DEVELOPMENT OF THE NEUROMUSCULAR JUNCTION

II. Cytological and Cytochemical Studies on the Neuromuscular Junction of Dedifferentiating Muscle in the Regenerating Limb of the Newt *Triturus*

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ABSTRACT

Following amputation of the limb of the newt, *Triturus viridescens*, muscle fibers dedifferentiate giving rise to mesenchymal cells. The earliest changes detected in neuromuscular junctions of dedifferentiating muscle fibers are the appearance of a few vacuoles and decrease in density of the terminal axoplasm. Later, synaptic vesicles become tightly clustered in the axon termination, and their content appears denser than normal. Then, vesicles diminish in number until few are seen in the ending. While these changes are occurring, the area of contact of nerve with muscle becomes smaller. Junctional folds persist only where the nerve maintains contact with muscle, but these are shorter than normal and appear as slight ridges on the muscle surface. Subsequently, the nerve withdraws from the muscle cell and is completely invested by Schwann cell cytoplasm, and all traces of junctional folds are lost at the former region of contact. Cholinesterase activity was localized with the thiolacetic acid–lead nitrate method. Even before marked morphological changes occur in the junction, DFP- and physostigmine-sensitive activity in the cleft between nerve and muscle is decreased in intensity. Activity continues to decrease as the area of nerve-muscle contact diminishes and junctional folds disappear. When the nerve has withdrawn from the muscle surface, only a few small deposits of lead are left in the intervening region. These results show that as muscle becomes less specialized during dedifferentiation, the neuromuscular junction also loses the cytological and cytochemical specializations associated with synaptic function.

INTRODUCTION

After the limb of the newt is amputated, muscle fibers adjacent to the wound undergo a series of changes during which they lose their specialized characteristics, especially myofilaments and associated membranous systems (Hay and Fischman, 1961; Hay, 1962; Lentz, 1969 *a*). This process of dedifferentiation gives rise to mesenchymal cells of the blastema that divide and later in regeneration differentiate to reform the specialized tissues of the limb. In contrast to muscle fibers that have been denervated, the dedifferentiating muscle fibers in the limb stump have not been deprived of their nerve supply. Since the integrity of the neuromuscular junction is thought to depend on an interaction between nerve and muscle (Zelená, 1962; Teräväinen, 1968; Kelly and Zacks, 1969; Lentz, 1969 *b*), it becomes important to know the changes occurring in the junction while muscle is losing its specialized characteristics but retains innervation. This type of modulation of the neuro-

muscular junction contrasts with the extensively investigated effects of nerve transection and has not been previously described.

This paper describes the cytological changes in the neuromuscular junction of the newt as muscle dedifferentiates early in limb regeneration. Changes in the localization of acetylcholinesterase (AChE) activity were also detected histochemically to correlate changes in enzymatic specialization with morphological alterations. The formation of the neuromuscular junction on differentiating muscle and the appearance of AChE activity have already been described (Lentz, 1969 *b*). Thus, this communication completes the study of the neuromuscular junction during the entire dedifferentiative and differentiative cycle undergone by muscle during limb regeneration. The present findings have been reported briefly (Lentz, 1969 *c*).

MATERIALS AND METHODS,

These studies were performed on adult newts, *Triturus viridescens*, which were maintained in large aquaria in the laboratory. The surgical, fixation, and histochemical methods employed were described in the previous paper (Lentz, 1969 *b*) and are not repeated in detail here. For study of motor end-plates on dedifferentiating muscle, newts were allowed to regenerate for 2 wk following amputation of the limb at the level of the lower third of the upper forelimb. At this time, all stages of muscle dedifferentiation can be found, with mesenchymal cells occurring distally in the stump and progressively less differentiated and finally normal-appearing muscle fibers occurring proximally. Enough tissue was removed for fixation to include all the stages of muscle dedifferentiation. The tissues were cut into small blocks, fixed for 1 hr in 3% glutaraldehyde in cacodylate buffer (pH 7.2) followed by 1% osmium tetroxide in cacodylate buffer (pH 7.2), and embedded in Maraglas. Sections were stained with lead hydroxide or lead citrate and examined with an RCA EMU 3F electron microscope.

The histochemical procedures for the demonstration of cholinesterase activity were the same as those used in the study of developing junctions, so that comparisons of intensity and sites of localization of enzyme activity during dedifferentiation and differentiation could be made. Glutaraldehyde-fixed tissue was incubated in a medium containing 0.0012 M thiolacetic acid (Eastman Organic Chemicals, Rochester, New York; redistilled in the laboratory) and 0.006 M $Pb(NO_3)_2$ in cacodylate buffer at pH 6.8 (Crevier and Bélanger, 1955; Barrnett, 1962). Tissues were incubated for 60 min at 4°C, rinsed, fixed for 1 hr in osmium tetroxide in cacodylate buffer (pH 7.2), and processed for electron microscopy in the usual manner. Control studies were performed by preincubation for 20 min and incubation in the presence of the inhibitors physostigmine (eserine) (10^{-4} M) or diisopropylfluorophosphate (DFP) (10^{-5} M), or by omission of substrate from the incubating medium.

RESULTS

Cytological Changes

Many motor end-plates in the 2-wk regenerates (Fig. 1) are similar to normal ones. These junctions are found on muscle cells that show few dedifferentiative changes. The axon termination covers an extensive area of muscle surface from which extend numerous junctional folds. The junctional folds are elongated as normally and are slightly constricted at their bases. An accumulation of moderately dense material occurs on the cytoplasmic side of the plasma membrane of the distal portion of the folds. Some vacuoles are located in the ending, and the axoplasm is of low density.

Other end-plates in the 2-wk regenerates show more noticeable changes involving loss of cytological specializations. The changes observed, however, did not always occur in a precise sequence, but occurred at different times or overlapped one another. Thus, the sequence described below is a general reconstruction based on the interpretation of many images.

Portions of the axon terminal become separated from the muscle by spaces greater than the usual synaptic cleft so that the area of close nerve-muscle contact is smaller (Fig. 2). A greater portion of the axon surface facing the muscle cell is enveloped by Schwann cell cytoplasm. Junctional folds are not so prominent, being reduced in size and number, especially where the nerve has become separated from the muscle surface. Within the axon, vesicles tend to become clustered in localized regions, leaving other portions of the axoplasm relatively devoid of vesicles. The vesicles accumulate toward the side of the axon facing the muscle cell.

Subsequent changes in the neuromuscular junction are continued aggregation of vesicles and then loss of vesicles, further separation of axon from muscle with consequent decrease in area of nerve-muscle contact, and reduction in height and number of junctional folds. The synaptic vesicles become highly concentrated in some regions of the endings (Fig. 3 *a*). The content of the tightly packed vesicles is of greater density than normal.

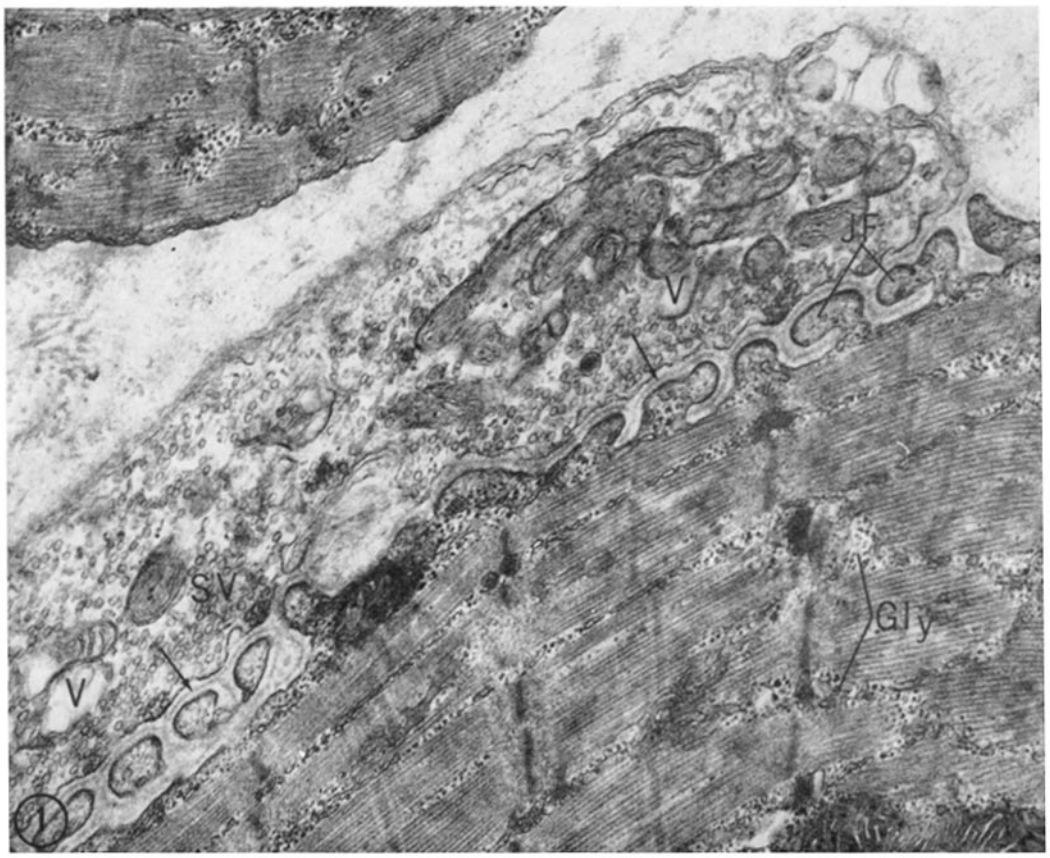


FIGURE 1 Neuromuscular junction in a 2-wk regenerate. The junction is similar to normal except for the presence of a few membranous sacs or vacuoles (*V*), possibly representing disrupted mitochondria, and the low density of the axoplasm. Synaptic vesicles (*SV*) are present in the axon ending. The ending covers a large area of muscle surface, and junctional folds (*JF*) are well-developed. Note the band of filamentous material in the synaptic cleft (arrows). In the muscle fiber, glycogen granules (*Gly*) occur in the sarcoplasm between myofibrils in greater numbers than normal, an early sign of dedifferentiation. × 26,000.

In most endings in which junctional folds are low or absent and the area of contact with muscle smaller, vesicles are not so abundant (Figs. 4, 5). A number of images were seen where the vesicle is continuous with the axolemma (Fig. 3 *b*).

As the vesicles become less numerous, the size of the junctional area decreases (Figs. 4, 5). Schwann cell cytoplasm covers the axon surface not in immediate contact with the muscle fiber. Junctional folds persist only where the nerve fiber ending is in contact with muscle, and none was seen outside the area of contact (Fig. 4). The folds are usually of lower height than normal, and may appear only as low ridges or elevations, especially toward the periphery of the junction. The submembranous density normally found at the ends of the folds remains even when the folds are reduced to low ridges (Figs. 4, 6, 9 *d*). When the ending is separated from the muscle, all traces of junctional folds disappear and the intercellular space is occupied by collagen fibrils (Figs. 5, 9 *e*, 9 *f*).

In endings in which the area of nerve-muscle contact is small, vesicles are usually few in number and occur in small aggregates toward the muscle fiber (Figs. 4, 5). Large vesicles with dense contents seem more abundant relative to the number of small vesicles present (Figs. 4, 5). The axoplasm

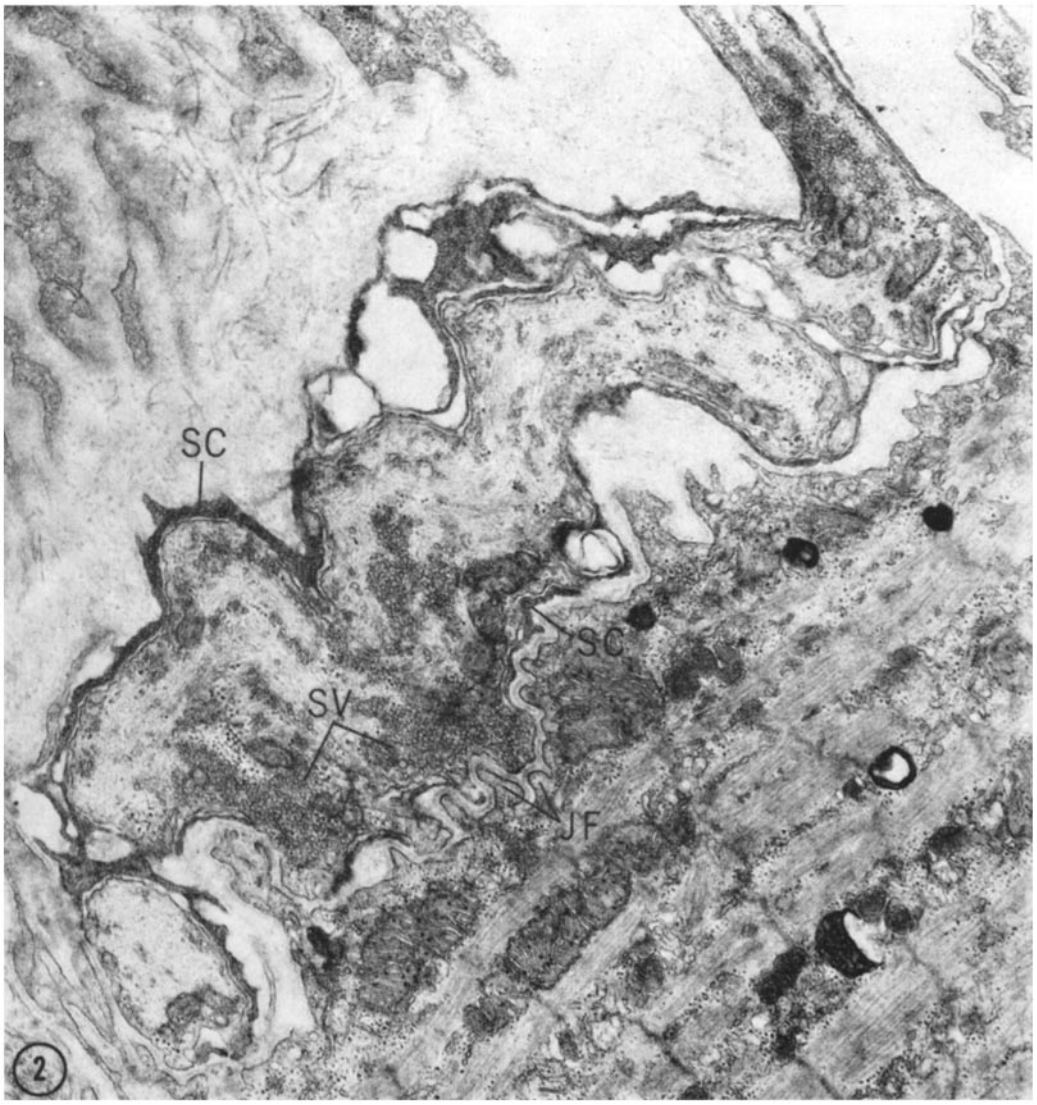


FIGURE 2 Neuromuscular junction 2 wk following limb transection. This junction differs from normal in several respects. The nerve fiber ending is not so closely applied to the muscle surface, being separated from it by wide spaces in some places. Junctional folds (*JF*) are not so high or so numerous, especially where spaces separate axon and muscle. Schwann cell cytoplasm (*SC*) covers portions of the axon surface facing the muscle fiber. In the axon, vesicles (*SV*) are tightly clustered in some regions leaving other areas of axoplasm devoid of vesicles. The content of some of the small vesicles is moderately dense. × 18,000.

is of low density and contains neurofilaments, neurotubules, and a few channels of smooth-surfaced endoplasmic reticulum (Fig. 5). Some endings contain small, dense granules resembling glycogen (Figs. 4, 5, 9 *d*).

The loss of cytological specializations results in endings that have relatively few synaptic vesicles and an empty-appearing axon terminal (Figs. 4, 5, 9 *f*). In a few instances, nerve fibers in contact with dedifferentiating muscle cells assume other features. These endings can be identified as neuromuscular junctions by the persistence of a

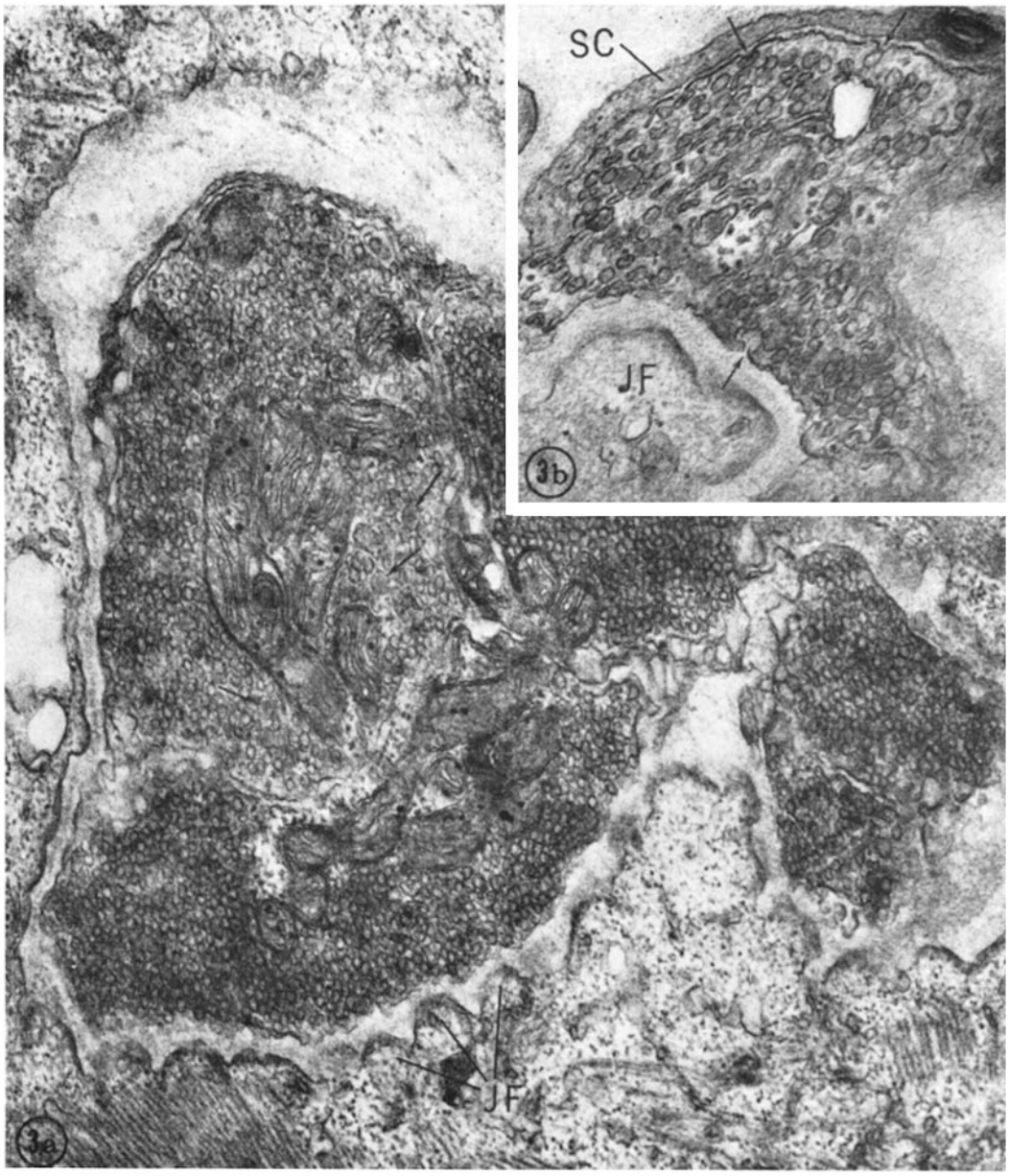


FIGURE 3 *a* Neuromuscular junction in a 2-wk regenerate. Note the tight packing of the small vesicles. A few larger dense-core vesicles (arrows) as well as some mitochondria are present among the small vesicles. The content of many of the smaller vesicles appears denser than normal (compare with Fig. 1). The junctional folds (*JF*) are no longer elongated but appear as low undulations or ridges. × 55,000.
FIGURE 3 *b* Junction containing synaptic vesicles with moderately dense content. Note that some vesicles (either synaptic or pinocytotic in nature) are fused with the axolemma (arrows), both on the side of the axon covered by Schwann cell cytoplasm (*SC*) and on the side facing the muscle surface. *JF*, junctional fold. × 50,000.

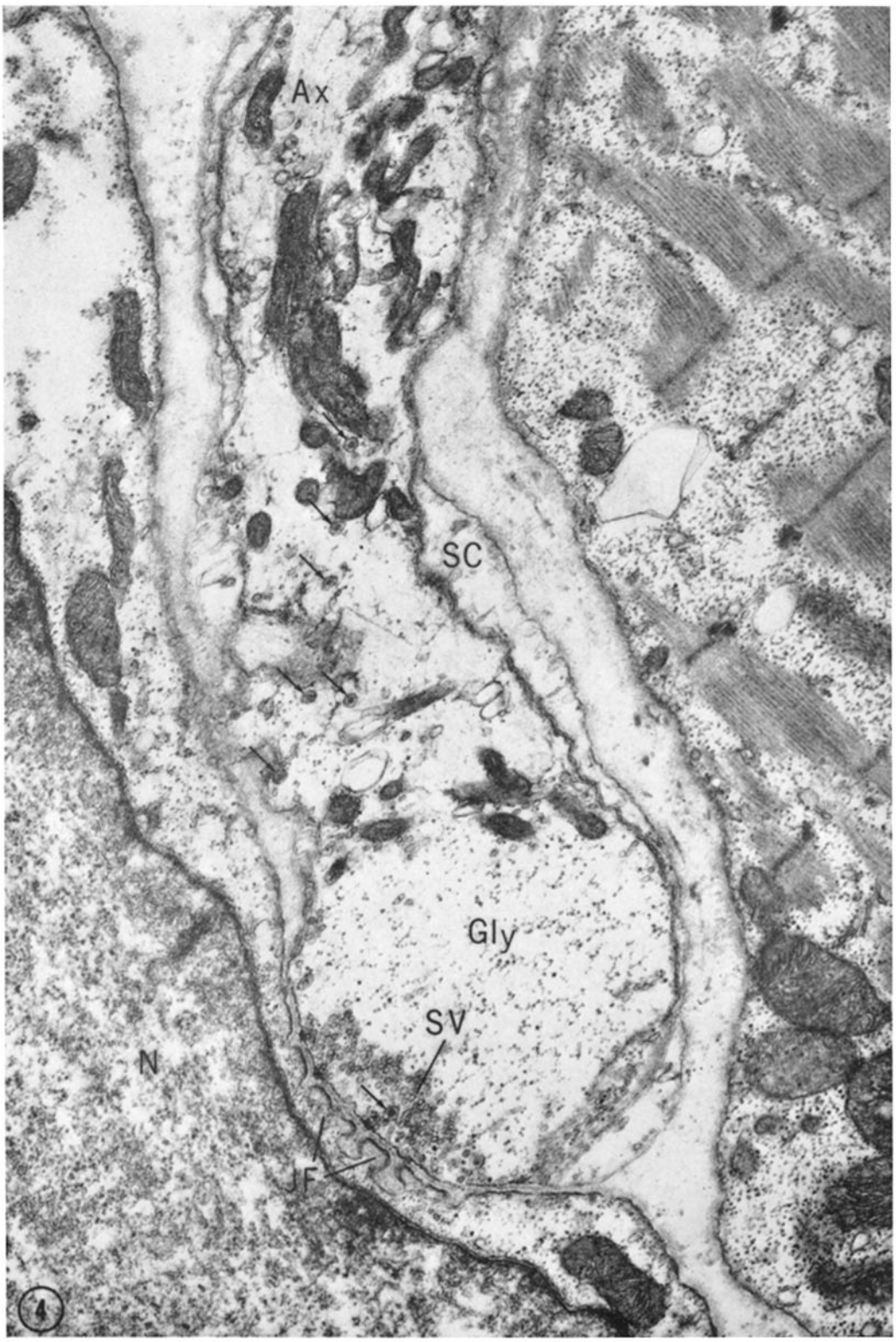


FIGURE 4 Nerve fiber terminating on a dedifferentiating muscle in a 2-wk regenerate. The bulblike terminal contains small dense particles resembling glycogen (*Gly*) and a small number of synaptic vesicles (*SV*) in the region nearest the muscle fiber. Mitochondria and dense granules or dense-core vesicles (arrows) are common in more proximal regions of the axon. The ending covers a relatively small area of muscle surface even though it is sectioned longitudinally as evidenced by the inclusion of more proximal regions of the axon (*Ax*). The junctional folds (*JF*) are highest at the center of the contact region but become lower proceeding laterally toward the edge of the junction. Junctional folds do not occur outside the region of close contact of nerve and muscle. Dense material is applied to the inner surface of the membrane of the tips of the folds. *N*, nucleus of muscle fiber; *SC*, Schwann cell. × 22,000.

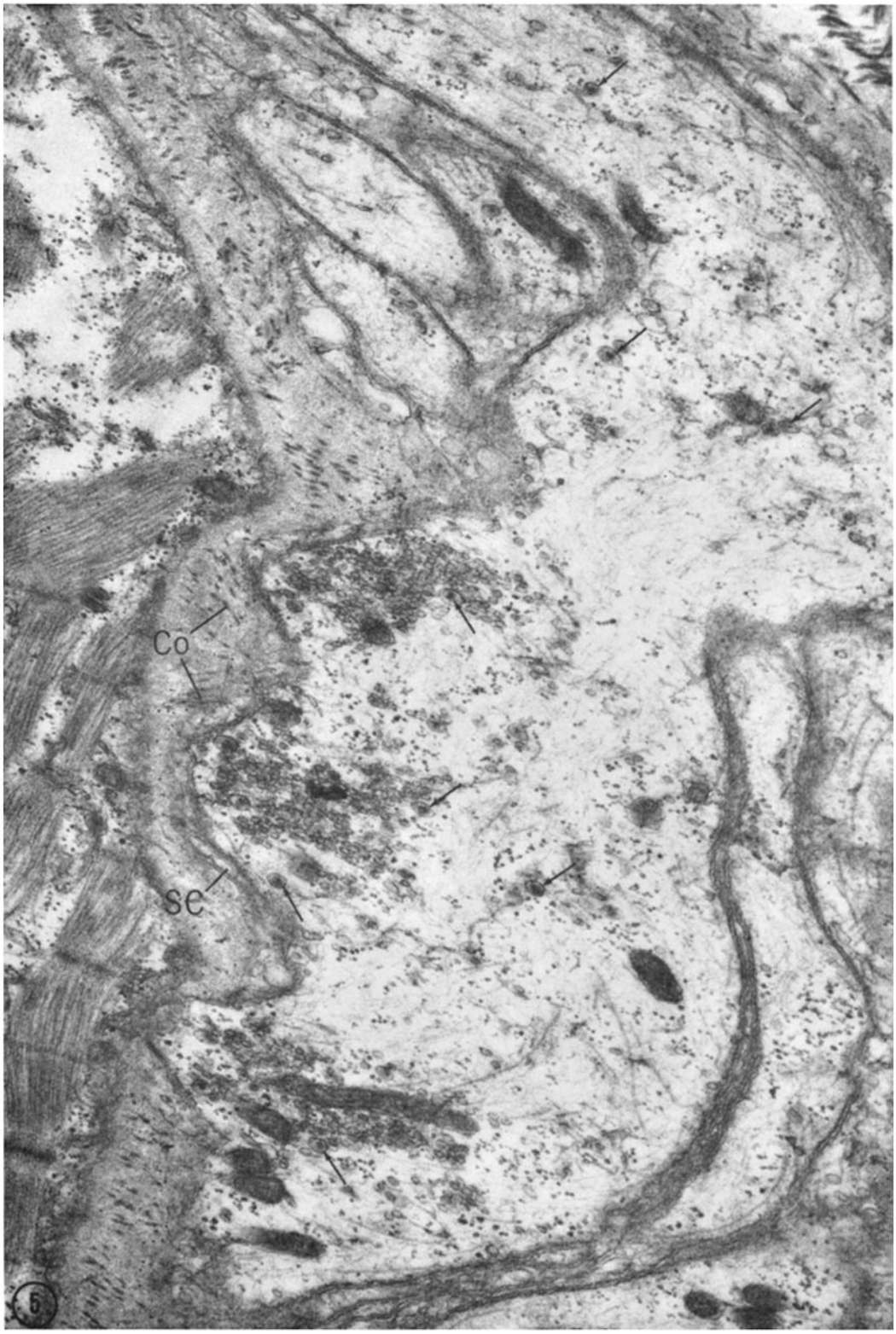


FIGURE 5 Nerve terminal near a muscle fiber of a 2-wk regenerate. The ending contains three small clusters of synaptic vesicles oriented toward the muscle. The axoplasm elsewhere is of low density and contains glycogen granules, mitochondria, tubules, channels, and filaments. Dense granules larger than synaptic vesicles occur throughout the nerve fiber (arrows). The axon in most places is separated from the muscle surface by a large space. Collagen fibrils (*Co*) are found in the space. Schwann cell cytoplasm (SC) covers much of the axon surface facing the muscle fiber. Junctional folds are not apparent on the muscle surface. × 26,000.

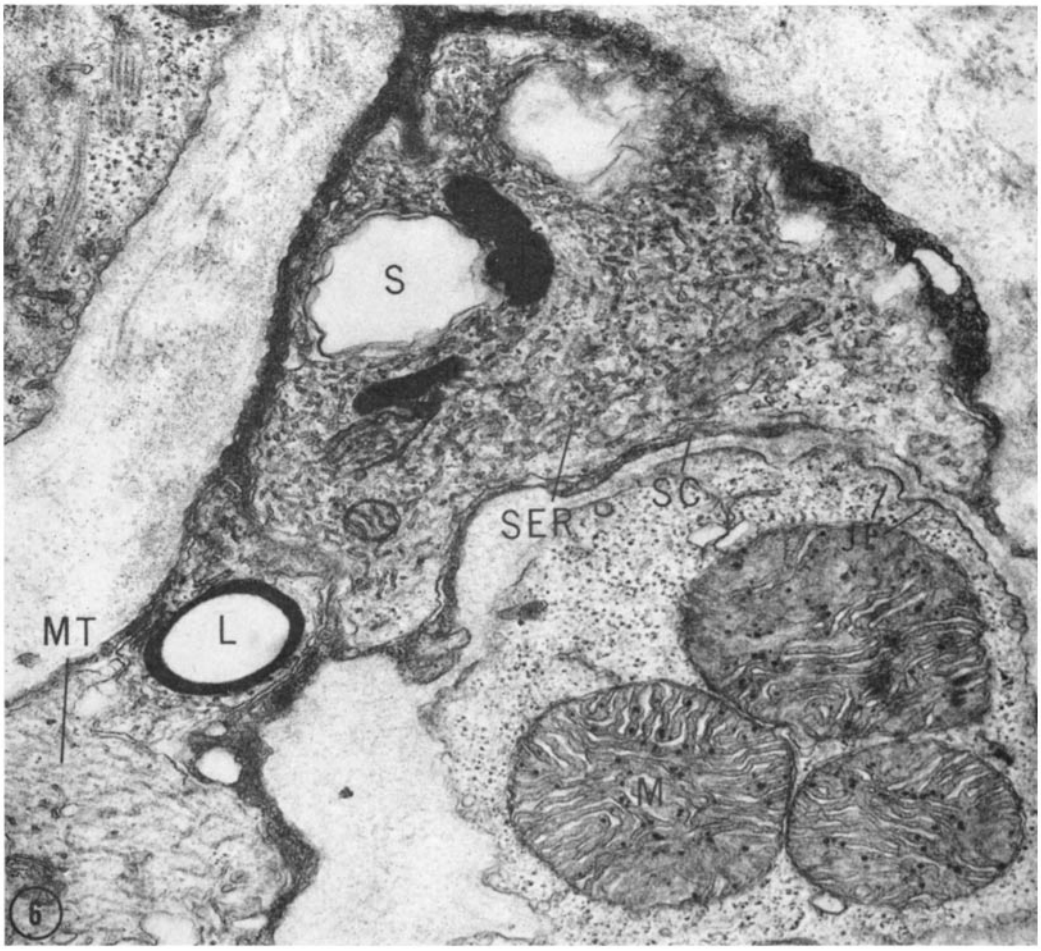


FIGURE 6 Nerve fiber and dedifferentiating muscle fiber 2 wk following limb transection. The axon is closely apposed to the muscle in one region. Where Schwann cell (*SC*) cytoplasm does not cover the axon surface, a few low junctional folds (*JF*) are found on the muscle surface. The nerve fiber has few vesicles but is filled with irregular channels of smooth endoplasmic reticulum (*SER*). Microtubules (*MT*) are common also, especially in more proximal regions of the axon. The nerve fiber also contains some large vacuoles or spaces (*S*), mitochondria, and lipid droplets (*L*). The band of filamentous material persists in the synaptic cleft. Cytoplasmic densities occur immediately beneath the plasma membrane of the junctional folds. Signs of dedifferentiation in the muscle fiber include reduction in number of myofibrils, increase in number of glycogen granules, and hypertrophied mitochondria (*M*). × 30,000.

few low junctional ridges at the small area of contact (Fig. 6). The densities persist in the sarcoplasm immediately adjacent to the membrane folds. In the axon, vesicles are greatly diminished in number while glycogen, irregular membranous sacs, lipid droplets, and a few mitochondria may be present. In addition, these terminations are filled with a tangle of irregular tubules or channels of smooth-surfaced endoplasmic reticulum (Fig. 6), typical of the growing nerve fibers of the blastema (Lentz, 1967). Microtubules (neurotubules) are also numerous, especially in more proximal regions of the axon.

Changes in Cholinesterase Activity

Normally, enzymatic activity is very intense, with reaction product completely filling the cleft

FIGURE 7 Cholinesterase activity in a motor end-plate of a 2-wk regenerate. The only morphological changes in the axon termination are the appearance of a few small vacuoles or spaces. Similarly, the muscle fiber closely resembles normal and shows few indications of dedifferentiation. Enzymatic activity is seen in its usual location in the synaptic cleft, but is not so densely accumulated as normal when it completely fills and obscures the cleft. Small amounts of reaction product occur in the axon. Precipitates over myofibrils and the Schwann cell are nonspecific as they sometimes occurred in control experiments. × 18,500.

between nerve fiber terminal and muscle surface (Lentz, 1969 *b*, Fig. 10). Activity in some end-plates of 2-wk regenerates is nearly as intense as normal (Fig. 9 *a*). These junctions also appear normal morphologically. In the end-plates showing the first morphological changes (appearance of a few vacuoles and spaces but otherwise closely resembling normal end-plates) in the 2-wk regenerates, enzymatic activity is reduced in intensity (Figs. 7, 9 *b*). Activity is primarily located in the synaptic cleft as usual, but reactive sites are not so numerous as to produce complete filling of the cleft with reaction product.

Enzymatic activity appears to diminish rapidly in intensity in the early stages of dedifferentiation. In general, loss of activity parallels the reduction in height of the junctional folds. Thus, in neuromuscular junctions of dedifferentiating muscle fibers, the most intense activity (still considerably less than normal) is found where the junctional folds show the least change (Fig. 8). Where the folds are low, only a few deposits of reaction product are seen (Fig. 8); and where the nerve is separated from the muscle surface and junctional folds have disappeared, little or no enzymatic activity is seen (Figs. 8, 9 *f*). At the same time, no reaction deposits in addition to those occurring in controls were seen in the sarcoplasm or over myofibrils.

Endings which maintain close contact with the muscle cell but where junctional folds are low show little enzymatic activity (Fig. 9 *c*). When junctional folds are not prominent at the stage when vesicles are tightly clustered in the ending, little activity is seen (Fig. 9 *d*). When the vesicles diminish and the axon begins to become separated from the muscle surface, only a few reaction deposits are found (Fib. 9 *e*). As the nerve fiber is further separated from muscle, only a few small lead deposits can be found in the intervening space (Fig. 9 *f*). In no cases were localized accumulations of enzymatic activity found on the surfaces of dedifferentiating muscle cells outside the area of nerve-muscle contact.

Results of control experiments during dedif-

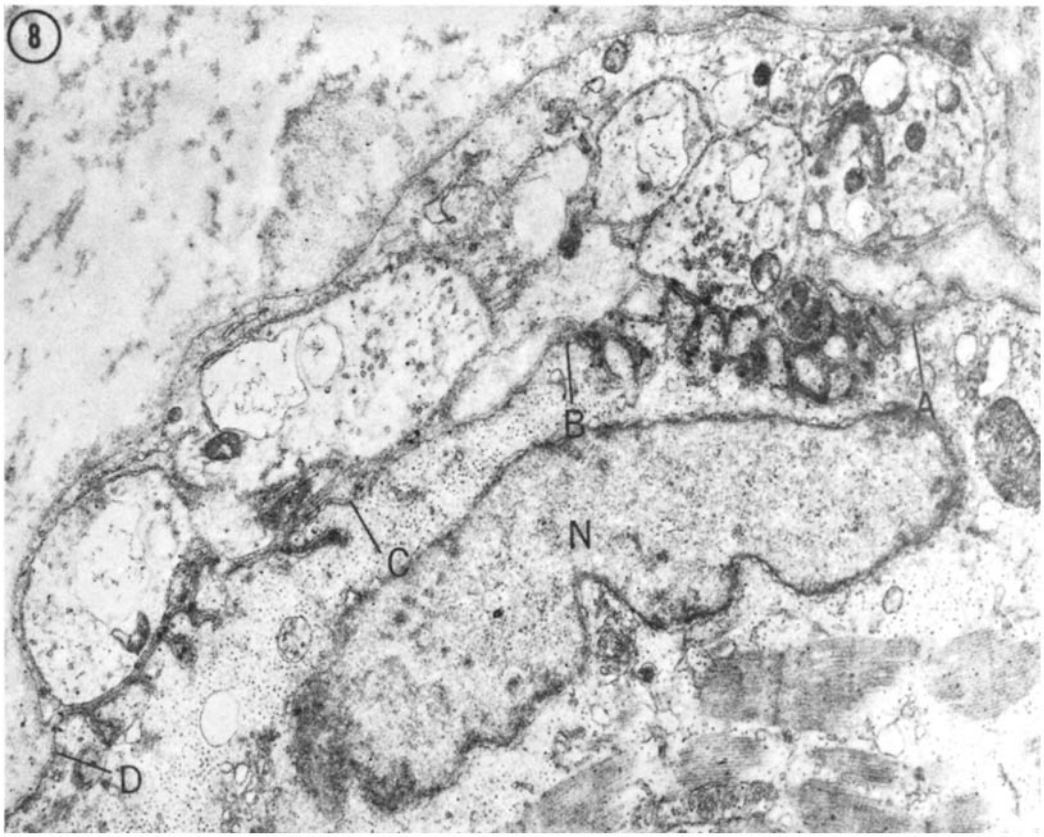


FIGURE 8 Cholinesterase activity in a neuromuscular junction in a 2-wk regenerate. The muscle fibers show more advanced signs of dedifferentiation as indicated by the separation of myofibrils by greater amounts of sarcoplasm, fragmentation of myofibrils, and abundance of glycogen. The axon terminal contains some vacuoles, has fewer vesicles, and in places is separated from the muscle surface. Note that enzymatic activity is most intense where the junctional folds are still elongated (*A* to *B*). Reaction product occurs in the synaptic cleft, but the number of reactive sites is greatly reduced in comparison to normal or to the junction illustrated in Fig. 7. Activity is even less intense, as evidenced by the number of reactive sites, in the portion of the junction in which junctional folds are reduced in height (*C* to *D*). Where the nerve fiber is not in close contact with the muscle fiber, virtually no activity is seen (*B* to *C*). Note also that the sarcoplasm is devoid of activity. Some nonspecific deposits occur over myofibrils. *N*, nucleus. × 13,000.

ferentiation were the same as those obtained in normal and regenerating muscle (Lentz, 1969 *b*). Activity in the junctional region during dedifferentiation was sensitive to DFP (10^{-5} M) and physostigmine (10^{-4} M). Activity was almost completely inhibited by these agents, indicating that it is due to a cholinesterase (Figs. 10, 11). Omission of substrate (incubation in lead medium) likewise produced no localized deposits in the end-plate although tiny lead deposits were sometimes widely scattered randomly or occurred over myofilaments and collagen fibrils or in synaptic vesicles.

DISCUSSION

Morphogenetic Relationships

As muscle loses its specialized characteristics during dedifferentiation, the neuromuscular junction also becomes less specialized. Even though the nerve is closely associated with the muscle cell, structural characteristics such as junctional folds disappear and chemical specializations (AChE) are lost. It is generally assumed that the nerve fiber exerts a morphogenetic effect on the motor end-plate and is responsible for its integrity

(Zelená, 1962; Teräväinen, 1968; Kelly and Zacks, 1969; Lentz, 1969 *b*). During development, both structural and chemical characteristics of the junction develop only where nerve endings and muscle become closely apposed. Muscle, however, must have reached a certain stage of differentiation before end-plate formation begins (Hirano, 1967; Lentz, 1969 *b*). Thus, in some respects, the present results are the converse of development, because as muscle loses its specialized characteristics during dedifferentiation, the junction becomes less specialized. It appears, then, that the integrity of the neuromuscular junction depends on a relationship between muscle fiber and nerve that have a certain degree of specialization. Degeneration of motor end-plates following nerve transection (Reger, 1959; Birks et al., 1960; Bauer et al. 1962; Nickel and Waser, 1968) and formation of end-plates only in the presence of vesicle-filled axon terminations (Lentz, 1969 *b*) have demonstrated the requirement for the nerve fiber. The present results emphasize the importance of the state of differentiation of the muscle fiber in the integrity of the end-plate.

The manner in which the nerve fiber exerts an inductive effect and maintains the integrity of the junction is not known, but it seems reasonable to assume the inductive interaction is linked to a mechanism of mutual recognition of information between nerve and muscle. As suggested previously (Lentz, 1969 *b*), it seems necessary that a certain degree of specialization of the nerve is necessary for the transfer of information. Moreover, the response to whatever information is supplied, whether it be at the level of genetic control and protein synthesis or of an effect on enzymatic activity, would also depend on the muscle cell being differentiated to a certain extent. Thus, in this case it is not surprising that the neuromuscular junction becomes less specialized as muscle dedifferentiates and loses its capacity to respond appropriately to the presence of the nerve.

Cytological Specializations

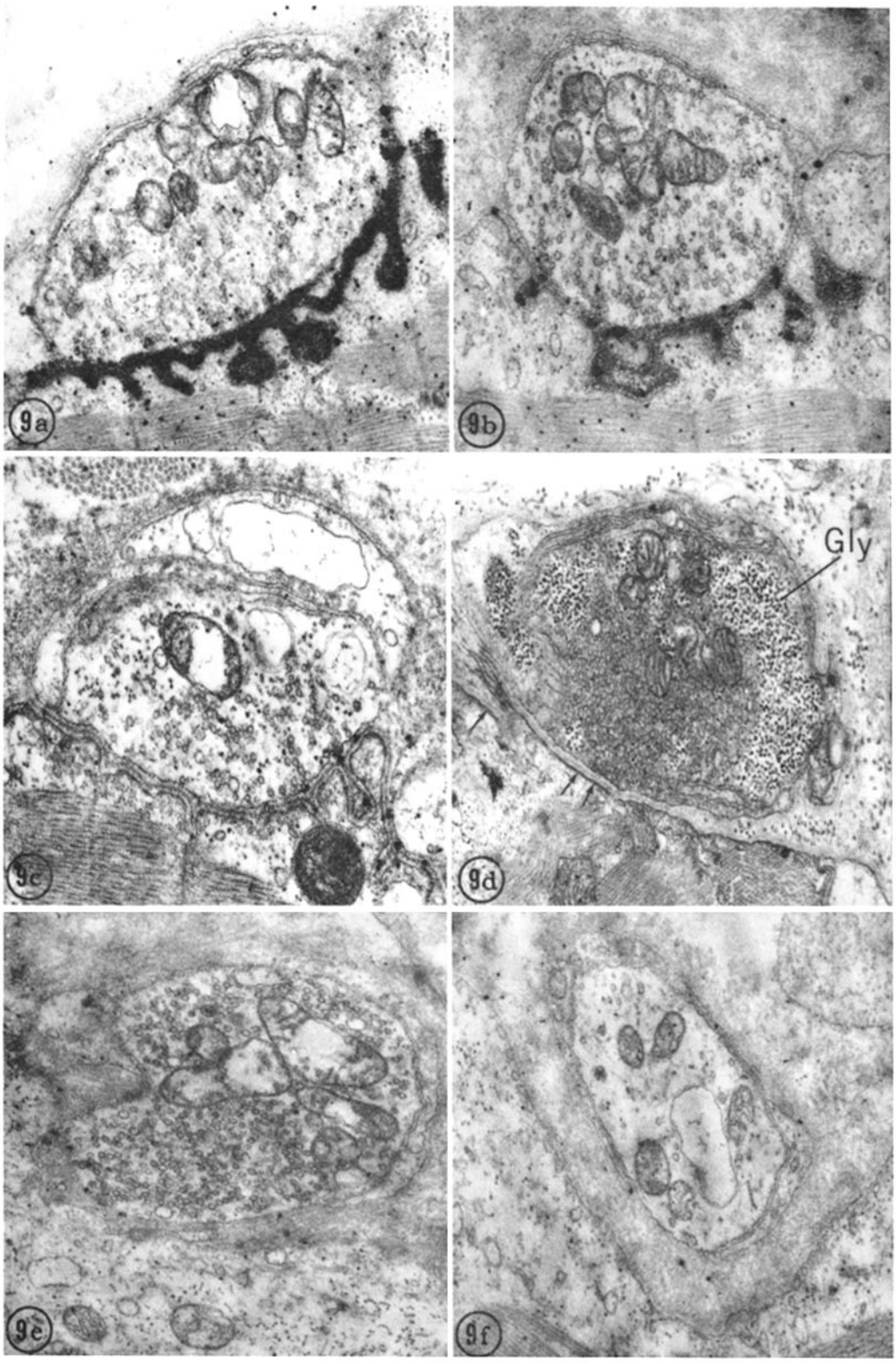
As muscle dedifferentiates, the axon termination becomes separated from the muscle surface, junctional folds disappear, and changes occur in the synaptic vesicles. In no cases did junctional folds persist outside the region of nerve-muscle contact. At some point during dedifferentiation, the synaptic vesicles become highly concentrated in the axoplasm similar to the clumping of vesicles to form honeycomb structures in degenerating endings (Birks et al., 1960). Later, the number of synaptic vesicles decreases and the clumped masses disappear, although it is not definitely known how these processes occur. Many images were seen in which vesicles were continuous with the axolemma, indicating that loss of vesicles could be accomplished at least in part by fusion with the axolemma. It seems doubtful, however, that this process can account for loss of all the vesicles. Furthermore, vesicles in continuity with the axolemma could alternatively be pinocytotic in nature.

As the area of contact of nerve with muscle becomes smaller, the side of the axon facing the muscle fiber is progressively enveloped by Schwann cell cytoplasm until the nerve terminal is completely surrounded when it is separated from the muscle. This process of withdrawal and Schwann cell investment is similar to the changes in synapses on the soma of chromatolytic motor neurons described by Kuno and Llinás (1970). They found a decrease in number of synaptic boutons and intervention of glial processes between the nerve terminal and cell body. In both cases, the changes in the nerve terminal do not appear to be degenerative but may be indicative of plasticity or capacity for modification of synapses.

After withdrawal from the muscle, the motor nerve fibers may undergo a phase of growth because they develop the characteristics of rapidly growing nerve fibers (microtubules, channels of endoplasmic reticulum, Peters and Vaughn, 1967; Lentz, 1967; Pellegrino de Iraldi and De Robertis, 1968). Later in regeneration, the intercellular nerve fibers of the blastema reinnervate differentiating myoblasts, but it is not known which of these fibers previously innervated muscle cells.

Enzymatic (AChE) Specialization

As in development of the junction, morphological changes are paralleled by changes in enzymatic activity during dedifferentiation. Thus, the number of reactive sites for cholinesterase decrease rapidly following amputation of the limb. In general, the loss of enzymatic activity parallels the reduction in plasma membrane presented as the number and size of junctional folds decrease. When the nerve has separated from the muscle fiber and junctional folds have disappeared, no enzymatic activity is found on the muscle surface. Cholinesterase activity similarly decreases in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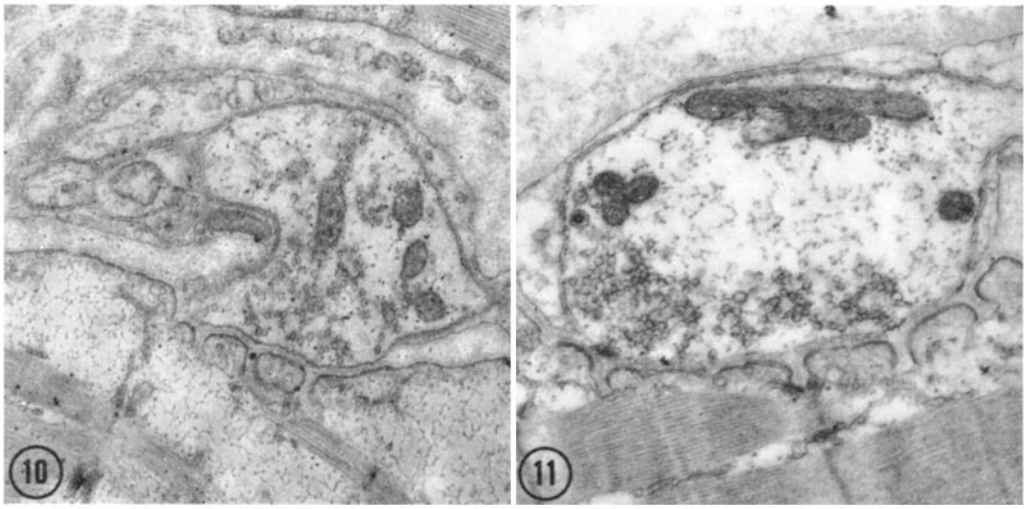

FIGURE 10 Neuromuscular junction incubated in the reaction medium which also contained DFP (10^{-5} M). Although some well-developed junctional folds are present, enzymatic activity is largely inhibited. × 21,000.

FIGURE 11 Neuromuscular junction of a 2-wk regenerate preincubated in physostigmine (10^{-4} M) and incubated in reaction medium containing physostigmine. Note the virtual absence of reaction product, indicating inhibition of enzymatic activity. × 22,000.

denervated end-plates but, in contrast to end-plates on dedifferentiating muscle, both enzymatic activity (Couteaux, 1955; Snell and McIntyre, 1956; Bergner, 1957; Brzin and Majcen-Tkačev, 1963; Guth et al., 1964; Eränkö and Teräväinen, 1967) and synaptic folds (Birks et al., 1960; Bauer et al., 1962; Nickel and Waser, 1968) seem to persist for a considerably longer time after nerve section. In the present situation in which end-plates are not denervated, activity is reduced or absent within 2 wk. This difference may reflect the more rapid changes occurring in muscle cells during dedifferentiation in comparison to the long-term atrophic alterations following denervation.

The loss of the structural and chemical specializations of the neuromuscular junction is progressive during muscle dedifferentiation. It does not seem to be associated with any events detectable with the techniques employed here that might account for the disappearance of these specializations. For example, lytic activity as would be evidenced by a preferential association of lysosomal structures in the end-plate region does not seem to be associated with breakdown of junctional folds or disappearance of enzymatic activity. In this

FIGURE 9 Series of micrographs of neuromuscular junctions from 2-wk regenerates reacted for cholinesterase activity. As more proximal regions of the axon are not included, these sections are considered to be transverse to the long axis of the nerve fiber termination. In some junctions, enzymatic activity is nearly as intense as normal (Fig. 9 *a*). In other junctions that are similar in structure to normal junctions and have well-developed junctional folds, enzymatic activity is reduced in intensity (Fig. 9 *b*). When the junctional folds are reduced in height, activity is considerably reduced (Fig. 9 *c*). In endings with tightly packed masses of vesicles, little activity is present if the junctional folds are low (Fig. 9 *d*). Note persistence of subsarcolemmal densities (arrows), although the folds have nearly disappeared (Fig. 9 *d*). When synaptic vesicles become reduced in number, junctional folds have disappeared, and the ending begins to separate from the muscle cell, only a few deposits of final reaction product are found (Fig. 9 *e*). Finally, the nerve fiber is separated from the muscle fiber by a relatively large intercellular cleft that contains collagen (Fig. 9 *f*). Only a few small lead deposits are found in the intervening space. Vesicles and junctional folds have largely disappeared. *Gly*, glycogen. *a*, × 18,000; *b*, × 22,000; *c*, × 20,000; *d*, × 20,000; *e*, × 21,000; *f*, × 20,000.

sense, the changes observed here are not degenerative but could be accounted for simply by failure of replacement of proteins (e.g., AChE) lost in normal turnover.

The end result of this process is a nerve fiber containing few synaptic vesicles and separated from muscle by a large cleft containing collagen. The postsynaptic membrane has become modified by loss of enzymatic activity and junctional folds. Thus, at this stage, the neuromuscular junction could be said to have dedifferentiated because it has lost those specializations characteristic of synaptic function.

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