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Cytochemical Localization of Lysosomal Enzyme Activity in Normal and Ischemic Dog Myocardium

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The effect of ischemia on the integrity of myocardial lysosomes was observed 3½ and 24 hours after the production of infarcts in 20 anesthetized closed-chest dogs by electrically induced thrombosis of the left anterior descending coronary artery. Biopsies from normal, marginal and infarcted areas were fixed and incubated to localize the lysosomal enzymes acid phosphatase and aryl sulphatase. Reaction product in normal cells was localized in small circular or oblong profiles between bundles of myofilaments and adjacent to mitochondria. In addition, curvilinear, membrane-bound profiles containing reaction product were found in close apposition to transverse tubules and near the free margins of the myocardial cells. Thus the distribution of acid phosphatase and aryl sulphatase activity in myocardium corresponds to the distribution of elements of the sarcoplasmic reticulum. Additional reaction product was also seen in residual bodies, on myelin figures, and in the few conventional appearing spherical lysosomes. Little or no acid phosphatase or aryl sulphatase reaction product was seen in the sarcoplasmic reticulum of infarcted myocardium. The degree of cellular degeneration correlated with disappearance of enzyme activity from the sarcoplasmic reticulum and included disruption of membranes and loss of mitochondrial matrix and erosion of I but not A bands. Marginal areas showed variable amounts of cellular degeneration. Separation of myofilament bundles and loss of glycogen correlated with the localized disappearance of acid phosphatase and aryl sulphatase activity in marginal tissue. Disruption of mitochondria and erosion of I bands correlated with extensive loss of these enzymes. The data suggest that degeneration of myocardial cells following ischemic injury is associated with release of endogenous lysosomal enzymes from the sarcoplasmic reticulum. (Am J Pathol 79:193–206, 1975)

ISCHEMIA and its consequent intracellular acidosis are known to disrupt lysosomes in various cell types,[1–3] permitting acid hydrolases

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to gain access to the cytosol. Such intracellular release of hydrolytic enzymes may be one of the mechanisms by which myocardial ischemia leads to cell injury and death. Fractionation studies of ischemic cardiac [4–8] and skeletal muscle [9] support this hypothesis by showing that lysosomal marker enzymes shift from the particulate to the soluble fraction within the first few hours of ischemia. Loss of membrane-bound acid phosphatase activity is detectable as early as 1 hour after initiation of ischemia.[8] The total activity of lysosomal enzymes in whole homogenates of ischemic myocardial tissue first decreases and then increases after 24 hours [4,5] as phagocytic cells migrate into the area of necrosis.[10]

The hypothesis that release of hydrolases from myocardial lysosomes is important in the pathogenesis of ischemic injury has been challenged, since some studies have shown that myocardial cells contain few lysosomes.[11–13] Most of the lysosomal enzyme activity localized by cytochemical procedures was found in residual or lipofuscin bodies in the perinuclear region.[11] Only a few lysosomes can be located in the distal regions of the cell.[13] Furthermore, electron microscopic observation of irreversibly injured ischemic myocardial cells seemed to show no alteration in residual bodies, and their limiting membranes appeared intact.[14] The redistribution of acid hydrolases from the particulate to the soluble subcellular fraction after ischemia might only reflect changes in lysosomes from nonmyocardial cells such as endothelial and interstitial cells.[14] However, fractionation [8,15–18] and cytochemical [13,19–21] studies of skeletal and myocardial tissue suggested the possibility that acid hydrolases might be located in the sarcoplasmic reticulum (SR) of muscle cells as well as in classic primary and secondary lysosomes and Golgi bodies. Our study was designed to determine whether the SR of myocardial cells does indeed normally contain acid hydrolases and whether these hydrolases are released during early ischemic injury.

Materials and Methods

Acute myocardial infarctions were induced in 20 anesthetized, closed-chest dogs by electrically induced coronary thrombosis.[22] Infarcted, normal (remote) and "marginal" samples were obtained with Vim-Silverman biopsy needles (No. 14) from the still-beating hearts 3½ or 24 hours after infarction and were fixed for electron microscopy. Selection of biopsy sites for infarcted and normal areas was simple, but selection of "marginal" areas was less precise. In the 3½-hour experiments, these sites were estimated primarily by their location with respect to the site of the thrombotic occlusion of the left anterior descending coronary artery used as the apex of an infarct "triangle." In the 24-hour experiments, "marginal" samples were taken 1 cm from the edge of the frank infarct. Two dogs were sham-operated, with insertion of coronary catheter, but no current was applied. Tissue samples were taken 24 or 96 hours later.

Electron Microscopy

Biopsy specimens were fixed for 1 hour at 0 to 4 C in a solution of 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, containing 0.1 M sucrose.[23] They were then washed three times in 0.1 M cacodylate buffer containing 5.13% sucrose and cut into small pieces with a sharp scalpel. The epicardial and endocardial pieces (2 mm each) were discarded and the remaining tissue washed in fresh buffered sucrose overnight at 4 C. After incubation in the appropriate cytochemical media the tissues were rinsed briefly in 0.1 M cacodylate buffer, postosmicated in 1% osmium tetroxide in 0.1 M cacodylate buffer at room temperature, dehydrated in acetone and embedded in Epon. Sections were cut with a diamond knife on a Porter Blum MT2B. Stained (lead citrate) and unstained sections were viewed on a Zeiss EM9S.

Cytochemistry

Acid Phosphatase

Two stock solutions were made up for the enzyme incubation. Solution A: 3.78 g β-glycerophosphate were dissolved in 50 ml 0.05 M acetate buffer, adjusted to pH 5.0 with 1.0 M acetic acid and diluted to 100 ml. Solution B: 1.1 g $PbNO_2$ was dissolved in 84 ml 0.05 M acetate buffer, the pH adjusted to 5 with 0.05 M NaOH, and H_2O added to 100 ml. Nine parts A and one part B were mixed, heated at 57 C for 1 hour, then filtered. Sucrose (0.68 g) was added for each 10 ml of filtrate, and 1 ml of dimethylsulfoxide (DMSO) was added for each 9 ml of reaction mixture. Tissue was incubated for 1 to 2 hours at 37 C with periodic agitation. Controls were incubated with 0.01 M NaF or without substrate.

Aryl Sulphatase

This procedure was modified from that of Hopsu-Havu *et al.*[24] One hundred and sixty milligrams *p*-nitrocatechol sulfate were dissolved in 4 ml H_2O, 12 ml 0.01 M acetate buffer, pH 5.5, and 4 ml of either 5% $BaCl_2$ or 8% $Pb(NO_3)_2$. The pH was adjusted to 5.5 with 0.2 M acetic acid, 513 mg sucrose was added for each 10 ml of solution, and DMSO was added for a final concentration of 10%. Controls were without substrate.

Results

Normal Acid Phosphatase

Membrane-bound acid phosphatase activity was detected cytochemically in numerous locations within myocardial cells. A coarse, crystalline precipitate of lead phosphate reaction product could be seen in residual bodies, a few Golgi cisternae and a few small primary lysosomes located near the reactive Golgi and in the mitochondrial rows. The most striking finding was the ubiquitous presence of acid phosphatase reaction product within elements of the SR (Figure 1). Dense masses of lead phosphate precipitate filled the lateral sac components of the diads and triads as well as those at the cell periphery, making them extremely conspicuous even at low magnification or in unstained sections. Reaction product was also visible filling round or elongated profiles of the longi-

tudinal SR components (Figures 1 and 2). No such precipitates were seen in control specimens incubated without substrate or in the presence of NaF. No differences were observed between tissue samples taken from sham-operated dogs or normal tissue taken from portions of ventricular wall remote from the ischemic area in infarcted hearts (Figure 3).

Aryl Sulphatase

When barium was used to trap the sulphate ions liberated by enzymatic activity, distinct reaction product was commonly seen in only primary lysosomes of the membranous variety described by Topping and Travis,[13] and the elements of the SR appeared to contain only a few fine, inconspicuous grains. However, on substitution of lead nitrate for barium chloride, a fine granular precipitate clearly different from the coarse acid phosphatase reaction product was localizable in the SR (Figure 4) and was not seen when the substrate, *p*-nitrocatechol sulphate, was omitted.

Aryl sulphatase reaction product was seen throughout the SR in both the lateral sacs and longitudinal elements. It was also seen in numerous discrete deposits near the mitochondrial outer membrane. This additional reaction product may represent the activity of aryl sulphatase C, a nonlysosomal enzyme that has a pH optimum of 8 but some activity at pH 5.5.[25] Attempts were made to increase this staining by incubating the tissue at pH 8 or to eliminate it by incubation at pH 4.2 (the pH optimum of the lysosomal aryl sulphatase A). Both attempts were unsuccessful since the ultrastructure of the cells was disrupted at these pHs.

Infarct

Severely ischemic cells from the center of 3½- and 24-hour infarcts were morphologically and cytochemically indistinguishable. Mitochondria were swollen, with amorphous matrix densities and vesiculated cristae. The spaces between myofibrils, which normally contain much glycogen, were electron lucent, devoid of stainable glycogen and retained very few vesiculated elements of the SR. Myofibrils themselves were frequently relaxed, with disrupted lines and partial or complete erosion of I bands (Figures 5 and 6).

Acid phosphatase activity was much reduced within ischemic cells as compared to normal cells. The residual enzyme activity had the same specific localization as in normal cells, but the sites at which it could be identified were sparser. For example, all T tubules in normal cells were conspicuously marked by lateral sacs filled with dense reaction product,

but only a few T tubules in ischemic cells were so outlined (Figure 5). Similarly, reaction product between the myofibrils was limited to a few rounded vesicles and was not seen in elongated profiles. A relationship appeared to exist between the state of preservation of sarcomere organization, particularly I bands, and the number of SR sites with cytochemically localizable acid phosphatase activity (Figure 7). Virtually no reaction product was visible in those cells or portions of cells in which I bands appeared eroded (Figures 5 and 6).

Similar results were obtained in tissue incubated for aryl sulphatase activity, that is the specific localization of the enzyme remained the same but the number of SR sites with enzyme activity were far fewer.

Marginal Zone

Partially injured tissue obtained from areas marginal to the severely ischemic zone receives a somewhat better collateral circulation and individual cells from this tissue show varying degrees of injury.[22,26,27] Such marginally injured tissue is relatively easy to obtain in early (3½ hour) ischemias, but by 24 hours this zone at the edge of the infarct has become very narrow and is easily missed when biopsies are taken. Thus, all of the sections cut from tissue at the edge of 24-hour infarcts consisted largely of cells with the morphologic and cytochemical characteristics of either completely normal or severely ischemic cells. Consequently the following description applies to the marginal zone of 3½-hour infarcts.

Ischemically injured cells from this zone showed evidence of severe cytoplasmic injury in the presence of mitochondria that were either normal or only slightly injured.[22] Frequently, the spacing between myofibrils was seen to be greater than in normal cells, and the cytoplasm between myofibrils around mitochondria and at the free margins of the cells became electron lucent. Stainable glycogen was no longer visible in those regions, although it could still sometimes be seen between myofilaments. Whole cells and, in some instances, parts of cells showed this kind of injury, and such cells could be seen adjacent to morphologically intact cells. Within these injured cells the frequency of acid-phosphatase- or aryl-sulphatase-positive SR elements was greatly reduced from that seen in nearby intact cells (Figures 8 and 9).

Discussion

The lysosomal enzymes acid phosphatase and aryl sulphatase appear to be normal constitutents of SR in canine myocardium. Their activity can be localized cytochemically in lateral sacs associated with the sarcolemma and with T tubules as well as in the longitudinal SR ele-

ments. So widespread is the distribution of SR in myocardial cells, and so ubiquitous the presence of acid phosphatase activity within the SR, that membrane-bound reaction product can be seen in orderly arrays throughout normal cells. Ischemically injured myocardial cells from 3½-hour and 24-hour infarctions show cytoplasmic damage that correlates with reduction in number of acid-hydrolase-positive elements of the SR. Thus, severely injured cells show little reaction product; that which is present is located within lateral sacs or vesiculated elements of the SR. Moderately damaged cells show a smaller reduction of these acid-hydrolase-positive vesicles than do severely injured cells. Morphologic features of ischemic injury to myocardial cells include intracellular edema and the loss of stainable glycogen.[14,22,26,27] Severely injured cells are completely devoid of glycogen, but moderately injured cells from the marginal zone of 3½ hour infarcts may show depletion of glycogen in only a few areas of cytoplasm. Those areas contain fewer acid-phosphatase-reactive SR elements than do adjacent areas of the same cell. These data, in addition to other work [22] which shows that such lytic injury to the cytoplasm can occur in the presence of an intact sarcolemma, suggest that at least some of the pathology found in ischemic myocardial cells may result from the intracellular release of lysosomal hydrolases from the SR.

The cytochemical localization of acid hydrolases in the SR of rat cardiac [13] and skeletal muscle [19,20] has previously been reported by other investigators. However, relatively few reaction-positive SR profiles were demonstrated. The essential difference between their methods and ours was that we have employed DMSO to increase the rate of deposition and amount of reaction product [28] so that small amounts of enzyme activity could be localized within restrictive limiting membranes.[15] DMSO presumably acts by increasing the accessibility of the substrate to the fixed enzyme without altering its specific localization.[28,29] Fractionation studies of skeletal muscle tend to support this localization of acid hydrolases by indicating that a population of lysosomes exists in this tissue that have properties distinct from those of phagocytic cells. These lysosomes contribute 95% of the cathepsin D and acid phosphatase activity and 75% of the β-glucuronidase and aryl sulphatase activity.[16] Other investigations have shown that the major peak of β-glucuronidase activity occurs in the same fractions of muscle homogenate that are enriched in fragmented SR, as demonstrated by enrichment of Ca^{++} accumulation [18] and Ca^{++} ATPase activity.[17] More closely related studies of normal and ischemic dog myocardium show a decrease within the ischemic tissue of specific activity of several lysosomal enzymes in both the heavy

mitochondrial lysosome fraction and in a lighter SR-enriched fraction.[8] These studies support the cytochemical evidence reported here which indicates that lysosomal enzymes are normally contained within myocardial SR but disappear from these locations in the course of ischemic injury.

These studies cannot be taken as proving or disproving a primary role for lysosomal enzymes in provoking the tissue injury of myocardial infarction. It is entirely possible that primary changes involving the plasma membrane, myofibrillar elements or mitochondria are responsible for the loss of tissue integrity during ischemia. Nevertheless, these studies provide clear evidence that lysosomes are among those organelles injured early in the course of myocardial infarctions.

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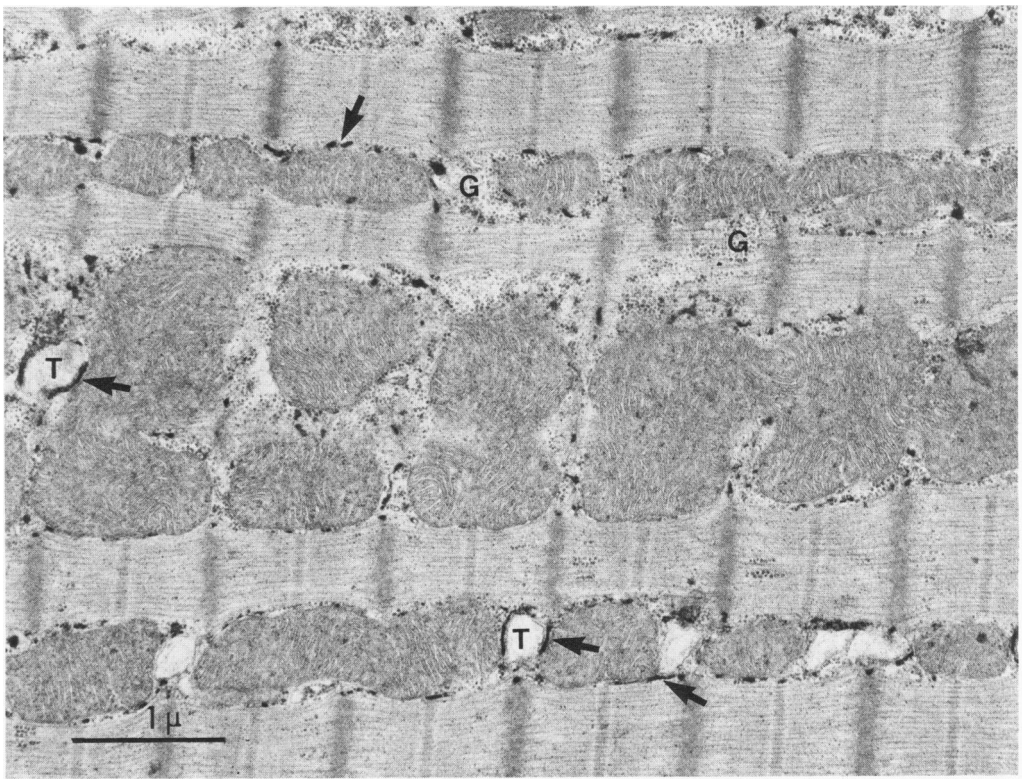

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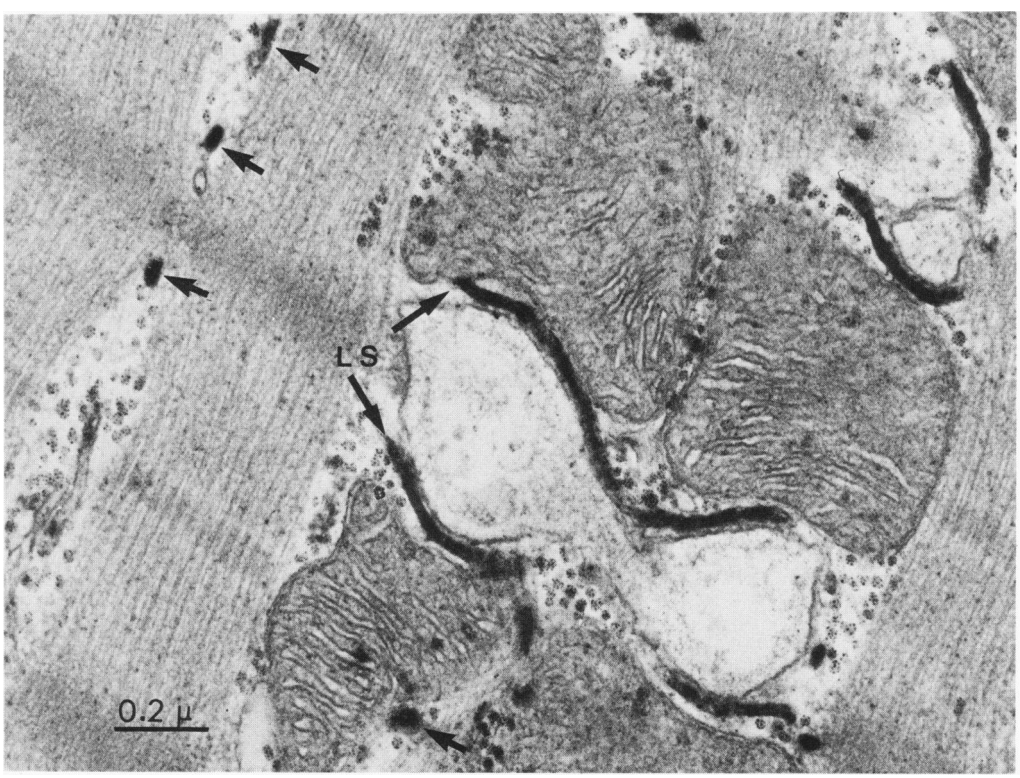

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Fig 1—A portion of a normal myocardial cell reacted to localize acid phosphatase activity. Reaction-product (*arrows*) can be seen adjacent to T tubules (*T*), around mitochondria and between myofibrils. Glycogen (*G*) is present in abundance (Uranyl acetate and lead citrate, × 20,400). **Fig 2**—A higher magnification view of another cell from the same specimen. Reaction-product is seen to lie within the lateral sacs (*LS*) of the SR as well as in other SR elements (*arrows*) (Uranyl acetate and lead citrate, × 60,800).

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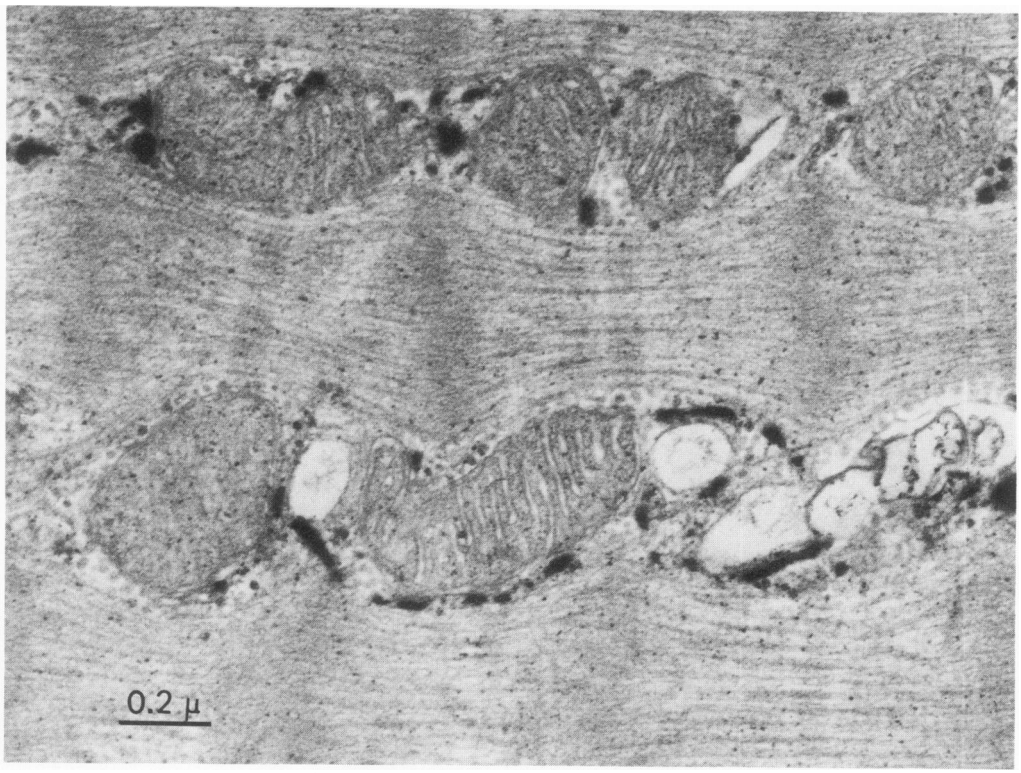

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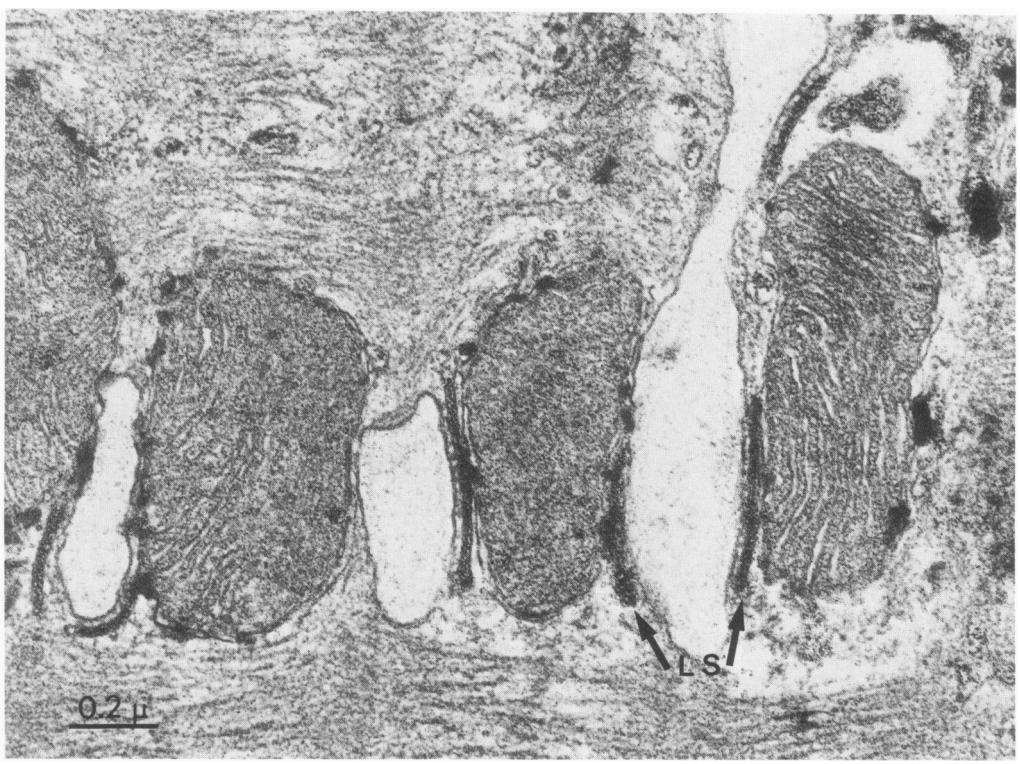

Fig 3—Part of a normal cell from another specimen showing the juxtaposition of mitochondria and acid-phosphatase-containing elements of the SR (Uranyl acetate and lead citrate, × 60,800). **Fig 4**—Part of a normal myocardial cell reacted for aryl sulphatase. Reaction product is conspicuous within the lateral sacs (*LS*) (Uranyl acetate and lead citrate, × 60,800).

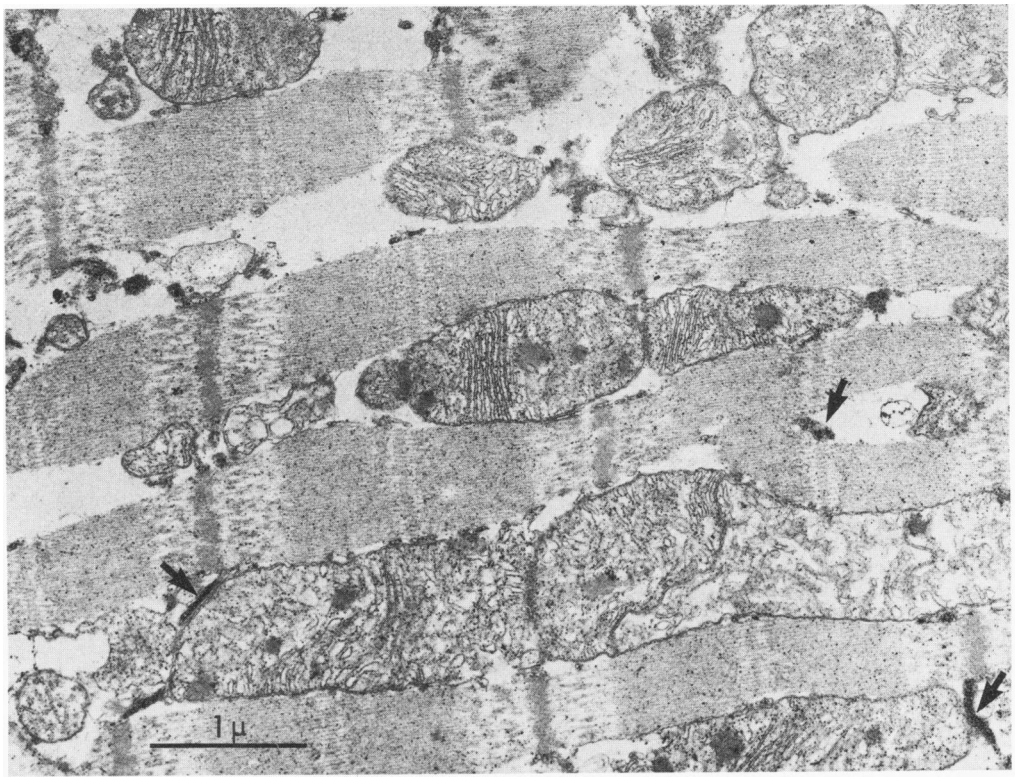

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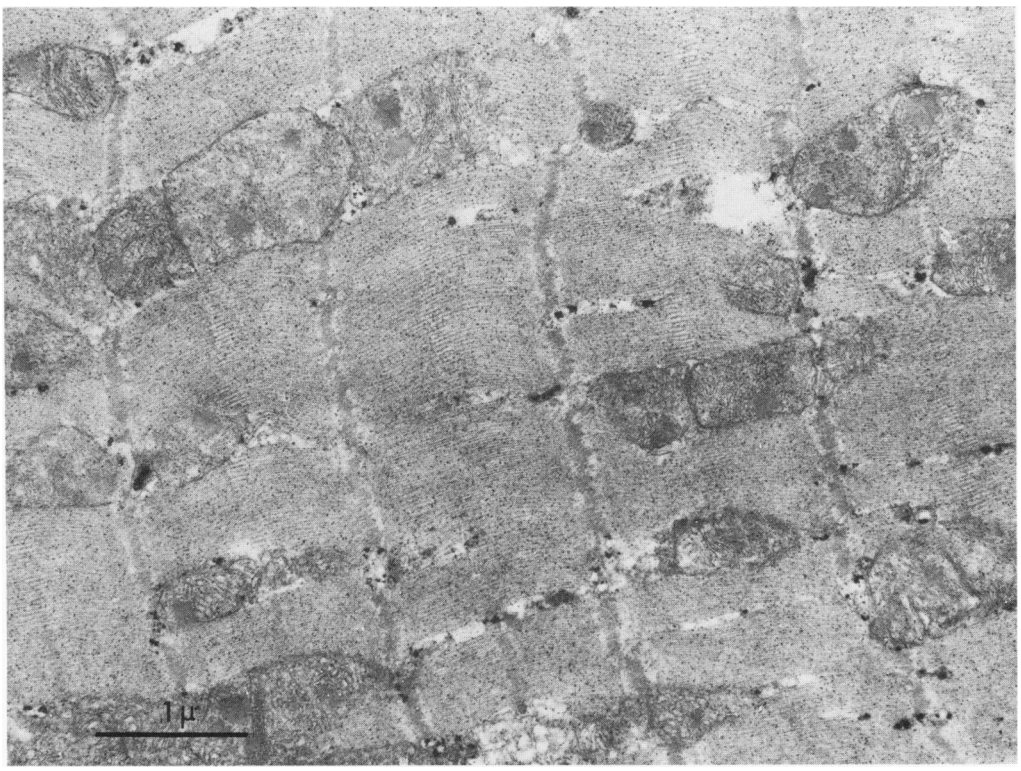
Fig 5—A cell from the center of a 3½-hour infarct. Acid phosphatase reaction-product (*arrows*) is visible in few places. The mitochondria are characteristic of infarcted myocardium, being swollen and containing amorphous matrix densities. The myofibrils are relaxed, and the I bands appear eroded (Uranyl acetate and lead citrate, × 20,400).
Fig 6—Part of a cell from a 24-hour infarct reacted for acid phosphatase. Some of these mitochondria are swollen, and all contain amorphous matrix densities. Myofibrils are relaxed, but the I bands cannot be resolved into filaments, and Z lines are not distinct. A small amount of membrane-bound reaction-product is visible in tiny vesicles (*arrows*) (Uranyl acetate and lead citrate, × 20,400).

Fig 7—A cell from another 24-hour infarct. Although the mitochondria contain amorphous matrix densities characteristic of irreversible ischemic injury, myofibrillar organization is relatively intact. Membrane-bound acid phosphatase reaction product is reduced below that seen in normal cells, but more is seen than in infarcted cells with greater myofibrillar disruption (Uranyl acetate and lead citrate, × 20,400).

Fig 8—A portion of a partially injured cell from the marginal ischemic zone of the same heart used for Figures 1 and 2. This specimen was incubated for acid phosphatase activity in an aliquot of the same cytochemical medium and for the same time as the normal, control specimen. Fewer deposits of membrane-bound reaction product can be seen here than in corresponding areas of control cells, and glycogen appears to be reduced in those portions of the cell with little reaction product *(arrows)* (Uranyl acetate and lead citrate, × 53,800).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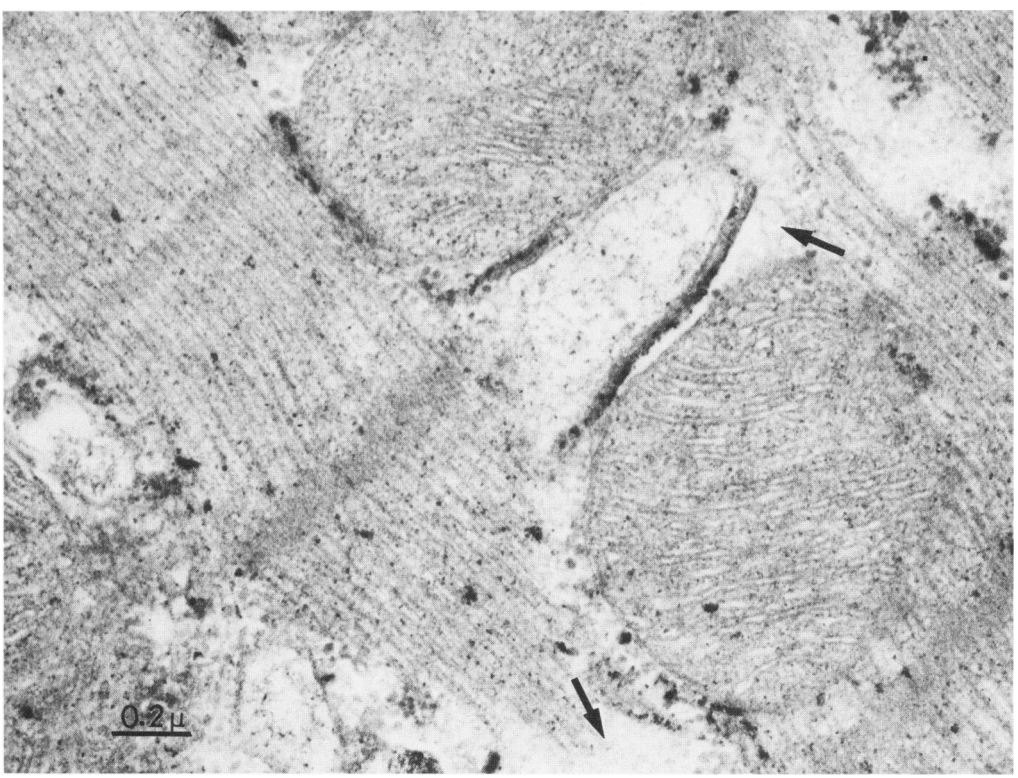
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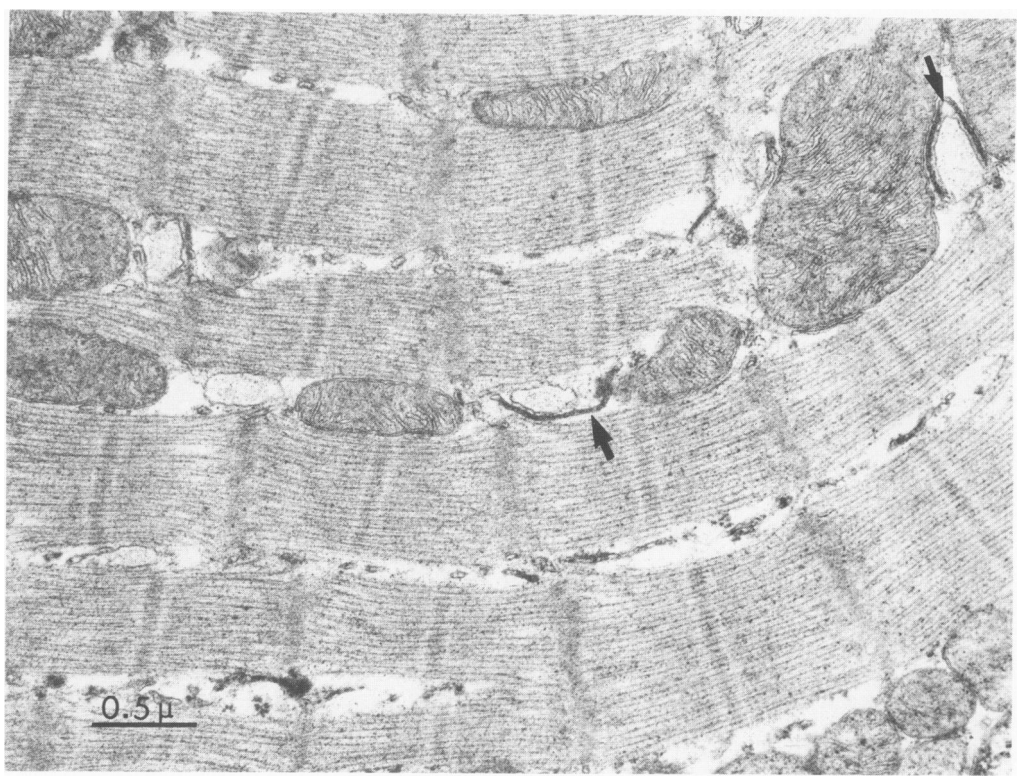

Fig 9—A cell from the marginal zone of another 3½-hour infarct. Although most of the mitochondria appear normal, the cell is depleted of glycogen. Acid phosphatase reaction product can be seen within the SR in fewer locations than in normal cells (*arrows*) (Uranyl acetate and lead citrate, × 26,800).