 0.25 LD$_{50}$ physostigmine, mitochondria were blistered, frothy, or grossly swollen. Likewise, presynaptic mitochondria were either multiply vesicated or exhibited numerous internal dilations. This characteristic lesion was previously found only in excessively stimulated endplates or in those exposed to near lethal levels of physostigmine (i.e., above 0.8 LD$_{50}$).

 Interestingly, a limited synergistic effect of physostigmine and increased nerve terminal activity was noted in endplates stimulated at 80 Hz for 15 or 30 minutes (Fig. 31). EDL (as well as soleus) myofibers exhibited severe alterations of pre- and post-synaptic mitochondria after either 15 or 30 minutes of stimulation plus drug (Fig. 31). Post-synaptic mitochondria were invariably blistered, frothy, or grossly swollen. Presynaptic mitochondria were either multiply vesicated or exhibited numerous internal dilations. Finally, despite severe pre- and post-synaptic alterations of mitochondria no evidence for supercontraction was observed following up to 30 minutes of stimulation at 80 Hz, with or without a subthreshold dose of physostigmine. Thus, these images suggest that excessive stimulation, even in the presence of 0.25 LD$_{50}$ (70% serum ChE inhibition), is not capable of mimicking all of the lesions characteristic of those seen following acute exposure to near lethal doses of physostigmine (i.e., >0.6 LD$_{50}$). These observations at first appear to contradict our previous suggestion that the threshold for physostigmine-induced damage is altered substantially by muscle use (see first and second Annual Reports.) However, additional data from stimulated and unstimulated EDL endplates fixed for electron microscopy at 15 and 30 minutes following acute high dose exposure to physostigmine and to pyridostigmine at similar dose levels provide an additional explanation for our original contention. Those data will comprise a substantial portion of the forthcoming Final Report.
Figure 26. Ultrastructure of EDL Neuromuscular Junctions after 15 and 30 Minutes of 20 Hz Stimulation of the Common Peroneal Nerve. Nerve terminals are partially depleted of synaptic vesicles (Fig. 26a) and contain slightly swollen mitochondria, whereas post-synaptic mitochondria are normal (i.e., no hypoxic swelling). Otherwise, endplates appear normal.
Figure 27. Ultrastructure of EDL Neuromuscular Junctions after 15 and 30 Minutes of 40 Hz Stimulation of the Common Peroneal Nerve. Nerve terminals are virtually depleted of synaptic vesicles and all pre-synaptic mitochondria are extensively "vesiclated". Post-synaptic mitochondria are relatively unaffected after 15 minutes of 40 hz stimulation. However, after 30 minutes of 40 hz stimulation (Figs. 27b and c), post-synaptic mitochondria are swollen or exploded.
Figure 28. Ultrastructure of EDL Neuromuscular Junctions after 15 and 30 Minutes of 80 Hz Stimulation of the Common Peroneal Nerve. Nerve terminals are virtually depleted of synaptic vesicles and all pre-synaptic mitochondria are extensively "vesicated". Post-synaptic mitochondria are relatively unaffected after 15 minutes of 80 hz stimulation. However, after 30 minutes of 80 hz stimulation (Figs. 28b), post-synaptic mitochondria are swollen or exploded. Note, however, that sarcomeres are of normal length and that there is no evidence for supercontraction.
Figure 29. Ultrastructure of EDL Neuromuscular Junctions after 15 and 30 Minutes of 20 Hz Stimulation in the Presence of 0.25 LD$_{50}$ Physostigmine. Except for a few swollen mitochondria, nerve terminals appear normal. Post-synaptically, sarcomeres and mitochondria appear normal despite 15 or 30 minutes of 20 Hz stimulation in the presence of a subthreshold dose of physostigmine.
0.25LD<sub>50</sub> physostigmine
20Hz-15min

0.25LD<sub>50</sub> physostigmine
20Hz-30min
Figure 30. Ultrastructure of EDL Neuromuscular Junctions after 15 and 30 Minutes of 40 Hz Stimulation in the Presence of 0.25 LD₅₀ Physostigmine. After 15 minutes of stimulation, pre- and post-synaptic components appear normal. However, after 30 minutes of 40 Hz stimulation in the presence of a subthreshold dose of physostigmine, nerve terminals are virtually depleted of synaptic vesicles and all pre-synaptic mitochondria are extensively "vesicated". Post-synaptic mitochondria are either swollen or exploded, yet sarcomeres of normal length and there is no evidence for supercontraction.
0.25LD50 physostigimine
40Hz-15min.
Figure 31. Ultrastructure of EDL Neuromuscular Junctions after 15 and 30 Minutes of 40 hz Stimulation in the Presence of 0.25 LD$_{50}$ Physostigmine. Nerve terminals contain numerous synaptic vesicles but all pre-synaptic mitochondria are extensively "vesicated". Post-synaptic mitochondria are relatively either swollen or exploded after 15 minutes of 80 hz stimulation. After 30 minutes of 80 hz stimulation (Figs. 31b), pre-synaptic mitochondria are extensively vesicated while post-synaptic mitochondria are frothy, swollen, or exploded. Sarcomeres are of normal length and there is no evidence for supercontraction.
7. Effects of Subacute Exposure to physostigmine

a. Physiology

Physiological analyses of subacute exposure experiments have been completed, were described in the second Annual Report, and are summarized in RESULTS, Section A.3-A.4.

b. Ultrastructure of Endplates

1) Controls

In well-perfused muscles from rats implanted with sham-filled mini-osmotic pumps, myofibers from diaphragm, soleus and EDL muscle (Fig. 32-36) resembled myofibers from all other "controls". Nerve terminals contained densely packed synaptic vesicles and either normal or slightly distended mitochondria, the latter being attributed to artifacts of hypoxic glutaraldehyde fixation. As in other control preparations, Schwann cell "fingers" were seen interposed between the nerve terminal plasma membranes and the junctional folds (Figs. 32b, 34c). Occasionally, junctional folds were extremely foreshortened (see Fig. 8d, from a control rat) or were associated with very small diameter nerve processes (compare Fig. 8b from control with Figs. 33b, 8b), while some junctional folds appeared to be without associated nerve terminal profiles (Fig. 34a; 8a, 8c). (For interpretive drawing, see Fig. 5b.) The frequency with which these images are encountered in myofibers in control rats requires cautious interpretation of similar images from drug treated animals. (See next section.)

2) High vs. Low Dose Subacute Exposure

Rats were implanted with Alzet pumps which maintained enzyme inhibitions of 40% ±10% (moderate dose) and 80% ±10% (high dose) for 3, 7, and 14 days. At low dose (40 ±10% ChE inhibition), no supercontractions, mitochondrial alterations, or other evidence for damage to the subjunctional cytoplasm were detected in any fibers at any exposure interval (Figs. 37-39). (The micrograph code "SM3" designates Subacute - Moderate dose - 3 day exposure.) [Where pre- or post-synaptic mitochondria were slightly swollen, mitochondria in Schwann cells were equally affected. Since similar alterations were common in control endplates (Figs. 33a-c; 34a-c; 35a,b; 36a-c), such alterations are attributed to artifacts characteristic of glutaraldehyde fixation.] As in control endplates (Fig. 8d), occasional examples were noted of nerve terminal branches either without associated junctional folds or with foreshortened junctional folds (Fig. 37b). At 14 days subacute exposure (Fig. 42), pre- and post-synaptic components of most myofibers appeared normal. Occasionally, however, the subjunctional cytoplasm of diaphragm and soleus (but not EDL) myofibers had become hypertrophied or "edematous" and contained swollen nuclei with compacted (thin) peripheral heterochromatin (shown in second Annual Report). On the other hand, "frothy" or swollen mitochondria were not observed. The source of the subjunctional hypertrophy/edema may reflect either a) response to prolonged endplate depolarization and associated ion and
water influx, b) increase in biosynthetic activity, or c) unknown factors. However, these data indicate near threshold expression of toxic effects in these high dose exposure experiments, as well as expression of cumulative effects of the drug over the 14 day high dose exposure period. These, too, are attributed to normal variability and not to drug effect.

In endplates exposed to subacute high doses (sustained 80% serum ChE inhibition), few changes were observed in diaphragm, soleus, or EDL myofibers after 3 days (Fig. 40). However, nerve terminals were often virtually depleted of synaptic vesicles and had increased numbers of coated vesicles and flattened cisternae (Fig. 40c, inset), as described by Miller and Heuser (54). Moreover, in one rat exposed to a subacute high dose for 7 days a few examples of soleus myofibers had severely damaged subjunctional sarcomeres (Fig. 41b). Most diaphragm and EDL muscles were also found normal at 7 days (Fig. 41a,c). These data are compatible with acute exposure experiments in which 80-90% ChE inhibition was found to be at (or just above) the "toxic threshold" for producing myofiber damage in the diaphragm. From these data we conclude that during subacute exposure at moderate doses (30-50% inhibition), supercontraction does not occur, nor is any other type of endplate damage apparent in thin sections examined by TEM. Therefore, extended low to moderate dose subacute exposure to physostigmine seems to be well tolerated by rats. However, 70-90% subacute exposure, yielding 70-90% serum ChE inhibition, appears to be very nearly a threshold level for producing pathophysiological alterations at the neuromuscular junctions. Alternative explanations for this temporal and pharmacological variability include a) limited endplate sampling size inherent to TEM, b) variations in enzyme inhibition in different animals, c) differential susceptibility of fast twitch vs. slow twitch fiber combined with different use patterns, d) different muscle use patterns in different rats, or e) or variable rates of osmotic pumping (i.e., "surges").
8. Recovery from Subacute Exposure

a. Physiology

The results of the physiological experiments were described in the second Annual Report and are summarized in RESULTS, Section VIII. A.4.

b. Ultrastructure

Rats receiving subacute exposure to physostigmine sufficient to produce sustained 40% (+10%) and 80% (+10%) serum ChE inhibition for 14 days were allowed to recover for 3, 7, 14, and 28 days. Except for typical artifacts of glutaraldehyde fixation (i.e., mitochondria swelling in nerves, muscles, and schwann cells, Figs. 43b,c; 46b; 47a; 49c; 50b,c) or of inadequate glutaraldehyde perfusion yielding primary fixation by OsO₄ (Fig. 49a), all myofibers in the "recovery" group appeared normal. No evidence for prior sarcomere alteration was detected, nor was there any evidence for denervation, re-innervation, or collateral sprouting. Thus, we conclude that in rats, "recovery" is rapid and complete following termination of subacute exposures that produce 14 days of sustained 40% and 80% serum ChE inhibition. Data for recovery from acute and subacute exposures are presented graphically (Figs. 51 and 52).
Figure 32. Comparison of Neuromuscular Junctions from Diaphragm, Soleus, and EDL Muscles 3 Days After Implantation of Alzet Pump Containing Sham Solution. Except for a few swollen mitochondria, the endplates are normal.
Figure 33. Comparison of Neuromuscular Junctions from Diaphragm, Soleus, and EDL Muscles 14 Days After Implantation of Alzet Pump Containing Sham Solution. Extensive mitochondria swelling is characteristic of fixation hypoxia.
Figure 34. Comparison of Neuromuscular Junctions from Diaphragm, Soleus, and EDL Muscles 3 Days After Removal of Alzet Pump Containing Sham Solution (14 Days Implant). Except for an occasional swollen mitochondrion, endplates are normal.
Figure 35. Comparison of Neuromuscular Junctions from Diaphragm, Soleus, and EDL Muscles 7 Days After Removal of Alzet Pump Containing Sham Solution (14 Days Implant). Except for an occasional swollen mitochondrion, endplates are normal.
Figure 36. Comparison of Neuromuscular Junctions from Diaphragm, Soleus, and EDL Muscles 14 Days After Removal of Alzet Pump Containing Sham Solution (14 Days Implant). Except for an occasional swollen mitochondrion, endplates are normal.
Figure 37. Comparison of Neuromuscular Junctions from Diaphragm, Soleus, and EDL Muscles 3 Days After Implantation of Alzet Pump Containing Moderate Dose of Physostigmine (Yielding Sustained 40% Serum ChE Inhibition) ("SM3"). Swollen mitochondria in the diaphragm myofibers (Fig. 37a) probably represent fixation artifacts. Apparent foreshortening of folds (Fig. 37b, arrowheads) are often seen in control endplates (see Fig. 8d).
Figure 38. Comparison of Neuromuscular Junctions from Diaphragm, Soleus, and EDL Muscles 7 Days After Implantation of Alzet Pump Containing Moderate Dose of Physostigmine (yielding sustained 40% Serum ChE Inhibition) ("SM7"). Except for an occasional swollen mitochondrion due to fixation hypoxia, endplates are normal.
Figure 39. Comparison of Neuromuscular Junctions from
Diaphragm, Soleus, and EDL Muscles 14 Days After Implantation of
Alzet Pump Containing Moderate Dose of Physostigmine (yielding
sustained 40% Serum ChE Inhibition) ("SM14"). Endplates appear
normal.
Figure 40. Comparison of Neuromuscular Junctions from Diaphragm, Soleus, and EDL Muscles 3 Days After Implantation of Alzet Pump Containing High Dose of Physostigmine (yielding sustained 80% Serum ChE Inhibition) ("SH3"). In some endplates (Fig. 40c, inset), nerve terminals are virtually depleted of synaptic vesicles and contain numerous coated vesicles (arrowhead). A few pre- and post-synaptic mitochondria are "blistered", frothy, or vesicated, presumably reflecting subthreshold expression of physostigmine toxicity.
Figure 41. Comparison of Neuromuscular Junctions from Diaphragm, Soleus, and EDL Muscles 7 Days After Implantation of Alzet Pump Containing High Dose of Physostigmine (yielding sustained 80% Serum ChE Inhibition) ("SH7"). Although most endplates appeared relatively unaffected by this dose of physostigmine, a few endplates exhibited supercontraction or dissolution of subjunctonal sarcomeres (Fig. 41b).
Figure 42. Comparison of Neuromuscular Junctions from Diaphragm, Soleus, and EDL Muscles 14 Days After Implantation of Alzet Pump Containing High Dose of Physostigmine (yielding sustained 80% Serum ChE Inhibition) ("SH14"). Except for an occasional swollen mitochondrion, most endplates appeared normal.
Figure 43. Comparison of Neuromuscular Junctions from Diaphragm, Soleus, and EDL Muscles 3 Days After Removal of Alzet Pump Containing Moderate Dose of Physostigmine (14 days implant) ("SM14R3"). Except for hypoxic mitochondria in the Soleus and EDL myofibers (Fig. 43b,c), endplates are normal.
Figure 44. Comparison of Neuromuscular Junctions from Diaphragm, Soleus, and EDL Muscles 7 Days After Removal of Alzet Pump Containing Moderate Dose of Physostigmine (14 days implant) ("SM14R7"). Endplates appear normal.
Figure 45. Comparison of Neuromuscular Junctions from Diaphragm, Soleus, and EDL Muscles 14 Days After Removal of Alzet Pump Containing Moderate Dose of Physostigmine (14 days implant) ("SM14R14"). Except for an occasional swollen mitochondrion, endplates are normal.
Figure 46. Comparison of Neuromuscular Junctions from Diaphragm, Soleus, and EDL Muscles 28 Days After Removal of Alzet Pump Containing Moderate Dose of Physostigmine (14 days implant) ("SM14R28"). Except for an occasional swollen mitochondrion, endplates are normal.
Figure 47. Comparison of Neuromuscular Junctions from Diaphragm, Soleus, and EDL Muscles 3 Days After Removal of Alzet Pump Containing High Dose of Physostigmine (14 days implant) ("SH14R3"). Endplates appear normal. Recovery appears complete.
Figure 48. Comparison of Neuromuscular Junctions from Diaphragm, Soleus, and EDL Muscles 7 Days After Removal of Alzet Pump Containing High Dose of Physostigmine (14 days implant) ("SH14R7"). Endplates appear normal.
Figure 49. Comparison of Neuromuscular Junctions from Diaphragm, Soleus, and EDL Muscles 14 Days After Removal of Alzet Pump Containing High Dose of Physostigmine (14 days implant) ("SH14RL4"). Except for occasional swollen mitochondria, endplates are normal.
Figure 50. Comparison of Neuromuscular Junctions from Diaphragm, Soleus, and EDL Muscles 28 Days After Removal of Alzet Pump Containing High Dose of Physostigmine (14 days implant) ("SH14R28"). Endplates appear normal.
Figure 51. Graph Depicting Time Course of Recovery from Ultrastructural Alterations Caused by Acute (Fig. 51A) and Subacute Physostigmine (Fig. 51B). Acute Exposures begin at 1/2 Hour PI*. Following termination of both acute and subacute exposures at very low to high dose, recovery is essentially complete within 3-7 days.

A. Recovery from Acute High Dose (0.8 LD$_{50}$)

- supercontraction
- exploded mitochondria
- frothy mitochondria
- blistered mitochondria
- hypoxic mitochondria
- unaltered mitochondria

Time (days)

B. Recovery from Subacute High Dose (80% Serum ChE Inhibition)

- supercontraction
- exploded mitochondria
- frothy mitochondria
- blistered mitochondria
- hypoxic mitochondria
- unaltered mitochondria

Time (days)
IX. INTERPRETATIONS

A. OVERVIEW

In this third Annual Report, we have completed the descriptions of the acute, subacute, delayed, and long-term effects on mammalian nerve, muscle, and neuromuscular junction physiology and ultrastructure following acute exposure to the anti-ChE compound physostigmine administered by subcutaneous injection at very low to very high doses (0.001-1.1 LD₅₀ or 0.00075-0.8 mg/kg) and following subacute exposure (up to 14 days) to moderate and high doses of physostigmine (40% +10% and 80% +10% sustained Che inhibition). We have also examined the extent of ultrastructural alterations in guinea pig neuromuscular junctions at high acute doses of physostigmine, and compared the changes to those seen in rat neuromuscular junctions at similar LD₅₀ dose levels. We have presented graphical data showing the time course and extent of reversibility of pathophysiological alterations following acute and subacute exposure to the same drug. We have also described the effect of neuromuscular activity on the "threshold" of drug-induced neuromuscular pathology, and show that muscle use may be an important factor in the interpretation of drug toxicity, habituation, and reversibility. From these data, we have shown a) that no alterations or relatively minor alteration of nerve-muscle ultrastructure or physiology occur following subacute (prolonged) exposure at relatively high dose, and 2) that there are no detectable alterations following moderate dose subacute exposure. Finally, we have confirmed the occurrence of extremely rapid mechanisms for repair and recovery of function of nerve terminals, muscle plasma membranes, and muscle cytoplasm following termination of high dose acute and subacute exposures.

In all rats that survived acute exposures to physostigmine, ChE activity returned to normal within 24 hours, but fluctuated greatly until termination of the experiment (as did control values). These data indicate that physostigmine-induced supercontraction (as well as other changes in myofiber ultrastructure) is dose-dependent, with severe damage occurring above a threshold of 75% ChE enzyme inhibition. Higher doses produced profound changes in endplate structure and physiology. These values for ChE inhibition at various partial LD₅₀s are essentially identical to those obtained by Hudson and coworkers for pyridostigmine (24-26). However, endplate damage was not demonstrable at serum ChE inhibition levels lower than 70% (except in myofibers that had been continuously stimulated at 20 hz for longer than 15 minutes or at 40 hz or 80 hz for 15 minutes or longer.

B. Comparative Effects of Acute High-Dose of Physostigmine in Rats and Guinea Pigs

Thirty minutes after single high-dose (0.8 LD₅₀ or 0.6 mg/kg) injections of physostigmine, all neuromuscular junctions of the constantly used diaphragm muscles of both rats and guinea pigs exhibited supercontraction of sarcomeres in the subjunctional sarcoplasm. Z bands were missing, destroyed, or grossly disrupted. Free thick and thin filaments were present in
disorganized masses, and a mixed population of frothy and grossly distended mitochondria plus distended sarcoplasmic reticulum cisternae was observed disrupting the subjunctional sarcoplasm. Interestingly, rat soleus (but not EDL) and guinea pig EDL (but not soleus) muscles showed similar supercontractions at $\frac{1}{2}$ hour PI. The reason for the apparent reversal of fiber-type specificity of drug effects in these two animal species is not known, but appears to be related to differences in muscle use patterns. Our reluctance to attribute the differences to inherent differences in fiber types arises in part because of the apparent reversal fiber-type effects in the two animal species, in part, from the observation that all fiber types are equally severely affected in the diaphragm (a "mixed muscle"), and in part because muscle use is shown to exert a profound effect on the threshold of neuromuscular pathology/toxicity.

C. Acute-Delayed Effects and Reversibility

We have determined that enzyme inhibition is rapidly reversible after a single acute dose. We presume that these changes in blood ChE are associated with similar changes in endplate ChE. However, it is possible that there are long-term compensatory changes in endplate ChE enzyme profiles that are not detected by the methods employed in this study.

The physiological effects being measured (EDL twitch potentiation and percent sustained high frequency contractions, or PSCs) returned to normal within one hour after acute physostigmine administration. In all cases, this return to normal physiology occurred when blood ChE inhibition levels were still 80% or higher. The relative extent of damage done to EDL during this period was readily reversible at least in terms of whole muscle physiology.

For the subacute/recovery exposures, at 3 days recovery and thereafter, EDL twitch tension and high frequency contraction properties were normal. This would be expected because a) ChE inhibition levels had returned to 0% (i.e., normal) and b) because these physiological properties were minimally altered in the period before recovery.

Ultrastructurally, there was rapid recovery from the major damage occurring during the first 24 hours after acute high dose exposure. For doses of 0.8–1.1 LD$_{50}$, the destructive effects observed in all myofibers of diaphragm and soleus fibers were partially reversed 24 hours PI, and blood ChE levels had returned to near normal. By 7 days, there was little remaining evidence for supercontraction; mitochondria appeared normal; and the sarcoplasmic reticulum appeared normal. Our data also revealed that extreme morphological damage occurred only at very high doses of physostigmine (0.8–1.1 LD$_{50}$) or at moderate doses (0.3 LD$_{50}$) only following sustained neuromuscular activity (i.e., normal respiration or sustained indirect stimulation). Since twitch potentiation responses were obtained in EDL muscles that had been treated at doses subthreshold for producing supercontraction and that had also been stimulated relatively frequently, these data are consistent with suggestions that muscle use exacerbates the effects of anticholinesterase exposure.
In the First Annual Report, we indicated that long-term changes in endplate morphology occurred in some myofibers ("simplification" of junctional folds, formation of vesicular membrane debris replacing some junctional folds, disappearance of nerve terminal branches, formation of collateral sprouts, and the apparent formation of new regions of nerve-muscle apposition in and near the original endplates). We concluded then that acute exposure to near LD₅₀ dose of physostigmine is rapidly reversible but that in the most severely affected fibers, physostigmine may exert substantial influences on biological control mechanisms regulating endplate morphology. However, no such long term effects were detectable following acute exposure to moderate to very low doses. Moreover, detailed examination of a very large sample size of treated vs. control endplates convinces us that previous evidence interpreted as consistent with denervation, collateral sprouting, and reinnervation must now be attributed primarily (if not exclusively) to "plane-of-section" artifacts and to inadequate sampling size for controls. We cannot confirm any type of long-term alteration of endplate structure or function. If such changes occur, they are either not resolvable by conventional thin-sectioning techniques or they are so rare as to be indetectable among the variabilities seen in normal endplates.

D. Effects of Subacute Physostigmine Exposure

For subacute exposure, EDL twitch tensions and high frequency contraction were not significantly different from normal. High dose pumps produced sustained ChE inhibitions of 80%, and thus, some damage to EDL neuromuscular junctions had been expected. However, with unprimed pumps, the gradual increase in serum ChE inhibition did not cause damage severe enough to substantially affect whole muscle function. The only observed effect on EDL physiology was a slight tendency towards greater fatigability during high dose treatment. This is in contrast to results of Adler et. al. (1984), who reported substantially increased EDL single twitch tensions, as well as a depression with 20 hz stimulation during subacute administration of pyridostigmine. Apparently, the response seen by Adler, et al. (1984) was due to interaction between pyridostigmine and the general anesthetic, chloral hydrate, used by them. As discussed in our first Annual Report, we abandoned the use of either chloral hydrate or ketamine after discovering interactive effects between those anesthetics and physostigmine.

Although the threshold for onset of twitch potentiation in the acute studies was at a ChE inhibition of about 80%, sustaining that level of inhibition does not necessarily sustain twitch potentiation. This was shown in the acute experiments where twitch tensions returned to normal while blood ChE inhibition levels were still well above 80%. Also, in the subacute experiments in which EDL physiology was tested while the pumps were still in place, normal twitch tensions were recorded when ChE inhibitions were greater than 80%. Later in the same experimental animal, with no significant change in inhibition level, substantial twitch potentiation occurred. These examples
emphasize that threshold for physostigmine-induced damage is dependent on the interaction of several incompletely characterized factors (e.g., drug/anesthetic interaction; humoral or other factors associated with animal stress).

E. Delayed Effects of Subacute Exposure

Following termination of moderate or high dose subacute 14 day exposure to physostigmine (40% or 80% serum ChE inhibition), no changes were detected in muscle physiology or endplate ultrastructure at 3, 7, 14, or 28 days following removal of Alzet mini-osmotic pumps. However, due to the limited sample size, we cannot exclude the possibility that a very small number of fibers that may have been damaged in the high dose subacute exposure group may have atrophied or become necrotic. Overall, however, there appeared to be rapid and complete recovery from any possible deleterious alterations occurring at high dose exposures. Moreover, since there were no detectable alterations following subacute exposure to moderate dose of physostigmine, and since there were no detectable "delayed alterations", the moderate dose regimen appears to be well tolerated in all rats.

F. Effects of Prolonged Stimulation on Muscle Physiology and Endplate Ultrastructure at a Subthreshold Dose of Physostigmine

In the absence of physostigmine, severe alterations were observed in mitochondria following prolonged moderate to high frequency stimulation. The extent of damage was found to be proportional to stimulation frequency. These changes are attributed to the cumulative effects of the 18,000 to 144,000 endplate depolarizations elicited. Although the NMJ has an enormous capacity for repolarization during normal rates and durations of stimulation, the levels of stimulation evoked in the presence and absence of physostigmine were 1 to 2 orders of magnitude greater than normally encountered during sustained neuromuscular activity. In rats treated with a subthreshold dose of physostigmine and whose common peroneal nerve had been stimulated at 20, 40, or 80 hz for 15 or 30 minutes, soleus and EDL myofibers exhibited equal or slightly increased damage to mitochondria and membrane-bound organelles, but myofibers were not supercontracted. In unstimulated myofibers of soleus and EDL muscles from the same rats, no ultrastructural alterations were observed. Thus, it must be inferred that the ultrastructural changes observed in stimulated fibers in the absence of physostigmine reflect ultrastructural alterations characteristic of severe neuromuscular fatigue. Under such circumstances, restoration of normal ionic and metabolite levels presumably requires hours or even days (certainly much more than the 15 minutes allotted for "recovery" in our experiments). Such ultrastructural damage and presumably the recovery from such hyperstimulation is qualitatively similar to endplate damage and repair following acute high dose exposures to physostigmine.
G. CONCLUSIONS AND RECOMMENDATIONS

We have demonstrated that:

1. Destructive supercontraction occurs in fast and slow twitch muscles in rats and guinea pigs at near LD$_{50}$ doses of physostigmine.

2. The threshold for producing supercontraction in muscles used for respiration is about 0.3 LD$_{50}$.

3. In the rat there is extremely rapid recovery from severe ultrastructural damage to myofibers produced by near lethal doses of physostigmine.

4. There are essentially no ultrastructural alterations produced in myofibers by moderate to very low acute doses of physostigmine. Previous reports suggesting mitochondrial alterations at low to very low doses of other anticholinesterase drugs are tentatively attributed to fixation artifacts rather than to drug effect.

5. Subacute administration of moderate to high doses of physostigmine does not produce significant changes in maximum muscle twitch tension but that resistance to fatigue may be lowered at moderate to high doses.

6. In auxiliary experiments, we have shown that under normal physiological stimulation conditions (low stimulation frequencies and/or short durations of stimulation), there were no ultrastructural alterations of soleus or EDL muscles. However, with sustained moderate to relatively high stimulation, gross muscle pathology was produced, whether or not the animals were exposed to physostigmine. At 0.25 LD$_{50}$, the pathological alterations did not include supercontraction, which apparently occurs only in the presence of anti-cholinesterase agents or at near threshold doses. The "threshold" of physostigmine for producing supercontraction appears to be lowered slightly by concurrent prolonged stimulation, and raised substantially (or eliminated) in the absence of intrinsic stimulation (i.e., during spinal neuromuscular blockade).

7. Based on the analysis of a much larger population of control samples, we have shown that subacute exposure to physostigmine at low to moderate doses produces no detectable alterations of endplate physiology or ultrastructure. This is in contrast to previous reports from this and other laboratories.

8. Finally, we have shown that guinea pigs undergo similar alterations at similar dose levels of physostigmine, allowing either animal to serve as a model system.

Physostimine has been suggested as a possible chemotherapeutic agent for the protection of military personnel against exposure to the toxic nerve agents. Our data are compatible with this suggestion. However, it is imperative that the effects of the two agents currently receiving closest scrutiny (physostigmine and pyridostigmine) be compared in a single laboratory using identical techniques. That study is in progress in this laboratory, and the data will be presented in our Final Report. However, based on the rapid repair of endplate ultrastructure and recovery of normal function during subacute exposure to physostigmine, and based on recent evidence for down
regulation of transmitter release efficacy as a normal control mechanism for neuromodulation, we also suggest that a combined physiological and ultrastructural (freeze-fracture) investigation be conducted to ascertain if the "protective" or compensatory alterations afforded by prior exposure to anticholinesterase agents reflect a decrease in transmitter release (i.e., decrease in quantal content), and if so, if alteration or partial disassembly of the "active zones" occurs in the period 0-12 hour following moderate to high dose exposures to anti-ChE agents. Such studies may prove of value in understanding the protective mechanisms to nerve agent exposure afforded by prior exposure to the reversible anti-ChE medications.
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