Myocytolysis and Mitochondrial Calcification in Rat Myocardium after Low Doses of Isoproterenol

Sherman Bloom, M.D., and Pasquale A. Cancilla, M.D.

HIGH DOSES of isoproterenol are known to produce myocardial necrosis in rats [1,2] but low doses, about 5 mg./kg. of body weight, have been reported to produce either focal myocardial necrosis [3] or myocardial hypertrophy without visible evidence of necrosis.[4] When preliminary experiments on the use of isoproterenol for the induction of myocardial hypertrophy in rats were carried out in this laboratory, it was found that the wet and dry weight of both ventricles increased after drug administration. Since this finding is difficult to explain on a hemodynamic basis, and because of the discrepancy described above, we undertook a detailed morphologic investigation of the ventricular myocardium following the intraperitoneal injection of isoproterenol at the low dosage level.

Materials and Methods

Male Sprague-Dawley rats weighing between 200 and 260 gm., maintained on standard Purina Rat Chow, were used for all experiments. DL-Isoproterenol hydrochloride (Sigma Chemical Company) was dissolved in 0.15 M NaCl to give a final concentration of 2 mg./ml. Solutions were stored frozen in tubes wrapped in aluminum foil; unused portions were discarded 48 hr. after preparation. The drug was administered by intraperitoneal injection, at a dose of 5 mg./kg. of body weight. In early experiments, the drug was given twice each day, at 8 A.M. and 5 P.M., but when it was clear that focal myocardial necrosis was occurring, rather than myocardial hypertrophy, the protocol was changed to a single injection at 8 A.M. Control animals always received 1.0 ml. of 0.15 M NaCl by intraperitoneal injection at the same time experimental animals were given injections.

Hearts were removed from ether-anesthetized animals and immediately covered with either plain Zenker fixative at room temperature or cold osmium tetroxide solution. Transverse sections about 1 mm. thick were then taken from the left ventricle at the apex and midway between the apex and base. Zenker-fixed tissue was used for the preparation of paraffin sections for light microscopy. Tissue for electron microscopy was kept in cold, buffered osmium tetroxide fixative and later divided into small pieces for final processing. After dehydration and clearing, the fragments were embedded in Epon 812. One-micron sections were stained

From the Departments of Pathology and Neurology, University of Utah College of Medicine, Salt Lake City, Utah.

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Address for reprint requests: Dr. Pasquale A. Cancilla, Department of Pathology, UCLA School of Medicine, Center for Health Sciences, Los Angeles, Calif. 90024.

with cresyl violet or toluidine blue and used for preliminary evaluation. Demonstration of lesions at short time intervals following isoproterenol administration required careful examination of these sections, since necrotic cells were often present singly and represented a relatively small proportion of the myocardium. Sections from selected blocks were obtained with a diamond knife, stained with lead citrate, and micrographs were made with an RCA EMU 3H or Siemens Elmiskop 1A electron microscope.

Results

Light Microscopy

Light microscopy of routine paraffin sections showed changes similar to those previously described.[3] There was no easily detectable evidence of necrosis or inflammation until 8 hr. after injection, when neutrophils could be seen on the endocardium. By 24 hr., focal clusters of lymphocytes, macrophages, and neutrophils could be seen throughout the myocardium of both ventricles and were especially concentrated near the apex. Within such clusters, single necrotic muscle cells could sometimes be seen, occasionally with a neutrophil or macrophage penetrating the fragmenting sarcolemma. Four days after drug administration, lymphocytes and macrophages were present as before, but few neutrophils could be found; interstitial edema, present earlier, had largely subsided. By 2 weeks focal fibrosis was the only evidence of the earlier necrosis and inflammation.

Light microscopy was also used to evaluate the effects of varied doses of isoproterenol. When single injections of the drug were given at a dose of 0.5, 1.0, 2.5, and 5 mg./kg., and all animals sacrificed 48 hr. later, lesions were found in all experimental animals. Although the foci of necrosis were somewhat smaller and less abundant in the animals receiving 0.5 mg./kg., it was not possible to determine the drug dosage a particular animal received by examining these sections. Figures 1 and 2 illustrate the effects of 0.5 and 5.0 mg. of isoproterenol per kilogram of body weight, respectively.

Lesions could be identified much earlier with 1-μ Epon-embedded sections than with the standard paraffin sections. Within 15 min. of isoproterenol administration, isolated myocardial cells were found with prominent contraction bands. Such cells showed a sequence of alterations which were partially identifiable in the 1-μ sections, but electron microscopy showed these and other changes in greater detail.

Electron Microscopy

Sections of apex from the hearts of 21 rats were selected, by light microscopy of 1-μ sections, from a much larger number of animals

sacrificed at various intervals between 2 min. and 4 days after receiving isoproterenol. The criteria of selection were freedom from recognizable artifact and the presence or suspicion of lesions. Additional blocks derived from animals given saline injections or no injections were also examined.

Sections from control animals showed various stages of contraction with sarcomere lengths between 1.6 and 2.2 μ (Fig. 3). Abundant glycogen, mitochondria with occasional dense granules, rare lipid droplets, infrequent lysosomes, and unremarkable transverse tubules and sarcoplasmic reticulum were additional relevant features of control myocardium.

The data on animals receiving 5 mg. isoproterenol intraperitoneally are summarized in Table 1. Animals sacrificed 2 and 4 min. after iso-

Table 1. Electron Microscopic Findings in the Myocardium after Isoproterenol Treatment

No. of animals	Time of killing	Glycogen	Contraction bands	Mitochondrial deposits	Loss of Z discs	Phagocytosis
3	2–4 min.	+	+			
1	8 min	±	+	+		
4	15–30 min.	±	+	+		
1	2 hr.	−	±	+	+	
5	8 hr.	−	−	+	+	+
7	12–24 hr.	−	−	+	+	+

DI-isoproterenol hydrochloride (5 mg./kg.) was injected intraperitoneally.
+ indicates "present;" ±, present but less than at earlier times; −, absent.

proterenol showed no identifiable lesions in the 1-μ sections. Electron micrographs of suspicious areas, where the myocardial cells appeared swollen or the cross striations appeared more prominent, occasionally revealed ultrastructural changes which may be significant. Hypercontraction, with sarcomere lengths of 0.58–1.5 μ, and dark, wide Z discs, such as those described by Caulfield and Klionsky [5] as "contraction bands" in ischemic myocardium, were noted in some cells. These same cells showed mitochondrial enlargement and pallor due to an increase in the intercristal space. The cristae themselves appeared unchanged. Such cells, as can be seen in Fig. 4, occurred singly, while adjacent cells appeared normal.

The heart obtained at 8 min. showed a clear reduction in glycogen in affected fibers. The mitochondria no longer showed the vesicular change noted earlier, but now contained deposits of electron-dense material which will be described in detail below. Caulfield-Klionsky type contraction bands were present in some cells (Fig. 5), while others (Fig. 6) showed contraction bands identical to those described by Herdson

et al.[6] The latter are thought to consist of several sarcomeres contracted into a single mass. We refer to these forms as "minor" and "major" contraction bands, respectively.

At 15 min. after drug administration, damaged cells were easily identified (Fig. 7). Although the changes varied greatly in extent among affected cells, they were qualitatively similar in nature. Glycogen was reduced. Major contraction bands were common, but minor contraction bands were sparse in altered cells. Between contraction bands the sarcoplasm was pale, and was traversed by only a few myofilaments which maintained their parallel array. Except for electron-dense deposits, the mitochondria appeared normal. Additional features noted in the animals sacrificed at 15 min. were the presence of small wisps of fibrin in the intercellular space and occasionally within a capillary lumen. A capillary filled with platelets was seen in only one section.

At 30 min. there was no essential difference from the lesions seen at 15 min. Rarely, dilatation of transverse tubules was noted in degenerating or intact cells (Fig. 8).

At 2 hr. the myofibrils of affected cells occasionally showed major contraction bands, but minor contraction bands were quite rare. In many of the affected cells the myofilaments had an extended parallel arrangement with little or no evidence of cross striations. In such cells the original position of the Z discs could be inferred from the periodic zones of decreased density and presence of tubular elements (Fig. 9 and 10). The distance between such zones was between 1.5 and 2.0 μ, further suggesting that they represented the Z-disc area.

By 8 hr. there was extensive breakdown of affected cells. The sarcolemma was disrupted in many areas and macrophages and polymorphonuclear leukocytes were present within the muscle cells. Macrophages often contained fragments of myofibrils (Fig. 11) or mitochondria from myocardial fibers within membrane-bound spaces. Such mitochondria could be easily distinguished from those of the inflammatory cells on the basis of their electron-dense deposits and large size. Mitochondria from inflammatory cells were about 0.6 μ in diameter, while mitochondria from myocardial cells were about 1.5 μ in diameter. The myofilaments of degenerating cells were often still visible in parallel array, but they usually lacked the normal cross striations, and were similar to the myofibrils lacking Z-disc material noted at 2 hr. (Fig. 12). In some cells, however, no details were visible in the myofilaments, and only a homogeneous sheet of material, comparable in electron density to normal myofilaments, was seen in place of the normal bundles.

Changes seen at 12 and 24 hr. after isoproterenol were more advanced

but qualitatively similar to those of 8 hr. The number of inflammatory cells increased, and those present appeared to possess a well-developed endoplasmic reticulum. The amount of phagocytosed muscle-cell material was also increased. Although dissolution of myofilaments was apparent at these times, it did not seem as important a means of removal as phagocytosis by macrophages; much of the phagocytosed material was easily recognized as being intact myofibrils. Intact myofilaments with loss of Z-line material were even more prominent than at 8 hr. Collections of material within macrophages could be recognized as being of mitochondrial origin by the presence of electron-dense structures.

Degenerating myocardial cells were not seen 4 days after a single injection and inflammatory cells were less common than at earlier times. Instead, fibroblasts with well-developed endoplasmic reticulum were abundant and were distributed in scattered foci where increased collagen was also noted.

Mitochondrial Densities

As early as 8 min. after isoproterenol treatment, scattered electron-dense deposits were apparent within the mitochondria. These densities were of two forms and differed from the dense bodies often seen in the mitochondria of normal rat heart. One type was an amorphous mass of electron-dense material which obscured the underlying mitochondrial architecture (Fig. 9–11). The second type consisted of electron-dense granules arranged in patterns that included (1) a circle with a pale center, (2) a circle with a pale center and a cluster of central granules, (3) a group of dense granules scattered in a pale field, and (4) a linear array of dense granules along one side of a crista (Fig. 5–12). These foci of amorphous or granular electron-dense deposits became increasingly abundant and prominent with time, until the mitochondria were phagocytosed. Aside from these deposits and the transient vesicular changes seen at 2 and 4 min., there was remarkable preservation of the normal mitochondrial morphology, which was lost after phagocytosis.

Discussion

The light microscopic findings described in this study are in essential agreement with those of Rona *et al.*[3] and support the concept that isoproterenol produces necrosis of myocardial fibers rather than myocardial hypertrophy. The progressive increase in heart weight, as well as the increase of DNA, RNA, and protein content during isoproterenol administration can all be explained on the basis of the inflammatory reaction.

That a necrotizing process, such as described here, should result in a fibrotic scar is consistent with general experience in pathology and the earlier findings of Rona *et al.*[3] but is contrary to the report of Maruffo[7] who found no fibrosis after isoproterenol-induced myocardial lesions in monkeys. Although a large dose of isoproterenol (100 mg./kg.) was used in the latter study, the myocardial changes observed did not clearly represent muscle necrosis and may have been reversible. While the findings of Maruffo may be explained as a species variation and may be more relevant to the situation in man, our study confirmed the irreversible nature of the myocardial lesion in rats.

The appearance of electron-dense material in the mitochondria of myocardial fibers within 8 min. of isoproterenol administration is of considerable interest. Why this change has not been noted previously in isoproterenol-treated rats is not clear. The dosage used here has not been studied in detail, and may explain the difference; but it is also possible that the care used in selecting the lesions to be studied was important. In any event, intramitochondrial deposits of electron-dense material similar to those seen here have been noted in a variety of experimental heart lesions,[6-11] and appear during perfusion of isolated dog hearts with calcium-containing solutions.[12] Incubation of mitochondria in solutions containing calcium and phosphate also result in the formation of such deposits of calcium and phosphorus in a molar ratio of 1.67 to 1.0; [13,14] our material is indistinguishable from such mitochondria. The normal appearance of the mitochondria while this deposit is forming is consistent with the fact that only functioning mitochondria are capable of accumulating calcium.[13] This has led us to the conclusion that within 8 min. of isoproterenol administration the mitochondria have a sufficiently intact Krebs cycle and electron transport system to allow copious uptake and storage of calcium, probably derived from the calcium-rich milieu of the sarcoplasmic reticulum. The periodic release of calcium from the sarcoplasmic reticulum is related to membrane depolarization and muscle contraction[15] so that the finding of contraction bands prior to the deposition of calcium within mitochondria suggests that the primary abnormality may be some disturbance in function of the sarcotubular system.

The strong contraction pattern seen in occasional cells 2 min. after the intraperitoneal administration of isoproterenol indicates an early, almost immediate effect on the contractile mechanism. This progresses to stages of pathologic contraction, with the formation of minor and then major contraction bands.

Minor contraction bands consist of sarcomeres which are between 0.5

and 1.5 μ in length and have wide Z discs. From consideration of the sliding-filament hypothesis and the fact that myosin filaments are 1.5 μ in length,[15] it is clear that myosin filaments have either penetrated Z discs or have become folded. The presence of only straight myofilaments parallel to the long myofibril axis suggests the former alternative. The increased Z-disc width may then be due to myosin filaments penetrating the Z disc.

Herdson *et al.*[6] have suggested that major contraction bands form by the coalescence of several sarcomeres. The sequence of events noted here supports this hypothesis. In the 8-min. preparations, in which minor contraction bands are being replaced by major contraction bands, extremely short sarcomeres are present with apparent coalescence of the wide Z discs of adjacent sarcomeres (Fig. 5).

The disappearance of contraction bands at about 2 hr. returns the myofilaments to a near normal condition, with sarcomere lengths of about 1.6–2.0 μ but with a remarkable absence of Z-disc material. This loss cannot be easily explained but may be similar to the solubilization of Z-disc material by sulfhydryl reagents under certain conditions.[16] If, indeed, vascular insufficiency plays a role in the development of this isoproterenol-induced lesion, as the studies of Handforth suggest,[17] then the resulting anoxia might duplicate the reducing environment of sulfhydryl reagents and cause the presumed disulfide reduction necessary for this phenomenon.

Summary and Conclusions

Low doses of isoproterenol were administered to rats with the hope of producing myocardial hypertrophy, but were found to produce focal myocytolysis. The resulting inflammation is presumably responsible for the increase in weight and content of DNA, RNA, and protein.

Hypercontraction of occasional cells could be demonstrated by electron microscopy within 2 min. of the intraperitoneal injection of isoproterenol. By 8 min. muscle cells showing contraction bands also showed foci of mitochondrial calcification. This sequence suggests early damage of, and release of calcium from, the sarcoplasmic reticulum, with uptake of this calcium by metabolically competent mitochondria.

As the lesions progressed, the contraction bands "relaxed," but Z discs were no longer present. It is therefore presumed that contraction band formation, or relaxation, is related to solubilization of Z-disc material, such as is seen when sulfhydryl reagents act on isolated muscle fibers.

By 8 hr. after drug administration, there were macrophages and poly-

morphonuclear cells within damaged muscle fibers, and phagocytosis of myofilaments and other cell components were apparent. This continued until cell fragments were no longer present and fibrosis had begun.

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[*Illustrations follow*]

Legends for Figures

Fig. 1 and 2. Myocardium 48 hr. after a single intraperitoneal injection of 0.5 and 5.0 mg. isoproterenol, respectively. In each illustration, there is a localized zone of inflammation. Necrosis of myocardial fibers is evident in Fig. 2. Hematoxylin and eosin. × 300.

Fig. 3. Portion of myocardial fiber from control animal. Mitochondria are compact and have regularly distributed cristae. Z bands *(arrows)* are uniformly spaced and are separated from A bands by pale I bands containing myofibrils. Glycogen is present as punctate dense granules. Lead citrate. × 17,000.

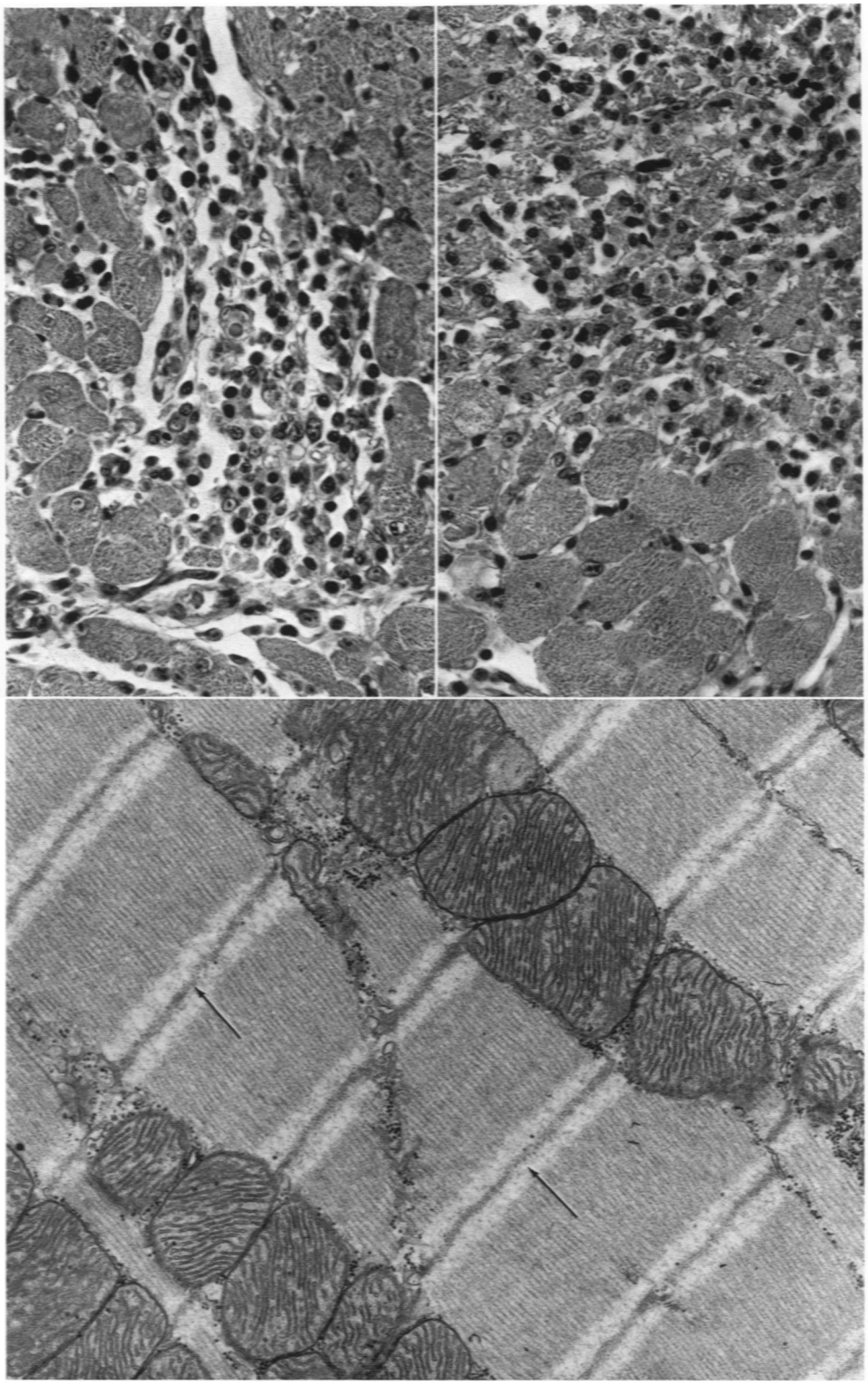
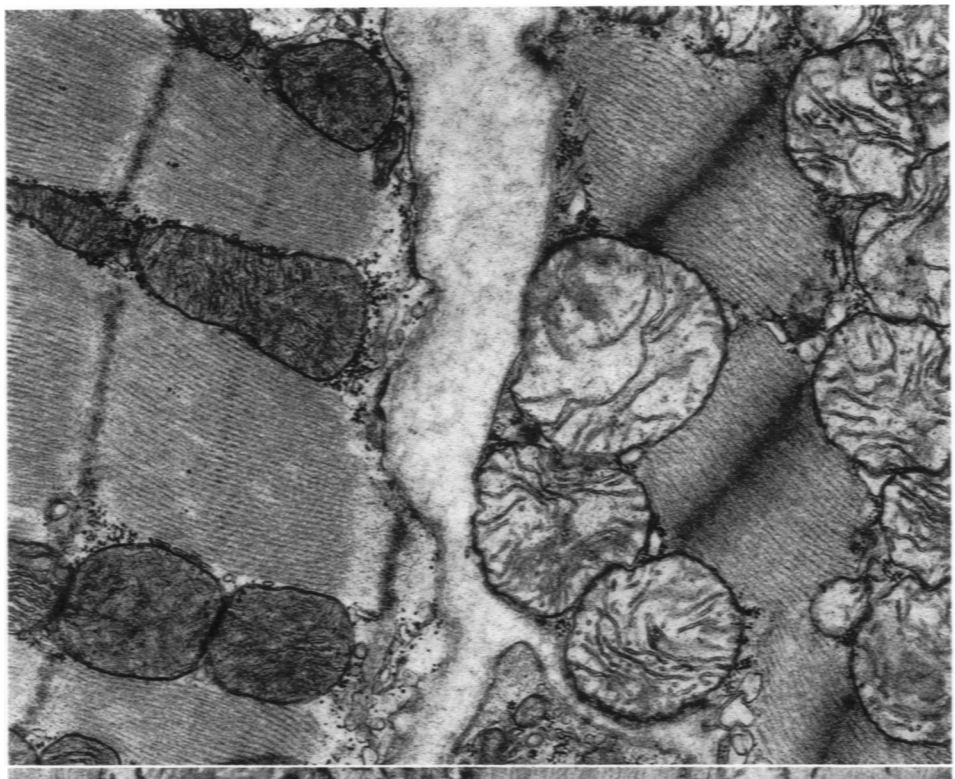


Fig. 4. Portion of 2 myocardial fibers 2 min. after administration of isoproterenol. Fiber at left is normal. There is swelling of mitochondria and thickening of Z discs in the fiber at right. Glycogen is normally distributed in each. Lead citrate. × 15,500.

Fig. 5. At 8 min. after isoproterenol, there is thickening and irregular approximation of Z discs. Discrete zones of increased density are present in mitochondrial matrix. Glycogen is diminished. Lead citrate. × 15,500.



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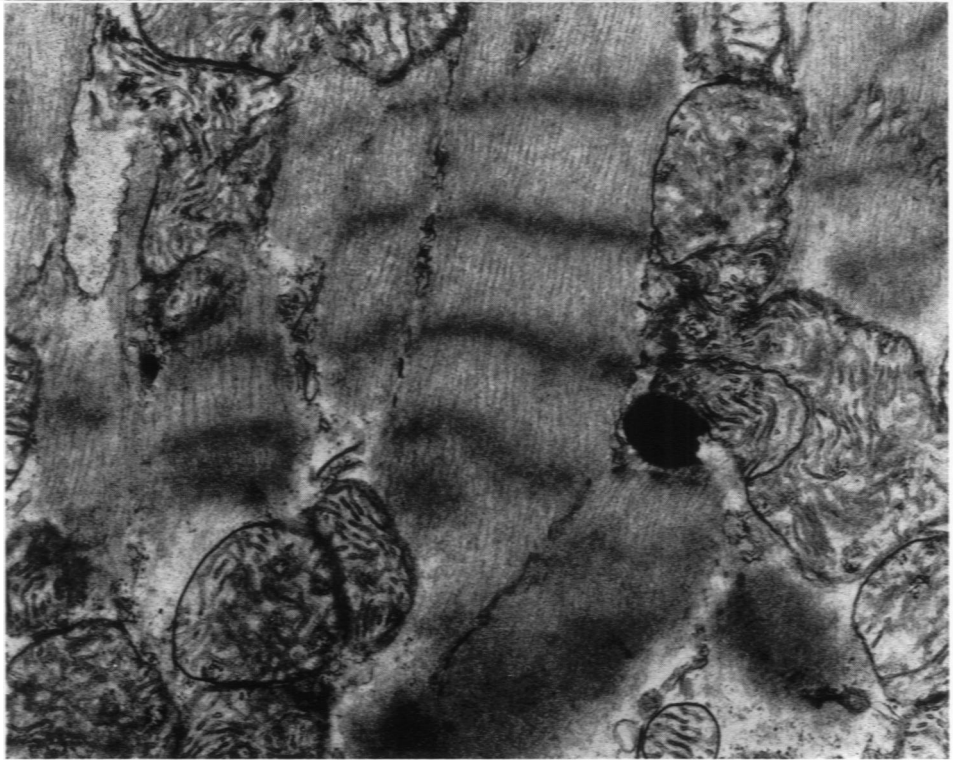

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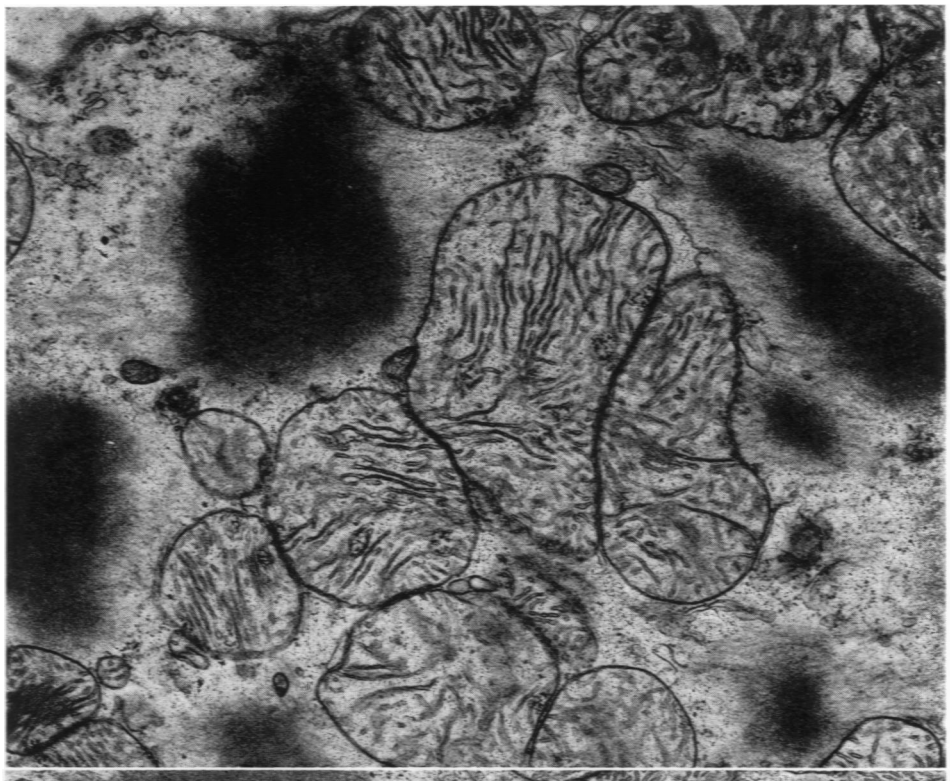
Fig. 6. At 8 min. after isoproterenol, fusion of Z discs has occurred, producing major contraction bands. Punctate densities are present in mitochondria. Mitochondrial cristae are intact. Glycogen is diminished. Lead citrate. × 15,500.

Fig. 7. At 15 min. after isoproterenol, major contraction bands are present. Some filaments extend out from the bands. Mitochondria are intact. Glycogen is reduced. Lead citrate. × 15,500.



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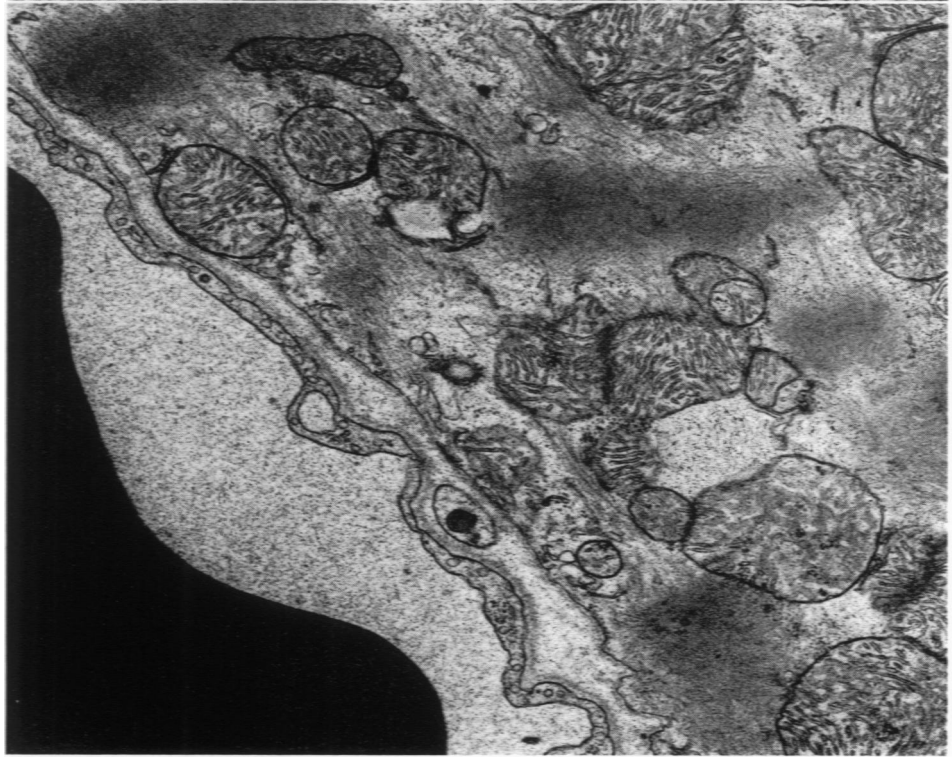

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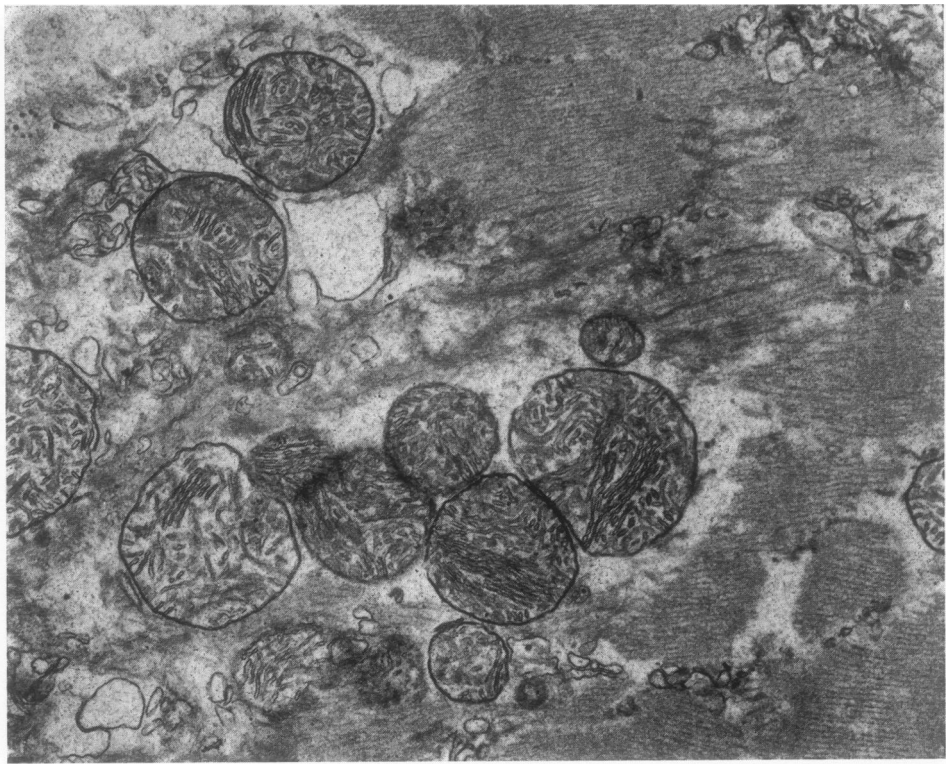

Fig. 8. At 30 min. after isoproterenol, there is disruption of myofibrils and dilation of some transverse tubules. Mitochondria with typical cristae are present although punctate dense deposits are scattered in the matrix. Glycogen is reduced. Lead citrate. × 15,500.

Fig. 9 and 10. At 2 hr. after isoproterenol, myofilaments are discernible. Z-disc material is absent. Major and minor contraction bands are not evident. Amorphous and punctate densities are present in the mitochondria. Lead citrate. × 15,500.

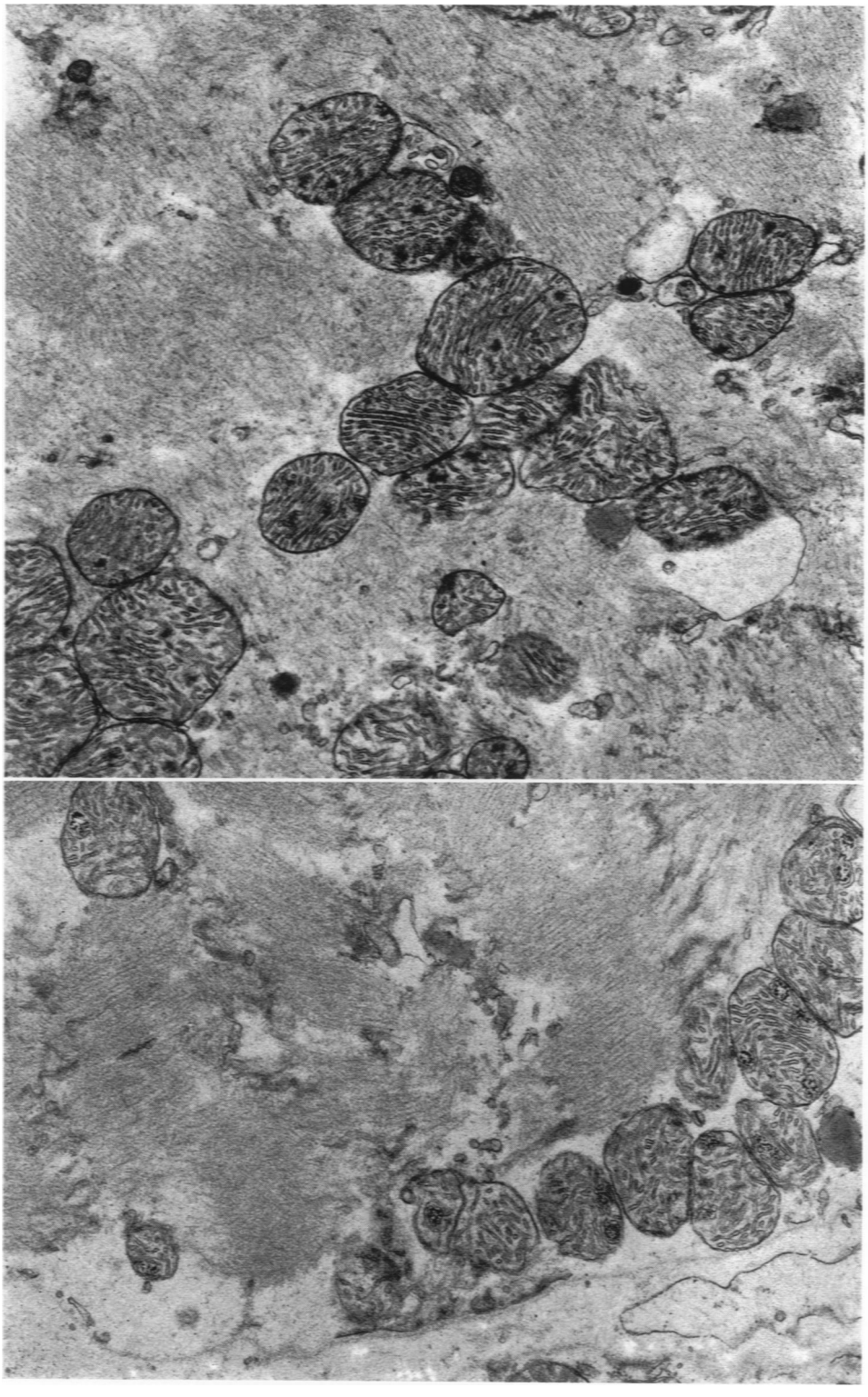

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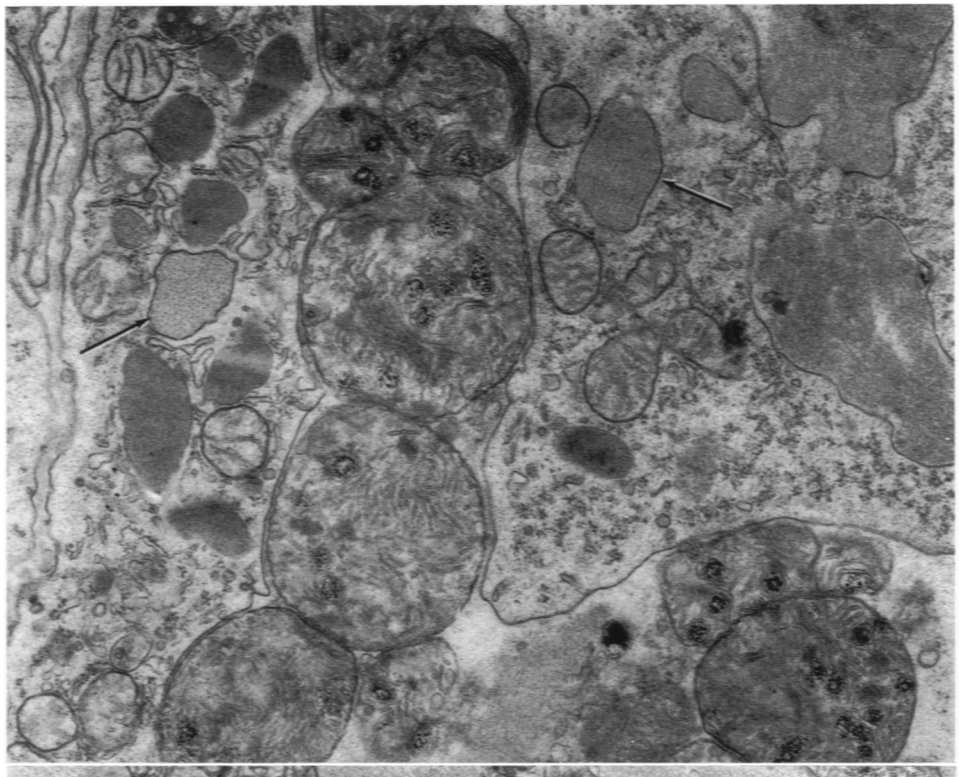
Fig. 11. At 8 hr. after isoproterenol, myofilaments are sequestered in membrane-bound vacuoles within cell processes *(arrows)*. There are myofilaments and mitochondria in the interstitial space between cell processes. These mitochondria are larger than those in the cell processes and contain amorphous and discrete densities. Lead citrate. × 15,500.

Fig. 12. At 8 hr. after isoproterenol, there is disruption of the sarcolemmal membrane. Myofilaments with loss of striations are evident. Mitochondria are well preserved and contain a variety of electron-dense deposits. Lead citrate. × 15,500.



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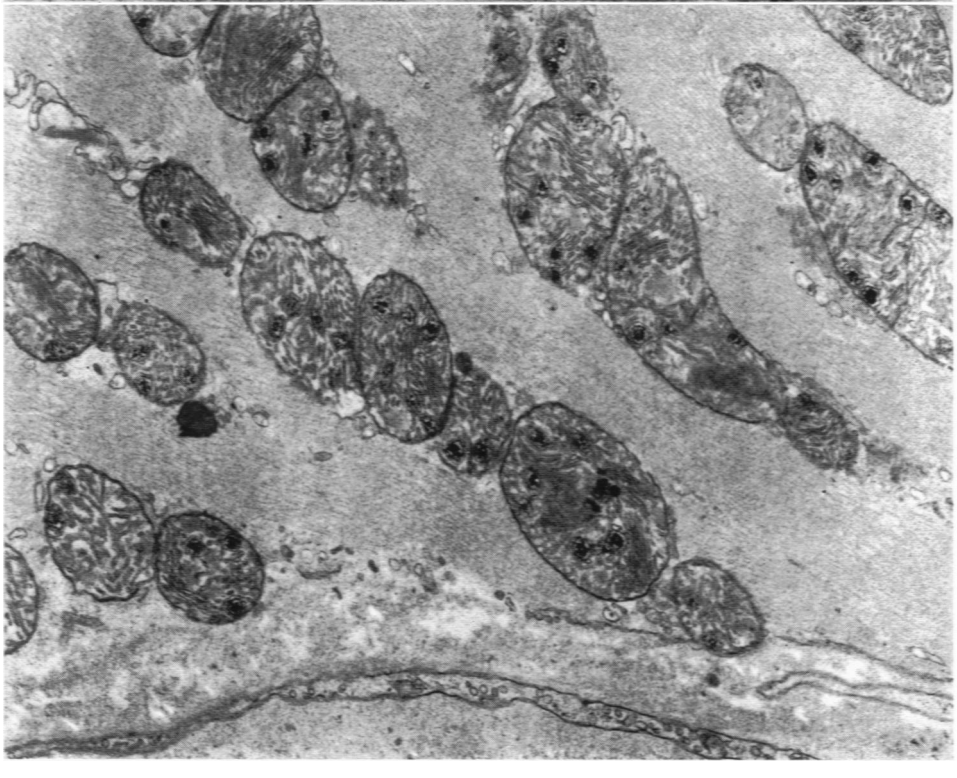

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