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IMPROVING TISSUE ENERGETICS IN COLD-STORED PORCINE HEARTS THROUGH ENHANCED BUFFERING OF PRESERVATION SOLUTIONS

By

Randy P. Pulis

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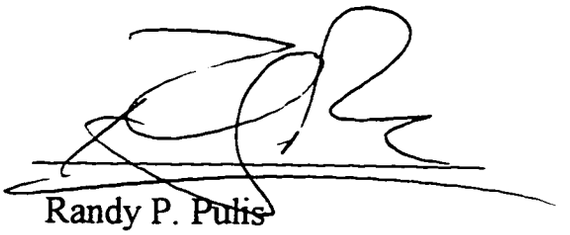

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Dedication

For Eleni and Nicholas who provide
the inspiration and support that I can always count on.
Με Αγαπη

Abstract

Two studies were undertaken to investigate the effects of enhanced buffering of flush-storage preservation solutions on glycolytic flux and tissue energetics in cold-stored ischemic pig hearts. In the first study, we hypothesized that increased buffering of preservation solutions would increase glycolytic flux during the cold ischemic period, and would therefore allow for better maintenance of the high energy compounds PCr, ATP, and the total adenylate pool within the cardiac tissue. Hearts from Landrace-Yorkshire pigs (35 -40 kg) were flushed and stored at 4°C for 10 hours with one of five solutions: St. Thomas Hospital solution II (STHS II) which served as control ; modified UW solution; and three other solutions comprised of modified UW solution plus one of 90mM histidine, bicine, or BES. Tissue energetics and anaerobic metabolism were assessed through assays of PCr, ATP, ADP, AMP, lactate and glycogen. Evidence of anaerobic metabolism was demonstrated by lactate accumulation in the tissue over the 10 h time course. All experimental groups, showed significantly greater lactate accumulation than the control group (STHS II) after 2 h of storage; by 10 h, values were 11.4 - 15.8 μmol/g higher ($p<.05$) in the enhanced buffer groups reflecting an increase in glycolytic flux of greater than 100%. Higher ATP and total adenylate levels after 10 h of cold storage ($p<.05$) were observed in all experimental groups except BES when compared to control; this increase in energetics positively correlated with the increase in glycolytic activity. Tissue energy charge, which ranged from 0.91 - 0.92 in the experimental groups at 10h were also significantly greater than control (0.85 at 10 h) ($p<0.05$). The data from this study suggests that enhanced buffering of cardiac flush/storage solutions results in increased glycolytic energy production with better

maintenance of cellular ATP and total adenylate levels during ischemic cold preservation. The finding that total buffering capacity did not linearly correlate with tissue energetics after 10 h suggests that under static storage conditions, the accumulation of end-products such as lactate may become an inhibitor of glycolysis independent of any pH changes.

In the second study, we studied the effects of solution buffering combined with an immediate 1 h period of continuous perfusion with oxygenated solution on tissue energetics. The effects of adding glucose plus insulin as substrate were also investigated. Four different solutions were used: STHS II (control), a modified UW solution, modified UW + 90 mmol/l histidine, and UW + 90 mmol/l histidine + 11.1 mmol/l glucose + 100U/l insulin (HGI). Similar to the results of the first experiment, enhanced buffering in the two histidine solutions resulted in stimulated glycolysis as evidenced by significant increases in lactate production in these two groups over 10 h of cold storage ($p<0.05$). Lactate accumulation during the perfusion period was negligible in all groups, suggesting that this end-product was either washed out of the tissue, or some residual oxidative metabolism was continuing because the perfusate was being oxygenated. Beyond 2 h of cold-ischemic storage, the histidine group was the only experimental group to show differences from control in terms of ATP, and total adenylates ($p<0.05$). Although glycolytic flux was stimulated in the HGI group, this was not reflected in greater ATP production, likely because glycogenolysis was decreased by the presence of glucose resulting in a lower yield of ATP per unit of substrate. This study supports the first study in providing evidence that buffering increases glycolytic energy production. The addition of glucose to the preservation solution may negate the benefit of buffering by

switching the substrate supply away from glycogen, which has a higher ATP yield per unit than exogenous glucose.

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List of Abbreviations

ABBREVIATION	FULL NAME
ADO	Adenosine
ADP	Adenosine Diphosphate
AMP	Adenosine Monophosphate
ATP	Adenosine Triphosphate
BES	N,N-bis[2-Hydroxyethyl]-2-aminoethane-sulfonic acid
Ca^{++}	Calcium
Ca^{++}_{I}	Calcium (Intracellular)
cAMP	Cyclic Adenosine Monophosphate
CCrP	Cyclocreatine Phosphate
CO_2	Carbon Dioxide
ECS	Euro-Collins Solution
G6PDH	Glucose 6-Phosphate Dehydrogenase
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
H^+	Hydrogen Ion
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HES	Hydroxyethyl Starch
HGI	Histidine-Glucose-Insulin Solution
HLR	Histidine-Lactobionate-Raffinose Solution

HTK	Histidine-Tryptophan-Ketoglutarate Solution (Bretschneider's)
IAA	Iodoacetate
IVC	Inferior Vena Cava
K^+	Potassium
KH	Krebs-Henseleit Solution
KH_2PO_4	Potassium Phosphate
Mg^{++}	Magnesium
MOPS	3-[N-Morpholino]propanesulfonic acid
N_2	Molecular Nitrogen
Na^+	Sodium
Na^+_I	Sodium (Intracellular)
NAD^+	Nicotinamide Adenine Dinucleotide (Oxidized Form)
NADH	Nicotinamide Adenine Dinucleotide (Reduced Form)
$NaHCO_3$	Sodium Bicarbonate
O_2	Molecular Oxygen
PCr	Phosphocreatine
PFK	Phosphofructokinase
P_i	Inorganic Phosphate
PIPES	Piperazine-N,N′-bis[2-ethanesulfonic acid]
PKC	Protein Kinase C
PLC	Phospholipase C

SGF	Silica Gel Fraction Solution
STHS	St. Thomas Hospital Solution
SVC	Superior Vena Cava
TRIS	Tris[hydroxymethyl]aminomethane hydrochloride
UW	University of Wisconsin Solution

Chapter I: Introduction

The concept of organ transplantation from one individual to another, or from one species to another is not a new one. Sanskrit text of India from the second and third century BC describes the autotransplantation of pedicled grafts from the forehead, neck and cheek to restore mutilations of the nose, ear, and lip [1]. Greek legend from 285 to 305 A.D. holds that Cosmos and Damian, the patron saints of medicine transplanted the leg of a recently dead Moor to a patient whose leg had been amputated. Modern transplantation began with the experiments of John Hunter who in the 18th century transplanted a human tooth to a cock's comb, and later a hen's spur to a cock's comb [1].

In the years following the initial experiments of Hunter, many obstacles remained to successful organ transplantation that medical and surgical science has only been able to overcome within the last century. In the late 1800's Lister described techniques of proper asepsis which allowed for a decrease in the infection rate. At the turn of the century, Carrel and Guthrie pioneered the technique of proper vascular anastomosis using fine continuous suture which penetrated all layers of the vessel wall. The problem of revascularizing explanted organs through effective vascular anastomosis without thrombosis, stenosis, and hemorrhage was therefore overcome, making solid visceral transplants possible [1].

While the rejection of allograft transplants remains a problem in modern transplantation, it is no longer an absolute barrier to success. Over the past 40 to 50 years, our knowledge of immunology and its role in tissue rejection has grown

tremendously. This has allowed for the development of pharmaceutical agents capable of suppressing the immune response to allografted tissue, and for devising strategies such as tissue typing which has lessened its incidence. While rejection has not been eliminated, the incidence has been reduced to acceptable levels, and when it does occur, it can in most cases be dealt with successfully.

The ultimate goal of organ transplantation is extending the life of patients whose life expectancy would otherwise be measured in weeks or months, as well as improving the quality of life. This applies to many people who suffer from end-stage disease of liver, kidneys, lungs, and heart. In North America, heart disease assumes a particularly prominent position because of its widespread prevalence, and transplantation for end-stage heart failure offers a form of surgical intervention that in many cases is the only hope of extending life significantly with excellent prospects for rehabilitation [2]. While the concepts outlined in the following discussion deal mainly with cardiac transplantation, many of them are also applicable in the setting of transplantation of other solid viscera such as liver, kidneys, pancreas, bowel, and lungs.

The first reported cardiac transplant was performed in 1905 by Carrel and Guthrie who heterotopically transplanted a canine cardiac allograft into the neck of a recipient dog [2]. With the development of cardiopulmonary bypass in 1953 by Gibbon, the groundwork necessary for orthotopic cardiac transplantation was laid, and in the 1960's, Shumway and Lower at Stanford University clearly demonstrated that a denervated dog heart could support a canine recipient's circulation when orthotopically transplanted [2]. Based upon the experiments of the Stanford group, Barnard performed

the first human orthotopic heart transplant in South Africa in December of 1967. Despite the fact that his patient died of gram negative sepsis less than 3 weeks later, this landmark event served as a catalyst for many other surgeons who collectively performed over 100 transplants in more than 60 centres in the next year [2]. Results were generally poor secondary to problems with graft rejection and immunosuppression. However, in the early 1980's cyclosporin was introduced and was subsequently shown by the Stanford group to improve survival with decreased morbidity in patients undergoing cardiac transplantation. By the end of 1984, 350 cardiac transplants had been performed, and currently the one-year survival of recipients exceeds 80% [2]. Cardiac transplantation has become a very real option in the treatment of end-stage cardiac disease.

D'Alessandro *et al* [3] have summarized the important advances in the last 30 years that have contributed to the enormous success of transplantation of many organs including the heart. They cite four factors: improved surgical techniques, improved immunosuppression, increased willingness of families to donate the organs of deceased loved ones, and the development of methods to successfully preserve organs. At present, the supply of hearts still falls far short of demand. With this in mind, the immense importance of optimal organ preservation in transplantation becomes very apparent. At present, cardiac preservation techniques are not at optimal levels. While safe storage of organs such as the liver and pancreas have seen a doubling of safe storage time over the past decade, the safe storage time limit of hearts remains only 4 to 6 hours. Improvement of cardiac graft preservation will benefit cardiac transplant

programs in several way. First of all the time available for graft transport between donor and recipient hospitals would be increased thus allowing for organ procurement from more distant centres. This would increase the donor pool and would therefore help lessen the problem of organ availability. Increased storage times would also allow for improved tissue matching, which could lead to less problems with rejection, and would also allow for better preparation of the recipient and the surgical team. Lastly, better organ preservation should have favorable impact on post-operative graft function which should make the post-transplant period more uneventful and cost effective. This is of benefit to both the transplant program, and the patient [3].

Cardiac preservation techniques that have developed over the past 30 years or so have a strong foundation in experimental studies. Much progress has been made in understanding the pathophysiology of cold ischemia, and its role in cellular injury. While we are still quite distant from fully understanding all of the mechanisms involved in cellular injury during warm and cold ischemia, researchers have provided a clearer understanding of some of the disturbances involved that lead to cell damage and death. For example, the ionic disturbances that occur during ischemia, particularly those involving calcium, sodium, and protons, are now recognized to play a significant role in injuring a cell. Understanding how cells are damaged during cold storage of a heart has allowed many investigators to devise strategies to minimize these pathophysiologic processes and to enhance organ function and survival for longer periods, the benefits of which have been outlined in the previous discussion. The discussion which follows will summarize and review the work of these investigators who have contributed to our

understanding of cardiac injury during preservation, and also those who have developed approaches to enhance cardiac preservation.

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Chapter II: Review of the Literature

Overview on Cardiac Preservation

Optimizing preservation techniques for the heart is critical, perhaps more so than for any other transplantable organ. The reason for this is that the recovery period for the heart after transplantation is extremely short. The heart must be fully capable of supporting the patient's circulation immediately following transplantation with very little tolerance for compromised function. Patients with transplanted kidneys can be supported with dialysis until recovery is optimized, and even the liver has several hours to resynthesize energy stores, correct tissue edema, and reestablish normal electrolyte concentrations [1]. In contrast, the heart must have an immediate and continual supply of high energy compounds, and it must be able to regulate calcium flux needed for normal excitation-contraction of the myocardium. The most effective way of accomplishing this is to ensure that the best possible preservation techniques are fully utilized. There are certain fundamental principles of effective organ preservation that apply to the heart as well as all other transplantable viscera.

The most widely utilized method currently for heart preservation is cold storage at 4°C. The appropriate use of hypothermia is a key for successful organ preservation. If an organ is rendered ischemic at warm temperatures, irreversible damage occurs after about 1 hour [1]. If the tissue is cooled however, metabolism is slowed, and the demand for oxygen and other metabolites is greatly reduced with conservation of chemical energy [2]. During procurement of the cardiac graft, rapid core cooling by vascular flushout is an essential factor in successful preservation. This considerably extends the

amount of time that the heart can withstand anoxia or ischemia [1]. The exact mechanism by which hypothermia is protective is not fully understood. Both warm and cold ischemia have been shown to results in rapid ATP depletion [3], yet organs such as the kidney are more viable by a factor of 6 to 72 times that of organs exposed to warm ischemia [1, 3]. Other factors such as intracellular pH drop caused by proton production and lactate accumulation also occur in the hypothermic state. Therefore, the suppression of ATP loss and pH decrease alone by hypothermia does not completely explain why hypothermia is protective [1].

Other key issues in successful cardiac preservation, as with other organs, are the prevention of cellular edema secondary to ischemia, maintenance of normal cellular ionic compositions, prevention of critical intracellular acidosis, and optimizing anaerobic energy resources [4]. Clearly, the problems associated with low temperature organ preservation cannot be attributed to a single mechanism, and each of these important factors will be discussed in detail in subsequent sections. As a result of the obvious complexities involved in designing an 'ideal' organ preservation solution, any novel preservation solution should be carefully formulated so that all of these important factors will be beneficially affected.

Baumgartner demonstrated in 1962 that simple cold saline flushout could yield successful preservation for as long as 5 to 7 hours [5]. Flushing out the organ is an important step in preserving it for transplantation, the major impact of which is removing residual red cells from the circulation during storage. As demonstrated by Weed [6] ischemic red cells become rigid, and could block the organ's microcirculation resulting in

the no-reflow phenomenon upon reperfusion [7]. Over the years since that time, many investigators have devised solutions in an effort to extend the safe preservation period, achievable by flush and cold storage. In the early and mid 1980's heart transplantation for end-stage cardiac failure became a standard treatment due to the development of improved immunosuppression. The common preservation solutions used at that time were intracellular type solutions with Na^+, K^+, and Ca^{++} concentrations similar to that found in the intracellular space [8]. This approach to organ preservation was pioneered by Collins in 1969 [9] for kidney preservation with the idea that the driving force behind Na^+-loading and K^+-depletion is the ionic gradient that exists between intracellular and extracellular fluid, and that these ionic changes can be minimized by using an appropriate solution that mimics the intracellular space. As discussed in the following section on ionic disturbances during ischemia, this is important because ischemic damage to organs is linked to these ionic changes, and it is therefore beneficial to minimize them as much as possible. Some of these earlier efforts are summarized in Table II-1.

Table II-1. Early attempts at preservation of the heart with intracellular-type flush storage solutions [1].

Solution	Duration	Function	Reference
Krebs Collins Sacks	26 hours	Orthotopic transplant; survival in Collins and Sacks for 8 hours to 5 days	Reitz et al[10]
Krebs Sacks	24-48 hours	Orthotopic transplantation; Sacks solution better than Krebs; function poor	Toledo-Pereyra et al[11]
SGF (high K^+)	48 hours	Heterotopic transplant; limited survival	Toledo-Pereyra et al[11]
DKS (modified Collins)	24 hours	Isolated reperfusion; good left ventricular function	Swanson et al[12]
Thomas (modified Sacks)	24 hours	Heterotopic transplantation; good function	Thomas et al[13]
Saline	7 hours	Orthotopic transplant; good function	Lower et al[14]

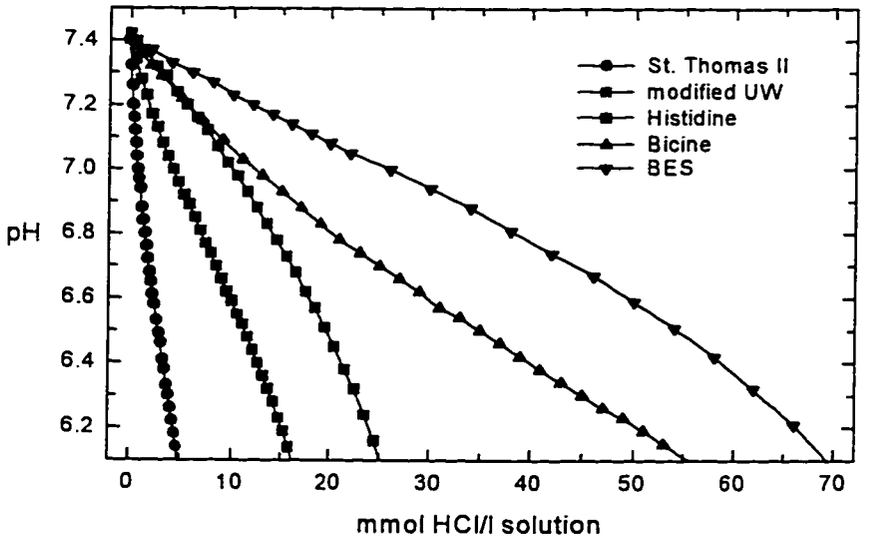
Collin's solution and other intracellular type solutions were used successfully for many years for renal preservation, but when applied in the venue of cardiac transplantation, the results were not satisfactory [15]. The extended preservation times summarized in Table II-1 did not translate to clinical practice and safe storage times remained in the range of 4 to 6 hours. Kohno [16] suggests that the reason for its lack of success in cardiac preservation is possibly secondary to Ca^{++} influx caused by high K^+ induced depolarization which activates the slow Ca^{++} channel, and Na^+-Ca^{++} exchange [15]. Other authors have found that high K^+ solutions are damaging to the coronary endothelium [17-20]. Based on this, some authors [15] have suggested that an extracellular type solution with high Na^+ and low K^+ is more appropriate for cardiac preservation. Several investigators have examined the effects modifying the University of Wisconsin solution by reversing the total Na^+ and K^+ concentrations (ie: Na^+ 120 mmol/L, and K^+ 30mmol/L) and have determined that this has no detrimental effect on liver preservation in either rat, or rabbit models [15, 21]. These results were confirmed for rat hearts by Okouchi and associates who showed that a low K^+ (30mmol/L) and high Na^+ (120mmol/L) was effective in preserving rat myocardium although they did not compare the effectiveness of their modified UW solution with the original Belzer UW solution [15]. A direct comparison of UW solution with either extracellular or intracellular type ion compositions on myocardial preservation was performed by Drinkwater [20]. Using pig hearts stored for 24 hours, they found no difference between the two solutions which is consistent with the findings of Okouchi. The authors explain their results by concluding that the prevention of cellular swelling through the addition of

impermeants, (ie: lactobionate, raffinose and hydroxyethyl-starch), to the University of Wisconsin solution eliminates the need for an intracellular composition [20].

The University of Wisconsin solution was developed in the mid 1980's by Southard and Belzer and was originally formulated for preservation of the pancreas, but was quickly adopted by many centres as an excellent solution for preservation of liver and kidney as well. UW solution had several components that were added on theoretical grounds, the most significant of which seems to be the addition of large impermeant molecules in the form of lactobionate, raffinose, and hydroxyethyl-starch [22]. These impermeants remain extracellular before and upon perfusion of the organ creating an osmotic force that offsets the cellular edema that occurs with ischemia. Glutathione was added as an antioxidant to minimize damage from oxygen radicals , and adenosine was added to enhance the ability of the cells to regenerate ATP upon reperfusion [22]. This formulation offered considerable advantages over the intracellular-type solutions in preserving liver, pancreas, and kidney with significant extension of the safe storage times of these organs. Although UW solution has been shown to be capable of preserving hearts [23, 24], its success in extending storage time of the heart has not been anywhere near that of its success in preserving intra-abdominal organs. Even with UW, the safe storage times in clinical practice are regarded as being in the order of 4 to 6 hours. One important note with regard to UW solution is that, a 0.2 μm filter must be used to prevent damage to the microcirculation of the organ as demonstrated by Walcher [25]. This is apparently caused by the presence of precipitated stearic and palmitic acids [26].

One solution used mainly in Europe was developed by Bretschneider in Germany in the early 1970's for the purposes of cardioplegia for open-heart surgery [4]. Since 1985, Bretschneider's solution has also been used in the setting of heart transplantation [4]. Bretschneider recognized the problem of intracellular acidosis that develops as a result of ischemia, and to address this problem his solution was designed to have a high buffering capacity. To accomplish this, histidine was added to act as an intracellular buffer. Figure II-1 [27] shows how adding different buffering agents to solution increases their buffering capacity . The data for this figure was generated by adding small amounts of HCl to the solution of interest. As demonstrated, a small acid load added to STHS II results in a steep drop in pH relative to the other solutions indicative of its poor buffering capacity when compared to solutions with more powerful buffering agents.

Figure II-1: Titration of Various Cardiac Preservation Solutions [27]



Tait and his colleagues [28] showed how Bretschneider's solution used during multi-dose cardioplegia in a canine model was superior to a less buffered bicarbonate solution in prevention of tissue acidosis. Tian and Mainwood [29] showed that a MOPS containing solution was effective in attenuating the drop in intracellular pH during cardioplegia of pig hearts, but that this did not translate into improved function or tissue energetics on reperfusion when compared to St. Thomas hospital solution II. These results are difficult to interpret however because their MOPS solution differed from the St. Thomas Hospital solution II in more ways than just buffering capacity. The most salient feature of Bretschneider's solution therefore is its improved buffering through histidine. In comparison, Belzer UW solution utilizes phosphate as its primary buffer and as a result has relatively lower buffering capacity.

What is apparent from the above discussion is that there are a multitude of solutions that have been promoted as being appropriate for cardiac preservation. Some of these solutions approach the problem of cardiac graft preservation from different perspectives as previously mentioned. UW solution for example addresses the problem of cellular swelling with less emphasis on tissue acidosis, whereas the opposite is true for Bretschneider's solution. Many studies have been performed comparing the efficacy of these solutions, and the results of several of these studies are summarized in Table II-2. Some studies focus mainly on functional recovery of the heart, others have looked mainly at biochemical parameters such as maintenance of tissue energetics, while still others have incorporated both end points .

Table II-2: Comparisons of the effectiveness of various different cardiac preservation solutions.

*Modified UW -- polyethylene glycol [20M] substituted for hydroxyethyl starch.

Solutions Compared	Model	Results	References
STSH II vs. Lactobionate-Raffinose (based on UW)	Rabbit	Lactobionate-Raffinose better in maintaining high energy phosphates.	Lockett et al [30]
Modified UW (high Na^+/low K^+) vs. Bretschneider's vs. glucose-insulin-potassium	Rat	Improved 1-week graft survival in modified UW group. Bretschneider's gave 2nd best result	Okouchi et al [15]
Euro-Collins vs. UW vs. Bretschneider's	In-vitro human atrium	Functional recovery much better in Bretschneider's after 24 hrs. UW was better than EC.	Hendry et al [8]
Fresh UW vs. UW. vs. Euro-Collins vs. Bretschneider's	Rat	Freshly prepared UW solution had better functional and metabolic recovery followed by Bretschneider's	Fischer and Jeschkeit[26]
Krebs-Henseleit vs. STSH I vs. UW	Rat	Functional recovery better with UW solution. ATP better preserved with St. Thomas solution when glucose is added. but repletion upon reperfusion was better with UW solution. Less tissue edema with UW.	Karck et al[31]
STHS II vs. UW	Rat	Functional recovery was much better with STHS. No differences were found in metabolic recovery.	Choong et al[32]
STHS vs. modified Krebs-Henseleit vs. saline vs. Euro-Collins	In-vitro human atrium	STHS gave best functional recovery.	Hendry et al[33]
STHS II vs. UW	Pig	STHS II was superior to UW solution in functional recovery and preservation of high energy phosphates at 12°C. No difference between solutions at 4°C	Tian et al[34]
STHS II vs. modified UW* vs. Bretschneider's	Rabbit	Functional recovery was best in modified UW solution.	Wicomb et al[35]
Stanford vs. UW vs. modified Collins	Canine	Functional recovery was best with UW solution. Less edema with UW solution.	Swanson et al[36]
STHS vs. UW	Human	Functional recovery was best with UW solution	Demertzis et al[37]

Reviewing the studies summarized in Table II-2, it is apparent that many solutions are capable of preserving cardiac function during short periods of hypothermic

storage, but that no one solution stands out as being clearly superior. These comparative studies are inconsistent in their results. What is clear however, is that in the clinical setting none of these solutions have been able to greatly prolong the safe storage interval past 4 to 6 hours. There is still quite considerable room for improvement although a completely novel approach may be necessary to achieve the advances in cardiac preservation that have been made in the past decade in liver, pancreas, and kidney preservation.

Ionic Disturbances During Myocardial Ischemia

It is important to understand the ionic changes that occur in the myocardium and other cells during an ischemic insult for two reasons. Firstly, it is ultimately at this level that disturbances incurred by ischemic insults ultimately lead to cellular damage, death, and contracture. Secondly, by understanding the pathologic ionic changes that occur, interventions aimed at minimizing these changes can be developed. A recent paper written by Pierce and Czubryt [38] is a good review summarizing the ionic imbalances that occur during ischemia, and their ultimate contribution to cellular injury.

With the onset of ischemia, oxygen delivery to the cell is cut off. In order for the cell to maintain ATP energy levels needed for maintenance of cellular structure and function, the cell undergoes a transition to anaerobic glycolysis whereby intracellular stores of glycogen are broken down and metabolized to lactate and ATP. As a result of this metabolic pathway, H^+ also accumulate resulting in a fall in intracellular pH. Since many of the intermediary enzymes involved in glycolysis such as glycogen phosphorylase

and particularly phosphofructokinase are pH sensitive, the pH changes that occur intracellularly with ischemia feed back and inhibit glycolysis, thus limiting the production of ATP.

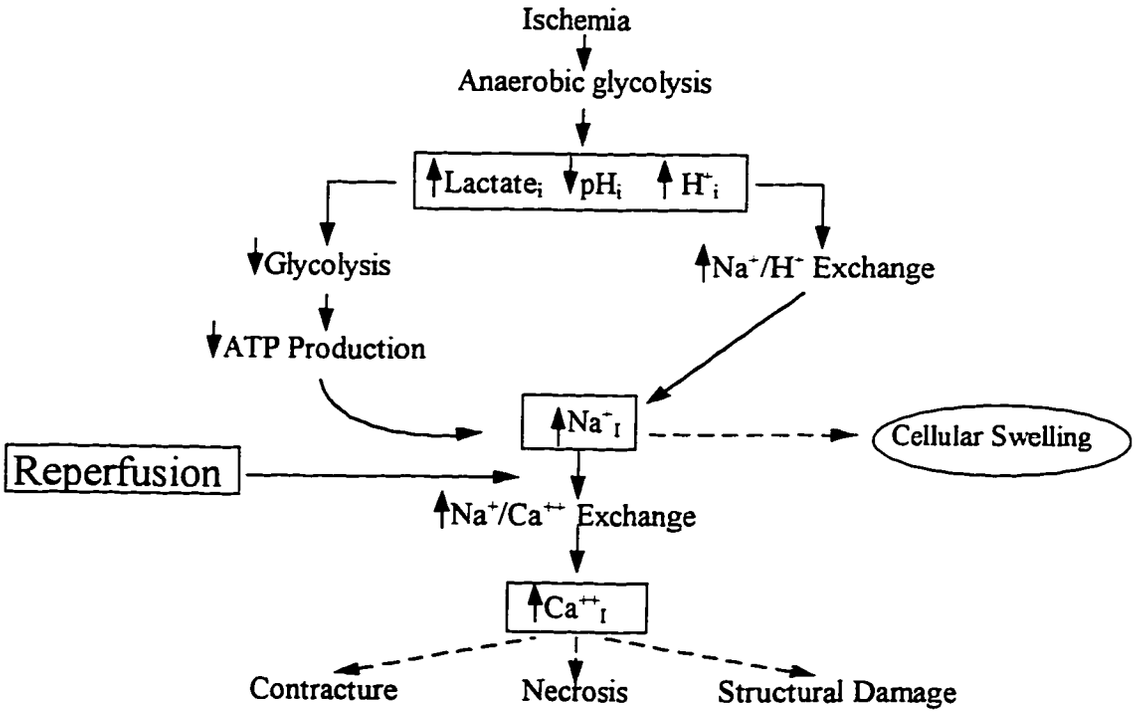
As our understanding of ionic dyshomeostasis during ischemia becomes clearer, two ions in particular are becoming the major focus of attention: Ca^{++} and Na^{+} [38, 39]. Historically, it was felt that Na^{+} overload intracellularly occurred during periods of hypoxia and ischemia secondary to impaired ATP production leading to a decrease in Na^{+}/K^{+} ATPase activity. The importance of this mechanism was demonstrated by Anderson who poisoned the Na^{+}/K^{+} ATPase with ouabain and showed that intracellular Na^{+} levels increased at a rate greater than 10 times that of normoxic controls [39]. A second mechanism by which intracellular Na^{+} overloading can occur has been proposed more recently. As stated previously, H^{+} accumulates as a result of anaerobic breakdown of glycogen. The cell, acting in a pH regulatory capacity extrudes these excess H^{+} in exchange for extracellular Na^{+} via a membrane bound Na^{+}/H^{+} exchanger [39]. Support for this hypothesis is founded in experimental evidence which shows that Na^{+}/H^{+} exchange inhibitors such as amiloride and its derivatives act in a cardioprotective manner during ischemia/reperfusion [38, 40, 41].

Intracellular Na^{+} loading has two consequences. First of all, an osmotic gradient between the intracellular and extracellular space is created leading to a shift of water from the extracellular compartment into the intracellular compartment resulting in cellular edema which is in itself damaging to organ function. The second sequelae of intracellular Na^{+} loading as outlined by Pierce and Czubryt is that extracellular Ca^{++} is

exchanged for intracellular Na^+ via a Na^+/Ca^{++} exchanger leading to Ca^{++} accumulation intracellularly [38]. As pointed out by Bolli [42] however, this usually occurs at the time of reperfusion as H^+, which inhibits the Na^+/Ca^{++} exchanger, is washed out of the tissue. It is this intracellular Ca^{++} accumulation which has been implicated most recently in causing myocardial contracture, damage to cellular components, and cellular necrosis.

These ionic changes and their implications on cellular function are summarized in the following flowsheet: See Figure 11-2.

Figure II-2. Ionic dyshomeostasis from myocardial ischemia



Referring to Figure II-2, insight can be gained into some of the mechanisms through which successful preservation solutions used today exert their positive effects. The University of Wisconsin solution which is used in many centres in North America for instance contains large impermeant molecules such as lactobionate and hydroxyethyl

starch [22]. These osmotic agents remain extracellular as a result of their size and help to offset cellular edema created by the intracellular accumulation of Na^+. Another popular solution used in Europe extensively for organ preservation, Bretschneider's HTK solution, includes histidine which acts as an intracellular buffering agent. By providing an intracellular H^+ sink, two benefits are realized. First of all, negative feedback on glycolysis through a drop in pH may be lessened allowing for increased glycolytic flux with enhanced energy production. This increased ATP generation may allow for better function of the Na^+/K^+ ATPase with a resultant decrease in intracellular Na^+ loading. Secondly, buffering can reduce intracellular Na^+ loading by reducing the amount of H^+ exchanged for Na^+ via the Na^+/H^+ exchanger. The overall net effect will be a decrease in flux through both pathways with less intracellular Na^+ accumulation and therefore less intracellular Ca^{++} loading.

Myocardial Stunning

The term "myocardial stunning" refers to the mechanical dysfunction that persists after an ischemic insult despite the absence of irreversible damage [42]. An essential point in this definition is that the dysfunction is completely reversible, and therefore the term does not apply to myocardium that demonstrates contractile abnormalities associated with infarcted, non-viable tissue. This concept is important in all settings where the heart is subjected to reversible non-lethal ischemia, such as coronary bypass and transplantation, because it predicts a certain degree of functional impairment of the post-ischemic myocardium, even when the cells are completely viable. This implies that enhancing viability through appropriate preservation solutions and

techniques may not necessarily lead to better function, at least in the short term, unless this issue of stunning is addressed. Bolli [42] wrote a comprehensive review on myocardial stunning. This paper suggests that through pharmacological manipulation, stunning can be reduced, leading to better myocardial function in the post-ischemic reperfusion period.

Many different mechanisms for myocardial stunning have been investigated and are summarized by Bolli. Some investigators in the early 1980's put forth the hypothesis that a deficiency of ATP is responsible for stunning [43-45]. Subsequent investigators have shown however that this is not likely the predominant mechanism, in that no correlation has been demonstrated between ATP levels and recovery of contractility during the period of stunning [46-48]. Furthermore, increasing ATP levels in stunned myocardium does not necessarily translate into increased mechanical performance [49]. It would seem therefore that while ATP levels must be maintained for cellular viability, simply preserving ATP alone is not sufficient to prevent myocardial stunning. This is consistent with the statement earlier that maintaining viability is important for function, but viability in and of itself does not necessarily ensure good graft function, at least in the short term.

Bolli suggests three major mechanisms involved in myocardial stunning: generation of oxygen free radicals, Ca^{++} overloading, and excitation-contraction uncoupling due to dysfunction of the sarcoplasmic reticulum [42]. Pharmacologic agents can be utilized to minimize stunning based upon these recognized mechanisms. For instance, Myers found that the addition of both superoxide dismutase and catalase,

both of which are free radical scavengers, enhanced myocardial function during reperfusion following a period of non-lethal ischemia [50]. Interestingly, both scavengers together had to be present in order for the benefit to be realized. With respect to Ca^{++} overload, the mechanism by which this occurs is outlined in a previous discussion dealing with ionic disturbances during ischemia. Evidence for the causal role of Ca^{++} overloading in stunning is provided by Porterfield who demonstrated that pre-treating a heart with ryanodine, an inhibitor of Ca^{++} overload, reduced the severity of stunning post-ischemia [51].

Anaerobic Metabolism During Ischemia

Explanting an organ for transplantation renders it ischemic, thus severely disrupting cellular metabolism by limiting energy production needed to sustain cellular reactions. The viability of the organ upon replantation as measured by its ability to recover pre-ischemic function will depend upon the integrity of its constituent cells. In the normal, aerobically perfused myocardium, energy in the form of ATP is derived from oxidative phosphorylation of several different substrates including glucose, lactate, and fatty acids [52]. Of these, fatty acids are utilized preferentially [52]. The time period between harvesting a heart for transplantation and reperfusion in the recipient is one of total oxygen deprivation when current storage techniques are utilized. Under these circumstances, cellular energy production depends entirely on anaerobic glycolysis to meet energy demand because fatty acids and lactate are required to undergo oxidative metabolism to generate ATP.

In a recent review, Opie reviewed the subject of metabolism during myocardial ischemia as well as the implications of increased glycolysis during such an insult [53]. Opie points out that in the absence of oxygen, high energy phosphates are depleted rapidly with a resultant increase in glycolytic flux. (See Appendix I for details of glycolytic pathway) Pyruvate and $NADH_2$ accumulate because they are formed faster than they can enter mitochondria for oxidation, and are then further converted to lactate and NAD^- which is the real end point of anaerobic glycolysis. The benefit of anaerobic glycolysis lies in the production of ATP needed by the cell to maintain homeostasis, but this also leads to the production of lactate and protons which are produced by ATP hydrolysis. Glycolysis therefore seems to be a double edged sword when lack of perfusion prevents washout of metabolites, and hence the harm caused by poor washout needs to be balanced against the benefits of increased anaerobic ATP production [53]. When this last point is considered, one might surmise continuous machine perfusion of explanted hearts may offer advantages over simple flush-storage in that continuous perfusion at least allows for some washout of protons and lactate. Opie also discusses control of glycolysis during ischemia. As he points out, there are metabolic control points that limit the "traffic" down the glycolytic path, one of which is the enzyme phosphofructokinase (PFK). This enzyme is inhibited during aerobic conditions when ATP levels in the myocardial cells are high. The block is relieved somewhat during anaerobic conditions which see ATP levels drop as ATP is converted to ADP and AMP. A second factor which serves to enhance the activity of this enzyme during anaerobic conditions is a drop in cellular citrate which occurs as a result of a decrease in

mitochondrial activity [53]. As mentioned previously however, protons accumulate during anaerobic states when perfusion is not adequate to wash them away. This serves to decrease PFK's activity and lessens glycolytic flux. This however does not mean that PFK's activity is completely inhibited. Churchill [54] demonstrated that stimulating factors may have more influence on this enzymes activity than pH inhibition leading to a net increase in PFK's activity despite the decrease in pH.

The importance of anaerobic glycolysis in the ischemic state with respect to functional recoverability and the maintenance of cellular structural integrity was demonstrated in an early study by Weissler *et al* [55]. They took isolated perfused rat hearts and inflicted an anoxic insult on them for a total of 30 minutes. During this time the hearts were continuously perfused with 5% albumin in Krebs-Ringer bicarbonate solution containing either anaerobic substrate in the form of glucose, or no anaerobic substrate. They found that hearts supplied with glucose during the anoxic period had much improved electrical and mechanical performance and enhanced recovery upon reoxygenation compared to hearts deprived of glucose during anoxia. In addition, cellular structural changes seen on electron microscopy in the form of alterations in mitochondrial morphology and dilatation of the longitudinal tubules were pronounced in the hearts perfused with solution lacking glucose, and were absent in hearts supplied with glucose. The conclusion drawn was that by supplying glucose, energy production through anaerobic glycolysis was enhanced and this improved cellular function and viability over those hearts that were unable to generate energy through glycolysis because of a lack of substrate.

Stringham *et al* [56] used University of Wisconsin perfusate which contained 3 mM of iodoacetate (IAA) which blocked glycolytic flux. They then measured ventricular compliance as an index of ischemic contracture and found that when glycolysis was blocked by IAA, the development of ischemic contracture was greatly accelerated, developing rapidly within 1 hour of cold ischemia. Measuring myocardial ATP levels in this group revealed a very rapid decline, with less than 15% remaining after 2 hours of storage. In contrast, hearts perfused with UW solution retained 85% of pre-ischemic ATP levels after 12 hours, and declined to 78% after 24 hours. These results are consistent with the important role that anaerobic glycolysis is thought to play in preserving organ viability under cold ischemic conditions. This study confirms the results of a similar experiment by Bing and Fishbein [57] who also showed that hypoxic contracture was worse when IAA was added to block glycolysis.

Other experimental evidence from several sources supports the hypothesis that increasing glycolytic flux during ischemia helps to decrease the severity of ischemic injury to myocardial cells. Maroko [58], Opie [59], and De Leiris [60], have shown decreased injury when exogenous glucose is provided to enhance glycolysis [53]. Insulin, which increases glucose uptake by cardiomyocytes has been shown by De Leiris and Feuvray to also have beneficial effects [61].

The Effect of Glycolytic End Products on Myocardial Preservation

As glycolytic flux progresses during ischemic cold storage of the myocardium, end-products of glycolysis accumulate. These end-products, (most

importantly lactate and H^+), accumulate because they are not being flushed out during static cold storage. It is therefore important to study the effects that they have on the intracellular biochemical processes, and more importantly on myocardial recovery following reperfusion.

Cross [62] studied the effects of exogenous lactate during low flow myocardial ischemia on tissue energetics and functional recovery during the post-ischemic period. These investigators were interested in whether myocardial damage was the result of lower intracellular pH which invariably develops secondary to anaerobic glycolysis in the ischemic myocardium, or whether this damage was caused by the accumulation of intracellular lactate. They found that hearts perfused with exogenous lactate, (thus decreasing lactate efflux from endogenous production causing an accumulation of intracellular lactate), demonstrated no functional recovery compared to control and pyruvate perfused hearts which regained 85% functional recovery despite the finding that the pyruvate perfused hearts had a significantly lower intra-cellular pH post-ischemia. They also found a significantly greater depletion of ATP in the lactate perfused hearts. The conclusions that were drawn from this study were that accumulation of intracellular lactate increased the $NADH/NAD^+$ ratio causing a block of glycolytic flux at GAPDH. They postulate that it is this decrease in anaerobic glycolysis and the resultant decrease in cellular energy production below critical levels that leads to irreversible myocardial injury. This study also highlights the importance of glycolysis during the ischemic period in maintaining functional potential.

Opie [63] investigated the effects of extracellular pH changes over the range 7.1-8.0 on myocardial mechanical function, and on the metabolic patterns of substrates such as pyruvate, lactate, acetate and palmitate which the myocardium oxidizes preferentially over glucose. He found that glucose and acetate were unaffected by decreasing pH to 7.1 but a rise in pH to 8.0 increased the rate of oxidation of these substrates, whereas oxidation of pyruvate and palmitate were not effected. Kreb's cycle activity was unaltered over the pH range studied. With respect to mechanical activity of the perfused , beating heart, he found that it was depressed at the lower pH range but unaffected by a rise in pH over 7.4. Possibly the most significant conclusion from this study is that mechanical deterioration of the myocardium by acidosis in the non-ischemic/anoxic state is not secondary to changes in oxidative metabolism.

A similar investigation to Opie's (1965) [63] on the effects of pH on tissue energetic status, and functional recovery post-ischemia was carried out by Bernard [64] with quite different results. The buffers used by these investigators were however markedly different (Opie used $NaHCO_3$ whereas in the current study they used chiefly glutamate). Using perfused working rat hearts, they demonstrated that after 2 hours of warm ischemia, PCr levels were higher and ATP levels recovered more quickly by intermittently perfusing the hearts with a glutamate buffered cardioplegia solution at an acidotic pH of 7.0 during the ischemic period when compared to a similar solutions at a pH of 7.7. With respect to left ventricular function, they found that hearts undergoing cardioplegia with a glutamate buffered acidotic perfusate (pH 7.0) recovered significantly greater aortic flow upon reoxygenation than a similar glutamate buffered

alkalotic cardioplegia solution (pH 7.7) at 3 minutes of reperfusion, but at 30 minutes there was no significant difference. In this study, various buffers in the perfusate used during the ischemic period (glutamate, histidine, TRIS, and $NaHCO_3$) were also compared at constant pH using the same parameters outlined above. They found that at pH 7.4, glutamate perfused hearts recovered aortic flow faster than TRIS and histidine perfused groups but ultimate recovery was similar. Also, ATP and PCr levels both at the end of ischemia and after reperfusion did not differ with the exception that $NaHCO_3$ buffered perfusate had an end reperfusion level of PCr significantly higher than the other groups. At a pH of 7.0 it was found that glutamate buffered cardioplegia gave higher levels of post-reperfusion ATP than the other buffers, but PCr levels were similar. Aortic flow post reperfusion was found to be lower in the histidine group than the groups at pH of 7.0. Overall, this study found the greatest myocardial preservation was provided by a glutamate containing cardioplegia solution at a pH of 7.0. The authors felt that glutamate was beneficial in that it possibly acted as a substrate for later reperfusion while at the same time being a good pH buffer. They also felt that mild intracellular acidosis lowers metabolic needs during arrest, most likely by limiting Ca^{--} overload. Previous studies showing a protective effect of alkalosis during pre-ischemic cooling utilized perfused beating hearts whereas this study worked with cold, cardioplegically arrested hearts.

Functional recovery of an ischemic myocardium after restoration of perfusion depends at least in part on maintenance of intracellular ATP stores. Bercot *et al* [65] used cardioplegia solutions of varying pH (6.0 - 7.8) and measured ATP depletion over

24 hours of ischemia at several intervals. They found that ATP depletion was lower in specimens perfused with acidic cardioplegic solution (pH 6.0) and that as the pH of the cardioplegic solution was raised, ATP was depleted at a faster rate. This study was performed on myocardium stored at 4°C.

The effect of changing the pH of St. Thomas Hospital cardioplegia solution through oxygenation on post-ischemic myocardial recovery was investigated by von Oppell [66]. They found that pH of the cardioplegic solutions shifted toward the alkaline end of the spectrum as temperature was decreased from 37°C to 4°C. In addition, solutions gassed with 5% CO_2 and either 95% O_2 or N_2, had a lowering of the pH in comparison to the rise in pH that occurs when 100% O_2 or N_2 are used to gas the solution. Functional recovery as measured by aortic flow, stroke volume, and cardiac output, were shown to be significantly improved by lowering the pH of the cardioplegia solution to approximately 7.0 through the addition of 5% CO_2. A significant decrease in lactate production by the heart was also noted in this group. Also, these investigator's findings confirmed the results of other authors (Bodenhamer (1983), Ledingham (1988)) [67, 68], who found improved post-ischemic recovery when the perfusate used during the ischemic period was oxygenated. According to this study the optimal gas content of the solution is 95% O_2 : 5% CO_2, the benefit of the CO_2 being the resulting lowering of pH. The addition of perfluorocarbon, (an O_2 carrying molecule), to the cardioplegia solution conferred no added benefit with respect to functional recovery.

The studies summarized in the preceeding discussion are strongly supportive of cardioplegic solutions more acidic than is physiological (ie: less than 7.4). By making

the extracellular space more acidic, the sarcolemmal Na^+/H^+ transporter which exchanges intracellular H^+ for extracellular Na^+ would be less active, resulting in lower intracellular Na^+ accumulation. The benefit of this would be less cellular edema, as well as less intracellular Ca^{++} loading upon reperfusion via the membrane bound Na^+/Ca^{++} exchanger as outlined in the discussion on ionic disturbances during ischemia (see figure II-2). The separation between intracellular and extracellular acidosis needs to be emphasized. While the aforementioned studies show a benefit by lowering the extracellular pH, the discussion conclusions are limited to the context of the *extracellular* space. No implication should be made, based on these studies that *intracellular* acidosis is beneficial to the heart.

Regulatory Enzymes of Glycolysis

The biochemical reactions of the glycolytic pathway are catalyzed by many different enzymes [see Appendix I]. Some of these enzymes such as phosphofructokinase act in a rate controlling capacity through a variety of intracellular signals that may either be rate limiting or rate enhancing. If one wishes to have any degree of control over glycolysis in the experimental setting, knowledge of these enzymes and their effectors is first necessary.

Phosphofructokinase plays a very important role in the control of glycolytic flux by catalyzing the phosphorylation of fructose 6-phosphate to fructose 1-6 bisphosphate. This is a rate limiting step in the glycolytic pathway, and therefore changes in PFK's activity will have a direct influence on the rate of glycolytic flux. This is especially

important when considering explanted organs being preserved for transplantation. These organs are rendered ischemic once removed from the donor, and anaerobic glycolysis is the only means of generating energy needed to sustain metabolism. The importance of PFK in sustaining cellular viability during ischemia is therefore apparent.

Dunaway *et al* [69] summarized what is known about PFK in human tissue. Three subunit types (L-type, M-type, and C-type) whose genes are located on chromosomes 21, 1, and 10 respectively are found in humans. Only the M-type subunit was originally thought to occur in human cardiac muscle, but these investigators showed that both the L-type and C-type were also present in small quantities. The importance of this lies in the fact that subunit composition of the various isoenzymes has a direct effect on the kinetic and regulatory properties of PFK. This would suggest that PFK activity is somewhat organ specific, and that studies of PFK kinetics and regulation in one organ cannot necessarily be extrapolated to other organs.

Hand and Carpenter [70] studied the effect of pH changes on PFK purified from rat myocardium. They showed that at a physiologic temperature of 37°C in vitro, decreasing the pH to 6.5 causes PFK to undergo a reversible hysteretic loss of activity. This raises the question of what happens to the action of this enzyme in the ischemic state where anaerobic glycolysis leads to a fall in intracellular pH? It must be remembered that pH is not the only effector of this enzyme. Increased citrate levels as well as ATP also inhibit PFK's activity, whereas increased levels of AMP, and inorganic phosphate (Pi) activate PFK [54]. During the ischemic period all of these factors play a role, and it is therefore inadequate to assume that PFK is inhibited simply because of a

drop in intracellular pH. Churchill *et al* [54] worked with rat liver in both the warm and cold ischemic states. They demonstrated that in both of these states fructose 6-phosphate levels declined with a subsequent rise in fructose 1,6-bisphosphate indicating an activation of glycolysis at the PFK locus. It seems from this study therefore that factors activating PFK may actually outweigh the pH inhibition that occurs resulting in a net increase in PFK activity. They do however point out that glycolytic flux requires the presence of NAD^+ in the oxidation of glyceraldehyde-3-phosphate, and that despite an activation of PFK, unless NAD^+ pools within the cell are maintained by continued conversion of pyruvate to lactate, glycolysis will be limited at this point further down the pathway. Also, as pointed out previously by Dunaway *et al* [69], kinetics and regulation of PFK depend on the isoenzymes that are present, and these isoenzymes differ between organs. Demonstration of PFK activation in liver therefore cannot necessarily be extrapolated to heart. Evidence of this is provided by Carpenter and Hand in a separate paper to the one cited above [71]. Using a rocket immunoelectrophoretic assay which they developed to determine whether PFK was in the active tetrameric form, or the inactive dimeric form, they demonstrated that rat hearts subjected to warm ischemia underwent a reversible depolymerization/inactivation of PFK. Furthermore, this deactivation was pH dependent and that realkalinizing the heart extract in vitro led to repolymerization of the inactive PFK dimers with a concomitant 86% recovery of the activity lost during the ischemic period. Of importance as well is that they found that post-ischemic reperfusion of the rat heart in vivo also resulted in repolymerization and recovery of function of PFK. Although PFK is an important regulatory enzyme of

glycolytic flux, Hand and Carpenter point out that glycolytic flux can be blocked at other sites along the pathway. Inhibition of the glyceraldehyde-3-phosphate dehydrogenase reaction may be more dominant than the one at PFK due to low NAD^+ levels [71]. They further suggest that PFK inhibition of glycolysis is most significant when severe intracellular acidosis results during prolonged ischemia [71].

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is a glycolytic enzyme that catalyzes the conversion of glyceraldehyde 3-phosphate to 3-phophoglycerol phosphate. According to Opie[53], this enzyme is also important in terms of rate control of glycolytic flux. NADH and H^+ accumulate during anaerobic glycolysis because of greatly decreased utilization by the mitochondria. Since these molecules are produced by the reaction catalyzed by GAPDH, feedback inhibition occurs on this enzyme. Also, GAPDH is also directly inhibited by lactate accumulation during anaerobic metabolism [53].

Cold Storage and Optimal Temperature of Preservation

In the setting of transplantation, an organ inevitably undergoes a period of ischemia as a result of currently used harvest and storage techniques. This period lasts from when the blood supply to the organ is first ligated upon removal from the donor until blood flow is reestablished in the recipient. Organs differ in their ability to tolerate this ischemic insult accounting for differences between organs in the time period from explantation to reimplantation that is considered safe in the clinical setting. Kidneys can be stored up to 48 hours, livers up to 24 hours, and pancreas up to 30 hours before being

reimplanted, whereas this period for hearts is only 4 to 6 hours[56]. The objective of current and past research dealing with organ preservation is to enhance the potential for recovery after this period of physiologic disruption. As techniques are devised that enhance functional recovery, the length of time that an organ can be stored safely is consequently prolonged. Maintenance of normal cellular physiology during the ischemic period is not at present within our ability. The alternative to this is to reversibly suppress normal physiologic function using physical and pharmacologic means to in effect achieve a state of 'suspended animation'. The use of hypothermia to suppress metabolism is the principle method used presently. One question that arises therefore is: what is the optimal temperature during storage that gives the best functional result?

Swanson *et al* [72] used a working canine heart to address this question of optimal temperature during preservation. Three different cardioplegia solutions were used by these investigators; a modified Collins, a modified extracellular solution (DKS) which they devised, and blood. Each of these groups underwent a period of ischemic storage at either 15°C or 4°C. A working heart model was then prepared measuring the return of left ventricular function as determined by stroke volume. They found that hearts preserved at 4°C functioned at levels equal to or greater than hearts stored at 15°C, even though hearts preserved at 4°C were stored for longer periods than those preserved at 15°C. Similar results were obtained by Masters and colleagues [73]. These investigators performed heterotopic cardiac transplants on dogs after 4 hours of ischemia at various temperature ranges from 0°C-3°C to 12°C-15°C. The cardioplegia solution used was not mentioned. The degree and rate of functional recovery as determined by

end-systolic elastance was followed, and myocardial metabolism was simultaneously studied by monitoring coronary flow, O_2, glucose, lactate, pyruvate, and free fatty acid uptake. The groups were then compared to controls who underwent immediate transplantation with no ischemic period. They found that cardiac function was depressed 30 minutes after transplantation, but were equal to control levels 2 hours later when the stored temperature was between 0°C and 6°C. In contrast, myocardial function remained significantly depressed over the 2 hour post-transplant recovery period when hearts were stored at temperatures between 6°C and 15°C. These studies support the temperatures used currently to preserve hearts in the clinical setting.

Not all studies performed to date have suggested that 4°C is the optimal temperature for storage of organs during the ischemic period. Flaherty suggested that heart preservation was optimal when stored between 15°C and 24°C [74]. Ohkado and his colleagues found that contractile function was best restored in hearts stored at 13°C in a histidine buffered solution for 8 and 16 hours [75]. Hendry and Keon have done much work on answering the question of optimal storage temperature for an explanted graft who feel that current temperatures used in the clinical setting are too cold [76-78]. These investigators used muscular trabeculae from human right atrial appendages removed during elective cardiac procedures [77]. These specimens were stored at variable temperature, (1°C, 4°C, 12°C, and 20°C), in Tyrode's solution for 1, 2, 4, 24, and 48 hours. Mechanical recovery was determined by measuring developed contractile force relative to pre-ischemic values, and was measured over a recovery period of 1 hour. They found that recovery was poor at 4°C over 1 hour, whereas muscle cooled to

12°C and 20°C did well in the recovery phase. The authors felt that at cooler temperatures, Na^+/K^+ ATPase activity is impaired even though absolute levels of ATP were well preserved at these temperatures. This loss of ATPase activity is postulated by the authors to result in intracellular accumulation of Na^+ with resultant cellular swelling and ultimately muscular dysfunction. They suggest that the optimal temperature of storage is 12°C based on their data. Masters and colleagues [73] have disputed this recommendation citing the fact that recovery was only monitored over a period of 1 hour. In their own experiments described previously, recovery of function continued well past the first hour. It is feasible that recovery in the 1°C and 4°C muscle is accelerated after 1 hour and possibly would have surpassed the performance of muscle stored at 12°C and 20°C. Another potential weakness with this study is that it did not entail the use of a working heart model, only isolated atrial muscle which the authors themselves point out is different from ventricular muscle.

This same group studied myocardial temperatures during the storage period by monitoring the drop in tissue temperature using probes placed in the ventricular walls and septum during storage utilizing the standard technique of placing the graft in a cooler containing saline and ice [76]. This technique presumably cools the myocardium down to 4°C. They found that within 4 hours, temperature was below 0°C throughout the myocardium and that under electron microscopy the cells exhibited signs of edema and mitochondrial calcium accumulation.

The successful cold storage of heart is highly ATP dependent as shown by Stringham *et al* [56] who found a near perfect negative correlation between ATP levels

and ventricular compliance. Unlike the kidney or liver, a transplanted heart must regain most of its pre-ischemic functional ability very shortly after reimplantation in order to sustain life. With the abdominal organs, there is a more extended time period allowable for recovery of function. The heart must maintain enough ATP to avoid the development of ischemic contracture. As they point out, use of the term "ischemic contracture" in this setting refers to the development of "stone heart" which describes a myocardium that is irreversibly thickened, contracted, and rigid [56]. This is not to be confused with the ischemic contracture of a perfused beating heart upon reperfusion, which presents as a rise in diastolic tension [56] The latter form of contracture is fully reversible implying the potential for recovery. Stringham *et al* suggest that the level at which the development of 'stone heart contracture' becomes a significant concern is when approximately 80% of pre-ischemic ATP levels are reached. Below this level, there is an accelerated decline in tissue ATP, with a consequent increasing contracture, and decreased potential for functional recovery [56].

Deslauriers [79], working with human atrial trabeculae excised during elective cardiac operations, measured the preservation of ATP levels in cardiac muscle. The samples were stored in saline at different temperatures (1°C, 4°C, 12°C, and 20°C) and then assayed for ATP levels using phosphorus 31-nuclear magnetic resonance imaging at intervals over a period of 6 hours or more. The results indicate ATP levels are better preserved at 1°C and 4°C, than at 12°C or 20°C.

When preserving organs for transplantation, one important point to consider with regard to temperature is that of thermal shock. This is a phenomenon whereby cells are

damaged by rapid cooling, even when freezing of the cell does not occur [2]. It has been suggested by some investigators that when an organ and its constituent cells are cooled too rapidly, (from 37°C to 4°C in the case of most clinical situations today), that mechanical fracture of the cell membrane occurs secondary to unequal cooling of the whole membrane simultaneously thus causing differences in thermal contraction [80]. Jacobsen [81] observed this phenomenon in rabbit kidneys cooled at 7.2°C/min but not when the cooling rate was slowed to 3.7°C/min. The rate of cooling of isolated tissue for transplant therefore needs to be carefully considered at the time of harvest.

In summary, the current practice of storing explanted hearts at 4°C has been shown by many investigators to be optimal with respect to preservation of functional potential and the preservation of high energy phosphates which have been shown to correlate strongly with recovery upon transplantation. It should be noted however that some authors have questioned this practice and have suggested that higher temperatures in the order of 12°C to 15°C may be more appropriate, although this has not been demonstrated conclusively in either the experimental, or clinical setting. It seems therefore that the issue of optimal graft storage temperature is not totally resolved. The rate of cooling of the organ is also of importance to prevent thermal shock with a resultant delay in recovery. Clinically this does not seem to be a problem at present although it should be kept in mind as new techniques are developed.

Cardiac Preconditioning

Murray in 1986 [82] and subsequent other investigators have noted that a brief ischemic episode preceding a more prolonged period of ischemia confers a degree of myocardial protection from injury during this sustained period [83]. The term coined for this phenomenon is cardiac preconditioning and has been shown to exist in many mammalian species including dogs, pigs, rats, and rabbits [83]. In the experiments by Murray, canine hearts were subjected to brief regional ischemia by occluding the circumflex artery for four 5 minute episodes with each episode separated by 5 minutes of reperfusion which was then followed by 40 minutes of sustained occlusion resulting in infarction. Interestingly, these authors found that infarction of the ischemic myocardium was 29.4% in control hearts compared to 7.3% in preconditioned hearts [83]. Other investigations have shown that preconditioning with a brief ischemic episode not only limits infarct size [82], but ultrastructural damage [84], and reperfusion arrhythmias [85] are reduced as well when compared to controls that were not subjected to a preconditioning insult [83]. The effect however is time limited as demonstrated by Miura [86]. They found a decrease in myocardial protective effects beyond 25 minutes after the initial preconditioning ischemic insult. Also, it seems that the duration of ischemia is also important as one would expect intuitively. Experimental evidence suggests that the protective effects of preconditioning are all but gone after 90 minutes of sustained warm ischemia [82]. It is possible that in the setting of transplant surgery where organs are subjected to cold ischemia, that this interval may be more prolonged.

The investigative efforts into the mediators of the cardiac preconditioning phenomenon have focused on several substances including adenosine receptors, α_1-adrenergic receptors, and protein kinase C (PKC) [83]. In myocardium, the adenosine receptor exists in two forms designated A_1 and A_2. Myocardial A_1 receptor stimulation causes a decrease in cellular cAMP levels resulting in negative chronotropic and inotropic effects. Adenosine A_2 receptors however predominantly cause vasodilation through direct effects on smooth muscle [83]. Support for the role of adenosine in cardiac preconditioning was offered by Liu who demonstrated that nonselective adenosine antagonists eliminated the preconditioning effect in rabbit myocardium [83, 87]. They then carried on to show that the administration of selective A_1 receptor agonists resulted in limited infarct size when given before a prolonged ischemic insult, but that A_2 receptor agonists alone afforded no additional protection. This points to the A_1 receptor as a mediator of the preconditioning phenomenon in rabbit heart. The exact mechanism of action remains unclear. Meldrum has suggested several possibilities including decreased calcium overload during reperfusion, decreased catecholamine induced cardiotoxicity, inhibition of platelet aggregation, and attenuated neutrophil activation [83, 88, 89]. Gross *et al* [90] have showed that blocking ATP dependent K^+ channels in dog myocardium eliminates preconditioning, while opening these channels enhances the effect.

Some researchers have shown the role of adenosine in myocardial preconditioning to be species dependent in that non-selective adenosine receptor inhibition in rats does not eliminate the benefit of preconditioning [83, 91]. Even in the

rat however, adenosine A_1 receptor agonists had beneficial effects on limiting infarct size [83, 91]. This suggests the presence of more than one mediator of the phenomenon, and the relative importance of these mediators may differ between species even though all may play a role in a given species.

Evidence for a role of α_1-adrenergeic receptor stimulation in the preconditioning phenomenon also exists. This hypothesis was based on the observation that some tissues such as skeletal muscle and gut have a greater tolerance to ischemic injury than the brain or heart, and that a major difference between these tissues is that during shock states, catecholamine release leads to vasoconstriction in these vascular beds [83]. The tolerance of certain tissues over others was therefore postulated to be mediated by catecholamine induced preconditioning of the tissue. Banerjee [92] provided support for this hypothesis. They infused norepinephrine into isolated rat left ventricle for 2 minutes followed by 10 minutes of normal perfusion and then 20 minutes of global ischemia at 37°C. After reperfusing these ischemic damaged ventricles for 40 minutes, these investigators found improved function as measured by increased developed pressure, and better contractility. They also found that these benefits were blocked by the addition of α_1-adrenergic receptor antagonists.

The distal effectors through which α_1-adrenergic and A_1-adenosine receptors exert their protective actions has not been elucidated, but Kitakaze has suggested that the actions of these two receptors are linked [93]. These authors maintain that 5′-nucleotidase activity is increased through phosphorylation by protein kinase C (PKC), and that the activation of PKC is mediated by α_1-adrenergic stimulation. They propose

that increased 5′-nucleotidase activity stimulates adenosine elaboration which then exerts its protective effects on the ischemic myocardium [83, 93]. More recently however, an alternate mechanism has been proposed and is summarized in a review article by Meldrum *et al.* According to these authors, adenosine and α_1-adrenergic agonists act as stress hormones and act synergistically to active PKC through phospholipase C (PLC) [89]. This activation of PKC results in stress protein synthesis which promotes resistance to reperfusion arrhythmias [89]. PLC activation also has effects on intracellular Ca^{++} homeostasis which also has some effects on PKC, however, the exact role that Ca^{++} plays in the preconditioning response has not yet been fully clarified.

In a recent paper by Brew [94], a role for bradykinin in preconditioning has been suggested which also acts through the activation of PKC according to the authors of this study. This group worked with isolated rat hearts at warm temperatures and looked at the effects of exogenous bradykinin compared with transient ischemia and non-preconditioned controls on functional recovery of hearts subjected to a period of global ischemia followed by reperfusion. They found that recovery was improved in both bradykinin administered and ischemic preconditioned hearts compared to controls. The authors indicate that bradykinin acts through β_2 receptors to initiate a series of intracellular events which eventually culminate with PKC activation [94]. This statement was based on the fact that bradykinin treated myocardium demonstrated increased activation of specific PKC isoforms as measured by immunoflorescent staining. Furthermore, β_2 receptor blockade and PKC blockade with chelerythrine chloride eliminated the beneficial effects of bradykinin. β_2 receptor blockade also eliminated the

effect of transient global ischemia. The overall conclusion of this study was that transient-ischemic mediated preconditioning involves intrinsic bradykinin stimulation, and that bradykinin acts through β_2 receptors to activate PKC which plays an important role in preconditioning.

Because cardiac preconditioning seems to exert protective effects on myocardium during ischemia, it has definite implications on cardiac transplantation where hearts are stored in a cooled, ischemic state. Once the exact mechanisms are understood, it should be possible to pharmacologically simulate the preconditioned state to further minimize ischemic injury during cold storage. At present, the vast majority of work on the preconditioning phenomenon has been done in the setting of warm ischemia, and there is a paucity of information dealing with the effects that cold temperatures have on preconditioning. For instance, as was mentioned previously, preconditioning effects have time limits; it is not presently clear whether hypothermia has any effect on these limits. Also, temperature may have an effect on the stimulus required for the preconditioning phenomenon to take place. For instance, is the time required for the initial preconditioning ischemic event altered by changes in temperature? These questions need to be answered before maximal benefit of this area of investigation can effectively be applied in the transplant setting. In any case, cardiac preconditioning may indeed play an important role in future techniques involved in enhancing cardiac allograft preservation.

Improving Flush Solutions Through the Inclusion of Novel Substrates

Several investigators have shown that provision of an exogenous supply of high energy phosphates and their precursors to in-situ hearts during total myocardial ischemia enhances functional recovery after reperfusion and with some substrates helps to maintain myocardial intracellular ATP [95-97]. Petsikas was interested in the possible protective effects of the high energy precursors adenosine and adenosine monophosphate (AMP) on ischemic myocardium [97]. The model used in this study was that of a continuously perfused canine cardiac allograft stored for 24 hours before being heterotopically transplanted into a recipient dog. Three solutions were used during continuous infusion, a modified Krebs-Henseleit solution (Group 1), a modified Krebs-Henseleit solution with the addition of 20 μmol/L of adenosine (Group 2), and a modified Krebs-Henseleit solution with 0.1 mmol/L of AMP (Group 3). After the preservation period the development of myocardial edema was assessed by noting increased wet weight of the grafts for each specimen. Coronary vascular resistance changes were also measured along with biochemical determinations of tissue ATP, and lactate. After the heart was transplanted they then measured functional recovery through developed pressure recordings (peak systolic pressure - diastolic pressure), and the rate of pressure development. In all groups, weight of the grafts increased by up to 41% indicating the presence of edema, but no significant differences between groups were noted. Coronary vascular resistance however was significantly lower for the groups containing either adenosine or AMP when compared with Group 1 (modified Krebs-Henseleit alone). Both developed pressure and the rate of pressure development were

also found to be significantly improved by the addition of either adenosine or AMP. Only group 3 (0.1 mmol/L AMP) was found to have post ischemic ATP levels greater than control. The lack of effect of adenosine on ATP is consistent with the results of Foker [98] who found no change in tissue ATP levels for canine hearts perfused with adenosine containing solution [97]. It is apparent therefore that the improvement in performance of grafts supplied with adenosine must be secondary to processes other than ATP conservation. The authors suggest that this was possibly due to enhanced aerobic metabolism and/or improved lactate washout through improved perfusion, but this was not proven by their study. This study therefore suggests that continuous hypothermic perfusion is improved by the addition of either adenosine or AMP to the perfusate, and that mechanisms other than simply ATP conservation are involved.

Elgebaly [99] studied the effects of cyclocreatine phosphate (CCrP) administration on cardiac function when this compound was administered before an ischemic insult. Rats were injected with either saline of 500 mg/kg of CCrP and after 2 hours were arrested with UW solution. The hearts of rats injected with saline were stored at 4°C in standard UW solution while hearts of rats initially injected with CCrP were stored at 4°C in UW containing 100 mg CCrP (referred to by the authors as Hartford Hospital solution). The hearts of both groups were then reperfused after 6 hours for 15minutes in the Langendorff mode and 30 minutes in the working heart mode. They found that cardiac function, as measured by aortic flow, coronary flow, cardiac output, stroke volume, and stroke work were significantly better in the CCrP group. Also, the saline injected group had significantly higher weight gains of the heart

compared to the CCrP group indicating more cellular edema. The conclusion of the authors from this study was that CCrP treatment improved functional recovery after prolonged hypothermic preservation. This conclusion is supported by a study by Roberts [100] who showed that when administered prior to ischemia, ATP levels were sustained for longer periods during ischemia, and that it also delays the onset of cardiac contracture. The author points out that the mechanism through which CCrP spares myocardial function is unknown, but suggests that CCrP may accumulate intracellularly and during ischemia it may continue to phosphorylate ADP at lower pH levels than creatine phosphate is able. This is not likely correct however since it is very doubtful that such a polar molecule could cross the cell membrane.

Several investigators have looked at the potential role that branch-chained amino acids may play in preserving cardiac function during cardioplegia. As pointed out by Schwalb [101], during ischemia there is a depletion of amino acid precursors, (which are Kreb's cycle intermediates), and that this depletion may become a limiting factor in aerobic ATP synthesis upon reperfusion. The evidence that he puts forth in support of this hypothesis is that oxygen consumption during reperfusion is increased when amino acid precursors are added to cardioplegic solutions indicating increased Kreb's cycle activity. Rosenkranz [102] showed that aspartate-glutamate enriched blood cardioplegia solutions enhanced functional recovery of dog myocardium that was reperfused after suffering an ischemic insult and delayed the onset of ischemic contracture [101]. Pausescu [103] devised a cardiac preservation solution of 16 amino acids with other electrolytes and claimed that successful storage was possible for up to 72 hours using a

continuous perfusion apparatus. These results however have not been duplicated by other independent investigators. Schwalb studied the effects of glucose and branched chain amino acids on functional recovery and maintenance of high energy phosphates of rat heart subjected to 68 minutes of normothermic global ischemia. The groups studied included modified St. Thomas hospital solution alone (Group 1), modified St. Thomas hospital solution plus 11.1 mmol/L glucose (Group 2), and modified St. Thomas hospital solution plus 11.1 mmol/L glucose plus the branched chain amino acids leucine, isoleucine, and valine (Group 3). They found that mechanical recovery, (measured by aortic flow, aortic pressure, cardiac output, stroke volume, and minute work), as well as ATP and creatine phosphate maintenance, were all significantly enhanced in group 3 over groups 1 and 2 [101]. They also found a significant decrease in the release of LDH from the myocardium upon reperfusion in group 3 implying improved cellular integrity. Also of note was that glucose without the presence of branched chain amino acids was also significantly superior to the base modified St. Thomas hospital solution when the solution was oxygenated. No such advantage was demonstrable when the solution was not oxygenated. The authors suggest that oxidative phosphorylation is able to continue to some degree with oxygenated solution and that glucose administration is beneficial by supplying substrate. Since oxygen is not required for glycolysis, the inference from this study is that exogenously supplied glucose is beneficial only when it can be metabolized aerobically. Under conditions where glycolysis is the the only energy producing pathway, (ie: strict anaerobic conditions), exogenous glucose does not confer any advantage in terms of functional recovery.

Hearse [104] found that adding glucose to unoxygenated St. Thomas hospital solution caused a dose dependent reduction in cardioprotection, and that insulin exacerbated this detrimental effect. A similar study by von Oppell [105] and associates showed that while high physiologic concentrations of glucose (11.1 mmol/L) were beneficial to the heart during multidose cardioplegia, higher concentrations (20-50 mmol/L) were harmful [106]. Similarly, Opie and his associates [106] studied the mechanism by which different glucose concentrations exert either beneficial or harmful effects when added to a crystalloid cardioplegia solution. They measured rates of glycolytic flux, glucose oxidation, glycogenolysis, lactate efflux, glycolytic ATP production, and effluent pH. They also measured functional recovery after 20 minutes of reperfusion. They found that functional recovery on reperfusion was greatest with a glucose concentration of 11 mmol/L, and ATP production was also greatest at this glucose concentration. Lactate and proton production during cardioplegia was also found to be greatest at 11mmol/L of glucose in keeping with enhanced glycolysis. At glucose concentrations between 11 and 50 mmol/L of glucose, glycolytic flux from extracellular glucose was increased, but glycogenolysis was markedly decreased with an overall reduction in total glycolytic ATP production. These authors therefore speculate that there is an optimal balance between glycogen derived ATP and exogenous glucose derived ATP, and that this balance is disrupted if the concentration of exogenous glucose is high enough to inhibit glycogen phosphorylase activity, thus decreasing glycogenolysis. One theory that they propose is that glucose derived ATP is used preferentially by the cardiac sarcolemma for ionic homeostasis. In contrast they feel that

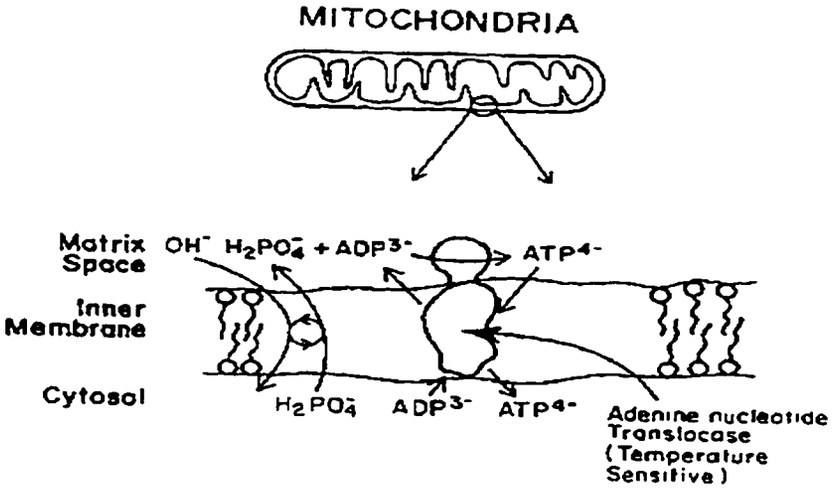
glycogen derived ATP may be utilized preferentially by the sarcoplasmic reticulum for calcium homeostasis [106]. This idea of compartmentalization within the cytosol of ATP is however speculation on the part of the authors and is not proven at this point.

Mitochondrial Functioning

In addition to detrimental effects at the cellular level, cold storage may adversely effect crucial subcellular organelles such as mitochondria. Maintaining the integrity of mitochondria during cold storage and upon reperfusion is vital in order to convert substrates into ATP for readily available energy during the reperfusion period. If a large enough proportion of mitochondria are damaged during the ischemic cold storage period, the transplanted heart may be unable to generate ATP levels sufficient for adequate performance. Belzer's group performed a study to determine the effect of cold storage in UW solution for up to 24 hours on mitochondrial function [107]. Results of this study indicated that mitochondrial function deteriorates within the first 6 hours of storage afterwhich no further deterioration is noted up to 24 hours. Their data also suggested that this functional deterioration occurred because of a reduction in electron transport in the inner mitochondrial membrane rather than uncoupling of the mitochondria. Furthermore, they found that this initial decrease in mitochondrial function was not of a sufficient magnitude to cause significant differences in mechanical performance when compared to control hearts. The overall conclusion of this study was than cold preservation techniques may protect the mitochondria from injury, and that mitochondrial function is not the limiting factor of extended cardiac preservation.

Southard and Belzer wrote a brief review of the effects of hypothermia on mitochondrial function as part of a chapter on organ preservation [1]. According to them, the primary effect of hypothermia on mitochondrial function is suppression of the rate of translocation of adenine nucleotides across the inner mitochondrial membrane: (see Figure II-3). This translocator exchanges intra-mitochondrial ATP for cytosolic ADP, and is not normally rate limiting for the formation of ATP under physiologic conditions. At 10°C or less however, it may become a limiting factor in oxidative phosphorylation with resultant impairment of ATP synthesis, although there is no recent evidence to support this [1, 108]. This is more likely of importance during the reperfusion period because during cold storage the major limiting factor on oxidative phosphorylation is a lack of oxygen, not nucleotide translocation.

Figure II-3: Schematic diagram of the mitochondrial adenine nucleotide translocase [4].



Buffering of Storage Solutions

The role of buffering in cardioplegic solutions was first proposed by Bretschneider and his associates [109]. It was their view that by limiting the tissue build up of lactate and hydrogen ions, glycolytic flux would be enhanced allowing for continual ATP production. Since then, many investigators have experimented with various buffers in both cardioplegic solutions used during cardiac surgery, and in preservation solutions used in the clinical transplant setting.

Kresh compared histidine with tromethamine (THAM) and bicarbonate in it's ability to act as a buffer in cardioplegic solutions and found that THAM and bicarbonate were both very limited in their ability to stabilize intracellular pH when compared to histidine [110].

In another experiment, Churchill and his collaborators studied the effect of amino acid buffers (histidine and carnosine) on glycolysis and energy levels in ischemic rat livers chilled on ice [111]. They showed that the addition of 180 mmol/l of histidine exhibited the most positive maintenance of adenylate levels when rat livers were flushed with preservation solution pre-ischemia, followed closely in performance by the addition of 90 mmol/l of carnosine. Histidine at a concentration of 90 mmol/L was least effective of the three buffered solutions tested. All three solutions were found to be superior in maintenance of tissue energetics when compared to a citrate based solution of minor buffering capacity. Buffering the solution also resulted in prolonged lactate production. The conclusion put forth by these authors was that the inclusion of efficient buffering

agents during periods of cold hypoxia supports flux through the glycolytic pathway and therefore has positive effects with regard to cellular energetic maintenance.

The University of Wisconsin solution utilizes phosphate as its primary buffer against intracellular H^+ accumulation secondary to anaerobiosis during ischemic storage [112]. Bretschneider's solution in comparison utilizes histidine which has substantially greater buffering capacity than phosphate. Sumimoto and his colleagues recognized that both Bretschneider's solution and UW solution had their own separate merits. UW solution for instance contains lactobionate which is a large impermeant molecule important for minimizing cellular edema. Bretschneider's solution on the other hand has almost twice the buffering capability of UW which may be important in increasing glycolytic flux [113]. The approach that Sumimoto and his colleagues took was to combine what they felt to be the most important constituents of each solution into a novel histidine-lactobionate solution. They modified UW solution by adding 90mmol of histidine to increase it's buffering capability and also omitting hydroxyethyl starch, adenosine, dexamethasone, and insulin. KH_2PO_4 (20 mM/L) was also added for buffering although in slightly lesser quantity than that found in the UW solution (25 mmol/L). As well, they altered Na^+ (increased) and K^+ (decreased) concentrations. They then compared graft survival rates of organs preserved in this new histidine lactobionate solution (HL) to organs preserved in standard UW solution. Experimental groups consisted of rats undergoing orthotopic liver or heterotopic heart transplantation. They found significantly higher graft survival for both livers and hearts preserved with the HL solution than for UW. They attributed this superiority to the

increased buffering capacity of the HL solution although biochemical data to support this is not presented in this study. Of note as well is that this new solution was found to have a much lower viscosity than standard UW solution (1.33 vs. 3.32) secondary to the omission of hydroxyethyl starch, making HL solution easier to flush.

Churchill extended the work of Sumimoto using rat livers to investigate the effects on tissue energy status of adding the histidine as a buffer to a preservation solution very similar to that formulated by Sumimoto [114]. These authors compared a histidine-lactobionate-raffinose solution (HLR) to University of Wisconsin (UW) solution in their ability to maintain tissue energetics in cold stored rat livers. In their experiments they showed increased levels of ATP, ADP and total adenylates with HLR over UW. Lactate production was also found to be twice as high in the HLR group supporting the hypothesis that increased buffering capability drives glycolysis. They also found beneficial effects of oxygenating the preservation solution before infusion.

del Nido [115] was interested in the effects of buffered cardioplegic solution on maintenance of tissue ATP levels, tissue pH, lactate production, and post arrest hemodynamic function of myocardium that was rendered ischemic at a temperature of 27°C. Their main interest was in optimizing cardioplegia within the venue of cardiac surgery, but their results have bearing on the issue of transplantation as well, in that the tissue effects of ischemia are of major importance in both situations. In their experiments they used four different cardioplegic solutions to maintain cardiac arrest in dog hearts rendered ischemic for a total of 4 1/2 hours. Two of the solutions used were unbuffered with either high K^+ (30 mEq/L), or low K^+ (10mEq/L). The other two solutions were

buffered with 195mmol/L of histidine with either high or low K^+ concentrations as in the unbuffered solutions. Results indicated that lactate production was markedly elevated in all groups but that they did not differ significantly despite the fact that exogenous glucose uptake was much greater in the unbuffered groups. Also, the pH of the coronary effluent was much closer to the physiologic range in the buffered group (~7.2) than in the unbuffered groups (6.6-6.8). ATP levels were also found to be much better preserved when the cardioplegic solution was buffered. The authors were dubious to conclude that the higher ATP levels were the result of enhanced glycolysis because of the fact that increased lactate production was not demonstrated. They were more of the opinion that the higher pH of the buffered group made the cell membrane less permeable to the high energy nucleotides and therefore buffering preserved ATP by preventing its loss to the extracellular compartment. With respect to functional recovery in the post-ischemic period, this study found that buffered low K^+ cardioplegia had significantly higher developed pressure during the first 15 minutes of reperfusion than the other 3 groups but did not improve over the rest of the reperfusion period. In contrast, the unbuffered groups continued to improve over the reperfusion period and ultimately reached levels that were not significantly different from the buffered low K^+ group. Of interest was that the combination of buffering and a high K^+ concentration seemed to have the worst recovery but this was not demonstrated to statistical significance. Consistent with this was the finding of increased myocardial necrosis histologically in this group. The explanation for this phenomenon was that the combination of a high K^+ and high pH increases the myocytes susceptibility to damage caused by low Ca^{++} in the

cardioplegia solution (ie: calcium paradox) which did not contain Ca^{++} salts. Overall this study highlighted the potential benefit of buffering capability of solutions used to perfuse the heart during ischemia. This study was however performed using temperatures significantly higher than those which are used in current transplant protocols and the results should be interpreted with this in mind.

Ohkado and his collaborators performed similar experiments with buffered cardioplegic solutions as del Nido but worked with hearts preserved at temperatures of 4°C, 13°C, and 21°C thus extending their results to cold ischemic hearts [75]. These investigators also used histidine as their intracellular buffer citing several reasons for this choice. With a pKa of 6.8 at 25°C l-histidine is a potent proton buffer (even at low temperatures), and is also the amino acid most responsible for normal intracellular buffering capacity. They also refer to the ability of histidine to bind calcium intracellularly which may be beneficial in that intracellular Ca^{++} overload has been shown by several authors to be harmful. Their solution consisted of a Krebs-Henseleit solution with the addition of 20mmol KCl and 100mmol of histidine. Initially they compared this solution with and without the histidine with respect to maintenance of ATP and PCr and found that the addition of buffer led to much higher cytosolic concentrations of histidine, better preservation of intracellular ATP and PCr, and much better maintenance of intracellular pH (6.8 unbuffered vs. 7.5 buffered). On the basis of these results they then compared their buffered Kreb's solution to University of Wisconsin solution in terms of development of contracture as measured by an increased diastolic pressure over an ischemic period of 8 and 16 hours, and of post-ischemic functional recovery after the

same time periods measured by left ventricular developed pressure. These solutions were compared at the temperatures mentioned previously. Results of this study indicate that contracture development was significantly delayed in buffered Kreb's solution compared to UW, and that left ventricular developed pressure after 30 minutes of reperfusion was greater in the modified Kreb's buffer solution. The authors attributed the better functional outcome of the buffered groups to superior maintenance of tissue energetics even though they did not demonstrate conclusively through direct assays that ATP and PCr levels are better preserved. Lactate production was however shown to be enhanced in the presence of buffer providing evidence that glycolysis is augmented. They also found that when buffered solution was compared over several temperatures, that both tissue energetics and functional recovery was better at 13°C than it was at 4°C and 21°C. This study therefore supports the use of buffered solutions in cardiac preservation which is likely at least in part due to improved glycolytic flux.

Concluding Statement

In the 20th Century, many great advances have been made by innovative scientists and clinicians in the field of organ transplantation. Cardiac transplantation has now become a standard therapy for selected patients suffering from end-stage heart failure. Many obstacles remain to be overcome however, despite these tremendous advances, before the full potential of cardiac transplantation as a therapeutic modality can be fully realized. One obvious limitation currently is the mismatch between the number of eligible patients who would benefit from cardiac transplantation, and the number of

donor grafts that are available. Other problems which are still unsolved include post-transplant cardiac failure and rejection. Beneficial effects in all of these problem areas could be obtained through improvements in methods used to preserve the graft during the ischemic period. With an improved storage time, not limited to the current 4-6 hours, hearts could be harvested from a much greater population base thus expanding the donor pool and making more hearts available to those requiring a transplant. In addition, impoved preservation of allografts may increase the size of the donor pool by allowing for more "marginal" hearts to be used. Another benefit of increasing the available time between harvest and transplantation is that this would allow for improved tissue matching between the donor and recipient leading to less problems with graft rejection, and better preservation of function would mean less mortality, secondary to lower incidence of post-operative graft failure.

It is clear that our understanding of the pathophysiology of myocardial ischemia is far from complete. As our knowledge about these processes grows, we will be able to gain insight into the underlying mechanisms involved in ischemic damage during hypothermic organ storage and subsequently devise preservation solutions to successfully prolong the safe storage time of cardiac grafts.

The overall objective of the experiments presented in this thesis is the improvement of solutions used clinically for cardiac preservation to allow for longer safe storage of allografts. The major focus is on enhancing the buffering capacity of solutions; the main hypothesis being that the provision of effective intracellular buffers will allow for increased glycolytic energy production by alleviating pH inhibition of

glycolytic flux resulting in better maintenence of tissue energetics during cold storage. Other possible beneficial manipulations in solution and harvesting technique are investigated. The effects of a brief perfusion period with oxygenated solution immediately following cardiac harvest were studied, as well as the addition of substrate in the form of exogenous glucose with insulin. The hope is that by improving the ability of the heart to maintian energetics during cold ischemia, damage to the myocardium will be attenuated, and that this will eventually allow for longer preservation periods.

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Chapter III: General Materials and Methods

Chemicals

All chemicals were of AR grade and were purchased from Sigma Chemicals or BDH.

Animals

Adult Yorkshire-Landrace pigs (35 - 40kg) obtained from the University of Alberta swine farm were used as cardiac donors. All animals were treated humanely in accordance with the regulations set forth by the Canadian Council on Animal Care (Animal Protection Act). The experiments were approved by the University of Alberta Health Sciences Animal Welfare committee under protocol #126/03/97.

Experimental Solutions

The components of the solutions used in the course of the experiments are outlined in Table III-1. Not every solution outlined was used in each individual experiment. In the instance where the same solution was used in each experiment, components were kept constant.

Table III-1: Components of experimental preservation solutions.

Component Compounds	St. Thomas Hospital Solution II (mmol/l)	Modified University of Wisconsin Solution (mmol/l)	Buffer**-Lactobionate-Raffinose Solution (mmol/l)	Histidine-Glucose-Insulin Solution (mmol/l)
Adenosine		5	5	5
Buffer**			90	
$CaCl_2$	1.2			
Glucose				11.1
Histidine				90
Insulin				100 U/l
K_2PO_4		25	25	25
KCl	16			
KOH		90	90	90
Lactobionate		90	90	90
$MgCl_2$	16			
$MgSO_4$		5	5	5
NaCl	110			
$NaHCO_3$	10			
NaOH		25	25	25
pH	7.4	7.4	7.4	7.4
Raffinose		25	25	25

** Experimental buffers used in the course of these experiments were bicine (N,N-bis[2-Hydroxyethyl]glycine), histidine, or BES (N,N-bis[2-Hydroxyethyl]-2-aminoethane-sulfonic acid)

Surgical Procedure for Cardiectomy

The pigs were anaesthetized with intramuscular ketamine 3500 mg (87.5-100 mg/kg) The animal's heart rate and oxygen saturation were continuously monitored with a Nellcor N-180 pulse oximeter attached to the ear to ensure an adequate oxygenation of 95 - 100%. A median sternotomy was then performed and the animal was ventilated by manual bagging once the chest was opened. The inferior vena-cava (IVC) and superior

vena cava (SVC) were then dissected circumferentially so that ligatures could be placed around them; the ligatures however were not tied at this time. The pericardium was then opened and the right atrial appendage was retracted to expose the aortic root. The adventitial layer was sharply removed to facilitate passage of the aortic cannula, and the aorta was subsequently cannulated. Once cannulation was complete the IVC and SVC were ligated with the previously placed ligatures, and the heart was emptied by allowing it to beat for several cycles. The aorta was then cross-clamped distal to the cannula and 350 cc of the experimental solution (previously cooled to 4 °C and stored on ice) was infused at a pressure head of approximately 100 cm H_2O. The time needed to infuse this volume of preservation solution was approximately 1 minute. The hearts arrested within several seconds of the start of the infusion and very quickly there was a noticeable lightening in the color of the myocardium. A small incision was made in the right atrium to allow drainage of the effluent. As the hearts were being perfused the apex was removed and immediately 'snap frozen' in liquid nitrogen using Wollenberger clamps. This sample was designated the initial t=0 sample. When the infusion was complete the hearts were rapidly excised and stored over a 24 hour period at 4 °C in 300 cc of the same solution used to flush the heart. Additional transmural tissue samples from the left ventricle were taken at 1, 2, 4, 10 and 24 hours and were also 'snap frozen' in liquid nitrogen using Wollenberger clamps. All samples were stored at -65°C until being processed.

Tissue Preparation for Metabolite Assays

The samples of heart tissue, (100-200 mg), were weighed out in polypropylene test tubes and were then stored on dry ice until being homogenized in 6% perchloric acid (containing 1mM EDTA) using an Ultra-Turrax homogenizer. A 50µl aliquot of the homogenate was removed and placed in 1 ml of 100 mM sodium acetate for glycogen determination. The samples were then immediately centrifuged at 4 °C for 15 min at 3000g to remove precipitated proteins. The supernatant was then transferred to a 1.5 ml microcentrifuge tube, neutralized with the addition of 3 M KOH/.4 M Tris/0.3 M KCl, and then recentrifuged. The supernatants were placed on ice and immediately assayed for PCr, ATP, ADP, and AMP. These assays were performed enzymatically based upon the absorbance of NADH at 340 nm using a Dynatech MRX plate reader. Glycogen determination in the homogenate/acetate solution was accomplished by incubating the solution in amyloglucosidase from Aspergillus niger at 55 °C for 24 h to allow the degradation of glycogen to glucose [1]. The solution was adjusted to pH 8.0 with the addition of 10 µl of 4 M Tris buffer, and total glycogen was then assayed as glucose utilizing the enzymatic glucose assay described by Passonneau and Lowry [2].

Metabolite Assays

All metabolite assays used are based on the oxidation or reduction of the purine nucleotide NAD(H) monitored spectrophotometrically at 340 nm using a Dynatech MRX plate reader. An aliquot of the sample extract of known volume was placed in 200 µL of the appropriate reaction mixture in a well of a 96 well plate. The initial absorbance of this

mixture was recorded, and the appropriate enzyme was then added to initiate the reaction. The progress of the reaction was monitored using standard amounts of the measured metabolite, and upon completion of the reaction, a final absorbance reading was recorded. Blank wells containing only added enzyme and reaction mixture with no sample extract were also recorded to be factored into the final calculation.

A calibration curve for the absorbance of NAD(H) for the plate reader was produced using standard amounts of NAD(H). The amount of NAD(H) converted in the well was then measured using the plate reader by calculating the absolute difference between the final and initial readings. The assays used in these experiments were based on either the oxidation or reduction of the substrates of interest based on the following general reaction:

(Oxidation of Substrate)

$$\mathbf{A + B \rightarrow C + D}$$

$$\mathbf{C + NAD^{+} \rightarrow E + NADH + H^{+}}$$

(Reduction of Substrate)

$$\mathbf{A + B \rightarrow C + D}$$

$$\mathbf{C + NADH \rightarrow E + NAD^{+}}$$

The molar quantity of NAD(H) converted, (as measured by the plate reader), was therefore equal to the molar quantity of the measured substrate, with the exception of AMP which was one half the molar quantity converted. The specific assays used for these experiments were as follows:

ATP and PCr

PCr + ADP → creatine + ATP (Creatine Kinase)

ATP + glucose → ADP + glucose-6-phosphate (Hexokinase)

glucose-6-phosphate + NAD^+ → 6-P-gluconolactone + NADH + H^+ (G6PDH)

Hexokinase (0.2 U) was added to a well containing Tris buffer (50 mM; pH = 8.0), NAD^+ (1 mM), $MgSO_4$ (5 mM), glucose-6-phosphate dehydrogenase from Leuconostoc mesenteroides (0.2 U), ADP (500 μM), and 20 μl of neutralized sample extract. The change in optical density due to ATP utilization was then recorded at the beginning and end of the reaction giving a value for the molar amount of ATP in the well. Creatine Kinase (1 U) was then added and the change in optical density secondary to creatine phosphate utilization was recorded.

ADP and AMP

AMP + ATP → 2AMP (Myokinase)

ADP + phosphoenolpyruvate → ATP + pyruvate (Pyruvate Kinase)

pyruvate + NADH + H^+ → lactate + NAD^+ + H_2O (Lactate Dehydrogenase)

Pyruvate kinase (0.3 U) was added to the well containing Tris buffer (50 mM; pH=7.0), NADH (200 μM), $MgSO_4$ (5 mM), KCl (50 mM), ATP (400 μM), phosphoenolpyruvate (500 μM), lactate dehydrogenase (1 U), and 20 μl of neutralized

sample extract. The optical density change due to ADP utilization was recorded giving a value for the molar quantity of ADP in the well. Myokinase (0.2 U) was then added and the change in optical density due to AMP utilization was recorded.

Lactate

lactate + NAD^+ → pyruvate + NADH + H^+ (Lactate Dehydrogenase)

pyruvate + hydrazine → pyruvate-hydrazone

L-Lactate dehydrogenase (1 U) from bovine heart was added to a well containing hydrazine buffer (150 mM; pH=9.6), NAD^+ (2 mM), and 10 µl neutralized extract. The change in optical density due to lactate utilization was recorded.

Glycogen

glycogen → glucose (Amyloglucosidase)

glucose + ATP → glucose-6-phosphate + ADP (Hexokinase)

glucose-6-phosphate + NAD^+ → 6-P-gluconolactone + NADH + H^+ (G6PDH)

Amyloglucosidase from Aspergillus niger was added to the acetate/homogenate solution (pH=4.5) and incubated for 24 h at 55 °C to allow the degradation of the glycogen to glucose[1]. Ten µl of Tris (4 M) was added to the acetate/homogenate solution upon completion of the conversion of glycogen to glucose. The total glycogen

was then assayed as glucose via the standard enzymatic glucose assay described by Lowry and Passonneau[2].

Glycogen levels are reported in terms of glucose units in μmol/g wet wt.; 1000 μmol/g wet wt. = 162 mg/g wet wt.

Statistics

Data are reported as means ± SEM for 4 to 6 hearts. Metabolite levels are reported in terms of μmol/g wet tissue weight. Metabolite measurements at the individual time points for each of the experimental groups were compared to the control group using an analysis of variance (ANOVA) followed by a Dunnett's *post hoc* comparison; $p<0.05$ are reported.

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Chapter V: Experiment #1

Effects of enhanced buffering of preservations solutions on glycolytic flux and tissue energetics in cold-stored pig hearts.

Introduction:

Cardiac transplantation has now assumed a position as a genuine therapeutic option for patients suffering from end-stage cardiac disease. The development of this mode of treatment has raised the need for safe storage methods of donor hearts. The University of Wisconsin (UW) solution, introduced in 1987, proved itself to be very successful in the preservation of liver, kidney, and pancreas, with significant prolongation of the safe cold-ischemic time of these organs [1]. While studies have shown that UW solution can be used for cardiac allograft preservation [2-4], the safe storage time remains a relatively short 4 to 6 hours [4, 5] Prolonging storage times would be advantageous by allowing for more distant organ procurement resulting in a larger effective donor pool, by allowing for better preparation of the recipient and surgical teams, and by providing a greater margin of safety for heart transplant procedures [1, 4]. Therefore, one objective in the development of any novel preservation solutions or techniques should be to extend the current acceptable time limitations.

In order to maintain myocardial viability during storage, tissue energy levels must be conserved. Stringham and colleagues showed that ischemic myocardial contracture correlated with a decrease in cellular ATP to less than 80% of pre-ischemic values [5]. In addition, high levels of ATP are needed by the heart in the early reperfusion period in

order for excitation-contraction coupling to function effectively. Therefore in order for the heart to sustain the patient's circulation upon re-implantation, the myocardium must either have an immediate supply of ATP, or the means to quickly regenerate ATP.

Tissue energy levels during the preservation period are reflected by a delicate balance between energy production by the organ, and energy utilization. Strategies to improve energetics, can take the form of increasing ATP synthesis, or decreasing its consumption. In clinical practice, both of these approaches are feasible and can be used together in a complimentary manner. Organs for transplantation are generally cooled during the storage period. Since all enzymatic reactions within the cell are temperature dependent, cooling the organ slows down cellular metabolism resulting in less ATP consumption by these reactions. This is often expressed in the literature as the Q_{10} effect which is the ratio of a reaction rate at two temperatures separated by 10 °C [6]. For instance, a Q_{10} of 2 indicates that a 10 °C decrease in temperature will half the rate of a given reaction. Decreasing the rate of metabolism through hypothermia is however not sufficient to maintain energetics, because ATP is still being consumed albeit at a slower rate. This serves to highlight the importance of enhancing energy production during cold-storage.

Currently utilized methods of cardiac transplantation necessitate a period of cold-ischemia lasting from the time the heart is removed from the donor, until perfusion is reestablished in the recipient. During this time, the only available source of energy needed by the cardiomyocyte to maintain viability is through anaerobic glycolysis. Energy production through glycolytic flux is limited however by the accumulation of

protons under anaerobic conditions. It has been shown that enzymes controlling flux through the glycolytic pathway, particularly phosphofructokinase (PFK), are inhibited by the pH decline that occurs during ischemia. It was hypothesized in the current study that increasing the buffering capacity of preservation solutions would enhance glycolytic energy production by relieving the pH inhibition of enzymes needed for continued metabolism through the pathway.

Materials and Methods:

Chemicals

All chemicals were of AR grade and were purchased from Sigma Chemicals or BDH.

Animals

Adult Landrace-Yorkshire pigs (35 - 40kg) obtained from the University of Alberta swine farm were used as cardiac donors. All animals were treated humanely in accordance with the regulations set forth by the Canadian Council on Animal Care (Animal Protection Act). The experiments were approved by the University of Alberta Health Sciences Animal Welfare committee under protocol #126/03/97.

Experimental Groups

The pigs used in these experiments were assigned to one of 5 experimental groups with an n=4-6 for each group. The groups differed only in the solution used to

flush and store the hearts with other factors being held constant. St. Thomas Hospital solution II served as the control group because of its wide-spread use in clinical transplantation, and because of its limited buffering capacity compared to the other experimental groups (see Figure II-1 for titration curves of solutions used). The solutions used for each of the experimental groups were adjusted to a pH of 7.4 and were composed as follows:

Group 1) (Control) St. Thomas Hospital solution II containing sodium chloride (110 mmol/l), potassium chloride (16 mmol/l), magnesium chloride (16 mmol/l), calcium chloride (1.2 mmol/l), and sodium bicarbonate (10 mmol/l)

Group 2) Modified UW solution containing lactobionate (90 mmol/l), raffinose (25 mmol/l), potassium hydroxide (90 mmol/l), sodium hydroxide (25 mmol/l), magnesium sulphate (5 mmol/l), potassium phosphate (25 mmol/l), and adenosine (5 mmol/l).

Group 3) BES solution which was identical to the modified UW solution with the addition of N,N-bis[2-Hydroxyethyl]-2-aminoethane-sulphonic acid (90 mmol/l).

Group 4) Bicine solution which was identical to the modified UW solution with the addition of bicine (90 mmol/l).

Group 5) Histidine solution which was identical to the modified UW solution with the addition of histidine (90 mmol/l).

Surgical Procedure for Cardiectomy

The pigs were anaesthetized with intramuscular ketamine 3500 mg (87.5-100 mg/kg). The animal's heart rate and oxygen saturation were continuously monitored with a Nellcor N-180 pulse oximeter attached to the ear to ensure an adequate oxygenation of 95 - 100%. A median sternotomy was then performed and the animal was ventilated by manual bagging once the chest was opened. The inferior vena-cava (IVC) and superior vena cava (SVC) were dissected circumferentially so that ligatures could be placed around them; the ligatures however were not tied at this time. The pericardium was opened and the right atrial appendage was retracted to expose the aortic root. The adventitial layer was sharply removed and the aorta was cannulated. Once cannulation was complete the IVC and SVC were ligated with the previously placed ligatures, and the heart was emptied by allowing it to beat for several cycles. The aorta was then cross-clamped distal to the cannula and 350 cc of the experimental solution (previously cooled to 4 °C and stored on ice) was infused at a pressure head of approximately 100 cm H_2O. The time needed to infuse this volume of preservation solution was approximately 1 minute. The heart arrested within several seconds of the start of the infusion and very quickly there was a noticeable blanching of the myocardium. A small incision was made in the right atrium to allow drainage of the effluent. As the heart was being perfused the apex was sharply excised and immediately

'snap frozen' in liquid nitrogen using Wollenberger clamps. This sample was designated the initial t=0 sample. When the infusion was complete the heart was rapidly excised and stored over a 10 hour period at 4 °C in 300 cc of the same solution used to flush the heart. Additional transmural tissue samples from the left ventricle were taken at 1, 2, 4, and 10 hours and were also 'snap frozen' in liquid nitrogen using Wollenberger clamps. All samples were stored at -65°C until being processed.

Sample Preparation and Metabolite Assays

The samples of heart tissue were weighed out and homogenized in 6% perchloric acid (containing 1mM EDTA). A 50µl aliquot of the homogenate was removed and placed in 1 ml of 100 mM sodium acetate for glycogen determination. The samples were then immediately centrifuged at 4 °C for 15 min at 3000g to remove precipitated proteins. The supernatant was then withdrawn, neutralized with the addition of 3 M KOH/.4 M TRIS/0.3 M KCl, and then recentrifuged. The protein pellet remaining was saved for determination of wet/dry weight ratios. The supernatants were placed on ice and immediately assayed for PCr, ATP, ADP, and AMP. These assays were performed enzymatically based upon the absorbance of NADH at 340 nm using a Dynatech MRX plate reader. Glycogen determination in the homogenate/acetate solution was accomplished by incubating the solution in amyloglucosidase from Aspergillus niger at 55 °C for 24 h to allow the degradation of glycogen to glucose[7]. The solution was adjusted to pH 8.0 with the addition of 20 µl of 4 M Tris buffer, and total glycogen

was then assayed as glucose, utilizing the enzymatic glucose assay described by Passonneau and Lowry[8].

The protein pellet remaining after the first centrifugation was dried for 24 h at 60 °C and weighed for determination of the sample's dry weight. Tissue H_2O content was calculated from the wet/dry weight ratios according to the formula:

$$\% H_2O = (\text{wet weight} - \text{dry weight})/\text{wet weight} * 100$$

Statistics

Data are reported as means ± SEM for 4 to 6 hearts. Metabolite levels are reported in terms of μmol/g wet tissue weight. Metabolite measurements at the individual time points for each of the experimental groups were compared to the control group using an analysis of variance (ANOVA) followed by a Dunnett's *post hoc* comparison; $p<0.05$ are reported.

Results:

Tissue Water Content:

Calculation of tissue water content revealed no significant changes over the 10 h experimental time course within or between groups. Water content of all samples pooled together averaged 64.7% ± 0.7.

Phosphocreatine (see Figure IV-1):

The phosphocreatine level in the control group (STHS II) was (6.4 μmol/g wet wt.) immediately following the flush (t=0). Initial levels in the modified UW, BES, bicine and histidine were 9.7, 11.3, 13.4, and 9.6 μmol/g wet wt. respectively which were significantly higher than control ($p<0.05$). After the first hour of storage, levels in all of the experimental groups showed a rapid decline of between 22% (modified UW) to 58% (BES) compared to initial values. Levels after 1 h of storage in the experimental groups ranged from 4.7 μmol/g wet wt. (BES), to 7.8 μmol/g wet wt. (Bicine); none were significantly different from control (6.5 μmol/g wet wt. at t=1 h). Over the remaining experimental time course, PCr decreased at a much slower rate in all groups. The modified UW group decreased a further 28% from initial values to 4.8 μmol/g wet wt. Further declines in the BES, bicine, and histidine groups were 9%, 22%, and 24%, respectively, to yield final values of 3.7, 4.9, and 4.6 μmol/g wet wt.. The control group in comparison declined to 4.6 μmol/g wet wt. representing an additional 30% decrease from t=0 for the time points after the first hour. In summary, although values in the experimental groups were 3-7 μmol/g wet wt. higher than control at t=0, subsequent values were not significantly different from PCr levels in the STHS II group.

ATP (see Figure IV-2):

The initial ATP levels did not differ between the control group (9.9 μmol/g wet wt.), and the experimental groups, which ranged in value from 9.0 μmol/g wet wt. (modified UW) to 14.9 μmol/g wet wt. (bicine). Over the experimental time course,

control group levels did not change significantly from the initial value, with a mean level of 11.2 μmol/g wet wt. at the end of the 10 h storage period. ATP levels were variable in all groups over the first 4 h of storage, but after 10 h storage, levels are significantly higher in 3 of the 4 experimental groups when compared to control. The bicine experimental group showed the greatest difference when compared to control with a 34% greater mean ATP level (15.93 μmol/g wet wt.) at 4 h ($p<0.05$), and a 72% greater mean (19.14 μmol/g wet wt.) at 10 h ($p<0.05$). The modified UW and histidine based solutions had mean levels 60% (17.78 μmol/g wet wt.), and 53% (17.08 μmol/g wet wt.) greater than control at 10 h respectively ($p<0.05$). These results are consistent with prolonged anaerobic energy production in the buffered experimental groups.

ADP and AMP (see Table IV-1):

All groups were found to have ADP and AMP levels which were relatively low compared to ATP levels. The initial ADP level in the control group was 2.3 μmol/g wet wt. compared to modified UW, BES, bicine, and histidine groups which were found to have levels of 1.8, 1.3, 2.4, and 1.6 μmol/g wet wt. respectively. BES was the only experimental group found to be different from control at t=0 ($p<0.05$). At the end of 10 h of storage, increases in mean ADP levels were found in the control, bicine, and histidine groups ($p<0.05$). The mean level at 10 h for the control group was 3.2 μmol/g wet wt. representing a 40% rise from initial values. ADP increases from initial levels in the bicine (3.0 μmol/g wet wt. at 10 h), and histidine (2.6 μmol/g wet wt. at 10 h) groups, were 27% and 60% respectively.

AMP levels at t=0 ranged from 0.22 μmol/g wet wt. (control) to 0.51 μmol/g wet wt. (bicine). The only group to show a significant change in value during the storage period was the modified UW group which increased from 0.30 μmol/g wet wt. at t=0 to 0.59 μmol/g wet wt. at 10 h ($p<0.05$). None of the experimental groups were significantly higher than control at the end of storage.

Total Adenylates (see Figure IV-3):

Trends found for total adenylates (ATP+ADP+AMP) were very similar to those seen for ATP because of its relative abundance compared to ADP and AMP in all tissue samples. Total adenylates at t=0 ranged from 11.0 μmol/g wet wt. (modified UW) to 17.8 μmol/g wet wt. (bicine) in the experimental groups. Control samples had an initial mean level of 12.4 μmol/g wet wt. which differed only from the bicine group ($p<0.05$). Over the 10 h storage period, no significant rise in total adenylates were found in the control and BES groups. In contrast, the modified UW group showed an 88% increase in levels from t=0 (20.6 μmol/g wet wt.) ($p<0.05$), while the bicine and histidine groups demonstrated increases of 36% (24.2 μmol/g wet wt.), and 33% (21.6 μmol/g wet wt.) respectively ($p<0.05$). The measured increases in these experimental groups reflect final total adenylate levels that were 47% higher than control in the modified UW group ($p<0.05$), 72% higher in the bicine group ($p<0.05$), and 54% higher in the histidine group ($p<0.05$).

Energy Charge (see Table IV-2):

Immediately following the myocardial flush (t=0), energy charge [(ATP+1/2ADP)/ATP+ADP+AMP] ranged from 0.89 (modified UW) to 0.93 (BES) in the experimental groups. Energy charge in the control group was 0.89 and differed significantly only from the BES group ($p<0.05$). At 10 h, energy charge in the control group had declined to 0.85, and was statistically lower ($p<0.05$) than all of the experimental groups which showed values between 0.90 (BES) and 0.92 (bicine).

Lactate Accumulation (see Figure IV-4):

Anaerobic metabolism in the myocardium was measured through the accumulation of lactate in the tissue over the 10 h experimental time course. All groups showed significant increases in lactate ($p<0.05$) from initial values within the first 2 hours of storage. Increases continued for all groups from 2 h to 10 h indicating constant flux through the glycolytic pathway. At 2 h of storage, lactate accumulation was 9.7, 13.1, 9.7, and 9.1 μmol/g wet wt. for modified UW, BES, bicine, and histidine groups respectively; these increases were all significantly higher than control ($p<0.05$) which showed a total accumulation of 4.2 μmol/g wet wt. over the first 2 h. At 10 h these significant differences were maintained; total accumulation in the experimental groups ranged between 23.1 (bicine), and 27.5 μmol/g wet wt. (BES), representing more than a 100% increase in glycolytic activity over STHS II (11.7 μmol/g wet wt.) ($p<0.05$). Among the experimental groups, no statistically significant differences were found at 10 h.

Glycolytic Substrate (see Figure IV-5):

Initial glycogen levels in the myocardium, (measured as glucose units), ranged from 26.5 μmol/g wet wt. (STHS II) to 39.5 μmol/g wet wt. (BES). Glycogen levels in all groups declined in a linear fashion, and was statistically significant at 10 h for all solutions ($p<0.05$). The total decline over 10 h ranged from 10.3 μmol/g wet wt. (bicine) to 12.4 μmol/g wet wt. (histidine). These values correlated well with the accumulation of lactate in all of the experimental groups, but not with the control group. In the case of the control group, glycogen decrease was approximately twice that expected from the total lactate accumulation measured in that group leaving approximately 5.5μmol/g wet wt. of glycogen unaccounted.

Discussion:

Current techniques utilized for cardiac transplantation involve a period of ischemic cold-storage during which time the nutrient, and oxygen supply to the organ is interrupted. Under these conditions, damage to the myocardium inevitably occurs and is cumulative with time. The injury sustained by the myocardium is at least in part related to the depletion of high energy molecules (ATP, PCr) during cold storage. Depletion of the tissues energy status leads to intracellular ionic dyshomeostasis, and is the result of a lack of fuel for the cellular ion pumps that regulate intracellular ion concentrations, (particularly Na^{+} and Ca^{++}) [9]. Experimental evidence suggests that ischemic

contracture of the graft with resultant non-function can occur with ATP drops as little as 20% from pre-ischemic levels [5, 10].

Levels of high energy molecules in the cold-stored myocardium reflect a balance between the production and utilization of these compounds. The current study addresses the production side of this delicate balance. In the anaerobic state, glycolysis becomes the primary source of energy production [11]. In terms of energy yield, glucose metabolism through this pathway is inefficient, producing only 5 - 8%, (depending upon the source of glucose), of the ATP produced by normal oxidative metabolism [12]. In order to compensate for this relative inefficiency, flux through glycolysis must increase in order to meet the organ's energy demands.

In this study, we investigated the effects of buffering capacity of preservation solutions on maintenance of high energy molecules (ATP, PCr) during ischemic cold storage over a 10 h experimental time course. We chose a 10 h storage time because this represents an approximate doubling of the 4 to 6 hours which is currently considered safe for cardiac allografts in the clinical transplant setting. Three key regulatory enzymes control flux through the glycolytic pathway when endogenous substrate is utilized under ischemic conditions: glycogen phosphorylase, phosphofructokinase (PFK), and glyceraldehyde 3-phosphate dehydrogenase [12]. Of these enzymes, the pH sensitivity of PFK in particular has been well documented [13, 14]. During anoxic storage, intracellular proton accumulate through anaerobic metabolism results in a decrease in intracellular pH causing PFK to undergo a reversible hysteretic loss of activity [14]. Glycolysis is therefore self-limiting when protons are allowed to accumulate in the tissue

by inhibiting enzymatic activity needed for continued flux through the pathway. By providing intracellular buffers, as proposed by Bretschneider in 1975 [15], we have the means available to 'soak up' some of these excess protons and therefore lessen the pH decline that results in an inhibition of glycolytic energy production.

The data presented in this study are consistent with our hypothesis that tissue energetics of cold-stored myocardium can be enhanced through increased buffering of the preservation solution. St. Thomas Hospital solution II (Group 1) was our control solution, and is widely used in clinical cardiac transplantation centres in Canada. This solution utilizes $NaHCO_3^-$ as its primary buffer [16]. We measured the buffering capacity of STHS II compared to our experimental solutions over a pH range of 6.0 to 7.4 to be considerably lower, ranging between 7% (BES) and 30% (modified UW) (See Figure II-1). Over the first 4 hours of storage, no definitive trends were found with respect to ATP levels and little difference was noted between the buffered solutions and control. Over longer periods of storage however, differences were definite. After 10 h of cold ischemia, 3 of the 4 experimental groups, (modified UW, bicine, and histidine), showed ATP levels that were 53% to 72% higher than control ($p<0.05$). We did not find a linear correlation between the absolute buffering capacity of the solution and final ATP levels at 10 h. Although the BES solution was the most highly buffered, no significant difference was found between it and control at the end of the storage period while differences were significant for the other experimental groups despite their lower buffering capacity. It is unclear as to why the increased glycolytic activity in the BES group, as shown by increased lactate production, did not translate into higher ATP levels

at the conclusion of the ischemic period. One possibility is that the presence of BES causes increased ATP utilization, perhaps by stimulating other energy employing reactions within the cardiomyocyte that the other buffers do not, or it may be that BES is actively transported across the membrane causing increased ATP usage .

Evidence that the increased myocardial ATP after 10 h of storage was secondary to enhanced glycolytic ATP production, is provided by lactate accumulation in the tissue over the cold storage period. Since lactate is the end point of the glycolytic pathway in anoxic tissue, increased lactate production by inference signifies increased flux through glycolysis. In the present study, lactate increased linearly in all groups over time consistent with the expected transition from aerobic to anaerobic metabolism. Total lactate accumulation was significantly higher ($p<0.05$) in all of the buffered groups compared to control at the conclusion of the storage period thus providing confirmation of augmented glycolytic activity in the buffered groups.

An important consideration with respect to lactate accumulation is the inhibitory effects that it has on anaerobic metabolism. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a glycolytic enzyme which catalyzes the conversion of glyceraldehyde-3-phosphate to 3-phosphoglyceroyl phosphate. Activity of this enzyme has been shown experimentally to be inhibited both by pH decline, and by lactate [17]. Mochizuki showed a 50% inhibition of GAPDH activity in hearts perfused with 20 mM lactate [17]. This may explain why little difference in ATP levels were found between the non-control groups in our study despite their wide range of buffering capacities. Similar to procurement methods used clinically, our study focused on a static storage model

whereby the heart is simply flushed with the preservation solution and then hypothermically stored. With no perfusion during storage, lactate accumulates as we and others have already shown. Although buffering addresses the problem of pH inhibition of glycolytic flux, metabolism will eventually be limited by other end-products such as lactate. One possible solution to this problem would be to either intermittently, or continuously perfuse the organ during storage. This would allow removal of inhibitory end-products such as lactate, therefore maximizing the potential benefits of solution buffering.

Since no exogenous glucose was supplied in the solutions used in this experiment, all glycolytic substrate came from intracellular glycogen stores. As expected, glycogen levels in the tissue declined in all groups over 10 h of storage ($p<0.05$). Based on the stoichiometric conversion of carbon from glycogen, 2 μmol of lactate should be produced for every 1 μmol of glucose from glycogen that enters glycolysis, assuming that all of the glycogen eventually is metabolized to lactate. Our buffered experimental solutions demonstrated a good correlation between lactate production and glycogen utilization with maintenance of the expected 2:1 ratio. This was not observed in the control group in which lactate increase was half of what was expected from the amount of glycogen that was mobilized. An explanation for this discrepancy is that alternative end-products such as glycerol or l-alanine were formed in lieu of lactate. Of these, glycerol seems the most likely alternative end-product under the conditions of the current study. As stated in the previous discussion, GAPDH catalyzes the conversion of glyceraldehyde-3-phosphate to 3-phosphoglyceroyl phosphate. In

situations where GAPDH is inhibited, glyceraldehyde-3-phosphate can be converted to glycerol. Investigators have shown that GAPDH is inhibited by not only by lactate, but by H^+ accumulation as well [17, 18]. Therefore, in the poorly buffered STHS II control group, a more rapid intracellular pH decline may lead to shunting of glycogen based carbon to glycerol as a result of GAPDH inhibition. This is important in terms of tissue energetics because glycolysis is ATP consuming up until this point in glycolysis, and therefore shunting of metabolic flux toward the formation of glycerol would be energy expensive for the cell.

Phosphocreatine (PCr) is an important molecule during cold-storage because it functions to maintain cellular ATP concentrations through phosphorylation of ADP [12]. It can be thought of as a source of potential ATP and a goal of cold-preservation should be to sustain high PCr levels. The data in this study revealed significantly higher myocardial PCr levels ($p<0.05$) in all of the buffered groups compared to control immediately following the flush. This may have been a reflection of rapid changes metabolite changes occurring during the flush period which occured more quickly in the control group. During the first hour of storage however, all groups showed a rapid decline to control levels for the remainder of the storage period. This suggests that intracellular buffering has little effect on the synthesis of PCr. The decline that was observed in the current study therefore likely reflects PCr consumption with little or no production.

ADP and AMP levels were low relative to ATP levels in all groups initially, and remained low over the experimental time course. After 10 h of storage, no differences

were found between the experimental groups and control with respect to AMP. With respect to ADP, the modified UW and BES experimental groups were lower than control, and although the differences were statistically significant ($p<0.05$), the actual magnitude of the differences were small, (1.0 μmol/g wet wt. for both groups), compared to the differences observed for ATP (6.3 to 8.7 μmol/g wet wt. for experimental groups significantly different from control). As a result, trends observed for total adenylate quantities (ATP+ADP+AMP) closely mirrored the trends seen for ATP. At the conclusion of the cold-storage interval, the modified UW, bicine, and histidine experimental groups had total adenylate pools that were 47% (modified UW) to 72% (bicine) higher than control ($p<0.05$), reflecting increased glycolytic ATP production in the buffered groups. This data strongly suggests that ATP is being synthesized *de novo* in these experimental groups. Substrate for *de novo* synthesis was provided through the inclusion of adenosine (5 mmol/l) in the experimental solutions.

Another index of tissue energetics during cold storage is the energy charge, [(ATP+1/2ADP)/ATP+ADP+AMP], described by Atkinson [19]. Pegg uses the analogy of a battery in describing the energy charge as being a measure of the "charged up" state of the adenylate pool [6]. If all adenylates existed in the form of ATP, the charge index would be 1. As ATP levels decline relative to ADP and AMP levels, the energy charge also drops, indicating that less of the total adenylate pool exists in a form that is immediately available for cellular 'work'. In the current experiment, ATP remained high relative to ADP and AMP in all groups, and as a result, large drops in energy charge were not observed. There was however a small but measurable difference between

energy charge in the STHS II (control) group (0.85) and all of the experimental buffered groups (ranging from 0.90 - 0.92) after 10 h of cold storage ($p<0.05$). This result is consistent with the study's hypothesis.

In summary, the data presented demonstrates that glycolytic flux in porcine myocardial tissue can be augmented by increasing the buffering capacity of preservation solutions. Furthermore, this increase in anaerobic metabolism results in improved myocardial energetics over 10 h of cold ischemia through greater tissue levels of ATP, total adenylates, and energy charge. Under static storage conditions however there does seem to be a limit to the benefits of increased buffering, the likely explanation being that as lactate accumulates, it in itself becomes an inhibitor of glycolysis independent of any pH changes. If inhibitory end-products of glycolysis such as lactate were 'washed out' by either intermittently, or continuously perfusing the organ during storage, the benefits of preservation solution buffering may become even more evident.

Figure IV-1:
Tissue Levels of PCr Over 10 h of Cold Storage

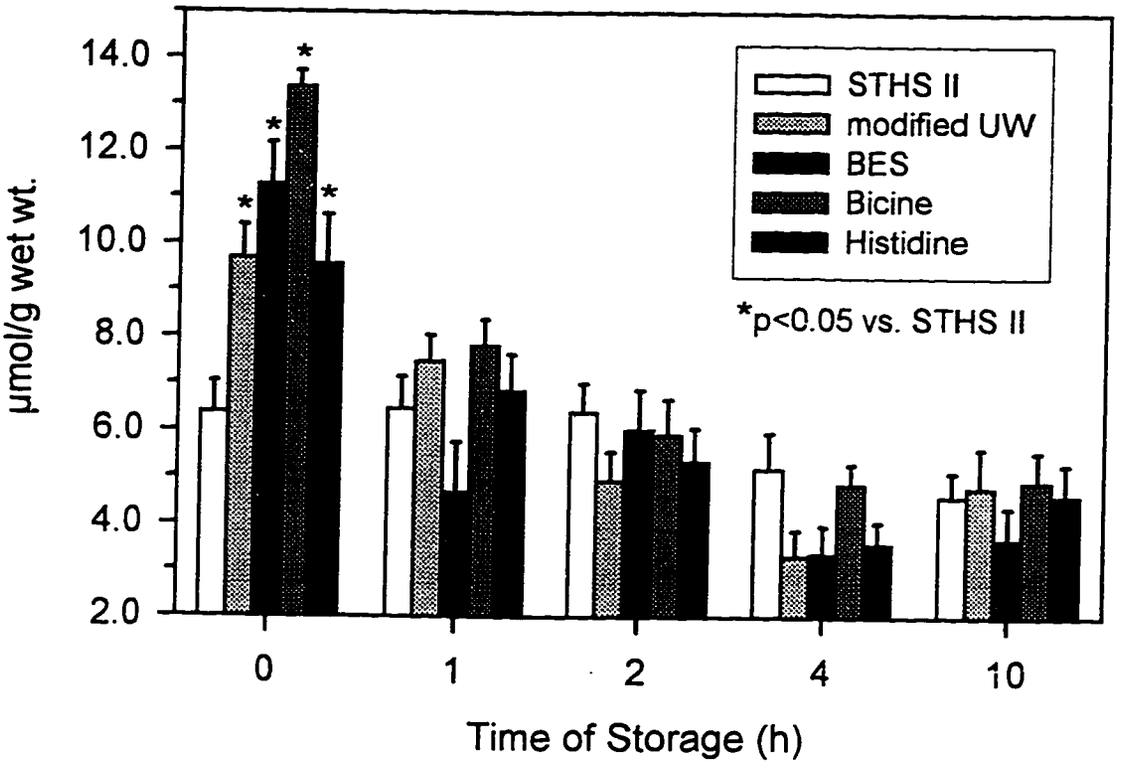


Figure IV-2:
Tissue Levels of ATP Over 10 h of Cold Storage

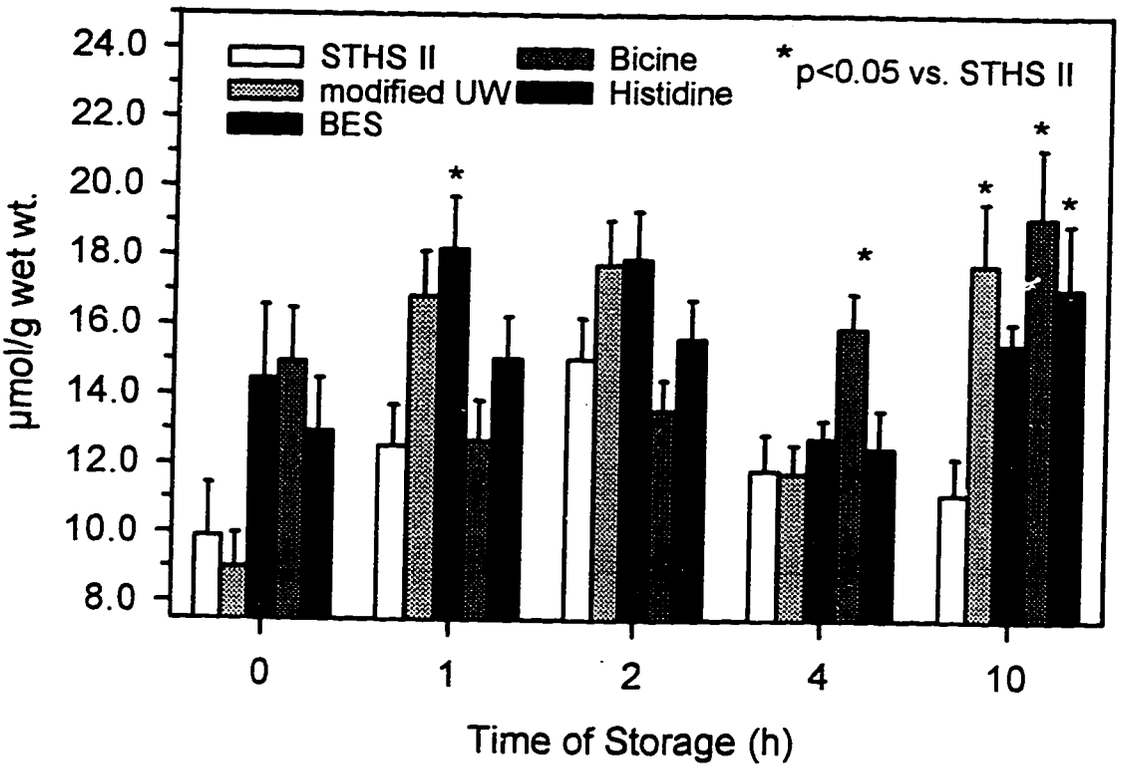


Table IV-1: Tissue ADP and AMP Levels Over 10 h of Cold Storage

	STHS II	Mod. UW	BES	Bicine	Histidine
	ADP				
t=0h	2.28 ± 0.29	1.78 ± 0.18	1.33 ± 0.21*	2.37 ± 0.11	1.63 ± 0.21
t=1h	3.05 ± 0.18	1.86 ± 0.10*	2.73 ± 0.59	2.32 ± 0.21	1.72 ± 0.11*
t=2h	2.26 ± 0.22	2.06 ± 0.17	1.82 ± 0.23	2.82 ± 0.20	2.21 ± 0.16
t=4h	2.65 ± 0.19	2.32 ± 0.22	1.92 ± 0.37	2.73 ± 0.16	1.77 ± 0.09*
t=10h	3.20 ± 0.20	2.16 ± 0.23*	2.16 ± 0.21*	3.02 ± 0.12	2.61 ± 0.25
	AMP				
t=0h	0.22 ± 0.06	0.30 ± 0.02	0.28 ± 0.05	0.51 ± 0.09*	0.37 ± 0.04
t=1h	0.41 ± 0.04	0.26 ± 0.06	0.39 ± 0.09	0.52 ± 0.07	0.33 ± 0.05
t=2h	0.29 ± 0.19	0.35 ± 0.08	0.43 ± 0.07	0.59 ± 0.11	0.37 ± 0.04
t=4h	0.16 ± 0.04	0.64 ± 0.11*	0.41 ± 0.07*	0.66 ± 0.05*	0.46 ± 0.09*
t=10h	0.54 ± 0.10	0.59 ± 0.06	0.55 ± 0.13	0.50 ± 0.04	0.40 ± 0.06

* denotes statistical significance with a $p < 0.05$ vs. control at given time point. Values are reported in µmol/g wet wt. as means ± SEM (n=4-6).

Figure IV-3:
Total Adenylates Over 10 h of Cold Storage

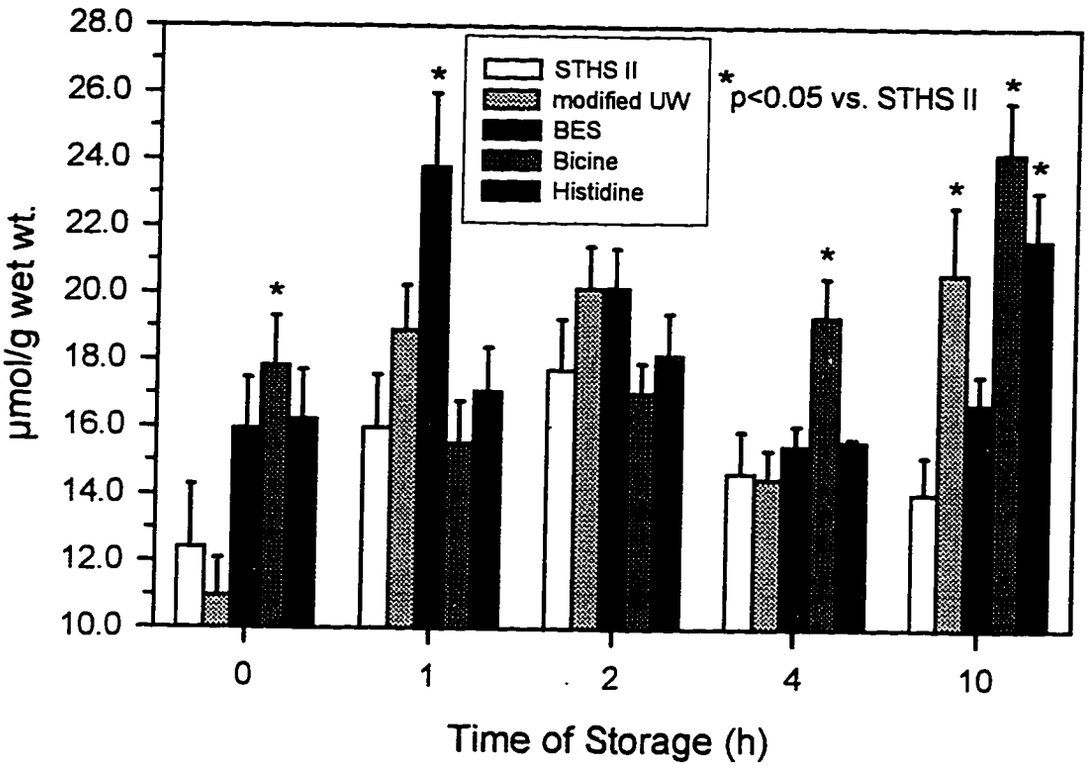


Table IV-2: Tissue Energy Charge Over 10 h of Cold Storage

	STHS II	Mod. UW	BES	Bicine	Histidine
t=0h	0.89 ± .01	0.89 ± .01	0.93 ± .02*	0.90 ± .01	0.92 ± .01
t=1h	0.88 ± .01	0.94 ± .01*	0.92 ± .01*	0.89 ± .01	0.93 ± .01*
t=2h	0.92 ± .02	0.93 ± .01	0.93 ± .01	0.88 ± .02	0.92 ± .01
t=4h	0.90 ± .01	0.88 ± .01	0.90 ± .02	0.89 ± .00	0.91 ± .01
t=10h	0.85 ± .02	0.91 ± .01*	0.90 ± .02*	0.92 ± .01*	0.91 ± .01*

* denotes statistical significance with a $p < 0.05$ vs. control at given time point. Values are reported as means ± SEM. (n=4-6)

Figure IV-4:
Tissue Lactate Accumulation Over 10h of Cold Storage

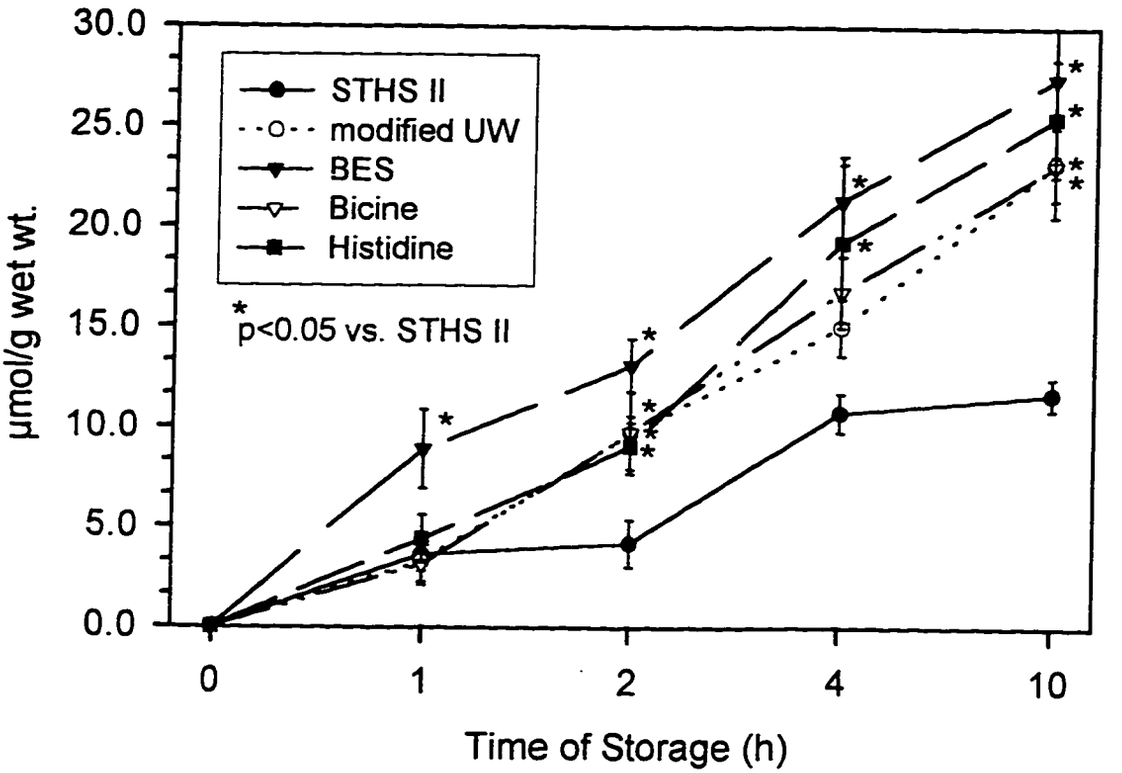
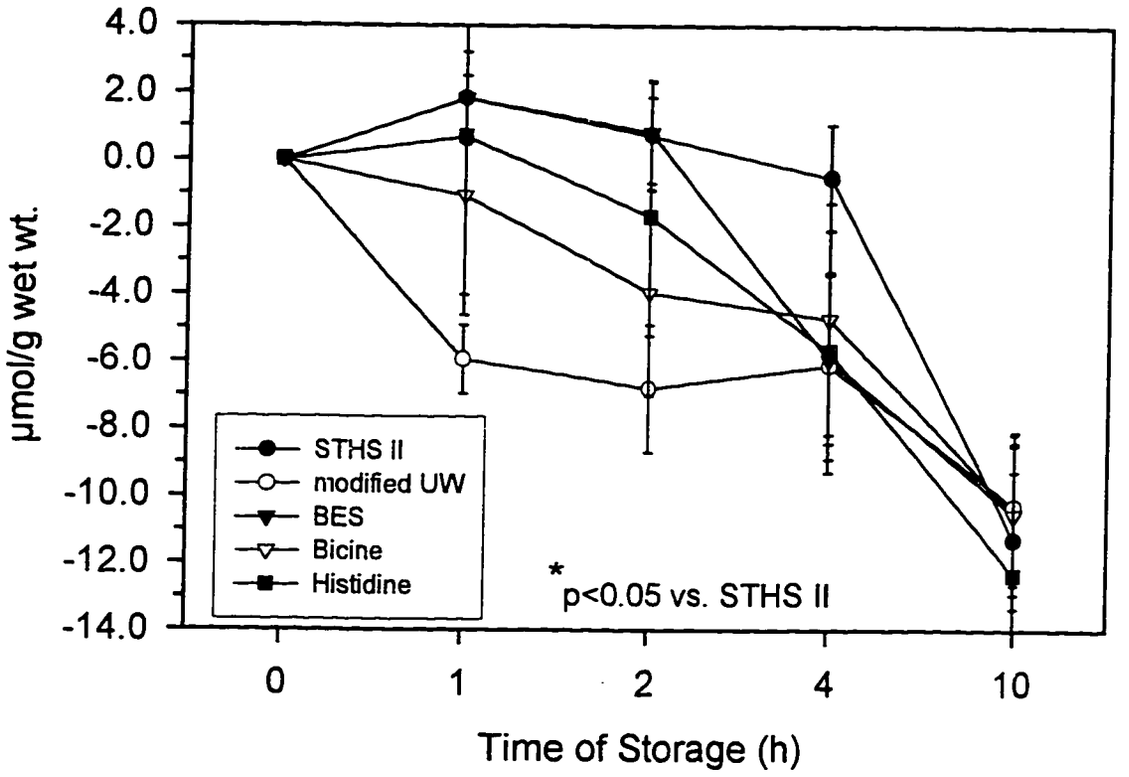


Figure IV-5:
Glycogen Decline Over 10 h of Cold Storage



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Chapter V: Experiment #2

Effects of Buffering, Glucose, and a Brief Hypothermic Perfusion Period on Glycolytic Energy Production in Ischemic Cold-Stored Pig Hearts

Introduction:

The ischemic insult suffered by cardiac allografts during storage is unavoidable at present. During cold storage, the oxygen and nutrient substrate supply is suspended. Under these conditions, damage to the myocardium inevitably occurs and is cumulative with time. The result of this is that hearts can be safely stored for only 4 to 6 hours before the risk of non-function becomes prohibitive [1, 2].

The role that preservation solutions play in minimizing the detrimental effects of ischemia, and therefore maximizing the safe storage time is momentous. This is highlighted by the introduction of the University of Wisconsin (UW) solution in 1987. Developed by Belzer, this solution more than doubled the safe storage times of liver, kidney, and pancreas [3]. Although this solution did not demonstrate the same success in cardiac preservation that it did with the intra-abdominal organs, it served to prove that the key to the successful development of preservation solutions is to understand the pathophysiologic changes that occur during ischemia, and then formulate a solution to counteract these changes.

At present, the effects of ischemia on myocardium are incompletely understood. One important issue appears to be the exhaustion of high energy molecules during ischemic cold storage. Previous investigators [2, 4] have shown that depletion of tissue energy stores (ATP, PCr, total adenylates) are associated with poor recovery of the graft

upon reperfusion. This is intuitive if one recognizes the fact that the heart must be able to carry out its mechanical function immediately upon transplantation, and that energy must be available in order for this to occur.

Levels of high energy molecules in the cold-stored myocardium reflect a balance between production and utilization. In general, hypothermia is globally applied to stored organs in the clinical setting in order to reduce the rate of energy consuming reactions, thus decreasing ATP utilization. The current study addresses the production side of this delicate balance. In the anaerobic state, glycolysis becomes the primary source of energy production [5]. In terms of energy yield, glucose metabolism through this pathway is inefficient, producing only 5 - 8%, (depending upon the source of glucose), of the ATP produced by normal oxidative metabolism [6]. In order to compensate for this relative inefficiency, flux through glycolysis must increase in order to meet the organ's energy demands.

This study investigates several potential avenues for improving glycolytic energy production during cold ischemia. These included the effects of solution buffering agents on energy production, the effect of a continuous perfusion period with oxygenated solution during the initial 1 h of storage, as well as the possible benefit of including glucose and insulin as substrate within the flush-storage solution.

Materials and Methods:

Chemicals

All chemicals were of AR grade and were purchased from Sigma Chemicals or BDH.

Animals

Adult Landrace-Yorkshire pigs (35 - 40kg) obtained from the University of Alberta swine farm were used as cardiac donors. All animals were treated humanely in accordance with the regulations set forth by the Canadian Council on Animal Care (Animal Protection Act). The experiments were approved by the University of Alberta Health Sciences Animal Welfare committee under protocol #126/03/97.

Experimental Groups

The pigs used in these experiments were assigned to one of 4 experimental groups with an n=4-6 for each group. The groups differed only in the solution used to flush and store the hearts with other factors being held constant. St. Thomas Hospital solution II (STHS II) served as the control group because of its ubiquitous use in clinical cardiac transplantation, and because of its limited buffering capacity compared to what is achievable through the addition of various buffering agents (see Figure II-1 for titration curves of other solutions). The solutions used for each of the experimental groups were adjusted to a pH of 7.4 and were composed as follows:

Group 1) (control) St. Thomas Hospital solution II containing sodium chloride (110 mmol/l), potassium chloride (16 mmol/l), magnesium chloride (16 mmol/l), calcium chloride (1.2 mmol/l), and sodium bicarbonate (10 mmol/l)

Group 2) Modified UW solution containing lactobionate (90 mmol/l), raffinose (25 mmol/l), potassium hydroxide (90 mmol/l), sodium hydroxide (25 mmol/l), magnesium sulphate (5 mmol/l), potassium phosphate (25 mmol/l), adenosine (5 mmol/l), and mannitol (90 mmol/l).

Group 3) Histidine solution which was identical to the modified UW solution with the addition of histidine (90 mmol/l).

Group 4) Histidine-Glucose-Insulin solution (HGI) which was identical to the modified UW solution with the addition of histidine (90 mmol/l), glucose (11.1 mmol/l), and insulin (100 U/l).

Surgical Proccdure for Cardiectomy and 1 h Continuous Perfusion

The pigs were anaesthetized with either inhalational halothane(2.5%) / oxygen(97.5%) or intramuscular ketamine 3500 mg (87.5-100 mg/kg) The animal's heart rate and oxygen saturation were continuously monitored with a Nellcor N-180 pulse oximeter attached to the ear to ensure an adequate oxygenation of 95 - 100%. A median sternotomy was performed and the animal was ventilated by manual bagging once the

chest was opened. The inferior vena cava (IVC) and superior vena cava (SVC) were then dissected circumferentially so that ligatures could be placed around them; the ligatures however were not tied at this time. The pericardium was opened and the right atrial appendage was retracted to expose the aortic root. The adventitial layer was sharply removed and the aorta was cannulated. Once cannulation was complete the IVC and SVC were ligated with the previously placed ligatures, and the heart was emptied by allowing it to beat for several cycles. The aorta was then cross-clamped distal to the cannula and 350 cc of the experimental solution was infused at a pressure head of approximately 100 cm H_2O. The solution was prepared 24 hours prior to use, was cooled to 4 °C, and was preoxygenated by bubbling 100% O_2 through it for 20 minutes. The solution was then stored on ice until being perfused. The time needed to infuse this volume of preservation solution was approximately 1 minute. The hearts arrested within several seconds of the start of the infusion and very quickly there was a noticeable blanching of the myocardium. A small incision was made in the right atrium to allow drainage of the effluent. As the heart was being perfused, the apex was removed and immediately 'snap frozen' in liquid nitrogen using Wollenberger clamps. This sample was designated the initial t=0 sample. When the infusion was complete, the heart was rapidly excised and was immediately transferred to a continuous perfusion apparatus (Figure V-1). During the 1 hour period of perfusion, the hearts were continuously perfused at a pressure of 100 cm H_2O with the same solution used to flush the hearts. The solution was continuously oxygenated by bubbling 100% O_2 through it. At the end of the 1 h continuous perfusion period, the heart was removed from the apparatus and stored for the remaining 9h in the flush/storage

solution at 4 °C. Additional transmural tissue samples from the left ventricle were taken at 1, 2, 4, and 10 hours and were also 'snap frozen' in liquid nitrogen using Wollenberger clamps. All samples were stored at -65°C until being processed.

Sample Preparation and Metabolite Assays

The samples of heart tissue were weighed out and homogenized in 6% perchloric acid (containing 1mM EDTA). A 50µl aliquot of the homogenate was removed and placed in 1 ml of 100 mM sodium acetate for glycogen determination. The samples were then immediately centrifuged at 4 °C for 15 min at 3000g to remove precipitated proteins. The supernatant was then withdrawn, neutralized with the addition of 3 M KOH/.4 M Tris/0.3 M KCl, and then recentrifuged. The protein pellet remaining was saved for determination of wet/dry weight ratios. The supernatants were placed on ice and immediately assayed for PCr, ATP, ADP, and AMP. These assays were performed enzymatically based upon the absorbance of NADH at 340 nm using a Dynatech MRX plate reader. Glycogen determination in the homogenate/acetate solution was accomplished by incubating the solution in amyloglucosidase from Aspergillus niger at 55 °C for 24 h to allow the degradation of glycogen to glucose [7]. The solution was adjusted to pH 8.0 with the addition of 20 µl of 4 M Tris buffer, and total glycogen was then assayed as glucose, utilizing the enzymatic glucose assay described by Passonneau and Lowry [8].

The protein pellet remaining after the first centrifugation was dried for 24 h at 60 °C and weighed for determination of the sample's dry weight. Tissue H_2O content was calculated from the wet/dry weight ratios according to the formula:

$$\% H_2O = (\text{wet weight} - \text{dry weight})/\text{wet weight} * 100$$

Statistics

Data are reported as means ± SEM for 4 to 6 hearts. Metabolite levels are reported in terms of μmol/g wet tissue weight. Metabolite measurements at the individual time points for each of the experimental groups were compared to the control group using an analysis of variance (ANOVA) followed by a Dunnett's *post hoc* comparison; $p<0.05$ are reported.

Results:

Tissue Water Content:

Calculation of tissue water content revealed no significant changes over the 10 h of ischemic cold-storage within or between groups. Water content of all samples from all groups averaged together was 65.3% ± 0.5.

Phosphocreatine (see Figure V-2):

Phosphocreatine levels in the control group immediately following the flush (t=0) averaged 8.6 μmol/g wet wt. and were not different from the experimental groups which showed initial values of 7.6, 7.9, and 8.5 μmol/g wet wt. for the modified UW, histidine, and HGI groups respectively. During the 1 h perfusion period with oxygenated solution, no group demonstrated a significant drop in PCr values. Over the 3h following cessation of the perfusion period, the experimental groups showed a rapid decline in PCr of between 45% (histidine) to 60% (modified UW) from initial values ($p<0.05$). This decrease was also rapid in the control group which dropped by 4 h of storage to 4.4 μmol/g wet wt. representing a 49% loss of PCr from levels measured at the beginning of the cold ischemic period ($p<0.05$). Over the remaining 6h of cold storage all groups showed a further progressive decline, although at a much slower rate than observed over the first 4 hours. Final PCr tissue concentrations (t=10 h) were between 39% (modified UW) and 48% (histidine) of initial values in the experimental groups. Mean levels were 2.2, 3.8, and 3.4 μmol/g wet wt. in the modified UW, histidine, and HGI groups respectively. These values were not significantly different from control which had a mean of 4.1 μmol/g wet wt. (47% of initial) at the conclusion of the experimental time course. In summary, ischemic cold storage of pig hearts results in a drop in mean PCr tissue levels that is independent of any of the solutions used in this study. The fact that no decrease in PCr was found in any group during the first hour of continuous perfusion suggests that PCr decline may be attenuated to some degree by continuous perfusion.

ATP (see Figure V-3):

ATP levels at t=0 did not differ between the control group and the experimental groups. Initial values for ATP were 11.3 μmol/g wet wt. for the control group, and were 10.8, 11.2, and 12.5 μmol/g wet wt. for the modified UW, histidine, and HGI groups respectively. In the first hour of storage, during which the hearts were continuously perfused with oxygenated solution, no group demonstrated significant changes in tissue ATP concentration. ATP levels in the control group declined 33% over the first 2 h of storage ($p<0.05$), but were not different from initial values at 4 and 10 h. By the end of the 10 h cold ischemic period, the histidine group showed a 63% increase in ATP levels compared to initial values to yield a final mean ATP tissue concentration of 18.1 μmol/g wet wt. ($p<0.01$); no change from initial was found for the modified UW, or HGI groups at any time point. Of the experimental groups, the histidine buffered solution showed the greatest difference from control with respect to ATP levels; values were significantly higher than control in this group at 2, 4, and 10 h ($p<0.05$). In contrast, the modified UW group showed significantly greater ATP only at 2 h (12.1 μmol/g wet wt. vs. 7.6 μmol/g wet wt. control), and the HGI group was greater only at 1 h (13.5 μmol/g wet wt. vs. 9.2 μmol/g wet wt. control), and 2 h (13.7 μmol/g wet wt.) ($p<0.05$). Therefore, beyond 2 h of ischemic cold storage, the histidine buffered solution was the only experimental solution demonstrated to be clearly superior to control in maintaining tissue ATP concentrations.

ADP and AMP (see Table V-1):

Both ADP and AMP values were low relative to ATP for each group at all time points. The initial ADP level in the control group was 2.3 μmol/g wet wt. and did not differ from the experimental groups which ranged from 1.4 μmol/g wet wt. (modified UW), to 1.6 μmol/g wet wt. (HGI). Values remained low in all groups over the 10 h storage interval and changed significantly only in the histidine group which increased from 1.4 μmol/g wet wt. at t=0 h to 2.3 μmol/g wet wt. at t=10 h ($p<0.05$) representing an absolute gain of 0.9 μmol/g wet wt. of ADP. At the conclusion of the cold-ischemic period, no difference was noted between control and any of the experimental groups with respect to ADP measurements. Final values ranged from 2.0 μmol/g wet wt. (HGI) to 3.0 μmol/g wet wt. (control).

Trends for AMP were similar to those seen for ADP. Initial levels did not differ between any of the groups, ranging from 0.1 μmol/g wet wt. (modified UW) to 0.2 μmol/g wet wt. (control, histidine, and HGI). AMP levels remained low over the perfusion period and during static cold storage in all groups. At t=10 h, AMP values ranged from 0.3 μmol/g wet wt. (modified UW, histidine, and HGI), to 0.4 μmol/g wet wt. (control), and were not significantly different.

Total Adenylates (see Figure V-4):

Due to the relative abundance of ATP compared to ADP and AMP, trends found for total adenylates (ATP+ADP+AMP) were very similar to those found for ATP. Total adenylates in the experimental groups at t=0 h ranged from 12.4 μmol/g wet wt. (modified

UW) to 14.3 μmol/g wet wt. (HGI). These values were not different from control which had an initial mean total adenylate concentration of 13.9 μmol/g wet wt.. During the continuous 1 h perfusion period no decrease in total adenylates were noted for either the experimental groups or the control group. After 2 h of cold ischemia the control group demonstrated a 33% decline in total adenylates (7.6 μmol/g wet wt.) from t=0 h ($p<0.05$), and was lower than all of the experimental groups at this time point ($p<0.05$). Levels in the control group rebounded at 4 and 10 h however, and were not significantly lower than initial values. Among the experimental groups, neither the modified UW or HGI solutions changed significantly in total adenylate concentrations over the 10 h cold ischemic period, and were not superior to control after 2 h of storage. In contrast, adenylate levels in the histidine buffered group were higher than control at 2, 4, and 10 h of storage ($p<0.05$). Mean levels in the histidine group increased to 18.1 μmol/g wet wt. at 10 h representing a 63% gain from t=0 h ($p<0.05$), and was 76% higher than control which had a final mean total adenylate level of 10.3 μmol/g wet wt. ($p<0.05$).

Energy Charge (see Table V-2):

Initial values for energy charge [(ATP+1/2ADP)/ATP+ADP+AMP] immediately following the myocardial flush (t=0), ranged from 0.90 (control) to 0.95 (histidine) ($p<0.05$). The modified UW solution and HGI solution both had initial values of 0.93 and were not statistically different from control. After the 1 h perfusion period, the experimental groups showed energy charge values between 0.91 (modified UW) to 0.93 (histidine and HGI groups), which were significantly higher than control (0.87) ($p<0.05$).

This measured increase was maintained for the hour following cessation of perfusion, ($p<0.05$) but was lost at 4 hours. At the end of the storage period however, energy charge was significantly higher in all experimental groups (0.91 to 0.93) compared to control (0.85) ($p<0.05$).

Lactate Accumulation (see Figure V-5):

The accumulation of lactate in the tissue over the 10 h experimental time course provided a quantitative measurement of anaerobic metabolism in the cold-stored myocardium. Lactate did not increase in any study group during the 1 h continuous perfusion period. The only group to show significant lactate accumulation during the first 2 h of cold ischemia was the modified UW group which accumulated a total of 4.0 μmol/g wet wt. at t=2 h ($p<0.05$ vs. t=0 h) compared to the control, histidine, and HGI groups which had a total accumulation of 0.3, 1.7, and 0.7 μmol/g wet wt. respectively over the same time period. By 4 h of cold ischemia however, all groups demonstrated measurable increases in tissue lactate ($p<0.05$), with continued increases in all groups over the remaining storage period indicating constant flux through the glycolytic pathway. At the 4 h time point, all experimental groups had amassed greater levels of lactate than the control group ($p<0.05$). At the conclusion of 10 h of cold storage, mean tissue lactate accumulation was 10.7 μmol/g wet wt. in the control group which was lower than the total accumulation measured for the histidine group (20.6 μmol/g wet wt.), and the HGI group (18.5 μmol/g wet wt.) ($p<0.05$), but was not significantly different from the modified UW group (14.6 μmol/g wet wt.). Extrapolating these values to calculate total

glycolytic activity during the 10 h storage period reveals a 73% increase in flux through the pathway for the HGI group compared to control, and a 93% increase in the histidine group.

Glycolytic Substrate (see Figure V-6):

Initial myocardial glycogen levels, (measured as glucose units), in the experimental groups ranged from 35.9 μmol/g wet wt. (modified UW) to 39.8 μmol/g wet wt. (histidine), and were not different from initial levels in the control group (34.0 μmol/g wet wt.). Glycogen levels declined in all groups in a linear fashion and were statistically significant by 10 h in all cases ($p<0.05$). The total decline between 1 h and 10 h storage was 5.9 μmol/g wet wt. in the control group; HGI and modified UW groups dropped by 6.2 and 8.5 μmol/g wet wt. respectively, and were not significantly different from control. Glycogen utilization over 10 hours in the histidine group was 13.3 μmol/g wet wt. and was statistically greater than control ($p<0.05$).

Discussion:

Previous investigators have shown that a decline in cellular ATP levels is associated with poor myocardial performance upon reperfusion [2, 9]. The likely mechanism behind this finding is that energy is needed by the cardiomyocyte for excitation-contraction coupling, and for fueling the membrane ion pumps that regulate normal intracellular ionic homeostasis [10]. This suggests that the injury sustained by the myocardium is at least in part related to the depletion of high energy molecules (ATP,

PCr) during cold storage. It follows therefore that an important aspect of any flush-storage solution should be its ability to preserve myocardial energetics, as well the ability to quickly replenish tissue energy levels in the early reperfusion period.

In the current study, several potential avenues for improving myocardial energetics during cold ischemia were investigated. These included the effects of solution buffering agents on energy production, the effect of a continuous perfusion period with oxygenated solution during the initial 1 h of storage, as well as the possible benefit of including glucose and insulin as substrate within the flush-storage solution.

With respect to buffering of the preservation solution, it was hypothesized that enhanced buffering would augment glycolytic energy production resulting in improved tissue energetics during cold-storage. The data presented are consistent with this hypothesis. St. Thomas Hospital solution II (Group 1) was the control solution, and is widely used in clinical cardiac transplantation centres in Canada. This solution utilizes $NaHCO_3^-$ as its primary buffer [11], and has a much lower buffering capacity than the modified UW or modified UW plus 90 mM histidine solutions employed in this study (see Figure II-1). The modified UW solution was not found to be superior to control in maintaining glycolytic energy production over the 10 h cold ischemic period as evidenced by a lack of significant differences in lactate accumulation, tissue ATP, and total adenylates. However, when the buffering capacity of the modified UW solution was increased through the addition of 90 mM of histidine, lactate production, tissue ATP, and total adenylates were all found to be significantly greater than control.

Interestingly, the benefit of solution buffering on maintaining ATP levels was lost with the addition of glucose (11.1 mmol/l) to the preservation solution. At the conclusion of storage no difference in ATP was found between the HGI group and control despite the fact that the HGI solution differed from the histidine solution only by the presence of glucose. This finding is consistent with those of Hearse [12] who found that adding glucose to St. Thomas Hospital solution resulted in a dose dependent reduction in cardioprotection, and that insulin exacerbated this detrimental effect. Owen and associates [13] showed that increasing glucose concentrations beyond 11 mmol/l resulted in extracellular glucose being utilized preferentially over endogenous glycogen through inhibition of glycogen phosphorylase. The ATP yield from glycolysis when extracellular glucose is metabolized is only 2/3 of what is produced when endogenous glycogen is the primary substrate. Thus, the presence of glucose and insulin in the HGI solution likely resulted in an overall reduction in total glycolytic ATP production through decreased glycogenolysis. This is supported by the finding of a significantly decline in glycogen stores over the entire period of static storage (1 h to 10 h post flush) in the histidine group only ($p<0.05$). This is in contrast to no significant decrease in glycogen levels in the HGI group despite an equivalent increase in lactate measured in the histidine group (Figure V-5 and 6).

The rationale behind the hypothesis that increased preservation solution buffering is beneficial in maintaining tissue energy levels comes from understanding the normal control mechanisms of anaerobic metabolism . Three key regulatory enzymes control flux through the glycolytic pathway: glycogen phosphorylase, phosphofructokinase (PFK), and

glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [6]. Of these enzymes, PFK is particularly sensitive to pH changes [14, 15]. Under conditions of anoxia, proton accumulation results in a decline in intracellular pH. This decline can be quite rapid as demonstrated by Anderson *et al* [16] who found that intracellular pH fell to 6.6 within 5 minutes of the onset of warm ischemia in rabbit myocardium. Other investigators [17] have shown that intracellular pH drops at a slower rate in the setting of hypothermic storage, but that the magnitude of the decline is still significant. Glycolysis is therefore self-limiting in the setting of cold storage because the protons that the pathway produces cause PFK to undergo a reversible hysteretic loss of activity [15]. By providing intracellular buffers, as proposed by Bretschneider in 1975 [18], the means are available to 'soak up' excess protons and lessen the pH decline that results in an inhibition of glycolytic energy production. The results obtained in the current study are consistent with this line of reasoning.

Evidence that the differences found in tissue energetics between the histidine based solution and control were secondary to enhanced anaerobic metabolism, is provided by lactate accumulation in the tissue. Since lactate is the end point of the glycolytic pathway in anoxic tissue, increased lactate production implies increased flux through glycolysis. Following the perfusion period lactate increased linearly in all groups over time consistent with the expected transition from aerobic to anaerobic metabolism. Total lactate accumulation was significantly higher ($p<0.05$) in the histidine buffered and HGI groups compared to control at the conclusion of the storage period providing confirmation of that glycolytic activity is augmented by improved buffering of the flush solutions. Glycolytic

substrate in the histidine group had to have come from endogenous glycogen stores because no exogenous glucose was supplied. Substrate in the form of exogenous glucose (11.1 mmol/l) was supplied in the HGI solution. Therefore, in the HGI group, two potential sources of glucose were present: endogenous glycogen, and exogenous glucose. In contrast to the histidine containing solutions, the less buffered modified UW solution did not show any difference from control.

In this study, lactate levels remained negligible in all groups during the 1 h perfusion period. One possible explanation for this is that lactate was flushed out of the tissue as it was being continuously perfused. Another possibility is that by oxygenating the solution, pyruvate was oxidized via the Kreb's Cycle instead of being converted to lactate. One way to sort out this problem would be to continuously perfuse the organ with solution gassed with 100% N_2 instead of O_2 and then measure tissue lactate levels. If no lactate accumulated under these conditions, then presumably the true mechanism is that lactate is being washed out of the tissue. Since lactate has been shown to decrease glycolytic flux through inhibition of the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [19-21], it may be possible to further enhance glycolytic activity by limiting lactate accumulation by continuously perfusing the heart during storage. It would therefore be of value to know the underlying mechanism behind the lack of lactate accumulation as well as the length of time that it can be sustained.

Phosphocreatine (PCr) is an important molecule during cold-storage because it functions to maintain cellular ATP concentrations through phosphorylation of ADP [6]. It can be thought of as a source of potential ATP and a goal of cold-preservation should

be to sustain high PCr levels. The data in this study revealed no significant differences between the control and experimental groups during the storage period During the first hour of continuous cold perfusion, no group demonstrated a loss of PCr. Once the perfusion period was complete however, all groups showed a rapid decline over the remainder of the storage period. This suggests that while intracellular buffering has little or no effect on the synthesis of PCr, continuous cold perfusion with oxygenated solution may be of some benefit in preserving PCr, perhaps because ATP is well maintained during the perfusion period.

Relative to ATP, ADP and AMP levels were low levels in all groups initially, and remained low over the experimental time course. After 10 h of storage, no differences were found between the experimental groups and control with respect to either ADP or AMP. As a result, trends observed for total adenylate quantities (ATP+ADP+AMP) closely reflected those observed for ATP. After 10 h of hypothermic ischemia, the histidine buffered solution had a total adenylate pool that was 76% higher than control ($p<0.05$) with the entire difference being accounted for by higher ATP levels in the histidine group.

Atkinson [22] described another useful measure of tissue energetics in what he called the 'energy charge', given by the formula: [EC=(ATP+1/2ADP)/ATP+ADP+AMP]. In illustrating the significance of this measurement, Pegg uses the analogy of a battery in describing the energy charge as being a measure of the "charged up" state of the adenylate pool [23]. If all adenylates existed in the form of ATP, the charge index would be 1. As ATP levels decline relative to ADP

and AMP levels, the energy charge also drops, indicating that less of the total adenylate pool exists in a form that is immediately available for cellular 'work'. In the current experiment, large drops in energy charge were not observed because ATP remained high relative to ADP and AMP in all groups. There was however a small but measurable difference between energy charge in the STHS II (control) group (0.85) and all of the experimental buffered groups (ranging from 0.91 - 0.93) after 10 h of cold storage ($p<0.05$). This result is consistent with the study's hypothesis.

In summary, the data presented supports the hypothesis that buffering against pH changes that occur during hypothermic ischemia improves myocardial tissue energetics through increased glycolytic energy production. Enhanced glycolytic flux was shown conclusively through significant increases in lactate production in myocardium flushed with histidine buffered solutions. The data also suggests that the addition of glucose and insulin results in decreased glycolytic energy production, likely by shifting the substrate supply away from endogenous glycogen to exogenous glucose resulting in a lower yield of ATP per unit of substrate. Finally, it was demonstrated that continuous perfusion with oxygenated solution during the first hour of storage resulted in no lactate accumulation in any group. Further study is needed to resolve the mechanism behind this observation, as well as to determine whether negligible lactate accumulation during cold-storage can be sustained indefinitely.

Figure V-1: Apparatus for 1 h Perfusion of Hearts

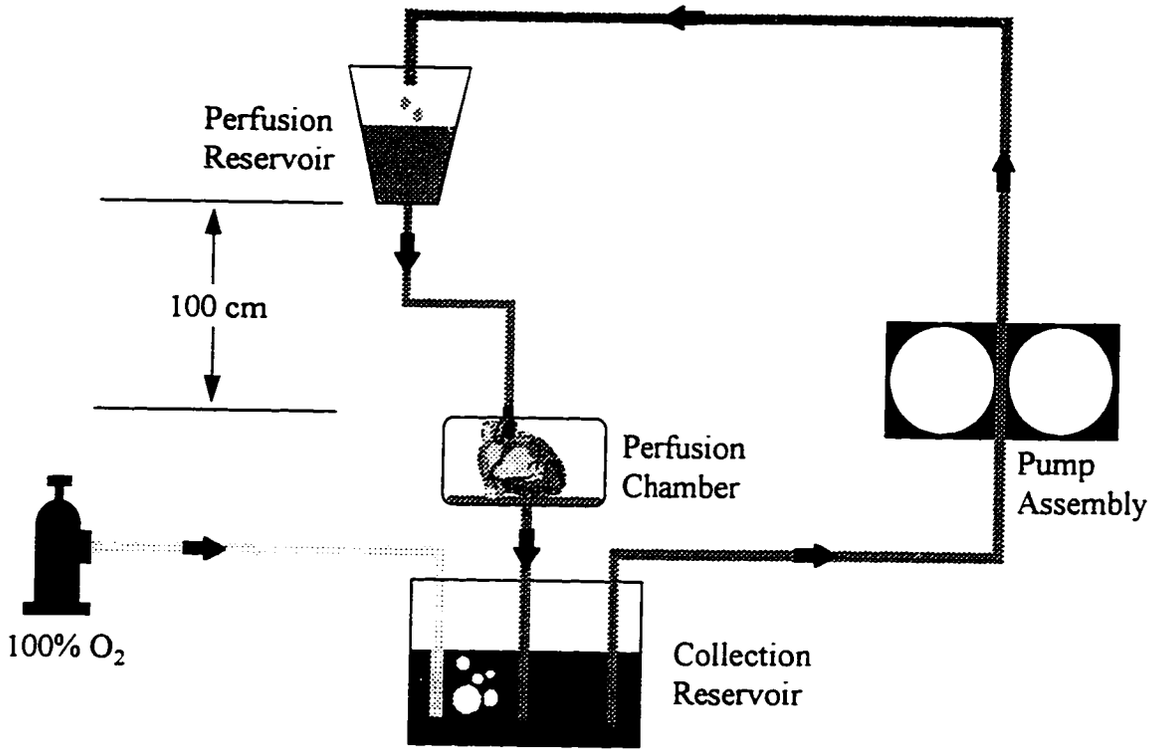


Figure V-2:
Tissue PCr Levels Over 10 h of Cold Storage

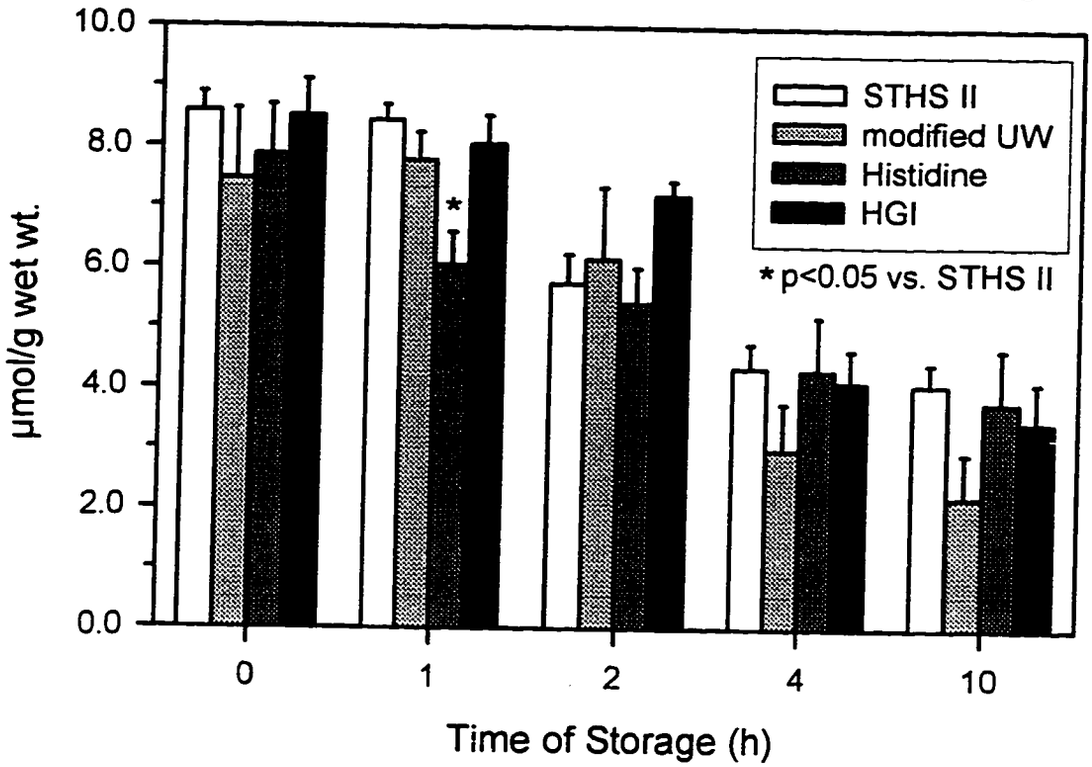


Figure V-3:
Tissue ATP Levels Over 10 h of Cold Storage

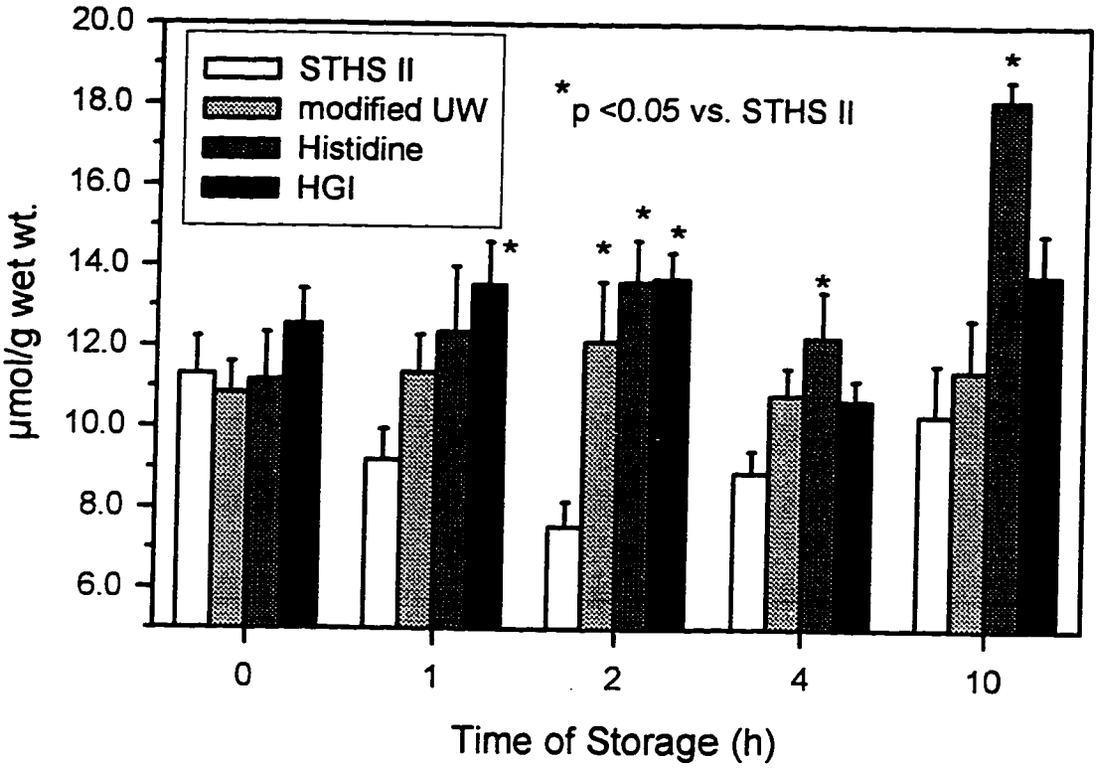


Table V-1: Tissue ADP and AMP Levels Over 10 h of Cold Storage

	STHS II	Mod. UW	Histidine	HGI
	ADP			
t=0h	2.26 ± 0.46	1.35 ± 0.16	1.37 ± 0.37	1.59 ± 0.27
t=1h	2.33 ± 0.29	1.58 ± 0.15*	1.30 ± 0.18*	1.73 ± 0.18
t=2h	2.07 ± 0.26	1.67 ± 0.29	1.44 ± 0.08	1.50 ± 0.11
t=4h	1.83 ± 0.21	2.13 ± 0.35	1.93 ± 0.25	1.66 ± 0.18
t=10h	2.95 ± 0.42	2.31 ± 0.32	2.29 ± 0.14	2.04 ± 0.11
	AMP			
t=0h	0.18 ± 0.08	0.11 ± 0.04	0.15 ± 0.06	0.18 ± 0.06
t=1h	0.38 ± 0.07	0.31 ± 0.02	0.15 ± 0.04*	0.35 ± 0.08
t=2h	0.31 ± 0.06	0.26 ± 0.07	0.12 ± 0.04	0.17 ± 0.04
t=4h	0.25 ± 0.03	0.34 ± 0.06	0.31 ± 0.03	0.32 ± 0.10
t=10h	0.36 ± 0.12	0.28 ± 0.05	0.28 ± 0.07	0.30 ± 0.03

* denotes statistical significance with a $p < 0.05$ vs. control at given time point.
Values are reported in µmol/g wet wt. as means ± SEM (n=4-6).

Figure V-4:
Total Adenylates Over 10 h of Cold Storage

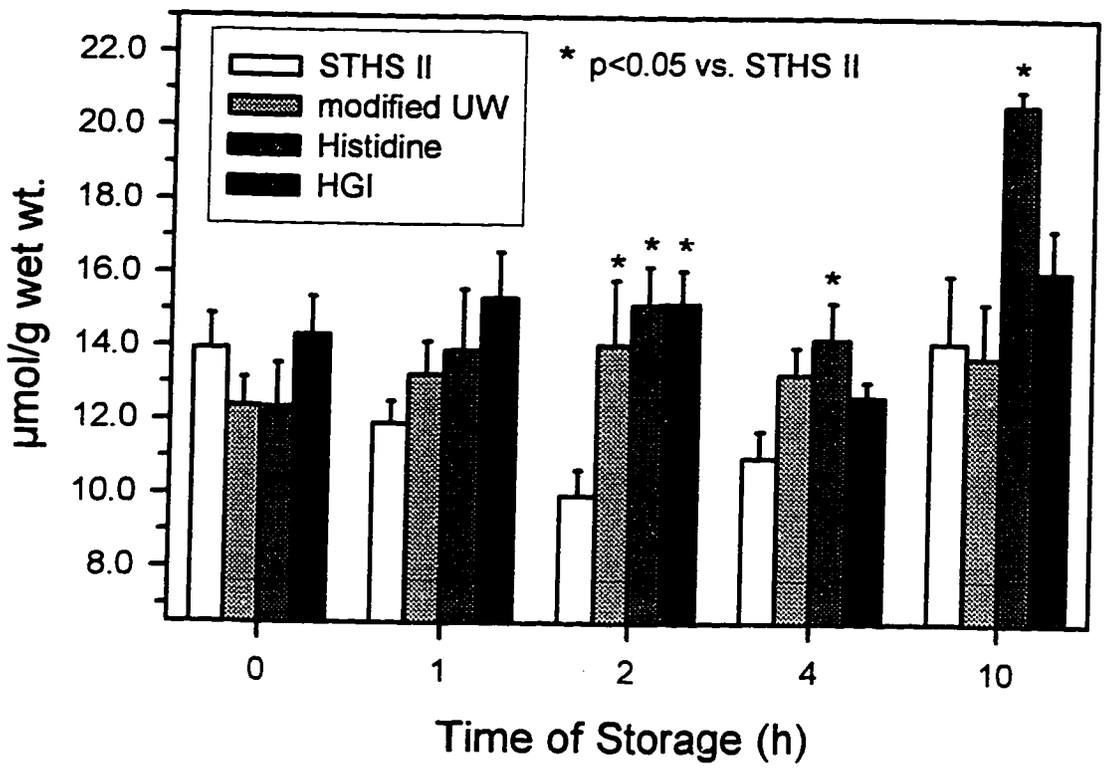


Table V-2: Tissue Energy Charge Over 10 h of Cold Storage

	STHS II	Mod. UW	Histidine	HCl
t=0h	0.90 ± .02	0.93 ± .01	0.95 ± .01*	0.93 ± .01
t=1h	0.87 ± .02	0.91 ± .01*	0.93 ± .01*	0.93 ± .01*
t=2h	0.86 ± .02	0.92 ± .01*	0.94± .01*	0.95 ± .00*
t=4h	0.89 ± .01	0.89 ± .02	0.92 ± .01	0.91 ± .01
t=10h	0.85 ± .01	0.91 ± .00*	0.93 ± .01*	0.92 ± .01*

* denotes statistical significance with a $p < 0.05$ vs. control at given time point.
Values are reported as means ± SEM. (n=4-6)

Figure V-5:
Lactate Accumulation Over 10 h of Cold Storage

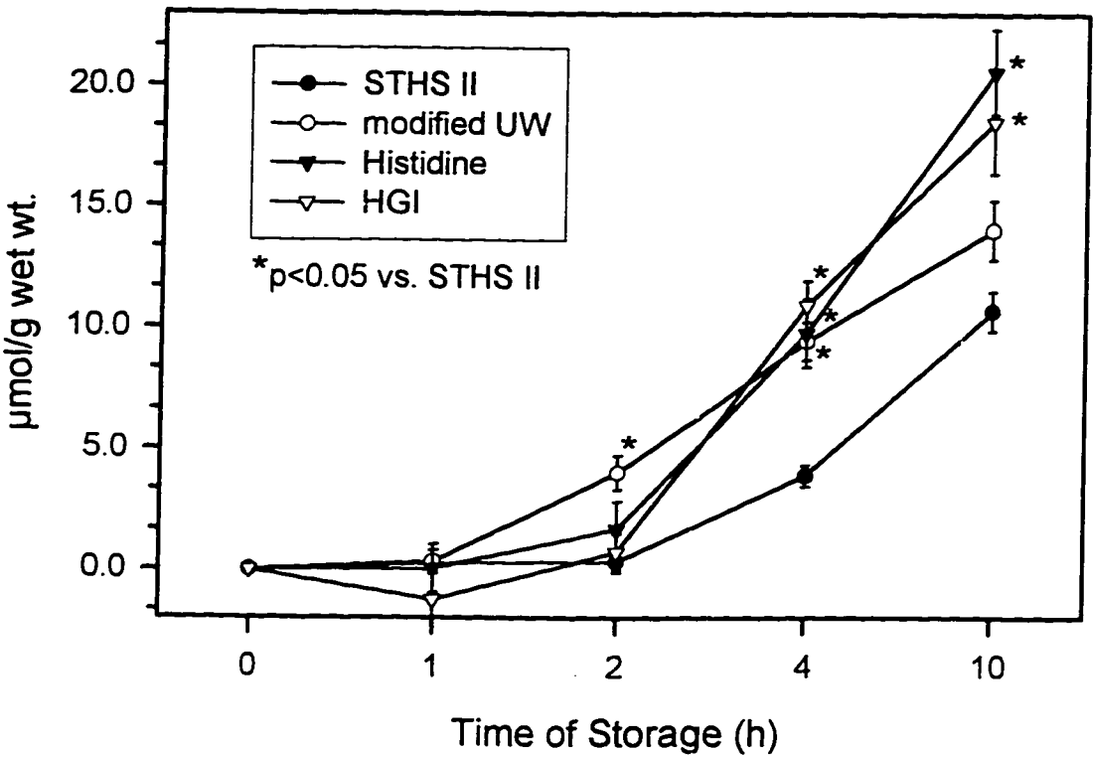
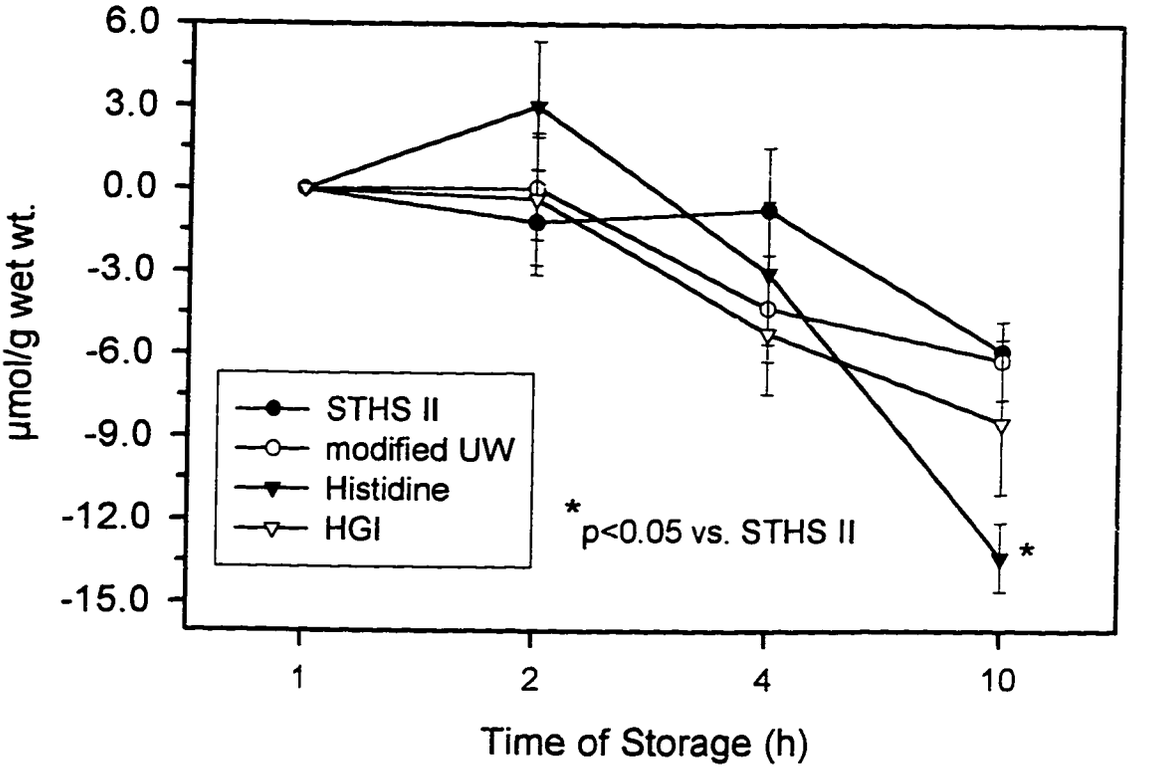


Figure V-6:
Glycogen Decline Between 1 h and 10 h of Cold Storage



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Chapter VI: Conclusions

The main focus of our current research was on the effect of enhanced buffering of preservation solutions on glycolytic energy production during ischemic cold storage. By allowing for anaerobic energy production to continue for longer periods of time, it may be possible to prolong the safe storage interval of cardiac allografts past the currently accepted limit of 4 to 6 hours.

In the first of two experiments presented, it was clearly demonstrated that 4 different solutions with augmented buffering capacity accumulated significantly higher tissue levels of lactate than the poorly buffered STHS II (control) over a 10 h cold ischemic period. This is consistent with the hypothesis that buffering increases glycolytic flux, likely by relieving pH inhibition of regulatory enzymes such as PFK. Furthermore, this measurable increase in glycolysis was shown to translate into improved myocardial tissue energetics after the conclusion of the storage period through significantly greater levels of ATP, total adenylates, and energy charge in the buffered groups. There was however a limit to the benefits of buffering. It was found that increasing buffering capacity past a certain point resulted in no added benefit in terms of tissue energetics. This was evidenced by the fact that no differences were found between the experimental groups despite differences in buffering ability. The explanation for this may be that under static storage conditions, the accumulation of end-products such as lactate may have become an inhibitor of glycolysis independent of any pH changes. Another possibility is that the buffers used possessed different membrane permeability characteristics. The

optimal buffer must be able to cross the cell membrane to achieve the desired intracellular buffering.

In the second experiment, the hearts were perfused with oxygenated preservation solution for the first hour of storage. Similar to the results of the first experiment, enhanced buffering with histidine resulted in a significant increase in glycolytic activity as shown by increased lactate production. This resulted in superior tissue energetics after 10 hours of cold storage with higher levels of ATP, total adenylates, and energy charge. Although the 1 hour perfusion period did not seem to compliment buffering in terms of tissue energetics, it was shown that tissue accumulation of lactate was impeded. The mechanism behind this was not clear, and further experimentation is needed to determine whether it was caused by simply washing the lactate from the tissue, or whether oxygenating the solution caused a delay in the onset of anaerobic metabolism. The importance of this is that end product build up can at least be delayed; possibly allowing for glycolysis to progress for longer periods as suggested by the findings of the first experiment. It was also demonstrated that glucose and insulin, when added to a highly buffered preservation solution, did not effect the buffers ability to increase glycolytic flux, but it did decrease total energy production. By providing exogenous glucose, the substrate supply is probably shifted away from endogenous glycogen resulting in lower ATP production per unit of substrate used.

In conclusion, buffering of preservation solutions does seem to enhance glycolytic energy production during cold storage resulting in improved tissue energetics. More

study is needed to find the optimal buffer, and to uncover the best means of maximizing the buffer's effect.

Chapter VII: Future Considerations

To those conducting scientific research, it seems that one of the most reliable outcomes of any study is that the number of questions answered by the investigation will be less than the number that are generated. This is undoubtedly a good thing in that it ensures that dead ends occur rarely, thus allowing for the continual production of new ideas. In the experiments presented here, questions arose that would benefit from further study.

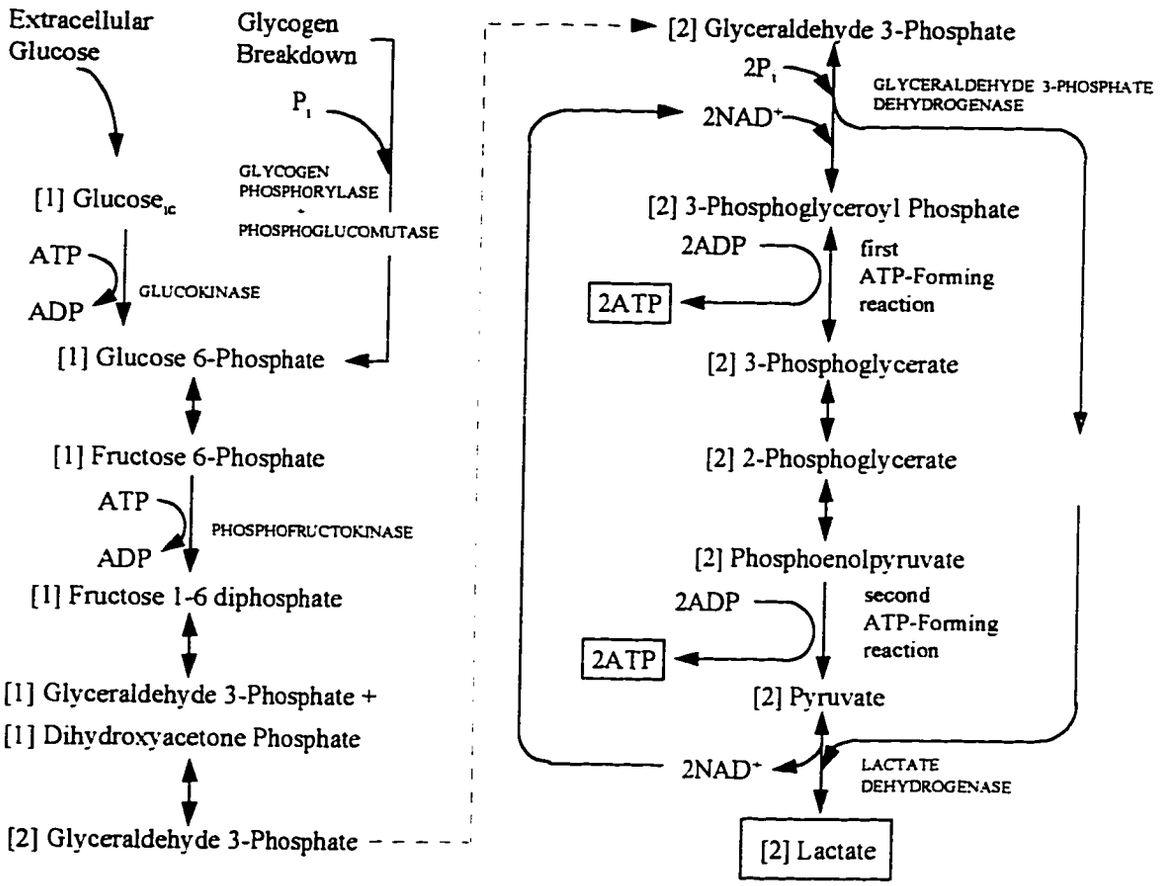
We found in the first experiment that buffering was of benefit in terms of tissue energetics, but that this benefit was likely limited by the accumulation of end-products such as lactate. It may therefore be that removal of glycolytic end-products would allow for solution buffering to achieve a maximal effect. In the second experiment it was demonstrated that lactate accumulation during a 1 hour hypothermic perfusion period with oxygenated preservation solution resulted in negligible lactate build-up in the tissue. Further experiments are need to determine whether this lack of accumulation was due to wash out of end-products, or from oxygenation of the solution. Repeating the experiment with solution gassed with 100% N_2 and looking at tissue lactate levels would answer this question. Also, continuously perfusing the heart for longer periods than 1 hour would reveal whether lactate accumulation can be avoided indefinitely, and also the effect that this would have on tissue energetics.

Another interesting line of study that should be pursued is in relation to a new artificial blood product (Hemolink®), developed in Canada by Hemosol Inc. The fundamental problem with static cold storage is the damage caused by anoxia. If oxygen

could be supplied to the graft during storage by either intermittently or continuously perfusing the graft with solutions capable of uptake and release of O_2, the problem may be circumvented. Hemolink® may be able to accomplish this, and solutions could be created which contain this product. These solutions can then be studied to reveal any possible benefits in terms of energy production, and organ function.

Finally, any novel preservation solutions shown to result in better tissue energetics need to be studied in the setting of a working heart model, as this is the ultimate test of the solutions utility. It is very exciting to show that buffering, (or some other manipulation), results in improved tissue energetics, but it needs to be demonstrated experimentally that this results in improved function. In the working heart model, the graft can be flushed and stored for varying intervals and then reperfused in a Langendorff manner, (ie: retrograde through the aorta), and allowed to beat. Functional parameters such as peak aortic pressure, cardiac output, and stroke volume could be measured, and would provide a means of directly testing the ability of the solution to preserve cardiac function. This model would also be able to compare solutions in terms of safe storage interval by demonstrating the length of cold-ischemic time it takes for graft function to be significantly impaired.

Appendix I:
The Glycolytic Pathway and Regulatory Enzymes



Appendix II:
Components of Common Preservation Solutions

University of Wisconsin Solution

Component	*Concetration (mM)*
K^+-Lactobionate	100
KH_2PO_4	25
$MgSO_4$	5
Raffinose	30
Adenosine	5
Glutathione	3
Pentastarch	5%
Heparin	10000 IU/L
Allopurinol	1
Insulin	100 U/L
Osmolarity	320
pH	7.4

Bretschneider's Solution

Component	*Concetration (mM)*
NaCl	15
KCl	9
$MgCl_2$	4
K^+-ketoglutarate	1
Histidine	180
Histidine HCl	18
Mannitol	30
Tryptophan	2

St. Thomas Hospital Solution II

Component	*Concetration (mM)*
NaCl	110
KCl	16
$MgCl_2$	16
$CaCl_2$	1.2
$NaHCO_3$	10
Osmolarity	280-300

Krebs-Henseleit Solution

Component	*Concetration (mM)*
NaCl	100
KCl	4.7
$MgSO_4$	1.2
Glucose	11
$NaHCO_3$	25
$CaCl_2$	1.75
EDTA	0.5
KH_2PO_4	1.2
Albumin	0.50%
Dextran	2%
Osmolarity	295
pH	7.4

Stanford Hospital Solution

Component	*Concetration (mM)*
KCl	27
$NaHCO_3$	20
Glucose	255
Mannitol	63
Heparin	1000 (IU/L)

Euro-Collins Solution

Component	*Concetration (mM)*
K_2HPO_4	42.3
KH_2PO_4	15.1
KCl	15
$NaHCO_3$	10