ELECTRON MICROSCOPIC AND HISTOCHEMICAL OBSERVATIONS OF MUSCLE DEGENERATION AFTER TOURNIQUET*

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Plates 200 to 209

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Muscle degeneration and a shock syndrome, which frequently results in death, follow the release of limb tourniquets. Albumin and other plasma components are transferred into the affected muscle tissue (1–4) while toxic products of autolysis and hemolytic substances appear in the circulating blood (5–7). It is possible that this abnormal transfer results in part from damage of capillaries and muscle fiber membranes. The application of cold delays autolysis and fluid exchange, modifies the shock symptoms, and reduces mortality (8). Chemical changes, such as the large decrease in phosphorus (9) and morphologic alterations, as revealed with the light microscope (9, 10), have been carefully studied. Wide interfibrillar spaces are observed in tissues taken immediately after release of the tourniquet, and some fibers show discoid decay of their fibrils. The primary alterations are followed several hours later by advancing granular and hyaline degeneration.

The present communication extends the light microscopic observations of the succinic dehydrogenase distribution in normal fibers (11) to the degenerated fibers. With the electron microscope an attempt is made to visualize the finer details in the development of discoid, hyaline, and granular degeneration seen after release of the tourniquet. The alterations thus produced are common to an extremely wide variety of muscle lesions. Further experience will determine whether degenerations of different etiology, such as those caused by bacterial toxins, vitamin E deficiency, or Coxsackie virus infection will expose specific details characteristic of each lesion.

Methods

As in antecedent electrophoretic studies of blood and tissue extracts after shock-producing injuries (2–4), the technique of Rosenthal (12) was used for traumatization of hind legs of C57

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black mice. In this technique, No. 30 Eberhard Faber rubber bands were lapped 10 times on a thin walled tube. The mouse's leg was pulled into the tube and the band pushed off high upon the thigh. The samples were taken far distal to the band location in order to avoid including lesions caused by the more complex effect of compression. Normal muscles, muscles immediately after release of 2-hour tourniquets (the time causing highest mortality) (12), and several hours after release were examined.

For electron microscopy samples were fixed in buffered osmium tetroxide (13) for ½ hour to 4 and even 24 hours and washed in buffer. Since prolonged fixation with osmium tetroxide loosens the connection between the muscle fibers and dissolves increasing amounts of organic material (14), causing a gradual disappearance of fibril substance and damage to the endoplasmic reticulum, short fixation for ½ hour was finally preferred. Except for the thick shadowed sections (Figs. 6, 8, and 15) which were fixed for 2 hours all other specimens shown were fixed 30 minutes. The specimens were then washed, dehydrated in alcohol and acetone, and embedded in 30 per cent methyl- 70 per cent butylmethacrylate or pure butylmethacrylate (15). Sections were cut with glass knives (16) on a microtome using thermal advance (17) and micrographs were taken in the RCA electron microscope, model EMU. Musculi tibialis anterior, gastrocnemius, soleus, distal parts of musculus quadriceps, and adductors were used. In early experiments the methacrylate was dissolved from relatively thick sections (0.1 to 0.3 μ) which were then shadowed with palladium. This obsolete method still offers certain advantages since, due to the great focal depth of the electron microscope, the thicker sections may be viewed in good focus and a closer correlation with the light micrographs can be made if studied at low magnification. The thin sections were from 0.03 to 0.05 μ.

For light microscopy fresh frozen 20 μ thick sections were stained for succinic dehydrogenase with neotetrazolium (18).

OBSERVATIONS

Normal Muscles.—Fibers of the same muscle differ considerably in the number of mitochondria they contain. Most small laboratory mammals have predominantly pale muscles with few red fibers. The use of neotetrazolium as hydrogen acceptor in the succinic dehydrogenase system of the mitochondria shows wide quantitative variation of these bodies from one fiber to another (11), (Fig. 1). Thin transverse sections of different fibers studied at low magnification with the electron microscope reveal the ratio of myofibrils to mitochondria. In the tibialis anterior, which is known to be relatively red, up to 50 mitochondria can be found in some fibers for each 100 myofibrils in cross-section, whereas in predominantly pale muscles many micrographs show only 5 or fewer mitochondria for each 100 myofibrils. In longitudinal sections the mitochondria may form chains parallel to the fibrils. More frequently they lie in conjugate positions near the I band in relaxed fibrils (Fig. 4) or adjacent to the reticulum at the level of the Z bands in shortened fibrils (Fig. 5). Some fibers show a thick subsarcolemmic layer of accumulated mitochondria surrounding the entire contractile material. The transverse and longitudinal arrangement of the endoplasmic reticulum can best be visualized in obliquely sectioned areas of contracted fibers (Fig. 5). For more details of normal muscle structure the reader is referred to the literature (19–27, 32).

Injured Muscles.—The variation in the histochemical neotetrazolium reaction of the fibers is still maintained after injury (Figs. 2 and 3). The total quantity of succinic dehydrogenase seemed to be slightly less in the injured muscle since a few fibers show areas without granules (Fig. 2). However, a comparison of greater amounts of tissue in homogenates of normal and injured specimens utilizing triphenyl tetrazolium chloride (28, 29) as indicator for succinic dehydrogenase established no constant difference.[1]

With the electron microscope incipient and advanced alterations of the fibrils are found 20 minutes after release of the 2-hour tourniquet and also 3 and 16 hours after release. Normal or nearly normal fibers are always present in the same specimen (Figs. 6 and 7). Figure 6, a thick metal-shadowed section 16 hours after tourniquet release, shows part of an intact fiber on the right. The Z lines are clearly visible and the sarcomeres are divided into the I, A, and H bands. Mitochondria occur in many places between the fibrils. The only unusual elements of this fiber are the dense bodies, presumably lipide accumulations, which are rarely seen in normal fibers. The adjacent fiber (left) separated in part by a capillary packed with red blood cells is in a state of marked degeneration showing both granular and hyaline areas. Discoid degeneration has taken place in the third fiber (bottom left) where the Z bands have been dissolved and apparently only the Q bands remain.[2] The thinner section (Fig. 7), taken from a different experimental animal, demonstrates one fibril in normal appearance except for the lipide body. Some mitochondria are arranged close to the Z bands and the endoplasmic reticulum is clearly visible between the fibrils. The other fiber at the lower right consists of disoriented fragments of the Q bands surrounded by sarcoplasmic debris. The disorientation results from the dissolution of the I bands, including the Z lines, as is clearly illustrated in the thicker section of Fig. 8 (methacrylate removed) in which the orientation of the Q bands is preserved, thus giving the appearance of typical discoid degeneration. The mitochondria are in the dissolved I bands and the tubules of the expanded endoplasmic reticulum are between the fibrils. The H bands are very prominent due to contiguous denser portions of the A bands. In some of the H discs the M line is visible as a thickening of each single filament. Essentially the same discoid degeneration is illustrated in greater detail in the thin section of Fig. 9 (embedding plastic not removed). An example of incipient discoid degeneration of a red fiber with many mitochondria is shown in Fig. 10. At the top left of this field the

[1] These measurements were kindly made by Dr. Frederick Agate of the Department of Anatomy, College of Physicians and Surgeons, Columbia University.

[2] With "Q" we designate the "Querband" consisting of A, H, including M and A as done by Szent-Györgyi. (33). For the other letters see the abbreviations used in figures, especially Figs. 4, 5, and 8.

Z bands are still preserved, but other sarcomeres are already disintegrated and slightly disoriented. Further signs of pathologic alterations are the lipide bodies, a considerable swelling of the mitochondria, and widened reticular tubules. At the bottom left a sarcolemma with attached canaliculi of endoplasmic reticulum in a highly degenerated fiber is visible.

In other fibers with obviously the same alterations of mitochondria and endoplasmic reticulum no discoid decay of myofibrils has developed. Fig. 11 shows part of a fibril with partially destroyed sarcolemma containing large roundish mitochondria, cross-sections of expanded reticulum, and longitudinal sections of shortened myofibrils. Wide Z bands are clearly visible in Fig. 11, but lacking in Figs. 12 and 13. In these micrographs of fibrils without I bands the myofilaments are continuous throughout the fibril length in contrast to those of Figs. 8 and 9. No sarcomeres can be recognized since the Z substance is dissolved as in discoid degenerated fibrils. Fig. 12 represents a fiber with many swollen mitochondria and expanded or ruptured tubules of the endoplasmic reticulum. In Fig. 13 the internal structure of the mitochondria between the tightly packed contractile material is in a state of dissolution. Fig. 14 shows a more advanced state of hyalinization of the contractile material. In the better preserved area it still appears filamentous, and closely spaced, vague Z bands indicate considerable shrinkage prior to dissolution (compare Figs. 6 and 15). The mitochondria are swollen outside the hyalinized material and in different states of dissolution inside. Furthermore the stroma between the mitochondrial membranes is greatly reduced. The relation between discoid and hyaline degeneration is clearly visible in the thicker section of Fig. 15, metal-shadowed after removal of the methacrylate. The central fiber is relatively stretched on the right but is increasingly compressed toward the left of the micrograph. The Z substance is removed throughout the fiber, but discoid splitting occurs only in the relaxed fibrils. In the shortened area next to the capillary the myofilaments remained continuous. Additional details of the figures are given in the legends.

DISCUSSION

It has previously been shown (4) that 2-hour tourniquets are followed by an increase of approximately 75 per cent of water in the ischemic tissue. From studies with the light microscope (10) it may be concluded that most of the water is stored in the widened interstitial spaces. The electron micrographs show, however, that muscle fibers in advancing degeneration must also imbibe fluid. The transfer from capillaries to the interstitial spaces and into the muscle fibers seems to be facilitated by visible damage to the capillary endothelium and the sarcolemma. Fibers in advanced degeneration frequently give the appearance of nearly empty (essentially fluid-filled) sarcolemmal sheaths

(Fig. 10). One should, however, always bear in mind that electron micrographs do not represent the actual distribution of substance in a given tissue. Especially in muscle fibers it is to be noted that most of the sarcoplasmic proteins must have been lost since interference microscopy (25) and other data (26) indicate in contrast to electron microscopy a higher protein concentration in the sarcoplasm than in the fibrils. Therefore the loss of substance in the sarcolemmal sheaths can only be evaluated comparatively.

The diameters of mitochondria in degenerated areas are two to four times larger than in normal muscle or in fibers which maintain normal structure. This indicates a 10- to 60-fold increase in mitochondrial volume, which is probably caused by hypotonicity of the fluid entering the muscle fiber. The tubules of the endoplasmic reticulum in many instances show osmotic swelling and rupture (Figs. 10 to 12, 14) but the myofibrils do not appear to change in volume, a factor which indicates the absence of hydrophilic swelling. The outer membranes of the mitochondria are generally closed, but openings or breaks leading into the spaces between the inner membranes occur with swelling (Figs. 10 to 12, 14). The inner membranes can be stretched by the mitochondrial swelling and by their attachment to the outer membrane they may prevent, in some cases, the deformation to spheres (Fig. 14). However, spherical shape and fragmentation of the inner membranes, followed by dissolution, are more frequent. Large mitochondria in normal fibers have a different inner structure from the swollen mitochondria of the degenerated fibers. In spite of the widely spread morphologic decomposition, the enzymatic activity of the mitochondrial succinic dehydrogenase is not essentially affected.

A change in the amount of mitochondria in fibers due to degeneration is difficult to evaluate from electron micrographs since in normal muscle there is such a wide variation from fiber to fiber. Sectioned muscle stained with neotetrazolium (for succinic dehydrogenase) showed the same wide variation in specimens of both injured and uninjured muscles. In many of the electron micrographs, particularly those of the thicker sections, at first there appeared to be a marked increase in mitochondria; after further studies of sections from many different experiments it was concluded that there probably is no definite increase in the quantity of mitochondria at any time up to 16 hours following ischemia. Failure to find any consistent difference in the amount of succinic dehydrogenase in homogenates of injured and uninjured limbs supports this conclusion.

In degenerated fibers the disappearance of the Z lines may be correlated with the decided decrease in acid soluble phosphorus, phosphocreatine, and adenosinetriphosphate (9). This would support the hypothesis that the high density of Z bands results from phosphorus components which are needed in the sequence of reactions started by activation of the fibrillar adenosinetri-

phosphatase (22). The disappearance of the Z lines during degeneration is independent of the sarcomere length. However, discoid degeneration takes place only in fibrils where the I bands are present. Since this band is very likely composed principally of actin (27), the myosin in the A bands seems to be more resistant to the damage caused by ischemia, as it is more resistant to tryptic digestion (19) and prolonged osmium tetroxide fixation.

Lack of correlation between the degree of degeneration and the interval of time after injury is rather striking, normal or almost normal elements appearing contiguous to those showing complete destruction at all time intervals examined (20 minutes to 16 hours after injury). One must remember in this connection that under normal conditions only a small percentage of the muscle capillaries is open for circulation. Thus the tourniquet affects fibers with different oxygen reserve from the very beginning. Two factors, however, which in all cases influence the form of degeneration are the presence of the I band mentioned above and the amount of mitochondria in the fiber. It is evident that only those fibers which are stretched develop discoid degeneration. Since the legs are paralyzed during ischemia and remain so for many hours afterwards, absence of the I band in the fibers with short sarcomeres indicates a passive state which inhibits discoid decay of the fibrils. Red fibers with many mitochondria give a granular appearance, while fibers with few mitochondria and far advanced degenerations appear homogeneous or hyaline. It seems, further, that one of the first symptoms of degeneration is the appearance of the lipide bodies which are about the same size and shape as mitochondria and occur only in sarcoplasmic spaces. It is possible that these bodies represent a special form of mitochondrial degeneration and also that lipide accumulates in the reticulum as seen in other cells (30, 31).

SUMMARY

As an experimental model for the different forms of muscle degeneration, injury caused by 2 hours' ischemia has been studied from 20 minutes to 16 hours after release of the tourniquet.

Discoid degeneration developed in stretched fibers by dissolution of the I bands (Z substances and actin). The discs represented the Q bands (A-H-A). In fibers which passively maintained contraction lengths during degeneration, the Z substances were dissolved, but the continuity of the fibrils was preserved, since the filaments are continuous over all sarcomeres under these conditions. Mitochondria and the tubules of the endoplasmic reticulum swelled, ruptured, and disintegrated. Granular degeneration developed in fibers where mitochondria were abundant. Unstretched degenerating fibers with few mitochondria gave a homogeneous or hyaline appearance. The different forms of degeneration therefore were dependent on the status of stretch and the fiber type. The

extent of degeneration was not a function of time after ischemia, there being both nearly normal and severely damaged fibers at 20 minutes and 16 hours after the release of tourniquets. When degeneration occurred, however, the basic alterations were the same in all fibers; there was mitochondrial and reticular swelling, dissolution of the Z substances, and finally disintegration of the contractile material. Some damage developed in the sarcolemmas and capillaries.

The mitochondrial disintegration was not linked with inactivation of the succinic dehydrogenase system.

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EXPLANATION OF PLATES

Abbreviations Used in Figures

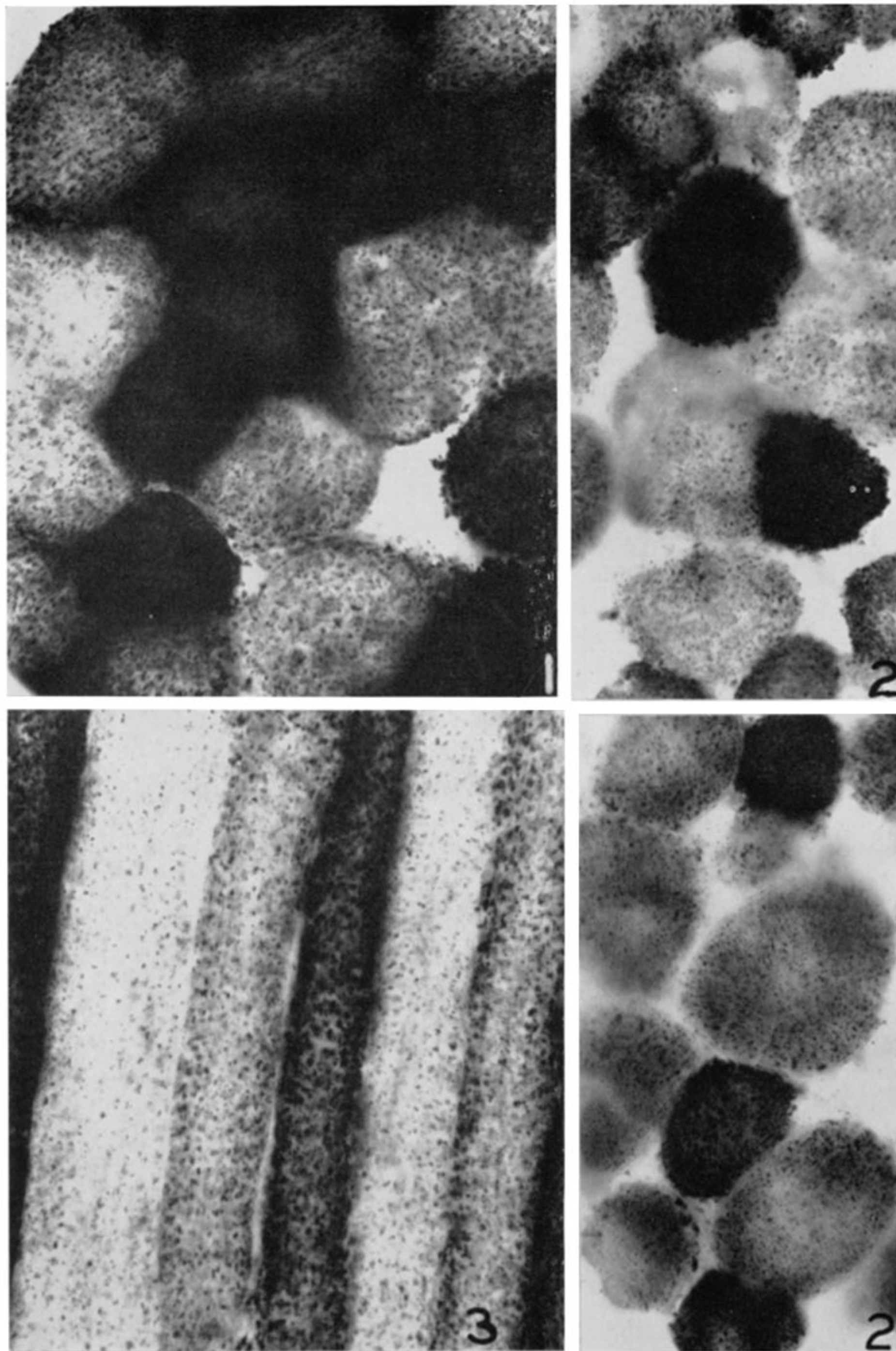
A, anisotropic band.
er, endoplasmic reticulum.
f, myofilaments.
F, myofibrils.
G, ground membrane (endoplasmic reticulum at the level of the Z bands).
H, Hensen's band (light band within Q).
I, isotropic band, including Z.
L, lipide bodies.
m, mitochondria.
M, M band (*Mittelscheibe*; mesophragma).
Q, Q band (*Querscheibe*).
S, sarcomere.
sl, sarcolemma.
Z, Z band (*Zwischenscheibe* or telophragma).

Arrows, special points of interest, explained in legend of figure.

PLATE 200

FIG. 1. Skeletal muscle (tibialis anterior) from normal mouse. Frozen section 20 μ in thickness were incubated for 2 hours in neotetrazolium. Formazan granules (black in photomicrographs) indicate mitochondrial succinic dehydrogenase. $\times$ 260.

FIGS. 2 and 3. Injured muscle (mouse, tibialis anterior) 50 minutes after release of tourniquet. Prepared as in Fig. 1. Two different fields are shown in Fig. 2. Portions of certain fibers appear void of grain. Fig. 3 is a longitudinal section indicating that, although the succinic dehydrogenase content varies from fiber to fiber and laterally in single fibers, it is uniform throughout the length of each fiber. $\times$ 260.

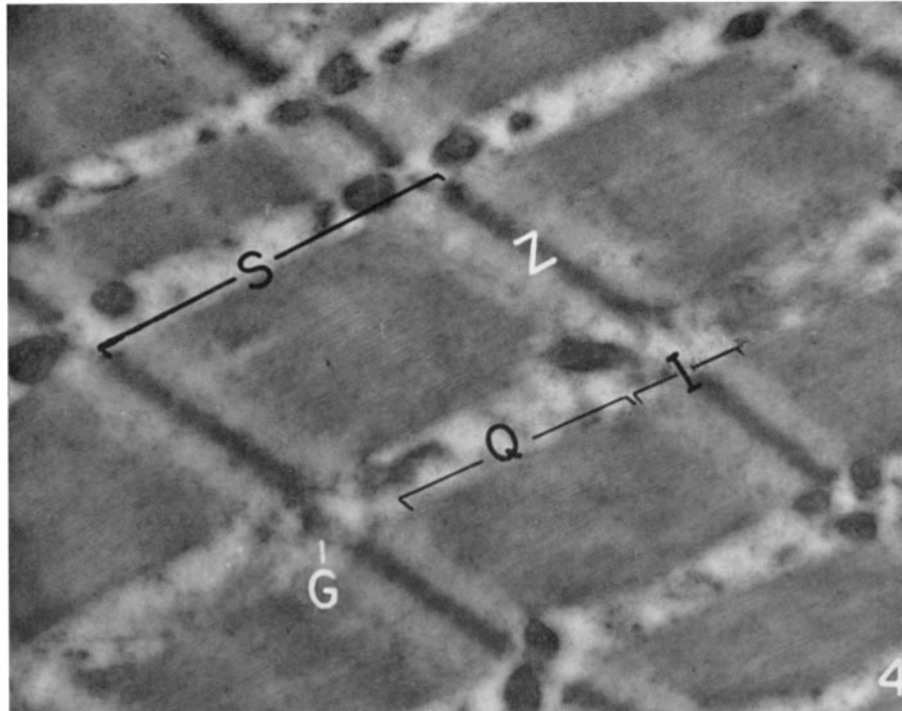


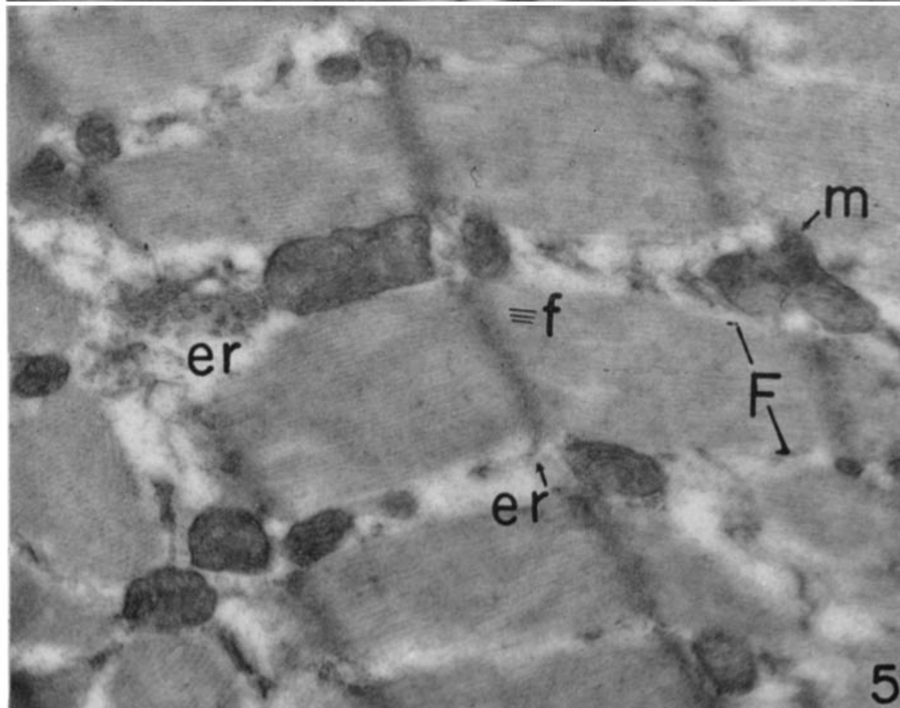
(Moore, Ruska, and Copenhaver: Muscle degeneration after tourniquet)

Plate 201

Fig. 4. Longitudinal section of normal muscle relaxed enough to show the I bands. *Q*, *Z*, and *S* are marked for general orientation. Mitochondria are often symmetrically placed on each side of endoplasmic reticulum at the level of the Z bands. The interfibrillar reticulum at this level forms the ground membranes of earlier authors, which also connect the sarcolemma (see Figs. 7, 10, and 11) and the cytoplasmic membrane of the nucleus (32). × 25,500.

Fig. 5. Longitudinal section of contracted normal muscle. Mitochondria of various size and shape lie between the fibrils. Many tubules of endoplasmic reticulum may be seen at left center (*er*). They enter the Z band where indicated by arrow. × 24,800.





(Moore, Ruska, and Copenhaver: Muscle degeneration after tourniquet)

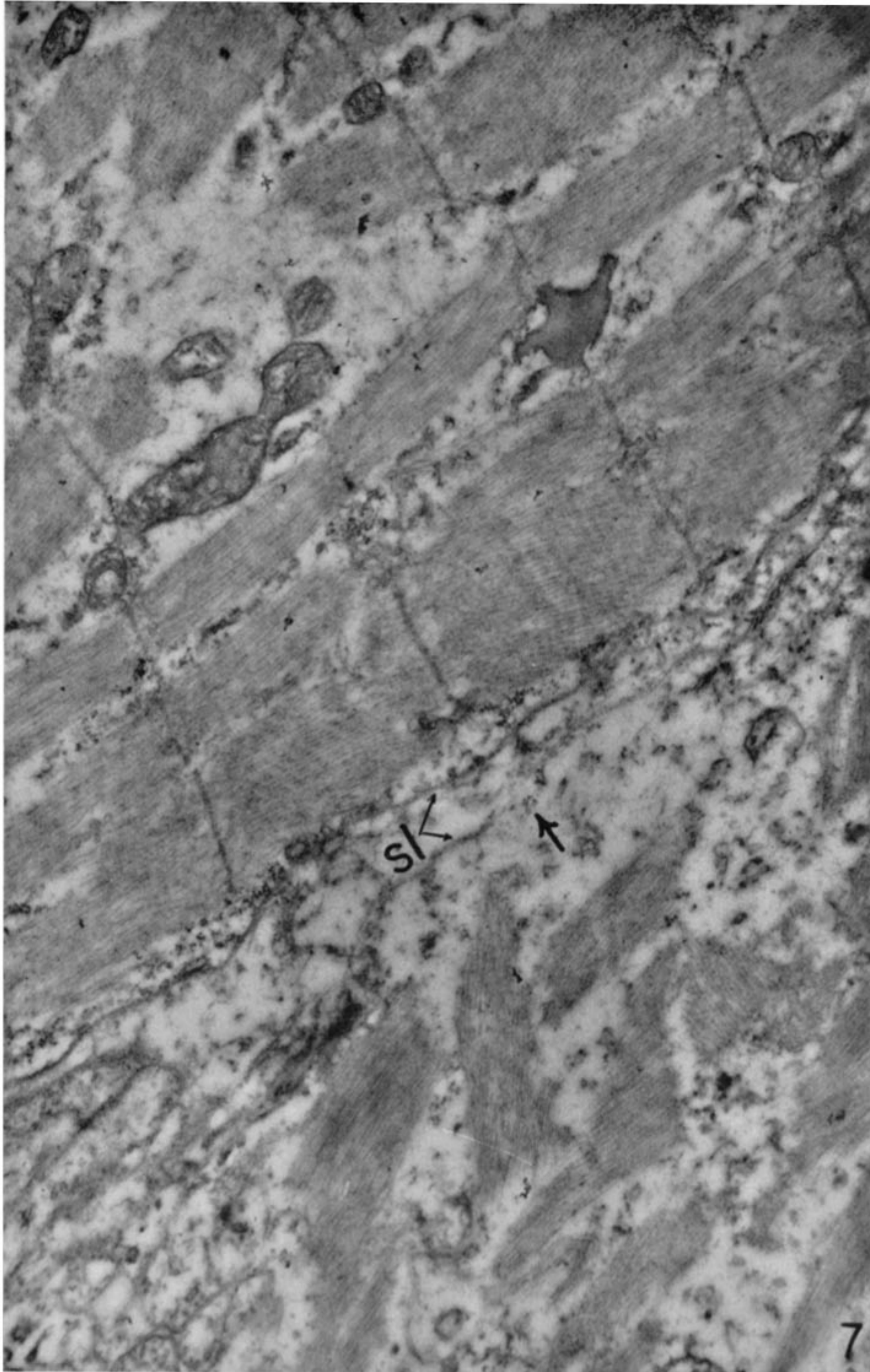
PLATE 202

FIG. 6. Sixteen hours after injury, an almost normal fiber on the right is separated in part by an erythrocyte-packed capillary from a severely necrotic fiber on the upper left. Lipide bodies and mitochondria can easily be distinguished in the almost normal fiber. The lipide bodies are larger in this fiber than in more degenerated adjacent fibers. Aggregates of mitochondria of various sizes can be seen between nearly homogeneous and shrunken contractile material in the most degenerated fiber in center. In the lower left fiber the substance of the Z band is dissolved. Methacrylate removed, palladium-shadowed. $\times$ 3,800.

(Moore, Ruska, and Copenhaver: Muscle degeneration after tourniquet)

PLATE 203

FIG. 7. Twenty minutes after injury. Longitudinal section of two adjacent fibers, one showing marked and the other only slight degeneration. In the degenerated fiber (lower right) only fragments of the Q bands (A-H-A) remain recognizable and they are oriented not only longitudinally but obliquely and transversely. A few swollen and fragmented mitochondria and elements of the endoplasmic reticulum are scattered throughout the area. In the other fiber (upper left) there are only local foci of slight alteration. In these foci mitochondria of various size and shape appear to be almost normal with clearly visible internal membranes. An irregularly shaped lipide body with an outer membrane is seen between two fibrils in contact with the Z bands. Arrow indicates damage of the sarcolemma of the markedly degenerated fiber. $\times$ 24,800.

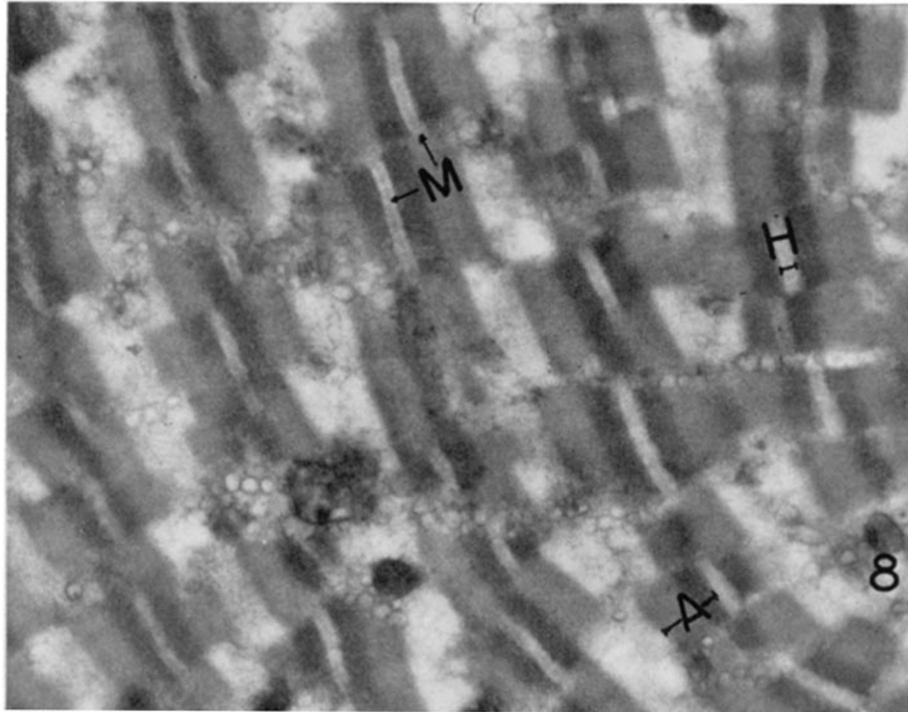


(Moore, Ruska, and Copenhaver: Muscle degeneration after tourniquet)

PLATE 204

FIG. 8. Three hours after injury. Nearly longitudinal section showing dissolution of I bands, some lipide bodies, mitochondria, and many cross-sectioned elements of widened endoplasmic reticulum. The H bands, between the denser portions of the A bands, appear in high contrast. Filaments of the H bands and M lines (as a thickening of H filaments) are clearly visible in the upper half of the figure. Thick section, methacrylate removed, palladium-shadowed. × 16,000.

FIG. 9. Sixteen hours after injury. Longitudinal section showing discoid degeneration in a fiber with few mitochondria. The I bands, including the Z bands, are completely absent. Individual myofilaments can be traced all through the remaining Q bands. The M line can again be seen as a thickening of filaments in the middle of each H band. Dense material is attached to the filaments on both sides of H. One mitochondrium and scattered endoplasmic reticulum are apparent. × 24,500.



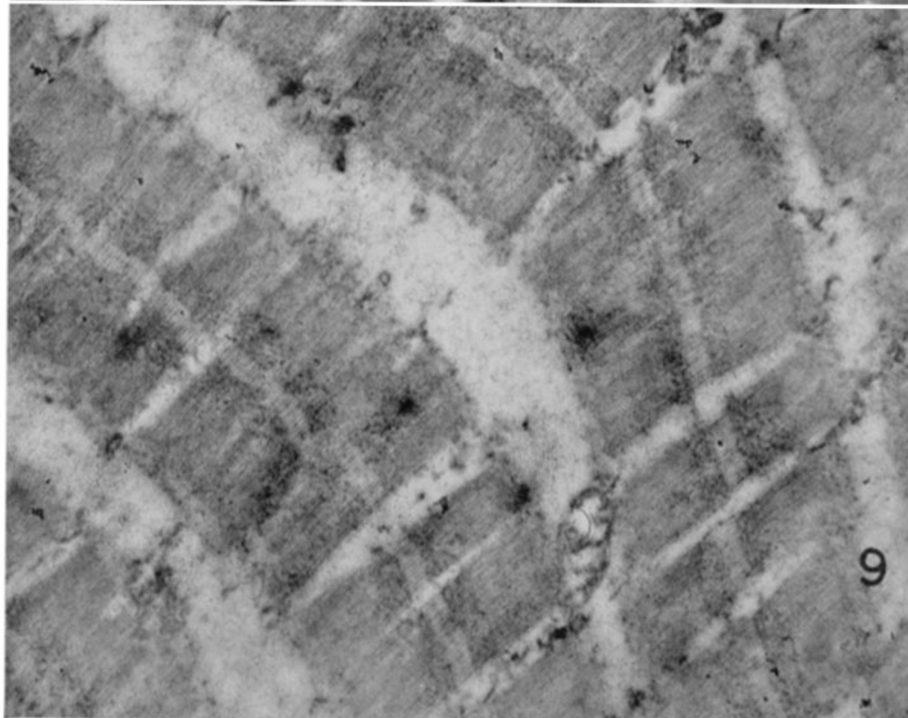


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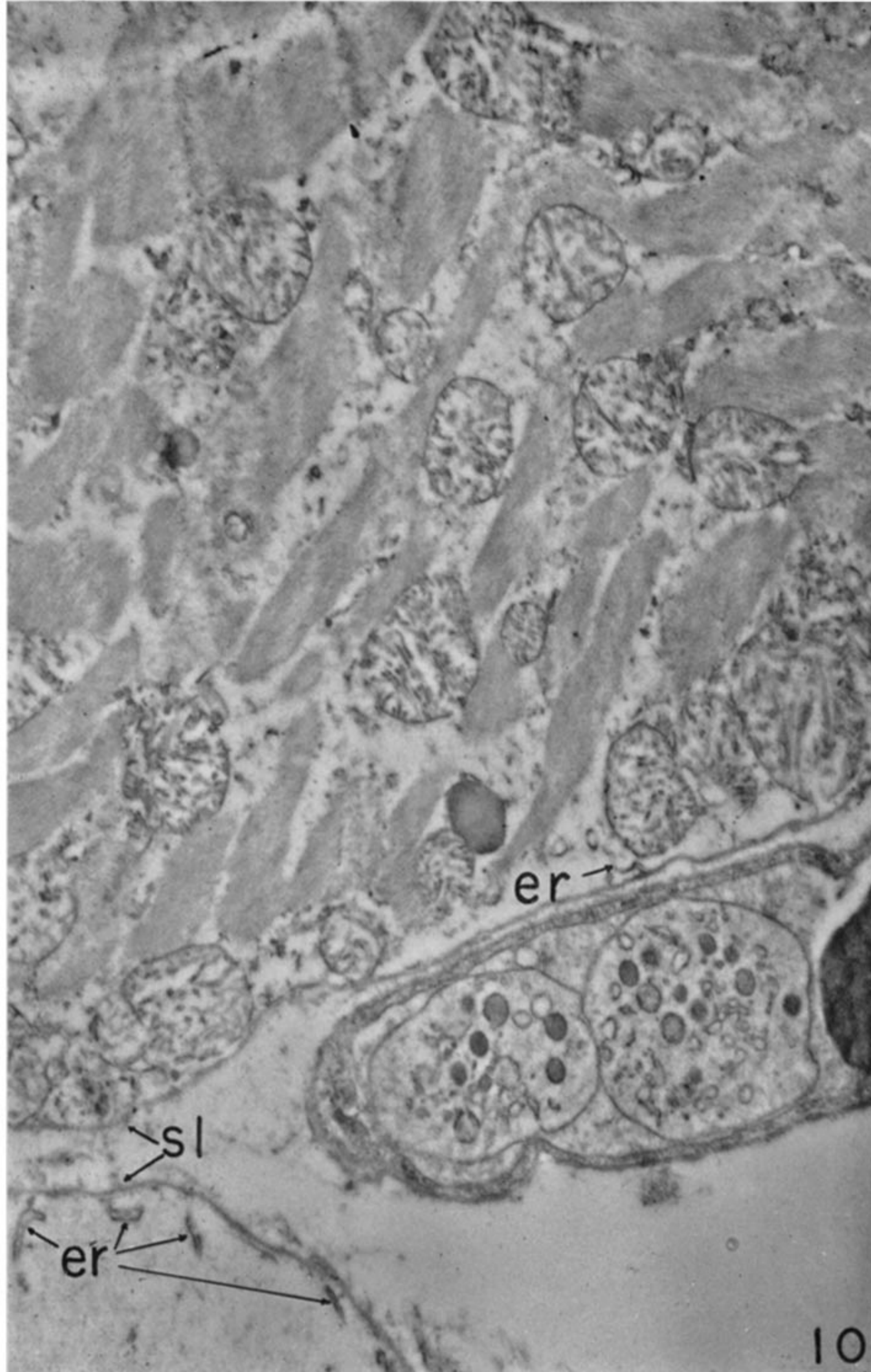
PLATE 205

FIG. 10. Twenty minutes after injury. Oblique section of a fiber with many swollen and partially ruptured mitochondria. Beginning discoid degeneration; I bands with Z only in upper left portion. Obliquely sectioned capillary with two platelets and the edge of a red cell at lower right (*cf.* Fig. 15). An empty sarcolemma from another fiber with traces of endoplasmic reticulum is at lower left. In both fibers elements of endoplasmic reticulum are attached to the inner sarcolemmal surface. × 24,500.

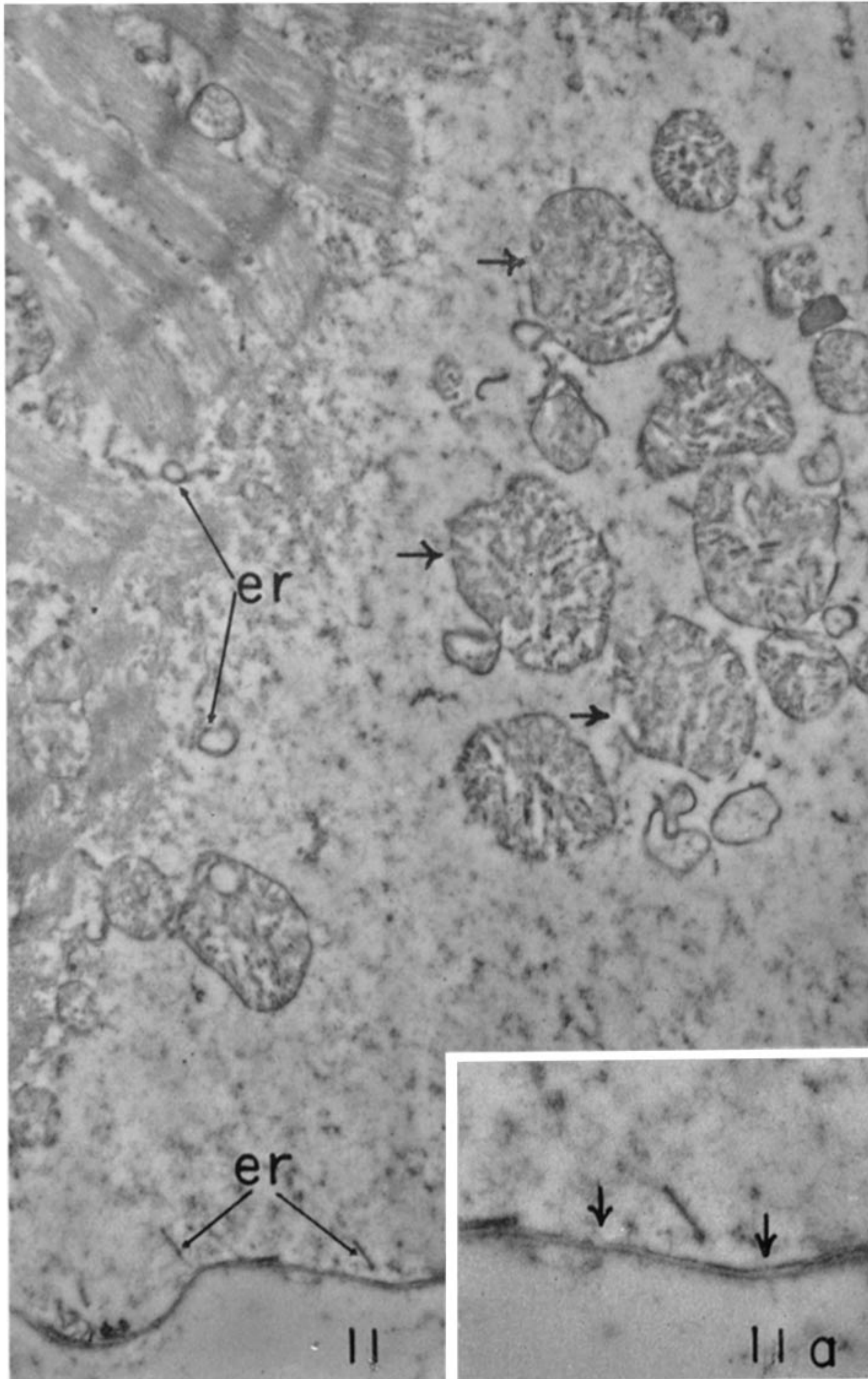


(Moore, Ruska, and Copenhaver: Muscle degeneration after tourniquet)

PLATE 206

FIG. 11. Twenty minutes after injury. On the left side fibrils with diffuse dark Z bands are visible, whereas only debris, swollen mitochondria, and distended tubules of the endoplasmic reticulum fill the remaining space within the sarcolemma. Many mitochondria have ruptured outer membranes (arrows). × 15,500.

The insert Fig. 11 *a* shows the sarcolemmal double membrane with some destruction of the inner layer (arrows) and fragments of reticulum (*cf.* Fig. 7). × 31,000.

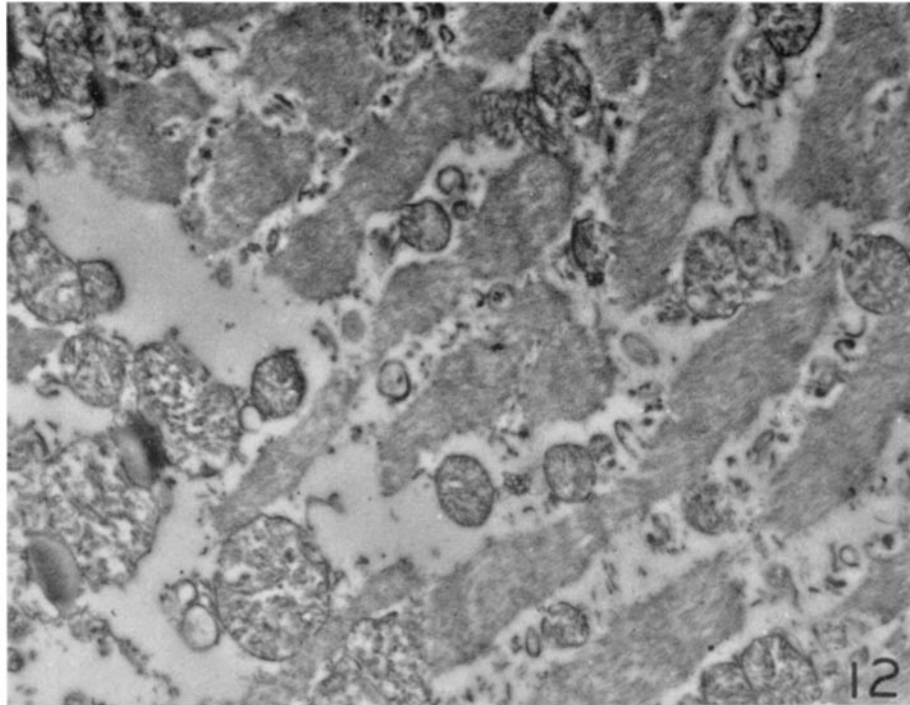


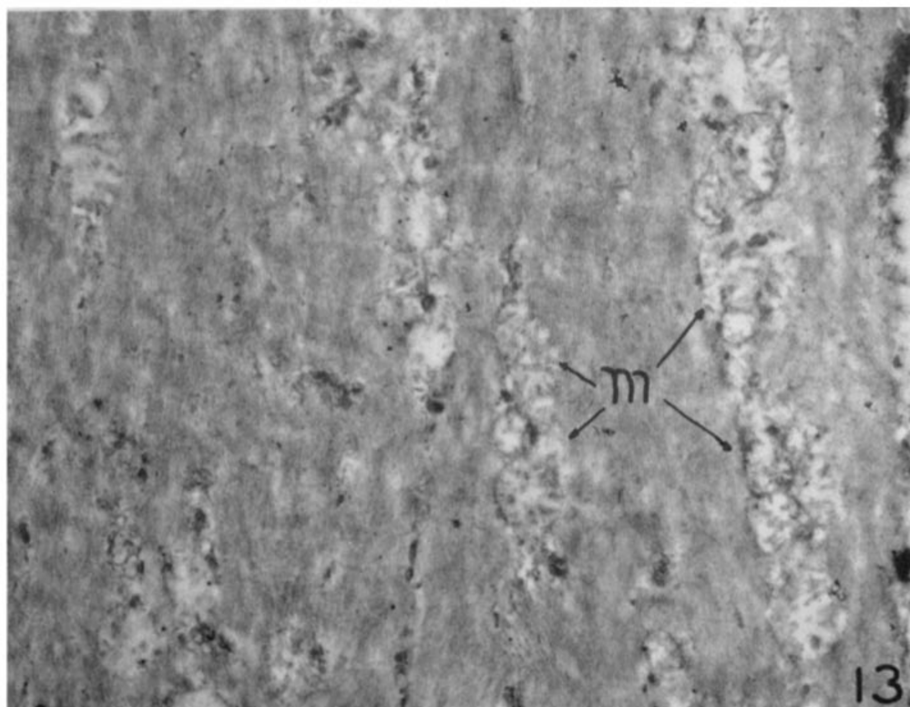
(Moore, Ruska, and Copenhaver: Muscle degeneration after tourniquet)

Plate 207

Fig. 12. Twenty minutes after injury. Almost homogeneous fibrils in a red fiber. Loss of Z substance makes sarcomeres unrecognizable. Swollen and fragmented elements of the endoplasmic reticulum are abundant. Several lipide bodies are in close association with mitochondria and mitochondrial fragments. Holes in the outer mitochondrial membrane are frequently observed. × 15,500.

Fig. 13. Twenty minutes after injury. Area illustrating hyaline degeneration of closely packed fibrils. Myofilaments, but no sarcomeres are visible. Mitochondrial debris (*m*) deprived of inner structure and dense reticular material are aligned between filamentous masses. × 15,500.

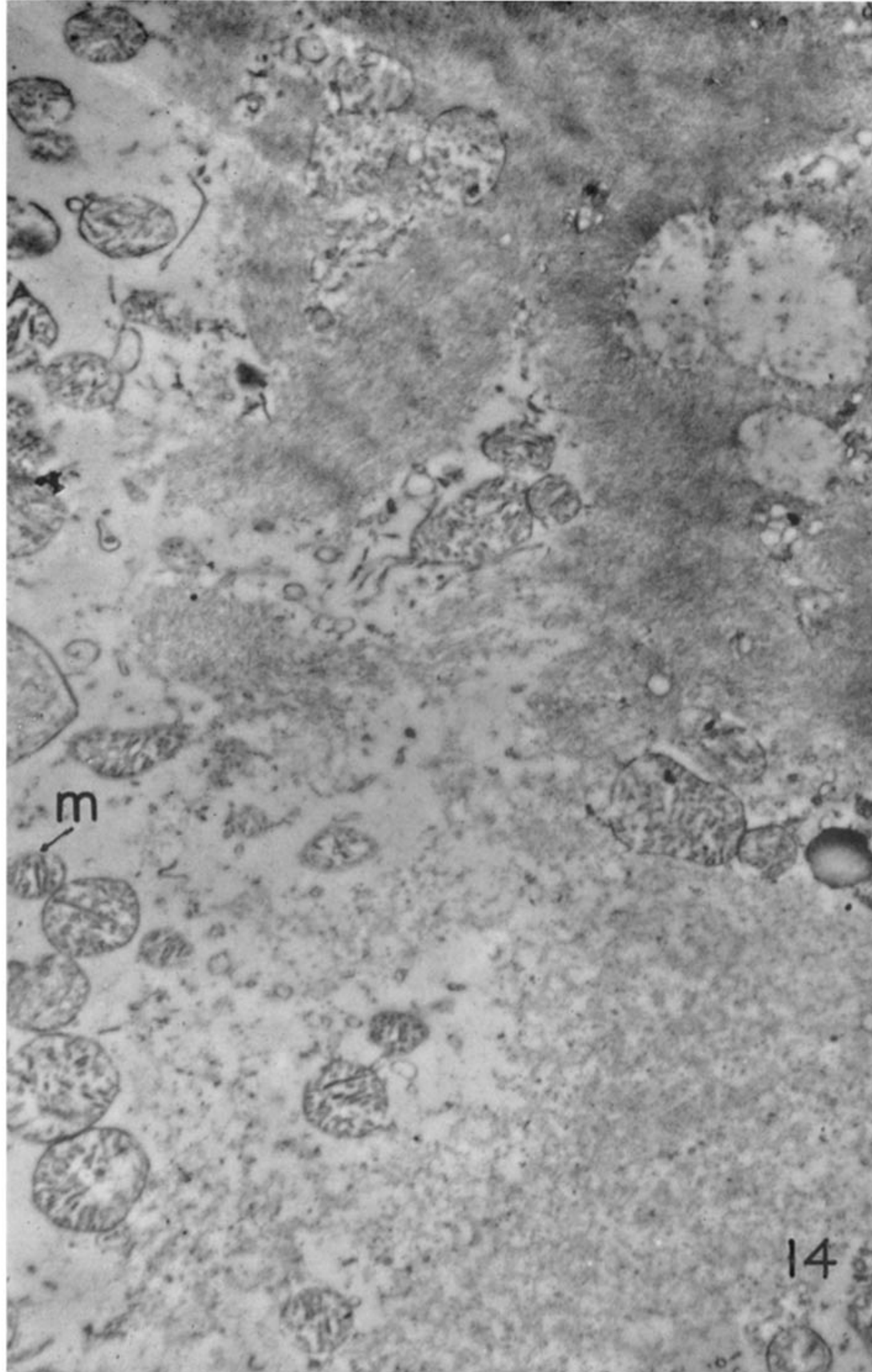




(Moore, Ruska, and Copenhaver: Muscle degeneration after tourniquet)

PLATE 208

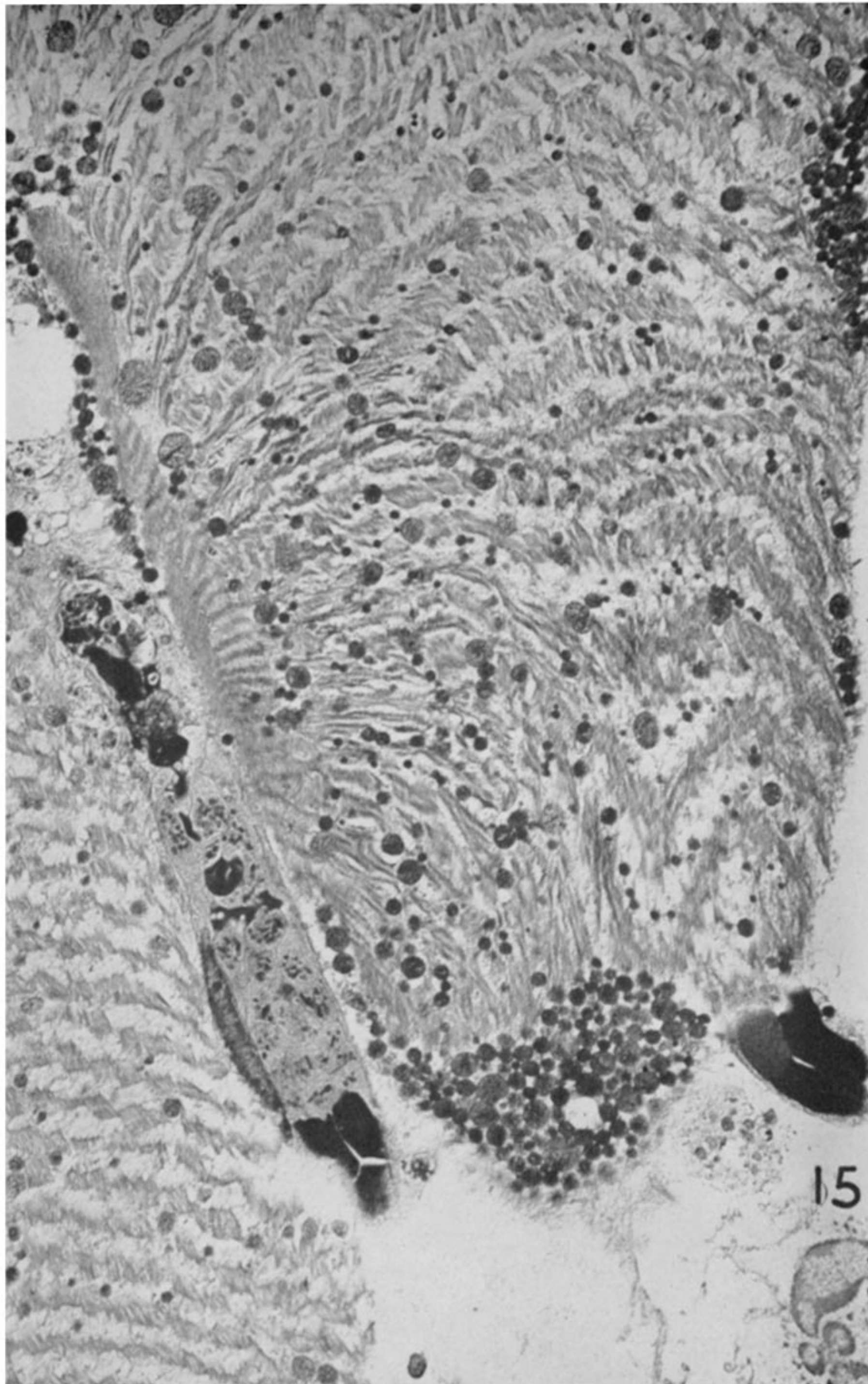
FIG. 14. Twenty minutes after injury. A marked degree of hyaline degeneration with almost complete destruction of filaments is apparent in the lower portion of the field. The large vacuoles at the upper right probably represent the space of completely destroyed mitochondria. Swollen mitochondria with stretched inner membranes are at left, one (*m*) in which the spherical swelling is prevented by attachment of an inner membrane. Distended endoplasmic reticulum is widely prevalent. $\times$ 15,500.



(Moore, Ruska, and Copenhaver: Muscle degeneration after tourniquet)

Plate 209

Fig. 15. Sixteen hours after injury. Different degrees of stretch are evident in the principal fiber of this field. Discoid degeneration is evident except for the unstretched region along the capillary. Subsarcolemmic concentrations of mitochondria (such as that at the lower extremity and the upper right of this fiber section) occur also in normal muscle. The capillary contains a few red cells but is packed predominantly with blood platelets measuring about 1.3 μ in diameter. An endothelial nucleus appears in the lower left side of the capillary segment. The lower right side shows a capillary with two red cells and a polynuclear white cell in the interstitial space. Discoid degeneration characterizes the left fiber. Thick section, embedding plastic removed, shadowed with palladium. $\times$ 3,300.



(Moore, Ruska, and Copenhaver: Muscle degeneration after tourniquet)