# BIOCHEMICAL, MORPHOLOGICAL AND FUNCTIONAL ALTERATIONS IN ACUTE MYOCARDIAL ISCHAEMIA

by

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## ABSTRACT

Changes in biochemical, functional and morphological indices of myocardial cell injury have been observed after short periods of ischaemia produced by coronary occlusion. These alterations are generally reversible if blood flow is re-instated within approximately 30 min post-ligation; however, reperfusion after this time cannot reverse the damage and actually exacerbates the existing injury. These studies have investigated ischaemia/reperfusion injury, assessing cell damage using several different techniques in order to avoid the inherent bias of any single method. The experiments concerned the molecular changes that occur during the transition from reversible to irreversible damage and their modification by a variety of drugs, especially the calcium channel-blocker, D-600 and the β-adrenergic antagonist, propranolol. Ischaemic or ischaemic/reperfused tissue was obtained by ligation (with or without release) of the left circumflex coronary artery of the open-chest, anaesthetized rabbit. This area was isolated and samples removed for the various biochemical, chemical and ultrastructural analyses. The functional integrity of mitochondrial (MITO) and sarcolemmal (SL) membranes was assessed using membrane fractions purified by differential and density gradient centrifugation.

Myocardial ischaemia was shown to produce progressive decreases in the activities of the membrane-bound MITO ATPase and SL $Na^+,K^+$-ATPase enzyme as well as a decline in tissue ATP. Interestingly, reperfusion fully restored only the MITO enzyme activity. The enzyme inhibition was not a generalized phenomenon as shown by the lack of effect of ischaemia on another MITO enzyme,

namely cytochrome c oxidase. The irreversible decrease in $Na^+,K^+$-ATPase activity was not accompanied by changes in $K^+$ and $Mg^{2+}$ affinity or alterations to the SL protein or phospholipid profiles although more subtle changes in the lipid or protein environment may have occurred. The specificity of the decrease to the SL membrane was shown by the concomitant increase in the tissue $Na^+/K^+$ ratio in the absence of $Ca^{2+}/Mg^{2+}$ changes.

Results from the 20 min ligation study in which D-600 pretreatment provided significant protection against ischaemia-induced ATP depletion and MITO ATPase inhibition prompted the examination of the efficacy of D-600 pretreatment prior to a 40 min ligation and 60 min reperfusion. Propranolol was also included in this study with the aim of determining the relative importance of decreased energy consumption vs. calcium channel-blockade in attenuating ischaemia/reperfusion injury. Both agents were shown to significantly reduce the biochemical and morphological damage, including $Ca^{2+}$ accumulation, that resulted from reperfusion of irreversibly-damaged tissue, although D-600 was consistently superior. Despite the beneficial effects on MITO and SL integrity, neither drug preserved ATP levels or contractile function. These data indicate that the predominant beneficial effects are probably the result of decreased energy requirements during ischaemia. This hypothesis was further supported by observations from the study in which D-600 was administered just prior to reperfusion. This method of drug administration reduced calcium influx to the same degree as when the drug was given prior to ischaemia. However, this intervention significantly preserved only MITO ATPase activity with no protection of SL function. MITO protection in the absence of preser-

vation of SL integrity would not be expected to provide sustained benefit to the ischaemic/reperfused myocardium. Even the observed protection of SL integrity in the drug-pretreated hearts was not accompanied by an improvement in contractility. Thus, the maintenance of intact permeability barriers does not necessarily result in an improvement of contractile function. Long-term studies would be necessary to determine the ultimate benefit of drug treatment in preventing or reducing the deleterious sequelae of myocardial ischaemia.

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Death and life are looked on as but transformations;
the myriad creation is all of a kind,
there is a kinship through all.

Huai Nan Tzu
2nd Century B.C.

# HISTORICAL OVERVIEW

Descriptions of the various clinical manifestations of coronary heart disease such as chest pain and syncope date from the earliest civilizations. However, until rigorous investigations of internal anatomy and physiology were initiated, the aetiology of this morbid condition could not be understood. Translation of several Egyptian papyri that date from ca. 3000 B.C. to ca. 1500 B.C. has revealed that this culture held some inaccurate and erroneous ideas concerning heart function in health and disease; however, the Egyptians did recognize that the heart was the central organ from which the blood vessels emanated and that these were connected to all parts of the body. In addition, they evaluated abnormalities of the pulse to ascertain the status of the heart - a practice known as "pulse lore".

During the period of Lao-Tse and Confucius (ca. 300 B.C.), the Chinese further advanced knowledge of cardiovascular medicine. However, by 1000 A.D. mysticism and pantheism flourished in Chinese medical practice and this trend, coupled with their poor grasp of anatomy, resulted in a retrogression of scientific thought. In the absence of an understanding of internal mechanisms, the Chinese, like the Egyptians, relied upon a complex pulse-lore to assess the status of the cardiovascular system.

In contrast to these earlier cultures, Greek scholars studied anatomy carefully and it was probably their practice of human and animal dissection that gave them an understanding of cardiovascular physiology that was hitherto unparalleled. However, the post-mortem dissections may also have been the source of some of their mistaken ideas concerning the mechanisms of heart function and blood circulation. These errors were perpetuated for centuries by uncritical scholars who regarded the Greek treatises as fact.

Nevertheless, the development of a rigorous and rational approach to the study of cardiovascular system in health and disease provided a framework within which later investigators could evaluate the validity of the Greek findings.

Probably the earliest significant contribution during the Greek civilization came from Alcmaeon of Crotona (ca. 500 B.C.), who practised anatomic dissection. He proposed that the brain was the seat of consciousness, thereby contradicting the commonly-held belief that the heart fulfilled this role. The later Hippocratic treatises (460-370 B.C.), a collection of manuscripts that were written mostly by experienced physicians, also propounded this view. In addition, this influential set of documents expanded the knowledge of external, and to a lesser extent, internal anatomy - including the heart. Interestingly, the Hippocratic doctrine maintained that the heart was not susceptible to disease owing to its "massive and compact" composition.

The work of Aristotle (384-332 B.C.) also had great influence and although he made some valuable contributions to anatomical knowlege, he made no attempt to investigate the aetiology of myocardial disease. His beliefs were influenced to a large extent by the Hippocratic treatises including the concept that the heart was the strongest of all organs and was therefore the least susceptible to disease. In addition, Aristotle had no concept of the action of the heart as a pump and he put forward the following theory of how the heart functions: the heart was the innate source of heat (heat distinguished life from death) and this heat liquified the blood and expanded it. This accounted for the movement of the chambers of the heart. Respiration not only nourished the vital spirit but also quenched the excess heat so that breathing was also associated with the passive movement of the heart

because the cooling reduced blood volume. Oddly, he believed that the heart, not the brain, was the seat of conciousness, reviving an idea that had long since been rejected. Given the above proposal for the mechanism of heart action, this heart-centred view was, in some sense, a logical necessity.

Many of Aristotle's ideas were refuted during the Alexandrian period (ca. 331 B.C.). During this period, human dissection was permitted by the authorities and, as a result of these dissections, the Alexandrian scholar Eristratus made the suggestion that the heart functioned as a pump. Unfortunately, the empty arteries of the post-mortem subjects led him to conclude that only the veins contained blood and that the arteries were filled with "pneuma" (vital spirit) - a life-giving substance derived from the atmosphere.

Subsequently, Ptolemy Physkon (146-117 B.C.) banned human dissection and expelled the physicians from Alexandria. This eradication of a centralized, scholarly institution fragmented the scientific community, and in the period that followed, the pneuma theory became more complex and entrenched and pulse-lore, not anatomic observation, become the more common medical practice. Therefore, in the midst of this scientific regression, it is not surprising that the scholarly work of Claudius Galen (131-201 A.D.) had a great impact not only on Greek medicine but on medicine in the centuries that followed. He refuted the existing theories of pneuma-filled arteries using experimental and rational investigation and showed that they contained blood. (However, he still incorporated pneuma into his concept of blood flow). Given that the understanding of normal cardiovascular physiology was sketchy, his ideas regarding heart pathology were not well-developed. Like Aristotle, Galen felt that the heart was the source of innate heat, therefore, he concluded that death must in some way be due to heart failure,

although he did not specifically investigate the nature of this failure. He had a superior knowledge of anatomy, yet his physiological interpretations were often wrong. The idea of the circulation of the blood eluded him and his own theory of blood flow forced him to postulate the existence of "invisible pores" that connected the left and right sides of the heart. In retrospect, it seems difficult to imagine that some of his erronous ideas were upheld until the 16th and 17th centuries. However, his apparently rigorous investigations spawned a workable model of cardiovascular function, albeit one that contained many inconsistencies. His theories were regarded as fact and thus later scholars did not feel that it was necessary to investigate the validity of his claims. A comment attributed to a contemporary of William Harvey reveals how much influence Galen had on medical thought until the 17th century. Somewhat facetiously, Jean Riolan wrote that "if dissections and observations differed from those of Galen, then nature must have changed".

The inevitable rejection of Galen's view of the cardiovascular system was initiated by the careful anatomical studies of Leonardo da Vinci (1452-1519) and Vesalius (1514-1564). By uncovering many of the errors of Galen's work, their findings laid the groundwork for the subsequent elucidation of the circulation of the blood by William Harvey in 1649. Using experimental and scientific method, Harvey not only demonstrated the continuous circulation of the blood - refuting the theory of the permeable intraventricular septum - but he also described a "third, extremely short circulation", i.e., the coronary circulation. A new understanding of the normal anatomy and physiology of the heart provided a framework within which abnormal conditions could then be identified and studied. Within 50 years of Harvey's discoveries, Lorenzo Bellini (1643-1704) documented calcification

of the coronary arteries and in 1698, Pierre Chirac produced cardiac standstill in the dog after performing an experimental coronary ligation. A textbook published in 1703 by Jean Baptist de Senac, served as a comprehensive reference in that it correlated case histories with post-mortem pathologic findings in the heart.

By the 18th century, the existence of coronary calcification was common knowledge; however, the connection between these post-mortem observations and the clinical signs of disease as well as an understanding of the genesis of the calcification were still to be discovered.

The classic description of anginal chest pain and its relation to the heart was first expressed by William Heberden in 1768, although he did not associate "pectoris dolor", later named angina, with any specific morbid pathology of the heart. This association was made by two of his contemporaries, C. H. Parry and W. Jenner, who, using careful bedside and post-mortem investigation, initiated the idea that angina was in some way related to the "ossification" of the coronary arteries. In addition, realizing that the pain was related to exertion, Parry suggested that the diseased heart was only capable of sustaining moderate workloads. In 1809, Allan Burns of Glasgow further refined this concept of an altered supply/demand ratio in angina with his elegant experiments in which he ligated an exercising limb and noted that pain was evoked when the muscle was fatigued. Extrapolating these results to cardiac muscle, he therefore concluded that pain was the result of insufficient blood supply to the heart under conditions of stress. Burns wrote the following to describe his findings:

> "If, however, we call into vigourous action, a limb round which we have with a moderate degree of tightness applied a ligature, we find then the member can only support its action for a very short time; for

> now its supply of energy and its expenditure do not balance each other: consequently, it soon from a deficiency of nervous influence and arterial blood fails and sinks into a state of quiescence. A heart, the coronary vessels of which are cartilaginous or ossified is in nearly a similar condition; it can like the limb, be girt with a moderately light ligature, discharge its functions so long as its action is moderate and equal. Increase, however, the action of the whole body and along with the rest, that of the heart, and you will soon see exemplified the truth of what has been said; with this difference, that as there is no interruption to the action of the cardial nerves, the heart will be able to hold out a little longer"[1]

Despite the pathological evidence of coronary occlusive disease in some patients with angina, the prevailing view was that the symptoms arose from a "nervous affliction" of the heart, possibly spasm. However, the concept that an occlusive disease (e.g. calcification) of the coronary arteries was responsible for the patients' symptoms was not universally accepted because careful post-mortem examination of the hearts of patients who had had symptoms of "pectoris dolor" did not always reveal coronary "ossification". These inconsistent pathological findings led many 19th century scientists including John Warren (1753-1815), his son, John Collins Warren (1778-1856), and Sir John Forbes (1787-1861) to vociferously criticize the coronary calcification theory of pain. As late as 1854, William Stokes wrote that "angina is a symptom of a weakened heart; the obstruction of the coronary arteries was a disease whose actions are remote and whose existence unnecessary". The alternative hypothesis, which was supported by most critics, maintained that vasospasm, not mechanical obstruction, was the cause of the chest pain. In 1867, Sir Thomas Lauder Brunton published an article in the Lancet describing the efficacy of the vasodilator, amyl nitrite, in relieving anginal pain. He ascribed the benefits of drug administration to the ability of the nitrites to relieve vasospasm. Early historians of medicine often minimized the importance of the observations of the critics and even suggested that they impeded the universal acceptance of the coronary occlu-

sion theory. It is interesting to view this attitude in the context of the current controversies regarding the role of vasospasm in the genesis of both angina and myocardial infarction. In retrospect, the restraint of some scholars in uncritically accepting the coronary occlusion theory was, it seems, the result of careful observation and not a stubborn refusal to "see the truth".

Another controversy that arose during the 19th century concerned the elucidation of the ischaemia-to-infarct progression in the myocardium. In 1852, the renowned German pathologist, Rudolf Virchow, attributed the fibrosis in infarcted hearts to chronic myocarditis. This view was accepted by many prominent investigators until 1880 when Virchow's student, Carl Weigart, after careful microscopic examination of hearts that had varying degrees of myocardial damage, described the entire infarction process, from coronary obstruction and muscle atrophy to the consequent necrosis and onset of fibrosis. Thus, by the end of the century, the genesis of myocardial infarction from coronary occlusion had been described in detail, and the relationship between coronary narrowing (either by mechanical obstruction or by spasm) and angina was known amongst the prominent investigators of the period.

Despite the relatively complete description of the disease by the end of the 19th century, its prevalence was still unrecognized until well into the 20th century. The details of the clinical signs of coronary heart disease and the post-mortem pathology, as well as the distinction between angina and myocardial infarction, were not commonly known amongst the majority of practising physicians. Thus, in the first years of the 20th century, the disease was not felt to be too important. Two papers, the first by W.P. Obrastzow and N.D. Straschesko (1910) and the second by James Herrick

(1912), were especially important in initiating the understanding of myocardial infarction as a clinically recognizable entity.[2,3] These gave coherent accounts of the current knowledge of heart disease and, moreover, the paper by Obrastozow and Straschesko clearly differentiated between angina pectoris and myocardial infarction. Clinical diagnosis was also aided by the development of the Einthoven string electrocardiograph (1908) and the association of myocardial ischaemia with easily measured ECG abnormalities probably played the greatest role in facilitating the clinical recognition of coronary heart disease.[4]

During the 1930s the ischaemia/infarction process was studied more rigorously with the aim of elucidating the early functional sequelae of coronary occlusion. The coronary ligation model of myocardial ischaemia was chosen by R. Tennant and C. Wiggers (1935) for their classic study of the contractile properties of the ischaemic canine left ventricle.[5] In addition to noting the immediate decline in contractile force caused by coronary occlusion, they also recognized that reperfusion could reverse the myographic abnormalities if the period of ischaemia was not too prolonged.

In 1941, this period of reversible coronary damage in dogs was more thoroughly assessed by Blumgart, Gilligan and Schlesinger.[6] Like Tennant and Wiggers, they recognized that temporary ischaemia may lead to myocardial necrosis and their study attempted to define the period of occlusion that produced irreversible myocardial damage, as assessed by the development of scar tissue. They showed that 5-20 minutes of occlusion produced injury that was reversed by reperfusion, whereas longer ligation periods (>30 minutes) resulted in subsequent fibrosis thereby confirming the suggestion that short periods of ischaemic damage were reversible.

While the basic scientific research into the ischaemia/infarction process was proceeding, studies aimed at reducing ischaemia-induced myocardial injury were already in progress. In 1932, C. S. Beck began performing revascularization surgery on dogs and the work culminated in his first such operation on a patient in 1949.[7] Furthermore, he correctly hypothesized that the "localized anoxia" produced by coronary occlusion could trigger ventricular fibrillation although he did not understand the underlying mechanism. Work is continuing in this field to further improve the conditions of bypass surgery in order to reduce the incidence of intra-operative, as well as post-operative myocardial damage.

In the 1930s, treatments other than revascularization surgery were attempted to limit the course of coronary artery disease. These included thyroidectomy and sympathectomy and, not surprisingly, these rather harsh treatments were quickly abandoned. Sympathectomy was later replaced by alcohol infusion paravertebrally along the sympathetic chain and even today, drug ablation of the left stellate ganglion is still rarely a used treatment for intractable angina. Thus, early investigators suspected that the sympathetic nervous system was involved in the development of ischaemic damage. In fact, the association between emotional stress and anginal pain was recognized even at the time of Heberden whose contemporary, John Hunter, suffered from stress-induced angina. (It was said that Hunter jokingly claimed that his life was in the hands of any rascal who chose to annoy or provoke him. Ironically, he died (presumably of a heart attack) during a particularly violent discussion at a board meeting). The advent of the β-adrenergic antagonists in the 1960s and 1970s, meant that sympathetic blockade became less traumatic and the administration of β-adrenergic antagonists in post-myocardial infarction has recently been shown to significant-

ly reduce one-year mortality rates.[8] A variety of drug interventions with very different pharmacological properties are currently being assessed for their ability to limit myocardial ischaemic damage.

In summary, the knowledge of the normal physiology of the human cardiovascular system attained by the 17th century set the stage for the subsequent integration of independent pathological and clinical observations of coronary heart disease. The connection between angina pectoris and coronary pathology was largely accepted by the 20th century although not without a contingent of investigators who felt that vasospasm was the only source of the pre-cordial pain. As the distinction between angina and myocardial infarction became clear, there was a realization that the "ossification" was not necessarily a terminal event, i.e., the patient might survive such an insult. Using improved clinical and laboratory diagnostic techniques, patients with the disease could be identified and with this advance, came the recognition of the prevalence of the disease. Subsequent research on the compromised myocardium has resulted not only in improved methods of treatment but it has also expanded the body of knowledge concerning the cellular physiology of both normal and ischaemic cardiac muscle.

# INTRODUCTION

Twentieth century studies of ischaemic heart disease have examined numerous indices of cellular damage and alterations in almost every aspect of cellular function have been measured. Because irreversible ischaemic injury results in necrosis and fibrosis of the myocardium, cessation of blood flow will ultimately affect all cellular processes. The important question then becomes: which effects of coronary ligation are responsible for the development of irreversible myocardial damage? If it were possible to sufficiently delay or prevent these critical changes, it might also be possible to reduce the extent of necrosis. This could be accomplished either by an intervention which affects a specific cellular process or by a general treatment which attenuates the effects of ischaemia by a more general action on cellular metabolism.

Attempts have been made to understand more fully the consequence of coronary ligation, including its effects on the contractile properties of the heart. The 17th and 18th century investigators often observed that coronary ligation produced cardiac standstill; however, it wasn't until 1935 that a detailed account of the ischaemia-induced contractile abnormalities was published by Tennant and Wiggers. By measuring the contractile force of the ischaemic canine left ventricle, they observed that coronary occlusion produced a rapid decrease in contraction that only slowly reversed upon reperfusion.[5] Subsequent studies have confirmed the validity of these observations and, in addition, have evaluated both the ischaemic and the normal left ventricle and in the former noted the presence of a transient marginal zone of partially impaired function in addition to the central zone of non-contracting fibres.[9,10] Thus, coronary ligation has been found to

produce an immediate decline in contraction that only slowly reverses with reperfusion.[11]

As mentioned previously, the electrocardiogram provided the first reliable and sensitive clinical method of detecting ischaemic damage.[4,12,13] However, although quantitation of the ECG wave changes (specifically S-T segment elevation) is a useful tool in assessing the efficacy of certain interventions on myocardial ischaemic injury, difficulty arises in relating these electrical abnormalities to alterations in intracellular potential changes.

In comparison to the electrocardiogram, the microscopic examination of the ischaemic myocardium reveals more directly the integrity of the cells, and, in spite of its disadvantages, microscopic analysis of myocardial tissue has been a valuable technique in the elucidation of the events following coronary occlusion. The use of the electron microscope revealed more subtle structural changes than had been detected by the 19th century pathologists. Jennings et al. observed early alterations in mitochondrial integrity, including clearing of the matrix and cristae fragmentation.[14] Glycogen depletion, the appearance of matrix densities, sarcolemmal disruption and nuclear chromatin clumping became apparent in tissue that had been subjected to longer periods of ischaemia.[14-16] This technique allowed investigators to study the time-course of morphologic changes in the organelles; however, Schaper et al. have shown that the appearance of ultrastructural defects lagged behind the immediate decrease in contractility and, conversely, cell morphology normalized with reperfusion despite the persistence of defects in biochemical and contractile function.[17] Thus, although microscopic analysis yields useful information regarding the effects of ischaemia on subcellular structure, it does not evaluate the functional

capability of the cell, e.g. the normal appearance of the mitochondrion on an electron micrograph does not preclude the existence of a defect in oxidative phosphorylation. Such ischaemia-induced decreases in mitochondrial oxidative phosphorylation have been measured in vitro by many investigators.[18,19] In addition, Rouslin and Millard have observed that ischaemia inhibits the activities of several of the electron transfer chain enzymes.[20] The dysfunction of oxidative phosphorylation would be expected to limit the supply of adenosine-5'-triphosphate (ATP) and, indeed, the rapid depletion of ATP after coronary occlusion is a well-documented phenomenon.[21,22] It has been speculated that measurement of tissue levels of ATP may not reflect the actual availability of ATP to the ATP-dependent processes in the cell because ATP is limited to certain subcellular areas or compartments. Such a compartmentalization of ATP has been invoked to explain the fact that contractility declines before a fall in ATP levels can be detected.[23] Several investigators have suggested that ATP availability could be reduced as a result of the ischaemia-induced inhibition of the mitochondrial adenine nucleotide translocase enzyme which exchanges ATP for extramitochondrial ADP. This inhibition may be mediated by accumulated metabolites of fatty acid oxidation, especially acyl-CoA derivatives.[24] Other factors that are thought to limit ATP supply in the ischaemic heart include decreased glycogen stores, decreased glucose supply and compromised anaerobic metabolism (glycolysis). The latter may be due to $H^+$-mediated inhibition of glycolytic enzymes.[22,25] It is evident from the foregoing that many of the deleterious effects of coronary occlusion arise not only from the depletion of $O_2$ and substrates, but also from the accumulation of metabolic products such as $H^+$ and fatty acid intermediates. Therefore, it is important to distinguish between the injury produced by ischaemia and

that associated with anoxia. Anoxia is the condition that arises when the $PO_2$ is negligible but blood flow to the myocardium is maintained, whereas ischaemia results from both low $PO_2$ and a critical decrease in blood flow. Ischaemic and anoxic hearts both depend on anaerobic metabolism for energy but anoxic injury is not as severe because substrate supply and metabolite removal are preserved. Thus, the results of studies examining anoxic injury cannot always be directly applied to the investigation of the molecular mechanisms of ischaemic injury.

Coronary occlusion not only disrupts mitochondrial function but also appears to affect the sarcolemmal membrane. Using extracellular $K^+$ electrodes, Weiss and Shine have demonstrated the existence of a small and rapidly reversible efflux of $K^+$ from the ischaemic portion of the myocardium in the first few minutes following coronary ligation.[26] If the ischaemic area is reperfused after approximately 15-30 min of coronary occlusion, the cells gradually accumulate $Na^+$ and $Ca^{2+}$ and lose $Mg^{2+}$ and $K^+$. There is also a concomitant gain in intracellular water.[27,28] In contrast to the initial $K^+$ efflux, these ionic changes are not easily reversed suggesting that the defects in sarcolemmal permeability are irreversible. The re-instatement of blood flow to irreversibly-damaged tissue probably induces these ionic abnormalities because the sarcolemma is no longer a barrier to the passive flow of ions down their concentration gradients.[29] Calcium influx is thought to be especially deleterious in that high cytosolic levels of calcium inhibit a variety of cell processes. It has been suggested that this calcium accumulation, in addition to the cell swelling, contributes to the rapid deterioration of cellular integrity that occurs with reperfusion.[30,31]

With increasing periods of coronary occlusion, the sarcolemmal disruption progresses until it is of a magnitude that allows leakage of larger components of the cytosol, including soluble enzymes. The phenomenon has been exploited clinically in that measurement of enzyme leakage is now used as an indicator of infarct size. La Due and Wroblewski showed that serum glutamic oxaloacetic transaminase (SGOT) levels were increased in the plasma of patients who had recently suffered a myocardial infarction.[32] More recently, the plasma level of the myocardial isoenzyme of SGOT as well as several other cytosolic enzymes, e.g., the creatine phosphokinase and lactate dehydrogenase MB-isoenzymes, have been used to confirm the presence of a myocardial infarct.[33] Although this technique is of clinical importance, the fact that enzyme leakage does not reach a maximum until several hours after coronary vessel occlusion indicates that it is a relatively late event in the development of ischaemic damage and heralds the development of an infarct in the ischaemic zone. Thus, although the measurement of plasma enzymes can be of clinical use to determine the presence of an infarct, it provides minimal information regarding the biochemical mechanism of sarcolemmal damage.

It is apparent that the critical events which determine the ultimate viability of the ischaemic myocardial cell occur within the first hour following coronary ligation and that the resulting plasma membrane damage would be expected to seriously compromise cellular homeostasis. The precise molecular events that are responsible for sarcolemmal damage are currently unknown, although several hypotheses have been formulated. For example, it has been speculated that the accumulation of the metabolites of fatty acid oxidation may contribute to cell injury.[34,35] At low concentrations, these detergent-like, amphipathic compounds have been shown to disrupt a

variety of cellular functions *in vitro*, including sarcolemmal $Na^+,K^+$-ATPase activity,[36,37] sarcoplasmic reticulum function[38] and, as noted earlier, mitochondrial function.[38,39] It is conceivable that even low concentrations of these agents may have widespread inhibitory effects on cellular metabolism. Other compounds have been implicated in the production of ischaemia-induced membrane disruption. Chien *et al.* have demonstrated an ischaemia-induced increase in lysosomal enzyme activity as well as an accelerated phospholipid degradation in both heart and liver tissue.[40-42] These data have led them to suggest that activation of lysosomal phospholipases may be responsible for the observed membrane damage. Moreover, further indirect evidence for phospholipid degradation has come from experiments demonstrating elevations in the tissue content of phospholipid breakdown-products, especially lysophosphatidylcholine and lysophosphatidylethanolamine. These agents are also potent detergents and, if present in critical concentrations in the membrane, could potentially disrupt metabolic processes. However, the magnitude and significance of the increase in phospholipid breakdown products is currently in dispute.[43-45] Thus, the role of phos- pholipid degradation and/or the accumulation of metabolic by-products in the genesis of ischaemic membrane damage is currently unclear.

From the foregoing, it is apparent that the alterations induced by ischaemia must ultimately involve virtually every aspect of cellular metabolism. Consequently, a diversity of therapeutic interventions have been tried empirically in an attempt to limit the damage. Moreover, the existence of tissue with intermediate damage bordering the severely ischaemic zone[9,46] has motivated researchers to identify treatments which might "salvage" this tissue, thereby reducing the extent of the infarction. In experimental myocardial ischaemia in the rat, Fishbein *et al.* showed that

this "border zone" is present for approximately 9 hours post-ligation after which the cells become irreversibly injured.[47] An apparent zone of intermediate damage may be formed when zones of normal and ischaemic myocardium interdigitate at the border of the occluded area. This pattern is seen in the ischaemic canine myocardium.[48] Thus, the timing of interventions aimed at reducing infarct size is crucial to their ultimate efficacy.

Reperfusion alone will reverse some of the deleterious effects of short (up to 15-20 min) periods of coronary occlusion.[6,49,50] However, reperfusion after more prolonged periods of ischaemia (>20-30 min) not only fails to reverse many of the biochemical and functional abnormalities but actually exacerbates the tissue damage.[51] This so-called "reperfusion injury" does not occur unless the tissue has already been made ischaemic, thus the label "reperfusion injury" is a misnomer. A more semantically-correct term would be ischaemic/reperfusion injury. As mentioned with regard to ionic changes after coronary occlusion, the accelerated damage is presumably caused by the sudden reinstatement of blood flow to cells which have non-functional permeability barriers.

A variety of treatments have been examined for their ability to limit the injury produced by both ischaemia and reperfusion. Generally, they possess at least one of the following actions; increasing blood flow to the ischaemic zone, altering ischaemic metabolism to reduce the production of deleterious metabolites, decreasing the work-load of the heart or directly modifying the conditions of reperfusion.

β-Adrenergic antagonists possess several actions some or all of which might attenuate ischaemic damage and these agents have been reported to reduce ischaemic injury with or without subsequent reperfusion in a variety of experimental models.[52-54] The reduction of myocardial oxygen consump-

tion ($M\dot{V}O_2$) which results from the beta-adrenergic blocking properties is thought to be the most important determinant of the beneficial effects of this class of drugs;[55,56] however, some investigators have shown that the non-β-blocking properties of these agents also contribute to the reduction in $M\dot{V}O_2$.[57,58] In addition, Hillis et al. showed that β-adrenergic antagonist treatment could also increase blood flow to the ischaemic area - an action which could potentially reduce infarct size.[59] However, this conclusion is not supported by the findings of Kloner, Reimer and Jennings who showed that the reduction in infarct size by β-blockers was probably not a result of increased collateral flow.[60] Thus, although these agents appear to significantly reduce tissue damage in the acute model of myocardial ischaemia, the relative contribution of the various drug actions to the beneficial effects is presently in dispute. The assessment of drug action is further complicated by problems in the definition and measurement of "infarct size" vs. "ischaemic zone" vs. "area of risk".

The organic calcium channel-blockers have also been shown to improve the functional, biochemical and morphological indices of ischaemic injury.[61-65] The calcium channel-blocking properties of these agents were first described in 1964 by Fleckenstein who stated that their effects on the heart were mimicked by exposing the heart to a hypocalcaemic perfusate.[66] The most-studied drugs include verapamil, diltiazem and nifedipine. This chemically heterogeneous group of compounds are all classified as calcium channel-blockers by virtue of their reduction in the calcium conductance which is partly responsible for the plateau phase of the action potential in the heart;[67-70] however, they have different effects on myocardial contractility and smooth muscle reactivity in the intact animal. For example, although all three drugs suppress atrioventricular nodal function

in the isolated rabbit heart, only verapamil and diltiazem are clinically useful in the treatment of atrial arrhythmias. In explanation, Kawai et al. have postulated that the greater vasodilatory properties of nifedipine produce a reflex activation of the sympathetic nervous system which overrides any direct effect on the A-V node.[71]

A consequence of their calcium channel-blocking ability is negative inotropism and this, in turn, results in a concomitant reduction in myocardial oxygen consumption in normal myocardium. Thus, one explanation for their beneficial effects in experimental ischaemic injury is that, like the β-adrenergic antagonists, calcium channel-blockers are capable of reducing the energy requirement of the ischaemic cell thereby preserving high energy phosphate levels for maintainance of cellular integrity. In addition, the vasodilatory properties off-load the heart, further reducing $M\dot{V}O_2$. The smooth muscle relaxation may also contribute to the beneficial effects of drug treatment via coronary vessel dilation with consequent increases in coronary blood flow.[72]

Besides these major effects on cardiac function and haemodynamics, which are for the most part associated with the decreased calcium conductance, these drugs have been shown to affect other aspects of cell function. Pang and Sperelakis have shown that $10^{-6}$-$10^{-5}$ M verapamil (but not nifedipine or diltiazem) inhibited calcium binding to the guinea pig sarcolemmal membrane.[73] Earlier experiments by Mas-Oliva and Nayler had also demonstrated that verapamil inhibited passive $^{45}Ca^{2+}$-binding to the sarcolemma and, in addition, showed that verapamil also interfered with the ATP-dependent transport of $^{45}Ca^{2+}$. Moreover, the decrease in $Ca^{2+}$ transport was associated with a decrease in sarcolemmal $Ca^{2+}$-ATPase activity.[74]

Nifedipine has also been shown to affect metabolism independent from its effects on calcium conductance. In experiments examining the effect of nifedipine on regional myocardial function in anaesthetized pigs, Verdouw et al. found that intracoronary administration of nifedipine produced decreases in regional $O_2$ consumption which were unaccompanied by a decrease in regional myocardial contractile function.[75] The authors postulated that the drug reduced $O_2$ consumption by a mechanism unrelated to its negative inotropic effects. Other evidence for direct effects of verapamil and diltiazem on mitochondrial function has been reported by Vaghy et al..[76]

Calcium channel-blockers have been shown to be effective not only in limiting the damage incurred during the ischaemic period, but also in preventing the sudden large influx of calcium that is associated with reperfusion. Whether these drugs prevent calcium influx via protective effects during the ischaemic period, direct calcium channel-blockade upon reperfusion or a combination of both of these mechanisms is a subject of current controversy.[61,77-79]

In contrast to the largely positive findings in the literature regarding the ability of calcium channel-blockers or ß-adrenergic antagonists to reduce ischaemic/reperfusion injury, the effectiveness of a variety of other agents in limiting ischaemic damage is less clearly established. For example, the glucocorticoids have been shown to preserve membrane structure under certain experimental conditions; however, their ability to preserve myocardial structure and function during ischaemia is, at most, marginal.[80-82] Similarly, the results obtained using prostaglandin (PG) synthetase inhibitors (such as indomethacin) have been inconclusive. Smith et al. postulated that the protective effects of PG synthetase inhibitors resulted from inhibition of thromboxane $A_2$ synthesis[83] whereas Miyazaki et al.

attributed the benefit to a reduction in the formation of prostaglandin $E_2$.[84] Conversely, Judgutt et al. have demonstrated that the inhibition of prostaglandin synthesis may actually increase infarct size, possibly by a direct effect on $O_2$ consumption.[85] Thus, it is not clear whether or not the non-specific inhibition of prostaglandin synthesis during acute myocardial ischaemia is beneficial. Other miscellaneous agents which have been shown to afford limited benefit to the ischaemic heart include: glucose-insulin-potassium solutions,[86,87] hyaluronidase,[88] and hyperosmolar mannitol.[89]

The use of this array of agents has not only provided information regarding potential therapeutic benefits of certain drug interventions but has indicated which of the sequelae of coronary occlusion may be involved in the production of cell damage. However, the "trigger" of irreversible cell damage is still unknown. A difficulty arises in separating the primary event(s) of ischaemic injury from the secondary and tertiary events.

The experiments discussed in this thesis have focussed on the elucidation of the early molecular alterations that result from experimental acute myocardial ischaemia and reperfusion in the anaesthetized rabbit. The preliminary studies examined the time course of ischaemic changes on several indices of cellular damage in order to determine the effects of coronary ligation on indirect measures of both sarcolemmal and mitochondrial functional integrity and their relationship to cellular energy levels. Subsequent experiments were undertaken to define the in vivo significance of the ischaemia-induced sarcolemmal enzyme changes that were measured in vitro. Having established the time-course and relative reversibility of these biochemical alterations, the effect of various pharmacological agents on the

changes produced by a 20 minute ligation period has been explored. The significant reduction in ischaemic damage by the calcium channel-blocker, D-600 (methoxyverapamil), as well as a desire to compare and contrast the effects of D-600 with the beta-blocking agent propranolol prompted us to study the ability of these drugs to attenuate ischaemic/reperfusion damage when administered prior to the ligation period. In this study, several levels of cellular organization ranging from whole organ function to sarcolemmal membrane integrity were monitored in the same animal thus enabling us to assess the effects of drug treatment without being restricted to the results of one technique. The aim of these studies concerning the effects of drug treatment was not only to investigate the mechanisms of beneficial drug action but also to attempt to identify the critical molecular events that are responsible for the production of irreversible cell injury.

## METHODS

### I. Surgical Technique

Male, New Zealand white rabbits 2-3 kg, were anaesthetized by injecting 60 mg/kg of sodium pentobarbital into the marginal ear vein. A tracheotomy was subsequently performed to permit positive-pressure ventilation using a Palmer pump. In the earlier studies, the animals were ventilated with room air but this was changed to room air enriched with 95% $O_2$/5% $CO_2$ in the last experiments concerning the effects of 0-60 min of occlusion on the sarcolemmal permeability. Blood gas measurements were done periodically and were in the normal range. A polyethylene cannula was inserted into the carotid artery and connected to a Bell and Howell pressure transducer (model 4-327-0010) in order to monitor blood pressure. The chest was opened by a sternotomy, the rib cage retracted, and the heart suspended in a pericardial cradle. In most of the later experiments, a 20-gauge Jelco catheter was inserted into the left ventricle through the apex and connected via polyethylene tubing to a Statham P23 ID pressure transducer. The output of this left ventricular pressure recording was fed into a calibrated differentiator (Grass Instruments 7P20C) to obtain the differentiated waveform - an indirect measure of cardiac contractility $(dP/dt)_{max}$.[90] Maximal rates of rise of left ventricular pressure were well within the response range of the pen recorder (see Appendix I). All pressure recordings and the electrocardiogram were recorded on a Grass Instruments Polygraph (model 7PCPB).

Subcutaneous electrodes measured lead II of the ECG and changes characteristic of ischaemia (e.g. S-T segment elevation, R wave enlargement) were monitored in order to assess whether or not the left circumflex coronary artery had been successfully ligated. The ECG also provided indirect evidence of the occurrence of reperfusion in our model because loosening the

ligature caused a gradual reversal of the ECG abnormalities induced by coronary occlusion.

Drug Infusion

All drugs were dissolved in saline and injected into the animal in 2-3 ml volumes via the marginal ear vein. Sham-operated animals received saline only. No drug was administered until the blood pressure had stabilized (at least 15 min after all surgery was completed). Drugs were infused over a 20 minute period prior to ligation.

Coronary Ligation, Reperfusion and Isolation of the Ischaemic Area

Just prior to insertion of the left ventricular catheter, a 4:0 silk cardiovascular suture was placed around the left circumflex coronary artery and surrounding muscle at its origin. The two free ends of the ligature were passed through a short length of polyethylene tubing and ischaemia was produced by pulling up and securing the ends with a haemostat. Clamping the suture in this way made the occlusion readily reversible when reperfusion was part of the study protocol. Tightening of the ligature resulted in an occluded zone of 55 ± 3% (mean ± SE, n = 15) of the left ventricle. The occluded

zone was measured as $\frac{\text{wet weight of occluded zone}}{\text{total left ventricular weight}} \times 100\%$

Reperfusion was accompanied by a decline in $(dP/dt)_{max}$, marked hyperaemia and, as stated previously, a gradual reversal of ECG abnormalities. Experiments with radioactively-labelled microspheres provided a quantitative analysis of the extent of occlusion and reperfusion. These studies are described in a later section.

If fibrillation occurred during the ligation or reperfusion periods, brass electrodes were placed directly on the heart and a 0.5 Watt-second

D.C. countershock was discharged from a defibrillator (American Optical Co.). This low level of stored energy was usually effective in restoring sinus rhythm and did so at a level well below the threshold shown by Koning et al. to produce tissue damage in isolated rabbit hearts.[91]

When the experiment was completed, the heart was quickly removed by cutting the aorta and other major vessels and the heart was immediately dropped into ice-cold buffer (.25 M KCl, 1 mM EDTA, 50 mM Tris, pH 7.4). Beating ceased within seconds. After removal of the heart, all procedures were carried out at 4°C. To visualize the occluded zone, this same buffer was perfused retrogradely via the aortic root (the ligature was re-clamped if the heart was removed during reperfusion). Thus, the ligated zone remained unperfused and could be separated from the surrounding, blanched, perfused tissue. An excess of tissue was trimmed from the border of the ischaemic zone to ensure that the isolated area contained only occluded tissue. Two transmural samples of ischaemic tissue (approximately 50 mg) were quickly frozen with liquid nitrogen for subsequent analysis of ATP, water and ion contents. Because of the small size of the rabbit heart, tissue for ATP analysis could not be routinely taken from the beating heart without some compromise in function. Therefore, samples for ATP and ion analysis from all groups were frozen in liquid nitrogen at the same time after removal of the heart. It has recently been demonstrated by Jennings et al. that the ATP content of the myocardium does not decrease when the tissue is stored in cold buffer for several minutes before freezing (although creatine phosphate levels are affected).[92] A study comparing the ATP levels in samples removed using the above protocol with samples removed directly from the beating heart confirmed Jennings' findings. The results of this study are shown in Table 1.

Table I. Comparison of ATP contents in tissue taken from the beating heart vs. KCl EDTA-Tris perfused heart

| Ligation Time (min) | ATP content (nmoles ATP / mg dry weight) | |
|---|---|---|
| | Perfused Heart[a] | Beating Heart[b] |
| Control | 13.7 ± 2.7 (2) | 9.2 ± 1.2 (3) |
| 10 min post-ligation | 3.9 ± 0.4 (2) | 4.3 ± 0.6 (2) |
| 60 min post-ligation | 0.8 (1) | 0.2 ± 0.1 (2) |

All values are expressed as mean ± S.E.

Number of animals per group shown in brackets

[a] Perfused heart - whole heart removed, placed in cold buffer, and perfused as described in the Methods section. Sample taken approximately 7 min after removal.

[b] Beating heart - tissue cut from ischaemic portion of left ventricle or comparable area in control heart, blotted to remove excess blood and immediately frozen in liquid nitrogen. Freezing occurred within 20 sec of removal.

In hearts that were to be analyzed by electron microscopy, a sample of approximately 3 $mm^3$ from the subendocardium was fixed in cold, buffered 3% glutaraldehyde solution. Further details regarding the procedures used in the ultrastructural analysis are contained in a later section and in Appendix II.

The remainder of the tissue from the occluded zone was subjected to a subcellular fractionation as outlined below.

Control tissue always was obtained from sham-operated animals which were subjected to all surgical procedures except tightening of the ligature. The comparable area of the left ventricle was used for tissue sampling after the appropriate time lag between removal and freezing of the tissue. Neither the control tissue nor the occluded zone were directly perfused via the coronaries with the KCl/EDTA/Tris buffer solution.

## II. Subcellular Fractionation

The preparation of mitochondrial and sarcolemmal-enriched fractions was performed using modifications of two previously published methods[93,94] which are summarized as follows. All procedures were performed at 4°C. Approximately 1-1.5 g of left ventricular normal or ischaemic myocardium was minced and homogenized as a 10% suspension (w/v) in buffer (1.25 M KCl, 0.5 mM $CaCl_2$, 2 mM dithiothreitol and 10 mM Tris, pH 7.4) using a Polytron PT-10 homogenizer (2 burst of 5 sec each at 1/4 maximum speed). The suspension was filtered through nylon mesh (0.5 $mm^2$) and rinsed through with an additional 10 ml of buffer. The filtrate was centrifuged for 10 min at 1200 g and the resulting pellet resuspended to the original volume. A second homogenization was performed using a Teflon-in-glass homogenizer (5 strokes), followed by centrifugation at 500g for 10 min. This second pellet

was resuspended in 10% (w/v) sucrose dissolved in 10 mM Tris, 2 mM dithiothreitol, pH 8.2, and hand-homogenized with a ground-glass homogenizer (Pyrex 7727) until all visible particulate matter was disrupted. One ml of this mixture was layered on each of six sucrose gradients consisting of 2.5 ml of 50, 52.5, and 60% (w/v) buffered sucrose, pH 8.2. Following centrifugation at 40,000 g for 60 min, the sarcolemmal-enriched fraction appeared at the 55-60% interface and the mitochondrial fraction appeared at the 10-50% interface. These two fractions were collected with a Pasteur pipette, diluted 5-fold with 10 mM Tris, pH 7.4 and centrifuged at 23,000g for 15 min. The final membrane pellets were resuspended in double-distilled water. Protein concentrations (approximately 3 mg/ml) were estimated by the method of Lowry et al..[95] Membrane fractions were also assayed the same day for cytochrome c oxidase activity (see Part III) and then frozen immediately in vials in dry ice/acetone and stored overnight at 20°C. All other enzyme assays were done on the following day.

## III. Characterization of Sarcolemmal and Mitochondrial Membrane Fractions

The activity of the mitochondrial marker enzyme cytochrome c oxidase, was measured in both the sarcolemmal and mitochondrial fractions in order to assess the contamination of sarcolemmal fraction with mitochondria. Specific details of the assay method are outline in Section IV.

Table II demonstrates that even prolonged ischaemia with or without reperfusion does not affect the fractionation characteristics of the sarcolemma as demonstrated by the consistent percentage contamination of the sarcolemmal fraction with mitochondria.

Similarly, tissue injury does not affect the purity of the mitochondria.

Table II. Percent contamination of sarcolemmal fraction ($F_4$) with mitochondria ($F_1$) as assessed by cytochrome c oxidase activity

| Treatment | n | Percent contamination $\frac{\text{activity } F_4}{\text{activity } F_1} \times 100$ [a] |
|---|---|---|
| Control | 9 | 12.7 ± 1.0 |
| 1 min ligation | 5 | 10.8 ± 1.8 |
| 5 min ligation | 10 | 12.1 ± 0.7 |
| 10 min ligation | 5 | 13.4 ± 0.9 |
| 20 min ligation | 3 | 14.2 ± 2.9 |
| 60 min ligation | 2 | 15.6 ± 2.5 |
| 10 min ligation + 60 min reperfusion | 2 | 11.5 ± 0.9 |
| 20 min ligation + 60 min reperfusion | 2 | 9.4 ± 2.6 |
| 60 min ligation + 60 min reperfusion | 2 | 10.8 ± 3.0 |

Mean ± S.E.

[a] $\text{activity} = \frac{\text{slope}}{\text{mg protein}}$

where 'slope' equals the slope of the regression line, log absorbance *vs.* time; This equals the rate of oxidation of reduced cytochrome C. See enzyme assay section in Methods for details.

The following calculation was used to estimate the purity of the mitochondrial fraction:

$$\frac{\text{azide-insensitive ATPase (NON-MITO)}}{\text{azide-sensitive ATPase activity (MITO)}} \times 100\%$$

The data shown in Table III demonstrate that neither the degree of tissue injury nor the presence of drugs affected the purity of the mitochondrial fraction.

The results shown in Table IV demonstrate that the fractionation of the membranes is unaffected by ischaemia alone. Thus, changes in specific enzyme activity of a particular marker enzyme during ischaemia are not caused by changes in the fractionation pattern of the organelle. This is in agreement with recent results of Bersohn et al.. Using rabbit myocardium made ischaemic in vitro, they showed that there was no difference in the percent increase in enzyme activity of the partially-purified fractions from the crude homogenate between control and ischaemic hearts.[96] Interestingly, there is a reduction in the yield of mitochondria after reperfusion of myocardium that has been made ischaemic for 40 minutes.

## IV. Enzyme Assays

### $Na^+,K^+$-ATPase

$Na^+,K^+$-ATPase is a reliable marker enzyme for the sarcolemma and the activity of this enzyme was measured in the sarcolemma-enriched fraction according to the method of Godin and Schrier[97] except that the reaction time was 15 min and each tube contained 150 µg of membrane protein. The reaction was started with the addition of the membranes to 3 ml (final volume) of assay medium at 37°C (55 mM Tris, 3 mM ATP, 3 mM $MgCl_2$, .03 mM EGTA, 80mM NaCl, 20 mM KCl, 5 mM $NaN_3$). Non-stimulated activity was

Table III. Purity of the mitochondrial fraction ($F_1$) after various treatments

| Treatment | n | Percent residual ATPase activity in the $F_1$ fraction[a] |
|---|---|---|
| Control | 8 | 3.0 ± 2.0 |
| 40 min ligation + 60 min reperfusion (L + R) | 8 | 4.1 ± 0.4 |
| D-600 pretreatment + L + R | 8 | 3.0 ± 0.3 |
| Propranolol pretreatment + L + R | 8 | 3.1 ± 0.3 |

[a] $\pm$ residual ATPase activity $= \frac{\text{azide-sensitive ATPase specific activity (mito)}}{\text{azide-insensitive ATPase specific activity (non-mito)}} \times 100\%$

Table IV. Purification of mitochondrial and sarcolemmal protein from wet heart weight

| Treatment | n | $\frac{\text{protein in purified fraction}}{\text{wet weight of occluded zone}} \times 10^3$ | |
|---|---|---|---|
| | | Mitochondria | Sarcolemmma |
| Control | 10 | 3.2 ± .2 | 7.2 ± .5 |
| 20' L | 7 | 2.8 ± .2 | 8.4 ± .6 |
| 40' L | 7 | 2.6 ± .3 | 7.6 ± .6 |
| 60' L | 8 | 3.4 ± .3 | 9.0 ± 1.1 |
| 40' L + 60' R | 9 | 1.6 ± .2 | 6.8 ± .9 |
| Propranolol + L+R | 7 | 2.0 ± .2 | 6.6 ± .6 |
| D-600 + L+R | 6 | 2.8 ± .4 | 8.2 ± .7 |

All values are expressed as mean ± S.E.

measured in tubes without NaCl and KCl. A tube containing 0.65 mM inorganic phosphate was used as a standard. After the 15 min incubation, the reaction was stopped with cold 10 trichloroacetic acid (TCA) and, after centrifugation at 1000 g to remove the membranes, an aliquot of the supernatant was assayed for inorganic phosphorus by the method of Fiske and Subbarow.[98] The activity is expressed in μmoles $P_i$/mg protein/hour. All tubes were kept on ice and the supernatant assayed as soon as possible to prevent non-specific hydrolysis of ATP in the acidic solution. As a further precaution, blanks with no membrane addition were subtracted from all readings to account for any non-specific hydrolysis.

$\underline{K^+\text{-NPPase}}$

The $K^+$-stimulated p-nitrophenylphosphatase ($K^+$-NPPase) enzyme is functionally-related to the $Na^+,K^+$-ATPase in that it catalyzes the terminal $K^+$-dependent dephosphorylation step of the sodium pump activity.[99] The enzyme activity was measured according to the method of Godin and Schrier.[97] The reaction time and amount of protein assayed was the same as for the $Na^+,K^+$-ATPase but the composition of the incubation medium differed (50 mM imidazole, pH 7.4, 3 mM $MgCl_2$, 3 mM p-nitrophenolphosphate, 30 mM KCl). The reaction was stopped with cold 20 TCA and the colourimetric reaction was initiated by alkalinization with 1 ml of 1.5 M Tris to each tube.

In both the $Na^+,K^+$-ATPase and $K^+$-NPPase assays, 30 mM ouabain was added to one tube to provide a measure of the ouabain-sensitivity of the enzyme. Ouabain-insensitive ATPase or NPPase activity was not significantly different from the activity measured in the absence of added $Na^+$ and $K^+$ (basal activity). Thus, the specific activity of the enzyme (μmoles NPP/mg protein/hour) could be expressed as the difference between the ion-stimulated and basal enzyme activity.

Cytochrome c oxidase

The activity of a mitochondrial marker enzyme, cytochrome c oxidase was measured in both the mitochondrial and sarcolemmal fractions for two reasons: firstly, to assess the function of this mitochondrial membrane enzyme; and secondly, to provide an estimate of the contamination of the sarcolemmal fraction with mitochondria (see Table II). Measurement of specific activity was performed using the spectrophotometric method of Cooperstein and Lazarow[100] in which aliquots of the diluted membrane fractions were added to buffered, reduced cytochrome c, pH 7.4, and the oxidation rate was followed over a 3 minute period by recording the decrease in absorbance at 550 nm. The slope of the regression line, log absorbance vs. time, was expressed per mg protein. The ratio of activity (log oxidation rate/mg protein) in the sarcolemmal fraction to the activity in the mitochondrial fraction was used as an index of contamination of the sarcolemmal fraction. Specific activity was expressed as μmoles cytochrome c oxidized/min/mg protein.

Azide-sensitive ATPase

Mitochondrial ATPase activity was assayed in the presence and absence of sodium azide ($NaN_3$, 5 mM) as described by Godin and Schrier[97] except that the reaction time was 15 min and each tube contained 15-30 μg of protein. The assay medium had the same composition as that for the $Na^+,K^+$-ATPase assay except that NaCl and KCl were omitted. Oligomycin is known to specifically inhibit the mitochondrial ATPase activity[101] and, in one experiment, either 0, .3, 1.0, or 3.0 μg/ml oligomycin was added to the assay mixture. The residual activity after addition of 3.0 μg/ml oligomycin was not significantly different from the level measured after $NaN_3$ treatment. Thus, the azide-sensitive ATPase activity is equivalent to the

oligomycin-sensitive ATPase and shall be henceforth referred to as azide-sensitive ATPase activity.

Triton X-100 Addition

For all but the earliest experiments (preliminary and 20 min ligation studies), 0.005% and 0.01% Triton X-100 was added to the assay medium for the mitochondrial and sarcolemmal fractions, respectively, in order to eliminate the effects of vesiculation on measured enzyme activity.[102,103] The effects of detergent on enzyme activity followed a bell-shaped curve, i.e. activation at low concentrations followed by inhibition at higher concentrations than those listed above.

## V. Chemical Assays

Water content

Transmural samples were blotted lightly with paper and frozen at -80°C. The frozen left ventricular tissue (approximately 50 mg) was weighed in porcelain crucibles, dried in the oven at 200°F and the dry weight determined by subtraction. Water content is expressed as a percent of total tissue weight.

ATP

A 50 mg transmural myocardial sample was weighed while still frozen, pulverized in a pre-cooled mortar with 4 volumes (w/v) of 6% perchloric acid and thawed on ice. Following the procedure of Jaworek et al., the extract was neutralized with 1.4 M $KHCO_3$, the precipitate centrifuged and the supernatant assayed for ATP content.[104] This was done spectrophotometrically at 340 nm using a coupled enzyme reaction in which the amount of ATP present is proportional to the amount of NADH oxidized. The levels are expressed as nmole ATP/mg dry weight.

Ions

Tissue levels of sodium, potassium, magnesium and calcium were measured by atomic absorption spectroscopy using a modification of the technique of James and Roufogalis.[105] The dried samples from the occluded zone or the comparable area of control hearts (approx. 10 mg) were dissolved in boiling 100% trichloroacetic acid:glacial acetic acid (v/v, 1:1) and 1 mM $LaCl_3$ was added to precipitate phosphate. After centrifugation, the supernatant was analyzed using a Varian Techtron atomic absorption spectrophotometer (Model AA5), calibrated with freshly prepared standard solutions. Standard solutions contained the same proportion of acetic acid, $LaCl_3$, KCl and CsCl as did the sample solutions as well as the appropriate volume of atomic absorption stock solution for each ion (Fisher Chemical Co., 1000 µg/ml). All solutions were prepared using freshly de-ionized water. Sample and standard blank solutions were also prepared in order to subtract the background readings. Air:acetylene fuel was used for the measurement of Na, K and Mg. Calcium absorbance was measured in a nitrous oxide:acetylene flame. Suppression of ionization was effected by the addition of KCl (2.5 mg/ml) to the solutions for Mg and Ca analysis and CsCl (1.0 mg/ml) to the solutions for K analysis. The amount of the ion in each sample was determined by interpolation from the standard curve and all results are expressed as nmoles element/mg dry weight.

SDS-gel electrophoresis

An aliquot of the mitochondrial or sarcolemmal fraction (100 µl) was solubilized in the following solution: 30 mM Tris, pH 8.0, 0.3% mercaptoethanol, 3% sodium dodecyl sulphate (SDS), 10% sucrose, 3 mM EDTA, 20 µg/ml bromphenol blue) and incubated at 37°C for 20 min to reduce sulfhydryl groups. The samples were loaded into one of six slots of a 5% polyacryla-

mide slab gel (75 μg protein/slot) (E-C Apparatus Corp.). The samples were electrophoresed at 150 V for approximately 3 hours by which time the marker dye had moved 10 cm. The SDS was removed by rinsing the gel overnight with distilled water:methanol:glacial acetic acid, 45:45:10 (v/v/v) and the gel was then stained with Coomassie Blue. Qualitative analysis was done by inspection of black and white photographs of the gels. For more quantitative analysis, the gels were scanned using a Gelman DCD 16 densitometer which recorded increased band density by greater pen deflection. Subsequently, these peaks were integrated using a Technicon Integrator Model AAG. Scans of the protein profiles from both of the mitochondrial and sarcolemmal membrane fractions showed considerable variability in the absolute areas of the peaks although the pattern of polypeptide distribution remained constant. As a consequence, all peak areas were normalized by expressing a particular peak area as a fraction of the area of a peak that was unaffected by the treatment, i.e.

$$\frac{\text{Peak area X}}{\text{Peak area B}} \times 100\%$$

Peak B was chosen as a reference for both fractions because it remained constant regardless of the treatment.

## VI. Blood Flow Studies

In order to measure the extent of blood flow reduction after ligation and, more importantly, the level of reperfusion when the ligature is released, radioactively-labelled microspheres were injected during the different treatment periods. Two isotopes were used, $^{113}Sn$ and $^{57}Co$. In order for each animal to serve as its own control, we divided the study into two sections. The first studied the reduction of blood flow as a

result of ligation and the second measured the extent of reperfusion after 40 min of ligation. In this way, three treatment periods could be studied (control, ligation, reperfusion) using only two isotopes. Two groups of 3 animals were subjected to the same surgical procedures as outlined in the section entitled "Operative Technique".

Microsphere preparation

$^{57}Co$ and $^{113}Sn$-labelled microspheres (15 ± 3 μ) were obtained from New England Nuclear suspended in physiological saline. These were vortexed for 20 min before adding them to a 10% Ficoll solution (with the addition of 0.05% chlorbutanol as a preservative) containing a drop of Tween 80 to prevent aggregation. Immediately before use, the $^{113}Sn$ microspheres were diluted again with the same vehicle so that each injection contained approximately 126,000 microspheres. Because of their higher activity, the $^{57}Co$-labelled microspheres were diluted to a lesser extent resulting in a total of 25,000 microspheres per injection.

When the haemodynamic variables had stabilized after surgery, $^{113}Sn$-labelled microspheres, prepared as described above, were injected directly into the left ventricular cannula and rinsed through with saline. Just prior to injection, reference blood from the carotid artery was withdrawn at a constant rate (0.7 ml/min) with a Harvard Apparatus pump and this was continued for at least one minute after completion of microsphere injection. This procedure provided the estimate of control blood flow. After 3-5 min had elapsed, the left circumflex coronary artery was occluded.

Group I (ligation study) - after 10 min of coronary ligation reference blood flow was withdrawn and the second isotope was injected ($^{57}Co$) in the same manner as the first. Three minutes later the heart was removed and plunged into cold, buffered high $K^+$ solution.

Group 2 (reperfusion study) - after 40 min of ligation and 10 min of reperfusion, the $^{57}Co$ was injected via the left ventricular cannula just after the withdrawal pump was switched on. A few minutes later, the heart was removed and immersed in cold, buffered, high $K^+$ solution.

After removal, both groups of hearts were re-ligated and perfused retrogradely with cold buffer to visualize the occluded zone. The radioactivity of the well-trimmed occluded zone, the non-occluded left ventricle, the right ventricle and the kidneys was counted on a Beckmann Gamma 8000 gamma radiation counter. A summary of the protocol and a sample calculation are shown in Appendix III.

As shown in Table V, this method of coronary ligation reduced the flow in the tissue supplied by the left circumflex artery (i.e., the occluded zone) to 6.2 ± 2.2% (mean ± S.E. n = 3) of the flow measured in non-ischaemic left ventricle. Release of the ligature permitted a return of blood flow; reperfusion resulted in flow rates of 110 ± 15% (mean ± S.E., n = 3) in this experimental model.

## VII. Electron Microscopy

Sample preparation for electron microscopy requires consistent technique and carefully prepared solutions in order to avoid generation of tissue artifacts. The techniques used in the ultrastructural study (the effect of D-600 and propranolol pretreatment on reperfusion injury) were a synthesis of procedures from several current references describing methods of tissue fixation.[106-108]

Additional information was received from Mr. Laszlo Veto, Senior Electron Microscopy Technician at the Department of Biology, University of

Table V. Blood flow in the ischaemic and reperfused left ventricle

| Treatment | Blood Flow[a] | |
|---|---|---|
| A. Control ($^{113}Sn$) --- Ligation ($^{57}Co$) | 85 ± 6.4 | 6.2 ± 2.2 |
| B. Control ($^{113}Sn$) --- Reperfusion ($^{57}Co$) | 91 ± 17 | 110 ± 15 |

Values are expressed as mean ± S.E.

n = 3 for each treatment

[a] Blood flow = $\frac{\text{BF ischaemic LV}}{\text{BF normal LV}} \times 100\%$

British Columbia. Mr. Veto kindly did the subsequent embedding, sectioning, staining and photography of the samples. He was given no information regarding the treatments and all samples were coded so that the photography was done in a blind manner.

Sample Preparation (For further details regarding solution composition and preparation, see Appendix II)

After the hearts were removed, perfused, and the occluded zone isolated, a small section (approx. 3 $mm^3$) of ischaemic or control subendocardium was placed in a Petri dish filled with 4°C buffered 3% glutaraldehyde and minced with a scalpel. After one hour at 4°C the tissue was transferred to small vials containing fresh 3% buffered glutaraldhyde using a wide-mouth Pasteur pipette and the vial was allowed to warm to room temperature for the second hour of fixation. After transfer, the tissue was minced again until the blocks were approximately 0.5 $mm.^3$ The small size of the tissue blocks permits maximum absorption of the poorly-penetrating secondary fixative, osmium tetroxide ($OsO_4$). At least 20 samples per heart were processed. The tissue was washed with 3-4 changes of 4°C buffer (no glutaraldehyde) and stored in the refrigerator until all samples were ready for $OsO_4$ fixation (approximately 3 weeks). The buffer was changed each week to prevent any microorganism growth, although this precaution may have been unnecessary because cacodylate is an arsenic compound and does not support bacterial growth.

After all 8 samples had been collected (2 per treatment group), all the tissue blocks were post-fixed in $OsO_4$, dehydrated and embedded in one day. All were subjected to the following treatments with the exception that one half of the tissue blocks (approx. 10) of each sample were stained for 45 min at room temperature with 0.8% ruthenium red - a dye that has an

affinity for mucopolysaccharide. The ruthenium red was removed with three washes of buffer, 30 min per wash. All samples were then post-fixed with the addition of cacodylate-buffered 1% $OsO_4$ for 30 min on ice followed by 30 min at room temperature. Excess $OsO_4$ was removed with two, 5 min changes of water.

Dehydration was accomplished by replacing the water with increasing concentrations of ethanol; 10, 20, 30, 40, 50, 60, 70, 85, 95% each for 2-3 min. For complete dehydration, the 95% ETOH was replaced with 2 changes of 100% ETOH for 30 min each. The embedding plastic is miscible with propylene oxide therefore 3:1, 1:1, 1:3 ETOH(100%):PrOX(100%) (v/v) solutions were added for 2-3 min each after the 100% ETOH. Finally 2 changes of 100% propylene oxide for 20 min each completed the tissue fixation. Samples were given to L. Veto within one hour after which they were embedded in Epon 812. From the 10 blocks, two were randomly chosen and sections were cut with a Porter-Blum MT-2 ultramicrotome, stained with uranyl acetate and examined on a Karl Zeiss EM-10 electron microscope at 8 KV. Five grids were randomly selected and photographed at various magnifications. Assessment of the photographs was performed by Dr. M. Todd, Department of Anatomy, University of British Columbia, using a randomized, blind protocol. Various features on the micrographs were scored according to a pre-arranged scoring system in which 0 = normal, 1 = mild abnormality, 2 = moderate abnormality, and 3 = severe abnormality.

Light Microsopy

In order to determine the uniformity of tissue sample preparation, i.e., that the myocardial sections did not contain mixed areas of damaged and normal tissue, thick sections were cut and stained with toluidine blue to be examined with a light microscope. Tissue blocks other than those used for electron microscopic analysis were sectioned. The sectioning and staining

were done by Mr. L. Veto of the Department of Zoology and the light microscopic photography by Dr. W. K. Ovalle, Department of Anatomy, University of British Columbia. The sections were photographed (negative magnifications 80X) on Kodak black and white film and printed on 3" x 5" paper for a final magnification of X260.

## VIII. Phospholipid Extraction and Separation

Two different procedures were used to separate the various classes of phospholipids. In the preliminary experiments, extraction was followed by a one-dimensional thin layer chromatographic (TLC) procedure. For the D-600 and propranolol and the 0-60 min ligation study, a two-dimensional TLC system was adopted in order to separate phosphatidylserine and phosphatidylinositol. Both procedures are outlined below.

### One-dimensional system

Extraction - One ml of membrane suspension was extracted with 2 ml chloroform:methanol ($CHCl_3$:MeOH, 2:1) (v/v) for 30 min on ice. The suspension was centrifuged at 12,000 g x 5 min and the supernatant decanted into a glass centrifuge tube. The pellet was extracted with an additional 2 ml of $CHCl_3$:MeOH for 30 min on ice, and, after centrifugation, the organic phases were pooled and washed three times using 0.5 ml of 0.75% NaCl. Each wash was followed by centrifugation and the aqueous phase discarded. The $CHCl_3$:MeOH extract was then evaporated to dryness under a stream of nitrogen and the residue resuspended with 200µl $CHCl_3$:MeOH and spotted on an aluminum-backed silica gel thin layer chromatographic plate (Merck, Silica gel 60 $F_{254}$, 0.2 mm thickness). The solvent system consisted of $CHCl_3$:MeOH:glacial $CH_3COOH$:$H_2O$ (v/v/v/v, 25:15:4:1). Ninhydrin and iodine vapour were used sequentially to visualize the spots.

Phosphorus Analysis

The plates were scraped and the gel analyzed for inorganic phosphorus using a modification of the method of Bartlett.[109]

Two-dimensional System

The extraction procedure was identical to that described above but the extract was chromatographed in 2 dimensions using a modification of the technique of Yavin and Zutra.[110] After the first solvent - $CHCl_3$:MeOH:$NH_3$ (v/v/v, 35:15:2.5) - had evaporated, the plate was put in the second solvent system 90° from the first direction. The second solvent system consisted of $CHCl_3$:$CO(CH_3)_2$:$CH_3OH$:glacial $CH_3COOH$:$H_2O$ (v/v/v, 25:10:5:7.5:2.5). The spots were visualized and analyzed for inorganic phosphorus as outlined in the previous section.

Statistical Analyses

Results were analyzed using a one-way analysis of variance followed by Tukey's test to assess specific group differences at $P < 0.05$. Percentage data were transformed with the arcsin square root transformation before any analysis was done.[111] The chi-square test for k independent variables was used to analyze the fibrillation data from the 20 minute ligation study.

## MATERIALS

Pentobarbitone sodium, $OsO_4$ ampoules, and propylene oxide were obtained from B.D.H.. Acids, solvents and standard ion solutions for atomic absorption spectrometry were all reagent grade (except the $CHCl_3$ and $CH_3OH$ which were spectrophotometric grade). Enzyme-grade sucrose (Ultra-pure) was from Schwarz-Mann. Gel electrophoresis materials came from E-C apparatus and the Silica gel plates (60 $F_{254}$) for thin-layer chromatography and electron microscopy-grade glutaraldehyde were obtained from Merck (Darm-stadt). J.B. EM Services was the source of the cacodylate. All other chemicals were reagent grade from Sigma Chemical Co.

# RESULTS

## I. Preliminary Studies

A preliminary study was undertaken in order to establish the effects of ischaemia on the activity of membrane-bound marker enzymes. The effects of ischaemia on two mitochondrial marker enzymes, namely cytochrome c oxidase and mitochondrial azide-sensitive ATPase are shown in Figure 1. The insensitivity of the cytochrome c oxidase activity to ischaemia contrasts markedly with the rapid decrease in the ATPase activity following coronary ligation. The decrease in mitochondrial ATPase activity reached a plateau of 40% of control at approximately 20 minutes post-ligation.

A similar pattern of progressive enzyme inhibition with increasing periods of ligation was seen with both the sarcolemmal $Na^+,K^+$-ATPase and $K^+$-NPPase activities (Figure 2). These measurements reflect the functional capability of the ouabain-sensitive sodium pump - the $Na^+$ and $K^+$-stimulated ATP hydrolysis and the terminal $K^+$-dependent step of ATP hydrolysis, respectively.[99] Both were affected to approximately the same extent by ischaemia.

In order to investigate the reversibility of these enzyme alterations, another group of animals were subjected to 60 minutes of reperfusion of the ischaemic area after various periods of coronary occlusion. The results, shown in Table VI, indicate that the effects of ischaemia on the mitochondrial ATPase activity are largely reversible. Even after 60 minutes of coronary ligation, reperfusion for one hour restored 80% of control mitochondrial ATPase activity. In contrast, recovery of the sarcolemmal $Na^+,K^+$-ATPase and $K^+$-NPPase activities with reperfusion was not as great as for the mitochondrial ATPase. In addition, as the period of ischaemia increased, the percent recovery of the sarcolemma enzyme activity

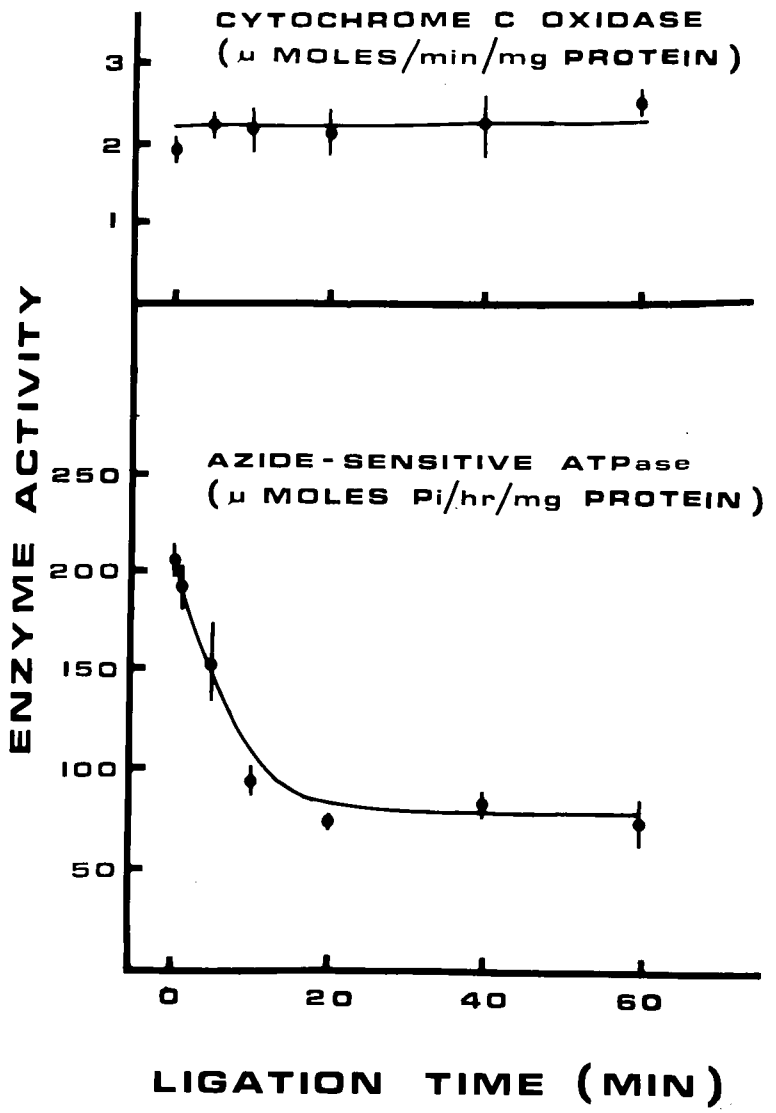



Figure 1. Mitochondrial membrane-bound enzyme activity after 0-60 min of ligation. Assays were performed on purified mitochondria and isolation procedures and details of the assays are described in the Methods section. No Triton X-100 addition was made to the assay medium. The top section shows the lack of effect of ischaemia on cytochrome c oxidase activity. In contrast, the ischaemia-induced inhibition of azide-sensitive ATPase activity is shown on the bottom half. All values are expressed as mean ± S.E., n = 4-6 for each point.

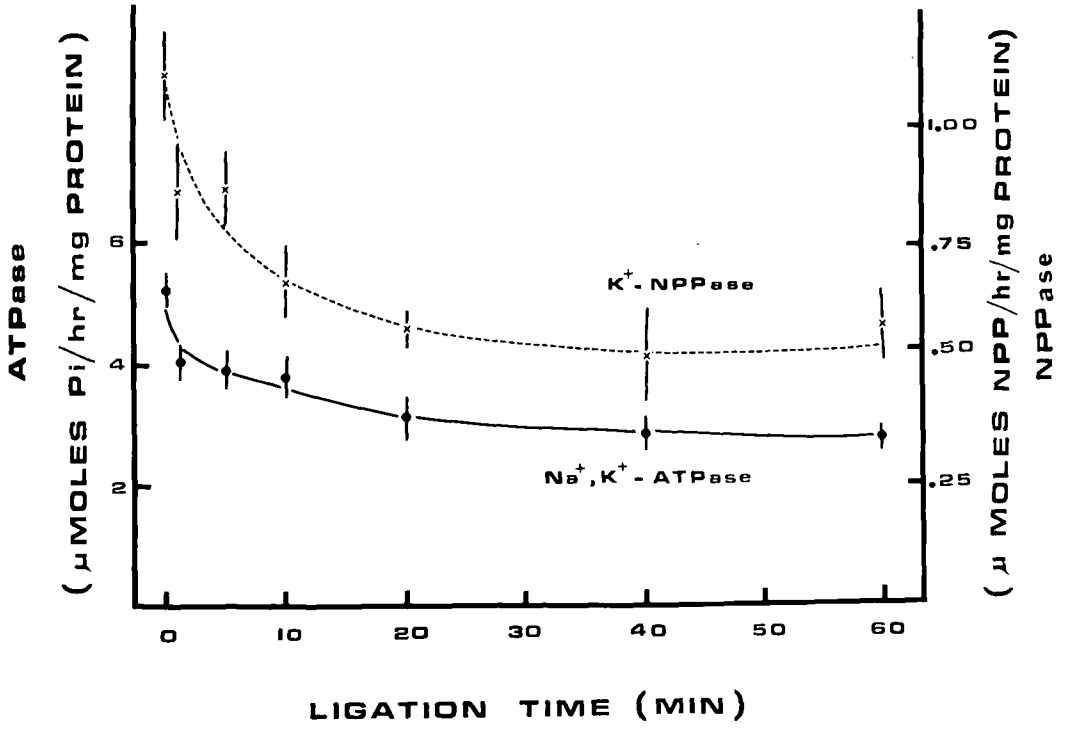



Figure 2. Sarcolemmal enzyme activity in response to ischaemia. Both $Na^+,K^+$-ATPase activity (•——•) and $K^+$-NPPase (x-----x) decrease with increasing periods of ligation. Results are expressed as mean ± S.E., and n = 4-6 for each group. No Triton X-100 was added to the assay medium.

Table VI. Degree of recovery of mitochondrial and sarcolemmal ATPase activities after various intervals of ischaemia followed by a 60 minute period of reperfusion

| Ligation Period (min) | % Recovery on reperfusion | | |
|---|---|---|---|
| | Mitochondria | Sarcolemma | |
| | azide-sensitive ATPase | $Na^+K^+$-ATPase | $K^+$-NNPase |
| 0 | 100 | 100 | 100 |
| 10 | 91 | 79 | 58 |
| 20 | 113 | 60 | 42 |
| 30 | 104 | 37 | 37 |
| 40 | 82 | 35 | 33 |
| 60 | 80 | 25 | 16 |

Each value represents the mean of 2 animals per group

For absolute values of enzyme activities, see Fig. 1 and 2.

decreased. Basal ($Mg^{++}$-dependent) enzyme activities also showed progressive, irreversible decreases in activity; however, these were not as severe as those seen with the $Na^{+}$ and $K^{+}$-stimulated activity (data not shown). For example, the inhibition of mitochondrial ATPase activity produced by 30 minutes of coronary ligation was completely reversed by a 60 minute reperfusion period, whereas after similar reperfusion $Mg^{++}$-dependent, sarcolemma ATPase and NPPase activities were restored to 70% and ion-stimulated ATPase activities recovered only 37% of control activity.

$Mg^{++}$ and $K^{+}$ interact with the $Na^{+},K^{+}$-ATPase enzyme complex at the inner and outer sides of the membrane respectively,[99] so that changes in the kinetic properties of ion stimulation could reveal the location of sarcolemmal enzyme alterations. Figure 3 ($K^{+}$) and Figure 4 ($Mg^{++}$) show that although 60 minutes of ischaemia reduced NPPase activity ($V_{max}$) at all concentrations of the substrate when compared with control, it did not cause any change in the apparent affinity ($K_M$) for either $K^{+}$ or $Mg^{++}$ (see insets of Figures 3 and 4). Furthermore, there was no significant difference between the ligated and the ligated/reperfused groups further supporting the hypothesis that the decrease in sarcolemmal enzyme activity after 60 minutes of occlusion is irreversible.

The tissue content of ATP was assessed to determine the time course of ATP depletion relative to alterations in the integrity of these sarcolemmal and mitochondrial enzymes. ATP content (expressed as nmoles ATP/mg dry weight) was measured in hearts subjected to 0-60 minutes of ligation with or without reperfusion. The results indicate that ischaemia causes a fall in ATP levels that reaches a plateau at approximately 30 minutes post-ligation (Table VII). Reperfusion does not restore ATP and after 20 to 40 minutes of ischaemia, ATP levels were even lower in the reperfused animals (although the difference was not statistically significant).

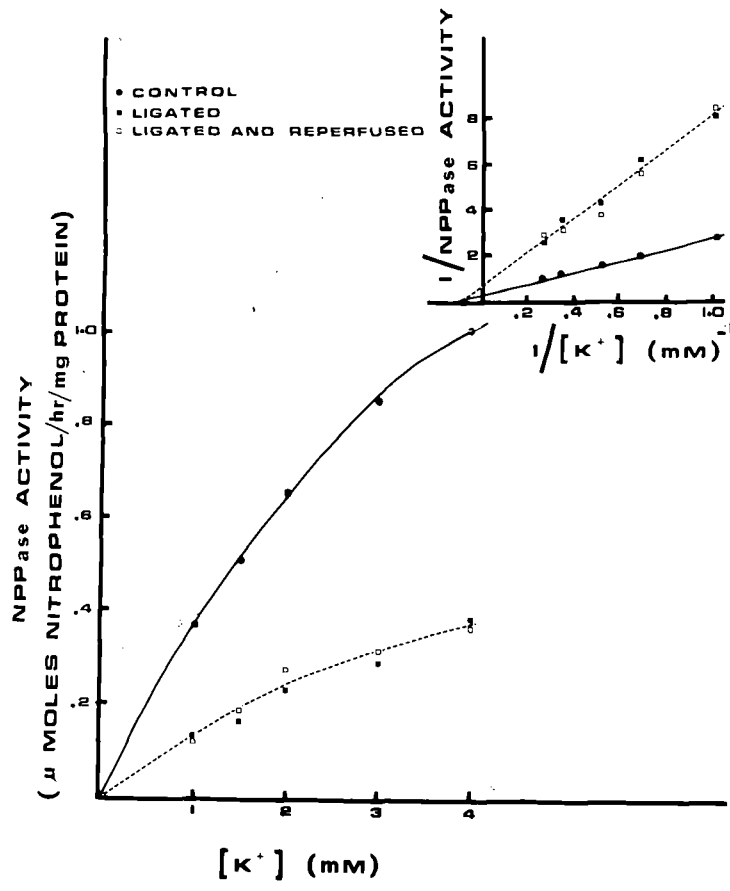



Figure 3. Stimulation by $K^+$ of $Mg^{2+}$-dependent NPPase activity in sarcolemmal vesicles isolated from control, 60 min ligated and 60 min ligated and reperfused myocardia. Each point represents the mean of two separate membrane preparations. Inset: double reciprocal plots of the same data.

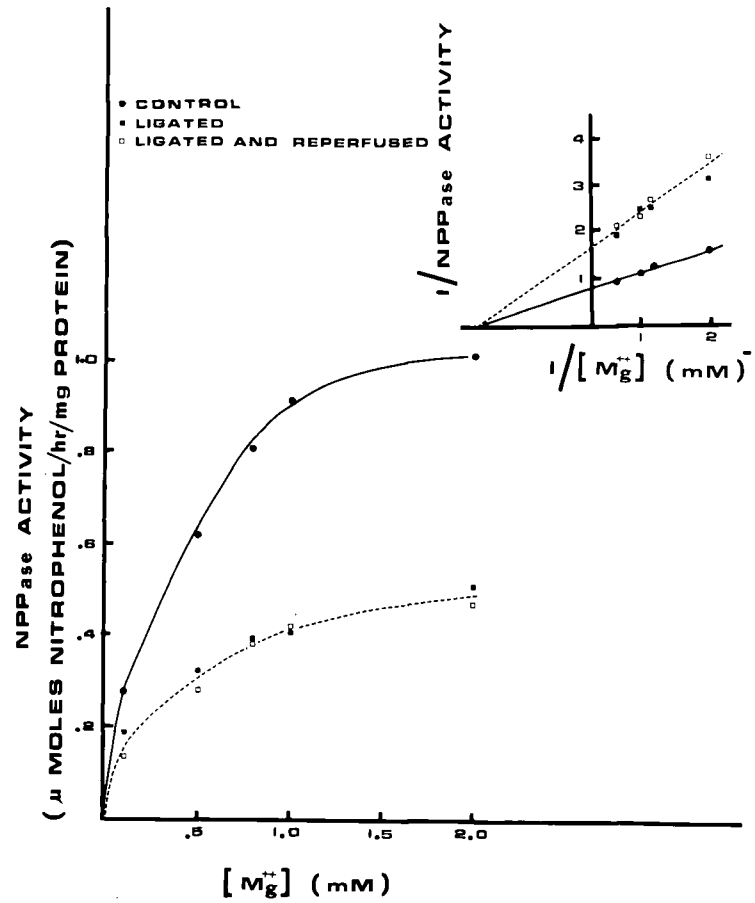



Figure 4. Activation by $Mg^{2+}$ of $K^{+}$-stimulated NPPase activity in sarcolemmal vesicles prepared from control; 60 min ligated and 60 min ligated and reperfused myocardia. Each point represents the mean of two separate membrane preparations. Inset: double reciprocal plots of the same data.

Table VII. ATP contents of hearts subjected to various periods of ligation and ligation plus reperfusion

| Ligation period (min) | ATP $\frac{\text{nmoles}}{\text{mg dry weight}}$ | |
|---|---|---|
| | Ligation | Ligation + Reperfusion[a] |
| Control | 11.6 ± 2.0 | |
| 5 | 9.8 ± 0.3 (2) | 12.1 ± 1.4 (2) |
| 10 | 6.9 ± 1.2 (3) | 7.0 ± 1.8 (2) |
| 20 | 4.9 ± 0.5 (4) | 3.1 ± 0.3 (2) |
| 40 | 3.1 ± 0.3 (3) | 1.4 ± 0.2 (2) |
| 60 | 0.6 ± 0.3 (3) | 1.7 ± 0.3 (3) |

All number are expressed as mean ± S.E.

( )= number of animals per group

[a] Reperfusion period was 60 min for all groups

The lack of restoration of the sarcolemmal enzyme activities suggested that they may have undergone an irreversible chemical modification during ischaemia; however, polyacrylamide gel electrophoresis of both mitochondrial and sarcolemmal membranes prepared from ligated or ligated/reperfused myocardia revealed no obvious qualitative differences in polypeptide profiles relative to control (data not shown).

The lipid-dependence of both the sarcolemmal and mitochondrial ATPase activity[112,113] prompted the examination of the effects of ischaemia on two of the major phospholipid classes of the myocardial membrane, namely phosphatidylcholine and phosphatidylethanolamine. Sixty minutes of ligation with or without reperfusion produced no quantitative change in either of these phospholipids in either the sarcolemmal or the mitochondrial membrane fractions (Table VIII - only sarcolemmal data shown).

II. 20 Minute Ligation with Drug Pretreatment

The effects of several different agents on the biochemical alterations produced by 20 minutes of coronary occlusion without subsequent reperfusion was investigated in this group of experiments. A 20 minute ligation period was chosen because the results of the previous study and work by other investigators[6,31] had suggested that this period of ischaemia is at the transition between reversible and irreversible cell injury and, indeed, marked membrane abnormalities were evident in both mitochondrial and sarcolemmal membranes after 20 minutes of coronary ligation. The ability of

Table VIII. Phospholipid content of sarcolemmal membranes isolated from control, ligated, and ligated and reperfused myocardia

| | % Total Lipid Phosphorus | |
|---|---|---|
| Treatment | Phosphatidylethanolamine | Phosphatidylcholine |
| Control | 45.4 ± 3.4 | 37.2 ± 6.9 |
| Ligation | 47.8 ± 6.8 | 40.8 ± 6.1 |
| Ligation and Reperfusion | 51.0 ± 3.2 | 32.2 ± 3.7 |

All values are expressed as mean ± S.D.

n = 2 for each group.

pretreatment with each of the following agents to reduce these biochemical alterations was studied:

| | |
|---|---|
| 3.0 mg/kg dl-propranolol HCl | (PROP) |
| 3.0 mg/kg pranolium Cl | (PRAN) |
| 0.15 mg/kg (±)-D-600 HCl | (D-600) |
| 30 mg/kg imidazole | (IMID) |
| 2.0 mg/kg chlorpromazine | (CPZ) |

The various rationale for the use of these agents in the treatment of myocardial ischaemic injury are listed in Table IX.

In order to eliminate the confounding effects of drug action on the variables independent of their possible benefit in reducing cell injury, the above dose of each drug was administered to one of 5 groups of animals (n = 4-6). These drug treated animals underwent all surgical procedures except ligation and are heretofore referred to as "drug controls". The results of the drug control study are shown in Table X. Interestingly, the administration of propranolol in the absence of myocardial ischaemia caused an increase in both the mitochondrial ATPase activity and the ATP content of the tissue, although only the rise in ATP content was statistically significant. The reduction of $(dP/dt)_{max}$ force and heart rate by propranolol pretreatment would be expected to reduce myocardial oxygen consumption $(\dot{M}VO_2)$[55] and this may account for the apparent "ATP-sparing" effect. The reduction in heart rate may be the major determinant because D-600 pretreatment also reduced contractility but did not increase ATP. No increase was seen with pranolium therefore non-β-blocking effects of propranolol were also not the cause of salutary effects on ATP content.

Inasmuch as these drugs (with the exception of propranolol) caused no alteration in the baseline biochemical measurements, we examined their

Table IX. Drugs used as pretreatment prior to a 20 minute period of coronary ligation

| Agent | Rationale |
|---|---|
| Propranolol | - β-adrenergic antagonist, "membrane stabilizer", antiarrhythmic.<br>- may block effects of catecholamine release in M.I. |
| Pranolium | - Quaternary derivative of propranolol, shares properties of parent compound except β-blockade. |
| Imidazole | - thromboxane synthesis inhibitor (thromboxanes may initiate coronary vasoconstriction and platelet aggregation). |
| Chlorpromazine | - "lysosomal membrane stabilization", inhibitor of intracellular phospholipases. |
| D-600 (methoxyverapamil) | - $Ca^{2+}$ channel-blocker, peripheral vasodilator, coronary dilator. |

Table X. Drug effects on biochemical variables after 20 min sham ligation.

"Drug controls"

| Treatment | Mito[a] ATPase | $K^+$-NPPase[b] | ATP nmoles ATP / mg dry wt | Water Content (%) |
|---|---|---|---|---|
| Control - Saline | 193 ± 6 | .99 ± .08 | 13.6 ± 1.9 | 79.3 ± .4 |
| Sham 20 min ligation, pretreatment with: | | | | |
| Propranolol (3 mg/kg) | 222 ± 5 | .95 ± .05 | 25.1 ± 2.3[c] | 79.1 ± .4 |
| D-600 HCl (.15 mg/kg) | 209 ± 3 | 1.00 ± .07 | 17.0 ± 2.5 | 80.0 ± .2 |
| Imidazole (30 mg/kg) | 208 ± 8 | .93 ± .07 | 19.7 ± .88 | 80.5 ± .6 |
| Chlorpromazine HCl (2 mg/kg) | 208 ± 14 | .86 ± .07 | 16.4 ± 1.4 | 80.3 ± .2 |
| Pranolium Cl (3 mg/kg) | 196 ± 7 | .91 ± .05 | 15.4 ± 1.6 | 80.3 ± .2 |

All values are expressed as mean ± S.E.

n = 4-6 per group except Control animals, where n = 7-8.

[a] enzyme activity = μmoles $P_i$/hr/mg protein.

[b] enzyme activity = μmoles nitrophenol/hr/mg protein.

[c] Significantly greater than control at $P < 0.05$

actions on the established indices of ischaemic injury. D-600 administration prior to ligation was the only treatment that significantly reduced the ischaemia-induced decrease in the mitochondrial ATPase activity as compared with that of saline-treated ligated animals. Enzyme activity was maintained at 75 % of control levels compared with the saline-treated group which retained only 35% of control activity (Figure 5), confirming the results of our earlier experiments. Propranolol and chlorpromazine pretreatment resulted in slight but non-significant protection of mitochondrial ATPase activity from the effects of ischaemia.

Drug effects on the sarcolemmal enzyme activities were more complex. D-600 significantly prevented the decrease in the $Mg^{++}$-ATPase activity (non-$Na^+$ and $K^+$-stimulated) of the sarcolemma (data not shown); however, it only slightly protected $Na^+$ and $K^+$-stimulated ATPase activity (Figure 6). Interestingly, despite the functional relationship between the $Na^+$,$K^+$-ATPase and $K^+$-NPPase enzymes, D-600 pretreatment did not reduce the ischaemia-induced decrease in $K^+$-NPPase activity.

As seen in the previous study, the ATP level of the myocardium is severely reduced after 20 min of occlusion. As shown in Figure 7, D-600 was the only drug which significantly preserved ATP levels in ischaemic myocardium; untreated hearts contained 30% of control levels whereas D-600-pretreated hearts maintained the level at 60% of control. In contrast, despite the higher pre-occlusion levels of ATP in propranolol-treated hearts, ischaemia reduced the ATP content to the same extent as in the saline-treated animals. Thus, the increase in high energy phosphate in the propranolol-treated drug controls appeared to confer no obvious benefit during the subsequent ligation period.

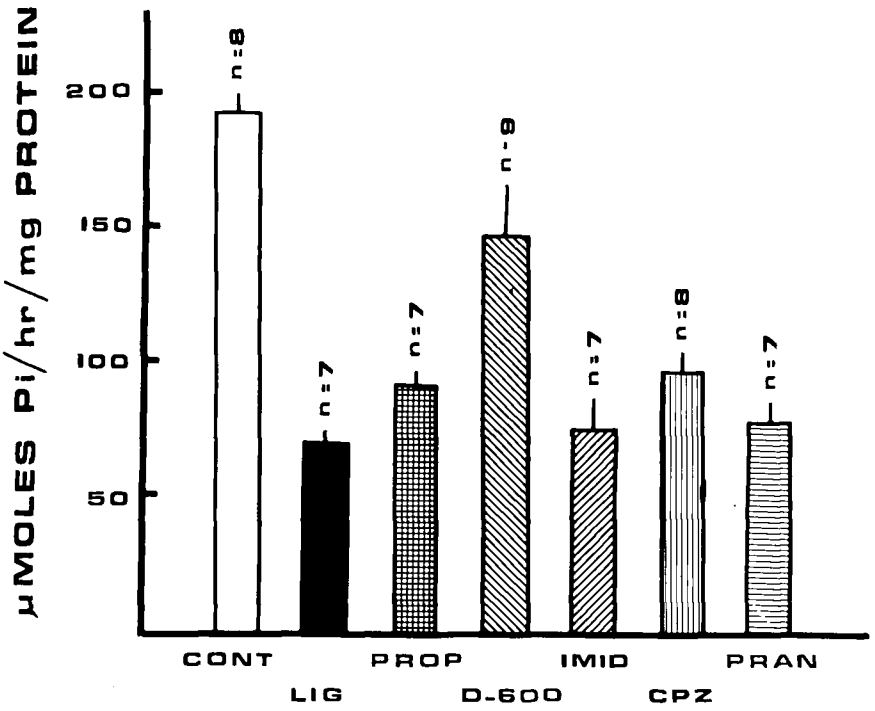



Figure 5. Mitochondrial azide-sensitive ATPase activity in mitochondria isolated from hearts subjected to 20 min of ligation with or without drug pretreatment. CONT = sham-operated control; LIG = ligated with saline pretreatment, 3 mg; D-600 = D-600 pretreatment, 0.15 mg/kg; IMID = imidazole pretreatment, 30 mg/kg; CPZ = chlorpromazine pretreatment, 2 mg/kg; PRAN = pranolium pretreatment, 3 mg/kg.

Drugs were administered as described in the Methods section. Bars represent mean ± S.E. Control and D-600 values are significantly different from all other groups but not significantly different from each other ($P < 0.05$). Statistical methods are outlined in the Methods section.

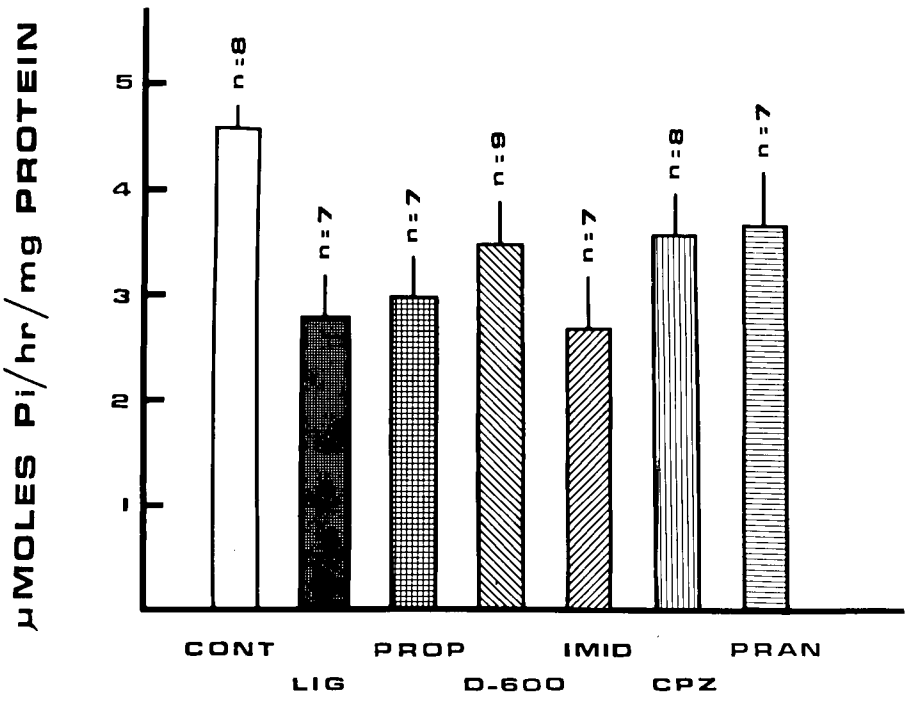



Figure 6. $Na^+,K^+$-ATPase activity in sarcolemma isolated from hearts subjected to 20 min of ligation with or without drug pretreatment. Abbreviations are the same as in Figure 5. Only LIG and IMID are significantly different from control ($P < 0.05$). Bars represent mean ± S.E.

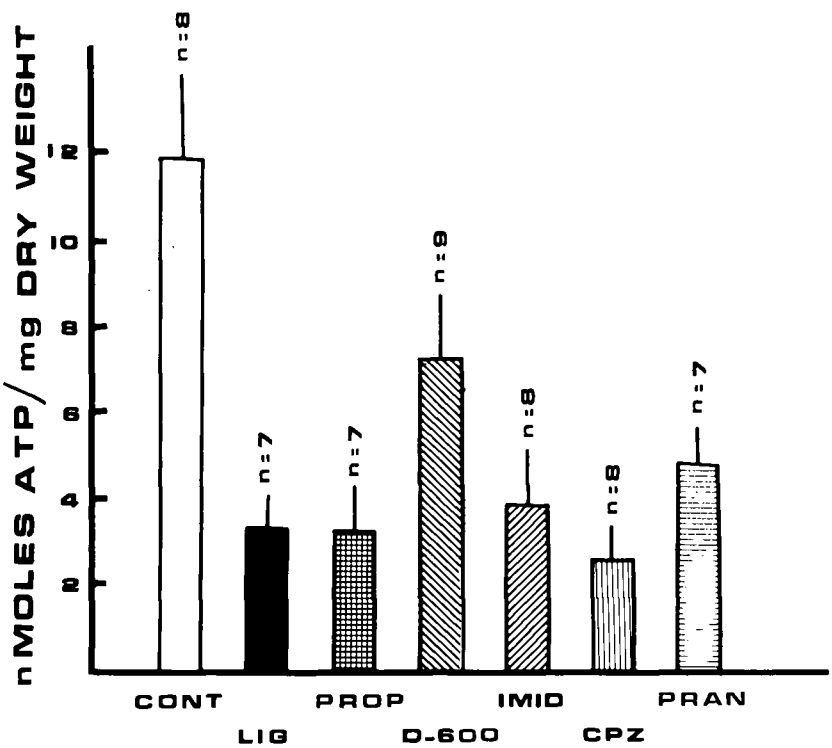



Figure 7. ATP content of hearts subjected to 20 min of ligation with or without drug pretreatment. Abbreviations are the same as in Figure 5. All groups except D-600 have significantly decreased ATP levels compared to control animals. Bars represent mean ± S.E.

Increases in tissue water were not apparent after this relatively short period of ischaemia, as shown by the equivalent water content of all groups (Table XI). However, this does not exclude the possibility that there was a re-distribution of water from the extracellular to intracellular space or vice versa.

Unexpectedly, these agents had variable effects on the incidence of ventricular fibrillation during the ligation period. Ventricular fibrillation was identified by the characteristic random electrical signal on lead II of the electrocardiogram coupled with a precipitous fall in systemic blood pressure to 0 mm Hg. An arbitrary incidence scale was devised and a score was given to each animal at the end of the experiment according to the following protocol:

0 = no fibrillation
1 = 1 episode
2 = 2-5 episodes
3 = >5 episodes

The mean score ± S.E.M. for each group is shown in Table XII. Of particular significance are the actions of D-600 and propranolol. Whereas pretreatment with D-600 completely abolished ventricular fibrillation, propranolol-treated animals consistently had greater than 5 episodes of this arrhythmia during the ischaemic period. This is significantly greater than the incidence of fibrillation in the animals which received only saline. Chlorpromazine also significantly increased the number of episodes; however, the frequency per animal was less and the results more variable when compared to propranolol. Imidazole and pranolium had no apparent effect on the mechanism of this. The frequency of fibrillation was not simply related to a lower systolic pressure in the propranolol group because the D-600 group had equivalent reductions in blood pressure and the incidence of fibrillation in this group was zero.

Table XI. Tissue water content in hearts subjected to 20 minutes of ligation with or without drug pretreatment

| Treatment | n | Water content (%) |
|---|---|---|
| Control | 7 | 79.3 ± 0.4 |
| 20 min ligation | 6 | 81.0 ± 0.5 |
| 20 min ligation, pretreatment with: | | |
| dl-propranolol HCl | 7 | 80.0 ± 1.2 |
| D-600 HCl | 8 | 81.7 ± 1.2 |
| Imidazole | 6 | 81.0 ± 0.4 |
| Chlorpromazine HCl | 8 | 81.6 ± 0.4 |
| Pranolium Cl | 6 | 80.8 ± 0.8 |

All values are expressed as mean ± S.E.

Table XII. Incidence of ventricular fibrillation in rabbits subjected to 20 minutes of ischaemia with or without drug pretreatment

| Treatment | n | Incidence of fibrillation (arbitrary scale)[a] |
|---|---|---|
| Control | 8 | 0.0 ± 0.0 |
| 20 min ligation | 7 | 0.4 ± 0.2 |
| 20 min ligation, pretreatment with: | | |
| dl-Propranolol HCl | 7 | 3.0 ± 0.0[b] |
| D-600 HCl | 8 | 0.0 ± 0.0[b] |
| Imidazole | 7 | 0.7 ± 0.4 |
| Chlorpromazine HCl | 8 | 2.1 ± 0.4[b] |
| Pranolium Cl | 7 | 0.4 ± 0.2 |

All values are mean ± S.E.

[a] scale: 0 = 0 episodes of fibrillation during ligation
1 = 1 episodes of " " "
2 = 2-5 episodes of " " "
3 = >5 episodes of " " "

[b] Significantly different from the expected value after 20 min ligation (0.4) at $P < 0.05$ using chi-squared test for k independent samples.

III. 40 Minute Ligation and 60 Minute Reperfusion with D-600 and Propranolol Pretreatment

This study concerned the ability of D-600 to attenuate the additional damage caused by 60 minutes of reperfusion after 40 min of ischaemia in view of this drug's salutary effects on 20 minutes of ischaemic injury. Furthermore, because rapid accumulation of calcium has been shown to accelerate ischaemic damage, the calcium channel-blocking properties of D-600 might be expected to provide additional benefit during the reperfusion period. In addition, many of the mechanisms whereby the calcium channel antagonists are thought to benefit the ischaemic heart are shared by the beta-adrenergic blocker, propranolol. Consequently, we also examined the actions of this agent on ischaemic/reperfusion damage using the same model of myocardial ischaemia as in our previous studies; however, a lower dose of this drug was used. The animals were randomly allocated into the following groups:

Sham-operated control (CONT)

40 min ligation followed by 60 min of reperfusion - saline pretreatment (L+R)

40 min ligation and 60 min reperfusion - 0.1 mg/kg D-600 HCl administered 20 min prior to ligation (D-600)

40 min ligation and 60 min reperfusion - 1.5 mg/kg propranolol HCl administered 20 min prior to ligation (PROP)

Mitochondrial ATPase activity

Previous experiments have demonstrated that 60 min of reperfusion restores 75% of mitochondrial ATPase activity after a 40 min ligation and this was confirmed by the present results (L+R; Figure 8). In these experiments, the decrease in mitochondrial azide-sensitive activity was prevented by pre-treatment with either D-600 or propranolol. Drug pretreatment

maintained the mitochondrial enzyme activity at 100% of control values, indicating significant preservation of mitochondrial membrane enzyme integrity (Figure 8). Recall that neither D-600 nor propranolol had any effect on the enzyme activity independent of their actions on the reperfused myocardium, i.e., administration of the drugs to the CONT group did not change the baseline enzyme activities.

Sarcolemmal $Na^+$,$K^+$-ATPase and $K^+$-NPPase activities

Ischaemia-induced decreases in sarcolemmal membrane-bound enzyme activities were shown to be less easily reversed by reperfusion compared to the mitochondrial ATPase. Results from the present experiments further support the hypothesis that the sarcolemma is more susceptible to irreversible ischaemic damage in that enzyme activity is minimally restored even after 60 min of reperfusion. In contrast to the beneficial effects of both drugs on the mitochondrial ATPase, the specific activity of the sarcolemmal enzymes is preserved only by D-600 (Figure 9). With no drug pre-treatment (L+R), the $Na^+$,$K^+$-ATPase activity was only 40% of the value of the CONT group, whereas D-600 pre-treatment resulted in a greater sarcolemmal enzyme activity - 73% of the CONT value. Although the effects of propranolol qualitatively paralleled those of D-600 (activity = 60% of CONT), they did not reach statistical significance. However, propranolol-treated hearts consistently required a greater number of defibrillating countershocks in comparison with any of the other groups which might have obscured a beneficial effect of the drug. Consequently, another group of sham-operated hearts (n = 5) were given 10 countershocks over a period of 10 minutes (one per minute) in order to assess the extent of shock-induced tissue damage. The low levels of stored energy involved in the D.C. countershocks (0.5 Watt-sec) are well below the threshold required to produce tissue damage

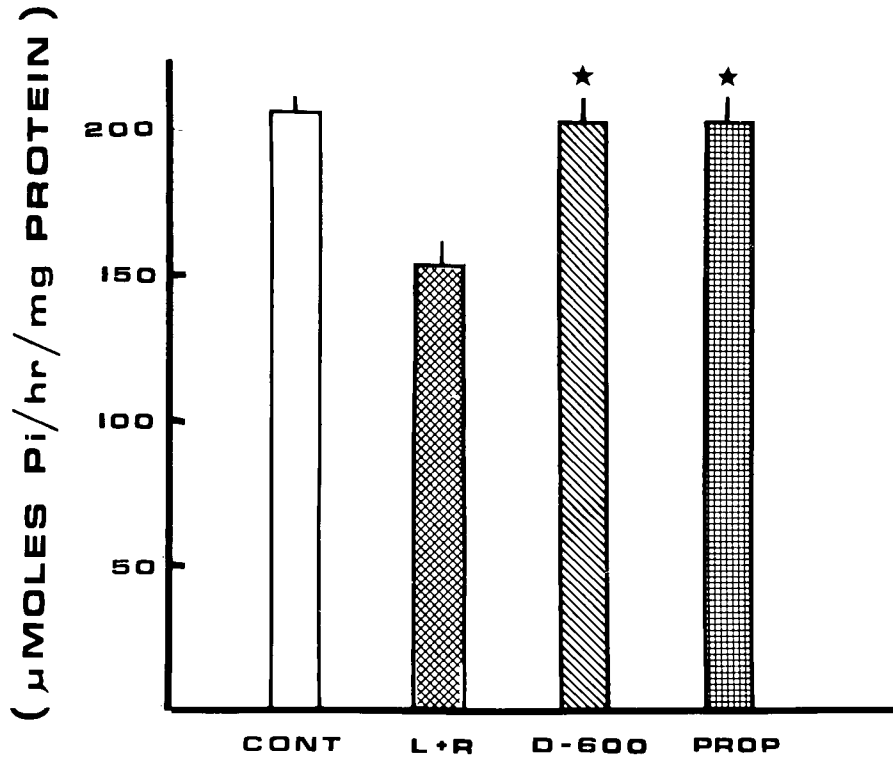



Figure 8. Mitochondrial azide-sensitive ATPase activity in mitochondria isoated from hearts subjected to a sham 40 min ligation plus 60 min reperfusion (CONT), 40 min ligation plus reperfusion with saline pretreatment (L+R), 40 min ligation plus reperfusion with D-600 (D-600) or propranolol (PROP) pretreatment. Bars represent the mean ± S.E. for each group. n = 8 for all groups. Only L+R is significantly decreased from CONT, ✱ = no significant difference from CONT at $P < 0.05$.

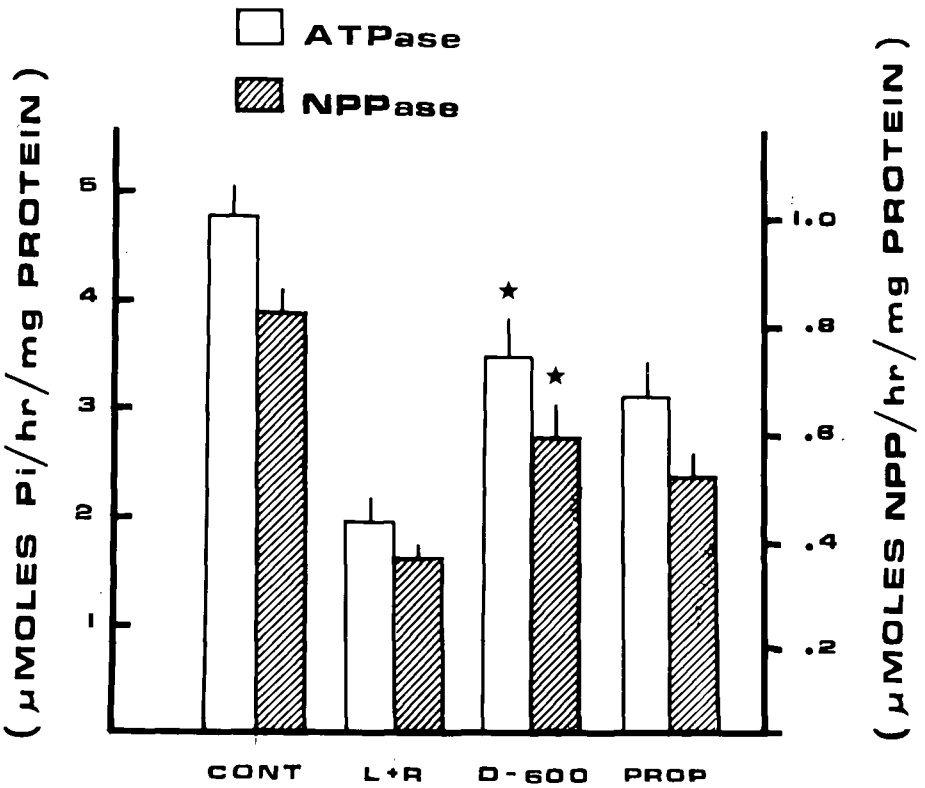



Figure 9. $Na^+,K^+$-ATPase and $K^+$-NPPase activity of sarcolemma isolated from hearts subjected to 40 min ligation followed by 60 min of reperfusion with or without drug pretreatment. Bars represent mean ± S.E. for each group, and n = 8-9 for each group. L+R and PROP significantly reduced from CONT. ✱ = D-600 significantly different from L+R ($P < 0.05$).

Table XIII. Membrane activities of hearts subjected to 10 D.C. countershocks of 0.5 Watt-sec each during sham-ligation and reperfusion.

| Treatment | Mitochondria | Sarcolemma | |
|---|---|---|---|
| | azide-sensitive ATPase[a] | $Na^+K^+$-ATPase[a] | $K^+$-NPPase[b] |
| Control | 206 ± 4 | 4.75 ± .79 | 1.09 ± .07 |
| Countershocked | 199 ± 8 | 5.58 ± .22 | 1.19 ± .18 |

All values are expressed as mean ± S.E., n=5

[a] enzyme activity = μmoles Pi/hr/mg protein.

[b] enzyme activity = μmoles nitrophenol/hr/mg protein.

directly.[91] The sarcolemmal enzyme activities were unaffected by the countershocks (Table XIII), so that the lack of protection of propranolol was not the result of an artifactual decrease caused by exposure to the defibrillating countershocks.

ATP

The negative inotropism and off-loading that results from administration of both of these drugs would be expected to decrease the $O_2$ consumption of the normal and ischaemic heart, thereby preserving stores of high energy phosphates. This, in turn, should be expected to allow the cell to maintain homeostasis with a concomitant protection of organelle integrity. Interestingly, despite the beneficial effect of these drugs on membrane enzyme activities, neither D-600 nor propranolol significantly preserved myocardial levels of ATP (Figure 10). Marked high energy phosphate depletion occurs very early in ischaemic injury [17,114] and, in our experiments, untreated hearts (L+R) retain only 6% of control ATP levels, i.e., over 90% of the ATP has been lost. The ATP levels of the D-600 and PROP groups were not significantly increased over those of the untreated (L+R) animals although D-600 pretreatment produced a slightly greater effect when compared with propranolol attaining 15% and 9% of CONT values, respectively.

Water content

Intracellular oedema can result from the reperfusion of damaged myocardial tissue and hearts from the L+R, D-600 and PROP groups all had some water gain upon reperfusion when compared to the CONT animals (assessed by gravimetric analysis). However, only the propranolol-treated hearts showed statistically significant increases in total tissue water (Table XIV). The water content of the CONT hearts was 80.3 ± 0.57%, in contrast to the PROP group - 84.7 ± 1.2%. The defibrillatory countershocks do not account for

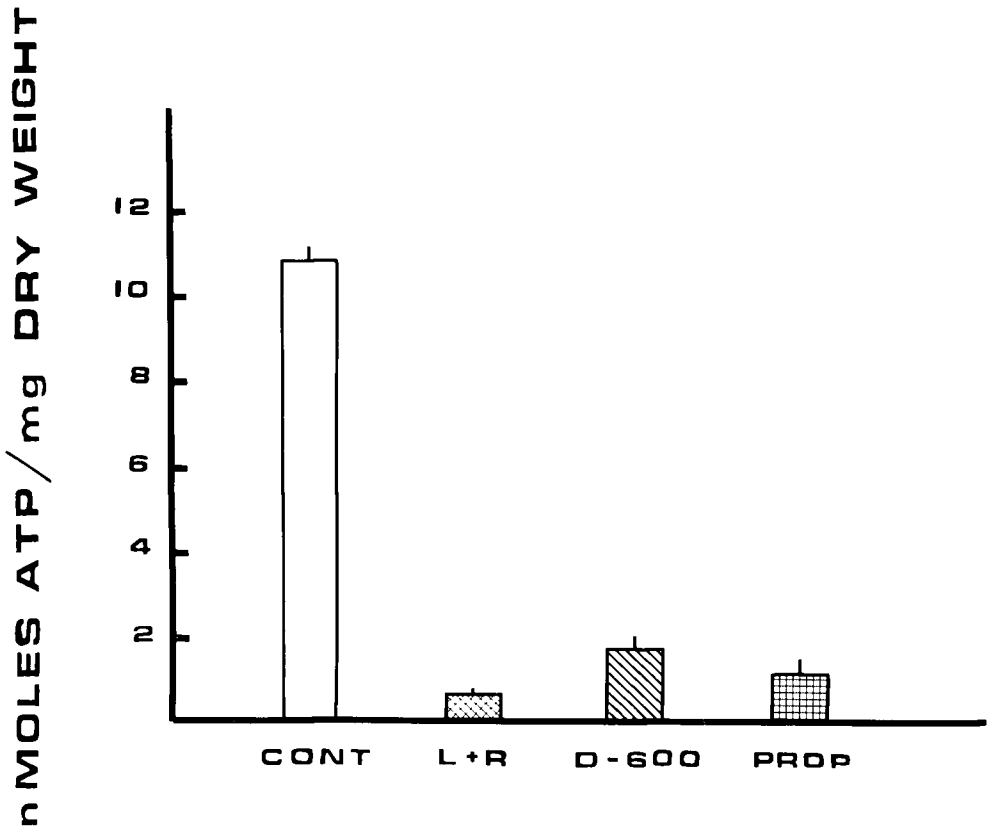



Figure 10. ATP content of hearts subjected to 40 min of ligation followed by 60 min of reperfusion with or without drug pretreatment. Bars represent mean ± S.E. for each group, and n = 8-9 for each group. All groups are significantly decreased from CONT at $P < 0.05$.

Table XIV. Tissue water content in hearts subjected to 40 min of ligation and 60 min of reperfusion with or without drug pretreatment

| Treatment | n | Water content (%) |
|---|---|---|
| CONT | 8 | 80.3 ± 0.6 |
| L+R | 7 | 82.3 ± 1.3 |
| D-600 + L+R | 8 | 82.6 ± 0.8 |
| PROP + L+R | 8 | 84.7 ± 1.2[a] |
| SHOCKS + L+R | 5 | 81.6 ± 0.9 |

All values are expressed as mean ± S.E.

[a] Significantly increased above control values at $P < 0.05$.

the elevated water content of these hearts because the water content in the SHOCKS group was not significantly increased above CONT values (81.6 ± 0.79%). The presence of large membrane-bound vacuoles within the muscle fibres seen in the electron micrographs of these tissue samples also suggest that the propranolol-treated hearts had water gain upon reperfusion (see Figure 19).

Ion levels

Intact membrane structure is intimately connected with the maintenance of ion levels within the myocardial cell. Therefore, drug interventions which preserve membrane integrity should reduce the passive flux of ions down their concentration gradients occurring as a result of ischaemic/reperfusion injury. In addition, both D-600 and propranolol would be expected to reduce the influx of calcium through the $I_{SI}$ channels: D-600 by a direct blockade of the slow channels and propranolol by an antagonism of β-adrenergic receptor activation.

Calcium - Pre-treatment of rabbits with either D-600 or dl-propranolol significantly reduced the calcium entry associated with reperfusion injury (Figure 11). CONT calcium levels were 12.2 ± 2.0 nmoles/mg dry weight (n = 8). Ligation followed by reperfusion resulted in a 4-fold increase in calcium levels to 48.5 ± 5.9, in contrast to the moderate increases seen in the D-600 and PROP hearts to levels of 23.2 ± 2.5 and 29.8 ± 6.2 nmoles/dry weight, respectively. Thus, D-600 and propranolol administration resulted in a 50% reduction in the amount of calcium taken up by the ischaemic myocardium.

Sodium - In contrast to the similar effects of the drugs on reducing the cell calcium concentration, only D-600 administration was effective in significantly preventing sodium accumulation (Figure 11). This may be

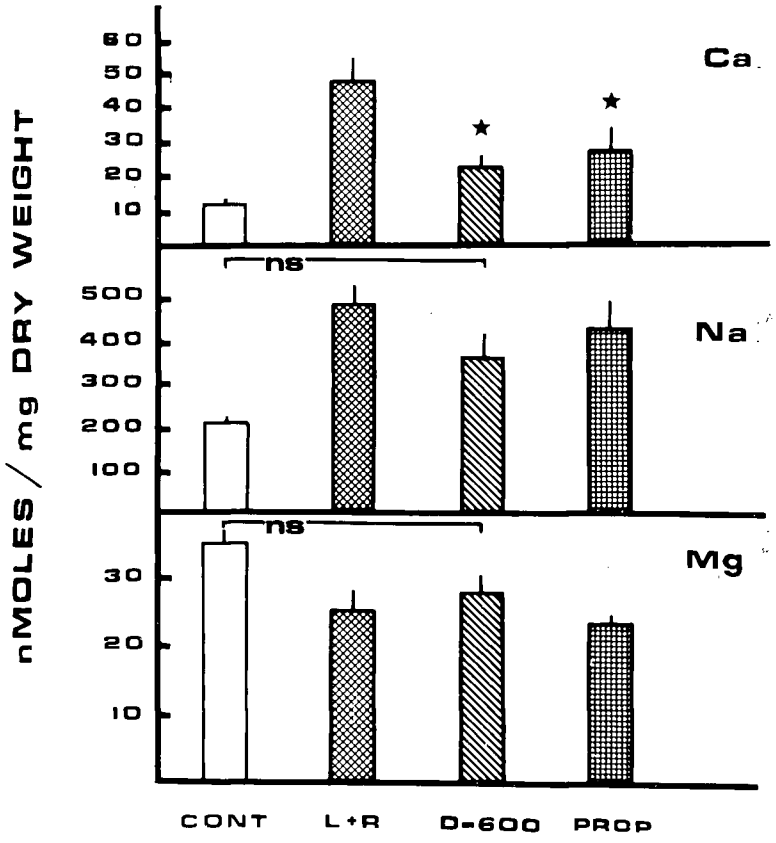



Figure 11. Ca , Na , and Mg levels of hearts subjected to 40 min of ligation plus 60 min of reperfusion with or without drug pretreatment. Bars represent mean ± S.E., and n = 8-9 for each group. Ca - ✱ = significantly reduced from L+R value. Na -D-600 group is not significantly different from control and L+R and PROP are significantly increased from control levels ($P < 0.05$). Mg -same as for $Na^+$.

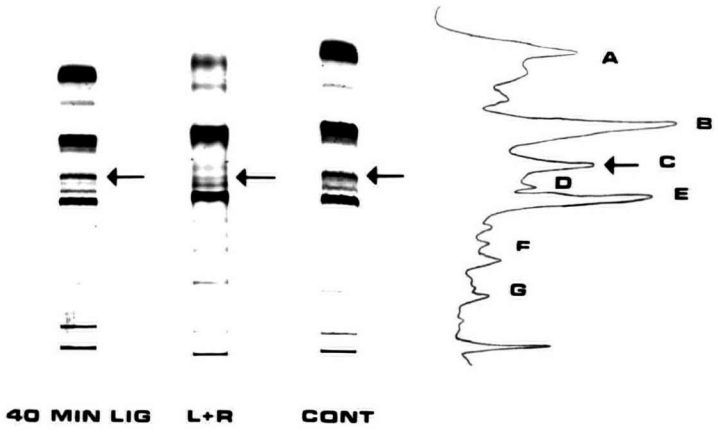
attributable to either its superior preservation of $Na^+,K^+$-ATPase activity or sarcolemmal integrity or both.

Magnesium - The effects of the drugs on the magnesium contents of these hearts were qualitatively similar to those obtained for the sodium ion (Figure 11). Reperfusion injury results in a loss of magnesium (see L+R) which was prevented by D-600 pretreatment but not by pretreatment with propranolol.

Potassium - Tissue levels of potassium did not differ from control, although the larger error seen in the treatment groups, in comparison with the control, may have masked subtle changes in potassium content. Given the significant decrease in sarcolemmal $Na^+,K^+$-ATPase activity due to reperfusion injury, one might have expected to see a significant decrease in the potassium concentrations. However, ischaemia-induced changes in tissue levels of $K^+$ (as opposed to changes in the extracellular concentration of this ion) have been shown by other investigators to be of a much smaller magnitude than those of sodium or calcium.[29,115]

SDS-gel electrophoresis

The irreversible decreases in enzyme activity and alterations in myocardial ion measurements suggested the possibly that the membrane protein and polypeptide profiles of these ischaemic hearts might be altered. Using more quantitative methods there was still no detectable change in the polypeptide profiles of the sarcolemma from any of the groups. However, an alteration was noted in the mitochondrial protein profile of the L+R group. The results of the densitometric scans of the gels containing the mitochondrial membrane fraction are shown in Figure 12. Solubilized protein from mitochondrial membranes from the ischaemic area show a significant decrease in




| | | Fractional Peak Area (Peak area X/Peak area B x 100) | | | | | |
|---|---|---|---|---|---|---|---|
| GROUP | n | A | C | D | E | F | G |
| CONT | 5 | 165 ± 16 | 24 ± 2.2 | 9.4 ± 3.7 | 58 ± 2.8 | 20 ± 1.1 | 16 ± 2.1 |
| L+R | 5 | 164 ± 38 | 5.0 ± 2.2* | 8.2 ± 1.3 | 55 ± 3.8 | 16 ± 3.7 | 15 ± 2.9 |
| D-600 | 4 | 183 ± 17 | 9.5 ± 2.9* | 6.0 ± 1.9 | 53 ± 4.2 | 17 ± 3.3 | 9.8 ± 1.1 |
| PROP | 4 | 189 ± 1.3 | 3.2 ± 0.7* | 11 ± 3.1 | 50 ± 5.5 | 21 ± 3.5 | 15 ± 1.3 |

Figure 12. SDS-gel electrophoresis of mitochondrial fractions isolated from hearts subjected to 40 min ligation only (40 MIN LIG), 40 min ligation plus 60 min reperfusion (L+R), sham 40 min ligation and reperfusion (CONT). Protein profiles are shown at the top left of the figure. The arrows show the band which is reduced after reperfusion (C) on both the gel and the densitometric scan (top right). The table at the bottom gives the normalized areas under the peaks of the scan for hearts subjected to ligation and reperfusion with or without drug pretreatment. Neither D-600 or PROP prevented the loss of band C.

the area of one of the protein peaks despite the equal amounts of protein applied to the gel. Neither D-600 nor propranolol pretreatment prevented the loss of this protein, whose molecular weight was estimated to be 55,000 by concurrent electrophoresis of protein standards of known molecular weight. As stated in the Methods section, the contamination of the mitochondrial membranes with sarcolemma was consistently 3% for all groups, thereby eliminating the possibility that differential sarcolemmal contamination could account for the observed decrease in a particular polypeptide.

Contractility

An indirect measure of the cardiac contractile force was obtained from the differentiated pressure wave signal of the left ventricle -- $dP/dt_{max}$ = the maximum rate of rise of left ventricular pressure development. Figure 13 shows the effect of drug treatment, ligation and reperfusion on $dP/dt_{max}$ as a percentage of the original contractility where initial $dP/dt_{max}$ = 100%. Both drugs reduced contractile force prior to ligation: D-600 to 55% of pre-drug value and propranolol to 80% of pre-drug $dP/dt_{max}$. Interestingly, despite the ability of the drugs to maintain subcellular structural integrity, there was no improvement in contractility upon reperfusion of ischaemic hearts. In all groups the $dP/dt_{max}$ reached only 50-60% of the CONT $dP/dt_{max}$, i.e., there was no significant difference in the contractility between hearts from the L+R, D-600 and PROP groups after 60 min of reperfusion. D-600 did seem to have a slightly salutary effect on contractility when compared with the other treatment groups, but this was not statistically significant. Table XV shows that the systolic arterial pressures of the L+R, D-600 and PROP groups were not significantly different so that changes in afterload probably did not affect the outcome. A confounding factor in this experiment arises when one considers that the

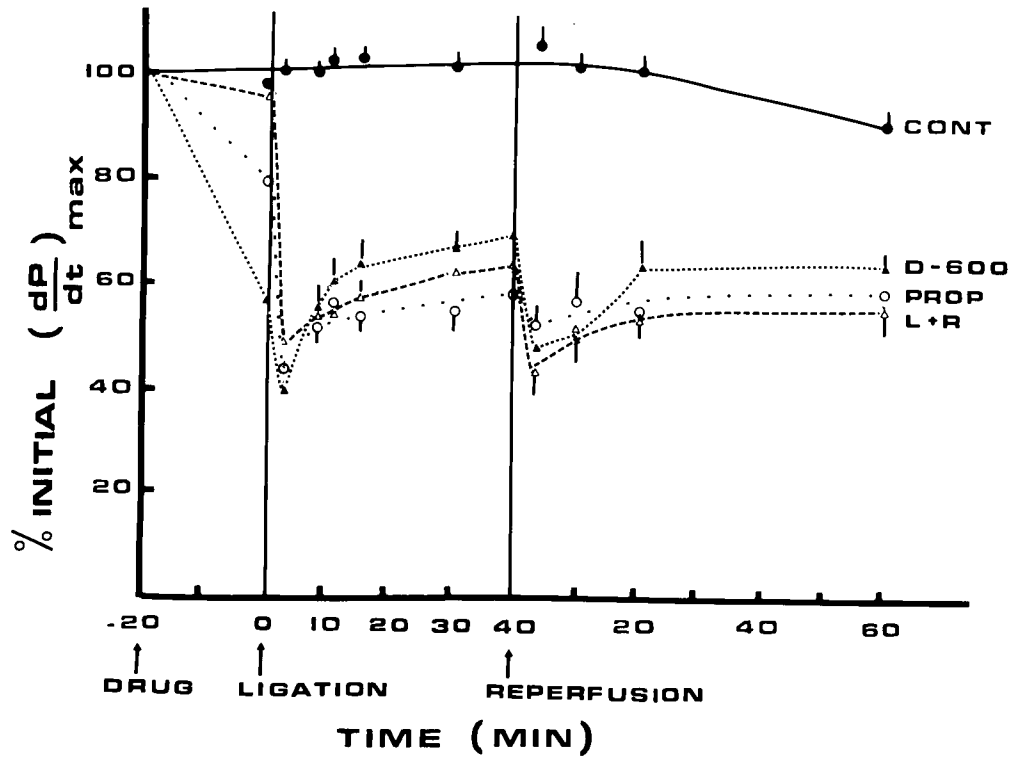



Figure 13. Percent initial $(dP/dt)_{max}$ of hearts during the period of drug treatment, ligation and reperfusion. Four groups are shown: ●—● (CONT), ▲·····▲ (D-600), o···o (PROP) and Δ---Δ (L+R). No significant difference in contractility was found between the L+R, D-600 and PROP groups at the end of the reperfusion period.

Table XV. Systolic blood pressure after various periods of reperfusion following 40 minutes of coronary ligation

| Treatment | n | Blood pressure (mm Hg) Minutes of reperfusion | | | |
|---|---|---|---|---|---|
| | | 3 | 10 | 20 | 60 |
| Control | 8 | 109 ± 4 | 109 ± 4 | 107 ± 4 | 104 ± 5 |
| L+R | 8 | 64 ± 5 | 69 ± 4 | 69 ± 3 | 70 ± 5 |
| D-600 | 8 | 63 ± 6 | 66 ± 6 | 74 ± 6 | 74 ± 4 |
| PROP | 8 | 64 ± 4 | 69 ± 5 | 70 ± 6 | 73 ± 6 |

All values are expressed as mean ± S.E.

negative inotropic actions of these agents may directly affect the contractility of the hearts at the end of the reperfusion period. In order to determine the time course of the negative inotropic effect of D-600 in our model, we administered D-600 (n = 5) to sham-operated rabbits using the same protocol as described in the Methods section. The study showed that $dP/dt_{max}$ had returned to control values 40 minutes after administration. Thus, for D-600 at least, the reduction in contractility at the end of the reperfusion period was probably not a result of direct effects of the drug on the myocardium (data not shown). However, it has been postulated that calcium channel-blockade is more effective on depolarized, ischaemic tissue.[116,117] Therefore, the reversal of the negative inotropic effects of D-600 in these sham-operated animals may have been more rapid than in the ischaemic rabbits. If D-600 was selectively depressing the contractility of the ischaemic zone, this effect may have masked any improvement in $dP/dt_{max}$.

Despite the reduced dose of propranolol in these animals when compared to the 20 min ligation study (1.5 mg/kg vs. 3.0 mg/kg), this group still had a greatly increased incidence of fibrillation that characteristically began at approximately 12 min post-ligation (similar to the L+R animals) and continued as an unstable fluctuation of sinus rhythm and fibrillation for approximately 10 min. This arrhythmia was not easily reversed by counter-shock (in contrast to the L+R animals). Similar to the previous study, the fibrillation was probably not the result of low perfusion pressure because the D-600 group had equally low systolic pressures and none of the hearts in the D-600 group fibrillated.

Ultrastructure

A potential problem in interpreting ultrastructural data arises when the experimental procedure creates inhomogeneous tissue damage. An advantage of using the rabbit model is that, unlike the dog, coronary ligation results in an ischaemic zone with a clearly defined border without interdigitation of normal tissue and ischaemic tissue.[118] This uniformity of damage was confirmed in our samples of thick sections examined by light microscopy (Figure 14 and 15).

The degree of ischaemic membrane damage determined by the biochemical measurements listed above was comparable with the appearance of the hearts in the electron micrographs. Sham-operated control hearts showed tightly-packed regular mitochondria with dense intramitochondrial matrices containing small distinct granules along the cristae and no evidence of oedema formation. The sarcotubular system appeared normal (Figures 16a, 16b). In addition, the sarcolemmal membrane and glycocalyx were not separated from the underlying structures and intercalated disks were closely apposed (Figure 16c).

L+R hearts showed great disruption with clearing of the mitochondrial space and fragmentation of the cristae and there were foci where complete destruction of the mitochondrion had taken place, as was evident by broken outer membranes (Figure 17a). In addition, ligation followed by reperfusion resulted in the appearance of amorphous intramitochondrial precipitates, which may be calcium salts or possibly denatured protein. Myofibrillar degeneration is evident and the sarcomeres are abnormally relaxed giving rise to prominent I-bands (Figure 17b). Figure 17c shows the areas where the glycocalyx had lifted entirely away from the cell surface. There is also severe clumping of nuclear chromatin.

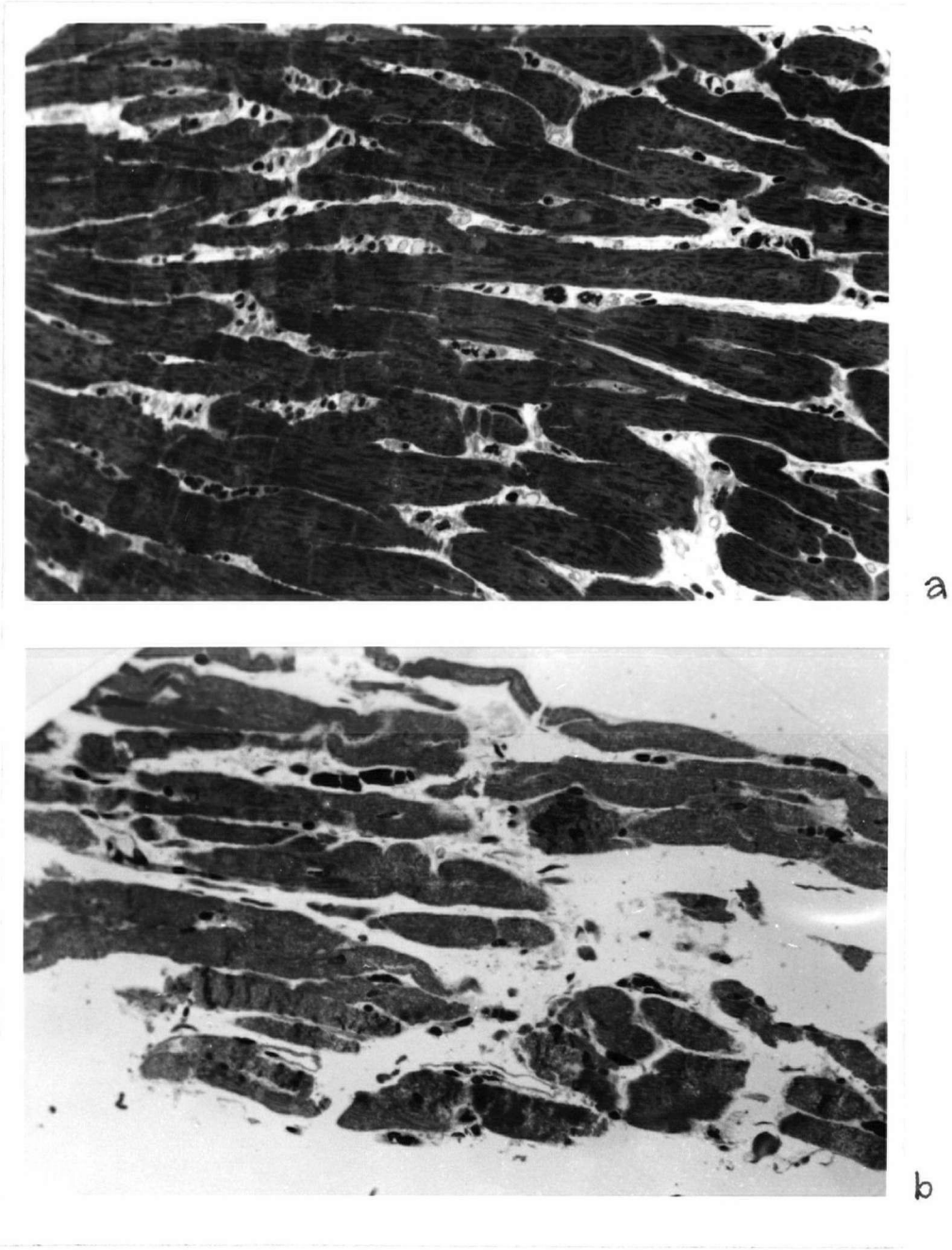
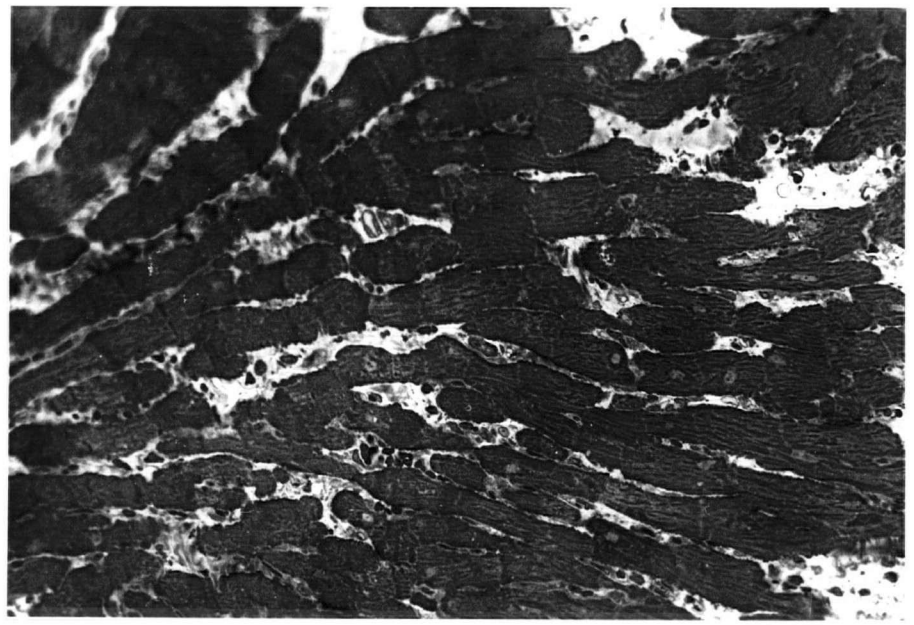



Figure 14a) Light micrograph of thick section from CONT tissue. X260.

b) Photograph (as above) of section of ligated and reperfused tissue (L+R)

Tissue has uniform appearance in both cases. Note the apparent loss of myofibrils in (b).



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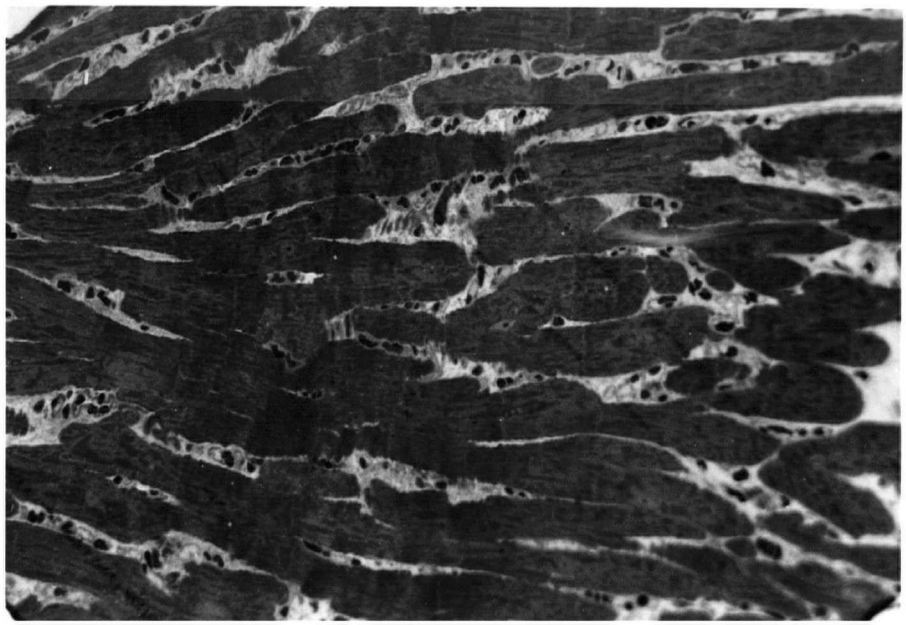


b

Figure 15a) Light micrograph of ligated and reperfused tissue pretreated with D-600. Staining was more difficult in this section. X260.

b) Light micrograph (as above) except that this heart was pretreated with propranolol.

Fairly homogenous damage to the tissue. Contraction bands are apparent in (b).

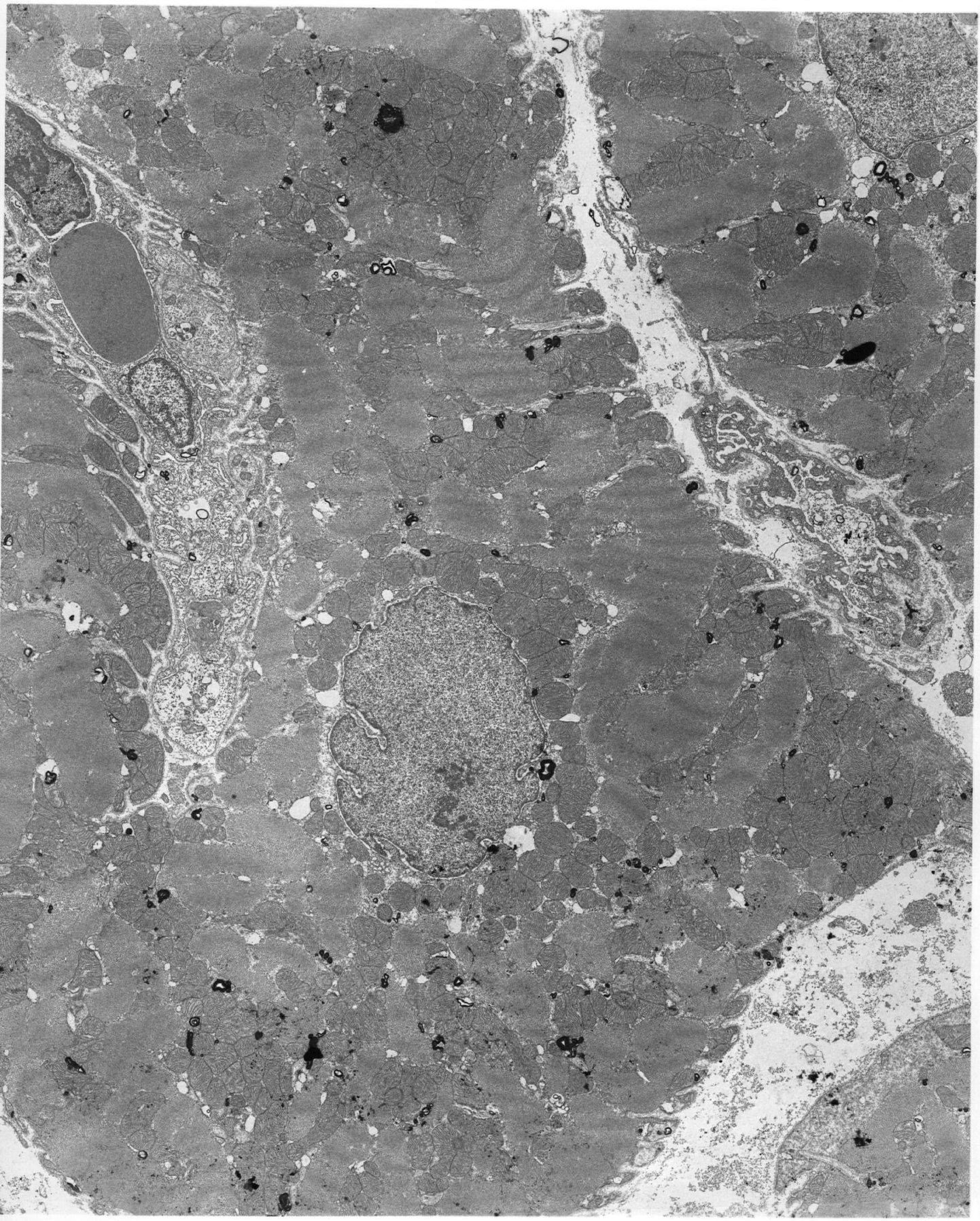
Figure 16. (CONT)

a) Electron micrograph of control tissue showing normal, tightly-packed mitochondria containing small, dense granules. Sarcotubular system is normal. Myofibrils do not appear to be abnormally contracted or relaxed and no myofibrillar degeneration is apparent. X30,000.

b) Control heart showing a large normal nucleus (centre) with diffuse chromatin. Sarcolemma and glycocalyx are attached to the underlying structure. Mitochondria have dense matrices and unbroken cristae. The dark myelin figures are artifacts produced by the ruthenium red stain. X6,000.

c) (top) Higher magnification of the sarcolemma and glycocalyx. X20,000. (bottom) Normal intercalated disk of a control heart. No separation of the cells is apparent. X20,000.

a



b

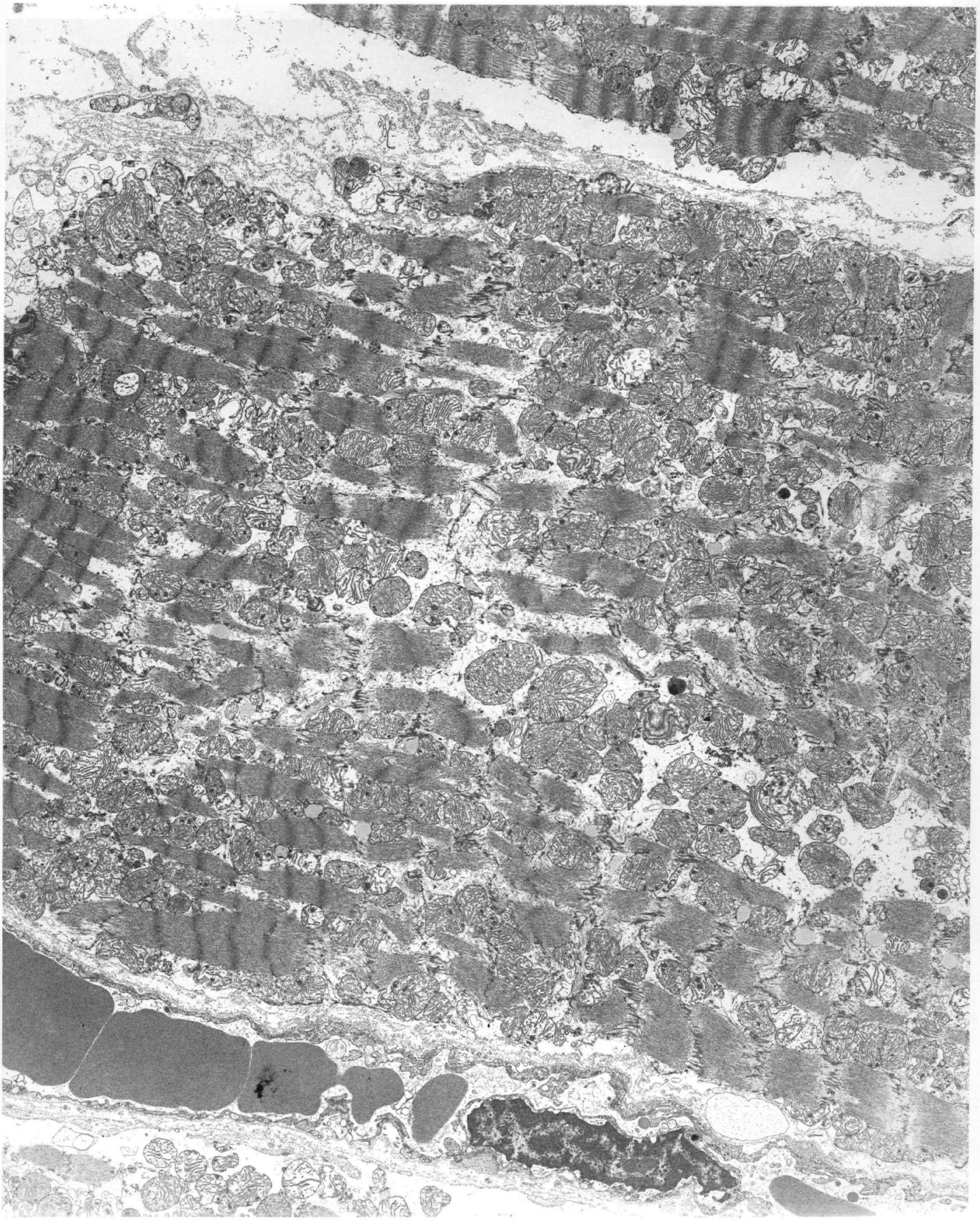
C

Figure 17. (L+R)

a) Electron micrograph of heart that received only saline pretreatment before 40 min ligation and 60 min reperfusion. Myofibrillar damage is evident. Mitochondria are grossly disrupted and some have been entirely lost. Intracellular oedema may be responsible for the increase in intramyofibrillar space. The intercalated disk (top right corner) appears normal. X6000.

b) Higher magnification showig the presence of amorphous intramitochondrial densities and relaxed myofibrils. X30,000.

c) In this photomicrograph, the sarcolemma/glycocalyx has pulled away from the underlying structures. There is a severely damaged nucleus with clumped chromatin shown in the centre of the page. Myofibrillar loss is apparent. X6,000.



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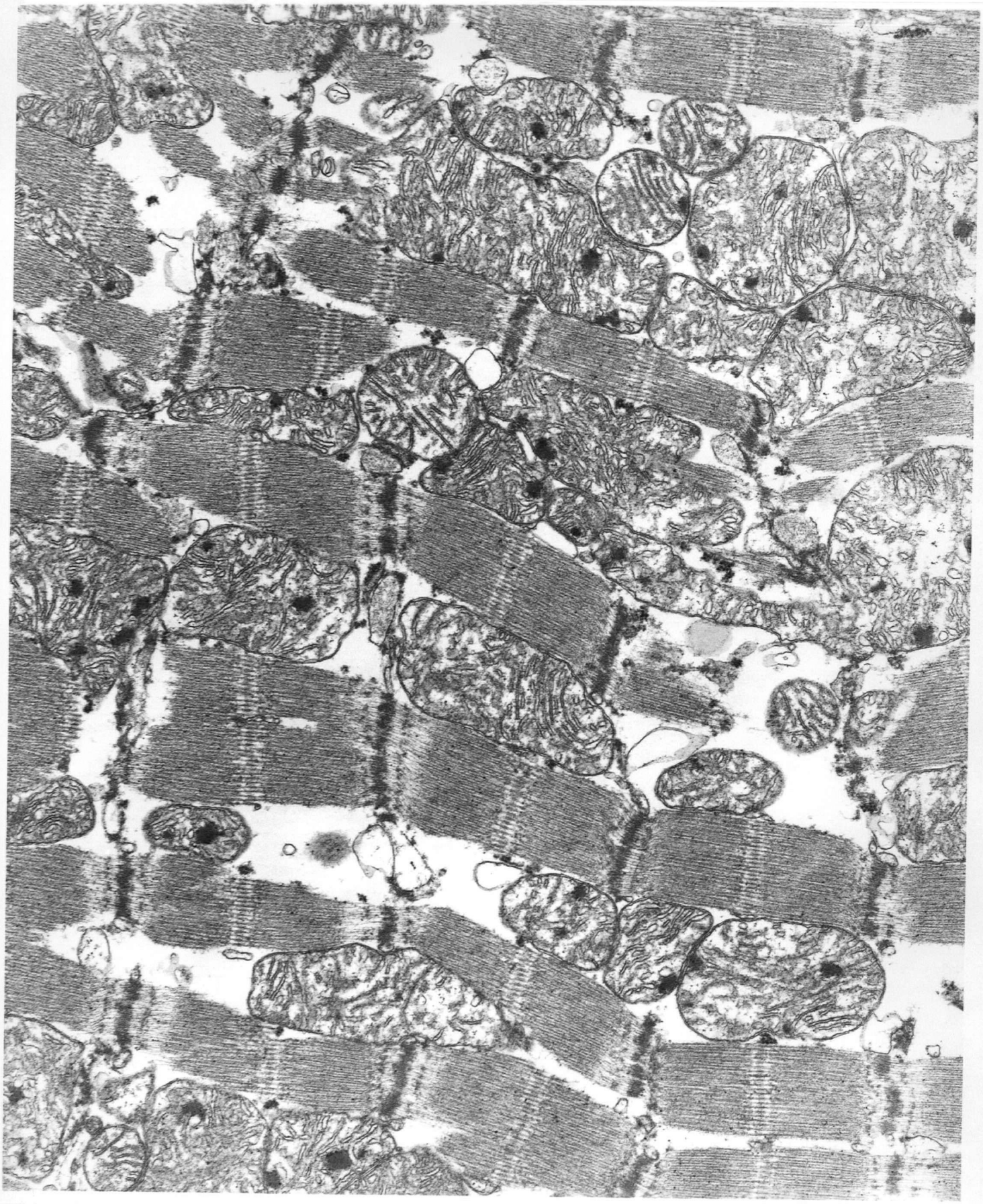


b

C

Samples from the D-600 treated hearts showed a marked improvement, when compared with the untreated hearts with dramatically less mitochondrial destruction and no separation of the sarcolemmal membrane from the underlying structures (Figure 18a and 18b). There was a noticeable clearing of the mitochondrial matrix in some cases, but the overall integrity was well preserved. In agreement with the decreased calcium levels in these hearts, there appeared to be fewer precipitates in the mitochondria of animals pre-treated with D-600 although myofibril contraction and relaxation was not entirely normal (Figure 18c).

The propranolol-treated rabbits also exhibited less myocardial damage from ligation and reperfusion than untreated animals (Figure 19a,19b). The maintenance of structural integrity appeared very similar to that of the D-600 hearts, i.e., near-normal mitochondrial structure, undisrupted sarcolemmal membrane, no intra-mitochondrial densities. However, more contraction bands appeared in this tissue. The feature of these hearts not seen in any of the other groups was the presence of membrane-bound vacuoles along the Z-lines (Figure 19c) implying an abnormality of the sarcotubular system and this was consistent with the increased tissue water in the propranolol-treated hearts.

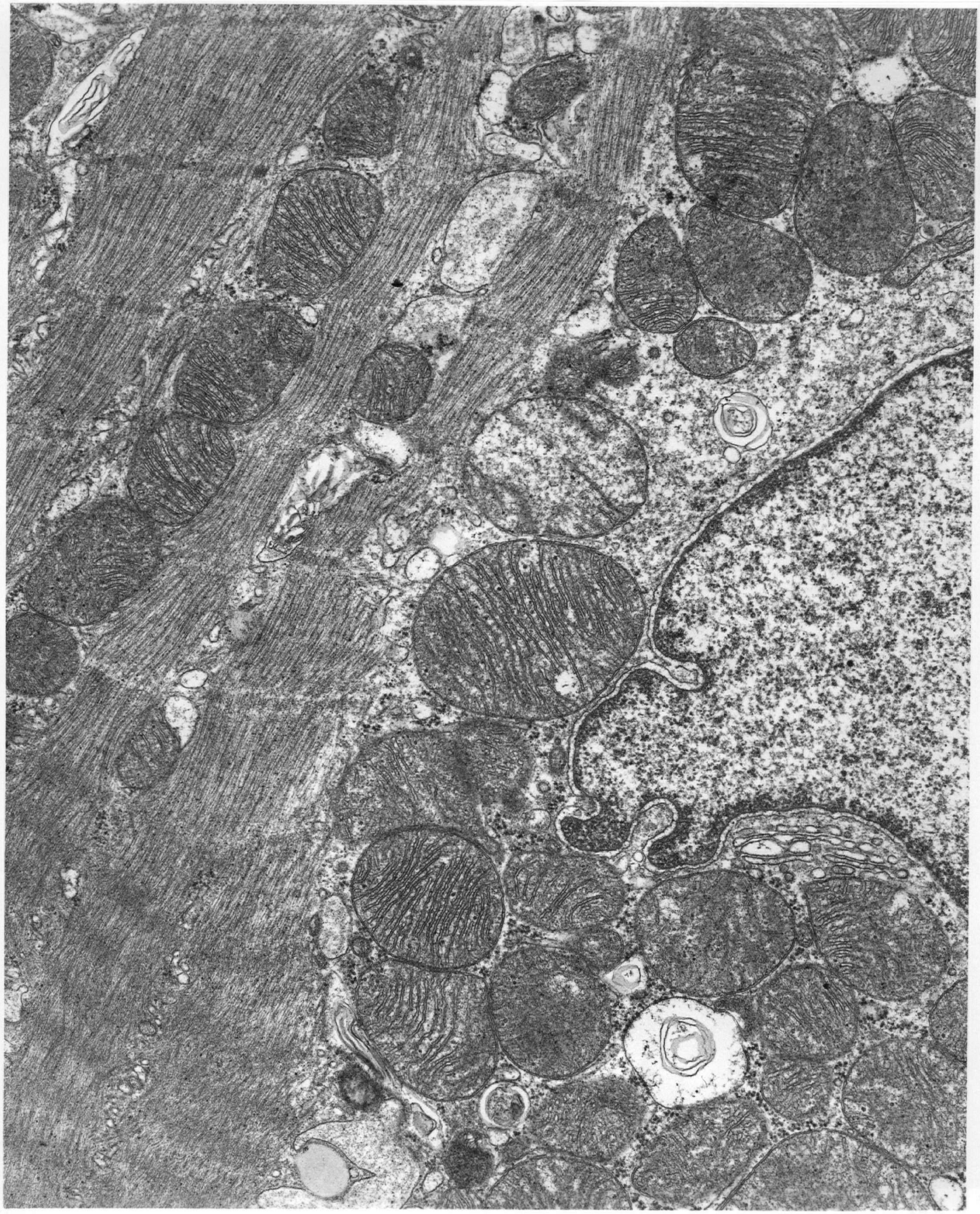
The qualitative analysis was confirmed by the more objective assessment of structural integrity. These results are summarized in Table XVI and these demonstrate a similar level of tissue preservation as that estimated by biochemical methods.

Figure 18. (D-600)

a) Photomicrograph of D-600 pretreated ligated/reperfused heart. Essentially normal mitochondrial structure with some clearing of matrices and occasional loss of this organelle (see myelin figure, bottom right). Nucleus essentially undamaged and myofibrillar degeneration is not apparent. Amorphorus intramitochondrial densities are not in evidence. X30,000.

b) Lower magnification of D-600 heart. Normal intercalated disk appears in the centre of the photograph. Mitochondrial structure and numbers are similar to control hearts. Myofibrils are not degraded; however, areas of dense contraction and relaxation exist within the same myofibrillar bundle. X6,000.

c) High magnification showing mitochondria with slightly cleared matrices. The intercalated disk is abnormally widened on the right side of the photomicrograph. X30,000.



a

b

C

Figure 19. (PROP)

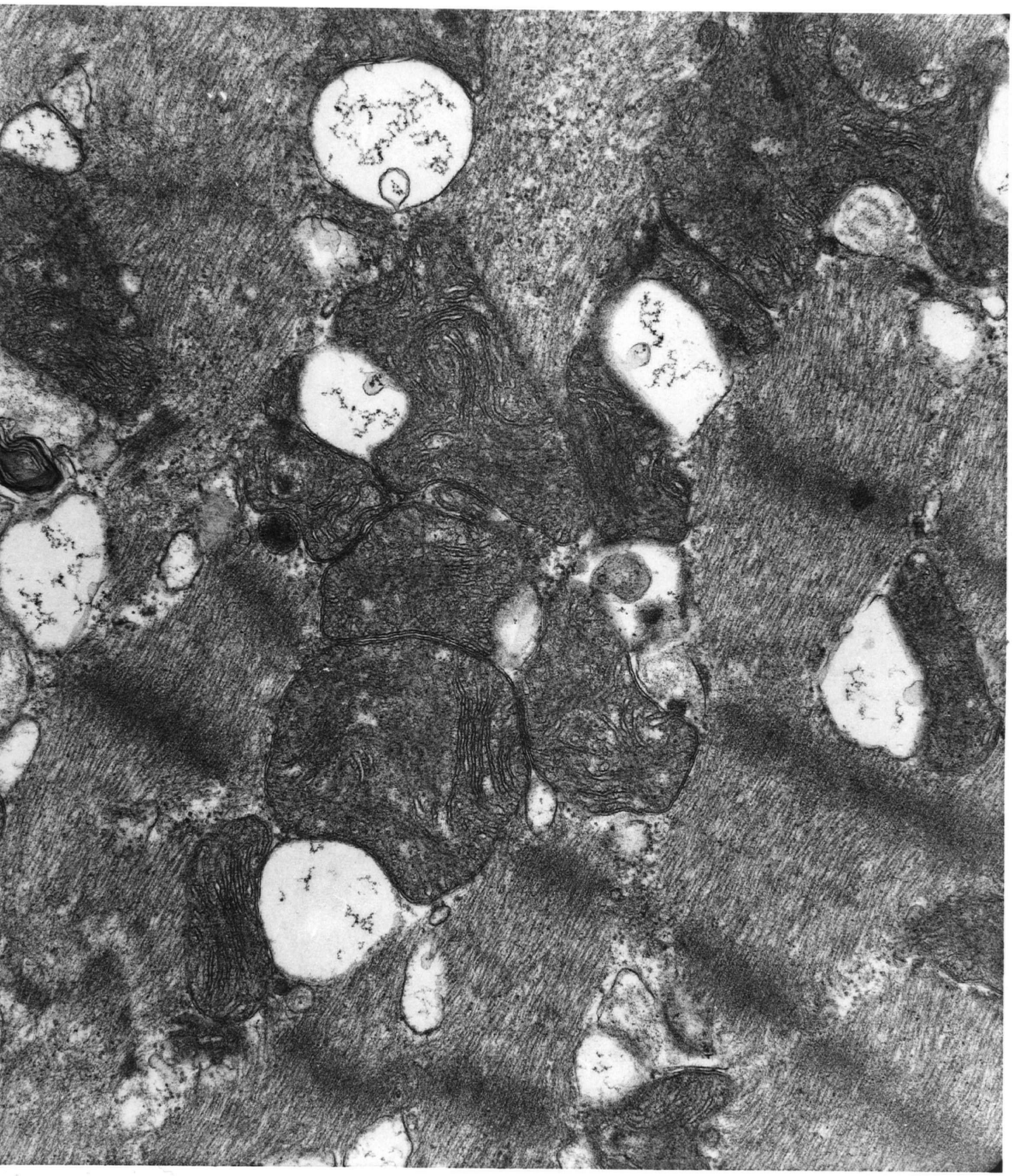
a) Electron micrograph of propranolol pretreated heart subjected to 40 min ligation followed by reperfusion. Myofibrils and mitochondria appear fairly normal except there are a few amorphous densities in some mitochondria (top right corner). The intercalated disk (bottom left) is normal. The sarcolemma/glycocalyx is not detached from underlying structures. The terminal cisternae of the sarcotubular system appear dilated. X30,000.

b) Lower magnification which shows some clearing of mitochondrial matrices and prominent contraction bands. X6,000.

c) Higher magnification of propranolol-treated hearts showing relatively normal mitochondrial structure. Prominent dilation of cisternae is seen at the Z-line. X60,000.

a

b



C

Table XVI. Objective assessment[a] of ultrastructural integrity of hearts subjected to ischaemia/reperfusion injury with or without drug pretreatment

| Treatment | Mitochondrial Membranes | Mitochondrial Densities | Sarcolemma & Glycocalyx | Myofibril Integrity | Intercalated Disk | Abnormal Contraction or Relaxation |
|---|---|---|---|---|---|---|
| Control | 0.4 ± 0.1[b] | 0.3 ± 0.3 | 0.2 ± 0.3 | 0.3 ± 0.3 | 0 | 0.6 ± 0.4 |
| L+R | 2.3 ± 0.3 | 2.9 ± 0.2 | 3.0 ± 0.0 | 2.8 ± 0.3 | 1.5 ± 0.2 | 2.2 ± 0.5[c] |
| D-600 | 0.8 ± 0.7 | 0.8 ± 0.7 | 0.3 ± 0.3 | 0.5 ± 0.6 | 0.4 ± 0.8 | 0.9 ± 1.0 |
| PROP | 1.2 ± 0.8 | 1.4 ± 0.7 | 1.5 ± 1.1 | 1.1 ± 1.4 | 0.6 ± 1.3 | 1.4 ± 1.1 |

All values expressed as mean ± S.D.

n = 2 for each group; number of photographs assessed per treatment group = 15-20.

[a] Scoring system: 0 = normal appearance
1 = mild abnormality
2 = moderate abnormality
3 = severe abnormality

[b] The S.D. was included to indicate the range of damage within each group.

[c] Abnormal relaxation with little evidence of contraction.

IV. 40 Minute Ligation and 60 Minute Reperfusion with D-600 Administration 5 Minutes Prior to Reperfusion

The results from the previous study suggested that D-600 pretreatment reduced the severity of the measured biochemical and ultrastructural abnormalities of the ischaemic/reperfused heart. If these actions were predominantly due to the ability of D-600 to block calcium entry during reperfusion one might expect that the presence of the drug in the reperfusate would result in a similar pattern of protection as that seen when the drug is given prior to ligation. We therefore compared the biochemical and functional consequences of 40 minute ligation period followed by 60 minutes of reperfusion on the following three groups of animals:

Sham-operated controls, as described previously (CONT)

Ligated and reperfused with saline administration at 35 minutes post-ligation (L+R)

Ligation and reperfused with 0.10 mg/kg D-600 HCl administered at 35 minutes post-ligation (i.e. 5 minutes prior to reperfusion) (D-600 REP)

The decrease in azide-sensitive mitochondrial ATPase activity was significantly attenuated by D-600 administration just prior to reperfusion (Figure 20). However, unlike the results obtained when D-600 was given before coronary occlusion, this method of drug administration did not completely restore the enzyme activity to control levels (see Figure 8).

In contrast to the beneficial effect of D-600 on mitochondrial ATPase and on sarcolemmal enzymes when D-600 was given prior to ligation, neither of the sarcolemmal marker enzyme activities was increased by this intervention - both saline-treated (L+R) and D-600-treated hearts had activities which remained at approximately 60% of sham-operated control (Figure 21). Similarly, the ischaemia-induced alterations in other assessments of sarco-

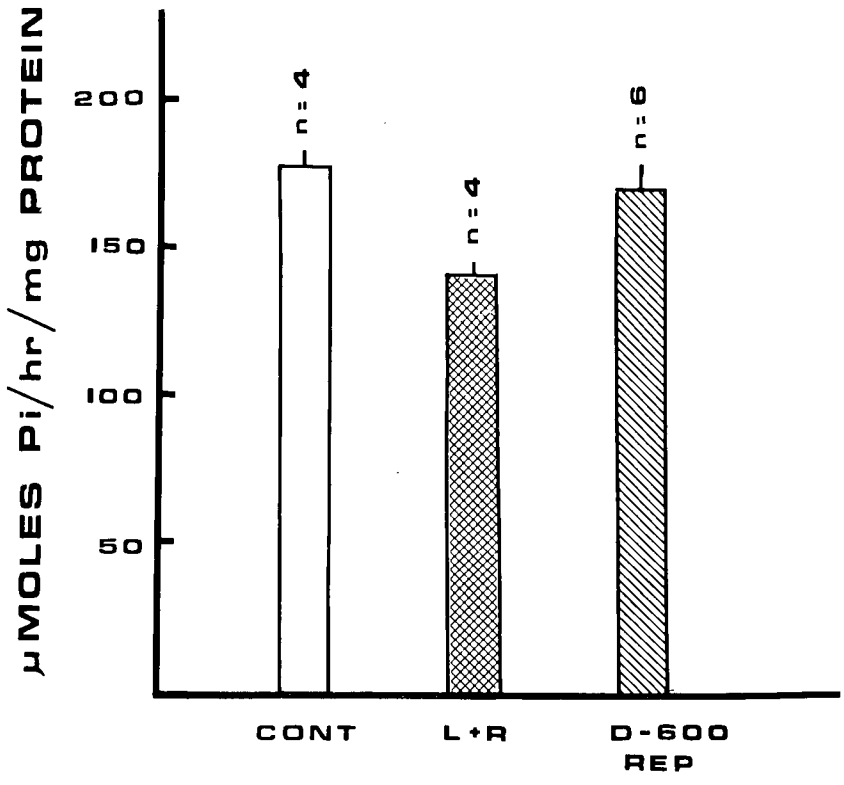



Figure 20. Mitochondrial azide-sensitive ATPase activity in sham-operated hearts (CONT), 40 min ligated and 60 min reperfused hearts (L+R), and ligated and reperfused hearts with D-600 administration 5 min prior to reperfusion (D-600 REP). Enzyme activity of D-600 REP is significantly increased over the L+R group ($P < 0.05$). Bars represent mean ± S.E. and n = 4-6 for each group.

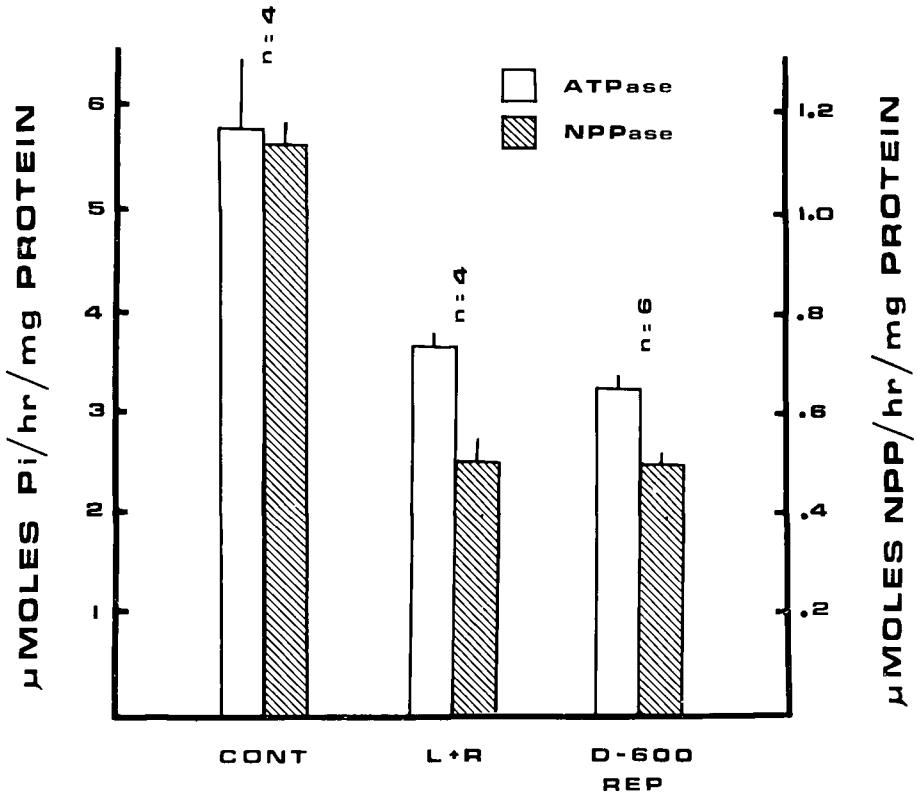



Figure 21. Sarcolemmal $Na^+,K^+$-ATPase and $K^+$-NPPase activity in CONT, L+R and D-600 REP groups. Bars represent mean ± S.E. and n = 4-6 for each group. Drug administration did not significantly increase the level of enzyme activity over that in the ligated/reperfused group (L+R).

lemmal integrity (L+R) were also unchanged by drug treatment just before reperfusion (D-600 REP). Losses of $Mg^{2+}$ and $K^{+}$ as well as accumulation of $Na^{+}$ occurred regardless of the presence of D-600 upon reperfusion. In addition, tissue water content increased significantly in the treated as well as the untreated hearts (Table XVII). Interestingly, despite the lack of effect of the calcium channel-blocker on $Na^{+}$, $K^{+}$ and $Mg^{2+}$ levels, the presence of D-600 during reperfusion significantly reduced the calcium accumulation that is the hallmark of irreversibly damaged, reperfused myocardial tissue. However, although a significantly lower accumulation of $Ca^{2+}$ occurred with drug treatment - 42.8 ± 4.5 (saline treated) compared with 25.3 ± 4.5 nmoles/mg dry weight in the D-600 group, the concentration of calcium in the D-600-treated hearts was still 5X that in the non-ischaemic myocardium (5.4 ± 0.5 nmoles/mg dry weight). It was, however, comparable to the level found in hearts receiving D-600 prior to ischaemia.

In view of the results demonstrating that D-600 preserves the mitochondrial ATPase activity and reduces intracellular calcium influx, it seemed possible that the drug could have a salutary effect on the reduction of high energy phosphate levels of the ishaemic/reperfused hearts. However, Figure 22 shows that the large reduction in ATP is not significantly prevented by the presence of D-600 during reperfusion. There was a small, non-significant enhancement in ATP levels to 3.0 ± 1.2 nmoles/mg dry weight in the drug treated animals (D-600 REP) when compared to the saline-treated (L+R) animals, 3.04 ± 1.2 compared to -.86 ± 0.33 nmoles/mg dry weight, although these values are both below 20% of control levels. The small enhancement of ATP by drug treatment upon reperfusion was not accompanied by any increase in contractile force $(dP/dt)_{max}$ of the D-600 REP group compared to L+R alone (Table XVIII).

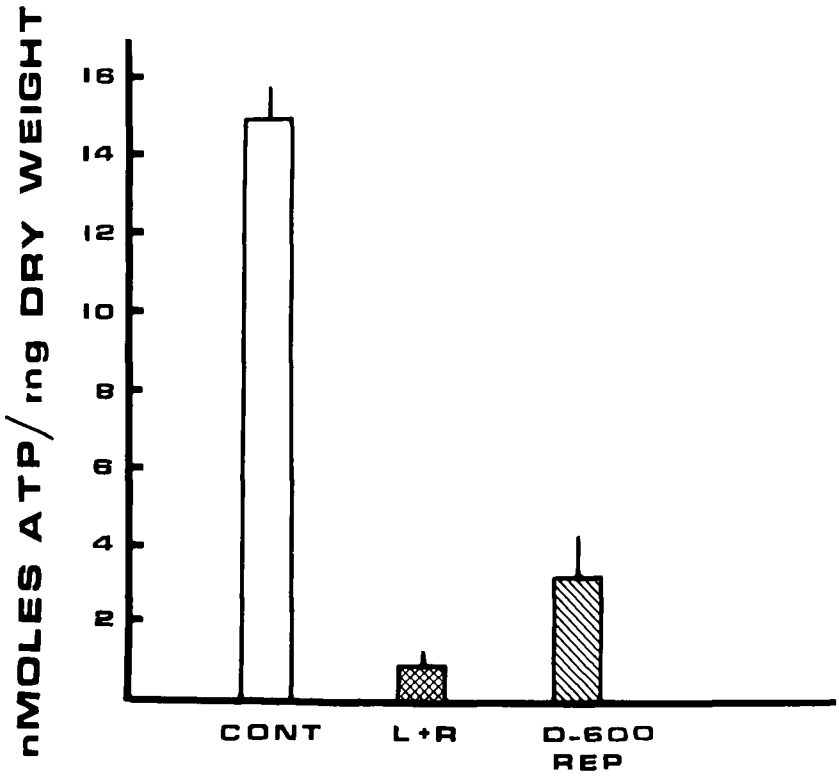



Figure 22. ATP content of hearts in CONT, L+R, and D-600 REP groups. D-600 REP is not significantly greater than L+R at $P < 0.05$. Bars represent mean ± S.E. and n = 4-6 for each group.

Table XVII. Tissue ion and water content in hearts given 0.10 mg/kg D-600 HCl 5 minutes prior to reperfusion.

| Treatment | n | Water content (%) | Ion Content (nmole/mg dry weight) | | | |
|---|---|---|---|---|---|---|
| | | | $Na^{+}$ | $K^{+}$ | $Mg^{+2}$ | $Ca^{+2}$ |
| Control | 5 | 80.8 ± 0.5 | 196 ± 17 | 492 ± 10 | 34.0 ± 0.9 | 5.4 ± 0.5 |
| L+R[b] | 4 | 84.8 ± 0.3 | 455 ± 19 | 360 ± 20 | 21.3 ± 1.0 | 42.8 ± 4.6 |
| D-600 REP | 6 | 84.2 ± 0.3 | 415 ± 28 | 422 ± 16[a] | 22.2 ± 0.6 | 25.3 ± 4.5[a] |

All values are expressed as mean ± S.E.

[a] Values of D-600 REP significantly different from L+R at $P < 0.05$.

[b] All L+R measurements are significantly different from control at $P < 0.05$.

Table XVIII. Percent initial $(dP/dt)_{max}$ [a] of hearts during reperfusion period after 40 minutes of ligation with or without D-600 prior to reperfusion

| | | Minutes of reperfusion | | | | | |
|---|---|---|---|---|---|---|---|
| Treatment | (n) | 3 | 5 | 10 | 20 | 40 | 60 |
| Control | (5) | 103 ± 4 | 102 ± 4 | 101 ± 3 | 102 ± 5 | 98 ± 9 | 97 ± 8 |
| L+R | (4) | 36 ± 3 | 43 ± 4 | 45 ± 5 | 56 ± 5 | 61 ± 5 | 63 ± 4 |
| D-600 Rep | (5) | 29 ± 4 | 33 ± 4 | 31 ± 5 | 49 ± 8 | 61 ± 5 | 60 ± 5 |

All values are expressed as mean ± S.E.

[a] Initial $(dP/dt)_{max}$ for each group (mm Hg/sec); mean ± S.E.

| | |
|---|---|
| Control | 3550 ± 320 = 100% |
| L+R | 3688 ± 426 = 100% |
| D-600 REP | 3650 ± 400 = 100% |

V. 0-60 Minute Ligation Study - Enzyme Activity vs. Ion Levels

The activity of azide-sensitive mitochondrial ATPase decreases rapidly with increasing periods of ligation (data not shown) as was seen in the preliminary study. The reduction is statistically significant as early as 5 minutes post-ligation and decreases further to a plateau at 40% of control activity after 20 minutes of ischaemia.

In contrast, $Na^+,K^+$-ATPase activity did not decrease in a continuous fashion but rather showed a small initial reduction after which the enzyme activity remained constant until 20 minutes post-ligation (Figure 23). Only after this time, when the mitochondrial ATPase already was maximally inhibited, did sarcolemmal enzyme activity decrease further. Similar behaviour was observed for the functionally-related $K^+$-stimulated p-nitrophenylphosphatase ($K^+$-NPPase) system. Recent experiments by Bersohn et al. using rabbit ventricular slices made ischaemic in vitro, also revealed a slight plateau in the fall in $Na^+,K^+$-ATPase activity within the first 20 minutes of ischaemia.[96] A comparison of Figures 2 and 23 show how the addition of Triton X-100 to the assay medium altered the pattern of enzyme activity with ligation time. The general decrease was still obvious but a plateau in activity until 20 minutes post-ligation was revealed by detergent treatment. Most likely the modification to the assay procedure caused an opening of vesicles in order to allow substrate availability to enzymatic sites sealed within an inside-out vesicle; however, the possibility that the Triton X-100 was itself having a direct effect on enzyme activity should not be discounted.

Table XIX shows the results of the tissue ion and water measurements. No apparent accumulation of water is evident ever after 60 minutes post-ligation but there was no change in $Mg^{2+}$ or $Ca^{2+}$ levels at the

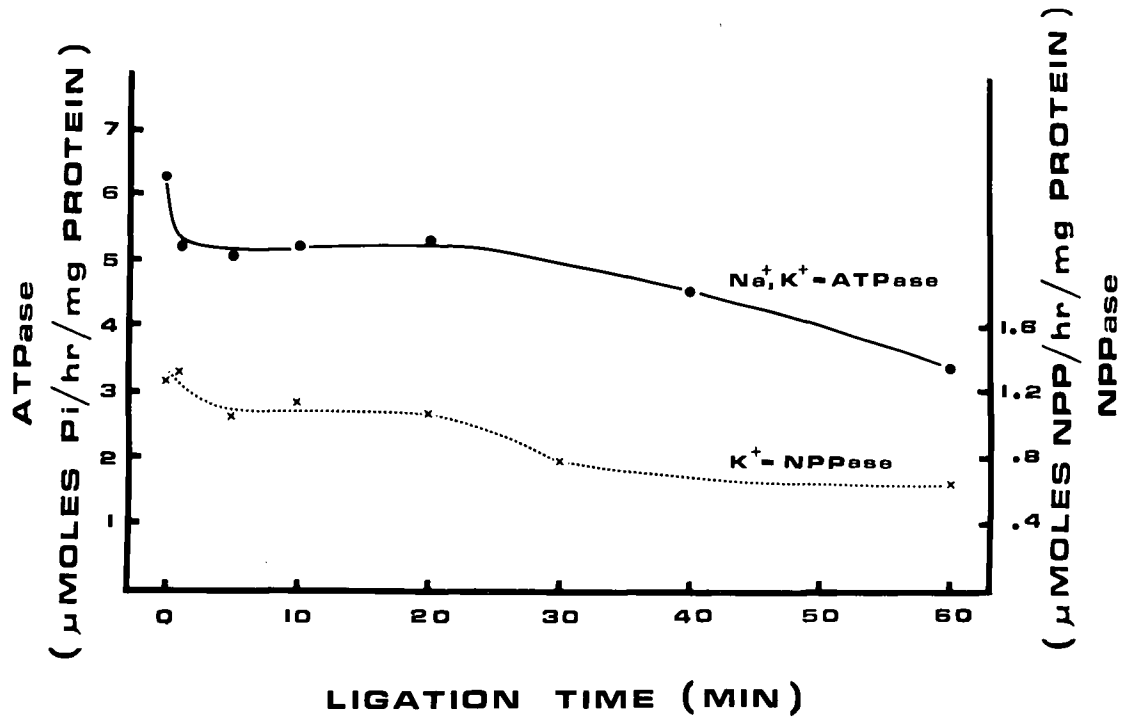



Figure 23. Sarcolemmal $Na^+,K^+$-ATPase and $K^+$-NPPase activity of hearts subjected to 0-60 min of ligation. Error bars were omitted for clarity - points represent mean of n = 5-7 animals. Enzyme activity is significantly reduced by 40 and 60 min post-ligation for both enzymes ($P < 0.05$). Triton X-100 was added to the assay medium (see Methods for further details).

Table XIX. Ion and water content of hearts subjected to 0-60 minutes of ligation

| Treatment (min of ligation) | n | Water content (%) | Na | K | Mg | Ca |
|---|---|---|---|---|---|---|
| | | | (nmoles/mg dry weight) | | | |
| 0 | 6 | 79.4 ± 0.5 | 217 ± 13 | 462 ± 14 | 31.4 ± 0.9 | 7.84 ± 0.58 |
| 1 | 2 | 80.3 ± 1.8 | 199 ± 10 | 430 ± 57 | 33.0 ± 2.9 | ND[b] |
| 5 | 6 | 79.8 ± 0.7 | 218 ± 17 | 484 ± 38 | 32.0 ± 1.5 | 8.26 ± 1.10 |
| 10 | 6 | 80.4 ± 0.3 | 231 ± 16 | 454 ± 15 | 32.3 ± 0.8 | 7.41 ± 0.86 |
| 20 | 7-9 | 80.4 ± 0.6 | 209 ± 16 | 407 ± 24 | 29.3 ± 2.5 | 8.12 ± 1.28 |
| 40 | 6 | 80.2 ± 0.7 | 262 ± 26 | 399 ± 26 | 28.4 ± 1.6 | 8.18 ± 0.94 |
| 60 | 6 | 79.6 ± 0.7 | 309 ± 14[a] | 389 ± 29 | 29.4 ± 1.4 | 8.95 ± 1.0 |

All values are expressed as mean ± S.E.

[a] Significantly different from control at $P < 0.05$.

[b] ND = not done.

time. $K^+$ loss was measurable but this did not achieve statistical significance. In order to see the effects of ischaemia on overall $Na^+$ and $K^+$ vs. $Ca^{2+}$ and $Mg^{2+}$ homeostasis, the $Na^+/K^+$ and $Ca^{2+}/Mg^{2+}$ were calculated for up to 60 minutes post-occlusion. The results are shown in Figure 24. In contrast to the minimal changes in the $Ca^{2+}/Mg^{2+}$ ratio, a progressive increase in the $Na^+/K^+$ ratio was observed after 20 minutes of ischaemia. Interestingly, this increase paralleled the fall in $Na^+,K^+$-ATPase activity (Figure 21), suggesting that the decreased sodium pump activity measured in vitro, reflects an in vivo phenomenon which is associated with an intracellular accumulation of sodium and a loss of potassium.

High energy phosphate levels are known to decrease rapidly following coronary ligation and ATP levels decrease to 50% of control after only 1 minute of coronary occlusion. This value is maintained for a further 10 minutes after which there is another drop in ATP to 17% of control, followed by a more gradual decline from 20 minutes until 60 minutes of ligation. Thus, most of the marked reduction in cellular ATP levels precedes the ischaemia-induced alterations in the sarcolemmal enzyme activity and the $Na^+/K^+$ ratio. In order to illustrate these temporal relationships more clearly, the individual graphs have been superimposed (Figure 25). The close association between $Na^+,K^+$-ATPase activity and the $Na^+/K^+$ ratio and their dissociation from the earlier fall in ATP are clearly apparent.

In order to explore the possible molecular basis of the observed membrane functional alterations, we have investigated the effect of ischaemic injury on myocardial phospholipid profiles. Two-dimensional thin-layer chromatography permitted the assessment of the major classes of phospholipids.

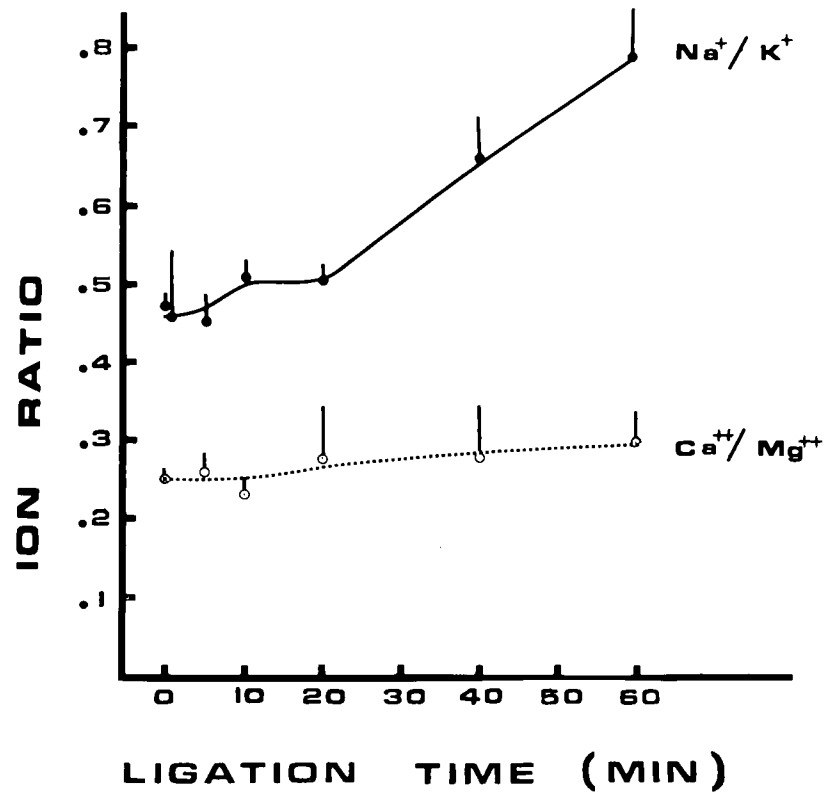



Figure 24. $Na^+/K^+$ and $Ca^{2+}/Mg^{2+}$ ratios in tissue obtained from hearts subjected to 0-60 min of ischaemia. The rise in the $Na^+/K^+$ ratio is statistically significant by 40 min post-ligation. $Ca^{2+}/Mg^{2+}$ is not significantly different from control even after 60 min of ligation ($P < 0.05$). Points represent mean ± S.E.

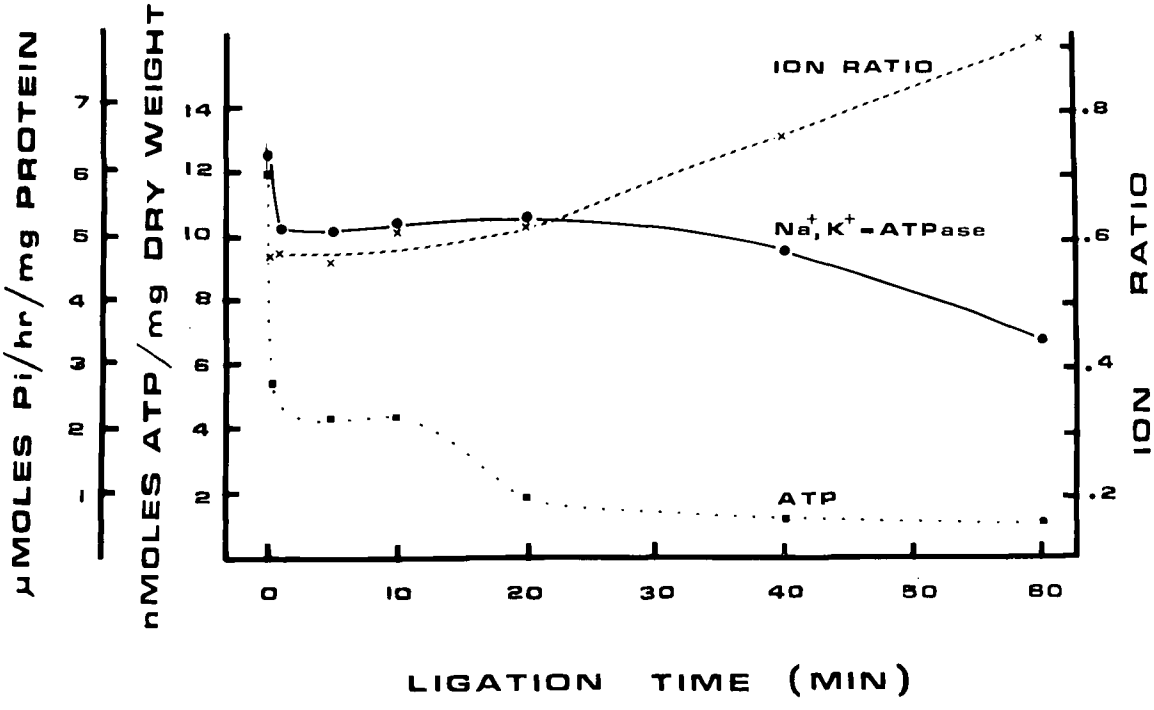



Figure 25. Superimposed graphs of the alteration in $Na^+/K^+$ ratio, $Na^+,K^+$-ATPase activity and ATP content with increasing ligation time. Note the concurrent rise of the $Na^+/K^+$ ion ratio with the decrease in sodium pump activity. (Error bars have been omitted for clarity).

Previously, no reduction in PC and PE were seen even after 60 minutes of ligation. This was confirmed using more sensitive analysis and furthermore, no significant alteration was seen in any of the other phospholipid components (Table XX). The stability of the two major phospholipids, phosphatidylcholine (PC) and phosphatidylethanolamine (PE), is significant in that these are thought to be the most susceptible to degradation under conditions of ischaemia[41].

The possible involvement of membrane protein alterations in the observed effects of ischaemia was explored by means of polyacrylamide gel electrophoresis which was quantitatively assessed by densitometry. The lack of detectable changes in the protein profiles of the mitochondrial and sarcolemmal fractions in the present study (data not shown) contrasts with the results from the reperfusion study and suggest that the alterations that result from reperfusion after ischaemia are responsible for the mitochondrial polypeptide alteration rather than the ischaemic insult per se.

Table XX. Phospholipid profiles of hearts subjected to various periods of ligation

| Treatment | n | Phospholipid content (% total lipid phosphorus) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Sph | PI | PS | PC | PE | CL |
| Control | 2 | 4.52 ± .18 | 2.95 ± .08 | 2.10 ± .40 | 41.0 ± .6 | 34.8 ± 1.7 | 14.6 ± .4 |
| 10' Ligation | 4 | 3.69 ± .94 | 3.05 ± .78 | 2.29 ± .33 | 43.2 ± 1.9 | 34.4 ± 2.3 | 13.0 ± 1.3 |
| 40' Ligation | 3 | 3.55 ± .29 | 2.87 ± .88 | 1.93 ± .29 | 43.0 ± 1.8 | 34.5 ± 1.7 | 13.1 ± 1.3 |
| 60' Ligation | 6 | 3.94 ± .85 | 2.79 ± .85 | 1.94 ± .57 | 44.0 ± 1.9 | 34.5 ± 2.1 | 13.1 ± .7 |

All values are expressed as mean ± S.E.

Sph = sphingomyelin

PI = phosphatidylinositol

PS = phosphatidylserine

PC = phosphatidylcholine

PE = phosphatidylethanolamine

CL = cardiolipin

# DISCUSSION

## Aims

The purpose of the work in this thesis was to investigate the biochemical alterations that occur during acute myocardial ischaemia and reperfusion. Specifically, these experiments have attempted to identify the onset of irreversible damage to sarcolemmal and mitochondrial membranes by examining the time course of decreases in membrane-bound enzyme activity and relating these to changes in high energy phosphate depletion, protein and phospholipid degradation and tissue ion levels. As discussed in the Introduction, it is evident that all cellular processes will eventually be affected as ischaemia progresses to infarction. Thus, these experiments concerned only the biochemical changes occurring within the first 60 minutes following coronary artery ligation.

Another aspect of the experiments described herein was to examine the effects of several drugs on our model of ischaemia/reperfusion injury in order to investigate the effects of specific pharmacological intervention on the biochemical indices of cell damage.

## Advantages and Disadvantages of the Model

The acute open-chest rabbit provides a stable, physiological preparation which allows both functional and biochemical measurements to be done in the same animal. The yield of ischaemic tissue is sufficient to allow sampling for ATP, ions, electron microscopy as well as separation into mitochondrial and sarcolemmal fractions without having to pool tissue from several animals. Fractionation permitted the evaluation of the dynamic function of membrane-bound sarcolemmal and mitochondrial enzymes. However, one must be cautious in extrapolating results from *in vitro* assays to the function of

the enzyme *in vivo* because organelle purification may obscure or alter the character of ischaemia-induced membrane alterations.

Total occlusion with subsequent release of the ligature around the left circumflex coronary artery was used as a model of myocardial ischaemia and reperfusion in man. Human post-mortem studies have not always demonstrated the existence of thrombi in coronary vessels supplying the infarcted tissue.[119] Angiographic evidence, however, has shown that a large percentage of patients with transmural infarction have thrombi in the implicated coronary vessel[120] although whether or not these platelet aggregates precipitate ischaemia/infarction or are formed as a result of ischaemia-induced local endothelial changes [121] is presently unclear. An explanation for the absence of thrombi upon post-mortem examination has arisen from recent studies that strongly suggest that spontaneous reperfusion of occluded coronary vessels occurs in patients as early as 6 hours after the initial symptoms.[122,123] Thus, ligation of a coronary artery followed by release is probably an adequate model of the human disease process.

A further advantage of using the rabbit as the experimental animal is that left circumflex coronary artery occlusion produces a well-defined zone of ischaemia without inter-digitating normal tissue[48] thus making for more reproducible and meaningful tissue sampling.

Although the *in situ* model is more physiological than the isolated heart, the pentobarbitone anaesthetic blunts reflex responses and, as a consequence, the conclusions regarding the effects of any treatment cannot be unreservedly applied to the conscious animal. Furthermore, the haemodynamics were not controlled in these experiments. Although this avoided complications from additional physiological or pharmacological manipulations, it was more difficult to dissociate direct and indirect effects of

drug treatment. The use of the in vivo preparation also did not readily permit the estimation of drug concentration in the myocardium. Effective concentrations were ascertained indirectly by measuring the drug-induced alterations in heart rate and $(dP/dt)_{max}$.

Coronary ligation produced a variety of effects on the biochemical, functional and morphological indices used to assess cellular damage. Both mitochondrial and sarcolemmal ATPase activities were reduced by short periods of coronary ligation; however, there were several important differences in the time course and reversibility of the alterations. The rapid fall in mitochondrial ATPase was maximal at 20 minutes post-ligation, a time when no significant decrease in $Na^+,K^+$-ATPase activity was apparent. The behaviour of these two enzymes upon reperfusion was of particular significance -- only the mitochondrial enzyme activity could be readily reversed by reperfusion. Even after 30 minutes of ischaemia, reperfusion reversed the decrease in mitochondrial ATPase activity in contrast to the small recovery of sarcolemmal $Na^+,K^+$-ATPase activity. Thus, it appears that the sarcolemmal ATPase is irreversibly damaged before any permanent depression in mitochondrial ATPase has occurred, indicating that ischaemia-induced decreases in the two enzyme activities may involve different mechanisms.

Other investigators have also demonstrated the relative reversibility of ischaemia-induced damage to mitochondrial structure and function.[49,50,124] In contrast, Nayler and Scott were not able to reverse the defect in mitochondrial oxidative phosphorylation with reperfusion after 90 minutes of global ischaemia in isolated rabbit hearts.[125] It is possible that this very long ischaemic period caused irreversible damage to the mitochondrion, preventing the restoration of function. On the other hand, oxidative phos-

phorylation will not necessarily return to control values even in the presence of normal ATPase activity. The co-ordinated activity of many enzymes is required for oxidative phosphorylation and the disturbance of a single process would affect the overall rate of ATP formation. Azide-sensitive ATPase activity and cellular respiration are functionally related[126,127] so that the depression of ATPase activity as well as the low oxygen tension during the ischaemic period would probably suppress oxidative phosphorylation in vivo. In vitro, the ATPase activity is still depressed, even if assayed in the presence of optimal concentrations of substrates. This indicates that the ischaemia-induced alteration is not simply due to a lack of substrate. The restoration of enzyme activity with reperfusion suggests the involvement of a reversible inhibitor accumulating during the course of ischaemia. (It should also be noted that the observed control values for enzyme activity measured in vitro does not prove that ATPase activity has been restored in vivo as many cofactors and substrates may have been depleted during the ischaemic period). The decrease in mitochondrial ATPase activity does not indicate a generalized depression of all respiratory chain enzyme activities as shown by the lack of effect of ischaemia on another inner mitochondrial membrane enzyme, namely cytochrome c oxidase. Sordahl and Stewart have also demonstrated that the respiratory chain damage caused by myocardial ischaemia does not involve cytochrome c oxidase.[128] Measurement of mitochondrial ATPase activity may, however, be a reasonable indicator of overall mitochondrial function. Rouslin and Millard have measured respiratory chain enzyme activities after coronary ligation, and found that the overall reduction in oxidative phosphorylation can be almost entirely accounted for by reductions in NADH-coenzyme Q and oligomycin-(azide-)sensitive ATPase activities.[129]

Although reperfusion of ischaemic mitochondria apparently reverses the decrease in the ATPase activity, the reduced mitochondrial recovery of the mitochondria fraction from ischaemic/reperfused hearts also suggests that there may have been selective recovery of undamaged mitochondria during the purification procedure. The restoration of ATPase activity to 75% of control values after a 40 min ligation would only be achieved if the proportion of recovered mitochondria was 50% damaged (activity = 40% of control) and 50% normal mitochondria (activity = 100% of control). If this were so, one would have expected to see equal proportions of normal and severely damaged mitochondria in the same tissue section; however, this was not apparent in the light and electron micrographs of the ligated and reperfused hearts (L+R). The degree of injury to mitochondria was relatively uniform within the L+R group and the number of normal mitochondria was extremely small compared to the damaged organelles. The L+R hearts did have an apparent loss of mitochondria when compared to the control and drug-treated hearts and this may have contributed to the decreased yield of this organelle from severely damaged tissue rather than a selective recovery of undamaged mitochondria. Furthermore, the mitochondrial membrane fraction isolated from ischaemic hearts has a missing protein band, indicating that the separation procedure probably does not preferentially eliminate damaged mitochondria.

Various investigators have also shown that ischaemia produces irreversible damage to the sarcolemmal membrane, although most studies have focussed on changes in sarcolemmal permeability rather than membrane-bound enzyme function. For example, Weiss and Shine have demonstrated an immediate and reversible increase in $K^+$ efflux from the ischaemic, arterially-perfused intraventricular rabbit septum. This reached a plateau at approximately 12

minutes post-ligation after which they found that the loss of $K^+$ from the cell was irreversible (25 minutes post-ligation).[26] Hill and Gettes have obtained similar results in ischaemic swine hearts.[130] They measured the extracellular $K^+$ activity and found that the rise followed a distinct triphasic pattern that closely paralleled the results of Weiss and Shine. The subtleties of $K^+$ efflux are somewhat obscured when total tissue $K^+$ levels are measured (as was done in the studies described herein) because of the large $K^+_{in}/K^+_{out}$ ratio. Despite this technical limitation, it is interesting to note the similarity of the time of onset of irreversible $K^+$ loss in the studies of Weiss and Shine and Hill and Gettes (25-30 minutes) with the onset of a significant decrease in $Na^+,K^+$-ATPase activity and concurrent increase in the $Na^+/K^+$ ratio (20-30 minutes) shown in the present experiments. In addition to the loss of potassium, net sodium influx has also been demonstrated by other investigators during the early ischaemic period using total tissue sodium measurements.[115] These ion changes take place in the absence of changes in the extracellular space (assessed by $^{51}$Cr-EDTA distribution).[28] Of particular interest is the observation of an increase in the $Na^+/K^+$ ratio in the absence of changes to the $Ca^{2+}/Mg^{2+}$ ratio indicating that ischaemia-induced plasma membrane damage consisted of a specific alteration to the plasma membrane involving $Na^+$ and $K^+$ permeability (probably related to a decrease in the $Na^+,K^+$-ATPase activity) rather than a generalized increase in permeability to all ions. Sarcolemmal calcium permeability during ischaemia and reperfusion is discussed in more detail under "Drug Studies" (see page 130). Other investigators have also attempted to relate decreases in $Na^+,K^+$-ATPase activity to the ion abnormalities observed after short periods of coronary occlusion. Measureable decreases in enzyme function have been

observed[96,131,132] although Schwartz et al. were not able to show any decrease in enzyme activity even after 60 minutes of ischaemia.[133] The lack of effect of ischaemia on sodium pump activity in the study of Schwartz et al. may be attributable to the fact that they examined highly purified sarcolemmal enzyme. Ischaemia-induced decreases in sodium pump activity may, therefore, be mediated via effects on the lipid environment of the enzyme rather than on the actual enzyme protein. There are several lines of evidence in support of this hypothesis. Firstly, the gel electrophoretic studies do not reveal any gross change in the sarcolemmal protein bands from hearts subjected to 60 minutes of ischaemia. This, of course, does not preclude the occurrence of changes in minor and functionally-important membrane proteins and/or subtle effects on enzyme conformation in vivo. Secondly, the $Na^+,K^+$-ATPase and $K^+$-NPPase enzymes were affected to the same degree regardless of the treatment. Finally, the reduction in $V_{max}$ of the $Na^+,K^+$-ATPase complex is not accompanied by decreases in the apparent affinities of $K^+$ and $Mg^{2+}$. The above would indicate an effect on the whole enzyme complex rather than an interference with one of its partial reactions. Furthermore, it is known that the activity of this enzyme depends on two distinct ligand-induced conformational transitions[134] and this change in conformation is dependent upon the lipid environment of the enzyme.[112,135] Thus, ischaemia-induced alterations in the lipid bilayer could be responsible for the observed decrease in sodium pump activity. Mitochondrial azide-sensitive ATPase has also been shown to have a specific lipid requirement[113] and alterations in cellular lipid could also affect its activity. Many attempts have been made to identify the presence of ischaemia-induced lipid disruption. Several hypotheses have been advanced and the more tenable of these will be discussed in connection with the results from the present studies.

It has been postulated that lysosomal disruption occurs as a consequence of ischaemia resulting in release of proteases and phospholipases into the cell which could produce, amongst other things, damage to the lipid bilayer.[136] Early studies by Decker *et al.* indicated that lysosomal enzyme release occurred during ischaemia[137] and Chien *et al.* have implicated such release as the possible cause of the phospholipid degradation seen after 3 hours of ischaemia.[42] Moreover, work from our laboratory has demonstrated that ischaemia results in a redistribution of two lysosomal enzymes (cathepsin D and N-acetyl-β-glucosaminidase) from the sedimentable to the supernatant portion of isolated lysosomal membranes. However, this redistribution does not reach statistical significance until marked decreases in both the sarcolemmal and mitochondrial enzyme activities have occurred. Moreover, it has not been possible to demonstrate the existence, in ischaemic tissue, of lysosomes showing an increased susceptibility to hypotonic lysis. No measureable phospholipid or protein degradation was detectable, even after 60 minutes of ischaemia making it unlikely that lysosomal enzyme release, if it does occur *in vivo*, is responsible for the observed decreases in membrane-bound enzyme activity.

If activated during ischaemia, extra-lysosomal phospholipases could also degrade membranes and produce membrane damage both by a reduction in phospholipid content and by the concomitant production of lysophospholipids. Such degradation might not be apparent by measurement of total phospholipid content because the percentage of lysophospholipid in cell membranes is very small in comparison to the other phospholipids (less than 1% of total lipid phosphorus) and the concentration thought to produce membrane disruption is only marginally greater. The involvement of these amphipathic, detergent-like compounds in the genesis of ischaemic damage was first suggested by

Hajdu, Weiss and Titus as early as 1957.[138] The level of lysolipids in ischaemic tissue was not actively investigated again until the 1970s when experiments by Sobel and his colleagues demonstrated lysophospholipid accumulation in ischaemic myocardium. They postulated that these agents were the cause of ischaemia-induced dysrhythmias.[44,129] Recently, Man et al. have also shown that several detergents (SDS, Triton X-100) as well as lysophosphoglycerides produce arrhythmias in the isolated, perfused hamster heart at concentrations below their critical micelle concentration.[140] The membrane-active properties of these compounds are also evident in their ability to inhibit $Na^+,K^+$-ATPase in vitro when added to adult, canine cardiac myocytes.[141] It would appear that these compounds are potential candidates in causing ischaemic membrane damage; however, despite the compelling evidence from isolated preparations, other evidence exists that casts doubt on the importance of these compounds in causing irreversible membrane damage. Shaikh and Downar have discredited earlier papers in which acidification of the extraction media was performed because this procedure produces a large artifactual increase in lyso-compounds. In their rigourous study, they found no degradation of phospholipid of swine hearts subjected to left anterior descending (LAD) coronary artery occlusion until 8 hours post-ligation and, more importantly, the increase in lysophosphatide levels (still less than 0.6% total lipid phosphorus) was maximal at 8 minutes post-occlusion with little change in levels after this time.[45] In view of the time course of membrane enzyme inhibition, then, lysophosphatides are an unlikely cause of membrane disruption seen after 20-40 minutes of coronary occlusion. In addition, although lysophosphatide-induced inhibition of many cellular functions is demonstrable in vitro, what impact such levels have in vivo is unknown. For example, a 350% increase in cardiac lysophosphatide

levels is seen in hibernating ground squirrels. The squirrels awaken each 8–12 days and their body temperature rises from 1 to 37°C, and, although the erythrocyte levels of these compounds fall rapidly upon arousal, a very significant content of lysophosphatides probably remains in cardiac tissue and this level is compatible with normal myocardial tissue function.[142] These latter considerations challenge the proposal that early ischaemic-induced membrane damage is the result of lysophosphatide accumulation.

Having largely discounted phospholipid or protein degradation as the major cause of early ischaemia-induced mitochondrial or sarcolemmal damage, another possible source of disruption to the lipid bilayer could arise from accumulation of intermediates of fatty acid metabolism.[24] Whitmer et al. have found that increases in acyl CoA levels reach a maximum at 5 minutes post-ligation whereas acylcarnitine levels are still increasing after 20 minutes of ischaemia.[35] Fatty acyl co-enzyme A and fatty acylcarnitine are amphipathic molecules with detergent properties and, as such, inhibit a variety of cellular processes at concentrations comparable to the lysophosphatides and other detergents, i.e., approximately 50 μM. However, Owens et al. using a modified method of membrane purification in which no detergents were added, have been unable to demonstrate inhibition of $Na^+,K^+$-ATPase activity by either palmitylcarnitine (0.4 mM) or palmityl CoA (0.1 mM) in vitro even though these compounds were shown to bind extensively to sarcolemmal vesicles.[141] In addition, results from this laboratory have shown that incubation of membrane fractions with 0.5 mM palmitylcarnitine (in the absence of Triton X-100) had no effect on mitochondrial ATPase and only reduced $Na^+,K^+$-ATPase activity by 25% (data not shown). Other evidence, albeit indirect, that further disputes the importance of palmitylcarnitine accumulation in ischaemia-induced $Na^+,K^+$-ATPase inhibition comes from a

study on diabetic rats done by Dr. D. V. Godin in collaboration with Drs. J. H. McNeill and G. Lopaschuk (Faculty of Pharmaceutical Sciences, U.B.C.) Briefly, they found that significant increases in palmitylcarnitine levels in diabetic hearts were not accompanied by decreases in either mitochondrial or sarcolemmal ATPase activity.[143] Thus, even if there is an increase in fatty acylcarnitine levels during ischaemia, there is not necessarily an *in vivo* inhibition of $Na^+,K^+$-ATPase activity. These agents have no effect on mitochondrial ATPase activity *in vitro*; however Kotaka *et al.* have shown that mitochondrial acyl CoA levels correlate well with decreases in another mitochondrial function, namely State 3 oxygen consumption ($r = -0.97$).[50] Interestingly, reperfusion after 30 minutes reversed the inhibition of mitochondrial function as well as lowering the level of acyl CoA. The association between the two events is interesting, but it does not prove that the events are causally related.

Although fatty acylcarnitine (and probably fatty acyl CoA) have no effect on mitochondrial ATPase activity *in vitro*, the accumulation and washout of an inhibitory metabolite does fit the pattern of mitochondrial ATPase inhibition. This is unlikely to be the case, however, in the mechanism of irreversible decreases in $Na^+,K^+$-ATPase and $K^+$-NPPase activity. There has not yet been any unequivocal evidence that some particular membrane-active agent is responsible and it is doubtful that lysophospholipids, fatty acid metabolites, or lysosomal enzymes are involved in early sarcolemmal membrane alterations. The aforementioned studies have only examined ischaemic damage in isolated membranes and it is possible that these compounds may have somewhat different properties in the intact myocardium.

ATPase activity would obviously be reduced *in vivo* because of the unavailability of ATP; however, enzyme activities were assayed *in vitro* with optimal concentrations of substrates. Thus, low ATP levels resulting from coronary occlusion do not *cause* the irreversible decrease in sarcolemmal $Na^+,K^+$-ATPase but may initiate a secondary or tertiary event that results in inhibition of enzyme function. The reduction of ATP content in our studies preceded the decrease in sarcolemmal $Na^+,K^+$-ATPase activity and the increase in the $Na^+/K^+$ ratio. Similarly, Reimer *et al.*, using posterior papillary muscles from dogs, has shown that depressed high energy phosphate synthesis preceded the development of overt sarcolemmal membrane damage.[144] A further temporal dissociation between low ATP levels and increases in sarcolemmal permeability has been made by Haworth *et al.*. Using intact, quiescent myocytes, they showed that the onset of ischaemia-induced contracture always followed a critical decrease in ATP content.[145] Jennings *et al.* have suggested that depletion of high energy phosphates to a critical level of <3-8% of control was associated with irreversible cell damage.[21] Whether or not low ATP levels and subsequent sarcolemmal damage are causally related is not certain although, as stated earlier, ATP depletion would probably result in irreversible plasma membrane damage via an intermediate step. Alternatively, membrane phosphorylation by ATP may directly preserve sarcolemmal membrane integrity. In this case, ATP depletion might have a direct effect on sarcolemmal structure and function.

The reduction in mitochondrial ATPase activity could account, in part, for the fall in ATP levels because the enzyme is also involved in ATP synthesis. However, unlike mitochondrial ATPase activity, the decrease in ATP levels is not reversed by reperfusion, even after short periods of ligation. Since decreases in mitochondrial function are largely reversible with

reperfusion, e.g., mitochondrial ATPase activity, State 3 oxygen consumption, and creatine phosphate levels attain pre-occlusion values,[146] it has been postulated that the lack of regeneration of ATP is due to an efflux of precursors for the synthesis of adenine nucleotides from the ischaemic zone.[92,147] The compound allopurinol preserves the purine pool by blocking the irreversible conversion of xanthine to uric acid by xanthine oxidase, and limited success in reducing ischaemic damage has been achieved with administration of this drug prior to coronary ligation.[148,149] However, the importance of this effect vs. the vasodilatory properties of the drug in protecting the myocardium from ischaemic damage is uncertain.

Decreases in cellular ATP have also been implicated as the cause of the prolonged depression of contractility.[17,150,151] In agreement with the reports in the literature, the present results showed that the depression of both mechanical function and high energy phosphate levels was not reversed upon reperfusion despite normalization of other structural and functional indices. The apparent co-dependence of ATP content and contractile function has led to the suggestion that the rapid decrease in ATP may be responsible for the immediate decline in tension seen after coronary ligation. However, Hearse has shown that within 5 seconds of anoxia, ATP has been reduced by 25% while contractility remains constant.[152] As well as demonstrating a dissociation of the two variables, it is interesting to compare these results from anoxic injury with those of Kloner et al. who have shown that the opposite situation occurs in ischaemia following ligation of a coronary artery, i.e., contractility has decreased despite normal levels of ATP.[23] It is clear, then, that the nature of the relationship between ATP levels and contractile force is not a simple one.

Another possible cause of the immediate decline in tension is the fall in pH within the ischaemic cell. Rapid decreases of pH (within 5 seconds) have been recorded by both pH-sensitive electrode[153] and $^{31}$phosphorus nuclear magnetic resonance techniques.[22] The major source of the $H^+$ production has been attributed to glycolytic lactate accumulation.[22] Again, although the parallel changes in tension and pH both with ischaemia and reperfusion suggest that these two occurrences may be related, this does not preclude a co-dependence of pH and tension on some other factor.

Interestingly, the fall in pH also closely parallels the decreases in mitochondrial ATPase activity, and both recover to control levels with reperfusion, i.e., recovery is a flow-dependent rather than ATP-dependent phenomenon. Whether or not the fall in pH is responsible for the observed decrease in mitochondrial enzyme activity is a matter of speculation and *in vitro* studies on the pH dependence of this enzyme would be of interest.

Drug Studies

Drug pretreatment prior to a 20 minute period of ligation had some effect on the measured indices of cellular injury. Of particular benefit was the calcium channel blocking agent D-600 HCl (methoxyverapamil) which significantly preserved mitochondrial ATPase activity and ATP levels. Propranolol, had some effect on preserving the mitochondrial enzyme activity although this did not reach statistical significance. Despite the fact that propranolol decreased rate and increased ATP levels in control hearts, this agent did not preserve ATP levels during 20 minutes of ligation. Neither propranolol nor pranolium, the quaternary derivative of propranolol, had significant effects on any of the measured indices of injury other than fibrillation. The relatively short period of ischaemia is just at the transition of reversible to irreversible sarcolemmal injury and consequently the

effects of drug pretreatment are not clear cut. In contrast to the results of the 20 minute ligation study shown here, some investigators have shown that propranolol reduces ischaemic damage (see Introduction). However, others have also failed to demonstrate any protective action of either pranolium[154,155] or d-propranolol[156] (the non-β-blocking optical isomer of l-propranolol) in preventing ischaemic injury. Thus, any beneficial actions of β-blockers probably arise from β-blockade and not other membrane-related properties. Contrary to expectations, one of the most interesting aspects of the 20 minute ligation study was the effect of drugs on the incidence of ventricular fibrillation. Propranolol and chlorpromazine significantly increased the incidence of fibrillation whereas D-600 abolished it. Pranolium did not alter the frequency of fibrillation from that observed with saline pretreatment. The ability of D-600 to decrease ventricular arrhythmias may be explained by the blockade of slow calcium channels which may be opened in depolarized, ischaemic ventricular tissue thereby initiating abnormal conduction. The effects of propranolol, chlorpromazine and pranolium are less easily explained. Propranolol and chlorpromazine both have quinidine- like effects, i.e., decreased automaticity of Purkinje tissue and decreased membrane responsiveness.[157,158,159] However, these effects would tend to oppose the development of aberrant conduction pathways rather than precipitate them. It is possible that the high doses used in this study in the presence of depolarized, ischaemic tissue may create an environment that promotes ventricular arrhythmias although at higher doses, propranolol reduces the inward fast sodium current and one would also expect this action to suppress abnormal ventricular rhythm. These agents also increase the P-R interval and reduce conduction through the atrioventricular (A-V) node. However, this property is also shared by D-600 which does not have the

deleterious effect on electrical activity. Thus, most of the above actions of these agents cannot readily account for the increase in fibrillation. A possible mechanism of propranolol may be related to the fact that it can increase the effective refractory period (ERP) of the A-V node with little effect on normal atrial and ventricular muscle, while at the same time substantially shortening the the ERP of Purkinje fibres.[161] One might therefore speculate that the reduction in the refractory period of Purkinje tissue may allow an escape phenomenon to develop in the ventricles, especially when coupled with the slowed conduction through the A-V node. Pranolium does not share the beta-blocking properties of propranolol although it has many of the same actions on isolated membranes[161]. From the data presented here, one might conclude that it is the beta-blocking actions of propranolol that are responsible for the arrhythmogenesis; however, non-beta-blocking properties of propranolol may contribute to the increased fibrillation frequency and whether or not these are also shared by pranolium is difficult to say. The real problem lies in the fact that the knowledge of the precise mechanisms underlying arrhythmogenesis are largely speculative in nature.

An alternative explanation may be found in the effects of these agents on the work of the heart. If propranolol or chlorpromazine reduced the supply/demand ratio to a critical level, one could imagine fibrillation resulting from the increased damage caused by an exacerbation of ischaemic injury. Propranolol, at least, probably diminished the work of the ischaemic heart by a reduction of rate and contractile force (see below) and this, in conjunction with the improved integrity of propranolol-treated hearts, make it unlikely that this is the mechanism of its action. Chlorpromazine, on the other hand, may have had its effects by exacerbating ischaemic tissue

damage. It is evident that the cause of the increased fibrillation frequency with these drugs is not easily explained and further studies are required in order to ascertain the underlying molecular alterations.

Both D-600 and propranolol might be expected to have salutary actions on ischaemia/reperfusion injury because of potential effects on the supply/demand ratio as well as calcium channel blockade. D-600 and propranolol are capable of reducing calcium entry although propranolol can do this directly only at pharmacological doses.[66,162] Reduction of calcium entry by functional antagonism by propranolol would be expected at doses that produce β-blockade. Calcium influx is thought to be deleterious for the following reasons; myofibrillar accumulation can result in contracture, $Ca^{2+}$-dependent degradative enzymes may be activated, $Ca^{2+}$ accumulation by mitochondria occurs at the expense of ATP synthesis,[127] and very high cellular calcium concentration will inhibit a variety of enzymatic reactions. These drugs also decrease the oxygen consumption of the heart by reducing some of the determinants of myocardial energy consumption, specifically heart rate, mean arterial pressure, stroke volume, and wall stress. Gibbs has determined that β-adrenergic blocking agents reduce energy consumption via a reduction in heart rate but that this saving will be off-set by an increase in end-diastolic volume (increasing wall stress). On balance, though, the decrease in heart rate, afterload and contractility produced by propranolol administration will more than compensate for any increase in end-diastolic volume. Calcium channel-blockers also reduce contractility, decrease the afterload and directly dilate the coronary vasculature[163] (thereby increasing the supply/demand ratio). Thus, although both of these agents would be expected to retard ischaemic damage, whether or not they actually reduce the final extent of ischaemic injury is presently not known. In the present

studies, when irreversible ischaemic damage (40 minutes) was followed by reperfusion, both D-600 and propranolol produced a parallel degree of protection to the ischaemic myocardium although D-600 was consistently superior. Both drugs prevented the decrease in mitochondrial ATPase activity as well as reducing the extent of sarcolemmal damage, including $Na^+,K^+$-ATPase activity and ion abnormalities. Despite these beneficial effects on membrane integrity, ATP levels were not significantly increased by either drug pretreatment, although there was a non-significant increase in ATP levels with D-600 pretreatment. Thus, the protective effects do not appear to be exclusively mediated by a preservation of ATP. This does not rule out the possibility that a specific "compartment" of ATP was increased with drug treatment or that the prolonged depression of high energy phosphate levels is reflecting a higher turnover of ATP for cell homeostasis.

The preservation of cellular integrity by drug pre-treatment was not accompanied by preservation of contractile force. This is in contrast to the work of other investigators who have shown that calcium channel blockers improve contractile function as well as other measures of cell viability.[63,79,164] In the present experiments, it is possible that the small non-significant increase in contractility in the D-600 group may have been enhanced if a larger dose of the drug had been used. Preliminary results using a dose of D-600 HCl twice that used in the studies presented here have demonstrated even greater protective effects on some of the indices of ischaemia/reperfusion injury (data not shown). The lack of any improvement in function by propranolol also contrasts with the biochemical and morphological protection observed. In fact, in contrast to the calcium channel-blockers, most studies have described benefits of propranolol administration at the molecular rather than functional level.[62,154,165] Moreover, our

results with D-600 and propranolol support the hypothesis that the level of ATP in the cell is somehow related to its contractile ability in light of the concomitant (albeit small) increase in ATP and contractility with D-600 and the lack of ATP preservation and contractile function with propranolol.

Preservation of cell integrity by drug pretreatment was also verified by electron microscopic analysis. The appearance of the myocardial sections from the ischaemic/reperfused zone generally agreed with the assessment of injury by biochemical techniques. For example, the calcium levels in the drug-treated hearts were only half the value of untreated ligated/reperfused hearts, although, their calcium content was still 5 times greater than control levels. Mitochondrial injury was apparent in the assessment of membrane structure and the presence of intra-mitochondrial densities and the evidence of sarcolemmal damage by ultrastructural analysis paralleled the results of the enzyme assays. In addition, both of the drug-treated groups had higher scores in the contractile abnormalities (presence of contraction band and/or relaxation) confirming that the sarcolemmal permeability to calcium was compromised. However, when interpreting the results of an electron microscopic study, one must be cautious in making conclusions about the state of the tissue in vivo, i.e., it is probable that some of the damage to the cells is the result of fixation artifact. For example, it is difficult to imagine that the severely damaged mitochondria from the L+R group (saline pretreatment) have an ATPase activity which is 75% of control. There is a further problem with the use of ultrastructural data alone because there is often a great difference between the apparently normal "mummified" cell and its viability as assessed by biochemical techniques. A striking example of the disparity between ultrastructural appearance and biochemical assessment of cell injury was shown by Ruigrok et al. in which they found that ischae-

mic myocardial tissue pretreated with DMSO appeared normal on electron micrographs even though the sarcolemma was permeable to the dye, trypan blue.[166] Therefore, a more accurate picture of the structural integrity of the tissue is obtained by a corroboration of several different methods of evaluation as was done in the present study.

The results from the reperfusion studies presented here have indicated that elevated calcium levels within the tissue are associated with a depression in mitochondrial ATPase activity. The accumulation of calcium by the mitochondrion is one of the processes of calcium homeostasis that maintain cytosolic calcium in the range of approximately $10^{-7}$ M although mitochondrial calcium uptake does not generally operate except at calcium concentrations far in excess of normal levels (approximately $10^{-5}$ M). The mitochondrion has a large capacity for calcium, which, in the presence of phosphate, will form calcium phosphate crystals. As stated previously, calcium uptake also occurs at the expense of ATP synthesis. This would have myriad effects on cell function in addition to compromising the active transport of more calcium out of the cell. Once the capacity of the mitochondrion is exceeded, however, calcium efflux occurs and there is a collapse of the mitochondrial membrane potential, presumably resulting in irreversible mitochondrial damage.[167] This type of damage occurs long after the decrease in other mitochondrial functions, and given the relative reversibility of mitochondrial structural and functional injury, it would appear that the ultimate irreversible depression of mitochondrial function may result from an increase in sarcolemmal permeability to calcium. Additionally, sodium accumulation (which in the present experiments occurs much earlier than calcium influx) will tend to create an influx of water into the cell, which,

if sufficiently severe, has also been postulated to perturb mitochondrial function by causing misalignment of spatially-oriented enzyme systems within the mitochondrial matrix.[168] Reperfusion per se causes massive $Na^+$ and $Ca^{2+}$ influx, thereby reducing mitochondrial function by at least two possible mechanisms. It would appear, then, that irreversible mitochondrial damage does not occur unless the sarcolemmal permeability is great enough to permit $Ca^{2+}$ to accumulate intracellularly to levels that are beyond the accumulating capacity of the mitochondrion and/or large influxes of $Na^+$ and water. The influence of $Na^+$ and water influx per se on mitochondrial function is not precisely known. However, the present experiments did demonstrate a relationship between the level of intracellular calcium and mitochondrial function. Reduction of calcium levels by pretreatment with either D-600 or propranolol resulted in preservation of mitochondrial ATPase activity. Furthermore, addition of D-600 prior to reperfusion also significantly prevented the decrease in mitochondrial ATPase activity and slightly preserved ATP levels despite the absence of any beneficial effect on other aspects of cell function including sarcolemmal permeability to other ions.

Is the mechanism of protection of cellular integrity by these agents related to their ability to directly prevent calcium influx rather than via their ability to reduce the metabolic requirements of the ischaemic cell ? In the experiments involving 40 minutes of ligation followed by 60 minutes of reperfusion, one would have expected only D-600 to reduce the calcium influx during reperfusion via a direct blockade of calcium channels. Propranolol would only reduce calcium accumulation if it preserved sarcolemmal integrity during the ischaemic period. Both agents were found to reduce calcium influx to the same extent and furthermore, both provided some protection against $Na^+$ influx. This result suggests that the mechanism of

protection occurs as a result of drug-induced decreases in myocardial energy requirements during the ischaemic period with consequent preservation of sarcolemmal integrity.

The importance of calcium channel blockade per se during reperfusion was examined in the study in which D-600 was administered immediately prior to reperfusion after a 40 minute ligation period. As mentioned above, this intervention reduced calcium influx and produced a concomitant preservation of mitochondrial ATPase activity (and ATP levels to a small extent). However, it had no effect on $Na^+$ or water influx, $K^+$ and $Mg^{2+}$ loss, $Na^+,K^+$-ATPase activity, or $(dP/dt)_{max}$, thereby confirming the hypothesis that the drugs' beneficial actions occur primarily during the ischaemic period.

There has been a great deal of controversy in the literature regarding the mechanism of β-adrenergic antagonist and calcium channel blocker protection against ischaemia/reperfusion injury.[61,79,169-171] The results of the present experiments may provide some insights into the reasons for differing opinions on the relative importance of $Ca^{2+}$ blockade vs. decreased energy requirements in preventing reperfusion damage. Administration of calcium antagonists after the ischaemic insult and prior to reperfusion directly reduces calcium overload thereby preserving mitochondrial function and ATP stores. If these indices are used as the exclusive determinants of cellular integrity, one would expect to observe a protective effect of calcium channel blocker administration in reperfusion injury, especially in the short-term. However, it is doubtful that the preservation of mitochondrial function would be of sustained benefit when, at the same time, there is irreversible sarcolemmal membrane damage. The present results indicate that the predominant beneficial effect is derived from a

reduction of $MVO_2$. This hypothesis receives further support from the work of Robb-Nicholson *et al.* in which either KCl (16 mEq/l) or verapamil (2 μg/ml) was added to reduce the energy requirements of perfused, isolated rat hearts. The results showed that KCl and verapamil provided equivalent protection against ischaemia/reperfusion injury.[172] Furthermore, consistent with the idea that calcium channel blockade may selectively improve ATP levels, these authors found that D-600 afforded slightly better protection than KCl against ATP losses. Similar results were obtained in the isolated, isovolumic feline heart by Magee *et al.* who also found similar protective effects of nifedipine (another calcium channel blocker) and KCl but only when the dose of nifedipine was sufficient to produce immediate asystole.[173]

The experiment wherein D-600 was added prior to reperfusion has shown that calcium influx is associated with ischaemic/reperfusion injury during the ischaemic period. The results of experiments presented herein have shown that even after 60 minutes of ischaemia in which coronary flow was reduced to approximately 5% of the blood flow in the non-ischaemic left ventricle, the occluded zone did not accumulate calcium to any significant degree (cf. $Na^+/K^+$ ratio). More sensitive techniques using radioactively-labelled $^{45}Ca^{2+}$ or $^{47}Ca^{2+}$ have also shown that the calcium does not accumulate during a 60 minute ischaemic period.[51,174] These studies would not be able to detect rapidly exchanging calcium so it is possible that a stimulation of efflux could have compensated for small increases in intracellular $Ca^{2+}$ levels. There are at least two other mechanisms for maintenance of the calcium gradient and both operate at lower calcium concentrations than the mitochondrial uptake. Sarcolemmal $Ca^{2+}$-ATPase has a high affinity for calcium ($K_M$ = .2 -.6 μM) although a lower transport rate as compared with the ATP-independent $Na^+$-$Ca^{2+}$ exchange.[175] The

limiting factor for calcium extrusion by $Ca^{2+}$-ATPase would be the availability of ATP and for $Na^+$-$Ca^{2+}$ exchange it would be a reduction in the $Na^+$ gradient. Thus, even with low concentrations of ATP, the cell could still utilize the $Na^+$ gradient to remove excess calcium. Therefore, increases in the $Na^+/K^+$ ratio in the absence of changes in $Ca^{2+}/Mg^{2+}$ content may be explained either by postulating that a specific alteration in $Na^+,K^+$-ATPase (with a concomitant increase in the $Na^+/K^+$ ratio) occurs without any increase in sarcolemmal calcium permeability or that the influx of calcium arising from a general increase in sarcolemmal permeability is compensated for by calcium efflux. In either case, the ischaemic myocardial cell can maintain low intracellular calcium levels. However, this does not take into account any re-distribution of calcium within the cell, e.g., release into the cytosol from the sarcoplasmic reticulum or mitochondrion. It is unlikely that calcium release would occur from the mitochondrion because the associated collapse of the mitochondrial membrane potential is more apt to be a consequence rather than a cause of cytoplasmic calcium accumulation.

This apparent preservation of the calcium gradient in irreversibly-injured ischaemic cells (exposed to periods of ischaemia greater than 30 min) is readily destroyed by the re-instatement of blood flow to the occluded zone. Some authors have simply explained this phenomenon as resulting from the large influx of plasma calcium entering the ischaemic tissue with the fresh blood of reperfusion. However, even with zero flow, there is still a very large calcium gradient across the membrane and, from the above evidence, it appears that the sarcolemmal barrier to the high extracellular concentration of this ion remains relatively intact. Thus, it is not just the presence of calcium in the reperfusing blood that causes calcium over-

load. Reperfusion of irreversibly damaged cells precipitates some event(s) that destroy the sarcolemmal permeability barrier. As yet, there has been no satisfactory answer as to what causes the sudden increase in permeability. It is interesting to note that at least some of the calcium influx during reperfusion is through voltage-sensitive calcium channels that can be blocked by D-600. After consideration of these data, a possible (albeit simplified) mechanism of reperfusion-induced calcium accumulation might be as follows:

After approximately 20-30 minutes of ischaemia, irreversible damage to the lipid environment of the plasma membrane-bound $Na^+,K^+$-ATPase occurs via some mechanism that is probably associated with low ATP levels. As ischaemia progresses, the gradients for $Na^+$ and $K^+$ run down. Other compensatory mechanisms for $Na^+$ and $K^+$ homeostasis apparently do not exist, and the cell gradually depolarizes.

During ischaemia, $Ca^{2+}$ does not enter the cell because sarcolemmal permeability to calcium is either unchanged or small influxes are compensated for by (e.g., $Ca^{2+}$-ATPase and $Na^+$-$Ca^{2+}$ exchange).

Upon reperfusion after irreversible injury, there is a large influx of water and $Na^+$ as well as efflux of $K^+$. Water will flow into the cell because of the increased internal osmotic pressure which results from the accumulation of $Na^+$ and $H^+$. Sodium influx exceeds potassium efflux and the cell would depolarize further. When the membrane potential becomes less negative (approx. -50 mV),[176] voltage-sensitive $Ca^{2+}$ channels would open and allow $Ca^{2+}$ influx. Low ATP levels would reduce uptake by the sarcoplasmic reticulum $Ca^{2+}$-ATPase and efflux by the sarcolemmal $Ca^{2+}$-ATPase. Furthermore, the $Na^+$ gradient would be abolished and therefore $Na^+$-$Ca^{2+}$ exchange would also be

inoperative. Mitochondria will take up calcium until their capacity is exceeded after which point the damaged mitochondria release $Ca^{2+}$ into the cytosol. This event, accompanied by intracellular oedema, would cause irreversible damage to most enzyme systems.

A logical implication of these data would be that if ischaemia is sufficiently prolonged (several hours) one might expect to see a gradual influx of calcium as the gradients for $Na^{+}$ and $K^{+}$ are destroyed and the cells depolarize. To my knowledge, serial measurements of tissue ions during periods of ischaemia greater than 2 hours have not been done.

The discussion of "calcium-induced reperfusion injury" would not be complete without the mention of the so-called "calcium paradox" phenomenon. It has been shown using isolated hearts that a period of calcium-free perfusion followed by perfusion with physiological $Ca^{2+}$ solution causes extensive damage to myocardial cells, including ion derangements similar to those seen with ischaemia/reperfusion injury.[177] Various investigators have examined the effects of calcium channel blockers on calcium entry during the "reperfusion" period with mostly negative results.[178,179] These results should not be extrapolated to the situation with ischaemia/reperfusion injury because the mechanism of sarcolemmal damage is probably quite different. During calcium-free perfusion the superficially-bound calcium of the glycocalyx is removed. The presence of this calcium is necessary for the maintenance of an intact sarcolemma/glycocalyx and subsequent reperfusion with calcium-containing solution causes profound changes in myocardial ion levels, morphology and biochemistry. Repletion of calcium in the perfusate after a 10 minute period of calcium-free perfusion, resulted in loss of cytosolic enzymes within 1 minute of "reperfusion".[78] Thus, although the

degree of calcium accumulation may be comparable, the time course and features of "calcium paradox" damage differ markedly from ischaemia/reperfusion injury. Some researchers have investigated the efficacy of interventions for calcium-induced reperfusion injury by using the "calcium paradox" technique; however, it is suggested that this is not an appropriate model of ischaemia/reperfusion injury.

The results of these experiments and those of many other investigators, have shown that a few agents, e.g. propranolol, calcium channel blockers, can limit the short-term damage produced by coronary occlusion and release. However, it is not clear that these interventions ultimately improve functional status. Few long-term studies have been performed. Bush et al. have shown that over a 4 week period of reperfusion, the initially-better functional status of diltiazem-pretreated hearts was not maintained[180] and all treatment groups had similar function by the end of a 4 week period. In the present studies, the slight benefit of D-600 on $(dP/dt)_{max}$ was in contrast to the significant biochemical and morphological improvement. The presence of intact permeability barriers would be necessary for contraction; however, their existence does not mean that contractile force would be able to return to control levels. Thus, it is important to emphasize that preservation of myocardial cell integrity does not necessarily mean that the function will also be preserved. Long-term studies of the dose and time-dependence of protective effects of both calcium channel blockers and beta-adrenergic antagonists in ischaemic injury would be valuable. The results of such studies may finally resolve the question as to whether or not the cardioprotective actions seen in acute models will ultimately result in sustained improvement of myocardial function and therefore be of value

clinically in preventing or reducing the degree of myocardial functional impairment resulting from ischaemic insults sustained either intraoperatively or following acute myocardial ischaemia.

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White PD (1951) Heart Disease New York, The MacMillan Co.

# APPENDIX I

FREQUENCY RESPONSES:

Grass Differentiator Model 7P20C

Input triangular wave: 1-100 volts/sec/cm

Output from differentiator on oscilloscope - "D"

Output from pen recorder - "P"

| Input (v/sec/cm) | Output "D" (v/sec/cm) | Output "P" (v/sec/cm) |
|---|---|---|
| 1 | 0.9 | 0.9 |
| 5 | 4.5 | 4.8 |
| 10 | 9.0 | 9.3 |
| 50 | 50 | 48 |
| 100 | 100 | 40 [a] |

[a] 54 Hz input frequency exceeds pen limit

Maximum setting used experimentally was 25 v/sec/cm

# APPENDIX II

## Electron Microscopy Procedures

Buffered glutaraldehyde: made fresh for each experiment - 25% glutaraldehyde was diluted with cold (4°C) cacodylate buffer (0.1 M cacodylate, 1 mM $CaCl_2$, pH 7.2) to a final concentration of 3% glutaraldehyde.

Ruthenium red: Dye crystals were diluted with the same buffer as described above to a final concentration of 0.8%. The solution was made one day before use and stored in a dark bottle in the refrigerator.

$OsO_4$ (osmium tetroxide): $OsO_4$ is light-sensitive and volatile. Consequently, a cleaned ampoule of $OsO_4$ crystals was dropped into an Erlenmeyer flask covered with aluminum and the ampoule broken open with a glass rod. Sufficient cacodylate buffer was added to produce a final concentration of 1% $OsO_4$. This was prepared one day in advance to allow all crystals to dissolve, and stored in the refrigerator.

Ethanol and Propylene Oxide: 95% ethanol (v/v) was diluted with double-distilled water to make up the desired final concentrations. To dehydrate the ethanol, ethanol/propylene oxide mixtures and the propylene oxide, molecular sieves (Davison Chemical Co.; 8-12 mesh) were added to the stock solutions to adsorb water. This precaution allowed these solvents in the tissue to be miscible with the embedding plastic thereby preventing the formation of water pockets within the sample.

## APPENDIX III

Radioactive microsphere technique for blood flow determination

Injection protocol:

| | Control | Ligation | Reperfusion |
|---|---|---|---|
| Group I | $^{113}Sn$ | $^{57}Co$ | -- |
| Group II | $^{113}Sn$ | -- | $^{57}Co$ |

After adjusting total and reference blood samples cpm values for losses in the injection and withdrawal syringes, as well as correcting the cobalt cpm for spill-over from the tin channel (approximately 17 ), the following calculation for the flow to the individual tissue could be carried out:

SAMPLE CALCULATIONS:

Experiment - February 2, 1983

Sample calculation:

| | 1st isotope (Sn) (Control) | 2nd isotope (Co) (After 10 min of ligation) |
|---|---|---|
| Amount given (cpm) | 33,997 | 247,528 |
| Withdrawal rate (ml/min) | 0.7 | 0.7 |
| Amount in blood (cpm) | 76 | 700 |

Total Cardiac Output:

$$\text{Control period} = \frac{33997}{76} \times 0.7 \text{ ml/min}$$
$$= 313 \text{ ml/min}$$

$$\text{Ligation period} = \frac{247,528}{700} \times 0.7 \text{ ml/min}$$
$$= 248 \text{ ml/min}$$

Flow calculations on following page.

TISSUE FLOW:

| Tissue | Wt (g) | Ch 1 (Co) [corrected cpm] | Ch 2 (Sn) | [a]Blood Flow (ml/min/g) Control | Ligation | Percent C.O. Control | Ligation |
|---|---|---|---|---|---|---|---|
| Ischaemic L.V. | 0.50 | 95 | 207 | 3.82 | 0.19 | 0.61 | 0.04 |
| Normal L.V. | 0.87 | 2143 | 416 | 4.40 | 2.46 | 1.22 | 0.86 |
| Right Ventricle | 1.15 | 3203 | 446 | 3.57 | 2.78 | 1.31 | 1.29 |
| Left Kidney | 8.04 | 10,737 | 2012 | 2.30 | 1.30 | 5.90 | 4.30 |
| Right Kidney | 7.71 | 10,985 | 2101 | 2.50 | 1.40 | 6.20 | 4.40 |

[a]Blood flow in ml/min/g = $\frac{\frac{\text{cpm sample}}{\text{cpm ref. blood}} \times \text{withdrawal rate}}{\text{tissue weight in g}}$