ALTERATIONS IN CARDIAC LYSOSOMAL ACID HYDROLASES FOLLOWING INDUCTION OF THE CALCIUM PARADOX

A Thesis

Presented to the

University of Manitoba

In Partial Fulfillment of the Requirements

for the Degree of

Master of Science

by

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March, 1984

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ACKNOWLEDGEMENTS

I would like to thank my advisor and mentor Dr. N.S. Dhalla for his guidance and encouragement. I would also like to thank the members of my advisory committee; Dr. V. Panagia and Dr. P.K. Singal for their help and advice during my studies. I would also like to express my appreciation to Mrs. Susie Petrychko for typing this thesis quickly and efficiently and putting up with me through the ordeal.

I would like to acknowledge the Canadian Heart Foundation for their financial support.

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ABSTRACT

Although perfusion of the heart with calcium-free medium for a brief period followed by reperfusion with calcium-containing medium (calcium paradox) has been shown to be associated with marked structural derangements, the mechanisms for this cell damage are far from clear. Since activation of lysosomal enzymes is known to occur under pathological conditions, it was the purpose of this study to examine alterations in the activities of several lysosomal enzymes in rat hearts subjected to calcium paradox. No significant changes in the specific activities of β-acetyl glucosaminidase, β-galactosidase, α-mannosidase and acid phosphatase were seen in the homogenates of calcium paradoxic hearts. However, there were dramatic alterations in the lysosomal enzyme activities in the sedimentable and nonsedimentable fractions of the hearts during calcium paradox. The lysosomal enzyme activities were also detected in the perfusate collected during reperfusing with calcium-containing medium. These changes seemed to occur during the reperfusion period as no alterations were apparent after just calcium-free perfusion. In addition, enzyme redistribution became more marked as time of reperfusion increased. Since the addition of EGTA in control heart homogenate or Ca^{2+} in Ca^{2+}-deprived heart homogenate did not produce any changes in lysosomal redistribution, the observed alterations in Ca^{2+}-paradoxic hearts may not be directly elicited by Ca^{2+}. These data indicate either an increase in the release of enzymes from the lysosomes or an increased lysosomal fragility occur as a result of calcium paradox in addition to enzyme leakage from the myocardium. The observed changes in the lysosomal enzyme activities may partially explain the cellular damage seen due to calcium paradox.

I. INTRODUCTION AND STATEMENT OF THE PROBLEM

The calcium paradox phenomenon was first described in 1966 by Zimmerman and Hulsmann (148). They observed that reintroduction of Ca^{2+} containing solution to an isolated rat heart perfused with a medium devoid of calcium resulted in an unexpected response. Rather than allowing for a recovery in contractile activity, reperfusion with Ca^{2+} resulted in an irreversible contracture, loss of intracellular proteins and severe morphological changes. Since these initial observations much effort has been expended by many investigators to completely characterize the events leading to, and arising from,the calcium paradox. It is now generally accepted that the basic underlying mechanism of the calcium paradox phenomenon is one of altered calcium regulation by the myocardium. In 1975, Yates and Dhalla (150) suggested that the isolated heart perfused with calcium free medium forms an interesting model for studying the pathogenesis of intracellular calcium deficiency, while reperfusion of calcium deprived hearts provides a model for the study of intracellular calcium overload. Since intracellular calcium overload and deficiency are believed to be related to the contractile failure and cardiac cell death seen in several pathological situations (162-167), this model provides a unique opportunity for studying the effects of these conditions in relation to clinically observed cardiomyopathies. Although alterations in lysosomal enzyme activities have been shown to occur concomitant with many of the mammalian cardiomyopathic conditions (85-111, 116-130, 136-139, 146,147), the eliciting stimulus remains unclear. This study was therefore designed to test if there were any changes in cardiac lysosomal enzyme activity accompanying the calcium paradox,

and if alterations in myocardial calcium contents could be implicated in the release of lysosomal enzymes.

II. REVIEW OF THE LITERATURE

A. The Discovery of Lysosomes

The study of cellular organelles usually begins with morphological observations of the cell followed by biochemical analysis of these structures and their molecular components. This pattern of approach was reversed for lysosomes as their nature and function were not recognized until these organelles had been characterized biochemically. It hardly needs to be pointed out that lysosomes are membrane limited organelles containing a variety of digestive enzymes which are active at acidic pH. Initially defined by the presence of a single enzyme, acid phosphatase, lysosomes are now known to contain at least fifty acid hydrolases including various phosphatases, nucleases, glycosidases, proteases, peptidases, sulfatases and lipases. Collectively these enzymes are capable of hydrolyzing almost all classes of macromolecules. The discovery of lysosomes began in 1947 by Dr. Christian de Duve and his colleagues. de Duve et al worked on localizing the enzyme glucose - 6 - phosphatase within the rat hepatocyte as part of a study involving the mechanisms of insulin action on the liver cell. By using differential centrifugation glucose - 6 - phosphatase activity was localized in the microsomal fraction. In addition to glucose - 6 - phosphatase, acid phosphatase activity was also measured for control purposes. It was surprising to find that the acid phosphatase activity in the homogenate was only ten percent of the anticipated value but when the same fraction was assayed 5 days after storage the activity of the homogenate was of the right order of magnitude. Further studies demonstrated that these results were not due to a technical error, but most of the enzyme in the "fresh" preparations was present in masked form and became activated upon storage. This latency of acid phosphatase was

attributable to a membrane like barrier limiting the accessibility of the enzyme to its substrate. The acid phosphatase containing particles were first believed to be mitochondria (1) but this fraction consisted of heavy and light subfractions containing cytochrome oxidase and acid phosphatase activity respectively (2). It was soon realized that the particles containing the acid phosphatase activity comprised a distinct group, different from both microsomes and mitochondria. Similar observations were made by Walker on β-glucoronidase (3). This stimulated de Duve's group to search for more enzymes and by 1955, five enzymes were localized in the "light mitochondrial" fraction, all of which proved to be hydrolytic enzymes with acidic pH optima (4). In addition, all of the hydrolytic enzymes acted on different sets of natural substrates. Such a coincidence suggested that the particles fulfilled some nonspecific hydrolytic function. Thus the term lysosomes, denoting lytic particles or bodies, was proposed (4).

Although the hydrolytic function of lysosomes was implied, the involvement of lysosomal enzymes in intracellular digestion was not widely accepted. The clue to the function of lysosomes came from the work of Straus, who succeeded in subfractioning "droplets" from the kidney which he had shown to be responsible for breakdown of resorbed proteins (5). It was discovered that these droplets contained acid phosphatase and other hydrolases similar to those described in liver lysosomes (6). This observation led de Duve in 1958 to postulate the function of lysosomal enzymes (7). Accordingly, their function included digestion of foreign material engulfed by pinocytosis, endocytosis or phagocytosis, physiologic autolysis and pathologic autolysis or necrosis. In this theory considerable importance was placed on the structure linked latency of the lysosomal hydrolases.

This provided the first satisfactory explanation for the fact that autolysis is largely held in check in most cells despite their highly active hydrolytic enzyme content. Further interest in lysosomes and lysosomal enzymes amplified. In the following eight years, the ubiquitous distribution of lysosomes in mammalian cells became apparent and it was realized that the lysosome is not a discrete body but part of a diverse and dynamic system. The various forms of lysosomes and related particles, together with the different types of interactions that may occur, are diagrammed in Figure 1. This schematic description was first proposed in 1966 by de Duve (8) when the presence of cardiac lysosomes was conclusively demonstrated (9).

It is now apparent that lysosomes, in combination with some other closely affiliated vacuolar structures, form an intracellular digestive system which is analogous to the digestive tracts of higher organisms. It has also been established that the material undergoing digestion may be associated with heterophagy or with autophagy. In heterophagy, the material to be degraded is external to the cell, whereas in autophagy, the material being degraded is of endogenous origin. The word "lysosome" was chosen on the basis of the classification illustrated in Figure 1; their identification is based primarily on the presence of acid hydrolases. Within the lysosomal group, the primary lysosomes are distinguished as those containing enzymes which have never been engaged in a digestive event, whereas secondary lysosomes represent sites of present or past digestive activity. The most important components of the lysosomal system which lack acid hydrolase activity are the prelysosomes, with their contents of undigested debris, generally destined for future digestion within lysosomes.

In addition to the invaluable work of de Duve and his co-workers,

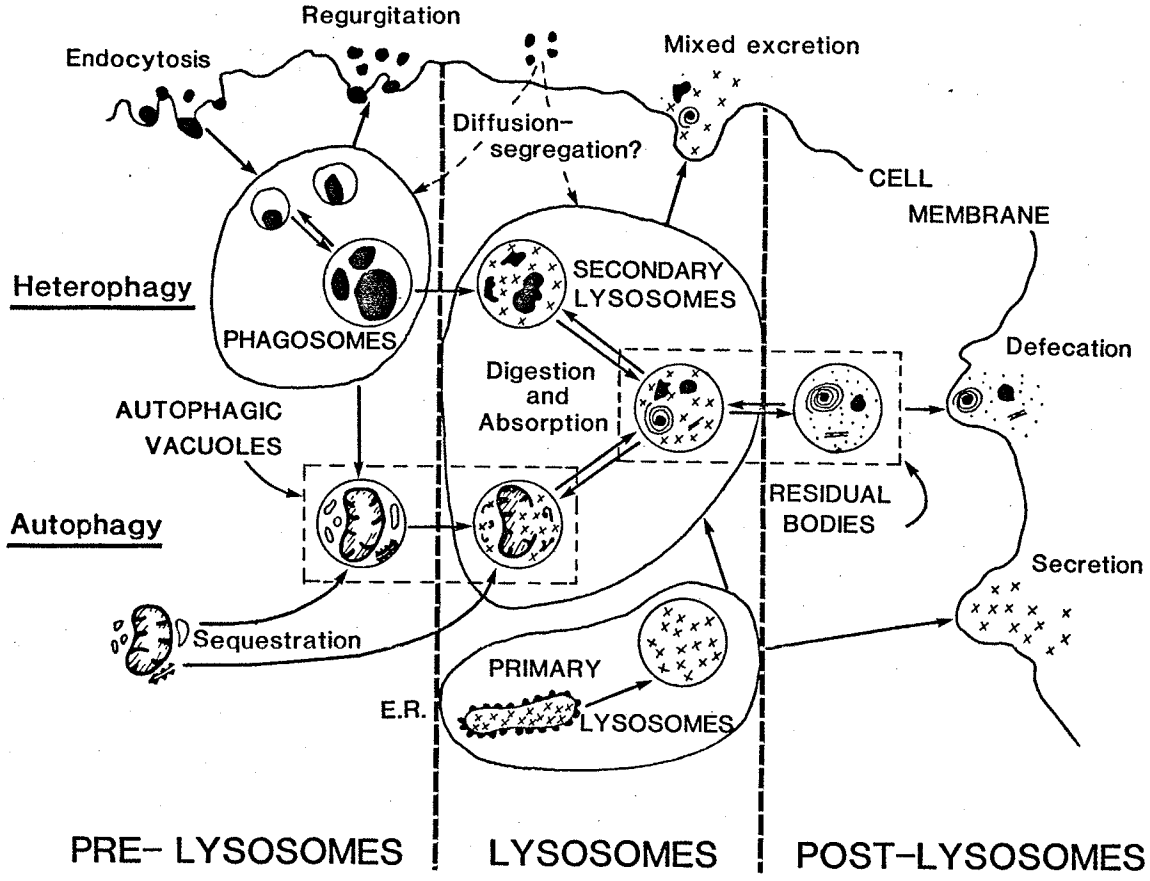

Figure 1. Schematic diagram illustrating various forms of lysosomes and related particles and the different types of interactions which may occur between them and the cell membrane. Crosses symbolize acid hydrolases [After de Duve and Wattiaux, (8)].

several other investigators have contributed significant information to the understanding of the lysosomal system. Stimulated by a review article (10) and later publications by Novikoff's group (11-14), many investigators have sought to determine the formation and identification of primary lysosomes and have queried their relationships to the Golgi apparatus and endoplasmic reticulum (ER). Novikoff introduced the acronym (GERL) (12,13) in reference to the specialized region of ER that is related to the Golgi saccule (G) and forms lysosomes (L) (12,14). Novikoff envisioned a system of direct formation of lysosomes from endoplasmic reticulum, without Golgi involvement. He maintained their existed endoplasmic regions rich in lysosomal enzymes. These regions form vesicles with two membranes containing lysosomal enzymes between the membranes, the inner of which is then digested. Since this hypothesis was first proposed, there has been histochemical and morphological evidence supporting this theory. Along with de Duve Novikoff played a central role in bringing the lysosomal system to the attention of a broad range of scientists. Cohn and his associates elucidated the role of lysosomes in digesting material engulfed by phagocytic leukocytes. They established the lysosomal nature of neutrophil granules, and demonstrated that these granules discharge their enzymes into the phagocytic vacuoles when the cells ingest foreign particles (15,16). It was shown that both neutrophils and macrophages were capable of degradation of the isotopically labelled bacteria (17). Hers and his co-workers (18) were the first to identify a true lysosomal storage disease, glycogen storage disease type II, where α-glycosidase, capable of degrading glycogen was absent. At least twenty-one conditions of similar etiology have now been described (19). Research on these pathological conditions has

yielded valuable information on the synthesis and transport of normal lysosomal enzymes. Farquhar and her colleagues (20) discovered that certain pituitary secretion granules fuse with lysosomes, and this mechanism serves to dispose of excess secretory products. This selective fusion between secretory granules and lysosomes was designated crinophagy to distinguish it from autophagy (20). Along with the discovery of this new type of autophagy, the origin and identification of several types of primary lysosomes was also established (20). Such developments in the field of lysosomal functions have progressed at a very rapid rate in the last decade. The focus of recent interest has been on the chemistry and biosynthesis of lysosomal enzymes and the pioneering work of the aforementioned scientists has left a strong foundation upon which innovative new research is being developed.

B. Structure of Lysosomes

1) Morphology

The earliest studies of cardiac ultrastructure that focused on lysosomal structures were published by Hibbs et al (21) and Wheat (22) who established the relative paucity of lysosomes in myocardial cells of young healthy animals compared to many other organs. As in other tissues, for an organelle to be classified as a lysosome, it must have two morphological features: the limiting membrane, like the plasma membrane, must be thicker than that of other organelles (23) and this membrane should be separated from the matrix by an electron-lucent halo (24,25). The free lysosomal structures observed in the myocardium are usually present primarily in perinuclear areas and resemble the ovoid "dense bodies" described in other organs (26). However, it is important to emphasize that especially in the heart, there are many different types of subcellular bodies with varying

structural and functional characteristics which have been appropriately termed "lysosomes". These structures may be associated with the Golgi apparatus, endoplasmic reticulum or they may exist as distinct organelles of varying appearances. Lysosomes can easily be distinguished cytochemically. Acid phosphatase histochemical staining was the first developed (27) and most often used method for the detection of lysosomes, but now the activity of various other hydrolases including β-glucuronidase (28), aryl sulfatase (29), β-acetylglucosaminidase (30-32), esterases (33) and napthylaminidases (34) can also reveal particulate localization of the enzymes (34). For several enzymes, it is difficult to obtain specific histochemical substrates which demonstrate unambiguously the localization of the enzyme and for this reason, immunological techniques are becoming increasingly important. Antibodies specific for single proteins can be obtained and these can be used directly or indirectly by employing a fluorochrome, enzyme, ferritin or radiochemical label to localize the antigen. The techniques and uses of immunohistochemistry in this regard have been reviewed by Poole (35). Other characteristics used to identify lysosomes including staining by heavy metal ions and with fluorescent dyes (36) are not always satisfactory as other organelles may also stain (37). Finally, transformations following phagocytosis, can be used to introduce identifiable particles such as colloidal gold, colloidal carbon, thorotrast, iron containing substances, silver, copper, mercury, horse radish peroxidase and zymosan into lysosomes (34).

2) Isolation

Lysosomes have a range of equilibrium densities, slightly higher than, but substantially overlapping those of mitochondria. Thus isolation of normal lysosomes in a pure state is not possible by centrifugation.

Their density can be altered *in vivo* by uptake of substances of low density like Triton WR1339 (38) or high density like Dextran 500 (39), iron (40) and colloidal gold (41). The only viable alternative technique involves differential centrifugation coupled with carrier free continuous electrophoresis (42). Two other methods for isolation have been proposed, but neither has received much acceptance (see 43,44). The heterogeneity of size as well as of density and variation in susceptibility to rupture during homogenization cause a purified lysosomal fraction to be a subpopulation that is not fully representative of the lysosomal system. This point must be considered when studying purified subfractions.

3) Composition

Although the lysosomes of some cell types such as polymorphonuclear leucocytes, platelets and sperm contain specialized enzymes, the lysosomes of most animal cells possess a common but diverse array of enzymes. About sixty-five acid hydrolases are known to be present in the lysosomes. Most have an acidic pH optimum, although some lysosomal enzymes are active at neutral pH (45). In general, the enzyme armoury of lysosomes is sufficient to degrade proteins to amino acids, carbohydrate moieties to monosaccharides, nucleic acids to nucleosides and phosphates, lipids to free fatty acids, and to remove phosphate groups. A comprehensive review of the digestive capacity of lysosomal enzymes is available (46). It should be noted however, that lysosomes, even in a single cell type, are quite variable in enzyme constitution. Smith and Bird (47) have described three major and distinct lysosomal subpopulations in rat heart with a unique complement of hydrolytic enzymes in each. This pronounced diversity may be due, in part, to the heterogeneity of cell types

in the whole heart.

Lipoproteins constitute about half of the protein of lysosomes isolated from rat kidney and liver (37). The phospholipid and cholesterol components of lysosomes have also been analyzed (39). Choline phospholipids and sphingomyelin predominant and the phospholipid to protein ratio is in the order of 0.3. The presence of cholesterol and sphingomyelin is interesting because these materials are virtually absent from other cytomembranes, with the exception of the plasma membrane. Several metallic ions are normally present in lysosomes at concentrations higher than in other organelles (48). Also, molecules like ferritin, which is resistant to lysosomal enzymes can often be observed in lysosomes (24). Lipofuscin is also indigestable by lysosomes and tends to accumulate.

4) Organization

The intralysosomal distribution of materials can be studied by breaking the lysosomes and separating the sedimentable and non-sedimentable material by centrifugation. The membrane is represented by the sedimentable fraction. It has been shown, that although plasma and lysosomal membranes share common features, they are quite distinct and lysosomal membranes possess unique autoantigens (49). Lysosomal membranes also share characteristics with other cytomembranes. For instance, ubiquinone, a mitochondrial component, has also been detected in the lysosomal membrane fraction (50). It has been shown that there is substantial carbohydrate material in the lysosomal membrane, including approximately sixteen micrograms sialic acid per milligram protein (51). Binding studies with lectins (52) suggest that most of the carbohydrate material is on the inner and outer surfaces of lysosomes, whereas there is a concentration of sialic acid on the

inside surface (53). It should be pointed out that information on the intralysosomal distribution of enzymes is still fragmentary. The requirement for sequestration of intralysosomal enzyme is not surprising, for proteinases within the lysosome could easily degrade other hydrolases within the matrix. Certain enzymes have been shown to be membrane bound (54) and some of these hydrolases like an esterase (55), fructose biphosphatase (56), β-acetylglucosaminidase (57), cathepsin M (58) and cathepsin B (58) may be located on the outside of intact lysosomes. The remaining membrane bound enzymes show the normal structure linked latency.

5) Stability and Permeability

Determination of the capacity of various compounds to afford osmotic protection to lysosomes has shown that disaccharides and anions do not penetrate lysosomal membranes. Permeation of compounds decreases with increasing molecular weight and molecules having molecular weights greater than three hundred are usually excluded (50). Lysosomal membranes show highly selective cation permeability. At 4°C, permeability for H^+ is much greater than for other cations such as K^+, which in turn, it is greater than for Na^+ (59). Cation permeability seems to be high at 4°C but much lower at higher temperatures (60). Agents which disrupt lysosomes include several bacterial toxins, steroids and substances with hydrogen bonding capacity (50). Metal ions both labilize and stabilize lysosomal membranes, depending on the ion species (61-72). It is now generally accepted that ATP is necessary for lysosomal membrane integrity (73-77) and cGMP labilizes while cAMP stabilizes these membranes (78).

C. Lysosomal Changes in Heart Disease

1) Myocardial Ischemia and Infarction

In recent years there has been much interest in the role of lysosomal enzymes in cardiac pathology. de Duve and Beaufay (79) observed several years ago that one of the early effects of ischemia in the liver was to reduce the proportion of lysosomal enzymes that could be recovered in the sedimentable fraction of the tissue homogenate. Their evidence and that of others (80-84) led to a general hypothesis that, in the liver, the acidotic and hypoxic conditions that accompany ischemia might lead to i) labilization of lysosomal membranes with the subsequent release of hydrolytic enzymes into the cytosol; ii) activation of the enzymes at the abnormally low pH that develops in ischemic cells; iii) degradation of intracellular molecules that otherwise would not be accessible to the enzymes, and iv) damage to the cell which may be permanent. Thus, through a sequence of lysosomal changes, reversible injury may proceed to irreversible necrosis. Such a hypothesis was viewed as an exciting model for understanding the evolution of irreversible myocardial necrosis during coronary ischemia. If it were true, it would open possibilities for treatment of early coronary occlusion by administration of agents to stabilize lysosomal integrity. Pioneering work by Leighty et al. (85) and Brachfeld and Gemba (86) presented evidence that both experimental asphyxia and ligation of coronary arteries could cause shifts of lysosomal enzyme activities from particulate to supernatant fractions of myocardial homogenates. Brachfeld (87) was responsible for popularizing the concept of a role for lysosomal enzymes during acute ischemia.

The mechanisms by which lysosomal membranes may be ruptured

during ischemia and intracellular acidosis have been studied by several investigators (88-90). The presence of endogenous phospholipases with pH optima in the acidic range have been demonstrated in cardiac tissue. These enzymes are lysosomal in origin and it appears likely that these lipases may contribute in a major way to break down and lysis of lysosomal and other membranes during ischemia. This may lead to a vicious cycle in which lysosomal rupture leads to release and activation of phospholipases which in turn contribute to the rupture of other lysosomes.

Since the pioneering work of Leighty et al (85), several investigators have shown that ischemia produces a redistribution of lysosomal hydrolase activity from the particulate or sedimentable fractions of the tissue homogenate to the supernatant or nonsedimentable fraction (87,91-102). Recently, studies of sequential enzyme redistribution have been performed and it has now been established that redistribution of cathepsin D and other lysosomal enzymes begin within fifteen to thirty minutes of coronary ligation and by forty-five minutes signs of irreversible necrosis appear (91, 102-107). Since the biochemical redistribution of enzyme could occur not only because of enzyme translocation, but also because intact lysosomes become more susceptible to rupture during homogenization, Wildenthal and his colleagues have performed a series of experiments employing an immunofluorescent technique for the localization of lysosomal cathepsin D (91, 102-104). This technique involves the use of a fluoresceinated monospecific antibody to purified cathepsin D, which when applied to tissue sections binds specifically to molecules of cathepsin D (108,109). In normal heart, cathepsin D is confined to discrete lysosomal particles, concentrated in perinuclear areas

(110). By thirty minutes of coronary ligation, cathepsin D containing lysosomes are enlarged in myocytes and in addition there appears to be a "diffusing out" of the enzyme yielding a halo-like effect (110). Redistribution of the enzyme occurs over the next ninety minutes. By two hours after occlusion, few intact lysosomes are visible and, instead, there is a diffuse staining throughout the cell (110). Thus, both biochemical and morphological data seem to indicate that early changes in the lysosomes occur in ischemic myocytes. However, these data do not prove that the lysosomal enzymes play a causal role in the cell damage since their changes might be merely concomitant with the observed necrosis (101). It should be pointed out that lysosomal alterations may occur not only during the development of irreversible damage, but also in the repair process of reversibly injured cells (111).

2) Cardiac Atrophy due to Starvation

Lysosomal enzymes were first shown to play an important role in the accelerated degradation and atrophy of liver tissue during starvation (112-114). Both skeletal muscle and heart also become atrophic during food deprivation; however, in skeletal muscle the activity of lysosomal cathepsin D does not appear to be altered (115). In contrast, studies on mice, rabbits and rats revealed that food deprivation for three days or more was accompanied by significant (20-25%) increases in total cathepsin D activity in the heart (116-118). Parallel changes were not observed in any other lysosomal enzyme studied. The increase in cardiac cathepsin D during starvation was predominantly in the nonsedimentable fraction of the tissue homogenate. To determine whether the observed redistribution of activity was due to increased size and/or fragility of lysosomes or to the existence of the enzyme in the cytosol, Wildenthal (26)

stained rabbit heart with fluoresceinated anticathepsin D and observed an increase in diffuse staining of the cytosol after starvation. This finding was interpreted as evidence for either increased enzyme in small primary lysosomes or free enzyme in the cytoplasm. In a recent communication it was conclusively shown that free cathepsin D was present in the cytosol (119). Since hearts of starved animals have a diminished capacity for proteolysis rather than an enhanced degradation of proteins (120, 121), the consistent changes observed in cathepsin D activity suggests this enzyme may not play a role in starvation mediated proteolytic capacity.

3) Cardiac hypertrophy and failure

The earliest studies on lysosomal alterations in hypertrophied cardiac tissue were performed by Kottmeier and Wheat in 1967 (22). These researchers investigated the ultrastructural changes that accompany the production of atrial septal defects in dogs. This study involves a volume overload on the right ventricle which induces right ventricular hypertrophy. Although enzyme activities were not measured, these researchers noted both increased size and number of lysosomes. Schneider et al (123) induced hypertrophy in rabbits by creating a volume overload on the left ventricle. They found a 30-60% elevation in the activities of total cardiac DNAase, RNAase and acid phosphatase with no change in the ratio of sedimentable to supernatant activities. Tolnai and Beznek (124) obtained similar results in rats subjected to aortic constriction; these researchers observed twenty to seventy percent increases in the total activities of acid phosphatase, β-acetylglucosaminidase and β-glucoronidase. Chronic exposure to atmospheric hypoxia, a potent stimulus for pulmonary hypertension and right ventricular hypertrophy, has been

reported by Meerson and his colleagues (125) to cause an increase in the left ventricular activities of DNAase, RNAase and acid phosphatase, especially in the particulate fraction. When Meerson and his colleagues (125) measured responses one day after aortic constriction, they observed significant decreases in the cardiac activities of the same enzymes in contrast to the increases demonstrated by Tolnai and Beznek (124). In a study by Martin et al (126) in which hypertrophy was induced by aortic constriction, no changes were observed in RNAase or cathepsin D after 12 days. Stoner et al (127) also observed no change in the activities of acid phosphatase, RNAase or cathepsin D in dog hearts after production of pulmonic stenosis and subsequent right ventricular hypertrophy.

The observations with cardiac hypertrophy seem confused mainly because the degree of hypertrophy has not been reported and it is difficult to differentiate lysosomal alterations that might be a specific accompaniment of the hypertrophic process from nonspecific responses to stress or injury. Furthermore, no attention has been made to distinguish non-failing hypertrophied hearts from failing hypertrophied hearts as well as physiologic versus pathologic hypertrophy. Interpretations are made even more difficult by the inability to distinguish intrinsic lysosomal changes in hypertrophied myocytes from alterations in nonmuscle cells.

4) Catecholamine-induced Cardiomyopathy

Large doses of isoproterenol have been shown to induce rapid cardiac hypertrophy in rats in addition to producing heart cell necrosis (128). Meerson et al (125) demonstrated a decrease in the activities of DNAase, RNAase and acid phosphatase 24 hours after subcutaneous administration of isoproterenol. Mueller et al (129)

reported similar decreases in β-acetylglucosaminidase and acid phosphatase activity 24 hours after isoproterenol injection. Results from this laboratory (Roman, Kutryk, Beamish and Dhalla, unpublished data) show increases in β-acetylglucosaminidase, β-galactosidase, acid phosphatase and α-mannosidase at 24 hours of isoproterenol induced cardiomyopathy. For all enzymes studied, except β-acetylglucosaminidase, these changes become apparent at 9 hours after the administration of isoproterenol. It is interesting to note that both Mueller et al (129) and Wexler and Judd (130) have reported increases in lysosomal enzymes at times greater than 24 hours. This discrepancy may be a result of differences in the degree of hypertrophy or cell injury produced by catecholamines.

5) Aging Myocardium

There are many reports in the literature which show increased levels of lysosomal enzyme activities in tissues (other than heart) of aged animals and man (131-135). However, few reports have been concerned with alterations in cardiac lysosomal activities with aging (136-139). Results of these studies indicate that major lysosomal enzymes undergo heterogenous changes with age, which resulted to be significant well before senescence (137). Hearts of aged animals also showed significant redistribution of some enzyme activities. Cathepsin D, for example, resulted to be recovered to a much greater extent in the nonsedimentable fraction of the tissue homogenate in older animals (137). However, immunofluorescence studies failed to reveal significant extra-lysosomal diffuse staining for cathepsin D. This suggests that the shift in enzyme distribution results from increased rupture of cathepsin D containing organelles during homogenization rather than

from translocation of enzyme outside lysosomes *in vivo*. These results emphasize the critical importance of specifying the age of the experimental animals and using age matched controls in all studies of cardiac lysosomes and lysosomal enzymes.

6) Diabetic Cardiomyopathy

It is well known that diabetes mellitus may be associated with cardiomegaly, left ventricular dysfunction and congestive heart failure in the absence of a significant degree of coronary artery disease (140-142). Decreased lysosomal enzyme activity has been reported previously in other tissues of diabetic rats (143-145) but only very recently has the involvement of lysosomal hydrolases been implicated in the genesis of pathologic changes observed in cardiac muscle cells. Comprehensive studies involving a good proportion of the lysosomal enzymes identified to date, indicate a consistent and progressive decrease in enzyme activities in the genetically diabetic mouse (146), alloxan diabetic rat (147), and streptozotocin diabetic rats (Kutryk, Pierce and Dhalla, unpublished data). The decrease in enzyme activity shows a rebound towards normal levels after sixteen weeks in streptozotocin induced diabetic animals (Kutryk *et al*, unpublished data) and twenty-two weeks in genetically diabetic mice (146). This rebound seems to correspond in time with the period in which myocytes exhibit necrosis identical with ischemic injury (146).

D. The Calcium Paradox

The calcium paradox phenomenon was first described by Zimmerman and Hulsmann in 1966 (148). Induction of the calcium paradox involves perfusion *in vitro* of an isolated beating heart with calcium free medium followed by reperfusion with the normal calcium containing

perfusate. Upon perfusion with calcium free medium, Zimmerman and Hulsmann (148) observed a rapid electromechanical dissociation of the electrical and contractile activities; the electrical activity of the heart was maintained while all contractions ceased. In addition, during the calcium free period, the heart cells retained myocardial proteins and the heart maintained its red color. When the normal perfusate was reintroduced after more than two minutes of calcium free perfusion, a series of changes occurred. The hearts became irreversibly contracted, electrical activity disappeared and the hearts became pale and mottled. This paradoxical response of the heart to the reintroduction of calcium became known as the "calcium paradox".

In a subsequent report, Zimmerman and his colleagues (149) maintained that no structural alterations were evident at the light microscope level after up to one hour of calcium free perfusion. Using the electron microscope, only a dilated transverse tubular system could be observed. Since these initial observations, more rigorous examinations of ultrastructural changes occurring during the calcium paradox have been performed. Various studies have revealed separation of the intercalated discs after 10-50 minutes of calcium free perfusion (150-153) and separation of the basal lamina from the sarcolemma after 20 to 40 minutes of calcium free perfusion (153-156). Calcium free exposure for 60 minutes has also been shown to disrupt myofibrillar integrity (157) and as little as five minutes of calcium free perfusion resulted in separation of the glycocalyx, a decrease in intramembrane particles, alterations in the middle lamina, intercalated disc and golgi apparatus as well as loss of heterogenic staining properties of the nucleoplasm

(158-159). Despite the controversy surrounding the sequence of morphological changes accompanying the calcium free period, the structural changes in reperfused hearts first described by Zimmerman et al (149) are generally accepted (150, 160-162). Upon reintroduction of calcium, mitochondria become spherical and swollen and the normal organization of the contractile apparatus is lost. Intercalated discs become unrecognizable and electron dense bodies are deposited in the mitochondria. These morphological alterations are usually explained on the basis that reperfusion results in an excessive influx of calcium.

Since calcium overload and deficiency are believed to be involved in the genesis of contractile failure and cardiac cell death in many cardiomyopathies (162-171), Yates and Dhalla (150) suggested that the isolated heart, perfused with calcium free medium forms an interesting model for studying the pathogenesis of intracellular calcium deficiency, while reperfusion of calcium deprived hearts provides a model for the study of intracellular calcium overload. This contention was later substantiated when it was demonstrated that calcium free perfusion does indeed deplete myocardial calcium contents (172,173) while reperfusion results in calcium overloading (173). The development of this experimental model which mimics abnormalities in myocardial calcium distribution in diseased hearts was critical in evaluating the existing hypothesis that irrespective of the pathological stimulus, one or more myocardial membrane system change in such a way that there occurs either an intracellular calcium deficiency or intracellular calcium overload (164,165). Although insufficient information regarding intracellular Ca^{2+} deficiency is available in the literature, it should be noted that increases in the concentration of intracellular calcium have been directly associated

with ultrastructural damage, and irreversible failure (164,169,174, 175). The mechanisms by which calcium overload can produce myocardial cell damage have not yet been fully elucidated, but several suggestions have been proposed. Increased calcium concentrations in the myocardium may initiate necrosis by depleting cellular high energy stores (165). Since the calcium uptake process of cardiac mitochondria may take precedence over their oxidative phosphorylation capabilities (176), increased mitochondrial calcium has been shown to depress respiratory activity and decrease cellular ATP levels (176, 177-181). Over stimulation of myofibrillar Ca^{2+}-ATPase due to intracellular calcium overload may also depress myocardial energy stores (182). It has also been postulated that elevated cellular calcium concentrations may stimulate the activation of several hydrolytic enzymes which may contribute to membrane defects. It is known that calcium increases protein turnover in muscle (183) and activates various proteases (184), and phospholipases (185). Increased levels of calcium have also been shown to stimulate lysosomal enzyme release from polymorphonuclear leukocytes (186-189). Thus it seems likely that marked alterations in lysosomal enzyme may become evident during the development of Ca^{2+}-paradox in the myocardium.

Upon exposure of the isolated perfused rat heart to calcium free medium, contractile force falls rapidly and cessation of mechanical activity occurs within the first thirty seconds (148,150,162,190,191). This loss of contractile function occurs without any alteration in the surface electrical activity of the heart (148,150,151). The observed loss of contractility may be due to decreased calcium entry, which may cause electromechanical dissociation (192-199) as well as alter intracellular calcium release (200-204). The dissociation of

electrical and mechanical events has been suggested to be due to the removal of a labile calcium component within the first thirty seconds of calcium free perfusion (205). During the loss of this calcium (approximately 32% of total cellular calcium), no structural alterations were apparent. A more stable compartment, representing 20% of myocardial calcium was depleted only after 10 minutes of calcium free exposure. These authors (205) reported that at this point, ultrastructural damage first appears, but as described earlier, ultrastructural alterations have been observed as early as five minutes after the introduction of calcium free perfusate (158, 159). Whether brief periods of calcium free perfusion (less than 5 minutes) affect the myocardium is still a contentious issue. Some investigators report no change (151) while others suggest increased sarcolemmal permeability occurs after short periods of calcium free perfusion (161,173,206,207). Since calcium has been shown to be involved in nonionic binding with carbohydrates (208) which can be envisioned to contribute to the structural stability of the membrane, removal of this labile calcium by calcium free perfusion may alter membrane function with respect to calcium movements.

In order for reperfusion to induce the calcium paradox phenomenon, the calcium free period must last for between 2 to 40 minutes depending on the animal species. In isolated perfused rat heart, reperfusion with calcium after 2 to 5 minutes of calcium free exposure results in paradoxical calcium necrosis and intracellular calcium overload (148, 150,173,210,211). Longer periods (10 to 20 minutes) of calcium free perfusion are required to demonstrate the calcium paradox in rabbit intraventricular septum (161,212) or in rabbit hearts (190). In rat heart, myocardial contracture developed with an elevation of resting

tension by 10 seconds of calcium repletion and reached its peak at around 20 seconds (209). The first detectable change in creatine kinase, protein and phospholipid occurred in the perfusate by 10 seconds, and reached its peak by 30 seconds (209). Reperfusion of calcium deprived hearts results in altered myocardial cation contents, notably intracellular calcium overload, massive enzyme and protein release, irreversible loss of active force generation and electrical activity, myocardial contracture, rapid depletion of high energy phosphate stores and extensive ultrastructural damage. Alterations in sarcolemmal calcium permeability and enzyme function (213) as well as derangements in subcellular calcium regulation (190) are all apparent during the calcium paradox, but the mechanisms of the calcium induced pathophysiologic changes are poorly understood. The present study was undertaken to examine alterations in cardiac lysosomal enzyme activities during the development of calcium paradox in rat heart.

III. METHODS

A. Isolated Heart Preparation

Healthy male Sprague Dawley rats weighing 250-300 grams were used in this study. All the animals were kept in environmentally controlled rooms and maintained on standard rat chow and water ad libitum. The animals were sacrificed by decapitation and their hearts quickly excised and washed in ice-cold, oxygenated Krebs-Henseleit (K-H) solution containing (mM), NaCl, 120; $NaHCO_3$, 25; KCl, 4.8; KH_2PO_4, 1.2; $MgSO_4$, 1.20; $CaCl_2$, 1.25; and glucose, 8.6 (pH 7.4). After removing the atria, extraneous fat and connective tissue, the ventricles were arranged for coronary perfusion according to the procedure of Langendorff as previously described (173,214-216). Equilibration perfusion was carried out for fifteen minutes with K-H medium gassed with 95% O_2 and 5% CO_2 and maintained at 37°C. Each preparation was electrically paced at 280 beats/minute with a Phipps and Bird 611 square wave stimulator (event duration: 1.5 msec, amplitude: 60 volts). The coronary perfusion rate was 7.8 ml/min. At the onset of each experiment a resting tension of two grams was applied to the heart and contractile force (developed tension) monitored on a Grass model 7 polygraph recorder with a force displacement transducer (FT.03). Calcium free perfusion was performed by introducing a calcium free K-H solution. Reperfusion was accomplished using K-H solution. Perfusing medium was collected after passage through the heart and maintained at 4°C.

B. Isolation of Lysosome-rich Subfraction

At the conclusion of each perfusion sequence, the heart was removed from the cannula and placed in a cold high potassium extraction buffer (217) containing (M) sucrose, 0.25; KCl,

0.6; imidazole, 0.01; $MgCl_2$, 0.001; EDTA, 0.001, pH 7.2. Isolation of a lysosome rich subfraction was performed using a modified method of Ruth et al (217) and Wildenthal and Mueller (218) described below. The heart was first crudely minced in 10 volumes of extraction buffer, using scissors, then homogenized using a loose fitting teflon glass homogenizer driven electrically at a medium speed of 12 strokes for 1 minute. An aliquot of the crude homogenate was diluted and homogenized using a Potter glass-glass homogenizer in an equal volume of extraction buffer containing 0.2% Triton X-100, and set aside for measurement of total lysosomal enzyme activity. The remaining homogenate was centrifuged at 350 x g for 10 minutes in a Sorvall RC2-B refrigerated centrifuge. The pellet obtained by this centrifugation represents the cellular debris fraction which is composed of unbroken cells and nuclear material from the ruptured myocytes. In some experiments, the lysosomal enzyme activity was monitored to ensure that no significant amount of the activity appeared in the cellular debris fraction.

No difference was seen between control and experimental groups. The particulate fraction was aspirated and subjected to centrifugation at 40,000 x g for 30 minutes on a Sorvall centrifuge. The supernatant (nonsedimentable fraction) thus obtained represented the (cytosolic fraction) and the pellet (sedimentable fraction) represented unbroken lysosomes, mitochondria and microsomes. The pellet was resuspended in extraction buffer containing 0.1% Triton X-100 and homogenized using a Potter teflon glass homogenizer. Assays for lysosomal enzyme activities were performed on both the nonsedimentable and sedimentable fractions. For determination of lysosomal enzyme activities in the perfusion medium, total perfusate was collected during control perfusion, calcium

free perfusion period, and during reperfusion with normal K-H solution. The perfusate samples from these periods were concentrated at 4°C using Amicon ultrafiltration cells equipped with Diaflo YM 10 ultrafiltration membranes to a final volume not exceeding one ml.

C. Heart Homogenate Studies

For homogenate studies, hearts were prepared for perfusion as described earlier. Control hearts were perfused for 10 minutes with normal K-H solution, removed from the cannula and homogenized in 10 volumes of cold extraction buffer as described earlier. The homogenate was divided into two equal parts, and ethyleneglycol-bis-(β-amino ethyl ether) N, N'-tetra-acetic acid (EGTA) in extraction buffer was added to one portion such that the final concentration of EGTA was 2 mM. To the other portion, a similar volume of extraction buffer was added. These mixtures were immediately put in a shaking water bath at 37^{o}C and incubated for 10 min. In another series of experiments, hearts were perfused with Ca^{2+}-free medium for 10 min and homogenized in 10 volumes of extraction buffer. The homogenate was separated into two equal portions, and to one portion calcium containing extraction buffer was added such that the final concentration of calcium in the homogenate was 2.5 mM. To the other portion a similar volume of extraction buffer was added. The tubes were immediately placed in a 37°C water bath and incubated for 10 minutes. After the incubation period, the tubes were placed on ice and the various subfractions prepared at 4°C for enzyme assay as described earlier.

D. Glycosidase Assays

1) β-acetylglucosaminidase, β-galactosidase and α-mannosidase.

Assays for the three glycosidases (β-acetylglucosaminidase, β-galactosidase and α-mannosidase) were performed using the appropriate glycoside of 4-nitrophenol as the substrate (p-nitrophenyl-N-acetyl-β-D-glucosaminidase, p-nitrophenyl-β-D-galactosidase and p-nitrophenyl-α-D-mannopyranoside) respectively. McIlvane (citric acid-sodium phosphate dibasic) buffer solutions were prepared such that the final pH values were: pH 4.3 for β-acetylglucosaminidase (β-NAG); pH 3.7 for β-galactosidase (β-GAL) and pH 4.6 for α-mannosidase (α-MAN). The appropriate substrate was added such that final concentration was 2.5 mM. These buffered substrate solutions were kept at -20°C for periods up to two weeks. For the assay, each tube contained; 400 µl of the buffered substrate solution, 50 µl 1% Triton X-100 and 25 µl of distilled water. The reaction was started by the addition of 25 µl of enzyme source and placing the tube into a 37°C water bath. After the appropriate incubation times (25 minutes for β-NAG, 120 min for β-GAL and 18 h for α-MAN). The reactions were terminated by adding 1.5 ml of ice cold glycine buffer (0.5 M, pH 10.2) and immediately placing the tubes on ice. The tubes were spun at 250 g for 10 minutes and the amount of the nitrophenol produced was determined colorimetrically using an Eppendorf spectrophotometer and measuring the absorbance at 405 nm. The absorbance values were converted to µmoles nitrophenol produced using the Beer-Lambert Law and a molar extinction coefficient $\varepsilon = 1.77 \times 10^4\ M^{-1}\ cm^{-1}$. Protein concentrations of each fraction were determined using the assay of Lowry et al (219) with modified reagents and columns as described by Miller (220).

2) Acid Phosphatase.

Acid phosphatase activities were determined by a slightly modified method of Barrett and Heath (221). In this method, 0.1 ml of enzyme sample is mixed with 0.2 ml distilled H_2O, and 1.2 ml 0.2 M sodium acetate-acetic acid buffer, pH 5.0. The mixture was incubated for 10 minutes at 37°C to destroy glucose-6-phosphatase. The reaction was initiated by the addition of 0.5 ml of 32 mM p-nitrophenyl phosphate (dicyclohexylammonium salt). After a 10 minute incubation, the reaction was stopped with 2.0 ml of ice-cold tris-phosphate reagent (M Tris-HCl, 0.4 M $K_2H\ PO_4$ pH 8.5). The tubes were spun at 750 g for 10 minutes and the E_{405} determined immediately after. The absorbance readings were converted to µmoles nitrophenol produced using the Beer-Lambert Law and a molar extinction coefficient $\varepsilon = 1.77 \times 10^4\ M^{-1}\ cm^{-1}$.

E. Statistical Analysis

Statistical analysis of the data was carried out using analysis of variance (ANOVA), and examined for significant difference with Duncan's New Multiple Range Post Hoc Test. A significant difference between two groups was detected using the Student's t-test. A P level of 0.05 was employed to determine significant differences.

IV. RESULTS

A. Triton Response Curves

In order to determine the optimum concentration of Triton X-100 for the glycosidase assays, the activities of the four glycosidases were determined in cardiac subfractions using various concentrations of Triton X-100. Using β-acetylglucosaminidase as an example, results from a typical series of experiments are presented in Figure 2 (A & B). Triton X-100 seems to have little effect on the nonsedimentable fraction indicating the paucity of patent lysosomes or membrane bound enzyme in this subfraction. The most dramatic increase in enzyme activity with increasing Triton X-100 concentration occurred in the sedimentable fraction indicating the presence of patent lysosomes in this fraction. The activity in the cellular debris, homogenate and particulate fractions increased to a maximum at 0.1% Triton. With concentrations over 0.1%, the activity decreased slightly. From all curves, it is evident that maximal activity was obtained at a Triton concentration of 0.1%. Similar results were obtained with all of the other enzymes studied. Thus all subsequent enzyme assays and homogenizations were performed in 0.1% Triton X-100.

B. Effect of Calcium Paradox on Lysosomal Enzyme Activity

1) Effect of time of calcium free perfusion on lysosomal enzyme activities.

The experiments in this series were performed in order to determine whether decreased intracellular calcium followed by calcium overload could cause alterations in myocardial lysosomal enzyme activities. Hearts perfused for 3 minutes of calcium free K-H medium followed by 5 minutes of reperfusion with calcium containing K-H solution exhibited 15 to 20% recovery of contractile force on reperfusion (Fig. 3A). There was a moderate increase in resting

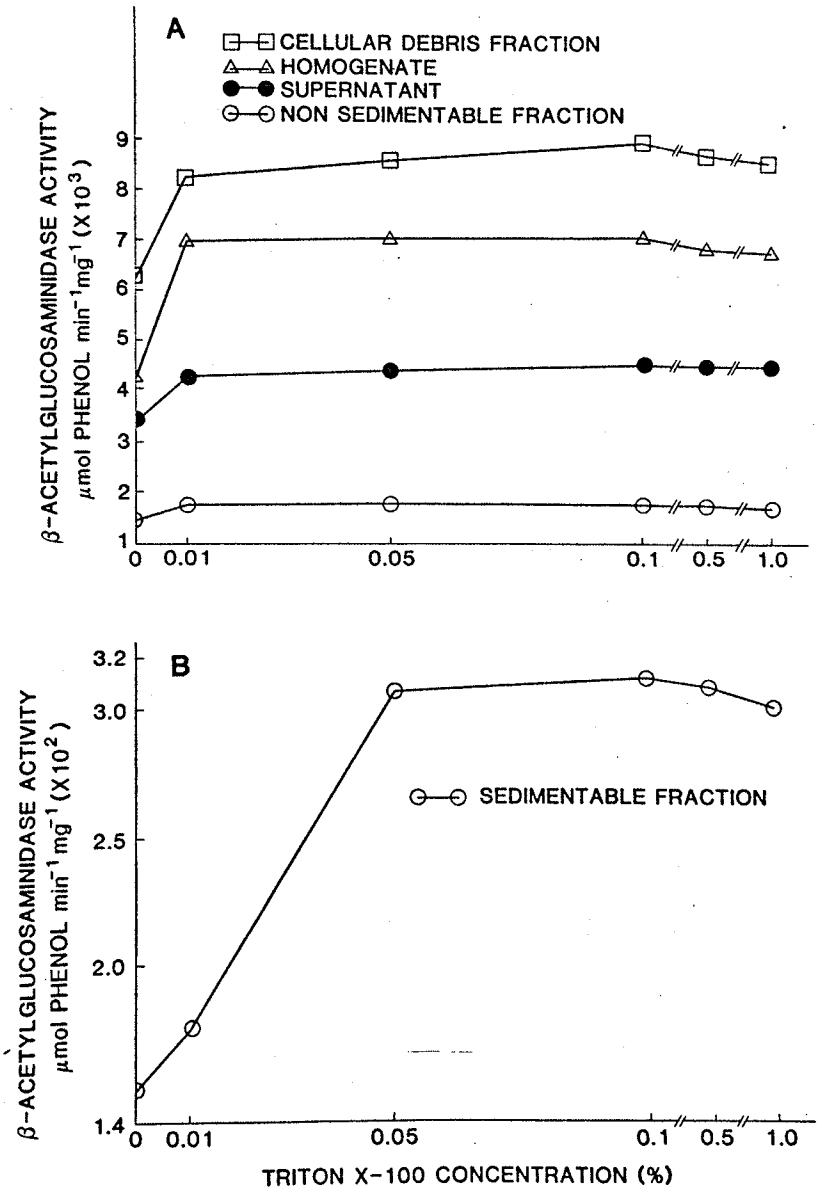

Figure 2. Effect of Triton X-100 on the activity of β-acetylglucosaminidase in various fractions isolated from rat liver. Supernatant fraction here refers to the particulate fraction.

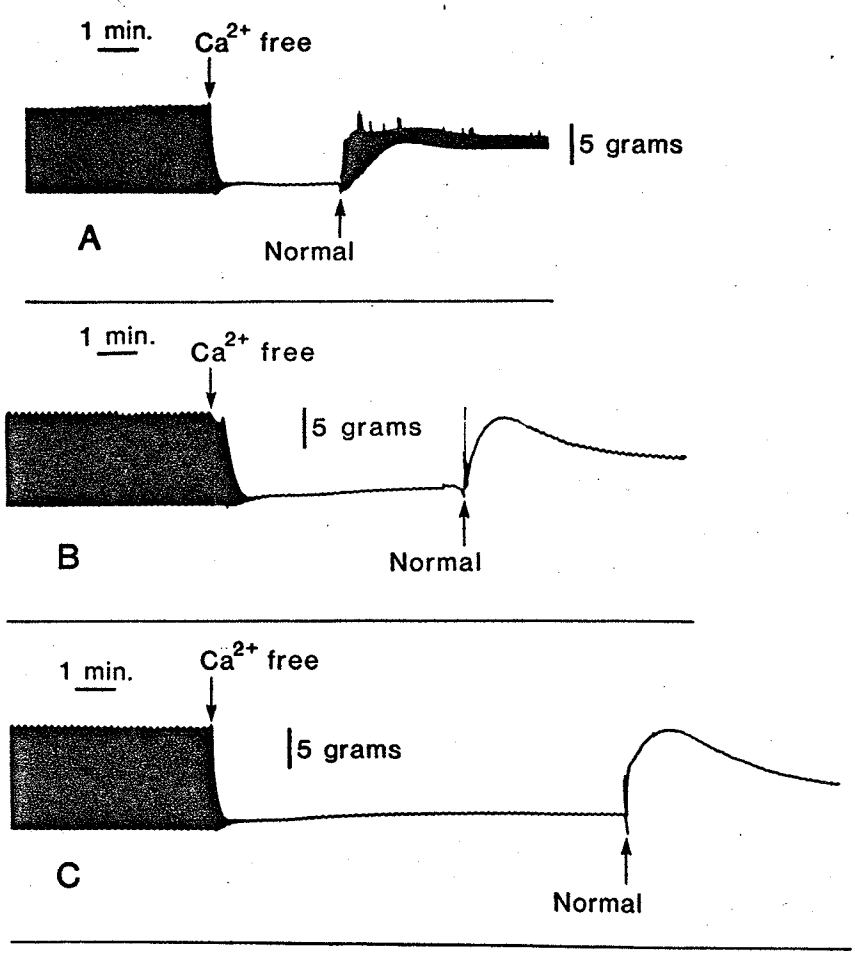

Figure 3. Polygraph tracings obtained from rat hearts subjected to the calcium paradox. Hearts were perfused for various times with calcium free medium. Calcium containing normal medium was reintroduced after the calcium free perfusion. A: 3 minutes calcium free perfusion; B: 5 minutes calcium free perfusion; C: 10 minutes calcium free perfusion.

tension in hearts subjected to 5 minutes of reperfusion. No significant changes were observed in the homogenate, sedimentable or non-sedimentable fraction in either β-acetylglucosaminidase (Fig. 4) or β-galactosidase (Fig. 5). For both enzymes, however, there was a significant increase in the enzyme activity recoverable from the perfusate. For α-mannosidase there was a significant increase in the enzyme activity assayed in the non-sedimentable fraction (Fig. 6). This increase was not associated with a concommitant significant decrease in sedimentable enzyme activity. Although there was an increase in the activity recovered from the perfusate, this increase was not significant. A similar pattern was seen for acid phosphatase activity (Fig. 7); there was a significant increase in the enzyme activity recoverable from the nonsedimentable fraction. This increase did not correspond with any decrease in enzyme activity recoverable from the sedimentable fraction. The enzyme activity recovered from the perfusate did not differ significantly from the activity recovered from the control perfusate. Figure 3B shows a typical polygraph tracing from a rat heart equilibrated with normal K-H buffer, followed by a 5 minute perfusion with calcium free K-H and reperfusion for 5 minutes with normal K-H solution. This tracing demonstrates the typical paradoxical response observed upon calcium repletion in the calcium deficient heart. During the equilibration period the contractile force was strong, the heart beated regularily, and resting tension remained constant. With the introduction of calcium free solution, contractile activity ceased and the resting tension of the heart increased gradually. Upon reperfusion with K-H solution, the resting tension of the heart increased dramatically, approaching 100% of the tension developed during the equilibration period.

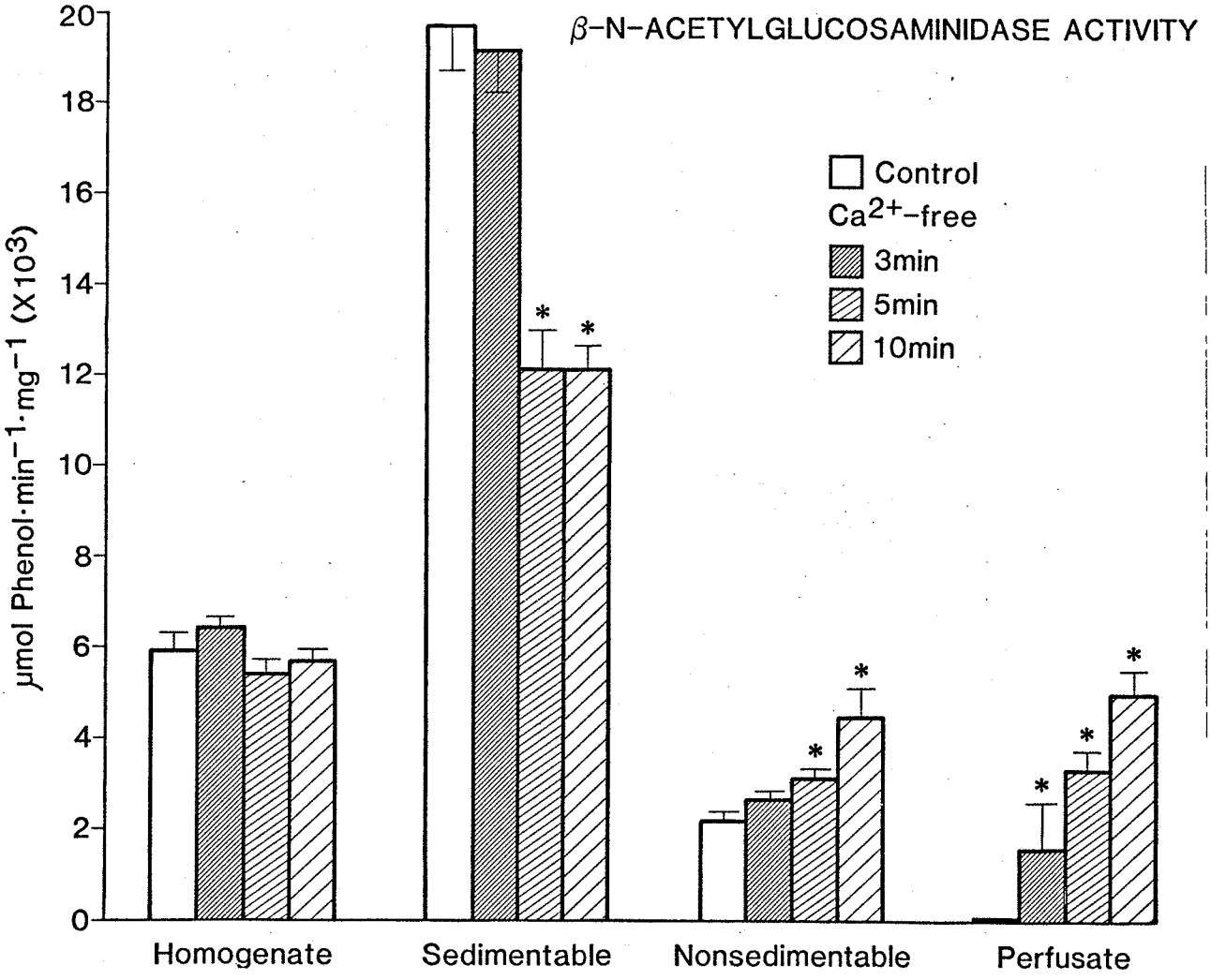

Figure 4. Effect of various times of calcium free perfusion on the distribution of β-acetylglucosaminidase activity in rat hearts subjected to the calcium paradox.by reperfusion with normal medium for 5 minutes. Each value is a mean ± S.E. of 4-6 experiments.

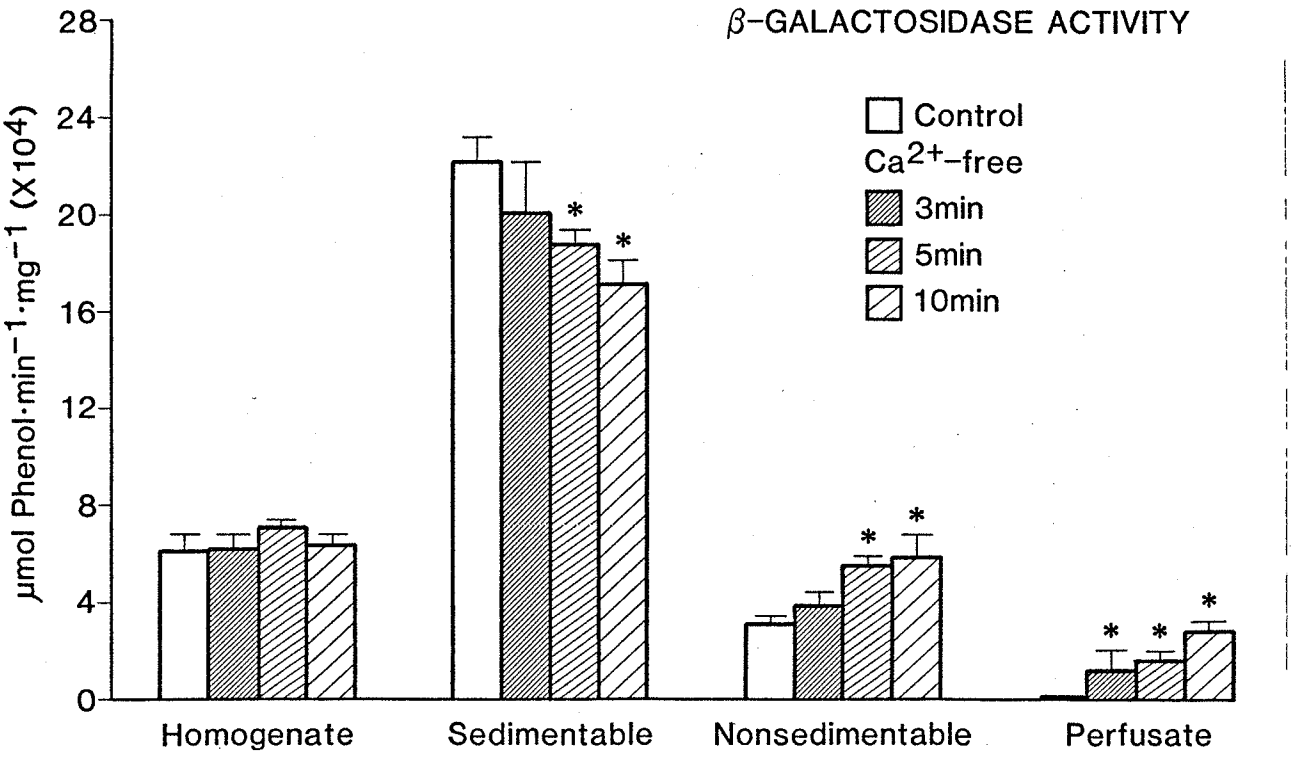

Figure 5. Effect of various times of calcium free perfusion on the distribution of β-galactosidase activity in rat hearts subjected to the calcium paradox by reperfusion with normal medium for 5 minutes. Each value is a mean ± S.E. of 4-6 experiments.

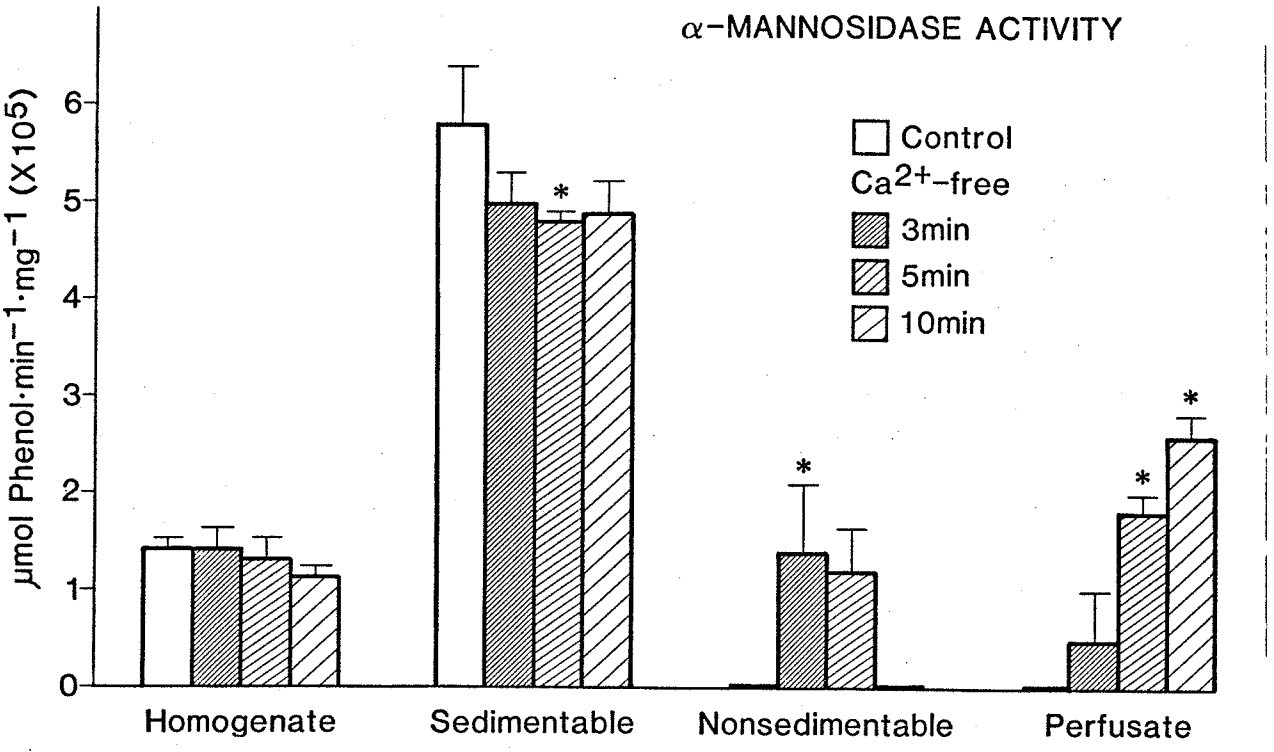

Figure 6. Effect of various times of calcium free perfusion on the distribution of α-mannosidase activity in rat hearts subjected to the calcium paradox by reperfusion with normal medium for 5 minutes. Each value is a mean ± S.E. of 4-6 experiments.

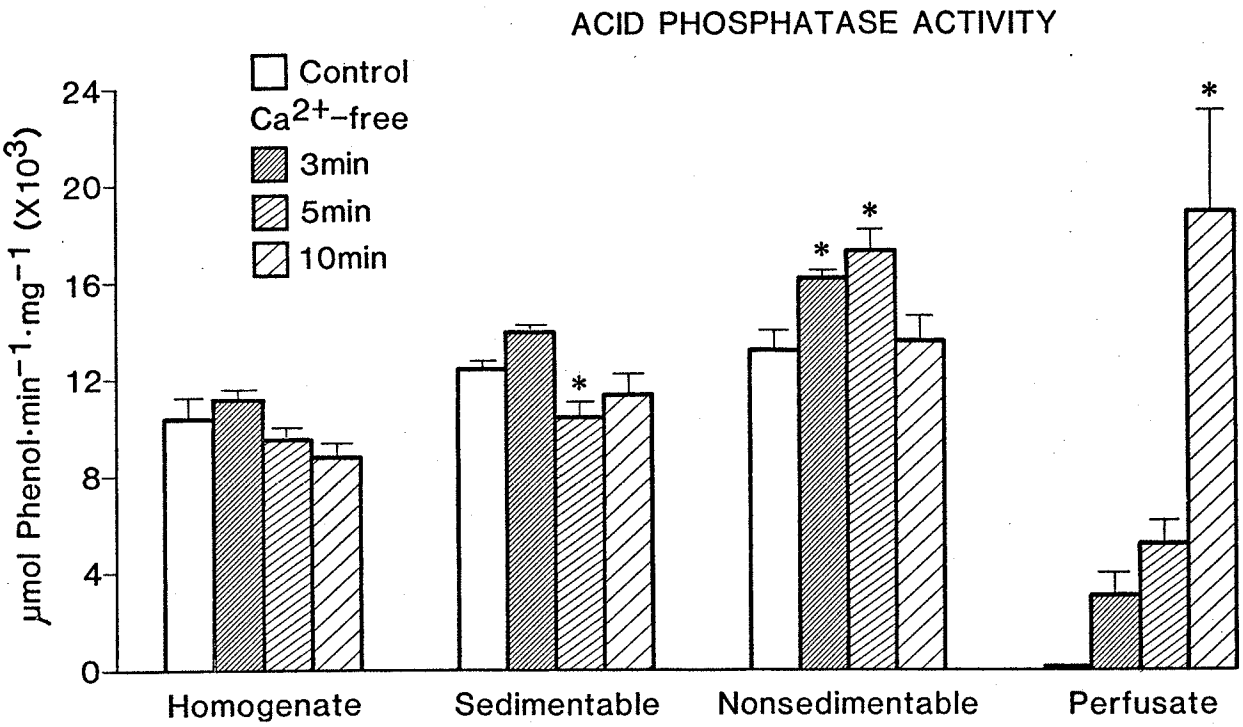

Figure 7. Effect of various times of calcium free perfusion on the distribution of acid phosphatase activity in rat hearts subjected to the calcium paradox by reperfusion with normal medium for 5 minutes. Each value is a mean ± S.E. of 4-6 experiments.

Contractile activity was not recovered, the heart lost its red color and appeared mottled and the perfusate became roseate during reperfusion with K-H solution. The specific activities of several lysosomal acid hydrolases were determined in hearts perfused for 10 minutes with normal K-H solution (control) and compared to specific activities obtained from hearts subjected to the calcium paradox by reperfusing Ca^{2+} deprived hearts with Ca^{2+} containing normal medium for 5 minutes. Figure 4 shows the β-acetylglucosaminidase activity recovered from control hearts and hearts subjected to Ca^{2+}-free perfusion and the calcium paradox. In control hearts, most of the lysosomal enzyme specific activity can be recovered from the sedimentable fraction, while little activity appeared in the nonsedimentable fraction. No activity was recovered from the perfusate collected in the last 5 minutes of tissue perfusion. In the heart subjected to calcium free perfusion for 5 minutes and reperfusion for 5 minutes, no significant changes were observed in the homogenate fraction when compared to control hearts. A significant decrease was observed however, in the amount of β-acetylglucosaminidase activity recoverable from the sedimentable fraction. This was coupled with a significant increase in the enzyme activity assayed in the nonsedimentable fraction. Significant enzyme activity was also detected in the perfusate collected during the reperfusion period. Enzyme activity could not be detected in the perfusate collected during exposure to calcium free medium for any of the enzymes in any experiment. An identical response was seen with β-galactosidase activity (Fig. 5). For α-mannosidase activity (Fig. 6) a similar pattern was evident and although the increase in the enzyme activity in the nonsedimentable fraction was not significant. Likewise for acid phosphatase activity

(Fig. 7), although the increase seen in the perfusate was not statistically significant, there was some activity recovered. Fig. 3C shows a typical polygraph recording obtained from a heart subjected to 10 minutes of calcium free perfusion, followed by reperfusion for 5 minutes with normal calcium containing K-H medium. Contractile force is not recovered during reperfusion and the resting tension approached one hundred per cent of the tension developed during the equilibration period.

Both β-acetylglucosaminidase (Fig. 4) and β-galactosidase (Fig. 5) showed a decrease in the activity recoverable from the sedimentable fraction with a corresponding increase in the activity recoverable from the nonsedimentable fraction; for both acid hydrolases significant activity was detectable in the perfusate. No significant alterations in the α-mannosidase (Fig. 6) were apparent except for the presence of activity in the perfusate. A similar pattern was evident for the acid phosphatase activity (Fig. 7).

2) Effect of time reperfusion on lysosomal enzyme activities.

In order to determine whether the time of reperfusion with normal calcium containing K-H buffer could affect the intracellular distribution of lysosomal enzymes, the activities of the various enzymes were measured in hearts subjected to zero, one and two minutes of reperfusion after five minutes of calcium free perfusion. The data from this series of experiments is presented in Tables 1 and 2. For β-acetylglycosaminidase (Table 1A), no significant difference from control in the enzyme activity recovered after 5 minutes of calcium free perfusion was observed in any of the fractions assayed. After one minute of reperfusion however, there was redistribution

Table 1

β-Acetylglucosaminidase and β-galactosidase activities in rat hearts reperfused with Ca^{2+} containing medium for various times following a 5 minute perfusion with Ca^{2+}-free medium.

	Control	5 min Ca^{2+} free	5 min Ca^{2+} free and reperfusion for	
			1 min	2 min
A. β-Acetylglucosaminidase Activity: (μmol phenol mg^{-1} min^{-1}) x 10^3				
Homogenate	5.8 ± 0.4	5.2 ± 0.5	5.4 ± 0.2	6.0 ± 0.3
Sedimentable	19.4 ± 1.0	19.0 ± 1.0	16.1 ± 0.3*	15.5 ± 0.6*
Nonsedimentable	1.9 ± 0.2	1.4 ± 0.1	2.6 ± 0.2*	3.3 ± 0.3*
Perfusate	ND	ND	ND	0.64 ± 0.09*
B. β-Galactosidase Activity: (μmol phenol mg^{-1} min^{-1}) x 10^4				
Homogenate	6.1 ± 0.5	5.2 ± 0.2	6.0 ± 0.3	6.8 ± 0.3
Sedimentable	23.5 ± 0.9	21.7 ± 1.0	19.6 ± 0.4*	17.4 ± 1.0*
Nonsedimentable	3.0 ± 0.3	2.6 ± 0.1	4.0 ± 0.2*	4.0 ± 0.3*
Perfusate	ND	ND	ND	ND

Values represent mean ± S.E. of 4-6 samples. * $P < 0.05$ vs control value. ND = Not Detectable.

Table 2

α-Mannosidase and acid phosphatase activities in rat heart reperfused with Ca^{2+} containing medium for various times following a 5 minute perfusion with Ca^{2+}-free medium.

	Control	5 min Ca^{2+} free	5 min Ca^{2+} free and reperfusion for	
			1 min	2 min
A. α-Mannosidase Activity: (μmol phenol mg^{-1} min^{-1}) x 10^5				
Homogenate	1.4 ± 0.1	1.1 ± 0.2	1.1 ± 0.1	1.1 ± 0.1
Sedimentable	5.8 ± 0.6	5.16 ± 0.7	5.5 ± 0.6	2.2 ± 0.8*
Nonsedimentable	0.14 ± 0.01	0.12 ± 0.03	0.23 ± 0.07	0.85 ± 0.01*
Perfusate	ND	ND	ND	ND
B. Acid Phosphatase Activity: (μmol phenol mg^{-1} min^{-1}) x 10^3				
Homogenate	10.3 ± 0.9	9.4 ± 0.3	8.5 ± 0.5	8.0 ± 1.0
Sedimentable	12.5 ± 0.3	11.9 ± 0.4	9.0 ± 0.1*	10.7 ± 0.6*
Nonsedimentable	13.3 ± 0.7	13.6 ± 0.5	14.4 ± 0.4	15.0 ± 4.0
Perfusate	ND	ND	ND	10.0 ± 5.0*

Values represent mean ± S.E. of 4-6 samples. * $P < 0.05$ vs control values. ND = Not Detectable.

of enzyme activity from the sedimentable fraction to the non-sedimentable fraction. This redistribution of activity became more marked after two minutes of reperfusion, and at this time enzyme activity is recoverable from the perfusate. Similar results were seen for β-galactosidase (Table 1B). Significant redistribution of activity was apparent after one minute of reperfusion and this shift in activity increased after two minutes. α-Mannosidase (Table 2A) exhibited redistribution of enzyme activity only after two minutes of reperfusion. Cardiac acid phosphatase activity decreased in the sedimentable fraction after both one and two minutes of reperfusion with calcium containing K-H solution. These decreases were not associated with increases in the nonsedimentable activity. Enzyme activity is detectable in the perfusate at two minutes of reperfusion.

3) Influence of Ca^{2+} on heart homogenate

In order to determine whether increased intracellular calcium itself was responsible for the redistribution of enzyme activity observed with the calcium paradox, an attempt was made to duplicate these results in vitro. Tables 3 and 4 show results from these studies. For all four enzymes studied, there appeared to be no significant increase or decrease in enzyme activity in any of the fractions assayed.

Table 3

Lysosomal acid hydrolase activity in rat hearts perfused with Ca^{2+} containing medium followed by addition of extraction buffer or EGTA containing extraction buffer *in vitro*.

		Enzyme Activity (μmol phenol mg^{-1} min^{-1})			
		β-NAG Activity ($\times 10^3$)	β-GAL Activity ($\times 10^4$)	α-MAN Activity ($\times 10^5$)	A.Ph Activity ($\times 10^3$)
Homogenate	Control	5.6 ± 0.4	5.8 ± 0.2	1.0 ± 0.1	8.2 ± 0.1
	2 mM EGTA	5.8 ± 0.6	6.2 ± 0.1	1.3 ± 0.4	8.3 ± 0.1
Sedimentable	Control	16.7 ± 2.0	21.6 ± 2.0	4.2 ± 1.0	9.8 ± 0.5
	2 mM EGTA	15.4 ± 1.0	20.7 ± 1.0	3.2 ± 0.3	8.8 ± 0.7
Nonsedimentable	Control	1.9 ± 0.3	2.8 ± 0.3	0.64 ± 0.07	9.5 ± 0.6
	2 mM EGTA	2.5 ± 0.4	3.0 ± 0.2	0.70 ± 0.01	9.5 ± 0.6

Values represent mean ± S.E. of 4-6 samples. * $P < 0.05$ vs control value. (β-NAG = β-N-acetylglucosaminidase, β-GAL = β-galactosidase, α-MAN = α-mannosidase, A.Ph = acid phosphatase).

Table 4

Lysosomal acid hydrolase activity in rat hearts perfused with Ca^{2+} containing medium followed by a ten minute perfusion with Ca^{2+}-free medium and addition of extraction buffer or calcium containing extraction buffer *in vitro*.

		Enzyme Activity (μmol phenol mg^{-1} min^{-1})			
		β-NAG Activity ($\times 10^3$)	β-GAL Activity ($\times 10^4$)	α-MAN Activity ($\times 10^5$)	A.Ph Activity ($\times 10^3$)
Homogenate	Control	5.2 ± 0.5	5.5 ± 0.1	1.3 ± 0.3	7.9 ± 0.1
	2.5 mM Ca^{2+}	5.3 ± 0.7	5.8 ± 0.4	1.2 ± 0.2	8.9 ± 0.5
Sedimentable	Control	12.0 ± 1.0	14.0 ± 0.8	3.9 ± 0.9	8.4 ± 0.1
	2.5 mM Ca^{2+}	12.0 ± 1.0	13.6 ± 0.8	3.5 ± 0.1	8.3 ± 0.5
Nonsedimentable	Control	1.7 ± 0.1	2.1 ± 0.4	0.51 ± 0.04	10.1 ± 0.1
	2.5 mM Ca^{2+}	1.9 ± 0.1	2.6 ± 0.1	0.68 ± 0.20	10.6 ± 0.2

Values represent mean ± S.E. of 4-6 samples. * $P < 0.05$ vs control value. (β-NAG = β-N-acetylglucosaminidase, β-GAL = β-galactosidase, α-MAN = α-mannosidase, A.Ph = acid phosphatase).

V. DISCUSSION

Induction of the calcium paradox in rat heart resulted in increases in nonsedimentable acid hydrolase activity with a concommitant decrease in the activity recoverable from the sedimentable fraction for β-acetylglucosaminidase, β-galactosidase, α-mannosidase and acid phosphatase. For β-acetylglucosaminidase and β-galactosidase, this redistribution of enzyme activity increased with increasing time of calcium free perfusion. For α-mannosidase and acid phosphatase, a similar trend was not obvious. Thus it appears that β-acetylglucosaminidase and β-galactosidase responses were different from those obtained with acid phosphatase and α-mannosidase. In this regard it should be noted that a marked disparity in the pattern of distribution of β-acetylglusominidase, β-galactosidase, α-mannosidase and acid phosphatase (222, 223) has been shown in rat liver. Framsen et al (88) and Smith and Bird (224) have shown that heart also contains several distinct populations of lysosomes, corresponding in part to different cell types (myocytes and interstitial cells). Topping and Travis (225) have reported that there are at least two morphologically and enzymatically distinct populations of lysosomes in the myocytes themselves. Thus the heterogeneity of cardiac lysosomes may explain the unique responses of the various lysosomal hydrolases to the Ca^{2+}-paradox phenomenon.

When the perfusate was assayed for the presence of lysosomal enzyme activity, no activity was recoverable from the perfusate at 3, 5, or 10 minutes of calcium free perfusion. This suggests that during calcium free perfusion, the sarcolemma maintains its structural integrity and the enzymes were unable to leak out. The possibility of an intracellular redistribution of the lysosomal activity

was ruled out because 5 minutes of calcium free perfusion did not induce any alterations in enzyme distribution. On the other hand, upon reperfusion with calcium containing medium, enzyme activity increased in the nonsedimentable fraction with a concomitant decrease in the activity recoverable from the sedimentable fraction. With increasing times of reperfusion, this redistribution became more prominent. It is possible that the observed redistribution of activity may simply be a result of enzymes being released during homogenization from lysosomes which are made more fragile by the calcium paradox process. However, it is pointed out that the presence of enzyme activity in the perfusate upon reperfusion suggests that enzyme leakage from lysosomes also occurred while the heart was still being perfused before homogenization. The increases in enzyme activities in the perfusate, which parallel increases in the activities of the nonsedimentable fraction, indicate that lysosomal enzyme leakage may reflect alteration in the sarcolemmal integrity in Ca^{2+}-paradoxic hearts. Leakage of other intracellular enzymes has also been reported to occur in Ca^{2+}-deprived hearts upon perfusion with medium containing Ca^{2+} (155).

It is known that myocardial calcium contents decrease during the calcium free perfusion but the myocardium is faced with calcium overload during reperfusion (173). This suggested that calcium may be involved directly as a mediator in the labilization of lysosomal membranes. Neither a calcium concentration of 2.5 mM nor addition of EGTA in heart homogenates caused any release of acid hydrolases from intact lysosomes. This indicates that the observed changes in lysosomal enzyme activities in the Ca^{2+} paradoxic hearts may not be directly dependent upon the massive increase in the intracellular

concentration of Ca^{2+} (173). However, the possibility of localized changes in Ca^{2+} concentrations in causing lysosome rupture and enzyme release cannot be ruled out. Furthermore, intracellular calcium overload occurring under Ca^{2+}-paradoxic conditions has been shown to be associated with dramatic alterations in other electrolytes such as Na^{+} and K^{+} (173). Thus it is probable that intracellular Ca^{2+} overload may indirectly affect the lysosomal activities in the myocardium. The redistribution of lysosomal enzymes seem to occur quite rapidly during the occurrence of Ca^{2+} paradox as the changes were apparent within 1 minute of perfusing the Ca^{2+}-deprived hearts with normal medium. In addition the extent of changes in lysosomal enzyme activities was dependent upon the severity of the Ca^{2+}-paradoxic process as seen by perfusing the hearts for 3 to 10 min with Ca^{2+}-free medium before starting the reperfusion. Such responses indicate that changes in lysosomal redistribution may play some role in the development of cell damage which has been shown to occur in Ca^{2+} paradoxic conditions (214). This view is consistant with the presence of lysosomal alterations in different cardiomyopathies showing ultrastructural abnormalities.

VI. REFERENCES

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