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An Ultrastructural Study of Degeneration and Necrosis of Muscle in the Dystrophic Mouse*

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Summary. Ultrastructural and histochemical features of three muscle fiber changes—branched fibers, large fibers and necrotic fibers—have been studied in Bar Harbor strain 129 dystrophic mice. The pathogenesis of these changes, which are considered to be responses to injury, have been discussed. Although these changes are not regarded as specific, they are characteristic of the histopathology of muscular dystrophy, so that an understanding of the mechanisms which produce these abnormal muscle fibers should contribute to the knowledge of the dystrophic process.

Zusammenfassung. Die ultrastrukturellen und histochemischen Merkmale von drei Muskelfaserveränderungen — Faserverzweigung, große Fasern und nekrotische Fasern — wurden an 129 dystrophischen Bar Harbor-Mäusen untersucht. Die Pathogenese dieser Veränderungen, die als Schädigungsfolgen aufgefaßt werden, wird diskutiert. Obwohl diese Veränderungen nicht als spezifisch angesehen werden, sind sie doch charakteristisch für die Histopathologie der Muskeldystrophie, so daß das Verständnis des Mechanismus, der diese abnormen Muskelfasern hervorbringt, zur Kenntnis des dystrophischen Prozesses beitragen dürfte.

Key-Words: Ultrastructure — Degeneration — Necrosis of Muscle — Dystrophy.

The skeletal muscles of Bar Harbor 129 dystrophic mice show a number of histopathological changes, consisting of a striking variation in muscle fiber diameter and shape, increased numbers of sarcolemmal nuclei — many of them centrally placed, coagulation necrosis with phagocytosis, branching of muscle fibers, regeneration and increased amounts of connective tissue and fat (West and Murphy; Banker, 1961). These are much the same histologic changes that characterize the various forms of human muscular dystrophy. While it is generally agreed that phagocytosis and connective tissue proliferation are not specific features of muscular dystrophy, opinion differs as to the significance of the other changes, and the mechanism of formation of the large and small fibers has not been explained adequately (Adams *et al.*, 1962; Adams, 1969). In this report, the ultrastructural and histochemical features of the large and small fibers, the

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branching fibers, as well as fibers showing coagulation necrosis will be described, and the mechanisms involved in the genesis of these changes will be discussed.

Methods

Eighteen dystrophic and ten non-dystrophic animals from nine litters of Bar Harbor strain 129 mice, ranging in age from three to nine weeks, were studied. All affected animals showed muscle weakness and weighed less than their unaffected litter mates. For ultrastructural studies, the adductor muscles of the hind limbs were fixed in glutaraldehyde, post-fixed with osmium tetroxide and embedded in Epon-812, as previously described (Banker, 1967). Sections 0.5 to 1 micron in thickness, either unstained or stained with hematoxylin, Luxol-fast blue, or Nile blue Sulphate, were studied with phase microscopy. Some sections were incubated for acid phosphatase according to the method of Gomori (Lillie). Blocks containing abnormal fibers were trimmed so that the abnormal fibers that had been detected by phase microscopy could be studied by electron microscopy. Ultra-thin sections of these fibers were stained with uranyl acetate or lead hydroxide and studied with an RCA EMU-3F, RCA EMU-4A, or Philips EM 100 electron microscopes.

For histochemical studies, the adductor muscles of the hind limbs were quenched in isopentane and cooled to $-160°$ C with liquid nitrogen. Sections seven microns in thickness were prepared in a cryostat and incubated for the following enzyme activities: myosin-adenosine triphosphatase (myosin-ATPase), phosphorylase, succinic dehydrogenase, cytochrome oxidase and acid phosphatase. In addition, sections prepared in the cryostat were stained by the following techniques: hematoxylin-eosin, periodic acid-Schiff, Scharlach R, and Nile-blue sulfate.

Results

I. Phase Microscopy

In the dystrophic mice, muscle fibers varied from 3 to 150 micra in cross sectional diameter, whereas the diameters of fibers from the unaffected litter mates varied from 30 to 40 micra. Many muscle fibers from the dystrophic animals showed normal longitudinal and cross-striations. Sarcolemmal nuclei were unusually prominent and increased in number. Proliferation of endomysial and perimysial connective tissue and fat were conspicuous. Muscle fibers in all stages of maturity could be identified; myoblasts and myocytes frequently formed chains.

In every section in which the muscle fibers were visualized in the longitudinal plane, a division of a fiber into two, three or four small fibers was evident (Fig. 1). The master fiber contained large mitochondria and often a chain of centrally placed nuclei. The division frequently appeared in the central portion of the fiber. Some of the resultant fibers also divided centrally, creating in this way, many smaller fibres. In cross section, the branched fibers were readily recognized because of their proximity to each other as well as the precise way in which their contours approximated one another (Fig. 2). A small capillary was frequently identified between the branching fibers. Each group of small fibers appeared to have replaced one single large fiber.

Necrotic muscle fiber, occurring either singly or in groups of two or three, were identified by their coagulated, homogeneous appearance. Although such fibers retained the general cylindrical shape of normal muscle fibers, both longitudinal and cross striations wer absent. At the earliest stage of necrosis, few reactive cells were detected in the coagulated muscle fibers (Fig. 3). At later stages of necrosis, numerous mononuclear cells infiltrated the pale, necrotic

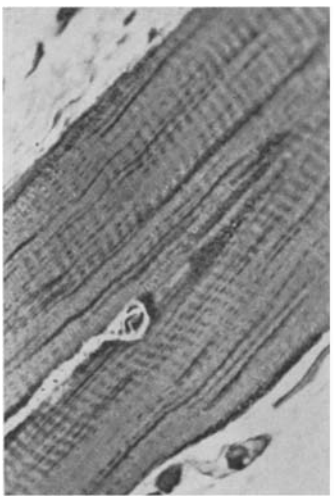

Fig. 1

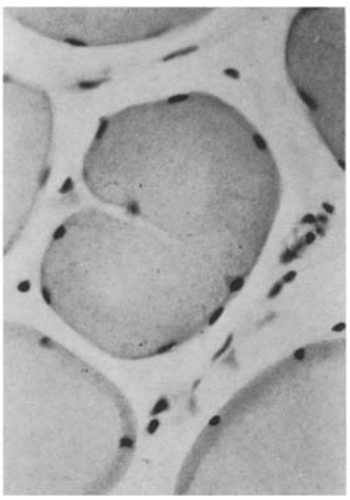

Fig. 2

Fig. 1. Master fiber branching into two smaller fibers. Phase Microscopy ×400

Fig. 2. In cross section the forked fibers approximate each other in shape. Phase microscopy ×400

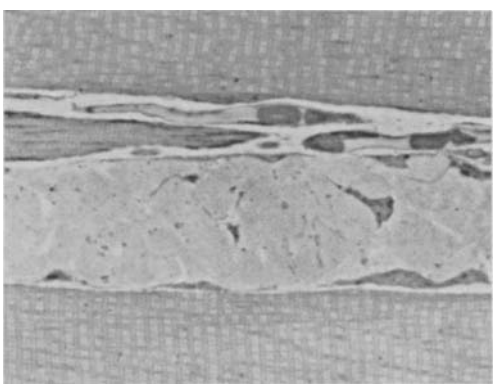

Fig. 3. Necrotic muscle fiber in longitudinal section showing absence of cross-striations and homogenization of myofibrils. Adjacent muscle fibers appear normal. Phase microscopy ×750

material and arranged themselves along its margins. Some of these cells were polygonal with scant cytoplasm and thin processes which projected into the necrotic material or along its margins; others, containing dense, round, osmophilic material, were clearly macrophages. Acid phosphatase activity was demonstrated only in the macrophage inclusions.

II. Histochemistry

Most small muscle fibers displayed increased activity when incubated for oxidative enzymes and decreased activity of myosin-ATPase and phosphorylase; their histochemical profile was that of Type I muscle fibers. On the other hand,

the largest muscle fibers frequently showed little oxidative enzyme activity but reacted strongly for myosin-ATPase and phosphorylase; their histochemical profile resembled Type II muscle fibers. In fibers resulting from longitudinal division, the oxidative enzyme, phosphorylase and myosin-ATPase activities were greatly diminished. In the necrotic muscle fibers, there was an absence of oxidative enzyme and glycolytic activity.

III. Electron Microscopy

The large muscle fibers, detected by phase microscopy, presented distinctly abnormal features when examined by electron microscopy. Many consisted of mature fibers to which were attached small primitive fibers in varying degrees of lateral apposition (Fig. 4). Their sarcolemmas could be seen in various stages of union. Vesicles and caveolae were prominent under the sarcolemma in these fibers. Other constituents of the mature component of the large fibers were normal. In some apposing fibers, the sarcolemmas were separated by a space containing membranous lamellar structures. There was interruption of both the plasma and basement membranes of the apposing muscle fibers bordering this cleavage space. The space between the apposing fibers was wide and frequently remained so beyond the point of separation.

Muscle fibers which had a forked or branched appearance by phase microscopy were examined by electron microscopy. The primary fiber possessed well preserved cross striations and the myofibrillar components were normal. At the point of cleavage in the center of the fiber, there was a space measuring $^1/_4$ to 1 μ. Within this space there were invariably one or more membranous lamellar structures (Fig. 5) and an occasional capillary. Frequently in the primary fiber the mitochondria and sarcoplasmic reticulum were unusually conspicuous at the point of separation. Pinocytosis was prominent under the sarcolemma of the branching fibers. Mitochondria were not enlarged nor increased in the small branched fibers. Otherwise, the constituents of these fibers were not remarkable.

In the necrotic fibers, pyknotic nuclei showing dense clumping of chromatin with relative preservation of the outer nuclear membrane were seen occasionally. Mitochondria were seen either collected in large aggregations (Fig. 6) or scattered throughout the necrotic fibrillar material (Fig. 7). Although many mitochondria appeared relatively normal, the matrix granules that are usually found in the organelles of normal muscle were not present in the necrotic fibers. Many mitochondria contained irregular, dense inclusions or vacuoles. Disintegration of the mitochondrial envelope and vesiculation of cristae occurred frequently.

The large areas of amorphous contractile elements, observed in the necrotic muscle fibers by phase microscopy, consisted mainly of irregular filamentous structures which were frequently clustered in masses of longitudinal strands (Fig. 7). In some areas the myofibrillar pattern was roughly retained while in other areas no filamentous structures could be identified. Instead, a fine granular or amorphous mass was all that remained. Cross striations could not be identified in any of the necrotic fibers.

Vacuoles and round dense structures lined by single membranes were frequently observed in the necrotic fibers (Fig. 6, 7). Although smaller than mitochondria, these structures varied in size. Some of the smaller vacuoles were

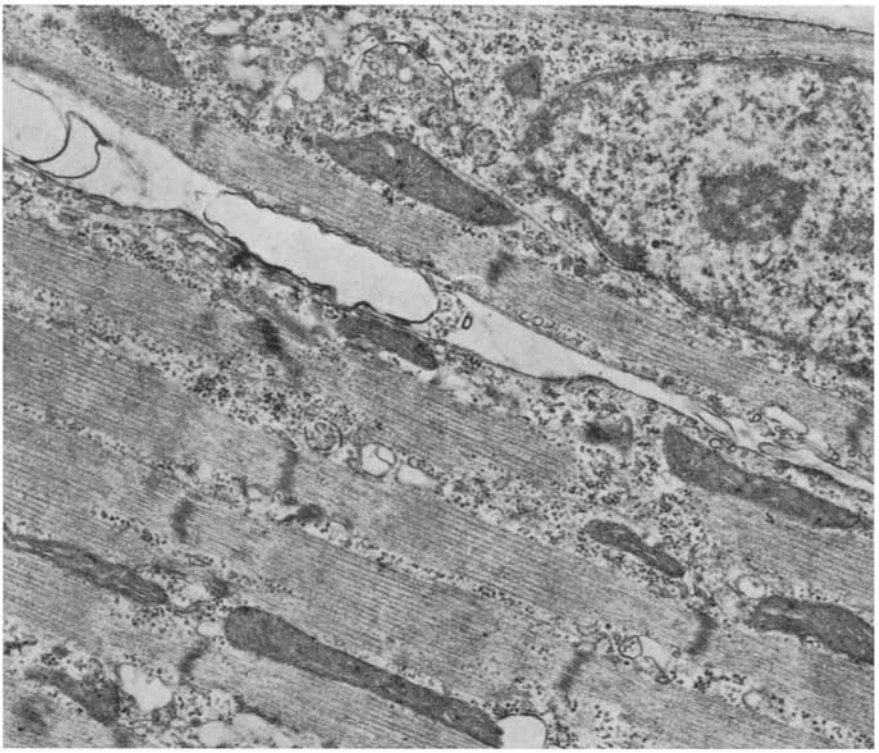

Fig. 4

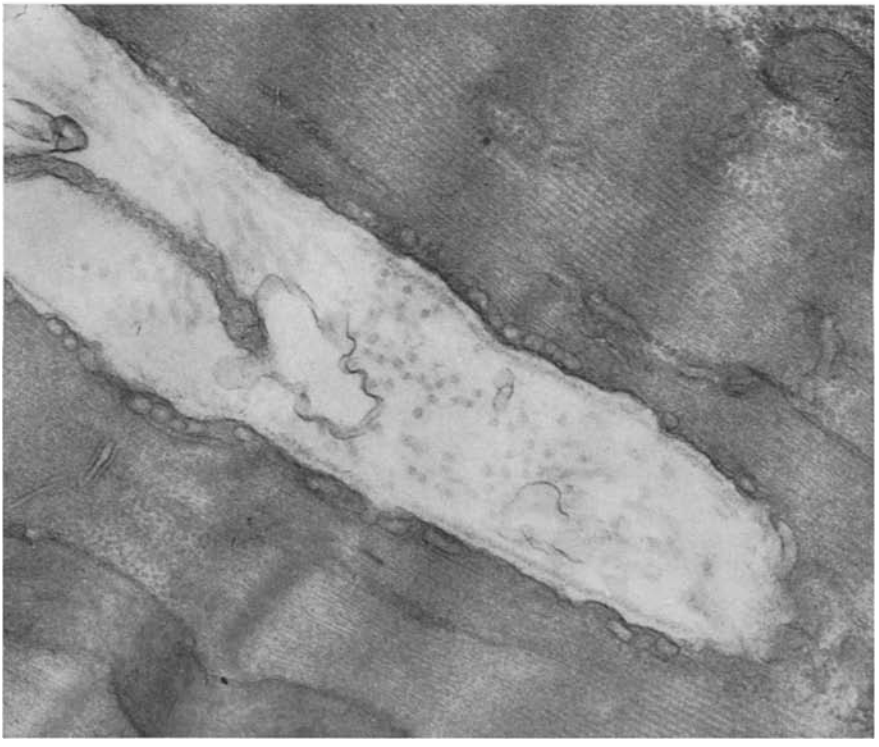

Fig. 5

outlined by electron-dense, ring-like structures (Fig. 6). The vacuoles appeared to represent distended and fragmented components of the sarcoplasmic reticulum. The origin of the round dense structures is uncertain.

Sarcolemmal plasma membranes were absent from the homogeneous necrotic fibers but a structure resembling the sarcolemmal basement membrane surrounded many of these fibers (Fig. 8). In other necrotic fibers, no sarcolemmal membranes could be identified. Occasionally there were membranous lamellar structures at the edge of the necrotic fibers in the region previously occupied by sarcolemma (Fig. 9). Cytoplasmic granules, Golgi networks, and lipid bodies were not seen within the necrotic muscle fibers. Except within macrophages, no structures in the necrotic fibers could be identified unequivocally as lysosomes rather than derivatives of the sarcoplasmic reticulum.

At more advanced stages of necrosis, numerous mononuclear cells were present within the necrotic muscle fibers, particularly along the margins. Many of these cells had large nuclei, one or two prominent nucleoli, scant cytoplasm, abundant rough endoplasmic reticulum and numerous thin processes which tended to overlap and envelop the necrotic fibers. Macrophages containing necrotic material and lysosome-like structures were also present at this stage.

Discussion

Cellular injury may result in reversible or irreversible changes. Changes that destroy architecture and cause cell death are designated as necrosis, while milder degrees of abnormality are referred to as degeneration. In the present study of muscle from dystrophic mice, the necrotic fibers were thought to result from severe injury, while the abnormally large fibers and the dividing fibers were considered to represent a response to sublethal injury. Although not specific, these changes of muscle necrosis, division of fibers and fiber size variation are the major histologic characteristics of the active stage of progressive muscular dystrophy.

The necrotic muscle fibers observed in this study have been compared with necrotic muscle fibers produced experimentally by ischaemia (Stenger *et al.*), crush (Allbrook), cold (Price *et al.*, 1964), heat (Shafiq and Gorycki), vitamin E deficiency (Howes *et al.*; Van Fleet *et al.*), plasmocid (Price *et al.*, 1962), and cortisone (D'Agostino). The changes in sarcolemmal nuclei, mitochondria and sarcoplasmic reticulum observed in the present study are similar to changes observed in these organelles in necrotic muscle fibers produced by a variety of experimental methods. The myofilament change in necrotic muscle was the same regardless of the type of injury, except that selective I-band dissolution, which is seen early in the necrosis resulting from ischaemia (Stenger *et al.*) or plasmocid toxicity (Price *et al.*, 1962) was not observed in the present investigation.

Following experimental necrosis of normal skeletal muscle, the sarcolemmal plasma membrane disintegrated and the basement membrane persists as a frame-

Fig. 4. Two adjacent fibers in the process of lateral apposition. Lead hydroxide ×21,320

Fig. 5. Forking is apparent. Concentric lamellar membranes persist in the cleavage space. Lead hydroxide ×20,800

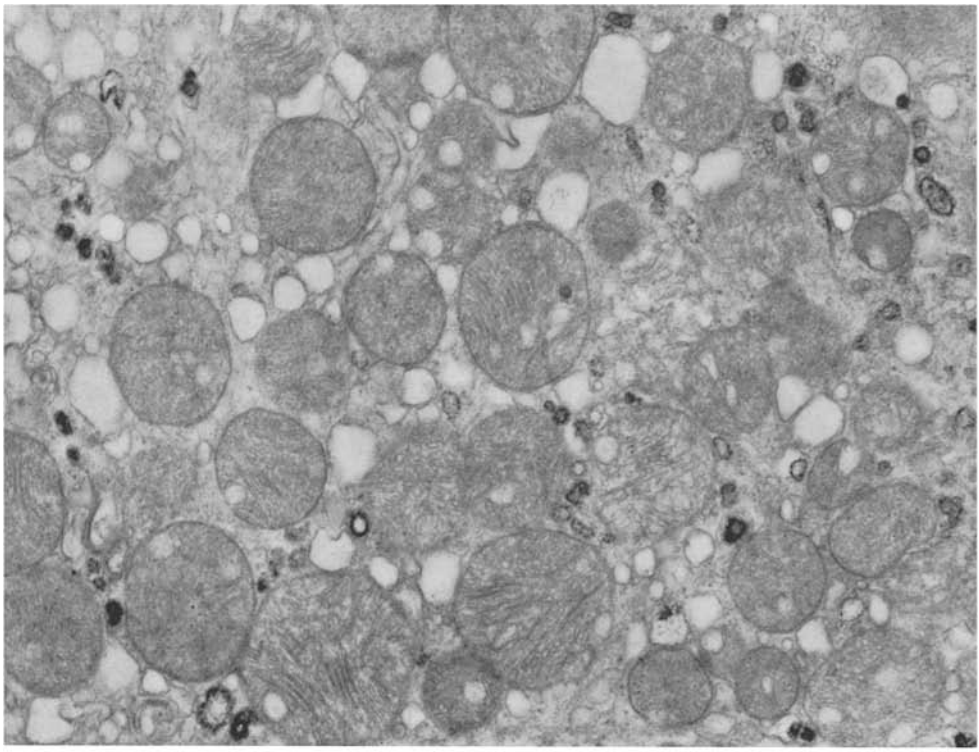

Fig. 6

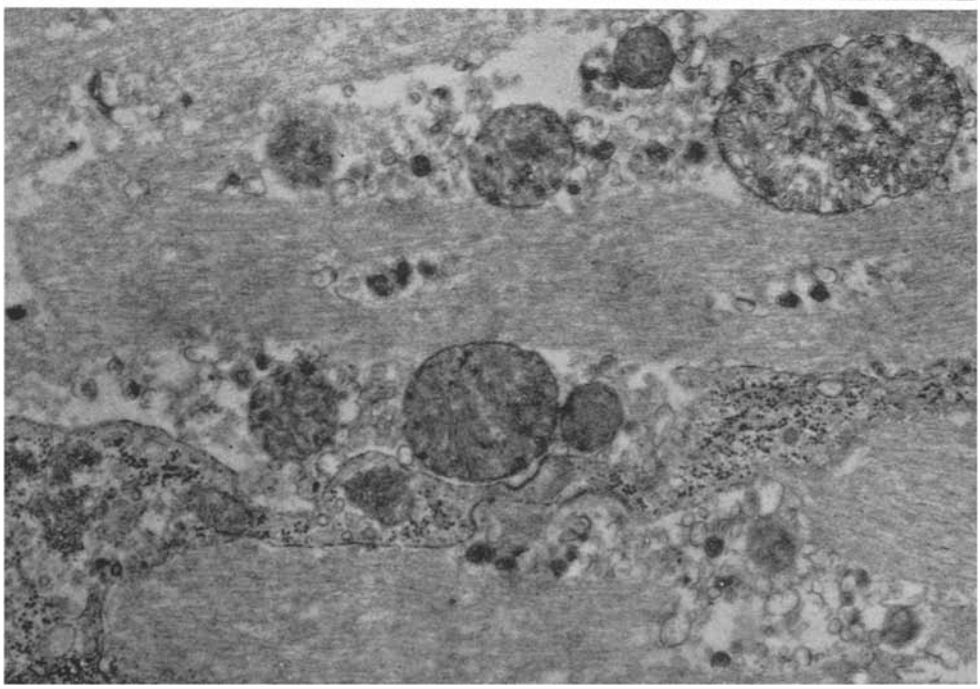

Fig. 7

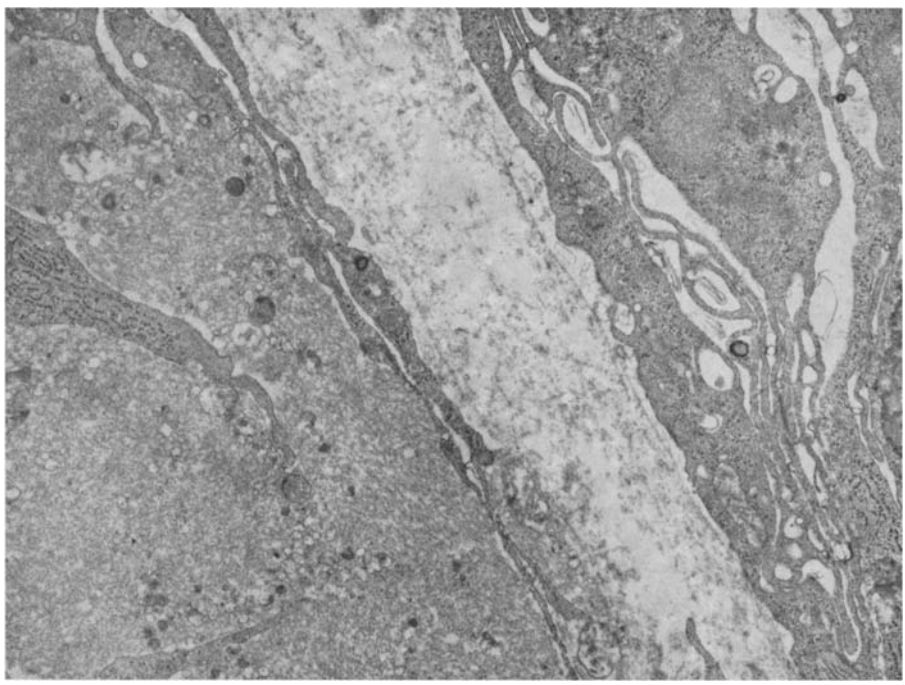

Fig. 8. Two adjacent necrotic muscle fibers. Each is enclosed by overlapping cellular processes. In addition, a structure resembling the sarcolemmal basement membrane is present adjacent to each necrotic fiber. Mitochondria and small, membrane-lined vacuoles and dense structures are seen in the necrotic material. Lead ×12,375

work for regeneration (Allbrook; Price *et al.*, 1964). In the present study, plasma membranes were absent from necrotic muscle fibers but a structure resembling the sarcolemmal basement membrane enclosed many necrotic fibers. Similar findings have been observed in necrotic fibers produced in dystrophic hamsters by exercise (Caulfield), and persistence of sarcolemmal basement membranes through the stages of necrosis, phagocytosis, and regeneration has been described in muscle biopsies from patients with Duchenne muscular dystrophy (Milhorat *et al.*; Shafiq *et al.*, 1967). However, Platzer and Chase reported that such membranes were absent in the necrotic muscle fibers of "preclinical" dystrophic mice. In the present study, the failure to detect sarcolemmal basement membranes in some of the necrotic fibers could be attributed to the proliferation of endomysial connective tissue fibrils, from which basement membranes could not be distinguished with certainty.

Fig. 6. Part of a necrotic muscle fiber showing various changes in mitochondria, distention of sarcoplasmic reticulum, and electron-dense, ring-like structures. Uranyl acetate ×23,150

Fig. 7. Necrotic muscle fiber showing remnants of the contractile substance, disintegrating mitochondria, and small, membrane-lined vacuoles and dense structures which may be derived from the sarcoplasmic reticulum. The outline of myofibrils is suggested but no cross-striations can be seen. A macrophage process containing phagocytosed necrotic material is also present. Lead ×21,825

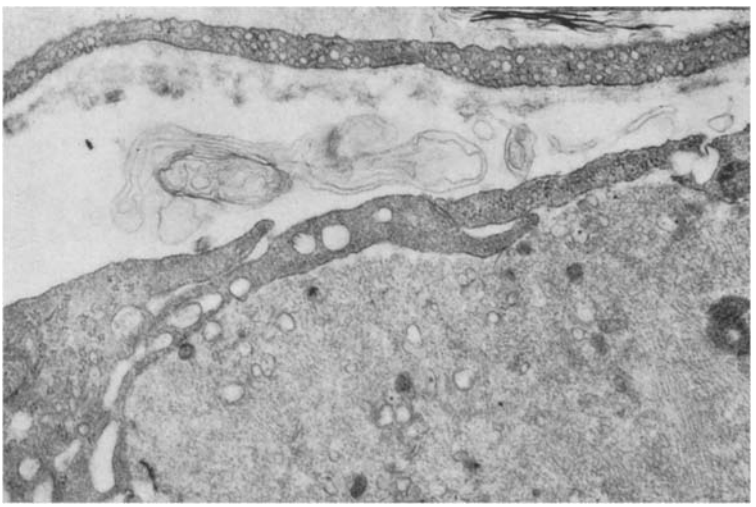

Fig. 9. Edge of a necrotic muscle fiber adjacent to capillary. Overlapping cellular processes enclose the necrotic material. No sarcolemmal membranes are seen. There are whorled lamellar structures adjacent to the necrotic fiber. Lead ×19,200

The concentric lamellar structures located adjacent to necrotic muscle fibers and between branching fibers, noted in the present study, have not been mentioned in previous descriptions of muscle fiber necrosis. It is possible that these extracellular lamellar structures were derived from sarcolemmal plasma membranes. The formation of similar structures from interrupted plasma membranes has been described in liver, stomach and kidney (Trump and Ericsson).

Of all the histopathological features of muscular dystrophy, variation in muscle fiber diameter within a fasciculus appears to be fundamental; there is no other primary muscle disease in which intrafascicular variation is so prominent. There appear to be two processes which produce the small fibers in dystrophic muscle: 1. ineffective regeneration, represented by various stages of embryonal muscle formation and by a failure of maturation beyond the myotubular stage (Banker, 1967) and 2. branching of muscle fibers, the result of incomplete lateral apposition.

The frequency with which branching fibers are encountered in dystrophic muscle has been emphasized previously (Wohlfart; Banker, 1969). In the present study, it was observed that the primary fiber and its branches are mature structures and no destructive ultrastructural changes were found at the point of division into smaller fibers. However, lamellar membranes were always prominent in the cleavage space. These findings suggest that there may have been focal damage to the two muscle fibers in the process of lateral apposition, resulting in incomplete fusion and the appearance of a branched fiber. Thus, the lamellar membranes can be regarded as scars of a previous focal destructive process.

The large fibers presented some unusual features which could explain their abnormally large diameter. Attached to many of these fibers were small primitive fibers in varying stages of lateral apposition. This fusion of muscle fibers at their

lateral margins represents a perpetuation of a normal fetal process by which muscle increases its lateral dimensions.

The foregoing observations suggest a unitary mechanism to explain two prominent histopathological features of muscular dystrophy—fiber size variation and fiber branching. Complete lateral apposition of muscle fibers results in the enlargement of diameter. Incomplete lateral apposition leads to the formation of fibers which appear branched or split in longitudinal section. In cross section the branches appear as small fibers.

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