Ischemic Myocardial Cell Injury

Prevention by Chlorpromazine of an Accelerated Phospholipid Degradation and Associated Membrane Dysfunction

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Ligation of the left coronary artery of an adult rat heart results in the reproducible ischemic cell death of the entire free wall of the left ventricular myocardium. The time course of the development of the cellular changes is biphasic. The subendocardial and subepicardial cells die within the first few hours. The main mass of free-wall myocardium reacts more slowly, with morphologic evidence of irreversible cell injury developing after 12 hours. Measurement of the increases in total free wall Ca^{++} reflected this biphasic pattern. There was a rapid 3-fold rise in total Ca^{++} during the first 4 hours. Between 4 and 12 hours the Ca^{++} was constant. Between 12 and 30 hours there was a second increase that reached a level some 8–10 times the control value. Treatment with chlorpromazine before and subsequent to surgery prevented the appearance of ischemic cell death in the main portion of the free-wall myocardium for at least 24 hours without affecting the reaction of the subepicardial and subendocardial cells. Chlorpromazine also inhibited the second phase of Ca^{++} accumulation. An accelerated degradation of phospholipids was observed with a 33% decrease in total phospholipids by 12 hours. Phosphatidylethanolamine was reduced by 50% and phosphatidylcholine by 25% without increases in the corresponding lysophospholipids. Chlorpromazine prevented the accelerated degradation and consequent loss of phospholipid. Isolated sarcoplasmic reticulum showed a time-dependent loss of phospholipid with a parallel loss of active Ca^{++} uptake that reached 60% with a total lipid depletion from these membranes of 33% by 12 hours. Twelve-hour ischemic sarcoplasmic reticulum exhibited a 6–7-fold increase in passive permeability to Ca^{++}. Chlorpromazine protected against the loss of phospholipids, the inhibition of Ca^{++} uptake, and the increased Ca^{++} permeability of the sarcoplasmic reticulum. These observations indicate that rat myocardial cells react to lethal doses of ischemia in a manner similar to the reaction of liver cells described previously. In both cases the evidence implies that a disturbance in phospholipid metabolism and its associated membrane dysfunction is the critical alteration that produces irreversible cell injury in ischemia. (Am J Pathol 97:505–530, 1979)

RECENT STUDIES in our laboratory using a rat liver model have indicated that a disturbance in phospholipid metabolism may be the critical alteration that produces irreversible cell injury in ischemia.[1] A progressive decrease in liver-cell phospholipids occurred secondary to an accelerated rate of degradation that reached 40–50% after 3 hours of

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ischemia and that had a time course closely parallel to the loss of reversibility of the cell injury. This loss of phospholipid from ischemic cells was associated with inhibited membrane function including a 25–50-fold increase in permeability to Ca^{++} ions.[1,2] Pretreatment with chlorpromazine prevented the phospholipid depletion associated with as much as 3 hours of ischemia.[1] At the same time chlorpromazine prevents the ischemic death of the liver cells.[3] These findings are relevant to the increasing evidence that disturbed membrane function underlies the development of irreversible ischemic cell damage.[4–15]

The brain and the heart are the major organs that undergo ischemic cell death in human disease. The question can be raised as to how similar the mechanisms of ischemic cell injury in these organs are to the sequence of events we have described in the liver. In the present study we have employed a rat heart model to investigate in myocardial ischemia the relationship between phospholipid metabolism, membrane function, and irreversible cell injury. With this model myocardial ischemia can be produced that is quite reproducible both with respect to its localization within the heart and with respect to the extent of cell injury actually produced. In addition, sarcoplasmic reticulum can be readily isolated and its reaction to ischemia used as a model for the effects of this hazard on myocardial membranes, in a manner analogous to our previous analysis of the effects of ischemia on liver-cell microsomal membranes.[1] In the present study we present data indicating that the myocardial cells react to ischemia in a manner very similar to that of the liver with an accelerated degradation of phospholipids and accompanying membrane dysfunction that can be prevented by chlorpromazine.

Materials and Methods

Female Wistar rats weighing 140–160 g were fasted 18 hours overnight prior to use. Myocardial ischemia was induced by the method of Selye et al.[16] The animals were placed under light ether anesthesia, and an incision was made into the fourth intercostal space, followed by costotomy of the fourth rib. The heart was exteriorized, and a ligature was placed over the left main coronary artery. The incisions were sutured, and the animals were killed at the times indicated in the text. Treated animals were given 45 mg/kg chlorpromazine by intraperitoneal injection of a 10 mg/ml solution 1 hour prior to surgery and every 6 hours thereafter. Histologic sections were obtained by fixing sections of the hearts in 10% buffered formalin, embedding them in paraffin, and processing them in the usual way. Histochemical localization of tissue calcium was done by staining frozen sections with a 2% alizarin red S (Fisher Scientific) solution for 30 seconds. Tissue calcium was measured as described previously.[17] Protein was determined by the method of Lowry et al.[18]

For the determination of total phospholipids, the entire left-ventricular free walls of the ischemic hearts were homogenized in 10 volumes of 5 mM histidine-imidazole buffer, pH 7.3. The phospholipid content of these homogenates and the concentrations of individual

phospholipid species were determined as previously described.[1] The rate of degradation of phospholipids in whole homogenates was determined by giving the rats 10 μCi of ^{14}C-ethanolamine by intraperitoneal injection. Twelve, 24, and 36 hours later, the phospholipids in homogenates of left-ventricular free wall were extracted and the radioactivity remaining in phosphatidylethanolamine determined as described.[1] In a separate group of animals, heart ischemia was induced 12 hours after injection of the isotope. These animals were killed 12 hours later, and the radioactivity remaining in phosphatidylethanolamine was determined.

Sarcoplasmic reticulum was prepared from the pooled left-ventricular free walls of 5–6 animals by the method of Martonosi et al.[19] In preparation for electron microscopy, pelleted sarcoplasmic reticulum was fixed in 2.5% glutaraldehyde in a 0.1 M phosphate buffer, pH 7.3, for 1.5 hours. The pellets were washed in the 0.1 M phosphate buffer and postfixed in 1.33% Os_2O_4 in a *s*-collidine buffer, pH 7.4, for 1 hour. After dehydration with ethanol, the samples were infiltrated with and embedded in an Epon–Araldite mixture. Sections were cut on a Sorval Porter-Blum Microtome and viewed in a Philips EM300 operating at 80 kV. 5′-Nucleotidase [17] and cytochrome oxidase [20] were measured as described previously. Calcium uptake activity and calcium permeability of the isolated sarcoplasmic reticulum were determined as previously described.[1,2] Membrane proteins were solubilized in 0.1% SDS in 10 mM histidine-imidazole, pH 7.4. SDS–polyacrylamide gel electrophoresis was performed according to Summers et al.[21] The gels were prerun for 30 minutes at 25 V and run at 25 V for 30 minutes and then 18 hours at 50 V.

Results

Characterization of the Rat Heart Model

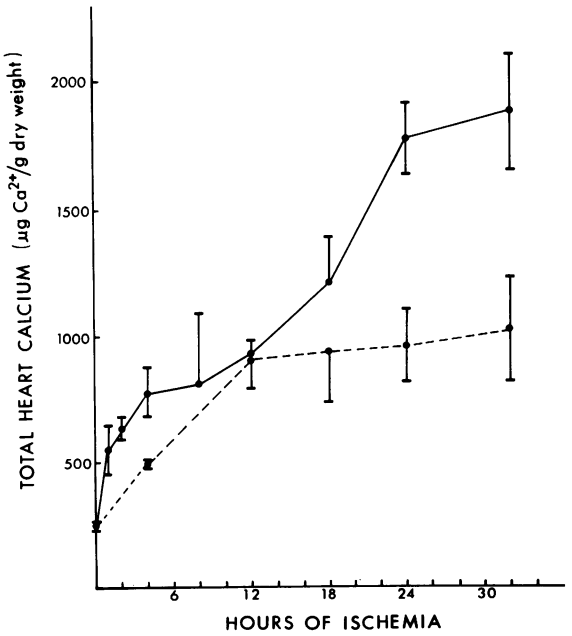
Figure 1 illustrates the typical extent of myocardial infarction 24 hours after ligation of the left coronary artery in an adult rat. Reproducibly there is ischemic cell death of the entire free wall of the left ventricle [22] with little or no mortality. Figure 2 is a higher-power photomicrograph of the distinct border between the zone of infarction and adjacent unaffected myocardium. As the free wall of the left ventricle is easily dissected from the remainder of the heart, such consistent localization of the ischemic cell injury should permit its experimental analysis, providing that all the cells are reacting somewhat in parallel during the first 24 hours.

Determination of the time course of the development of the changes in the free-wall myocardium during the first 24 hours revealed two distinct patterns. The subepicardial and subendocardial myocardial cells are very susceptible to the effects of ligation of the left coronary artery and react very rapidly. Within 4 hours they manifest the usual histologic changes of ischemic cell death. At this time there is no histologic alteration of the mass of the free-wall myocardium lying between these two areas and constituting over 75% of the thickness of the ventricular wall. Histologic evidence of the ischemic death of this portion of the ventricular free wall does not appear until after 12 hours. By 18 hours the cells are clearly irreversibly injured, with pyknotic nuclei and eosinophilic, coagulated cytoplasm on hematoxylin–eosin staining. By 24 hours the entire free wall of

the left ventricle is not beating, appears yellow when sectioned, and is dead when examined histologically (Figures 1 and 2). The left-ventricular chamber is dilated.

Increases in total Ca^{++} content parallel the development of irreversible ischemic cell injury in both rat liver [3] and the dog heart.[14] Text-figure 1 illustrates the time course of the changes in total Ca^{++} during the development of ischemic cell death in the free wall of the rat left ventricle. These data reflect the biphasic pattern noted above. There is a rapid 3-fold rise in the total Ca^{++} content during the first 4 hours. Between 4 and 12 hours, the Ca^{++} content does not increase further. Between 12 and 30 hours, there is a second increase that reaches a level 8–10 times the control by 24 hours. Histochemical localization of the early increase in Ca^{++} content showed that it is confined to the subendocardial and subepicardial cells. At 24 hours the increased Ca^{++} content is distributed throughout the thickness of the free-wall myocardium.

Treatment of the rats with chlorpromazine before ligation of the left coronary artery and during the course of the first 24 hours following surgery prevents the development of much of the ischemic death of the main mass of free-wall myocardium between unprotected subendocardial and subepicardial cells. Figure 3 illustrates the typical extent of this protective effect of chlorpromazine. Figures 4 and 5 show in greater detail the inter-



TEXT-FIGURE 1—Time course of the changes in total myocardial Ca^{++} during the development of ischemic cell death in untreated (*solid line*) and chlorpromazine-treated (*dashed line*) rats. Chlorpromazine (45 mg/kg) was given 1 hour before surgery and every 6 hours thereafter. Results are the mean ± SD of 3 separate determinations on the left-ventricular free wall of from 3–10 separate hearts.

face between protected myocardium and the unprotected subepicardial (Figure 4) and subendocardial cells (Figure 5). The effect of chlorpromazine treatment on total free-wall Ca^{++} content is indicated by the dashed line in Text-figure 1. The second phase of Ca^{++} accumulation does not occur. The differences between the Ca^{++} content with and that without chlorpromazine treatment are significant: $P < 0.001$ and $P < 0.01$ at 24 and 30 hours, respectively. The early increase in Ca^{++} accumulation still occurs with chlorpromazine treatment, although at a slower rate. By 8 hours there is no difference between the Ca^{++} levels in the presence or absence of chlorpromazine.

Phospholipid Metabolism in Ischemic Myocardial Cells

After ischemic periods of increasing duration, the left-ventricular free-wall myocardium was removed, homogenized, and the content of protein and phospholipid measured. There was a 10–15% decrease in the total protein content during the first 12 hours and a greater decrease in the total phospholipid content. The change in protein content was not reflected in any detectable change in the gram dry weight of the myocardium, which bore a reproducible relationship to the wet weight. Table 1 illustrates the time-dependent change in total and individual phospholipids normalized to gram dry weight. There is only a very slight change through the eighth hour. By 12 hours, however, there is a highly significant decrease (33%) in the phospholipid content ($P < 0.001$). This represents a 45% loss of phosphatidylethanolamine and a 25% loss of phosphatidylcholine without accumulation of either lysophosphatidylethanolamine or lysophosphatidylcholine. Pretreatment of the rats with chlorpromazine prevented the loss of phospholipids produced by 12 hours of ischemia (Table 1).

The inability to demonstrate accumulations of lysophospholipids can-

Table 1—Phospholipids in Ischemic Heart Tissue

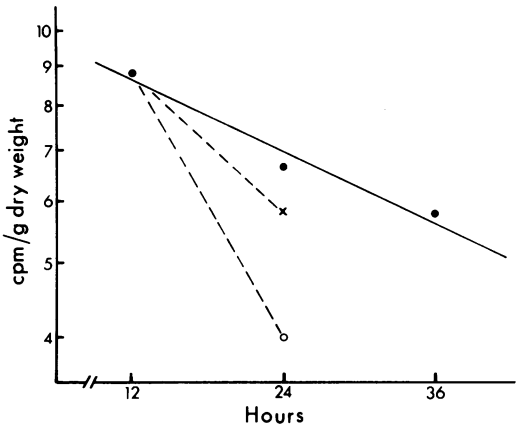
Treatment	Total phospholipids	Phosphatidylethanolamine†	Phosphatidylcholine†
		μmol PO_4^{-3}/g dry weight	
Control	104 ± 1.6*	41.0†	31.5†
8 hours of ischemia	93 ± 2.0 ($P < 0.001$)	—	—
12 hours of ischemia	70.4 ± 2.0 ($P < 0.001$)	22.7	24.2
Chlorpromazine plus 12 hours ischemia	107.0 ± 3.5	—	—

* Results are the mean ± SD of separate determinations on each of three animals. P values were computed on the basis of the Student t distribution for the comparison between two means of independent samples.[39]

† Results are the mean of separate determinations on each of two animals.

not be explained by a failure to extract these lipids into the chloroform phase with the other phospholipids by the lipid extraction procedure described previously.[1] Lysophosphatidylcholine added to liver microsomes is quantitatively recovered in the chloroform phase.[23] If rat liver microsomes prelabeled with ^{14}C-ethanolamine *in vivo* are treated at 4 C with *Naja naja* phospholipase A_2, all of the original radioactivity is recovered as lysophosphatidylethanolamine in the chloroform phase, despite a greater than 50% reduction in phosphatidylethanolamine.[23] If these microsomes are incubated with a lower concentration of the same phospholipase at 37 C for longer times, there is now a 50% reduction in total radioactivity in the chloroform phase accompanying a now 90% reduction in phosphatidylethanolamine. The radioactivity in the methanol (aqueous) phase is glycerophosphatidylethanolamine. These studies are consistent with a lysophospholipase active at 37 C over a 3-hour period but not at 4 C for much shorter times. More importantly for the present purposes, they document clearly that lysophospholipids under the conditions used to extract phospholipids are recovered in the chloroform phase.

The depletion of phospholipid could result from an inhibition of synthesis or a stimulation of degradation. In order to assess the role of an accelerated degradation, cellular phospholipids were labeled *in vivo* with ^{14}C-ethanolamine. At 12, 24, and 36 hours after this injection, phospholipids in homogenates of free-wall myocardium were extracted and chromatographed, and the radioactivity remaining in phosphatidylethanolamine was measured with respect to gram dry weight. Under these conditions there was no detectable labeling of phosphatidylcholine consistent with the previously documented inability of ethanolamine to act as a precursor of phosphatidylcholine in extrahepatic tissues of the rat.[24] Text-figure 2 (closed circles) indicates that the rate of loss of radioactivity from phosphatidylethanolamine is linear on a semi-log graph with a half-life of approximately 48 hours. In contrast, if after an initial 12-hour period following administration of ^{14}C-ethanolamine, the free-wall myocardium is made ischemic for 12 hours, there is a markedly accelerated loss of radioactivity in phosphatidylethanolamine (open circle in Text-figure 2). The extent of loss of radioactivity after 12 hours of ischemia (approximately 50%) correlates very closely with the extent of loss of phosphatidylethanolamine determined chemically in Table 1. This suggests that the depletion of phospholipid from ischemic myocardium is entirely the consequence of an accelerated rate of degradation with a half-life some 4 times faster than in normal cells. Pretreatment with chlorpromazine significantly reduces the rate of loss of radioactivity from phosphatidylethanolamine associated with 12 hours of ischemia (X in Figure 2) in agree-



TEXT-FIGURE 2—Degradation of total-cell phosphatidylethanolamine in control (*closed circles*), ischemic (*open circle*), and ischemic plus chlorpromazine-treated (*X*) rat hearts. All animals were given an intraperitoneal injection of 10 μCi of ^{14}C-ethanolamine at 0 hours. The control animals were killed at 12, 24, and 36 hours, and the specific radioactivity (cpm/mg dry weight) of phosphatidylethanolamine in homogenates of the left-ventricular free wall was determined. Another group of labeled animals was made ischemic at 12 hours and killed at 12 hours. A third group of animals was pretreated with chlorpromazine (45 mg/kg) 1 hour before surgery and again at 6 hours. These animals were killed 12 hours after the induction of heart ischemia. All values are the average of separate determinations on the entire left-ventricular free wall of each of 2 animals.

ment with its protection of the loss of heart phospholipids documented above (Table 1).

Sarcoplasmic Reticulum in Ischemic Heart Cells

There is increasing evidence that changes in membrane function are crucial to the development of irreversible cell injury in ischemia.[1-15] Previous study of liver-cell ischemia showed a close association between the accelerated degradation of phospholipid and the appearance of membrane dysfunction.[1,2]

These experiments made use of liver-cell microsomes as a model of the effect of ischemia on cellular membranes.[1,2] Marked changes in the structure and function of microsomal membrane were associated with the time-dependent loss of phospholipids. Such alterations could be prevented by chlorpromazine.

Following the example of these previous studies, we have used the sarcoplasmic reticulum as a model for the effects of myocardial ischemia on cellular membranes. Sarcoplasmic reticulum was prepared from control and 12-hour ischemic hearts. The purity of these preparations was monitored by electron microscopy and by the activity of marker enzymes for mitochondria and plasma membranes. Figures 6 and 7 are electron photomicrographs of control and ischemic sarcoplasmic reticulum, respectively. In both cases the preparations consist almost entirely of round and flattened vesicles characteristic of sarcoplasmic reticulum. There is only an

occasional mitochondrion and no obvious fibrillar debris. In Table 2 there are no differences in the specific activities of 5′-nucleotidase (plasma membrane marker enzyme) and cytochrome oxidase (mitochondrial marker enzyme) between sarcoplasmic reticulum prepared from control and from ischemic myocardium. These data indicate that the preparations of sarcoplasmic reticulum are relatively clean. Sarcoplasmic reticulum derived from 12-hour ischemic myocardium is not preferentially enriched with mitochondria or plasma membranes.

Text-figure 3 shows that the phospholipid content of isolated sarcoplasmic reticulum progressively declined with increasing duration of ischemia. With 12 hours of ischemia there was a 30% decrease in the ratio of phospholipid to protein. Chromatography of the extracted lipids after 12 hours of ischemia revealed a 50% decrease in phosphatidylethanolamine and a 25% decrease in phosphatidylcholine (data not shown). Table 3 summarizes the phospholipid changes at 12 hours. Table 3 also indicates that pretreatment of the rats with chlorpromazine prevented the loss of phospholipid from the membranes of the sarcoplasmic reticulum associated with 12 hours of ischemia.

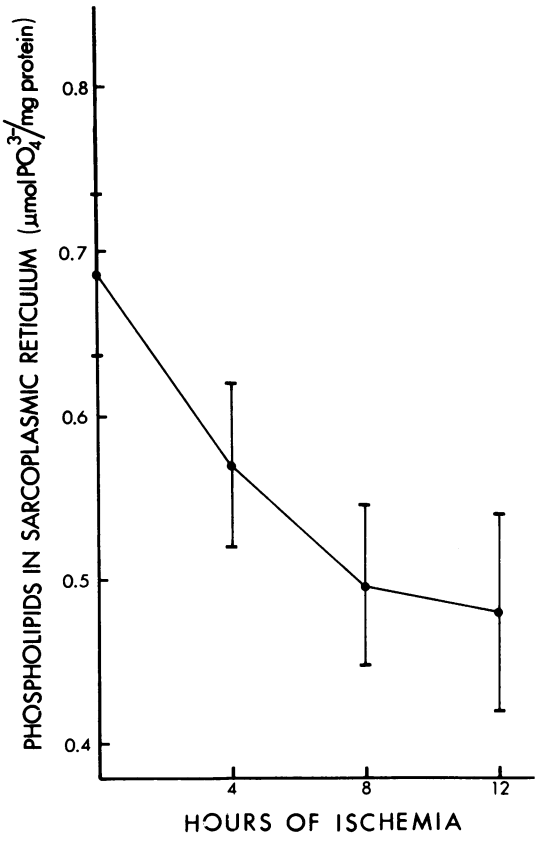
Loss of phospholipid from the sarcoplasmic reticulum is accompanied by evidence of membrane dysfunction with an inhibition of active Ca^{++} uptake (Table 4). The Ca^{++} uptake assay of both ischemic and control vesicles was augmented by oxalate and was linear with respect to time and concentration of protein. Table 1 also indicates that the residual Ca^{++} pump activity in ischemic sarcoplasmic reticulum, like the control activity, is not inhibited by sodium azide, a potent inhibitor of mitochondrial Ca^{+} uptake. This supports the conclusion reached above that neither preparation is significantly contaminated with mitochondria.

In order to explore further the relationship between the inhibition of sarcoplasmic reticulum function and the changes in membrane phospholipid content, the time course of the loss of Ca^{++} uptake activity was determined. In Text-figure 4 the relationship between the duration of ischemia and the Ca^{++} transport activity of isolated sarcoplasmic reticulum is very similar to that of the loss of phospholipids in Text-figure 3. It is, of

Table 2—Marker Enzymes in Control and Ischemic Sarcoplasmic Reticulum

	5′-Nucleotidase (μmol PO_4^3/mg protein/hr)	Cytochrome oxidase (μmols cyt c oxidized/ mg protein/min)
Control	3.6 ± 1.4*	0.063 ± 0.013
12-hour ischemic	3.4 ± 0.9	0.081 ± 0.011

* Results are the mean ± SD of separate determination on each of three preparations.



TEXT-FIGURE 3—Phospholipid content of sarcoplasmic reticulum isolated from left-ventricular free walls made ischemic for the times indicated. Results are the mean ± SD of three separate preparations from the pooled left-ventricular free walls of 5–6 rat hearts.

course, well known that depletion of the sarcoplasmic reticulum of phospholipids will inhibit Ca^{++} uptake activity.[19,25] This fact plus the data in Table 3 and in Text-figures 3 and 4 suggest that the loss of function of the sarcoplasmic reticulum is a consequence of the loss of phospholipids. Prevention by chlorpromazine of the loss of phospholipids from the sarcoplas-

Table 3—Phospholipids in Ischemic Sarcoplasmic Reticulum

Treatment	Phospholipids
	μmol PO_4^{-3}/mg protein
Control	0.69 ± 0.05*
12 hours of ischemia	0.48 ± 0.06 ($P < 0.025$)
Chlorpromazine plus 12 hours of ischemia	0.67 ± 0.03 ($P < 0.010$)

* Results are the mean ± SD of three separate preparations from the pooled left-ventricular free walls of 5–6 animals. The P values refer to the difference between each result and the result above.

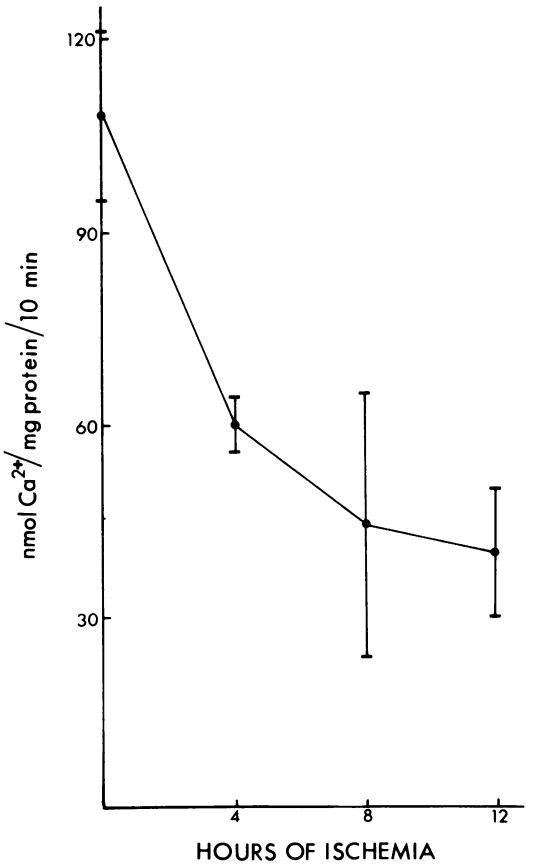
Table 4—Calcium Uptake Activity of Ischemic Sarcoplasmic Reticulum

Treatment	Calcium uptake	
	Minus azide	Plus azide (1 mM)
	nmol $^{45}Ca^{++}$/mg protein	
Control	107.5 ± 14.5*	110
12 hours of ischemia	40.0 ± 11.3 ($P < 0.001$)	36
Chlorpromazine plus 12 hours of ischemia	80.0 ± 6.0 ($P < 0.001$)	—

* Results are the mean ± SD of three separate preparations from the pooled left-ventricular free walls of 5–6 animals. The P values refer to the difference between each result and the control result.

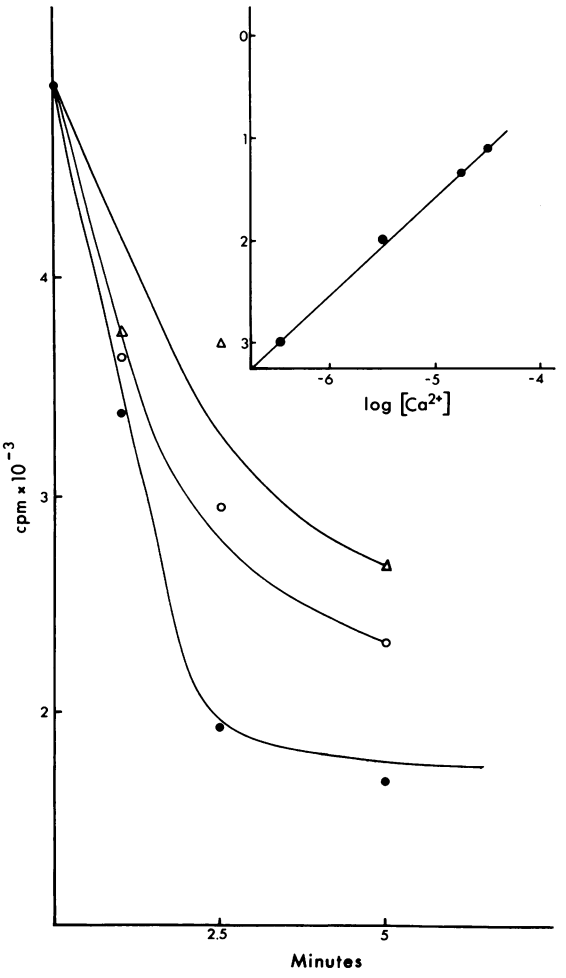
mic reticulum (Table 3) protects this membrane from the loss of Ca^{++} uptake activity produced by 12 hours of ischemia (Table 4).

The critical membrane function lost during ischemic cell injury may be the passive barrier to Ca^{++} movement down the very steep electrochemi-



TEXT-FIGURE 4—Time course of the loss of calcium uptake activity of sarcoplasmic reticulum isolated from ischemic free-wall ventricular myocardium. The same samples used to determine the data in Text-figure 1 were assayed for ATP-dependent, oxalate-augmented calcium uptake activity. Results are the mean ± SD of three separate preparations from the pooled tissue of 5–6 rat hearts.

cal gradient that exists between the inside and outside of most cells. It became of interest, therefore, to determine the Ca^{++} permeability properties of ischemically damaged sarcoplasmic reticulum. We measured the Ca^{++} permeability of isolated control and ischemic (12 hours) sarcoplasmic reticulum by loading the vesicles with various concentrations of $^{45}Ca^{++}$ and then diluting the vesicles into a $-Ca^{++}$ buffer and measuring the rate of loss of the isotope from the vesicles.[1] Text-figure 5 shows that with this assay the rate of Ca^{++} efflux is dependent upon the external Ca^{++} concentration and that there is a linear relationship between the log of the internal Ca^{++} concentration and the log of the rate of Ca^{++} efflux (inset, Text-figure 5). The assay is, therefore, measuring passive Ca^{++} per-



TEXT-FIGURE 5—Calcium permeability of rat heart sarcoplasmic reticulum vesicles. Control sarcoplasmic reticulum vesicles were loaded with 0.33×10^{-4} M $^{45}CaCl_2$ and diluted into solutions containing 10^{-6} (*closed circles*), 10^{-5}M (*open circles*), or 10^{-4} (*open triangles*) $CaCl_2$, and the extent of efflux was determined at the times indicated. The inset shows the relationship between the concentration of $^{45}CaCl_2$ inside the vesicles and the rate of efflux into calcium-free medium determined at 2.5 minutes.

meability. With this assay there is a 6–7-fold increase in the Ca^{++} permeability of sarcoplasmic reticulum vesicles isolated from myocardium ischemic for 12 hours (Table 5). Treatment of the rats with chlorpromazine protects against this increase in Ca^{++} permeability (Table 5).

The loss of phospholipid, the inhibition of active calcium uptake, and the increased Ca^{++} permeability of ischemic sarcoplasmic reticulum are not accompanied by any readily apparent change, either quantitatively or qualitatively, in the membrane proteins. SDS–polyacrylamide gel electrophoresis of the proteins solubilized from control and ischemic sarcoplasmic reticulum (12 hours) demonstrated essentially identical membrane protein composition in the two preparations (Text-figure 6).

Discussion

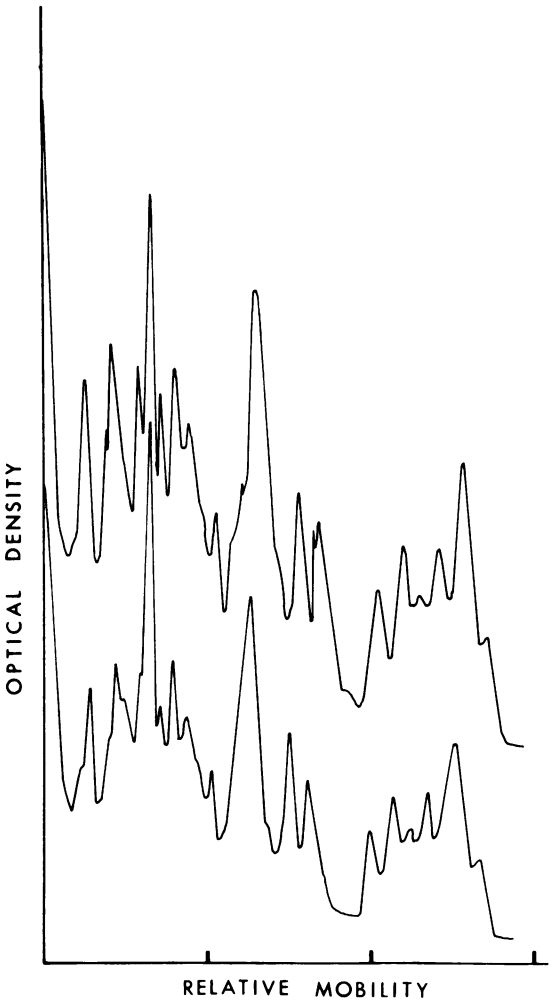
In the rat heart ischemia is easily induced in a reproducible manner to a clearly delimited and accessible portion of the myocardium. The surgery is simple, with no necessity for elaborate equipment or operative support of the animals. Despite the emergence of relatively large areas of ischemic cell death, there is little or no mortality during the first 24 hours. In the present report we have used this model to examine the relationship between the time course of the development of ischemic necrosis, changes in Ca^{++} content, and the metabolism of phospholipids. The resulting data support the conclusion that a disturbance in phospholipid metabolism and its associated membrane dysfunction is most likely the critical alteration that produces irreversible cell injury in myocardial ischemia.

Within the ischemic free wall of the rat left ventricle there are two populations of cells differing in their sensitivity to ligation of the left coronary artery. A minor proportion consisting of the immediate subendocardial and subepicardial zones reacts relatively rapidly and dies within the first few hours. The remainder of the major mass of the free-wall myocardium reacts more slowly. Histologic evidence of its death occurs after 12 hours of ischemia (Figures 1 and 2).

Table 5—Calcium Permeability of Ischemic Sarcoplasmic Reticulum

Treatment	Calcium efflux from sarcoplasmic reticulum preloaded with $^{45}Ca^{++}$ $-$ 3.3×10^{-5} M
	nmol Ca^{++}/mg protein/min
Control	0.067 ± 0.027*
12 hours ischemia	0.477 ± 0.14 ($P < 0.01$)
Chlorpromazine plus 12 hours of ischemia	0.081 ± 0.04 ($P < 0.01$)

* All values are the mean ± the range of two separate experiments based in each case on sarcoplasmic reticulum prepared from the pooled left-ventricular free walls from 5–6 hearts. The *P* values refer to the difference between each result and the control result.



TEXT-FIGURE 6—SDS-polyacrylamide gel electrophoresis of the proteins (50 μg) solubilized from control (*top tracing*) and 12-hour ischemic (*botton tracing*) sarcoplasmic reticulum. Sarcoplasmic reticulum was prepared, the membrane proteins solubilized and electrophoresced as described in Materials and Methods. The gels were stained with Coomassie blue and scanned at 563 nm.

This biphasic pattern of the morphologic alterations is reflected in a similar biphasic pattern of the changes in Ca^{++} content over the same time course (Text-figure 1). There is an initial rise that is essentially complete during the first 4 hours, resulting in a 3–4-fold increase in the total Ca^{++} content. Histochemical localization of this Ca^{++} showed that it was essentially confined to the endocardial and epicardial zones of the ischemic myocardium. The second phase of calcium accumulation occurs after 12 hours and results in an 8–10-fold increase in the total Ca^{++} content by 24 hours. Histochemically, this increased Ca^{++} is now localized throughout the ischemic free-wall myocardium. Chlorpromazine treat-

ment prevents the histologic manifestations of irreversible ischemic injury in the major portion of the ventricular free wall without having any effect on the early reaction of the subepicardial and subendocardial cells (Text-figure 1; Figures 3 and 4). In parallel to this protection, chlorpromazine prevents the second phase of calcium accumulation without having any significant effect on the first phase. It would seem reasonable to conclude that the biphasic changes in Ca^{++} content are a manifestation of the biphasic pattern of the evolution of the ischemic cell death. Whether the increases in Ca^{++} are causally related to each phase of irreversible cell injury or are simply the effect of the death of the cells is not evident from these data.

An attempt was then made to relate changes in phospholipid metabolism to the time course of the development of ischemic cell injury. There was only a very slight change in the phospholipid content in homogenates prepared from the entire free wall during the course of the first phase of ischemic cell death. However, an accelerated degradation of phospholipids (Table 1 and Text-figure 2) preceded the apparent loss of viability of the main mass of free-wall myocardium as assessed by morphologic changes and increased Ca^{++} content. Treatment with chlorpromazine prevented this alteration in phospholipid metabolism (Text-figure 2 and Table 1), again along with its prevention of the death of the main mass of free-wall myocardium.

The absence of a greater decrease in phospholipids during the initial phase of the reaction of the free-wall myocardium to ischemia is not necessarily inconsistent with the argument that phospholipid changes underlie the development of irreversible cell injury. The subepicardial and subendocardial zones represent too small a percentage of the total free-wall myocardium for changes in their phospholipid content to be readily detected. If one assumes that these zones represent at most 20% of the free-wall myocardium, a 33% decrease in their phospholipid content would produce only a 6–7% decrease in the phospholipid content of samples of the entire free-wall myocardium.

It might also be argued that the changes in phospholipid metabolism that develop between 8 and 12 hours are not necessarily causally related to the second phase of ischemic cell death. Rather, they could be a manifestation of a repair process initiated by the first phase of injury. Such an argument would imply that chlorpromazine, on the one hand, prevents this repair process and, on the other, does something unrelated to lipid metabolism that inhibits the biochemical mechanisms underlying the death of the main portion of the free-wall myocardium. Such an argument would predict that there should be a second phase of phospholipid degra-

dation after the death of the main portion of the free-wall myocardium. Further, this second phase of lipid loss should be considerably greater than the first. There is no evidence for such a second phase of phospholipid degradation. Furthermore, against such an argument is the fact that such a degradation of phospholipids related to repair would surely have to be basically inflammatory in origin, and there is little or no inflammatory infiltrate as early as 8–10 hours after ligation of the left coronary artery. It would, therefore, seem reasonable to conclude that the accelerated degradation of phospholipid described above is related to the reaction of the main portion of the free-wall myocardium.

This conclusion is supported by the occurrence of alterations in membrane structure and function accompanying the disturbed phospholipid metabolism. Disturbances in membrane structure and function have been reported to characterize the loss of reversibility of the cell injury associated with myocardial ischemia.[4–15] In the present report isolated sarcoplasmic reticulum displayed a time-dependent loss of phospholipid that was similar in pattern and extent to that observed in the whole-cell homogenates (Text-figure 3). Morphologic (Figures 6 and 7) and biochemical (Table 2) characterization of the control and ischemic preparations of sarcoplasmic reticulum indicated that such phospholipid depletion could not be ascribed to differences in the purity of the samples. Parallel to the time course of the lipid depletion, there was a progressive loss of active Ca^{++} uptake by the sarcoplasmic reticulum that reached 60% by 12 hours (Text-figure 4 and Table 4). In addition, 12-hour ischemic sarcoplasmic reticulum exhibited a 6–7-fold increase in passive permeability to Ca^{++} ions (Table 5). These changes in membrane phospholipid content and in membrane function were not accompanied by any detectable change in membrane protein composition as revealed by SDS–polyacrylamide gel electrophoresis (Text-figure 6). Chlorpromazine protected against the loss of phospholipid (Table 3), the inhibition of Ca^{++} uptake (Table 4), and the increased Ca^{++} permeability (Table 5).

The major conclusion to be derived from these results is that rat myocardial cells react to lethal doses of ischemia in a manner very similar to the reaction of ischemic rat liver cells. In both cases, our data indicate that a disturbance in phospholipid metabolism and its associated membrane dysfunction is most likely the critical alteration producing irreversible injury in ischemia. The similarity of this disturbance in both cells suggests they have a common pathogenesis.

At least three mechanisms can be considered to account for the accelerated phospholipid degradation in ischemic cells. We have previously suggested that the accelerated phospholipid degradation in liver-cell isch-

emia is the result of the activation of endogenous, membrane-bound phospholipases by an increased cytosolic Ca^{++} concentration.[1] Phospholipase A activity has similarly been demonstrated in rat heart "microsomes" (sarcoplasmic reticulum).[26] Phospholipase A_1 and A_2 activities were optimal at pH 7.5 in the presence of 1.0 mM Ca^{++}. Phosphatidylethanolamine was preferred to phosphatidylcholine as the substrate, consistent with the substrate specificities of similar activities in other tissues.[27] Treatment of skeletal muscle sarcoplasmic reticulum with snake venom phospholipase A *in vitro* increased permeability for calcium and abolished the ATP-driven calcium storage.[25] The loss reported here from ischemic sarcoplasmic reticulum of predominantly phosphatidylethanolamine and then phosphatidylcholine accompanied by an increased Ca^{++} permeability and an inhibition of active Ca^{++} uptake are probable consequences of the Ca^{++} activation of endogenous, membrane-bound phospholipases present in the myocardial cells. An elevated cytosolic free Ca^{++} concentration in ischemic heart cells could result from intracellular acidosis and the discharge into the cytoplasm as a result of the declining ATP levels of Ca^{++} stored in mitochondria and the sarcoplasmic reticulum.

Release of lysosomal enzymes is a potential alternative to the activation of endogenous phospholipases as the mechanism responsible for the accelerated phospholipid degradation. There have been several recent reports of biochemical alterations in cardiac lysosomes subsequent to coronary occlusion,[28–33] and the potential certainly exists for activation of multiple lysosomal enzymes early in ischemia. Direct evidence, however, that any of these enzymes actually contributes to cellular damage is not available.

The third and final mechanism that can be considered as possibly playing a role in the accelerated phospholipid degradation is the accumulation of potentially toxic concentrations of normal metabolic intermediates. The recently reported high levels of both long-chain acyl CoA esters and long-chain acyl carnitine in ischemic cells [34–36] suggest that these intermediates may have generalized detergent effects that could disrupt membranes. There is very little data available on the membrane effects of exposure to these compounds. Long-chain acyl carnitine inhibits Na^+,K^+-ATPase activity at concentrations that are likely to be present in ischemic tissue.[37]

The data presented in this report do not allow a decision as to the particular role any of the above three potential mechanisms play in the pathogenesis of the disturbance in phospholipid metabolism in ischemia. Clearly more work is needed with respect to each of these. The data presented in the present report, however, serve to more specifically focus future studies on the role each may play in mediating membrane injury, in general, and an accelerated phospholipid degradation in particular.

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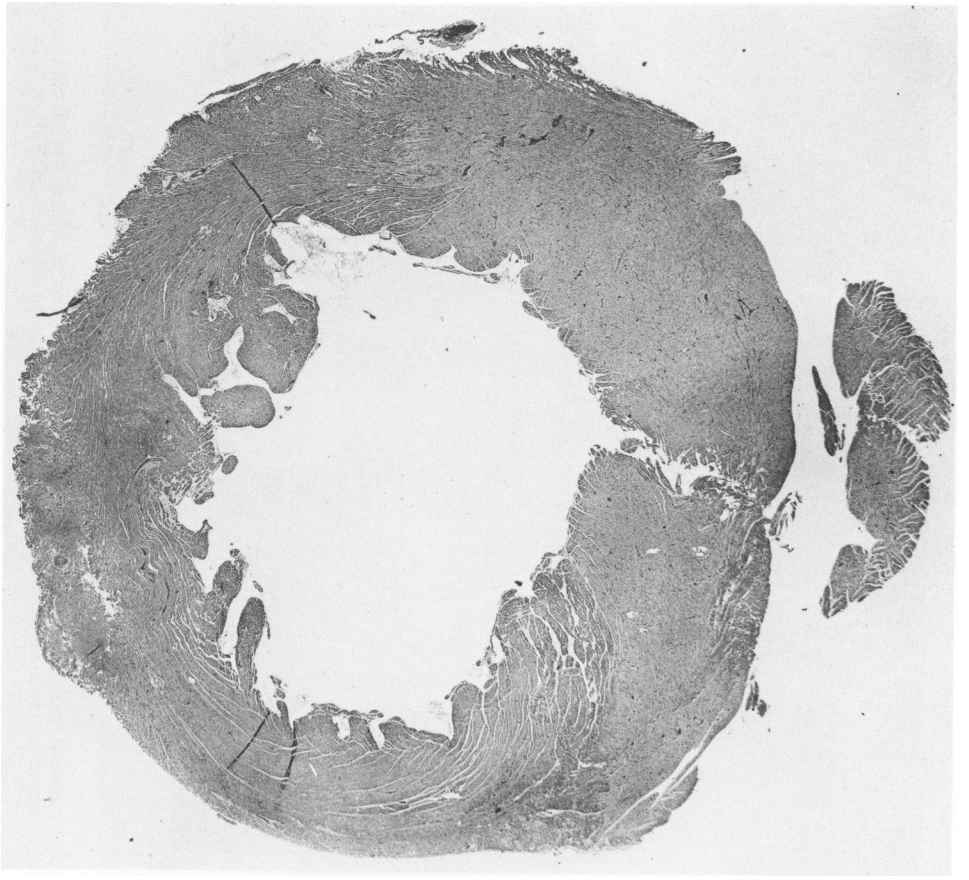

Figure 1—Ischemic necrosis in the free wall of the left ventricle 24 hours after ligation of the left coronary artery. The figure is of a cross-section through the mid-portion of the heart, with the left-ventricular free wall on the left and a portion of the right-ventricular free wall on the right. A sharply defined zone of ischemic necrosis begins at the base of the interventricular septum (5 o'clock) and extends around to the left to the top of the septum at 12 o'clock. The entire free wall is necrotic. (H&E, ×14)

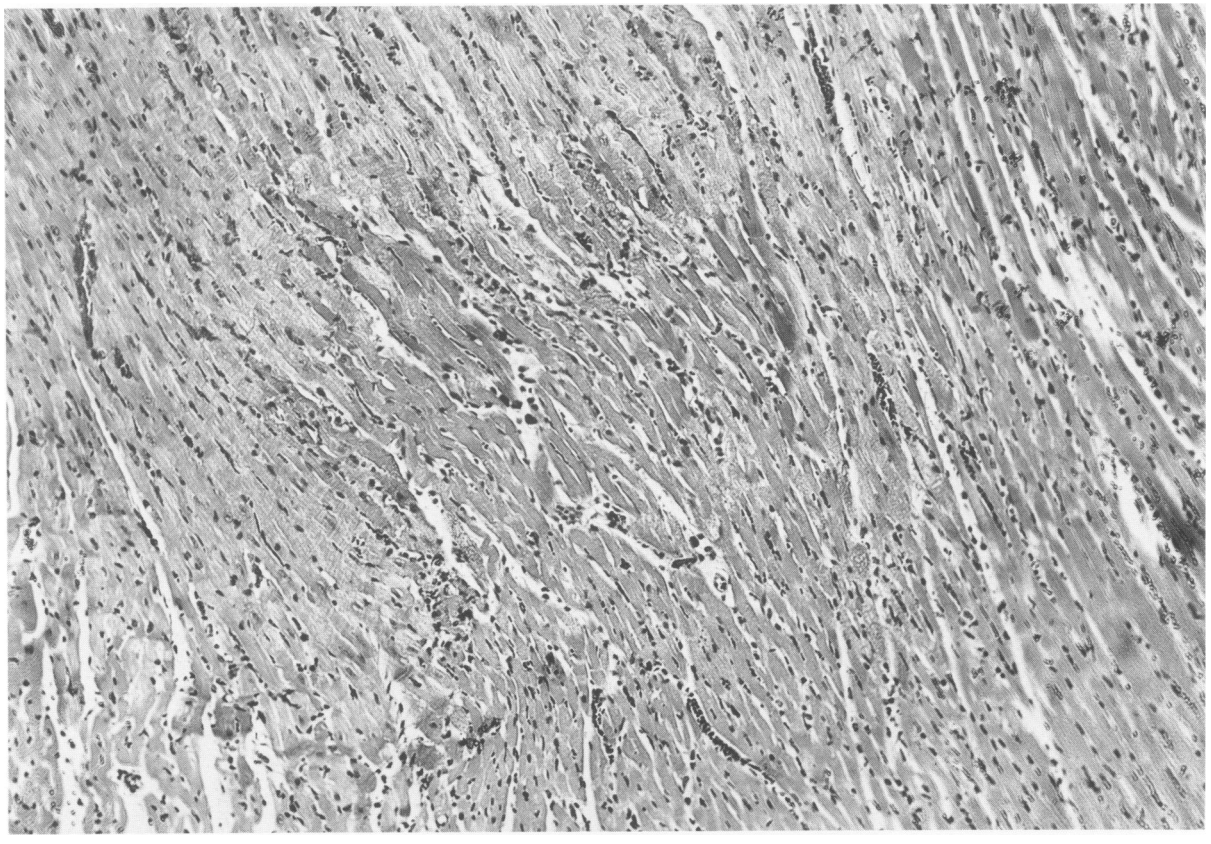

Figure 2—Detail of the interface between normal and necrotic myocardium 24 hours after ligation of the left coronary artery. A ragged, sharply defined border separates normal myocardium (*upper left*) from necrotic myocardium. H&E, ×142)

Figure 3—Prevention by chlorpromazine of the ischemic necrosis produced by ligation of the left coronary artery. The figure is of a cross-section of a rat heart 24 hours after coronary occlusion with chlorpromazine treatment as described in Materials and Methods. Three small foci of necrosis (3 o'clock in the septum; 6 o'clock at the base of the septum and on the lateral surface of the right-ventricular free wall) are evident. The major portion of the left-ventricular free wall is free of necrosis. (H&E, ×14)

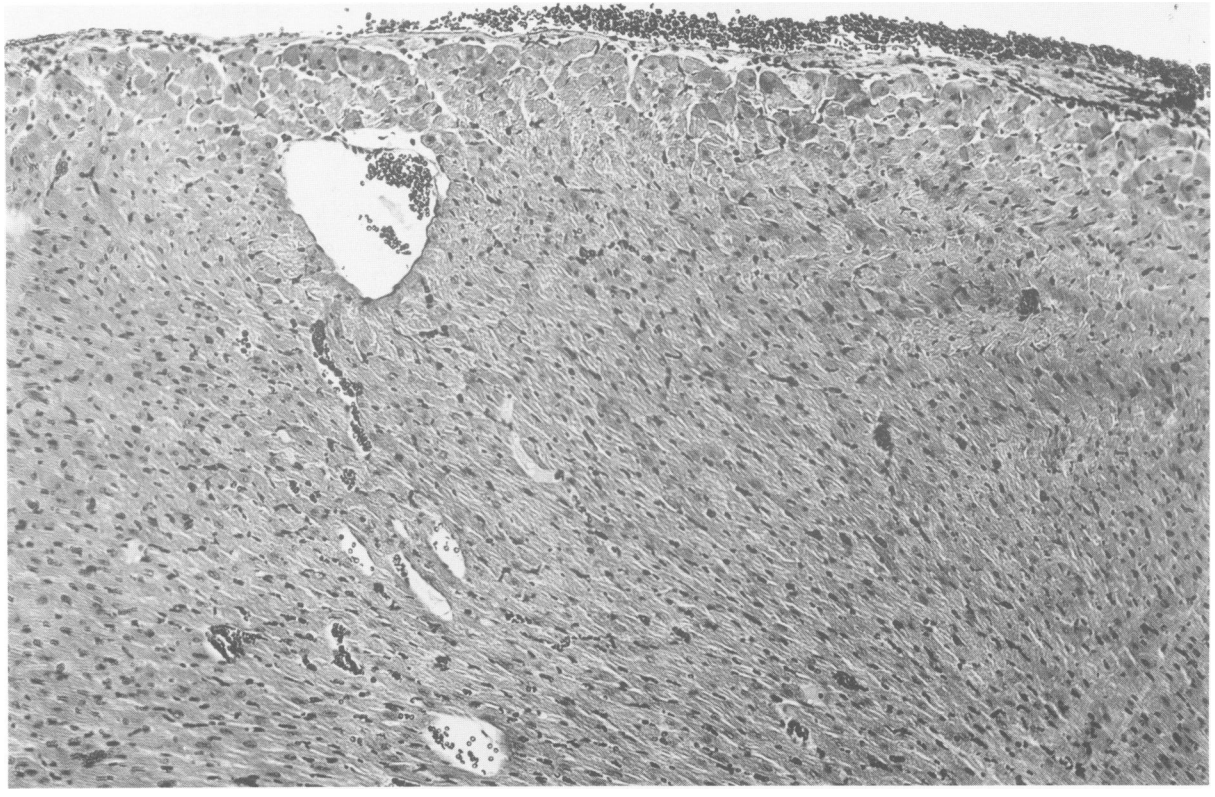

Figure 4—Detail of the left-ventricular free wall 24 hours after ligation of the left coronary artery in a rat treated with chlorpromazine. A thin zone of ischemic necrosis of the subepicardial cells can be seen overlying an intact layer of myocardium. (H&E, ×142)

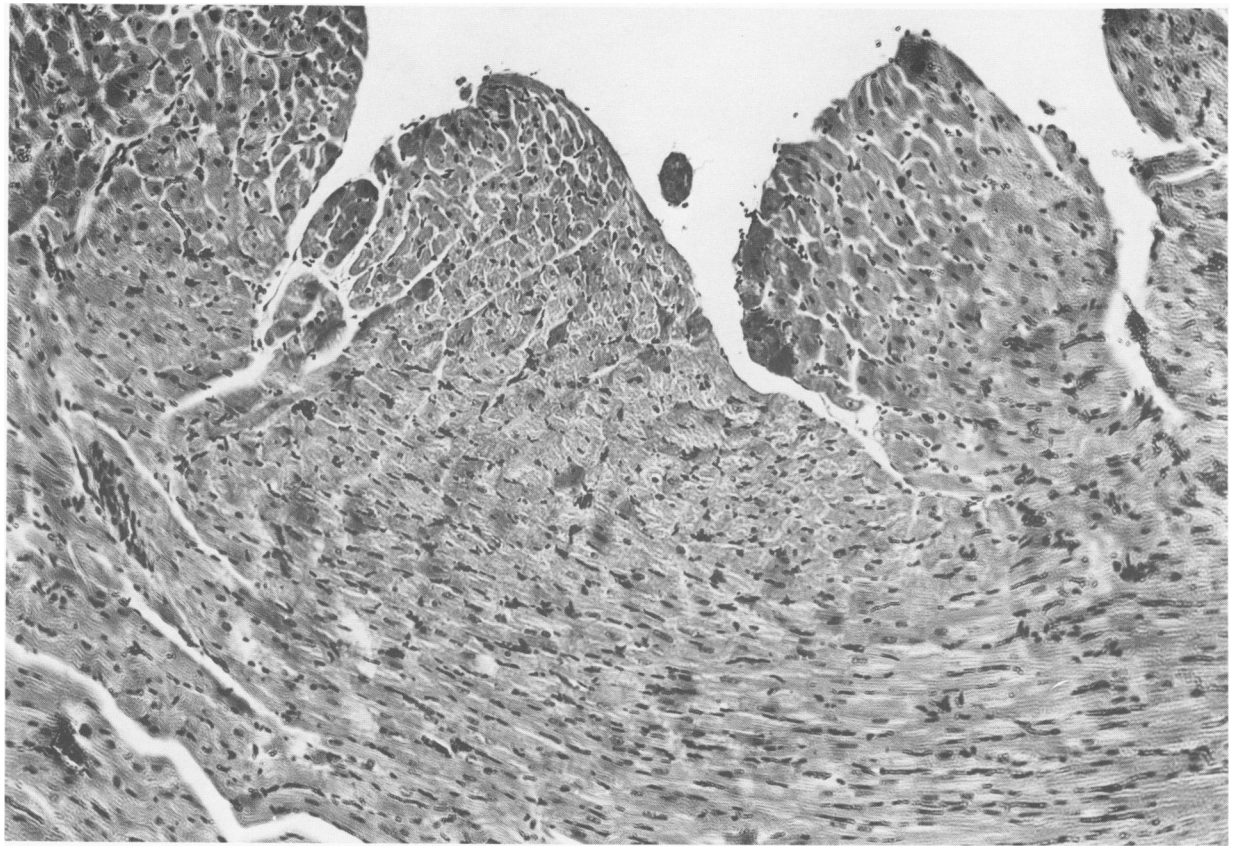

Figure 5—Detail of the left-ventricular free wall 24 hours after ligation of the left coronary artery in a rat treated with chlorpromazine. A thin zone of ischemic necrosis of the subendocardial cells can be seen overlying an intact layer of myocardium. (H&E, ×142)

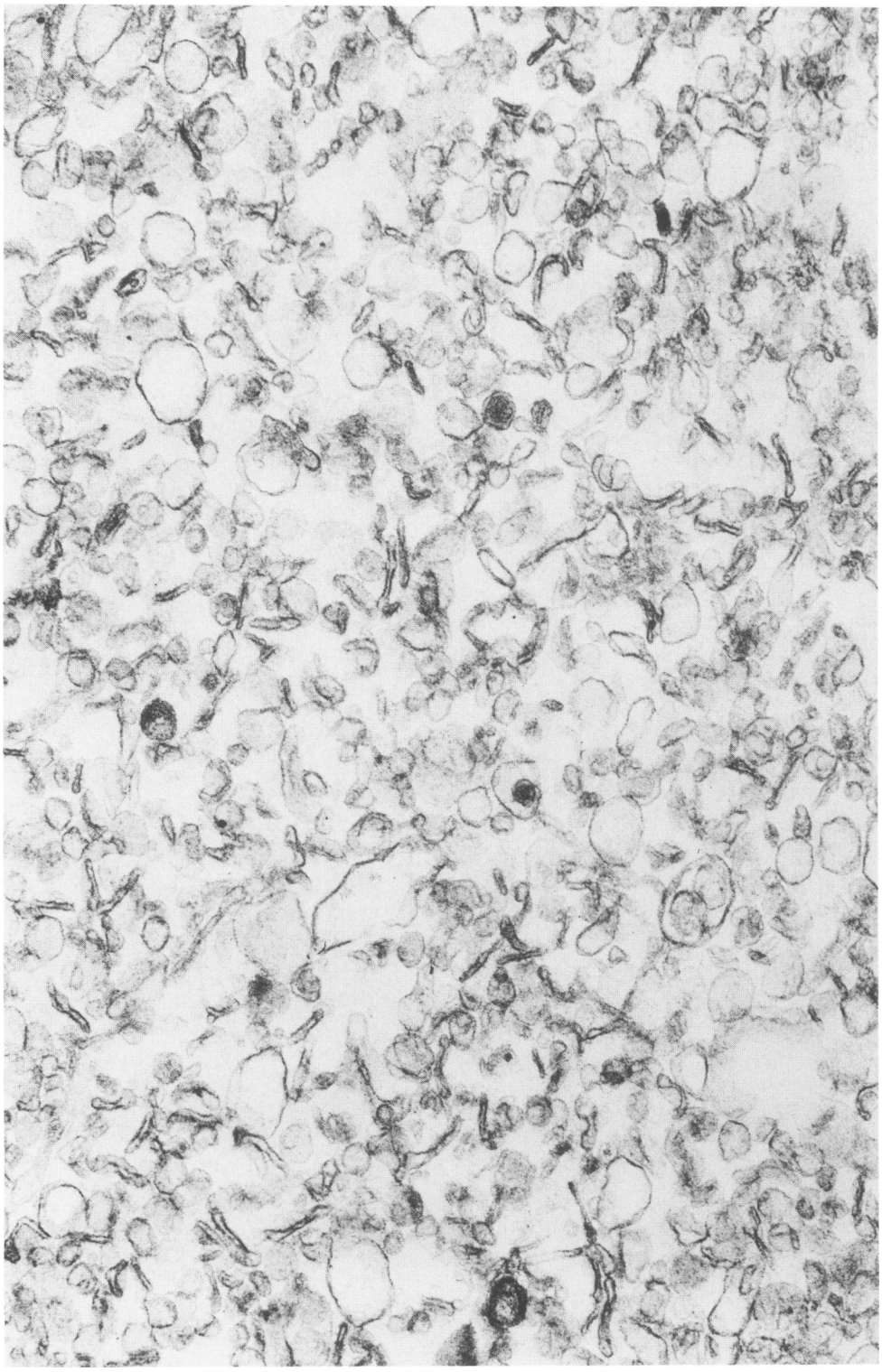

Figure 6—A control preparation of rat heart sarcoplasmic reticulum. (×46,000)

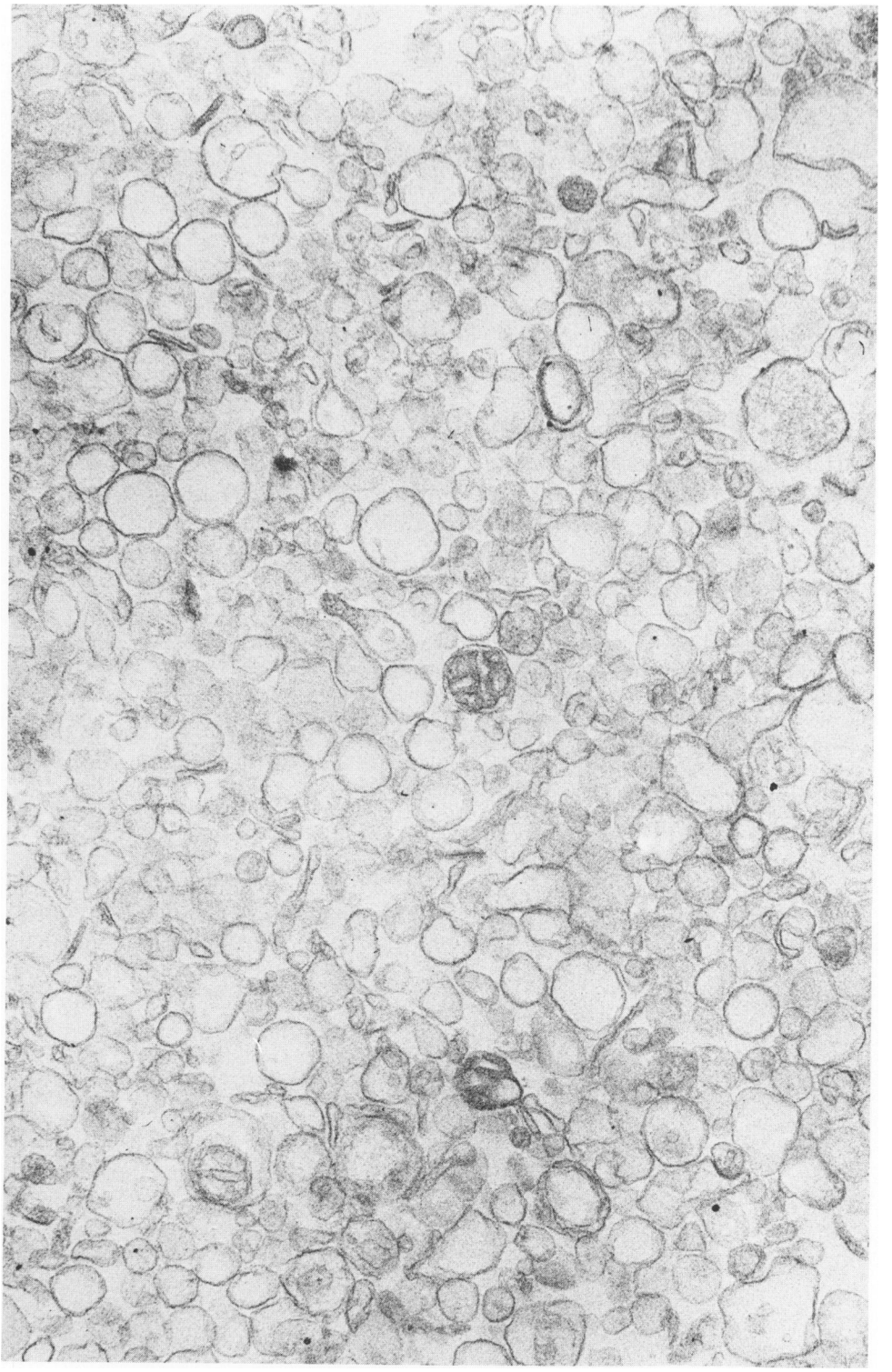

Figure 7—A preparation of sarcoplasmic reticulum from a 12-hour ischemic heart. (×46,000)