METABOLISM IN MYOCARDIAL ISCHAEMIA AND REPERFUSION WITH SPECIFIC REFERENCE TO THE ROLE OF GLUCOSE

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Abstract

Metabolism in myocardial ischaemia and reperfusion with specific reference to the role of glucose.
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Hypothesis: Glucose is known to be protective in moderate low flow ischaemia due to the production of glycolytic ATP. However, it is questioned whether glucose would still be protective in ultra low flow ischaemia. Firstly, glycolysis is thought to be inhibited, and secondly, deleterious glycolytic metabolites accumulate. Our hypothesis was that in ultra low flow ischaemia, glucose utilisation is not inhibited at the level of glycolysis, but by delivery. Increased delivery of glucose should result in increased production of protective glycolytic ATP, but the rate of metabolite accumulation would also increase. Using ultra low flow rates, I wished to investigate how to achieve optimal rates of glycolysis, and how such rates would be balanced by any detrimental component of metabolite accumulation.

Methods: The isolated Langendorff-perfused rat heart, with a left ventricular balloon to record ischaemic contracture and reperfusion stunning, was used, with severe flow restriction. Glucose concentrations were changed and pre-ischaemic glycogen contents were altered by perfusion with different substrates (acetate - depletion; glucose + insulin - loading) or by preconditioning, with 5 min ischaemia and 5 min reperfusion prior to sustained ischaemia.

Results: Analysis of glucose uptake relative to delivery showed that in severe low flow ischaemia, the extraction of glucose was increased, and glycolysis was thus limited more by substrate supply than by enzyme inhibition. Analysis of metabolites confirmed this concept. The optimal glucose concentration during severe low flow ischaemia was 11 mM, giving maximal recovery on reperfusion. Both lower and higher glucose concentrations increased ischaemic contracture. Changes in pre-ischaemic glycogen levels correlated with the time to onset of contracture, such that a reduction in glycogen accelerated contracture. Prior glycogen depletion or loading did not improve functional recovery. The benefits of preconditioning on reperfusion function following sustained total global ischaemia could not be related to glycogen depletion. If preconditioning were followed by sustained low flow ischaemia, glucose uptake was increased, but no benefit was found, possibly because a low residual flow abolished the effects of preconditioning. Many of the above results are consistent with the hypothesis that too low a rate of glycolysis results in insufficient ATP production for protection, while excess glycolytic rates lead to excess metabolite accumulation with detrimental effects.

Conclusions: Provision of glucose at the correct concentration, when the benefit associated with glycolytic ATP outweighs the detriment associated with moderate metabolite accumulation, is protective to the low-flow ischaemic myocardium, which can upregulate its ability to extract glucose. Improved residual flow enhances this benefit. Prior glycogen depletion is not beneficial, despite a reduced metabolite accumulation. This mechanism cannot be related to the protective effect of preconditioning.

Table of contents

Summary

Glucose is generally considered to be protective to the ischaemic myocardium. Glucose reduces ischaemic contracture and arrhythmias, and increases functional recovery on reperfusion, possibly by maintaining the sarcolemmal pump function by production of glycolytic ATP and thus regulating ionic concentrations in the cytosol. Glucose has been administered clinically to patients with myocardial infarction, in the form of glucose-insulin-potassium therapy, but this has generally been under-utilised. Glycolysis is thought to be inhibited in ischaemia at the level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and some studies have indicated that there may be detrimental effects associated with increased glycolysis, due to increased accumulation of the metabolites of the pathway, specifically lactate and protons. These detrimental effects may be present especially under ultra low flow conditions, when the detriment of the metabolites may outweigh the benefit of increased glycolytic ATP. These ultra low flow rates (0.015 to 0.2 ml/g wet wt/min), are of particular relevance given that *in vivo* flow rates in severe ischaemia in larger animals are in this range. Study of any therapy for ischaemia should therefore be undertaken with experimental models using similar rates of delivery and washout. I wished to test the hypothesis that the residual coronary flow may be a crucial determinant of the effectiveness of the provision of glucose, and then to determine the optimal conditions for provision of glucose to the ischaemic myocardium.

I used the Langendorff isolated perfused rat heart, with a left ventricular balloon to record ischaemic contracture (a rise in resting tension during ischaemia, associated with ATP depletion and calcium entry) and functional recovery on reperfusion.

My first goal was to establish at what low residual flow rate would the detriments associated with glucose provision (at the standard concentration of 11 mM) outweigh the benefits, such that the absence of glucose would lead to better tolerance of ischaemia. However, at no residual flow rate (even as low as 0.015 ml/g wet wt/min) was the presence of glucose more detrimental than the absence of glucose during the ischaemic period. Having established that the presence of glucose was beneficial, I then wished to determine the optimal concentration of glucose under a variety of low flow conditions, looking at functional recovery. I found that the standard glucose concentration of 11 mM, used in almost all perfusions with the isolated rat heart (in the absence of any alternate substrate) was optimal over a range of ultra low flows. Even when an alternate substrate (acetate) was present, 11 mM glucose was optimal. At higher flow rates (0.5 ml/g wet wt/min) the hearts perfused with 11 mM glucose resulted in almost 100% recovery after 30 min ischaemia and reperfusion.

At low flow rates (0.1 and 0.2 ml/g wet wt/min) the use of 22 mM glucose exacerbated ischaemic contracture and reduced functional recovery. These findings pointed to an impairment of function associated with excess glycolytic substrate. Even with a residual low flow of 0.5 ml/g wet wt/min, the effects of 22 mM glucose with and without insulin were worse than 11 mM glucose hearts. Thus, at a

flow rate of 0.2 ml/g wet wt/min, provision of 11 mM glucose appears to be optimal. I used this as the standard reference model for further work.

No difference in the time to onset of ischaemic contracture was found with different glucose concentrations at a low flow of 0.2 ml/g wet wt/min. I then reasoned that a change in glycogen may alter the time to onset of contracture. Glycogen levels were altered by perfusion with different substrates (acetate or substrate-free to reduce glycogen; addition of insulin to glucose-containing perfusate to increase glycogen). Increased glycogen was associated with a delay in time to onset; reduced tissue glycogen reduced time to onset of contracture. The provision of glucose also reduced the peak contracture in all hearts. I was thus able to draw inferences regarding the respective roles of glucose-derived versus glycogen-derived ATP, acting at different sites in the myocyte. Glucose-derived ATP may regulate the Na^+/K^+ ATPase pumps in the sarcolemma (and thus the cytosolic Ca^{2+} gradient following inhibition of the Na^+/Ca^{2+} exchange), as well as the SR Ca^{2+} ATPase pump, and thus be responsible for maintaining ion gradients. Glycogen-derived ATP may be present mainly at the sarcoplasmic reticulum (SR) and the myofibrils, maintaining SR Ca^{2+} ATPase-mediated Ca^{2+} uptake and ensuring relaxation of actin-myosin bonds. These mechanisms needs to be defined further on a molecular level.

I analysed the kinetics of glucose uptake in ischaemia, over a range of glucose concentrations and residual flow rates. I established that if glucose uptake is expressed as a function of the glucose delivered to the myocardium (determined by the glucose concentration and the coronary flow) that in fact glucose extraction increases as the residual flow rate decreases below about 1-2 ml/g wet wt/min. Thus the ischaemic myocardium appears to be able to upregulate its ability to take up glucose from the perfusate delivered to the cells. This may be achieved by increased translocation of GLUT4 insulin-sensitive glucose transporters to the membrane, or an upregulation of transporters already in the membrane. The kinetics of glucose uptake over the range of glucose concentrations and low flows I studied establishes that glucose uptake follows Michaelis Menten kinetics, consistent with enzyme-driven glucose uptake i.e. by facilitated diffusion. In addition, measurement of glycolytic metabolites did not confirm inhibition at GAPDH, although there may be a bottle neck effect at phosphofructokinase, especially when glycolytic substrate in excess, resulting in increased sugar phosphate accumulation. Thus the concept that glycolysis is inhibited in ischaemia is challenged by these results. Rather, I suggest that glycolysis is limited in ischaemia by the availability of substrate. Increased substrate availability should thus increase the benefit. However, an excess glycolytic substrate is detrimental by producing excess metabolites. Thus an optimal concentration of 11 mM glucose is confirmed, at which delivery of glucose and subsequent rates of ATP production are balanced by any detrimental effect of metabolite accumulation. Increased delivery of glucose and washout of metabolites by increased residual flow is of additional benefit, such that at 0.5 ml/g wet wt/min, functional recovery after 30 min approaches pre-ischaemic values.

Glycogen is the endogenous glycolytic substrate, and its content at the onset of ischaemia is thus a primary determinant of the rate of glycolytic ATP production, metabolite accumulation, and ischaemic injury. Preconditioning, whereby one or more brief episodes of ischaemia and reperfusion protect the heart against the effects of a subsequent sustained ischaemic period, has received much attention in the literature. The preconditioning protocol (one 5 min episode of ischaemia and 5 min reperfusion) results in a depletion of pre-ischaemic glycogen, and this has been proposed as one of the mechanisms whereby preconditioning may exert its protective effects. A much-quoted paper [387] suggests that the accumulation of lactate and protons from the breakdown of glycogen is deleterious to the ischaemic myocardium. This finding is in contrast to our results which found that provision of glucose is beneficial, even at very low residual flows. I investigated the effect of changes in pre-ischaemic glycogen content (glucose - control; acetate - depleted; glucose + insulin - loaded) on the subsequent response to total global ischaemia with reperfusion, and preconditioned similar hearts to determine any correlation between pre-ischaemic glycogen content, preconditioning, and functional recovery. I could find no benefit associated with increased glycogen levels, with a significant increase in sugar phosphate levels during ischaemia to account for this detrimental effect. In addition, no benefit was associated with glycogen depletion, with exacerbation of ischaemic contracture. Preconditioning resulted in a doubling in functional recovery in hearts perfused with glucose alone (standard pre-ischaemic glycogen content) but preconditioning had no effect on either glycogen-loaded or glycogen depleted hearts, correlated with maintained high sugar phosphate levels, and severely reduced ATP levels respectively. thus for preconditioning to occur, a "normal" level of glycogen is required, and glycogen depletion is not related to the protective effects of preconditioning.

I subsequently tested the effects of preconditioning (5 min total global ischaemia + 5 min reperfusion) on sustained low flow ischaemia (0.2 ml/g wet wt/min) with 11 mM glucose. Preconditioning significantly increased glucose uptake, and attenuated contracture compared to preconditioning zero flow hearts, but contracture was no different compared to control low flow hearts. In addition, functional recovery was not altered by preconditioning. If pre-ischaemic glycogen content and ischaemic glucose uptake were modulated as described above, I could find no relationship between changes in glycolysis and the effects of preconditioning. No benefits were associated with preconditioning on low flow ischaemia under any circumstance. Unaltered functional recovery after preconditioning and low flow ischaemia could be related, firstly, to an increase in glucose uptake with preconditioning to a value higher than optimal, which resulted in excess accumulation of glycolytic metabolites despite a reduction in pre-ischaemic glycogen levels, and thus outweighed any possibly benefit associated with preconditioning. Preconditioning did not additionally increase glucose uptake in insulin-treated hearts, suggesting that preconditioning may exert effects on glucose uptake in a similar fashion to insulin i.e. translocation of the GLUT4 transporter. However, these hearts showed

increased sugar phosphate accumulation because of a high pre-ischaemic glycogen. Secondly, reduced glucose uptake and reduced tissue glycogen in acetate preconditioned hearts resulted in exacerbated contracture with poor recoveries because of low ATP production.

Alternative explanations for these findings are that in the presence of a low residual flow, preconditioning may not exert any protective effects, and the subsequent changes in glycolytic flux (from both glucose and glycogen) are independent of any possible protective effects brought about by preconditioning. Other workers support the concept that a residual low flow abolishes the protective effect of preconditioning, in terms of functional recovery in rats, and infarct size in larger animals.

Finally, I wished to confirm previous results showing that the addition of lactate to a glucose-containing perfusate infused at a low residual flow of 0.5 ml/g wet wt/min is deleterious to the myocardium. However, I found that only at a low residual flow of 0.2 ml/g wet wt/min was any detrimental effect with lactate noted. Thus I could not confirm a detrimental effect of the lactate produced in the presence of sufficient glycolytic ATP i.e. with an optimal concentration of 11 mM glucose. In addition, the role of lactate as a major inhibitor of glycolysis in ischaemia is questioned, following results obtained from: direct application of extracellular lactate; analysis of glucose uptake in ischaemia which suggests that glycolysis is limited mainly by the availability of substrate and not by enzyme inhibition; and metabolite accumulation which does not suggest inhibition at GAPDH, and shows a large accumulation of lactate directly related to glucose uptake.

Thus glucose provision, at an optimal concentration of 11 mM, is beneficial to the ischaemic myocardium. The benefits associated with glycogen loading are not as clear. Rather, moderate glycogen, together with an optimal glucose is required for ensuring recovery. The addition of insulin would lower the optimal concentration *in vivo*, as would the presence of fatty acids and other substrates. These factors need to be investigated further prior to application of glucose-insulin-potassium therapy in patients.

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Publications arising from this work

Articles

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Abbreviations

A list of abbreviations commonly used throughout this thesis are given below.

1,3-BPG - 1,3-bisphosphoglycerate

A(1)(2)(3) - adenosine receptor subtypes

α1 - alpha 1 adrenergic receptor

ADH - alanine dehydrogenase

ADP - adenosine diphosphate

αGP - αglycerophosphate (also glycerol 3-phosphate)

αKG - αketoglutarate (also 2-oxoglutarate)

AMP - adenosine monophosphate

ATP - adenosine triphosphate

C_6 - 6-carbon units (glucose moieties of glycogen)

Ca^{2+} - calcium ion

cADPR - cyclic adenosine diphosphate ribose

cAMP - cyclic adenosine monophosphate

CF - coronary flow

cGMP - cyclic guanosine monophosphate

CK - creatine kinase

CL - citrate lyase

CP - creatine phosphate (PCr - phosphocreatine)

Cr - creatine

DAG - diacylglycerol

DHAP - dihydroxyacetone phosphate

DOD - difference in optical density

DP - diastolic pressure

dpm - disintegrations per min

ECG - electrocardiogram

EDTA - (ethylenedinitrilo)-tetraacetic acid

F 2,6-P_2 - fructose 2,6 -bisphosphate

F6P - fructose 6-phosphate

FAD(H_2) - flavin adenine dinucleotide (reduced)

FDP - fructose 1,6-diphosphate

$G(_s)(_i)$ - (stimulatory) and (inhibitory) GTP binding proteins

G1P- glucose 1-phosphate

G6P - glucose 6-phosphate

G6PDH - glucose 6-phosphate dehydrogenase

GAP - glyceraldehyde 3-phosphate

GAPDH - glyceraldehyde 3-phosphate dehydrogenase

GDH - glycerol 3-phosphate dehydrogenase (αGP dehydrogenase)

GDP - guanosine diphosphate

GIK - glucose-insulin-potassium therapy

GLUT - glucose transporter (1-6)

GOT - Glutamate-oxaloacetate transaminase

GTP - guanosine triphosphate

H^+ - proton

HK - hexokinase

HR - heart rate

IP_3 - inositol trisphosphate

IRS-1 - insulin receptor substrate 1

K_{ATP} - ATP-dependent potassium channel

LDH - lactate dehydrogenase

LV - left ventricle

LVDP - Left ventricular developed pressure

LVEDP - left ventricular end diastolic pressure

MDH - malate dehydrogenase

MW - molecular weight

Na^+ - sodium ion

Na^+/Ca^{2+} - sodium-calcium exchanger

Na^+/H^+ exchanger - sodium-proton exchanger

Na^+/K^+ ATPase pump - ATP-dependent sodium-potassium pump

NAD(P)(H) - nicotinamide adenine dinucleotide (phosphate) (reduced)

NO - nitric oxide

PCA - perchloric acid

PEP - phosphoenolpyruvate (TCHA - tricyclohexylammonium salt)

PFK - phosphofructokinase

PGI - phosphoglucoisomerase

pHi - intracellular pH

pHo - extracellular pH

Pi - inorganic phosphate (PO_4^-)

PK - pyruvate kinase

PKA - protein kinase A

PKC - protein kinase C

PPi - pyrophosphate

rpm - revolutions per min

SP - systolic pressure

SR - sarcoplasmic reticulum

SR Ca^{2+} ATPase - sarcoplasmic reticulum ATP-dependent calcium pump (also sarcolemmal Ca ATPase)

TCA cycle - tricarboxylic acid cycle

TIM - triosephosphate isomerase

Tn (T)(C) - troponin T or C

TRAM - triethanolamine HCl

Tris - Tris(hydroxymethyl-methylamine)

UDP - uridine diphosphate

UTP - uridine triphosphate

Overview - aims and organisation of thesis

1) AIMS AND GENERAL HYPOTHESIS OF THE THESIS

Glucose is a simple hexose sugar utilised by all cell types, especially the brain and erythrocytes. In the isolated perfused rat heart, glucose at a concentration of 11 mM is generally the sole extrinsic substrate.

Besides its physiological role, provision of glucose may have a protective effect on the ischaemic myocardium by reducing arrhythmias and ischaemic contracture, and improving functional recovery on reperfusion. These benefits are hypothetically mediated by increased glycolytic ATP production, which may be involved in control of SR and membrane pumps. However, the advantages of increased ATP could theoretically be outweighed by the accumulation of possibly deleterious end-products including lactate, NADH, protons and sugar phosphates. The extent of harm caused by these end-products is not clear, particularly when balanced against the benefits of glycolytic ATP.

The major aim of this thesis was to clarify the role of glycolytic flux under ischaemic conditions more representative of those in the *in vivo* myocardium.

I wished to determine an optimal glucose concentration, at which the production of glycolytic ATP is maximal, and its benefit is not outweighed by excess deleterious metabolite accumulation. A major determinant of both the amount of glucose made available to the cells, and the rate of washout of end products, is the coronary flow. Some residual flow is usually present in ischaemic tissue *in vivo*, but this factor is not usually considered in an experimental isolated rat heart. Another major determinant of the rate of glycolysis is the amount of glycogen, the endogenous glycolytic substrate, available in the tissue at the onset of ischaemia. Tissue glycogen can be modified by perfusion with different substrates, and also by preconditioning, a fairly recent phenomenon whereby an initial ischaemic episode(s) with subsequent reperfusion can protect against the consequences of a subsequent sustained ischaemic period. Glycogen is reduced by the initial ischaemic episode(s), and a reduction in glycogen has been shown to be beneficial by reducing the accumulation of glycolytic end products. Thus glycogen reduction may be a mechanism involved in preconditioning. However, preconditioning is also known to stimulate glucose uptake in a model of sustained low flow ischaemia.

Our basic hypothesis was that provision of glycolytic substrate to the ischaemic heart should always be beneficial except under circumstances where the accumulation of metabolites from excess substrate could outweigh the benefits of increased ATP production. I thus investigated changes in glucose uptake over a range of low coronary flows and different glucose concentrations, and modifications in glycogen by a number of mechanisms, and determined the response of the tissue to ischaemia.

2) ORGANISATION OF THESIS

The thesis is divided into several sections. The literature review describes the validity of the ischaemic model used in the majority of studies presented in this thesis, with reference to the clinical correlates. A broad overview of the changes brought about in the tissue by ischaemia is given. Ischaemic contracture, the main functional index of ischaemic injury used in the studies, is discussed in terms of the mechanisms which trigger contracture and potentiate its effects. The changes on reperfusion, and functional recovery, are also discussed. The specific actions of glucose on the ischaemic heart are then discussed in depth, expanding the argument over the benefit vs. the detrimental effects of glucose, extending into the role of glycogen. Some of the postulates of the thesis are elaborated in this section. The concept of compartmentation, as the possible mechanism for distinguishing the benefits of ATP from glucose, glycogen and oxidative phosphorylation, is discussed. An extensive review of preconditioning is then given, a much investigated phenomenon, but with little indication that the mechanism has yet been fully clarified. One hypothesis, that glycolysis is involved in preconditioning, is discussed with reference to the various proposals for the effector of preconditioning. A summary of each chapter is added at the end.

There are six sections of results, each of which describes a complete study (as published, or in preparation for publication). The first two reports deal largely with experimental data describing glucose uptake at a range of low flows with or without insulin, the consequences to the heart in terms of ischaemic contracture and functional recovery, and the role of glycogen which appears to be involved specifically in determining the time to onset of contracture. The third paper focuses specifically on glucose uptake, reviewing old concepts of regulation of glucose utilisation under ischaemic conditions.

The next two reports look at preconditioning, firstly concentrating on glycogen, and secondly on glucose uptake. Both of these are dissociated from the mechanism of preconditioning by these studies, but the findings explain further the roles of glucose and glycogen in ischaemia. The final report is a brief investigation of the role of lactate, as a major end product of glycolysis. This study directly refutes a previous report, suggesting that lactate is always deleterious.

The discussion focuses on different aspects emphasised by the results. The first chapter expands on Ch III of the Results section, with a more in-depth analysis of previous concepts that glycolysis is inhibited in ischaemia, which are challenged by the present findings. The second chapter focuses on ischaemic contracture, and its modification by glycolytic ATP. Several points of contradiction were brought out in the studies, which are hopefully clarified in this section. A brief reappraisal of preconditioning is then given, based on the experiences in trying to obtain a consistent model of preconditioning.

Finally, a brief description of the possible clinical applications of the use of glucose in ischaemia are described, to place the findings in a clinical context. An appendix is included, describing uptake and metabolism of glucose and of glycogen, for reference purposes.

Literature review

I. ISCHAEMIA

1) ISCHAEMIC HEART DISEASE

a) Definition

"Ischaemia" is derived from the Greek, "ισχειν" - to hold back, and "αιμα" - blood, and can be defined simply as a local and temporary deficiency of blood supply to a tissue (although even this simple definition has been challenged [187]). Ischaemia is thus a reduction in blood flow with metabolic consequences for the tissue. Thus a more exact definition of myocardial ischaemia in terms of metabolic demand is "whenever the flow of arterial blood through the diseased vessels is reduced to a volume below that required by the myocardium for adequate function" [221], (although the vessels themselves may not be diseased) and on a molecular level "whenever the arterial blood flow is insufficient to provide enough oxygen to prevent intracellular respiration from shifting from the aerobic to the anaerobic form" [221]. Myocardial ischaemia can thus arise either from a reduction in, or complete stoppage of flow, or an imbalance between supply and demand. Within the limits of this definition, there is a wide range in severity, and extent of "ischaemia" with differing consequences for the metabolism of the tissue.

The biochemical consequences of ischaemia are many and varied, stemming mainly from a reduction in energy supply and an accumulation of harmful end products, leading to mechanical and electrical changes in heart function. Prolonged ischaemia may develop into a pathological state. The associated clinical manifestations include angina pectoris or chest pain, shortness of breath, ECG changes and arrhythmias, contractile dysfunction with left-sided heart failure, and myocardial infarction.

"Ischaemia" is a heterogeneous state, both in its genesis and at a cellular level. The different degrees to which supply and demand are altered are reflected in the variety of laboratory models used to study the pathological changes associated with ischaemia. This thesis is concerned with models of global zero flow or very low residual flow, representative of compromised tissue at the centre of an underperfused region with some degree of residual flow, as is usually found *in vivo*. After a brief description of clinical ischaemia, the validity of this model in terms of its clinical relevance are discussed. A broad review of the consequences of ischaemia for the heart is given, discussed in terms of cellular and tissue dysfunction during ischaemia and on restoration of flow. The following chapters then focus on specific aspects of ischaemia and reperfusion affected by glucose, glycogen, and preconditioning.

b) Causes of ischaemic heart disease

Ischaemic heart disease is the clinical entity of coronary vessel disease, the underlying pathology of which is mostly atherosclerosis. This is an chronic, age-related degenerative disease with fibrous, fatty plaques or atheromas, developing in the arterial walls of the coronary circulation. Narrowing of the vessel, or complete occlusion, results, especially if a thrombus develops at the constriction. Other causes of ischaemia from changes in the coronary circulation include reflex coronary spasm in response to emotion, cold, upper gastrointestinal tract disease, or smoking, and hypoxia. The standard experimental models mimic some degree of occlusion of the arteries, although hypoxia has also been used extensively.

c) Clinical complications - causes and consequences

i) Angina pectoris

Angina pectoris indicates chronic compromised flow to a portion of the myocardium. The symptoms - chiefly chest pain - may become evident only under strain, e.g. exercise - angina of effort; or may be persistent at rest - unstable angina. These differences are due to variations in the degree of obstruction across the artery, and the extent of tissue perfused by the affected artery. Unstable angina is characterised by ST segment elevation. In rare cases, vasospasm may result in Prizmetal's angina, which is generally distinguished by changes in the ECG including T wave inversion, although this is variable.

ii) Myocardial infarction

Myocardial infarction is due to partial or complete occlusion of a coronary artery, possibly by a thrombus, resulting in an ischaemic insult. Chest pain and ECG changes are evident, followed by more severe manifestations, including enzyme loss from ruptured cells. Cell death, or necrosis, occurs after about 15-20 min, with the beginning of an infarct. After 6 hrs, the infarct is probably fully developed i.e. the total area at risk (that perfused by the affected artery below the site of occlusion) is affected. Infarct size must be limited by reperfusing as much of the remaining viable tissue as possible. A myocardial infarct consists of a region of no perfusion with dead tissue, surrounded by tissue with a gradient of underperfusion. Collateral flow from the surrounding arteries, and residual flow through the affected area determine the limits of the infarct. The area surrounding an established infarct, or scar tissue, develops increased collateral arteries to improve perfusion.

iii) Ventricular fibrillation and sudden death

The generation of ischaemia arrhythmias is complex, largely involving cAMP accumulation and subsequent cytosolic Ca^{2+} overload, resulting in disturbances in ion currents across the membrane [329]. Arrhythmias result from ischaemia of Purkinje cells, which become the focus of

arrhythmogenesis, with a disruption in atrio-ventricular conduction. Ventricular arrhythmias have also been attributed to electrically disturbed cells at the "border" of the ischaemic zone, arrhythmogenic substances released from the ischaemic zone, and circus movement, as the path of conduction is altered, and the refractory periods are disturbed [90, 336]. Ventricular arrhythmias may then develop into fibrillation, resulting in complete cardiac failure and sudden death. The presence of an old infarct predisposes to arrhythmias, as the route of electrical conduction around the myocardium is affected.

d) Experimental models of ischaemia

Ischaemia has been investigated using a number of experimental models, each with specific characteristics. Ischaemia can be broadly categorised as either supply or demand ischaemia. These categories merge as deprivation of oxygen is prolonged, leading to irreversible changes. Hypoxic/anoxic models are also frequently used. However, despite the validity of most models, care must be taken in extrapolating between models, and to the clinical condition.

i) Hypoxia vs. ischaemia

The consequences of a reduced blood supply during ischaemia are an inadequate supply of oxygen and impaired washout of metabolic products. The effect of drugs, or changes in the composition of the blood in ischaemia are difficult to study because of the absence of flow in most models; therefore many studies have used hypoxia or anoxia which enable the effects of oxygen deprivation to be studied. In addition, isolated cell models can only mimic ischaemia, using hypoxia and metabolic inhibition. The continued delivery of substrate (unless removed) and other substances, as well as continued washout of metabolites, modulates the response of the hypoxic tissue compared to that of "true" ischaemia. These differences must be taken into account.

ii) "Demand" ischaemia

"Demand" ischaemia results from insufficient supply of oxygen to the heart following a reduction in blood oxygen content (from pulmonary complications - hypoxia; anaemia), or when the oxygen demand of the heart is increased (extreme exercise, excitement), generally with some degree of coronary narrowing [15]. The latter is typical of angina of effort. Washout of metabolites is still present, which reduces the impairment. Pathologic increases in heart rate, metabolic rate (thyrotoxicosis, increased catecholamine secretion), left ventricular wall stress, inotropic state and preload (aortic stenosis, aortic insufficiency, diastolic hypertension, hypertension), all increase myocardial oxygen consumption and result in ischaemic heart disease if the coronary flow reserve is impaired. While this definition may now be a little outdated, it still has important implications. Experimental models of "demand" ischaemia include rapid pacing, increased preload, and hypertrophy.

iii) Supply ischaemia

"Supply" ischaemia arises from reduced supply of oxygen to the tissues because of a reduced coronary flow. This is typical of coronary occlusion and resultant myocardial infarction [36, 530] and is by far the most commonly used experimental model.

Regional ischaemia - results from a narrowing or obstruction of an artery, reducing perfusion in the area distal to the constriction [36]. Experimentally, regional ischaemia is induced by ligation, usually of the left descending coronary artery. While this blockage may be fully reversible, a central area of necrosis may develop if sustained, surrounded by cells with varying severity of ischaemia. This model predisposes to the development of arrhythmias, and depression of left ventricular function, with reduced cardiac output and aortic pressure. The heart does not cease to beat.

Global ischaemia - results from a shut down of total coronary blood supply. This is generally only found in experimental conditions, but occurs clinically following aortic cross clamp and cardiopulmonary bypass. This model in an isolated perfused heart allows study of a reasonably homogenous ischaemic tissue. Global ischaemia can either be total (complete absence of coronary flow), or a low residual flow may be maintained using a lowered constant pressure or a constant flow infusion. This low flow can be termed the sum of residual and collateral flow to an ischaemic region *in vivo*. The term, residual, henceforth applies to the low flow supplied to the globally ischaemic isolated perfused rat heart model.

iv) Model of global low flow ischaemia used in this thesis

While the majority of isolated heart studies use total global ischaemia, low flow ischaemia allows a more clinically relevant evaluation of changes in ischaemia. However, the degree of low flow used is also important. The extent of residual and collateral coronary flow is important in determining the eventual recovery [485], and some flow is usually present, even with very severe *in vivo* ischaemia. In *in situ* pig or dog hearts, the normal coronary flow is about 1-2 ml/g wet wt/min, with differences between the endo-and epicardium, the latter usually better perfused. With a flow restriction, the endocardium is more at risk with flow restricted to 0.05-0.15 ml/g wet wt/min [360, 421, 507], about 2.5-15% of normal flow rates.

In vivo flow rates in a rat heart are about 5-6 ml/min/g wet wt [279]. In excised, buffer-perfused hearts, coronary flow is about twice to three times the flow *in vivo* (Langendorff mode less than working mode). The reduced oxygen-carrying capacity of the crystalline solution results in maximal dilation of the vessels, which in turn lack autoregulation [39]. The flow rate is determined largely by the perfusion pressure. If the residual flow in ischaemia is set at about 10-20% of pre-ischaemic perfusion flow rates, this is equivalent to 20-40% or more of normal *in vivo* flows - enough to produce hypoxia, but not necessarily "true" ischaemia with greatly impaired washout. Lower flow rates more representative

of true "*in vivo*" ischaemic flow rates need to be investigated. This premise is the basis of the standard model used in this thesis. I have chosen 0.2 ml/g wet wt/min as a reference point, a rate which delivers sufficient coronary flow and substrate to allow some residual cell function, such that after a relatively long period of ischaemia, the hearts recover about 50-60% of pre-ischaemic mechanical function. However, under these conditions (with 11 mM glucose), a moderate but marked contracture (about 40-50%) is seen. Thus both contracture and functional recovery can be either improved, or exacerbated. This flow rate represents a flow reduction to about 3-5% of normal *in vivo* flow rates in the rat, and about 1-2% of *in vitro* flow rates in the isolated organ. This flow rate is thus comparable with those in subendocardial ischaemia in larger animals, and more truly representative of ischaemia than flow rates more often used (0.5-0.6 ml/g wet wt/min [14, 123, 218, 389, 390, 424, 468, 469]). While differences certainly exist between species (cf. *in vivo* flow rates of rats vs. larger animals above) and there is also a significant difference between hearts perfused with crystalloid solution, compared to blood, at these very low flows, the rate of delivery and of washout between the different models becomes negligible. At flow rates of 0.5-0.6 ml/g wet wt/min, more divergence between the different models would be present as other factors could affect the response of the tissue (oxygen content, friction forces, oedema, other substrates, degree of ischaemia, autoregulation).

e) Consequences of ischaemia

i) Systolic vs. diastolic contractile failure

"Demand" ischaemia leads to a rise in diastolic tension with reduced compliance. Insufficient high energy phosphates from increased utilisation leads to intracellular calcium accumulation and a reduced relaxation. Diastolic dysfunction leads to pulmonary dysfunction with shortness of breath, reduced relaxation, an upward shift of the diastolic pressure-volume curve and an absence of increased late systolic filling as compensation. A reduced cardiac output results. Systolic failure may also be present, with a shift in the pressure-volume relationship (less pressure generated for a given diastolic volume). Demand ischaemia may thus cause predominantly diastolic dysfunction with pulmonary congestion, with a reduced return of blood from the lungs to the heart because of reduced filling time.

Systolic failure following supply ischaemia is accompanied by low cardiac output, left ventricular pump failure and reduced pump function. With total global ischaemia, the accumulation of metabolites including Pi and protons leads to contractile failure (see below), and the tissue becomes flaccid. As ATP falls, an increase in resting tension, or contracture (see below), follows, compounded by an increased intracellular Ca^{2+}. On reperfusion, diastolic failure is often present as a result of contracture, and of calcium-induced depression of relaxation. Thus measurement of contracture can be used as a rough index of ischaemic injury.

ii) Functional recovery, stunning and necrosis

The degree of functional recovery after an ischaemic period (supply ischaemia) is determined by three entities. On reperfusion, some function is usually restored immediately (non-injured tissue). Some tissue may be stunned. Stunning is strictly defined a transient, reversible depression in function following a brief period of ischaemia (< 15-20 min), insufficient to cause necrosis [44]. Reperfusion for a sustained period should result in full recovery of function. The period of reperfusion required can vary depending on the species and severity of ischaemia. The third determinant of recovery is the degree of injury to the tissue, i.e. alterations in ultrastructure, which may be reversible on reperfusion, or irreversible leading to necrosis. Irreversible injury occurs after 20-25 min or more. Broadly speaking, the relative degrees of fully recovered tissue, stunning, and of necrosis (infarct size), will determine the eventual outcome of a more prolonged period of ischaemia. In addition, reperfusion injury, that which occurs on reperfusion, but which appears to be directly related to the degree of ischaemic injury, determines the degree of recovery. Thus functional recovery is an index of ischaemic and reperfusion injury. The issues of reversible vs. irreversible injury, and reperfusion injury, are discussed more fully below.

iii) Preconditioning

Preconditioning is defined as one or more brief periods of ischaemia providing protection against the consequences of a subsequent sustained ischaemic episode. The interest in preconditioning has been extensive, as an endogenous mechanism of protection. This phenomenon alters the concept of ischaemia as a purely deleterious event, and it can now be seen to trigger protective actions. The concept therefore is that if an ischaemic episode must occur, it is preferential for this to be preceded by a brief episode of ischaemia to precondition the heart. A more clinically applicable method of preconditioning has been much researched, and is one of the issues discusses in this thesis with respect to glucose.

2) ISCHAEMIC INJURY

a) Ultrastructural changes in ischaemia

Ischaemic injury ranges from fully reversible to irreversible cellular modifications, dependent on the duration and severity of ischaemia. Manifestations of ischaemic injury include changes in the cellular ultrastructure with membrane rupture, mitochondrial swelling, oedema, and shortening of the sarcomeres, with an overall reduction in contractile function [222, 484, 540]. Patches of necrosis may be present, weakening the myocardial wall, and acting as a focus for arrhythmogenesis. Changes to the myocardium are caused by depletion of energy stores, accumulation of waste products, membrane

disruption, intracellular acidosis and Ca^{2+} accumulation, free radical activity, and activation of catalytic enzymes. The functional changes broadly reflect cellular changes.

b) Reversible vs. irreversible injury

Reversible injury implies that restoration of flow will lead to full recovery of tissue. Changes may be seen in the mitochondria and other organelles, but these disappear on reperfusion, although possibly with a lag time. Irreversible injury occurs with changes in the cellular components which are not reversed by reperfusion. Irreversible injury may be termed the changes in the cellular ultrastructure such that the cell will not recover full function, which can lead to cell death and permanent scarring. There is an continuum of ischaemia/reperfusion injury, from reversible to irreversible changes, and ultimately cell death. An increased duration of ischaemia means that a greater proportion of the area at risk becomes irreversibly injured. Using a totally globally ischaemic dog heart, with severe ischaemia (60 min), histological and biochemical evidence of cell damage is apparent (contraction bands, swollen mitochondria with loss of dense granules, leakage of extracellular enzymes). On reperfusion, recovery is very slow [484]. These changes do not necessarily precipitate cell death. After 90 min ischaemia, the damage is irreversible, with severe cellular distortion [456, 484]. Cell death, or necrosis, is associated with loss of membrane integrity [456], occurring at the level of the individual cells and spreading to adjacent cells if ischaemia is maintained. Derangement of the cellular structures occurs, resulting in the development of an infarction with scar tissue [148].

3) MECHANICAL CHANGES WITH FLOW REDUCTION

a) Early contractile failure

After the onset of total global ischaemia, developed pressure falls rapidly. The mechanism for early contractile failure is as yet not fully understood. A reduction in ATP is an obvious candidate - however, the fairly slow rate of ATP depletion is not temporally correlated with rapid contractile failure [7]. Inorganic phosphate (Pi) and protons (H^+) can also reduce contractility [31, 284], and are likely candidates for pump failure. Pi binds to sites in the myofilaments inhibiting actin-myosin binding, resulting in less cross-bridge formation and reduced force development. Pi may also decrease SR Ca^{2+} loading and thus decrease Ca^{2+} dependent Ca^{2+} release, and thus reduce contractility [592]. H^+ compete for Ca^{2+} binding sites, such that for a given concentration of Ca^{2+}, less tension is developed [288]. The mechanical influences of a reduced vessel turgidity following vascular collapse may also be important [560]. With a reduction in filling pressure, the stiffness and stretch are reduced - the so-called "garden hose" effect.

The trigger for contraction, the action potential, is affected by ischaemia. A slowed rate of rise of the upstroke of the action potential and slower conduction velocity reduces Ca^{2+} influx into the cell and Ca^{2+}-induced Ca^{2+} release from the SR [130]. Ischaemia induces a rapid membrane depolarisation attributed to rapid K^+ efflux, mediated in part by the K_{ATP} channel [233]. Extracellular K^+ may increase to about 15 mM, changing the membrane potential to -50 mV [258] at which the membrane is inexcitable. Cell-to-cell uncoupling also occurs reducing excitability.

b) Ischaemic contracture

After 5-10 minutes of total global ischaemia in an isolated perfused heart, developed pressure is usually absent. After 10-15 min a rise in resting tension (ischaemic contracture) develops [189]. Ischaemic contracture is defined as a rise in resting tension over time, with shortening of the muscle and increased stiffness. Contracture is a useful index of cell injury, linked to ATP depletion and Ca^{2+} accumulation [42, 184, 189, 271, 511], and is a possible predictor of functional recovery [32] (see below for discussion). If perfusion is restored before the onset, or in the early stages, of ischaemic contracture, recovery should be virtually complete [7, 484]. However, if contracture is well established, developed pressure does not recover fully on reperfusion.

Cell damage may be accelerated by reperfusion, with extensive enzyme release [192, 406]. Reperfusion may lead to contraction band formation, with excess Ca^{2+} entry through fragile membranes [148]. Contraction bands are a pathological feature of severely injured myocytes [147]. Regions of the sarcomere become disrupted, with aggregated clumps of actomyosin.

4) CHANGES IN METABOLISM IN ISCHAEMIA

a) Hypoxia vs. ischaemia - the role of oxidative phosphorylation

In hypoxic hearts, developed pressure falls by about 70%, but may remain stable for about 30 min. The immediate consequence of a reduced oxygen supply is a rapid inhibition of oxidative phosphorylation, and reduced ATP production. However, anaerobic glycolysis is stimulated 20-fold in the first minute (in dogs) via reversal of the Pasteur effect, and can be maintained at this level if substrate is available [276]. With only 2 ATP mol vs. a maximum of 38 mol per mol glucose produced under aerobic conditions, this is a temporary measure. Hypoxic hearts gradually weaken as ATP production cannot keep up with demand. Alternatively, pump function may be lowered to meet reduced supply but eventually hearts are impaired by insufficient ATP.

In ischaemia, the reduction in function is rapid. NADH increases rapidly, lowering the redox potential. Some residual oxidative phosphorylation may continue, with CO_2 accumulation. Glycolysis

may be stimulated initially, but declines rapidly. While cellular strategies exist to reduce ATP utilisation, ATP content falls rapidly.

b) Energy balance

Protective mechanisms activated to conserve available energy include K^+ efflux to inhibit electrical activity, a fall in pHi which reduces Ca^{2+} binding, a decrease in contractile force, and cessation of contractile function mediated by Pi. The balance of supply and demand may be maintained for a brief period, especially with the breakdown of CP [50]. Energy production is dependent on substrate availability. Neither free fatty acids nor lactate can be utilised because oxygen is required, thus glucose and glycogen become the primary substrates. As ischaemia progresses, energy production is lowered as substrate levels fall.

i) Creatine phosphate

Although ATP is rapidly utilised with ischaemia, the levels do not fall as fast as would be expected. This is due to creatine phosphate utilisation (CP), which buffers the level of ATP for a few minutes via creatine kinase (CK) activity (see appendix). The fluxes through this reaction are very rapid. Reductions in ADP and Pi lead to marked shifts from CP to ATP. CP falls very rapidly as ATP is hydrolysed, to 20% of normal values in 1-3 min ischaemia [276]. Once CP is depleted, ATP levels drop rapidly. Pi levels increase linearly with a breakdown in CP [276], affecting contractile function. Thus rates of CP breakdown may be a major determinant of the rate of contractile failure.

ii) ATP production and utilisation

ATP is required for virtually all cellular processes, including contraction, ion regulation, and membrane integrity. In the first few minutes of total global ischaemia in an isolated rat heart, ATP falls to about 3 μmol/g wet wt (about 40-70% of basal). This level remains fairly stable for a few minutes, until a second rapid decrease occurs [189]. For many years, a "critical level" of ATP was sought as a determinant of recovery [189, 456]. However, values for this critical level of ATP differed widely (2 μmol/g wet wt [189, 276]; 0.4-0.6 μmol/g wet wt [223] etc. - see [408] for further discussion). Total tissue ATP appears to give little indication of the ability of the tissue to recover [278, 387]. Neely et al. [387] reported a wide range of functional recoveries after 30 min ischaemia, but with little difference in adenine nucleotide levels. This lack of correlation has been attributed in part to compartmentation of ATP (see Ch. II), and the involvement of the many other factors altered by ischaemia (see below). Only if ATP values are very low can it be reasonably surely predicted that recovery will be largely impaired.

In addition to the absence of correlation of ATP levels and recovery, several processes are known to occur in ischaemia e.g. opening of K_{ATP} channels, and formation of rigor cross-bridges, occur *in vitro*

only at very low ATP concentrations, far less than gross tissue levels e.g. the Km for rigor formation is 0.05 mM [131]. This effect can be attributed to the heterogeneity of ischaemia, with different rates of ATP depletion in different cells. In addition, different rates of ATP utilisation within the cell, and breakdown of processes for ATP transport, can result in localised areas of ATP depletion below critical levels. Total tissue ATP reflects the mean value of many cells at different stages.

Functional compartmentation within the cell also affects ATP utilisation (see Ch II). Certain membrane processes, including inhibition of the K_{ATP} channel [571], maintenance of the integrity of the membrane [49], and maintenance of the Na^+/K^+ ATPase pump [167], appear to utilise glycolytic ATP preferentially. The location of glycolytic enzymes, attached to the sarcolemma [434], may account for this preference. The mitochondria are closer to the sarcomeres - thus it is postulated that ATP from oxidative phosphorylation is utilised more by the contractile elements [570]. The concept of compartmentation is crucial to understanding many of the observations of protection by glucose and is discussed in depth in Ch II.

In addition to compartmentation, a given amount of ATP in ischaemia may be less efficient when broken down than it would be under normal conditions. The free energy change with hydrolysis of ATP is reduced as ADP and Pi rise, even though there may be no change in the concentration of ATP [7, 235] (see appendix). A fall in ATP will further reduce the free energy change. Each ATP-dependent process requires a different free energy change [7], thus some processes are more affected by others. The affinity of various cellular processes for ATP may also be altered in ischaemia, such that ATP binding is reduced, and the efficacy of ATP lessened. These effects all contribute to the lack of correlation of ATP levels with cellular changes.

iii) Adenosine production

With continued ATP hydrolysis without metabolite recycling, adenosine accumulates. Adenosine is an important regulator of Ca^{2+} entry, glycolysis, and vessel contraction [372]. As ischaemia is prolonged, adenosine is broken down into inosine, xanthine and hypoxanthine, which may contribute to free radical genesis on the reintroduction of oxygen [346], although this mechanism has been disputed in the human heart because of the lack of xanthine oxidoreductase activity [436]. On reperfusion, the adenine nucleotides are washed out reducing their availability for build-up of the depleted stores of ATP [455]. This effect contributes to poor recovery on reperfusion.

Breakdown pathway of ATP to adenosine and other products:

$$ATP \rightarrow ADP + PO_4^- + H^+ \rightarrow AMP + PO_4^- + H^+ \rightarrow Adenosine + PO_4^- + H^+$$

$$Adenosine \rightarrow Inosine \rightarrow Xanthine \rightarrow Hypoxanthine$$

c) Glycolytic ATP production

i) Glycolysis

Glycolysis and glycogenolysis are an important source of energy in compromised cells. In hypoxia, or with a relatively high residual flow, glycolytic metabolites are washed out, allowing glycolysis to proceed at a high rate [389]. In severe or total ischaemia, glycolysis falls rapidly within 2-3 min, although a residual flow can provide glucose to maintain flux but at a rate lower than control. The small amount of ATP produced from residual glucose delivery and endogenous glycogen is crucial for recovery of the ischaemic tissue [404]. The rate of glycolysis then becomes dependent on the availability of glucose and glycogen.

ii) Glucose vs. glycogen

Glucose availability is determined by the coronary flow and its concentration in the perfusate. When a molecule of glucose is anaerobically broken down, 2 lactate + 2 ATP are produced. After breakdown of ATP, the total sum is 2 lactate + 2 ADP + 2 H^+. When glycogen is broken down into C_6 units and subsequently catabolised by glycolysis, the end products are 2 lactate + 3 ATP, or 2 lactate + 3 ADP + 2 H^+. Glycogen should therefore be more advantageous as an energy source. However, glycogen depletion prior to ischaemia has been shown to increase recovery of function [387] possibly by a reduction in lactate and H^+ accumulation, although this is very controversial (see Ch. II). Many studies indicate a beneficial role of glucose, also a source of lactate and H^+. This paradox remains to be explained. The roles of glucose and glycogen are discussed in depth in Ch. II.

5) ION REGULATION IN ISCHAEMIA

a) pH

i) Source and removal of protons

pH falls rapidly from the onset of ischaemia (after an initial transitory rise due to CP breakdown) from about 7.2 to 7.05 by 5 min, and 6.5 by 15 min [76]. After an hour, intracellular pH may drop as low as 5.8 [76]. H^+ accumulation is due mainly to continued ATP hydrolysis with reduced ATP regeneration [107, 156]. While there is some controversy over this point [584] with the view that H^+ come from lactic acid production, the pKa of lactic acid (3.9) suggest that only 0.08% of the total is lactic acid at physiological pH [156]. The sum for ATP production from glycolysis shows that H^+ production is balanced by consumption [107, 156]. H^+ may also come from CO_2 produced by residual oxidation, from adenosine breakdown, and from increased NADH + H^+ production without re-oxidation to NAD^+ [107].

Intracellular pH is partially dependent on the buffering capacity of the myocyte, which is determined by the CO_2/HCO_3^- system, phosphate concentration, lactate and pyruvate, and proteins. The myofilaments, SR and mitochondria also buffer pH. The total buffering capacity (62 mM) is made up of the intrinsic buffering capacity (mainly from histidyl residues - about 40 mM), from CO_2 (14 mM), and from Pi (8 mM) [545]. Increases in Pi and lactate should increase the buffering capacity in ischaemia, but this is counteracted by the increased H^+ load.

Mechanisms to remove H^+ include the Na^+/H^+ and the Cl^-/HCO_3^- exchangers of the sarcolemma. At normal pH, the Na^+/H^+ exchange removes intracellular H^+, while the Cl^-/HCO_3^- exchange mediates H^+ influx. The Na^+/H^+ exchanger becomes important particularly on reperfusion, when a $[Na^+]i$ load results from acidosis [119]. Lactate and H^+ also exit the cell in a co-transport. An increased extracellular lactate inhibits this efflux [239], resulting in intracellular acidosis [102].

ii) Deleterious effect of proton accumulation

A drop in pHi may initially be protective by combating the effects of increased $[Ca^{2+}]i$, as H^+ compete with Ca^{2+} for intracellular binding sites [288]. Acidosis may protective in hypoxia [31] by reducing contractility and conserving ATP, although there is little correlation between the time course and extent of depression of mechanical function with acidosis [345], suggesting that acidosis is not involved in contractile failure in these hearts [7]. In ischaemia, an acidotic cardioplegic solution offers more protection than an alkaline solution [564], while reperfusion with an acidotic solution is also beneficial [257, 306]. Acidosis may inhibit 5' nucleotidase, and thus help to preserve adenine nucleotide levels in ischaemia, and enhance resynthesis of ATP on reperfusion [20]. However, too great a drop in pH activates proteases and lipases, reduces sarcolemmal binding, and alters many pH-dependent processes [89]. Increased intracellular H^+ accumulation may also increase Ca^{2+} influx via the Na^+/H^+ and Na^+/Ca^{2+} exchange mechanisms [303], a major factor in reperfusion injury. On reperfusion, while restoration of intracellular pH is mediated primarily by lactate and CO_2 washout, with Na^+/H^+ exchange contributing only about 35% to total H^+ efflux [545], about 5 mmol/l/min of Na^+ enters in exchange for the H^+, which would make a large contribution to Ca^{2+} overload [545].

Despite many reports implicating H^+ accumulation in ischaemic injury [239, 240, 387], the drop in pHi during ischaemia is not well correlated with recovery of function [92, 151, 306]. While a drop in pHi may contribute to ischaemic injury, this effect may be outweighed other, more significant factors [306].

b) Calcium

i) Cellular role of calcium

Ca^{2+} is central in the regulation of myocyte function [157, 330, 441] and is thought to be the major mediator of ischaemic and reperfusion injury [405, 412, 413]. Ca^{2+} modulates the rate of ATP utilisation and contractile activity, and maintains the integrity of the plasma membranes [144] and the intercalated discs [461]. Ca^{2+} regulates excitation-contraction coupling, transmembrane ionic current flow, and mitochondrial respiration. The main cellular compartments of Ca^{2+} are the myoplasm, the SR and the mitochondria, and that bound to membrane phospholipids and proteins.

In ischaemia, an excess influx of Ca^{2+} is thought to occur [4, 251, 374], and the ability of the cells to handle the load is reduced [356]. Although absolute values do not necessarily increase, particularly in the first 5-10 min, the distribution of Ca^{2+} within the cell may change with release of Ca^{2+} into the cytosol [230, 356]. Cytosolic Ca^{2+} subsequently increases in an almost linear fashion [510]. However, due to technical difficulties associated with measuring intracellular concentrations, the precise changes that take place are not fully understood. Glycolytic flux may be crucial in ischaemic Ca^{2+} regulation [87, 229] as re-uptake mechanisms are dependent on ATP [593].

ii) Ca^{2+} release into cytosol

Ca^{2+} enters the cell via the L-type Ca^{2+} channel during the action potential [78], and stimulates Ca^{2+}-induced Ca^{2+} release from the SR [130], the Ca^{2+} thought to be responsible for initiation of contraction. The size of the Ca^{2+} current determines the amount of Ca^{2+} released by the SR. The SR Ca^{2+} release channel is the site of ryanodine binding [467]. Ca^{2+} release by this mechanism is altered by a number of factors including pH (acidosis inhibitory) and ATP (stimulatory). Inositol (1,4,5)-trisphosphate (IP_3) from stimulation of α receptors, also induces Ca^{2+} release from internal stores, but acts on a different receptor [124]. Cyclic ADP-ribose, a compound derived from NAD^+ stimulated by cGMP, may also enhance Ca^{2+} release from the SR [146], although its role in the heart has not been clarified. Free fatty acids may exert some harm by increased Ca^{2+} entry. Long chain fatty acids activate sarcolemmal Ca^{2+} channels [207] as does lysophosphatidylcholine, a fatty acid metabolite [589]. Release of Ca^{2+} from the SR may contribute to stunning [356] although this has been disputed [238].

iii) Ca^{2+} removal from the cytosol

Intracellular Ca^{2+} levels are tightly controlled against a large extracellular concentration. The Ca^{2+} entering via the channels must be all extruded if equilibrium is to exist. Ca^{2+} can be removed from the cytosol in several ways; either extruded from the cell into the extracellular space, or taken up into

intracellular stores (mainly SR). Ca^{2+} can also bind to intracellular buffers, or be taken up by the mitochondria.

Extrusion of Ca^{2+} from the cell is accomplished by two mechanisms - the sarcolemmal Ca^{2+} ATPase pump, and the Na^{+}/ Ca^{2+} exchange.

The sarcolemmal Ca^{2+} *ATPase pump* has a low maximum transport rate with a low capacity, but a high affinity for Ca^{2+}. The sarcolemmal Ca^{2+} pump seems relatively unimportant in cardiac muscle.

The Na^{+}*/* Ca^{2+} *exchange* has a relatively low affinity for Ca^{2+}, but a high capacity, with a stoichiometry of 3 Na^{+}:1 Ca^{2+} [125]. The Na^{+}/ Ca^{2+} exchanger serves mainly as a means of Ca^{2+} extrusion under normal conditions, removing about 30% of the Ca^{2+} from the cytoplasm during relaxation, approximately the amount of Ca^{2+} entering via the Ca^{2+} channels of the sarcolemma - a requirement for steady state. A change in $[Na^{+}]_i$ has a direct and immediate effect on contraction. The Na^{+}/ Ca^{2+} exchange can also mediate Ca^{2+} influx when the gradients for Na^{+} and Ca^{2+} are reversed, as occurs in ischaemia.

The exchange is activated by depolarisation leading to increased Ca^{2+} entry, ATP [183] (decreases the Km for Ca^{2+} possibly by phosphorylation), and phospholipase C and D activity. The exchange is inhibited by a reduced pH [433], and inorganic cations including Mg^{2+}.

SR Ca^{2+} *ATPase pump* - the SR Ca^{2+} pump has a high affinity and high capacity, and utilises 1 ATP per 2 Ca^{2+}. The amount of Ca^{2+} available for release from the SR pool for contraction is dependent on cytosolic Ca^{2+} availability, and the activity of the Ca^{2+} re-uptake mechanisms. This pump is largely responsible for lowering cytosolic Ca^{2+} levels (70% of total), and its activity determines the rate of relaxation. Phospholamban, activated by protein kinase A in response to catecholamines, stimulates the pump by lowering the Km (Ca^{2+}) [520], causing release of Ca^{2+} into the lumen of the SR, increasing pump efficiency and accelerating relaxation. β stimulation may bias Ca^{2+} extrusion such that uptake by the SR pump exceeds extrusion to the extracellular space. The size of the SR Ca^{2+} pool will increase, allowing increased Ca^{2+} release on stimulation, and enhanced contractility [54].

SR Ca^{2+} pumps are highly dependent on ATP [56]. The large concentration gradient between the cytosol and the SR (9-14 mM vs. 125 nM in diastole) requires a highly efficient, but highly energy-dependent pump. If SR Ca^{2+} ATPase pump activity is reduced (reduced ATP concentration, reduced free energy of hydrolysis by ischaemia), the SR Ca^{2+} pool becomes depleted and cytosolic Ca^{2+} may rise [499], with a slowing in relaxation. The SR pump is also sensitive to pH as H^{+} compete with Ca^{2+} for binding sites. The SR Ca^{2+} ATPase can be inactivated by altered phospholamban phosphorylation as well as a specific loss in ATPase activity [492].

iv) Changes in intracellular Ca^{2+} in ischaemia

An increased cytosolic Ca^{2+} is a primary candidate for ischaemic injury [4, 374, 405, 412, 413]. Inhibition of Ca^{2+} re-uptake or extrusion mechanisms increase cytosolic Ca^{2+} [72, 356, 559]. In addition, Ca^{2+} enters down its diffusion gradient through membranes made leaky in ischaemia by reduced phosphorylation [70, 383] and acidosis lowers the affinity of the Ca^{2+} binding sites of the various Ca^{2+} buffering proteins [30, 263]. Intracellular Ca^{2+} overload also reduces the stability of the cell membranes, and Ca^{2+}-dependent phospholipases in the membrane may be activated [461]. Lipid peroxidation ensues, with the appearance of holes in the lipid bilayer, through which intracellular enzymes are lost. Conformational changes in the membrane may also occur with inhibition of sarcolemmal ATPase activity [315]. Ca^{2+}-sensitive proteases may also be activated [383, 461].

v) Mitochondria

A significant consequence of intracellular Ca^{2+} overload is changes in the mitochondria. A Na^+/ Ca^{2+} exchanger is present on the inner membrane of the mitochondria, as well as a Na^+/H^+ exchange mechanism and a Ca^{2+} uniport [7]. Ca^{2+} entry via the uniport exhibits a sigmoid dependence on intracellular Ca^{2+} concentration, with a Km >30 μM for Ca^{2+}. At normal $[Ca^{2+}]i$, (0.1-1 μM) the influx pathway is at a relatively low level. Ca^{2+} accumulates in the mitochondria at the expense of ATP via the H^+ gradient of the electron transport chain [56] (the proton motive force), the same as that used to convert ADP to ATP. Thus, when intramitochondrial Ca^{2+} increases, ATP production is reduced.

If ischaemia is prolonged, calcium phosphate granules may be deposited in the mitochondria, uncoupling oxidative phosphorylation and inhibiting mitochondrial recovery on reperfusion [222]. Energy production on reperfusion is affected, as well as predisposing to free radical generation as the oxygen levels are not reduced by mitochondrial respiration [602].

vi) pH and Ca^{2+}

pH is a major modulator of Ca^{2+} action in the cell, and thus in ischaemia [52, 382, 383, 597]. Ca^{2+} and H^+ compete for binding sites [25, 288, 442], such that acidosis has a negative inotropic effect [126] by binding to the troponin C of the myofibrils and decreasing the sensitivity of the troponin-tropomyosin complexes to Ca^{2+} [23, 69]. A fall in pHi of 0.2 units reduces developed tension by about 50% [440]. Even at saturating levels of Ca^{2+}, H^+ reduce the maximum tension developed [126, 288].

SR Ca^{2+} ATPase activity is reduced at low pH [6, 25] and the Na^+/Ca^{2+} exchanger is also inhibited by acidosis [433]. Ca^{2+} binding to phospholipids is reduced with a lower pH [288] with release of Ca^{2+} into the cytosol. Cell-to-cell coupling may be affected by acidosis with a reduction in junctional

conductance [105, 442]. Thus under ischaemic conditions, H^+ may only partially antagonise the deleterious effects of Ca^{2+}.

c) Sodium

Intracellular Na^+ is normally at a concentration of 10 mM. Influx of Na^+ occurs mainly via voltage-dependent channels, and exchangers, such as the Na^+/Ca^{2+} and Na^+/H^+ exchange mechanisms. These exchangers are reversible, depending on the gradients, and membrane potential. The Na^+/K^+ ATPase pump is responsible for maintaining the gradient by extruding Na^+ in exchange for K^+ (3 Na^+/2 K^+ per ATP). The pump activity is largely dependent on ATP. Inhibition of the pump by decreased ATP levels will tend to increase $[Na^+]i$. The major consequence of an increase in $[Na^+]i$ is the subsequent increase in $[Ca^{2+}]i$. Developed tension is thus strongly dependent on Na^+.

A decreased intracellular pH increases $[Na^+]i$ via the Na^+/H^+ exchanger, which leads to subsequent Ca^{2+} accumulation, when Na^+ is extruded in exchange for Ca^{2+} via the Na^+/Ca^{2+} exchange [30, 303]. During ischaemia, with a reduced intra- and extracellular pH, the Na^+/H^+ exchanger may be inhibited [212]. On reperfusion, however, this mechanism becomes extremely important [119]. $[Na^+]i$ accumulation may have other detrimental effects including increased osmolarity and cell swelling, as well as disturbing the ionic gradients for the generation of action potentials.

d) Potassium

K^+ efflux and extracellular accumulation are primary consequences of ischaemia [139, 258]. Increased extracellular K^+ lowers the resting membrane potential and reduces the amplitude and duration of the action potentials [232, 258, 336, 366]. Loss of K^+ is complex [583], but partially attributed to an efflux of K^+ down its concentration gradient through the semi-permeable membrane and the K_{ATP} channels which are normally blocked by ATP [236]. In addition, the Na^+/K^+ ATPase pump, which normally restores the K^+ gradient, is inhibited by a reduced ATP.

Provision of glycolytic ATP has been closely linked to inhibition of the K_{ATP} [571]. With a reduction of glycolytic activity, the channel opens, despite a high overall ATP concentration [571]. Specific blockers for this channel e.g. glibenclamide may be protective by reducing arrhythmias [236]. Maintaining inhibition of this channel may be an important function of glycolysis. However, the role of this channel is unclear, given that K_{ATP} openers e.g. chromakalim, pinacidil, have cardioprotective effects [170] and preconditioning may also act by opening K_{ATP} channels [172]. K^+ efflux via this channel, by depolarising the membrane and inhibiting excitable activity, may be a defensive mechanism whereby energy is conserved - the heart is effectively "arrested".

e) Inorganic phosphate

Pi starts to rise as soon as there is a net breakdown of CP and ATP, and exerts a major depressant effect on tension [246, 284]. Pi accumulation decreases maximal force output for a given increase in Ca^{2+}. The most prominent effect is observed at concentrations below 10 mM. If normal Pi is 1-3 mM, increasing to 20 mM in ischaemia or hypoxia, maximum Ca^{2+} activated tension falls by about 50%. Pi also shifts the pCa curve rightward, although not to a large extent [245]. Pi reduces force development, possibly by reducing the percentage of force generating cross-bridges. Accumulation of Pi also reduces the free energy of hydrolysis from the available ATP [234], and may thus reduce SR Ca^{2+} loading and decrease Ca^{2+} induced Ca^{2+} release [592].

6) SIGNAL TRANSDUCTION PATHWAYS IN ISCHAEMIA

Most of the cell signalling systems regulate intracellular Ca^{2+}, which rise in ischaemia. The signalling systems are affected by ischaemia, and contribute largely to ischaemic injury. Preconditioning (see Ch III) acts on several of these signalling pathways, which in turn are also involved in regulation of metabolism.

a) β adrenergic stimulation and cAMP

β adrenergic receptor stimulation activates a GTP binding protein (Gs) which activates adenyl cyclase and produces cAMP. cAMP in turn activates the cAMP-dependent protein kinase (PKA), which is the final messenger in the signal pathway initiated by β receptor stimulation. The enhanced myocardial contractility associated with β stimulation is mediated via β1 receptors. PKA has several effects, including phosphorylation of the L-type Ca^{2+} channel, increasing the opening probability of the channel and hence increasing the Ca^{2+} current. Gs may also exert a direct effect on Ca^{2+} channel activity [277]. While single channel conductance is not altered, the number of active channels in the sarcolemma may be increased [277, 531]. The result is that the Ca^{2+} transient is much larger and faster, with a subsequent increase in cardiac contraction. An anomaly in the effect of β stimulation is that cAMP reduces the Ca^{2+} sensitivity of the myofilaments by phosphorylation of troponin I, and thereby promote the rate of relaxation. The increased I_{Ca} increases the amount of Ca^{2+} released by the SR via Ca^{2+}-induced Ca^{2+} release [54, 130]. Because more Ca^{2+} enters the cytosol with each action potential, SR Ca^{2+} ATPase activity must be increased to ensure relaxation. PKA phosphorylates phospholamban, the regulatory protein of the SR pump, thus a larger fraction of the cytosolic Ca^{2+} pool is taken up during relaxation, which is subsequently available to enhance contraction on stimulation.

The increased energy demand with the increased inotropic state can increase oxygen consumption dramatically. β receptor activation increases the metabolic rate and rate of glycogenolysis via PKA. PKA activates phosphorylase b kinase, which in turn activates phosphorylase b, resulting in increased glycogen breakdown for high energy phosphate production.

In ischaemia, the lack of ATP causes release of noradrenaline from neuronal storage vesicles [490]. The density of β receptors on the sarcolemma is increased, and adenyl cyclase activity, possibly mediated by protein kinase C (PKC) [277], is enhanced. Thus cAMP levels rise in ischaemia after five to ten minutes of coronary occlusion [531], peaking at 10-20 min. An increased cAMP is hypothetically deleterious by increasing cytosolic Ca^{2+} and predisposing to arrhythmias [329, 437, 531]. The increased energy utilisation following increased contractility with cAMP may increase the cytosolic free fatty acid concentration, with the production of arrhythmogenic lysophospholipids [531] which in turn increase cytosolic Ca^{2+} levels by acting on Ca^{2+} channels [207, 589]. cAMP levels are also decreased in preconditioned myocardium [479] (see Ch III).

b) α adrenergic stimulation and the IP_3/protein kinase C pathway

α adrenergic receptor stimulation initiates the inositol polyphosphate cascade acting via a Gs protein [209]. Gs binds to phospholipase C which catalyses the conversion of phosphatidylinositol 4,5-bisphosphate (PIP_2) to 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). DAG remains in the membrane, where it activates protein kinase C (PKC) [526], a Ser-Thr kinase with many cellular actions. IP_3 mediates the release of Ca^{2+} from intracellular stores acting on a specific IP_3 receptor [213]. IP_3 is then broken down in a cascade of reactions to inositol. Several other phosphoinositols (IP_4, IP_5, IP_6) exist, the functions of which are not fully clarified. IP_3 is an important regulator of intracellular Ca^{2+}. IP_3 levels fall during ischaemia, but rise rapidly on reperfusion [369], and may be involved in the deleterious rise in Ca^{2+}. α1 blockade has been shown to decrease the rise in cytosolic Ca^{2+} in ischaemia, and attenuate reperfusion injury [365].

PKC has a number of isozymes (at least eleven) [514], which regulate cellular processes by phosphorylation. PKC requires phospholipids such as phosphatidyl serine and may also need Ca^{2+}. PKC regulates cellular Ca^{2+} levels, and has a feedback effect on the phosphoinositol system [396]. PKC may act on a large number of substrate proteins including Ca^{2+} ATPase, Na^+/K^+ ATPase, Na^+/H^+ exchange system, the glucose transporter, troponin T and I, myosin light chain, glycogen synthase, glycogen phosphorylase kinase, phosphofructokinase, β-adrenergic receptor, insulin receptor and many others [396]. PKC has been implicated in the regulation of contraction, by phosphorylation of substrates and modulation of calcium and other ion levels with inotropic and chronotropic effects,

gene expression, secretion of cardiac factors and hypertrophy. PKC often has effects similar to PKA, for instance PKC also phosphorylates phospholamban. PKC activation may sensitise the adenyl cyclase, resulting in cross talk between the two systems [515]. PKC may modulate cAMP and calmodulin-dependent protein kinases. Increased α1 receptor stimulation also increases adenosine production [255].

In ischaemia, the number of α receptors increases [443]. α stimulation in ischaemia has arrhythmic effects [443]. α1 blockade attenuates adenosine release from ischaemic myocardium, possibly via reduced PKC activation which would normally activate 5'-nucleotidase (enzyme responsible for adenosine production from 5'-AMP) [256]. PKC can also be translocated to the membrane in ischaemia independent of α1-stimulation [515]. PKC activation aggravates hypoxic injury by stimulating Ca^{2+} overload, possibly secondarily to accelerated Na^+/H^+ exchange activity [211]. However, preconditioning induces PKC translocation, and this has been linked to its protective effects [601] (see Ch III).

c) Adenosine

Adenosine, formed from the breakdown of ATP, is an important cellular messenger, with both intra- and extracellular effects. Adenosine is a potent local vasodilator, increasing oxygen delivery to the heart [26, 277]. It has a very short half life (1s), therefore its effects are very localised [371]. Adenosine acts via A1 or A2 receptors (an A3 receptor has also been proposed [16]). A1 receptors are generally found on the myocyte and nodes, while A2 receptors are located in vascular smooth muscle. A1 receptors bind to Gi proteins, which affect a variety of systems, including adenyl cyclase, phospholipase A2 and C (activates PKC), K^+ and Ca^{2+} channels, guanylate cyclase and Na^+/Ca^{2+} exchange. K_{ATP} channels are specifically activated by adenosine [254]. Adenosine is anti-adrenergic by inhibiting both norepinephrine release [460], and adenyl cyclase activity [255]. These energy-sparing effects inhibit ATP degradation [596]. A2 receptor activation, however, stimulates adenyl cyclase, enhances phospholipase C stimulation, and K_{ATP} channel opening. Adenosine may also inhibit neutrophil activation and preserve endothelial cell integrity.

Increased adenosine may be protective in ischaemia, an effect mediated by A1 rather than A2 receptors [292]. A1 stimulation decreases cAMP in nodal tissue and the myocytes, whereas A2 stimulation increases cAMP in vascular smooth muscle. Adenosine reduces energy utilisation by reducing contraction, and depressing heart rate [372], and may also be involved in glucose uptake and stimulation of glycolysis, although this is controversial. Some reports find that adenosine stimulates glucose utilisation [333, 591]; others finds that adenosine limits or inhibits glycolysis [134, 135, 141,

546, 573 or fail to show any difference in glucose uptake with adenosine 96. Stimulation of glycolysis by adenosine may only occur in the presence of insulin 295, 296.

Determination of ATP, glycogen and lactate in dog hearts show that adenosine slows the rate of ATP depletion, glycogen utilisation and lactate accumulation during ischaemia 546. The potent vasodilatory effect of adenosine increases the delivery of substrate to the myocardium, and improves washout. Inhibition of adenosine breakdown by an adenosine deaminase inhibitor, and reduced washout by using a nucleoside transport blocker, is also beneficial with reduced stunning in dogs 606. This finding suggests that enhanced levels of endogenous adenosine, acting intracellularly, is beneficial. Adenosine is one of the most studied mechanisms of preconditioning protection (see Ch III).

d) Nitric oxide and cGMP

Nitric oxide is a short-lived molecule freely permeable to cell membranes, inactivated by oxidation. NO is produced by endothelial cells by NO synthase acting on L-arginine, and causes vasodilation (endothelium-derived relaxing factor - EDRF). Many of the effects of NO are mediated by Ca^{2+} and cGMP. cGMP is derived from GTP by the action of guanylate cyclase, which is stimulated by NO 138. cGMP regulates levels of cyclic ADP-ribose (cADPR) 29 which is derived from NAD^+. cADPR mobilises Ca^{2+}, by causing release of Ca^{2+} from internal stores, possibly acting on the ryanodine receptor 146. The role of this compound in the heart is not as yet clear.

Depré et al 109 reported a rise in cGMP in ischaemia and hypoxia. An inhibitor of NO synthase, L-NAME, reduced cGMP levels in ischaemic tissue, and was protective to the ischaemic heart 110 delaying the onset of ischaemic contracture and increasing functional recovery. These effects were associated with increased glucose uptake. However, administration of 8-bromo-cGMP, an analogue of cGMP had no effect, implying that these effects were not mediated by cGMP. These results were corroborated 118, but the role of the NO pathway is still largely unresolved.

7) ISCHAEMIC CONTRACTURE

I have used contracture as an index of ischaemic injury throughout this thesis, but its initiation and development are not simple indices of injury. Because of the complexity of the phenomenon, contracture is discussed in depth with reference to available literature. A hypothetical model of contracture, with specific reference to the role of glycolysis based on the findings of this thesis, is presented in the discussion.

a) What is contracture?

i) Definition

Contracture is defined as an increase in resting myocardial stiffness with shortening of myofibrils. In total global experimental ischaemia, the whole heart contracts slowly over time, with a rise in intraventricular pressure. Contracture develops in ischaemia or with metabolic inhibition.

ii) Clinical correlate

Myocardial ischaemic contracture was first described as a clinical manifestation, the "stone heart" [86]. Following open heart surgery with cardiopulmonary bypass, the development of "a small spastic heart" "literally frozen in systole" was described in some patients following prolonged ischaemia [86]. This condition was said to be analogous to rigor in skeletal muscle [242], and is linked to rigor mortis [327]. Increased myocardial stiffness was reported in patients with angina, with increased ventricular end-diastolic pressure [22]. These changes were reversible, but indicative of sustained contraction, or failure of relaxation of a portion of the left ventricle during angina. This finding was attributed to contracture of the affected tissue.

iii) Time course of contracture

Following initiation of total global ischaemia in an isolated heart, contractile activity ceases, and pressure development is absent. After about 5-15 min (dependent on model), the resting tension begins to rise (= onset of contracture), and reaches a peak within minutes, with a sigmoid curve. A decline in tension then follows if ischaemia is maintained. Reperfusion at this stage shows incomplete recovery, an indication of irreversible injury. The time to onset of contracture has been used extensively as an index of ischaemic injury with a delay in time to onset implying protection [110, 189, 537]. Peak contracture may also be an important determinant of the extent of ischaemic injury, but has not been used as frequently as time to onset.

iv) Ultrastructural changes in the myocardium during contracture

Papillary muscles which have undergone contracture show focal irregularities of A, I and Z bands with sarcomere mal-alignment, hypercontracture and fibre disruption [32]. In fully developed contracture, severe disruption of the myocardial fibres is observed, with distortion of adjacent structures, especially at the intercalated discs. Morphological changes correlate to mechanical changes of increased tension [32]. In tissue from hearts perfused with acetate (leads to high peak contracture [424]), at 5% of maximum contracture (100%), foci of contracted myocytes are found, predominantly in the subendocardium and papillary muscle [181]. With 30% contracture, damage spreads to the subepicardium, with increased number and size of foci of contracture. Swelling of the tubular

sarcolemmal system is found. At 50% contracture, lanthanum uptake is increased, indicating increased membrane permeability. At full contracture, the majority of cells are contracted.

b) Mechanism of contracture - drop in ATP, or rise in Ca^{2+}

i) Possible mechanisms

Initiation of contracture has been attributed to two mechanisms: i) a decrease in ATP at the contractile proteins, with formation of fixed cross-bridge attachments and increased stiffness, i.e. rigor bonds; ii) an increase in diastolic Ca^{2+} that maintains the contractile system in a partially or completely activated state, i.e. tetanic contracture (tension development without associated cyclic changes in membrane potential). While there is some controversy over whether a rise in Ca^{2+} or a depletion of ATP precipitates ischaemic contracture, both appear to be determinants of the extent of contracture, and its subsequent deleterious effects on the myocardium. Contracture occurs at the level of the cross-bridge cycle.

ii) The cross-bridge cycle

The cross-bridge cycle (see Fig I.1) is closely regulated by ATP, ADP, Ca^{2+}, H^+ and Pi. For normal contraction, an increased cytosolic Ca^{2+} is required to remove troponin (Tn) inhibition of actin-myosin binding. The troponin-tropomyosin (Tn-Tm) complex consists of 26 components, including troponin C (Ca^{2+}-binding), troponin I (inhibitory component), and tropomyosin which binds the complex to the actin filament. Ca^{2+} binding engenders a conformational change in the complex such that actin is exposed to myosin, to allow binding of the myosin S1 heads to the actin. Actin binding to myosin enhances myosin ATPase activity [45] and ATP hydrolysis, and the release of the Pi thus generated results in the power stroke. Cross-bridge attachment serves as a positive feedback mechanism that transiently increases the Ca^{2+} affinity of TnC [176, 179]. TnC is an essential element in the chain of Tn-Tm molecules that transmit co-operative signals along the strand [43]. Thus one cross-bridge relays the signal for contraction down the myofibril via the troponin-tropomyosin complex. With increased Ca^{2+}, more sites on Tn will be occupied, further removing inhibition of binding.

ATP when bound to myosin at rest inhibits actin binding in the absence of Ca^{2+}. ATP is also required for relaxation, and by hydrolysis provides the energy for contraction. In the absence of ATP, actin and myosin bind strongly forming rigor cross-bridges. There are several models of the cross-bridge cycle which vary in the detail. One of these is described in Fig I.1, showing states of normal contraction, and of rigor.

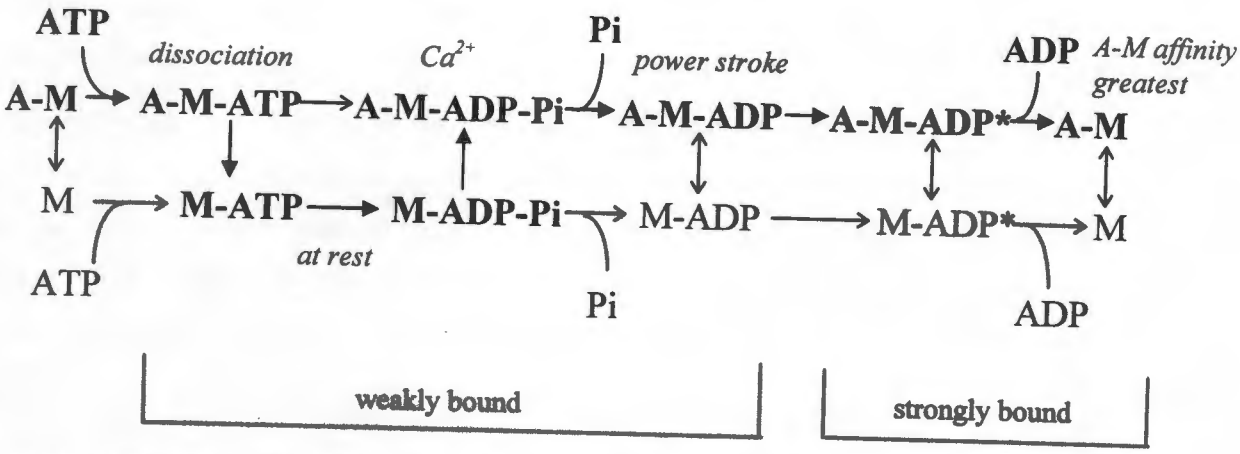

Fig I.1: A chemical model of the steps in the cross-bridge cycle based on Goldman and Brenner [275]. The top row shows the states in which actin (A) interacts with myosin (M). The heavy arrows indicate the normal reaction pathway, but all the reactions can occur and are reversible. Two energetically different states of A-M-ADP are indicated by the asterisk on the more strongly bound.

At rest myosin (M) is mostly complexed with ATP (M-ATP) or in the rapidly equilibrated M-ADP-Pi where ATP is hydrolysed by myosin ATPase, but the energy has not been used. As Ca^{2+} rises and binds to Tn C, M-ADP-Pi interacts with actin, and the phosphate group is rapidly released. This step may represent the "power stroke" or myosin head rotation, with an increase in the affinity of binding represented by the two energetic states of A-M-ADP, as the angle of the head changes from 90° to 45°. The affinity of myosin for actin increases along this series of steps, and is strongest after ADP dissociates (A-M). At normal ATP, A-M binds ATP rapidly, inducing the dissociation of actin and M-ATP. The cycle then continues until Ca^{2+} declines, thereby stopping myofilament interaction (in the M-ADP-Pi state) or until ATP is depleted (rigor, with cycle stopped in the A-M state).

iii) Rigor development

Rigor tension develops when the ATP concentration is depleted to micromolar levels (≤ 100 μM [307]) at the myofibrils. Relaxation can be restored by increased ATP. Increased tension, increased stiffness, and muscle shortening, are observed on withdrawal of ATP from skinned muscle, in the absence of Ca^{2+} [580]. The final tension developed is independent of the initial ATP concentration [580]. The contraction is induced by actomyosin complex formation in the absence of ATP. The rigor complex then stimulates actin cofactor activity of adjacent myosin ATPase molecules [45], which in turn depletes ATP further, increasing actin-myosin affinity. Rigor complex formation also increases the affinity between Ca^{2+} and troponin [176], such that a small rise in Ca^{2+} may result in considerable Ca^{2+}-dependent tension production. Rigor bonds also alter the conformation of TnC and elicit contraction in a similar manner to Ca^{2+} binding [179]. The rigor bonds also act on the Tn-Tm complex, with the subsequent co-operative spread of transmission of the signal down the length of the myofibril [43].

Rigor increases the stiffness of muscle i.e. the number of heads attached, compared to the normally contracting state, an indication of increased cross-bridge attachment. 40-60% [595] or 94-100% [85]

attachment of cross-bridges in rigor vs. 30% in normal cells have been estimated. These rigor cross-bridges exist in two states of myosin head attachment - at 90° and 45° - thought to represent myosin heads "trapped" at different stages of the power stoke [529] (see Fig I.2). The 45° heads are those though to bind first, and complete the power stroke before "seizing" in the fixed 45° position. The 90° heads represent heads which have bound but still need to complete the power stroke - i.e. they exert a positive force, and will switch to 45° if released. The 45° heads prevent slippage of the 90° heads, fixing the myofibril in a rigor state. Thus the muscle in rigor exerts a positive tension as well as increased stiffness.

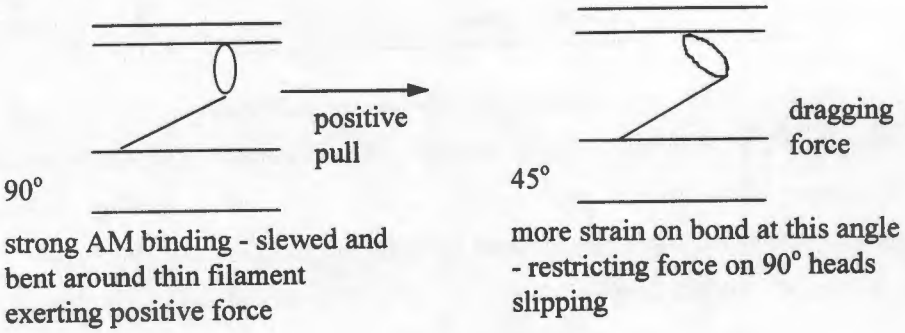

Fig I.2: 90° and 45° heads in rigor showing opposing force [529]

iv) Contracture and ATP depletion

Rigor mortis in dog hearts is temporally associated with ATP depletion [327]. In an ischaemic heart, interventions which increase ATP consumption increase resting tension [384], while those which reduce ATP consumption or increase production, delay time to onset of contracture [189]. The fall in tissue ATP follows a biphasic pattern. Time to onset is correlated with the second phase of depletion [189]. In isolated rat myocytes, this second phase of ATP depletion was correlated with an increase in the number of cells which had undergone contracture, suggesting that in any cell, contracture is preceded by a loss in ATP (possibly following glycogen depletion) [184, 185]. These results suggest the involvement of rigor cross-bridges in ischaemic contracture.

Further evidence for the involvement of rigor bridges in contracture comes from studies with hypoxia and metabolic inhibition. Muscle with hypoxic contracture shows increased stiffness, indicative of rigor bonds [309]. In a model using quick alterations in length of isolated papillary muscles, the rise in tension with metabolic inhibition is shown to be maintained by rigor bonds rather than cycling cross-bridges[557]. Heat generation during hypoxic contracture in papillary muscles, once peak tension is reached, is significantly less than that produced by cross-bridge cycling contraction induced by KCl [205], which implies that ATP is not utilised in maintaining tension. The heat developed during the development of ionic contracture (induced by low Na^+, high K^+) was attributed to the ATP required for Ca^{2+} homeostasis, while the tension was attributed to rigor formation [74].

v) ATP levels required for rigor bond formation

The arguments against rigor bonds as the mechanism of contracture come from the lack of correlation between absolute ATP levels and those required for rigor bond formation (≤100 μM [307]). 100 μM approximates to 0.02 μmol/g dry wt, much lower than average ATP levels in the tissue at the onset of contracture (12 μmol/g dry wt [189]). However, the lack of correlation may be due to tissue inhomogeneity of ATP, and intracellular compartmentation(see Ch II). A transmural gradient of ischaemia, and of ATP, exists in the globally ischaemic heart [327]. ATP depletion occurs fastest in the subendocardial layer, in which contracture initially occurs. As the depletion of ATP spreads to the epicardium, the layer of contracture also spreads. The heterogeneous dispersion of rigor complexes, and of individual cells with contracture [181], would increase overall resting tension of the heart, despite an overall maintained level of tissue ATP. Haworth et al. [185] used the terms "synchronous" and "asynchronous" to describe ATP depletion in single cells. Asynchronous ATP depletion means that the ATP levels in individual cells could be depleted to 90% of their original levels without undergoing contracture, if glycolysis were maintained. In a whole heart, this approximates to a slow increase in resting tension with reasonably high ATP levels, as is found [189]. A "synchronous" depletion of ATP in all cells at once would precipitate contracture coincident with total ATP depletion in a whole heart, which only occurs when ATP is rapidly depleted e.g. by glycolytic inhibition [189].

In an individual cell, ATP levels are approximately 2.2 nmol/mg cell weight or less (approx 1.5 mM) when contracture is initiated [185], which is still higher than that at which rigor is thought to occur (approx 100 μM [307]). However, this ATP measurement was made in a gross sample of many cells [185]. In individual cells, a far lower value was attained [42]. Cell shortening occurred at approximately 150 μM ATP [42]. This value corresponds far more closely to the ATP required for rigor formation but there is also the component of compartmentation within the cell [48] (Ch II), including a large proportion of the cellular ATP retained in the mitochondria [153]. This factor increases the total cell ATP, but does not reflect the local ATP content e.g. at the myofibrils which may be depleted relative to the rest of the cell.

Other factors which may affect contracture at a given ATP are ADP, Pi and pH, as well as Ca^{2+}.

vi) Increased cytosolic calcium

An increased intracellular Ca^{2+} is implicated in ischaemic injury [441] although the exact relationship between the rise in $[Ca^{2+}]i$ and injury is unclear. A consistent relationship between the degree of stunning and the rise in intracellular Ca^{2+} has been found [509]. Evidence to suggest that cytosolic $Ca2^{+}$ accumulation precipitates contracture was found following administration of nifedipine, a Ca^{2+}

channel blocker, during low flow ischaemia, which prevented contracture and led to almost complete recovery [197], as does nisoldipine [474]. A low Ca^{2+} (0.05 mM) delays and reduces contracture in isolated hearts [210]. However, whether Ca^{2+} is a precipitating factor in contracture, or only modifies contracture once initiated by rigor formation, is still a matter of debate.

Increased $[Ca^{2+}]i$ was thought to contribute to contracture development early after metabolic inhibition in isolated myocytes, in that a Ca^{2+}-dependent phase preceded ATP-depletion rigor [23]. This finding did not, however, show that Ca^{2+} is necessary for the onset of contracture, and is not always reproducible but appears to be protocol-dependent [9]. The role of Ca^{2+} in the onset of contracture was further discounted in isolated myocytes, given that the sigmoid fall in % rod-shaped cells vs. time was not affected by the presence or absence of Ca^{2+} [184].

Several authors have reported isolated cell shortening before a rise in $[Ca^{2+}]i$, which supports the argument that contracture is initiated by ATP depletion. Severe injury in metabolically-poisoned myocytes precedes a rise in $[Ca^{2+}]i$ [77]. Spontaneous shortening also occurred prior to a sharp rise in $[Ca^{2+}]i$ in cells subjected to glucose-free anoxia [8] and to metabolic inhibition [311]. A fall in ATP was coincident with cell shortening, and this preceded a rise in $[Na^{+}]i$, and presumably in $[Ca^{2+}]i$ [42]. The rise in Ca^{2+} would deplete ATP further [189]. $[Na^{+}]i$ increases with metabolic inhibition, following increased Na^{+}/H^{+} exchange activity with a reduced pHi, and inhibition of Na^{+}/K^{+} ATPase [481]. No rise in $[Ca^{2+}]i$ was found. Cells underwent contracture, following which $[Ca^{2+}]i$ began to rise. Restoration of glucose at this point resulted in a large increase in $[Ca^{2+}]i$, possibly from increased Na^{+}/Ca^{2+} exchange which is inhibited by severe energy depletion [183]. Contracture was therefore linked to rigor, and not to Ca^{2+} overload [481].

In isolated perfused hearts, arrest with K^{+} or Mg^{2+} to achieve total Ca^{2+} block prior to ischaemia also affected the rate of ATP depletion, and the rise in ischaemic contracture [511]. In all cases, contracture development was initiated after depletion of total ATP to less than 50% control, with a progressive rise in Ca^{2+} during and after contracture development. These findings in whole hearts do not determine whether Ca^{2+} leads to contracture, or whether the rise in Ca^{2+} is a result of ATP depletion, and only modifies contracture. Evidence that a rise in cytosolic Ca^{2+} may precede contracture was provided by using a fluorescent probe to determine the distribution of Ca^{2+} between cytosolic and internal membrane stores [230]. Membrane-bound Ca^{2+} and Ca^{2+} from the SR were released into the cytosol prior to the onset of contracture. Similarly, in isolated ferret hearts, $[Ca^{2+}]i$ was increased greatly by 20-25 min, prior to onset of contracture at 40 min [271]. However, only after ATP was depleted to less than 10% of basal levels, did contracture occur. In hearts with glycolytic inhibition (iodoacetate), onset of contracture was much sooner, and preceded the rise in $[Ca^{2+}]i$ [271]. At close to

maximal contracture, the $[Ca^{2+}]i$ started to rise. These results thus dissociate the rise in $[Ca^{2+}]i$ from the initiation of contracture.

vii) Relationship between ATP and Ca^{2+}

Ischaemia increases $[Ca^{2+}]i$, and reduces high energy phosphate levels. Attenuated $[Ca^{2+}]i$ accumulation has been shown to reduce peak contracture [189, 197, 210], but this may be due to the ATP-preserving effects rather than a direct effect of Ca^{2+}. An increased $[Ca^{2+}]i$ increases ATP consumption by contractile proteins, while a reduced ATP concentration slows the energy-dependent sequestration of Ca^{2+} from the cytosol, contributing to intracellular Ca^{2+} accumulation [182]. Release of Ca^{2+} into the cytosol may activate Ca^{2+}-dependent ATPases, further accelerating the rate of ATP depletion, and increasing contracture [182]. Interventions which lower Ca^{2+} entry affect the development of ischaemic contracture but are associated with a change in ATP; thus inhibition of Ca^{2+}-induced ATP utilisation may delay contracture by conserving ATP [189].

The time course of Ca^{2+} increase is dependent on the metabolic status of the heart [189, 271]. When ATP falls below a critical level, especially when glycolysis is inhibited, Ca^{2+} rises quite rapidly (within minutes), possibly because of failure of the various Ca^{2+} pumps (Km (ATP) - SR Ca^{2+} ATPase - 0.18 mM) [7]. While onset of contracture is associated with a 50% depletion in ATP, a rise in $[Ca^{2+}]i$ is always associated with the development of contracture [511]. A modification in Ca^{2+} entry modifies the subsequent contracture.

Compartmentation affects the role of ATP, such that glucose-derived ATP maintains ion homeostasis and attenuates the positive feedback between ATP depletion and Ca^{2+} overload. Glycolytic ATP also delays the onset of contracture, possibly by affecting overall ATP concentration. This issue is discussed in depth in Ch II.

c) Rise in tension and peak contracture

Once contracture is initiated, the tension may be amplified by a rise in intracellular Ca^{2+}. Ca^{2+} entry can occur via several mechanisms (discussed previously). As Ca^{2+} levels rise, the probability of it binding to Tn increases, thereby increasing actin-myosin binding. Rigor bonds increase the affinity of TnC for Ca^{2+} [176], so a small increase in Ca^{2+} will have a potentiated effect on removal of Tn inhibition of actin-myosin binding. A Ca^{2+}-contraction will be imposed on the rigor "contraction". This effect is pH-dependent, given the inhibitory effects of H^+ on Ca^{2+} binding. An additive effect ensues, especially as an increase in contraction of the myofilaments further depletes ATP. As contracture of the myofibrils increases, the cell-to-cell attachments, already weakened by an increase in cytosolic Ca^{2+} and H^+, are strained. As the membranes stretch, the leakiness increases, adding to

Ca^{2+} entry. A positive feedback cycle is then set up, which, if leading to excess contracture, will result in irreversible injury.

Peak contracture appears to be related to a number of factors, including i) the rate of ATP depletion (relative rates of production and utilisation) ii) the drop in pH, and iii) rise in Pi (both modulate contractile force in the normally beating myocardium), and iv) Ca^{2+}.

d) Modifiers of contracture

i) pHi

A drop in pH modifies virtually every cellular system involved in Ca^{2+} regulation and force development because of competition with Ca^{2+} for binding sites. Tn C buffers about 40% of the Ca^{2+} at normal systolic levels. An increased $[H^+]i$ may thus displace this Ca^{2+}, increasing free $[Ca^{2+}]i$, although this should only be a transient effect. A lowered pH may also increase cytosolic Ca^{2+} accumulation via the Na^+/Ca^{2+} exchange [303], although this may only be active on reperfusion [413]. A drop in pH reduces the myofilament sensitivity to Ca^{2+} and decreases maximum force production [417], possibly by decreasing the maximum force generated by each cross-bridge as well as the turnover rate of the ATPase. Acidosis depresses myocardial contractility [31] and has a larger depressing action on rigor tension than on stiffness [558]. H^+ decrease the number of cross-bridges attached. Trimetazidine, an "anti-ischaemic " drug which may act as an intracellular buffer, reducing acidosis, reduces contracture at therapeutic doses [41].

Myosin ATPase activity may also be modified by acidification. H^+ are released at the Pi dissociation step, when ATP binds to myosin, while two H^+ are taken up when ADP dissociates. The ATP at which cell shortening occurs is 120 μM at a pH of 7.2, and 185 μM at pH 6.7 [42]. Thus as pH falls, the amount of ATP required to inhibit rigor development increases. However this effect is counteracted by effects of increased H^+ on Ca^{2+} binding and cross-bridge attachment described above. A fall in pH partially inhibits the development of contracture, but only if a low pH is present during the development of tension, and not once tension is fully developed [500]. With a drop in pH from 7 to 6.5, a reduction in peak tension of about 40% is found.

The onset of contracture has been linked to a levelling off in the drop in pH, attributed to cessation of glycolytic activity [253]. Glycogen depletion reduced the drop in intracellular pH but also reduced time to onset of contracture. There was no relationship between the extent of the drop in intracellular pH and peak contracture under these conditions. The role of pH in ischaemic contracture is thus dependent largely on other factors, particularly ATP.

ii) Pi

Pi is the major depressant of contractile force at the onset of ischaemia [284]. Pi reduces the maximal Ca^{2+}-regulated force, ascribed to changes at the level of the cross-bridge cycle, and not to reduced free energy of hydrolysis of ATP [247]. Pi shifts the sigmoid curve of force vs. Ca^{2+} to higher Ca^{2+} concentrations, but increase the cross-bridge cycling rate in normally contracting myocardium. In skinned ventricular fibres with rigor tension, Pi does not affect maximal tension development, as release of Mg^{2+}-ADP becomes the main rate-limiting step in the actomyosin reaction, minimising the effects of Pi accumulation [558]. Pi may decrease the sensitivity of the rigor tension development to a decrease in Mg^{2+}-ATP [558]. In skinned ventricular trabeculae, Pi has a similar, if lesser, effect to pH, by reducing maximal rigor tension, but only if changed prior to peak tension [500]. This has consequences for the development of ischaemic contracture - once initiated, its course cannot be altered.

iii) Mg^{2+}-ADP and CP

Local ATP regeneration by the CK mechanism is important in maintaining SR Ca^{2+}-ATPase function, and therefore cytosolic Ca^{2+} levels [272]. Myofibrillar CK activity may also be important in preventing ischaemic contracture by sustaining a high local ATP/ADP ratio [558]. Because of the importance of ADP in the cross-bridge cycle (release of ADP appears to limit the rate of cross-bridge detachment thus limiting shortening velocity), increased [Mg^{2+}-ADP]i potentiates isometric tension and slows kinetics [558] by binding strongly to cardiac myosin, slowing the detachment rate. Mg^{2+}-ADP increases maximal rigor force when ATP is reduced, and increases the susceptibility of rigor tension development to decreased levels of Mg^{2+}-ATP. A decreased CP and local Mg^{2+}-ADP accumulation increase stiffness [558]. Mg^{2+}-ADP may inhibit dissociation and/or further binding of Mg^{2+}-ATP and cross-bridge detachment, even in the presence of Pi and acidosis. In addition, the free energy of hydrolysis of ATP (determined by ADP and Pi concentrations) [235] may alter the effectiveness of a given amount of ATP. High CP levels remove the effect of Mg^{2+}-ADP by the action of CK. A creatine analogue delays ATP depletion and also the onset of ischaemic contracture [462].

e) Fall in tension

After contracture reaches a peak, the tension falls off. The rate of decline appears to be related to the peak reached - the greater the peak, and the sooner it is reached, the more rapid the fall-off. The decline in tension can be attributed to rupture of the myofibrils, as the highly contracted myofibrils pull the cells apart [32]. Cell membranes rupture, with severe and irreversible injury, precipitating cell death. Morphological changes observed in contracture involve the disruption of the myofibrils and

intercalated discs, followed by separation of sarcomeres and a decline in developed tension. Physical stress is increased with contracture, leading to increased cell fragility and injury [148]. Thus peak contracture may also reflect the ability of the heart to recover - the less the peak, the less rupture of myofibrils, and the less irreversible damage.

f) Reperfusion of contracted hearts

Ischaemic contracture compresses subendocardial blood vessels, enhancing ischaemic injury. This may result in non-reperfusion of these vessels when blood flow is restored, thereby contributing to reperfusion injury. The subendocardial vessels are the first to be compressed by contracture, an effect which spreads through to the epicardium as ischaemia progresses [210]. The vessels lose their ability to dilate and allow reperfusion, with reduced compliance, possibly accounting, at least in part, for the "no-reflow" phenomenon. The extent of reflow is increased if pre-ischaemic Ca^{2+} is lowered, while iodoacetate (glycolytic inhibitor) increases the loss of vascular compliance from an increased ischaemic contracture.

2,3-butanedione monoxime (BDM) inhibits excitation-contraction coupling, and thereby inhibits contracture. This compound improves functional recovery, an effect which may be related to reduced contracture or its effects on attenuating ATP depletion [551]. When the ischaemic period is extended, there is still no evidence of contracture, with preservation of ATP, if BDM is present, but membrane damage, and contractile failure on reperfusion are found. If the rate of glycolysis is increased during ischaemia, a reduced CK leakage and improved recovery of function are found with BDM. Thus insufficient ATP production results in ischaemic injury, while only a very small component can be attributed to mechanical changes following of contracture [551]. However, this study looked only at the effects of reducing contracture from a relatively small amount (50% of peak). If contracture were exacerbated, then a more detrimental effect of contracture by mechanical stresses might be found.

Reperfusion in itself may increase diastolic tension above that seen prior to reperfusion. This "contracture" is Ca^{2+}-dependent in that it results from a rise in cytosolic Ca^{2+} with active shortening of the myofibrils [210]. On reoxygenation, the stiffness component is reduced although tension is maintained or increased, indicative of Ca^{2+}-induced active force [309]. A repletion of ATP is required for relaxation of the myofibrils, as well as restoration of the Ca^{2+} homeostasis. Reoxygenation or reperfusion tend to increase $[Ca^{2+}]i$ [405]. If cytosolic Ca^{2+} levels do not rise above a critical point during ischaemia, they can be restored to normal on reperfusion. If the Ca^{2+} load is too high, with inhibited mitochondrial activity and increased cell fragility, the injury will be irreversible. The cell undergoes hypercontracture, leading to cell death. This concept was confirmed by Allshire et al. [8]

who found that reoxygenation of isolated cells with Ca^{2+} less than 2-3 μM restored function; a higher Ca^{2+} precipitated cell shortening and cell death.

8) REPERFUSION INJURY

a) Recovery of hearts after short term ischaemia

Restoration of flow after a period of ischaemia is essential for recovery. However, both the extent of injury during the ischaemic period, and the consequences of reperfusion itself may impair the degree of recovery. After a brief period of ischaemia (< 20 min), mechanical function may be depressed, but can be restored with maintained reperfusion (stunning). Increased duration of ischaemia (> 20-25 min), increased severity (determined by extent of residual flow) and an increased area at risk, increase the degree of ischaemic injury and of irreversible functional impairment (see above).

The main end-points of recovery after ischaemia are reperfusion arrhythmias, infarct size and functional recovery. In an isolated rat heart, the latter is the primary means of determining ischaemic injury. Functional recovery is determined by the relative proportions of fully functional, stunned, irreversibly injured, and dead cells. The number of cells in each state is determined by the length of ischaemia, and the modifications made to protect the heart, both during ischaemia, and on reperfusion.

b) Reperfusion injury

Restoration of flow after ischaemia, while essential to maintain viability, may actually exacerbate cellular injury [186]. While it is impossible to dissociate entirely the consequences of ischaemic from those on reperfusion there are three points to consider: 1) the extent of ischaemic injury directly influences the degree of functional recovery on reperfusion and 2) events can be modified only on reperfusion which suggests that distinct mechanisms are brought into play by the act of restoring coronary flow but 3) restoration of coronary flow as soon as possible is an absolute requirement for an ischaemic heart. There is some controversy over whether reperfusion kills cells that would otherwise have lived. This issue is, however, difficult to resolve because one cannot determine cell viability without some form of reperfusion.

Reperfusion-associated events include 1) reperfusion arrhythmias, 2) vascular damage and no-reflow, 3) myocardial stunning, 4) acceleration of necrosis in non-lethally injured cells, possibly by cell swelling and 5) acceleration of necrosis in cells irreversibly injured by ischaemia [406]. Ultrastructural cell damage, release of cytoplasmic enzymes, massive influxes of Ca^{2+} and Na^{+}, Ca^{2+} deposition in the mitochondria, and contraction band necrosis all contribute to reperfusion injury. Accumulated metabolites increase the osmotic load, such that on reperfusion, there are large transmembrane gradients and cell swelling.

The possible mechanisms of reperfusion injury include facets of the calcium and oxygen "paradoxes". The calcium paradox [604] occurs when Ca^{2+} is removed from cells, and then restored, leading to cell-cell uncoupling and disintegration of the membranes. This phenomenon is largely experimental, but there may be areas in the ischaemic tissue where this may occur, and the findings from calcium paradox studies apply to reperfusion injury, implicating Ca^{2+} in many of the deleterious effects found on reperfusion. The oxygen paradox (formation of free radicals on reintroduction of oxygen to anoxic tissue) [191] has also been implicated in the genesis of stunning, and may also precipitate cell death. While there has been some controversy over whether Ca^{2+} overload or free radicals are the main determinants of "reperfusion injury", the two phenomena are likely to be closely interrelated.

c) Calcium overload

The increase in cytosolic Ca^{2+} is a primary mechanism of ischaemic and reperfusion injury. Prevention of Ca^{2+} overload during ischaemia is beneficial to the heart, while reduction of Ca^{2+} entry immediately on reperfusion, using either reduced Ca^{2+} levels, Ca^{2+} channel antagonists [120], reduced SR Ca^{2+} release [121] or inhibition of the Na^+/H^+ exchange mechanism [119] are all beneficial by reducing reperfusion arrhythmias and improving recovery of function.

Ca^{2+} may enter the cell during ischaemia, but the rate of entry appears to be accelerated on reperfusion [353, 413]. Ca^{2+} enters through sarcolemmal Ca^{2+} channels and permeabilised membranes, while SR Ca^{2+} re-uptake is reduced, and the release of Ca^{2+} from intracellular stores is increased. During ischaemia, intracellular Ca^{2+} rises slowly in a more or less linear fashion [305, 353]. Thus the duration of ischaemia should roughly determine the amount of Ca^{2+} available at the start of reperfusion [353]. An increased intracellular Ca^{2+} is associated with increased tissue damage [305, 509]. On reperfusion, a further accumulation of intracellular Ca^{2+} can result from the Na^+/H^+- Na^+/Ca^{2+} exchange interaction [303]. A rise in cytosolic H^+ concentration ischaemia, mainly from reduced ATP turnover [156] activates the Na^+/H^+ exchange on reperfusion, leading to Na^+ influx. Reduced activity of the Na^+/K^+ ATPase with depleted ATP may also contribute to increased cytosolic Na^+. In addition, the fast Na^+ channel may be activated in ischaemia. Na^+ is extruded in exchange for Ca^{2+} via the Na^+/Ca^{2+} exchange, resulting in greatly increased intracellular Ca^{2+} levels [528]. Inhibition of the Na^+/H^+ exchange by amiloride or its derivatives has a significantly protective effects on reperfusion function, confirming this hypothesis [119]. SR Ca^{2+} ATPase function is also impaired by ischaemia [273, 315, 353] and is limited by ATP availability. Thus increased $[Ca^{2+}]i$ cannot be removed efficiently in reperfused tissue [353].

d) Free radicals

Oxygen free radicals have been implicated as the causative agent in a number of pathological conditions [177]. Oxygen free radicals have an unpaired electron which makes these species highly reactive. These include the superoxide anion ($\tilde{O}_2^-$) and the hydroxyl radical ($\tilde{O}H$). In addition, hydrogen peroxide (H_2O_2), and singlet oxygen are also highly reactive oxidising species. Superoxide dismutase (SOD) is a ubiquitous enzyme which catalyses the conversion of $\tilde{O}_2^- + 2\ H^+$ to $H_2O_2 + O_2$. H_2O_2 can then be reduced to H_2O by catalase (produces O_2) or glutathione peroxidase which converts GSH to GSSG. This change affects the redox state in the cell and many of the proteins which are activated by changes in SH binding [602].

$\tilde{O}_2^-$ can be generated from endothelial cells by one or more pathways (xanthine oxidase, cyclo-oxygenase, catecholamine oxidation, or mitochondria) [602]. $\tilde{O}_2^-$ formation can then initiate a series of reactions leading to neutrophil adherence (not in isolated rat heart) which subsequently release a variety of toxic products including free radical species. The release of these substances can lead to membrane permeability and damage to the myocyte [320]. Damage by free radicals can occur as a result of metabolic and structural changes including peroxidation of lipids.

Myocardial stunning can be partially relieved by the presence of free radical scavengers [38]. How free radicals result in stunning is not fully understood, but may be linked to injury to the SR or sarcolemma, possibly via lipid peroxidation, resulting in uncoupling of excitation-contracture and an overload of cytosolic Ca^{2+}. A predisposition to arrhythmias has also been attributed to free radicals, although this is not the most important mechanism [594].

Oxidant stress may exacerbate intracellular Ca^{2+} overload by changes in membrane phospholipids, impaired Ca^{2+} regulation by mitochondria, increased ATP depletion, changes in the Ca^{2+} release channel of SR, reduced uptake of Ca^{2+} by the SR, changes in the Na^+/K^+ ATPase pump and Na^+/Ca^{2+} exchange, and a slowed rate of inactivation of Ca^{2+} ATPases [413]. Ca^{2+} overload can initiate oxidation by damaging the mitochondria [222] which may affect their ability to reduce O_2 to H_2O. Changes in the SR induced by free radicals affect Ca^{2+} flux across the sarcolemma. The role of free radicals is, however, controversial, as the magnitude of the effects does not appear to be sufficient to explain all the deleterious consequences associated with reperfusion [594]. However, there is little doubt that free radicals do exert some effect, but whether this is triggered by increased Ca^{2+}, as one of the deleterious effects of cytosolic Ca^{2+} overload, or whether free radicals precipitate the increased cytosolic Ca^{2+}, is still questioned.

9) FUNCTIONAL RECOVERY

a) Myocardial stunning

i) Definition

Stunning is a consequence of relatively short periods of ischaemia (<20 min) and is defined as a fully reversible defect of myocardial mechanical function developing on reperfusion, in the absence of necrosis [37]. This statement implies that if reperfusion is sustained for a sufficient period of time the hearts will recover full function. The mechanism of stunning is as yet unclear, but may involve both cytosolic Ca^{2+} overload and oxygen free radical generation (see above).

Post-ischaemic stunning may occur in two phases [413]. On reperfusion, over a period of 0-5 min, cytosolic Ca^{2+} is in excess, during which a "normal" mechanical function may be found. Inotropic interventions at this time which increase cytosolic Ca^{2+} exacerbate eventual recovery (after 20-30 min reperfusion) [120]. After 5 min the more prolonged phase of 'classical" stunning is found, with underfunctioning and hypocontractile myocardium [413]. At this point, interventions with positive inotropic effects improve function [120]. Ca^{2+} availability does not appear to be the problem in stunning, as cytosolic Ca^{2+} levels are high.

ii) Dissociation of function and oxygen consumption

In stunned myocardium, oxygen consumption is maintained at levels equivalent to those in control hearts, but functional activity is depressed [397]. ATP levels in stunned myocardium take time to recover, although there appears to be little correlation with recovery of ATP and recovery of function. Some ATP is required; however, full restoration of ATP does not necessarily result in full functional recovery [37]. The source of ATP appears to be important i.e. glucose-derived vs. oxidative substrates [229, 325] (discussed Ch II). Reperfusion after differing severities of ischaemia showed similar levels of oxygen consumption, despite large differences in severity of injury [165]. The recovery in oxygen consumption was disproportionately high, and dissociated from recovery of contractile recovery. This dissociation was attributed not to metabolic inefficiency but rather to abnormalities in contraction or electromechanical coupling [293]. In addition, an increased intracellular Ca^{2+} should increase the rate of cross-bridge cycling, but instead the work rate and pump function are reduced in stunned myocardium. The myofibrils may be partially disrupted, but this is insufficient to account for the reduced recovery of function. In addition, arrhythmias are prevalent on reperfusion which predispose to reduced function. These findings imply some mechanism acting at the level of excitation-contraction coupling is responsible for reduced function.

iii) Myofilament sensitivity to Ca^{2+} and excitation-contraction coupling

Myofilament Ca^{2+} responsiveness is expressed as the relationship between free $[Ca^{2+}]i$ and force, such that a decrease in the Ca^{2+} transient results in reduced force. A decrease in myofilament responsiveness also results in reduced force, even if Ca^{2+} transients are unchanged. A shift in calcium sensitivity may occur, such that the range of contractile activation shifts to a higher Ca^{2+}, or a decrease in maximal Ca^{2+}-activated force scales down the overall response.

A decrease in the maximal Ca^{2+}-activated force and decreased sensitivity to extracellular Ca^{2+} of the myofilaments from stunned myocardium imply reduced sensitivity to Ca^{2+} [283]. A reduced sensitivity is likely given the increased cytosolic Ca^{2+} in stunned myocardium, but reduced force development. A possible explanation for decreased activity of the myofilaments may be due to activation of Ca^{2+}-dependent proteases by increased cytosolic Ca^{2+} which may attack the myofilaments and decrease their responsiveness [281].

However, stunned myocardium has an inotropic reserve which can be utilised with inotropic stimuli in "late" reperfusion [120]. A decrease in maximum calcium-activated force (Fmax) has been observed, but desensitisation of myofilament was discounted because of no change in the slope of the curve of force versus Ca^{2+} [58]. No depression of Ca^{2+} transients was found. The decrease in Fmax was attributed to increased Pi levels, a change in cross-bridge kinetics, a decrease in the relative force generation per attached cross-bridge and interaction with the inositol polyphosphate-diacylglycerol system which may affect excitation-contraction coupling [58]. Soei et al. [503] however, suggest that Ca^{2+} desensitisation of the myofibrils is involved in stunning, as a calcium sensitiser increases systolic segment length shortening in stunned myocardium, more so than in control myocardium. An increased myosin ATPase activity could then be responsible for the decreased mechanical efficiency, with excess oxygen consumption for the work output [293]. Gao et al. [149] found evidence for decreased myofilament responsiveness and altered diastolic function in stunned myocardium. At any given Ca^{2+}, force generation was significantly lower in stunned tissue, with no differences in calcium transients. A decrease in maximal Ca^{2+}-activated force and an increased $[Ca^{2+}]i$ was required for 50% activation. An accelerated rate of diastolic relaxation was found. With high Ca^{2+} concentrations, diastolic tone increased more in stunned tissue. Thus the primary lesion of excitation-contraction coupling appears to be at the myofilaments [149]. Stunning primarily involves decreased cycling rates of myofibrillar cross bridges, with a reduction in myofilament Ca^{2+} sensitivity after more severe ischaemia [347].

iv) Preconditioning

An important possible consequence of "stunning" is the recently described phenomenon of preconditioning, whereby brief periods of ischaemia, which may lead to stunning, protect the heart against a subsequent sustained period of ischaemia. Some protective mechanism may be switched on

during the preceding ischaemic episode, which may also be involved in stunning [491]. However, the effects of stunning and preconditioning have been dissociated [358].

b) Diastolic dysfunction and irreversible injury

A transient, reversible rise in LV diastolic pressure is commonly observed in angina pectoris. This may be due to an increased end diastolic volume because of reduced cardiac function, or because of a reduced diastolic distensibility of the LV chamber. Reoxygenation or reperfusion may bring about, or exacerbate, an existing diastolic tension or contracture, largely mediated by a rise in resting Ca^{2+} on reperfusion [229, 283]. An increased end-diastolic resting tension is undesirable because of a reduced left ventricular filling, physical occlusion of coronary blood vessels, and possible damage to the sarcolemma induced by increased shortening of the cells with stretch of the membranes. Increased resting tension can exacerbate cell damage and precipitate death. The ability of the heart to recover is limited by the extent of irreversible damage, such that only the reversibly injured tissue will recover.
A lower Ca^{2+} on reperfusion, or application of Ca^{2+} blockers at this time reduce Ca^{2+} entry, thereby inhibiting contracture development, and subsequent injury [229]. Glucose is also beneficial by improving Ca^{2+} homeostasis and increasing ATP availability. ATP on reperfusion may be limited by Ca^{2+} accumulation in the mitochondria during ischaemia, and glycolysis may provide essential ATP at that point. Acidosis is beneficial on reperfusion by inhibiting Ca^{2+} binding and Ca^{2+} entry [257].

c) Reperfusion arrhythmias

An increased incidence and duration of reperfusion arrhythmias leads to a decline in function and a reduced ability of the tissue to recover from ischaemia. Increased cytosolic calcium has been implicated in the development of reperfusion arrhythmias [270]. Free radical generation leading to loss of membrane integrity and Ca^{2+} overload has also been implicated [590, 594]. Arrhythmias can also be attributed to re-entrant circus effects due to the presence of an infarcted area [336]. Increased Ca^{2+} oscillations (delayed after depolarisations - DADs) on reperfusion increase the excitability of the membranes and may precipitate arrhythmias [80]. A reduction in DADs has been noted with increased glycolytic ATP availability [79]. An interesting point is that arrhythmias cannot arise from dead cells; a certain degree of viability is necessary to generate arrhythmias.

d) Myocardial infarction

Infarct size is dependent on the extent and severity of ischaemia, and the degree of reduction in oxygen delivery, determined by residual and collateral coronary flow [194, 485] The size of the area at risk i.e. the area perfused by the affected artery, and variation of ischaemia within this area also

determine eventual infarct size. The severity of ischaemia can be determined by the imbalance between the metabolic requirements of the myocardium and the supply of oxygen and substrates by any residual flow to the tissue. Collateral blood flow is thus an important determinant of infarction, the extent of which varies greatly between species. Increased subendocardial pressure restricts flow to the area at risk, thus the subendocardium is generally more ischaemic than outer regions [485, 507] (especially if an intraventricular balloon is present), and more prone to early infarction. Infarction spreads as a wave from the endo- to the epicardium [327]. Preconditioning has been shown to be the most effective mechanism of protection against infarct size, but the mechanism and its consequences are not yet fully understood.

e) Chronic sublethal ischaemia

Coronary perfusion pressure, coronary flow, myocardial oxygen consumption and contractile performance are tightly coupled [39, 464]. Thus even a relatively small reduction in blood flow (10-20%) will result in a significant compensatory reduction in contractile performance [552]. The term, "hibernation", has been coined to describe a prolonged subacute or chronic reduction in coronary blood flow, in which the myocardial contractility, metabolism and ventricular function are reduced to match the reduced blood supply [448]. Restoration of normal blood flow should result in rapid, complete restoration of myocardial function [497].

However, there is much dispute as to whether the coronary flow is in fact reduced in hibernating tissue. It has been suggested that "hibernating" myocardium may in fact be tissue exposed to repeated brief, sublethal episodes of ischaemia, followed by restoration of blood flow [155, 464]. Over time there may be an overall reduction in contractile performance, with repetitive stunning [155, 464]. Thus a reduction in blood flow in chronically dysfunctional segments may not be present [340]. Positron emission tomography is used to predict viable tissue, usually indicated by a "mismatch" between coronary flow (unchanged or reduced) and glucose extraction (increased). This concept contradicts the accepted inhibition of glycolysis in ischaemia, and indicates an adaptation of the tissue to a chronic condition.

10) SUMMARY

"Ischaemia" describes an imbalance between supply of energy substrates and demand. Most experimental models of the isolated perfused heart use total global ischaemia i.e. complete cessation of flow. However, *in vivo* some residual flow is almost always present. Thus a low flow model of ischaemia is more representative.

On reduction or cessation of flow, the globally ischaemic heart undergoes contractile failure, and ceases to beat. After a few minutes, a rise in resting tension, or contracture is found. The time to onset and extent or peak of contracture are dependent of the availability of ATP and of $Ca^{2+}i$, which in turn are related to changes in $[Na^{+}]i$ and pHi. The ion contents of the cytosol are closely regulated by pathways which are disturbed by ischaemia, including the metabolic and signalling pathways. Intermediates or metabolites of these pathways can seriously affect the compromised tissue.

On reperfusion, the extent of injury caused by cellular perturbation results in ischaemic injury which may be exacerbated on reperfusion. Thus the resultant functional recovery is the sum of ischaemic and reperfusion injury. In addition, arrhythmias and infarct size can reflect ischaemic injury and are in turn important determinants of eventual outcome. Glucose modifies many of the cellular changes induced by ischaemia, which is reflected in a change in contracture and function on reperfusion. Modifications of glycogen also alter the response to ischaemia, as does preconditioning. These issues are discussed in detail in the following chapters.

II. GLUCOSE IN ISCHAEMIA

1) THE "GLUCOSE HYPOTHESIS"

Following on from early work establishing a role for enhancement of substrate supply in ischaemia, Opie [401] propounded the "glucose hypothesis". Several possible beneficial actions of glucose in the ischaemic myocardium were proposed, including increased energy production, reduced loss of K^+ ions and attenuated arrhythmias, inhibition of changes in the transmembrane action potential, altered extracellular volume, and decreased circulating free fatty acids which are toxic to the ischaemic heart. These mechanisms have been reflected in reduced incidence of arrhythmias, reduced ischaemic contracture, and improved recovery of function following administration of glucose.

Despite many laboratory studies showing benefits of increased glucose, there are several important exceptions. These include 1) the British Medical Research Council trial published in the Lancet which did not find a beneficial effect associated with glucose-insulin-potassium (GIK) treatment of patients with infarction [352], 2) addition of glucose in cardioplegic solutions which was detrimental to the myocardium [193], and 3) reduction in glycogen was found to be beneficial to the globally ischaemic rat heart, an effect attributed to reduced lactate accumulation [387]. These studies have contributed to the controversy over the therapeutic use of glucose, and in particular have resulted in the discontinuance of the use of GIK therapy for patients with myocardial infarction. However, these deleterious effects may be largely determined by the incorrect dosage and rate of removal of metabolites, Our hypothesis is that glucose is beneficial at an optimal concentration, and that increased glucose, possibly with insulin, may indeed be deleterious by increased metabolite accumulation. Removal of the metabolites by increased residual flow should result in increased recovery with higher glucose concentrations.

2) GLUCOSE METABOLISM IN ISCHAEMIA

a) Glucose in hypoxia/anoxia

Hypoxia stimulates glucose utilisation by reversal of the Pasteur effect, with a 20-fold increase in glycolytic flux in dogs *in vivo* [276], and a 3-fold increase in isolated rat hearts perfused with 11 mM glucose [469]. Glycolytic inhibition at the level of PFK by citrate and ATP is removed, and glycolysis is stimulated [586] (see appendix). If hypoxia is severe, heart function declines slowly, from insufficient ATP. If glycolysis is inhibited, developed tension dissipates completely [7]. Contracture may develop following ATP depletion [5, 9].

Hypoxia depletes tissue glycogen by increasing glycolytic rates to maintain ATP levels. If high glucose concentrations are present, heart failure is attenuated [470]. However, similar rates of lactate

production are found, and thus similar rates of glycolysis. A high glucose thus inhibits glycogenolysis, as well as increasing glucose utilisation by glycolysis. Utilisation of glucose appears to be better than glycogen utilisation, despite similar rates of ATP production [470]. The mechanism for this preference may lie in the localisation of glycogen granules in the cell, with functional compartmentation of the ATP produced, and of localisation of the metabolites (see below). Glucose in hypoxic hearts prevents contracture [116] and reduces enzyme leakage in the rat [190].

b) Energy production in ischaemia

Reserves of high energy phosphates (ATP and CP) are sufficient only for 3-4 contractions of the normal heart. However a rapid cessation of contractile function reduces the demand for high energy phosphates.

Jennings and Reimer [225] have described the metabolic changes occurring in ischaemia as cessation of aerobic metabolism, depletion of CP, onset of anaerobic glycolysis, and accumulation of glycolytic products (lactate, α-glycerophosphate, adenine nucleotide breakdown). The demands of the myocardium for ATP exceed supply, such that net ATP decreases. These changes lead to contractile failure, as Pi increases. In the absence of flow, the majority of ATP is derived from anaerobic glycolysis. Pyruvate is converted to the dead-end molecule of lactate, a temporary measure to reduce accumulation of NADH, and restore NAD^+ levels. If washout is not maintained, the end products accumulate, which may be deleterious. After a short period (a few minutes) glycolysis in inhibited in ischaemia despite an initial stimulation.

c) Rate-limiting steps in glycolysis - original concepts

Kübler and Spieckermann [276] found that when pO_2 becomes critical (less than 5 mmHg) in ischaemic myocardium in dogs *in vivo*, glycolysis is initially stimulated by reversal of the Pasteur effect, with a build-up of lactate. The subsequent decline in glycolysis was attributed to the limiting effect of reduced ATP for the phosphofructokinase step, i.e. the conversion of F6P to FDP (see appendix). The level of ATP (3.5 μmol/g wet wt) at this point was said to be critical, with ischaemic injury occurring at lower values.

This concept of glycolytic inhibition was revised by Neely and Rovetto [389, 390, 468, 469] who proposed that glycolytic flux in ischaemia was inhibited at GAPDH by an accumulation of end products, specifically lactate, H^+, and NADH. This concept was derived from work on the isolated working rat heart. A 60% reduction in coronary flow (from 15 ml/min to 6 ml/min) reduced oxygen consumption and accelerated glucose utilisation by 100%. At a flow rate of 0.6 ml/min, glucose utilisation was less than in control conditions (about 50%). After 16 min of low flow ischaemia, the hearts were clamped and levels of metabolites assessed. Lactate values were high, suggesting that

NADH was increased, because of the equilibrium of the LDH reaction. Increased NADH would, in turn, inhibit GAPDH, which is regulated by the NADH/NAD^+ ratio [362] (see appendix). GAPDH inhibition was shown by an increase in DHAP levels [468] and application of the crossover theorem [68]. Following on from these publications, it has been widely accepted that glycolytic enzymes are inhibited in ischaemia by a build-up of glycolytic metabolites, and glycolytic flux thus inhibited. For the purposes of the following discussion, this view is applied. However, our analysis of glucose uptake in low flow ischaemia indicates that this interpretation i.e. that glycolysis is inhibited in ischaemia, is not strictly true (See Results 1,2,3); rather, glycolysis is limited by substrate supply. The limitations of the above interpretations are discussed further in the Discussion of the thesis.

3) Role of glucose in ischaemic myocardium

a) Specific role of glycolytic ATP

Despite the limitation of glycolytic flux in ischaemia, cell viability may be determined by the residual rate of glycolytic flux [404]. Glycolytic ATP appears to have a preferentially effective role as opposed to ATP derived from oxidative phosphorylation [49]. A basal level of glycolytic activity is required to prevent irreversible injury [227], with control of cytosolic Ca^{2+} during ischaemia [441] and on reperfusion [229], prevention of ischaemic contracture [48, 424], inhibition of enzyme release [190], and inhibition of free radical activity [405]. Glycolytic ATP is thought to have several effects on membrane functions: blocking the K_{ATP} channel [571], maintaining the activity of the Na^+/K^+ ATPase pumps in the sarcolemma [167], sustaining membrane integrity [49, 203], and maintaining Ca^{2+} homeostasis by the SR Ca^{2+} ATPase pumps [593]. These cellular effects modify functional responses to ischaemia with reduced diastolic tension [14, 48, 316, 424], and improved functional recovery on reperfusion [14, 123, 228, 550]. Much indirect evidence suggests that ATP production within the cell is spatially compartmented [49, 428, 593], accounting for differences in the effectiveness of different sources of ATP. In ischaemia, the processes for transport of ATP within the cell may break down. Thus provision of ATP near to the sites of utilisation becomes a crucial determinant of its effectiveness.

The role of glycolytically-derived ATP therefore appears clear-cut in terms of its beneficial effects. However, during the breakdown of glucose and glycogen, a number of metabolites accumulate which may contribute to some of the deleterious effects of ischaemia. The benefit of glycogen in particular has been questioned in this regard, especially given the interest generated in the recently described phenomenon of preconditioning. A reduction in glycogen is postulated as one of the mechanisms whereby preconditioning may exert its beneficial effects [224] and is investigated in this thesis (see Ch III).

My proposal is that a crucial balance exists between rates of ATP production and rates of metabolite washout. This relationship is determined largely by the rate of residual coronary flow and the glucose concentration, as well as the level of the endogenous glycolytic substrate, glycogen. The role of coronary flow in determining the benefit of glucose is crucial, and is one of the focal points of this thesis.

b) Membrane integrity

The role of glucose in maintaining the integrity of the cell membrane was illustrated initially by de Leiris et al. [104]. Enzyme release from severely damaged cells is a frequently-used indicator of myocardial cell death and infarction in patients. These markers include the isozymes of CK and LDH. In isolated rat hearts perfused with high concentrations of free fatty acids, enzyme release is attenuated in the presence of glucose [104], as shown in hypoxic hearts [190, 192]. In a low flow ischaemic model, glucose provision also reduces the release of LDH, compared to pyruvate, despite similar ATP levels [49]. The effectiveness of glycolytically-derived ATP appears greater than that derived from residual oxidative phosphorylation, accounted for by spatial compartmentation of ATP [49]. Glucose-derived ATP may maintain the phosphatidic acid cycle, thereby preventing lysophospholipid accumulation which leads to membrane breakdown [89]. Glucose limits phospholipase C degradation of membranes [203], and increased phosphorylation with increased ATP availability may enhance the stability of the phospholipid membrane [202]. Cell swelling in ischaemia may be reduced by the osmotic effects of glucose, which also helps to reduce cell rupture [225]. In addition, reduced contracture with glucose will limit cell rupture from stretching.

c) Membrane pump and channel activity

Glucose plays a major role in ion homeostasis. When cells are made ischaemic, a large efflux of K^+ results, which depolarises the membrane, and renders the cell inexcitable, a positive effect with depleted ATP. However, the excess extracellular K^+ can precipitate arrhythmias if the efflux is maintained [328]. The ATP-dependent K^+ (K_{ATP}) channel, one of the major channels involved in the efflux of K^+ in ischaemia [233, 236], is blocked by ATP. However, the experimental levels to which ATP must fall to allow channel opening in excised patches [233] are well below those in ischaemic tissue with a noted K^+ efflux [233]. The observed changes in K^+ efflux through this channel despite relatively small changes in ATP levels can be explained firstly by the high density of channels present such that a small change in maximal conductance can result in a large change in action potential shortening and K^+ loss [572]; and secondly, a localised fall in ATP near the membrane allowing for individual channel opening. Glucose-derived ATP, as opposed to that from oxidative phosphorylation,

preferentially maintains closure of the K_{ATP} channel supporting the concept that this ATP is localised near the membrane [570, 571]. Sarcolemmal-associated glycolytic enzymes may maintain the ratio of ATP/ADP in the vicinity of the K_{ATP} channel [572].

Recent studies have shown that Na^+/K^+ ATPase pump inhibition by ouabain abolishes protection by glucose [94, 167]. A reduced energy supply from glucose inhibits Na^+/K^+ ATPase, and increases osmolarity and cell swelling [94]. Eventually, Ca^{2+} overload may occur from an increased $[Na^+]i$. In addition, glycolytic enzymes are functionally coupled to SR Ca^{2+} transport [593]. Ca^{2+} transport could thus be maintained specifically by ATP produced by glycolysis. Glycogenolytic enzymes and glycogen granules are also associated with the SR [128, 593]. A preferential role for glycolytic ATP in preserving Ca^{2+} homeostasis by maintained Ca^{2+} re-uptake is postulated. Jeremy et al. [229] report that glycolysis plays a central role in maintenance of Ca^{2+} homeostasis on reperfusion, possibly by activation of the SR Ca^{2+} ATPase pump, the activity of which is affected in stunned hearts [273, 315] (see Ch I).

Glycolytic ATP has been associated with maintenance of Ca^{2+} homeostasis during β-adrenergic stimulation, by continued activity of the SR Ca^{2+} ATPase pump [380]. Maintained glycolysis also inhibits the release of noradrenaline from nerve terminals [57], which should attenuate the effect of increased catecholamines on cellular damage in ischaemia (see Ch I).

d) Arrhythmias and free fatty acids

Ventricular arrhythmias generated during ischaemia or on reperfusion are complex in origin. Changes in cell membrane integrity, ionic fluxes across the membrane, and impairment of conduction, all result in arrhythmias. Reperfusion arrhythmias are attenuated in hearts perfused with glucose, compared to those with acetate or palmitate [49]. The vulnerability of the dog heart to arrhythmias is reduced when glucose is administered intravenously [471]. Glucose also protects against arrhythmias in a model of regional ischaemia in the isolated perfused rat heart [28].

Glucose may reduce the arrhythmoticity of ischaemic tissue by a number of factors, mainly those involved in membrane integrity and pump and channel activity, as discussed above. Glucose maintains action potential duration in the isolated perfused rat heart [416], presumably by blocking the K_{ATP} channel, thereby limiting K^+ loss [571]. Glycolytic ATP also reduces cAMP accumulation, and lowers LDH release, an effect associated with reduced arrhythmias on reperfusion [49]. In addition, glycolysis reduces cytosolic Ca^{2+} accumulation on reperfusion [229], a precipitating factor in reperfusion arrhythmias [412, 539]. Preserved Na^+/K^+ ATPase function [94, 167] would attenuate the increase in $[Na^+]i$, which in turn would reduce $[Ca^{2+}]i$ [303]. Glucose may also have a free radical

scavenging effect [199], important especially on reperfusion in maintaining membrane integrity and reducing arrhythmias [594].

A recent editorial has highlighted the benefit of glucose and insulin in lowering the levels of circulating free fatty acids [399]. Glucose and fatty acid utilisation are closely related - the so-called glucose fatty acid cycle, whereby glycolysis provides glycerol 3-phosphate (αGP) required for the formation of triglycerides[451]. Glucose uptake inhibits free fatty acid release from adipose tissue, an effect enhanced by insulin [451]. Free fatty acids have a toxic effect on ischaemic hearts, and lead to increased arrhythmias [399]. This effect may be due to the accumulation of intracellular acylcarnitine and acyl Co-A which are thought to promote intracellular Ca^{2+} overload [207, 399, 589]. A recent paper has described detrimental effects of acyl Co-A on contracture, an effect attenuated by glucose [75]. Lysophospholipids from the breakdown of lipids are arrhythmogenic, with a detergent effect on the membranes [88]. Lipid compounds may also inhibit Ca^{2+} reuptake mechanisms and activate Ca^{2+} channels [207, 399, 589]. Glucose provision counters many of the deleterious effects associated with free fatty acids in ischaemia, by reducing free fatty acid availability in the circulating blood, as well as combating the deleterious effects of free fatty acids on Ca^{2+} overload in the cell [399].

e) Glycolytic ATP and ischaemic contracture

Another important role for provision of glucose during low flow ischaemia is a reduction in ischaemic contracture. There is some controversy over whether ischaemic contracture is indicative of injury or not [551], given that under certain conditions increased contracture is associated with improved functional recovery on reperfusion e.g. preconditioning [266] (see Ch III). However, increased contracture is generally considered to reflect increased cell injury [110, 189], and may in itself add to cell damage, thereby further impairing functional recovery.

The level of ATP is thought to be a major determinant of the time to onset of contracture [189] (see Ch II). In general, a depletion in ATP levels reduces the time to onset; increased ATP availability delays contracture. Glucose + atractyloside (inhibits translocation of mitochondrial ATP) lessens contracture, compared to mitochondrial substrates (acetate and pyruvate) with inhibited glycolysis (iodoacetate or 2-deoxyglucose) [48], despite similar levels of ATP production. Thus ATP from glycolysis appears better able to prevent contracture. Similar results were obtained in acetate-perfused hearts without glycolytic inhibition [316]. Time to onset of contracture was greatly reduced compared to glucose-perfused hearts despite much higher ATP levels [316].

In moderate low flow (50%) ischaemia (termed "hibernation" by the authors), a preference for glucose rather than pyruvate has been observed in terms of reducing contracture [483]. Glycolytic ATP

was essential to ensure recovery of CP. ATP derived from oxidative phosphorylation (pyruvate) was not sufficient to promote metabolic recovery or to maintain diastolic function [483]. Apstein et al. [14, 123] showed that provision of glucose in rat models of low flow ischaemia (0.5 ml/min/g wet wt or 0.06 ml/min/g wet wt) maintains diastolic function, and improves recovery of function on reperfusion. A higher concentration of glucose (27 mM vs. 5 mM) with insulin, and a higher of residual flow, were more effective.

The onset of contracture has been linked to cessation of glycolysis, as measured by a levelling-off in the drop in intracellular pH [253]. These studies were performed in hearts exposed to total global ischaemia, where glycolytic flux is determined by the level of endogenous glycogen. With low flow ischaemia (0.5 ml/g wet wt/min), a minimum rate of at least 2 μmol/min/g wet wt glycolytic ATP production is required to prevent contracture [424]. Similar or larger amounts of ATP derived from glycogen or non-glycolytic substrates were not effective in delaying contracture, which showed a graded response to increased glucose concentrations [424]. Therefore the onset of contracture is not associated so much with cessation of glycolysis, as with a fall in glycolytic flux below a threshold. In total global ischaemia, this occurs rapidly. Vanoverschelde et al. found that the rate of glycolysis during ischaemia was directly related to the degree of ischaemic injury i.e. contracture, and functional recovery [550]. A high glucose (11 mM) + insulin prevented contracture in rabbit hearts with a low flow of 0.4-0.5 ml/g wet wt/min. Increased pre-ischaemic glycogen also reduced contracture. Lessened contracture was associated with improved functional recovery.

Other mechanisms which increase glycolysis, including a slower glycogen depletion and less severe acidosis [12, 189], inosine (putatively acting by increasing pyruvate-to-alanine conversion, and removing glycolytic inhibition by lactate) [308], adenosine [291] (See Ch I) and reduced free fatty acids [550] delay the onset of contracture. Glycogen depletion tends to reduce time to onset of contracture [278]; glycogen loading delays time to onset of contracture [189]. Glucose attenuates the deleterious effects of long-chain acyl carnitine (LCAC) on contracture [75]. LCAC's may disrupt Ca^{2+} homeostasis [75, 399], an effect counteracted by glucose-derived ATP.

Again, in considering the role of glucose on contracture, the local concentration of ATP appears to be crucial, rather than total ATP content. Intracellular compartmentation of ATP can explain many observations (see below). Contracture is triggered by the formation of rigor complexes, which in experimental conditions, bond only at very low ATP concentrations (<100 μM [307]). Individual cells can have very low total ATP levels without contracting as long as glycolysis (from glycogen) is maintained [185]. However, a drop in ATP to less than 150 μM in isolated myocytes precipitates contracture [42]. Glycolytic ATP may also be essential for direct relaxation of the actin-myosin complexes, the so-called "plasticising" effect, reversing the formation of rigor complexes [13].

However, there are some difficulties associated with this concept, namely that glycolytic i.e. glucose-derived ATP, appears to be functionally associated with the sarcolemma and SR rather than the myofibrils (see above and below for further discussion). Glycolytic ATP may therefore be more important in maintaining ion homeostasis, rather than ensuring direct relaxation of the cross-bridges by binding to myosin. Only under conditions of high glycolytic flux rates, may the diffusion of glycolytic ATP to the myofibrils be sufficient to increase the rate of relaxation and prevent rigor formation. However, this would only be under conditions of relatively unimpaired function i.e. a fairly high residual flow rate, and a high glucose concentration, ensuring a large intracellular ATP production [14, 123]. Glycolytic ATP may reduce contracture mainly by reducing intracellular Ca^{2+} accumulation by maintaining activity of the Na^+/K^+ ATPase and SR Ca^{2+} ATPases and attenuating intracellular Ca^{2+} accumulation. While Eberli et al. [122] propose that Ca^{2+} is not involved in ischaemic diastolic dysfunction, in a low flow model with maintained developed pressure, these results do not translate directly to the truly ischaemic heart, which has severely impaired flow. The exact mechanism of glycolytic ATP protection against ischaemic contracture is unclear, and is investigated further in this thesis.

f) Mechanical function on reperfusion

Recovery of mechanical function is determined by the degree of ischaemic injury, and the conditions on reperfusion (see Ch I). At both periods, substrate provision can be altered, affecting eventual recovery. Increased glucose provision during low flow ischaemia increases the functional recovery of hearts [14, 49, 123, 550], mainly by reducing diastolic pressure on reperfusion, a consequence of reduced contracture during ischaemia, and improved relaxation on reperfusion [13].

Sufficient levels of ATP are essential for complete restoration of function. Sako et al. suggest that optimal recovery on reperfusion requires the presence of several compounds, one of which should be glucose [476]. Glucose is necessary to ensure recovery as opposed to mitochondrial substrates including pyruvate (in glycogen-depleted hearts) [334], or palmitate [325]. Pyruvate does, however, add to the beneficial effect of glucose, but glycogen or glucose appear essential to ensure a basal recovery [334]. Pyruvate may be beneficial by providing anaplerotic substrates [198]. Glycolysis in early reperfusion is therefore required to prevent energetic and contractile collapse. Jeremy et al [228, 229] found that glycolysis is specifically required on reperfusion to facilitate Ca^{2+} homeostasis. Alternatively, free radicals, one of the possible causes of stunning and arrhythmias, inhibit glycolysis, and thus impair Ca^{2+} homeostasis [87]. Inhibition of glycolysis, despite the presence of pyruvate and oxygen, depressed functional recovery severely, with a persistent Ca^{2+} overload [228, 229]. The glycolytic ATP may required to reduce $[Na^+]i$ by maintaining the activity of the Na^+/K^+ ATPase, thereby resulting in reduced Ca^{2+} overload [228]. Na^+/K^+ ATPase inhibition during ischaemia has been

shown to remove the protective effect of glucose on functional recovery, possibly by increasing cytosolic Ca^{2+} via the Na^{+}/Ca^{2+} exchange [94, 167]. ATP derived from glycolysis is also functionally associated with the sarcoplasmic reticulum [593], thereby providing a means of restoring Ca^{2+} overload on reperfusion by enhanced SR Ca^{2+} ATPase function.

The role of glucose versus pyruvate on reperfusion does not seem clear cut. Hearts pre-treated for 1 h with 5.5 mM glucose and 5 U/l insulin, or pyruvate, subjected to ischaemia, and then reperfused with pyruvate, lactate, acetate (all at 5 mM), or glucose and insulin, showed that reperfusion of the pre-ischaemic glucose hearts was maximal with 5 mM pyruvate as opposed to glucose (these would have high glycogen) [60]. Pre-ischaemic perfusion with pyruvate increased ischaemic contracture (following reduced glycogen), but subsequent recoveries with pyruvate and glucose were similarly high. This finding was similar to that reported by Kupriyanov et al [278], with a similar improvement in functional recovery in hearts subjected to 2h pre-ischaemic perfusion with pyruvate. This effect was attributed to a decreased glycogen. Deboer et al [106] found that pyruvate enhanced recovery on reperfusion, an effect attributed to reduced free radical generation. Pyruvate may also replenish the Krebs cycle (anaplerotic effects), reduce the $NADH/NAD^{+}$ ratio, and increase the activity of pyruvate dehydrogenase, an important regulator of the Krebs cycle (see appendix).

While palmitate is the preferred substrate on reperfusion, accounting for over 90% of ATP from exogenous substrates, inhibition of palmitate oxidation with increased glucose oxidation improves functional recovery [325]. High levels of fatty acids on reperfusion limit glucose utilisation by competitive inhibition [459]. This deleterious effect is primarily due to inhibition of glucose oxidation and not of glycolysis [326]. Increased glucose oxidation is beneficial while insulin has no effect as lack of substrate is not the problem. A recent review has resolved two main hypotheses as to why increased glucose oxidation should be beneficial on reperfusion [354]. Stimulation of glucose oxidation would speed the initial rate of return to total oxidative metabolism, resulting in a more rapid return to normal levels of high energy phosphates, which is then sustained by fatty acid or pyruvate oxidation. Secondly, a reduced intracellular acidosis from increased glucose oxidation versus glycolysis may be beneficial by reducing intracellular Ca^{2+} overload.

g) Preconditioning

Glycolytic flux is stimulated in preconditioned hearts subjected to low flow ischaemia, and may exert its positive effects by this mechanism [218]. Several preconditioning studies have used different substrates on reperfusion and found that the use of pyruvate with or without glucose [142, 400], or deoxyglucose+acetate [142], eliminates any protective effect of preconditioning on functional recovery. This reduction in function is associated with impaired calcium homeostasis. Finegan et al. [136] found

that glucose oxidation is increased relative to glycolysis in preconditioned hearts, which may be beneficial (see above). However, preconditioning is associated with a reduced glycogen, which attenuates the fall in pHi and the rise in lactate, and may be beneficial by these mechanisms. This phenomenon adds to the controversy over glycogen and glycolysis, and is investigated in this thesis (see Ch III).

4) DELETERIOUS EFFECTS ASSOCIATED WITH INCREASED GLYCOLYSIS

a) Benefits associated with depleted glycogen

While there is much evidence to suggest that ATP derived from glycolysis is beneficial to the ischaemic myocardium, this may be outweighed by the accumulation of deleterious end products. A great deal of controversy arose from a much-quoted paper published in 1984 by Neely and Grotyohann [387]. Beneficial effects were associated with glycogen depletion prior to sustained total global ischaemia. A greatly improved recovery of function was found when hearts were exposed to 10 min anoxia prior to the ischaemic period, with or without an intervening period of reoxygenation. The improved recovery was correlated with a reduced lactate content at the end of ischaemia. Thus it was hypothesised that increased glycolysis is associated with a detrimental effect due to an increase in lactate and other end products. The concept then arose that increased glucose should be detrimental.

Several other studies have shown a beneficial effect of reduced glycogen levels. 2h perfusion with pyruvate depleted tissue glycogen by 40-50 %, which reduced the fall in pHi but lowered ATP levels [278]. However, glycogen-depleted hearts showed a similar peak contracture to glucose hearts, although time to onset of contracture was greatly reduced. Functional recovery after 25 min total global ischaemia was significantly improved in the glycogen-depleted hearts, attributed to reduced $[H^+]i$ accumulation. Glycogen reduction by hypoxic perfusion, followed by 25 min total global ischaemia, reduced lactate accumulation, and improved function [3]. 10 min perfusion prior to ischaemia with the glycolytic inhibitor, 2-deoxyglucose, improved recovery of function only in hypertrophied hearts [3].

The possible benefits of glycogen depletion also apply to preconditioning. The brief episode(s) of ischaemia deplete tissue glycogen prior to the sustained ischaemic period. An increased duration of intervening perfusion was correlated with repletion of glycogen stores but loss of recovery [588].

A more complicated protocol compared hearts subjected to 10 min substrate-free perfusion followed by 30 min perfusion with pyruvate (50% glycogen depletion) with and without a subsequent period of glucose perfusion, hearts perfused with glucose throughout the pre-ischaemic period, and preconditioned hearts [482]. Recovery of function was improved in the preconditioned and the pyruvate glycogen-depleted hearts, but only if glucose was not provided to the latter. The drop in pHi was lessened by interventions to reduce pre-ischaemic glycogen, but the fall in ATP was greater in these hearts. The conclusion reached was that glycogen depletion per se was not sufficient to explain the

beneficial effects of preconditioning, but interventions which limited glycolysis and thus H^+ accumulation were beneficial.

b) Mechanism of benefit of glycogen depletion

i) Protons

While intracellular pH always falls in ischaemic hearts, the extent of the drop can be modified by changes in the amount of glycogen in the tissue at the onset of ischaemia. Glycogen-depleted hearts show less of a fall in intracellular pH during ischaemia [151]. Increased $[H^+]i$ accumulation increases $[Na^+]i$, and in turn increases $[Ca^{2+}]i$ via the Na^+/H^+ and Na^+/Ca^{2+} exchange mechanisms [303]. Glycogen depletion is associated with reduced $[Na^+]i$ accumulation, linked to reduced H^+ accumulation [528]. Recovery of function is improved, with reduced cytosolic Ca^{2+} overload. However, while some reports have correlated intracellular pH with ischaemic injury [99], others have dissociated these effects [92, 151]. The relationship between increased acidosis and increased ischaemic injury is not clear (in cardioplegia, for example, a lower pHo is protective [564]).

ii) Lactate

Lactate is utilised as a substrate by the heart if oxygen is present. If the blood supply is reduced, stimulation of glycolysis increases intracellular lactate levels. Lactate added to the perfusate of an isolated heart has been shown to impair recovery of an ischaemic heart [92, 239, 387]. Lactate exits from the cell in a H^+-coupled efflux [439] such that increased extracellular lactate would reduce the gradient, preventing H^+ extrusion which may in turn lead to intracellular Ca^{2+} accumulation. A Na^+/H^+ exchange inhibitor diminishes the deleterious effects of increased extracellular lactate [239], presumably by attenuating the consequences of H^+ overload. Cross et al. [92], however, dispute this hypothesis, given that no change in pHi during ischaemia with increased extracellular lactate was found. The effects of lactate were attributed more to glycolytic inhibition, accounting for the reduced H^+ load, but also for its deleterious effects by reduced ATP production. Lactate may inhibit glycolysis at the level of GAPDH [362, 468], both directly and by a build-up of NADH (see appendix). As lactate accumulates, the conversion of pyruvate to lactate is inhibited. This also prevents the oxidation of NADH, which then accumulates [362]. The altered redox balance may affect many other enzyme reactions. Lactate in itself may harm cells by imposing an osmotic load, and possibly contributing to mitochondrial damage [225]. Lactate can leak into the extracellular space and affect the membrane, thereby shortening the action potential [342, 478] by affecting the K_{ATP} channel [249] or by increased Na^+ channel conductance [174] (see Ch II for further discussion on role of lactate).

Lactate may activate K_{ATP} channels [249], thereby shortening the action potential and predisposing to arrhythmias. However, in a myocyte model of anoxia and reoxygenation, Geisbuhler et al [154] reported no deleterious effect of high concentrations of lactate (10 mM and 50 mM) on anoxic myocytes. Damage was, however, found if pH was lowered. The role of lactate is unclear (see Ch I). Studies altering pre-ischaemic glycogen have found no correlation between end-ischaemic tissue lactate and functional recovery [163, 286]. In addition, the addition of 10 mM lactate together with 11 mM glucose on reperfusion improves functional recovery by 75% [163]. I have briefly attempted to clarify these issues by repeating previous work [92] and do not find that the addition of lactate with a reasonably high residual low flow (0.5 ml/g/min) confers any particular disadvantage (see Results 6).

iii) Sugar phosphates

Kusuoka and Marban [282] found increased diastolic dysfunction with glycolytic inhibition in normally perfused hearts. This effect was associated with increased sugar phosphates, which may impair Ca^{2+} homeostasis. Phosphomonoester resonance, which reflects the phosphorylated compounds including G6P, F6P, α-GP and AMP, increased during ischaemia and has been correlated with reduced recovery [219]. A similar predictive correlation was found by Schaefer et al. [483]. However, glycolytic ATP is linked to Ca^{2+} homeostasis by functional coupling with SR Ca^{2+} ATPase re-uptake [593]. A decreased Ca^{2+} would be expected to reduce diastolic impairment. The relative rates of sugar phosphate accumulation vs. ATP production may thus be crucial in determining the efficacy of glucose. Increased G6P production would also lower ATP levels, as this step (from glucose) consumes an ATP (see appendix). If glycolysis were inhibited, this would be detrimental as no replacement ATP would be provided [28].

The above factors may account for any detrimental effects of a high glucose concentration, and for some of the controversy surrounding glucose and glycogen (see Results).

c) Evidence contradicting benefit of glycolytic inhibition

i) Failure of glycogen depletion to provide benefit

Taegtmeyer et al. tried to replicate the findings of Neely and Grotyohann [387], with little success. Lagerstrom et al. [286] reported that a period of anoxia or substrate-free perfusion prior to 30 min total global ischaemia reduced tissue glycogen, but did not improve recovery of function on reperfusion; rather, function was significantly impaired. Interestingly, the anoxic hearts did not show a reduction in tissue lactate despite a 60% decrease in glycogen. This lactate could be accounted for by the residual glucose remaining in the tissue following cessation of flow. In substrate-free hearts with no residual glucose, lactate levels decreased substantially. A later publication by the same group [163] found similar results with a shorter period of ischaemia (15 min). There was no correlation of lactate

build-up with functional recovery, but a depletion of glycogen was detrimental. Postischaemic glycogen availability and functional recovery were well correlated.

A possible reason for discrepancies between results may be differences in the actual tissue glycogen levels due to differences in perfusion conditions and protocols. Reported values of normal tissue glycogen after a period of perfusion range from 40 [3, 286, 487] to 120 [93, 348, 387] μmol/g dry weight (8 - 24 μmol/g wet weight). However, there is no consistency in findings from different reports with the same pre-ischaemic tissue glycogen levels. For example, Neely et al. [387] found that glycogen depletion from a high level was beneficial. Cross et al. [93], with a similar pre-ischaemic high glycogen level and the same duration of ischaemia (albeit with a residual flow rate) found that recovery was impaired with reduced tissue glycogen. Alternatively, McElroy et al. [348] found that glycogen loading to values twice that of Neely et al. [387] was beneficial.

ii) Increased glycogen

Contrary to studies which suggest that glycogen depletion is beneficial, a number of reports show that glycogen loading can benefit hearts exposed to ischaemia. Oldfield et al. [398] noted that preoperative glucose-insulin-potassium administration increased tissue glycogen levels, and this was associated with a lower incidence of postoperative hypotension, reduced arrhythmias and fewer complications. Glycogen loading with insulin greatly improves the tolerance of isolated rabbit hearts to ischaemia, while glycogen depletion by epinephrine infusion severely impairs ischaemic tolerance [348]. Fasting, which increases glycogen content, also increases the resistance of hearts to an ischaemic episode [487]. Improved recovery of function, reduced membrane damage and reduced loss of adenine nucleotides were recorded in hearts from fasted animals. Vanoverschelde et al. [549] reported that increased glycogen by adding palmitate to the perfusate of isolated rabbit hearts significantly improved recovery of function while Doenst et al. [114] reported that glycogen loading and lactate perfusion mimicked the protective effects of preconditioning, improving tolerance to ischaemia.

A recent paper by Cross et al. [93] purports to explain the controversy over glycogen, suggesting that while glycogen is being utilised, the ATP provided supplies necessary energy. Reperfusion at this stage results in good recoveries. However, once the glycogen has been used up (in the absence of glucose), the accumulation of intracellular metabolites outweighs the advantages conveyed by ATP, with impaired functional recovery. Provision of a Na^+/H^+ exchange inhibitor on reperfusion after extended ischaemia resulted in improved recovery of function, suggesting that H^+ concentration was high following glycogen depletion [93]. This finding was reported in an ischaemic model of relatively "high" coronary flow (0.5 ml/min/g wet wt) in isolated rat hearts. In a model of ultra low flow ischaemia, the results may differ slightly due to differences in the relative rates of ATP production and metabolite accumulation.

iii) Glucose versus glycogen

Major concerns with studies purporting to show deleterious effects of glycolysis are 1) glycogen is confused with glucose as the source of glycolytic ATP and 2) most studies have been done using models of total global ischaemia, where glycolysis is limited by substrate and may also be inhibited by metabolite build up. Glucose is therefore not supplied throughout the totally ischaemic period. If glycogen loading is detrimental, this does not necessarily imply that increased glucose is detrimental; alternatively, if glycogen depletion is beneficial, glucose should not necessarily be removed. In addition, many of the early studies using brief periods of anoxia can now be said to mimic preconditioning [83, 290, 498, 603] (see Ch III), which may act by completely different pathways other than glycolytic involvement. I have attempted to correlate changes induced by preconditioning with changes in glycolysis and find no significant relationship (see results 4 and 5). Studies implicating a deleterious effect of glucose must be considered carefully in terms of the experimental model, and the source of glycolytic substrate. The hypothetical basis for the effects of the different sources of ATP lies partly in the functional compartmentation of ATP within the cell.

d) Changes in glucose concentration and coronary flow

High concentrations of glucose have been used in several different models. Runnman et al [470] found that in hypoxic hearts an increased glucose concentration lowered glycogen utilisation with no change in ATP production, and was associated with improved cardiac function. On the contrary, Owen et al [425] found that a high glucose concentration in a cardioplegic solution administered at intervals during the arrest period was detrimental to the hearts, also linked to inhibited glycogen breakdown. Reduced glycogen utilisation reduced overall ATP production from glycolysis. This effect was detrimental despite a reduced lactate production. Different mechanisms exist in hypoxia compared to the heart arrested and maintained with cardioplegia which would account for different responses to glycogen.

Apstein et al. [14] compared the effects of a high concentration of glucose (28 mM) with insulin (100 U/l) compared to a low glucose concentration (5.5 mM) without insulin, at two different low coronary flow rates (0.6 and 0.5 ml/g/min). The higher glucose concentration inhibited the development of contracture at both flow rates, but improved functional recovery on reperfusion was found only with a higher flow rate. Vanoverschelde et al. [550] also found that a high glucose concentration + insulin (in the presence of palmitate) abolished contracture (10 mM glucose + 140 mU/L insulin) compared to a lower glucose concentration (5 mM glucose + 70 mU/l insulin) at a low flow rate of 0.4-0.5 ml/g/min. Recovery of function was also significantly improved.

5) PROPOSED COMPARTMENTATION OF GLYCOLYTIC ATP

a) Compartmentation of ATP

Many cell components are compartmented - e.g. intracellular Ca^{2+} is highly compartmented in the myocyte, distributed among the SR, the Ca^{2+}-binding proteins and the cytosol. The adenine nucleotides including ATP are also strictly compartmented, as highlighted in ischaemia [153]. Functional compartmentation of the high energy phosphates, CP and ATP, was proposed by Gudbjarnason et al. [173], with CP used as a shuttle of ATP to the myofibrils. A reduction in CP inhibits the transfer of ATP. Local ATP regeneration near the SR Ca^{2+} ATPase pump in skeletal muscle [272] and maintenance of a local high ATP/ADP ratio at the myofibrils [558] show a preferential diversion of ATP to sites of utilisation. In liver cells, clear gradient of ATP have been shown, with nonuniform distribution of ATP-generating and ATP-consuming systems [19]. Substrates may also be compartmented, such that two intracellular "pools" of lactate may exist, with little interchange between these [71]. One pool may be tightly bound to macromolecules, while the other is free to diffuse out of the cell, or to exchange with pyruvate.

Ottaway and Mowbray [419], in an extensive review, provided evidence for the compartmentation of glycolysis, with "microenvironments" within the cell. A high degree of functional organisation of the glycolytic enzymes, particularly in muscle cells, would allow for efficient "channelling" of the substrate through its catabolic pathway [576]. Other studies have provided evidence of compartmentation of glycolytic ATP versus oxidative ATP in different cell types e.g. liver [19], vascular smooth muscle [427], myometrium [453]. A further separation can be made between ATP derived from glucose and from glycogen on the basis of cellular location [331, 332], and preferential oxidation of glycogen in the normoxic heart [161].

In myocardial ischaemia, it has been postulated that ATP derived from the anaerobic breakdown of glucose (glycolysis) is of particular benefit to the myocyte, in that the ATP is synthesised near the membrane, and thus can be utilised by the membrane processes, with maintenance of membrane integrity and ionic homeostasis [410]. In addition, ATP near the cell membrane may be preferentially involved in maintenance of the activity of the sarcolemmal pumps which help to maintain the normal ionic composition of the cell (see above). While compartmentation is difficult to prove, this concept explains many observations and has been invoked by a number of authors [49, 108, 424, 570, 571].

Lynch and Paul [332] suggested that glucose and glycogen metabolism form two functionally exclusive compartments in the cytoplasm of vascular smooth muscle. The pathways are coupled to the specific cellular processes which utilise the ATP. Oxidative phosphorylation and glycogen (which supplies substrate for the oxidative pathway) may supply ATP for contraction, while glycolysis (glucose) may

be required to maintain sarcolemmal pump function and membrane integrity [570]. Evidence to support this hypothesis in the isolated rat heart comes from the observation that glycogen is preferentially oxidised, compared to glucose, with glycogen mobilised following an increase in contraction [161]. The explanation for the functional distinction of ATP may be the physical location within the cell of the different substrates and enzymes.

b) Location of enzymes within the cell - basis for compartmentation theory

i) Oxidative phosphorylation

Oxidative phosphorylation occurs in the mitochondria, which are localised in the myocyte near the myofibrils [381]. The ATP produced in the mitochondria needs to be transported to the sites of utilisation. In the muscle cell, the myofibrils are the primary energy consumers. Transport of ATP to the myofibrils is carried out by the CK shuttle, which is required to maintain a local ratio of ATP/ADP around the myofibrils [173, 272, 558], thereby maintaining contraction. In ischaemia, CP levels fall and mitochondrial activity is inhibited. ATP becomes trapped in the mitochondria [153], which in turn become major sites of ATP utilisation as the mitochondrial ATP synthase reverses, and becomes an ATPase [466] (this mechanism is not thought to be important in the rat [465]). Inhibition of oxidative phosphorylation has a major depressant effect on tension [7, 116, 571], an effect slowed down by the presence of glucose. Inhibition of glycolysis with maintained oxidative phosphorylation has a much lesser effect on reduction in tension [571]. These findings and others [410] suggest that ATP from oxidative phosphorylation is used preferentially by the contractile apparatus. In vascular smooth muscle, the evidence is very convincing for compartmentation of glycolytic vs. oxidative ATP in terms of membrane function (glycolytic) and contraction (oxidative phosphorylation) [332, 427], findings which presumably can extend to the myocytes. Hasin et al. [182] showed a functional compartmentation of glycolysis (Na^+/K^+ ATPase function) and oxidative phosphorylation (contraction) in isolated cardiac myocytes during metabolic inhibition.

ii) Glucose breakdown

In vascular smooth muscle, an unexpectedly high proportion of glucose is converted to lactate under fully oxygenated conditions. Paul [427, 428] suggested that this was due to a preferential utilisation of glycolytic ATP by the Na^+/K^+ ATPase membrane pump. Following Na^+/K^+ ATPase inhibition by ouabain, glycolysis (in terms of lactate production) was reduced, with no change in force development. When Na^+/K^+ ATPase activity was stimulated, lactate production was increased [427]. While this mechanism requires a feedback regulation of ATP utilisation on glycolytic flux which is difficult to understand, the evidence for association of glycolysis with the membrane is fairly convincing. Paul et al. [429] found that the enzymes of the glycolytic cascade are associated with the

plasma membrane in smooth muscle, allowing isolated vesicles to produce ATP and maintain Ca^{2+} pump function. Pump function was blocked by iodoacetate.

It was initially thought that the enzymes and substrates of glycolysis were freely dispersed in the aqueous medium of the cytoplasm [419]. However, subsequent studies have shown specific localisation of the glycolytic enzymes within the cell [419]. The glycolytic enzymes, GAPDH and PK, have been shown to bind to sarcolemma and SR membranes, more so to the former [434]. The binding was attributed to attraction to charged phospholipids, which occur in greater amounts in sarcolemmal membranes. Further evidence for physical compartmentation comes from excised inside-out patches of sarcolemmal membrane. Addition of substrates for the ATP-producing steps of glycolysis (phophoglycerokinase and pyruvate kinase) block the K_{ATP} channel [571], by a localised production of ATP. This finding suggest that key glycolytic enzymes are located in or near the sarcolemma. Glycolytic enzymes are also functionally coupled to the SR, maintaining Ca^{2+} homeostasis [593]. The coupling of glycolytic enzymes to the sites of ATP utilisation, to allow efficient utilisation of glucose, substantiates the hypothesis of functional compartmentation.

Glycolytic ATP appears to be utilised preferentially by the membrane functions, on the basis of measurements of K^+ efflux. If glycolysis were inhibited in myocytes with maintained oxidative phosphorylation, a large efflux of K^+ was found, with little change in tension [571]. However, if oxidative phosphorylation were inhibited, tension was depressed, but K^+ efflux was much lower. While glycolytic ATP therefore appears to prevent K^+ efflux, this does not imply that glycolytic ATP cannot be distributed to the contractile apparatus. However, the means of ATP transport may be limited in conditions of metabolic impairment such as hypoxia and ischaemia. Glycolytic enzymes are not found near myofibrils in any great quantity [419], suggesting that glycolytic ATP is not synthesised in these regions, and thus is of less importance than oxidatively derived ATP for the contractile apparatus.

Higgins et al. [203] found that individual myocytes incubated with phospholipase C were more resistant to enzyme loss if glucose were present. Glucose may inhibit membrane degradation, possibly by increasing the degree of phosphorylation of the phospholipids, thought to be important in maintaining membrane stability. Energy is also required for membrane repair. Extending this finding to normoxic isolated rat hearts, iodoacetate increased enzyme release, while cyanide had no effect [202].

iii) Glycogen

Glycogen particles are located along the mitochondrial columns between myofibrils, and are also present in the perinuclear sarcoplasm and the SR including the sub-sarcolemmal cisterns [381]. The enzymes for glycogen breakdown (phosphorylases etc.) have been found near the sarcoplasmic reticulum in cardiac muscle [128]. Two forms of glycogen are found, proglycogen, and macromolecular

glycogen (see appendix). The former constitutes about 50% glucose moieties, with the rest consisting of glycogen enzymes. This form is thought to be the precursor of macromolecular glycogen [322]. Classic macromolecular glycogen particles also contain enzymes for glycogen synthesis and breakdown [506], including all the glycolytic enzymes. Thus the glycogen-enzyme complexes form "metabolons" [506] for the efficient synthesis and breakdown of glycogen

The substrate supply from glycogen is co-ordinated with mitochondrial substrate utilisation whereas glucose utilisation correlates with Na^+/K^+ ATPase activity in smooth muscle cells [427]. This arrangement would maximise the efficiency and response time of energy transduction within the smooth muscle cell. In the myocyte, the distances are greater because the cell is much larger, and the demands on energy are also increased with the much greater contractile component of the cell. Thus compartmentation within the myocyte is plausible given the requirement for sustained efficiency of contraction. In hepatocytes, Aw et al. [19] also suggest that there are 2 pools of ATP - one cytosolic, and one near the membrane, on the basis of ATP utilisation by membrane (Na^+/K^+ ATPase) and cytosolic (ATP-sulfurylase) enzymes. Differences in the mechanisms of ATP depletion show that the different elements are not exposed to the same average overall ATP level. Intracellular inhomogeneities of ATP can be attributed to different rates of ATP-producing and ATP-consuming sites, such that ATP produced in large amounts from the mitochondria supply the cytosolic elements. These elements reduce the amount of ATP at the membrane because of rapid utilisation of ATP diffusing from the mitochondria, such that at further distances the ATP is depleted. Thus mitochondrial ATP utilisation by the membrane components appears to be limited by diffusion and steepness of gradients within the cell. A localisation of energy-producing pathways near energy consuming pathways thereby ensures efficient supply and utilisation of energy. Paul et al [429] suggest that glycogenolysis is linked to the contractile and cytosolic elements in vascular smooth muscles, rather than membrane functions. In conditions when ATP supply is plentiful, the different systems may compensate for each other. However, if energy supply is limited, the source of energy is crucial depending on the relative importance of the various cell components. In ischaemia, the different effects of glycogen-derived, glucose-derived and oxidative ATP on contracture and on functional recovery observed in the present studies may be explained by these differences.

6) SUMMARY

Glucose has many benefits for the ischaemic heart, including maintenance of membrane activity, inhibition of contracture, reduced arrhythmias, and improved functional recovery. However, the possible deleterious effects associated with increased glycolysis cannot be ignored, and may explain some of the controversial findings reported in the literature. In addition, glucose must be distinguished from glycogen on the basis of the effects of modified pre-ischaemic glycogen levels vs. changes in

glucose concentration on subsequent ischaemic tolerance. These differences may be explained by functional compartmentation within the cell.

Many of the studies above support the hypothesis of the importance of an optimal balance between glucose delivery and removal of metabolites as a crucial determinant of ischaemic and reperfusion damage. While at moderate low coronary flow rates, the benefit of glycolysis appear extensive, the controversy arises at very low flow rates, or in the absence of flow; and when glycolytic substrate may be provided in excess. There is good evidence that glycolytic substrate can be in excess during ischaemia, which would prove deleterious. Under conditions of total global ischaemia, an excess accumulation of lactate, H^+, NADH, sugar phosphates and Pi may indeed prove detrimental, by a variety of mechanisms. However, an optimal balance between the rate of ATP production and rate of accumulation of metabolites (determined by the glycolytic rate, and removal of metabolites by the rate of washout), may ensure optimal recovery. I wished to test this hypothesis under conditions of ultra low flow ischaemia, where the balance between the rates of glycolytic ATP production and metabolite accumulation may be especially crucial in determining the tolerance of the myocardium to ischaemia.

III. PRECONDITIONING

1) WHAT IS PRECONDITIONING?

a) Introduction

Preconditioning is defined as one or more brief periods of ischaemia and reperfusion (preconditioning period) which protect against the effects of a subsequent sustained ischaemic episode. Manipulations which mimic preconditioning i.e. improved tolerance of ischaemia, have also been defined as preconditioning. The strongest, and most reproducible, result of preconditioning is a reduction in infarct size.

The phenomenon of preconditioning, or so-called "endogenous" protection, was first described by the group of Reimer, Murry and Jennings [457], where contrary to expectations, repeated brief episodes of ischaemia in the dog did not further deplete the tissue of ATP, and did not cause necrosis. This followed on from earlier work showing inhibited necrosis with repeated ischaemic events despite an overall occlusion time of 200 min [152]. The protective effects of four 5-min episodes of ischaemia (I), with intervening 5-min periods of reperfusion (R) were then tested on coronary occlusion sustained for 40 min or 3 hrs [375]. The degree of necrosis associated with the shorter, but not with the longer period of sustained ischaemia, was attenuated. While ATP is depleted by the initial ischaemic period [378], the subsequent rate of fall in ATP is slower. Two general mechanisms whereby preconditioning could afford protection were mooted: slowed ATP depletion, and limited catabolite accumulation during sustained ischaemia [375]. Reduced glycolysis following glycogen depletion by a preceding period of anoxia is beneficial, an effect attributed to reduced accumulation of end products [387]. Our hypothesis focuses on the possible role played by a reduction in glycolysis brought about as a result of glycogen depletion following preconditioning. In addition, the role of glycolysis from glucose was investigated (see Results 4 and 5).

These early studies were followed by a burst of publications particularly from 1991 to the present. Preconditioning has been shown to protect against many of the consequences of ischaemia. Many different hypotheses of the mechanism of preconditioning have been proposed, several of which revolve around the initial observation of preserved energy supply. A number of possible "triggers" have been investigated, including stunning, free radicals, PKC activation, cAMP, adenosine, K_{ATP} channel activity, and glycogen depletion (see Ch I for background). Many of these are interrelated, and in order to put our particular hypothesis in context, a full understanding of possible mechanisms, and interrelated events is required.

Despite all this work on preconditioning, there is still some controversy over the degree and reproducibility of protection, and the mechanisms involved. While reduction in infarct size appears to

be a consistent result, some other end points do not show the same degree of reliability. Sufficient evidence from a number of researchers suggests that the effects of preconditioning on these alternate end points are not always certain. However, the interdependent relationship between the different end points (as discussed below) should imply that if one shows a difference, the others should also be affected. A clear understanding of the mechanism of protection for each end point is required. In addition, the equivocal findings with regard to the mechanism of preconditioning suggests that either the phenomenon is not as remarkable as has been suggested, or else that the experimental approach has often been inappropriate. The choice of end point, an appropriate protocol, and interpretation of the evidence must be correct. These issues are analysed in depth, with particular reference to the proposed role of glycolysis, ATP and glycolytic metabolites in the mechanisms associated with preconditioning. A figure (Fig III.1) is included to illustrate different terms and concepts discussed below.

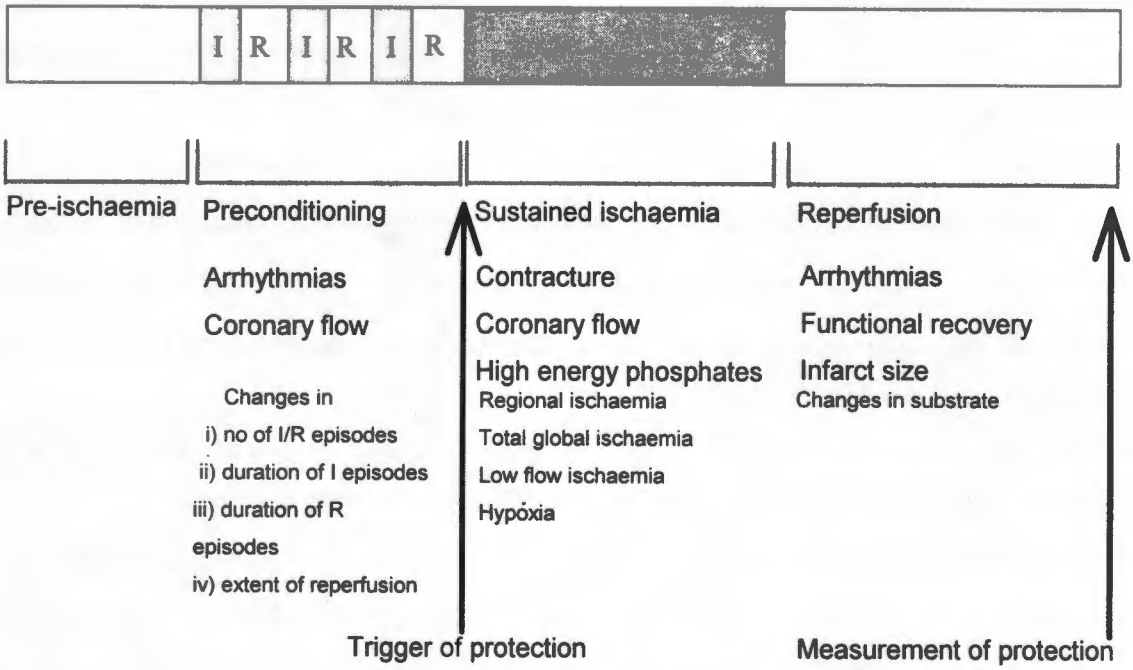

Fig III.1. Diagram of preconditioning showing points of measurement of indices of protection, changes in protocols of preconditioning, and changes in sustained, or "test" ischaemia

b) Protection conferred by preconditioning

i) Infarct size

The most significant and reproducible effect of preconditioning is a reduction in infarct size after a period of reperfusion, the main predictors of which are the size of the area at risk, the collateral blood flow, and myocardial oxygen consumption [194, 485].

Preconditioned dog hearts (4x5 min I + 5 min R prior to 40 min sustained occlusion followed by reperfusion) have a 75% reduction in infarct size compared to control animals [375]. Most other studies

with preconditioning have shown a reduction in infarct size, in dogs [169, 172, 421], pigs [269], rabbits [82, 318] and rats [220, 313, 319, 504, 588, 598], with coronary occlusion models or with total global ischaemia.

ii) Ischaemic contracture and diastolic dysfunction on reperfusion

Despite improved infarct size and functional recovery (see below), preconditioning paradoxically appears to increase contracture in the majority of cases. Attenuated time to onset of ischaemic contracture in an isolated perfused heart following preconditioning was first described by Asimakis et al., with one or two episodes of preconditioning (5 min I + 5 min R) [18]. While both preconditioning protocols reduced time to onset of contracture, two periods of preconditioning had the greater effect. Peak contracture during ischaemia was also increased with increased preconditioning. Both groups however, recovered function to a similar degree on reperfusion, which was better than in control hearts (see also [66]). Preconditioning with either 5 min I or 5 min hypoxia (hypoxic preconditioning - see below) also reduced time to onset of contracture, but reduced diastolic pressure on reperfusion [290], possibly reflecting improved Ca^{2+} handling on reperfusion [528].

In contrast, Steenbergen et al. found that with an increased number of cycles (4x5 min I + 5 min R), the time to onset of contracture was not significantly affected by preconditioning, and in fact slightly delayed [512]. These results have not always been reproduced, in that using a similar protocol resulted in a significant reduction in time to onset of contracture in preconditioned rat hearts [171], with improved functional recovery.

Contracture and functional recovery are thus not well-correlated in preconditioned hearts, and highlight the problem of using contracture as an index of ischaemic injury in all cases.

The effects of preconditioning on the precipitators of contracture are contradictory. Preconditioning tends to reduce both ATP and intracellular Ca^{2+}, which have opposing effects on contracture [512]. While preconditioning is thought to attenuate high energy phosphate utilisation [224], an increased ATP depletion in early ischaemia (<20 min) has been found in preconditioned rat hearts, and correlated with accelerated contracture [266]. Provision of glycolytically-derived ATP is thought to be especially crucial in delaying the onset of contracture [424] and improving recovery, and yet glycolysis is usually attenuated in preconditioned hearts [136, 224], largely as a result of glycogen depletion [588] or slowing in the rate of glycogenolysis [574]. In preconditioned rabbit hearts exposed to sustained low flow ischaemia (10% of normal - 0.4 ml/min/g wet wt), however, contracture is reduced, followed by improved recovery of function [218]. During the low flow ischaemia, however, preconditioning increased glucose uptake and ATP production, which could explain the lessened contracture [424]. Thus precipitation of contracture by preconditioning is consistent with previous findings of the level

(or rate of production) of ATP as a determining factor in contracture [189, 271] (see Ch I). Reduced intracellular Ca^{2+} overload found in preconditioned myocardium [512] is inconsistent with increased peak contracture [189]; however, the decreased Ca^{2+} is only evident after 15 min ischaemia, at which time contracture has usually occurred [512].

The question remains as to how the heart is protected despite an increase in contracture with preconditioning. The answer may lie partially in the degree of contracture which the heart can tolerate. Increased contracture (within certain limits) may be well tolerated by improved protection with preconditioning. If the contracture precipitated by preconditioning were excessive, this could be deleterious. I also wished to confirm the role of glycolysis in preconditioning, and the effects of increased glucose uptake by preconditioning on ischaemic contracture and functional recovery.

iii)Functional recovery and stunning - an index of protection

There is little evidence to suggest that preconditioning improves functional recovery after the sustained ischaemic period in large animals. The early studies of preconditioning, in the dog, did not record recovery of myocardial function [224, 375, 377, 457]. In a subsequent study, also in the dog, no difference in functional recovery between control and PC hearts was found, after 15 min sustained ischaemia and 3 hrs reperfusion, despite marked protection against infarction [421]. The short ischaemic period was chosen to induce stunning, against which preconditioning was not effective. All hearts with an ischaemic subendocardial blood flow of more than 0.2 ml/min/g were excluded from the study (as in the Murry, Reimer and Jennings studies) (normal *in vivo* flow rates - 1-1.6 ml/min/g). Preconditioning with a longer period of ischaemia which would induce necrosis (4x3 min occlusion + 5 min R, followed by 60 min occlusion and subsequent reperfusion), showed a similar lack of protection against stunning and/or reversible injury with improved functional recovery, despite marked reduction in infarct size in dogs [420]. Ischaemic preconditioning also does not improve systolic shortening on reperfusion in dogs [498]. Preconditioning in the pig heart, which has a low collateral flow during ischaemia, also showed no difference in segment shortening between control and preconditioned hearts [360], although CP levels were improved in ischaemia and reperfusion, and recovery of ATP was better. pHi was also higher in preconditioned hearts during ischaemia.

The only evidence of protection against stunning in dog models is from a model using repeated brief episodes of ischaemia and reperfusion, showing an attenuated decline in function with succeeding episodes [1, 81]. These findings do not, however, necessarily extend to a "true" preconditioning protocol, with improved mechanical recovery following a sustained ischaemic episode.

In smaller animals, improved functional recovery is commonly used as an index of protection with preconditioning. In the isolated rabbit heart, improved developed tension has been found on reperfusion after 5 min I + 5 min R followed by 60 min total global ischaemia, increasing from 16%

in controls to 74% with preconditioning [400]. Improved wall motion on reperfusion after sustained coronary occlusion has been observed in preconditioned hearts, with a significant improvement in segment shortening [82]. End-diastolic pressure, or "diastolic stiffness" (change in end diastolic pressure with incremental changes in intraventricular balloon volume) is also attenuated following reperfusion in the preconditioned rabbit heart [368]. However, several reports testing preconditioning effects on ischaemia induced by coronary occlusion [358, 532] or with total global ischaemia [447, 479] have found no improvement in functional recovery on reperfusion in rabbits.

Cave and Hearse purported to be the first to examine the role of preconditioning in an isolated globally ischaemic rat heart. Using one cycle of 5 min I + 5 min R, an improved cardiac output during recovery from varying periods of sustained ischaemia (10-25 min) was observed [65]. A significant difference between control and preconditioned hearts was observed when these were subjected to 15 min or more sustained ischaemia. The "half-life" of the curve relating reduction in function vs. time of ischaemia, was delayed by preconditioning, from 15.6 min to 22 min. These findings were corroborated by Zhai et al. [603], also in an isolated working rat heart. Asimakis et al. also reported improved functional recovery following preconditioning with one or two cycles, in an isolated Langendorff-perfused rat heart with a left ventricular balloon [18]. However, two reports [97, 577] did not find improved recovery with only one or two cycles of preconditioning in the rat. At least 4 cycles were required for protection against postischaemic mechanical dysfunction. In general, in larger animals preconditioning does not appear to improve functional recovery; in rabbits, the results are equivocal; and in rats, the tendency has generally been for improved recovery of function.

iv) Functional recovery vs. infarct size

An important question is whether a reduction in infarct size explains improved functional recovery in preconditioned rat hearts. These variables were correlated in isolated rat hearts, with changes in the duration of sustained ischaemia following preconditioning. Recovery was directly correlated with infarct size, suggesting that the improved function is due to reduced infarct size, and not to reduced stunning [220]. Thus if either functional recovery or infarct size are affected, the other index should be likewise altered. This report does not, however, offer conclusive evidence that preconditioning does not protect against stunning, but is consistent with observations on infarct size protection by preconditioning. A working hypothesis is that preconditioning delays the onset of necrosis and thus irreversible injury, rather than the short term effects of stunning, which do not involve the mechanisms of necrosis, but rather have a transient effect at the level of the myofilament (see Ch I). However, results with shorter periods of ischaemia in rats (15 min [65]) or measurement of segment shortening in rabbits [82] suggest that stunning may be partially attenuated by preconditioning. (Stunning has also been proposed as one of the mechanisms triggering preconditioning - see below).

v) Changes on reperfusion in preconditioned hearts

Some investigators have looked at the changes on reperfusion in preconditioned versus control hearts. A lesser fall in pHi was recorded in preconditioned myocardium following sustained ischaemia, together with a higher coronary flow [98]. A higher coronary flow on reperfusion was also reported following total global ischaemia with preconditioning in the rat heart [18, 237].

Substitution of pyruvate for glucose upon reperfusion after sustained ischaemia eliminates any beneficial effects of preconditioning on functional recovery [400], despite the benefits usually associated with pyruvate on reperfusion (see Ch I and II). Pyruvate given in addition to glucose on reperfusion also attenuates the effect of preconditioning on functional recovery, Ca^{2+} accumulation and pH [140]. This points to a beneficial effect of glycolysis on reperfusion of preconditioned hearts. If preconditioned hearts were reperfused after sustained ischaemia with lactate + glucose, no effect was found. However, if preconditioned hearts were reperfused with pyruvate or 2-deoxyglucose + acetate, high energy phosphate levels were lower and functional recovery was impaired [142]. Glycolytic substrates may therefore be better on reperfusion in preconditioned hearts, although restoration of mitochondrial function appears essential. Glucose provision could be a stop-gap measure, prior to the delayed recovery of mitochondrial function following ischaemia (see above for controversy over pyruvate on reperfusion). However, in contrast Finegan et al. found that glycolysis (in the presence of palmitate) was inhibited by preconditioning on reperfusion after sustained ischaemia, although glucose oxidation was unchanged [136]. The reduced glycolysis with preconditioning was associated with a reduced H^+ accumulation, which may be beneficial by reducing Ca^{2+} influx. The effects of different substrates on reperfusion in preconditioned hearts are a point of controversy, and difficult to understand.

vi) Ventricular arrhythmias

Following 5 min ischaemia with increasing durations of reperfusion in rat hearts *in vivo*, the incidence of postischaemic ventricular arrhythmias was recorded after a second episode of 5 min regional ischaemia [495]. With intervening periods of 10 to 20 min, the incidence of ventricular arrhythmias after the second ischaemic period was very low. Although an increased duration of reperfusion increased the incidence of arrhythmias after the second ischaemic period, a substantial antiarrhythmic effect was still evident. The incidence of arrhythmias is also reduced with increased numbers of ischaemic episodes [418].

Preconditioning also substantially reduces the arrhythmias during the ischaemic period in rat models of regional ischaemia [299, 313, 319, 504] and in the dog [555]. One study reports an increased incidence of arrhythmias in the preconditioning reperfusion period (following 5 min coronary occlusion), but not at any later stage [598]. This effect was surmised to be due to the duration of ischaemia associated

with vulnerability to arrhythmogenesis [27]. A "dose" dependency of protection against arrhythmias has been found in the blood-perfused rat heart, with reduced arrhythmias with increased episodes of preconditioning [299]. The mechanism of reduced arrhythmias may be related to reduced cAMP found in preconditioned hearts [479] (see below).

vii) Hypoxic injury and low residual flow

The effect of 5 min I + 5 min R was tested on 30 min total global ischaemia, 25 min substrate free hypoxia, or 60 or 90 min hypoxia with substrate [66]. No protection by preconditioning against hypoxia-induced injury was found, with functional recovery as the index of protection. During hypoxia, any accumulated metabolite possibly responsible for the preconditioning effect may be washed out, accounting for the absence of protection. However, Janier et al. did find a small but significant improvement in functional recovery in rabbit hearts preconditioned with 3 min total global I + 12 min R, followed by 60 min global ischaemia with a low flow of 0.4-0.45 ml/g wet wt/min [218]. These results must be dissociated from the protection which can be induced by hypoxic preconditioning (discussed below), as opposed to the protection afforded by preconditioning against a sustained hypoxic period.

Infarct size and collateral flow during sustained ischaemia have been correlated, with a significant difference between control and PC hearts with collateral flows less than 0.07 ml/min/g [310], as previously described [375]. At higher flow rates, the results for the two groups converged [310]. These results were taken to mean that factors other than collateral flow were responsible for protecting the myocardium, but also imply that at higher coronary flows the preconditioning effect is not apparent. In addition, the observation that preconditioning can occur in pigs, which have very little collateral flow, and in isolated rat hearts subjected to total global ischaemia, suggest that improved collateral flow in not a determinant of protection in a preconditioned heart [313, 491]. However, as described above, whether protection is apparent when collateral flow is improved is not clear.

The discrepancies in these results is unexplained, and is one of the points investigated indirectly in this thesis.

c) Protocols of preconditioning

i) Number and duration of ischaemic episodes

The original "preconditioning" studies [375, 378] used a protocol of 4 cycles of 5 min ischaemia + 5 min reperfusion, based on findings from previous studies [152, 457]. The importance of the number and duration of preconditioning cycles has since been tested. One episode of ischaemia versus 6 or 12 cycles of 5 min duration were compared prior to 60 min sustained occlusion in dogs [310]. No differences in haemodynamic parameters was noted on reperfusion among the three groups. Infarct

size was significantly reduced in all protocols of preconditioning, but with no difference among the three groups. 0, 1, 2 and 4 cycles of 5 min I + 5 min R were compared in rabbits prior to 30 min ischaemia [359]. Repetitive episodes were no more advantageous than a single episode in terms of infarct limitation. In addition, no difference in infarct size limitation among 2x2-min I, 1x5-min I or 2x5-min I was found in rabbits [358]. Isolated rat hearts subjected to either one or two cycles of 5 min I + 5 min R, followed by 40 min sustained ischaemia showed significantly improved function compared to controls, but with no difference between the two groups [18]. 2.5 min I (no stunning) compared to 5 min I (with stunning) followed by 5 min R was equally efficacious against infarct development in the dog heart [423].

On the contrary, rats exposed to 3x2 min total global ischaemia + 10 min R vs. 3x5 min I + 10 min R [561] show a proportional increase in recovery of function after 20 min ischaemia with increased duration of preconditioning ischaemia. Ischaemic periods of 1,2 and 4 min followed by 10 min R prior to 18 min sustained total global ischaemia in an isolated rat heart show a gradual increase in the degree of protection afforded in terms of functional recovery, related to the duration of preconditioning ischaemia [486]. A pronounced reduction in infarct size with 3 cycles (5 min I + 5 min R) but not with a single episode of preconditioning prior to 30 min coronary occlusion was found in the *in situ* rat heart [319]. 1 and 4 cycles of 5 min I + 5 min R both showed protection in terms of reperfusion-induced arrhythmias after 30 min global ischaemia, but this was significant only with 4 cycles of preconditioning [538].

In general, functional recovery after short periods of ischaemia (stunning) seems to be better with increased preconditioning ischaemia duration, made up of an increased number of episodes. Protection against infarct development seems less sensitive to the preconditioning protocol. With long term ischaemia (and development of infarction) increased preconditioning ischaemia does not seem necessary because the recovery is determined more by infarct size [220]. This relationship is tenuous, and dependent on many contributory factors, but may partially explain the lack of reproducibility between protocols. I have chosen a protocol of 5 min ischaemia + 5 min reperfusion, as this resulted in a significant improvement of functional recovery on reperfusion after 30 min sustained total global ischaemia in the isolated rat heart.

ii) Duration of reperfusion between intermittent ischaemic episodes and prior to sustained ischaemia

The duration of reperfusion between the intermittent episodes of preconditioning ischaemia, determines the extent of metabolite washout, substrate restoration, and return of function prior to sustained ischaemia. Murry et al. suggested that an extended reperfusion between successive episodes of ischaemia would gradually reduce the effectiveness of preconditioning [376]. However, the time

course of decay in protection may be largely species-dependent, as well as determined by the protocol and model used.

Protection against infarction is similar with 1 min and 10 min reperfusion, but is lost when only 30 secs reperfusion is used [2]. Protection conferred by 5 min I prior to 30 min ischaemia in rabbit hearts is lost if reperfusion is longer than 15 min [359]. If reperfusion is extended to 1 hr between preconditioning and sustained ischaemia [313], any benefit associated with preconditioning in rats is lost. Similar results were reported by Wolfe et al, who correlated the loss of protection with restoration of glycogen levels [588]. In dogs, 2 hrs reperfusion prior to occlusion attenuated the effect of preconditioning on infarct size, although there was still some slight protection compared to control animals [377]. In an open-chest pig model, 10 min occlusion followed by 15, 60, 120 180 or 240 min reperfusion prior to 60 min occlusion showed no gradual decline in protection, but rather an "all-or-none" response, the incidence of which did decline with increased duration of intervening reperfusion [269]. Repeat preconditioning after 1 hr has similar effects to preconditioning followed immediately by occlusion [312], and can be regarded as a separate event.

A so-called "second window of protection" has been demonstrated following preconditioning, whereby dog hearts are shown to tolerate ischaemia better after 24 h than after 3 h or 12 h reperfusion [285]. This protection was comparable to that found with immediate (5 min R) re-occlusion, but is attributed to different mechanisms. Stimulation by the initial ischaemic episode may lead to DNA transcription or RNA translation of heat shock and other stress proteins, which are protective to the heart over the long term [599].

iii) Restoration of flow in intermittent reperfusion periods

Most models have complete restoration of blood flow following the preconditioning ischaemic period. Collateral flow in the 10-min reperfusion period after a single 5 min ischaemic episode was well correlated with functional recovery after sustained ischaemia in dogs [81]. However, this relationship was reduced with increasing numbers of cycles. Partial stenosis (50% reduction in flow) followed by 60 min total coronary occlusion did not confer protection compared to control dogs, but if an intervening restoration of flow was present, a significant protection against infarction was seen [422]. No difference in stunning was observed. This finding suggests that full restoration of blood flow is required after an ischaemic period to precondition hearts, but that the preconditioning ischaemia itself need not be severe (see below for different mechanisms of mimicking preconditioning protection).

2) POSSIBLE MECHANISMS OF PROTECTION BY PRECONDITIONING

a) Introduction

The possible mechanisms involved in preconditioning suggested to date include: 1) changes in myocardial blood flow; 2) energy sparing effects - depressed contractility (stunning), maintained ATP levels, improved glycogen synthesis; 3) limitation of acidosis; 4) oxygen free radicals; 5) modified release of endogenous myocardial substances (adenosine, nitric oxide, catecholamines etc.) which affect phospholipase C, G proteins, protein kinase C and protein phosphorylation; 6) reduced release of possibly injurious substances; 7) K_{ATP} channel activation; 8) induction of protective proteins or enzymes [426].

The search for the "mechanism" of preconditioning can be divided into several components, namely:

1) induction of endogenous protective mechanisms by the initial ischaemic period(s). The duration of the preconditioning ischaemic episodes should not be too long, otherwise irreversible damage may occur, and the natural protective mechanisms triggered may be overwhelmed by adverse changes. The ischaemia needs to be relieved by reperfusion.

2) washout of substances in reperfusion/upregulation of mechanisms stimulated by ischaemia with the reperfusion stimulus. The number of ischaemia/reperfusion cycles may determine the effectiveness of "reperfusion" stimuli, either by relieving the heart of the burden of ischaemia - giving it a rest before the next onslaught and thereby allowing increased duration of exposure to ischaemia; or by providing a stimulus in itself, of a rapid re-infusion of blood/perfusate, which can switch on mechanisms, or by flushing out harmful metabolites.

(The above components may explain why the different preconditioning protocols have different effects, but it is difficult to differentiate between these factors)

3) onset of sustained ischaemia, where after a reasonable length of time (greater than 10-15 min ischaemia) mechanisms induced by preconditioning exert their effects i.e. more long term consequences of changes brought about by prior ischaemia/reperfusion. These changes could be initiated in the previous episodes, and then over time the myocardium would adapt to ischaemia, with deferment of necrosis. A delay in the onset of necrosis would ensure improved recovery of function if the ischaemia were of sufficiently short duration (less than 3 hrs [375]) i.e. the extent of irreversible injury is not complete (see Ch I).

In seeking the mechanism of preconditioning, certain criteria need to be fulfilled, and a careful distinction between changes involved in preconditioning, and those attributed to other effects must be made:

1) the mechanism should be apparent in all species in which preconditioning works (while there may be multiple mechanisms, several of each working together in different models and species, the ultimate aim is one single effector)

2) when mimicking the effects of preconditioning, a careful analysis of the mechanisms involved is required to ensure that the many interventions (see below for those which have been said to mimic preconditioning, but which can include any modification made prior to ischaemia and found to be protective) found to be protective when given prior to ischaemia which may result in similar changes in the end-point measured, are not confused with the "endogenous" mechanism of preconditioning

3) a distinction between cause and effect must be made - while many factors appear to be altered in the preconditioned heart, these may not be at all involved in the mechanism of preconditioning, but may occur simply as side effects. While these side effects may in turn contribute to the beneficial effects of preconditioning, this does not necessarily mean that these are the mechanisms. In addition, many changes can result in a single convergent point e.g. many cellular changes affect pHi. Alternatively, a change in pHi can affect many cellular processes. Thus, is reduced acidosis a protective mechanism in preconditioning, or a consequence of many of the changes induced by preconditioning, but not required for protection? Many of the observed changes with preconditioning appear to contradict some of the accepted concepts of ischaemic injury, particularly the controversy over glycolysis, glucose and glycogen. Whether these changes are involved in the protective effects, or are simply outweighed by the benefits of preconditioning, is largely unresolved in the majority of cases.

4) the choice of end-point is crucial in the study of preconditioning. The changes in an ischaemic heart, while closely interacting, are also distinct from each other. Consistent protection against a given index does not extend to other indices. The question then to be asked is whether preconditioning protection against one index e.g. infarct size, is worthwhile in the absence of protection against another index e.g. functional recovery. The mechanisms involved in protection for the different indices may be very different, and may only related as cause and effect i.e. infarct size may partially determine the extent of functional recovery.

The majority of the hypotheses presented below are those of the authors of the respective manuscripts, and do not necessarily reflect the consensus opinion. Some of these concepts are difficult to resolve with other findings. The lack of consistency contributes to the confusion regarding preconditioning. While an attempt has been made to review the subject as thoroughly as possible, so many manuscripts are published and it is impossible to mention every one. The more important aspects of each topic discussed have hopefully been covered.

b) Mechanisms other than ischaemia resulting in or mimicking "preconditioning"

The mechanism of preconditioning can be studied by mimicking the protection conferred by preconditioning. Several manipulations have been tested, using mainly infarct size and functional recovery as indices of protection. The validity of comparing these manipulations with preconditioning is not, however, always justified - any manipulation prior to ischaemia which improves the end points

can be regarded as mimicking preconditioning and therefore pointing to the mechanism of preconditioning. A major point in this regard is the study by Neely and Grotyohann [387] which purported to show the deleterious effects of glycolysis in total global ischaemia (see Ch II). The protocol used in this study has subsequently been used to initiate preconditioning (see below). Thus the conclusions of the original study i.e. that glycolysis is detrimental, are not necessarily valid given that pre-ischaemic hypoxia may induce preconditioning by completely different mechanisms, and the effects on glycolysis may be coincidental. A positive balance between the detriment brought about by glycolytic inhibition versus the benefit conferred by other simultaneously active preconditioning mechanisms may lead to improved recovery. Alternatively, "hypoxic" preconditioning may act simply by depleting glycogen, and may not be "true" preconditioning. While glycolytic inhibition is one of the major proposals for the mechanism of preconditioning, there is also a large body of evidence to contradict this possibility. Under conditions when "preconditioning" is not present, the inhibition of glycolysis would normally be considered detrimental.

i) Anoxia, hypoxia and underperfusion

Neely and Grotyohann [387] found that a pre-ischaemic period of anoxia conferred protection to the heart in terms of functional recovery. Four different protocols were used - control, 10 or 15 min anoxia, and 10 min anoxia + 10 min reoxygenation, prior to 30 min total global ischaemia in the rat. The recoveries (% rate-pressure product) were 28%, 68%, 92% and 75% respectively. Similar protocols (with preconditioning under investigation), with 5 min hypoxia + 5 min R, conferred protection in *in vivo* dog hearts [498], or isolated rat [290, 603] and rabbit hearts [83]. In dogs, this protocol was more effective than 5 min I + 5 min R, with improved functional recovery. Reoxygenation after the initial hypoxic period is not required for protection [567], contrary to the need for reperfusion after an ischaemic period (see above).

However, there is some controversy over these findings. In particular, Lagerstrom et al. [286] and Goodwin et al. [163] were not able to replicate the findings of Neely and Grotyohann [387] using similar protocols and measuring functional recovery. The main controversy arises from the debate about the role of glycogen (see previous discussion, Ch. II). Thus the involvement of glycogen in preconditioning needs to be addressed (see below for further discussion).

Dog hearts can be preconditioned with cyclical variations in flow with maintained stenosis [422], and with a 15 min period of partial stenosis, with a 50% reduction in blood supply [422]. The oxygen balance is disturbed by these manipulations. However, while significant protection against infarct size was found, none of these protocols were able to reduce stunning. In the rat heart, the effects of preconditioning by hypoxia, low flow ischaemia (1.6 ml/min/g) or total ischaemia, each of 5 min duration, and with 5 min reperfusion, were compared [603]. Equivalent protection against reperfusion

injury, in terms of functional recovery, was found in all three preconditioned groups. Isolated myocytes subjected to short periods of hypoxia, and a subsequent sustained hypoxic period followed by reoxygenation, showed reduced cell damage [569]. These studies suggest that the accumulation of catabolites is not required for preconditioning, but rather that the lack of oxygen triggers the protective mechanism, and that accumulated metabolites cannot be the mechanism responsible. However, Cohen et al. suggest that hypoxia preconditions rabbit myocardium by adenosine and catecholamine release during the hypoxic period, as these are present in sufficient amounts to account for preconditioning [83]. Blockade of both these effects abolished preconditioning.

ii) Pre-ischaemic changes in metabolism

While hypoxia can induce preconditioning, the specific component of hypoxia involved has not been elucidated. Several authors [364, 569, 603] suggest that oxygen deprivation and not catabolite accumulation triggers preconditioning, but the effect of inhibition of mitochondrial activity was not investigated directly. Cyanide preconditioning (2x5 min infusion, followed by 10 min washout) was compared to ischaemia-preconditioned and control hearts [99]. Over 30 min global ischaemia, cyanide hearts showed the highest end-diastolic pressure, but this was lowest on reperfusion. Developed pressure was highest with cyanide hearts, while control hearts had very little recovery. Recovery was correlated with improved CP and ATP, and with less acidosis. Interventions to lower pHi during ischaemia significantly attenuated the effect of preconditioning, both with ischaemia and cyanide. The benefits of preconditioning were thus attributed largely to an attenuated fall in pHi, and not to mitochondrial activity *per se*.

A recent publication reports that a transient inhibition of glucose uptake mimics the effect of preconditioning on infarct size [166]. 15 min glucose-free perfusion followed by 30 min with glucose present prior to ischaemia, significantly reduced infarct size, an effect which could be blocked by a PKC inhibitor (see role of PKC below). Transient addition of 20 mM pyruvate or 1 mM acetate (both presumably inhibiting glucose uptake) to glucose buffer prior to ischaemia also decreased ultimate infarct size. Glycolytic inhibition was proposed to open the K_{ATP} channel, which could subsequently trigger PKC activation. If acetate were present throughout, no protective effect was evident, suggesting that a substantial basal rate of glycolysis is required to enable protection to be evident. This concept is unexplained. In *in situ* hearts, pre-treatment with pyruvate did not precondition the hearts, possibly due to the presence of free fatty acids, which have an effect similar to that of acetate in perfused hearts. The mechanisms involved in these findings are, however, largely unclear.

Alternatively, pyruvate abolishes the effect of preconditioning if present prior to ischaemia [140, 480] and on reperfusion [142]. However, if the preconditioning protocol was changed from 4 x 5-min cycles of ischaemia and reperfusion, to 4 x 7-min I + 5 min R, with a 40% increase in total preconditioning ischaemia, protection in the presence of pyruvate was found to be similar to results from the initial

protocol with glucose only present [480]. Pyruvate thus appears to increase the threshold for preconditioning. The inhibition of preconditioning by pyruvate on reperfusion was associated with the requirement for glucose [142]. However, this has been disputed in the literature in non-preconditioned hearts (see Ch II).

iii) Supply-demand imbalance

Cardiac adrenergic nerve stimulation raises energy demand. If energy supply were concomitantly increased by increasing the blood flow prior to ischaemia, no change in postischaemic infarct size was found [215]. If blood flow, and thus energy supply, were not increased with increased adrenergic activity, infarct size was attenuated. The transient energy-supply imbalance may thus trigger preconditioning-mediated protection. An absolute reduction in energy supply does not appear to be necessary i.e. a relative flow deficit of 36% can induce protection against infarction (comparable to effects of partial stenosis [422]). These results also partially corroborate the effects of a transient inhibition of glucose uptake in rats which would be expected to reduce energy supply [166], as well as the effects of hypoxic preconditioning.

Rapid cardiac pacing ("demand" ischaemia) prior to a sustained ischaemic episode can also induce protection in dogs [556]. Pacing a dog ventricle at 300 beats/min for two 2 min periods, with intermittent periods of 5 min (normal heart rate = 140 beats/min) followed by 25 min occlusion reduced ST segment elevation, and lowered the incidence and reduced the severity of arrhythmias. Increased pacing can also induce preconditioning in the rabbit, and is associated with increased tissue cGMP, and reduced tissue cAMP levels [519].

iv) Acidosis

A transient acidosis, one of the components of ischaemia, was used to precondition the isolated rat heart [603]. However, no change in recovery after ischaemia compared to control hearts was seen, suggesting that pre-ischaemic changes in pH are not a mechanism of preconditioning protection. Pre-ischaemic changes must be distinguished from changes in pHi during sustained ischaemia, which have been mooted as being of importance in preconditioning [99].

c) Stunning - a possible protective mechanism?

A brief period of ischaemia (5-20 min) leads to stunning (see Ch I), which could play a role in the protective effects of preconditioning. Stunning can reduce ATP utilisation and catabolite accumulation due to the depressed contractile state, similar to the effects of preconditioning on energy balance. If stunning is involved in preconditioning, the protective effect should last as long as stunning is present [377]. Protection against infarct size is evident if hearts are reperfused for 5 min prior to sustained occlusion. However, if reperfusion is continued for 120 min prior to re-occlusion,

the protection against infarct size is 50% less, despite persistence of severe stunning. No correlation between the degree of "pre-ischaemic" stunning with different preconditioning protocols and subsequent infarct size limitation was observed [358]. In addition, stunning was precluded as the mechanism of preconditioning by showing an equivalent protection with 2.5 min as opposed to 5 min I followed by 5 min R, despite a lack of stunning in the former protocol [423]. Preconditioning ischaemia followed by hyperkalaemic perfusion prior to sustained ischaemia, which eliminates contractile activity, indicated that the reduced energy demand in PC hearts is not due to stunning-induced mechanisms [224]. The use of hypoxia to precondition hearts also negates the role of stunning, as a short period of hypoxia does not show reduced function on reoxygenation, but does protect the heart [83, 290, 387, 498, 603].

d) Changes in energy metabolism

i) Glycolytic inhibition and high energy phosphate preservation

Changes in glycolytic intermediates were monitored in dog hearts during total global ischaemia, preceded by 4 x 5 min I (or control) and hyperkalaemic perfusion [224]. ATP levels were initially lower in preconditioned tissue. Almost 40 μmol/g more high energy phosphates were used in the control myocardium in the first 15 min. The differences between the two groups became less evident with an extended duration of ischaemia. Lactate, G6P, G1P and αGP accumulated substantially in the first 60 min, but thereafter G1P and αGP declined. In each case, control tissue had a higher metabolite content than did the preconditioned hearts. Tissue glucose levels, normally very low, increased initially in preconditioned hearts, but then declined, whereas control hearts showed a continued increase. Glycogen was depleted in the preconditioned hearts prior to ischaemia, and glycogen utilisation was also reduced. The increased tissue glucose was attributed to cleavage by debranching enzymes of α,1-6 linkages of glycogen, which releases glucose rather than G1P. Thus preconditioned hearts had decreased high energy phosphate utilisation and decreased glycolytic metabolite accumulation. Intermittent aortic cross-clamping in dogs also reduces the loss of high energy phosphates compared to an equivalent sustained ischaemic period [1]. The addition of a nucleoside transport inhibitor and an adenosine deaminase inhibitor had similar effects. Both groups were associated with maintained function. Preconditioning may therefore slow ATP utilisation and reduce the glycolytic rate, attributed to a reduced energy demand in ischaemia.

A CP "overshoot" occurs after a brief period of ischaemia and reperfusion [525], and is a consistent observation in preconditioned hearts. In pig hearts with 4x5 min cycles of coronary occlusion, followed by 20 or 60 min sustained ischaemia, CP was significantly higher than control hearts after preconditioning, and declined at a slower rate during ischaemia [250]. ATP is depleted in

preconditioned hearts, but may decline subsequently at a slower rate, partly due to increased CP. However, the CP effect can only be relatively short-lived because of a rapid breakdown.

In contrast, increased ATP depletion has been temporally related to increased contracture in preconditioned hearts [266]. The preceding ischaemic period depletes ATP, a difference which is maintained over the first 20 min total global ischaemia (the absolute amounts utilised must be distinguished from the rate of utilisation i.e. the slope). After 20 min ischaemia, no difference was observed between the 2 groups, with very low residual values of tissue ATP (although preserved ATP has been found after 25 min total global ischaemia following preconditioning, compared to control hearts [237]).

Metabolism may also be slowed by hypothermia, or by cardioplegia. Preconditioning has an additive effect to the degree of protection conferred by hypothermia, allowing much longer maintenance of function [65]. However, this is a transient effect in that after an extended time, no additional protection is found. With cardioplegia, no additive benefit with preconditioning is seen [265]. While preservation of high energy phosphates would normally be considered beneficial, high ATP levels do not always imply protection (see Ch I and II for discussion on "critical" levels of ATP). Other factors must also be considered.

ii) Glycolysis and glycogenolysis

The role of glycolysis in preconditioning is very controversial. Provision of glucose to the ischaemic heart may generally be considered advantageous; inhibition of glycolysis should therefore be detrimental. Many reports [18, 99, 250, 512] show that the fall in pHi during sustained ischaemia following preconditioning is reduced, presumably because of reduced glycogen breakdown. If a fall in pHi contributes to ischaemic injury, an attenuated fall should be beneficial. Preconditioning reduces total tissue glycogen prior to the sustained ischaemic period, and attenuates the rate of glycogenolysis during sustained ischaemia [224, 574], consistent with the reduced acidosis. A reduced glycogen breakdown also lowers tissue lactate accumulation, which may be protective [387]. However, these results have not always been replicated [163, 286]. A positive relationship between glycogen content at the end of ischaemia and improved recovery is found [163]. Further controversy is added by the findings of the preconditioning effects of hypoxia [83, 290, 498, 603] (see above).

The precise role of glycogen in preconditioning (as being separate from hypoxia-induced changes - see above for argument) has not been fully elucidated. Wolfe et al. drew specific attention to the role of glycogen depletion in preconditioning [588]. Rats were subjected to 4 cycles of 5 min regional I + 5 min R, followed by immediate re-occlusion for 45 min, or with an additional 30, 60 or 360 min reperfusion prior to re-occlusion. Myocardial glycogen content of the ischaemic tissue was determined prior to sustained re-occlusion. Longer reperfusion increased repletion of glycogen stores,

such that by 60 min, full restoration of glycogen was found. Glycogen repletion was correlated with loss of protection in terms of infarct size. However, this study did not necessarily take into account previously documented evidence that the time of intervening perfusion between preconditioning and sustained ischaemia is an important determinant of the efficacy of preconditioning [269, 359, 377], and that the protection from preconditioning wanes with time [285, 375]. Thus these findings did not fully dissociate changes in glycogen from other changes associated with maintained reperfusion. Another report showed that the total amount of glycogen broken down during ischaemia is reduced by preconditioning, with reduced lactate and H^+ accumulation [574]. However, these changes were not necessarily correlated with the effect of preconditioning. More recently, Asimakis correlated the diurnal change in myocardial glycogen content with improved functional recovery on reperfusion after sustained ischaemia following a preconditioning protocol . No correlation was found, implying that glycogen is not involved in preconditioning [17].

Contrary to these findings, glycogen-loading by fasting, together with pre-ischaemic perfusion with lactate, improved tolerance to ischaemia to a similar extent as that found by preconditioning [114]. This study followed on from previous observations, that fasting protects the heart against ischaemia-induced injury [487]. There is thus still some controversy over the role of glycogen, both in itself, and in tandem with preconditioning, which I have attempted to clarify in this thesis.

Utilisation of glucose in preconditioned hearts has not been much investigated because the majority of studies have used total global ischaemia. In hearts subjected to 2 x 5 min I + 5 min R, followed by 35 min aerobic perfusion, glycolysis from glucose was inhibited (in the presence of palmitate) [136]. If these hearts were subjected to 30 min total global ischaemia and reperfusion, glycogen utilisation during ischaemia was reduced, and glycolysis was lower on reperfusion. Glucose oxidation was similar in the two groups, indicating improved coupling of glycolysis with oxidation. ATP and adenosine were higher in PC hearts at the end of ischaemia, with improved functional recovery. The reduced glycolysis is consistent with a reduced $[H^+]i$ in preconditioned hearts, which would attenuate Ca^{2+} overload as recorded in preconditioned hearts [142]. However, glycolysis is stimulated during low flow ischaemia by preconditioning [218], which challenges this finding. Reduced contracture and improved functional recovery were recorded in rabbit hearts after preconditioning with 3 min I + 12 min R and 60 min low flow ischaemia (0.4 ml/min/g wet wt) [218]. The perfusate also contained palmitate, insulin, and 5 mM glucose (cf [136]). Preconditioning increased glucose uptake and lactate production during ischaemia, with higher ATP levels at the end of ischaemia. Glycogen levels prior to ischaemia were no different between control and preconditioned hearts, but there was a significant preservation of glycogen at the end of ischaemia in preconditioned hearts, presumably from increased glycogen synthesis, or from decreased glycogenolysis. Thus preconditioning increased glucose but not

glycogen utilisation. The mechanism whereby glucose uptake is stimulated by preconditioning is unclear, but may be related to GLUT4 translocation by the initial ischaemic episodes [517]. This hypothesis is partially corroborated in this thesis.

iii) Inhibition of mitochondrial ATPase

Mitochondria are a major site of ATP utilisation in ischaemia [465]. During ischaemia, the cytosolic $[H^+]$ increases, reducing the H^+ gradient across the inner mitochondrial membrane (matrix pH is usually less than cytosolic pH and provides the proton-motive force to allow for ATP extrusion from the mitochondria). ATP synthase function may be reversed when the pH gradient is affected, and then may function as an ATPase, consuming an ATP and pumping H^+ out of rather than into the mitochondria (although this is controversial [488]). Mitochondria contain an endogenous ATPase inhibitor protein which binds to the ATP synthase in ischaemia [465, 466] and can prevent this loss in ATP. The inhibitor is triggered by a low pH and has been implicated in preconditioning, particularly in species with slow hearts rates (pigs, dogs, rabbits) [226]. This mechanism would prevent the large wastage of ATP associated with mitochondrial activity in ischaemia [226]. However, this hypothesis has been disputed, given that preconditioning can occur in the rat which has very low concentrations of this protein [319, 598]. However, despite these low levels, the inhibitor could be activated by preconditioning [598], and thus exert a greater effect than would be predicted.

e) Ionic changes

i) pHi, Na^+i and $Ca^{2+}i$

pHi falls in ischaemia, leading to $[Na^+]i$ accumulation, and Ca^{2+} entry by activation of the Na^+/H^+ and Na^+/Ca^{2+} exchanges [303]. If pHi is reduced during sustained ischaemia, the protective effects of preconditioning are removed [99]. In preconditioned hearts, the pHi does not fall as low. The lesser fall in pHi in preconditioning [18, 99, 250, 512] may be a major point of convergence for many "preconditioning mechanisms", possibly leading to a reduced $[Ca^{2+}]i$ [303]. A reduced acidosis may be attributed to reduced ATP hydrolysis [156], or reduced glycogenolysis [151] (which indirectly reduces ATP hydrolysis). Lactate accumulation during ischaemia is reduced in preconditioned hearts [18, 224], suggesting that the change in pHi is related to reduced glycogenolysis. These issues are thus intimately related with the investigations carried out in this thesis.

4 cycles of 5 min I + 5 min R prior to 30 min global ischaemia in rats and guinea pigs did not affect ATP levels during sustained ischaemia and reperfusion, but CP levels recovered slightly better in the preconditioned hearts [512]. Levels of $[Na^+]i$ were generally higher towards the end of ischaemia in control hearts, but not significantly so, while $[Ca^{2+}]i$ and $[H^+]i$ were significantly reduced. pHi

levelled off in both groups, but at different values, while $[Ca^{2+}]i$ continued to increase [512]. Mg^{2+}, which blocks Ca^{2+} entry via the Na^+/Ca^{2+} exchange, and is beneficial by itself when given prior to ischaemia, has no additive effect on preconditioning, suggesting that preconditioning does retard Ca^{2+} entry [512], possibly by reducing the fall in pHi.

Another puzzling study showed that preconditioning significantly increased $[Na^+]i$ prior to sustained ischaemia, but did not alter $[Na^+]i$ during ischaemia [449]. On reperfusion, the decline in $[Na^+]i$ in preconditioned hearts was far greater than in control hearts. Na^+/H^+ exchange inhibition prior to sustained ischaemia limited $[Na^+]i$ accumulation, and reduced the beneficial effects of preconditioning. Transient acid load prior to sustained ischaemia greatly increased $[H^+]i$ in preconditioned hearts compared to control, but these hearts showed a faster recovery of pHi. This recovery was also abolished by Na^+/H^+ exchange inhibitors. Thus preconditioning appeared to stimulate Na^+/H^+ exchange, although this seems contrary to the accepted benefits of Na^+/H^+ exchange inhibitors during ischaemia and on reperfusion [119, 489]. The difference may lie in the absolute loads of Na^+, H^+ and Ca^{2+} at different times, although this is difficult to reconcile.

ii) Ca^{2+} release channels

Preconditioning may alter the ryanodine SR Ca^{2+} release channels such that the channel density is reduced, with reduced Ca^{2+} efflux [605]. The time course of changes in channel density is correlated with the protection conferred by preconditioning. This observation remains to be explored

iii) ATP-dependent potassium channels

Opening of K_{ATP} channels may provide a cardioprotective effect by exerting a localised mechanical arrest, although this may also precipitate arrhythmias [236] (see Ch I). Preconditioning is thought to increase opening of these channels via adenosine (see below), an effect usually counteracted by glycolytic ATP [571] (see Ch II). The role of these channels in usual models of ischaemia is equivocal (see Ch I, II). In preconditioned hearts, glibenclamide administered to dogs prior to preconditioning, abolished any protective effect, compared to pre-treated control hearts [169, 172]. A pre-ischaemic coronary infusion of adenosine resulted in a degree of protection against necrosis similar to that with ischaemic preconditioning [596], but this protection was abolished by glibenclamide, suggesting a role for the K_{ATP} channel. The possible effect of preconditioning in attenuating glycolysis (see above) may also result in opening of these channels. This effect does not agree with concepts of protection conferred by glycolytic ATP (see Ch II). However, glibenclamide has no effect on preconditioning in rats [143, 171, 319], suggesting that K_{ATP} channel activation is not a universal mechanism of preconditioning in all species, but rather an epiphenomenon which when it occurs may exert positive effects. A recent paper has implicated opening of this channel in preconditioning in human atrial tissue [565].

K_{ATP} channel opening does not appear to be involved in the pronounced antiarrhythmic effects of preconditioning, as administration of glibenclamide to preconditioned dog hearts failed to affect the reduction in ventricular fibrillation and increased survival [555]. In preconditioned rabbit hearts, however, activation of the K_{ATP} channel, which reduces action potential duration, may be responsible for the delay in the onset of electrical uncoupling [527]. Therefore the role of the K_{ATP} channel in preconditioning seems controversial and species-dependent.

f) Receptor activation

i) Adenosine

Adenosine released in the preceding ischaemic period(s) can bind to receptors, and exert a beneficial effect on subsequent sustained ischaemia. This has been one of the more thoroughly investigated mechanisms of preconditioning. A significant reduction in interstitial adenosine accumulation during sustained ischaemia is seen in preconditioned hearts, despite a large release in the first reperfusion period (with 2 cycles of preconditioning) [544]. A significant transient increase in adenosine is seen in control hearts during sustained ischaemia. No difference in the release of the other purines i.e. xanthine, hypoxanthine and inosine is found.

Adenosine acts in a number of ways which are beneficial to the ischaemic heart (see Ch I). Adenosine may be a key regulator of energy metabolism in the heart, but its actions are still unclear. Adenosine may stimulate glycolysis, an effect noted in hypoxic hearts [591]. However, this effect in preconditioned hearts is contradicted by the observations of a reduced rate of glycolysis [224]. The actions of adenosine on glycolysis are, however, quite controversial (see Ch. I, II).

Adenosine exerts its actions by binding to several receptors on the myocyte and endothelial cells (see Ch. I). Infusions of 8-phenyltheophylline (8-PT) (non-selective adenosine antagonist) and PD 115,199 (A1/A2 antagonist) block preconditioning, while adenosine pre-treatment mimics the protective effect of preconditioning on infarct size in rabbits [318]. 8-PT also abolishes the protective effect of preconditioning on stunning in rabbits [542]. More recently, Armstrong and Ganote have added to the debate using specific A1 and A3 antagonists on isolated rabbit myocytes undergoing contracture. Only A3 antagonists (A3 receptor still putative) were effective in delaying cell contracture, an effect which could be blocked by PKC inhibitors [16].

Most beneficial effects of adenosine appear to be modulated via A1 activation [291], the receptors found mainly on the myocytes. A1 activation causes translocation of PKC to the membranes in close association with the adenosine receptor. When PKC is removed from the membranes, the protective effect of preconditioning is lost [355, 601]. A1 receptor stimulation also activates the K_{ATP} channel via Gi proteins [254]. However, these channels are not involved in preconditioning in all species (see

above). Adenosine also has many effects on energy metabolism, although the reports are somewhat contradictory (see Ch I).

While adenosine may be central in the mechanism of preconditioning in dogs, pigs and rabbits, a large amount of evidence refutes this mechanism in the rat. Administration of adenosine prior to ischaemia does not improve functional recovery in isolated rat hearts, while administration of 8-PT does not abolish preconditioning [64, 373]. In rats, PD 115,199 was not effective, although an A2 specific blocker did reduce infarct size [313]. Induction of preconditioning by a period of hypoxia or low flow ischaemia also disputes the adenosine proposal in a rat [603]. A maintained coronary flow should prevent any accumulation of adenosine, inhibiting stimulation of the receptors. Hypoxic preconditioning is also effective in dogs, suggesting that adenosine is also not required for preconditioning in this species [498]. However, a contradiction to this particular argument has been provided by Cohen et al., albeit in rabbits where adenosine has been shown to be important [318]. Sufficient amounts of both catecholamines and adenosine were released during hypoxia to account for preconditioning protection [83].

ii) α1-adrenergic receptor stimulation, protein kinase C translocation and preconditioning

Reserpinised rabbits (with reduced catecholamine stores) subjected to ischaemia with or without a preconditioning protocol show no difference in infarct size, suggesting that catecholamine release is crucial for preconditioning [536]. More specifically, α1-adrenergic stimulation was identified as a prerequisite for preconditioning in the rat heart [21]. A pre-ischaemic infusion of norepinephrine or phenylephrine reduced infarct size in normal and reserpinised hearts, while α1-adrenergic blockade inhibited preconditioning. Increased PKC translocation to the membranes following α1-adrenergic activation (or adenosine) may be protective to the heart, and thus a possible mediator of preconditioning [601].

Preconditioning may, by a number of pathways, cause PKC translocation, thereby activating protective mechanisms. Both the trigger for PKC translocation, and the subsequent benefits to the heart, are unclear. In addition, there are several isoenzymes of PKC [514], which may have opposing effects. In the study of PKC and preconditioning, little attempt has been made to dissociate the effects of the different isoenzymes (see Ch I for further discussion on PKC and α1 activation).

PKC translocation exacerbates hypoxia-induced injury [211], and sensitises adenyl cyclase [515], leading to accumulation of cAMP, stimulation of Na^+/H^+ exchange, and an increase in Ca^{2+} influx [211]. PKC phosphorylation is also energy-utilising (see Ch I for summary of effects - note particularly effects on glycogen, glucose uptake and glycolysis). In addition, phorbol esters are very pro-arrhythmic by reducing action potential duration [33]. K_{ATP} channels may be phosphorylated by PKC translocation, with increased opening probability, and increased ion perturbation. Alternatively, K_{ATP} channel

opening is thought to be involved in preconditioning in some species, but this may be a consequence, not a cause. There are many isoenzymes of PKC [514], and phorbol esters may target those not necessarily involved in preconditioning. The protective effect of PKC activators against infarction was tested in rabbits [601] and rats [355]. Preconditioning is abolished by PKC antagonists, while reduced infarct size (in rabbits) and improved functional recovery (in rats) follows infusion of diacylglycerol (DAG) and phenylephrine, which induces PKC translocation to the cell membranes. Phorbol 12-myristate 13-acetate (PMA - phorbol ester - potent activator of PKC) confers a comparable protection to that afforded by a brief period of "preconditioning" hypoxia on isolated hypoxic myocytes [569].

While tyramine-induced catecholamine release prior to sustained ischaemia is protective, additional adenosine A1 receptor stimulation appears to be necessary for preconditioning in rabbits [533]. Conversely, infarct size limitation by A1 stimulation is mediated by PKC activation in rabbits [475]. These findings have not always been replicated in the rat. Reserpinisation did not affect the response to preconditioning in terms of infarct size, suggesting that endogenous catecholamines are not required [577] (contrary to previous findings [21]). In addition, chelerythrine, a specific PKC inhibitor, failed to inhibit preconditioning in the isolated rat heart in terms of functional recovery [264]. Moolman et al. also could not find an effect of α receptor blockade or PKC inhibition on functional recovery in preconditioned rat hearts [363]. However, PKC inhibitors did abolish preconditioning effects in terms of infarct size reduction in the rat heart [504]. In dogs, the effect of PKC translocation on infarct size reduction has been disputed [445].

The role of PKC in normal and preconditioned hearts is unclear, and appears to be species-specific, as well as involving different isoenzymes. However, this remains the most tenable hypothesis as yet presented, and remains the major focus of investigation of preconditioning.

iii) β-adrenergic receptor stimulation

An increased cAMP is implicated in the deleterious effects of ischaemia (see Ch. I). A reduced cAMP was noted by Sandhu et al. in preconditioned rabbit hearts [479]. Adenosine A1 receptor activation inhibits adenyl cyclase through Gi, which inhibits cAMP formation. However, the reduced cAMP may be more a consequence of preconditioning rather than a mediator of the effect [363, 479]. Thus the attenuated adenyl cyclase activity following PKC or adenosine A1 activation may not be relevant. However, the reduction in cAMP may well explain the attenuated arrhythmias noted with preconditioning (see Ch I). A reduction in cAMP may also explain a reduction in the rate of glycogenolysis during sustained ischaemia following preconditioning [574].

iv) Gi protein function in ischaemia and preconditioning

Following adenosine A1 receptor stimulation, Gi proteins are activated, which may thus be the primary factor in preconditioning, as many putative effectors activate these proteins. Pertussis toxin, which catalyses ADP ribosylation of Gi proteins, thereby uncoupling them from receptors, abolishes preconditioning [534]. Carbachol can mimic preconditioning in a rabbit heart, in terms of ischaemic contracture and functional recovery, by binding to M2 receptors which act via Gi proteins [195]. In ischaemia, β adrenergic receptor activation increases cAMP levels by increasing adenyl cyclase activity. cAMP increases energy demand, and potentiates arrhythmias. Gi proteins, activated by a number of receptors, antagonise the adrenergic system by inhibiting adenyl cyclase, activating K_{ATP} channels, and inhibiting Ca^{2+} channels. Increased Gi regulation would be protective by reducing excessive Ca^{2+} loading and energy demand, and by increasing electrical stability. Alternatively an impairment of Gi protein-mediated regulation in ischaemia by a functional alteration of the proteins should be detrimental. Preconditioning may enhance the responsiveness of the adenosine A1 receptor pathway, thereby stimulating Gi function. A sufficient amount of adenosine is usually released in the first few minutes of ischaemia to ensure maximal stimulation of all available A1 receptors and thus Gi proteins [394]. An increased responsiveness of the Gi proteins to receptor activation is found after an initial ischaemic period, but an intervening reperfusion period is necessary for this mechanism [394]. Increased Gi protein activation would increase adenyl cyclase inhibition. The reduction in cAMP by preconditioning [479] supports this concept. However, Lawson et al. found that the antiarrhythmic effect of preconditioning does not involve Gi proteins, although this does not exclude the role of Gi proteins in the other protective end points of preconditioning [298]. Further clarification of the role of Gi proteins in preconditioning is needed.

g) Stimulation of protein synthesis

While synthesis of heat shock proteins is a protective mechanism associated with ischaemia [115], the rapidity with which preconditioning exerts its protective effects is far quicker than can be accounted for by any gene process. The detection of heat shock proteins following ischaemia occurs after about 8 hrs [115], but this is still a small effect. Hearts can still be preconditioned despite protein synthesis inhibition [117]. However, the "second window of protection" [285, 599] may well be due to influences on gene transcription and translation, as this phenomenon is consistent with the time frame of heat stress protein induction. The mechanism whereby heat stress proteins exert protection is as yet unclear, but there is accumulating evidence to suggest that increased expression of these proteins reduces infarct size and enzyme efflux and improves functional recovery after ischaemia [339].

h) Free radicals and redox state

Brief coronary occlusion results in a burst of free radical production. These would therefore be present at the onset of the sustained ischaemic episode, and should be detrimental. An antioxidant (NAC), which prevents depletion of glutathione stores, does not affect the normal response to ischaemia [73]. In preconditioned hearts, glutathione is depleted, with a more oxidised state of the tissue. If NAC is used to prevent glutathione depletion, the effects of preconditioning on functional recovery are abolished. Prevention of thiol oxidation is normally protective, but in preconditioned hearts this mechanism may be responsible for adaptive responses of the myocardium to subsequent injury [73].

i) Vascular changes

While most focus is on changes in myocytes with preconditioning, important sources of possible mediators are the endothelium and smooth muscle cells. Because preconditioning is initiated following a transient reduction in flow or oxygen supply, the endothelium is directly affected, both mechanically and metabolically, which can result in release of important endogenous mediators, including endothelin, atrial natriuretic peptide, etc.

A fairly recent hypothesis regarding preconditioning involves the release of bradykinin by the endothelial cells during ischaemia [554]. Bradykinin activates L-arginine nitric oxide and cyclo-oxygenase pathways, with production of NO and prostacyclin [138, 304, 568]. NO may depress myocardial contractility and reduce energy demand. NO stimulates guanylate cyclase and increases cGMP, which in turn may reduce Ca^{2+} influx through L-type channels [516], stimulates cGMP-sensitive phosphodiesterase which reduces cAMP levels, and lowers oxygen consumption. The ratio of cAMP to cGMP may be an important protective mechanism. cGMP is increased, inversely related to a decreased cAMP, in ventricular overdrive pacing-induced preconditioning [519]. The NO-cGMP pathway may be also an important modulator of glucose uptake [110, 494] (see Ch I), and a reduction in cGMP may be one of the mechanisms involved in regulation of glycolysis in preconditioning.

j) Diffusion of a protective substance

An alternative concept to those discussed above has been proposed, with diffusion of a protective substance from the ischaemic zone which could exert effects in "virgin" tissue [444, 582]. Data from studies in the rat heart (used because of low collateral flow) using 3x3 min I + 5 min R followed by 90 min coronary occlusion, and measuring infarct size was analysed using a mathematical model [582]. The average infarct size was smaller in the preconditioned group (28% vs. 60% of area at risk), with a direct relationship between area at risk and infarct size in preconditioned hearts but not in controls. The data are consistent with the hypothesis that inward diffusion of some "protective" substance

occurs, which suggests that tissue remote from the site of original occlusion would be protected against an ischaemic insult. This hypothesis was supported by findings in dogs, where infarct size in remote "virgin" myocardium was smaller if the myocardium had been preconditioned at a different site [444]. This mechanism could be explained by diffusion of e.g. adenosine, catecholamines etc. from the original ischaemic zone, although the degree of protection would be limited by the rate of diffusion through the interstitium and the half-life of the various compounds, or by the area perfused downstream from the original site.

3) SUMMARY

Preconditioning can be defined as one or more brief episodes of ischaemia which result in improved tolerance of the myocardium to a subsequent sustained ischaemic episode. The most reproducible effect of preconditioning is a reduction in infarct size measured at the end of the reperfusion period. Preconditioning alters many pathways in the cell, and thus the determination of the mechanism of preconditioning is difficult. Many of these cellular effects appear to act synergistically to bring about preconditioning, and the particular pathways involved seem to be partially species-dependent. The contribution of several factors means that a given pathway can be limited, and another upregulated to compensate. This confuses the issue of the mechanism of preconditioning.

Another important consideration in determining the mechanism of preconditioning is the end point chosen. Much confusion results from different interpretations of the "protection" conferred by preconditioning, as can be seen from the above discussion of the various mechanisms putatively involved in preconditioning.

The most accepted "common" mechanism of preconditioning to date is the involvement of the PKC system, activated either by adenosine activation of A1 receptors, the α1-adrenergic receptors, or by cytosolic-mediated events. The trigger mechanism appears to be species-dependent. In the rabbit, dog and other species excluding the rat, adenosine appears to stimulate the A1 receptor and Gi proteins. These in turn inhibit adenyl cyclase activity, which is also modulated by PKC. PKC may be directly affected by Gi protein activity. The ability of PKC to phosphorylate proteins is dependent on ATP levels, and is in turn responsible for increased re-energisation on reperfusion. While many reports find that blockade of PKC does not abolish preconditioning, there are many isoenzymes of the enzyme, and it is unclear which is involved. Of note is that the evidence for the PKC hypothesis comes from indirect observations using activators and inhibitors of PKC translocation. Attempts to ascertain whether PKC molecules are indeed found in the sarcolemma following preconditioning in the dog have found no evidence of this occurring [445].

I have investigated the role of glycogen and of glucose in preconditioning, both i) because of the apparent contradiction in an attenuation of glycolysis by preconditioning but increased protection despite the large amount of evidence to suggest that glucose provision is beneficial to the ischaemic

heart; and ii) to determine how preconditioning may modulate glycolysis. Preconditioning was thus investigated both as a "mechanism" of protection, and as a "modifier" of glycolysis. In addition, the effect of preconditioning on hearts subjected to low flow ischaemia (as opposed to total global ischaemia) was tested, in order firstly to allow for the determination of glucose uptake in preconditioned hearts, and secondly to determine the effect of preconditioning on low flow ischaemia, a more physiological model and one which has seldom been used.

Methods

1) THE ISOLATED HEART APPARATUS

i) Animals

The isolated Langendorff perfused rat heart was used for all experiments reported in this thesis [287]. Male Long Evans rats weighing 250 to 300g were used (some experiments were conducted in London, when Wistar rats were used). The rats were fed *ad libitum* on standard rat chow. They were anaesthetised with ether vapour. Once the rats were asleep, an inguinal incision was made to allow exposure of the femoral vein into which 200 i.u. heparin was injected. An abdominal incision was then made, and the diaphragm and ribs were cut to allow excision of the heart. The hearts were immediately placed in ice cold buffer. All animals were cared for according to the Recommendations of the Declaration of Helsinki and the Guiding Principles in the care and use of animals.

ii) Perfusion apparatus

The hearts were mounted within one minute of excision via the aorta on a standard Langendorff perfusion apparatus (see Fig M.1), with immediate initiation of perfusion. A perfusion pressure of 100 cm H_2O (76 mmHg) was used, with standard modified Krebs Henseleit solution (118.5 mM NaCl, 25 mM $NaHCO_3$, 5.75 mM KCl, 1.19 mM $MgSO_4$, 1.18 mM KH_2PO_4, 1.36 mM $CaCl_2$ and 11 mM glucose except where stated) [274]. The non-recirculated perfusate was gassed throughout with 95% O_2 5% CO_2, to maintain a pO_2 of 500-550 mmHg and a pH of 7.4. Temperature was maintained at 37°C in all experiments by water-jacketing, and monitored by a thermistor probe inserted into the right ventricle. A compliant balloon made of cling film was inserted into the left ventricle, and inflated to allow recording of a diastolic pressure of 4 mmHg on the multi-channel recorder (Grass Model 79D), via a Statham P23XL pressure transducer. This resulted in systolic pressures of 100-140 mmHg, with heart rates of 180-400 beats/min, and coronary flows 8-16 ml/min. Some hearts were paced, after excision of the right atrium, using a Grass S88 stimulator, at a rate of 360 beats/min.

iii) Protocol

The protocol varied between studies, and is elaborated in the methods in each section of the results. The standard protocol had a 15-20 min stabilisation period of perfusion prior to cessation of flow. Ischaemia was maintained for 30 min, mostly with a residual flow rate infused via a side arm, and maintained by a Gilson Minipuls 2 pump (see Fig M.1). The hearts were then reperfused for 20-30 min. The preparation was stable over the whole duration of the experiment (90 min) with a maximum of 5 % deterioration in function in the absence of ischaemia. Coronary effluent was collected and frozen immediately to allow for analysis of glucose utilisation and lactate washout. Parallel series of hearts were clamped with Wollenberger tongs kept in liquid nitrogen and the tissue then freeze-dried (Christ Alpha 1-4 freeze dryer) to allow analysis of tissue metabolites.

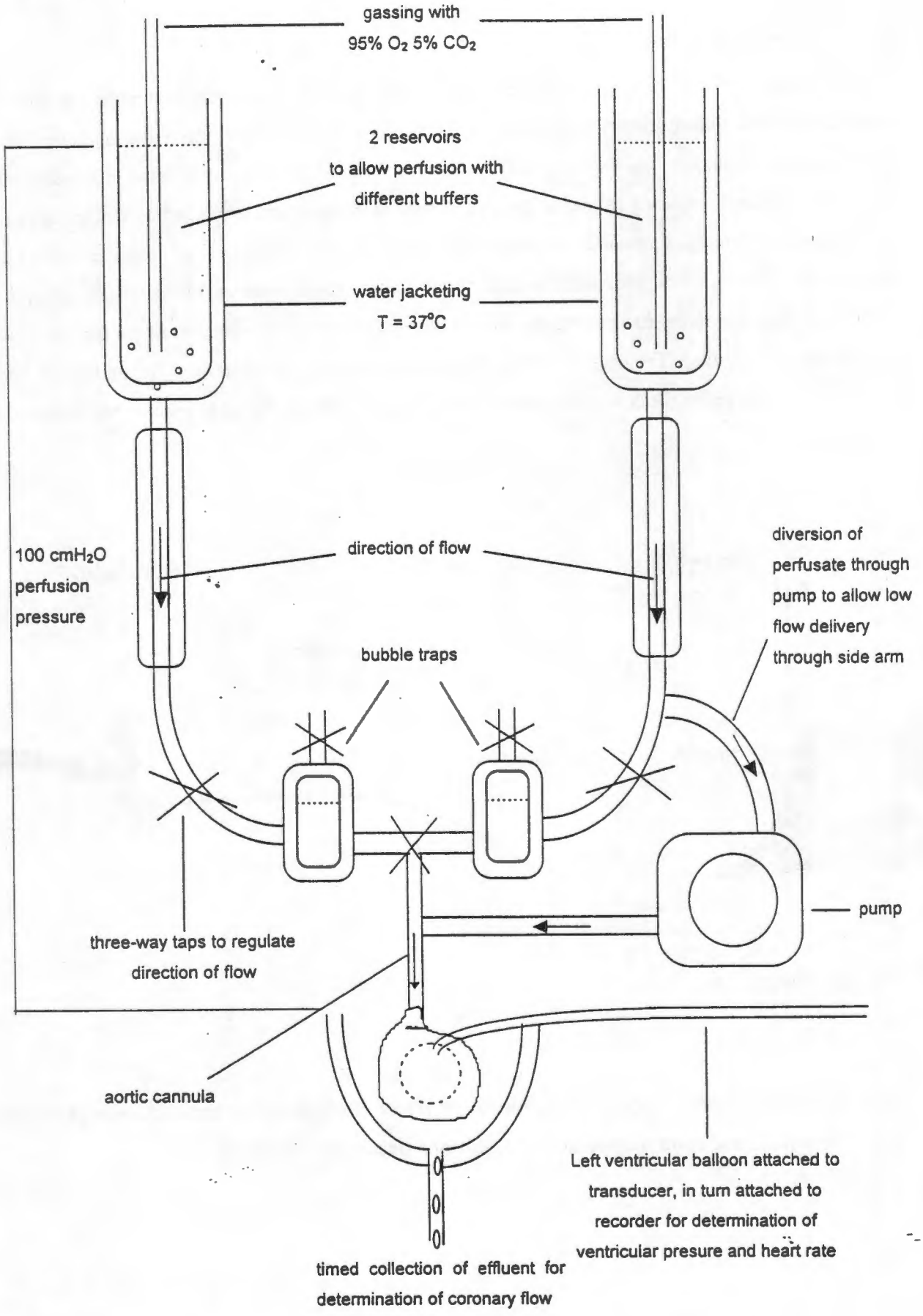

Fig M.1. Langendorff apparatus for perfusion of isolated rat heart

iv) Functional measurements

A representative trace showing pre-ischaemic, ischaemic and reperfusion left ventricular pressure is shown in Figure M.2. Coronary flow, heart rate, diastolic and systolic pressure were monitored at 5 min intervals throughout the experiments. Developed pressure (the difference between systolic and diastolic pressure) was used as the main index of functional recovery, expressed as a percentage of pre-ischaemic developed pressure (measured at the end of the stabilisation period). During ischaemia, developed pressure fell to zero within a few minutes, after which a rise in resting tension generally occurred. The time to the rise in diastolic tension above basal levels was defined as the time to onset of contracture (in min). The peak of resting tension, or contracture was recorded (in mmHg), as was the time to peak (min). Peak contracture was expressed as a percentage of pre-ischaemic developed pressure.

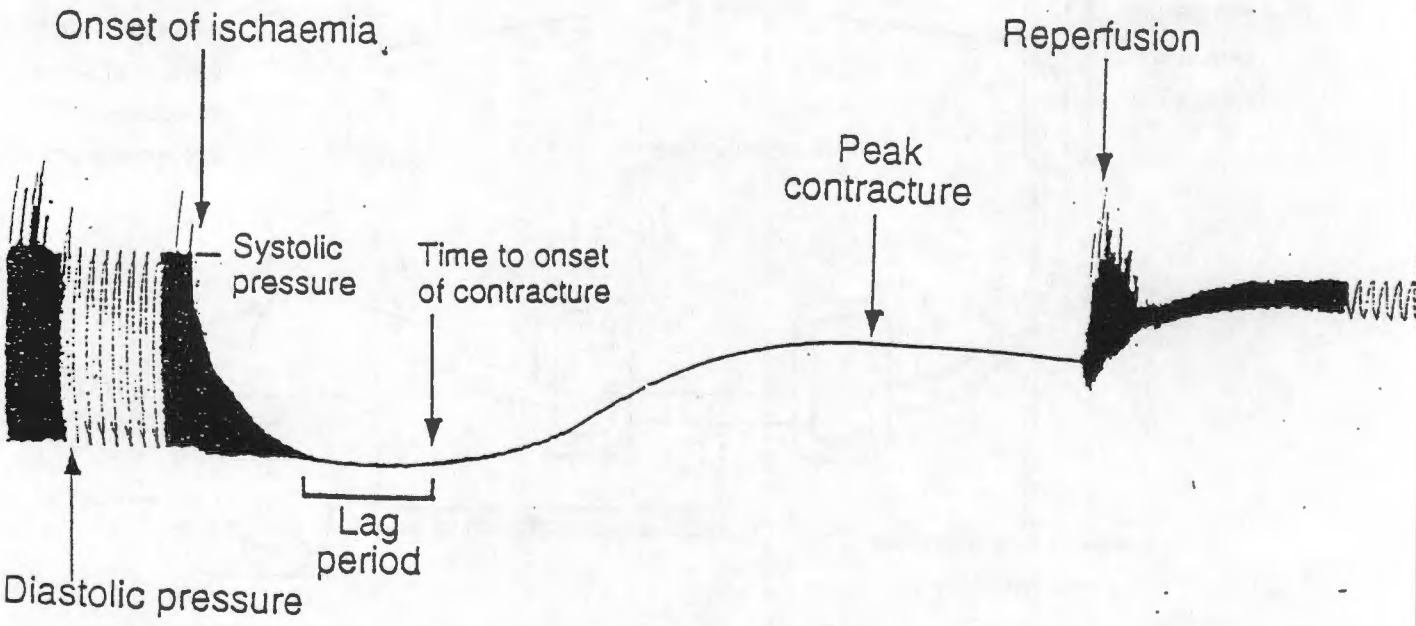

Fig. M.2. Representative trace of pre-ischaemic, ischaemic and reperfusion left ventricular pressure, showing systolic and diastolic pressure, heart rate and ischaemic contracture.

2) BIOCHEMICAL ANALYSES

After freeze-drying, the atria and connective tissue were removed, and the ventricular tissue was teased to allow a more homogenous sample to be obtained. The tissue metabolites were then extracted either using perchloric acid (for the majority of essays) or by alcohol, for the determination of tissue glycogen levels. Tissue metabolites were expressed as μmol/g wet wt, where wet weight = 5 x dry weight (a ratio determined from hearts weighed before and after drying - data not shown).

a) Perchloric acid extraction

i) Solutions

5 % perchloric acid - 71.4 ml of 70% solution in 1L distilled water

Tris/KOH/KCl: 0.2 M Tris - 24.22 g/L - adjust pH to 7.5 with concentrated HCl.

40% w/v KOH - 40g/100 ml

saturated KCl - saturate 40 % KOH with KCl, then mix with 0.2 M Tris in ratio of 6:4.

ii) Method

1) Use 5 ml flat-bottomed plastic tubes (Greyward without EDTA) and Eppendorff reaction vials. Label one set of 5 ml tubes and two sets of Eppendorffs
2) For freeze-dried rat heart extraction, put 1.2 ml cold 5% PCA into the 5 ml tubes. Keep on ice. Add 20-25 mg powdered (teased) freeze-dried tissue to PCA.
3) Homogenise immediately on adding tissue to PCA, keeping tube on ice all the time. Homogenise for 15-30 secs at about 300 rpm.
4) Spin homogenised samples in centrifuge (Sigma 202MK) at 5000 rpm for 1 mins at 4°C.
5) Take of 1 ml of supernatant into a labelled Eppendorff tube (volume for neutralisation).
6) Neutralise the acid extract with Tris/KOH/KCl buffer using 5 μl universal indicator (reverse titration with PCA). Solution becomes cloudy. Approximately 300 μl buffer is required to obtain a pH 6.5-7.5 (greenish-blue). The volume of buffer added is the neutralising volume.
7) Allow to precipitate for 10 mins. Centrifuge in Eppendorff rotor in Sigma 202MK for 5 mins at 5000 rpm and decant the supernatant into new labelled Eppendorff tubes. Freeze until ready to assay. (majority of assays should be done within one week of extraction, and preferably on same day)

iii) Calculations

Dilution factor for neutralising = F_1 = [(1.2 * total volume)/dry heart weight (g)] / 5

where total volume = neutralising volume + volume for neutralisation + universal indicator

Metabolites (μmol/g wet wt) = concentration (μmol/ml (mM) from assay) * F_1

b) Glycogen extraction

i) Solutions

40% KOH - 40 g/100 ml

2N HCl - conc HCl about 10 N - dilute 1/5

2N NaOH - MW 40 - 80 g/100 ml

Tris buffer - MW 121 0.2 M - 24.22 g/L - adjust to pH 7.5 with conc HCl

ii) Method

(adapted from [160])

1) Weigh Eppendorff tubes (3 ml) and add 10 mg freeze-dried tissue (teased)
2) Add 0.2 ml 40% cold KOH to digest tissue
3) Heat to 95°C in water bath or Eppendorff heating unit for 30 mins, shaking at 10 min intervals. Place weight on lids to prevent bursting open. Ensure tissue is broken up. Allow to cool.
4) Add 0.8 ml 95 % EtOH (dilute KOH 1/5). Glycogen precipitates overnight in fridge.
5) Eppendorff centrifuge - spin 2.5 mins at 3200 rpm
6) Aspirate supernatant, wash sediment 3 times with 1 ml 95 % EtOH each time. Aspirate.
7) Add 0.2 ml 2N HCl to sediment, to hydrolyse glycogen to glucose moieties. Boil at 95°C for 3 hrs. Allow to cool.
8) Add 5 µl universal indicator and neutralise with 0.1 ml Tris buffer (pH 7.5) and increments of 2N NaOH (about 0.2 ml total) until pH 7.5-7.7 (greenish blue). Note volume added, and weigh tubes.
9) Measure glucose (carbon-6 units) - standard assay

iii) Calculations

Factor for multiplication F_2 = {[Final wt (tube + neut vol) - tube weight]/dry heart wt (g)} / 5

or F_2 = {[5 µl + 0.1 ml + vol for neutralisation]/heart weight (g)} / 5

(compare above factors - should be within 5%)

Glycogen (µmol carbon-6 units/g wet wt) = glucose (µmol/ml (mM) from assay) * F_2

c) Adaptation of macrocuvette spectrophotometric methods for the Cobas Fara II Clinical analyser

The Cobas Fara II multichannel analyser (Roche Diagnostics) was used to measure the concentrations of metabolites. The micro-cuvuette methods utilise far less sample per assay, allowing many more assays to be done per extract. Many assays can be done within a short time, and are far more cost-effective in terms of enzymes, reagent volumes etc. The methods were adapted from those previously used on the normal spectrophotometer, with standard assay volumes of 3 ml per cuvette [24]. The steps taken to establish the techniques, and the methods used are described in Appendix II.

d) Adenosine triphosphate (ATP) and creatine phosphate (CP)

Glucose 6-phosphate dehydrogenase

$$G6P + NADP \leftrightarrow 6\text{-phosphoglucono-}\delta\text{-lactone} + NADPH + H^+$$

Hexokinase

$$ATP + glucose \leftrightarrow ADP + G6P$$

Creatine kinase

$$CP + ADP \leftrightarrow ATP + creatine$$

i) Reagent mixture

Solution	Molec Wt	Stocks Conc (mM)	Content	Other	Cobas /cuvette Vol (µl)	/35 cuv (µl)
Glucose	180.162	100	1.802 g/10 ml		2	70
NADP	743.4	13.45	0.1 g/10 ml	1 %	4.46	156
Tris (HCl)	121.4	200	24.22g/l	pH 7.5	40	1400
$MgCl_2$	203.3	1	0.203 g/l		4	140
H_2O					69.54	2434
G6PDH			1 mg/ml		1	35
Total					120+1	4235

ii) Start reagents

1. G6PDH (350 U/mg - 5 mg/ml) included with reagent (1 µl/sample) (dilute 5 mg/ml stock 1/5)
2. HK (450 U/mg - 1500 U/ml) 1 µl/sample (20 µl of 1/20 dilution - 40 µl HK + 760 µl H_2O (in large diluent cup - in R position - change after first pipetting)
3. $ADP.Na_3$ (493.2 MW) - stocks 10 mM (0.049 g/10 ml) 5 µl/sample (200 µl in cup - P1)
4. CK (350 U/mg) - 2 mg/200 µl (make up fresh daily) 5 µl/sample (200 µl in cup - P2)

iii) Standards

0.0625-0.5 mM ATP (605.2 MW) and CP (363.2 MW) in same cups

Stocks 10 mM (0.061g/10 ml and 0.036g/10 ml - dilute 1/10)

iv) Calculations

(M2 - M1)/slope = ATP mM (A1 - M3)/slope = CP mM

e) Adenosine diphosphate (ADP) and adenosine monophosphate (AMP)

Lactate dehydrogenase

Pyruvate + NADH + H^+ ↔ Lactate + NAD^+

Pyruvate kinase *Myokinase*

ADP + phosphoenolpyruvate ↔ ATP + pyruvate AMP + ATP ↔ 2 ADP

i) Reagent mixture

Solution	Molec Wt	Stocks Conc (mM)	Content	Other	Cobas /cuvette Vol (µl)	/35 cuv (µl)
TRAM -	185.65	400	14.85g/200 ml	pH 7.5	24	840
EDTA	372.25	100	0.185 g	(after)		
KCl	74.56	500	37.28 g/l		20	700
$MgCl_2$	203.3	1000	203.3 g/l		4	140
$NADH.Na_2$	709.4	14.1	0.1g/10 ml	1%	3.11	109
PEP-TCHA	168	10	16.8 mg/10 ml		8	280
$ATP.Na_2$	605.2	10	60.5 mg/10 ml		0.4	14
H_2O					60.49	2117
LDH					1	35
Total					120+1	4235

ii) Solutions

TRAM buffer - 1/2 vol TRAM. pH to 7.5 (NaOH), add EDTA and make up to vol (200 ml)

iii) Start reagents

1. LDH (250 U/mg - 100 mg/10 ml) included with reagent (1 µl/sample)
2. PK (200 U/mg - 10 mg/ml) 0.5 µl/sample (5 µl of 1:10 - 20 µl +180 µl H_2O in P1)
3. Myokinase (360 U/mg - 5 mg/ml) 0.5 µl/sample (5 µl of 1:10 - 20 µl +180 µl H_2O in P2)

iv) Standards

0.03125-0.25 mM $ADP.Na_2$ (471.2 MW) and $AMP.Na_2$. $6H_2O$ (499.2 MW) in same cups.

Stocks 10 mM (0.047g/10 ml and 0.049g/10 ml)

v) Calculations

(M2 - M1)/slope = ADP mM (A10 - M2)/slope = AMP mM

f) Inorganic phosphate

Phosphate ions + ammonium molybdate ↔ phosphomolybdate

i) Reagent mixture

Solution	Stocks Conc	Other	Cobas /cuvette Vol (µl)	/35 cuv
Ammonium molybdate + Sulfuric acid	2.1 750	use as is	150	5250
Total			150	5250

ii) Standards

0.125 - 1.0 mM $Na_2HPO_4.12H_2O$ (358.14 MW)

Stocks 10 mM (0.0358 g/10 ml)

iii) Calculations

(A25 - A1)/slope = Pi (as printed)

iv) Notes

Linearity limit 5 mM

Measures concentration of phosphomolybdate complex photometrically

Inorganic phosphorus Roche Unimate 7 PHOS 3677

g) Glucose

Hexokinase

Glucose + ATP ↔ Glucose 6-phosphate + ADP

Glucose 6-phosphate dehydrogenase

G6P + NADP ↔ 6 phosphoglucono-δ-lactone + NADPH + H^+

i) Reagent mixture

Solution	Stocks Conc (mM)	Other	Cobas /cuvette Vol (µl)	/35 cuv (µl)
Tris	85			
+ ADP	3.6			
+ NAD^+	1.8			
+ HK	20 µkat/L			
+ G6PDH	10 µkat/L	solid (R1)		
Tris	10	+30 ml (R2)	200	7000
Total			200	7000

ii) Standards

0.0625 - 5 mM glucose (180.6 MW)

Stocks 10 mM (0.0180 g/10 ml)

iii) Calculations

(A19 - A1)/slope = Glucose mM (as printed)

iv) Notes

Range of assay up to 33 mM

If expected range <1 mM, use 10 or 20 µl sample

Change standards for effluent sample.

Roche kit Unimate 5 GLUC HK 3672 4

h) Glucose 6-phosphate (G6P) and fructose 6-phosphate (F6P)

Glucose 6-phosphate dehydrogenase

Glucose 6-phosphate + NADP↔ 6-phosphoglucono-δ-lactone + NADPH

Phosphoglucoisomerase

Fructose 6-phosphate ↔ Glucose 6-phosphate

i) Reagent mixture

Solution	Molec Wt	Stocks Conc (mM)	Content	Other	Cobas /cuvette Vol (µl)	/35 cuv (µl)
Tris (HCl)	121.4	200	24.22g/l	pH 7.5	40	1400
ATP	605.2	10	0.0605/10 ml		8	280
$MgCl_2$	203.3	1000	203.3g/l		4	140
NADP	743.4	13.5	0.1g/10 ml	1%	4.44	156
H_2O					63.56	2224
Total					120	4200

ii) Start reagents

1. G6PDH (350 U/mg - 5 mg/ml) 0.5 µl/sample (5 µl of 1:10 - 20 µl +180 µl H_2O in P1)
2. PGI (350 U/mg - 2 mg/ml) 0.5 µl/sample (5 µl of 1:10 - 20 µl +180 µl H_2O in P2)

iii) Standards

0.03125 - 0.25 mM G6P (304 1 MW) and F6P (304.1 MW) - in same cups

Stocks 10 mM (0.0304g/10 ml and 0.0304g/10 ml)

iv) Calculations

(M2 - M1)/slope = G6P mM (A5 - M2)/slope = F6P mM

v) Notes

Range of assay < 0.5 mM

i) Dihydroxyacetone phosphate (DHAP) and Glyceraldehyde 3-phosphate (GAP) and Fructose 1,6-diphosphate (FDP)

Aldolase

FDP ↔ GAP

Triosephosphate isomerase

2 GAP ↔ 2 DHAP + 2 NADH + 2 H^+

Glycerol 3-phosphate dehydrogenase

2 DHAP + 2 NADH + 2H^+ ↔ 2 αGP + 2 NAD^+

i) Reagent mixture

Solution	Molec Wt	Stocks Conc (mM)	Content	Other	Cobas /cuvette Vol (µl)	/35 cuv (µl)
$NADH.Na_2$	709.4	14.1	0.1g/10 ml	1%	0.048	109
Tris (HCl)	121.1	200	24.22 g/l	pH 7.5	12	2100
H_2O					107.9	1991
Total					120	4200

ii) Start reagents

1. GDH (170 U/mg - 2 mg/ml) 1 µl/sample (20 µl of 1:20 - 40 µl + 760 µl H_2O in R - change after first pipetting)

2. TIM (5000 U/mg - 10 mg/ml) 0.5 µl/sample (5 µl of 1:10 - 20 µl +180 µl H_2O in P1)

3. Aldolase (9 U/mg - 10 mg/ml) 0.5 µl/sample (5 µl of 1:10 - 20 µl +180 µl H_2O in P2)

iii) Standards

0.03125 - 0.25 mM DHAP (170.1 MW), GAP (170.1 MW) and FDP (550.2 MW) in same cups

Stocks 10 mM (0.0701 g/10 ml; 0.0170 g/10 ml; 0.0550 g/10 ml)

iv) Calculations

(M2 - M1)/slope = DHAP mM (M3 - M2)/slope = GAP mM (A5 - M3)/slope = FDP mM

j) Pyruvate

Lactate dehydrogenase

$$\text{Pyruvate} + \text{NADH} + H^+ \leftrightarrow \text{Lactate} + NAD^+$$

i) Reagent mixture

Solution	Molec Wt	Stocks Conc (mM)	Content	Other	Cobas /cuvette Vol (µl)	/35 cuv (µl)
$NADH.Na_2$	709.4	14.1	0.1g/10 ml	1%	3.41	109
Tris (HCl)		200		pH 7.6	60	2100
H_2O					56.6	1991
Total					120	4200

ii) Start reagents

1. LDH (250 U/mg - 100 mg/10 ml) 0.5 µl/sample (5 µl of 1:10 - 20 µl +180 µl H_2O in P1)

iii) Standards

0.03125 - 0.25 mM pyruvate (110 MW)

Use fresh standard each day

iv) Calculations

(A3 - M1)/slope = Pyruvate mM (as printed)

v) Notes

Assay on same day as extraction

k) L-Lactate

Lactate dehydrogenase

$$\text{Pyruvate} + \text{NADH} + H^+ \leftrightarrow \text{Lactate} + NAD^+$$

i) Reagent mixture

Solution	Molec wt	Stocks Conc (mM)	Content (stocks)	Other	Cobas /cuvette Vol (µl)	/35 cuv (µl)
Hydrazine SO_4	130.1	799	20.8 g			
+ EDTA	404.47	9.9	0.8 g			
+ glycine	75.07	1200	30 g/ 200 ml	pH 9.3		
+ NaOH	40	2 N	80 g/l	- 9.5	60	2100
NAD^+	663.4	15.1	0.1 g/10 ml	0.01	7.99	280
H_2O					52	1820
Total					120	4200

ii) Solutions

Hydrazine buffer - weigh out solutes and add water to final volume of 200 ml. Titrate with 2 N NaOH, with approximately equal volumes to pH 9.3-9.5.

iii) Start reagents

1. LDH (250 U/ml - 100 mg/10 ml) 0.5 µl/sample (5 µl of 1:10 - 20 µl +180 µl H_2O in P1)

iv) Standards

0.0625 - 0.5 mM Na-Lactate (112.1 MW)

Stocks 10 mM (0.0112 g/10 ml)

v) Calculations

(A10 - M1)/slope = Lactate mM

vi) Notes

Limit of assay 5 mM

(For higher concentrations increase diluent volume/decrease sample volume)

l) L-glycerol 3-phosphate (αglycerophosphate - αGP)

Glycerol 3-phosphate dehydrogenase

αglycerophosphate + NAD^+ ↔ glycine 1-phosphate + NADH + H^+

i) Reagent mixture

Solution	Molec Wt	Stocks Conc (mM)	Content	Other	Cobas /cuvette Vol (µl)	/35 cuv (µl)
Hydrazine SO_4	130.1	160	20.8 g			
+ EDTA	404.47	2	0.8 g			
+ glycine	75.07	400	30 g/200 ml		60	2100
+ NaOH	40	2N	80 g/l	pH 9.5		
NAD^+	663.4	15.1	0.1 g/10 ml	1%	8	280
H_2O					52	1820
Total					120	4200

ii) Start reagents

1. GDH (170 U/mg - 2 mg/1 ml) 0.5 µl/sample (5 µl of 1:10 - 20 µl +180 µl H_2O in P1)

iii) Standards

0.03125 - 0.25 mM αGP (216.1 MW)

Stocks 10 mM (0.0216g/10 ml)

iv) Calculations

(M2 - M1)/slope = αGP mM (as printed)

m) L-alanine

Alanine dehydrogenase

$$\text{L-alanine} + NAD^+ + H_2O \leftrightarrow \text{Pyruvate} + NADH + NH_4^+$$

i) Reagent mixture

Solution	Molec Wt	Stocks Conc (mM)	Content	Other	Cobas /cuvette Vol (µl)	/35 cuv (µl)
Hydrazine SO_4	130.1	160	20.8 g			
+ EDTA	404.47	2	0.8 g			
+ glycine	75.07	400	30 g/200 ml			
+ NaOH	40	2N	80 g/l	pH 10	100	3500
NAD^+	663.4	15.1	0.1g/10 ml	1%	20	700
H_2O						
Total					120	4200

ii) Start reagents

1. ADH (20 U/mg) 0.5 µl/sample (5 µl of 1:10 - 20 µl +180 µl H_2O in P1)

iii) Standards

0.03125 - 0.25 mM L-Alanine (89.1 MW)

Stocks 10 mM (0.0089 g/10 ml)

iv) Calculations

(A6 - M1)/slope = L-alanine mM

n) Citrate

Citrate lyase

Citrate ↔ oxaloacetate + acetate

Malate dehydrogenase

Oxaloacetate + NADH + H^+ ↔ malate + NAD^+

Oxaloacetate decarboxylase

Oxaloacetate ↔pyruvate + CO_2

Lactate dehydrogenase

Pyruvate + NADH + H^+ ↔ Lactate + NAD^+

i) Reagent mixture

Solution	Molec Wt	Stocks Conc (mM)	Content	Other	Cobas /cuvette Vol (µl)	/30 cuv (µl)
Glycylglycine	132.1	0.5		pH 7.8		
+ MDH			136 U			
+ LDH			280 U			
+ $NADH.Na_2$	709.4	10	6 mg	1.4 g/12 ml H_2O	57.5	2012
H_2O					62.5	2188
Total					120	4200

ii) Start reagents

1. CL (12 U/50 mg) dilute with 300 µl H_2O in P1

iii) Standards

0.0625 - 0.5 mM Citrate (192.1 MW)

Stocks - 0.395 g/l in kit (2.056 mM)

iv) Calculations

(A2 - M1)/slope = Citrate mM

v) Notes

Citric acid Boehringer Mannheim kit no 139 076

o) Malate

Malate dehydrogenase

Malate + NAD^+ ↔ Oxaloacetate + NADH + H^+

Glutamate-oxaloacetate transaminase

Oxaloacetate+ L-glutamate ↔ L-aspartate + 2-oxoglutarate

i) Reagent mixture

Solution	Molec Wt	Stock Conc (mM)	Content	Other	Cobas /cuvette Vol (µl)	/35 cuv (µl)
Glycylglycine	132.1			pH 10		
+ L-glutamic acid	147.1		440 mg	/30 ml	57.14	2000
NAD^+	663.4		210 mg	/6 ml H_2O	11.43	400
H_2O					51.43	1800
Total					120	4200

ii) Start reagents

1. GOT (160 U/0.4 ml) as is in kit
2. MDH (2400 U/0.4 ml) as is in kit

iii) Standards

0.0625 - 0.5 mM Malate (134.1 MW)

Stocks 0.204 g/l in kit - 1.5 mM

iv) Calculations

(A10 - M1)/slope = L-malate mM

v) Notes

L-malic acid Boehringer Mannheim kit no 139 068

p) Glycolytic flux

Glycolytic flux is measured as the rate of cleavage of a H^+ from the glucose molecule to form water. The H^+ is traced using tritium (modified from [386, 468]). With D [2-^{3}H] glucose, the $^3H^+$ is cleaved in the phosphoglucoisomerase reaction (G6P to F6P).

i) Materials

D [2-^{3}H] glucose (1 mCi/ml - supplied by Amersham) was added to the perfusate. 220 µl of D [2-^{3}H] glucose/L Krebs (11 mM glucose) was used with an activity of 20 µCi/mmol glucose

Glass Pasteur pipettes (short form) were inserted into racks, and stoppered with cotton wool. Dowex-1 borate was used to retard the glucose and enable ^3H_2O to be collected.

Dowex-1 borate was made from Dowex-1 (chloride form; 4% cross-linked, 100-200 dry mesh from Sigma) which comes as a dry powder. The powder was saturated with 2 N Na-borate (50 g/l NaOH + 100 g/l boric acid). The solution was washed several times with distilled water (3-4 volumes of water, allow to settle and pour off) to remove excess NaCl until the pH of the resin was that of water. (The resin can be stored in the fridge in a suspension of water to retard bacterial growth. For long term storage, the resin must be filtered and stored as a dry paste in the freezer.)

ii) Method

1) Place approximately 1 ml of the resin in the column, ensuring that there are no bubbles. Wash the column twice with distilled water and allow to drain.
2) Add 200 µl of coronary effluent sample (deproteinise blood if this is used).
3) Wash column with 2 x 0.5 ml distilled water.
4) Collect effluent in 10 ml scintillation cocktail in 20 ml vials and vortex (must be sufficient cocktail to the aqueous samples, such that cocktail does not remain cloudy).

iii) Counting

The sample is counted on a beta counter (Packard Tri-Carb 460) using a quench curve for tritium to obtain the dpm.

Blank - non-radioactive Krebs - 45-65 dpm

Total specific activity of 200 µl radioactive Krebs (not through Dowex columns)

Standards - 200 µl radioactive Krebs through Dowex columns (activity absent or very minimal)

iv) Calculations

Rate of glycolysis (g) = (A/F)

where A = measured rate of production of ^3H_2O in dpm/min/g wet (dry) wt

F = specific activity of glucose in dpm/µmol

A = [Sample dpm (in 200 µl) - standard dpm] * 5

= dpm/ml

= dpm/ml * coronary flow (ml/min) * heart weight (g)

= dpm/min/g heart weight

F = [Total dpm (in 200 µl)- standard dpm] * 5

= (dpm/ml)

= (dpm/ml) / glucose concentration (µmol/ml)

= dpm/µmol

3) SUPPLIERS

The Grass Model 79D recorder and S88 stimulator was supplied by Grass Instruments Co. (Quincy, MA, USA). The Gilson Minipuls 2 pump was obtained from Gilson International (Villers-le Bel, France). The Christ Alpha 1-4 freeze dryer was obtained from Martin Christ Gefriertrocknungsanlagen GmbH (Osterode am Harz, Germany). The Sigma 202MK centrifuge was supplied by SIGMA Chemical Company (St Louis, MO, USA). The COBAS Fara II was supplied by Hoffmann-La Roche Diagnostics Division (Basel, Switzerland). The Packard Tri-Carb 460 beta counter was obtained from Packard Instrument Co. (Meriden, CT, USA).

The majority of enzymes were supplied by Boehringer Mannheim GmbH (Mannheim, Germany), in the concentrations and activities quoted. Substrates were also supplied by Boehringer Mannheim, or else by Sigma Chemical Company (St Louis, MO, USA). Some standard chemicals were supplied by BDH Chemicals Ltd (Poole, England), Saarchem (Pty) Ltd (Krugersdorp, South Africa) and E. Merck (Darmstadt, Germany). D[2-^{3}H] glucose was supplied by Amersham Life Sciences (Little Chalfont, Buckinghamshire, UK). Ready-Safe Scintillation cocktail was supplied by Beckman Instruments Inc. (Fullerton, CA, USA). Scintillation vials were supplied by Packard Instrument Co. (Meriden, CT, USA).

4) STATISTICS

The ANOVA two-way analysis of variance was used to determine significance among groups, followed by application of the modified t-test for comparison of individual groups. The modified student's t-test was used to determine significance between two groups. A $p<0.05$ was taken as the level of significance. At least 6 hearts were used in each group. GraphPAD Instat™ computer programme was used for statistics, and MicroCal Origin™ for regression analyses.

Results 1. Coronary flow and glucose delivery as determinants of contracture in the ischaemic myocardium

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ABSTRACT

Ischaemic contracture may be avoided by the provision of glucose under low flow conditions [424]. However, accumulation of harmful metabolic end products may inhibit glycolytic flux and lessened the benefit of glucose. We assessed whether during increasingly severe flow restriction, provision of glucose might be harmful rather than beneficial, using the Langendorff perfused rat heart. Ischaemic contracture (resting tension expressed as percent of pre-ischaemic developed pressure) was measured via a left ventricular balloon. Reductions in flow to 0, 0.015, 0.03, 0.06, 0.1, 0.2 or 0.4 ml/g wet wt/min over 60 min were tested. At zero flow, peak contracture was 61.4 ± 3.5% (± S.E.) but fell to 15.6 ± 6.3% with 0.4 ml/g wet wt/min ($p<0.05$) in the presence of 11 mM glucose. Time-to-onset of contracture was significantly delayed by the higher coronary flows. At coronary flows down to zero, the effect of glucose was inconstant or absent, but not harmful. With the residual flow at 0.2 ml/g wet wt/min, a dose response to glucose in ischaemia was elicited, using concentrations of 0, 2.5, 5.5, 11 or 22 mM. Maximum protection against ischaemic contracture was found with 11 mM glucose. However, once contracture occurred, functional recovery was severely impaired in all cases. Reducing glycogen prior to low flow ischaemia (0.2 ml/g wet wt/min) with 11 mM glucose increased peak contracture, and reduced the time-to-onset of contracture. Increased pre-ischaemic glycogen had little effect on contracture.

Glycolytic flux fell in proportion to the coronary flow. However, there was an increased glucose extraction at lower flows of 0.1 and 0.2 ml/g wet wt/min, suggesting that it is the rate of delivery (i.e. coronary flow) which is the rate limiting step rather than enzyme inhibition by accumulated metabolites. If flow were further reduced, metabolite accumulation would become more important, such that with no flow, this would be the determinant of glycolytic flux rate.

In our model, the two requirements for optimal protection from ischaemia were (i) provision of glucose (11 mM was optimal) and (ii) an adequate coronary flow to deliver the glucose and remove end product inhibition (greater than 0.06 ml/g wet wt/min).

INTRODUCTION

Ample experimental evidence shows that the provision of glucose (or glucose-insulin-potassium) may have therapeutic potential in the treatment of myocardial ischaemia and infarction [14, 123, 401, 502]. Indices of protection in the isolated perfused heart include: reduction of enzyme leakage [104], decreased reperfusion-induced arrhythmias [28], attenuation of stunning [133] as well as reduced ischaemic contracture [123, 424]. The mechanisms involved may include an increased production of glycolytic ATP [123, 424] with a resulting improvement in the control of cell calcium [229, 405] inhibition of the ATP-sensitive K channel [571], and a decrease of tissue cyclic AMP in the ischaemic zone [438]. In addition, glucose plus insulin given over 180 min in isolated rabbit hearts perfused at only 1 ml/g wet wt/min with an erythrocyte suspension was markedly protective [123]. Recent clinical studies have also shown a beneficial effect of glucose (with insulin and potassium - GIK) when given to patients with refractory left ventricular failure after aortocoronary bypass surgery [84].

Despite such convincing evidence for a protective role of glucose, the more general clinical use of GIK has in part been restrained by the finding of Neely and Grotyohann [387] that a greater rate of glycolysis from glycogen had detrimental effects in zero flow global ischaemia. The harm was attributed to the accumulation of metabolites, especially lactate and protons, which with zero flow could not be washed out of the tissue. The phenomenon of preconditioning also suggests a possible protective effect of glycogen depletion resulting from the initial period(s) of ischaemia [378, 588]. This finding has been corroborated by studies showing the protective effect of preconditioning is linked to an attenuated rise in proton [512] and lactate [18] concentrations in ischaemia, which in turn are attributed to reduced breakdown of glycogen. In addition, when lactate is added to the perfusate prior to ischaemia, a reduction in functional recovery has been found [239, 387]. However, these models investigated utilisation of glycogen as the glycolytic substrate, and not glucose. A difference in the protective role of glucose versus glycogen has been suggested [424, 470].

The potential role of a severely restricted residual coronary flow, and its effect on removal of the potentially harmful end products of glycolysis, has not been investigated. Hearse et al. [193] added glucose to a cardioplegic solution used to arrest hearts, and found a deleterious effect, contrary to subsequent observations where glucose in a multidose protocol (i.e. with washout of metabolites) was used [563]. While the classic studies of Neely et al. [390] and Rovetto et al. [468] showed an increase in glucose utilisation at reduced coronary flow rates compared to normal flows in an isolated perfused rat heart, the flows used in these studies were relatively high (0.6 - 6 ml/g wet wt/min), albeit restricted, and thus may not reflect true *in vivo* ischaemia, where normal coronary flows are lower

than in an isolated organ. The role of glucose in severe low flow conditions in protecting against ischaemic damage has not been fully clarified.

We, therefore, tested the hypothesis that the residual coronary flow (less than 4% of control flows in perfused hearts, and less than 7% of normal *in vivo* coronary flows) to the ischaemic myocardium could be the crucial factor determining whether provision of glucose would be beneficial or harmful in the development of ischaemic contracture. The latter is potentially an irreversible event and a cause of "no-reflow" on reperfusion [210]. We used the isolated globally ischaemic rat heart to investigate the possible benefit of addition of various concentrations of glucose over a range of low coronary flows, much lower than those previously used [424]. Lactate washout and glycolytic flux were measured, and ischaemic injury was assessed by the rate and extent of ischaemic contracture. In addition, reperfusion arrhythmias and functional recovery were recorded in some studies, to assess any correlation between ischaemic contracture and recovery. Because of the potential contribution of glycogen to the glycolytic pathway during ischaemia, we also altered tissue glycogen content prior to low flow ischaemia.

The major finding of this study was that the severity of coronary flow restriction plays a critical role in determining the rate of delivery of glucose and hence its beneficial effect. At no flow was a detrimental effect of glucose observed. In addition, there was a greater benefit during ischaemia of glycolysis from glucose than from glycogen, confirming the proposal of Owen et al. [424].

METHODS

ANIMALS AND ISOLATED RAT HEART APPARATUS

The isolated Langendorff-perfused rat model with a left ventricular balloon was used, as described in Methods.

PROTOCOLS

Low flow ischaemia. Three different protocols were employed:

(i) An initial comparison of two ischaemic low flows, and the absence or presence of glucose at each flow, was made in terms of ischaemic contracture, functional recovery and glucose utilisation. After 15 min normoxic perfusion, hearts were made globally ischaemic for 30 min at low flows of 0.1 or 0.2 ml/g wet wt/min. These residual flows of oxygenated buffer were delivered by a Gilson Minipuls pump. Glucose was either absent, or present at a concentration of 11 mM at each flow. The hearts were then reperfused for 25 min with perfusate containing 11 mM glucose, and post-ischaemic functional recovery was recorded.

During ischaemia, the coronary effluent was collected over each 5 min period, and glycolytic flux and lactate washout were measured (see later). A sample of coronary effluent was also taken just before ischaemia as control. On reperfusion, the total coronary effluent was collected over the first 2 min of reperfusion, and this was analysed for lactate content. The extent of glycogen breakdown and ATP production during ischaemia was calculated (see later). Ten hearts were used in each group.

(ii) The role of residual flow in determining the extent of ischaemic contracture was investigated, and the requirement for glucose evaluated at each flow. After 15 min normoxic perfusion, hearts were rendered ischaemic for 60 min, during which time, oxygenated perfusate containing 11 mM glucose was infused at different flows (0, 0.015, 0.03, 0.06, 0.1, 0.2, and 0.4 ml/g wet wt/min). Initial experiments with a flow of 0.4 ml/g wet wt/min with 11 mM glucose showed that ischaemic contracture was markedly delayed in this group; thus a period of 60 min ischaemia was chosen to ensure attainment of peak contracture. The hearts were not reperfused after this extended period of ischaemia. Six hearts were used in each group.

In a second series of experiments with the above flows, glucose was omitted throughout the ischaemic period. In addition, to ensure that no glucose was trapped in the heart during ischaemia, the hearts were perfused with glucose-free medium for 1 min just prior to the onset of ischaemia. Six hearts were used in each group.

(iii) A dose response curve to glucose was elicited at two flows, in terms of ischaemic contracture and functional recovery. After 15 min normoxic perfusion, hearts were made ischaemic for 30 min at low flows of 0.06 or 0.2 ml/g wet wt/min, with glucose concentrations of 0, 2.5, 5.5, 11 or 22 mM. The hearts were then reperfused for 25 min with perfusate containing 11 mM glucose and functional

recovery was recorded. An ECG electrode was placed in the apex of the hearts in order to record the incidence of ventricular arrhythmias. Six to eight hearts were used in each group.

Pre-ischaemic modification of tissue glycogen content. In a separate series of experiments the role of glycogenolysis in determining the degree of ischaemic contracture in the absence and presence of glucose was investigated. Different glycogen levels prior to the start of ischaemia were achieved by perfusing hearts as follows:

(i) 20 min perfusion with 11 mM glucose

(ii) 20 min perfusion with 5 mM acetate

(NaCl was reduced by 5 mM to accommodate Na-acetate)

(iii) 20 min substrate-free perfusion

(iv) 20 min perfusion with 11 mM glucose + 1 U/L insulin.

All hearts were then subjected to ischaemia for 60 min, at a coronary flow of 0.2 ml/g wet wt/min, in the absence or presence of 11 mM glucose, following which the hearts were clamped. A parallel series of experiments were carried out, in which the hearts were freeze-clamped just prior to the onset of ischaemia. The frozen tissue was analysed for glycogen, lactate, adenosine triphosphate (ATP) and creatine phosphate (CP) contents. Six hearts were used in each group.

MEASUREMENT OF ISCHAEMIC CONTRACTURE, VENTRICULAR ARRHYTHMIAS AND FUNCTIONAL RECOVERY

Peak ischaemic contracture and time to onset of contracture were recorded as described in Methods. Arrhythmias were defined as the occurrence of ventricular tachycardia and/or ventricular fibrillation. These were quantified according to the Lambeth conventions [566] and the incidence of arrhythmic hearts following reperfusion was expressed as a percentage of the total number of hearts in each group. No arrhythmias were observed during ischaemia in any hearts.

Functional recovery, where measured, was indicated by developed pressure after 5 min (early) and 25 min (late) reperfusion expressed as a percentage of pre-ischaemic developed pressure, measured at the end of the stabilisation period. Early and late reperfusion are used to indicate the rate of return to function, or the degree of stunning. Heart rates were little changed, thus developed pressure was sufficient to express functional recovery.

BIOCHEMICAL MEASUREMENTS

Glycolytic flux and glycogenolysis. Glycolytic flux (from glucose) was determined as described in Methods. Effluent lactate was assayed according to Bergmeyer [24]. Both lactate washout and glycolytic flux are expressed as μmol 6-carbon (C_6) units to allow for comparison of equivalent units. From the average wet heart weight, (1.05 ± 0.003 g), all values were expressed as per g wet weight. The rate of utilisation (production) /g wet wt/min corrected for coronary flow is calculated by

$$\mu mol\ C_6\ units/ml \times coronary\ flow\ (ml/g\ wet\ wt/min) = \mu mol\ C_6/g\ wet\ wt/min$$

Glycogenolysis (expressed in μmol C_6 units) was estimated from the lactate in excess of that accounted for by glycolytic flux, where total lactate production in ischaemia was derived from the sum of the lactate efflux in ischaemia plus that during the first 2 min of reperfusion (as an estimate of tissue lactate at the end of ischaemia, corrected for by subtracting the rate of lactate efflux in normal perfusion, which was an average of 0.5 ± 0.1 μmol C_6/g wet wt/min). No allowance was made for the input of glycolytically derived pyruvate into the Krebs cycle or to alanine, any contribution to which would reduce total lactate efflux and thus give an underestimation of glycogenolysis. Anaerobic ATP production during ischaemia was calculated using 2 mol ATP per mol glucose utilised, and 3 mol ATP per mol of C_6 units of glycogen broken down.

Contribution of oxidative phosphorylation. The pO_2 of the solution delivered to the hearts during ischaemia was measured at 500-550 mmHg. Assuming an average uptake of 95% of the delivered oxygen (measured in a representative number of hearts - data not shown), this resulted in an arterio-venous difference in pO_2 of 475-520 mmHg. Using the relationship

$$PV = nRT$$

the μmol O_2/ml solution at a given pO_2 at 37°C can be calculated using

$$\text{no of mol } O_2/L \text{ soln} = \frac{pO_2/760\ mmHg \times Vg}{0.08205\ L.atm.mol^{-1}.K^{-1} \times 310K}$$

where $Vg = \alpha\ pO_2/760\ mmHg \times Vol\ soln$

where α = solubility coefficient of O_2 in saline solution (0.155 N NaCl) at 37°C

= 0.02273 ml O_2/ml saline per atmosphere [11]

The maximum amount of O_2 taken up per min can then be calculated from the coronary flow. At a flow of 0.2 ml/g wet wt/min, and a pO_2 difference of 500 mmHg, this means an uptake of 0.08 μmol O_2/g wet wt/min. Assuming that glucose (or C_6 units from glycogen) is the sole substrate contributing to acetyl Co A formation, this would mean a maximum production of 0.46 μmol ATP/g wet wt/min from oxidative phosphorylation (given that 6 mol O_2 are required per mol glucose to give an additional 36 mol ATP under aerobic conditions). Over 30 min ischaemia, this would be a maximum contribution of 13.9 μmol ATP. At a flow of 0.1 ml/g wet wt/min, these values would be halved. If fatty acids from the breakdown of endogenous triglycerides were the sole substrate contributing to oxidative phosphorylation, a maximum of 13.6 μmol ATP over 30 min ischaemia would be produced, given a P:O ratio of 2.8 [424]. Thus any contribution of endogenous fatty acids would only serve to reduce oxidative phosphorylation of glucose or glycogen, while not affecting total maximum production of ATP from aerobic metabolism.

Analysis of tissue metabolites. Hearts taken for analysis were freeze dried before extraction with perchloric acid to determine tissue levels of adenosine triphosphate (ATP), creatine phosphate (CP)

and lactate, assayed according to Bergmeyer [24]. Tissue glycogen was measured by assaying the C_6 unit content (glucose assay) after potassium hydroxide degradation according to Good et al. [160]. Tissue levels are expressed as μmol/g wet weight, where wet weight is calculated as dry weight x 5.

STATISTICAL PROCEDURES

All results are expressed as mean ± S.E. Analysis of variance (ANOVA) tests were performed to determine whether any significant differences existed among groups, after which the modified Student's t test was used to obtain levels of significance for individual comparisons within each group. The t test was used to examine individual differences between groups. The Fisher's Exact test was used to determine statistical differences in incidence of ventricular arrhythmias. $p<0.05$ was taken to indicate significance.

RESULTS

LOW FLOW ISCHAEMIA WITH AND WITHOUT GLUCOSE - ISCHAEMIC CONTRACTURE WITH METABOLIC MEASUREMENTS

In the first set of experiments, a basic two way comparison of flow (0.1 vs. 0.2 ml/g wet wt/min) and glucose (either zero or 11 mM) was made. Ischaemic contracture and recovery on reperfusion were recorded, and glycolytic flux rates and lactate washout determined. Ischaemic contracture, illustrated by the change in diastolic pressure (mmHg) (Figure 1), was influenced both by coronary flow and the presence of glucose.

Mechanical function. Left ventricular developed pressure prior to ischaemia was no different between groups, with a mean of 124.3 ± 2.5 mmHg (Figure 1). Heart rate was 325.6 ± 9.4 beats/min, and coronary flow 12.2 ± 0.2 ml/g/min.

With a reduction in flow to 0.1 ml/g/min, the rise in diastolic pressure in ischaemia was significantly greater in the absence of glucose (Figure 1). Peak contracture was 51.0 ± 2.2% of pre-ischaemic developed pressure in the absence of glucose , compared with 36.5 ± 4.5% in the presence of glucose ($p<0.02$). However, the time-to-onset of contracture was similar in both groups (13.2 ± 1.2 and. 11.3 ± 0.9 min respectively). At the higher coronary flow (0.2 ml/g/min), the times-to-onset of contracture again were similar in the absence and presence of glucose (10.0 ± 0.8 and 11.5 ± 1.1 min respectively) and did not differ significantly from the values observed in the 0.1 ml/g/min flow groups. Peak contracture in the absence of glucose was 65.9 ± 2.9%, ($p<0.01$ vs. 0.1 ml/g/min) but the addition of glucose markedly reduced the severity of contracture (to 26.1 ± 3.5%, $p<0.01$ vs. zero glucose at 0.2 ml/g/min; $p<0.02$ vs. 11 mM glucose at 0.1 ml/g/min). At the higher flow, glucose had a greater inhibitory effect on contracture, and the absence of glucose was more detrimental.

On reperfusion, coronary flow returned to pre-ischaemic values in all groups but then diminished slightly with time. Heart rates during the early minutes of reperfusion were also slightly depressed but these returned to pre-ischaemic control values by 10 min reperfusion. Diastolic pressures increased slightly in all groups on reperfusion from values attained at 30 min ischaemia, and then fell gradually (see Figure 1). There were no differences between the groups. However, the initial recovery of developed pressure was significantly higher in the glucose-infused groups due to better recovery of systolic pressure, as shown in Figure 1. The hearts perfused with no glucose during ischaemia showed a significantly reduced systolic pressure, and a very poor recovery of developed pressure, in early reperfusion (14.7 ± 9.9 % at 0.1 ml/g/min; 4.9 ± 4.0 % at 0.2 ml/g/min), compared to hearts with glucose present in ischaemia (40.2 ± 10.9 % and 53.8 ± 11.2 % for the two flows, $p<0.05$ vs. lower flow). By late reperfusion, all hearts had recovered to a similar extent (41.6 ± 9.4 % and 55.5 ± 3.6 % (no glucose), and 40.3 ± 8.7 % and 58.9 ± 3.9% (11 mM glucose).

Metabolic data. Mean pre-ischaemic glycolytic flux rate in all groups was 1.5 ± 0.1 μmol/g/min. Such flux was rapidly and substantially decreased at values of 0.1 and 0.2 ml/g/min (Figure 2). Thereafter in the presence of glucose, flux rate increased with each 5 min period, reaching a plateau at 10-15 min of ischaemia. This plateau value was proportional to the residual coronary flow, being 0.51 μmol/g/min at 0.2 ml/g/min and 0.26 μmol/g/min at 0.1 ml/g/min. Total glucose utilisation over the 30 min ischaemic period was 5.7 ± 0.6 μmol/g (0.1 ml/g/min) and 12.6 ± 1.3 μmol/g (0.2 ml/g/min; $p<0.01$ vs. lower flow).

The percentage of glucose taken up of that delivered can be calculated as follows. Glucose uptake (μmol/g/min) was assumed to equal the measured glycolytic flux rate from glucose and can be related to the glucose delivery rate (coronary flow (ml/g/min) x 11 μmol/ml). The percentage uptake of glucose (Figure 3) was greatly increased in ischaemia, eventually to a plateau of about 24% of delivered glucose (compared with a pre-ischaemic uptake of 0.9 ± 0.1%). Thus although the absolute amount of glucose taken up was reduced during ischaemia, the relative quantity of glucose taken up from that available to the ischaemic myocardium was greatly increased. The percentage uptake reached a plateau after 10-15 min ischaemia and was not influenced by the different low coronary flows (0.1 or 0.2 ml/g/min).

Lactate washout /g/min was initially reduced in all groups when compared to pre-ischaemic values. However, this was followed by a subsequent increase to a plateau level in glucose-perfused hearts. In contrast, in glucose-free hearts the lactate production from the breakdown of glycogen did not achieve a steady state and declined after 10-15 min of ischaemia. At a coronary flow of 0.1 ml/g/min, total lactate washout during ischaemia was not affected by the addition of glucose (7.1 ± 0.7 μmol C_6 units/30 min with zero glucose, versus 7.9 ± 1.0 μmol C_6 units/30 min with 11 mM glucose). At a coronary flow of 0.2 ml/g/min, lactate washout values peaked early and were higher than those seen in the lower flow group ($p<0.01$). In addition, lactate output was increased by the presence of glucose (10.5 ±0.6 μmol C_6 units/30 min with zero glucose vs. 15.2 ± 0.7 μmol C_6 units/30 min with glucose, $p<0.01$).

In the glucose-perfused groups, the measured glycolytic flux rates could account for most of the lactate produced (see Figure 2). There was, however, an excess lactate washout of 20-30% during ischaemia that could not be accounted for by the measured glycolytic flux from glucose, and was presumably derived from the breakdown of glycogen [424]. A more detailed analysis of total lactate production during ischaemia was achieved by measuring the amount of lactate flushed out of the heart at the time of reperfusion (an indication of total lactate remaining in the tissue at the onset of reperfusion) and adding this value to the total lactate washed out of the heart during ischaemia. The washout values for lactate during reperfusion in the 0.1 ml/g/min group were 8.3 ± 1.2 μmol C_6 units for zero glucose and 9.1 ± 1.6 μmol C_6 units for 11 mM glucose. In the 0.2 ml/g/min group the values

were 4.3 ± 0.6 μmol C_6 units with zero glucose and 6.4 ± 0.8 μmol C_6 units with 11 mM glucose, indicating better washout of the tissue at the higher flow during ischaemia. Once the lactate that could be accounted for by glycolytic flux in ischaemia was subtracted, the remaining lactate was presumed to be derived from the breakdown of glycogen (Figure 4). The estimated breakdown of glycogen was greater in the absence of glucose (15.4 ± 1.1 and 14.8 ± 0.7 μmol C_6 units/30 min at 0.1 and 0.2 ml/g/min respectively), while a greater coronary flow in the presence of glucose inhibited glycogen breakdown (11.3 ± 1.7 (0.1 ml/g/min) vs. 8.9 ± 2.2 μmol C_6 units/30 min (0.2 ml/g/min), $p<0.01$ vs. zero glucose groups).

The total estimated production of ATP from the anaerobic breakdown of glucose and glycogen over the whole ischaemic period was similar in all groups (Figure 5), with values of 46.3 ± 3.3 and 44.3 ± 2.0 μmol/g with no glucose, and 45.2 ± 5.33 and 52.0 ± 4.8 μmol/g with 11 mM glucose for the two flows. However, when the contribution of glucose to anaerobic ATP production is taken into account, the importance of the source of glycolytic ATP becomes evident in terms of protection against ischaemic contracture. At a low flow of 0.1 ml/g/min, glycolytic flux from glucose contributed 28.0 ± 4.1 % of total anaerobic ATP, while at a flow of 0.2 ml/g/min, this was increased to 54.1 ± 7.6 % ($p<0.01$), with a corresponding reduction in contracture.

The total ATP production in low flow ischaemia also includes the contribution of residual oxidation from the continued delivery of oxygen, even at very low coronary flows. This could utilise some of the pyruvate derived from glycolysis, introducing an error into the above calculations. ATP production from oxidative phosphorylation could account for as much as 13.9 μmol/30 min at the higher flow (0.2 ml/g/min) and half of this value at the lower flow (see Methods). Under optimal conditions, aerobic production of ATP may have contributed a maximum of 12-25 % of total ATP production during ischaemia. Assuming that all of the oxygen taken up was utilised in the oxidative phosphorylation of glucose, the calculated oxygen uptake suggests that a maximum of 4.0 ± 0.6 % at 0.1 ml/g/min, and 3.4 ± 0.4 % at 0.2 ml/g/min of the measured glycolytic flux could enter the Krebs cycle, with the balance being converted to lactate. When glycogen breakdown is taken into account, the contribution from glucose is even less, with less than 3 % of total glycolytic substrate going on to oxidative phosphorylation. The aerobic utilisation of triglycerides would reduce this amount even further, by utilising some of the available oxygen. Thus, the residual oxidative phosphorylation would only account for only negligible amounts of the substrate going through the glycolytic pathway, reducing the significance of error in the above calculations.

CONTRACTURE WITH CHANGES IN FLOW WITH AND WITHOUT GLUCOSE

The initial experiments indicated that ischaemic contracture was influenced both by coronary flow and glucose. We next determined the relationship of contracture to coronary flows in the range of zero to 0.4 ml/g/min in the absence or presence of glucose (11 mM). In comparing the times-to-onset of

contracture, no significant difference between any of the groups was observed, except with the highest flow of 0.4 ml/g/min with 11 mM glucose, where a prolonged delay (28.8 ± 8.8 min; $p< 0.01$ vs. all other groups) was observed (Table 1A). Peak contracture, in the absence of glucose, increased as the flow was increased from zero to 0.4 ml/g/min (65.5 ± 3.1% vs. 82.8 ± 4.2%, $p<0.05$), although the incremental changes between the different flows were slight (Table 1A). In the presence of 11 mM glucose, the opposite trend was observed, such that peak contracture decreased as flow increased (61.4 ± 3.5% at zero flow, vs. 15.6 ± 6.3% at 0.4 ml/g/min, $p<0.05$). This glucose-dependent reduction was significant when flows of 0.1 ml/g/min and higher were compared with zero flow (Table 1A). When the zero and 11 mM glucose groups were compared at each flow, marked differences in peak contracture were observed with flows of 0.03 ml/g/min and higher ($p<0.05$).

From these data it can be concluded that (i) glucose, at a concentration of 11 mM, reduced peak contracture compared to the absence of glucose, (ii) the benefit was most apparent at a flow of 0.4 ml/g/min, although still detectable even at 0.03 ml/g/min; and (iii) at the lowest coronary flow tested (0.015 ml/g/min), no effect of glucose on ischaemic contracture was found.

DOSE RESPONSE TO GLUCOSE

A dose response to glucose at two different low flows was elicited, in terms of ischaemic contracture (Table 1B) and reperfusion recovery (Table 2B), with 30 min of ischaemia, by which time peak contracture had been reached in all groups. Functional data for all groups prior to ischaemia are shown in Table 2A. No significant differences in heart rate, systolic pressure or diastolic pressure between groups were seen in the control period.

Coronary flow of 0.06 ml/g/min. At this coronary flow (approximately 0.5% of the control pre-ischaemic values - 12.9 ± 0.5 ml/g/min), changes in glucose concentration ranging from zero to 22 mM in ischaemia had little effect on peak contracture (Table 1B). A large increase in diastolic pressure was observed in all hearts on reperfusion, approximately double that at the end of ischaemia (from about 50 to 100 mmHg). Recovery of developed pressure after 5 min reperfusion was very poor in all groups (Table 2B). With time, this failure of diastolic relaxation on reperfusion improved. While systolic pressures were reduced by about 20-30% from control pre-ischaemic values, they remained stable throughout the reperfusion period. After 25 min reperfusion, recovery of developed pressure was only about 18-25% in all glucose groups (Table 2B). There was, however, a significant reduction in the incidence of reperfusion ventricular arrhythmias with increasing glucose concentrations (Table 2B). When data from both sets of 0.06 ml/g/min groups with and without 11 mM glucose were combined ($n=12$) (Table 1A and 1B), a small but significant difference ($p<0.05$) in peak contracture was found (57.8 ± 2.3% - zero glucose vs. 50.6 ± 2.5% - 11 mM glucose).

Coronary flow of 0.2 ml/g/min. At this flow (approximately 1.5% of pre-ischaemic coronary flow - 13.8 ± 0.7), a dose response effect of glucose with respect to peak contracture was observed (Table

1B). Time-to-onset of contracture was shortest with 2.5 mM glucose, but no significant differences were found within the series (Table 1). Peak contracture was reduced at the glucose concentration range 5.5-22 mM, with an optimal response at 11 mM glucose (27.6 ± 3.7 % vs. 58.3 ± 3.2 % - zero glucose, $p<0.05$).

On reperfusion, diastolic pressures increased slightly compared to the level reached by 30 min ischaemia, although not to the extent seen with the lower flow of 0.06 ml/g/min. Systolic pressures were generally slightly depressed throughout reperfusion in all groups, being about 16% lower than control pre-ischaemic values in all hearts. Changes in heart rates did not contribute to changes in function. However, the incidence of reperfusion arrhythmias was significantly reduced by increasing glucose concentrations (Table 2B).

Developed pressure after 5 min reperfusion correlated well with peak contracture. Following glucose-free ischaemia, early recovery was 20.8 ± 5.8 %, while 11 mM glucose in ischaemia resulted in a rapid recovery to 54.8 ± 10.9 % by 5 min. There was some recovery over time, especially in the glucose-free group (to 57.5 ± 6.0%) (Table 2B), while the 11 mM group reached a final developed pressure of 68.3 ± 5.3% by 25 min. The groups with glucose at higher or lower concentrations did not recover as well, but the differences were not significant. Early recovery on reperfusion correlated well with ischaemic contracture and incidence of reperfusion arrhythmias, while recovery late in reperfusion did not.

When all peak contracture data obtained with 0.1 or 0.2 ml/g/min, with or without 11 mM glucose, were combined, significant differences were still found with the addition of glucose at both flows ($p<0.01$).

PRE-ISCHAEMIC ALTERATION OF GLYCOGEN LEVELS

A 20 min period of substrate free aerobic perfusion to reduce glycogen levels in the myocardium resulted in an increased diastolic pressure from 3.9 ± 0.4 mmHg (control) to 10.7 ± 1.3 mmHg (substrate free), and a reduced systolic pressure (95.0 ± 3.8 vs. 132.5 ± 4.3 mmHg) (Table 3B) prior to ischaemia. Developed pressures were therefore significantly reduced, from 128.6 ± 4.3 to 84.4 ± 4.4 mmHg ($p<0.05$). Acetate pre-perfusion was also used as a means of reducing glycogen levels (Table 3), as this had no effect on function (developed pressures 125.5 ± 2.8 mmHg), but did not reduce glycogen as much. Glucose + insulin pre-ischaemic perfusion, used to increase glycogen levels, also showed no differences in function compared to control (developed pressures 128.7 ± 11.9 mmHg). No differences in coronary flow or heart rate were seen between groups (Table 3).

Glycogen levels prior to ischaemia were reduced by acetate (10.0 ± 1.2 μmol C_6/g) or substrate-free aerobic perfusion (4.0 ± 0.7 μmol C_6/g) compared to control values with 11 mM glucose (16.1 ± 1.4 μmol C_6/g, $p<0.05$). Glycogen levels were significantly increased by 20 min glucose + insulin perfusion to 20.5 ± 1.1 μmol C_6/g ($p<0.05$). While no differences in ATP levels were seen

(Table 3A), acetate pre-perfusion resulted in increased tissue CP (6.4 ± 0.8 μmol/g), while substrate free perfusion reduced tissue CP levels (2.8 ± 0.3 μmol/g) compared to control hearts (4.7 ± 0.4 μmol/g, $p<0.05$). Following alteration in glycogen levels, the coronary flow was reduced to 0.2 ml/g wet wt/min, with or without glucose 11 mM present in the ischaemic period. The changes in diastolic pressure are illustrated in Figure 6.

Times-to-onset of contracture were profoundly affected by reduced glycogen levels (Table 3B), from 9.7 ± 1.7 min (control) to 3.1 ± 1.0 min (acetate) and 2.1 ± 0.1 min (substrate-free) when no glucose was present in ischaemia ($p<0.05$ vs. control). Similar values were found when glucose 11 mM was present during ischaemia (Table 3). An increased pre-ischaemic glycogen content, however, had little effect on time-to-onset (Table 3).

Peak contracture values were also altered by prior changes in glycogen levels. When no glucose was present during the ischaemic period, peak contractures increased to 88.5 ± 1.8% (acetate) and 160.2 ± 19.3% (substrate free) compared to 63.8 ± 1.9% of pre-ischaemic developed pressures (control glycogen levels with 11 mM glucose) (Table 3). If, however, glucose was present during ischaemia, peak contractures were 60.9 ± 6.7% and 137.0 ± 8.5% compared to 30.0 ± 4.0% respectively. Thus the presence of glucose helped to reduce the peak contracture with a low glycogen. In addition, the higher glycogen levels inhibited the fall off in diastolic pressure following peak contracture, which may be due to rupture of the cells.

Post-ischaemic levels of glycogen were significantly reduced by 60 min ischaemia in all groups. However, as would be expected, a higher pre-ischaemic glycogen resulted in a higher post-ischaemic glycogen, and a higher tissue lactate content. The presence of glucose also resulted in higher tissue glycogen content, indicating inhibition of glycogen breakdown in the presence of glucose. There is good agreement between the calculated rates of glycogen breakdown from the first series of experiments, and tissue levels of glycogen measured before the start of ischaemia, such that in substrate free hearts, most of the available glycogen was broken down. When glucose was present, the breakdown of glycogen was inhibited, in agreement with the present results. The possible contribution of pyruvate to alanine production has been excluded from the above calculations, and must be taken into account [521]. However, given the good agreement between ischaemic glycogen breakdown measured by tissue analysis, and estimated rates of glycogenolysis from the first series of experiments, the significance of the formation of alanine would be relatively small. Utilisation of glucose throughout 60 min ischaemia was shown by (i) the higher tissue lactate when glucose was available, despite reduced glycogenolysis and (ii) the fact that most of the lactate produced during ischaemia would be washed out under low flow conditions.

DISCUSSION

ISCHAEMIC CONTRACTURE AND FUNCTIONAL RECOVERY

Ischaemic contracture, or the clinical equivalent, the "stone heart" [86], is an important complication of severe ischaemia, hypothetically developing as a consequence of decreased ATP availability and/or high cytosolic Ca^{2+} levels (see [259] for review). Ischaemic contracture is associated with severe microscopic damage [14], and may, by mechanical compression, further compromise coronary flows already critically low, thereby precipitating irreversibility [123, 194, 210]. This observation may explain why the degree of residual or collateral flow is a major determinant of the rate and extent of ultimate cell death [194, 485] and the degree of recovery upon reperfusion.

Criteria for the benefit or harm of an intervention. Two main parameters - ischaemic contracture and recovery on reperfusion - were used to determine benefit or harm of an intervention. Ischaemic contracture, delimited by time-to-onset and peak, was used to indicate ischaemic injury. However, time-to-onset of contracture did not differ greatly between groups with two exceptions. Only when flow was increased to a "moderate" level i.e. 0.4 ml/g wet wt/min (Table 1), or when pre-ischaemic glycogen was significantly reduced (Table 3), was time-to-onset of contracture affected. Peak contracture was found to be a better indicator of ischaemic injury. In our studies, functional recoveries were very poor in all hearts after 25 min reperfusion, with a maximum recovery of about 60%, indicating a large degree of irreversibility in the reperfused tissue. The early rate of return of function (at 5 min), however, varied significantly between groups. The variability of early recovery indicates different degrees of stunning [413], and thus different degrees of reperfusion injury. In addition, the incidence of reperfusion arrhythmias differed significantly between groups. Thus interventions which increased peak contracture and the incidence of reperfusion arrhythmias, and reduced the rate of mechanical recovery of hearts were deemed to be deleterious.

Interaction of glucose and coronary flow. The data presented here indicate that the degree of protection conferred by glucose in an isolated perfused rat heart is dependent on the severity of flow reduction. At lesser degrees of ischaemia (flow: 0.4 ml/g wet wt/min) glucose was able to delay the onset of contracture and substantially reduce the severity of peak contracture. At flows of 0.2, 0.1 or 0.06 ml/g wet wt/min, the severity of contracture was still glucose-sensitive but the onset of contracture could not be delayed. The best results were found when glucose was present at a high physiological concentration (11 mM). At progressively lower flows (below 0.06 ml/g wet wt/min), glucose effects were marginal or absent without any evidence of harmful effects. The optimal condition ensuring maximal protection was at the high end of the range studied, together with a glucose concentration of 11 mM. We will make the novel proposition that the benefit of glucose is

lost at very low coronary flows, not because glycolytic flux becomes inhibited as has been proposed [387, 468], but because the rate of delivery of glucose is too low and therefore rate-limiting.

GLUCOSE UPTAKE AT LOW CORONARY FLOWS

Glycolytic flux from glucose was measured at two very low coronary flows of 0.1 ml/g wet wt/min and 0.2 ml/g wet wt/min, with or without 11 mM glucose (Figure 2). A flow of 0.2 ml/g wet wt/min is a reduction to about 2-4 % of normal *in vivo* values, which is severe enough to be potentially lethal if sufficiently prolonged [194]. A reduction in measured rates of glycolysis was found with low flow ischaemia compared to control values measured prior to ischaemia, in good agreement with previous reports [390, 468].

When the rate of glycolysis was expressed as a function of the glucose concentration and ischaemic coronary flow, i.e. glucose delivery, no evidence of glycolytic inhibition was found when examining the effect of a progressively decreased flow (Figure 3). Although absolute rates of uptake were depressed in a flow-dependent manner, the extraction of the available glucose was greatly increased, as also found in the pig model by Stanley et al. [507]. This finding would substantiate the computer-based calculations that, although the absolute rate of glycolysis is reduced in ischaemia, the actual limiting factor is the rate of delivery of glucose to the heart [262]. However, despite greatly increased extraction of glucose observed in low flow ischaemia, we cannot exclude some concurrent enzymatic inhibition of glycolysis by accumulated metabolites [468]. In the absence of any such inhibition, glycolytic rates would possibly have been greater at low flows, with increased extraction.

The two factors thus governing glycolytic flux rate in ischaemia, closely linked in an inverse ratio, are the rate of delivery of glucose, and enzymatic inhibition by metabolite accumulation. With complete absence of flow, there would be no delivery, and metabolite accumulation would be the major determinant of glycolytic flux rate. If coronary flow were increased from zero, at some point the measured glycolytic flux rate (glucose uptake) would be equivalent to that measured at normoxic flows. Further increases in coronary flow would result in glycolytic flux rates greater than normoxic rates, as found by Neely et al. [390] and Rovetto et al. [468]. In their data, at a coronary flow of 0.6 ml/g/min, glycolytic flux was 0.4 μmol/g/min, while with a flow of 2.3 ml/g/min, glycolytic flux rate was 1.1 μmol/g/min. Control glycolytic flux rate was intermediate at 0.8 μmol/g/min with a coronary flow of 15 ml/g/min [390, 468]. These data suggest that the point at which measured glycolytic flux rates would be equivalent to those in control hearts is between 1 and 2 ml/g wet wt/min. In addition, when expressed as percentage of delivered glucose, with increasing flows, the percentage extraction is 6.1%, 4.3% and 0.5% respectively for the above glycolytic flux rates. This relationship between

glucose uptake and coronary flow explains why glycolytic flux may be increased in "moderate" ischaemia, and reduced in "severe" ischaemia [14, 123, 390, 468, 507].

Uptake of glucose into the cell occurs via facilitative transporters, in a diffusion-dependent process driven by the concentration gradient across the cell membrane. The GLUT1 transporter has a Km of 21-26 mM, while that of the GLUT4 is 4.3 mM [395]. In ischaemia, there is an increased translocation of the GLUT4 glucose transporters to the membrane in ischaemia [517], allowing increased extraction of glucose, as observed in our experiments (Figure 3). However, there would still be a limit to glucose transport across the membrane at low concentrations. The consequence would be a reduced rate of formation of glycolytic ATP, with less protective effect (see Table 1B and 2B with 2.5 mM glucose). A much higher glucose concentration (e.g. 22 mM as tested in the present study), however, may result in a faster rate of accumulation of lactate and protons from a higher glycolytic flux rate than could be washed out by severe low flows, imposing a metabolic burden on the cells and compromising recovery on reperfusion (Table 1B and 2B). In our experiments, an optimal glucose concentration of about 11 mM was found (Tables 1 and 2), approximately double the Km of the GLUT4 transporter. This optimal concentration could possibly be altered by insulin, by the provision of alternate substrates, or by the nutritional state of the rat (fed or fasted).

GLYCOLYTIC FLUX RATES AND ATP PRODUCTION

While glucose uptake and enzymatic regulation serve to regulate the rate of glycolysis, it has been proposed that it is the rate of ATP production that is the eventual determining factor in the protective effect of glucose [424]. Anaerobic ATP (glycolytic ATP) contributed more than 75% of total ATP production in low flow ischaemia at 0.1 or 0.2 ml/g wet wt/min (Figure 5). However, our measured rates of glucose utilisation show that ATP from glucose at a coronary flow of 0.2 ml/g wet wt/min was well below 2 μmol/g/min, the minimal rate required to prevent ischaemic contracture in this model [424]. The average rate of glycolytic flux from glucose was 0.43 ± 0.04 μmol/g wet wt/min (in the group with 11 mM glucose at a flow of 0.2 ml/g wet wt/min). Estimated rates of ATP production from glucose thus never rose much above 0.9 μmol/g wet wt/min which would explain why contracture, although partially inhibited by glucose, still occurred [424].

At the highest coronary flow tested (0.4 ml/g wet wt/min), contracture was still observed, although to a lesser extent, and the onset was greatly delayed when compared with lower flows. Although we did not measure glycolytic flux at this flow, by extrapolation of the calculated ATP production at 0.2 ml/g wet wt/min, and assuming that coronary flow remains limiting, the probable rate of ATP production at a flow of 0.4 ml/g/min was about 1.8 μmol/g wet wt/min, at which rate contracture still developed, albeit greatly reduced. Our previous data showing that 11 mM glucose totally inhibits contracture was at a slightly higher flow (0.5 ml/g wet wt/min), with a minimal required rate of ATP production from

glucose of 2.0 μmol/g wet wt/min [424]. The crucial coronary flow and corresponding rate of delivery of glucose required to prevent contracture totally would thus appear to lie between 0.4 and 0.5 ml/g wet wt/min in the isolated buffer-perfused rat heart.

BALANCE OF GLUCOSE DELIVERY VERSUS LACTATE WASHOUT - THE CRUCIAL ROLE OF RESIDUAL CORONARY FLOW

While rate of delivery of glucose was crucial in determining the rate of glycolysis in our experiments, the concurrent washout of lactate and protons by residual coronary flow is also required to reduce the extent of metabolic inhibition. While the total amount of lactate produced with glucose present was greater at the higher flow (21.5 ± 1.4 vs. 16.9 ± 1.8 μmol C_6/30 min), the greater portion of the lactate was washed out during ischaemia (70% vs. 46%) at the higher flow (0.2 vs. 0.1 ml/g wet wt/min). Similar rates of lactate washout were seen with no added glucose in ischaemia when there could be no contribution of glucose-derived ATP to total ATP production (Figure 5). Even at very low coronary flows, there were no detrimental effects of 11 mM glucose when compared to zero glucose in our experiments. The lack of any harmful effect of a glucose-containing perfusate could be explained by the supposition that any flow bringing adequate glucose would be sufficient to wash out metabolites. However, if an additional metabolic load were placed on the myocardium, e.g. excess glucose (22 mM) or the addition of lactate to the perfusate [91, 387], the critical balance between delivery of glucose and washout of metabolites would be disturbed, resulting in a deleterious effect on recovery following end product accumulation. In the complete absence of flow, this would explain why enhanced glycolysis in no flow conditions may be deleterious [193, 278, 378, 387], although caution must be taken when extrapolating the effects of the utilisation of glycogen to that of glucose as the glycolytic substrate.

Some estimates may be made of how these flows relate to those found *in vivo*. While the flow of the buffer-perfused isolated rat heart ranges from 8-12 ml/g wet wt/min, the *in vivo* blood flow is only 4-6 ml/g wet wt/min [279]. Thus a reduction to 0.5 ml/g wet wt/min would be about 5% of the control for the isolated heart and about 10% of the *in vivo* value. Other workers have used the blood-perfused rabbit heart to show that a flow reduction of 10 -20% is associated with a protective rate of ATP production from glycolysis of about 1.7 μmol/g wet wt/min [123] in good agreement with our protective values of 1.8-2.0 μmol/g wet wt/min [424]. Therefore our coronary flow values, although obtained on buffer-perfused non-working hearts, may provide data applicable in principle to blood-perfused hearts.

GLYCOGEN AND CONTRACTURE

The role of glycogen in ischaemia remains controversial. Depletion of glycogen prior to ischaemia improved functional recovery by reducing the lactate and proton load in some studies [278, 387] but not in others [286]. In addition, glycogen loading prior to ischaemia by various methods is reported to be either harmful [387] or protective [487]. Confounding evidence regarding the role of glycogen in ischaemia is also provided by the phenomenon of preconditioning. The brief periods of ischaemia prior to preconditioning reduce glycogen levels by up to 50 % prior to the longer period of ischaemia [378], and this change has been associated with a reduced proton load and improved recovery [512].

A reduction in pre-ischaemic glycogen levels in our model of low flow ischaemia hastened the time-to-onset of contracture and increased peak contracture both in the presence and in the absence of glucose, as also found by Kupriyanov et al. [278] and Kingsley et al. [253] in total global ischaemia. If the amount of ATP produced during the first ten minutes of ischaemia, prior to the onset of contracture, were calculated (from the first series of experiments - Figure 2), glycogen, with glucose present, contributed 45% of the total anaerobic ATP produced (at a flow of 0.2 ml/g wet wt/min, with a rate of production of total glycolytic ATP of 10.3 ± 0.6 μmol/ 10 min and contribution from glycogen of 4.9 ± 1.0 μmol/ 10 min). In the absence of glucose, ATP production over the first ten minutes was 13.0 ± 0.9 μmol/ 10 min. Thus glycogen breakdown contributed significantly to ATP production in the first ten minutes of ischaemia. However, the contribution of glycogen to ATP production is rapidly reduced with extended ischaemia as its levels fall, and the utilisation of glucose as substrate is accelerated following glycogen depletion [390]. Glycogen may therefore exert its protective effect primarily in the first ten minutes or so of ischaemia, playing a role in determining the time-to-onset of contracture. This finding may explain the observations of Asimakis et al. [18], of a reduced time to onset of contracture and an increased peak contracture in preconditioned hearts with reduced pre-ischaemic glycogen. In preconditioning, glycogen depletion during the first episode of ischaemia is followed by reduced proton overload in the second period of ischaemia [512], which may participate in the protective effects of preconditioning. Nevertheless, in our experiments, glycogen breakdown helped to protect from the very early effects of ischaemia, in the first 5 - 10 minutes before glucose extraction could increase. It must be noted that these experiments were conducted under low flow conditions, with continuous removal of potentially harmful metabolites. In the absence of flow, glycogen breakdown could well be harmful by allowing accumulation of metabolites [278, 387].

In the series of experiments measuring glycolytic flux, large increases in peak contracture were seen when comparing glycogenolysis (glucose-free hearts) with both glucose and glycogen utilisation (11 mM glucose), despite similar rates of total ATP production (Figure 5). A greater percentage contribution from glucose-derived ATP to total ATP production was associated with reduced peak

contracture, and better rate of recovery of function. Glucose-derived ATP (from 11 mM glucose) was thus more effective in inhibiting contracture. In addition, the presence of glucose inhibited glycogen breakdown (Figure 4, Table 3). The reason for the inability of glycogen breakdown in the absence of glucose to protect against contracture is a matter of speculation, especially because the rate of ATP production from glycogen was in the protective range (see Figure 5). Intracellular compartmentation of energy (glucose vs. glycogen) may be invoked [424, 470].

RECOVERY FROM ION LOAD DURING ISCHAEMIA

Recovery from ischaemia is a complex event which depends in part on the operation of various energy-dependent pumps and exchange systems. The major ions that build up in ischaemia are calcium, sodium and protons. We did not measure any of these, but existing data allow a reasonable inference of that could be expected in our experimental conditions. It is already known that glycolytic flux from glucose is required for restitution of the calcium load [229]. Therefore it is extremely unlikely that severely ischaemic hearts perfused with glucose in the ischaemic period would have had any recovery at all in the absence of glucose in the reperfusion period (all hearts were reperfused with 11 mM glucose).

A major ionic load is that of protons. Promotion of glycolysis during subtotal ischaemia leads to a low intracellular pH [278, 512], and therefore a greater activity of the Na^+/H^+ and other exchanges during reperfusion [545]. Inhibition of the Na^+/H^+ exchanger also helps to relieve cytosolic calcium overload [119]. Extrusion of protons leads to sodium gain, which requires energy for its restitution. The increased proton load as a result of enhanced glycolysis during ischaemia is associated with a lessened development of ischaemic contracture, which may be due to increased ATP production and reduced Ca^{2+} binding to myofilaments at a lower pH [259]. Despite the increased proton load, recovery with glucose 11 mM was enhanced compared with the absence of glucose [91]. These studies were done at an ischaemic flow of 0.5 ml/g/min. Our data show that at lower ischaemic flows (0.06 and 0.2 ml/g wet wt/min) early recovery at 5 min reperfusion was glucose-dependent with optimal developed pressures at glucose 11 mM and 22 mM. Late recoveries, however, showed no difference between the provision of 11 mM glucose or no glucose during the ischaemic period (both groups of hearts being reperfused with glucose). We propose that at these very low coronary flows the ultimate extent of recovery is set by the ischaemic flow i.e. by irreversible damage during ischaemia with, as expected, a greater recovery at the higher (0.2 ml/g/min) than at the lower (0.06 ml/g/min) flow (Table 2B). It needs re-emphasising that in our study the coronary flow was artificially fixed during ischaemia, thereby preventing a contracture-induced decrease in flow [123, 210]. If coronary flow were allowed to fall, for example from 0.2 to 0.06 ml/g/min as a result of ischaemic contracture, then it would be

likely that the absence of glucose during ischaemia would result in a more severely impaired recovery also at 25 min reperfusion.

RESERVATIONS TO STUDY

Several assumptions regarding the calculations of glycogenolysis and ATP production were made. We did not directly measure glycogen and tissue lactate levels or O_2 utilisation and CO_2 production during ischaemia. In addition, the technique of measuring glycolytic flux using D-[2-^{3}H]-glucose may be questioned. The measurement depends on the washout of ^3H_2O from the tissue, which may be inhibited at low flows. In addition, Neely et al. [386] used D-[5-^{3}H]-glucose, which may have theoretical advantages. However, a comparison of both isotopes failed to find any differences in the rates of glycolytic flux [468]. We feel that within the limitations of the model, the data with D-[2-^{3}H]-glucose may be accepted. The present study also did not investigate recovery on reperfusion in detail, because of the large degree of irreversible injury observed after such long periods of ischaemia (30 min), which was required to ensure maximum contracture.

This study was performed using an isolated rat heart perfused with a crystalloid solution. Blood flows *in vivo* are very different to those found with an isolated preparation, and may affect the results. However, Eberli et al. [123], using isolated rabbit hearts perfused with an erythrocyte suspension, also showed a protective effect of increased glycolytic flux. Their baseline coronary flows were similar to those found *in vivo* in the rabbit (approximately 1.1 ml/g wet wt/min), and were reduced in one arm of their study to approximately 0.2 ml/g wet wt/min, which is in a range similar to those used in the present study. Blood-perfused models would be required to elucidate more exactly the relationship of glucose concentration to its efficacy at very low coronary flows.

Direct extrapolations to clinical therapy of patients with acute myocardial infarction by glucose-containing solutions such as GIK, cannot be made. We perfused isolated rat hearts with varying concentrations of glucose in the absence of other added substrates such a as free fatty acids. In the reperfusion period all hearts received a fixed glucose concentration of 11 mM. *In vivo*, provision of glucose plus insulin, for example as part of GIK therapy, decreases the levels of circulating blood free fatty acids, which may lessen the several deleterious effects of fatty acids on the ischaemic myocardium [399]. Nonetheless, Eberli et al. [123] have established that glucose and insulin are still beneficial even in the presence of high fixed levels of free fatty acids. Further work in conditions more closely approximating those in human myocardial infarction including a consideration of insulin resistance would be required to asses the optimal glucose concentration to protect the severely ischaemic myocardium.

CONCLUSIONS

i) Avoidance of ischaemic contracture in this model requires a coronary flow of about 0.4 - 0.5 ml/g wet wt/min plus external glucose. At flows of 0.1 - 0.2 ml/g wet wt/min, ischaemic contracture consistently develops, but provision of glucose lessens development of peak contracture. At even lower flows where the glucose effect on contracture is minimal or variable, glucose is not harmful. Depletion of glycogen in the pre-ischaemic period is, however, disadvantageous under low flow conditions.

ii) Glycolytic flux under ischaemic conditions is regulated both by the severity of metabolic inhibition and the restricted delivery of glucose. Even in severe low flows of 0.1 and 0.2 ml/g/min, the delivery is the rate limiting step. As the coronary flow is further reduced, the influence of metabolic inhibition should become more important, such that in the absence of any delivery at all (zero coronary flow) such inhibition would be the rate limiting step for glycolytic flux.

iii) While some quantity of tissue glycogen is required for optimal protection against ischaemia, the preferred substrate is still glucose. ATP production from glycogen is less able than that from glucose and glycogen to inhibit ischaemic contracture. However, some glycogen breakdown is required, particularly in the early stages of low flow ischaemia, to delay the onset of ischaemic contracture.

The above study investigated changes in contracture and functional recovery in hearts subjected to modifications during sustained global ischaemia. However, glucose concentrations *in vivo* remain constant during pre-ischaemia, ischaemia and reperfusion. We wished to investigate the effect of different glucose concentrations maintained throughout the experiment, and establish a tight dose response of the hearts to changes in glucose concentration in terms of ischaemic contracture and functional recovery, with changes in residual coronary flow.

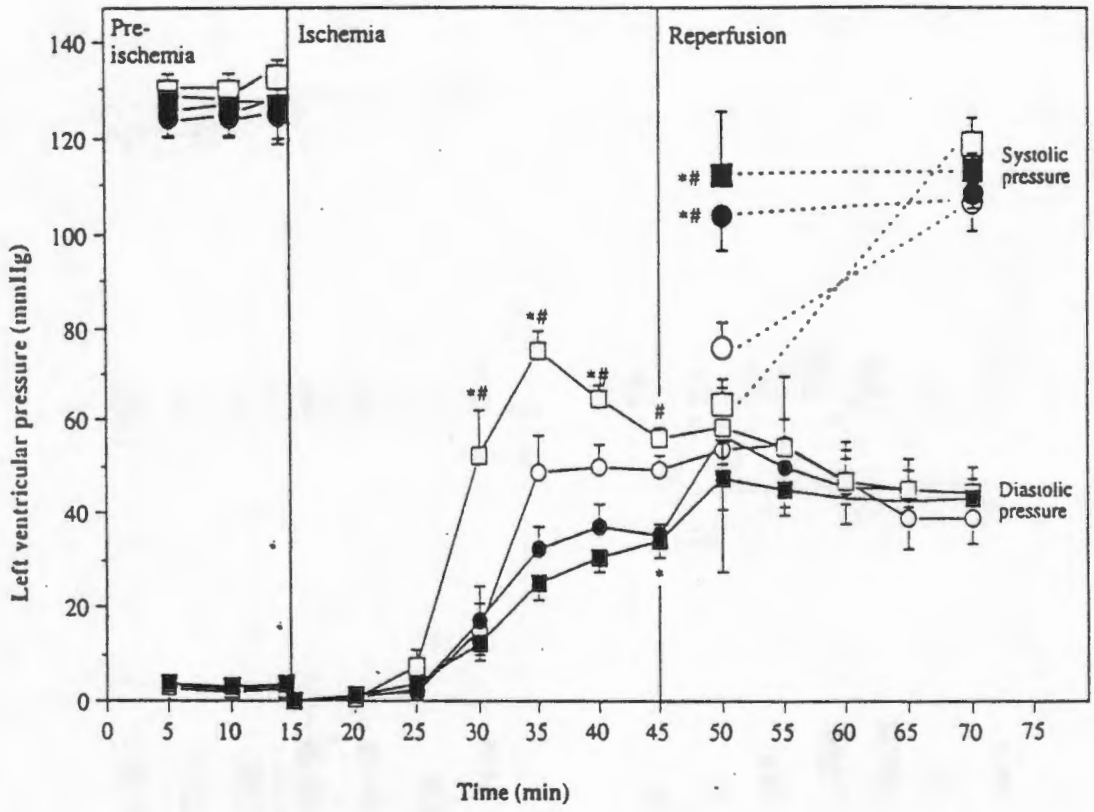


Fig 1: Systolic and diastolic pressure (mmHg) in ischaemia and reperfusion with glucose concentrations of zero (open symbols) or 11 mM (closed symbols) at flows of 0.1 ml/g wet wt/min (circles) or 0.2 ml/g wet wt/min (squares) during ischaemia. Diastolic pressure was set initially at 4-5 mmHg (pre-ischaemia). Ischaemia was for 30 min followed by 25 min reperfusion with 11 mM glucose.

Systolic pressures (larger symbols) are shown before ischaemia, and with 5 min and 25 min reperfusion. The dotted lines follow the changes in systolic pressure over time. The actual data is not shown for clarity. Recovery of developed pressure at 5 min was 14.2 ± 9.9 % (zero glucose) and 40.2 ± 10.9 % (11 mM glucose) with 0.1 ml/g wet wt/min, and 4.9 ± 4.0 % (zero glucose) and 53.8 ± 11.2 % (11 mM glucose) with 0.2ml/g wet wt/min. By 25 min reperfusion, recoveries of developed pressure were 41.6 ± 9.4 %, 40.3 ± 8.7 %, 55.5 ± 3.6 % and 58.9 ± 3.9 % respectively (n=10 in each group).

* *p<0.05 vs. zero glucose/0.1 ml/g wet wt/min*

\# *p<0.05 vs. 11 mM glucose/0.2 ml/g wet wt/min*

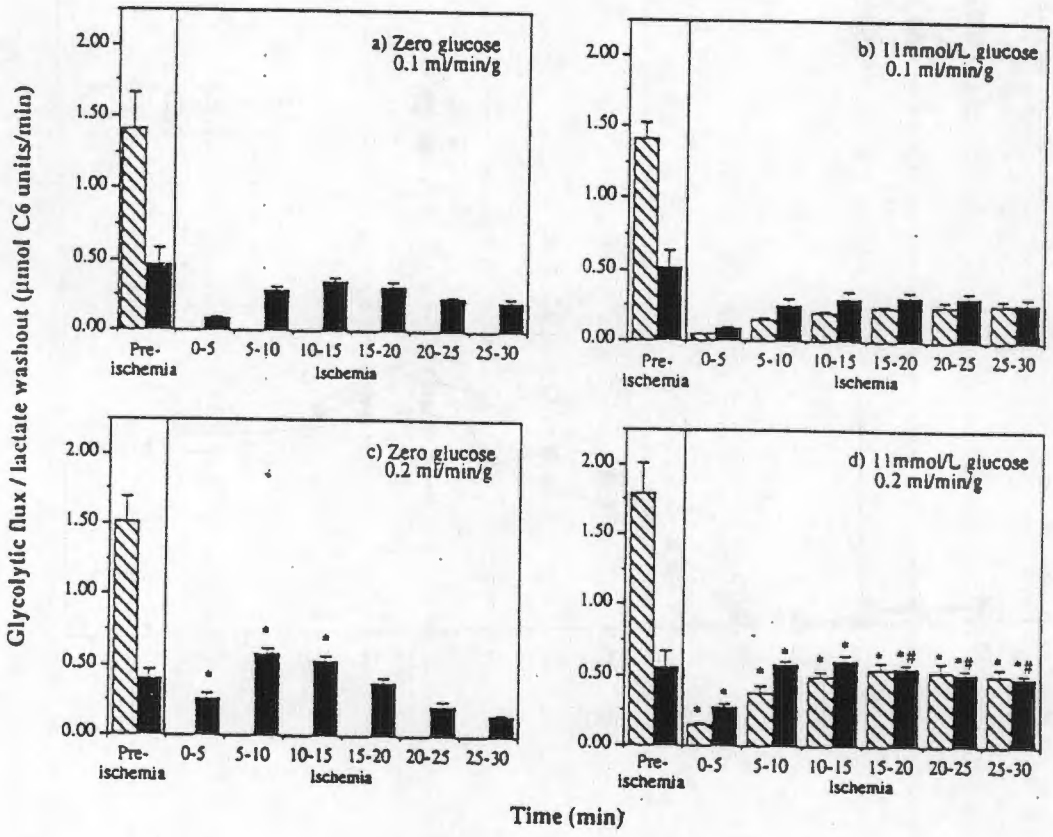


Fig 2: Glycolytic flux (■)and lactate washout (■) in µmol C_6 units per minute before and during ischaemia with glucose concentrations of zero (a and c) or 11 mM glucose (b and d) at flows of 0.1 ml/g wet wt/min (a and b) or 0.2 ml/g wet wt/min (c and d) during ischaemia. Effluent was collected over each five minute period during ischaemia, and corrected for coronary flow (n=10 in each group).

* *p<0.01 0.1 ml/g wet wt/min vs. 0.2 ml/g wet wt/min*

\# *p<0.03 zero glucose vs. 11 mM glucose*

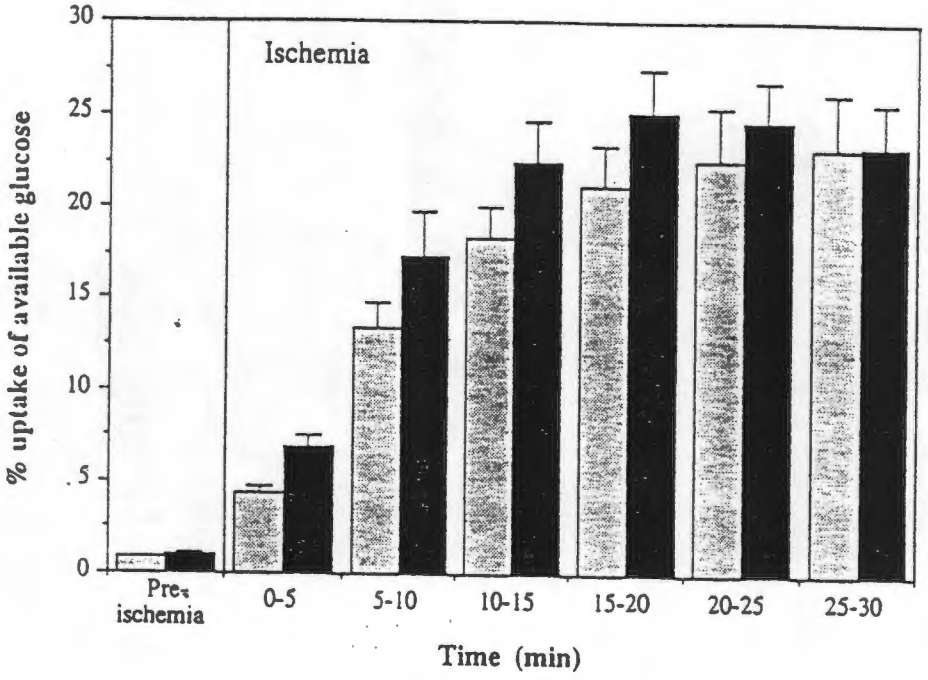


Figure 3: The percentage of glucose uptake before and during ischaemia calculated from the glycolytic flux rate (utilisation of D-[2-^{3}H]-glucose), in µmol/g wet wt/min, expressed as a percentage of delivered glucose, where delivered glucose = concentration of glucose x coronary flow rate. With a concentration of 11 mM glucose, delivery at flow rates of

12-15 ml/g/min (pre-ischaemia) = 132-165 µmol glucose /g/min

0.1 ml/g/min (ischaemia) = 1.1 µmol glucose /g/min (▩)

0.2 ml/g/min (ischaemia) = 2.2 µmol glucose /g/min (■)

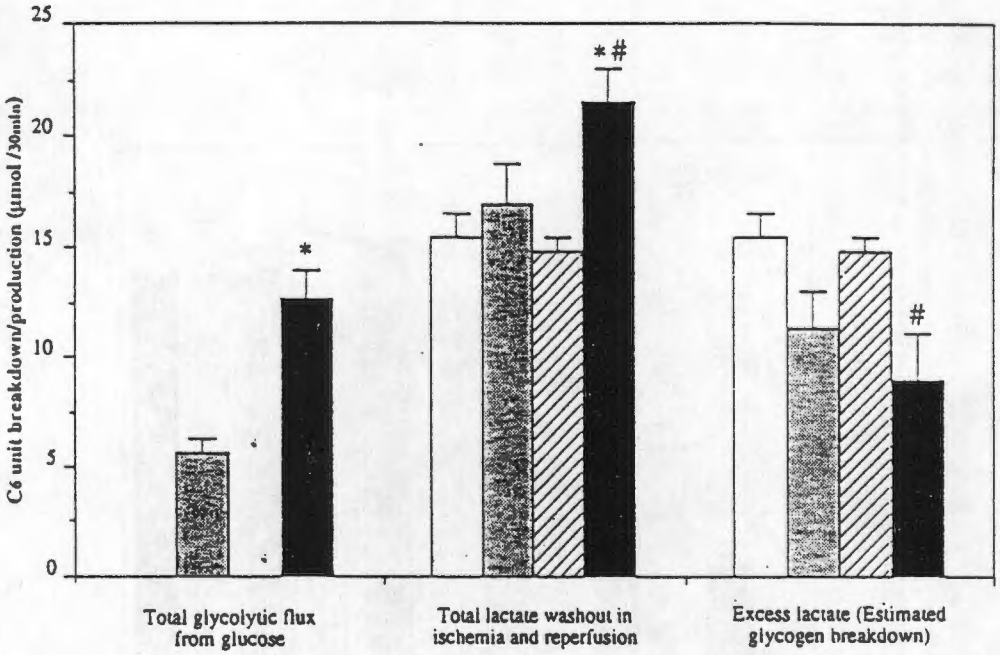


Figure 4: Lactate in excess of that accounted for by glycolytic flux as an estimation of glycogen breakdown (in µmol C_6 units) over 30 min by ischaemic hearts perfused under different conditions.

□ *Zero glucose, 0.1 ml/min* ■ *Zero glucose, 0.2 ml/min*
■ *11 mM glucose, 0.1 ml/min* ■ *11 mM glucose, 0.2 ml/min*

The excess lactate, presumed to come from glycogen breakdown, was calculated as the difference between

a) the total lactate produced in ischaemia, calculated from the total washout over 30 min ischaemia + washout during the first 2 min reperfusion, and

b) the total glycolytic flux from glucose over the 30 min period of ischaemia (µmol/30 min) contributing to 1 mol C_6 units lactate/mol glucose utilised.

* *$p<0.05$ 0.1 ml/min vs. 0.2 ml/g wet wt/min*

\# *$p<0.05$ zero glucose vs. 11 mM glucose*

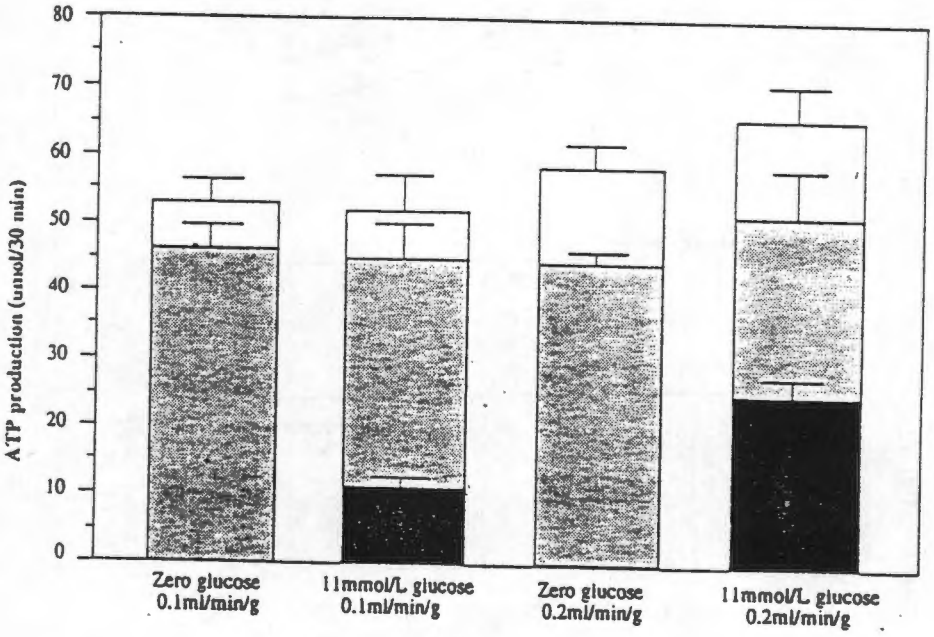


Fig 5: Estimated total ATP production over 30 min ischaemia. Total ATP production in each group is made up of ATP from the anaerobic breakdown of glucose (■ - 2 mol ATP/mol glucose) and glycogen (▦ - 3 mol ATP/mol C_6 units), together with the maximum possible subsequent generation of ATP by oxidative phosphorylation (□).

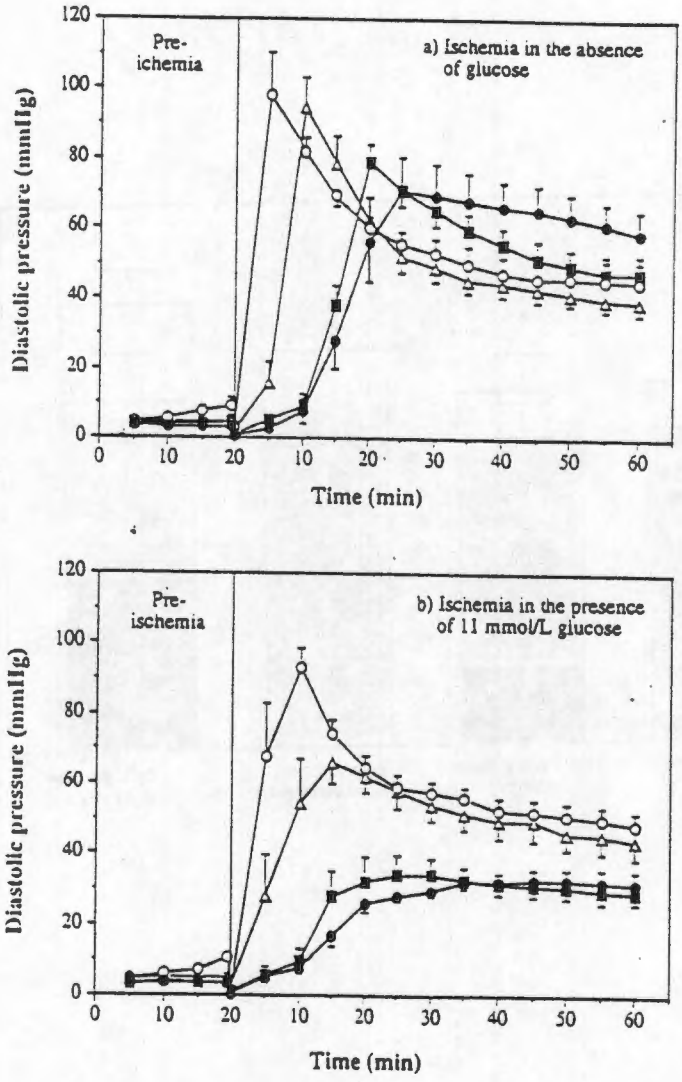


Fig 6: Diastolic pressure before and during ischaemia with 20 min pre-ischaemic perfusion with 11 mM glucose (■), 5 mM acetate (Δ), substrate free (O) or 11 mM glucose + insulin (●). Ischaemia was for 60 min, at a low flow of 0.2 ml/min, with zero (a) or 11 mM glucose (b).

Table 1: Time to onset and peak contracture with changes in flow rate with and without 11 mM glucose (A) , and changes in glucose concentration at flow rates of 0.06 and 0.2 ml/g wet wt/min (B).

A.	Flow rates	Time to onset (min)		Peak contracture (% developed pressure)	
	(ml/g/min)	0 mM	11 mM	0 mM	11 mM
	0	8.5 ± 1.4	7.6 ± 0.6 *	65.5 ± 3.1 *§	61.4 ± 3.5 *§†
	0.015	8.7 ± 0.8	7.7 ± 1.5 *	63.9 ± 2.8 *§	57.7 ± 3.0 *§†
	0.03	4.8 ± 1.8	8.4 ± 0.9 *	73.1 ± 3.9	54.0 ± 3.4 *§†#
	0.06	9.3 ± 0.6	6.8 ± 1.5 *	63.5 ± 2.8 *§	51.7 ± 3.5 *§ #
	0.1	8.8 ± 0.9	8.1 ± 1.6 *	71.0 ± 2.0 *	42.3 ± 5.0 * #
	0.2	7.2 ± 0.9	7.7 ± 2.1 *	76.4 ± 5.8	31.0 ± 2.2 * #
	0.4	6.7 ± 1.5	28.8 ± 8.8 #	82.8 ± 4.2	15.6 ± 6.3 #

† $p<0.05$ vs. 0.1 ml/g wet wt/min, § $p<0.05$ vs. 0.2 ml/g wet wt/min
* $p<0.05$ vs. 0.4 ml/g wet wt/min, # $p<0.05$ zero vs. 11 mM glucose

B.	Glucose conc	Time to onset (min)		Peak contracture (% developed pressure)	
	(mM)	0.06 ml/g/min	0.2 ml/g/min	0.06 ml/g/min	0.2 ml/g/min
	0	8.3 ± 0.7	12.4 ± 0.8 #	52.3 ± 1.7	58.3 ± 3.2 *
	2.5	7.3 ± 1.4	8.5 ± 1.7	47.6 ± 3.5	58.3 ± 4.3 *
	5.5	9.3 ± 0.8	11.3 ± 1.1	46.2 ± 4.1	44.7 ± 5.0 *§
	11	9.4 ± 1.3	14.4 ± 2.0	49.5 ± 3.6	27.6 ± 3.7 §#
	22	7.3 ± 0.9	12.3 ± 1.2 #	42.8 ± 6.1	37.4 ± 7.1 §

* $p<0.05$ vs. 11 mM glucose, § $p<0.05$ vs. zero glucose, # $p<0.05$ 0.06 ml/g wet wt/min vs. 0.2 ml/g wet wt/min; n = 6-8 per group

Table 2: Changes in flow and glucose concentration in ischaemia. Heart rate, systolic and diastolic pressure before ischaemia (A), and incidence of ventricular reperfusion arrhythmias and recovery of function (B).

A.	Glucose conc	Heart rate		Systolic Pressure		Diastolic Pressure	
	(mM)	0.06 ml/g/min	0.2 ml/g/min	0.06 ml/g/min	0.2 ml/g/min	0.06 ml/g/min	0.2 ml/g/min
	0	282.0 ± 10.9	322.5 ± 27.0	130.0 ± 5.1	126.6 ± 5.9	3.3 ± 0.4	4.3 ± 0.5
	2.5	306.0 ± 10.9	329.1 ± 25.4	133.0 ± 6.9	120.6 ± 3.1	4.2 ± 0.2	4.1 ± 0.3
	5.5	288.6 ± 18.8	334.3 ± 14.9	133.8 ± 4.6	123.3 ± 1.9	4.2± 0.2	3.0 ± 0.7
	11	330.3 ± 7.9	322.5 ± 17.3	121.7 ± 4.1	116.8 ± 5.2	4.0 ± 0.0	3.5 ± 0.3
	22	310.7 ± 20.1	342.9 ± 14.1	129.7 ± 5.0	119.0 ± 5.5	3.5 ± 0.4	4.0 ± 0.2

B.	Glucose conc	Incidence of arrhythmias (%)		Developed Pressure (% 5 min)		Developed Pressure (% 25 min)	
	(mM)	0.06 ml/g/min	0.2 ml/g/min	0.06 ml/g/min	0.2 ml/g/min	0.06 ml/g/min	0.2 ml/g/min
	0	83	100	11.3 ± 4.5	20.8 ± 5.8 *	21.2 ± 8.9	57.5 ± 6.0 #†
	2.5	50	85.7	8.5 ± 0.5	25.3 ± 6.0 *#	25.9 ± 5.6 †	46.0 ± 8.1
	5.5	16 §	28.6 §	10.6 ± 2.2	35.7 ± 11.8	18.7 ± 4.2	43.3 ± 10.4
	11	16 §	50 §	11.3 ± 2.2	54.8 ± 10.9 #	21.0 ± 4.8	68.3 ± 5.3 #
	22	0 §	14.3 §	10.5 ± 0.8	51.2 ± 11.0 §#	24.7 ± 7.7	43.3 ± 9.9

* $p<0.05$ vs. 11 mM, § $p<0.05$ vs. 0 mM, # $p<0.05$ 0.06 ml/g wet wt/min vs. 0.2 ml/g wet wt/min, † $p<0.05$ 25 min vs 5 min

Developed pressure, expressed as the percentage of pre-ischaemic developed pressure, after 5 min (early) and 25 min (late) reperfusion following 30 min ischaemia with changes in glucose concentration, at low flows of 0.06 ml/g wet wt/min (n=6) and 0.2 ml/g wet wt/min (n=7, 8 hearts in each group).

Table 3: Effects of different pre-ischaemic perfusion substrates to alter glycogen levels on tissue metabolites before and after ischaemia (A.) and on left ventricular function and ischaemic contracture (B.).

A. Pre-ischaemic perfusion substrates to alter glycogen levels	Pre-ischaemic tissue content				Post-ischaemic tissue content			
	ATP (μmol/g wet wt)	CP	Glycogen	Lactate (μmol C_6/g wet wt)	Glycogen (μmol C_6/g wet wt)		Lactate	(μmol C_6/ g wet wt)
					No glucose	Glucose	No glucose	Glucose
Control								
11 mM glucose	5.2 ± 0.8	4.7 ± 0.4	16.1 ± 1.4	0.6 ± 0.1	2.7 ± 0.4	5.5 ± 0.8 #	4.0 ± 0.4	4.8 ± 0.5
Low glycogen								
5 mM acetate	5.2 ± 0.3	6.4 ± 0.8 *	10.0 ± 1.2 *	0.5 ± 0.1	0.4 ± 0.1 *	1.6 ± 0.3 *	2.6 ± 0.4 *	3.2 ± 0.3 *
Substrate free	5.4 ± 0.2	2.8 ± 0.3 *§	4.0 ± 0.7 *	0.5 ± 0.1	0.6 ± 0.1 *	2.9 ± 0.2 *#	1.2 ± 0.1 *§	1.9 ± 0.2 *§#
High glycogen								
Glucose + Insulin	5.0 ± 0.4	4.4 ± 0.2	20.5 ± 1.1 *	0.7 ± 0.1	4.9 ± 0.5 *	9.3 ± 1.4 *#	4.1 ± 0.2	6.0 ± 0.3 * #

B. Pre-ischaemic perfusion substrates to alter glycogen levels	Pre-ischaemic left ventricular function				Ischaemic contracture			
	SP (mmHg)	DP (mmHg)	Dev P (mmHg)	Heart rate (beats/min)	Time to onset (min)		Peak contracture (% developed pressure)	
					No glucose	Glucose	No glucose	Glucose
Control								
11 mM glucose	132.5 ± 4.3	3.9 ± 0.4	128.6 ± 4.3	278.0 ± 13.0	9.7 ± 1.7	8.6 ± 2.7	63.8 ± 1.9	29.9 ± 3.9 #
Low glycogen								
5 mM acetate	131.3 ± 2.6	5.0 ± 0.5	125.5 ± 2.8	288.0 ± 11.1	3.1 ± 1.0 *	1.8 ± 1.3 *	88.5 ± 1.8	60.9 ± 6.7 *#
Substrate free	95.0 ± 3.8 *#	10.7 ± 1.3 *#	84.4 ± 4.4 *#	296.1 ± 8.3	2.1 ± 0.1 *	2.3 ± 0.3 *	160.2 ± 19.3 *§	137.0 ± 8.5 *§
High glycogen								
Glucose + Insulin	140.7 ± 2.5	3.7 ± 0.3	128.7 ± 11.9	294.2 ± 16.3	9.6 ± 1.6	8.9 ± 1.6	51.6 ± 5.7	26.6 ± 1.2 #

* $p<0.05$ vs. control, § $p<0.05$ vs. acetate, # $p<0.05$ 11 mM vs. 0 mM glucose all postischaemicue levels significantly different from pre-ischaemic levels ($p<0.02$).

20 min pre-ischaemic perfusion with different perfusate substrate was used to alter glycogen levels. Hearts were then clamped, or made ischaemic for 60 min, with a low flow of 0.2 ml/g wet wt/min, either with or without glucose 11 mM (n=6 in each group). At the end of ischaemia, the hearts were clamped. Pre-ischaemic functional data is the combined average of both no glucose and glucose ischaemic hearts (n=12 in each group). ATP and CP levels at the end of ischaemia were negligible and are not shown.

ATP - adenosine triphosphate, CP - creatine phosphate, SP - systolic pressure, DP - diastolic pressure, Dev P - developed pressure

Results 2: Relationship between glycolytic flux rates and protection against ischaemic/reperfusion damage in isolated perfused rat hearts

ABSTRACT

Background: Hypothetically, glycolytic flux reduces ischaemic contracture by increased ATP production, but also results in metabolite accumulation which may impair recovery. We wished to determine whether enhanced glycolytic flux was consistently of benefit over a range of reduced coronary flows, with changes in glucose concentration, in the presence of an alternate substrate.

Methods: Isolated Langendorff rat hearts were perfused with increasing glucose concentrations (0, 2.75, 5.5, 11, and 22 mM) with 5 mM acetate in all experiments. Hearts were subjected to 30 min low flow ischaemia (0.1, 0.2 or 0.5 ml/g/min), and reperfused for 30 min. In a separate set of experiments, insulin was added at each glucose concentration, with a flow rate of 0.2 ml/g wet wt/min. Function was monitored by a left ventricular balloon. Glycolytic flux was assessed by [2-^{3}H]-glucose detritiation.

Results: At each low flow rate, glycolytic flux was sustained at higher glucose concentrations, but fell with lower values. Hearts with residual flows of 0.1 and 0.2 ml/g wet wt/min did not recover well, although 11 mM glucose reduced peak contracture compared to other groups. 22 mM glucose was deleterious during severe ischaemia when compared to 11 mM glucose, presumably due to excess metabolite accumulation. With less severe ischaemia (0.5 ml/g wet wt/min - 3% of control), low glucose concentrations (0 and 2.75 mM) were associated with high peak contracture values and poor mechanical recoveries. Hearts perfused with the higher glucose concentrations had little contracture and good recoveries, with 11 mM glucose again having optimal recovery.

Conclusions: Provision of glucose at a high physiological concentration (11 mM) was beneficial at all flow rates by attenuating ischaemic contracture. Improved functional recovery was more apparent with less severe coronary flow restrictions. There was an optimal rate of glycolysis, below which contracture was enhanced (reduced ATP), and above which (supraphysiologic glucose) recovery was reduced (possibly increased metabolite accumulation).

INTRODUCTION

Provision of glucose during ischaemia, with increased rates of glycolysis, is beneficial to the heart in several ways [401]. Glucose inhibits the genesis of arrhythmias [28], preserves cellular ultrastructure [192] with reduced contracture [424], reduced infarct size [95, 341], and improved cardiac output on reperfusion [14].

However, a possible problem attributed to increased glucose provision is an excess accumulation of glycolytic end-products, which may be deleterious to the ischaemic myocardium [193, 387]. We have found that no deleterious effect was associated with 11 mM glucose, compared to hearts with no glucose, at flow rates of 0.4 ml/g wet wt/min and less (Results Ch 1). In addition, 11 mM glucose was optimal compared to lesser or higher glucose concentrations during low flow ischaemia. However, in that study (Results Ch 1), all hearts were perfused prior to ischaemia and throughout reperfusion with 11 mM glucose, a concentration used in most isolated rat heart perfusions. No alternative substrate was present.

We wished to mimic the *in vivo* situation more closely, when glucose concentrations prior to, during and after ischaemia with a residual flow, remain constant, in the presence of an alternate substrate. In addition, a major clinical use of glucose which has recently come into consideration after a lapse of several years [399] is glucose-insulin-potassium therapy (GIK), which was originally devised by Sodi Pallares in the 1960s [501]. The regimes for utilisation of this therapy vary widely. It is given to patients with infarction, and post-thrombolysis, with varying glucose and insulin concentrations. While the systemic effects of GIK of decreasing free fatty acid levels is possibly crucial [399], the consequences to the heart, and in particular to the ischaemic zone, are not as clear. Another application of glucose is in the more modern approach of warm blood cardioplegia, with a constant infusion of a low residual flow (about 0.55 ml/g wet wt/min [55]) at normothermia [178, 314]. Hypothermia in itself is known to impair membrane function [55], and cause an increase in the cytosolic Ca^{2+} level, and result in impaired recovery on rewarming [513]. However, warm cardioplegia results in up to 10% residual ATP utilisation [55]. Enhanced glucose in the warm cardioplegia may increase ATP production, and prove favourable. It is important to understand the consequences of different glucose concentrations under low flow conditions, where the balance between ATP production and metabolite accumulation may affect the recovery of the hearts.

We tested the effects of different glucose concentrations used throughout the experiment, with a variety of low flow rates during the ischaemic period. *In vivo* myocardial blood flow in the rat is about 4-6 ml/g/min [279]. Flow rates of 0.1, 0.2 and 0.5 ml/g/min were used in the present study, which represent reductions to 1.7-8.3% of *in vivo* flows, and are comparable to those in *in vivo* ischaemia

(0.07-0.15 ml/g wet wt/min). Different glucose concentrations were used, from 0 to 22 mM. Acetate was included in all experiments to provide an alternative non-carbohydrate substrate [424].

METHODS

EXPERIMENTAL APPARATUS

The isolated Langendorff-perfused rat model with a left ventricular balloon was used, as described in Methods. The hearts were paced at 350 beats/min by a monopolar electrode attached to the left ventricle. The sinus node was removed by excising the left atrium, and the atrioventricular node was isolated by cutting the atrioventricular septum. The basal substrate was 5 mM Na-acetate (to make a total Na^+ concentration of 143.5 mM), and glucose was added in varying concentrations according to the experiment.

EXPERIMENTAL PROTOCOL

Hearts were subjected to 15 min aerobic perfusion, followed by 30 min global subtotal ischaemia by reducing coronary flow to i) 0.1 ml/g wet wt/min, ii) 0.2 ml/g wet wt/min or iii) 0.5 ml/g wet wt/min. Hearts were perfused with glucose concentrations of zero, 2.75, 5.5, 11, and 22 mM throughout the experiment for each of the above flow rates.

Time to onset of contracture (TOC - min), and peak contracture (mmHg) were recorded as described in Methods. At the end of 30 min the hearts were reperfused for 20 min with the same buffer. The recovery of contractile function was monitored. Developed pressures after 20 min reperfusion were expressed as a percentage of pre-ischaemic developed pressure after 15 min perfusion.

Coronary effluents were collected immediately prior to and over each 5 minute period during ischaemia, and at each five minutes on reperfusion. Glycolytic flux rates were assessed immediately after collection of the effluents. Lactate washout was determined in hearts with a low residual flow rate of 0.2 ml/g wet wt/min.

BIOCHEMISTRY

Glycolytic flux rates were measured as described in Methods. Lactate and high energy phosphates were determined by standard photometric assays [24].

STATISTICS

6 hearts were used in each group. Because of the mean wet heart weight of 1.05 ± 0.01 g, results could be expressed as per g wet weight. All results are expressed as mean ± SEM. Results were compared using ANOVA two way analysis of variance following which individual t tests between groups were performed, with the Bonferroni correction. A $p<0.05$ was taken as the level of significance.

RESULTS

PRE-ISCHAEMIC GLYCOLYTIC FLUX RATES AND FUNCTION

Pre-ischaemic function data from each glucose concentration group were lumped together, with a total of 18 hearts per group, as there were no differences within each glucose group. Pre-ischaemic function was not altered significantly by different concentrations of glucose (Table 1). Pre-ischaemic glycolytic flux rates were dependent on glucose concentration, with no significant differences in coronary flow (Table 3).

CHANGES IN GLYCOLYTIC FLUX AND DIASTOLIC PRESSURE IN ISCHAEMIA

Changes in flow rate with different glucose concentrations

Changes in glycolytic flux and diastolic pressure during ischaemia for each flow rate and glucose concentration are illustrated in Fig 1. In the absence of glucose, TOC was not affected by increasing flow rate (Table 2), while peak contracture was significantly increased, from 93.8 ± 1.3 mmHg at 0.1 ml/g wet wt/min, to 126.2 ± 2.3 at 0.5 ml/g wet wt/min (Table 2; Fig 1a). With glucose present, glycolytic flux (from glucose) was depressed initially, and then increased, to peak at 15-20 min, the value of which was dependent on flow rate and glucose concentration (Fig 1). With 2.75 mM glucose, glycolytic flux fell after 15 min ischaemia at each flow rate, such that by 30 min ischaemia, the rates of glycolysis were similar in each group (Fig 1b). Mean glycolytic flux during ischaemia increased with increased flow rate (Table 3). A higher flow delayed TOC, with a significant difference between 0.1 and 0.5 ml/g wet wt/min (10.0 ± 1.1 vs. 15.4 ± 0.5 min; $p<0.01$), but peak contracture was significantly increased, from 68.3 ± 7.7 to 101.7 ± 4.2 mmHg ($p<0.05$) (Table 2; Fig 1b).

With a glucose concentration of 5.5 mM (Fig 1c), a decline in glycolytic flux was seen only with a flow rate of 0.2 ml/g wet wt/min This decline may have been due to the high rate reached in the first 15 min ischaemia, which exceeded the rates with 11 mM. The rate of glycolysis at the end of ischaemia, however, was proportionate to the concentration, as were mean values (Table 3). An increase in flow rate significantly delayed TOC, (12.4 ± 0.9 min vs. 25.7 ± 2.2 min, 0.1 vs. 0.5 ml/g wet wt/min respectively) and peak contracture was also significantly reduced at the higher flow rate (10.3 ± 1.3 mmHg) compared to the lower flow rates (51.3 ± 2.4 mmHg - 0.1 ml/g wet wt/min) (Table 2).

With 11 mM, glycolytic flux was maintained at each flow rate (Fig 1d). With a flow rate of 0.5 ml/g wet wt/min, and 11 mM glucose, glycolytic flux rates reached levels similar to those in pre-ischaemic hearts (Table 3 and Fig 1d). A significant difference in TOC was observed between 0.1 and 0.2 ml/g wet wt/min (13.6 ± 1.2 min vs. 16.9 ± 0.9 min, $p<0.05$). Peak contracture was also reduced (48.2 ± 6.0

mmHg to 30.5 ± 3.4 mmHg, 0.1 and 0.2 ml/g wet wt/min respectively - Table 2). With 0.5 ml/g wet wt/min, contracture occurred in only one heart, with a mean of 6.8 ± 0.3 mmHg (Table 2; Fig 1d).

A higher glucose concentration of 22 mM was less protective than 11 mM during severe low flow ischaemia, with higher peak contracture (50.3 ± 2.7 and 54.5 ± 6.2 mmHg for 0.1 and 0.2 ml/g wet wt/min respectively) and slightly reduced TOC (11.5 ± 1.6 and 13.7 ± 1.1 min) (Table 2). With a low flow of 0.5 ml/g wet wt/min, no hearts perfused with 22 mM glucose showed contracture (Table 2, Fig 1e). Mean glycolytic flux rates increased proportionately with the increase in residual flow rate (Table 3).

Changes in glucose concentration with different residual coronary flow rates

With a low flow of 0.1 ml/g wet wt/min, time to onset of contracture (TOC) was significantly delayed in the 2.75 mM glucose group versus zero glucose (10.0 ± 1.1 min vs. 4.5 ± 0.7 min, $p<0.01$ - Fig 1). Peak contracture was highest with zero glucose (93.8 ± 1.3 mmHg) (Table 2). Contracture was significantly reduced with 2.75 mM (68.3 ± 7.7 mmHg; $p<0.01$) and 5.5 mM glucose (51.3 ± 2.4 mmHg; $p<0.01$ vs. zero and 2.75 mM glucose). There were no differences in peak contracture between the 5.5 and 11 mM glucose groups (Table 2; Fig 1). Higher glucose concentrations did not further alter TOC (Table 2; Fig 1). Glycolytic flux rates doubled with increased glucose concentrations from 2.5 to 5.5 to 11 mM. However, rates with 22 mM were not different from those with 11 mM (Table 3).

With a residual flow rate of 0.2 ml/g wet wt/min, a clearer dependence of TOC and peak contracture on glycolytic flux was seen (Table 2; Fig 1). With zero glucose, TOC was 5.5 ± 0.5 min, with a peak of 104.0 ± 3.0 mmHg. With 2.75 mM glucose, TOC was delayed until 12..3 ± 1.1 min, with a peak contracture of 70.3 ± 7.8 mmHg. 11 mM was the most effective concentration of glucose, delaying TOC to 16.9 ± 0.9 min, with a low peak contracture of 30.5 ± 3.4 mmHg (Table 2; Fig 1). With 22 mM glucose, TOC was reduced compared to 11 mM glucose hearts, while peak contracture was increased (Table 2, Fig 1). Mean glycolytic flux rates during ischaemia showed a similar relationship to glucose concentration as at 0.1 ml/g wet wt/min (Table 3).

At the highest residual flow tested (0.5 ml/g wet wt/min), peak contracture was very high (126.2 ± 3.3 mmHg) with a rapid TOC (3.1 ± 0.8 min) when no glucose was present. A low glucose concentration of 2.75 mM delayed the onset of contracture (15.4 ± 0.5 min; $p<0.02$), but was ineffective in reducing peak contracture (Table 2; Fig 1). Very little contracture was observed with 5.5, 11 or 22 mM glucose, with high rates of glycolysis (Table 3), although no significant differences were found between then latter concentrations.

Lactate washout

Lactate washout was measured in hearts with a residual flow rate of 0.2 ml/g wet wt/min at each glucose concentration. With 2.75 and 5.5 mM glucose, lactate washout was low initially, but increased to a steady state of about 0.5 and 0.8 μmol/g/min respectively. Values declined slightly by the end of ischaemia, as glucose uptake also decreased. Some of this lactate could be accounted for by glycogen breakdown which would be greater in the presence of low glucose concentrations. Lactate washout was significantly higher in the 11 mM and 22 mM glucose groups, reaching a steady state after 5-10 min. A slightly higher rate of washout was found in the 22 mM glucose group, although this was not significant (Fig 2), which agreed with the non-significant increase in glycolytic flux between these two glucose concentrations. The majority of lactate in these hearts would come from glucose uptake, as the glucose would inhibit glycogen breakdown (see Results Ch 1).

High energy phosphate levels

Myocardial ATP, ADP and AMP levels were measured at the end of 30 min low flow (0.2 ml/g wet wt/min) ischaemia with glucose 0, and 11 mM, and 22 mM. With no glucose present, ATP and ADP levels were very low, with high AMP (Fig 3). 11 mM showed the highest ATP levels. 22 mM glucose hearts had slightly lower ATP levels than 11 mM.

RECOVERY OF MECHANICAL FUNCTION AND GLYCOLYSIS

Recovery after 30 min ischaemia with varying residual flow rates

Recovery of developed pressure after 30 min ischaemia with a residual flow rate of 0.1 ml/g wet wt/min was not affected by the glucose concentration, with recoveries of only 20 to 30% (Table 1). The poorest recovery (24.6 ± 6.6 %%) was with 22 mM glucose. Diastolic pressure was elevated in all hearts throughout reperfusion (Table 1). Glycolytic flux rates at the end of 20 min reperfusion tended to be lower than pre-ischaemic rates, except with 2.75 mM (Table 3). Coronary flow rates were also reduced compared to pre-ischaemic values, possibly indicating no-reflow following high ischaemic contracture and high diastolic pressure on reperfusion (Table 3).

With a flow rate of 0.2 ml/g wet wt/min, there were no significant differences in recovery of function in the majority of groups, except that hearts with 11 mM glucose were significantly improved compared to all other groups (Table 1 - 69.4 ± 8%; $p<0.05$), in agreement with previous results (Results Ch 1). There were no differences in glycolytic flux rates on reperfusion between the different glucose groups (Table 3).

With a flow rate of 0.5 ml/g wet wt/min, recovery of function with zero glucose was very poor (20.4 ± 2.1%) but with 2.75 mM glucose, recovery was increased to 62.6 ± 0.9% ($p<0.01$). However, the glycolytic flux rate after 20 min reperfusion was very low (Table 3), while coronary flow was also

reduced (Table 3). With glucose concentrations of 5.5 mM and 11 mM, recoveries were 93.2 ± 2.5 and 97.0 ± 0.6 % respectively ($p<0.01$ vs. 0 mM and 2.75 mM - Table 1). Coronary flows were improved compared to other postischaemic groups, but glycolytic flux rates were lower than pre-ischaemic rates (Table 3). Recoveries with 22 mM glucose were slightly lower than with 5.5 mM and 11 mM glucose (Table 1). Glycolytic flux was also slightly lower than with 11 mM glucose.

Recovery after 30 min ischaemia - effect of different glucose concentrations

With zero glucose, increases in flow rate had no effect on recovery of function (all 20-30%). With a glucose concentration of 2.75 mM, an increasing flow rate improved recovery (from 33.8 ± 7.5 to 62.6 ± 0.9% - 0.1 to 0.5 ml/g wet wt/min; $p<0.01$), although the difference between 0.1 and 0.2 was not significant (Table 1). Glycolytic flux rates on reperfusion decreased with increased residual ischaemic coronary flow rate. With 5.5 mM glucose, the recovery attained with 0.5 ml/g wet wt/min was significantly higher than those with both lower flow rates (Table 1), although glycolytic flux was also decreased compared to 0.1 and 0.2 ml/g wet wt/min groups. With 11 mM glucose, results with each flow rate were significantly better than that lower (from 28.1 9.3 % to 69.4 ± 8.0 % to 97.0 ± 0.6 % respectively; $p<0.05$) - again, glycolytic flux on reperfusion was lowest with 0.5 ml/g wet wt/min during ischaemia (Table 3). The higher flow rate (0.5 ml/g wet wt/min) was also beneficial for hearts with 22 mM glucose, with a significant improvement compared to the lower flow rates.

DISCUSSION

Previous studies of glucose and low flow ischaemia have used different substrates in combination, glucose by itself, or changes in substrate only during pre-ischaemia, ischaemia, or reperfusion. We tested the effect of different glucose concentrations with an alternate substrate present throughout the experiment. The use of an alternate substrate reflects a more physiological system, where fatty acids are the main substrate utilised by the normoxic heart. Acetate is a homologue of fatty acids without the detergent effect, and supplies acetyl CoA directly to the citric acid cycle.

The findings of this study are: 1) the optimal glucose concentration for protection throughout the experiment is shown to be 11 mM; 2) even at this concentration, a very low coronary flow rate removes the benefit of glucose during reperfusion, although protection during ischaemia is still apparent in terms of reduced ischaemic contracture; and 3) enhanced glycolytic flux (by a high glucose concentration) at very low coronary flows may not be an advantage, even if the residual flow is relatively high (0.5 ml/g wet wt/min). This may be attributed to increased metabolite accumulation, and/or to inhibition of glycogen breakdown [425].

ACETATE AS A SUBSTRATE - INTERACTION WITH GLUCOSE

Acetate as sole substrate did not affect pre-ischaemic function, but 20 min perfusion with 5 mM acetate alone reduces glycogen content by 40% compared to glucose hearts (Results Ch 1). Acetate as the sole exogenous substrate accounts for approximately 75% of oxidation at concentrations of 1-10 mM, with the residual oxidation accounted for by endogenous substrate including glycogen [585]. An increased glucose concentration would reduce the amount of glycogen broken down. A concentration of 5 mM acetate decreased glucose utilisation in the presence of 11 mM glucose by about 20% in normoxic hearts (compare with Results Ch 1). However, acetate did not appear to affect glucose utilisation during ischaemia. Acetate perfusion sustains adenosine triphosphate (ATP) levels, and increases levels of creatine phosphate (CP) compared to 11 mM glucose hearts (Results Ch 1). Use of acetate only may eventually result in some impairment of function because of the reduced contribution to anaplerotic pathways with no contribution to oxaloacetate as there would be from glucose-derived pyruvate. Acetate by itself increases LDH washout during low flow ischaemia, indicative of increased tissue damage [49], consistent with the effects of free fatty acids on membrane function in ischaemia [399]. However, even with only acetate present pre-ischaemically, during low flow ischaemia and on reperfusion, functional recoveries were 20-30%, indicating the role of TCA cycle activity in maintaining ischaemic and reperfusion function. In addition, endogenous fatty acids may contribute to energy production in these conditions. The addition of glucose enhanced the ability of the hearts to tolerate ischaemia, with reduced contracture at all flow rates, and resulted in improved functional recovery, especially at the higher flow rates. A proposed benefit of glucose in ischaemia is

inhibition of the deleterious effects of fatty acids [399], and this effect may be apparent in the presence of acetate.

GLYCOLYSIS AND TOLERANCE OF HEARTS TO ISCHAEMIA

Glycolysis throughout the experiment with different glucose concentrations

Pre-ischaemic glycolytic flux rates were dependent on the glucose concentration, with evidence of saturation at the higher glucose concentrations. This indicates that an enzyme-regulated process may be involved in glucose uptake, i.e. the membrane glucose transporters which act by facilitative diffusion (this issue is discussed in Results Ch 3). In most hearts, glycolytic flux during ischaemia reached a plateau after 10-15 min. In the hearts perfused with 2.75 mM at flow rates of 0.1 and 0.2 ml/g wet wt/min, glucose uptake then fell significantly. A similar fall in glycolytic flux rates has been noted previously in rabbits [14, 110, 218] with 5-6 mM glucose. Higher glucose concentrations and insulin maintain glycolytic flux [14], despite a significant increase in lactate production which is said to inhibit glycolysis [468].

On reperfusion, there was little relationship between glycolytic flux and glucose concentration, and between glycolytic flux and functional recovery. It has been suggested that on reperfusion after a period of ischaemia, glycolytic flux and glucose oxidation may be uncoupled, resulting in excess glycolysis, and thus increased proton accumulation, which may be detrimental by resulting in increased Ca^{2+} entry [136, 326]. Fatty acids in particular appear to uncouple glycolysis and glucose oxidation [326], which may partially account for the deleterious effects of fatty acids, or acetate. Thus the rates of glycolytic flux measured on reperfusion may not reflect the rate of glucose subsequently utilised by oxidation, as is presumably the case in the pre-ischaemic hearts. The mitochondria are known to be affected by ischaemia [137], and rates of oxygen utilisation are altered in the stunned myocardium relative to contractility [293, 397], with reduced efficiency. In hearts with better functional recovery, the coupling between glycolytic flux and glucose oxidation may have been better, contributing to improved recovery. Thus the enzyme-determined relationship between glucose concentration and glucose utilisation is altered on reperfusion, as is the relationship between glycolytic flux and mechanical function. The provision of glucose on reperfusion, and improved glucose oxidation, results in optimal recovery, but it must also be remembered that reperfusion function is determined largely by the extent of ischaemic injury. Ischaemic injury appears particularly susceptible to a lack of glucose, or an excess of glycolytic metabolites.

Change in glucose concentration and residual coronary flow rate

Time to onset of contracture has previously been correlated with cessation of glycolytic activity [253]. However, only with a glucose concentration of 2.75 mM was any reduction in the rate of glycolysis

noted during ischaemia, although contracture was present in most hearts. An increase in peak contracture with increased flow was found in the 2.75 mM glucose hearts, showing that there was insufficient ATP production to prevent contracture, with an increased ATP demand in the face of increased coronary flow. A reduction in peak contracture was found with increasing glucose up to a concentration of 11 mM, and an increase in residual flow also increased the tolerance of the myocardium to 30 min global ischaemia at higher glucose concentrations. At a flow rate of 0.5 ml/g wet wt/min, 11 mM glucose abolished contracture in all but one heart, in agreement with a previous report [424]. The rate of glycolysis in these hearts reached 1.02 ± 0.06 μmol/g wet wt/min by 15 min, which would result in a glycolytic ATP production of 2.03 ± 0.11 μmol/g wet wt/min. A minimum rate of glycolytic ATP of 2 μmol/g wet wt/min was also found to be necessary by Owen et al. [424] to prevent contracture. Reperfusion recoveries with 0.5 ml/g wet wt/min and 11 mM glucose were close to 100%, following reduced contracture and sustained glycolysis.

With a concentration of 22 mM glucose at very low flows, however, peak contracture was increased, similar to previous findings (Results Ch 1). TOC was also delayed with increasing glucose up to 11 mM, but was reduced by 22 mM. Increased contracture with a high glucose suggests that this was disadvantageous to the hearts during the ischaemic period with low residual flows of 0.1 and 0.2 ml/g wet wt/min. At a flow rate of 0.5 ml/g wet wt/min, no contracture was recorded in 22 mM glucose hearts, but recovery of function was slightly depressed compared to 11 mM glucose hearts, indicating that even with the high flow rate, some detriment was associated with excess glycolysis.

Apstein et al. [14] compared the protective effects of 5.5 mM glucose and 28 mM glucose + 100 i.u./l insulin, at flow rates of 0.5 or 0.06 ml/g wet wt/min in rabbits. A reduction in contracture with the higher glucose and insulin was found at both flow rates, but protective effects of glucose + insulin versus glucose only on reperfusion function were found only at the higher flow rate (0.5 ml/g/min vs. 0.06 ml/g/min). Glucose + insulin was associated with a higher effluent lactate concentration after 35-40 min ischaemia, indicative of sustained glycolysis. In the 5.5 mM groups, independent of flow rate, lactate production decreased substantially during ischaemia, to levels similar to those found in non-ischaemic hearts. This result suggests that either glucose uptake or glycolysis was inhibited at the lower concentration of glucose.

Increased glucose uptake and pre-ischaemic glycogen loading by insulin treatment result in substantial glycolytic metabolite accumulation during ischaemia, including G6P, F6P, and lactate (Results Ch 3). Sugar phosphate accumulation has been associated with a reduction in diastolic relaxation [282], supposedly because of impaired Ca^{2+} homeostasis, while lactate is thought to inhibit glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and glycolysis by increasing NADH [362]. We could find no deleterious effect of 11 mM glucose even at very low flows. If flow and washout were maintained, the benefits of glucose are apparent by increasing glycolytic ATP production [49, 570]. However, excess

glycolytic substrate from higher glucose concentrations, insulin and increased glycogen may be deleterious if these metabolites are not washed out.

CLINICAL IMPLICATIONS OF FINDINGS

Glucose-insulin-potassium therapy

Glucose-insulin-potassium therapy was widely studied in the 1960s and 1970s with interest initially stimulated by the proposals of Sodi Pallares [501, 502]. However, early clinical trials suggested that GIK did not confer any additional benefit to the patient recovering from myocardial infarction. These trials were poorly designed, with incorrect dosages [132]. The present study details the effect of altered glucose concentration present throughout the duration of normoxia, ischaemia and reperfusion, with an alternative substrate. A positive effect of glucose was found, especially if flow rates were maintained during ischaemia. An increased provision of glucose was beneficial as long as it is not in excess. The use of insulin with high concentrations of glucose may only be beneficial to ischaemic tissue if sufficient washout was maintained. Despite the adverse effects of high glucose, major clinical benefits in 1) zones of lesser ischaemia and 2) reduced systemic blood free fatty acids may be found. The adverse effects do not apply to the use of GIK post infarction, with higher coronary flows.

Cardioplegia

Recent advances in cardioplegia utilise a warm blood solution continually infused throughout a procedure [55]. The glucose content of the perfusate can be manipulated during ischaemia in the case of blood cardioplegia, or pre-ischaemically by glucose-insulin-potassium pre-treatment [302]. In such cases, the glucose content remains relatively constant, while the degree of residual flow is also fixed. Previous studies in isolated rat hearts have shown an optimal concentration of glucose of 11 mM in a cold cardioplegic solution (10°C) without any additional substrate, delivered in a multidose protocol [425, 563]. These findings contradcited a previous report that provision of glucose in the St Thomas' Hospital cardioplegic solution was detrimental. However, the latter study used a single dose at the onset of 70 min arrest at 28°C. The continuous flushing with multidose infusions confers protection in the presence of glucose. The findings of the present study substantiate the concept that delivery of glucose prior to, during ischaemia, and on reperfusion is beneficial, especially if some degree of residual flow is maintained.

GLYCOLYTIC FLUX - RESERVATIONS OF METHODOLOGY

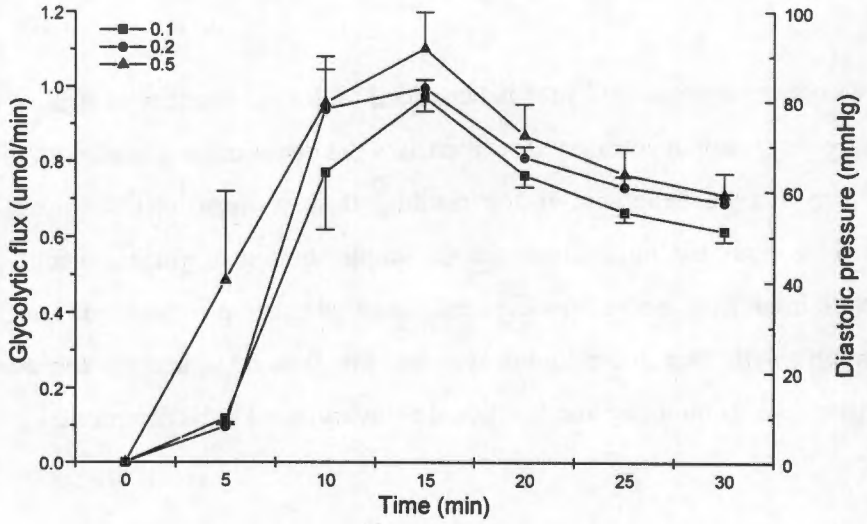
There is some controversy as to whether the use of [2-^{3}H] glucose reflects the true glycolytic flux rate. The ^3H_2O is cleaved off at the phosphoglucose isomerase step, and therefore does not measure the rate of glycolysis further down the pathway. We compared [2-^{3}H] glucose with [5-^{3}H] glucose (^3H_2O

cleaved off at enolase step) and found no difference in the measurement of glycolytic flux at a low flow rate of 0.2 ml/g wet wt/min (data not shown) (see Results Ch 3 and Discussion A and D for further discussion on technique).

SUMMARY

Glucose at a concentration of 5-11 mM is beneficial to the ischaemic myocardium (in the absence of insulin). Improving residual coronary flow increases the benefits associated with glucose. High doses of glucose may not be beneficial if the residual flow is insufficient to wash out accumulated metabolites. This study has implications for the administration of glucose-insulin-potassium therapy to patients with infarction, where flow/dose relationships must be considered. The role of insulin, and its effects on glycolytic flux in conditions of severe low flow rates, and the consequences to the heart in terms of ischaemic contracture and functional recovery, need to be determined.

Having established the dependency of ischaemic contracture and functional recovery on glucose concentration and residual coronary flow, we examined the kinetics of the glycolytic flux/glucose uptake data derived in this study which is discussed in depth in the following chapter.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Glycolytic flux (umol/min)
Diastolic pressure (mmHg)
Time (min)
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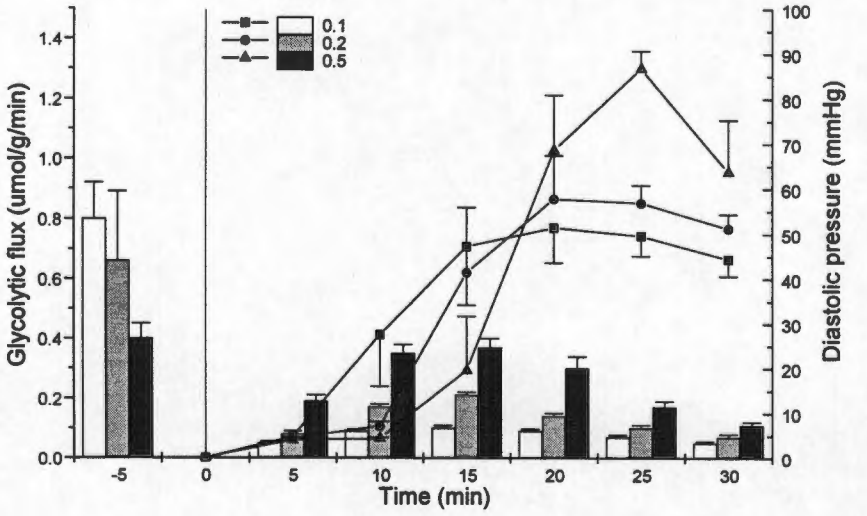
Fig 1a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Glycolytic flux (umol/g/min)
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Fig 1b

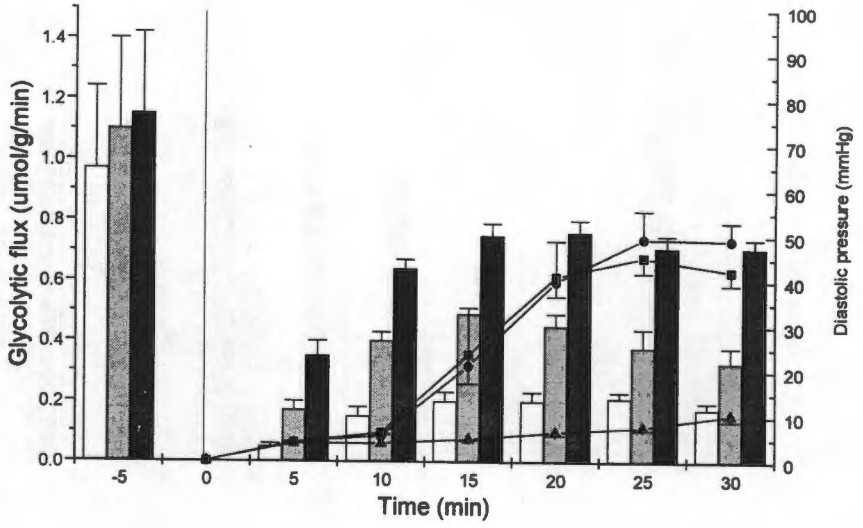
Glycolytic flux (umol/g/min)
Diastolic pressure (mmHg)
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Fig 1c

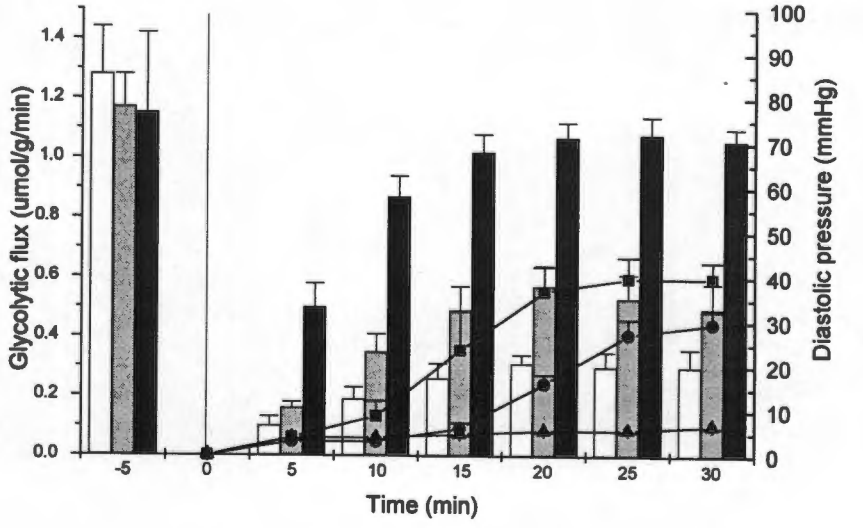
Glycolytic flux (umol/g/min)
Diastolic pressure (mmHg)
Time (min)
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Fig 1d

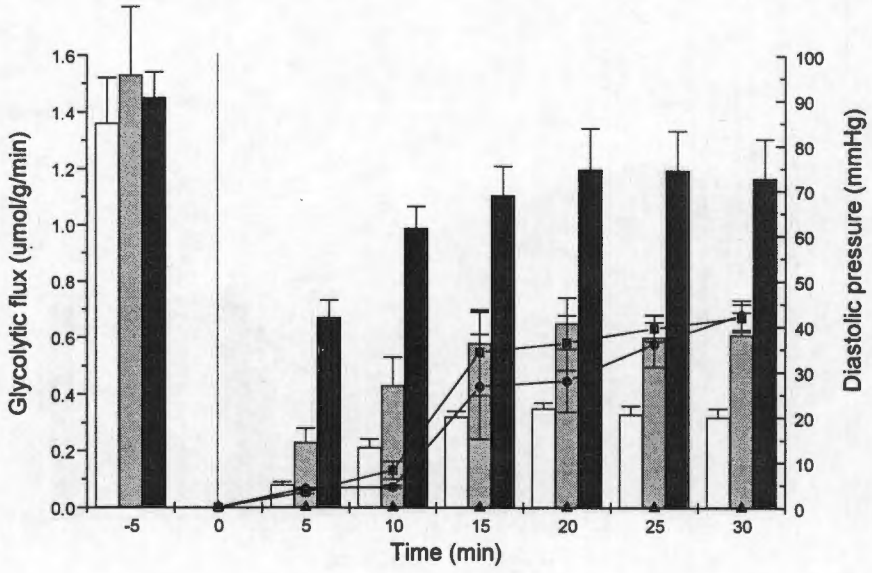

Fig 1e

Fig 1: Glycolytic flux versus time prior to and during low flow ischaemia, and changes in diastolic pressure reflecting ischaemic contracture with different flow rates and glucose concentrations (Fig 1a - 0 mM, Fig 1b - 2.75 mM; Fig 1c - 5.5 mM; Fig 1d - 11 mM; Fig 1e - 22 mM).

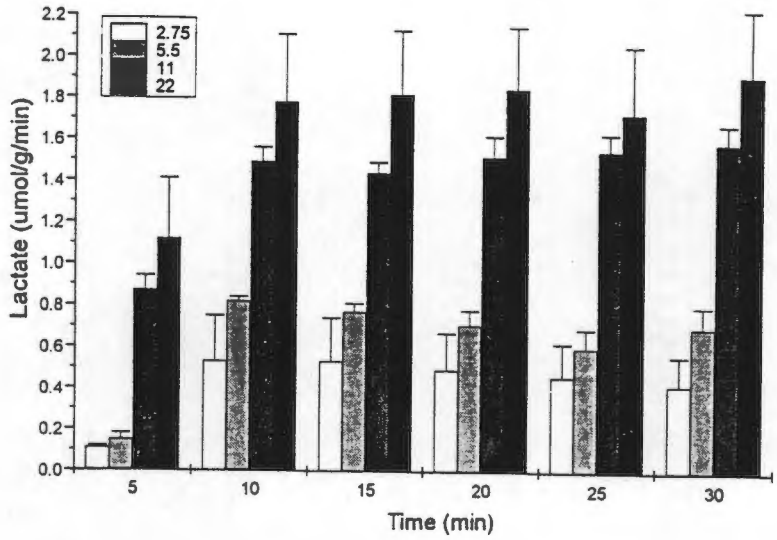

Fig 2: Lactate washout before and during ischaemia at a flow rate of 0.2 ml/g wet wt/min, with glucose concentrations of 2.75 mM, 5.5 mM, 11 mM, or 22 mM,.

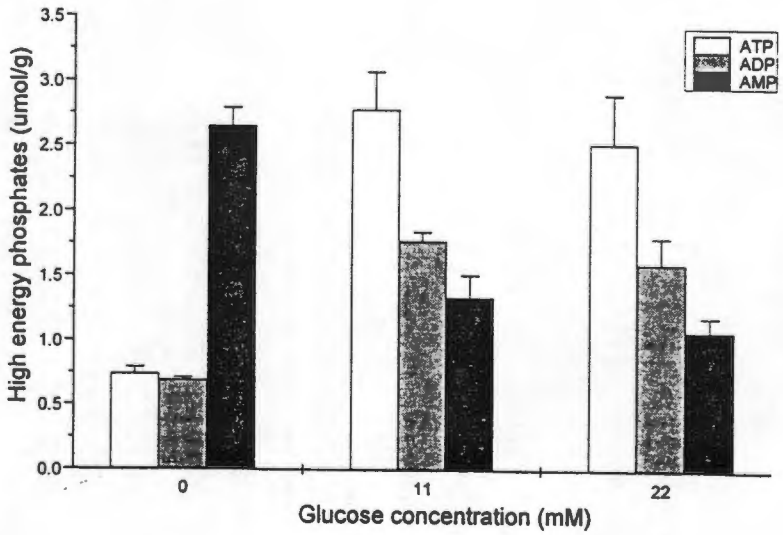

Fig 3: Tissue high energy phosphates after 30 min low flow (0.2 ml/g wet wt/min) ischaemia with changes in glucose concentration.

Table 1: Pre-ischaemic and reperfusion function in hearts perfused throughout with different glucose concentrations and basal substrate of 5 mM acetate, after 30 min ischaemia with varying flow rates and 20 min reperfusion

		Glucose concentration				
		0 mM	2.75 mM	5.5 mM	11 mM	22 mM
Pre-ischaemia	SP(mmHg)	117.7 ± 1.6	108.4 ± 3.9	108.4 ± 3.0	108.4 ± 4.1	104.9 ± 2.8
	Dev P (mmHg)	111.8 ± 1.8	104.2 ± 3.9	104.2 ± 3.0	104.4 ± 4.1	100.8 ± 2.8
Reperfusion						
0.1 ml/g wet wt/min	SP (mmHg)	93.7 ± 2.7	82.2 ± 2.8	87.3 ± 2.8	86.7 ± 2.9	84.8 ± 4.5
	DP (mmHg)	62.2 ± 4.0	50.7 ± 5.3	57.7 ± 5.3	59.5 ± 6.7	60.0 ± 5.1
	Dev P (%)	28.6 ± 4.0	33.8 ± 7.5	30.2 ± 8.7	28.1 ± 9.3	24.6 ± 6.6
0.2 ml/g wet wt/min	SP (mmHg)	90.6 ± 1.8	86.7 ± 4.6	87.5 ± 6.1	97.1 ± 6.5	93.2 ± 6.1
	DP (mmHg)	59.1 ± 3.9	44.2 ± 5.7	47.5 ± 2.7	24.9 ± 5.0	58.0 ± 5.7
	Dev P (%)	28.4 ± 4.0	42.9 ± 9.0	41.2 ± 8.5	69.4 ± 8.0 $**	33.5 ± 9.8
0.5 ml/g wet wt/min	SP (mmHg)	90.5 ± 1.9	101.5 ± 2.3	116.3 ± 4.4	127.2 ± 1.9	96.9 ± 6.6
	DP (mmHg)	66.7 ± 2.4	28.0 ± 3.5	8.0 ± 1.7	7.6 ± 1.4	8.0 ± 3.2
	Dev P (%)	20.4 ± 2.1	62.6 ± .9 $	93.2 ± 2.5 $&	97.0 ± 0.6 $&	89.4 ± 7.2 $&

* p<0.05 vs 2.75 mM; # p<0.05 vs 5.5 mM; ** p<0.05 vs all concs; $ p<0.05 vs 0.1 ml/g wet wt/min; & p<0.05 vs 0.2 ml/g wet wt/min

Table 2: Time to onset (TOC) and peak contracture with changes in glucose concentration and residual flow rate

		Glucose concentration				
		0 mM	2.75 mM	5.5 mM	11 mM	22 mM
0.1 ml/g wet wt/min	TOC (min)	4.5 ± 0.7 **	10.0 ± 1.1	12.4 ± 0.9	13.6 ± 1.2 *	11.5 ± 1.6
	Peak (mmHg)	93.8 ± 1.3 **	68.3 ± 7.7	51.3 ± 2.4 *	48.2 ± 6.0 *	50.3 ± 2.7 *
0.2 ml/g wet wt/min	TOC (min)	5.5 ± 0.5 **	12.3 ± 1.1	14.3 ± 1.1	16.9 ± 0.9 $*	13.7 ± 1.1 @
	Peak (mmHg)	104.0 ± 3.0 **$	70.3 ± 7.8	57.7 ± 6.5	30.5 ± 3.4 $*#	54.5 ± 6.2 @
0.5 ml/g wet wt/min	TOC (min)	3.1 ± 0.8 **	15.4 ± 0.5 $&	25.7 ± 2.2 $&	30.0 ± 0.0 $&*	30.0 ± 0.0
	Peak (mmHg)	126.2 ± 2.3 **$&	101.7 ± 4.2 $&	10.3 ± 1.3 $&*	6.8 ± 0.3 $&*	0.0 ± 0.0

* $p<0.05$ vs 2.75 mM; # $p<0.05$ vs 5.5 mM; @ $p<0.05$ vs 11 mM; ** $p<0.05$ vs all concs

$ $p<0.05$ vs 0.1 ml/g wet wt/min; & $p<0.05$ vs 0.2 ml/g wet wt/min

Table 3: Coronary flow and glycolytic flux in pre-ischaemia, mean flux during 30 min ischaemia, and coronary flow and glycolytic flux after 20 min reperfusion in hearts perfused with increasing concentrations of glucose.

		Glucose concentration			
		2.75 mM	5.5 mM	11 mM	22 mM
Pre-ischaemia	CF (ml/min)	15.5 ± 0.5	15.3 ± 0.4	16.4 ± 0.6	16.8 ± 0.6
	Glyc flux (μmol/min)	0.62 ± 0.06	1.03 ± 0.18 *	1.17 ± 0.11 *#	1.45 ± 0.09 *
Ischaemia					
0.1 ml/g wet wt/min	Glyc flux (μmol/min)	0.08± 0.00	0.16 ± 0.01 *	0.24± 0.02 *#	0.27 ± 0.02 *#
0.2 ml/g wet wt/min	Glyc flux (μmol/min)	0.13 ± 0.01	0.37 ± 0.02 *$	0.43 ± 0.04 *$	0.52 ± 0.04 *#$
0.5 ml/g wet wt/min	Glyc flux (μmol/min)	0.25 ± 0.02 $	0.65 ± 0.03 *$	0.93 ± 0.04 *#$	1.05 ± 0.06 *#@‡
Reperfusion					
0.1 ml/g wet wt/min	CF (ml/min)	11.42 ± 1.44	11.50 ± 0.82	13.00 ± 1.46	9.67 ± 0.67
	Glyc flux (μmol/min)	0.87 ± 0.16	0.76 ± 0.09	1.00 ± 0.11	0.73 ± 0.18 ‡
0.2 ml/g wet wt/min	CF (ml/min)	12.42 ± 0.81	12.42 ± 0.69	12.42 ± 0.55	12.08 ± 0.67
	Glyc flux (μmol/min)	0.80 ± 0.12	0.95 ± 0.07	0.82 ± 0.11 ‡	0.86 ± 0.13 ‡
0.5 ml/g wet wt/min	CF (ml/min)	10.27 ± 0.72	11.72 ± 0.89	14.20 ± 0.22	12.10 ± 0.61
	Glyc flux (μmol/min)	0.48 ± 0.03	0.61 ± 0.05	0.76 ± 0.07 * ‡	0.72 ± 0.05 ‡

* $p<0.05$ vs 2.75 mM; # $p<0.05$ vs 5.5 mM; @ $p<0.05$ vs 11 mM; † $p<0.05$ vs 22 mM; ‡ $p<0.05$ vs pre-isch; all isch rates $p<0.05$ vs pre-isch.

$ $p<0.05$ vs 0.1 ml/g wet wt/min; & $p<0.05$ vs 0.2 ml/g wet wt/min

Results 3. Reappraisal of glucose uptake by the myocardium - relationship between glucose concentration, coronary flow and glucose uptake.

Relevance to PET "mismatch" concept

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ABSTRACT

Background: Glycolysis is generally thought to be stimulated by hypoxia, and inhibited in severe myocardial ischaemia by glycolytic metabolite inhibition of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). However, observations with positron emission tomography (PET) in patients with myocardial ischaemia have shown increased rather than decreased fluoro-deoxyglucose (18FDG) uptake relative to coronary flow in ischaemic tissue, or "mismatch". To clarify this apparent contradiction, we reassessed glucose uptake by the isolated rat heart using 1) data from our own laboratory dealing with ultra low flow rates and 2) data previously published that claimed to demonstrate glycolytic inhibition.

Methods and Results: Absolute glucose uptake plotted against coronary flow showed that, as coronary flow fell, glucose uptake increased initially due to reversal of the Pasteur effect, but then fell to levels below those obtained at control flow rates, as expected. The percent glucose extraction was calculated from glucose uptake at each flow rate expressed as a percentage of that delivered to the myocardium, where delivery = concentration x coronary flow. At coronary flows less than about 1-2 ml/g wet wt/min in the rat heart, the % extraction of glucose was greatly increased. Analysis of glycolytic metabolite accumulation did not indicate inhibition of GAPDH.

Conclusions: These results challenge the concept that glycolysis is limited by enzyme inhibition under severe low flow conditions, but rather show that glucose uptake is markedly dependent on glucose delivery. Even though absolute uptake decreased, the extraction of glucose rose as coronary flow fell. These findings are consistent with PET "mismatch" observations.

INTRODUCTION

Glucose protects the ischaemic myocardium, with reduced reperfusion arrhythmias and ischaemic contracture, and improved reperfusion recovery[14, 49, 123, 424] (Results Ch 1), by provision of glycolytic ATP. The rate of glycolysis is dependent on substrate supply and enzyme reactions. Glycolysis is generally thought to be inhibited in ischaemia at the glyceraldehyde 3-phosphate dehydrogenase step (GAPDH)[362, 468] by a build up of metabolites[389, 390, 468, 469], a concept derived from observations of significantly lower rates of glycolysis in isolated rat hearts perfused with a low coronary flow (0.6 ml/g wet wt/min) compared to control (12-15 ml/g wet wt/min)[389, 390, 468, 469]. Thus an increase in substrate at a given low flow should not be able to overcome the restraint on ischaemic glycolytic flux[468], and the relationship between glucose utilisation and coronary flow would be expected to be proportionate. This hypothesis, however, is contrary to observations using PET, which suggest that glucose extraction is increased at low flow rates in viable tissue ("mismatch")[112, 343, 535, 562]. In addition, in the in vivo pig model, absolute glucose uptake in the subendocardium of an ischaemic zone is not less than that in the non-ischaemic epicardium, but extraction is increased[507].

We re-examined data from the literature describing glucose uptake over a range of coronary flows in the isolated perfused rat heart, combined with new data concentrating on low flows more comparable to 'true' in vivo ischaemic flows (0.07-0.15 ml/g wet wt in pigs and dogs[507]). Under ischaemic conditions, the substrate supply is severely impaired, which is directly reflected in a reduced rate of glycolysis. At each flow rate, glucose uptake was related to glucose concentration by a relationship equivalent to Michaelis Menten kinetics. However, the rate of glycolysis (or glucose uptake) is not reduced proportionately to the reduction in flow, and in fact is upregulated relative to flow as shown by an increased % glucose extraction. In addition, analysis of glycolytic metabolites did not show evidence of inhibition of GAPDH. On the basis of these findings, we conclude that, during severe ischaemia, inhibition of glycolytic enzymes is not the rate limiting factor in determining glycolytic flux rate, but that the rate of glucose utilisation is determined primarily by the delivery of glucose to the cells, and its subsequent rate of transport across the cell membrane. While enzyme inhibition is not excluded as a modulator of glycolysis, its importance must be understood relative to delivery of substrate, a factor which has not previously been taken into consideration. These data support the "mismatch" concept, of increased glucose extraction under low flow conditions.

METHODS

ISOLATED RAT HEART PERFUSIONS

The isolated Langendorff-perfused rat model with a left ventricular balloon was used, as described in Methods. 5 mM acetate was used to provide an alternative substrate when glucose was absent or at very low concentrations, to prevent adverse effects associated with substrate free perfusion[424] (Results Ch 1). In low flow conditions, acetate does not affect glucose uptake (data not shown). The solution was gassed with 95% O_2 5% CO_2 to maintain pH at 7.4.

Experimental protocol

Hearts were subjected to 15 min aerobic perfusion, followed by 30 min global subtotal ischaemia with coronary flows of i) 0.1, ii) 0.2 or iii) 0.5 ml/g wet wt /min, with different glucose concentrations (0. 2.75, 5.5, 11 and 22 mM) for each flow rate. 6 hearts were used in each group. Coronary effluents were collected over 15 secs after 15 min control perfusion and over each 5 min period during ischaemia.

In a second series of experiments to determine changes in metabolites with ischaemia, hearts were perfused with 11 mM glucose with or without insulin (1 U/l), and subjected to 30 min global ischaemia with no flow or a low flow of 0.2 ml/g wet wt/min. Glucose uptake was measured during low flow ischaemia. Hearts were clamped with Wollenberger tongs kept in liquid nitrogen at the onset, after 15 min and after 30 min ischaemia. 6 hearts were used for each time point.

Biochemistry

Glucose uptake was measured by the rate of $^3H_20$ production from D-[2-^{3}H] glucose[389, 468], as described in Methods.

Heart tissue was freeze-dried, and metabolites extracted with perchloric acid[24], or with ethanol and NaOH for analysis of glycogen content[35]. The extracts were then assayed using spectrophotometric assays adapted for use on a Cobas Fara centrifugal analyser (Roche Diagnostics, Switzerland)[24]. Values were expressed as µmol/g wet wt. Glycogen was expressed as µmol 6-carbon units/g wet wt.

LITERATURE ANALYSIS

Data from articles measuring glucose uptake in the isolated perfused rat heart was analysed, specifically focusing on a series of articles published by the group of Neely[388-390, 468, 469]. The model used in these studies was the working heart (perfusion via the pulmonary veins - 7.5 mmHg preload, 60 mmHg afterload) with a one way valve. The hearts ejected against the valve which subsequently trapped the effluent, preventing reflux perfusion of the coronaries and resulting in

gradual ischaemia. Glucose utilisation, measured by D-[2-^{3}H] or D-[5-^{3}H]-glucose, was taken to be equivalent to glucose uptake.

EXPRESSION OF RESULTS AND CURVE FITTING

Results were expressed as mean ± SEM. All data were expressed as per gram wet weight (wet weight = 5 * dry weight - previous determinations). The data were fitted using Origin™ (MicroCal) computer programme. Continuous reiterations were performed until the best fit was obtained. The goodness of fit of non-linear regression was assessed by the χ^2 value.

RESULTS

GLUCOSE UPTAKE AT DIFFERENT FLOW RATES AND GLUCOSE CONCENTRATIONS

Changes in glucose uptake in control and ischaemic hearts

Pre-ischaemic glucose uptake rates were dependent on glucose concentration, as shown in Figs 1 and 2. At each low coronary flow rate, glucose uptake fell initially from control levels, and then increased to peak at 15-20 min (see Fig 1a - 0.1, Fig 1b - 0.2; Fig 1c - 0.5 ml/g wet wt/min). With 2.75 mM glucose, glucose uptake fell after 15 min ischaemia at each low coronary flow, such that by 30 min ischaemia, the rates of glucose uptake were similar in each group (cf. Fig 1 a, b, c). With a glucose concentration of 5.5 mM, a decline in glucose uptake was seen only with a flow rate of 0.2 ml/g wet wt /min (Fig 1b). This decline may have been due to the high uptake reached in the first 15 min ischaemia, which exceeded that with 11 mM The rate of glucose uptake at the end of ischaemia, however, was proportionate to the concentration. With both 11 mM and 22 mM, glucose uptake was maintained at a plateau level throughout ischaemia at each flow rate (Fig 1a, b, c). With a flow rate of 0.5 ml/g wet wt/min, glucose uptake rates with 11 and 22 mM glucose reached levels similar to those in pre-ischaemic hearts (Fig 1 c).

Mean glucose uptake - effect of concentration and flow rate

The mean glucose uptake rate over the total ischaemic period of 30 min was calculated, and plotted against glucose concentration for each flow rate (mean value used because of lack of steady state for all concentrations). Double rectangular hyperbolic relationships were obtained (Fig 2), equivalent to Michaelis Menten kinetics, such that glucose uptake increased linearly from 0-5 mM, and was saturated at concentrations greater than 11 mM. Similar trends were observed at flow rates of 0.2 and 0.5 ml/g wet wt /min, as well as with control coronary flows (Fig 2), although the curves were shifted upwards, and to the left.

Mean glucose uptake plotted against flow rate showed an apparent positive linear relationship for each glucose concentration within the range of low flows investigated (Fig 3). The slope of the line increased with increasing glucose concentration. However, control values (at control coronary flows) were in a similar range, suggesting that the curves would decline after reaching a peak in order to fall to these values, giving an inverted-U shape.

DATA AT A GLUCOSE CONCENTRATION OF 11 MM

Absolute glucose uptake

The standard concentration of glucose used in isolated rat heart perfusions in the absence of insulin is 11 mM. The results from the above perfusions for glucose uptake with 11 mM glucose, together with

data taken from reports by Neely et al.[388-390, 468, 469], and previous data from our laboratory (Results Ch 1), were plotted as a function of coronary flow (Fig 4). Despite differences in models (a one-way valve resulted in a gradual fall in coronary flow, and a gradual reduction in glucose uptake at low flows[389], compared to abrupt changes in Langendorff model), steady state rates of glucose uptake were reached. The plateau, or steady state values for glucose uptake were used for further calculations (mean glucose uptake not always given; steady state rates more easily comparable between models). With 11 mM glucose, uptake was sustained over at least 30 min at each flow rate (Fig 1). A total of 29 points, each representative of at least 6 hearts, was obtained. The points followed an inverted-U pattern, as predicted from Fig 3 (see above).

Percentage glucose extraction

While absolute glucose uptake was reduced at low flow rates, the uptake relative to that available to the tissue has not previously been reported for isolated rat hearts. In order to establish the relationship between glucose uptake and delivery of glucose, where

delivery (μmol/min/g wet wt) = glucose concentration (μmol/ml) * coronary flow (ml/g wet wt/min) eqn 1

(glucose concentration - of the perfusate) the term percentage glucose extraction was derived, defined as

% Glucose extraction = [glucose uptake (μmol/min/g wet wt) / delivery (μmol/min/g wet wt)] * 100 eqn 2

Because

glucose uptake = Δ glucose content (μmol/ml) / coronary flow (ml/g wet wt/min) eqn 3

coronary flow appears to be cancelled out of eqn 2. However, because glucose uptake is dependent on coronary flow (according to Fig 4), extraction is a function of coronary flow. When percentage extraction of each data point in Fig 4 was plotted against coronary flow, a negative double exponential relationship was found (shown in Fig 5a).

$$\% \text{ extraction} = 5.76\, e^{(-x/7.54)} + 28.69\, e^{(-x/0.35)} \qquad \text{eqn 4}$$

with $\chi^2 = 6.64$ where x = coronary flow (ml/g wet wt/min). At the "normal" range of flows for an isolated perfused heart (8-16 ml/g wet wt/min), % extraction was very low, below 3% of that delivered to the myocardium, even though glucose was the sole external substrate in the majority of hearts. When the double exponential curve was plotted as 2 separate components, a slow rise in glucose uptake was seen as flow rate was reduced over the whole range of flow rates for the first component. However, the second component showed a sharp increase in glucose uptake as flows fell below 1-2 ml/g wet wt/min, with a much shorter half life, of 0.35 versus 7.54 min. The extraction of glucose rose from below 3% up to 25-28% at low flow rates of 0.1 - 0.2 ml/g wet wt/min.

% extraction versus delivery

When the percentage glucose extraction for different glucose concentrations (from Fig 2) was plotted against delivery of glucose (flow rate * concentration) rather than coronary flow, to allow comparison of the data, a similar negative exponential relationship was found (Fig 5b), with a sharp increase in glucose extraction at reduced delivery. The graph is plotted showing different ranges of glucose concentrations.

MATHEMATICAL MODEL OF GLUCOSE UPTAKE

Predicted glucose uptake from extraction

If the curve of % extraction versus coronary flow generated above (eqn 4; Fig 5a) were expressed as a function of glucose uptake, where

$$\textbf{[glucose uptake/(glucose conc * coronary flow)] / 100 = f(coronary flow)} \qquad \text{eqn 5}$$

the predicted uptake became

$$\textbf{glucose uptake (}\mu\textbf{mol/min/g wet wt)} = 0.63 \times e^{(-x/7.54)} + 3.16 \times e^{(-x/0.35)} \qquad \text{eqn 6}$$

where x = coronary flow (Fig 4 - derived fit from Fig 5a).

If this function were used to fit the data in Fig 4, the variables changed slightly to

$$\textbf{glucose uptake} = 0.80 \times e^{(-x/6.15)} + 4.79 \times e^{(-x/0.19)} \qquad \text{eqn 7}$$

where x = coronary flow, and $\chi^2 = 0.165$ (shown in Fig 4 - adjusted fit). There was a very good agreement between the predicted and the fitted curves indicating the consistency of the model.

Non-linear regression

If the data were fitted by non-linear regression using standard equations, the best fit was obtained with a polynomial function, with a $\chi^2 = 0.09$. The relationship was described by

$$\textbf{Glucose uptake (}\mu\textbf{mol/min/g wet wt)} = 0.306 + 0.507\,x - 0.049\,x^2 + 0.001\,x^3 \qquad \text{eqn 8}$$

where x = coronary flow (ml/g wet wt/min) (see Fig 4 - polynomial regression curve). There was also good agreement between the curves generated from the % extraction, and that described by the polynomial.

With a reduction in coronary flow, a peak glucose uptake was reached at a flow rate of about 6 ml/g wet wt/min, where a reduced oxygen availability would stimulate glucose uptake. At lower flow rates, the curve showed a reduced uptake as flow fell, rather than continuing to increase. Given a control normoxic uptake of 1-1.5 μmol/min/g wet wt, glucose uptake was lower than control values at flow rates less than about 2 ml/g wet wt/min. Flow rates of 5-6 ml/g wet wt/min are equivalent to those *in vivo* in the normoxic rat heart.

TISSUE METABOLITE VALUES

The values for the glycolytic metabolites measured, as well as glycogen, are shown in Table 1. Metabolites can be broadly grouped into substrates (G6P, F6P, FDP, GAP, DHAP, pyruvate) and end products (L-alanine, α-glycerophosphate, lactate). If the "substrates" are expressed as a percentage of the total (substrate+products), this value declines during ischaemia, as the product accumulation greatly outweighs the accumulation of the metabolites (Table 1). If the cross-over theorem is applied[68, 468], G6P and F6P are shown to accumulate significantly in ischaemia, as do the end products, lactate and α-glycerophosphate (Fig 6). However, no significant accumulation of other glycolytic metabolites was found, contrary to previous reports[468]. Only GAP increased by 30 min with glucose low flow ischaemia (Fig 6), but these values were widely spread and thus not of significance (see Table 1). Values of "substrates" tended to be higher at 15 min than at 30 min in zero flow ischaemia, indicating that glycolysis was limited after 30 min ischaemia by reduced substrate, with all available metabolites used up to provide additional ATP (Table 1; Fig 6). Only 2.5 μmol/g wet wt glycogen was utilised during the last 15 min, compared to 12.4 μmol/g wet wt in the first 15 min. With maintained flow rates, this trend was not observed, with maintained glycolysis throughout 30 min ischaemia. Glycogen breakdown was also significantly greater in the absence of flow (91% vs. 61.5% over 30 min).

If insulin was present, glycogen was increased prior to ischaemia (Table 1), as was glucose uptake (to 0.9 ± 0.1 vs. 0.5 ± 0.0 μmol/g wet wt/min; $p<0.05$). G6P and F6P increased significantly during ischaemia, from increased glucose uptake. A "bottle-neck" effect was seen. Values of FDP, GAP and DHAP were lower after 30 min compared to the 15 min values despite high G6P and F6P values, possibly indicating some enzyme inhibition with total global ischaemia and excess glycolytic substrate. With a maintained low flow, G6P and F6P values were lower, while the differences between 15 and 30 min for the other metabolites were also not as apparent.

Lactate production was significantly greater in insulin hearts. The % substrate to total metabolites was subsequently higher in these hearts, although there was little evidence of GAPDH inhibition.

DISCUSSION

The main finding presented in this paper is that the rate of glycolysis is not inhibited in ischaemia; rather, glycolysis is limited by the availability of substrate and rates of transport into the cell. We do not fully exclude the contribution of enzyme inhibition, but its importance appears secondary to limitation of substrate supply. These results are in agreement with standard concepts of substrate - product relationships determining flux through a pathway[391], and support the observations of mismatch as measured by ^{18}FDG uptake. A scheme illustrating glycolytic regulation at different levels is shown in Fig 7.

TECHNIQUES, DEFINITIONS AND RESERVATIONS

Data from Langendorff and working hearts have been included together in Figs 4 and 5. While differences in the perfusion model certainly affect glucose uptake at normal flow rates, given that the working heart has a much higher demand, the values were found to be quite comparable, particularly at the low flow range in which we were interested. The model used by Neely et al. used lower perfusion pressures than others (preload - 10 cm H_2O; afterload - 80 cm H_2O vs. 20 cm H_2O and 100 cm H_2O respectively[414, 425]) which reduces the demand for glucose, and resulted in values comparable to those in Langendorff hearts. Factors such as oxygen uptake, perfusion pressure, and work rate, will also modify glucose uptake under normal conditions. The values at higher flow rates are thus open to interpretation (see below). However, under ischaemic conditions, both working and Langendorff hearts show virtual cessation of pump function, and the metabolic rates are thus comparable.

We measured the amount of D-[2-^{3}H] glucose detritiated in the phosphoglucoisomerase reaction (G6P to F6P), and equated this to glucose uptake. These data do not differ significantly from measurements of arteriovenous glucose differences (data not shown), although the radioactive methods are more precise. D-[2-^{3}H] glucose detritiation has been referred to as the "gold standard" for measuring glucose uptake[180]. This method has also been used to measure glycolytic flux rates[468] (Results Ch 1), although D-[5-^{3}H] glucose may give a more accurate result as this compound is detritiated "further down" the glycolytic pathway, at the enolase step[337]. However, Rovetto et al.[468] used both forms of glucose indiscriminately, and we found no difference in results using the two isotopes in low flow ischaemia (data not shown). Thus the majority of glucose phosphorylated in ischaemia appears to be converted to pyruvate.

D-[2-^{3}H] glucose detritiation does not distinguish between rates of glucose transport across the sarcolemma, and subsequent phosphorylation. These processes are taken as a single step, although the implications are discussed. Alternative fates of glucose including glycogen and intermediate metabolite accumulation are also not considered in the gross analysis of glucose uptake/glycolysis, for reasons as follows. Only about 5% of glucose goes to glycogen turnover in the normally beating heart

in the absence of insulin[162], and 11.6% with insulin and free fatty acids present[196]. These values are presumably even lower in the ischaemic heart. Tissue glycogen levels during ischaemia are rapidly depleted, more so when it is the only substrate present, and the sole contributor to glycolysis (see Table 1). More glycogen is utilised in 30 min ischaemia in the absence of any exogenous glucose, and in the absence of a residual coronary flow (Table 1). Any residual glycogen is likely to be proglycogen, a form of glycogen more resistant to catabolism[40, 103]. These findings contribute to the argument that glycolysis is not limited by enzyme inhibition in ischaemia.

The sum of glycolytic metabolites, other than the final end products of lactate and αGP, does not account for more than 7.5% of the total metabolite accumulation (see Table 1, and from[468]). In addition, with any residual flow, much of the lactate will be washed out and there may be some residual mitochondrial oxidation, which further reduces this percentage. While αGP accumulation also accounts for some of the glycolytic substrate, the values are relatively small compared to both total glycolytic substrate, and overall lactate production.

Thus, for the purposes of this discussion, glucose uptake equates to glycolytic flux and to glucose utilisation (terms used interchangeably in the literature, but each of which has specific implications which must be considered when applied in context), i.e. glucose entering the cell, and subsequently phosphorylated is assumed to be converted to pyruvate, and subsequently to lactate under anaerobic conditions. This assumption also invokes the concept of glycolysis as a "metabolon", or single unit made up of multiple enzymes[505, 506], which allows efficient "channelling" of the product of one reaction to the next, where it becomes the substrate[576]. Thus glycolysis is like a "funnel" - entry of substrate into the pathway virtually ensures eventual exit (see Fig 7).

KINETICS OF GLUCOSE UPTAKE WITH DIFFERENT FLOW RATES AND GLUCOSE CONCENTRATIONS

In most hearts, glucose uptake reached a peak at about 10-15 min. In hearts perfused with 2.75 mM, glucose uptake then decreased significantly. Hearts with 5.5 mM glucose showed similar trends, although not as marked. A similar decline has been noted previously in rabbits perfused with 5-6 mM glucose in low flow ischaemia[14, 110, 550]. Glucose uptake occurs by a process of facilitated diffusion, which implies that a certain minimal glucose gradient is required to ensure transport across the membrane[59]. In addition, if glucose supply is limited, the rate of ATP production will decrease, which will inhibit cell function. As the cells deteriorate, the failing heart will require less ATP, and thus less glucose. Higher glucose concentrations were able to maintain glucose uptake at a steady state throughout ischaemia. These concentrations in turn generally result in improved recovery of heart function[14, 550] (Results Ch 1).

The data for mean glucose uptake versus glucose concentration for each flow rate were fitted by double rectangular hyperbolas, equivalent to the Michaelis - Menten relationship used to describe

enzyme activity. At control flow rates, the apparent Km of glucose uptake for the whole heart was 5 mM, with a Vmax of 1.8 μmol/min. Therefore at least 5 mM glucose was required to sustain glucose uptake in normally perfused hearts, and preferably double this concentration in the absence of insulin. This result agrees with the other studies on the isolated heart, showing similar kinetics, where the optimal perfusion concentration was around 8-10 mM[367, 415].

The Km was shifted to the right at low flow rates (Fig 2) suggesting that, in ischaemia, a higher minimum glucose concentration (at least 11 mM glucose) is required to sustain glucose uptake than is necessary in normoxia. This shift may be explained by the increased transit time of perfusate through the coronary vasculature, with a reduced coronary flow. The available glucose may be taken up rapidly, such that the tissue perfused further downstream would be exposed to a lower glucose concentration, insufficient to maintain uptake. A higher concentration may thus be required when coronary flow is very low, to sustain uptake throughout the passage of the perfusate through the heart.

The maximum rate of glucose uptake (Vmax) was dependent on coronary flow (Fig 2), and thus on delivery of substrate. In the absence of insulin, an increase in glucose concentration much above 11 mM had little effect on absolute glucose uptake, showing saturation of glucose uptake at these high concentrations. Only an increased coronary flow could increase absolute glucose uptake at each glucose concentration. The mechanisms for this finding may include; improved metabolism within the cell, such that the intracellular glucose content is continually reduced by utilisation; better perfusion by the coronary system allowing more tissue to take up glucose;, and reduced transit time such that at any given time more tissue is exposed to a high glucose concentration. The Vmax for 0.5 ml/g wet wt/min was greater than that for control flow rates, indicating that at higher "ischaemic" flow rates, the rate of glucose uptake could exceed that at control flows.

GLUCOSE UPTAKE AND EXTRACTION VERSUS CORONARY FLOW AT AN OPTIMAL GLUCOSE CONCENTRATION

Glucose uptake, at a fixed standard concentration of 11 mM, was increased at a coronary flow rate of 6 ml/g wet wt/min compared to that in control hearts, and declined with a further fall in the coronary flow rate[388] as delivery became limiting (see Fig 4). However, extraction increased sharply as coronary flows fell below about 1-2 ml/g wet wt/min, showing a disproportionate increase in glucose uptake relative to flow and challenging the concept of limitation of glycolysis in ischaemia. Coronary flow can be broadly separated into three ranges.

Normal coronary flow rates

At 'normal" coronary flows in the isolated rat heart (8-16 ml/g wet wt/min - Fig 4), glucose uptake is modified by factors including the model used (see above), work rate, heart rate, perfusion pressure, oxygen availability, alternate substrates, and status of the heart (diabetic, hypertrophied, starved) (e.g.

see refs[367, 414, 415, 424]). Rates of glucose uptake are therefore not closely dependent on coronary flow at "normal" flow rates. The line shown in Fig 4 over this range of flows is thus only applicable to the data presented. However, despite variations in absolute glucose uptake with different models, the % extraction of available glucose is still very low in control conditions (Fig 5).

Intermediate reduction in coronary flow rates

At "moderate" coronary flow rates in the isolated rat heart (2-8 ml/g wet wt/min), glucose uptakes may also differ from the curve illustrated (Fig 4). In the isolated rat heart model shown, glucose uptake was high at flow rates in the range of 4-10 ml/g wet wt/min, peaking at a flow rate of 6-7 ml/g wet wt/min with a glucose uptake of 1.75 to 2 μmol/min/g wet wt. The increased glucose uptake (and glycolysis) at these flow rates was presumably due to reversal of the Pasteur effect, with a relative hypoxia at lower flow rates, and a fall in citrate and ATP levels. At coronary flow rates of 6-7 ml/g wet wt/min, the heart thus presumably has optimal stimulation of glycolysis, and adequate delivery of substrate and removal of metabolites. As flow falls, delivery of substrate becomes limiting, resulting in the decline of glucose uptake. If delivery were not limiting, glucose uptake should continue to rise as flow falls.

"Ischaemic" coronary flow rates

If enzymatic inhibition were the major determinant of glycolytic flux in ischaemia, as flow falls, the rate of glucose uptake should be proportionate to the fall in coronary flow[468], such that % extraction should remain reasonably constant (as shown by the first component of the exponential curve - Fig 5a), or possibly even decrease with reduced flow rate (tending to the origin). Glycolysis was initially said to be inhibited because glucose utilisation in an isolated rat heart at a flow rate of 0.6 ml/g wet wt /min was less than that in the normally perfused myocardium[390]. However, as flow decreases below 1-2 ml/g wet wt/min ("ischaemic), % extraction rose sharply (Fig 5). We also did not find evidence of accumulation of GAP and DHAP during ischaemia, to support the concept of GAPDH inhibition[388-390, 468, 469] (see Fig 7). We therefore propose that glucose uptake is limited mainly by delivery of glucose to the myocardium, which, at an optimal concentration of 11 mM, is determined by coronary flow; and that the heart in fact compensates for reduced delivery by increasing glucose extraction (see Fig 7 for illustration of concepts). We do not, however, exclude the contribution of enzyme inhibition at low coronary flows. If no inhibition were present, the curves of uptake (Fig 4) and extraction (Fig 5) would be shifted upwards. Delivery and enzyme inhibition thus compete to determine the final rate of glucose uptake and utilisation. In zero flow conditions, some distributive control of glycolysis may occur, when the amount of available substrate is fixed, and the regulation of glycolysis then lies entirely with enzyme regulation.

Does this relationship occur in vivo?

Normal *in vivo* flow rates in the rat are about 5-6 ml/g wet wt/min[279], thus in the middle portion of the glucose uptake curve (Fig 4), while in the dog, normal flow rates are about 2 ml/g wet wt/min[507] i.e. nearing the "ischaemic" portion of the curve. In ischaemia, flow rates in the subendocardium in large animals *in vivo* are in the range of 0.07-0.15 ml/g wet wt/min[507] - about 3-8% of normal flow rates. Thus flow rates used in the present study (0.1-0.5 ml/g wet wt/min - about 2-8% of normal *in vivo* flow rates in the rat[279]) more closely reflect "true" ischaemia than do flow rates of 0.6 ml/g wet wt/min and higher[388-390, 468, 469].

The relationship between glucose uptake and coronary flow (Fig 4) would be altered in larger animals and in man, because the range of flows is much lower than in the isolated perfused rat heart. Measurements of glucose uptake in the *in vivo* pig heart showed no change in absolute glucose uptake with a fall in flow rate, compared to that found in the rat (Fig 4), but a negative logarithmic relationship between glucose extraction and coronary flow was found[507], similar to the present findings (Fig 5).

GLUCOSE TRANSPORT ACROSS THE MEMBRANE AND THE ROLE OF INSULIN

Glucose uptake is determined by the concentration difference across the membrane, the number of pores, or glucose transporters, in the membrane, and the affinity of the glucose transporters. Whether the rate limiting step in glycolysis in ischaemia (when glucose concentration is maximal) is the rate of delivery of glucose to the cells (determined by coronary flow rate), or the subsequent rate of transport across the cell membrane is difficult to establish. The rate of glucose phosphorylation by hexokinase may also be important, as this has been shown to be the most important factor in regulating glycolysis in normoxic conditions[241, 335].

The double exponential curve of extraction suggests that some mechanism is present, that results in an increased glucose uptake as flow falls below about 1-2 ml/g wet wt/min (Fig 5a). Kohn and Garfinkel proposed an increased affinity of glucose transport in ischaemia enabling increased glucose extraction in a computer generated model[262]. A more recent hypothesis suggests that translocation of GLUT 4 molecules (insulin sensitive glucose transporters) to the sarcolemma is triggered by ischaemia[517]. Alternatively, the cell surface accessibility of the glucose transporters already in the membrane may be increased[548] or they may be upregulated[454]. The apparent Km of the GLUT 4 transporter in *Xenopus* oocytes is 4.3 mM as opposed to 26.2 mM of GLUT 1[395], which would result in an overall increased affinity for glucose if the GLUT 4 membrane density were increased.

The addition of insulin to hearts perfused with 11 or 22 mM glucose at a low flow of 0.2 ml/g wet wt/min increased ischaemic glucose uptake by 65% and 85% respectively (see Results 2). Thus insulin shifts the glucose uptake versus glucose concentration curve significantly upwards (increased

Vmax), indicating that membrane transport is rate-limiting when sufficient substrate is available. Insulin increases the number of membrane "pores", as well as increasing the overall affinity for glucose transport by increasing membrane GLUT 4 density. Insulin-treated hearts also showed an increased accumulation of tissue G6P and F6P levels in ischaemia, possibly indicating inhibition of glycolysis at the level of phosphofructokinase (PFK) when substrate and product (lactate) are both in excess (see Fig 7). The response of the tissue to excess substrate adds to the concept of glycolysis as a "funnel" - at some point the amount of substrate entering the pathway may transiently exceed the amount which can exit. However, eventual output will be increased because of increased input.

These results highlight membrane glucose transport as a major rate-limiting factor in determining rates of glucose utilisation in ischaemic tissue (if external glucose present in sufficient or saturating concentration), as it is in non-ischaemic tissue[241].

POSITRON EMISSION TOMOGRAPHY

PET scanning enables non-invasive measurement of regional myocardial blood flow, substrate flux, and biochemical reaction rates. A reduction in blood flow is frequently associated with relative or absolute increases in ^{18}FDG uptake, or "mismatch", which is thought to indicate viability[112, 343, 535, 562]. The evidence presented in the present paper may explains the mechanisms involved in "mismatch", or increased extraction. While absolute glucose uptake may not be increased with reduced flow in ischaemic myocardium *in vivo*[507], a maintained glucose uptake despite a reduced coronary flow would show increased extraction[507], and indicate viability[111, 562] (Results Ch 1). Hypothetically, only if glucose uptake were proportionately more reduced than the reduction in flow rate, with reduced extraction, would the myocardium be non-viable[111]. The capacity of the non-viable tissue to take up glucose is presumably impaired, and the subsequent rate of ATP production would be reduced.

Two recent papers have introduced some concerns over the validity of PET and "mismatch". Hariharan et al.[180] found that uptake of ^{18}FDG was not as accurate a measure of glucose uptake as was the use of [2-^{3}H] glucose, as used in the present study. While this may question some of the measurements made by PET, this does not contradict the findings in the present paper. The concept of reduced blood flow and maintained glucose uptake in hibernating myocardium has also been challenged by Marinho et al.[340] who observed that glucose uptake and coronary flow in hibernating segments (not with scar tissue) was unchanged despite a reduction in left ventricular function. Thus extraction was not affected. This finding is not contrary to the concepts presented in the present paper, but emphasises the involvement in chronic left ventricular dysfunction of changes other than in glucose uptake and coronary flow.

SUMMARY

At low glucose concentrations, glucose uptake is limited by an insufficient gradient for facilitated diffusion, and the glucose concentration is rate limiting. At higher concentrations (> 11 mM), glucose uptake is saturated and is more dependent on coronary flow. The addition of insulin to perfusate with high glucose concentrations increases glucose uptake by increasing the ability of the membrane to transport glucose. Thus if sufficient or excess glucose is present, the transport of glucose through the membrane becomes rates limiting i.e. the number of transporters in the membrane determines the rate of uptake, which can be increased by insulin and/or ischaemia. A low coronary flow (<1-2 ml/g wet wt/min) appears to trigger an adaptive response in the myocardium allowing increased extraction of glucose at low flow rates. While absolute glucose uptake remains the same (*in vivo*) or falls (in isolated rat heart) with low coronary flows, the percentage extraction increases greatly when flow is reduced, indicating an ability of the myocardium to upregulate its capacity to transport glucose and thereby provide ATP essential for maintained cell function. Similar conclusions have been reached by others using different methods of analysis[110, 262].An increased extraction substantiates the concept of PET "mismatch".

Our findings suggest that the concept that glycolysis is inhibited in ischaemia is not complete. Rather, glycolysis is limited primarily by delivery of glucose, and/or membrane transport, and may be modified secondarily by enzyme activity and ATP demand.

The consequences to the heart of the different rates of glucose uptake/glycolysis are presented in Results Ch 2.

Glucose utilisation is closely dependent on the delivery of glucose, but the rate of glycolysis is also closely related to the tissue glycogen level, the endogenous storage form of glucose. The phenomenon of preconditioning has aroused much interest, as the intervention of a preceding ischaemic period confers protection against a subsequent ischaemic period. One of the many proposals for the protective mechanism of preconditioning is a pre-ischaemic depletion of glycogen. However, this concept is contrary to the findings presented in the above studies, where all results suggest that provision of glycolytic substrate is of benefit to the heart. We wished to test whether glycogen depletion is of benefit to the ischaemic heart, and whether the protective effects of preconditioning can be related to glycogen depletion.

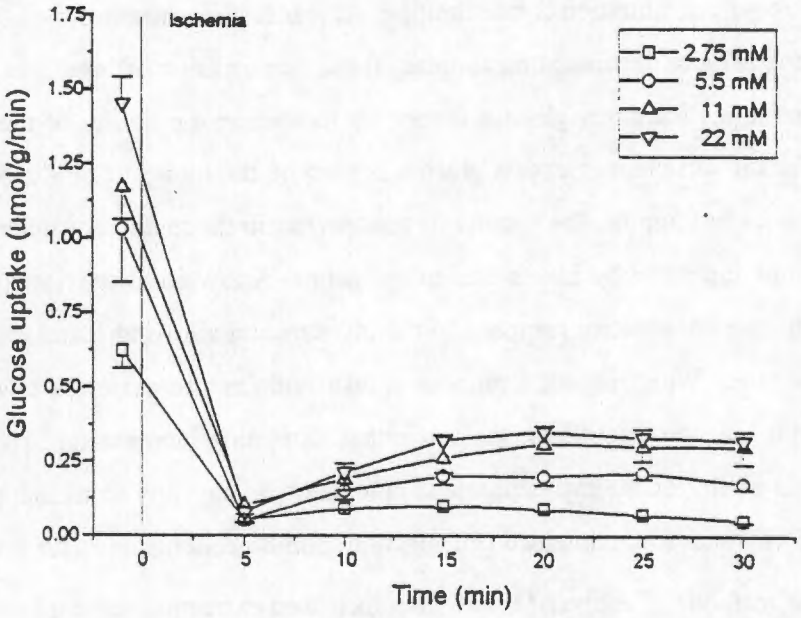

Ischemia
2.75 mM
5.5 mM
11 mM
22 mM
Glucose uptake (umol/g/min)
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
0
5
10
15
20
25
30
Time (min)

Fig 1a

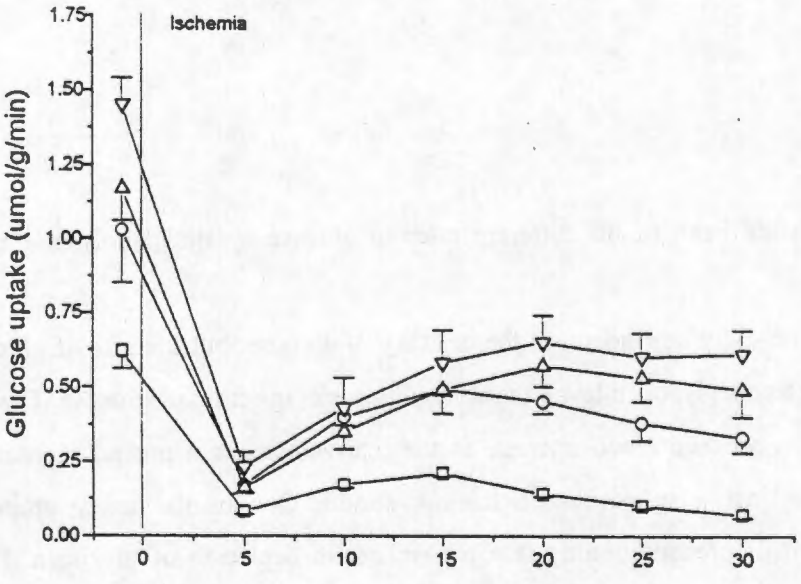

Ischemia
Glucose uptake (umol/g/min)
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
0
5
10
15
20
25
30

Fig 1b

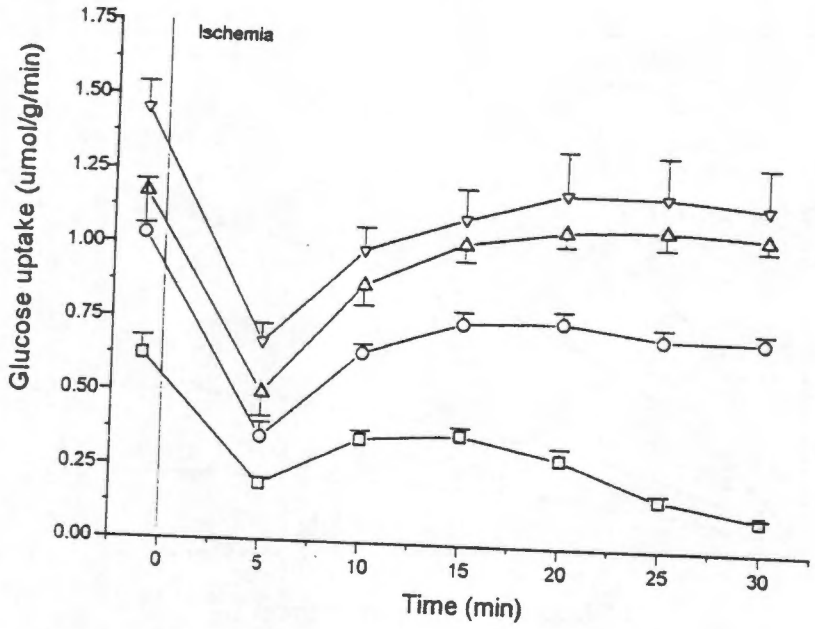


Fig 1c

Fig 1. Glucose uptake versus time before and during low flow ischaemia, with different glucose concentrations at different flow rates (Fig 1a - 0.1; Fig 1b - 0.2; Fig 1c - 0.5 ml/g wet wt /min). Coronary flows in control hearts were 15.5 ± 0.5, 15.3 ± 0.4, 16.4 ± 0.6, and 16.8 ± 0.6 ml/g wet wt/min for glucose concentrations of 2.75, 5.5, 11 and 22 mM respectively.

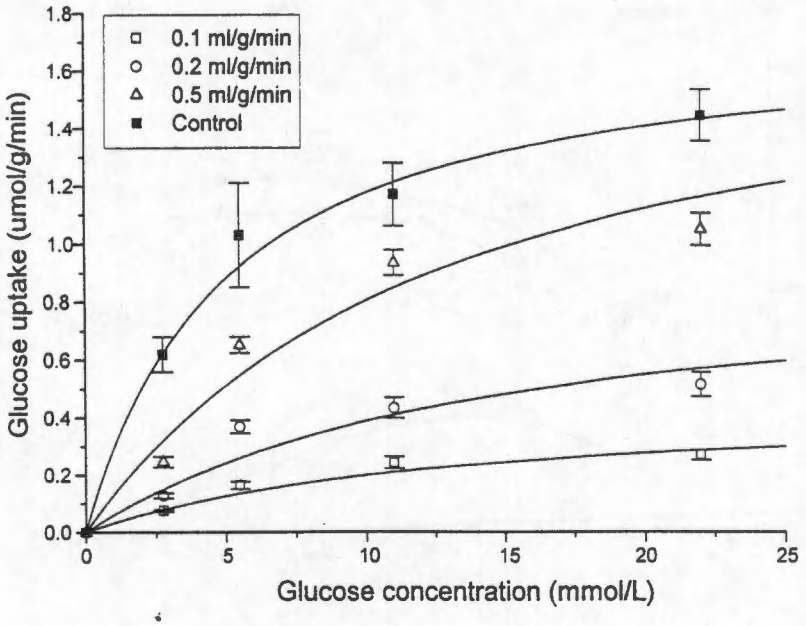


Fig 2: Mean glucose uptake versus glucose concentration for a range of flow rates. At each flow rate, glucose uptake was described by a double rectangular hyperbola, equivalent to Michaelis-Menten kinetics, where v=Vmax S/(Km+S) where v=rate of reaction (uptake); S=substrate (glucose) concentration, Vmax=maximum rate of reaction (uptake), Km=S where v=1/2 Vmax (measure of affinity where lower Km = increased affinity).

15 ml/g wet wt /min $v = 1.76\ S/(5.04 + S)$, $\chi^2 = 0.18$

0.5 ml/g wet wt/min $v = 1.86\ S/(13.23 + S)$, $\chi^2 = 12.48$

0.2 ml/g wet wt /min $v = 0.95\ S/(14.60 + S)$, $\chi^2 = 10.33$

0.1 ml/g wet wt /min $v = 0.46\ S/(13.05 + S)$, $\chi^2 = 3.13$

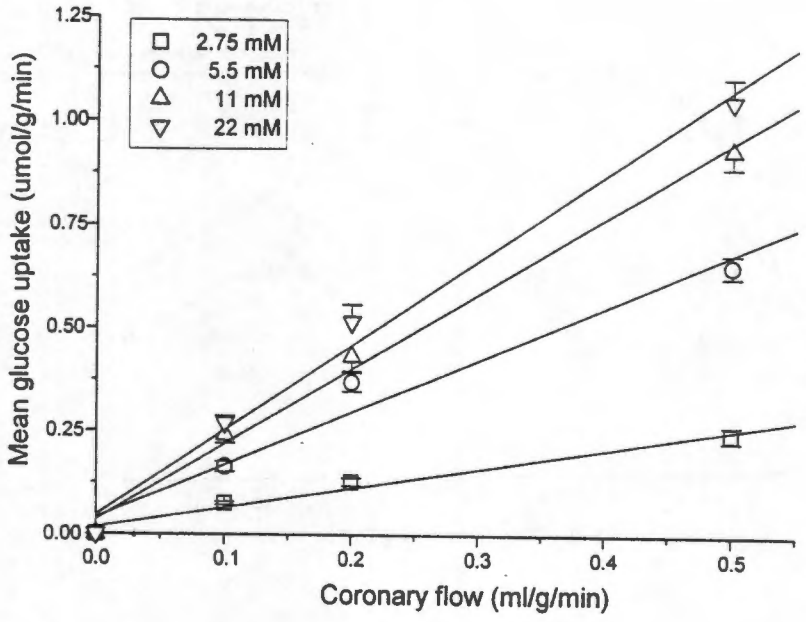


Fig 3: Mean glucose uptake versus coronary flow for each glucose concentration. Note that while relationship is apparently linear, this only applies in the range of low flows. At control flow rates, values are in the same range (see Fig 1), implying a parabolic relationship, with a peak glucose uptake at a flow rate of about 7-9 ml/g wet wt/min (given a control flow of 15-16 ml/g wet wt/min). R=0.99 for all curves.

2.75 mM y = 0.47x+0.02; 5.5 mM y = 1.28x+0.04;

11 mM y = 1.83x+0.04; 22 mM y = 2.06x+0.04

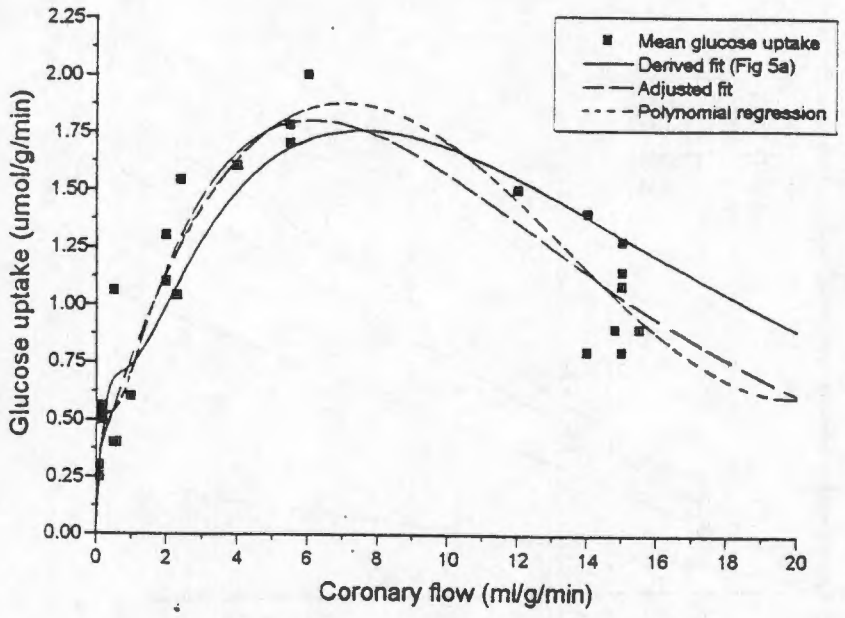


Fig 4: Glucose uptake versus coronary flow in isolated rat hearts perfused with buffer containing 11 mM glucose. Data was taken from a number of publications in the literature[388-390, 468, 469] *and from Results Ch 1 and 2. A total of 29 points (means only) were obtained. Using the relationship from Fig 5a, the predicted change in glucose uptake was plotted. The derived equation was* $y = 0.63 \times e^{(-x/7.54)} + 3.16 \times e^{(-x/0.35)}$.

When this form of equation was used to fit the data, the curve was adjusted to

$$y = 0.80 \times e^{(-x/6.15)} + 4.79 \times e^{(-x/0.19)}$$

with $\chi^2 = 0.17$. *The data was then fitted using a polynomial expression. The resulting equation was*

$$y = 0.305 + 0.507\,x - 0.049\,x^2 + 0.001\,x^3,$$

with $R^2 = 0.85$. *At coronary flows greater than 12 ml/g wet wt/min, factors other than coronary flow determine glucose uptake, and these values may vary widely (see text). At lower flow rates, there is a sharp fall in glucose uptake as coronary flow rate falls.*

This graph shows that the point of division between inhibition of glucose uptake at "ischaemic" flow rates, versus stimulation relative to control[389] *is arbitrary. The point at which "ischaemic" glucose uptake is less than control is dependent on the initial control values.*

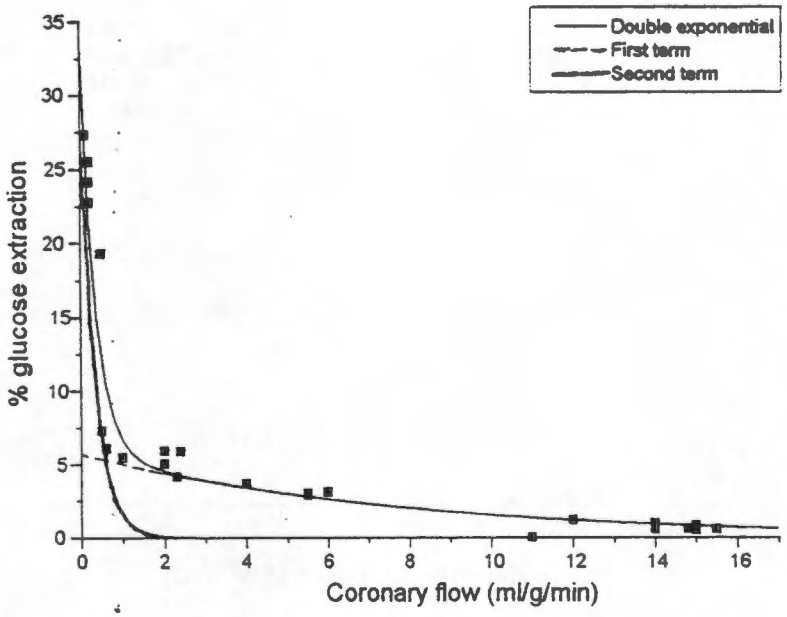


Fig 5a: Individual data points from Fig 4 expressed as a percentage of glucose delivery i.e. extraction (delivery = flow x concentration), with a glucose concentration of 11 mM, versus coronary flow. The data were fitted with a double negative exponential equation, with $\chi^2 = 6.64$ *where* $y = 28.69\, e^{(-x/0.35)} + 5.75\, e^{(-x/7.54)}$. *The individual components of this curve are plotted. The second component, showing % extraction with low coronary flows, indicates the point below which glucose uptake is upregulated. A value of 1-2 ml/g wet wt/min is indicated (points of intersection of curve with abscissa, and with curve of first component).*

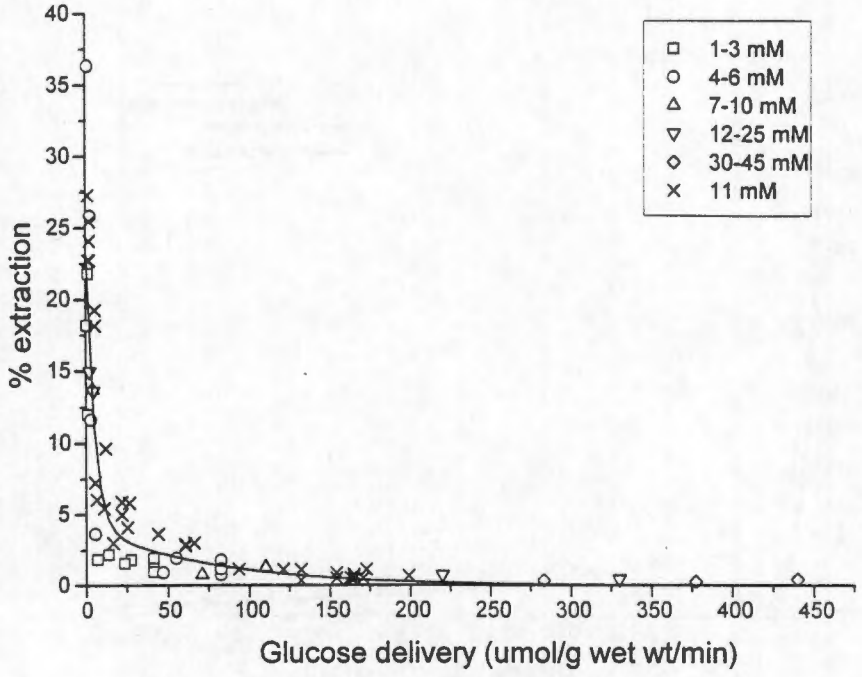


Fig 5b: Mean glucose uptake derived from data shown in Fig 1 were calculated as a function of delivery of glucose to allow for different glucose concentrations and coronary flow. Different ranges of glucose concentrations used are distinguished. The % extraction for each point was then calculated, and plotted against delivery. The data points from Fig 5a (11 mM glucose) were also replotted as a function of delivery. All the points were then fitted with a negative exponential relationship, described by $y = 20.15\, e^{(-x/4.76)} + 4.23\, e^{(-x/75.42)}$, $\chi^2 = 14.58$

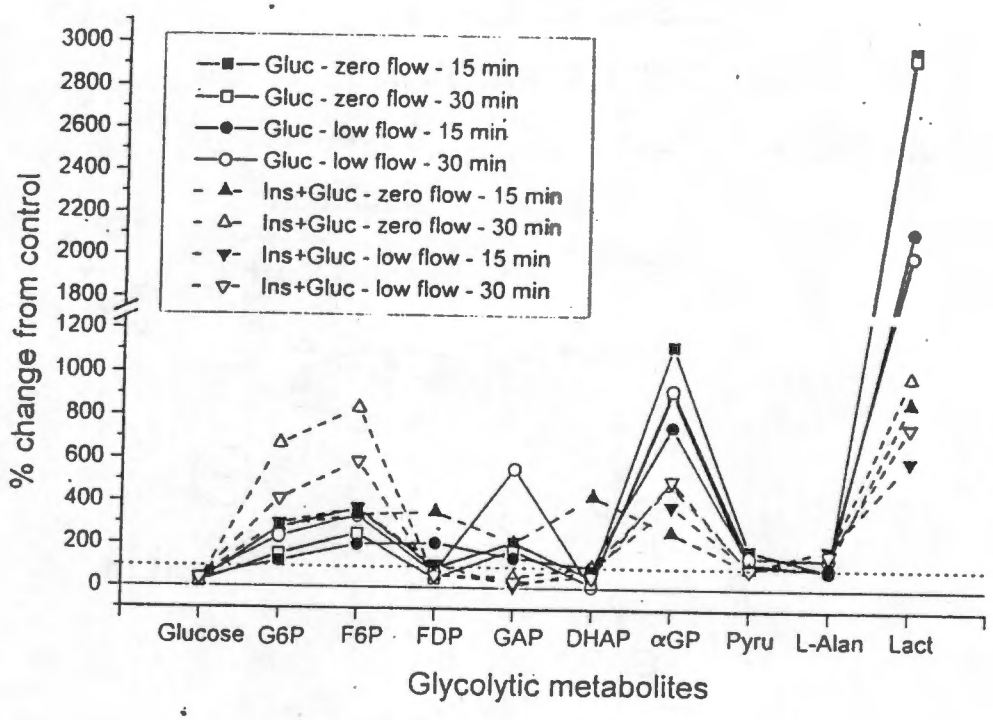


Fig 6: Percentage change from control values of tissue metabolites from hearts perfused with 11 mM glucose with and without 1 U/l insulin. Hearts were clamped at the onset, after 15 min and after 30 min ischaemia. The dotted line represents control values (100%). See Table 1 for abbreviations.

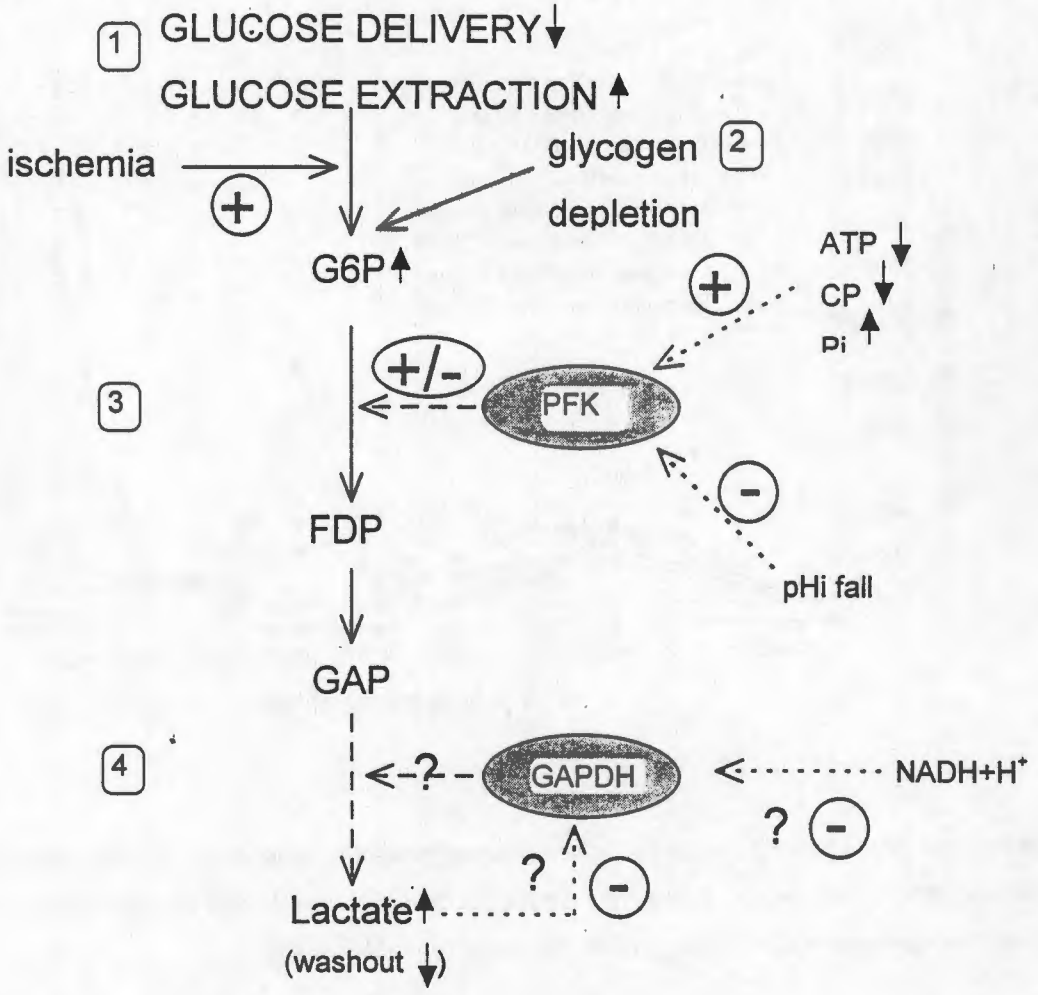


Fig 7: Possible sites of regulation of glycolysis.

(1) - glucose delivery is decreased in ischaemia as the flow rate falls, resulting in reduced glucose uptake. A reduction in coronary flow below 1-2 ml/g wet wt/min appears to stimulate glucose uptake, resulting in increased extraction of glucose delivered to the cells.

(2) - glycogen is rapidly depleted during ischaemia (within the first 15 minutes, most has been utilised - Table 1). Once glycogen is depleted, it can no longer contribute to glycolysis. The residual glycogen remaining may consist largely of proglycogen, a more stable intermediate of glycogen synthesis which is more resistant to breakdown in ischaemia[40, 103]. Under total global ischaemic conditions, or when glucose is present only in very low concentrations, glycogen is the sole glycolytic substrate, and the tissue content at the onset of ischaemia determines subsequent glycolytic rates.

(3) - phosphofructokinase (PFK) was thought to be the major site of glycolytic inhibition under normal conditions when ATP and CP levels are sufficient[541]. When ATP and CP levels are depleted, inhibition of PFK is removed allowing glycolysis to continue. However, a more recent analysis, in conditions of normoxia with sufficient substrate (glucose and insulin), has found that control of glycolysis at all sites below phophoglucoisomerase, including PFK, is limited to less than 25% of the total[241]. Glucose uptake and phosphorylation are the main rate limiting factors.

In ischaemia, the site of glycolytic regulation was though to be GAPDH rather than PFK[468] (see below). However, if high glucose and insulin are present, regulation of glycolysis at the level of PFK by a drop in pH[541]

may become more important. A large accumulation of G6P and F6P was found especially in insulin hearts, increasing with increased duration of ischaemia (see Table 1, Fig 6), suggesting that with sufficient substrate, some feedback is present to prevent too excessive an accumulation of end product.

(4) GAPDH catalyses the conversion of GAP to 2,3-bisphosphoglycerate, with the reduction of NAD^+ to $NADH+H^+$. Thus a build up in NADH and H^+ inhibits this reaction. In ischaemia, as lactate accumulates because of increased production and reduced washout, balancing the lactate dehydrogenase equilibrium should theoretically lead to an accumulation of NADH and H^+. This mechanism is then thought to inhibit glycolysis[362, 468]. However, the importance of this mechanism in the overall regulation of glycolysis in ischaemia is challenged by the findings that: glycolysis is limited by supply of substrate, in accordance with normal substrate-product relationships[391] (Fig 3); at low coronary flows, glucose extraction is increased (Fig 5); and analysis of glycolytic metabolites does not point to inhibition at the level of GAPDH (Table 1, Fig 6). In addition, with high glucose and insulin at low coronary flows, lactate washout has been shown to remain at a steady state for an extended period, without any evidence of attenuation of glycolysis by feedback inhibition[14]. Tissue lactate levels were increased if insulin were present (Table 1). While the role of lactate-mediated inhibition of glycolysis cannot be excluded, its importance appears relatively small. Only if excess external lactate is added[92], may GAPDH inhibition regulate glycolysis, although this has not been shown directly.

The process illustrated in the figure can be regarded as a "funnel". Hypothetically, in ischaemia, if glucose is taken into the cell and phosphorylated, its eventual fate must be lactate if the cell is still viable. Some constriction may be present to slow down the rate, but this does not affect the eventual outcome.

Table 1: Glycogen (μmol C6/g wet wt) and glycolytic metabolites (μmol/g wet wt) in hearts perfused with 11 mM with and without 1 U/l insulin. Hearts were clamped at the onset, and after 15 and 30 min zero flow or low flow (0.2 ml/g wet wt/min) ischaemia.

	Glucose	Zero flow		Low flow (0.2)		**Gluc+ Insulin**	Zero flow		Low flow (0.2)	
	Control	15 min	30 min	15 min	30 min	Control	15 min	30 min	15 min	30 min
Glyc	16.272 ± 0.440	3.857 ± 0.700	1.358 ± 0.126	5.505 ± 1.212	6.266 ± 0.998	21.398 ± 1.083	10.901 ± 1.378	3.852 ± 0.662	17.064 ± 1.620	11.863 ± 2.260
G6P	0.081 ± 0.015	0.224 ± 0.059	0.125 ± 0.035	0.099 ± 0.044	0.190 ± 0.046	0.167 ± 0.049	0.466 ± 0.077	1.116 ± 0.213	0.500 ± 0.104	0.689 ± 0.205
F6P	0.018 ± 0.003	0.065 ± 0.018	0.044 ± 0.012	0.035 ± 0.011	0.059 ± 0.016	0.040 ± 0.011	0.137 ± 0.023	0.337 ± 0.09	0.149 ± 0.035	0.238 ± 0.075
FDP	0.077 ± 0.031	0.073 ± 0.012	0.035 ± 0.010	0.162 ± 0.058	0.058 ± 0.014	0.078 ± 0.056	0.276 ± 0.149	0.045 ± 0.018	0.088 ± 0.030	0.046 ± 0.010
GAP	0.060 ± 0.011	0.128 ± 0.038	0.107 ± 0.053	0.085 ± 0.022	0.336 ± 0.161	0.082 ± 0.042	0.174 ± 0.050	0.043 ± 0.031	0.171 ± 0.033	0.025 ± 0.011
DHAP	0.058 ± 0.013	0.028 ± 0.005	0.009 ± 0.006	0.058 ± 0.009	0.008 ± 0.007	0.035 ± 0.008	0.151 ± 0.075	0.035 ± 0.010	0.032 ± 0.008	0.019 ± 0.008
Pyru	0.078 ± 0.005	0.142 ± 0.014	0.088 ± 0.029	0.101 ± 0.009	0.118 ± 0.013	0.122 ± 0.010	0.115 ± 0.023	0.123 ± 0.010	0.127 ± 0.012	0.117 ± 0.022
αGP	0.285 ± 0.082	3.206 ± 0.693	2.584 ± 0.748	2.154 ± 0.910	2.644 ± 0.874	0.870 ± 0.331	2.289 ± 0.883	4.228 ± 0.810	3.392 ± 0.539	4.443 ± 0.933
L-Alan	3.501 ± 0.949	3.197 ± 0.453	3.253 ± 0.380	2.984 ± 0.163	4.971 ± 0.93	2.687 ± 0.724	3.119 ± 0.543	4.361 ± 0.137	5.127 ± 0.664	4.394 ± 0.166
Lact	0.807 ± 0.032	24.006 ± 0.766	23.755 ± 0.740	17.156 ± 2.252	16.271 ± 0.536	2.719 ± 1.122	23.802 ± 2.416	27.041 ± 1.105	16.546 ± 1.330	20.801 ± 1.858
% S/(S+P)	7.49	2.12	1.36	2.36	3.12	7.71	4.32	4.55	4.08	3.68

Glyc - glycogen; G6P - glucose 6-phosphate; F6P - fructose 6-phosphate; FDP - fructose 1,6-diphosphate; GAP - glyceraldehyde 3-phosphate; DHAP - dihydroxyacetone phosphate; Pyru - pyruvate; αGP - α glycerophosphate (glycerol 3-phosphate); L-Alan - L-alanine; Lact - lactate; %S/(S+P) - % substrate/(substrate + product) where S = G6P+F6P+FDP+GAP+DHAP+Pyru and P=αGP+L-Alan+Pyru. 1,3-bisphosphoglycerate, 2-phosphoglycerate and phosphoenolpyruvate were not measured as these values are very low in tissue[468].

Results 4. Does preconditioning act by glycogen depletion in the isolated rat heart?

In press J Mol Cell Cardiol 1996

ABSTRACT

Preconditioning hastens the time to onset of ischaemic contracture and increases peak contracture in an isolated perfused rat heart, but improves recovery of function. The preconditioning ischaemic episode is also known to deplete glycogen stores. We tested whether a depletion in glycogen is related to the protection conferred by preconditioning.

Methods: The isolated Langendorff perfused rat heart, with a left ventricular balloon to record function, was perfused with either glucose 11 mM, acetate 5 mM, or glucose 11 mM + insulin to alter pre-ischaemic glycogen levels prior to 30 min total global ischaemia. In addition, hearts were preconditioned by on episode of 5 min ischaemia and 5 min reperfusion. Time to onset of contracture (TOC - min), peak contracture and recovery of developed pressure after 20 min reperfusion with glucose-containing perfusate (both expressed as % pre-ischaemic developed pressure) were measured (n = 9-10). Parallel groups of hearts were clamped at various times for assessment of tissue metabolites.

Results: Acetate pre-perfusion reduced glycogen levels compared to glucose hearts, from 16.27 ± 0.44 to 10.77 ± 0.96 μmol/g wet wt. TOC was reduced and peak contracture increased, with poor functional recovery. Glucose+insulin pre-perfusion increased glycogen (21.39 ± 1.08 μmol/g wet wt) with opposite effects on contracture, but functional recovery was still poor.

Preconditioning hastened the time to onset of contracture, which could be partially attributed to glycogen depletion. Preconditioning significantly improved functional recovery in glucose hearts, but had little or no effect in the other groups. Thus the protective effect of preconditioning on functional recovery could not be linked to glycogen depletion.

Conclusions: Pre-ischaemic glycogen appeared to play a dual role. When low, preconditioning was ineffective, presumably because of lack of production of glycolytic ATP, and severe contracture. When pre-ischaemic glycogen was increased, preconditioning was also relatively ineffective, presumably because of excess accumulation of the metabolites of glycogenolysis.

INTRODUCTION

Preconditioning can be defined as one or more brief periods of ischaemia with intermittent reperfusion that protects against a sustained period of subsequent ischaemia. Protection has been observed in terms of reduction in infarct size [313, 319, 375, 598], reduction in ischaemia- and reperfusion-induced arrhythmias [298, 495] and an improvement in functional recovery on reperfusion [18, 61, 528]. This is generally associated with a reduced utilisation of high energy phosphates, a reduced intracellular lactate accumulation, and reduced fall in intracellular pH. Primarily, preconditioning can be said to delay the onset of irreversible injury or necrosis, such that with timely reperfusion, there is an increased degree of residual functional tissue, resulting in improved recovery of mechanical function. However, there is a time limit on the protection conferred by preconditioning, such that if the hearts are reperfused after an extended period of ischaemia, no difference between control and preconditioned hearts will be observed [375].

Because a brief period of ischaemia reduces the level of glycogen in the tissue [525], and following on from the much-quoted report by Neely and Grotyohann [387], an obvious hypothesis is that a reduction in glycogen levels brought about by preconditioning is protective. This was attributed to reduced lactate and proton accumulation, factors that have been noted in preconditioned hearts.

There have been a number of studies which have attempted to answer the question whether glycogen depletion is in itself beneficial to the heart. The results are have yielded conflicting results. Kupriyanov et al [278] reported improved recoveries following ischaemia preceded by 2h perfusion with pyruvate to reduce tissue glycogen, despite a reduced time to onset of contracture (TOC). Lagerstrom et al found that 15 min anoxia (as used in the Neely paper) or substrate free perfusion prior to 30 min total ischaemia to reduce tissue glycogen impaired functional recovery compared to control hearts, with no change in lactate accumulation in the former case, but a significant reduction in the latter [286]. There was thus no correlation of recovery with lactate accumulation. These discrepancies may be due in part to the different levels of pre-ischaemic glycogen in the different studies. However, studies on glycogen depletion from different reports, with the same control pre-ischaemic glycogen levels, do not give consistent comparable results in terms of functional recovery (see Ch. II).

The involvement of glycogen depletion in preconditioning has been investigated, the most notable publication that of Wolfe et al [588]. Loss of protection against infarct development during a prolonged ischaemic period was correlated with a return to control tissue glycogen levels, during extended reperfusion after preconditioning. No differences in infarct development between control and preconditioned hearts were observed after 1 hour reperfusion, when glycogen levels were similar. However, as has been noted in a number of publications, the protection conferred by preconditioning wanes with time [313, 375, 377, 596]. The time dependence also appears to be species dependent with

shorter time periods of protection available in rats [313] than in dogs [375] or pigs [269]. The study by Wolfe et al. therefore does not offer conclusive evidence that a reduction in glycogen prior to the sustained ischaemic episode is a prerequisite for preconditioning, or that this can be defined as the protective mechanism. In addition, functional recovery, an important component of determining the effectiveness of protection, was not recorded. Studies of the role of glycogen have generally used this as the end point.

We wished to test two hypotheses 1) whether a reduction in glycogen levels prior to ischaemia, by perfusion with acetate, is protective to the heart, and 2) whether glycogen depletion can be linked to the protection conferred by preconditioning. In addition, glycogen-loaded hearts (perfused with glucose + insulin) were investigated. If the hypothesis were true that glycogen depletion is beneficial, glycogen loading should be detrimental, and preconditioning should improve function in these hearts compared to controls by reducing glycogen.

5 min ischaemia + 5 min reperfusion was used to precondition the hearts. A similar protocol has been used in a number of other studies [18, 66, 290, 364, 603]. A 30 min period of sustained ischaemia was chosen to ensure sufficient degrees of both reversible and irreversible injury, and to enable discernment of a protective effect of preconditioning. While preconditioning did result in improved functional recovery, this was dissociated from glycogen depletion.

METHODS

PERFUSION APPARATUS

The isolated Langendorff-perfused rat model with a left ventricular balloon was used, as described in Methods. Glucose 11 mM was the substrate used unless otherwise stated.

PROTOCOL

At least 9 hearts were used in each group. The standard protocol was a 30 min period of perfusion, followed by 30 min ischaemia, and subsequently 20 min reperfusion (see Figure 1). In the 30 min period prior to ischaemia, two major modifications were made. The hearts were perfused with the standard glucose containing substrate (at a concentration of 11 mM), with 5 mM acetate instead of glucose (the NaCl concentration was reduced by 5 mM to allow for the addition of 5 mM Na-acetate), or with 11 mM glucose + 1U/l insulin (Humulin). Acetate reduces tissue glycogen because of its reduced P/O ratio (2.3 - [402]) which despite an increase in citrate results in glycogen breakdown [585]. The presence of insulin stimulates glucose uptake and steers glucose towards glycogen synthesis by stimulation of glycogen synthase and inhibition of phosphorylase. A high insulin concentration was used to ensure maximal glycogen synthesis during the pre-ischaemic period.

In each of the above three groups, hearts were perfused either for the entire 30 min, or after 20 min were subjected to 5 min total global ischaemia followed by 5 min reperfusion to precondition the hearts.

After the pre-ischaemic perfusion period, all hearts were made totally ischaemic by complete cessation of flow, for 30 min. After 30 min ischaemia, all hearts were reperfused with the standard oxygenated glucose-containing solution for a further 20 min.

Parallel groups of hearts were perfused as above but for each group, hearts were clamped with Wollenberg tongs kept in liquid nitrogen, immediately prior to sustained ischaemia, after 15 min and 30 min ischaemia, and at the end of the reperfusion period. These hearts were freeze dried for later biochemical analysis. 6 hearts in each group for each time point were used.

FUNCTIONAL MEASUREMENTS

Indices of contracture and functional recovery were recorded as described in Methods.

BIOCHEMICAL MEASUREMENTS

The freeze dried samples were extracted using perchloric acid. Levels of tissue metabolites including high energy phosphates, glycolytic metabolites and citric acid cycle intermediates, were determined used spectrophotometric assays [24] adapted for use on a centrifugal analyzer (Cobas Fara, Roche Diagnostics, Switzerland), as described in Methods. This enabled many determinations to be made with small sample volumes. Glycogen levels were determined by measuring the glucose content of

samples following alcohol and alkali extraction. All tissue metabolites were expressed as μmol/g wet wt, where wet weight = 5 x dry weight (using previously determined ratios). Glycogen was expressed as μmol glucose units (C6 units)/g wet wt.

STATISTICS

The Anova two-way analysis of variance was used to determine significance among groups, after which the modified t-test with the Bonferroni correction was used for comparison between individual groups. A value of $p<0.05$ was used as the level of significance.

RESULTS

MODEL OF PRECONDITIONING

The standard model used in this study was 30 min zero flow ischaemia with or without a preceding 5 min ischaemia + 5 min reperfusion for preconditioned hearts. Table 1 shows the measurements recorded after 30 min perfusion with and without preconditioning in glucose-perfused hearts. Preconditioning significantly increased coronary flow, but reduced systolic pressure and developed pressure before sustained ischaemia (Table 1). The changes in systolic and diastolic pressure throughout the experiments are shown in Figure 2. Preconditioned hearts showed faster TOC and a slightly increased peak contracture (Table 2), but there was still an increased recovery of developed pressure (47.6 ± 7.3% vs 22.5 ± 7.0%; $p<0.05$) due to improved diastolic pressure on reperfusion (Table 2). Coronary flow on reperfusion was significantly improved in preconditioned hearts compared to control values (Table 2).

Pre-ischaemic tissue values of adenosine triphosphate (ATP) showed no difference between control and preconditioned hearts, and there was a small increase in creatine phosphate (CP) in preconditioned hearts (Figure 5). Following preconditioning, there was a significant reduction in glycogen (11.19 ± 1.05 vs 16.27 ± 0.44 μmol C6/g wet wt; $p<0.05$) (Figure 3a) and a significant increase in G6P (Figure 6). An increased citrate (0.26 ± 0.01 vs 0.16 ± 0.01 μmol/g wet wt; $p<0.001$) and malate (0.42 ± 0.08 vs 0.18 ± 0.04 μmol/g wet wt; $p<0.01$) were also found in preconditioned hearts.

During ischaemia, glycogen levels at 15 min and 30 min were similar in the two groups (Figure 3b), indicating a reduced glycogen utilisation in preconditioned hearts (9.4 vs 14.9 μmol C6/g wet wt/30 min). ATP and CP levels were no different during ischaemia (Figure 5), nor was there a significant reduction in tissue lactate accumulation (Figure 7). Tissue glucose measured at the onset of ischaemia (indicative of the amount of glucose available for subsequent utilisation) was 5.2 ± 0.3 and 5.7 ± 0.4 μmol/g wet wt for control and preconditioned hearts respectively, and when taken together with glycogen was sufficient to account for the lactate produced.

Glycolytic metabolite accumulation (data not shown) during ischaemia was reduced at 30 min compared to 15 min ischaemia in normal hearts. In preconditioned hearts an increased accumulation by 30 min was found (see G6P levels - Figure 6). Inorganic phosphate (Pi) levels were similar in both groups.

A significant recovery of CP levels was found in preconditioned hearts after 20 min reperfusion (Figure 5), together with a significant reduction in Pi (Figure 6), presumably because of maintained CP. There was also a trend towards improved ATP levels, and reduced AMP, suggestive of better adenine nucleotide regeneration in preconditioned hearts. Glycogen levels were no different after reperfusion (Figure 3b).

PRE-ISCHAEMIC REDUCTION OF GLYCOGEN CONTENT - ACETATE PREPERFUSION

Control hearts

Acetate perfusion for 30 min did not affect cardiac function except for a small non-significant increase in coronary flow, a consequence of increased oxygen requirements (Table 1). There was an increase in CP compared to glucose perfused hearts (Figure 5), and a reduction in tissue glycogen (10.77 ± 0.96 vs 16.27 ± 0.44 μmol/g wet wt; $p<0.05$) (Figure 3a). There was also a significant increase in citrate levels (0.26 ± 0.02 vs 0.16 ± 0.01 μmol/g wet wt), a consequence of increased acetyl Co A formation directly from acetate inhibiting pyruvate dehydrogenase and activating pyruvate carboxylase.

On cessation of flow, acetate perfused hearts underwent a rapid contracture and the extent of contracture was greatly increased (Figure 4a, Table 2) versus glucose hearts. This resulted in a poor recovery of developed pressure ($8.2 \pm 3.0\%$) due to reduced systolic pressure compared to glucose hearts. Diastolic pressure was no different between acetate- and glucose-perfused hearts (Table 2).

Tissue high energy phosphate levels in acetate hearts showed a greater fall in ATP (Figure 5) and an increase in AMP, although glycogen was similar to glucose hearts after 30 min ischaemia (1.13 ± 0.19 vs 1.36 ± 0.13 μmol/g wet wt; $p<0.05$) (Figure 3b). Citrate levels declined during ischaemia in acetate hearts (0.18 ± 0.06 μmol/g wet wt by 30 min). Glycolytic metabolite levels were reduced in acetate hearts throughout ischaemia (G6P - 0.02 ± 0.00 vs 0.13 ± 0.04 μmol/g wet wt in glucose hearts by 30 min ischaemia; $p<0.01$) (Figure 6). There was a significant reduction in lactate accumulation in the acetate hearts (Figure 7), while Pi accumulation was high (Figure 8) due to increased breakdown of high energy phosphates, and reduced sugar phosphate formation from glycogen.

Preconditioning

When acetate hearts were preconditioned, glycogen levels prior to ischaemia were even further reduced (5.98 ± 1.04 μmol C6/g wet wt) (Figure 3a) with very little glycogen available in the tissue by 30 min ischaemia (0.81 ± 0.23 μmol C6/g wet wt) (Figure 3b). However, prior to ischaemia, acetate preconditioned hearts show an even greater CP overshoot (Figure 5), and an increased citrate level (0.78 ± 0.06 μmol/g wet wt) compared to acetate control hearts, and both glucose control and preconditioned hearts. However, during ischaemia, the high energy phosphates were rapidly depleted to values lower than in any other hearts (see Figure 5). Glycolytic metabolite levels were also very low (see Figure 6). A rise in diastolic pressure frequently occurred during the preconditioning episode of ischaemia, such that diastolic pressure immediately prior to sustained ischaemia was increased (Table 1). Thus ischaemic contracture was almost immediate in acetate preconditioned hearts, with a large peak contracture, both significantly different from acetate control, and glucose hearts (Table 2).

Recovery of acetate preconditioned hearts was also poor (Table 2), and no different to acetate control hearts.

No difference in coronary flow after 20 min reperfusion was evident in the acetate preconditioned hearts (Table 2).

PRE-ISCHAEMIC INCREASE IN GLYCOGEN CONTENT

Control hearts

Pre-ischaemic perfusion with glucose-containing buffer (11 mM) with the addition of 1 U/L of insulin resulted in increased glycogen synthesis and storage. After 30 min perfusion, glycogen levels were 21.39 ± 1.08 μmol/g wet wt compared to 16.27 ± 0.44 μmol/g wet wt in glucose perfused hearts (Figure 3a). ATP levels were no different, but there was a slight increase in CP (Figure 5). Citrate levels were also increased (0.24 ± 0.01 μmol/g wet wt; $p<0.05$). There was a general trend to an increase in glycolytic metabolites (data not shown), but with continued perfusion, glycolysis would be efficient, without undue accumulation. Developed pressures were higher in insulin hearts compared to glucose perfused hearts (Table 1).

TOC was significantly delayed in insulin hearts and peak contracture was slightly lower than glucose hearts (Figure 4b, Table 2). Glycogen levels after 30 min ischaemia were significantly higher in insulin treated hearts than in glucose hearts (5.03 ± 1.29 vs 1.36 ± 0.13 μmol/g wet wt; $p<0.05$) (Figure 3b). G6P increased substantially during ischaemia in insulin hearts (from 0.17 ± 0.05 to 1.12±0.23 μmol/g wet wt after 30 min ischaemia) compared to glucose hearts (Figure 6). Lactate was also significantly increased in insulin-treated hearts by 30 min ischaemia (Figure 7). Citrate levels declined significantly (to 0.10 ± 0.01 μmol/g wet wt) during ischaemia. Pi was high initially but decreased slightly as glycogen was broken down, and the sugar phosphate content increased.

Despite a reduced contracture and a higher rate of glycolysis, recovery of insulin-perfused hearts after 30 min total global ischaemia was not, however, improved compared to glucose hearts, and were in fact slightly worse (11.6 ± 4.3%).

Preconditioning

When hearts pre-treated with glucose + insulin were subjected to 5 min ischaemia + 5 min reperfusion, an overshoot in CP was found, and a reduction in glycogen (15.53 ± 1.55 μmol/g wet wt, $p<0.05$ vs control) (Figure 3a). When these hearts were subjected to a further 30 min total global ischaemia, TOC was much reduced compared to control insulin hearts), although peak contracture was not affected (Figure 4b, Table 2). Recovery of developed pressure was not improved (18.6 ± 8.1%), although diastolic pressure was reduced and coronary flow was improved (Table 2).

ATP levels in insulin preconditioned hearts after 15 min ischaemia were higher than those in other hearts - the only group which showed a reduced ATP utilisation (Figure 5). In addition, AMP levels

after 15 min ischaemia were lowest in insulin preconditioned hearts, although after 30 min ischaemia, these values were the highest. However, this would maintain AMP availability for restoration of ATP. While G6P was also greatly increased in preconditioned insulin treated hearts, preconditioning tended to reduced accumulation compared to control insulin hearts (0.8 ± 0.3 μmol/g wet wt at 30 min) (Figure 6). Lactate at the end of ischaemia was not however, different between control and preconditioned insulin treated hearts (Figure 7), again accounted for by tissue glucose available at the onset of ischaemia (6.93 ± 0.88 μmol/g wet wt in control and 7.26 ± 0.44 μmol/g wet wt in preconditioned hearts). Pi in the insulin preconditioned hearts was higher than control, presumably because of reduced sugar phosphate accumulation.

GLYCOGEN UTILISATION AND ISCHAEMIC CONTRACTURE

While it was difficult to make accurate calculations of glycogen utilisation, because each time point consisted of a different group of hearts, some conclusions could be drawn. Glycogen utilisation occurred mainly in the first 15 min of total global ischaemia in the glucose and acetate hearts, but still continued to be broken down after 15 min in the insulin hearts.

By the end of ischaemia, tissue glycogen levels were higher in the insulin treated hearts, but overall breakdown of glycogen was not greatly increased compared to glucose control hearts (16.4 μmol vs 14.9 μmol). However, the percentage breakdown was less in the insulin treated hearts (76.5 vs 91.6%) despite a significant increase in pre-ischaemic glycogen, which may be attributed to the inhibitory effect of insulin on glycogenolysis. Tissue lactate accumulation after 30 min ischaemia was significantly increased in the insulin treated hearts, which could be accounted for by the increased glycogen breakdown as well as increased utilisation of residual glucose in the tissue. There was also a significant increase in tissue G6P and fructose 6-phosphate (F6P), as well as in most of the other glycolytic metabolites.

There was a significant reduction in glycogen utilisation in all preconditioned hearts compared to controls, presumably because of reduced initial glycogen levels. Tissue levels of glycogen in the glucose and acetate preconditioned hearts after 15 and 30 min ischaemia were similar to controls. Only in the insulin treated hearts was there a significant difference in tissue glycogen between control and preconditioned hearts after 15 min ischaemia. There was therefore a low rate of glycogen utilisation in the insulin preconditioned hearts, slightly less than in the glucose preconditioned hearts (6.5 vs 6.7 μmol/15 min - 41.8 vs 59.5%) despite an increased tissue glycogen. By the end of ischaemia, glycogen levels in control and preconditioned insulin hearts were similar. However, percentage utilisation of glycogen was still less in the insulin preconditioned as opposed to glucose preconditioned hearts (67.5 vs 84.3%).

When glycogen utilisation over 30 min ischaemia was plotted against TOC (Figure 9), a linear relationship was observed for control hearts, indicating that tissue glycogen availability may be one on the major determinants of the TOC. Peak contracture was also related to tissue glycogen, with

increasing peak correlated to reduced glycogen. When TOC was plotted against glycogen utilisation for preconditioned hearts, a less convincing linear relationship was found, given that glycogen utilisation in glucose and insulin and hearts was similar. A similar TOC was also found in these two groups. However, this slope was almost exactly parallel to the slope in non-preconditioned hearts, but shifted to the left. Thus in preconditioned hearts, a reduced time to onset would occur compared to control hearts, despite similar degrees of glycogen utilisation.

DISCUSSION

Previous studies have attributed improved functional recovery with a number of different protocols to a pre-ischaemic glycogen depletion limiting proton accumulation. While we did not measure changes in pHi, the model of glycogen depletion in this study was not beneficial to the heart subjected to 30 min total global ischaemia. Reduced tissue glycogen was associated with significantly reduced TOC, greatly increased peak contracture, and a poor recovery of function. Glycogen therefore appeared to be necessary to inhibit contracture during ischaemia. On reperfusion, however, the status of the hearts in terms of ATP and metabolite accumulation, determined functional recovery.

Preconditioning with one cycle of 5 min ischaemia + 5 min reperfusion, which also depleted pre-ischaemic glycogen, was protective in glucose-perfused rat hearts with increased functional recovery, despite increased contracture. This is similar to other reports [18, 66]. Preconditioning was ineffective when the hearts were metabolically stressed, but its protective effects were not related to a depletion of glycogen. The effects on ischaemia and on reperfusion, of glycogen manipulation, and of preconditioning, were dissociated.

GLYCOGEN AND GLYCOLYSIS IN ISCHAEMIA

The role of glycogen in ischaemia is very controversial, with glycogen either reported to be deleterious to the heart [3, 278, 387], or beneficial [163, 348, 487, 551]. We have attempted to resolve the debate, with an intermediate view - that glycogen depletion is detrimental by reducing substrate availability for ATP production, but that glycogen loading does not necessarily convey additional protection because of undue metabolite accumulation. There was a marked accumulation of end products in the insulin-treated hearts, possibly responsible for the deleterious effects. Manipulation of glycogen levels appears to be unnecessary. We can compare acetate control with glucose preconditioned hearts, as well as insulin preconditioned and glucose control hearts, on the basis of equivalent glycogen contents prior to sustained ischaemia.

GLYCOGEN DEPLETION - REDUCED TOLERANCE TO ISCHAEMIA

Glycogen can be depleted by a brief period of anoxia [163, 286, 387], epinephrine infusion [348], long term perfusion with pyruvate [278], and substrate free perfusion [189, 253, 286], each of which may modify heart function. After 30 min perfusion with acetate as the sole substrate, glycogen levels were reduced by 35%. A similar level of ATP was found compared to glucose hearts, but CP was significantly increased, as were citrate levels. Heart function was slightly better than control glucose perfused hearts (see Table 1). However, when subjected to ischaemia, there was a rapid onset of contracture, and a large peak contracture (Table 2) in these hearts. This may be attributed to inhibition of glycolysis, which has been shown to be essential in delaying contracture [424]. Glycogen depletion was in no way beneficial to the heart, contrary to the studies of Neely and Grotyohann [387], and others

[3, 278], despite a significant reduction in tissue lactate. A more recent study comparing the effects of glycogen depletion with preconditioning have shown a similar reduction in pH by both interventions, but glycogen depletion by itself was not effective in protecting against ischaemic injury [482].

Acetate control and glucose preconditioned hearts could be compared on the basis of similar glycogen levels prior to the sustained ischaemic episode, a similar CP overshoot, as well as an increase in citrate levels, although glycolytic metabolites were significantly lower in the acetate hearts. Both groups showed a significant reduction in TOC, and an increase in peak contracture, although these parameters were much more severe in the acetate hearts. On reperfusion however, there was a significant diversion between the two groups, with a good recovery in the preconditioned glucose hearts and very poor recovery in the control acetate hearts.

Preconditioning acetate hearts, however, did not lead to an improvement in functional recovery despite the presumed induction of the preconditioning mechanism. These hearts underwent an even more rapid contracture, and the peak reached was even greater than in acetate control hearts. The glycogen levels in these hearts prior to sustained ischaemia were very low. While glycogen depletion does not appear to be the mechanism whereby preconditioning exerts its protective effect, glycogen was required to ensure continued ATP production, to inhibit the development of severe contracture, which impaired functional recovery.

GLYCOGEN LOADED HEARTS - DELETERIOUS ACCUMULATION OF METABOLITES

Hearts perfused with glucose and insulin to increase glycogen were more tolerant to ischaemia than glucose hearts, as shown by a significant delay in TOC and a reduced peak contracture. Recovery of function was not, however, improved in these hearts. Preconditioning insulin hearts hastened TOC but peak contracture was further reduced. The slight improvement in recovery of function due to reduced diastolic pressure was indicative of some protection against irreversible injury. The large increase in G6P and F6P levels in the control insulin hearts was slightly reduced by preconditioning.

An accumulation of sugar phosphates in hypoxia has been implicated in intracellular Ca^{2+} overload [282], which may account for some of the deleterious effects of insulin pre-treatment, especially on reperfusion. An accumulation of phosphomonoesters, which includes G6P, glucose 1-phosphate, α-glycerophosphate, and other phosphate compounds, has been linked to increased ischaemic injury, reflected by CK release [219, 482]. In addition, there was an increase in lactate which was not greatly reduced by preconditioning in the glycogen loaded hearts. This may be linked to a greater fall in pHi, a well known consequence of increased glycogen [151]. Preconditioning with one episode was not sufficient to overcome the effects of increased metabolites following glycogen load.

Glycogen-loaded hearts with preconditioning had similar glycogen levels to glucose control hearts prior to sustained ischaemia, although no similarities in the response to sustained ischaemia were observed. TOC was significantly lower in the preconditioned insulin hearts, although peak contracture

was reduced. Recoveries of developed pressure were equally poor, although the preconditioned insulin hearts did show a reduced diastolic dysfunction. Further episodes of preconditioning prior to sustained ischaemia may have shown a beneficial effect, with a more reduced glycogen and less metabolite accumulation, although our other results dissociate a reduced glycogen from the beneficial effects of preconditioning

PRECONDITIONING, ISCHAEMIC CONTRACTURE AND FUNCTIONAL RECOVERY

Time to onset of ischaemic contracture

The reduced TOC in preconditioned hearts could be attributed partially to reduced glycogen. The relationship between glycogen utilisation and TOC held true in both control and preconditioned hearts (Figure 9), although the curve was shifted to the left in preconditioned hearts. TOC is also determined by ATP and intracellular Ca^{2+} accumulation [189, 511, 558]. Preconditioned hearts have been shown to have a reduced Ca^{2+} and proton accumulation [512], which would be expected to result in reduced contracture. However, preconditioned hearts generally have a lower ATP level because of the depletion by the preconditioning ischaemic episodes. A recent paper by Kolocassides et al. [266] shows a temporal relationship between ATP depletion and contracture, with reduced ATP and increased contracture in preconditioned hearts. However, by 15 and 30 min ischaemia (equivalent to the times in the present study) there was no difference in ATP levels. The initial 10-15 min appear to be crucial in determining the onset and peak of contracture.

We did not find a reduced tissue ATP in preconditioned hearts prior to sustained ischaemia, contrary to other studies [266, 378, 512]. A previous study, however, also found that one episode of 5 min preconditioning does not reduce ATP levels [18]. This finding may be attributed to the use of a single ischaemic episode, and to the model involved. The fact that preconditioning was obtained in both this and the present study suggests that a pre-ischaemic reduction in ATP is not involved in preconditioning. A recent paper by Kolocassides et al. [266] also dissociates the reduction in ATP from the effect of preconditioning. The issue of reduced TOC in our study despite no reduction in ATP may be partially resolved by examining the role of glycolytic ATP in determining time to onset and peak contracture. Owen et al. [424] correlated onset of contracture (in a low-flow model) with reduced ATP production from glucose. In the present study, where flow was absent, the sole substrate was glycogen (with some residual glucose in the glucose perfused groups). With reduced glycogen levels, rates of glycolytic ATP production were decreased, with reduced TOC. Other factors that may be involved include inorganic phosphate and intracellular pH, which would in turn differ depending on initial glycogen levels.

Peak contracture

In our hands, preconditioning did not significantly increase peak contracture compared to control glucose hearts, although the values were higher. This differs from other reports where a large increase in contracture in preconditioned hearts was observed [18, 66]. This may be due to the preconditioning protocol, where the more periods of preconditioning used the greater the degree of contracture in the sustained ischaemic episode [18].

The role of contracture as either an index or a mediator of ischaemic injury is still controversial. Vanoverschelde et al. [551] suggest that myocardial energy metabolism (specifically maintenance of glycolysis) is more important than ischaemic contracture as a determinant of survival. However, in their study, peak contracture was only about 48 mmHg (about 50% of pre-ischaemic developed pressure) and did not decrease after reaching peak i.e. the actin-myosin bonds did not break, with subsequent rupture of cells. If contracture were more severe, as in the case of the acetate hearts in the present study (from 90-120% peak contracture), a significant fall-off in tension after peak contracture was seen (see Figure 4a). This relaxation in tension may indicate cell rupture, due to disruption of the myofibrils following extended stretch, leading to cell death and irreversible injury [181]. The heart appears to tolerate a degree of contracture, especially if protected by some mechanism e.g. the effector of preconditioning. Preconditioning did not exacerbate contracture greatly in the glucose hearts or the insulin hearts, and contracture therefore did not modify functional recovery. However, contracture was greatly exacerbated by preconditioning in the acetate hearts, which had poor recoveries.

Functional recovery

Despite a presumed increase in ischaemic injury shown by the reduced time to onset and increased peak contracture, recovery of developed pressure in the preconditioned glucose hearts was substantially improved compared to control hearts. This discrepancy between the effects on contracture and recovery is unclear. Generally it has been argued that an increase in contracture, with a reduced time to onset, is detrimental to the heart [189], and has been used as a predictor of functional recovery. However, this correlation is disrupted by preconditioning [18, 66]. The effects of preconditioning on ischaemia and on reperfusion should therefore be dissociated.

Preconditioning is thought to protect against the development of irreversible injury [338]. Improved functional recovery conferred by preconditioning was recorded mainly as a result of reduced diastolic dysfunction, an index of the extent of rigor development or irreversible injury. A recent report by Jenkins et al. finds that preconditioning improves functional recovery in an isolated rabbit heart by reducing infarct size, and not by a reduction in stunning [220]. Our findings support this concept, in that preconditioning reduced diastolic pressure in glucose and glucose-insulin hearts. 30 minutes of global

ischaemia ensures a component of reversibly (stunned) as well as irreversibly injured (infarcted) tissue. There was still a large component of irreversible injury (about 45-50%) which could not be limited by preconditioning.

When glycolysis was inhibited (acetate hearts) preconditioning did not improve functional recovery, indicating the requirement for glycolytic ATP. However, when glycolysis was increased by glycogen loading, slight detrimental effects were also noted in that, despite an improvement in diastolic function, systolic function was impaired, presumably because of increased metabolite accumulation.

Different factors appear to determine contracture as opposed to functional recovery i.e. ischaemic vs. reperfusion injury. Preconditioning is generally thought to delay the onset of necrosis, with improved recovery on reperfusion [61]. Contracture predisposes to necrosis, by cell rupture, and preconditioning may protect against this effect. However, contracture is generally not advantageous. There must be a critical balance between the deleterious effects of preconditioning i.e. energy depletion at the onset of ischaemia and increased contracture, versus the protective effects on reperfusion function. These mechanisms may well be distinct from one another.

INHIBITION OF GLYCOLYSIS, LACTATE ACCUMULATION AND PRECONDITIONING

Neely and Grotyohann [387] proposed that a reduction in glycogen was beneficial because of reduced lactate accumulation. No reduction in lactate accumulation with one episode of preconditioning was found in the present study, despite a significant reduction in glycogen. Lagerstrom et al. [286] also reported no reduction in ischaemic lactate accumulation following a 60% glycogen depletion by anoxia. We could find no correlation between lactate accumulation and functional recovery, except that glycogen loaded hearts did not respond well to preconditioning. A reduction in lactate by glycogen depletion was not beneficial.

Goodwin and Taegtmeyer [163], could not replicate the results of Neely, with a short period (15 min) of sustained ischaemia. Similarly, no benefit of glycogen depletion by acetate was apparent with 30 min ischaemia. The Neely paper used a period of anoxia before a sustained period of ischaemia. More recent use of similar protocols has been termed hypoxic preconditioning [290, 498]. The benefits associated with glycogen depletion in previous studies may therefore be attributed to the "preconditioning" effect of a brief hypoxic period, which may stimulate a number of possible protective mechanisms e.g. protein kinase C translocation [601] and is dissociated from glycogen depletion. A more recent paper from Downey's group [166] found that a transient inhibition of glucose uptake by introduction of 1 mM acetate to glucose-containing perfusate for 15 min 30 min prior to sustained ischaemia reduced infarct size, similar to the effect of preconditioning. This was hypothesised to be due to translocation of PKC. The protocol was different from the present study, where a longer duration of acetate perfusion with acetate as sole substrate was used, with a higher concentration of acetate. The results of this paper [166], while interesting, offer no metabolic data to

substantiate their claims of reduced glucose uptake, while glycogen levels were not mentioned. In our studies, where glycogen was severely depleted by long term acetate perfusion, preconditioning was not effective in terms of improved functional recovery.

Finegan et al. [136] report that preconditioned hearts have reduced glycolysis on reperfusion, and therefore reduced proton accumulation, which will in turn reduce Ca^{2+} overload on reperfusion. In addition, they report similar findings to the present report, that a reduced glycogen breakdown is found during ischaemia with preconditioning. However, the results of the present study suggest that the role of glycogen utilisation during ischaemia is not involved in the protective effect of preconditioning.

RESERVATIONS

Validity of preconditioning protocol with one cycle of ischaemia and reperfusion

The results of the present study, together with a number of others [447, 577] indicate that preconditioning is not always effective. One possible reason is the metabolic status of the subject. A similar observation was made by Sargent et al. [480] who noted a requirement for increased ischaemia to precondition hearts perfused with pyruvate. We did not investigate the effect of increased episodes of ischaemia. While more episodes of ischaemia may have resulted in greater protection, the study by Asimakis et al. [18] showed that 2 episodes of preconditioning exacerbated contracture more than did one episode, and had no additional effect on functional recovery. Cave and Apstein [62] also recorded no significant benefit of increased episodes (3 vs 1). Because of the rapid onset of contracture in the acetate hearts with one episode of preconditioning, we did not wish to increase the number of cycles. In addition, if the protective effect could not be observed with one cycle when the metabolic status was altered, we questioned the validity of increasing the number of cycles, in terms of our hypothesis, that glycogen reduction is not involved in preconditioning. The findings of this study raise concerns as to the widespread applicability of preconditioning, and the fact that it may be deleterious by increasing contracture.

Functional recovery vs infarct size

In the present study, we only measured functional recovery as the index of protection. The majority of preconditioning studies using the rat heart have used this as the index, as have most isolated heart studies. While preconditioning has been shown to be most effective against infarct development [375], functional recovery can be regarded as the critical end point in short term experiments [61]. A reduction in infarct size without good functional recovery is of limited value in a short term experiment. The inability of preconditioning to improve functional recovery under conditions of metabolic stress shows a limitation of preconditioning. While infarct size may have been reduced in these hearts, this

is not important given the inability of the heart to pump efficiently. A longer reperfusion would possibly indicate the benefits of reduced infarct size on functional recovery in preconditioned hearts; however, with the in vivo dog and pig heart, there have been, to our knowledge, no reports of improved functional recovery with ischaemic preconditioning, despite reduced infarct size. Our primary interest was whether a depletion of glycogen by preconditioning was beneficial. With an equivalent reduction in glycogen by acetate perfusion, we found no improvement in recovery of function, the index used in other glycogen studies.

IN CONCLUSION

A pre-ischaemic reduction in glycogen was detrimental to the myocardium. The protective effect of preconditioning could not be linked to glycogen depletion. A moderate level of glycogen is required by the hearts either with or without preconditioning, to allow maintained ATP production in the absence of any other exogenous substrate. The reduced TOC in preconditioned hearts could be attributed at least in part to the reduction in tissue glycogen. Glycogen-loaded hearts did not recover better than glucose hearts subjected to long term ischaemia, nor do they respond well to preconditioning, possibly due to sugar phosphate accumulation. These findings are supported by Finegan et al. [136] who also suggest that an "optimal" glycogen level is required. The effects of preconditioning on ischaemic injury and on reperfusion injury must be dissociated.

Preconditioning depletes pre-ischaemic glycogen, but this is not related to the beneficial effect of preconditioning. Maintaining a low residual flow is protective to the heart compared to total global ischaemia, acting partially by providing glucose. Thus preconditioning and low flow ischaemia with provision of glucose should be additionally protective.

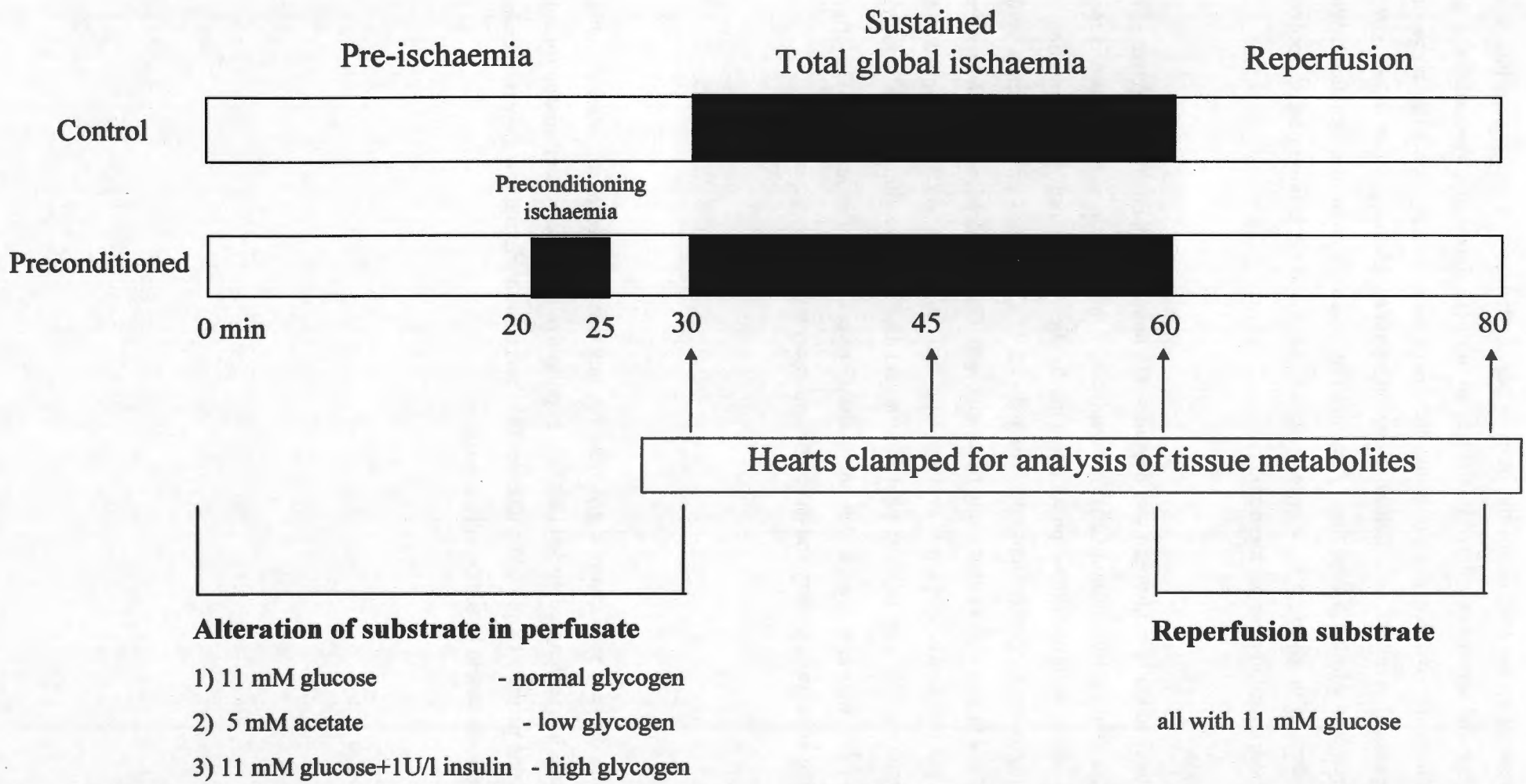


Fig 1: Protocol of experiments with control and preconditioned hearts with different substrates prior to sustained ischaemia. A left ventricular balloon was used to record function. Developed pressure at 20 min reperfusion (80 min) and peak contracture were normalised against developed pressure at 20 min pre-ischaemic perfusion. 9-10 hearts were used in each group. 6 hearts in each group were clamped at each time point for analysis of tissue metabolites.

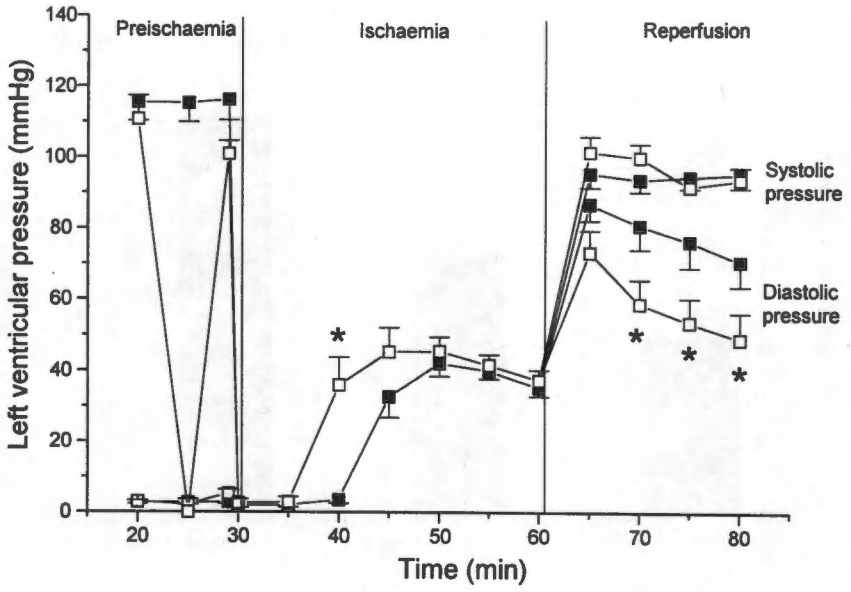


Fig 2: Systolic and diastolic pressure throughout the experiments for control and preconditioned hearts with glucose 11 mM. Ischaemic contracture was hastened with a slight increase in peak. Systolic-diastolic pressure gave developed pressure which was significantly increased in preconditioned hearts on reperfusion, mainly due to reduced diastolic pressure.

Control - filled symbols. Preconditioned - open symbols.

** $p<0.05$ control vs preconditioned*

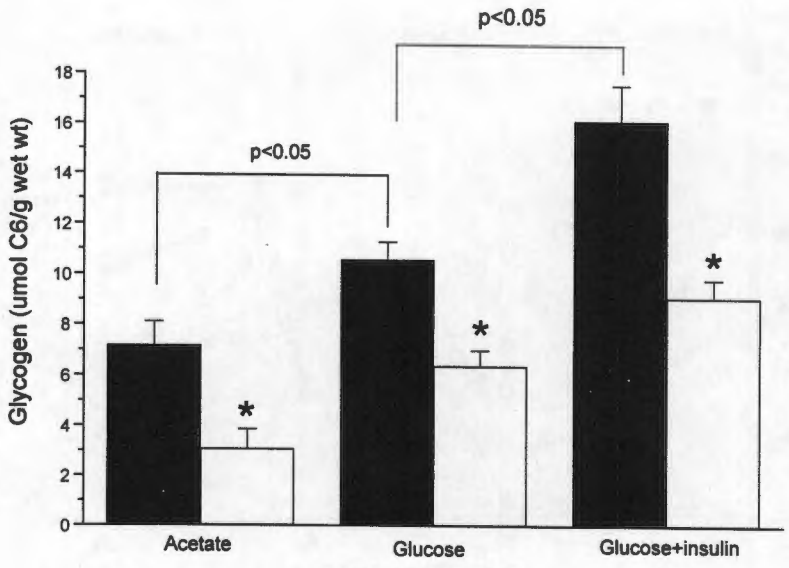


Fig 3a

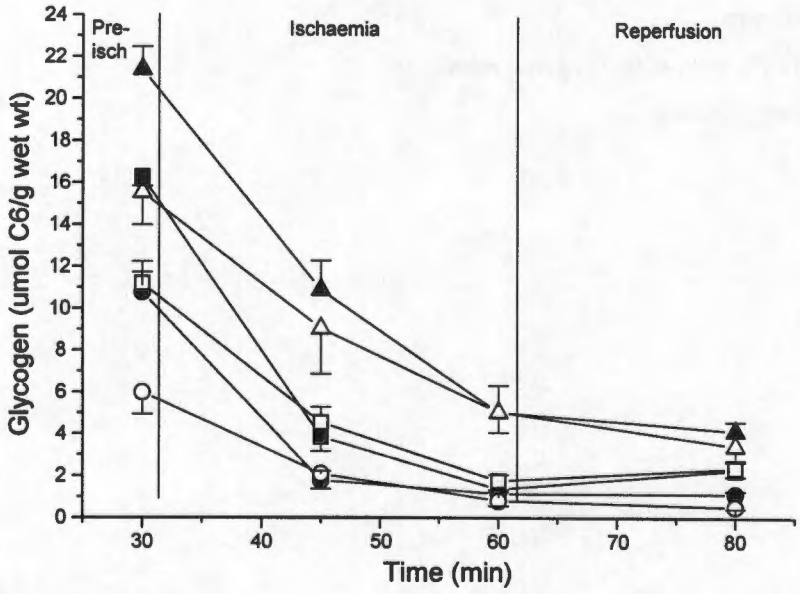


Fig 3b.

*Fig 3a: Glycogen levels prior to sustained ischaemia with different pre-ischaemic substrates, with and without preconditioning. Lines show significant differences ($p<0.01$) between groups. * $p<0.05$ between control and preconditioned hearts. Filled bars -control. Open bars - preconditioned*

Fig 3b: Changes in tissue glycogen during ischaemia and reperfusion with different pre-ischaemic substrates, and control vs preconditioned hearts. Control - filled symbols, Preconditioned - open symbols. Glucose - squares, Acetate - circles, Glucose+insulin - triangles

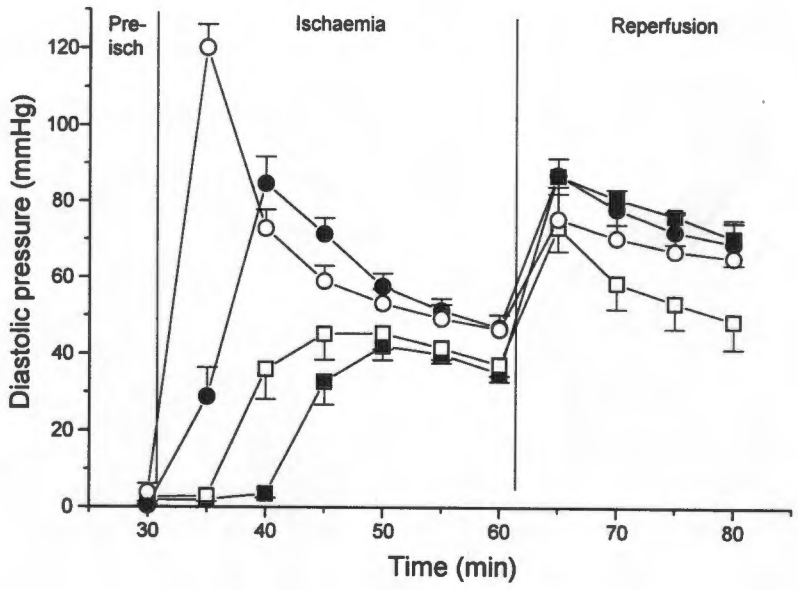
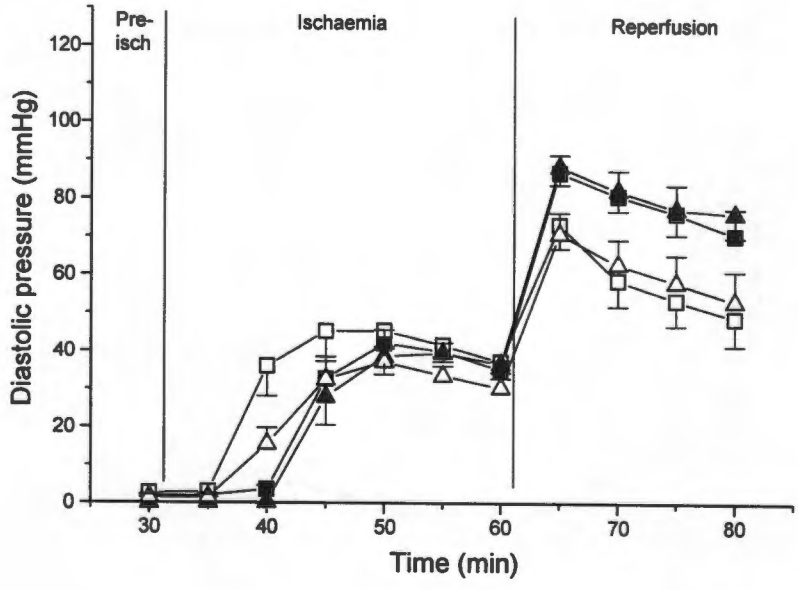


Fig 4a



Fug 4b

Fig 4: Changes in diastolic pressure during ischaemia and reperfusion with acetate perfusion (Fig 4a) and insulin pre-treatment (Fig 4b), in control and preconditioned hearts. Changes in diastolic pressure for glucose control and preconditioned hearts are shown for comparison. Control - filled symbols, Preconditioned - open symbols. Glucose - squares, Acetate - circles, Glucose+insulin - triangles

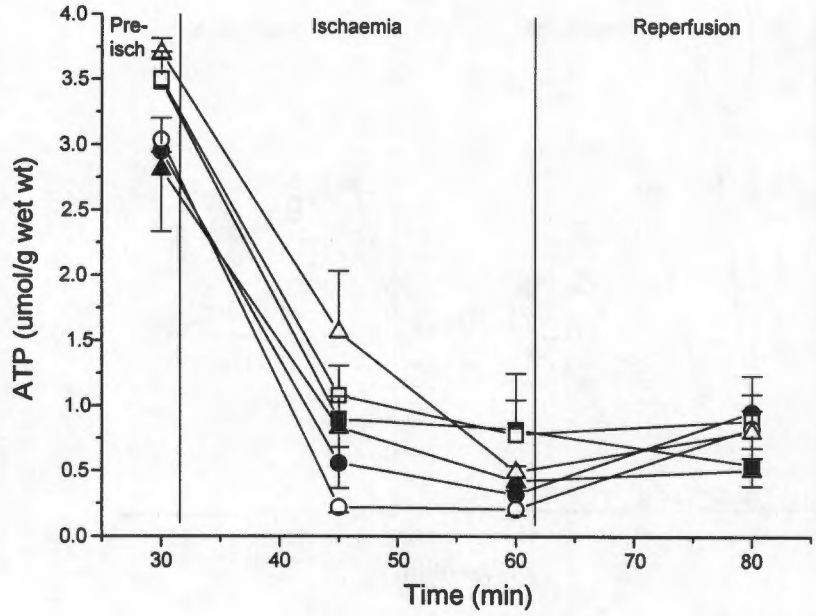


Fig 5a

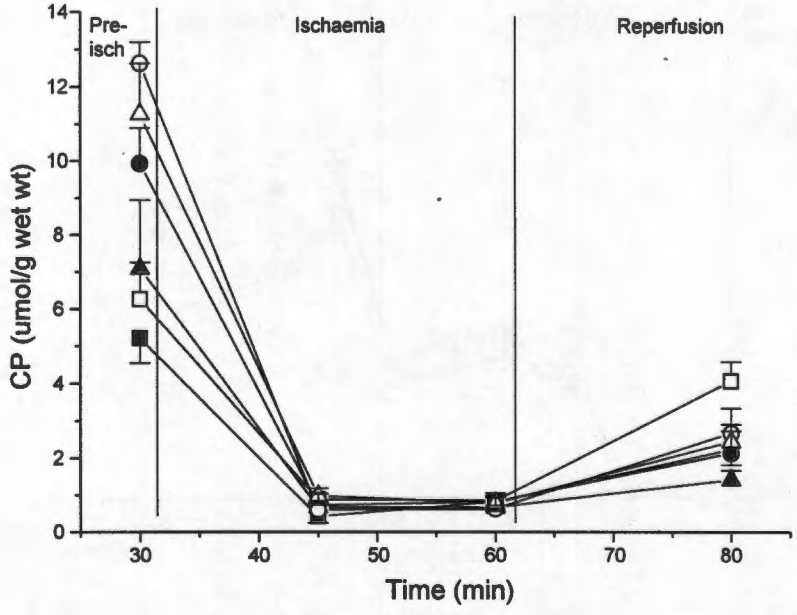


Fig 5b

Fig 5: Tissue adenosine triphosphate (ATP) (Fig 5a) and creatine phosphate (CP) (Fig 5b) levels prior to and during sustained ischaemia and reperfusion with changes in pre-ischaemic substrate, with an without preconditioning. Control - filled symbols, Preconditioned - open symbols. Glucose - squares, Acetate - circles, Glucose+insulin - triangles

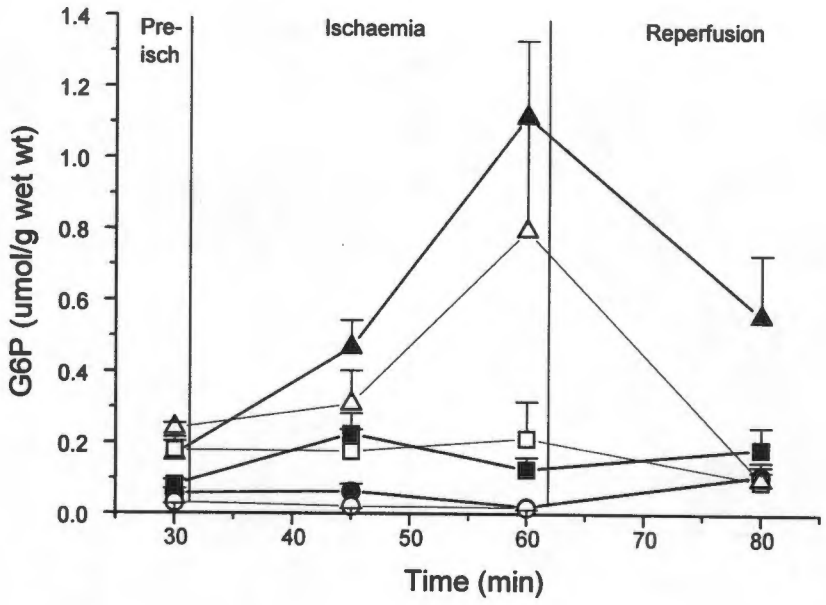


Fig 6: Changes in tissue glucose 6-phosphate (G6P) prior to and during ischaemia and reperfusion with changes in pre-ischaemic substrate, and in control and preconditioned hearts. Control - filled symbols, Preconditioned - open symbols. Glucose - squares, Acetate - circles, Glucose+insulin - triangles

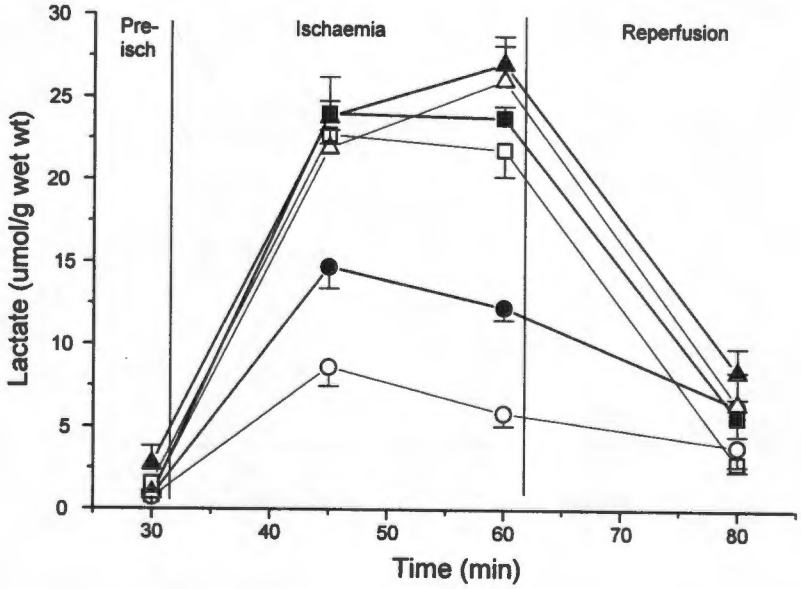


Fig 7: Changes in tissue lactate prior to and during ischaemia and reperfusion with changes in pre-ischaemic substrate, and in control and preconditioned hearts. Control - filled symbols, Preconditioned - open symbols. Glucose - squares, Acetate - circles, Glucose+insulin - triangles

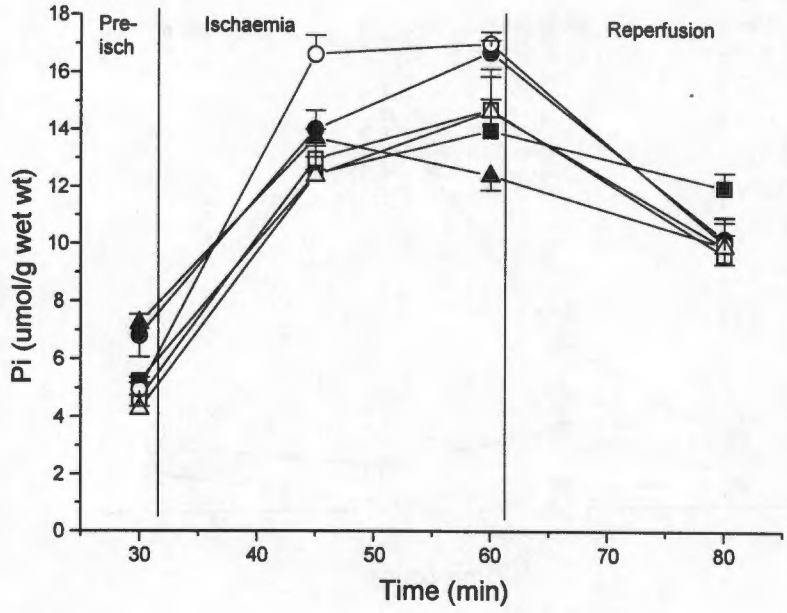


Fig 8: Changes in tissue levels of inorganic phosphate (Pi) prior to and during ischaemia and reperfusion with changes in pre-ischaemic substrate, and in control and preconditioned hearts. Control - filled symbols, Preconditioned - open symbols. Glucose - squares, Acetate - circles, Glucose+insulin - triangles

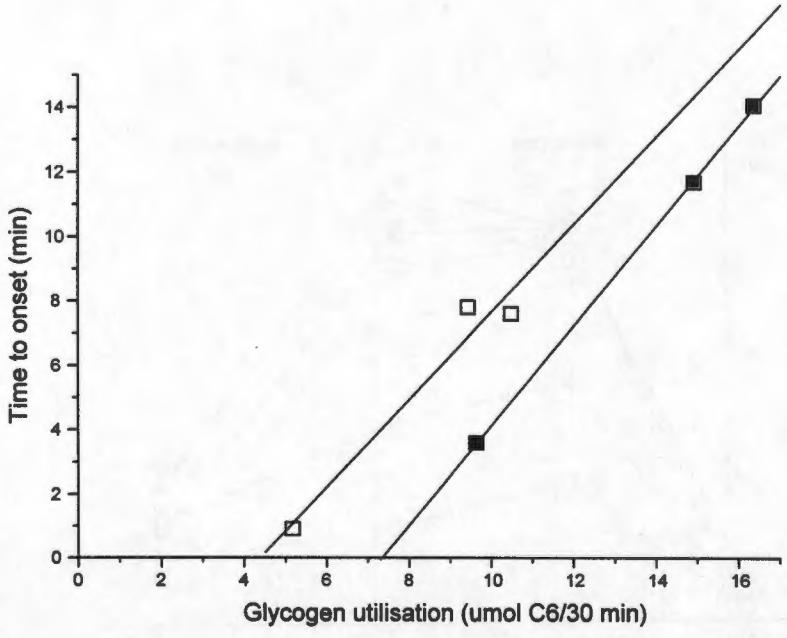


Fig 9: Correlation between time to onset of contracture and glycogen utilisation over 30 min ischaemia in control and preconditioned hearts. There was a linear relationship in both groups, showing a dependency of time to onset of contracture on glycogen availability. This curve was shifted to the left in preconditioned hearts, indicating a reduced time to onset at equivalent rates of glycogen utilisation. -

Control - filled symbols. $y = 1.55x - 11.41$ $R = 0.99$; $P=0.01$

Preconditioned - open symbols. $y = 1.36x - 5.98$ $R = 0.97$; $P=0.14$

Table 1: Functional parameters after 30 min perfusion prior to sustained ischaemia in control and preconditioned hearts with altered glycogen levels. n=9-10.

	Glucose		Acetate		Glucose+Insulin	
	Control	Precond	Control	Precond	Control	Precond
Coronary flow (ml/min)	12.6 ± 0.8	15.3 ± 0.4 *	14.5 ± 1.0	15.7 ± 0.8	11.9 ± 0.5	16.2 ± 0.6 *
Heart Rate (beats/min)	255.0 ± 23.0	243.0 ± 16.6	201.0 ± 17.0	222.4 ± 18.9	246.4 ± 19.5	297.6 ± 12.0 *
Systolic pressure (mmHg)	116.1 ± 5.8	100.8 ± 3.8 *	128.6 ± 4.4	110.8 ± 5.3 *	142.3 ± 5.2	114.7 ± 3.0
Diastolic pressure (mmHg)	2.9 ± 0.5	5.2 ± 1.2	1.4 ± 0.6	9.9 ± 3.9 *	0.9 ± 0.6	4.1 ± 1.9
Developed pressure (mmHg)	113.3 ± 5.7	95.6 ± 4.2 *	127.2 ± 4.4	100.9 ± 7.2 *	141.4 ± 5.3 #	110.5 ± 3.9 #

* $p<0.05$ vs control: # $p<0.05$ vs glucose

Table 2: Ischaemic contracture and functional recovery after 20 min reperfusion in control and preconditioned hearts with altered glycogen levels. n=9-10.

	Glucose		Acetate		Glucose+Insuli n	
	Control	Precond	Control	Precond	Control	Precond
Contracture						
Time to onset (min)	11.7 ± 0.7	7.8 ± 1.1 *	3.6 ± 0.3 #	0.9 ± 0.1 *#	14.1 ± 0.9 #	7.6 ± 0.8 *
Peak (%)	44.1 ± 3.8	52.7 ± 5.0	91.8 ± 4.9 #	113.3 ± 3.2 *#	38.1 ± 3.6	31.7 ± 2.6 #
Functional recovery						
Coronary flow (ml/min)	8.5 ± 0.5	11.0 ± 0.6 *	9.1 ± 0.8	8.2 ± 1.2	7.6 ± 0.6	10.2 ± 1.1
Developed pressure (%)	22.5 ± 7.0	47.6 ± 7.3 *	8.2 ± 3.0	11.7 ± 6.5 #	11.6 ± 4.3	18.6 ± 8.8 #
Diastolic pressure (mmHg)	70.4 ± 7.0	48.7 ± 7.3 *	68.8 ± 5.4	64.9 ± 9.9	76.0 ± 6.2	53.0 ± 7.9 *

* $p<0.05$ vs control; # $p<0.05$ vs glucose

Results 5. Dissociation between effects of preconditioning on glycolysis and functional recovery in low flow ischaemia

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ABSTRACT

A current hypothesis is that preconditioning (PC) is protective by decreasing glycolysis during sustained ischaemia, thereby lessening metabolite accumulation. To test this hypothesis, pre-ischaemic glycogen levels and glucose uptake (GU) during low flow (0.2 ml/g wet wt/min with 11 mM glucose) ischaemia were altered in isolated rat hearts by perfusion with glucose 11 mM (G), acetate 5 mM (A) or G + insulin (G+I). PC hearts in each group were subjected to 5 min ischaemia + 5 min reperfusion before ischaemia. Ischaemic contracture and postischaemic recovery were measured on reperfusion with 11 mM G. Glycogen utilisation was reduced in G PC hearts, but overall metabolite accumulation was greater because ischaemic GU increased from 0.52 ± 0.08 to 0.83 ± 0.05 μmol/g/min by PC ($p<0.01$). However, functional recovery was not improved. If glycogen were reduced by A perfusion, contracture was exacerbated, with no improvement in recovery. If A hearts were preconditioned, glycogen levels were further depleted, and GU was inhibited. However, contracture was worse, as was functional recovery. Increased glycogen and GU with G+I perfusion also did not alter functional recovery in control or PC hearts, although contracture was reduced. Thus in low flow ischaemia, decreased glycolytic rates could not be related to any beneficial effects; and glycolytic flux was dissociated from PC.

INTRODUCTION

Preconditioning, with one or more brief episodes of ischaemia and reperfusion, reduces infarct size and arrhythmias [319, 504], and improves functional recovery [18, 66, 290] of rat hearts exposed to subsequent sustained ischaemia. Preconditioning depletes tissue glycogen levels, and reduces tissue lactate, proton, glucose 6-phosphate (G6P) and α-glycerophosphate (αGP) accumulation during ischaemia [18, 224], while high energy phosphate levels are better maintained [224]. Preconditioning has also been shown to inhibit glycolysis in rat hearts during subsequent aerobic perfusion, and on reperfusion after sustained total global ischaemia [136]. From these observations, it has been suggested that preconditioning is beneficial because of reduced energy demand, and reduced glycolytic catabolite accumulation [224]. Thus by implication, reduced glycolysis is beneficial to the ischaemic myocardium [387].

In contrast, a residual coronary flow with enhanced glycolytic flux is beneficial to the globally ischaemic myocardium, with reduced contracture and improved functional recovery [14, 123] (Results Ch 1). Preconditioning together with maintained residual coronary flow during sustained ischaemia should therefore be additionally beneficial. However the effect of preconditioning on low flow ischaemia has not been fully elucidated. In one report of preconditioning followed by sustained low flow ischaemia in rabbit hearts, Janier et al. reported increased glycolytic flux during the sustained ischaemic period associated with improved functional recovery [218]. This finding is contrary to accepted concepts of glycolytic inhibition by preconditioning [136, 224].

We have dissociated the effects of glycogen depletion from the effects of preconditioning prior to sustained total global ischaemia (Results Ch 4). The aim of the present study was to confirm the changes in glycolysis in hearts preconditioned prior to sustained low flow ischaemia, as reported previously [218], and to determine the effect of modifications in glycolysis on preconditioning, thereby attempting to establish clearly whether there is any relationship between glycolysis and preconditioning.

METHODS

PERFUSION APPARATUS

The isolated Langendorff-perfused rat model with a left ventricular balloon was used, as described in Methods.

PROTOCOL

9-10 hearts were used in each group. The standard protocol was a 30 min period of perfusion, followed by 30 min ischaemia, and 20 min reperfusion (see Fig. 1). In the 30 min period before sustained ischaemia, hearts were perfused with the standard glucose-containing solution (at a concentration of 11 mM) either for the entire 30 min, or were subjected to preconditioning of 5 min total global ischaemia followed by 5 min reperfusion after 20 min. All hearts were then subjected to 30 min global ischaemia, with a residual flow of 0.2 ml/g wet wt/min, following which reperfusion was maintained for 20 min.

In the second set of experiments, hearts were perfused prior to the sustained episode of ischaemia with either 5 mM acetate instead of glucose, or with 11 mM glucose + 1U/l insulin (Humulin) prior to the sustained ischaemic episode. The NaCl concentration was reduced by 5 mM to allow for the addition of 5 mM Na-acetate. Control and preconditioned hearts were then subjected to 30 min low flow ischaemia (0.2 ml/g wet wt/min), with glucose 11 mM during ischaemia and for 20 min reperfusion.

Parallel groups of hearts were perfused as above for each group, with hearts clamped before ischaemia, after 30 min ischaemia, and at the end of the reperfusion period, using Wollenberger tongs kept in liquid nitrogen. 6 hearts were used in each group at each time point. These hearts were freeze dried for later biochemical analysis.

In addition samples of coronary effluent were collected over each 5 min period of low flow ischaemia. These samples were analysed for glucose uptake and lactate washout.

FUNCTIONAL MEASUREMENTS

Indices of contracture and functional recovery were recorded as described in Methods.

BIOCHEMICAL MEASUREMENTS

The freeze dried samples were extracted using perchloric acid. Levels of tissue metabolites, including high energy phosphates and glycolytic intermediates, were determined using spectrophotometric assays adapted for use on a centrifugal analyser (Cobas Fara, Roche Diagnostics, Switzerland) as descrbed in Methods. Effluent glucose and lactate were also measured using these methods. Glycogen levels were determined by measuring the glucose content of samples following alcohol extraction. All tissue metabolites were expressed as μmol/g wet wt, where wet weight = 5x dry weight. Washout

values were expressed as μmol carbon-6 units (C6) /g wet wt/min, corrected for coronary flow, and an assumed wet heart weight of 1 g (previously determined), to allow comparison between glucose uptake and lactate washout values. % glucose extraction was determined by the relationship:

$$\%\text{ glucose extraction} = (\text{glucose uptake/delivery}) * 100$$

where delivery = glucose concentration * coronary flow (Results Ch 1).

Glycogen was expressed as μmol glucose (C6) units/g wet wt.

STATISTICS

The Anova two-way analysis of variance was used to determine significance among groups, after which the modified t-test with the Bonferroni correction was used for comparison between individual groups. A value of $p<0.05$ was used as the level of significance.

RESULTS

GLUCOSE PERFUSED HEARTS WITH LOW FLOW ISCHAEMIA

Control - when control glucose hearts were subjected to a low residual flow of 0.2 ml/g wet wt/min (less than 2% of normal *in vitro* flow rates, about 3-7% of *in vivo* flow rats), onset of ischaemic contracture occurred after 12.0 ± 1.0 min, with a peak contracture of 37.0 ± 6.6 % (Table 1). Recovery of developed pressure was 55.1 ± 8.6 % (Table 1). There was a small reduction in systolic pressure compared to control levels, but the main determinant of functional recovery was diastolic pressure (Fig. 2).

Glycogen breakdown during ischaemia was approximately 10 μmol C6/g wet wt/30 min. Glucose uptake increased during ischaemia to a plateau of 0.59 ± 0.11 μmol/g wet wt/min by 10-15 min (27.0 ± 5.2 % extraction of delivered glucose) (Fig. 3). Mean glucose uptake over 30 min ischaemia was 0.52 ± 0.08 μmol/g wet wt/min, with a mean extraction of 25.45 ± 6.27 %. Mean lactate washout was 0.85± 0.09 μmol/g wet wt/min, which equates to 0.42 ± 0.04 μmol C6 units/g wet wt/min (Fig. 3). The lactate washout was less than could be accounted for by glucose uptake. There was, however, a substantial tissue lactate accumulation (Table 2),and an accumulation of glycolytic metabolites by the end of ischaemia (Table 2) which accounted for the uptake of glucose and the breakdown of glycogen. The contribution of oxidative phosphorylation in low flow would also reduce lactate production (this can account for about 2-3 μmol glucose/g wet wt/30 min low flow ischaemia (Results Ch 1)).

Preconditioned - when glucose preconditioned hearts were subjected to 30 min low flow ischaemia, no difference in time to onset of contracture or in peak contracture versus control hearts was seen (Fig. 2; Table 1). Recovery of coronary flow, diastolic or developed pressure was not affected by preconditioning (Table 1; Fig. 2).

Glucose uptake was significantly increased in preconditioned hearts throughout sustained ischaemia compared to control hearts (mean 0.83 ± 0.05 vs. 0.52 ± 0.08 μmol/g wet wt/min; $p<0.01$ vs. control; Fig. 3), with an extraction of 37.9 ± 1.97 % vs. 25.45 ± 6.27 % ($p<0.01$). Lactate washout was not, however, different between the two groups, although preconditioned hearts had the higher value (mean 0.47 ± 0.03 vs. 0.42 ± 0.04 μmol C6/g wet wt/min; Fig. 3). Tissue lactate at the end of ischaemia was higher in preconditioned hearts than in control hearts (Table 2). A significant reduction in pre-ischaemic glycogen levels was found with preconditioning, from 16.27 ± 0.44 to 11.19 ± 1.05 ($p<0.05$; Table 2). After 30 min ischaemia, however, total tissue levels of glycogen were similar between control and preconditioned hearts (Table 2). There was a difference in the amount of glycogen utilised, with approximately 6.3 μmol C6/30 min vs. 10.0 μmol/ C6/30 min in the preconditioned versus control hearts. The lowered glycogen utilisation therefore reduced the total lactate production despite the increased glucose uptake, although lactate was still higher in

preconditioned hearts. Increases in both G6P and αGP were found in preconditioned hearts compared to controls (Table 2), although these were not significant.

End ischaemic ATP and CP in preconditioned hearts were significantly lower than in control hearts (Table 3). After 20 min reperfusion, high energy phosphate levels tended to be higher in the preconditioned hearts but this was not significant (Table 3).

INSULIN-TREATED HEARTS AND LOW FLOW ISCHAEMIA

Control - insulin pre-treated hearts showed no difference in pre-ischaemic function compared to glucose hearts (Table 1). When insulin hearts were made ischaemic with a residual low flow, no significant difference in time to onset of contracture was observed compared to glucose hearts, although peak contracture was reduced (Table 1; Fig. 4). Functional recovery was no different (Table 1). Pre-ischaemic glycogen was significantly increased by insulin pre-treatment (Table 2), and levels were higher at the end of ischaemia compared to control hearts. Total glycogen breakdown was the same in glucose and insulin hearts (9.53 vs. 10 μmol C6/g wet wt/30 min). Glucose uptake (Fig. 5) was significantly increased compared to glucose perfused hearts (mean 0.86 ± 0.11 vs. 0.52 ± 0.08 μmol/g wet wt/min; $p<0.05$ - 38.97 ± 2.38 % and 23.56 ± 1.85 % extraction). No differences in high energy phosphates at the end of ischaemia were observed (Table 3).

Lactate washout was higher throughout ischaemia in the insulin treated hearts due to increased glucose uptake (Fig. 5), as was total tissue lactate at the end of ischaemia (Table 2). G6P and αGP accumulation was significantly greater in the insulin-perfused hearts by the end of ischaemia (Table 2). Insulin and sustained glucose utilisation with low flow suppressed glycogen breakdown, although total glycolytic flux was increased due to increased glucose uptake.

Preconditioned - when glycogen-loaded hearts were preconditioned, and subjected to low flow ischaemia, time to onset of contracture was slightly reduced compared to control insulin low flow hearts, but peak contracture was further reduced (Fig. 4; Table 1). Recovery was slightly less in preconditioned hearts but this was not significant (Table 1). While a small reduction in diastolic pressure was seen with preconditioning (Fig. 4; Table 1), recovery of systolic pressure was poor, resulting in the reduced developed pressure (Table 1).

At the end of ischaemia, glycogen levels were similar in control and preconditioned low flow hearts despite an initial difference (Table 2). These levels were substantially higher than those seen in glucose perfused hearts (Table 2). By the end of ischaemia, approximately 6 μmol glycogen had been utilised in preconditioned hearts, compared to 9.5 μmol in control insulin hearts and 6 μmol in glucose preconditioned hearts.

Glucose uptake was not increased in insulin-treated hearts by preconditioning compared to controls (mean 0.77 ± 0.12 μmol/g wet wt/min; Fig. 5), and the rates were similar to the values measured in glucose preconditioned low flow hearts (Fig. 3). Lactate washout during ischaemia was also no different between control and preconditioned insulin hearts (Fig. 5) while end-ischaemic tissue lactate

was the same in both groups (Table 2). Preconditioning and low flow also reduced G6P and αGP accumulation in the tissue (Table 2), with a lower overall glycolytic metabolite accumulation (data not shown) compared to control glucose + insulin hearts, due to reduced glycogen breakdown.

There was no reduction in ATP levels prior to ischaemia following preconditioning, although CP was increased (Table 3). End ischaemic ATP in preconditioned hearts was slightly less than in control insulin hearts (Table 3). Reperfusion elicited no differences in recovery of high energy phosphate between control and preconditioned insulin hearts (Table 3).

ACETATE PREPERFUSED HEARTS WITH LOW FLOW ISCHAEMIA

Control- acetate perfusion did not affect pre-ischaemic function (Table 1). During low flow ischaemia, the acetate pre-perfused hearts showed a reduced time to onset of contracture and an increased peak contracture compared to glucose hearts (Table 1; Fig. 4). Recovery of function was reduced, although this was not significant due to the large variability (Table 1).

When acetate hearts were switched to low flow ischaemia with glucose there was a large initial uptake of glucose, reaching 84% extraction in the first 5 min (Fig. 5). Mean glucose uptake was therefore 1.11 ± 0.10 μmol/g wet wt/min. However, this value was probably largely artefactual, given that the tissue was glucose-free which would dilute the effluent, and the low glucose content of the tissue would lead to a large uptake down the concentration gradient. After 30 min ischaemia, however, glucose uptake was still quite high (0.77 ± 0.14 μmol/g wet wt/min - 35.22 ± 6.31% extraction; Fig. 5), comparable to that in glucose hearts. The reduced glycogen and G6P (Table 2) content may have stimulated glucose uptake. Lactate washout showed a rise initially (reaching 0.51 ± 0.08 μmol C6/g wet wt/min at 5-10 min) but this then decreased to 0.24 ± 0.04 μmol C6/g wet wt/min after 25-30 min (Fig. 5), a value half that of glucose low flow hearts (Fig. 3). Tissue lactate accumulation was reduced in the acetate hearts (Table 2), with glycogen utilisation over 30 min low flow ischaemia about 7.9 μmol/g wet wt (Table 2)compared to 10 μmol /g wet wt in glucose hearts. Tissue G6P and αGP levels were maintained at levels equivalent to those in glucose hearts, indicating efficient utilisation of glucose and glycogen.

A large CP content prior to sustained ischaemia was found (Table 3), but end-ischaemic high energy phosphate levels in the acetate hearts were lower than in glucose hearts (Table 3). Recovery of high energy phosphate levels after 20 min reperfusion was no different.

Preconditioned - when acetate pre-perfused hearts were preconditioned, and then subjected to low flow ischaemia, the time to onset of contracture was immediate, and significantly reduced compared to control low flow hearts (Table 1; Fig. 4). Peak contracture was high, with a significant increase compared to control hearts (Table 1; Fig. 4). Recovery in preconditioned low flow acetate-pre-perfused hearts was 38.0 ± 9.7%, which no different to that obtained with control acetate hearts (Table 1).

Glucose uptake in preconditioned acetate hearts was significantly reduced compared to control acetate hearts, with a mean washout of 0.82 ± 0.08 μmol/g wet wt/min. At the end of ischaemia, glucose uptake was significantly reduced compared to all other groups (0.39 ± 0.09 μmol/g wet wt/min) (Fig. 5), as was lactate washout (Fig. 5). Tissue lactate was also higher in control hearts at the end of the ischaemic period (Table 2). Tissue values of G6P and αGP were reduced by preconditioning in acetate hearts (Table 2). Glycogen breakdown in preconditioned acetate hearts was only 4.5 μmol /g wet wt/30 min.

Preconditioned acetate hearts showed an even higher CP level prior to sustained ischaemia than control hearts, and a reasonable recovery of CP after 20 min reperfusion, although there was no difference in high energy phosphate levels between control and preconditioned hearts during ischaemia (Table 3). Pre-ischaemic ATP was not reduced by one episode of preconditioning.

DISCUSSION

We aimed to test the hypothesis that decreased rates of glycolysis could explain preconditioning. We applied our previously established model of preconditioning with one episode of 5 min total global ischaemia + 5 min reperfusion, followed by 30 min sustained total global ischaemia (Results Ch 4), which has been shown to be efficacious in a number of studies [18, 66, 603]. In that model, preconditioning reduced the time to onset of contracture and increased peak contracture, but significantly improved functional recovery following total global ischaemia (Results Ch 4). In the present study, when a low residual flow was maintained during the sustained ischaemic period, no protective effect of preconditioning on postischaemic function was discerned, despite large changes in the rate of glycolysis, one of the mechanisms proposed to explain preconditioning. Thus no relationship between preconditioning and changes in glycolytic flux rate could be established.

CHANGES IN GLYCOLYTIC FLUX INDUCED BY PRECONDITIONING

We confirm that glucose uptake during low flow ischaemia was increased by preconditioning, as reported previously [218]. This finding, together with unchanged or increased end ischaemic tissue levels of lactate, G6P and αGP (Table 2), challenges previous concepts that glycolysis is inhibited by preconditioning [224]. Only in total or near total ischaemia may glycolysis be limited in preconditioned hearts by the availability of substrate, specifically because of glycogen depletion. In addition, the present study somewhat contradicts previous findings that preconditioning inhibits glycolysis during aerobic perfusion after an initial preconditioning period, and on reperfusion after sustained total global ischaemia [136]. Preconditioning thus appears to affect aerobic (normal) and anaerobic (ischaemic) glucose utilisation differently, although the mechanism involved is unclear.

Glucose uptake - possible mechanisms of increased glucose uptake in low flow ischaemia following preconditioning include the lowered glycogen levels during ischaemia, and increased activity of glucose transporters in the membrane. A reduced glycogen level should result in increased glucose uptake, because of the reduced glycolytic substrate [206]. In acetate hearts with a low pre-ischaemic glycogen, when the hearts were switched to a low flow with glucose present, glucose uptake rates were high especially in the first 15-20 min of ischaemia. By the end of ischaemia, glucose uptake was equivalent to that in glucose preconditioned hearts (cf. Fig 3 and Fig 5a). Lactate production was low in acetate hearts because of reduced glycogen levels (Fig 5b). However, glucose uptake in preconditioned acetate hearts was severely reduced after 15 min ischaemia, despite an even lower glycogen content at the onset of ischaemia. The reduced glucose uptake may be explained by marked ischaemic hypercontracture with compression of the vessels, and increased mechanical failure in these hearts. Thus, glycogen depletion can be dissociated from an increased glucose uptake. This finding is strengthened by the observations of Depré et al. [111], who found that increased glucose uptake in

dysfunctional but salvageable myocardium ("mismatch") was correlated with increased glycogen in the tissue.

Preconditioning of insulin-treated hearts resulted in no additional stimulation of glucose uptake. Possibly, preconditioning and insulin may act on glucose uptake in a similar fashion. Insulin stimulates glucose uptake mainly via increased translocation of the insulin-sensitive glucose transporter, GLUT 4, to the membrane, and upregulation of transporters in the membrane [548]. Increased sarcolemmal GLUT 4 density has also been found in ischaemic tissue [517], together with increased glucose extraction [507] (Results Ch 1). A speculative hypothesis is that preconditioning ischaemia induces GLUT 4 translocation to the sarcolemma, or upregulates function of GLUT 4 already in the external membranes, such that by the onset of the sustained ischaemic period, the transporters would be available to increase glucose uptake. A number of factors modified by the preconditioning ischaemic episode (adenosine, cAMP, cGMP, PKC) may affect glucose transporter number and/or activity and thus increase glucose uptake. This proposed mechanism remains to be investigated.

Glycogen - the rate of glycogenolysis appears to be slowed in preconditioned hearts [574]. Our results confirmed this concept, in that there was no significant difference in tissue levels of glycogen after 30 min ischaemia between control and preconditioned hearts, despite large pre-ischaemic differences. Preconditioned hearts did tend to have lower end ischaemic glycogen levels in each group. Given that glucose utilisation is stimulated by preconditioning, the apparent concomitant inhibition of glycogen utilisation is difficult to explain. Reduced glycogenolysis may be attributed to a direct attenuation of phosphorylase activity following reduction of cAMP levels in preconditioned hearts [479]. Alternatively, the structure of glycogen may be relevant.

After NaOH extraction, we measured total tissue glycogen, which is made up of classic "macromolecular glycogen" or macroglycogen (about 10^7 Da), and proglycogen, a 400 kDa protein-rich form of glycogen insoluble in acid [322]. The residual glycogen at the end of ischaemia may consist mainly of core glycogen (α–1,6 linkages in macroglycogen requiring an additional enzyme step for breakdown), or proglycogen, which makes up about 15-50% of total glycogen levels and is more resistant to breakdown [10]. De Jong et al. [103] reported that proglycogen levels were unchanged by intermittent periods of ischaemia, although total glycogen levels were depleted. Thus, differences in pro- and macroglycogen levels at the onset of ischaemia may determine the subsequent rates of ischaemic glycogenolysis, and the amount of residual glycogen remaining after a period of ischaemia. The faster rate of glycogen breakdown found in control hearts compared to preconditioned hearts may reflect the rapid breakdown of the macroglycogen [10, 322] which has already occurred in preconditioned hearts prior to ischaemia. Insulin-treated hearts showed a slower rate of glycogen utilisation (Table 2), which may be attributed to increased proglycogen synthesis with insulin prior to ischaemia. In addition, both insulin and glucose appear to retard glycogen breakdown in low flow

ischaemia (Table 2) (Results Ch 1). Increased glycogenolysis with acetate or preconditioning could reduce pre-ischaemic proglycogen levels, resulting in lower end ischaemic tissue glycogen levels.

PRECONDITIONING AND CONTRACTURE WITH A LOW RESIDUAL FLOW

With sustained zero flow ischaemia, contracture is increased in preconditioned hearts compared to control hearts, but paradoxically functional recovery is improved [18, 66] (Results Ch 4). A temporal correlation between depleted ATP levels and increased contracture has been found in preconditioned hearts [266].

When there is maintained glucose delivery in the presence of a low residual coronary flow, contracture is reduced (Results Ch 1) and there is a better recovery of function in control hearts, compared to hearts with zero flow ischaemia (Results Ch 4). A low coronary flow also attenuated the exacerbation of contracture by preconditioning (Fig 2, Table 1), presumably due to increased glucose uptake and glycolysis, and maintained ATP production [424] (Results Ch 1). In acetate hearts, the ATP production from glucose was insufficient, resulting in increased contracture, made worse by preconditioning which inhibited glucose uptake. Functional recovery tended to be worse in these hearts.

The overall increase in ATP production with insulin present (calculated from glucose uptake - 2 μmol ATP/μmol glucose/min; and see Table 3) reduced contracture in control and preconditioned hearts compared to other low flow groups. However, sugar phosphate accumulation has been implicated in a loss of Ca^{2+} homeostasis [282], and may thus exacerbate contracture. In insulin hearts, preconditioning lessened contracture, possibly by reducing metabolite accumulation [18, 224, 282] compared to control insulin hearts (Table 2), although ATP production was also slightly less (Table 2). The reduced contracture was not, however, reflected in a better functional recovery (Table 1). Contracture thus does not always reflect the degree of ischaemic injury.

PRECONDITIONING AND FUNCTIONAL RECOVERY AFTER SUSTAINED LOW FLOW ISCHAEMIA

There was no improvement in functional recovery with preconditioning in any of the groups of low flow hearts, contrary to previous findings [218]. A significant, albeit small improvement in recovery of function (of 14%) was found with preconditioning in rabbits exposed to 60 min low flow ischaemia (0.4-0.45 ml/g wet wt/min), together with increased glucose uptake [218]. Differences from the present study lie in the species and in the perfusion solutions used.

The increased glucose uptake previously noted with preconditioning, and the benefits associated with this effect [218] may have been masked by the use of a perfusate with double the glucose concentration (5 mM glucose, with palmitate and 70 mU/l insulin [218] compared to 11 mM glucose in the present study). Of note is that our standard glucose concentration (11 mM) is optimal in ischaemic rat hearts with a residual flow of 0.2 ml/g wet wt/min without insulin (Results Ch 1), while a higher glucose

concentration (22 mM) increases contracture and impairs functional recovery (Results Ch 1). In the previous study [218], with the lower glucose concentration (5 mM), glucose uptake decreased markedly during ischaemia despite the presence of insulin, compared to a steady state maintained with a higher glucose concentration (11 mM) (see Fig 3). A lower glucose concentration leads to insufficient ATP production, increased contracture and poorer functional recovery [424] (Results Ch 1). Thus, in the present study, glucose hearts may have had optimal glycolytic flux with 11 mM glucose, while with a lower glucose concentration, glycolytic flux was less than optimal in control hearts, and the stimulation by preconditioning resulted in a higher rate of glycolysis, which was protective. In the present study, increased glycolytic flux with preconditioning above the optimal rate may have led to an imbalance between excess metabolite accumulation and ATP production, such that recoveries were not improved.

This hypothesis is substantiated by the effects of increased glucose uptake (and tissue glycogen) in the presence of insulin, which were detrimental possibly due to excess metabolite accumulation [219, 282, 387] (Table 2), despite the calculated increase in ATP production. Thus insulin treatment did not lead to improved recovery of function in the present study. When insulin-treated hearts were preconditioned, glucose uptake was not additionally increased nor was functional recovery improved, compared to the previous report [218]. Insulin-treated hearts in the present study (15-fold increase in insulin and double glucose concentration compared to previous [218]) may have had maximal stimulation of glucose transporters, such that preconditioning would have no additive effect. Janier et al. reported an increase in glucose uptake of about 20-25% with preconditioning, compared to a 60% increase in the present study, while basal rates were relatively higher. A possible greater lactate and sugar phosphate accumulation with a high glucose + insulin and a lower flow rate (0.2 vs. 0.4-0.45 ml/g wet wt/min) could also be more deleterious in the present model, partially accounting for the discrepancies.

Confirmation of the findings of the present study come from two previous reports using rat hearts. Preconditioning (5 min total global ischaemia + 5 min reperfusion) was ineffective against hypoxia-induced damage (sustained hypoxia 60-90 min) in terms of functional recovery [66]. While it may be argued that low flow may increase the threshold of preconditioning, so that "more preconditioning" is required to discern an effect, 3 cycles of preconditioning (5 min isch + 5 min reperf) did not improve recoveries after 90 min low flow ischaemia (0.3 ml/g wet wt/min) in a blood-perfused isolated rat heart [67]. Speculatively, low residual flow may wash out the "effector" of preconditioning, such as adenosine [63]. A residual coronary flow as low as 0.1 ml/g wet wt/min has also been shown to attenuate the protective effects of preconditioning on functional recovery (F. R. Boucher - personal communication).

DISSOCIATION OF PRECONDITIONING AND GLYCOLYSIS

Increased glycolytic substrate with glucose and insulin was not beneficial, and was associated with increased metabolite accumulation. When glycolytic substrate, and thus metabolite accumulation, were limited by acetate preperfusion, hearts subjected to sustained low flow ischaemia showed similar recoveries to glucose hearts, and similar end-ischaemic glucose uptakes. However, contracture was significantly increased. These hearts also responded badly to preconditioning, with a rapid time to onset of contracture and high peak contracture, and reductions in both glucose uptake and glycogen availability. Recoveries were generally poorer than controls, although the large variability precluded significance. Thus reduced glycolytic metabolite accumulation with or without preconditioning was not advantageous in the presence of a reduced ATP production, despite the possible presence of the preconditioning "effector".

The proposal that the net beneficial effect of glycolysis in ischaemia is determined by the balance between the requirement for sufficient glucose for ATP production, versus an overload of glycolytic substrate (glucose and glycogen) with the result of excess metabolite accumulation, is substantiated by the present results. We could also dissociate the effects of preconditioning on glycolysis from those on recovery of function. With modifications in glycolysis similar to those seen in preconditioned hearts subjected to low flow ischaemia (increased glucose uptake, reduced glycogen), preconditioning was still not effective. The lack of correlation between preconditioning and glycolysis, and effects on contracture and functional recovery, suggest that decreased glycolysis is not involved in the effects of preconditioning, contrary to previous opinion [136, 218, 224]. Increased glycolysis in preconditioned low flow hearts was beneficial by counteracting the effect of preconditioning on contracture. The beneficial effect on functional recovery with preconditioning in the previous report using a model of low flow ischaemia [218] may be attributed solely to the stimulation of glycolysis by preconditioning in the face of a less than optimal basal level of glucose uptake, rather than preconditioning *per se* (see above).

The argument could be made that if preconditioning acts by inhibition of glycolysis and reduction of metabolite accumulation, then when glycolysis is stimulated (by preconditioning), the benefits are no longer apparent, suggesting that increased glycolysis is detrimental [387]. While we agree that excess glycolysis is indeed detrimental to ischaemic hearts, this does not mean that the presence of glucose or glycogen is bad. An optimal concentration of glucose is required, usually 11 mM (Results Ch 1). Additional counterpoints to the above argument are: i) in low flow conditions, glycolysis is essential for reduced contracture and improved functional recovery [14, 48, 424] (Results Ch 1); ii) glucose was present throughout low flow ischaemia and reperfusion in all hearts - removal of glucose would have been severely detrimental (Results Ch 1); iii) if preconditioning exerts benefit by inhibiting glycolysis, but glycolysis is stimulated in low flow ischaemia, this outcome strengthens the concept that preconditioning is ineffective in low flow ischaemia; iv) hearts with inhibited glycolysis (acetate

perfused) had exacerbated contracture, particularly when preconditioned (contrary to other glycogen groups), with poorer recoveries.

FUNCTIONAL RECOVERY VERSUS INFARCT SIZE AS THE END-POINT OF PROTECTION BY PRECONDITIONING

Studies in larger animals (pigs, dogs) have generally only measured infarct size as the index of protection by preconditioning. When functional recovery has been measured, ischaemic preconditioning is ineffective against an ischaemia-induced reduction in systolic shortening [498], or stunning [360, 421], despite significant reductions in infarct size. Subendocardial ischaemic blood flow in these models ranges from 0.05 to 0.15 ml/g wet wt/min [375, 421], about 5-15% of control flows.

Improved recovery of function with preconditioning has only been shown in rabbit [218, 368, 542] and rat hearts [66, 136, 143, 290, 588]. The majority of these studies have used total global ischaemia, and have shown a consistent improvement in functional recovery in rat hearts, as have we (Results Ch 4). Less consistent results have been reported for the rabbit, in that a number of reports find no protection against reperfusion dysfunction [358, 447, 532].

In the present study using the isolated rat heart model, a total global flow rate of 0.2 ml/g wet wt/min was used, a % reduction (about 5% of normal *in vivo* flow rates [279]) equivalent to that found in subendocardial ischaemia *in vivo* in larger animals [360, 375, 421, 507]. No improvement in functional recovery was found with preconditioning under these conditions. While we did not measure infarct size, the effect of residual flow in our model may indicate why in larger animals with collateral flow, preconditioning is not effective against impaired recovery of function.

A recent paper by Jenkins et al. [220] correlates reduced infarct size with improved recovery of function in preconditioned rabbit hearts. The authors suggest that preconditioning does not protect against stunning, but that improved functional recovery is a direct consequence of reduced infarct size. Thus if recovery is not improved, infarct size is presumably not modified. In our results, no effect on functional recovery was found, implying either that preconditioning is not always effective, or that functional recovery is dissociated from infarct size, assuming infarct size was limited. This relationship, however, requires further clarification.

Interestingly, Li et al. [310] found that infarct size was similar in control and preconditioned dog hearts with subendocardial flow rates of 0.07 ml/g wet wt/min and greater, suggesting that a higher residual flow also limits the effect of preconditioning on infarct size (see Cave and Apstein [63] for further discussion of this concept). The mechanism for this observation is unclear, and remains to be investigated.

It may also be argued that, given that there was no improvement in functional recovery with preconditioning followed by a sustained ischaemic period, we did not have a model of true preconditioning. However, while this argument may be valid when drawing inferences regarding the

role of glycolysis in preconditioning, I believe that the observations presented in this paper are of particular concern when the application of preconditioning as a possible clinical therapy is considered.

CONCLUSIONS

The hypothesis that preconditioning acts via inhibition of glycolysis and attenuation of metabolite accumulation is opposed by the present results. We found that preconditioning leads to increased glucose uptake during low flow ischaemia, which reduces the effects of preconditioning on contracture found in zero flow conditions. Our data show that any benefit of preconditioning can be dissociated from its effects on glycolysis, either from glucose or from glycogen, and that a residual low flow abolishes the protection associated with preconditioning.

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A low residual flow abolishes the protection conferred by preconditioning. This effect may be attributed to an overload of glycolytic metabolites, following preconditioning-induced enhanced glucose uptake. The data from the previous studies in the thesis also suggest that increased glycolytic flux (whether from a high extracellular glucose concentration, or high tissue glycogen) may be deleterious by an accumulation of glycolytic metabolites. One of the primary metabolites of anaerobic glycolysis is lactate, which has been previously implicated as a mediator of ischaemic injury. However, the results from provision of lactate in various protocols are contradictory. We investigated whether the addition of increasing lactate concentrations was detrimental to the heart exposed to sustained low flow ischaemia with and without glucose.

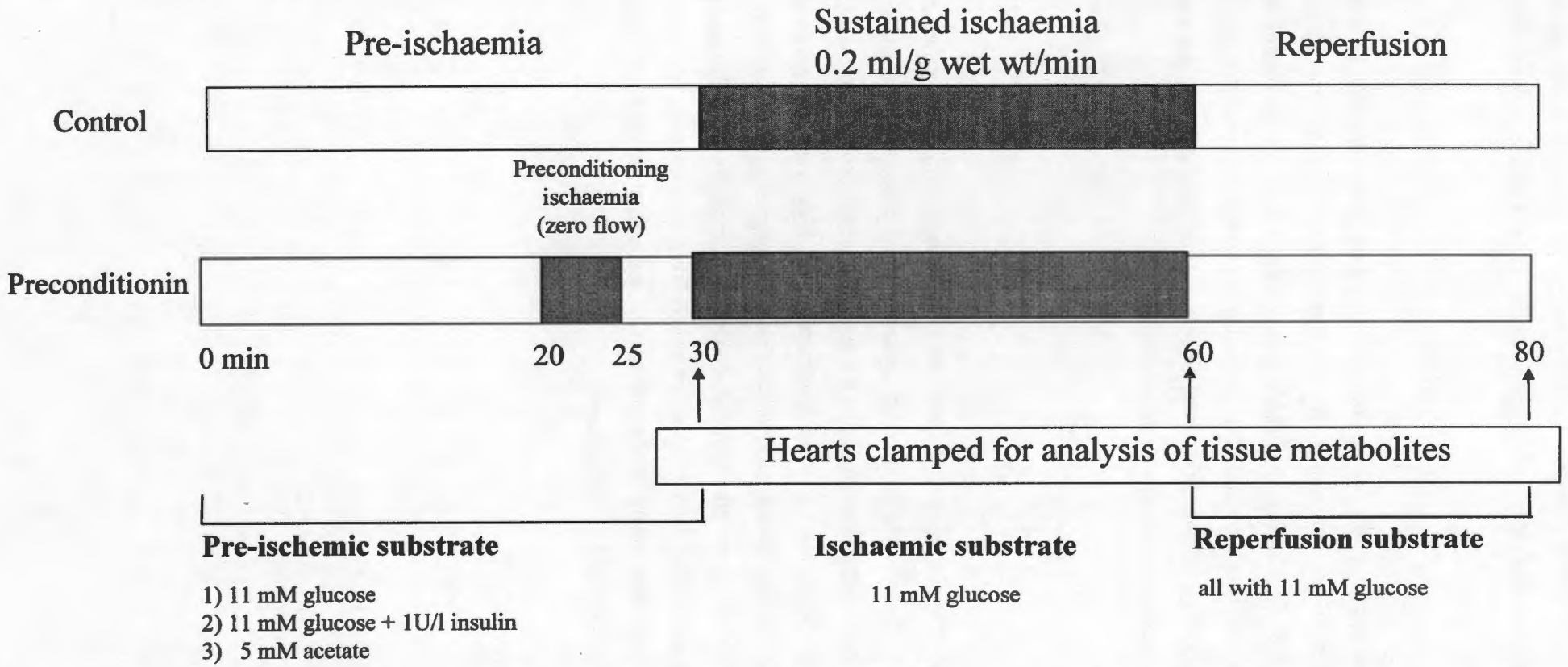


Fig. 1: Protocol of experiments with control and preconditioned hearts prior to sustained low flow (0.2 ml/g wet wt/min) ischaemia with 11 mM glucose throughout; and hearts pre-perfused with either 5 mM acetate or 11 mM glucose + 1 U/l insulin prior to low flow ischaemia (0.2 ml/g wet wt/min with 11 mM glucose) with and without preconditioning. A left ventricular balloon was used to record function. Preconditioning ischaemia was 5 min total global ischaemia + 5 min reperfusion.

Coronary effluent was collected during low flow ischaemia over each 5 min period for analysis of glucose uptake and lactate washout. Developed pressure at 20 min reperfusion (80 min) and peak contracture were normalised against developed pressure at 20 min pre-ischaemic perfusion. 9-10 hearts were used in each group. 6 hearts in each group were clamped at each time point for analysis of tissue metabolites.

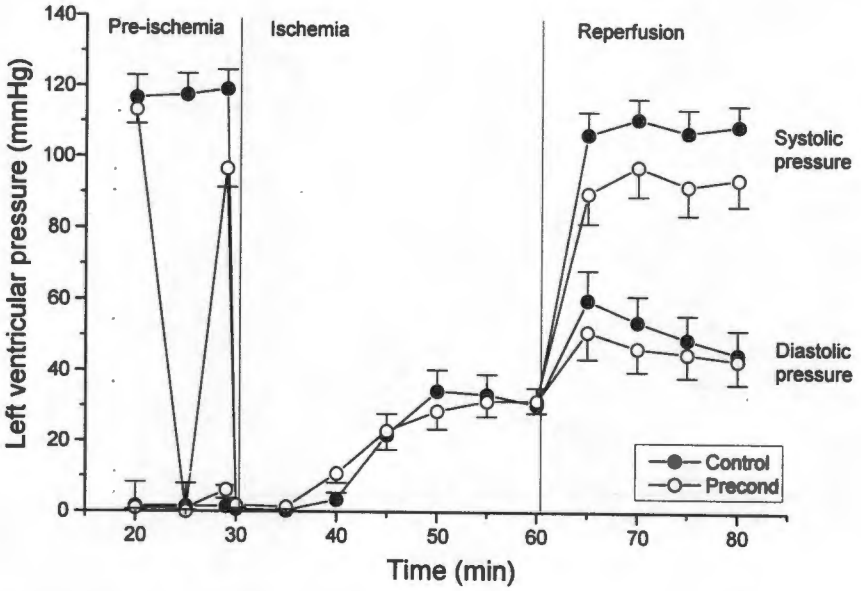


Fig. 2: Systolic and diastolic pressure throughout the experiments for control and preconditioned hearts with glucose 11 mM, with low flow sustained ischaemia. Systolic - diastolic pressure gave developed pressure. There was no difference in recovery between control and preconditioned low flow hearts, although the latter tended to have a reduced systolic function.

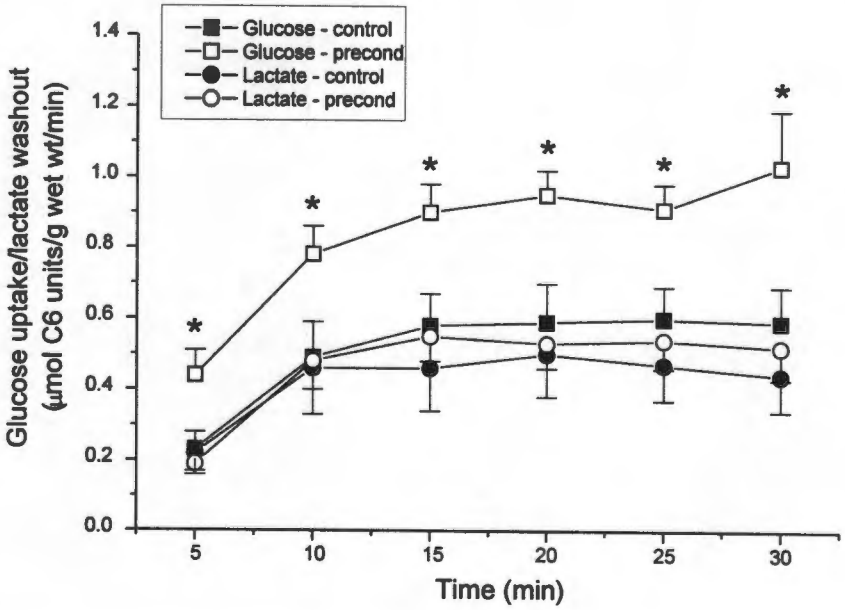


*Fig. 3: Glucose uptake and lactate washout during low flow ischaemia in glucose hearts subjected to 30 min low flow (0.2 ml/g wet wt/min) ischaemia with and without preconditioning expressed as µmol C6/g wet wt/min. * $p<0.05$ precond vs. control*

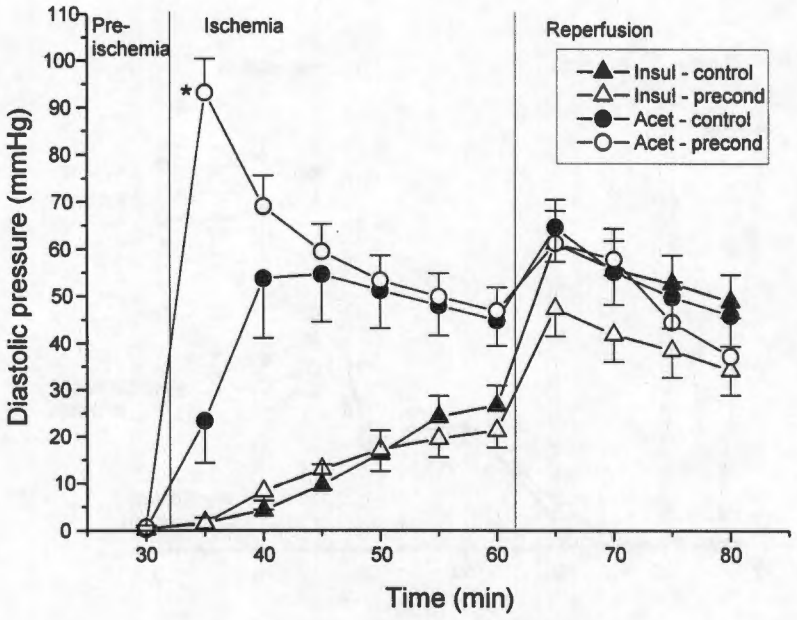


Fig. 4: Changes in diastolic pressure during ischaemia and reperfusion with acetate pre-perfusion and insulin pre-treatment, in control and preconditioned hearts subjected to low flow ischaemia.

** $p<0.05$ precond vs. control*

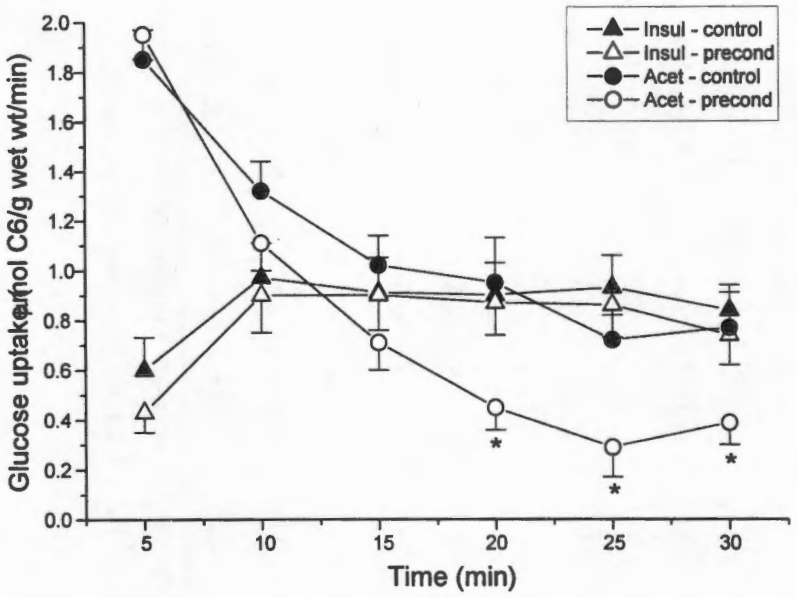


Fig 5a

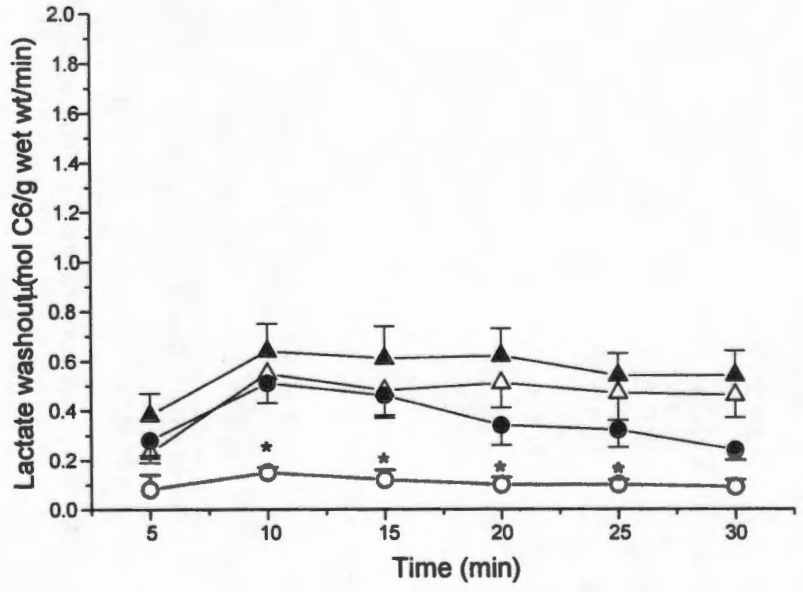


Fig 5b

Fig. 5: Glucose uptake (a) and lactate washout (b) during low flow ischaemia in acetate and glucose+insulin pre-perfused hearts subjected to 30 min low flow (0.2 ml/g wet wt/min) ischaemia with 11 mM glucose with and without preconditioning expressed as µmol C6/g wet wt/min.* $p<0.05$ precond vs. control

Table 1: Pre- and post-ischaemic function and ischaemic contracture hearts with low flow (0.2 ml/g wet wt/min with 11 mM glucose) ischaemia, with and without preconditioning, perfused with 11 mM glucose, 11 mM glucose + 1U/l insulin, or 5 mM acetate prior to ischaemia.

		Glucose		Glucose + Insulin		Acetate	
		Control	Precond	Control	Precond	Control	Precond
Pre-ischaemia	CF (ml/min)	12.7 ± 0.6	16.1 ± 0.7	12.2 ± 0.6	15.8 ± 1.0 *	13.4 ± 0.6	16.3 ± 1.0 *
	HR (beats/min)	220.0 ± 18.7	289.7 ± 20.0	231.0 ± 21.2	260.0 ± 20.1	216.9 ± 17.9	211.0 ± 19.5
	SP (mmHg)	119.0 ± 5.5	96.5 ± 5.3 *	129.0 ± 5.4	110.8 ± 3.8 *	120.5 ± 3.7	116.4 ± 8.0
	DP (mmHg)	1.4 ± 0.5	6.1 ± 2.4	0.8 ± 0.5	0.5 ± 0.4	1.5 ± 0.6	4.0 ± 2.0
	Dev P (mmHg)	117.6 ± 5.8	90.4 ± 6.2 *	128.2 ± 5.5	110.3 ± 3.7 *	119.4 ± 3.7	112.4 ± 7.9
Ischaemia	TOC (min)	12.0 ± 1.0	10.5 ± 1.5	15.5 ± 2.3	12.3 ± 2.2	6.8 ± 1.8 #	0.8 ± 0.2 *#
	Peak (%)	37.0 ± 6.6	32.0 ± 3.8	23.1 ± 4.2	18.1 ± 3.2 #	61.5 ± 10.6 #	96.7 ± 8.6 *#
Reperfusion	CF (ml/min)	10.9 ± 0.6	11.1 ± 0.6	9.1 ± 0.6	11.1 ± 0.7	10.8 ± 0.9	13.2 ± 1.4
	Dev P (%)	55.1 ± 8.6	47.5 ± 11.8	51.4 ± 8.5	41.1 ± 8.0	40.2 ± 9.7	38.0 ± 9.7
	DP (mmHg)	44.8 ± 6.9	43.1 ± 6.6	48.9 ± 5.6	34.2 ± 5.3	45.9 ± 6.6	37.2 ± 8.3

Pre-ischaemic measurements after 30 min perfusion. Reperfusion function after 20 min reperfusion. Reperfusion developed pressure expressed as percentage of pre-ischaemic function at 20 min perfusion. CF - coronary flow; HR - heart rate; SP - systolic pressure; DP - diastolic pressure; Dev P - developed pressure; TOC - time to onset of contracture; Peak - peak contracture. * $p<0.05$ vs. control; # $p<0.05$ vs. glucose.

Table 2: Tissue levels of glycogen and glycolytic metabolites in hearts perfused with 11 mM glucose, 11 mM glucose + 1U/l insulin or 5 mM acetate hearts with and without preconditioning followed by low flow ischaemia (0.2 ml/g wet wt/min with 11 mM glucose).

		Glucose		Glucose+ Insulin		Acetate	
		Control	Precond	Control	Precond	Control	Precond
Pre-ischaemia	Glycogen	16.27 ± 0.44	11.19 ± 1.05 *	21.39 ± 1.08 #	15.53 ± 1.55 *#	10.77 ± 0.96 #	5.98 ± 1.04 *#
	G6P	0.08 ± 0.02	0.18 ± 0.03 *	0.17 ± 0.05	0.24 ± 0.15	0.06 ± 0.01	0.03 ± 0.00
	αGP	0.29 ± 0.08	0.52 ± 0.14	0.87 ± 0.33	0.61 ± 0.25	0.39 ± 0.18	0.36 ± 0.06
	Lactate	0.81± 0.03	1.56 ± 0.44	2.72 ± 1.12 #	0.95 ± 0.04 *	0.79 ± 0.09	0.71 ± 0.05
Ischaemia	Glycogen	6.27 ± 0.99 †	4.86 ± 0.86 †	11.86 ± 2.26 †#	9.51 ± 2.35 †#	2.89 ± 0.58 †#	1.46 ± 0.22 †#
	G6P	0.20 ± 0.04	0.25 ± 0.07	0.67 ± 0.21 †#	0.34 ± 0.09	0.09 ± 0.05	0.17 ± 0.13
	αGP	2.64 ± 0.87 †	2.85 ± 0.59 †	4.44 ± 0.93 †	2.82 ± 0.76 †	2.25 ± 0.78 †	2.12 ± 0.76 †
	Lactate	16.27 ± 0.54 †	20.84 ± 2.67†	20.80 ± 1.86 †	22.33 ± 1.93 †	8.46 ± 1.04 †#	5.84 ± 1.49 †#
Reperfusion	Glycogen	4.58 ± 0.88 †	4.33 ± 0.86 †	10.11 ± 1.80 †#	8.64 ± 1.08 †#	2.88 ± 0.46 †	1.73 ± 0.74 †
	G6P	0.08 ± 0.04	0.07 ± 0.03 †	0.06 ± 0.03	0.07 ± 0.03	0.06 ± 0.03	0.13 ± 0.03
	αGP	1.03 ± 0.25	1.24 ± 0.26	1.12 ± 0.21	1.35 ± 0.20	1.06 ± 0.34	1.39 ± 0.50
	Lactate	6.77 ± 1.37 †	3.67 ± 0.74	4.54 ± 0.95	6.23 ± 1.80	2.14 ± 0.60 #	4.35 ± 2.98

Tissue levels in μmol/g wet wt after 30 min pre-ischaemic perfusion, at the end of 30 min ischaemia, and after 20 min reperfusion.

G6P - glucose 6-phosphate; αGP - α-glycerophosphate. * $p<0.05$ vs. control; # $p<0.05$ vs. glucose; † $p<0.05$ vs. pre-ischaemic values

Table 3: High energy phosphates levels in hearts perfused with 11 mM glucose, 11 mM glucose + 1U/l insulin or 5 mM acetate hearts with and without preconditioning followed by low flow ischaemia (0.2 ml/g wet wt/min with 11 mM glucose).

		Glucose		Glucose + Insulin		Acetate	
		Control	Precond	Control	Precond	Control	Precond
Pre-ischaemia	ATP	3.48 ± 0.23	3.50 ± 0.15	2.81 ± 0.47	3.70 ± 0.11 *	2.95 ± 0.25	3.04 ± 0.10
	CP	5.21 ± 0.67	6.26 ± 1.00	7.09 ± 1.86	11.27 ± 1.36 #*	9.94 ± 0.96 #	12.63 ± 0.56 #*
Ischaemia	ATP	1.53 ± 0.11	0.70 ± 0.23 *	1.63 ± 0.28	1.23 ± 0.25 #	0.79 ± 0.31 #	0.56 ± 0.19
	CP	1.51 ± 0.36	0.61 ± 0.18 *	1.06 ± 0.06	1.00 ± 0.21	1.03 ± 0.29	1.23 ± 0.23
Reperfusion	ATP	0.55 ± 0.15	1.17 ± 0.37	1.31 ± 0.63	0.95 ± 0.32	0.91 ± 0.44	1.48 ± 0.40
	CP	2.36 ± 0.51	3.58 ± 0.84	2.00 ± 0.83	2.65 ± 0.79	2.52 ± 0.98	3.94 ± 1.04

Levels after 30 min pre-ischaemic perfusion, at the end of 30 min ischaemia, and after 20 min reperfusion. ATP and CP in μmol/g wet wt.

* $p<0.05$ vs. control; # $p<0.05$ vs. glucose; all end-ischaemia and reperfusion values significantly different from pre-ischaemic values

Results 6: Lactate: is it detrimental to the ischaemic myocardium?

ABSTRACT

Increased lactate accumulation is thought to be the major culprit in determining a deleterious effect of increased glycolysis [387]. Increased extracellular lactate, provided in the perfusate, should therefore result in an attenuation of recovery. We used the isolated Langendorff rat heart with a left ventricular balloon, perfused with 11 mM glucose at a pressure of 76 mmHg. The perfusate was then changed to 10 mM lactate + 11 mM glucose, or 40 mM lactate + 11 mM glucose 3 min prior to low flow (0.5 or 0.2 ml/g wet wt/min) ischaemia for 30 min with the same substrates. The hearts were then reperfused for 30 min with 11 mM glucose perfusate. With a low flow of 0.5 ml/g wet wt/min, the addition of lactate did not result in any impairment of function, either in terms of ischaemic contracture or functional recovery. Only with a low flow of 0.2 ml/g wet wt/min, was an increase in peak contracture noted, with a reduction in functional recovery. Thus only when lactate was in excess, at a very low residual flow rate, was any deleterious effect noted in the presence of glucose. The effect of lactate accumulation as a major factor in ischaemic injury, when lactate is not in excess, is not substantiated by these results .

INTRODUCTION

The role of lactate accumulation in ischaemic injury is of great interest, arising from the controversies of whether endogenous glycogen should be augmented prior to ischaemia, and whether glucose should be provided to the ischaemic myocardium. The opponents to both of these strategies suggest that an accumulation of lactate in the tissue, and more specifically in the cytosol, is detrimental, largely by inhibiting glycolysis. The most frequently quoted report supporting this concept is that by Neely and Grotyohann [387], who found that a depletion of glycogen by a period of anoxia prior to ischaemia was beneficial, an effect attributed in part to reduced lactate accumulation.

Alternatively, many studies, in particular those involved with glucose-insulin-potassium therapy, and others which have focused more on the role of glucose alone, suggest that glucose is beneficial when supplied to the ischaemic myocardium [407], and is essential on reperfusion [228, 334, 476]. An increased residual flow adds to the beneficial effect of glucose, but at no flow is glucose more detrimental than the absence of glucose (Results Ch 1). Thus at an optimal glucose concentration (11 mM), lactate accumulation does not appear to outweigh the benefits of increased ATP production. However, an increased glucose concentration (>11 mM), especially if insulin is present, is associated with detrimental effects, in terms of increased contracture and reduced recovery of function at severe low flows (<0.5 ml/g wet wt/min) (Results Ch 1, 2). A higher flow rate (0.5 ml/g wet wt/min) removes many of these deleterious effects (Results Ch 2), although functional recovery is still relatively impaired (Results Ch 2). These deleterious effects can be attributed to a large increase in lactate production, as well as to the accumulation of other metabolites including glucose 6-phosphate (G6P) and fructose 6-phosphate (F6P) (Results Ch 5). Sugar phosphates have been implicated in the deleterious effects of ischaemia by increasing Ca^{2+} overload [219, 380, 482].

Looking specifically at the effects of lactate, Cross et al. found that lactate at a concentration of 10 mM was deleterious when infused at a flow rate of 0.5 ml/g wet wt/min in the presence of 11 mM glucose, resulting in increased contracture and reduced recovery of function [92]. This finding was attributed to a build-up of intracellular lactate which would inhibit the conversion of pyruvate to lactate and the co-conversion of NADH to NAD^+. An increased build up of NADH was predicted, which would affect many of the redox reactions in the cell, including glyceraldehyde 3-phosphate dehydrogenase (GAPDH), one of the main regulatory enzymes in the glycolytic pathway [362, 468]. A reduced lactate efflux, and thus presumably reduced glycolysis, was found in the lactate + glucose hearts [92]. These findings are contrary to our concepts that, with a residual flow rate of 0.5 ml/g wet wt/min, there should be sufficient washout of metabolites, while provision of glucose by increasing ATP production should override the deleterious effects of lactate.

We attempted to repeat the observations of Cross et al, both at a residual coronary flow rate of 0.5 ml/g wet wt/min, at a lower flow rate of 0.2 ml/g wet wt/min with reduced washout, and with a higher lactate concentration. When glucose was present no deleterious effect of lactate was observed at a flow rate of 0.5 ml/g wet wt/min even with 40 mM lactate present, while at a flow rate of 0.2 ml/g wet wt/min, recoveries in the glucose and lactate hearts were depressed compared to those with glucose alone. Thus only when intracellular lactate accumulation was very high, with reduced washout and more severe ischaemia, was any deleterious effect noted.

METHODS

Experimental apparatus

The isolated Langendorff-perfused rat model with a left ventricular balloon was used, as described in Methods. 11 mM glucose was included in the perfusate except where stated.

Protocol

The hearts were perfused for 20 minutes prior to ischaemia. Standard glucose-containing buffer solution was used until 3 minutes prior to ischaemia, when it was changed to one of the test solutions as shown in Table 1. The hearts were then made ischaemic with residual flow rates of 0.5 or 0.2 ml/g wet wt/min using a roller pump (Gilson Minipuls 2) with infusion of test solution via a side arm. Global ischaemia was maintained for 30 min following which the hearts were reperfused for 30 minutes with the standard glucose-containing solution. Measurements were made at five minute intervals for assessment of function. Recovery of function was expressed as a percentage of pre-ischaemic function measured at 15 minutes perfusion.

Coronary effluent was collected prior to, during ischaemia and for the first two minutes of reperfusion, for assessment of glucose uptake and lactate washout, by arteriovenous difference. During ischaemia, the effluent was collected over each five minute period. Glucose and lactate were measured by standard spectrophotometric assays [24]. Because of a mean wet heart weight of 1 g,, all values where relevant can be taken to be per g wet wt.

RESULTS

Ischaemic contracture

0.5 ml/g wet wt/min

With a low flow of 0.5 ml/g wet wt/min, time to onset of contracture was delayed in all glucose-containing hearts, despite the presence of lactate (Fig 1). In substrate free and lactate 10 mM hearts, time to onset was significantly reduced, but to a similar degree in both groups. Peak contracture was also very low in all glucose groups (Fig 2), although the glucose 11 mM/lactate 40 mM group showed an increased peak compared to the other glucose groups. Substrate-free and lactate 10 mM hearts had severe contracture, reaching pre-ischaemic developed pressures (Fig 2).

0.2 ml/g wet wt/min

With a low flow of 0.2 ml/g wet wt/min, time to onset of contracture was similar in all groups (Fig 1). Values in the glucose hearts were significantly reduced compared to the 0.5 ml/g wet wt/min groups. In the glucose groups, peak contracture was greater than in the respective 0.5 ml/g wet wt/min hearts, but peak contracture in the substrate-free hearts was less than with 0.5 ml/g wet wt/min (Fig 2). There was a relationship between lactate and peak contracture with increased peak contracture associated with increased lactate concentration in the presence of glucose.

Functional recovery

0.5 ml/g wet wt/min

Functional recovery in the 0.5 ml/g wet wt/min hearts was similar in all glucose groups irrespective of the addition of lactate. Substrate free and lactate 10 mM hearts showed poor recoveries (Fig 3). Coronary flow was highest in glucose 11 mM hearts after 30 min reperfusion, while lactate 10 mM and substrate free hearts had the lowest values (Fig 4)

0.2 ml/g wet wt/min

Hearts subjected to low flow ischaemia of 0.2 ml/g wet wt/min had good recoveries of function only with 11 mM glucose, which were not significantly different from those attained with 0.5 ml/g wet wt/min although more variable. The presence of lactate significantly reduced functional recovery compared to the glucose-only hearts at a flow rate of 0.2 ml/g wet wt/min, and also compared to the respective lactate groups with 0.5 ml/g wet wt/min, although there was no difference between the two lactate groups (Fig 3). All hearts with 0.2 ml/g wet wt/min ischaemia showed a tendency to have lower coronary flow values after 30 min reperfusion compared to 0.5 ml/g wet wt/min hearts, with similar values between groups (Fig 4).

Incidence of ventricular fibrillation

0.5 ml/g wet wt/min

With a low flow of 0.5 ml/g wet wt/min, the only group with a significant incidence of ventricular fibrillation (VF) were the substrate free hearts, with 50% incidence. 1 out of 6 hearts in the lactate 10 mM group had VF (Table 2).

0.2 ml/g wet wt/min

With 0.2 ml/g wet wt/min, all groups had hearts with VF (Table 2). The group with the lowest incidence of VF was glucose 11 mM/lactate 40 mM, but there were no significant differences between groups. The difference in VF between the two flow rates was only significant in the glucose 11 mM/lactate 10 mM hearts, increasing from 0% to 66% ($p<0.03$).

Glucose uptake and lactate washout

0.5 ml/g wet wt/min

Glucose uptake at a low flow of 0.5 ml/g wet wt/min was maintained at levels of 1.25 - 1.75 μmol/g wet wt/min throughout ischaemia in the control group, with a mean of 1.50 ± 0.13 μmol/g wet wt/min over 30 min (Fig 5a; Table 3). Lactate 10 mM suppressed glucose uptake slightly throughout ischaemia, to 1.17 ± 0.24 μmol/g wet wt/min (Table 3). Hearts with glucose 11 mM/lactate 40 mM showed suppressed glucose uptake in the first 15 min ischaemia, following which levels were similar to those attained with glucose 11 mM/lactate 10 mM (Fig 5a). Mean glucose uptake over 30 min was significantly reduced in these hearts compared to glucose only hearts (0.84 ± 0.11 μmol/g wet wt/min; $p<0.05$; Table 3). Lactate washout was sustained at a mean of 1.67 ± 0.18 μmol/g wet wt/min with 11 mM glucose, and increased to 2.01 ± 0.14 and 2.42 ± 0.31 μmol/g wet wt/min for 10 and 40 mM lactate (both with glucose) respectively (Fig 5b, Table 3). In the absence of glucose, hearts perfused with lactate 10 mM showed highly variable lactate washout of 0.89 ± 0.68 μmol/g wet wt/min. Substrate free hearts showed a significant reduction in lactate washout from 0.75 μmol/g wet wt/min at the start of ischaemia, to less than 0.1 μmol/g wet wt/min by the end of ischaemia, with a mean rate of 0.40 ± 0.09 μmol/g wet wt/min, indicative of glycogen utilisation (Fig 5b; Table 3).

On reperfusion, the presence of lactate greatly increased lactate washout compared to glucose only and substrate free hearts (Table 3).

0.2 ml/g wet wt/min

With a low flow of 0.2 ml/g wet wt/min, the presence of lactate reduced mean glucose uptake slightly (Table 3) although significant differences were only found at the end of ischaemia (Fig 6a). Rates of lactate washout were decreased by the presence of lactate compared to glucose only hearts at a flow of 0.2 ml/g wet wt/min (Table 3), while substrate free hearts again showed a drop in lactate washout

to very low levels by the end of ischaemia (Fig 6b). Glucose uptake and lactate washout were significantly reduced in almost all hearts at the lower flow rate compared to those with the higher (Table 3). On reperfusion, glucose only hearts showed a greater lactate washout than glucose hearts with lactate 10 mM, as a result of higher glucose uptake rates. Hearts with lactate 40 mM and glucose showed a high rate of lactate washout on reperfusion, and thus presumably greater interstitial and intracellular accumulation during ischaemia. Lactate washout tended to be higher on reperfusion with the higher flow rate (Table 3), although glucose only hearts showed a similar washout between the two flow rates, possibly indicative of increased anaerobic glycolysis from glucose and glycogen at the lower flow, and increased oxidation of glucose at the higher flow rate.

DISCUSSION

Functional recovery with glucose and lactate

A previous study found that recovery of function was abolished in hearts perfused with 11 mM glucose and 10 mM lactate at a flow rate of 0.5 ml/g wet wt/min. Glucose 11 mM hearts under the same conditions had a recovery of 88 ± 3 % (rate pressure product) [92]. With a low flow of 0.5 ml/g wet wt/min, and glucose 11 mM as sole substrate, we obtained a similar good recovery. However, if lactate 10 mM were added, no significant reduction in functional recovery was found, nor was contracture exacerbated. Even if 40 mM lactate was added to glucose hearts, no deleterious effect was noted. Only when flow was reduced to 0.2 ml/g wet wt/min, was there a deleterious effect associated with lactate provision. At this flow rate, there was no difference in recovery between the 10 mM and 40 mM lactate groups, although the 40 mM hearts showed a greater contracture but a lesser incidence of reperfusion arrhythmias.

We therefore cannot agree with previous findings that the presence of lactate *per se* is deleterious to ischaemic heart i.e. any detrimental effects from the lactate derived from an optimal rate of glycolysis are outweighed by the concomitant ATP production. Only when flow is severely reduced may an excess extracellular lactate accumulate intracellularly, with deleterious effects. We did not find a marked inhibition of glucose uptake in the presence of lactate 10 mM at either flow rate, although 40 mM lactate did reduce glucose uptake significantly in 0.5 ml/g wet wt/min low flow hearts. We did not measure tissue high energy phosphates, but there was no evidence of markedly inhibited glycolysis (to the extent of 60% as found previously [92]) from glucose and glycogen, given the increased lactate washout. The effects of lactate were thus largely dissociated from glycolytic inhibition.

Additional studies in this laboratory using the working rat heart model have also not found any deleterious effect with the addition of lactate to a glucose-containing perfusate with a residual coronary flow of 0.5 ml/g wet wt/min (J. van Rooyen, personal communication). Even if the residual low flow was reduced to 0.2 ml/g wet wt/min, recovery of function after 30 min ischaemia and 30 min reperfusion was 71 % with glucose and lactate 10 mM. Glucose uptake was also not severely affected, with a value of 0.5 μmol/g wet wt/min, which is equivalent to that in non-lactate hearts [543]. ATP levels at the end of ischaemia was well preserved when glucose was present.

Possible discrepancies with the previously published study [92] may arise from the use of larger rats (393 ± 10 g), with a perfusion pressure of 100 mmHg, and a calcium concentration of 1.75 mM, compared to the model used in the present study (see Methods). However, even if these conditions were replicated we could not repeat the observations of an abolished recovery of function with

glucose and lactate with a residual coronary flow of 0.5 ml/g wet wt/min. The lack of recovery previously reported could have been due to the presence of severe arrhythmias which could not be monitored by the system in use (using NMR). However, we could not find any indication of irreversible ventricular fibrillation with glucose present at a flow rate of 0.5 ml/g wet wt/min, despite the addition of lactate.

In the absence of glucose, lactate 10 mM was deleterious compared to glucose 11 mM in hearts with a flow rate of 0.5 ml/g wet wt/min. While we did not look at hearts with lactate only at the lower flow rate, because of the deleterious effects at the higher flow, a recovery of only 7.5% has been recorded with lactate only at a flow rate of 0.2 ml/g wet wt/min in working hearts [543]. The complete absence of substrate was equally disadvantageous, in terms of ischaemic contracture and functional recovery, and even more so in terms of reperfusion arrhythmias (Table 2). Thus lactate only did not confer any additional harm to that found with a complete lack of substrate. Both groups of hearts had continued utilisation of glycogen, as shown by lactate production in these hearts during ischaemia, which resulted in some protection.

Function of lactate in the normoxic heart

Lactate can be utilised efficiently by the normoxic heart as a substrate. Under conditions of increased blood concentration, e.g. exercise, lactate may be a primary substrate of the heart, thereby serving to lower excess blood lactate levels [579]. When lactate is added to a glucose-containing perfusate, it accounts for 90% of oxygen utilised by the heart [101]. Lactate also inhibits fatty acid oxidation, possibly by increasing the $NADH/NAD^+$ ratio as it is converted to pyruvate under oxygenated conditions (see Fig 7). However, lactate stimulates triacylglycerol turnover, with increased glycerol release, but maintained triacylglycerol levels [101], resulting in an active pool of triacylglycerol readily accessibly to hydrolysis.

Lactate-pyruvate conversion is one of the three systems involved in maintaining the $NADH/NAD^+$ ratio. This ratio is kept relatively constant under normal conditions, at about 0.002, by the action of the lactate, α-glycerophosphate (αGP) and malate dehydrogenases. In the presence of oxygen, cytosolic NADH (produced by glycolysis) is transported across the mitochondrial wall by the malate-aspartate shuttle [473], and subsequently undergoes oxidative phosphorylation. The rate of disposal of cytosolic NADH may regulate the maximal rate of glycolysis at the level of GAPDH [261] (see Fig 7). If oxygen tension falls and NADH accumulates in the cytosol, lactate dehydrogenase (LDH - step *c* in Fig 7) ensures that sufficient NAD^+ is regenerated by converting pyruvate to lactate to maintain the rate of conversion of glyceraldehyde 3-phosphate (GAP) to 3-phosphoglycerate (3-PG -step *a* in Fig 7), with the continued formation of glycolytic ATP. The LDH equilibrium is far to the right i.e. to lactate formation. Glucose to lactate has a ΔG (pH 7) = -196 $kJ.mol^{-1}$. (Glucose to oxidative

phosphorylation has ΔG (pH7) = -2872 kJ.mol^{-1}, and this reaction would therefore be favoured when conditions optimal). An alkaline pH, a high NAD^+ content and very low pyruvate levels are required to reverse the relationship i.e. highly oxygenated conditions. The conversion of dihydroxyacetone phosphate (DHAP) to αGP by αGP dehydrogenase also ensures regeneration of NAD^+ (step *d* in Fig 7). Thus both lactate and αGP accumulate in conditions of ischaemia and hypoxia [468] (see Results Ch 1).

Apart from its metabolic effects, lactate also reduces the duration of the action potential [478], possibly by increasing the opening probability of the K_{ATP} channel [249].

Lactate and intracellular pH regulation

Lactate passes through the cell membrane via a lactate$^-$/H^+ co-transporter, which is driven by the gradients of the ions [439]. The co-transporter has a Km of 2.2 mM for lactate, and 200 μM for pyruvate. Thus transport of pyruvate will be preferred to that of lactate, although cytosolic pyruvate concentrations are generally very low. Increased extracellular lactate thus increases intracellular lactate content [361], and also reduces the pHi [102]. If isolated rat myocytes are exposed to an external lactate concentration of 20 mM and pHo of 7.4, the pHi falls rapidly by 0.24 pH units [53]. This drop in pHi recovers by 0.16 pH units. If the pHo is 6.4, the drop in pHi induced by lactate is about 0.70 pH units, with no recovery in pHi. Na^+/H^+ exchange inhibitors abolish the recovery of pHi, or increase acidosis further. The addition of lactate results in a rise in $[Ca^{2+}]i$, attributed largely to a rise in $[H^+]i$ acting on the Na^+/H^+ exchanger [303]. In turn, increased intracellular acidosis should result in lactate efflux if the extracellular pH is higher. Vandenberg et al. found that lactate$^-$/H^+ efflux was the main factor involved in restoration of pH on reperfusion after a brief period (10 min) of ischaemia [545].

In ischaemic hearts, in the absence of coronary flow, intracellular lactate accumulates rapidly from increased anaerobic glycolysis, although the final level is dependent on the initial availability of glycolytic substrate [387]. With increased flow, the rate of removal of lactate should increase, unless extracellular lactate is supplied, in which case the efflux across the membrane should be inhibited with a rise in cytosolic levels. Increased cytosolic levels of lactate are thought to be deleterious to the ischaemic myocardium.

Evidence that lactate is deleterious to hearts subjected to total global ischaemia was provided by Neely and Grotyohann [387], who found that increasing lactate concentration in the perfusate 10 min prior to total global ischaemia was directly correlated with reduced recovery of function on reperfusion. Thus lactate in the tissue was increased prior to the onset of total global ischaemia. This would serve to reduce pHi [102]. Increased intracellular H^+ accumulation brought about by increased extracellular lactate would increase Ca^{2+} entry [303] prior to the onset of ischaemia [53]. Karmazyn [239]

found that Na^+/H^+ exchange inhibitors given with the lactate reversed the effects of 20 min pre-ischaemic lactate perfusion prior to total global ischaemia on the subsequent depression of postischaemic ventricular recovery. This effect would substantiate the concept of increased $[Ca^{2+}]i$ following lactate perfusion, which would be deleterious to the ischaemic heart [406], and separate from a direct effect of lactate in ischaemia.

Contrary to the findings with total global ischaemia and lactate preperfusion, Cross et al. [92] did not find a drop in pHi in glucose+lactate hearts with low flow ischaemia (with protocol as repeated in the present study), and could thus not account for the protective effect of Na^+/H^+ inhibitors by a reduction in $[H^+]i$ accumulation as suggested by Karmazyn [239]. In these hearts, lactate accumulation prior to the onset of ischaemia was limited by the shorter (3 min) pre-ischaemic infusion of lactate, compared to other reports (10 and 20 min - see above). Cross et al. suggested that ATP from glucose prevents $[Na^+]i$ build-up by maintaining the activity of the sarcolemmal Na^+/K^+ ATPase, an effect inhibited by lactate which reduced the rate of glycolysis. By inhibition of glycolysis (and reduced turnover of ATP), the effects of lactate on intracellular pH via the $lactate^-/H^+$ co-transporter were nullified [92].

Lactate and glycolytic inhibition

The major deleterious effect of lactate in ischaemia is thought to be its effects as a feedback inhibitor of glycolysis. However, in terms of glucose uptake, and lactate washout we found no evidence of severe glycolytic inhibition, even with 40 mM lactate in the presence of glucose (only 40% reduction). We therefore cannot substantiate the findings of Cross et al. [92] in terms of inhibited glycolytic flux rates (by 60%), and thus abolished functional recovery in the presence of glucose and lactate at a fairly high residual flow rate of 0.5 ml/g wet wt/min.

Cross et al. explained their observations of reduced glycolysis by the effects of lactate on NADH levels. Mochizuki et al. [361] found that at a flow rate of 6 ml/g wet wt/min (about 40% of control coronary flows i.e. hypoxia), 20 mM lactate with 10 mM glucose inhibited glycolysis by about 60%. 20 mM pyruvate had a similar effect on glycolysis. The maximum inhibitory effect was reached at an intracellular lactate concentration of 25-30 mM. Lactate may inhibit GAPDH directly (20 mM lactate has a 50% inhibitory effect on enzyme activity) and indirectly (NADH found to be a potent inhibitor) [362].

In ischaemia, lactate and αGP accumulate significantly [468] (Results Ch 3), allowing continued production of glycolytic ATP with maintained NAD^+ regeneration, as shown in Fig 7. However, this process is not able to prevent the eventual maximal reduction of NAD^+ to $NADH+H^+$. NADH and H^+ accumulate rapidly in ischaemic tissue, presumably largely because of inhibited mitochondrial oxidation. While normal $NADH/NAD^+$ is fairly low, ischaemic conditions (isolated cells with metabolic inhibition, pH 6.5 and 20 mM lactate) induce maximal conversion of NAD^+ to NADH

within 1 min (maximal amount shown by application of amytal - NADH dehydrogenase inhibitor, and FCCP - uncoupler of oxidative phosphorylation [129]). At a residual flow rate of 0.6 ml/g wet wt/min, the increase in NADH/NAD^+ was slower because of a relatively high residual oxygenation at this flow rate, increasing from 3.2 x 10^{-3} in control conditions to 36.9 x 10^{-3} after 16 min low flow (0.6 ml/g wet wt/min)ischaemia (calculated from lactate:pyruvate ratio which increased from 27:1 to 309:1 [468]). The increased NADH will favour the formation of lactate, but the relative distribution, if the enzyme is in equilibrium, means that NADH will increase. Lactate to pyruvate oxidation may only occur if the H^+ produced from the process are removed, or pyruvate or NADH or both. While the lactate concentration at end of ischaemia in hearts perfused with glucose only at a low flow rate of 0.5 ml/g wet wt/min was 20 mM [92], the conditions mimicked in the single cell preparation could be reached much sooner e.g. pHi falls to about 6.6 by 6 mins [92]. Any further increase in extracellular lactate is thus unlikely to increase NADH further. In addition, the conversion of DHAP to αGP also increases the NADH/NAD^+ ratio. We found that with the addition of insulin, which substantially increased glucose uptake and lactate production, the ratio of lactate to pyruvate, and of DHAP/αGP, were unchanged compared to glucose only hearts with less lactate production (see Results Ch 3). Thus the calculated NADH/NAD^+ was not increased. Thus the addition of lactate in the extracellular medium is unlikely to alter the NADH/NAD^+ ratio significantly in favour of greater NADH formation more than is already present. In addition, hypoxia is associated with an increase in NADH and in lactate from stimulated anaerobic glycolysis [585], which would in turn be expected to inhibit glycolysis at the level of GAPDH (step *a* [362, 468]). However, hypoxia is associated with a greatly increased rate of glycolysis. Thus while overall NADH levels may rise, these are not necessarily associated with inhibited glycolysis as long as there is continued regeneration of NAD^+ by αGP dehydrogenase and LDH. Each NAD(H) molecule can be used over and over again.

The production of ATP outweighs the detrimental effects of lactate, and lactate does not appear to inhibit glycolytic flux at sufficiently high coronary flows, where glycolytic flux is determined by delivery of substrate. The ischaemic heart appears able to tolerate a relatively large extracellular lactate concentration as long as ATP production is high. Geisbuhler and Rovetto [154] found that the addition of 10 and 50 mM lactate does not add to anoxia/reoxygenation damage in isolated myocytes, measured in terms of cell contraction. Lactate had no effect on high energy phosphate levels, nor did a lower pH affect the role of lactate. With a high glucose concentration delivered at a low flow, particularly in the presence of insulin (Results Ch 2), the increased lactate production, as well as accumulation of other deleterious metabolites of glycolysis, such as sugar phosphates [282] may outweigh the benefits of increased glycolytic ATP. In these circumstances, an increased lactate would be deleterious, as shown by the present results obtained with a low flow of 0.2 ml/g wet wt/min.

Lactate accumulation and preconditioning

The possible benefits of reduced lactate accumulation have been highlighted by the phenomenon of preconditioning, whereby one or more brief periods of ischaemia with intermittent reperfusion are beneficial to the heart in terms of reduction in infarct size and increased functional recovery. A depleted glycogen following preconditioning tends to lower end ischaemic tissue lactate values, which was proposed to be a mechanism of preconditioning [224] However, we could not find a correlation between glycogen depletion and improved functional recovery, with or without preconditioning (Results Ch 4, 5) in agreement with previous findings [17, 18, 588]. No correlation between end ischaemic tissue lactate and functional recovery was found, as has also been previously observed [163]. The pre-ischaemic anoxic period used to deplete tissue glycogen [387] has been shown to "precondition" hearts, and the effects of subsequent reduced lactate accumulation [387] may thus be thus secondary, and not involved in any protective effect *per se*. In low flow ischaemia, preconditioning increases glucose uptake and lactate accumulation, but does not result in an increased recovery of function, possibly because the rate of glycolysis, and subsequently of lactate and sugar phosphate accumulation, then outweighs the benefits of the increased ATP production (Results Ch 5). Thus lactate accumulation must not be in excess of a certain threshold rate of glycolytic ATP production, as yet undetermined but likely to be dependent on several other factors, including insulin status, other substrates, health of the heart etc.

Conclusions

Contrary to previous results, we could find no detrimental effect of lactate added to glucose at a low flow of 0.5 ml/g wet wt/min, and at a flow of 0.2 ml/g wet wt/min, recoveries were reduced in the Langendorff hearts, but not in working hearts. This could be attributed to peak contracture in Langendorff hearts which was exacerbated by lactate at a low flow of 0.2 ml/g wet wt/min, but would not have as severe an effect in the working heart, in the absence of a balloon. Thus flow rate has an important effect on the role of lactate, as well as of glucose (Results Ch 1, 2).

Discrepancies with the previous study on low flow ischaemia with glucose and lactate [92] are difficult to resolve. Each study is internally consistent, with reasonably plausible explanations for the observations, resting mainly on differences in glycolytic flux rates. Further work is required to clarify these issues. The best correlate of increased glycolysis and ischaemic injury appears to be accumulation of the sugar phosphates [219, 482] (Results Ch 4, 5) which may impair Ca^{2+} homeostasis [282], rather than lactate. The involvement of sugar phosphates, pHi, Ca^{2+} and glycolytic ATP in determining the eventual outcome from an ischaemic episode still needs to be determined on a cellular level.

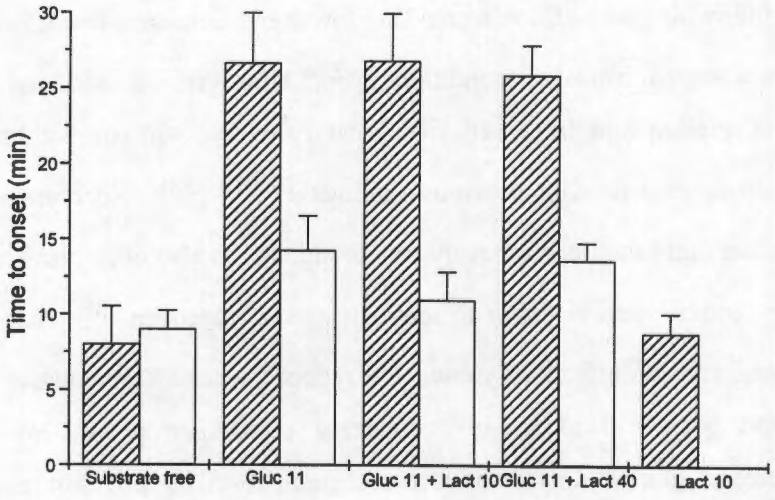


*Fig 1. Time to onset of contracture in min after onset of ischaemia in hearts with a low residual flow (0.5 (shaded bars) or 0.2 (open bars) ml/g wet wt/min) perfused for 3 min prior to and throughout ischaemia with different substrates (see Table 1). * $p<0.05$ vs substrate free; # $p<0.05$ vs 0.5 ml/g wet wt/min*

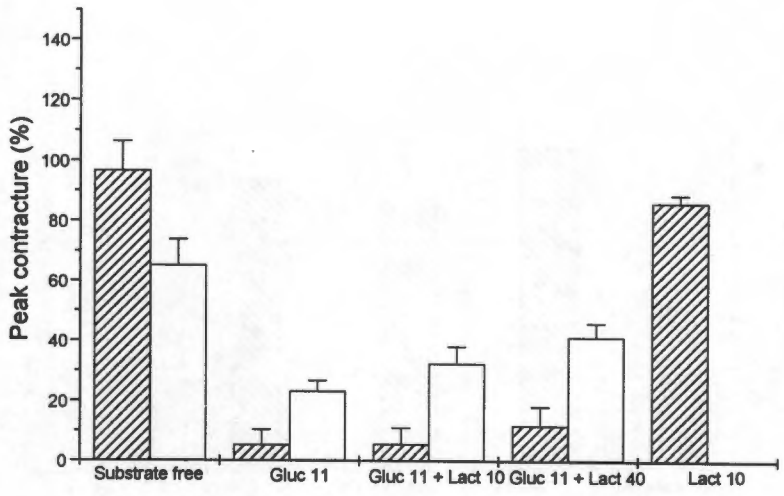


*Fig 2. Peak contracture (% pre-ischaemic developed pressure) in hearts with a low residual flow (0.5 or 0.2 ml/g wet wt/min) perfused for 3 min prior to and throughout ischaemia with different substrates (see Table 1). Legend - see Fig 1. * $p<0.05$ vs substrate free/lactate 10; # $p<0.05$ vs 0.5 ml/g wet wt/min; @ $p<0.05$ vs glucose 11.*

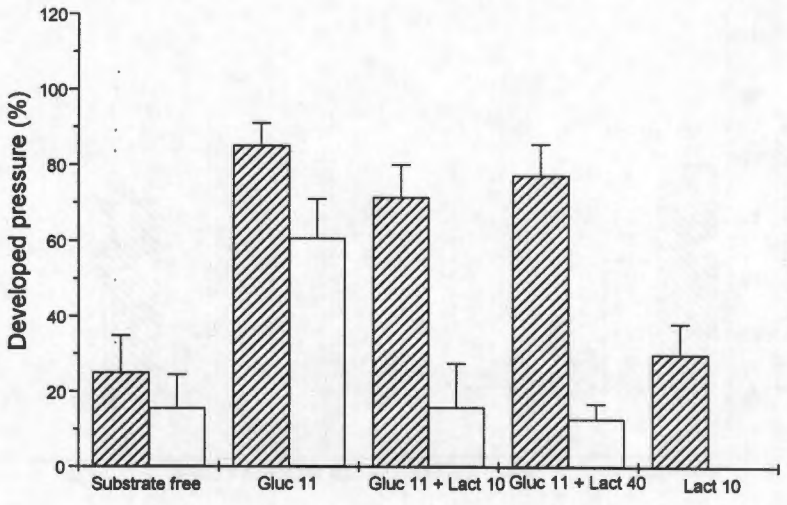


*Fig 3. Recovery of developed pressure (% pre-ischaemic developed pressure) after 30 min reperfusion following 30 min ischaemia with a low residual flow (0.5 or 0.2 ml/g wet wt/min). Hearts were perfused for 3 min prior to and throughout ischaemia with different substrates (see Table 1). All hearts were reperfused with 11 mM glucose. Legend - see Fig 1. * $p<0.05$ vs substrate free; @ $p<0.05$ vs glucose 11*

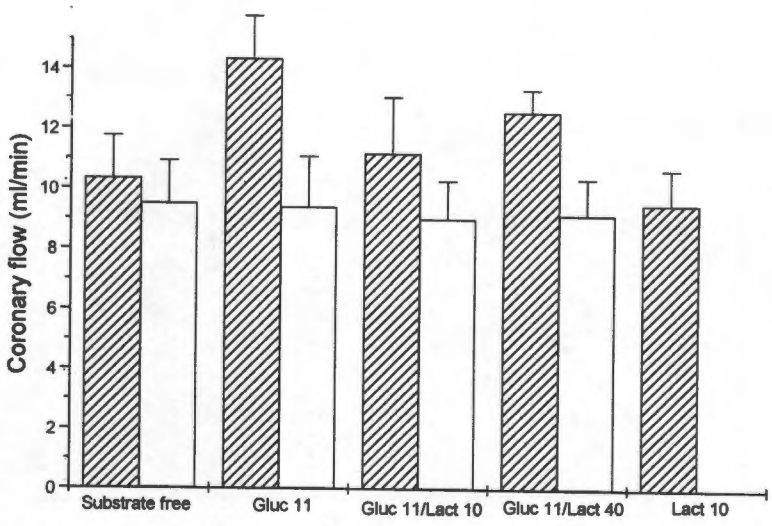


Fig 4. Coronary flow (ml/g wet wt/min) after 30 min reperfusion following 30 min ischaemia with a low residual flow (0.5 or 0.2 ml/g wet wt/min). Hearts were perfused for 3 min prior to and throughout ischaemia with different substrates (see Table 1). All hearts were reperfused with 11 mM glucose. Legend -see Fig 1

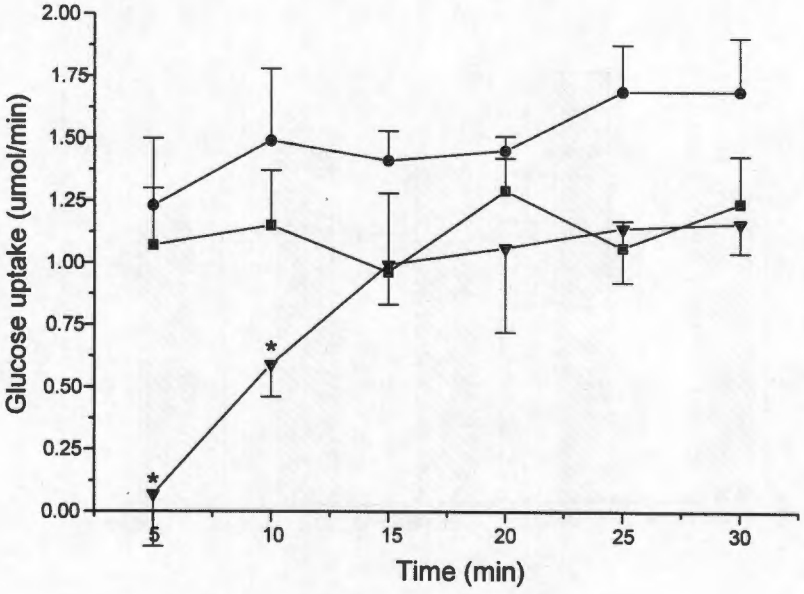


Fig 5a

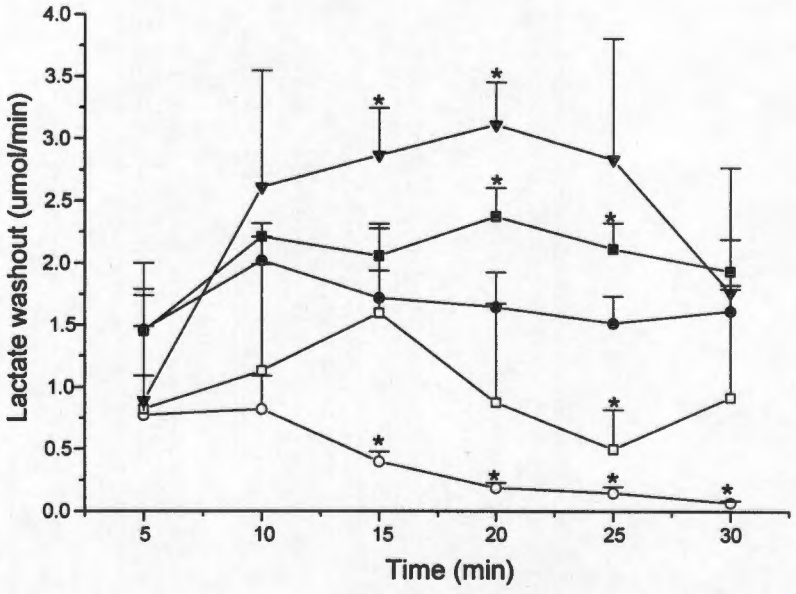


Fig 5b

*Fig 5. Glucose uptake (a) and lactate washout (b) over 30 min ischaemia in hearts perfused with different substrates (see Table 1) for 3 min prior to and throughout ischaemia at a residual flow rate of 0.5 ml/g wet wt/min. Open circles - substrate free; closed circles - glucose 11; closed squares - gluc 11, lact 10; closed triangles - gluc 11, lact 40; open squares - lactate 10. * $p<0.05$ vs glucose 11*

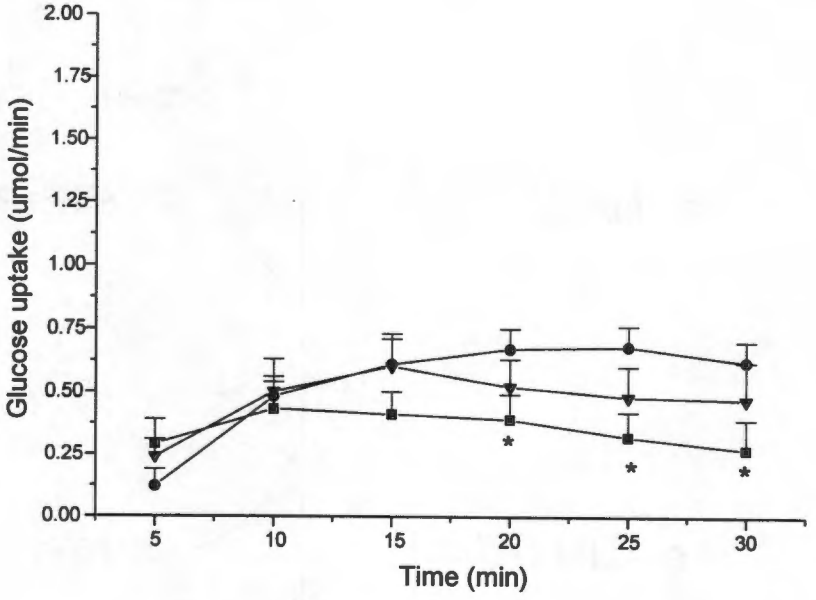


Fig 6a

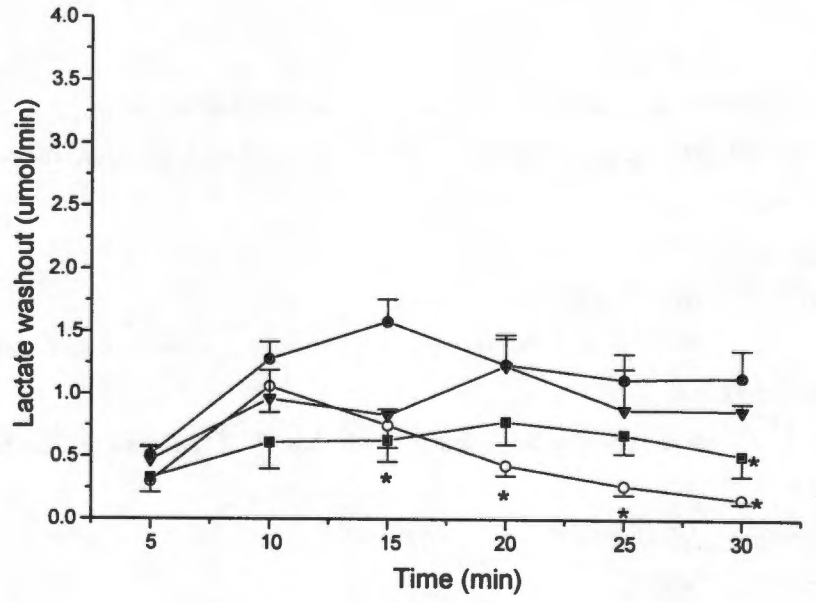


Fig 6b

*Fig 6. Glucose uptake (a) and lactate washout (b) over 30 min ischaemia in hearts perfused with different substrates (see Table 1) for 3 min prior to and throughout ischaemia at a residual flow rate of 0.2 ml/g wet wt/min. Open circles - substrate free; closed circles - glucose 11; closed squares - gluc 11, lact 10; closed triangles - gluc 11, lact 40; open squares - lactate 10. * $p<0.05$ vs glucose 11*

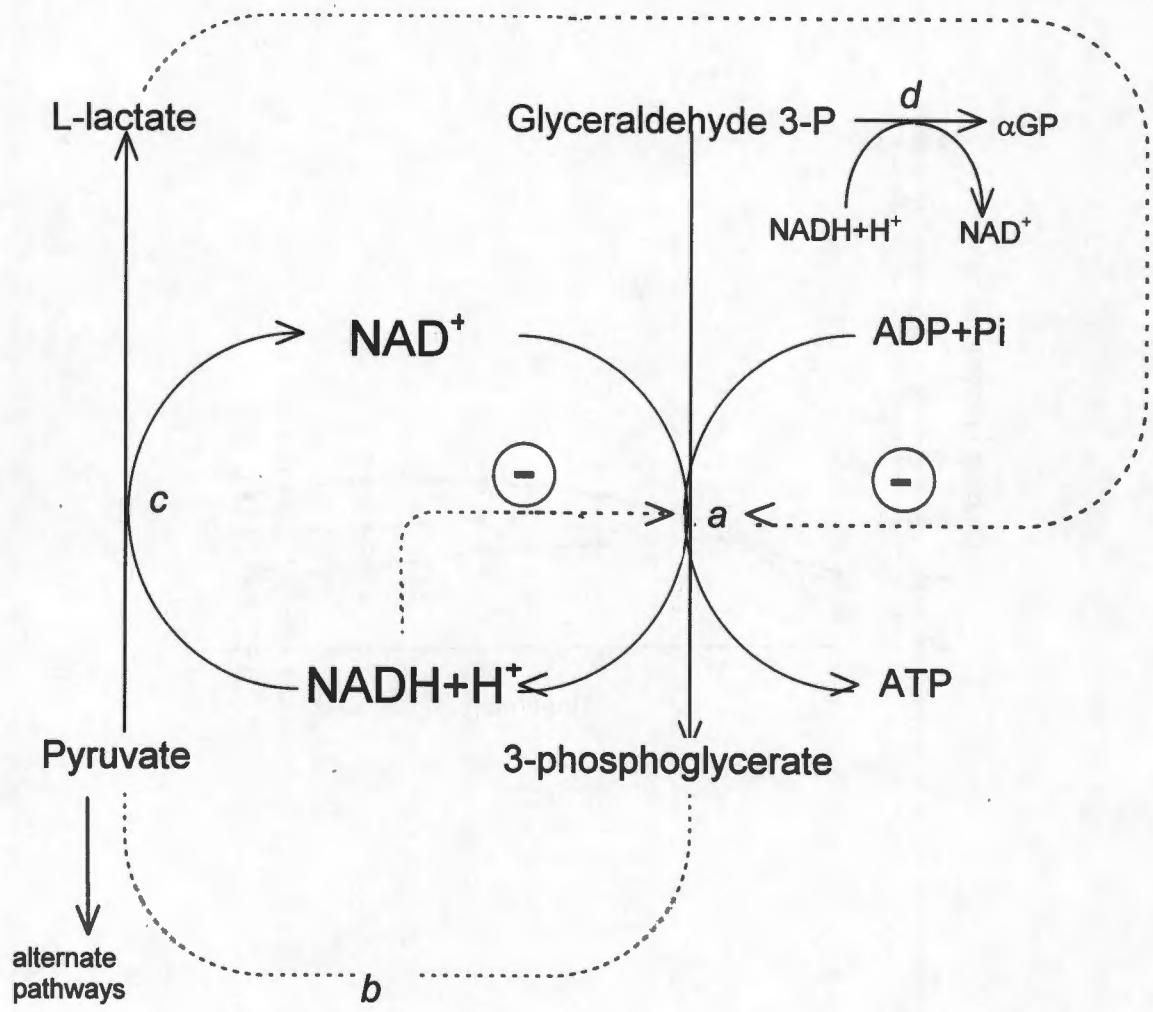


Fig 7. Regulation of NADH/NAD$^+$ by lactate dehydrogenase and regulation of glycolysis.

a - *reaction catalysed by GAPDH, requiring oxidation by NAD$^+$, and by phosphoglycerate kinase which requires ADP and Pi for ATP formation.*

$$Ka' \text{ (pH7, 38°C)} = \frac{[ATP]}{[ADP][Pi]} \frac{[3\text{-PG}]}{[GAP]} \frac{[NAD^+]}{[NADH]} = 62$$

b - *slow reactions to form pyruvate from 3-PG (3 enzymes). Pyruvate can then go to lactate or to further oxidative metabolism (acetyl Co A)*

c - *reaction catalysed by lactate dehydrogenase, requiring reduction by NADH and H$^+$. Lactate formation is highly favoured by LDH.*

$$Kc' \text{ (pH7, 38°c)} = \frac{[lactate]}{[pyruvate]} \frac{[NAD^+]}{[NADH]} = 8.8 \times 10^3$$

d - *reaction catalysed by aglycerophosphate dehydrogenase which converts dihydroxyacetone phosphate (DHAP) to aglycerophosphate (aGP). Glyceraldehyde 3-phosphate is converted to DHAP by triose phosphate isomerase*

Reactions c and d may be coupled by channeling of NADH between enzymes complexes [600] enabling rapid equilibrium of the reactions.

Table 1: Glucose and lactate concentrations of perfusate used 3 min prior to ischaemia and during low flow ischaemia (0.5 or 0.2 ml/g wet wt/min). Prior to change in substrate, and on reperfusion, all hearts were perfused with perfusate containing glucose 11 mM only.

	0.5 ml/g wet wt/min		0.2 ml/g wet wt/min	
	Glucose	Lactate	Glucose	Lactate
Subs free	0 mM	0 mM	0 mM	0 mM
Gluc 11	11 mM	0 mM	11 mM	0 mM
Gluc 11 Lact 10	11 mM	10 mM	11 mM	10 mM
Gluc 11 Lact 40	11 mM	40 mM	11 mM	40 mM
Lact 10	0 mM	10 mM		

Table 2: Incidence of ventricular fibrillation on reperfusion after 30 min low flow ischaemia (0.5 or 0.2 ml/g wet wt/min) with different substrates (see Table 1).

	0.5 ml/g wet wt/min		0.2 ml/g wet wt/min	
	Incidence	%	Incidence	%
Subs free	0/6	0	3/8	37.5
Gluc 11	0/6	0	4/6	66.6 *
Gluc 11 Lact 10	0/9	0	1/7	14.3
Gluc 11 Lact 40	3/6	50	6/6	100
Lact 10	1/6	16.7		

* $p<0.03$

Table 3: Glucose uptake and lactate washout during low flow ischaemia with different substrate (see Table 1), and lactate washout and coronary flow for the first two minutes of reperfusion.

	0.5 ml/g wet wt/min				0.2 ml/g wet wt/min			
	Ischaemia		Reperfusion		Ischaemia		Reperfusion	
	Gluc uptake (µmol/g wet wt/min)	Lact washout (µmol/g wet wt/min)	Lact washout (µmol/2 min)	Coron flow (ml/min)	Gluc uptake (µmol/g wet wt/min)	Lact washout (µmol/g wet wt/min)	Lact washout (µmol /2 min)	Coron flow (ml/ min)
Subs free	-	0.40 ± 0.09	7.81 ± 3.31	9.92 ± 1.51	-	0.50 ± 0.07	5.70 ± 1.23	4.54 ± 0.55
Gluc 11	1.50 ± 0.13	1.67 ± 0.18	13.92 ± 1.36	16.54 ± 1.05	0.53 ± 0.07	1.14 ± 0.12	14.66 ± 3.18	12.29 ± 1.70
Gluc 11 Lact 10	1.17 ± 0.24	2.01 ± 0.14	80.78 ± 2.74	11.58 ± 1.96	0.35 ± 0.10	0.59 ± 0.14	10.68 ± 4.59	6.29 ± 0.95
Gluc 11 Lact 40	0.84 ± 0.11	2.42 ± 0.31	73.46 ± 18.13	13.19 ± 2.41	0.47 ± 0.10	0.89 ± 0.09	37.0 ± 15.90	7.75 ± 1.42
Lact 10	-	0.89 ± 0.68	11.48 ± 3.60	10.67 ± 0.64				

Discussion

A. REGULATION OF GLYCOLYSIS IN CONTROL AND ISCHAEMIC MYOCARDIUM

1) SUMMARY OF FINDINGS OF THESIS, WITH SPECIFIC REFERENCE TO GLUCOSE METABOLISM

This thesis provides evidence for novel views on the control of glycolysis in ischaemia. A general summary of the findings of this thesis suggests that:

i) the rate of glycolysis is a crucial determinant of ischaemic contracture and functional recovery

ii) the rate of glycolysis is determined primarily by the extracellular glucose concentration, the coronary flow, and the endogenous glycogen level

iii) glycolysis is limited by availability of substrate, and not by enzyme inhibition, although enzyme regulation is not excluded as a modifying factor

iv) glucose utilisation in ischaemia is increased relative to that in normal conditions, when the relative glucose extraction is compared. This may be attributed to an ischaemia-induced translocation of glucose transporters to the membrane, or an upregulation of transporters already in the sarcolemma

v) pre-ischaemic glycogen levels determine the time to onset of contracture, and may thus partially modify the recovery of function. However, glucose is more important than glycogen. An excess glycogen may be deleterious because of excess metabolite accumulation

v) preconditioning reduces pre-ischaemic glycogen levels, and thus reduces glycolytic flux in total global ischaemia. Preconditioning also reduces time to onset of contracture, most probably because of the reduction in pre-ischaemic glycogen. However, this glycogen depletion is not related to any beneficial effect, despite a reduction in end products. Glycogen depletion in the absence of preconditioning may be detrimental because of reduced ATP production.

vi) preconditioning with a brief period of ischaemia increases the rate of glucose uptake in sustained low flow ischaemia, possibly following ischaemia-induced translocation of glucose transporters to the membrane. However, with 11 mM glucose as the substrate, this may lead to excess glycolysis, with detrimental effects which may outweigh any benefit associated with preconditioning. Alternatively a low residual flow may abolish the protection found with preconditioning.

The data presented in this thesis therefore show that glucose uptake is reduced in low flow ischaemia but that glucose extraction increases as the coronary flow rate falls. These findings are contrary to the earlier reports from Neely [389, 390] and Rovetto [468, 469] who proposed that glycolysis was inhibited during ischaemia because the rates of uptake, at a residual flow rate of 0.6 ml/min/g wet wt, were lower than in control hearts. This conclusion was substantiated by measurement of glycolytic metabolites, which, on application of the cross-over theorem [68] suggested inhibition at the level of GAPDH. To clarify, the reasons for the differences between the views here proposed and those that are usually accepted, requires a brief review of the principles of metabolic regulation in general,

followed by specific application to glycolysis. The relevance of changes in glucose uptake influencing the extent of recovery of the ischaemic myocardium must also be noted (see Results Ch 1 and 2).

2) PRINCIPLES OF METABOLIC REGULATION

A classical definition of a metabolic pathway is as a series of reactions initiated by a flux-generating step, where the latter is defined as a step which "initiates a flux to which all other participant reactions in a pathway must respond to reach a steady state" [391]. This pathway is subject to regulation by changes in substrate supply, product removal, allosteric regulation, and covalent modification of enzymes [248, 392].

These original principles of metabolic regulation conform to the existence of a single "rate-limiting step" as the major control point in a given pathway. However, metabolic control analysis [231] suggests that control is distributed throughout the component enzymes of a system, with more than one enzyme exerting significant control over flux [231, 391, 446]. The distribution of control can shift with changes in metabolic state [241, 446]. Thus neither control nor regulation of a pathway must be viewed as occurring at a single "rate-limiting step" [393]. These different concepts are illustrated in Fig. A.2.

More recent analysis of metabolic control suggest that "control" and "regulation" of a pathway must be clearly distinguished [51, 523]. A pathway can be controlled by factors including the workload, oxygen availability, substrate availability, coronary flow etc., namely factors which affect the overall availability of substrate, and the requirement for energy provided by the substrate utilisation, with the concept of product removal modifying the pathway. The pathway can be controlled from either end, or from the middle [51]. Alternatively, the pathway can be regulated by factors affecting individual enzyme activity, including levels of ions and cofactors, and signal transduction pathway second messengers [51, 523].

While metabolic control analysis gives useful information, modification of the activity of a specific enzyme is necessary. The recent method of "top-down analysis" (also "bottom-up" [51, 446]) is simpler to apply to a variety of systems including metabolism. This method of analysis selects a section of a pathway, to determine the contribution and importance of this section to control of the whole pathway. Summation of flux control coefficients can be used to derive the overall flux coefficient for a chosen section of the pathway. Using these techniques in an isolated working rat heart (top-down and bottom-up analysis), the control of flux in glucose utilisation is found not to be exerted by a single enzyme, but distributed among several enzymes [241].

This analysis allows determination of the distributive control of a pathway, which invokes the concepts of channelling, and metabolons. Together, the concepts of "channelling" and "metabolons" (see below) confirm distributive control of glycolysis. Additional information regarding metabolic

regulation can be obtained by utilising recombinant DNA technology to alter specific sites of enzymes to determine their individual contribution to metabolism [393].

3) GLUCOSE METABOLISM

a) Integration of metabolism

Glucose utilisation involves a number of major pathways, each of which is modified by, and in turn has a modifying influence on the others. The major pathways include glycolysis, glycogen synthesis/breakdown (Cori cycle), the tricarboxylic acid (TCA) cycle (also called the citric acid or Krebs cycle), and oxidative phosphorylation. Intersecting pathways include mechanisms to reduce the redox potential (the malate-aspartate and the α-glycerophosphate shuttles), anaplerotic pathways to replenish the TCA cycle, and the contribution of a number of amino acids, as well as fatty acid synthesis/breakdown (see Appendix I for discussion of these pathways). These cycles in turn are governed by substrate balance, hormones (insulin, adrenaline, glucagon), ions (Mg^{2+}, Ca^{2+}, H^{+}), and the energy status of the cell (levels of high energy phosphate metabolites and the cytosolic phosphorylation potential). Michaelis Menten relationships and mass action ratios are used to understand and explain points of regulation, but it must be remembered that each pathway is controlled as a whole , not merely at one or two points along the way, and each pathway in turn is dependent on the others [150, 505]. The more modern approaches of "top-down" and "bottom-up" analyses [51, 241, 446] allow better determination of sites of control, and suggest distributive control of glycolysis throughout the pathway, with different sites becoming more important as conditions change.

b) Glucose uptake

i) Facilitated diffusion

Certain sugars penetrate cells at rates several orders of magnitude faster than would be predicted for free diffusion through the lipid bilayer from regions of high concentration to low concentration. Thus sugar transport is mediated by carriers, presumably proteins [59]. Once glucose is taken up into a cell, it is rapidly phosphorylated. This prevents glucose leaving the cell, and ensures a constant gradient for glucose uptake.

The rate of facilitated diffusion is determined by the concentration gradient, the number of pores available in the membrane, and the affinity of the carriers. Formation of a carrier-substrate complex similar in concept to an enzyme-substrate complex is assumed. This concept is consistent with the observation that the carrier exhibits Michaelis Menten kinetics, in which the number of interactions with a carrier or enzyme reaches a maximum when the carrier or enzyme molecules are all occupied by substrate molecules. The relationship between sugar flux and sugar concentration is linear only at

low sugar concentrations, and can be seen to saturate at higher concentrations. Such Michaelis-Menten kinetics are characterised by two constants: Km and Vmax.

$$V = Vmax.[S]/(Km+[S])$$

V = rate of reaction, [S]= substrate concentration. Vmax (the maximum rate of transport) is related to both the number of transporters and the translocational ability of the individual transfer proteins. Km ([S] where V is 1/2 Vmax) is an indirect measurement of the affinity of the transporter for the substrate - the lower the Km the higher the affinity.

ii) Glucose transporters

Two types of facilitated glucose uptake mechanisms are found. The Na^+-dependent co-transport of glucose, which is driven by the Na^+ gradient, does not occur in heart cells [430]. In the absence of insulin, most tissues take up glucose by the facilitative transporters, designated GLUT. Up to 6 isoforms of these transporters are found, and are expressed according to the requirements of the individual tissue for glucose [370, 430]. GLUT1 is found in the cell membrane in tissues which do not respond to insulin, and are particularly abundant in red blood cells as well as in myocytes. The insulin-sensitive glucose transporter is designated GLUT4 [217] and these are distributed between vesicles in the cytosolic pool, and the plasma membrane. These transporters are specific for glucose, and glucose analogues [260], and can be inhibited by cytochalasin B [260] which is used in assays for determination of GLUT4.

Insulin acutely stimulates glucose transport in muscle and fat by recruiting GLUT4 to the plasma membrane [260, 294]. About 40% of GLUT4 transporters are translocated to the membrane, compared to a normal level of 1%, with a 10-40 fold increase in glucose transport following insulin stimulation. GLUT1 translocation may also occur with insulin stimulation [127]. A recent finding using a transgenic mouse model is that ablation of the GLUT4 gene results in cardiac hypertrophy [243], suggesting that this mechanism of metabolic regulation is an important determinant of cell growth.

GLUT1 has a low affinity (apparent Km = 26 mM [395], although this may be lower (5-10 mM) in different cell lines [216]), but a high capacity for glucose (apparent Vmax = 3.5 nmol/min/cell) while GLUT4 has a low capacity (0.7 nmol/min/cell) and high affinity (4.3 mM) [395]. However, the relative numbers of transporters in the cell indicate that GLUT4 is largely responsible for insulin-stimulated glucose uptake, with a far greater efficiency [395].

iii) Regulation of glucose uptake

The rate of glucose uptake is dependent on the metabolic requirement of the cell, the availability of the different substrates, and the number and activity of transporters in the membrane. Glucose utilisation is regulated by the dietary state, oxygen availability, and hormones (insulin, glucagon, catecholamines, thyroid) [367, 523]. Glucose uptake is increased with increased work rate [385] or

exercise [164] in direct response to increased energy requirements. The majority of glucose is oxidised. An increased glucose concentration in a perfused rat heart increases glucose uptake sharply in the range of 1.25-5 mM glucose, and levels off above 11 mM [415].

A fall in metabolic rate has the opposite effect. Fatty acids compete with glucose as the substrate of choice [385], and limit entry of glucose when present in high concentrations - the so-called "glucose-sparing" effect [450]. *In vivo*, glucose is directed to the liver for storage as glycogen when blood free fatty acids are elevated. Insulin enhances glucose uptake and lowers free fatty acid level in the blood [451].

Insulin is the major regulator of glucose uptake, and directs glucose towards glycogen synthesis [523]. Glucose uptake is increased if glycogen is depleted in the normally perfused heart [206]. Adenosine is also an important regulator of glucose utilisation, although its effects are controversial (see Ch I). Adenosine may specifically enhance insulin-stimulated glucose uptake, an effect inhibited by isoprenaline [548]. cAMP stimulates glucose uptake following β adrenergic stimulation [244, 454] by increased translocation of glucose transporters [454]. cGMP may also stimulate glucose transport, although the mechanism is unclear [494]. Hypoxia and anoxia are potent stimulators of glucose transporter translocation [578]. Ischaemia may also induce GLUT4 translocation [517]. In transgenic mice, increased expression of GLUT1 increases glucose uptake and glycogen storage, implying that glucose transport is the rate-limiting step of glucose utilisation.

While most factors regulating glucose uptake act via GLUT4 translocation to the membrane, GLUT4 carriers in the membrane must also be correctly orientated to ensure uptake. Adenosine and isoprenaline do not appear to affect the subcellular distribution of GLUT4, but rather their function within the membrane [548]. Other factors may also modulate glucose uptake by affecting orientation of transporters in the membrane, such that glucose uptake may be increased without any measurable change in sarcolemmal GLUT4 density.

iv) Insulin

Insulin increases the rate of synthesis of glycogen, fatty acids and proteins, promotes glucose entry and stimulates glycolysis, and inhibits glycogen and fat utilisation. Insulin binds to membrane receptors stimulating an intrinsic tyrosine kinase activity, with autophosphorylation of the receptor [477]. The activated receptors phosphorylate the insulin receptor substrate 1 (IRS-1) [477] which binds with Src homology 2 proteins (SH2-phosphotyrosine binding sites). SH2 proteins include phosphoinositide 3-kinase (PI 3-kinase), Ras GTPase-activating protein, phospholipase C and others involved in the mitogenic role of insulin [379]. PI 3-kinase phosphorylates phosphoinositides, and is involved in growth factor stimulation. In addition, PI 3-kinase may mediate GLUT4 translocation to the membrane [379] and increase glycogen synthesis. The insulin receptor in turn can be

phosphorylated by protein kinase Cα [317]. How this affects the insulin receptor is not clear, but implies regulation of insulin activity by mechanisms involved in calcium regulation (IP3 pathway).

c) Glucose utilisation

i) Glycogen

Glycogen is the storage form of glucose, and as such contributes significantly to glycolysis (see Appendix I). Glycogen synthesis is stimulated by insulin, and an increased glucose or G6P, while glycogenolysis is strongly governed by cAMP, energy requirements and glucose. There are two distinct pathways for glycogen synthesis and breakdown, each regulated by hormones which stimulate one pathway, while inhibiting the other (see Appendix I for full pathways). Glycogen synthesis and utilisation is thought to follow the "last on, first off" principle, whereby the last carbon molecule to be attached is the first to be cleaved off, although this has recently been disputed [162]. Production of glucose 1-phosphate (G1P) from glycogen occurs without the expenditure of an ATP molecule, and prevents diffusion out of the cell. The complete oxidation of G1P (or G6P) yields about 37 ATP, while storage consumes slightly more than one ATP. The equilibrium favours glycogen breakdown, thus the energy yield is very efficient, about 97%.

The utilisation of glycogen can be separated from that of glucose, on the basis of cellular localisation of glycogen particles (see Ch II) which contain the enzymes for glycogen metabolism [10, 128], and form so-called "metabolons" [505].

More recently, a new acid-precipitable "form" of glycogen, called proglycogen, was described [322]. This 400 kDa molecule has a high protein content (10% vs. 0.35% in macromolecular form) accounting for its response to acid. It constitutes about 3% - 50% of total glycogen, depending on the tissue type (heart muscle - as much as 50% [10]). Proglycogen acts as an efficient receptor of glucose residues from UDP-glucose and may be an intermediate in the synthesis and degradation of glycogen [10, 322]. Glycogenin, a self-glycosylating protein, is thought to be the primer, or backbone of glycogen synthesis, which then becomes part of the proglycogen and eventually of the macroglycogen molecule [10]. These three forms are important in the regulation of glycogen synthesis and degradation.

Gross measurement of glycogen, as used in this thesis (see Methods) usually detects both forms. Acid-extractable glycogen was found to reflect changes in ischaemia more closely, representing a subfraction of glycogen more responsive to degradation i.e. macromolecular glycogen. Glycogen in perchloric precipitate remained unchanged during ischaemia, i.e. proglycogen [40, 103]. An estimated 20% of initial glycogen stores may remain in the tissue, consisting mainly of proglycogen and core macromolecular glycogen [40].

ii) The glycolytic pathway

The glycolytic pathway is shown in Fig A.1. G6P is the entry point of both glucose and glycogen to glycolysis. Glucose is phosphorylated by hexokinase (HK) with the hydrolysis of ATP, an essentially irreversible reaction. An additional ATP is consumed, but 4 ATP are produced if glycolysis goes through to pyruvate, together with 2 $NADH+H^+$. The net ATP production is 2 ATP. Pyruvate can then follow a number of options (see Appendix I), which determines the total amount of ATP derived from a glucose molecule. In hypoxia or ischaemia, lactate is formed from pyruvate, to regenerate NAD^+ and allow continued glycolysis If glycogen is broken down, the net production is 3 ATP, as a molecule of Pi is utilised to produce G6P, rather than ATP.

iii) Levels of regulation of glycolysis

Several points along the glycolytic pathway are subject to regulation. These include glucose transport and glycogenolysis (discussed above), and reactions catalysed by HK, PFK, GAPDH, and PK. In addition, the activity of pyruvate dehydrogenase (PDH), which converts pyruvate to acetyl Co A, is tightly controlled by a number of factors (see Appendix I).

Hexokinase is stimulated by an increased glucose, and inhibited by G6P, which builds up when PFK is blocked resulting in increased F6P. If HK is inhibited, the glucose can pass out of the cell again, or if the extracellular concentration is high, glucose can accumulate intracellularly. If G6P accumulates, glycogen can be synthesised. The role of G6P in determining the activity of HK plays an important role in determining glucose utilisation in the muscle. Recent studies [241, 335] have shown that in the presence of physiological concentrations of glucose and insulin i.e. adequate supply of substrate, the rate-limiting step in glucose utilisation is phosphorylation by hexokinase. Glucose transport is not limited under these conditions. As extracellular glucose increases, intracellular glucose 6-phosphate (G6P) reaches a plateau, which does not change with glucose concentrations greater than 2 mM. The estimated intracellular glucose at this point is about 300 μM. Hexokinase is then saturated with glucose, limiting glycolysis. The increased G6P stimulates glycogen synthesis. Regulation of glycolysis at steps below phosphoglucoisomerase is limited to less than 25% under these conditions.

Phosphofructokinase is inhibited allosterically by a high ATP content, an effect enhanced by citrate (from the TCA cycle) and reversed by cAMP. The reaction catalysed by this enzyme is virtually irreversible, thus this becomes an important step in the regulation of glycolysis [541] (see Fig A.2). The rate of glucose breakdown is signalled by the need for ATP as determined by the ATP/AMP ratio. The reaction catalysed by adenylate kinase

$$ATP + AMP \leftrightarrow ADP$$

amplifies the signal. If ATP falls by 15%, ADP levels increase 2-fold, with a greater than 5-fold increase in AMP. A relatively small decrease in total tissue ATP markedly stimulates glycolysis (and glycogenolysis). PFK is also inhibited by an increased $[H^+]i$ [541], which may prevent excessive lactate and H^+ accumulation. The $NADH/NAD^+$ ratio is also an important determinant of activity where an

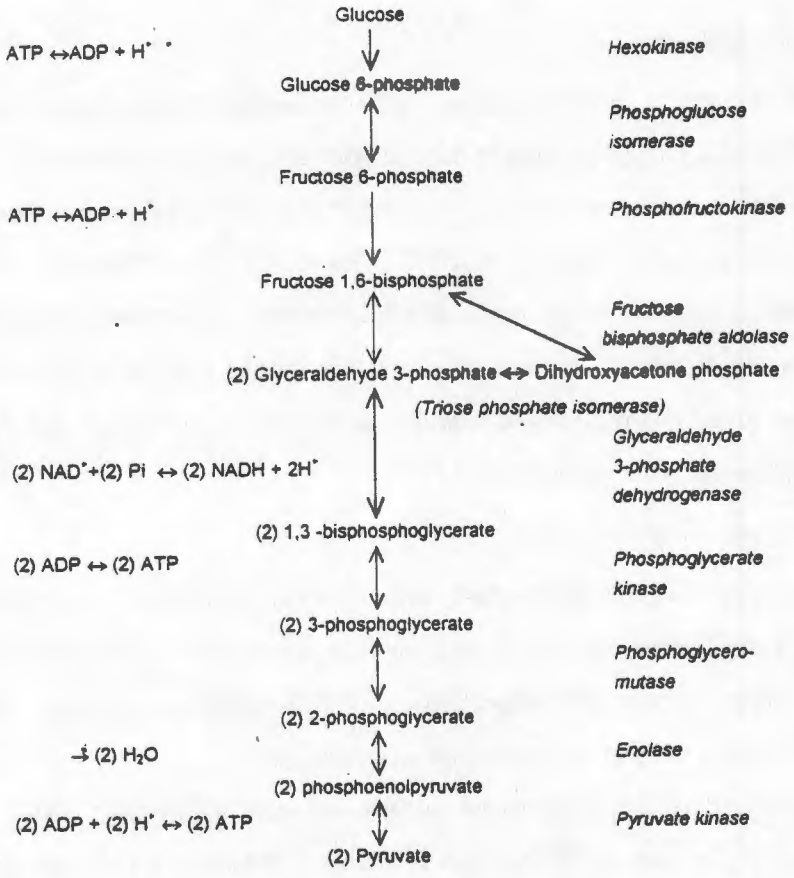


Fig A.1. The glycolytic pathway. HK phosphorylates glucose after entering the cell. G6P then undergoes a conformational change to fructose 6-phosphate (F6P), which is modulated by phosphoglucose isomerase. This reaction is fully reversible, with concentrations of G6P generally 10-fold higher than F6P. F6P is phosphorylated to fructose 1,6-bisphosphate (FDP) by phosphofructokinase (PFK), with an additional hydrolysis of ATP. The utilisation of glucose has thus consumed two ATP molecules by this stage, whereas glycogenolysis has consumed only one ATP per glucose residue, and a Pi. Because this reaction uses the energy of ATP hydrolysis, the equilibrium favours the formation of FDP.

The above compounds are all hexose sugars. However, with 2 phosphate groups attached, the 6-C chain can be symmetrically broken down to two phosphorylated 3-C chains. Two distinct molecules are formed - glyceraldehyde 3-phosphate (GAP), and dihydroxyacetone phosphate (DHAP). These molecules are interconverted by triose phosphate isomerase. DHAP is either converted to GAP or to α–glycerophosphate (αGP). To continue glycolysis, GAP is simultaneously oxidised and phosphorylated to 1,3-bisphosphoglycerate by glyceraldehyde 3-phosphate dehydrogenase (GAPDH), with the conversion of NAD^+ to $NADH+H^+$, and the consumption of an additional Pi molecule per 3-carbon. One phosphate group on each 3-C molecule is subsequently cleaved off to form ATP, when 1,3-bisphosphoglycerate is converted to 3-phosphoglycerate (3PG) by phosphoglycerate kinase. 3PG undergoes a conformational change to 2-phosphoglycerate, which is dehydrated with the formation of an enol group, to form phosphoenolpyruvate (PEP). The high phosphoryl-transfer potential of PEP allows the transfer of the remaining high energy phosphate group to $ADP+H^+$, with the end products of pyruvate and ATP. This reaction, catalysed by pyruvate kinase (PK), is virtually irreversible. The net ATP production from the breakdown of glucose to pyruvate is 2 ATP; from glycogen, 3 ATP are produced.

increase in NADH inhibits the enzyme [362]. Mechanisms which lower NADH are crucial for the regulation of glycolysis (see Appendix I). cAMP stimulates PFK, possibly by stimulating F2,6-P_2 formation. This compound may be the most important regulator of the enzyme [208, 301].

F2,6-P_2 is formed from F6P by hydrolysis of ATP, catalysed by an enzyme called PFK-2 (to distinguish from PFK-1) [208]. This reaction is stimulated by F6P. F2,6-P_2 is a potent stimulator of PFK, with a feed forward effect [301]. The reconversion of F2,6-P_2 to F6P releases a Pi molecule, allowing continued glycolysis with F6P (See Appendix I).

Glyceraldehyde 3-phosphate dehydrogenase is the next major regulatory step, especially in ischaemia [468]; otherwise the control is largely attributed to PFK (although this has been disputed - see HK). GAPDH is regulated mainly by the NADH/NAD^+ ratio (inhibited by an accumulation of NADH), and lactate [361, 362]. Again, mechanisms to restore NAD^+ levels are important. The product of the reaction, 1,3-bisphosphoglycerate, is also a potent inhibitor of the enzyme. Breakdown products of ATP (i.e. ADP, AMP and Pi) may stimulate the enzyme.

Pyruvate kinase may also be regulated, with inhibition by ATP, and stimulation by FDP. However, the importance of regulation of this enzyme may be outweighed by the other glycolytic enzymes, as well as pyruvate dehydrogenase (see Appendix I).

iv) Distributive control of glycolysis in the normal heart

There is some controversy over whether the control of glycolysis exists at certain defined points, as discussed above, or whether control is distributed along the entire length of the pathway [505]. Under conditions of ischaemia, the level of control was thought to be shifted from PFK to GAPDH [468]. However, more recent concepts and analysis, as well as the present work, suggest that under control conditions with sufficient substrate, the rate of glycolysis is largely determined by the rate of glucose uptake [241] (see Fig A.2).

"Top-down" and "bottom-up" analysis of glucose utilisation in an isolated working rat heart established control points including glucose transport, hexokinase, enolase and pyruvate kinase [241]. The relative importance of each of the control steps was found to be dependent on the conditions, including the presence of lactate, pyruvate, and/or glucose. However, despite slight differences with different substrates, all enzymes below phosphoglucoisomerase, including PFK, together contributed less than 25% to glycolytic regulation under any condition (although the branch points - fructose 6-phosphate, glyceraldehyde 3-phosphate, hexose monophosphate pathway - see Appendix I - were not considered in this analysis). In the normoxic working rat heart with sufficient external substrate and insulin, glucose transport is not the main rate-limiting step, but rather that transport combines with hexokinase to control glucose utilisation [241]. Glucose transport and phosphorylation exerted 58% of the control of glycolysis. Additionally, increased expression of GLUT1 transporters in transgenic mice increased

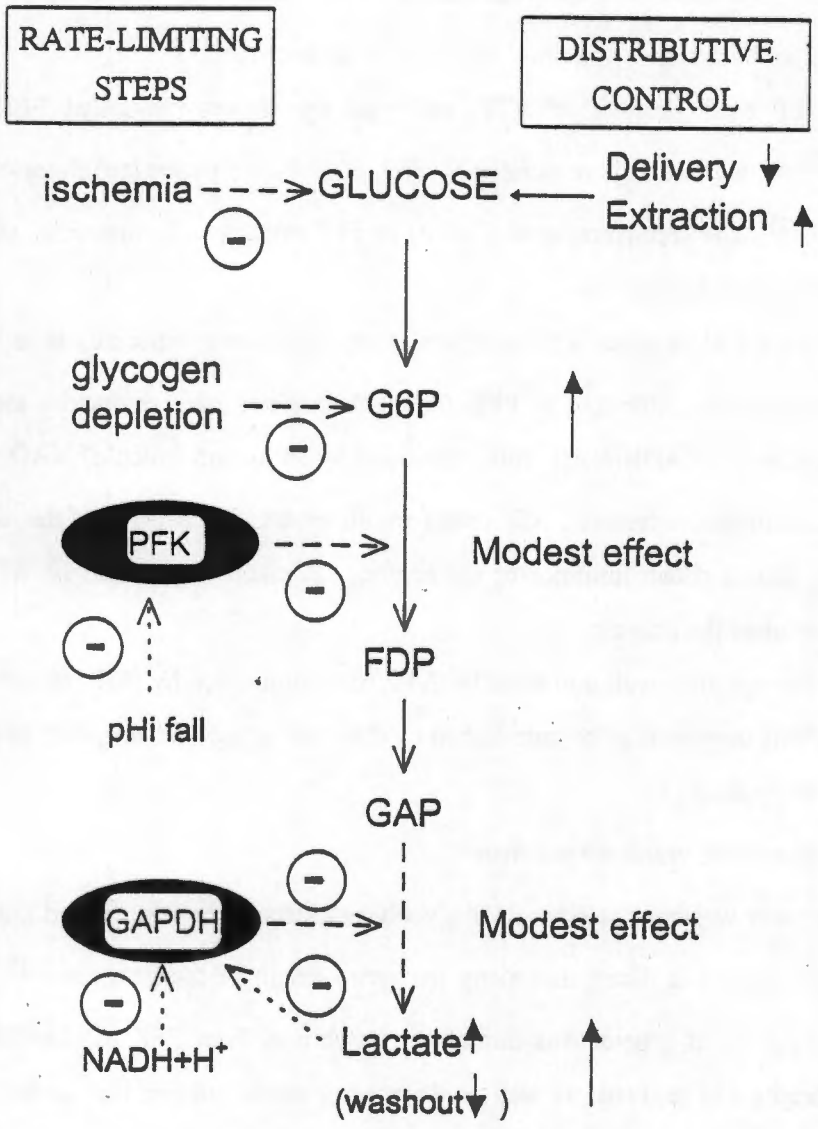


Fig A.2. Rate limiting steps regulating the rate of glycolysis in ischaemia vs the concept of distributive control, where the supply of substrate is the major determinant of the subsequent rate of product (lactate) accumulation (see text for further discussion of concepts)

glycogen deposition and glycolysis in skeletal muscle [458], suggesting that glucose transport is rate-limiting under conditions when intracellular utilisation of glucose was optimal.

Glycolysis (as well as several other metabolic pathways including glycogen metabolism) should not be viewed as a number of single entities or enzyme reactions acting together, but rather as a cohesive unit, or "metabolon", whereby all the glycolytic enzymes are grouped together, by protein-protein, protein-membrane, and physico-chemical interactions [505, 506] .The concept of "channelling", whereby enzymes are spatially associated to allow efficient transfer of product from one enzyme reaction to the next as substrate [576], also substantiates this argument.

These concepts also suggest that glycolysis is controlled throughout its "length"; such that while the activity of each enzyme contributes to regulation of the pathway to a varying extent, the pathway as a whole is ultimately driven by the flux-generating step i.e. substrate (glucose or glycogen) provision. When a metabolic pathway is inhibited or stimulated, the activities of all the enzymes of the pathway are co-ordinately increased or decreased. Thus glycolysis may be viewed as analogous to a car's engine. When the car runs out of petrol, the engine will not work. This does not mean that the engine is broken - if petrol is resupplied, the car will run. However, the maximum speed attained will be the same whether the tank is full or only half-full.

4) Reappraisal of glucose uptake and utilisation in ischaemia

Given that control of glucose utilisation may rest mainly with provision of substrate, the previous assumptions regarding glycolysis in ischaemia must be reassessed. The evidence presented in this thesis suggest that glycolytic flux is determined primarily by the availability of substrate, determined by substrate concentration and coronary flow. The subsequent modification of rates of glycolysis by enzyme regulation appears secondary to substrate supply.

i) Enzyme inhibition and glucose transport

The concept of enzyme inhibition at specific sites as the major modulator of the glycolytic flux rate in ischaemia is flawed in several respects. Firstly, glucose extraction is increased in ischaemia (see Results Ch 3 for elaboration of this point). Secondly, we could find no evidence of GAPDH inhibition by analysis of metabolite accumulation (see Results Ch 3 for data and further discussion). Thirdly, limiting control of glycolysis to only two steps, specifically PFK and GAPDH, has come to be recognised as a simplification, and possibly erroneous [51, 241, 505]. Glycolysis is controlled along the pathway, with distributive control affected by the metabolic state (see above). That this concept can be extended to glycolysis in ischaemia needs to be confirmed, but is supported by the present findings.

Conceptually, if the number of glucose transporters in the membrane remains constant, the external substrate concentration should become "rate-limiting", until saturating concentrations are reached

(around 11 mM). If the number of transporters can be increased by a stimulus, such as insulin or ischaemia [472, 517], then a higher rate of glycolysis can be reached, again levelling off at saturating glucose concentrations independent of the status of the heart (normoxic or ischaemic). Once the intracellular glucose concentration exceeds 2 mM (e.g. when insulin and sufficient glucose are present), the rate of glycolysis may be controlled by hexokinase, with some regulation by other enzymes including PFK and PK (see above). This substrate-dependent regulation of glycolysis appears independent of other factors when the demand for glycolysis is high e.g. hypoxia, ischaemia, no alternate substrates. When oxygen saturation is high, or other substrates are present, factors such as citrate levels, work rate, ATP content etc. may be more important in regulating the rate of glycolysis.
In ischaemia, the coronary flow rate and glucose concentration are fixed. However, the heart appears able to increase the extraction of glucose from that available to it, possibly by an upregulation of glucose transporters in the sarcolemma. The protective effect of glucose provision can thus be enhanced by intrinsic mechanisms to increase glycolytic flux, despite any enzyme inhibition.

ii) Lactate production *in vivo*

Lactate was thought to inhibit GAPDH, both directly, and by increasing NADH and H^+ levels, which inhibit the reduction of NAD^+. These effects were thought to result in end-product inhibition of glycolysis. However, considerable lactate accumulates in ischaemic tissue, despite a supposed inhibition of glycolysis. *In vivo*, lactate accumulates inversely with a reduction in coronary flow [175], and increases in a linear fashion in the ischaemic zone over 120 minutes [403]. Glycolytic substrate under these conditions can only be derived from residual glucose delivery by collateral vessels, and from tissue glycogen. Once the substrate has been utilised, glycolysis should cease.
If glycolysis were inhibited, both glucose and glycogen utilisation should be reduced. However, if glucose is available in limited amounts or is absent, glycogen breakdown is increased (Results Ch 1), resulting in the large amount of lactate observed in the tissue after 16 minutes ischaemia [468]. In addition, α-glycerophosphate, another product of glycolysis accumulates markedly in ischaemic tissue [403, 468] (Results Ch 3). Continued metabolite accumulation indicates that there is continued activity of glycolytic enzymes throughout ischaemia. Otherwise early production of lactate etc. would lead to later inhibition, and a slowing in the rate of glucose utilisation with time. Apstein et al. demonstrated this concept at a low residual flow with a high glucose (28 mM) and insulin able to sustain glycolytic flux i.e. lactate production over 3 hrs ischaemia. Alternatively, with a concentration of 5.5 mM glucose, lactate washout rates declined substantially [14], presumably because of the inability of the low glucose concentration to sustain glucose uptake (see Results Ch 2 3).
The glycolytic pathway thus continues to produce lactate for a period well past that which would be expected if glycolysis were limited by enzyme inhibition. We have also found that addition of 10 mM lactate to glucose-containing perfusate infused at a flow rate of 0.5 ml/g/min does not affect glucose

uptake or recovery. Only if the lactate concentration is increased to 40 mM, or the flow rate is reduced, is some effect seen on glucose uptake and recovery after 30 min ischaemia (see Results Ch 6). These findings further confirm the concept that glycolysis is not inhibited in ischaemia.

iii) Glycogen

It has been suggested that glycolysis is inhibited, because glycogen breakdown occurs only in the first period of ischaemia, and some residual glycogen is left in the tissue [225]. In addition, increased G6P levels in ischaemia may be said to show inhibition; however, increased glycogen breakdown may also result in an excess of G6P which would saturate phosphoglucoisomerase. While the rate of glycolysis is certainly limited by substrate availability, and glycogen may be the sole source of glycolytic substrate, the fact that some glycogen remains in the ischaemic tissue is not evidence for cessation of glycolysis, but rather may indicate the presence of residual core macromolecular glycogen, or proglycogen, which is more resistant to ischaemia [103]. In addition, the breakdown of glycogen is complex, requiring the actions of glycogen phosphorylase, and its activators. This enzyme may be modulated in severe ischaemia, as phosphorylation is required for activation (see Appendix I). The activity of this enzyme is inhibited in preconditioned hearts [575].

In zero flow ischaemia, lactate accumulation occurs as glycogen is broken down. The increased lactate would be expected to inhibit glycolysis. However, there is less tissue glycogen remaining after 30 min total global ischaemia than after subtotal ischaemia (Results Ch 1, 3). Thus the increased lactate is not associated with decreased glycogenolysis. In low flow ischaemia, the presence of glucose inhibits glycogen breakdown, while lactate build-up is reduced by washout.

5) OPTIMAL RATES OF GLYCOLYTIC FLUX IN THE ISCHAEMIC MYOCARDIUM

While the kinetics of glucose uptake indicate that the ischaemic heart can increase extraction of glucose, the ultimate determinant of viability is the rate of glycolytic ATP production [424]. We have found that functional recovery of the heart over a range of low flows is optimal with a glucose concentration of 11 mM (Results Ch 1,2), which results in sufficient ATP production to reduce ischaemic injury, but does not lead to excess glycolytic metabolite accumulation. Obviously, if the excess metabolites could be removed, this would increase the benefit associated with increased glycolysis. However, the only candidate mechanism apparent at this point is an increase in the residual flow rate.

Reducing the glucose concentration results in suboptimal delivery of glucose, and thus suboptimal ATP production; equally, increasing glucose entry into the cell by increasing the glucose concentration and/or adding insulin can impair recovery relative to the 11 mM hearts, because of excess metabolite accumulation, specifically sugar phosphates (see Results Ch 1,2, 4, 5). We do not find that glucose uptake relative to delivery is decreased, and thus that glycolysis is inhibited in

ischaemia. Rather the rate of glycolysis is limited in ischaemia by substrate availability. Alternatively, an ischaemic heart (with viable tissue) appears able to upregulate glucose uptake relative to control hearts. If glucose uptake is increased above a certain threshold level, e.g. by the factors above, or by preconditioning (see Results Ch 5), the glycolytic metabolites may again accumulate in excess, with detrimental effects. There is a complex interaction between glucose uptake, glycolytic ATP production and glycolytic metabolite accumulation.

6) CONCLUSIONS

In conclusion, while a build-up of metabolites does occur in ischaemia, and may have some inhibitory effect on glycolysis, accumulation of metabolites may also indicate upregulation of glycolysis, rather than inhibition. While an overload of the glycolytic cascade may occur, with a subsequent bottleneck effect, the overall flux cannot be said to be limited. This applies especially to ischaemia with any degree of residual flow, with continued delivery of substrate, as well as removal of inhibitory metabolites, ensuring continued glycolysis. Only in the extreme situation of total global ischaemia, is glycolysis fully limited by a lack of substrate. In low flow ischaemia, glucose extraction is *increased* relative to glucose delivery. We have found that tissue glycolytic metabolite content actually decreases as ischaemia is prolonged, indicating utilisation of all available glycolytic metabolites for production of ATP, rather than any cessation of glycolysis (Results Ch 3). These data call for more precise metabolic control analysis of glycolysis in ischaemia and reperfusion, as has been done in normoxic isolated working rat hearts [241].

The consequences to the heart of increased glucose uptake are dependent on the subsequent degree of metabolite accumulation, ATP production, and the modifying influence of these factors on cytosolic Ca^{2+} accumulation, membrane stability etc. Thus while increased glucose concentrations may result in increased glucose uptake, a high glucose concentration (with or without insulin) may prove deleterious, as shown in Results Chapters 1 and 2, as a consequence possibly of increased sugar phosphate accumulation [219, 282, 482]. Increased contracture, increased incidence of arrhythmias, and reduced functional recovery were consequences of excess glycolytic substrate, as shown in Results Chapters 1, 2, 4, and 5. Thus an optimal delivery of glucose is required to ensure maximal ATP production, but this can be outweighed by excess metabolite accumulation. However, it must be stressed that this hypothesis is in slight contradiction with previous concepts, in that an accumulation of lactate, H^+ and NADH is not necessarily involved in deleterious effects by inhibiting glycolysis, but that other metabolites of glycolysis accumulating intracellularly have an effect on other factors including Ca^{2+} homeostasis [282].

B. HYPOTHETICAL MODEL OF ISCHAEMIC CONTRACTURE

Based on the concepts elaborated in the literature review, and on the work presented in this thesis, the following conclusions have been reached regarding the genesis of contracture, its modulation, and finally its role as a predictor of reperfusion recovery i.e. its effect on, and index of ischaemic injury. There is some speculation in this discussion, indicating possible avenues of further research. In addition, some points are still not clear, despite fairly extensive research. The precise role of glycolytic ATP in contracture is not yet fully understood, as is highlighted in the following discussion.

a) Genesis of contracture

i) Unifying hypothesis of onset of contracture

The seminal event in shortening of single hypoxic cells is rigor, which is soon followed by a rise in $[Ca^{2+}]i$ [9, 42] (see Ch I). Rigor complexes co-operatively activate adjacent myosin ATPases, accelerating ATP utilisation. In addition, ATP depletion limits activity of the ion pumps, leading to increased cytosolic Ca^{2+}. Cell contracture can only occur in the presence of low ATP; contracture is inhibited in the absence of ATP (no myosin ATPase activity to allow shortening) and high ATP (inhibits rigor bond formation) [42].

If one extends the findings in isolated myocytes to the whole heart, contracture can be seen as a co-operative response, both intra- and intercellularly, involving ATP depletion triggering initial rigor bond formation, and Ca^{2+} entry, which in turn exacerbates contracture and ischaemic injury (see Fig B.1).

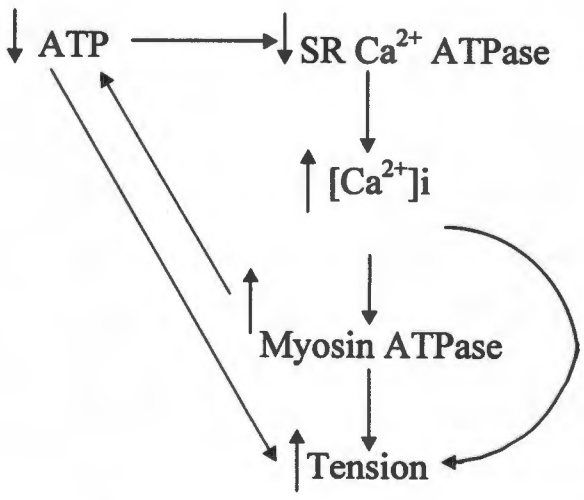


Fig B.1. Diagrammatic illustration of positive feeback relationship betwen ATP depletion and rise in cytosolic Ca^{2+} levels leading to an eventual rise is resting tension.

Heterogeneous dispersion of rigor complexes from intracellular localised depletion of ATP, and localised foci of contraction of individual cells with depleted ATP, lead to shortening of the whole muscle, and ischaemic contracture.

Intracellular - local areas of ATP depletion → single rigor bond formation → co-operative bond formation → increased adjacent myosin ATPase activity → depleted ATP → increased Ca^{2+} → increased TnC affinity → increased myofibril contraction → further ATP depletion

Intercellular - increased stretch of individual cells → increases stretch in adjacent cells → induced rupture of cell membranes

ii) Heterogeneous contracture

The evidence for heterogeneity of contracture is manifold, firstly on the basis of microscopic examination of tissue changes with contracture (see Ch I). In addition, Steenbergen [508] found a heterogeneous distribution of anoxic zones in low flow ischaemia. In the whole heart, with different substrates (oxidative versus glycolytic) in low flow ischaemia, residual oxidative phosphorylation in the absence of glycolysis would continue in the better-perfused zones, while the non-perfused zones would be more ischaemic and undergo contracture faster. With maintained glycolysis, there would be a more homogenous spread of ATP production with anaerobic glycolytic ATP in anoxic zones preventing contracture of these cells. The slower onset of contracture would be less damaging. This hypothesis could explain the data of Bricknell et al. [49], who showed that anaerobic glycolysis prevented contracture better than the equivalent rates of ATP produced by oxidative phosphorylation. From these findings, it was assumed that glycolytically-derived ATP is more effective than oxidative ATP in inhibiting contracture at a cellular level. Subsequently, Vanderwee et al. [547] found that the variability in glycogen content was the likely contributor to variability in ATP content and thus, time to onset of contracture. This hypothesis is confirmed by the present results.

iii) Role of compartmentation of ATP

With oxygen deprivation, the mitochondria are shut down. These are the powerhouses of the cell, in particular providing ATP to the myofibrils, near to which they are spatially located. In addition, glycogen granules located near the sarcoplasmic reticulum and the myofibrils, provide ATP for contraction (see Ch II). When these local sources of ATP are depleted, adjacent actin and myosin molecules bond. These bonds cause a co-operative binding of actomyosin in neighbouring molecules, thereby spreading bond formation outwards from a focal point. This effect may be relatively slow if glycolytic ATP is still available, providing some degree of relief of tension. However, the ATP derived from glucose must travel from the sarcolemma or SR, where the majority of the glycolytic enzymes are located [434, 593].

Glycolytic ATP also serves an important function by providing ATP to maintain Na^+/K^+ ATPase function in the sarcolemma [94, 167], and membrane integrity [49, 203] preventing membrane leakiness,

both major mechanisms of Ca^{2+} entry. Both glucose- and glycogen-derived ATP, produced near the SR [128, 593], may prevent cytosolic Ca^{2+} overload by maintaining activity of the SR Ca^{2+} ATPase, the main sink for cytosolic ATP. Compartmentation of glycolytic ATP production (glucose vs. glycogen), as well as of oxidative phosphorylation (see Ch II) is an essential concept in explaining the development of contracture. ATP from glycogen may be linked to the myofibril activity, as is oxidatively-derived ATP, while glucose-derived ATP is produced near the sarcolemma and SR. Thus there is an important functional dissociation between forms of ATP, important in ischaemia, when cellular transport process for ATP may break down. ATP derived from glycogen may be involved in determining myofilament binding, and thus glycogen-derived ATP would be important in maintaining diastolic relaxation in ischaemia, whereas glucose-derived ATP would be more important in maintaining levels of $[Na^+]i$ and $[Ca^{2+}]i$, thereby inhibiting ion overload and reducing the deleterious effects associated with increased cytosolic Ca^{2+}. In addition, the metabolites from glucose and glycogen utilisation would accumulate at different regions in the cell where they may exert different effects. H^+ produced at the myofibrils may reduce Ca^{2+} binding to the myofilaments, whereas increased H^+ accumulation at the membrane will increase the capacity for Na^+/H^+ exchange thereby increasing intracellular Ca^{2+}, especially on reperfusion.

Once glycogen is depleted, rigor bonds may form, with the onset of rigor [253]. When glucose is depleted, the drop in ATP leads to increased intracellular Ca^{2+} accumulation. The increased $[Ca^{2+}]i$ binds to Tn C, removing inhibition of actin-myosin bonding. Thus there is an active shortening of the myofibrils induced by Ca^{2+}, following on from the initial trigger of rigor bond formation. In addition, the increased Ca^{2+} hastens ATP depletion, which in turn increases $[Ca^{2+}]i$ accumulation by reduced re-uptake by the SR Ca^{2+} ATPase. However, maintained ATP production from glucose may prevent attenuated SR Ca^{2+} uptake, and thus reduce peak contracture.

Apstein proposed that glycolytic ATP has a "plasticising" effect i.e. it exert its effects by enhancing relaxation of contracted myofibrils [13]. However, this is not likely given compartmentation of ATP, except under conditions when ATP content is relatively high e.g. under relatively high flow conditions, and when glucose is present in reasonably high concentrations (at least 11 mM). Only then can the ATP diffuse in sufficient amounts from its sites of production near the sarcolemma and SR to affect myofibril contraction. We (Results Ch 1,2) and others [14, 424] have found that with a higher flow rate (0.5 ml/g wet wt/min) and with 11 mM glucose, there is very little or no contracture, indicating that glucose-derived ATP is preventing rigor bond formation, by diffusion to the sites on the myofibrils, as well as preventing cytosolic Ca^{2+} accumulation. At lower flow rates, changes in glucose concentration do not alter the time to onset of contracture, but do attenuate the rise in peak contracture (see Results Ch 1). However, an excess glucose concentration at low flow rates does increase contracture more than with 11 mM glucose (see Results, Ch 1, 2), suggesting that some other

factor, possibly sugar phosphates which have been implicated in a loss of Ca^{2+} homeostasis and a rise in diastolic pressure [281], is involved. While there are still some points which are not clear regarding the precise role of glycolytic ATP in determining the onset of contracture, a direct effect of this ATP on contracting myofibrils in severe ischaemia is unlikely.

iv) Glycolytic vs glycogenolytic ATP

Apstein et al. [14] and more directly Owen et al. [424], provided evidence that a certain minimum rate of glycolytic ATP production is required to prevent the onset of contracture. A rate of 2 μmol/g wet wt/min in an isolated heart perfused at a low flow rate of 0.5 ml/g wet wt/min was required. This finding appears to be contradictory to the hypothesis presented that a reduction in glycogenolytic ATP production is the main determinant of the onset of contracture. However, the point is still valid for several reasons: firstly, a larger flow rate ensures that the heart function is better maintained. Thus the capacity of the cells to transport ATP within the cytosol is improved. By simple diffusion, a higher rate of ATP production to the sarcolemma will ensure that the ATP is translocated to the myofibrils. Secondly, the increased ATP ensures maintenance of Ca^{2+} homeostasis, both by maintaining the Na^+/K^+ ATPase and preventing cytosolic Na^+ accumulation, preserving membrane integrity, and also to a lesser extent, by maintaining the sarcolemmal Ca^{2+} ATPase pump.

The concept that inhibition of glycogenolytic ATP production is coincident with the onset of contracture is supported by the findings of Kingsley et al. [253] and Cross et al. [93], the former in total global ischaemia where glycogen is the sole glycolytic substrate, and in the latter with low flow ischaemia (0.5 ml/g wet wt/min) where glucose was absent, and glycogen again thus the sole glycolytic substrate. In each case, the onset of contracture occurred with cessation of glycolysis, measured by a steady state in the fall of pHi during ischaemia [253], and a fall-off in lactate washout [93]. However, it must be emphasised that glycolytic ATP from glucose was not present in either case. When glucose is present in low flow ischaemia, contracture is still present (see Results Ch 1, 2, 5), despite a rate of glycolytic ATP production above 2 μmol/g wet wt/min (the threshold value given by Owen et al. [424]). Thus under severe low flow conditions with glucose, the initiation and potentiation of contracture are more complex, and affected by other factors such as metabolite accumulation, and Ca^{2+} and H^+ levels.

The hypothesis that glycogen depletion will reduce the time to onset of contracture is also challenged by the findings in preconditioned low flow hearts (Results Ch 5). In zero flow ischaemia, the reduced time to onset of contracture in preconditioned hearts is explained by a reduced pre-ischaemic glycogen, and reduced high energy phosphate levels [266]. However, in low flow hearts, despite a reduced glycogen levels with preconditioning, which we would have expected to reduce the time to onset of contracture (see Results Ch 1) there was no difference in time to onset compared to control

low flow hearts. However, the preconditioned hearts did show a significantly increased glucose uptake, prior to onset of contracture, and the greatly increased glycolytic ATP produced at the sarcolemma may have been able to diffuse to the myofibrils, and exert a "plasticising" effect [13]. However the benefits of increased glucose uptake on ischaemic contracture in the preconditioned hearts was counteracted by a higher metabolite accumulation, such that final recovery was no different from control hearts i.e. there was no protection associated with preconditioning in low flow hearts

b) Modulation of peak contracture

ADP is an important modifier of cross-bridge cycling, but may not play an important role in ischaemia, as it is broken down rapidly. While a lower pH should lessen peak contracture by decreasing Ca^{2+} binding, this beneficial effect may be counteracted by the deleterious consequences of Na^+/H^+ and Na^+/Ca^{2+} exchange stimulation by increased $[H^+]i$ accumulation. This mechanism may only be important on reperfusion [119], when reperfusion leads to increased contracture i.e. increased diastolic tension (see Results Ch 1), due to Ca^{2+} overload inducing contraction of the cells. In addition, in low flow ischaemia with maintained glycolytic ATP, the Na^+/K^+ ATPase pump continues to function, removing excess Na^+. Inhibition of this pump abolishes the protective effect of glucose [94, 167]. Pi may also modify contracture, by reducing the extent of cross-bridge cycling [280]. However, increased Pi is a direct reflection of reduced high energy phosphates, and also reduces the affinity of ATP and its free energy change on hydrolysis [235]. Thus this mechanism also has contradictory effects. We found that tissue with high Pi levels i.e. acetate-perfused hearts, showed the highest contracture (see Results Ch 1, 4 and 5).

Finally the most important modifiers of contracture are ATP and Ca^{2+}. While contracture may be precipitated despite continued production of glycolysis (as we found in low flow ischaemia - Results Ch 1, 2, 5) which appears to be contrary to the findings of Kingsley et al. in total global ischaemia, where cessation of glycolysis was associated with the onset of contracture [253]), the extent of contracture will be reduced if some residual glycolytic ATP is present. Owen et al. found a minimum rate of 2 μmol/g wet wt/min (from glucose) was required to prevent contracture [424]. Equivalent amounts of glycogenolytic ATP were inadequate, a finding confirmed by our results with modulation of pre-ischaemic glycogen contents (Results Ch 1), and in preconditioned hearts (Results Ch 4 and 5). In the absence of glucose, despite sufficient ATP from glycogen, we found a large contracture - especially if the metabolic rate was increased by an increased flow rate (Results Ch 1, 2, 5). The greater demand on ATP depleted the ATP levels at a faster rate. Thus the time to onset of contracture appears to be mainly dependent on glycogenolytic ATP (the ATP present in the globally ischaemic rat heart - i.e. Kingsley et al. [253]), although of course glycolytic (glucose-derived) ATP will also play

some role in this - a cell deprived of glycolytic ATP will utilise its other sources of ATP faster. Oxidative ATP may also help to reduce ischaemic contracture, but the contribution of this source of ATP is fairly small, and only relevant when the residual coronary flow is increased significantly.
The peak contracture is modified by the extent of Ca^{2+} entry, the accumulation of H^+ and Pi, and residual rates of ATP utilisation. Increased glycogen can reduce peak contracture (see Results Ch 4 and 5) but not as much as the presence vs. the absence of glucose (see Results Ch 1). A depletion of glycogen will precipitate contracture, but this can be modified by the presence of glucose (Results Ch 1). Thus the source of ATP is crucial in determining the onset and the peak of contracture, again emphasising the important role of glycolytic ATP.

c) Time course of contracture - consequences of altered rate of rise of tension

The faster the onset of contracture, the higher the peak of contracture (see Results Ch 1, 2, 4 and 5), and the more deleterious the consequences in that the higher the peak, the greater the tension, and the greater the cell rupture. Thus a high contracture should be deleterious. A delay in time to onset of contracture generally ensures that the peak of contracture will also be reduced. Mechanisms during the lag period in which heart adjusts to ischaemia may allow the heart to tolerate the extended period better. If perturbations occur largely in these early periods, the tolerance to ischaemia appears to be lessened. This relationship can be seen if time to peak, time to onset of contracture, and peak contracture are correlated (see Results Ch 1, 2, 4 and 5). Relationship of time to onset of contracture with functional recovery not always as clear cut (see Results Ch 1, 2, 4 and 5). This may be attributed to other modifying influences e.g. preconditioned hearts in total global ischaemia tend to have increased contracture, but recovery is significantly improved (see Results Ch 4).

d) Contracture as a determinant of recovery

Recovery on reperfusion is more or less determined by the extent of ischaemic injury (see Ch I). Increased contracture should reflect increased injury, as increased stress on the cells by the mechanical stretch would be expected to lead to cell rupture. In addition, increased contracture presumably indicates a state of reduced ATP and increased Ca^{2+} overload, both precipitators of reperfusion injury. However, there appears to be about three "ranges" of peak contracture, within each range there is no direct correlation with recovery. The first range is that where there is very little contracture, or a complete absence - as a very rough guide 0-20% of pre-ischaemic developed pressure. The recoveries should be good - more than 60%, possibly up to 100%. This state is only really possible with either very brief ischaemia (less than 15 -20 min), with reperfusion prior to the onset of contracture, or else with less severe ischaemia, i.e. maintained residual flow of at least 0.5 ml/g wet wt/min, and a glucose concentration of 11 mM (or 5 mM with insulin) (see Results Ch 1, 2). The second "range" of contracture is a peak of about 20-60%. Recoveries will generally be in the

range of about 40-60% (see Results Ch 1, 2). Thus a significant difference in contracture within this range will not greatly affect recovery of function, and there may in fact be a lack of correlation between groups i.e. increased contracture but increased recovery as in preconditioned hearts (Results Ch 4, 5). The third range, with peak contractures greater than 60%, will generally lead to recoveries less than 40% (Results Ch 1, 2, 4, 5). In this range the predictive value of contracture becomes more accurate. This is due to the increased contracture precipitating cell rupture, indicated by the rapid fall off in tension seen after the attainment of peak values e.g. in acetate hearts. This reduction in tension can be attributed to cell rupture, indicative of cell death and irreversible injury. The greater the fall off in tension, the greater the irreversible injury. However, contracture is a heterogeneous phenomenon i.e. individual cells contract, and cause contracture and rupture in adjacent cells. Foci of contracture develop [181], leading to shortening of the whole myocardium. However despite quite severe shortening there may still be some degree of salvageable myocardium e.g. acetate preconditioned hearts subjected to low flow ischaemia show reasonable recoveries despite a large peak contracture (Results Ch 5). The amount of residual salvageable myocardium is dependent on other modifiers of ischaemic injury as well as the amount of tissue still perfused after a period of time. In the contracting myocardium, the endocardium is more severely ischaemic. This is the normal occurrence in any ischaemic heart, but with an intraventricular balloon, as the heart contracts, the endocardium is more compressed by the epicardium, against the fixed volume of the balloon. The perfusate is thus forced to the epicardium. The degree of residual flow is an important determinant of functional recovery (see Results Ch 1), presumably limiting infarction, and ensuring some degree of functional recovery.

On reperfusion, an increase in diastolic pressure is often seen if contracture occurs during ischaemia (see Results Ch 1). This effect can be partially attributed to the "garden hose" effect i.e. increased turgidity due to filling of the vascular bed [210, 560]. However, this explanation does not fully explain the phenomenon. On reperfusion, there is a large influx of Ca^{2+} via the Na^{+}/Ca^{2+} exchange mechanism, and through leaky membranes [353]. This Ca^{2+} influx causes contraction of the myofibrils i.e. active shortening, which then go into a state of tetany because of the increased Ca^{2+} and the reduction of ATP which is required for relaxation [147-149]. This increase in diastolic tension on reperfusion may be dependent on the degree of injury during ischaemia - an increased H^{+} and Na^{+} load during ischaemia, increased ATP depletion, and increased fragility of the membranes, as well as the percentage of contracted cells, all contribute to the degree of diastolic tension on reperfusion. i.e. the greater the changes in ions and high energy phosphates the greater the chance of precipitating irreversible contracture and cell rupture (leading to cell death) on reperfusion. If these changes are reduced, the chances of recovery are greatly improved. Increased duration of reperfusion should then result in a gradual increase in recovery. If, however, the changes are irreversible i.e. cell death, extended reperfusion will make no difference, with impaired diastolic function maintained. This will

result in the presence of an infarct, as measured after extended reperfusion. Preconditioning appears to exert its main effect by delaying the initiation of necrosis, and thus reducing the extent of irreversibly injured tissue on reperfusion and reducing the increased diastolic tension on reperfusion (see Discussion C for further discussion on this point). However, this effect is somewhat contradicted by the exacerbation of contracture noted in the majority of preconditioned hearts (see Ch III).

The dichotomy of the exacerbation of contracture by preconditioning, but the subsequent improvement in recovery of function in these hearts, as observed by ourselves, and a number of other investigators [18, 266-268] suggests that ischaemic contracture may not always be a good index of ischaemic injury. However, preconditioning only increases contracture by a relatively small degree, in the order of 10-20 mmHg [18, 266-268], i.e. within the intermediate range described above. Preconditioning may confer an ability to tolerate the increased contracture, although excess contracture with preconditioning (as found with acetate-perfused hearts - Results Ch. 4) may not be as easily tolerated, and may attenuate the protective effects of preconditioning, as observed.

Contracture (ATP and Ca^{2+}-dependent) during ischaemia can precipitate increased contracture on reperfusion (mainly Ca^{2+}-dependant). This contracture is essentially irreversible (see reperfusion after 30 min severe ischaemia - maintained diastolic dysfunction reaching a steady state - Results Ch 1, 2, 4 and 5) and may indicate the degree of infarction, as implied by Jenkins et al. (in preconditioned hearts, the degree of functional recovery was associated with infarct size [220]). However, it must be noted that use of ischaemic contracture as an absolute index of ischaemic injury, and a predictor of functional recovery can be misleading. To reiterate, a full understanding of the mechanism of ischaemic contracture, and its effects on the tissue, is required to interpret the results.

C. PRECONDITIONING - A REAPPRAISAL OF PROTECTION

In Basic Res Cardiol 91:5-7, 1996.

a) Preconditioning - not always beneficial?

Preconditioning has been described as the most potent form of protection against myocardial necrosis yet described [300]. The protection conferred by preconditioning has been found against most of the deleterious effects induced by ischaemia and reperfusion, and in most animal models as well as in humans. While preconditioning undoubtedly delays infarct development, and offers intriguing mechanisms of endogenous protection, a word of caution is required before assuming that preconditioning could be the basis of a new-found therapy for patients. We attempt to take a critical look at the literature and to emphasise that the reduction of infarct size, often praised as an important consequence of preconditioning, has two important limitations. First, it is a delay in the development of necrosis that is achieved; thus preconditioning buys time but does not cheat death. Secondly, almost all the models used in the studies of infarct size reduction use regional ischaemia followed by reperfusion, so that the benefits of preconditioning could have occurred in either the ischaemic or in the reperfusion period, which is an important distinction. We will emphasise that preconditioning can have different end-points, and that not all of its effects are favourable. Specifically, there may be adverse effects during the ischaemic period, which differ from those on reperfusion.

b) The mechanism of preconditioning - no consensus after 10 years of research

Many proposals as to the mechanism of preconditioning have been made, without consensus being reached (see Ch III). Many diverse routes to preconditioning have been proposed, including limitation of glycolysis, maintenance of high-energy phosphate compounds, enhanced activity of ATP-sensitive potassium channels, and increased activity of the inhibitory G protein [300], besides an extensive literature implicating adenosine and protein kinase C activity (see Ch III for further discussion). Although the role of adenosine has been well studied, and it was one of the first major hypotheses for preconditioning, it can be eliminated as the mechanism in the rat [64], and it therefore can not be the universal mediator. Another tenable hypothesis involves protein kinase C translocation. However, the particular isoenzyme involved has not been identified; each may have very different actions and different responses to preconditioning ischaemia. Thus in the absence of any agreed single mechanism it becomes important to examine carefully the experimental conditions chosen for the various studies and to understand their limitations.

i) Variability of protection

A major problem with attaining a consensus on preconditioning is reproducibility. Some authors claim that preconditioning is highly reproducible and very protective; others find the results so variable and that to obtain a consistent model, a number of different protocols must be tested. Such an attempt has been described by Weselcouch et al. [577] who reported that 4 episodes of preconditioning each of 5 min ischaemia followed by 5 min reperfusion were required, before any noticeable protection could be achieved. This is in contrast to previous reports using rat hearts, which suggested that one episode of ischaemia was as efficacious as two episodes [18].

ii) Indices of protection - infarct size vs stunning and functional recovery

A major point of difficulty lies in the choice of end points. Preconditioning was originally shown to reduce infarct size by 75% compared to control hearts [375]. This observation has been confirmed in many subsequent studies and is indeed the most consistent, and most potent effect of preconditioning. While a reduction in infarct size is very important, in the context of the shorter laboratory experiment, recovery of function has generally been used. Preconditioning can be said to delay the time to onset of necrosis such that at any given time during the sustained ischaemic period (between onset of injury and final development of total necrosis) if the heart is reperfused, recovery of function would be expected to be greater because of a smaller infarct (see Fig C.1a and b). The results, however, have been very contradictory.

Preconditioning does not protect against stunning, the reversible component of injury, but rather delays the development of necrosis [220]. A possible point of confusion lies in the definition of functional recovery. Stunning is defined as a transient postischaemic condition in which the cells have full viability, but are poorly functioning (Ch I). This is usually associated with reperfusion after short term ischaemia. Once there is a component of irreversible injury, as after long term ischaemia, then preconditioning may be more protective.

However, in larger animals (pigs and dogs), no improvement in recovery of function with preconditioning has been reported to our knowledge, despite a significant reduction in infarct size. Preconditioning prior to short periods of ischaemia (15 min coronary occlusion) showed no benefit on functional recovery [421]. Shizukuda et al. used a 60 min period of coronary occlusion, and showed that segment shortening during reperfusion was not improved by ischaemic preconditioning, despite a reduced ultimate infarct size [498]. Some studies in rabbits found no improvement in functional recovery [532] despite reduced infarct size after 30 min ischaemia (a time which would be expected to induce at least some necrosis). Other studies have recorded improved function in rabbits [82], but after 5 to 20 min sustained coronary occlusion, which would be expected to produce more stunning and

less necrosis. We have found that preconditioning is not always protective against reperfusion mechanical dysfunction in a rat heart model (see Results Ch. 4, 5). Changes in pre-ischaemic glycogen can abolish the protective effects (Results Ch 4), while no additional protection with preconditioning is observed with a sustained residual coronary flow for the test ischaemia (Results Ch. 5). Thus, unlike the effects on infarct size, preconditioning does not consistently induce benefits on postischaemic mechanical function.

iii) Ischaemic contracture

The importance of the end-point selected for preconditioning is emphasised by the finding that preconditioning exacerbates ischaemic contracture [18] even when functional recovery is improved (see Results Ch 4). Infarct size is generally measured after a period of reperfusion, which implies that both the components of ischaemia and reperfusion injury are present. Preconditioning may actually exacerbate ischaemic injury by increased contracture, but be beneficial in reperfusion by stimulation of the Na^+/H^+ exchanger [449]. The mechanism for this difference may lie in the following speculative sequences:

1) the preconditioning ischaemic period depletes the heart of glycogen (see Results Ch 1)

2) ischaemic contracture is therefore more likely to occur

3) the mechanism may involve inhibition of the Na^+/K^+ ATPase pump, which would explain the increased cell Na^+ values found by Ramasamy et al [449].

4) in the reperfusion period Na^+ and H^+ exchange is enhanced, so that Ca^{2+}-mediated necrosis would be diminished and the ultimate infarct size after reperfusion would be less.

iv) Effects in absence of full reperfusion

Reduction in infarct size can be found even in the apparent absence of reperfusion, when there is an initial period of total ischaemia followed by low flow ischaemia [493]. Two comments are appropriate; firstly, the low flow ischaemia does represent reperfusion when compared with no flow; secondly, during the subsequent low flow ischaemia there was a substantially reduced work index which could have contributed to infarct size reduction.

c) Concluding comments

While the undoubted benefits of preconditioning on reduction of infarct size after reperfusion are important, it would be prudent to maintain a degree of circumspection because of the variability of the protocol and the differing results according to the end-points chosen. Preconditioning is not always beneficial (see Results Ch 4, 5), and may have some detrimental effects especially in the ischaemic as opposed to the reperfusion period. Thus there should be due care in extrapolating from experiments to any possible clinical application.

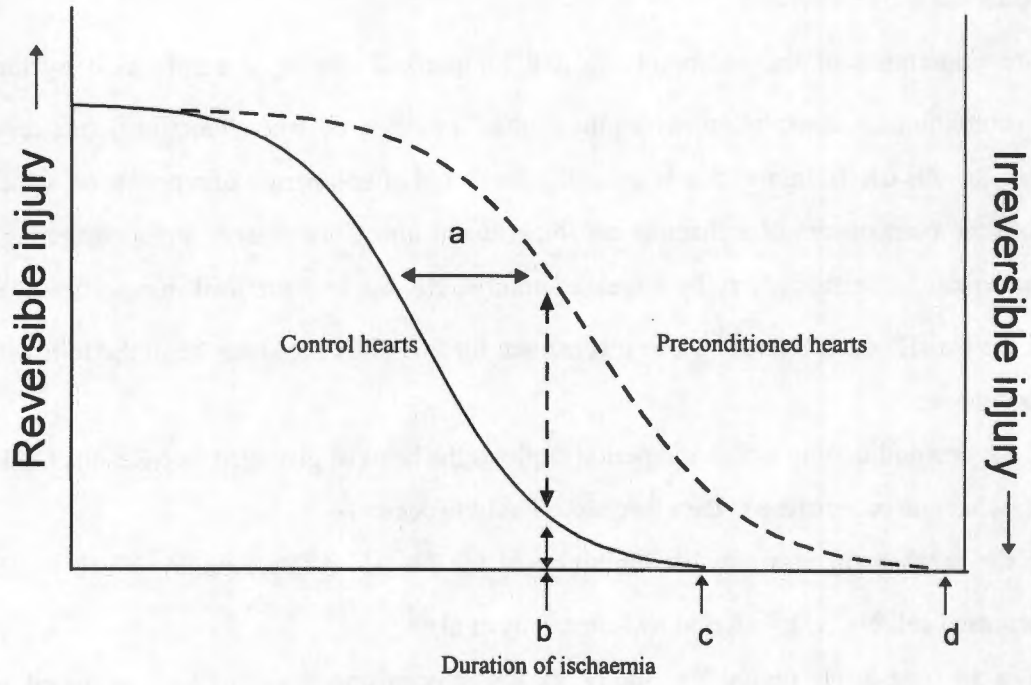


Fig C.1a: Effect of preconditioning on degree of residual function in the tissue with duration of ischaemia, as a ratio of reversible to irreversible injury

a - rightward shift in preconditioned hearts of protection against irreversible injury

b - at point b residual function is much greater in preconditioned hearts

c - at point c control hearts have no residual function, with total infarction

d - at point d the duration of tolerated ischaemia has increased but preconditioned hearts end up with the same degree of infarction as control hearts

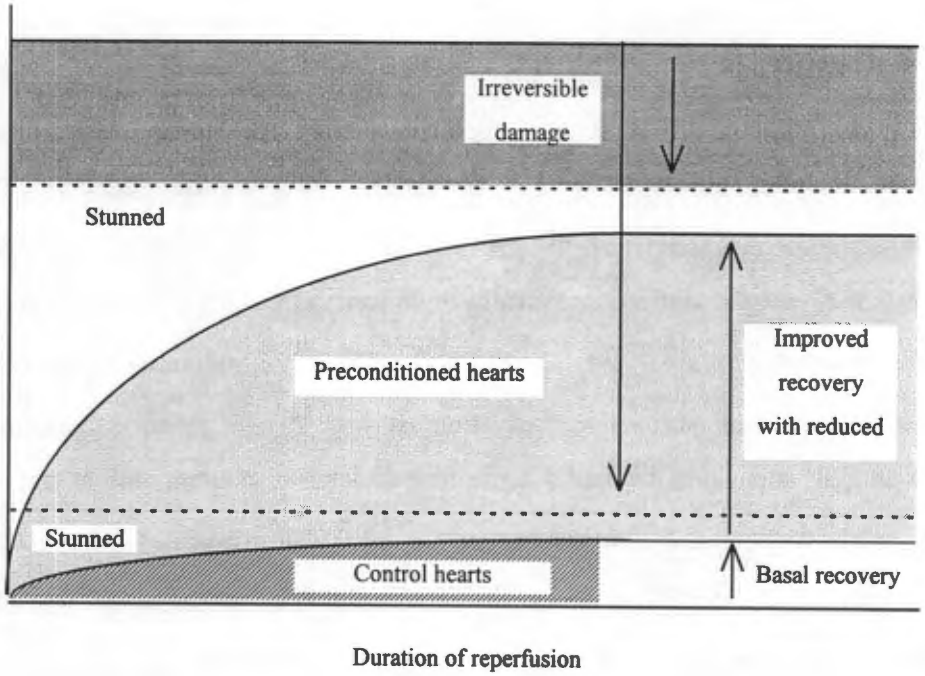


Fig C.1b. Presumed recovery of function if control and preconditioned hearts are reperfused at point b. There would still be a component of stunned myocardium, which may not be affected by preconditioning. If overall functional recovery is improved, this may be attributed to reduced infarction; alternatively, if infarct size is reduced, there should be better recovery of function. However, this does not always hold true, and raises concerns as to the validity of preconditioning as a therapy.

D. RESERVATIONS TO STUDIES

The isolated rat heart perfused in the Langendorff mode was used in all studies presented in this thesis. While this model is frequently used in studies of metabolism and ischaemia, there are certain limitations, which, while not detracting from the present observations, must be taken into consideration before a direct clinical application can be made. Reservations regarding the measurement of glycolysis/glucose uptake are discussed in Results Ch 3.

a) Choice of species

The rat myocardium is different in many respects from the human myocardium. Fundamental differences include metabolic rate, contractile performance, enzyme functioning, and electrophysiological characteristics [46, 145]. The rat has a high metabolic rate inversely correlated with a low body weight, and a consequently high heart rate. This is reflected in a higher coronary flow (5-6 ml/g wet wt/min [279] vs. 1-2 ml/g wet wt/min [507]) and more rapid enzyme activities, in particular those of the oxidative phosphorylation pathway [34]. The rat has a characteristic short action potential with an attenuated plateau, a high intracellular Na^+ content, and an excitation-contraction coupling mechanism unusually dependent on Ca^{2+} [145]. The myosin ATPase isoform of the rat is predominantly V_1, i.e. the faster enzyme [113]. The rat also has different Ca^{2+}-handling mechanisms. Susceptibility to ischaemia also varies between species [145]. In addition, with respect to preconditioning, some pathways common to other species, e.g. K_{ATP} channel opening, adenosine receptor activation etc. have been found not to be active in the rat despite the finding that there is a protective effect exerted by preconditioning (see Ch III).

b) Choice of rats

In the majority of studies using the isolated rat heart, the hearts are from young, healthy animals, without any coronary heart disease or other factor which may affect the response to ischaemia. Hearts from older rats are well known to show reduced tolerance to ischaemia, and thus more variability. These rats show a predominance of the V_3 myosin ATPase isoform, which is slower than V_1 [113]. In the interests of tighter data, and thus more precise information, younger rats are more frequently used (i.e. between 250-350 g). However, we wish to further our studies on the role of glucose in ischaemia in pathological models, concentrating particularly on hearts from diabetic animals, which show reduced glucose utilisation, and increased dependence of fatty acids. Fatty acids induce a toxic effect on the ischaemic heart, which may be counteracted by increased glucose uptake [399].

c) The isolated organ perfusion system

The utilisation of an isolated organ is associated with problems not found *in vivo*. When removing the heart, it may be damaged in some way, while the heart is arrested i.e. ischaemic, for a brief period. This may induce preconditioning, glycogen depletion, and regional irreversible patches of ischaemia. While the time from excision of the heart until cannulation and restoration of flow is reduced (less than one minute), and the hearts are arrested in cold buffer to reduce the metabolic changes, there may still be more variation than would be found *in vivo*, and the response to ischaemia may be affected. However, the isolated organ allows for more precise and easy measurement of cardiac function under different stresses, with far easier administration of drugs etc. and easily manipulated changes in the perfusate, inducing changes in substrate. In addition, factors such as catecholamines released into the blood, and circulating anaesthetics are eliminated in this model. Strict exclusion criteria are used to ensure maximal consistency between hearts.

We used the Langendorff mode in all the perfusions for this thesis. While this mode allows many experiments to be done within a reasonably short space of time, with a good reproducibility, and low exclusion rate, there are disadvantages in terms of the physiological application of the model. The heart does not work in this mode i.e. the heart does not eject perfusate against an afterload. However, in our studies dealing with ultra low flows, the hearts do not beat and thus would perform no work even if the working mode is used. Thus in comparing the changes in glucose uptake, glycogen utilisation etc. at extreme low coronary flow rates, there should be no differences between Langendorff and working hearts. In addition, with a relatively long period of ischaemia, very small degrees of recovery can be distinguished. In the working heart, there is a threshold of recovery, determined by the pre- and after-loads, below which no recordings can be made.

We were also interested in measuring ischaemic contracture, as an index of ischaemic injury, and relative ATP and Ca^{2+} levels. This can only be measured with an intraventricular balloon, or with a tension transducer attached to the apex of the heart. The former technique cannot be applied in the working heart for obvious reasons; the latter technique imposes a tension on the heart which would interfere with the pump function, and may tear the tissue.

We used a model of global ischaemia, whereas ischaemia *in vivo* usually only involves a portion of the myocardium. However, we believe that a low flow model is more representative of *in vivo* ischaemia. In addition, we used a fixed low flow rate, which may prevent some of the deleterious effects of contracture which may compress the vessels, and result in no-reflow. A low perfusion pressure system would be more physiological, with a reduction in blood flow over time. However, this system is more variable, and more difficult to interpret.

d) Perfusion solution

We used a crystalloid solution with glucose 11 mM as the sole substrate in the majority of cases. This perfusion solution has been used frequently, and can maintain heart function for several hours, longer than was needed in the present protocols. However, a crystalloid solution results in maximal dilation of the arteries, because of the reduced oxygen-carrying capacity, resulting in a greatly increased coronary flow compared to *in vivo* hearts. In addition, myocardial contractile performance is tightly coupled to the perfusion pressure and oxygen availability. Thus function in "control" hearts may vary widely depending on the apparatus and conditions used. However, we were interested mainly in the response of hearts subjected to a low residual flow, at which point we believe that the differences between *in vivo* and *in vitro* hearts are reduced. While there will always be room for criticism, we have tried to allow for these differences. We believe that the range of coronary flow used in these studies (0.015 - 0.5 ml/g wet wt/min) more closely represents true *in vivo* ischaemia (with residual flow rates of 0.07-0.15 ml/g wet wt/min [360, 375, 507]) than previous studies (0.5 - 6 ml/g wet wt/min [389, 390, 424, 468, 469].

An alternate substrate, acetate, was included when a period of glucose-free normoxic perfusion was used, when we wished to deplete tissue glycogen levels, but did not wish for adverse effects associated with substrate-free perfusion. Acetate is not a truly a physiological substrate (although derived from ethanol) although it can be used in isolated heart perfusions in the absence of glucose, with no reduction in function over at least 30 min [524]. While there may be some eventual decline in function because of the lack of anaplerosis from acetate in the absence of glucose, this effect is more likely to impair reperfusion function. However, even with hearts perfused throughout with 5 mM acetate only, (which reduces pre-ischaemic glycogen levels and thus further impairs anaplerosis) recovery of function was 15-30% (depending on ischaemic conditions) suggesting that in the absence of glucose, this substrate can supply some residual ATP production, and maintain some heart function.

The addition of free fatty acids and insulin would have made the model more physiological, but the use of free fatty acids add another dimension of complexity, in that excess free fatty acids contribute to ischaemic injury. If we wish to extrapolate our findings and apply the principles to a clinical situation, for instance as therapy for patients with myocardial infarction, further work is required on the effect of glucose in the presence of alternate substrates, insulin etc. in ischaemic hearts. In particular, a primary mechanism of protection by insulin is a reduction in circulating free fatty acids [399]. This effect was not investigated. For more complete information, these experiments should be done in hearts perfused with blood, either *in vitro* (paracorporeal blood-perfusion model) or *in situ.*

However, in these cases, the substrate concentrations are difficult to maintain at exact levels. We wish to confirm our simple observations in one of these more physiological models.

e) Preconditioning

We used one five minute episode of preconditioning, which may be insufficient to ensure complete protection. However, with the Langendorff model, two or more episodes of preconditioning ischaemia tend to impair function prior to the sustained period. In preliminary work, we have found no difference in protection with two or more episodes, especially with a low residual flow rate during ischaemia. These observations remain to be confirmed.

We also used an insulin-free perfusate for our control hearts. After 30 min perfusion with 11 mM glucose only, there is some glycogen depletion compared to control (*in situ*) hearts. Thus our hearts were already relatively glycogen-depleted, which may have conferred some protection according to the Neely hypothesis [387]. A further depletion in glycogen by acetate perfusion may have depleted glycogen in these hearts to an extent below which the hearts could recover. However, the preconditioned hearts had glycogen levels comparable to the preconditioned glucose-only hearts, and yet these hearts showed improved functional recovery. While there may be some outstanding question regarding a difference in the absolute levels of glycogen reported by different authors, and the meaning of the different levels, I believe that the conclusion that preconditioning is unrelated to glycogen depletion holds true. This proposal has been confirmed by a number of other authors, using different experimental conditions.

The choice of end point for preconditioning studies can also be questioned.

f) Choice of end point

The choice of end point and the time of measurement are critical in terms of clinical application, where patients are generally only seen some considerable time after the ischaemic insult, which thus may also vary significantly in duration. In the isolated perfused heart, functional recovery and infarct size etc. are recorded after a relatively brief period of reperfusion, of 20-60 min (sometimes longer). While immediate functional recovery may indeed be a necessary requirement for the patient, the effects of reduced infarct size may become more important in the long term, with reduced scarring of the myocardium. This issue is particularly relevant to the discussion regarding preconditioning. While we and others do not find improved functional recovery with maintained low flow with preconditioning (see Results C 5), the effects on ultimate infarct development may be more important from the clinician's point of view. This can only be decided when preconditioning is applied in patients, but this can only be done after more research to clarify the issues still under debate.

Unfortunately, it is difficult to record infarct size reliably in an isolated rat heart (Derek Yellon, personal communication). A larger animal model is required for these assessments.

g) Biochemical measurements

We used a relatively crude model, the whole heart, to obtain tissue for biochemical measurements. With low flow ischaemia, there may be some degree of heterogeneity between totally ischaemic, and better perfused areas. We are then taking the whole heart, and making an estimate of average levels of the metabolites. This method may blunt the changes in the more extreme tissue. However, any attempt to investigate glycolysis under low residual flow conditions (which we believe to be more physiological) will have this flaw. The involved analysis by Kashiwaya et al. [241] of glucose metabolism in the normal working heart needs to be applied to a low flow model (and total global ischaemia) in order to determine precisely the points of regulation of glucose utilisation under these conditions. In addition, the techniques of nuclear magnetic resonance and positron emission tomography may allow a clearer determination of glucose uptake and coronary flow, and the consequences to the tissue in terms of ion changes, changes in high energy phosphate etc.

h) Summary

Despite all the above reservations, we feel that the observations made clarify some important issues, and contribute to our knowledge on the kinetics of glucose utilisation in ischaemic myocardium, and thus to the potential clinical application of substrate supplementation, including glucose-insulin-potassium therapy in patients with myocardial infarction.

E. POSSIBLE THERAPEUTIC STRATEGIES UTILISING GLUCOSE

a) Glucose insulin potassium (GIK) therapy

i) Historical background

Despite all the experimental evidence regarding the benefits of glucose provision to the ischaemic myocardium, its utilisation as a therapy for myocardial infarction has not been widely exploited. Acute myocardial infarction leads to major metabolic changes in heart muscle and affects the whole body. Impaired oxygenation, reduced substrate supply and inhibited washout of end products impairs membrane ionic transport, increases free fatty acid concentrations and reduces ATP availability. Increased substrate provision should attenuate these changes.

The development of glucose insulin potassium (GIK) therapy for use in patients with cardiac problems is attributed to Demetrio Sodi Pallares. In 1944, his mother developed a serious heart condition, for which he prescribed a low Na^+, high K^+ diet. This treatment greatly improved her health, and was applied in other patients [501]. At that time there was very little support for his treatment. He believed in a harmful role of Na^+, and a protective one for K^+ in the development of cardiac necrosis. In 1960, the "polarising solution" of Henri Laborit, consisting of glucose, insulin and K^+ was described for use with anaesthesia. K^+ enters the myocardial fibres, increasing Na^+ efflux, while insulin enhances glucose uptake into the cells. The effects of the diet developed by Sodi Pallares were linked to the benefits associated with the polarising solution. The GIK solution was then tested on a dog with a recent myocardial infarction. Intra- and extracellular Na^+ and K^+ levels were measured. The optimum effects were found with 20% glucose (in 1000 ml), 40 mEq KCl, and 40 U insulin administered intravenously at a rate of 40 drops per minute. A reduction in Na^+ and an increase in K^+ was found.

The possible benefits of the polarising solution include repolarisation, increased anaerobic glycolysis, increased tissue glycogen, increased tissue α glycerophosphate for free fatty acid esterification, decreased circulating free fatty acids, membrane effects, hyperosmolar effects, increased oxidative metabolism of glucose, decreased lysosomal activity, accelerated wound healing [401, 501]. The K^+ concentration is the major determinant of transmembrane potential, which in turn regulates most of cell functioning.

ii) Early experimental studies

The effects of GIK have been tested on many of the changes brought about by ischaemia, including infarct size, electrolyte balance, arrhythmias, myocardial contraction, healing, cardiac shock, mortality rate, prevention of myocardial infarction, and treatment of severe angina pectoris [501]. An early clinical study by Sodi Pallares showed favourable ECG changes following intravenous

administration of GIK in patients with acute MI and other coronary syndromes. Mittra et al. [357] published the results of a trial in the Lancet, in which insulin was given subcutaneously, with oral doses of glucose. A 50% reduction in mortality when given to patients with myocardial infarction was found. However, the British Medical Research Council subsequently published results of a similar trial in the Lancet in 1968. No significant benefit was associated with a GIK regimen in terms of mortality and arrhythmias. This publication resulted in a loss of interest in GIK therapy. However, a number of faults were apparent in this study, namely very low doses of insulin, glucose and potassium, poor selection of patients, and late administration of the therapy [401].

Despite the clinical setback, further experimental evidence was presented to show the benefits of GIK. GIK infusion 30 min after coronary occlusion exerted beneficial effects against myocardial ischaemia, and reduced the extent of necrosis in the region distal to the ligation [341]. GIK infused 60 min after coronary ligation increased glycogen, CP and ATP in the central infarct zone and decreased Pi [411]. GIK thus prevented early tissue metabolic deterioration in the infarcting heart. Similar results were reported by Dalby et al. [95] in dogs.

iii) Renewed interest in GIK

Apstein published two important papers detailing the effects of high and low glucose concentrations under low flow conditions in rabbit hearts [14, 123]. A high glucose concentration (28 mM) + insulin improved functional recovery and reduced ischaemic contracture at a higher flow rate (0.5 ml/min/g wet wt). Tissue high energy phosphates after 180 minutes of "moderate" ischaemia were higher with glucose supplement, while lactate production was greatly increased. Cellular ultrastructure was better preserved with substrate enhancement. With a lower flow rate (0.06 ml/min/g wet wt), high glucose + insulin reduced ischaemic contracture but did not affect recoveries. Lactate washout was maintained during ischaemia, indicating sustained glycolysis. High energy phosphate levels were no different. Eberli confirmed these findings in a model of the rat heart perfused with an erythrocyte suspension [123]. These findings, and others, led to a slow resurgence of interest in GIK.

GIK has a glycogen-sparing effect, thought to prolong the availability of metabolic substrate for high energy phosphate metabolism [204], with increased glycogen available if given prior to ischaemia [398]. Glucose and insulin (30% glucose + 300 U insulin + 5 g KCl in 500 ml at a rate of 1.66 ml/kg/hr) infused 1 hr prior to cardiopulmonary bypass [159] improved protection of hearts during operations and on reperfusion.

Metabolic support for the postischaemic heart has recently been advocated by Taegtmeyer [198], specifically indicated for refractory left ventricular failure following cardiopulmonary bypass. A significant improvement in time to recovery of function is found with GIK. In addition, the use of other metabolic substrates e.g. anaplerotic substrates is proposed. Fibrinolytic therapy allows

controlled reperfusion of an ischaemic zone. Reperfusion reduces mortality, but may also potentiate injury. Glucose attenuates experimental reperfusion arrhythmias and other deleterious effects, specifically increased Ca^{2+} [229].

A recent abstract [132] reports the results of a meta-analysis of studies using GIK. While most of these studies had very low sample numbers, the overall results showed reduced mortality with GIK after acute MI. It was also suggested that this regimen would be complimentary to thrombolysis by reducing the risk of reperfusion injury. A need for a large randomised trial on the effects of GIK with thrombolysis was indicated.

iv) Other applications

In an experimental model of anoxic cardiac arrest, GIK retrogradely infused into the coronary circulation, greatly improved mechanical function and cardiac rhythm [321]. GIK administered prior to 60 min hypothermic arrest, significantly reduced SR Ca^{2+} uptake and protected the excitation-contraction coupling system, possibly by free radical scavenging effects [200].

Lazar [302] suggested that substrate enhancement in "surgical" stunning may be advantageous, with application in surgical revascularisation of acute ischaemia. Reduced necrosis and stunning were found. GIK (high dose regimen) was infused during coronary occlusion (with cardioplegic arrest) and reperfusion. GIK hearts had significantly less tissue acidosis and higher wall motion scores, and less necrosis.

v) Concentrations of glucose, insulin and potassium

The major problem with GIK has been the establishment of a regimen which is practical, beneficial with limited side effects. A wide range of regimens of GIK have been used, including oral supplements and intravenous solutions with high concentrations of glucose. While sufficient glucose is required to provide ATP, the possible side effects of a high glucose concentration in a severely ischaemic zone must be taken into consideration. Reported adverse effects of GIK include hypoglycaemia [435], hyperglycaemia and hyperkalaemia [463], hypophosphalaemia [344], and effects on the conduction system [168]. In severe ischaemia, with low coronary flow, glycolysis may be impaired to such an extent that the addition of exogenous glucose may not benefit the ischaemic zone. In this case the benefit would be limited to reduction of circulating free fatty acids. The findings presented in this thesis advocate the careful utilisation of glucose, at a range of concentrations consistent with those found to be optimal in the isolated heart. While systemic effects may indeed play an important role, a careful assessment of the relative detriment of a high glucose and insulin to the ischaemic tissue, versus the systemic effects, of reducing free fatty acid levels in the blood, is required. Results from this thesis suggest that maintaining a (fasting) blood concentration of 5-10 mM glucose (the higher end of the physiological range), with insulin, may be beneficial in patients with myocardial

infarction. In addition, the possible resistance to insulin in patients with chronic heart failure argue for substrate supplementation [518]. In general terms, less glucose would have little effect; more glucose would be detrimental to the disadvantaged heart. However, hyperglycaemia should be avoided. However, caution must be taken before extrapolating the results of the present thesis directly to the clinical situation, because of reservations discussed above (see Discussion D).

b) Cardioplegia

i) Cold cardioplegia and glucose

Many different formulations of cardioplegic solutions have been described. The most commonly used cardioplegic solutions include the St Thomas' Hospital solution (STH) [188], the University of Wisconsin (UW) solution and Bretschneider's HTK [47], none of which have glucose in their standard formulation. Addition of glucose to STH was detrimental when given in a bolus dose at the onset of 70 min arrest at a temperature of 28°C [193]. If STH were administered at the onset of 3 hrs arrest, at a temperature of 10°C, the addition of 11 mM glucose also had no benefit; when administered in a multidose regimen, especially if gassed with 95% O_2 5% CO_2, the recoveries were substantially improved with glucose [252, 563]. The optimal glucose concentration was 11 mM, with excess glucose (20 and 50 mM) proving deleterious to the hearts [425]. Higher glucose concentrations inhibited glycogenolysis, reducing total glycolysis and ATP production [425]. These results are in agreement with concepts presented in this thesis.

ii) Warm blood cardioplegia

A more recent advance in cardiac preservation has been the introduction of warm blood cardioplegia [314]. The concept works on the premise that if the heart is arrested, cessation of electromechanical function accounts for 90% of myocardial oxygen consumption. An additional 10% saving in energy is outweighed by the benefits of the more physiological temperature, and thus a reduction in temperature is not required if mechanical arrest is maintained. Continued perfusion with warm blood supports continued aerobic metabolism and also allows for myocardial substrate replenishment. Warm blood cardioplegia preserves function and metabolism at near pre-arrest levels [55]. Glucose was present at a concentration of 56.6 mM (after admixture with blood), delivered at a flow rate of 3.14 ± 0.37 ml/kg/min i.e. 0.78 μmol/g/min. Hanafy et al [178] used warm cardioplegia with glucose (0.222 M - 250 -300 ml/min for 2 min - total 13 mmol) to induce arrest followed by use of cold cardioplegia. This regimen was especially beneficial in patients with severe underlying coronary disease.

The findings presented in this thesis are applicable to warm blood cardioplegia administered continuously. The findings also suggest that glucose would be beneficial to the heart. but that the

concentrations should be carefully monitored. While the "stone heart" [86, 242] is a consequence of surgery to be avoided, largely by glucose provision, excess glucose at low flow rates may be equally deleterious.

c) Other interventions

Recently, interest in manipulating carbohydrate metabolism e.g. by inhibiting fatty acid oxidation and increasing glucose oxidation, or activating pyruvate dehydrogenase (PDH) and thus also increasing glucose oxidation have received attention. Fatty acid oxidation can be inhibited by carnitine palmitoyl transferase I (CPT-I) inhibitors such as etomoxir and oxfenicine, which inhibits fatty acyl Co A transport into the mitochondria [201, 323]. Thus, glucose oxidation can be increased, and presumably increase ATP production if residual oxygen is present. Increased pyruvate or stimulation of PDH (by dichloroacetate [351, 581]) may also increase ATP production by increased oxidation, but again this is dependent on the availability of oxygen [92]. The benefits of the above mechanisms should thus only be present if oxygen is available, delivered by a residual coronary flow. Alternatively, a simple increase in residual flow may prove as beneficial. The benefits of the above interventions have been shown particularly on reperfusion, when oxygen is resupplied [324, 326, 351].

Alternative strategies would be to modulate the effects of the metabolites associated with increased glucose metabolism. For instance, a decrease in NADH levels or H^+ accumulation may enhance ATP production. Trimetazidine, an anti-ischaemic agent, may act as an intracellular buffer [41], thereby exerting protective effects. The deleterious effects of sugar phosphates on Ca^{2+} homeostasis also need to be investigated, as a focus of possible avenues of therapy.

Appendix I: Aspects of glucose metabolism

1) PATHWAYS OF GLYCOGEN SYNTHESIS AND BREAKDOWN

a) Glycogen synthesis

Glycogen is synthesised from UDP-glucose (uridine diphosphate glucose), an activated form of glucose derived from G1P and uridine triphosphate (UTP), with formation of pyrophosphate (PPi) (see Fig AI. 2). Pyrophosphate is hydrolysed to 2 Pi, an essentially irreversible reaction which drives glycogen synthesis. The UDP is then cleaved from the glucose by glycogen synthase and the glucose moiety is attached to the non-reducing end of a glycogen branch (see Fig AI. 1).

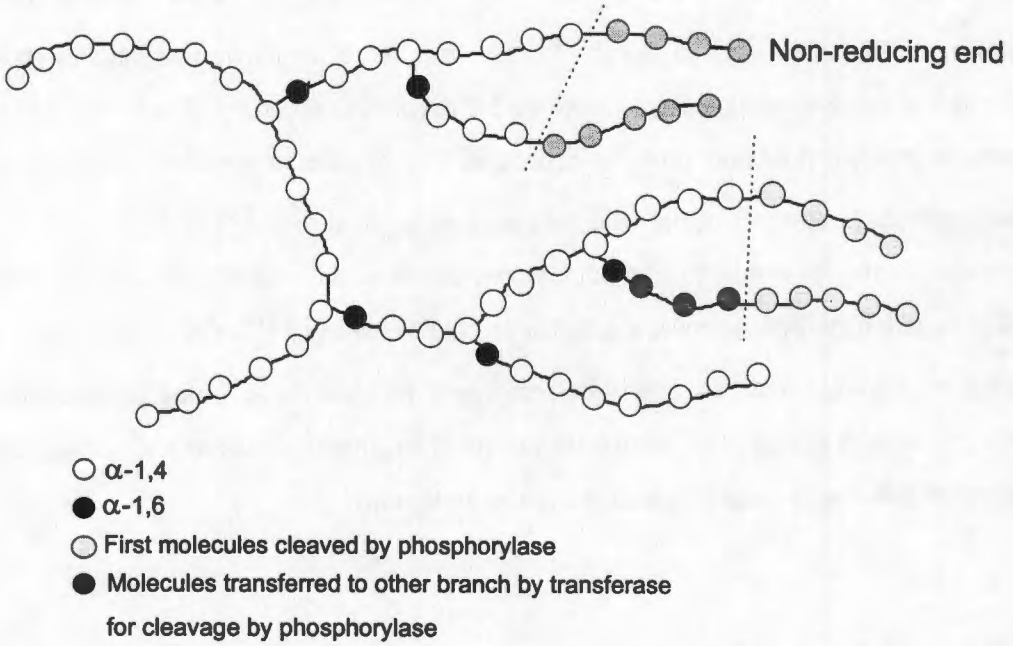


Fig AI.1: Classic macromolecular glycogen is a branched molecule, with 93% α-1,4 linkages, and 7% α-1,6 linkages. Glycogen consists of about 10 000-30 000 molecules of glucose, with a molecular weight of 10^7 Da and constitutes about 1% of muscle mass. It forms large granules in the cytoplasm which can be clearly seen under the electron microscope. A portion of the glycogen molecule is illustrated showing points of cleavage. Phosphorylase (see Fig AI.2) stops cleaving when there are 4 terminal residues from the branch point. A transferase transfers the remaining α-1,4 residues to the linear branch. The remaining α-1,6 bond is hydrolysed by amylo-1,6 glucosidase. Each circle represents a C6 molecule.

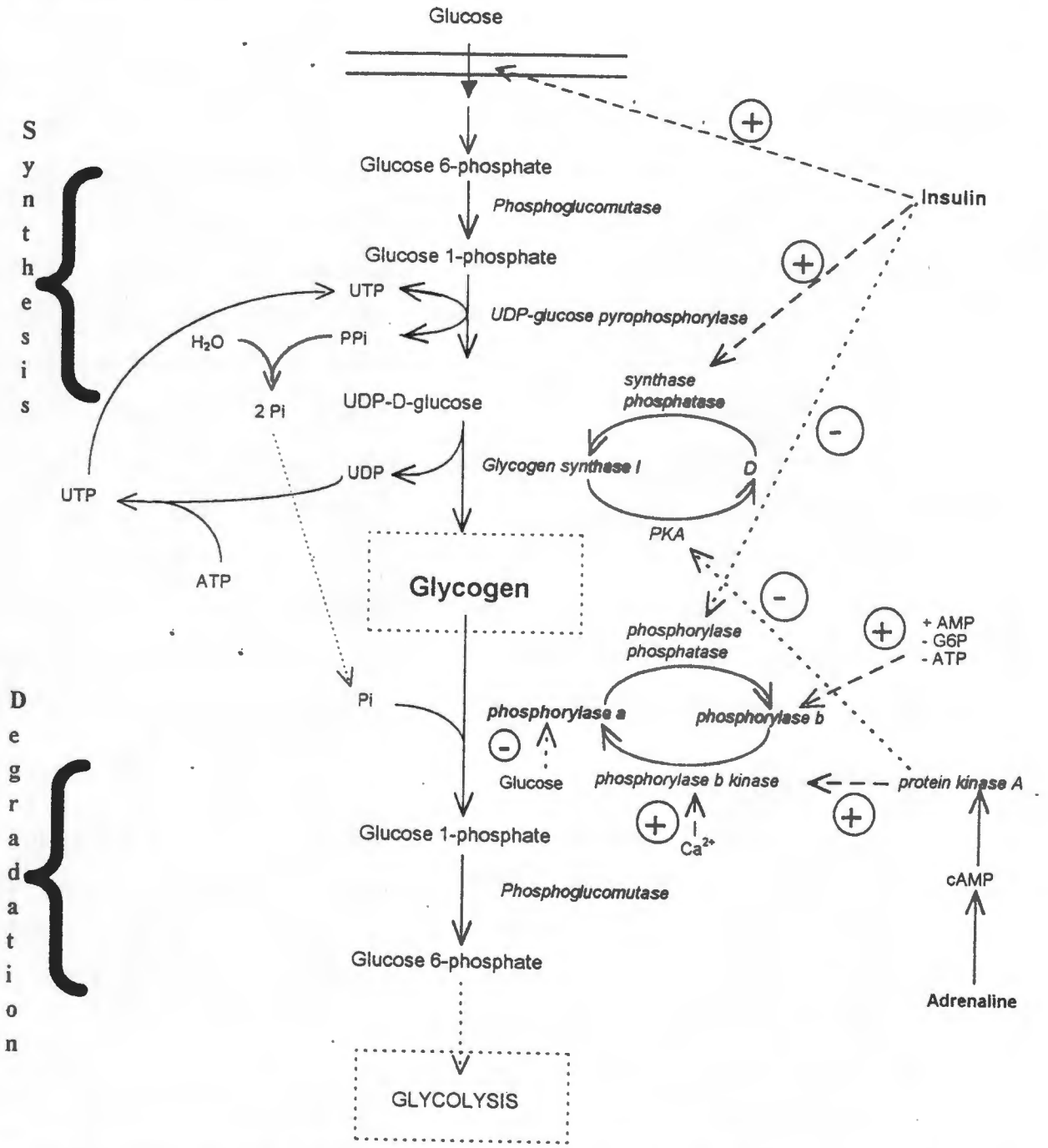


Fig AI.2. Glycogen synthesis - Glucose is activated by the addition of UDP. Glycogen synthase then cleaves UDP from UDP-glucose, and attaches the glucose moiety to the non-reducing end of a glycogen branch. Synthase phosphatase activates glycogen synthase by converting the enzyme from an inactive phosphorylated (b or D) to an active dephosphorylated form (a or I) (D - dependent on; I - independent of G6P). PKA phosphorylates and thus inhibits glycogen synthase. G6P is a potent stimulator of glycogen synthase activity (from 10, 522).

Glycogen breakdown - Phosphorylase is the main enzyme of glycogen breakdown. Phosphorylase activity is upregulated by phosphorylation (from b - inactive to a - active) by a Ca-dependent phosphorylase b kinase, using a molecule of ATP. The Ca-dependence of phosphorylase b kinase ensures a close co-operation between contraction and glycogen breakdown. This kinase is in turn activated by phosphorylation, which is catalysed by cAMP-dependent protein kinase following β-stimulation. PKA simultaneously inhibits glycogen synthesis. α-1,6 bonds, however, cannot be degraded by phosphorylase. Debranching is required for the exposure of single chain residues for phosphorylation (see Fig AI.1).

Phosphorylase phosphatase inhibits phosphorylase by dephosphorylation 100, 297. Inactive phosphorylase b can be directly stimulated by increases in AMP, and decreases in G6P and ATP, while phosphorylase a is inhibited by high concentrations of glucose.

About 9 protein kinases also phosphorylate/dephosphorylate the enzyme [297], the most important being the cAMP-dependent protein kinase A [100]. PKA phosphorylates glycogen synthase, inhibiting glycogen synthesis [289]. Protein kinase C and phosphorylase kinase also inhibit the enzyme.
Insulin stimulates glycogen synthesis by enhanced glycogen synthase dephosphorylation, via a number of protein kinases [297]. Insulin promotes dephosphorylation, and thus inactivation, of phosphorylase, the primary enzyme in glycogen breakdown. Insulin also greatly increases glucose uptake, increasing substrate levels for glycogen synthesis. Fasting (short term) increases glycogen deposition by enhanced fatty acid oxidation, and inhibition of glycolysis [487]. Transient ischaemia also activates glycogen synthase, possibly via G6P-mediated activation of phosphatase [350], thereby stimulating glycogen synthesis. This is contrary to the normal concept of ischaemia-induced glycogen breakdown. Two forms of glycogen synthase, one acting on proglycogen another on macromolecular glycogen, have been identified [10], possibly accounting for different rates of synthesis of the different forms. The rates of breakdown may also be different [103]. Glycogen appears to oscillate between proglycogen and macromolecular glycogen, with proglycogen as a stable intermediate [10].

b) Glycogen breakdown

Glycogen breakdown follows a simple pathway (Fig AI.2), with a molecule of Pi consumed per glycogen residue, catalysed by phosphorylase. G1P is produced, which is converted to G6P by phosphoglucomutase, an enzyme which favours the formation of G6P, unless this is in high concentrations. G6P then enters the glycolytic pathway, for conversion to pyruvate. Glycogenolysis is stimulated by hypoxia and ischaemia, glucagon, and epinephrine.

2)ALTERNATE FATES OF METABOLITES OF GLYCOLYSIS

a) Glucose 6-phosphate

G6P is one of the main branch points of carbohydrate metabolism (see Fig AI.3). G6P is the precursor of glycogen synthesis, as well as the entry point of glycogen breakdown into glycolysis. G6P can also be used to restore levels of NADPH and D-ribose 5-phosphate by the pentose phosphate pathway.

b) Glyceraldehyde 3-phosphate and dihydroxyacetone phosphate.

GAP and DHAP are interconverted by triose phosphate isomerase (see Fig AI.3). GAP can be converted to glycerol, and then glycerol 3-phosphate (α-glycerophosphate - αGP) but the more usual reaction is the conversion of DHAP to αGP, which can then be incorporated into triglycerides.

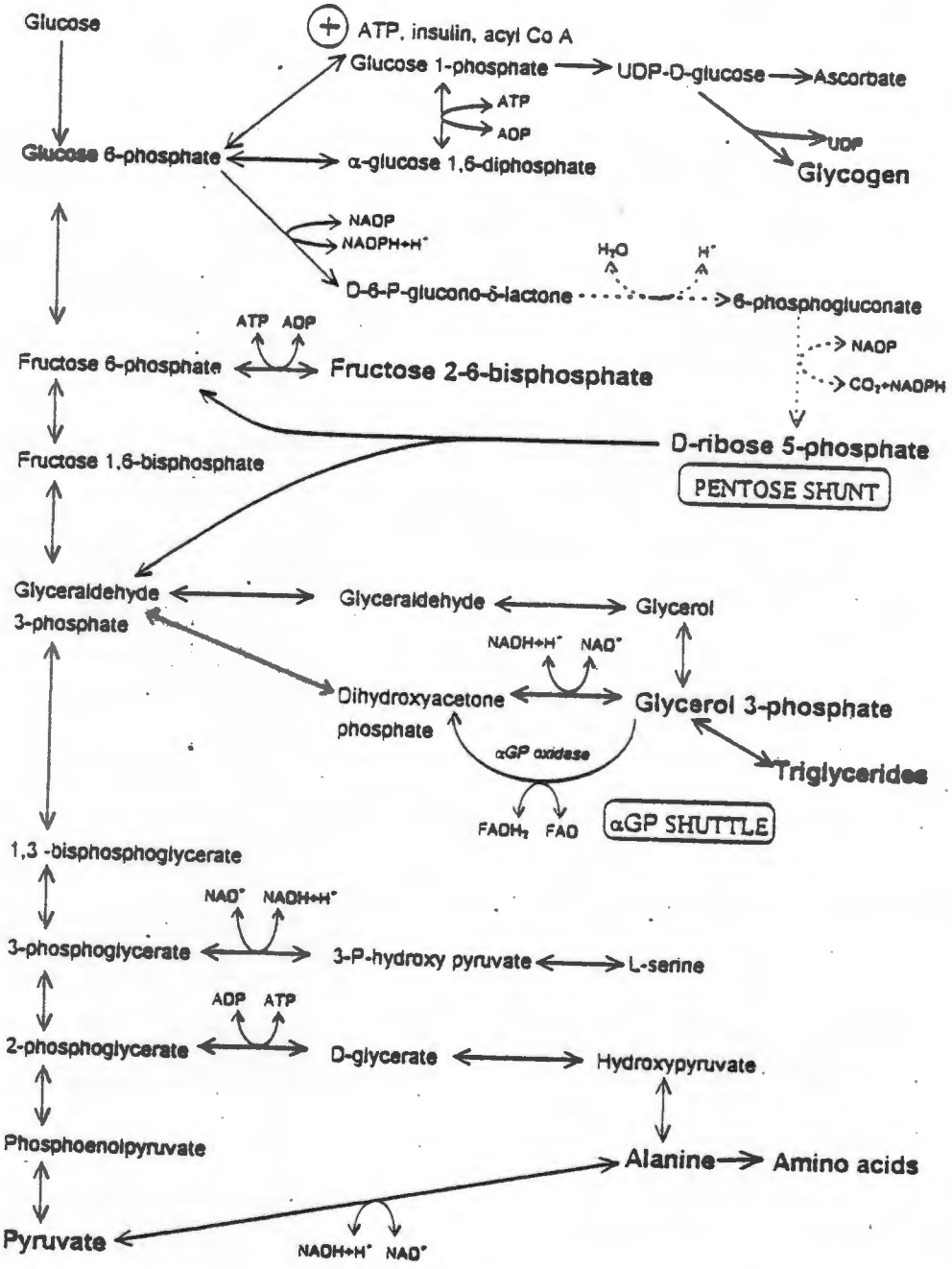


Fig AI.3. Alternative fates of metabolites of glycolysis.
G6P is a junction for the formation and breakdown of glycogen, as well as the pentose shunt. G6P is converted to D-6-P glucono-δ-lactone by glucose 6-phosphate dehydrogenase, which uses NADP. This compound is hydrolysed to 6-phosphogluconate which is converted to D-ribose 5-phosphate, with an additional NADP utilised. 2 molecules of NADPH are thus regenerated. D-ribose 5-phosphate can then be converted to F6P or GAP, thereby re-entering the glycolytic pathway. The proportions which flow along the pathway are dependent on the requirement of the cell for D-ribose 5-phosphate, NADPH, and continued glycolysis.
αGP shuttle - conversion of DHAP to αGP is catalysed by α-glycerophosphate dehydrogenase, with reduction of NADH to NAD^+. αGP, readily permeable to the mitochondrial membrane, can subsequently be re-oxidised to DHAP by αGP oxidase, a transmembrane mitochondrial protein linked to FAD with the consumption of a molecule of O_2, and production of H_2O.. This system allows transport of NADH into the mitochondria against a high concentration gradient, although the cost is an ATP molecule, given that only 2 ATP are produced per $FADH_2$, rather than 3 per NADH. However, the level of αGP oxidase is low in muscle [473]. αGP increases during ischaemia as a product of glycolysis. In addition, αGP is required in the formation of triglycerides.
Other glycolytic metabolites contribute to various pathways as shown. These are not considered important in ischaemia. For fates of pyruvate see Fig AI.4.

c) Pyruvate

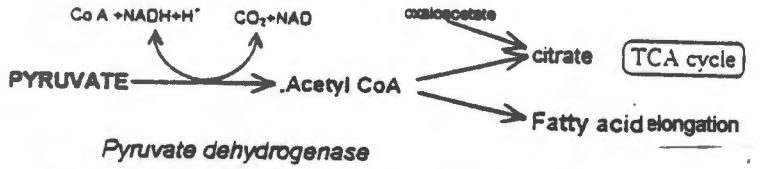
The major product of glycolysis is pyruvate. From this point there are a number of options, dependent on the status of the cell (see Fig AI.4). Under normal conditions, pyruvate is converted to acetyl Co A by pyruvate dehydrogenase (PDH). Acetyl CO A then enters the TCA cycle by combining with oxaloacetate to form citrate (Fig AI.5), or goes to the formation of fatty acids. Pyruvate can also be exported from the cell, together with a H^+.

In anaerobic conditions, or with exercise, the TCA cycle is inhibited by an accumulation of NADH. Pyruvate is then converted to lactate by lactate dehydrogenase, with the regeneration of NAD. Lactate is a "dead end", its only possible fate reconversion to pyruvate under conditions of adequate oxygenation, or else export from the cell (also with a H^+). However, the regeneration of NAD^+ during the pyruvate-to-lactate conversion allows glycolysis to continue. When sufficient oxygen is available, NADH will be regenerated, with the oxidation of lactate to pyruvate. NADH can then be transported into the mitochondria, and utilised by oxidative phosphorylation. If glycolysis continues in the absence of oxygen, H^+ accumulate from a reduced turnover of ATP [156]. Thus lactate and H^+ accumulate as a result of increased anaerobic glycolysis. Pyruvate is also important as an anaplerotic substrate (mechanisms which "top up" the TCA cycle intermediates), supplying α-ketoglutarate and oxaloacetate to the TCA cycle (see Fig AI.5). Pyruvate is converted to oxaloacetate in the mitochondria by pyruvate carboxylase (see Fig AI.4). While activity of this enzyme is low in heart muscle, pyruvate carboxylation and subsequent contribution to anaplerotic pathways has been found [431]. Phosphoenolpyruvate can be converted to oxaloacetate in the cytosol by phosphopyruvate carboxylase. Alanine is an important product of glycolysis, and accumulates during ischaemia [432]. Alanine can also contribute to anaplerotic mechanisms, and to amino acid synthesis from glycolysis.

3) SEQUENTIAL PATHWAYS AND ALTERNATE SUBSTRATES

1) TRICARBOXYLIC ACID CYCLE

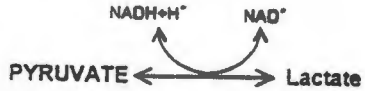
The TCA cycle (see Fig AI.5) is the meeting point of substrate metabolism, whereby reducing equivalents for subsequent generation of ATP by the respiratory chain are produced. Acetyl Co A is the entry point of most substrates into the TCA cycle, and NADH and $FADH_2$ are the end products, which enter oxidative phosphorylation with the formation of high energy phosphates. The TCA cycle is the most important generator of ATP of each substrate, such that an additional 36 ATP can be produced from glucose, and for a fatty acid such as palmitate, 130 ATP can be formed (after oxidative phosphorylation). Completion of the TCA cycle is required for the complete oxidation of these substrates. Several glycolytic metabolites are important as anaplerotic substrates, to replenish the TCA substrates (see above).



Pyruvate dehydrogenase

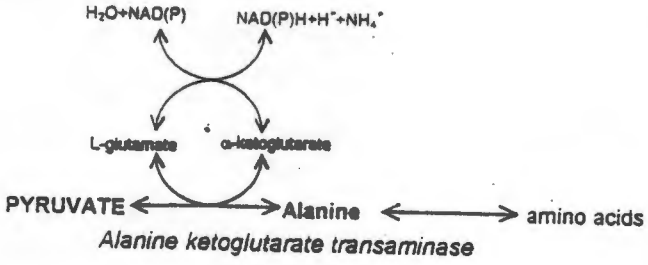
⊕ pyruvate, ADP, AMP, CoA, NAD, GMP, Mg^{2+}, Ca^{2+}, catecholamines
dephosphorylation (*PDH phosphate phosphatase*)

⊖ ATP, Acetyl Co A, NADH, fatty acids, ketone bodies
phosphorylation (*PDH kinase*)

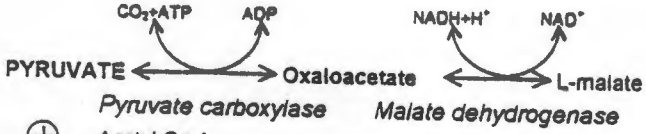


Lactate dehydrogenase

⊕ NADH, acetyl Co A

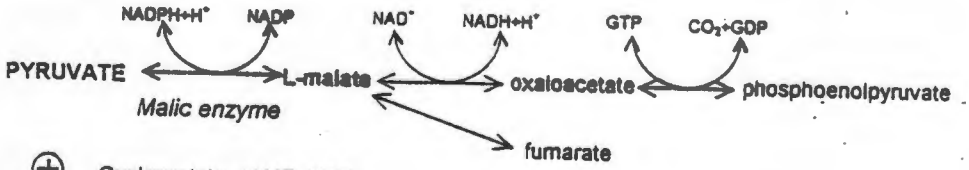


Alanine ketoglutarate transaminase



Pyruvate carboxylase *Malate dehydrogenase*

⊕ Acetyl Co A
⊖ ADP



Malic enzyme

⊕ Oxaloacetate, cAMP, NAD
⊖ Co A

Fig AI.4. Fates of pyruvate. Pyruvate is normally converted to acetyl Co A by pyruvate dehydrogenase (PDH). PDH is a complex of three enzymes which require a number of cofactors. The enzyme complex is tightly regulated by the ratios of NADH/NAD^+, acetyl Co A/Co A, and ATP/AMP ratios (increases in which inhibit the enzyme - end product inhibition), as well as phosphorylation (inactivation - by PDH kinase) and dephosphorylation (activation - by PDH phosphate phosphatase).

Under anaerobic conditions, pyruvate is converted to lactate to allow regeneration of NAD^+, and continuation of glycolysis. Pyruvate can also be converted to alanine, with the concomitant transamination of glutamate, and the formation of α-ketoglutarate. Pyruvate can also be converted to oxaloacetate by pyruvate carboxylase, and then to malate, or directly to malate by malate dehydrogenase.

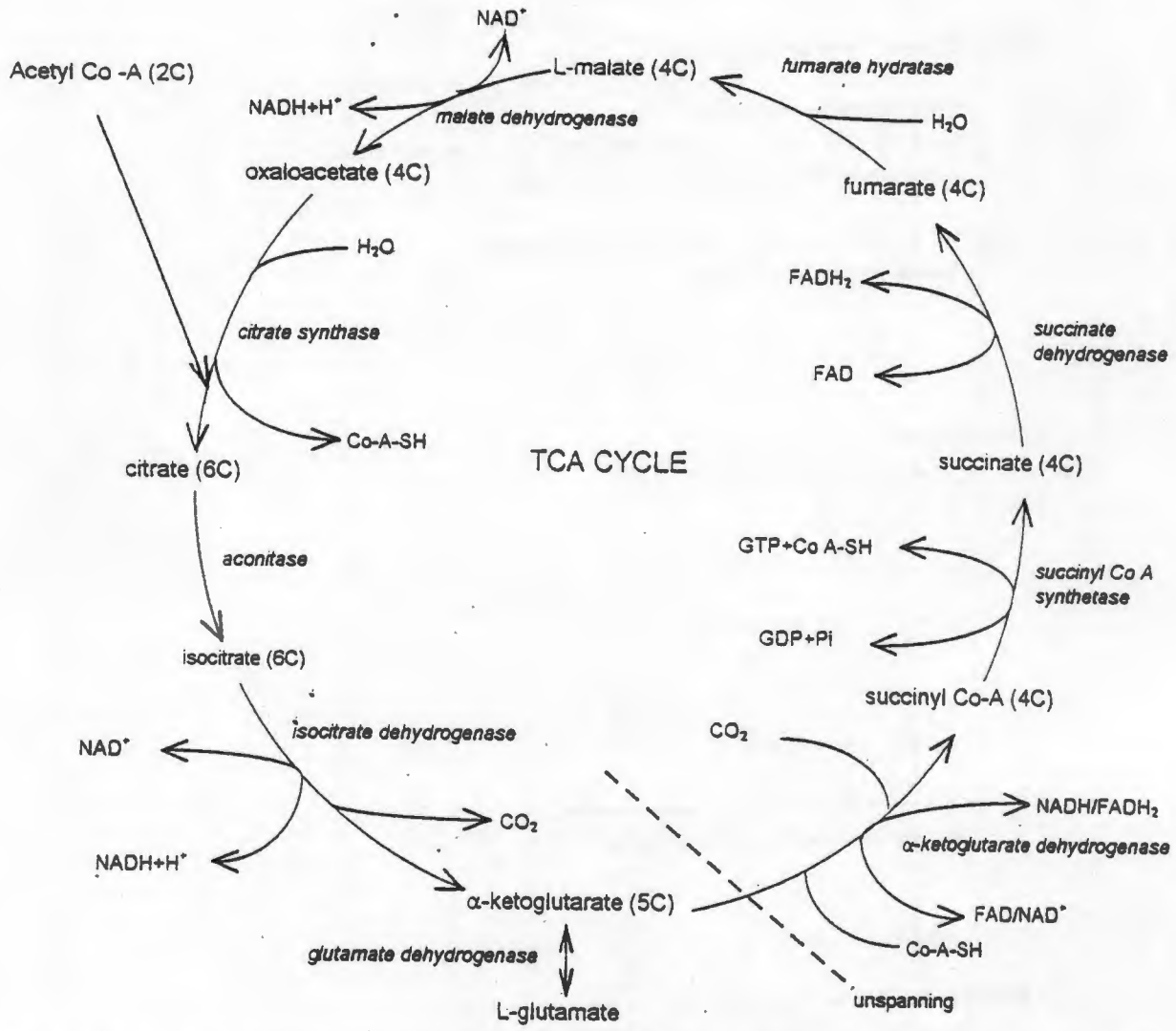


Fig AI.5. Acetyl Co A crosses the mitochondrial barrier, and then combines with oxaloacetate, with the release of Co A. The reaction is catalysed by citrate synthase, one of the regulatory enzymes of the TCA cycle, dependent largely on the level of oxaloacetate (OAA) and acetyl Co A. Citrate is transformed by aconitase to isocitrate, which is then oxidised to α-ketoglutarate (αKG or 2-oxoglutarate), with the release of CO_2, and the formation of NADH. αKG is the second regulated compound of the TCA cycle, as it can be used for the transamination of many amino acids, with the concomitant production of glutamate. αKG is involved in the anaplerotic pathways, and regeneration of NAD^+ and is the entry point for some of the 5-C amino acids into the TCA cycle.

The conversion of αKG to succinyl Co A is catalysed by αKG dehydrogenase. This step can be inhibited leading to unspanning of the TCA cycle. NADH and $FADH_2$ are released, with the release of CO_2. The Co A picked up in this reaction is lost in the next in the sequence, with the conversion of succinyl Co A to succinate, with the concomitant conversion of GDP + Pi to GTP. Succinate is then oxidised to fumarate, with the production of $FADH_2$. Fumarate is hydrolysed to malate, another important regulated metabolite. Malate is oxidised to oxaloacetate by malate dehydrogenase (MDH) using NAD^+. This enzyme has a high activity, with the equilibrium largely towards malate formation. MDH and citrate synthase compete for OAA as substrate. Cytosolic and mitochondrial MDH is important in the regulation of redox potential, and cell metabolism.

Following a surge in metabolic activity, e.g. increased work load, change in substrate etc., the isolated heart demonstrates that the TCA cycle operates in two "spans" - one regulated by citrate synthase (from citrate to αKG) and one by αKG synthase (αKG to OAA). If there is a large increase in acetyl Co A, flux through citrate synthase is increased while that through αKG synthase is reduced [587]. αKG in the mitochondria increases and can be exported out of the mitochondria in exchange for malate (Fig AI.6). Malate is then converted to OAA to supply substrate for citrate synthase. The TCA cycle is thus "unspanned". αKG synthase is possibly inhibited by end product inhibition i.e., $NADH+H^+$ and succinyl Co A. Balance is restored by glutamate-aspartate exchange, with increased formation of αKG from OAA overcoming inhibition of αKG synthase (Fig AI..6).

The "rate" of the TCA cycle is controlled by the rate of ATP production by oxidative phosphorylation of ADP. The determinants of mitochondrial respiration are the extra-mitochondrial [ATP]/[ADP]x[Pi] (cytosolic phosphorylation potential [553]) and the intramitochondrial $NADH/NAD^+$ as well as the oxygen availability. If ATP increases, NADH and $FADH_2$ will not be oxidised, and the lack of FAD and NAD^+ will slow down the TCA cycle.

2) Regulation of NAD^+/NADH, glycolysis and the TCA cycle

a) Lactate and α-glycerophosphate

Under conditions where acetyl Co A accumulates, when there is insufficient oxygen, or when NADH accumulates, NAD^+ must be regenerated, both to ensure continued glycolysis at the levels of PFK and GAPDH, and maintain turnover of the TCA cycle. The former is cytosolic, the latter mitochondrial. Cytosolic NADH can be converted to NAD^+ by the pyruvate-lactate reaction catalysed by lactate dehydrogenase (Fig AI.4). As mentioned above, this is a dead end - a temporary mechanism whereby NAD^+ can be regenerated to allow continued glycolysis. Levels of cytosolic NADH can also be lowered by the reduction of DHAP to αGP by αGP dehydrogenase (Fig AI.3). However, the importance of this shuttle in the heart has been disputed [473], given the low activity of αGP dehydrogenase in heart muscle [349]. In addition, under normal conditions, the level of αGP is far below the Km for αGP oxidase. In ischaemia, αGP would be expected to increase [468] and may be important under these conditions [214, 473].

b) Malate-aspartate shuttle

The malate-aspartate shuttle is perhaps the most important mechanism whereby the cytosolic and mitochondrial levels of NADH are regulated [473] (Fig AI.6). There is an overall shift of malate into the mitochondria, and aspartate out of the organelle. This exchange is also involved in regulation of the TCA cycle (see Fig AI.5).

This flux is readily reversible. Its direction is determined by the cytosolic to mitochondrial NADH/NAD, such that a higher cytosolic ratio drives NADH transport into the mitochondria. This transfer does not utilise energy, but allows production of 3 ATP per NADH. If oxygen is limiting, NADH will accumulate in the mitochondria, and will prevent transport of NADH from the cytosol. Cytosolic NADH will then accumulate, which may affect redox-dependent reactions including glycolysis. The malate-aspartate shuttle can be reversed if there is an increase in NADH in the mitochondria. with the formation of malate. Malate can be transported out of the mitochondria, and converted to pyruvate by NADP-dependent malic enzyme, or to oxaloacetate by MDH (Fig AI.6). The formation of alanine from pyruvate, with the concomitant transamination of glutamate to α-

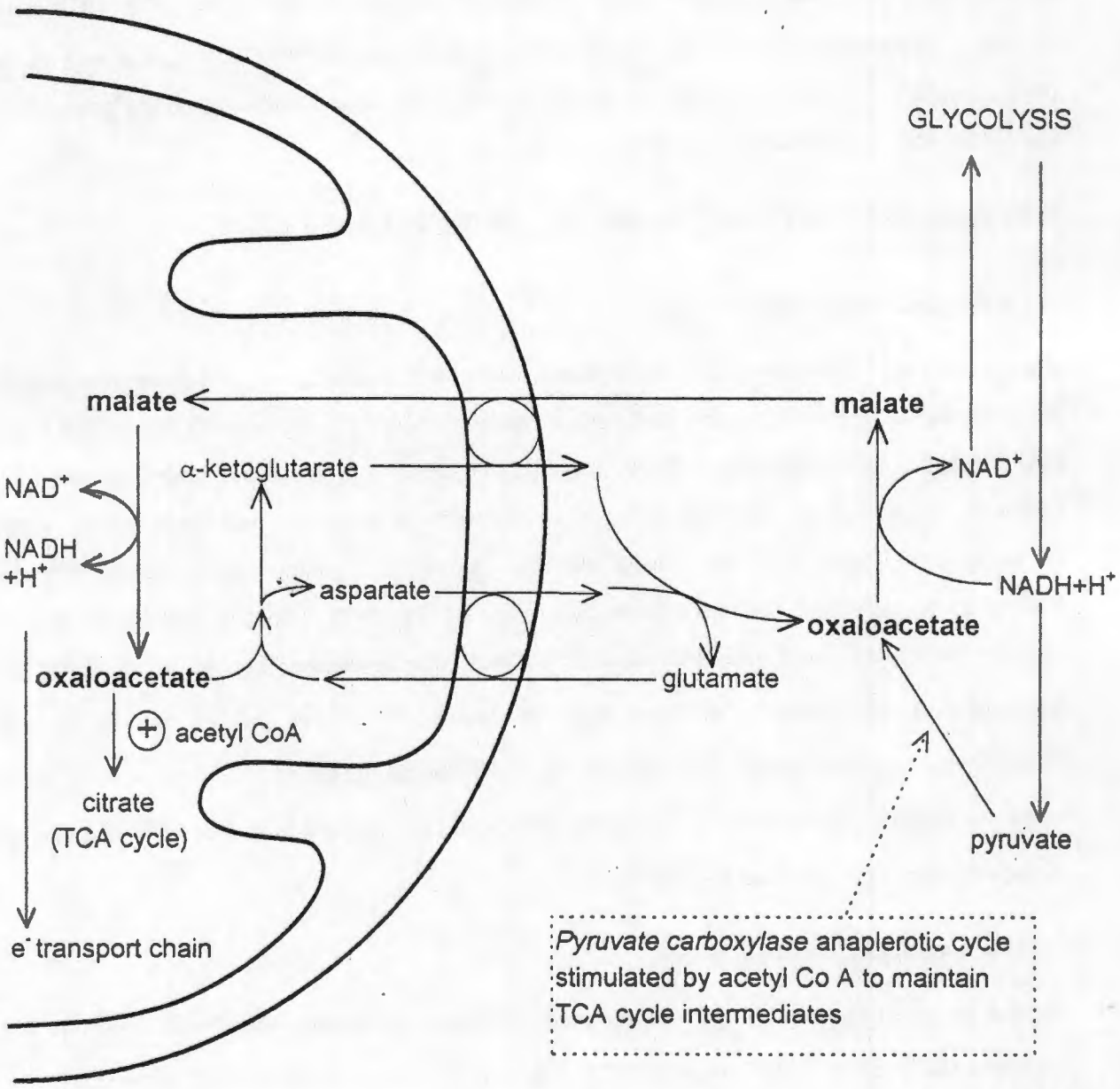


Fig AI.6. The malate-aspartate shuttle. A cytosolic accumulation of NADH following increased glycolysis, shifts the activity of MDH in the direction of malate formation from oxaloacetate. The malate crosses the mitochondrial membrane in exchange for α-ketoglutarate, and is then reconverted to oxaloacetate by mitochondrial MDH. NADH is thus regenerated in the mitochondria and can enter the respiratory chain. In addition, oxaloacetate replenishes the TCA cycle, combining with acetyl Co A to form citrate. Oxaloacetate can also combine with glutamate to form α-ketoglutarate and aspartate. α-ketoglutarate then continues to drive the entry of malate, as aspartate exits the cell in exchange for glutamate. In the cytosol, the α-ketoglutarate and aspartate recombine to form oxaloacetate and glutamate. There is thus an overall shift of malate into the mitochondria, and aspartate out of the organelle when cytosolic NADH increases. The whole shuttle can reverse, such that malate is transported out of the mitochondria, converted to oxaloacetate, and then to pyruvate. Pyruvate can also contribute to oxaloacetate formation via pyruvate carboxylase, or to malate by malic enzyme. These mechanisms, and the malate-aspartate shuttle, result in the anaplerotic contributions of pyruvate.

ketoglutarate, replenishes the latter as well, in an important anaplerotic mechanism. The levels of α-ketoglutarate are important in maintaining the second "span" of the TCA cycle (see Fig AI.5).

3) OTHER SUBSTRATES

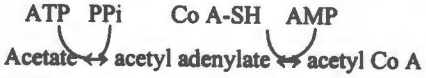
a) Fatty acid metabolism

Free fatty acids are the major substrate in the normal heart, especially in the fasted state. Increased free fatty acids reduce glucose utilisation [388, 452, 496]. Fatty acids are taken up across the cell membrane in a concentration-dependent process. Triglycerides are formed from acyl Co A and αglycerophosphate, the latter a product of glycolysis. For each mol of triglyceride produced, 3 mol H^+ are produced, which can increase acidosis in ischaemia [107]. Endogenous lipids are not normally used as an energy source, given sufficient external substrate, but may provide energy substrate e.g. in a perfused heart with limited external substrate [409]. When required as substrates, free fatty acids are activated by Co A and ATP, and converted to acyl Co A. Acyl Co A is transported into the mitochondria by carnitine, and converted to acetyl Co A by β oxidation. β oxidation is stimulated by falls in $NADH+H^+$ and $FADH_2$, and inhibited by increased reducing equivalents which occurs when oxygen falls.

Fatty acids have a higher requirement for oxygen than other substrates, but yield a higher ATP per molecule. Fatty acids are therefore useful when oxygenation is high, but are "oxygen-wasting". In conditions of ischaemia, fatty acids exert a toxic effect, as well as consuming available oxygen rapidly. The breakdown products of fatty acids (acyl Co A, acyl carnitine, and lysophosphoglycerides) may be involved in many of the deleterious effects in ischaemia, particularly arrhythmogenesis [409].

b) Acetate metabolism

Acetate is a 2-carbon compound which can be utilised as a substrate under aerobic conditions. It is broken down to acetyl Co A by a two step process catalysed by acetyl Co A synthetase.



Acetate is the metabolite of ethanol which can be utilised by the TCA cycle. Two high energy phosphate bonds from a molecule of ATP are required for acetate utilisation, which may be detrimental when ATP is depleted e.g. ischaemia. Acetate has a lower P/O ratio, implying reduced efficiency as a substrate, and a greater requirement for oxygen. Acetate perfusion increases the tissue content of acetyl Co-A, citrate, glutamate and malate, increasing the TCA cycle turnover by 50-60%, and inhibits glycolysis by increasing citrate levels [386]. However, acetate can be used to deplete glycogen in an isolated perfused heart, in the absence of any additional substrate. The mechanism

may be the lower P/O ratio which reduces the efficiency of ATP production, stimulating glycogen utilisation to prevent a shortfall in energy.

4) OXIDATIVE PHOSPHORYLATION

The end products of substrate utilisation are $NADH+H^+$ and $FADH_2$. These reducing equivalents provide the energy for the coupled phosphorylation of ATP from ADP + Pi via the oxidation

$$NADH + H^+ + \tfrac{1}{2}O_2 \leftrightarrow H_2O + NAD$$

This reaction is driven by the proton-motive force across the mitochondrial membrane i.e., a pH gradient.

$$ADP + Pi + NADH + H^+ \leftrightarrow ATP + H_2O + NAD$$

Three electrons are associated with NADH + H+, each of which allows phosphorylation of an ADP. $FADH_2$ only generates 2 ATP because its electrons enter lower down the respiratory chain. Ca^{2+} can compete with H^+, and enter the mitochondria in this way.

ADP and ATP are not freely permeable to the membrane, and are moved by a ATP-ADP translocase, the activity of which may be inhibited by acyl Co A. The amount of ADP is a critical regulator of oxidative phosphorylation, and therefore of the TCA cycle. This is usually described as the energy charge, or phosphorylation potential [ATP]/[ADP]*[Pi] [158, 553].

Appendix II: Adaptation of macrocuvette spectrophotometric methods for the Cobas Fara II Clinical Analyser

a) Adaptation of biochemical methods for the Cobas Fara

A standard reagent volume of 120 µl was chosen for the majority of adapted assays, as this gives allows for a greater substrate content. This allows for a total end-reaction volume (reagent + sample + diluent + enzymes) of less than 250 µl, which gives absorbance data approximately equivalent to a 1 cm fixed light path reading. The sample to volume ratio was maintained initially, but altered according to different concentrations in the samples. A standard sample volume of 10 µl was chosen initially. The diluent volume should initially be set at twice the sample volume, or a minimum of 20 µl. The principles of each reaction were considered, and the above calculations taken into account. The manual techniques were used, with selection of the appropriate reaction mode on the Cobas Fara II. The appropriate calibration mode was chosen (usually linear regression with a series of incremental standards). A reagent blank was included for all assays (usually distilled H_2O). The wavelength was usually set at 340 nm, either at room temperature or at 37°C.

If pre-made kits were used (cited in notes), the reagents were sometimes reconstituted at a factor less than that recommended by the manufacturers for the macro-cuvette methods. The factor was calculated from the diluent (at least 20 µl * no of samples in kit for macro method). The volumes used to reconstitute the reagents are given with the methods. The incubation periods were generally shorter because of much smaller volumes. A standard curve was generated for each assay to determine changes in substrates, reagents enzymes etc. While one standard curve can be used for several runs on the same day, it is recommended that new standards are run each day for the majority of the assays. Most standards are stored in the freezer (except where stated) in 1 mM aliquots, made from a 10 mM stock solution. Most solutions used in the assays can be stored in the fridge or freezer (unless stated otherwise).

Given that each rotor has 30 cuvettes, 1 air + 1 blank + 4 standards allows 24 samples to be determined in each run. For each assay, the amount of reagent for 35 samples is calculated to allow for dead space. If more or less samples are to be done, this can be calculated from the volume/cuvette for each reagent, taking into account dead space volumes (see below).

b) Standard procedure

Reagents - the reagent cocktail (for 35 samples) is placed into large reagent container in position R on reagent rack (see Fig M.6).

Start reagent 1 (enzyme) into cup in position P1

Reagent 2 (enzyme) into cup in position P2.

If three reagents are added (see ADP + AMP assay), the first reagent can be placed in position R once initial reagent pipetting is completed, and then reagent 2 is placed in P1 and reagent 3 in P2. If 4 reagents are used (see ATP + CP assay), the first reagent is added to the reagent mixture (if this does not affect baseline reading), and the others in R, P1 and P2 respectively.

Enzymes - place in diluent cups in larger reagent cups to reduce dead volume. At least 70 µl dead volume + additional enzyme adequate for total number of samples assayed is needed (usually 200 µl added). A standard volume of 5 µl enzyme per sample was used in the assays. Only 1 µl enzyme was generally needed, thus enzymes were diluted 1:5 on day of use.

If the start reagent was placed in R position, the total volume must be increased because the larger pipette tip is used, which is less accurate. A minimum volume which can be taken up by the reagent pipette is 20 µl (see ATP + CP assay). A 1:20 dilution is used in these cases.

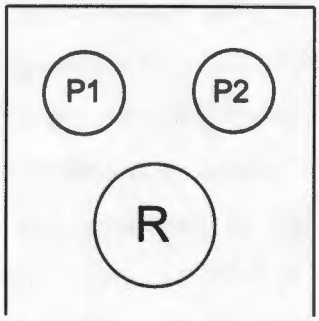


Fig M.6. Illustration of reagent positions on reagent rack.

Samples - Approximately 200 µl of sample (from extractions or effluent) is pipetted into sample cups (dead volume of 90 µl, so add more sample volume if greater amount taken for each assay, if assay to be run several times, or several assays to be run on each sample).

Standards - for assays of multiple substrates, include all standards in each standard cup, i.e. for two standards (see ATP + CP assay) make sure that final concentration is as required for each standard, taking into account dilution by other standard. The standards as shown below can be altered according to predicted values of samples.

Blanks - distilled water from source in machine

Methods - the programmes as set up on the Cobas Fara II are included below.

Calculations - while the Cobas Fara II prints out final concentrations calculated on the basis of the calibration mode chosen (usually linear regression) in the interests of saving reagent, samples etc. multiple reactions were carried out in some cases (e.g. ATP + CP, ADP + AMP). In these cases. the linear regression for each standard needs to be calculated, and the subsequent sample concentrations calculated from the absorbances measured at time points as specified below for each assay.

Appendix III. Programmes for Cobas Fara II for spectrophotometric analyses

i) Programme on Cobas Fara for ATP and CP

General		
Measurement mode:		Absorbance
Reaction mode:		P I R I SR1 I W I SR2 I W A
Calibration mode:		Linear regression
Reagent blank:		Reagent/diluent
Wavelength:		340 nm
Temperature:		25 °C
Decimal position:		4
Unit:		mmol/L
Analysis		
P	Sample volume:	30 µl
	Diluent name:	H_2O
	Volume:	20 µl
	Reagent volume:	120 µl
I	Incubation:	180 secs
	M1:	175 secs
R1	Reagent 1:	20 µl
I	Incubation:	180 secs
	M2:	175 secs
SR1	Start Reagent 1:	5 µl
	Diluent name:	H_2O
	Volume:	5 µl
I	Incubation:	120 secs
W	Wait time:	185 secs
I	Incubation:	60 secs
	M3:	55 secs
SR2	Start Reagent 2:	5 µl
	Diluent name:	H_2O
	Volume:	5 µl
I	Incubation:	200 secs
W	Wait time:	400 secs
A	Readings	
	First:	400 secs
	Number:	1
	Interval:	5 secs
Calculation:		Manual
Calibration		
Std 1:		0.0625
Std 2:		0.125
Std 3:		0.25
Std 4:		0.5

ii) Programme on Cobas Fara for ADP + AMP

General		
Measurement mode:		Absorbance
Reaction mode:		P I SR1 I SR2 A
Calibration mode:		Linear regression
Reagent blank:		Reagent/diluent
Wavelength:		340 nm
Temperature:		25 °C
Decimal position:		4
Unit:		mmol/l
Analysis		
P	Sample volume:	40 µl
	Diluent name:	H_2O
	Volume:	20 µl
	Reagent volume:	120 µl
I	Incubation:	10 secs
	M1:	10 secs
SR1	Start Reagent 1:	5 µl
	Diluent name:	H_2O
	Volume:	5 µl
I	Incubation:	60 secs
	M2:	60 secs
SR2	Start Reagent 2:	5 µl
	Diluent name:	H_2O
	Volume:	5 µl
A	Readings:	
	First:	30 secs
	Number:	5
	Interval:	20 secs
Calculation:		Manual
Calibration		
Std 1:		0.03125
Std 2:		0.0625
Std 3:		0.125
Std 4:		0.25

iii) Programme on Cobas Fara for Pi

General		
Measurement mode:		Absorbance
Reaction mode:		P A
Calibration mode:		Linear regression
Reagent blank:		Reagent/diluent
Wavelength:		340 nm
Temperature:		37 °C
Decimal position:		4
Unit:		mmol/l
Analysis		
P	Sample volume:	4 µl
	Diluent name:	H_2O
	Volume:	25 µl
	Reagent volume:	150 µl
A	Readings:	
	First:	0.5 secs
	Number:	25
	Interval:	10 secs
Calculation:		A25 - A1
Calibration		
Std 1:		0.125
Std 2:		0.25
Std 3:		0.25
Std 4:		1.0

iv) Programme on Cobas Fara for glucose

General		
Measurement mode:		Absorbance
Reaction mode:		P A
Calibration mode:		Linear regression
Reagent blank:		Reagent/diluent
Wavelength:		340 nm
Temperature:		37 °C
Decimal position:		4
Unit:		mmol/l
Analysis		
P	Sample volume:	3 µl
	Diluent name:	H_2O
	Volume:	50 µl
	Reagent volume:	200 µl
A	Readings:	
	First:	0.5 secs
	Number:	19
	Interval:	10 secs
Calculation:		A19 - A1
Calibration		
Std 1:		0.0625
Std 2:		0.125
Std 3:		0.25
Std 4:		0.5

v) Programme on Cobas Fara for G6P + F6P

General		
Measurement mode:		Absorbance
Reaction mode:		P I SR1 I SR2 A
Calibration mode:		Linear regression
Reagent blank:		Reagent/diluent
Wavelength:		340 nm
Temperature:		25 °C
Decimal position:		4
Unit:		mmol/l
Analysis		
P	Sample volume:	40 µl
	Diluent name:	H_2O
	Volume:	20 µl
	Reagent volume:	120 µl
I	Incubation:	100 secs
	M1:	100 secs
SR1	Start Reagent 1:	5 µl
	Diluent name:	H_2O
	Volume:	5 µl
I	Incubation:	150 secs
	M2:	150 secs
SR2	Start Reagent 2:	5 µl
	Diluent name:	H_2O
	Volume:	5 µl
A	Readings:	
	First:	100 secs
	Number:	5
	Interval:	40 secs
Calculation:		Manual
Calibration		
Std 1:		0.03125
Std 2:		0.0625
Std 3:		0.125
Std 4:		0.25

vi) Programme on Cobas Fara for DHAP + GAP + FDP

General		
Measurement mode:		Absorbance
Reaction mode:		P I R I SR1 I SR2 A
Calibration mode:		Linear regression
Reagent blank:		Reagent/diluent
Wavelength:		340 nm
Temperature:		25 °C
Decimal position:		4
Unit:		mmol/l
Analysis		
P	Sample volume:	40 µl
	Diluent name:	H_2O
	Volume:	20 µl
	Reagent volume:	120 µl
I	Incubation:	45 secs
	M1:	45 secs
R1	Reagent 1:	20 µl
I	Incubation:	45 secs
	M2:	45 secs
SR1	Start Reagent 1:	5 µl
	Diluent name:	H_2O
	Volume:	5 µl
I	Incubation:	180 secs
	M2:	180 secs
SR2	Start Reagent 2:	5 µl
	Diluent name:	H_2O
	Volume:	5 µl
A	Readings:	
	First:	100 secs
	Number:	4
	Interval:	30 secs
Calculation:		Manual
Calibration		
Std 1:		0.03125
Std 2:		0.0625
Std 3:		0.125
Std 4:		0.25

vii) Programme on Cobas Fara for pyruvate

General		
Measurement mode:		Absorbance
Reaction mode:		P I SR1 A
Calibration mode:		Linear regression
Reagent blank:		Reagent/diluent
Wavelength:		340 nm
Temperature:		25 °C
Decimal position:		4
Unit:		mmol/l
Analysis		
P	Sample volume:	30 µl
	Diluent name:	H_2O
	Volume:	20 µl
	Reagent volume:	120 µl
I	Incubation:	10 secs
	M1:	10 secs
SR1	Start Reagent 1:	5 µl
	Diluent name:	H_2O
	Volume:	5 µl
A	Readings:	
	First:	120 secs
	Number:	3
	Interval:	20 secs
Calculation:		Manual
Calibration		
Std 1:		0.03125
Std 2:		0.0625
Std 3:		0.125
Std 4:		0.25

viii) Programme on Cobas Fara for lactate

General		
Measurement mode:		Absorbance
Reaction mode:		P I SR1 A
Calibration mode:		Linear regression
Reagent blank:		Reagent/diluent
Wavelength:		340 nm
Temperature:		25 °C
Decimal position:		4
Unit:		mmol/l
Analysis		
P	Sample volume:	20 µl
	Diluent name:	H_2O
	Volume:	20 µl
	Reagent volume:	120 µl
I	Incubation:	80 secs
	M1:	80 secs
SR1	Start Reagent 1:	5 µl
	Diluent name:	H_2O
	Volume:	5 µl
A	Readings:	
	First:	120 secs
	Number:	10
	Interval:	90 secs
Calculation:		Manual
Calibration		
Std 1:		0.0625
Std 2:		0.125
Std 3:		0.25
Std 4:		0.5

ix) Programme on Cobas Fara for αGP

General		
Measurement mode:		Absorbance
Reaction mode:		P I SR1 A
Calibration mode:		Linear regression
Reagent blank:		Reagent/diluent
Wavelength:		340 nm
Temperature:		25 °C
Decimal position:		4
Unit:		mmol/l
Analysis		
P	Sample volume:	20 µl
	Diluent name:	H_2O
	Volume:	20 µl
	Reagent volume:	120 µl
I	Incubation:	120 secs
	M1:	120 secs
SR1	Start Reagent 1:	5 µl
	Diluent name:	H_2O
	Volume:	5 µl
A	Readings:	
	First:	900 secs
	Number:	10
	Interval:	120 secs
Calculation:		Manual
Calibration		
Std 1:		0.03125
Std 2:		0.06125
Std 3:		0.125
Std 4:		0.25

x) Programme on Cobas Fara for L-alanine

General		
Measurement mode:		Absorbance
Reaction mode:		P I SR1 A
Calibration mode:		Linear regression
Reagent blank:		Reagent/diluent
Wavelength:		340 nm
Temperature:		37 °C
Decimal position:		4
Unit:		mmol/l
Analysis		
P	Sample volume:	30 µl
	Diluent name:	H_2O
	Volume:	5 µl
	Reagent volume:	120 µl
I	Incubation:	10 secs
	M1:	10 secs
SR1	Start Reagent 1:	5 µl
	Diluent name:	H_2O
	Volume:	5 µl
A	Readings:	
	First:	900 secs
	Number:	3
	Interval:	150 secs
Calculation:		Manual
Calibration		
Std 1:		0.03125
Std 2:		0.0625
Std 3:		0.125
Std 4:		0.25

xi) Programme on Cobas Fara for citrate

General		
Measurement mode:		Absorbance
Reaction mode:		P I SR1 A
Calibration mode:		Linear regression
Reagent blank:		Reagent/diluent
Wavelength:		340 nm
Temperature:		30 °C
Decimal position:		4
Unit:		mmol/l
Analysis		
P	Sample volume:	20 µl
	Diluent name:	H_2O
	Volume:	20 µl
	Reagent volume:	120 µl
I	Incubation:	60 secs
	M1:	60 secs
SR1	Start Reagent 1:	2 µl
	Diluent name:	H_2O
	Volume:	30 µl
A	Readings:	
	First:	90 secs
	Number:	3
	Interval:	300 secs
Calculation:		Manual
Calibration		
Std 1:		0.0625
Std 2:		0.125
Std 3:		0.25
Std 4:		0.5

xii) Programme on Cobas Fara for malate

General		
Measurement mode:		Absorbance
Reaction mode:		P I SR1 I SR2 A
Calibration mode:		Linear regression
Reagent blank:		Reagent/diluent
Wavelength:		340 nm
Temperature:		30 °C
Decimal position:		4
Unit:		mmol/l
Analysis		
P	Sample volume:	20 µl
	Diluent name:	H_2O
	Volume:	20 µl
	Reagent volume:	120 µl
I	Incubation:	10 secs
	M:	no
SR1	Start Reagent 1:	5 µl
	Diluent name:	H_2O
	Volume:	30 µl
I	Incubation:	150 secs
	M2:	140 secs
SR2	Start Reagent 2:	5 µl
	Diluent name:	H_2O
	Volume:	10 µl
A	Readings:	
	First:	0.5 secs
	Number:	2
	Interval:	30 secs
Calculation:		Machine
Calibration		
Std 1:		0.0625
Std 2:		0.125
Std 3:		0.25
Std 4:		0.5

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