Effect of Collateral Flow on Epicardial and Endocardial Lysosomal Hydrolases in Acute Myocardial Ischemia

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ABSTRACT Early changes in lysosomal enzymes must occur if their role is significant in irreversible myocardial injury. Therefore, we ligated the anterior descending coronary artery in 14 dogs and after 60 min excised epicardial and endocardial samples from the ischemic and adjacent normal heart. The collateral flow measured with radioactive microspheres in the endocardial samples averaged 19% of control. The muscle was disrupted and fractionated by ultracentrifugation into nuclear pellet (NP), heavy lysosomal pellet (HL), light lysosomal pellet (LL), microsomal pellet (M) and supernate (S). Electron microscopy demonstrated changes characteristic of ischemia in whole tissues and sedimented fractions. Acid phosphatase reaction product was present in residual bodies in the HL fraction and membrane-bound vesicles in the LL fraction and in the intact tissue. Significant decreases in the specific activity of *N*-acetyl-β-glucosaminidase and β-glucuronidase occurred in the endocardial LL fraction, while significant increases in both were found in the S fraction ($P < 0.05$). Losses of acid phosphatase occurred in both LL and S fractions. Moreover, decreases of total *N*-acetyl-β-glucosaminidase in the HL fraction and of total β-glucuronidase and acid phosphatase in the LL fraction were positively correlated ($P < 0.01$) with the degree of ischemia measured with radioactive microspheres. Only insignificant enzymatic changes were found when the collateral flow was greater than 40%, and the differences were less significant in epicardial samples where the flow averaged 29%. The early loss of enzymes from the lysosomal fractions in severe ischemia suggests a role for lysosomal hydrolases in the necrosis that follows coronary occlusion.

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INTRODUCTION

The biochemical mechanism of injury and death of the myocardial cell after acute ischemia and infarction is unresolved. Ischemia has been postulated to cause the release of acid hydrolases from lysosomes in hepatic tissue (1) and skeletal muscle (2), and acid hydrolases have been used as markers of lysosomal integrity during ischemia of cardiac tissue (3–7). Recently, with histochemical techniques, the early depletion of particle-bound acid phosphatase in ischemic myocardium has been reported (8). Loss of acid phosphatase has been found along with morphological alterations shown to represent ischemic damage to the cellular structure (8–10). These results deserve consideration in the study of the mechanism of death of the myocardial cell, since lysosomal enzymes have been shown capable of hydrolyzing a multitude of cellular components (11).

Recent work has revealed the inhomogeneity of myocardial blood flow during ischemia and has provided evidence that a gradient of flow extends from the epicardium to the endocardium (12, 13). Thus, the endocardium should be affected maximally by the ischemia, and regional differences of cellular injury across the ischemic myocardium should be detectable. Therefore, we chose a model of myocardial ischemia in which the blood flow could be measured in epicardial and endocardial sections of the left ventricle. At the same time we performed electron microscope histochemical studies of control and ischemic tissues and tissue fractions to evaluate morphological changes in the distribution of acid phosphatase within the myocardial cell. To quantify changes in acid hydrolases during early ischemia, we selected three acid hydrolases reported to be located in lysosomes. These enzymes were studied comparatively in five tissue fractions to localize further the particle-bound and free enzymatic activities. This approach allowed us to correlate changes of enzymatic activity with

blood flow and to compare morphological, physiological, and biochemical alterations during early ischemia of the myocardium.

METHODS

Model of experimental ischemia. 15 randomly selected mongrel dogs were anesthetized with sodium pentobarbital (27 mg/kg). Respiration was maintained at a constant rate by a Harvard respirator (Harvard Apparatus Co., Inc., Millis, Mass.) with 100% oxygen delivered via tracheostomy. A left thoracotomy was performed in the fifth intercostal space, exposing the left atrium and ventricle. The left anterior descending coronary artery (LAD) [1] was dissected free of fat approximately 1.5 cm distal to its origin and ligated. Systemic and left ventricular pressures were monitored throughout the experiment. A transient decrease of pressure was seen frequently after ligation of the LAD and, in one animal, ectopic ventricular activity was followed by ventricular fibrillation and death. In three more animals lidocaine HCl was used as a 100-mg bolus within 5 min after occlusion to suppress ventricular ectopic activity.

To measure myocardial flow 15 min after ligation of the anterior descending coronary artery, 1×10^6 microspheres of 15 μm diameter (3M Co., St. Paul, Minn.), labeled with ^{85}Sr, were injected into the left atrium over a period of 15–30 s. An arterial reference sample was withdrawn at a constant rate starting at the time of microsphere injection and continuing for 30 s after completion of injection (14). 1 h after occlusion, the heart was excised and ischemic tissue was sampled within the boundaries of the tissue supplied by the ligated artery. A sample of normal tissue was obtained from the posterior base of the left ventricle, which was distant from the ischemic border. The normal and ischemic samples were sectioned into epicardial and endocardial halves and immediately immersed in ice-cold extraction buffer (0.15 M KCl, 0.005 M histidine, pH 7.0).

Tissue fractionation. Each tissue sample was trimmed to an approximate weight of 3 g, minced with scissors, washed twice in extraction buffer, and then homogenized in 40 ml of ice-cold buffer with two 5-s bursts at position eight in a Sorvall Omni-mixer (Sorvall-Dupont Instruments, Sorvall Operations, Newtown, Conn.). Five fractions were obtained by centrifugation in a Beckman 42.1 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.): nuclear pellet (NP) fraction, 1,000 *g* for 10 min; heavy lysosomal-mitochondrial pellet (HL) fraction, 9,000 *g* for 15 min; light lysosomal-microsomal pellet (LL) fraction, 60,000 *g* for 30 min; light microsomal pellet (M) fraction, 120,000 *g* for 30 min; and supernatant (S) fraction.

The pellets were rehomogenized by five up-and-down strokes in a glass-Teflon homogenizer in 3 ml of extraction buffer (0.15 M KCl, 0.005 M histidine, pH 7.0) at 0–4°C, and immediately incubated to determine activity of lysosomal hydrolases. The influence of storage on our fractions at 4°C was examined and the results indicated that no significant loss of activity occurred in particulate or supernatant fractions after 4 h under the described conditions.

[1] *Abbreviations used in this paper:* HL, heavy lysosomal-mitochondrial pellet; LAD, left anterior descending coronary artery; LL, light lysosomal-microsomal pellet; M, light microsomal pellet; NP, nuclear pellet; S, supernate; SR, sarcoplasmic reticulum.

Myocardial flow. The microsphere method for measurement of myocardial flow is based on the assumption that a volume of tissue, trapping the same number of microspheres as found in the arterial reference sample (A), must have a flow equal to the volume of blood withdrawn in the syringe (W). Thus the myocardial blood flow (MBF) in any tissue sample is given by the equation $MBF = W(M/A)$, where M is given in number of microspheres per gram and W in milliliters per minute (14). Normal tissue samples contained in excess of 400 microspheres/sample. Comparison of counts from adjacent normal samples indicated that all microspheres of the intact tissue were recovered in the NP fraction and in the tissue washes. Thus myocardial blood flow could be calculated on the basis of counts recovered for each sample subjected to biochemical analysis. The samples were counted in an automatic well-type gamma counter for enough time to reduce errors due to the statistics of counting to less than 3%.

Enzyme assays. *N*-Acetyl-β-glucosaminidase (15) was assayed, with *p*-nitrophenyl-acetyl-β-glucosaminide (Sigma Chemical Co., Inc., St. Louis, Mo.) (3.6 mM) as substrate, and sodium citrate buffer containing 1 mg/ml bovine serum albumin at pH 4.5. The reaction was stopped with addition of 0.5 ml trichloroacetic acid (25% wt/vol). After centrifugation, 1 ml of the supernate was added to 1.5 ml of 2 N NH_4OH HCl solution at pH 10.7 and the optical density was recorded at a spectrophotometric setting of 420 nm. β-Glucuronidase (16) was assayed in 1 ml of 0.1 M sodium acetate buffer at pH 5.0, containing 1% bovine serum albumin. Phenolphthalein glucuronide (Sigma Chemical Co.) (1 mM) was the substrate. The reaction was stopped and the color developed by the addition of 3.5 ml glycine-NaCl-NaOH buffer (pH 10.4). After centrifugation the supernate was read at 540 nm. Acid phosphatase (17) was assayed with 0.2 M sodium acetate buffer at pH 4.8, containing 0.1 M KCl in a total volume of 1 ml. The substrate was *p*-nitrophenylphosphate (Sigma Chemical Co.) in 5 mM solution. The reaction was stopped by the addition of 0.5 ml trichloroacetic acid (25%, wt/vol). After centrifugation, 1 ml of the supernate was added to 1.5 ml of 2 N NH_4OH-HCl solutions at pH 10.7 and read at 420 nm. The protein was determined for each sample in all fractions (18). The HL and LL fractions were examined for mitochondrial contamination with cytochrome *c* oxidase as a marker (19). The HL fraction contained 34.7% of the total cytochrome oxidase recovered per gram of tissue while the LL fraction contained only 3.3%–4.7%; no significant differences in mitochondrial contamination were seen between normal and ischemic epicardial or endocardial tissue.

Latency of lysosomal enzymes. "Latency" is considered to be a property of lysosomes where the enzymes are inaccessible to substrate due to the intact lysosomal membrane. Detergents have been used to disrupt the lysosomes and release the enzymes. We examined latency in membrane-bound fractions of six paired experiments. Normal and ischemic fractions were treated with Triton-X-100 while the protein concentration was kept constant. Seven concentrations of detergent (between 0.1 and 0.001%) were used since a dose relation to the concentration of Triton X-100 was apparent from initial studies. The unmasking or activation of enzymatic activities with Triton X-100 was expressed as percent of control activity: percentage of latency = (activity with Triton X-100/activity without Triton). Generally, a higher degree of maximal latency was found in control fractions than in the ischemic

TABLE I
Total Activity of Acid Hydrolases of Ischemic Myocardium

Fraction	*N*-Acetyl-β-glucosaminidase			β-Glucuronidase			Acid phosphatase		
	Normal SEM	Ischemic SEM	*P*	Normal SEM	Ischemic SEM	*P*	Normal SEM	Ischemic SEM	*P*
	μmol/100g tissue-min			*μmol/100g tissue-h*			*μmol/100g tissue-min*		
Epicardial section									
NP	23.90±1.67	21.71±1.05	<0.05	14.71±2.03	14.92±2.27	NS	53.90±4.56	52.80±2.83	NS
HL	7.47±0.71	6.44±0.57	NS	2.92±0.27	2.68±0.27	NS	6.00±0.52	5.73±0.41	NS
LL	2.32±0.20	2.69±0.19	NS	1.10±0.12	1.08±0.08	NS	6.85±0.68	6.70±0.52	NS
M	0.45±0.05	0.40±0.04	NS	0.32±0.04	0.23±0.04	<0.05	1.47±0.46	1.16±0.26	<0.05
S	14.75±0.71	14.27±1.10	NS	7.75±1.61	7.30±1.08	NS	36.85±2.48	34.21±2.02	NS
Endocardial section									
NP	25.67±1.88	22.01±1.25	<0.05	16.05±2.33	18.91±3.74	NS	58.75±3.73	52.73±4.73	<0.05
HL	7.53±0.79	5.47±0.80	<0.001	2.63±1.18	2.50±0.27	NS	5.57±0.40	5.60±0.45	NS
LL	1.91±0.19	1.45±0.20	<0.005	1.21±0.31	0.74±0.09	<0.005	6.40±0.53	4.64±0.49	<0.005
M	0.44±0.05	0.49±0.04	NS	0.34±0.06	0.28±0.08	NS	1.43±0.08	1.05±0.19	<0.05
S	13.95±1.16	16.97±1.29	<0.005	7.83±0.92	9.15±1.06	<0.05	38.00±2.22	31.08±1.97	<00.1

The values are the mean of 14 experiments±SEM. *P* values are calculated according to the Student *t* test for paired data, $P < 0.05$ indicating a significant difference.

fractions, with the exception of *N*-acetyl-β-glucosaminidase in the HL fraction.

Statistical evaluation of the results was performed for paired data with Student's *t* test, and $P < 0.05$ was considered to be significant (20).

Electron microscopy. In three experiments small pieces were cut from normal and ischemic epicardial and endocardial tissues before homogenization. These were immersed in cold fixative and cut with a razor into blocks (2 × 2 mm wide, and no more than 1 mm thick). Both tissue slices and the HL and LL pellets were fixed for 1 h at 0–4°C and washed overnight in buffered sucrose (21). They were then reacted for 90 min in a Gomori-type medium (22) modified by the addition of 10% dimethylsulfoxide (23) for cytochemical localization of acid phosphatase activity. Controls were incubated without substrate or with 0.01 M NaF. After incubation the specimens were washed, postfixed in 2% OsO_4 in H_2O, dehydrated in acetone, and embedded in Epon 812 (Shell Chemical Co., New York). Lead citrate-stained and unstained sections were examined with a Zeiss EM 95 (Carl Zeiss, Inc., New York).

RESULTS

Table I lists enzymatic changes found in all fractions. Comparison of the epicardial fractions from the ischemic tissue revealed activities only slightly different from the corresponding normal samples, while most of the differences in the endocardial fractions were highly significant. Thus, similar enzymatic changes occurred in both but were larger in endocardial sections. A significant loss of enzymatic activity was a common finding in the endocardial LL fraction for all three enzymes. In addition, *N*-acetyl-β-glucosaminidase was decreased significantly in the HL fraction. The NP fraction showed significant losses of *N*-acetyl-β-glucosaminidase and acid phosphatase. Increases were found for *N*-acetyl-β-glucosaminidase and β-glucuronidase in the S and M fractions. Acid phosphatase showed a significant loss of total activity in the S and M fractions. *p*-Nitrophenyl phosphate has been shown to be a less specific substrate for lysosomal hydrolases than the two other substrates (11). The differences in changes of acid phosphatase may be due to localization to lysosomes and other organelles.

Table II shows the protein content (determined in triplicate) of the fractions related to tissue weight. This indicates that during 1 h of ischemia, the protein content did not change in the NP, HL, LL, or S fractions. The only significant decrease in protein content was found in the endocardial M fraction, which contained less than 1% of the total protein. These minimal changes in the protein indicate that changes in specific and total

TABLE II
Total Protein Content

Fraction	Normal	Ischemic	*P*
	mg/g tissue		
Epicardial			
NP	85.5±6.5	80.2±6.1	NS
HL	7.6±0.7	7.4±0.7	NS
LL	4.4±0.4	3.6±0.4	NS
M	2.9±0.3	2.7±0.3	NS
S	32.2±3.1	29.4±4.2	NS
Endocardial			
NP	80.6±7.4	75.9±11.1	NS
HL	6.4±0.6	6.1±0.4	NS
LL	3.1±0.4	2.9±0.3	NS
M	2.8±0.3	1.8±0.2	<0.05
S	29.2±2.1	29.1±0.3	NS

Total protein content of epicardial and endocardial tissue fractions. The values represent means of 14 determinations ±SEM.

TABLE III

Specific Activity of Acid Hydrolases of Ischemic Myocardium

Fraction	*N*-Acetyl-β-glucosaminidase			β-Glucuronidase			Acid phosphatase		
	Normal SEM	Ischemic SEM	*P*	Normal SEM	Ischemic SEM	*P*	Normal SEM	Ischemic SEM	*P*
	μmol/mg protein-min			*μmol/mg protein-h*			*μmol/mg protein-min*		
Epicardial section									
NP	0.286±0.026	0.302±0.037	NS	0.181±0.029	0.215±0.053	NS	0.645±0.043	0.700±0.082	NS
HL	1.008±0.061	0.994±0.098	NS	0.391±0.037	0.392±0.065	NS	0.810±0.069	0.843±0.053	NS
LL	0.593±0.062	0.590±0.069	NS	0.299±0.023	0.288±0.031	NS	1.833±0.111	1.752±0.120	NS
M	0.179±0.018	0.162±0.017	NS	0.126±0.022	0.107±0.021	NS	0.542±0.043	0.488±0.042	NS
S	0.496±0.043	0.519±0.043	NS	0.275±0.082	0.248±0.044	NS	1.205±0.090	1.291±0.091	NS
Endocardial section									
NP	0.345±0.041	0.344±0.039	NS	0.201±0.032	0.393±0.184	NS	0.625±0.089	0.783±0.072	NS
HL	1.157±0.086	0.856±0.069	<0.001	0.395±0.037	0.392±0.044	NS	0.775±0.061	0.920±0.110	NS
LL	0.673±0.072	0.437±0.061	<0.001	0.399±0.048	0.238±0.029	<0.001	2.133±0.152	1.481±0.151	<0.005
M	0.171±0.013	0.244±0.033	<0.05	0.194±0.060	0.207±0.050	NS	0.508±0.056	0.535±0.068	NS
S	0.483±0.039	0.628±0.049	<0.001	0.299±0.044	0.372±0.055	<0.05	1.224±0.128	0.994±0.120	<0.005

The values are the mean of 14 experiments±SEM. *P* values are calculated according to the Student *t* test for paired data, $P < 0.05$ indicating a significant difference.

activities were not caused by changes in the consistency of the tissue that might alter the efficiency of disruption of normal and ischemic tissues. Because of this fact, the data, when expressed as specific activity (Table III), is not substantially altered from that presented in Table I, based on tissue wet weight. However, variance introduced by the protein determination alters the magnitude of the statistical significance.

Fig. 1 shows the influence of seven different concentrations of Triton X-100 (between 0.001 and 0.1%) on the activity of *N*-acetyl-β-glucosaminidase, β-glucuronidase, and acid phosphatase in six experiments (±SEM) for the LL fraction of normal and ischemic tissues. The changes caused by the addition of Triton X-100 were different for each single concentration of detergent and did not increase in a predictable fashion with increasing increments of detergent. In Table IV the maximal differences of enzymatic activities with and without Triton X-100 are listed as a percentage of control. It is apparent that different concentrations of Triton X-100 cause variable increases in enzymatic activity so that maximal effects could not be obtained with a single standardized procedure. The decrease in latency of β-glucuronidase and acid phosphatase was significant ($P < 0.01$) in the particulate fractions of ischemic endocardial tissues, while the latency of the ischemic HL fraction of *N*-acetyl-β-glucosaminidase was slightly increased.

The most severe ischemia was shown in the endocardial sections, where the collateral flow averaged 19±4% of control. The flow to the epicardial sections averaged 29±3% and the difference between epicardial and endocardial sections was highly significant ($P < 0.005$, Fig. 2).

Fig. 3 illustrates the relationship between the decrease of enzymatic activities of endocardial samples of particle-bound fractions and collateral flow as a percentage of control. Significant correlations were obtained for the changes in endocardial levels of *N*-acetyl-β-glucosaminidase of the HL fraction ($r = 0.90$, $P < 0.001$) and for β-glucuronidase of the LL fraction ($r = 0.74$, $P < 0.01$).

TABLE IV

Latency of Normal and Ischemic Myocardial Fractions with Triton X-100

Fraction	*N*-Acetyl-β-Glucosaminidase		β-Glucuronidase		Acid phosphatase	
	Normal	Ischemic	Normal	Ischemic	Normal	Ischemic
HL	126±3*	137±8*	140±3*	132±2*	185±12*	149±9*
LL	201±2‡	170±20‡	155±13*	135±6*	123±5‡	111±2‡

The values represent latency in percent of enzymatic activity, i.e., increment of lysosomal hydrolytic activity after addition at detergent under otherwise stable conditions, where hydrolytic activity without detergent was given as 100%. Maximal latency was found at different detergent concentrations for the individual enzymes and fractions. Therefore, concentrations of Triton X-100 contained in each preparation are indicated.

* 0.04%.

‡ 0.075%.

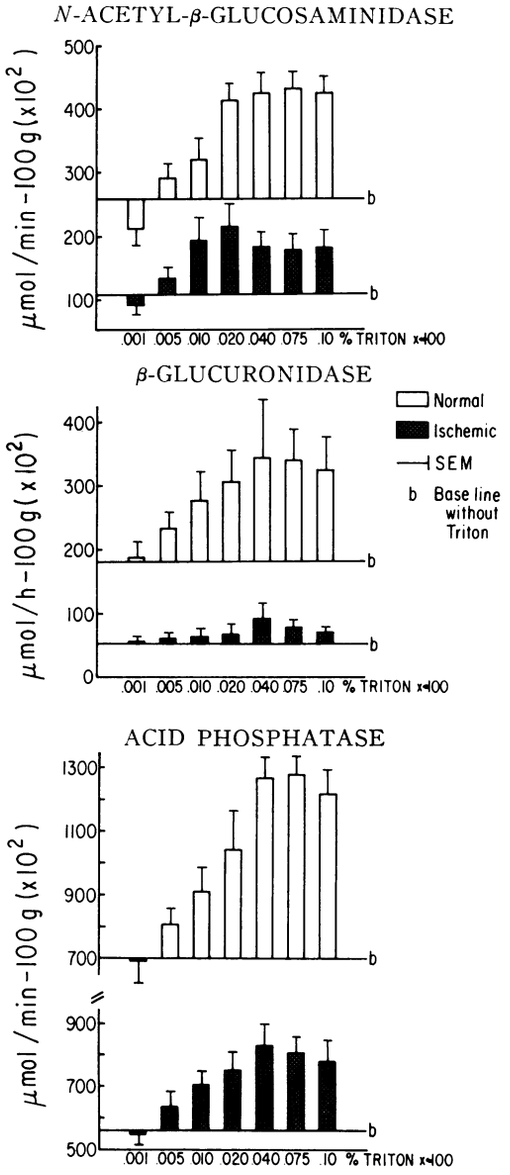


FIGURE 1 Latency of the particle-bound enzymes of the LL fraction as an increment of hydrolytic activity over the base line (b). The units are given as micromoles per minute per 100 g ($\times 10^2$) tissue for *N*-acetyl-β-glucosaminidase and acid phosphatase, and as micromoles per hour per 100 g tissue for β-glucuronidase. The values include six experiments±SEM. Different latency is shown for normal and ischemic fractions at seven different concentrations of Triton X-100 (between 0.001 and 0.1%).

The correlation for acid phosphatase was less significant ($r = 0.54$, $P < 0.05$). The correlation for *N*-acetyl-β-glucosaminidase in the LL fraction did not reach significance ($r = 0.45$, $P > 0.05$). However, with elimination of one atypical value the correlation could attain significance ($r = 0.61$, $P < 0.05$).

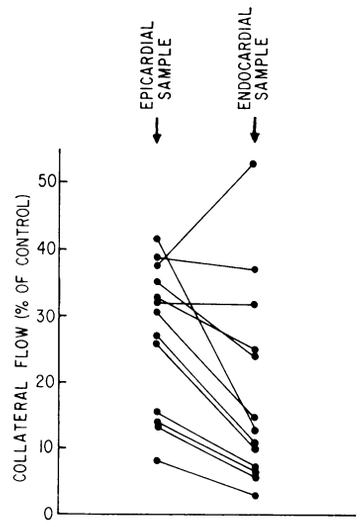


FIGURE 2 Flow gradient from epicardial samples to corresponding endocardial samples within the ischemic area after ligation of the LAD. The average endocardial flow is 48% of the epicardial flow. $P < 0.005$.

In the epicardial sections enzymatic changes were much smaller; however, several of the changes correlated with flow, indicating a rather sensitive relationship between enzymatic changes and decreased flow. Loss of β-glucuronidase in the epicardial HL fraction correlated with flow ($r = 0.68$, $P < 0.01$). Also, a similar relationship was seen in the LL fraction ($r = 0.71$, $P < 0.01$). Loss of *N*-acetyl-β-glucosaminidase showed a borderline correlation with flow in the LL fraction ($r = 0.51$, $P < 0.05$). *N*-Acetyl-β-glucosaminidase in the HL fraction and acid phosphatase did not correlate with flow in the epicardial sections. When epicardial and endocardial samples with flow values less than 40% were combined ($n = 24$), significant correlations between the loss of enzymatic activity and the decrease in flow could be attained for all three enzymes in the LL fractions and for *N*-acetyl-β-glucosaminidase in the HL fraction. All correlations were significant at $P < 0.01$, with the exception of acid phosphatase at $P < 0.05$.

Electron microscope examination in three experiments showed acid phosphatase activity within the sarcoplasmic reticulum (SR) throughout normal myocardial cells. Dense masses of lead phosphate reaction product filled the lateral sacs adjacent to both transverse and longitudinal T-tubules as well as those adjacent to the cell membrane, making them extremely conspicuous even at low magnification in unstained sections. Reaction product was also visible and filled round or elongated profiles of the longitudinal sarcoplasmic reticulum components (Fig. 4*a*). No such precipitates were seen in control specimens incubated without substrate or in the pres-

ence of NaF. Acid phosphatase activity was also localized in residual bodies, a few Golgi cisternae, and small primary lysosomes located near the reactive Golgi and the mitochondrial row. Irreversibly injured ischemic myocardial cells were characterized by swollen mitochondria with clear matrices. The spaces between myofibrils were electron lucent and devoid of stainable glycogen. The myofibrils were frequently more relaxed than in normal control tissue (9). When such tissue was incubated to localized acid phosphatase, much less activity was evident than in normal cells. The residual enzymatic activity had the same specific localization as the normal cells but the sites in which it could be identified were more sparse. Specifically, all T-tubules in normal cells were conspicuously marked by lateral sacs filled with dense reaction product but only a few T-tubules in ischemic cells were so outlined (Fig. 4*b*). Similarly, reaction product between the myofibrils was limited to a few rounded vesicles.

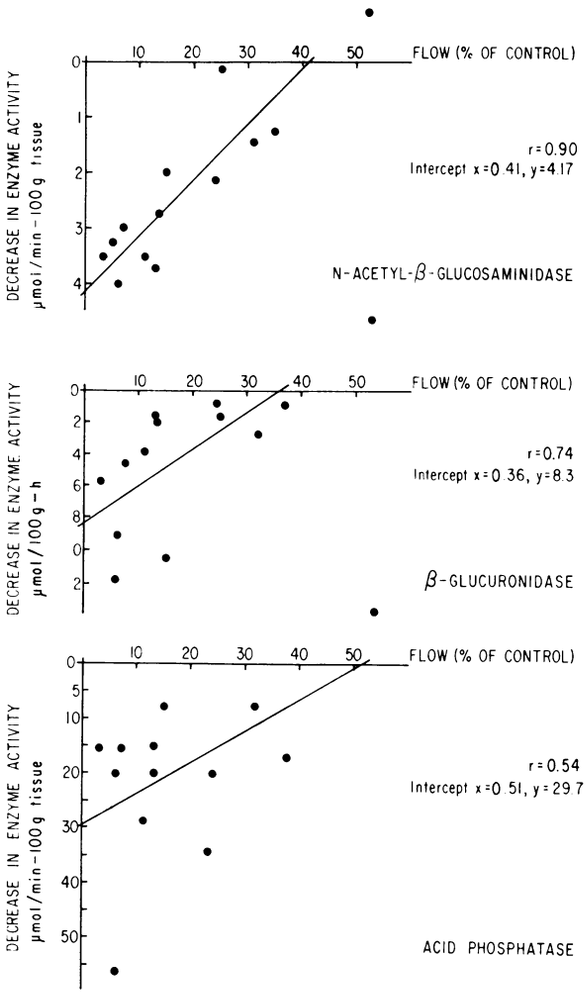


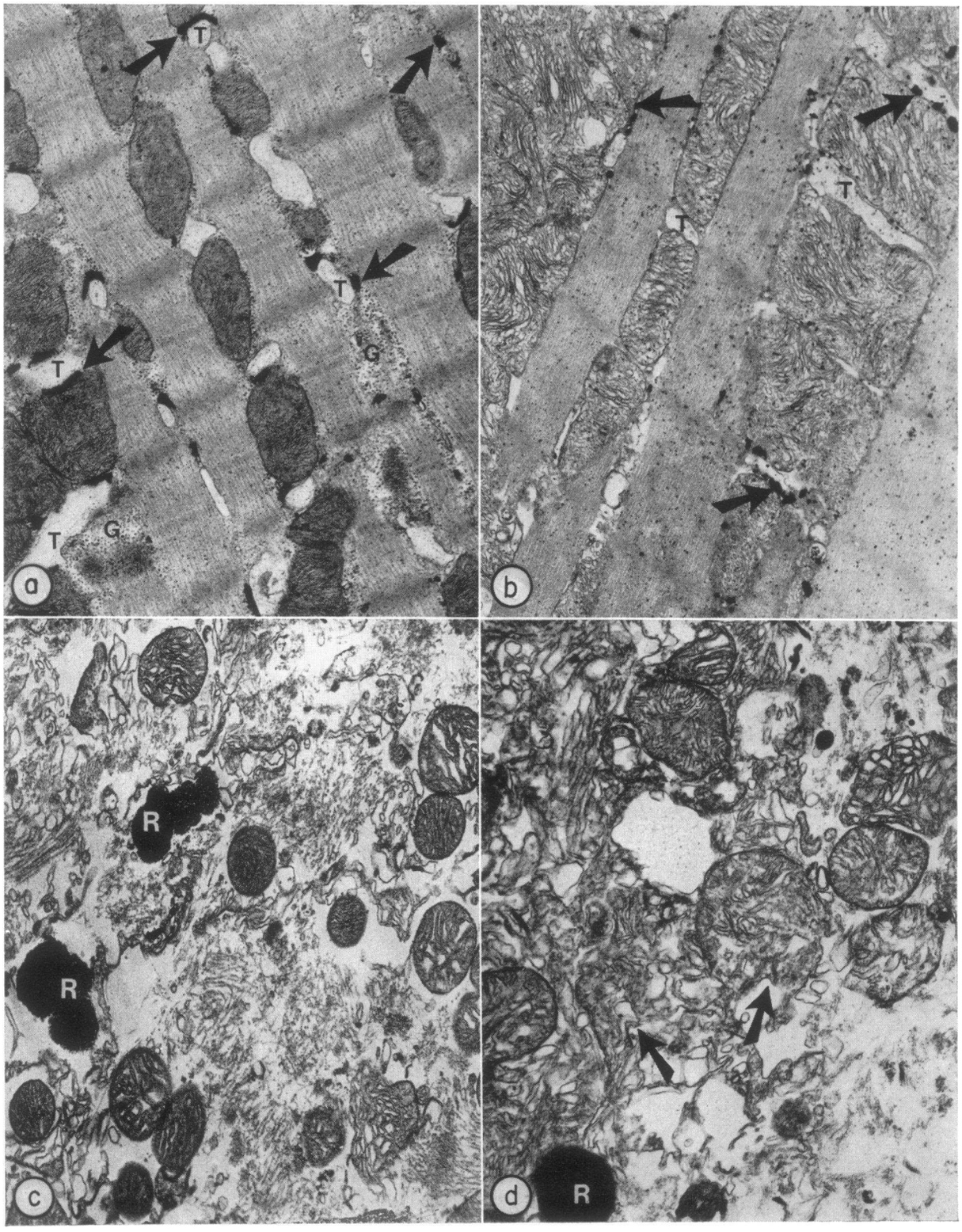
FIGURE 3 Correlation of flow and enzyme activity of *n*-acetyl-β-glucosaminidase in the HL fraction and of β-glucuronidase and acid phosphatase in the LL fraction of the ischemic myocardium. The collateral flow is expressed as a percentage of its normal control: the enzyme in micromoles of substrate released per min-100 g of tissue. $n = 13$.

Ultrastructural cytochemical studies of subcellular fractions prepared from normal and ischemic tissue showed differences in the isolated organelles that corresponded with the differences seen in the whole tissue. The HL fraction from both normal and ischemic tissues contained numerous mitochondria and residual bodies, as well as some SR and other myofibrillar fragments. The mitochondria in the pellet derived from control tissue had the normal appearance of freshly isolated organelles (Fig. 4*c*), whereas those from ischemic tissue were frequently ruptured and devoid of matrix (Fig. 4*d*). Similarly, differences were seen in the LL fractions related to changes seen in the corresponding tissues, in that the LL pellet from normal tissue was enriched in SR fragments that stained positively for acid phosphatase and contained much glycogen (Fig. 4*e*). The LL pellet from ischemic tissue showed fewer SR fragments with such staining and very little glycogen (Fig. 4*f*).

DISCUSSION

In recent years a number of studies of the pathophysiology of ischemia have focused on lysosomal alterations that may accompany cellular damage. DeDuve and Beaufay (1) observed previously that after several hours of ischemia in hepatic tissue there was a reduction of lysosomal enzymatic activity in the particle-bound fractions of the disrupted tissue. These results led to the hypothesis that the acidotic condition that accompanies ischemia might cause labilization of lysosomes with subsequent release of hydrolytic enzymes into the cytosol of the cell.

In the present study we report a decrease of particle-bound lysosomal enzymes after 1 h of ischemia of the myocardium. This decrease correlates significantly with the diminished collateral flow, and is accompanied by a decrease in stainable acid phosphatase on electron microscopy. We chose a 1-h period of ischemia because Jennings et al. reported the onset of irreversible morphological changes after 40 min of ischemia (9, 10, 24). By ligating the anterior descending coronary artery, we were able to use left ventricular tissue from the non-ischemic area as a control. Epicardial and endocardial sections were examined to assess the effect of the flow gradient across the myocardium during ischemia. The examination of five fractions of the disrupted myocardium for lysosomal enzymatic activity appeared appropriate, since multiple localization of lysosomal enzymes has been described in muscle tissue (25). Several populations of lysosomes have been identified during sedimentation by zonal centrifugation (26). In addition,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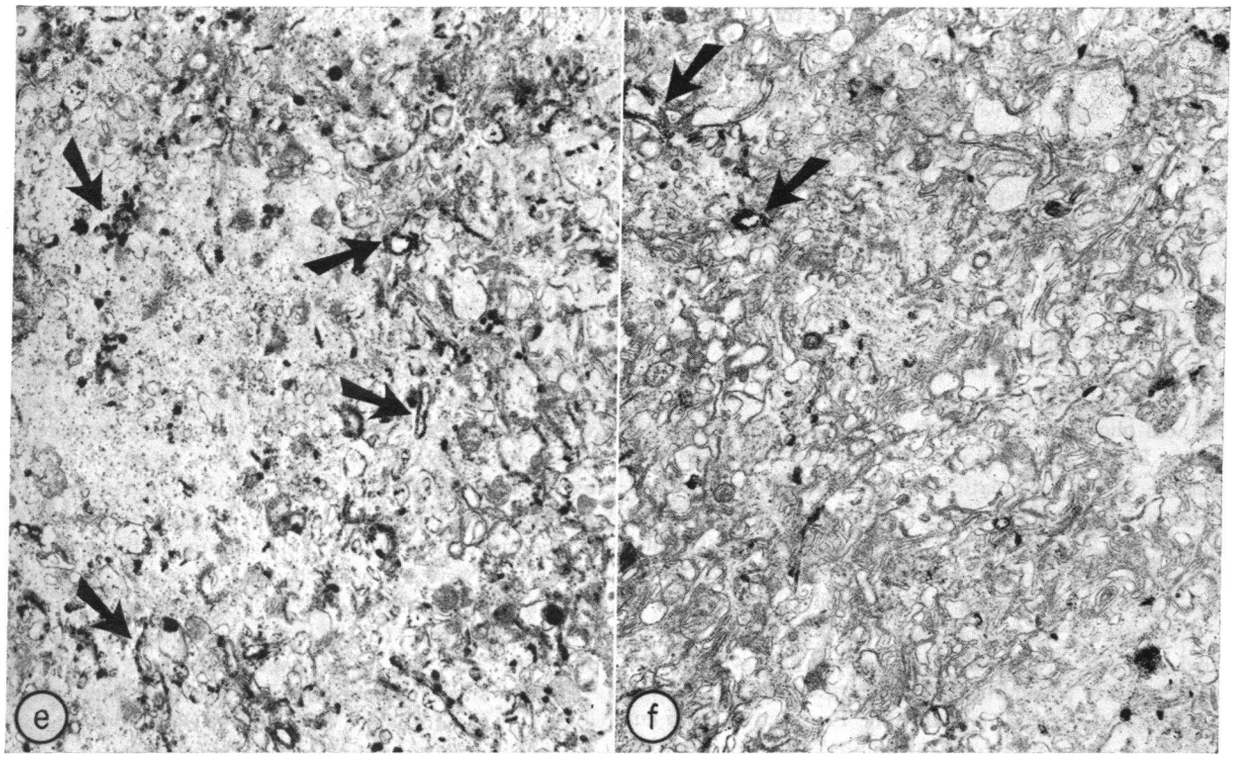

FIGURE 4 *a.* Normal, left ventricular endocardial cell reacted for acid phosphatase. Much glycogen (G) is present and mitochondria appear normal with packed cristae and dense matrices. Acid phosphatase reaction-product (arrows) is visible in numerous elements of the SR including lateral sacs adjacent to T-tubules (T). Magnification in *a–f* is 20,000 ×. *b.* A cell from the ischemic endocardium of the same animal reacted for acid phosphatase. The collateral flow was determined to be 4% of control in this sample. Glycogen is absent and the mitochondria are swollen and devoid of matrix with vesiculated cristae. Acid phosphatase reaction-product is visible in only a few places (arrows). *c.* The heavy lysosomal fraction from a homogenate of the normal endocardium reacted for acid phosphatase. Residual bodies (R) and mitochondria are present along with other membrane and myofibril fragments. The mitochondria have the condensed appearance normal for freshly isolated mitochondria. *d.* The heavy lysosomal fraction from the corresponding ischemic endocardium. Many of the mitochondria are swollen and without matrix density as in the ischemic tissue (*d*). Some mitochondria appear to be ruptured (arrows). In addition, some membrane fragments and a residual body (R) can be seen. *e.* The light lysosomal fraction from normal endocardium. Numerous electron-dense, acid phosphatase-reactive SR elements (arrows) can be seen looking much as they do in the whole normal tissue (*a*) and glycogen is present in abundance. *f.* The light lysosomal fraction from the corresponding ischemic endocardium. Fewer acid phosphatase-reactive SR elements (arrows) are present in this fraction than in that shown in *e*. Glycogen as well is markedly reduced.

the distribution of lysosomal enzymes may be altered by a release of enzymes during disruption of tissue and readsorption to different subcellular structures; this phenomenon may affect certain hydrolases, e.g. *N*-acetyl-β-glucosaminidase, more than others, e.g. β-glucuronidase (27). Nevertheless, the changes that might be due to nonischemia-related perturbations of the lysosomal hydrolases should be consistent for normal and ischemic portions of the myocardium, so that the differences that we report should reflect ischemic alterations in the lysosomes.

All three lysosomal hydrolases were decreased significantly in the endocardial LL fraction after 1 h of ischemia. The data for acid phosphatase and β-glucuronidase confirmed the results of Ricciutti (5), who used a 4-h model of ischemia, and also the results of Spath et al. (6), who used a model with a 5-h occlusion. Ricciutti (4) was able to show changes of acid phosphatase after 1 h of partial occlusion of the circumflex artery; however, the significant decrease in activity could be shown only in the supernatant fraction, while the sedimentible lysosomal enzymatic activity was in-

creased when compared to the control group. Brachfeld (3) reported an increase in cathepsin activity of the supernate; however, the "large granule activity" did not change significantly at postmortem analysis.

Our changes in enzymatic activity in the supernate correspond to the findings of Ricciutti (4), showing a decrease of acid phosphatase after 1 h; the increases in *N*-acetyl-β-glucosaminidase and β-glucuronidase are similar to findings reported for cathepsin by Bachfeld (3). Similar data were previously described in skeletal muscle after several hours of ischemia (2). In these experiments initial increases of enzymatic activity in the supernate were followed by losses in the supernate; this occurred at different rates for individual enzymes but acid phosphatase was lost earlier than the other enzymes. Recent results from our laboratory (28) indicate that the loss of enzymatic activity from the supernatant fraction may be a function of collateral flow; however, these results were obtained after a 2-h period of ischemia. Denaturation of acid hydrolases may be another factor that should be considered in such studies.

In studies of lysosomal enzymes, it has been a standard procedure to enhance the release of lysosomal enzyme activity by addition of a detergent such as Triton X-100 to a lysosomal preparation. The increment in enzymatic activity that could be elicited after the addition of a detergent was considered due to membrane labilization and is called latency. Baggiolini [2] stated that the increment in lysosomal enzymatic activity of the white blood cell may be a function of the protein content of the preparation. The changes that resulted from the addition of Triton X-100 to our particulate fractions of lysosomes were found to be somewhat unpredictable. For example, *N*-acetyl-β-glucosaminidase was inhibited by very small concentrations (0.001%); acid phosphatase, with the addition of 0.1% Triton X-100, was inhibited in both particulate fractions. These results indicate that the addition of a fixed amount of Triton X-100 may not result in maximal release of all enzymatic activities. Nevertheless, a higher degree of latency was found for normal than for ischemic tissues, and optimal concentrations of Triton X-100 actually augment the differences in lysosomal and enzymatic activities between normal and ischemic fractions.

When normal and ischemic tissues were compared after 1 h of ischemia, only small changes were observed in the protein content of the single fractions per gram of wet tissue. This indicates that the described enzymatic changes do not represent artifacts that might be caused by dilution resulting from alterations in edema or turgor of ischemic tissue.

The electron microscopic and histochemical changes observed in intact tissue and in HL and LL subcellular fractions supported our biochemical data. Morphological changes characteristic of the irreversibly injured cell (9) were represented by swollen mitochondria with clear matrices and loss of stainable glycogen. The acid phosphatase in the ischemic tissue was less evident than in the controls, indicating loss of particle-bound enzyme in tissue samples and in fractions. Similar findings could not be obtained in samples of epicardium and samples of normal tissue; therefore, we believe these changes were caused by ischemia and were not concomitant postmortem alteration of the tissue.

It could be argued that the microsphere method, used to measure myocardial flow in this experimental approach, caused microembolization of the myocardium, which by itself might induce myocardial damage. If this would be the case, a more severe artifact should be expected in the normal tissue than in the ischemic tissue, since more microspheres are trapped in this portion of the myocardium. This should diminish the enzymatic differences between normal and ischemic tissue. In addition, six experiments were performed without injection of microspheres and the enzymatic changes were not different from the experiments described. We believe that flow measurements with radioactive microspheres are most suitable, since this method allows measurement of flow in the tissue undergoing biochemical and cytochemical analysis.

The correlation of collateral flow with the decrease of sedimentable enzymatic activity revealed a threshold for enzymatic release in the vicinity of 40% of control for all three enzymes. This may represent a level of ischemia, at which the integrity of lysosomal membranes cannot be maintained for 1 h, resulting in activation and release of hydrolytic activity.

We propose that the anaerobic metabolism and acidosis induced by ischemia may lead to an activation and release of lysosomal enzymes within the myocardial tissue within 1 h of restricted flow. The contribution of lysosomal enzymes that might be released from white blood cells, macrophages, and other cells that reside in or migrate to the area of hypoxia during 1 h of ischemia could not be quantified in the present studies. Recent experiments in our laboratory (28) in which the enzymatic levels of arterial samples have been compared with those from regional coronary veins indicate that lysosomal enzymes are released into veins draining the ischemic area between 30 min and 1 h after occlusion.

Our cytochemical studies revealed early ultrastructural changes in the myocardial cell, and the apparent loss of membrane-bound acid phosphatase at this early stage of the ischemic injury suggests that the hydrolytic activity released within the myocardial cell may take part in the primary processes of ischemic injury. It appears conceivable that physiological or pharma-

[2] M. Baggiolini. Personal communication.

cological manipulation of the described release of hydrolytic enzymes may provide a model to assess the effects of such interventions on the evolution of myocardial infarction. Our results provide evidence for significant labilization of lysosomes during the 1st h of myocardial ischemia. Whether these changes are primary or secondary in the process of myocardial injury must be resolved by more extensive studies of the mechanism of cellular injury and death.

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