Irreversible Myocardial Injury in Anoxic Perfused Rat Hearts

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Isolated rat hearts were perfused at 37 C on a double reservoir, nonrecirculating Langendorff apparatus. For aerobic perfusion, Krebs-Henseleit medium containing 10 mM glucose was gassed with 95% O_2–5% CO_2; for anoxic perfusion, glucose was replaced with 10 mM mannitol, a nonmetabolizable substrate, and the medium was equilibrated with 95% N_2–5% CO_2. Heart effluent was serially collected during perfusion for creatine phosphokinase activity (CPK) analysis. Fixation was with 1% glutaraldehyde for morphologic studies. With aerobic control perfusion, hearts continued contracting, released no CPK, and were morphologically normal by light and electron microscopy examination after 120 minutes. With anoxic perfusion, contractions soon ceased, and by 60 minutes a sustained slow release of CPK was first observed. By electron microscopy, cells at 60 or 90 minutes were swollen and contained amorphous matrix densities in mitochondria; a few cells showed breaks in cell plasma membrane. When anoxic hearts were challenged with reoxygenation, there was a sudden change in color to a pale opaque appearance, CPK was rapidly released, and there was massive cellular swelling. By electron microscopy, damaged cells showed contraction bands, clumping of nuclear chromatin, both amorphous densities and granular dense bodies in mitochondria, and prominent disruptions of cell plasma membranes. The number of damaged cells observed increased as a linear function of time between 30 and 55 minutes of anoxia. The results show that anoxic perfusion *in vitro* produces irreversible myocardial injury and that this injury is closely associated with loss of cell volume control, release of intracellular enzymes, and striking structural defects in the plasma membrane of the sarcolemma. Reoxygenation accelerates the development of lesions in irreversibly injured cells but protects reversibly injured cells. (Am J Pathol 80:419–450, 1975)

THE ISOLATED PERFUSED HEART has been used widely to study the effects of anoxia on myocardial metabolism and function.[1-7] However, it has been difficult to apply the results of these studies to the pathogenesis of *in vivo* irreversible myocardial injury because there has been no way to characterize the irreversible state *in vitro*.

In the intact animal, irreversible myocardial ischemic injury is defined unequivocally by the appearance of cell necrosis or fibrous repair 4 to 7 days after a temporary vascular occlusion.[8] Similar long-term perfusion experiments cannot be conducted *in vitro*. However, irreversible myocar-

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dial cell injury can be recognized *in vivo* by characteristic ultrastructural and biochemical lesions. These lesions develop after a few minutes of reperfusion of the ischemic tissue with arterial blood. The irreversibly injured cells swell and show clumped nuclear chromatin, contraction bands, granular and amorphous intramitochondrial matrix densities, and, eventually, a breakdown of all membrane organization.[9-12] Biochemically, they lose intracellular cations, gain Na^+, Cl^-, and H_2O, and release intracellular enzymes such as creatine phosphokinase to the extracellular fluids.[13-16]

In the present study, isolated rat hearts perfused with anoxic media were studied by morphologic and biochemical techniques to determine if changes similar to those observed in irreversible myocardial cellular injury *in vivo* could be produced under controlled conditions *in vitro*. The structural and biochemical changes which occurred following brief periods of anoxic injury were characteristic of irreversibly injured cells *in vivo* and demonstrate that irreversible myocardial injury can be a consequence of anoxic perfusion *in vitro*.

Materials and Methods

Thirty-two hearts were obtained from male specific-pathogen-free Sprague Dawley rats weighing between 200 and 500 g that were fed *ad libitum*. The animals were anesthetized by intraperitoneal injection of 40 mg/kg sodium pentobarbital (Diabutal, Diamond Laboratories Inc., Des Moines, Iowa), and their hearts were rapidly removed and immersed in ice-cold perfusion fluid. After dissection of extraneous tissue, hearts were blotted and weighed, and the aortas cannulated to the perfusion apparatus.

Experimental Design

All hearts were perfused at 37 C with oxygenated Krebs-Henseleit medium (O_2-medium) for an initial 15-minute control period. After equilibration, three experimental protocols were used: a) perfusion with O_2-medium for 120 minutes (controls); b) perfusion with medium equilibrated with 95% N_2–5% CO_2 gas (N_2-medium) for periods of 30, 60, or 90 minutes; c) perfusion of hearts with N_2-medium for 30, 35, 40, 45, 50, or 55 minutes followed by perfusion with O_2-medium, for a total duration of 120 minutes of perfusion.

Perfusion Apparatus

The perfusion was accomplished on a modified double reservoir, nonrecirculating, Langendorff apparatus [17] with both sides connected to the aortic cannula by a three-way stopcock. One reservoir was filled with O_2-medium and was continuously gassed with 95% O_2–5% CO_2, the other was filled with N_2-medium and was continuously gassed with 95% N_2-5% CO_2. Both reservoirs were maintained at 37 C in a water bath. Using a Harvard peristaltic pump, fluid was pumped through tygon tubing to a bubble trap connected by an air pressure reservoir to a mercury manometer. The bubble trap consisted of a water jacketed glass coil connected to a separate circulating water bath. Final perfusion temperature control was accomplished by a thermal probe inserted near the outflow tip of the perfusion cannula. The probe was connected to a Yellow Springs Instruments (Model 73) temperature controller which maintained the circulating water-bath temperature so that the perfusion fluid passing the tip of the aortic cannula was at 37 ± 0.5 C. Perfusion

could be rapidly switched from N_2-medium to O_2-medium and back with little loss of perfusion pressure.

Perfusion pressure was maintained between 60 and 100 mmHg by manual control of the pump rate. Two hearts were perfused at a time using a Y-shaped aortic cannula. Flow rates were individually measured by timed collection of heart effluent in a graduated cylinder. After the 15-minute control perfusion period, flow rates averaged 17.9 ± 0.8 ml/min. With onset of anoxic perfusion, flow rates increased within the first minute to average about 22 ± 0.8 ml/min and gradually decreased thereafter to reach control levels by 30 to 40 minutes of perfusion. With reoxygenation of the anoxic hearts, the flow rates immediately before reoxygenation were 19.3 ± 1.1 ml/min, dropping to 17.8 ± 1.5 ml/min within 1 to 5 minutes of reoxygenation.

Perfusion Fluid

Perfusion fluid was a standard Krebs-Henseleit-bicarbonate medium [18] containing 2.5 mM calcium and 10 mM D-glucose (O_2-medium). Medium for anoxic perfusion (N_2-medium) was the same except that 10 mM mannitol was used in the place of glucose. Osmolality, measured by freezing point depression was 290 to 295 mOsmoles/liter and pH was 7.3 to 7.4. Both media were equilibrated with gas for at least 45 minutes prior to beginning the experiment. This period previously has been shown to reduce the pO_2 of N_2-gassed media to 0 to 5 mmHg.

CPK Analysis

One- to two-ml samples of effluent were collected serially in glass or plastic vials and were stored in crushed ice until the end of the experiments. Creatine phosphokinase activity was measured on 0.1-ml aliquots by the method of Oliver [19] in a Gilford model 3400 automatic enzyme analyzer using reagents obtained from Boehringer Mannheim GMBH (Mannheim, Germany). The results of CPK activity expressed in international units per minute per gram were obtained from the formula given below:

$$\text{IU/min/g} = \frac{\text{CPK activity (IU/ml)} \times \text{flow (ml/min)}}{\text{initial heart weight (g)}}$$

Morphologic Studies

At the end of the experiment, hearts were fixed by perfusion through the aortic cannula with 1% glutaraldehyde in modified Tyrode's solution.[20] Following initial fixation, hearts were removed, and the apex and atria were discarded. Four equally spaced horizontal sections of the heart were taken and processed for light microscopy. In addition, four 0.5-mm thick sections which comprised the entire thickness of the free wall of the left ventricle were cut from tissue adjacent to the light microscope sections and were placed in glutaraldehyde for an additional 1 hour. Following postfixation in osmium tetroxide [21] and staining in aqueous uranyl acetate, these blocks were flat-embedded in Epon 812 (Shell Chemical Co., New York, N.Y.).[22] Semithin sections were stained with toluidine blue for light microscopy, and areas were selected for thin sectioning using diamond knives. Thin sections were mounted on plain copper grids and, following staining with 7.7% aqueous uranyl acetate and lead citrate, were examined in an Hitachi HU-12 electron microscope.

Estimation of Cell Damage

Comparison of adjacent sections of tissue by routine light microscopy and by light and electron microscopy of plastic-embedded tissue confirmed that severely injured myocardial cells, the lateral limits of cells being the intercalated discs,[23] could be identified easily by routine light microscopy. The severely damaged cells were characterized by swelling,

loss of staining intensity, granularity, and the presence of contraction bands. The percent of damaged cells was estimated by direct counts of successive nonoverlapping high-power fields across the entire thickness of the left ventricular free wall on each of the four hematoxylin- and eosin-stained sections from each heart. Approximately 5000 cells were counted from each heart.

Results

Gross Observations

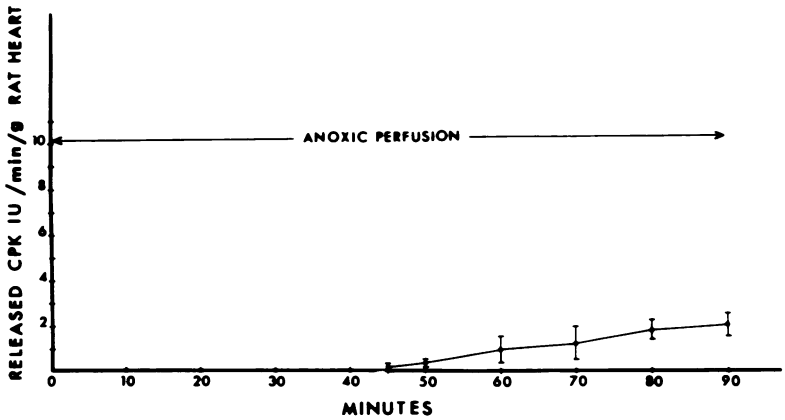
Control hearts perfused with oxygenated medium maintained vigorous spontaneous contractions and a deep reddish color for the full 120 minutes of perfusion. With onset of anoxic perfusion, the myocardium assumed a darkened cyanotic appearance, and contractions briefly increased in force and then gradually decreased in both force and frequency, ceasing after 5 minutes. The anoxic hearts then remained quiescent, with dilated ventricles, for the duration of the perfusion. Beginning after 60 to 90 minutes of anoxia, hearts developed pale, opaque streaks and patches which gradually enlarged, encompassing both ventricles by 120 minutes.

Brief periods of anoxia (30 to 35 minutes) followed by reoxygenation usually resulted in resumption of spontaneous contractions which, however, were often weak compared to control hearts. After longer periods of anoxia there was no resumption of ventricular contractile activity. A striking change occurred in hearts made anoxic for 45 to 55 minutes that were reoxygenated. With reoxygenation, there was a sudden change in color from the dark anoxic appearance to a pale opaque appearance characteristic of necrotic muscle tissue. This change occurred within the first 1 to 3 minutes of reoxygenation and was fully developed by 5 minutes. The result was that hearts subjected to 55 minutes of anoxia and only 3 to 5 minutes of reoxygenation usually appeared more severely injured grossly than other hearts made continuously anoxic for 120 minutes. With briefer periods of anoxia, the color change was progressively less pronounced, so that hearts subjected to only 30 or 35 minutes of anoxia and subsequent reoxygenation had only a few whitish streaks at the end of the perfusion period.

Enzyme Release Studies

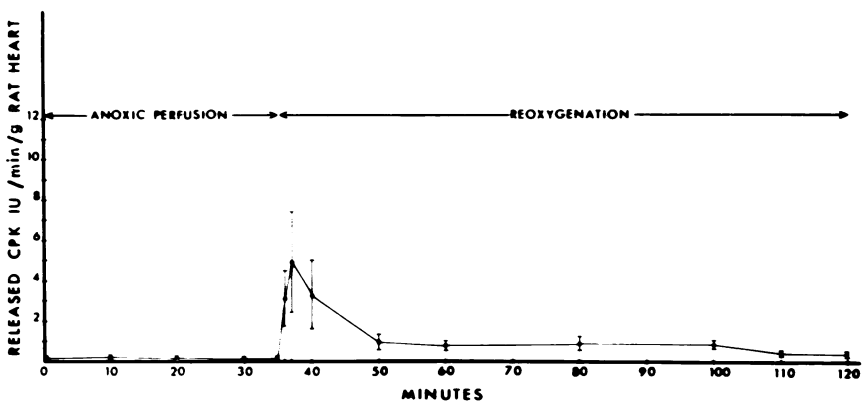
Control hearts perfused with O_2-medium did not release measurable amounts of CPK over a 120-minute perfusion period. During continuous anoxic perfusion of six hearts, a slow, sustained release of CPK activity began at 60.8 ± 2.7 minutes and gradually increased as the anoxic perfusion continued (Text-figure 1).

Anoxic perfusion of hearts followed by reoxygenation resulted in a sudden peak of CPK release which occurred within the first few minutes of

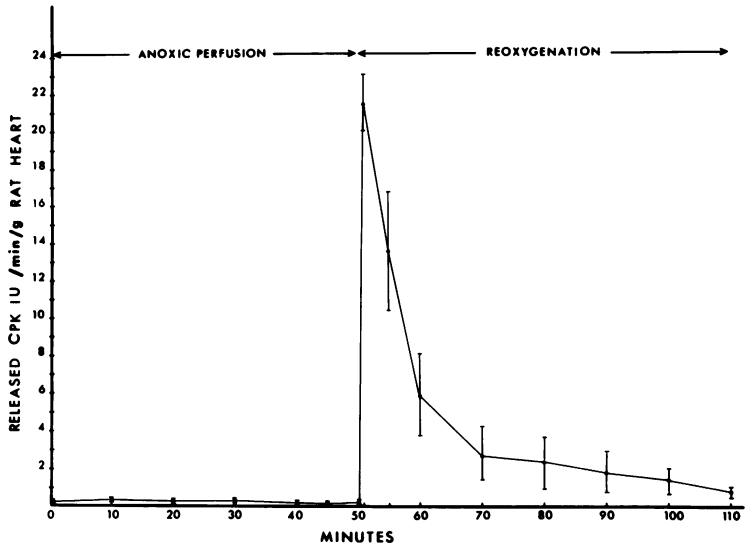


TEXT-FIGURE 1—Creatine phosphokinase (CPK) release from six rat hearts during continuous anoxic perfusion at 37 C in the absence of exogenous substrate. CPK was measured on aliquots of heart effluent. Prior to 45 minutes, all values were 0 or 1 and were not plotted at this scale. Only two hearts released measurable quantities of CPK prior to 60 minutes, accounting for the small rise at 50 minutes. After this time, there was a continuous slow increase in rate of CPK release in all hearts. Control hearts did not release measurable CPK for 120 minutes of aerobic perfusion. Values are given as mean ± SE.

reoxygenation. The CPK release corresponded in time to the abrupt change in color of the hearts, described above. With continuous anoxia, enzyme release began only after 60 minutes, while with reoxygenation, small peaks were observed after as little as 30 minutes of anoxia (Text-figure 2). Shorter intervals were not studied in this experiment. With



TEXT-FIGURE 2—CPK release from four hearts following 35 minutes of anoxic perfusion and then reoxygenation for a total of 120 minutes. Although with continuous anoxia hearts did not release CPK until about 60 minutes, with reoxygenation hearts showed a sudden CPK release after only 35 minutes of anoxia. At the end of the perfusion period, it was estimated by histologic analysis that about 25% of cells were severely injured in these hearts. Values are given as mean ± SE.



TEXT-FIGURE 3—CPK release from four hearts following 50 minutes of anoxic perfusion and then reoxygenation for 70 minutes. The rate and amount of CPK release upon reoxygenation was greater in hearts with longer periods of anoxia. After 50 minutes of anoxia and reoxygenation, it was estimated by histologic analysis that about 76% of cells were severely damaged. Values are given as mean ± SE.

longer periods of anoxic perfusion the peaks of enzyme release at reperfusion became progressively larger and by 50 minutes or longer were quite prominent (Text-figure 3). To determine if the amount of peak enzyme release correlated with histologic evidence of cellular injury, hearts releasing small and large amounts of CPK were examined by light microscopy, and the percent of severely damaged cells was estimated in samples from the left ventricular free wall. Grouped together, hearts made anoxic for 30 to 40 minutes averaged 24.0 ± 5.0% damaged cells, while hearts made anoxic for 45 to 55 minutes averaged 76.5 ± 5% damaged cells ($P < 0.001$). The peak CPK release in the 30 to 40 minute group was 4.6 ± 1.3 IU/min/g, while that for the 45 to 55 minute group was 14.4 ± 2.8 IU/min/g ($P < 0.01$). The threefold increase in both CPK release and damaged cells between the two groups suggests that cell damage and CPK release are related events.

Light Microscopy of Hematoxylin- and Eosin-Stained Sections

Control Hearts

Hearts perfused with O_2-medium for 120 minutes were composed of uniformly dense cells with regular cross striations.

Anoxic Hearts

Cells of hearts fixed after anoxic perfusion for 30 minutes were difficult to distinguish from controls. After 60 or 90 minutes of anoxic perfusion, the cells appeared slightly swollen, and occasional contraction bands were found. In general, the changes were difficult to distinguish from control but seemed somewhat more prominent at 90 than 60 minutes.

Anoxic Hearts Followed by Reoxygenation

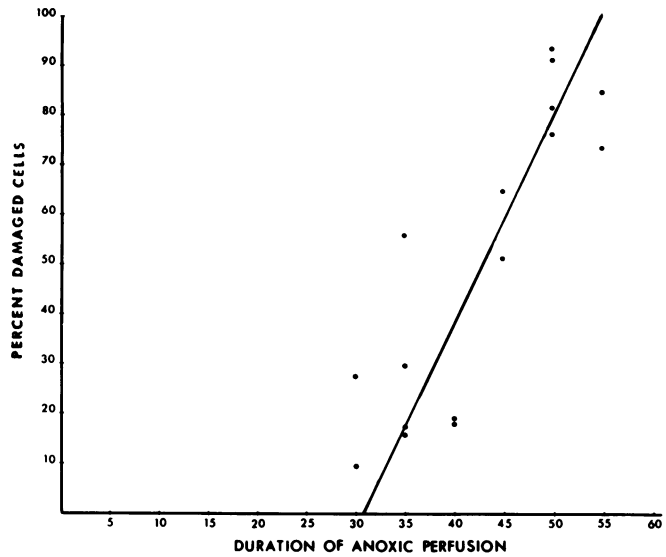
By light microscopy, hearts subjected to varying periods of anoxia followed by reoxygenation for a total of 120 minutes of perfusion showed two populations of cells. The first were uniformly dense and were similar to cells in normal control hearts except that cytoplasmic vacuoles were usually present. The second type of cell was swollen, had granular cytoplasm, loss of normal striations, and often contained prominent contraction bands.

Because in every heart the damaged cells were randomly distributed throughout both the right and left ventricles, it was considered valid to sample the population of cells in the left ventricular free wall and to estimate the percent damaged cells in the heart. When the percent damaged cells was plotted against duration of anoxic perfusion, regression analysis revealed a highly significant linear correlation ($r = 0.87$) (Text-figure 4). From these data, we estimate that about half of the cells of a heart will show histologic evidence of severe injury after 45 minutes of anoxia. Most cells are injured by 55 minutes of anoxia, and relatively few cells are injured by periods of anoxic perfusion of 30 minutes or less.

Although cellular injury was randomly distributed in both ventricular walls, it was observed that in a narrow zone of left ventricular subendocardium and the papillary muscles, myocardial cells usually showed only slight swelling regardless of the duration of anoxia and presence of absence of reoxygenation. Previous studies of regional blood flow in anoxic hearts have shown that injected intravascular colloidal carbon often did not stain the subendocardium or papillary muscles of anoxic hearts. It seems likely that there were narrow subendocardial zones of poor perfusion and that cells in the subendocardium in anoxic hearts are permanently ischemic. A small population of ischemic cells would not significantly affect the results of these studies since we were careful not to include this zone in our samples for analysis.

Light Microscopy of Plastic-Embedded Tissue

Light microscopy of toluidine-blue-stained, plastic-embedded tissue showed changes similar to those described above at a much higher level of



TEXT-FIGURE 4—The percent of severely damaged cells in hearts subjected to 30 to 55 minutes of anoxia and then reoxygenated for a total of 120 minutes perfusion was estimated by direct counts from four equally spaced light microscopic sections of each heart. The percent of damaged cells correlated linearly by regression analysis ($r = 0.861$, $P < 0.001$) with duration of anoxic perfusion between 30 and 55 minutes.

resolution. Control hearts were composed of uniform cells with regular cross striations in longitudinal sections and regular profiles in cross sections. In general, these hearts were indistinguishable from normal controls except that the interstitial spaces were widened in perfused hearts (Figures 1 and 2). Following anoxic perfusion and reoxygenation, two populations of cells were present, corresponding again to the results of routine light microscopy. The first cell type, which predominated in hearts made anoxic for longer than 45 minutes, were greatly swollen, stained pale, and contained numerous granules (Figure 3). In longitudinal sections these cells often contained dense contraction bands (Figure 4). The second cell type, which was most prominent in hearts with brief anoxic perfusions, appeared similar to control myocardial cells except for the presence of cytoplasmic vacuoles.

Electron Microscopy

Control Hearts

All cells in hearts perfused aerobically for 120 minutes closely resembled normal myocardium. Nuclear chromatin was distributed

evenly throughout the nucleus. Sarcomeres were in register, and moderate amounts of glycogen were distributed throughout the myoplasm. Transverse tubules were distended but were usually not dilated. Mitochondria were uniformly dense and contained moderate numbers of normal matrix granules (Figures 5 and 6). Intercalated discs were intact. The plasma membrane was continuous and closely applied to the cell. There were numerous small pinocytotic vesicles.

Anoxic Hearts

The changes seen after substrate-free anoxic perfusion varied according to the duration of perfusion. After 30 minutes of anoxia, overall cell structure was maintained. However, mitochondria of most cells appeared slightly swollen and were less dense than controls. Also, normal matrix granules were less prominent. Occasionally, focal cytoplasmic swelling was observed, and vacuoles were randomly distributed in the cytoplasm (Figure 7). The number of pinocytotic vesicles were reduced from that seen in control hearts.

Following 60 minutes of anoxic perfusion, when most cells were destined to show progressive changes despite reoxygenation, additional and more prominent changes were present. Nuclear chromatin was clumped, leaving clear areas in the nucleoplasm. Mitochondria often were moderately to greatly swollen, and contained amorphous matrix densities (Figure 8) which, however, were small and scattered. Along with the mitochondrial changes, the cytoplasm usually appeared swollen, and glycogen stores were diminished. The sarcoplasmic reticulum often was dilated and formed small vesicular structures (Figures 8 and 9). Sarcomeres were often out of register; in some cells, there was thickening of Z-lines (Figure 10). In a small number of cells, contraction bands were present (Figure 11). The transverse tubular system often was greatly dilated. In many cells the plasma membrane focally separated from the surrounding basement membrane (Figure 9). Where the separation of basement and plasma membranes occurred, the space often contained small vesicular whorls and blebs of membranous material. Following 90 minutes of anoxic perfusion the cellular changes consisted of moderate accentuation of the changes described at 60 minutes. Cell swelling was more pronounced, amorphous matrix densities were found commonly, and clumping of nuclear chromatin was more marked (Figure 12). In addition, there were more cells showing severe swelling and contraction bands than were observed at 60 minutes. Cells showing severe changes, however, accounted for only a small percentage of the total number of cells even after 90 minutes of continuous anoxic perfusion.

Anoxia Followed by Brief Reoxygenation

In contrast to the relatively slow progression of cellular changes seen with continued anoxic perfusion, there were sudden and striking changes in hearts subjected to reoxygenation. Hearts subject to 50 minutes of anoxia and only 3 minutes of reoxygenation appeared quite different than those subjected to 60 or 90 minutes of anoxia without reoxygenation. The most striking change was massive cellular swelling (Figures 13 and 14). Swollen cells could be classified by their mitochondrial morphology. Some cells had massive swelling of both mitochondria and cell cytoplasm. In these cells, amorphous mitochondrial matrix densities were usually evident (Figures 13 and 14). Other cells were also swollen, but the swelling was due largely to prominent dilatation of both sarcoplasmic reticulum and transverse tubules. In the latter cells, there was a mixture of swollen mitochondria and contracted mitochondria with a dense matrix space and dilatation of the intracristal spaces (Figure 15). The changes may represent either different stages of swelling or cells with different degrees of mitochondrial damage prior to reoxygenation. Discontinuities in sarcolemmal membranes were observed commonly in greatly swollen cells (Figure 16).

Anoxia Followed by Long Periods of Reoxygenation

Consistent with light microscopy, there were two types of cells. Hearts subjected to periods of anoxia from 30 to 55 minutes with subsequent reoxygenation for a total perfusion period of 120 minutes were composed of two distinct populations of cells.

The *first cell type* was the severely damaged cell that was swollen massively and usually contained contraction bands (Figures 17 and 24). In almost all cases there was a bleb-like space beneath the sarcolemma in which membranous vesicles and greatly swollen mitochondria appeared to be floating. In most swollen cells in any given section, the plasma membrane was focally discontinuous, and only remnants of membranous vesicles remained attached to the cellular surface of the basement membrane (Figures 18 and 19). Along with the cytoplasmic swelling was massive uniform swelling of all mitochondria, which in contrast to cells of hearts with brief reoxygenation, contained large, amorphous, matrix densities (Figures 20 and 21). In addition to the amorphous densities many mitochondria contained distinctly different granular inclusions. These appeared either as annular dense bodies (Figures 20 and 22) or as focal area of matrix, partially surrounded by cristae membranes (Figure 21), which contained dense granules (Figures 20–22). Both types of granular dense bodies had the same structure as calcium accumulation granules which oc-

cur in myocardial,[9-11] renal,[24] or liver [25] mitochondria during calcium accumulation.

In addition to swelling and matrix inclusions there was a third mitochondria lesion consisting of material in the intercristal space. This appeared as a periodic double line when viewed in properly oriented sections (Figure 23) and in tangential sections had a paracrystalline structure (Figure 20).

The *second cell type* resembled normal control myocardial cells (Figures 24–26). There was no apparent cytoplasmic or mitochondrial swelling, and the nuclear chromatin was dispersed normally (Figure 25). The sarcolemma was intact, transverse tubules and sarcoplasmic reticulum were normal or only slightly dilated, and mitochondria contained normal matrix granules (Figure 25). The major differences from control myocardium were that sarcomeres were sometimes out of register, and most cells contained cytoplasmic vacuoles (Figures 24 and 26). Often these vacuoles appeared empty, but they sometimes were filled by a cytoplasmic protrusion containing granular and flocculant material. With increasing periods of anoxic perfusion, an increasing proportion of Type 2 cells showed additional changes, consisting of focal myofibrillar degeneration and loss of register of sarcomeres, or they contained a mixture of swollen mitochondria and relatively normal-appearing mitochondria. Occasionally, cells were observed which appeared to be shrunken and more dense than normal.

Discussion

To the best of our knowledge, the present study establishes for the first time that myocardial cells were irreversibly injured by relatively brief periods of substrate-free anoxic perfusion *in vitro*. The evidence for this is discussed below, but in view of the fact that the metabolic and ultrastructural effects of anoxia on isolated perfused hearts have been the subject of numerous past studies, it would seem important to first consider why the present results may seem to differ from those previously reported. Current literature adequately supports the view that prolonged anoxia produces severe metabolic injury to myocardial cells. With anoxia or severe hypoxia, myocardial cell metabolism rapidly converts to anaerobic glycolysis.[26-28] Glycogen is the chief substrate and lactate is the end product of metabolism. Glycogen and cellular stores of creatine phosphate and ATP are rapidly depleted, and hearts soon cease contraction.[29,30] After only 20 to 30 minutes of anoxia, cellular ATP levels may be so low that even with residual glycogen, critical steps in the glycolytic pathway cannot be maintained, and anaerobic metabolism may cease.[30] Functional

recovery of anoxic hearts is seriously impaired.[29] Supply of exogenous substrate during anoxic perfusion greatly enhances functional and metabolic recovery of the heart. Cellular rates of anaerobic metabolism and ATP and creatine phosphate levels are enhanced by exogenous substrate, and the duration of anoxic perfusion which a heart supplied with substrate can withstand is considerably prolonged.[1,3,4,29,31] Hearts also can be protected from injury by cooling, and a combination of hypothermic perfusion and substrate has been shown to preserve hearts for considerable periods of time.[32,33] Previous ultrastructural studies of anoxic injury have often stressed the changes occurring during relatively brief periods of anoxic perfusion.[5] In other studies, substrate has been present during the period of anoxic perfusion [34-36] or the temperature of perfusion may have been lower than the 37 C. Cellular and mitochondrial swelling, loss of normal mitochondrial matrix granules, and dilatations of transverse tubular systems are the principal changes reported to occur as a result of anoxic injury in isolated perfused hearts.[5,36]

In the present study we chose experimental conditions which would produce the most severe metabolic stress on the heart. The temperature during anoxic perfusion was maintained rigorously at 37 C. Substrate was omitted from the perfusion fluid, and perfusion was maintained until definite structural and biochemical changes developed. In addition, hearts subjected to varying periods of anoxic perfusion were challenged with reoxygenation to determine if recovery was possible. Under these conditions, it was found that some cells are severely injured by as little as 30 minutes of anoxia and that these cells released CPK and showed progressive ultrastructural changes despite reoxygenation. Other cells showed minor degrees of structural damage and, with reoxygenation, remained intact for the duration of the experimental period. The observation that only a small percentage of cells were severely injured by 30 minutes of anoxia, while most were injured by 55 to 60 minutes, also may account for the results of some previous studies which could have dismissed a small number of severely injured cells as artifacts particularly if tissue were fixed for electron microscopy by immersion techniques.

Enzyme Release From Anoxic Hearts

Measurement of serum enzyme levels is currently an important clinical [37-39] and experimental method of determining the presence of myocardial infarction.[40] Because creatine phosphokinase is one of the first enzymes released after infarction, it has been widely used in both clinical and experimental studies.[40] Tissue CPK activity is rapidly diminished in areas of cell death in experimental myocardial infarcts, and the amount

and rate of enzyme released into the blood has been used to estimate the size of experimental myocardial infarctions.[40,41] Sudden release of CPK has been observed soon after reflow of blood to severely injured ischemic myocardium.[42] Thus, there is considerable evidence that release of intracellular enzymes is closely associated with impending or actual death of myocardial cells.[43] Presumably, the release of cellular proteins is a result of increased permeability or loss of structural integrity of cell plasma membranes.[29,43,44]

In the present study, measurable amounts of CPK activity were first detected in the effluent after about 60 minutes of continuous anoxic perfusion. As perfusion continued, the rate of enzyme release continuously increased; and in other experiments we, as well as others,[29,44] have shown that CPK release peaks at 150 to 200 minutes, slowly declining thereafter. With reoxygenation there was a sudden peak of CPK release after only brief periods of anoxia. The amount of enzyme released correlated both with duration of anoxia between 30 and 55 minutes and the percentage of injured cells in the left ventricle as determined by histologic methods. This evidence along with the results of the ultrastructural studies shows that severe and irreversible cell injury occurs in hearts after 30 to 55 minutes of *in vitro* anoxic perfusion and that reoxygenation invokes a rapid release of CPK from these severely damaged cells.

Morphologic Changes

Hearts perfused with oxygenated media containing glucose maintained their structural integrity for long periods of time.[5,35,45,46] On the other hand, anoxic, substrate-free perfusion was associated with myocardial cells which were depleted of glycogen and appeared to be swollen. Most of the cells contained vacuoles in the cytoplasm and had enlarged mitochondria containing decreased members of normal matrix granules. If anoxia was prolonged, additional changes occurred; these included severe cellular swelling and the development of tiny, amorphous matrix densities in mitochondria. By 60 and 90 minutes, when CPK release was first noted in the effluent, small numbers of cells also contained hypercontracted sarcomeres and discontinuties in cell plasma membranes.

With reoxygenation, hearts subjected to from 30 to 55 minutes of anoxia showed both CPK release and increasing numbers of cells which suddenly developed severe and rapidly progressive ultrastructural changes within the first few minutes of reoxygenation. The affected myocardial cells were markedly swollen; also, most showed disrupted cell membranes. With longer periods of reoxygenation, these severely damaged cells showed additional and progressive degenerative changes which included formation

of prominent amorphous densities in mitochondria as well as granular dense bodies of the type associated with calcium accumulation. They also demonstrated prominent contraction bands and a striking loss of continuity of plasma membranes. The progressive nature of these changes despite reoxygenation, as well as their similarity to irreversible injury *in vivo* demonstrate that these cells were no longer capable of maintaining cell integrity and were irreversibly injured.

Evidence that the changes described indicate irreversible cellular injury is provided by detailed studies of myocardium subjected to irreversible ischemic injury. Fifteen minutes of temporary coronary artery occlusion in dogs, followed by reflow to the injured area, has been associated with full recovery of all cells. If ischemia is prolonged past 20 minutes, an increasing percentage of cells are destined to die despite blood reflow. Approximately 55% of cells in the posterior papillary muscle die after 40 minutes, and 80 to 90% die after 60 minutes of ischemia. Permanently ischemic cells show loss of glycogen stores and moderate cellular and mitochondrial swelling if ischemia is extended past irreversibility, they also develop amorphous mitochondrial matrix densities. These changes slowly progress during permanent ischemia but are severe after 120 minutes.[47] With reflow, however, cells irreversibly injured by only 40 minutes of ischemia show rapidly progressive changes.[9,48,49] There is explosive cellular swelling with formation of subsarcolemmal blebs of edema fluid.[10] Mitochondria develop prominent granular dense bodies which almost certainly contain calcium phosphate [51] in addition to the small amorphous matrix densities which were present prior to reflow. Prominent contraction bands form, and cellular membrane systems are disorganized. Frequent disruptions of plasma membranes are observed, especially in the sarcolemma overlying edematous blebs. Similar changes occur in other cellular systems irreversibly injured by a variety of means. In renal tubular cells,[15,16,51] isolated Ehrlich ascites tumor cells,[16] or liver parenchymal cells,[52] irreversible injury is associated with many of the changes described above. Two are considered particularly characteristic of irreversible cellular injury. These are: a) formation of large amorphous densities in the mitochondrial matrix,[47-49,52] and if cells are exposed to extracellular fluid early in the course of the lesion, mitochondrial calcification [9-11,16,49,51]; and b) plasma membrane degeneration with loss of cell volume control and finally loss of structural and functional identity of cell membrane.[14-16,24,50,52] Since the cellular changes after anoxic injury are essentially identical to those occuring in other irreversibly injured cells, the conclusion that anoxic injury *in vitro* is capable of irreversibly injurying myocardial cells now seems firmly established.

Changes in Reversible Injury

Other cells were less severely damaged in the same hearts and with reoxygenation resumed normal cell density and resembled normal myocardial cells. The latter cells, which were capable of maintaining structural integrity if reoxygenated, are presumed to have been reversibly injured and to be potentially capable of full recovery.

Brief anoxic perfusion reduced cellular glycogen and produced cellular swelling and formation of vacuoles in the cytoplasm. Three types of vacuoles were observed: a) focal "empty" areas of the cytoplasm not bounded by limiting membrane, b) definite membrane-limited vacuoles possibly derived from the endoplasmic reticulum, and c) vacuoles containing recognizable cytoplasmic components. The first two types of vacuoles probably are related to intracellular collections of edema fluid and are similar to vacuoles which occur following dinitrophenol poisoning of Langendorff-perfused rat hearts.[35] The third type of vacuole may be a digestive vacuole to provide endogenous substrate or possibly represent cell to cell protrusions or interdigitations as have been described in cardiac muscle cells injured by isoproterenol.[53] Similar vacuoles containing glycogen have been described in pig hearts [54] after partial ischemia. Other changes were reversible. Mitochondrial swelling and loss of normal matrix granules are reversible events and were no longer evident in reoxygenated myocardial cells. The ability of reversibly injured cells, although previously anoxic, to maintain cell structure for long perfusion periods provides an internal control for these experiments, again showing that the damaged cells were irreversibly injured.

Anoxic Versus Ischemic Injury

In this study, while maintaining continuous perfusion, anoxia was induced by reducing the perfusate oxygen tension to very low levels. Anoxia with continuous perfusion differs from ischemia. In ischemia, low tissue oxygen tension results from a reduction in flow rate. Along with the reduced supply of oxygen resulting from the decrease in flow, there is a reduced supply of substrate and also a local accumulation of metabolic products, including lactate and hydrogen ions.[55] Under the conditions reported here, irreversible injury was found to result from deprivation of oxygen and substrate alone. Lactate is rapidly washed from anoxic hearts, if flow is maintained. The cells are bathed with a constantly renewed buffered perfusate which should maintain near normal extracellular pH levels. Thus, the results suggest that decreased supply of materials to maintain cellular energy requirements is sufficient to induce irreversible injury. The possible role of accumulation of lactate and hydrogen ions in

the pathogenesis of irreversible ischemic injury remains unknown, but the present study suggests that reduced flow rates and tissue accumulation of toxic metabolites are not necessary to cause irreversibility.

Pathogenesis of Irreversible Myocardial Injury

Some myocardial cells become irreversibly injured by relatively short periods of substrate-free anoxic perfusion. With reoxygenation, irreversibly injured cells suddenly swell, release intracellular proteins, and show large defects in plasma membranes by electron microscopy. The suddenness of these changes suggests that metabolic events during the anoxic interval somehow alter irreversibly injured cells so that they react violently to reoxygenation. That reoxygenation itself is the crucial event producing these changes, and not readmission of substrate or resumption of mechanical activity, has been shown by enzyme release studies of potassium-arrested hearts in which substrate was removed from both the anoxic and oxygenated perfusion media.[44] Why reoxygenation produces such a violent reaction in these critically altered cells is not known. That oxygen is directly toxic to anoxic myocardial cells [44] must be considered. The fact that oxygen induces damage to cells only after they have been preconditioned by a suitably long anoxic interval, while it allows structural and functional recovery of less (reversibly) injured cells, suggests that the critical event in irreversible injury occurs during the anoxic interval, and reoxygenation only provides a suitable environment in which injured cells can be readily identified. It seems likely that oxygen would permit reactivation of aerobic cellular metabolism and that this somehow produces a stress on unstable (irreversibly injured) cells that leads to swelling and possible rupture of plasma membranes.

The instability may be related to changes in calcium transport or binding during the period of anoxia. Of great interest, in this regard, is the so-called calcium paradox. Almost identical functional, cellular, and biochemical changes to those noted in our anoxia-perfusion experiments are induced in rat hearts at 37 C by a period of calcium-free perfusion followed by reperfusion with calcium-containing media.[56-58]

That rupture of the sarcolemma actually occurred in the anoxia reperfusion experiments is inferred from electron microscopy of processed tissue. It cannot be ruled out that the discontinuities in plasma membranes were an artifact caused by inadequate fixation of altered plasma membranes. The close correlation of the observed structural defect and CPK release, however, suggests that the damaged cells did indeed have defects in cell membrane permeability and that it was the damaged cells that accounted for the enzyme release.

Although cell swelling and increased membrane permeability were surely closely related events, it cannot be determined from these studies which occurred first. Cellular swelling could stretch membranes, causing increased permeability. Conversely, a sudden increase in cell membrane permeability could in itself lead to cellular swelling.

After ischemia *in vivo*, explosive cellular swelling also has been observed upon reflow of blood to myocardium irreversibly injured by temporary coronary artery occlusion.[10,50] These studies have shown that loss of ability of cells to regulate cell volume is a prominent early event after irreversible ischemic myocardial injury. The nature of the cell injury responsible for this defect has not been determined. Its cause could lie in metabolic defects in cell energy metabolism, loss of sodium–potassium pump function, presence of toxic metabolic inhibitor substances or increased cell plasma membrane permeability. Increased cell membrane permeability or actual membrane rupture has been proposed as an important early event in ischemic injury which allows entry of calcium into irreversibly injured cells.[11,50,59] Massive calcium influx could induce contraction bands and accumulation of calcium in mitochondria as granular dense bodies.

The close similarity of the morphologic events following both irreversible ischemic injury *in vivo* and irreversible anoxic injury *in vitro* suggests that the isolated perfused heart will be a useful and valid model for study of the pathogenesis of irreversible myocardial injury. Present evidence, derived from both the *in vivo* studies in dogs and *in vitro* models of both dog heart slices [60] and rat hearts, suggests that loss of integrity of cell membranes does occur in irreversible injury and could in itself lead to cell death. Further studies will, however, be necessary to determine if membrane changes are a primary or secondary event in the pathogenesis of irreversible myocardial cell injury.

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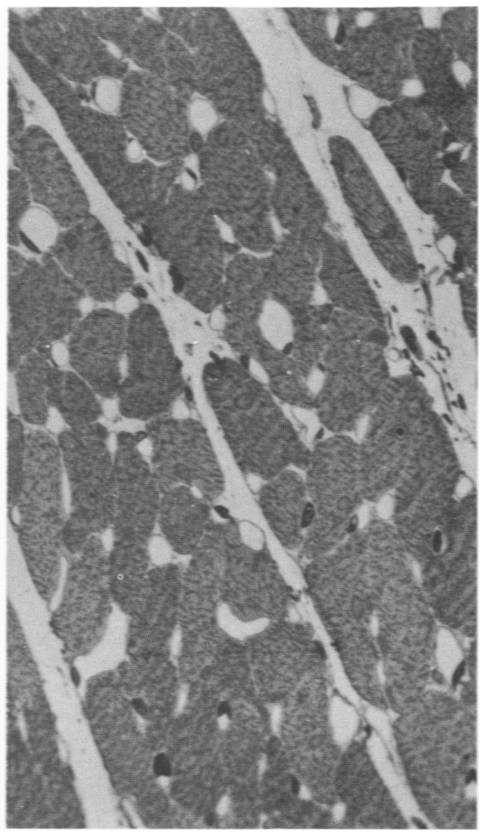
Legends for Figures

Figure 1—Control heart perfused for 120 minutes with O_2-medium. This cross section shows the uniform appearance of control myocardium. Capillaries are patent, and the only difference from perfusion-fixed *in vivo* control hearts is an increase in the width of the interstitial space. (Perfusion fixation, toluidine-blue-stained, plastic-embedded section, × 1300)

Figure 2—Control heart perfused for 120 minutes with O_2-media. In longitudinal sections, myocardial cells have uniform cross striations and cellular density. (Perfusion fixation, toluidine-blue-stained, plastic-embedded section, × 1300)

Figure 3—Heart perfused with N_2-medium for 35 minutes and then reoxygenated for 85 minutes. Two populations of cells are present. One resembles control myocardium except that vacuoles are present in the cytoplasm. The other appears as swollen, pale-staining cellular ghosts (*arrows*). Damaged and undamaged cells were randomly distributed throughout both right and left ventricles. (Perfusion fixation, toluidine-blue-stained, plastic-embedded section, × 1300)

Figure 4—Heart perfused with N_2-medium for 50 minutes and then reoxygenated for 70 minutes. Most cells appear severely damaged. There is cellular swelling and contraction bands (*arrows*). The cytoplasm has a granular appearance between contractures, due to rounded, swollen mitochondria. (Perfusion fixation, toluidine-blue-stained, plastic-embedded section, × 1300)



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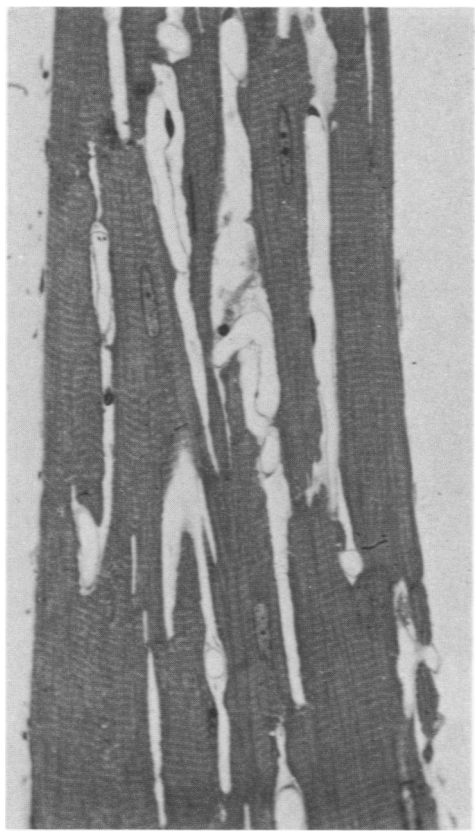

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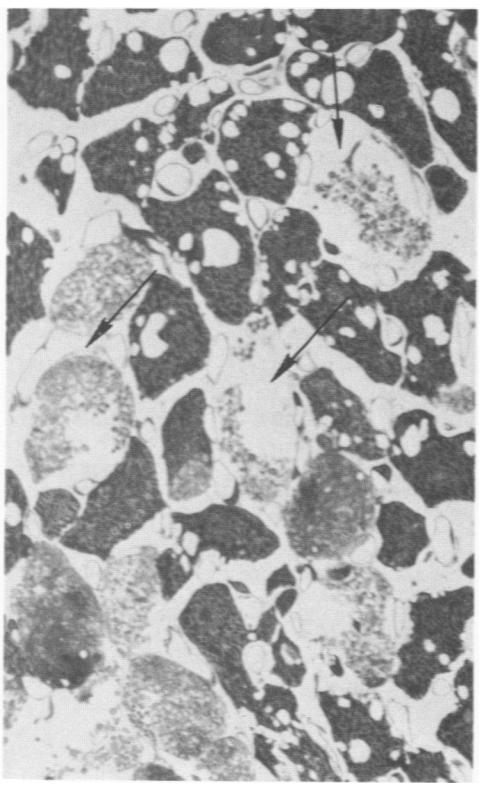

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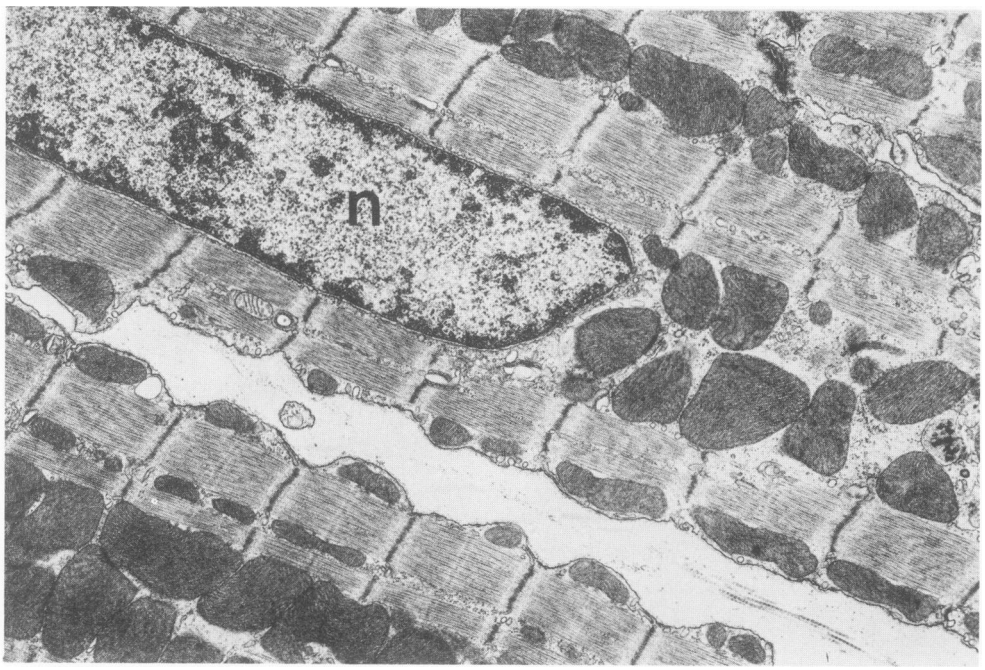

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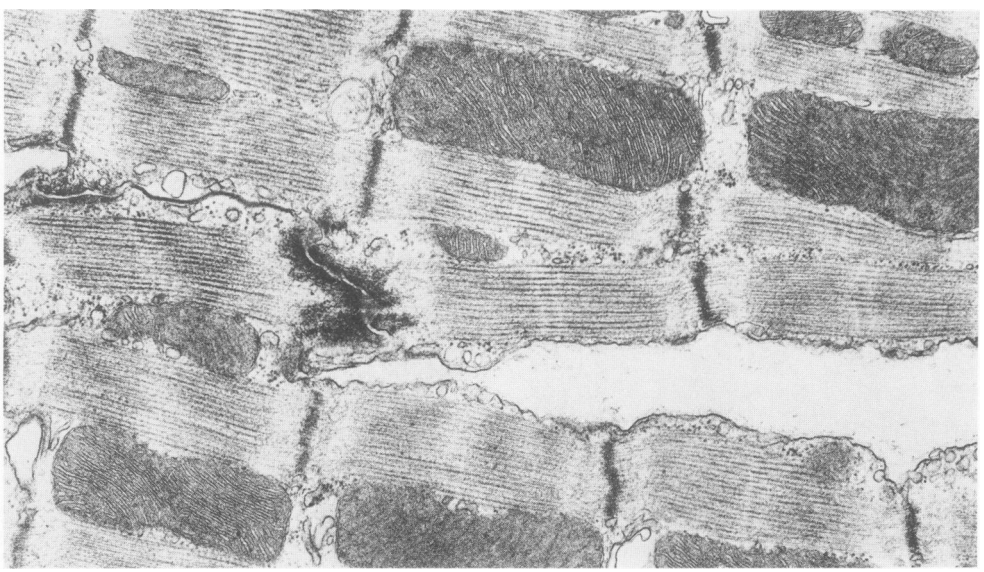

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Figure 5—Control heart perfused for 120 minutes with O_2-medium. All cells from control hearts resembled normal myocardium. The nuclei have uniform density (*n*), and sarcomeres are in nearly perfect register. The presence of I bands is due to dilation of the heart as it ceases contracting during perfusion fixation and is a normal feature of perfusion-fixed cardiac muscle. Mitochondria vary in shape near the nucleus but in the cytoplasm are elongated and of a uniform, moderate density. The plasma membrane appears continuous, is closely applied to the myofibrils and mitochondria, and shows numerous pinocytotic vesicles. (× 10,000) **Figure 6**—Control heart perfused for 120 minutes with O_2-medium. At this magnification, glycogen granules can be seen beneath the plasma membrane and between myofibrils. Sarcoplasmic reticulum and transverse tubular systems are not significantly dilated. Mitochondria contain a moderate number of normal matrix granules. (× 20,000)

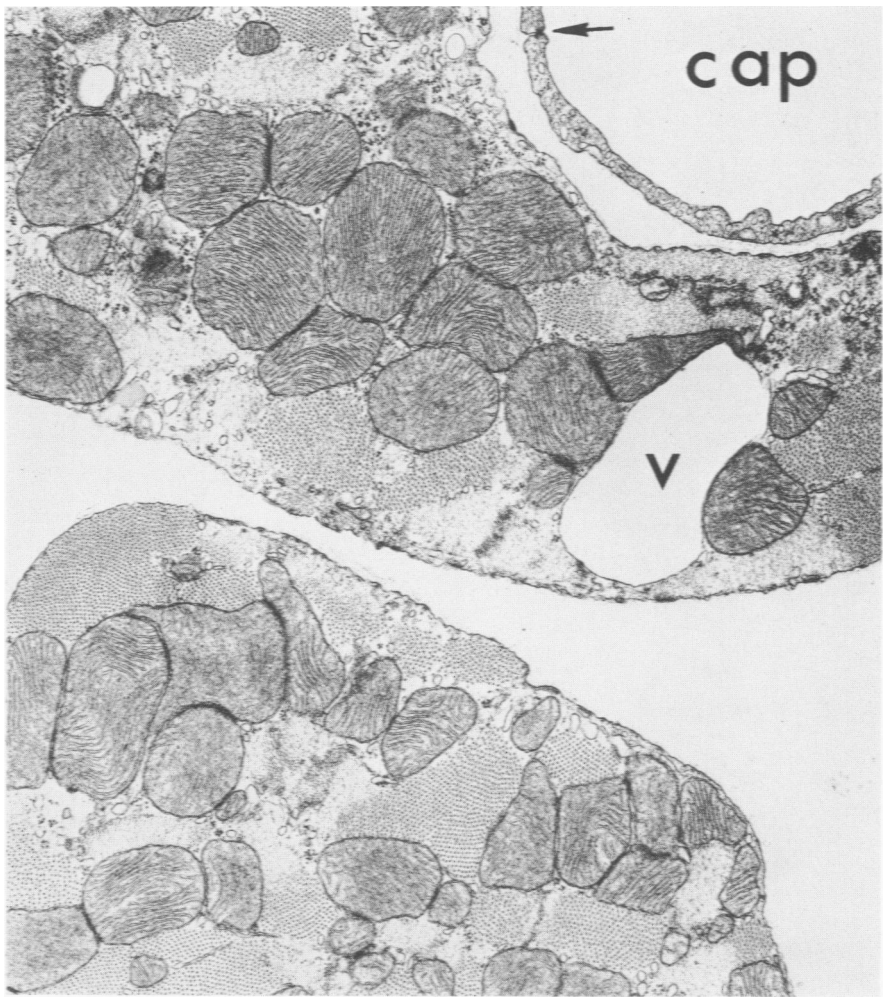


Figure 7—Heart perfused with N_2-medium for 30 minutes and immediately fixed. In cross section, myocardial cells show mild swelling and occasional small vacuoles (*v*). The mitochondria are moderately swollen and have decreased numbers of normal matrix granules. The cell plasma membrane appears continuous, but a decreased number of pinocytotic vesicles was a consistent feature of anoxic cells. The interstitial space is enlarged. A patent capillary (*cap*) shows intact intracellular junctions (*arrows*). (× 16,000)

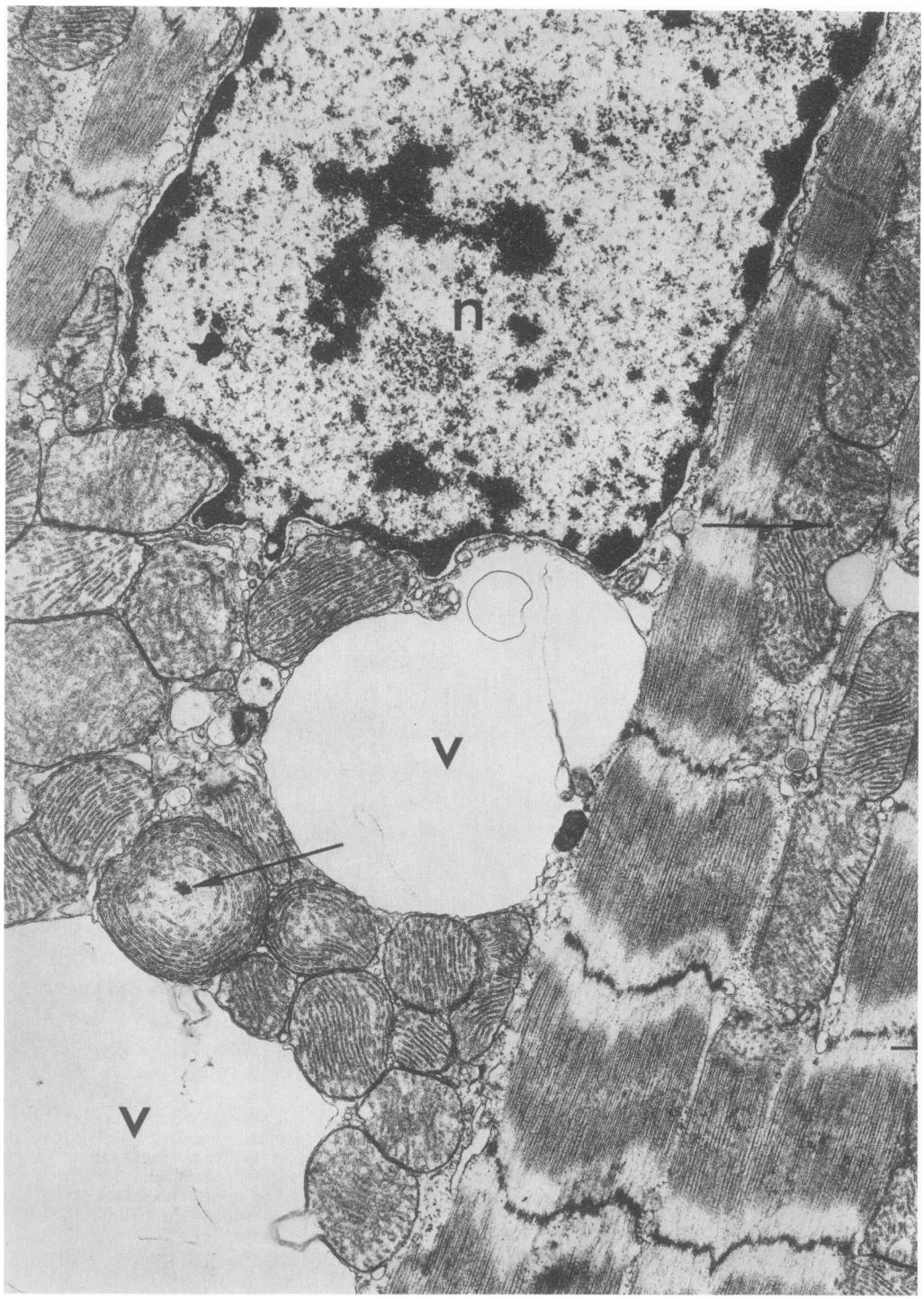
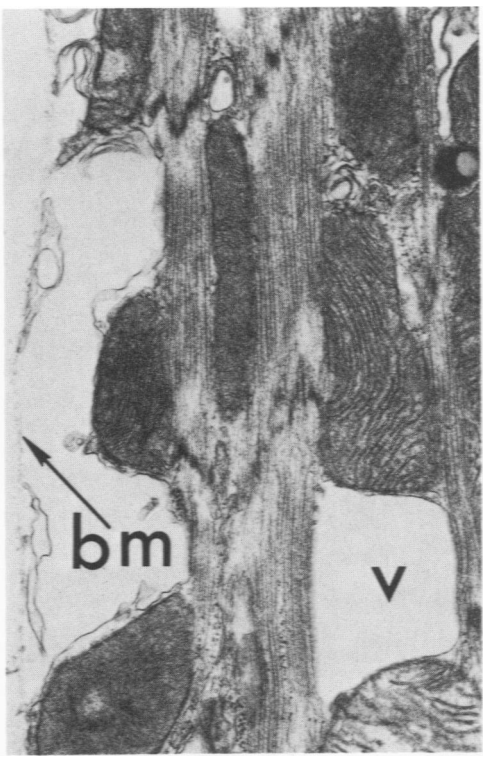


Figure 8—Heart perfused with N_2-medium for 60 minutes and immediately fixed. There is early clearing and clumping of nuclear chromatin. Cytoplasmic vacuoles (*v*) add to the moderate mitochondrial and cytoplasmic swelling. Mitochondria have decreased matrix density and loss of normal matrix granules. Glycogen is depleted compared to control cells and occasional mitochondria contain amorphous densities *(arrows). n* = nucleus. (× 14,600)



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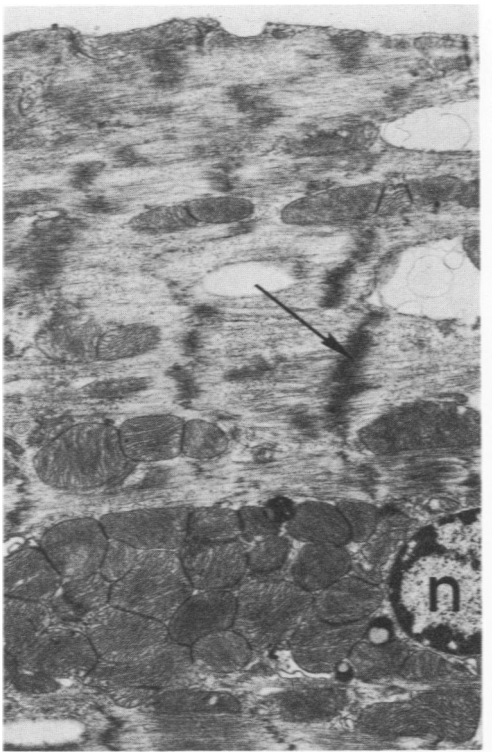

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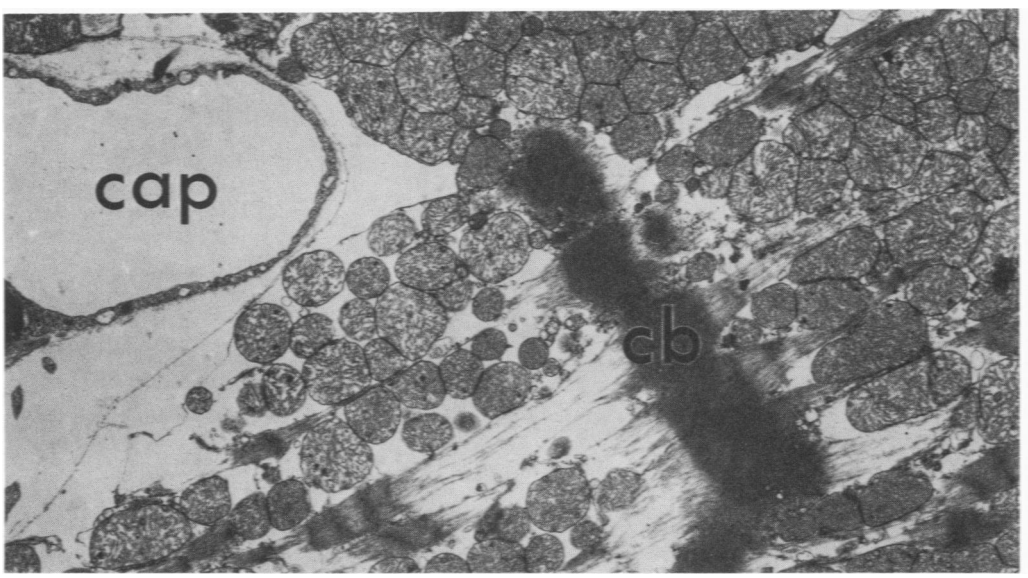

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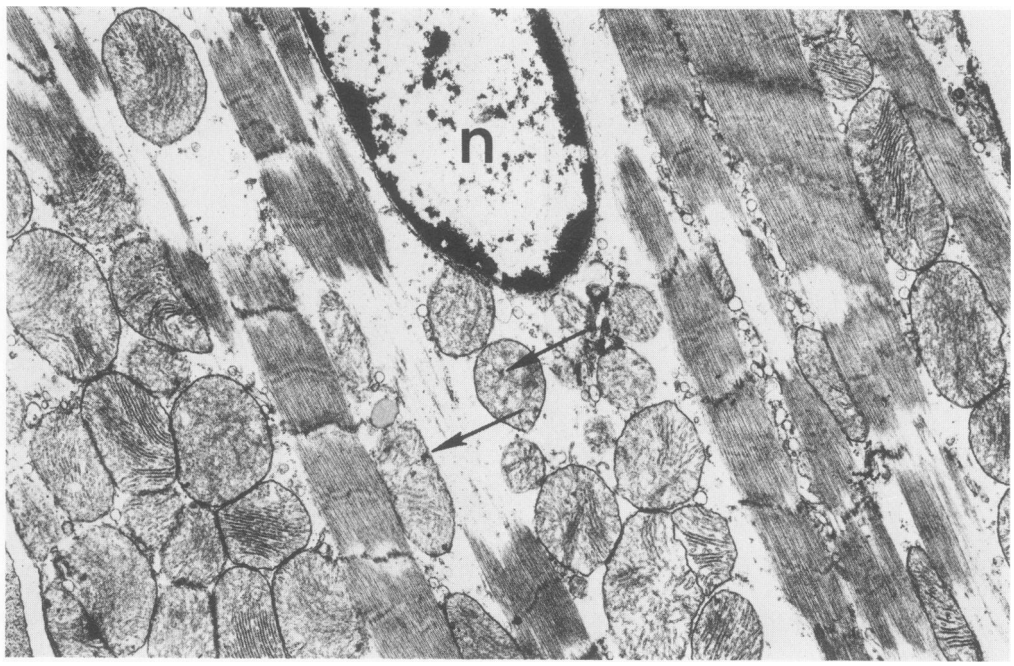
Figure 9—Heart perfused with N_2-medium for 60 minutes and immediately fixed. The cell plasma membrane appears scalloped and has separated from the basement membrane (*BM*). Some membranous debris is present in the space so formed. A vacuole (*v*) and dilated sarcoplasmic reticulum are present. Mitochondria show varying degrees of mild swelling. (× 16,000) **Figure 10**—Heart perfused with N_2-medium for 60 minutes and immediately fixed. In addition to cytoplasmic vacuoles and mild degrees of mitochondrial swelling, there is prominent thickening of Z bands (*arrow*). *n* = nucleus. (× 6700) **Figure 11**—Heart perfused with N_2-medium for 60 minutes and immediately fixed. At 60 minutes, and more often after 90 minutes of anoxia, a few cells were greatly swollen, had contraction bands (*cb*), and often discontinuous plasma membranes. It is thought that these cells may account for the low levels of CPK activity which could be detected in the effluent at these times. *cap* = capillary. (× 5900)



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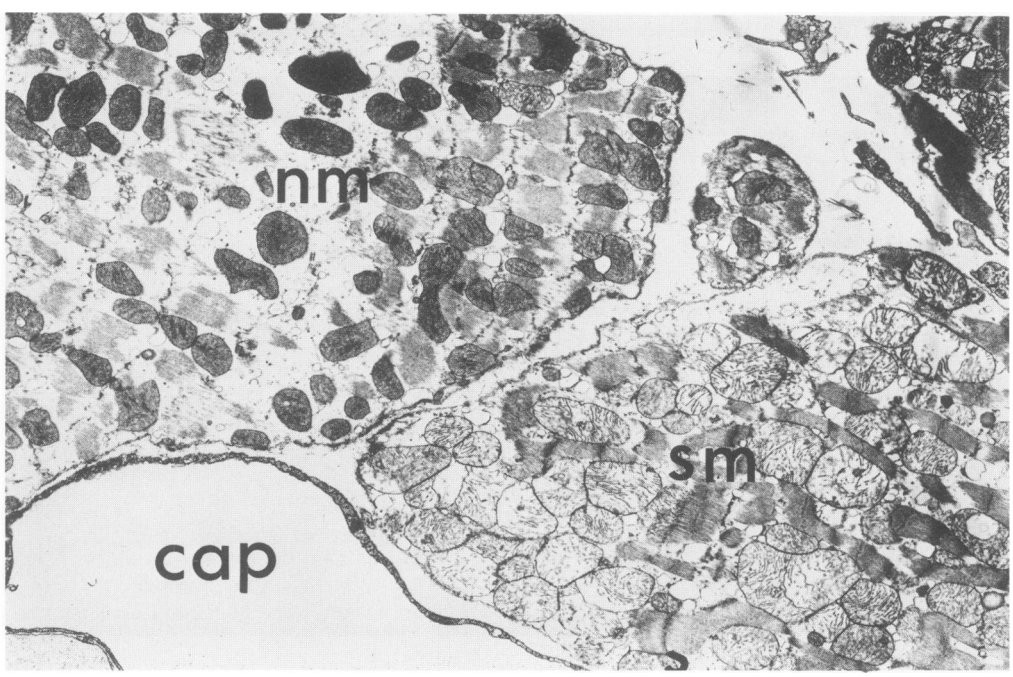

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Figure 12–Heart perfused with N_2-medium for 90 minutes and immediately fixed. This is a typical cell appearance at 90 minutes, which is probably long after irreversible injury has occurred. The lesions are similar to but more advanced than those at 60 minutes. There is prominent clumping of nuclear chromatin (*n*), almost total loss of glycogen, and obvious cellular swelling. Mitochondria are swollen and many contain amorphous densities (*arrows*). (× 10,400) **Figure 13**—Heart perfused with N_2-medium for 50 minutes and then reoxygenated for 3 minutes. During peak enzyme release, cells were greatly swollen. Mitochondria were either greatly swollen in some cells (*sm*) or in others showed only mild swelling (*nm*). *cap* = capillary. (× 4800)

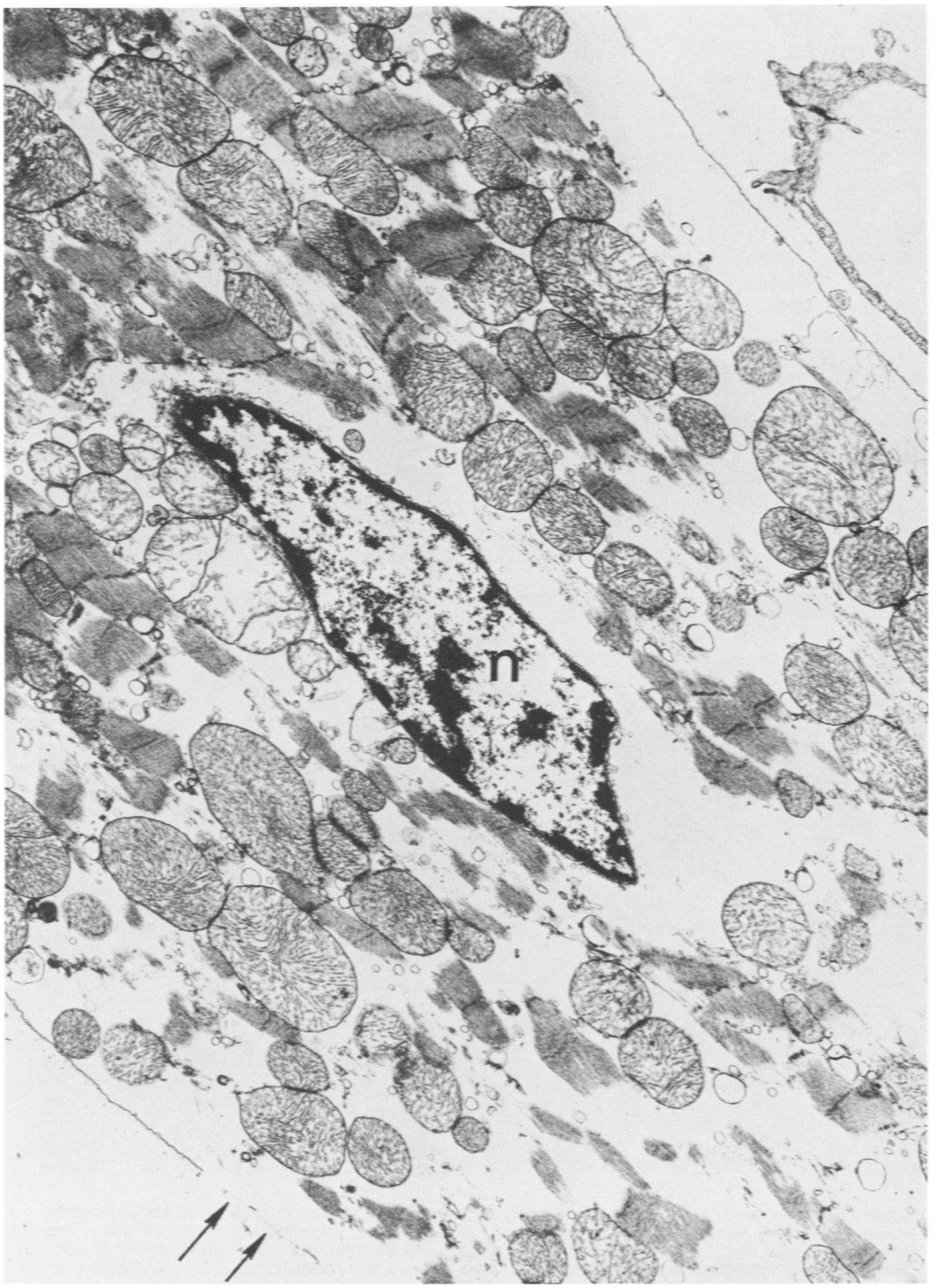

Figure 14—Heart perfused with N_2-medium for 50 minutes and then reoxygenated for 3 minutes. After only 3 minutes of reoxygenation, irreversibly injured cells were markedly swollen with formation of irregular, empty, cytoplasmic and subsarcolemmar spaces. The nuclear chromatin (*n*) is markedly clumped, and mitochondria are massively swollen. Amorphous densities were present in many but not all mitochondria. The plasma membrane often was discontinuous in many of these swollen cells (*arrows*). This lesion is shown at a higher magnification in Figure 16. (× 8300)

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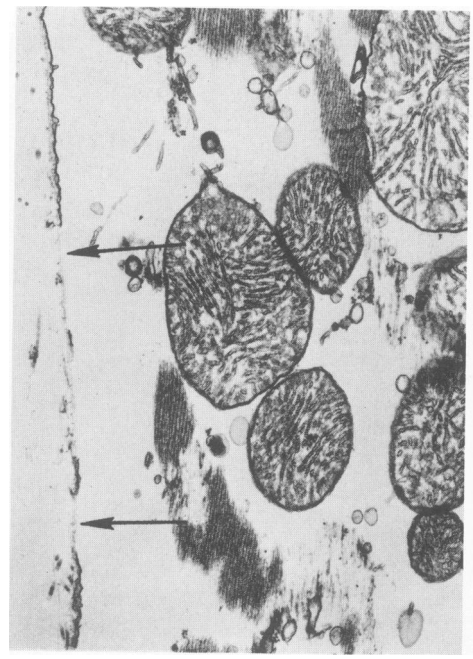

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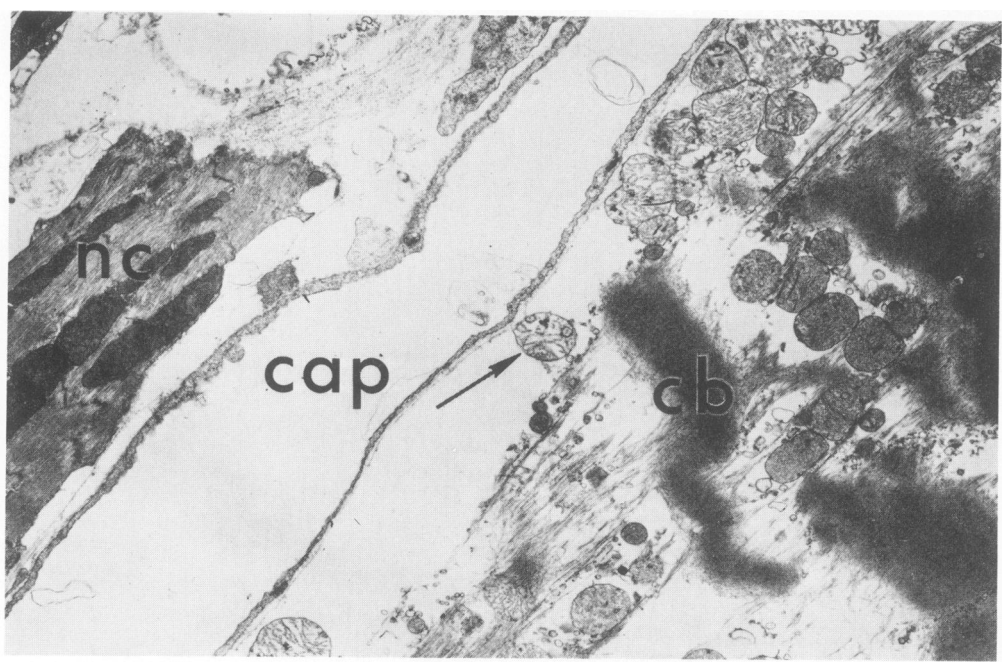

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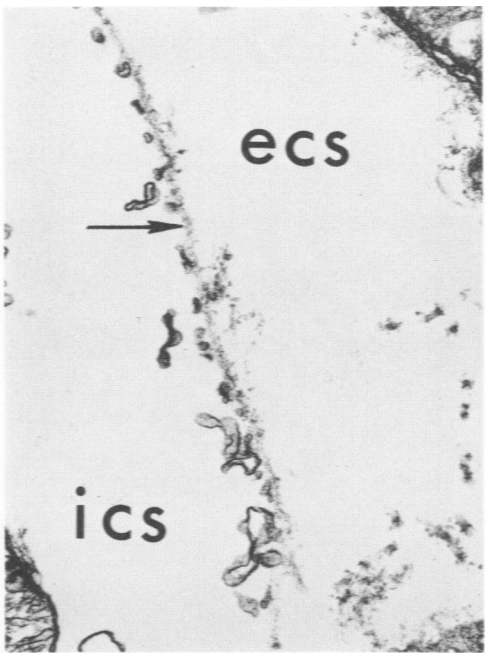
Figure 15—Heart perfused with N_2-medium for 50 minutes and then reoxygenated for 3 minutes. Some cells were swollen by numerous membrane-bounded vacuoles (*v*), many of which are thought to be derived from sarcoplasmic reticulum. Cells swollen in this manner sometimes contained dense contracted mitochondria with dilated intracristal spaces. (× 22,000) **Figure 16**—Same cell as shown in Figure 14. Focal discontinuities in the plasma membrane were a common feature of swollen cells. There is marked intracellular edema. Mitochondria are swollen but often contained no amorphous or granular densities. (× 14,300) **Figure 17**—Heart perfused with N_2-medium for 50 minutes and then reoxygenated for 70 minutes. After long periods of reoxygenation, severely damaged cells had this typical appearance. There were large subsarcolemmal blebs of edema fluid in which mitochondria appeared to be floating (*arrows*). Dense contraction bands (*cb*) alternated with areas of cytoplasm containing only a few stretched myofilaments. A small percentage of cells appeared relatively normal (*nc*). *cap* = capillary. (× 6400)



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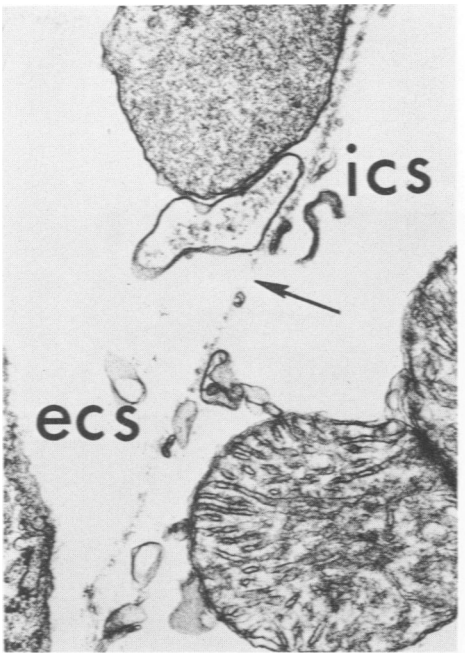

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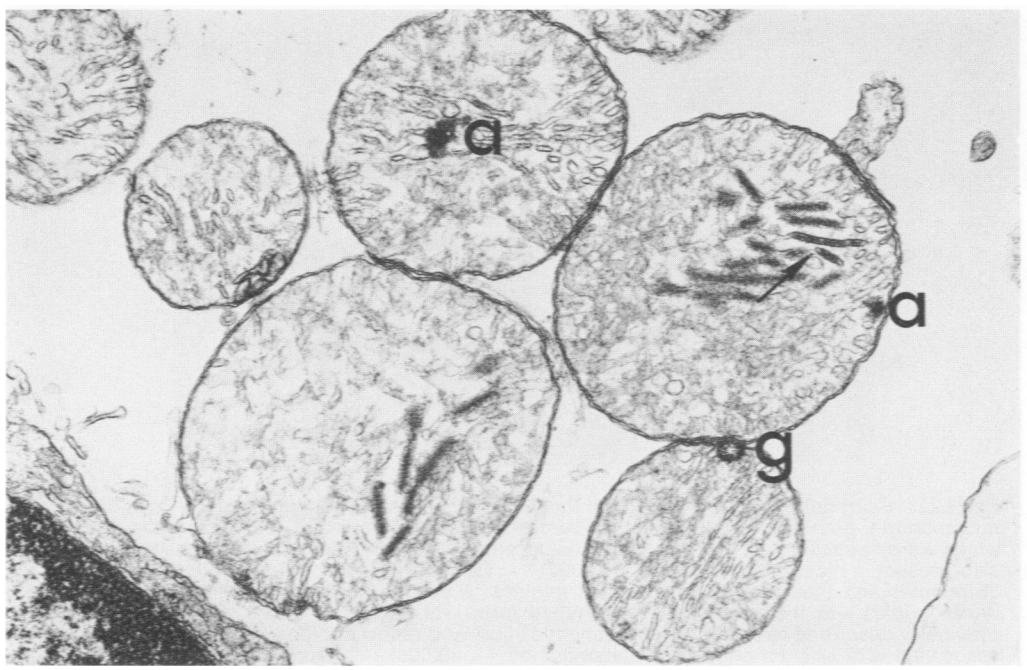

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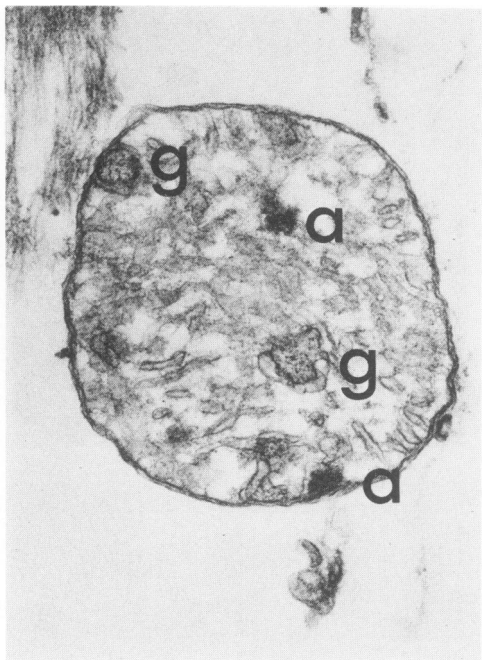
Figure 18—Heart perfused with N_2-medium for 35 minutes and then reoxygenated for 85 minutes. Severely damaged cells usually had focal loss of cell plasma membrane with only vesicular membrane remnants attached to the basement membrane. The basement membrane (*arrows*) appeared as the only barrier between the intracellular space (*ics*) and the extracellular space (*ecs*). (× 28,300) **Figure 19**—Heart perfused with N_2-medium for 35 minutes and then reoxygenated for 85 minutes. This micrograph shows another area in which there were large discontinuities in the cell plasma membrane. In some areas, even the basement membrane appeared ruptured (*arrows*), and there was no apparent separation of the intracellular space (*ics*) and extracellular space (*ecs*). (× 28,100) **Figure 20**—Heart perfused with N_2-medium for 35 minutes and then reoxygenated for 85 minutes. In irreversibly injured cells, mitochondria showed prominent lesions. In addition to swelling and loss of matrix granules, there were large amorphous matrix densities (*a*), annular granular dense bodies (*g*), and electron-dense material between cristal membranes (*arrow*). A portion of a capillary cell cytoplasm and nucleus is present in the extreme lower left of this micrograph. (× 28,600)



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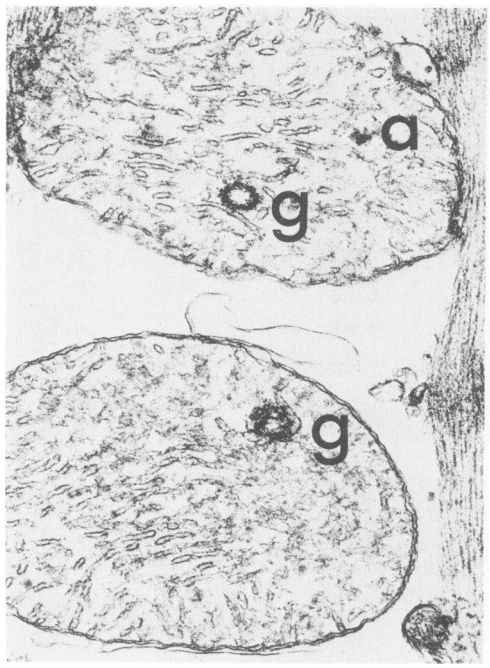

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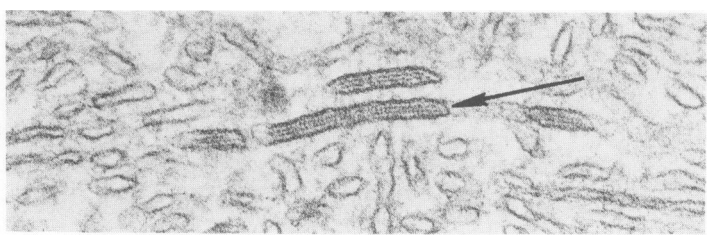

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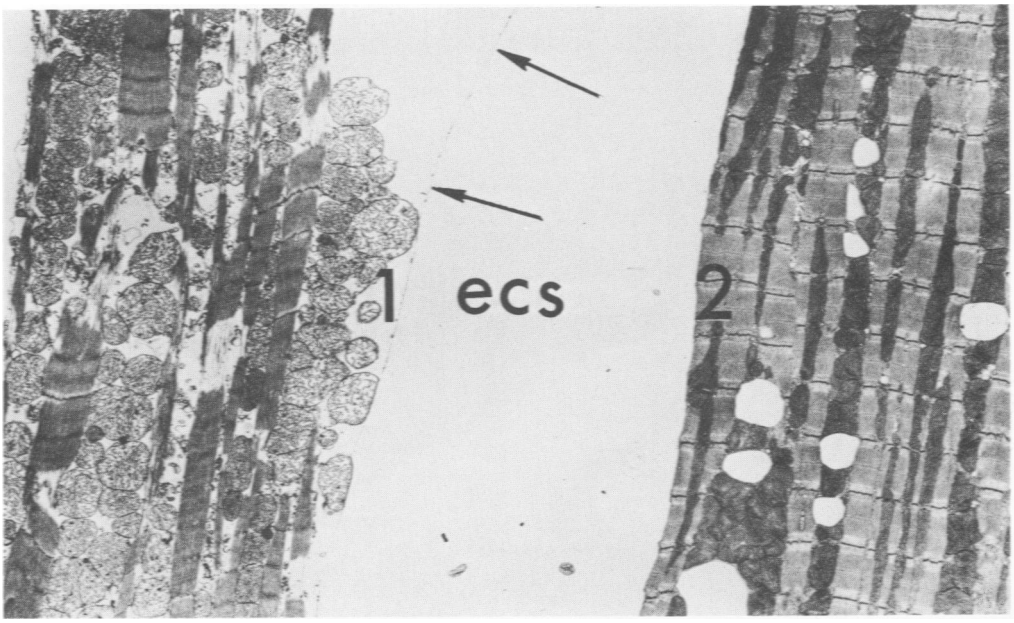
Figure 21—Heart perfused with N_2-medium for 50 minutes and then reoxygenated for 70 minutes. In mitochondria, granular dense bodies sometimes appeared as round or oval focal areas of matrix which were speckled with tiny electron-dense granules (*g*). Amorphous matrix densities (*a*) were also present in most mitochondria. (× 40,000) **Figure 22**—Heart perfused with N_2-medium for 35 minutes and then reoxygenated for 85 minutes. A second common appearance of granular dense bodies was the annular or ring-shaped form. This is typical of the type of dense body previously described after irreversible ischemic injury and reflow of blood in dogs which has been associated with calcium phosphate accumulation. (× 29,000) **Figure 23**—Heart perfused with N_2-medium for 40 minutes and then reoxygenated for 80 minutes. In properly oriented sections, the intracristal material appears as a periodic double line (*arrow*). (× 81,000)



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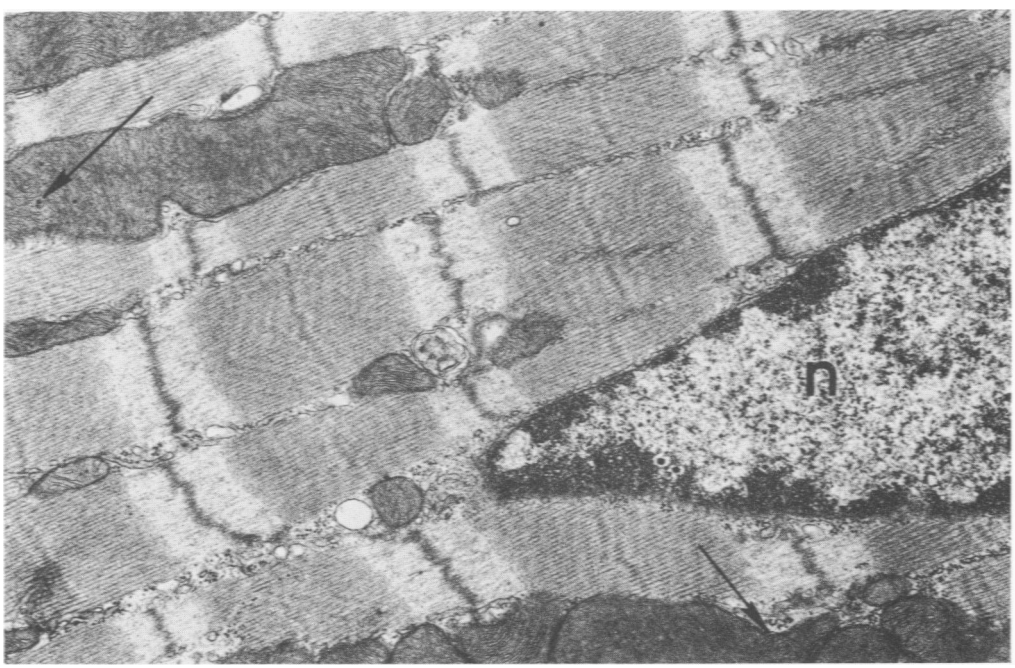

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Figure 24—Heart perfused with N_2-medium for 50 minutes and then reoxygenated for 70 minutes. With reoxygenation, two cell populations were present. The first was the swollen cells, which are considered to be irreversibly injured (*1*). The second was similar in most respects to control myocardial cells except for the presence of cytoplasmic vacuoles and is considered to have to be reversibly injured (*2*). In perfused hearts there was often considerable enlargement of the interstitial space (*ecs*). *Arrows* point to the sarcolemmal membrane of the swollen cell. (× 3500)
Figure 25—Heart perfused with N_2-medium for 35 minutes and then reoxygenated for 85 minutes. Type 2 or reversibly injured cells were often virtually indistinguishable from normal. The nucleus (*n*) had a normally distributed chromatin pattern, the sarcomeres were regularly spaced, and mitochondria had normal density and contained normal matrix granules (*arrows*).

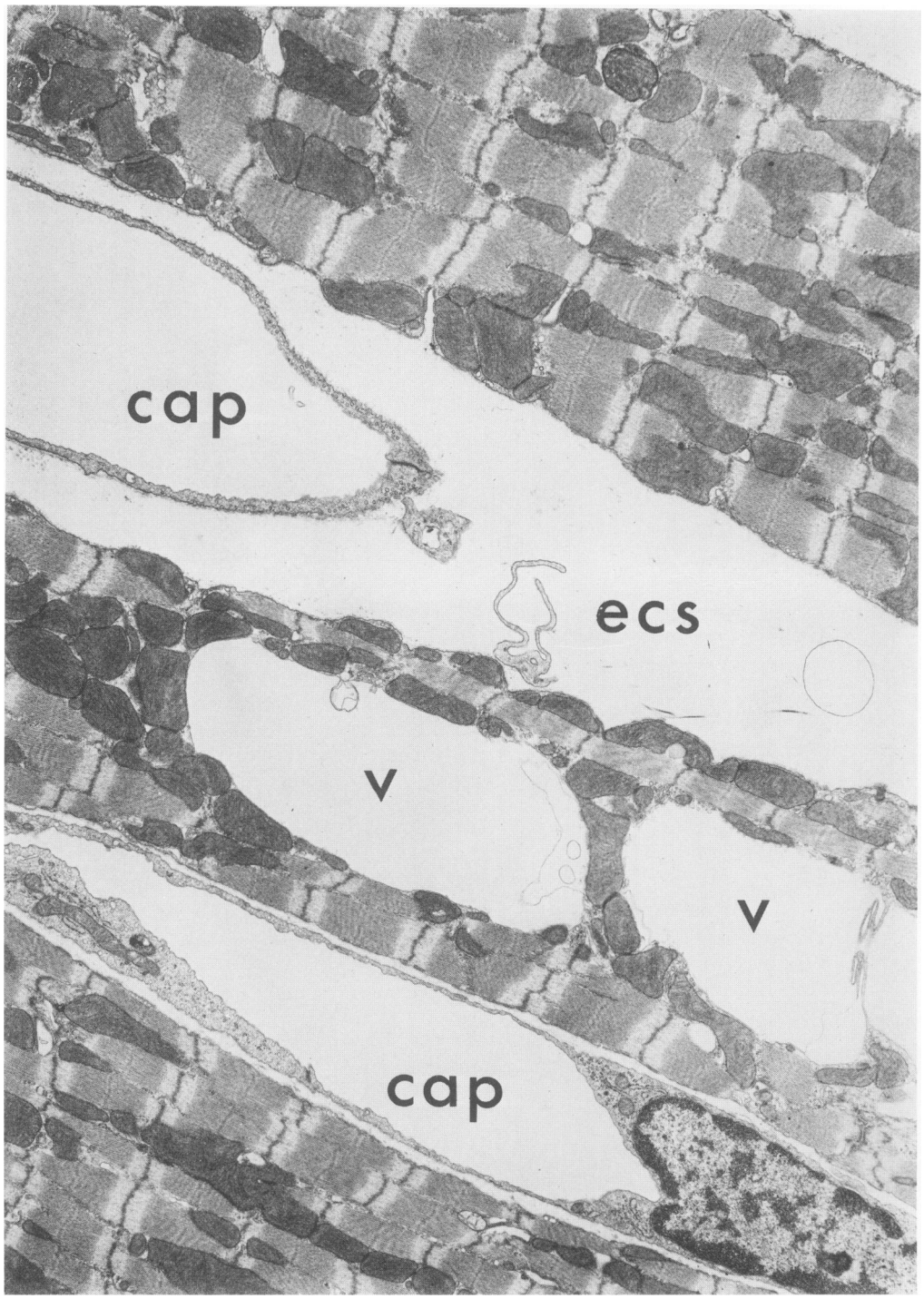

Figure 26—Heart perfused with N_2-medium for 35 minutes and then reoxygenated for 85 minutes. Type 2 or reversibly injured cells were similar to those from control hearts except that they frequently contained often large cytoplasmic vacuoles (*v*). *ecs* = interstitial space, *cap* = capillary lumen. (× 7300)