# The Effects of Exogenous L-Aspartate and L-Glutamate During Ischemia-Reperfusion for Cardiac Surgery. A Magnetic Resonance Study in Isolated Pig Hearts.

By

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A thesis

submitted for the degree of

**Master of Science**

Department of Physiology, University of Manitoba

September, 1998

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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# THE EFFECTS OF EXOGENOUS L-ASPARTATE AND L-GLUTAMATE DURING ISCHEMIA-REPERFUSION FOR CARDIAC SURGERY. A MAGNETIC RESONANCE STUDY IN ISOLATED PIG HEARTS

BY

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

*To my parents, whose encouragement and sacrifices have made it all possible, and to my love, Leah, for her patience and support throughout my studies.*

# 1. FRONT MATERIAL

## 1.1 Table of Contents

## 1.2 Abstract


### A) Background

Reperfusion of ischemic hearts with warm blood cardioplegia enriched with L-aspartate (asp) and L-glutamate (glu) has been reported to alleviate post-ischemic metabolic and functional derrangements. Two studies were performed to investigate this possibility using phosphorus-31 ($^{31}P$) magnetic resonance spectroscopy (MRS) in isolated pig hearts. The first study tests the effects of exogenous asp and glu on myocardial energy metabolism during ischemia-reperfusion. The second tests the ability of the Buckberg cardioplegic protocol to reverse myocardial stunning in ischemic hearts. $^{31}P$ MRS was used to observe cellular energetics and intracellular pH throughout the protocols. Left-ventricular function and oxygen consumption were evaluated before and after ischemia.

### B) Study #1

Isolated pig hearts were perfused with blood (Group A, n=8) or blood enriched with 13 mmol/L each of asp and glu (Group B, n=6). The hearts were subjected to 30 minutes total normothermic ischemia and then reperfused for 40 minutes. Two hearts from each group were inotropically stimulated by titration with calcium after normokalemic reperfusion.

MRS showed no decrease in the rate of energy decline during ischemia for Group B versus Group A. There were also no significant differences between the two groups in terms of myocardial function, oxygen consumption, or the rate or extent of high-energy-phosphate recovery after normokalemic reperfusion or subsequent inotropic stimulation.

Inotropic stimulation of post-ischemic hearts however lead to dramatic improvement in myocardial function in both groups ($p<0.05$ for all parameters) and significant improvement in oxygen consumption ($p=0.01$).

## C) Study #2

Fifteen blood-perfused Langendorff pig hearts were subjected to 30 minutes of total, normothermic ischemia. Control hearts (Group A, n=8) were reperfused with blood for 40 minutes as described above. Experimental hearts (Group C, n=7) received 20 minutes of asp/glu enriched blood cardioplegic solution according to Buckberg's "warm reperfusate" recipe, followed by 20 minutes of normal blood.

The MR spectra showed no improvement in rate or extent of high energy phosphate recovery with asp/glu cardioplegia, but showed a transient increase in intracellular pH during cardioplegic perfusion ($p<0.05$). This, however, did not affect post-ischemic recovery of myocardial function or oxygen consumption.

## D) Conclusions

In a normal, isolated, blood-perfused pig heart subjected to 30 minutes of total normothermic ischemia:

(1) Enrichment of the perfusate with asp/glu prior to and following ischemia affects neither myocardial energy metabolism during ischemia-reperfusion nor post-ischemic recovery of myocardial function or oxygen consumption.

(2) Inotropic stimulation can recruit significant post-ischemic function and sufficient aerobic respiration to support it. irrespective of asp/glu enrichment.

(3) Buckberg's asp/glu enriched secondary cardioplegia has no beneficial effects on functional or metabolic status of stunned pig hearts.

## 1.3 Acknowledgments

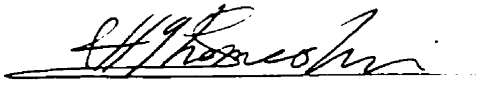
A great many people have contributed to this project. I would first like to thank my supervisors, Drs. Roxanne Deslauriers and Tomás Salerno for their encouragement, support, and patience. Roxanne, you've been more patient and accommodating than I could have asked for! I also owe much to Dr. Ganghong Tian who in many ways acted as a co-supervisor and friend, and taught me much of what I know about experimentation. Many thanks to Dr. Carlos Filgueiras for his surgical teachings and to Mr. Edward Hoffenberg for his friendship, resourcefulness, and the last-minute salvage of this document! I must give special thanks to Dr. Jian Ye, Mr. Jiankang Sun, Mrs. Shelley Germscheid and Mrs. Lori Gregorash for endless hours of technical assistance during extremely demanding experiments. Last, but not least, I thank Mr. Randy Summers for his statistical expertise, Ms. Lori Shoemaker for expert biochemical analyses, and Mrs. Monique St. Jean for patiently coordinating all experiments-a truly daunting task!

## 1.4 Statement

The work reported in this thesis was undertaken in the Institute for Biodiagnostics of the National Research Council of Canada in conjunction with the Department of Physiology at the University of Manitoba, and the Department of Cardiothoracic Surgery at the State University of New York at Buffalo. Supervision of this work was provided by Drs. Roxanne Deslauriers and Tomás Salerno.

I declare that the work presented in this thesis is original and that this material has not previously been submitted, either in part or in full, for a degree at this or any other university.

September, 1998



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# 2. INTRODUCTION

Since its humble origins around the turn of the century, the science and art of cardiac surgery has developed to such a point that most diseases of the heart have been affected to some extent by this practice [4]. There are currently nearly one million cases of open-heart surgery performed every year in North America. The incredible success and growth of this relatively new field has been largely due to advances that have allowed surgeons access to the heart and thoracic vessels without excessively compromising the physiological processes that depend on the heart. Two such advances have been cardiopulmonary bypass and cardioplegia. Cardiopulmonary bypass effectively takes over the heart's primary function of delivering oxygenated blood to tissues (including the heart itself), and removing deoxygenated blood. This allows the heart to be arrested and thus provides stationary, bloodless access to the heart chambers, valves, coronary blood vessels, and the great vessels. Cardioplegia, or elective cardiac arrest, is the means by which the heart is arrested during surgery, while protecting it from excessive myocardial injury during periods of ischemia accompanying this arrest.

Cardioplegia gained widespread acceptance in the 1970's. Despite many advances in this field during the past few decades, intraoperative myocardial injury is still an important cause of mortality and morbidity in cardiac patients after technically successful operations [5]. The development of better techniques for intraoperative cardioprotection is therefore a continuing priority in open-heart surgery. One of the problems that has plagued developments in cardioplegia has been a surprising amount of controversy in determining which technique provides the most protection. This has been due to a variety

of factors including unavailability of adequate experimental models for studying cardioplegia and a lack of sufficient controlled randomized trials for many newer techniques. One such area of controversy is that surrounding the use of the amino acids L-aspartate (asp) and L-glutamate (glu) in cardioplegic solutions.

Asp and glu were introduced into cardioplegic solutions in the 1980's by Buckberg's group at the University of California, Los Angeles (UCLA), as a means of improving myocardial energy production during and after cardioplegic arrest [6,7]. Although Buckberg and associates have published many studies advocating the use of these agents, their benefits during ischemia-reperfusion remain controversial. Nevertheless, Buckberg's asp/glu-enriched blood cardioplegic solutions have been made commercially available and are routinely used in some centres during elective and emergency cardiac surgery [8-10], with possible potential for use during cardiac harvesting [11,12] and for neonatal cardiac surgery [13].

Resolution of the asp/glu controversy is of prime interest to many cardiac surgeons as there are potential disadvantages to using these agents. Both asp and glu are excitatory neurotransmitters and are neurotoxic at sufficiently high concentrations [14]. In addition, the delivery of asp/glu cardioplegia brings increased complexity to the perfusion techniques and thus increases the risk of complications. Furthermore, there are increased costs associated with the specialized equipment needed for delivery of Buckberg's asp/glu cardioplegia.

The main mechanisms suggested for the beneficial effects of asp and glu in cardioplegic solutions involve improved anaerobic energy production during ischemia and faster

recovery of efficient aerobic respiration during reperfusion. Therefore, one could hypothesize that continuous measurement of myocardial energy levels during ischemia-reperfusion should allow one to determine the value of asp/glu enrichment.

Nuclear magnetic resonance is a powerful tool that allows direct, continuous observation of certain metabolic events non-invasively and non-destructively. Phosphorus-31 ($^{31}P$) magnetic resonance spectroscopy (MRS) can be used to measure tissue levels of high energy phosphates such as adenosine triphosphate (ATP) and phosphocreatine (PCr), and to determine intracellular pH. In this study, we investigated the benefits of asp and glu by evaluating myocardial function, oxygen consumption, and energy metabolism using $^{31}P$ MRS in isolated, blood-perfused pig hearts. Two separate experiments were performed in order to answer the following two questions:

1. Does enrichment of the perfusate with asp and glu improve myocardial energy metabolism during ischemia and/or reperfusion?

2. Can Buckberg's asp/glu-enriched cardioplegia resuscitate ischemically stunned myocardium?

To evaluate the effects of asp and glu on myocardial energy metabolism during cardiac surgery, an understanding of the underlying phenomena is essential. Specifically, one must comprehend normal myocardial physiology and metabolism, the processes involved in ischemia-reperfusion injury, and the mechanisms by which cardioplegia can and does affect these processes. Furthermore, an understanding of relevant experimental models and the assumptions behind their use is critical to the success of the study and adequacy

of any conclusions drawn. It is based on these principles that the chapters in the 'Background Information' section have been organized. The first chapter includes a discussion of the principles of myocardial energy and substrate metabolism, followed by a chapter describing the cellular changes observed during ischemia and subsequent reperfusion of myocardium. An introduction to cardiac surgery, cardioplegia, and asp/glu enrichment is provided in the third chapter. Finally, there is a chapter describing the experimental models used for studying myocardial protection and the principles of magnetic resonance spectroscopy.

# 3. BACKGROUND INFORMATION

## 3.1 Myocardial Energy and Substrate Metabolism

### A) Overview

The true function of the heart was not known until 1628 when William Harvey refuted the theories of Galen, describing the circulation of blood and the role of the heart in propelling it [15]. Opie describes the heart well when he says: "the heart is a restless organ, in a constant state of mechanical or metabolic flux" [15]. In performing its task of supplying all body tissues with the prerequisites of normal cellular function, the heart relentlessly pumps nutrient-containing, oxygen-rich blood to tissues, deriving the energy for this task from the breakdown of energy-rich ATP to adenosine diphosphate (ADP) and inorganic phosphate (Pi). So enormous are the energy requirements of the heart, that an estimated 4-8% of the total ATP pool in the heart is broken down to ADP during a single contraction [16]. Nevertheless, myocytes normally maintain remarkably constant ATP concentrations under different conditions of work, as the metabolic functions of the heart are geared primarily to continuous regeneration of this vital energy source by oxidation of energy-rich substrates. This chapter provides a brief review of the processes involved in the delicate balance between myocardial energy generation and utilization.

### B) ATP Utilization

Under normal beating conditions, the great majority of energy consumption in the heart is for contraction. When an action potential reaches a myocyte, $Ca^{2+}$ ions enter the cell

through channels in the sarcoplasm, and more $Ca^{2+}$ is released from the sarcoplasmic reticulum into the cytosol. This leads to a nearly 100 fold increase in intracellular $Ca^{2+}$ concentration, which is responsible for initiation of myofibrilar contraction. In short, the $Ca^{2+}$ ions cause a change in the conformation of the troponin complex, ultimately leading to formation of cross bridges between actin and myosin molecules, which in turn is responsible for providing the contractile force of the myofilaments. The role of ATP in this process is two fold. First, each myosin head ATPase requires the attachment of one ATP molecule and its break down to ADP and Pi before detaching from actin. Thus, in the absence of abundant ATP, the cyclic formation of actin-myosin cross-bridges cannot occur. The other crucial role of ATP is in supplying the energy that eventually removes the released calcium from the cytosol, effectively ending one cycle of contraction in preparation for the next. ATP enables $Ca^{2+}$ removal directly via $Ca^{2+}$ ATPases in the sarcolemma and sarcoplasmic reticulum, and indirectly via the $Na^{+}/Ca^{2+}$ exchanger on the sarcolemma [17] (see below).

In addition to the energy required for contractile function, the heart has basal energy requirements arising from the need for structural preservation and for metabolic and electrophysiologic readiness for function. This energy is used mainly to maintain essential ionic gradients by ion pumps such as the membrane $Na^{+}/K^{+}$ ATPase and $Ca^{2+}$ ATPase, and for synthesis and degradation of biomolecules. The ATP-dependent maintenance of normal ionic gradients is not only a prerequisite for action potential generation and contraction, but also enables many routine cellular processes, such as co-transport of essential molecules across the membrane. In addition, the normal activity of

exchangers such as the $Na^+/Ca^{2+}$ exchanger mentioned above depends on these ionic gradients.

## C) Mitochondrial ATP Production

The sources of ATP in heart muscle are oxidative phosphorylation of ADP in the respiratory chain and substrate level phosphorylation in the glycolytic pathway or citric acid (or citrate or Krebs or tricarboxylic acid) cycle. Under normal conditions, oxidative phosphorylation is by far the most important mechanism. For instance, complete oxidation of a single molecule of glucose-6-phosphate under aerobic conditions produces a total of 38 ATP, whereas oxidation of the same molecule under anaerobic conditions can normally produce only 3 ATP [18]. Thus, in light of the very rapid ATP turnover mentioned above, the heart is clearly dependent on a continuous supply of oxygen in order to carry out its functions.

### *i) Citric acid cycle and oxidative phosphorylation*

Oxidative phosphorylation of ADP to ATP depends on the production of reducing equivalents and the passage of electrons along the mitochondrial respiratory chain. In short, energy-rich substrates are converted to acetyl-CoA, which is then oxidized in the citric acid cycle (Figure 1), whose enzymes are located in the mitochondria.

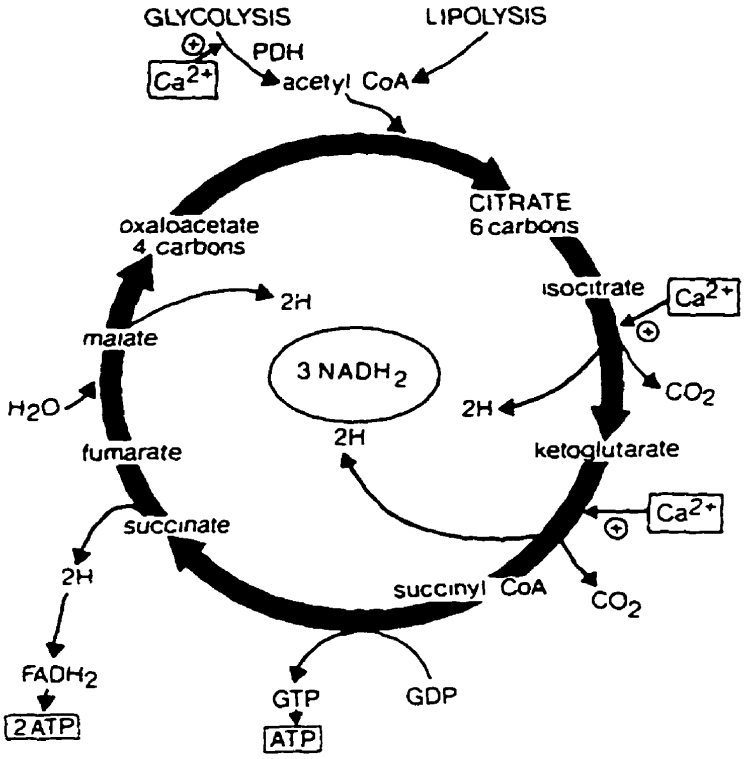


**Figure 1: The Citric Acid Cycle of Krebs (from: Opie LH, 1991 [19]).**

At many stages in their catabolism, the substrates generate reducing equivalents in the form of nicotinamide adenine dinucleotide, reduced form ($NADH_2$ or $NADH^- H^-$) and/or flavin adenine dinucleotide, reduced form ($FADH_2$). In addition, one ATP equivalent per turn of the citrate cycle is produced in the form of guanosine triphosphate (GTP) via substrate level phosphorylation at the stage of conversion of succinyl-CoA to succinate (Figure 1). $NADH_2$ and $FADH_2$ then transfer their electrons to the electron transport chain on the inner mitochondrial membrane where they ultimately react with molecular oxygen to produce water. In the process, as the electrons pass through the cytochromes (b, c, and a), ATP is produced via a mechanism involving creation of a proton gradient

across the mitochondrial membrane by pumping protons out at each cytochrome level. This transmitochondrial gradient forces protons to re-enter the mitochondrial matrix via a membrane protein called ATP synthetase, causing phosphorylation of ADP to ATP in the process [18]:

$$2NADH + 2H^{+} + O_2 \Rightarrow 2NAD^{+} + 2H_2O$$

**and**

$$6ADP + 6Pi \Rightarrow 6ATP$$

Thus, re-oxidation of each $NADH_2$ to $NAD^+$ produces 3 ATP molecules while using up one oxygen atom, and re-oxidation of $FADH_2$ produces 2 ATP per oxygen atom used (because electrons from $FADH_2$ enter the electron transport chain after the first step). In other words, $NADH_2$ has a phosphorylation-oxidation (P/O) ratio of 3 while the P/O ratio of $NADH_2$ is 2 [18]. This means that fuels, such as fatty acids, which produce a relatively higher proportion of $FADH_2$ to $NADH_2$, also utilize more oxygen to produce the same amount of energy. The has important implications for substrate selection in the heart, especially under situations of oxygen deficiency (see *section 3.1-E-iii)*.

### *ii) The creatine kinase reaction*

Both the citrate cycle and the events of oxidative phosphorylation take place in the mitochondria. Thus, the large amounts of ATP produced by the mitochondrial ATP synthetase must be transported to the cytosol. This is achieved by the creatine kinase (CK) system consisting of two isoenzymes. First, the mitochondrial membrane CK isoenzyme transfers the high energy phosphate bond from ATP to a molecule of creatine, thus forming the high energy phosphate phosphocreatine (PCr) in the cytoplasm:

**ATP + Creatine <=> ADP + PCr**

In the cytoplasm, PCr is in equilibrium with ATP and ADP via the above CK reaction, and is thought to play two possible roles. First, PCr can act as an energy reservoir, buffering the cytoplasmic ATP level according to the above equilibrium, catalyzed by a cytoplasmic CK isoenzyme [2,18]. This helps keep ATP levels remarkably constant under many conditions, such that during an acute work jump, PCr falls but ATP is kept virtually constant [20]. Secondly, PCr may have a role as an energy shuttle, transporting the energy produced by the mitochondria to the sites of utilization such as myofibrils, where ATP is again regenerated by the CK isoenzyme for use by the myofibrils [2,18].

## D) Myocardial Substrates

The heart, in spite of its extremely rapid energy turnover, is very versatile in its choice of substrates. This allows it to use the most available or most advantageous substrate under various conditions. Strictly speaking, the word substrate refers to a chemical compound that an enzyme catalytically converts to the product of that reaction. In the heart, however, as each energy source needs a series of catalytic reactions before it can be converted to acetyl-CoA, substrate simply means a fuel for the myocardium. The main types of fuels used in the heart are carbohydrates, fatty acids, and to a lesser extent, ketone bodies and amino acids.

### *i) Carbohydrates*

The main carbohydrates involved in cardiac energy metabolism are glucose, glycogen, lactate and pyruvate. Glucose is taken up via a facilitated passive transport system and

once inside the cell is phosphorylated to glucose-6-phosphate (G-6-P) which is then either metabolized via glycolysis (Embden-Meyerhof pathway), converted to glycogen, or metabolized via the pentose-phosphate pathway, the latter being of little importance in the heart [1].

Synthesis and degradation of glycogen happen via two different pathways with separate regulation. The balance between these two pathways determines whether there is net breakdown or synthesis. Glycogen synthesis begins with conversion of G-6-P to glucose-1-phosphate (G-1-P). The subsequent transfer of G-1-P to a pre-existing glycogen chain is an active process (requires uridine triphosphate) and so cannot take place during energy depletion [18]. Glycogen breakdown, on the other hand, is energy neutral (i.e. no HEP used) and is set in motion via a cyclic-AMP (adenosine monophosphate) dependent process and thus is enhanced during low energy states when cellular AMP levels are increased [18]. The breakdown product of glycogen (G-1-P) can be converted to G-6-P for breakdown in glycolysis. Although the exact role of cardiac glycogen is still under debate, it may act as a storage for glucose taken up from the circulation, especially during fasting when the main fuel tends to be FFA [18]. G-6-P from glucose or glycogen is metabolized in the glycolytic pathway (Figure 2) producing 2 net moles of ATP and two moles of $NADH_2$ which can lead to production of ATP in the mitochondria by passing of reducing equivalents into the mitochondria (see section 3.1-D-iv).

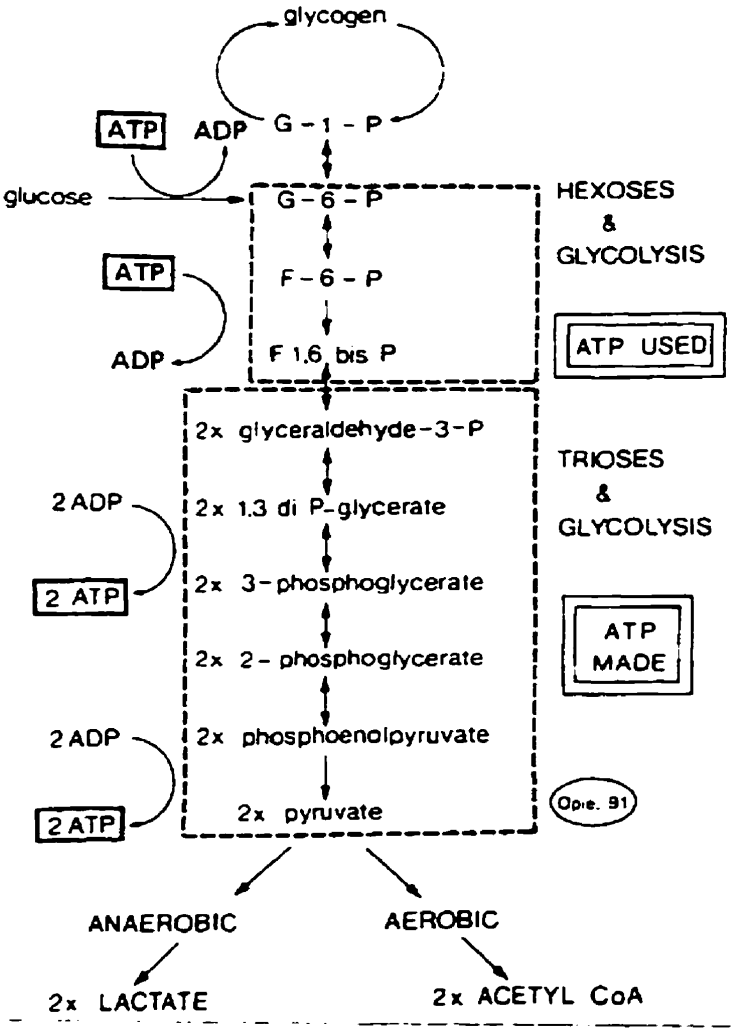


**Figure 2: Glycolytic (Embden-Meyerhof) pathway (from: Opie LH, 1991 [19]).**

Under normal aerobic conditions, the product of glycolysis is pyruvate. Indeed, this is the main source of pyruvate in heart muscle, as circulating pyruvate levels are normally low. Thus pyruvate uptake per se only accounts for a minor portion of total $O_2$ uptake. Some pyruvate may be metabolized by transamination to alanine or carboxylation to malate or oxaloacetate [1]. However, under normal oxygenation, the majority of pyruvate enters the mitochondria via a specific carrier, is converted to acetyl-CoA via pyruvate

dehydrogenase enzyme, and then enters the citrate cycle [1]. Under anaerobic conditions, most pyruvate is reduced to lactate, thus completing the glycolytic pathway (Figure 2):

$$\text{Pyruvate} + \text{NADH} + H^+ \Leftrightarrow \text{lactate} + NAD^+$$

This reaction is catalyzed by lactate dehydrogenase and is an essential step in anaerobic glycolysis, as it means that anaerobic glycolysis produces no net $NADH_2$. Although glycolysis per se is not normally an important energy source in the heart, the latter has two important implications for conditions such as ischemia, when glycolysis could be a potentially important energy source independently of oxidative phosphorylation. First, without regeneration of $NAD^+$ by this reaction, anaerobic glycolysis could not take place as there would be accumulation of NADH. Secondly, despite the common belief that lactate production causes acidosis, anaerobic glycolysis produces no net $H^+$ and thus is not directly responsible for development of acidosis during ischemia [21] (see chapter 3.2).

Lactate uptake can account for 10% of total $O_2$ uptake in the heart of a resting, fasting person, and up to 60% during vigorous exercise when blood lactate levels are high [18]. During adequate oxygenation, lactate is converted to pyruvate via the above lactate dehydrogenase reaction and then metabolized as discussed above. This process is dependent on a supply of $NAD^+$. During oxygen deprivation, lactate accumulates as a result of anaerobic glycolysis, and if the ischemic/hypoxic insult is sufficient in intensity, there may be a net lactate release from the myocardium [18].

### *ii) Free fatty acids*

Free fatty acids (FFA) are the primary source of energy in the heart, especially under fasting conditions. This is partly because FFA contain considerably more reducing equivalents per unit weight than does glucose and partly because of the large amounts of FFA that can be stored in the body as triglycerides. In addition, FFA inhibit glucose metabolism at several sites [18]. FFA are carried in plasma bound to albumin and cross the cell membrane, probably by simple diffusion [18]. The first step in FFA oxidation is activation on the outer mitochondrial membrane by esterification with CoASH to form fatty acyl-CoA (FA-CoA). The fatty acyl is then transferred from the CoA to carnitine, which is part of a special transport system for fatty acyl entry into the mitochondria. For long chain fatty acids, transport into the mitochondria is the rate-limiting step in oxidation [1]. Inside the mitochondria, the fatty acyl is re-esterified with CoASH and then undergoes β-oxidation to form acetyl-CoA for oxidation in the citrate cycle. A small amount of activated fatty acid may stay in the cytosol where it can be esterified with glycerol to form triglycerides for storage.

### *iii) Ketone bodies*

Ketone bodies such as acetoacetate and α-hydroxybutyrate are produced by the liver when supply of FFA exceeds the liver's capacity to completely oxidize them in the citrate cycle [1]. Although they are not considered a major substrate in the normal human heart, ketone bodies can be metabolized in a few simple steps to acetyl-CoA for oxidation in the citrate cycle. The blood levels of these compounds can rise up to 50 fold, even under physiological conditions such as exercise or short-term starvation, when their oxidation

by the heart may have a glucose sparing effect [1]. Nevertheless, ketone bodies are not considered a major substrate for the human heart, as, even at high blood concentrations, their oxidation only accounts for 2-9% of total myocardial oxygen uptake ($MVO_2$) [22]. They, however, contribute significantly to energy production during severe diabetic ketosis [22].

### *iv) Amino acids and the malate-aspartate shuttle*

In addition to forming the "building blocks" of myocardial proteins, amino acids play an integral part in myocardial energy metabolism. However, under normal conditions, their role as a myocardial fuel is minimal, accounting for a negligible portion of the total $O_2$ uptake [18]. Rather, the importance of amino acids appears to be mainly as intermediates in some metabolic pathways. It should be noted that in spite of the variety of compounds named "amino acids", each one behaves differently from the others with respect to its metabolism. For example, some amino acids such as glutamate, glutamine, aspartate, alanine, and leucine may be synthesized or degraded in the heart depending on the conditions, while others such as phenylalanine and tyrosine are not metabolized at all [1].

The first step in degradation of amino acids is normally transamination via amino-transferase enzymes. One function of amino-transferases is provision of carbon skeletons for the citrate cycle [1]. Another important function involves the passage of reducing equivalents across the mitochondrial membrane, which is an important role of asp and glu. As previously discussed, oxidative phosphorylation is driven by production of reducing equivalents in the form of $NADH_2$ or $FADH_2$. These reducing equivalents are obtained from oxidation of acetyl-CoA in the mitochondria, or from extramitochondrial

oxidation of fuels prior to formation of acetyl-CoA. The latter is normally $NADH_2$ from glycolysis at the stage of conversion of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate, or from conversion of lactate taken up from the circulation to pyruvate. $NADH_2$ cannot cross the mitochondrial membrane. Therefore, unless there is a mechanism for transport of the extra-mitochondrially produced $NADH_2$ into the mitochondria for re-oxidation, accumulation of $NADH_2$ in the cytosol will inhibit glycolysis and lactate uptake, thus leaving FFA as the only fuel. Fortunately, there are mechanisms for transferring of these reducing equivalents into the mitochondria, the most important of which in the heart is the malate-aspartate cycle or shuttle [18] (Figure 3).

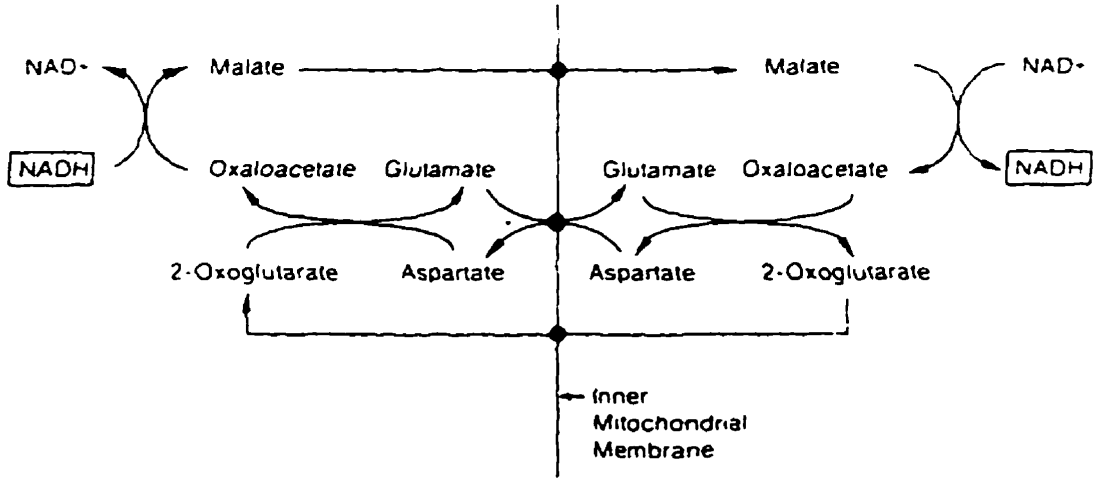


**Figure 3: The malate-aspartate shuttle (from: Taegtmeyer H, 1988 [1]).**

The malate-aspartate cycle begins with oxidation of $NADH_2$ coupled to reduction of oxaloacetate to malate. Malate enters the mitochondria and is oxidized to oxaloacetate, thus regenerating $NADH_2$ from $NAD^+$. Oxaloacetate is then transaminated with glu to

form asp and α-ketoglutarate (2-oxoglutarate), which then leave the mitochondria and are transaminated to regenerate the glu and oxaloacetate in the cytosol, the net effect being a transfer of hydrogen ions across the mitochondrial membrane. It should be noted that asp/glu or malate/oxaloacetate passage across the mitochondrial membrane are paired and each via a specific, complex transport system on the mitochondrial membrane [23].

The significance of the malate-aspartate cycle in maintenance of cytosolic $NAD^+$ levels is obvious. However, the presence of key common metabolic intermediates between the malate-aspartate and citric acid cycles has led to suggestions that the malate-aspartate cycle may also be an important coordinator of cytosolic and mitochondrial metabolism [24]. Both of the above roles are potentially important in determining the metabolic significance of the key amino acids asp and glu during normal or pathologic conditions, as will be discussed in following chapters.

## E) Metabolic Control Mechanisms

The metabolic control processes in the heart are extremely complex and in many cases incompletely understood at this time. Thus, the following is only a brief description of some key concepts in metabolic regulation.

### *i) Glycolytic regulation*

Glycolysis is regulated mainly at the enzymes phosphofructokinase (PFK) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). PFK catalyzes the irreversible conversion of fructose-6-phosphate to fructose-1,6-bisphosphate, using one ATP molecule in the process. The enzyme is subject to significant allosteric control, its activity

being increased by breakdown products of ATP, cyclic AMP, and ammonium ions, and inhibited by high $H^+$, ATP, PCr, and citrate [1]. GAPDH converts glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate, reducing one $NAD^+$ to $NADH_2$. The significance of this enzyme appears to be during extreme conditions such as ischemia when it appears to be mostly responsible for slowing down glycolysis. Although the mechanism for its inhibition is not clear, NADH and possibly lactate, the product of anaerobic glycolysis, appear to be the most important inhibitors [1].

### *ii) Regulation of mitochondrial respiration*

The remarkably constant [ATP] in the normoxic myocyte under different conditions of work suggests a tight coupling between ATP production by oxidative phosphorylation in the mitochondria and ATP consumption by the contractile apparatus. Although the exact mechanisms of control are still under debate [2], it is thought that ATP, ADP, or Pi play a major role in mitochondrial regulation either directly as [ADP], or through cytosolic ratios such as the phosphate potential ([ATP]/[ADP][Pi]), the [ATP]/[ADP] ratio, or the energy charge ([ATP + 0.5 [ADP])/([ATP] + [ADP] + [AMP]) [2]. Another important candidate involved in mitochondrial regulation is the intramitochondrial $NAD^+$ /NADH ratio [2]. If the myocardial work load decreases, this could increase the [ATP]/[ADP] which would reduce the rate of ATP synthesis by shifting the equilibrium of the phosphorylation reaction, thus leading to reduced oxidation of $NADH_2$ and $FADH_2$. This, in turn, slows down the reactions of the citrate cycle, which depend on a supply of $NAD^+$ and $FAD^+$, preventing excessive oxidation of acetyl-CoA and the fuels it is derived from [1]. Conversely, increased work load could lead to increased respiration by the same

mechanisms. On the other hand, calcium ions are also thought to play a role in citrate cycle regulation. For instance, if increased work load is due to increased contractility associated with higher cytosolic [$Ca^{2+}$], it is suggested that the intramitochondrial [$Ca^{2+}$] may also increase, which would have excitatory effects on certain citrate cycle reactions and on pyruvate oxidation to acetyl-CoA [25].

### *iii) Fuel selection*

The exact choice of fuels to be metabolized depends on a number of factors [1,18]. First, the utilization of each substrate from a mixture of substrates is affected by its plasma concentrations. Glucose concentrations are constant under most physiological conditions, but the levels of fatty acids, ketone bodies, or lactate can fluctuate. Thus, after an overnight fast when plasma FFA levels are high, they are the preferred fuel for respiration. Similarly, during exercise when lactate levels rise, they can become the preferred fuel. Secondly, the ease of access of fuels to the enzymes of oxidative metabolism can affect their selection. Thus, when available in sufficient concentrations, ketone bodies and lactate can become significant contributors to acetyl-CoA production, due to the relatively few steps involved in their metabolism to acetyl-CoA. The third important factor affecting fuel selection is the $O_2$ supply and the P/O ratio of the fuel. Thus, in situations where the $O_2$ reserve is limited, fuels with lower P/O ratio (such as FFA) are less likely to be metabolized than those with a higher P/O ratio, such as glucose or pyruvate. Another important factor is the interaction of intermediary metabolites of substrate degradation with the metabolism of other substrates. For example, products of FFA β-oxidation are thought to inhibit glucose metabolism at several key sites in

glycolysis [18]. Finally, hormonal levels in the body can affect substrate selection. For example, insulin can both increase tissue uptake of glucose and inhibit FFA metabolism, resulting in selection for glucose when its levels are high [1].

## 3.2 Ischemia-Reperfusion in Heart Muscle

### A) Overview

Ischemia (from the Greek "to hold back blood") has traditionally been defined as an imbalance between supply and demand of oxygen or substrates caused by lack of adequate blood flow [26,27]. This view of ischemia, however, ignores another important aspect of insufficient blood flow, that is the inadequate washout of metabolites. Thus, a more appropriate definition of myocardial ischemia provided by Hearse [27] is: "...a condition in which coronary blood flow is inadequate to permit the maintenance of a steady-state metabolism". The two main types of myocardial ischemia are regional and global (low-flow or total) ischemia. Clinically, regional ischemia is most common and caused by coronary artery diseases such as atherosclerosis, thrombosis, or arterial spasm. Although global ischemia (low-flow) is also possible clinically, such as in acute heart failure, global ischemia (total) is observed most commonly during aortic cross-clamping in open-heart surgery (see following chapter).

As discussed in the previous chapter, the heart is strongly dependent on a continuous supply of oxygen. Thus, the primary effect of ischemia is disturbance of oxidative metabolism leading to an imbalance between ATP production and utilization. This energetic imbalance then directly or indirectly causes a number of disruptions to normal

cellular homeostasis, which are discussed in some detail in this chapter. Reperfusion (i.e. re-establishment of adequate blood flow) of ischemic tissue is an absolute pre-requisite to its long-term survival [27]. However, reperfusion also appears to have an injurious component that accelerates myocardial injury caused during ischemia, and possibly even causes further injury independent of ischemia [27]. The consequences of reperfusion can range from severe, irreversible abnormalities such as cell death (i.e. infarction), to transient, completely reversible abnormalities such as reperfusion arrhythmias or myocardial stunning. Myocardial stunning is considered a mild, sublethal consequence of ischemia-reperfusion. It can be defined as a prolonged, but eventually fully reversible, functional and metabolic dysfunction in the absence of any cell necrosis [27,28]. The major outcomes of ischemia-reperfusion and the main processes responsible are summarized in this chapter.

## B) Substrate and Energy Metabolism

Figure 4 provides a schematic view of the processes affecting ATP metabolism during normoxia, ischemia, and reperfusion. As discussed in the previous chapter, during normal conditions the levels of ATP are kept constant, with balance between ATP producing and consuming processes. In order of significance, the main sources of ATP are oxidative phosphorylation, glycolysis, and the cytosolic PCr reserve, while the main consuming processes are contraction, ionic pumps, and biosynthesis (Figure 4, top panel). During ischemia, flow restriction leads to diminished oxygen delivery to the myocytes, and hence significant reductions in oxidative ATP production. Initially, there is a coincident increase in glycolytic flux due primarily to acceleration of phosphofructokinase (PFK)

activity [26]. Increased PFK activity occurs as a consequence of reduced inhibition by ATP, increased stimulation by breakdown products of ATP and PCr, and reduced inhibition by citrate, the levels of which drop secondary to decreased oxidative metabolism of non-glucose fuels [26]. As ischemia progresses, however, glycolytic flux decreases due to inhibition of PFK by accumulating $H^+$ (see section 3.2-C) and inhibition of glyceraldehyde-3-phosphate dehydrogenase by accumulating lactate and NADH [26]. Thus, in severe ischemia when anaerobic glycolysis is most needed to produce ATP, it is the least able to perform this vital function [18]. The cessation of glycolysis during severe ischemia leads to severe imbalance in ATP metabolism and can be linked to irreversible ischemic damage [18,29].

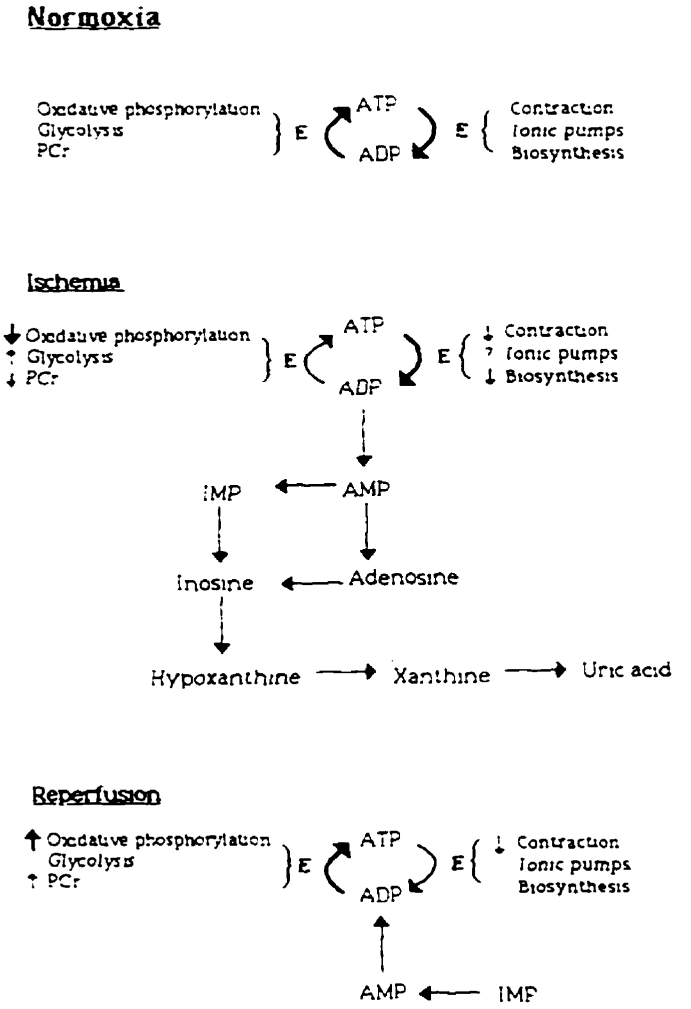


**Figure 4: ATP metabolism during normoxia, ischemia, and reperfusion (from: Harmsen E and Seymour AML, 1988 [2]). E = energy.**

The onset of ischemia is also associated with an immediate reduction in energy demand which is responsible for a significantly slower loss of high-energy phosphates (HEPs) than would be expected [30]. In the initial phase of oxygen deprivation, the main cause for reduction of energy demand is the onset of mechanical failure [30], which has been attributed to a number of processes including inadequate ATP production or delivery, increased ADP and Pi, decreased phosphate potential, acidosis, or low oxygen tension [2]. However, it has been suggested that there are also non-mechanical mechanisms for

reduction in demand which have not yet been identified [30], and which may include reduced activity of biosynthetic pathways (Figure 4, middle panel).

Ischemia is a relative term, its severity depending primarily on the extent of imbalance between ATP production and utilization [2]. Despite the reduction in energy demand, increased glycolytic flux, and buffering by PCr via the CK reaction, there is a fall in ATP levels and a more pronounced fall in PCr as it buffers ATP levels. The breakdown of these HEPs naturally leads to increased ADP and Pi levels, and then to increased AMP levels as rapid breakdown of ADP is catalyzed via the enzyme myokinase [31]. AMP is then broken down by 5`-nucleotidase to adenosine which can diffuse out of the cell and is further metabolized to inosine, hypoxanthine, xanthine, and urate (Figure 4, middle panel) [31]. The alternative pathway of deamination of AMP to inosine monophosphate (IMP) is relatively inactive in heart muscle, but could have a role in preserving the purine ring as non-diffusable IMP [31]. The loss of purines from the cell, together with the decline in HEPs, is considered by some as a true reflection of ischemic severity [2].

During reperfusion, when both circulation of blood and adequate oxygen supply are re-established, the remaining IMP, AMP, and ADP are rapidly phosphorylated and converted to ATP (Figure 4, bottom panel). If the mitochondria are unable to generate sufficient energy, as reflected by persistently elevated ADP and low PCr concentrations, then reperfusion may be incomplete and the mitochondria are most likely irreversibly damaged and cell death will result [2]. However, if mitochondrial function is well-preserved, as is often the case when ischemic damage is reversible, PCr levels recover rapidly, often to supranormal levels [2]. This post-ischemic "PCr overshoot" often occurs

despite sub-normal ATP concentrations. The mechanisms for this increased PCr/ATP ratio, which often accompanies contractile dysfunction following ischemia, are still under debate. The most widely accepted theory is that post-ischemic ATP recovery is limited by a diminished adenine nucleotide pool caused by loss of adenine nucleotides during ischemia combined with slow resynthesis after reperfusion [32-35]. This limitation on ATP synthesis would mean insufficient ADP precursor for phosphate transfer from PCr, thus explaining the high PCr/ATP ratio. Another theory has been that the PCr overshoot is simply due to reduced myocardial work load after ischemia, as is observed in skeletal muscle, but a recent study by Kaplan, et al [36] has provided evidence that this is not the case in cardiac muscle. Moreover, another study by Kaplan and associates [37] found that reperfusion produces hydrogen peroxide ($H_2O_2$), a tissue oxygen metabolite that has shown activity against CK enzyme both *in vitro* and *in vivo*. They detected significant inactivation of cytoplasmic CK during reperfusion after a 25 minute ischemic episode in rat hearts. They concluded that contractile dysfunction in spite of high PCr levels after reperfusion (i.e. stunning) is caused by inactivation of cytoplasmic CK, which they suggest prevents adequate delivery of ATP to myofibrils for contraction. They further argued that this inactivation of cytoplasmic CK may be responsible for the increased PCr/ATP ratio (i.e. PCr overshoot) observed after ischemia-reperfusion, and that this condition is avoidable by pharmacological protection of CK.

The above theory, however, has several flaws. First, the rapid, supranormal recovery of cytoplasmic PCr levels would indicate that mitochondrial CK enzyme remained functional despite inactivity of cytoplasmic CK. Kaplan, et al proposed that $H_2O_2$ selectively inhibited cytoplasmic CK and not mitochondrial CK, but were unable to

demonstrate this due to technical limitations of the study [37]. Secondly, these conclusions are inconsistent with many studies that have demonstrated reversibility of post-ischemic contractile dysfunction by inotropic stimulation [33,38-39], suggesting that ATP utilization and not ATP supply is the culprit in the contractile and metabolic dysfunctions observed during myocardial stunning. Finally, as mentioned above, there is significant evidence that post-ischemic ATP recovery is limited by the size of conserved adenine nucleotide pool, and speed of resynthesis of purines. Furthermore, there is recent evidence that species or age differences with respect to myocardial stunning may be a result of a deficiency, differences in activity, or feedback inhibition of 5'-nucleotidase responsible for AMP degradation [40], further supporting the adenine nucleotide pool theory.

## C) Ionic Imbalance

During ischemia, there is a marked increase in intracellular $Na^+$ and $H^+$ and possibly $Ca^{2+}$, while intracellular $K^+$ decreases. Potassium loss from the cell starts early, and ultimately leads to depolarization of the membrane potential and $Na^+$ channel inactivation. One possible mechanism for this is reduction of $Na^+/K^+$ ATPase activity due to the decline in ATP levels and in the $\Delta G_{ATP}$ [41], which is affected by concentrations of ADP, ATP, and Pi. The $\Delta G_{ATP}$ will eventually decrease to the point that the thermodynamically available energy from ATP breakdown will not be sufficient to drive the $Na^+/K^+$ and other ATPases [41]. $K^+$ loss has also been attributed to a possible opening of ATP-sensitive $K^+$ channels [18].

The rise in $Na^+$ is probably initially due to inactivity of the $Na^+/K^+$ ATPase, which fails to pump out the $Na^+$ that continues to enter via fast $Na^+$ channels during initial ischemia [42]. Another important mechanism is increased $Na^+$ entry via reverse $Na^+/H^+$ exchange resulting from $H^+$ accumulation [43].

Protons during ischemia come mainly from glycolytic ATP turnover, increased $CO_2$ levels, and eventually from the net breakdown of ATP [21]. Proton accumulation can have an important role during ischemia, and even more during reperfusion, by causing further $Na^+$ entry via the sarcolemmal $Na^+/H^+$ exchanger. This in turn leads to $Ca^{2+}$ overload during reperfusion via the activity of the $Na^+/Ca^{2+}$ exchanger [43]. Interestingly, acidosis may also have a beneficial role during ischemia. Firstly, acidosis depresses cardiac contractility [21] thus helping ATP preservation. Secondly, acidosis may improve the capacity for ATP resynthesis during reperfusion by $H^+$ inhibition of 5'-nucleotidase during ischemia, reducing AMP breakdown and thus improving preservation of the adenine nucleotide pool during ischemia [35].

It is now accepted that some increase in cytosolic $Ca^{2+}$ does occur during ischemia, and is probably largely due to reversed $Na^+/Ca^{2+}$ exchange secondary to $Na^+$ overload, but there may be other mechanisms involved as well [43,44]. The major increases in cytosolic $Ca^{2+}$ are however observed during reperfusion when $Ca^{2+}$ overload may have a primary role in irreversible cell injury and necrosis [42]. The mechanisms of $Ca^{2+}$ overload are complex and as yet incompletely understood. A main factor may be perturbation of sarcolemmal transport mechanisms, possibly including $Na^+/Ca^{2+}$ exchange, the slow $Ca^{2+}$ channel, and the $Ca^{2+}$ ATPase [42]. The $Na^+/Ca^{2+}$ exchanger appears to be the most significant of these

[42,43], leading to mass $Ca^{2+}$ influx secondary to $H^+$ and $Na^+$ accumulation as explained above. The role of the slow $Ca^{2+}$ channel is still unclear; it may be inactivated during ischemia due to acidosis, and its blockage by antagonists during reperfusion does not appear to reduce $Ca^{2+}$ accumulation [42]. Inactivity of $Ca^{2+}$ ATPase is also unlikely to be a significant contributor to $Ca^{2+}$ accumulation, partly because its capacity for $Ca^{2+}$ efflux is much lower than that of the $Na^+/Ca^{2+}$ exchanger [42]. Other factors that may exacerbate $Ca^{2+}$ overload are catecholamines, oxygen-derived free radicals, and lipid degradation, all of which may affect the structure or function of the sarcolemmal membrane [42].

In myocardial stunning, elevated intracellular $Ca^{2+}$ may bring about negative effects by activating protein kinases to change $Ca^{2+}$ sensitivity and/or maximal $Ca^{2+}$ activated force through phosphorylation of one or more of the contractile proteins [45]. Another possibility is degradation of contractile proteins by $Ca^{2+}$-activated proteases [45]. The latter would help explain why stunning may last several days, as it takes substantial time for protein degradation and subsequent resynthesis.

## D) Free-Radical Generation

Oxygen derived free radicals (ODFR) may arise from a number of intracellular and extracellular sites, including the arachidonic acid pathway, xanthine oxidase pathway, leukocytes, oxidation of catecholamines, and mitochondrial activity [46]. A number of naturally occurring defense systems, such as glutathione peroxidase, normally protect cells against the harmful effects of ODFR. It is suggested that reoxygenation of ischemic tissue leads to abundant production of oxyradicals such as superoxide anion, hydrogen

peroxide, and hydroxyl radical. These oxyradicals have been suggested to be important in causing myocardial injury during reperfusion, and are thought to be largely responsible for the "oxygen paradox" phenomenon [28]. Oxygen paradox refers to observations that restoration of oxygen supply to ischemic/hypoxic tissue can cause sudden and massive damage, that appears to be far greater than might have occurred had ischemia/hypoxia continued [46].

Although the role of ODFR in causing irreversible injury is still being questioned [27,45], there is more agreement over their role in causing myocardial stunning [28,45]. The mechanisms are still unclear. A suggestion for which there is some evidence is that ODFR may actually cause injury via exacerbation of $Ca^{2+}$ overload [45], though the mechanism by which ODFR may achieve this are also unclear. One recent proposal is that ODFR inhibit glycolysis by inactivating glyceraldehyde-3-P dehydrogenase, which in turn leads to deranged $Ca^{2+}$ metabolism, and the resultant $Ca^{2+}$ overload produces dysfunction [45]. ODFR may also affect $Ca^{2+}$ influx by altering transmembrane transport systems through damage to membranes via lipid peroxidation [47] or to membrane proteins involved in ion transport [28].

## E) Significance of Reperfusion Injury

Reperfusion of acutely ischemic myocardium is associated with complex structural and functional derangements that vary significantly in severity. The mechanisms of reperfusion injury are not fully understood, but $Ca^{2+}$ overload and ODFR production, which were briefly described above, are thought to be important factors. Post-ischemic injury has often been arbitrarily divided into reversible and irreversible injury, although it

has been difficult to identify a specific event beyond which injury is irreversible [48]. More recently, Hearse [27] has suggested that the effects of reperfusion injury may manifest themselves in one of four distinct forms, namely reperfusion arrhythmias, myocardial stunning, accelerated necrosis, and lethal injury.

Reperfusion arrhythmias, such as premature ventricular beats or ventricular fibrillation, occur within seconds of reflow, and have been observed in all species [27]. In humans, they may occur after thrombolytic therapy or cardiac surgery, and are potentially lethal, but normally preventable or reversible. Myocardial stunning (MS) was already described briefly as functional and metabolic dysfunction that may develop after a brief period of ischemia, but which is eventually fully reversible, although it may last several days. The difficulty in studying MS is that by definition it has to be fully reversible, something that is difficult to ascertain in most experimental models where reperfusion is carried out for a limited amount of time. Nevertheless, MS is of great potential significance clinically, especially in cardiac surgery, where it is commonly observed after both normothermic and hypothermic cardioplegic arrest [49] (see chapter 3.3). The third manifestation of reperfusion may be accelerated necrosis in tissue that has already sustained irreversible injury during ischemia, and the fourth is lethal injury caused by reperfusion in tissue that was potentially viable prior to reperfusion. Hearse suggests that the latter two manifestations would be extremely difficult to distinguish due to limitations in most experimental protocols, such that the existence of lethal reperfusion injury may still be questionable [27].

The concept of reperfusion injury has far reaching practical implications. If reperfusion of tissue can bring about damage that was not present before restoration of flow, it would be logical to suggest that changing the conditions and composition of reperfusion could affect the extent of such injury. Not only could this be significant for myocardial performance immediately after medical or surgical reperfusion, but it could also affect long-term morbidity and mortality, if indeed lethal reperfusion injury truly exists. These possibilities led some to suggest specific modifications to reperfusion conditions and the reperfusate composition, in an attempt to counter some of the perceived mechanisms of reperfusion injury. Possible modifications include the use of antioxidants, transient hypocalcemia, modifications to pH and osmolarity, the provision of substrates (such as asp and glu), the rate of initial reperfusion, and more. The applicability of such modifications is particularly attractive in the surgical setting, where these factors can be well controlled. This has led to a great deal of activity by many groups, and particularly that of Buckberg, in attempts to devise the "ideal" reperfusion conditions for open-heart surgery.

## 3.3 *Cardioplegia*

### A) Overview

Cardioplegia is defined as an elective, temporary stopping of cardiac activity. This elective cardiac arrest can be induced by injection of chemicals, selective hypothermia, or electrical stimuli. Cardioplegia is most commonly brought about chemically by use of cardioplegic solutions. Cardioplegic techniques combined with Cardiopulmonary bypass are used in nearly all elective and emergency open-heart surgery performed today. When

used as such, cardioplegia provides the surgeon with adequate access to the heart, while providing some protection against myocardial damage during ischemia, and possibly during reperfusion. Cardioplegia is also used for ex-vivo preservation of donor hearts for transplantation, although most of the following discussions will concentrate on its application during open-heart surgery.

The properties and methods of delivery of cardioplegic solutions vary according to the desired effects and particular use, as well as personal preferences of surgeons. In fact, it is difficult to find identical cardioplegic techniques in different institutions, or even by different surgeons in the same institution. Even when the techniques are basically the same, there are often small differences in the perfusion equipment used, the exact composition of the cardioplegic solution, or the protocol by which the technique is applied. Nevertheless, the majority of the differences are minor ones and most cardioplegic protocols fall within a few major groups, some of which are discussed below.

Cardioplegia not only aims to arrest the heart by suppression of its electrical and mechanical activities (i.e. 'cardioplegia'), but to protect the heart against ischemia-reperfusion induced injuries; thus the term 'myocardial protection' may be more accurate [50]. The mechanisms by which different cardioplegic techniques protect the heart depend on the specific features of the technique and its desired effects, and in many cases are not clearly understood. As discussed in previous chapters, there is a delicate balance between energy supply and demand in the normally beating heart. The disruption of this balance, directly or indirectly, causes the various metabolic and ionic derangements

observed during myocardial ischemia. The primary mechanism by which cardioplegic solutions protect the heart is by reducing its energy demands during ischemia, thus improving the supply/demand ratio. This reduction in energy demand is achieved primarily by suspension of work output through induction of rapid diastolic arrest; and secondarily by reduction of basal metabolic rate using hypothermia.

## B) History and Development

The origins of cardiac surgery are attributed to closure of a stab wound in the right ventricle of a heart by Ludwig Rehn in 1896 [4]. Many similar operations were performed by others in subsequent years, but it was not until the experiences of World War II that full maturation of this surgical field began [4]. Although the exact history behind the development of Cardiopulmonary bypass (CPB) is not easily described, the first successful use of a heart-lung machine for full CPB is attributed to John Gibbon in 1953 [51], while the systematic use of a heart lung machine (pump-oxygenator) was pioneered by John Kirklin in a series of clinical operations beginning in March 1955 [4]. In the 40 years since the advent of CPB, cardiac surgery has developed such that almost all congenital and acquired diseases of the heart have come under the scrutiny of cardiac surgeons and the great majority have been alleviated to some degree by surgical interventions [4].

Cardiopulmonary bypass consists of removal of some or all returning venous blood from the right atrium (or venae cavae) via a blood pump, re-oxygenation of the blood by passage through an artificial oxygenator, and re-introduction of the blood into the proximal aorta, effectively bypassing the heart and lungs. This, combined with placement

of a cross-clamp on the aorta, allows bloodless access to the heart, the coronary vessels, and the aorta, without compromising systemic circulation. Unfortunately, this convenience comes at the cost of global ischemia to the heart during each cross-clamp period. During the early years of cardiac surgery, little attention was paid to this intraoperative ischemic period as a potential source of fatal or non-fatal low post-operative cardiac output [52]. Full realization of the importance of myocardial protection began in 1967, when Najafi and associates suggested that acute, diffuse, subendocardial myocardial infarction found frequently in patients who died early after valve replacement might be due to inadequate intraoperative myocardial management [52].

The concept of "elective cardiac arrest" by use of chemicals was introduced by Melrose in 1955, out of a desire for better intracardiac exposure, rather than as a cardioprotective measure [52,53]. Melrose achieved this by use of a cold, hypertonic potassium citrate blood solution, but later stopped its use due to poor results in subsequent operations [54]. In the late 1950's, discussions of the concept of reducing global ischemic myocardial damage by immediate cessation of electromechanical activity led Kirklin and associates to attempt using Melrose's solution for this purpose at the Mayo Clinic, a practice that stopped due to lack of any apparent benefits [52]. Prior to the 1970's, the main method of cardioprotection was to use topical hypothermia to lower myocardial temperature and metabolic rate and thus improve the energy supply/demand ratio during aortic clamping. Hypothermia was introduced into cardiac surgery by Bigelow et al and Shumway et al in the 1950's, and still remains one of the most important factors in most cardioprotective techniques [52,55]. However, re-emergence of chemical cardioplegia began in the 1970's with the wide-spread use of crystalloid cardioplegic solutions [52]. This re-emergence of

chemical cardioplegia was in large due to the persistent research by investigators such as Bretschneider, Kirsch, Hearse, Gay, and Ebert during the late 1960's and early 1970's, and by Tyers' report that the problem with Melrose's solution was not the components but their concentrations [55]. Randomized trials soon confirmed the advantage of using cold cardioplegia over hypothermia alone [52]. Intermittent ischemic arrest induced by hypothermic crystalloid cardioplegic solutions ('conventional cardioplegia') long remained the standard for cardioprotection and is still used today in many centres [52,55].

In 1979, Buckberg suggested that blood could be used as a vehicle for the cardioplegic components and reported that this provided superior myocardial protection to crystalloid cardioplegia [56]. This slowly led to the use of intermittent cold blood cardioplegia in many centres, although others still continue to use crystalloid cardioplegia. Intermittent cold blood cardioplegia later led to the use of continuous cold blood cardioplegia by Bomfim, and then by the team at the University of Toronto, in an attempt to eliminate intraoperative ischemia altogether [57]. Continuous normothermic blood cardioplegia was subsequently pioneered at the University of Toronto with the aim of eliminating complications from hypothermia and providing the resting heart with a chance at cellular repair during the operation [57]. The advent of so called 'warm heart surgery' has led to a tremendous amount of enthusiasm and to an equal amount of controversy [58-62]. Ironically, 40 years after Melrose introduced chemical cardiac arrest to create convenient, bloodless access to the heart, that convenience has been sacrificed by some in search of better myocardial protection.

## C) Ionic Principles of Cardiac Arrest

Although a number of different pharmacological agents such as tetrodotoxin, acetylcholine, local anaesthetics (eg. Procaine), and calcium antagonists, can be used to bring about cardiac arrest, these agents are often temperature sensitive, exhibit a narrow therapeutic range, or accumulate in the myocardium, leading to at least a delay in cardiac recovery upon reperfusion [53]. Therefore, most cardioplegic methods currently in use are based on three ionic principles of cardiac arrest [50,53,63]. The first, and by far the most common, is increased extracellular potassium concentration, $[K^+]_o$; the second is increased extracellular ionized magnesium concentration, $[Mg^{2+}]_o$; and the last is simultaneous reduction of the extracellular sodium, $[Na^+]_o$, and calcium, $[Ca^{2+}]_o$, concentrations. The full details of the effects of each of these ionic conditions is complex and depends on the exact concentrations of these and other components of the solution [63]. In simple terms, increased $[K^+]_o$ in the usual cardioplegic range of 15-30 mmol/L [53,63] can cause a decrease in the reversal potential and membrane potential according to the Nernst equation [64], leading to a depolarization of the membrane in both myocytes and pacemaker cells. This in turn inactivates both the $Na^+$ and the $Ca^{2+}$ channels, thus preventing the generation of an intracellular $Ca^{2+}$ spike and the subsequent $Ca^{2+}$-dependent $Ca^{2+}$ release, ending in diastolic arrest [53,63].

High myocardial $[Mg^{2+}]_o$ can induce diastolic arrest by inactivation of fast $Na^+$-channels and competitive inhibition of cellular calcium influx via the slow $Ca^{2+}$-$Na^+$ channel in the sarcolemma [53]. Magnesium is, however, not as effective as potassium in inducing cardiac arrest and its inclusion in potassium cardioplegic solutions is usually due to its

ability to provide additional myocardial protection [63], possibly by preventing loss of intracellular $Mg^{2+}$ and attenuating intracellular $Ca^{2+}$ overload [65]. The optimal $[Mg^{2+}]_o$ depends on the $[Na^+]_o$, $[K^+]_o$, and $[Ca^{2+}]_o$ and was determined to be approximately 16 mmol/L in dose-response studies on high potassium cardioplegia by Hearse & associates [66]. The benefits of increased $[Mg^{2+}]_o$ in high potassium cardioplegic solutions have, however, been questioned by Tian and co-workers [65].

Decreasing myocardial $[Na^+]_o$ and $[Ca^{2+}]_o$ levels to close to their cytosolic concentrations can also cause cardiac arrest. Calcium-free solutions abolish the pacemaker potential of the sino-atrial node cells and uncouple the electrical and mechanical components of myocytes, while the lack of sodium prevents action potential generation [53]. These components must, however, be depleted jointly in order for adequate diastolic arrest and protection to occur. For instance, decreasing $[Na^+]_o$ without $[Ca^{2+}]_o$ can lead to increased myocardial tone by increasing $Ca^{2+}$ influx via the $Na^+/Ca^{2+}$ exchanger [53]. On the other hand, low $[Na^+]_o$ reduces the risk of a calcium paradox, presumably by enhancing $Ca^{2+}$ binding at the sarcolemma [53]. This phenomenon may be responsible for the failure to observe any calcium paradox with some intracellular-type cardioplegic solutions (see below), despite lower $Ca^{2+}$ levels than the threshold identified by many investigators at 0.15-0.5 mmol/L [67].

## D) Classification of cardioplegic solutions

### *i) Ionic Composition*

Most cardioplegic solutions can be divided into one of two types based on their ionic composition, namely extracellular and intracellular type solutions [63]. Extracellular type solutions are the most common and include both purely crystalloid solutions such as St. Thomas Hospital II solution [68] and blood cardioplegic solutions, which are those in which blood acts as the vehicle for the cardioplegic components (see below). Extracellular type solutions generally have an ionic composition similar to that of extracellular fluids, except for a high [$K^+$] that acts as the cardioplegic agent [63], but some (eg. St. Thomas Hospital II solution) may have increased $Mg^{2+}$ as well. Intracellular type cardioplegic solutions, on the other hand, have an ionic composition similar to the intracellular medium, that is little or no sodium or calcium, but very high potassium [63]. These solutions use the first and third of the three cardioplegic principles discussed above, while extracellular type solution use one or both of the first two principle. An example of intracellular cardioplegic solutions is the University of Wisconsin solution, which has been advocated by some for use in *ex-vivo* heart preservation [69].

### *ii) Cardioplegic Vehicle*

Another method of classification of cardioplegic solutions is based on the cardioplegic vehicle which is usually blood or crystalloid fluids, but can theoretically be of a fluorocarbon base as well [55]. Although Melrose's original cardioplegia was a modified blood solution, re-emergence of cardioplegia in the 1970's was with a variety of

crystalloid-based solutions. These solutions offer a number of advantages over blood: they are easy to prepare, relatively inexpensive, yet quite effective. During surgery, they have the advantage of being available in large quantities, add no risk of blood borne infections, are not damaged by the roller pump during prolonged infusion, and eliminate the risk of capillary obstruction observed with hypothermic blood infusion [70]. On the other hand, blood-based solutions offers a number of advantages over crystalloid cardioplegia. First and foremost, the presence of red blood cells greatly enhances oxygen-carrying capacity which is of prime importance in cardioplegia: although crystalloid solutions can theoretically carry enough oxygen to meet the greatly reduced needs of an arrested heart, it has been suggested that they may not be able to provide sufficient oxygen to a damaged heart in which oxygen requirements are often increased [71]. Furthermore, erythrocytes contain abundant endogenous free radical scavengers, such as superoxide dismutase, catalase, and glutathione, which can potentially reduce oxygen-mediated injury during reperfusion [71]. Other advantages of blood include improved buffering capacity from the histidine and imidazole groups of blood proteins, reduced chance of myocardial edema due to high intrinsic oncosity, and rheologic benefits on the microvasculature [71]. These and other benefits of using blood as a cardioplegic vehicle have led to increased acceptance of blood cardioplegic solutions as an alternative to crystalloid cardioplegia [72].

### *iii) Temperature*

Hypothermia has played a role in myocardial protection since the beginning of modern cardiac surgery, and remained the only generally accepted mode of cardioplegic delivery

until the recent emergence of warm heart surgery as an alternative. The popularity of hypothermia is based primarily on its ability to decrease myocardial metabolic demands, reducing oxygen uptake to less than five percent of normal at temperatures of 15-20°C [55]. This provides some protection against ischemic damage, removes the need for continuous perfusion with oxygenated cardioplegia, and allows intermittent periods of ischemia to better visualize the surgical field during vascular anastomoses [61]. It also provides a margin of safety in case of complications [61]. Despite these benefits, a number of disadvantages of hypothermia have been identified including detrimental effects on membrane stability, calcium sequestration, the generation and utilization of glucose and high energy phosphates, tissue oxygen uptake, and cellular osmotic homeostasis [57,61]. In addition, intermittent perfusion with cold cardioplegic solutions subjects the myocardium to several periods of ischemia and possible subsequent reperfusion injury [57,61]. There were also reports of post-operative arrhythmias and conduction disturbances, as well as coagulation disorders due to systemic hypothermia [61].

Normothermic blood perfusion was initially advocated by Buckberg and co-workers for use in secondary cardioplegia [73] (see below) for "resuscitation" of hearts responding poorly to reperfusion after cold blood cardioplegia. This led to the subsequent development of continuous normothermic blood cardioplegia by Salerno, Lichtenstein and associates in an attempt to remove ischemia from the cardioplegic protocol, bypass the deleterious effects of hypothermia, and take advantage of the resuscitative potential of warm blood cardioplegia during the operation [57,74,75]. The use of normothermia may initially seem unwise. First, use of continuous blood perfusion hampers visualization

during construction of critical coronary anastomoses, although some simple methods of improving visualization have been suggested [76]. Secondly, one may lose the margin of safety afforded by hypothermic reduction in myocardial metabolic demands. Indeed, some investigators have warned against the potential hazards of interrupting flow during warm blood cardioplegia [77]. However, the effects of hypothermia on myocardial oxygen uptake are not as important when electromechanical arrest is maintained by chemical cardioplegia. In fact, chemical arrest is reported to reduce oxygen uptake by 90%, with hypothermia (15°C) only providing a further 5% reduction [70]. This argument was recently supported in an experimental study by Tian and co-workers [78] who showed the safety of using warm blood cardioplegia intermittently in isolated pig hearts, as long as adequate flow rates are used during intermittent perfusion. However, the situation in diseased human hearts may be substantially different. As explained above, warm blood cardioplegia depends largely on chemical arrest to provide myocardial protection to the normothermic heart. Thus, in cases where coronary obstruction in a diseased heart prevents adequate delivery of cardioplegia to all tissues, some areas of the heart could be particularly susceptible to ischemic injury during interruptions of flow. This has led to the use of retrograde perfusion of the heart through the coronary sinus for the administration of normothermic cardioplegia [79-82], and more recently to simultaneous antegrade (through the coronary arteries) and retrograde perfusion [83].

Continuous normothermic blood cardioplegia is a novel technique that has generated overwhelming interest in recent years. However, as experience with this technique increases, a number of potential problems are being identified that warrant more

investigation and caution. A number of recent articles have reviewed the benefits and pitfalls of warm heart surgery [58,62].

### *iv) Cardioplegic Additives*

A number of metabolic and non-metabolic additives have been used to improve the protection afforded by cardioplegia, and new agents are continuously being tested for this purpose. Non-metabolic additives have included volume expanders such as mannitol, free radical scavengers, and calcium channel blockers [70,84]. Metabolic additives have included glucose with or without insulin, adenine nucleotides, and a number of amino acids such as taurine, histidine, methionine, and cysteine [84,85]. The most common metabolic additives, however, are asp and glu, which are discussed in some detail in the following section.

## E) Substrate-Enhanced Cardioplegia

Although the term "substrate" can refer to a wide variety of compounds, in the context of cardioplegia it most often refers to asp and glu. This is due to the use by Buckberg use of the term "substrate-enriched" in referring to his much advertised cardioplegic recipe, which contains asp/glu as well as several other features. The following sections will discuss the development of Buckberg's cardioplegic solution, potential mechanisms for the effects of asp/glu, and controversies surrounding substrate-enhanced cardioplegia.

### *i) History and Development*

The majority of the above discussions of cardioplegia generally refer to techniques that can be classified as primary cardioplegia, that is cardioplegia used to prevent intraoperative myocardial injury. In 1979, Buckberg's group suggested that it may be possible to use secondary blood cardioplegia, that is cardioplegia applied subsequent to the ischemic insult, in order to reverse ischemic injury ("resuscitate") and limit reperfusion injury [73]. In their original study, Lazar, Buckberg, and associates [73] reported substantial improvement of $MVO_2$ and functional parameters with 5 minutes of secondary, warm (37°C), blood cardioplegia delivered before aortic unclamping. The rationale was that cardioplegic arrest in hearts that cannot otherwise maintain circulation after aortic unclamping would allow extracted oxygen to be used for reparative processes rather than "needless electromechanical work" [73] during CPB prolongation.

Extended global ischemia has been shown to result in impaired myocardial oxygen utilization in both experimental and clinical studies [86]. Low $MVO_2$ after global ischemia has been shown not to be due to flow problems or $O_2$ extraction, but probably results from impaired cellular utilization of oxygen [86]. These observations led to speculations that low $MVO_2$ in post-ischemic hearts was due to loss of Krebs cycle intermediates, prompting Buckberg and colleagues to look for a means of replenishing such intermediates by adding amino acids to the perfusate. The original study to this effect [6] chose L-glutamate citing studies showing that the myocardial glu concentration was depleted during anoxia and a study by Rau [87] showing that supplementation with glu and other amino acids in rabbit septal preparations improved functional recovery. The

study used 15 minutes of normothermic ischemia in dog hearts on CPB, followed by 30 minutes of blood reperfusion with or without glu. Glu had no effects on any parameters during normal control perfusion in non-ischemic hearts. However, addition of 26 mmol/L glu to the reperfusate (after an initial 5 minutes of reperfusion with normal blood) caused near-complete functional recovery, above-normal post-ischemic $MVO_2$, and improved ATP recovery. These superb results were quickly followed by a second study evaluating the effect of glu in secondary cardioplegia [7]. In this second study, dog hearts subjected to 45 minutes global ischemia were unable to support the systemic circulation 15 minutes after aortic unclamping. Treatment of these hearts with 10 minutes of secondary cardioplegic infusion improved functional recovery, $MVO_2$, and ATP levels. The best results were obtained when the secondary cardioplegic solution contained 26 mmol/L glu, a result that prompted the authors to advocate the use of this and other amino acids in cardioplegic solutions.

The next important study in development of Buckberg's cardioplegic strategy was a study of reperfusate ionic composition [88]. This important study determined the ideal ionic and osmotic conditions for reperfusion of dog hearts subjected to 1 hour cold (16°C) ischemia. Reduced reperfusate $Ca^{2+}$ (0.5 mmol/L), increased pH (7.8), high $K^+$ (~30 mmol/L), and high osmolarity (~360 mOsm) each improved post-ischemic functional recovery and $MVO_2$, and reduced myocardial edema. When all these factors were combined, the greatest benefit was observed with near-complete recovery of function and $MVO_2$, and significantly reduced tissue edema.

The first clinical experience using all the cardioplegic principles discussed above was in 1983 when Rosenkranz and associates induced cardioplegic arrest in high-risk coronary patients with a warm, glu-enriched, hyperkalemic, hypocalcemic, hyperosmotic, hyperglycemic, alkalotic blood cardioplegic solution [89]. These patients, who were in cardiogenic shock prior to surgery, experienced reduced morbidity and mortality compared to similar patients receiving standard cold blood cardioplegia.

In 1986, the addition of L-aspartate was the last major step in the development of the Buckberg solution. Asp enrichment of glu blood cardioplegia used to arrest hearts previously subjected to ischemia-reperfusion injury (45 minutes of global ischemia followed by 5 minutes blood perfusion) resulted in full functional recovery and improved $MVO_2$ compared to glu enrichment alone. Any further modifications to the recipe have been minor ones, such as slight variations in the composition of the specific solutions used for cardioplegic induction, maintenance, or reperfusion. Subsequently, Buckberg and associates published a series of 17 articles in the Journal of Thoracic and Cardiovascular Surgery entitled "Studies of Controlled Reperfusion after Ischemia" [143] in which they detailed their cardioplegic strategy and rationale in a very systematic manner.

Two very important clinical papers in the early 90's provided further support for this much advertised cardioplegic strategy. The first was a case study of 14 patients with perioperative cardiac arrest who were "resuscitated" by 20 minutes of perfusion with Buckberg's "warm reperfusate" cardioplegic recipe [9]. The authors claimed that many patients who would have otherwise likely been lost were saved because of their

aggressive approach in these cases. They suggested that by "reversal" of ischemic damage, their substrate-enriched cardioplegia could salvage hearts that might otherwise be considered irreversibly injured. It should be noted that no control group was used for this study. The second clinical study showed reduced morbidity and mortality when using asp/glu cardioplegic reperfusion in patients with acute coronary occlusion when compared to results reported from transluminal coronary percutaneous angioplasty (TCPA) [8]. Studies such as these have led Buckberg's group to adapt their cardioplegic strategy for all adult cardiac operations [10].

### *ii) Controversy*

The above account has been but a brief summary of the work presented by Buckberg's group in support of their cardioplegic strategy. Despite the apparent overwhelming amount of evidence supporting asp/glu cardioplegia, there remains considerable controversy over many aspects of their cardioplegic solution and especially over asp/glu enrichment, as studies from other groups have shown significant variability in results. Some investigators using asp and/or glu enriched cardioplegic solutions have reported extraordinary benefits [90-92], while other studies have failed to show any benefit whatsoever [93-96]. Yet others observed benefits that were only short-lived [97,98] or attributed to coincident changes in the perfusate ionic composition [99]. Similar disagreements can be seen among studies using asp/glu enriched perfusates without cardioplegia with some observing positive effects [6,100,101], others no effects at all [102], and some even seeing negative effects [14].

There is also much confusion on specific issues related to asp/glu. For instance, some studies supporting the concept of substrate enrichment point to reduced ischemic tissue levels or increased post-ischemic extraction of glu, while others have suggested a relation between the size of the tissue asp/glu pool, and both the energy supply and the recovery of contractile function in post-ischemic hearts. Most such observations, however, are inconclusive. For example, Wiesner, et al. [103] showed that flow deprivation in dog hearts causes reductions in tissue glu levels, but has no effect on asp. On the other hand, a study by Pisarenko [104] correlated post-ischemic recovery of function and energy status in rat hearts with maintenance of tissue asp, not glu, an observation that contradicts other studies, including a previous clinical study by the same investigator [105]. In fact, such inconsistencies are the rule rather than the exception among the asp/glu literature, making objective interpretation of the various results extremely difficult.

The variability in experimental results relating to asp/glu enrichment may stem largely from variations in experimental design. These variations include the choice of experimental species, the particular model used, the extent and type of ischemic insult, the composition of the perfusate and reperfusate, concentration of amino acid used, the temperature during various stages of the protocol, experimental parameters, diagnostic tools, and methods of data analysis. Two such factors have contributed significantly to the conflicting results in the literature on asp/glu enrichment. First is the lack of adequate controls in many studies. For example, Galiñanes and coworkers [99] have shown the importance of correcting for coincidental changes in ionic composition of cardioplegic solutions when using salts of amino acids, a factor overlooked in many studies [7,106-108]. Second is the choice of diagnostic tools and parameters. The failure to provide a

solution to the asp/glu controversy may be largely due to a lack of adequate tools for direct observation of metabolic events throughout an episode of ischemia-reperfusion.

### *iii) Mechanisms*

Various mechanisms have been suggested for the beneficial effects of asp and/or glu, many of which are discussed in a recent review by Pisarenko [109]. Of the many mechanisms suggested, improved myocardial energy metabolism during ischemia and/or reperfusion have been generally regarded as the primary rationale for their use in cardioplegic solutions.

Asp and glu may protect myocytes during ischemia by anaerobic energy production through substrate-level phosphorylation in the Krebs cycle. Glu can be converted to 2-oxoglutarate (α-ketoglutarate) by cytosolic alanine aminotransferase. The 2-oxoglutarate can in turn enter the Krebs cycle (Figure 1) and follow through to succinate with production of one GTP by substrate-level phosphorylation. This process is however limited because of the need for a supply of $NAD^+$ (in the step between 2-oxoglutarate and succinyl-CoA) which depends on oxidative phosphorylation. Asp can be converted to oxaloacetate by cytosolic asp aminotransferase. Oxaloacetate can then be converted to malate, fumarate, and finally to succinate, in the process replenishing one mole of $NAD^+$ and one mole of $FAD^+$. In theory, if coupled, glu and asp metabolism should be able to continue because of the replenishment of $NAD^+$ by asp, thus producing ATP (via GTP) and a build up of succinate or alanine. Several investigators have provided indirect evidence for the above mechanisms in anoxic or ischemic hearts [87,101,110].

Theoretically, asp and glu may also increase glycolytic flux during ischemia by one of two mechanisms [109]. First, they could help replenish cytosolic $NAD^+$ through their role in the malate-aspartate shuttle [23], which may in turn improve glycolytic flux through glyceraldehyde-3-phosphate dehydrogenase. Secondly, transamination of glu with pyruvate may reduce lactate accumulation and thus attenuate inhibition of glycolysis at the lactate dehydrogenase step. Stimulation of glycolysis through this mechanism or others may also have an "oxygen sparing" effect by diverting substrate selection away from fatty acids [85].

Perhaps the most commonly quoted mechanism for asp/glu use in cardioplegia is that they may replenish important metabolites during ischemia-reperfusion, and thus improve the potential for aerobic respiration upon reperfusion. Interestingly, this is also the mechanism for which there is the least amount of evidence. Nevertheless, asp/glu could stimulate anaplerosis, a term used to describe pathways that replenish Krebs cycle intermediates [111], provide $NADH_2$ to the mitochondria as discussed above, or provide accumulated succinate from the anaerobic reaction previously mentioned. All of these metabolites could theoretically help "kick start" mitochondrial oxidative metabolism upon reperfusion.

Other possible roles for asp/glu are reduction of oxidant injury [112], binding of excess ammonia in glutamine or asparagine synthesis, or maintenance of ionic homeostasis [109].

## 3.4 Studying Myocardial Protection

### A) Experimental Heart Models

A variety of models have been used for studying heart preservation, including *in vivo* preparations, isolated heart preparations, and *in vitro* preparations with heart fragments. The following discussion will concentrate mainly on the isolated heart models, as they are most relevant to the current study.

#### *i) In vivo models*

A number of *in vivo* models have been described in the literature for studying myocardial protection, normally consisting of an animal on cardiopulmonary bypass, possibly combined with left coronary occlusion. These models have the great advantage of being the most clinically relevant, thus making extrapolation of some results to the clinical setting easier. However, one major disadvantage is that secondary neural or endocrine systemic effects can complicate results making studies of myocardial mechanisms difficult. In addition, the complexity of these models prevents their use in many settings, and makes experimental manipulations more difficult.

#### *ii) Isolated heart preparations*

In 1895, in order to study the uptake of glucose and other fuels by the mammalian heart, Langendorff designed one of the first *in vitro* isolated heart systems that is still widely used today [113]. For a successful preparation, provision of an adequate oxygen supply and maintenance of the correct ionic and pH conditions was essential. The latter had to

wait until 1932 when Krebs and Henseleit described the detailed composition of mammalian blood. The Krebs-Henseleit bicarbonate buffer system resembled that in human blood and is now standard for most heart perfusion systems [113].

Isolated heart preparations are considered *in vitro* preparations, but unlike *in vitro* preparations with heart fragments, the heart is perfused through its own coronary distribution and subject to relatively normal heart rate regulation. These preparations have been used extensively because the effects of various treatments can be tested without interference from complicating systemic factors. On the other hand, the fact that these models are separated from nervous and endocrine influences may affect the way in which they respond to various perturbations and thus limit their usefulness for studying cardiovascular mechanisms [114]. There are two main types of isolated heart systems, namely the Langendorff and working models.

- *Langendorff Heart Model*

In a Langendorff heart system, the perfusion fluid is provided to the heart through the aorta, as a result of which the aortic valves are closed due to the pressure in the aorta and the perfusion fluid is forced into the coronary arteries, thus perfusing the heart. As the left ventricle of the Langendorff heart is not filling normally, the heart is not pumping any fluid, and thus there is no external work being performed. This is why such preparations are often called 'non-working' heart preparations. Despite this deficiency, Langendorff hearts have been used extensively for studies of myocardial protection because they allow full control of perfusion conditions such as coronary flow and pressure, temperature, and perfusate substrate or ionic composition. The total coronary flow and oxygen

consumption can be very easily measured, making these models attractive for studying myocardial metabolism. In addition, mechanical function of the heart can be evaluated when using an intraventricular balloon (see following section). However, it has been noted that the use of such balloons can result in subendocardial ischemia, which may then affect the metabolism of the preparation [114].

- *Working Heart Model*

The so called 'working' isolated heart model as it is now known was first described by Neely in 1967 [113]. In a working heart preparation, the perfusate fluid fills the left atrium under pressure, the left atrium then empties into the left ventricle, which then pumps the fluid into the aorta, following the normal pathways of circulation. This preparation is somewhat more physiological than a Langendorff model because the heart is performing work by pumping the fluid, and because the perfusion of the heart itself is dependent on its ability to pump, as would be the case *in vivo*. In these preparations, preload and afterload can be manipulated separately, giving these models a further advantage. However, the dependence of the heart on itself as a pump can also be a major disadvantage of this system, limiting its usefulness for studies of significant atrial flow reduction or oxygen deprivation. Under such conditions, a severe decrease in cardiac function will result, ultimately ending in reduced coronary flow and then cardiac arrest.

### *iii) Heart Fragment preparations*

*In vitro* preparations consisting of ventricular or septal strips, isolated atria, or papillary muscles have been used for studies of ischemia, substrate metabolism, cardioplegia, etc.

These models have the advantage of simplicity, and allow studies of certain tissue types independently of other heart functions. However, there are a number of disadvantages, the most important being that oxygen and substrate delivery (or waste removal) is limited by diffusion through the tissue [122].

## B) Evaluation of Myocardial Condition

### *i) Ventricular Function*

The ultimate determinant of a patient's survival after cardiac surgery is the ability of the heart to perform its function as a blood pump. Although evaluation of myocardial metabolism, electrophysiology, and morphology all provide valuable information regarding the condition of the heart, these parameters alone cannot adequately predict post-ischemic cardiac function [5]. Therefore, independent assessment of myocardial function is crucial in the evaluation of any myocardial protective strategy.

#### ◆ *Determinants of contractile function*

*In vivo*, cardiac function consists of a periodic contraction, relaxation, and filling of the right and left ventricles. The result of this cycle is ejection of a volume of blood from the ventricle (stroke volume), which when multiplied by the heart rate gives the cardiac output, a true measure of the heart's work. Cardiac output is dependent on a number of factors, namely preload, afterload, and contractility. Preload, normally determined by the end-diastolic volume, relates to the muscle fibre length or the overlap between actin and myosin filaments before the muscle contracts. This, according to the Frank-Starling Law [115], determines the degree of fibre shortening and force development during the

subsequent systole. Afterload is the force that opposes the shortening of muscle fibers during contraction, normally determined by the arterial impedance, and is related to the wall stress developed in the myocardium during systole. Contractility is related to the wall stress developed in the myocardium during systole. Contractility relates to the extent and frequency of cross-bridge formation between actin and myosin filaments independent of their overlap, and thus relates to intrinsic muscle properties.

When judging the success of an operative intervention or cardioplegia on ventricular and regional myocardial function, it is preferable to measure parameters that depend only on contractility rather than loading conditions. Ideally, diastolic and systolic function are characterized by the simultaneous assessment of ventricular pressure, volume, and time [5], but this can be methodologically difficult. One simple alternative is the use of an isolated heart preparation, where preload and afterload can be kept constant, as they are both dependent on the intraventricular balloon volume. Under these conditions, ventricular developed pressure reasonably reflects the contractility and heart rate. To remove any effects of heart rate, one can either pace the hearts using an electronic pacemaker, or use the rate-pressure-product (RPP), which already reflects the heart rate [116]. Another standard reference for the assessment of ventricular contractility is the maximum first derivative of left ventricular pressure ($dP/dt_{max}$ or +dP/dt) [117]. Although +dP/dt is not a very specific measure of contractility, it is highly sensitive to changes in contractility. This is especially the case if +dP/dt is reached during isovolumic systole [5], which is by definition the case in an isolated heart preparation with intraventricular balloon.

- *Diastolic Function*

Normal cardiac function depends not only on systolic contraction, but also on isovolumic relaxation and ventricular filling during diastole. Isovolumic relaxation is primarily an active energy dependent process, as calcium is actively transported back into the sarcoplasmic reticulum [5, 118]. The subsequent ventricular filling phase normally involves remodeling of the myocardium by passive mechanisms [5]. The resistance of the ventricle to stretch determines ventricular stiffness, which is defined as the ratio of change in pressure to change in volume during the filling phase.

In a Langendorff isolated heart preparation, diastolic function is assessed mainly by the rate of myocardial relaxation ($dP/dt_{min}$ or $-dP/dt$), that is the maximum rate of pressure decrease during diastole. As with $+dP/dt$, $-dP/dt$ is calculated by taking the first derivative of the developed pressure curve and measuring the value at its most negative point. In addition to $-dP/dt$, measuring ventricular end-diastolic pressure provides some information on ventricular stiffness. If the volume in the ventricular balloon is kept constant, an increase in end-diastolic pressure likely indicates increased ventricular stiffness, and vice versa.

### *ii) Metabolic Status*

- *High Energy Phosphates*

The dependence of the heart on a continual supply of HEPs and the changes in levels of these compounds during ischemia-reperfusion were discussed in previous chapters. Although a correlation between high energy phosphate levels and myocardial function is

questionable [31]. the level of HEPs can be a good indicator of the severity of ischemia and provide some insight into the metabolic status of the post-ischemic myocardium [2].

There are currently two main methods for quantification of HEPs and inorganic phosphate in the heart. The first is to freeze the tissue rapidly to arrest metabolism. conduct appropriate extraction (eg. with perchloric acid or tricloroacetic acid), and then measure HEP levels by enzymatic assay, bioiluminescence, isotachophoresis, or high performance liquid chromatography (HPLC). The main disadvantages of this method are that the tissue is destroyed in the process and that there can be significant errors in the measurement due to the unstable nature of nucleotides [2]. The second method is using $^{31}P$ nuclear magnetic resonance spectroscopy. The main advantages of NMR are that it is non-invasive and non-destructive. which makes it ideal for serial studies on a single heart. $^{31}P$ NMR is discussed further in section 3.4-C.

Compartmentalization is an important consideration in the measurement of HEP levels. HEP levels in the heart may differ in various cell types and in specific subcellular compartments, such as the mitochondria [2]. Measurements may also be affected by the proportion of nucleotides in bound form. For instance, up to 80% of the ADP in myocytes may be bound to actin [2]. Finally, there may be multi-enzyme complexes that channel the product of one enzyme for use by another enzyme, possibly affecting gross measurements of HEP levels [2].

- *Oxygen Consumption*

The heart can use a variety of carbon substrates for its energy supply and depends on a continuous supply of oxygen to maintain energy metabolism. Since normal extraction of carbon substrate is quite low, and extraction of oxygen very high, oxygen is essentially the limiting metabolite for ATP production [119]. Therefore, under most normal conditions, $MVO_2$ can provide an indirect measure of energy production and utilization. This can be particularly useful under conditions (eg. myocardial stunning) where measurements of high energy phosphates may not accurately reflect the ability of the mitochondria to produce energy [120]. However, $MVO_2$ must be correlated with other parameters in any study, as under some severe conditions uncoupling of the mitochondria could lead to artificially high $MVO_2$ values without any ATP turnover.

- *Tissue pH*

Changes in myocardial pH during ischemia were discussed in section 3.2-C. Measurement of myocardial pH can provide a sensitive indicator of tissue ischemia and is commonly used in cardiac surgery for that purpose [119]. Although most electrodes available for tissue pH measurement normally measure extracellular pH, NMR spectroscopy can provide a direct measure of intracellular pH.

### *iii) Other*

- *Enzyme Leakage*

Loss of membrane integrity appears to be a crucial event in irreversibility of ischemic damage and can lead to large-scale loss of cardiac enzymes into the circulation [121]. The

levels of enzymes leakage into the perfusate can thus be used to evaluate myocardial injury. The MB isoenzyme of creatine kinase is most commonly used due to its specificity to the heart. Alcohol dehydrogenase is also used, although less commonly. Infarct size can be estimated by extrapolation from the rate of myocardial enzyme leakage. The main disadvantage of this technique is that rapid breakdown of the enzyme at the site of injury and a variable rate of decay in the blood can be a significant source of error [121].

- *Edema*

During ischemia there is a significant intracellular build-up of $Na^+$, lactate, phosphate, and other metabolites. This can result in significant increases in intracellular osmolarity, which can lead to excessive movement of water into the cell during reperfusion, and cause myocardial edema. Myocardial water content is a general indicator of the condition of the myocardium after reperfusion, especially since excessive edema in the myocytes and capillary endothelium can cause an increase in coronary vascular resistance and reduced myocardial blood flow during reperfusion [122].

## C) Magnetic Resonance Spectroscopy

Nuclear magnetic resonance (NMR), or magnetic resonance (MR) for short, is a powerful technique with applications in many fields. Although the applications of NMR in medicine have mostly been in the area of imaging (MRI), magnetic resonance spectroscopic (MRS) techniques are rapidly evolving.

### *i) Principles of NMR*

The NMR phenomenon depends on the fact that the nuclei of some atoms have a "spin" that gives them a magnetic moment. NMR uses a powerful magnet to apply a static, uniform magnetic field ($B_0$) that orientates these nuclear spins in the direction of the $B_0$. These nuclei can then be excited by a second oscillating magnetic field ($B_1$), typically in the form of a radio-frequency (r-f) pulse near the resonant frequency for the particular atomic species under study. This r-f pulse causes the excited nuclei to be tipped off of the $B_0$ axis. When the r-f pulse is removed, the nuclei relax back to their original orientation, in the process releasing energy in the form of an r-f signal that can be detected by an r-f coil (or probe), which normally also produces the $B_1$ r-f pulse. This r-f "signal" is known as the free induction decay (FID) which is then analyzed for its various frequency components by Fourier transformation, a process that converts the time-dependent FID to a frequency dependent spectrum [3].

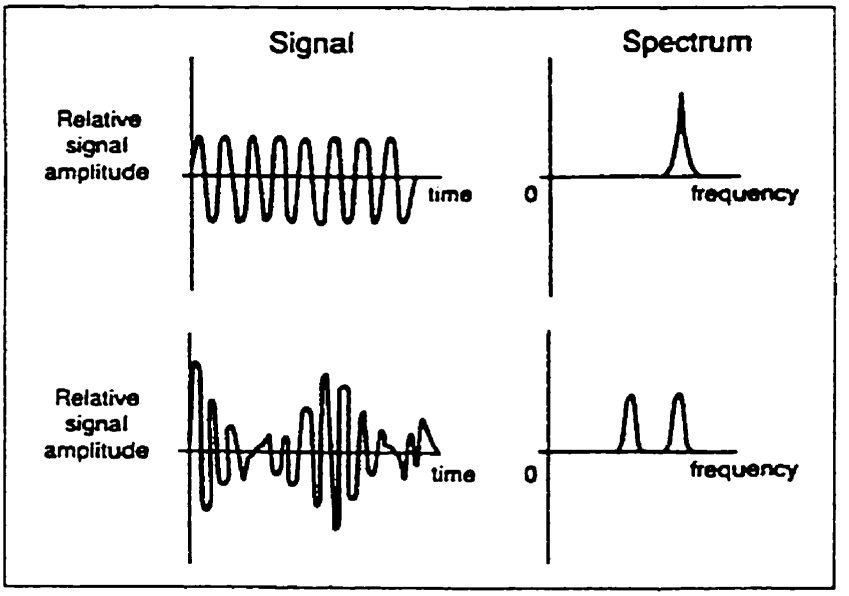


**Figure 5: Diagrammatic representation of Fourier-transformation of FID signals (from: Schaefer S, 1989 [3]).**

The different chemical environments of nuclei in one or more compounds causes the nuclei to emit an r-f signal at a slightly different frequency called a "chemical shift". In this manner, various peaks in the spectrum can represent different compounds, and the peak area is proportional to the quantity of the compound in the sample.

A number of different elements have nuclei with spin that can be useful for NMR studies in biological systems [123]. Of these, $^{1}H$, and $^{13}C$ have been most commonly used.

### *ii) $^{31}P$ MRS*

The use of $^{31}P$ MRS has grown significantly over the past two decades [124,125]. $^{31}P$ MRS allows real-time determination of myocardial HEPs, inorganic phosphate, and intracellular pH. A typical $^{31}P$ spectrum from a blood-perfused pig heart can be seen in the top panel of Figure 8. Of note are the reference peak, the PCr peak, and the three ATP

peaks (α, β, γ). Of the three ATP peaks, the β-ATP has the least overlap with resonances from other metabolites such as ADP and NADP and is thus the most reliable peak for determination of ATP levels. Another important peak arises from Pi and has considerable overlap with peaks from phosphomonoesters (PME) and phosphodiesters (PDE) This overlap makes determination of the Pi peak size and exact position difficult. This is particularly important as the ability of $^{31}P$ MRS to determine intracellular pH depends on accurate determination of the Pi and PCr peak positions.

The relative shift of the Pi peak can be used as a measure of pHi [126,127]. Pi can exist in the cell as either $HPO_4^{2-}$ or $H_2PO_4^-$, each with a different chemical shift. The exchange between these two ionic forms is rapid, giving rise to a single Pi peak in $^{31}P$ spectra. The position of this Pi peak depends on the relative concentrations of the two forms, which is pH dependent.

# 4. MATERIALS AND METHODS

These studies were performed at the Institute for Biodiagnostics and all animals received humane care in accordance with the Canadian Council on Animal Care's Guide to the Care and Use of Experimental Animals [128]. All experimental protocols were approved by the Animal Care Committee at the Institute for Biodiagnostics.

## *4.1 Experimental Design*

Two related questions are addressed by the experiments:

1. Does enrichment of the perfusate with asp and glu improve myocardial energy metabolism during ischemia or reperfusion?

2. Can Buckberg's asp/glu enriched cardioplegia resuscitate ischemically stunned myocardium?

Thus, the experiments have been organized into two separate "studies", the results of which are presented separately. A total of 21 isolated pig hearts were used, divided into one control and two experimental groups. The experimental timelines for the two studies are discussed below, followed by several sections detailing the methods.

## A) Study #1: The effects of asp/glu on energy metabolism during ischemia-reperfusion

The protocol for the first study is summarized in Figure 6. Control hearts (Group A, n=8) were perfused with blood without added substrate. Experimental hearts (Group B, n=6) were perfused with blood enriched with 13 mmol/L each of asp and glu[1], and will be referred to as the "asp/glu" group. Upon filling of the intraventricular balloon, all hearts were perfused for 20 minutes (control perfusion), subjected to 30 minutes of total normothermic ischemia[2], and then reperfused for 40 minutes using the respective perfusate for each group (Stage I).

To assess possible causes for the observed contractile dysfunction, after normal reperfusion (stage I), two hearts from each group were subjected to inotropic testing by titration with calcium. The perfusate [$Ca^{2+}$] was gradually increased over a 20 minute period through addition of aqueous $CaCl_2$ (2 mEq boluses) to the reservoir, until myocardial function reached a plateau. Heart function was then allowed to stabilize over a 10 minute period, after which data collection was resumed (Stage II). The hearts were then perfused for an additional 10 minutes (for a total of 40 minutes).

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[1] This dose is commonly suggested to be beneficial both experimentally [107-108] and clinically [8, 10].

[2] Although the intentions were to maintain heart temperature at 37°C, the temperature decreased gradually during 30 minutes of ischemia to 32-35°C.

Group A (control): blood without added substrate

Group B (asp/glu): blood + 13 mmol/L each of asp and glu

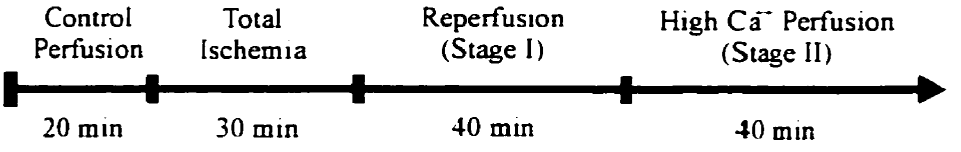


**Figure 6: Outline of experimental protocol for Study #1.**

## B) Study #2: Asp/glu-enriched secondary blood cardioplegia for myocardial resuscitation

The timelines for the second study appear in Figure 7. After 20 minutes of control perfusion with blood (37°C), all hearts were subjected to 30 minutes of normothermic, zero-flow ischemia. The hearts were then divided into two groups. Control hearts (Group A, n=8) were reperfused with blood for 40 minutes. Experimental hearts (Group C, n=7) were reperfused with warm asp/glu blood cardioplegia (see section 4.2) for 20 minutes[3], followed by 20 minutes perfusion with blood. This group is refereed to as the "cardioplegia" group.

[3] This time period was used in a clinical study advocating the use of warm, asp/glu cardioplegia for resuscitation of "stunned" hearts [9].

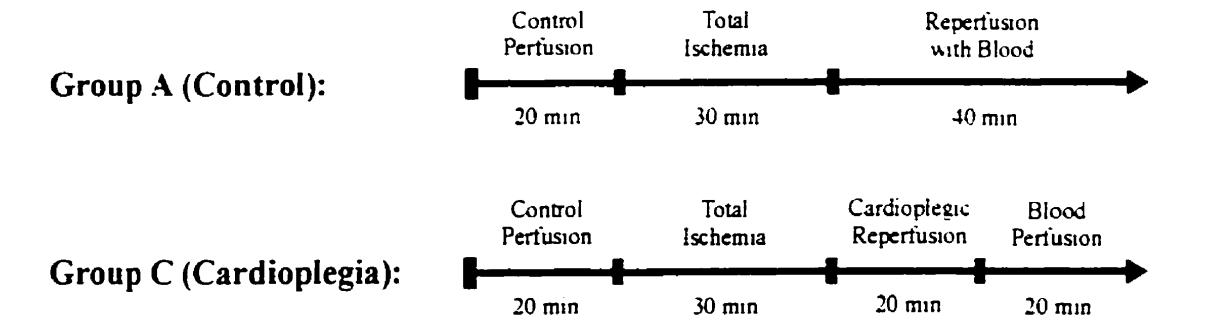


**Figure 7: Outline of experimental protocols for Study #2.**

## 4.2 *Solutions and Perfusates*

All solutions were prepared prior to experiments using ultrapure water (NanoPure, Barnstead/Thermolyne, Dubuque, Iowa) and solutes obtained from Sigma Chemical Company, St. Louis, MO.

St. Thomas Hospital II (STH) cardioplegic solution was used to arrest the hearts during the isolation procedure and contained (in mmol/L): NaCl 100, $NaHCO_3$ 25, KCl 26, $MgCl_2$ 16, and $CaCl_2$ 1.2; the pH adjusted to 7.4 by HCl.

Krebs-Henseleit (K-H) solution with a modified composition was used for topical hypothermia during heart isolation, for hemodilution, and to compensate for changes in ionic composition of the blood perfusate. The K-H composition (in mmol/L) for control hearts was: NaCl 118, $MgSO_4$ 1.2, $NaH_2PO_4$ 1.2, $Na_2$ EDTA 0.5, D-glucose 11, $CaCl_2$ 1.75, $NaHCO_3$ 25, and bovine serum albumin 5g/L. For the asp/glu group, the K-H composition was similar except that the NaCl content was lowered to 25 mmol/L. This

was to correct for the increase in [$Na^+$] that would result from addition of the monosodium salts of the amino acids (see below).

The following stock aqueous solutions were also prepared: Asp/glu (1.0 M each: monosodium salts), D-glucose (0.5 M), NaCl (2.0 M), KCl (0.5 M), and $CaCl_2$ (0.5 M).

Blood collected from the chest by suction after heart excision (see below) had a hematocrit of 23-29% as it contained some K-H solution (normal pig hematocrit is ~30%). Blood perfusate for initial perfusion of all hearts, and for reperfusion of control hearts, was prepared by adding K-H solution to adjust the hematocrit to 20-25%. In both groups, KCl was omitted to compensate for any rise in [$K^+$] from residual mixing of crystalloid cardioplegic solution with the blood during cardioplegic infusion. The ionic composition and osmolarity (270-280 mOsm) of this blood/K-H mixture (refereed to as "blood" hereon) was similar to that of the pig's own blood.

In group B, the low $Na^+$ content in the K-H solution lowered the total [$Na^+$] below 120 mmol/L to allow for addition of 13 mmol/L each of monosodium asp and monosodium glu (from asp/glu stock solution). The final [$Na^+$] was adjusted to 140±5 mmol/L. The final [$K^+$] was adjusted to 4.5±0.5 mmol/L.

In Group C, asp/glu-enriched blood cardioplegic solution was prepared by mixing the collected blood in a 4:1 proportion with concentrated cardioplegic additive solution prepared according to Table 1, which reflects Buckberg's recipe for "warm reperfusate" [10]. Discrepancies in Table 1 between desired and actual blood modifications represent

actual values obtained in this experiment after following the recipe, except in the case of asp and glu where the difference is due to miscalculations in Buckberg's recipe.

The osmolarity of the prepared perfusates (3WII osmometer, Advanced Instruments, Needham Heights, MA) was similar in all experiments and comparable to that of the pig's own blood (270-280 mOsm/L). The cardioplegic solution in Group C was the exception, it was not meant to have normal osmolarity.

**Table 1: Composition of Blood-Cardioplegic Solution for Controlled Reperfusion**

| | Concentration in cardioplegic additive to be mixed with 4 equivalents of blood | Desired modifications of blood (from Buckberg's recipe) | Actual modifications of blood |
|---|---|---|---|
| Potassium chloride | 31 mmol/L (0.23%) | $K^+$ to 8-10 mmol/L | $K^+$ to 8-10 mmol/L |
| THAM[4] | 70 mmol/L (0.85%) | pH to 7.5-7.6 | pH to 7.4-7.6 |
| CPD solution[5] | 23.6% by volume | $Ca^{2+}$ to 0.15-0.25 mmol/L | $Ca^{2+}$ to 0.21-0.28 mmol/L |
| L-Aspartate (monosodium salt) | 60 mmol/L (0.93%) | substrate - 13 mmol/L | substrate added: 12 mmol/L |
| L-Glutamate (monosodium salt) | 60 mmol/L (1.01%) | substrate - 13 mmol/L | substrate added: 12 mmol/L |
| D-Glucose (anhydrous) | 175 mmol/L (3.15%) | glucose to >22 mmol/L (400 mg/dl) and osmolarity to 380-400 mOsm/L | glucose to ~45 mmol/L and osmolarity to 365-375 mOsm/L |

[4] tris(hydroxymethyl)aminomethane, also known as tromethamine.

[5] citrate-phosphate-dextrose solution (Sigma Chemical) composed of 0.3% citric acid (anhydrous); 2.63% $Na_3$ citrate (dihydrate); 0.193% $NaH_2PO_4$ (anhydrous); 2.32% D-glucose (anhydrous).

## 4.3 Surgical Procedures

Domestic pigs weighing 40-55 kg were obtained from a local supplier and housed in the animal facility at the Institute for Biodiagnostics for a minimum two week acclimatization period. The experimental animal was fasted overnight and pre-medicated with an intramuscular injection of ketamine hydrochloride (25 mg/kg), midazolam (400 μg/Kg) and atropine sulphate (0.05 mg/kg). The animal was then transferred to the operating room. Surgical anesthesia was induced and maintained with isoflurane (1.5-2.0%) in a 1:1 mixture of oxygen and air, initially administered through a face mask. Anesthesia and ventilation were maintained and monitored using an Excel 210 anesthesia system with a 5250 respiratory gas monitor (Ohmeda, Madison, MI). After intubation and initiation of mechanical ventilation, the right common carotid artery was cannulated and a catheter positioned at the aortic root for monitoring arterial pressure, blood sampling, and subsequent infusion of cardioplegic solution. Blood-gas analysis (StatProfile 7+, NOVA Biochemical) was performed to verify the adequacy of ventilation.

A median thoracotomy was performed and the pericardium was opened longitudinally along the midline. The superior and inferior venae cavae, brachiocephalic artery, left subclavian artery, and descending aorta were dissected and isolated by threading umbilical tape around each vessel. Following heparinization (600 IU/kg), the right atrium was cannulated. The carotid artery cannula was secured in position by tying the umbilical tape around the brachiocephalic trunk. After ligating the great arteries and clamping the venae cavae, the heart was arrested using heparinized (3 IU/ml), cold STH solution (7 ml/Kg; 4°C) infused through the carotid artery cannula. The right atrial cannula was

opened to drain collecting cardioplegic solution and prevent its mixture with the pig's blood. Concurrently, the left atrium was cut open to prevent the return of warm blood from the lungs to the heart. Once the heart was arrested, 700 ml of cold, modified K-H solution were slowly poured over the heart for topical hypothermia; the composition of the K-H solution for the two groups was as described above. The heart and lungs were then excised from the chest en-bloc and maintained in St. Thomas solution at 4°C. All clamps were removed from the great vessels and the pig was exsanguinated.

Once the heart was excised from the chest, the lungs and pericardium were cut away to free the heart. Subsequently, each ventricle was punctured with a small piece of tubing and a compliant balloon (unstressed volume > 50 ml) was inserted into the left ventricle (LV), held in place by a purse-string suture closing the mitral valve. A small (1.0 ml) round-bottom flask containing phenyl phosphonic acid, an MR intensity reference, was placed inside the right ventricle and tied in place with a suture. The brachiocephalic and left subclavian branches of the aorta were cannulated for perfusion and pressure monitoring, respectively. Finally, a catheter was loosely placed inside the coronary sinus (for collection of venous blood) and secured to the wall of the atrium. The heart preparation was then transferred to the MR instrument.

After removal of the heart from the chest, all clamps were removed from the great vessels and the blood collecting in the chest was removed by suction. This blood was then processed as detailed above to prepare the various perfusates.

## 4.4 Perfusion System

The perfusion system was designed to minimize prime volume (<400 ml) despite the large length of tubing required to penetrate a Faraday cage and reach into the bore of the MR magnet. Thus, the smallest possible pediatric components and tubing were used. In addition, the use of magnetic components in proximity to the MR instrument was avoided.

Blood was pumped from the reservoir using a Precision Blood Pump (Cobe) through a Capiox 308 hollow-fiber oxygenator (Terumo Corp., Tokyo, Japan) followed by a 20 μm arterial blood filter (model D735, Dideco, Mirandola, Italy). The arterial line entered the Faraday cage through a specially built radio-frequency (r-f) filter and connected to the brachiocephalic artery cannula via plastic quick disconnect fittings (Cole-Parmer, Chicago, IL). The blood was collected at the bottom of the MR probe container and returned to the reservoir by suction. All tubing used was Tygon (Cole-Parmer, Chicago IL).

For group C, a two-reservoir perfusion system was used, allowing easy switching of perfusion source from one reservoir (cardioplegia) to the other (blood).

The system allowed blood to recirculate independently of the arterial line, to prevent excessive settling or clotting during periods of ischemia. Anti-coagulation was maintained by an additional bolus of heparin (5,000 IU) every hour. Arterial blood temperature was maintained at 37°C by use of two water baths (model RM6, Lauda, Germany), one connected to heat exchangers in the reservoir and oxygenator, and the

other to a glass heat exchanger placed outside the bore of the magnet. $O_2$ and $CO_2$ gases were supplied to the oxygenator from separate sources and mixed in appropriate proportions to maintain blood gas partial pressures (P) as follows: $PO_2$>200 mmHg and 35<$PCO_2$<45 mmHg.

After each experiment, the reservoir, oxygenator, filter, and tubing were rinsed with cold tap water followed by dilute bleach, and then again rinsed extensively with tap water followed by filtered water. The tubing and reservoir were drained and stored in a freezer. The oxygenator and filter were dried by pressurized air for several hours and then stored. All components were replaced every fourth experiment.

## *4.5 Heart Perfusion*

After transfer to the MR instrument, the heart preparation was hung by the aortic arch and the brachiocephalic artery cannula connected to the arterial line from the perfusion apparatus. The aorta was clamped at the root and the perfusion pump turned on, using the subclavian artery cannula as a vent to remove air as the aorta filled with perfusate. Once all air was removed, the aorta was unclamped and heart perfusion began. The subclavian artery cannula and the LV balloon were then connected to pressure transducers (Cobe) which were calibrated and linked to a multichannel polygraph recorder with digital output (model TA 5000, Gould, Valley View, OH). The arterial flow was increased gradually over a 5-minute period and adjusted to maintain the perfusion pressure at 60 mmHg until initiation of data acquisition, after which the flow rate remained constant (~1.0-1.5 ml/g heart tissue) for the remainder of the experiment. During the initial warming period, the hearts were monitored for fibrillation and defibrillated as required using an HP

CodeMaster XL+ (Hewlett Packard, McMinnville, Oregon). Once stable, the heart preparation was placed inside the MR probe and the probe was inserted into the bore of the MR magnet. The LV balloon was gradually filled with filtered water to achieve a stable diastolic pressure of 0-5 mmHg, after which the balloon volume remained constant throughout the experimental protocol.

## 4.6 Data Acquisition

Upon filling the intraventricular balloon, MR magnet shim values were adjusted where necessary, after which continuous acquisition of $^{31}P$ MR spectra was initiated. Functional data from the LV balloon were collected continuously throughout the protocols. Arterial and venous (from coronary sinus) blood samples were taken every 10 minutes to measure blood gases (StatProfile 7+, NOVA Biomedical and OSM3 hemoximeter, Radiometer A/S, Copenhagen, Denmark), pH, electrolytes, and glucose (StatProfile 6, NOVA Biomedical), and to calculate myocardial oxygen consumption ($MVO_2$). At the end of each protocol, LV biopsy samples were taken for high performance liquid chromatography (HPLC) quantification of metabolites. Finally, a piece of LV tissue was taken to determine the dry/wet weight ratio as a measure of myocardial edema.

## 4.7 MRS Methods

MR spectroscopy was performed in a 40 cm horizontal bore magnet at 7 Tesla using a Bruker Biospec spectrometer (Bruker, Karlsruhe, Germany). The heart was suspended inside a home-built MR probe consisting of an r-f coil surrounding a glass container. After tuning the coil and using the $^{1}H$ signal to optimize the magnetic field homogeneity

(shimming), the coil was switched to a frequency of 121.47 MHz for acquisition of $^{31}P$ spectra. Free induction decay (FID) signals were obtained using 2 K data points and a sweep width of 12 KHz. Single r-f pulses were used with a pulse length and repetition time of 80 μsec and 2 seconds, respectively. Sixty FIDs were accumulated for each spectrum, thus allowing a 2-minute time resolution.

## 4.8 HPLC Analysis

The HPLC system consisted of two Waters Model 510 pumps, a Waters System Interface Module, a Waters U6K Injector, and a Waters 996 PDA Detector, all controlled by Waters Millennium Chromatography Software. Separation was achieved with a Nucleosil LC18 column (5 μm, 250 mm x 4.6 mm ID) using binary gradient ion-pair chromatography.

Heart biopsy samples were stored at -80°C until lyophilized. Once the samples were freeze-dried, they were stored at -80°C until analyzed by HPLC. Samples (approx. 2-4 mg dry weight) were ground with silica, extracted with perchloric acid, and neutralized according to S. Lareau, et al [129]. Immediately after this step, 50 μl of the sample were injected onto the HPLC system. Data was collected for 27 minutes and the system allowed to re-equilibrate for 11 minutes prior to the next injection.

## 4.9 Data Analysis

Accumulated FIDs were transferred to in-house software (Xprep) where, after Fourier transformation, they underwent automatic phase correction and manual baseline

correction. To improve the signal-to-noise ratio, the spectra were then subjected to 20 Hz line broadening by exponential multiplication of the time function. The resulting spectra were then transferred to another in-house analysis software package (X-Allfit) where they were fitted with Lorentzian curves and quantified using the integrals of the curves. The data were sent to Microsoft Excel 5.0 spreadsheets for further processing. In order to compensate for changes in spectral intensity during various stages of the protocol, peak integral values for the β-ATP, PCr, and Pi peaks were corrected with respect to the reference (phenyl phosphonic acid) peak. Due to the difficulty in absolute quantification of metabolites from $^{31}P$ spectra, the metabolite levels were expressed as a percentage of the initial β-ATP peak obtained during control perfusion. The spectra from different experiments in the same group were arranged according to time points and averaged.

Intracellular pH (pHi) was determined from the difference in position between the Pi and PCr peaks [127]. To increase the accuracy of pHi calculations, in-house software (Xprep) was used to manually determine the exact positions of the Pi and PCr peaks for each spectrum[6].

LV balloon and blood gas data were entered into Microsoft Excel 5.0 spreadsheets for calculation of the extent of post-ischemic recovery of myocardial function and $MVO_2$.

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[6] The exact position of the Pi peak is often difficult to determine due to its overlap with peaks from phosphomonoesters and phosphodiesters (from blood), thereby limiting the accuracy of pHi determination under certain conditions. This problem is particularly acute during periods of normal metabolism, such as control perfusion, when the Pi peak is small. Automatic fitting programs thus have significant difficulty in this area.

Statistical analyses were performed using Statistica 5.0 software (Statsoft. Tulsa. OK). Student's t-test and the Mann-Whitney U test were used to compare data between groups. The paired Student t-test was used to compare data across time within groups. Statistical significance was attributed to p values below 0.05.

# 5. RESULTS

The results have been organized into two separate studies. Study #1 has been further split into two sections based on stage I (all hearts) and stage II (inotropically tested hearts) reperfusion. All data are presented as means ± standard errors of the means, unless otherwise indicated.

## 5.1 Study #1: Stage I

### A) Myocardial Energy Phosphates

#### *i) Magnetic Resonance Spectroscopy*

Figure 8 shows representative $^{31}P$ spectra from a control heart subjected to 30 minutes normothermic ischemia and then reperfused. The spectra show decreased PCr levels immediately upon initiation of ischemia, an equally rapid increase in Pi levels, and a slight decrease in the three ATP peaks. In addition, the position of the Pi peak shows a slight upfield shift (i.e. toward the PCr peak) indicating decreased intracellular pH (pHi). By the end of 30 minutes of normothermic ischemia, the PCr peak has all but vanished, the Pi peak is very large and shifted significantly due to acidification of the medium, and the ATP peaks are significantly reduced in size. These parameters return to normal levels during the first few minutes of reperfusion, with the exception of the ATP peaks which show only modest recovery.

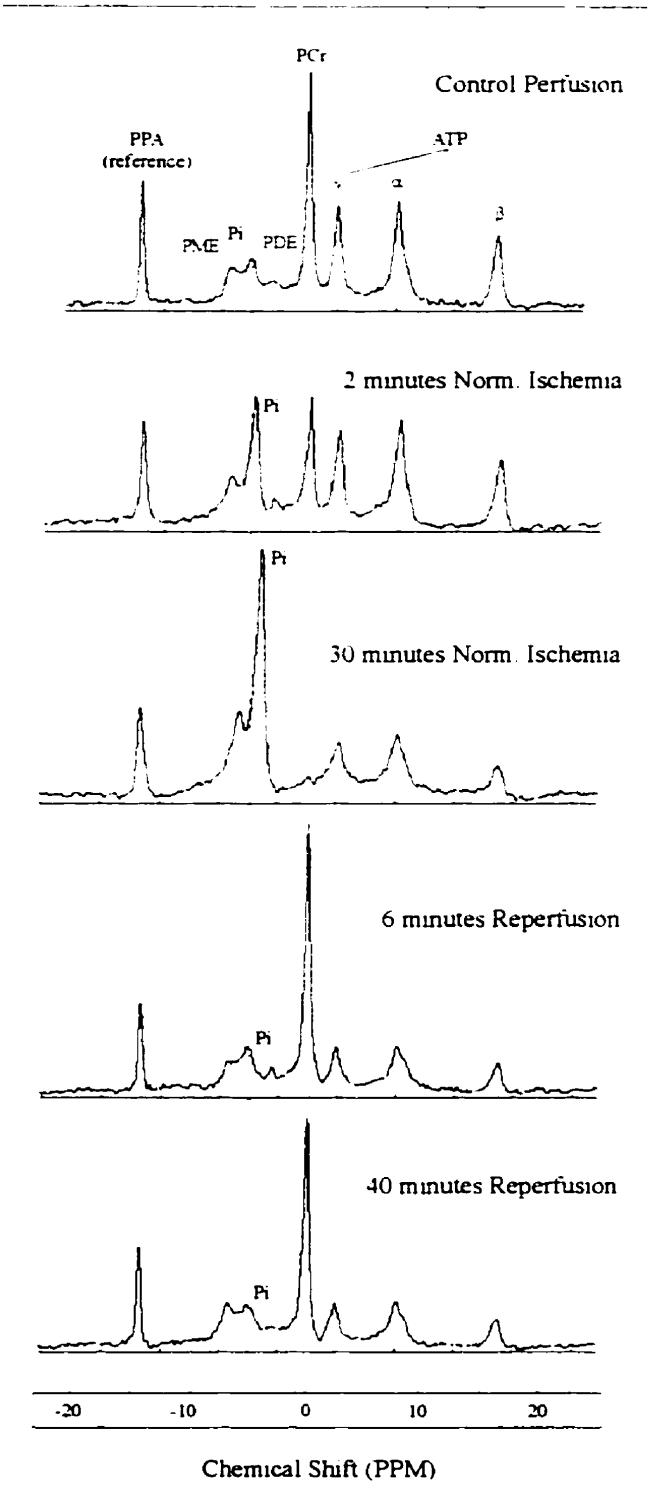


**Figure 8: Representative 31P MR spectra from blood perfused pig hearts. The main resonances are from phenyl phosphonic acid (PPA, spectrum intensity reference), phosphomonoesters (PME), inorganic phosphate (Pi), phosphodiesters (PDE), phosphocreatine (PCr), and the three phosphate groups (α, β, and γ) of adenosine triphosphate (ATP). The chemical shift is expressed as parts per million (ppm) relative to the PCr peak.**

The time course of the changes in myocardial energy phosphates for the two groups are shown in Figure 9. Presented as a percentage of initial ATP levels[7], the rate and extent of energetic changes throughout ischemia-reperfusion appear to be identical in the two groups and follow a pattern consistent with the spectra in Figure 8. The only apparent difference between the two groups is in levels of Pi during control perfusion, which was not statistically significant and was most likely due to difficulties in fitting of the Pi peak created by its overlap with peaks arising from phosphomonoesters (PME) and phosphodiesters (PDE)[8].

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[7] Absolute quantification of metabolites from $^{31}P$ spectra is difficult, though not impossible. Presentation of metabolite levels as a ratio of a relatively stable peak, such as β-ATP during control perfusion, allows comparison of changes between different hearts without determination of actual concentrations.

[8] In order to allow the automatic fitting software (X-Allfit) to distinguish between the Pi peak and the sugar phosphate peaks (PME and PDE), certain restraints had to be placed on the size of the peak. These restraints allowed accurate detection of any changes in Pi levels, but limited the accuracy with which the absolute value of the Pi peak could be calculated during periods (such as control perfusion) when the size of the peak was minimal.

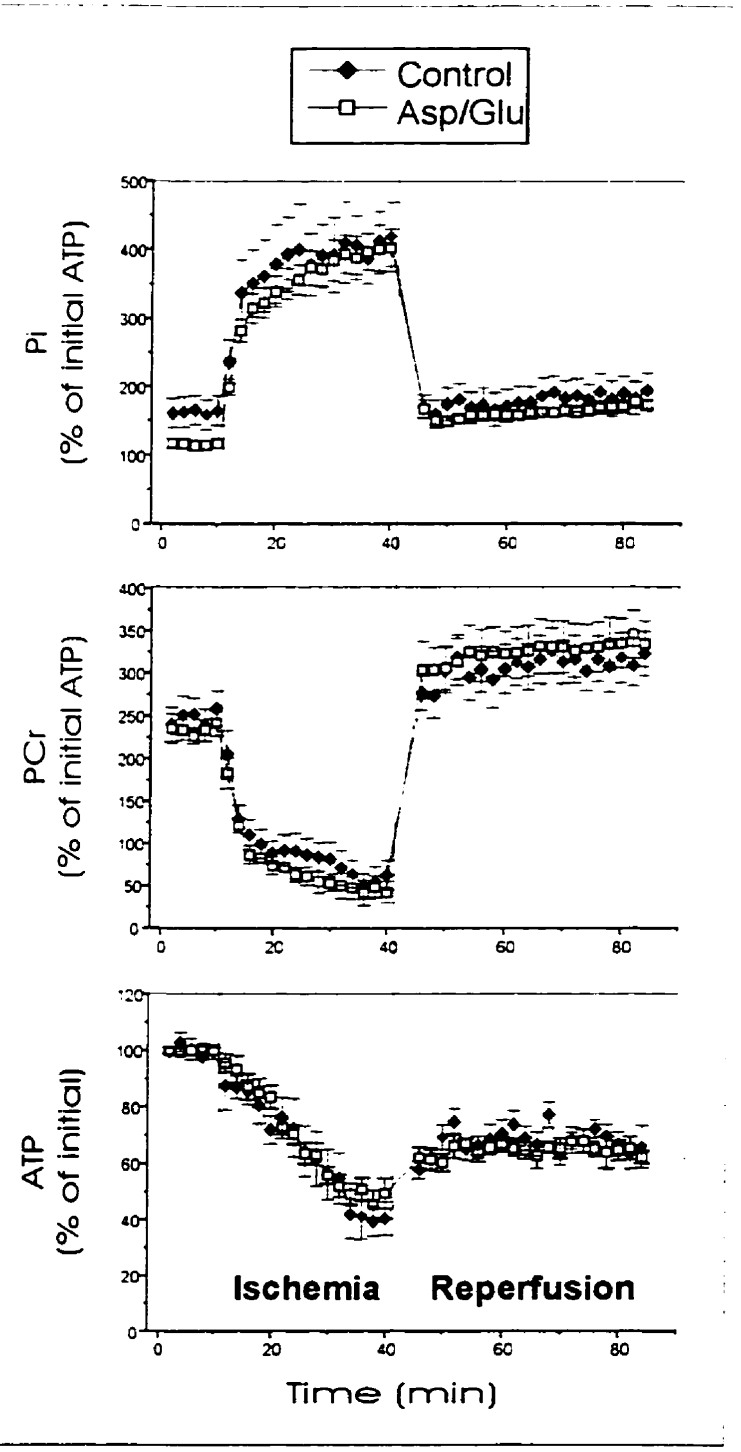


**Figure 9: Changes in myocardial energy phosphates during ischemia-reperfusion. ATP=adenosine triphosphate; PCr=phosphocreatine; Pi=inorganic phosphate. Time points represent average metabolites over a two-minute period. Data points represent means ± standard errors of the means.**

In both groups, the onset of ischemia is marked by a very rapid decrease in PCr levels during the initial 5-6 minutes, followed by a more gradual decline. Despite the expected buffering by PCr, ATP levels begin a gradual decline immediately upon onset of ischemia and continue at the same rate, though there appears to be a slightly lower rate of decline during the last 10 minutes of ischemia. The changes in Pi levels during ischemia appear to follow a similar two-stage pattern to PCr, increasing at a rate corresponding to the combined breakdown of PCr and ATP.

Upon reperfusion (Figure 9), PCr and Pi show a dramatic initial recovery, reaching normal levels within five minutes[9]. PCr levels, in fact, reach higher values than control ($p<0.05$), possibly due to increased inorganic phosphate availability (from ATP breakdown) and reduced contractile demand (see below). ATP levels also show recovery during the initial reperfusion period, though the recovery is modest. After the initial energy recovery, there is no apparent change in ATP levels, although there is a slight gradual increase in both Pi and PCr levels. The former is possibly due to increasing myocardial edema with continuous perfusion, while the latter may indicate slowly improving supply/demand ratio.

The extent of energetic changes in the two groups is summarized in Table 2. Comparison of ATP, PCr, and Pi levels shows no significant differences between groups. These MRS

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[9] Due to post-ischemic fibrillation in many hearts, acquisition of spectra was often interrupted at the start of reperfusion, leading to a data gap of 1-2 time points. The first observed time point upon reperfusion (Figure 3) therefore corresponds on average to 3-5 minutes of reperfusion.

results indicate that asp/glu enrichment of the perfusate did not decrease the rate of energetic decline during ischemia, or improve energy production upon reperfusion.

**Table 2: Changes in Myocardial Energy Phosphates and Intracellular pH During Ischemia-Reperfusion.**

| | | Control Perfusion | End of Ischemia | Initial Reperfusion | End of Reperfusion |
|---|---|---|---|---|---|
| **Group A** | **ATP** | 100±3 | 47±7 | 65±4 | 66±5 |
| **(control)** | **PCr** | 260±22 | 75±21 | 298±23* | 318±24* |
| **n=8** | **Pi** | 171±21 | 463±72 | 182±21 | 194±22 |
| | **pHi** | 7.22±0.15 | 6.33±0.16 | 7.18±0.16 | 7.20±0.07 |
| **Group B** | **ATP** | 100±1 | 49±4 | 63±3 | 65±4 |
| **(asp/glu)** | **PCr** | 233±13 | 46±14 | 310±29* | 334±29* |
| **n=6** | **Pi** | 116±7 | 396±29 | 155±8 | 170±9 |
| | **pHi** | 7.30±0.07 | 6.44±0.19 | 7.28±0.11 | 7.35±0.09 |

ATP=adenosine triphosphate; PCr=phosphocreatine; Pi=inorganic phosphate; pHi=intracellular pH. ATP, PCr, and Pi are expressed as a percentage of initial ATP levels. The values shown are averages over a ten minute period (six minutes for pHi) at the indicated stage of the protocol. There were no significant differences between groups ($p>0.05$). *$p<0.01$ with respect to control perfusion

### *ii) High Performance Liquid Chromatography*

To complement MRS data, HPLC was used to determine energy metabolite concentrations in biopsy samples taken from the left ventricle at the end of each protocol. The results for Stage I (normal reperfusion) hearts are summarized in Table 3. The ATP and PCr levels, normalized to the total tissue creatine, were similar in the two groups, suggesting that the hearts were in equivalent energetic states [130]. Furthermore, using the ATP concentrations determined by HPLC and the initial and final high energy phosphate levels as measured by MRS, the initial ATP concentration (in μM/g dry wt) is

estimated at 28±3 and 26±1 for groups A and B, respectively. These values are in good agreement with those obtained for pig hearts in other studies [131,132] and indicate that asp/glu enrichment of the perfusate had no effect on myocardial energetics during normal perfusion.

**Table 3: High Energy Metabolites Measured by HPLC at the End of Stage I Reperfusion**

| | ATP (μmol/g dry wt.) | ATP (%total Cr) | PCr (μmol/g dry wt.) | PCr (%total Cr) |
|---|---|---|---|---|
| Group A (control) | 17.0±1.7 | 10.1±0.5 | 106±6 | 62.7±4.5 |
| Group B (asp/glu) | 15.1±0.6 | 9.4±0.6 | 104±7 | 63.8±2.9 |

ATP=adenosine triphosphate; PCr=phosphocreatine; Cr=creatine. Total creatine is equal to the sum of PCr and Cr. All data presented as mean ± standard error of the mean. There were no significant differences between groups ($p>0.05$).

## B) Intracellular pH

Myocardial intracellular pH (from MRS) during control perfusion averaged at 7.22±0.15 and 7.30±0.07 for the control and asp/glu groups, respectively, which was in good agreement with other studies using a similar model [78]. The changes in pHi during the protocols are shown in Figure 10 and summarized in Table 2. At the onset of ischemia, pHi in all hearts decreases rapidly during the first 10 minutes, followed by a slower rate of decline during the subsequent 20 minutes. As observed in the case of high energy phosphates, the pHi in both groups recovers quickly upon reperfusion. One-way ANOVA showed no significant differences between groups at any of the time points in Figure 10. This observation would indicate that the presence of an enriched extracellular source of

asp/glu did not reduce the severity of the ischemic insult or improve cellular recovery from this insult.

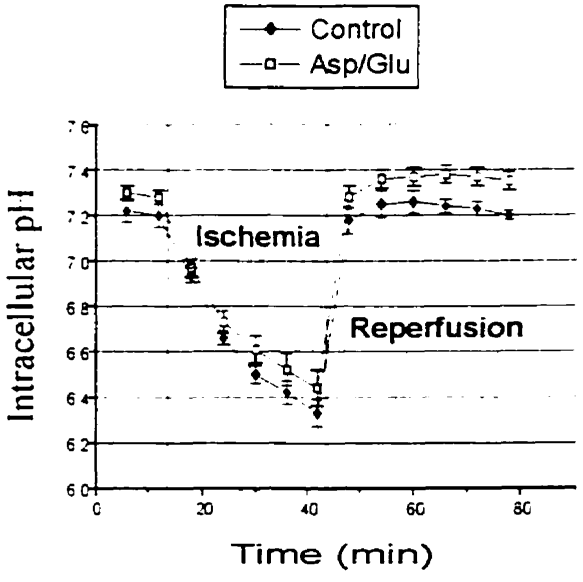


**Figure 10: Myocardial intracellular pH during ischemia-reperfusion. Each data point represents the average pH value over a six minute period. Data points represent means ± standard errors of the means. There were no statistically significant differences between the groups ($p>0.05$) at any time points.**

## C) Myocardial Function

The isolated blood-perfused pig hearts showed good LV function during control perfusion. The average pre-ischemic functional parameters were similar for the two groups (Table 4). The post-ischemic recovery of myocardial functional parameters is shown in Figure 11. A comparison of LV rate-pressure product (RPP), +dP/dt, and -dP/dt shows poor recovery of contractile function in all hearts, with no significant differences between the groups. Asp/glu enrichment of the perfusate did not affect myocardial function during control perfusion or improve functional recovery after ischemia.

**Table 4: Pre-ischemic Myocardial Function and Oxygen Consumption**

| | RPP (mmHg/min) | +dP/dt (mmHg/sec) | -dP/dt (mmHg/sec) | $MVO_2$ (ml $O_2$/100g/min) | RPP/$MVO_2$ (mmHg/ml) |
|---|---|---|---|---|---|
| Group A (control) | 10900±1300 | 1220±90 | 1040±100 | 5.5±1.4 | 1100±200 |
| Group B (asp/glu) | 12100±800 | 1200±90 | 1090±80 | 6.3±1.1 | 950±90 |

RPP=rate-pressure product; +dP/dt=rate of contraction; -dP/dt=rate of relaxation; $MVO_2$=myocardial oxygen consumption. All data are presented as mean ± standard error of the mean. There were no significant differences between the groups ($p>0.05$).

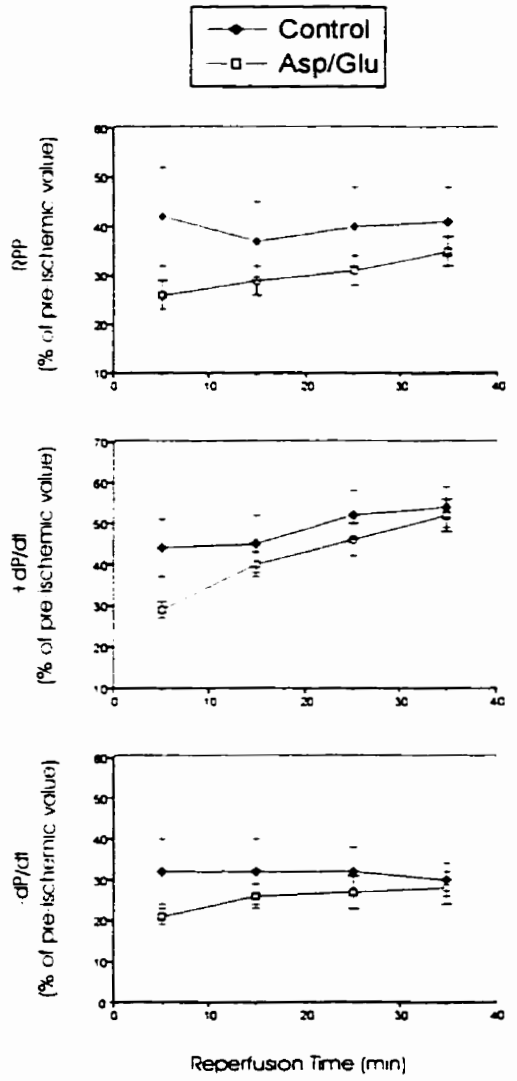


**Figure 11: Post-ischemic recovery of left-ventricular functional parameters. RPP=rate-pressure-product; +dP/dt=the rate of increase in systolic ventricular pressure; -dP/dt=the rate of decrease in diastolic ventricular pressure. Data points represent means ± standard errors of the means. There were no statistically significant differences between the groups (p>0.05).**

## D) Oxygen Consumption

As seen in Table 4, addition of asp/glu to the perfusate did not have a significant effect on pre-ischemic $MVO_2$ or the efficiency of conversion of chemical energy into mechanical

function ($RPP/MVO_2$). Furthermore, the post-ischemic recovery of these parameters (Figure 12) was similar in the two groups. These results give no indication that exogenous asp/glu can improve mitochondrial function during reperfusion.

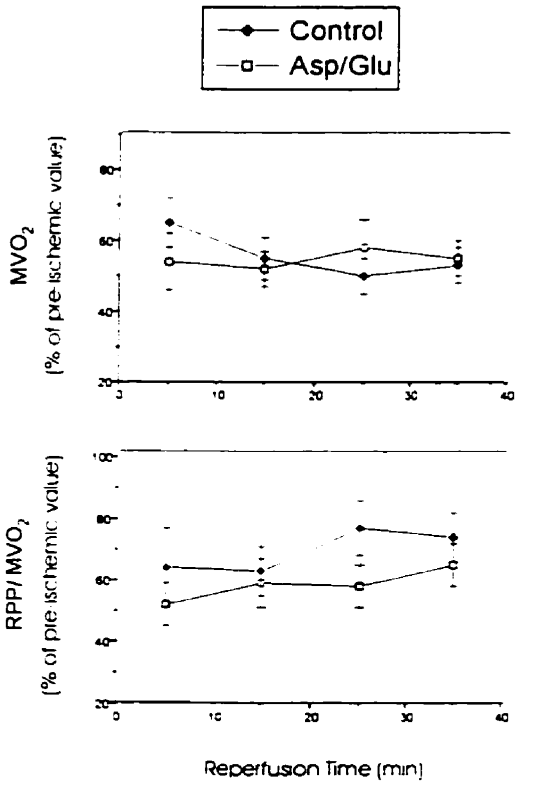


**Figure 12: Post-ischemic recovery of myocardial oxygen consumption ($MVO_2$) and the efficiency of conversion of chemical energy to mechanical work ($RPP/MVO_2$). The data points represent means ± standard errors of the means. There were no statistically significant differences between the groups ($p>0.05$).**

## E) Myocardial Edema

The total water content at the end of reperfusion was similar for the two groups ($p>0.05$), with dry/wet weights of 16.1±0.4% and 17.6±1.3% for groups A and B, respectively.

## 5.2 Study #1: Stage II

This section comprises hearts subjected to inotropic testing (n=4 total) after the normal reperfusion period. The perfusate [$Ca^{2+}$] in these hearts was increased gradually until myocardial function was saturated. The initial response to the addition of the $CaCl_2$ bolus was dramatic, with myocardial function quickly reaching well above control (pre-ischemic) levels. Functional parameters then declined slowly with decreasing [$Ca^{2+}$] caused by mixing of the perfusate, and stabilized at a level that was higher with each subsequent $CaCl_2$ bolus. The final [$Ca^{2+}$] varied between hearts (mean [$Ca^{2+}$] = 3.07 and 3.35 mmol/L for Groups A and B, respectively) but showed no significant difference among groups (p=0.33).

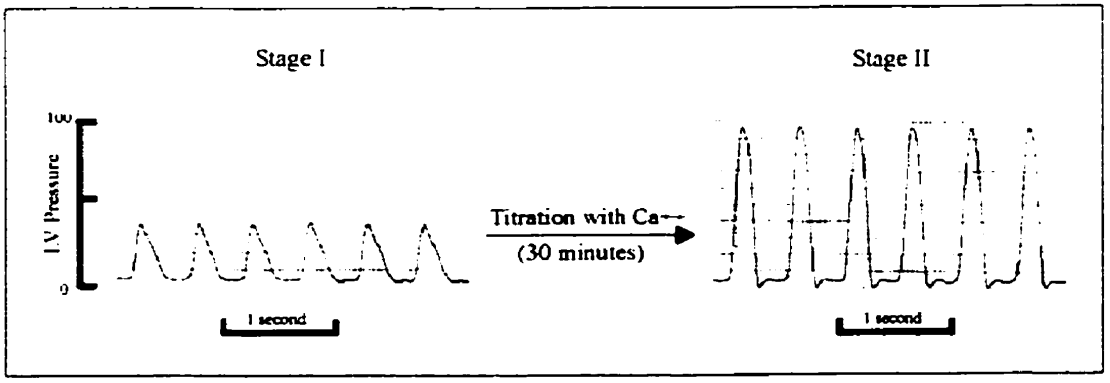


**Figure 13: Representative polygraph tracing showing myocardial function in post-ischemic hearts before (Stage I) and after (Stage II) inotropic stimulation.**

Figure 13 shows representative tracings of LV pressure in a heart before and after titration with $Ca^{2+}$. The tracings show that LV developed pressure as well as the rate of contraction and relaxation increased dramatically in stage II. The improvement in myocardial function from stage I to stage II is compared for the two groups in Figure 14. The Figure shows that after inotropic stimulation, myocardial rate-pressure product

(RPP), +dP/dt, and -dP/dt increased significantly ($p<0.01$) in both control and asp/glu hearts. The hearts showed dramatic recovery of functional parameters which was sustained for the duration of high $Ca^{2+}$ perfusion.

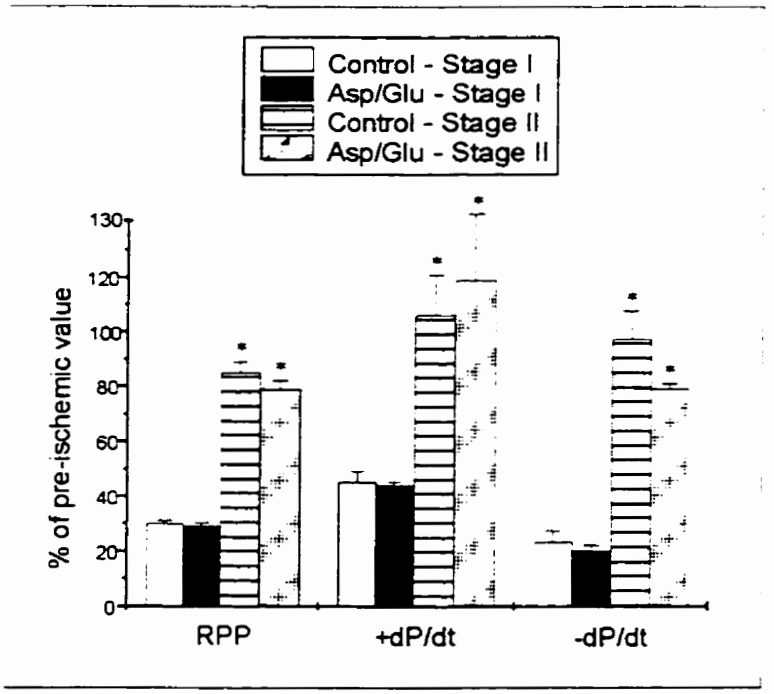


**Figure 14: Improvements in post-ischemic myocardial function after inotropic stimulation (stage II). The error bars represent standard errors of the means. *P<0.05 with respect to stage I.**

The high level of myocardial function observed during stage II was relatively stable for the entire duration of heart perfusion. The ability of the post-ischemic hearts to recruit and maintain near-complete recovery of LV function when stimulated suggests that the lack of adequate functional recovery during normal reperfusion was not limited by the energy supply. This statement is consistent with the observed changes in $MVO_2$ during stage II (Figure 15). $MVO_2$ in both control and asp/glu hearts improved considerably in stage II. Furthermore, the efficiency of conversion of chemical energy to mechanical function ($RPP/MVO_2$) also improved, which would explain the dramatic changes in

myocardial function. Although the improvements in $MVO_2$ and $RPP/MVO_2$ were significant only for the control group ($p<0.05$), the lack of significance in the asp/glu group is due to the small number of hearts. When the data for the inotropically tested control and asp/glu hearts are combined (n=4), $MVO_2$ (as % of control) is $52\pm7$ and $86\pm7$ ($p=0.01$) while $RPP/MVO_2$ is $59\pm7$ and $102\pm10$ ($p=0.01$) for stage I and stage II, respectively.

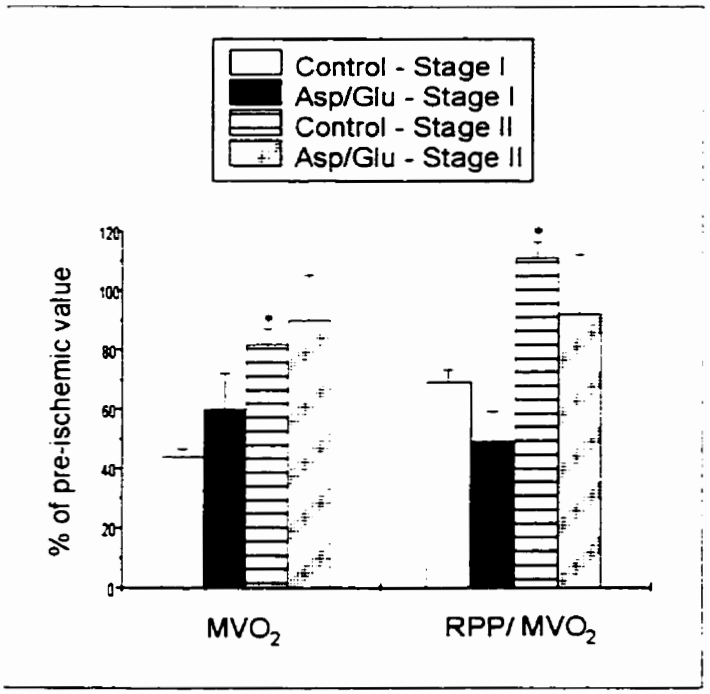


**Figure 15: Improvements in myocardial oxygen consumption ($MVO_2$) and the efficiency of conversion of chemical energy to mechanical work ($RPP/MVO_2$) with inotropic stimulation (stage II). The error bars represent standard errors of the means. *P=0.01 with respect to stage I.**

The improvement in $MVO_2$ and $RPP/MVO_2$ are not surprising when observing the energetic state of the hearts in stage II. Figure 16 shows the levels of myocardial energy phosphates in inotropically tested hearts (n=4) before and after titration with $Ca^{2+}$. The spectra show a decrease in PCr levels from $307\pm9\%$ in stage I to $273\pm8\%$ in stage II

($p<0.01$), but no significant changes in ATP ($66\pm3$ vs. $66\pm2$) or Pi ($144\pm5$ vs. $150\pm5$). These observations are consistent with biochemical data from stage I (n=8) and stage II (n=4), showing no significant differences in ATP/total Cr ($9.6\pm0.4\%$ vs. $10.7\pm0.4\%$, $p>0.05$), but a decrease in PCr/total Cr ($63.4\pm1.1\%$ vs. $54.8\pm2.7\%$, $p<0.05$). The decrease in PCr levels, however, does not indicate energetic stress in stage II hearts, as PCr was still higher than pre-ischemic levels (Table 2). Furthermore, the pHi in stage II was $7.23\pm0.02$, which is in good agreement with control values obtained in this ($7.25\pm0.11$) and other studies [78]. The maintenance of myocardial pHi negates significant anaerobic metabolism and further supports the hypothesis that the post-ischemic hearts were able to recruit sufficient aerobic respiration to meet the increased demands in stage II.

## 5.3 Study #2

The changes in phosphorus metabolites during ischemia and reperfusion have been quantified and are presented in Figure 17. As in study #1, all hearts showed a rapid recovery of Pi and PCr to near control values during reperfusion, but only slight recovery of ATP. The rate or extent of energetic recovery in post-ischemic hearts appears unaffected by the composition of the reperfusate. HPLC determination of high energy phosphates in the left ventricle confirms that the hearts were in similar energetic states after 40 minutes of reperfusion, with final ATP levels of $17\pm2$ vs. $16\pm1$ $\mu$mol/g dry wt and PCr levels of $106\pm6$ vs. $113\pm4$ $\mu$mol/g dry wt for Group A vs. Group C, respectively.

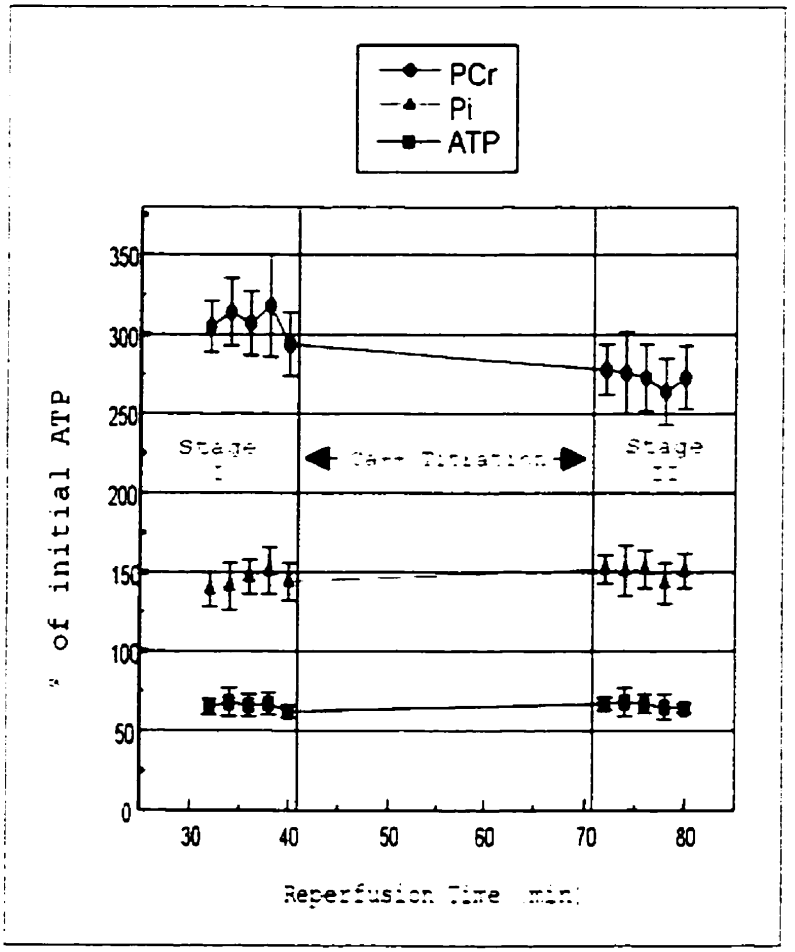


**Figure 16: Changes in myocardial energy phosphates after 30 minutes of inotropic stimulation (titration with calcium). ATP=adenosine triphosphate; PCr=phosphocreatine; Pi=inorganic phosphate. The error bars represent standard errors of the means.**

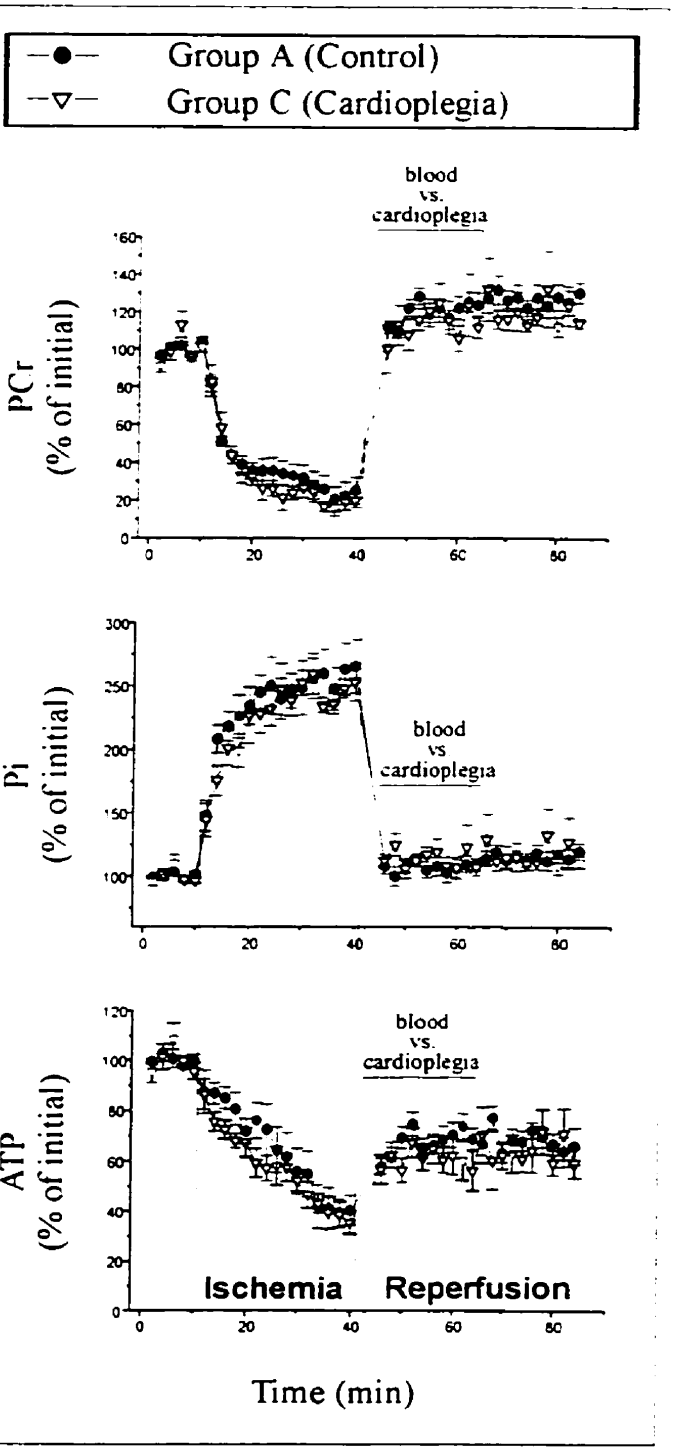


**Figure 17: Changes in myocardial energy phosphates during ischemia-reperfusion. ATP=adenosine triphosphate; PCr=phosphocreatine; Pi=inorganic phosphate. The time points represent mean metabolites over a two-minute period ± standard error of the mean.**

The changes in myocardial pHi for Groups A and C are shown in Figure 18. The pHi during control perfusion is estimated at 7.22±0.03, which agrees well with previous studies [78]. The pHi during ischemia declined rapidly to an average of 6.37±0.04 for all hearts. In Group A, the pHi recovered to 7.18±0.06 within 4-6 minutes of reperfusion. Cardioplegic reperfusion of Group C hearts caused an increase of pHi to above pre-ischemic values ($p<0.05$), reaching 7.49±0.03 within approximately 10-12 minutes of reperfusion. As shown in Figure 18, the higher pHi in Group C was maintained throughout 20 minutes of cardioplegic reperfusion, but declined to normal levels upon perfusion with normal blood. The difference in pHi between the groups was, however, no longer significant at 16-18 minutes of reperfusion.

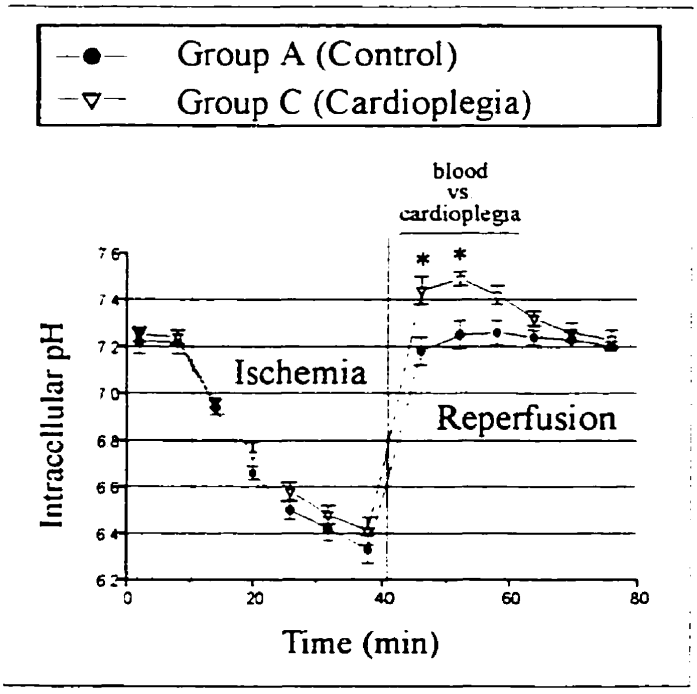


**Figure 18: Intracellular pH during ischemia-reperfusion. Each data point represents mean pH value over a six minute period ± standard error of the mean. *$p<0.05$ with respect to control hearts**

Reperfusion of the hearts with asp/glu cardioplegia did not affect post-ischemic recovery of myocardial functional parameters compared to control hearts (Figure 19). There were also no effects on the post-ischemic $MVO_2$ or on the RPP/$MVO_2$ ratio (Figure 20). In addition, the left-ventricular dry/wet weight ratio at the end of the protocols was similar for the two groups of hearts (16.1±0.4% and 16.3±0.4% for Groups A and C, respectively).

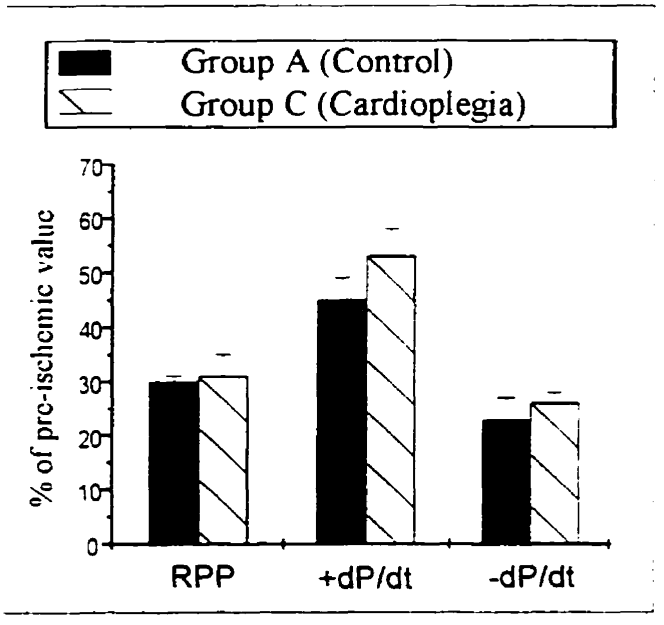


**Figure 19: Post-ischemic recovery of left-ventricular functional parameters. RPP=rate-pressure-product; +dP/dt=the rate of increase in systolic ventricular pressure; -dP/dt=the rate of decrease in diastolic ventricular pressure. The error bars represent standard errors of the means. There were no statistically significant differences between the groups.**

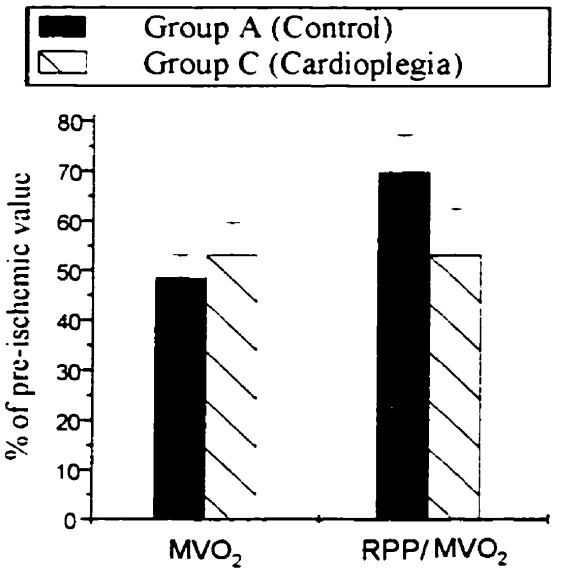


**Figure 20: Post-ischemic recovery of myocardial oxygen consumption ($MVO_2$) and the efficiency of conversion of chemical energy to mechanical work ($RPP/MVO_2$). The error bars represent standard errors of the means. There were no statistically significant differences between the groups.**

# 6. DISCUSSION

## 6.1 *Interpretation of the results*

### A) The effects of asp/glu on energy metabolism during ischemia-reperfusion

This study investigated the effects of an enriched exogenous source of asp/glu on normal, isolated blood-perfused pig hearts subjected to a period of normothermic ischemia. The results showed no beneficial effects of these amino acids on myocardial energy levels or intracellular pH before, during, or after ischemia. Furthermore, there were neither any effects on myocardial function nor on oxygen consumption at anytime during the experimental protocol.

The potential mechanisms for beneficial effects of asp and glu were previously reviewed. The hypothesis in this study was that the suggested mechanisms should improve the energy profile of the myocardium during either ischemia or reperfusion, and that continuous observation of high energy phosphate levels should thus detect any beneficial effects of asp/glu. Hearts in this study were subjected to 30 minutes of total normothermic ischemia, resulting in extensive depletion of high energy phosphates and poor recovery of $MVO_2$ and contractile function. The severity of ischemia is confirmed by the significant decline in intracellular pH. Therefore, the choice of ischemic insult was such that improvements in functional or metabolic parameters with asp/glu should have been detected.

Both groups of hearts showed minimal recovery of ATP after ischemia, but rapid recovery of PCr to above normal levels (Table 2). While the lack of recovery of ATP to normal levels is most likely due to a reduction in the adenine nucleotide pool (see section 3.2-B), the rapid recovery of PCr during early reperfusion suggests that mitochondrial function was well-preserved [36], despite the poor recovery of contractile function and $MVO_2$. This is further supported by the response of the hearts to inotropic stimulation. During stage II reperfusion, the hearts showed significant recovery of myocardial function and $MVO_2$, with good maintenance of high energy phosphates. In addition, there was significant improvement of $RPP/MVO_2$, which is an indicator of the efficiency of conversion of chemical energy to mechanical function [131]. These changes suggest that the poor contractile function and $MVO_2$ during stage I reperfusion were due to an inability of the contractile machinery to use the available chemical energy, possibly because of problems with calcium handling [131]. These results show that lower post-ischemic $MVO_2$ does not necessarily indicate lack of potential for efficient aerobic respiration, and poor myocardial function is not necessarily due to energy depletion. Therefore, the use of agents (such as asp/glu) intended to improve aerobic respiration during reperfusion may be misguided in instances when poor contractile function or $MVO_2$ do not represent an inability to produce energy. Indeed, of the mechanisms suggested to be responsible for the beneficial effects of asp/glu, the often quoted replenishment of Kreb's cycle intermediates and improved $MVO_2$ are those for which there is the least evidence [109].

## B) Asp/glu enriched secondary blood cardioplegia for myocardial resuscitation

Buckberg's cardioplegic solutions have a number of features designed to prevent reperfusion injury, as previously discussed. The creators of the solution have frequently commented that the solutions were meant to be used as designed, and that all the components must be present for the benefits to be seen. Thus, the purpose of this second study was to test the components as they are used clinically. The results have failed to confirm any benefits of using the "warm reperfusate" cardioplegic solution for reperfusion of ischemic pig hearts.

The warm reperfusate cardioplegic recipe was chosen because a recent report suggested it may be useful for reversal of myocardial stunning [9]. The inotropic stimulation of hearts in study #1 has satisfied the definition of myocardial stunning by showing reversal of the functional and metabolic ($MVO_2$) dysfunction. However, no reversal of this stunning was observed in the cardioplegic reperfusion group before inotropic stimulation.

The characteristics of the cardioplegic solution were: asp/glu as substrate, low $Ca^{2+}$ to reduce $Ca^{2+}$ overload, high $K^+$ to maintain electromechanical arrest, alkaline pH to reverse acidosis, and high osmolarity to reduce edema. Asp/glu were discussed in the previous section. Although the extent of $Ca^{2+}$ overload and the adequacy of electromechanical arrest could not be evaluated in this study, intracellular pH and tissue edema were measured. The results provided no evidence that the high osmolarity of the cardioplegic solution actually reduced tissue edema. Intracellular pH, however, was

clearly elevated during the 20 minute cardioplegic reperfusion, but no lasting effect was seen on myocardial function or metabolism.

The above results are consistent with a previous study by Tian and associates that used high buffer cardioplegia in pig hearts for long-term hypothermic storage [133]. In that study, high buffer cardioplegia increased intracellular pH during arrest, but had no effect on high energy phosphates or function after reperfusion. These studies suggest that acidosis may in fact not be harmful under some circumstances. Indeed, acidosis depresses cardiac contractility [21] and may help preserve ATP. Furthermore, acidosis may actually improve the capacity for ATP resynthesis during reperfusion by $H^+$ inhibition of 5'-nucleotidase during ischemia, reducing AMP breakdown and thus improving preservation of the adenine nucleotide pool during ischemia [35]. The study by Tian and associates also found that a dose of secondary cardioplegic solution before reperfusion reduced the incidence of ventricular fibrillation and improved contractile function, but had no effect on high energy phosphates [133].

## 6.2 Evaluation of Methodology

There are several shortcomings to this work that may affect application of the results clinically. Some of these limitations are discussed below.

The pig heart was chosen for this study because, with the exception of primates, it most closely resembles the human heart in cardiovascular function, size, anatomy, coronary distribution, collateral circulation, enzyme spectrum, and conduction systems [134-139]. Nevertheless, most other studies evaluating asp/glu have been done in dogs, rats, and

rabbits. These species have higher metabolic rates than pigs and human hearts, and there may also be differences with respect to membrane permeability to asp/glu or transaminases needed for asp/glu incorporation into biochemical pathways. These factors could all contribute to the differences observed in the other studies, and particularly studies from Buckberg's group which always used dog hearts.

One factor that may have affected the results is the use of a Langendorff heart preparation. A working heart models may have had higher energy demands, which may in turn have provided a better baseline for asp/glu enrichment to show any beneficial effects on energetics. Furthermore, some have cautioned against extrapolation of data regarding myocardial stunning from isolated hearts to intact animals, in light of the short-lived nature of these experiments and the difficulty in assessing cellular viability [28]. In addition, the cold cardioplegic method of heart isolation that was used may in fact have led to some ischemic preconditioning, which may in turn have protected all hearts to such an extent that any protective effects of asp/glu were undetected. Lastly, the hearts used for this study were initially normal hearts. Diseased hearts have been suggested to have altered metabolism of asp/glu [140] and thus could respond differently to asp/glu enrichment.

The MR probe used for this study observed the whole-heart probe and not specific regions of the heart. It is possible that asp/glu could have had some effects on specific small regions of the heart, but the effect was not large enough to be detected in a spectrum from the whole heart. Furthermore, although there is good evidence that determination of ATP levels by $^{31}P$ MRS is equivalent to HPLC in accuracy [141,142],

MRS on whole hearts cannot address the issue of compartmentalization of ATP. For instance, $^{31}P$ MRS cannot distinguish between cytosolic or mitochondrial ATP, but asp/glu may stimulate production of ATP via substrate-level phosphorylation in the mitochondria and thus protect the mitochondria without significant effects on overall cellular energetics [100]. Lastly, $^{31}P$ MRS was unable to distinguish between intracellular and extracellular phosphate, which may lead to inaccuracies in the measurements.

Functional recovery might have been better evaluated with a load-independent parameter such as e-max [31]. However, attempts at doing pressure/volume curves would have increased the time required for the protocols and may have affected shimming of the NMR magnetic field, which is the reason they were not done. As NMR was the most important aspect of this study, the model and protocol were adjusted to best suit the collection of the NMR data.

It's possibe that the heart model used here may have been unstable, and there may have been a decline in function had hearts been subjected to an extended perfusion period without ischemia. Although no such experiments were done as part of this work, a previous study assessed the stability of our beating, isolated, blood-perfused pig heart model over a period of 150 minutes [78]. Despite a slight gradual decrease in functional parameters over time, the hearts in that study showed reasonable stability over this extended perfusion period. Furthermore, there were no changes in high energy phosphate levels during the 150 minutes. These results suggest that our model, in and of itself, is unlikely to have caused the serious level of functional or metabolic injuries that would preclude a useful assessment of myocardial protective agents during ischemia.

## 6.3 The Aspartate/Glutamate Controversy

Since the introduction of crystalloid cardioplegia during the early 1970's, there has been a great deal of development that has significantly improved the surgeon's ability to manage intraoperative myocardial damage. Unfortunately, the rapid development of new, exciting techniques has also lead to much disagreement and controversy over which methods provide the most protection. There is currently a large number of different cardioplegic solutions and protocols, none of which is universally accepted. This lack of agreement reflects the differing needs of various clinical cases, the lack of a clear understanding of cellular changes resulting from cardioplegia, and the significant difficulties in conducting cardioplegic research.

The existence of so many controversies in the field of intraoperative myocardial protection is largely due to the difficulties in studying these techniques objectively. It is practically impossible to design an experimental model that accurately reflects the clinical situation but is still practical from a technical and ethical point of view. Not only are there significant species differences between humans and most common laboratory animals, but the fact that most patient hearts are diseased hearts further complicates the issue. It is difficult to design a model that accurately reflects the many disease conditions observed clinically as well as the individual differences between patients. Furthermore, there is an inability to mimic various types of ischemia accurately, and the parameters that are most objectively measured are often not the most applicable clinically. All these factors have led to the development of many different experimental models, which

complicates comparisons between various studies and leads to uncertainty, controversy, and an inability to apply experimental results to the clinical situation.

Buckberg and associates have carried out hundreds of studies over the past three decades regarding almost every aspect of cardiac surgery. Many of these studies have been parts of series that systematically addressed one question or another, such as the series on controlled reperfusion after ischemia [143]. Of these studies, well over a hundred have been published since their much advertised asp/glu enriched cardioplegic solution was put into regular use. However, only a few of the early studies provided any scientific evaluation of the individual components of Buckberg's cardioplegic solution, such as pH, osmolarity, $[K^+]$, $[Ca^{2+}]$, [glucose], and in particular the use of asp and glu. Even in those studies, the models are often complicated, with much potential for confounding factors, including coincident changes in ionic composition as previously discussed. Since adopting the formulation of their cardioplegic solution as the standard solution used, not a single randomized trial has been performed to show the superiority of asp/glu cardioplegia, with many clinical studies either having no control group [9] or using historical control groups [8,10]. The unwillingness of many investigators to conduct randomized trials is understandable due to the ethical dilemma of subjecting patients to techniques which the investigators believe may be inferior, solely for the purpose of research. Nevertheless, such unwillingness is largely responsible for many of the unresolved controversies in the field.

Although the majority of studies on asp/glu seem to support the concept that they may be beneficial during ischemia-reperfusion, it is my belief that many of these studies have

been affected by the apparent overwhelming amount of evidence, particularly from Buckberg's group, advocating the use of asp/glu clinically. Indeed, the experimental design in most studies seems to assume that asp/glu improve energy production, as recently noted by Suleiman and colleagues [144]. The current study used $^{31}P$ MRS as a tool of choice for observation of myocardial energy metabolism. $^{31}P$ MRS, however, did not give any insight into the metabolic reactions involving asp/glu and whether or not they were in any way affected. Such information could have been provided by carbon-13 ($^{13}C$) magnetic resonance spectroscopy, which is well-suited to the study of myocardial metabolism, without the need for assuming metabolic or isotopic steady state [145]. Jessen and coworkers [102] recently used $^{13}C$ isotopomer analysis to study the effects of 5 mmol/L asp/glu on substrate selection and anaplerosis in rat hearts subjected to 25 minutes of total normothermic ischemia. The results showed no effects of asp/glu on functional recovery, substrate selection, or anaplerosis. In fact, they could not detect any metabolism of $^{13}C$-labeled asp or glu. These observations are consistent with the results of the current study and suggest that under certain conditions, exogenous asp/glu may not be able to enter the cell. Additional support for this possibility comes from another study in isolated pig hearts in which there was a net release of glu during reperfusion of hearts subjected to cold cardioplegic ischemia [146]. Suleiman and colleagues [144] observed a similar drop in both asp and glu levels during reperfusion in patients subjected to cold blood cardioplegic arrest. They suggested that $Na^+$ accumulation during ischemia may have been responsible for the decrease in asp/glu becauseas the transport of these amino acids appears to be $Na^+$ dependent.

Unlike many other amino acids, very little is known about the kinetics of asp and glu transport in cardiomyocytes. Recent reports by Dinkelborg et al have provided some insight into glu transport in isolated rat cardiomyocytes [147-149]. First, glu appears to be actively transported into cells, as apparent from the large gradient across the sarcolemma: [glu] in rat blood is 0.1 mmol/L while [glu] inside myocytes is ~3 mmol/L. These numbers are comparable to those obtained by other investigators [144]. Secondly, glu transport appears to occur via a high affinity/low capacity system and a low affinity/high capacity system that did not seem to saturate at concentrations of up to 10 mmol/L. Thirdly, glu levels seemed to drop rapidly during a period of anoxia, despite increased glu uptake from the medium. Upon reoxygenation, however, glu levels returned rapidly to normal values. Interestingly, only 2.4% of the glu increase after reoxygenation was from increased glu uptake, suggesting that myocytes replenish glu mainly from intracellular sources such as proteolysis. Further evidence for this hypothesis was provided when competitive inhibition of transaminases using aminooxyacetate prevented both the observed intracellular decrease in glu during anoxia and the increase in intracellular glu after reoxygenation [148].

Although few studies have demonstrated any negative effects of asp/glu on the myocardium, it is still important that a clear benefit be demonstrated before using these agents in cardioplegia, as there are potential risks associated with their use. Both asp and glu are excitatory neurotransmitters and are neurotoxic at sufficiently high concentrations [14]. Although delivery of cardioplegia is theoretically isolated from the systemic circulation, in practice some leakage of cardioplegic solution into the circulation is unavoidable. Therefore, usage of asp/glu in cardioplegia may pose a risk, especially in

hypoxic/ischemic conditions when these neurotransmitters may accumulate. This results in increased cellular permeability to $Na^+$ and $Ca^{2+}$-mediated cell death [14]. In addition, the delivery of asp/glu cardioplegia brings increased complexity to the perfusion techniques and thus increases the risk of complications. Furthermore, as with any new technique, the increased expense associated with its use must be justifiable.

## 6.4 Summary

Stunned myocardium can and does occur after cardiac surgery using both normothermic and hypothermic cardioplegia [49]. Thus, the development of methods of myocardial protection or reperfusion that can reduce post-ischemic dysfunction is important in reducing post-operative morbidity and mortality. The continued controversy surrounding the concept of substrate enhancement in cardiac surgery indicates the need and desire to devise better ways of reversing pre-operative myocardial injury, while minimizing intra-operative insult. The conflicting results of experimental studies over the past decade, nevertheless, raise questions concerning the use of such controversial agents in the clinical setting, where the possibility of adequate controls is further reduced. A solution to the controversy can only come from a better understanding of fundamental concepts. More basic research is needed to clarify issues such as details of the kinetics of asp/glu transport into cells and interactions of various experimental conditions, such as composition of the perfusate, with these kinetics. In addition, any species differences in asp/glu transport or in other relevant factors, such as transaminases required for incorporation of these amino acids into various metabolic processes, must be determined. Such information would help explain the conflicting results obtained from different

experimental designs, and facilitate application of experimental findings to the clinical setting.

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# IMAGE EVALUATION
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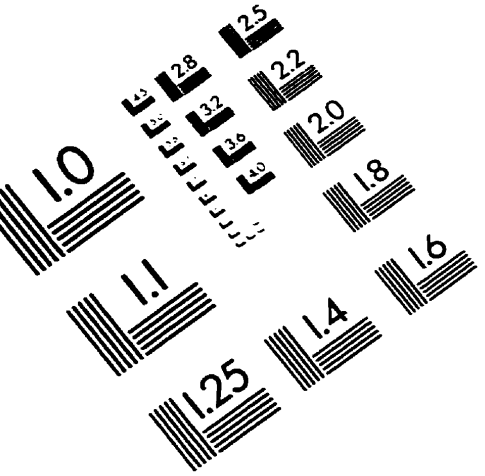


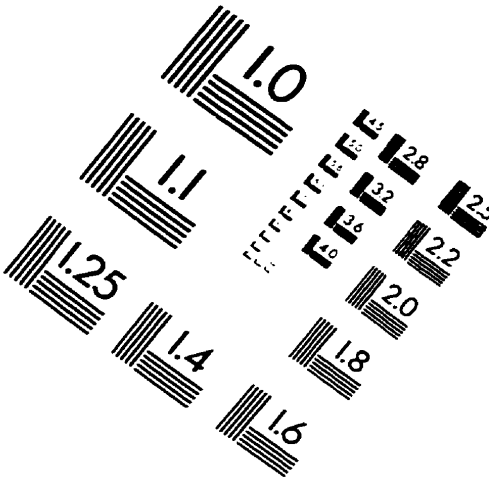


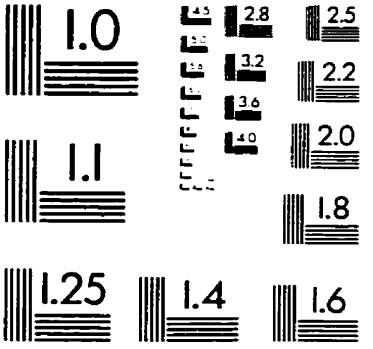


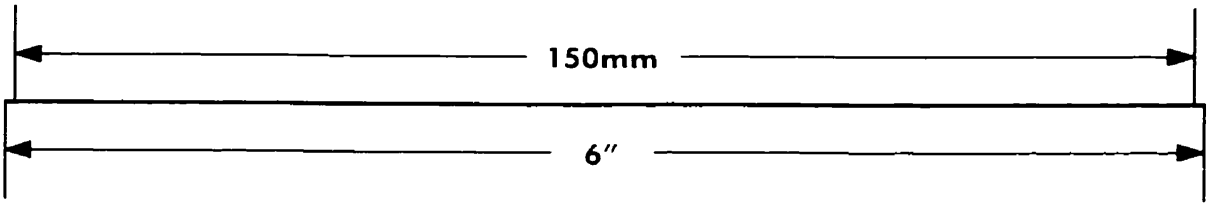


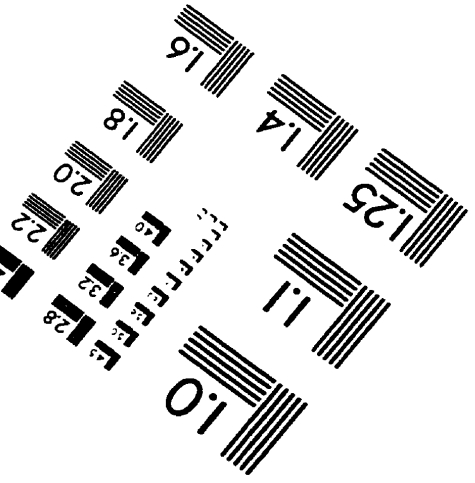


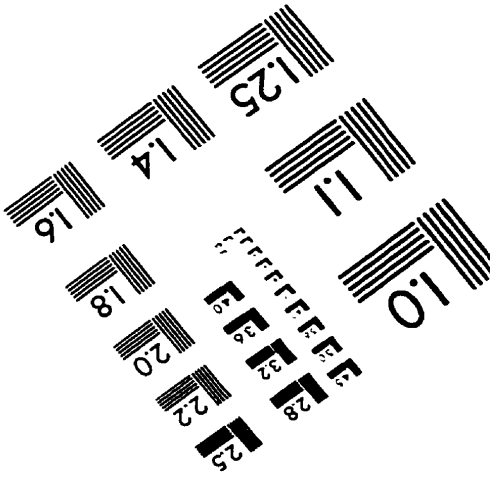


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