Quantitative Correlation between Cell Swelling and Necrosis in Myocardial Ischemia in Dogs

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SUMMARY Previously, we demonstrated that there were effects of elevated plasma osmolality on both early cell swelling and eventual cell necrosis in ischemic cardiac muscle. The present study quantifies the extent of cell volume derangement, determines whether or not there is a quantitative relationship between cell swelling and eventual necrosis, and defines the time limits of ischemia within which prevention of early swelling by hyperosmotic intervention can reduce eventual necrosis. Computer-assisted analysis of tissue pathology was used for quantification of myocardial cell swelling early during ischemia. The results then were correlated with the extent of eventual necrosis. When canine posterior papillary muscle was sampled for electron microscopy soon after the restoration of blood flow following proximal circumflex artery occlusion, stereological methods revealed a substantial increase in myocardial cell volume. The data define the spectrum of volume gain in ischemic myocardial cells. The effectiveness of an osmotic intervention with mannitol in preventing cell swelling and eventual necrosis was limited to ischemic periods of less than 90 minutes. There was a strong linear correlation between the fractions of cells with increased volume (whether the increase was measured as cytoplasmic space, mitochondrial volume, or overall volume expansion), and the fraction of cells necrotic 12 hours after restoration of blood flow. The pattern of action of osmotic intervention in the prevention of ischemic cell swelling and in diminishing eventual necrosis in this model suggests strongly that there is an important relationship between a failure in cell volume regulation and eventual cell death in myocardial ischemia. Circ Res 47: 653-665, 1980

RECENTLY, the search for agents and interventions to salvage ischemic myocardial tissue has led to emphasis on the events that occur early during myocardial ischemia (Braunwald, 1976). Our approach to this problem has been based on the finding in other organ systems that an early and potentially dangerous consequence of tissue ischemia is a failure in cell volume regulation (Chiang et al., 1968; Flores et al., 1972; Leaf, 1970). This important process is impaired with ischemic inhibition of metabolism, since a supply of metabolic energy is required for the continuous active extrusion of sodium on which cellular volume control depends (Macknight and Leaf, 1977). Earlier work suggested, within the confines of the model employed, that cell swelling, determined qualitatively, occurs during myocardial ischemia (Kloner et al., 1974a; Powell et al., 1973, 1976a). The administration of hyperosmotic mannitol prevents early cell swelling and reduces the extent of eventual necrosis (Powell et al., 1973, 1976a). It is recognized that the potential clinical utility of this particular intervention, hyperosmotic mannitol, has limitations, and it has since been noted by others (Fixler et al., 1977; Härzel and Kirk, 1977) that the general applicability of the above findings to other experimental situations might be limited as well.

In light of the above considerations, the present study was designed to investigate specifically the validity of the emergent hypothesis that the degree to which myocardial cells swell after reflow of arterial blood in an area of ischemia has a direct effect on the probability of their eventual death. The possibility of a correlation was suggested earlier (Powell et al., 1976a), but it was recognized, at that time, that statistical comparisons were unwarranted because objectivity was provided only by blind examination with purely subjective criteria. Although estimations of necrosis were valid, no attempt had been made to quantify the state of individual cells with respect to their volume, and cell swelling was, therefore, largely undefined. This report details a quantitative examination of the swelling of papillary muscle cells following transient circumflex artery occlusion and correlates the extent to which the degree of swelling in individual cells is related to the probability of their eventual necrosis. The extent to which hyperosmotic mannitol can alter the degree of cell swelling which develops after an episode of ischemia followed by reflow is quantified. With systematic variations of the period of arterial
occlusion and with the use of increased plasma osmolality as an experimental variable, it has also been possible to determine the maximum duration of an ischemic insult which myocardial cells can tolerate without incurring damage that is irreversible by osmotic means.

Methods

All experiments were performed on adult mongrel dogs (weighing between 20 and 24 kg) which were anesthetized with intravenous sodium pentobarbital (30 mg/kg). After endotracheal intubation, the animals were ventilated with 95% O2/5% CO2 at rates of 14–18 cycles/min; a left thoracotomy was then performed. We used the model of ischemia which we have used previously (Powell et al., 1976a), clamping the proximal circumflex branch of the left coronary artery within 1 cm of its origin for a predetermined time to interrupt blood flow to the posterior papillary muscle of the left ventricle (Jennings et al., 1960). With this model, the anterior papillary muscle provides a nonischemic control. Prior to the occlusion, procainamide hydrochloride (Pronestyl, Squibb & Sons), 50 mg/kg, was administered intramuscularly at three separate sites. With this agent, there was no mortality due to arrhythmias either during the occlusion or during the reflow period. During each of the experiments a manniot, saline, or control plasma infusion was administered intravenously during the last 15 minutes of the period of occlusion and during the first 15 minutes of the period of reflow. The manniot solution was infused at 15.3 ml/min for 10 minutes and then 7.64 ml/min for 20 minutes. This rate of manniot infusion elevated the serum osmolality to 30–40 mOsm/Kg H2O above control. The control infusions were carried out at 38.2 ml/min for 10 minutes and then 15.3 ml/min for 20 minutes—a rate which was sufficient to lower the hematocrit by the same amount as the manniot. Aortic pressures, blood gases and serum ion content and osmolality were monitored throughout each experiment (Powell et al., 1976a). The protocols employed were of two types: for evaluation of necrosis, the reflow period following release of the proximal circumflex clamp was continued for 12 or 24 hours. Six dogs were in each of the six groups (40, 60 or 90 minutes of the circumflex artery occlusion with or without manniot administration) of experiments in which reflow was prolonged. For assessment of swelling, the dogs were killed after brief periods of reflow of either 15 or 45 minutes. There were six animals in each of the four groups that underwent 40 or 60 minutes of circumflex artery occlusion—either with or without manniot—and brief reflow of blood. The two 90-minute occlusion groups which had brief reflow periods had four animals in each.

Tissue Preparation and Examination

When the reflow had been allowed to proceed for the time prescribed by the experimental protocol, the heart was removed and the papillary muscles were quickly excised. Immersion fixation was the procedure of choice for these experiments for the reasons previously described (Powell et al., 1976a). Cross-sectional slices (1–2 mm in thickness) were obtained from the distal thirds of the papillary muscles. These slices were immersed in fixative (2.5% glutaraldehyde in an isotonic phosphate buffer, pH = 7.4) and cut into cubes. Care was taken to avoid tissue which was within 2 mm of the endocardium. This process required less than 45 seconds from the time the heart was excised to the time when approximately ten 1- to 2-mm cubes of tissue were placed in bottles of fixative. Samples remained in this fixative overnight at 4°C and were subsequently postfixed in OsO4, dehydrated, and embedded in an Epon-Araldite mixture (DiBona et al., 1969). Sections for light and electron microscopy were cut on an LKB Ultratome III microtome (LKB Produkter). Thin sections (500–800 Å) were double-stained with uranyl acetate and lead citrate (Reynolds, 1963). Three to six cubes of tissue were selected randomly from the cubes from each papillary muscle for electron microscopy. Sections were examined with a Philips EM-200 electron microscope (Phillips Instruments) when conventional photographic recording of images was to be employed. An RCA EMU-4 electron microscope which was fitted with a transmitting phosphorescent screen was used for direct observation by video methods. Video images were collected both from this machine and from a light microscope with a Minicon camera (Sierra Scientific Products) and stored on a 500-channel video disc recorder (Data Memories, Inc.). Video images were collected routinely at a scanning frequency of 1029 lines/frame to optimize vertical resolution.

Quantitative Analysis of Tissue

Analysis of papillary muscle morphology was carried out with computer-assisted morphometric methods. Specimen images stored on the disc recorder were examined on the video display tube of a Princeton 801 computer graphics terminal (Princeton Electronic Products) which was interfaced to a PDP 11/10 computer (Digital Equipment Corp.). Programs for extraction of quantitative information were written in BASIC. The general format employed was based on the frequency of occurrence of the components of interest within the displayed sample. This was determined by compiling the distribution of “hits” on structures in the field that resulted from a spraying of cross-hair images at randomly generated positions. This is similar to methods that employ a fixed rectangular grid (Page and McCallister, 1973) but offers the advantages that point selection is completely objective and that the resolution of the grid is limited only by the resolution of the graphics display tube (1024 × 1024 points). The number of targets required to determine distribution frequencies accu-
rately was assessed in preliminary studies by examination of the fluctuation of results with increasing numbers of hits.

Necrosis

The extent of necrosis in papillary muscles was evaluated as the percentage of cells which appeared necrotic by light microscopy of Toluidine blue-stained 1-μm thick sections. These were examined at the level of the graphics terminal by direct video transmission from a light microscope. Final magnification was 1000X. A minimum of 10 sections was examined for each muscle in question; no more than two sections were taken from any one block and when two were taken, the second section was cut after the block face was retrimmed to assure a separation of sections by at least 0.5 mm. Four to six fields per section were sufficient to encompass completely the material in each section. Examination of accuracy in the measurements established that 20 cells/field, 4 fields/section, 10 sections/animal yielded a reproducibility to within 3.0%. The reproducibility was verified by doubling the sample size (i.e., 20 sections) on at least one occasion for each experimental protocol. Necrotic cells were identified by pale, unstained cytoplasmic content and pyknotic nuclei. Determination of necrosis was periodically cross-checked by examination of adjacent thin sections with the electron microscope; cells that had been specified as necrotic routinely contained contraction bands, disrupted mitochondria, and ruptured plasma membranes, confirming the light microscopic determinations.

Cell Volumes

Changes in myocardial morphology which occurred shortly after reflow (prior to evidence of necrosis) required the use of electron microscopy. To measure the relative volumes of subcellular compartments in a quantitative way, a program was used which determined the distribution of randomly generated points on displayed electron microscopic images. In this way, the volume fractions of mitochondria, myofibrils, and cytoplasmic ground substance were assessed. The extent of sampling required to gain a quantitative estimate of nuclei and the transverse tubular (T) system would have been prohibitive, since the nuclei do not occur in each cell section and the T-system is composed of very small profiles rarely hit by a randomly generated point. The collected data were in the form of numbers of hits on mitochondria (M), fibrils (F), and cytoplasmic space free of organelles (C). It then was possible to calculate the volume fractions of the various compartments. F, the number of hits on fibrils, was treated as a normalizing quantity. It was assumed that the volume of myofibrils would not be sensitive to change, since these organelles are not surrounded by individual membranes and, therefore, should be less sensitive to osmotic influence. If fibrillar volume is reduced in the presence of a hyperosmotic perfusate (as suggested by Huxley et al., 1963), it would be expected that the changes in the determined ratios would be minimized, since fibrillar volume would then move in the direction of the other measured parameters. Data were thus reduced in the following three ways. With the assumption that fibrillar volume does not change from one state to another, the ratio (C + M)/F is a sensitive index of total cell volume apart from the nuclear fraction.

Mitochondrial volume was assessed as M/F so that no cytoplasmic component would influence the ratio. The cytoplasmic component was examined as (C + M + F)/(M + F). This fraction was employed to obtain ratios which varied from 1.00 for control anterior muscles (where C = 0) through a range of finite proportions. Using this approach, 3-6 sections of muscle were examined with 12-18 fields of view being analyzed from each. Individual views were examined at a magnification of 7500X. Repeated examination of individual samples confirmed that reproducibility ranged from 1 to 6% in terms of actual percentages of hits/cellular compartment when 60 hits/cell were used. The number of cells/field varied from 2 to 5, with cells included for analysis only when the plasma membrane was observed to completely surround the sectioned profile.

Results

Necrosis

Quantitative analysis of the extent of necrosis after 12 hours of reflow for each of the protocols is shown in Figure 1. In every case, necrosis was absent in the control anterior papillary muscle, and the extent of necrosis within posterior papillary muscles increased with the duration of applied ischemia. Hyperosmotic perfusion was effective in protecting a significant fraction of the posterior papillary myocardium in each of the shorter periods of ischemia. Necrosis was almost abolished in the 40-minute experiments (reduction from 27% with plasma infusion to less than 3% with a mannitol infusion) and substantially reduced (from 63% to 17%) in the 60-minute protocol. Nonetheless, when the period of ischemia was extended to 90 minutes, it was observed that hyperosmotic perfusion was completely without effect. With or without elevation of serum osmolality, approximately 90% of the cells were found to be necrotic (plasma 89%, mannitol 91%). These percentages for the 90-minute studies were virtually unchanged when the posterior papillary muscle tissue was examined after longer reflow periods (19-24 hours); in a series of four dogs to which mannitol was administered early
Morris and associates (1972) have described a brief period of reflow following coronary occlusion as contributing to ischemia-reperfusion injury. They demonstrated that this injury was observed only when a delay between reperfusion and return of perfusing blood to the ischemic heart was present, and that the injury was dose-dependent with increased injury occurring at greater delays. They also demonstrated that this injury was not observed when perfusion with norepinephrine was continued during the delay between occlusion and reperfusion.

We have described in a previous publication qualitative morphological changes in the posterior papillary muscle of the canine left ventricle which accompany ischemia as produced by circumflex occlusion of 40 and 60 minutes (Powell et al., 1976a, 1976b). This qualitative examination consisted of a description of the presence or absence of subsarcolemmal blebs. In the present study, these changes and those following a 90-minute occlusive period have been evaluated quantitatively, but a brief description of tissue appearance is necessary to provide a structural framework within which to appreciate the accumulated numerical data.

Figure 2 provides comparative electron microscopic views of control anterior (Fig. 2a) and posterior (Fig. 2b) papillary muscle cells sampled 45 minutes after release of a circumflex artery clamp that had been in place for 1 hour. As seen in Figure 2a, anterior papillary muscle tissue was unaffected by a 1-hour period of circumflex occlusion; myofibrils are highly organized, mitochondria are compact, the T-tubular system is patent, membrane profiles are continuous, and there is neither evident elevation of the sarcolemma nor cytoplasmic space which is free of formed organelles. This appearance was found consistently for anterior muscle cells even when the occlusive period was extended to 90 minutes. Tissue from the posterior papillary muscle of the same heart is illustrated in Figure 2b. Here there are qualitative changes in the cell architecture which are expressions of an increased intracellular volume. In contrast to the image in Figure 2a, cytoplasmic space is evident between the contained organelles, the sarcolemma is elevated from the enclosed array of mitochondria and myofibrils, and the T-tubular system is no longer visible. This contrasting appearance was noted as well for samples taken 15 minutes after release of a 40-minute circumflex occlusion, but evidence of swelling in the posterior muscle cells was less striking. As reported earlier (Powell et al., 1976a, 1976b), administration of hyperosmotic mannitol substantially reduced the fraction of swollen (presence of subsarcolemmal blebs) posterior muscle cells in both the 40- and 60-minute samples. Details of these effects are described below in a quantitative way. With the rapid fixation technique described in the Methods section, contraction bands were not seen in the 40- and 60-minute samples from either the anterior or posterior papillary muscles. Aside from cross-sections, many sections of the posterior papillary muscles were examined in longitudinal view. In these longitudinal sections, there was no evidence of contraction bands in spite of there being no difficulty in identifying contraction bands in the 12-hour reflow samples.

Tissue sampled 45 minutes after a 90-minute occlusive period revealed a still more pronounced contrast between anterior and posterior papillary muscle cells and no apparent morphological effect of hyperosmotic mannitol administration. Anterior muscle cells were indistinguishable from that shown in Figure 2a; as expected, this tissue was unaffected by a circumflex occlusion even of 1-1.5 hours duration. Figure 3 is an electron micrograph of posterior muscle cells from an experiment with 90 min-
FIGURE 2 Comparative electron micrographs of anterior and posterior papillary muscles fixed 45 minutes after release of a circumflex artery occlusion of 60 minutes duration and in the absence of elevated plasma osmolality. a: Anterior papillary muscle. Myofibrils (f) and mitochondria (m) are closely packed; profiles of the T-tubular system (t) are patent and clearly visible, whereas no free cytoplasmic space can be seen. The surrounding sarcolemma (SL) is closely adherent to the cellular contents. b: Posterior papillary muscle. Myofibrils (f) and mitochondria (m) are here somewhat dispersed; the T-tubular system is no longer visible. Cytoplasmic space (c), free of formed organelles, separates the mitochondria and fibrils and is evident at the cell periphery where the sarcolemma (SL) is elevated above the interior array. Note also that several mitochondria (*) are grossly distended and show a pale matrix. Each figure is magnified 8300x.
FIGURE 3  Electron micrograph of posterior papillary muscle fixed 45 minutes after release of a circumflex artery occlusion of 90 minutes duration and where reperfusion was with plasma made hypertonic by administration of mannitol. Myofibrils (f) show a wide variation in banding pattern and evidence of some disruption; Z-lines (z) are occasionally very widely separated. Mitochondria (m) appear swollen and contain dense inclusion granules; many mitochondria (*) contain disrupted cristae. Cytoplasmic space (c) is abundant and no longer granular as in 60-minute occlusion experiments (Fig. 2b). The sarcolemma (sl) is now markedly elevated. This view and those from isosmotically perfused preparations after a 90-minute occlusion were largely indistinguishable as is subsequently demonstrated by quantitative morphometric analysis. Magnification is 7900x.
minute occlusive periods); mitochondria show a pale matrix and are often disrupted, myofibrils are no longer uniform in appearance and, in several places, show evidence of disruption, and the sarcolemma is elevated in a pronounced way. This appearance was noted consistently in 90-minute occlusion experiments with no evident difference between isosmotic and hyperosmotic perfusion protocols.

Quantification of Myocardial Cell Volume Distribution

Analysis of the distribution of volume in both nonischemic anterior and ischemic posterior papillary muscles was carried out for each of the six protocols. Results were collected as the fractional volumes of cytoplasmic space, mitochondria, and fibrils in the manner described in detail under Methods. The accumulated data were expressed in three different ways.

The first of these was to assess the overall swelling of cells. The most direct available measure of whole-cell volume change was the volume distribution between the sum of cytoplasmic and mitochondrial compartments and the volume occupied solely by myofibrils, the most stable component. This is expressed as a histogram in Figure 4 as (cyto + mito)/fibrils. In Figure 4a are data from control

![Figure 4a](http://circres.ahajournals.org/)

**Figure 4** Histograms describing the percentage distribution of cells with regard to the partition of cellular volume expressed as the sum of cytoplasmic and mitochondrial components divided by the myofibrillar component [(cyto + mito)/fibrils]. This ratio is chosen as an index of overall cellular swelling. Each bar represents the mean percentage among the four to six dogs examined) of the cells which revealed a volume ratio between that specified on the abscissa and the next lower value. Posterior PM median indicates the median cell volume of the posterior papillary muscle. a: Isosmotic perfusion. More than 70% of control anterior muscle cells had volume ratios of 0.50 or less in each of the 40-, 60-, or 90-minute protocols. With extension of the ischemic period, the distribution of posterior muscles shifts to the right reflecting increased intracellular volume; the median cell position changes from 0.75 to 1.00 to 1.25 from 40 to 60 to 90 minutes of occlusion. b: Hyperosmotic perfusion. Control anterior muscles are distributed as in Figure 4a. The distribution of ischemic posterior muscle cells is closer to that of the anterior muscles than with isosmotic perfusion for both 40- and 60-minute protocols (compare with Fig. 4a) but still skewed to the right, since some cells reflect increased intracellular volume after hyperosmotic perfusion. The median cell position has shifted left toward control values in each of these two experimental samples (dashed arrows denote median position with isosmotic perfusion). With 90 minutes of ischemia, cells sampled early in the reflow period are distributed in similar fashion to that found with isosmotic perfusion.
(plasma perfused) animals; Figure 4b shows the data from those animals treated with hyperosmotic mannitol perfusion. The nonischemic anterior papillary muscles are unaffected by the length of the ischemic period. In each case, the distribution of volumes suggests that a normal ratio of cytoplasmic plus mitochondrial volume to that of myofibrils is about 1:2. (There is, however, little or no cytoplasmic contribution to this ratio in nonischemic tissue.) This ratio is in close agreement with the recent data of Legato (Legato, 1979), which demonstrated, also using quantitative stereology, a mitochondrial-to-sarcomere ratio of 0.482 ± 0.46. The mitochondrial volume fraction (mitochondrial volume-to-cell volume) of 1/3 is also in good correspondence to that measured by Page et al. in rats and rabbits (Page et al., 1972). While the latter authors measured the myofibrillar volume as approximately 50% of cell volume as opposed to approximately 65% in our study, we have not taken into account the nuclear volume which may account, along with species-to-species variation, for this discrepancy. In the ischemic posterior papillary muscles (Fig. 4a) there is a clear increase in the combined cytoplasmic and mitochondrial compartments with increased duration of circumflex occlusion. The median cell position moves to an increased volume with increased time of occlusion (0.75 to 1.0 to 1.25 with 40, 60, and 90 minutes, respectively). By 90 minutes, a substantial number of cells have less than 1/3 of their total volume contained within the fibrillar mass; note those cells where (cyto + mito)/fibrils > 2.0 at 90 minutes. In Figure 4b, the consequences of hyperosmotic perfusion are detailed. In both 40- and 60-minute samples, posterior papillary muscle cells exhibit a decided shift in distribution toward the control anterior muscle values. The results with 90 minutes of occlusion do not show displacement of the median or any obvious changes in the overall distribution of values compared with those in Figure 4a. The data demonstrate that hyperosmotic perfusion had a distinct effect on the swelling of cells after both 40- and 60-minute ischemic periods, but was largely without effect when applied after 90 minutes of circumflex occlusion. In each of the six groups, the anterior papillary muscles showed identical median cell positions and virtually identical volume ratio distribution; it must be concluded that neither the duration of ischemia nor the administration of mannitol had any significant effect on anterior papillary muscle cell volume. There was no shift in the median cell position of the anterior papillary muscle.

The suggested correlation between the magnitude of swelling and the extent of necrosis was examined directly. Figure 5 illustrates the results of that examination where the sample of affected cells chosen consisted of those with a value for (cyto + mito)/fibrils > 1.0, a value well beyond those found for the normal anterior papillary muscle cells. The data were generated from the three periods of occlusion both with and without hyperosmotic mannitol. Regression analysis shows that a strong linear correlation exists between the cells so affected early in the reflow period and the number of cells which eventually become necrotic. Since the index of swelling included both cytoplasmic and mitochondrial components, it was of interest to examine the volume data for each of the components alone.

Figure 6, a and b, shows the distribution of cells that occurs when the collected data are assessed with respect to mitochondrial vs. fibrillar volume. As before, there was a reproducible pattern of cell distribution among the nonischemic anterior muscle samples. It is also apparent that the duration of applied ischemia shifts the distribution of posterior papillary muscle cells to the right (Fig. 6a), indicating an increase in mitochondrial volumes. There was a displacement of the median value from 40 to 60 to 90 minutes in those samples where the reperfusion was isosmotic. The presence or absence of shifts in the median values obtained with mannitol administration (Fig. 6b) are similar to those noted when the cytoplasm and mitochondria were considered together (Fig. 4b). Mannitol administration had a clear effect on mitochondrial volume in the 40-minute samples, a more pronounced effect in the 60-minute samples, and no effect after 90 minutes of ischemia. In each of the six groups, the anterior...
papillary muscles again showed identical median cell positions.

Correlation of these findings with necrosis data is provided in Figure 7. A value for mito/fibrils ≥ 0.7 was chosen, since that constituted the point beyond which virtually no anterior papillary muscle tissue fell. The relationship by regression analysis is again very strong (R = 0.996, P < 0.001).

Finally, the data were examined with emphasis on the fluid taken up by the cells and not incorporated into organelles but remaining as a part of the "free" cytoplasmic volume. The ratio (cyto + mito + fibrils)/(mito + fibrils) was used specifically to avoid the wide nonlinear range of values which would be encountered because of the lack of measurable cytoplasm in normal cardiac cells. These results are illustrated in Figure 8, a and b. Figure 8a points out that this index of cell volume also increases with the duration of ischemia. (Anterior muscles, if plotted here, would fall entirely on the 1.0 meridian, since they reveal no cytoplasmic space by these methods.) In Figure 8b, with the administration of mannitol, the 40-, 60-, and 90-minute samples all show a partial restoration to normal values in terms of the shift of the median. Although there is a shift from 1.3 to 1.2 in the value for the medial cells in the 90-minute experiments, this shift...
Figure 8 Histograms describing the percentages of cell with respect to cytoplasmic volume expressed as (cyto + mito + fibrils)/(mito + fibrils). Anterior muscles are not plotted here, as all observed cells showed a value of 1.00 for this ratio. Plotted bars depict means ± standard errors of the mean between dogs for the posterior papillary muscle cells. a: Isosmotic perfusion. Posterior muscle cells are distributed toward higher values with longer occlusion time; note the shift in position of the median cell. b: Hyperosmotic perfusion. With 40- and 60-minute protocols, cell distribution is shifted left from that seen with isosmotic perfusion (Fig. 8a). Note also that the number of cells whose mito/fibril ratios is 1.00 is approximately twice that found with isosmotic perfusion for both 40- and 60-minute experiments. In the 90-minute experiments, a shift of the median position toward normal values was also noted but there was no apparent increase in the percentage of cells which had mito/fibril ratios of 1.00.

Discussion

The findings of this study permit conclusions which appear valid within the confines of the experimental protocols and which appear useful to a more complete understanding of the pathophysiology of myocardial ischemia. The most important


### Figure 9
Relationship of percentages of cells with increased cytoplasmic space (free of organelles) during early reflow, i.e. (cyto + mito-fibrils)/(mito + fibrils) ≥ 1.2, to percentages of necrotic cells 12 hours after release of occlusion. Equation of line by linear regression analysis: $y = 0.712x + 8.56$, correlation coefficient ($r$) = 0.958. Symbols are as in Figure 5.

### Conclusion
The conclusion is that myocardial cells which will eventually become necrotic exhibit an expanded intracellular volume during reflow as an early consequence of an ischemic insult. This previously postulated view (Powell et al., 1973; Powell et al., 1976a) is now quantitatively established. It is apparent that an elevation of plasma osmolality with a nonpermeating solute during reperfusion effectively minimizes the extent of swelling in individual cells and that the degree to which this intervention results in a smaller population of swollen cells is quantitatively related to its effectiveness in the prevention of eventual cell necrosis. Furthermore, the time period of total ischemia after which myocardial cells may be salvaged by hyperosmotic means is defined.

A functional defect in cell volume regulation is an early consequence of ischemia which, in the absence of intervention, appears to correlate closely with an irreversible lesion. The number of posterior papillary muscle cells which were morphometrically assessed as swollen within the first hour after release of a circumflex occlusion of fixed duration was found to be nearly identical to the number of cells which were rendered necrotic by 12 hours after such an occlusive period. Each of the methods by which the data were reduced to generate indices of increased volume yielded positive, statistically meaningful correlations between early swelling and eventual necrosis (Fig. 5, 7, and 9).

However, these results do not establish whether mitochondrial or cytoplasmic swelling constitutes the critical lesion or whether markedly swollen cells are predestined to necrosis. Against the latter are two observations. First, some redistribution of fluid into the intracellular space of all the cells occurs during total interruption of blood flow for 40 or 60 minutes (Powell et al., 1976a), and yet substantial numbers of these cells are still salvageable during the reflow period (Powell et al., 1976a). Second, as can be seen in Figures 5 and 7, some cells exhibit an initial substantial increase in intracellular space and do not progress to death. These are cells with either an increase in total intracellular space or an increase in mitochondrial volume. The potential consequences of damaged mitochondrial architecture are evident, but there should be equivalent concern over distortions of the cytoplasmic ground substance; the spatial distribution of critical enzymes and substrates is necessarily highly organized (Katchalsky and Curran, 1969) and disruption of this organization may lead to impairment of essential metabolic processes. An overall expansion of cell volume also might prove lethal if it should result in enhanced nonspecific permeability of the sarcolemma; this phenomenon has been observed, for example, in retinal ischemia (Parks et al., 1976). Increased membrane permeability may lead to enhanced uptake of calcium and phosphate which have been proposed as potentially instrumental factors in ischemic cell death (Whalen et al., 1974). An alternative explanation of the connection between cell swelling and necrosis is based on the premise that swelling results in an impairment of blood flow which remains for some time after release of the circumflex clamp, so that ischemic conditions are perpetuated (Leaf, 1970; Powell et al., 1973). This mechanism also appears to be operative in the brain (Chiang et al., 1968). However, in the kidney, where initial studies suggested a persistent perfusion deficit following removal of the ischemic insult (Flores et al., 1972), subsequent studies indicated that if this mechanism exists, its applicability is limited to the subcortical tissue (Frega et al., 1976).

Whether or not a perpetuation of ischemia takes place (Leaf, 1970; Powell et al., 1973), it seems clear that cellular uptake of fluid is an early consequence of the initial insult. The use of three periods of arterial occlusion (40, 60, and 90 minutes), both with and without the administration of mannitol, resulted in a determination of the quantitative extent of cell swelling early in the reflow period with high reproducibility for each of the six protocols. Whether total cell volume, mitochondrial volume, or the extent of cytoplasmic space free of organelles was selected, the resulting six values yielded a statistically significant linear correlation when plotted against the set of values obtained for resulting necrosis from each of six protocols of clamping and reperfusion. Whereas this close correlation strengthens the possibility of a cause and effect...
relationship between ischemic cell swelling and necrosis, the data neither conclusively prove the causal relationship nor exclude other possibilities.

Regression analysis of the numbers of swollen cells (by any of the indices chosen) vs. the number of cells eventually necrotic did not result in a zero intercept; this is apparent in Figures 5, 7, and 9. In each case, more cells were swollen than become eventually necrotic in, at least, the 40-minute occlusion experiments. This is not a completely unexpected finding, for it must be appreciated that cell swelling, per se, is probably not the immediate cause of cell death. The data imply that a substantial fraction of those cells which appear swollen shortly (15 minutes) after release of a 40-minute circumflex occlusion are not irreversibly damaged and will recover their volume during isosmotic perfusion to appear normal some 12 hours later.

In the present study, the experimental protocol involved interruption of blood flow followed by reflow. This model was chosen to enable the results from a reproducible ischemic lesion to be compared from animal to animal. Data by Whalen et al. (1974), Kloner et al. (1974a, 1974b), and Willerson et al. (1977) obtained from the same experimental model or one similar to that employed in the present study indicate that a rapid gain in cell volume occurs early during the reflow period. The purpose of the present study was to compare the ischemia-related gain in intracellular volume with eventual necrosis. It should be pointed out that the gain in cell volume is not dependent solely on reflow of blood, as we have shown previously that there is evidence of cell swelling in the posterior papillary muscle before release of the circumflex artery ligation (Powell et al., 1976a), but without a change in water content (Powell et al., 1977). Furthermore, we recently have demonstrated that substantial increases in intracellular volume without a significant change in extracellular volume occur under conditions of low-flow ischemia (Powers et al., 1978).

An objective of this study was a definition of the limits of the efficacy of hyperosmotic intervention in myocardial ischemia. The tested intervention—addition of mannitol to plasma in an amount sufficient to raise osmolality to 330–340 mOsm/kg H2O—provided a substantial salvage of cells that had been rendered ischemia for either 40 or 60 minutes. Since the hyperosmotic plasma could not have reached the tissue prior to clamp release (Jennings et al., 1975), it is clear that the ischemic insult, including the development of cell swelling (Powell et al., 1976a), was comparable in both treated and untreated muscles during the time of vascular occlusion. After 90 minutes of circumflex occlusion, this hyperosmotic intervention was completely ineffective. It is nonetheless premature to conclude that necrosis is inevitable for cells after this extended ischemic period, since only a single therapeutic intervention was assessed. Others have demonstrated therapeutic efficacy in ischemic myocardium with the use of other agents such as propranolol (Maroko and Braunwald, 1973) and hyaluronidase (Maroko et al., 1972). Further investigation of the duration of ischemia after which myocardial cells may survive might locally involve application of discrete combinations of these agents with their varying mechanisms of action.

There are several possible mechanisms whereby hyperosmotic mannitol can prevent early cell swelling and increase viability. The most direct of these mechanisms is related to the obligatory extracellular location of mannitol providing a direct osmotic restoration of cell volume. Alternatively, the well described (Krishnamurtty et al., 1978; Willerson et al., 1972) effect of mannitol in lowering coronary vascular resistance also might be operative. In this latter instance, a return to improved environmental conditions for the swollen cells should result in a restoration of normal function and structure and enhanced viability.

The observation that hyperosmotic intervention with mannitol after 90 minutes of ischemia is ineffective in either the preservation of cell volume or in the prevention of necrosis warrants consideration. Central to the conclusions of this study is the fact that cell volume expansion was not corrected in a situation in which necrosis was not reduced. The appearance of cells 60 minutes after a 90-minute occlusion suggests more damage than a simple expansion due to excess intracellular fluid. There was considerable evidence for the loss of band registration among the myofibrils and the disruption of mitochondrial cristae (see Fig. 3). These changes and the very irregular profiles of both cells and mitochondria imply that these structures, distended beyond normal dimensions, have become sufficiently leaky so that they cannot remain turgid, as cells appeared to do early after shorter periods of occlusion. Such an increased permeability of the sarcolemma would markedly lower the reflection coefficient for mannitol (mol. wt. 182) and reduce its effectiveness as an osmotic agent. It is possible that elevation of the plasma osmolality with a much larger solute, such as polyethylene glycol (mol. wt. 6000), might prove more effective. Recently, it has been noted that this solute (PEG-6000) is effective in preservation of structure and function in experiments with renal ischemia (Frega et al., 1979).

The model used in this study was chosen for the specific advantages it offers to the examination of the relationship between early changes in tissue morphology and the eventual death of cells following a reproducible ischemic insult. Extension of these findings to a clinical situation must await a more detailed understanding of other variables (e.g., collateral blood flow) which were deliberately minimized in our choice of an experimental system.

We conclude that an early consequence of ischemia in the canine myocardium is an impairment of the processes which regulate myocardial cell volume. The degree of swelling in individual cells
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and the number of cells swollen increases with the duration of applied ischemia. It is likely that these cells constitute the population which will eventually become necrotic. Osmotic reversal (or prevention) of cell swelling through addition to the plasma of a solute which fails to penetrate cell membranes provides substantial benefit if the myocardial tissue has not been rendered ischemic for more than 1 hour. This finding suggests strongly that preservation of cell volume is a valuable means by which to enhance myocardial cell viability.

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