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Effect of a Transient Period of Ischemia on Myocardial Cells

I. Effects on Cell Volume Regulation

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The effect of temporary periods of ischemia on the electrolytes and water of myocardial cells were studied in groups of mongrel dogs. Myocardial tissue exposed to 40 minutes of ischemia induced by occlusion of the circumflex branch of the left coronary artery developed no changes in water or electrolytes when compared to nonischemic left ventricle of the same or sham-operated animals, even though this period of ischemia is known to produce irreversible injury to many of the damaged cells. However, reperfusion of the affected myocardium with arterial blood for only 2 minutes resulted in striking increases in tissue H_2O, Na^+, Cl^- and Ca^{2+}. These changes in electrolytes increased in severity with longer periods of reflow, and tissue K^+ was decreased significantly after 10 minutes of reflow had passed. Analysis of the results suggested that the tissue edema was primarily the result of cellular swelling. Myocardium exposed to 15 minutes of ischemia followed by 2 minutes of reflow showed no significant changes aside from a slight increase in Na^+. These studies demonstrate that defects in cell volume regulation occur early in severe ischemic injury (Am J Pathol 74:381–398, 1974).

DESPITE REFLOW OF ARTERIAL BLOOD into the area of injury, myocardial cell death develops in the subendocardial myocardium of dogs given 40 minutes of transient ischemia. Even though necrosis later

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develops in the affected tissue, its electrolytes and water at the end of 40 minutes of ischemia are similar to that of control myocardium. However, reperfusion of the damaged tissue with arterial blood for 20 minutes produces marked alterations in electrolytes and water, including increases in sodium, calcium and water, and significant decreases of potassium and magnesium.[1,2] Furthermore, periods of reflow of up to 4 hours do not result in greater increases in the electrolyte maldistribution in the damaged tissue.[2]

Recent studies of the kinetics of radioactive calcium uptake of cells damaged by 40 minutes of ischemia have shown that tissue calcium increases markedly after only 2 minutes of arterial reflow and that the source of the increased calcium is the plasma.[3] This observation suggests that the capacity of the ischemic cells to regulate Ca^{2+} concentration and perhaps cell volume is defective and that reflow provides an optimal milieu for the detection of this defect. The studies reported in this paper were designed to determine the sequential changes in tissue water and electrolytes occurring after brief periods of reflow of arterial blood into dog myocardium containing large numbers of cells irreversibly injured by 40 minutes of ischemia. Our aim was to establish the characteristics of cell volume regulation in this form of ischemic injury and to establish whether the cellular Ca^{2+} change was an isolated finding or part of a generalized defect involving all electrolytes and water. The results show that cell swelling developed quickly, almost explosively, after only 2 minutes of arterial reflow. The magnitude and rapidity of the changes found suggest that loss of ability to maintain cellular electrolytes and volume is a striking feature of the early stages of the development of irreversibility in ischemic injury.

Materials and Methods

Experimental Animals

Seventy four mongrel dogs of both sexes, weighing between 20 to 55 pounds were kept in air conditioned quarters, fed Purina Dog Chow and given water *ad libitum*. In all animals, the circumflex branch of the left coronary artery was occluded temporarily or permanently by technics described previously.[1,2] Briefly, the animals were anesthetized with sufficient intravenous sodium pentobarbital (Diabutal®) to eliminate the corneal reflex. Each received 50 mg/kg body weight of procaine amide HCl, (ER Squibb) by intramuscular injection 30 minutes prior to occlusion. Respiration was maintained through a cuffed endotracheal tube by a Harvard (model 1063) positive pressure respirator at a rate of about 300 ml kg min. The chest was opened through the left fourth intercostal space; the circumflex branch of the left coronary artery was isolated near its origin and was occluded proximal to the first atrial branch, usually 0.8 to 1.5 cm from the origin. A Goldblatt clamp was used for temporary and permanent occlusions. Sham occlusions in-

cluded placement of the clamp without tightening it. The circumflex artery was occluded for 40 minutes in 4 dogs; it was occluded temporarily for 40 minutes in groups of 4 dogs allowed 2, 5 or 10 minutes of arterial reflow. Two dogs were sham-occluded for 40 minutes, and the circumflex artery of 3 dogs was occluded temporarily for 15 minutes followed by 2 minutes of reflow.

Dogs from each group were scattered at random in the study so that control and various occlusion periods were interspersed. When a specific time interval was chosen, however, consecutive dogs were run until a survivor was obtained. About one-half of the dogs developed ventricular fibrillation while the artery was occluded or at the time of reflow. These animals are not included in this report.

After occlusion, the sequential EKG changes were recorded continuously for the first 5 minutes on lead II of a Grass Model 5P17 polygraph and intermittently thereafter. Animals included showed elevation of the ST segment in leads II as well as III and aVF and myocardial cyanosis which extended to within a minimum of 1.5 cm of the apex of the heart posteriorly. Arterial pressure was monitored in 10 dogs with an indwelling polyethylene catheter in the right femoral artery connected to a Statham model P234C pressure transducer. After 2 to 4 minutes of direct observation, the left lung was allowed to reinflate, the retractors were removed and the wound was covered. Following the desired interval of ischemia, the heart was exposed and either excised immediately or the Goldblatt clamp was released and reflow was allowed for 2, 5 or 10 minutes before excising. In all animals allowed reflow, the cyanotic myocardium became pink within a period of seconds and the ST segment elevation quickly disappeared. At the completion of the experiment, the heart was excised quickly; the left ventricle was opened anteriorly and the endocardium was examined. Usually a pale area could be seen in the tip or body of the posterior axillary muscle (PP). In reflow experiments, localized swelling often was observed. A median longitudinal section of the PP was taken for light microscopic study and was examined for gross signs of injury and then placed in formalin. The area to be sampled was identified within the PP by the fact that it failed to contract either spontaneously or upon mechanical stimulation, was pale and swollen when compared to the adjacent myocardium and corresponded to the portion of the longitudinal section of PP which turns quite pale shortly after being placed in 10% formalin.

Duplicate or triplicate 0.4- to 0.6-g samples of the PP were taken. In 25 dogs, corresponding samples of damaged tissue were fixed by immersion fixation for electron microscopy. The fine structural studies are the subject of another paper.[4] Control samples were obtained in each case from the anterior superior portion of the left ventricular septum.

In addition, arterial blood was drawn from the pulmonary artery 30 seconds prior to sacrifice for analysis of serum for H_2O, Na, K and Cl.

Tissue Analysis

Samples for electrolyte and water analysis were trimmed of endocardium and discernible fat and connective tissue, blotted free of surface blood with filter paper and weighed in a 20-ml scintillation vial. Samples were then dried to constant weight in a 105 C oven. After drying, electrolytes were extracted in 5 ml of 0.75 N nitric acid in a boiling water bath for 1 hour.[1] During extraction, vials were sealed with parafilm held in place by a plastic screw-on cap with a Teflon® lining. Teflon and glass were the only substances which touched the solution. The digest then was transferred to a 15-ml graduated centrifuge tube and centrifuged at 500 *g* for 15 minutes. For sodium, potassium and magnesium determinations, a 1.0-ml aliquot of the digest was diluted with a lanthanum chloride-nitirc acid solution ($LaCl_3 \cdot 6H_2O$, Fisher Scientific Products, purified lanthanium chloride lot

No. 9) to a final volume of 25 ml, containing 10,000 ppm lanthanum and 1 N nitric acid.[5] For calcium, a 2-ml aliquot was diluted similarly but to 5-ml final volume.

A water standard containing Na^+, K^+, Cl^-, PO_4^{3-}, Mg^{2+} and Ca^{2+} was used according to methods described previously.[1,2,5] It was prepared fresh each day. All solutions were prepared with deionized water with a resistance of more than 10^6 Ω. All glassware was cleaned by special technics described previously.[5] Standards and unknown samples were analyzed on a Jarrel Ash model 82-500 atomic absorption spectrophotometer using an air-acetylene flame and a laminar-flow burner with a 10-cm slot. Wave lengths used were 4227 Å for Ca^{2+}, 2852 Å for Mg^{2+}, 7665 Å for K^+ and 5890 Å for Na^+. Results were calculated and are generally expressed as millimoles per 100 grams dry weight of tissue sample.

Tissue chloride was determined by a modification of the Volhard procedure[2] in which 1 ml of the tissue digest is added to a known quantity of silver nitrate. The chloride reacts to form an insoluble colloidal suspension of silver chloride. The remaining silver nitrate is determined by back titration with sodium thiocyanate in the presence of ferric ammonium sulphate as an indicator. Nitrobenzene was included in the reaction in order to facilitate the determination of the end-point of the titration.[6]

Serum water was determined by drying to constant weight, and serum Na and K were measured in a SMA model 6 60.

Results

Severe Ischemic Injury

The electrolytes and water of the PP were not altered by 40 minutes of ischemic injury (Table 1, line 40 − 0) and were indistinguishable from those found in sham-operated control tissue. However, 2 minutes of reperfusion of the tissue with arterial blood caused marked increases in water, Na^+, Ca^{2+} and Cl^-. The Na^+ and chloride were increased by more than 50% and the Ca^{2+} was almost doubled. After 5 and 10 minutes of reflow, there were further increases in tissue water, Na^+ and Cl^- shown graphically in Text-figure 1.

There was a gradual decrease in the K^+ of the ischemic tissue (Text-figure 2), but the decrease was not statistically significant until 10 minutes of reperfusion. Mg^{2+} content was not decreased significantly, although 20 minutes of reperfusion is known to decrease Mg^{2+} by about 20%.[1]

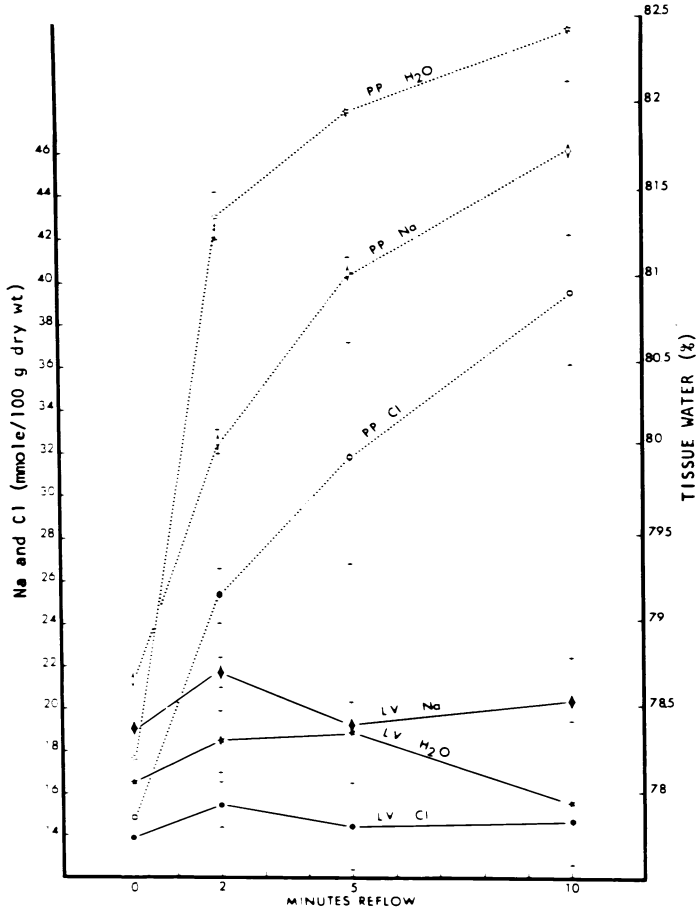
The Ca^{2+} of the PP increased abruptly at 2 minutes, remained stable through 5 minutes and increased somewhat at 10 minutes. Previous studies [1] have shown that the Ca^{2+} increases to approximately ten times control [1] values after 20 minutes of reperfusion.

The water and electrolytes of nonischemic LV (control tissue) remained constant throughout the period of reperfusion of the injured tissue.

Table 1 Myocardial Electrolytes and Water (Mean ± SE)

Minutes occlusion + reflow	No. of dogs	Sample site	H_2O (% wet weight)	Electrolyte concentrations (mmoles/100 g dry wt) Na	K	Mg	Ca	Cl
15 + 2	3	LV	78.61 ±0.02	19.8 ±1.2	42.0 ±0.62	4.7 ±0.38	0.54 ±0.02	14.7 ±2.2
		PP	79.49 ±0.38	23.2† ±1.4	41.0 ±1.6	4.9 ±0.13	0.56 ±0.02	16.2 ±2.6
Sham occlusion (40 min)	2	LV	78.16 ±0.43	22.3 ±2.3	37.0 ±0.65	4.6 ±0.15	0.48 ±0.02	—
		PP	78.02 ±0.20	23.4 ±2.5	34.8 ±0.15	4.7 ±0.20	0.50 ±0.02	—
40 + 0	4	LV	78.07 ±0.07	19.1 ±1.0	41.5 ±2.1	5.0 ±.16	0.46 ±.02	13.8 ±2.0
		PP	78.20 ±0.41	21.2 ±1.6	40.1 ±1.1	5.0 ±.19	0.50 ±.04	14.7 ±0.9
40 + 2	4	LV	78.34 ±.22	21.6 ±.07	39.9 ±1.9	5.0 ±.16	0.60 ±.07	15.4 ±1.2
		PP	81.39§ ±.13	32.5§ ±.55	37.1 ±4.3	5.3 ±.25	1.06* ±.24	25.3 ±1.3§
40 + 5	4	LV	78.37 ±.45	19.0 ±1.3	41.1 ±2.5	4.9 ±.14	0.40 ±.07	14.4 ±2.1
		PP	81.98† ±.83	40.2 ±8.7	33.5 ±5.4	4.9 ±.56	1.05* ±.34	31.9 ±5.2*
40 + 10	4	LV	77.93 ±.49	20.2 ±2.1	40.6 ±1.9	4.9 ±.16	0.55 ±.08	14.7 ±2.2
		PP	82.42‡ ±.31	45.9‡ ±4.1	31.3* ±4.5	4.8 ±.20	1.44* ±.26	39.2 ±3.3‡

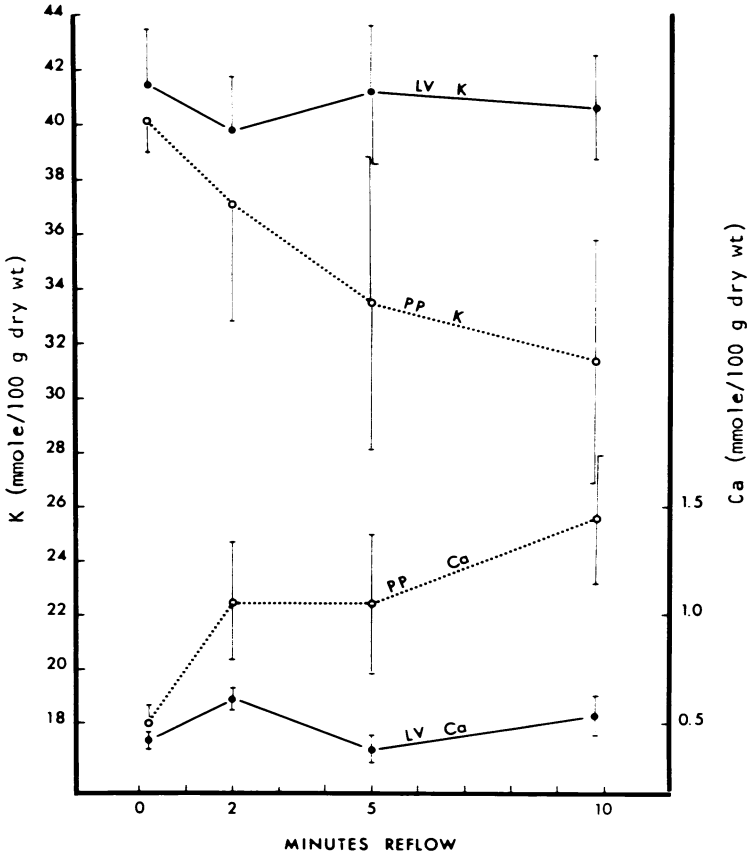
Differences are between the PP values and the corresponding LV values within the same group. The one-tailed paired *t* test was used.
* **P** < .05
† **P** < .01
‡ **P** < .005
§ **P** < .0005



TEXT-FIG 1—Plot of the content of water, sodium and chloride in PP after 2, 5 and 10 minutes of reperfusion with arterial blood following 40 minutes of ischemia. Non-ischemic LV contents also are included.

Reversible Ischemic Injury

Episodes of ischemia of 15 minutes duration have not been associated with cell death [7] although chemical changes consequent to anaerobic metabolism are found in the PP [8] at this time. Two minutes of arterial reflow following 15 minutes of ischemia had no effect on the electrolytes of the ischemic tissue aside from a slight increase in tissue Na and slight but insignificant increases in water and Cl^-. These changes stand in marked contrast to those found in 40 minute ischemic myocardial cells after 2 minutes of arterial reflow, at which time there are marked increases in H_2O, Na^+, Cl^- and Ca^{2+}.



TEXT-FIG 2—Plot of the content of potassium and calcium in PP after 2, 5 and 10 minutes of reperfusion with arterial blood following 40 minutes of ischemia. Nonischemic LV contents also are included.

Interpretation

The marked swelling of the severely ischemic tissue after only 2 minutes of reflow presents some questions which are not answered by the raw data presented in Table 1 and Text-figures 1 and 2. For example, is the swelling isotonic for the electrolytes which are principally extracellular prior to injury? To attempt to answer this question, the water and electrolyte data should be referred to the dry weight of the tissue (Table 2), since the dry weight is the only reference base that we can assume remains unchanged in both control and edematous tissue. The water present per 100 grams of dry weight in control nonischemic LV or in PP and LV of sham operated animals is quite constant at a mean of 356 ± 1.5 ml. It includes about 70% intracellular and 30% extracellular water.[9] The total H_2O increases by 76 ± 7.6 ml/100 g

Table 2—Changes of Cell H_2O and Theoretical Electrolyte Content of Damaged Tissue

Group	Serum		ml H_2O/100 g dry wt*		Increased ml of PP H_2O/100 g dry wt	mmoles/100 g dry weight					
						Na		Cl		Ca	
	Na	Cl	LV	PP		T†	O‡	T	O	T	O
40	149.8	106.5	356	359	—	—	21.2	—	14.7	—	0.5
	±2.5	±4.3	±1.5	±8.6							
40 + 2	146.8	109.3	363	438	76	32.8	32.5	23.7	25.3	0.8	1.1
	±1.1	±0.6	±4.8	±3.8	±7.6						
40 + 5	149.5	111.3	363	459	95	33.0	40.2	24.4	31.9	0.6	1.1
	±1.3	±0.8	±9.5	±2.5	±20.9						
40 + 10	150.5	115.3	354	470	116	37.7	45.9	28.0	39.2	0.8	1.5
	±2.5	±1.0	±10.0	±9.7	±8.7						
40 + 20§	146.7	111.1	374	539	165	43.2	62.2	—	—	0.8	4.2
	±1.6	±0.9	±25.3	±50.3	±34.3						

* Milliliters of H_2O per gram of dry weight is obtained by dividing the water measured per sample by the dry weight of the sample and multiplying by 100 to place the results in units of 100 grams.

† T = theoretical values calculated by assuming all of the increase in H_2O in the damaged tissue represented fluid from the extracellular compartment. Therefore, the theoretical concentration of any extracellular electrolyte in mmoles/100 grams of dry tissue is the product of the increased water per 100 grams of dry tissue and the serum concentration of the electrolyte in mmoles per milliliter plus the electrolyte in mmoles per 100 grams in the nonischemic LV of the same group. PP and LV electrolytes are known to be identical prior to the onset of onset of ischemia and at the end of 40 minutes of ischemia,[2] Table 1. The mean serum Ca was not measured in these experiments. The value of 2.57 mmoles Ca/kg of serum from Moulder et al was used.[10]

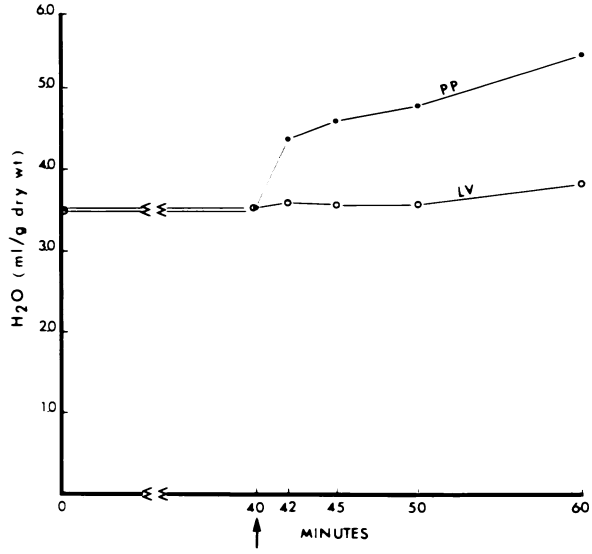
‡ Observed values from Table 1.

§ The 40 20 group includes 6 dogs treated similarly and reported earlier[1,2] for Na and water and 4 dogs from Jennings et al[2] for Ca.

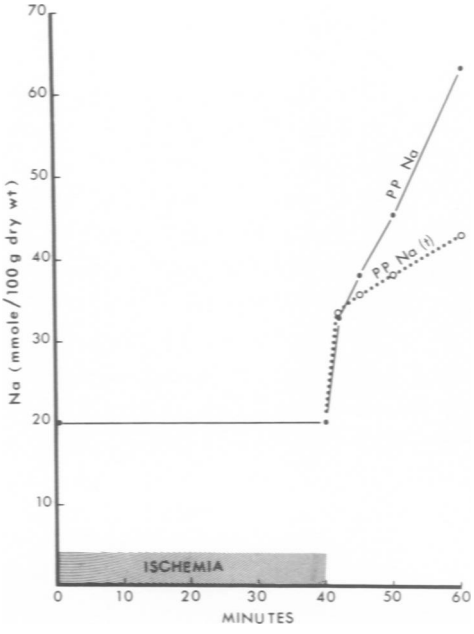
of dry tissue after only 2 minutes of reflow and slowly increases thereafter as the period of reflow is increased. These data are given in Table 2 and are plotted in Text-figure 3.

The abrupt increase in water is associated with an equivalent increase in Na^+. Assuming that the Na^+ all originated in 76 ml of plasma water which entered the damaged tissue, the Na^+ content of the tissue would be 32.8 mmoles/100 g dry weight. In fact it was 32.5 mmoles, which indicates that the initial swelling occurred because of an influx of plasma water (Table 2). The same calculation with chloride gives similar results. At 5 minutes of reflow, the theoretical Na^+ content based on the increase in total water was 33 mmoles, while the measured content was 7.2 mmoles greater, a disparity which progresses with increasing periods of reflow (Text-figure 4). Again, findings with chloride were similar. These results indicate that the first swelling is isotonic and is due simply to an influx of plasma water, while later, sodium and chloride accumulate in greater quantities than can be explained by the increased water content of the tissue.

The discrepancy between the observed and theoretical values for Na^+ and Cl^- (Table 2) may be partially explained by an exchange of extracellular NaCl for intracellular K^+. If the concentration of Na^+ and K^+ are calculated in the H_2O of 100 g of dry tissue, these values can be considered to represent the potential total major cation concentration in the cells. The concentration of Na^+ and K^+ remains constant in the



TEXT-FIG 3—Plot of the water content of PP and LV on the basis of ml H_2O/g of dry weight. Each point is the mean of the H_2O of PP and LV of groups of 4 dogs. Note the abrupt increase in H_2O after 2 minutes of arterial reflow and the gradual increase in H_2O thereafter as the period of reflow is extended to 4, 10 and 20 minutes.



TEXT-FIG 4—Plot of the content of sodium in PP (PPNa) as a function of the time. The Na^+ content does not change during the 40 minute ischemic period. After 2, 5, 10 and 20 minutes of arterial reflow, the Na^+ of the PP triples. The dotted line was made using data from Table 2, in which the theoretical Na^+ content of the PP was calculated [PPNa(t)] assuming that the increase in Na all came from plasma water. Note that the lines diverge at 5, 10 and 20 minutes, suggesting that Na has entered the tissue in excess of plasma H_2O.

left ventricle at a mean of 55.8 for Na^+ and 114 for K^+, and a total Na^+ and K^+ concentration of about 170 mmoles/liter of water. Actually, the intracellular K^+ concentration * is estimated to be about 165 mmoles/liter and the extracellular Na^+ concentration is 148.9 mmoles/liter, when the values are based on estimates of the volume of intra- and extracellular water. The final concentrations of Na^+ and K^+, calculated by the technic used in Table 3, thus represent dilution of both ions by the fluid in the extracellular and intracellular compartments. The results in Table 3 show that even though the Na^+ in the damaged tissue increases more than can be accounted for by the increase of total water, the con-

* Calculation of concentration of K^+ in intracellular H_2O: Using data from LV in the 40 + 10 group and calculation methods given by Jennings *et al*[2] and Manery,[9] the extracellular space, as estimated by chloride, was 254 ml/kg of wet muscle. The total tissue H_2O was 780.1 ml. The difference of 526 ml is the intracellular H_2O. The plasma K^+ concentration was 4.3 mmoles/liter in a volume of 254 ml, which means there is 1.1 mmoles of K^+ in the extracellular space. The total tissue K^+ was 88 mmoles/kg wet weight less the K^+ in the extracellular space, which yields 86.9 mmoles of K^+ in 526 ml of intracellular water. Thus, the estimated intracellular K^+ concentration is 165 mmoles K^+/liter of cell water. Continuing this calculation in the nomenclature used in Table 2, there are 780/220 or 3.55 ml H_2O/g dry weight; 254/220 = 1.16 ml extracellular fluid (ECF)/g dry wt and 526/220 = 2.39 ml intracellular fluid (ICF)/g dry weight. In 1 g of dry tissue there are 0.406 mmoles K; the contribution of the ECF is 0.00499 mmoles K^+ (1.16 × 0.0043) which leaves an ICF concentration of K^+ of 0.40101 mmoles K^+ in 2.39 ml or 0.168 mmoles K^+/ml of ICF or 168 mmoles K^+/liter of ICF.

Table 3—Tissue Sodium and Potassium Concentration Based on Tissue Water per Gram of Dry Weight

Electrolyte concentration* (mmoles/liter tissue water)	Period of Ischemia and Reflow in Minutes†							
	40 + 0		40 + 2		40 + 5		40 + 10	
	LV	PP	LV	PP	LV	PP	LV	PP
Na	53.6	58.8	59.7	74.1	52.3	86.1	57.5	98.1
	±2.7	±3.6	±2.2	±1.3	±4.3	±14.7	±6.6	±9.3
K	117.4	112.1	110.2	84.8	113.3	75.3	115.0	66.6
	±5.3	±5.3	±5.8	±9.9	±2.8	±15.1	±5.8	±9.2
Na + K	171.0	170.9	169.9	168.9	165.7	161.3	172.5	164.7
	±3.1	±4.9	±5.1	±9.7	±6.4	±6.2	±11.4	±11.4

*These concentrations were obtained by dividing the tissue electrolyte content in mmoles per gram of dry weight by the H_2O per gram of dry weight ,which yields mmoles of Na or K. per milliliter of tissue water. This value multipled by 1000 yields mmoles/liter of tissue water.

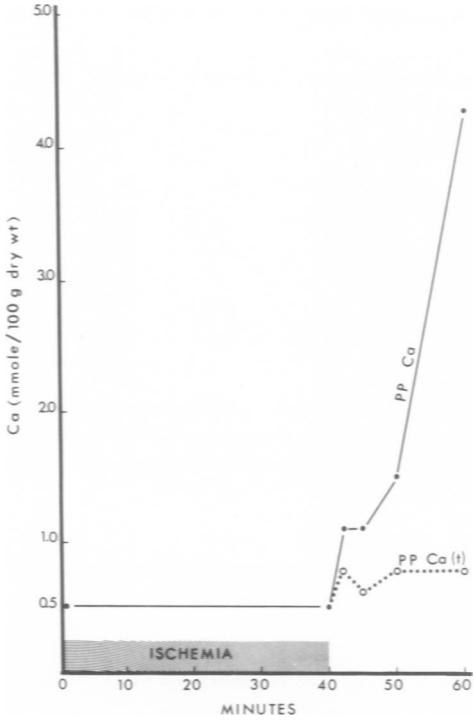
† Period of ischemia is 40 minutes plus 2, 5 and 10 minutes of arterial reflow.

comitant decrease in K^+ yields a situation in which the net total cation concentration is similar to control at 2, 5 and 10 minutes of reflow. These data imply that after Na^+ and H_2O have entered the cells to produce the initial rapid phase of swelling, extracellular Na^+ exchanges passively and in the direction of its concentration gradient for K^+ in the intracellular water and, thus appears to be accumulating in the tissue in quantities which are in excess of the observed increases in extracellular water in the tissue. If this explanation is correct, our findings indicate that the swelling involves intact cells.

Most of the Ca^{2+} which accumulates in excess of the theoretical amount (Table 2, Text-figure 5) that could be derived from the increased H_2O, is known to be present as intramitochondrial deposits of calcium phosphate.[1] Thus, the great disparity between the theoretical and observed content with this ion was predicted.

Discussion

The tissue water and measured electrolytes of the PP were unaffected by 40 minutes of ischemia and remained virtually identical to values found in nonischemic LV or the PP and LV of sham-operated control dogs. Upon release of the occlusion and subsequent reflow of blood, there were marked alterations in the composition of the previously ischemic tissue. It swelled, almost explosively, with a 21% increase in H_2O after only 2 minutes of reflow. This increase at 2 minutes of reflow represents 70% of the increase in tissue water found at 10 minutes and about 50% of the maximum total rise which has been reported to occur



TEXT-FIG 5—Plot of the content of calcium in PP (PPCa) as a function of time. The PPCa does not change during the 40-minute ischemic period. After, 2, 5 and 10 minutes of arterial reflow, it is increased markedly, and at 20 minutes, the content is about eight times the content of nonischemic control myocardium. The dotted line was made using data from Table 2, in which the theoretical Ca content of the PP was calculated [PPCa(t)] assuming that the increase in Ca all came from plasma water. This latter assumption probably is valid since there is almost 4 g% protein in dog cardiac lymph in health [11] and presumably even more is present after 40 minutes of ischemic injury. To the extent that this assumption is incorrect, the PPCa(t) is high. Note the marked increase in PPCa. Much of this increase undoubtedly is due to mitochondrial accumulation of calcium phosphate.[1]

after 20 minutes of reflow.[1,2] Similarly, after 2 minutes of reflow, tissue Na and Cl had increased to about half the total increase in these ions found at 10 minutes. Potassium, on the other hand, was not reduced significantly after 2 minutes of reflow, when the data was calculated on the basis of dry weight. Calcium showed a sharp initial rise at 2 minutes but only a small further rise between 2 and 10 minutes. Tissue magnesium remained constant for the first 10 minutes of reflow.

The changes in electrolyte and water of the myocardial tissue severely damaged by 40 minutes of ischemia and allowed 2 to 10 minutes of arterial reperfusion are believed to be underestimates of the values one would observe in pure samples of injured cells. Although tissue samples were taken from areas grossly identifiable as damaged, significant numbers of reversibly injured cells are known to be present in these areas. Reimer, Rasmussen and Jennings [12] have shown that only half of the PP is irreversibly injured by 40 minutes of ischemia. Since few changes were observed in the electrolytes of reversibly injured cells (15 minutes ischemia) after reflow, it seems likely that reversibly injured cells in the PP would dilute the electrolyte changes occurring in more severely injured cells in the same area. Thus, pure samples of injured cells would

have shown that the water, Na^+, Cl^- Ca^{2+} were higher, and the K^+ and Mg^{2+} were lower than those found experimentally. Actually, evidence supporting this speculation can be obtained from data on individual dogs in this experiment. One or 2 dogs in each group of 4 showed more marked changes than the others, which we attribute to the fact samples in these animals included a greater proportion of severely injured cells.

Location of Tissue Edema

The rapid early increases in tissue Na^+, Cl^- and water, as well as initial increases in Ca^{2+}, could have occurred either by a rapid increase in extracellular space or by an isotonic influx of water and electrolytes into cells from the large circulating plasma volume. An increased space was seen separating myocardial cells in a light microscopic study of hematoxylin and eosin-stained paraffin sections following similar periods of temporary ischemia.[13] However, a concurrent study (using many of the same dogs) of the ultrastructural changes occurring in early reflow times is reported in the following paper[4] and has shown that there is early and rapid cellular swelling including organelles such as mitochondria as well as formation of large blebs of fluid beneath the sarcolemma. By light microscopy these blebs probably would appear as extracellular swelling, since the very thin sarcolemma can not be resolved by this technic. Since there is no direct physiologic or morphologic evidence of significant enlargement of the extracellular space, and since the chemical data reported here and the morphologic data reported elsewhere[4] are consistent with cellular swelling, we believe that much or most of the tissue found after reflow is due to cellular swelling. Small to moderate increases in vascular or tissue extracellular spaces, however, cannot be ruled out with the present data.

Irreversible Cellular Injury

We have shown previously that cells injured by 15 minutes of ischemia followed by extended periods of reflow do not die. In fact, all evidence shows that they quickly begin functioning shortly after reflow begins.[4,7] Our findings in this paper further support this observation since, aside from a slight increase in Na^+ content, the electrolytes and water of these injured cells are unchanged by ischemia and reflow.

An important unanswered question raised by these experiments is the following: What happens to the myocardial cells when the period of ischemia is extended from 15 to 40 minutes? It is known that this period of ischemia causes irreversible injury, in that reflow fails to prevent death of many of the ischemic cells. The data in this paper

show that the irreversible state of injury is associated with acute swelling as soon as reflow begins, along with accumulation of intramitochondrial Ca^{2+}. It seems clear that the capacity of the injured cells to prevent these electrolyte changes and regulate their volume was defective prior to reflow, but was not detected because of insufficient extracellular fluid to allow swelling. Thus, inability to regulate cell volume may be an important factor in the pathogenesis of irreversible cell injury.

Mechanisms of Cell Swelling

Failure of Pump

The damaged cells during ischemia have been functioning primarily by anaerobic glycolysis for 40 minutes. Cellular glycogen and high energy phosphate supplies are low, while lactate and inorganic phosphate contents are high.[8,14–16] When new arterial blood first reaches the damaged cells, the cells respond with an influx of NaCl and H_2O. The mechanism behind this influx is not known. Current concepts suggest that cell volume is normally maintained by an active Na–K pump which utilizes ATP to extrude Na^+ from the cell in exchange for K^+. This pump balances a small passive leak for Na^+ and K^+ which is largely determined by the cell membrane permeability.[17] These same mechanisms also account for a negative intracellular electrical potential, while changes in the passive flux of Na^+ and K^+ produce the action potential and allow electrical conduction.[18] Consequently, either a failure of the Na–K pump or a marked increase in cell membrane permeability overcoming the capacity of the pump to extrude Na could cause cell swelling.

There is no direct evidence of pump failure in this system and this possibility has to be considered in relation to the state of the cells during the time they become edematous. Immediately after reflow begins, cellular supplies of high energy compounds are depleted, and the Na–K pump as well as other enzyme systems are in an enviroment high in lactate and other acidic intermediates. As reflow continues, O_2 tension increases and the possibility of generating energy by oxidative phosphorylation to drive the pump develops. If the pump and membrane were intact and could function in the acidic environment, the cells then might extrude Na^+ and H_2O and concentrate K^+. The question of the capacity of the mitochondria of this tissue to generate ATP at this time thus becomes an important issue. The mitochondria of the damaged cells prior to reflow are swollen, contain small amorphous matrix densi-

ties and may or may not be able to provide ATP by oxidative phosphorylation.[4] There is evidence, after 60 minutes of ischemia, that mitochondrial functional capacity is defective [19] and early defects probably are present at 40 minutes. Also, mitochondria are accumulating Ca at this time, a process which, *in vitro,* utilizes energy which could have been converted to ATP by oxidative phosphorylation.[20] The capacity of the mitochondria to synthesize ATP is compromised during the process of calcium accumulation or when the accumulation is massive. Thus, there are at least two ways that ischemia could induce cell swelling through direct effects on the Na–K pump—first, by denaturation or inhibition of the enzymes involved and, second, by inadequate supplies of high energy phosphate to drive the pump.

Altered Membrane Permeability

Myocardial sarcolemmal membranes normally become transiently highly permeable to Na^+ and Ca^{2+} during conduction of the action potential as cell electronegativity rises to near neutrality.[18] Since ischemic muscle may be in a depolarized state, an increased membrane permeability to cations could be due to physiologic mechanisms. Alternatively, rapid increases of the cell membrane area by sudden cell swelling during the early reflow period or breakdown of membrane structural integrity during the initial period of ischemia could both produce increased sarcolemmal membrane permeability and the subsequent observed water and electrolyte changes.

The relatively slow loss of K^+ from the tissue as compared to the rapid increases of NaCl and water would appear to argue against loss of cell membrane integrity to cations during the initial ischemic period. If intracellular potassium equilibrated with extracellular fluid during the ischemic period, and assuming only a 20% extracellular space (including vascular lumina), a rapid washout of K^+ during the first seconds of reflow could amount to as much as 8 mmoles K/100 g dry weight. According to the data presented here, actual loss was only about 3 mmoles/100 g dry weight after 2 minutes of blood reflow. These results could be explained, however, by assuming that part of intracellular K^+ is either bound or sequestered, perhaps in mitochondria and is not freely diffusable even though the sarcolemma becomes highly permeable to ions.

Role of Calcium Accumulation

Evidence of increases in cell membrane permeability currently is indirect. Calcium has been shown in this and previous studies to rapidly

accumulate in tissues with blood reflow following irreversible periods of ischemia. There is normally a large concentration gradient for calcium inward which is presumed to be maintained by a low membrane permeability to calcium and an as yet uncharacterized active calcium efflux mechanism. Increased membrane permeability could thus explain the rapid influx of calcium and subsequent accumulation by mitochondria. Previous *in vivo* studies, using isotopic methods, have shown a linear accumulation of calcium with time of reflow for at least 10 minutes [3] and tissue levels of about 4 to 5 mmoles/100 g dry weight after 20 minutes of reflow.[1] Increases in Ca^{2+} at 10 minutes found in the present study were modest but the results confirm the occurrence of acute significant tissue calcium accumulation following ischemic injury. Translocation of calcium into cells theoretically could produce mitochondrial damage and lead to irreversible cellular injury. Also increases in intracellular calcium could contribute to the formation of contraction bands seen with myocardial injury in the presence of blood flow by an exaggerated response of the contractile mechanism to excess calcium.[3] The relationship between the changes in tissue Ca^{2+} and membrane permeability remains hypothetical but is of great potential interest.

Changes in tissue magnesium were not observed during the initial 10 minutes of reflow but significant decreases have been reported after 20 minutes of reflow.[1] A possible explanation for the delay in the exit of Mg is the fact that most of it is not freely diffusable, but rather is bound to enzymes and nucleotides.[20] Rapid losses then would not be expected until significant autolytic breakdown of large molecules had occurred.

Pathogenesis of Ischemic Necrosis

The rapidity and magnitude of the changes observed in water and electrolytes of injured myocardium suggests that defects in ability to regulate normal water and electrolytes of myocardial cells may be important or even primary events in the pathogenesis of irreversible cellular injury and that the injury leading to this failure occurs during the initial ischemic period.

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