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PATHOPHYSIOLOGY OF ANTICHOLINESTERASE AGENTS

Annual and Final Report

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

July 7, 1988

Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-84-C-4010

Department of Anatomy and Neurobiology
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I. SUMMARY

In this Annual and Final Report, we describe 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 describe dose-response curves for cholinesterase (ChE) inhibition and relate those changes to observed ultrastructural pathology and altered physiology. (In additional correlative experiments using guinea pigs as alternative models to rats, we also show that the effects of sub-lethal but acute high doses of physostigmine produce similar alterations of endplate ultrastructure at similar dose levels.) In the second major group of experiments, we describe the immediate and long-term effects on rats following 3-14 days of subacute exposures to moderate and high doses of physostigmine, and document the extent and time course of the reversibility of effects during recovery for 3-28 days following termination of subacute exposure. In the third group of experiments, we compare the effects of acute physostigmine vs. pyridostigmine exposure and show that similar ultrastructural alterations are induced at similar blood ChE inhibition levels. Finally, we present physiological and ultrastructural data showing that sustained neuromuscular activity mimics many of the ultrastructural alterations produced by physostigmine and pyridostigmine, but that the alterations induced by prolonged high frequency neuromuscular activity do not result in the supercontraction of subjunctional sarcomeres that characterizes acute high-dose exposures to these potent anti-ChE agents. These data concerning the synergistic interactions of sustained neuromuscular activity plus exposure to anti-ChE agents may be of value in evaluating and comparing anti-ChE medications and dosages used for protection against the irreversible anti-ChE agents.

Specifically, in our study of acute exposures to physostigmine and pyridostigmine, we have:

1) completed the examination of a large number of "control" myofibers in the sham-injected (as well as sham-implanted rats) and have established criteria that allow effects of drug exposure to be distinguished a) from previously undescribed but normal variability in control endplates, and b) from common artifacts of specimen preparation;

2) established the LD_{50} of acute subcutaneous injections of physostigmine as 0.75 mg/kg for adult albino rats.

Based on this value for LD_{50}, we have:

3) injected 24 groups of 3 "experimental" and 2 "control" rats with 0.001 LD_{50} to 1.1 LD_{50} ("very low" to "high" doses), and subsequently obtained samples for electron microscopic analysis from diaphragm, soleus and extensor digitorum longus (EDL) muscles at ½ hour and 1, 7, 14, 21, and 56 days post-injection (PI). [From the 72 experimental and 48 control rats, we examined 360 different perfusion-fixed muscles (3-5 samples from each muscle), yielding more than 1000 specimens.

Of these, more than 10,000 myofibers were examined and more than 2500 NMJs were photographed, of which nearly 300 are presented here.];

4) obtained blood samples before and at ½ hour and 1, 3, 7, 14, 28 and 56 days PI for ChE inhibition analysis and subsequent correlation with drug-induced cytopathology;

5) completed ultrastructural analyses of acute very low to high dose exposures and identified "supercontraction" of subjunctional myofibers as the most reliable diagnostic marker for identifying acute physostigmine toxicity in skeletal muscle.

In an alternative model, the guinea pig, we have:

6) verified the occurrence of supercontraction of subjunctional myofibers following acute high-dose exposures equivalent to those producing supercontraction in the rat, and

7) obtained data concerning the time course and extent of "reversibility" of cytopathological alterations from 1 day to 56 days following a single acute injection of physostigmine.

In these experiments, we show that presumed "alterations" in endplate morphology (including Schwann cell "fingers" interposed between nerve terminals and junctional folds, junctional folds devoid of nerve terminals, and very small diameter nerve terminals near shallow junctional folds or without apposed junctional folds, all previously described as collateral sprouts or other manifestations of endplate damage and "remodeling") are equally common in endplates from control (sham-injected) rats. 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 physostigmine, pyridostigmine (see below), or (presumably) any other anti-ChE agent.

In the acute exposure experiments, changes in muscle and nerve ultrastructure and physiology were correlated with temporal profiles of the changes in blood ChE inhibition levels for the same rat. We then compared possible ultrastructural alterations of the low (0.01 LD_{50}) to very low dose (0.001 LD_{50}) acute exposure experiments with images from the large sample of control endplates from sham-injected animals. At ½ hour PI, blood ChE was inhibited approximately 33% at 0.01 LD_{50}, 67% at 0.1 LD_{50}, and 89% at 0.8 LD_{50}. Levels of enzyme inhibition up to 67% were not associated with major changes in myofiber ultrastructure in EDL, soleus, or diaphragm myofibers. Moreover, acute exposure to physostigmine at low to very low doses (0.01-0.001 LD_{50} or <35% blood ChE inhibition) produced no detectable alterations of endplate ultrastructure, at least as can be discerned in thin section electron micrographs. [Nor were ultrastructural alterations indicative of drug-induced pathology observed during the recovery phase 1-56 days following acute moderate to very low dose exposure to physostigmine. (See below.)]

In contrast, at ½ hour after injection of 0.8 LD_{50} (89% +7% ChE inhibition), all neuromuscular junctions of the constantly used diaphragm and soleus myofibers exhibited supercontraction of sarcomeres in the subjunctional sarcoplasm. Often, Z bands were missing, free thick and thin filaments were present in disorganized masses, and a mixed population of frothy and grossly

distended mitochondria disrupted the subjunctional sarcoplasm. EDL muscles from the same rats were much less affected at ½ hour than soleus or diaphragm myofibers. Although some EDL fibers exhibited mitochondria that appeared "blistered" or "frothy" in the immediate subsynaptic regions, most EDL fibers exhibited no supercontraction or other detectable changes in sarcomere ultrastructure.

By 24 hours after acute exposure to single doses of 0.8-1.1 LD_{50}, the destructive effects observed in all myofibers of diaphragm and soleus fibers were partially reversed, and blood ChE levels had returned to near normal. In contrast, at 24 hours PI, EDL myofibers exhibited increased damage to subjunctional sarcomeres and increased damage to subjunctional mitochondria. Since the same rats had recovered their respiratory capabilities and exhibited partially repaired diaphragm myofibers, the increased damage to EDL fibers at 24 hours PI was attributed to the resumption of voluntary muscle activity after the first samples were taken and during the initial 6-12 hour period of low esterase activity. (To assess the possible synergistic effects of muscle use and anti-ChE toxicity, additional experiments examined the maintenance of muscle contractility at different stimulation frequencies during acute exposure to physostigmine and to pyridostigmine.) Based on the rapid recovery of severely damaged muscles (especially diaphragm and soleus), we conclude that recovery from the effects of high and low dose exposure is much more rapid and complete than previously suggested.

In the analysis of neuromuscular repair and recovery of contractile function, subjunctional sarcomeres in diaphragm and soleus fibers from rats that had been exposed to a single acute high dose of physostigmine were substantially reorganized by 24 hours PI and were virtually indistinguishable from normal subjunctional sarcomeres by 7 and 14 days PI, and thereafter. EDL fibers exposed to high doses of physostigmine showed only slightly increased damage by 24 hours, but appeared normal at 7 days PI and thereafter. Thus, recovery from an acute high dose of physostigmine was remarkably rapid and complete, with very few fibers showing any abnormalities after 7 days of recovery.

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 ½ hour PI of 1.2 mg/kg physostigmine, virtually all neuromuscular junctions of the diaphragm and EDL myofibers exhibited supercontraction of sarcomeres in the subjunctional sarcoplasm. As in rat myofibers, Z bands were missing and free thick and thin filaments were present in disorganized masses. Similar concentric zones reflecting a continuum of severely to slightly damaged mitochondria were observed in the subjunctional sarcoplasm. Progressing from the junctional folds to adjacent undamaged perijunctional regions, the sequence of mitochondrial morphologies progressed from "exploded" to "swollen" to "frothy" to "blistered" mitochondria. However, the EDL muscles of guinea pigs were more severely affected than the EDL muscles of rats; the diaphragms equally affected, and the soleus muscles were less affected than 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, with only minor differences noted among the several muscles examined.

In correlative physiological experiments, we:

8) identified a synergistic effect of physostigmine and certain types of general anesthetic on muscle contractile properties. After changing to the use of spinal block anesthesia, we were able to:

9) assess the effects of low to high doses (0.001 to 0.8 LD_{50}) of physostigmine on muscle twitch tension and resistance to fatigue.

Physiological effects on skeletal muscle contractility, including potentiation of EDL twitch tensions and grossly observable fasciculations, were observed 20-50 minutes PI at doses greater than 0.05 LD_{50} but not at 0.025 LD_{50} or lower. It is interesting that twitch potentiation was directly correlated with the magnitude of visually observed and mechanically recorded fasciculations.

In the studies of the effects of subacute exposure to physostigmine, we have:

10) established "partial priming" procedures (used in conjunction with surgically implanted Alzet mini-osmotic pumps) which yielded continuous levels of blood ChE enzyme inhibitions of 40 $\pm$10% (low dose) and 80 $\pm$10% (high dose) for up to 14 days;

11) completed ultrastructural analysis of diaphragm, soleus, and EDL muscles from these subacute exposure groups at 3, 7, and 14 days post implantation and during recovery at 3, 7, 14 and 28 days after surgical removal of the pumps. We have found that there were virtually no detectable ultrastructural changes following either high or low dose subacute exposure, provided that the initial onset of exposure was gradual (i.e., gradual increase in blood ChE inhibition over a period of at least 3 hours).

In these subacute exposure experiments in rats, we show that low to moderate doses (yielding sustained 40 $\pm$10% inhibition) produce no (or very few) detectable alterations in endplate ultrastructure after 3, 7 or 14 days of continuous exposure, nor were any alterations detected during the recovery period 3-28 days following termination of moderate dose subacute exposure. Likewise, at relatively high-dose subacute exposures (80% $\pm$10% sustained blood ChE inhibition), most fibers exhibited no obvious changes in endplate fine structure, even though the dose level was equivalent to an exposure of 10 to 30 LD_{50}s per day. However, after 3 and 14 days continuous exposure, a few nerve terminals were found in which terminal branches were substantially 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. In contrast, when pre-primed pumps were employed (rather than unprimed pumps), toxicity (including

lethality) in rats was observed at much lower dose levels (1/10 to 1/30) than when unprimed or partially primed pumps were imiplanted. We concluded that previously primed pumps release high levels of drug immediately upon implantation, thereby producing immediate high levels of inhibition of blood ChE. The rapid onset of ChE inhibition results in alterations equivalent to those caused by acute high dose exposures. In contrast, ultrastructural pathology was greatly reduced when a gradual inhibition of blood (and presumably endplate) ChE activity was produced by the gradual onset of exposure afforded by unprimed or partially primed pumps. By introducing an even more gradual onset of exposure (e.g., 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, we found that:

12) muscle contractile properties were minimally affected during the subacute exposure regimens employed, and that muscle structure and function 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 ultrastructure occur very rapidly in the rat following sublethal subacute exposure to physostigmine. However, a few severely affected fibers were observed, indicating either a variability of effect during initial exposure or that a small percentage of fibers were slower to recover. However, because of the low percentage of fibers affected, the effects of damage to such a small fraction of fibers were not discernible in the recordings of whole muscle contractile properties.

In supplementary studies comparing the effects of pyridostigmine and physostigmine, we:

13) confirmed the occurrence of supercontraction of subjunctional myofibers following acute exposure to both anti-ChE agents at doses producing similar blood ChE inhibition levels.

However, a cautionary note concerning attributions of damage to synergistic effects of anti-ChE agents and neuromuscular activity seems appropriate because we also observed:

14) that many of the alterations induced by prolonged high frequency neuromuscular stimulation mimic many of the cytopathological changes induced by acute high dose exposure to physostigmine and pyridostigmine.

Endplates from rats that were not exposed to anti-ChE agents but that were physiologically stimulated at 20, 40, or 80 Hz for 15 or 30 minutes revealed many of the ultrastructural changes seen after an acute high dose of physostigmine alone (i.e., in the absence of neuromuscular stimulation). Of the major alterations associated with acute high-dose exposure, all except supercontraction of subjunctional sarcomeres were evoked by 30 minutes of 80 Hz stimulation. (Similar alterations included blistered, frothy, and swollen subjunctional mitochondria, as well as blistered and swollen pre-synaptic mitochondria.)

Finally, in studies of the effect of muscle use on anti-ChE

toxicity, we:

15) analyzed endplates following prolonged 40-Hz stimulation in the presence and absence of physostigmine or pyridostigmine; and

16) established that the threshold of physostigmine and pyridostigmine cytopathology is lowered substantially (by almost two orders of magnitude of exposure dose) by prolonged neuromuscular stimulation.

The presence of both supercontraction and severe mitochondrial alterations during acute exposure to low to high doses of anti-ChE agents appears not only to reflect nearly continuous endplate depolarizations, but also to indicate that such changes occur only during the simultaneous presence of anti-ChE drugs plus sustained nerve activity. These data may help to explain why the constantly activated diaphragm myofibers are rapidly and severely affected in animals exposed to near lethal doses of physostigmine, whereas seldom used voluntary muscles (e.g., EDL) in the same animal show little or no damage. Muscle use is thus shown to be an important factor in the expression of anti-ChE toxicity at the motor endplate. However, since non-quantal release mechanisms account for 50-95% of the total acetylcholine (ACh) released at motor endplates (in active vs. resting muscles, respectively), muscle use is not the only factor affecting the expression of endplate cytopathology.

Taken together, these data indicate that:

a) In normal, unrestrained, unstressed rats, physostigmine-induced supercontraction, as well as other changes in myofiber ultrastructure, is dose dependent, with severe damage occurring above a threshold of 70% enzyme inhibition.

b) The time course of repair and/or recovery from acute physostigmine exposure is relatively rapid, with substantial repair noted in 24 hours and virtually complete recovery of normal ultrastructure and function within 1-2 weeks.

c) Muscle use during the period of maximal ChE inhibition is an important factor in the expression of drug toxicity, as well as in the rate of subsequent neuromuscular recovery.

d) Subacute exposure to physostigmine (using devices which produce a gradual onset of drug delivery) minimizes alterations of endplate physiology and ultrastructure.

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-cholinesterase (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 synthesis and introduction of neostigmine, which has superceded physostigmine 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 anti-ChE "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 cholinesterase (ChE), whereas a quaternary compound, pyridostigmine, afforded little or no protection for brain ChE. However, there have been no published descriptions from a single laboratory comparing the physiological and ultrastructural alterations of muscle produced by equivalent subacute sublethal doses of these two potent anti-ChE agents. Thus, direct comparison of pathophysiological alterations at the motor nerve endings has not been possible. One portion of this report addresses a portion of that deficit.

A. ANTI-CHOLINESTERASE AGENTS PRODUCE DEPOLARIZING NEUROMUSCULAR BLOCKADE AND SUPERCONTRACTION AT ENDPLATES

It has been known since early in this century that anti-ChE agents such as neostigmine and physostigmine, and depolarizing neuromuscular blocking agents such as nicotine and succinylcholine, produce profound myopathies and neuropathies, as well as distinctive pathophysiologies of neuromuscular transmission (6-30). The pathophysiological alterations of neuromuscular transmission caused by anti-ChE agents are attributed primarily to severe and prolonged endplate depolarization, which is thought to cause the observed supercontraction and destruction of subjunctional myofibrils. Moreover, some authors suggest that calcium-activated proteases are activated by the influx of calcium, resulting in myofiber destruction and disappearance of Z-bands (26,27). According to this proposal, the proteolytic alterations are essentially irreversible or only slowly reversible. If the proposed proteolytic digestion of substantial numbers of subjunctional sarcomeres were to occur (26,27), a substantial period would be required for gene reactivation and for the synthesis of the requisite amounts of actin, myosin, troponin, actinin, tropomyosin, and other myofibrillar proteins. Thus, we have paid particular attention to the early processes of myofiber repair and recovery of function, especially at 24 hours post-injection (PI), when it is unlikely that sufficient time has elapsed to permit repair based on gene reactivation and protein synthesis.

B. REFINING THE ESTIMATED "THRESHOLD" FOR PRODUCING NEUROMUSCULAR CYTOPATHOLOGY

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 acetylcholinesterase (AChE) at a given site" (1). Hudson, et al., (28-30) refine this estimate by showing that with pyridostigmine (a quaternary anti-ChE 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, as well as greater invasion of synaptic clefts by Schwann cell "fingers".) 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 listed. In addition to the proposed "plane-of-section" and sampling artifacts, we note that skeletal and cardiac muscles 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). Such artifactually swollen mitochondria closely resemble the swollen mitochondria identified by Hudson and coworkers as representing the first signs of anti-ChE toxicity. Since these changes were detected even at extremely low exposure levels (0.001 LD_{50}), we were concerned that the perceived alterations might instead be attributable to other, possibly secondary effects of anti-ChE agents. For example, as a group, the anti-ChE agents produce profound bradycardia, hypotension, bronchoconstriction, and release of epinephrine, resulting in substantial systemic tissue hypoxia (1). Thus, we began this study with the awareness that current glutaraldehyde fixation techniques, [which also greatly increase tissue hypoxia (34)], may yield ambiguous images of swollen mitochondria that may mimic or camouflage low-dose drug effects. Consequently, we have devised improved methods of fixation (including the use of well-oxygenated clearing solutions and fixatives, both of which were perfused under pressure with 95% O_2, 5% CO_2). These improved methods usually eliminated glutaraldehyde-induced hypoxic alterations of mitochondria, thereby allowing at least some of the physostigmine- and pyridostigmine-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 those attributed to effects of anti-ChE. In current parlance, we have attempted not

only to increase the "signal-to-noise" ratio by increasing the signal (i.e., increased number of samples) and decreasing the "noise" (i.e., by reducing the number and extent of fixation artifacts), we have also devised methods whereby much of the remaining noise can be identified and subtracted from the overall "message" (i.e., "signal + noise").

C. ACUTE VS. DELAYED EFFECTS OF ANTI-CHOLINESTERASE TOXICITY

Numerous ultrastructural studies of acute anti-ChE toxicity report subtle to profound alterations of the neuromuscular junction at near-LD_{50} 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. Following a single acute exposure, these initial changes appear to be partially ameliorated within 24 hours, with recovery 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). As above, more detailed examination of endplates from sham-injected (control) rats allows several of the signs of delayed toxicity to be reassigned to atypical but normal morphologies and allows more definitive discrimination of pathology from normal variability (i.e., allows some of the images to be identified as noise and subtracted from the presumed signal).

D. ANTI-CHOLINESTERASE AGENTS AS POSSIBLE PROPHYLACTIC AGENTS FOR SOMAN INTOXICATION

Following exposure to the irreversible anti-ChE agent soman, endplate 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 LD_{50}), compensatory motor nerve activity results in normal or increased release of acetylcholine (ACh), which because of reduced ACh hydrolysis and prolonged activation of ACh receptors, results in prolonged and profound endplate depolarizations. Severe pathological alterations in endplate physiology and ultrastructure ensue, potentially resulting in neuromuscular (including respiratory) failure. A quaternary carbamate anti-ChE, pyridostigmine, has been shown to be a moderately effective prophylactic agent against exposure to lethal doses of soman (5), while a tertiary carbamate, physostigmine (which crosses the blood-brain barrier), may prove even more effective (cf. 2-5). 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 a "protective" reversible anti-ChE agent, the AChE sites protected by prior or concomitant exposure to the reversible anti-ChE agents are thought to be unmasked by decarbamylation reactions, thereby 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), the two anti-ChE drugs currently under study. 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 or both drugs. Since a substantial number of rats survive a dose of soman of several LD_{50}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, physiological, and ultrastructural approaches to identify the acute and delayed effects on neuromuscular junctions following exposure to very low to near-lethal doses of physostigmine. The rat was selected as a model system for analyzing the chemoprophylactic agents proposed for protecting against 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).

E. PURPOSES 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 those alterations persisted with "chronic" [sic] (i.e., multiple-injection) exposures. No long-term recovery or delayed effects following 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 extensor digitorum longus (EDL) muscles of rats after a) acute and b) subacute exposures, and from the same muscles during c) recovery (1-56 days of recovery after single acute drug exposure)

and d) up to 28 days following termination of subacute exposure). An additional 6 guinea pigs were also examined following exposure to high acute doses of physostigmine (4 experimental, 2 control animals), and essentially identical alterations were observed as in the rat.

In each rat, changes in nerve and muscle ultrastructure and physiology were correlated with temporal changes in blood ChE inhibition levels. Blood ChE levels were measured periodically in each animal as an independent measure of the effect and distribution of physostigmine. Physiological studies of rats included an assessment of twitch potentiation of EDL muscles following acute exposure to physostigmine. Rapid alterations in neuromuscular physiology, as well as rapid recovery of function were correlated with parallel changes in blood ChE inhibition levels. Thin section transmission electron microscopy was used to assess pre- and post-synaptic alterations of neuromuscular junctions. In the second phase of that study, we monitored delayed effects, recovery, and reversibility of ultrastructural alterations following very low to high doses of physostigmine.

In parallel studies of the effects of subacute exposure to physostigmine, we have described the extent of neuromuscular damage and time course of recovery. We also compared endplates from those rats to a large sample of control myofibers from rats implanted with sham-filled mini-osmotic pumps. In so doing, we have established criteria that allow effects of subacute drug exposure to be distinguished from most artifacts of specimen preparation.

In the fifth series of experiments, we used combined physiological, ultrastructural and biochemical experiments to identify potent synergistic effects of increased neuromuscular activity on drug toxicity. In addition, we show that prolonged moderate to high frequency nerve stimulation in the absence of drug mimics many of the alterations seen during acute moderate to high-dose physostigmine exposure.

Finally, we assessed the synergistic effects of prolonged high frequency stimulation in the presence and absence of physostigmine or pyridostigmine (0.01-0.6 LD_{50}). We show that similar pathophysiological and ultrastructural alterations occur at similar blood ChE inhibition levels. Moreover, the potent synergistic effects of neuromuscular activity and increased drug toxicity are demonstrated after exposure to both drugs.

The biochemical, ultrastructural, and physiological data obtained during this study provide a better understanding of the pathophysiology of physostigmine and pyridostigmine. From these data, others may be able to make a more informed decision concerning use of these anti-ChE medications as possible prophylactic agents against the irreversible nerve agents.

VII. EXPERIMENTAL METHODS

A. CARRIER SOLUTIONS FOR PHYSOSTIGMINE AND PYRIDOSTIGMINE

Physostigmine sulfate in aqueous solution is labile above pH 6 (42). Thus, to facilitate comparison of data from acute exposure experiments with data from subacute exposure regimens (where stability of drug for up to 14 days is required), similar diluents consisting of rat Ringer's solution made slightly acidic were used in both acute and subacute exposure 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 one 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.) For consistency of effect in the comparisons of acute exposures to physostigmine and pyridostigmine (see **RESULTS, Section N.2**), pyridostigmine was also prepared in acetic acid-sodium citrate buffer, as described above for physostigmine solutions.

B. ESTABLISHMENT OF LD_{50} OF PHYSOSTIGMINE IN RATS; USE OF PUBLISHED VALUES FOR LD_{50} OF PYRIDOSTIGMINE

The approximate LD_{50} of the acid-stabilized solutions of physostigmine was established in preliminary experiments involving two small test groups of rats. An initial group of 6 rats was injected subcutaneously (s.c.) over a wide range of dose levels (0.4 to 2.0 mg/kg) to establish an approximate LD_{50}, while the second group was used to refine the estimate. (For details, see **RESULTS, Section A.**) Based on these preliminary experiments, an LD_{50} of approximately 0.75 mg/kg was determined. (Subsequent experimentation confirmed that estimate in acclimated rats, but also revealed an unexpected source of variability in response to the drug. See **RESULTS, Section C**, describing the effects of stress on LD_{50}.) For all acute exposure experiments, the experimental dose levels for physostigmine were standardized as follows:

high dose	= 0.8	LD_{50}	(0.6 mg/kg)
moderate dose	= 0.1	LD_{50}	(0.075 mg/kg)
low dose	= 0.01	LD_{50}	(0.0075 mg/kg)
very low dose	= 0.001	LD_{50}	(0.75 u/kg)

For correlation with studies of this and other anti-ChE agents, relative inhibitions of blood ChE enzyme levels were measured for all rats before injection (control) and at selected times after injection.

Published values for LD_{50} of pyridostigmine (29,30) were used for calculating dose levels required to obtain 40% and 80% blood ChE inhibition. The resulting enzyme inhibitions were within the range projected in all experiments (see **RESULTS, Section N**). For

all acute exposure experiments, the experimental dose levels for pyridostigmine were standardized as follows:

high dose	= 0.8	LD_{50} (2.96 mg/kg)
moderate dose	= 0.1	LD_{50} (0.37 mg/kg)
low dose	= 0.01	LD_{50} (0.037 mg/kg)

Intermediate doses were based on an LD_{50} of 3.7 mg/kg.

C. PROCEDURE FOR ACUTE ADMINISTRATION OF PHYSOSTIGMINE AND PYRIDOSTIGMINE

In the acute physostigmine exposure experiments, "high," "moderate," "low," and "very low" doses corresponding to 0.8, 0.1, 0.01, and 0.001 LD_{50} (0.6, 0.075, 0.0075, and 0.00075 mg/kg) were tested. Seventy-three male Wistar rats weighing 180 to 250 g were given single s.c. injections of 0.001 to 1.1 LD_{50} in the rostral mid-back region. Blood samples were obtained immediately before injection (control), at 30 minutes and at 1, 7, 14, 28, and/or 56 days PI, as well as immediately before perfusion fixation. Blood and tissue samples were coded to conceal dose and sampling time. Blood samples were analyzed for ChE inhibition and the results correlated with ultrastructural analyses of the tissue samples. Forty-eight sham-injected (control) rats were obtained for examination after the same exposure intervals (30 minutes to 56 days PI; age- and sex-matched controls). [For each experimental protocol or exposure interval, diaphragm, soleus, and EDL muscles from 3 treated and 2 control (sham-injected) rats were prepared for ultrastructural analysis. See Sections G-J, below.]

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. Serum ChE analyses were not obtained for the 4 treated guinea pigs because of equipment malfunction. Consequently, dose levels (in mg/kg) were correlated with ultrastructural alterations and with observed behavioral signs. Additional correlations were provided by ultrastructural analyses, in which guinea pigs surviving near lethal doses had similar endplate alterations to those observed in rats at similar near lethal doses (see RESULTS).

Preliminary comparisons of the effects of acute exposure to physostigmine and pyridostigmine on normally active rats employed 7 groups of 5 rats each (3 drug-injected "experimental" and 2 sham-injected "controls"). Rats were injected with 0.01, 0.1 or 0.3-0.8 LD_{50} and their diaphragm, soleus and EDL muscles examined ultrastructurally.

In initial studies of the synergistic effects of neuromuscular stimulation on the expression of anti-ChE toxicity, 6 groups of 5 rats (3 experimental, 2 control) were injected with 0.25 LD_{50} of physostigmine or with a sham solution and subjected to 20, 40, or 80 Hz continuous stimulation for 15 or 30 minutes (see Section F and Table 1, Section F, below). Once appropriate stimulation frequencies and durations were established, more detailed studies of the synergistic effects of neuromuscular stimulation and anti-ChE toxicity were completed. In these

experiments, 6 groups of 3 rats each were injected with sufficient physostigmine or pyridostigmine to obtain 30%; 70%, and 90% blood ChE inhibition (0.01, 0.1 and 0.3-0.8 LD_{50}) and the EDL and soleus muscles were stimulated at 40 Hz for 15 min as described in Section F (below). Stimulated EDL and soleus muscles were compared with spinal-blocked contralateral controls and with normally stimulated diaphragm myofibers.

D. SUBACUTE ADMINISTRATION OF PHYSOSTIGMINE TO RATS

Rats were anesthetized with ether, a small incision was made in the rostral midback region, and an implantation cavity was made by blunt dissection. Alzet mini-pumps (Alza, Palo Alto, CA) were implanted in the cavity, which was then closed and sutured using wound clips.

1. Determination of Doses Required to Obtain Pre-determined Enzyme Inhibition Levels

To determine the doses required to obtain 40% and 80% blood ChE enzyme inhibition, 10 rats were implanted with Alzet pumps containing the following dilutions of physostigmine: 1.0 mg/ml; 0.6 mg/ml; 0.5 mg/ml; 0.4 mg/ml; 0.3 mg/ml; 0.2 mg/ml; 0.1 mg/ml; 0.01 mg/ml, 0.001 mg/ml; and 0.0001 mg/ml. Each Alzet pump delivers 2.5 ul/hour or 60 ul/day of the designated concentration. As in the acute exposure experiments, blood enzyme levels were measured before implantation of the pumps (controls) and after 24 hours, 2, 5, and 14 days of exposure and in the subacute recovery experiments at 7, 14, 28, and 56 days after pump removal. Data from those preliminary experiments are described in greater detail in **RESULTS**. After initial difficulties traced to the use of "pre-primed" pumps (see **RESULTS, Section K.1**), final experiments employed "partially pre-primed" pumps, which were prepared by briefly placing them in rat Ringer's solution for 30 minutes to 1 hour. Using this procedure, Alzet mini-osmotic pumps loaded with 0.25 mg/ml and 25 mg/ml and delivered at a rate of 2.5 ul/hour yielded sustained serum ChE inhibitions of 30-50% and 70-90%, respectively. All subsequent subacute exposure experiments were based on these estimates and dose levels.

2. Measurement of Pumping Rate; Determination of Sources of Pump Failure

Since significant decreases in average enzyme inhibition were measured during preliminary subacute exposure experiments (especially in the high-dose groups; see **RESULTS, Section K1**), it was necessary to determine whether the decreases resulted from diminished rates of osmotic pumping or different responses in individual rats. Therefore, in all cases, the pumps were removed from each animal before perfusion fixation and their contents and pumping rates examined by at least three of four independent methods: 1) volumetric and weight measurement of residual pump contents, 2) examination of solution for tell-tale changes in color associated with loss of biological activity (42), 3) analysis of coded samples for necrosis or other signs of surgical fault at the implant site, and 4) injection of residual

contents of selected pumps into untreated animals and measurement of the resulting ChE enzyme depressions. Pumps that were found not to have released the proper dose of physostigmine were identified and data for those animals discarded from the study. (Note: In preliminary experiments, we found that pump failure occurred only when the implantation cavity was not sufficiently large. The resulting compression of blood vessels in the skin over the pump resulted in inadequate vascular perfusion and localized tissue necrosis. It was also assumed that the tissue compression may have resulted in partial pump blockage, which may have contributed to inadequate drug absorption.) Animals with evident surgical fault were also omitted from the study prior to enzyme analysis and their tissues were not examined ultrastructurally. Fewer than 8 animals were discarded because of pump failure and/or surgical fault. These were replaced by additional rats treated at the appropriate dose.

E. WHOLE BLOOD CHOLINESTERASE ENZYME ASSAY

We have used the radioisotope assay method of Siakotos, et al. (43) to determine blood ChE enzyme activity levels. Using ^{14}C-ACh as the substrate, we have measured the relative activity of blood ChE after acute and subacute exposure to physostigmine. Blood samples were obtained a) before injection of physostigmine and at 30 minutes, 1, 7, 14, 28, and 56 days PI, or b) before surgical implantation of osmotic pumps, and c) at 3, 7, 14, and 28 days of recovery (after pump removal). Two 50-ml samples of whole blood were drawn into heparinized capillary tubes from a small incision in the tail vein. Samples were diluted 1:9 (50 ml:450 ml) 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. (43), blood ChE enzyme activity was determined by measuring the amount of ^{14}C-acetate generated from ^{14}C-ACh after 5 minutes in the reaction mixture. Diluted whole blood (100 ml) was added to 100 ml of 0.1 M sodium phosphate (pH 7.38) and 100 ml of ^{14}C-ACh (containing 0.3 mmoles of total ACh and 0.10 mCi of ^{14}C-ACh). 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-dioxane slurry was prepared by adding 200 ml of dioxane to 50 g of AG 50W-X8 cation exchange resin (200-400 mesh from Bio-Rad Laboratories, Richmond, CA). The resin was previously converted to the Na^+ form, washed with acetone, and dried <u>in vacuo</u>. Five ml of dioxane was added to this mixture, which was then mixed thoroughly and 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 (Beckman Instruments, Inc., Irvine, CA). For each assay, background radioactivity was determined by sham incubation with distilled H_20 replacing blood (to assess the condition of the radiosubstrate). The total amount of radioactive isotope used in each assay was also determined.

Normal (control) blood 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 3,000 enzyme assays have been obtained. Thus, enzyme histories are available for virtually every rat, permitting direct correlation of altered ultrastructure with ChE enzyme inhibition.

F. PHYSIOLOGY

In vivo twitch tensions were recorded from EDL muscles in rats from 4 experimental groups: a) following acute exposure to physostigmine, b) during and after recovery from subacute physostigmine administration by Alzet pumps; c) in non-injected rats receiving prolonged muscle stimulation at 20, 40, or 80 Hz, and d) in rats receiving both acute exposure to drug (either physostigmine or pyridostigmine), followed by prolonged muscle stimulation.

1. Physiological Monitoring of the Interactions of General Anesthetics with Physostigmine

In preliminary experiments, the interaction of general anesthetics with acute physostigmine administration was documented. General anesthetics included chloralhydrate (400 mg/kg i.p. initial dose, supplemental doses i.v. to effect) or ketamine/xylazine (87 mg/kg and 13 mg/kg i.m. initial dose, supplemental doses i.v. to effect). In subsequent experiments, spinal block anesthesia was employed to avoid interaction between general anesthetic and physostigmine. (For descriptions of interactions of chloral hydrate and of ketamine-xylazine with physostigmine, see **RESULTS, Section E.**)

2. Spinal Block Anesthesia Used to Minimize Interactions of Physostigmine with Other Drugs

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.

3. Methods for Measuring Twitch Tensions and Percent Sustained Contractions

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 (Grass Instrument Company, Quincy, MA). 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 s.c. in the rostral mid-back region. Doses varied from 0.0025 LD_{50} to 0.5 LD_{50}, each 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, initially at 10-15 minute intervals to establish the time course of blood ChE inhibition.

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. Maximum twitch tensions were measured, and from these, "percent sustained contraction" (PSC) values were used to assess overall performance of the contractile mechanism. PSC 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 group of rats described in experimental group c, above, 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. After measurement of initial PSCs in the experimental group d, above, physostigmine was administered and single EDL twitches (0.1-Hz stimulus) were monitored on a Graphtec WR3101 chart recorder (Western Graphtec, Irvine, CA). An increase in single twitch tension signaled the onset of the whole muscle response to physostigmine. 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 PI). Table 1 (below) presents the number of animals in each protocol of continuous stimulation with and without physostigmine administration.

TABLE 1. Number of Animals in Each Experimental Group for the Study of Continuous Stimulation Without and With Physostigmine

Stimulus Duration	15 Min.			30 Min.		
Stimulus Frequency	20 Hz	40 Hz	80 Hz	20 Hz	40 Hz	80 Hz
Stimulation only	3	3	3	2	2	2
Physostigmine plus stimulation	3	3	3	1	1	1

4. Comparison of Physiological Alterations Induced by Continuous Stimulation During Acute Exposure to Physostigmine vs. Pyridostigmine

Methods for spinal block anesthesia, surgical preparation, and recording of EDL twitch tensions and PSC values were as described in Sections F1-F3, above. The protocol for continuous stimulation was also the same as above, except that the stimulation was always 40 Hz for 15 minutes because those were determined to be optimum values for yielding profound alterations in endplate physiology and ultrastructure. Final PSCs were determined 30 seconds to 60 minutes after stimulus

termination (corresponding to 30-100 minutes PI of physostigmine or pyridostigmine.) The 24 rats used in this part of the study were divided into experimental groups as follows: 3 each for each dose level (3 x 3 = 9), times two drugs (2 x 9 = 18), plus 6 stimulated but non-injected controls. Low, medium, and high dose levels corresponded to 30%, 70% and 90% blood ChE inhibitions, respectively. In each stimulated, drug-treated rat, the unstimulated contralateral muscles were removed for examination as a drug-treated, non-stimulated control.

G. 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. The number of animals and number of samples examined in the ultrastructural and physiological studies of acute and subacute physostigmine exposures is presented in Table 2, below. In addition to the 2500 images described therein, over 500 images of endplates were obtained from EDL, soleus, and diaphragm muscles of rats examined physiologically.

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 and pyridostigmine on muscles with relatively pure populations of fast twitch (EDL) and slow twitch (soleus) myofibers. However, the relevant factor in expression of anti-ChE toxicity may not be twitch contraction time (44-47) 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 instead may be related to the amount (frequency and duration) of muscle activity. (The soleus, a postural muscle, is activated for extended periods to

TABLE 2. Sources of Ultrastructural Data: Acute vs. Subacute Exposure to Physostigmine

Subject of Study	Acute	Subacute	Total
# of Rats in Study	120	70	190
# of Groups (5/group)	24	14	38
# of ChE Assays	1450	800	2250
# of Muscles Examined	360	210	570
# of Endplates Photographed	>1500	>1000	>2500
# of Micrographs	>2000	>1000	>3000

maintain balance and position, whereas the EDL muscle, normally used to extend the digits during walking, is seldom activated during the period of quiescence that is characteristic of rats that survive a near-lethal dose of physostigmine.) Finally, 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 (44-46), all of which are activated frequently to maintain breathing. Thus, the close proximity of three fiber types in the same muscle (diaphragm) provides for an internal fiber-type control. Presumably, under respiratory insufficiency, all fiber types in the diaphragm (fast, slow, and intermediate myofibers) are activated at near maximum levels. The fast twitch fibers in the diaphragm may then be contrasted with fast twitch myofibers in the EDL muscle, which are voluntarily rested for prolonged periods during the initial period of neuromuscular distress produced by acute high doses of physostigmine. By comparison, under physostigmine-induced respiratory distress, the fast twitch fibers in the diaphragm have relatively continuous firing of myofibers, presumably equal to or greater than that in postural muscles, such as the soleus (47). Consequently, both diaphragm and soleus muscles (but not EDL) are relatively continuously activated until the animal collapses from exhaustion and/or 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.

H. FIXATION FOR ELECTRON MICROSCOPY

1. Perfusion Fixation

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, but are described in **RESULTS, Section F.**] 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. [In preliminary experiments using control animals, we determined that pressurization of the perfusion apparatus with 100% nitrogen gas (as described by Hudson and coworkers, 24) substantially decreased O_2 tension in the perfusate, resulting in severe mitochondrial swelling as an artifact of hypoxic fixation. Oxygenated perfusion solutions used in our experiments greatly diminished but did not always prevent mitochondrial swelling.] After tissue clearing, a fixative 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 yellowed 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 for 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 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 based on the occurrence of similar changes in control samples fixed by immersion only (next section).

2. Immersion Fixation; Identification of Artifacts

To identify the most common artifacts of inadequate perfusion fixation, whole EDL muscles from untreated rats were excised and placed into 37°C oxygenated 2.5% glutaraldehyde in rat Ringer's buffer (pH 7.2). After 3-5 minutes, the intact muscles were chilled to 4°C and fixed for 16-20 hours in 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). These wedges from the immersion-fixed samples were used to identify mitochondrial alterations characteristic of delayed fixative penetration and/or hypoxia (31, 32) and were instrumental in discriminating artifacts of fixation from low-dose drug effects. (See RESULTS.) Results from this ancillary study of immersion vs. perfusion fixation are described in Lee, et al., (32). None of the experimental data in this study were obtained from immersion-fixed muscles.

I. LOCATING ENDPLATES; EMBEDDING SAMPLES IN PLASTIC

After primary glutaraldehyde fixation, endplate regions were stained using a modified <u>in vitro</u> ChE stain (48,49). 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. Subsequent reactivation of AChE (1) yielded positive staining by the ChE reaction products. Rinsing for 1-3 days in drug-free fixative (see Section H,1, above) or in drug-free Ringer's solution restored rapid stainability.]

Small segments containing opalescent endplates were dissected free and post-fixed for 1 hour in 1% OsO_4 in Ringer's buffer. 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 (50), and polymerized at 70°C for 24 hours. (Reagents for electron microscopy were purchased from Tousimis Research Corporation, Rockville, MD, Ted Pella, Inc., Tustin, CA, and Polysciences, Inc., Warrington, PA.)

J. MICROTOMY AND ELECTRON MICROSCOPY

Thin (gray to pale gold) and thick (1-micron) sections were cut with EMS or Dupont diamond knives using Dupont Sorvall MT-2B, MT-2, and LKB Model IV ultramicrotomes (Electron Microscopy Sciences, Fort Washington, PA; E. I. Dupont de Nemours, and Co, Inc., Newtown, CN; and LKB Instruments, Inc., Rockville, MD). Thick sections were stained with toluidine blue and photographed at 400-900X using a Zeiss Photoscope (Carl Zeiss, Inc., Thornwood, NY). Thin sections were collected on 200 or 700 mesh copper grids, and post-stained with lead citrate (51,52). Specimens were examined at 80 kV on Philips 400T or Philips EM 200 transmission electron microscopes (Philips Electronics Instruments, Inc., Mahwah, NJ) and photographed at initial magnifications of 1,000X to 40,000X using Electron Microscope Film (Kodak, Rochester, NY).

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 coding 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 were photographed and analyzed, almost 300 of which are reproduced in **RESULTS**.

VIII. RESULTS

A. ESTABLISHMENT OF LD_{50} FOR ACUTE PHYSOSTIGMINE EXPOSURE

To ascertain an approximate LD_{50} of the acid-stabilized solutions of physostigmine that were to be used in all subsequent acute and subacute exposure experiments, two small groups of rats were tested. The first group of rats was injected s.c. with physostigmine over a wide range of dose levels to establish an approximate LD_{50}, while the second group was used to refine the estimate. The first group of 6 rats was tested in the following sequence: At 2.0 mg/kg, the rat died in 80 seconds. At 0.4 mg/kg, the rat lived and showed only moderate signs of anti-ChE intoxication. (See Section B, below, for a description of behavioral responses to toxic doses of physostigmine.) At 1.0 mg/kg, the third rat experienced severe signs of anti-ChE intoxication, but survived (and thus was presumed to have received a near-LD_{50} dose). At 1.5 mg/kg, the fourth rat died in 93 seconds. At 0.625 mg/kg, the rat exhibited signs of severe anti-ChE intoxication, but recovered rapidly. The final rat in the initial test survived a dose of 0.75 mg/kg, but exhibited moderate to severe symptoms of toxicity. In the second test series, a test dose of 0.8 mg/kg was administered to well-acclimated rats. Three of 5 rats (60%) died within 22 minutes PI. The remainder survived. Thus, 0.75 mg/kg was presumed to represent an approximate LD_{50} for acclimated rats. (Subsequent experimentation confirmed that estimate, but also revealed an unexpected source of variability in response to the drug. See Section C, below, describing the effects of stress on LD_{50}.)

Based on an approximate LD_{50} of 0.75 mg/kg, the experimental dose levels were standardized as follows:

high dose	= 0.8 LD_{50}	(0.6 mg/kg)
moderate dose	= 0.1 LD_{50}	(0.075 mg/kg)
low dose	= 0.01 LD_{50}	(0.0075 mg/kg)
very low dose	= 0.001 LD_{50}	(0.75 mcg/kg)

Additional rats were given acute exposures of 1.0 and 1.1 LD_{50}.

B. BEHAVIORAL RESPONSES TO ACUTE PHYSOSTIGMINE EXPOSURE

1. Initial Responses to High and Low Doses

Within 3-10 minutes after injection of 0.8-2.67 LD_{50} (or 0.6-2.0 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. Rats that did not survive the initial acute exposure exhibited pronounced hyperexcitability, uncoordinated lunging and running motions, extensive piloerection, and extreme respiratory distress, leading to convulsions and death within 1-30 minutes. On the other hand, animals surviving an acute high dose usually retired to a corner of the cage and exhibited minimal muscle activity. Rats exposed to low to very low doses (0.01-0.001 LD_{50}) exhibited no overt signs of drug intoxication after they were released from the restraint (or in the initial experiments, after they had recovered from ether anesthesia).

2. Recovery

After 30 minutes of exposure to 0.8-1.1 LD_{50}, surviving rats had greatly diminished or nearly normal startle reflexes. By 1 hour PI, the rats were resuming normal neuromuscular activity (i.e., brief walking excursions, drinking, and occasional feeding). Within 6-24 hours, surviving rats exposed to 0.8-1.1 LD_{50} had resumed (near) normal activity. In the acute-recovery experiments, rats were weighed periodically, as well as immediately before perfusion fixation. Normal weight gain was noted in all acute exposure groups.

C. EFFECTS OF STRESS ON LD_{50} FOR ACUTE PHYSOSTIGMINE EXPOSURE

Rats recently delivered to the Animal Care Facility were much more susceptible to physostigmine-induced lethality than rats acclimated for 7-10 days. Handling the rats for several minutes each day during the acclimation period (i.e., transferring them from cage to cage, gentle stroking) produced more rapid acclimation. That is, acclimated rats exhibited reduced excitability in their new surroundings (i.e., reduced startle reflex) and noticeably reduced susceptibility to drug lethality. For example, the LD_{50} was determined in acclimated rats to be 0.8 mg/kg. At that level, only 5% (1/19) died after injection of 0.8 LD_{50} (0.6 mg/kg). On the other hand, approximately 70% (5/7) non-acclimated rats died at an even lower dose of 0.65 LD_{50} (0.5 mg/kg). This is strong suggestive evidence that the LD_{50} is lowered substantially (by 30-50%) in non-acclimated, stressed animals. However, since it was not the purpose of this investigation to measure possible changes in LD_{50} in response to stress, more detailed comparisons were not attempted. Nevertheless, since serum ChE inhibition levels were similar for acclimated and non-acclimated animals exposed to similar amounts of physostigmine, we attribute the increased susceptibility in the non-acclimated rats to hyperexcitability and increased respiratory and voluntary muscle activity under conditions of stress. This observation may have implications for exposure to other anti-ChE agents, including nerve agents (see INTERPRETATIONS). We emphasize, however, that for consistency, all acute exposure experiments in this study utilized acclimated rats and were based on an LD_{50} level of 0.8 mg/kg, as established by acute injection into acclimated rats.

D. MEASUREMENT OF WHOLE BLOOD ChE LEVELS BEFORE AND AFTER ACUTE EXPOSURES TO PHYSOSTIGMINE

1. Determination of Blood ChE Inhibitions for Control and Experimental Rats

Blood ChE assays were completed on all rats in the acute exposure experiments. Blood samples were taken immediately before injection of physostigmine and at the following intervals PI: 30 minutes and 1, 7, 14, 28, and 56 days (or until the animals were prepared by perfusion fixation). Normal or control blood ChE activity was measured for each animal just prior to exposure to physostigmine. The pre-drug enzyme activity was assigned the value of 100% activity. Thirty minutes after exposure to physostigmine, blood ChE activity was measured and

compared with the pre-drug ChE activity. The inhibition of blood ChE (30 minutes PI) was linearly proportional to the log of the physostigmine dose between 0.01-0.8 LD_{50} (0.0075-0.6 mg/kg) (Fig. 1). At 30 minutes PI, the following blood ChE inhibitions were measured:

0.8 LD_{50}	=	89% (± 3%)
0.1 LD_{50}	=	67% (± 7%)
0.01 LD_{50}	=	33% (± 7%)
0.001 LD_{50}	=	28% (± 15%)

These values for blood ChE inhibition are consistent with blood ChE inhibitions measured at similar doses of other anti-ChE agents (24-26). However, individual variation and stress were found to exert profound effects on LD_{50} (see Sections A and C, above).

2. **Reproducibility of Blood ChE Measurements**

We found high reproducibility between two samples taken simultaneously from the same animal (i.e., less than 7% variability). There was equally low (less than 10%) variability between samples examined immediately vs. those frozen and thawed two times. In addition, control blood samples held for 65 minutes at room temperature had the same enzyme activity as fresh samples. The control ChE activity was therefore quite stable in terms of minor variations in time intervals between sample collection, dilution, freezing, thawing, and enzyme analysis. Thus, the differences and variabilities in blood ChE activities measured in this project are thought to reflect accurately drug-induced changes, as well as normal fluctuations in enzyme levels.

3. **Temporal Fluctuations in Blood ChE Levels**

Blood ChE activity of animals not exposed to physostigmine (i.e., controls) fluctuated substantially from day to day (Fig. 2, dotted line). (One standard deviation for control values of blood ChE activity determined at 30 minutes, 1, 7, 14 and 28 days after sham injection included values from 65% to 140% of initial activity.) Thus, the large changes observed in enzyme activity levels in both the experimental and control rats reflect not only specific inhibition of blood ChE by physostigmine, but also normal temporal variation within individual rats and normal variation from animal to animal. [Although similar large-scale fluctuations in whole blood ChE levels for both control and experimental animals have not been reported by other investigators, the values reported here were confirmed in duplicate analyses run at different times. Moreover, the ultrastructural alterations observed in virtually all high- and moderate-dose experiments (see RESULTS, Sections H1, H3, H4, and M3) corresponded closely to the ChE inhibitions measured. Thus, we believe the temporal fluctuations in blood ChE levels are real. In any case, blood ChE levels were measured before and after experimental treatment for each rat, and were not calculated against an idealized or average ChE level.] Despite the large fluctuations in whole blood ChE activities measured in individual control rats, some obvious features emerge

from the ChE activity profiles of the physostigmine-treated rats:

a) exposure to the high dose (0.8 LD_{50} or 0.6 mg/kg) sharply depressed blood cholinesterase activity to 11% ±3% of normal (i.e., average inhibition of 89% ± 3%) at 30 minutes PI (Fig. 2, solid line).

b) blood ChE activities from these high-dose rats recovered to the normal range within 24 hours (Figs. 2-4), but showed continued widespread fluctuations over the next 56 days (Fig. 4).

c) At lower doses of physostigmine (0.01-0.1 LD_{50} or 0.0075-0.75 mg/kg), blood ChE activity was depressed to 67% (±7%) and 33% (±7%) of normal activity, respectively), but was followed by recovery to the normal range of activity after 24 hours (Figs. 5-6).

We also note that after exposure to $\leq$0.01 LD_{50}, the range of variation at 30 minutes PI was far less than the daily fluctuation observed for sham-treated rats (Fig. 2). Likewise, although the range of blood ChE activity levels for rats receiving 0.001 LD_{50} (Fig. 7) appears to be distinguishable from that of rats receiving no drug, the apparent differences are not considered statistically significant because they differ by less than one standard deviation from values obtained from control rats (compare Fig. 7 with Fig. 2, dotted lines). Moreover, because physostigmine at low concentration is first exposed to and presumably bound preferentially to blood ChE, blood ChE inhibition data from very low dose exposures may not pertain directly to endplate AChE inhibition (see INTERPRETATIONS).

E. MUSCLE PHYSIOLOGY BEFORE AND AFTER ACUTE HIGH-DOSE EXPOSURE TO PHYSOSTIGMINE

Physiological effects of acute physostigmine administration on <u>in vivo</u> muscle contractility have been studied in 28 rats; 4 animals were anesthetized with ketamine-xylazine, 10 with chloral hydrate, and 14 by spinal block with lidocaine. Administration of physostigmine produced varying degrees of muscular tremor and fasciculations, excess salivation, hemorrhage of mucous membranes around eyes and nose, and respiratory distress. Symptoms ranged from isolated fasciculations of facial muscles with mild respiratory congestion at low doses (0.05 LD_{50} or 0.0035 mg/kg), to whole body tremor and fasciculations with death by respiratory paralysis at high doses (0.5 LD_{50} or 0.375 mg/kg).

Average EDL twitch tension for normal rats (i.e., prior to physostigmine administration) was 30 ±7 g (mean ± one standard deviation), and did not vary significantly with different anesthesia methods.

A comparison of results from different anesthesia methods is summarized in Figure 8. The figure shows the fraction of EDL muscles responding to physostigmine with measurable twitch potentiation, as also reported by others (53). Figure 8 also shows the maximum potentiation achieved at different dose levels for different anesthetics. Consistent potentiation response at relatively low doses of physostigmine has been obtained only with use of the spinal block procedure.

Measurable potentiation of EDL twitch tension began 7 to

30 minutes after s.c. injection of a high dose of physostigmine, with time of onset of potentiation corresponding directly 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 inhibition 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. Examples of potentiation responses at low and high doses of physostigmine are shown in Figure 9. For the 18 EDL muscles showing twitch potentiation, average time of onset of potentiation was 22 $\pm$7 minutes after s.c. injection. Average duration of the response (time elapsed before return to pre-injection tensions) was 23 $\pm$12 minutes. At peak potentiation the magnitude of the twitch response ranged from 121% of normal at 0.1 LD_{50} to 484% at 0.5 LD_{50} (both under lidocaine block).

As a measure of possible physostigmine damage to contractile properties, EDL muscle was monitored for its ability to sustain tension with 20 Hz stimulation (Fig. 10). The data are converted to and discussed as PSC values. As described in METHODS, Section F.3, a smaller PSC indicates decreased ability to sustain contraction. Figure 10 is an example of a chart record demonstrating the effect of physostigmine on PSCs. The first 20-Hz, 10-second stimulus train delivered prior to physostigmine administration (Segment A-B in Fig. 10) produced a PSC of 91%, indicating that the EDL was sustaining contraction at a level near the maximum twitch attained at the beginning of the stimulus train. (If the stimulus train had been longer than 10 seconds in duration, the PSC would have reached 100% or more, as the continued partial tetany of the muscle produced greater tension.)

The actual pattern of sustained contraction observed on chart records, as well as calculated PSC values, varied from animal to animal, even prior to physostigmine administration. This was attributed in part to differences in the condition of nerves and muscles after surgical preparation. For example, the PSC value for control rats ranged from 90% to 120% during 10 seconds of 20-Hz stimulation. The relevant point to note is that PSC values decreased during drug response and returned to normal or near normal values during recovery.

About 20 minutes after physostigmine administration, the potentiation of EDL single twitches (Segment D in Fig. 10) indicated strong positive response to the drug. During this response, another 20-Hz, 10-second stimulus train produced a PSC of 48% (point E in Fig. 10), demonstrating the reduced ability of EDL to sustain a high-frequency contraction while under the influence of physostigmine. About 1 hour after physostigmine injection, the PSC was 67% (point F in Fig. 10), as the EDL began to recover its normal contractile properties.

Measurements of PSCs during the physostigmine response interfered with other parameters being monitored, and therefore were made on only a few animals. However, PSCs were measured for all rats prior to and during recovery from acute exposure to

physostigmine. For rats under spinal block anesthesia, the average PSC value before physostigmine administration was 90%. The average PSC value measured 45-70 minutes post-physostigmine injection was 92%, indicating good recovery of contractile properties by EDL in a short time period. Although some rats receiving higher doses of physostigmine showed poorer recovery 1 hour PI than those receiving lower doses, no statistical correlation between dose level (or ChE inhibition) and PSC recovery could be demonstrated.

One further observation, of a qualitative nature, was made concerning the potentiation response. For all of the physostigmine doses and all of the anesthesia methods employed, potentiation of twitch tensions occurred only when there was a background level of muscular activity, as demonstrated by noticeable fasciculations of the EDL (see physiological record, Fig. 9). Moreover, the level of potentiation appeared to be directly proportional to the degree of fasciculation. In four experiments using spinal block anesthesia and a physostigmine dose of 0.25 LD_{50}, the initial tension on the EDL was set higher than the usual 4 g. In one experiment with 8 g and two with 12 g initial tension, no measurable fasciculations occurred in the EDL and no potentiation response was observed, even though blood assays showed that ChE inhibition reached >85% in all cases. In two other experiments, 8 g of initial tension failed to prevent EDL fasciculations, and maximum potentiations of 211% and 216% of normal were obtained.

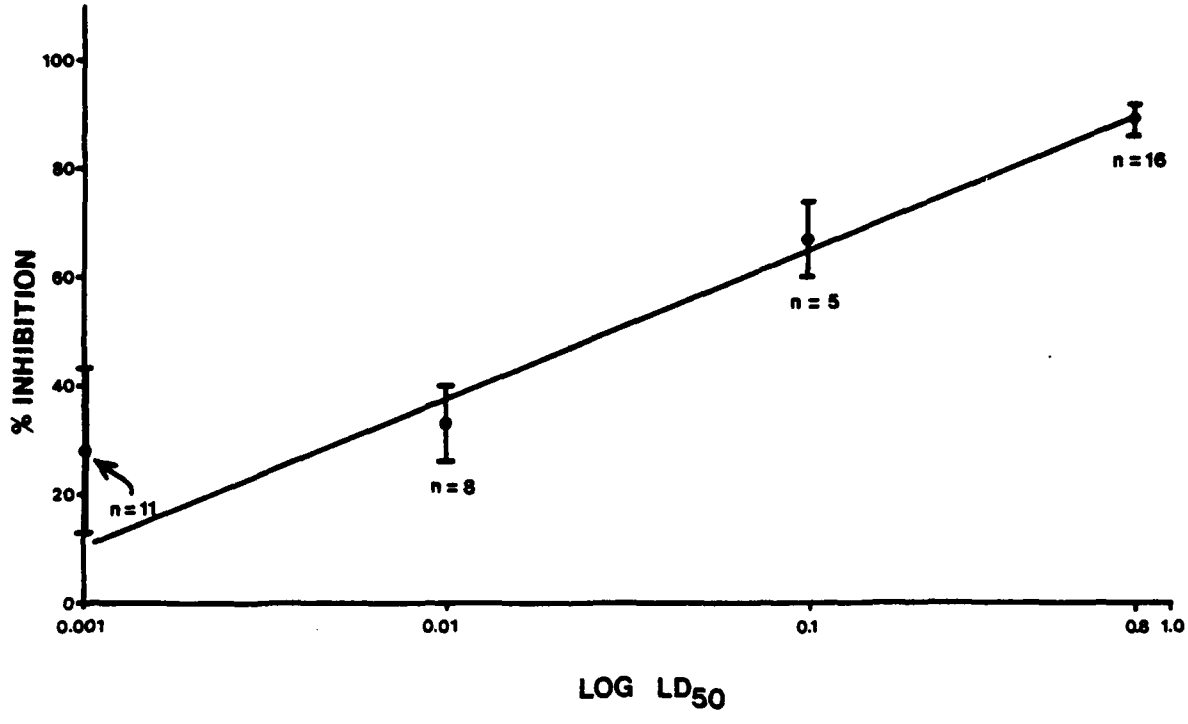


Figure 1. Dose-Response Relationship: Blood Cholinesterase Inhibitions at Different Physostigmine Doses 30 Minutes After Injection. In Figures 1-7, the error bars represent 1 standard deviation; n = number of rats from which blood samples were analyzed. Analyses were done in duplicate. (The curve was drawn as a straight line to indicate approximate values. No inferences should be made concerning the slope of the curve at the very low dose.)

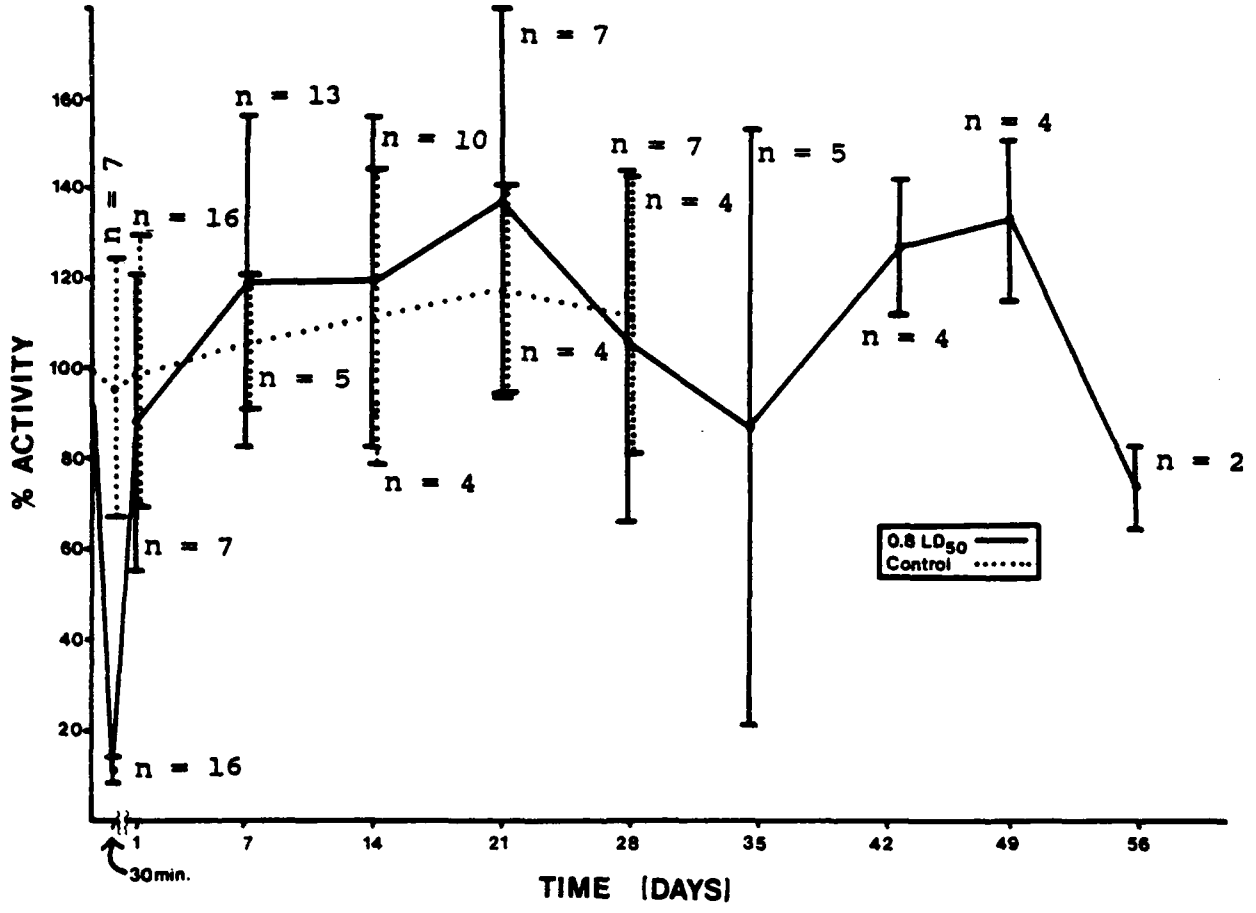


Figure 2. Blood Cholinesterase Activity vs. Time After Injection of a High Dose (0.8 LD_{50} or 0.6 mg/kg) of Physostigmine. (Each measurement represents the average of two measurements for each animal; n = number of animals at each data point.)

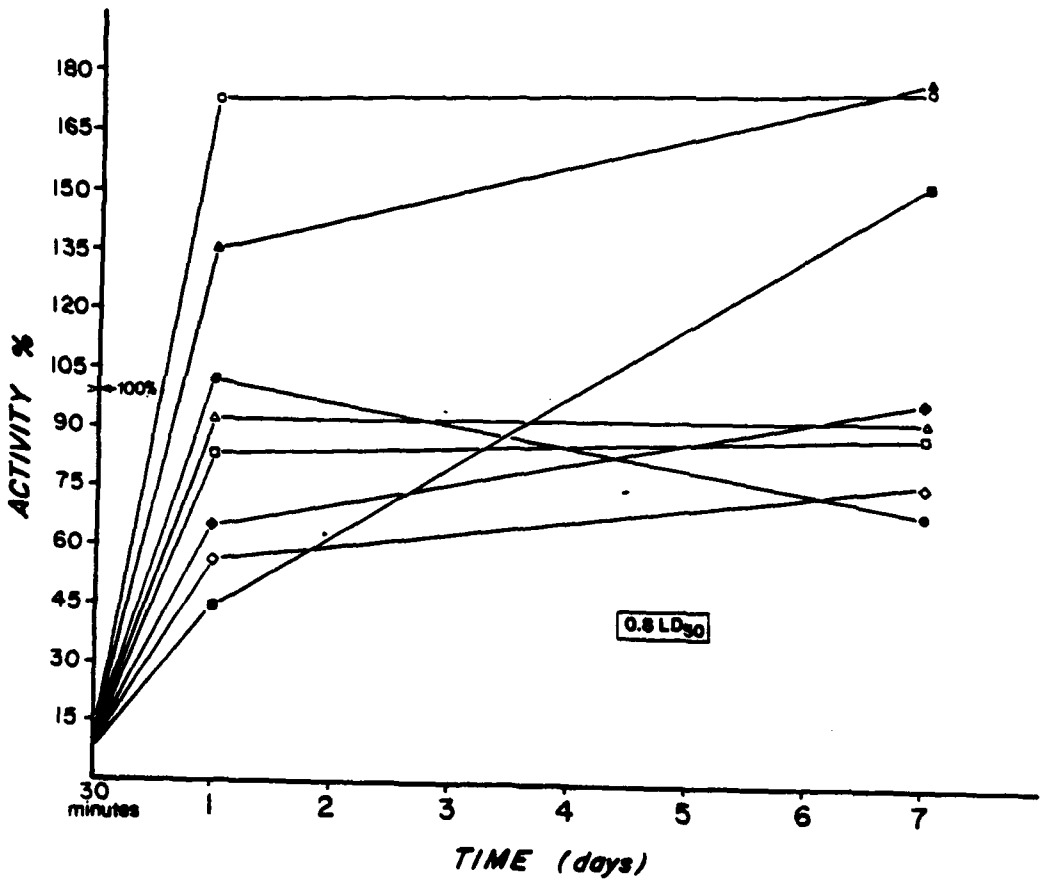


Figure 3. Short-term Variations in Blood Cholinesterase Activity 7 days After Injection of a High Dose of Physostigmine (0.8 LD_{50}). Enzyme profiles are shown for individual rats over 1 week PI. (Each measurement represents the average of two measurements for each animal.)

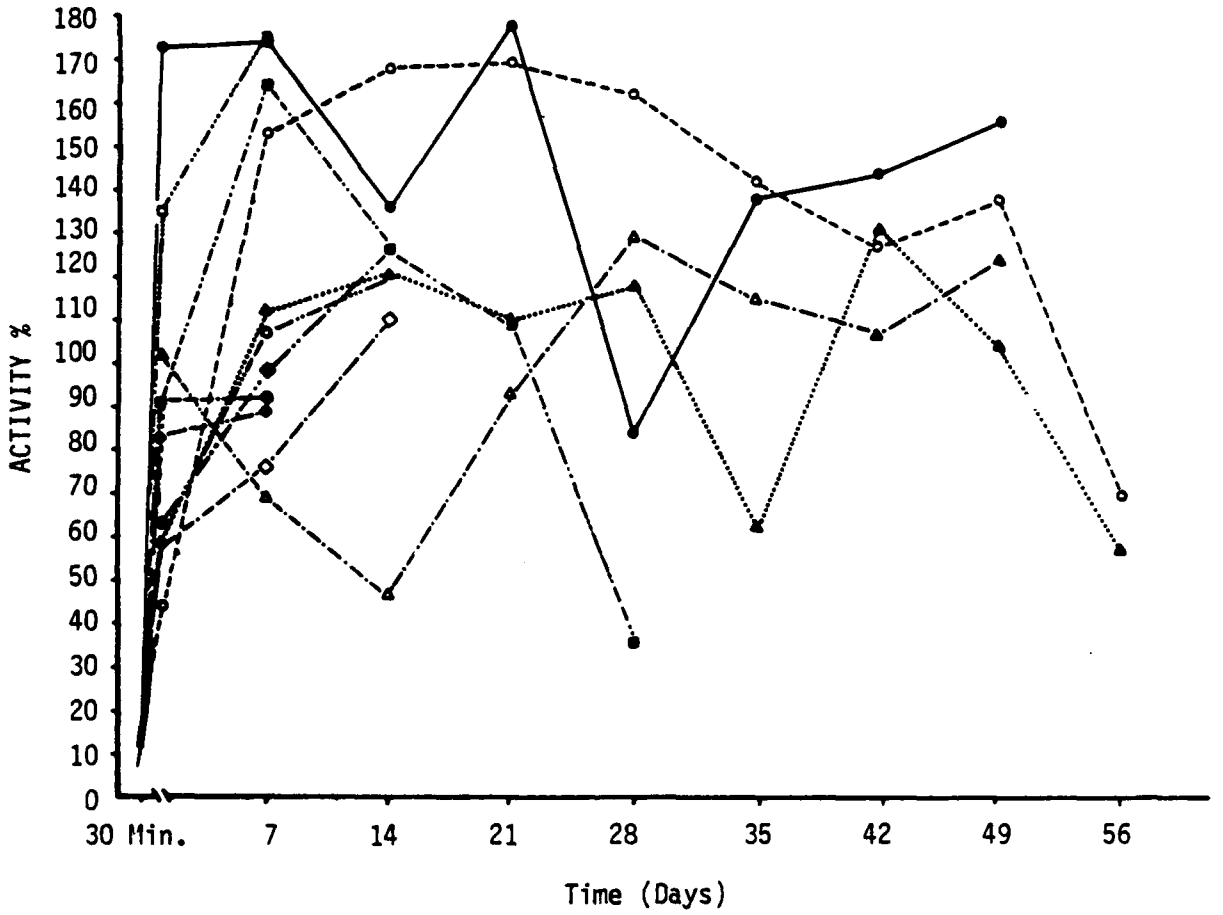


Figure 4. Long-term Variations in Blood Cholinesterase Activity from 1-56 days After Injection of a High Dose of Physostigmine (0.8 LD_{50}). Enzyme profiles are shown for individual rats up to 8 weeks PI. (Each measurement represents the average of two measurements for each animal.)

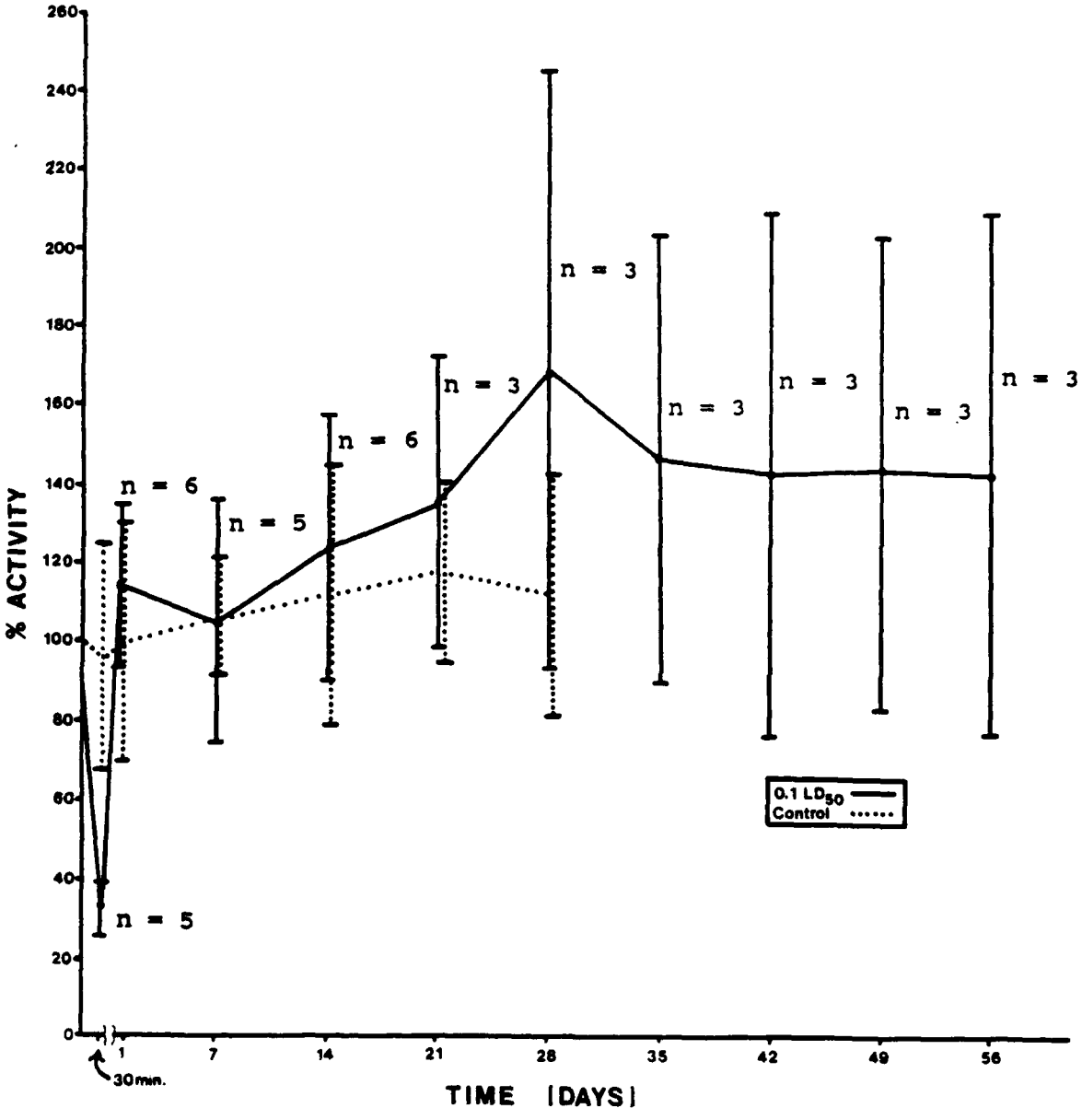


Figure 5. Blood Cholinesterase Activity vs. Time After Injection of 0.1 LD_{50} Physostigmine. (Each measurement represents the average of two measurements for each animal; n = number of animals at each data point. For controls, n is as described for the same data points shown in Figure 2.)

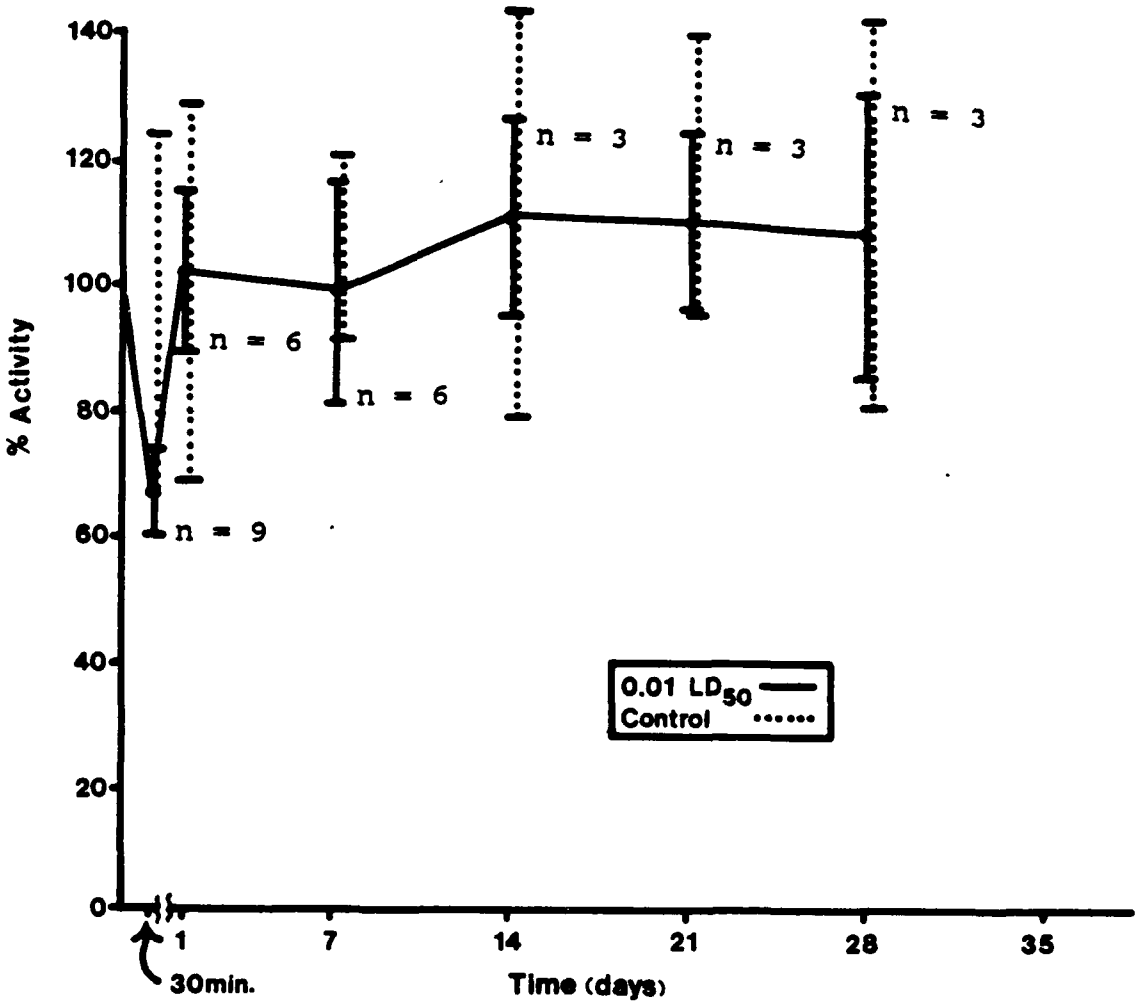


Figure 6. Blood Cholinesterase Activity vs. Time After Injection of 0.01 LD_{50} Physostigmine. (Each measurement represents the average of two measurements for each animal; n = number of animals at each data point. For controls, n is as described for the same data points shown in Figure 2.)

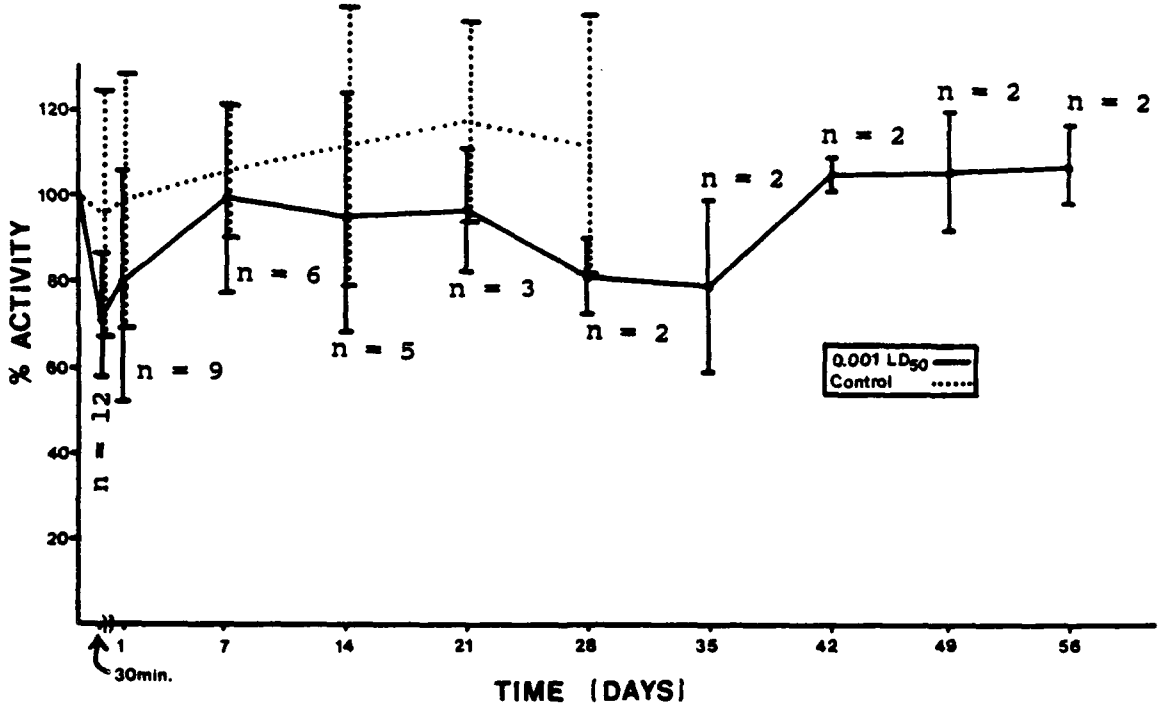


Figure 7. Blood Cholinesterase Activity vs. Time After Injection of 0.001 LD_{50} Physostigmine. (Each measurement represents the average of two measurements for each animal; n = number of animals at each data point. For controls, n is as described for the same data points shown in Figure 2.)

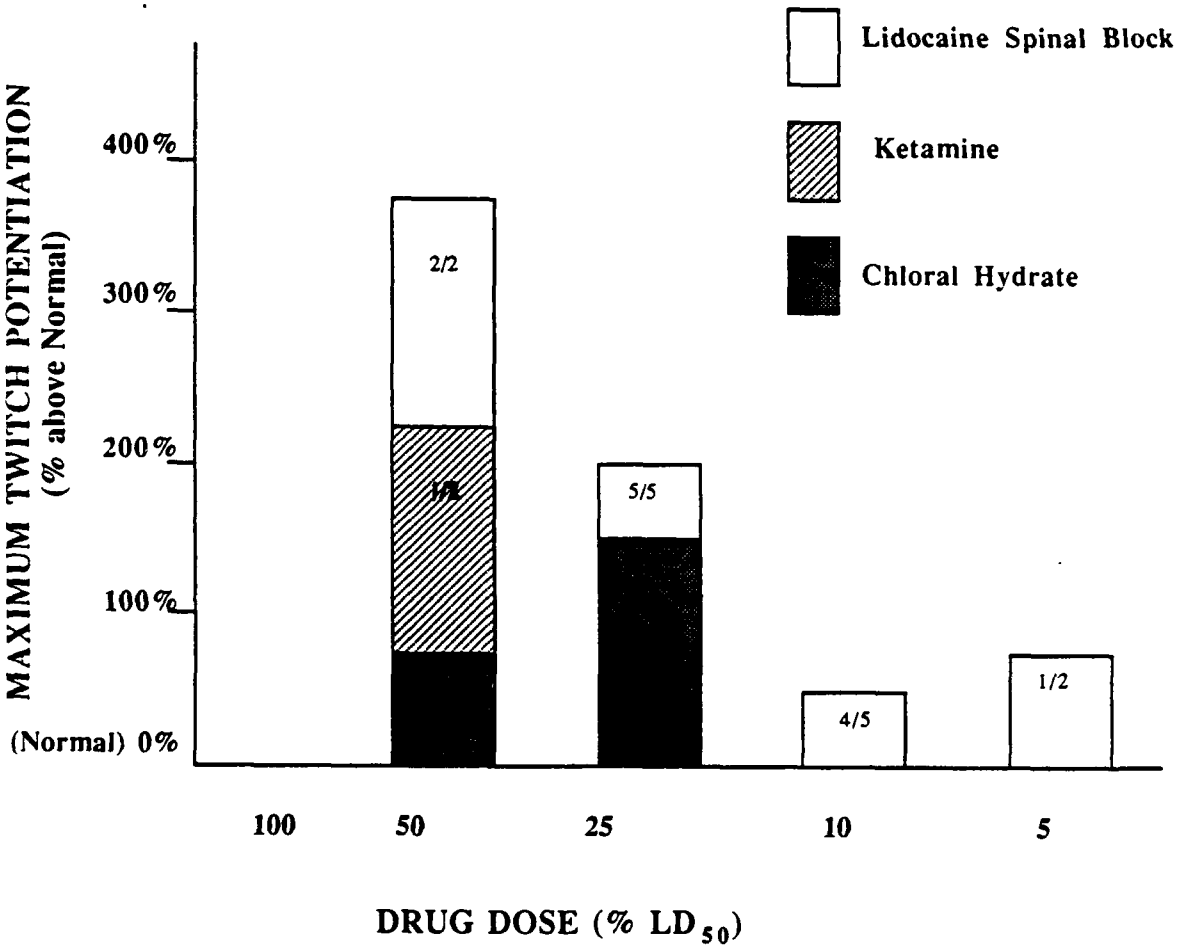


Figure 8. Maximum <u>In Vivo</u> Twitch Potentiation of EDL Muscles in Response to Acute Physostigmine Administration Under Different Anesthetics. Physostigmine was injected s.c. Numbers on the graph indicate total number of EDL muscles showing a potentiation response vs. number tested. Maximum percent twitch potentiation for each croup of EDL muscles is an average value for those responding with a twitch potentiation. There was no twitch potentiation for ketamine anesthesia at 0.25 LD_{50} physostigmine (0/1) and 0.1 LD_{50} physostigmine (0/1) or for chloral hydrate at 0.1 LD_{50} physostigmine (0/3).

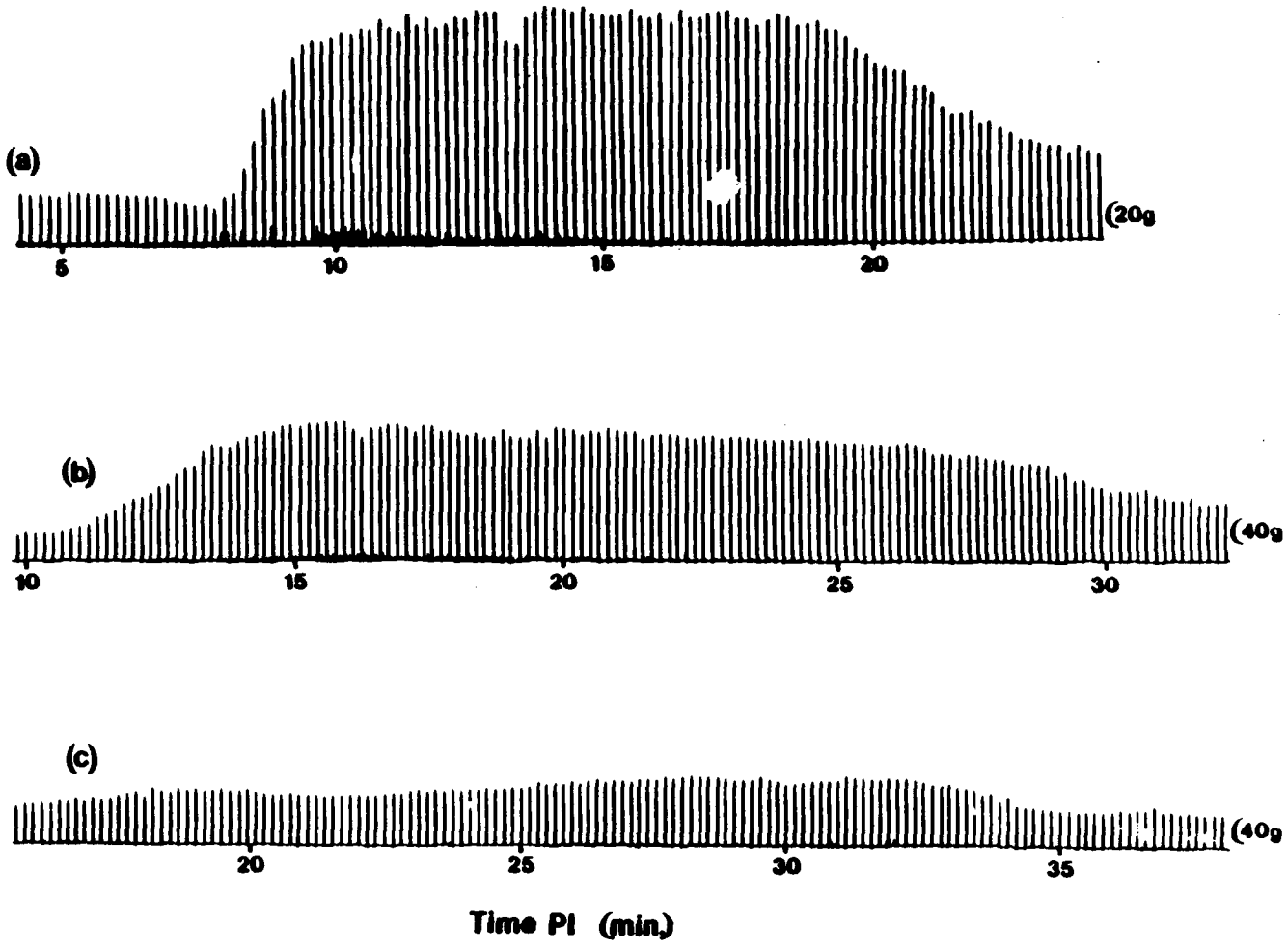


Figure 9. Examples of Effects of Physostigmine on EDL Contractility In Vivo. Physostigmine was injected s.c. Chart records show EDL twitch contractions in response to supramaximal stimulation of the peroneal nerve at 0.1 Hz. Twitch potentiation responses at (a) 0.5 LD_{50}; (b) 0.25 LD_{50}; and (c) 0.05 LD_{50}. All data are from experiments using spinal block with lidocaine.

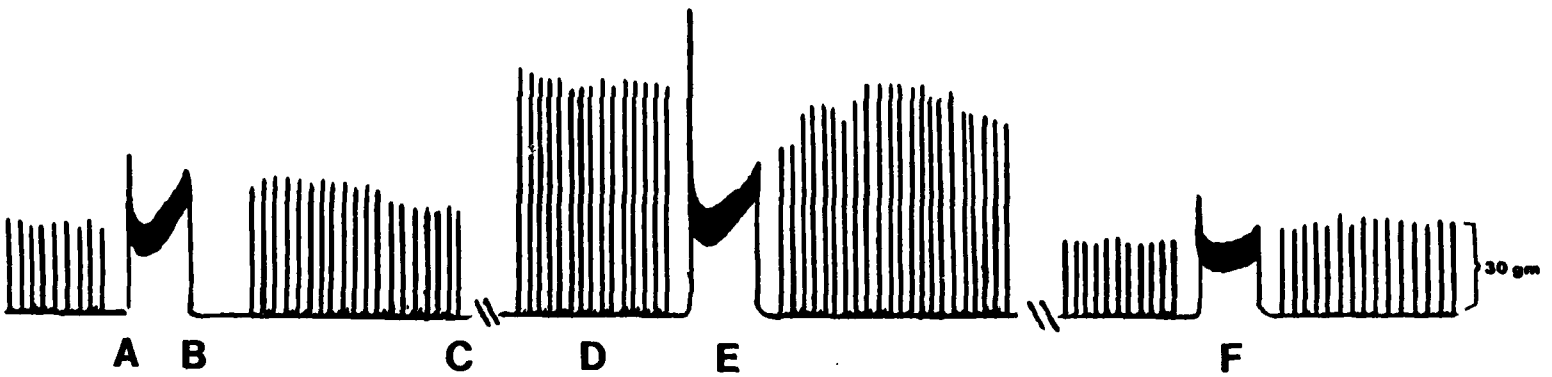


Figure 10. Effect of Acute Physostigmine Administration on Ability of EDL to Sustain Contraction <u>In Vivo</u>. PSC values were calculated from these data. Example of chart record showing EDL response to supramaximal stimulation of the peroneal nerve. Physostigmine dose was 0.25 LD_{50}, administered s.c. under spinal block lidocaine anesthesia.

A & B Prior to physostigmine administration, 20-Hz, 10-second stimulus train produced EDL contraction response shown. PSC is tension at end of train divided by tension at beginning of train (B/A), converted to a percentage.

C Physostigmine was administered s.c.

D Potentiation of EDL twitch tension (stimulation at 0.1 Hz) indicates response to physostigmine.

E Another 20-Hz, 10-second stimulus train during physostigmine response elicited EDL contraction pattern that resulted in a lower PSC value, indicating reduced ability to sustain high frequency contraction.

F About 1 hour PI, PSC was returning to pre-physostigmine value as EDL recovered its ability to sustain contraction.

F. VARIABILITY OF ULTRASTRUCTURE IN NORMAL NEUROMUSCULAR JUNCTIONS

In well-perfused muscles from sham-injected rats (diaphragm, soleus and EDL muscle, Figs. 11-17), 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 mm (Figs. 11a-17a). 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 mm (Figs. 11c-17c), while the myofibrils in the soleus were foreshortened to slightly less than rest length, or 2.1 mm (Figs. 11b-17b).

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. 12c, 15a, 18b, and 19a&b). (Similar images were also seen in control endplates following implantation of sham-filled mini-osmotic pumps, see Section K.4). In several endplates, junctional folds adjacent to some nerve branches were extremely foreshortened (Figs. 12c, 13b, and 15a) or appeared to be present without an adjacent nerve terminal profile (Figs. 11c, 15a, and 18a). 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. 16c). In other areas, junctional folds were associated with small-diameter nerve processes (Figs. 11c, 18b), similar to the "collateral sprouts" seen during nerve re-growth and associated with endplate repair and remodeling. Finally, some normal endplates exhibited clusters of axon terminals (Figs. 15a, 16d). These observations are in contrast to those of Hudson, et al. (28-30), who reported a) Schwann cell fingers between nerve and muscle <u>only</u> in anti-ChE-treated tissue and b) junctional folds apparently devoid of nerve terminals <u>only</u> 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.

G. ARTIFACTS OF FIXATION

1. Artifacts of Inadequate or Poor Perfusion

We have investigated several methods for transcardiac (i.e., ventricular) perfusion. When conventional thoracotomy techniques are used, the vasculature supplying the diaphragm is often damaged during surgery, thereby yielding a higher probability of poor perfusion. The resulting artifacts may be misinterpreted as drug effects. Such poor perfusions usually are characterized by the presence of numerous erythrocytes in most capillaries (Fig. 20a) and by the presence of characteristic swollen or exploded mitochondria in all areas of the myofibers including extrajunctional regions (Fig. 20b), in the nerve terminals (Fig. 20c), in their myelinated axons, and in adjacent non-contractile cells (e.g., Schwann cells and fibroblasts, Figs. 12c, 17c, 20b). (For clarification of mitochondrial descriptive terminology, see Section G.2., below.) We have also documented that mitochondria with normal condensed matrices interrupted at irregular intervals by abrupt lateral swellings (Fig. 20b,c) represent identifiable artifacts of poor perfusion. As artifacts, these laterally distended mitochondria are present in limited areas even in the highest rated perfusions, and are now known to result in part because of systemic (metabolic) hypoxia, but are also increased substantially by hypoxia arising from the oxygen-requiring reactions of glutaraldehyde fixation chemistry (34). In severe cases of such fixation hypoxia, mitochondrial swelling and swelling of sarcoplasmic reticulum superficially resemble the effects of acute physostigmine exposure. Thus, criteria have been identified which allow these and similar artifacts of poor fixation to be distinguished from drug effects. (See RESULTS Section G.2.)

2. Artifacts of Immersion Fixation

To duplicate common artifacts of glutaraldehyde fixation, an extreme case of metabolic hypoxia was induced in central fibers by immersion-fixation of the muscle (Fig. 21). Mitochondria throughout the myofiber sarcoplasm (not just the endplate region), as well as mitochondria in non-contractile cells (Schwann cells and fibroblasts) had extended regions of condensed matrix material and regularly spaced cristae. However, they also exhibited numerous large dilations and distinctive lateral swellings (Fig. 21, arrowhead) not normally seen in most perfusion-fixed myofibers. Moreover, in some immersion-fixed fibers, the sarcoplasmic reticulum was distended into a discontinuous series of clear vesicles. If unrecognized, this characteristic artifact of hypoxic fixation may be attributed improperly to drug effect, and thereby complicate the analysis of the primary and secondary effects of physostigmine toxicity. Consequently, the pronounced artifacts of hypoxia (which invariably occur during fixation by immersion in glutaraldehyde) make immersion fixation unacceptable in the analysis of anti-ChE effects on cell ultrastructure. Moreover, immersion fixation in glutaraldehyde cannot be (and was not) used as a "backup" protocol when perfusion fixations fail(ed). (Ultrastructural

alterations with increasing depth of immersion fixation are described in greater detail in Lee, et al. [32].)

Figure 11. 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. Because of the position of the hind limbs during perfusion fixation, the respective 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 (Figure 11c, arrow. Also see Figures 69a, 18a,c, and 19c). Nerve terminals have numerous synaptic vesicles and either normal or slightly distended mitochondria. Mitochondria in control endplates contain numerous calcium phosphate granules (Figure 11a, inset). In addition, about 30% of normal endplates exhibit swollen mitochondria, presumably reflecting artifacts of fixation resulting from normal variability in fibers stimulated during and/or by glutaraldehyde fixation.

The non-synaptic surface of the nerve terminal is covered by cytoplasmic processes of Schwann cells (*). Occasionally, Schwann cell processes are within the primary synaptic cleft, interposed between the nerve terminal and the junctional folds (Figures 11b, 12c, 15, 16b, 68a,b and 69c). In all electron micrographs, scale bars represent 1 um.

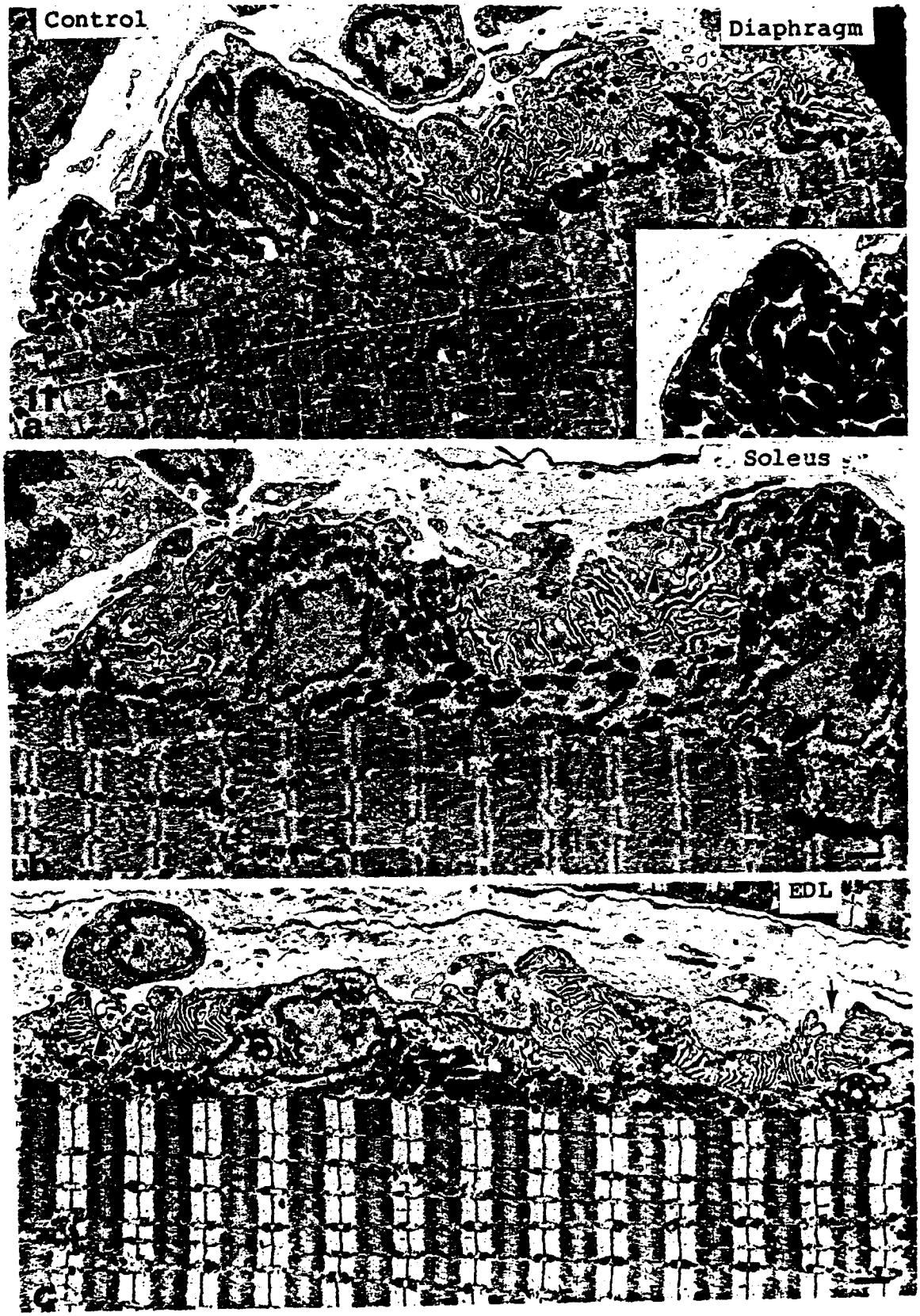

Control
Diaphragm
a
Soleus
b
EDL
c

Figure 12. 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 (swollen mitochondria in the nerve terminals of Figure 12b,c) reflect artifacts commonly observed after perfusion fixation. Although the perfusion fixation solutions were carefully oxygenated, they unfortunately carry inadequate oxygen both for the oxygen-requiring chemical reactions of glutaraldehyde fixation and for the metabolic requirements of active tissues, such as nerve and muscle.

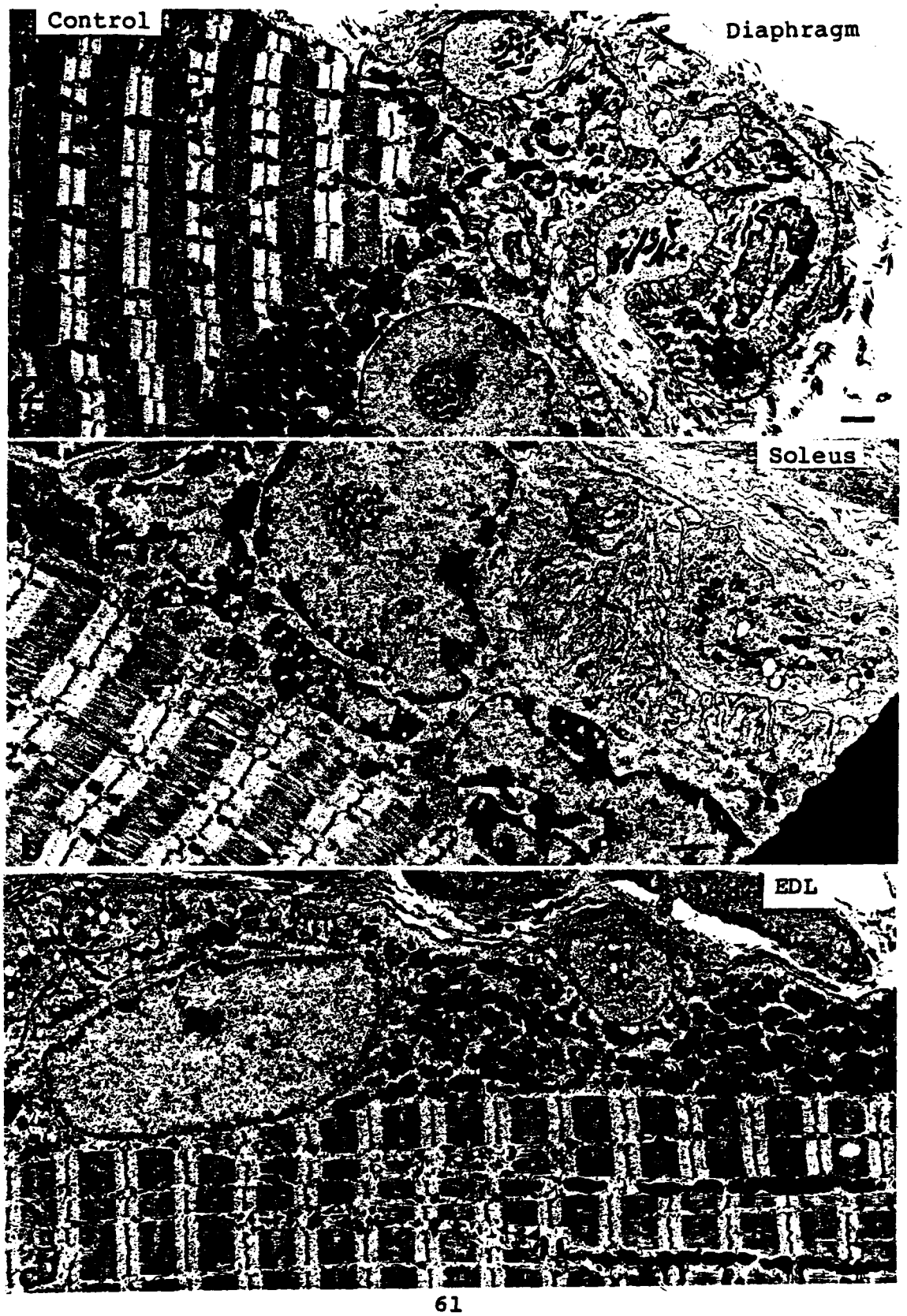

Control
Diaphragm
Soleus
EDL

Figure 13. 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 Figure 13b, arrows) and some from normal variability of endplate ultrastructure (Figure 13b, arrowhead, and at higher magnification in Figure 18d). For interpretive drawing, see Figure 19c.

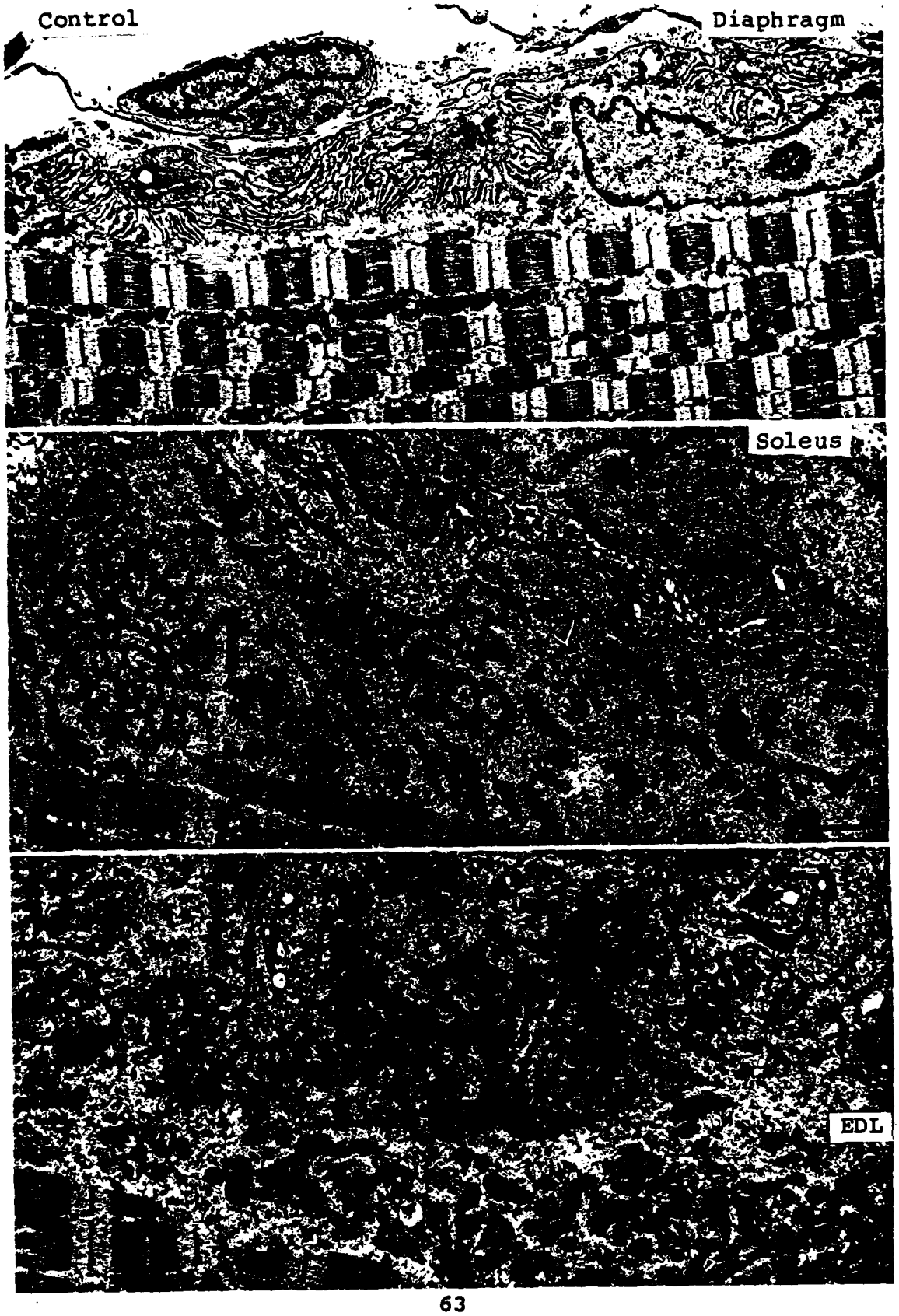

Control
Diaphragm
Soleus
EDL

Figure 14. Comparison of Neuromuscular Junctions from Diaphragm, Soleus,, and EDL muscles 14 Days After Sham Injection (Control). Misalignment of sarcomeres (Figure 14a) and occasional swollen mitochondria, especially in Schwann cells, reflect inadequacies of chemical fixation. A portion of Figure 14a (arrowhead) is shown at higher magnification in Figure 15.

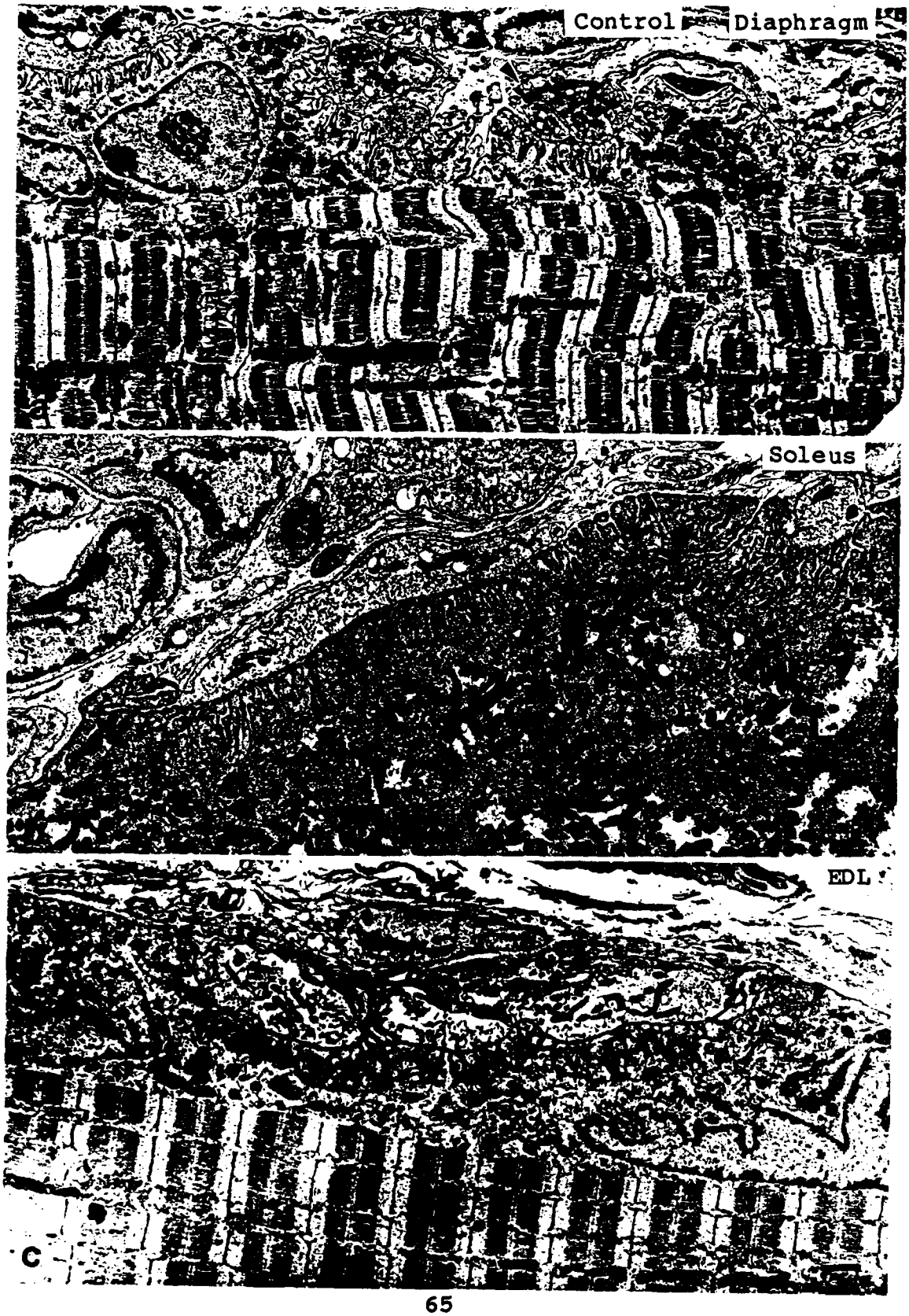

Control
Diaphragm
Soleus
EDL
C

Figure 15. Higher Magnification Image from Figure 14a. The single lateral swellings of the presynaptic mitochondria are characteristic of hypoxia during fixation, while the multiple "blisters" seen following prolonged nerve stimulation (Figures 92,93) are diagnostic for and proportional to the amount of prolonged nerve activity.


Diaphragm
a

Figure 16. Comparison of Neuromuscular Junctions from Diaphragm, Soleus,, and EDL muscles 28 Days after Sham Injection (control). Note minor variations in endplate preservation.

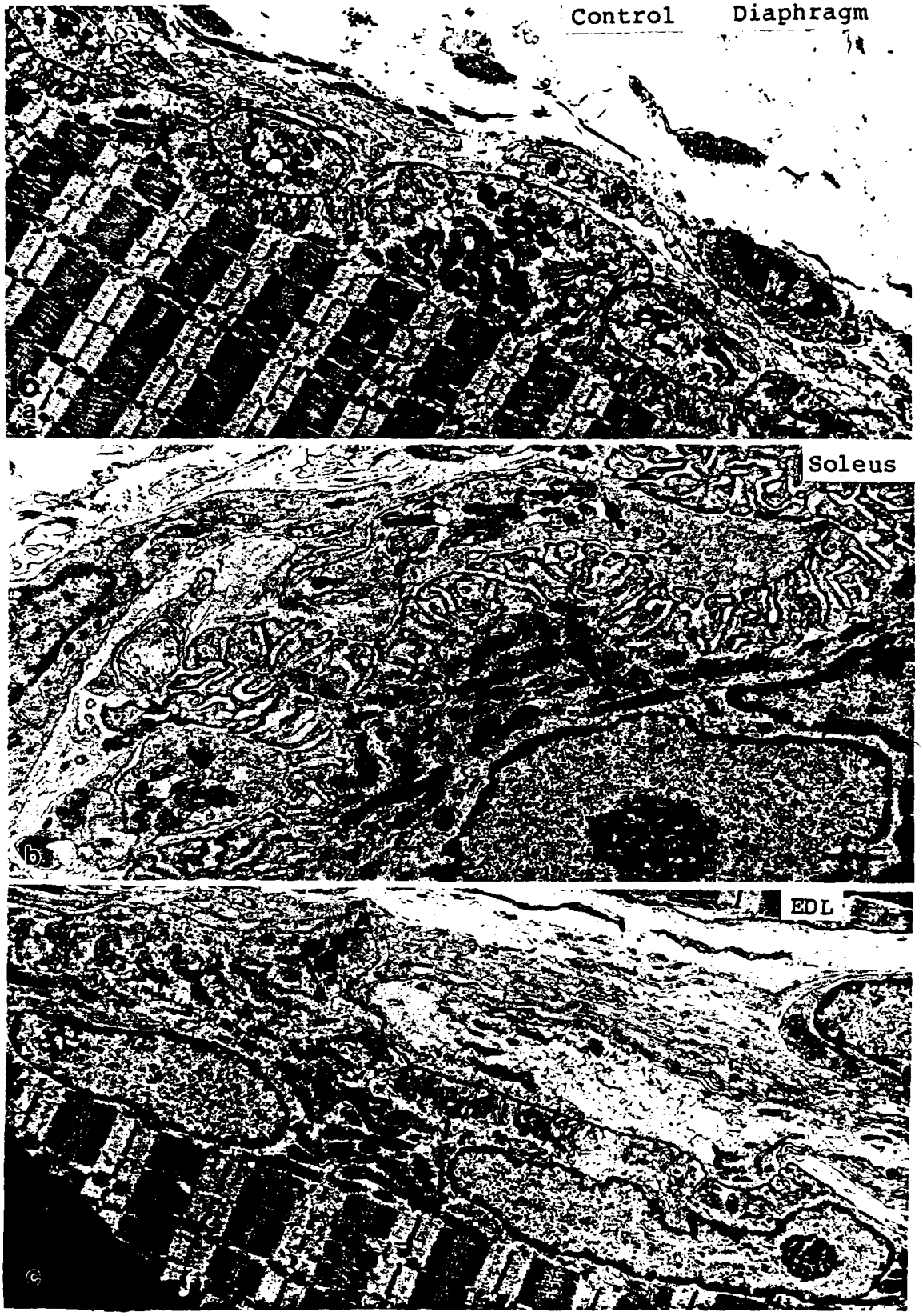

Control
Diaphragm
a
Soleus
b
EDL
c

Figure 17. Comparison of Neuromuscular Junctions from Diaphragm, Soleus,, and EDL muscles 56 Days After Sham Injection (Control). Note hypoxic mitochondria in Figure 17a,c.

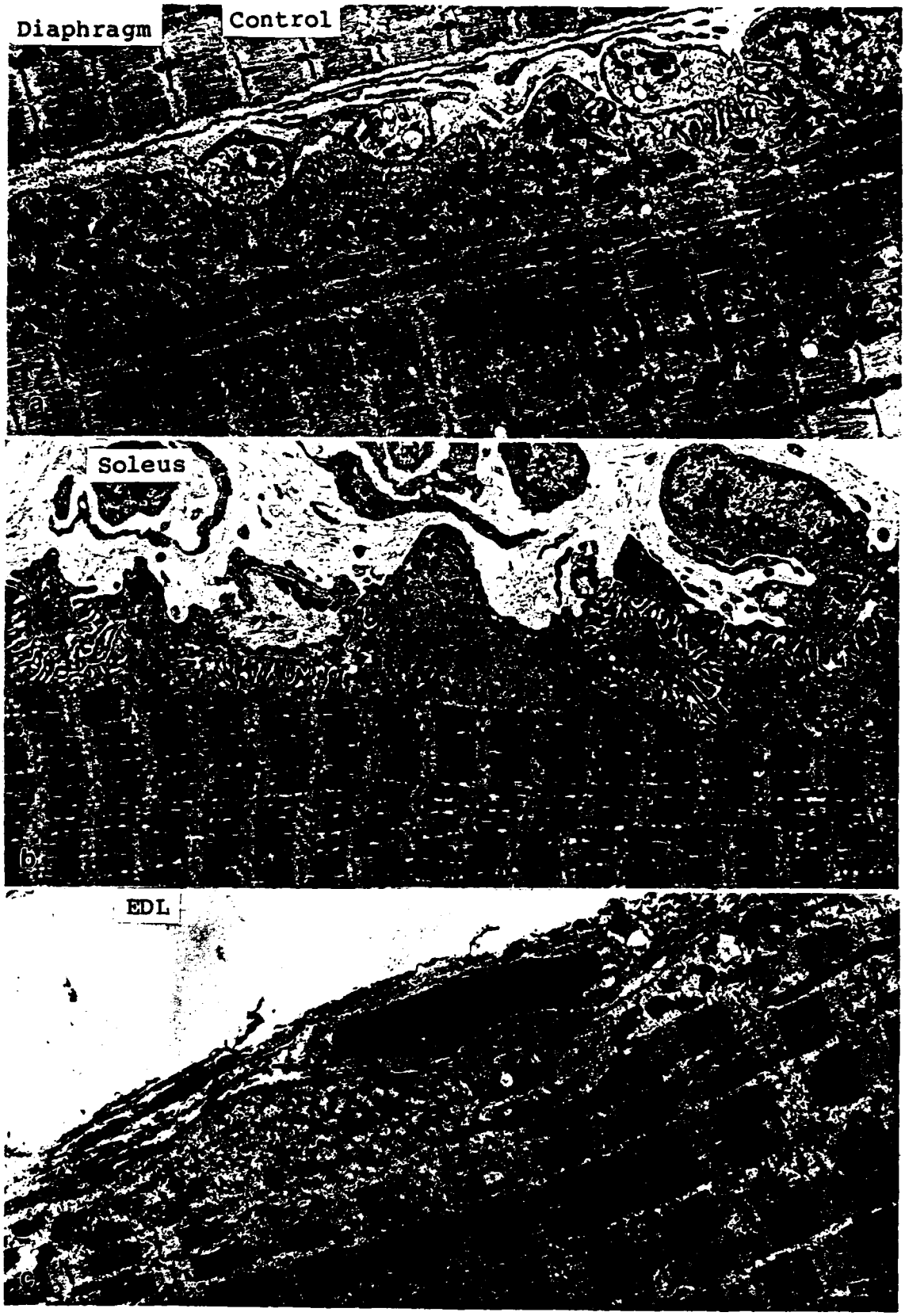

Diaphragm
Control
Soleus
EDL

Figure 18. Atypical Variations Seen in Nerve Terminals of Sham-injected Rats. Junctional folds are seen without (apparent) association with axon terminals (Figure 18a,c, arrowheads). In some regions, the depth of junctional infoldings is less than 1/2 um (Figure 18d, arrow), while in other areas, the near tangential plane of section causes junctional folds to appear to be several micrometers in depth (Figure 18b, arrow).

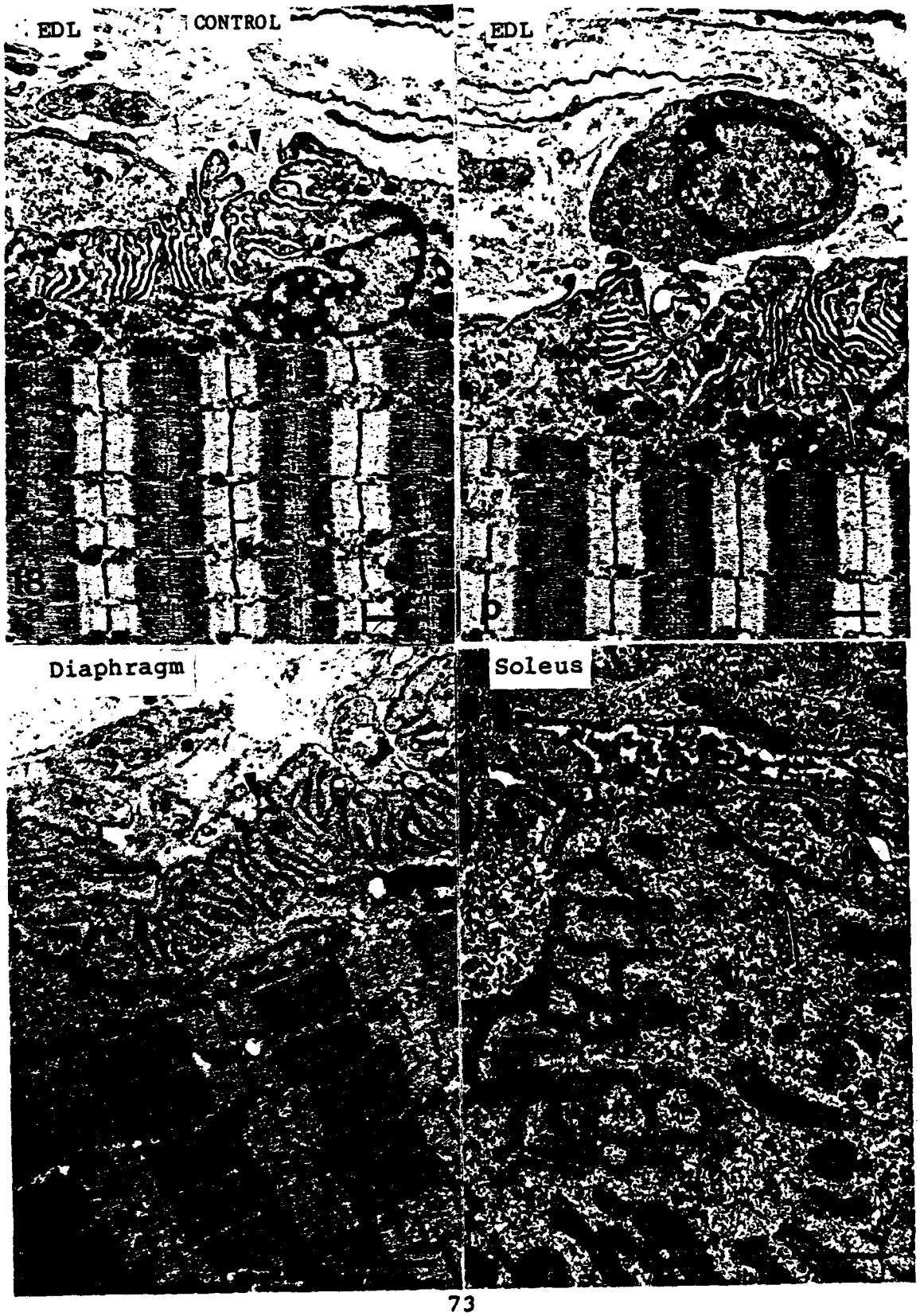
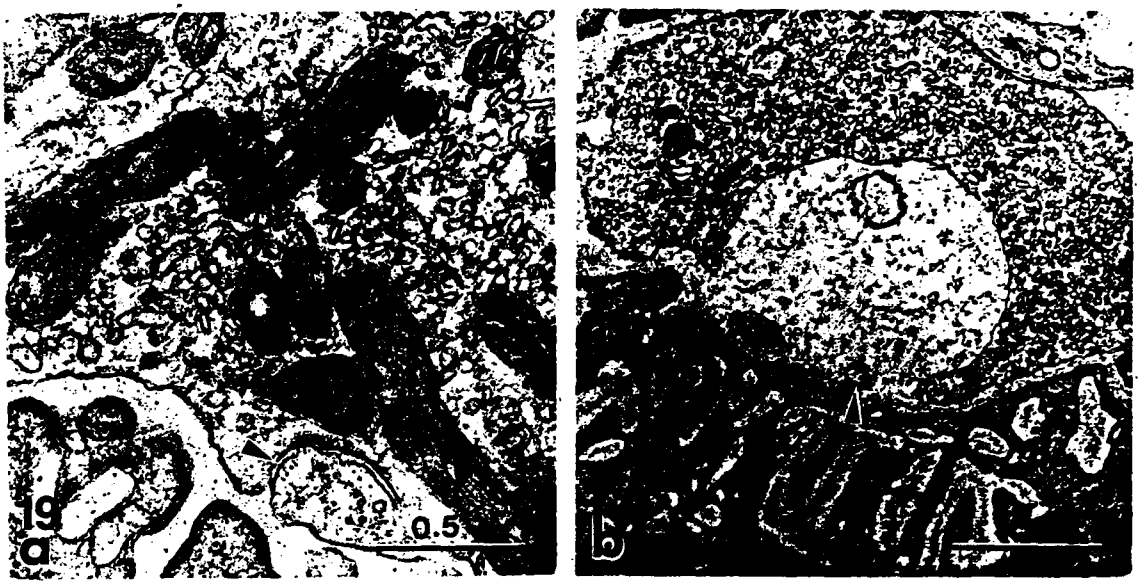

EDL
CONTROL
EDL
Diaphragm
Soleus

Figure 19. Variations Seen in Nerve Terminals of Control Muscle, Including Plane-of-Section Artifacts; and Diagram Illustrating How Such Images Arise. In normal nerve terminals, Schwann cell fingers (Figures 19a,b, arrowheads) are frequently observed interposed between junctional folds and the nerve terminal plasma membrane. Likewise, normal nerve terminals are occasionally found to be widely separated from the junctional folds (Figure 19b). Finally, small extensions of nerve terminal [Figure 19b, (*)] may resemble Schwann cell fingers, especially if they are locally devoid of synaptic vesicles. Figure 19c is a diagram illustrating sources of several plane-of-section artifacts, including images of nerve terminals that appear to be widely separated from the junctional folds, junctional folds that appear to be present without an associated nerve terminal profile (large arrow), and nerves opposite regions apparently devoid of junctional folds (profile on right side). (See also Figure 18a-d).

CONTROL



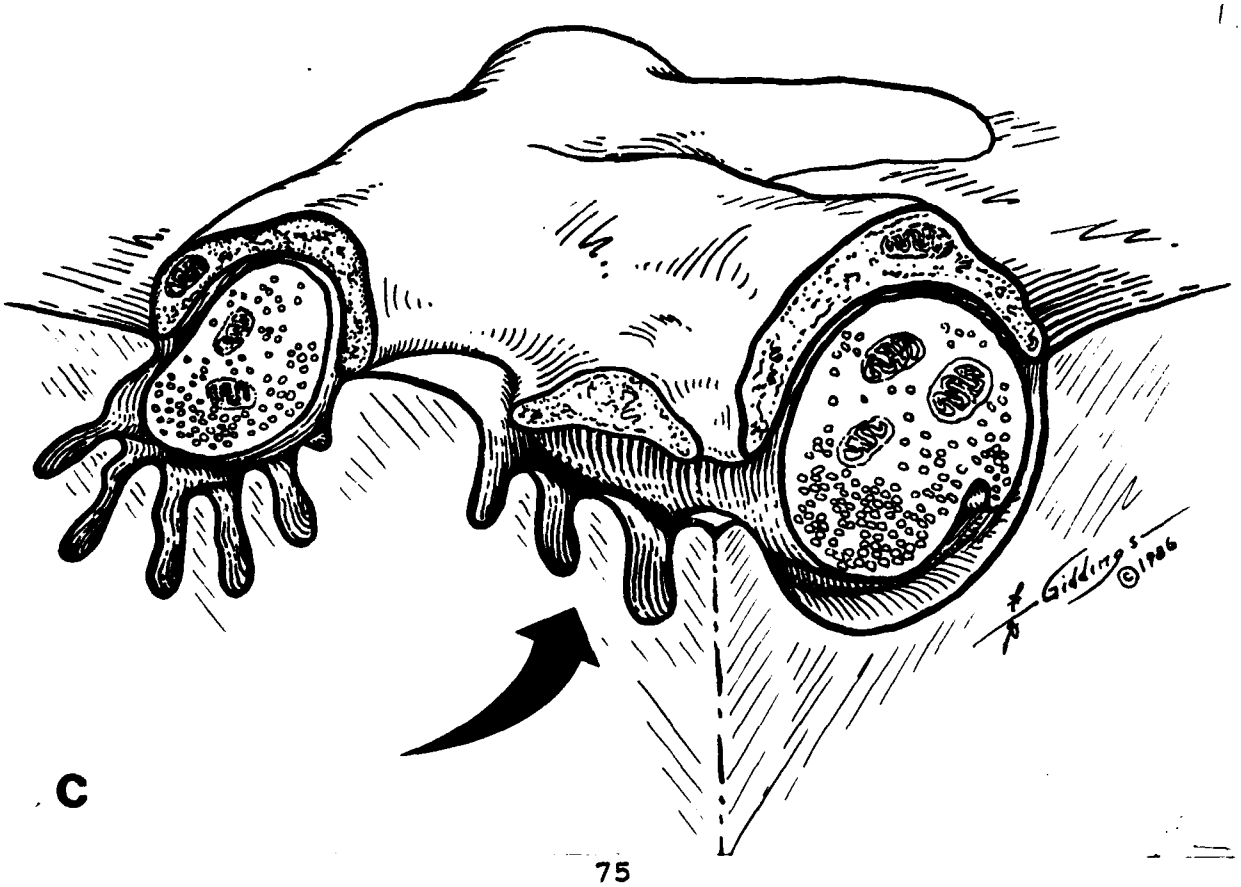


Figure 20. Artifacts of Perfusion Fixation. Poor perfusions are characterized by the presence of numerous erythrocytes in many capillaries (Figure 20a) and by the presence of characteristic swollen or exploded mitochondria in all areas of the myofibers, in the nerve terminals, and in their myelinated axons (Figure 20b, arrowheads). These markers are useful in confirming the existence of structural alterations caused by physostigmine vs. those resulting from fixation artifacts.

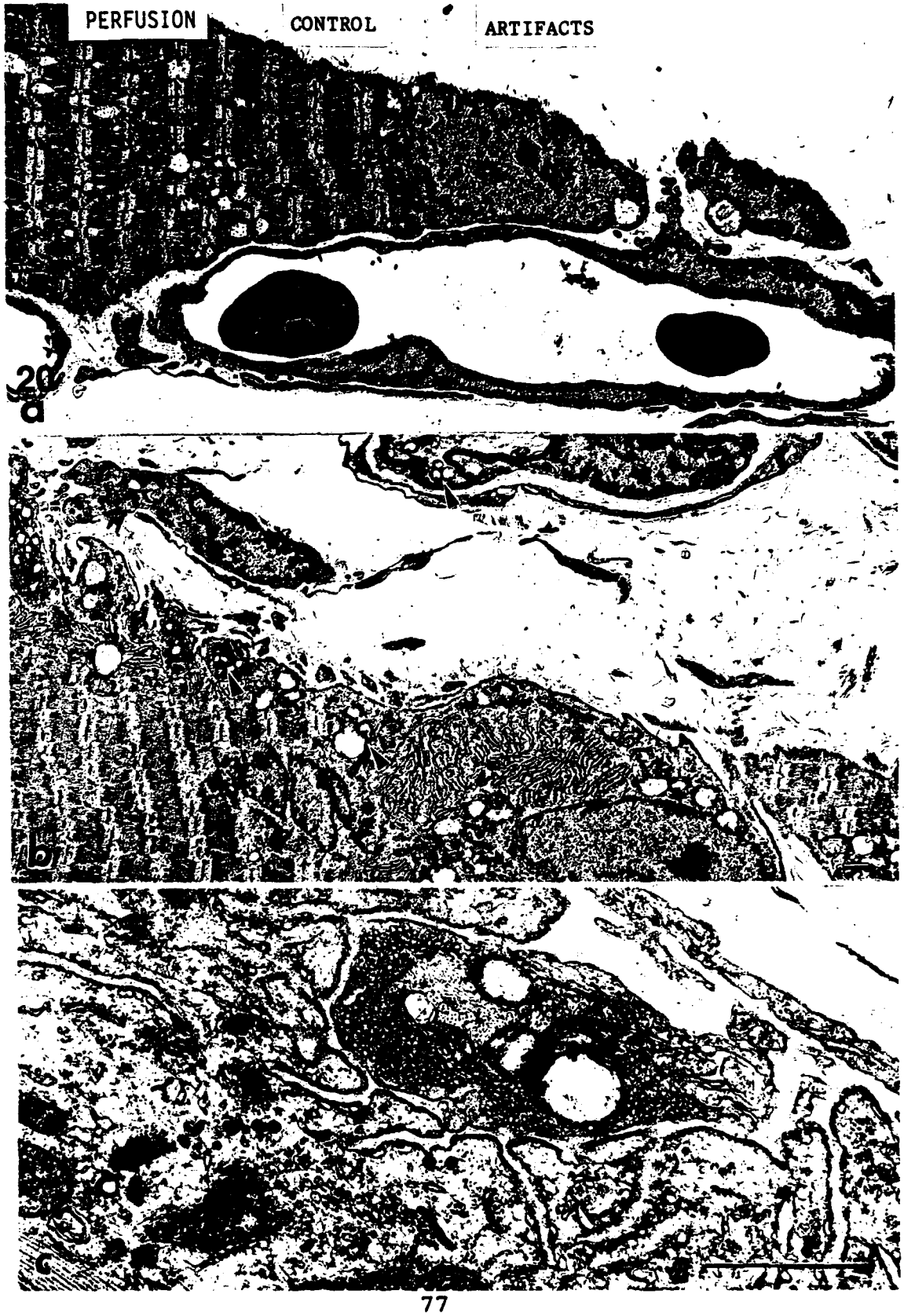

PERFUSION
CONTROL
ARTIFACTS
a
b
c

Figure 21. Artifacts of Immersion Fixation. In all but the outermost fibers in immersion-fixed control muscles, many swollen or exploded mitochondria are seen in all areas of the myofibers and in the nerve terminals (arrowheads). Some swollen cisternae of sarcoplasmic reticulum are also seen. These artifacts of immersion fixation resemble some of the ultrastructural changes sometimes attributed to physostigmine. Thus, immersion fixation cannot be used for characterizing anti-ChE toxicity, even as a backup procedure.

IMMERSION CONTROL ARTIFACTS

H. MUSCLE STRUCTURE AND ULTRASTRUCTURE AFTER ACUTE EXPOSURE TO PHYSOSTIGMINE

1. Effects of Acute High-Dose Exposure (0.8-1.1 LD_{50})

a. Observations by Light Microscopy

In thick plastic sections (0.5-1.0 um) of diaphragm and soleus muscles from rats fixed by perfusion 30 minutes after s.c. injection of a high dose of physostigmine (0.8-1.1 LD_{50}), supercontractions were easily recognized in the subjunctional sarcoplasms (Figs. 22a-b). Such areas were devoid of the regular cross-striated patterns observed in adjacent nonjunctional regions. Clear or refractile areas in the subjunctional sarcoplasm and in the nerve terminals were observed (Fig. 22a-c, arrow), but required electron microscopic analysis to ascertain that they represented extremely swollen mitochondria (see Section G.3, below). In the EDL muscle (Fig. 22c), no supercontraction was observed, but small clear areas (later identified by electron microscopy as slightly swollen mitochondria) were seen in the subjunctional sarcoplasm. It was not possible to determine by light microscopy whether myofibrils in the supercontracted area were severely damaged, whether the sarcoplasmic reticulum and/or the mitochondria were swollen, or whether the nerve terminals were degenerated. Thus, in the remainder of this study, electron micrographs were utilized because they exhibit much finer detail, even at magnifications equivalent to those used in light microscopy (i.e., 400x-2000x).

b. Ultrastructural Analysis of Post-synaptic Alterations

Electron microscopic examination of myofibers fixed by perfusion fixation 30 minutes after injection of a single acute sub-lethal but high-dose of physostigmine (0.8-1.1 LD_{50} or 0.6-0.8 mg/kg) revealed a characteristic lesion of the neuromuscular junctions of all frequently used myofibers. Diaphragm fibers were rapidly, consistently, and grossly affected (Figs. 23a, 24); soleus fibers were somewhat less affected (Figs. 23b, 25); and EDL fibers were consistently the least affected (Figs. 23c, 26). All (10 of 10) neuromuscular junctions of diaphragm myofibers (Figs. 23a, 24, including both fast twitch and slow twitch fibers) and all neuromuscular junctions of soleus myofibers (6 of 6; Fig. 23b, slow twitch fibers only) exhibited supercontraction restricted to the subjunctional sarcoplasm. In contrast, only 1 of 6 EDL myofibers showed even a limited area of supercontraction (Figs. 23c and 26).

In regions of maximum supercontraction in the diaphragm and soleus muscles, partial or complete destruction of the myofibrillar apparatus was observed 30 minutes PI, including the disappearance of Z-bands (Figs. 24, 25, 27) and the dissolution and/or disorganization of recognizable sarcomeres. Moreover, a continuum of severely to slightly damaged mitochondria was observed in the subjunctional sarcoplasm (Figs. 24, 25). The most severely affected mitochondria (designated as "swollen" and "exploded," see Figs. 27, 28) were immediately adjacent to the junctional folds; less affected (or

"frothy") mitochondria were at a greater distance; the least affected mitochondria ("blistered" mitochondria and those enriched in calcium phosphate granules, called "granular") were at the margins of the supercontracted areas. (We define a blistered mitochondrion as one that has smooth, regular margins but whose matrix has only one or only a few scattered "bubbles" or lens-like areas of low electron density. No obvious lateral swellings are observed in blistered mitochondria, clearly distinguishing them from the "laterally distended" mitochondria of hypoxic myofibers. "Frothy" mitochondria are those with numerous blisters that do not distend the surface contour. "Swollen" mitochondria are grossly distended, often with irregular surface contours, separations of inner and outer mitochondrial membranes, and occasional internal saccules. The most extreme alterations seen in this study are "exploded" mitochondria, which have obvious discontinuities in their external membranes, and are recognizable as mitochondria only because of the presence of double limiting membranes and/or the presence of widely separated but identifiable cristae.)

From these images, we conclude that high-dose exposures to physostigmine (i.e., 0.8 LD_{50} or 0.6 mg/kg) produce a characteristic lesion -- concentric hemispheres surrounding the endplate region, each successive zone toward the endplate exhibiting increased damage to sarcomeres, mitochondria, and sarcoplasmic reticulum. Starting from the unaffected nonjunctional cytoplasm and terminating at the junctional folds, this apparent continuum of mitochondrial changes associated with severe endplate depolarization includes the following sequence: granular mitochondria --> blistered mitochondria --> frothy mitochondria --> swollen mitochondria --> exploded mitochondria. Supercontraction of subjunctional sarcomeres and the associated formation of exploded, swollen, frothy, and blistered subjunctional mitochondria were thus confirmed as the most obvious markers for the characteristic toxic lesion of the neuromuscular junction produced by this anti-ChE agent.

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 were seen under hypoxic conditions (Fig. 13b, c). 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). Moreover, in the absence of drug, frothy mitochondria are never observed under hypoxic conditions. 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 and/or glutaraldehyde fixation. (See Section N for a description of alterations induced by prolonged stimulation in the absence of drug.)

In the soleus muscle (Figs. 25, 27), mitochondria were somewhat less affected than endplates in the diaphragm. In contrast, EDL fibers (Fig. 26), which were the least affected at

½ hour, exhibited only blistered and granular mitochondria, distributed throughout the subjunctional cytoplasm (Fig. 28d). Many EDL fibers revealed little or no changes in ultrastructure. In only 1 of 6 EDL neuromuscular junction regions were the myofibrils even partially misaligned (Fig. 26). However, slight to moderate swelling of post-synaptic mitochondria was observed (Fig. 26b), with a few subsynaptic mitochondria exhibiting internal blisters characteristic of the first recognizable stage in endplate damage.

c. Ultrastructural Analysis of Pre-synaptic Alterations

At high doses of physostigmine, changes in nerve terminals were also observed. When compared to the normal variability in mitochondrial preservation in control endplates (Figs. 8-14 and 29a), mitochondria in the nerve terminals from rats exposed to 0.8 LD_{50} of physostigmine were swollen and distorted (Figs. 29b-d). (By comparison, however, the postsynaptic mitochondria were much more severely affected. See Figs. 28a-c.) 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 (for review of neuromuscular transmission, see 55). Thus, we support in part the conclusion of Hudson (28-30) that nerve terminal alterations indicate the occurrence of high or sustained rates of nerve terminal depolarization, while the postsynaptic changes (including severe super-contraction restricted to subjunctional sarcomeres) indicate profound and prolonged sub-synaptic depolarization. However, during the ChE-induced depolarizing neuromuscular blockade (1), it is not yet clear whether increased presynaptic activity precedes, coincides with, or follows the failure of neuromuscular transmission, nor is it clear in this sequence when swelling of presynaptic mitochondria occurs.

2. Differential Effects on Fiber Type are Related to Muscle Activity Patterns

It has been suggested that because predominantly slow twitch muscles (e.g., soleus) are affected much more severely than predominantly fast twitch muscles (e.g., EDL), slow twitch myofibers are inherently much more susceptible to anti-ChE toxicity than fast twitch myofibers (17,24). However, since the diaphragm is a mixed muscle containing fast twitch, intermediate, and slow twitch myofibers, and since all three fiber types in the diaphragm were equally severely affected (Figs. 23a, 24), it is clear that the damaging effects of physostigmine are not restricted to any one fiber type in that muscle. Instead, the relative amount of damage in fast twitch vs slow twitch muscles appears to be related to muscle use patterns. (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 periodically but not constantly [because during the period of maximum drug effect, the rat supported much of its weight on its

abdomen, thereby temporarily reducing the amount of neuromuscular activity]; and the EDL muscles [which extend the digits during walking] were virtually unused because walking was minimal during the initial exposure period.) Moreover, ultrastructural changes in diaphragm myofibers immediately following acute high-dose exposures appear to reflect damage induced by the relatively continuous muscle activity required by respiration. This possibility is supported by the observation that at only slightly higher doses (1.1-1.5 LD_{50}), virtually all rats died, usually within 15 minutes, reflecting, in part, catastrophic failure of respiratory muscles. Diaphragm myofibers thus appear to provide the best model for analyzing ultrastructural alterations associated with one of the immediate life-threatening effects of physostigmine intoxication (i.e., blockage of respiration). To investigate the proposed synergistic effects of muscle use on the apparent threshold for anti-ChE toxicity, additional experiments involving prolonged stimulation of soleus and EDL muscles were conducted (Sections M, N).

3. Establishing The Threshold Dose for Producing Endplate Cytopathology in Normally Active Rats

To identify the dose level required to produce identifiable myofiber damage in diaphragm, soleus, or EDL muscles, rats were administered acute s.c. injections of physostigmine at 0.001, 0.1, 0.3, 0.6, and 0.8 LD_{50}. Titration of responses revealed an abrupt increase in ultrastructural alterations between 0.1 LD_{50} (67% $\pm$7% inhibition) to 0.3 LD_{50} (75% $\pm$5% inhibition) to 0.8 LD_{50} (89% $\pm$3% inhibition). At 0.1 LD_{50}, no supercontraction or other evidence for endplate cytopathology was observed. (See Section H.4, below.) At 0.3 LD_{50}, approximately 60% of diaphragm myofibers were supercontracted (Fig. 30), while soleus and EDL myofibers showed no evidence for supercontraction (Fig. 31). Consequently, using supercontraction and frothy mitochondria as the two morphological criteria for establishing the desired dose-response curve for physostigmine toxicity, it is apparent that there is a steep onset resembling a threshold for the development of cytopathology. In resting, non-stressed animals, threshold for cytopathology occurs at 0.3 LD_{50} or 75% ($\pm$5%) enzyme inhibition. However, threshold in soleus and EDL muscles varied according to indirectly elicited neuromuscular activity. In subsequent physiological experiments, the effect of muscle use on the threshold dose was examined, and a distinct positive synergism was detected (see Section N, below).

Figure 22. Light Micrographs Revealing the Effects of an Acute High Dose of Physostigmine (0.8-1.1 LD_{50}) on Neuromuscular Junctions of Diaphragm, Soleus, and EDL 30 Minutes PI. Supercontraction and disruption of myofibrils in the subjunctional sarcoplasm of diaphragm and soleus fibers are evident, as is mitochondrial swelling (arrowheads) in all three fiber types. However, at light-microscopic magnification and resolution, endplates are relatively difficult to identify, and resolution of pathological details is not possible.

LIGHT
MICROGRAPHS

HIGH DOSE

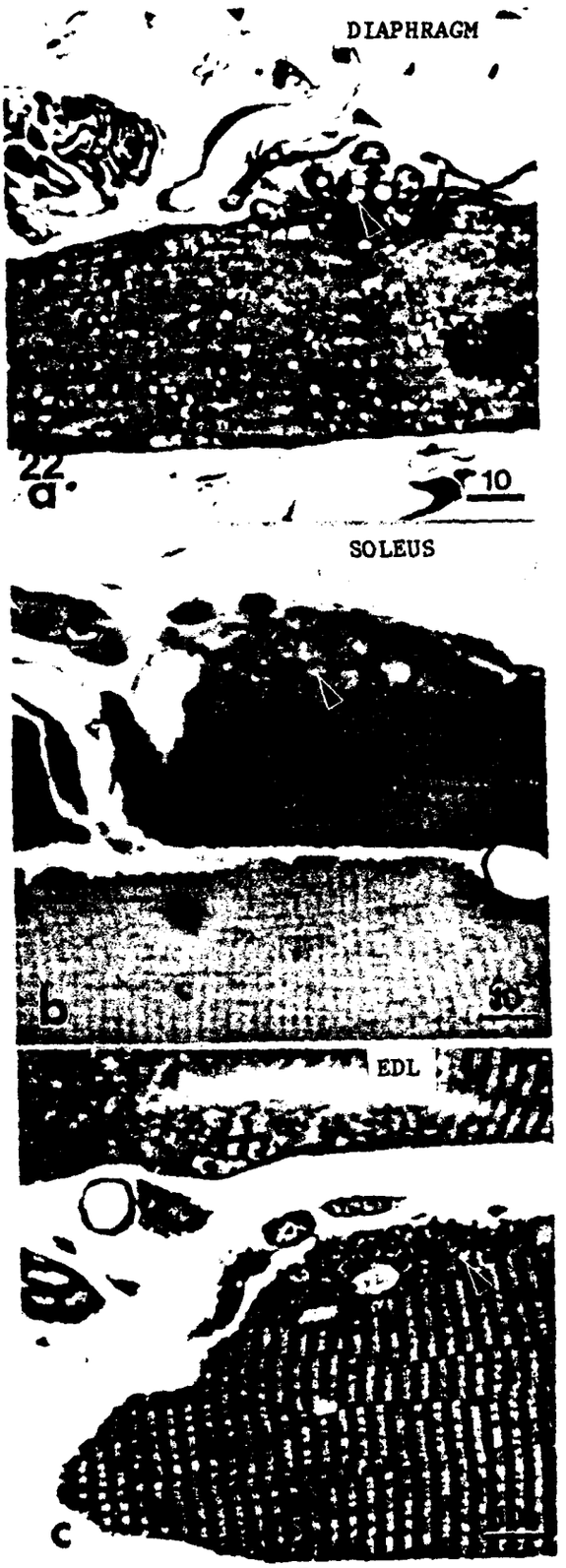


Figure 23. Comparison of the Effects of an Acute High Dose of Physostigmine (0.8-1.1 LD_{50}) on Neuromuscular Junctions of Diaphragm, Soleus, and EDL 30 Minutes PI. These three electron micrographs were taken from the same specimens used for light microscopy (Figures 22a-c). Structural alterations can be identified more quickly and visualized at much greater resolution by electron microscopy than by light microscopy. Supercontraction with associated exploded, swollen, frothy and blistered mitochondria (Figures 23a,b) provide unambiguous evidence for physostigmine toxicity at the neuromuscular junction of diaphragm and soleus muscle. EDL myofibers in unstressed rats were little affected at 30 minutes PI.

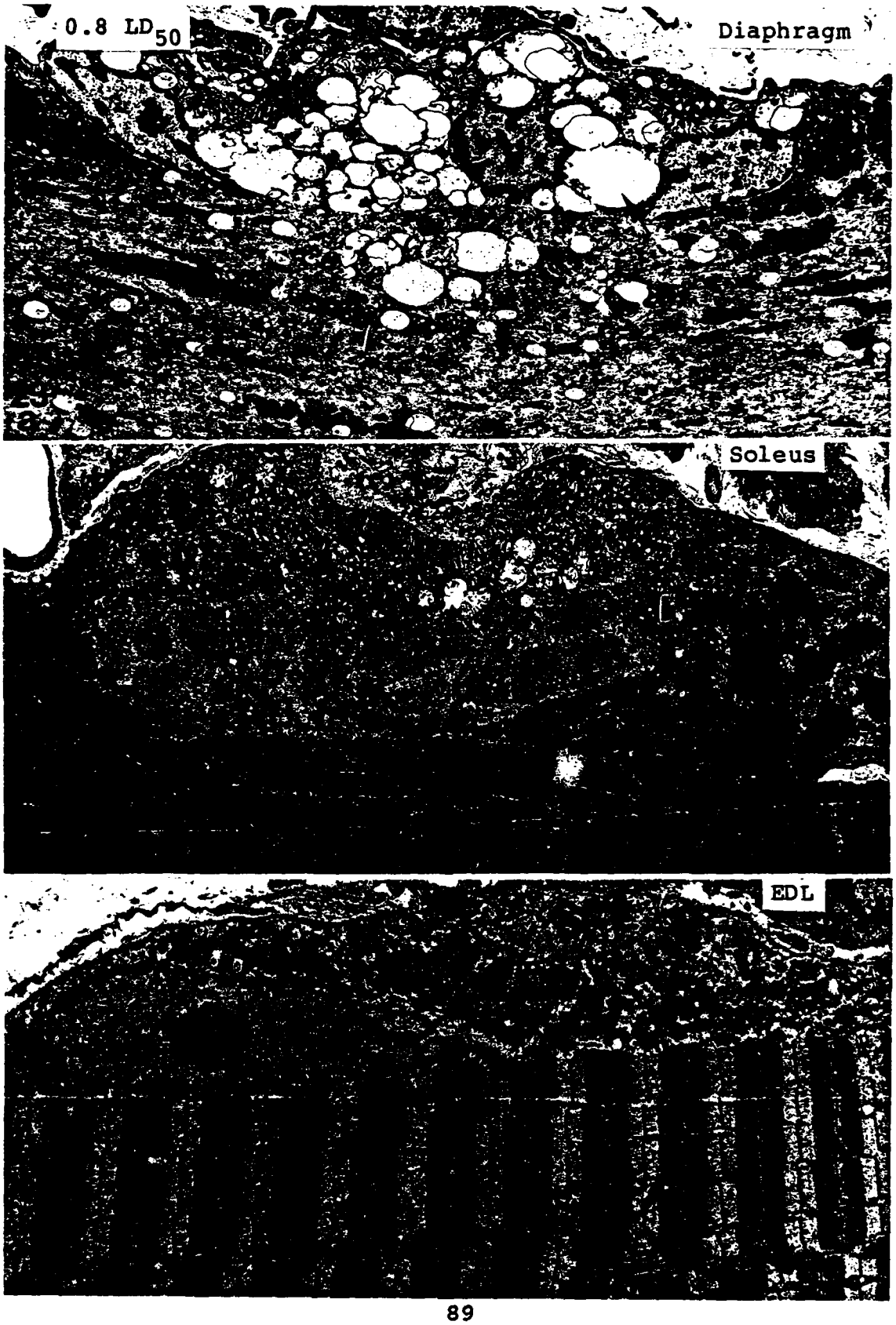

0.8 LD_{50}
Diaphragm
Soleus
EDL

Figure 24. Higher-magnification Images of the Effects of an Acute High Dose of Physostigmine (0.8-1.1 LD_{50}) on the Neuromuscular Junctions of Diaphragm Myofibers 30 Minutes PI. Supercontraction of subjunctional myofibrils was consistently observed in all neuromuscular junctions examined. However, the degree of supercontraction was highly variable. Exploded, swollen, and frothy mitochondria were present within, as well as just below, the raised endplate region. Swelling and vesiculation of the subjunctional sarcoplasmic reticulum was also evident.

HIGH DOSE

DIAPHRAGM

Figure 25. Effects of an Acute High Dose of Physostigmine (0.8-1.1 LD_{50}) on the Neuromuscular Junctions of the Soleus Muscle 30 Minutes PI. Supercontraction of subjunctional myofibrils was consistently observed in all junctions examined, but the extent and degree of supercontraction was somewhat variable (compare Fig. 25a with Fig 25b). In all cases, however, subjunctional mitochondria were found to be swollen, frothy, or blistered. Swelling and vesiculation of the sarcoplasmic reticulum was also evident in most myofibers.

HIGH DOSE

SOLEUS

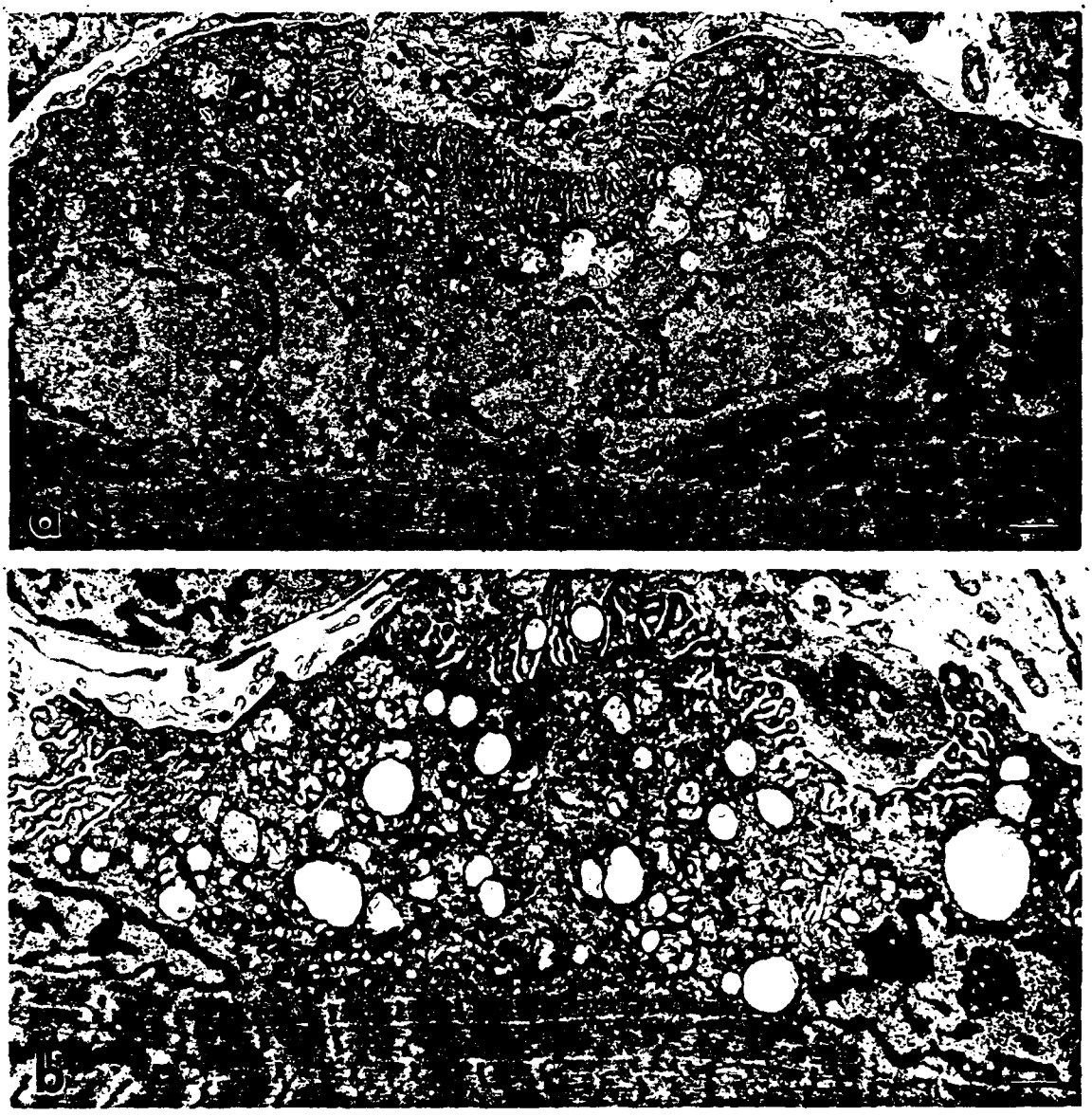


Figure 26. Effects of an Acute High Dose of Physostigmine (0.8-1.1 LD_{50}) on the Neuromuscular Junctions of EDL Muscles 30 Minutes PI. EDL endplates closely resembled those of control muscle. Very few fibers exhibited supercontraction of subjunctional myofibrils. In one instance (Fig. 26b, arrowheads), a partially damaged area of myofibrils (localized supercontraction) was present. Often subjunctional mitochondria were frothy (Figure 26a,b), but swollen or exploded mitochondria were seldom observed.

HIGH DOSE

EDL

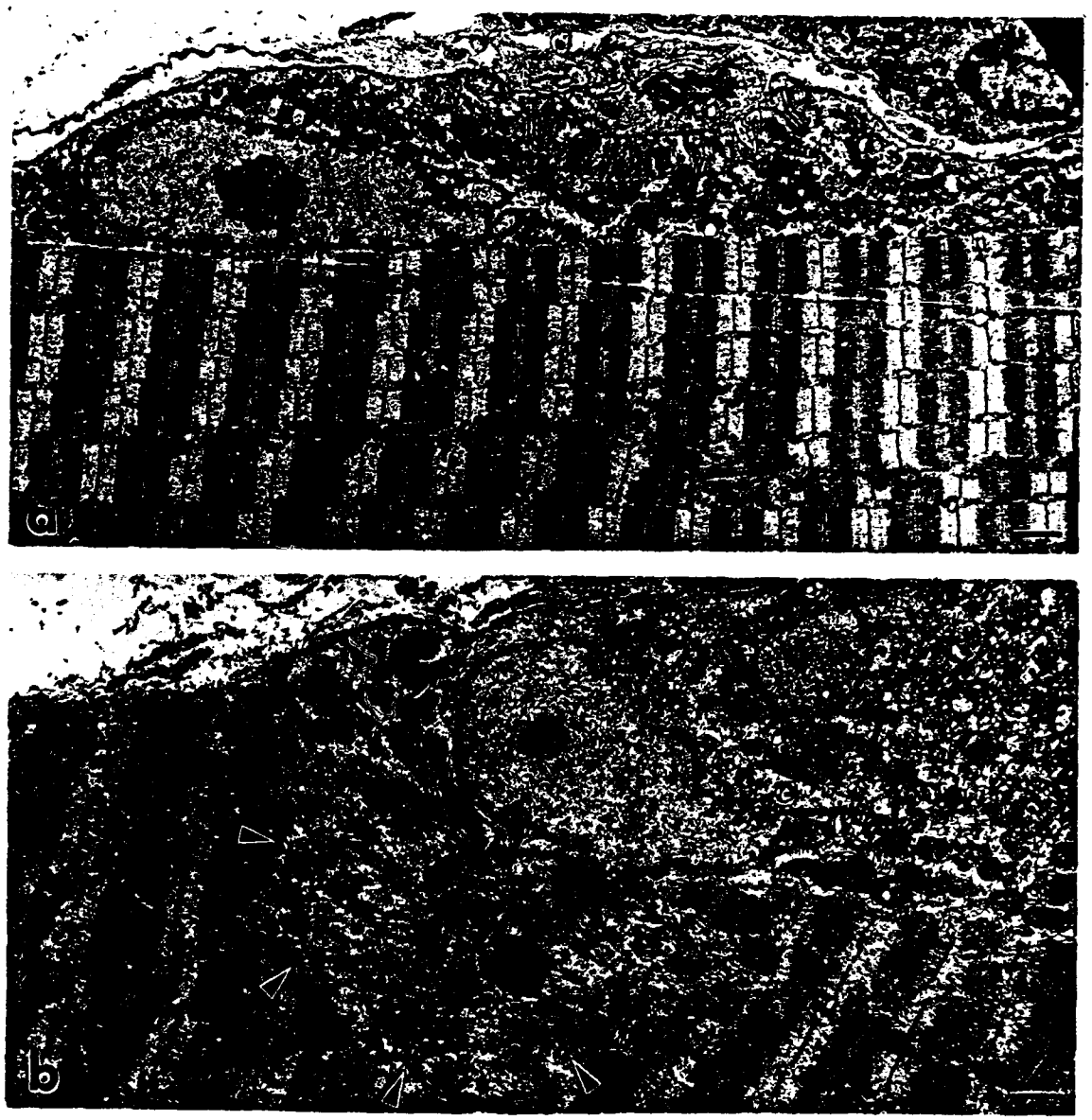


Figure 27. Broad Overview of the Effects of an Acute High Dose (0.8 LD_{50}) of Physostigmine on Ultrastructure of Soleus Neuromuscular Junctions. (Enlarged from Figure 25a.) Note the exploded mitochondria beneath junctional folds, as well as the frothy mitochondria at 2-10 um beneath the folds.

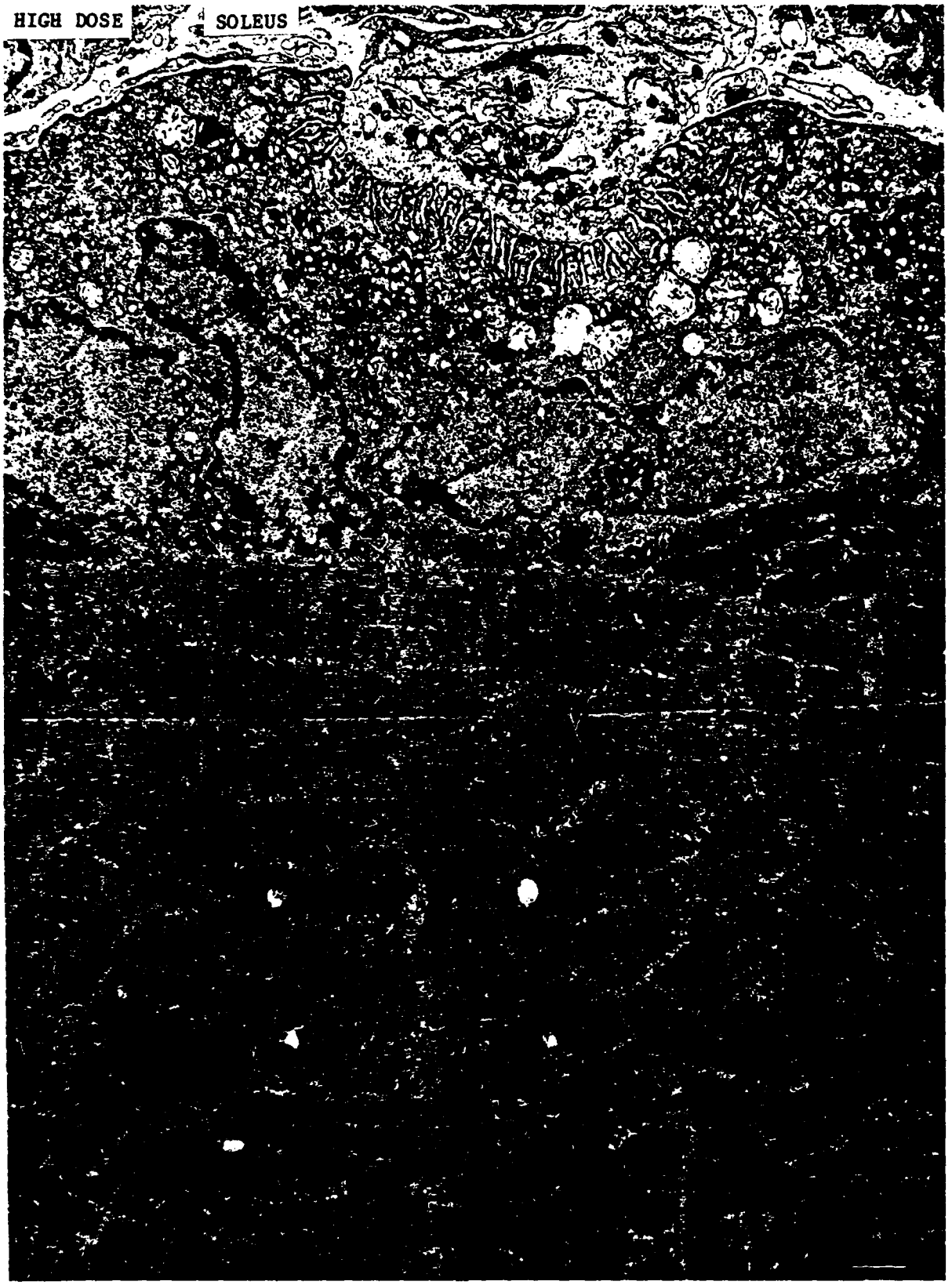

HIGH DOSE
SOLEUS

Figure 28. Comparison of the Effects of Sham Injection and High Acute Dose Exposure to Physostigmine (0.8-1.1 LD_{50}) on Endplate Mitochondria. Mitochondria in sham-injected animals appear normal. At 30 minutes after physostigmine injection, numerous extremely swollen mitochondria (S) are seen in the subjunctional sarcoplasm (Figures 28b,c). Sarcomere damage is evident. Frothy mitochondria (F) are also observed deeper in the subjunctional sarcoplasm. In soleus muscle (Figure 28c), swollen mitochondria are seen near the junctional folds, while frothy mitochondria appear at greater distances from the folds. Thus, the mitochondrial alterations are arranged in concentric layers, with progressively reduced damage at greater distances from the junctional folds. In the EDL muscle (Figure 28d), numerous frothy mitochondria and a few blistered or swollen mitochondria are seen, but are restricted to the subjunctional sarcoplasm. Presynaptic mitochondria were dilated or "vesicated," an observation consistent with prolonged presynaptic activity (cf. Section N and Figures 92, 93).

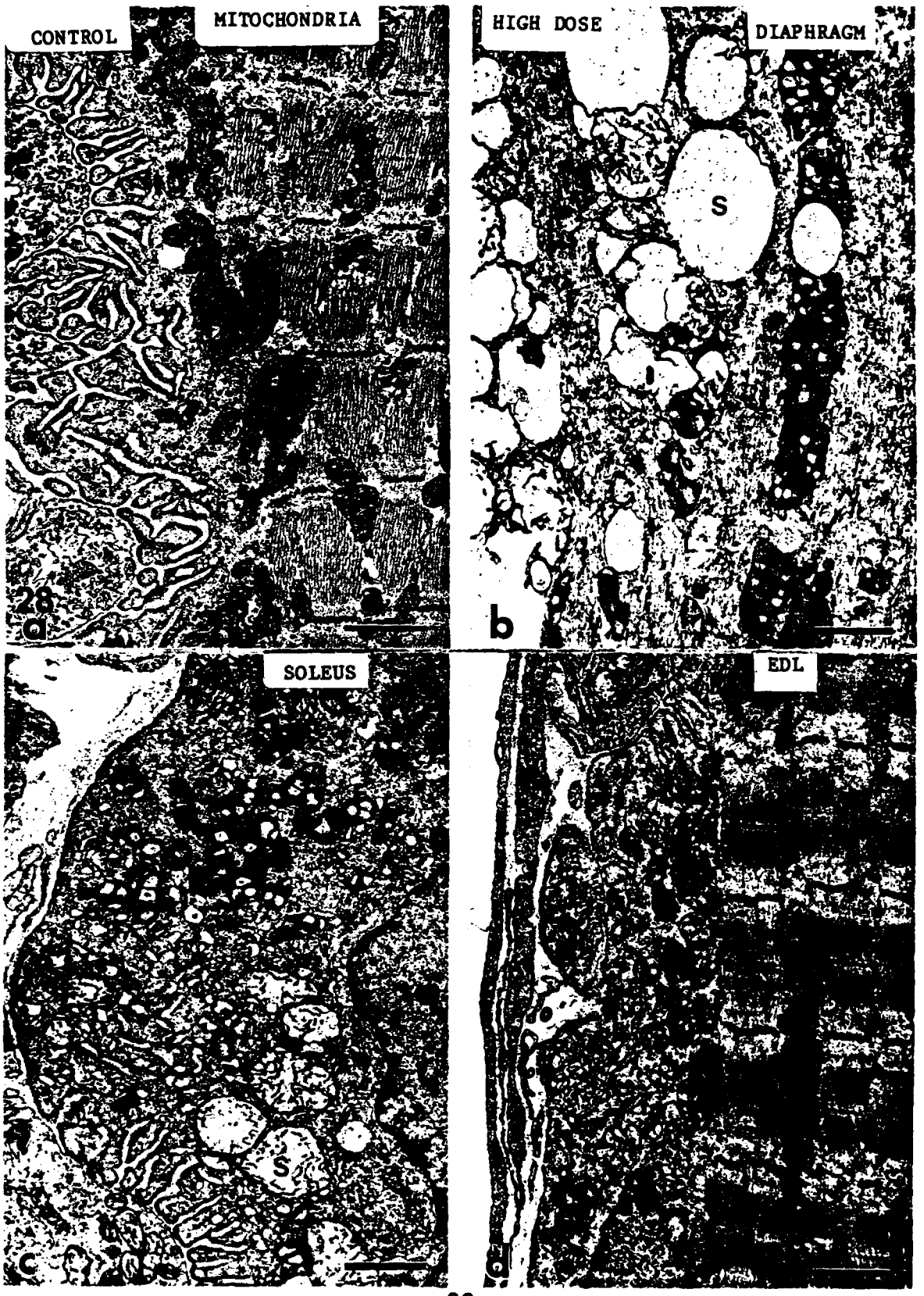

CONTROL
MITOCHONDRIA
a
HIGH DOSE
DIAPHRAGM
S
b
SOLEUS
S
c
EDL
d

Figure 29. Effects of an Acute High Dose of Physostigmine (0.8-1.1 LD_{50}) on Nerve Terminals of Diaphragm, Soleus, and EDL Muscles 30 Minutes PI. Figure 29a is from a control diaphragm. Mitochondria are characteristically dilated within the nerve terminals of the drug-treated muscles (cf. Figure 29b, arrowhead). Swollen mitochondria (S) are also seen in the control nerve terminal. Without additional criteria, it would be difficult to determine to what extent the swollen mitochondria in the drug-treated nerve terminals were due to primary drug effect, to artifact, or to secondary effect of hyperactivity. Thus, the reader is referred to Section N and Figures 92, 93.

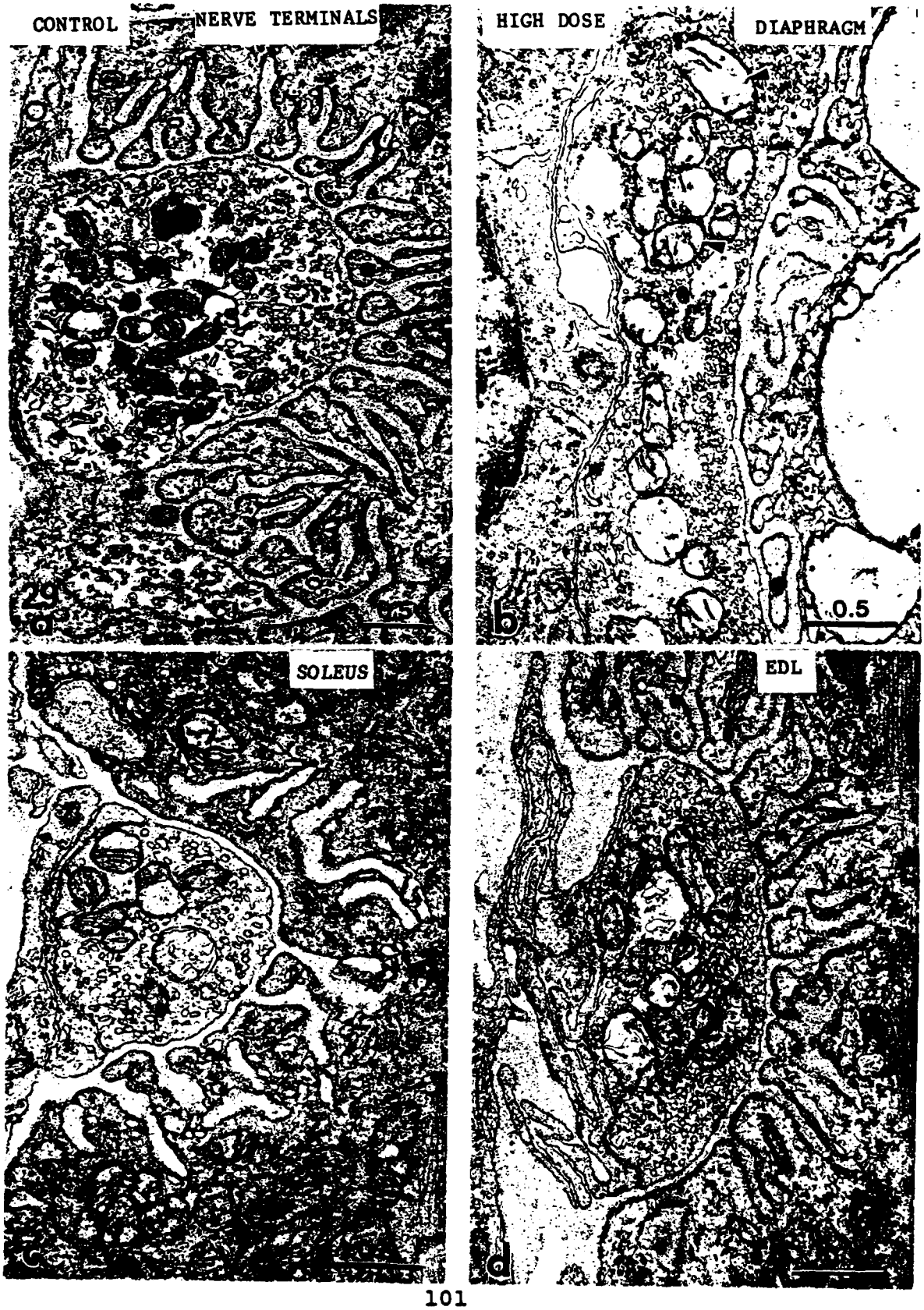

CONTROL
NERVE TERMINALS
HIGH DOSE
DIAPHRAGM
a
b
0.5
SOLEUS
EDL
c
d

Figure 30. Comparison of the Effects of an Acute Near-threshold Dose of Physostigmine (0.3 LD_{50}) on Neuromuscular Junctions of Rat Diaphragm Muscles 30 Minutes PI. Concentric hemispherical zones of decreasing destruction surround the neuromuscular junctions.

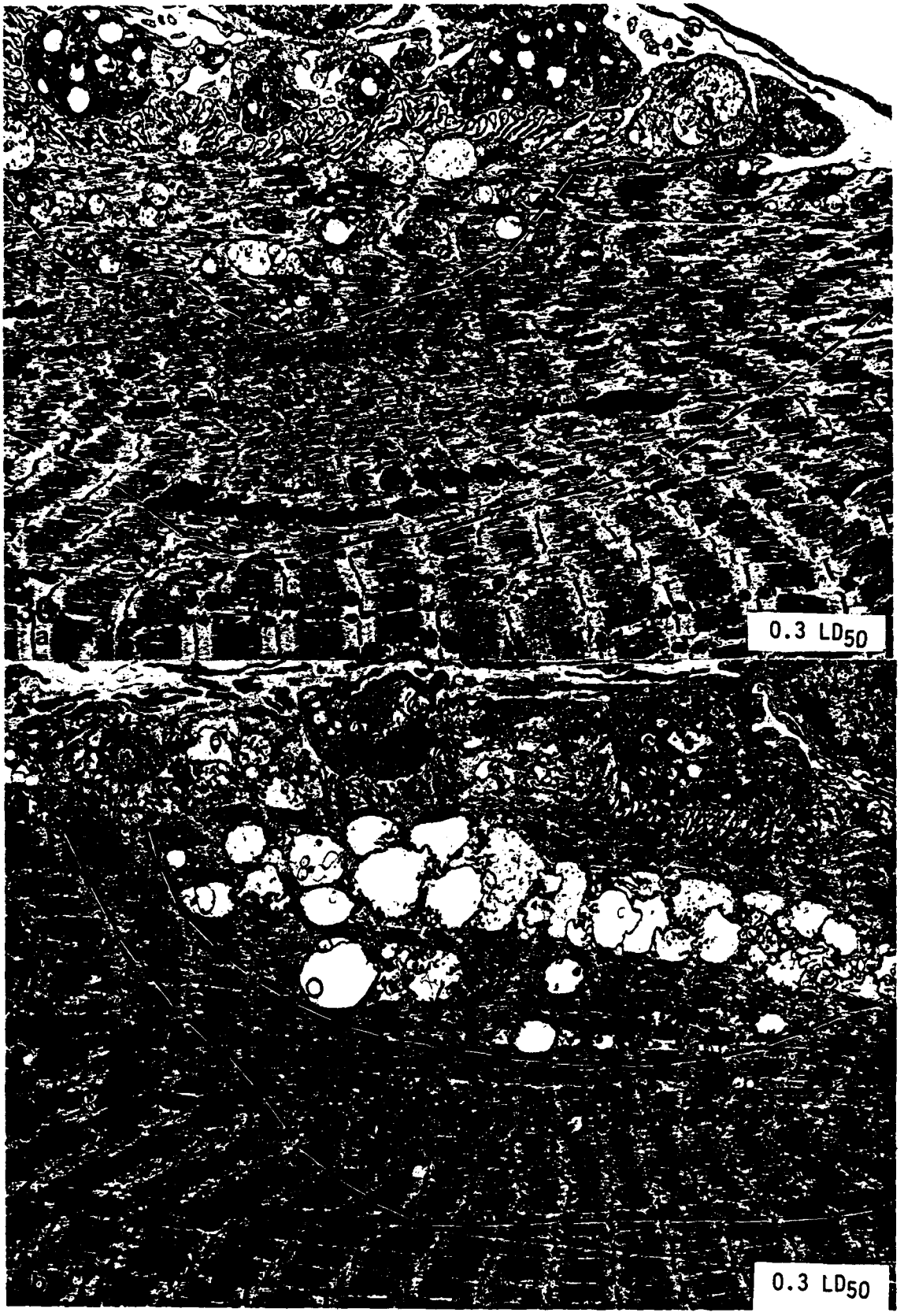
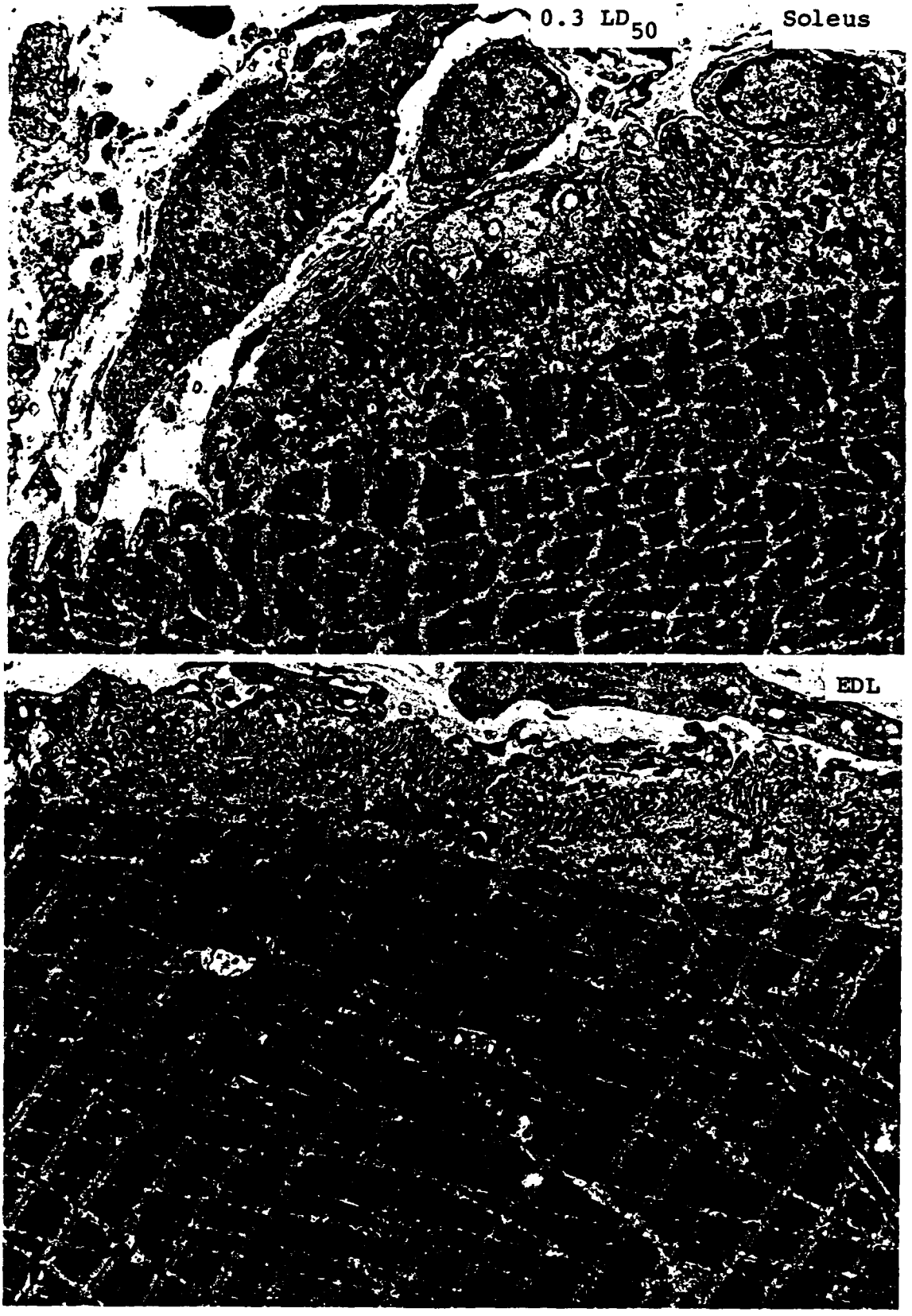

0.3 LD50
0.3 LD50

Figure 31. Variability of Effects of Acute Near-threshold Dose of Physostigmine (0.3 LD_{50}) on Rat Soleus and EDL Muscles 30 minutes PI. No obvious ultrastructural alterations are evident even though these muscles are from the same rat as illustrated in Figure 30, above.


0.3 LD_{50}
Soleus
EDL

4. Rat Neuromuscular Junctions After an Acute Moderate Dose (0.1 LD_{50}) of Physostigmine

After 30 minutes' exposure to a moderate dose of physostigmine (0.1 LD_{50}, 0.075 mg/kg; ChE inhibition of 67% ± 7%), the nerve terminals of most endplates in diaphragm, soleus, and EDL (Fig. 32) appeared normal. They contained 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 (54-56), presumably as an effect of drug toxicity. In some cases (Fig. 32a), both pre- and post-synaptic mitochondria, as well as those in adjacent myofibers, were swollen and distorted. [Such widely distributed mitochondrial changes are not attributed to drug effect, but instead, are attributed primarily to generalized tissue hypoxia during aldehyde fixation. The hypoxia may arise from non-specific systemic drug effect (1) and/or glutaraldehyde fixation-induced hypoxia (32,34).]

Post-synaptically, diaphragm myofibers revealed few gross changes in their myofibrils (Fig. 32a). None (0%) of the myofibers in diaphragm, soleus, or EDL exhibited any evidence of supercontraction. 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 alterations that are seen in the high dose acute exposures. Thus, 0.1 LD_{50} (or 67% enzyme inhibition) appears to represent a near- but sub-threshold level for induction of endplate cytopathology in unstressed animals. (Evidence that the threshold is not static at 0.3 LD_{50} but is altered substantially by normal muscle use or by prolonged indirect stimulation is described in Sections N, below.)

5. Rat Neuromuscular Junctions After Acute Low and Very Low Doses (0.01 and 0.001 LD_{50}) of Physostigmine

After 30 minutes of exposure to low and very low doses of physostigmine [0.01 and 0.001 LD_{50}, yielding 33% (±7%) and 28% (±15%) inhibition of blood ChE, respectively], myofibers from diaphragm, soleus, and EDL had no detectable changes in nerve terminals or in the subjunctional cytoplasms (Figs. 33,34). 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. 9c, 14c, 4b, 17b], swollen mitochondria were also observed in nerve terminals (Fig. 34a,c)

and in nearby fibroblasts and Schwann cells (Fig. 34a). Since other stages in the continuum of physostigmine-induced alterations (blisters, frothy and swollen mitochondria) were not present, the swollen mitochondria were attributed to artifacts of hypoxia and of glutaraldehyde fixation rather than to drug effect on neuromuscular junction ultrastructure. Thus, we concluded that following low and very low acute doses of physostigmine (0.01 and 0.001 LD_{50}), there were no detectable changes in nerve terminals or in the subjunctional cytoplasm.

Dose-response curves for supercontraction and for frothy mitochondria are presented in Figures 35 and 36. A threshold of effect of these alterations occurs at about 0.3 LD_{50}, with minor variations attributed to differences in use patterns for the three muscles (i.e., diaphragm, soleus, and EDL).

I. ULTRASTRUCTURE OF GUINEA PIG NEUROMUSCULAR JUNCTIONS AFTER HIGH-DOSE ACUTE EXPOSURE (0.3-0.8 LD_{50}) OF PHYSOSTIGMINE

In myofibers obtained from a guinea pig 30 minutes after injection of 1.2 mg/kg (approximately 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. 37a, 37c). In contrast to those in rats, guinea pig soleus fibers (Fig. 37b) were less affected than EDL fibers. Few soleus myofibers had supercontracted sarcomeres and none exhibited exploded mitochondria. Thus, at 30 minutes PI in guinea pigs (4 animals tested), EDL fibers appear to be more sensitive to the toxic effects of physostigmine than soleus fibers. At 0.75 mg/kg (approximately 0.5 LD_{50}), myofibers appeared nearly normal (Fig. 38). Sarcomeres were of normal length in all myofibers, but subjunctional mitochondria in diaphragm and soleus were enlarged and had occasional blisters between cristae. The alterations seen at these two dose levels are consistent with alterations seen in rats at 0.8 and 0.3 LD_{50}, respectively. Thus, the LD_{50} dose in guinea pigs as measured in milligrams per kilogram appears to be approximately 50-75% larger than in rats, but the small sample size does not permit conclusions concerning the sources of the differences between rats and guinea pigs. However, the apparent reversal of fiber-type specificity for anti-ChE effect at 0.8 LD_{50} but not at 0.5 LD_{50} may be due to differing muscle use patterns between rats and guinea pigs, species differences in susceptibility to anti-ChE agents, or normal variations not accurately sampled because of the 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 suggest 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 equal or better model for predicting pathophysiological alterations of skeletal muscle because they

are easier to maintain, procedures for obtaining blood samples for ChE analysis are simpler, perfusion fixations require less reagents, and they are purchased and maintained at a substantial cost savings.

Figure 32. Effects of an Acute Moderate Dose of Physostigmine (0.1 LD_{50}) on Neuromuscular Junctions 30 Minutes PI. Myofibers of diaphragm (Figure 32a) and EDL (Figure 32c) exhibit nearly normal ultrastructure, while the subjunctional sarcomeres of soleus (Figure 32b) show evidence of irregular or zigzag Z-bands. At this dose, most mitochondria appear normal in both subjunctional sarcoplasm and nerve terminals. Occasionally, unusual bundles of Schwann cell fingers are interposed between the nerve terminal and junctional folds (Figure 32b, arrowheads). This may represent normal variability or short-term drug effect.

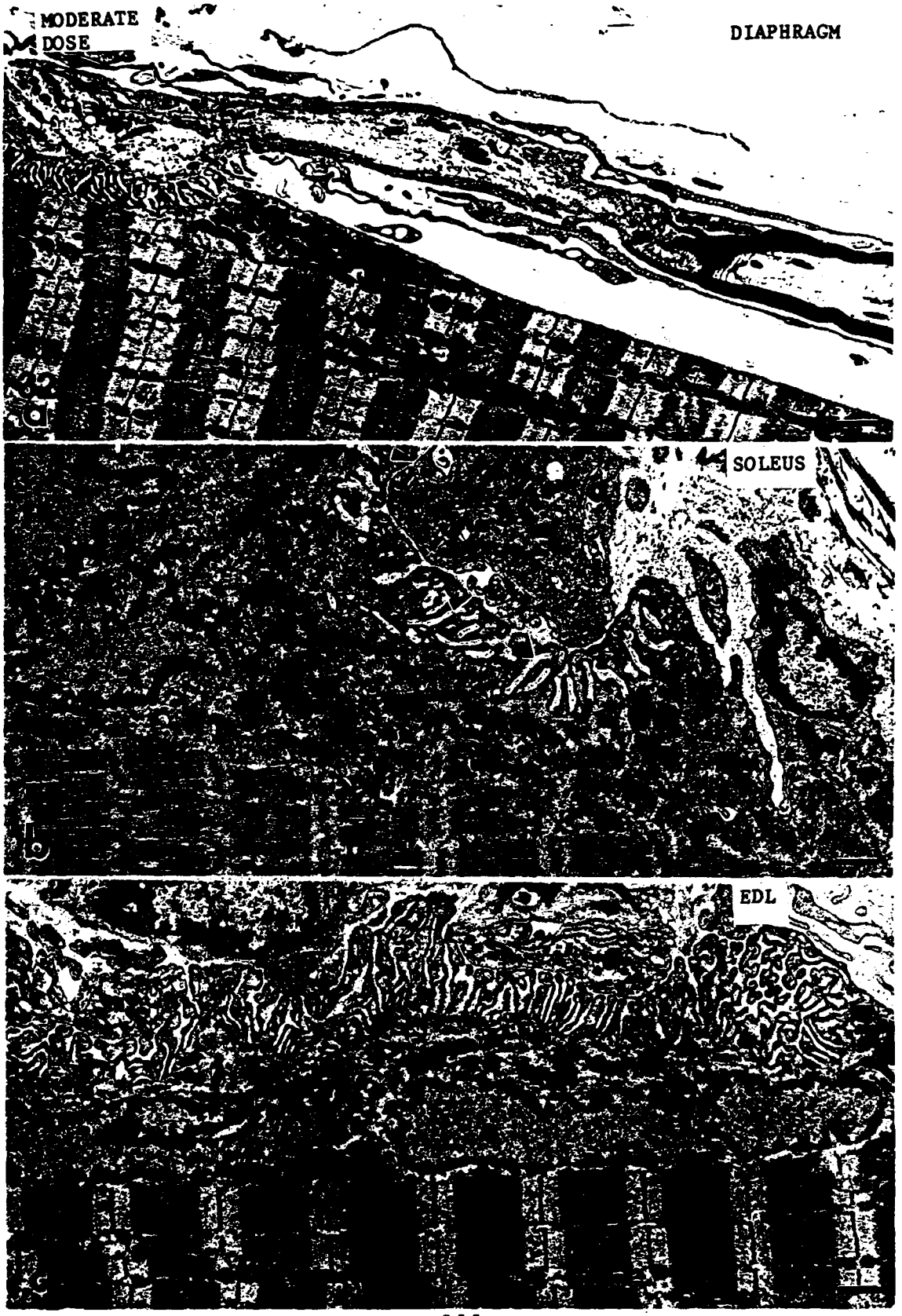

MODERATE
DOSE
DIAPHRAGM
SOLEUS
EDL

Figure 33. Effects of an Acute Low Dose of Physostigmine (0.01 LD_{50}) on Neuromuscular Junctions 30 Minutes PI. No changes in endplate ultrastructure were noted.

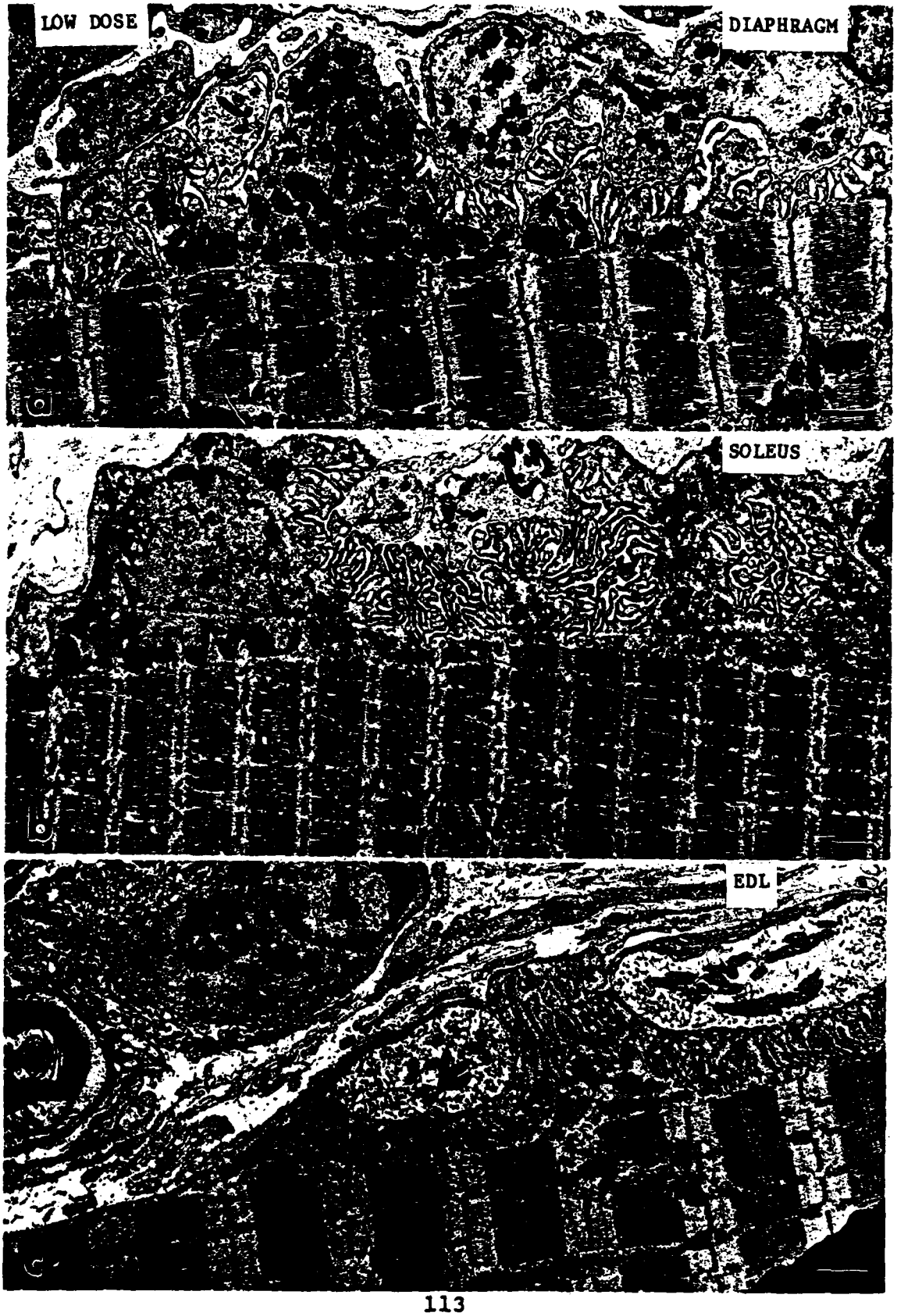
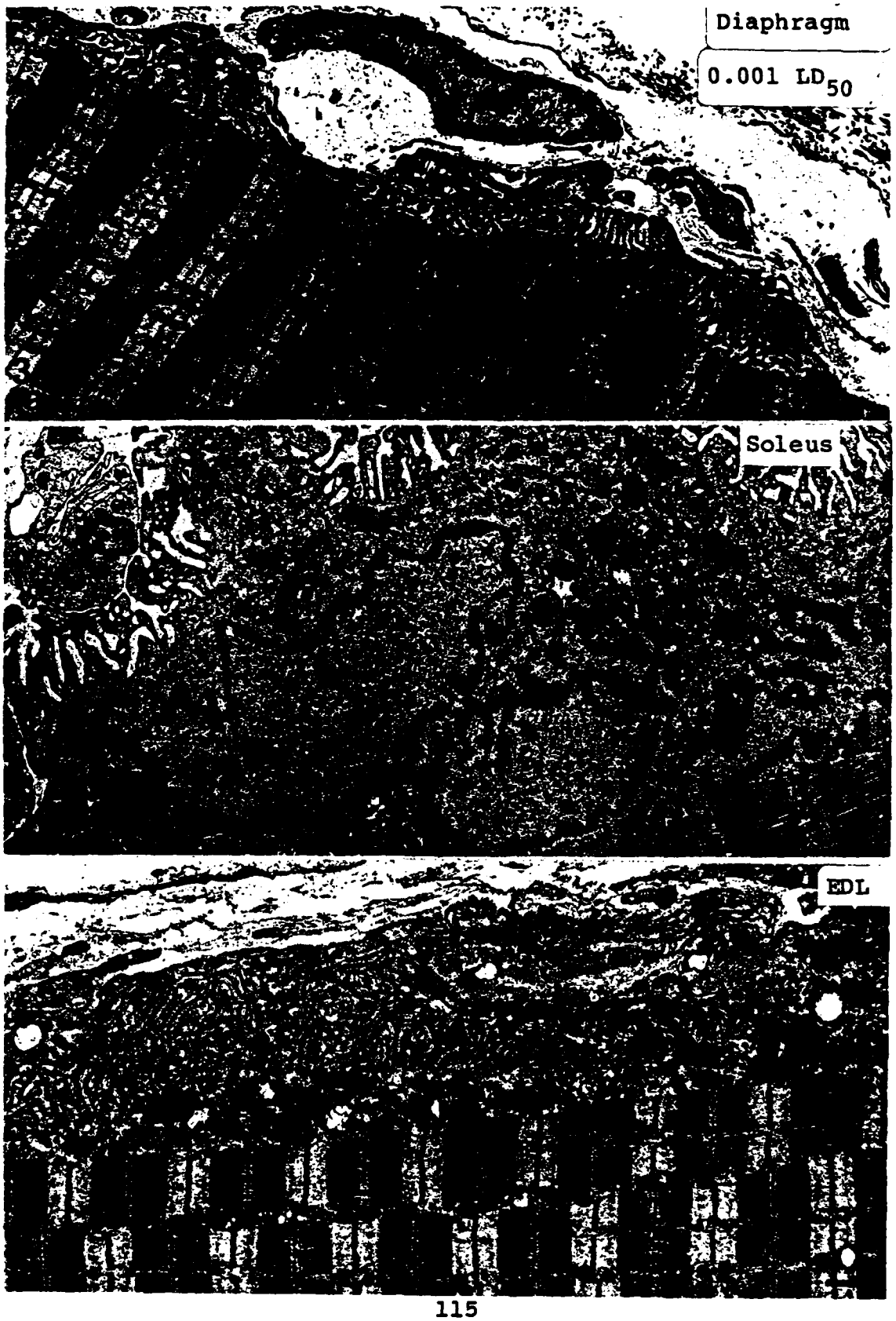

LOW DOSE
DIAPHRAGM
a
SOLEUS
b
EDL
c

Figure 34. Effects of an Acute Very Low Dose of Physostigmine (0.001 LD_{50}) on Neuromuscular Junctions 30 Minutes PI. Except for hypoxic mitochondria (Figures 34a, 34c), the endplates appeared normal.


Diaphragm
0.001 LD_{50}
Soleus
EDL

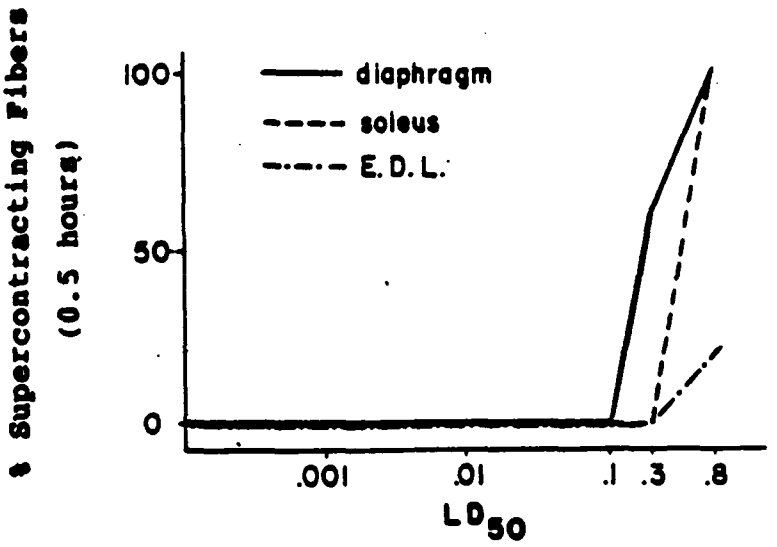


Figure 35. Dose-response Curve for Percent of Fibers with Supercontraction 30 Minutes PI at Different LD_{50} Levels of Physostigmine. About 60% of fibers in the diaphragm muscles were supercontracted at a 0.3 LD_{50} dose of physostigmine, while soleus and EDL showed no supercontraction. At a very high dose (0.8-1.1 LD_{50}), diaphragm and soleus muscles showed 100% supercontraction, whereas EDL muscle showed only 20% supercontraction. (Estimates were made based on an analysis of all electron micrographs from all rats fixed by perfusion 30 minutes after acute exposure to physostigmine at each of the indicated doses.) Fibers were considered to be supercontracted when 3 or more myofibrils had supercontraction of 3 or more sarcomeres each. Virtually all myofibers that scored positive for supercontraction had 20 or more supercontracted sarcomeres. Only those fibers with an identifiable endplate were included in the analysis. In almost all myofibers with identifiable endplates, supercontraction was restricted to the subjunctional sarcoplasm.

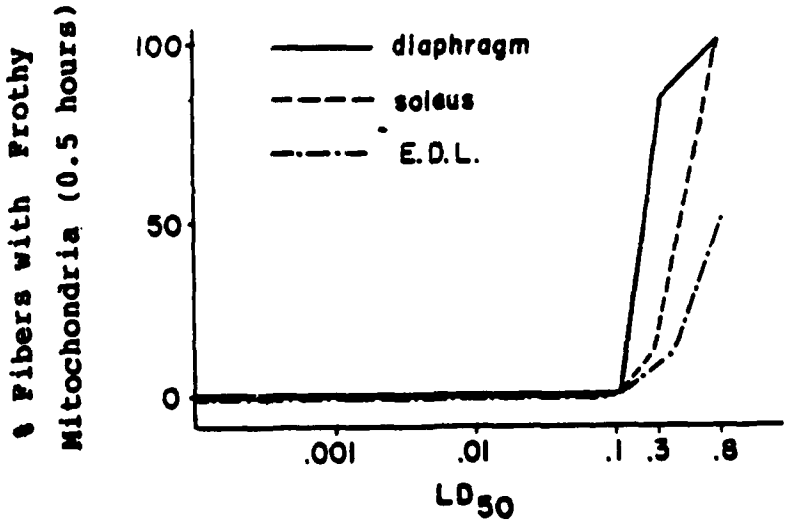


Figure 36. Dose-response Curves Based on Percent of Fibers with Frothy Mitochondria After 30 Minutes Acute Exposure to Each of the Indicated Dose Levels of Physostigmine. (Frothy mitochondria were identified by the presence of three or more electron-lucent areas within the same mitochondrial profile. The fiber was considered "positive" for frothy mitochondria when 2 or more mitochondrial profiles were found to be frothy or if a single profile had 6 or more blisters.) At 0.3 LD_{50}, frothy mitochondria were found in 90% of subjunctional cytoplasm of diaphragm muscles, while only 10% of soleus and EDL had frothy mitochondria. At 0.8 LD_{50}, 100% of fibers in diaphragm and soleus had frothy mitochondria in the subjunctional cytoplasm, while about 50% of mitochondria were frothy in the EDL. At 0.8 LD_{50}, virtually all frothy myofibers had more than 5 frothy mitochondria and often had 20 or more.

Figure 37. Comparison of the Effects of an Acute High Dose of Physostigmine (1.2 mg/kg) on Neuromuscular Junctions of Guinea Pig Diaphragm, Soleus, and EDL Muscles 30 Minutes PI. Note the characteristic continuum of mitochondrial changes associated with supercontraction (Figure 37a,c) as well as the vesicated mitochondria in the nerve terminals (Figure 37b).

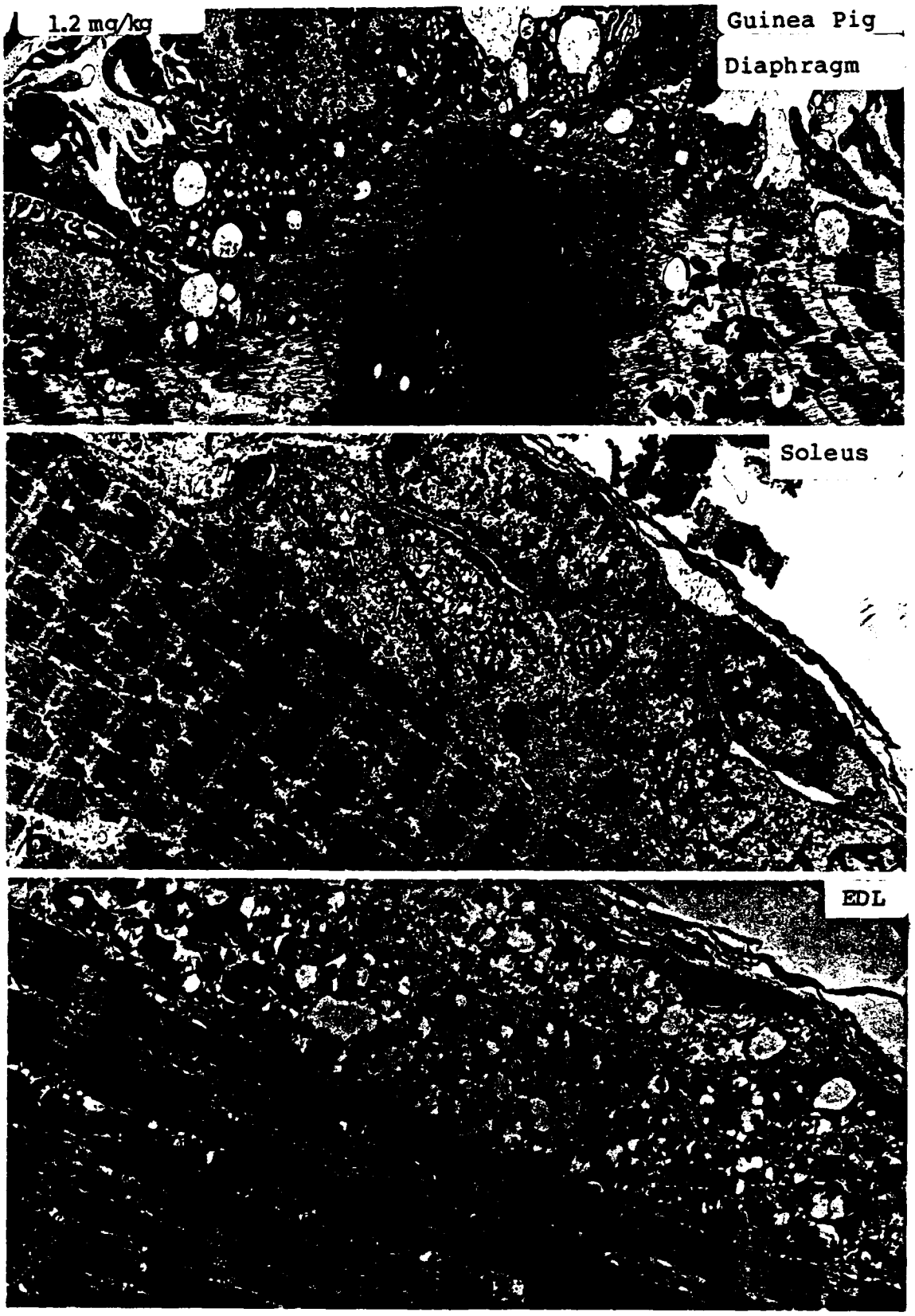
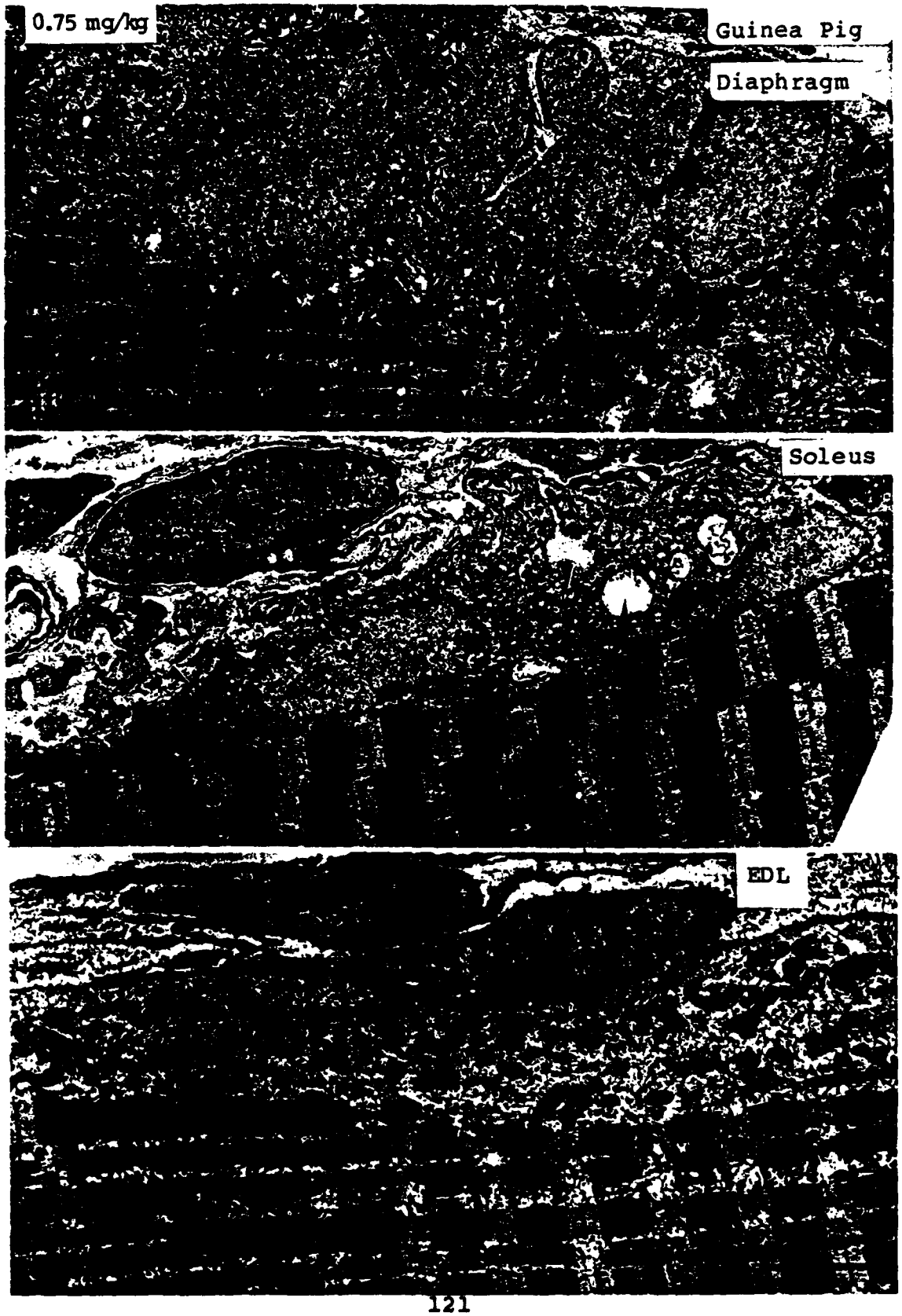

1.2 mg/kg
Guinea Pig
Diaphragm
Soleus
EDL

Figure 38. Comparison of the Effects of an Acute Near-threshold Dose of Physostigmine (0.75 mg/kg) on Neuromuscular Junctions of Guinea Pig Diaphragm, Soleus, and EDL Muscles 30 Minutes PI. Note the blistered, frothy (Figure 38b, arrowheads), and swollen mitochondria (Figure 38a,b, arrows). Consequently, 0.75 mg/kg of physostigmine is considered a near- but sub-threshold dose in the guinea pig.


0.75 mg/kg
Guinea Pig
Diaphragm
Soleus
EDL

J. DELAYED EFFECTS OF AND RECOVERY FROM ACUTE EXPOSURE TO PHYSOSTIGMINE

1. Blood ChE Inhibition Levels

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 (Figs. 1-7). (Similar fluctuations in blood ChE activity were also seen in controls; see Section D, above.)

2. Physiology

The physiological alterations (EDL twitch potentiation and sustained high frequency contraction) returned to normal within 1 hour after acute physostigmine administration (Fig. 9 and Fig. 10, segment F). In all cases, this return to normal physiology occurred when blood ChE inhibition levels were still 80% or higher.

3. Delayed Ultrastructural Alterations Following Acute Exposure to Physostigmine

a. Delayed Effects of Acute High Dose

During the period 1-56 days after a single high-dose injection (0.8-1.1 LD_{50}) of physostigmine, few unusual morphologies were noted. In contrast to the destructive effects on sarcomeres and subjunctional mitochondria that were consistently observed in the neuromuscular junctions of diaphragm and soleus muscles 30 minutes after a single high dose (0.8-1.1 LD_{50}) injection of physostigmine, most alterations were partially or completely reversed 24 hours PI (Fig. 39a,b.) Likewise, blood ChE levels had returned to near normal. (The micrograph code "AH-R1" in Fig. 39 designates that the sample was from an experiment entitled "Acute High dose - Recovery 1 day." Similar codes identify each micrograph in this and following sections.)

EDL myofibers, which were scarcely damaged at 30 minutes after an injection of 0.8-1.1 LD_{50} (See Fig. 26, above), exhibited extensive myofibril disruption and damage to junctional folds at 24 hours PI (Fig. 39c). Thus, in contrast to measurements of EDL whole muscle physiology (which showed rapid recovery of twitch tension, usually within 1 hour), endplate ultrastructure was maximally altered sometime between ½ hour and 24 hours PI. This ½-hour to 1-day delay in the appearance of ultrastructural alterations in EDL myofibers is temporally associated with the resumption of normal ambulatory behavior (i.e., normal activation of EDL muscles).

In our first Annual Report (57), we also noted that at 24 hours PI, clusters of closely packed nerve terminal branches were present in the neuromuscular junctions of a few diaphragm muscles (Fig. 40a). In the absence of evidence for similar images from control endplates, these unusual morphologies were originally attributed to delayed drug effects (cf. 24). Subsequently, however, similar images have been observed equally frequently in control endplates (cf. Figs. 14a, 15a). Thus, clusters of nerve terminal profiles have been eliminated as a

criterion for assigning drug toxicity.

In myofibers from 3 of the rats, subjunctional sarcomeres were disorganized. The presence of intact sarcoplasmic reticulum, triads, and mitochondria, and the absence of true supercontraction suggest that these regions were in the process of membrane reorganization, realignment of myofilaments, and repair of sarcomeres. However, focal areas of supercontraction and/or sarcomere dissolution (Fig. 40b) remain as evidence of incomplete recovery of diaphragm myofibers at 24 hours PI.

Seven days after acute exposure to a 0.8 LD_{50} of physostigmine, junctional folds in some diaphragm and soleus myofibers appeared to be devoid of attached nerve terminal branches (Figs. 41, 42), an alteration potentially attributable to drug effect. However, since similar images have been observed in normal endplates (cf. Figs. 11c, 13b, 14a,b, 15a, 18a-d, 19b), these images are now considered to represent either endplate remodeling as a normal process (58) or perhaps a slightly increased rate of remodeling induced by exposure to anti-ChE agents (cf. 24).

At 14, 28, and 56 days after a single high dose injection, most myofibers in diaphragm, soleus, and EDL muscle appeared normal (Figs. 43, 44, 45, respectively). Post-synaptically, sarcomeres, mitochondria, and nuclei were indistinguishable from controls. However, in a few diaphragm and soleus myofibers, a small proportion of nuclei in the soleplate region were necrotic (Fig. 44), presumably reflecting irreversible damage to some nuclei during the initial period of profound endplate depolarization. In other fibers, junctional folds appeared to be devoid of attached nerve terminals (Figs. 42a,b). However, subsequent analyses of a much larger number of images from control endplates revealed similar frequencies of small-diameter nerve branches, some not embedded within a primary synaptic cleft (Figs. 13b, 18d). Thus, such images may not reflect denervation or the aberrant formation of new nerve terminals by collateral sprouting as previously proposed (28-30). Finally, several endplates from the diaphragm and soleus muscles at 14, 28, and 56 (Figs. 44a, 46) days PI had vesicular debris in the synaptic clefts and areas of apparent junctional fold simplification similar to that in neuromuscular junctions of patients with myasthenia gravis (35-38, 40, 41, 59, 60) and after treatment with other anti-ChE agents (11-30). We note, too, that in analogous images from normal endplates having areas apparently devoid of nerve terminals (Fig. 47, see also Figs. 11c, 13b, 14a,b, 15a, 18a-d, and 19b), junctional fold debris was never observed. Thus, normal endplate remodeling is to be contrasted with the that seen occasionally following subacute exposure to high doses of physostigmine.

From these data, we conclude that at recovery periods greater than 7 days following sublethal but high-dose acute exposures to physostigmine, most fibers in all surviving animals were indistinguishable from normal myofibers. However, a few fibers were damaged sufficiently to require a prolonged period of recovery, or in some cases, were irreversibly damaged.

Nevertheless, for most fibers in surviving rats, recovery and repair processes were both rapid and efficient, and were essentially complete within 7-14 days in most myofibers and in their innervating nerves.

b. Absence of Delayed Effects from Moderate to Very Low Doses of Physostigmine

At 1-56 days after injection of moderate to very low doses of physostigmine (0.1 LD_{50}-0.001·LD_{50}), no damage to endplates was detected and no delayed effects were noted in any of several hundred myofibers examined (Figs. 48-62). 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 had the same variability as normal endplates; junctional fold depth, number, and distribution appeared normal; and Schwann cells had the same variability as in untreated controls. In contrast to data presented by Hudson and coworkers (28-30) concerning delayed effects from moderate and low doses of pyridostigmine, no increase was noted in the normal frequency of separations of nerve terminal membranes from junctional folds in physostigmine-treated rats. Moreover, the endplates of control rats revealed frequent areas with Schwann cell fingers interposed between nerve terminal and junctional folds similar to those previously described as alterations due to pyridostigmine (28-30). 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) often resulted when the plane-of-section transected the crests of junctional folds but did not include the closely associated axon end-bulbs (see Figs. 18c, 19c). In addition, junctional folds without associated nerve terminal branches were observed in both normal and drug-treated muscles. 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.

4. Reversibility of Ultrastructural Alterations

a. Acute High Dose

The acute effects of high-dose exposure (0.8-1.1 LD_{50}; 0.6-0.8 mg/kg) to physostigmine seen at ½ hour and 24 hours PI were substantially reversed by 7 days and were virtually undetectable by 14 days and thereafter. Since at ½ hour after injection of 0.8 LD_{50} of physostigmine, only a very small proportion of fibers sustained irreversible damage, the relatively small number of fibers examined in this portion of the study (<300) and the very small number of endplates examined (approximately 30) would permit an estimate sufficiently accurate only to conclude that the proportion of irreversibly damaged fibers is at most only a few percent.

b. Acute Moderate and Low Doses

After moderate to very low doses [0.1 to 0.001 LD_{50}, 67% (±7%) to 28% (±15%) blood ChE enzyme inhibition], the junctional ultrastructure of diaphragm muscle at 1-56 days PI (Figs. 48-62) 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. (See Section J, above.) The relative extent of ultrastructural alterations and their time course of recovery for 0-56 days following high to very low doses of physostigmine are summarized in Figures. 63, 64.

Figure 39. Effects of an Acute High Dose of Physostigmine (0.8 LD_{50}) on Diaphragm, Soleus, and EDL Neuromuscular Junctions 1 day PI (AH-R1). In the diaphragm (Figure 39a), the myofibrils appear almost fully recovered in this area, but an irregular arrangement of myofibrils is seen just below the endplate. Junctional folds appear slightly broadened. There are no swollen or frothy mitochondria in evidence. In the soleus muscle (Figure 39b), the neuromuscular junctions and myofibrils appeared normal. However, in the EDL muscle (Figure 39c), the junctional folds appear abnormally broadened and subjunctional myofibrils were disorganized. The presence of intact (apparently normal) sarcoplasmic reticulum, triads, and mitochondria suggests that these regions were in the process of membrane reorganization, realignment of myofilaments, and repair of sarcomeres.

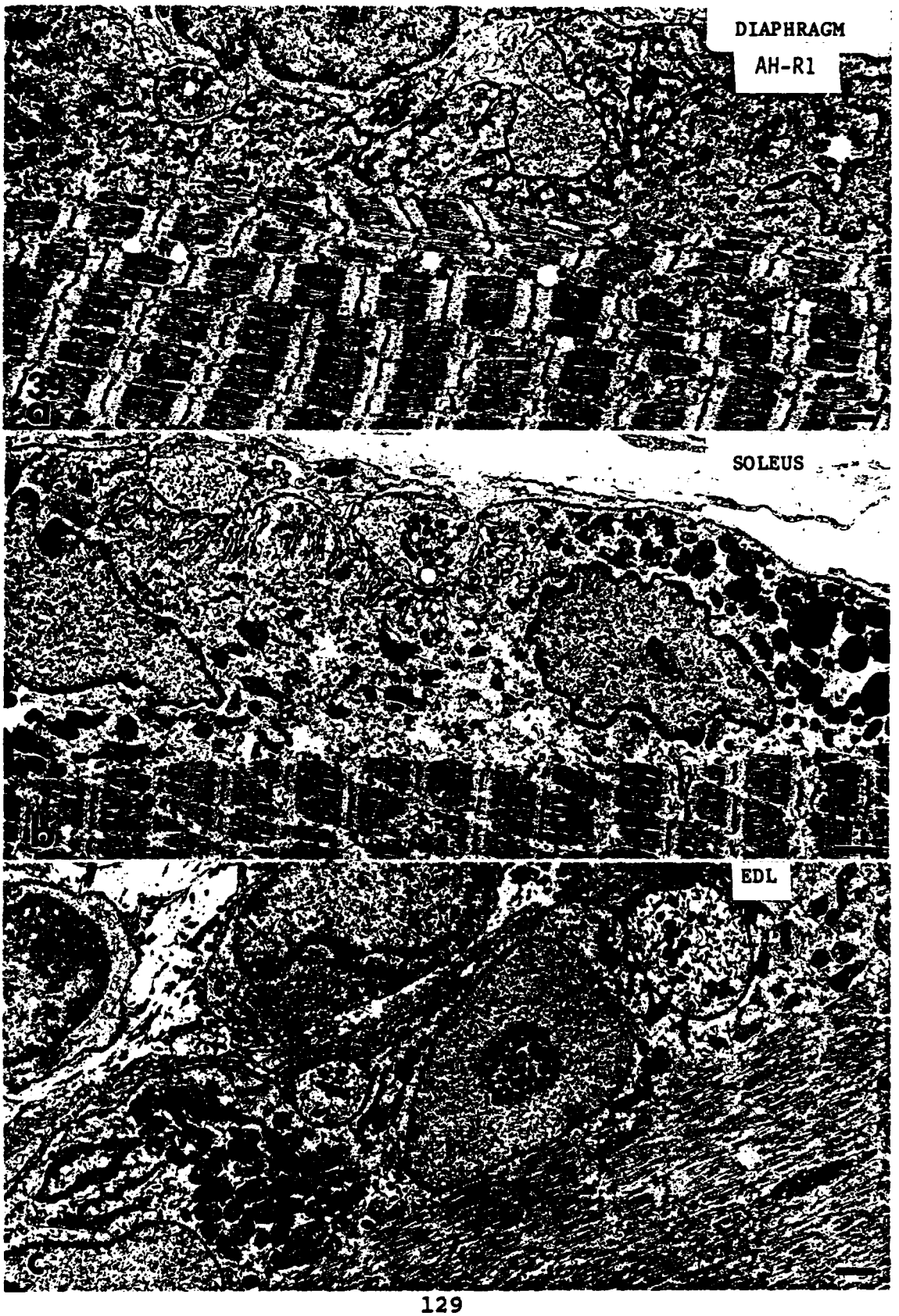

DIAPHRAGM
AH-R1
a
SOLEUS
b
EDL
c

Figure 40. Recovery of Diaphragm Neuromuscular Junction 1 Day After a High Dose of Physostigmine (0.8 LD_{50}). At 1 day PI, the mitochondria appeared normal. However, continuing evidence of myofibril destruction both at the endplate (Figure 40a) and away from the neuromuscular junction (Figure 40b) is observed. Unusual clusters of nerve terminals (Figure 40a, arrowheads) are seen at 1 day PI, but similar images were occasionally observed in normal endplates.

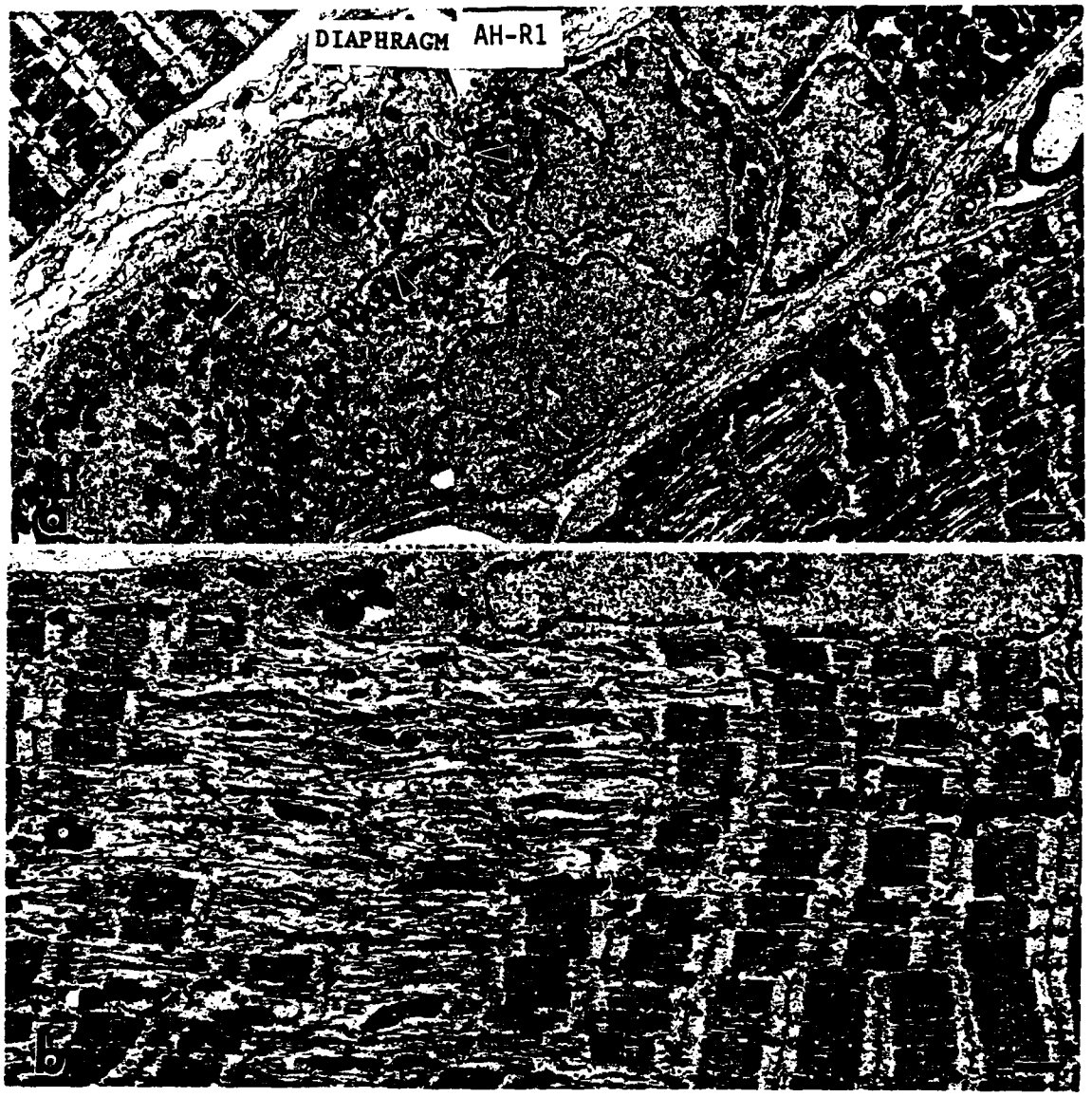

DIAPHRAGM AH-R1

Figure 41. Recovery of Neuromuscular Junctions of Diaphragm, Soleus, and EDL Muscles 7 Days After an Acute High Dose of Physostigmine (AH-R7). Myofibrils, mitochondria, and nerve terminals appear normal. Since 100% of diaphragm and soleus myofibers had been affected at 30 minutes (Figures 22-27), it is clear that the muscle fibers are capable of extremely rapid recovery following a single high-dose injection.

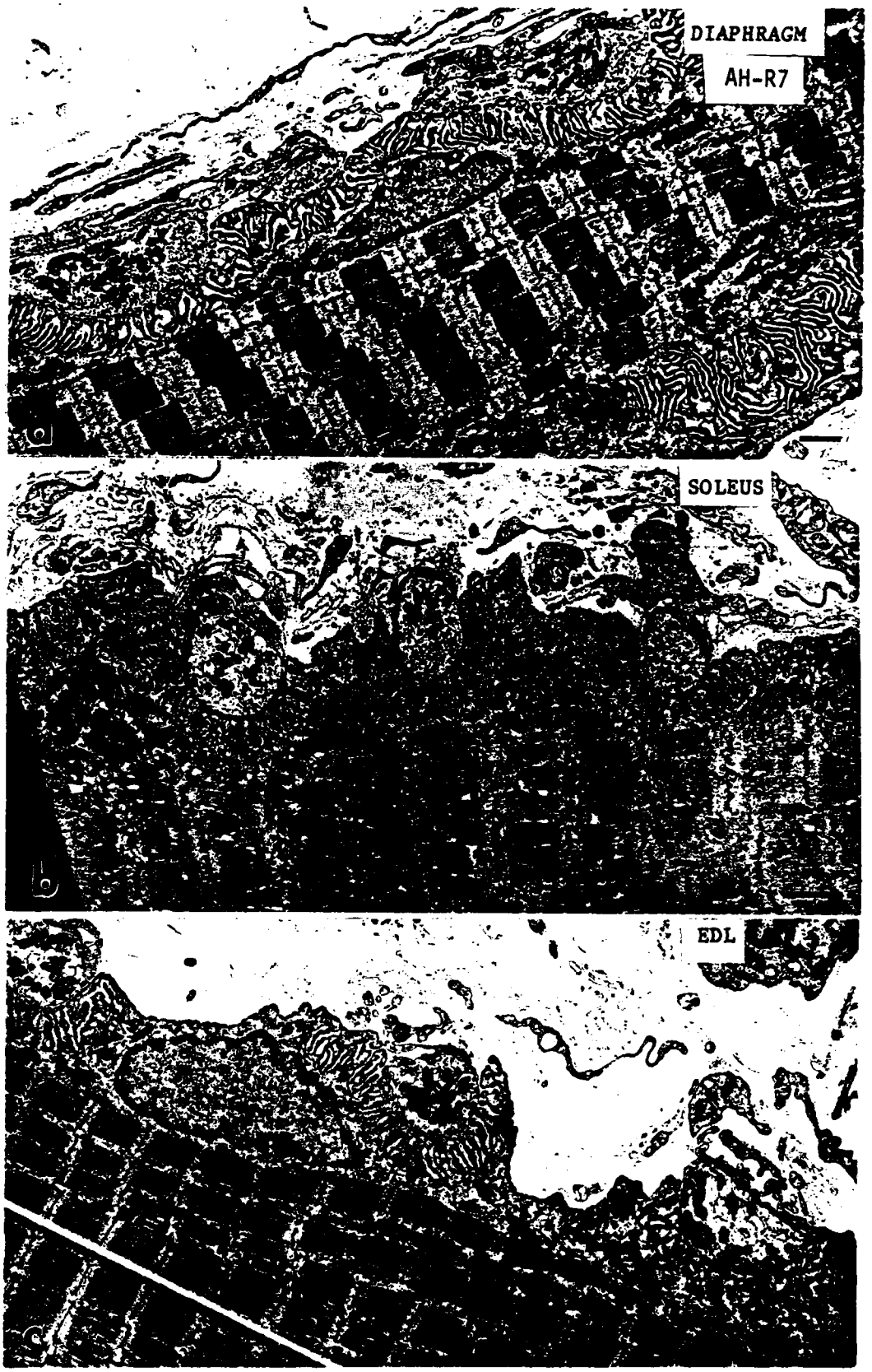

DIAPHRAGM
AH-R7
a
SOLEUS
b
EDL
c

Figure 42. Images of Possible Delayed Effects on Nerve Terminals of Diaphragm, Soleus, and EDL Muscles 1 Day After an Acute High Dose of Physostigmine (0.8 LD_{50}). Small nerve terminals (small arrowheads) and larger nerve terminals (large arrowheads) are observed on and adjacent to the flattened denervated portions of the junction. Although these micrographs are consistent with processes of denervation and reinnervation of nerve terminals by collateral sprouting, similar images have been observed repeatedly in normal neuromuscular junctions (compare to Figures 11c, 13b, 14a,b, 15a, 18a-d, 19b and Figures 68a, 69a, 70b).

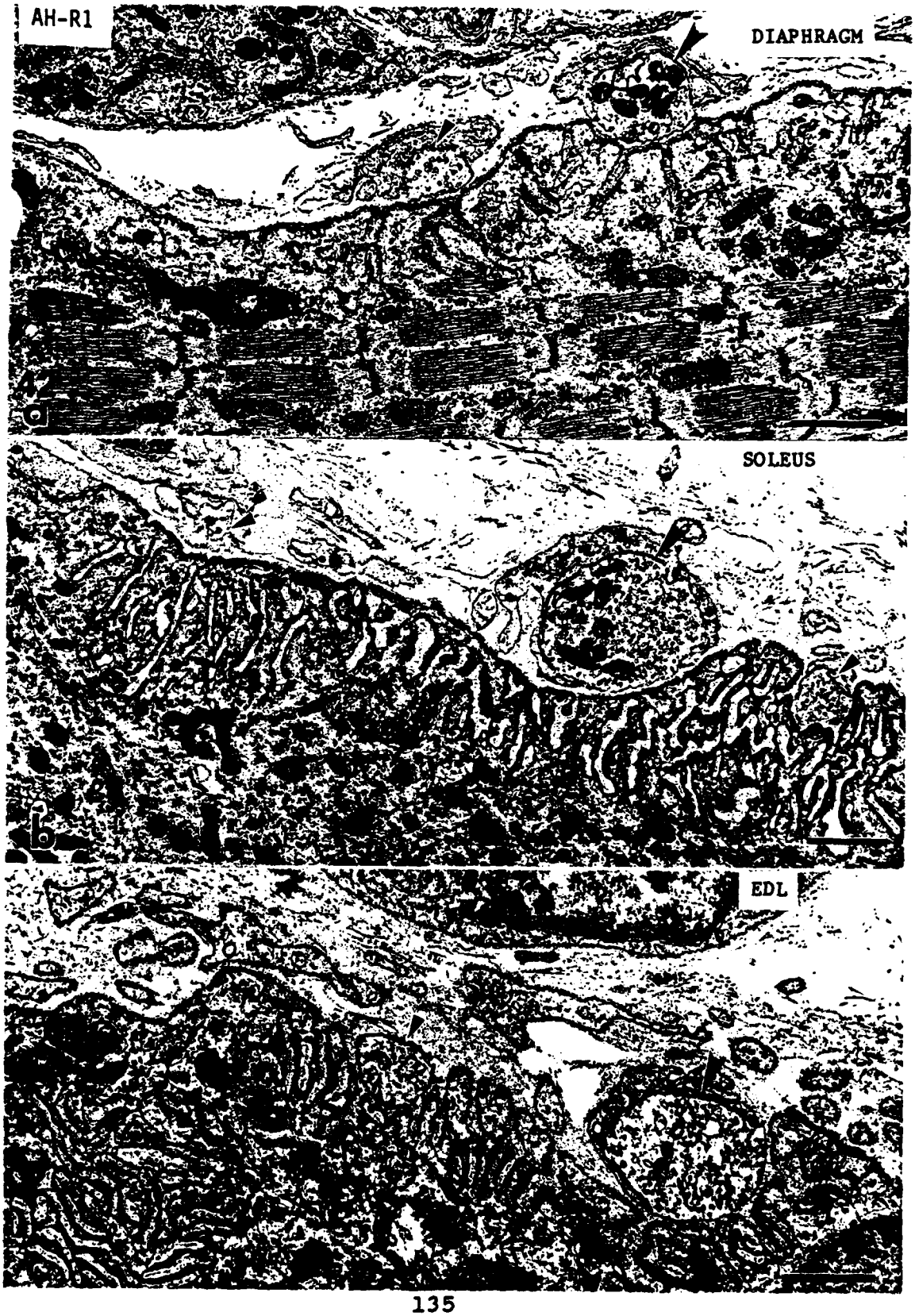

AH-R1
DIAPHRAGM
a
SOLEUS
b
EDL

Figure 43. Recovery of Neuromuscular Junctions in Diaphragm, soleus, and EDL 14 Days After an Acute High dose of Physostigmine (0.8-1.1 LD_{50}). All myofibrils, mitochondria, and nerve terminals appeared normal.

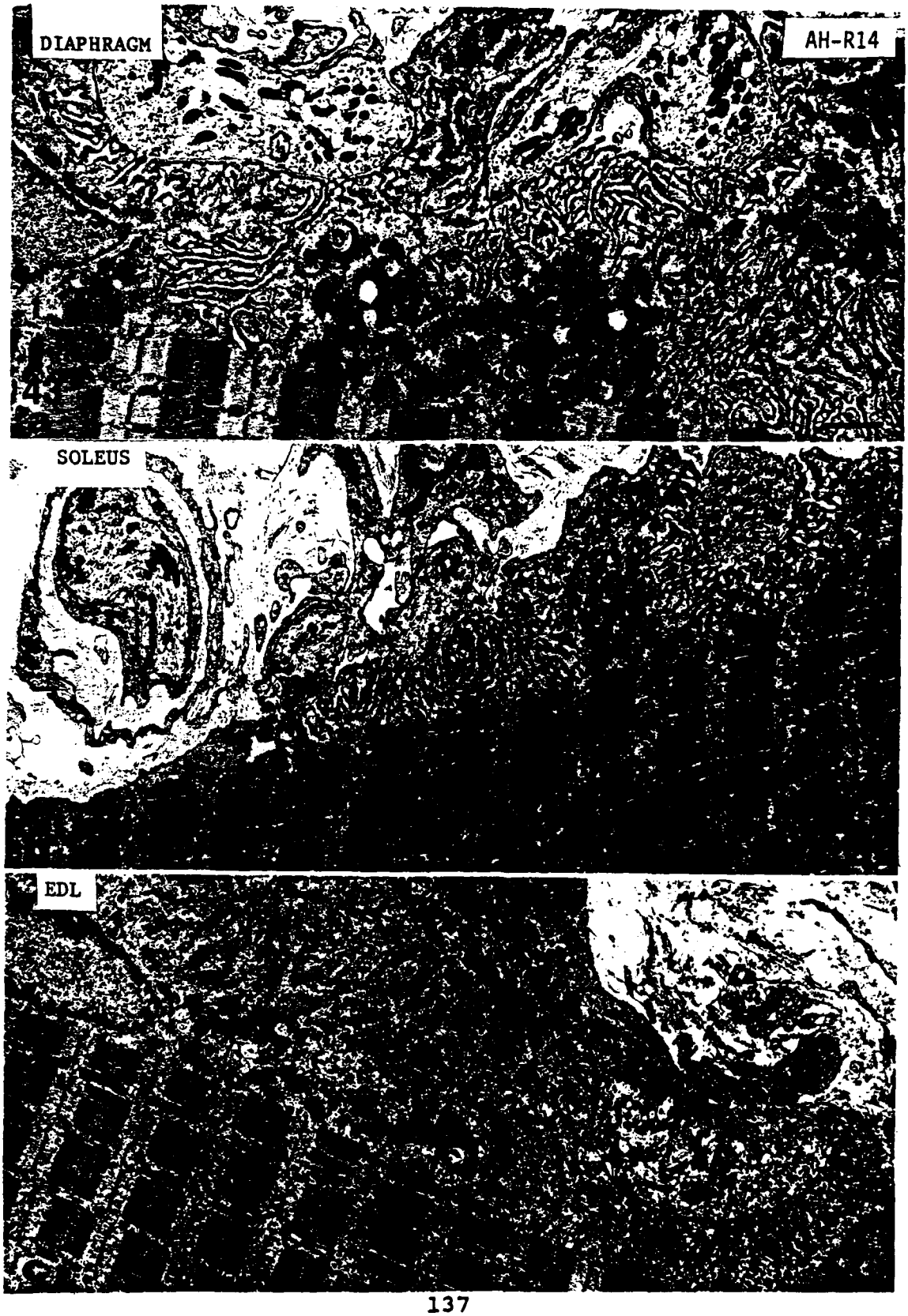

DIAPHRAGM
AH-R14
SOLEUS
EDL

Figure 44. Recovery of Neuromuscular Junctions in Diaphragm, soleus, and EDL 28 Days After Acute High Dose of Physostigmine (0.8-1.1 LD_{50}). Myofibrils, mitochondria, and nerve terminals were normal. (The swollen mitochondria in Figure 44b,c are typical fixation artifacts). The arrow in Figure 44a points to the necrotic residue of a nucleus, indicating that at least some nuclei were irreversibly damaged by the initial prolonged and severe endplate depolarizations. The arrowheads in Figure 44a indicate vesicular debris similar to that reported in neuromuscular junctions from human patients with myasthenia gravis. (See Figure 46.)

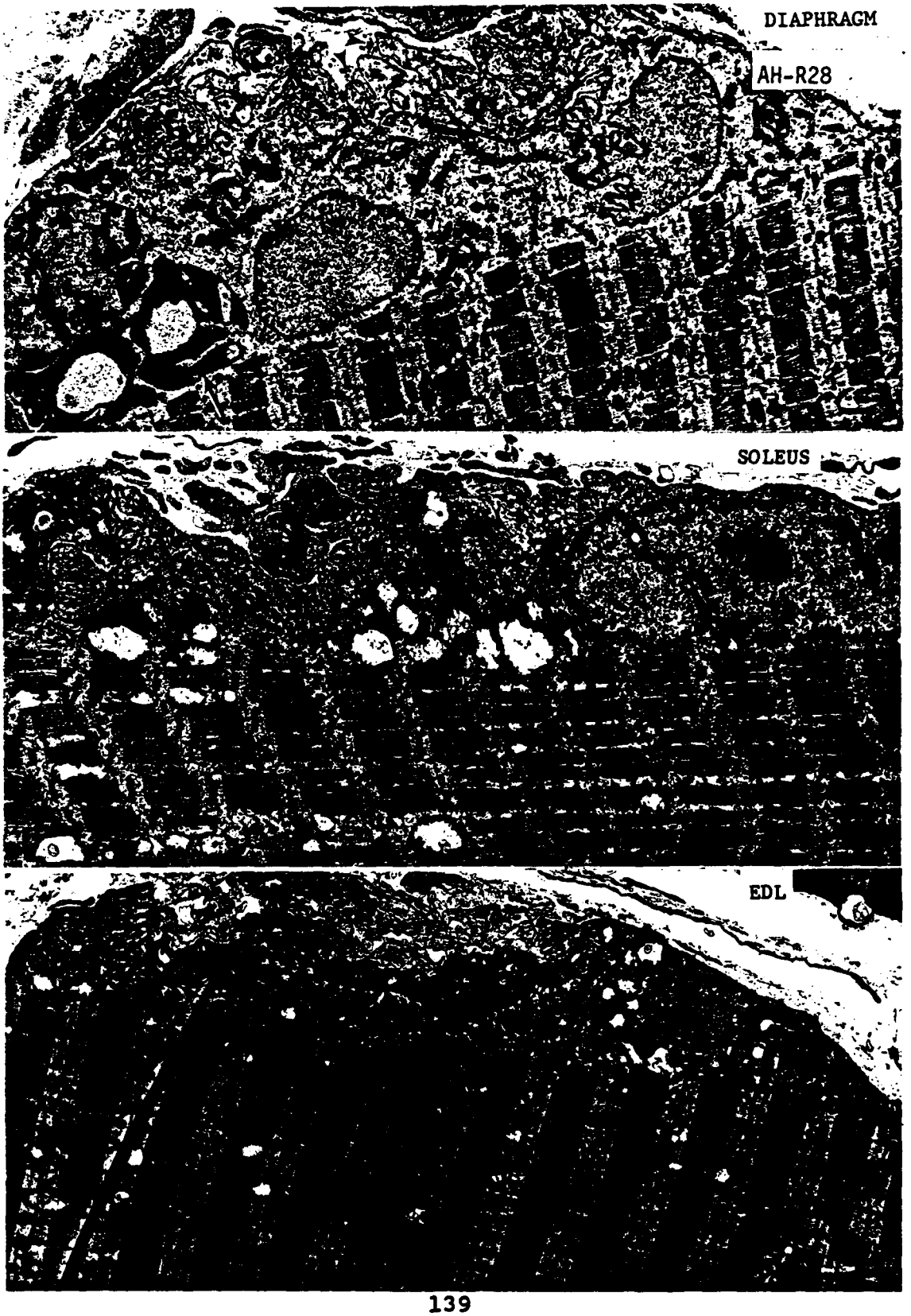

DIAPHRAGM
AH-R28
SOLEUS
EDL

Figure 45. Recovery of Neuromuscular Junctions in Diaphragm, Soleus, and EDL 56 Days After an Acute High Dose of Physostigmine (0.8-1.1 LD_{50}). No detectable alterations were found in the myofibrils, mitochondria, or nerve terminals. An unusual multivesicular inclusion is present in one subjunctional nucleus (Figure 45b, arrow).

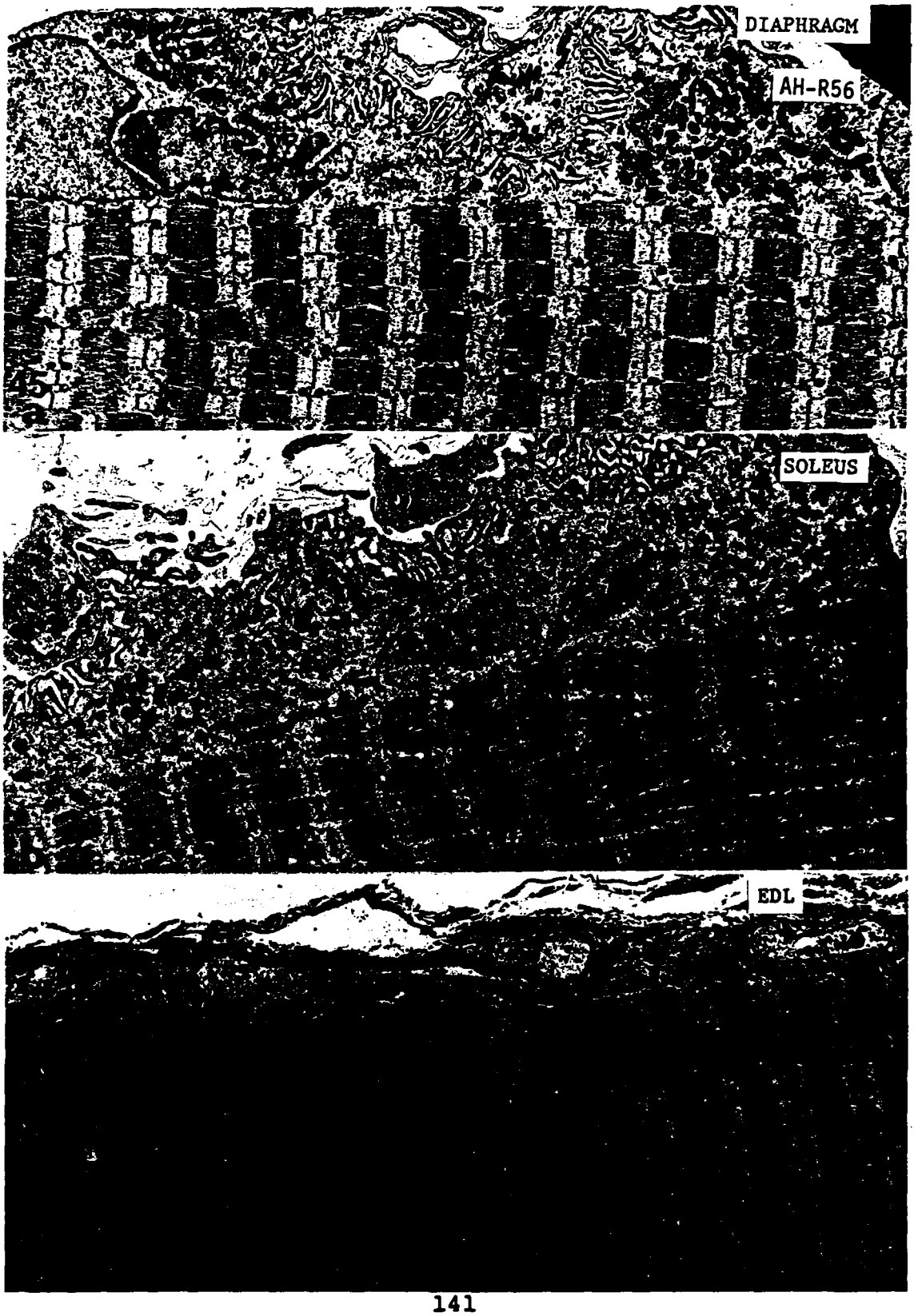

DIAPHRAGM
AH-R56
SOLEUS
EDL

Figure 46. Delayed Effects on Nerve Terminals of Diaphragm Muscle After a Single High Dose of Physostigmine. Micrographs show neuromuscular junctions of diaphragm muscles 14 days (Figure 46a), 28 days (Figure 46b), and 56 days PI (Figure 46c). Debris (arrowheads) similar to that reported in neuromuscular junctions of human patients with myasthenia gravis and some rats treated with high doses of anti-ChE agents (see text for references) is found in the synaptic cleft (Figure 46a), on the degenerating crests of the junctional folds (Figure 46b), and on the denervated portion of a partially recovered endplate (Figure 46c). However, junctional fold debris was never observed in normal endplates, even in those apparently undergoing endplate remodeling.

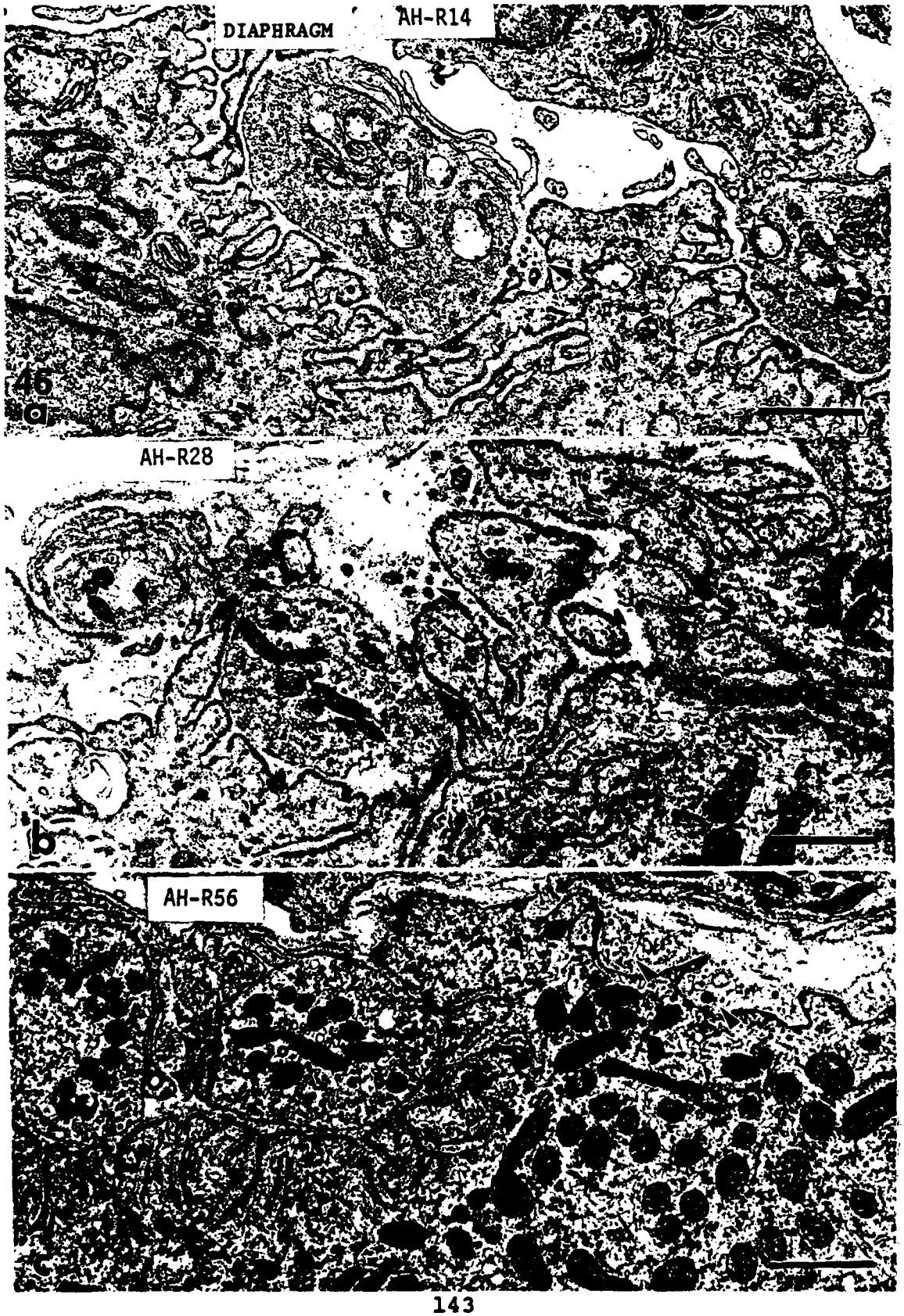

DIAPHRAGM
AH-R14
46
a
AH-R28
b
AH-R56
c

Figure 47. Thin Section Images of Control Endplates Showing Junction Folds Without Associated Nerve Terminal Profiles. (SC = Schwann cells; N = Nerve terminal.)

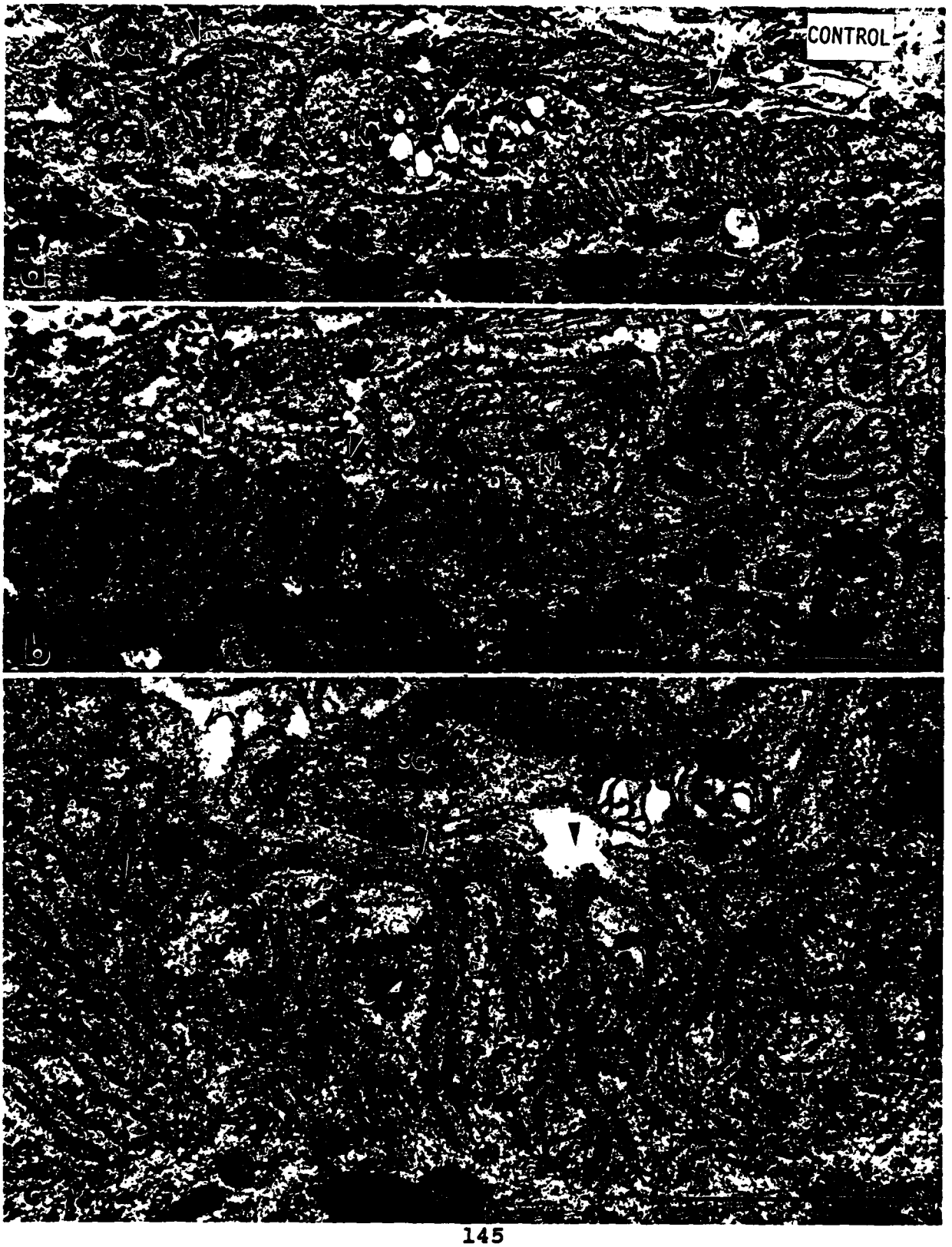

CONTROL
a
b
SC

Figure 48. Recovery of Diaphragm, Soleus, and EDL Neuromuscular Junctions 1 Day After an Acute Moderate Dose of Physostigmine (0.1 LD_{50}). Myofibrils, mitochondria, and nerve terminals appear normal. Although diaphragm and soleus myofibers had been slightly affected at 30 minutes PI (Figure 32a,b), it is clear that these fibers have virtually recovered from any minor alterations within the subsequent 24 hours.

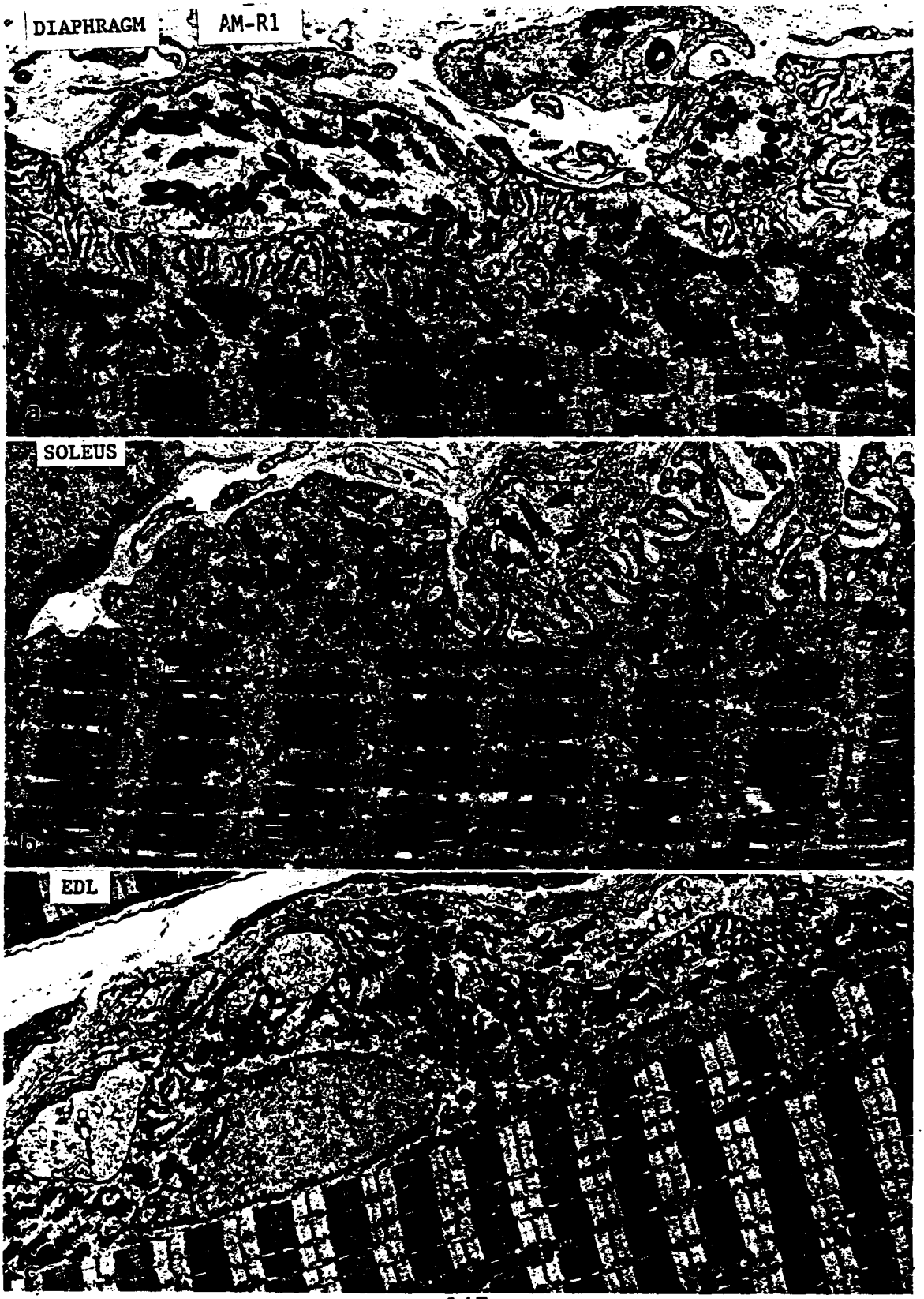

DIAPHRAGM
AM-R1
a
SOLEUS
b
EDL

Figure 49. Neuromuscular Junctions of Diaphragm, Soleus and EDL Muscles 7 Days After an Acute Moderate Dose of Physostigmine (0.1 LD_{50}). Endplate and myofiber morphology appear normal. No structural alterations were detected in any of the muscles treated at this dose.

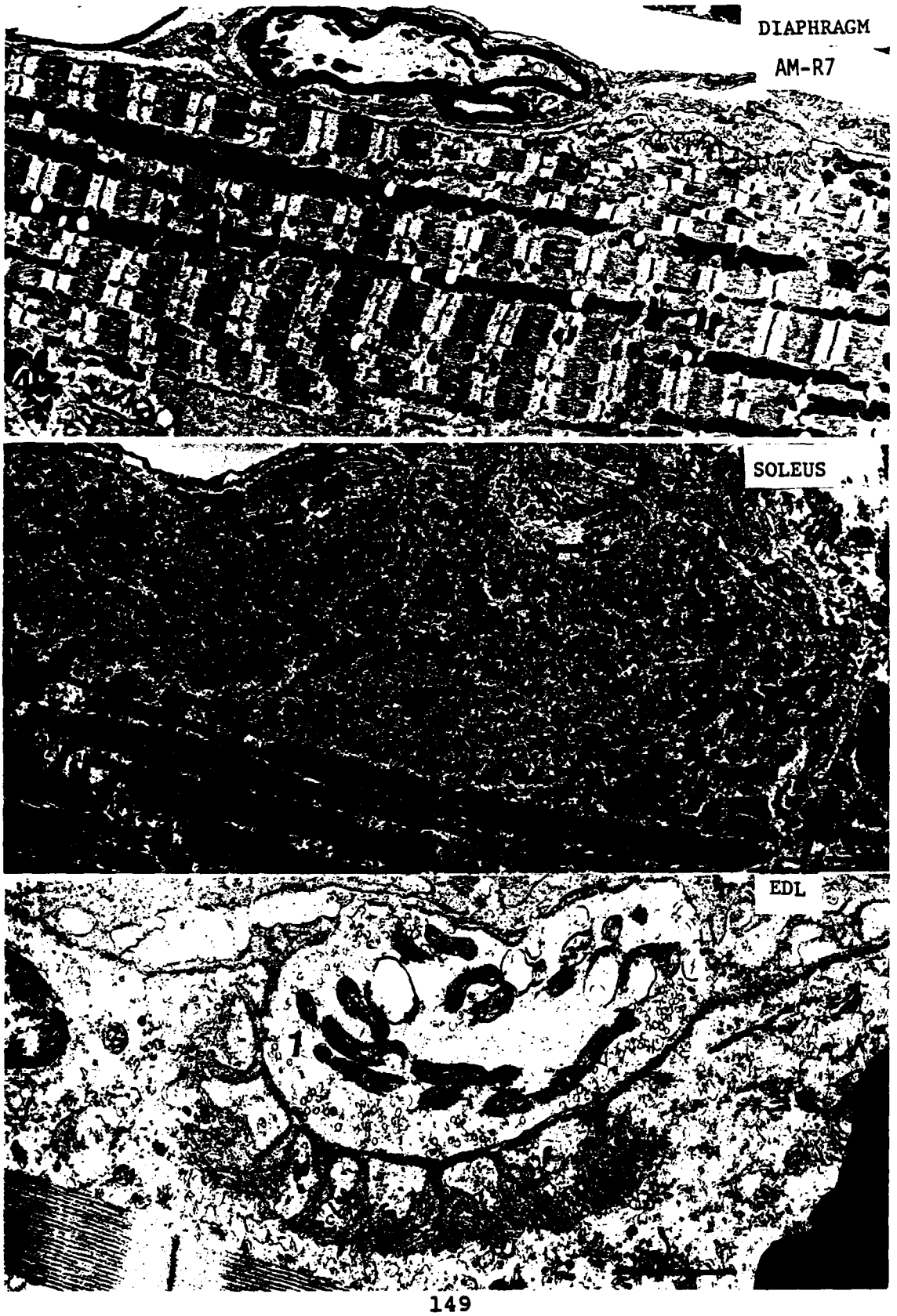

DIAPHRAGM
AM-R7
SOLEUS
EDL

Figure 50. Recovery of Rat Diaphragm, Soleus, and EDL Neuromuscular Junctions 14 days After an Acute Moderate Dose of Physostigmine (0.1 LD_{50}). Myofibers, mitochondria, and nerve terminals appear normal.

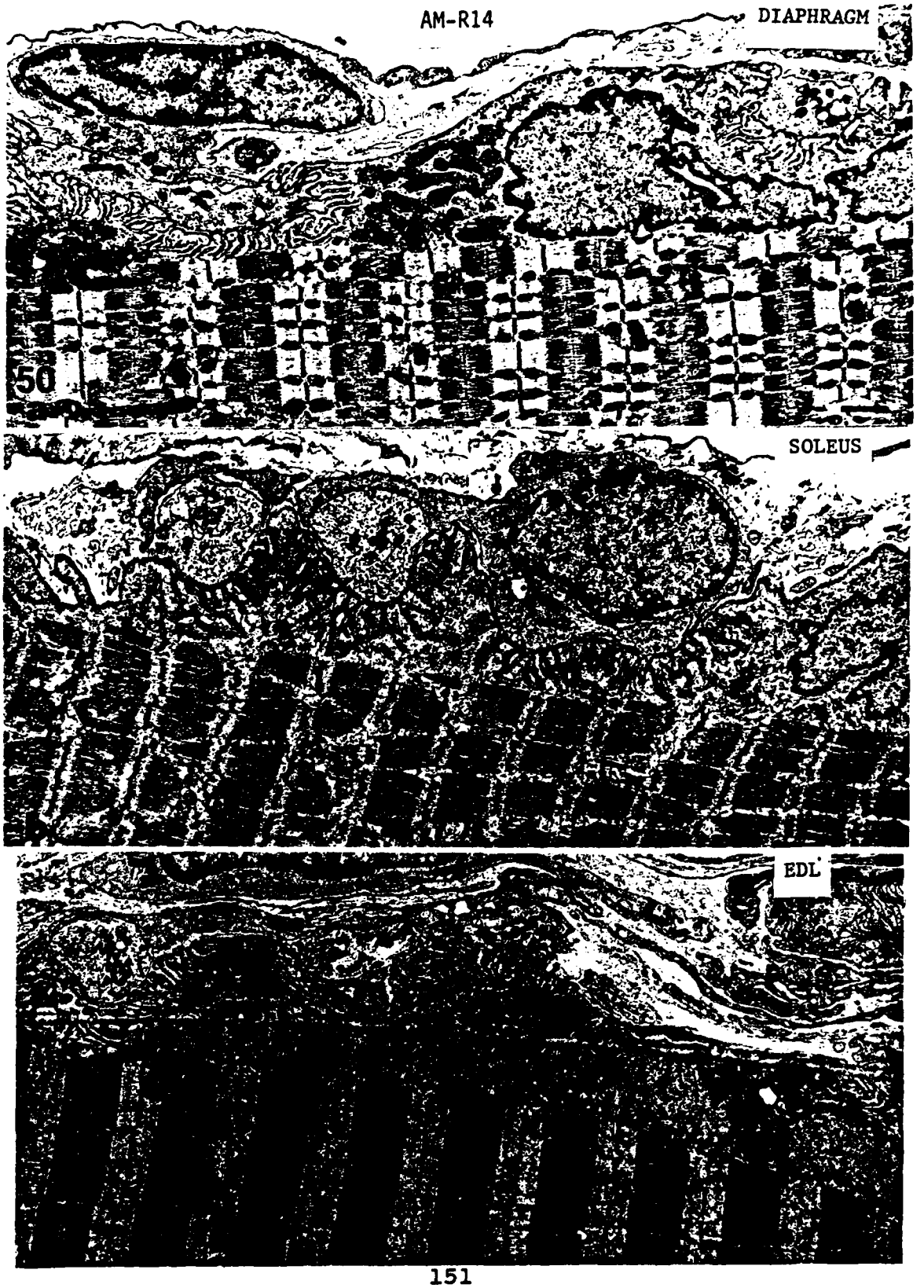

AM-R14
DIAPHRAGM
50
SOLEUS
EDL

Figure 51. Recovery of Diaphragm, Soleus, and EDL Neuromuscular Junctions 28 days After an Acute Moderate Dose of Physostigmine (0.1 LD_{50}). No detectable alterations were found in the myofibrils, mitochondria, or nerve terminal. (This series of muscle fixations was stained improperly, resulting in low density staining of Z bands and precipitation of uranyl phosphate throughout the tissue. Despite their unusual staining characteristics, however, cytostructural details were sufficiently preserved by the initial glutaraldehyde/osmium fixation to allow the requisite ultrastructural analysis.)

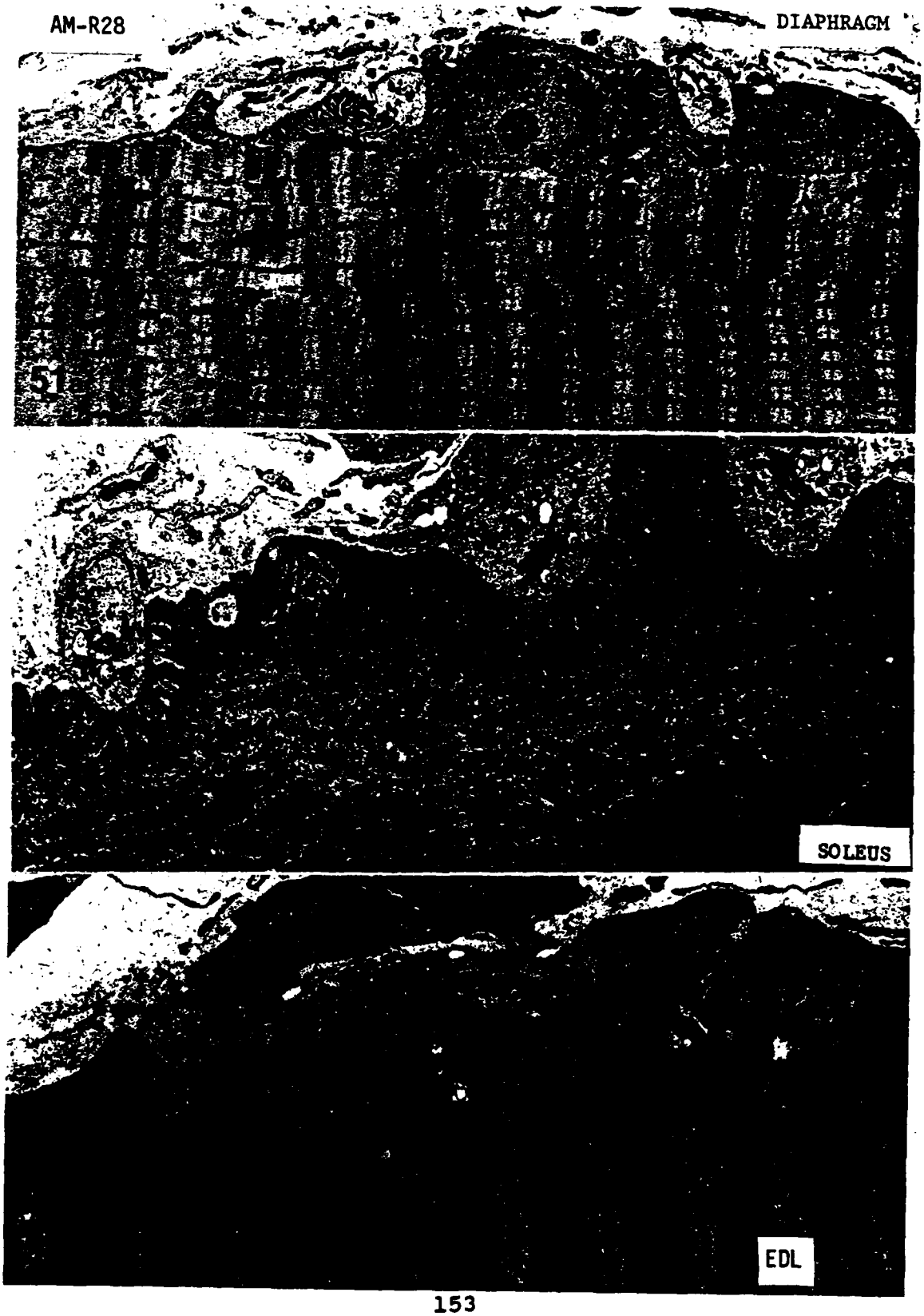

AM-R28
DIAPHRAGM
SOLEUS
EDL

Figure 52. Recovery of Diaphragm, Soleus, and EDL Neuromuscular Junctions 56 days After an Acute Moderate Dose of Physostigmine (0.1 LD_{50}). Myofibers, mitochondria, and nerve terminals appear normal. The few swollen mitochondria are believed to represent fixation artifacts.

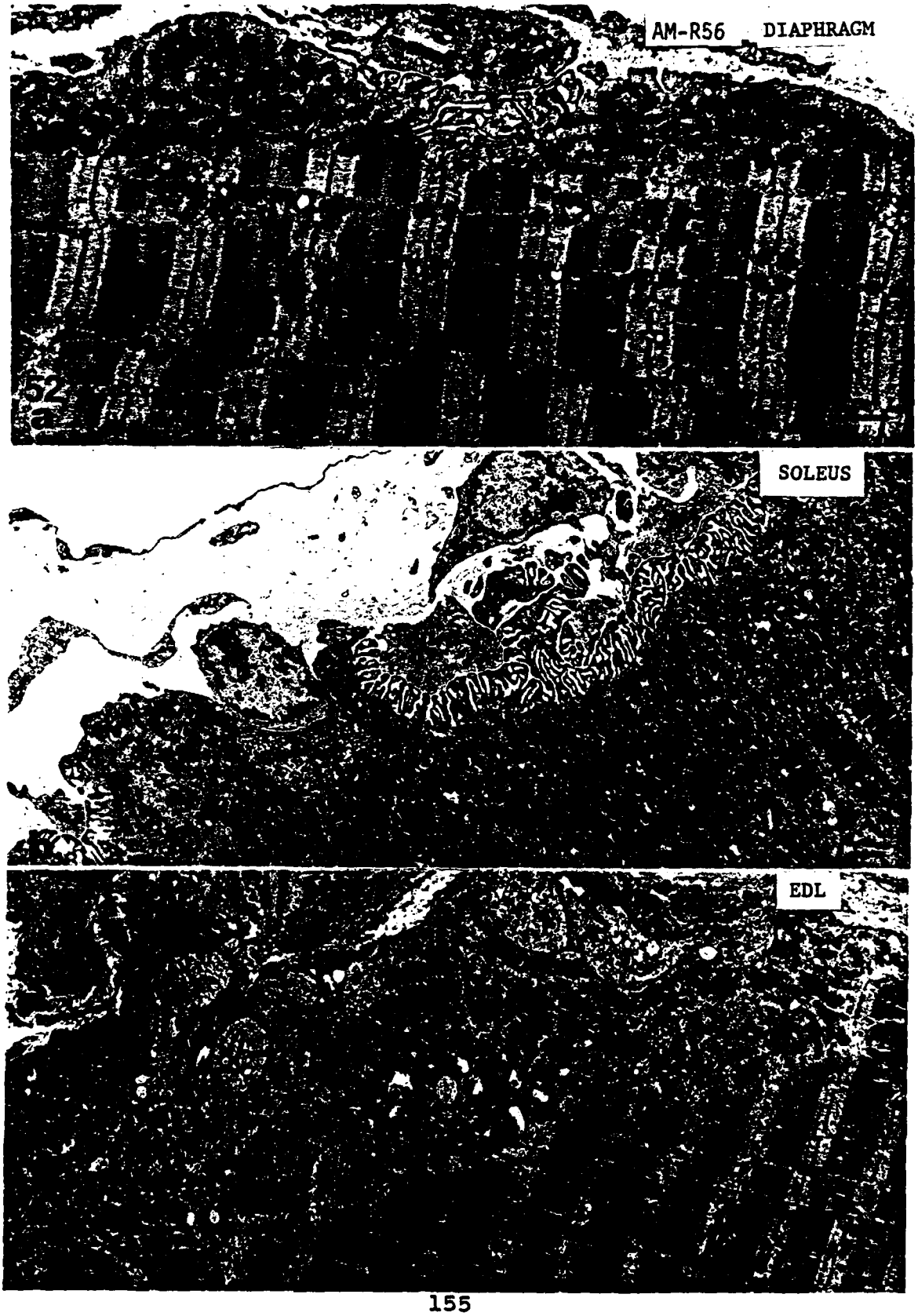

AM-R56 DIAPHRAGM
SOLEUS
EDL

Figure 53. Recovery of Diaphragm, Soleus, and EDL Neuromuscular Junctions 1 day After an Acute Low Dose of Physostigmine (0.01 LD_{50}). Myofibrils, mitochondria, and nerve terminals appear normal. Swollen mitochondria in some nerve terminals are probably fixation artifacts.


DIAPHRAGM
AL-R1
SOLEUS
EDL

Figure 54. Recovery of Rat Diaphragm, Soleus, and EDL Neuromuscular Junction 7 Days After an Acute Low Dose of Physostigmine (0.01 LD_{50}). Except for hypoxic mitochondria (Figure 54a,c), myofibers, mitochondria, and nerve terminals appear normal.

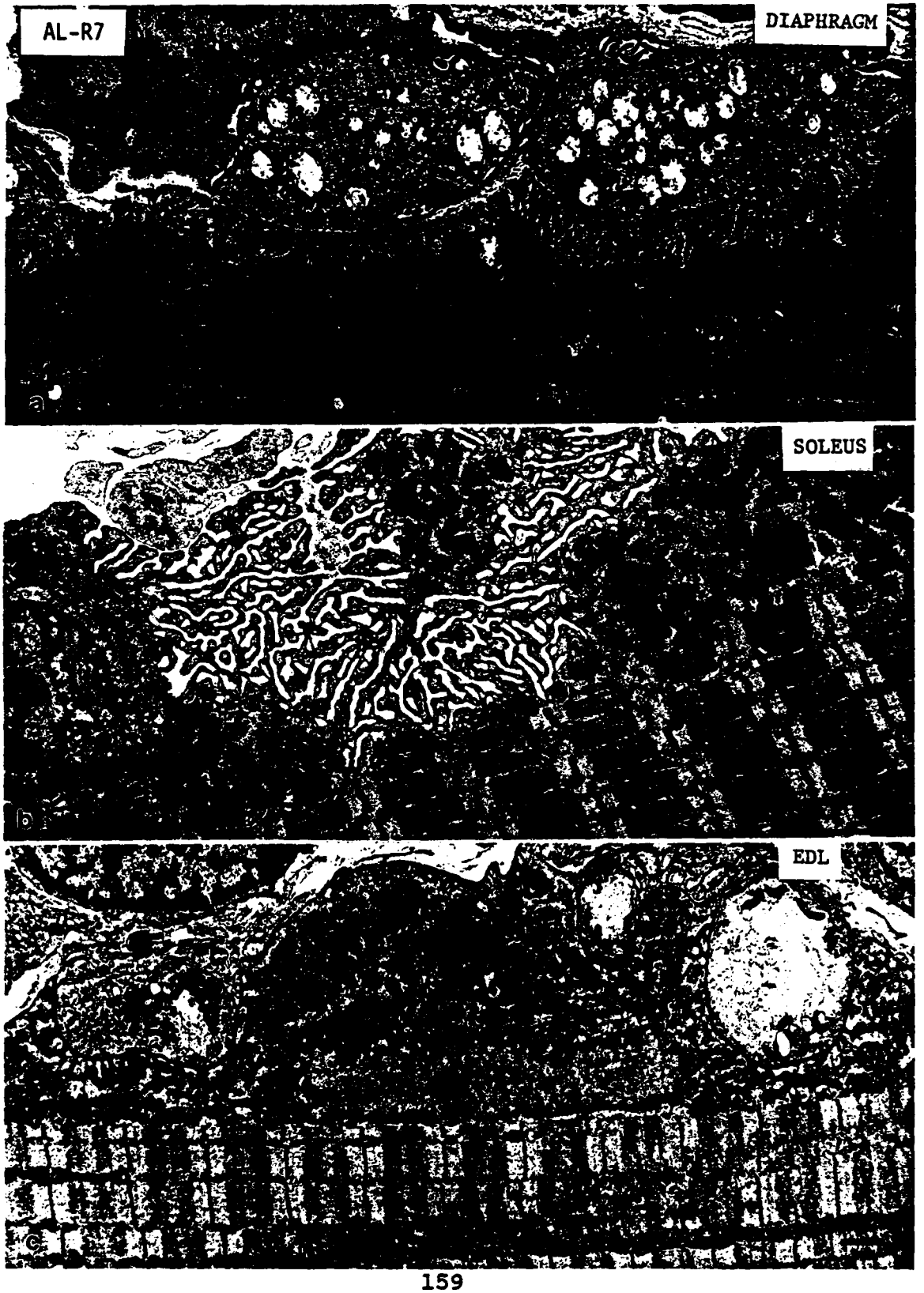

AL-R7
DIAPHRAGM
SOLEUS
EDL

Figure 55. Recovery of Rat Diaphragm, Soleus, and EDL Neuromuscular Junctions 14 Days After an Acute Low Dose of Physostigmine (0.01 LD_{50}). Endplates have no recognizable alterations.

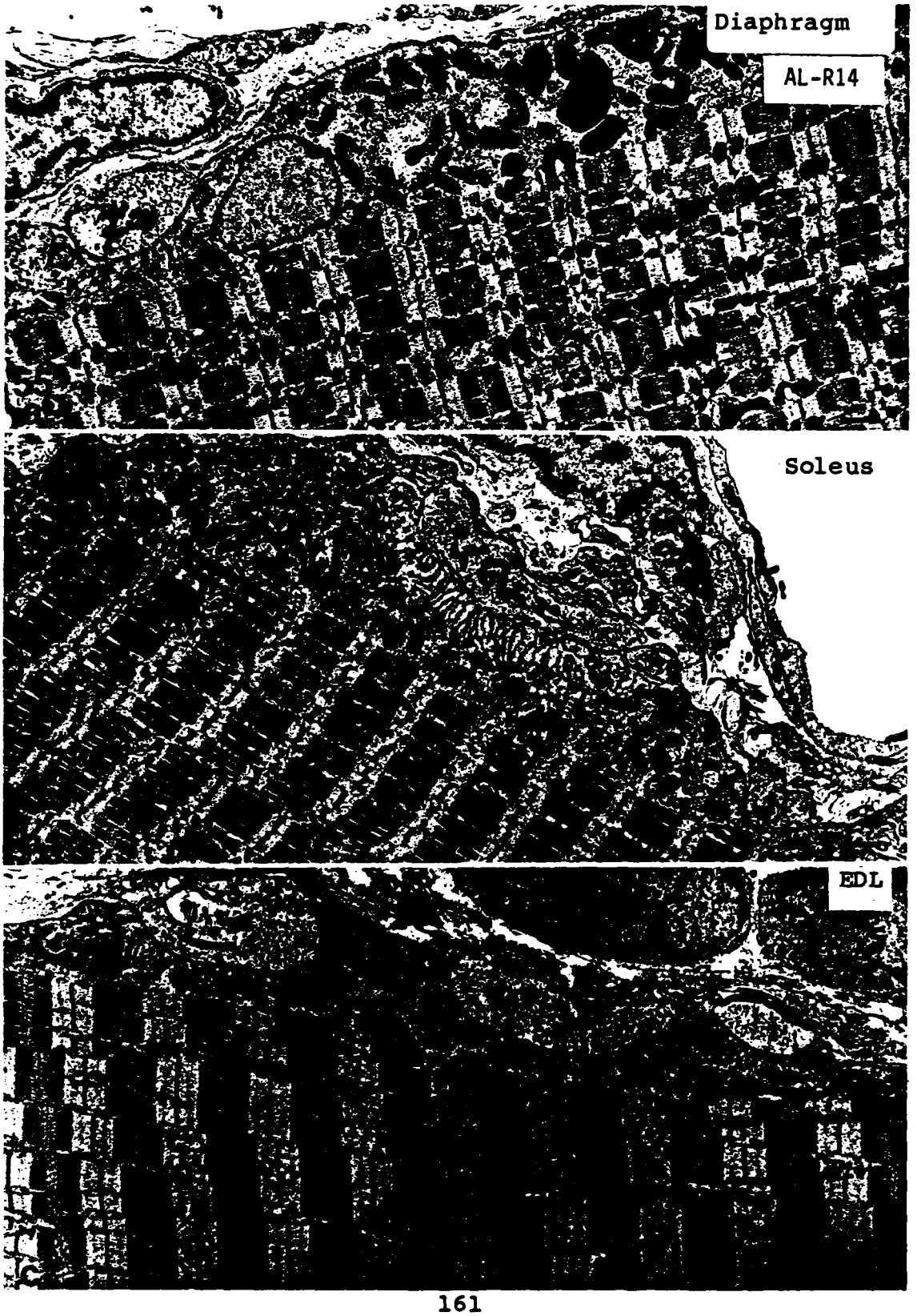

Diaphragm
AL-R14
Soleus
EDL

Figure 56. Recovery of Diaphragm, Soleus, and EDL Neuromuscular Junctions 28 Days After an Acute Low Dose of Physostigmine (0.01 LD_{50}). Except for hypoxic mitochondria, endplates appear normal.

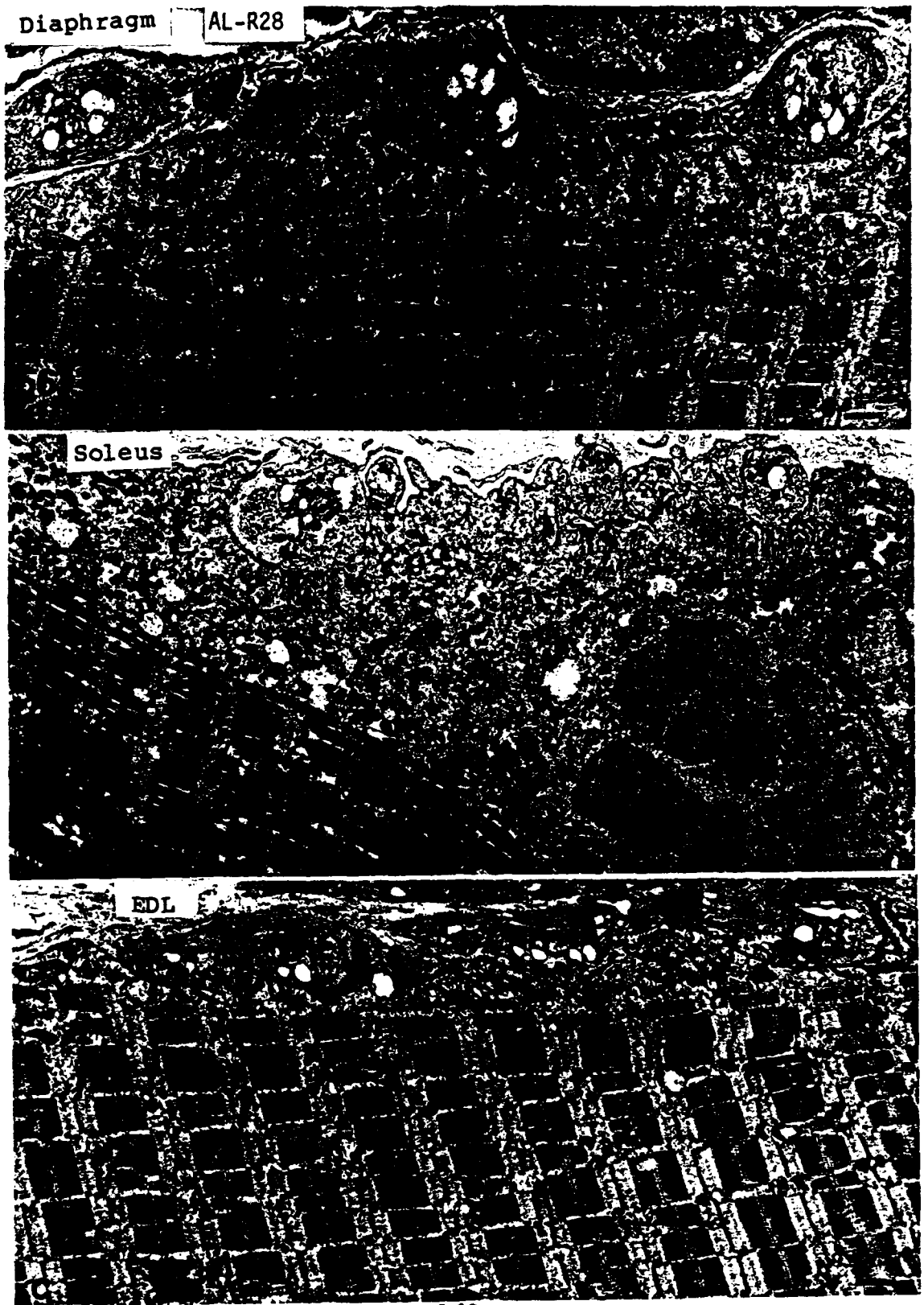

Diaphragm
AL-R28
Soleus
EDL

Figure 57. Recovery of Diaphragm, Soleus, and EDL Neuromuscular Junctions 56 Days After an Acute Low Dose of Physostigmine (0.01 LD_{50}). Except for slightly to severely hypoxic mitochondria, endplates appear normal.

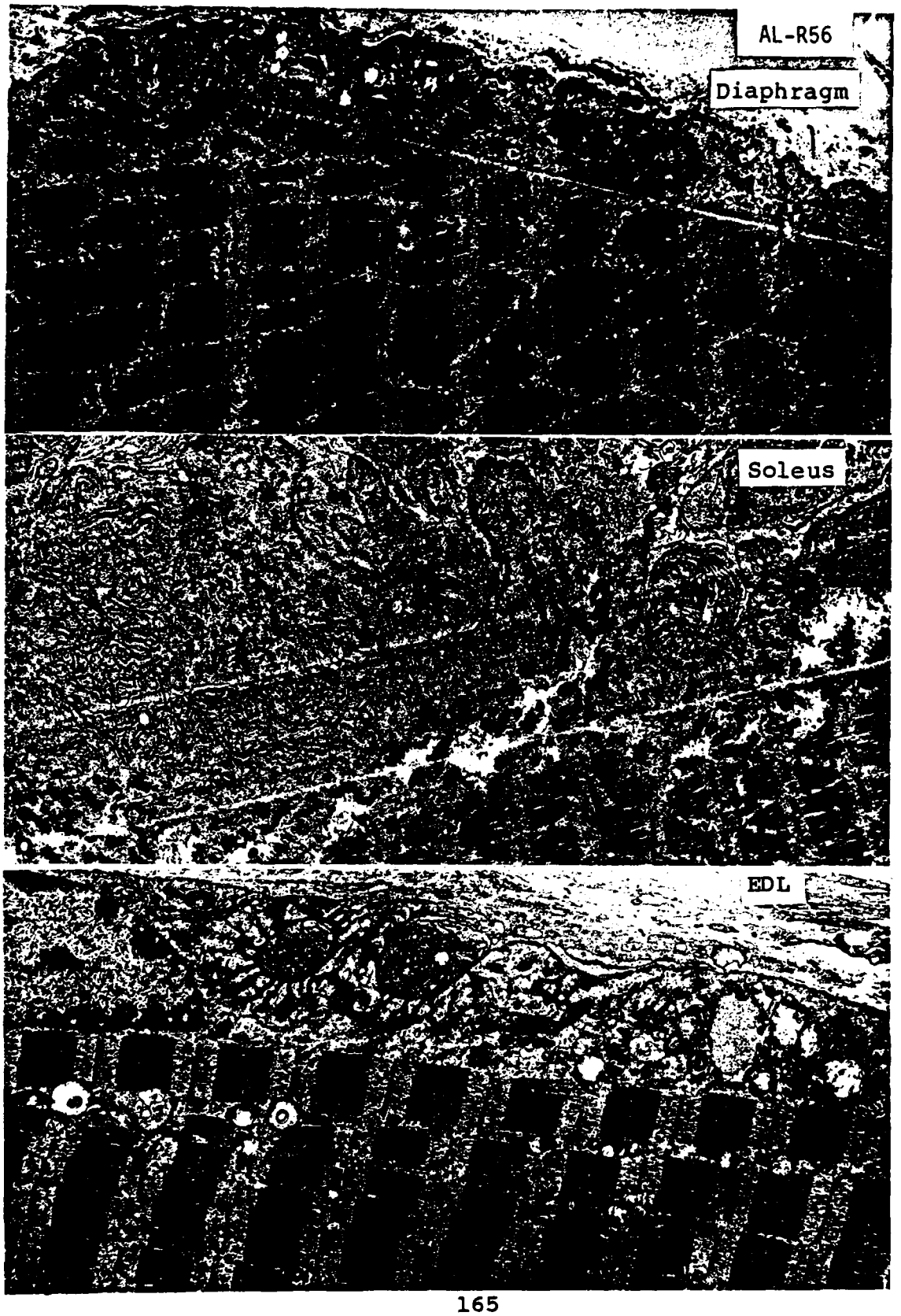

AL-R56
Diaphragm
Soleus
EDL

Figure 58. Recovery of Diaphragm and EDL Neuromuscular Junctions 1 Day After an Acute Very Low Dose of Physostigmine (0.001 LD_{50}). Myofibrils, mitochondria, and nerve terminals appear normal. Thus, no signs of delayed toxicity were observed in the muscles of animals treated with low doses of physostigmine.

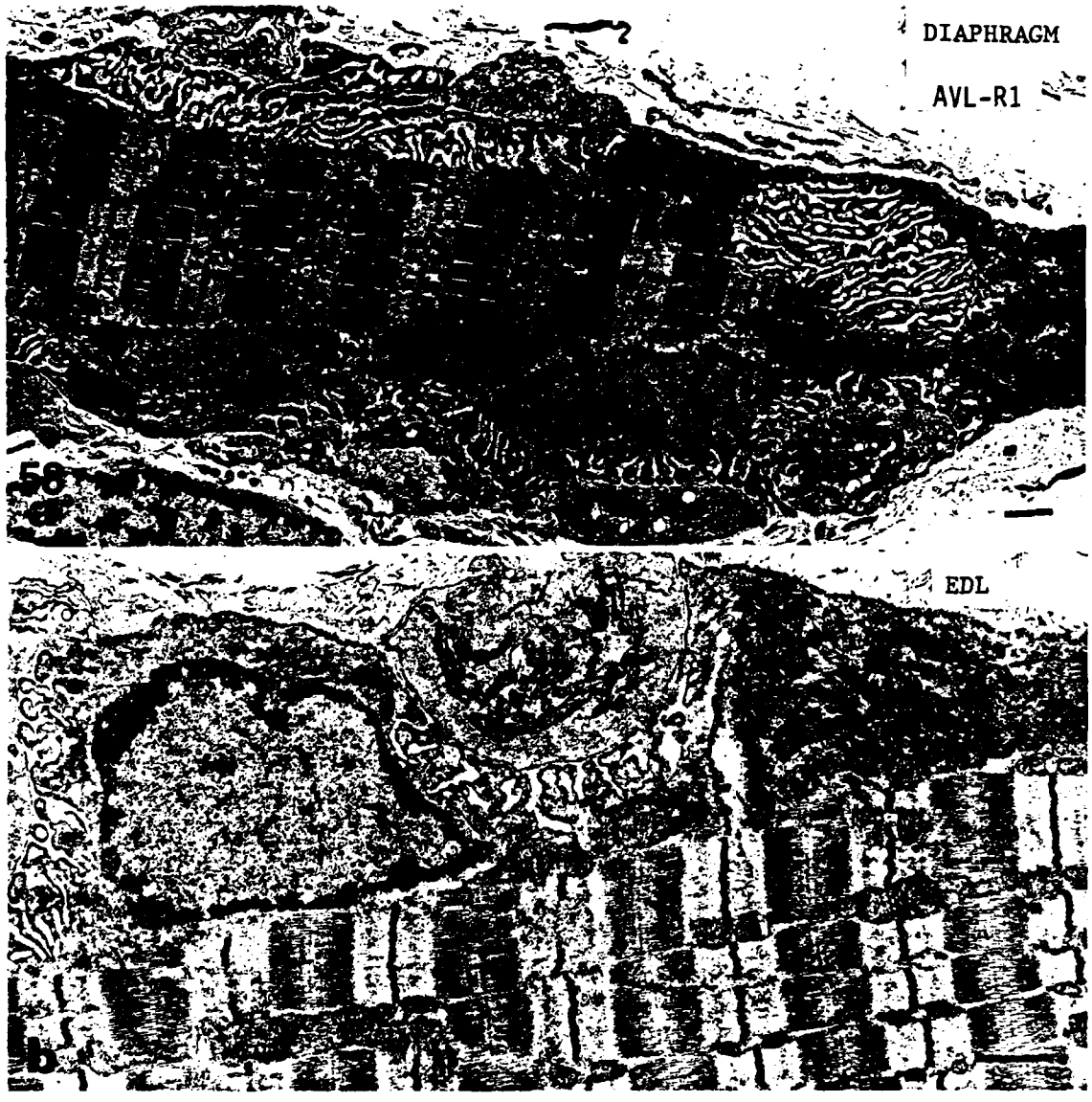

DIAPHRAGM
AVL-R1
EDL

Figure 59. 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).

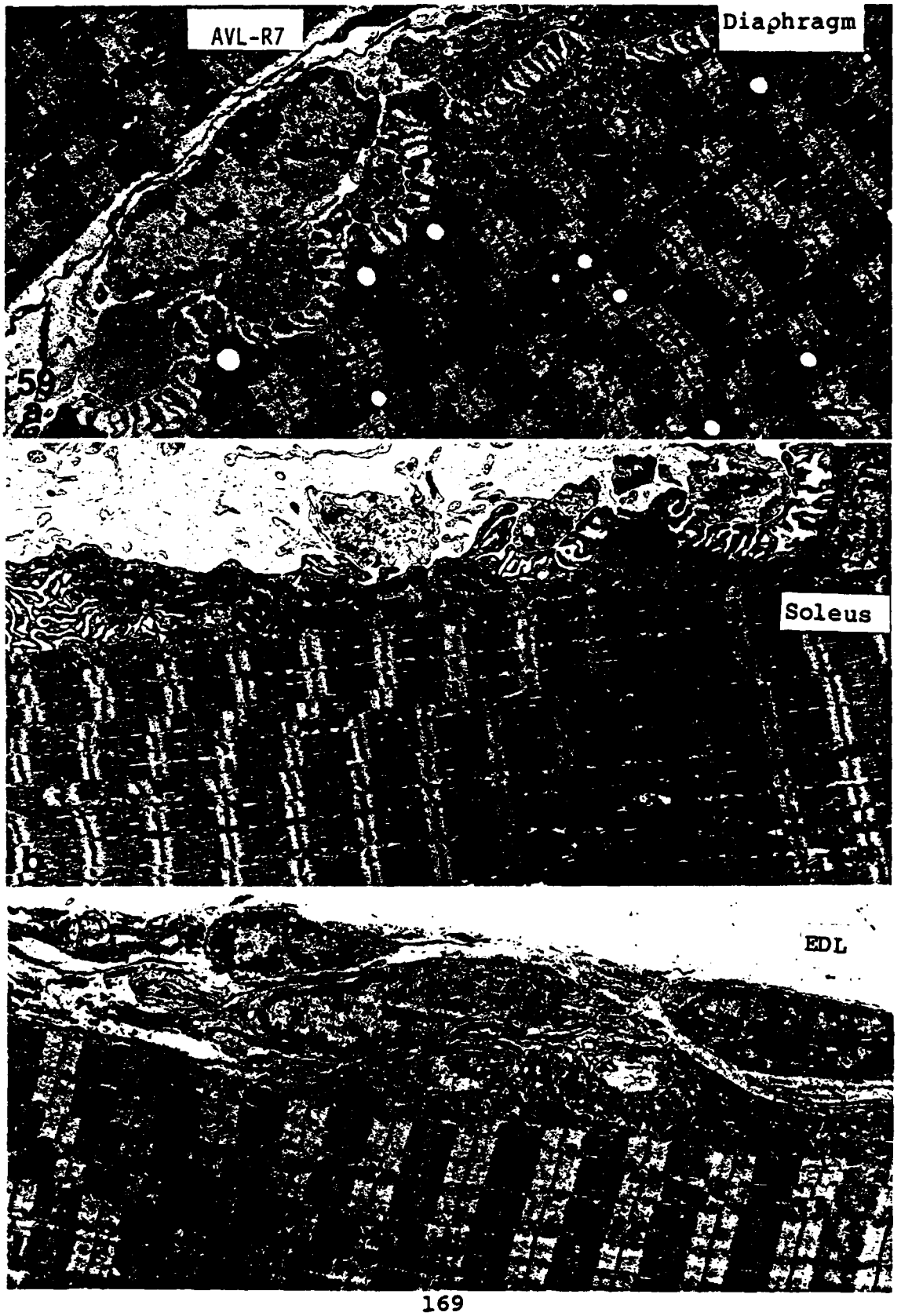

AVL-R7
Diaphragm
Soleus
EDL

Figure 60. Recovery of Diaphragm, Soleus, and EDL Neuromuscular Junctions 14 Days After a Very Low Dose of Physostigmine (0.001 LD_{50}). Endplates appear normal.

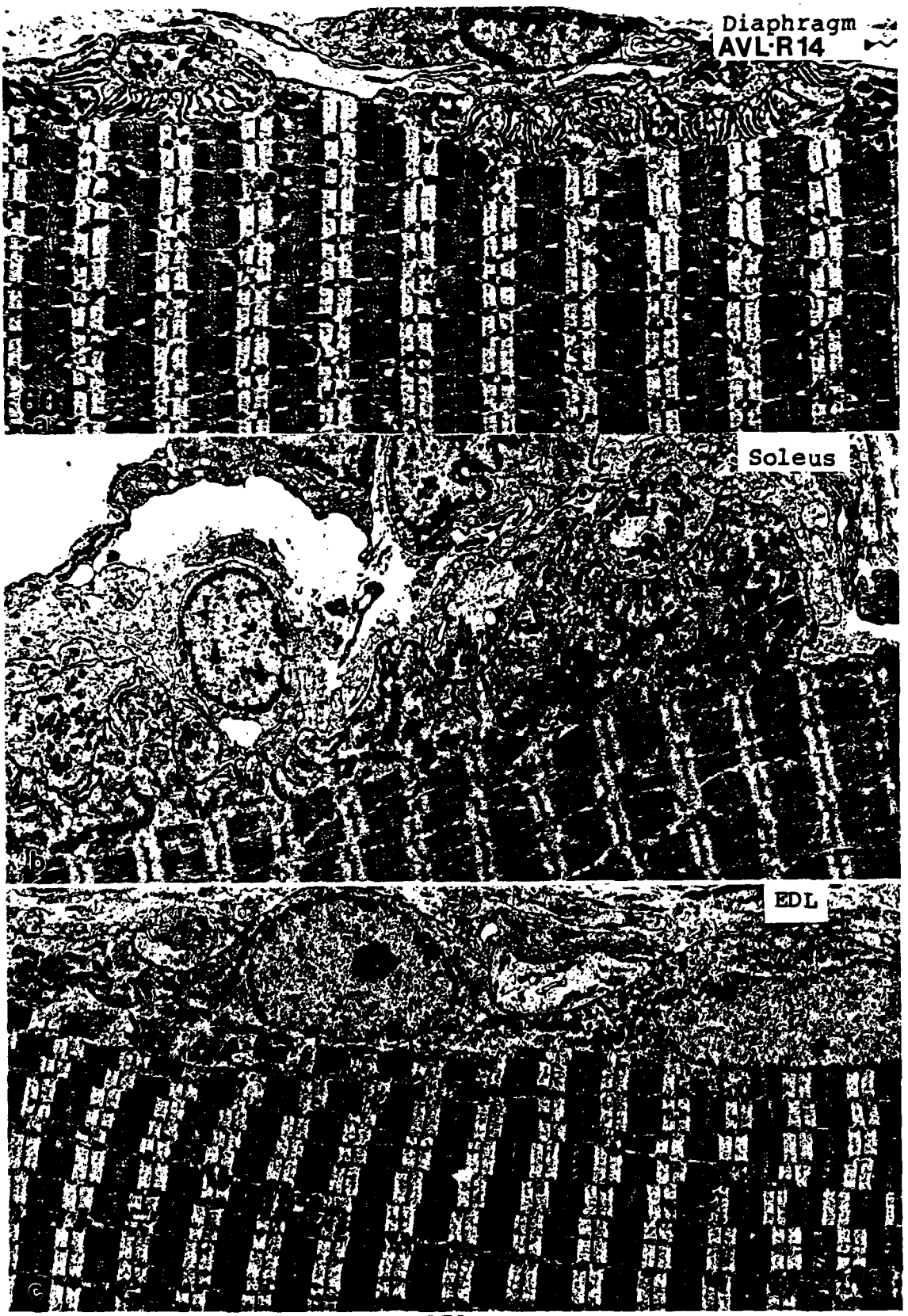

Diaphragm
AVL-R14
Soleus
EDL

Figure 61. Recovery of Diaphragm, Soleus, and EDL Neuromuscular Junctions 28 Days After a Very Low Dose of Physostigmine (0.001 LD_{50}). Endplates appear normal.

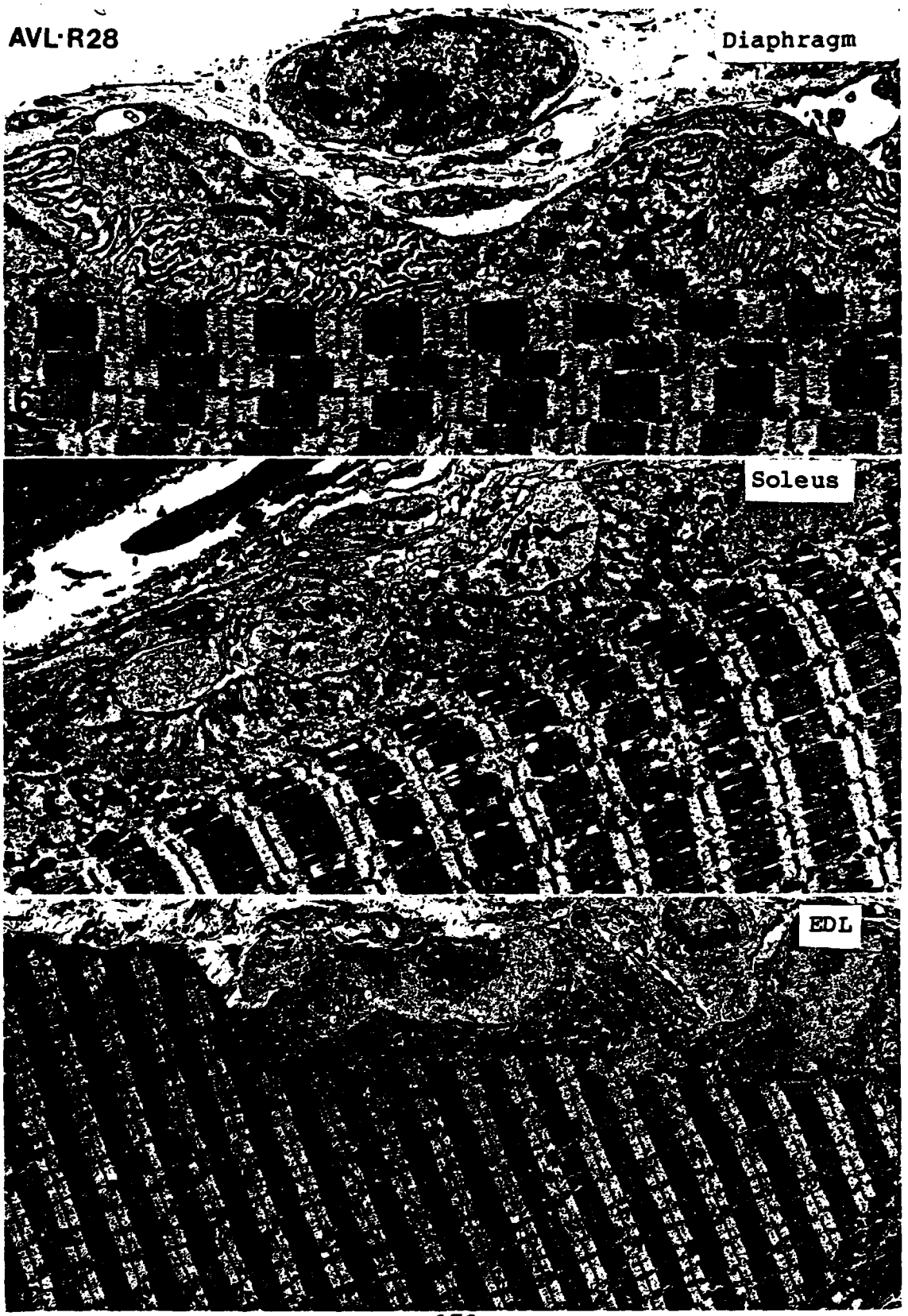
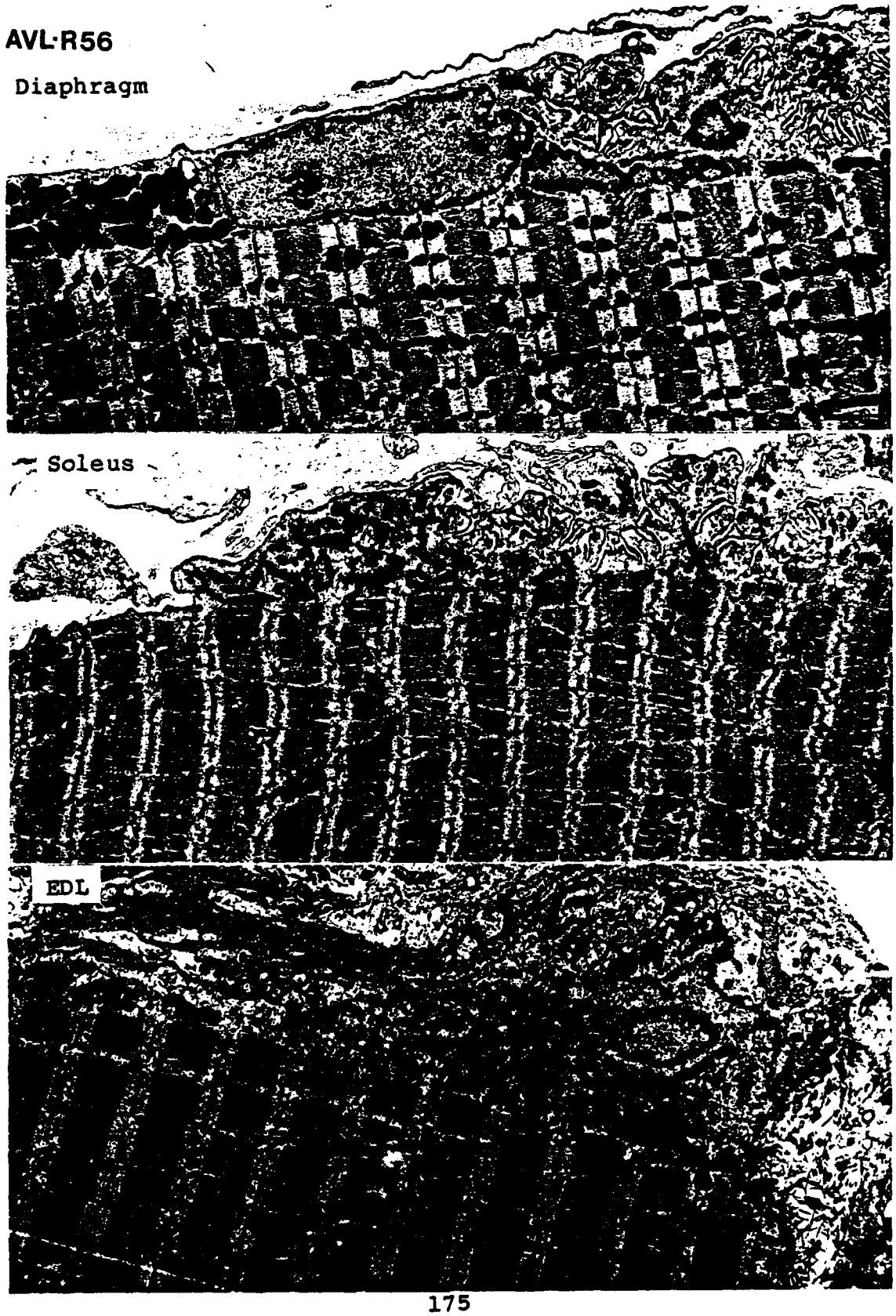

AVL·R28
Diaphragm
Soleus
EDL

Figure 62. Recovery of Diaphragm, Soleus, and EDL Neuromuscular Junctions 56 Days After a Very Low Dose of Physostigmine (0.001 LD_{50}). Endplates appear normal.


AVL-R56
Diaphragm
Soleus
EDL

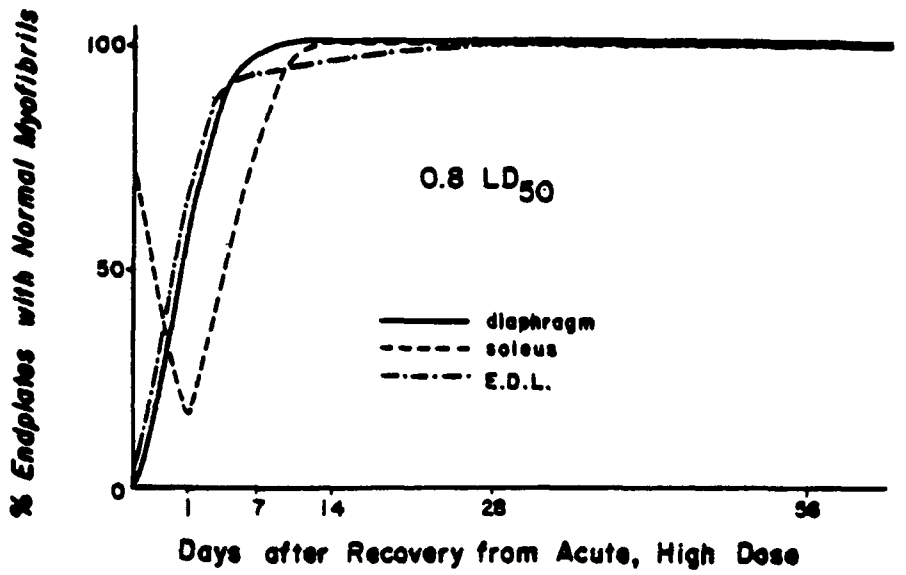


Figure 63. Graph Depicting Time Course of Recovery from Ultrastructural Alterations Caused by Acute High-dose Exposure to Physostigmine. Temporal response curves reflect relative number (%) of fibers with normal subjunctional myofibrils in diaphragm, soleus, and EDL muscles after high-dose (0.8-1.1 LD_{50}) injections of physostigmine. One hundred percent of diaphragm and soleus myofibers showed supercontraction at 30 minutes PI. By 7 days, virtually all myofibers appeared normal. In contrast, EDL myofibers had more damage at 1 day PI and had a slightly slower rate of recovery. However, virtually all EDL fibers were completely recovered by 14 days PI.

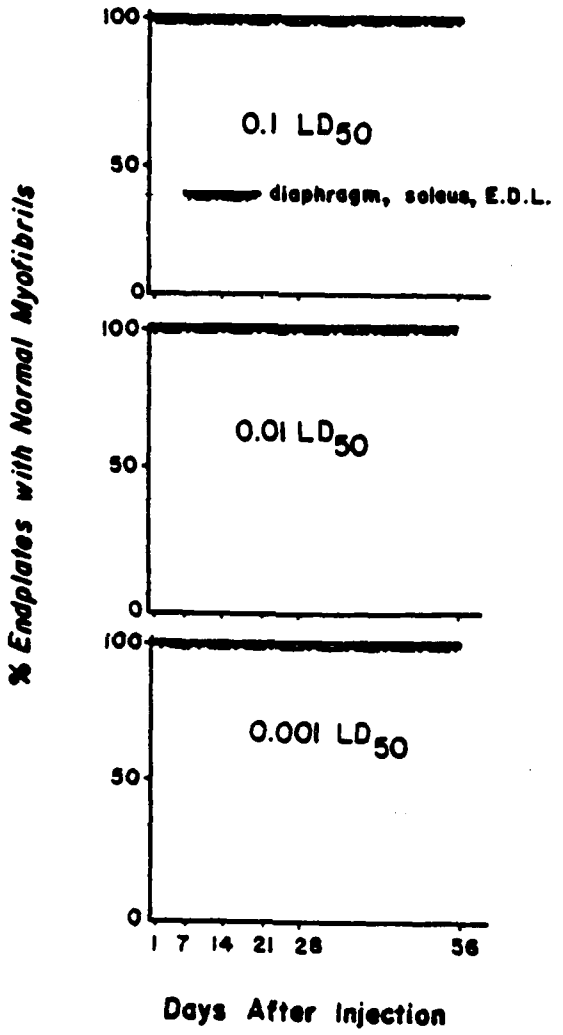


Figure 64. Graph Depicting Time Course of Recovery from Ultrastructural Alterations Caused by Acute Moderate to Very Low Doses of Physostigmine. At lower doses of physostigmine (0.1-00.1 LD_{50}), all diaphragm, soleus and EDL myofibers were normal at all stages of recovery.

K. EFFECTS OF SUBACUTE EXPOSURE

1. A Constant Rate of Physostigmine Infusion Yields a Constant Rate of Serum ChE Inhibition

For subacute exposure experiments in rats, preliminary experiments were performed to determine the doses and implantation regimens required to obtain and maintain low and high blood ChE enzyme inhibition levels (for example, 30% and 80%). Alzet pumps were filled with physostigmine at concentrations of 0.001 mg/ml to 1 mg/ml and pre-primed to deliver at a rate of 2.5 ul/hour. Whole blood ChE levels were monitored before implantation and at assigned intervals thereafter. The minipumps were implanted surgically in the rostral mid-back. (See **EXPERIMENTAL METHODS** for monitoring schedules.) Initially, the desired level of 30% ChE inhibition was obtained after 24 hours of exposure at 0.1 mg/ml, delivered at a rate of 60 ul/day (0.006 mg/day or ca. 0.03 mg/kg/day), and that value of inhibition was maintained for up to 14 days of subacute exposure. However, 80-90% inhibition was obtained initially at subacute delivery levels of 0.3 mg/ml (0.018 mg/day or 0.1 mg/kg/day), but the 80% inhibition level was not maintained. (By comparison, these delivery rates correspond to approximately 0.005 and 0.015 LD_{50} per day for 30% and 80% inhibitions.) The lower subacute dose level was reasonable for that predicted to maintain the desired inhibition level (i.e., the daily dose was equivalent to 2-3 times a single acute dose required to obtain an initial 35% blood ChE inhibition level). The higher subacute dose, although determined empirically, was at least one order of magnitude lower than the predicted value, and was inconsistent with all acute exposure data (i.e., was about 1/3 of a single acute dose required to achieve the same level of ChE inhibition). However, at significantly higher dose levels, pre-primed pumps caused signs of severe toxicity, including the deaths of several animals. The sources of these unexpected results required further investigation prior to initiating the final subacute exposure experiments.

The failure of the pumps to maintain 80% inhibition was consistent with initial near-saturation of blood ChE, but with subsequent redistribution and/or re-equilibration with other tissue esterases. The source of the anomalous decrease in blood ChE levels was investigated as follows: 1) To ascertain whether pumping rates decreased after the first day, volumetric and weight measurements were made of the residual pump contents, whereas 2) to ascertain whether physostigmine potency decreased rapidly following implantation, the contents of the pumps were examined for telltale changes in color associated with loss of biological activity (39), and 3) residual pump contents were injected into untreated animals and the resulting ChE inhibitions were measured. Finally, to ascertain whether absorption was compromised, 4) all rats were examined for necrosis or other signs of surgical fault at the implant site. Thus, we determined that the drift toward normal values did not occur because of detectable pump failure, inactivation of physostigmine within the pump, or surgical failure. The major sources of error were identified as arising from the use of pre-primed pumps rather

than unprimed pumps. [The initial high rate of drug delivery from pre-primed pumps was equivalent to an initial acute high dose injection. In contrast, the gradual onset of delivery from unprimed pumps resulted in lower initial ChE inhibitions and concomitant lowered toxicity. Thus, the use of unprimed pumps filled with much higher concentrations of physostigmine yielded lowered toxicity while simultaneously maintaining high ChE inhibition.]

After modification of procedures (primarily the use of partially primed pumps), levels of 40% and 80% ChE inhibition (see Figs. 65, 66) were obtained and maintained for up to 14 days using mini-osmotic pumps filled at concentrations of 0.25 mg/ml and 25 mg/ml (i.e., 100-fold differences in concentrations. For comparison, if these doses had been administered as single acute doses, these values would be equivalent to a delivery of 0.003 LD_{50}/hour and 0.3 LD_{50}/hour, or 0.72 and 7.2 LD_{50}/day.) It is interesting that this 100-fold difference in drug delivery between the two subacute exposure doses yielded only a 2-3-fold difference in blood ChE inhibition. This difference is roughly consistent with data from acute exposure experiments, which suggest a log-linear relationship between physostigmine exposure and blood ChE inhibition. (See **RESULTS, Sections A-D.**) Nevertheless, we note that blood ChE inhibitions were less than predicted at high doses. Consequently, confirmation of potency of the residual contents of each pump was performed as described in **EXPERIMENTAL METHODS** and in the paragraph above. The acetic acid carrier solutions proved very effective in maintaining physostigmine potency and/or ChE inhibition for the entire exposure period.

The implantation of control Alzet pumps (which contained buffer but not physostigmine) and their removal had no observable effect on blood ChE (**Figs. 65,66**, see control values).

2. Behavioral Response to Subacute Exposure

Upon recovery from the ether anesthesia (usually within 10 minutes), the animals showed severe toxic reactions only in the rats implanted with high-dose mini-pumps. Signs of drug toxicity included prolonged respiratory distress, muscle fasciculations, bloody tears and muscle spasms. To minimize stress-induced damage during the initial hours of exposure, the room lights were darkened and the rats were left alone and undisturbed. At 24 hours, when the second blood sample was taken, the high-dose rats remained hypersensitive and vocal, but showed no respiratory distress or bloody tears. By that time, they were also mobile and were eating and drinking. During the next 13 days, the only obvious effects of the high-dose subacute exposures were a gradual weight loss and a continuing malaise (i.e., rats were subdued and had reduced muscle activity). Other long-term behavioral and gross physiological effects were not detectable in high-dose animals 7 days or more after Alzet pump implantation. In contrast, the low-dose and control animals steadily gained weight and showed no signs of drug toxicity.

It is interesting that during the stress of prolonged physiological monitoring (see Section K.3., below), some animals

in the high-dose group re-developed obvious signs of physostigmine toxicity after about 1 hour of recording. Presumably, this represented synergistic effects of stress and increased neuromuscular activity on physostigmine toxicity. In contrast, low-dose animals showed no altered behavioral response at any time during physiological recording.

For the high-dose group, blood ChE inhibitions of 70-80% were maintained for 14 days (in one case up to 21 days). After the Alzet minipumps were removed, ChE enzyme levels returned to normal or above normal and remained at that level for the entire 28-day recovery period.

For the low-dose group (Fig. 65), 35-50% ChE inhibitions were obtained in most animals and maintained for up to 14 days. A second anomalous group, which showed no apparent enzyme inhibition at the same dose level, was considered separately in this study. This group was presumed to result from either a) pump failure in a few animals, or b) low initial control values. (The latter explanation is favored because those few animals responded to pump removal by a rebound of ChE level to about 160% of normal. If the latter value is presumed to be the true control value, the enzyme inhibitions measured in the anomalous responders were near the projected 30-50% level.) In addition, some animals developed thickened capsules around the pump implants. However, variation in blood ChE did not correlate with capsule formation. Regardless, both low dose groups (ChE responders and ChE-non responders) were identical in their lack of ultrastructural alterations at all durations of exposure and in all recovery stages.

3. **Physiological Effects of Subacute Exposure are Minimal.**

Effects of subacute physostigmine administration on <u>in vivo</u> muscle contractility were obtained from 22 rats. Ten animals had either high- or low-dose Alzet pumps inserted for 3, 7 or 14 days before testing. An additional 10 rats had high or low dose pumps implanted for 14 days followed by 3, 7 or 14 days' recovery after pump removal (subacute-->recovery). Two additional rats were implanted with Alzet pumps containing acetic acid carrier solution and were designated controls. (These rats were in addition to controls prepared exclusively for ultra-structural analysis.)

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 3, 7, or 14 days of recovery) and compared to rats receiving sham-filled implants. (For ultrastructural analysis, additional rats were fixed following up to 28 days of recovery.) Blood samples taken either immediately or within 15 minutes after the start of recording gave average enzyme inhibition levels of 79% for high-dose-->non-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 immediately before fixation.

During surgical preparation and the first hour of

recording, none of the rats from either the low-dose-->no recovery or the high-dose-->recovery group showed any overt signs of physostigmine intoxication (i.e., no muscular fasciculations, respiratory difficulty, chromodacryorrhea, etc.). Likewise, high-dose-->non-recovery animals that were tested either with pumps still in place or after pump removal initially showed no signs of physostigmine toxicity. However, toxic symptoms did appear in several animals later in the recording session. The latter animals showed enzyme inhibition levels >80%, even up to 2 hours after pump removal.

Average EDL twitch tensions, measured during the first 10 minutes of recording, are summarized in Table 3. The overall average twitch tension for all subacute exposure regimens was 28 ±4 g, (i.e., not significantly different from averages for any of the subgroups in Table 3 or from the normal population value of 30 g ±7 g obtained during the acute exposure experiments).

TABLE 3. Average <u>In Vivo</u> EDL Twitch Tension With and Without Subacute Administration of Physostigmine

Treatment	Twitch Tension (g)
High dose/Non-recovery	28 ±2 (N=10)
High dose/Recovery	26 ±8 (N=5)
Low dose/Non-recovery	28 ±9 (N=3)
Low dose/Recovery (3-14 days)	29 ±6 (N=5)
Control Population	30 ±7 (N=28)

PSCs were measured during the first 10-15 minutes of recording and again 15-20 minutes later. So that a larger control population could be used for comparison to the subacute exposure animals, data from the acute exposure physiology experiments were included. [It had already been demonstrated (see **RESULTS, Section N.1**) that the PSCs measured at the end of these experiments (45-70 minutes PI) were completely recovered (i.e., not significantly different) when compared to PSCs measured prior to physostigmine administration.]

For the Alzet pump animals (both high- and low-dose, recovery and non-recovery), PSCs measured during the first 10-15 minutes of recording were not significantly different ($P<.01$) from the control population. These early PSCs also showed no significant difference ($P<.01$) between recovery and non-recovery rats. However, in both recovery and non-recovery high-dose animals, the EDL muscles appeared to fatigue more easily than normal and to lose some ability to sustain a contraction. This was demonstrated by significantly lower ($P<.01$) PSC values measured 15 to 20 minutes after the early PSCs. (Between the

early and late PSC measurements, a period of 20-30 minutes, the EDL was continuously stimulated at 0.1 Hz.)

4. Ultrastructure of Endplates Following Subacute Exposure

a. Controls

In well-perfused muscles from rats implanted with sham-filled mini-osmotic pumps (i.e., "implant controls"), myofibers from diaphragm, soleus and EDL muscles (Figs. 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. 67b, 68, 69c). Occasionally, junctional folds were extremely foreshortened (as shown previously in Fig. 11d) or appeared to be without associated nerve terminal profiles (Fig. 69a; 11a, 11c). (For interpretive drawing, see Fig. 19c.) 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.)

b. 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 was detected in any fibers at any exposure interval (Figs. 72-73). (The micrograph code "SM3," as an example, designates Subacute Moderate dose, 3-day exposure.) [In those samples where pre- or post-synaptic mitochondria were slightly swollen, mitochondria in Schwann cells and fibroblasts were equally affected. Since similar alterations were equally common in control endplates (Figs. 68a-c; 69a-c; 70a,b; 71a-c), such alterations are attributed to artifacts characteristic of glutaraldehyde fixation rather than to drug effect.] As in control endplates (Fig. 11d), occasional examples were noted of nerve terminal branches either without associated junctional folds (Fig. 72b) or with foreshortened junctional folds (Fig. 72b).

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 (Figs. 75, 76b,c). However, nerve terminals were often virtually depleted of synaptic vesicles and had increased numbers of coated vesicles and flattened cisternae (Fig. 75c, inset), similar to images of stimulated endplates as described by numerous others (see 54-56, 60, 61). 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. 76b), demonstrating that prolonged exposure to 80 ±10% ChE inhibition is at a near-threshold level for producing overt endplate cytopathology, and that endplate damage is exacerbated by prolonged neuromuscular activity (see Sections M-N).

At 14 days subacute exposure (Fig. 77), 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 (not shown). It is noteworthy that frothy or swollen mitochondria were not observed. The source of the subjunctional hypertrophy or edema may reflect either a) response to prolonged endplate depolarization and associated ion and water influx, b) increase in biosynthetic activity, c) fixation fault, d) myofiber damage due to prior physical trauma, and/or e) unknown factors. However, these ultrastructural data combined with evidence for weight loss and for induction of pathophysiology during electrophysiological monitoring indicate near threshold expression of toxic effects in these high-dose exposure experiments, and provide evidence for cumulative effects of the drug over the 14-day, high-dose exposure period. [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 fibers, d) different muscle use patterns in different rats, and e) or variable rates of osmotic pumping (i.e., "surges").] Finally, these data (summarized in Figs. 78, 79) are to be compared with acute exposure experiments in which 70% ChE inhibition was at (or just above) the toxic threshold for producing myofiber damage in the diaphragm (cf. Figs. 30, 31). However, the reduced damage following sustained ChE inhibition at significantly higher blood ChE inhibition levels (and at approximately 7.2 LD_{50}/day) suggests the occurrence of compensatory and/or protective mechanisms causing either a) a decrease in the number of postsynaptic ACh receptors or b) a decrease in transmitter release efficacy [i.e., decrease in quantal content of the miniature endplate potential (MEPP)].

In conclusion, subacute exposure at moderate doses (30-50% inhibition) does not produce supercontraction, nor is any other type of endplate damage apparent in thin sections examined by transmission electron microscopy. Therefore, extended low- to moderate-dose subacute exposure to physostigmine seems to be well tolerated by rats. In contrast, subacute exposures yielding 80 $\pm$10% sustained serum ChE inhibition appear to be near the threshold level for producing both pathophysiological and ultrastructural alterations at the neuromuscular junctions.

L. RECOVERY FROM SUBACUTE PHYSOSTIGMINE EXPOSURE

1. Physiology

For rats in the subacute-recovery experiments, at 3, 7, and 14 days after pump removal, EDL twitch tensions and early PSCs (see Section K.3, above) were normal. This would be expected because a) blood ChE inhibition levels had returned to 0% (ie, normal) and b) because these physiological properties had been altered minimally in the period before recovery. However (as discussed above in Section K.3), resistance to fatigue,as indicated by reduced late PSC values, was slightly lowered at high subacute doses. This was true for both recovery and non-recovery animals.

2. 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. 80b,c; 83b; 84a-c; 86c; 87b,c) and for artifacts of inadequate glutaraldehyde perfusion, which yield primary fixation by OsO_4 (e.g., the extracted cytoplasm in Figs. 86a, 87b), 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. (Data for recovery from acute and subacute exposures are presented graphically in Figure 88a,b.) Thus, we conclude that in rats, long-term effects on muscle ultrastructure and physiology appeared minimal following prolonged (subacute) exposure to relatively high doses of physostigmine. Moreover, recovery was rapid and complete following termination of subacute exposures that produced 14 days of sustained 40% and 80% serum ChE inhibition.

3. Dose-Response Effects and Recovery

No alterations were detected in any of the myofibers from either the low-dose-->recovery or high-dose->recovery groups. This suggests either that the few damaged soleus fibers in the high-dose-->non-recovery group (Fig. 76) resulted from initial surges in physostigmine delivery (osmotic pumping from partially primed pumps), damage from increased neuromuscular activity in some motor units, or other unknown factors. Since there were no differences in the low-dose vs high-dose recovery groups, a dose-response curve is not presented.

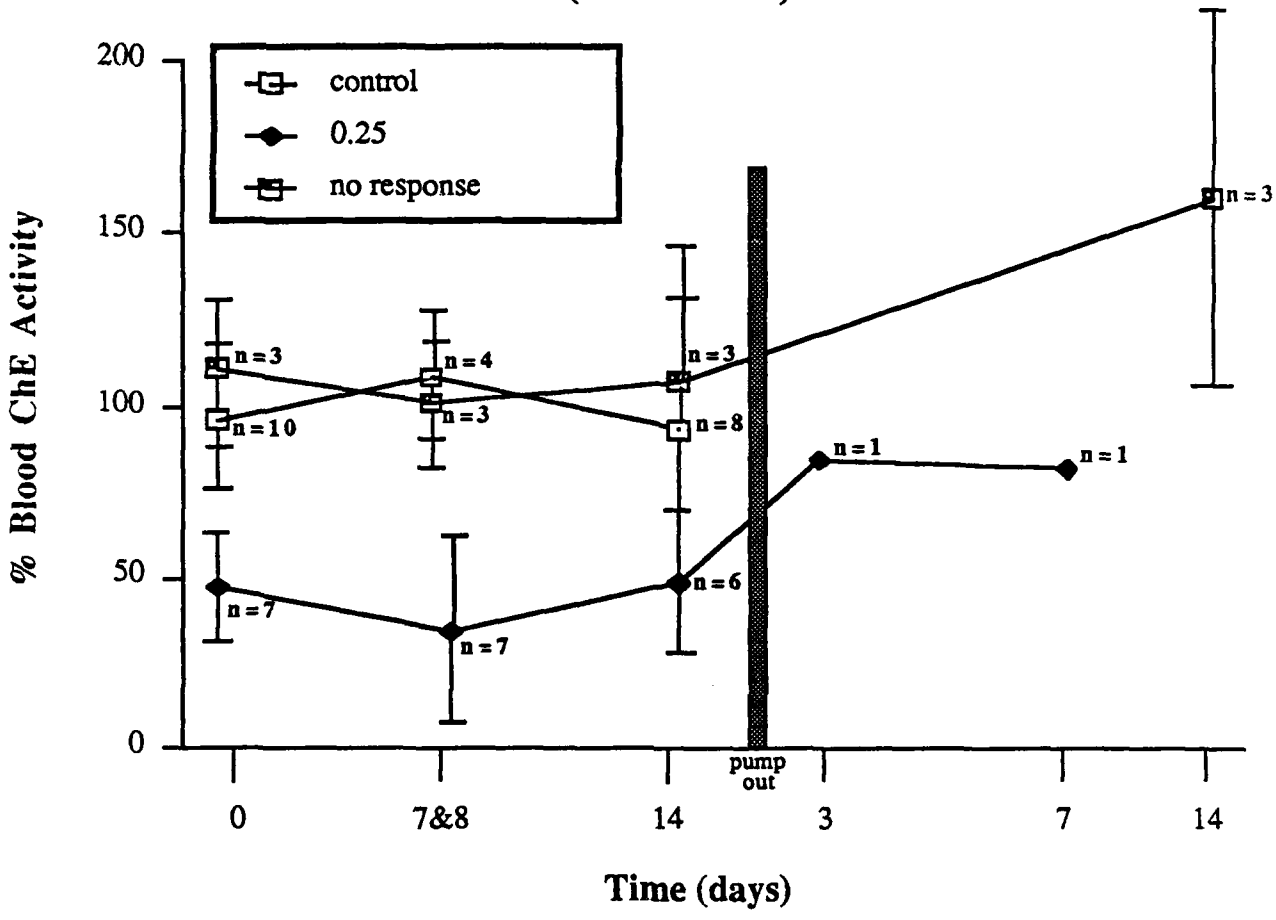


Figure 65. Blood Cholinesterase Inhibition of 40% Obtained by Subacute Exposure to 0.25 mg/ml Delivered at 25 ul/hour (Low Dose). The error bar represents one standard deviation. The return to normal ChE activity after Alzet pump removal ("pump out", vertical stippled bar) is consistent with little or no long term effect on blood ChE activity. (The "no response" group corresponds to the "anomalous responders" described in Section K.2, page 181.)

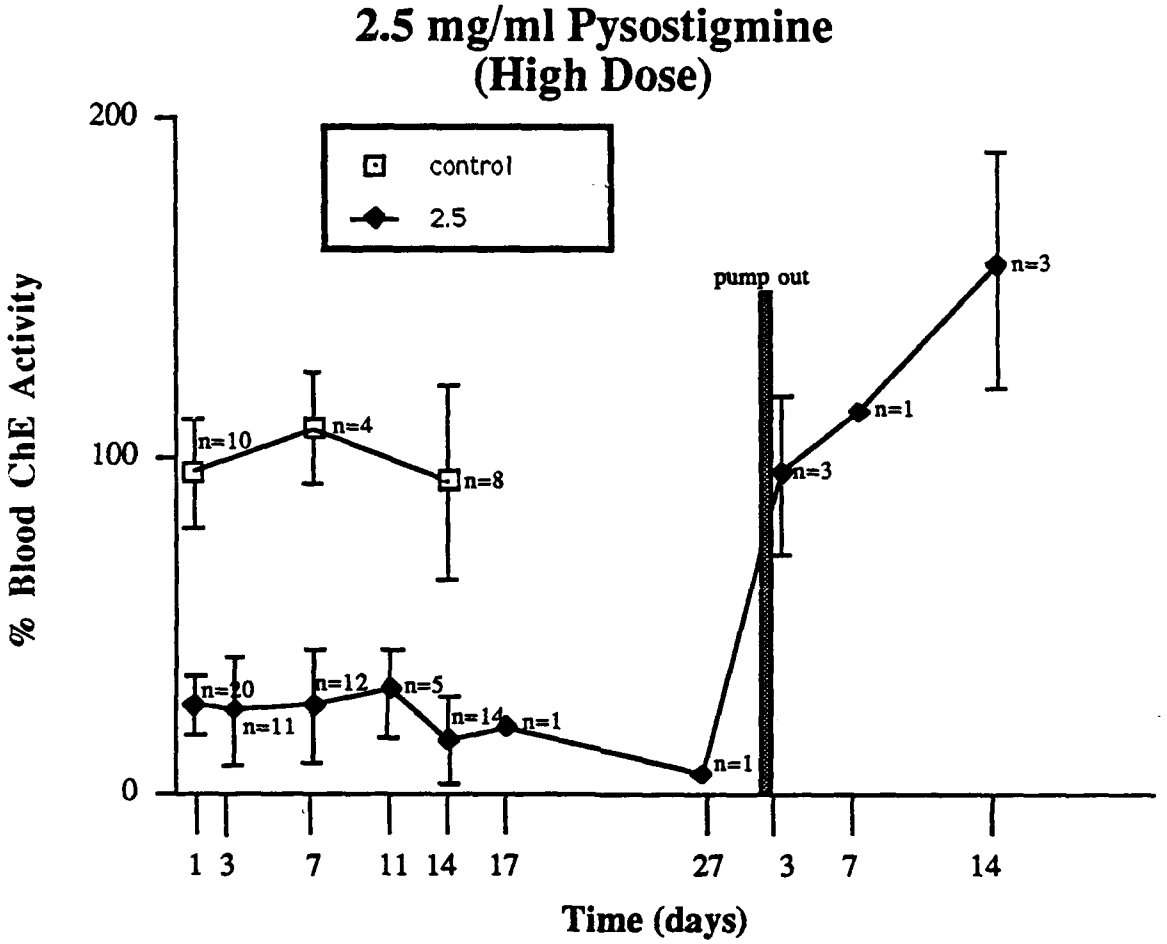


Figure 66. Blood Cholinesterase Inhibition of 80% Obtained by Subacute Exposure to 25 mg/ml Delivered at 25 ul/hour (High Dose). The error bar represents one standard deviation. The return to normal ChE activity within 3 days after Alzet pump removal is consistent with little or no long term effect on blood ChE activity following subacute high dose exposure to physostigmine. (The apparent "rebound" at 14 days after pump removal is consistent with long-term variations observed in control rats, Figures 1-7.) For the animals in which the pumps were heavily encapsulated by tissue, the inhibition of ChE activity was anomalously low (approximately 50% of that in animals that had non-encapsulated pumps).

Figure 67. Neuromuscular Junctions from Diaphragm, Soleus, and EDL Muscles 3 Days After Implantation of Alzet Pump Containing Sham Solution (SC3). (The code designates Subacute Control 3 days.) Except for a few swollen mitochondria, the endplates are normal.

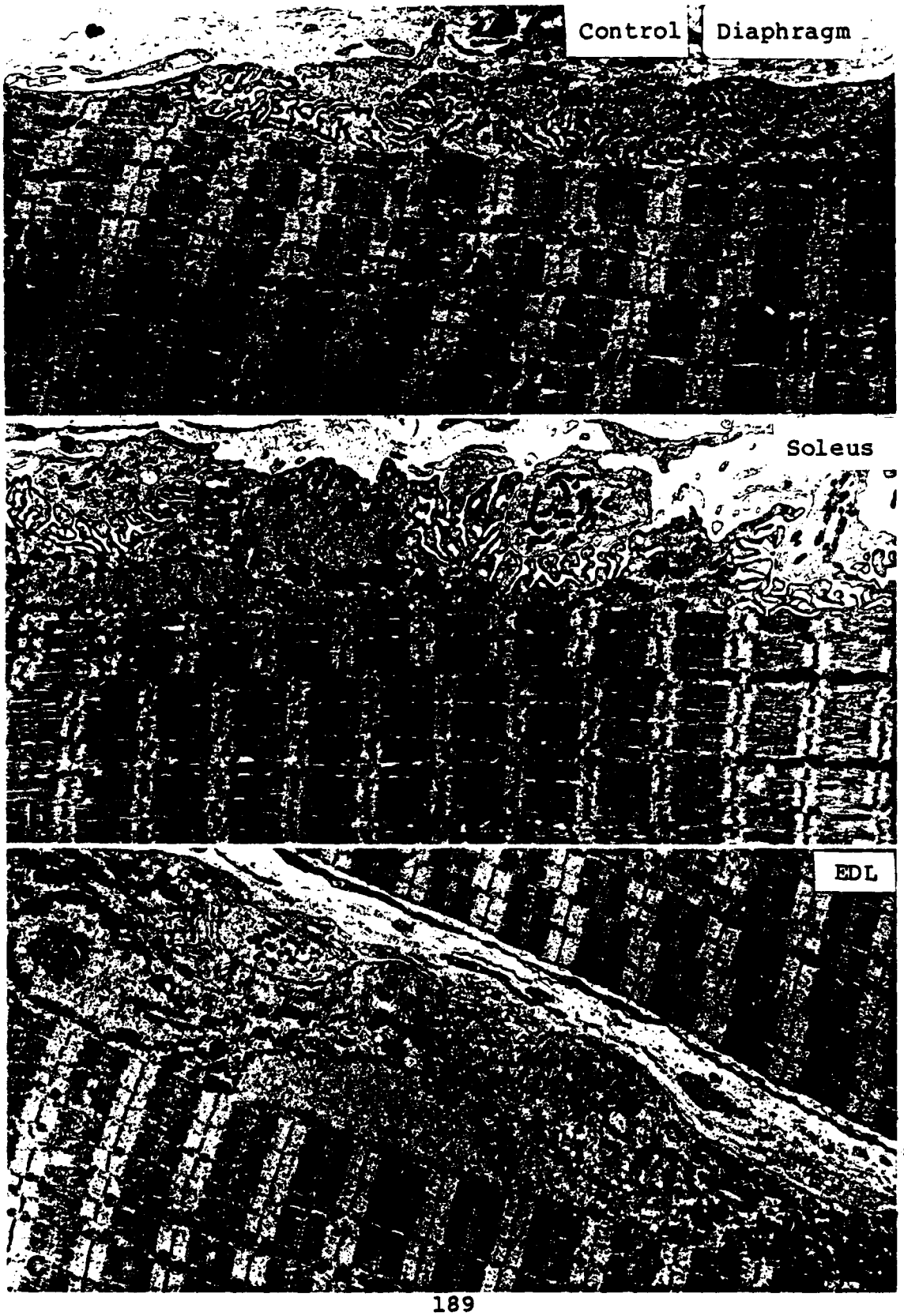

Control
Diaphragm
Soleus
EDL

Figure 68. Comparison of Neuromuscular Junctions from Diaphragm, Soleus, and EDL Muscles 14 Days After Implantation of Alzet Pump Containing Sham Solution (SC14). Swelling of pre- and postsynaptic mitochondria is characteristic of fixation hypoxia. Note, also, the junctional folds without associated nerve terminal profiles (arrowheads, Figures 68a, 69a, 70b).


Diaphragm
Control
68
a
Soleus
b
EDL

Figure 69. Comparison of Neuromuscular Junctions from Diaphragm, Soleus, and EDL Muscles 3 Days After Removal of Alzet Pump Containing Sham Solution (14-Day Implant) (SC14-3). Except for an occasional swollen mitochondrion, endplates are normal.

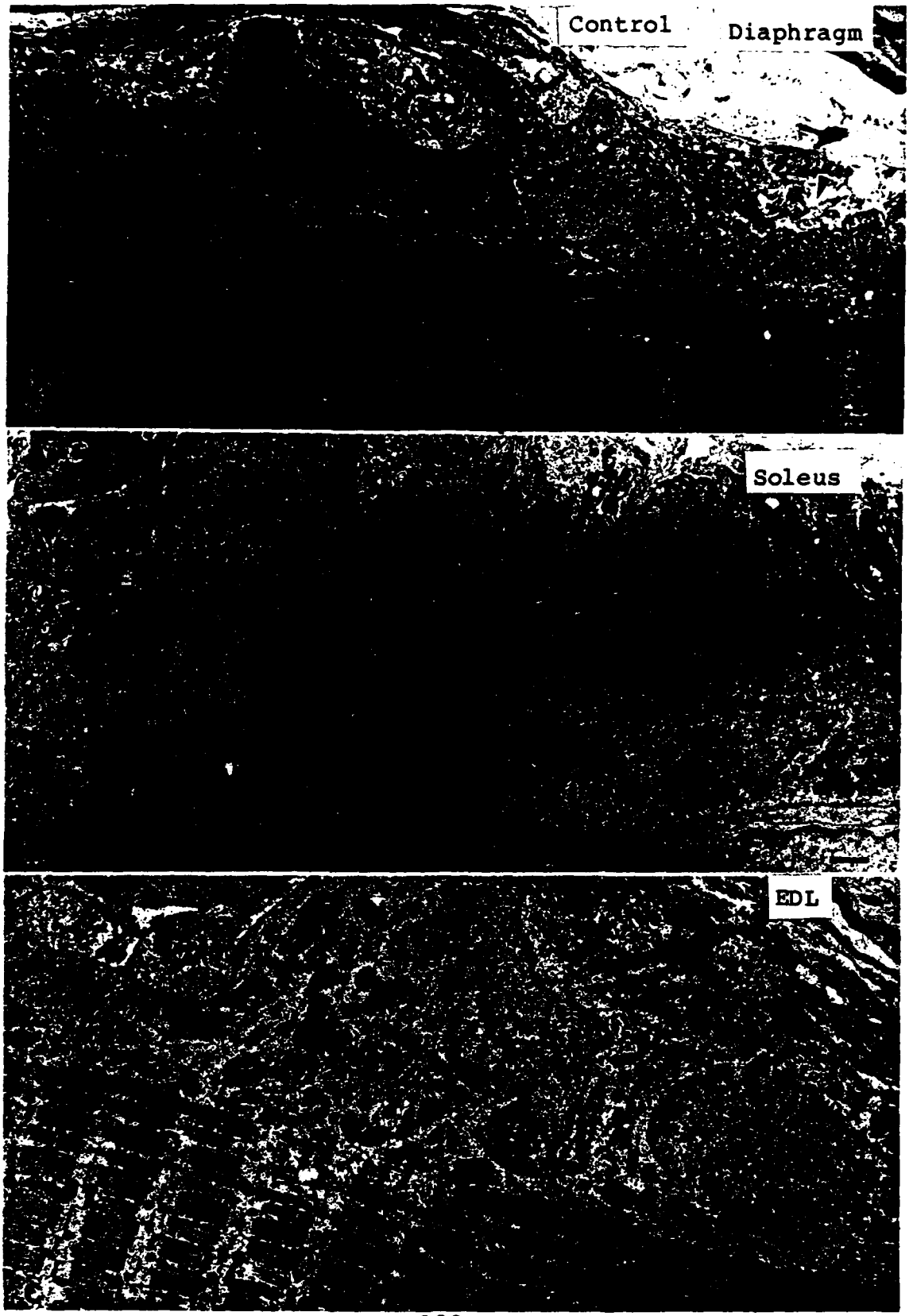

Control
Diaphragm
Soleus
EDL

Figure 70. Comparison of Neuromuscular Junctions from Diaphragm, Soleus, and EDL Muscles 7 Days After Removal of Alzet Pump Containing Sham Solution (14-day Implant) (SC14-7). Except for an occasional swollen mitochondrion, endplates are normal.

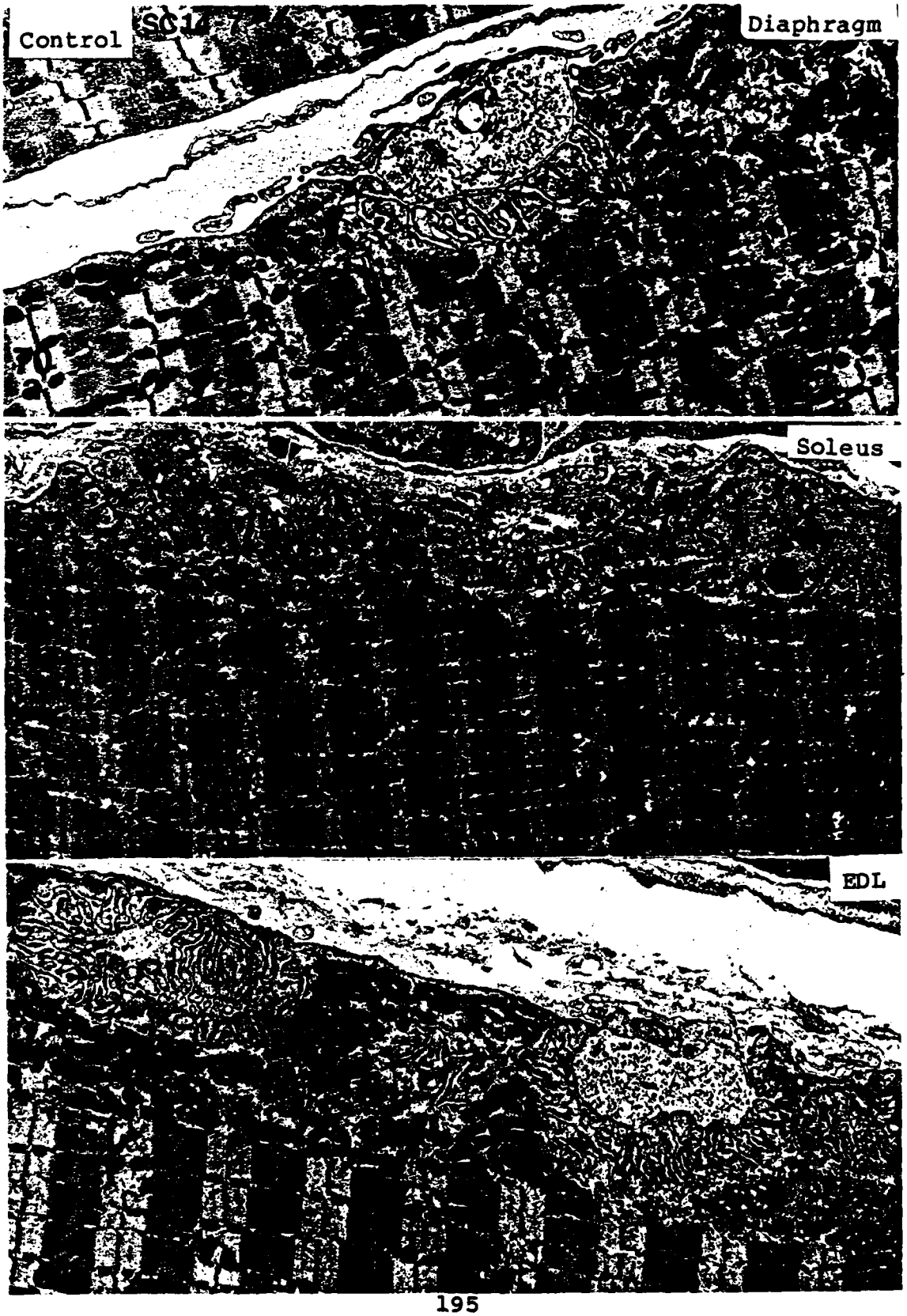

Control
SCI
Diaphragm
Soleus
EDL

Figure 71. Comparison of Neuromuscular Junctions from Diaphragm, Soleus, and EDL Muscles 14 Days After Removal of Alzet Pump Containing Sham Solution (14-day Implant) (SC14-14). Except for an occasional swollen mitochondrion, endplates are normal.

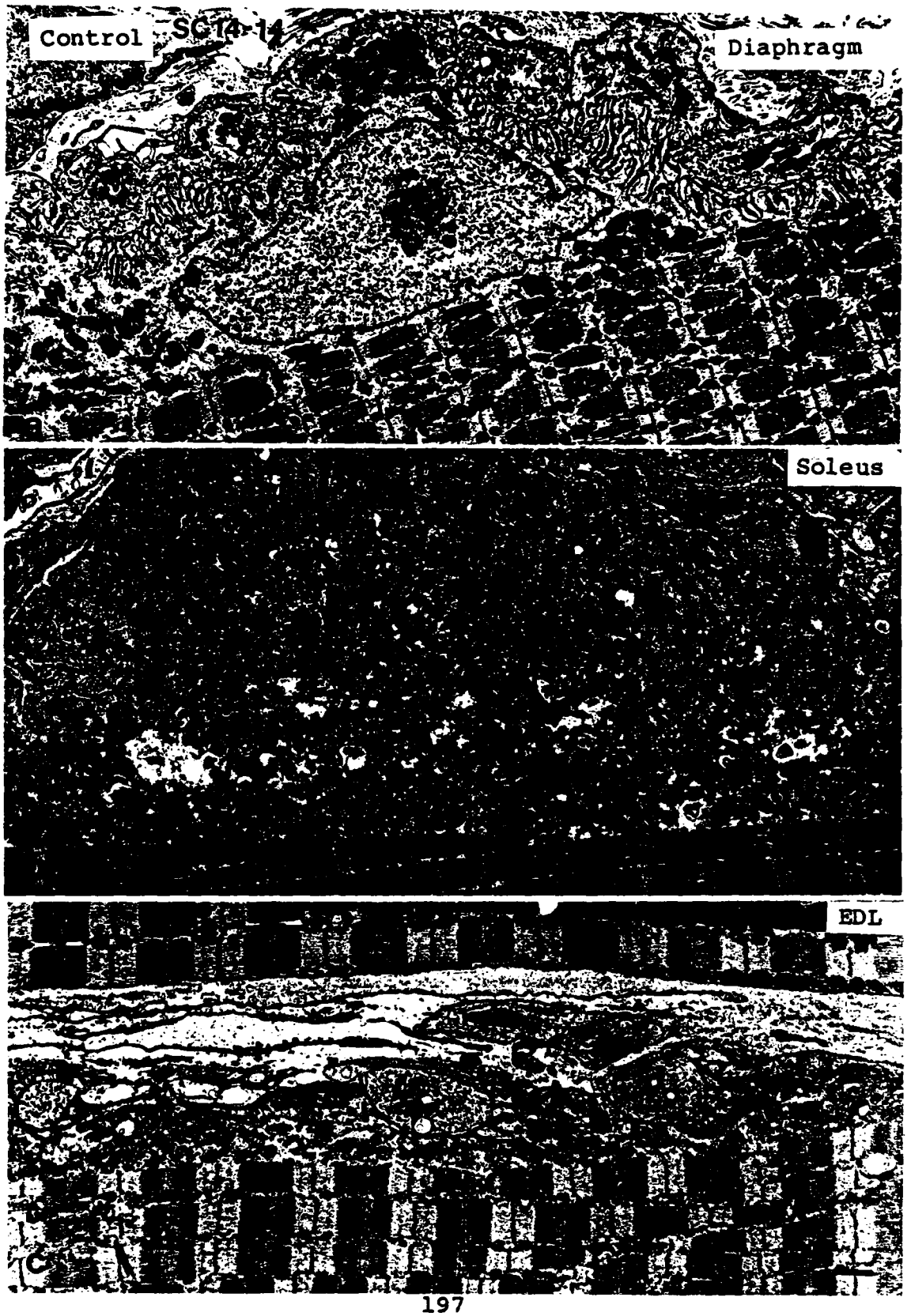

Control
SCTA-1
Diaphragm
Soleus
EDL

Figure 72. Neuromuscular Junctions from Diaphragm, Soleus, and EDL Muscles 3 Days After Implantation of Alzet Pump Containing Moderate Dose of Physostigmine (Yielding Sustained 40% Blood ChE Inhibition) (SM3). (SM3 designates Subacute Moderate Dose, 3 days implantation.) Swollen mitochondria in the diaphragm myofibers (Figure 72a) probably represent fixation artifacts. Apparent foreshortening of folds (Figure 72b, arrowheads) is often seen in control endplates (see Figure 18d).

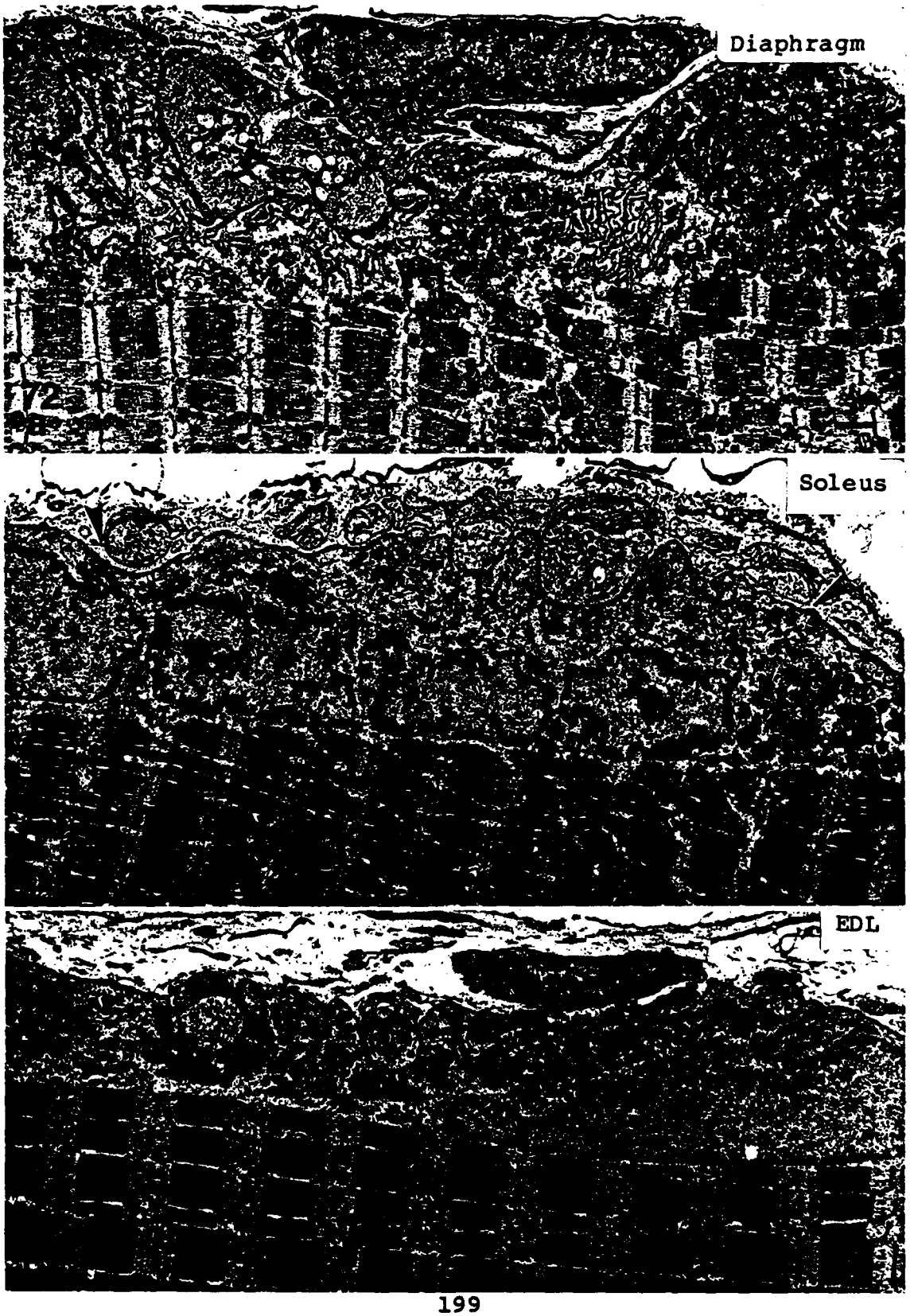

Diaphragm
Soleus
EDL

Figure 73. Neuromuscular Junctions from Diaphragm, Soleus, and EDL Muscles 7 Days After Implantation of Alzet Pump Containing Moderate Dose of Physostigmine (Yielding Sustained 40% Blood ChE Inhibition) (SM7). Except for an occasional swollen mitochondrion due to fixation hypoxia, endplates are normal.

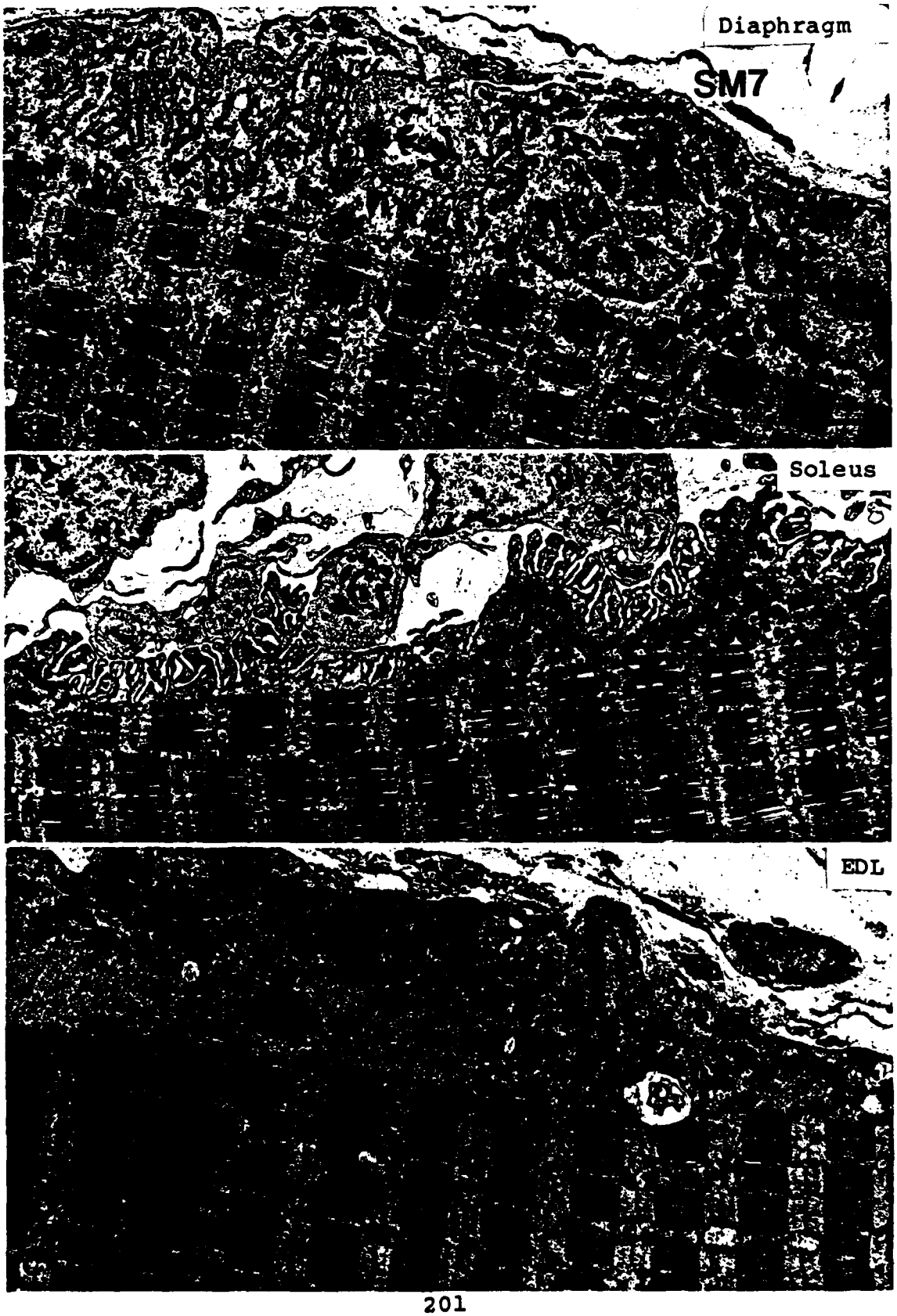

Diaphragm
SM7
Soleus
EDL

Figure 74. Neuromuscular Junctions from Diaphragm, Soleus, and EDL Muscles 14 Days After Implantation of Alzet Pump Containing Moderate Dose of Physostigmine (Yielding Sustained 40% Blood ChE Inhibition) (SM14). Endplates appear normal.

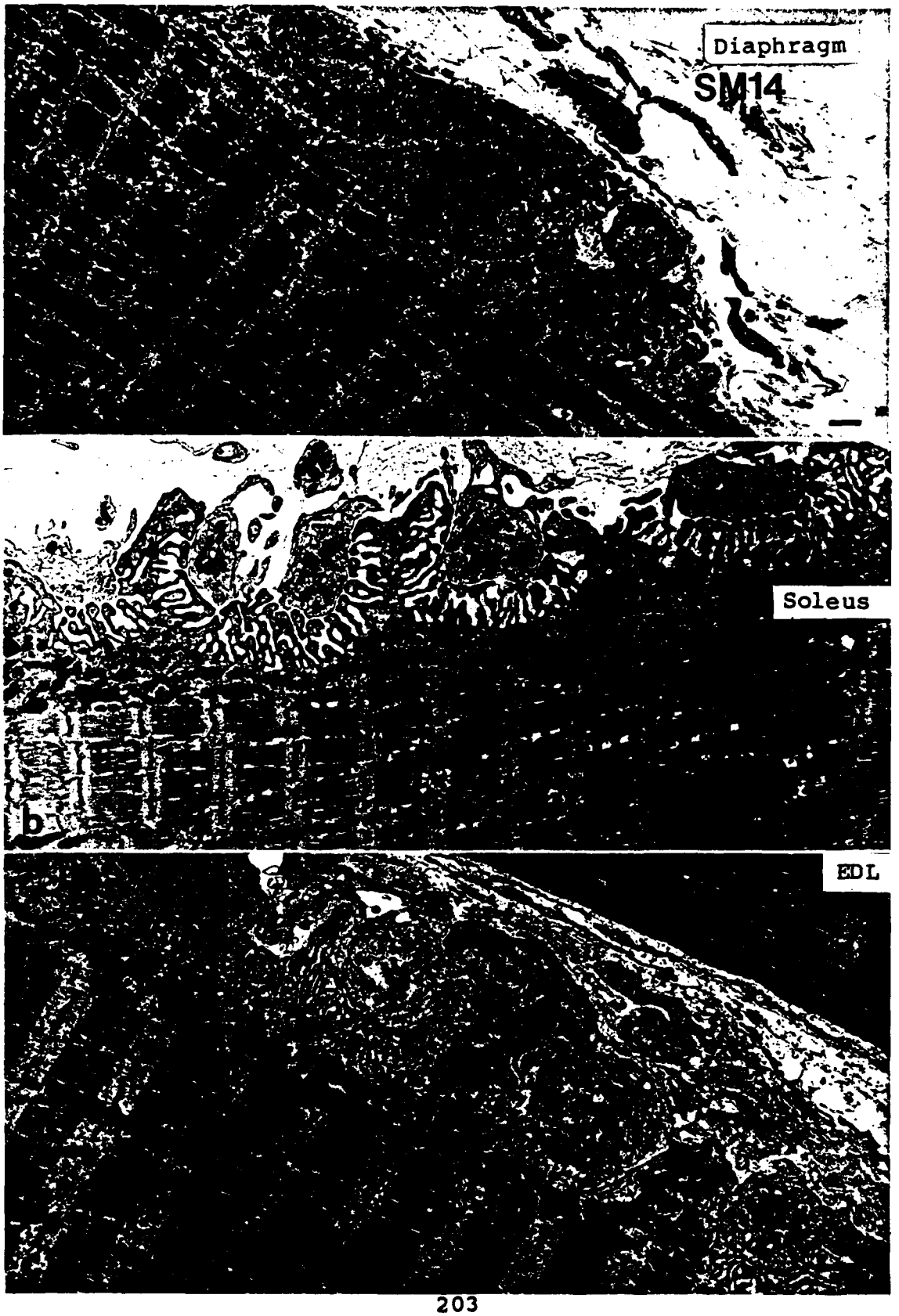

Diaphragm
SM14
Soleus
b
EDL

Figure 75. Neuromuscular Junctions from Diaphragm, Soleus, and EDL Muscles 3 Days After Implantation of Alzet Pump Containing High Dose of Physostigmine (Sustained 80% Blood ChE Inhibition) (SH3). In some endplates (Figure 75c, inset), nerve terminals are virtually depleted of synaptic vesicles and contain numerous coated vesicles (Figure 75c inset, arrowhead). A few pre- and post-synaptic mitochondria are blistered, frothy, or vesicated, presumably reflecting subthreshold expression of physostigmine toxicity.

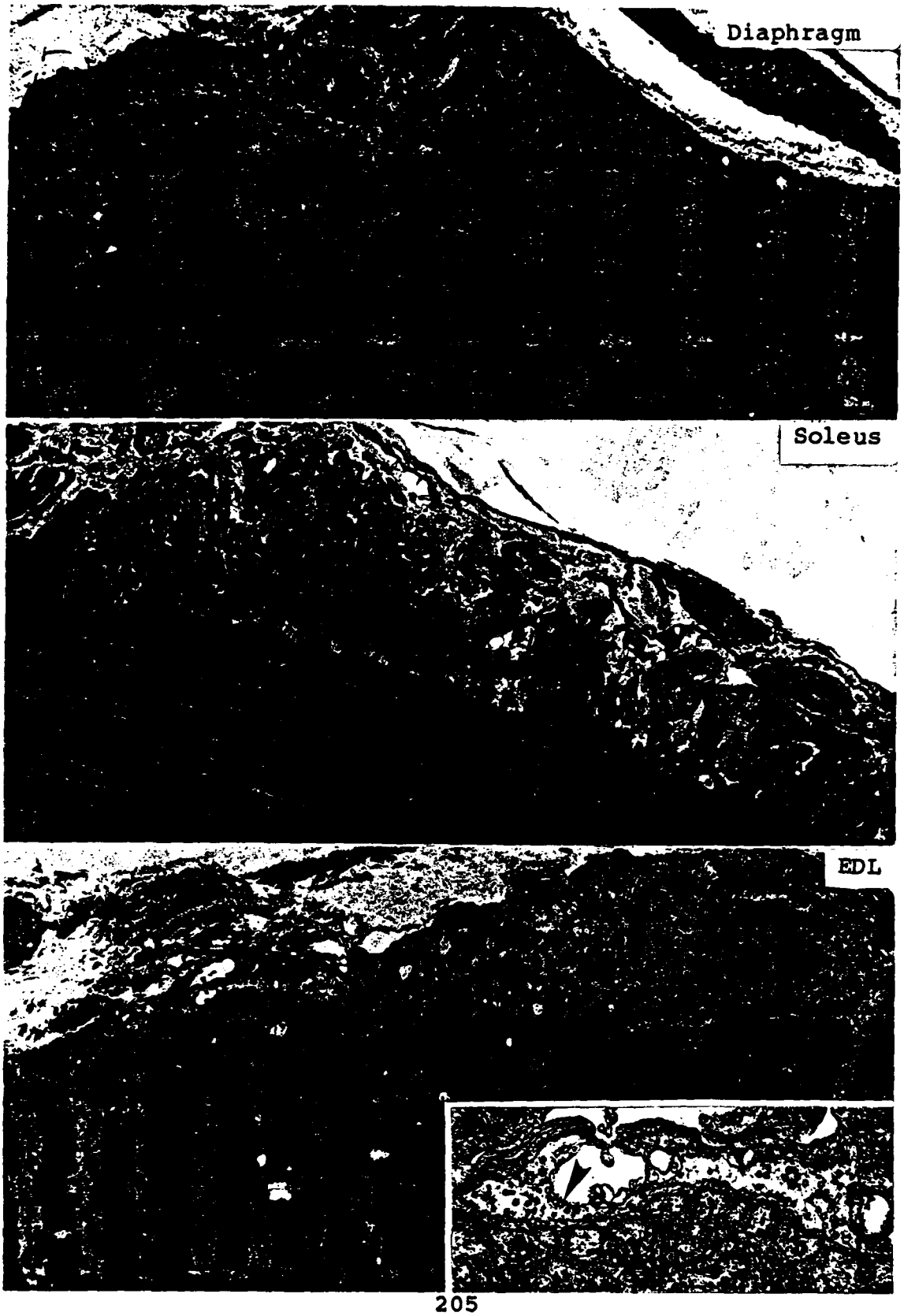

Diaphragm
Soleus
EDL

Figure 76. Neuromuscular Junctions from Diaphragm, Soleus, and EDL Muscles 7 Days After Implantation of Alzet Pump Containing High Dose of Physostigmine (SH7). Although most endplates appeared relatively unaffected by this dose of physostigmine, a few endplates exhibited supercontraction or dissolution of subjunctional sarcomeres (Figure 76b).

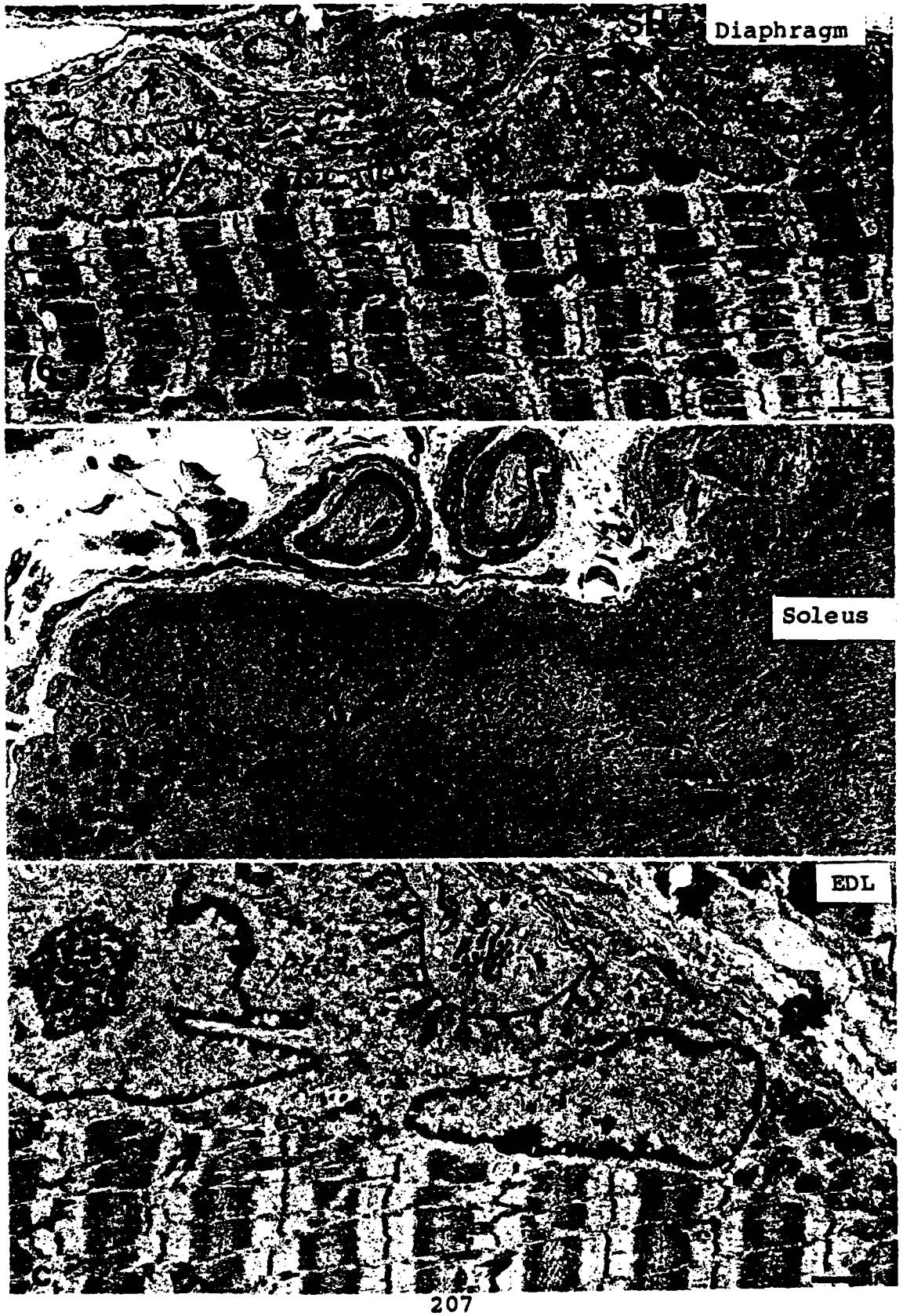
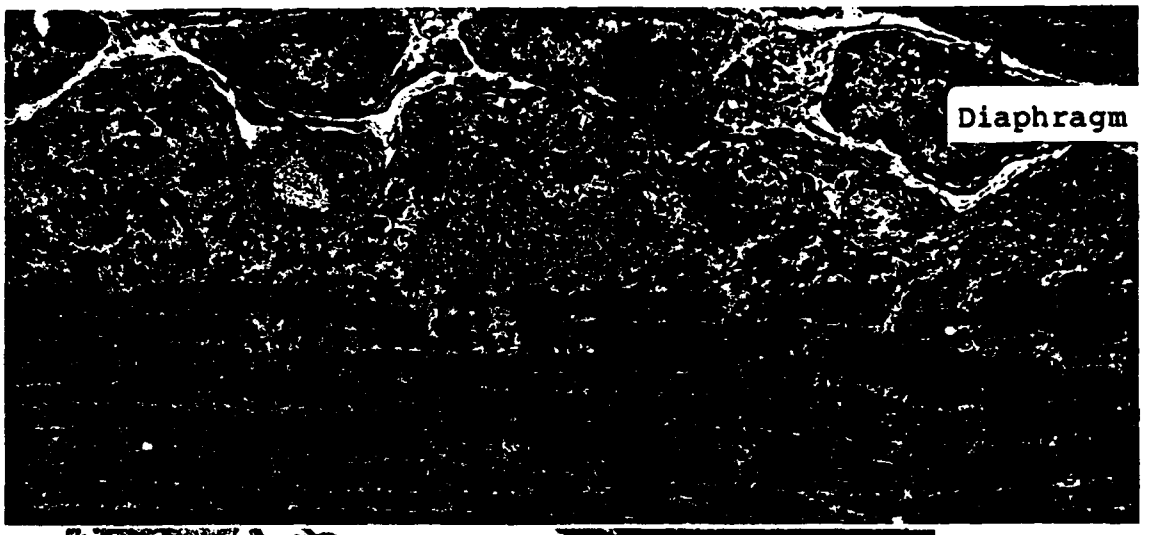
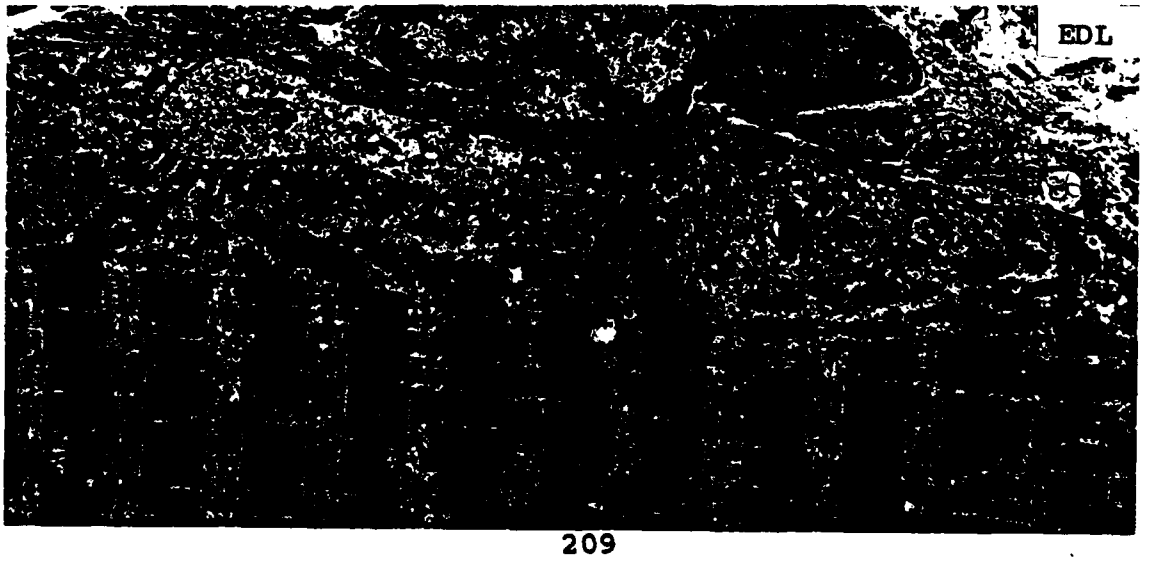

Diaphragm
Soleus
EDL

Figure 77. Neuromuscular Junctions from Diaphragm, Soleus, and EDL Muscles 14 Days After Implantation of Alzet Pump Containing High Dose of Physostigmine (SH14). Except for an occasional swollen mitochondrion, most endplates appeared normal.


Diaphragm


Soleus


EDL

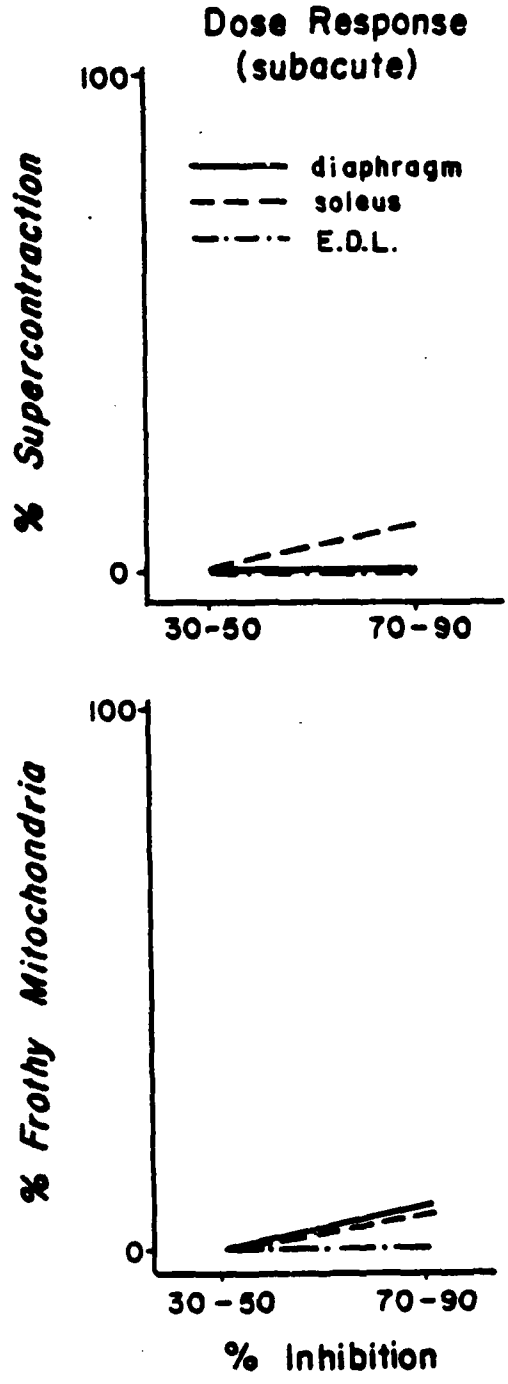


Figure 78. Dose-Response Curves for Low- and High-dose Subacute Exposures to Physostigmine. At low dose, no changes were observed in sarcomere or mitochondrial ultrastructure. At high dose, approximately 10% of sarcomeres were at least partially disrupted and most of the same fibers had damaged mitochondria.

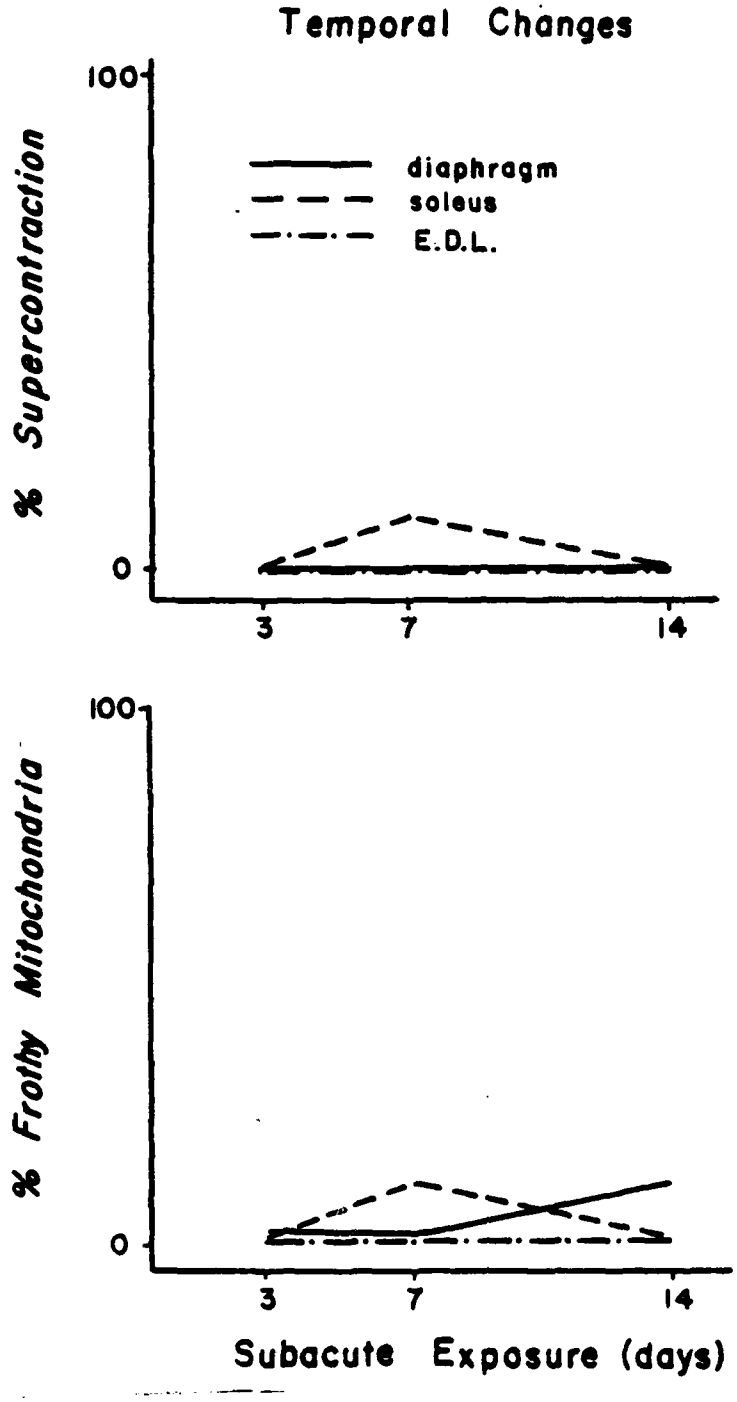


Figure 79. Graph Depicting Changes in Myofibers After 3, 7, and 14 Days of Subacute Exposure to a High Dose of Physostigmine (7 LD_{50}/day or 0.3 LD_{50}/hour). Surprisingly few myofibers showed evidence for cytopathology.

Figure 80. Neuromuscular Junctions from Diaphragm, Soleus, and EDL Muscles 3 Days After Removal of Alzet Pump Implanted for 14 Days and Containing a Moderate Dose of Physostigmine (SM14R3). (SM14R3 is the abbreviation for Subacute, Moderate dose, 14-day implant, Recovery, 3 days). Except for hypoxic mitochondria in the soleus and EDL myofibers (Figure 80b,c), endplates are normal.

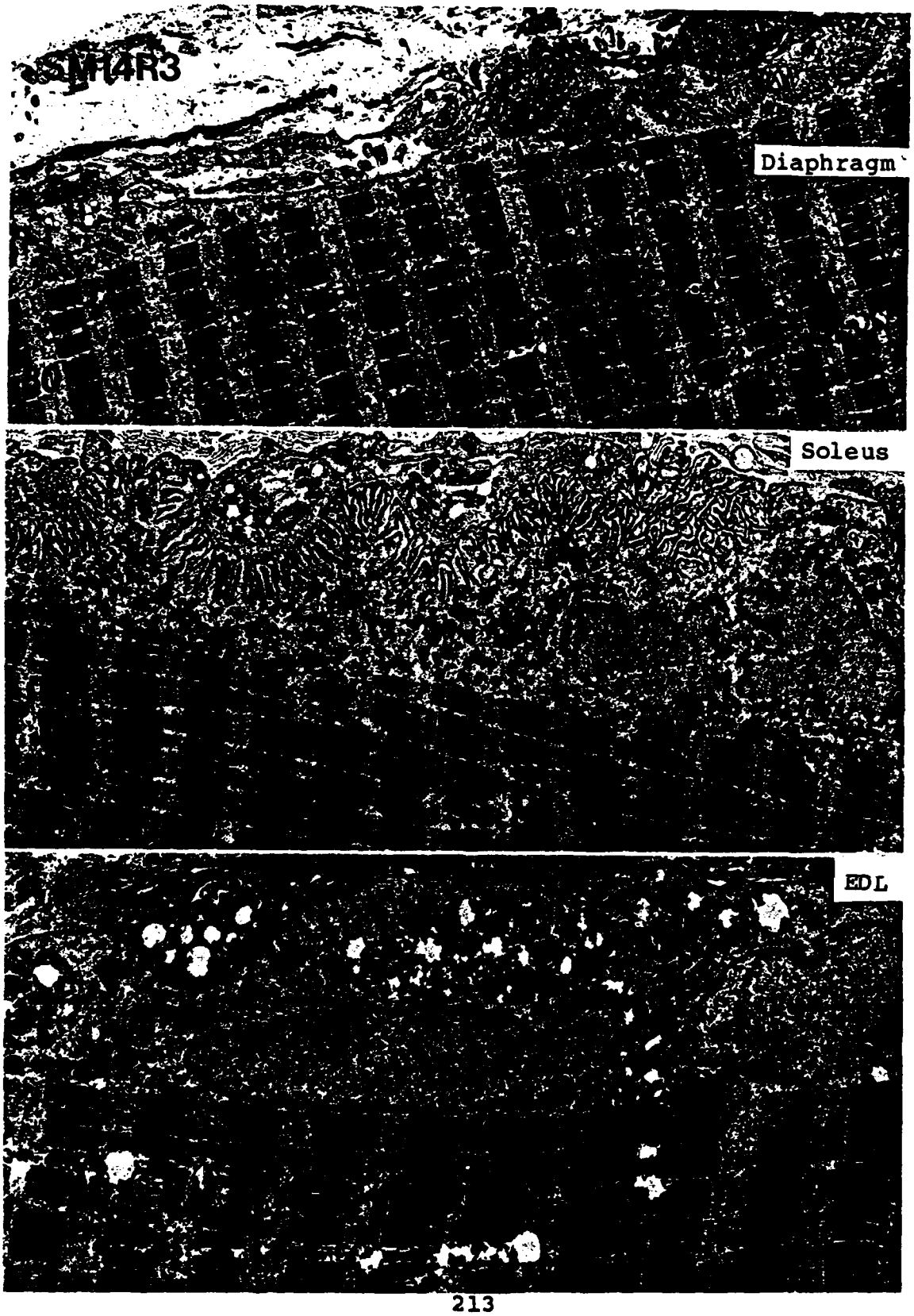

SMH4R3
Diaphragm
Soleus
EDL

Figure 81. Neuromuscular Junctions from Diaphragm, Soleus, and EDL Muscles 7 Days After Removal of Alzet Pump Containing Moderate Dose of Physostigmine (SM14R7). Endplates appear normal.

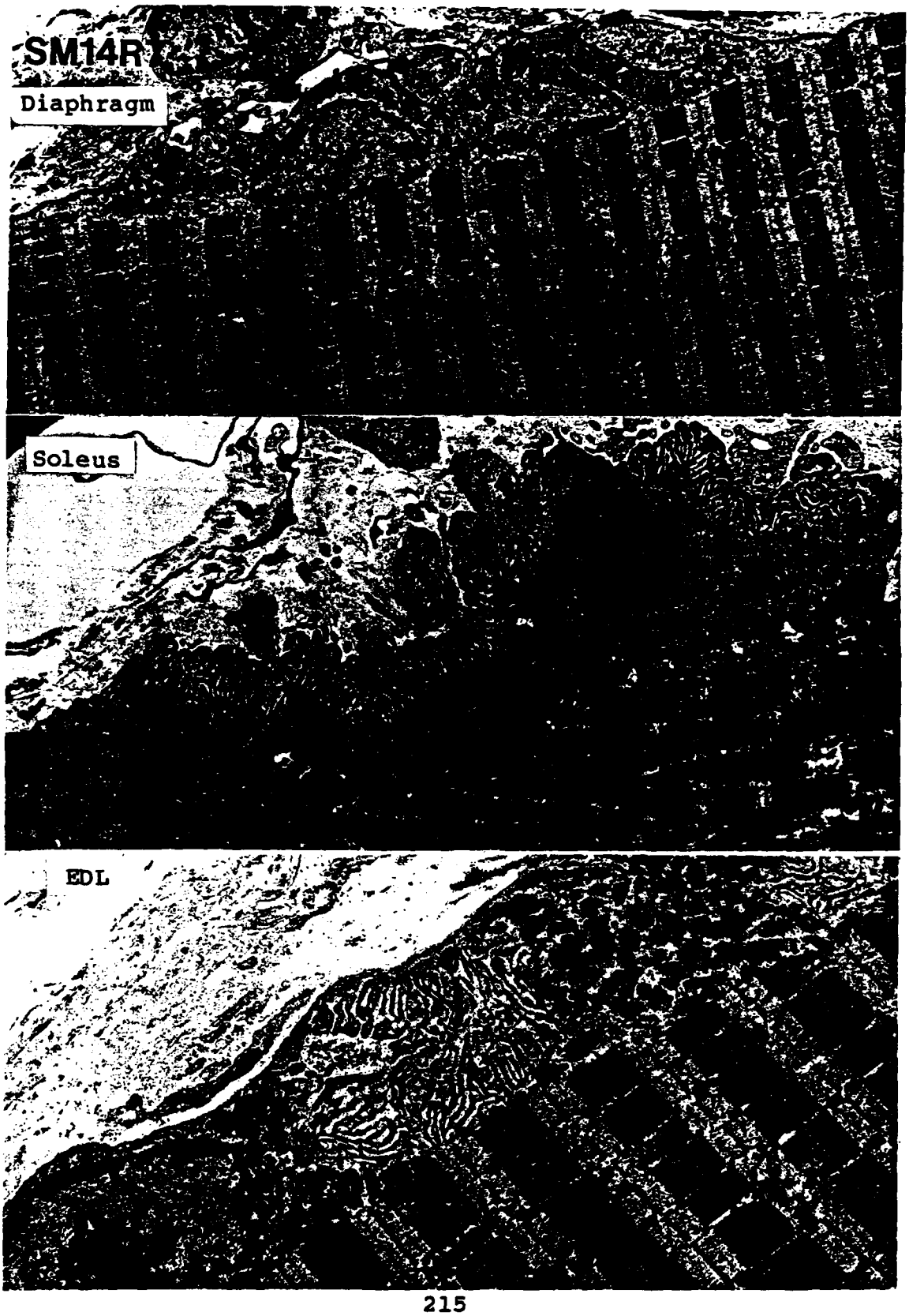

SM14R
Diaphragm
Soleus
EDL

Figure 82. Neuromuscular Junctions from Diaphragm, Soleus, and EDL Muscles 14 Days After Removal of Alzet Pump Containing Moderate Dose of Physostigmine (Sustained 40% ChE Inhibition) (SM14R14). Except for an occasional swollen mitochondrion, endplates are normal.

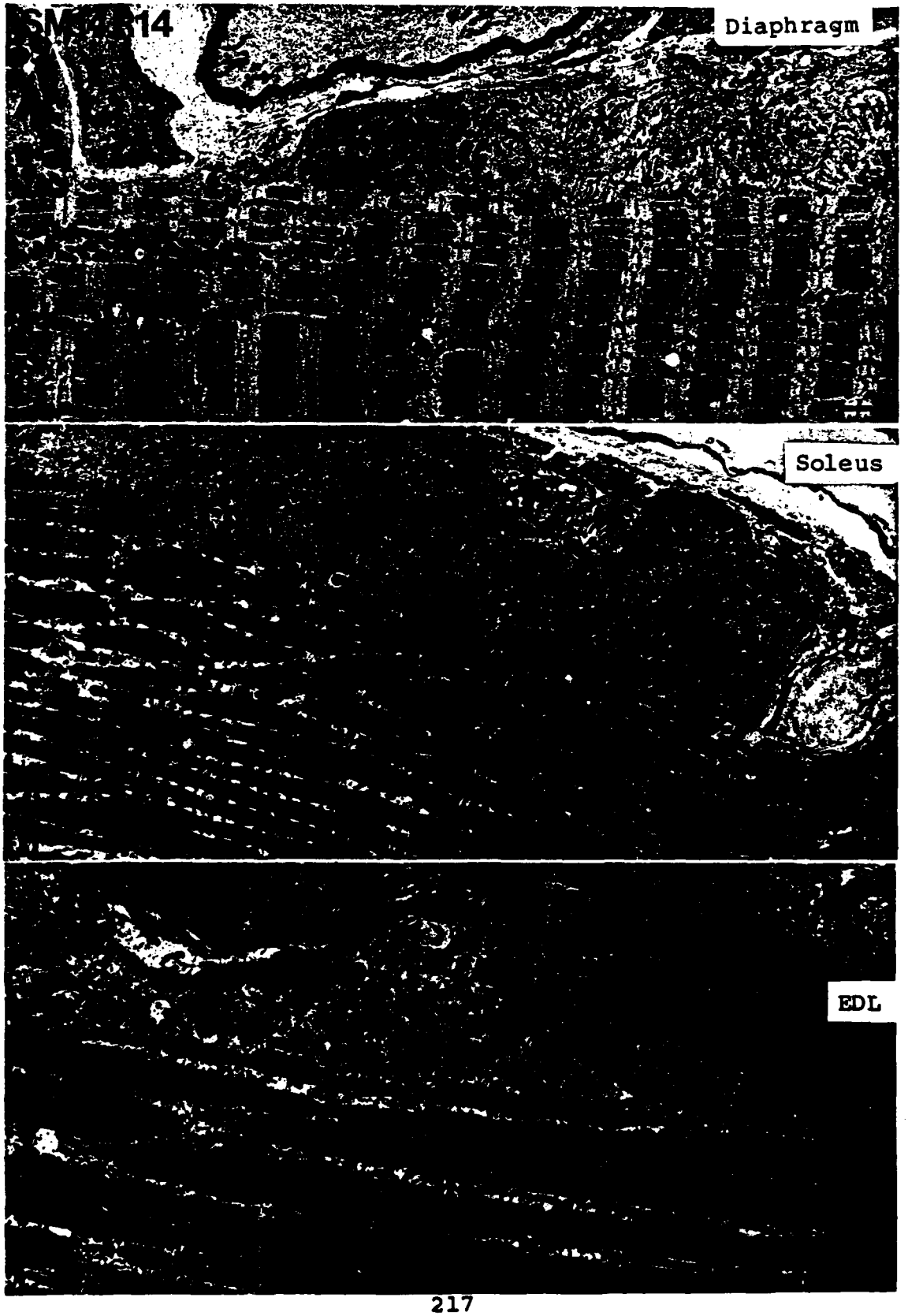

Diaphragm
Soleus
EDL

Figure 83. Neuromuscular Junctions from Diaphragm, Soleus, and EDL Muscles 28 Days After Removal of Alzet Pump Containing Moderate Dose of Physostigmine (Sustained 40% ChE Inhibition) (SM14R28). Except for an occasional swollen mitochondrion, endplates are normal.

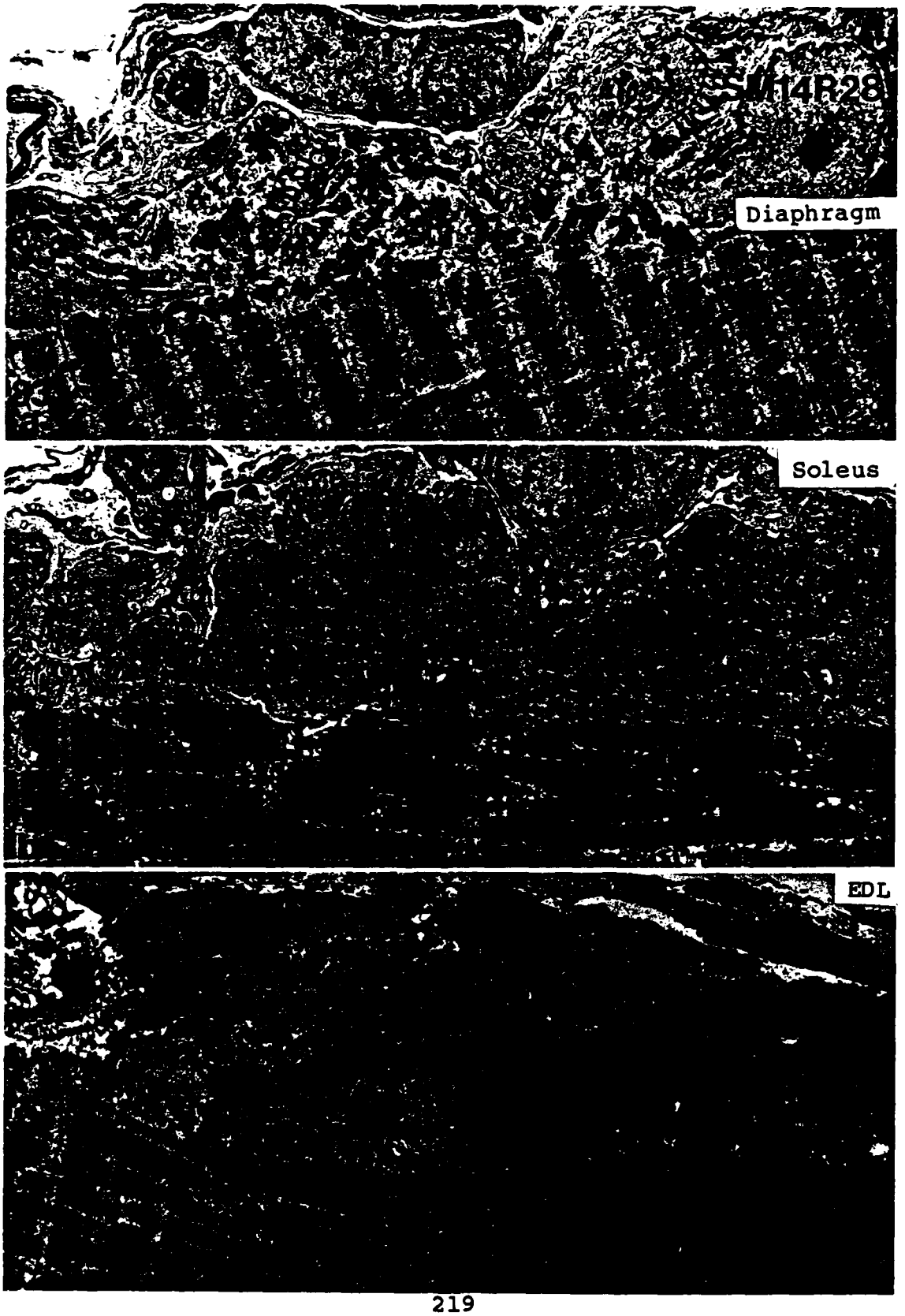

M14R28
Diaphragm
Soleus
EDL

Figure 84. Neuromuscular Junctions from Diaphragm, Soleus, and EDL Muscles 3 Days After Removal of Alzet Pump Containing High Dose of Physostigmine (Sustained 80% ChE Inhibition) (SH14R3). Endplates appear normal. Recovery appears complete.

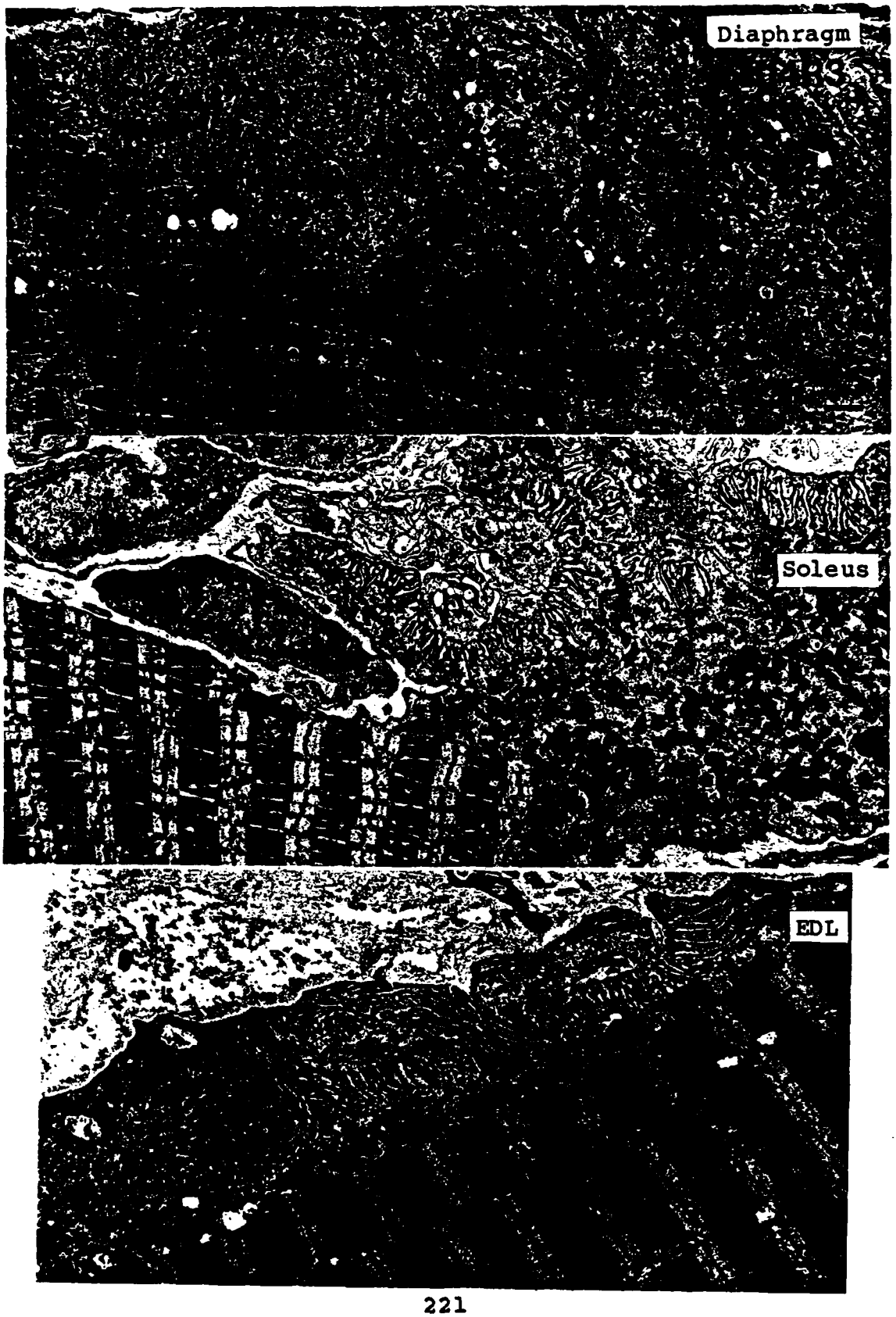

Diaphragm
Soleus
EDL

Figure 85. Neuromuscular Junctions from Diaphragm, Soleus, and EDL Muscles 7 Days After Removal of Alzet Pump Containing High Dose of Physostigmine (Sustained 80% ChE Inhibition) (SH14R7). Endplates appear normal.

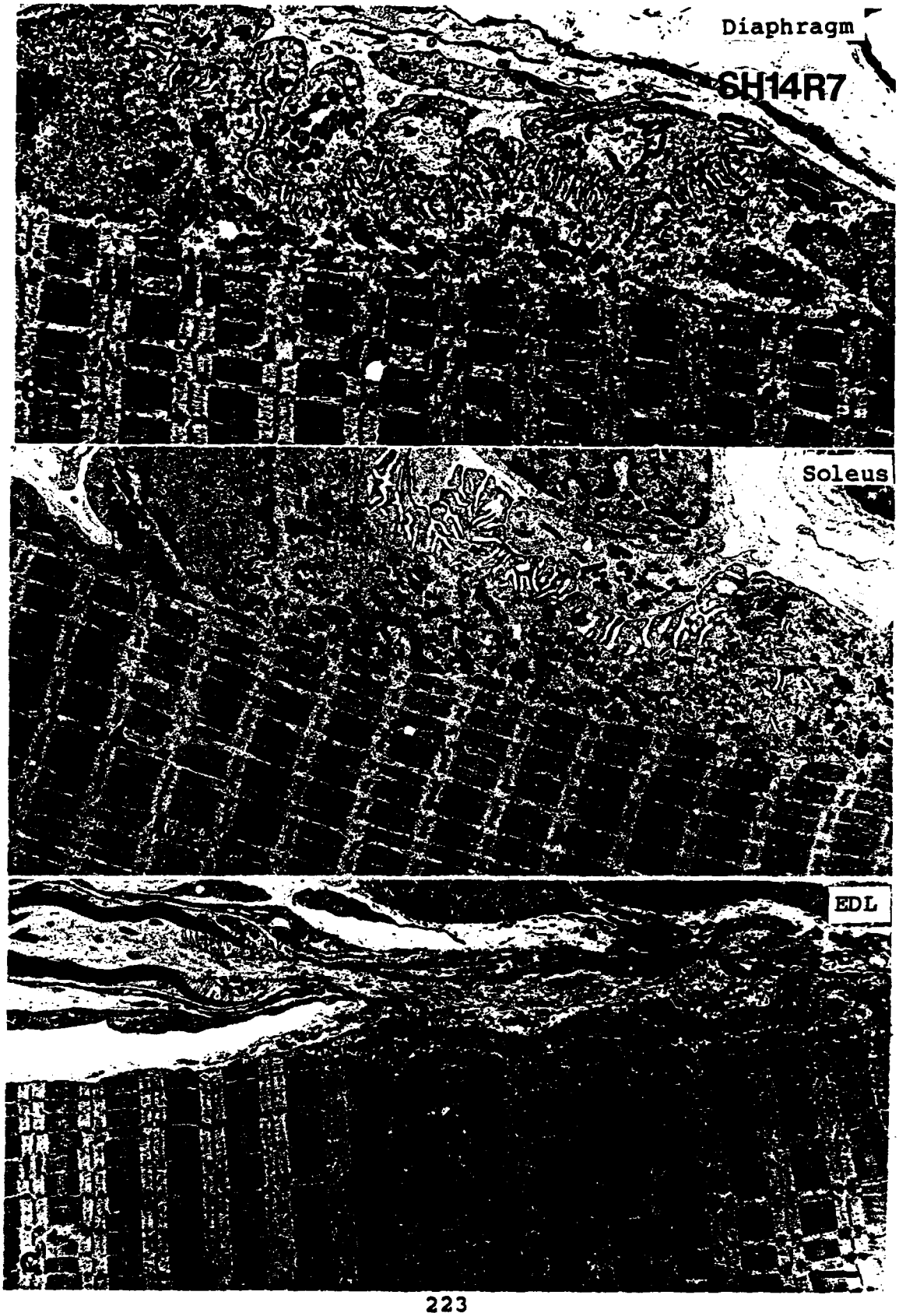

Diaphragm
SH14R7
Soleus
EDL

Figure 86. Neuromuscular Junctions from Diaphragm, Soleus, and EDL Muscles 14 Days After Removal of Alzet Pump Containing High Dose of Physostigmine (Sustained 80% ChE Inhibition) (SH14R14). Except for occasional swollen mitochondria, endplates are normal.

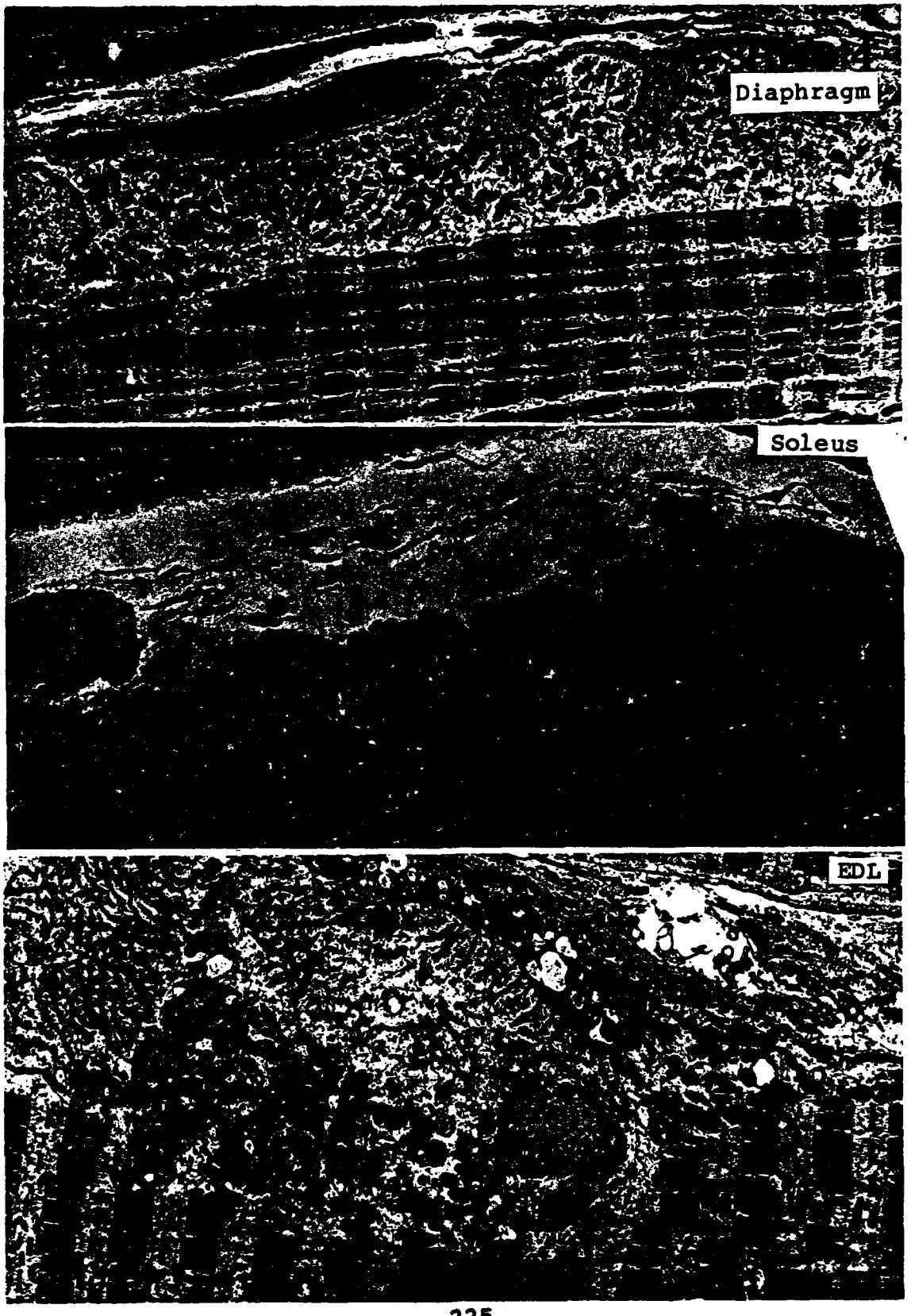
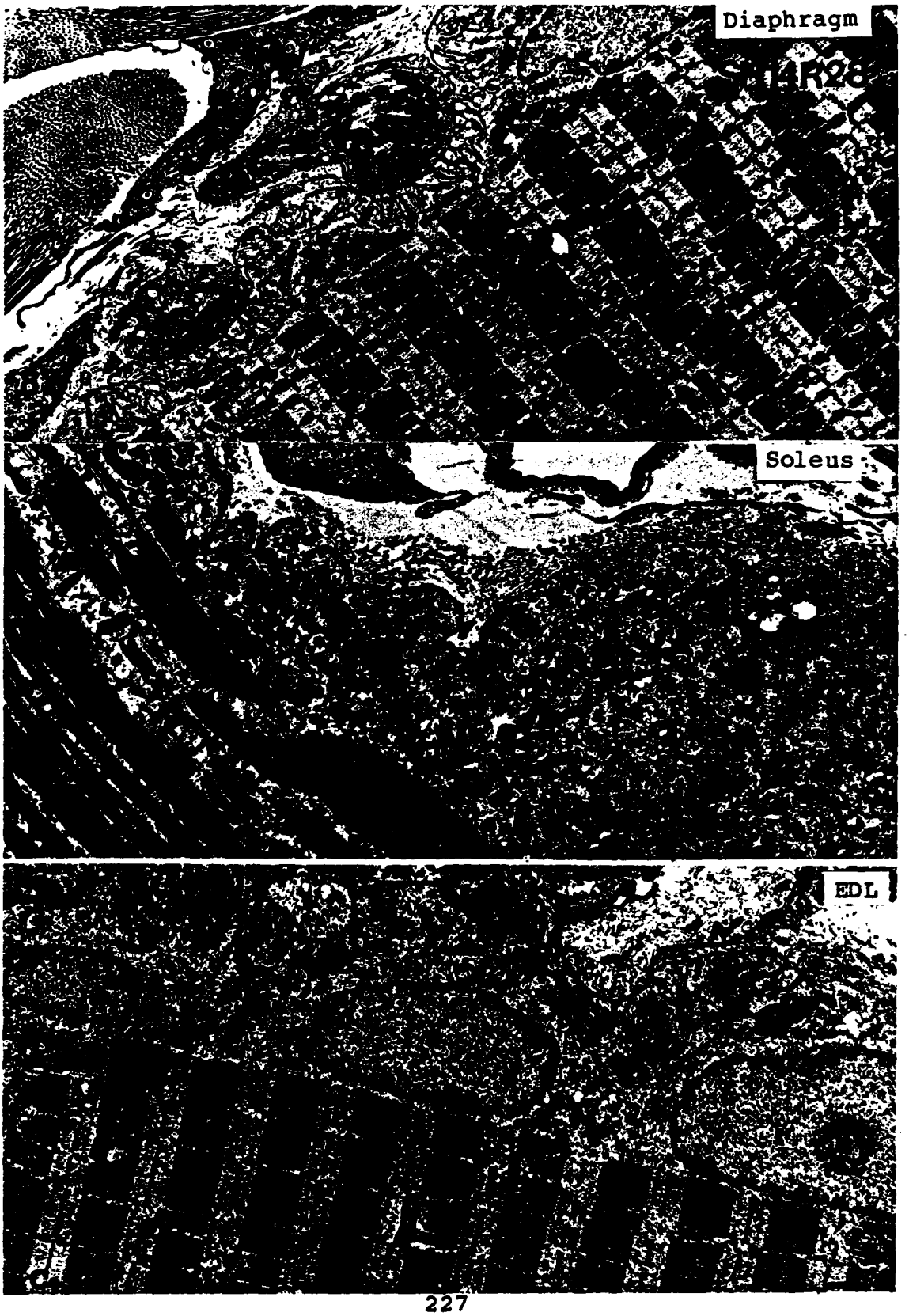

Diaphragm
Soleus
EDL

Figure 87. Neuromuscular Junctions from Diaphragm, Soleus, and EDL Muscles 28 Days After Removal of Alzet Pump Containing High Dose of Physostigmine (Sustained 80% ChE Inhibition) (SH14R28). Endplates appear normal.


Diaphragm
Soleus
EDL

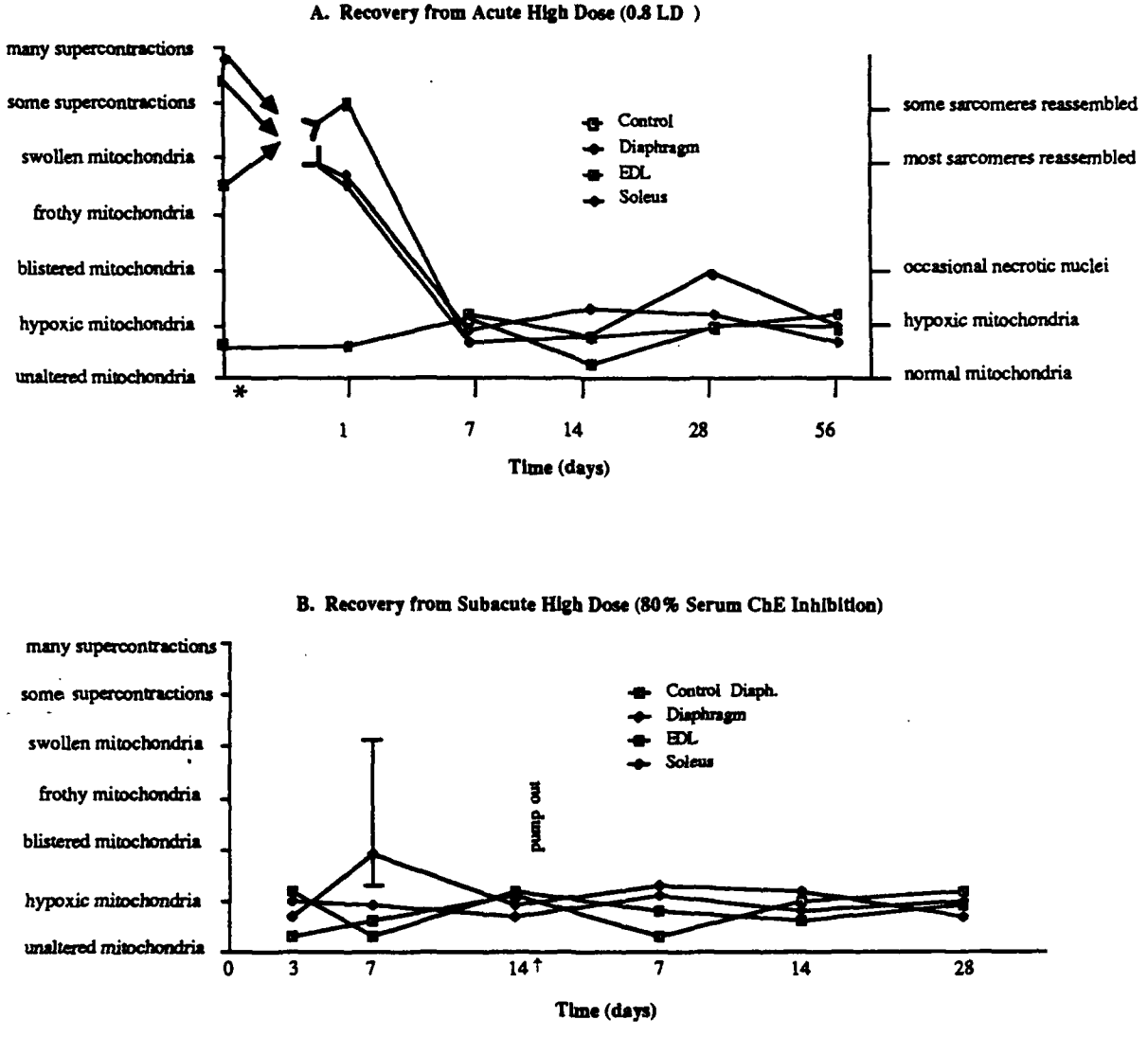


Figure 88. Graphs Comparing Time Course of Recovery From Alterations Caused by Acute and Subacute Exposure to Physostigmine. Recovery from acute exposure begins at 1/2 hour PI (Fig. 88A, *), while recovery from subacute exposure begins at pump removal (Fig. 88B, ↑) (i.e., after 14 days of continuous exposure). Following termination of both acute and subacute exposures at very low to high doses, recovery is essentially complete within 3-7 days. [The curves are broken in Figure 88A because ChE inhibition levels remain elevated for 6-12 hours after acute exposure to physostigmine. Since substantial damage and subsequent repair are known to occur during this period (see Section J.4), the sampling intervals were not brief enough to resolve the changes likely to occur during the initial 24-hour recovery period. Thus, continuous curves would be misleading.]

M. THE EFFECTS OF NEUROMUSCULAR STIMULATION ON THRESHOLD OF TOXICITY

The possible synergistic effects of muscle use on the expression of anti-ChE toxicity at the neuromuscular junction have been assessed in four series of experiments using prolonged low- to-high frequency stimulation of the common peroneal nerve in the presence or absence of low to high doses of physostigmine and of pyridostigmine. The stimulation experiments were performed under spinal block anesthesia to characterize a) endplate alterations induced by prolonged neuromuscular activity in the absence of anti-ChE agents ("stimulation only"), b) effects on endplate ultrastructure of low to high doses of anti-ChE agents in the absence of neuromuscular stimulation ("drug only"), c) effects on endplate ultrastructure of "normal" neuromuscular activity at low to high doses of physostigmine vs. pyridostigmine ("drug plus normal activity"), and d) the effects of sustained high frequency neuromuscular stimulation at low to high doses of the same two anti-ChE agents ("drug plus hyperactivity").

1. Effects on Muscle Physiology of Sustained Stimulation in the Absence and Presence of a Moderate Dose of Physostigmine

In the first series of experiments designed to identify the simulation patterns that yield optimum sustainable contraction without drug vs. maximum sustainable rate of contraction at a near-threshold dose, stimulation patterns of 20, 40, or 80 Hz trains of 15 or 30 minutes' duration were delivered via the common peroneal nerve. (As defined in Section H.3, in normally active, acclimated, unstressed animals, a threshold dose produces only very limited supercontraction in only a few sub-junctional myofibers. Most myofibers are not supercontracted.) Rats either received no anti-ChE drugs ("stimulation only") or were injected with a threshold dose (0.25 LD_{50}) of physostigmine ("stimulation plus drug'").

At a subthreshold dose of 0.25 LD_{50}, blood samples obtained during stimulation (26 samples from 7 animals) averaged 76% ChE inhibition. Of the 27 rats included in this part of the study, 15 received continuous stimulation without drug, while 12 received continuous stimulation plus 0.25 LD_{50} of physostigmine. (The number of rats in each experimental group is shown in Table 1, EXPERIMENTAL METHODS).

Average twitch tension (single twitches elicited at 0.1 Hz prior to continuous stimulation, with or without physostigmine administration) was 32 $\pm$9 g (n=27). (Initial twitch tension is illustrated as segment "A" in Fig. 89.) A large reduction in strength of contraction occurred over the 15 or 30 minutes of continuous stimulation in the presence or absence of physostigmine, with the decrease in strength proportional to stimulation frequency. (The nature of this decrementing response is illustrated in Fig. 89, "G.") The decrease in strength of contraction in the presence or absence of physostigmine averaged 82% during 20 Hz, 92% during 40 Hz, and 95% during 80 Hz stimulation. (See ultrastructural correlates, next section.)

As an alternative method for analyzing neuromuscular

function, PSCs were compared prior to and at selected times after continuous stimulation and/or physostigmine administration (Fig. 89, "B" and "I"). For stimulation only (i.e., 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. However, an ANOVA comparing these stimulation controls to rats treated with continuous stimulation plus physostigmine (stimulation plus drug) showed a significant ($P<.01$) decrease in PSC when compared to stimulation only. (Ultrastructural correlates are presented in the next section.) For the physostigmine-treated rats, neither variation in stimulus frequency nor interaction between stimulus frequency and drug treatment was found to cause a significant decrease in PSC.

Final PSCs were measured at selected times after termination of continuous stimulation and/or physostigmine administration. Recovery times in stimulation control rats ranged from 6 to 30 minutes following the termination of continuous stimulation, with some rats being tested at both 15 and 30 minutes post-stimulation. For physostigmine-treated rats (stimulation plus drug), recovery times ranged from 8 to 45 minutes after termination of continuous stimulation, corresponding to 45 to 80 minutes after physostigmine administration. Within the recovery times investigated (from the initial improvement seen at 6 minutes), PSCs did not recover significantly. Thus, recovery of contractile properties was a two-phase process--the initial phase reflected a partial recovery within a few minutes, while the second phase was much slower and could not be analyzed in this portion of the physiological study, which also required immediate fixation for electron microscopy.

2. Alterations of Endplate Ultrastructure by Stimulation in the Absence of Drug

In myofibers continuously stimulated for 15 or 30 minutes at 20, 40, and 80 Hz in the absence of drug (stimulation control), a continuum of changes of endplate ultrastructure was noted, with the changes directly proportional to duration and frequency of stimulation. After 15 minutes of 20-Hz stimulation (Fig. 91a), mitochondria in the nerve terminals of EDL myofibers exhibited a few vesications. [These contiguous bubbles differ from the smaller, more widely separated blisters observed in post-synaptic mitochondria of physostigmine treated endplates, and are characteristic of and proportional to the frequency and duration (i.e., total number of endplate depolarizations).] Post-synaptically, the myofibers appeared normal or contained a few blistered and/or frothy mitochondria, restricted solely to the immediate subjunctional sarcoplasm (Fig. 91). After 30 minutes of 20-Hz stimulation, mitochondria in the nerve terminals were frequently vesicated but the subjunctional sarcoplasm remained relatively unaltered (Fig. 91b).

After 15 and 30 minutes' stimulation at 40 Hz and at 80 Hz (Figs. 92 and 93, respectively), EDL myofibers exhibited substantial increases in vesication of pre-synaptic mitochondria. Most notable, however, was the appearance of blistered, 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, they exhibited no supercontraction of subjunctional sarcomeres.

After 30 minutes of stimulation at a given frequency (e.g., 40 Hz), endplates were altered approximately the same as endplates in animals receiving stimulation at twice that frequency but for half that duration (i.e., In "blind" comparisons, endplates stimulated at 40 Hz for 30 minutes were indistinguishable from those in endplates stimulated at 80 Hz for only 15 minutes. Similarly, stimulation at 20 Hz for 30 minutes produced changes equivalent to those seen after 40-Hz stimulation for 15 minutes. (At these frequencies and durations, 18,000 endplate depolarizations are evoked in 15 minutes at 20 Hz, while 144,000 synaptic activations are evoked in 30 minutes at 80 Hz). 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, we were concerned that the ultrastructural alterations produced by prolonged and continuous stimulation might be reversed substantially during that delay. Therefore, additional samples were prepared a) by 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 variable number of evoked endplate depolarizations. Moreover, 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 that induced during normal neuromuscular activity in phasic myofibers (44,47). Apparently, these ultrastructural changes reflect the morphological correlate for severe endplate fatigue. Moreover, they closely resemble the sequence of ultrastructural alterations produced by increasing doses of physostigmine, up to but not including threshold (0.25 LD_{50}). 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, endplate damage and normal repair mechanisms are qualitatively similar to those occurring after acute high-dose exposures to physostigmine.

3. Alterations of Endplate Ultrastructure by Combined Stimulation and Acute Exposure to a Threshold Dose (0.25 LD_{50}) of Physostigmine

Preliminary experiments examining the possible synergistic effect of neuromuscular stimulation on expression of endplate cytopathology were somewhat variable, in part reflecting inadequacies of sampling for electron microscopy, inherent tissue variability, and increasing experience in the surgical procedures utilized. Consequently, the data presented should be considered as establishing boundary conditions for follow-up experiments which more accurately compared the synergistic effects of muscle stimulation over a wide range of exposures of both physostigmine and pyridostigmine (see Section N, below).

In rats exposed to 0.25 LD_{50} of physostigmine (i.e., a threshold dose, as defined in Section H.3), soleus and EDL muscles stimulated at 20 Hz for 15 minutes (Fig. 94) were essentially identical to those in rats stimulated for 30 minutes in the absence of drug. In many fibers, presynaptic mitochondria were vesicated, while postsynaptic mitochondria were frothy or severely swollen. Likewise, endplates stimulated at 40 Hz for 30 minutes (Fig. 95) or at 80 Hz for 15 or 30 minutes (Fig. 96) were also relatively severely affected (i.e., were essentially identical to those receiving similar stimulation in the absence of drug). Mitochondria were blistered, frothy, or grossly swollen. Presynaptic mitochondria were either multiply vesicated or exhibited numerous internal dilations. [This characteristic presynaptic 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}).] Thus, following 0.25 LD_{50} of physostigmine, 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. 96). EDL (as well as soleus) myofibers exhibited severe alterations of pre- and post-synaptic mitochondria (Figs. 95, 96). After 80-Hz stimulation (Fig. 96), post-synaptic mitochondria were invariably blistered, frothy, or grossly swollen. Presynaptic mitochondria were either multiply vesicated or exhibited numerous internal dilations. However, there was wide variability of effect in the relatively few endplates examined (3-10 each for each muscle from each protocol), with a few fibers showing no effect of stimulation plus drug, while others showed alterations consistent with the first signs of supercontraction. Thus, 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 threshold dose of physostigmine.

If taken at face value, these images suggest that excessive stimulation, even in the presence of a 0.25 LD_{50} of physostigmine (70% serum ChE inhibition), would not be capable of mimicking all of the lesions characteristic of those seen following acute exposure to near-lethal doses of the same drug (i.e., >0.6 LD_{50}). Thus, these observations appeared to contradict our previous suggestion that the threshold for physostigmine-induced damage is altered substantially by muscle

use (see 57, 62). Nevertheless, the alterations observed at the stimulation patterns and single-dose level used in this preliminary investigation were deemed sufficiently informative to warrant more detailed investigation. As a result, additional data were obtained from endplates fixed after 15 minutes' stimulation at 40 Hz during acute exposure to either physostigmine or pyridostigmine at low- to high-dose levels (see next section). Since data from those improved experimental conditions provide evidence for a strong synergistic effect of neuromuscular stimulation on the expression of drug toxicity, we presume that in the above experiments, fibers from different motor units may have been differentially activated. If so, this would account for the wide variability of effects in different myofibers.

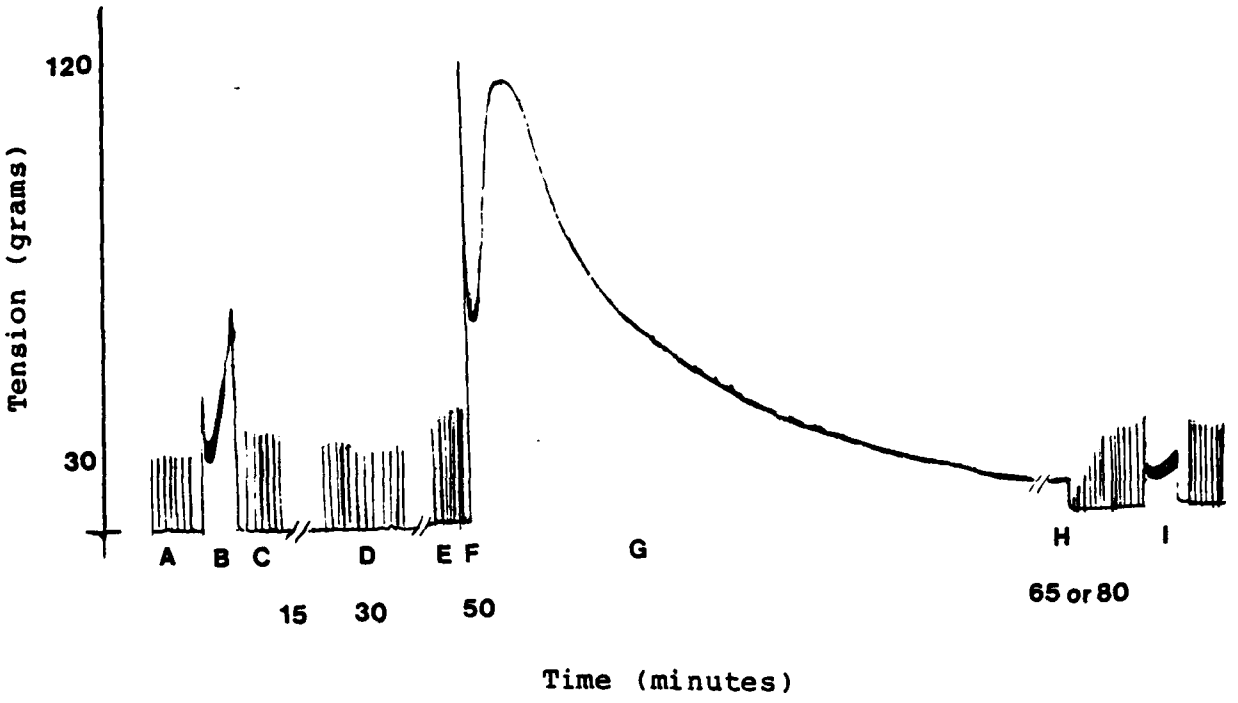


Figure 89. Chart Recording Illustrating Experimental Protocol for Identifying Effects of Physostigmine Intoxication Plus High-frequency Stimulation on rat EDL Muscles. Rats were anesthetized by spinal block with 2% lidocaine and immobilized as described in **EXPERIMENTAL METHODS**. The segments labeled below correspond to results from the 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-second stimulus train.
C) Temporary twitch potentiation following sustained contraction elicited in B.
D) When twitch tension returned to normal, physostigmine was administered (0.25 LD_{50} given s.c.).
E) Approximately 10-25 minutes PI, twitch potentiation indicated beginning of whole muscle response to physostigmine.
F) When potentiation in E was observed, stimulation at 20, 40 or 80 Hz was begun.
G) Strength of contraction gradually decreased 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 returned to normal, the final PSC was determined with 20-Hz, 10-second stimulus train.

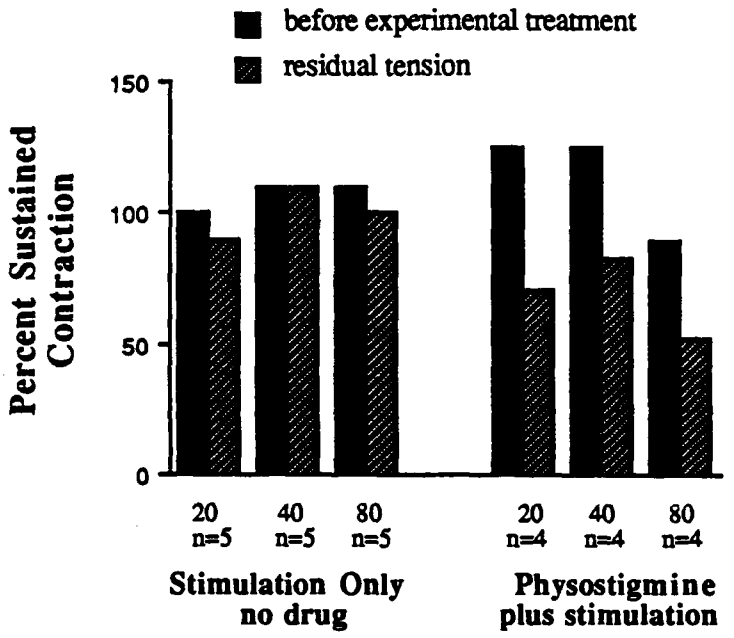


Figure 90. Effect of Physostigmine and Continuous Stimulation on Percent Sustained Contraction in EDL Muscles. PSC is the tension (in grams) at the end of a 10-second, 20-Hz stimulus train divided by the tension at the beginning of the train. A Lower PSC indicates a decreased ability of the muscle to 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 s.c. at a dose of 0.25 LD_{50}.

Figure 91. 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 (Figure 91a) and contain slightly swollen mitochondria, whereas post-synaptic mitochondria are normal (i.e., no hypoxic swelling). Otherwise, endplates appear normal.

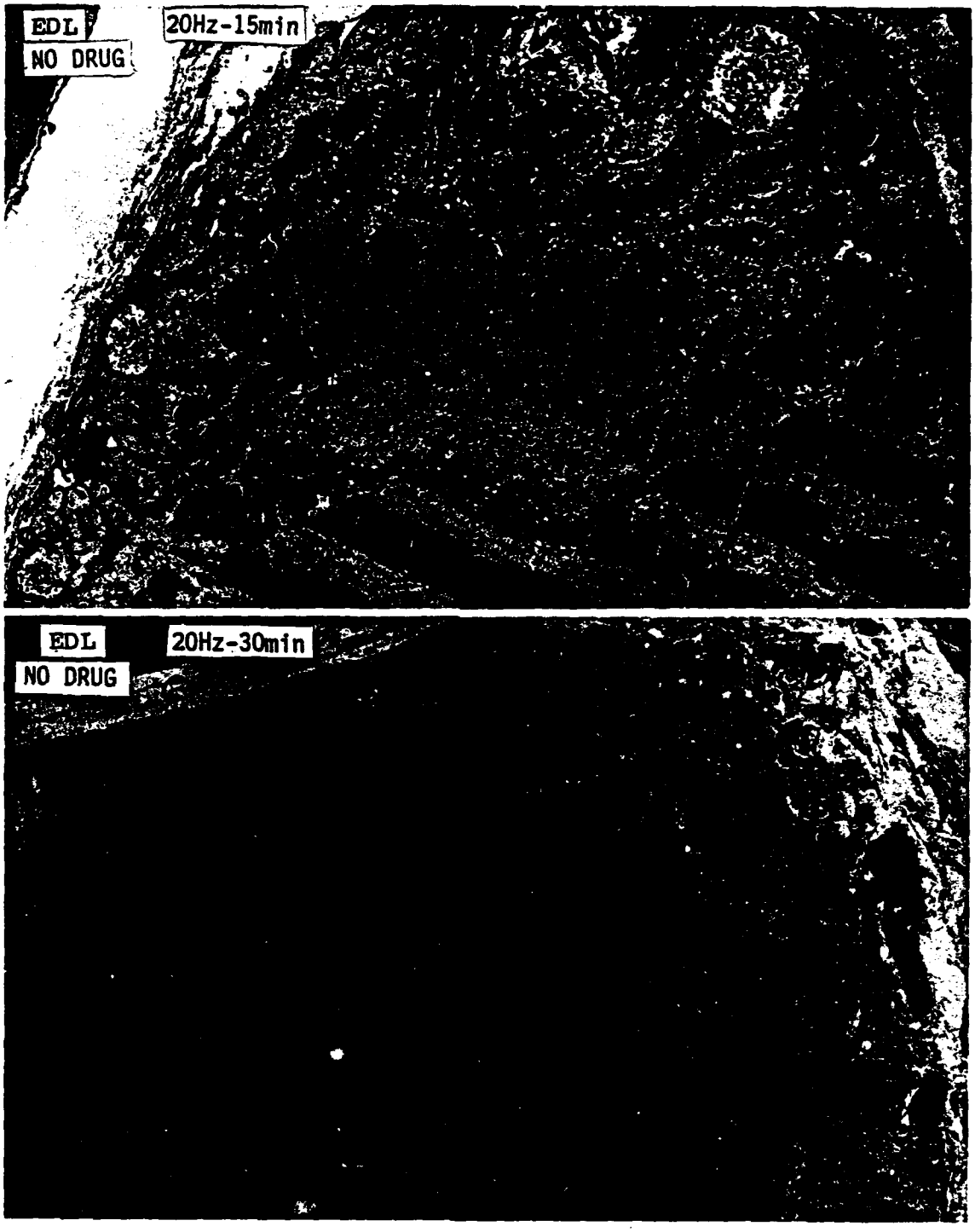

EDL
NO DRUG
20Hz-15min
EDL
NO DRUG
20Hz-30min

Figure 92. 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 vesicated. Post-synaptic mitochondria are relatively unaffected after 15 minutes of 40-Hz stimulation. However, after 30 minutes of 40-Hz stimulation (Figure 92b,c), post-synaptic mitochondria are swollen or exploded.

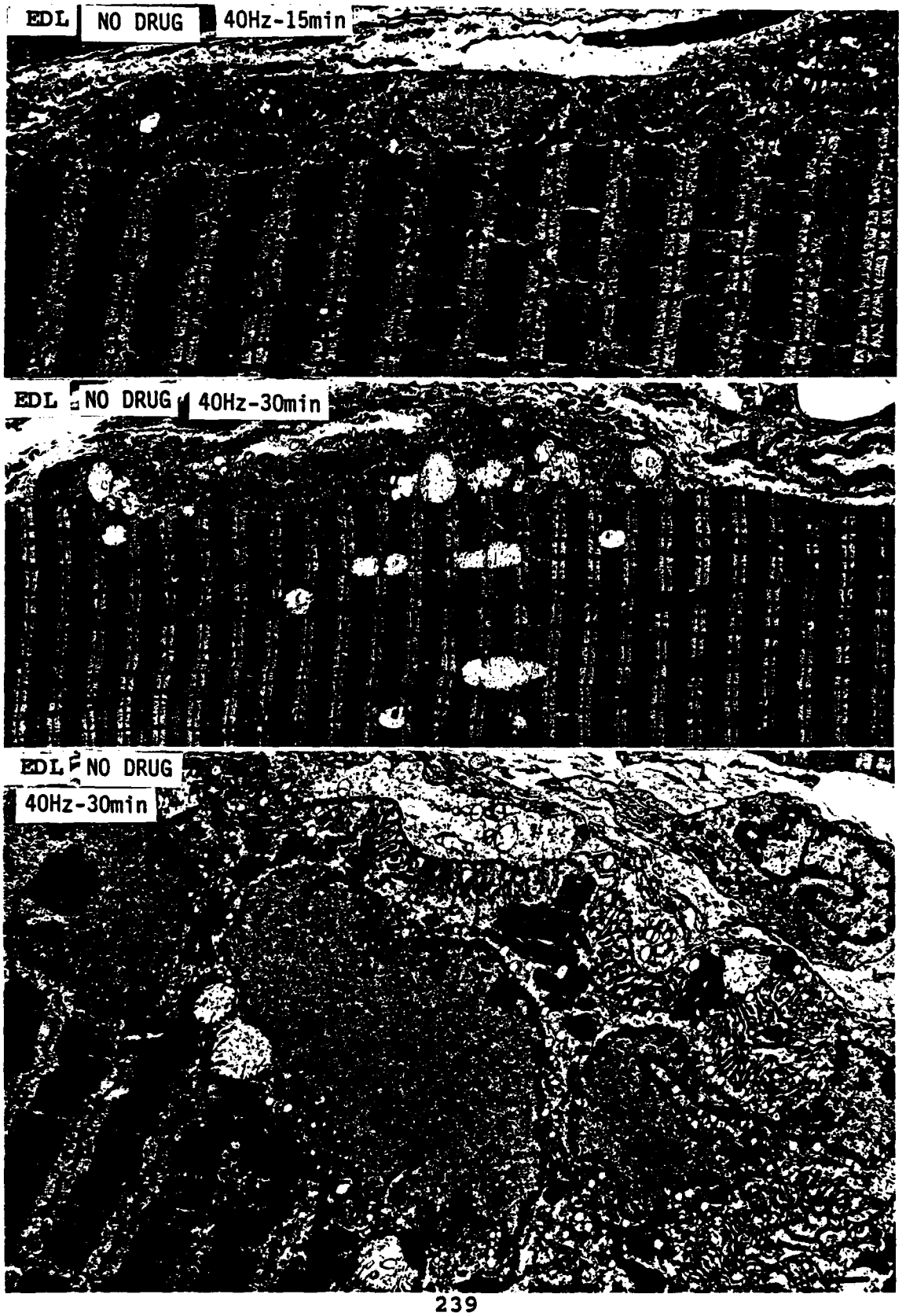
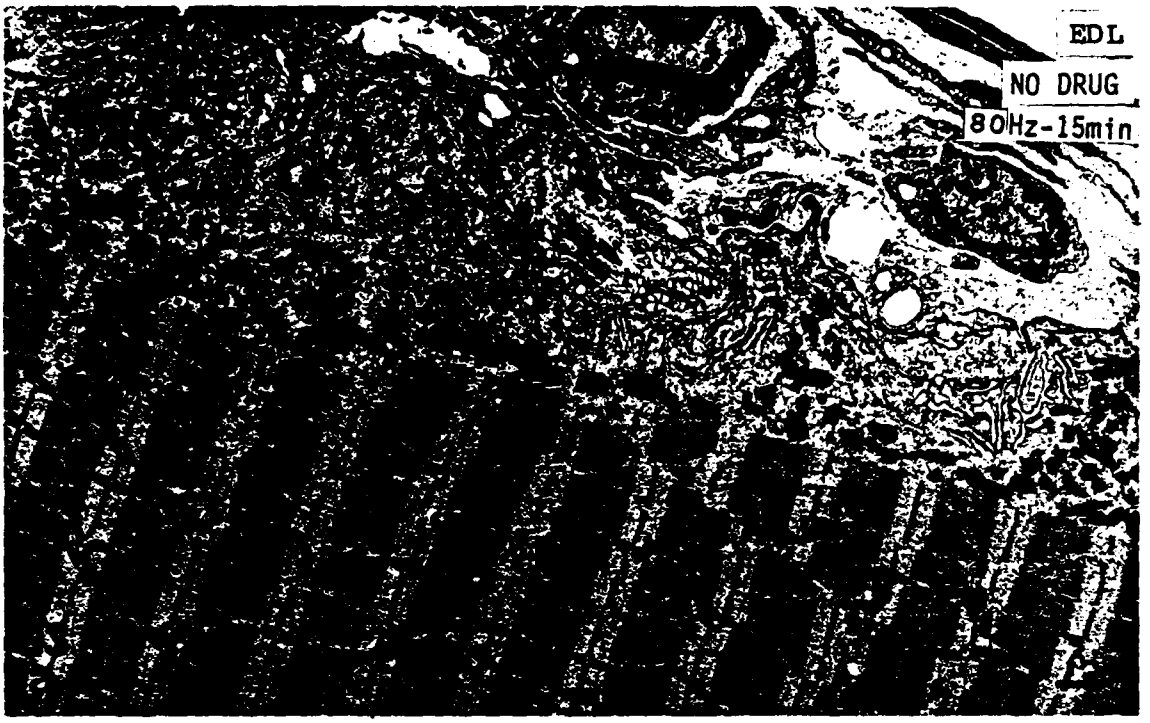

EDL
NO DRUG
40Hz-15min
EDL
NO DRUG
40Hz-30min
EDL
NO DRUG
40Hz-30min

Figure 93. 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 (Figure 93b), post-synaptic mitochondria are swollen or exploded. Note, however, that sarcomeres are of normal length and that there is no evidence for supercontraction.


EDL
NO DRUG
80Hz-15min

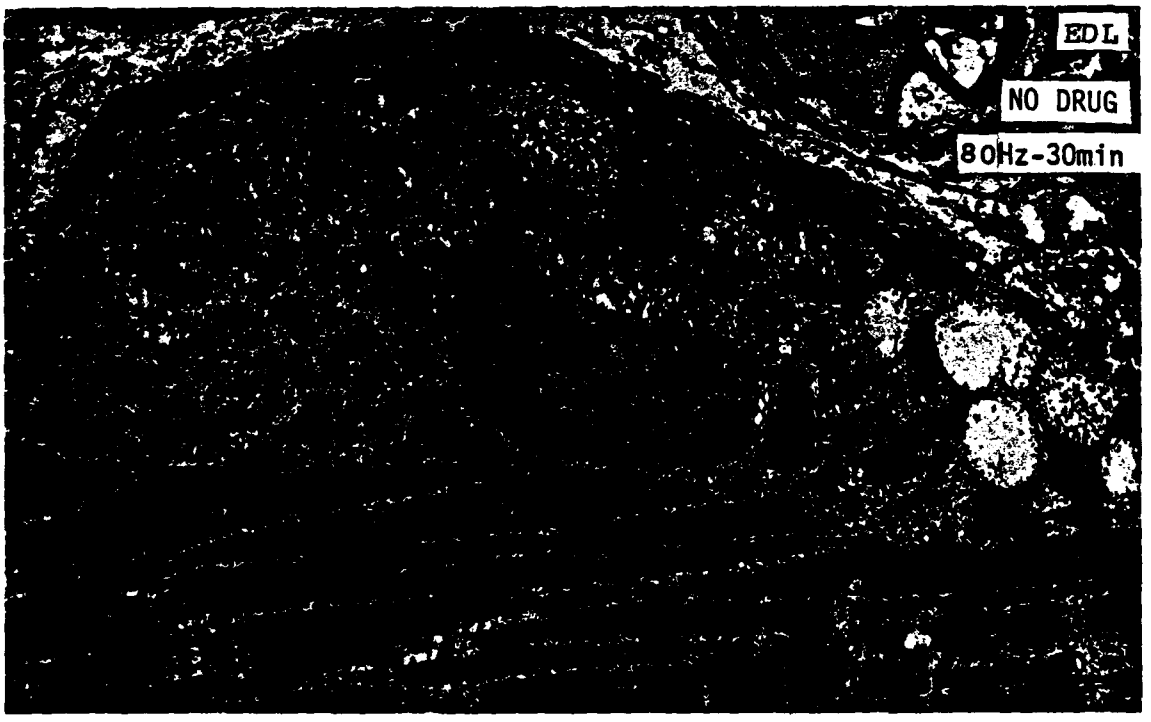
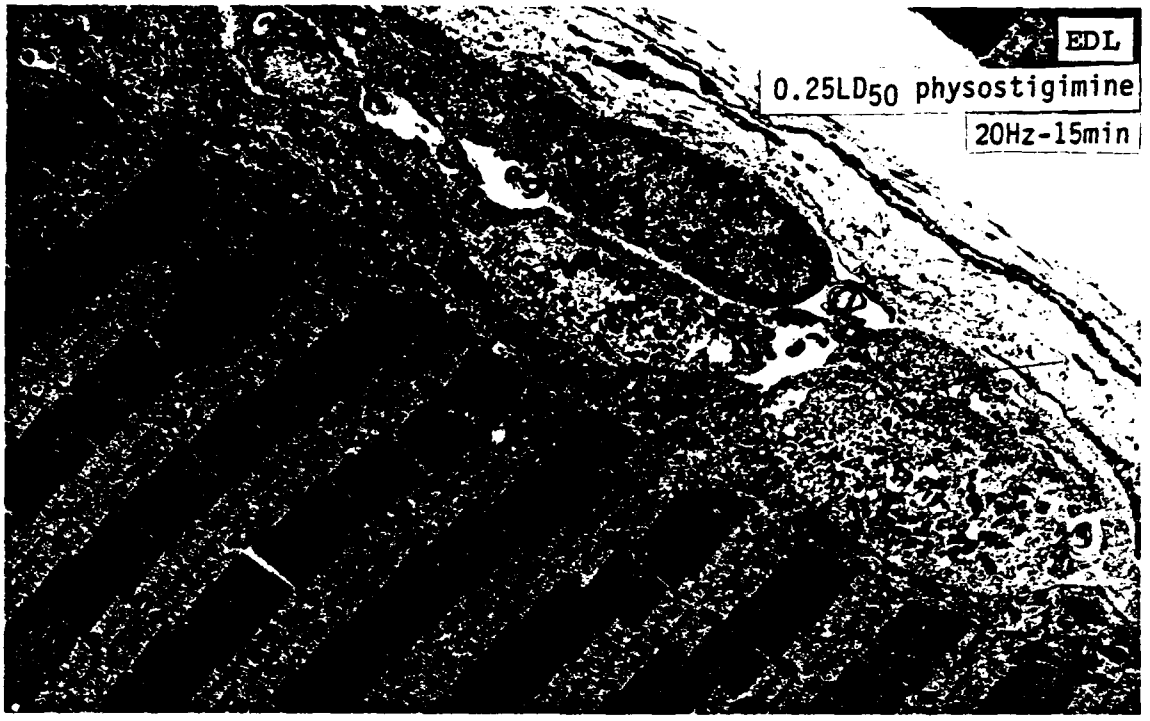

EDL
NO DRUG
80Hz-30min

Figure 94. 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.


EDL
0.25LD50 physostigimine
20Hz-15min

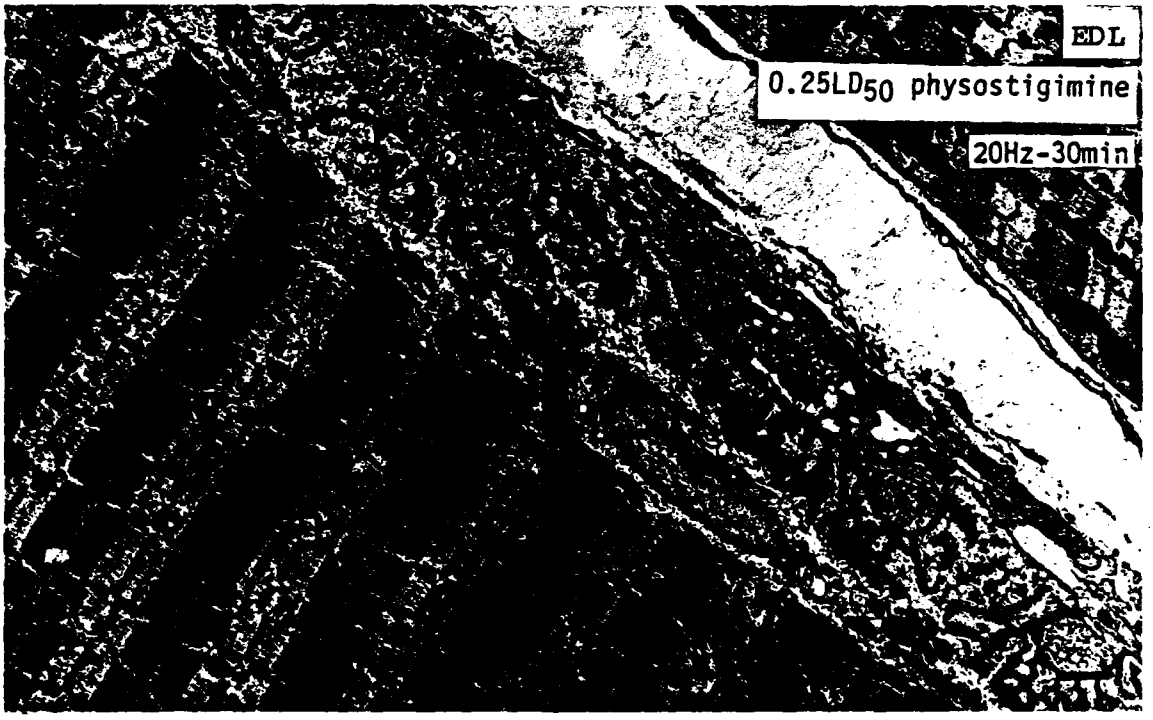
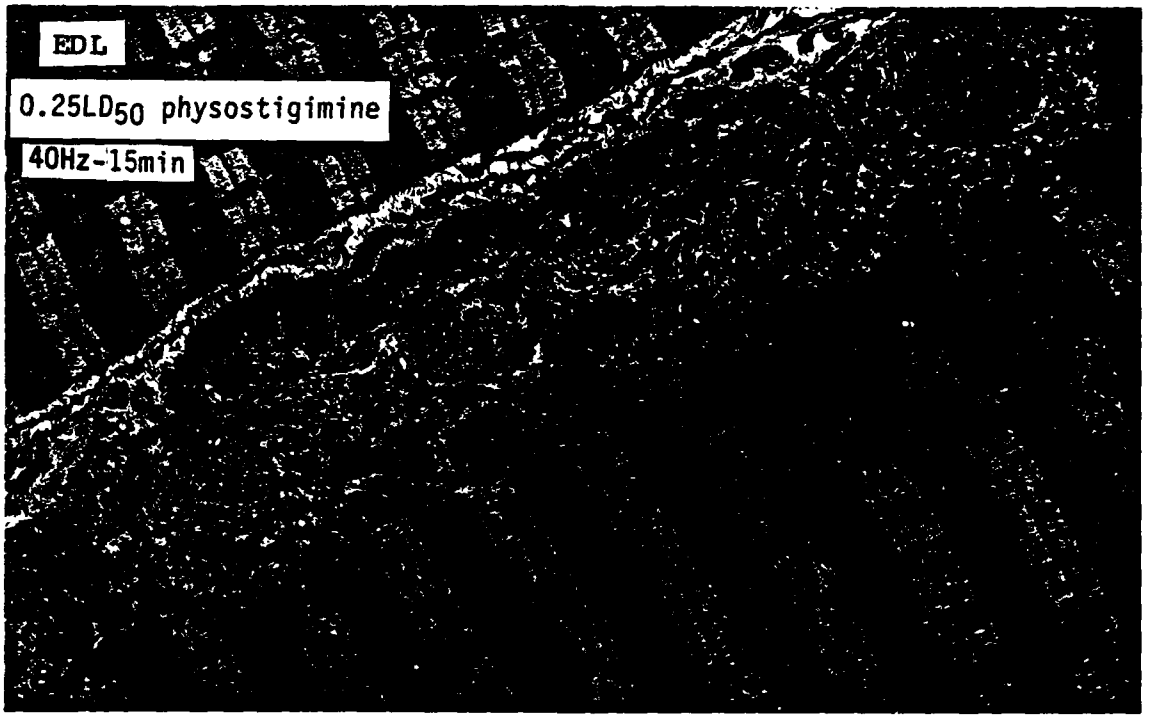

EDL
0.25LD50 physostigimine
20Hz-30min

Figure 95. Ultrastructure of EDL Neuromuscular Junctions After 15 and 30 Minutes of 40-Hz Stimulation in the Presence of 0.25 LD_{50} Physostigmine. After 15 minutes of stimulation, endplates exhibited variable morphology. In many endplates, pre- and post-synaptic components appear normal (Figure 95a), possibly indicating failure of nerve conduction to some endplates. However, after 30 minutes of 40-Hz stimulation in the presence of a subthreshold dose of physostigmine, nerve terminals were virtually depleted of synaptic vesicles and all pre-synaptic mitochondria were extensively vesicated. Post-synaptic mitochondria were either swollen or exploded, yet sarcomeres were of normal length and there was no evidence for supercontraction in any of the six endplates examined.


EDL
0.25LD50 physostigimine
40Hz-15min

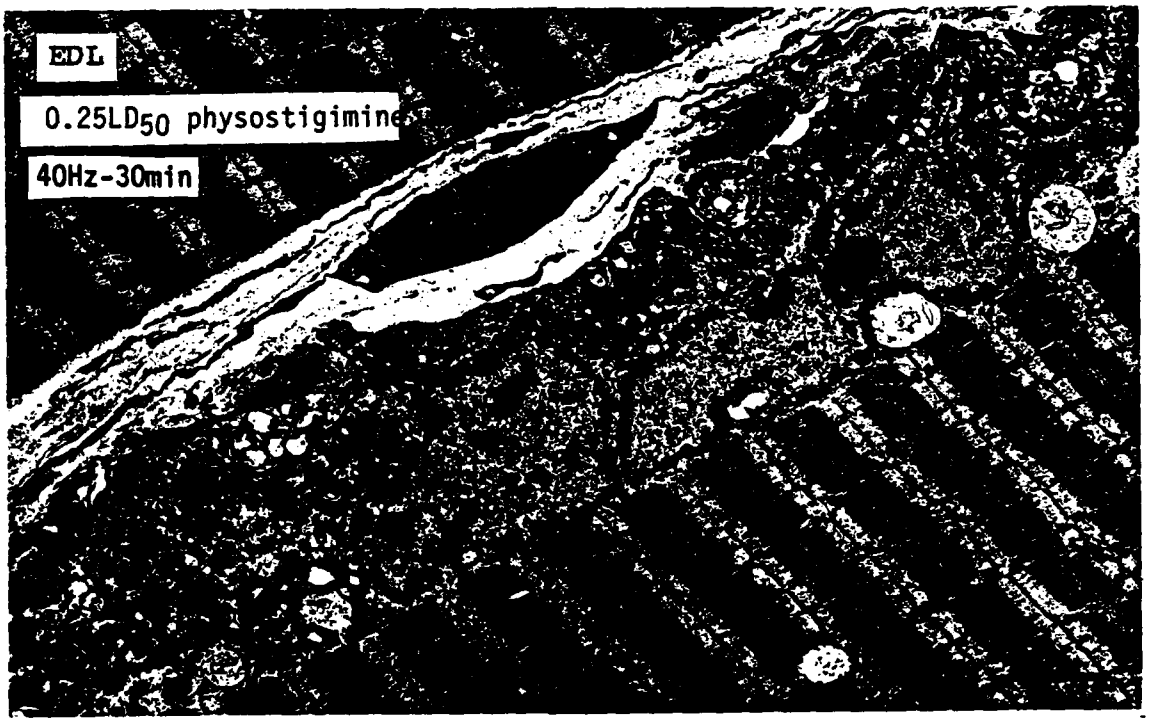
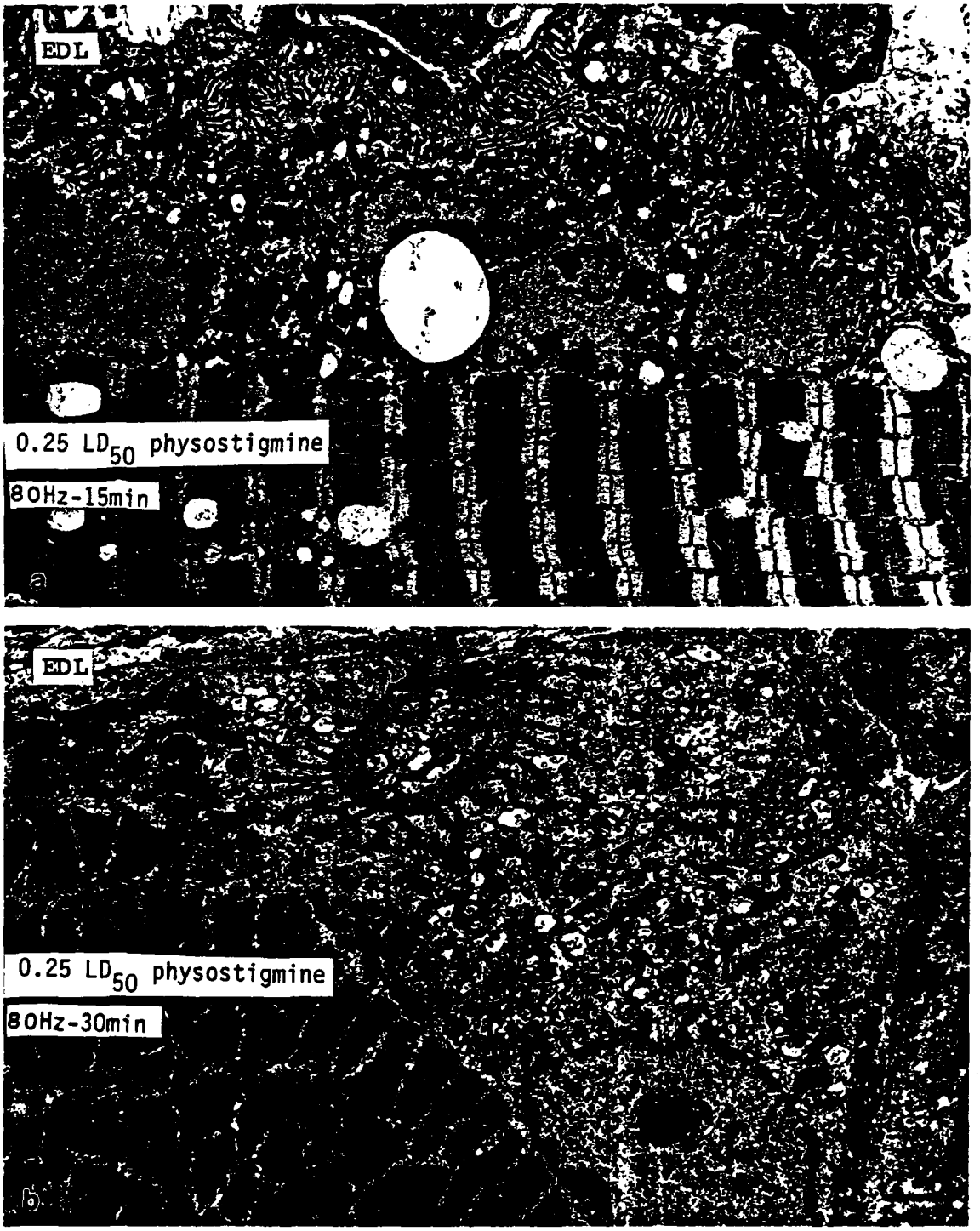

EDL
0.25LD50 physostigimine
40Hz-30min

Figure 96. Ultrastructure of EDL Neuromuscular Junctions After 15 and 30 Minutes of 80-Hz Stimulation in the Presence of 0.25 LD_{50} Physostigmine. Nerve terminals contained numerous synaptic vesicles but all pre-synaptic mitochondria were extensively vesicated. Post-synaptic mitochondria were either swollen or exploded after 15 minutes of 80 Hz-stimulation. After 30 minutes of 80-Hz stimulation (Figure 96b), pre-synaptic mitochondria were extensively vesicated, while post-synaptic mitochondria were frothy, swollen, or exploded. Sarcomeres were of normal length and there was no evidence for supercontraction.


EDL
0.25 LD50 physostigmine
80Hz-15min
a
EDL
0.25 LD50 physostigmine
80Hz-30min
b

N. DEMONSTRATION OF A STRONG POSITIVE SYNERGISTIC EFFECT OF STIMULATION ON ANTI-CHOLINESTERASE TOXICITY

1. Functional Changes Produced by Sustained Stimulation plus Acute Low to High Doses of Pyridostigmine vs. Physostigmine

A comparison of the effects of muscle stimulation combined with acute exposure either to physostigmine or to pyridostigmine was made using 21 rats. For each protocol, 3 rats were injected with low to high doses (0.01, 0.1, and 0.5-0.8 LD_{50}) of either physostigmine or pyridostigmine (yielding 30%, 70%, or 90-95% blood ChE inhibition) and, after maximum inhibition of blood ChE was obtained (less than 15 minutes), stimulated at 40 Hz for 15 minutes. (Stimulation procedures are described in **EXPERIMENTAL METHODS, Section F.**) This frequency and duration were described in the previous experiment as producing a near-threshold effect -- i.e., reproducible alterations in pre- and postsynaptic mitochondria, but not causing supercontraction of subjunctional sarcomeres. The animals were analyzed in groups of 3 rats each as detailed in **Table 1.** Average EDL twitch tension prior to drug administration was 34 $\pm$6 g. In addition, PSC values were again used as an indicator of possible effects of stimulation and drug treatment on whole muscle EDL physiology. Average PSC values measured before and after treatment are given in **Table 4.**

Correlated t tests showed that both physostigmine and pyridostigmine decreased PSC values significantly ($P<.05$), and that there was no significant difference between the effects of physostigmine vs. pyridostigmine treatment. The 20% reduction in

TABLE 4. Percent Sustained Contraction After Continuous Stimulation in the Presence and Absence of Low to High Doses of Physostigmine and Pyridostigmine

	Physostigmine			Pyridostigmine		
Dose	PSC Before*	PSC After	% Reduction#	PSC Before*	PSC After	% Reduction#
Low	116	68	41	108	80	26
Medium	111	73	34	95	93	2
High	110	57	48	97	37	62
Control	123	96	20			

*Initial PSCs (measured at "B" in Fig. 89) were usually greater than 100% (always greater than 95%).

#The % reduction in PSCs reflects the sum of drug effects and deterioration of the muscle. (For example, see Control.)

PSCs shown for the control group in Table 4 can be attributed to the 40-Hz 15 minute continuous stimulation and also to general deterioration of the surgical preparation with time. Any PSC reduction greater than 20%, then, reflects the effect of physostigmine or pyridostigmine and possibly a synergistic effect of stimulation on drug toxicity. While the results from rats exposed to physostigmine showed noticeably decreased PSCs for all three dose levels, the PSC was reduced substantially only at the highest dose of pyridostigmine. An ANOVA using pooled physostigmine and pyridostigmine data confirmed that the change in PSCs varied directly and significantly ($P<.05$) with dose levels.

In almost all cases, the post-treatment (final) PSCs were measured less than 1 minute after the continuous stimulation was terminated so that the animals could be perfused immediately for ultrastructural analysis. Consequently, the effect of recovery time (post-stimulation or post-injection) on PSC values could not be analyzed.

2. Ultrastructure of Continuously Stimulated, Intermittently Stimulated, and Unstimulated Muscles in Rats Receiving Low to High Doses of Pyridostigmine vs. Physostigmine

In a more detailed analysis and comparison of possible synergistic effects of sustained neuromuscular activity on endplate cytotoxicity of anti-ChE agents, soleus and EDL muscles were continuously stimulated at 40 Hz for 15 minutes in the presence and absence of low to high doses of physostigmine or pyridostigmine. Muscles in the contralateral (unstimulated, spinal-blocked) leg served as control (complete absence of nerve-evoked stimulation), whereas the diaphragm served as a normally stimulated control (albeit probably stimulated at higher than normal rates because of the stresses imposed during recording from the alert animal).

a. Severe Alterations in Continuously Stimulated Muscles: Demonstration of a Strong Synergistic Effect of Motor Activity on Endplate Damage

Severe supercontraction of subjunctional myofibrils was observed in stimulated fibers in soleus and EDL muscles in rats exposed to low, medium, and high doses of pyridostigmine (Figs. 97,98) and in medium and high doses of physostigmine (Fig. 99), but not in low doses of physostigmine (Fig. 100). The failure to observe supercontraction in fibers from rats exposed to low and moderate doses of physostigmine, while supercontraction was observed in stimulated fibers following low, moderate, and high doses of pyridostigmine, may represent, in part, a) selection artifact based on the low number of fibers sampled or b) inherent differences in the toxicities expressed by tertiary vs. quaternary anti-ChE agents. The former interpretation is supported by the observation that PSC values were significantly more depressed following exposure to physostigmine than following exposure to pyridostigmine, suggesting equal or greater physiological effect on muscle by physostigmine. Alternatively, the latter interpretation is supported by the repeated failure to elicit supercontraction of

subjunctional sarcomeres at 20-, 40-, or 80-Hz stimulation following exposure to 0.25 LD_{50} physostigmine (see Section M), whereas supercontraction was induced following 15 minutes of stimulation at 40 Hz following acute exposure to even lower doses (0.01 and 0.1 LD_{50}) of pyridostigmine (this experiment). Regardless, the development of severe supercontraction, even at low dose levels of pyridostigmine after 40-Hz stimulation for 15 minutes demonstrates that neuromuscular stimulation exerts a strong synergistic effect on the expression of anti-ChE toxicity, and moreover, that the synergism is more pronounced with pyridostigmine than with physostigmine. Finally, the induction of supercontraction at much lower blood ChE inhibition levels with pyridostigmine than with physostigmine suggests that endplate ChE may be inactivated at different drug concentrations from those producing equal levels of inactivation of blood ChE.

b. Inactivated (Spinal-blocked) Contralateral Muscles as Internal Controls

In rats exposed to 0.01, 0.1, and 0.5-0.8 LD_{50}, unstimulated myofibers (those maintained in a quiescent state by spinal block anesthesia) exhibited no discernible ultrastructural alterations of sarcomeres and only minor alterations of endplate mitochondria (Figs. 101, 102). It is important to point out that in continuously stimulated fibers from the same animals, supercontraction was the norm at all doses tested (Figs. 97b, 98). Moreover, in animals treated with the same dose [i.e., above 0.3 LD_{50} (see Section a, above, and Section c, below)] and allowed to stand and to walk, supercontraction is usually observed in most diaphragm and soleus myofibers (compare Fig. 103 at 30% ChE inhibition with Fig. 104 at 70 and 98% inhibition). Consequently, the absence of supercontraction at the higher doses in unstimulated but functionally innervated soleus fibers is attributed to the virtual absence of nerve-evoked endplate depolarization during spinal blockade. Since rats treated by spinal block anesthesia recover all function shortly after recovery from the anesthesia, the absence of alterations in these functionally denervated but structurally innervated endplates indicates that alterations commonly observed in recently denervated animals exposed to anti-ChE agents (cf. 24) likely reflect increased transmitter release as a result of denervation damage rather than direct antidromic stimulation of motor units. Finally, the absence of alteration in the inactive but innervated muscles indicates that the alterations seen in surgically denervated endplates do not arise from antidromic activation of motor units by anti-ChE agents.

c. Intermediate Alterations in the Internally-Stimulated Diaphragm

In rats exposed to 0.01 LD_{50} physostigmine or pyridostigmine and maintained in an alert state by spinal block anesthesia, endplates in the self-stimulated diaphragm myofibers (constantly used in breathing) had normal pre- and postsynaptic mitochondria. At 0.1 LD_{50}, subjunctional mitochondria in most diaphragm fibers were blistered or frothy (Figs. 104a, 105),

indicating that presynaptic activity was at or near normal levels, but that the prolongation of normal postsynaptic depolarizations in the presence of physostigmine resulted in slightly altered subjunctional mitochondria. (These data for extent of neuromuscular damage at 0, 30, 70, and 90% ChE inhibition are presented in graphical form in Figs. 105 and 106.) In rats exposed to 0.5-0.8 LD_{50} physostigmine or pyridostigmine, most of the diaphragm myofibers (i.e., muscles constantly used in breathing) had subjunctional sarcomeres that were supercontracted, as well as mitochondria that were blistered, frothy, and/or grossly swollen (Figs. 104b, 105c). These data are consistent with ultrastructural alteration observed in acute exposure experiments described in Section H in which endplate damage was routinely observed in myofibers of muscles activated at normal rates in the presence of high doses of anti-ChE agents (Fig. 23), but not present in similar muscle exposed to moderate or low doses (Figs. 32, 33). As myofibers and motor units fail, recruitment of additional fibers and their rapid failure produces a "positive feedback" that results in catastrophic failure of whole muscles. If those muscles are used in breathing, for example, respiratory failure is likely.

In summary, these data from stimulated and unstimulated soleus and EDL muscles suggest that moderate to high rates of neuromuscular transmission are required for the production of the characteristic lesions seen at the motor endplate following acute exposure to near-lethal doses of this potent anti-ChE agent. The presence or absence of supercontraction is thus directly related to both the inactivation of endplate ChE and the occurrence and rate of (current or recent) nerve terminal activity. These observations may be especially relevant to proposed uses of these agents as possible chemoprophylactic drugs for treating exposure to the irreversible anti-ChE agents.

Figure 97. Ultrastructure of EDL Neuromuscular Junctions Whose Common Peroneal Nerves Had Been Stimulated at 40 Hz for 15 Minutes in the Absence of Pyridostigmine (Stimulation Control) and Following Subacute Exposure to a Low Dose of Pyridostigmine. Stimulation plus a low dose of pyridostigmine produced supercontraction of subjunctional sarcomeres in most EDL and soleus myofibers (Figure 97b), whereas stimulation in the absence of drug produced no supercontraction (Figure 97a). Vesicated mitochondria in the nerve terminals (Figure 97a) represent alterations characteristic of hyperstimulation.

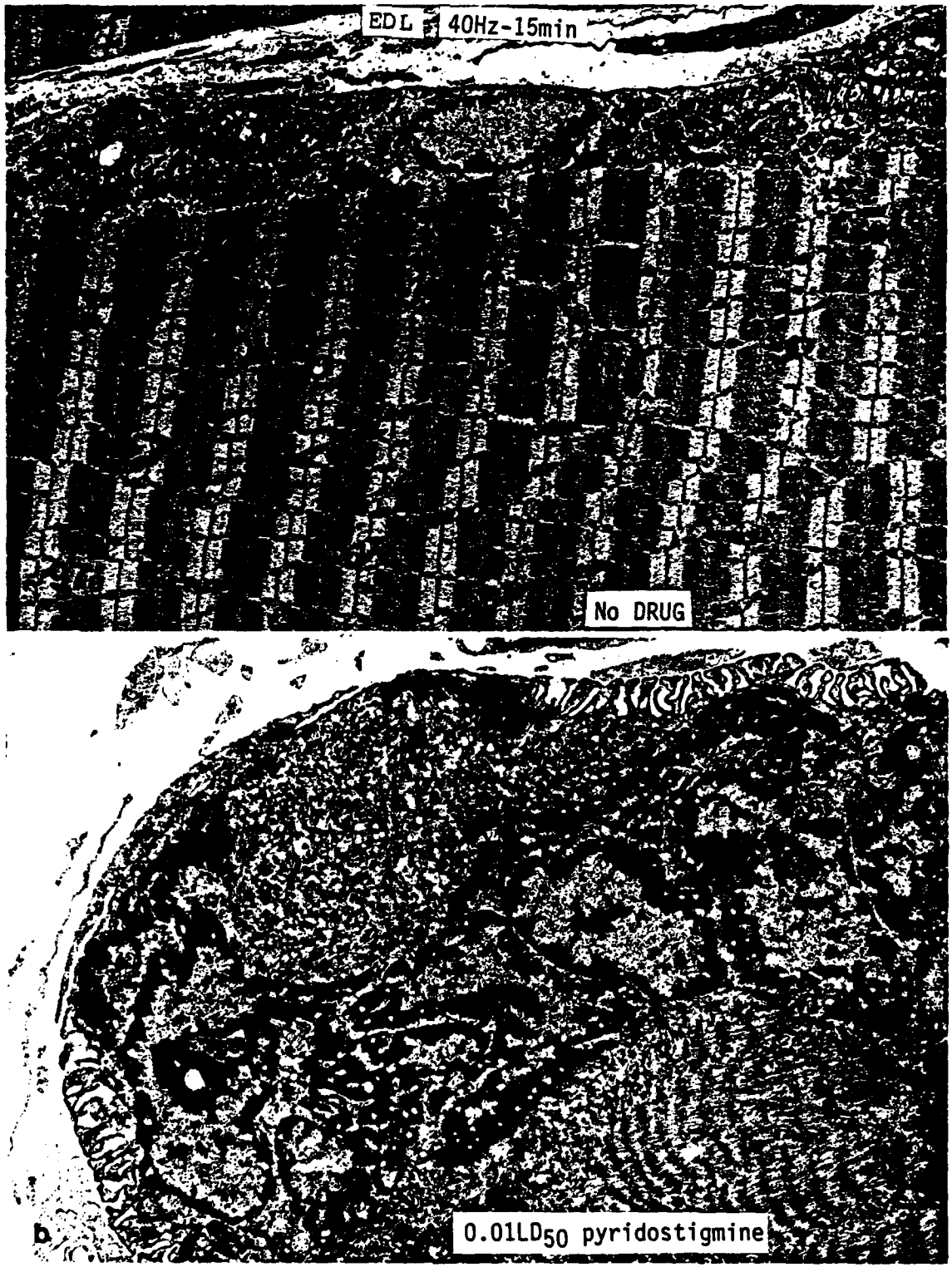

EDL 40Hz-15min
No DRUG
b
0.01LD50 pyridostigmine

Figure 98. Ultrastructure of EDL Neuromuscular Junctions Whose Common Peroneal Nerves Had Been Stimulated at 40 Hz for 15 Minutes in the Presence of 0.1 or 0.8 LD_{50} Pyridostigmine. Pronounced supercontraction of subjunctional sarcomeres was observed in most hyperstimulated EDL and soleus myofibers at 70% blood ChE inhibition (Figure 98a) and in all myofibers examined at 90% blood ChE inhibition (Figure 98b). Supercontraction was always accompanied by exploded subjunctional mitochondria and by vesicated mitochondria in the nerve terminals (Figure 97a). Presynaptic alterations characteristic of hyperstimulation were absent from myofibers that were not supercontracted, suggesting that some variability may result from nerve failure during prolonged moderate-frequency stimulation of the common peroneal nerve.

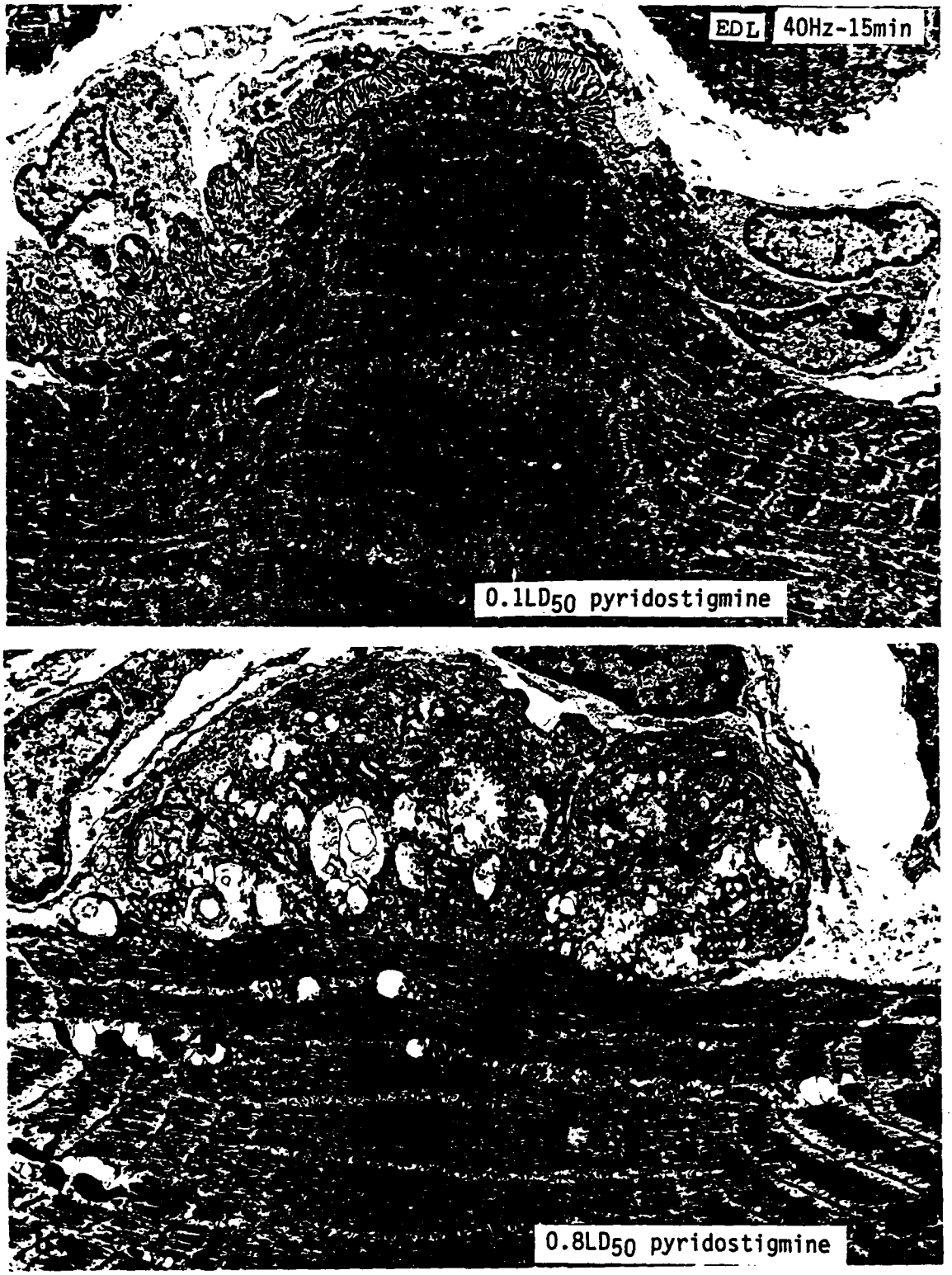

EDL 40Hz-15min
0.1LD50 pyridostigmine
0.8LD50 pyridostigmine

Figure 99. Ultrastructure of EDL and Solues Neuromuscular Junctions Whose Common Peroneal Nerves Had Been Stimulated at 40 Hz for 15 Minutes in the Absence of Physostigmine and Following Subacute Exposure to a Low Dose of Physostigmine. Stimulation in the absence or presence of a low dose of physostigmine produced no detectable supercontraction of subjunctional sarcomeres in EDL or soleus myofibers. Vesicated mitochondria in the nerve terminals (arrowheads) confirm the efficacy of the stimulation regimen in these non-supercontracted fibers.

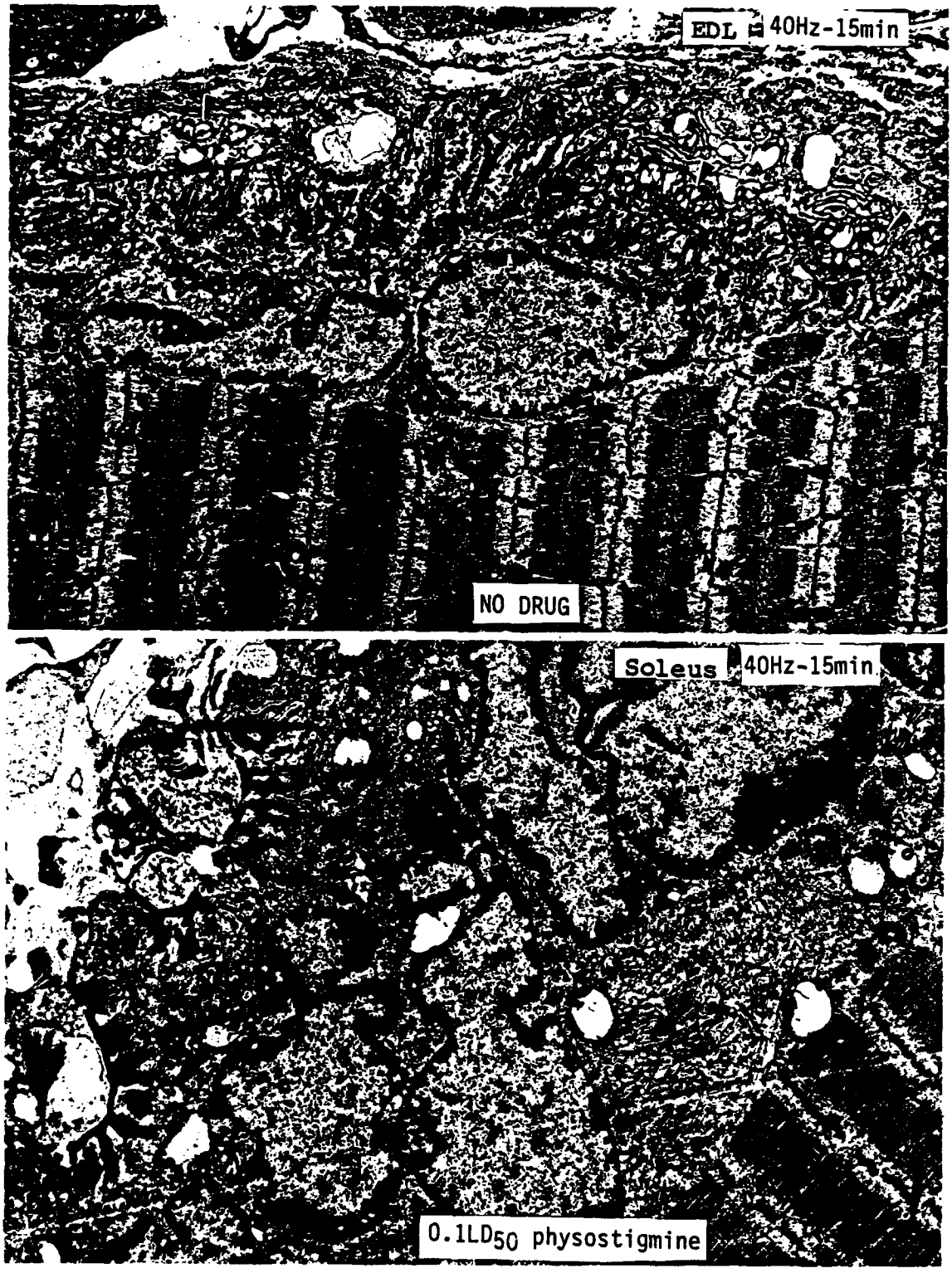

EDL 40Hz-15min
NO DRUG
Soleus 40Hz-15min
0.1LD50 physostigmine

Figure 100. Ultrastructure of EDL and Soleus Neuromuscular Junctions Whose Common Peroneal Nerves Had Been Stimulated at 40 Hz for 15 Minutes in the Presence of 0.1 or 0.8 LD_{50} Physostigmine. Pronounced supercontraction of subjunctional sarcomeres was observed only rarely after 70% blood ChE inhibition (Figure 100a shows a typical non-supercontracted myofiber) but was present in all myofibers examined at 90% blood ChE inhibition, Figure 100b). Supercontraction was always accompanied by exploded subjunctional mitochondria and by vesicated mitochondria in the nerve terminals (see Figure 97a). The variability observed at 70% blood ChE inhibition between pyridostigmine and physostigmine may result from differences in drug redistribution between blood and endplate ChE, differential effects of drug on endplate ChE, different time courses to maximum drug effect at the endplate, or to undetected variations in sampling procedures for electron microscopy.

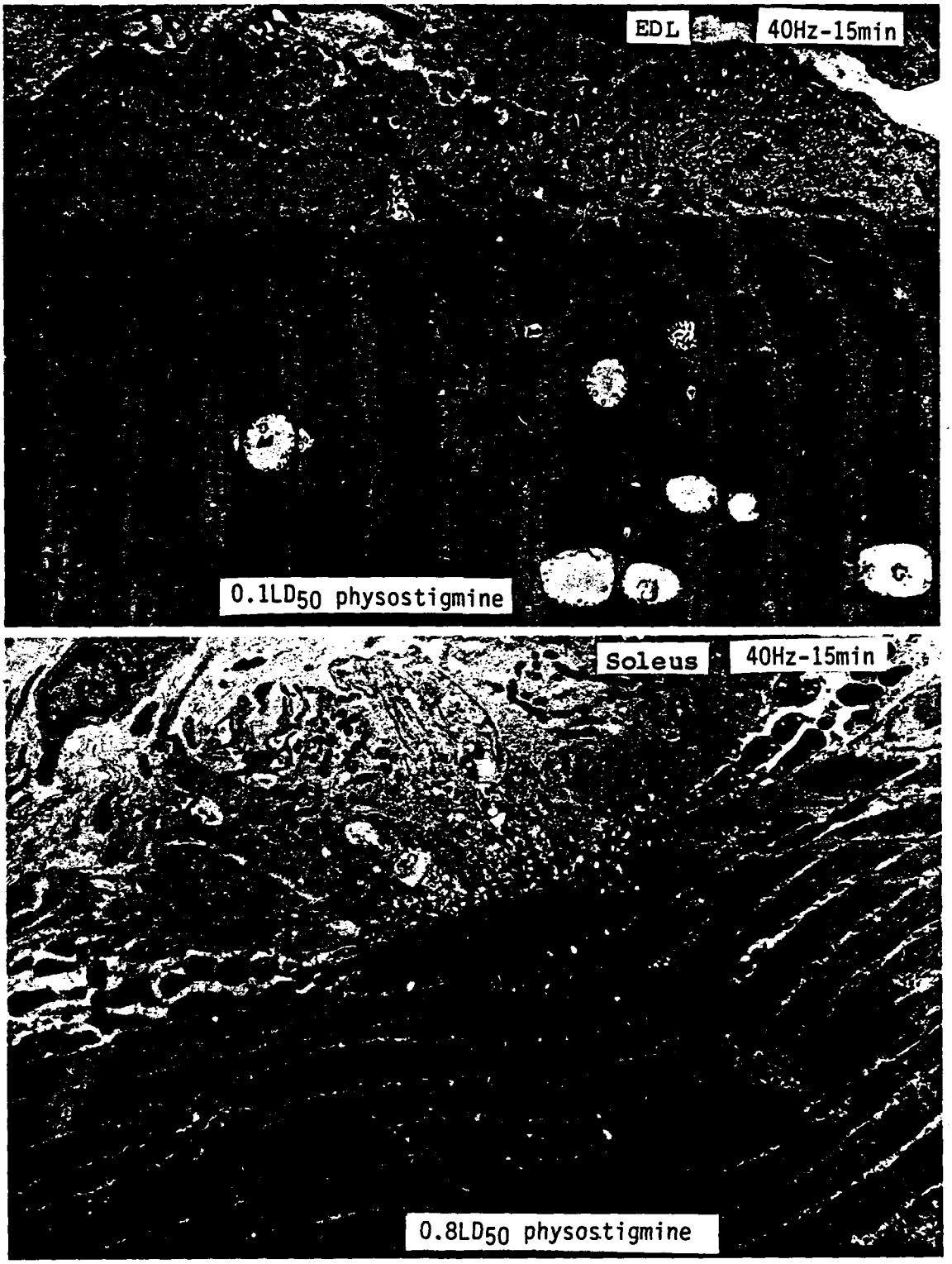

EDL
40Hz-15min
0.1LD50 physostigmine
Soleus
40Hz-15min
0.8LD50 physostigmine

Figure 101. Ultrastructure of Soleus Neuromuscular Junctions From Spinal-Blocked Animals Exposed to Low, Moderate, and High Doses of Pyridostigmine. Except for hypoxic mitochondria in nerves and Schwann cells (Figure 101b), known to represent an artifact of inadequate perfusion fixation, all myofibers were normal (i.e., were in the resting state). No evidence for damage was detected, either from direct effects of high dose exposure on endplate ultrastructure or from antidromic activation of nerve terminals or axons.

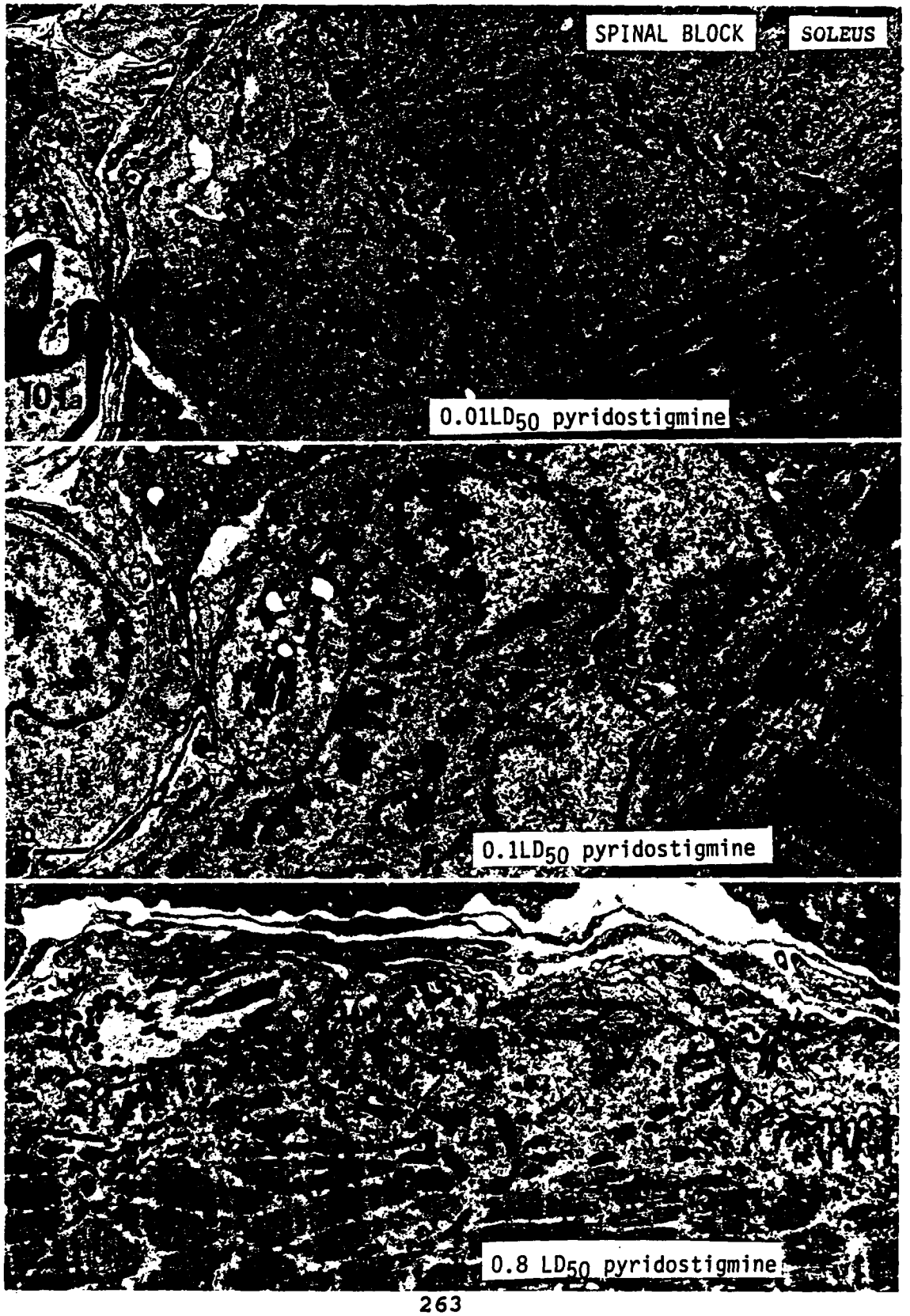

SPINAL BLOCK
SOLEUS
0.01LD50 pyridostigmine
0.1LD50 pyridostigmine
0.8 LD50 pyridostigmine

Figure 102. Ultrastructure of Soleus Neuromuscular Junctions From Spinal-Blocked Animals Exposed to Low, Moderate, and High Doses of Physostigmine. All myofibers were normal (i.e., were in the resting state). No evidence for damage was detected, either from direct effects of high dose exposure on endplate ultrastructure or from antidromic activation of nerve terminals or axons.

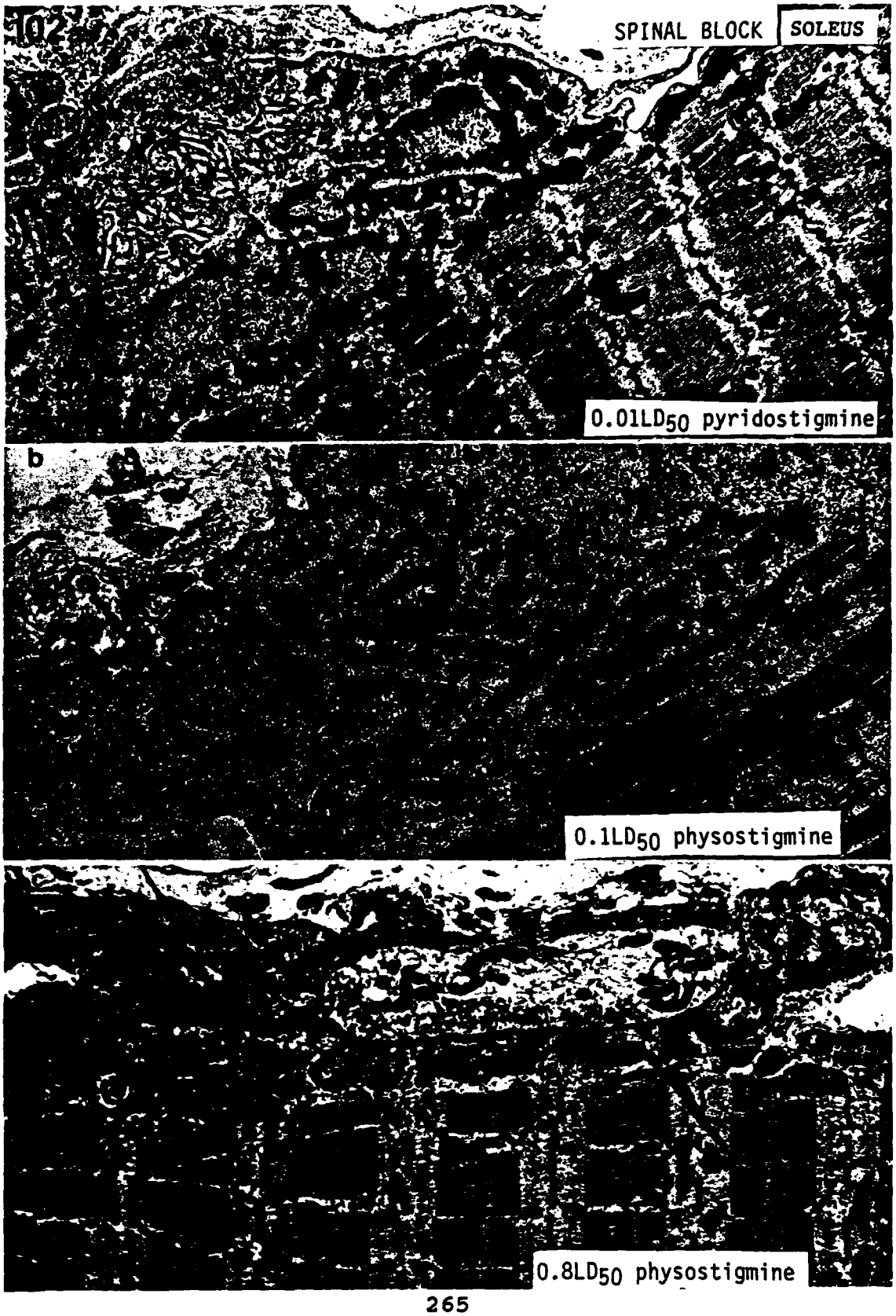

SPINAL BLOCK
SOLEUS
0.01LD50 pyridostigmine
b
0.1LD50 physostigmine
0.8LD50 physostigmine

Figure 103. Ultrastructure of Diaphragm Neuromuscular Junctions Undergoing Normal Rates of Neuromuscular Stimulation in the Absence of Drug and After Acute Exposure to a Low Dose of Pyridostigmine. Normal rates of stimulation in the absence or presence of a low dose of pyridostigmine produced no detectable supercontraction of subjunctional sarcomeres (Figure 103b), whereas hyperstimulation at the same dose produced severe supercontraction (Figure 97b).

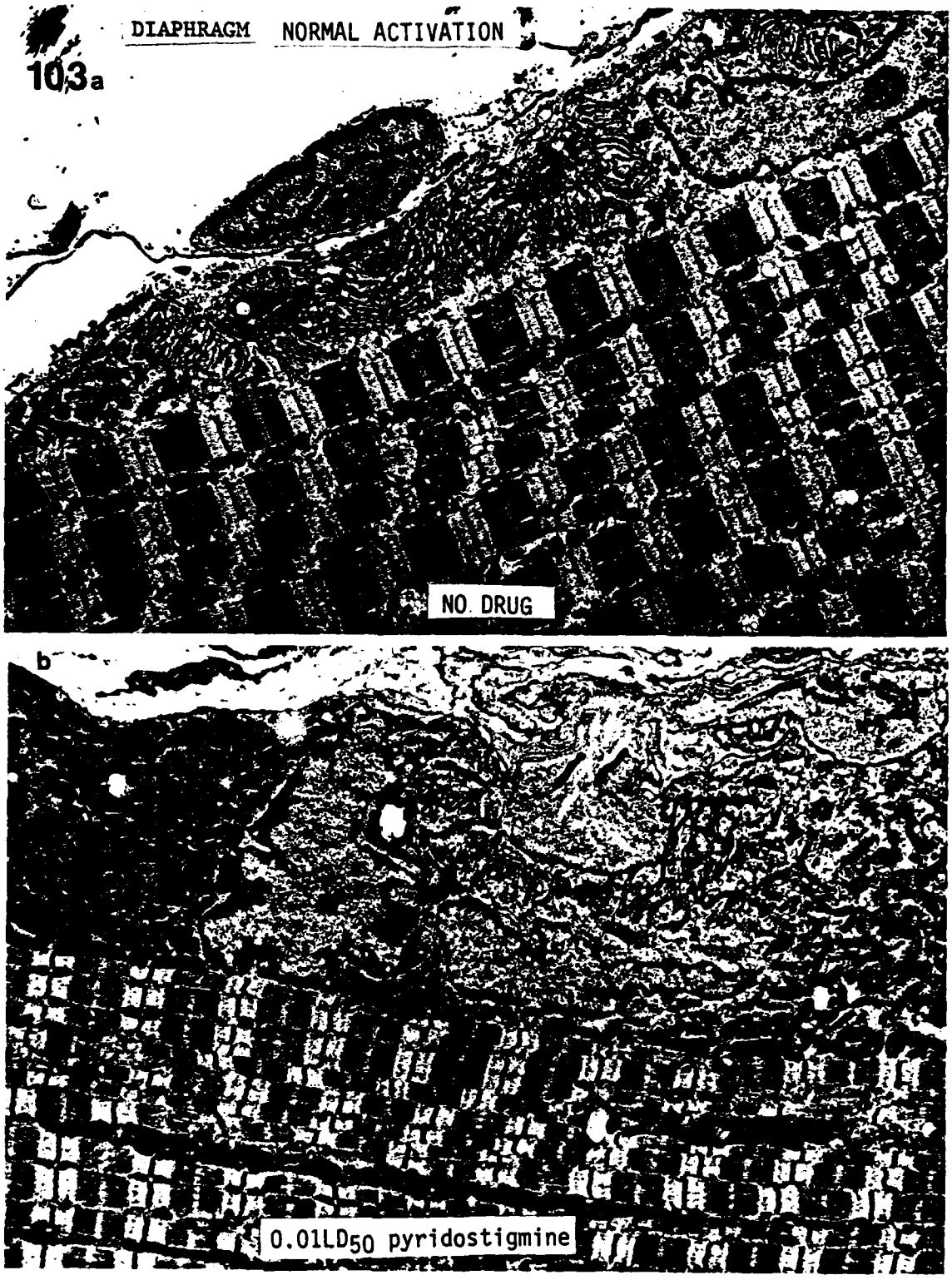
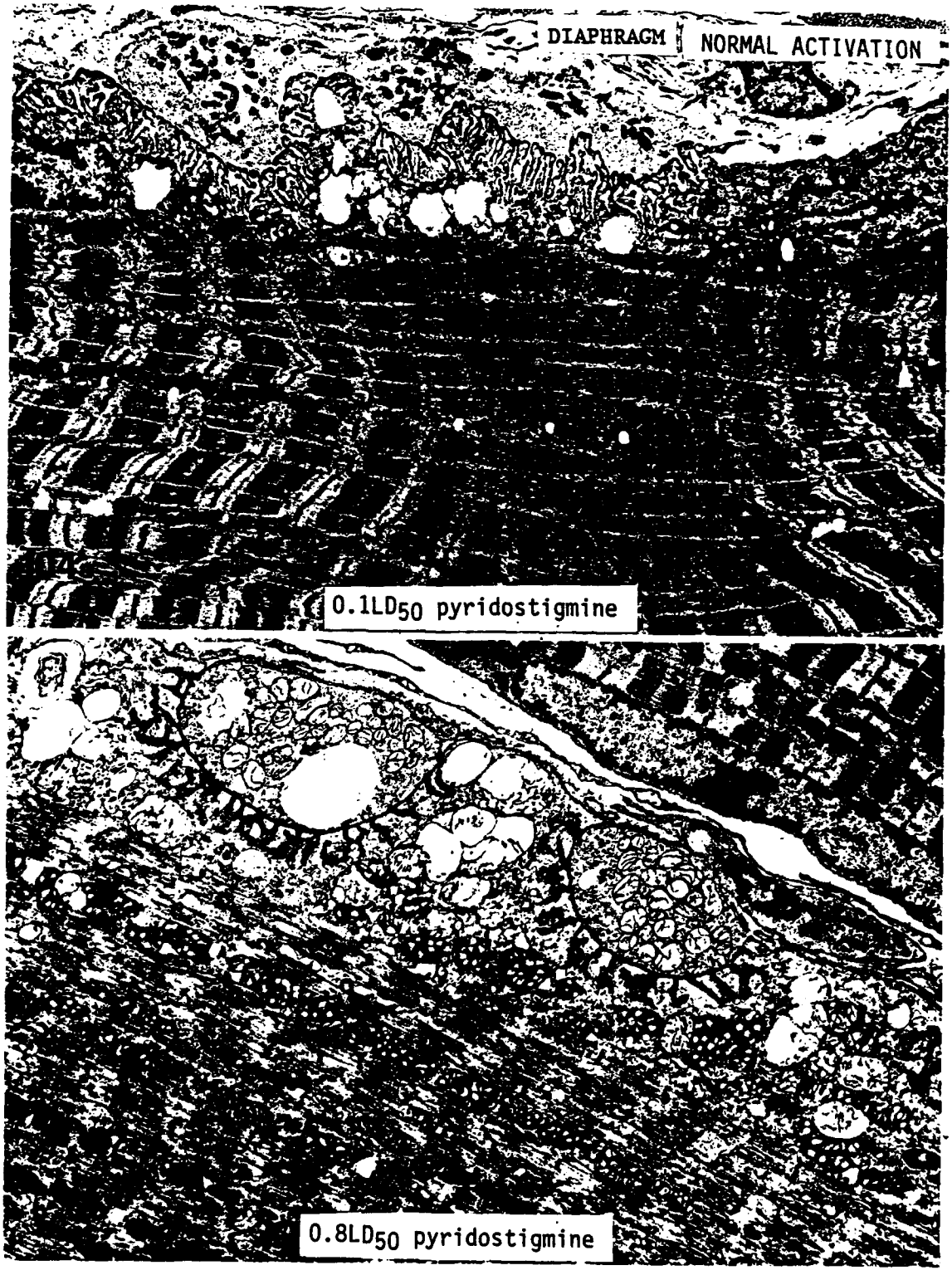

DIAPHRAGM NORMAL ACTIVATION
103a
NO DRUG
b
0.01LD50 pyridostigmine

Figure 104. Ultrastructure of Diaphragm Neuromuscular Junctions Undergoing Normal Rates of Neuromuscular Stimulation After Acute Exposure To Moderate and High Doses of Pyridostigmine. By comparison to Figure 101 (endplates from spinal-blocked animals), where supercontraction of subjunctional sarcomeres was not observed, supercontraction was produced by relatively moderate rates of neuromuscular activity at 70% blood ChE inhibition (Figure 104a) and by hyperactivity at 30% blood ChE inhibition (Figure 97b, above). This provides strong evidence for the synergistic effect of the rate and duration of neuromuscular activity on the expression of anti-ChE toxicity at the rat neuromuscular junction. These data from pyridostigmine and physostigmine are summarized graphically in Figures 105 and 106.


DIAPHRAGM
NORMAL ACTIVATION
0.1LD50 pyridostigmine
0.8LD50 pyridostigmine

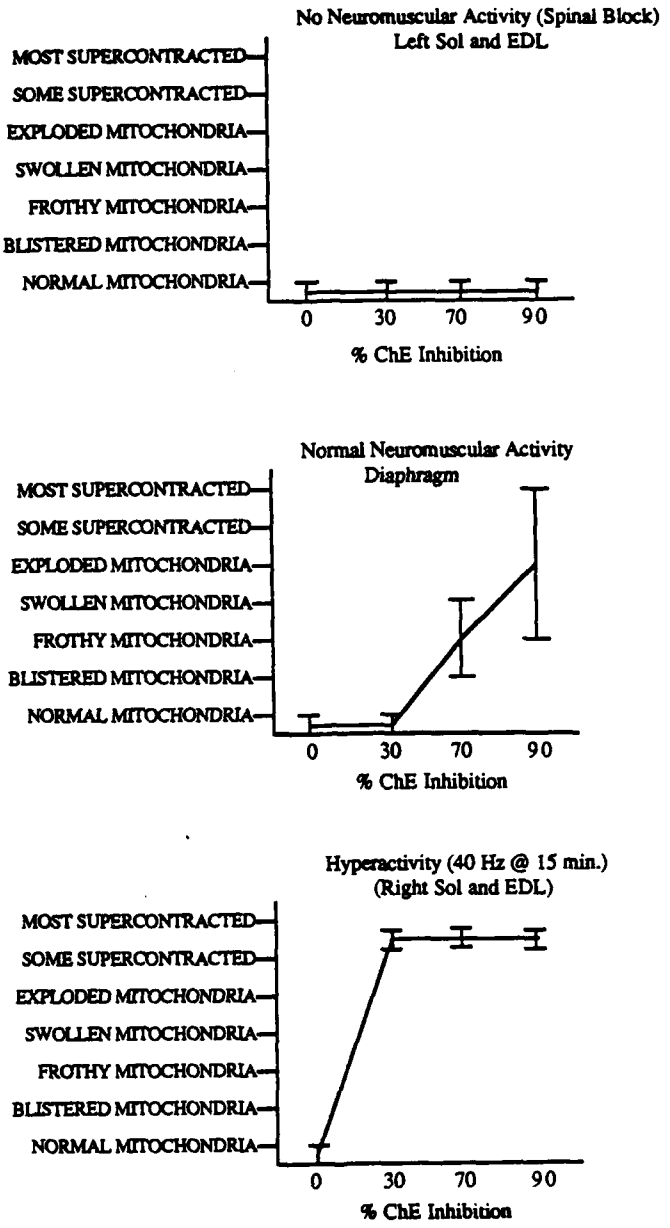


Figure 105. Effect of Neuromuscular Activity on Endplate Ultrastructure as an Expression of Pyridostigmine Toxicity at Selected Blood ChE Inhibition Levels. The "error bars" represent the range of responses observed at each level of ChE inhibition. Muscles are grouped according to their stimulation and/or use patterns. Muscles of the left hindlimb (top graph) were blocked for 15 minutes ("No Neuromuscular Activity"); the diaphragm was used in normal respiration during the 15-minute experiment (middle graph); and muscles of the right hindlimb were stimulated at 40 Hz for 15 minutes (bottom graph). Ultrastructural responses in grouped muscles were similar.

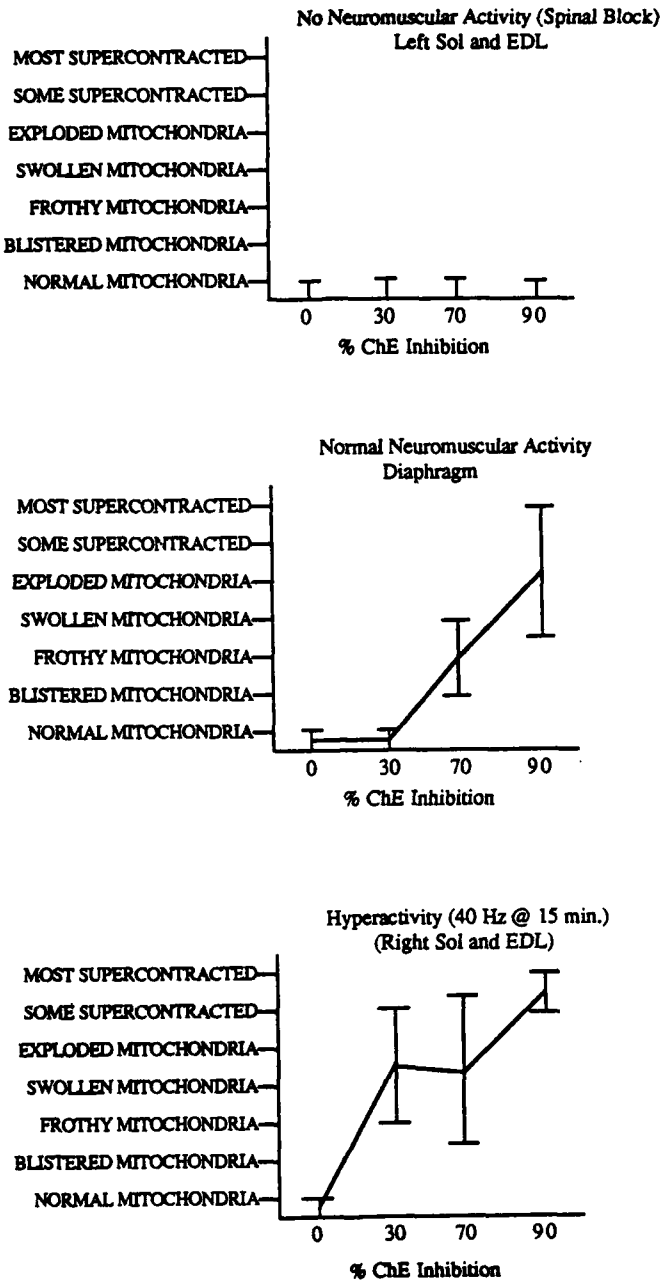


Figure 106. Effect of Neuromuscular Activity on Endplate Ultrastructure as an Expression of Physostigmine Toxicity at Selected Blood ChE Inhibition Levels. The "error bars" represent the range of responses observed at a given level of ChE inhibition. Muscles are grouped according to their stimulation and/or use patterns. Muscles of the left hindlimb (top graph) were blocked for 15 minutes ("No Neuromuscular Activity"); the diaphragm was used in normal respiration during the 15-minute experiment (middle graph); and muscles of the right hindlimb were stimulated at 40 Hz for 15 minutes (bottom graph). Ultrastructural responses in grouped muscles were similar.

IX. INTERPRETATIONS

A. Overview

In this Annual and Final Report, we have described four related investigations of the pathophysiology of two potent anti-ChE agents, physostigmine and pyridostigmine. In the first group of experiments, we described the immediate, delayed, and long-term effects on rat nerve, muscle, and neuromuscular junction physiology and ultrastructure following acute exposure to the anti-ChE compound physostigmine administered by s.c. injection at very low to very high doses (0.001-1.1 LD_{50} or 0.00075-0.8 mg/kg). 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 LD_{50} dose levels. We verified supercontraction of subjunctional myofibers as the primary diagnostic feature for neuromuscular cytopathology following acute high-dose physostigmine exposure in both the rat and guinea pig. Our data revealed that extreme morphological damage occurred in quiescent rats following very high doses of physostigmine (0.8-1.1 LD_{50}) and in active rats following high or moderate doses (0.8 or 0.3 LD_{50}). During these experiments, we also obtained preliminary physiological and ultrastructural evidence suggesting that muscle use exacerbates the effects of anti-ChE exposure. (Additional data from studies of the synergism of neuromuscular activity and drug exposure levels are summarized below.) Finally, in the acute exposure experiments, we showed that recovery from the effects of high- and low-dose exposure is much more rapid and complete than previously suggested.

In the second major project, we have used biochemical, physiological, and ultrastructural techniques to characterize changes in neuromuscular function and ultrastructure following subacute (up to 14 days) exposure to moderate and high doses of physostigmine (40% ±10% and 80% ±10% sustained Che inhibition) and have used the same techniques to analyze the time course and extent of reversibility of pathophysiological alterations for up to 28 days following termination of subacute drug exposure. From these data, we have shown a) that there are no detectable alterations of nerve-muscle ultrastructure or physiology following moderate-dose subacute exposure, and b) that there are few detectable alterations following subacute exposure at a relatively high dose. We have also confirmed the rapid repair and recovery of function of nerve and muscle following termination of high-dose subacute exposures.

In the third group of experiments, we compared the effects of acute exposure to physostigmine vs pyridostigmine at equitoxic doses (0.01, 0.1, and 0.3-0.6 $LD5_0$, or 30%, 70% and 90-95% blood ChE inhibition levels). We showed that supercontraction of subjunctional myofibers and a characteristic sequence of mitochondrial alterations occurs over the same time course and at similar blood ChE inhibition levels for both drugs, with the effects of pyridostigmine equal to or slightly greater than those of equivalent doses of physostigmine (differences not analyzed statistically).

In the fourth group of experiments, we have described the effect of neuromuscular activity on the threshold of drug-induced neuromuscular pathology, and showed that muscle use is a major factor in the expression of primary drug toxicity. We documented a strong positive synergism between the amount of muscle use and the level of blood ChE inhibition on endplate cytopathology. The extent of muscle cytopathology, in turn, was correlated with the time course of reversibility of ultra-structural alterations.

The primary conclusion obtained from these several groups of experiments is that the neuromuscular pathophysiology and cytopathology found following acute physostigmine exposure results directly and indirectly from the primary action of anti-ChE's on endplate ChE. All of the major cytopathological changes are consistent with the following sequence:

1. Inactivation of cleft AChE [and butyrylcholinesterase (BuChE)], which results in...
2. Prolongation of the nerve-evoked endplate depolarization that results from normal vesicular (quantal) release.
3. In addition, the substantial amount of ACh released by non-vesicular ("non-quantal") mechanisms contributes enormously to increased and prolonged endplate currents through relatively continuous activation of post-synaptic ACh receptors.
4. If depolarization remains above the threshold for activating the muscle action potential, primary depolarizing neuromuscular blockade ensues. [Initially, this blockade may be intermittent, as in the first stages of blockade by succinylcholine(1).]
5. To compensate for the resulting decrease in force generation in each muscle (for example, in respiration or in postural control), neuronal activity is increased, both in frequency and by the recruitment of additional motor units.
6. In turn, the increased rate of nerve terminal depolarizations and increased release of vesicular ACh increases the number and probability of depolarizing blocks in additional endplates and motor units.
7. As a consequence of the increased rate of synaptic vesicle fusion with the nerve terminal plasma membrane (NTPM), a disproportionate number of the ACh transport molecules (present in the synaptic vesicle membrane to fill them with ACh) become incorporated into the NTPM. These continue to transport ACh across the membrane, but because of their new location in the NTPM, transport is now into the synaptic cleft. The resulting increased transmembrane transport is reflected as an increase in non-vesicular or non-quantal release. After prolonged nerve terminal activity, non-vesicular release [ACh "leakage", which represents 50%-95% of total ACh release (63-65, reviewed in 56)], becomes increasingly important in producing and maintaining endplate depolarization. [Note, however, that "desensitization" of acetylcholine receptors (AChR) may limit the period of endplate depolarization and contribute to the partial restoration of resting membrane potential.]
8. The increasing degree of postsynaptic

depolarization and accompanying influx of calcium ions produces the characteristic sequence of morphological changes in subjunctional mitochondria: granular --> blistered --> frothy --> swollen --> exploded mitochondria. In its extreme state, severe and prolonged endplate depolarization produces mitochondrial "explosion," massive release of sequestered calcium ions, and the progressive supercontraction of subjunctional sarcomeres, yielding gross myofiber damage. At this stage, supercontraction (which is slowly reversible) is maintained for an extended period, whether or not endplate resting potential is restored to normal.

9. If death does not ensue quickly following acute exposure, recovery of muscle function and endplate ultrastructure is relatively rapid and complete. Moreover, the rapid recovery does not support previous suggestions (26,27) for widespread proteolysis and recovery based on de novo protein synthesis. Rather than molecular synthesis and assembly of new myofibrillar proteins, the repair mechanisms appear to involve primarily the reassembly of pre-existing sarcomere components that were disassembled during the period of maximum supercontraction.

10. The threshold for neuromuscular pathology is lowered substantially (by well over one order of magnitude) by prolonged neuromuscular stimulation. In that regard, the expression at the neuromuscular junction of anti-ChE toxicity (from both physostigmine and pyridostigmine) is blocked by motor neuron inactivation (i.e., reversible spinal block anesthesia), even when the drug is administered at 2-3x suprathreshold levels. Thus, the wide latitude for expression of cytopathology was shown to be strongly dependent on muscle activity, thereby confirming the proposed synergistic effect of nerve terminal activity on the expression of neuromuscular damage and toxicity. Moreover, the functional denervation experiments (with maintained anatomical innervation) cast strong doubt on models of toxic expression mediated by antidromic activation of nerves or their motor units. Those earlier experiments demonstrating fibrillations and fasciculations following exposure of recently denervated preparations to high doses of anti-ChE agents are reinterpreted as reflecting in large part the effects of increased vesicular and non-vesicular release at motor endplates made unstable and/or hyperactive by recent denervation. The ensuing muscle contractions result from random activation of endplates previously brought to near threshold by the inactivation of endplate ChE in the presence of normal or increased rates of vesicular and non-vesicular release (cf. 56, 63-65).

Evidence for each of these postulated stages is summarized below.

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

In all rats that survived acute high-dose exposures to physostigmine, ChE activity returned to normal within 24 hours, but fluctuated greatly until termination of the experiment. (Likewise, control values fluctuated substantially during an equivalent period). Thirty minutes after single high-dose (0.8

LD_{50} 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 and amorphous material were present in disorganized masses. Dose-response data from quiescent rats and guinea pigs indicated that physostigmine-induced supercontraction (as well as other changes in myofiber ultrastructure) was dose-dependent, with severe damage occurring above a threshold of 75% ChE enzyme inhibition. Higher doses produced profound changes in endplate structure and physiology. The values for ChE inhibition at various partial LD_{50}s are essentially identical to those obtained by Hudson and coworkers for pyridostigmine (28-30). However, endplate damage was not demonstrable at blood ChE inhibition levels lower than 70% (except in myofibers that had been continuously stimulated for prolonged periods at moderate to high frequencies; see Sections F and G, below).

In addition to supercontraction, a mixed population of frothy and grossly distended mitochondria plus distended sarcoplasmic reticulum cisternae was observed disrupting the subjunctional sarcoplasm at doses above 0.3 LD_{50} (70% ChE inhibition). 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 depolarization is thought to result in flooding of the endplate region with Na^+ and Ca^{++} ions and associated water molecules, as well as in the efflux of K+ ions. Mitochondria initially sequester Ca^{++}, but once they become saturated with calcium phosphate granules, they develop numerous internal clear blisters and frothy areas of as yet undetermined origin. As ion and water influx continue, the frothy areas expand and coalesce, and the mitochondria begin to swell. At much higher dose levels, severe and prolonged endplate depolarization ultimately result in rupture or explosion of the subjunctional mitochondria, and presumably, in the explosive release of toxic levels of Ca^{++}.

Similar ultrastructural alterations were observed in rat and guinea pig muscles at similar LD_{50} levels. It is interesting that at ½ hour PI, rat soleus (but not EDL) and guinea pig EDL (but not soleus) muscles showed similar ultrastructural alterations. The reason for the apparent reversal of fiber-type specificity of drug effects in these two animal species is not known, but may be related to differences in muscle use patterns or to inadequacies of sampling procedures and/or number of samples examined by electron microscopy. Our reluctance to attribute the differences to inherent differences in fiber types arises in part because of the apparent reversal of 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 the amount and duration of muscle use is shown to exert a profound effect on the threshold of neuromuscular pathology. (See below.)

C. Acute-delayed Effects and Reversibility

We have determined that enzyme inhibition is virtually completely reversed within 24 hours 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 measured (EDL twitch potentiation and PSCs following high frequency stimulation) returned to normal within 1 hour after acute physostigmine administration. In all cases, this return to normal physiology occurred when blood ChE inhibition levels were still 80% or higher. This is consistent with suggestions that transmitter release is decreased substantially following exposure to other anti-ChE agents (66), possibly by affecting the efficacy of transmitter release, thereby decreasing the quantal content of the EPP. In addition, the post-synaptic membrane may become less sensitive to ACh (by "desensitization block" or by removal of ACh receptors).

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 exhibited normal condensed morphology, and the sarcoplasmic reticulum appeared as the normal network of thin tubules and flattened oval profiles. Since the repair processes, including complete reassembly of subjunctional sarcomeres and reestablishment of normal contractile responses, occurred very quickly (often in less than 24 hours after acute exposure), we consider it unlikely that the widespread proteolytic activity proposed by others (27) could have been reversed by the limited amount of protein synthesis possible in less than 24 hours. Rather, a process of dissolution and reassembly of the same molecular subunits seems more likely.

In our first Annual Report (57), 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 a near LD_{50}-dose of physostigmine is rapidly reversible but that in the most severely affected fibers, physostigmine may exert substantial influences on long-term biological control mechanisms regulating endplate morphology. However, more detailed examination of a much larger sample size of treated vs. control endplates convinces us that those images previously interpreted as consistent with denervation, collateral sprouting, and reinnervation must now be reinterpreted as arising primarily (if not exclusively) from normal endplate remodeling (cf. 58 vs. 67) and to plane-of-

section artifacts not previously recognized because of inadequate sampling size for controls. Likewise, we were unable to detect any type of long-term alteration of endplate structure or function following moderate to very low doses. If such changes occurred, they were either not resolvable by conventional thin-sectioning techniques or they were so rare as to be undetectable among the variabilities seen in normal endplates.

D. Effects of Subacute Physostigmine Exposure

For the subacute-recovery exposures, at 3 days' recovery and thereafter, EDL twitch tension and high-frequency contraction properties were not significantly different from 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 minimally altered during the subacute exposure period before recovery. On the other hand, high-dose pumps had produced sustained ChE inhibitions of 80%, and thus, some damage to EDL neuromuscular junctions had been expected. However, in rats implanted with unprimed pumps, we detected no major alterations in whole muscle function. Thus, the gradual increase in blood ChE inhibition minimized physiological and ultrastructural alterations. 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. (53), who reported that during subacute administration of pyridostigmine, EDL single twitch tensions were decreased substantially, and that there was a depression of contractility during 20-Hz stimulation. Apparently, the response seen by Adler, et al. (53) was due to interaction between pyridostigmine and the general anesthetic that they used (i.e., chloral hydrate). As shown in Fig. 10 and 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. Thus, all experiments described in this Final Report were done under local spinal block anesthesia.

The threshold for onset of twitch potentiation in the acute studies was shown to occur at a blood ChE inhibition of about 80%. Nevertheless, sustaining that level of inhibition did not necessarily sustain twitch potentiation. This was shown in the acute exposure experiments when twitch tensions returned to normal while blood ChE inhibition levels were still well above 80%. Likewise, in the subacute exposure 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, there was substantial potentiation of twitch. These examples emphasize that threshold for physostigmine-induced damage is dependent on the interaction of several incompletely characterized factors (e.g., drug-anesthetic interaction; synergistic interaction of drug and neuromuscular activation, and/or on 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% blood 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, because of the limited sample size, we cannot exclude the possibility that a very small portion of fibers may have been damaged in the high-dose subacute exposure group and that they 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 anti-ChE agents, prolonged moderate- to high-frequency stimulation induced severe alterations in both pre- and postsynaptic mitochondria. The extent of damage was proportional to stimulation frequency and duration. These changes are attributed to the cumulative effects of the 18,000 to 144,000 endplate depolarizations elicited. Although the neuromuscular junction has an enormous capacity for repolarization (and associated ion pumping and ion sequestration) during normal rates and durations of stimulation, the levels of stimulation evoked in the presence and absence of physostigmine were one to two 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 other membrane-bound organelles, but few myofibers were super-contracted. In unstimulated myofibers of contralateral soleus and EDL muscles from the same rats, no ultrastructural alterations were observed. Thus, the ultrastructural changes observed in stimulated fibers in the absence of physostigmine (stimulation only) 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 1-15 minutes alloted for recovery in our experiments). Such ultrastructural damage from stimulation in the absence of drug (and presumably the time course of recovery from such hyperstimulation) is qualitatively similar to endplate damage (and of repair) following acute high-dose exposures to physostigmine.

G. SYNERGISM OF NEUROMUSCULAR ACTIVITY AND ANTI-CHOLINESTERASE TOXICITY

A detailed examination of the effects of stimulation on the expression of drug toxicity for both physostigmine and pyridostigmine revealed that supercontraction of subjunctional myofibers could be induced by prolonged stimulation in the presence of either physostigmine or pyridostigmine, even at doses which normally do not produce any detectable change in endplate ultrastructure. Moreover, supercontraction did not occur in the contralateral muscles of these spinal-blocked animals, and mitochondria remained normal. Even at high doses (which normally produced gross alteration of the subjunctional sarcoplasm), endplates in the contralateral (unstimulated) leg remained normal. Moreover, no evidence for antidromic activation of these intact and functional (but spinal-blocked) nerve-muscle units was detected. Finally, normally activated endplates in diaphragm myofibers of the same animals revealed the typical dose-dependent alteration of endplates. These data are consistent with activity-dependent expression of anti-ChE toxicity. An additional modulating factor may be the amount of non-vesicular (or non-quantal) release, which, depending on recent rates of neuromuscular activity, may vary from 50% to 95% of the total amount of ACh released in stimulated vs. resting endplates, respectively (56, 63-65). Thus, in a related observation, the observation of a few fibers with blistered postsynaptic mitochondria in EDL muscles of high-dose, spinal-blocked animals provides evidence for the proposed magnitude of non-vesicular release of ACh, even in quiescent endplates.

H. CONCLUSIONS AND RECOMMENDATIONS

We have demonstrated that:

1. Destructive supercontraction occurs in normally activated fast and slow twitch muscles in rats and guinea pigs at near-LD_{50} doses of physostigmine.

2. The threshold for producing supercontraction in normally activated muscles (e.g., those used for respiration) is about 0.3 LD_{50} (or 75% blood ChE inhibition).

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. Reports of mitochondrial alterations at low to very low doses of other anti-ChE drugs are now attributed to fixation artifacts rather than to drug effect.

5. Guinea pigs and rats undergo similar alterations at similar dose levels of physostigmine, allowing either animal to serve as a model system.

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

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. In auxiliary experiments, we have shown that in the absence of drug and 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 or pyridostigmine.

9. In more detailed experiments comparing the synergistic effect of stimulation on the expression of drug toxicity for both physostigmine and pyridostigmine, the threshold for producing supercontraction was lowered substantially by concurrent prolonged stimulation. Conversely, the toxic threshold was raised substantially in the absence of intrinsic stimulation (i.e., during spinal neuromuscular blockade).

Recommendations

Physostigmine and pyridostigmine have been suggested as possible chemotherapeutic agents for the protection of military personnel against exposure to the toxic nerve agents. This study (which is the first combined biochemical, physiological, and ultrastructure investigation of those two agents conducted in the same laboratory) is consistent with suggestions that pyridostigmine and physostigmine are not precisely equivalent in their actions at the neuromuscular junction. At doses that yield

the same blood ChE inhibition levels, pyridostigmine plus prolonged neuromuscular activation produced supercontraction at substantially lower doses than physostigmine. Evidence was also presented for rapid recovery of neurotransmission in the period 0-12 hours following moderate- to high-dose exposures of physostigmine (pyridostigmine not monitored). Based on the rapid repair of endplate ultrastructure and recovery of normal function during subacute exposure to physostigmine, on recent evidence for down-regulation of transmitter release efficacy as a normal control mechanism for neuromodulation (68-70), and on evidence that the number of active zone particles are proportional to the amount of vesicular transmitter release in normal (68-70) and diseased motor endplates (71), we suggest that a combined physiological and freeze-fracture ultrastructural investigation be conducted to ascertain whether the protective or compensatory alterations afforded by prior exposure to anti-ChE agents (66) reflect a decrease in transmitter release efficacy. The proposed study should determine whether alteration or partial disassembly of the active zones occurs in the relevant period 0-12 hours following moderate- to high-dose exposures to anti-ChE agents. If so, these data would prove of value in understanding the molecular basis for the protection against nerve agent exposure afforded by prior exposure to the reversible anti-ChE agents.

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XI. GLOSSARY

A-band -- That portion of the sarcomere containing the thick (myosin) filaments.

acetylcholine (ACh) -- the neurotransmitter chemical at the neuromuscular junction

acetylcholinesterase (AChE) -- The enzyme that hydrolyzes acetylcholine.

ACh receptor -- Chemically sensitive ion channels localized on the crests of the junctional folds. They respond to acetylcholine by opening and allowing ions to cross the muscle plasma membrane at the NMJ.

Anticholinesterase (anti-ChE) -- Chemicals that bind to and block the action of cholinesterases (including AChE and butyryl ChE)

I-band -- That portion of a sarcomere containing only thin (actin) filaments.

Junctional folds -- The specialized infoldings of the sarcolemma within the neuromuscular junction. Junctional folds contain high densities of ACh receptors.

Motor endplate -- Equivalent to neuromuscular junction (see below).

Myofiber -- A muscle cell. (Not equivalent to a myofibril, see below.)

Myofibril -- A contractile strand of sarcomeres. There are several hundred myofibrils in each myofiber.

Myofilaments -- Each sarcomere (see below) consists of hundreds of thick (myosin) filaments and thin (actin) filaments. Not to be confused with myofibers (cells) or myofibrils (chains of sarcomeres).

Neuromuscular junction -- The region of apposition of the motor nerve and muscle plasma membranes, the site where the muscle receives the chemical message from the nerve.

Percent sustained contraction (PSC) -- tension developed by a muscle at the end of a 20-Hz, 10-sec train divided by the tension at the beginning of the train. A decrease in PSC reflects a decreased ability to sustain a tetanic contraction.

Physostigmine -- A reversible tertiary amine carbamate anti-ChE.

Pyridostigmine -- A reversible quatenary amine carbamate anti-ChE.

Sarcolemma -- The muscle cell plasma membrane (plus associated external coats of glycoproteins).

Sarcomere -- The fundamental repeating unit of the myofibril; one sarcomere is the portion of a myofibril from one Z-band to the next Z-band.

Sarcoplasmic reticulum -- A network of membranes surrounding each myofibril; one component surrounds the A-band while a second surrounds the I- and Z-bands. The two elements are coupled at the triad via the T-system tubules (see T-system and triad).

Supercontraction -- A pathological state in which sarcomeres in (a portion of) the myofibril contract to the point that thick filaments are thrust against or into the Z-bands, resulting in crumpling and/or disassembly of thick filaments. Sarcomere dissolution often accompanies supercontraction.

T-tubules / T-system -- A branching system of tubules that are continuous with and conduct muscle action potentials from the sarcolemma.

Triad -- The three-part junction consisting of one T-tubule and two attached sacs of sarcoplasmic reticulum.

Twitch tension -- The maximum force exerted by an entire twitch muscle following a supramaximal stimulation of the innervating nerve bundle.

Z-band -- The very narrow electron dense bands traversing the myofibril at the middle of the I-band. The Z-bands separate the myofibril into successive sarcomeres.

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