DEVELOPMENT OF THE NEUROMUSCULAR JUNCTION

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

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ABSTRACT

Development of the neuromuscular junction on differentiating muscle was investigated in the regenerating limb of the newt *Triturus*. Motor end-plate formation begins when vesicle-filled axon terminations approach differentiating muscle cells that have reached the stage of a multinucleate cell containing myofibrils. Slight ridges or elevations occur on the muscle surface, and there is an increase in density of the cytoplasm immediately beneath the plasma membrane of the elevation. The axon becomes more closely approximated to the muscle cell and comes to lie in a shallow depression or gutter on the surface of the muscle. The surface ridges increase in length and constrict at their bases to form junctional folds. In the axon terminal, focal accumulations of vesicles are found where the axon contour projects slightly opposite the secondary synaptic clefts. Cholinesterase activity in the developing junctions was demonstrated by the thiolacetic acid–lead nitrate method. Enzymatic activity is not found on intercellular nerve fibers or the muscle surface prior to close approximation of axon endings and muscle. Eserine- and DFP-sensitive activity appears concurrently with morphological differentiation. Activity occurs in membranous tubulovesicles in the sarcoplasm subjacent to the neuromuscular junction and in association with the sarcolemma. The largest reaction deposits occur at the tips of the emerging junctional folds. Smaller and less numerous localizations occur on the axon membrane and within the axoplasm. It is concluded from these studies that the nerve endings have an inductive effect on both the morphological and chemical specializations of the neuromuscular junction.

INTRODUCTION

Following amputation of the limb of the newt, muscle and other specialized tissues of the limb dedifferentiate to form a blastema composed of mesenchymal cells (Butler, 1933; Thornton, 1938; Singer, 1952; Chalkley, 1954; Hay and Fischman, 1961; Hay, 1962; Trampusch and Harrebomée, 1965; Lentz, 1969). Subsequently, the undifferentiated mesenchymal cells differentiate to reform muscle, bone, and connective tissue. Thus, during limb regeneration, muscle undergoes a complete developmental cycle in which all of its differentiative states are represented. This system affords an

opportunity to correlate the degree of specialization of the neuromuscular junction with the developmental stage of the muscle cell.

This paper describes reinnervation of differentiating muscle and formation of the neuromuscular junction in the newt. In addition, the appearance and localization of acetylcholinesterase (AChE) activity was determined histochemically to correlate enzymatic specialization with morphological development. Other studies have been performed on development of the neuromuscular junction in the rat (Kelly, 1966; Teräväinen, 1968) and chick (Hirano, 1967). As a prior step to this investigation, the cytological aspects of muscle dedifferentiation and differentiation in the newt limb have been described in detail (Lentz, 1969). It will be shown here that both morphological and chemical (AChE) specializations characteristic of the neuromuscular junction develop only where axon endings and muscle become closely apposed. The present findings have been reported briefly (Lentz, 1968).

MATERIALS AND METHODS

Adult newts, *Triturus viridescens*, used in these experiments, were maintained in the laboratory in large aquaria. For study of motor end-plate development, newts were anesthetized in 1% chloretone and the limb was amputated at the level of the lower third of the upper forelimb. The newts were then placed on damp paper in covered finger bowls, fed chopped beef liver three times a week, and the limb was allowed to regenerate for periods of 6–12 wk. Although muscle differentiation begins as early as 2 wk after limb transection, the later time periods were selected because all stages in muscle differentiation from mesenchymal cell to fully differentiated muscle can be found in the regenerate at this time. After about 8 wk, more advanced stages of differentiation predominate. The newts were then anesthetized, and the regenerating limb was amputated for fixation. Muscle and overlying epidermis of the regenerate was trimmed from the cartilage and cut into small blocks. The tissue blocks were placed in cold 3% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2) and fixed for 1 hr. The blocks were rinsed briefly

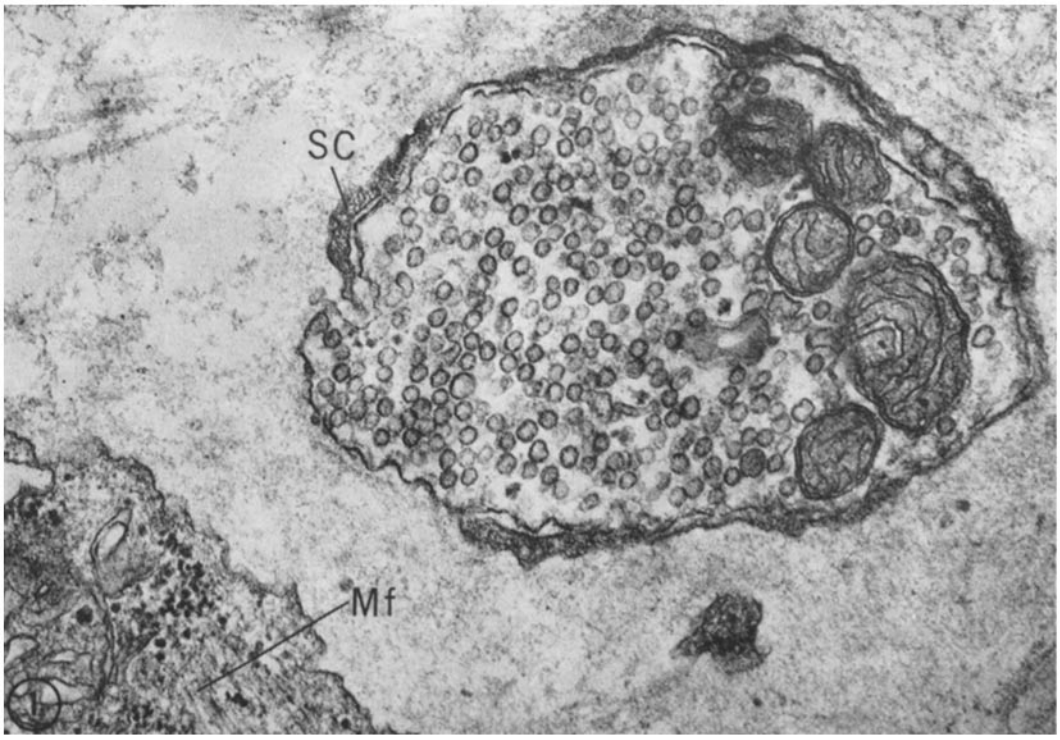


FIGURE 1 A vesicle-filled axon is located in the intercellular space near a differentiating muscle cell containing myofilaments (*Mf*). The Schwann cell (*SC*) covering is deficient over the side of the axon facing the muscle cell. No changes have occurred on the muscle surface opposite the axon. 6-wk regenerate. × 58,500.

in cold buffer and fixed for an additional hour in cold 1% osmium tetroxide in 0.05 M cacodylate buffer at pH 7.2. The tissues were dehydrated rapidly in ethanol, infiltrated with Maraglas (Freeman and Spurlock, 1962), and polymerized at 49°C. Thick, 1–2 μ sections were cut with glass knives on a Porter-Blum MT-1 microtome and stained with 0.1% toluidine blue for light microscopic orientation. Thin sections were cut from blocks containing muscle, stained with lead hydroxide (Feldman, 1962), and examined with an RCA EMU 3F electron microscope.

The thiolacetic acid–lead nitrate method (Crevier and Bélanger, 1955; Barrnett and Palade, 1959; Barrnett, 1962) was chosen for the demonstration of cholinesterase in the developing motor end plate. Comparison of this method with several other available procedures has shown that it results in a sharp and accurate localization at the electron microscopic level (Bloom and Barrnett, 1966; Koelle and Gromadzski, 1966; Davis and Koelle, 1967). A limitation of the method is its low specificity, which can be obviated to some degree by the use of appropriate inhibitors.

Tissue was obtained in the same manner for the histochemical investigations, rinsed in buffer after glutaraldehyde fixation, and placed in the incubating media for 60 min at 4°C. The incubating media contained 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 a final pH of 6.8. After incubation, the tissues were rinsed in three changes of buffer, 5 min each. They were then placed in osmium tetroxide and prepared for electron microscopy in the manner described above.

Control procedures consisted of omission of substrate from the medium or, alternatively, addition of physostigmine (eserine) (10^{-4} M) or diisopropylfluorophosphate (DFP) (10^{-5} M and 10^{-7} M) to the incubating medium. The tissues were placed in buffer containing inhibitor for 20 min prior to exposure to the incubating medium with inhibitor. In the case of eserine, the buffer rinse prior to osmium tetroxide fixation contained inhibitor.

RESULTS

Cytology of Neuromuscular Junction Formation

Structures comparable to axon terminations are first seen in close relationship to muscle only when differentiation of the latter has progressed to the stage of a multinucleate cell containing myofibrils. These axons are dilated and contain some small

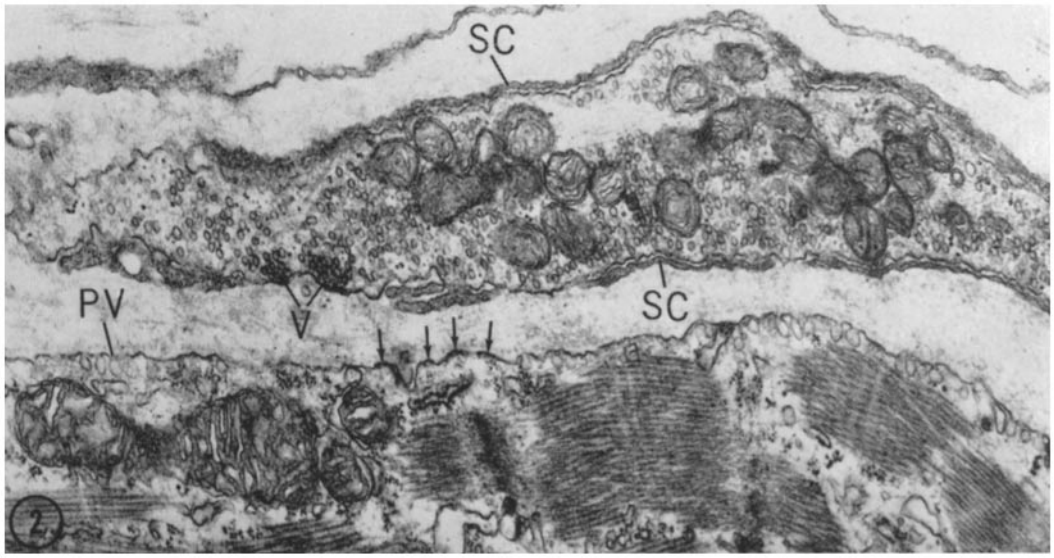


FIGURE 2 Axon containing vesicles and mitochondria near a differentiating muscle cell in a 6-wk regenerate. The Schwann cell (*SC*) covering is complete over the outer surface of the fiber, but is discontinuous over the side facing the muscle cell. A few slight elevations or ridges (arrows) with underlying densities occur on the muscle surface. Pinocytotic vesicles (*PV*) are not so abundant on the muscle surface facing the center of the nerve fiber termination as they are laterally. In the nerve fiber, there are two clusters of vesicles (*V*) beneath projections from the surface. × 26,000.

mitochondria, large numbers of small vesicles (500 A) with contents of low density, and smaller numbers of large vesicles with dense contents (Fig. 1). The Schwann cell cytoplasm is deficient or incomplete with gaps in it over the side of the axon facing the differentiating muscle cell but continues to envelop the opposite side (Figs. 1, 2).

The first morphological changes indicative of motor end-plate formation develop at the apposing surfaces of differentiating muscle and axon terminal where the Schwann cell covering is incomplete (Fig. 2). On the muscle surface, a few low elevations or ridges occur (Fig. 2). There is a slight increase in density of the cytoplasm immediately beneath the plasma membrane of the elevations. Pinocytotic vesicles are not so common along the muscle surface facing the axon as over the rest of the muscle surface. On the surface of the axon, elevations or evaginations similarly project outward, but there is no cytoplasmic density associated with them. Synaptic vesicles, abundant throughout the nerve terminal, seem more concentrated adjacent to the plasma membrane facing the muscle cell and are tightly packed beneath the elevations on the axon surface (Fig. 2).

In ensuing stages, the axon becomes more

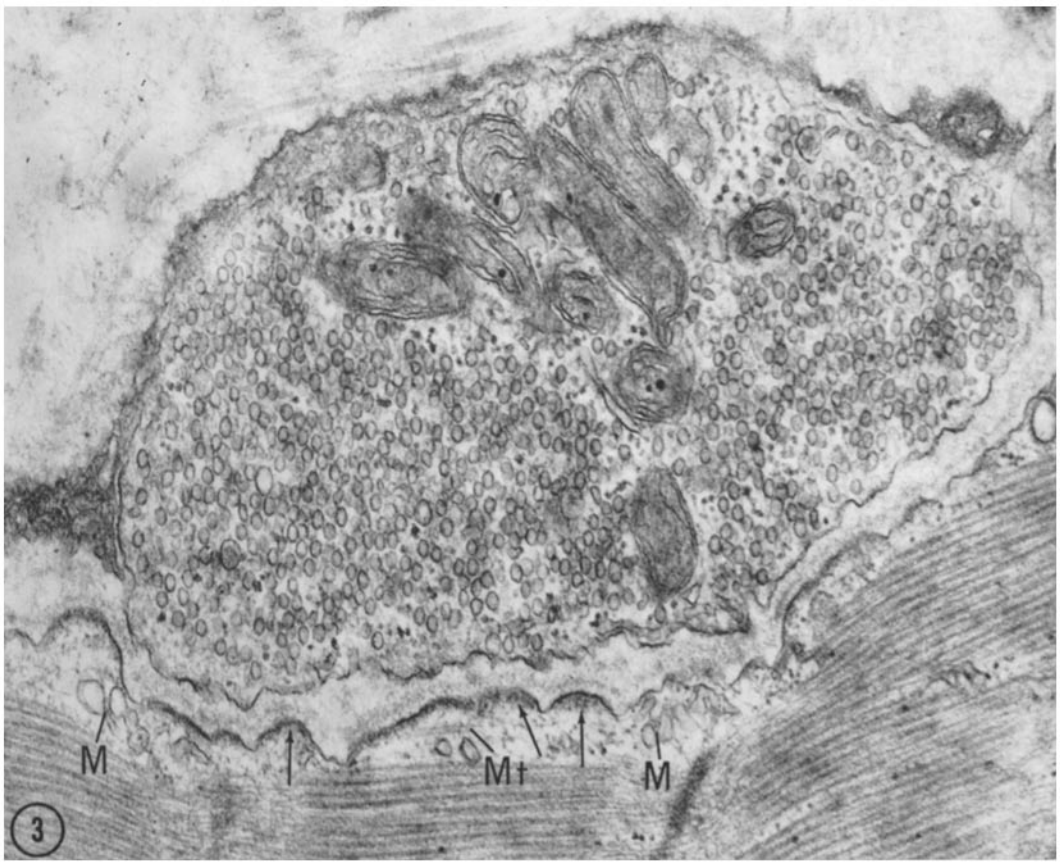


FIGURE 3 Nerve fiber termination on muscle. 6-wk regenerate. The axon is more closely apposed to the muscle cell than in the previous stage, and its contour roughly follows that of the muscle surface. Schwann cell cytoplasm is now completely absent over the inner surface, and the filamentous coating of muscle (basement lamina) occupies the intercellular space. The ridges (arrows) on the muscle surface are more prominent, and dense material is accumulated on the inner aspect of the sarcolemma of the folds. Pinocytotic vesicles are diminished in the region of the junction while some irregularly shaped membranous structures (*M*) and a microtubule (*Mt*) occupy the sarcoplasm between myofilaments and plasma membrane. × 43,500.

closely approximated to the muscle cell, coming to lie in a shallow depression or gutter on the surface of the muscle (Fig. 3). Schwann cell cytoplasm is now completely absent on the side of the axon-facing muscle. The contour of the ending generally follows that of the muscle, producing a relatively constant synaptic cleft. The cleft is occupied by filamentous material that has a denser central band somewhat closer to the muscle than to the axon surface. At the same time, the ridges on the muscle surface become more numerous and prominent and can definitely be identified as early junctional folds (Fig. 3). The subplasmalemmal densities are also more apparent and are due to the accumulation of fine-textured material (Fig. 3).

Subsequently, the junctional folds increase in length and the intercellular space is reduced further (Fig. 4). The bases of the folds are nar-

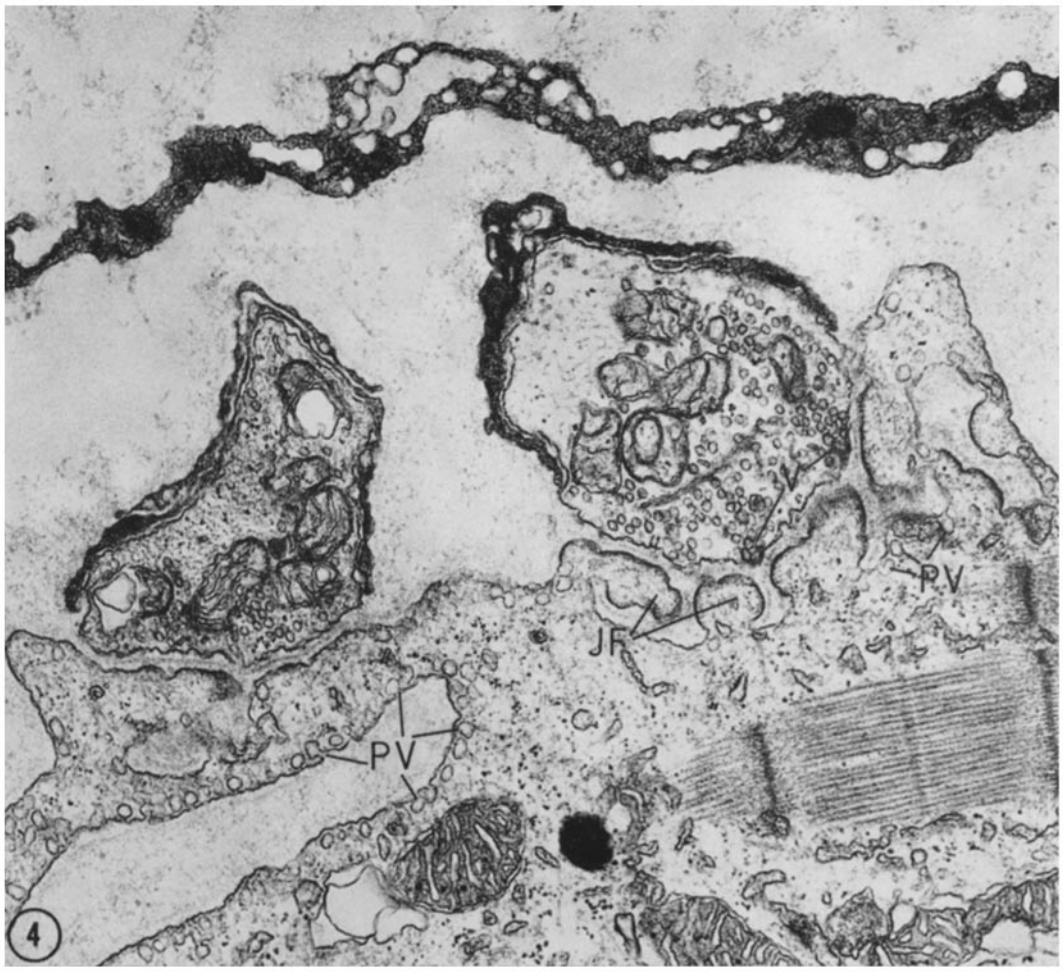


FIGURE 4 Developing neuromuscular junction on a differentiating muscle cell in a 6-wk regenerate. The axon is closely apposed to the muscle cell, with the densest part of the filamentous intercellular material being situated roughly equidistant between nerve and muscle membranes. In the axoplasm, vesicles are segregated toward the muscle. The right hand portion of the junction appears more advanced than the region to the left. In the former, junctional folds (*JF*) are apparent; in the axon, vesicles (*V*) accumulate beneath projections opposite the clefts between junctional folds. Pinocytotic vesicles (*PV*) are absent in the immediate region of the junction except at the bases of the folds, but are numerous along the rest of the muscle surface. Membranous structures and tubulovesicles are found in the sarcoplasm beneath the junction. × 37,500.

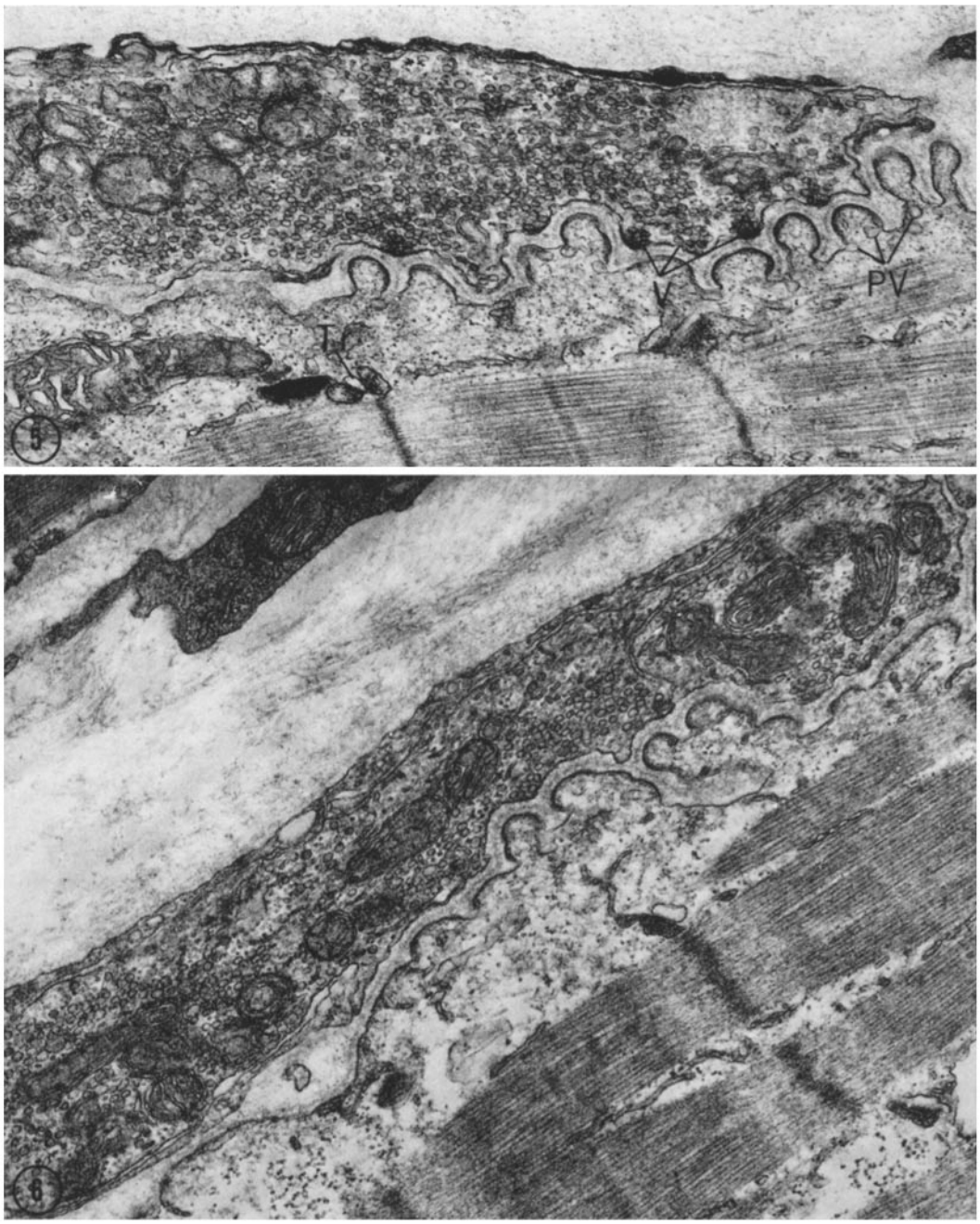


FIGURE 5 Neuromuscular junction from a limb allowed to regenerate for 6 wk. Junctional folds are well developed and have pinocytotic vesicles (*PV*) at their bases. The intercellular space is ~600 Å across and occupied by a band of filamentous material. Vesicles (*V*) are abundant in the axon and are especially concentrated beneath projections of the nerve surface toward the secondary clefts. This junction is morphologically mature and indistinguishable from normal junctions (Fig. 6). Note also that the muscle cell is highly differentiated, containing well organized myofibrils with triads (*Tr*) associated with them. × 28,500.

FIGURE 6 Motor end plate from the upper forelimb of a normal newt. Compare the structural features of this junction and the sarcoplasm with those in Fig. 5. × 29,000.

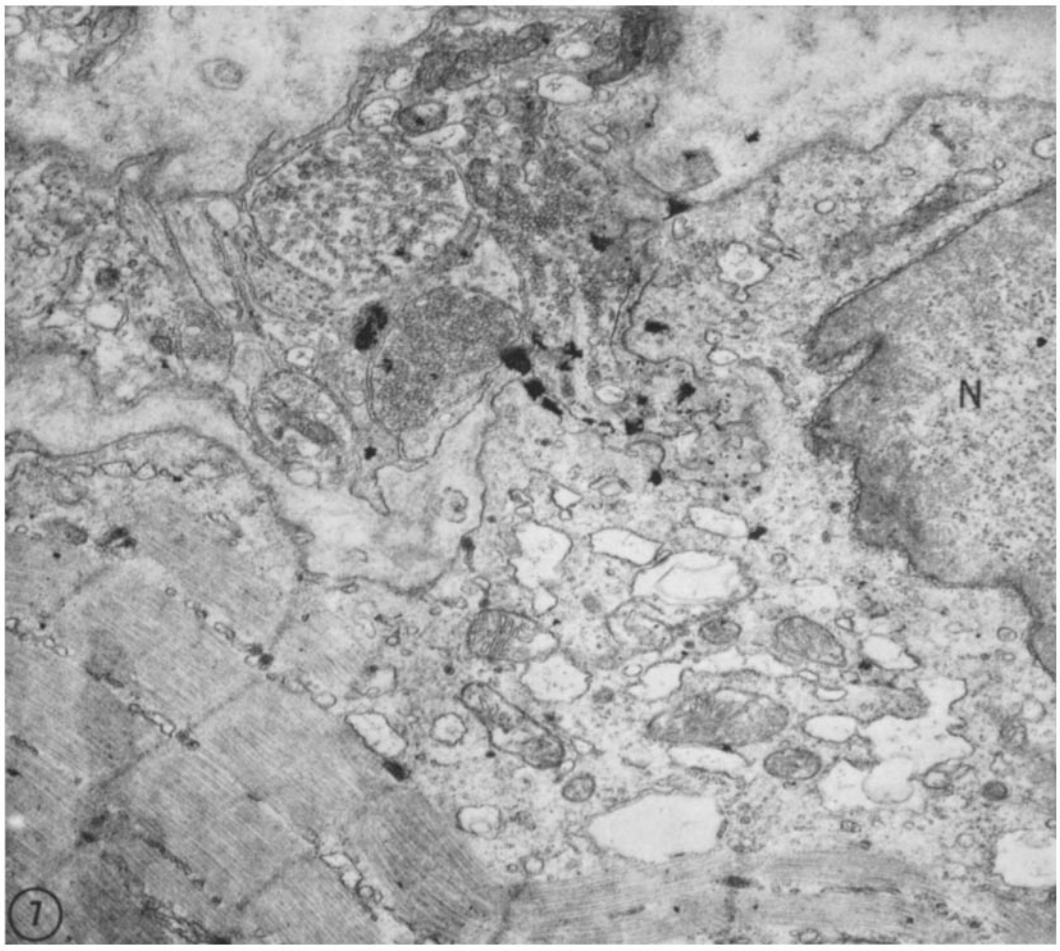


FIGURE 7 Low magnification of an early neuromuscular junction reacted for cholinesterase activity. Note that enzymatic activity is largely restricted to the region of close contact between the vesicle-filled axon termination and the muscle cell surface. Junctional folds are emerging in the same area. A few reaction deposits are present in the axoplasm of the nerve fiber and in the enveloping Schwann cell cytoplasm. Irregular membranous structures (some with associated activity), mitochondria, and small granules resembling ribosomes occur in the subjunctional sacroplasm. Myofibrils are found outside this region. *N*, nucleus. × 17,500.

rower than the distal region, giving the folds a club-shaped appearance. With the closer apposition of axon to muscle, the dense band in the filamentous material occupying the synaptic cleft is situated roughly equidistant between adjacent plasma membranes. The subplasmalemmal cytoplasmic density in the muscle cell is found only in the junctional folds, being absent in other regions of the junction, for example, at the bases of the clefts between folds. Pinocytotic vesicles are largely restricted to the bases of the junctional folds and are rarely seen along the sides and ends of the folds (Fig. 4). Outside the end-plate region, pinocytotic vesicles in association with the sarcolemma are numerous. In the axon terminal, synaptic vesicles are segregated in the pole adjacent to the muscle cell. Focal accumulations of synaptic vesicles immediately beneath the axon membrane are more common (Fig. 4). These tightly packed masses of vesicles occur where the axon contour projects slightly opposite the secondary synaptic clefts. In the early stages of junction

formation, only a small amount of sarcoplasm separates the myofibrils from the sarcolemma (Figs. 1–3). This area increases in size as development proceeds, and it contains irregularly shaped membranous profiles, an occasional rough-surfaced cisterna, mitochondria, glycogen granules, free ribosomes, and sparsely distributed microtubules (Figs. 3, 4).

Increase in the area of axon contact with muscle results in the formation of myoneural junctions (Fig. 5) indistinguishable from those observed in normal animals (Fig. 6). The fully formed motor end plates are found on muscle cells that are also fully differentiated, or nearly so. Myofibrils, previously short and separated by wide stretches of cytoplasm, are now long and tightly packed. The membranous elements of the sarcoplasm (transverse tubules and sarcoplasmic reticulum) are also developed and oriented in the usual relation to each other and the myofibrils (e.g., triad in Fig. 5).

Appearance of Cholinesterase Activity

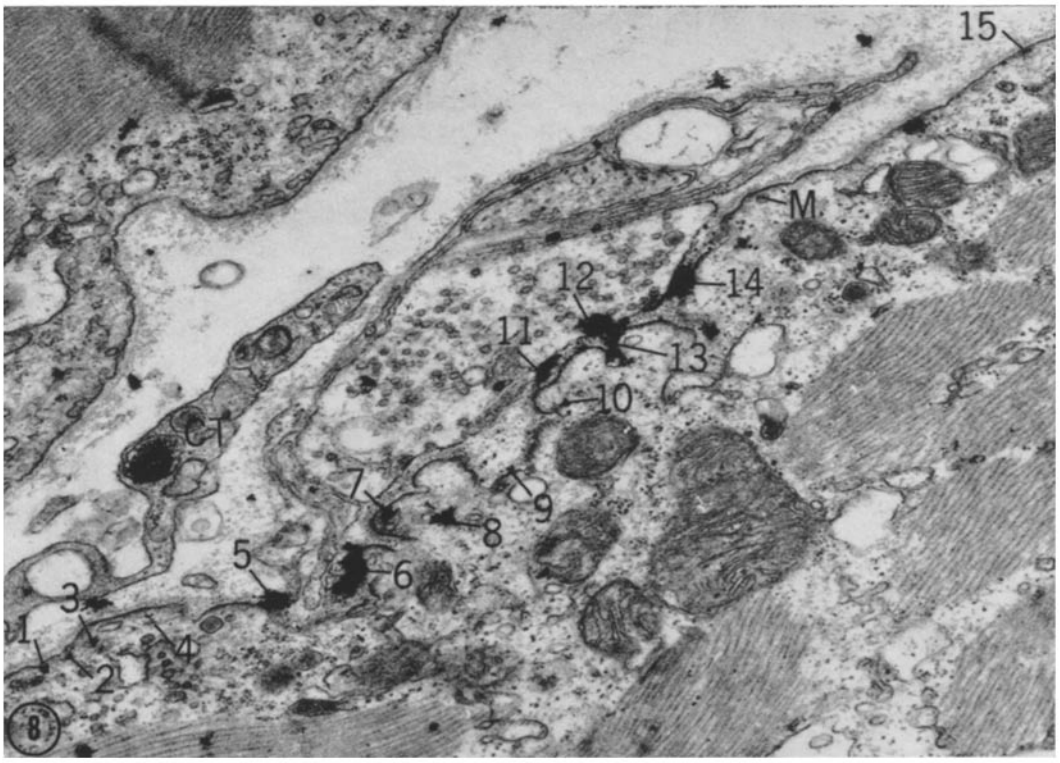
The final reaction product (PbS) of the thiolacetic acid–lead nitrate method for cholinesterase occurs as discrete, electron-opaque particles. The particles are variable in shape and size, depending on the amount of final product precipitated, but can be accurately localized to subcellular structures and organelles (Bloom and Barrnett, 1966; Davis and Koelle, 1967). Because the final product is precipitated at or near the site of substrate hydrolysis, the number of deposits would seem to depend on the number of enzymatic sites. Thus, it is not surprising that relatively few reaction deposits are observed in the early stages of junction formation while more are seen in the later developmental stages.

Enzyme activity was not found on intercellular nerve fiber endings or on the muscle surface prior to the appearance of morphological indications of motor end-plate formation. Enzymatic activity was demonstrated only where axon terminations are closely approximated to the muscle surface and where junctional folds are beginning to emerge (Fig. 7). Physostigmine- and DFP-sensitive activity appears in the following locations: within membranous structures in the muscle cytoplasm, on the plasma membranes of muscle and axon, and within the axoplasm of the terminations.

In the muscle cell, deposits of final product are found on small membranous tubules or vesicles (tubulovesicles) (Figs. 8, 9). Although membranous structures of this type occur all along the cortical sarcoplasm, only those subjacent to the end-plate region contain activity. Some of these tubulovesicles containing reaction product appear to be connected with or to have fused with the plasma membrane (Fig. 8). Serial sections show that some of the apparently isolated tubulovesicles beneath the plasma membrane are actually connected to the membrane (Figs. 8, 9). These tubulovesicles seem to differ from the pinocytotic invaginations that occur along the muscle surface and at the bases of the junctional folds; they are variable in size, not as regular in shape, and not restricted to the bases of the folds. Other small deposits, not enclosed by a vesicle, are applied to the inner aspect of the plasma membrane (Fig. 8).

Larger deposits of final product occur on the

FIGURES 8 and 9 Serial sections through a developing neuromuscular junction in an 8-wk regenerate reacted for cholinesterase activity. Corresponding deposits of final reaction product in the two figures are numbered. The smallest deposits occur in membranous structures or tubulovesicles beneath or applied to the inner surface of the sarcolemma (Fig. 8, *1, 2, 3, 4, 8, 10, 15, M*; Fig. 9, *1, 2, 4, 8, 10, 13, M*). Comparison of the two figures shows that some of the apparently isolated subsarcolemmal deposits are connected to the plasma membrane (*1, 2, 4, 10*) and that those on the inside of the membrane may project into the intercellular cleft (*3, 13, 15*). Some of the small vesicles containing reaction product are fused with the plasma membrane (Fig. 8, *2*; Fig. 9, *4*). Larger reaction deposits occur on the outer surface of the sarcolemma and may fill the intercellular cleft (*5, 6, 13, 14*). Activity is present on both the inner (*12*) and outer (*11*) surfaces of the nerve membrane and also in the axoplasm. A dense dot occurs in the center of some of the synaptic vesicles. A few small deposits occur in the Schwann cells enveloping the axon and in a membrane-bounded structure, resembling a lysosome, within the process of a connective tissue (*CT*) cell. × 26,000.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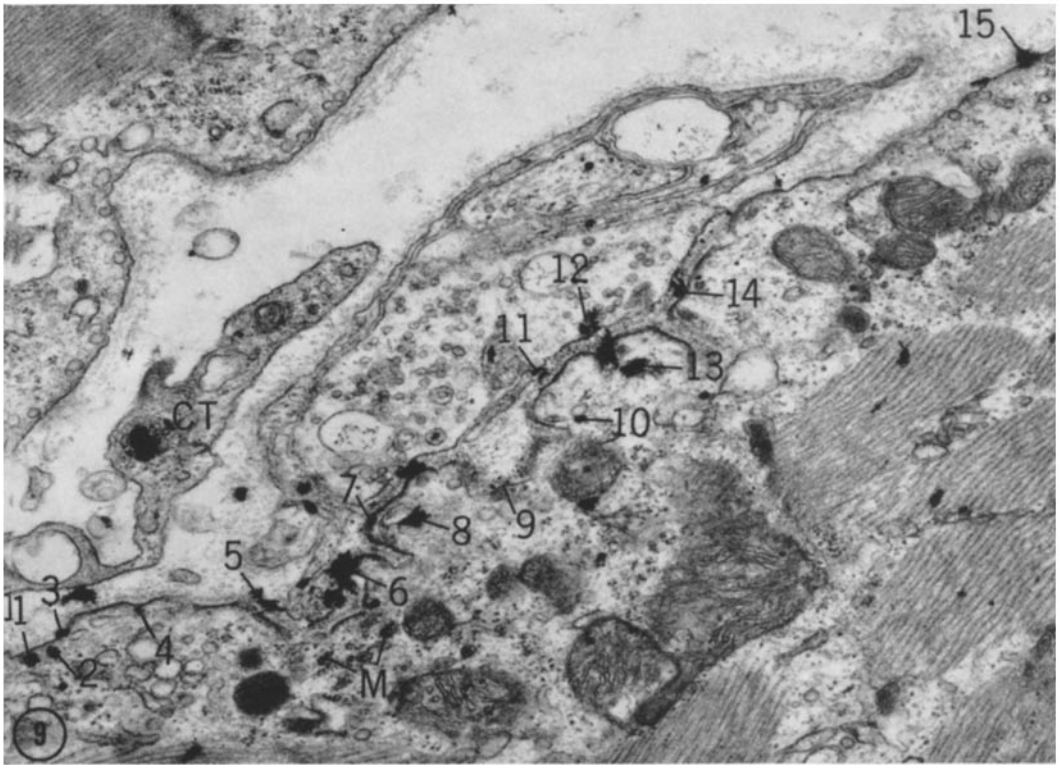
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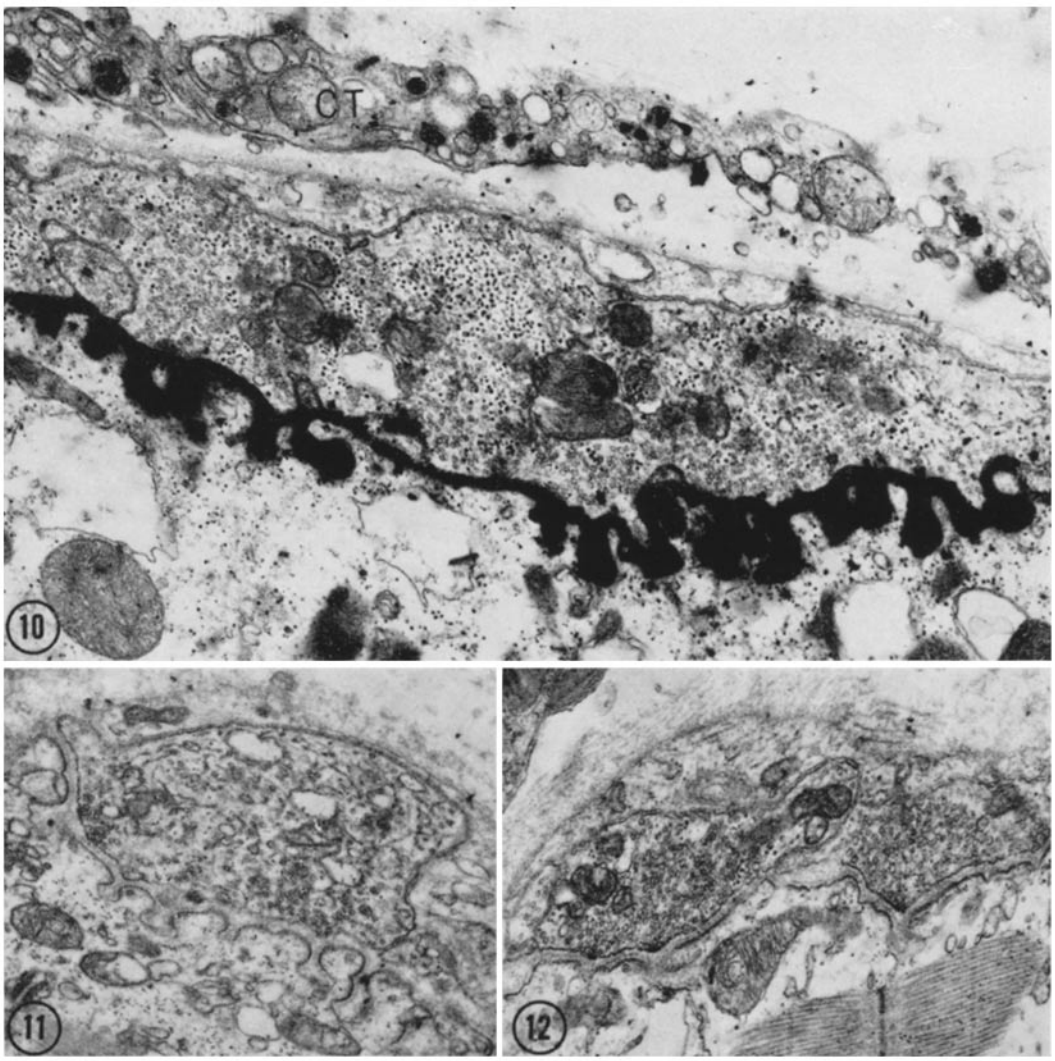

FIGURE 10 Neuromuscular junction from a normal limb reacted for cholinesterase activity. Activity in this junction is unusually intense, with reaction product filling the junctional clefts. Lysosome-like bodies in the connective tissue (*CT*) cell process overlying the nerve terminal are reactive. × 17,000.

FIGURE 11 Highly developed motor end plate preincubated in physostigmine (10^{-4} M) and incubated in the reaction media for cholinesterase which also contained physostigmine. 8-wk regenerate. The reaction in the end-plate region has been almost completely inhibited. Most of the persisting lead deposits occur in the intercellular spaces around the junction. × 16,000.

FIGURE 12 Neuromuscular junction after incubation in the reaction media for AChE which contained DFP (10^{-5} M). Activity in the motor end plate is inhibited. Lead deposits do occur in association with extracellular collagen fibrils and dispersed over the myofibrils. 8-wk regenerate. × 19,500.

outer surface of the sarcolemma (Figs. 8, 9). Most of these are located at the tips of the ridges and more fully developed junctional folds. Some deposits are so large that they extend across the intercellular cleft, but the serial sections show that most of them originate on the muscle surface (Figs. 8, 9).

Reaction product is less abundant on the axon

surface and is localized to both sides of the axon membrane (Figs. 8, 9). The deposits on the inner side of the membrane could not be associated with vesicles or other structures. Deposits on the outer axon surface are not numerous and not so large as those on the outer surface of the sarcolemma. Activity also occurs in the axoplasm, but could not be associated consistently with a specific organelle (Figs. 8, 9).

As development of the motor end plate progresses, the reactive sites on the plasma membrane increase in size and number. Although morphologically well developed junctions have a normal localization of enzyme activity, the activity is not so intense, as evidenced by the number of reaction deposits, as in motor end plates of normal animals (Fig. 10), even after 12 wk of regeneration. In normal animals, the reaction is sometimes so intense as to fill the intercellular space and structures such as tubulovesicles, pinocytotic vesicles, and synaptic vesicles that might be continuous with the space.

Occurrence of reaction product at the sites described above was almost completely prevented by preincubation of the tissues in physostigmine (10^{-4} M) (Fig. 11) and DFP (10^{-5} M) (Fig. 12). Inhibition of activity at these sites occurred at all stages of end-plate formation. Activity was only partially inhibited, however, by DFP at a concentration of 10^{-7} M. Small lead deposits in Schwann cell cytoplasm (Figs. 7, 8) were not significantly affected by inhibitors and are presumably due to an enzyme other than a cholinesterase. Large membrane-bounded granules, resembling lysosomes, in connective tissue cells are reactive (Figs. 8, 10). This activity was resistant to inhibition and is probably also due to another enzyme, possibly cathepsin C. A small dense deposit was variably observed in synaptic vesicles of both experimental and control incubations. Incubation in media without substrate sometimes produced small dense deposits throughout the section, especially over myofilaments and collagen fibrils. These deposits could be reduced or eliminated, however, by adequately rinsing the tissues in buffer prior to osmium fixation.

DISCUSSION

Morphogenetic Relationships

In differentiating muscle, both morphological (e.g., junctional folds) and chemical (AChE) specializations characteristic of the neuromuscular junction develop only where axon terminals become closely apposed to the muscle surface. In no cases were postjunctional structures or localized concentrations of enzyme activity found on the muscle surface outside the area of contact. Thus, these findings support the conclusion of others (Zelená, 1962; Kelly, 1966; Teräväinen, 1968), that motor nerve fibers exert a morphogenetic effect on the formation of the subneural apparatus.

It was observed here that nerve and muscle are already differentiated to some extent before end-plate formation begins. As noted by Hirano (1967), muscle differentiation progresses through the myoblast and early myofiber stages before neuromuscular junctions are formed. The intercellular nerve fiber terminations change from growing end bulbs that invade the early regenerate to expansions filled with synaptic vesicles. The early end bulbs differ from the vesicle-filled terminals of later regenerates in that they have fewer small vesicles, but more membranous channels and tubules and larger vesicles containing dense material (Lentz, 1967). Junctions develop only in relation to the vesicle-filled endings. If either the axon or muscle is less differentiated, the motor end plate does not form, even if the axon passes near the muscle cell. Formation of the neuromuscular ujnction, then, depends on a relationship between nerve fiber and muscle that have each attained a required degree of specialization. If the inductive interaction between nerve and muscle is linked to a mechanism of mutual recognition which depends on transfer of information, it seems likely that a certain degree of specialization is necessary for transfer and/or reception of the information.

A significant question involves the time of appearance of cholinesterase activity in relationship to innervation of the muscle cell. Some investigators have found that cholinesterase appears before innervation and suggest that prior appearance of the enzyme may have a chemotactic influence on the approaching motor nerve fiber (Kupfer and Koelle, 1951; Beckett and Bourne, 1958; Shen, 1958). Other investigators have concluded that concentration of enzyme activity at the end-plate region occurs only after axon contact with muscle has been established (Mumenthaler and Engel, 1961; Zelená, 1962; Khera and Laham, 1965; Kelly, 1966). The present findings support the latter conclusion and show that cholinesterase activity develops concurrently with morphological

differentiation. This conclusion is supported further by studies of muscle cells in tissue culture; the uninnervated cells do not develop cholinesterase-containing motor end-plate structures (Engel, 1961). On this evidence, it appears that the motor nerve fiber directly influences the formation of the chemical, as well as the morphological, specialization of the neuromuscular junction.

Cytological Specializations

The most conspicuous event in motor end-plate development is the formation of the junctional folds. The folds differ somewhat in structure from the adjacent muscle surface, indicating that they may form by outgrowth from, rather than folding of, the muscle surface. Pinocytotic vesicles, abundant all along the muscle surface, are not associated with the membrane of the folds but persist only at their bases. Similarly, the cytoplasm of the folds is largely devoid of structures such as ribosomes or glycogen granules found throughout the rest of the cytoplasm. On the other hand, dense material, not found elsewhere, accumulates beneath the plasma membrane of the fold. Dense material has also been described in the junctional folds of intrafusal motor myoneural junctions of the frog (Karlsson and Andersson-Cedergren, 1966). The subsarcolemmal densities in the motor end plate are similar in appearance to accumulations of material beneath the postsynaptic membrane of synapses from several areas including the central nervous system (Bloom and Aghajanian, 1966). Although the exact nature of this material is unknown, the material is thought to be proteinaceous and presumably is involved in synaptic function. If this is the case also for the motor end plate, its appearance is significant as a further indication of acquisition of functional capacity.

Enzymatic (AChE) Specialization

In the earliest stages of junction formation, enzymatic activity is associated with tubulovesicles in the muscle cytoplasm only in the region beneath the axon. These reactive structures also seem to be a component of the normal motor end plate (Miledi, 1964). Some of the tubulovesicles are connected to the sarcolemma, indicating their possible role in delivery of the enzyme from deeper levels to the cell surface. A somewhat similar process has been suggested for the electroplaque of the eel in which reactive tubulovesicles containing mucoid, extracellular-space substance are continuous with the surface (Bloom and Barrnett, 1966). The reverse process, inward migration of the reactive sites by a process of pinocytotic invagination, does not seem as likely because of the sparse surface localization of enzyme at this time and the fact that unreactive pinocytotic vesicles can be distinguished from the reactive membranous structures. Larger deposits are found in association with the sarcolemma, indicating accumulation of enzyme at this site.

The origin of AChE within the muscle cell is unknown. Structures such as mitochondria, glycogen, a few rough-surfaced cisternae of endoplasmic reticulum, and free ribosomes are found in the sarcoplasm near the developing end plate. No unusual or special relationships were found between these structures and demonstrable activity that might suggest a role in enzyme formation. Ribosomes are associated with protein synthesis, and their persistence in the end-plate region (Padykula and Gauthier, 1967) while decreasing in the rest of the muscle cell during development (Lentz, 1969) might implicate them as sites of synthesis.

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