

THE APPEARANCE OF ACETYLCHOLINESTERASE IN THE MYOTOME OF THE EMBRYONIC RABBIT

An Electron Microscope Cytochemical and Biochemical Study

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ABSTRACT

Acetylcholinesterase (AChE) activity has been studied in the myoblast of skeletal muscle of the 9–13 day fetal rabbit. Cytochemical activity is present in the nuclear envelope and the endoplasmic reticulum, including its derivatives the subsurface reticulum and the sarcoplasmic reticulum. End product is also found in the Golgi complex of the more differentiated myoblasts. The formation of reticulum-bound acetylcholinesterase in the myoblast appears to be independent of nerve-muscle contact, since the enzyme is present before the outgrowth of the spinal nerve. The nerve lacks cytochemical end product until the myoblast is well differentiated. Possible mechanisms of spontaneous muscle contraction have been discussed. A second type of myotomal cell, which exhibits a poorly localized end product of AChE activity, has been described. The ready solubility of the enzyme or diffusibility of its end product suggests that the enzyme may be a lyoesterase. This cell may be the precursor of the morphologically undifferentiated cell which is closely apposed to the myotubes in later stages of skeletal muscle development. Biochemical studies show a significant increase in AChE activity in the dermomyotome by day 12, when many of the myoblasts are well differentiated and the second type of myotomal cell is prominent. Cytochemical studies have indicated that many of the cells in the sample lack reaction product of enzymic activity, whereas others are very active. Biochemical values, therefore, reflect the amount of enzyme in the dermomyotome as a whole, but give little information on the enzymic content of individual cells.

INTRODUCTION

An initial formation of cholinesterase (ChE)¹ occurs in developing skeletal muscle before in-

nervation (Nachmansohn, 1938, 1939, 1940; Kupfer and Koelle, 1951; Zacks, 1954; Mumen-

¹Abbreviations: ACh-ChE, acetylcholine-cholinesterase; AChE, acetylcholinesterase; AThCh, acetylthiocholine iodide; BuChE, butyrylcholinesterase;

BuThCh, butyrylthiocholine iodide; ChE, cholinesterase; iso-OMPA, tetraisopropyl pyrophosphor-tetramide.

thaler and Engel, 1961; Karczmar, 1963). The enzyme appears in embryonic amphibian muscle deprived of its nerve supply (Shen, 1958), as well as in nerveless muscle cells grown in tissue culture (Engel, 1961). Histochemical studies have shown that myogenic ChE is located diffusely in the sarcoplasm of uninucleated myoblasts, and is found in greater quantities in the early plurinucleated myotubes (Bonichon, 1957; Gerebtzoff, 1957, 1959; Mumenthaler and Engel, 1961; Filogamo, 1964; Gabella, 1964; Veneroni and Murray, 1969). A perinuclear localization of enzymic activity has been reported (Mumenthaler and Engel, 1961; Engel, 1961) as well as deposition of reaction product within the nuclei at later stages of development (Kupfer and Koelle, 1951).

Electron microscope cytochemical studies have been done on the appearance of ChE during the formation of the motor end plate (Hirano, 1967; Lentz, 1969), but detailed information on the intracellular localization of this enzyme during myoblast differentiation is lacking. A preliminary study from this laboratory (Slotwiner and Tennyson, 1969) reported that acetylcholinesterase (AChE) activity is localized in the nuclear envelope and in elements of the endoplasmic reticulum of the myoblast. These results are reported in full here. In addition, a second type of myotomal cell containing AChE activity is also described. This cell might be the precursor of the morphologically undifferentiated cell which accompanies the myotube, and might also be related to the muscle satellite cell (Mauro, 1961). Biochemical studies were done on myotomes at different stages of development to estimate the amount of enzyme present. Spinal nerves adjacent to the myotomes were examined cytochemically as a frame of reference for a discussion of the induction of the enzyme in skeletal muscle.

MATERIALS AND METHODS

Fixation and Cytochemical Procedure

Brachial and thoracic dermomyotomes of 84 Dutch rabbit embryos from day 9 to day 13 of gestation were used. At least one cytochemical procedure was performed on multiple sections from each embryo. In control studies either adjacent sections of the same embryo or similar areas from littermates were used depending on the amount of tissue available at a given age. Specimens of peripheral nerve adjacent to some of the myotomes were also examined. Details of the fixation and cytochemical procedure have been

described elsewhere (Tennyson, 1970; Tennyson and Brzin, 1970). The embryos were fixed by perfusion with 1% glutaraldehyde (Sabatini et al., 1963) or a mixture of aldehydes in a phosphate buffer. Some embryos were perfused with saline before fixation to remove blood cells, since preliminary studies showed that these cells contained ChE activity. After fixation, the tissue was washed in several changes of buffer for at least 1 hr. Specimens were cut freehand into 0.2 mm blocks or were embedded in agar and cut at 60 μ on a Smith-Farquhar microchopper. The latter procedure permitted a more satisfactory penetration of substrates and inhibitors into the tissue, and resulted in a consistent deposition of reaction product in the areas illustrated in Fig. 1. Cytochemical studies were also done on unfixed tissue, but cellular preservation was so poor that the results will not be emphasized.

INCUBATION MEDIUM: The copper thiocholine technique for the localization of AChE activity (Koelle and Friedenwald, 1949) was used as modified by Brzin et al. (1966). The specimens were incubated at pH 6 for 5–50 min at 4°C in a freshly prepared medium containing 3.46×10^{-3} M acetylthiocholine iodide (AThCh) (Sigma Chemical Co., St. Louis, Mo.), 0.04 M glycine, 0.008 M copper sulfate, 0.12 M sodium chloride, and 0.03 M magnesium sulfate in 0.02 M sodium hydrogen maleate buffer. Before incubation the tissue was preincubated for 30–60 min in the copper glycinate medium lacking the substrate, but containing the appropriate inhibitor. After incubation, the tissue was washed briefly in cold distilled water and postfixed in a freshly prepared solution of cold 3% potassium permanganate for 30 min. The tissue was rapidly dehydrated and embedded in Durcupan (International Chemical & Nuclear Corp., Burbank, Calif.).

The Koelle-Friedenwald technique (1949), including copper sulfide conversion of the end product, was done on whole dermomyotomes and nerve, and the histochemical reaction was examined with the dissecting microscope.

CONTROL STUDIES: To determine the effect of inhibitors on AChE activity, the following substances were added to the preincubation and incubation medium: BW 284 C 51 (1,5-bis-[4-allyl-dimethylammoniumphenyl]pentan-3-one-dibromide) 2×10^{-4} M (Burroughs Wellcome & Co., Tuckahoe, N. Y.), an inhibitor of AChE, and eserine (physostigmine sulfate 1×10^{-4} M (K & K Lab., Plainview, N. Y.), an inhibitor of ChE in general. To eliminate the possibility of hydrolysis of AThCh by butyrylcholinesterase (BuChE), tetraisopropyl pyrophosphotetramide (iso-OMPA) 2×10^{-4} M (Pierce Chemical Co., Rockford, Ill.), an inhibitor of BuChE, was used in most experiments. To determine whether sites of nonspecific ChE activity were present, butyrylthiocholine iodide (BuThCh) 3.1×10^{-3} M (K & K

Labs.), with or without the inhibitor BW 284 C 51, was used. To determine whether nonspecific binding occurs between the tissue and the components of the medium, specimens were incubated in the medium lacking substrate for 85 min.

Since the use of saturated sodium sulfate has been recommended for the elimination of diffusion artifact in ChE studies (Koelle, 1951), this substance was tried in some experiments. Concentrations varying from 0.1 to 1.5 cc of a saturated sodium sulfate solution per 5 cc of solution were used either in the incubation medium, the fixative, or in a saline perfusion before fixation.

Somites and peripheral nerve adjacent to the areas examined cytochemically were postfixed with 2% OsO₄ (Palade, 1952) in a phosphate buffer to study organelles not optimally preserved by potassium permanganate.

Thick sections for phase microscopy and thin sections for electron microscopy were cut with glass or diamond knives on a Proter-Blum (Ivan Sorvall, Inc. Norwalk, Conn.) microtome. Thin sections were placed on 200-mesh copper grids, blotted dry, and then stained with uranyl acetate for 5 min (Watson, 1958), and with lead citrate for 30 min (Reynolds, 1963). Electron micrographs were taken with a Siemens Elmiskop I.

Quantitative Procedure

The embryos were perfused with saline to remove all blood before dissection. Tissue from 57 embryos was isolated in cold saline by freehand technique with the aid of a dissecting microscope. Four or five somites in a row were collected from each side of the embryos during days 9 and 10. From days 11 to 13, individual dermomyotomes were large enough so that they could be dissected free of ectoderm and much of the intersegmental connective tissue. Individual dermomyotomes were then pooled into groups of 3–6 per sample. Immediately after isolation the specimens were frozen and then dried. The dry weight of the specimens (measured on a torsion electromagnetic balance having a sensitivity of $\pm 0.04 \mu\text{g}$) was between 2 and 80 μg , depending on the stage of development and number of pooled specimens.

The radiometric assay for AChE described by McCaman et al. (1968) was followed using acetyl-¹⁴C-choline iodide (The Radiochemical Centre, Amersham, England), except that the volumes throughout the procedure were doubled and the enzymic reaction was performed at 19°C. Specimens weighing less than 20 μg were introduced directly into the reaction tube and hydrated in Ringer's solution for at least 2 hr. Then the tissue was dispersed with a fine glass needle with the aid of a dissecting microscope. Samples weighing more than 20 μg were either cut into smaller pieces, each of which was suspended

as described above, or were homogenized in an appropriate volume of Ringer's solution (5–50 μl) using small glass homogenizers. Samples of 5 μl of homogenate were used for the ChE determination.

OBSERVATIONS

Phase microscope sections and a brief description of osmium-fixed material will be given for each developmental period as a frame of reference for the cytochemical study. The ultrastructural changes taking place during embryonic development of the myoblast of skeletal muscle in the rabbit are very similar to those occurring in other species (Bergman, 1962; Hay, 1963, 1968; Allen and Pepe, 1965; Dessouky and Hibbs, 1965; Heuson-Stiennon, 1965; Przybylski and Blumberg, 1966; Fischman, 1967; Auber, 1969), and thus they will not be illustrated in detail here. Ribosomes, filaments, and cell junctions, moreover, are not optimally visualized in permanganate-treated specimens, which are used in most of this study.

GROSS OBSERVATIONS OF THE HISTOCHEMICAL REACTION: The distribution of AChE activity in the dermomyotome from days 10 to 13, as seen by gross observation, is illustrated in the diagram in Fig. 1. The enzyme-active area of

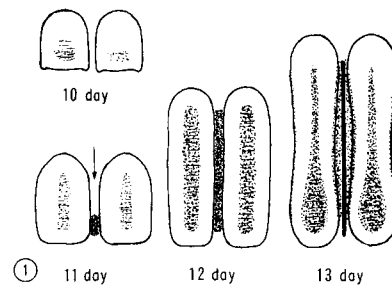


FIGURE 1 Diagram showing the distribution of AChE activity in two adjacent dermomyotomes and the intersegmental septum (arrow) viewed from the lateral surface. Dorsal (top of diagram). The dermatomal cells (unstippled) lack end product. The areas of the myotome containing the heaviest amount of reaction product (stippled) can be seen through the dermatome by transillumination. The precipitate of AChE activity is present in the ventral portion of the intersegmental septum at day 11 (dense stipple) and throughout most of the septum during day 12. A dense precipitate is found along the margin of the dermomyotome and scattered within the septum at day 13. A branch of the spinal nerve running through the septum during this period exhibits end product.

the myoblast is more extensive when viewed from the surface of the myotome. The dermatome (unstippled) and dorsal portion of the myotome lack cytochemical end product. At day 10 the ventral portion of the myotome exhibits a light histochemical reaction which increases somewhat in intensity and extends throughout the midportion of the myotome during day 11. In the intersegmental septum (arrow) a deeper staining precipitate is present in the ventral portion (dense stipple). The histochemical end product occupies a larger area of the myotome by day 12 and the precipitate is denser. An intense reaction extends throughout most of the septum. The enzyme-active area is concentrated in the ventral regions of the myotome during day 13, but end product extends dorsally in a narrow central zone. A dense reaction is present at the edge of the myotome bordering the septum, in areas within the septum, and along the nerve.

Myoblasts

NONFILAMENTED MYOBLAST: The ovoid or pyramidal cells of the somite exhibit very little morphologic differentiation during day 9 of gestation. They are filled with polyribosomes, but have

only a few rough-surfaced cisternae of the endoplasmic reticulum and scattered mitochondria. The Golgi complex is small and consists of flattened sacs and vesicles. The cells are joined apically by primitive desmosome-like junctions (macula adherens diminuta, Hay, 1968), and they rest on a thin basal lamina.

Only some of the myoblasts have AChE activity. They are usually located in the presumptive myotomal region (Fig. 2, *inset*, arrow), but a few others are scattered throughout the somite. A patchy distribution of AChE activity is present in the nuclear envelope (Fig. 2, *Ne*) and in the endoplasmic reticulum (*Er*). Rarely, a sclerotomal cell exhibits a cisterna containing end product surrounding a moderately dense droplet. Although only a small proportion of the cells in the somite exhibit AChE activity at this period, a few are seen in every section.

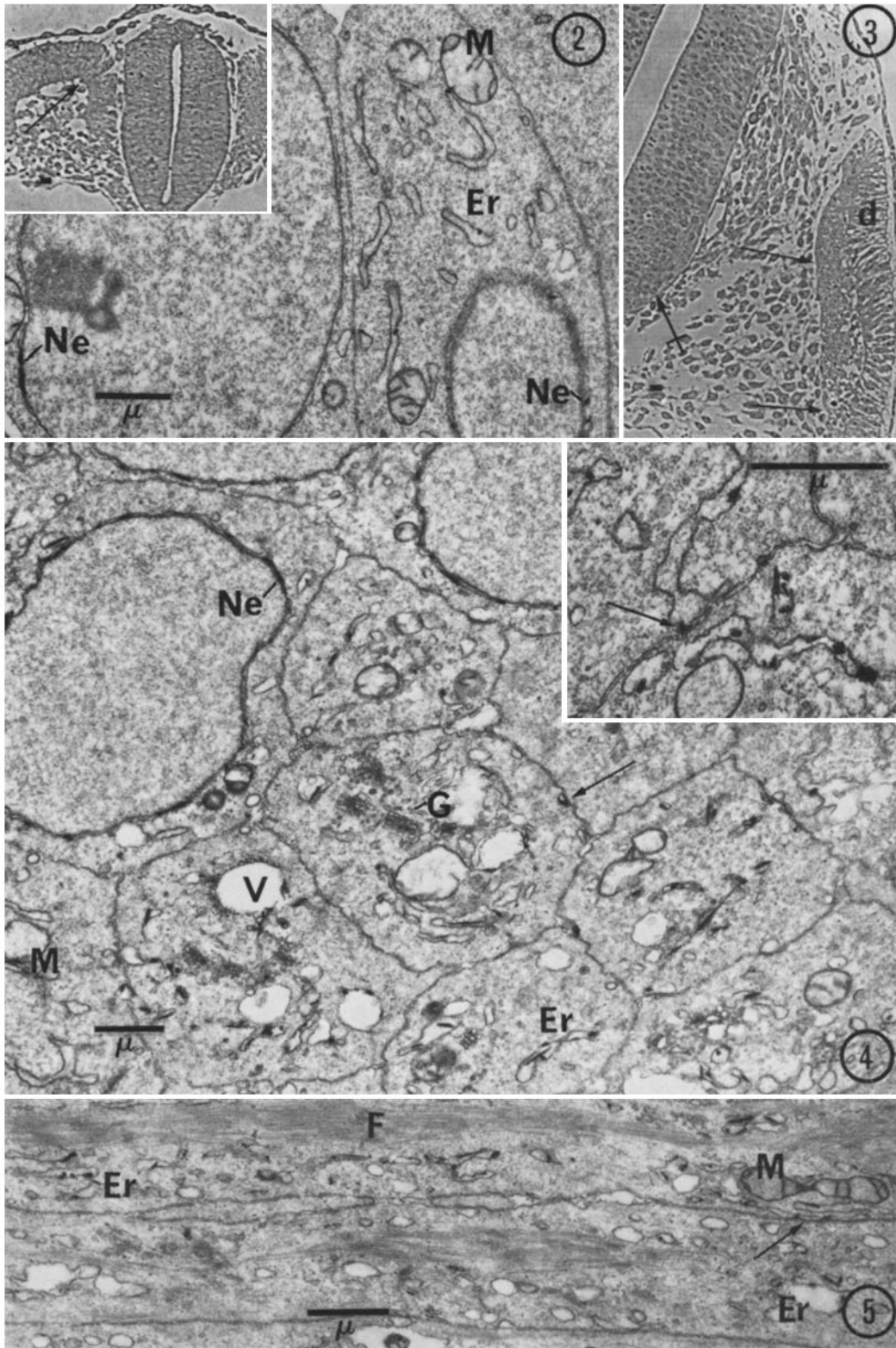
EARLY FILAMENTED MYOBLAST: The rostro-caudally oriented cells in the middle and ventral portions of the myotome (Fig. 3, between the arrows) differentiate into elongated spindle-shaped myoblasts during day 10. Patches of thick ~ 150 A and thin ~ 60 A filaments in association with one another are found in areas containing polyribosomes. Randomly oriented intermediate-

FIGURE 2 Cytochemical end product is found in the nuclear envelope (*Ne*) and endoplasmic reticulum (*Er*) of some cells in the somite at day 9 of gestation. The mitochondria (*M*) are ovoid and contain few cristae. AThCh + iso-OMPA, 75 min. Scale marker: 1μ . $\times 11,200$. *Inset:* phase micrograph of the neural tube and somite at day 9 of gestation. Most of the enzyme-active cells are found in the presumptive myotomal region (arrow). 19 somite embryo. Scale marker: 10μ . $\times 150$.

FIGURE 3 Phase micrograph of the neural tube and dermomyotomal plate before the formation of the spinal nerve. The cranio-caudally oriented myoblasts in the middle and ventral portion of the myotome (between arrows) are elongated cells with cigar-shaped nuclei and prominent nucleoli. Although some neuroblasts are differentiating in the ventral horn (crossed arrow), only a few short ventral root axons could be found in a sequential section. Bipolar neuroblasts are present in the presumptive dorsal root ganglion, but their processes are short and do not form a peripheral root. Dermatome (*d*). Day 10 of gestation, 29 somite embryo. Scale marker: 10μ . $\times 150$.

FIGURE 4 Cross-section through the perinuclear region of myoblasts from the ventral region of the myotome at day 10. Cytochemical end product is present in the nuclear envelope (*Ne*) and endoplasmic reticulum (*Er*). Enzyme-active cisternae are closely apposed to the cell surface (arrow). The Golgi complex (*G*) rarely contains end product at this stage. Vacuoles (*V*) and ovoid mitochondria (*M*) with few cristae are present. Scale marker: 1μ . $\times 10,500$. *Inset:* cisternae of the endoplasmic reticulum containing end product (arrow) approximate the surface membrane. AThCh + iso-OMPA, 60 min. Scale marker: 1μ . $\times 20,800$.

FIGURE 5 Longitudinal section of the distal prolongations of myoblasts from a $10\frac{1}{2}$ day embryo. Filaments (*F*) are forming in the cytoplasm. Cytochemical end product is present in the endoplasmic reticulum (*Er*), which is located close to the filaments and at the cell surface (arrow). The constricted mitochondrion (*M*) with few cristae may be undergoing division. AThCh + iso-OMPA, 40 min. Scale marker: 1μ . $\times 12,400$.



sized filaments ~ 100 A (Ishikawa et al., 1968), microtubules, and glycogen granules are conspicuous in ground substance of low density. The cisternae of the endoplasmic reticulum are studded with ribosomes over much of the surface, but a considerable surface area lacks them. Primitive desmosomal-like junctions are seen between myoblasts.

The few myoblasts having AChE activity at the beginning of day 10 are located ventrally (Fig. 3, lower arrow), but as their numbers increase, they are found as far as the middle zone (upper arrow). Neither the dermatomal cells (*d*) nor cells in the dorsal edge of the myotome contain end product. Initially, most of the enzymic activity of the myoblast is found in the perinuclear region, i.e., in portions of the nuclear envelope (Fig. 4, *Ne*) and in the endoplasmic reticulum (*Er*). As the myoblasts elongate and form more filaments (Fig. 5, *F*), narrow channels of endoplasmic reticulum (*Er*) containing end product are found in the distal prolongations of the cell. The enzyme-active reticulum is often closely approximated to the surface of the myoblast (Fig. 4, arrow and *inset*; Fig. 5, arrow). Study of osmium-fixed tissue from adjacent somites confirms that the subsurface

reticulum at this stage is derived from ribosome-studded cisternae of the endoplasmic reticulum (Fig. 6, arrow) rather than from invaginations of the surface membrane. The Golgi complex (Fig. 4, *G*) rarely contains end product during the early stage. Pinocytotic vesicles are also devoid of AChE activity.

Enzyme-active myoblasts are distributed throughout most of the myotome by day 11 (Fig. 7, ventral to arrow) and by day 12, with the exception of the dorsal edge. The latter area and the dermatome (*d*) lack cytochemical end product. Filaments (Fig. 8, *F*) are still irregularly disposed in the myoblast, but a larger number of thick and thin filaments assume a parallel orientation with respect to one another and become associated with Z-line material (arrow). The flattened reticulum in the perinuclear region has attached ribosomes in osmium-fixed tissue. The subsurface reticulum and other channels in the distal prolongations of the myoblast have large areas devoid of ribosomes. The distal tip of the myoblast which extends into the intersegmental septum often has collections of small and large ovoid bodies (Fig. 9, arrow), ranging from 500 to 1500 A in diameter, and irregular elongate profiles (crossed arrow). Both of

FIGURE 6 Subsurface reticulum has a few ribosomes (arrow) attached to its cytoplasmic surface. The surface apposed to the cell membrane (crossed arrow) is smooth. Nucleus (*N*). Day 10 of gestation, 29 somite embryo. Osmium postfixation. Scale marker: $0.1 \mu \times 58,000$.

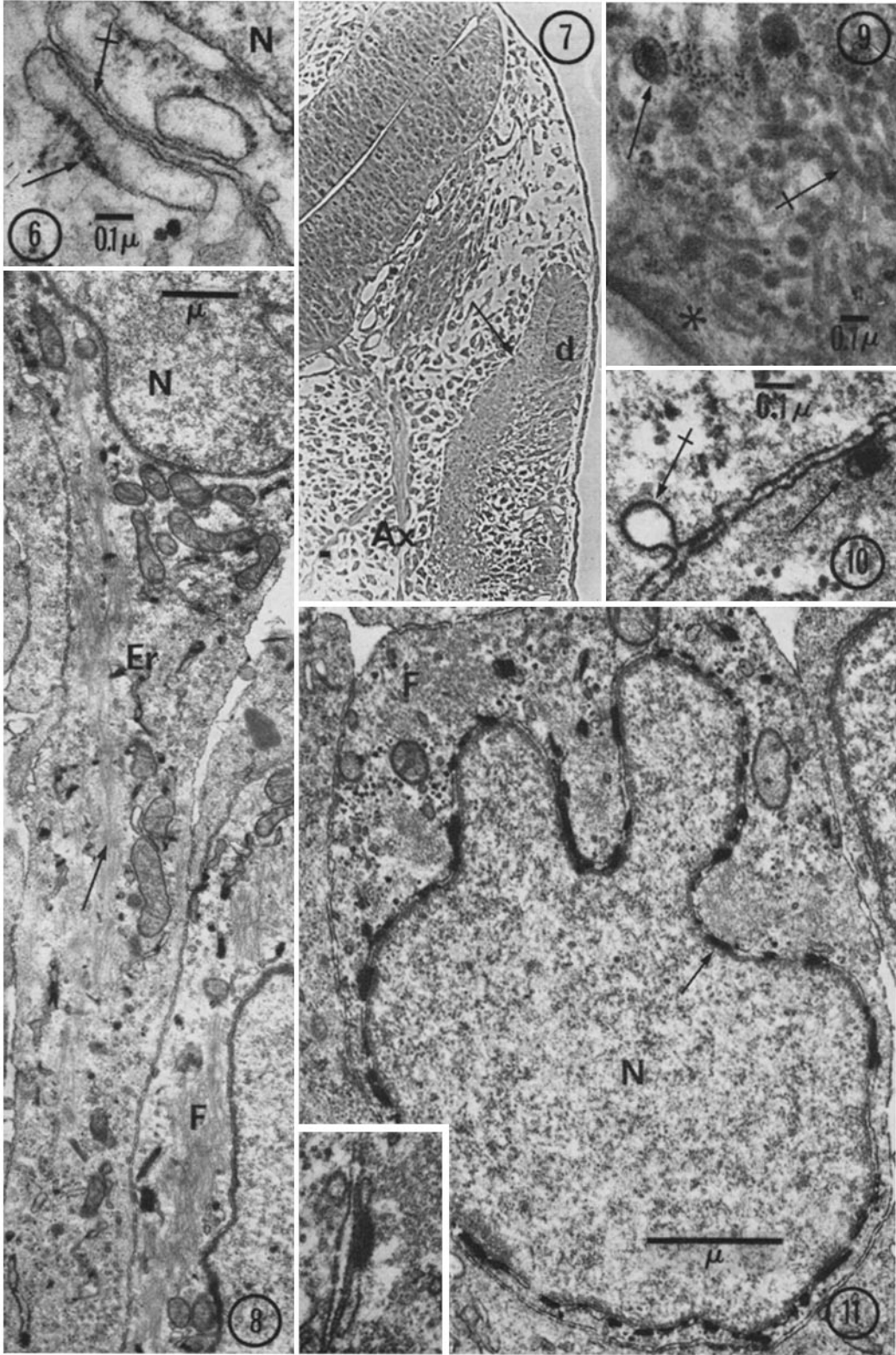
FIGURE 7 Phase micrograph of the neural tube, dorsal root ganglion and dermomyotome just after the outgrowth of the spinal nerve. The myoblasts (ventral to arrow) show considerable differentiation and enzyme activity. The dorsal edge of the myotome is relatively undifferentiated. Dermatome (*d*). The spinal nerve (*Ax*) runs about 20μ away from the tip of the ventral edge of the myotome. Day 11 of gestation. Scale marker: $10 \mu \times 150$.

FIGURE 8 A greatly elongated myoblast at day 12 of gestation shows a partial alignment of filaments in association with Z-line material (arrow). Some filaments (*F*) are still randomly oriented. The flattened cisternae (*Er*), containing cytochemical end product, probably correspond to ribosome-studded endoplasmic reticulum. Nucleus (*N*). AThCh 50 min. Scale marker: $1 \mu \times 11,600$.

FIGURE 9 Dense ovoid (arrow) and elongate bodies (crossed arrow) fill the tip of the myoblast which projects into the intersegmental septum. There is an increased density of the cell membrane and an undercoating of finely filamentous material (*). A basal lamina coats the exterior. Day 11 of gestation. Osmium postfixation. Scale marker: $0.1 \mu \times 40,000$.

FIGURE 10 Invaginations of the cell membrane (crossed arrow) lacking end product often have elongated connections to the surface. Enzyme-active subsurface reticulum (arrow) is present in the adjacent filamentous myoblast. Day 12 of gestation. AThCh, 50 min. Scale marker: $0.1 \mu \times 60,000$.

FIGURE 11 A considerable amount of end product (arrow) is present in the nuclear envelope of the deeply indented myoblast nucleus (*N*), and in a few cisternae in the cytoplasm. Bundles of filaments (*F*) are cut in cross section. Scale marker: $1 \mu \times 21,600$. *Inset*: cytochemical end product is in narrow channels, some of which are closely apposed to the surface membrane of the peripheral pole of the myoblast. Day 12 of gestation. AThCh, 50 min. $\times 41,600$.



these structures contain a moderately dense material, but a clear zone is often seen beneath the membrane of the larger ovoid bodies. Filamentous material (*) undercoats the surface membrane. Invaginations of the cell membrane to form caveolae (Fig. 10, crossed arrow) are common. Some of them exhibit elongated connections with the surface.

The nucleus (Figs. 8 and 11, *N*) of these greatly elongated myoblasts frequently exhibits deep indentations. The nuclear envelope often contains more end product of AChE activity (Fig. 11, arrow) than at earlier periods. Cytochemical end product can be seen from time to time in the Golgi complex of the more differentiated myoblasts. Enzyme-active reticulum is found in the central cytoplasm (Fig. 8, *Er*) and beneath the cell membrane (Fig. 10, arrow; Fig. 11, *inset*). Caveolae invaginating from the cell membrane (Fig. 10, crossed arrow) lack end product of AChE activity as do many other vesicles and saccules of unknown origin.

LATE MYOBLAST-EARLY SARCOMERE STAGE: The myotome consists of greatly elongated spindle-shaped myoblasts (Fig. 12, *m*) and other mesodermal cells by day 13. Very little, if any, dermatomal epithelium or epithelioid elements of the dorsal edge are evident, but there are many mesodermal cells between the myoblasts which lack end product of AChE activity. Other mesenchymal-like cells which contain the enzyme will be discussed in the next section. The more advanced myoblasts show early sarcomere formation (Figs. 13 and 14, *S*).

The elongated nuclei of the myoblasts are frequently indented; and they have end product of AChE activity in the nuclear envelope (Fig. 13, *Ne*). A few tubules of the Golgi complex (arrow) also exhibit end product at this stage. As in earlier stages, the endoplasmic reticulum (*Er*) in the perinuclear region, as well as the reticulum beneath the surface membrane of more distal parts of the cell (Fig. 14, arrow), have cytochemical activity. It is likely that portions of the enzyme-active reticulum which are aligned parallel to the sarcomeres have begun to differentiate into sarcoplasmic reticulum (*sr*), since corresponding channels in osmium-fixed tissue are smooth surfaced. As in the preceding stage, caveolae lacking end product are common, but there is little or no evidence of extensive T-system formation as yet.

A Second Type of Myotomal Cell

A cell type distinct from the myoblast is recognizable in the myotome as early as day 10 of gestation, but is more common by day 11. It is first found bordering the intersegmental septum close to the ventral margin of the myotome (Fig. 15, arrows). In the more differentiated myotome, it is present along the length of the septum and also between the myoblasts in deeper portions of the myotome, but it appears to be absent from the dorsal edge (*D*) of the myotome proper. Morphologically, this cell resembles the relatively undifferentiated mesenchymal cell described by others (Bergman, 1962; Hay, 1963; Allen and Pepe, 1965; Dessouky and Hibbs, 1965; Przybylski and Blumberg, 1966). It has a large ovoid nucleus (Fig. 16, *N*), prominent nucleolus, and scant perikaryal cytoplasm. There are few organelles, but sometimes a centriole is present in a Golgi region. The cytoplasm lacks filaments, but is filled with polyribosomes in osmium-fixed tissue. The endoplasmic reticulum is sparse. Junctions are not obvious in permanganate-fixed tissue in which this cell can most readily be identified. The cell varies from early undifferentiated mesoderm and mesenchymal cells elsewhere in that it exhibits AChE activity. The distribution of the enzyme, however, is different from the membrane-bound pattern seen in the nonfilamented and filamented myoblasts. The end product in the second type of myotomal cell is scattered throughout the nucleus and cytoplasm in a random manner (arrows), suggesting that the enzyme or the end product may be loosely bound in these cells.

Near the end of day 10 and the early part of day 11, small groups of free cells, which resemble the second type of myotomal cell, are found in the ventral portion of the intersegmental septum (Fig. 15,*) close to the walls of the blood vessel (*Bv*). During the latter part of this period, similar cells are found deeper within the septum (arrowhead), and by day 12 they are spread throughout the septum. These cells have large nuclei (Fig. 17), scant cytoplasm, and few organelles. Cytochemical end product (arrows) is scattered throughout the nucleus and cytoplasm. It is common to find random end product in neighboring cells in mitosis. As will be pointed out in the discussion, it is likely that the mesenchymal-like cell containing AChE activity originates from the second type of myotomal cell.

The second type of myotomal cell and its probable derivative, the enzyme-containing cell free in

the septum, seem to have developed a very high level of AChE activity after day 11, since short incubation periods are optimal for demonstrating the end product within the cell (Fig. 17). With

somewhat longer incubation times, i.e. periods appropriate for demonstrating AChE in myoblasts, the cytochemical end product diffuses into the connective tissue space of the septum (Fig. 15,

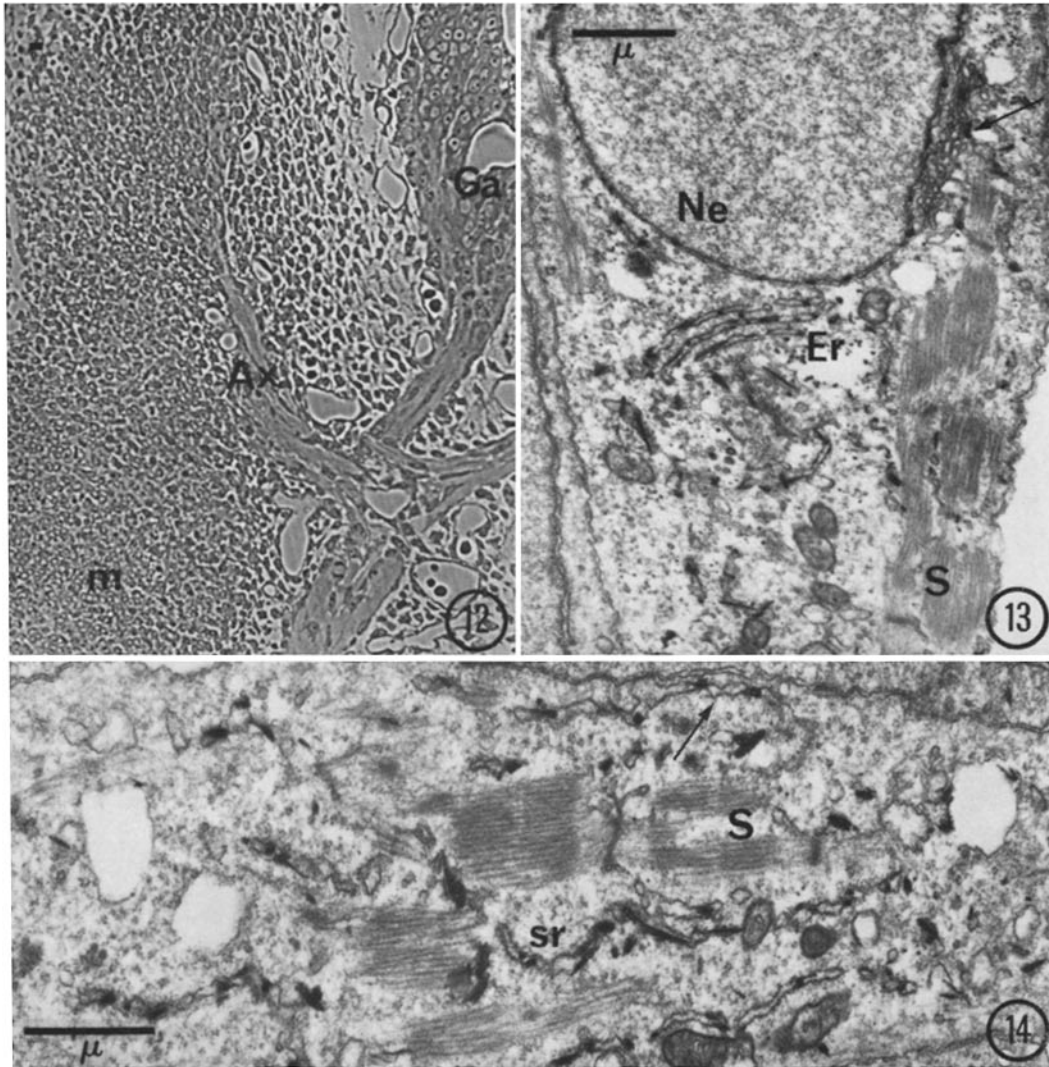


FIGURE 12 A phase micrograph of the myotome during day 13 of gestation showing the cranial-caudal orientation of myoblasts (*m*) and mesenchymal-like cells. Epithelioid cells are no longer evident. The posterior ramus of the spinal nerve (*Ax*) closely invests the dorsal medial surface of the myotome and penetrates the intersegmental septum. Dorsal root ganglion (*Ga*). Scale marker: 10 μ . \times 165.

FIGURE 13 End product of AChE activity is present in the nuclear envelope (*Ne*), endoplasmic reticulum (*Er*), and a few tubules of the Golgi complex (arrow). Developing sarcomeres (*S*) in myoblast at day 13 of gestation. ATHCh + iso-OMPA, 25 min. Scale marker: 1 μ . \times 13,200.

FIGURE 14 End product of AChE activity is present in the sarcoplasmic reticulum (*sr*), which is longitudinally oriented with respect to the developing sarcomere (*S*). Some reticulum (arrow) is closely apposed to the cell membrane. Day 13 of gestation. ATHCh + iso-OMPA, 25 min. Scale marker: 1 μ . \times 17,400.

crossed arrow) and into the distal processes of the myoblasts bordering it. The second type of myotomal cell contains somewhat more endoplasmic reticulum by day 13 (Fig. 18, *Er*), but the cytochemical end product (arrows) remains dispersed. The cells at this stage are most commonly found adjacent to the distal poles of the myoblasts bordering the intersegmental septum and in groups within the septum, as well as within the myotome proper adjacent to myoblasts. A diffuse distribution of end product of AChE activity is also seen in other embryonic cells, such as developing red blood cells (Fig. 19) and Schwann cells (Fig. 20, *Sc*), differentiating along the spinal nerve after day 12 of gestation. AChE has been found biochemically in human red blood cells (Richter and Croft, 1942; Bellhorn et al., 1970).

Spinal Nerve

None of the cells of the neural tube at day 9 (Fig. 2, *inset*) have differentiated into neuroblasts. At day 10, i.e. the 29 somite stage, neuroblasts are present in the ventrolateral neural tube (Fig. 3, crossed arrow), but only occasionally are there neurites which extend into the mesenchyme. These axons are about 20 μ in length at most, and do not join the short cytoplasmic prolongations of the loosely arranged spindle-shaped neuroblasts, which are forming the presumptive dorsal root ganglion. After the middle of day 10, i.e. the 34 somite stage, the motor axonal processes are longer and they

ramify within the mesenchyme just medial to the myotome. Some axons have been traced to within 10–20 μ of the central area of the myotome, i.e. the nuclear zone of the longitudinally oriented myoblasts, but then the axons turn and run ventrally, forming a distinct motor root by day 11 (Fig. 7, *Ax*). No axonal-myoblast contacts have been observed. The peripheral axons of the dorsal root ganglia are rudimentary until day 11 $\frac{1}{4}$. They join the motor root, forming a distinct spinal nerve by day 11 $\frac{1}{2}$. Some axons wander in the mesenchyme close to the myotome, but do not appear to enter it. A few axons of the posterior ramus enter the medial entrance of the intersegmental septum at day 12 $\frac{1}{4}$. A well-formed nerve (Fig. 12, *Ax*) penetrates the medial and dorsal edges of the myotome, as well as the septum from day 12 $\frac{1}{2}$ to 13. Presumptive Schwann cells separate the nerve from the bulk of the myotomal cells, but it is not known whether scattered exploratory nerve-muscle contacts occur or not. Most of the cells in the dorsal edge of the myotome during this period resemble mesenchymal cells and lack cytochemical end product.

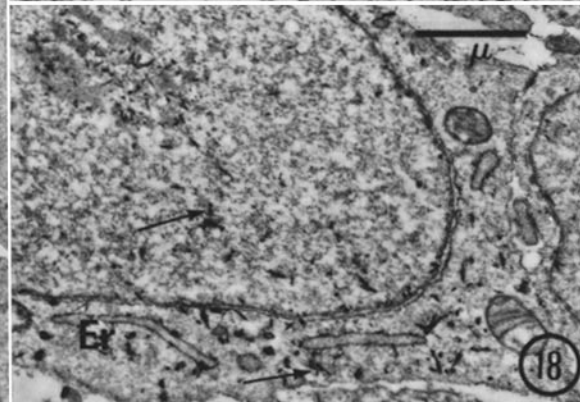
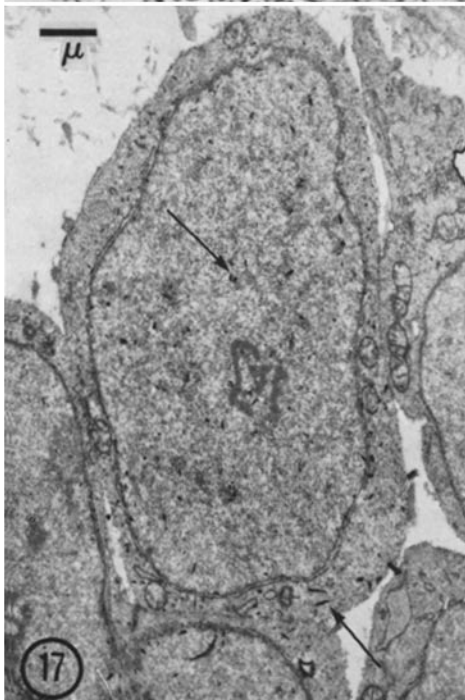
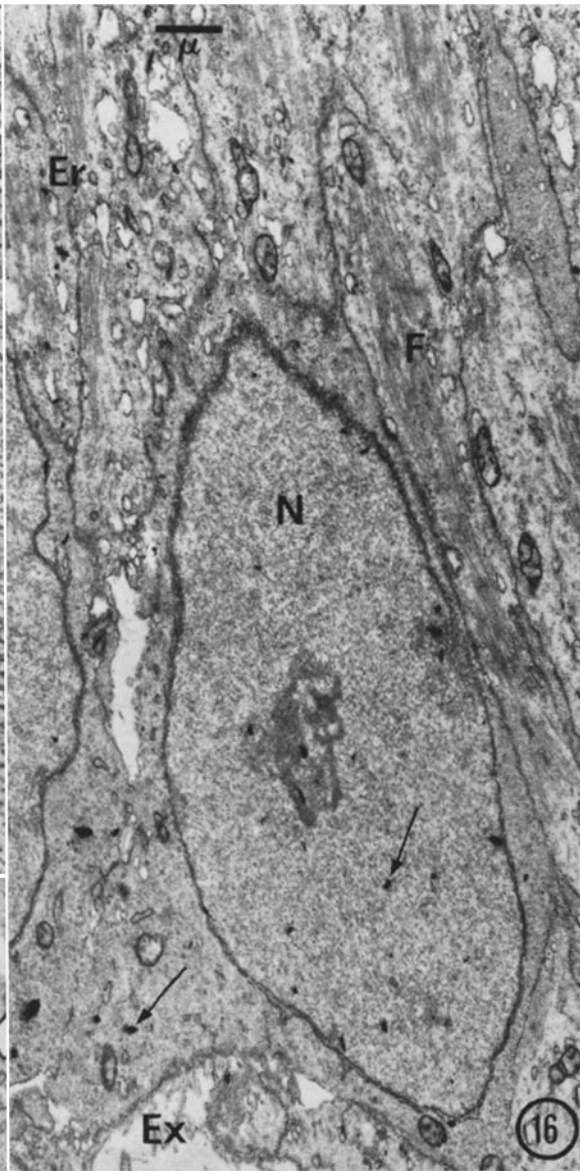
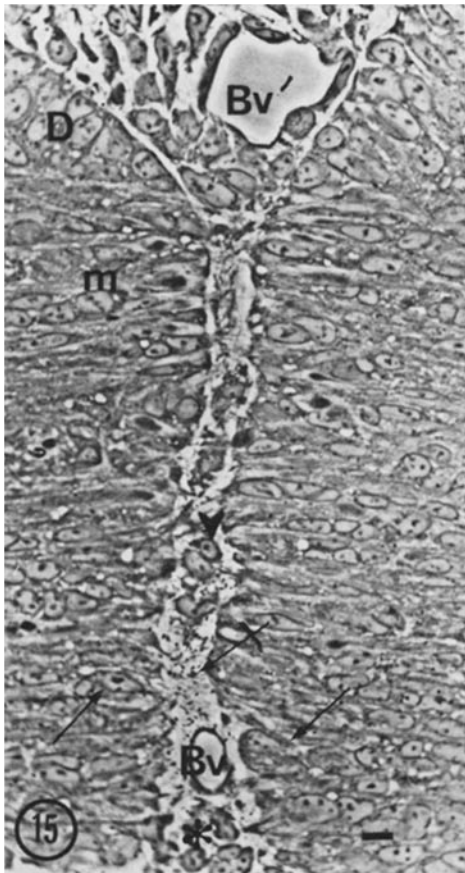
A detailed account of the development of AChE in the spinal nerve will be the subject of another paper, but the following data will be given since contamination of the myotomal sample by AChE-containing nerve may affect the biochemical results. There is very little AChE activity in the early nerve fibers (Fig. 21, *Ax*) or Schwann cells

FIGURE 15 Phase micrograph of a longitudinal section of portions of two myotomes and the intersegmental septum at day 11 $\frac{1}{4}$ of gestation. Elongated filament-containing distal processes of the myoblasts (*m*) are evident. A second type of myotomal cell (arrows) border the ventral portion of the septum. These cells, as well as free cells (*) which appear first near the entrance of the capillary (*Bv*) and later within the septum (arrowhead), are the source of the cytochemical end product (crossed arrow) in the connective tissue area. No end product is present in the dorsal edge of the myotome (*D*), or in the dorsal portion of the septum or around the distal extension of the capillary (*Bv'*). AThCh + iso-OMPA, 20 min. Scale marker: 10 μ . \times 400.

FIGURE 16 A second type of myotomal cell having a large nucleus (*N*), scant cytoplasm, and few organelles borders the extracellular space (*Ex*) close to the ventral portion of the intersegmental septum. Cytochemical end product (arrows) is randomly distributed throughout the nucleus and cytoplasm of these cells. In the filamented (*F*) myoblast, end product is most frequently associated with the reticulum (*Er*). Day 10 $\frac{1}{2}$ of gestation. AThCh + iso-OMPA, 40 min. Scale marker: 1 μ . \times 8700.

FIGURE 17 A group of cells within the intersegmental septum that closely resemble the AChE-containing second type of myotomal cell. Cytochemical end product (arrows) is scattered throughout the nucleus and cytoplasm. Day 11 $\frac{1}{2}$ of gestation. AThCh + iso-OMPA, 10 min. Scale marker: 1 μ . \times 7200.

FIGURE 18 Mesenchymal-like cells containing random end product (arrows) of AChE are present in the intersegmental septum at day 13. Elongated channels of endoplasmic reticulum (*Er*) are evident. AThCh + iso-OMPA, 25 min. Scale marker: 1 μ . \times 14,400.



which course past the myotome during day 11 (Fig. 7, *Ax*). Only occasionally has end product been seen, and that was found in the agranular reticulum of growth cones. When enzymic activity develops in the spinal nerve, it seems to appear in a distal to proximal direction, as was found in the dorsal root (Tennyson and Brzin, 1970). The Schwann cells exhibit a significant amount of cytochemical end product by day 12½ (Fig. 20, *Sc*),

but the reaction is diffuse. AChE activity can be seen at the surfaces of some axons (Fig. 22, arrows) at day 13, but the Schwann cells are more consistently active. It is likely that the axons and Schwann cells containing AChE activity in the spinal nerve are derived from the sensory nerve. The peripheral nerve of the dorsal root ganglion exhibits AChE activity at day 13, but the anterior root of the spinal cord does not.

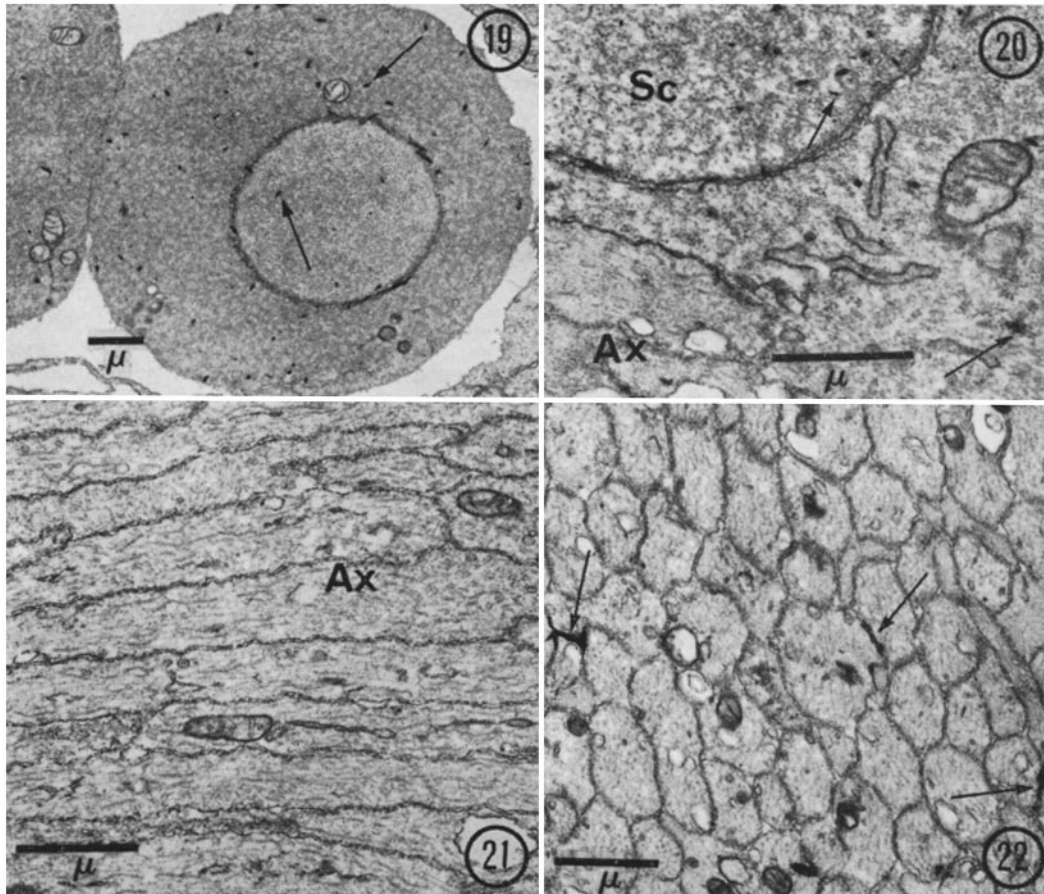


FIGURE 19 Developing red blood cells also show a random distribution of end product of AChE activity (arrows). Day 11 of gestation. AThCh + iso-OMPA, 45 min. Scale marker: 1 μ . \times 6300.

FIGURE 20 Random end product (arrows) is present in the nucleus and cytoplasm of many of the Schwann cells (*Sc*), in sensory nerve fibers joining the spinal nerve at day 12½. The axons (*Ax*) less frequently contain cytochemical activity. AThCh + iso-OMPA, 15 min. Scale marker: 1 μ . \times 18,600.

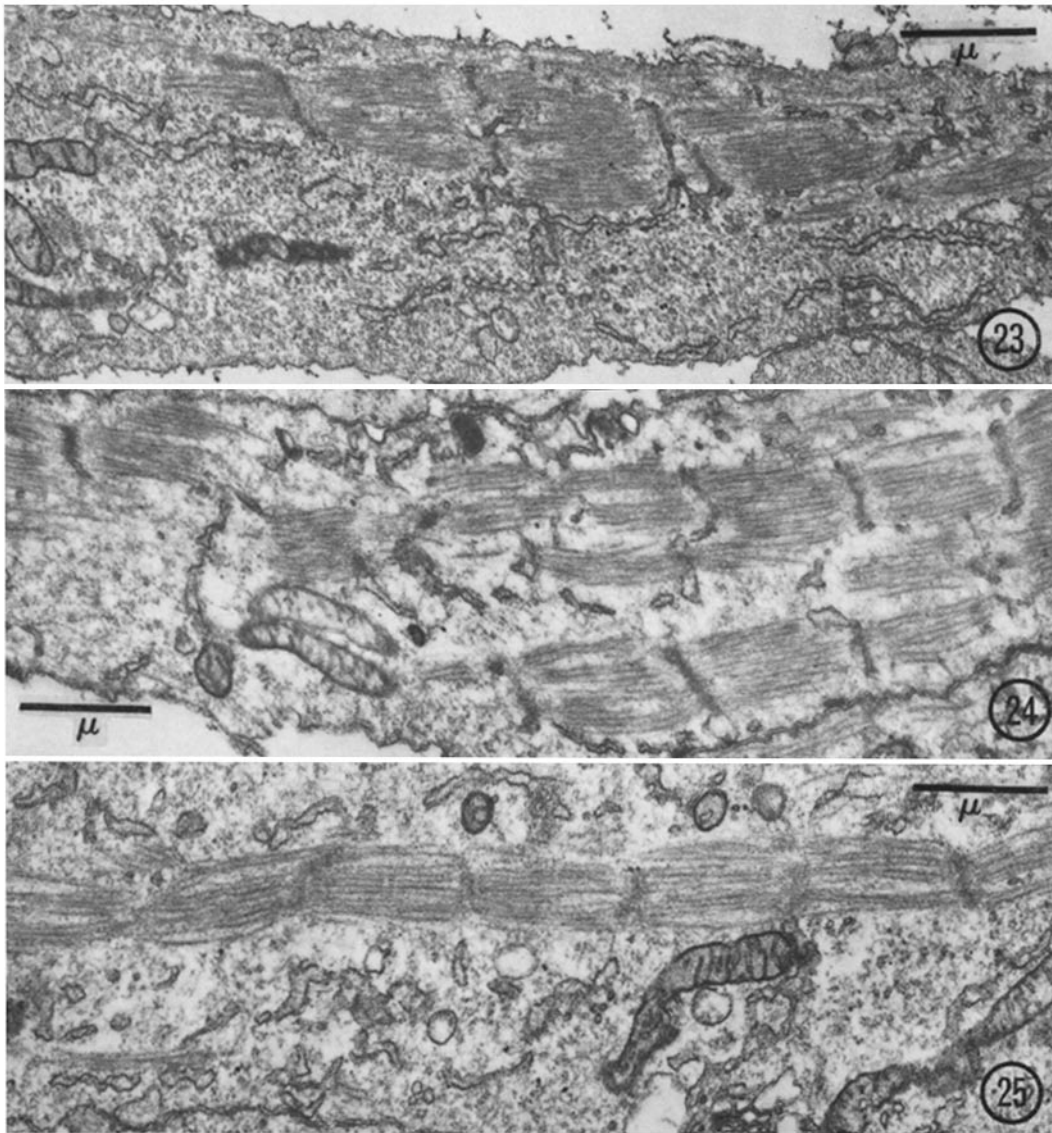
FIGURE 21 Axons (*Ax*) of the spinal nerve passing within 20 μ of the somite at day 11 lack cytochemical end product. AThCh + iso-OMPA, 20 min. Scale marker: 1 μ . \times 15,900.

FIGURE 22 Some axons of the posterior ramus of the spinal nerve, which are closely associated with the myotome at day 13, have some end product of AChE activity at their surfaces (arrows). Most of the axons lack end product. AThCh + iso-OMPA, 30 min. Scale marker: 1 μ . \times 13,500.

Control Studies

The specificity of the enzyme was examined cytochemically in specimens from day 9 through day 13 of gestation. Myoblasts, the second type of

myotomal cell, and spinal nerve reacted similarly at all ages studied. The myoblast of the 13 day fetus was selected to illustrate the control studies since it consistently contains end product after incubation in AThCh and it is readily recognized



FIGURES 23–25 show the effect of inhibitors on AChE activity in the reticulum of elongated processes of the late myoblast at day 13 of gestation. Scale marker: 1 μ . \times 17,400.

FIGURE 23 Cytochemical activity is inhibited by treatment with BW 284 C 51. AThCh + BW 284 C 51, 25 min.

FIGURE 24 Cytochemical activity is inhibited by treatment with eserine. AThCh + eserine, 25 min.

FIGURE 25 Cytochemical activity is inhibited after incubation in BuThCh + BW 284 C 51 for 25 min.

TABLE I
AChE Activity of the Dermomyotome Expressed as $\mu\text{M ACh} \times 10^{-4}/\text{hr}$ per Dry Weight of Tissue ($\pm\text{SD}$)

Gestation day				
9	10	11	12	13
0.31 \pm 0.07 (n = 7)*	1.13 \pm 0.38 (n = 7)	2.69 \pm 0.54 (n = 8)	10.29 \pm 0.80 (n = 5)	14.52 \pm 1.81 (n = 5)

* n = number of experiments.

by its filaments. The second type of myotomal cell, on the other hand, can only be distinguished from a mesenchymal cell by the presence of end product of AChE activity. The statement on the effect of inhibitors on these cells, therefore, is based on the absence of end product in mesenchymal-like cells in the specific areas of the myotome and inter-segmental septum where these cells are normally found.

There is little or no effect on enzymic activity when specimens are treated with iso-OMPA (2×10^{-4}), an inhibitor of nonspecific ChE. The distribution and the relative number of deposits of cytochemical end product (Fig. 14) is the same as in experiments with AThCh without inhibitors. There was inhibition of cytochemical activity when the tissue was incubated in AThCh in the presence of either BW 284 C 51 (2×10^{-4} M), an inhibitor of AChE (Fig. 23), or eserine (1×10^{-4} M), an inhibitor of both specific and nonspecific ChE (Fig. 24). Preincubation for 1 hr of 60 μ sections in the medium without substrate, but containing the inhibitors, is necessary to effect complete inhibition of the enzyme. Cytochemical end product was occasionally observed when specimens were incubated with BuThCh (3.1×10^{-3} M) as substrate, but these deposits are abolished by the use of BW 284 C 51 (Fig. 25). There was no precipitate if the tissue was incubated in the copper maleate buffer medium without the substrate.

Attempts made to alter the diffuse distribution of end product in the second type of myotomal cell had no effect on its localization. The use of high concentrations of sodium sulfate in (a) a saline or buffer perfusate before fixation, (b) in the perfusing fixative itself, or (c) in the incubation medium caused tissue shrinkage but did not confine the end product to membranes. The random distribution of end product is present in both unfixed and fixed tissue. It is not present if AThCh is omitted from the incubation medium, and thus the precipitate cannot be due to a nonenzymatic binding of copper (Langley and Landon, 1969).

Quantitative Studies

Chemical measurements for AChE activity in somites taken from 16 to 19 somite embryos at day 9 of gestation are low (Table I). Enzymic activity has increased almost fourfold by day 10 and almost ninefold by day 11. A sharp rise in AChE activity occurs during day 12, and it reaches a peak at day 13, when enzymic activity is almost 50-fold that of day 9.

DISCUSSION

AChE activity has been demonstrated in the nuclear envelope and in elements of the endoplasmic reticulum of the myoblast of the rabbit embryo by use of the substrate AThCh in conjunction with selective inhibitors. Although it is not possible to distinguish with certainty between rough and smooth reticulum in permanganate-treated specimens, study of osmium-fixed tissue shows that most of the reticulum during early myoblast development is studded with ribosomes. In later stages some of the reticulum is devoid of ribosomes, particularly that closely associated with developing sarcomeres. Although the Golgi complex begins to show a small amount of cytochemical end product in later stages, the preponderance of AChE activity first in the endoplasmic reticulum then in areas known to contain smooth reticulum supports the evidence showing that the sarcoplasmic reticulum is derived from the endoplasmic reticulum (Ezerman and Ishikawa, 1967). It should be pointed out that cytochemical end product is abundant in the honeycomb structures clearly identifiable as sarcoplasmic reticulum in myotubes (unpublished observations). Study of osmium-fixed tissue also shows that the enzyme-containing subsurface reticulum is derived from the rough surfaced reticulum rather than from the T system.

Ezerman and Ishikawa (1967) and Ishikawa (1968) have presented evidence showing that T-tubule formation begins *in vitro* in the myotube

stage. There is an inpocketing of the sarcolemma to form a caveola, which resembles a micropinocytotic vesicle having a tunnel-like connection to the surface. Then secondary caveolae extend from the inpocketing and the structure becomes branched. A well-formed, branching T system is not apparent in the present study of myoblasts before fusion, although there are numerous vesicles of unknown origin in the cytoplasm. Caveolae are present at all stages, so it is likely that many of them are pinocytotic vesicles. Caveolae are encountered which exhibit an elongated neck suggestive of T-tubule formation. End product has not been found in any of the caveolae. A study of later stages of development is necessary, therefore, to determine whether the T system has AChE activity.

Second Type of Myotomal Cell

A second type of myotomal cell which exhibits a poorly localized cytochemical reaction for AChE has been described in the intersegmental septum and along the medial border of the myotome with the septum. It is derived from the mesodermal somite, as is the nonfilamented myoblast, but it differentiates slightly later. The second type of myotomal cell differs from the nonfilamented myoblast in that it lacks primitive desmosomal-like junctions, exhibits a random end product of AChE activity, and initially appears only in a localized part of the myotome, i.e., along the ventral border of the septum. Similar cells containing random end product are found along the surfaces of myotubes at later stages (unpublished observations) and also in dystrophic muscle (Miranda, 1971). Fibroblasts lack the reaction product. The second type of myotomal cell, therefore, may be the precursor of the cell associated with myotubes and referred to as the satellite cell (Muir et al., 1965; Ishikawa, 1966; Church, 1969) or undifferentiated cell (Kelly and Zacks, 1969), and may ultimately be related to the satellite cell of mature muscle (Mauro, 1961).

It is not possible to state with the present technique the precise intracellular localization in vivo of AChE in the second type of myotomal cell. Attempts made to limit diffusion artifact were unsuccessful. Koelle and coworkers (1970) have discussed the possibility that differences with respect to solubility or diffusibility of this enzyme at motor end plates as opposed to autonomic ganglia, might be explained by the recent findings of isoenzymes of AChE (Bernsohn et al., 1962; Eränko et al., 1964; Ecobichon and Israel, 1967).

Histochemical studies (Eränko et al., 1964; Koelle et al., 1970) have dealt with the question of tissue-bound esterases vs. diffusible esterases. Eränko et al. (1964) have distinguished desmesterases, which are tightly bound and are demonstrable by histochemistry, and lyoesterases, which are readily solubilized in fresh tissue sections, but are immobilized by formalin fixation. The random cytochemical end product in the second type of myotomal cell, the developing red blood cell, and the developing Schwann cell in this study and in a study of the dorsal root (Tennyson and Brzin, 1970) suggests that the enzyme in these cells may be closely related to a lyoesterase. Although a certain amount of the enzyme is retained by fixation, the solubility of the enzyme or the diffusibility of its end product is so great that its ultrastructural localization may be meaningless. The electron microscope does show, however, that this AChE-containing cell from the myotome is distinct from the myoblast in which the enzyme appears to be more tightly bound to membranes.

It is probable that the enzyme-active mesenchymal cells in the septum are derived from the second type of myotomal cell. Although they aggregate around the endothelial cells, it is unlikely that they originate from the wall of the blood vessel. Pericapillary cells around the same blood vessel in the dorsal portion of the septum lack the end product during the early stage. Later, after the second type of myotomal cell differentiates along the dorsal margins of the septum, the enzyme-active mesenchymal cell can be found in the adjacent intersegmental area.

Chemical Measurements

The cytochemical results as diagrammed in Fig. 1 have shown that AChE is not distributed uniformly throughout the tissue studied. The biochemical measurements, therefore, represent the enzymic activity in the dermomyotome as a whole, but give little information concerning the amount of enzyme within the individual cells. At day 10, there is a considerable amount of cytochemical activity in a few myoblasts in the middle and ventral myotome, but the rest of the myotome and the dermatome are inactive. The values for day 10 are rather low because the sample consists of a sparse population of AChE-containing myoblasts and many inactive dermatomal cells. The marked increase in biochemical values during days 11, 12, and 13 is in part due to the larger number of differentiated myoblasts, but probably

even more to the second type of myotomal cell, which shows intense histochemical activity. The spinal nerve may also have contributed to the measurements of AChE activity in the myotome at day 13. Although care was taken to remove all of the spinal nerve visible to the eye, it is possible that microscope nerve fibers and sheath cells, which now contain AChE activity, may have been adherent to the sample measured biochemically.

Nerve-Muscle Interaction

A direct contact between nerve and muscle has been postulated as a necessary prerequisite for the formation of AChE at the neuromuscular junction (Mumenthaler and Engel, 1961; Zelená, 1962; Lentz, 1969), but differentiation of muscle is not directly dependent on innervation (Zelená, 1962). The myoblast, moreover, can form intracellular AChE at very early stages of development in the absence of nerves as indicated by light microscope studies (Zacks, 1954; Bonichon, 1957; Shen, 1958; Engel, 1961; Mumenthaler and Engel, 1961), and as confirmed here both cytochemically and biochemically. Even after nerve outgrowth, the formation of AChE within elements of the endoplasmic reticulum of the myoblast appears to be independent of direct nerve-muscle contact. There is a considerable amount of enzymic activity in the myotome by day 11, but the spinal nerve coursing within 10–20 μ of it does not appear to enter the myotome proper. The nerve itself does not exhibit AChE at this time, nor were motor end plates encountered. Using silver impregnation, Romanes (1941) noted that a short posterior primary ramus first penetrated the dorsal musculature of the rabbit during day 12, but no definite neuromuscular endings were seen until day 19. He noted, however, that reflex contraction on stimulation of the snout could be elicited by day 16½ and suggested that the earliest endings might not be impregnated by silver.

Visintini and Levi-Montalcini (1939) have demonstrated fine argentophilic nerve fibers directed toward the medial surface of the myotome of the chick embryo of 68–96 hr. This period would correspond roughly to days 10½–12 in the rabbit embryo. Since motor end plates are not present at the end of day 4 in the chick, Visintini and Levi-Montalcini (1939) postulated the existence of provisional contacts between terminal boutons

and myoblasts to explain spontaneous movements in myoblasts. They also showed inhibition of these movements by curare. Hamburger (1963) has confirmed the observations concerning curarization and assumes that the earliest motility in the embryo is neurogenic. Engel (1961) has pointed out that the presence of spontaneous movements in young embryos does not necessarily demonstrate functional neuromuscular transmission of stimuli, since Li et al. (1959) have found strong and rhythmic spontaneous contractions of chick embryo skeletal muscle in tissue culture in the absence of innervation. Hagopian et al. (1970) have shown that AChE is present in the reticulum of spontaneously contracting cardiac muscle before the formation of T tubules or the ingrowth of nerve fibers. It was suggested that an acetylcholine (ACh)-ChE system of myogenic origin may be involved in ionic events underlying spontaneous contractions of cardiac muscle. Skeletal myoblasts exhibit similar conditions in early stages, i.e., AChE is present in the reticulum before the formation of T tubules and before neuromuscular contacts are made. An ACh-ChE system of myogenic origin may be postulated for spontaneous contractions in skeletal muscle, if future studies can demonstrate ACh within these cells.

On the other hand, if ACh is present within early axons, a neurogenic origin for spontaneous contraction may be possible even in the absence of direct nerve-muscle contact. ACh may be released from early neurites and diffuse to the myotome. This possibility has been suggested for a later stage in myotube development (Kupfer and Koelle, 1951). Burt (1968) has shown that choline acetyltransferase, the enzyme necessary for ACh synthesis, is present in the chick spinal cord after the middle of day 3. The onset of spontaneous activity in the chick occurs at this time (Hamburger et al., 1965). Although there is AChE activity in the cell bodies of the motor horn of the chick, this enzyme seems to be absent from the ventral root fibers during their early outgrowth (Gerebtzoff, 1959). The latter observation was confirmed in the present study of the rabbit embryo. Since there is little or no AChE in the early neurites to inactivate ACh and the axons are incompletely ensheathed, there would be no barrier to the diffusion of ACh 10–20 μ through the mesenchyme to the neighboring myotome. The AChE-containing subsurface re-

ticulum in the myoblast might serve as a site of inactivation of ACh. This reticulum closely resembles subsurface cisternae in the neuron (Rosenbluth, 1962). Rosenbluth (1962) has suggested that the close apposition of these cisternae to the cell membrane may change the properties of the neuronal plasmalemma, possibly altering its permeability and affecting electrophysiological behavior in these regions. A similar mechanism may operate in the myoblast before the formation of an extensive T system. AChE in the subsurface reticulum may play a role in spontaneous contraction of the myoblast as part of an ACh-receptor protein-AChE system. Nachmansohn (1970) has suggested that ACh may induce a conformational change in the receptor protein thereby releasing previously bound calcium ions, which are involved in the excitability of muscle. The AChE rapidly hydrolyzes ACh, thereby permitting the return of the receptor protein to its original conformation and reestablishing the barrier for ion movements (Nachmansohn, 1970).

However tenable the theory of free diffusion of ACh from early neurites to myotomes may be, one must still take into account the recent evidence from electrophysiological studies of developing neuromuscular junctions in culture (Robbins and Yonezawa, 1971). This investigation indicates that chemical transmission begins about the time when nerve-muscle contacts are first visible by light microscopy. Stimulation of spinal cord fragments or nerve fibers results in excitatory junctional potentials in innervated myotubes. No response is detected in noninnervated myotubes, even though they are sensitive to iontophoretically applied ACh. These data seem to show that free diffusion of ACh does not occur through the culture at this stage after stimulation. The absence of free diffusion of ACh can most easily be explained by assuming that the nerve fibers in the culture have matured, and are probably ensheathed and have AChE activity at their junctions. The study by Robbins and Yonezawa (1971), which depends on the response of motor end plates to demonstrate ACh release, cannot be expected to provide information on a much earlier stage of axonal-myoblast interaction. On the other hand, their evidence that noninnervated myotubes are sensitive to iontophoretically applied ACh is pertinent to the previous discussion of a possible mechanism for spontaneous contraction involving AChE-containing subsurface

reticulum and ACh diffusing from sparsely sheathed, AChE-free neurites. AChE-containing subsurface reticulum and sarcoplasmic reticulum are prominent in early myotubes, but decrease in later myotube stages before motor innervation (unpublished observations). This may suggest that the mechanism for spontaneous activity is no longer operational and has been replaced by another.

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