Structural Changes in Myocardium During Acute Ischemia

By Robert B. Jennings and Charles E. Ganote

ABSTRACT

The structural changes occurring in severely ischemic myocardial cells were described as a function of the duration of ischemia. Tissue for study was obtained from dog myocardia following acute occlusion of a major branch of a coronary artery. Ischemia was defined as being present when arterial flow was insufficient to maintain aerobic metabolism and was described as "total" (no flow), mild (anerobic metabolism present but flow sufficient to allow cells to survive), and severe (marked anerobic metabolism because of low flow; cell death occurs if ischemia persists).

Characteristic structural changes were found in severely ischemic myocardial cells after 30 to 40 minutes of coronary occlusion. These changes included virtual depletion of glycogen supplies, relaxation of myofibrils, and nuclear and mitochondrial changes. The mitochondria were swollen, exhibited decreased matrix density, and developed amorphous densities in the matrix space. These densities increased in size and number as the period of ischemia was prolonged. Mitochondria exhibiting these changes were very fragile and showed marked functional defects.

The effect of reperfusion of the area of ischemia with arterial blood was described in detail. Reversibly injured cells resumed contractile function quickly after reperfusion. Irreversibly injured cells, on the other hand, swelled enormously after only two minutes of reflow. They also accumulated Ca** in mitochondria and had marked contraction bands. These findings suggest that failure of cell volume regulation may be one of the earliest signs of the development of the irreversible phase of ischemic injury.

KEY WORDS irreversible cellular injury reversible cellular injury permanent ischemia temporary ischemia mitochondrial defects cell swelling mitochondrial amorphous matrix densities membrane permeability

The structural changes occurring during acute myocardial infarction from the stage of overt necrosis to scarring are well known. Less is known about the early effects of ischemia on myocardial cells, but electron microscopic studies of ischemic animal myocardial tissue have revealed highly characteristic sequential alterations in the ultrastructure of myocardial cells as they pass from a phase of reversible injury past the "point of no return" to overt cell death. Before presenting our findings on the acute effects of myocardial ischemia in vivo, it is necessary to review some of the biological features of acute myocardial ischemic injury in order to provide the reader with some perspective on the information available in this complex system.

Biological Features of Myocardial Ischemia

Sudden occlusion of a coronary artery in the dog results in reduced arterial flow to the myocardium supplied by this artery. If the reduction in flow is sufficient to induce hypoxia, the metabolism of the muscle shifts from the aerobic to the anerobic form, with a great reduction in the amount of energy produced per unit time. After 10 to 15 seconds of reduced flow, the affected myocardium becomes cyanotic, electrocardiographic changes appear, and the hypoxic cells cease contracting. Thus, myocardial ischemia is present when arterial flow is insufficient to maintain specialized cardiac functions, and its presence is most easily detected by noting that metabolism in the affected myocardium is predominantly anerobic (Fig. 1).

Acute occlusion of the circumflex branch of the left coronary artery of anesthetized dogs produces a large area of ischemia involving the posterolateral surface of the left ventricle (LV); the ischemic myocardium is easily recognized in vivo by the fact that it is cyanotic and acontractile. However, it is clear from the radioactive microsphere studies of Becker, Fortuin, and Pitt that all portions of the affected myocardium are not equally ischemic. These workers have shown that the instantaneous decrease in arterial flow 30 seconds after occlusion of this vessel is much greater in the subendocardial
MYOCARDIAL METABOLISM

ISCHEMIC INJURY

1. DECREASE IN ARTERIAL FLOW

2. HYPOXIA — ANAEROBIC METABOLISM

3. DECREASE IN CELLULAR ENERGY LEVEL

     CESSATION OF
     SPECIALIZED FUNCTIONS

     IRREVERSIBILITY

     CELL DEATH

**FIGURE 1**

Diagram of the sequence of events occurring in acute myocardial ischemic injury. (Reprinted from *New Perspective in Diagnosis and Management,* by permission.)

than in the subepicardial myocardium. Recent studies of Kloner et al.* with the fluorescent dye, thioflavin S (methyldehydrothio-p-toluidine-sulfonate), have provided a qualitative visual estimate of the local distribution of arterial flow in ischemic myocardium. Thioflavin, when injected intravenously one circulation time prior to excision of the heart, stains the endothelium of all vessels which were perfused with arterial blood after the injection. Tissue receiving flow is identified by the bright fluorescence of the dye under ultraviolet light (Fig. 2).

The thioflavin technique shows that after high occlusion of the circumflex artery, except for the endothelium of some larger arteries, the subendocardial myocardium and the posterior papillary muscle (PP) receive insufficient blood containing thioflavin S to allow staining. Conversely, fluorescence often is preserved in the subepicardium. Thus, although the entire thickness of the posterolateral wall of the left ventricle is ischemic after occlusion of this vessel, it is clear that parts of the outer wall of the ischemic LV are receiving arterial blood. Obviously, the amount of collateral arterial flow is insufficient to allow aerobic metabolism, or the tissue would not be cyanotic and acontractile. However, the damaged subepicardial cells rarely die in the infarct. Flow apparently is adequate to maintain the life of these ischemic cells.

Contributing to regionalization of flow in areas of ischemia is the fact that subendocardial blood flow occurs during diastole while subepicardial blood flow occurs throughout the cardiac cycle. Thus, cardiac rate and end-diastolic pressure be-

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*Kloner R, Reimer KA, Ganote CE, Jennings RB: Unpublished observations.

**FIGURE 2**

These photographs are of longitudinal sections cut parallel to the long axis of the anterior papillary muscle (top) and the posterior papillary muscle (bottom) of dogs injected intravenously with 1 ml/kg body weight of a 4% solution of the dye thioflavin S ten seconds before excision of the heart. The epicardial region is at the bottom of both photographs, and the papillary muscles are at the top. Both photographs were made under ultraviolet light. The even distribution of thioflavin S throughout the capillary endothelium of control nonischemic left ventricle is seen at the top, while the pattern of blood flow after 60 minutes of occlusion of the circumflex branch of the left coronary artery is shown at the bottom. Most of the subendocardial myocardium is free of thioflavin S, indicating that it is a low flow area. Note the subepicardial myocardium did receive sufficient blood to allow some staining. The endothelium of some of the larger arteries also stained. Some of the bright refractile opaque pale zones which appear to be stained in the PP in this black and white photograph are highlights.
ischemia generally is found in the subendocardial myocardium. Ischemia usually is mild in the superficial myocardium, although cell death can occur in this region.

Our general aim has been to determine the structural and functional effects of severe ischemia on myocardial cells with the particular goal of learning what intracellular events lead to the development of irreversibility (Fig. 1). The focus of severe ischemia used in most of our studies has been the posterior papillary muscle (PP) infarct of the dog. This tissue, which is easily identified for sampling, becomes severely ischemic after high occlusion of the circumflex branch of the left coronary artery. The superficial, less ischemic myocardium has not been studied in detail by electron microscopy although many authors have used this tissue for chemical studies of ischemic injury.

Reversible and Irreversible Ischemic Injury

As noted earlier, ischemia alone quickly produces failure of specialized functions in the involved myocardium; contraction, in particular, ceases. However, these ischemic, contractile cells are not dead. Restoration of arterial flow after periods of ischemia of up to 18 minutes is followed by prompt restoration of function of all cells. Injury to these cells, thus, is reversible. Prolongation of the period of ischemia to 20 minutes results in isolated cell death or tiny islands of cell death in the PP of about 50% of animals, regardless of whether arterial flow is restored. The injury to these cells is irreversible (Fig. 1). These cells have passed the point of no return. If the period of ischemia is prolonged to 40 minutes, cell death occurs in spite of reflow in about 50% of the cells of the PP. After 60 minutes of ischemia, most of the cells in the PP and the subendocardial myocardium are irreversibly injured.

No changes in the gross appearance of the myocardium can be seen in the PP during the phase of reversible injury. However, the irreversibly injured tissue often is visible after 40 and almost always is visible after 60 minutes of ischemia; it is pale gray in contrast to the deep red control myocardium (Fig. 3).

Structural Changes in Ischemic Injury

Healthy myocardial tissue removed from well-oxygenated left ventricle and fixed by immersion fixation shows contracted cells (Fig. 4). The myofibrils exhibit A and Z bands; the sarcolemma is scalloped and is covered with a fuzzy basement membrane. The nucleus usually is centrally located, and nuclear chromatin is evenly distributed. There are abundant mitochondria in the sarcoplasm. In the rat, for example, 35.8% of the volume of the myocardial cell is occupied by these organelles. In sections there is usually one per sarcomere. The cristae are tightly packed; the matrix space is small. Abundant granules of glycogen are present in the sarcoplasm of most cells. Occasionally, lysosomes are present, particularly in the perinuclear region. Droplets of neutral fat are uncommon and, when present, are found at random in the sarcoplasm. The T-tubule system is prominent at many Z bands, and sarcoplasmic reticulum is best seen covering the myofibrils in sections through the periphery of these contractile elements.

Tissue fixed by perfusion in vivo with glutaraldehyde shows relaxed myocardial cells; the myofibrils have prominent I bands. The relaxation occurs because the heart stops beating shortly after the start of perfusion and dilates, with the result that the cells become fixed in a position of extreme stretch. This form of fixation shows the close proximity of capillaries and sarcolemma in myocardium and demonstrates that the morphological

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**FIGURE 3**

Myocardium after 60 minutes of ischemia due to permanent occlusion of the circumflex branch of the left coronary artery. This longitudinal section includes the middle of the posterior papillary (PP) muscle. Note that the pale gray, irreversibly injured tissue is present throughout the entire PP and much of the adjacent subendocardial myocardium. The grossly normal subepicardial myocardium was cyanotic and acontractile throughout the period of ischemia. This section was fixed for 60 seconds in formalin prior to photography. Magnification × 55. (Reprinted from Effect of Acute Ischaemia on Myocardial Function, by permission.)

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features of myocardial cells do not change much as a function of the slower fixation which occurs in osmium.21

REVERSIBLE ISCHEMIC INJURY

With low arterial flow providing little or no substrate and depressed tissue pO₂ allowing little oxidative metabolism, severely ischemic myocardial cells function primarily by anaerobic glycolysis. Glycogen is the principal fuel for this process, and its content continuously decreases as the period of ischemia is prolonged.4 Intermediates of glycolysis accumulate, as does lactic acid, inorganic phosphate, creatine, hydrogen, and other metabolites. The myocardium ceases contracting and bulges with each systole, as a function either of depleted supplies of high energy phosphate7 or because of altered intracellular pH.14 The two principal changes found ultrastructurally, diminished cellular glycogen and relaxed myofibrils, are reflections of the metabolic state of the cell (Fig. 5). Even though relaxed, the myofibrils remain in register. These changes are not sufficiently marked during the first few minutes after occlusion to allow detection of reversible injury but usually are seen after 10 to 15 minutes of ischemia. These early changes are not necessarily uniform. Some cells are identical to control; others show glycogen depletion and relaxation. This finding may reflect difficulties in sampling or variation in the degree of injury. By the time the cells have been ischemic for 15 minutes, some cells contain mitochondria which
Fifteen minutes ischemia, reversible injury. This sample of PP shows relaxed myofibrils with I bands on either side of the Z band (Z). Little glycogen is present. The mitochondria are similar to those found in nonischemic control left ventricle. Osmium fixation. Magnification × 66,400. (Reprinted from Effect of Acute Ischaemia on Myocardial Function, by permission.)

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appear to be slightly swollen, and there is a suggestion of margination of nuclear chromatin.

**IRREVERSIBLE ISCHEMIC INJURY**

The changes found in the early phase of irreversible injury are described best in tissue which has been subjected to 60 minutes of ischemia because irreversibly injured tissue can be identified grossly and accurately sampled at this time (Fig. 3). Such tissue exhibits all the changes found in severe reversible injury, but the changes are more marked (Fig. 6). Glycogen is virtually absent. The myofibrils are stretched but to a greater extent; N bands are common. Moreover, the cells appear to be swollen and exhibit an increased sarcoplasmic space. The sarcolemma occasionally is lifted off the involved cells and occasionally exhibits tiny defects in the plasma membrane. Mitochondria also are swollen and have an enlarged matrix. Some mitochondria are broken; others have disorganized cristae. The most striking mitochondrial finding is the appearance of one or more amorphous densities in the matrix space of each mitochondrial profile (Figs. 6 and 7). The nuclear chromatin pattern also shows striking changes, primarily peripheral aggregation of the chromatin. Lysosomes, the T-tubules, and sarcoplasmic reticulum are morphologically indistinguishable from those found in control non-ischemic myocardium. Also, there is no change in the number of fat droplets in ischemic vs. perfused myocardium of the same animal.

Similar changes are seen in the PP after 40 minutes of ischemia (Fig. 8) but are less severe. For example, swollen and occasionally fragmented mitochondria are present but occur in a smaller number of cells. The amorphous matrix densities are smaller and less frequent. All cells which have marked mitochondrial changes exhibit marked

**FIGURE 6**

Sixty minutes of ischemia, irreversible injury. This section was obtained from the pale gray tissue illustrated in Figure 3. Note the margination of the chromatin in the nucleus (Nu). The myofibrils are relaxed and have prominent I bands (I); an N band also is present in the I bands. The mitochondria are swollen and contain amorphous matrix densities at the arrows. Little or no glycogen is expected to be present at this time, but this tissue cannot be used to illustrate the fact because the fixative extracts glycogen during processing. Osmium fixation with uranyl acetate in block. Magnification x 16,658. (Reprinted from Effect of Acute Ischaemia on Myocardial Function, by permission.)

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nuclear margination, glycogen depletion, marked relaxation of myofibrils, etc.

After 40 minutes of ischemia, some cells of the PP are not irreversibly injured. These are difficult to identify absolutely. However, we believe the absence of mitochondrial matrix densities indicates reversible injury. At 30 minutes, when even fewer cells are irreversibly injured, the likelihood of any block of eight to ten cells including irreversibly injured cells is small. Samples at this time generally have shown the changes of severe reversible ischemic injury but with more marked nuclear and mitochondrial changes.

All the changes noted at one hour are present in irreversibly injured cells subjected to longer periods of ischemia. The number and size of the mitochondrial matrix densities appear to increase up to three to four hours, but we have not studied longer periods of ischemia. Breaks in the unit membrane of the sarcolemma are common after two or more hours of ischemia.  

TOTAL ISCHEMIA OR AUTOLYSIS

It seems likely that some arterial flow is provided to most portions of the ischemic myocardium for an undefined period after occlusion of a major branch of a coronary artery in the dog. The extent of this flow to the areas of severe ischemia such as the PP is unknown at present. If no flow were received by the PP and subendocardial myocardium, the environment of the ischemic tissue in vivo could easily be duplicated in vitro by simply incubating (autolyzing) the tissue at 37°C in a moist environment. This would be the most extreme form of ischemia in the sense that the ischemia is total, but it still differs from the in vivo form produced by occlusion of a single vessel in one other way: the ischemic tissue in vivo is being stretched with each contraction by the volume load during systolic ejection.

Autolysis experiments have shown that all the changes occurring in severely ischemic tissue in vivo also occur in vitro with two exceptions: In vitro the myofibrils are contracted instead of being relaxed, and mitochondrial amorphous matrix densities appear but at a slightly later time (Fig. 9). The stretching of myofibrils in vivo probably occurs because of the dilatation of the acontractile ischemic tissue. The cause of the difference in the rate of appearance of the mitochondrial changes is not known. It is noteworthy that ischemic cell death appears to occur more quickly in myocardium than in other well-differentiated tissues such as kidney. Perhaps, partial oxygenation and the rhythmic stretching of the damaged tissue contribute to the severity of ischemic injury occurring in myocardium in vivo.

EFFECT OF TEMPORARY EPISODES OF ISCHEMIA ON CELLULAR STRUCTURE

Ischemic injury has been defined as being reversible if restoration of arterial flow after an episode of occlusion is followed by restoration of function. Cells of the PP after 15 minutes of ischemia are reversibly injured and after two minutes of reflow are indistinguishable by structural and chemical techniques from nonischemic left ventricular myocardium of the same animal.

Irreversibly injured cells, on the other hand, show striking changes when arterial flow is restored to them. For example, two minutes of arterial reperfusion after 40 minutes of ischemia result in marked swelling, almost explosive in character, in

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**FIGURE 7**

Sixty minutes of ischemia, irreversible injury. This mitochondrion is from the same tissue shown in Figure 6. The outer membrane is intact. The cristae are widely separated, and four amorphous matrix densities (a) are present. The sarcolemma is at S. Osmium fixation with uranyl acetate in block. Magnification × 75,000. (Reprinted from *Effect of Acute Ischaemia on Myocardial Function*, by permission.)

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those cells which contain amorphous mitochondrial matrix densities. This swelling is visible grossly and is readily detectable chemically. The ultrastructural manifestations of swelling include the appearance of vacuoles within the cell, enlargement of the mitochondria, and, finally, enlargement of the cell both because of the aforementioned features and the fact that the sarcolemma often is lifted off the myofibrils (Fig. 10). Other striking features of temporary ischemia are the appearance of mitochondrial deposits of calcium phosphate (Fig. 10, insert) and myofibrillar contraction bands. These bands include six to eight sarcomeres and result in the disruption of myofibrils between the bands. Lastly, all the changes which were present prior to reflow persist following reflow. These include glycogen depletion, nuclear changes, and mitochondrial amorphous matrix densities (Fig. 10, insert).

The source of the mitochondrial Ca\textsuperscript{2+} accumulation is the plasma reperfusing the area. We have shown that myocardial Ca\textsuperscript{2+} uptake is linear between two and ten minutes of arterial reflow (Fig. 11). The tissue calcium content increases to as much as ten times the concentration found in control tissue after 20 minutes of reflow. Much of this calcium is accounted for in the mitochondria in the form of granular dense aggregates. The speed of the process is remarkable.

It should be noted that these calcium changes do not occur as isolated phenomena but are a part of the process of swelling, contraction band forma-
slightly during the phase of reversible injury. With continued ischemia they develop small amorphous matrix densities. These are first seen at 30 to 40 minutes and increase in size and number in the next 200 minutes.

These amorphous matrix densities are characteristic of irreversible ischemic injury, but the cause of their development is unknown. These densities have been concentrated and purified several-fold by sonication and differential centrifugation. Preliminary results of these experiments have shown that they consist primarily of lipid although Ca²⁺ also is increased slightly within them.

The capacity of mitochondria isolated from PP after 60 minutes of permanent ischemia to metabolize NADH-linked substrates is depressed greatly. In addition, these mitochondria are more fragile than mitochondria of nonischemic myocardium. However, damaged mitochondria containing the early small amorphous matrix densities can accumulate CaPO₄ when blood flow to the injured cells is restored. Sustained and marked mitochondrial Ca accumulation requires substrate, ATP, and oxygen in vitro, and the fact that it occurs in damaged mitochondria in vivo indicates that part or all of the electron transport system of these mitochondria still is intact at the time of arterial reperfusion. The accumulated Ca²⁺ precipitates as calcium phosphate, a phenomenon which suggests that Ca²⁺ is present in excess in the sarcoplasm adjacent to the mitochondrion. These aggregates appear to be unrelated to the amorphous matrix densities.

The significance of these various mitochondrial changes relative to the development of the irreversible state remains unknown.

Summary

The sequential changes occurring in dog heart myocardial cells as a consequence of ischemia are described. During the phase of reversible ischemic injury, the myocardial cells show swelling, some glycogen depletion, and mild nuclear and mitochondrial changes. The early phase of irreversible injury is characterized by stretching of myofibrils, mitochondrial swelling, increased mitochondrial fragility, the appearance of amorphous densities in the matrix space of the mitochondria, margination of nuclear chromatin, and the virtual absence of glycogen. Similar structural changes occur in myocardial cells subjected to autolysis in vitro except that myofibrils remain contracted and the other changes appear later than they do after permanent

Mitochondrial Changes in Ischemic Injury

Since mitochondria are critical for normal cardiac function and since these organelles show extensive structural changes in severe ischemic injury, the changes in these organelles deserve special consideration. The sequence of events occurring in mitochondria of severely ischemic tissue is depicted in Figure 12. The mitochondria swell
FIGURE 10

Forty minutes of ischemia and 20 minutes of arterial reflow in PP. Note the disruption of cellular architecture with prominent contraction bands adjacent to the nucleus (Nu). The myofibrils between the contraction bands are disrupted. Mitochondria (M) are swollen and contain dense granules (arrows). The cells are swollen. Vacuoles (V) are present, and the plasma membrane of the sarcolemma (S) often is lifted off the myofibrils. Osmium fixation. Magnification × 4,000. The insert shows the interior of a mitochondrion with a typical granular density (g). These contain much calcium phosphate and are in the matrix. However, nearby cristae often contain granular dense material. An amorphous matrix density is at (a), and the arrow points to the unit membrane of a cristal. Osmium fixation. Magnification × 172,000.
Graph illustrating calcium accumulation in control and damaged dog myocardium as a function of time. **Ca was injected 30 minutes prior to occlusion of the circumflex branch of the left coronary artery for 40 minutes followed by the periods of arterial reflow noted on the abscissa. Tissue **Ca has been adjusted for the level of serum **Ca found 45 minutes after intravenous injection of the **Ca and is reported as 10^8 cpm/g wet heart per 10^5 cpm/ml plasma. The damaged tissue (PP) is indicated by solid circles, and the control tissue (LV) is indicated by open circles. The magnitude of the standard error is represented by the brackets. (Reprinted from American Journal of Pathology, by permission.)

Sequential changes in mitochondrial morphology as a function of ischemia with and without arterial reflow. With permanent ischemia (no reflow), the amorphous matrix densities first appear at 40 minutes. They enlarge and increase in number for the next three to four hours. Also, the mitochondria swell and often show ruptured outer membranes. If arterial reflow is allowed at 40 minutes, granular densities of calcium phosphate appear near the cristae of the involved mitochondria. (Diagram modified from one of Dr. A. C. Shen and based on experiments described in references 32 and 33.)
ischemia. Reperfusion of arterial blood into a focus of irreversibly injured myocardial cells produces explosive cell swelling, contraction bands, and the accumulation of calcium phosphate within the mitochondria. These latter changes suggest that defects in cell membrane permeability or cell volume regulation are a prominent feature of the early phase of irreversible injury.

Acknowledgment

Over the course of these experiments, P. B. Herdson, M.B., Ch.B., Ph.D.,* 11, 12, 13, 14 and A. C. Shen, M.D., Ph.D.,* 15 have contributed much to the evaluation of the changes in ultrastructure associated with ischemic injury. Hannah Schellin, W. B. Taylor, Barbara Green, and Saideh Safavi have provided expert technical assistance.

References


Discussion

Dr. E. I. Chazov, Moscow, U.S.S.R.: You have described the structural changes occurring with varying periods of ischemia. Your data seem to indicate that after 40 minutes of ischemia, there is little that can be done by the clinician to reverse the initial necrosis. We are interested in methods to prevent damage at the periphery of the infarct. Could you comment, not on the time frame, but on the influence of the degree of ischemia on cell size? What cellular changes would be seen in a case of sudden death?

Dr. Jennings: First, let me say that it is very difficult to translate these data from dogs to man. In addition to species differences, these dogs were anesthetized and had a major operation. On the other hand, I am certain that the events occurring in the myocardium of a man with sudden occlusion of a major coronary artery are generally similar to those seen in dogs.

I think that the changes that lead to sudden death in man probably include a constellation of events. There are individuals who simply have an episode of minor ischemia and develop an arrhythmia and die. The myocardium of these individuals would be indistinguishable from normal myocardium in which ventricular fibrillation had developed. There are other patients who have a large area of ischemia and who, after a period of time varying from minutes to hours, develop an arrhythmia. Areas of ultrastructural change, such as I have shown here, would be found in such hearts, even though overt necrosis had not yet developed, but identification of such cells would be a slow process since one can only examine a few cells at a time.

I should note that the severe permanent ischemia found in the posterior papillary muscle infarct is essentially equivalent to total ischemia or autolysis. If we simply remove a bit of control myocardium and incubate it at 37° C, all of the changes which I have shown you in permanent severe ischemia in vivo will develop in vitro with two exceptions: First, there is no fiber stretching because there is no systolic tension to stretch the fibers, and second, the changes appear at a slightly slower rate. The changes present at 60 minutes in vivo are present at about 90 minutes in vitro. The difference may result from oxygen entering the area from collateral flow.

Also, I think that it is certain that not all cells in an area of reduced flow are equally ischemic. It is clear that some myocardium on the periphery of the infarct dies at a slower rate or survives the ischemic episode. We have assumed that the changes occurring in this tissue are similar to those found in severely or totally ischemic tissue but have no data on this point. At any rate the effects of mild to moderate ischemia on myocardial cells need investigation not only for general interest, but because of their clinical relevance.

Dr. Chazov: Since ventricular fibrillation is the most common cause of sudden death in patients with acute ischemia, should we not be more concerned with changes in electrolyte balance than in cell morphology? What changes in calcium occur during acute ischemia?

Dr. Jennings: I believe that ischemic cells swell. However, in severe ischemia they swell from a finite volume. For example, 1 kg of totally ischemic tissue contains 750 ml of water—550 ml of intracellular water and 200 ml of extracellular water. If there is no flow, the only electrolyte changes that can occur result from redistribution between the intracellular and extracellular fluid. The only calcium available for electrolyte shifts in totally ischemic tissue is the amount present intracellularly, which as free ion is about 10^-7 M, and ionized calcium in the extracellular fluid. There are about 2.5 mM of calcium per liter of blood. Assuming that it all was ionized, there would be 0.0025 mM Ca** per ml × 200 ml of extracellular fluid or 0.5 mM of calcium to shift. Thus, the amount of calcium
available to shift in ischemic tissue is extremely small.

These calculations can be made from any ion present in the tissue. I was greatly surprised by the results with potassium. I initially postulated that after 40 minutes of ischemia, potassium would have equilibrated between the intracellular and the extracellular fluid in the fashion described above. Under these conditions reperfusion of the area has produced a large potassium efflux. As you have seen, the potassium washout was slow. My assumption that potassium would equilibrate was not supported by this analysis.

The first situation I described relative to Ca\(^{2+}\) was one in which ischemia was total, i.e., no arterial flow. The second situation was one in which there was reperfusion of an area of severe ischemia. The third situation is more complicated and is that represented by tissue receiving a significant arterial flow but in a volume inadequate to support aerobic metabolism. By definition this tissue is ischemic, but because of the arterial flow, greater electrolyte shifts are possible. The significance of these shifts is unknown because it is difficult to identify such areas. Moreover, if we could identify them, quantitation of electrolyte shifts would be difficult because there is currently no technique available to monitor local arterial flow continuously.

Dr. A. M. Vikhert, Moscow, U.S.S.R.: I should like to comment on two particular points. First, you described a relative decrease of subendocardial as opposed to subepicardial myocardial blood flow during ischemia. These observations correlate well with the clinical data, first reported in the United States in 1931, describing subendocardial infarction during hypoxia, anemia, and general circulatory disturbances. Second, I should like to point out the usefulness of the appearance of the amorphous matrix densities which apparently signify that irreversible change has occurred.

I also have five questions: (1) Does the pH change in the zone of ischemia, and if so, how rapidly does it change? (2) Do you have any information on the permeability of the blood vessels? (3) What is the composition of the amorphous material? (4) When does the potassium loss begin? Are the calcium shifts for dogs specific since it is known that in dogs myocardial necrosis is very frequently accompanied by calcification, while in other mammals, such as rabbits, calcification does not take place? Could the inclusion of calcium in these amorphous densities be a characteristic of the canine species only? (5) Did you study the effect, not of complete ischemia, but of partial chronic ischemia on muscle fibers? Such observations would relate to the unresolved clinical question of whether chronic coronary insufficiency per se leads to hypertrophy of muscle fibers.

Dr. Jennings: (1) As the cell converts from aerobic to anaerobic metabolism, a hydrogen ion excess rapidly develops. Drs. Neely and Morgan have demonstrated the fall in pH very clearly in their ischemic perfusion model. (2) We have no good data on the permeability of blood vessels. During the first 40 or even 60 minutes of ischemia, I can see no changes in blood vessel morphology, aside from some membranous masses which occasionally appear to be projecting into the lumen of the vessel. (3) We have partially isolated the amorphous matrix densities. We have concentrated them and attempted to purify them. About all I know at this time is that much of them is lipid because they disappear as we try to dissolve the contaminating mitochondrial cristae that are found with our isolated dense bodies. These amorphous matrix densities are found in reversibly injured cells in other systems. They are found with ischemic or mercury injury to the kidney or with carbon tetrachloride injury to the liver. I think it is a fairly general phenomenon. (4) As for accumulation of calcium, the ion will accumulate in myocardial cells quite rapidly in a variety of species—monkeys, rats, guinea pigs—after toxic doses of catecholamines, such as isoproterenol. Also, the terminal electron transport system of isolated mitochondria in the presence of calcium, substrate, and high energy phosphate will take up calcium and deposit it as calcium phosphate in the matrix of mitochondria. Thus, Ca\(^{2+}\) accumulation is a fairly general phenomenon which is not restricted to the dog. (5) We have done no experiments on partial ischemia or on hypertrophy associated with ischemia, so I cannot respond to your question concerning muscle fibers.

Dr. G. A. Langer, Los Angeles, California: Dr. Jennings, you did not comment directly on the characteristics of the sarcotubular system under the circumstances of ischemia. What did you find to be the state of the sarcoplasmic reticulum (SR) prior to reperfusion and then following reperfusion in the irreversible situation?

Dr. Jennings: It is difficult to see changes in the SR in cells that have just entered a state of irreversible injury. Occasionally, it appears to be vacuolated. I can comment that the transverse tubular system still contains basement membrane in the early phases of irreversible injury. These
irreversibly injured cells swell enormously during reflow. I showed you those large vesicles which developed. The landmarks are so disrupted that I do not know whether this represents a swollen transverse tubular system or a swollen SR.

**Dr. Langer:** When we intentionally calcium load cells via low sodium, high calcium solutions or whatever, the mitochondrial response is similar to that which you have described. The cristae are disrupted, and calcium phosphate or calcium phosphate-carbonate complexes appear.

In isolated perfused myocardium the SR also dilates to a great extent. We undertook a series of studies with the surgical department on heart preservation in which hearts were removed and then maintained perfused for 12, 18, or 24 hours. It was striking that if one did not control the level of calcium in the reperfusion medium and even in the preservation medium, these hearts would rapidly assume a rigor state with marked contracture. Perfusates and preservative solutions with low calcium concentrations virtually eliminated this rigor.

I think your figures demonstrate that the cells prior to reperfusion handle whatever calcium load that is present very well and, in fact, that the sarcomeres are in a relaxed state. Two processes might be responsible for calcium uptake during reperfusion: (1) The calcium pumping for mitochondrial storage could be affected, and (2) the prevention of calcium influx by the plasma membrane might be greatly impaired. Have you reperfused with solutions of very low calcium concentration, such as 50 μM, which is enough to preserve or re-establish integrity of the membrane, but not enough to permit marked calcium influx?

**Dr. Jennings:** That is a very interesting set of observations and an excellent idea. We have reperfused only with blood. I think those of you who have worked with this kind of system will recognize that there is a very high incidence of ventricular fibrillation on reflow. Since these hearts are so fragile, it would be a difficult, but not impossible experiment.

**Dr. Robert M. Berne, Charlottesville, Virginia:** Have you tried to correlate irreversibility with any specific chemical changes, particularly with the level of high energy phosphate stores? As I mentioned yesterday, in the studies of Gerlach and his associates, hypoxia is a strong stimulus to de novo synthesis of nucleotides. This finding is particularly striking in the kidney where hypoxia produces a 700% increase in nucleotide synthesis. In the brain there is no increase in de novo synthesis of nucleotides following hypoxia. Perhaps this lack of increased nucleotide synthesis relates to the early development of irreversible change in the brain during hypoxia. With respect to the heart, I am curious to know whether there was any correlation between the adenine nucleotide level of the myocardium and irreversibility of ischemic damage.

**Dr. Jennings:** I cannot answer that question from our own work. However, in the experiments of Gudbjarnason and Bing, in areas that I would define as ischemia, it is quite clear that creatine phosphatase disappears after about 30 seconds. ATP levels drop to about 50% of normal in a period of minutes, but further decrease in the ATP level is quite slow. I am quite certain, although I have not measured it directly, that there is still a significant amount of ATP present in the posterior papillary muscle in the early stages of irreversible injury. We have estimated creatine phosphatase, which is absent, and inorganic phosphates, which are quite high.

**Dr. Berne:** We did similar studies some years ago but not for such long periods of time, and our findings are identical with yours. We noted an almost immediate disappearance of creatine phosphate followed by a sharp decline in ATP and then a leveling off of the ATP content. Interestingly enough, when we reperfused the heart, the ATP levels were rather slow to return to control levels. We were dealing with a reversible injury. I am curious to know how long ischemia could be prolonged before the depression of ATP content becomes irreversible?

**Dr. Jennings:** I do not know.

**Dr. J. Gergely, Boston, Massachusetts:** I wonder if you could go back to a point you made earlier. I did not quite follow the argument about the presence of 10⁻⁷ M free calcium.

**Dr. Jennings:** I was attempting to make the point that myocardial tissue calcium is almost infinitely small. There is calcium present, but the intracellular concentration is about 10⁻⁷ M, according to the data we heard yesterday.

**Dr. Gergely:** I think there may be some misunderstanding of that point. That may well be the free ionized calcium, but the total myocardial calcium would be on the order of 0.5 to 1 or 2 mM when the amount sequestered in mitochondria and the reticulum is considered.

**Dr. Jennings:** I cannot remember the exact figures, but we have measured total tissue calcium, and it is quite small. I shall look them up for you.

[Note added in proof: Dog left ventricle contains 0.86 mmole Ca/kg wet weight. Part of this Ca is}
intracellular, and a part is extracellular. If one estimates the extracellular fluid (ECF) volume as 225 ml and assumes that all of the 2.5 mmole of Ca in a liter of plasma is in the ECF and is ionized, one can estimate that there are 0.56 mmole Ca in the ECF of each kg of heart. This means that there is about 0.86 minus 0.56 or 0.3 mmole Ca inside the cells. The intracellular Ca is distributed in 560 ml of intracellular fluid (ICF) which yields a concentration of 0.54 mmole Ca/L of ICF or 5.4 \times 10^{-4} \text{ M}. Actually, much of this Ca surely is bound since the intracellular Ca concentration is believed to be 10^{-7} \text{ M}. Thus, the Ca available to shift in an area of total ischemia is the amount found in the ECF to the extent that it is available for transport intracellularly plus the bound intracellular Ca. The amounts involved seem very small, particularly in view of the capacity of mitochondria to accumulate this ion even in the absence of oxygen.\]

**Dr. Eugene Braunwald, Boston, Massachusetts:** For those of us who are interested in preserving myocardium in patients, 40 minutes is too short a period of time to intervene between the onset of ischemia and the time an intervention can be applied to be useful clinically. As I will be indicating later this morning, we have reason to believe that this time period can be extended. So, my question is, what is the evidence for the statement that the myocardium was irreversibly damaged by 40 minutes of ischemia?

**Dr. Jennings:** I have been discussing the changes occurring in an area of severe ischemia comprising about one third of the total area of ischemia. It is quite likely that cells in this area will die. As you probably know, we can prevent cell death, even in the severely ischemic cells, by pretreatment of the animals with propranolol. I really do not know the mechanism of this protection by propranolol, but at any rate, it occurs. I think that some of the cells in the other two-thirds of the area of ischemia die as long as three or four hours postocclusion. Later events, such as an arrhythmia, could lead to delayed cell death. I want to emphasize that I am talking about the changes occurring in severely, not mildly ischemic tissue.

**Dr. Braunwald:** Have you examined the injured myocardium a week after ischemic injury?

**Dr. Jennings:** Yes, we have studied this tissue a week later and found most of the dead cells are either gone or are undergoing phagocytosis. Eventually, there is replacement of the posterior papillary muscle and the subendocardial myocardium with scar tissue. There is a picture of this in the *Annals of the New York Academy of Science*.

**Dr. Braunwald:** With 40 minutes of ischemia?

**Dr. Jennings:** Yes.

**Dr. Braunwald:** And then reperfusion?

**Dr. Jennings:** Yes. Even with reperfusion about half the cells in the subendocardial myocardium die, become necrotic, and are removed by phagocytosis.

**Dr. Braunwald:** Dr. Leaf and his colleagues at the Massachusetts General Hospital have been very interested in cell swelling after ischemia. They began with the observation by Dr. Ames that after occlusion of a cerebral vessel, there is sufficient swelling of the nerve cells that the microcirculation is blocked during reperfusion. They then extended this observation to the kidney and found that after ischemia there was sufficient swelling of renal tubular cells to obstruct renal blood flow during reperfusion. Finally, they have extended their observations to cardiac cells after coronary occlusion. It is postulated that ischemia damages the pumping mechanism in the cell membrane with resultant cell swelling. Drs. Powell, Leaf, and their colleagues have utilized a hypertonic mannitol solution in the kidney to reduce cell size and thereby increase perfusion at the level of the microcirculation. They have made similar observations in the heart. I wondered if Dr. Jennings would like to comment on these findings which seem to correlate well with the cell swelling he has described.

**Dr. Jennings:** Ames, Majno, et al., who first described the no reflow phenomenon in the brain, demonstrated quite clearly that only five minutes of total ischemia were required to limit the amount of reflow in this organ. It seems likely that this failure of reflow contributed to additional cell death. However, since the cranium limits expansion of the brain, these findings may not be generally applicable to tissues, such as heart and kidney, in which swelling can occur to a greater extent. In the rat kidney events following occlusion of the renal artery are more complex. After occlusion the kidney continues to swell. Since there is no significant de novo formation of water in ischemic kidney, the source of the water must be from blood flow still entering the kidney. If there is flow to an ischemic area, the cells obviously will be able to swell to a greater extent. Moreover, it seems likely that reflow eventually will be slowed in greatly swollen tissue. Dr. Leaf and his colleagues have postulated that swelling could be slowed or prevented if the tissue was kept hypertonic during the ischemic phase. Mannitol infusions were used for this purpose and did, in fact, improve reflow.
think that some renal cells die during the initial ischemic period, but I agree that failure of reflow will greatly increase the severity of the injury.

In the reflow model in the heart that I have just shown you, there is obvious cell swelling and occlusion of capillaries after 40 minutes of ischemia and two to five minutes of reflow. However, there is no alteration in the thioflavin S staining pattern; calcium is accumulated out of the plasma in a linear manner, and the swelling itself indicates that a significant amount of reflow is occurring when the clamp is opened at 40 minutes. Longer periods of ischemia, such as 90 minutes, do result in no reflow. We think that this is due to direct capillary damage. I believe mannitol pretreatment should improve reflow in severely ischemic tissue. However, this belief remains to be verified along with establishment of the period of time during which this treatment will be effective.