THE UNIVERSITY OF HULL

Studies of ischaemia and reperfusion in cardiac hypertrophy

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ABSTRACT

Cardiac hypertrophy may be associated with an enhanced susceptibility to ischaemic/reperfusion injury but the mechanisms remain unresolved. There is evidence for an increased dependence on glucose metabolism in cardiac hypertrophy, which may be beneficial in normoxia but detrimental in ischaemia. The role of glycogen, the major endogenous substrate during ischaemia, to the enhanced susceptibility of the hypertrophied heart to ischaemic/reperfusion injury is unclear. Work in this thesis investigates the role of glycogenolysis to the severity of ischaemia, and assesses oxidative substrate utilisation following reperfusion, in the hypertrophied heart.

Pressure overload cardiac hypertrophy was induced surgically in male Sprague-Dawley rats by intra-renal constriction. A moderate hypertrophy was observed nine weeks post surgery as evidenced by between a 4 and 25 % increase in heart weight: tibia length ratio. Hearts were perfused in an isovolumic mode, and function was recorded. ^{13}C-NMR spectroscopy was performed on extracts from hypertrophied and control hearts reperfused with ^{13}C labelled substrates to determine the profile of substrate use.

Glycogen content was unchanged in hypertrophied hearts compared to control hearts and there was no evidence for glycogen loading in the presence of physiological substrates and insulin. In addition, no further glycogen loading occurred when insulin concentrations were increased to pharmacological levels. Provision of other carbohydrate substrates, such as lactate, did result in a further increase in myocardial glycogen content.

Hypertrophied hearts experienced the same extent of ischaemia as controls with no evidence of increased ischaemic injury, implying that a compensated model of hypertrophy was generated in this study. Myocardial function decreased during

low flow ischaemia and stopped during global ischaemia, but contracture was not observed. The severity of ischaemia was the determining factor in the degree of glycogen degradation. Increased glycogen degradation during ischaemia did not correlate with increased ischaemic injury, suggesting that the availability of glycogen for energy provision limited ischaemic injury.

Recovery on reperfusion was markedly improved in the presence of insulin. This improvement appeared to be mediated by the inotropic actions of insulin rather than by alterations in substrate provision. The profile of substrate use in hypertrophied hearts during reperfusion was found to be the same as that in controls.

No metabolic alterations were observed in the hypertrophied heart that enhanced susceptibility to ischaemic/reperfusion injury, implying that compensated hypertrophy is a beneficial response of the heart.

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ABBREVIATIONS

ACC	Acetyl CoA carboxylase
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AngII	Angiotensin II
ATP	Adenosine triphosphate
AV	Atrioventricular
BSA	Bovine serum albumin
BWt	Body weight
cAMP	Cyclic adenosine monophosphate
CAT	Carnitine acylcarnitine translocase
CF	Coronary flow
CH	Cardiac hypertrophy
CK	Creatine kinase
CO	Cardiac output
CPT 1	Carnitine palmitoyltransferase 1
CPT 2	Carnitine palmitoyltransferase 2
D_2O	Deuterated water
DCA	Dichloroacetate
ECM	Extracellular matrix
ET	Endothelin
FABP	Fatty acid-binding protein
$FADH_2$	Flavin adenine dinucleotide (reduced)
FID	Free induction decay
g	Gravity
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GI	Global ischaemia
GIK	Glucose, Insulin, Potassium
GLUT	Glucose transporter
h	Wall thickness
HCM	Hypertrophic cardiomyopathy
HPLC	High performance liquid chromotography
HR	Heart rate
K-H	Krebs-Henseleit
lCPT 1	Liver carnitine palmitoyltransferase 1
LDH	Lactate dehydrogenase
LFI	Low flow ischaemia
LPL	Lipoprotein lipase
LV	Left ventricle
LVDP	Left ventricular developed pressure
mCPT 1	Muscle carnitine palmitoyltransferase 1
NAD^+	Nicotinamide adenine dinucleotide (oxidised)
NADH	Nicotinamide adenine dinucleotide (reduced)
NMR	Nuclear magnetic resonance

NOE	Nuclear overhauser effect
OD	Outer diameter
Pr	Pressure
PCA	Perchloric acid
PCr	Phosphocreatine
PDC	Pyruvate dehydrogenase complex
PFK	Phosphofructokinase
P_i	Inorganic phosphate
PI3-K	Phosphatidylinositol 3-kinase
PPARα	Peroxisome proliferator activated receptor α-isoform
R	Radius of the curvature of the wall
RF	Radio frequency
RPP	Rate pressure product
S	Sham
SA	Sinoatrial
SDS	Sodium dodecyl sulphate
SHR	Spontaneously hypertensive rat
SERCA	Smooth endoplasmic reticulum Ca^{2+}-ATPase
$S.O_2$	Solubility of O_2 at 37°C
SV	Stroke Volume
T	Wall tension
TCA	Tricarboxylic acid cycle
TGFβ	Transforming growth factor β
TMS	Tetramethylsilane
T-tubules	Transverse tubules
UDP	Uridine diphosphate
UDPG	Uridine diphosphoglucose
VLDL	Very low-density lipoprotein
Wt	Weight
Y	Anaplerotic flux

PERFUSION PROTOCOLS

G	Glucose only
G+P	Glucose with palmitate
GD	Glycogen depletion
GDR	Glycogen depletion/repletion
GPLP	Glucose, palmitate, lactate and pyruvate with insulin
NP	No perfusion
REP	Glucose and palmitate in the absence of insulin
REPI	Glucose and palmitate in the presence of insulin
REPI13C	Glucose and palmitate in the presence of insulin with ^{13}C-glucose and ^{13}C-palmitate during reperfusion
REPIR	Glucose and palmitate with insulin added at the time of reperfusion
REPLPI	Glucose, palmitate, lactate and pyruvate present with insulin throughout

1: INTRODUCTION

1:1 Cardiac function

The heart is central to all functions in the body. Its roles are i) to pump blood via the systemic arteries to provide oxygen and nutrients to all the tissues of the body and ii) to pump blood to the lungs for gaseous exchange [Katz 1992a]. The cycle of cardiac activity is divided into two phases, a contracting phase (systole) and a resting phase (diastole) [Sherwood 1993]. Systole can be sub-divided into contraction and ejection phases. The amount of blood pumped out of each ventricle with each contraction is known as the stroke volume (SV). Cardiac output (CO) is the volume of blood pumped by each ventricle per minute [Sherwood 1993]. Thus, CO is a function of heart rate (HR) and SV.

$$CO = HR \times SV$$

1:2 Basic cardiac structure

The heart is a muscular organ that contracts rhythmically. It is located in the thoracic cavity surrounded by a fibrous sac, the pericardium [Berne and Levy 1993]. It consists of four pumping chambers: the left and right atria and the left and right ventricles (Figure 1.1). Venous blood from the systemic circulation enters the right atrium and right ventricle, where it is then pumped into the lungs for the exchange of carbon dioxide with oxygen. The left atrium and ventricle then receive the oxygenated blood via the pulmonary vein and deliver it to the systemic arteries via the aorta [Berne and Levy 1993]. Valves, tough endothelium covered fibrous tissue, maintain unidirectional blood flow through the heart.

The walls of the ventricles consist of three regions, the epicardium (external), the myocardium (middle) and the endocardium (internal) [Junqueria *et al.* 1995].

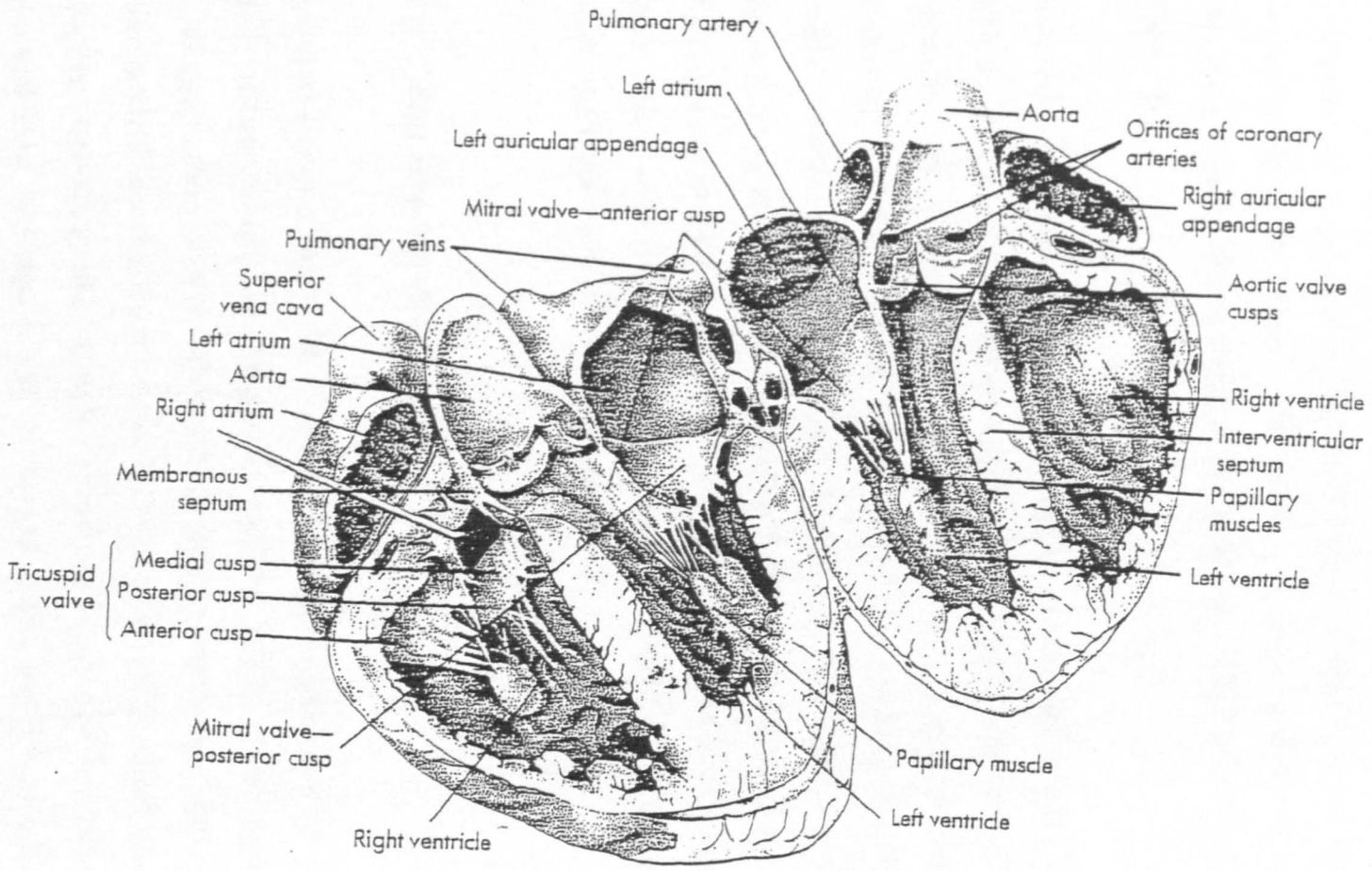


Figure 1.1: Drawing of a heart split perpendicular to the interventricular septum to illustrate anatomical relationships [Berne and Levy 1993]

The myocardium is the thickest region of the heart and is composed of cardiac muscle cells (myocytes) and fibroblasts interspersed with blood vessels containing endothelial and smooth muscle cells [Junqueria *et al.* 1995]. Cardiac fibroblasts are responsible for the production and deposition of the extracellular matrix (ECM) of the heart. The ECM contains a scaffold of non-contractile proteins, in which other structures are embedded. [Brilla *et al.* 1992, Booz and Baker 1995]

Wall thickness between the two ventricles is markedly different due to the different pressures under which they function [Berne and Levy 1993]. The left ventricle wall is the thickest. However, the general architecture of all components of the ventricle (i.e. endothelium, conduction cells, capillaries, arteries, veins, nerves) is similar in both left and right ventricles. Atrial architecture is, by and large, identical in both atria except that the sinoatrial (SA) node (pacemaker cells) and atrioventricular (AV) node are located in the right atrium close to the superior vena cava and near the atrial septum close to the AV junction, respectively [Berne and Levy 1993].

1:3 Fine myocyte structure

Myocytes only comprise 25 % of the cardiac cell population but account for the bulk of heart tissue mass [Bugaisky and Zak 1989]. Each myocyte is packed with longitudinal bundles of fibres (myofibrils) consisting of a number of smaller contractile filaments, the thick and thin filaments. These thick and thin filaments display alternating dark bands (A Bands) and light bands (I bands). Visible in the middle of the I band is a dense vertical Z line [Harrington and Rodgers 1984]. The area between two Z lines is called a sarcomere, the fundamental unit of muscle contraction (Figure 1.2).

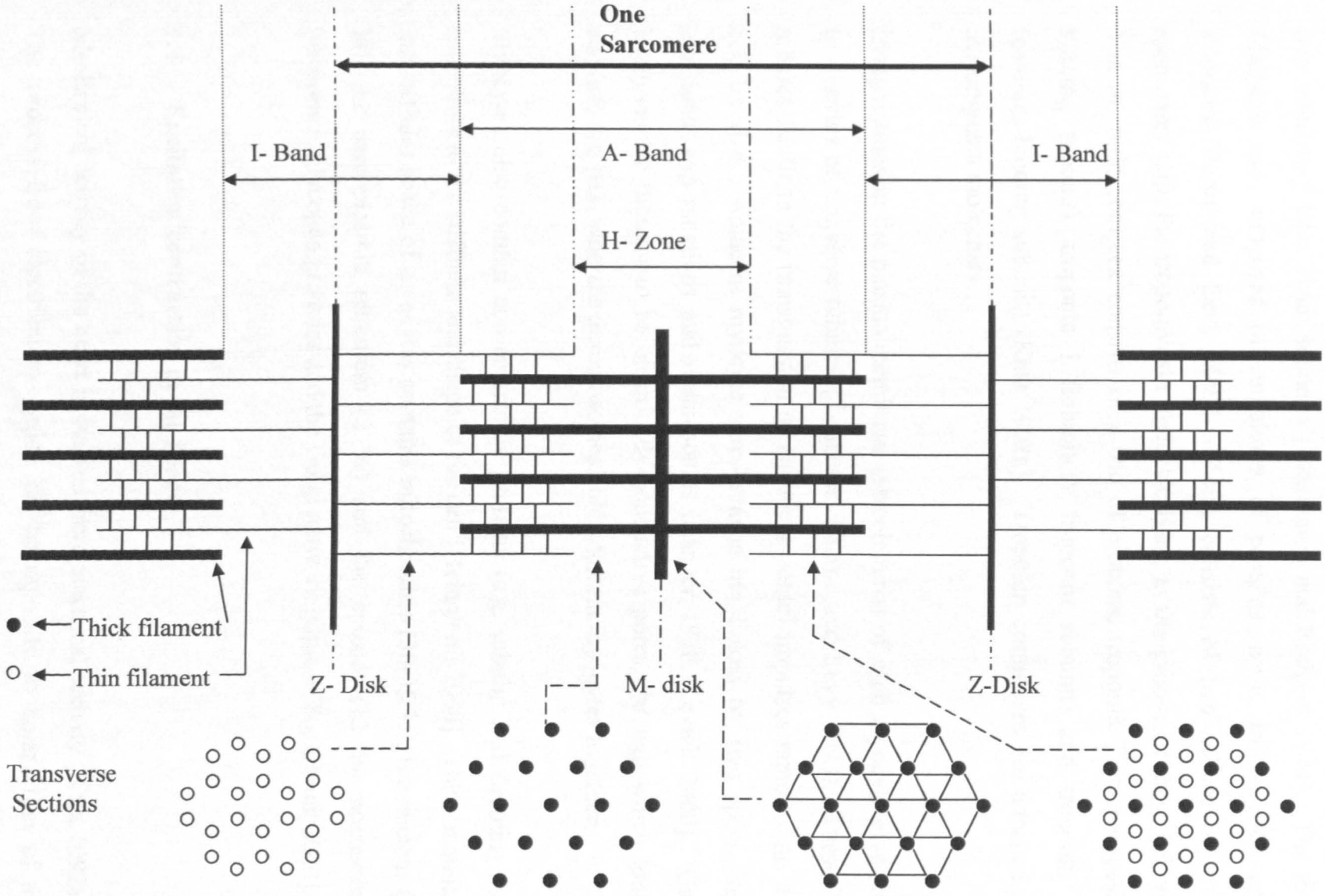


Figure 1.2: Longitudinal and cross sectional diagrams of thick and thin muscle filaments [Berne and Levy1993]

Within the sarcomere, thick filaments are composed of myosin, a large hexameric structure, consisting of two heavy chain, two essential light chain and two regulatory light chain subunits [Harrington and Rodgers 1984]. The thin filaments are composed of complexes of proteins, actin, tropomyosin and troponin [Berne and Levy 1993]. Actin consists of two helices of actin monomers with the tropomyosin molecule lying in the groove of these helices. The troponin complex consists of a trio of proteins, troponin T (tropomyosin binding subunit), troponin I (inhibitory troponin subunit) and troponin C (calcium binding subunit) [Katz 1992a]. Troponin complexes are attached to tropomyosin molecules.

Invaginations in the plasma membrane (sarcolemma) of each myocyte give rise to a series of tranverse tubules (T-tubules) [Berne and Levy 1993]. These T-tubules facilitate the transmission of chemical (ionic) impulses rapidly into the cell interior. Adjacent myocyte sarcolemmas are linked by two specialised junctions, gap junctions and desmosomes [Langer 1978, Levick 2000]. Gap junctions are thought to be electrically conductive pores, through which ionic currents can pass whereas desomosomes hold adjacent myocytes together.

Myocytes also contain non-contractile proteins (e.g. tubulin and desmin) that contribute to the stiffness and shape of the cell [Hein *et al.* 2000]. The remaining intracellular space of myocytes contains mitochondria (30-35 %), the nucleus (5 %), the sarcoplasmic reticulum (2 %) and the cytosol (12 %) possessing lysosomes, glycogen granules and the Golgi network [Opie 1986, Levick 2000].

1:4 Excitation/contraction in the heart

Mechanical activity of the heart is derived from electrical activity [Katz 1992a]. The process from electrical excitation of the myocyte to contraction of the myocyte is known as excitation-contraction coupling [Bers 2002]. The

mammalian cardiac contraction cycle is initiated by the SA node. SA nodal cells spontaneously depolarise in an autorhythmic fashion and transmit a depolarising (excitation) current to neighbouring atria cells to trigger the heart beat [Berne and Levy 1993]. The electrical impulse then reaches the AV node. The AV node is the only electrical connection between the atria and the ventricles as the annulus fibrosis completely insulates the two from one other [Levick 2000]. The dense fibrous tissue of the AV node causes the electrical impulse to slow down, delaying transfer of current to the ventricles, allowing the atria to contract before depolarisation of the ventricles [Levick 2000]. The impulse is then transmitted down the bundle of His, the bundle branches, the Purkinje network, and finally to the ventricular myocytes causing their depolarisation. As the wave of depolarisation spreads over the sarcolemmal surface and into the t-tubules it activates the voltage-dependent dihydropyridine calcium (Ca^{2+}) channels (L-type channels), allowing extracellular Ca^{2+} to flow into the myocyte [Fozzard and Arnsdorf 1991]. Influx of Ca^{2+} into the myocyte serves as a trigger to release more Ca^{2+} from the sarcoplasmic reticulum through the ryanodine receptors [Fabiato 1983], a process known as calcium induced calcium release. This combination of Ca^{2+} influx and release raises the free cytosolic Ca^{2+} concentration.

The activation of cardiac contraction is initiated by the binding of Ca^{2+} to troponin C of the troponin complex, resulting in conformational changes of troponin I and T, which alters the position of the adjacent tropomyosin molecule. Displacement of tropomyosin exposes the active sites of actin filaments, allowing it to interact with myosin to form new cross-bridges [Leavis and Gergely 1984]. The repeated making and breaking (cycling) of cross bridges between the two filaments propels them past each other, the so called sliding filament hypothesis [Sugiura 1999]. The mechanical energy required for contraction (systole) is obtained from adenosine triphosphate (ATP) hydrolysis by an ATPase site on the head of the myosin molecule [Lowey *et al.* 1969] resulting in the formation of adenosine diphosphate (ADP), inorganic phosphate (P_i) and free energy,

elements essential for contraction. The rate of ATP hydrolysis and its availability determine the maximum shortening velocity of the sarcomere. Maximum force-generating capacity of the sarcomere is proportional to the number of thick and thin filament cross bridge attachments and is governed by availability of cytosolic Ca^{2+} for troponin C [Fabiato and Fabiato 1975].

For relaxation to occur cytosolic Ca^{2+} must be removed from the cytosol to allow the dissociation of Ca^{2+} from troponin C. This permits troponin I via its interaction with tropomyosin to inhibit further interaction between the actin and myosin filaments, allowing relaxation (diastole). Four pathways facilitate Ca^{2+} transport out of the cytosol, which are i) sequestration of Ca^{2+} into the sarcoplasmic reticulum through the smooth endoplasmic reticulum Ca^{2+}-ATPase (SERCA) (that is controlled by the cardiac specific protein phospholamban [James *et al.* 1989]) where it binds to calsequestrin [Slupsky *et al.* 1987], ii) calcium exchanged out of the myocyte via the sarcolemmal sodium (Na^{+})/Ca^{+} exchangers [Blaustein 1989], iii) calcium extrusion from the myocyte via the sarcolemmal Ca^{2+}ATPase [Blaustein 1989] and iv) uptake of Ca^{2+} into mitochondria via the mitochondrial Ca^{2+} uniport [Bers 2002].

1:5 Myocardial energy metabolism

ATP is the energy currency of the heart. Its hydrolysis provides energy for contractile function and the maintenance of cellular homeostasis in the heart. Due to the rate at which it is consumed, ATP has to be continuously re-synthesised from its breakdown products ADP and P_i. The greater the work output of the heart, the higher the ATP turnover [Taegtmeyer 1994].

The primary means for ATP synthesis is by oxidative metabolism in the mitochondria. Myocardial ATP production results from the oxidation of a variety of substrates (e.g. non-esterified fatty acids derived from lipids, glucose,

lactate, ketone bodies and under certain circumstances amino acids, Figure. 1.3), the selection of which is mainly due to their blood concentration [Taegtmeyer 1994]. Due to this choice, the heart has been termed an omnivore, as it is an organ that functions best when it oxidises different substrates simultaneously [Taegtmeyer 1994]. It has also been proposed that the fuels for energy metabolism can be divided into two groups; i) essential fuels – those that can provide both acetyl CoA and anaplerotic substrates (e.g. glucose, lactate, pyruvate and certain amino acids) and ii) non-essential fuels – those that provide only acetyl CoA (e.g. fatty acids, ketone bodies and the amino acid leucine) [Taegtmeyer *et al.* 1993].

Acetyl CoA is the common intermediate for oxidative metabolism from carbohydrate and fatty acid metabolism (Figure 1.3) [Heineman and Balaban 1992]. Metabolism of acetyl CoA through the tricarboxylic acid (TCA) cycle generates carbon dioxide and the reducing equivalents nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$). NADH and $FADH_2$ are delivered to the respiratory chain, driving oxidative phosphorylation with the subsequent generation of ATP. NADH-linked dehydrogenases [M^{c}Cormack and Denton 1989, Heineman and Balaban 1992], NADH concentration and to a lesser extent ADP concentration are all thought to control myocardial oxidative phosphorylation [From *et al.* 1990].

The adenine nucleotide transporter allows the exchange of ATP for ADP across the mitochondrial membrane [Klingenberg 1979]. It is vital in allowing ATP exit from mitochondria to sites of extra-mitochondrial utilisation and in allowing the return of ADP for ATP production within mitochondria. The phosphocreatine shuttle is one mechanism that can transport ATP out of mitochondria once it is in the inter-mitochondrial membrane space [Ingwall *et al.* 1985]. The high-energy phosphate bond of ATP may be transferred to creatine to yield phosphocreatine and ADP, a reaction catalysed by mitochondrial creatine kinase (CK) located at the inner mitochondrial membrane. Phosphocreatine serves as a high-energy

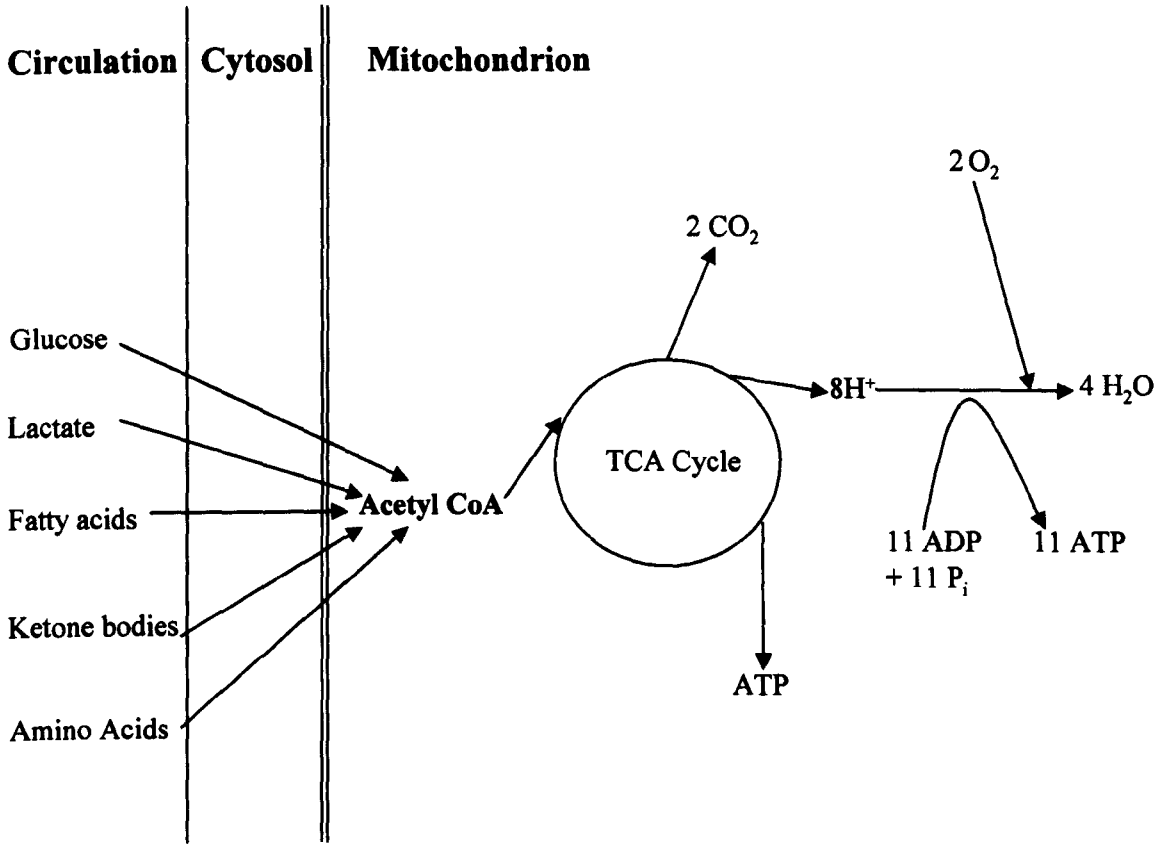


Figure 1.3: Myocardial energy substrate metabolism overview [Taegtmeyer 1994]

intermediate as it is transported to the cytosol and the contractile elements where it is converted by myofibrillar CK into ATP and creatine. ATP production is tightly coupled to ATP use, therefore cardiac work is tightly coupled to ATP turnover [Taegtmeyer 1994].

1:5:1 Glucose metabolism

1:5:1:1 Glucose transport

A family of intrinsic membrane proteins called the facilitated glucose transporters (GLUT) transport glucose into cells. [Pessin and Bell 1992]. There are two GLUT isoforms expressed in the heart, GLUT 1 and 4, which reside at different tissue locations under normal myocardial function. GLUT 1 is readily available at the sarcolemma and is primarily involved in basal metabolic glucose transport as its activity is generally regarded as insulin-independent. GLUT 4 is responsive to insulin and is normally sequestered in intracellular vesicles close to the Golgi region [Slot *et al.* 1991]. Upon insulin stimulation [James *et al.* 1988] GLUT4 moves to the sarcolemma [Cushman and Wardzala 1980, Watanabe *et al.* 1984]. Glucose is rapidly phosphorylated to glucose 6-phosphate by hexokinase (often termed glucose uptake or extraction). At this point, glucose 6-phosphate can either be converted to glycogen for storage, or enter the glycolytic pathway [Depre *et al.* 1999].

1:5:1:2 Glycogen metabolism

In an adult myocyte glycogen occupies up to 2 % of the cell volume [Taegtmeyer 1994]. Glycogen consists of chains of glucose monomers linked together by α-[1-4] glycosidic bonds, each α-[1-4] chain having an average length of 13 glucose residues [Laughlin 1993, Meléndez-Hevia *et al.* 1993, Meléndez *et al* 1998]. Each chain has two branching points by means of α-[1-6] glycosidic bonds, from which new chains can originate. These new chains form the next tier of the glycogen particle [Laughlin 1993]. The full molecule of glycogen (called macroglycogen) has a spherical shape, organised in concentric tiers, with

the full molecule having up to 12 tiers [Laughlin 1993, Meléndez-Hevia *et al.* 1993]. Molecules of glycogen are most commonly found localised in the interstices surrounding mitochondria, interfibrillar sarcolemma, perinuclear areas and around the sub-sarcolemmal cisternae [Berne and Levy 1993]. The surface of the glycogen particle is associated with the enzymes that metabolise glycogen, and their regulatory enzymes [Laughlin 1993].

Glycogen synthesis is primed by the self-glucosylating protein glycogenin [Whelan 1986]. Initially, glycogenin adds glucose from uridine diphosphate (UDP) glucose to itself at Tyr-194 before adding further glucose residues via α-[1-4] linkages to generate a maltosaccharide. This maltosaccharide serves as a primer for the synthesis of macroglycogen by glycogen synthases and branching enzymes via an intermediary known as proglycogen [Lomako *et al.* 1993]. The synthesis of macroglycogen is depicted in Figure 1.4.

Two independent forms of macroglycogen synthase exist, which are interconvertible by phosphorylation. Glycogen synthase *i* is the active form of the enzyme, and contains relatively little covalently bound phosphate. Its activity is largely independent of allosteric activators [Ramachandran *et al.* 1983]. Phosphorylation of synthase *i* by glycogen synthase kinase at one or more sites, modifies the enzyme to the less active synthase *d* whose activity is primarily determined by glucose 6-phosphate. Epinephrine, glucagon or β-agonists inactivate synthase in the *i* state by phosphorylation mediated through cyclic adenosine monophosphate (cAMP) dependent kinase [Ramachandran *et al.* 1983]. Differences in phosphorylation states of the same glycogen synthase have also been postulated to explain the differences in activity observed between the rates of synthesis of proglycogen and macroglycogen [Lomako *et al.* 1993].

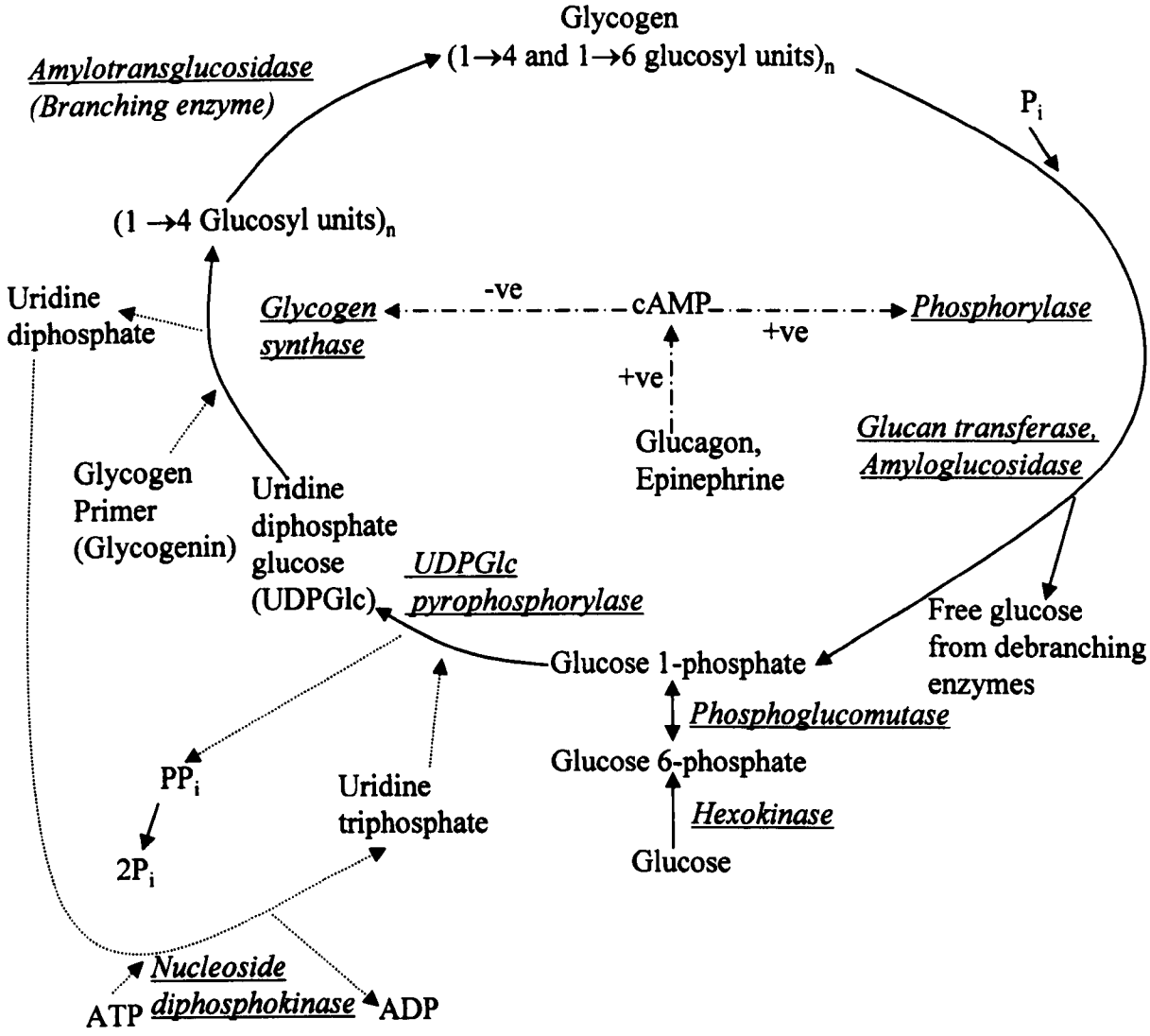


Figure 1.4: Glycogen synthesis and degradation.

The enzyme, amylo α-[1→4]α-[1→6]-transglucosidase establishes branch points in glycogen by transferring parts of an α-[1→4] glycogen chain to a neighbouring chain forming an α-[1→6] linkage [Stryer 1988]. Whether there are distinct forms of the branching enzyme is not known [Alonso *et al.* 1995].

Glycogen stores are normally increased by insulin, through the simultaneous stimulation of glucose transport and an increase in glycogen synthase activity by synthase phosphatases [Slot *et al.* 1991, Lawrence *et al.* 1992]. *In vivo*, myocardial glycogen synthesis can also be activated by increased oxidation of an alternative substrate, such as lactate [Laughlin *et al.* 1992] re-routing glucose away from glycolysis towards glycogen synthesis.

Glycogen phosphorylase catalyses glycogen phosphorolysis (α-[1→4]-bond cleavage by the substitution of a phosphate group) to yield glucose 1-phosphate. There are two forms of the phosphorylase enzyme, *a* and *b* [Morgan and Parmeggiani 1964, Newgard *et al.* 1989]. Phosphorylase *b* requires adenosine monophosphate (AMP) for activity [Morgan and Parmeggiani 1964]. ATP and glucose 6-phosphate depress the activity of phosphorylase *b* [Morgan and Parmeggiani 1964] but do not affect phosphorylase *a* activity. Phosphorylase *b* can be converted to the more active phosphorylated form, phosphorylase *a*, by glycogen phosphorylase kinase. Increased myocyte calcium concentrations can stimulate phosphorylase kinase or cAMP-dependent protein kinase activity, both of which promote the conversion of phosphorylase *b* to *a* [Rasmussen 1986].

Terminal glucosyl residues from the outermost chains of the glycogen molecule are removed sequentially until approximately four glucose residues remain on either side of an α-[1→6] branch [Laughlin 1993]. The α-[1→6] branch point is exposed by α-[1→4]→ α-[1→4] glucan transferase transferring a tri-saccharide unit, proximate to the branch point, to another chain. Amylo α-[1→4]α-[1→6]-glucosidase hydrolytically splits the branch point.

1:5:1:3 Glycolysis

Glucose is catabolised via the glycolytic pathway with the final step in aerobic glycolysis being the conversion of pyruvate to acetyl-CoA by the pyruvate dehydrogenase complex (PDC). PDC consists of three enzymes (pyruvate dihydrolipoyl dehydrogenase, dihydrolipoyl transacetylase and decarboxylase) and five coenzymes or prosthetic groups that are organised into a multienzyme complex located on the inner side of the inner mitochondrial membrane [Kerbey *et al.* 1976]. PDC is regulated by both its own substrates and products and by covalent modification (Figure 1.5) [Randle 1981]. Pyruvate dehydrogenase kinase inhibits PDC activity and is itself activated by acetyl-CoA and NADH but inhibited by pyruvate [Reinauer and Muller-Rucholtz 1976]. Ca^{2+} stimulates pyruvate dehydrogenase phosphatase, which activates PDC [M^{c}Cormack *et al.* 1990]. The regulation of PDC plays a major role in fuel selection by the heart [Seymour 1994, Taegtmeyer 1994].

1:5:2 Lactate metabolism

Lactate transport across the sarcolemmal membrane from the circulation is facilitated by a monocarboxylate transporter [Garcia 1994]. In the aerobic heart, lactate is rapidly metabolised by lactate dehydrogenase, decarboxylated by the PDC and oxidised in the TCA cycle. Circulating levels of lactate are the major determinant of lactate uptake in the heart [Gertz *et al.* 1981]. Under conditions of lactate production (e.g. strenuous physical activity) lactate can become the predominant fuel for the heart [Stanley 1991]. Indeed, work has shown that the heart can extract lactate from, as well as release lactate into, the circulation under normal conditions [Gertz *et al.* 1981, Chatham *et al.* 2001].

1:5:3 Lipid metabolism

Lipids utilised by the heart can come from exogenous or endogenous sources [Van der Vusse *et al.* 1992]. The main endogenous source is thought to be

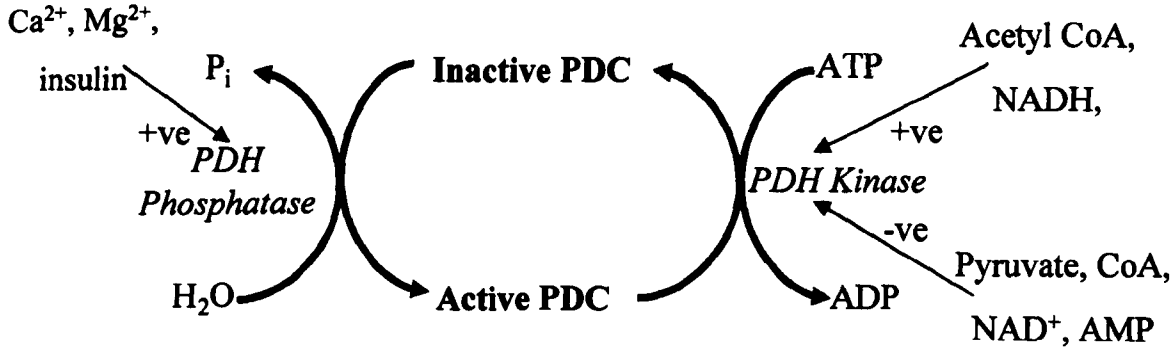


Figure 1.5: Regulation of PDC

triacylglycerol, present in lysosomes and as lipid droplets in the sarcoplasm [Stam *et al.* 1980]. Several lipolytic enzymes are involved in the degradation of myocardial triacylglycerol [Severson 1979]. These include the exogenous lipoprotein lipase (LPL) and endogenous di-monoglyceride lipases [Stam *et al.* 1986].

1:5:3:1 Fatty acid metabolism

The main source of exogenously derived fatty acids are free circulating fatty acids bound to albumin, and fatty esters present in chylomicrons and very low-density lipoproteins (VLDLs). LPL located on the luminal side of endothelial cells liberates fatty acids from chylomicrons and VLDLs by hydrolysis which then bind to albumin. The heart is very efficient at extracting fatty acids. The pathways by which fatty acid-albumin complexes present in the vascular lumen are transported across the myocyte membrane have not been fully defined and it is not clear if free fatty acids are taken up by the myocytes by a passive diffusion process or by a carrier mediated process [Van der Vusse *et al.* 1992]. Once inside the myocyte, fatty acids bind to fatty acid-binding proteins (FABPs), a class of low molecular weight (14-15kDa) proteins [Glatz *et al.* 1993]. FABPs aid the transfer of the fatty acid from a favourable micellar environment to an unfavourable aqueous one. The fatty acids are 'activated' to long-chain acyl-CoA by a family of acyl-CoA synthase enzymes. Synthases convert the carboxylic head of the fatty acid to a more reactive CoA thioester at the expense of two, high-energy phosphoanhydride bonds from one ATP molecule [Waku 1992]. This 'activation' is imperative for the subsequent catabolic breakdown of the fatty acids.

Before the long chain acyl molecule can be further metabolised, it has to be transported into the mitochondrial matrix via the concerted efforts of three carnitine dependent enzymes: carnitine palmitoyltransferase 1 (CPT 1), carnitine-acylcarnitine translocase (CAT) and carnitine palmitoyltransferase 2 (CPT 2)

[Lopaschuk *et al.* 1994]. This three-step membrane transport process is the rate-controlling step for long-chain fatty acid oxidation (Figure 1.6).

Once inside the mitochondrial matrix, the long chain acyl CoA is degraded by a system of enzymes known as the β-oxidation spiral, which sequentially degrades long chain acyl CoA, resulting in the generation of acetyl CoA that is subsequently metabolised in the TCA cycle [Lopaschuk *et al.* 1994].

The oxidation of fatty acids in the normal heart supplies 60-70 % of the overall energy requirements [Neely and Morgan 1974]. Oleic and palmitic acid are the main fatty acids oxidised by the heart. Theoretically, oxidative metabolism of fatty acids yields more than twice as much ATP as oxidative metabolism of an equal mass of glucose [Stanley and Chandler 2002].

1:6 Myocardial hypertrophy

Cardiac hypertrophy (CH) is the adaptive mechanism where the heart increases in mass in response to a chronic increase in workload, as is observed following a myocardial infarction [Morgan *et al.* 1987]. However, as the mature heart is unable to regenerate new myocytes (hyperplasia) the observed increase in mass is primarily a result of an increase in size of the myocytes (hypertrophy). The reason(s) why cardiac myocytes lose their ability to divide during development remains unknown [Brooks *et al.* 1998], but it has been proposed that the majority of myocytes appear to be locked in either the G_o or G_1 phases of the cell cycle [Capasso *et al.* 1992].

Induction of CH involves a sequence of events (initiating signals, coupling mechanisms, and regulation of gene expression) that result in an increase in myocardial cell size, proliferation of supportive tissues, and other associated structural changes [Swynghedauw *et al.* 1990]. Hypertrophy can be subdivided

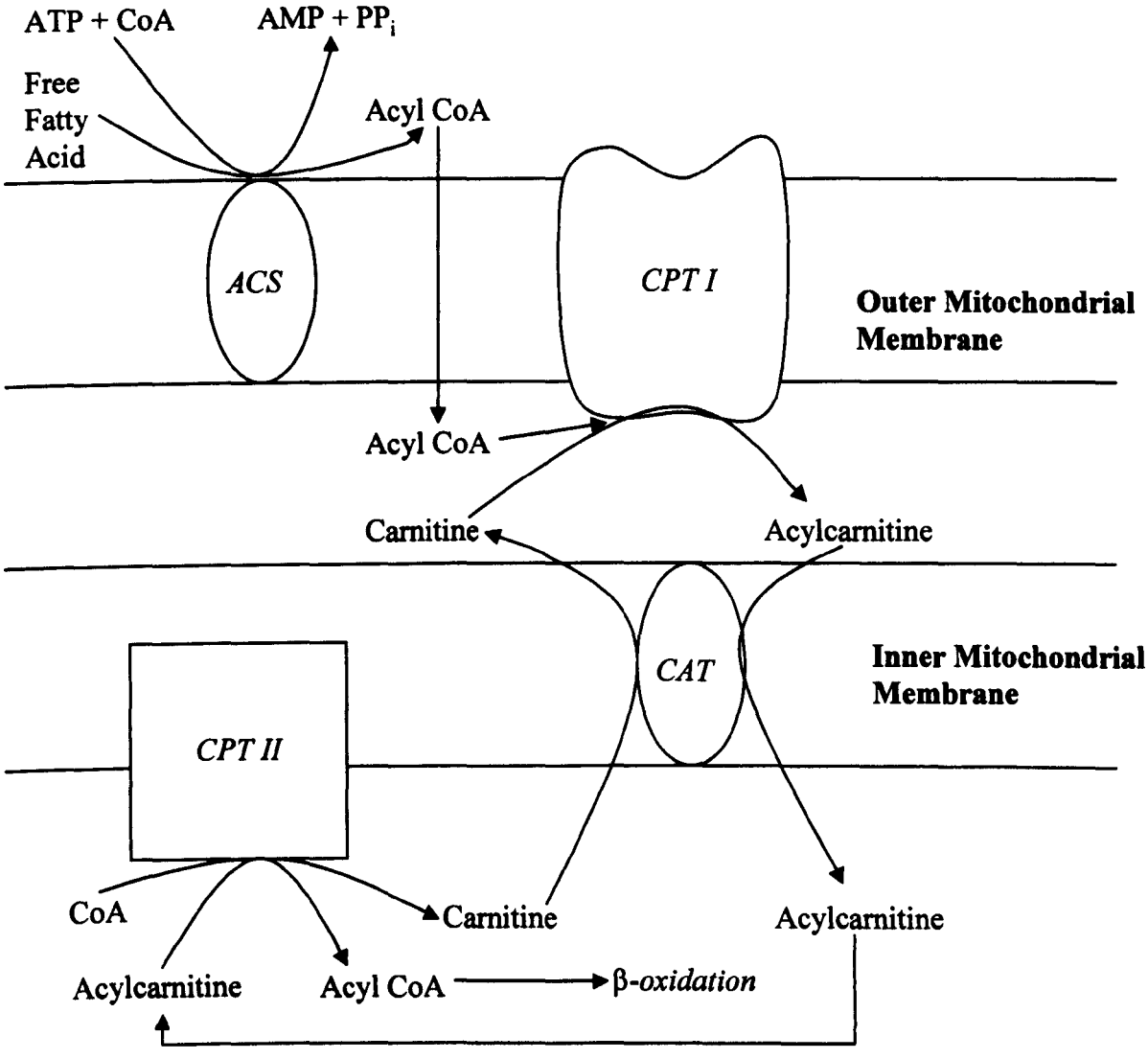


Figure 1.6: Transport of long- chain fatty acids through the mitochondrial membrane

into pathological hypertrophy, resulting from a disease state or physiological hypertrophy, as seen in the hearts of athletes.

1:6:1 Pathological CH

Pathological CH can be considered to be a maladaptation of the heart to a chronic increase in workload. It can be divided into three different phases [Meerson 1961]. The immediate phase is one of acute heart failure; the unexpected demands of an overloaded heart overwhelm the functional reserves of the as yet non-hypertrophied myocardium. During the secondary phase, 'compensatory hyperfunction', the myocardial hypertrophy increases the ability of the heart to match the demands of the overload. Deleterious effects are seen during the tertiary phase, as the cells of the overloaded heart deteriorate and die. These three phases have been observed in patients after myocardial infarction [Katz 1992b].

There are two common types of pathological left ventricular hypertrophy, concentric and eccentric [Grossman *et al.* 1975]. The increase in ventricular mass seen with concentric hypertrophy is usually a result of a chronic pressure overload (e.g. resulting from essential hypertension, aortic stenosis). There is a marked increase in the ventricular wall thickness with a chamber volume that is normal or slightly reduced [Anversa *et al.* 1993]. This chamber volume alters in heart failure as the ventricle dilates. Eccentric hypertrophy is produced by a chronic volume overload (e.g. resulting from incompetent heart valve leaflets, anaemia) and increases ventricular weight. The chamber volume is increased in proportion to mass, and may occur without alteration to wall thickness [Anversa *et al.* 1993].

By increasing ventricular wall thickness (or chamber size), the hypertrophy process distributes the pressure or volume overload over a greater myocardial cross-sectional area (increased number of sarcomeres) such that wall stress can

remain normal, according to the law of Laplace [Katz 1992a]. In its simplest form, the law of Laplace states that the wall tension (T) of a cylinder is equal to the pressure (Pr) within multiplied by the radius of the curvature of the wall (R).

$$T = Pr \times R \qquad \text{The law of Laplace}$$

The increased thickness of the cylinder wall in CH can reduce the wall stress by distributing the increased tension among a greater number of active sarcomeres in the overloaded heart. The law of Laplace for a thick walled cylinder can be formulated [Katz 1992a].

$$T = \frac{(Pr \times R)}{h} \qquad \text{(where h is the wall thickness)}$$

1:6:2 Triggers and signalling pathways of myocardial hypertrophy

Stretch overload has been identified as a key factor in stimulating a hypertrophic response [Anversa *et al.* 1993]. Increased stretch can directly cause changes in myocyte gene expression and protein transcription [Yamazaki *et al.* 1998]. In addition these changes may be stimulated directly or indirectly via various hormones or growth factors [Sadoshima and Izumo1997, Bloom *et al.* 1996]. Hypertrophic stimuli may activate a number of different intracellular signalling pathways that may be inter-linked [Sadoshima and Izumo 1997]. The signalling pathways activated in a hypertrophic response are initiated mainly from the stimulation of; G protein coupled receptors [Sadoshima and Izumo 1997], unidentified stretch receptors [Sadoshima *et al.* 1992] and sarcolemma tyrosine kinases [Thorburn *et al.* 1993]. Stimulation of G-protein or stretch receptors is thought to result in activation of a mitogen-activated cascade [Sadoshima *et al.* 1992] that amplifies the hypertrophic signal.

1:6:3 Altered gene expression in hypertrophy

Overall there is an upregulation of protein synthesis in the hypertrophied heart [Chien *et al.* 1991]. Quantitative and qualitative adaptations in gene expression play a critical role in the hypertrophic response [Sadoshima and Izumo 1997, Depre *et al.* 1998a]. This adaptation is characterised in part by the re-expression of a 'foetal gene program' [Boheler and Schwartz 1992]. Results of this are a switching of proteins from the adult to the foetal isoforms, the re-expression of proto-oncogenes (such as c-fos) and the induction of growth factors, such as transforming growth factor β (TGFβ). Adaptations in protein expression may involve a selective repression, non-activation or induction of any one gene [Boheler and Schwartz 1992].

The immediate early gene program is one of the initial sets of genes to be upregulated at the start of hypertrophy and specifically involves the expression of proto-oncogenes [Komuro *et al.* 1988]. Proto-oncogenes are transcriptional factors that participate in normal growth induction of the cell in response to growth stimuli [Johnson and McKnight 1989]. Many proto-oncogenes control the re-entry of resting cells into the cell division cycle and regulate the transcription of a variety of genes [Reddy 1997]. The precise hypertrophic role of the proto-oncogene products of these genes in non-myocytes (*c-myc*) and myocytes (*nur 77*, *junB*, *c-fos* and *c-jun*) are yet to be determined [Sadoshima and Izumo 1997, Brooks *et al.* 1998] but they are thought to switch on the cascade of events leading to hypertrophy. Recently, it has been reported in the human heart that re-expression of the foetal metabolic gene profile does not involve inducing foetal metabolic genes but occurs by repression of adult metabolic genes [Razeghi *et al.* 2001].

1:6:4 Functional adaptation

The myocardium alters its mechanical properties to maintain normal function when subject to a chronic increase in pressure or volume overload. Modifications of the excitation-contraction-relaxation coupling i.e. length of the muscle fibres, adrenergic stimulation [Pouleur 1990] and frequency of stimulation are modifications typically seen after an increase in myocardial workload.

1:6:4:1 Diastolic dysfunction

Diastolic dysfunction is an early change observed in hypertensive patients, pre-dating clinically detectable ventricular hypertrophy [Dianzumba *et al.* 1986]. Diastolic dysfunction arises from a combination of structural remodelling of the myocardial collagen matrix, disproportionate accumulation of fibrous tissue and biochemical energy defects as the duration of hypertension increases [Weber *et al.* 1993].

Relaxation of the left ventricle (LV) is impaired in hypertrophy. It is manifested by a longer isovolumic relaxation time, as it takes longer for LV pressure to decrease to a level, which is lower than left atrial pressure (allowing the mitral valve to open). There is a progression in diastolic dysfunction in pathological LV hypertrophy from initially impaired relaxation to additional decreasing ventricular compliance [Mayet and Foale 1998] or myocardial stiffening resulting from an increase in collagen deposition in the ventricle [Weber *et al.* 1993].

1:6:4:2 Systolic dysfunction

There is clear evidence that systolic dysfunction occurs in CH. Animal models of pathological hypertrophy determining preload, afterload and myocardial contractility to quantify ventricular ejection, demonstrate that reduced myocardial contractility is an early feature of CH [Dubus *et al.* 1993]. It has

been shown that although endocardium shortening and ejection fraction are normal in patients with LV hypertrophy, mid wall shortening is depressed [Aurigemma *et al.* 1995]. Therefore, the hypertrophied ventricular wall is maintaining a normal chamber function in spite of an abnormal cardiac function, indicating that abnormal systolic function may be an early characteristic in patients with CH, as observed in animal models of CH.

1:6:5 Cellular adaptations in pathological hypertrophy

Hypertrophy of myocardial cells can have both beneficial and harmful consequences. Although the initial changes in architecture and cellular composition contribute a remarkable adaptation of form to function [Katz and Katz 1989] that helps the heart to meet an acute overload, long standing hypertrophy is accompanied by alterations in gene expression that appear to accelerate deterioration.

1:6:5:1 Myocytes

Adult ventricular myocytes are terminally differentiated cells, having withdrawn from the cell cycle around parturition. They respond to an increase in workload by an increase in cell size (hypertrophy), and not by an increase in cell number (hyperplasia). However, there is evidence that may indicate cardiac myocytes retain their capacity to synthesise DNA and re-enter the cell cycle [Anversa *et al.* 1996].

Hypertrophic myocyte growth is largely dependent on myocyte work. Chronic volume overload stretches cardiac myocytes and increases their length-dependent work. Myocytes respond by an in-series addition of contractile proteins (sarcomeres) and increased cell length and volume, increasing myocardial mass [Anversa *et al.* 1993]. Chronic pressure overload raises systolic tension development. Myocytes respond with an in-parallel addition of contractile

proteins and increased cell width and volume, increasing myocardial mass [Anversa *et al.* 1993].

1:6:5:2 Subcellular structural changes in myocytes

Cells from a hypertrophied myocardium (without degeneration) often appear to be normal apart from their size. In volume overload CH, the volume fractions of subcellular components are usually unchanged. In contrast, there is a decrease in the mitochondrial volume fraction in severe pressure overload hypertrophy. The decrease in the ratio of mitochondria volume to myofibrillar volume has a profound impact on the energy homeostasis of the cell [Rakusan 1998].

The cell surface-to-volume ratio and the proportion of external sarcolemma decrease with increasing myocyte size. Increased T-tubule and smooth endoplasmic reticulum surface area compensate for this [Anversa *et al.* 1986, Rakusan 1998], providing greater cellular capacity for calcium storage and handling.

1:6:5:3 Isoform changes in myofibrillar proteins

Tonic changes in myocardial contractility that arise from alterations in gene expression develop relatively slowly. Expression of the β-heavy-chain myosin isoform has a negative inotropic effect, by reducing the maximal velocity of contraction [Lompré *et al.* 1979]. This isoform switch has an important effect on muscle energy expenditure as muscles containing the slow β-myosin heavy chains generate tension more efficiently than muscles with the faster α-isoform, thus increasing the amount of work that can be done for a given amount of ATP. No differences are observed in the expression of the intracellular contractile protein troponin I in experimental rat models of hypertrophy [Cumming *et al.* 1995] or in end stage heart failure in humans [Sasse *et al.* 1993]. In contrast, troponin T isoform changes have been reported in heart failure in humans [Sasse *et al.* 1993] and in rabbit [Chen *et al.* 1997], but not rat [Cumming *et al.* 1995], models of pressure overload CH. Changes in isoform expression of troponin T

are associated with alterations to Ca^{2+} binding with troponin C [M^{c}Auliffe *et al.* 1990].

1:6:5:4 Alterations in calcium handling proteins

Abnormal intracellular calcium handling is a major factor associated with the dysfunction observed in CH and heart failure [Dhalla *et al.* 1991]. Expression of SERCA 2a (the major Ca^{2+} ATPase isoform in the sarcoplasmic reticulum) is down regulated in pressure overloaded hearts [De La Bastille *et al.* 1990]. Impaired Ca^{2+} sequestration by the sarcoplasmic reticulum [Limas *et al.* 1987, Boateng *et al.* 1998] causes a slowing in the relaxation of the heart, contributing to diastolic dysfunction. Due to poor re-sequestration of Ca^{2+}, cytosolic Ca^{2+} levels remain elevated, also reducing the force of contraction of the heart.

The activity of SERCA 2a is modulated through its interaction with phospholamban [Tada and Katz 1982]. A decrease in basal cAMP production may also slow relaxation by decreasing the tonic phosphorylation of phospholamban, and therefore indirectly inhibit SERCA 2a. Low basal cAMP was reported in hypertensive rats [Hilal-Dandan and Khalirallah 1991]. Alterations are also observed in the expression of the Na^{+}/Ca^{2+} exchanger [Boheler and Schwartz 1992]. Work with hypertrophied myocyte preparations has shown that Na^{+}/Ca^{2+} exchangers were compromised more than SERCA 2a [Naqvi *et al.* 1994]. In addition, alterations have also been observed in calcium-release channels (ryanodine and dihydropiridine receptors) in CH and heart failure [Arai *et al.* 1993, Brillantes *et al.* 1992]. Alterations in the expression of these proteins may also contribute to functional abnormalities in CH by prolonging calcium transient times.

1:6:5:5 Metabolic energy changes in the hypertrophied myocardium

In animal models of hypertrophy, controversy exists regarding changes in ATP concentration. A reduction in ATP concentration has been observed in some studies [Massie *et al.* 1994] but not in others [Regitz and Fleck 1992]. Phosphocreatine (PCr) serves as a high-energy phosphate reserve to maintain cytosolic ATP levels. Decreases in the PCr: ATP ratio have been reported in animal models of cardiac hypertrophy [Ingwall *et al.* 1985, Seymour *et al.* 1990, Ingwall *et al.* 1990, Massie *et al.* 1995]. Reduced PCr: ATP ratios have also been reported in patients showing signs of heart failure [Conway *et al.* 1991] and in patients with dilated cardiomyopathy [Neubauer *et al.* 1992].

Reduced levels of PCr are primarily thought to be a result of a decrease in intracellular creatine. Creatine pool depletion with a concomitant reduction in PCr levels have been observed in animal models of cardiac hypertrophy [Zhang *et al.* 1993] and in patients [Conway *et al.* 1991].

In decompensated cardiac hypertophy, decreases in PCr may also result from altered activity of CK, the enzyme responsible for the transfer of high energy phosphate from PCr to ATP. Decreases in total CK activity have been observed in the failing spontaneously hypertensive rat (SHR) heart [Bittl and Ingwall 1987] and the cardiomyopathic hamster [Khuchua *et al.* 1989], a model of heart failure. Decreases in the amount of mitochondrial creatine kinase were also observed in the failing heart [Ingwall 1984].

Taken collectively, the above studies indicate that a decreased energy reserve in the hypertrophied heart may limit the ability of the myocardium to perform work resulting in a progression to contractile failure [Ingwall 1993].

The use of substrates for energy production changes in the hypertrophied heart and becomes similar to that of the foetal heart. Increased rates of glucose uptake [Bhutta *et al.* 1996], glucose oxidation and glycolytic capacity have been shown

experimentally in hypertrophied hearts [Seymour *et al.* 1990, Allard *et al.* 1994]. These observations are consistent with the view the hypertrophied heart switches its substrate selection from predominantly fatty acid oxidation to glucose metabolism [Taegtmeyer and Overturf 1988, Christe and Rodgers 1994].

In addition, decreased long chain fatty acid oxidation has also been demonstrated in the hypertrophied heart [El Alaoui-Talibi *et al.* 1992]. A number of possibilities have been postulated to explain mechanisms for the observed decreases in fatty acid oxidation in hypertrophied hearts.

Firstly, decreased levels of myocardial carnitine have been observed in pressure [Reibel *et al.* 1983] and volume overload hypertrophy [El Alaoui-Talibi *et al.* 1992]. Carnitine is an essential co-factor for the transport of long chain fatty acyl moieties into mitochondria, as it regulates CPT 1 activity in the heart [Reibel *et al.* 1983, El Alaoui-Talibi *et al.* 1997]. The rate of myocardial fatty acid oxidation is controlled by the rate of fatty acid transfer into the mitochondria through CPT 1 [Lopaschuk *et al.* 1994].

Secondly, a down regulation in myocardial levels of the muscle (adult) CPT 1 (mCPT 1) but not the liver (foetal) CPT 1 (lCPT 1) isoform has been observed in pressure overload hypertrophy [Depre *et al.* 1998a]. In the adult heart, mCPT 1 accounts for over 90 % of CPT 1 activity [Brown *et al.* 1995].

Thirdly, the expression of the transcription factor peroxisome proliferator activated receptor α-isoform (PPARα) is significantly down regulated during pressure overload hypertrophy [Sack *et al.* 1996, Sack *et al.* 1997]. This may also contribute to the decreased levels of fatty acid oxidation observed in myocardial hypertrophy as PPARα co-ordinates the genes encoding mitochondrial fatty acid transporters and oxidation enzymes [Barger and Kelly 2000]. However, studies in the volume overloaded heart with short chain fatty acids, which do not require CPT 1 or carnitine for transport into mitochondria,

showed no signs of impaired fatty acid oxidation [El Alaoui-Talibi *et al.* 1992], thus indicating that alterations in β-oxidation enzymes are not major factors in the decreased fatty acid utilisation observed in myocardial hypertrophy.

Finally, fatty acid oxidation may be inhibited at CPT 1 by the production of malonyl CoA (Figure 1.7) [M^{c}Garry *et al.* 1983], formed from acetyl CoA by acetyl CoA carboxylase (ACC) [Awan and Saggerson 1993]. Increases in glucose or lactate metabolism will result in an increase in acetyl CoA from pyruvate that will stimulate the production of malonyl CoA [Saddik *et al.* 1993], thus inhibiting fatty acid oxidation.

1:6:5:6 Non-myocyte cellular changes

The heart also contains other cells such as fibroblasts, endothelial cells lining the vasculature and endocardium, and vascular smooth muscle cells [Weber *et al.* 1991]. These non-myocyte cells are important in CH because, in addition to undergoing a mixed hyperplasic/hypertrophic response, they may release local signalling factors that may further trigger the hypertrophy of cardiac myocytes [Rozengurt 1986, Baker *et al.* 1992]. Such factors include endothelins (ET) released from the endothelial cells, noradrenaline derived from sympathetic innervation, and angiotensin II (AngII) produced by cardiac fibroblasts and cardiac myocytes [Baker *et al.* 1992, Weber 1997]. Excessive non-myocyte hyperplasia may also distort the normal heart structure [Katz 1992a].

1:6:5:7 Vascular adaptations

Intramural small arterial microvessels perfuse the myocardium. As coronary vessels penetrate the ventricular wall, intramyocardial pressure influences the supply of blood, especially to the endocardial region. Coronary flow occurs primarily during diastole, as during systole the high intramyocardial pressure limits flow [Katz 1992b]. Diastolic pressure is raised in pressure overload, which could further restrict the blood supply to the heart [Katz 1992b]. This

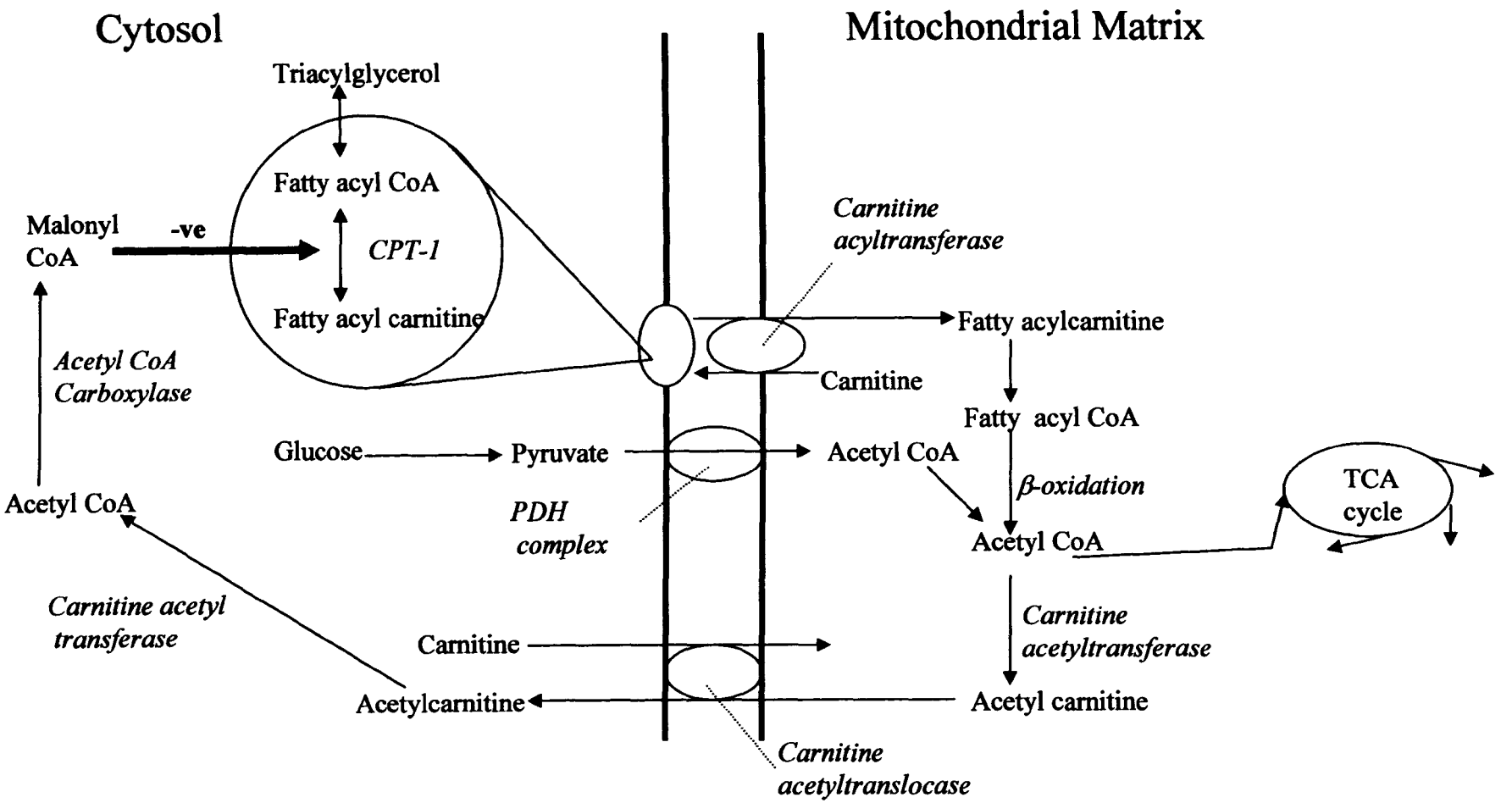


Figure 1.7: CPT-1 inhibition by malonyl CoA resulting from an increased production of acetyl CoA

could lead to an ischaemic myocardium, resulting in oxygen and energy substrate deprivation.

Alterations to the cardiac microvascular induced by a pressure-overload are determined by two factors: coronary arterial hypertension and ventricular pressure overload [Ito *et al.* 1993]. The former, resulting from high blood pressure, causes vascular hypertrophy with peri-vascular collagen deposition in arteries and larger arterial microvessels [Isoyama 1998], while ventricular pressure overload causes peri-vascular collagen deposition without vascular hypertrophy in small microvessels [Isoyama 1998]. Both these changes are observed together in hearts with coronary arterial hypertension and ventricular pressure overload leading to a detrimental blood supply to the heart.

Whether or not pressure-overload CH is accompanied by appropriate angiogenesis still remains controversial. Capillary angiogenesis occurs in parallel with an increase in myocardial mass during the normal development of the heart [Rakusan 1992]. In contrast, a decrease is observed in the capillary density of hypertrophied hearts from adults with acquired aortic stenosis, leading to an impaired blood supply to provide for normal myocardial function [Rakusan 1992].

In addition, there is also an increase in the intercapillary distance in the hypertrophied adult heart due mainly to the increase in myocyte size. This may result in increased diffusion distances for nutrients of the heart, adding support to the proposal that the hypertrophied and failing heart may be energy starved and therefore more susceptible to ischaemic damage [Katz 1992b].

1:7 Transition to heart failure

Heart failure can be simply defined as an inability of the heart to maintain adequate perfusion for the haemodynamic demands of tissues at normal filling pressures. Heart failure develops at the end-stage of any heart disease with chronic mechanical overload. Most cases of heart failure are preceded by cell and chamber hypertrophy (myocardial remodelling). CH initially represents an important adaptive mechanism to haemodynamic stresses [Katz and Katz 1989], the compensatory phase. When the stimulus to hypertrophy is sustained for months and years important deleterious effects begin to appear in the architecture, blood supply and ultrastructure of the heart [Katz 1992a], the decompensatory phase. Several factors are implicated in heart failure and each may contribute to varying degrees to the development of heart failure. Heart failure is characterised by ventricular dilatation, reductions in thickness of the left ventricle wall, decline in left ventricular function, increased systemic vascular resistance and activation of compensatory neuro-endocrine systems [Katz 1992a, Braunwald and Bristow 2000].

In hypertensive patients diastolic dysfunction is prevalent, particularly in patients with CH [Betocchi and Hess 2000]. Dysfunction occurs in all phases of diastole and can be recognised by an increase in left ventricular end diastolic pressure with a normal end diastolic volume, a reduced systolic volume but a preserved cardiac output. Diastolic isovolumic relaxation is prolonged in CH [Betocchi *et al.* 1993] and is sometimes incomplete when filling resumes [Mirsky and Pasipoularides 1990], which increases diastolic pressure. Increased diastolic pressure results in an increase in cardiac work according to Starling's law of the heart [Levick 2000], thus preserving cardiac output in patients with diastolic dysfunction.

Systolic dysfunction is characterised by a decreased left ventricular ejection fraction and an increase in end systolic and diastolic volume in the failing heart.

Although systolic ejection is impaired, dilation of the ventricle initially helps to maintain adequate perfusion as it increases end systolic volume, raising atrial pressure and increasing cardiac work [Levick 2000]. However, ventricular dilation also increases wall tension, even when intraventricular pressure remains constant, by increasing the radius of the chamber and decreasing the chamber wall thickness, resulting in an increase in tension on each muscle fibre in its walls, according to the law of Laplace [Katz 1992a]. This establishes a vicious circle that further overloads the heart. Symptoms of heart failure may develop in the presence of 'normal' systolic function [Dougherty *et al.* 1984] but once heart failure due to systolic dysfunction occurs, the prognosis is bleak [Betocchi 2000].

CH may also increase myocyte necrosis and apoptosis [Teiger *et al.* 1996, Sharov *et al.* 1996], which contribute to the pathogenesis of heart failure [Elsässer *et al.* 2000]. Myocyte apoptosis and necrosis increase the burden on surviving myocytes, accelerating their deaths, setting up a detrimental loop. In addition, if myocytes of the hypertrophied heart necrose (e.g. after a myocardial infarction), fibroblast proliferation is stimulated and muscle is replaced by connective tissue [Linzbach 1960]. This also contributes to an increased wall tension in the failing heart due to increasing the stiffness of the heart [Weber *et al.* 1993].

The sequence of events leading to the development of decompensated hypertrophy and ultimately heart failure are still not fully understood. Biochemical and biophysical disorders of myocytes in the hypertrophied heart produce a depression in the contractility and relaxation of the myocardium. It is unlikely that any one such biochemical alteration can trigger, or characterise, heart failure due to tight regulation of metabolic processes within cells. Heart failure is most likely to be a result of several molecular disruptions occurring in quick succession.

1:8 Myocardial ischaemia

Reduction of blood flow to the myocardium, termed ischaemia, is detrimental to contractile function and cell viability [Reimer and Jennings 1992]. The most significant effects of ischaemia are due to impaired coronary flow resulting in oxygen deprivation (hypoxia) and a reduced supply, or removal, of metabolites, leading to impaired pump function [Katz 1992a]. The exact mechanisms of ischaemic cell death still remain to be elucidated. Myocardial ischaemic injury is currently divided into two areas, reversible or irreversible cellular injury [Jennings 1970]. Injury from which the cells can recover after ischaemia is alleviated (reperfusion) is termed reversible. Injury is irreversible if restoration of arterial flow does not prevent cell death. The perpetrator(s) of the transition from reversible to irreversible injury is (are) not yet established.

Ischaemic episodes are termed mild, moderate or severe, reflecting the degree of imbalance between oxygen supply to, and demand of, the tissue [Reimer and Jennings 1992]. Total ischaemia defines the complete cessation of blood flow to a region of myocardium. Ischaemia of sufficient severity and duration can irreversibly injure myocytes resulting in their necrosis [Jennings *et al.* 1990]. Myocyte necrosis in the heart, resulting from ischaemia, is observed following a myocardial infarction [Theroux 1999].

Many studies have shown that CH is associated with impaired contractile function and an enhanced susceptibility to ischaemia [Bache 1988, Gaasch *et al.* 1990, Anderson *et al.* 1990]. Abnormalities involving the left ventricle give rise to the major haemodynamic consequences seen with ischaemia, e.g. reduced cardiac output.

1:8:1 Early features of the ischaemic heart

A severe and sustained reduction in blood flow to the myocardium leads to a cessation in function [Katz 1992a]. Almost immediately there is a reduction in oxidative phosphorylation leading to a failure to re-synthesise energy-rich phosphates including ATP and creatine phosphate [Jennings *et al.* 1990, Jennings *et al.* 1991]. With the profound reduction in energy stores active tension generation is reduced, ion homeostasis is lost with the leakage of potassium ions into the extracellular environment and the accumulation of calcium ions in the cytoplasm [Kléber 1984]. These changes contribute to electrical instability in the cells and a failure of relaxation. Ischaemia characteristics continue to develop, as long as there is no or limited flow to the heart, until the myocytes are swollen, acidotic, and show signs of structural disorganisation.

1:8:2 Alterations in conductivity

Myocardial ischaemia causes changes in membrane permeability resulting in a loss of intracellular homeostasis. Development of local in-excitability (due to elevated extracellular potassium (K^+)) or blocked conduction (due to cellular acidosis) may prevent myocardial cells from being excited and contracting [Downar *et al.* 1977]. Therefore interruption to an initial component in the chain of events leading to contraction may produce complete cardiac failure despite the viability of subsequent processes [Katz 1992b].

Depolarisation due to myocardial ischaemia is associated with an initial rapid loss of intracellular K^+, with a corresponding increase in extracellular K^+ up to 15mM after 15min ischaemia [Kléber 1984]. Inhibition of the sodium pump does not explain the loss of intracellular K^+ due to the sharp onset and the high rate of loss [Kléber 1983]. It is proposed that anions, e.g. P_i or lactate, are simultaneously transported out of the cell with K^+, as depolarisation of the cell occurs at the same time [Kléber 1984]. The loss of K^+ is biphasic, after the initial

loss there is a plateau phase where the K^+ levels remain constant before a further loss that is indicative of irreversible damage.

Increased levels of extracellular K^+, resulting from intracellular K^+ loss, inactivate the Na^+/K^+ ATPase by reducing the membrane potential of the cell [Kléber 1983]. Ischaemic myocardial cells ultimately lose so much potassium as to cease either to initiate or propagate a sodium action potential, giving rise to arrhythmias. The depressed excitability in depolarised ischaemic cells affects mechanical function by reducing the number of contracting myofibrils as well as causing contraction and relaxation inhomogeneities that impair ventricular ejection and filling. Approximately half the deaths caused by ischaemic heart disease result from disordered cardiac rate and rhythm [Katz 1992a].

1:8:3 Glucose metabolism in the ischaemic heart

During ischaemia, the impaired supply of oxygen causes an increased reliance on anaerobic glycolysis from either exogenous glucose or endogenous glycogen for energy provision [Morgan *et al.* 1961]. The degree of ischaemia determines the contribution of glucose to energy production. Ischaemic glycolysis is important in providing a source of ATP. This residual production of ATP sustains the activity of ATP requiring enzymes, mainly the sarcolemmal Na^+/K^+ ATPase and the sarcoendoplasmic Ca^{2+} ATPase [Goudemant *et al.* 1995].

Following the induction of ischaemia there is a rapid recruitment of both GLUT 1 and GLUT 4 transporters from intracellular stores to the sarcolemma [Young *et al.* 1997], resulting in an initial enhancement in glucose transport into myocytes. However, even when there is a constant substrate supply, e.g. during low flow ischaemia, glucose uptake progressively decreases throughout ischaemia [Bricknell *et al.* 1981].

Ischaemic stimulation of glucose transport [Young et al. 1997] is coupled to accelerated glycolytic flux [Morgan *et al.* 1961]. The increased flux is attributed to the activation of phosphofructokinase-1 (PFK-1) by an increase in AMP (an activator of PFK-1) and a decrease in ATP (an inhibitor of PFK-1) [Newsholme 1971]. The change in ratio of AMP: ATP controls the ischaemic activation of PFK-1 as there is no change in citrate and fructose-2, 6-diphosphate concentrations (two prominent regulators of PFK-1 activity) from normoxic levels [Depre *et al.* 1993].

Under ischaemic conditions, inhibition of glycolysis developed at the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [Rovetto *et al.* 1975]. An increase in myocardial levels of NADH, a strong inhibitor of GAPDH, was observed under ischaemic conditions. However, when the changes in NADH and oxidised nicotinamide adenine dinucleotide (NAD^+) were taken into account, the GAPDH reaction was displaced from equilibrium, indicating inhibition of this enzyme was aided by factors additive to the mass action effect of increased NADH [Rovetto *et al.* 1975]. It has been postulated that glycolytic flux may be limited at GAPDH, through an accumulation of protons [Neely and Grotyohann 1984], although no allosteric control of GAPDH has been found in a purified enzyme preparation [Mochizuki and Neely 1979].

Myocardial glycolysis is eventually inhibited by the accumulation of its products (mainly NADH) and accompanying acidosis in severe ischaemia [Neely and Grotyohann 1984]. Glucose uptake progressively decreases once glycolysis is inhibited, while protons and Ca^{2+} continue to accumulate [Vanoverschelde *et al.* 1994]. Increased supply of exogenous glucose, or the presence of insulin, attenuates the decline of glucose uptake during prolonged severe myocardial ischaemia [Apstein *et al.* 1983]. Such interventions promote glucose uptake to meet the increased demand for glucose moieties as an energy source.

Increased utilisation of exogenous glucose during ischaemia does not increase the overall rate of glycolytic flux but limits the participation of endogenous glycogen stores to this flux [Runnman *et al.* 1990]. In the model of no-flow ischaemia, glucose uptake is interrupted so glycogen breakdown supports glycolytic flux [Liu *et al.* 1996a].

1:8:3:1 Glycogen metabolism

Glycogen degradation occurs during anoxia and ischaemia when ATP levels fall and the concentrations of P_i and AMP are increased [Mellgren and Coulson 1983]. The conversion of phosphorylase *b* to *a*, is too slow to account for the increased rates of glycogen degradation, and it is thought that the elevated P_i and AMP concentrations also independently promote breakdown through phosphorylase *b* activation [Laughlin 1993]. This complex regulatory scheme allows for the very fast mobilisation of glycogen required for glycolytic ATP production under circumstances where energy demand can outstrip the supply of oxygen for oxidative phosphorylation. The ability of the heart to use glycogen rapidly compared to the increase in exogenous glucose use in response to acute changes suggests that glycogen provides a buffer for abrupt changes in substrate demand [Laughlin 1993].

1:8:4 Fatty acid metabolism in the ischaemic heart

Under conditions of severe ischaemia, where limited oxygen (O_2) is available for oxidative phosphorylation, there is a shift in energy production away from oxidative metabolism towards anaerobic production of energy via glycolysis [Lopaschuk *et al.* 1994]. Although fatty acids still represent the major residual substrate source for oxidative metabolism under ischaemic conditions [Fox *et al.* 1983] the increased $NADH/NAD^+$ ratio, as a result of the lack of oxygen, leads to a depression in fatty acid β-oxidation. This results in increased long chain acyl-CoA, long chain acylcarnitine and fatty acid intermediates of β-oxidation, which are detrimental to myocardial function [Fox *et al.* 1983, Liedtke *et al.* 1975].

1:8:5 Changes in myocardial levels of high energy phosphates

Maximal ATP production from glycogenolysis and anaerobic glycolysis is only 10 % of that produced by oxidative phosphorylation in the normal contracting heart [Kobayashi and Neely 1979]. ATP synthesis from oxidative phosphorylation is thought to cease immediately after coronary occlusion (ischaemia) [Barlow and Chance 1976]. As the heart has virtually no stores of oxygen, dramatic decreases in tissue oxygen tension levels are also observed in the heart shortly after ischaemia.

The rate of ADP phosphorylation is markedly diminished at the onset of anaerobic conditions and becomes further depressed as the duration of ischaemia increases. If ATP consumption were to continue at the pre-ischaemic rate, reserves of high energy phosphate would be depleted within the first minute of ischaemia [Jennings and Steenbergen 1985]. However, the rate of ATP utilisation is reduced in the ischaemic cell due to reductions in contractile work [Jennings and Steenbergen 1985].

Ischaemic levels of ATP are initially maintained by the flux of phosphate into cytosolic ATP synthesis from phosphocreatine and subsequently anaerobic glycolysis. Creatine phosphate levels are dramatically reduced after the onset of ischaemia [Fiolet et al. 1984]. The dissociation of creatine phosphate is practically in equilibrium with ATP synthesis. Small increases in ADP and P_i concentration lead to dramatic shifts of phosphate from creatine phosphate to ATP [Fiolet et al. 1984].

The activation of adenyl kinase (myokinase) is a defence mechanism of the heart to maintain ATP production. Myokinase converts ADP back to ATP at the expense of AMP formation [Jennings *et al.* 1981]. The result of this, together with the phosphate flux from phosphocreatine, is that enough ATP is present within the ischaemic myocytes to maintain, for a limited amount of time, their

ionic homeostasis and thus remain in a state in which cellular function can be reversed to normal upon reoxygenation. However, there is a persistent deficit between ADP phosphorylation and ATP hydrolysis that causes the overall ATP content to decrease as the duration of ischaemia is prolonged [Neely *et al.* 1973]. Eventually the purine precursors that are used for the re-synthesis of ATP are lost [Ronca-Testoni and Borghini 1982].

1:8:6 Ischaemic acidosis

Intracellular pH measurements have shown that the pH decreases from around 7.2 to 6.5 in the rat myocardium after 12mins of total ischaemia [Garlick *et al.* 1979]. Most of the protons that accumulate in the ischaemic heart are released by the hydrolysis of glycolytic ATP [Dennis *et al.* 1991]. As oxidative metabolism markedly decreases during ischaemia this proton release is no longer compensated by the consumption of protons that occurs with mitochondrial ATP regeneration leading to a net increase in proton production [Dennis *et al.* 1991]. As the duration of ischaemia lengthens the degree of tissue acidification increases.

Large amounts of inorganic phosphate (P_i) are also released upon the net hydrolysis of high energy phosphate compounds. The main effect of P_i accumulation is a decrease in maximal force development of the heart in early ischaemia as P_i reduces the calcium sensitivity of the cardiac contractile proteins [Fabiato and Fabiato 1978]. However, P_i is also a weak acid that liberates protons; with the role of P_i in cellular acidification becoming more prominent once the intracellular pH is below 6.9, the pK_a value of P_i [Dennis *et al.* 1991].

The main effect of acidosis is a negative inotropic effect during ischaemia. The acidosis increases the Ca^{2+} concentrations required to produce a given amount of tension in the myocardial wall [Fabiato and Fabiato 1978]. Protons can displace calcium from anionic binding sites of proteins, such as in troponin I involved in

the interaction between the contractile elements actin and myosin. The uptake of Ca^{2+} into the sarcoplasmic reticulum is also depressed by acidosis [Fabiato and Fabiato 1978]. In terms of cell survival, a rapid fall in cytosolic pH may therefore be of some initial benefit to ischaemic tissue, by helping it to decrease contractility and thereby retarding the breakdown of ATP [Poole-Wilson 1978].

Acidosis also inhibits several acidotic-sensitive enzymes in the glycolytic pathway. The most notable inhibition occurs at the rate limiting PFK-1 reaction. A decrease of glycolysis has also been shown by depletion of glycogen stores prior to ischaemia, leading to a marked reduction of tissue acidification [Garlick *et al.* 1979].

The overall buffering capacity of ischaemic tissue from the various buffering systems has been calculated from relationships between lactate production, pH, and changes in high energy phosphates. The main buffering systems are; ATP/P_i/PCr, intracellular proteins, and bicarbonate system in the pH range 6.4 to 7.0 [Wolfe *et al.* 1988]. It has been observed that the intrinsic cardiac buffering capacity in myocytes increases at lower tissue pH values [Wolfe *et al.* 1988]. Wolfe went on to suggest that the greater intracellular buffering capacity at lower pH may actually be protective by limiting the degree of intracellular acidosis, subsequently limiting further functional deterioration and irreversible damage [Wolfe *et al.* 1988].

1:8:7 Transition from reversible to irreversible ischaemic damage

Loss of membrane integrity is widely recognised as the event that signifies ischaemic injury has entered the lethal phase [Jennings *et al.* 1983]. Membrane damage may be a result of phospholipase action by ATP depletion, or a calcium overload. As the overall phospholipid concentration is only minimally altered it is thought that ischaemic membrane disruption can occur without significant phospholipid breakdown [Reimer and Jennings 1992]. Phospholipase activity

probably contributes more to the late membrane damage occurring in necrotic cells than to primary ischaemic membrane changes [Katz 1992a]. A significant increase in the by-products of phospholipid breakdown has been observed after 20 minutes of myocardial ischaemia [Chien *et al.* 1984].

Amphipathic compounds, e.g. fatty acyl derivatives, accumulate during ischaemia [Katz and Messineo 1981]. It is known that the detergent effects of high concentrations of these compounds damage membranes [Katz and Messineo 1981]. However, levels reached in the ischaemic myocardium are not sufficiently high enough to play an important role in disruption of the sarcolemma but may play a role in ionic homeostasis of the cell.

Cell swelling as a result of osmotic overload may also cause sarcolemma disruption by physically rupturing it [Jennings *et al.* 1990]. Firstly, disaggregation of the attachment of the cytoskeleton complexes, or protease (activated in a low energy environment) mediated breakdown of membrane or cytoskeletal proteins weaken the sarcolemma. Secondly, swelling of the cell causes the weakened membrane apparatus to rupture. Cellular swelling may also be the result of depletion of high-energy phosphates, resulting in insufficient energy to maintain cellular homeostasis.

The depletion of high-energy phosphates is hypothesised to be involved in the development of irreversible injury. Work by Jennings has shown that reversibly injured myocytes tolerate ATP levels as low as 10 μmoles/g dry weight, whereas when ATP levels were below 5 μmoles/g dry weight in irreversibly damaged myocytes [Jennings and Steenbergen. 1985]. Delayed or increased ATP depletion is known to delay/increase ischaemic cell death respectively. However, ATP depletion must be present for a period of time before irreversibility ensues, indicating an indirect relationship between ATP depletion and irreversibility.

1:9 Mechanisms of reperfusion injury

The reversible and irreversible manifestations of injury after a transient episode of ischaemia may not be the result of cellular conditions prior to reperfusion but may be a consequence of a detrimental component of the reperfusion itself. Whether reperfusion exacerbates tissue damage, destroying potentially salvageable tissue, or whether it merely accelerates the destiny of cells already doomed by injury is still a matter of debate. Several mechanisms have been proposed to explain reperfusion injury. The two most prominent mechanisms are the free radical hypothesis and the calcium paradox.

1:9:1 Free radical hypothesis

Irreversibly induced sarcolemma damage by oxygen free radicals, of which there are many sources in the reperfused myocardium, remains the subject of debate. In the absence of reperfusion it does not appear likely that the amount of oxygen free radicals generated during ischaemia would be significant [Reimer and Jennings 1992]. This is explained by a lack of oxygen for free radical production, and high intracellular levels of reduced cytochrome oxidase, an oxygen scavenger.

Free radicals are generated upon myocardial reperfusion in animals and humans. This free radical production remains to be defined as a basis of injury or merely a secondary feature arising from severely disrupted tissue upon reperfusion [Maxwell and Lip 1997].

The reduced resynthesis of ATP during ischaemia, due to interruption of oxidative phosphorylation, leads to a build up of adenine nucleotides such as AMP. This is rapidly converted to adenosine, which is irreversibly deaminated to yield inosine, hypoxanthine and subsequently xanthine (Figure 1.8) during ischaemia [Maxwell and Lip 1997]. The latter two molecules are substrates for

xanthine oxidase, but rapid production of the superoxide ion will only occur in the presence of oxygen, i.e. at the time of reperfusion.

Free radicals can also be formed during the breakdown (autooxidation) of catecholamines, and also from the metabolism of arachidonic acid via the cyclo-oxygenase system. The nicotinamide adenine dinucleotide phosphate oxidase system, located in phagocytotic neutrophils, is also an important source of oxygen free radicals. Following stimulation, the neutrophils rapidly consume oxygen with the simultaneous digestion of necrotic cells, producing superoxide radicals and subsequently hydrogen peroxide [Timbrell 1991].

Hydrogen peroxide can act as a source of further oxygen free radicals as it is relatively stable, and freely diffuses around cells. It can be broken down into the highly toxic hydroxyl ion by the Fenton reaction, [Timbrell 1991] and therefore disseminate injury over a wider range.

1:9:2 Calcium paradox

In both reversible and irreversible reperfusion injury, derangements in calcium homeostasis appear to play an important role [Tani 1990]. Reperfusion after prolonged ischaemia is associated with a large increase in the Ca^{2+} content in the severely damaged myocardium [Grinwald 1982] but not in myocardium that is not irreversibly injured [Jennings *et al.* 1985].

In reversible reperfusion injury cytosolic Ca^{2+} levels are rapidly normalised upon reperfusion, in contrast to contractility, which can remain depressed for several hours or days. The reason for this electro-mechanical uncoupling remains elusive [Kusuok and Marban 1992]. Contractile force was restored to pre-ischaemic levels by interventions that increased cytosolic Ca^{2+} levels [Ito *et al.* 1987]. Therefore, it has been postulated that the contractile elements are desensitised to Ca^{2+} [Kusuok and Marban 1992].

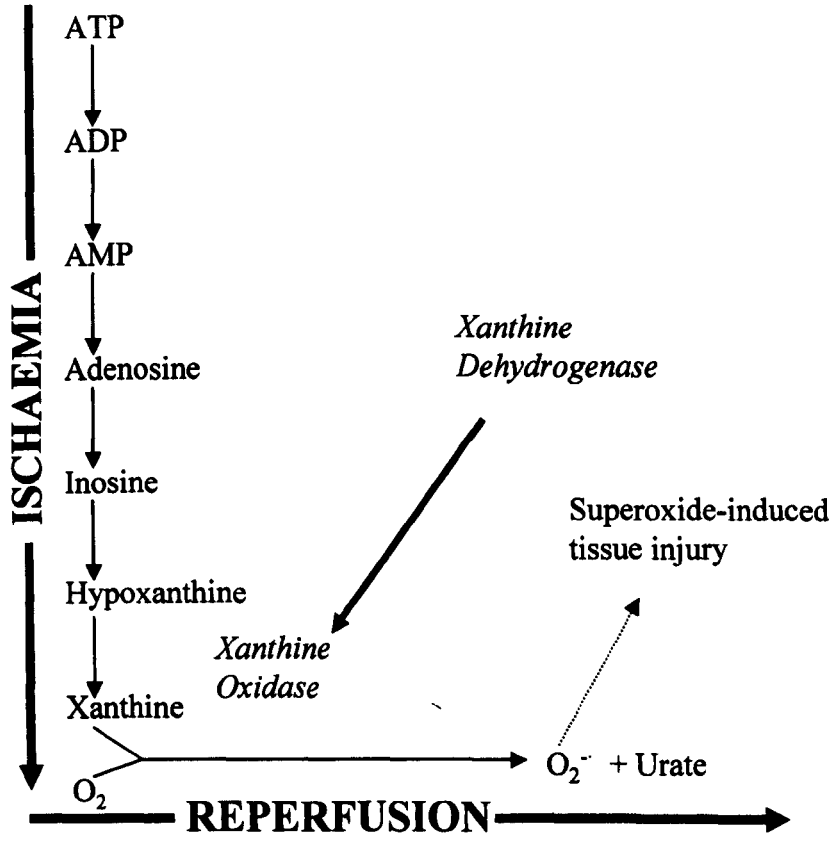


Figure 1.8: Purine nucleotide degradation [Maxwell and Lip 1997]

In irreversible injury there is an increased influx of Ca^{2+} during reperfusion in excess of the handling capacity of systems for Ca^{2+} extrusion from the cytosol, resulting in an elevation of cytosolic free Ca^{2+} concentration. The exact mechanisms responsible for increased Ca^{2+} entry upon reperfusion have not been defined, but the consequences of Ca^{2+} overload for myocyte integrity are well known [Tani 1990, Maxwell and Lip 1997].

1:9:3 Metabolism of glucose and glycogen at reperfusion

The role of glucose and glycogen as substrates during reperfusion is uncertain [M^{c}Nulty *et al.* 1996, M^{c}Nulty *et al.* 2000]. After brief periods of global ischaemia oxidative metabolism rapidly returns, well before the restoration of contractility [Liu *et al.* 1996a]. Stimulation of glucose oxidation at reperfusion accelerates functional recovery whereas inhibition of glucose utilisation impairs post-ischaemic contractility [Tamm *et al.* 1994].

1:9:4 Fatty acid metabolism in the reperfused heart

Upon reperfusion, the myocardium quickly regains the ability to oxidise fatty acids [Saddik and Lopaschuk 1992]. After an initial burst of endogenous fatty acid oxidation during reperfusion, the heart predominantly relies upon exogenously derived fatty acids for oxidation [Saddik and Lopaschuk 1992]. High rates of fatty acid oxidation, at the expense of glucose oxidation, may be detrimental to functional recovery of the heart during reperfusion [Lopaschuk *et al.* 1990].

1:10 Nuclear magnetic resonance

Nuclear magnetic resonance (NMR) spectroscopy is a powerful non-invasive technique that can be used to study metabolism *in situ*. NMR uses a strong external magnetic field and radiofrequency pulses to detect the magnetic resonance signal of nuclei that possess magnetic spin. It can be used to study a wide variety of systems from molecules, to isolated cells and organs, to *in vivo* [Gadian 1995]. Several biologically interesting atomic nuclei are suitable for NMR, such as hydrogen (^{1}H), carbon (^{13}C), sodium (^{23}Na) and phosphorus (^{31}P) [Gadian 1995]. Although it has many advantages, NMR has one serious disadvantage: it is insensitive. Typically, NMR-visible nuclei must be millimolar before they can be observed within physiologically relevant timeframes.

1:10:1 ^{31}P-NMR

The phosphorus-31 (^{31}P) isotope is 100 % naturally abundant, and many small molecular weight phosphorylated metabolites are visible by NMR within cells. Compared to hydrogen (^{1}H), ^{31}P is less sensitive, but has a reasonable chemical shift range (~30ppm for biological phosphates) [Gadian 1995].

^{31}P spectra are relatively simple containing only a small number of signals. Narrow signals in ^{31}P-NMR spectra are only observed from relatively mobile compounds. Membrane phospholipids are highly immobilised and produce very broad signals that are either NMR invisible or appear as broad components underlying the narrow metabolite signals. A result of this is that it is commonly assumed that ^{31}P-NMR monitors cytoplasmic metabolites [Gadian 1995]. The most significant NMR visible phosphorus signals in cardiac tissue come from the three phosphates of ATP, (P_i) and PCr [Gadian 1995], metabolites that play central roles in energetics, and to a lesser extent phosphomonoesters and phosphodiesters.

^{31}P-NMR is ideally suited to investigating the changes in high energy phosphate metabolism, which can be used to estimate the energetic status of the heart [Jung and Dietze 1999]. In addition, intracellular pH can be quantified from the differences in chemical shifts between PCr and P_i, as the P_i signal is pH sensitive. [Moon and Richards 1973, Bailey *et al.* 1982].

1:10:2 ^{13}C-NMR

Despite the low natural abundance (only 1.1 %) and relative insensitivity of the ^{13}C nucleus, observation is possible of some molecules without selective enrichment. These natural abundance studies are primarily of storage molecules, such as glycogen [Bottomley *et al.* 1989]. Most ^{13}C-NMR work is performed using selective enrichment of the compound of interest to investigate specific metabolic pathways. The chemical shift range of the ^{13}C nucleus is 0-220 ppm.

The low isotopic abundance of ^{13}C can also be used to advantage with the application of labelled precursors. For example, cells can be fed with glucose labelled with ^{13}C in position 1. As the glucose is metabolised, label appears in the carbons of various metabolites and intermediates that can be observed in a single spectrum. The rate of label appearance, the steady state levels of labels, and the isotopomeric distribution of end products can all be used to determine the identity and activity of the metabolic pathway used [Malloy *et al.* 1988, Malloy *et al.* 1990a, Weiss *et al.* 1992, Weiss *et al.* 1993, Chatham *et al.* 1995, Jeffrey *et al.* 1996]. ^{13}C-NMR is an important tool with which to study the metabolic fluxes of [Weiss *et al.* 1992, Weiss *et al.*1993, Chatham and Forder 1995], and substrate selection in [Malloy *et al.* 1988, Malloy *et al.* 1990a, Jeffrey *et al.* 1996], the TCA cycle.

1:10:2:1 ^{13}C-NMR and its application to studies with myocardial glycogen

Myocardial glycogen metabolism has been observed *in vivo* in the rat [Laughlin *et al.* 1988] and canine heart [Laughlin *et al.* 1992], as well as in the perfused

heart [Brainard *et al.* 1989] using proton-decoupled ^{13}C-NMR. Glycogen visibility in perfusions is routinely enhanced with ^{13}C-labelled glucose and insulin [Taegtmeyer 1994]. Technical advances in spatial localisation and safe decoupling practices have resulted in the spectra of naturally occurring ^{13}C-glycogen from human hearts [Bottomley *et al.* 1989].

In 1983, Sillerud and Schulman reported that glycogen gave a well-resolved ^{13}C NMR spectrum in which the carbons are almost completely NMR visible [Sillerud and Schulman 1983]. These findings were surprising as normally, large molecules with relatively little mobility such as glycogen, give very broad NMR signals. The NMR relaxation properties of glycogen observed in tissue have been found to be a function of water content and temperature. At 4 °C, the ^{13}C NMR signal has a broad and narrow component. Upon raising the temperature to 30 °C, the signal is homogeneous, narrow and Lorenzian [Zang *et al.* 1990]. Each glucosyl unit of glycogen is associated with water molecules. When the degree of hydration of glycogen passes 60 %, as in the cell matrix, it appears in the NMR spectra as effectively homogenous [Jackson and Bryant 1989]. The increase in hydration of glycogen primarily reduces the spin-lattice relaxation time (which gives rise to the broad component of the observed NMR signal), due to an increase in the presence of other nuclei that can absorb the radio frequency (RF) energy from the ^{13}C nuclei.

^{13}C-NMR has been used as a tool to investigate the synthesis and turnover of glycogen in the heart. Primarily, these experiments have used [1-^{13}C]-glucose labelling to shorten the time necessary to achieve a spectrum for glycogen with acceptable signal to noise, although [2-^{13}C]-glucose has also been used [Brainard *et al.* 1989]. ^{13}C Glucose labelled in the C1 carbon atom has been the most popular used substrate because the C1 carbon of both glucose (α-C1, 92.7ppm; β-C1, 96.6ppm) and glycogen (100.6ppm) resonate in an otherwise unoccupied part of the spectrum, and are easily resolved using current B_0 fields [Malloy *et al.*1990a]. ^{13}C glucose is also relatively inexpensive (compared to other ^{13}C

labelled compounds) and is readily available. The location of a single label of one type (e.g. ^{13}C) within a molecule can easily be followed in metabolic pathways, as it will be assimilated into subsequent metabolites in a very defined place. The label of choice can either be injected or infused into the animal, or included in the perfusate of an isolated organ.

By measuring the changes in the concentration of ^{13}C-glycogen and the ^{13}C enrichment of uridine diphosphoglucose (UDPG) from ^{13}C-glucose, one can determine the rate of flow of the labelled glucose into glycogen and the enrichment of the label in the precursor pool [Laughlin *et al.* 1990]. Two characteristics of glycogen metabolism in the heart make these two measurements relatively straightforward. Firstly, UDPG is synthesised only from glucose that enters the tissue from the circulation as the heart lacks a gluconeogenic pathway. As a result, the ^{13}C enrichment of UDPG has to be equal to that of the plasma or perfusate glucose. Secondly, glycogen synthesis and breakdown rates are slow enough to be measured by NMR, but sufficiently rapid to be studied in short experiments [Laughlin 1993].

Similarly, the breakdown of glycogen can be witnessed by observing the depletion of labelled ^{13}C-glycogen signal. Glycogen turnover can be measured with NMR by using [1-^{13}C]-labelled glucose followed either with ^{13}C glucose labelled in a different position (e.g. [2-^{13}C]), or with unlabelled glucose. As the first label is washed from the bloodstream or the perfusate, the NMR signal of glycogen made from this glucose falls as a function of the rate of glycogen turnover.

1:10:2:2 ^{13}C-NMR analysis of the myocardial TCA cycle

Isotopes of carbon have been used as tracers to study the TCA cycle and its regulation for over 40 years [Ajl and Kames 1951]. However, the first NMR detection of ^{13}C in intact cells was not reported until 1972 [Eakin *et al.* 1972].

Over the last 20 years, ^{13}C NMR spectroscopy has developed as a powerful tool for investigating the numerous pathways that intersect in the TCA cycle [Walsh and Koshland 1984, Malloy *et al.* 1987, Jeffrey *et al.* 1991]. ^{13}C NMR has three distinct advantages when compared to traditional carbon tracers used in animal and tissue studies. First, all labelled metabolites present at sufficient concentration are detected in a single spectrum, not just those selected for study. Second, the information obtained is the same as the classical studies but the experiments are simpler to perform as no further purification and chemical degradation is required. By directly observing a ^{13}C NMR spectrum the relative fractional enrichments can be measured at each carbon atom by comparing signal peak areas. Third, by measuring the fractional enrichments at individual carbons, the enrichment patterns of adjacent carbons are also detected. These patterns provide a considerable amount of information on the relative activity of the pathways investigated, and also allow the simultaneous use of different labelled substrates [Malloy *et al.* 1988, Malloy *et al.* 1990a, Weiss *et al.* 1992, Weiss *et al.* 1993].

Recent studies have further developed approaches to interpreting the patterns produced in isotopomer analysis of the TCA cycle. Computer simulations [Malloy *et al.* 1990a] can provide a turn-by-turn analysis of the fate of ^{13}C entering the TCA cycle. Mathematical representations of the TCA cycle [Malloy *et al.*1988] allow the measurement of carbon flow into the cycle via anaplerotic pathways as well as through acetyl CoA. Methods to directly measure how much of the acetyl CoA entering the cycle is derived from glucose, acetate or lactate have been developed [Malloy *et al.* 1990a], providing a direct measurement of the substrates that are selected for oxidation.

Of special interest to this study are the simple multiplet patterns of glutamate, which can be easily observed in the ^{13}C spectrum as they appear in an otherwise unoccupied region of the spectrum.

1:11 Objectives

Myocardial hypertrophy may lead to functional and biochemical abnormalities that can enhance the susceptibility of the hypertrophied heart to ischaemic injury, which may impair recovery of function at reperfusion. There is evidence for an increased dependence upon glucose metabolism in CH that may be detrimental to the heart under ischaemic conditions. During ischaemia oxidative metabolism decreases, and glycolysis becomes a more significant source of ATP production. Studies have reported that enhanced glucose use and glycolytic rates may be detrimental in the hypertrophied heart due to a greater than normal accumulation of damaging glycolytic end products. During ischaemia glycogen is the major endogenous substrate for glycolysis. Studies in normal hearts have reported benefits of glycogen depletion prior to ischaemia, limiting glycolysis and the accumulation of toxic end products, where as other work has shown that the maintenance of glycogenolysis, providing ATP during ischaemia, is beneficial for myocardial recovery post-ischaemia. The role of glycogen in ischaemic/reperfusion injury remains unclear in the hypertrophied heart.

The objectives of this study were to;

i) assess the susceptibility to ischaemic/reperfusion injury in pressure overload cardiac hypertrophy. Aortic constriction was performed surgically in rats to develop a model of pressure overload cardiac hypertrophy for investigation. Ischaemic/reperfusion injury was assessed by functional recovery and inosine (a breakdown product of ATP) release on reperfusion. Two models of ischaemia were investigated, low flow ischaemia and global ischaemia.

ii) assess the contribution of glycogenolysis to the severity of the ischaemic insult in the hypertrophied heart. In particular, the impact of insulin on glycogen loading and how this relates to subsequent ischaemic injury. Glycogen loading protocols were developed using increasing levels of insulin, and by providing alternative energy

substrates in perfusion buffers. A depletion/repletion perfusion protocol was employed to try and enable the labelling of the glycogen stores using 1-^{13}C-glucose.

iii) assess the extent of oxidative metabolism under normoxia and during reperfusion in the hypertrophied heart and how it relates to functional recovery. Oxidative metabolism was assessed at the end of perfusions using ^{13}C-labelled energy substrates by analysing the isotopomeric distribution of ^{13}C-label in glutamate. Both steady state and non-steady state methods were utilised to determine the relative contributions of substrates to oxidative metabolism in the TCA cycle.

iv) investigate the role of insulin on myocardial recovery post-ischaemia by assessing its effects on functional recovery as well as oxidative substrate metabolism.

2: MATERIALS AND METHODS

2:1 Materials

A list of chemicals, biochemicals and the relevant suppliers used in this thesis is given in Tables 2.1 and 2.2. All chemicals were of AnalaR grade.

2:2 Surgical induction of pressure overload left ventricular cardiac hypertrophy by abdominal aortic banding

Pressure overload left ventricular cardiac hypertrophy was induced surgically as described previously [Boateng *et al.* 1997, Boateng *et al.* 1998]. All procedures were performed using aseptic techniques and were in accordance with the Home Office Guidance on the Operation of Animals (Scientific Procedures) Act 1986.

Adult male Sprague Dawley rats (220-270 g) were anaesthetised with an intraperitoneal injection of Ketaset: Domitor: sterile water, ratios 2.4: 1: 9 respectively (dose – 960 mg/Kg body weight (BWt) ketamine hydrochloride and 4 mg/Kg BWt medetomidine hydrochloride). A subcutaneous injection of Rimadyl (4 mg/Kg BWt), an analgesic agent, was also administered at this time for post-operative pain relief. A midline incision was made and the abdominal aorta was exposed and isolated. The aorta was ligated between the left and right renal artery branches (Figure 2.1) using a 0.6 or 0.7 mm outer diameter (OD) blunted needle (NHS Supplies, England) with suture (Ethilon Mersilk 0/0 suture. Ethicon, Somerville, U.S.A.). Ligation of the aorta caused the left kidney to blanch. Removal of the needle restored the blood flow and re-colouration of the left kidney was used to verify re-establishment of circulation. Sham (S) animals underwent the same procedure but without constriction of the aorta. Sterile isotonic saline (Animal Care Ltd., York, England) was administered directly into the abdominal cavity to compensate for any fluid loss during the procedure. The muscle layer of the abdominal wall was closed with Chromic suture (Ethicon) and the epidermal layer with blue suture thread (Ethicon). Two subcutaneous

Description	Chemical	Supplier
Chemicals	AMP, $NH_4H_2PO_4$, Ammonium molybdate tetrahydrate, Amyloglucosidase, Ascorbic acid, Caffeine, Dithiothreitol, Glucose 1-phosphate (G 1-P), Glycine, Glycogen, K^+HEPES, Hydrazine, Hypoxanthiine, Inosine, NAD^+, NaF, Palmitic acid -sodium salt, Perchloric acid, Silica gel Uric acid, Xanthine	Sigma, Poole, England
	$CaCl_2.2H_2O$, EDTA, Ethanol, Glucose, Glycerol, Methanol, $MgSO_4.7H_2O$, K_2CO_3, KCl, K_2HPO_4, KH_2PO_4, KOH, NaCl, $NaHCO_3$, Silica gel, Na_2SO_4, Sodium dodecyl sulphate (SDS)	BDH, Poole, England
	Acetic acid	Vickers Laboratories, Pudsey, England
	Lactate dehydrogenase (LDH)	Boehringer Mannheim, Lewes, England
	Deuterated water (D_2O)	Goss Scientific Instruments, Great Baddow, England
	Bovine serum albumin-fatty acid free (BSA)	Intergen, Purchase, USA

Table 2.1: Chemicals and supplier information.

Description	Chemical	Supplier
Assay Kit	Glucose assay kit (N° 510)	Sigma, Poole, England
^{13}C-Chemicals	D-[1-^{13}C] glucose, [U-^{13}C] palmitic acid	Cambridge Isotopes, Massachusetts, USA
Drugs	Antisedan (5 mg/ml atipamezole hydrochloride), Domitor (1 mg/ml Medetomidine hydrochloride), Rimadyl (5% w/v carprofen)	Pfizer Ltd., Sandwich, England
	Ketaset (100 mg/ml ketamine hydrochloride)	Fort Dodge Animal Health Ltd., Southampton, England
	Amfipen (150 mg/ml anhydrous ampicillin)	Intervet UK Ltd., Cambridge, England
	Thiovet (0.025 g/ml thiopentone sodium)	Vericore Ltd., Marlow, England
	Heparin	CP Pharmaceuticals Ltd., Wrexham, Wales

Table 2.2: Assay kits, ^{13}C-chemicals, veterinary drugs and supplier information.

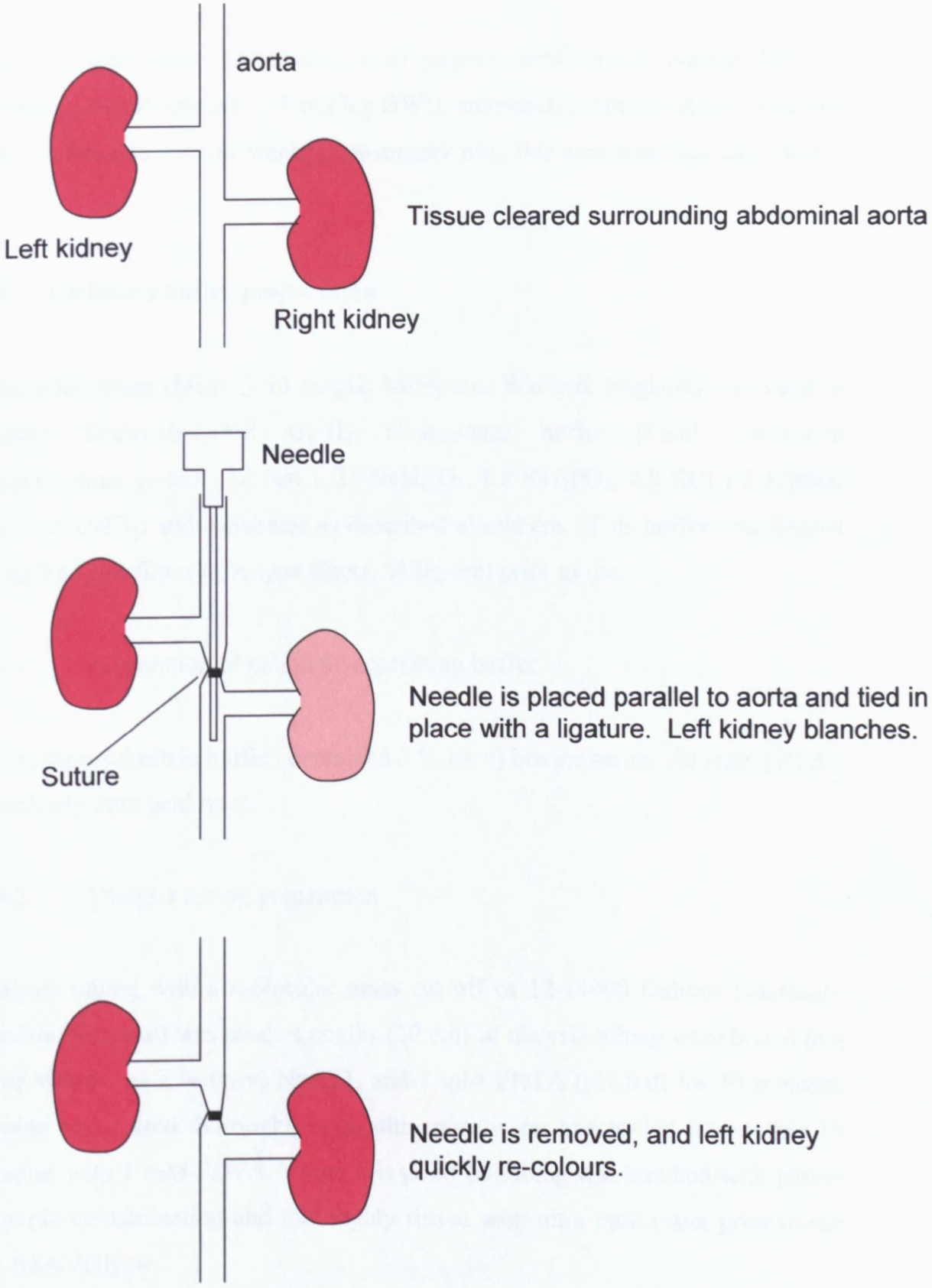


Figure 2.1: Illustration of the position of the aortic ligature

injections were given immediately after surgery, Amfipen (42 mg/Kg BWt) an antibiotic, and Antisedan (0.8 mg/Kg BWt), anaesthetic reversal agent. Animals were maintained for nine weeks post-surgery with free access to food and water.

2:3 Perfusion buffer preparation

Ultra-pure water (Milli Q 18 megΩ, Millipore, Watford, England) was used to prepare Krebs-Henseleit (K-H) bicarbonate buffer (Final electrolyte concentrations (mM); 118 NaCl, 25 $NaHCO_3$, 1.2 KH_2PO_4, 4.8 KCl 1.2 $MgSO_4$ and 1.25 $CaCl_2$) and substrates as described elsewhere. This buffer was filtered using 0.45 μm filters (HA type filters, Millipore) prior to use.

2:3:1 Preparation of palmitate containing buffer

All sodium palmitate buffers contained 3 % (w/v) bovine serum albumin ((BSA), essentially fatty acid free).

2:3:2 Dialysis tubing preparation

Dialysis tubing with a molecular mass cut off of 12-14000 Daltons (Medicell, London, England) was used. Lengths (20 cm) of dialysis tubing were boiled in a large volume of 2 % (w/v) Na_2CO_3 and 1 mM EDTA (pH 8.0) for 10 minutes. Tubing was rinsed thoroughly with ultra pure water and boiled for another 10 minutes with 1 mM EDTA. From this point on tubing was handled with gloves to avoid contamination and thoroughly rinsed with ultra pure water prior to use for BSA dialysis.

2:3:3 BSA dialysis

BSA solutions were prepared by dialysing against 118 mM NaCl and 2.5 mM

$CaCl_2$ prior to use. This removed any small molecular weight impurities from BSA, as well as saturating the calcium binding sites. A 30 % (w/v) solution of BSA in 118 mM NaCl and 2.5 mM $CaCl_2$ was dialysed against a 20 times volume of 118 mM NaCl and 2.5 mM $CaCl_2$ solution at 4 °C for 48 hours. After dialysis, the volume of BSA solution was recorded prior to storage at -20 °C until required.

2:3:4 BSA buffer containing palmitate

BSA containing buffer was prepared freshly as required. Sodium palmitate (unlabelled or uniformly ^{13}C labelled) was initially dissolved in hot water before adding to the 30 % (w/v) BSA stock solution. K-H buffer was added to this stock to give a final concentration of 3 % (w/v) BSA. Appropriate corrections were applied for NaCl and $CaCl_2$ in the K-H buffer to account for the 118 mM NaCl and 2.5 mM $CaCl_2$ already present in the BSA stock. The K-H buffer containing BSA was filtered through 5 μm and 0.45 μm low protein binding filters (HVLP and SVLP type filters respectively, Millipore) before use.

2:4 Sodium palmitate preparation from palmitate

Uniformly ^{13}C labelled-([U-^{13}C]-) sodium palmitate was prepared from the free [U-^{13}C]-palmitic acid using the method of Wolfe *et al.* 1980. Briefly, a 30.9 mM NaOH solution was prepared in 80 % (v/v) methanol. 0.3 mmol of [U-^{13}C]-palmitic acid was dissolved in 25 ml of hexane, and 10 ml of the NaOH/methanol solution. This mixture was heated to 60 °C and evaporated to dryness with nitrogen gas. The resulting [U-^{13}C]-sodium palmitate salt was stored desiccated (Silica gel) at 0-4 °C until required.

2:5 Isovolumic heart perfusion

Male Sprague-Dawley rats were anaesthetised with an intraperitoneal injection of Thiovet (100 mg/Kg BWt sodium thiopentone). Body weights were recorded and heparin (1000 IU/Kg BWt) was injected into the femoral vein. Hearts were immediately excised and placed in ice-cold K-H bicarbonate buffer containing 5 mM glucose. Hearts were cannulated via the aorta, for retrograde perfusion in a modified isovolumic Langendorff mode, Figure 2.2 [Chain *et al.* 1969, Ogino *et al.* 1996]. The apex of the left ventricular wall was pierced to prevent accumulation of fluid by allowing Thebesian drainage.

An oxygenator rig (Figure 2.3, [Gamcsik *et al.* 1996]) was used for all perfusion experiments at a constant flow rate of 14 ml/min (MHRE/22 Mk3 Flow Inducer, Watson-Marlow, Leeds, England). This flow rate was selected to match a perfusion pressure of 85 cm H_2O in Langendorff perfused hearts (63.5 mmHg, Clarke 2001). All buffers were maintained at 37 °C and equilibrated with 95 % O_2/5 % CO_2 to pH 7.4.

2:6 Physiological measurements during perfusion

Left ventricular function was monitored using a cling-film water-filled balloon [Ogino *et al.* 1996]. This balloon, attached via a fluid filled polyethylene tube (Cole-Parmer, Illinois, USA) to a physiological pressure transducer (SensoNor, Horten, Norway), was inserted into the left ventricular cavity via the mitral valve (Figure 2.2). The pressure transducer was connected to a MacLab bridge amp linked to a two channel MacLab/2e system (AD Instruments, Hastings, England) to record left ventricular diastolic and systolic pressures, and heart rate (Figure 2.4). The balloon was inflated using a 2.0 ml micrometer syringe (Gilmont Instruments, Barrington, U.S.A.) to give an end diastolic pressure of ~5 mmHg [Ogino *et al.* 1996].

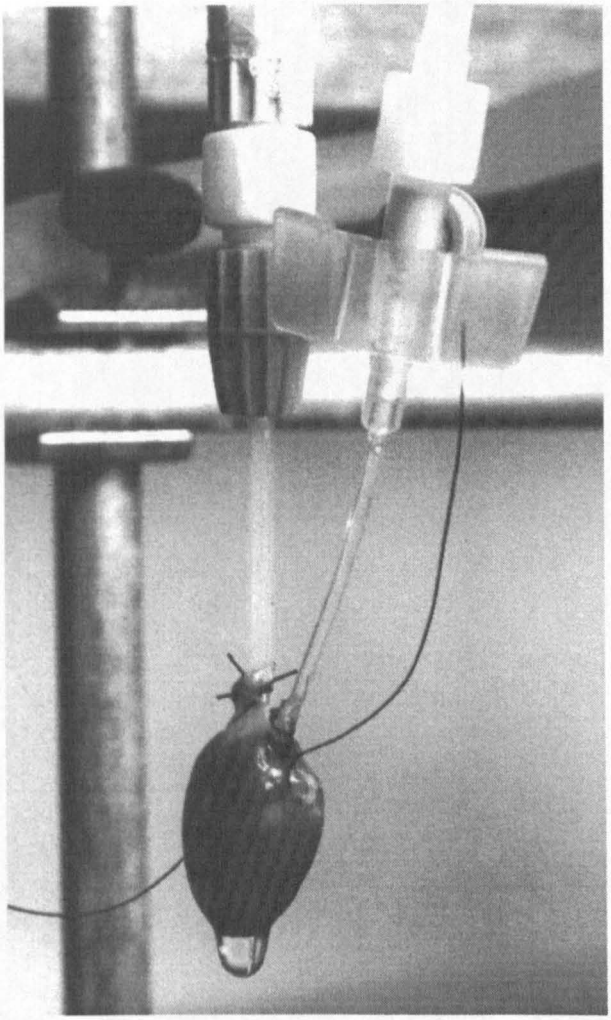


Figure 2.2: Cannulated heart with balloon inserted into left ventricle.

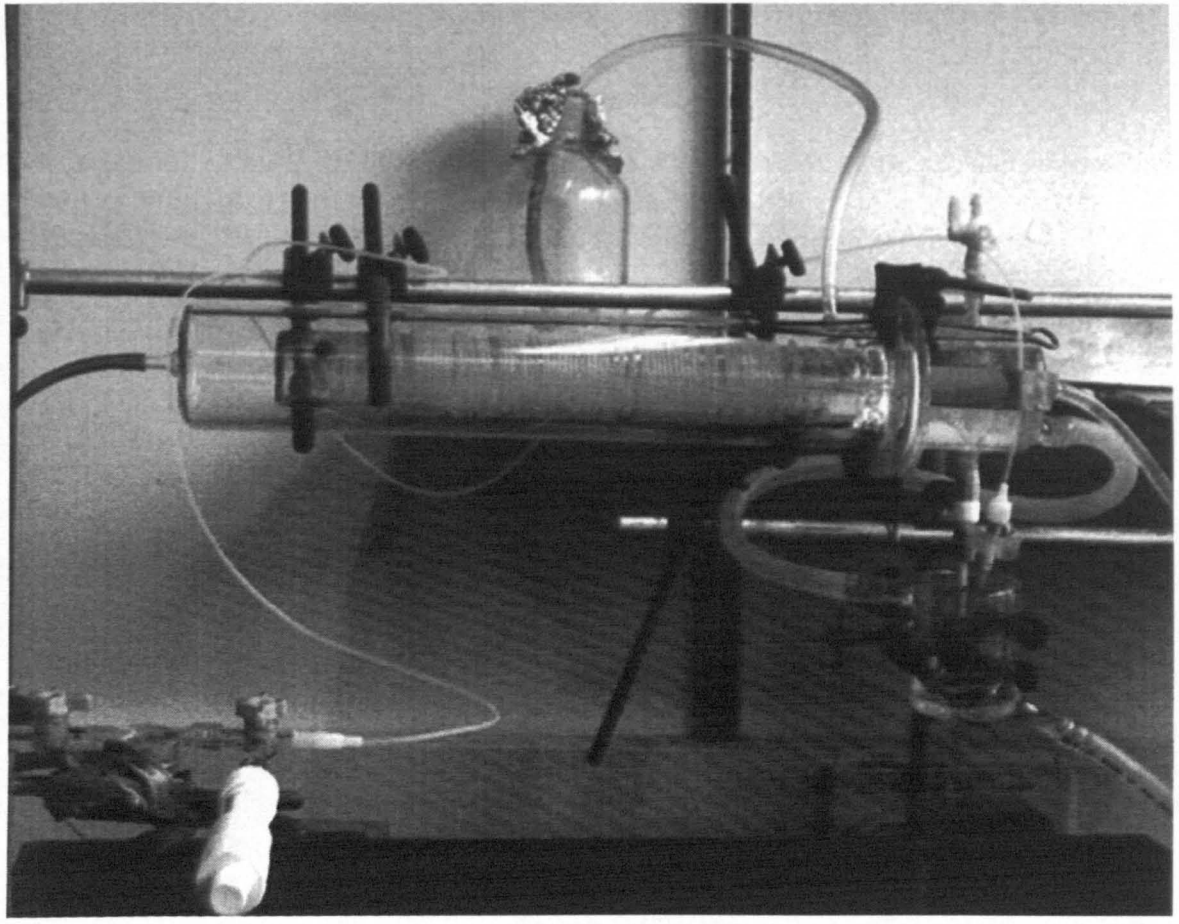


Figure 2.3: Oxygenator perfusion rig.

Physiological measurements were continuously recorded during each experiment. Left ventricular developed pressure (LVDP) was calculated (LVDP = end systolic pressure (ESP) - end diastolic pressure (EDP), Figure 2.5) at 10 minute intervals and averaged over the whole perfusion duration. Cardiac contractile function was gauged by calculating the rate pressure product (RPP), where RPP = LVDP x heart rate. Hearts with an average normoxic RPP of less than 15 x 10^3 mmHg.min were excluded from studies, of which there was only one.

2:7 General perfusion protocol

All hearts were perfused initially with K-H buffer containing only unlabelled (^{12}C) substrates for a 20 minute equilibration period. The equilibration period commenced once the balloon was positioned and left ventricular end diastolic pressure set. Subsequently, perfusion buffer was switched as required for each experimental protocol. During all experimental protocols, coronary flow rates were recorded every 15 minutes. At the end of each perfusion protocol, hearts were freeze-clamped using Wollenberger tongs cooled to the temperature of liquid nitrogen and were stored under liquid nitrogen until further analysis.

2:7:1 Perfusion buffer analysis and oxygen consumption calculation

Perfusion buffer samples were analysed prior to hanging of hearts. Effluent samples were also analysed at 20 and 40 minutes during normoxia and at 5, 15 and 25 minute time points during reperfusion where appropriate. Samples were analysed using an ABL 70 blood gas analyser machine (Radiometer Ltd., Crawley, England) for the determination of pH, pCO_2, pO_2, Na^+, K^+, and Ca^{2+}. Oxygen consumption was determined for hearts where perfusate samples were analysed based on a calculation described by Neely [Neely *et al.* 1967].

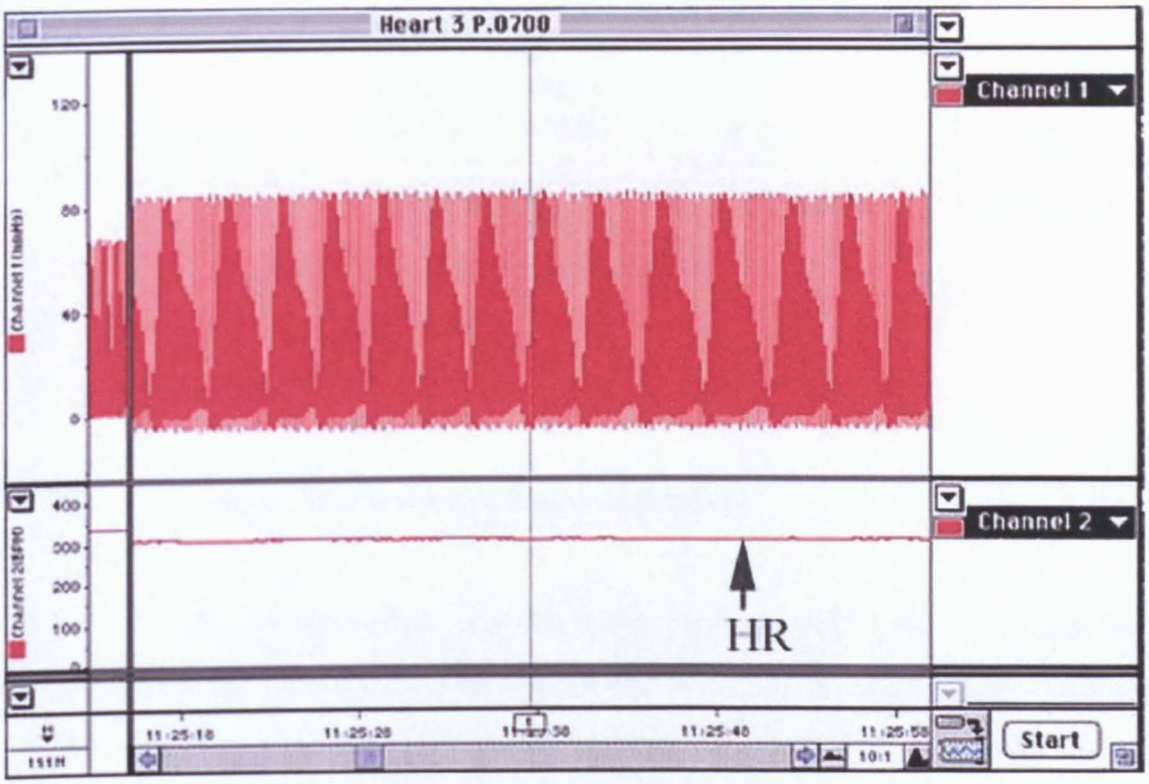


Figure 2.4: Pressure trace during normoxia.

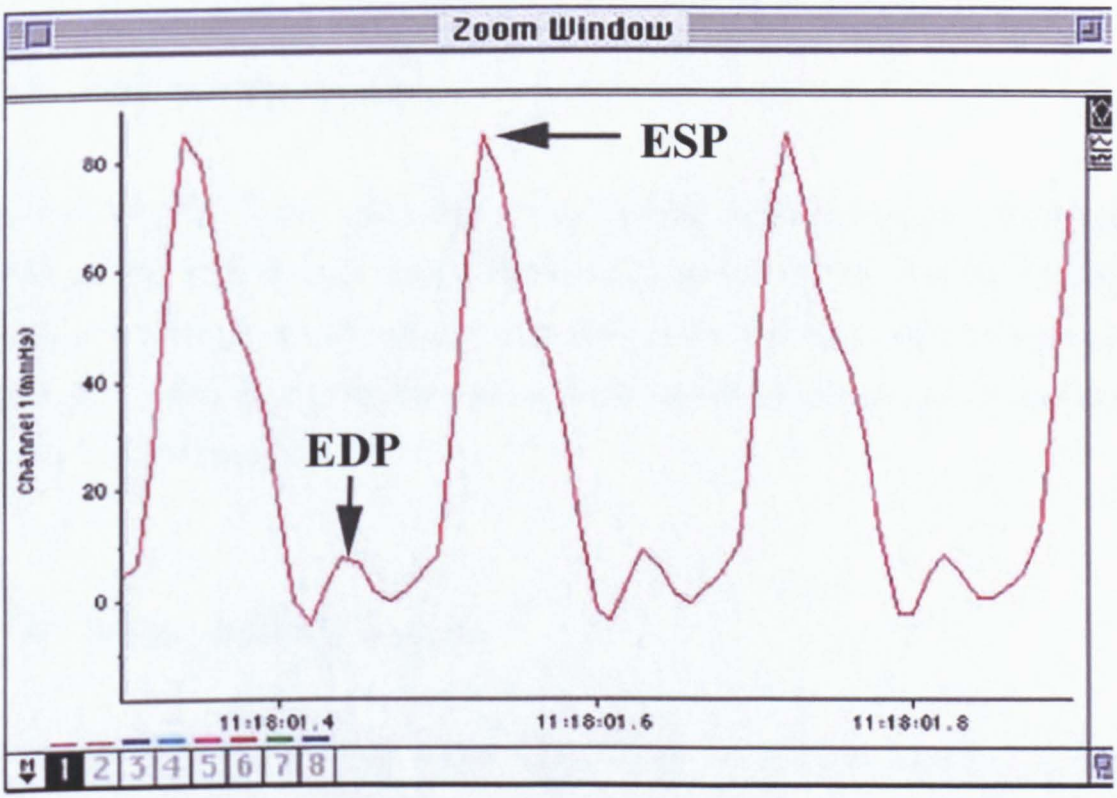


Figure 2.5: Enlarged portion of myocardial function trace showing individual beats.

$$O_2 \text{ consumption (mmol/min.g dry wt.)} = \frac{pO_2 \text{ buffer (mmHg)} - pO_2 \text{ effluent (mmHg)}}{760\text{mmHg}} \times \frac{S.\,O_2}{22.4 \text{ (ml/mmol)}} \times \frac{CF}{\text{Dry Heart Wt.}}$$

S. O_2 – solubility of O_2 at 37 °C (ml/ml H_2O)

CF – coronary flow (ml/min)

Wt. - weight

2:7:2 Reperfusion coronary effluent collection

Coronary effluent was collected at the following time points during reperfusion; at 30 second intervals for the first minute and at minute intervals up to 5 minutes. Samples were stored at –70 °C, until required for analysis by high performance liquid chromatography (HPLC) of nucleoside content.

2:8 Indices of hypertrophy

The extent of CH was determined by calculating the heart weight: tibia length ratio and the body weight: heart weight ratio [Yin *et al.* 1982]. Right: left kidney weight ratios were used to assess the severity of constriction caused by the aortic ligature. Tibias were removed and cleaned before the lengths were measured using a pair of callipers.

2:9 Tissue extraction methods

Frozen hearts were weighed before being ground to a fine powder with a pestle and mortar under liquid nitrogen. Approximately 100 mg of the powdered tissue was removed, thawed at room temperature to determine the wet weight, and was then left to dry at 40 °C until constant weight to determine the dry weight. The wet: dry weight ratios for each heart were subsequently calculated.

2:9:1 Acid extraction for metabolite analysis

A known weight of frozen ground tissue was extracted at 4 °C as described previously [Seymour *et al.* 1990]. In brief, 6 % (v/v) perchloric acid (PCA) was added at a ratio of 5:1 (e.g. 5 ml of PCA per 1g tissue), mixed thoroughly and left on ice for 10 minutes. The suspension was centrifuged in a bench centrifuge (General laboratory centrifuge-4, Sorvall instruments, Bishop's Stortford, England) at 4 °C for 10 minutes at 800 times gravity (*g*) to remove the precipitated protein. A known volume of the resulting supernatant was decanted into a clean, cooled, centrifuge tube and neutralised to pH of 7 using a 6 M KOH/0.7 M K_2CO_3 solution at 4 °C. All volumes were accurately recorded. The pH was monitored using universal and narrow range (pH 6.8 to pH 8.3) pH papers (Whatmann International Ltd., Maidstone, England). Subsequently, the sample was centrifuged at 800 *g* for 10 minutes at 4 °C to remove the perchlorate salt and the resulting supernatant was decanted and stored at –70 °C until further analysis.

2:9:2 Acid extraction for ^{13}C-NMR analysis

Extraction for ^{13}C-NMR analysis was as described above (Section 2:5:2), except 6 M KOH was used to neutralise the sample. The resulting supernatant was transferred to a 50 ml tube (Becton Dickinson) and lyophilised using a freeze drier (Freeze Drier Modulyo. Edwards, Crawley, England). This sample was stored with desiccant (Silica Gel) at –20 °C until further analysis.

2:9:3 Preparation of coronary effluent samples for HPLC analysis

PCA (6 % v/v) was added to the coronary volume samples collected in a 1:1 ratio and the samples were extracted as described above (Section 2:9:1).

2:9:4 Alkali extraction for glycogen analysis

A known weight of frozen ground tissue (~400 mg) was digested with 0.5 ml of 30 % (w/v) KOH for 30 min at 100 °C for glycogen precipitation as described previously [Walaas and Walaas 1950, Bergmeyer 1983]. Subsequently, 0.2 ml of 2 % w/v Na_2SO_4 was added to the cooled digest and a sufficient volume of 100 % ethanol to give a final concentration of 75 % ethanol, assuming the heart to have a specific gravity of 1. The resulting solution was transferred to a centrifuge tube, sealed, and left overnight at 0-5 °C to allow glycogen precipitation. Subsequently the precipitate was separated by centrifugation at 4 °C for 10 minutes at 38000 *g* and was washed in 80 % ethanol. The washed solution was re-spun at 38000 *g*, and the remaining pellet was dried in an oven at 40 °C for 2 hours.

The precipitated glycogen was digested to glucose using 0.5 ml acetate buffer (1 M sodium acetate/acetic acid buffer pH 5.0), 0.1 ml amyloglucosidase (500 μg/ml amylo α-1,4 α-1,6-glucosidase, made up in acetate buffer) and 1.5 ml distilled water, at 37 °C for 1 hour. The volume was made up to 2.5 ml with distilled water, and centrifuged at 8000 *g* for 20 minutes. The supernatant was decanted, and used for the assay of glucose (Section 2:10:1).

2:9:5 Extraction method for determination of glycogen phosphorylase activity

Heart tissue was extracted in a buffer designed to prevent the inter-conversion of a and b forms of phosphorylase [Kalil-Filho *et al.* 1991]. Approximately 300 mg of frozen tissue was mixed with 1 ml extraction buffer (containing 60 % (v/v) glycerol, 5 mM EDTA, 20 mM NaF, 20 mM K^+HEPES and 1 mM dithiothreitol, pH 7) at 4 °C. Added to the suspension was 2ml of dilution buffer (5 mM EDTA, 20 mM NaF, 20 mM K^+HEPES and 1 mM dithiothreitol, pH 7). The extract was centrifuged at 12000 *g* for 10 minutes and the supernatant removed

and kept. The remaining pellet was re-extracted with 0.5 ml of extraction buffer, spun and the supernatant removed. Supernatants were combined and diluted with dilution buffer to 5 ml for determination of glycogen phosphorylase activity (Section 2:10:3).

2:10 Metabolite assays

2:10:1 Glucose assay

Glucosyl units from glycogen digests were determined using a Sigma glucose kit (N° 510-A) based upon the method of Raabo and Terkilsden [Raabo and Terkilsden 1960]. Absorbance was measured at λ = 450 nm (Philips PU8720 UV/VS Scanning Spectrometer, Spectronic Analytical Instruments, Leeds, England). A standard curve for the assay is shown in Figure 2.6.

$$\text{Glucose} + H_2O \xrightarrow{\text{Glucose oxidase}} \text{Gluconic Acid} + H_2O_2$$

$$H_2O_2 + \text{o-Dianisidine} \xrightarrow{\text{Peroxidase}} \text{Oxidised o-Dianisidine}$$

(Colourless) (Brown)

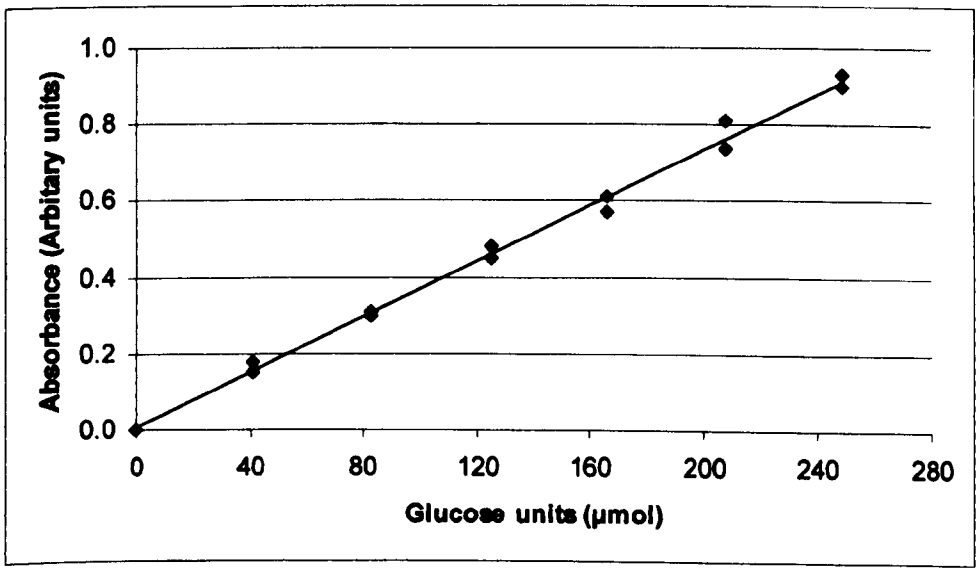


Figure 2.6: Glucose assay kit standard curve

2:10:2 Lactate assay

Myocardial lactate was determined in neutralised PCA acid extraction according to the method of Gutmann and Wahlefeld [Gutmann and Wahlefeld 1978].

$$\text{L-(+)-Lactate} + NAD^+ \xrightarrow{LDH} \text{Pyruvate} + NADH + H^+$$

Following PCA extraction, 80 µl was added to 1 ml of lactate assay buffer (containing 0.4 M hydrazine; 0.5 M glycine; pH 9.0) and 80 µl of 40 mM NAD^+. A further 8 µl of 5 mg/ml lactate dehydrogenase (LDH) suspension was added and samples were incubated at 37 °C for 30 minutes. Absorbance was read at 340 nm. A standard curve for the lactate assay is shown in Figure 2.7.

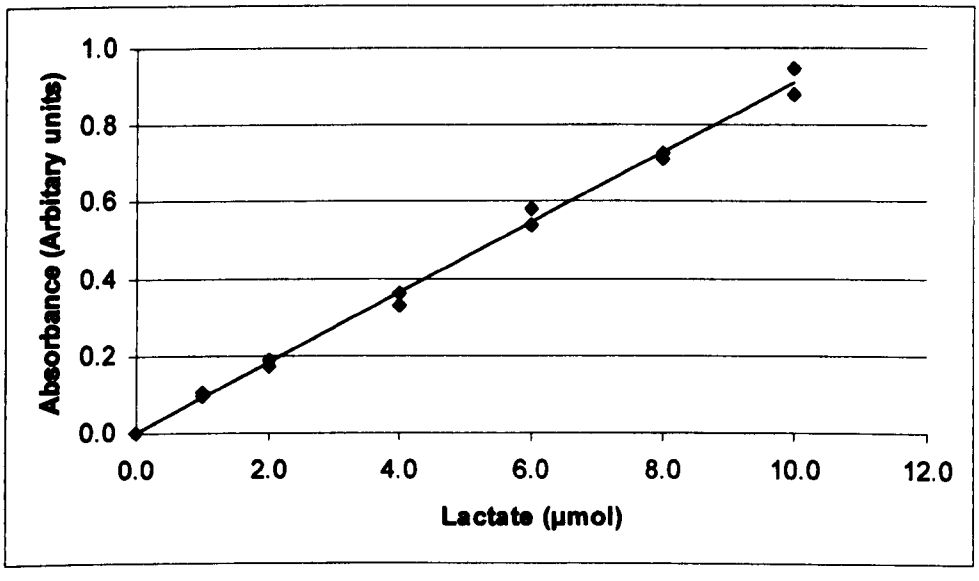


Figure 2.7: Lactate standard curve

2:10:3 Glycogen phosphorylase assay

Phosphorylase activity was determined in the reverse direction by detecting the release of P_i from glucose 1-phosphate.

$$\text{Glucose 1-phosphate} + \text{Glycogen}_{(n-1)} \xrightarrow{\text{Phosphorylase}} \text{Glycogen}_{(n)} + P_i$$

To determine total phosphorylase activity (the *a* plus *b* forms), 0.05 ml of supernatant extract (Section 2:9:5) was added to 0.5 ml of a reaction buffer (containing 50 mM glucose 1-phosphate, 0.15 M NaF, 1 mM EDTA, 1.4 mM AMP, of pH 6.5; and 0.2 ml of 1 % (w/v) glycogen). For the determination of phosphorylase *a*, 0.1 ml of extract was used and the reaction buffer contained 0.85 mM caffeine in place of AMP [Kalil-Filho *et al.* 1991]. Reactions were incubated for 5 minutes at 30 °C, before termination through the addition of 50 µl of 20 % (w/v) sodium dodecyl sulphate (SDS). Blanks were analysed by adding SDS at zero time.

Liberated P_i was measured by the molybdate assay [Saheki *et al.* 1985]. This colourimetric assay determines the amount of phosphate present by a reaction between P_i, ammonium molybdate tetrahydrate and ascorbic acid. The ascorbic acid reduces ammonium molybdate facilitating it to interact with P_i and the resulting complex is a chromophore with maximal absorption at $\lambda = 850$ nm.

Two millilitres of molybdate reagent (15 mM ammonium molybdate, 0.1 M zinc acetate, pH 6) and 0.5 ml of 10 % (w/v) ascorbic acid solution (pH 5) was added to 200 µl of sample. This mixture was incubated at room temperature for 20 minutes. Absorbance was measured at $\lambda = 850$ nm and a standard curve for this assay is given in Figure 2.8.

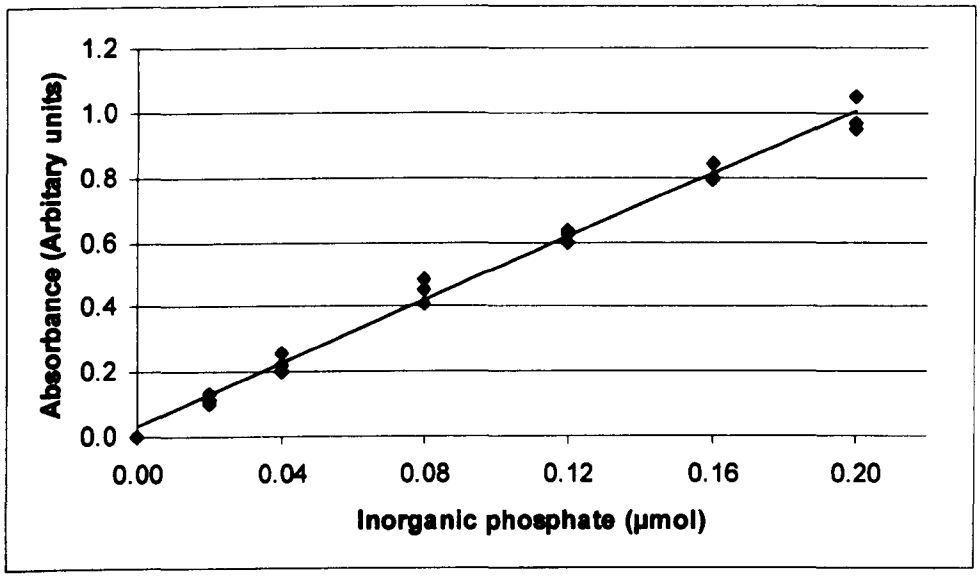


Figure 2.8: Inorganic phosphate standard curve

2:11 NMR experiments

2:11:1 Preparation of NMR buffer

Fifty millilitres of 50 mM KH_2PO_4 phosphate buffer was prepared, adjusted to pH 7.0, and lyophilised using a freeze drier (Freeze Drier Modulyo). Deuterated phosphate buffer was prepared by re-constituting the lyophilised KH_2PO_4 buffer in 50 ml deuterated water (D_2O).

2:11:2 Preparation of NMR samples

Samples for NMR analysis were reconstituted in 1 ml of deuterated phosphate buffer. A small spatula of chelating resin (Sigma) was added to remove any paramagnetic ions. Samples were filtered through a 0.22 µm syringe filter (Millipore) into a 5 mm NMR tube for ^{13}C-NMR analyses.

2:12 ^{13}C-NMR analysis

Myocardial oxidative metabolism was studied using ^{13}C-NMR in hearts supplied with ^{13}C-labelled substrates by following the incorporation of ^{13}C label into glutamate.

2:12:1 ^{13}C-NMR spectra acquisition

Proton decoupled ^{13}C spectra on cardiac extracts were acquired in a 9.4 Tesla vertical bore superconducting magnet with a JEOL JMN-LA400 FT NMR system (JEOL, Welwyn Garden City, England) at 101 MHz using a WALTZ decoupling sequence. Free induction decays (FIDs) were accumulated over 12000 scans using a pulse width of 8 μsec, a 3 second inter-pulse delay, and a sweep width of 200 ppm. All ^{13}C-NMR chemical shifts were expressed relative to the standard tetramethylsilane (TMS) assigned a chemical shift of 0 ppm. Sample temperature was controlled at 25 °C during NMR analysis by a thermocouple built into the NMR probe. FIDs were processed with a line broadening factor of 0.5 Hz.

2:12:2 ^{13}C-Glutamate and isotopomer patterns from [1-^{13}C]-glucose

The ^{13}C label from [1-^{13}C]-glucose enters the TCA cycle via C3 (number denotes enriched carbon) of pyruvate and the methyl carbon (C2) of acetyl CoA (Figure 2.9). The label is transferred to the C4 of citrate following condensation of acetyl CoA with oxaloacetate as it enters the TCA cycle. During the first turn of the cycle the label appears at C4 in α–ketoglutarate and subsequently in glutamate. As C4 labelled α–ketoglutarate is further metabolised in the TCA cycle, the label is randomly scrambled into the C2 or C3 positions of succinate and/or fumarate. The first complete turn of the TCA cycle thus generates oxaloacetate labelled at either C2 or C3. As the next turn of the TCA cycle begins, ^{13}C labelled oxaloacetate is condensed with another acetyl CoA that may

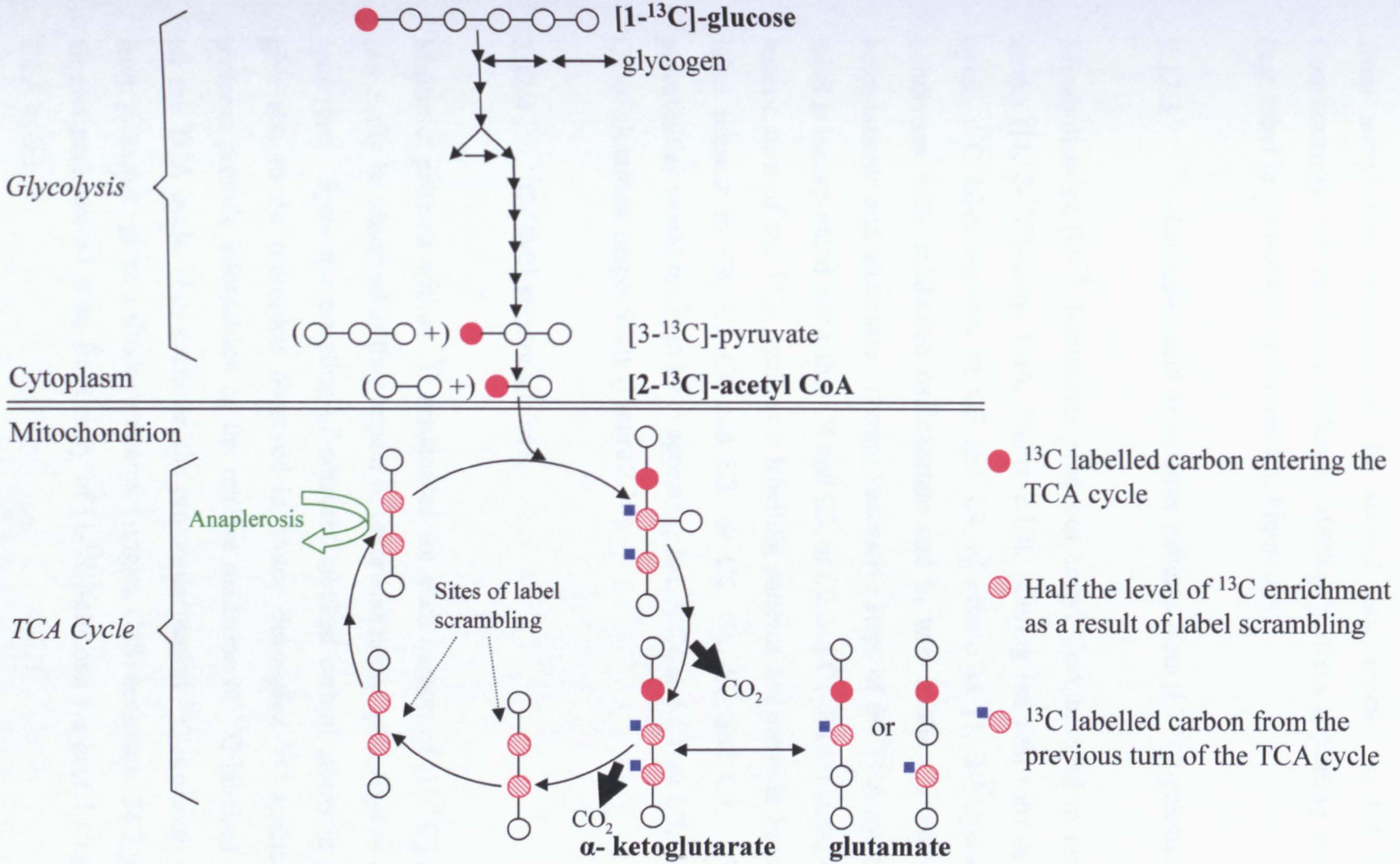


Figure 2.9: Flow diagram illustrating the position of label (^{13}C) in acetyl CoA from [1-^{13}C]-glucose degradation and the resulting ^{13}C-isotope distribution during the first one and a half turns of the TCA cycle.

or may not be labelled. Label is now present in the C3 or C2 position of citrate from label present during the first turn of the TCA cycle and any additional label from acetyl CoA entering on the second turn appears in C4 of citrate. Consequently, this results in different labelling patterns appearing in glutamate (e.g. label in C4 and C2, or C4 and C3, Figure 2.9).

2:12:3 ^{13}C-Glutamate and isotopomer patterns from [U-^{13}C]-palmitate

Metabolism of [U-^{13}C]-palmitate produces acetyl CoA labelled in both carbon atoms ([1, 2-^{13}C]-acetyl CoA, Figure 2.10). During the first turn of the TCA cycle, ^{13}C label appears in C5 and C4 of citrate as [1, 2-^{13}C]-acetyl CoA condenses with unlabelled oxaloacetate and in turn in the C5 and C4 of α-ketoglutarate and glutamate. During successive steps of the TCA cycle, this ^{13}C label is incorporated into either C4 and C3, or C2 and C1 of oxaloacetate. On the second turn of the TCA cycle two labelling patterns are possible in citrate, ^{13}C label present in C6, C5 C4 and C3, or C5, C4, C2 and C1. These two possibilities would result in label appearing in C5, C4 and C3, or C5, C4, C2 and C1 of glutamate respectively (Figure 2.10).

2:12:4 ^{13}C-NMR spectra analysis

Multiplet patterns within ^{13}C resonances for each carbon of [U-^{13}C]-glutamate can easily be observed as they appear in otherwise unoccupied regions of the ^{13}C spectrum. Spin-spin coupling of adjacent labelled carbon atoms in glutamate give rise to the multiplets observed in proton decoupled ^{13}C spectra. These patterns provide information on the relative oxidation of ^{13}C labelled substrates by the TCA cycle. For example, the proton-decoupled ^{13}C spectrum of [4-^{13}C] from glutamate gives a single resonance (singlet, C4S) centred at 34.2 ppm. This singlet peak would arise from entry of [1-^{13}C]-glucose via acetyl CoA into the TCA cycle.

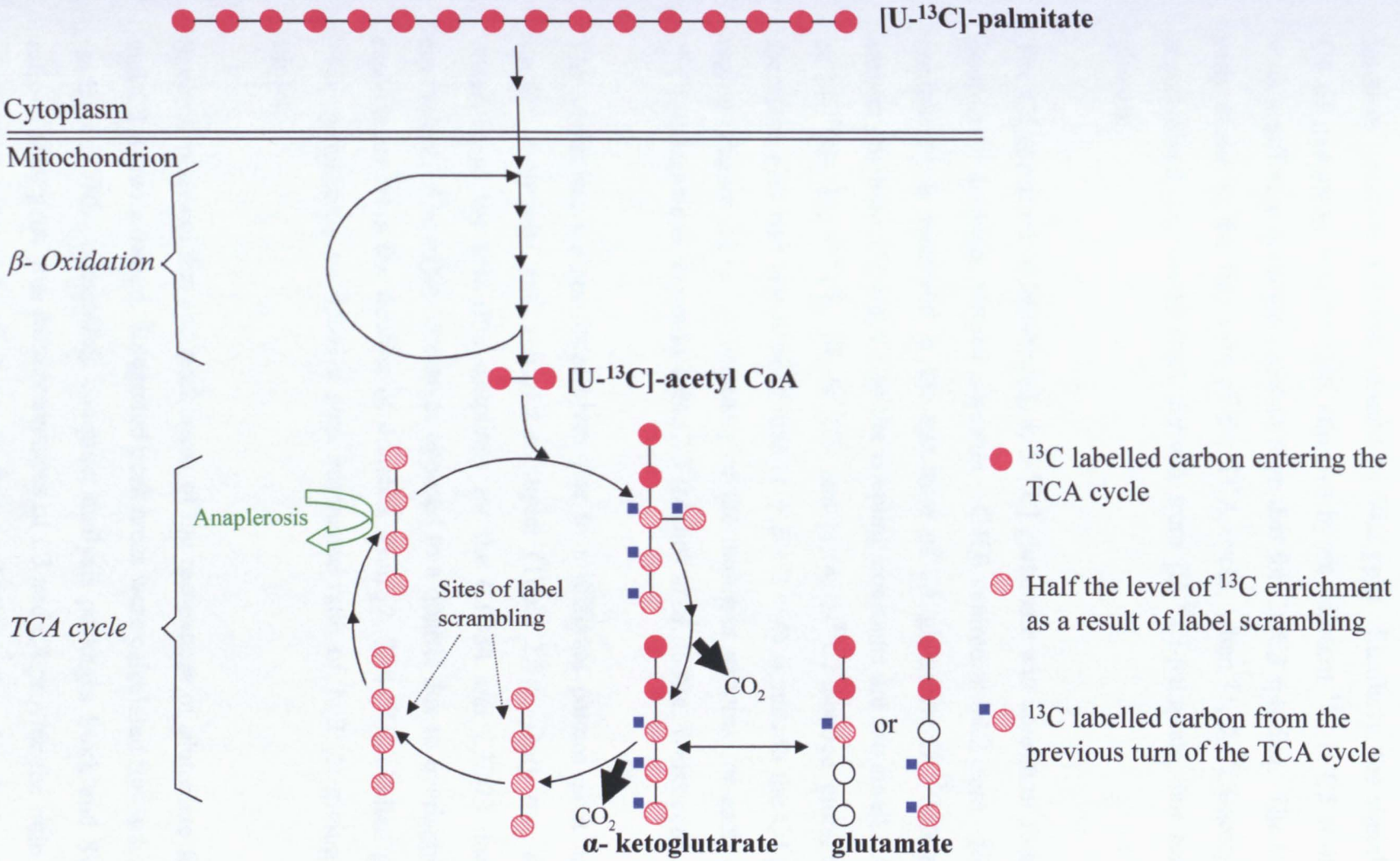


Figure 2.10: Flow diagram illustrating the position of label (^{13}C) in acetyl CoA from [U-^{13}C]-palmitate degradation and the resulting ^{13}C-isotope distribution during the first one and a half turns of the TCA cycle

If the adjacent C3 carbon is also labelled with ^{13}C, the signal from C4 will be split by spin-spin coupling with the C3 carbon, producing two signals of equal intensity (doublet, C4D34) centred on 34.2 ppm. Similarly, the signal from the C4 of glutamate may be split into two by an adjacent ^{13}C at C5 (C4D45), but with a different coupling constant from that for C4-C3 splitting. The C4D45 can only occur in the first turn of the TCA cycle, when [1, 2-^{13}C]-acetyl CoA is metabolised, i.e. acetyl CoA derived from [U-^{13}C]-palmitate, but not [1-^{13}C]-glucose.

The C4 spectrum of labelled [3, 4, 5-^{13}C] glutamate will consist of four peaks (a doublet of doublets, termed a quartet – C4Q) centred at 34.2 ppm. No spectral complexity is observed in the spectrum of C4 glutamate if ^{13}C is present at carbon positions C2 and C1 as the coupling constants are too small. A mixture of [4-^{13}C]-, [3, 4-^{13}C]-, [4, 5-^{13}C]- and [3, 4, 5-^{13}C]-labelled glutamate could therefore give rise to a total of nine (1 + 2 + 2 + 4) signals in the C4 glutamate region (Figure 2.11). A summary of the multiplet patterns for each carbon of [^{13}C]-glutamate is shown in Table 2.3 [Malloy *et al.* 1990a, Weiss *et al.* 1992].

The glutamate C3 spectrum gives rise to a different pattern as it only has a singlet, a doublet and a triplet multiplet (Table 2.3). Only one doublet is observed as the spin-spin couplings for the C3D34 and C3D23 doublets are equivalent. The triplet occurs as opposed to a quartet due to overlapping of the resonances from the doublet of doublets from [2, 3, 4-^{13}C]-labelled glutamate. This overlapping produces a peak resonance ratio of 1: 2: 1, giving rise to a triplet.

Spectral peak heights and peak areas of the resonances of glutamate for C4, C3 and C2 were measured. Integrated peak areas were calculated for each resonance using the UNIX compatible computer analysis packages Stork and Swan. The ratio of total peak area from resonances of C3 and C4 provide the ratio of carbon atom fractional enrichment between the two. Individual peak heights of each C4,

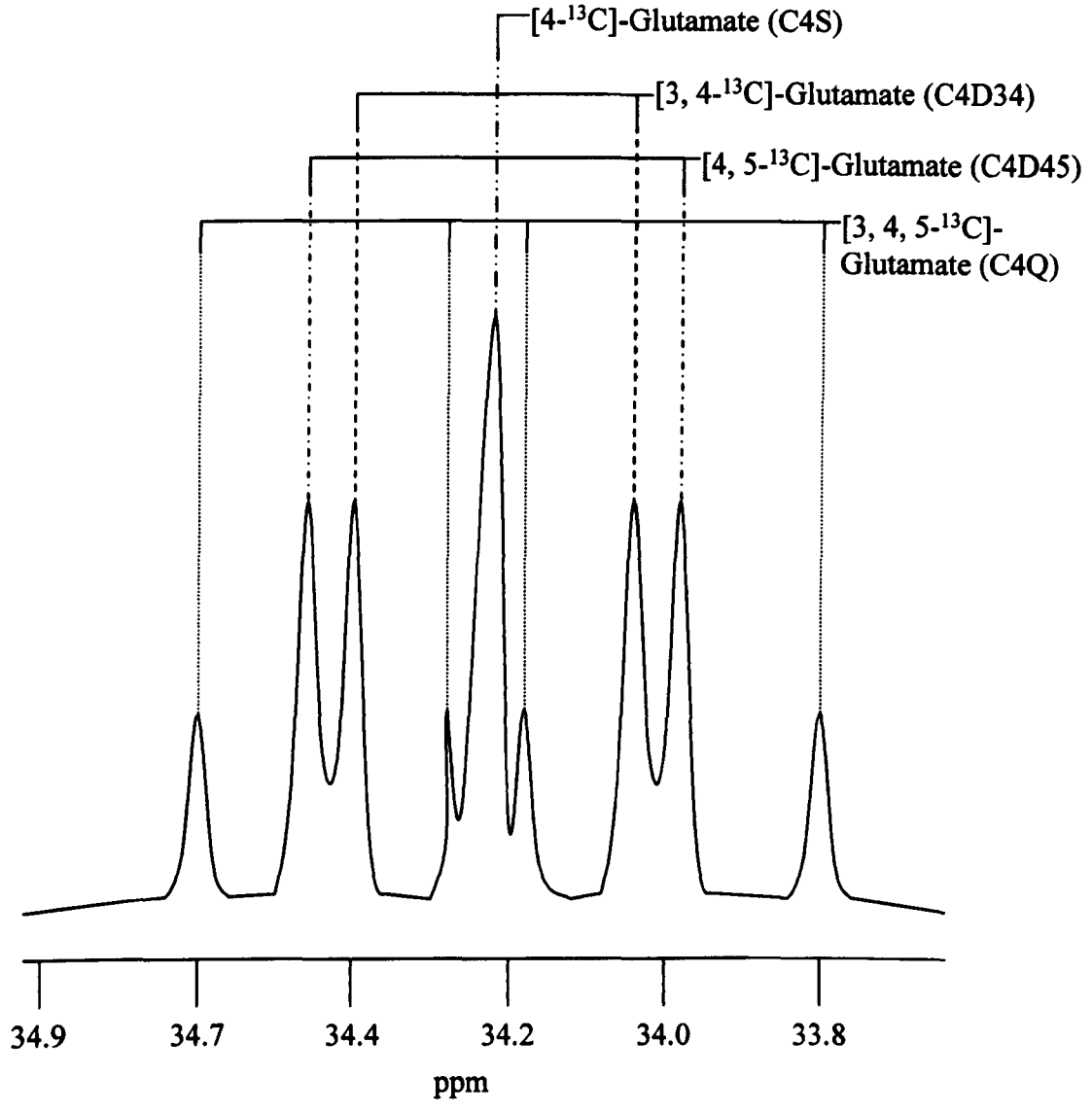


Figure 2.11: Simulated spectrum of the possible multiplet patterns for C4 from metabolism of [U-^{13}C]-palmitate using the TCA_{sim} program [Malloy *et al.* 1990].

Glutamate Carbon Atom Number	Number Of Possible Signal Peaks	Multiplets
5	3	C5S (Singlet) C5D45 (Doublet)
4	9	C4S (Singlet) C4D34 (Doublet) C4D45 (Doublet) C4Q (Quartet)
3	5	C3S (Singlet) C3D (Doublet) C3T (Triplet)
2	9	C2S (Singlet) C2D12 (Doublet) C2D23 (Doublet) C2Q (Quartet)
1	3	C1S (Singlet) C1D12 (Doublet)

Table 2.3: The possible multiplet patterns for each carbon of glutamate

C3 and C2 multiplet were measured and expressed as a percentage of the sum of all peak heights for the corresponding carbon atom. These results gave the fractional intensity values of each multiplet. The maximum fractional intensity of any peak within a single carbon atom from glutamate was 1.

2:12:5 Steady state ^{13}C-NMR analysis using TCA_{calc}

The mathematical modelling program TCA_{calc} [Malloy *et al.* 1990a] was used to assess relative substrate contribution to myocardial energy production in the TCA cycle under steady state conditions. TCA_{calc} is based upon algebraic equations that describe a ^{13}C-NMR spectrum in terms of experimental (substrate enrichment and labelling patterns) and metabolic (pathway fluxes) parameters.

The following data from ^{13}C-NMR spectra of glutamate were entered into the TCA_{calc} program for isotopomer analysis;

- C3: C4 total peak area ratio

- Fractional enrichment values for each multiplet of C4, C3 and C2 resonance
- Fractional enrichment of acetyl CoA from different ^{13}C labelled substrates
- Ratio of flux through anaplerosis (y) relative to that through citrate synthase is negligible.

The following set of parameters generated by the TCA_{calc} program define the relative fractional enrichments of the acetyl CoA pool supplying the TCA cycle [Malloy *et al.* 1990a];

- Fc0 - Fraction unlabelled at both carbon atoms
- Fc1 - Fraction labelled at carbonyl carbon atom (C1)
- Fc2 - Fraction labelled at methyl carbon atom (C2)
- Fc3 - Fraction labelled at carbon atoms C1 and C2

  where Fc0 + Fc1 + Fc2 + Fc3 is defined as equalling to 1

Myocardial metabolism of a molecule of [1-^{13}C]-glucose generates two acetyl CoA molecules (one labelled and one unlabelled) that can enter the TCA cycle (Figure 2.9). Therefore, the fractional enrichment value obtained by TCA_{calc} for Fc2 was multiplied by two to correct for the actual relative amount of glucose supplying the TCA cycle.

2:12:6 Assumptions of TCA_{calc} model

The TCA_{calc} model is based upon the following assumptions regarding carbon entry into and out of the TCA cycle [Malloy *et al.* 1990a];

- All carbon flows into the TCA cycle via acetyl-CoA or anaplerosis
- All metabolic reactions are at steady state [Katz 1985]
- Concentrations and fractional enrichment of acetyl CoA, anaplerotic substrates, TCA cycle intermediates and the exchanging pools are constant
- α-Ketoglutarate and glutamate are in rapid exchange between each other

relative to total TCA cycle flux [Randle *et al.* 1970]

- Randomisation occurs for all ^{13}C entering oxaloacetate between C1 and C4 and between C2 and C3
- Values for pathway fluxes are relative to TCA cycle turnover, which is considered to be 1 [Malloy *et al.* 1990a]
- Entry of $^{13}CO_2$ into the TCA cycle is inconsequential
- Natural ^{13}C abundance does not influence the NMR signal

2:12:7 Non-steady state ^{13}C-NMR analysis

Relative substrate contributions to the TCA cycle under non-steady state conditions (reperfusion experimental ^{13}C-NMR spectra) were calculated using the ^{13}C isotopomer analysis method of Malloy [Malloy *et al.* 1990b]. This non-steady state analysis is based upon the possibility that a ^{13}C labelled C2 oxaloacetate molecule condensing with a given acetyl CoA isotopomer corresponds to the relative concentration of that isotopomer in the acetyl CoA pool.

After half a turn of the TCA cycle the carbonyl carbon (C2) of oxaloacetate becomes C3 of glutamate. C1 of oxaloacetate is lost as CO_2 at the isocitrate dehydrogenase step of the TCA cycle. C1 and C2 of glutamate, which are not used in this analysis, originate from C3 and C4 of oxaloacetate. Also, as relative enrichments in acetyl CoA are being investigated, the absolute enrichment in C2 of oxaloacetate need not be known.

As a result of the ^{13}C labelled substrates used during reperfusion ([U-^{13}C]-palmitate and [1-^{13}C]-glucose), three different acetyl CoA isotopomers may occur; [U-^{13}C]-acetyl CoA, [2-^{13}C]-acetyl CoA and unlabelled acetyl CoA. The relative concentrations of these are defined as Fc3, Fc2 and Fc0 respectively (as already mentioned above, Section 2:12:5). Only 24 out of a maximum of 32 glutamate isotopomers are possible from the metabolism of these three acetyl

CoA isotopomers as [1-^{13}C]-acetyl CoA cannot be generated. Therefore, by definition Fc0 + Fc2 + Fc3 = 1. The possible glutamate isotopomers resulting from the condensation of the three acetyl CoA ^{13}C isotopomers with ^{13}C labelled C2 oxaloacetate are shown in Figure 2.12.

By combining the ^{13}C glutamate multiplet and fractional enrichment ratio relationships with the probabilities that a given acetyl CoA isotopomer enters the TCA cycle, the following two simple equations have been demonstrated [Malloy *et al.* 1990b];

$$C4D34.(C4/C3) = Fc2 \qquad (1)$$

$$C4Q.(C4/C3) = Fc3 \qquad (2)$$

Therefore, non-steady state analysis was performed on ^{13}C spectra obtained from reperfusion studies by measuring the relative areas of the C4D34 and C4Q multiplets and the enrichment ratio between C4 and C3 of glutamate. These values were entered into equations 1 and 2 (shown above) to give Fc2 and Fc3 respectively. Fc0 was subsequently worked out by difference, as Fc0 + Fc2 + Fc3 = 1 under the perfusion conditions used.

2:12:8 ^{13}C-NMR correction factors

Relative intensities of individual carbon resonances in glutamate may not directly reflect the relative enrichment of ^{13}C at each site due to nuclear overhauser effects (NOEs) and relaxation effects [London 1988]. These fractions can be allowed for if appropriate corrections are applied.

To determine correction factors, a ^{13}C-NMR spectrum of 500 mM sodium glutamate was acquired using identical experimental conditions to that used for extract samples. Due to the natural abundance of ^{13}C, a peak area ratio of 1: 1: 1: 1: 1 for C1: C2: C3: C4: C5 of glutamate should be observed if there are no

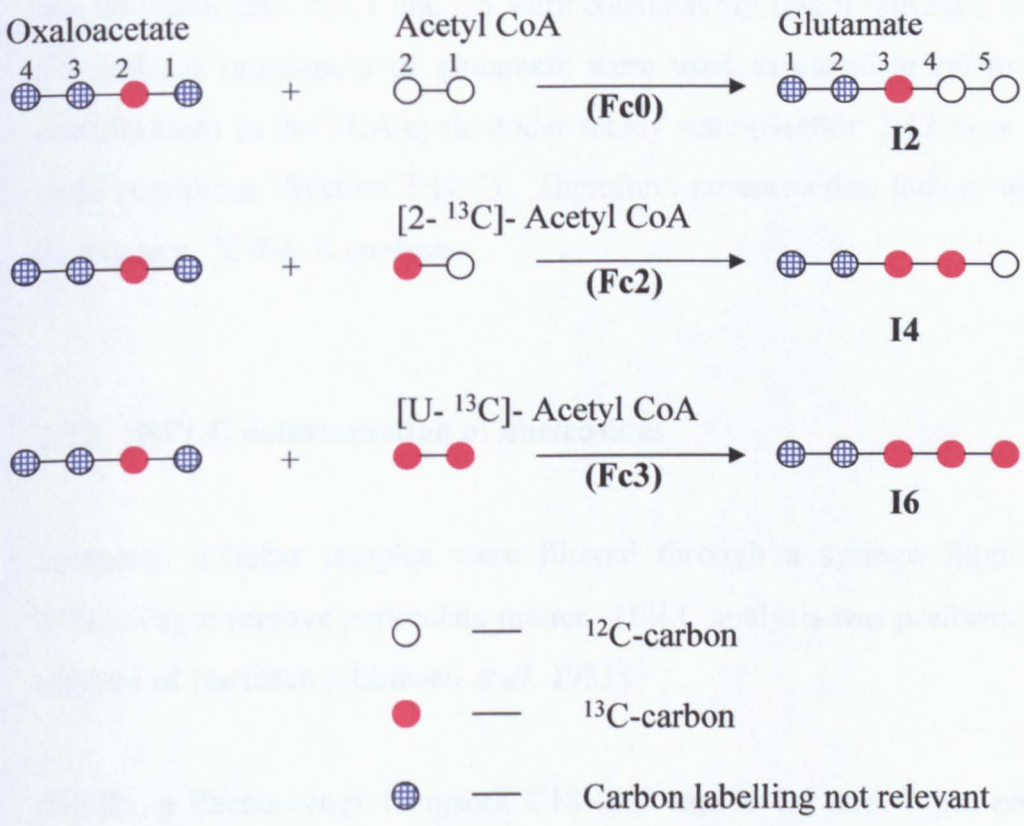


Figure 2.12: Possible glutamate isotopomers from condensation of the three acetyl CoA ^{13}C isotopomers with oxaloacetate ^{13}C labelled at C2

differences in the relative intensities of each carbon resonance due to experimental protocols.

The glutamate control relative intensities for C2: C3: C4 were all found to be 1 but the intensities of C1 and C5 were considerably less (Figure 2.13). Only C2, C3 and C4 resonances of glutamate were used to calculate relative substrate contributions to the TCA cycle under steady state (Section 2:12:5) or non-steady state conditions (Section 2:12:7). Therefore, no correction factors were applied during any ^{13}C-NMR analysis.

2:13 HPLC determination of nucleosides

Extracted effluent samples were filtered through a syringe filter (0.22 μm, Millipore) to remove particulate matter. HPLC analysis was performed using the method of Harmsen [Harmsen *et al.* 1981].

Briefly, a Phenomenex Kingsorb C18 (250 mm x 4.6 mm, 5 μm particle size) column (Phenomenex, Macclesfield, England) was used for separation with a flow rate of 1ml/min. Running buffer was 10 mM $NH_4H_2PO_4/CH_3OH$ (10:1 v/v, pH 5.5), filtered under a water vacuum using a Nylon 66 0.45 μm filter (Supelco, Poole, England) prior to use. A continuous helium purge system in the buffer reservoir was operational throughout.

Analysis was performed using a Hewlett Packard Series 1050 HPLC machine (Hewlett Packard, Bracknell, England). Metabolites were detected at 254 nm using an ultra violet, diode array detection system (Hewlett Packard 1040 Series II) using a sample volume of 100 μl. Standards containing 100 μM adenosine, inosine, uric acid, hypoxanthine and xanthine were run to identify peaks (Figure 2.14). Standards were run at the start of HPLC analysis, and after every five samples.

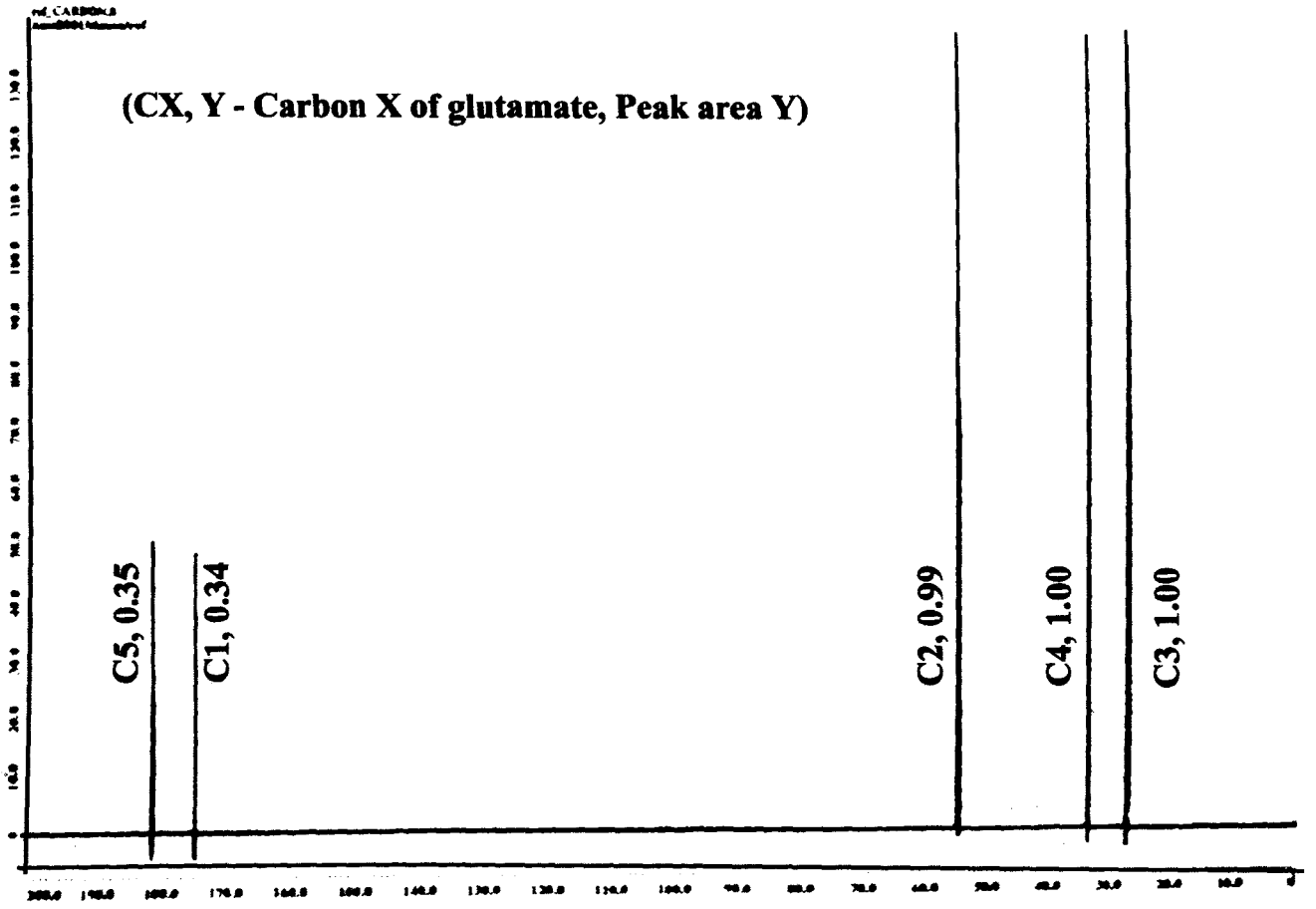
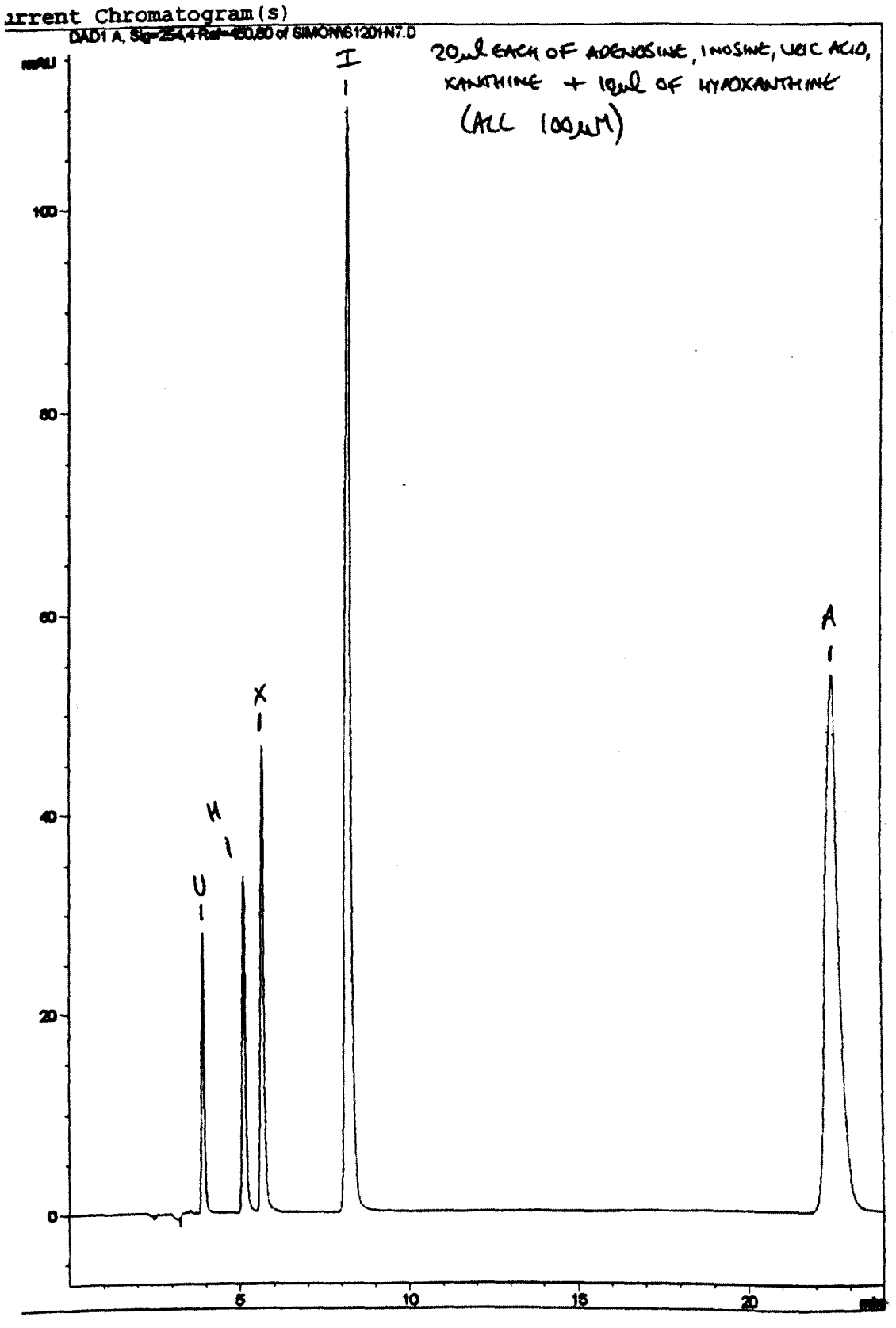


Figure 2.13: ^{13}C-NMR spectrum of unlabelled glutamate (500 mM)



U – uric acid, A – adenosine, I – inosine, X –xanthine and H -hypoxanthine

Figure 2.14: HPLC chromatogram of nucleoside standards

2:14 Expression of results

All results are expressed as mean ± the standard error of the mean. Significance was determined using an unpaired Student's t-test or by analysis of variance with post hoc comparison by Newman-Keuls test where appropriate. A probability of less than 5 % was considered to be of statistical significance.

3: GLYCOGEN LOADING IN THE HYPERTROPHIED HEART

3:1 Introduction

To meet its substantial energy requirements the heart is capable of metabolising a variety of substrates. Cardiac function is very tightly linked to substrate uptake and utilisation. Impairment in the myocardium of the provision of energy for mechanical work results in functional and metabolic irregularities for every other organ in the body, which is commonly referred to as heart failure.

3:1:1 Energy metabolism in the normal heart

Energy (ATP) generation in the normal adult heart relies on the oxidation of long chain fatty acids by the mitochondria. Fatty acid oxidation provides 60-70 % of the overall energy requirements of the normal heart [Neely and Morgan 1974, Saddik and Lopaschuk 1991, Schönekess *et al.* 1997]. The remaining portion of myocardial energy provision comes primarily from carbohydrate sources. Under normal conditions, glucose is thought to be the carbohydrate of choice due its continual supply to the heart, as a result of the tight regulation of blood glucose concentration *in vivo* [Newsholme 1976]. However, there is a growing body of evidence that lactate oxidation may represent a significant source of energy for the heart [Drake *et al.* 1980, Gertz *et al.* 1988, Bartelds *et al.* 1999].

3:1:2 Energy metabolism in the hypertrophied heart

The hypertrophied heart switches its energy substrate selection from predominantly fatty acid oxidation to an increased reliance upon glucose metabolism [Taegtmeyer and Overturf 1988, Seymour *et al.* 1990, Allard *et al.* 1994, Christe and Rodgers 1994].

3:1:2:1 Glycolysis and glucose oxidation in hypertrophied hearts

Increased rates of glucose uptake have been demonstrated in pressure overloaded hypertrophied hearts [Zhang *et al* 1995, Bhutta *et al.* 1996]. Intracellular

accumulation of the glucose analogue 2-deoxyglucose 6-phosphate was observed to positively correlate with the degree of hypertrophy observed in the pressure overloaded heart [Zhang *et al* 1995]. Myocardial glucose uptake has also been observed to increase in the hypertensive rat [Leipala *et al.* 1989]

Increases in glycolytic rates of hypertrophied rat hearts resulting from pressure overload [Allard *et al.* 1994] and hyperthyroidism [Seymour *et al.* 1990] have been reported. The levels of glycolytic enzymes have also been observed to increase in hypertrophied hearts [Bishop and Altschuld 1970, Seymour *et al.* 1990]. These results indicate that there is a change in the balance of acetyl CoA supplied from fatty acid breakdown to that supplied from glucose metabolism in myocardial hypertrophy. In support of this, a moderate increase in glucose oxidation has been observed in SHR hearts [Christe and Rodgers 1994] and hypertrophied rabbit hearts [Taegtmeyer and Overturf 1988].

3:1:2:2 Fatty acid oxidation in hypertrophied hearts

Decreased long chain fatty acid oxidation has been demonstrated in both pressure and volume overloaded hypertrophied rat hearts [El Alaoui-Talibi *et al.* 1992, Christe and Rodgers 1994]. In the clinical setting, patients with hypertrophic cardiomyopathy (HCM) showing no signs of myocardial dysfunction were also observed to have decreased levels of fatty acid oxidation [Nakata *et al.* 1996].

3:1:3 Alterations in energy metabolism in hypertrophied and failing hearts

Alterations in energy metabolism may be a critical factor in the progression from compensated hypertrophy to heart failure [Seymour 1994]. The sequence of events leading to the development of decompensated hypertrophy and ultimately heart failure are not fully understood. One hypothesis for this transition to failure proposes that the hypertrophied failing heart is in an energy-depleted state [Ingwall *et al.* 1990, Seymour *et al.* 1990, Neubauer *et al.* 1992] due to imbalances between energy production and consumption [Katz 1990, Ingwall

1993].

3:1:3:1 Inadequate substrate provision in the hypertrophied heart

Alterations in the profile of myocardial energy substrate preference in the hypertrophied heart could play a crucial role in the development of pathophysiological hypertrophy and heart failure, by resulting in an inadequate provision of substrate.

Provision of acetyl CoA from increased glucose metabolism is also known to inhibit fatty acid oxidation [Saddik *et al.* 1993]. Inhibition of myocardial fatty acid oxidation results in the intracellular accumulation of fatty acyl molecules [Russell and Taegtmeyer 1991, Brady *et al.* 1993].

Intracellular accumulation of fatty acyl moieties will sequester any available CoA, which in turn may affect the rate of glucose oxidation and contractile function [Russell and Taegtmeyer 1992, Timmons *et al.* 1996]. A reduced availability of CoA will result in an increase of the acetyl CoA: CoA ratio, an important regulator of PDC [Garland and Randle 1964]. Pyruvate dehydrogenase kinase, which inhibits PDC, is stimulated by an increase in acetyl CoA: CoA ratio [Reinauer and Muller-Rucholtz 1976, Kerbey *et al.* 1976]. Reductions in the active form of PDC have been observed in hypertrophied rat hearts induced by a pressure overload [Seymour and Chatham 1997] and in the hamster model of heart failure [Di Lisa *et al.* 1993]. Inhibition of PDC could have detrimental effects on glucose oxidation in the hypertrophied heart.

Therefore, in spite of an increased dependence on glucose metabolism to supply energy to the hypertrophied heart, there may be a limited capacity to use glucose. Together with the reduction in fatty acid oxidation, the hypertrophied heart may become energy depleted resulting in adverse effects on function [Seymour 1994].

3:1:4 Susceptibility of the hypertrophied heart to ischaemia

A further factor that may compound the decreased energy reserve in the hypertrophied heart further is an inadequate perfusion of the myocardium resulting in insufficient oxygen substrate delivery (ischaemia). Cardiac hypertrophy has been shown to have an enhanced susceptibility to ischaemic injury in studies of both animal models and humans [Schaper *et al.* 1978, Bache *et al.* 1981, Anderson *et al.* 1990, Gaasch *et al.* 1990].

Inadequate supply of oxygen and exogenous substrates may occur in hypertrophied hearts due to alterations in the myocardial vasculature [Bache *et al.* 1981, Katz 1992a, Rakusan 1992]. During hypertrophic growth in the adult heart there is insufficient angiogenesis due to a loss of angiogenic capacity with age [Rakusan *et al.* 1992]. In addition, inter-capillary distance also increases in the hypertrophied heart [Katz 1992a], resulting in increased diffusion distances for substrates for the heart [Bache *et al.* 1981].

However, controversy still exists concerning alterations to coronary reserve in the hypertrophied heart. Reductions in overall diffusion distances were observed in a hypertensive animal model of hypertrophy [Anversa and Capasso 1991], as well as abnormalities of coronary reserve in non-hypertrophied and hypertrophied regions of the heart in patients with hypertrophic cardiomyopathy [Camici *et al.* 1991].

3:1:5 Energy provision during ischaemia

During ischaemia, the impaired supply of oxygen results in a shift towards anaerobic glycolysis derived from exogenous glucose or endogenous glycogen [Morgan *et al.* 1961]. The more severe the cessation in coronary flow (ischaemia) the greater the reliance there is in the heart on anaerobic metabolism of glucose and the endogenous store of glucose, glycogen, for energy production

[Stanley *et al.* 1992]. During total global ischaemia (complete cessation of coronary flow) oxidative metabolism decreases immediately as the heart contains virtually no stores of oxygen. Glucose uptake is negligible during global ischaemia [Depre *et al.* 1998b], so glycogen is the sole substrate in the heart capable of providing energy.

Recent work has shown that the chain length and degree of branching within the glycogen molecule are those that provide the maximum efficiency for glucose storage and release [Melendez-Hevia *et al.* 1993, Melendez *et al.* 1998]. The structure of glycogen is optimised to, i) ensure the amount of glucose stored is in the smallest possible volume, ii) maximise the proportion of glucose that can be directly released by phosphorylase before debranching is necessary and iii) provide the greatest number of points of attack (non-reducing ends) for phosphorylase mediated breakdown. Therefore, the structure of the glycogen molecule is one that allows the rapid release of large amounts of glucose in accordance with its metabolic role as a very effective, quick supplier of fuel.

However, controversy exists over the effect of glycogen levels on the ischaemic myocardium. Studies have proposed that there is a detrimental effect of glycogen in ischaemic hearts, due to the accumulation of protons and lactate, and have highlighted the beneficial consequences of depleting glycogen stores prior to an ischaemic episode [Neely and Grotyohann 1984]. Other studies have shown that glycogen availability is related to protection against ischaemic injury [Taegtmeyer *et al.*1985, Lagerstrom *et al.* 1988, Goodwin and Taegtmeyer 1994, Cross *et al.* 1996].

3:1:6 Objectives

In order to study the effects of increased glycogenolysis during ischaemia/reperfusion under physiological substrate conditions, a glycogen loading protocol was required. Three different methods reported in the literature

are generally employed to modify glycogen content in isolated perfusions. First, increasing perfusate levels of insulin to pharmacological levels has been shown to markedly elevate myocardial glycogen [Cross *et al.* 1996, Moule and Denton 1997, Fraser *et al.* 1998, Cave *et al.* 2000,]. Insulin stimulates glucose transport as well as increasing activation of glycogen synthase activity [Villar-Pallasi and Larner 1970, Cushman and Wardzala 1980]. Second, the presence of an additional carbohydrate source e.g. lactate, has been shown to increase glycogen synthesis [Bolukoglu *et al.* 1996]. Lactate is preferentially oxidised over glucose in the heart allowing the uptake of exogenous glucose to be re-directed to glycogen synthesis [Bolukoglu *et al.* 1996]. Third, several studies use a substrate free period to deplete glycogen stores [Henning *et al.* 1996, Goodwin *et al.* 1998]. Following the substrate free period glycogen synthesis is up-regulated allowing the glycogen pool to be labelled by exogenous glucose containing a nuclear probe (e.g. ^{13}C).

The aim of this chapter was to develop methods that could modulate the size of the glycogen pool and label the glycogen with ^{13}C to determine subsequently the extent of glycogen utilisation. This was undertaken by the following methods;

1. Maximally loading isolated perfused hearts with glycogen by increasing levels of insulin, and by providing lactate as an alternative carbohydrate source in the perfusate.
2. Depleting glycogen stores using a substrate free period, to allow ^{13}C labelling of the myocardial glycogen pool by [1-^{13}C]-glucose in order to study glycogen utilisation.

3:2 Methods

3:2:1 *In vivo* glycogen levels

Hearts from hypertrophied or sham operated animals were removed 9 weeks post surgery (Section 2:5), immediately freeze clamped and stored under liquid nitrogen. Myocardial glycogen was assayed (Sections 2:9 and 2:10) to determine *in vivo* tissue levels by an *ex vivo* method. Hearts from this group were termed no perfusion (NP).

3:2:2 Perfusion protocols

3:2:2:1 Insulin induced glycogen loading

Increasing concentrations of insulin were used to promote glycogen synthesis and deposition. Hearts were perfused as previously described (Section 2:7). Myocardial glycogen deposition was investigated under the following perfusion conditions (Figure 3.1):

- 5 mM glucose and increasing insulin concentrations (G1, G2 and G3)
- 5 mM glucose, 0.3 mM palmitate and increasing concentrations of insulin (G+P1, G+P2, G+P3 and G+P4, Figure 3.1)

The glucose (5 mM) and palmitate (0.3 mM) concentrations were chosen to represent the fed state *in vivo* [Connolly *et al.* 1993].

3:2:2:2 Depletion/repletion protocol for glycogen pool labelling

A 20 minute substrate free perfusion was initially used to deplete myocardial glycogen (Figure 3.2). One experimental group was freeze clamped at the end of the substrate free period (GD, Figure 3.2). Two groups were perfused for a further 45 minutes with 5 mM [1-^{13}C]-glucose, 0.3 mM palmitate and 5 mU/ml insulin concentrations to promote glycogen loading (GDR1 and GDR2, Figure 3.2).

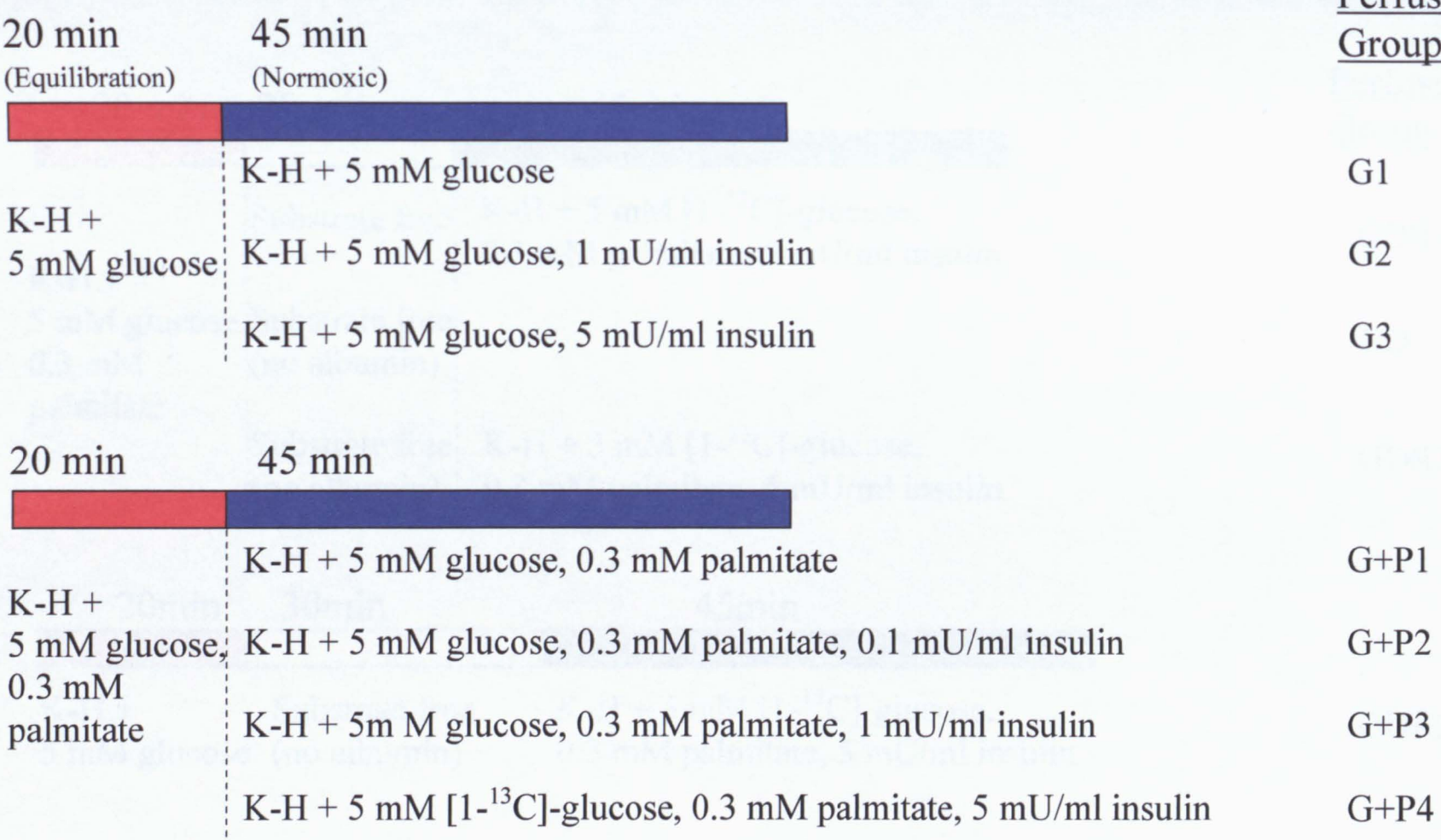


Figure 3.1: Insulin induced glycogen loading perfusion protocols

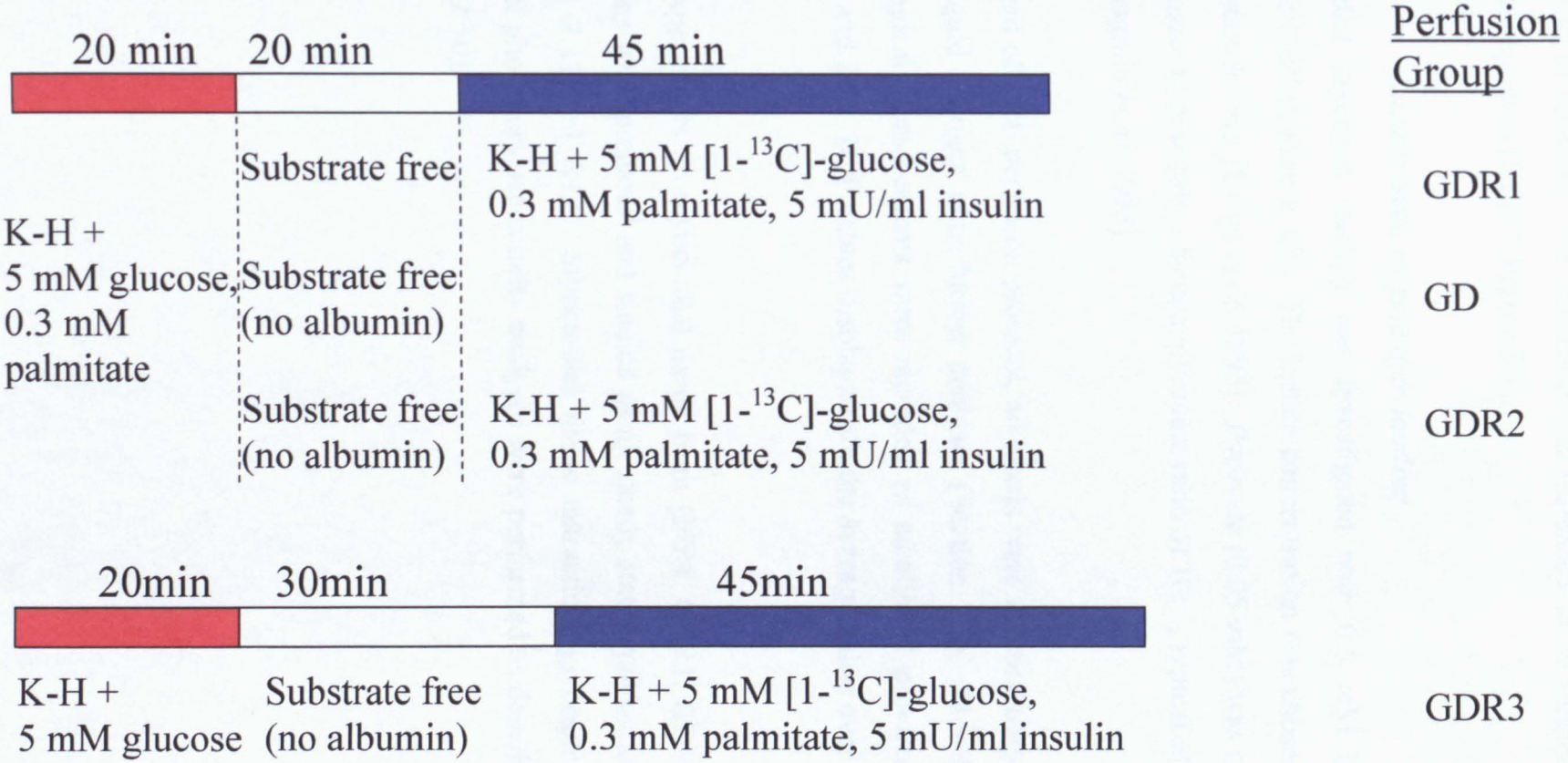


Figure 3.2: Glycogen depletion/repletion perfusion protocols

The period of substrate free perfusion was increased to 30 minutes to enhance glycogen depletion (GDR3, Figure 3.2).

3:2:2:3 Lactate induced glycogen loading

Myocardial glycogen loading was investigated with 0.5 mM lactate in the perfusate (GPLP, Figure 3.3). The lactate concentration was chosen to represent the fed state *in vivo* [Large *et al.* 1995]. Pyruvate (0.05 mM) was also present in the perfusate to maintain a lactate: pyruvate ratio of 10: 1, typical of that found *in vivo* [Laughlin *et al.* 1993].

At the end of each perfusion protocol, all hearts were freeze clamped and stored under liquid nitrogen until further analyses (Section 2:7). Morphological and physiological measurements were recorded as mentioned previously (Sections 2:6, 2:7 and 2:8). RPP values displayed are the average value over the perfusion period.

NMR experiments on myocardial tissue from G+P4, GDR1, GDR2 and GDR3 perfusions were prepared and studied using steady state analysis as described in Sections 2:11 and 2:12. Myocardial tissue extraction, glycogen content and glycogen phosphorylase activity analyses were performed as described (Sections 2:9 and 2:10).

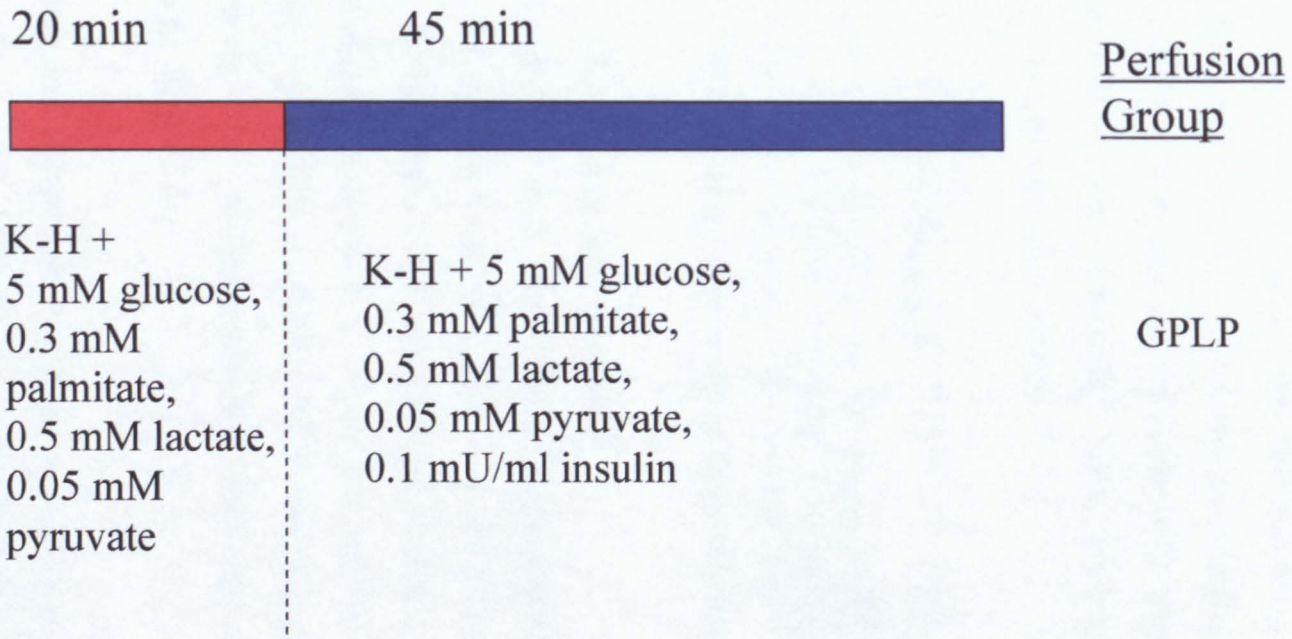


Figure 3.3: Lactate induced glycogen loading perfusion protocol

3:3 Results

3:3:1 Myocardial glycogen content

3:3:1:1 Hearts freeze clamped immediately after removal (NP)

Following nine weeks of aortic constriction, no significant change in myocardial glycogen content was observed between S and CH animals (Table 3.1) *in situ.* Indeed, no differences were observed in glycogen content between S and CH hearts in any of the experimental groups.

Model	**Number**	**Glycogen (μmol/g dry wt.)**
S	5	55 ± 4
CH	5	58 ± 4

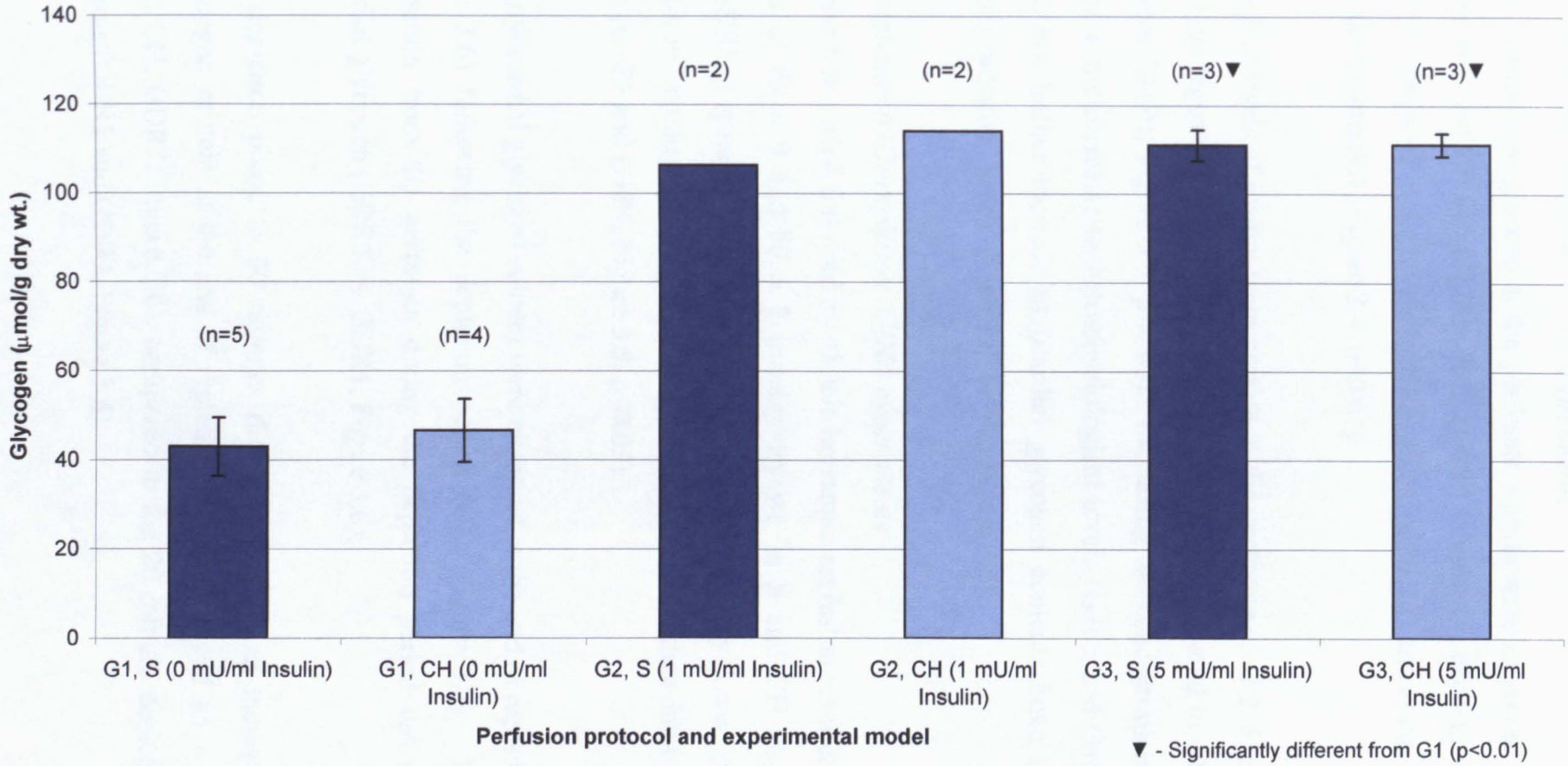
Table 3.1: Myocardial glycogen content from NP experiments

3:3:1:2 Glucose only (G) experiments

Following a period of normoxic perfusion without insulin, 43 ± 7 and 47 ± 7 μmol/g dry wt. glycogen was observed in S and CH hearts respectively (G1, Figure 3.4). In the presence of 1 and 5 mU/ml insulin, glycogen levels increased to 106 and 114 μmol/g dry wt. (G2, Figure 3.4) and 110 ± 4 and 111 ± 3 μmol/g dry wt. (G3, Figure 3.4) in S and CH hearts respectively. The increase in glycogen content with 5 mU/ml insulin was significant when compared to the G1 group (Figure 3.4, $p<0.01$).

A marked decrease in myocardial glycogen content was observed comparing the group with glucose only (G1, Figure 3.1) to that freeze clamped immediately (NP, Table 3.1 $p<0.05$). The presence of insulin (G3, Figure 3.1) in the perfusate dramatically increased myocardial glycogen content compared to that in NP hearts (Table 3.1, $p<0.01$).

Figure 3.4: Myocardial glycogen levels from glucose only (G) perfusions with varying insulin concentrations



3:3:1:3 Glucose with palmitate (G+P) experiments

The addition of palmitate to glucose in the perfusate significantly enhanced the glycogen content in S and CH hearts (79 ± 7; S, and 80 ± 5 μmol/g dry wt.; CH, G+P1, Figure 3.5) compared to the levels found *in vivo* (Table 3.1) and at the end of glucose only perfusions (G1, Figure 3.4. $p<0.01$).

When physiological levels of insulin were present in the perfusate (G+P2, Figure 3.5) myocardial glycogen content increased by another 10 % compared to when insulin was absent (G+P1, Figure 3.5, $p<0.05$). Increasing the concentration of levels of insulin in the perfusate to supraphysiological levels (G+P3 and G+P4, Figure 3.5) did not further increase myocardial glycogen content above that observed with physiological levels of insulin (G+P2, Figure 3.5).

3:3:1:4 Depletion (GD)/repletion (GDR) experiments

A 20min substrate free period followed by 45 min normoxic perfusion resulted in glycogen levels of 69 ± 9 and 80 ± 7 μmol/g dry wt. in S and CH hearts respectively (GDR1, Figure 3.6). These levels were considerably lower than those reported for normoxic perfusions of hearts with glucose, palmitate and high levels of insulin (G+P3 and G+P4, Figure 3.5, $p<0.05$).

No changes in myocardial glycogen content were observed at the end of repletion (GDR2, Figure 3.6) following the depletion period (GD, Figure 3.6). The removal of albumin from the perfusate during the depletion period did not modify myocardial glycogen (GDR2 vs. GDR1, Figure 3.6).

Extending the depletion phase to 30 minutes did not result in an increased myocardial glycogen content at the end of repletion (89 ± 6; S, and 85 ± 10 μmol/g dry wt.; CH, GDR3, Figure 3.6), compared to the 20 minute depletion repletion protocols (GDR1 and GDR2, Figure 3.6).

Figure 3.5: Myocardial glycogen content from glucose and palmitate (G+P) perfusions with varying insulin concentrations

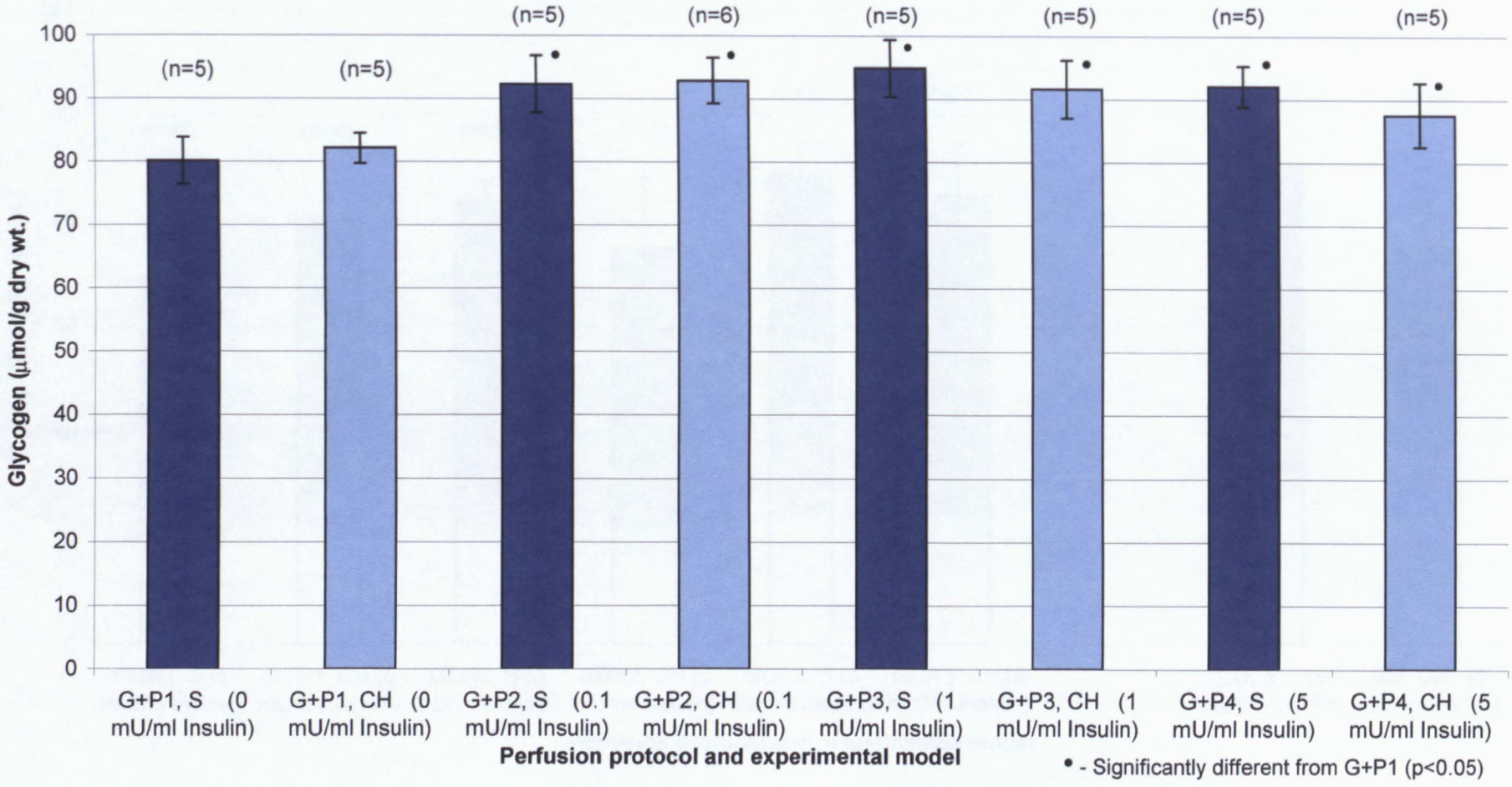
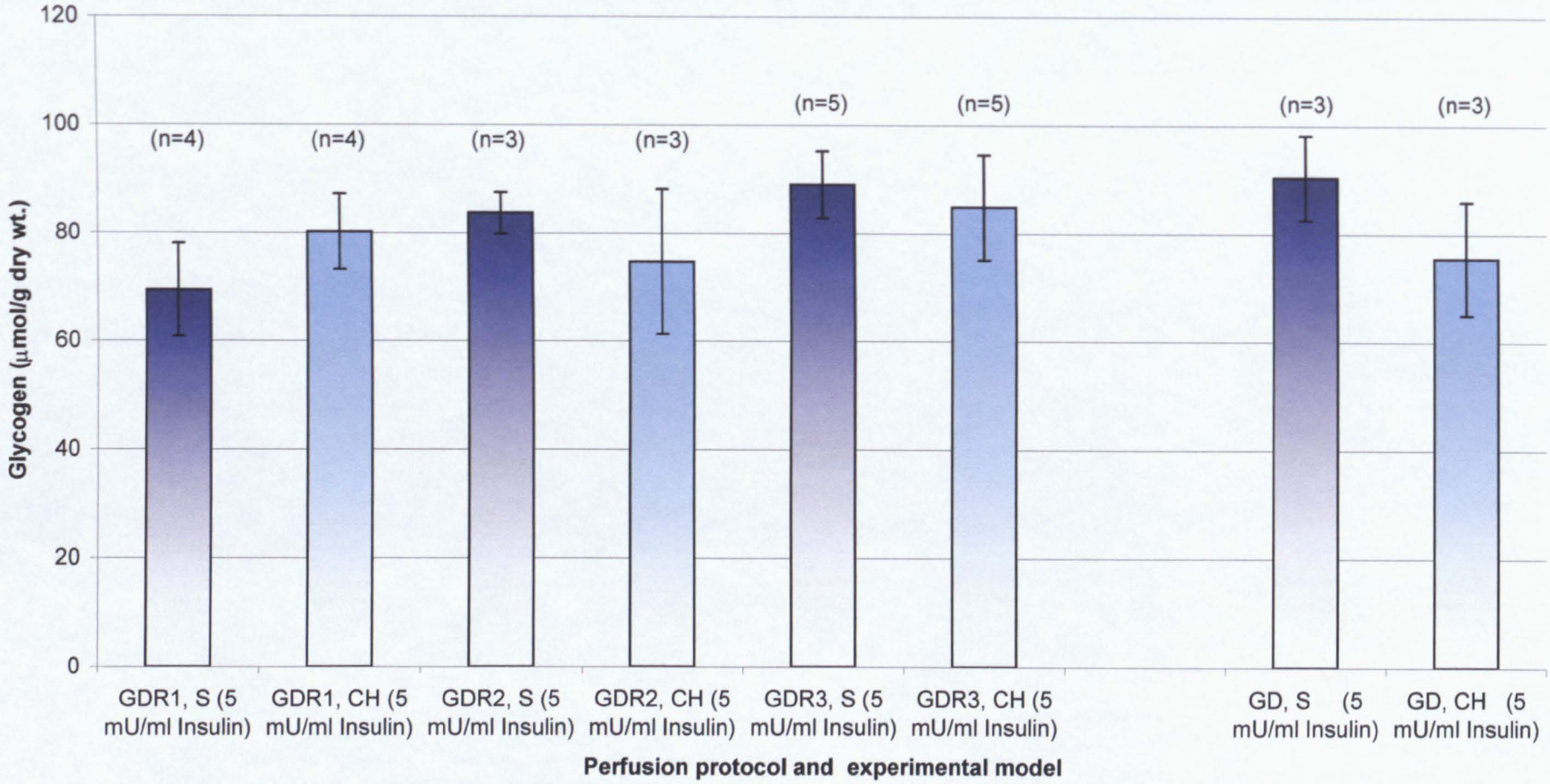


Figure 3.6: Myocardial glycogen content from glucose and palmitate depletion/ repletion (GD/ GDR) perfusions with supraphysiological insulin concentrations



Following the GDR2 and GDR3 depletion/repletion perfusion protocols, myocardial glycogen content in these hearts (Figure 3.6) was significantly higher than the levels reported for NP hearts (Table 3.1, $p<0.05$). However, no further myocardial glycogen loading was observed when comparing any GDR data (Figure 3.6) to that of other protocols with high concentrations of insulin (G3, Figure 3.4 and G+P3, Figure 3.5) in the perfusate.

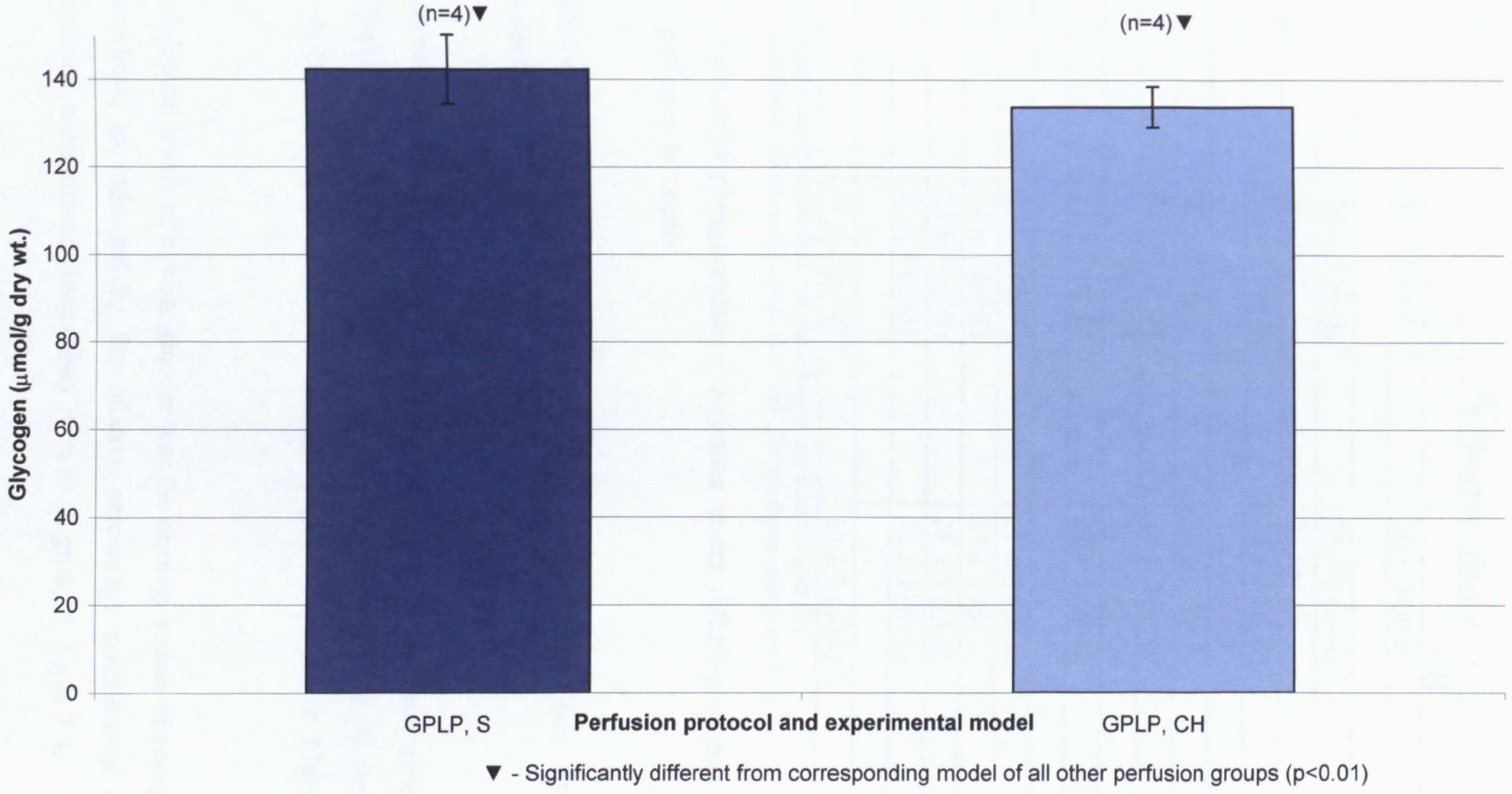
3:3:1:5 Lactate induced glycogen loading

The presence of lactate in the perfusate significantly increased the myocardial glycogen content (142 ± 8; S, and 134 ± 7 μmol/g dry wt.; CH, GPLP, Figure 3.7) above all other glycogen loading perfusion protocols (Figures 3.4, 3.5 and 3.6, $p<0.01$).

3:3:2 Myocardial phosphorylase activities

The percentage phosphorylase *a* activities out of total phosphorylase activity are given in Table 3.2. *In vivo*, phosphorylase *a* activity was greater than that observed in perfusions with glucose or glucose and palmitate as substrates (NP, Table 3.2, $p<0.05$). Percentage phosphorylase *a* activity was not different between S and CH hearts at the end of any of the perfusion protocols (Table 3.2). Increased phosphorylase *a* activity was observed in hearts perfused with glucose in the absence of insulin (G1, Table 3.2) compared to glucose with insulin (G3, Table 3.2, $p<0.05$). The presence of palmitate did not modify phosphorylase *a* activity nor did the presence of insulin with palmitate (G+P1 to 4 inclusive, Table 3.2). No increases in phosphorylase *a* activity were observed following any of the glycogen depletion/repletion perfusion protocols (GDR1-3 and GD, Table 3.2). In the presence of lactate phosphorylase *a* activity levels (GPL, Table 3.2) were above all other perfusions and similar to those found *in vivo* (NP, Table 3.2, $p<0.05$).

Figure 3.7: Myocardial glycogen content from glucose, palmitate, lactate and pyruvate with insulin (GPLP) perfusions



Perfusion Group	% Phosphorylase *a*	
	S	CH
NP	31 ± 3 (n=5) •	30 ± 2 (n=5) •
G1	26 ± 2 (n=5) ○	25 ± 2 (n=4) ○
G2	24 (n=2)	21 (n=2)
G3	21 ± 3 (n=3)	21 ± 2 (n=3)
G+P1	23 ± 2 (n=5)	23 ± 2 (n=5)
G+P2	22 ± 4 (n=5)	23 ± 3 (n=6)
G+P3	21 ± 4 (n=5)	22 ± 2 (n=5)
G+P4	22 ± 3 (n=5)	22 ± 4 (n=5)
GDR1	22 ± 4 (n=4)	21 ± 3 (n=4)
GD	24 ± 5 (n=3)	24 ± 4 (n=3)
GDR2	25 ± 3 (n=3)	24 ± 3 (n=3)
GDR3	23 ± 3 (n=5)	22 ± 3 (n=5)
GPLP	30 ± 2 (n=4) •	30 ± 2 (n=4) •

○ - Significantly different from corresponding model from G3 ($p<0.05$)
• - Significantly different from all G, G+P and GDR perfusion groups ($p<0.05$)

Table 3.2: Myocardial phosphorylase *a* activities under differing normoxic perfusion protocols

3:3:3 Relative contribution of glucose to oxidative metabolism pre-ischaemia

^{13}C-NMR spectroscopy was performed on myocardial extracts, from perfusions using [1-^{13}C]-glucose substrate, to assess glucose oxidation under steady state conditions. A typical experimental ^{13}C-NMR spectrum is displayed in Figure 3.8.

At supraphysiological levels of insulin, glucose was the primary source of energy in the myocardium, as indicated by the relative percentage contribution of glucose to oxidative metabolism (greater than 79 % in all groups), Table 3.3.

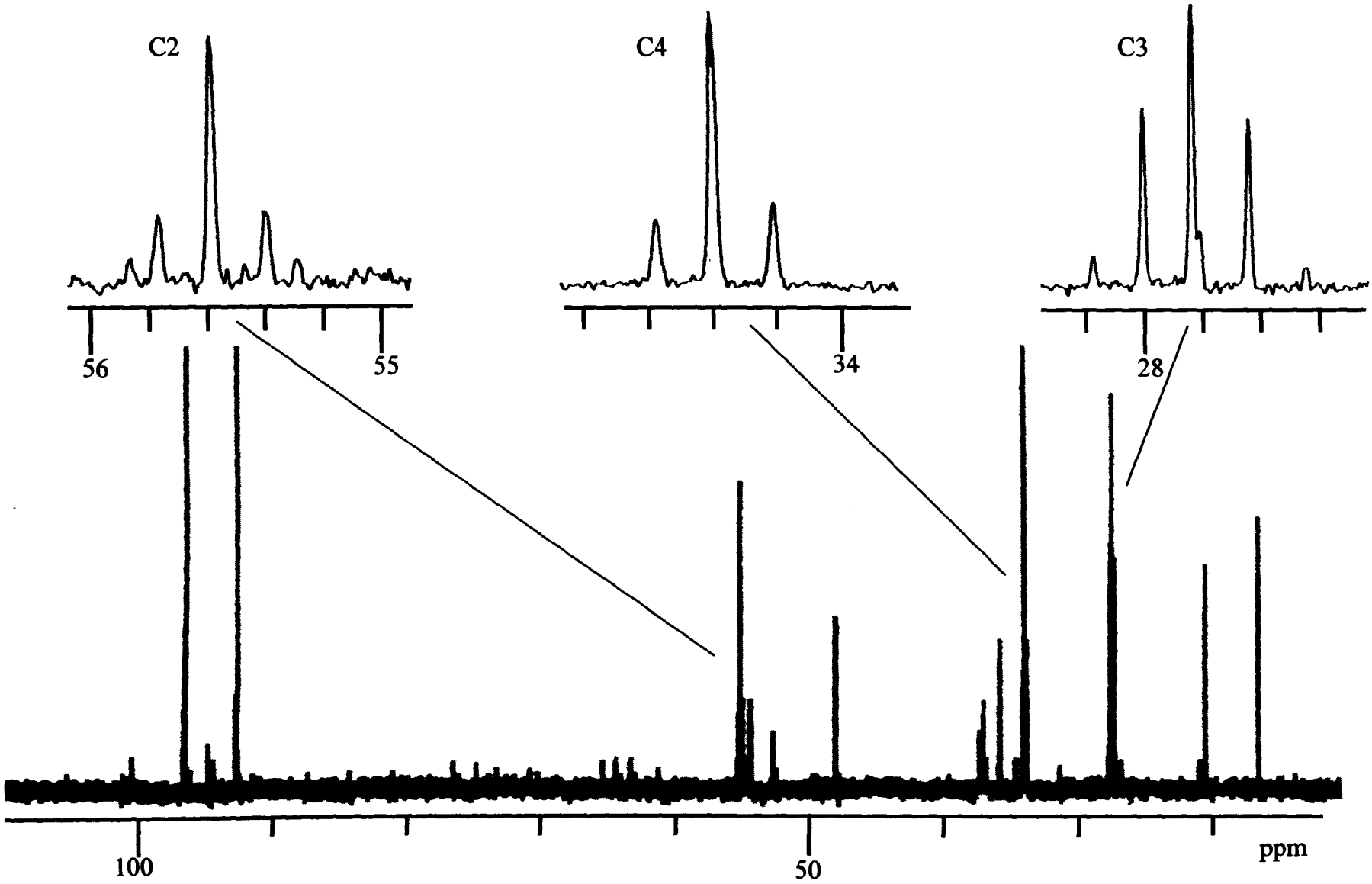


Figure 3.8: A typical steady state spectrum from a heart perfused with [1-^{13}C]-glucose and unlabelled palmitate. Isotopomer patterns for the C2, C3 and C4 of glutamate are shown in the expanded regions.

Model	Nº	Perfusion Group	Relative Percentage Contribution of Glucose to Oxidative Metabolism
S	5	G+P4	86 ± 2
CH	5	G+P4	83 ± 3
S	4	GDR1	87 ± 2
CH	4	GDR1	84 ± 3
S	3	GDR2	85 ± 3
CH	3	GDR2	80 ± 4
S	5	GDR3	84 ± 2
CH	5	GDR3	88 ± 1

Table 3.3: Relative contribution of glucose to oxidative metabolism (TCA Cycle)

No significant differences were observed between the relative contributions of glucose to the TCA cycle in S or CH hearts from any of the perfusion groups (Table 3.3).

In the GD group the relative contribution of glucose could not be measured due to the lack of detectable NMR signal from ^{13}C-glutamate isotopomers. Similarly, little ^{13}C labelling of glycogen was observed in acquired ^{13}C-NMR spectra.

3:3:4 Morphological results

Heart weight: tibia length and heart weight: body weight (Table 3.4), were used to assess the degree of hypertrophy in the CH model for each experimental group.

At the start of this study a 0.7 mm OD needle was used with the ligature during the surgical procedure to induce myocardial hypertrophy. In later experiments a smaller needle, 0.6 mm OD, was used to try and increase the degree of hypertrophy observed.

Model	Perfusion Groups	Number	Outside Diameter of Banding Needle (mm)	Heart Weight: Body Weight ($x10^{-3}$ g/g)	Heart Weight: Tibia Length (g/cm)	Right: Left Kidney Ratio (g/g)
S	NP	5	n/a	2.88 ± 0.13	0.31 ± 0.02	0.99 ± 0.01
	G1, G2 and G3 Combined	10	n/a	4.29 ± 0.10	0.52 ± 0.01	0.98 ± 0.31
	G+P1, G+P2, G+P3 and G+P4 combined	21	n/a	4.32 ± 0.10	0.56 ± 0.01	1.00 ± 0.01
	GDR1	4	n/a	4.50 ± 0.40	0.51 ± 0.04	0.99 ± 0.02
	GD, GDR2 and GDR3 combined	11	n/a	4.30 ± 0.20	0.56 ± 0.03	0.99 ± 0.10
	GPLP	4	n/a	4.11 ± 0.08	0.55 ± 0.01	0.98 ± 0.01
CH	NP	5	0.6	3.34 ± 0.10○	0.41 ± 0.02▼	1.57 ± 0.37○
	G1, G2 and G3 Combined	9	0.6	4.60 ± 0.01○	0.58 ± 0.01○	1.67 ± 0.44○
	G+P1, G+P2, G+P3 and G+P4 combined	24	0.7	4.62 ± 0.02○	0.59 ± 0.01	1.29 ± 0.01○
	GDR1	4	0.6	5.10 ± 0.60	0.60 ± 0.08	1.85 ± 0.50○
	GD, GDR2 and GDR3 combined	11	0.7	4.90 ± 0.10○	0.61 ± 0.02○	1.84 ± 0.39○
	GPLP	4	0.6	4.17 ± 0.10	0.61 ± 0.02○	1.19 ± 0.03○

▼ - Significantly different from corresponding sham ($p<0.01$)

○ - Significantly different from corresponding sham ($p<0.05$)

Table 3.4: Morphological ratio data

Aortic banding with a 0.7 mm OD needle produced an increase in heart weight: tibia length ratio of between 5 and 8 % (Table 3.4). Reducing the diameter of the needle to 0.6 mm OD produced a greater degree of CH. Increases of between 10 and 25 % of the heart weight: tibia length ratio, were observed in animals banded with a 0.6 mm OD needle (Table 3.4).

Right: left kidney weight ratios are also shown in Table 3.4 to show the effect of the surgical band on blood flow above and below the band in the CH model. Right kidney weights were observed to increase in weight, whereas left kidney weights were observed to decrease in weight, in the CH model compared to controls. Increases were observed in the right: left kidney weight ratio of CH animals from all perfusion groups, indicating that the ligature had created a pressure difference across the two kidneys (Table 3.4, $p<0.05$). Animals with a kidney ratio greater than three to one were excluded from the study, as under these conditions the left kidney may have undergone necrosis.

3:3:5 Physiological results

Following nine weeks of aortic constriction there was no significant difference between the myocardial work done (RPP) by S and CH hearts from any of the perfusion groups, Tables 3.5, 3.6 and 3.7.

No increases in myocardial work was observed when comparing RPP's between glucose only perfusions in the presence of (G3, Table 3.5), to those in the absence (G1, Table 3.5) of, 5 mU/ml insulin. The presence of dual physiological substrate (G+P1, Table 3.5) did not increase the myocardial work done (RPP) by S and CH hearts when comparing the RPP to that from hearts perfused with glucose alone (G1, Table 3.5).

Perfusion Group	Model	LVDP (mmHg)	HR (beats per minute (bpm))	Normoxic RPP (x10^3 mmHg.min)
G1	S (n=4)	90.8 ± 7.7	299 ± 20	26.8 ± 2.0
	CH (n=5)	104.6 ± 8.4	249 ± 23	25.5 ± 1.9
G2	S (n=2)	91.3	290	26.3
	CH (n=2)	89.2	268	24.9
G3	S (n=3)	98.0 ± 4.7	289 ± 8	27.8 ± 0.6
	CH (n=3)	111.1 ± 2.5	268 ± 12	29.7 ± 1.4
G+P1	S (n=6)	102.7 ± 8.5	258 ± 11	26.3 ± 2.0
	CH (n=8)	112.5 ± 10.9	250 ± 7	28.0 ± 2.3
G+P2	S (n=5)	98.7 ± 9.5	268 ± 14	26.1 ± 1.8
	CH (n=5)	118.5 ± 11.6	252 ± 7	30.0 ± 3.3
G+P3	S (n=5)	108.6 ± 6.1	247 ± 19	27.3 ± 2.4
	CH (n=6)	122.5. ± 8.9	249 ± 15	30.1 ± 1.6
G+P4	S (n=5)	96.0 ± 5.1	256 ± 10	24.5 ± 1.5
	CH (n=5)	94.6 ± 7.3	234 ± 29	22.6 ± 3.4

Table 3.5: Physiological data for G and G+P Perfusions

No increases in myocardial work from hearts perfused with dual substrate was observed when insulin concentrations increased from 0.1 mU/ml (G+P2, Table 3.5) up to 5 mU/ml (G+P2 and G+P3, Table 3.5).

No signs of myocardial function impairment was observed during any of the 20 minute substrate free periods (GDR1 and 2 and GD, Tables 3.6 and 3.7). Myocardial function was compromised when the substrate free period was extended to 30 minutes as shown by a significant decrease in RPP of both S and CH hearts (GDR3, Table 3.7, $p<0.05$). Following the substrate free period myocardial function recovered within 5 minutes from the start of the repletion perfusion to workload levels observed prior to the substrate free period (Tables 3.6 and 3.7) in all perfusion groups. No further impairment of myocardial

Perfusion Group	Model	Physiological Parameter	Time During Depletion (min)			Repletion
			0	10	20	
GDR1	S (n=4)	LVDP (mmHg)	81.3 ± 10.2	81.8 ± 6.1	83.4 ± 7.9	79.6 ± 6.5
	CH (n=4)		98.6 ± 8.0	107.8 ± 5.4	113.3 ± 5.3	118.7 ± 6.0
	S (n=4)	HR (bpm)	262 ± 20	257 ± 5	261 ± 14	273 ± 16
	CH (n=4)		230 ± 15	248 ± 3	238 ± 13	252 ± 15
	S (n=4)	RPP ($x10^3$, mmHg.min)	21.5 ± 2.5	21.0 ± 1.6	21.7 ± 1.9	21.7 ± 1.7
	CH (n=4)		22.4 ± 1.2	26.8 ± 1.5	26.7 ± 1.1	29.8 ± 1.7
GDR2	S (n=3)	LVDP (mmHg)	85.7 ± 4.0	84.9 ± 11.1	83.0 ± 10.2	76.3 ± 11.7
	CH (n=3)		85.8 ± 3.7	87.0 ± 6.4	91.2 ± 4.6	87.3 ± 4.7
	S (n=3)	HR (bpm)	233 ± 18	246 ± 5	264 ± 16	271 ± 7
	CH (n=3)		249 ± 8	244 ± 10	247 ± 13	238 ± 9
	S (n=3)	RPP ($x10^3$, mmHg.min)	19.7 ± 1.9	20.6 ± 2.4	21.6 ± 1.8	20.8 ±3.3
	CH (n=3)		19.2 ± 1.5	20.5 ±2.2	22.7 ± 2.2	20.9 ± 1.7

Table 3.6: Physiological data for 20 minute depletion/repletion perfusions

Perfusion Group	Model	Physiological Parameter	Time During Depletion (min)				Repletion
			0	10	20	30	
GD	S (n=3)	LVDP (mmHg)	72.1 ± 8.1	74.5 ± 7.7	77.2 ± 8.1	n/a	n/a
	CH (n=3)		68.6 ± 6.9	73.5 ± 7.3	81.9 ± 8.9	n/a	n/a
	S (n=3)	HR (bpm)	273 ± 8	276 ± 5	264 ± 16	n/a	n/a
	CH (n=3)		278 ± 8	280 ± 10	275 ± 13	n/a	n/a
	S (n=3)	RPP ($x10^3$, mmHg.min)	19.6 ± 1.9	20.7 ± 2.4	21.6 ± 1.8	n/a	n/a
	CH (n=3)		19.2 ± 1.5	20.5 ±2.2	22.7 ± 2.2	n/a	n/a
GDR3	S (n=5)	LVDP (mmHg)	93.4 ± 2.3	87.9 ± 3.6	84.6 ± 4.1	78.6 ± 3.3$^{\bigcirc}$	98.5 ± 4.6
	CH (n=5)		93.2 ± 3.6	87.2 ± 2.0	81.6 ± 4.4$^{\bigcirc}$	72.1 ± 5.8$^{\bigcirc}$	93.8 ± 8.3
	S (n=5)	HR (bpm)	263 ± 5	265 ± 7	268 ± 4	272 ± 5	265 ± 4
	CH (n=5)		274 ± 12	270 ± 10	252 ± 20	246 ± 20	250 ± 5
	S (n=5)	RPP ($x10^3$, mmHg.min)	24.6 ± 0.8	23.3 ± 0.9	22.6 ± 1.1	21.3 ± 0.8$^{\bigcirc}$	26.2 ± 1.2
	CH (n=5)		25.4 ± 0.4	23.5 ± 0.6	20.2 ± 1.2$^{\bigcirc}$	19.4 ± 1.5$^{\bigcirc}$	24.6 ± 1.3

$\bigcirc$ – significantly different from corresponding value prior to depletion, T = 0min ($p<0.05$)

Table 3.7: Physiological data for 20 minute depletion and 30 minute depletion/repletion perfusions

function was observed during the remaining repletion period in either the S or CH groups.

The presence of lactate and pyruvate in addition to glucose and palmitate in the perfusate did not influence myocardial work done in S and CH hearts (Table 3.8) compared to hearts perfused with glucose and palmitate at similar insulin concentrations (G+P 2 Table 3.5).

Perfusion Group	**Model**	**LVDP (mmHg)**	**HR (bpm)**	**Normoxic RPP ($x10^3$, mmHg.min)**
GPLP	S (n=4)	97.2 ± 4.5	261 ± 11	25.3 ± 1.4
	CH (n=4)	105.2 ± 6.7	248 ± 18	26.0 ± 1.8

Table 3.8: Physiological data for GPLP perfusions

3:4 Discussion

Overall, glycogen loading was achieved in all hearts perfused with insulin. The percentage of glycogen loading by insulin was greatest when glucose was the only substrate. Nevertheless, the maximum myocardial glycogen content observed was similar under all perfusions with glucose, ± palmitate, and insulin. Glycogen loading appeared to reach a maximum with physiological levels of glucose, palmitate and insulin as no further loading was observed at higher levels of insulin. Myocardial glycogen content was greatest when lactate was present in the perfusate with glucose, palmitate and insulin. Under perfusion conditions with high levels of insulin, glucose was the primary fuel supplying acetyl CoA for oxidative metabolism. ^{13}C labelling of the glycogen pool was insufficient to be observed by NMR methods.

3:4:1 Myocardial glycogen content

3:4:1:1 In vivo glycogen levels

No differences were observed in the glycogen content of S or CH hearts freeze clamping (NP group, Table 3.1) shortly after removal. Myocardial glycogen content reported here is markedly lower, at least 40 % lower, on comparison with gram wet wt. (12 ± 1; S, and 13 ± 1 μmol glucosyl units/g wet wt.; CH) to those reported in the literature [Laughlin *et al.* 1987, Wolfe *et al.* 1993].

Laughlin reports *in vivo* myocardial glycogen content of 22.7 ± 0.6 μmol glucosyl units/g wet wt. in extracts from normal Sprague Dawley rat hearts [Laughlin *et al.* 1988]. Another study reports the enzymatic determination of *in vivo* rat myocardial glycogen content to be 31.5 ± 9.6 μmol glucosyl units/g wet wt. [Wolfe *et al.* 1993], which equates to myocardial glycogen levels between 120 and 150 μmol glucosyl units/g dry wt. [Fraser *et al.* 1998].

A possible explanation for these differences in glycogen content is that in this

study hearts were placed in ice cold K-H buffer containing 5 mM glucose immediately after removal from the animal to allow trimming of excess tissue. Whereas in the studies of Wolfe and Laughlin, portions of ventricular tissue were removed and immediately freeze clamped [Laughlin *et al.* 1987, Wolfe *et al.* 1993]. Trimming was required prior to freeze clamping in this study to determine the heart weight accurately. Although this trimming took no longer than two minutes, it was possible that glycogen degradation might occur during this time for energy provision. Even though the contractile function of the excised hearts ceased very quickly when placed in the ice cold K-H buffer, energy will be required to maintain cellular ionic homeostasis. As limited substrates were being supplied to the heart, energy provision would have to originate from an endogenous source and only glycogen could provide energy under these potentially anaerobic conditions.

Throughout this study, metabolite concentrations and enzyme activities measured are expressed relative to gram dry weight. However, comparisons between results in this study and those from other research groups will be made per gram wet weight when the results have been published in this form by these researchers. Expressing results per unit dry weight is considered more accurate owing to differences observed in myocardial tissue water content. Water content of the heart has been shown to increase during *ex vivo* perfusion [Hjalmarson *et al.* 1969]. Indeed, a 20 % increase in tissue wet weight has been reported when comparing *ex vivo* heart perfusions with crystalloid media against *ex vivo* blood perfusions [Taegtmeyer *et al.* 1980]. In addition to this, the technique of freeze clamping the heart while it is still being perfused may increase the water content of the frozen myocardial tissue [Adolfsson *et al.* 1972] as perfusate will be present within the vasculature of the heart.

3:4:1:2 Glycogen content from glucose only (G) perfusions

Glycogen content in S and CH hearts in all of the perfusion groups was unchanged. This observation may indicate that in this model of CH there was no

deficit or enhancement of carbohydrate storage in hearts from CH compared to those from S animals.

The total glycogen content in the glucose only perfusion group (G1, Figure 3.4) was markedly lower than that reported *in vivo* in the rat heart [Wolfe *et al.* 1993, Fraser *et al.* 1998]. It has been shown, and our data support this, that perfusions in the absence of fatty acids or insulin lead to abnormally low glycogen levels, 51.3 ± 5.2 μmol glucosyl units/g dry wt. [Lagerstrom *et al.* 1988] and 52.8 ± 4.4 μmol glucosyl units/g dry wt. [Goodwin *et al.* 1995]. Goodwin *et al.* report that, in the absence of added hormones, glycogen breakdown was linear in hearts perfused with glucose only over a 30 minute study period [Goodwin *et al.* 1995], indicating that glycogen was being used as a supplementary energy substrate. This suggests that myocardial glucose uptake is limiting under these perfusion conditions as net glycogenolysis is required for energy provision.

The presence of supraphysiological levels of insulin increased myocardial glycogen content by over two fold (G2 + G3, Figure 3.4) to levels close to those reported for isolated working heart preparations, 106 ± 6 μmol glucosyl units/g dry wt [Goodwin *et al.* 1995]. However, the glycogen levels observed (G2 + G3, Figure 3.4) were still lower than those reported *in vivo* by other workers [Wolfe *et al.* 1993, Fraser *et al.* 1998].

3:4:1:3 Effects of insulin on glycogen metabolism

Myocardial glycogen content is known to be increased by the action of insulin stimulating both glucose transport and glycogen synthase activity [Moule and Denton 1997]. Insulin has also been shown to decrease myocardial glycogenolysis in isolated normal [Goodwin *et al.* 1995 and Allard *et al.* 2000] and hypertrophied perfusions [Allard *et al.* 2000].

Insulin activates glycogen synthase (the enzyme responsible for α-[1-4] bond synthesis in glycogen) but does not simultaneously stimulate branching enzyme

activity (responsible for the synthesis of α-[1-6] bonds in glycogen) [Villar-Palasi and Larner 1960, Adolfsson *et al.* 1972]. This could lead to a structural defect within the glycogen molecule by preventing further synthesis of the molecule as further chains cannot be added because a new tier would not be started. It may be that insulin stimulation of glycogen synthase without a concomitant increase in branching enzyme activity is a factor resulting in the lower maximum glycogen contents observed in these perfusions.

The low glycogen levels observed in G perfusions (G1, G2 and G3, Figure 3.4) may be due to the perfusion conditions (Figure 3.1). In perfusion studies of normal hearts with K-H buffer containing supraphysiological insulin concentrations (1 mU/ml and greater) with elevated glucose levels (10 mM and greater), a greater myocardial glycogen content was reported (162 ± 39 μmol glucosyl units/g dry wt. [Chen *et al.* 1997], 173.4 ± 9.4 μmol glucosyl units/g dry wt. [Cross *et al.* 1996]) than determined in this study (111.0 ± 3.5; S, vs 111.1 ± 2.6 μmol glucosyl units/g dry wt.; CH, Figure 3.4). This suggests that in this study perfusing with high levels of insulin and 5 mM glucose, the glucose buffer concentration may have been a limiting factor for glucose uptake. This might explain the lower glycogen levels observed in this study than those reported in the literature.

3:4:1:4 Glycogen content from glucose and palmitate (G+P) perfusions

Inclusion of both glucose and palmitate as substrates (G+P1, Figure 3.1) did not modify glycogen content between S and CH hearts (78 ± 7; S, vs 80 ± 5 μmol glucosyl units/g dry wt.; CH, Figure 3.5). This is in contrast to recent work by Allard who reported a 15% increase in glycogen content in CH compared to S hearts (98.2 ± 2.7; S, vs 112.9 ± 2.2 μmol glucosyl units/g dry wt.; CH) using isolated working heart perfusions [Allard *et al.* 2000]. This difference in glycogen content may be due to the more severe surgical model of CH involved in the Allard study. A clip was placed around the aorta that constricted the aorta to a 0.4 mm diameter [Allard *et al.* 2000], as opposed to the 0.6 mm or 0.7 mm

constriction used in this study. Also, Allard's model differed from the model used in this study as the constriction was placed closer to the heart. The combination of a smaller band, and its placement closer to the heart, should create a greater myocardial hypertrophic response compared to that observed in this study. This is confirmed by Allard *et al.* reporting a 22 % increase in heart weight: body weight ratio in the CH group compared to the S group [Allard *et al.* 2000]. Another contributing factor to the observed differences is that the banding procedure in the Allard study was on three week old animals. Although the animals were left for a similar period of time post surgery (8 weeks) their age may have effects on glycogen metabolism and storage as there is a greater dependence on glucose metabolism for energy provision in the immature heart than the normal adult heart [Lopaschuk *et al.* 1992]. Banding so close to parturition may upregulate glucose use to a greater extent as the animal will not have matured to the adult phenotype where the dependency upon glucose for energy production is less [Lopaschuk *et al.* 1992].

The myocardial glycogen content when fatty acids and glucose were present in the perfusate (G+P1, Figure 3.5) was close to those levels reported *in vivo* [Wolfe *et al.* 1993, Fraser *et al.* 1998]. Fatty acids, the predominant fuel of the heart, are known to inhibit glucose oxidation, glycolysis and glucose uptake [Randle *et al.* 1963]. This is thought to provide a sparing effect upon glucose use by the heart allowing it to be more readily available for organs with little carbohydrate storage (e.g. the brain). Fatty acid inhibition of glucose oxidation is greater than the inhibition of glycolysis which is greater than the inhibition of glucose uptake [Randle *et al.* 1964], thus a re-routing of glucose towards glycogen synthesis can be expected. However, glycogen levels from the G+P1 perfusion group were still 30 % lower compared to those reported *in vivo*. This is not surprising as only two substrates are present whereas *in vivo* there are several additional substrates present (e.g. lactate and ketone bodies) as well as the hormonal milieu controlling myocardial glycogen levels.

Although there was a 10 % increase in glycogen when insulin was present (G+P2 Figure 3.5), no further glycogen loading was observed when insulin levels were increased above 0.1 mU/ml (G+P3 and G+P4, Figure 3.5). This demonstrates that glycogen loading reached a maximum at physiological levels of insulin. Higher myocardial glycogen levels from similar normoxic perfusions using the working heart perfusion model (0.1 mU/ml [insulin]) have been reported [Allard *et al.* 2000]. However, in this study, high levels of fatty acids (1.2 mM) in addition to lactate were also present in the perfusion buffer, which could increase glycogen synthesis via a re-routing of glucose metabolism.

3:4:1:5 Glycogen content from depletion/repletion (GD, GDR) perfusions

A 20 minute depletion period was originally chosen as other workers have reported a 52 % decrease in myocardial glycogen content of rat hearts after the depletion protocol [Goodwin and Taegtmeyer 1994], followed by an 18 % increase in content above baseline amounts following 45 minutes of repletion with 5 mM glucose, 10 mM lactate and 10 mU/ml [insulin] present in the perfusion buffer [Goodwin *et al.* 1995]. The increased glycogen loading observed by Goodwin *et al.* compared to this study could be explained by hearts preferentially oxidising lactate resulting in increased glycogen synthesis and a greater effect of insulin promoting glycogen synthesis.

Albumin (which was supplied as fatty acid free) was removed from the perfusate during the substrate free period in case there was a small amount of fatty acids associated with the protein that could serve as an energy source for the heart. In contrast to the work of Goodwin [Goodwin *et al.* 1995], glycogen content did not fall in hearts freeze clamped after this substrate free period (GD, Figure 3.6) or increase above normoxic levels (G+P4, Figure 3.5) following the repletion period (GDR2, Figure 3.6).

One explanation for the lack of glycogen depletion following the substrate free period may lie in the perfusion model. Goodwin *et al.* use a working heart

preparation in their study in contrast to the isovolumic Langendorff preparation used in this study [Goodwin *et al.* 1995]. One criticism of previous studies using the Langendorff preparation is that the workload of the heart is often well below physiological levels [Taegtmeyer *et al.* 1980]. This is not the case with a working heart preparation where physiological levels of workload have been achieved [Taegtmeyer *et al.* 1980, Henning *et al.* 1996]. Workload is a major factor controlling substrate utilisation by the heart [Neely *et al.* 1972, Taegtmeyer *et al.* 1980]. As myocardial workload increases, oxygen consumption and substrate utilisation increase to satisfy the increased demand for energy. As a result, during a substrate free perfusion period, an isolated heart preparation at a near physiological workload will utilise a greater proportion of its endogenous substrate stores to provide the required energy.

Extending the substrate free period to 30 minutes (GDR3, Figure 3.6) did not further enhance glycogen content after repletion (G+P4, Figure 3.5). Henning has showed that in the Langendorff heart preparation, following a 30 minute substrate free period, over 60 % of baseline glycogen is degraded [Henning *et al.* 1996] with re-synthesis to baseline content after 60 minutes repletion in the working heart mode with 1.2 mM palmitate, 11 mM glucose, 0.5 mM lactate and 0.1 mU/ml [insulin]. Taking the glycogen levels they report (115 μmol glucosyl units/g dry wt) and the high levels of fatty acid, glucose with the presence of lactate in their re-synthesis perfusate, this depletion/repletion protocol does not appear to offer any gain in loading the heart with glycogen when perfusing with a mix of fatty acid and carbohydrate substrates.

Interestingly, Henning reported that when the perfusate contained insulin and physiological levels of fatty acid (0.4 mmol/L palmitate), net glycogen loss occurred from working hearts [Henning *et al.* 1996]. It has also been reported that the myocardial triacylglycerol pool decreased in size in perfusions with physiological levels of fatty acid [Saddik and Lopaschuk 1991]. Saddik went on to speculate that 0.4 mmol/L palmitate was not the concentration of fatty acid in

the interstitial compartment seen by the heart *in vivo*, and therefore, endogenous pools of triacylglycerol were reduced. In light of this, Henning postulates that the same may hold true for glycogen metabolism, in that glycogen was used to generate energy in the face of subphysiological fatty acid levels in the perfusate. This may account for the low glycogen content observed with physiological levels of glucose and palmitate (G+P1, G+P2, G+P3 and G+P4, Figure 3.5 and GDR1, GDR2 and GDR3, Figure 3.6) compared to that reported *in vivo* [Wolfe *et al.* 1993, Fraser *et al.* 1998].

3:4:1:6 Glycogen content from glucose, palmitate, lactate and pyruvate (GPLP) perfusions

The addition of lactate and pyruvate to perfusate containing physiological levels of glucose, palmitate and insulin resulted in glycogen levels (142 ± 8; S and 134 ± 7 μmol glucosyl units/g dry wt.; CH, GPLP, Figure 3.7) analogous to those reported *in vivo* [Wolfe *et al.* 1993, Fraser *et al.* 1998]. The presence of lactate resulted in the greatest myocardial glycogen content out of all the glycogen loading perfusion protocols.

Studies have shown that when lactate is present in the perfusate *in vitro*, myocardial glycogen content is very close to that reported *in vivo* [Wolfe *et al.* 1993, Fraser *et al.* 1998, Allard *et al.* 2000]. Glycogen synthesis is reported to occur *in vitro* when lactate is a fuel for the heart [Depre *et al.* 1993, Laughlin *et al.* 1994]. Increased oxidation of lactate, a preferred substrate of the heart [Taegtmeyer 1994, Bolukoglu *et al.* 1996], enhances citrate levels that result in inhibition of glycolysis at the PFK step [Depre *et al.* 1993]. Glucose uptake was barely affected by increased lactate oxidation [Depre *et al.* 1993], implying that myocardial glucose metabolism is re-directed towards glycogen synthesis when lactate is present in the perfusion buffer.

3:4:2 Phosphorylase *a* activities

The greatest levels of phosphorylase *a* activity were observed in hearts *in vivo* (NP, Table 3.2). As glycogen levels were decreased in these hearts compared to those reported *in vivo* [Laughlin *et al.* 1987, Wolfe *et al.* 1993] it may give further weight to the possibility that glycogen degradation was occurring prior to freeze clamping as described above (Section 3:4:1:1).

Little differences were observed in the percentage activities of phosphorylase *a* between perfusion protocols (Table 3.2). An increase was observed in phosphorylase *a* activity when hearts were perfused with glucose alone (G1, Table 3.2) that correlated with a decrease in glycogen content (G1, Figure 3.4) at the end of the perfusion protocol. This indicates that glycogen was being used as a fuel to supplement the energy demands of the heart under these perfusion conditions. The utilisation by the heart of 5 mM glucose may be a limiting concentration as in other studies the additional presence of insulin has been required for adequate substrate provision [Opie *et al.* 1962]. No increases in phosphorylase *a* activity were reported at the end of the substrate depletion period (GD, Table 3.2). Taken together with no observed decreases in glycogen content at the end of the GD perfusion protocol (GD, Figure 3.6), this indicates that under these depletion conditions other sources of substrate are sufficient to supply the mechanical requirements of the heart. No increase in phosphorylase *a* activity was observed when palmitate and/or insulin were present in the perfusate (G+P perfusions, Table 3.2) and glycogen levels are maintained, indicating that the presence of either does not increase the cycling of glucose moieties into and out of (turnover of) glycogen. Phosphorylase *a* activity was highest from all perfusion groups in the presence of lactate and pyruvate (GPLP, Table 3.2). Since glycogen content increased in hearts from GPLP perfusions, it implies that lactate stimulates glycogen deposition via a separate mechanism to a decrease in phosphorylase *a* activity. In addition, it may indicate that *in vivo* when the heart is supplied with several substrates glycogen turnover rates may play an important

role in ischaemic injury, in addition to the overall glycogen content.

3:4:3 Glucose oxidation

The contribution of exogenous glucose to myocardial oxidative metabolism in the TCA cycle was very high (>79 % in all cases, Table 3.3) in all perfusion conditions containing 5 mU/ml insulin. These results are comparable to those reported by Bradamante and co-workers [Bradamante *et al.* 2000] under similar perfusion conditions. The high levels of exogenous glucose contributing to acetyl CoA entering the TCA cycle highlight that the PDC must be activated. The mechanism by which insulin activates PDC is not fully understood at present [Brownsey *et al.* 1997]. It is thought that the activation of the complex by insulin is explained by increased PDC-phosphatase activity [Hughes and Denton 1976] by a mechanism distinct from the activation of PDC-phosphatase by calcium [Hughes and Denton 1976, Rutter *et al.* 1992]. The observed high rates of glucose oxidation with high levels of insulin will have masked any subtle differences between the S and CH hearts. In support of this, hypertrophied hearts have been observed to have an increased reliance on glucose oxidation in the absence of insulin, but when physiological levels of insulin were present no differences in glucose oxidation were observed between control and hypertrophied hearts [Clarke 2001].

3:4:3:1 ^{13}C Labelling of glycogen

In extracts of myocardial tissue (*in vitro*), little ^{13}C-labelling of glycogen was observed in any of the ^{13}C-NMR spectra obtained. Although glycogen is a large molecule, it is visible *in vivo* by NMR [Laughlin *et al.* 1988] and its ^{13}C-NMR signal is routinely enhanced with ^{13}C-labelled glucose and insulin in isolated myocardial perfusions performed within NMR machines with wide bore magnets [Brainard *et al.* 1989]. The absence of a glycogen signal in heart extracts is most likely due to a lack of sufficient glycogen synthesis from ^{13}C-glucose. However it may also be due, in part, to the extraction procedure. It has previously been

demonstrated in our laboratory that acid extraction with PCA, does not extract all of the glycogen from heart tissue (only ~80 %). Any glycogen that remains in the tissue after PCA extraction may contain ^{13}C-NMR visible glycosyl residues. However, the contribution of incomplete extraction of glycogen may be minimal as new glycogen chains synthesised from 1-^{13}C glucose are going to be in the outermost tiers of the molecule. These chains will be the first to be degraded during the acid extraction and as a result most of the ^{13}C-NMR visible glycosyl residues will be extracted.

3:4:4 Model of hypertrophy

The experimental model used in this study generated a moderate degree of CH at nine weeks post surgery. Tibia length, used as an indicator of growth, was not affected by the banding or sham procedure. Increased myocardial growth resulting from aortic banding was assessed from the heart weight: tibia length ratio, as increases in this ratio will reflect an increase in overall myocardial weight [Yin et al. 1982]. Evaluating hypertrophy from the heart weight: tibia length ratio is regarded as a more accurate measurement of cardiac hypertrophy than the heart weight: body weight ratio [Yin et al. 1982]. Body weight may vary significantly due to differences in appetite, as well as the pecking order of the animals within the cage that they are housed.

Kidney weights were also used as an indicator of the pressure overload in this model. An increase in the right: left kidney weight ratio was observed in CH animals compared to shams in all experimental groups (Table 3.4, $p<0.05$). After banding, the right kidney (above the intra-renal ligature) was hyper-perfused, resulting in renal hypertrophy. In contrast, the left kidney (below the ligature) was hypo-perfused resulting in atrophy of the left kidney compared to those from sham animals. Maximal hypertrophy in this model has been reported when the right: left kidney ratio is 2: 1 [Boateng *et al.* 1997].

Comparing animals between the different experimental groups, there was variation in the degree of hypertrophy observed (Table 3.4). Increases of between 4 % and 8 % were observed in the heart weight: tibia length ratio in CH rats banded with a 0.7 mm OD needle (Table 3.4). This was lower than that previously reported for this model [Seymour & Chatham 1997, Boateng *et al.* 1998] but similar to more recent studies at Hull [Clarke 2001]. Overall hypertrophy of the whole heart was much less than the actual myocyte hypertrophy. It has been shown that this model induces a 38 % hypertrophy of CH myocytes compared to S myocytes [Linehan 2001]. However, this extent of hypertrophy may not be observed across the whole heart (even though myocytes contribute the bulk of tissue mass) because they only compromise 25 % of the cardiac cell population [Bugaisky and Zak 1989].

In consequence, a 0.6 mm OD needle was used subsequently to increase the extent of hypertrophy observed in this model. Banding with the smaller needle should increase aortic constriction, creating a greater pressure overload on the heart that should result in a greater degree of myocardial hypertrophy. Increases of between 10 and 25 % were observed in the heart weight: tibia length ratio from CH animals banded with a 0.6 mm OD needle (Table 3.4).

3:4:5 Function

No significant differences in physiological functional performance were observed in any of the perfusion groups between S and CH hearts (Tables 3.5 to 3.8), irrespective of substrates used.

No inotropic effect was observed when insulin was present in the buffer (Table 3.5). This is in contrast to studies *in vivo* [Baron 1994, Scherrer and Sartori 1997] and *in vitro* [Doenst *et al.*1999, Allard *et al.* 2000] where inotropic effects of insulin have been reported. Recently it has been demonstrated in the isolated working heart that insulin has a direct inotropic effect that precedes changes in

glucose metabolism suggesting that there are independent pathways mediated by insulin in the heart [Doenst *et al.*1999].

In the depletion/repletion studies myocardial function was not modified during the 20 minute depletion period (Tables 3.6 and 3.7). In contrast, in the working heart a decrease in myocardial function occurs during the depletion period [Goodwin *et al.* 1995]. This observed decrease is probably due to the greater myocardial work rate of a working heart preparation than a Langendorff preparation making it more susceptible to an impairment of energy substrate supply. A decrease in myocardial function was observed in S and CH hearts during the 30 minute depletion period, giving an indication that this perfusion protocol exerted more stress on the hearts than the 20 minute depletion period perfusion protocols.

The presence of lactate and pyruvate did not modify myocardial function (Table 3.8) from that observed with other perfusion protocols (Table 3.5). This suggests that the provision of a physiological substrate mix closer to that observed *in vivo* does not affect normoxic myocardial function.

3:4:6 Conclusions

There was no difference between physiological parameters for S and CH hearts under any perfusion conditions. This implies that during the time frame of this experiment, intra-renal banding with either needle produced a beneficial stage of hypertrophy i.e. a model of compensated hypertrophy. Myocardial glycogen content maximised in the presence of physiological concentrations of glucose and palmitate, which was not further modified by the level of insulin present. This may suggest that the maximum space available for glycogen deposition was taken up. However, in the presence of lactate, further glycogen loading was observed. This indicates that the space for glycogen loading was not limiting but that the stimulation of glycogen deposition by insulin alone (in the presence of

glucose and palmitate) was not sufficient to maximally load the heart. Indeed, the effects of insulin may exert a greater effect on glucose oxidation than on glycogen deposition, as the presence of pharmacological levels of insulin resulted in glucose being the principal fuel metabolised in the TCA cycle.

4: ISCHAEMIC STUDIES IN CARDIAC HYPERTROPHY

4:1 Introduction

4:1:1 Susceptibility of the hypertrophied heart to ischaemic injury

There is no clear reason why hypertrophied hearts should be intrinsically more sensitive to ischaemic injury than normal hearts. Indeed some data indicate that pressure overload CH results in an efficient biochemical and contractile adaptation that is well suited to increased pressure work [Swynghedauw *et al.* 1984]. However, there are clinical and experimental data that indicate an impaired tolerance to ischaemia in CH [Iyengar *et al.* 1973, Hearse *et al.* 1978, Shaper *et al.* 1978]. This could be related to one of many factors; biochemical adaptations in the hypertrophied myocardium [Fenchel *et al.* 1986, Wambolt *et al.* 1999], abnormalities in the coronary vasculature causing mild ischaemic injury before the ischaemic arrest [Sink *et al.* 1981, Menasche *et al.* 1985], or to the effects of ventricular fibrillation at the onset of ischaemia [Spadaro *et al.* 1982]. Many studies have shown that the enhanced susceptibility to ischaemic injury in pressure overload CH is associated with alterations in glycolysis [Anderson *et al.* 1990, Gaasch *et al.* 1990, Schönekess *et al.* 1996, Wambolt *et al.* 1999].

4:1:2 Metabolism in the ischaemic hypertrophied heart

Oxidative metabolism decreases during ischaemia and glycolysis becomes a more significant source of ATP production [Neely and Morgan 1974]. The role of glycolysis in the enhanced susceptibility of the hypertrophied heart to ischaemic injury remains unclear [Anderson *et al.* 1990, Gaasch *et al.* 1990].

It has been suggested that in CH there is a decreased capacity of glycolysis for ATP production during ischaemia, which may enhance ischaemic injury [Gaasch *et al.* 1990]. In support of this, stimulation of glycolysis in hypertrophied hearts during ischaemia increased functional recovery on reperfusion [Takeuchi *et al.*

1995].

In contrast, the enhanced susceptibility to ischaemic injury may be a result of accelerated glycolytic flux in CH [Anderson *et al.* 1990, Wambolt *et al.* 1999]. Although increased glycolysis will increase ATP availability during severe ischaemia in CH, it may also exacerbate injury due to a greater than normal accumulation of glycolytic products and H^+ [Anderson *et al.* 1990, Allard *et al.* 1994]. In support of this, reducing ischaemic glycolytic rates [Allard *et al.* 1994] and increasing the removal of glycolytic products and H^+ from the heart during ischaemia [Munfakh *et al.* 1991] have been shown to be beneficial for recovery on reperfusion in CH.

4:1:3 Glycogen metabolism in ischaemia

The metabolic consequences of glycogenolysis during ischaemia/ reperfusion remain controversial [Bailey *et al.* 1982, Neely and Grotyohann 1984, Goodwin and Taegtmeyer 1994]. Studies have highlighted deleterious effects of glycogen breakdown on cellular integrity [Neely and Grotyohann 1984, Allard *et al.* 1994]. In support, studies in which glycogen stores were depleted prior to ischaemia resulted in improved recovery during reperfusion [Neely and Grotyohann 1984]. Myocardial contracture (irreversible injury) has been observed in ischaemic hearts after the exhaustion of glycogen stores [Steenbergen *et al.* 1990], indicating that the ischaemic heart is better preserved as long as glycogenolysis is providing energy [Cross *et al.* 1996] In support of this, studies have shown that maintenance of glycogenolysis during ischaemia is beneficial, with hearts showing good recovery during reperfusion [Bailey *et al.* 1982, Taegtmeyer *et al.* 1985, Lagerstrom *et al.* 1988, Goodwin and Taegtmeyer 1994]. These contradictory findings may be due to the extent of glycogen depletion during ischaemia [Cross *et al.* 1996]. On the one hand, the greater the glycogen degradation during ischaemia the greater the provision of energy and hence more energetically favourable conditions for the myocardium. On the other hand, is

the reasoning that the greater the glycogen degradation, the greater accumulation of toxic end products, which could exacerbate ischaemic damage to the myocardium.

As in normoxia [Goodwin *et al.* 1996], under moderate [Schönekess *et al.* 1997] and severe [Wambolt *et al.* 1999] low flow ischaemic conditions, glucose from myocardial glycogen is preferentially oxidised compared to exogenous glucose. Therefore, under moderate low flow ischaemic conditions where small amounts of TCA cycle activity occur, glycogen may be a more beneficial energy substrate than exogenous glucose as its use will result in less glycolytic product accumulation in contrast to earlier studies [Neely and Grotyohann 1984]. However, during severe low flow ischaemia (when TCA activity is negligible), glycogenolytic rates were higher in hypertrophied hearts compared to controls [Wambolt *et al.* 1999]. This resulted in an accelerated proton production in the hypertrophied heart, which may have contributed to the exaggerated ventricular dysfunction observed and may be a contributing factor in the enhanced susceptibility to ischaemic injury in CH.

4:1:4 Objectives

A number of investigators have shown that the increased susceptibility of the hypertrophied heart to ischaemic injury is associated with increased glucose use for energy production. The metabolic consequences of increased glycogenolysis/glycolysis during ischaemia remain controversial in normal and hypertrophied hearts.

Work was undertaken in this chapter to;

1) Investigate the role of glycogen in CH under low flow and total global ischaemic conditions.
2) Assess the susceptibility of the hypertrophied heart to ischaemic injury.

4.2 Methods

4:2:1 Perfusion protocols

Hearts were removed from animals 9 weeks post surgery and perfused as previously described (Section 2:5).

4:2:1:1 Low flow ischaemia

For low flow ischaemia (LFI) studies, flow was reduced to either 1 ml/min (LFI 1, LFI 2 and LFI 3, Figure 4.1), or 0.5 ml/min (LFI GD, Figure 4.2), by means of an in line perfusion pump.

4:2:1:2 Global ischaemia

During the global ischaemia (GI) phase, flow was reduced to zero by switching off the perfusion pump (GI 1 and GI 2, Figure 4.2). Foil was placed around the perfusion chamber and cannula to minimise temperature loss during ischaemia.

At the end of each perfusion protocol, hearts were freeze clamped and stored under liquid nitrogen until further analyses (Section 2:7). Physiological measurements were taken throughout each perfusion as described previously (Sections 2:6 and 2:7). RPP values reported are the mean value over the perfusion period.

Myocardial tissue extraction, glycogen content, lactate content, glycogen phosphorylase activity and NMR analyses were performed as described (Sections 2:9, 2:10, 2:11 and 2:12).

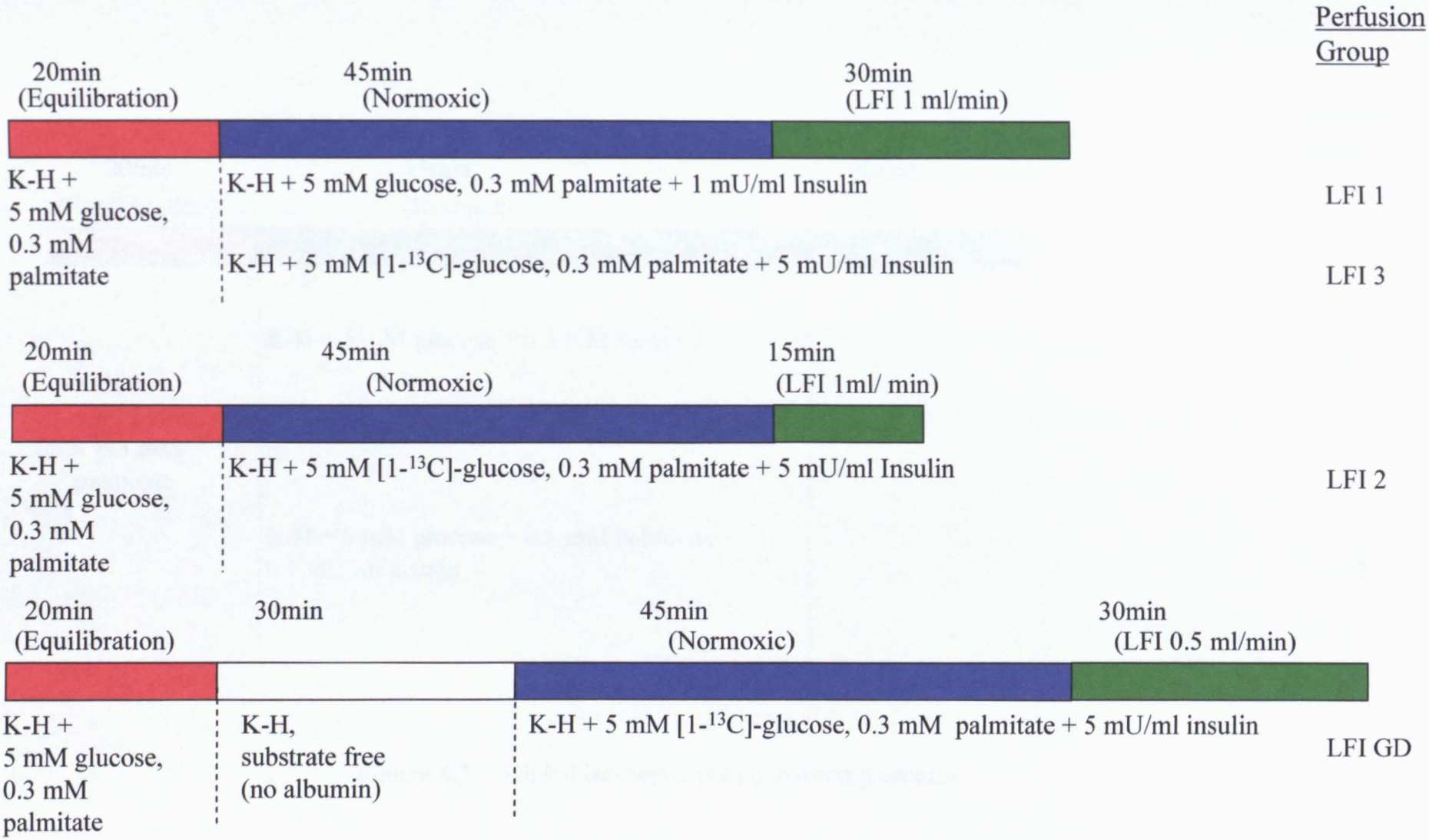


Figure 4.1: Low flow ischaemia (LFI) perfusion protocols

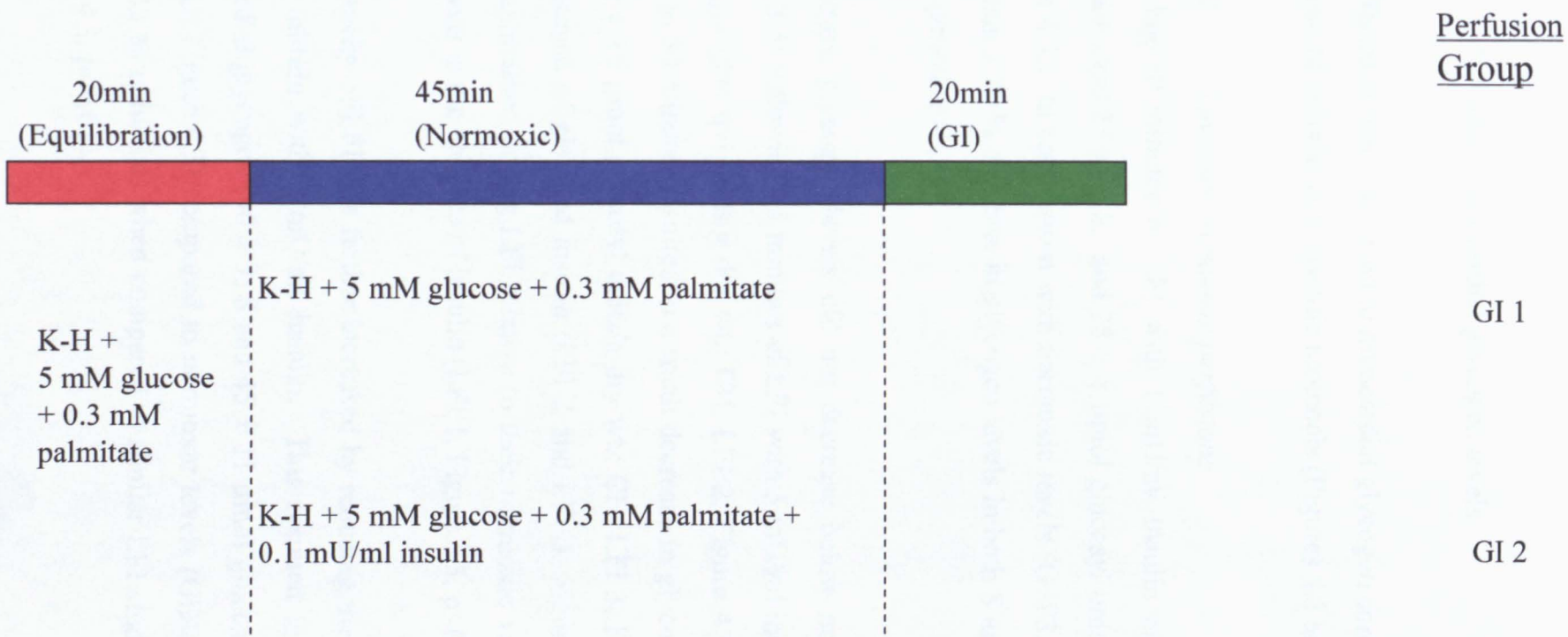


Figure 4.2: Global ischaemia (GI) perfusion protocols

4:3 Results

4:3:1 Ischaemic myocardial glycogen levels

No differences were observed in myocardial glycogen content between S and CH at the end of individual ischaemic protocols (Figures 4.3 and 4.4).

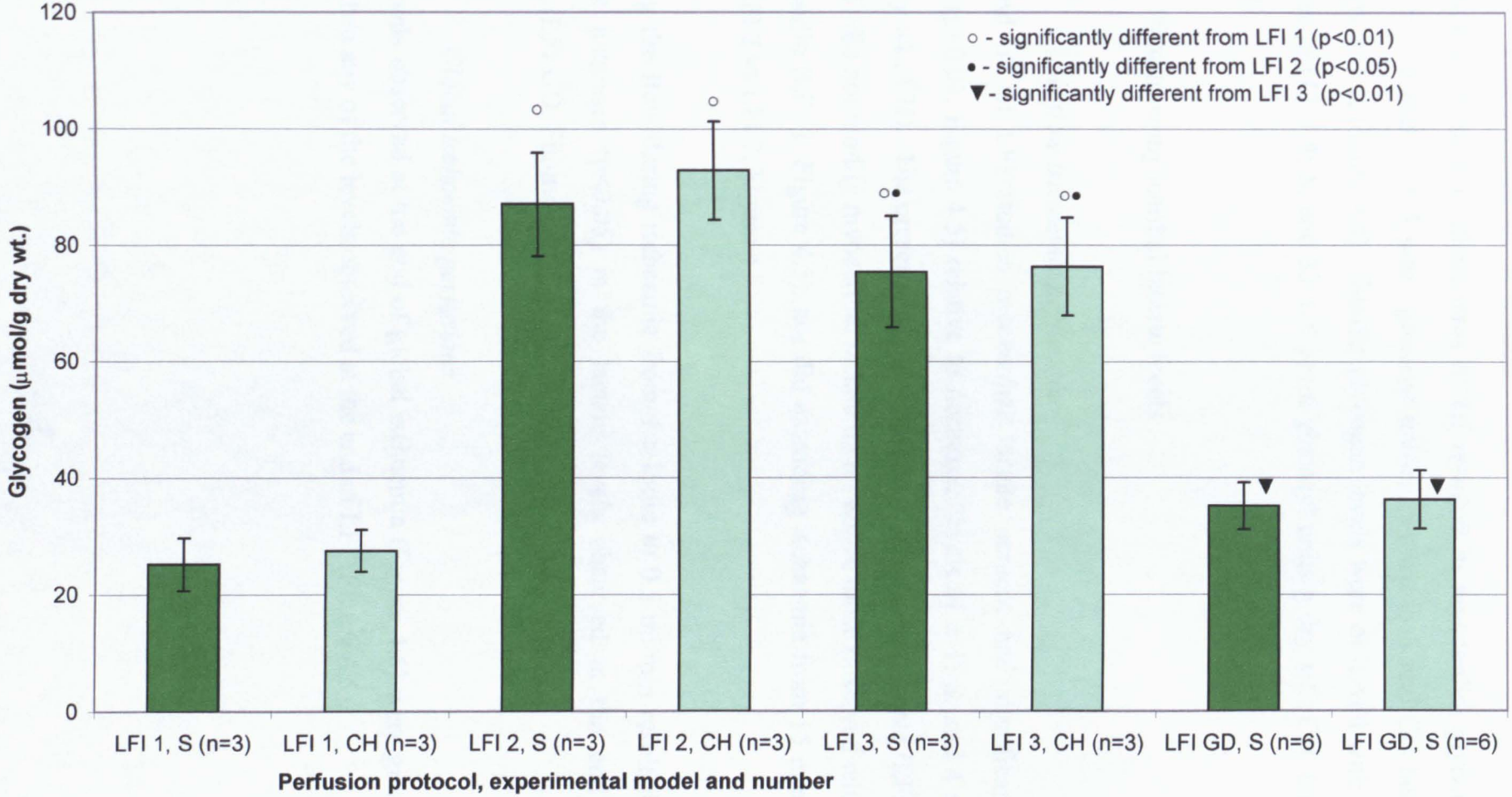
4:3:1:1 Low flow ischaemic perfusions

Following 30 minutes of LFI with 1 mU/ml insulin present, glycogen levels observed were 25 ± 7; S, and 28 ± 8 μmol glucosyl units/g dry wt.; CH, LFI 1 (Figure 4.3). In comparison with normoxic levels (G+P3, Figure 3.5), there was more than a 70 % decrease in glycogen levels in both S and CH hearts at the end of LFI period ($p<0.01$).

In contrast, glycogen levels did not decrease below normoxic levels (G+P4, Figure 3.5) following 15 minutes of LFI with 5 mU/ml insulin (87 ± 5; S and 93 ± 7 μmol glucosyl units/g dry wt.; CH, LFI 2, Figure 4.3). Increasing the LFI period to 30 minutes resulted in a small decrease in glycogen content (75 ± 9; S and 76 ± 10 μmol glucosyl units/g dry wt.; CH, LFI 3, Figure 4.3, $p<0.05$). In the presence of elevated insulin (LFI 2 and LFI 3, Figure 4.3) glycogen levels were maintained during LFI relative to their normoxic values compared to LFI perfusions in the absence of insulin (LFI 1, Figure 4.3, $p<0.01$).

The severity of LFI was further increased by reducing the perfusion flow from 1 to 0.5 ml/min with 5 mU/ml insulin. This resulted in a 45 % reduction in myocardial glycogen (40 ± 9; S and 45 ± 11 μmol glucosyl units/g dry wt.; CH, LFI GD, Figure 4.3) compared to normoxic levels (GDR3, Figure 3.5, $p<0.01$) and a 43 % reduction when compared to similar LFI studies at 1 ml/min (LFI3, Figure 4.3, $p<0.01$).

Figure 4.3: Myocardial glycogen content from varying low flow ischaemic perfusions



4:3:1:2 Global ischaemic perfusions

In the absence of insulin, 20 minutes of GI resulted in myocardial glycogen levels of 35 ± 3 and 36 ± 5 μmol glucosyl units/g dry wt. in S and CH hearts respectively (GI 1, Figure 4.4). Similar glycogen levels were observed with 0.1 mU/ml insulin (43 ± 8; S, and 33 ± 5 μmol glucosyl units/g dry wt.; CH, GI 2, Figure 4.4).

4:3:2 Ischaemic myocardial lactate levels

4:3:2:1 Low flow ischaemic perfusions

At the end of all LFI studies myocardial lactate content had significantly increased ($p<0.01$, Figure 4.5) relative to normoxic levels (4 ± 1; S, and 4 ± 1 μmol/g dry wt.; CH). The presence of insulin (5 mU/ml) in the perfusate (LFI 3, Figure 4.5) did not modify myocardial lactate levels above those observed with 1 mU/ml insulin (LFI 1, Figure 4.5), nor did extending ischaemia from 15 min to 30 min (LFI 2 vs LFI 3, Figure 4.5).

Decreasing the flow during ischaemia from 1 ml/min to 0.5 ml/min resulted in significant increases ($p<0.05$) in the lactate levels observed at the end of ischaemia (LFI GD, Figure 4.5).

4:3:2:2 Global ischaemic perfusions

Lactate levels observed at the end of global ischaemia (Figure 4.6) were greater ($p<0.01$) than any of the levels observed at the end of LFI (Figure 4.5).

Figure 4.4: Myocardial glycogen content from global ischaemic perfusions with varying insulin concentrations

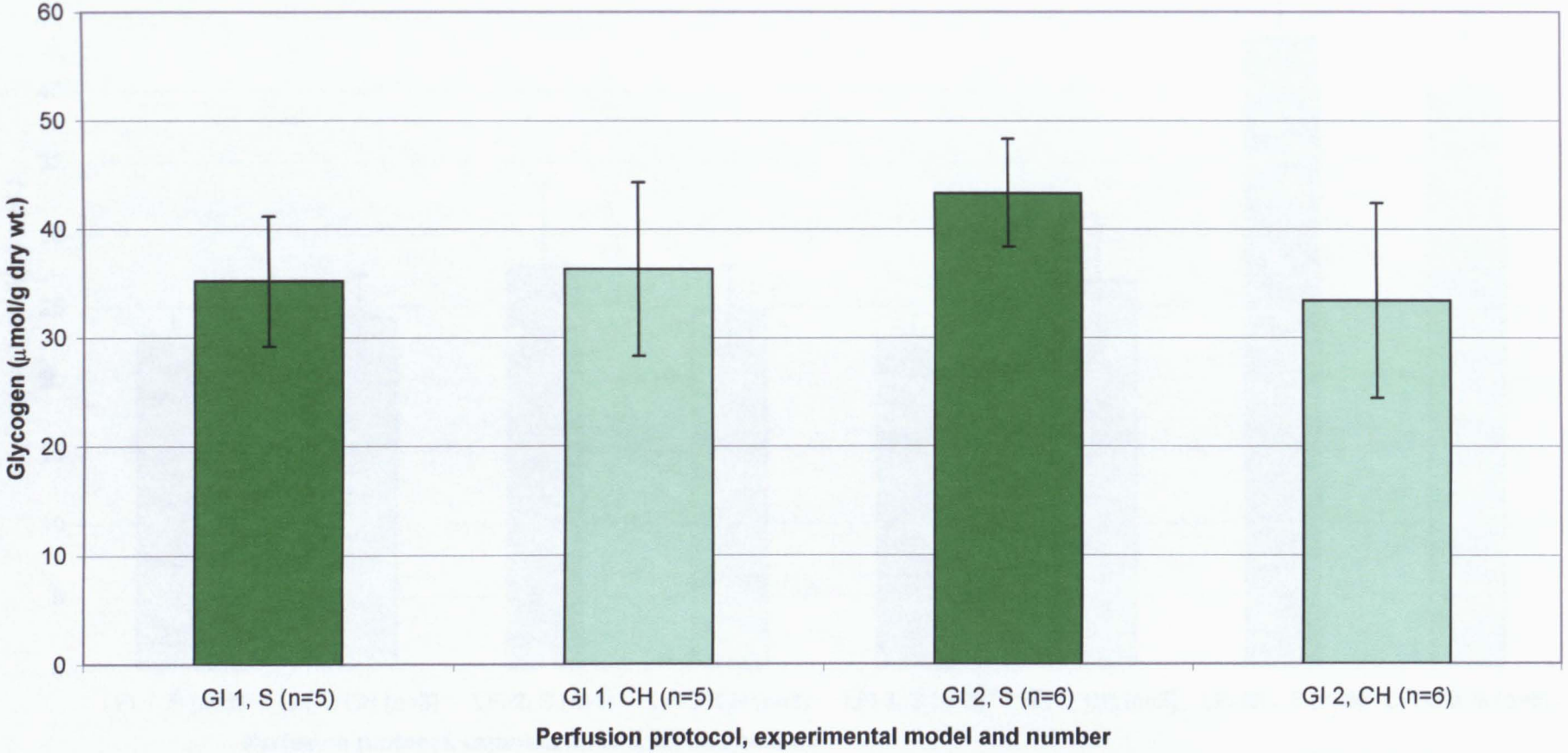


Figure 4.5: Myocardial lactate from varying low flow ischaemic perfusions

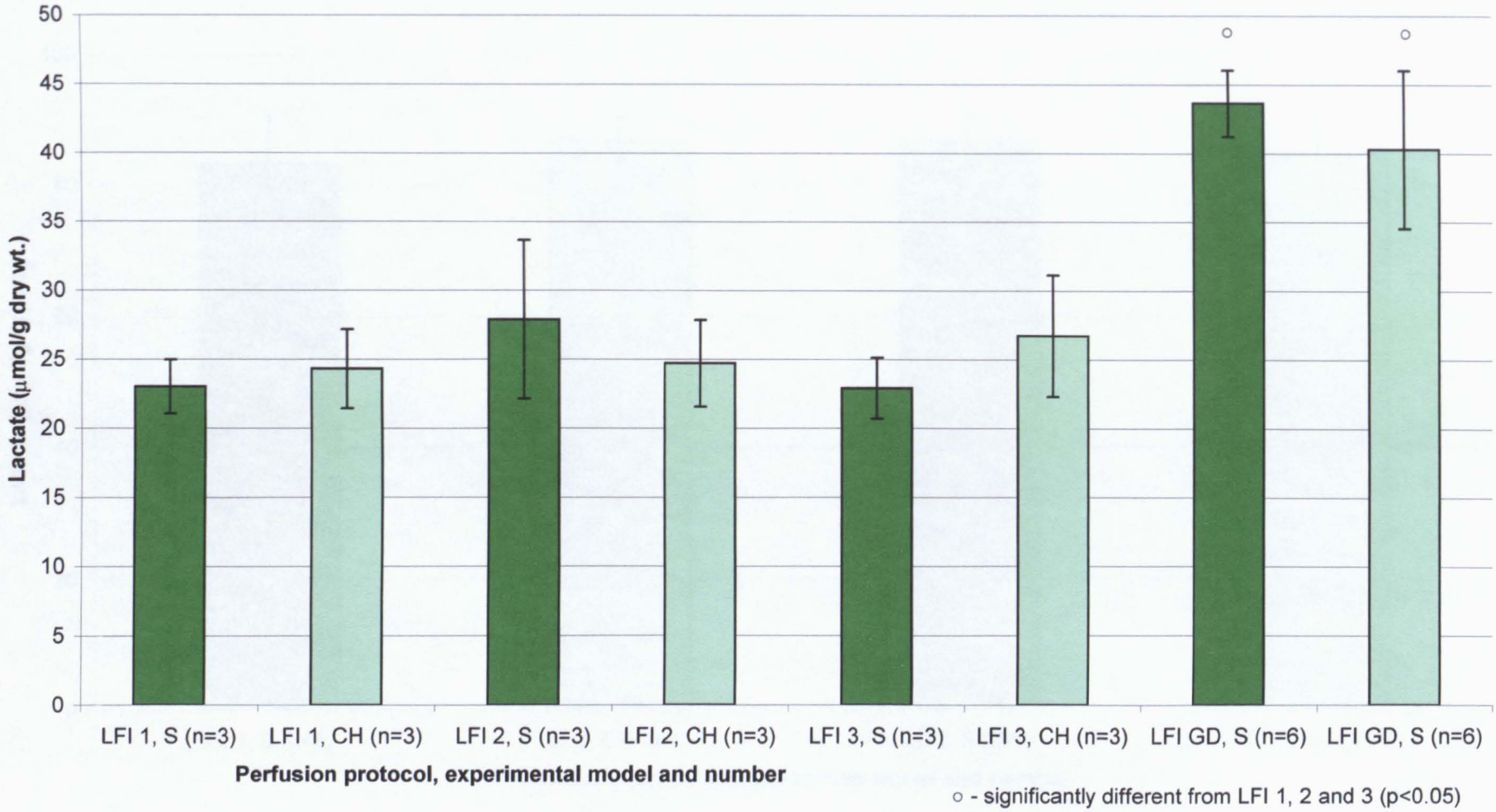
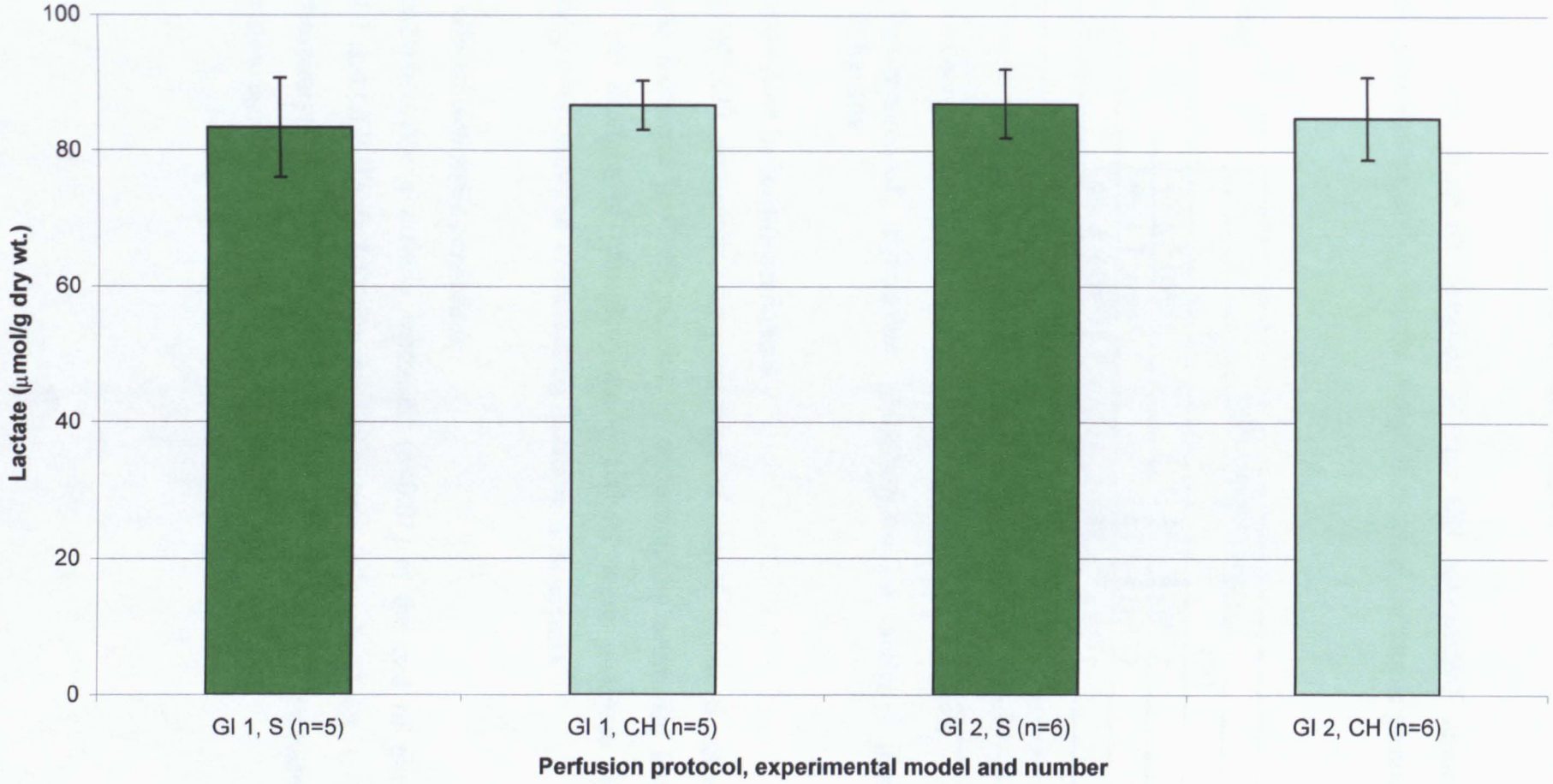


Figure 4.6: Myocardial lactate content from global ischaemic perfusions with varying insulin concentrations



4:3:3 Ischaemic myocardial phosphorylase activities

No differences were observed between S and CH myocardial glycogen percentage phosphorylase *a* activity at the end of individual ischaemic protocols (Table 4.1).

Perfusion Group	% Phosphorylase *a*	
	S	CH
LFI 1	46 ± 5 (n=3)	44 ± 4 (n=3)
LFI 2	44 ± 7 (n=3)	45 ± 4 (n=3)
LFI 3	46. ± 4 (n=3)	48 ± 7 (n=3)
LFI GD	50 ± 8 (n=3)	49 ± 6 (n=3)
GI 1	57 ± 4 (n=5)•	55 ± 5 (n=5)•
GI 2	55 ± 7 (n=6)•	56 ± 5 (n=6)•

• - significantly different from corresponding groups LFI1 to 3 inclusive ($p<0.05$)

Table 4.1: Percentage of myocardial phosphorylase *a* activity during ischaemia

4:3:3:1 Low flow ischaemic perfusions

At the end of all LFI perfusions, the percentage of phosphorylase *a* activity (Table 4.1) was increased ($p<0.05$) relative to corresponding normoxic levels (Table 3.2). No changes in phosphorylase *a* activity were observed with increasing insulin concentrations or increasing duration of ischaemia.

4:3:3:2 Global ischaemic perfusions

Myocardial phosphorylase *a* activity increased ($p<0.01$) at the end of global ischaemia (GI 1 and GI 2, Table 4.1) compared to normoxic levels (G+P1, G+P2 Table 3.2). Phosphorylase *a* activity was elevated at the end of global ischaemia relative to low flow ischaemia (1 ml/min) studies (Table 4.1, $p<0.05$).

4:3:4 Morphological results

A 0.7 mm OD needle was used for the banding procedure for groups LFI 1 and LFI 2. This produced a moderate degree of myocardial hypertrophy, as seen by a 17 % increase in the heart weight: tibia length ratio (Table 4.2, $p<0.01$). For all remaining ischaemia groups, a 0.6 mm OD needle was used. A greater degree of hypertrophy was produced, giving a 21 % increase in the heart weight: tibia length ratio (Table 4.2, $p<0.01$).

4:3:5 Physiological results

4:3:5:1 Low flow ischaemic perfusions

During LFI experiments, myocardial workload decreased rapidly at the onset of ischaemia (Figure 4.7). After 15 minutes LFI, workload (RPP) had decreased by over 90 % (Table 4.3, $p<0.01$) and by 30 minutes only a small residual myocardial function was still detected. No cessation of myocardial function was observed during LFI when perfusing with 5 mU/ml insulin buffer (LFI 2, Table 4.3).

Increasing the severity of ischaemia by reducing the perfusate flow from 1 to 0.5 ml/min did not result in a further decline in function after 30 min LFI (Table 4.4)

Model	Perfusion Groups	Number	Outside Diameter of Banding Needle (mm)	Heart Weight: Body Weight ($x10^{-3}$ g/g)	Heart Weight: Tibia Length (g/cm)	Right: Left Kidney Ratio (g/g)
SHAM (S)	LFI 1 and LFI 2 combined	9	n/a	3.81 ± 0.12	0.48 ± 0.01	1.00 ± 0.01
	LFI 3, GI 1 and GI 2 combined	17	n/a	3.59 ± 0.11	0.43 ± 0.01	1.02 ± 0.01
CARDIAC HYPERTROPHY (CH)	LFI 1 and LFI 2 combined	9	0.7	4.44 ± 0.06°	0.56 ± 0.01$^{\blacktriangledown}$	1.28 ± 0.05$^{\blacktriangledown}$
	LFI 3, GI 1 and GI 2 combined	17	0.6	4.23 ± 0.12°	0.52 ± 0.01$^{\blacktriangledown}$	1.43 ± 0.05$^{\blacktriangledown}$

▼ - Significantly different from sham ($p<0.01$)
o - Significantly different from sham ($p<0.05$)

Table 4.2: Morphological indices for ischaemic perfusions

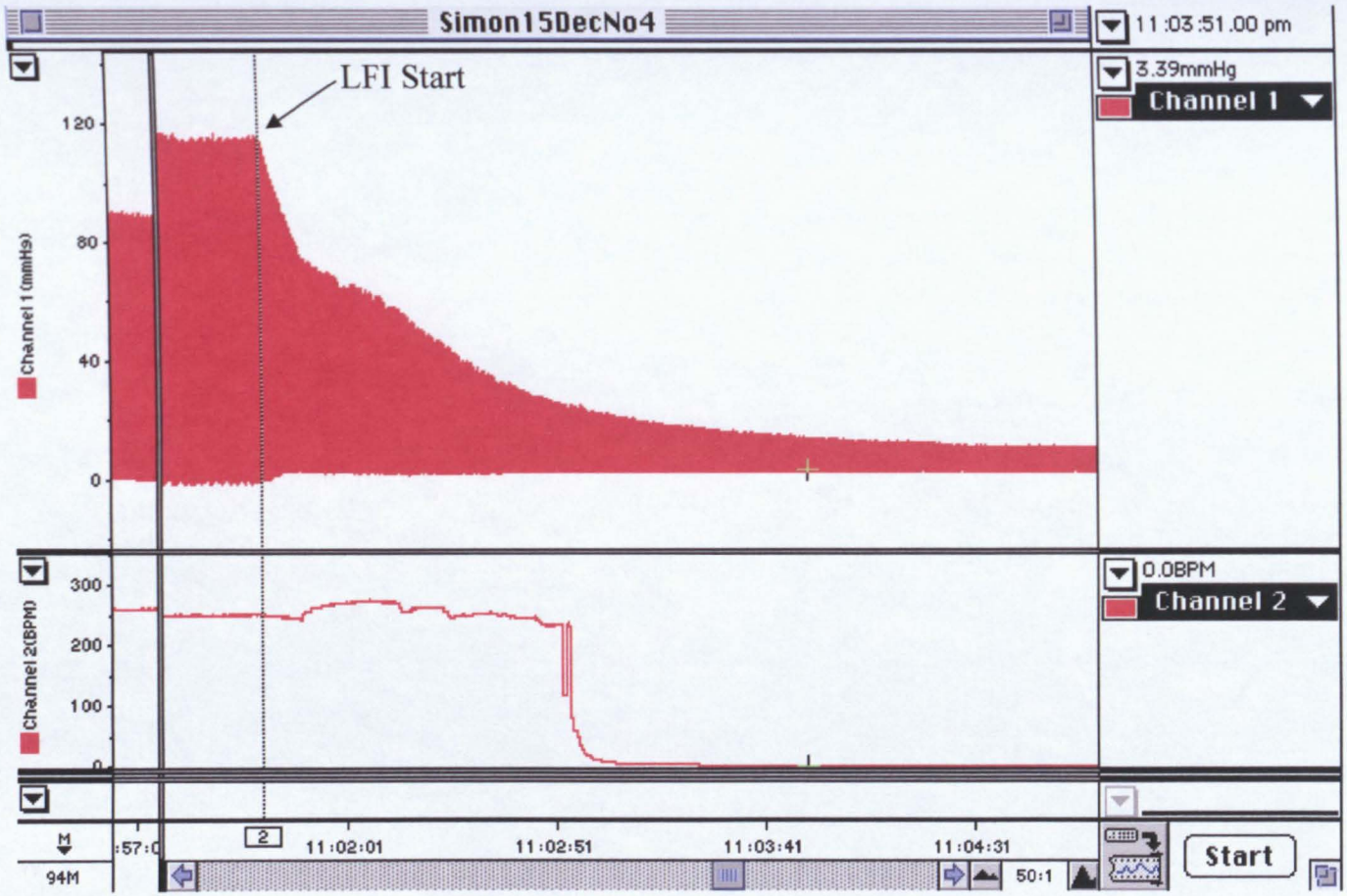


Figure 4.7: Functional trace from a typical LFI (1 ml/min) experiment showing the decrease in function during ischaemia

Perfusion Protocol	Model (Number)	Physiological Parameter	Perfusion Time Period		
			Normoxic	15min LFI (1ml/min)	30min LFI (1ml/min)
LFI 1	S (n=3)	LVDP (mmHg)	100 ± 6.8	17.9 ± 3.6▼	19.1 (n=2, function ceased in one heart)
		HR (bpm)	253 ± 11	109 ± 23▼	62 (n=2,function ceased in one heart)
		RPP (mmHg.min)	25.1 ± 1.2	1.9 ± 0.5▼	1.2 (n=2, function ceased in one heart)
	CH (n=3)	LVDP (mmHg)	122.7 ± 8.9	22.3 ± 5.8▼	47.1 (n=2,function ceased in one heart)
		HR (bpm)	240 ± 17	122 ± 27▼	57 (n=2,function ceased in one heart)
		RPP (mmHg.min)	29.4 ± 2.9	2.5 ± 0.3▼	1.5 (n=2, function ceased in one heart)
LFI 2 and LFI 3 Combined	S (n=6)	LVDP (mmHg)	102.5 ± 4.5	12.2 ± 1.9▼	15.7 ± 2.2 (n=3)
		HR (bpm)	251 ± 12	86 ± 14▼	63 ± 5 (n=3)
		RPP (mmHg.min)	25.5 ± 1.1	1.0 ± 0.1▼	0.9 ± 0.1 (n=3)
	CH (n=6)	LVDP (mmHg)	100.0 ± 4.5	19.2 ± 4.4▼	28.7 ± 10.4 (n=3)
		HR (bpm)	258 ± 5	56 ± 9▼	32 ± 14 (n=3)
		RPP (mmHg.min)	25.3 ± 0.9	0.9 ± 0.2▼	0.7 ± 0.1 (n=3)

▼ - significantly different from corresponding normoxic value ($P<0.01$)

Table 4.3: Physiological parameters for LFI (1ml/min) experiments

Perfusion Protocol	Model (Number)	Physiological Parameter	Depletion Time (Time from start of substrate free period, min)			
			0	10	20	30
LFI GD	S (n=6)	LVDP(mmHg)	109.7 ± 8.3	102.1 ± 7.4	96.6 ± 8.1	86.0 ± 8.1
		HR(bpm)	272 ± 31	264 ± 27	262 ± 22	257 ± 23
		RPP(mmHg.min)	29.6 ± 3.5	26.7 ± 2.6	24.8 ± 1.6	21.5 ± 1.0
	CH (n=6)	LVDP(mmHg)	113.8 ± 5.4	109.1 ± 6.6	100.6 ± 7.8	95.7 ± 4.9
		HR(bpm)	267 ± 5	276 ± 11	302 ± 22	271 ± 10
		RPP(mmHg.min)	30.0 ± 1.9	30 ± 1.4	30 ± 2.2	25.9 ± 1.6
			Perfusion Time Period			
			Normoxic	15min LFI (0.5ml/min)	30min LFI (0.5ml/min)	
LFI GD	S (n=6)	LVDP(mmHg)	123.6 ± 10.2	16.5 ± 3.6	21.0 ± 3.9	
		HR(bpm)	253 ± 25	67 ± 4	56 ± 6	
		RPP(mmHg.min)	30.4 ± 1.5	1.1 ± 0.2	1.2 ± 0.3	
	CH (n=6)	LVDP(mmHg)	128.2 ± 9.3	10.0 ± 2.4	10.6 ± 2.5	
		HR(bpm)	254 ± 10	75 ± 10	71 ± 7.1	
		RPP(mmHg.min)	32.5 ± 2.4	0.7 ± 0.2	0.8 ± 0.2	

Table 4.4: Physiological parameters for LFI GD experiments

4:3:5:2 Global ischaemic perfusions

During GI experiments, myocardial workload decreased more rapidly than observed in LFI experiments (Figure 4.8). In all experiments, myocardial function came to an abrupt halt within 7 minutes from the onset of GI (Figure 4.8). Normoxic physiological measurements are displayed in Table 4.5. After myocardial function ceased, contracture was not observed during the remaining period of ischaemia.

Perfusion Protocol	**Model (Number)**	**Physiological Parameter**	**Perfusion Time Period**
			Normoxic
GI 1	S (n=6)	LVDP (mmHg)	95.9 ± 2.3
		HR (bpm)	251 ± 14
		RPP (mmHg.min)	24.1 ± 1.6
	CH (n=6)	LVDP (mmHg)	102.5 ± 3.9
		HR (bpm)	253 ± 28
		RPP (mmHg.min)	25.8 ± 2.3
GI 2	S (n=6)	LVDP (mmHg)	95.7 ± 3.9
		HR (bpm)	261 ± 9
		RPP (mmHg.min)	25.0 ± 1.1
	CH (n=6)	LVDP (mmHg)	101.3 ± 4.6
		HR (bpm)	253 ± 17
		RPP (mmHg.min)	25.4 ± 1.0

Table 4.5: Physiological parameters for GI experiments

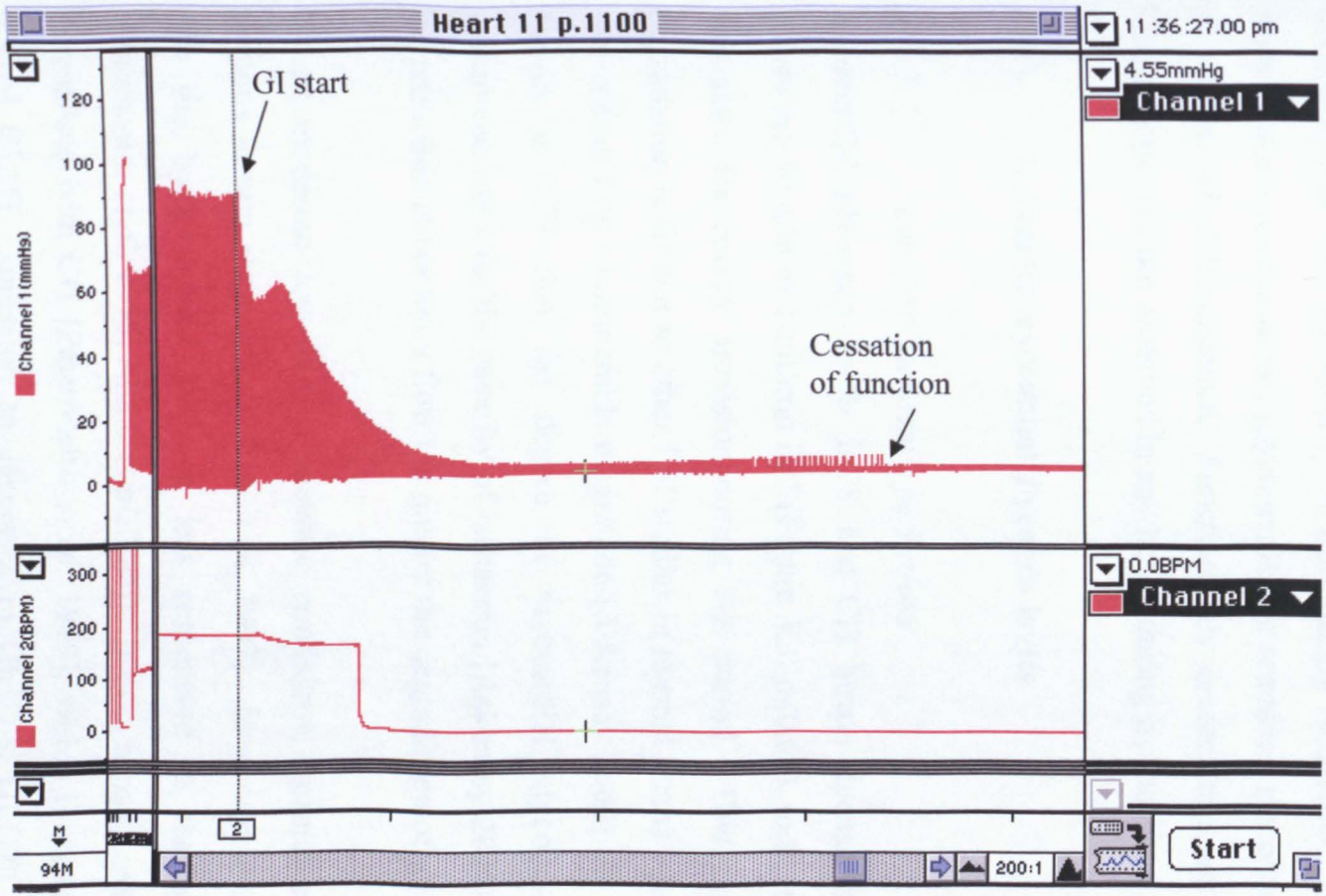


Figure 4.8: Functional trace from a typical GI experiment showing cessation of function during ischaemia

4:4 Discussion

Glycogen was utilised equally in S and CH hearts during all ischaemic conditions. The degree of glycogen degradation and lactate accumulation was commensurate with the severity of flow during ischaemia. In the presence of high insulin concentrations, glycogen levels remained closer to normoxic levels at the end of LFI ischaemia. Function only ceased in hearts from GI studies. Contracture was not observed in any hearts during ischaemia.

4:4:1 Ischaemic myocardial glycogen levels

4:4:1:1 Low flow ischaemic perfusions

Myocardial glycogen levels in S and CH hearts decreased by over 70 % following 30 min of 1 ml/min LFI (Figure 4.3, $p<0.01$), indicating that glycogen was used for energy provision during this period. This degree of glycogen degradation is similar to other LFI studies in normal hearts where coronary flow was reduced by a comparable magnitude [Askensay 2000]. It has recently been shown in LFI that the degree of myocardial glycogen degradation is commensurate with the severity of ischaemia [Askensay 2000]; the more severe the reduction in coronary flow the greater the degradation of glycogen.

Under moderate low flow ischaemic conditions, normal myocardial tissue remains insulin responsive [Chen *et al.* 1997]. However, it has been reported that the hypertrophied heart is less responsive to the effects of insulin [Paternostro *et al.* 1995, Allard *et al.* 2000]. Insulin resistance has been observed in patients with CH [Paternostro *et al.* 1999], which is explained, in part, by altered GLUT expression as decreases in GLUT4: GLUT1 ratios have been observed [Paternostro *et al.* 1995, Depre *et al* 1998b]. In addition, the translocation of the insulin sensitive GLUT4 to the sarcolemma is increased by ischaemia [Sun *et al.* 1994]. Therefore in CH, down regulation of expression and reduced recruitment of GLUT4 may limit glucose availability under ischaemic

conditions. However, no evidence for insulin resistance in CH was detected as glycogen levels were similar between S and CH hearts at the end of LFI (Figure 4.3).

The presence of pharmacological levels (5 mU/ml) of insulin during LFI maintained myocardial glycogen levels similar to normoxic (LFI 2, Figure 4.3 vs G+P4, Figure 3.5). Indeed, following 15 min LFI with 5 mU/ml insulin, glycogen levels did not differ from normoxic levels (LFI 2, Figure 4.3 vs G+P4, Figure 3.5) but after 30 min of LFI glycogen content decreased by 30% compared to normoxic values (LFI 3, Figure 4.3 vs G+P4, Figure 3.5, $p<0.05$). This may suggest that elevated levels of insulin preserve myocardial glycogen levels during LFI. Alternatively it could imply that during LFI there was sufficient flow to deliver an alternate substrate for energy provision, preserving glycogen levels. During LFI, energy could be provided from the residual oxidative metabolism. As well as increasing myocardial glucose uptake [Morgan et al. 1961, Paternostro *et al.* 1995, Bolukoglu et al. 1996, Russell et al. 1998, Verma *et al.* 1999], insulin is also known to stimulate glucose oxidation [Allard *et al.* 2000] by increasing PDC activity [Randle *et al.* 1995]. Therefore under these low flow perfusion conditions, the presence of pharmacological levels of insulin in the perfusate may maintain glycogen levels indirectly by increasing glycolytic and oxidative energy production from extracellular glucose. Insulin has also been shown to increase glycogen synthesis and decrease myocardial glycogenolysis concomitantly, in normal [Goodwin et al. 1995, Allard et al. 2000] and hypertrophied hearts [Allard et al. 2000]. Under the perfusion conditions used in this study, elevated levels of insulin may maintain glycogen levels during LFI by providing an adequate supply of exogenous glucose for glycolysis, or by stimulating glycogen synthesis close to that of its degradation.

Increasing the severity of ischaemia (by a further reduction in flow to 0.5 ml/min) resulted in end ischaemic myocardial glycogen levels similar to those reported previously [Wambolt *et al.* 1999]. The levels were markedly lower than

those from corresponding 1ml/min LFI studies (LFI GD vs LFI 3, Figure 4.3, $p<0.01$), thus indicating that the more severe the ischaemia the greater the degree of degradation of glycogen. Indeed, during a study using a 60 min 0.5 ml/min LFI period, glycogenolysis was found to be an important source of energy throughout the entire ischaemic period [Schönekess *et al.* 1997].

4:4:1:2 Global ischaemic perfusions

GI is a more severe ischaemic insult than LFI. During GI, anaerobic glycolysis or glycogenolysis provides the only source of energy. Glycogen levels decreased below normoxic values following 20 min GI whether in the absence or presence of 0.1 mU/ml insulin (Figure 4.4, $p<0.05$). No preservation of myocardial glycogen levels was observed at the end of GI in the presence of insulin. This could be explained by an inhibition of the insulin response due to increased tissue acidification [Beauloye *et al.* 2001].

In GI experiments, intracellular pH decreases to 6.2 within 13 minutes from the onset of GI [Garlick *et al.* 1979]. The extent of this acidosis is proportional to the degree of glycogen degradation [Garlick *et al.* 1979]. It has recently been reported that inhibition of insulin stimulated-glucose transport occurs below intracellular pH 6.75, with insulin response abolished at pH 6.0 [Beauloye *et al.* 2001].

The decreases in glycogen levels at the end of GI (Figure 4.4) were of a similar magnitude to those observed after LFI at higher insulin levels (LFI 1, Figure 4.3). This may be due to a more rapid accumulation of toxic metabolites causing a more rapid inhibition of glycolysis in GI than LFI. As there is no flow in GI studies, metabolites accumulate in the tissue rapidly, in contrast to LFI. Indeed, many LFI studies monitor the amount of lactate released into perfusate effluent to aid the assessment of anaerobic glycolysis during ischaemia [Cave *et al.* 1994, Massie *et al.* 1995, Bolukoglu *et al.* 1996, de Jonge *et al.* 1998].

4:4:2 Ischaemic myocardial lactate levels

Myocardial lactate levels increased markedly at the end of 1 ml/min LFI (Figure 4.5, $p<0.01$) compared to normoxic values (4 ± 1; S, and 4 ± 1 μmol/g dry wt.; CH) indicating that anaerobic glycolysis and glycogenolysis are occurring comparable to other LFI studies [Cross *et al.* 1995]. When the flow was reduced to 0.5 ml/min from 1 ml/min greater myocardial lactate levels were observed (LFI GD, Figure 4.5) but this was not accompanied by a greater degradation of glycogen (LFI GD, Figure 4.3, $p<0.05$). This implies that the increase in lactate was due to reduced wash-out of glycolytic products at the lower flow. This was supported by the greatest myocardial lactate levels of all ischaemic perfusions being observed in hearts from GI perfusions (Figure 4.6).

The role of lactate in ischaemic contracture remains unclear. The deleterious effects of elevated lactate are thought to be mediated by an increased lactate: pyruvate ratio resulting in an increased NADH/NAD ratio [Cross *et al.* 1995], inhibiting GAPDH [Neely and Grotyohann 1984]. Studies have also proposed that exogenous lactate may be detrimental during ischaemia due to increased Na^+/H^+ exchange as lactate exerts a proton load on the cell [Karmazyn 1993]. However, further work found no changes in pH with increased extracellular lactate [Cross *et al.* 1995], and that NADH accumulation was the important factor in detrimental effects of lactate in ischaemia.

4:4:3 Ischaemic myocardial phosphorylase levels

Glycogen content measurements are not necessarily good indicators of actual glycogen use during ischaemia due to continuous turnover of glycogen during severe LFI [Schönekess *et al.* 1997]. Although ischaemia induces high rates of degradation of glycogen, overall turnover rates of the glycogen pool may also be a contributing factor in the beneficial/detrimental effects of anaerobic glycogenolysis during ischaemia. The determination of phosphorylase activity

was aimed at investigating this possibility.

The percentage of phosphorylase in the more active *a* form was increased at the end of LFI studies (Table 4.1) compared to normoxia (Table 3.3, $p<0.05$). This corresponded to a decrease in the myocardial glycogen content of the tissue. In the presence of high insulin concentrations no differences were observed in percentage phosphorylase *a* activities at the end of LFI but glycogen levels remained elevated. This may be explained by the fact that insulin is known to stimulate glycogen synthase. If insulin stimulated glycogen synthesis to a similar level to that of its degradation there would be little net glycogen degradation.

Comparable levels of phosphorylase *a* activity were found after 15 and 30 minutes LFI. This implies that a new steady state in the percentage of phosphorylase in the *a* form occurs within 15 minutes of ischaemia and, if ischaemia progresses at a constant rate, further changes in activity do not occur. Indeed, changes in the forms of phosphorylase have been shown to occur in less than a minute during ischaemia [Askensay 2000].

As the severity of ischaemia was increased, small increases in the percentage of phosphorylase *a* were observed ($p<0.05$). The greatest proportion of phosphorylase *a* was observed in hearts after GI perfusions. During GI glycogen is the only endogenous substrate that can supply energy once intracellular glucose is no longer accessible therefore the greatest rates of glycogen degradation are expected.

4:4:4 Morphological results

A moderate degree of cardiac hypertrophy was generated by the surgical procedure used in this study. The heart weight: tibia length ratio was significantly increased by 21 % (Table 4.1, $p<0.01$) and 17 % (Table 4.1, $p<0.01$) when aortic constriction was performed using a 0.6 or 0.7 mm OD needle respectively

The degree of hypertrophy reported here is similar to that observed in hearts weighed immediately after excision in this study (Section 3:4:6) and reported elsewhere for this model of CH [Seymour and Chatham 1997, Boateng *et al.* 1997]. The marked increase in hypertrophy compared to previous normoxic crystalloid perfusions in this study may be attributed to a myocardial fluid loss during ischaemia as evidenced by a decrease in wet: dry weight ratios of ischaemic hearts. This may provide further weight for the argument proposing that myocardial oedema resulting from crystalloid perfusions masks the real extent of hypertrophy in this model (Section 3:4:10). During ischaemia fluid retention would be expected due to increased concentrations of metabolites within cells of the heart, increasing the cellular osmotic potential. However, in this study where the temperature is maintained during ischaemia, fluid loss may occur during the ischaemic period due to rates of evaporation exceeding rates of fluid uptake from the minimal or no flow of the perfusate.

4:4:5 Physiological results

4:4:5:1 Low flow ischaemic perfusions

The decline in myocardial workload after the onset of LFI was similar to that reported in recent LFI work [Askensay 2000]. Initially, at the onset of ischaemia there was a rapid decrease in function, followed by a gradual decrease over the remaining ischaemic period. Residual myocardial contraction was detectable at the end of all 1 ml/min LFI experiments (bar 1 S and 1 CH heart from LFI 1, Table 4.2). This decrease in function is in agreement with other LFI work where a graded mechanical depression was observed, proportional to the decline in aerobic energy production [Askensay 2000].

An increase in ischaemic stress upon the myocardium did not result in the cessation of myocardial function. Indeed, in all of the hearts subjected to 30 min LFI at 0.5 ml/min, residual ventricular contraction was detected at the end of ischaemia. This is in contrast to the studies of Wambolt and Schönekess on the

working heart with similar perfusate, as they did not observe any mechanical function during LFI at 0.5 ml/min [Schönekess *et al.* 1997 and Wambolt *et al.* 1999]. This difference in the detection of function during severe LFI can be explained by differences in the perfusion model. Myocardial function is monitored in Langendorff perfusions via a balloon (connected to a pressure transducer) placed in the left ventricle. This is not possible in the working heart where function is measured distant from the heart via a pressure transducer placed in the aortic outflow line. Therefore, the slightest contraction of the left ventricle will be detected more easily in the Langendorff mode.

Contracture was not observed in any of the LFI studies. This is in agreement with the work of Cross who only saw myocardial contracture during LFI upon depletion of glycogen stores when glucose was not present in the perfusate [Cross *et al.* 1996].

4:4:5:2 Global ischaemic perfusions

In all GI studies, myocardial function ceased within 7 minutes from the start of ischaemia. No inotropic effects of insulin were observed when insulin was present during GI, nor was myocardial function prolonged. The more rapid decrease in, and cessation of, function observed during GI compared to LFI was most likely due to inhibition of glycolysis in GI due to no washing out of glycolytic end products. Contracture was not observed in GI studies. The onset of contracture in GI has been observed to occur only when glycogen levels become fully depleted [King *et al.* 1995].

In a review by King and Opie, it was postulated that energy production from anaerobic glycolysis and glycogenolysis may be used for distinct purposes under ischaemic conditions [King and Opie 1998]. The intracellular location of glycolytic enzymes are found close to the sarcolemma [Ottaway and Mowbray 1977], whereas glycogen particles are located near to myofibrils [Berne and Levy 1993]. However, during ischaemia the mechanisms of ATP transport produced

from either source may be limited. It was therefore proposed that glycolytically derived ATP appears to be closely involved with regulating ionic homeostasis at the membranes whereas glycogen derived ATP is present primarily at the myofibrils and may attenuate contracture [King and Opie 1998]. Therefore in this study, contracture may not be observed due to the availability of glycogen to provide energy to the myofibrils.

4:4:6 Conclusions

Physiological responses to ischaemic insults were similar in control and hypertrophy hearts, indicating that there was not an increased susceptibility to ischaemic injury in the hypertrophied heart. Glycogen utilisation during different ischaemic protocols was comparable between S and CH hearts. The severity of ischaemic flow was the major determinant for glycogen degradation, but insulin may preserve glycogen levels during LFI. Contracture was not observed in any of the ischaemic experiments. Therefore the provision of energy from glycogen during ischaemia appeared to outweigh the detrimental accumulation of end products in this compensated model of hypertrophy.

5: REPERFUSION STUDIES IN CARDIAC HYPERTROPHY

5:1 Introduction

5:1:1 Susceptibility of the hypertrophied heart to reperfusion injury

The presence of pressure overload hypertrophy increases the severity of mechanical dysfunction during reperfusion of hearts following ischaemia [Anderson *et al.* 1987, Gaasch *et al.* 1990]. Alterations in energy metabolism have been implicated as contributing to this enhanced deterioration of function in the hypertrophied heart [Anderson *et al.* 1987, Gaasch *et al.* 1990, El Alaoui-Talibi *et al.* 1992].

5:1:2 Carbohydrate metabolism at reperfusion

The contribution of glucose to myocardial energy provision during reperfusion is uncertain. The factors that determine whether glucose taken up by the post-ischaemic myocardium is energetically metabolised, as opposed to be being stored as glycogen, remain unclear [M^{c}Nulty *et al.* 1996, M^{c}Nulty *et al.* 2000].

Turnover of glycogen is normally slow in the heart [Goodwin *et al.* 1995], but it is rapidly consumed during ischaemia. During reperfusion following a brief coronary occlusion [M^{c}Nulty *et al.* 1995], as well as in prolonged low flow ischaemia [M^{c}Nulty *et al.* 1996], glycogen synthase was activated, catalysing increased glycogen resynthesis. In contrast in a recent ^{13}C-NMR study myocardial glycogen synthase was not activated in regions reperfused after 20 minutes of coronary occlusion [M^{c}Nulty *et al.* 2000]. An increase in ^{13}C labelling of glycolytic end products was observed in the reperfused regions indicating that there was a shunting of imported ^{13}C-glucose from glycogen repletion towards energy generating pathways [M^{c}Nulty *et al.* 2000].

Several studies have suggested that a rapid recovery of glycolysis is important for the recovery of heart function following ischaemia [Mallet *et al.* 1990, Eberli

et al. 1991, Jeremy *et al.* 1992]. Evidence suggests that glycolytically derived ATP, formed in the cytosol, is used preferentially to support cytosolic ionic homeostasis whereas oxidatively formed ATP is thought to support mechanical work [Weiss and Hiltbrand 1985]. Glycolysis may in fact be coupled to sarcoplasmic reticulum calcium transport [Xu *et al.* 1995] as investigations with Ca^{2+} antagonists during reperfusion decreased post ischaemic myocardial dysfunction [du Toit and Opie 1992]. Indeed, stimulation of glycolysis in the reperfused myocardium reduced cytosolic Ca^{2+} accumulation [Jeremy *et al.* 1992]. Thus, increased glycolytic flux during reperfusion may contribute to maintaining cellular viability.

Controversy exists as to whether glycolytic capacity is decreased or increased during reperfusion in the hypertrophied heart [Cunningham *et al.* 1990, Allard *et al.* 1994, Schönekess *et al.* 1996]. Glycolytic capacity was reported to be impaired in hypertrophied hearts during hypoxia and reoxygenation, which contributes to contractile dysfunction [Cunningham *et al.* 1990]. This is in contrast to the situation during normoxia, where increased glycolytic rates have been observed in hypertrophied myocardia [Allard *et al.* 1994]. Schönekess suggests that there is no impairment in the recovery of glycolysis or oxidative metabolism in the hypertrophied heart during post-ischaemic reperfusion [Schönekess *et al.* 1996], but rather the efficiency of converting ATP produced into mechanical function decreases.

After brief periods of global ischaemia, oxidative metabolism rapidly returns, well before the restoration of contractility [Liu *et al.* 1996a]. Stimulation of glucose oxidation at reperfusion has been shown to accelerate functional recovery whereas inhibition impairs post-ischaemic contractility [Tamm *et al.* 1994].

Improvements are observed in cellular ionic homeostasis upon stimulation of PDC, increasing glucose oxidation, by preventing the accumulation of glycolytic

protons [M^{c}Veigh and Lopaschuk 1990]. PDC consumes the net protons that are produced with the breakdown of glycolytically synthesised ATP. When glycolysis is not coupled to glucose oxidation and pyruvate derived from glycolysis is metabolised to lactate, there is a net production of 2 H^+ from each glucose molecule, which originates from the hydrolysis of glycolytically derived ATP [Stanley *et al.* 1997]. The resulting accumulation of protons may stimulate the Na^+/H^+ transporter during ischaemia and at reperfusion resulting in an accumulation of Na^+ that activates the sarcolemmal Na^+/Ca^{2+} exchanger [Tani 1990]. A high rate of Na^+/Ca^{2+} exchange could consequently lead to Ca^{2+} overload and cell death [Tani 1990].

5:1:3 Fatty acid metabolism in the reperfused heart

Viable myocardium regains the ability to oxidise fatty acids at reperfusion [Saddik and Lopaschuk 1992]. After an initial burst of endogenous fatty acid oxidation during reperfusion, the heart predominantly relies upon exogenously derived fatty acids for oxidation [Saddik and Lopaschuk 1992]. Studies have demonstrated that there is an overshoot in the rate of fatty acid oxidation during reperfusion at the expense of pyruvate oxidation [Saddik and Lopaschuk 1992].

The reason for impaired myocardial pyruvate oxidation during reperfusion may be due to low tissue malonyl CoA levels [Stanley *et al.* 1997]. A decrease in malonyl-CoA levels relieves the inhibition at CPT 1 [Kudo *et al.* 1995] and fatty acid oxidation. The fall in malonyl CoA levels corresponds to a fall in ACC activity [Kudo *et al.* 1995]. AMP accumulation during ischaemia stimulates AMP-activated kinase, which phosphorylates ACC, decreasing the activity of ACC [Kudo *et al.* 1995]. Therefore, the decrease in malonyl CoA levels relieves the inhibition on CPT 1, increasing the transport of fatty acids into the mitochondria for oxidation. Increased fatty acid oxidation increases the mitochondrial NADH/NAD^+ and acetyl CoA/CoA ratios, which enhance PDC kinase activity, resulting in inhibition of PDC [Patel and Roche 1990].

High levels of fatty acids, which were observed during reperfusion, can also contribute to the severity of ischaemic injury [Saddik and Lopaschuk 1992]. In addition to the detergent effects of these amphipathic molecules, the detrimental effects of fatty acids following ischaemia appear to be more closely related to their ability to inhibit glucose oxidation [Lopaschuk *et al.* 1990]. In support of this, stimulation of glucose oxidation during reperfusion in normal hearts directly, by the inhibition of PDC kinase with dichloroacetate [M^{c}Veigh and Lopaschuk 1990], or indirectly with CPT 1 inhibitors [Lopaschuk *et al.* 1990] improved functional recovery. Beneficial effects were also observed in functional recovery when pyruvate was the sole energy providing substrate at reperfusion [Bunger *et al.* 1989]. Therefore, this may indicate that anaplerotic sequences may need to be activated upon reperfusion to replenish TCA cycle intermediates depleted during ischaemia.

In addition to the above, fatty acid oxidation can be depressed in CH (even in the absence of ischaemia), which may be due to a decrease in myocardial carnitine content [El Alaoui-Talibi *et al.* 1992]. This may lead to a further decrease in energy reserve in the hypertrophied heart during reperfusion [Ingwall 1984], as evidenced by a decreased intra-mitochondrial NADH: NAD^{+} ratio [Cheikh *et al.* 1994] that may further exacerbate injury.

5:1:4 Objectives

Post-ischaemic glucose metabolism may be beneficial for recovery of function during reperfusion, whereas the reliance on fatty acids as energy substrate may be detrimental. Alterations in the utilisation of fatty acids with an increased reliance on glucose metabolism have been observed in CH, but the effects of this during reperfusion are unclear. Similarly, insulin can cause a shift from utilisation of fatty acids to glucose which may prove beneficial for myocardial functional recovery on reperfusion.

Work was undertaken in this chapter to;

1) Assess the susceptibility of the hypertrophied heart to reperfusion injury
2) Investigate the role of insulin in myocardial recovery on reperfusion

5:2 Methods

Hearts were studied 9 weeks post surgery as previously described (Section 2.5).

After a 20 minute equilibration period, hearts were perfused for 45 minutes of normoxic perfusion, followed by 20 minutes of global ischaemia (GI) and 30 minutes of reperfusion. Perfusion protocols are displayed in Figures 5.1 and 5.3. In summary, hearts were perfused with physiological levels of;

- glucose and palmitate in the absence of insulin (REP, Figure 5.1)
- glucose and palmitate in the presence of insulin (REPI, Figure 5.1)
- glucose and palmitate in the presence of insulin with [1-^{13}C]-glucose and [1-^{13}C]-palmitate during reperfusion (REPI13C, Figure 5.1)
- insulin added at the time of reperfusion with the presence of [1-^{13}C]-glucose and [1-^{13}C]-palmitate during reperfusion (REPIR, Figure 5.2)
- glucose, palmitate, lactate and pyruvate present with insulin throughout with [1-^{13}C]-glucose and [1-^{13}C]-palmitate during reperfusion (REPLPI, Figure 5.2)

^{13}C Labelled substrates were present during the reperfusion phase of REPIR and REPLPI to enable the assessment of the relative contributions of substrates to energy provision during this phase (Figure 5.2).

During the GI phase flow was reduced to zero. Immediately after switching off the pump, foil was placed completely round the perfusion chamber and cannula to minimise temperature loss during ischaemia. Flow was re-established by switching the pump on. All insulating foil was removed at the start of reperfusion.

Effluent samples from REP, REPI13C and REPLPI were taken at 20 and

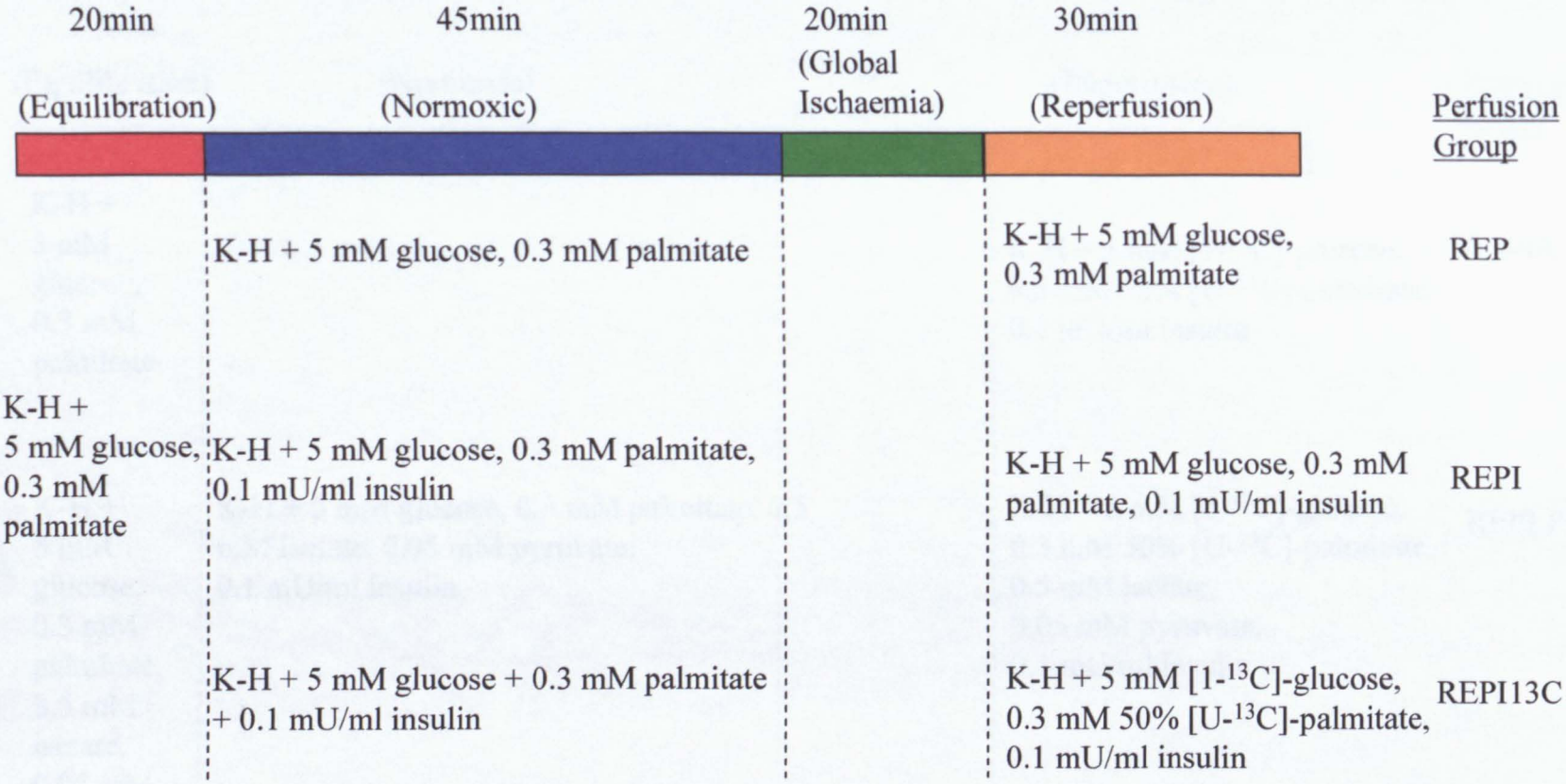


Figure 5.1: Reperfusion (REP) perfusion protocols

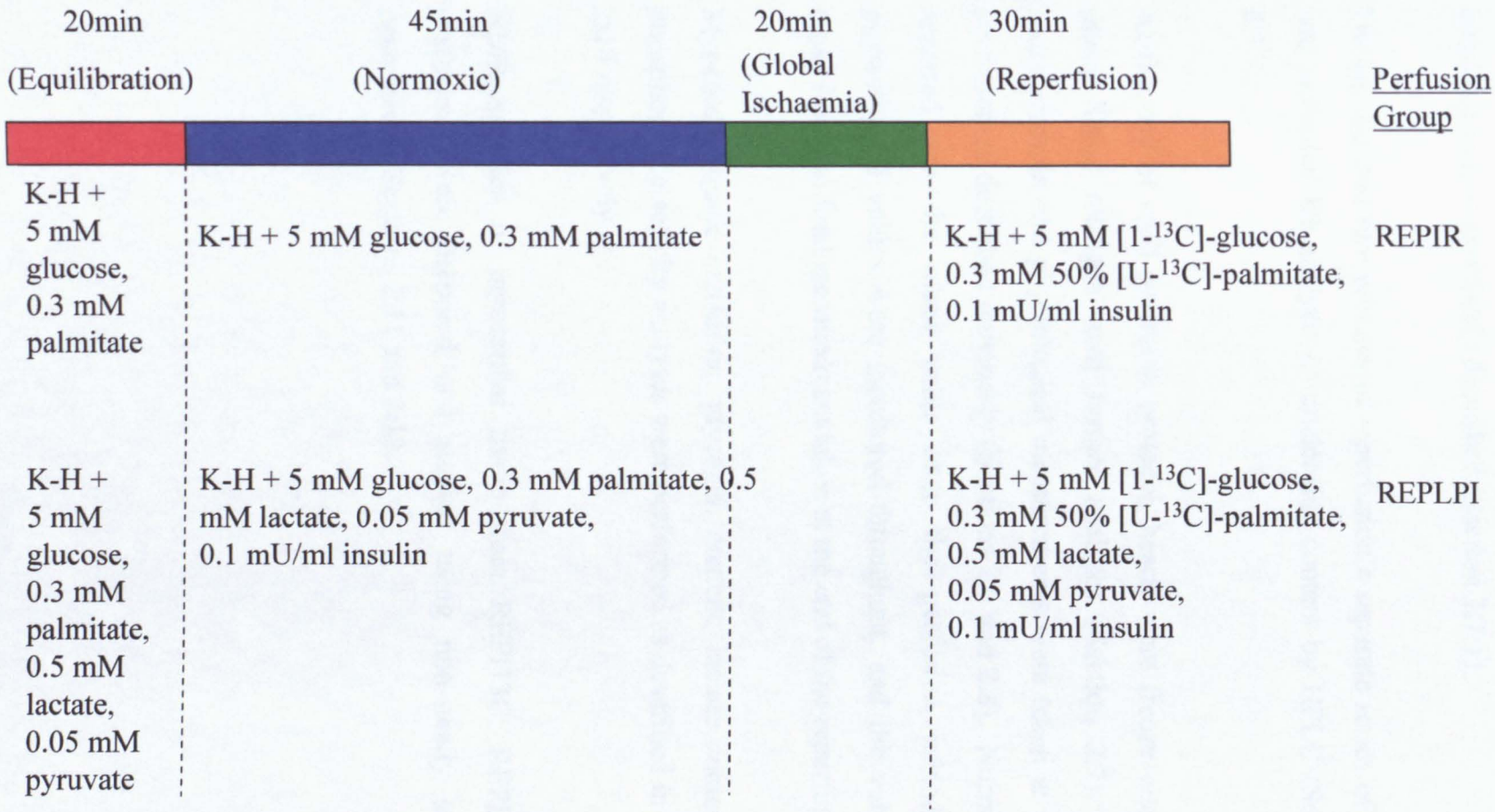


Figure 5.2: Reperfusion (REP) perfusion protocols

40 minutes during normoxia and at 5, 15 and 25 minute reperfusion time points for analysis on a blood gas analyser to determine myocardial oxygen consumption, as previously described (Section 2:7:1).

During the first five minutes of reperfusion a separate series of effluent samples was collected for analysis of nucleoside content by HPLC (Sections 2:7:2 and 2:13).

At the end of each perfusion protocol, hearts were freeze clamped and stored under liquid nitrogen until further analyses (Section 2.7). Morphological measurements and physiological measurements were taken at the time of each perfusion as described previously (Sections 2.6 and 2.8). Normoxic RPP values reported are the mean value over the perfusion period. Reperfusion physiological values were monitored throughout, and the values reported are those from the final measurement taken at the end of the reperfusion period.

Myocardial tissue extraction, glycogen content, lactate content and glycogen phosphorylase activity analyses were performed as described in Sections 2:9 and 2:10 respectively.

NMR samples of myocardial tissue from REPI13C, REPIR and REPLPI perfusions were prepared and studied using non-steady state analysis as described in Sections 2:11 and 2:12.

5:3 Results

5:3:1 Functional status and physiological results

Recovery of myocardial function was deemed to have occurred when the RPP at the end of reperfusion was equal to or greater than 50 % of that observed during normoxia. Recovery of each heart was assessed against individual normoxic physiological measurements rather than compared to the mean of the group. Whereas, all other results are expressed as mean ± sem where statistically possible.

5:3:1:1 Glucose and palmitate only

No significant differences were observed in physiological parameters (LVDP, HR and RPP) between S and CH hearts during normoxia (REP, Table 5.1). Function rapidly ceased in all reperfusion groups during GI. Upon restoration of flow, recovery of myocardial function (Figure 5.3) was only observed in 25 % of S and CH hearts.

5:3:1:2 Glucose and palmitate with insulin throughout

Results from perfusion groups REPI and REPI13C were combined as they differed only by the inclusion of ^{13}C-labelled substrates in the REPI13C group.

An increase in normoxic LVDP was observed in CH compared to S hearts when insulin was present in the perfusate (REPI + REPI13C combined, Table 5.1, $p<0.05$). However, this did not translate to an increase in myocardial workload in CH hearts as the RPP calculated for S and CH hearts was not found to be statistically different. The normoxic RPP did not increase in S and CH hearts in the presence of insulin (REPI + REPI13C combined versus REP, Table 5.1).

The presence of insulin markedly improved the proportion of S and CH hearts recovering function at the end of reperfusion to 75 % and greater (REPI

Perfusion Group	Model (Number)	Perfusion Phase	LVDP (mmHg)	HR (bpm)	RPP ($x10^3$ mmHg.min)	% Recovery of RPP at end of Reperfusion
REP	S (n=4)	Normoxia	90.7 ± 11.9	237 ± 8	21.2 ± 3.3	
	CH (n=4)		97.8 ± 6.8	233 ± 11	23.1 ± 1.7	
	S (n=1)	Reperfusion (recovered)	113.3	231	26.1	97.0
	CH (n=1)		82.5	237	19.5	66.6
	S (n=3)	Reperfusion (did not recover)	14.0 ± 6.4▼	299 ± 45	4.9 ± 2.6▼	
	CH (n=3)*		9.0	83	0.8	
REPI + REPI13C combined	S (n=12)	Normoxia	96.2 ± 3.7	253 ± 11	24.1 ± 1.1	
	CH (n=10)		109.8 ± 3.6°	247 ± 12	27.1 ± 1.6	
	S (n=9)	Reperfusion (recovered)	88.5 ± 11.8	256 ± 15	23.9 ± 1.5	106.3 ± 8.4
	CH (n=7)		118.2 ± 10.1	257 ± 10	30.0 ± 2.6	111.5 ± 7.7
	S (n=3)	Reperfusion (did not recover)	21.5 ± 9.9▼	476 ± 93	7.5 ± 1.6▼	
	CH (n=3)		24.0 ± 10.7▼	446 ± 80	8.4 ± 2.1▼	

* - Of the three CH hearts that did not recover, two did not recover any function at all, therefore only values for one CH heart are shown.
° - Significantly different from corresponding sham ($p<0.05$)
▼ - Significantly different from corresponding model in normoxia ($p<0.01$)

Table 5.1: Physiological parameters during normoxia and reperfusion in the presence and absence of insulin

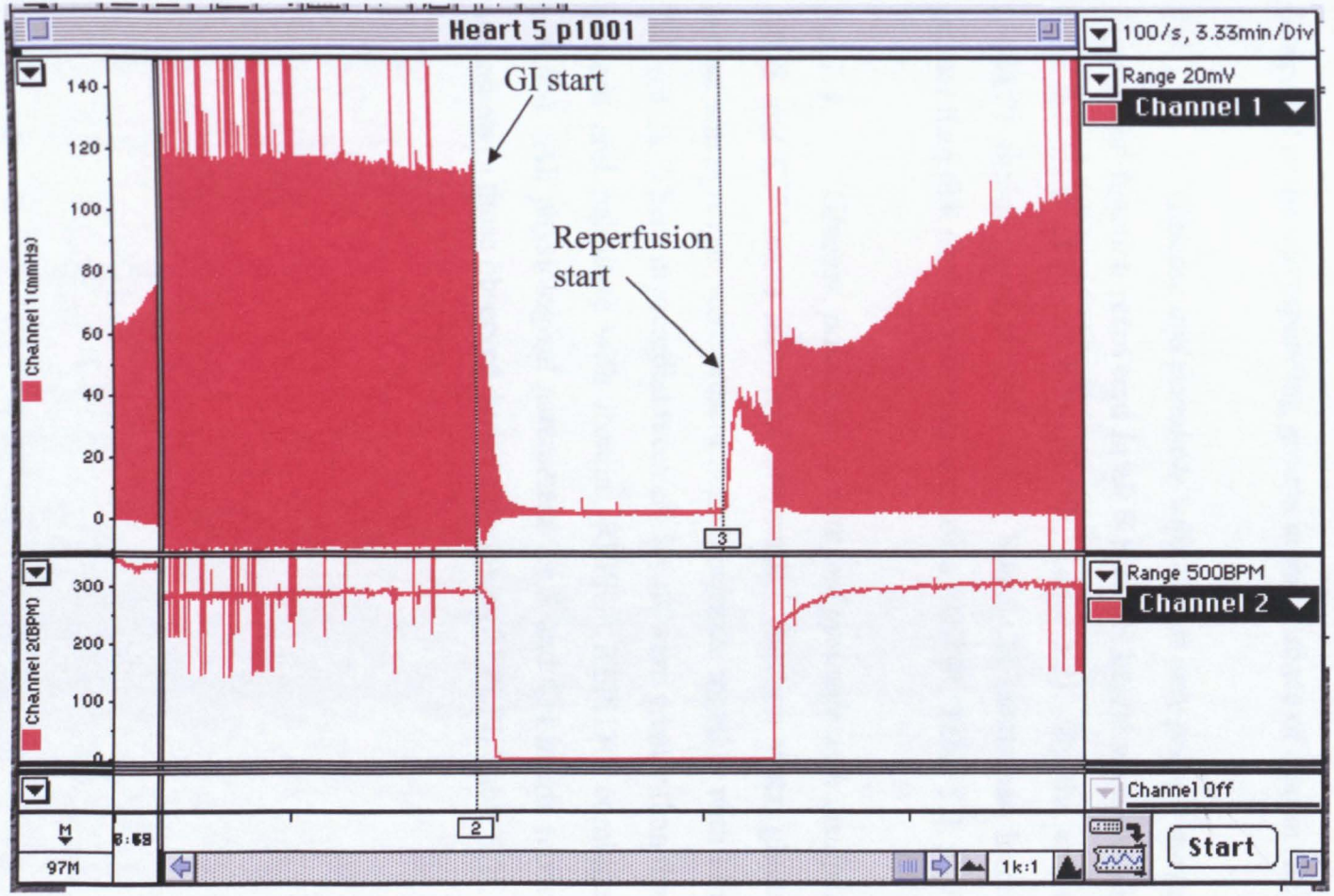


Figure 5.3: A typical perfusion trace showing myocardial functional recovery in the presence of insulin during reperfusion

+REPI13C combined, Table 5.1). Complete functional recovery to normoxic levels was observed in these hearts. Although approximately 25 % of S and CH hearts did not recover to 50 % of normoxic function, improved levels of recovery were observed in these groups (REPI + REPI13C combined, Table 5.1, $p<0.01$) compared to the corresponding groups in the absence of insulin (REP, Table 5.1).

5:3:1:3 Glucose and palmitate with insulin only present during reperfusion

Myocardial function recovered in all S and CH hearts when insulin was present from the onset of reperfusion (REPIR, Table 5.2). By the end of reperfusion ($p<0.05$), the myocardial workload of S and CH hearts had increased to levels greater than that observed during normoxia (REPIR, Table 5.2, $p<0.05$).

5:3:1:4 Glucose, palmitate, lactate and pyruvate with insulin throughout

All S and CH hearts recovered myocardial function when glucose, palmitate, lactate and pyruvate were present in the perfusate together with insulin (REPLPI, Table 5.2). These myocardial recovery levels were greater than that observed for glucose and palmitate with insulin (REPI + REPI13C combined, Table 5.1, $p<0.05$). All physiological parameters for S and CH hearts recovered to levels analogous to those observed during normoxia (REPLPI, Table 5.2).

Perfusion Group	Model (Number)	Perfusion Phase	LVDP (mmHg)	HR (bpm)	RPP ($x10^3$ mmHg.min)	% Recovery of RPP at end of Reperfusion
REPIR	S (n=5)	Normoxia	100.7 ± 9.6	221 ± 21	19.2 ± 1.8	
	CH (n=5)		103.6 ± 3.8	227 ± 12	20.3 ± 2.3	
	S (n=5)	Reperfusion (recovered)	96.1 ± 8.8	242 ± 30	23.6 ± 2.1•	102.5 ± 7.2
	CH (n=5)		109 ± 3.6	249 ± 14	28.3 ± 2.9•	121.0 ± 6.9
REPLPI	S (n=4)	Normoxia	95.5 ± 1.0	288 ± 15	27.5 ± 1.2	
	CH (n=4)		93.1 ± 7.4	246 ± 19	22.7 ± 2.2	
	S (n=4)	Reperfusion (recovered)	86.6 ± 15.3	281 ± 19	26.7 ± 2.4	97.7 ± 8.9
	CH (n=4)		94.8 ± 8.8	249 ± 18	23.5 ± 2.3	104.1 ± 7.4

• - Significantly different from corresponding model in normoxia ($p<0.05$)

Table 5.2: Physiological parameters during normoxia and reperfusion

5:3:2 ^{13}C-NMR substrate utilisation studies

^{13}C-NMR spectroscopy was performed on myocardial extracts, from perfusions using 1-^{13}C glucose and U-^{13}C palmitate, to assess glucose and palmitate oxidation under non-steady state conditions. An experimental non steady state ^{13}C-NMR spectrum is displayed in Figure 5.4.

The relative contributions of glucose and palmitate to oxidative metabolism during reperfusion for REPI13C, REPIR and REPLPI are displayed in Figures 5.5, 5.6 and 5.7 respectively. No differences were observed between the relative contributions of glucose or palmitate between S and CH hearts within any of the experimental groups.

Decreases of more than 47 % in the relative contribution of glucose during reperfusion were observed in S and CH hearts when insulin was only present from the time of reperfusion (REPIR, Figure 5.6) compared to when insulin was present throughout (REPI13C, Figure 5.5, $p<0.01$). There was no alterations in the relative contributions of palmitate to the TCA cycle between REPIR (Figure 5.6) and REPI13C (Figure 5.5) indicating that a greater proportion of unlabelled substrates were contributing to energy production from the TCA cycle when insulin was administered at the time of reperfusion (REPIR, Figure 5.6, $p<0.05$).

The relative contributions of glucose to the TCA cycle in S and CH hearts were also markedly decreased (by over 35 %) when glucose, palmitate, lactate, pyruvate and insulin were present in the perfusate (REPLPI, Figure 5.7, $p<0.05$) with no changes in the contribution from palmitate.

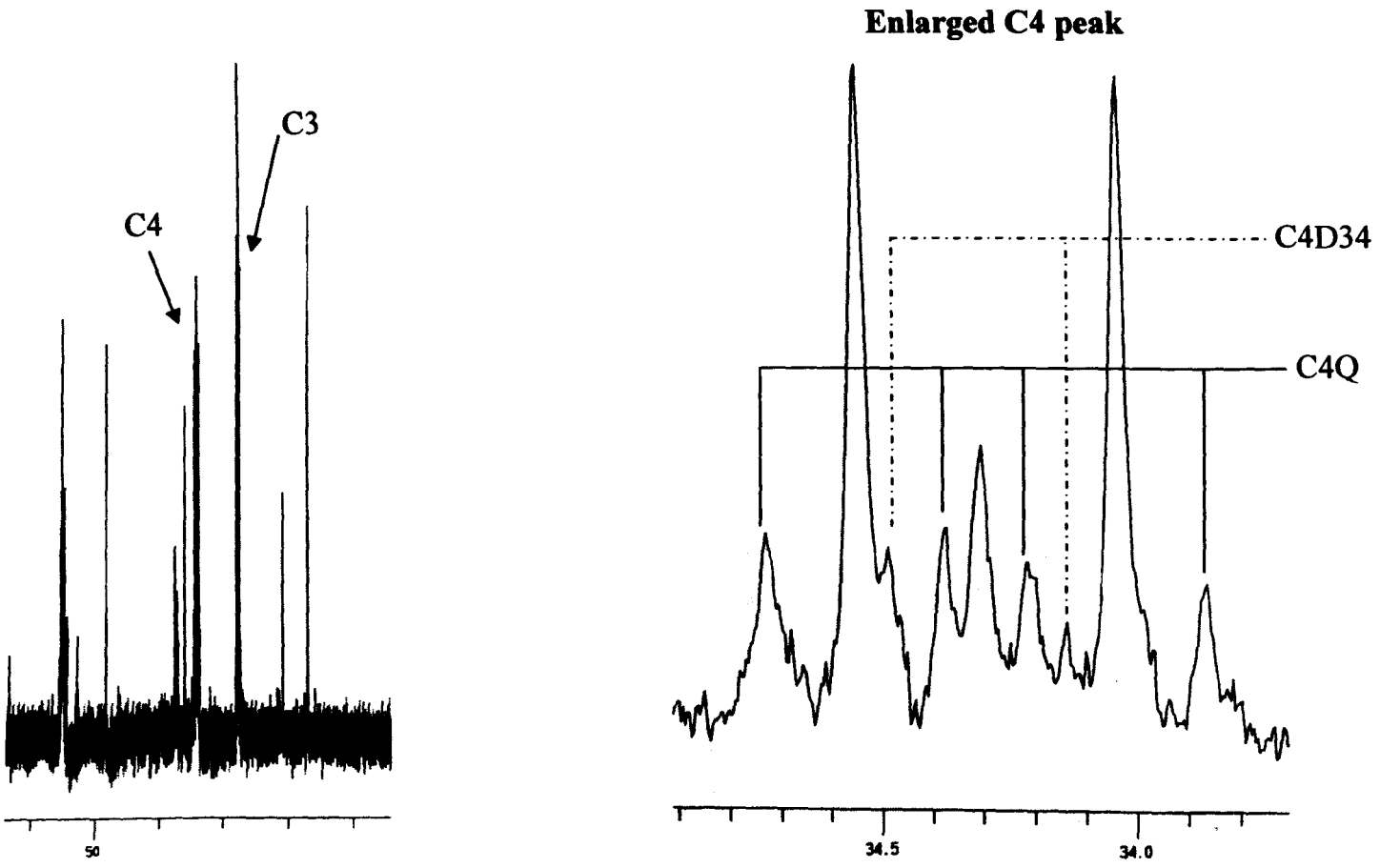


Figure 5.4: ^{13}C-NMR spectrum of C3 and C4 of glutamate from a reperfusion experiment, the enlarged C4 peak area shows the multiplet patterns used for non-steady state analysis

Figure 5.5: Relative contribution of various energy providing substrates to the TCA cycle during reperfusion from REPI13C

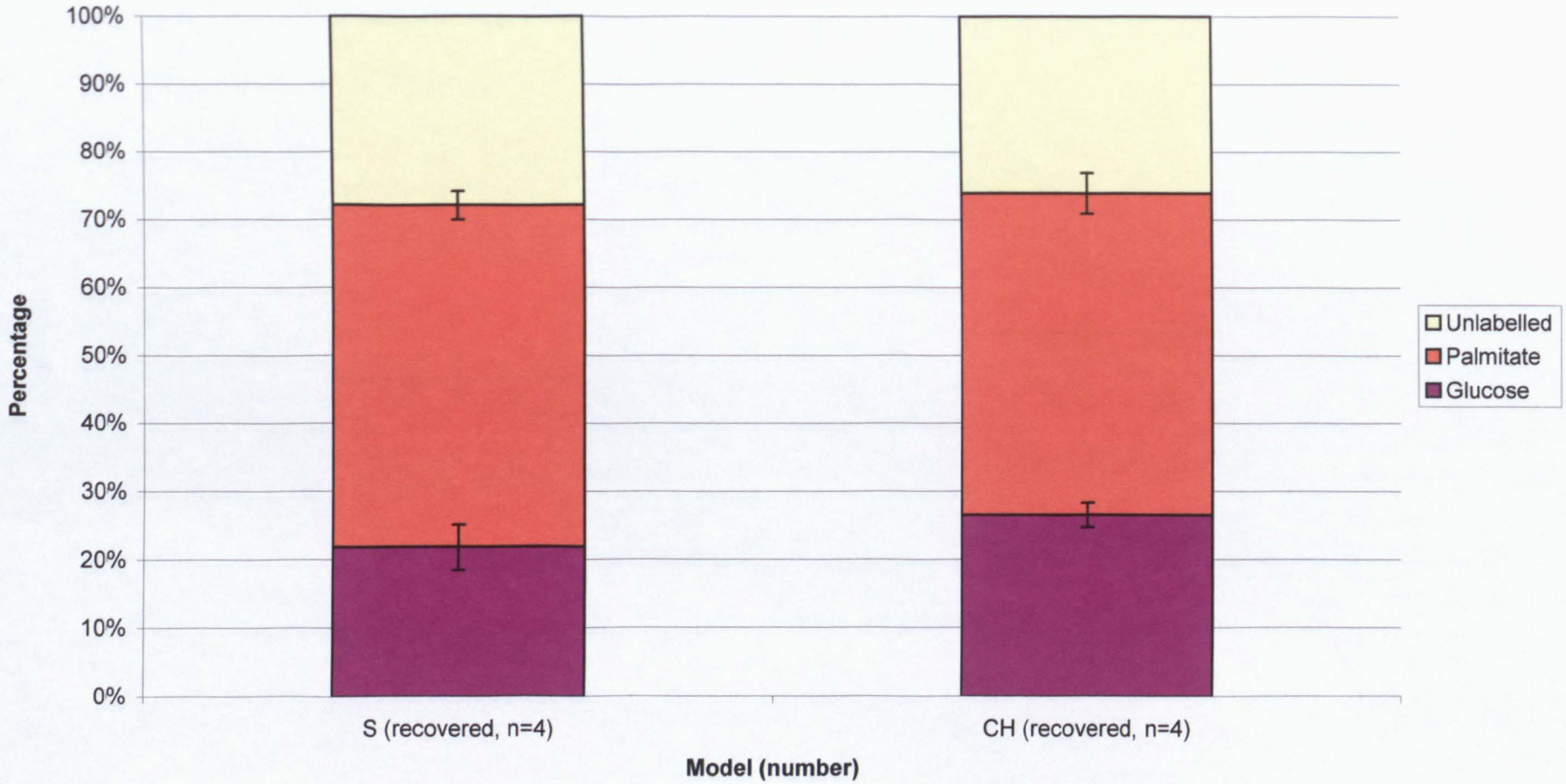


Figure 5.6: Relative contribution of various energy providing substrates to the TCA cycle during reperfusion from REPIR

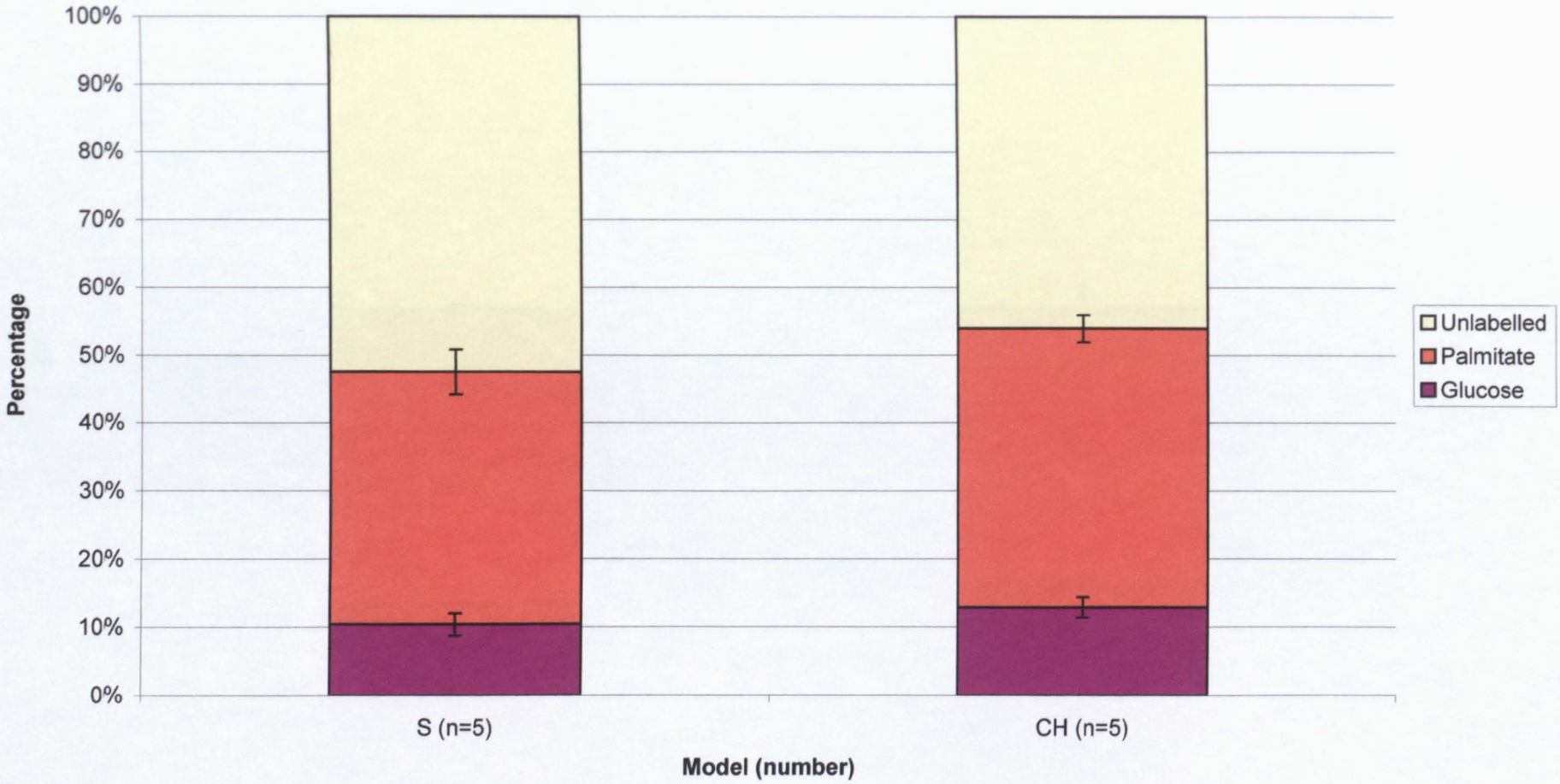
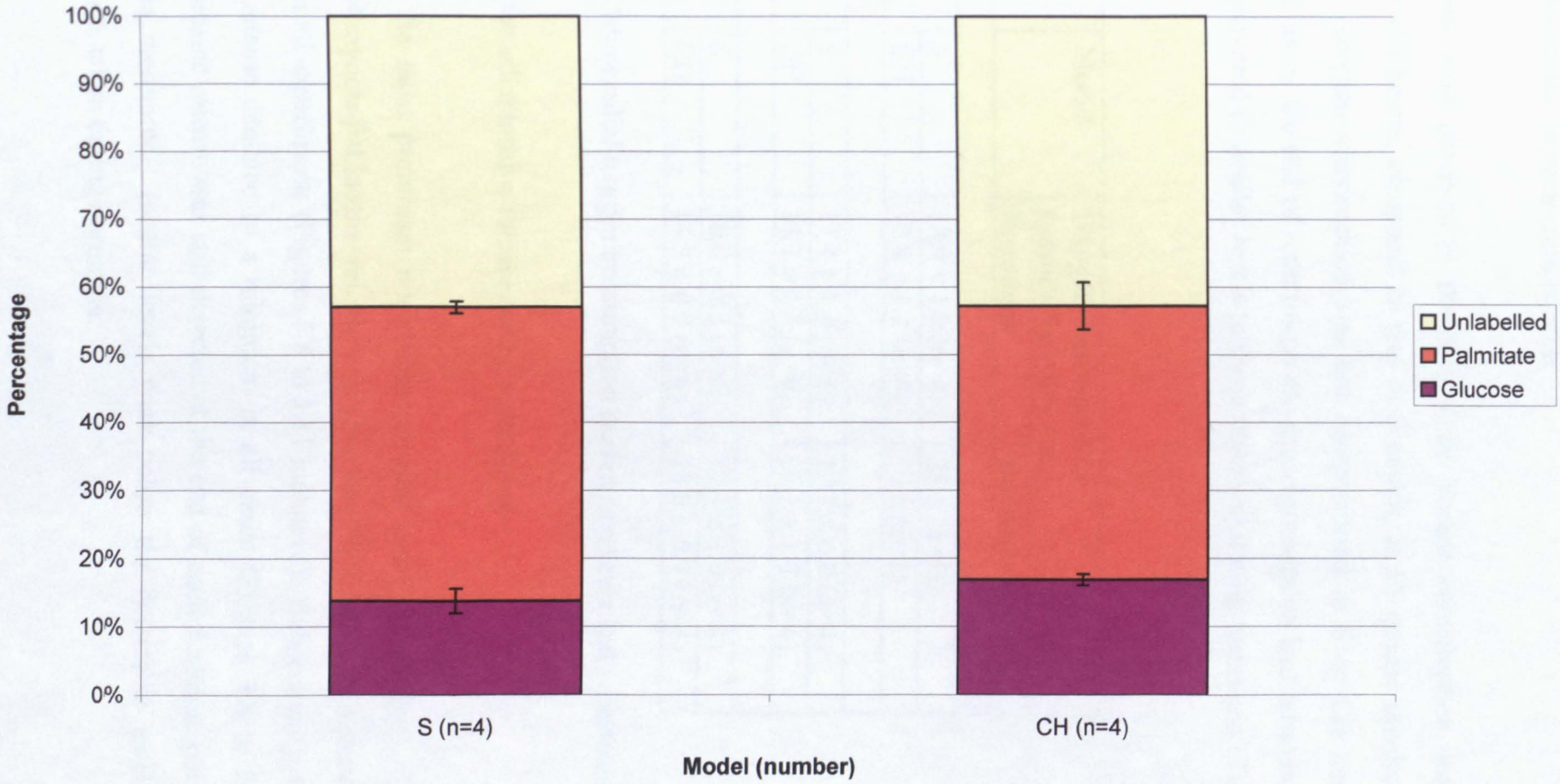


Figure 5.7: Relative contribution of various energy providing substrates to the TCA cycle during reperfusion from REPLPI



5:3:3 Myocardial oxygen consumption

No differences were observed in the amount of oxygen consumption during normoxia by CH hearts, compared to that of S hearts, in all groups monitored (Table 5.3). Oxygen consumption was not compromised in S or CH hearts following GI, as by the end of reperfusion oxygen consumption had returned in hearts that recovered to similar levels to those observed during normoxia (Table 5.3).

Perfusion Group	**Model**	**Oxygen Consumption (μmol/min/g dry wt.)**	
		Normoxia	**Reperfusion**
REP	S	18.0 ± 1.0 (n=4)	28.1 (n=1)
	CH	16.4 ± 1.1 (n=4)	19.6 (n=1)
REPI13C	S	17.4 ± 1.4 (n=5)	17.7 ± 0.8 (n=4)
	CH	14.7 ± 1.3 (n=6)	16.1 ± 1.3 (n=4)
REPLPI	S	16.0 ± 0.3 (n=4)	17.1 ± 1.0 (n=4)
	CH	14.9 ± 0.9 (n=4)	15.0 ± 0.7 (n=4)

Table 5.3: Myocardial oxygen consumption during normoxia and reperfusion

5:3:4 Myocardial inosine release during reperfusion

Inosine was the most prominent nucleoside released upon reperfusion. The highest rate of myocardial inosine release was observed within the first minute of reperfusion in all experiments (Figures 5.8 to 5.11 inclusive). Subsequently, this initial rapid release declined to a minimum in all cases (Figures 5.8 to 5.11 inclusive). Inosine release was still detected at the end of each 5 minute period of reperfusion monitored. Inosine levels were below the detectable level in effluent samples taken during normoxia.

Figure 5.8: Myocardial inosine release of hearts that recovered during reperfusion from REP

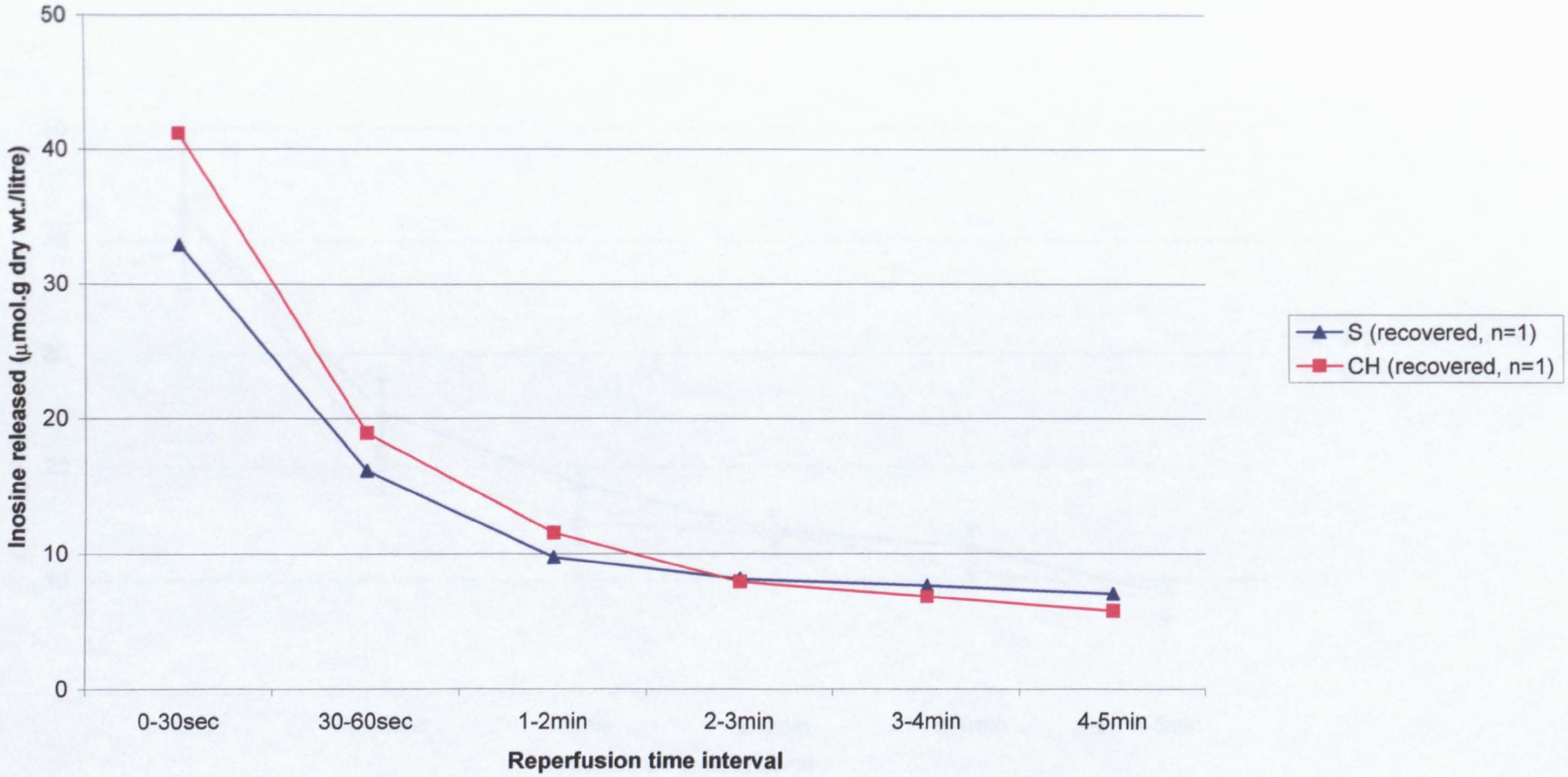


Figure 5.9: Myocardial inosine release of hearts that recovered during reperfusion from REPI and REPI13C combined

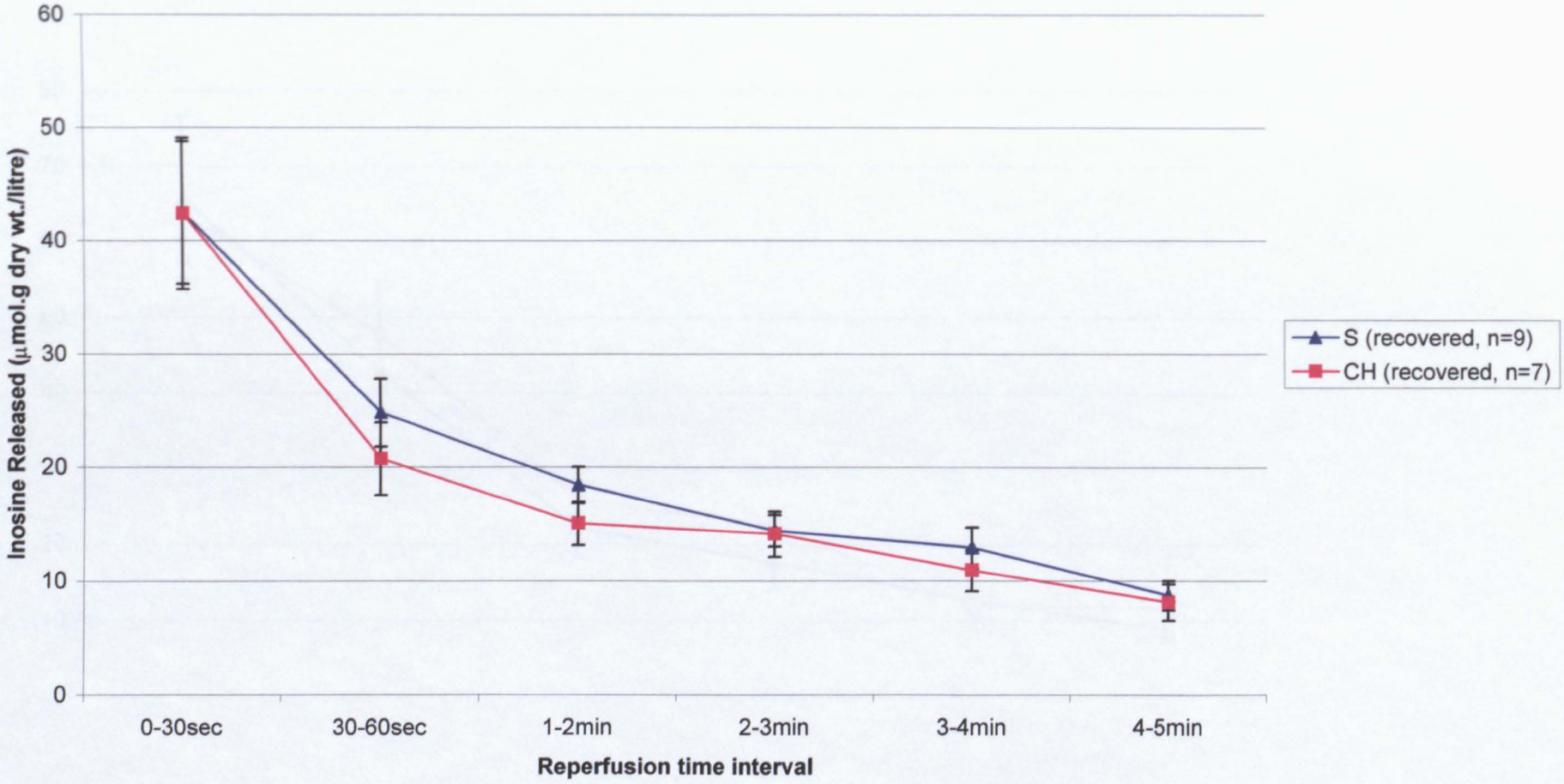


Figure 5.10: Myocardial inosine release of hearts that recovered during reperfusion from REPIR

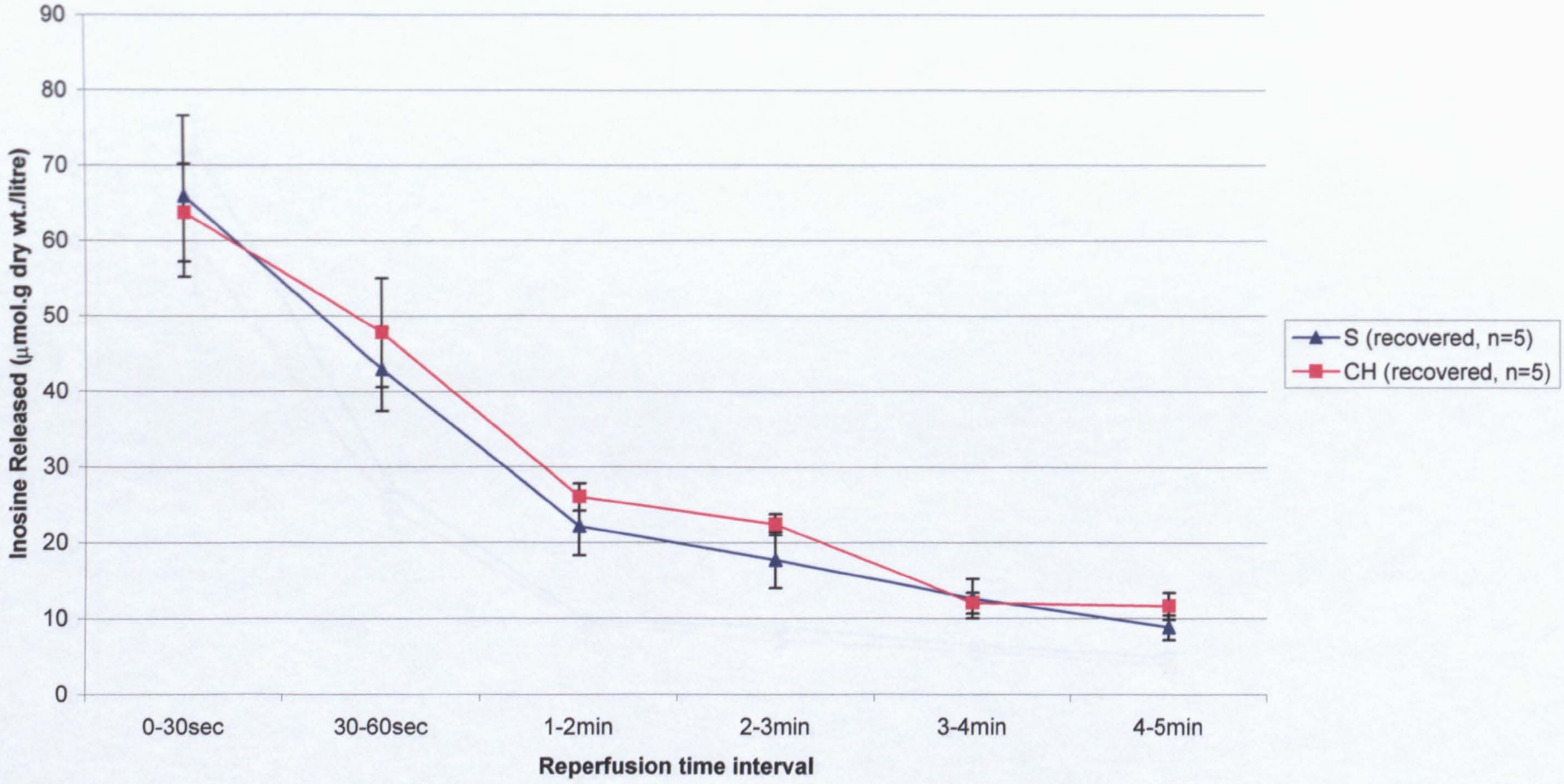
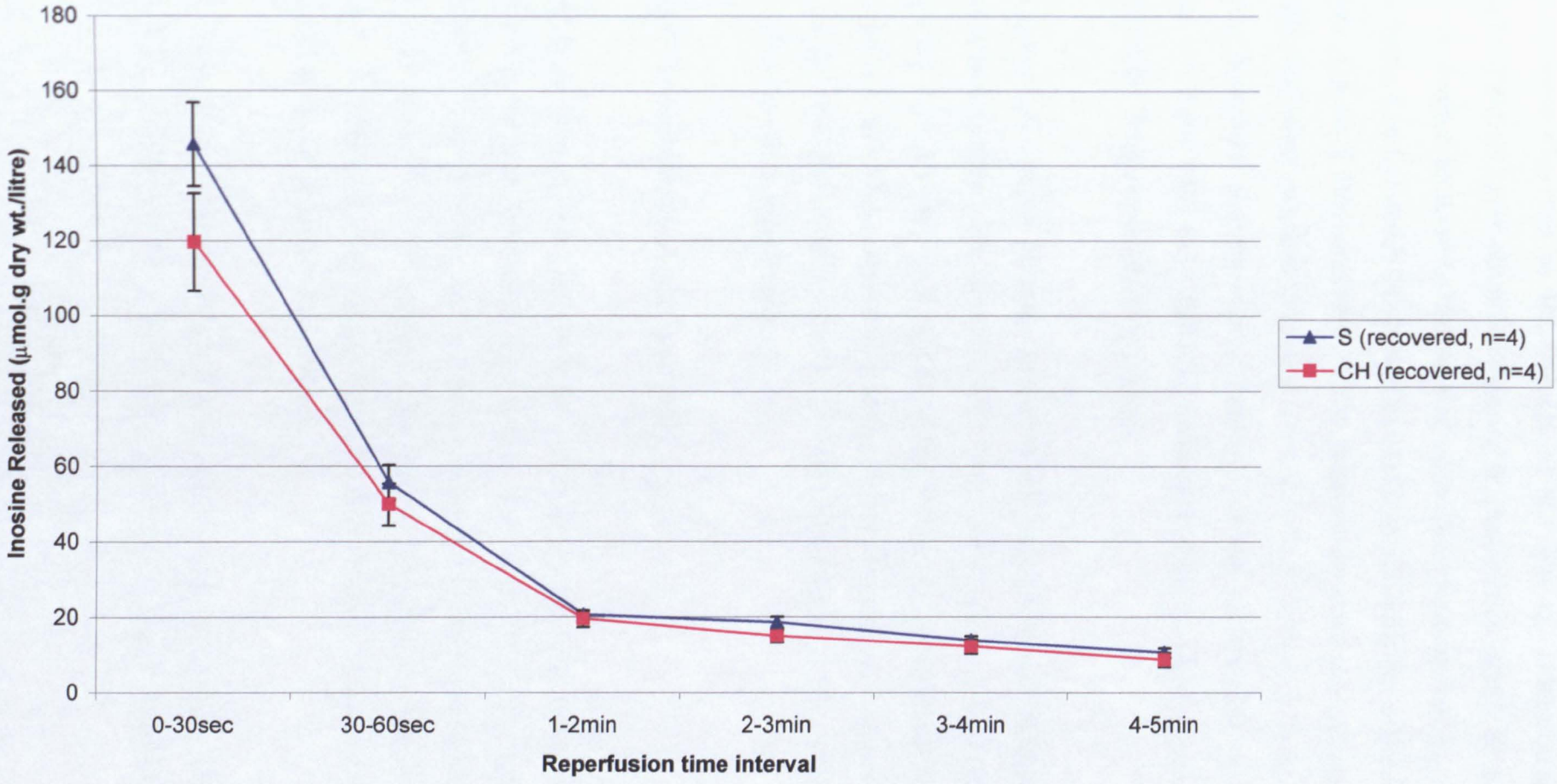


Figure 5.11: Myocardial inosine release of hearts that recovered during reperfusion from REPLPI



No differences were observed in the amount or the rate of inosine release between S and CH hearts in the absence of insulin or presence of insulin (Figures 5.8 and 5.9). A marked increase in the amount of inosine released over the first minute was observed in CH and S hearts when insulin was added only at the time of reperfusion (Figure 5.10) compared to the absence or presence of insulin throughout the perfusion protocol (Figures 5.8 and 5.9, $p<0.05$). Myocardial inosine release decreased sharply after 1 minute and was comparable to that released by hearts from REP and REPI13C perfusions after 2 minutes, and for the remainder of the 5 minute monitoring period.

The greatest extent of inosine release was observed when glucose, palmitate, pyruvate, lactate and insulin were present in the perfusate (Figure 5.11, $p<0.05$). Amounts released over the first half minute were three times as great ($p<0.01$) compared to perfusions with glucose and palmitate alone (Figure 5.8). Amounts of inosine released dropped sharply after the first minute (Figure 5.11) to levels similar to those already observed (Figure 5.10).

5:3:5 Reperfusion myocardial glycogen levels

Myocardial glycogen stores were replenished to varying degrees at the end of reperfusion in all hearts that recovered (Figures 5.12 and 5.13). In hearts that did not recover (Figure 5.12) glycogen levels remained very similar to those reported at the end of GI (Figure 4.4). No further degradation of glycogen was observed in these hearts. No difference was observed in glycogen content between S and CH hearts of any group at the end of reperfusion.

When insulin was present throughout glycogen levels increased by over 80 % (REPI & REPI13C combined, Figure 5.12) from GI levels (Figure 4.4, $p<0.05$).

Figure 5.12: Myocardial glycogen content of hearts that recovered function at the end of reperfusion (REP and REPI)

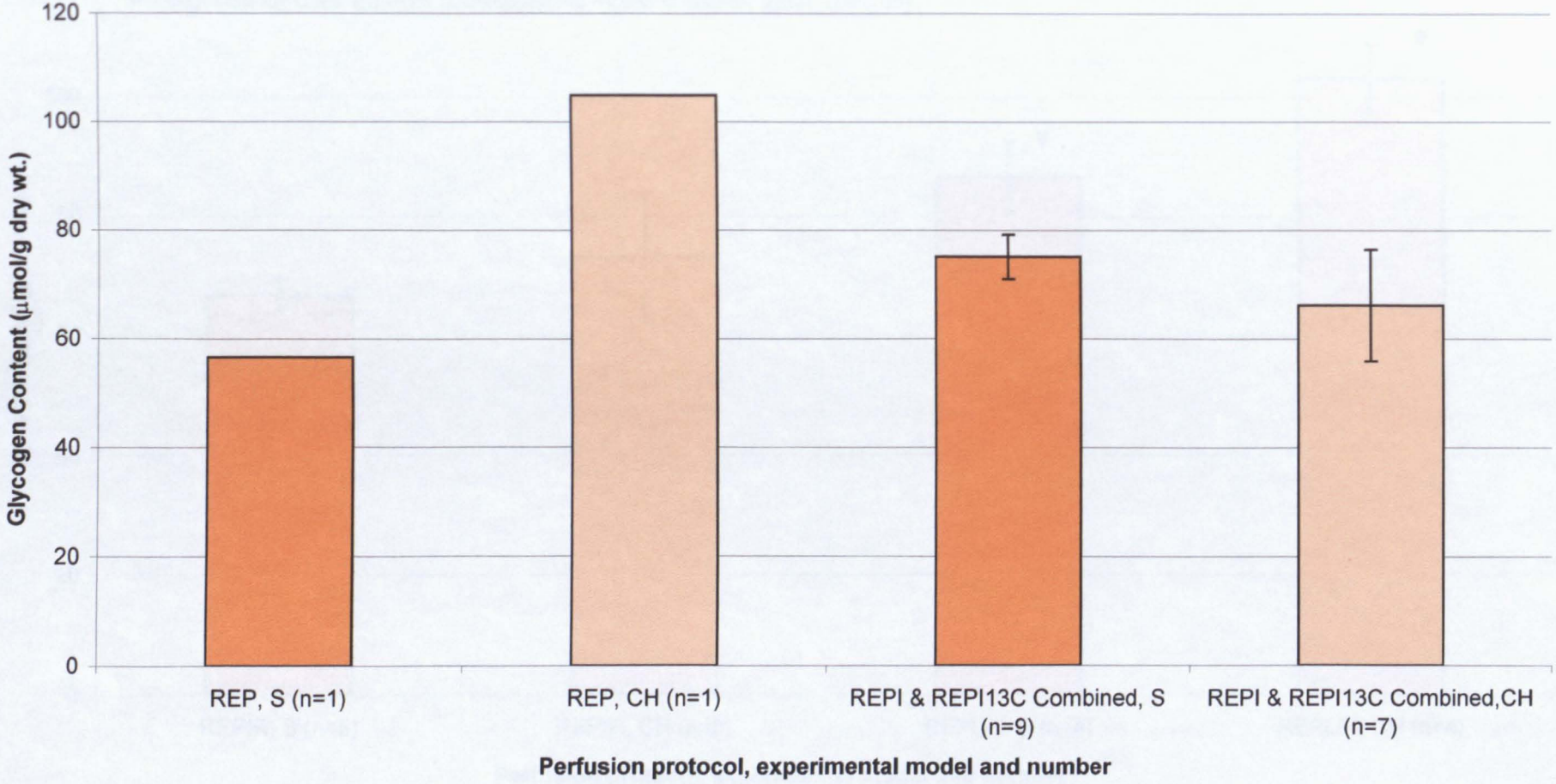
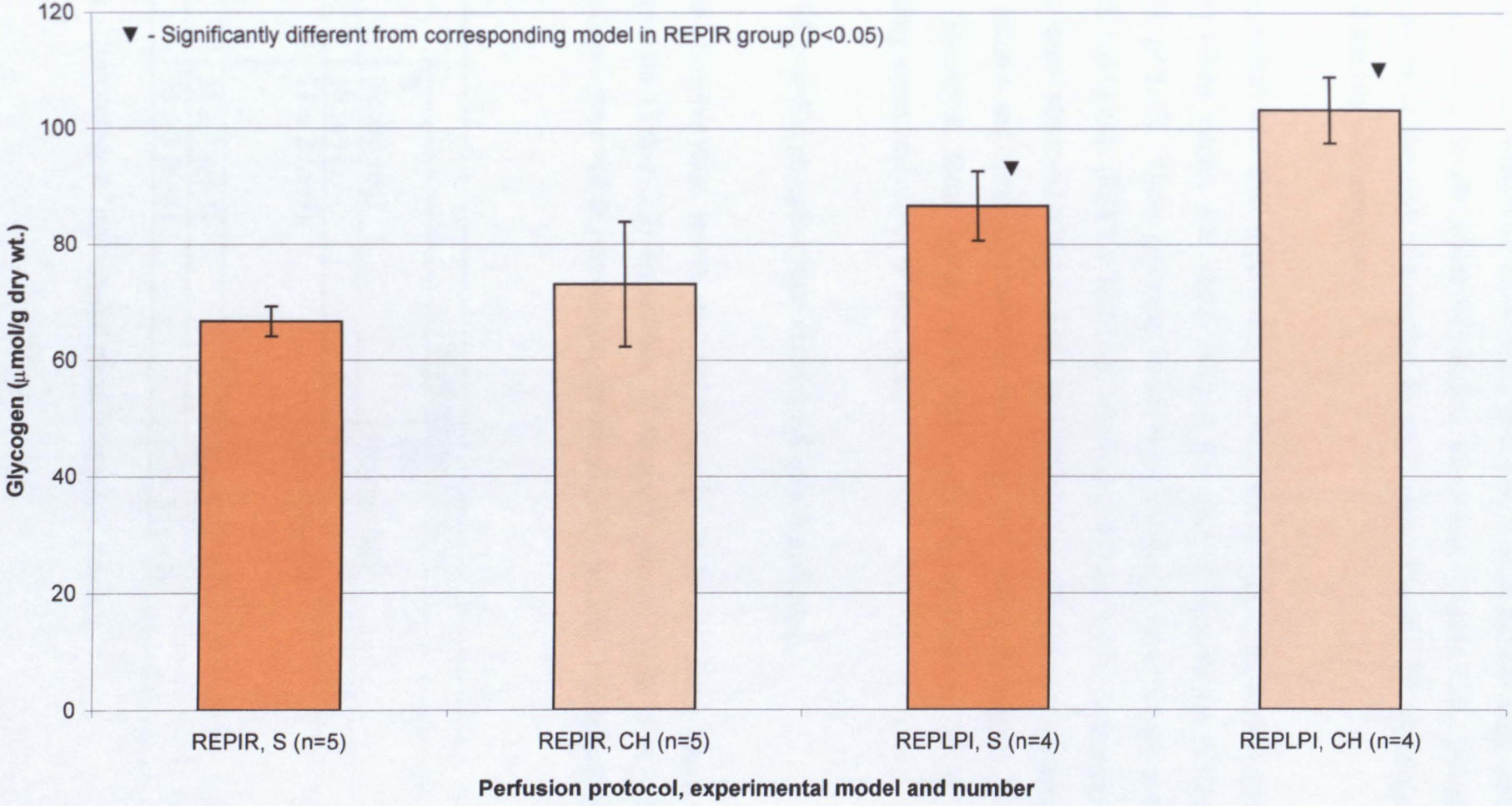


Figure 5.13: Myocardial glycogen content of hearts at the end of reperfusion (REPIR and REPLPI)



However, this replenishment of myocardial glycogen content did not fully replete glycogen to those levels observed during normoxia (Figure 3.4, $p<0.05$). Glycogen levels at the end of reperfusion were approximately 75 % of those observed at the end of normoxia.

Glycogen content was also observed to increase above ischaemic levels (LFI1, Figure 4.3) when insulin was added only at the time of reperfusion (REPIR, Figure 5.13, $p<0.05$). These glycogen levels were similar to those when insulin was present throughout (REPI & REPI13C combined, Figure 5.12). The greatest glycogen content observed at the end of reperfusion was with glucose, palmitate, pyruvate, lactate and insulin present in the perfusate (REPLPI, Figure 5.13, $p<0.05$). However, these levels were still only replenished to 70 % of corresponding normoxic values (Figure 4.4).

5:3:6 Myocardial phosphorylase activities during reperfusion

Myocardial phosphorylase levels returned to levels similar to those observed during normoxia (Table 3.2) regardless of recovery status (Table 5.4). No differences were observed in percentage phosphorylase *a* activity between S and CH hearts.

Perfusion Group	% Phosphorylase *a*	
	S (recovered)	CH (recovered)
REP	19 (n=1)	18 (n=1)
REP + REPI13C Combined	21 ± 2 (n=9)	23 ± 2 (n=7)
REPIR	21 ± 2 (n=5)	22 ± 2 (n=5)
REPLPI	33 ± 3 (n=4)	32 ± 3 (n=4)

Table 5.4: Percentage of myocardial phosphorylase *a* activity

5:3:7 Morphological results

Aortic constriction using a 0.6 mm OD needle only produced an 8 % increase in heart weight: tibia length ratio in CH compared to S hearts (Table 5.5, $p<0.05$).

Perfusion Group	Model	Number	Heart Weight: Body Weight ($x10^{-3}$ g/g)	Heart Weight: Tibia Length (g/cm)	Right: Left Kidney Ratio (g/g)
All groups combined	S	25	4.89 ± 0.01	0.62 ± 0.01	0.99 ± 0.01
	CH	23	5.41 ± 0.11°	0.67 ± 0.01°	1.50 ± 0.11$^{\nabla}$

° - Significantly different from corresponding sham ($p<0.05$)
$^{\nabla}$ - Significantly different from corresponding sham ($p<0.01$)

Table 5.5: Morphological indices for reperfusion experiments

5:4 Discussion

Overall, the presence of insulin improved functional recovery during reperfusion following GI. Differences in the profile of substrate oxidation were not found between S and CH hearts during reperfusion. Myocardial recovery on reperfusion was markedly improved when insulin was administered at the time of reperfusion. However, only the presence of insulin prior to GI increased the reliance on glucose oxidation for energy production in S and CH hearts during reperfusion. Glycogen re-synthesis occurred during reperfusion in recovering hearts under all perfusion conditions. All other determined metabolites and enzyme activities returned to values comparable to those observed during normoxia.

5:4:1 Functional recovery

Myocardial functional recovery during reperfusion of S and CH hearts was poor in the absence of insulin (REP, Table 5.1). Depressed functional recovery following total global ischaemia has also been observed with similar perfusion conditions in the working heart [Lopaschuk *et al.* 1992, Doenst *et al.* 1999]. In one study, stable myocardial function during reperfusion was not achieved when insulin was absent form the perfusate [Bailey *et al.* 1981]. It has been postulated that poor recovery during reperfusion is related to a decrease in efficiency of coupling energy metabolism to contractile function [Stanley *et al.* 1997]. Although mitochondrial oxidative phosphorylation returns to pre-ischaemic levels during reperfusion, contractile power is transiently impaired. This phenomenon is termed myocardial stunning.

In contrast, when insulin was present throughout the experiment, improved functional recovery (75 % recovered) was observed in both S and CH hearts during reperfusion (REPI + REPI13C combined, Table 5.1). Comparable levels of functional improvement have been observed in both the hypertrophied [Allard

et al. 2000] and normal heart [Bailey *et al.* 1981, Doenst *et al.* 1999], in the presence of insulin. The inotropic effects of insulin on myocardial function are well known, having been reported in hearts *in vivo* and *in vitro* [Ferranini *et al.* 1993, Freestone *et al.* 1996, Vetter *et al.* 1988]. One explanation for improved functional recovery could be the known inotropic effects of insulin [Doenst *et al.* 1999]. These arise either as a result of vasodilation decreasing myocardial afterload [Barrett *et al.* 1984], or by increasing adrenergic tone [Scherrer and Sartori 1997].

However in this study, no inotropic effects of insulin were observed in hearts during normoxia compared to that previously reported in the working heart [Doenst *et al.* 1999]. Similarly no inotropic differences were observed during reperfusion between S and CH hearts when insulin was present. This is in contrast with a recent study, where CH hearts have been reported to be less sensitive to the functional effects of insulin [Allard *et al.* 2000]. These differing results may be due to differences between the CH model used by Allard and that of this study. Allard used a tighter band for aortic constriction, which was placed closer to the heart, and banding was in younger animals [Allard *et al.* 2000]. A greater degree of CH (>30 %) was reported by Allard as a result of a more severe constriction on younger animals [Allard *et al.* 2000]. This extent of CH may generate insulin resistance at an earlier stage, modifying GLUT transport. The immature heart has a greater reliance on glucose for energy production [Lopaschuk *et al.* 1992]. The predominant isoform for glucose transport into the myocyte is GLUT1 in the immature heart [Santalucia *et al.* 1992]. During the transition from the immature heart to the adult heart, levels of the insulin sensitive GLUT4 increase [Santalucia *et al.* 1992] resulting in a relative dilution of the levels of GLUT1 in the adult heart [Razeghi *et al.* 2001]. Insulin resistance, as occurs in CH, is proposed to be a result of a decreased expression of GLUT4 in the hypertrophied heart reducing the GLUT4: GLUT1 ratio [Paternostro *et al.* 1995]. Therefore, banding in the immature/developing heart may exacerbate the decrease in GLUT4 expression observed in CH as the greater

adult levels of GLUT4 may not be expressed before the development of CH. This may lead to a greater insulin resistance observed in the model used by Allard [Allard *et al.* 2000] compared to the model used in this study.

When insulin was administered at the time of reperfusion hearts completely recovered function (REPIR, Table 5.2), indicating that S and CH hearts remained responsive to insulin at reperfusion. When insulin was present throughout the perfusion protocol, myocardial tissue may be desensitised to the effects of insulin due to prolonged exposure. In effect, the administration of insulin only at the time of reperfusion may be of more benefit for the return of myocardial function.

Complete functional recovery was also observed when a complex mixture of substrates and insulin was present (REPLP, Table 5.2). The addition of lactate and pyruvate in the perfusate has been reported to improve post-ischaemic myocardial functional recovery [Bunger *et al.* 1989, Goodwin and Taegtmeyer 1994], whereas the utilisation of fatty acids instead of glucose strongly impairs function of the reperfused heart [Tamm *et al.* 1994]. This suggests that anaplerotic pathways are stimulated at reperfusion to replenish TCA cycle metabolite pools that have been depleted during ischaemia. During ischaemia succinate levels increase that promote its efflux from the mitochondria, resulting in a depletion of TCA cycle intermediates [Taegtmeyer 1978]. Glucose, lactate and pyruvate, but not fatty acids, can serve as anaplerotic fuels to replenish TCA cycle intermediates, thus allowing the quick return of energy production by oxidative metabolism on reperfusion [Goodwin and Taegtmeyer 1994].

5:4:2 Relative substrate contribution to the TCA cycle during reperfusion and oxygen consumption

The relative contribution of substrates (glucose, palmitate and unlabelled sources) to the TCA cycle was similar between control and hypertrophied hearts for all reperfusion experiments [Figures 5.5 to 5.7]. This is in contrast to other

work where the contribution of fatty acids to the TCA cycle was decreased in hypertrophied hearts [Allard *et al.* 1994]. The differences in fatty acid utilisation between this study and that of Allard may be a result of different models used generating different stages of CH. The myocardial overload created by the tighter aortic constriction, and the placement of the ligature, in the model used by Allard may have resulted in a more severe model of CH that may have been in a decompensated phase. Previous work using the model employed in this study has shown that as CH progressed from a compensated to a decompensated state, reductions in the contribution of fatty acid oxidation were observed [Clarke 2001]. In support of this decreased levels of fatty acid oxidation have been observed in failing hearts of animals [Recchia *et al.* 1998]and decreased levels of mRNA encoding fatty acid oxidation enzymes have been observed in heart failure patients [Sack *et al.* 1996].

The relative contribution of glucose oxidation by the TCA cycle was lower when insulin was added at the time of reperfusion compared to the presence of insulin throughout (Figures 5.4 and 5.5). However, no changes were observed in the relative contribution of palmitate to energy production in the TCA cycle regardless of the time at which insulin was added. The decrease in glucose oxidation when insulin was added on reperfusion was balanced by an increase in oxidation of unlabelled substrates in the TCA cycle (Figure 5.5). This may result from an increased use of unlabelled lactate on reperfusion, as it has been shown to accumulate during GI (Figure 4.6). Although improved recovery was observed when insulin was added at the time of reperfusion, the enhanced recovery cannot be attributed to beneficial effects of increased glucose oxidation. Recently, it has been shown that the inotropic effects of insulin are immediate, but increases in the rates of glucose oxidation are more protracted [Doenst *et al.* 1999].

The relative contribution of glucose to oxidative energy metabolism was reduced when lactate and pyruvate were present in the perfusate. Studies have shown that

lactate can be a significant source of energy in the heart [Jeffrey *et al.* 1995], directly competing with glucose. Increased rates of lactate oxidation from lactate present in the perfusate may account for the increased use of unlabelled substrates.

Oxygen consumption did not change markedly during reperfusion compared to normoxia in hearts that recovered and those that did not (data not shown). This suggests that, in those hearts that did not recover fully, there was a mismatch between oxygen consumption and myocardial work. Respiratory inefficiency of contractile performance in post-ischaemia is a core feature of ischaemic/reperfusion injury [Liu *et al.* 1996b, Lewandowski *et al.* 1997]. Activation of glucose oxidation by stimulation of PDC with dichloroacetate at reperfusion has been shown to result in a significant increase in both cardiac work and cardiac efficiency during reperfusion [Liu *et al.* 1996b]. These data demonstrate that mitochondrial function and overall myocardial ATP production quickly recover in rat hearts after a 30 minute period of global ischemia. However it appears that mitochondrial ATP production is not efficiently translated into mechanical work in the reperfused heart, possibly leading to myocardial stunning. Hearts that are stunned have a relative excess of oxygen consumption for a given rate of contractile force [Stanley *et al.* 1997]. This was clearly highlighted in this study when calculating the ratio of myocardial oxygen consumption to RPP in hearts that do and do not recover (Tables 5.1 and 5.3). A significant decrease in this ratio was observed in hearts that do not recover indicating an excess of oxygen consumption per contractile work.

5:4:3 Reperfusion injury (nucleoside release)

Inosine release can act as a sensitive marker of ATP catabolism as the amount of inosine released at reperfusion reflects the degree of catabolism of ATP during ischaemia. However, in this study little difference was observed in inosine

release between hearts that recovered during reperfusion and those that did not (data not shown), thus implying that ATP depletion occurred at similar rates.

Significant differences were not observed in the amount of inosine released between S and CH in any perfusion protocol (Figures 5.7, 5.8 and 5.9). This may indicate that there was no enhanced susceptibility to ischaemic/reperfusion injury in this model of CH due to a decreased energy reserve.

When lactate and pyruvate were present in the perfusate the greatest amount of inosine was released during reperfusion (Figure 5.9). The increased levels of inosine release may reflect a greater amount of glycogen degradation during ischaemia. At the end of normoxia, glycogen content was the greatest in the presence of lactate from all glycogen loading protocols (GPLP, Figure 3.7). This could result in a larger amount of glycogen degraded anaerobically to produce ATP during ischaemia than in the other experiments. Since these hearts fully recovered function by the end of reperfusion, it implies that the maintenance of energy provision in the form of ATP during ischaemia is of primary importance. Indeed, studies have previously shown that the maintenance of ATP in ischaemia is of more importance to reperfusion recovery than the maintenance of intracellular pH [Seymour *et al.* 1983].

5:4:4 End reperfusion myocardial metabolite levels/activities

5:4:4:1 Glycogen

Re-synthesis of glycogen occurred during all reperfusion protocols in all hearts that recovered myocardial function but did not reach pre-ischaemic normoxic levels. Glycogen levels were not observed to increase above ischaemic levels in hearts that did not recover. Net glycogen synthesis during reperfusion has been observed to be associated with improved functional recovery [Doenst *et al.* 1996].

Insulin stimulated myocardial glycogen re-synthesis is mediated through a phosphatidylinositol 3-kinase (PI3-K) signalling pathway [Saltiel 1996]. Recently, glycogen re-synthesis during reperfusion has been shown to be independent of PI3-K [Doenst and Taegtmeyer 1999], thus demonstrating a direct pathway exists for replenishment of myocardial glycogen stores during reperfusion. During reperfusion fatty acids once again become the primary fuel for oxidation, thus limiting glucose oxidation, allowing glucose to be used for glycogen resynthesis. Enhanced rates of glucose uptake and glycogen synthase activity during ischaemia may still prevail during early reperfusion allowing for a rapid replenishment of glycogen stores [Doenst *et al.* 1996].

The largest myocardial glycogen content at the end of reperfusion was observed in hearts perfused with glucose, palmitate, lactate, pyruvate and insulin. Lactate plus glucose has been shown to promote glycogen re-synthesis in the post-ischaemic heart beyond the rate of glycogen re-synthesis observed previously in hearts re-perfused with glucose as the sole substrate [Bolukoglu *et al.* 1996]. Lactate is preferentially oxidised over glucose allowing the uptake of exogenous glucose to be directed primarily to glycogen synthesis in the heart when lactate and glucose are supplied [Bolukoglu *et al.* 1996].

5:4:4:2 Glycogen phosphorylase

The amount of phosphorylase in the *a* form at the end of reperfusion (Table 5.4) returned to levels similar to those observed during normoxia (Table 3.2). As in the normoxic situation, the amount of phosphorylase *a* activity was greatest when lactate and pyruvate were present in the perfusate. This indicates a greater degree of glycogen turnover during reperfusion in these hearts as glycogen content was elevated (Figure 5.13), as was the situation in normoxia. It has been proposed that glycogen turnover rates may play a role in the recovery of contractile function in the post-ischaemic heart [Doenst *et al.* 1996].

5:4:5 Conclusions

Myocardial functional recovery on reperfusion was similar in control and hypertrophied hearts. Similar rates of inosine release at reperfusion were also observed in control and hypertrophied hearts. This implies that in this model of CH, there was not an increased susceptibility in the hypertrophied heart to ischaemic/reperfusion injury due to a decreased energy reserve in the hypertrophied heart. Insulin improved myocardial functional recovery on reperfusion. The presence of insulin prior to ischaemia increased oxidative glucose metabolism compared to the administration of insulin only at the time of reperfusion. Therefore, improved myocardial recovery on reperfusion was not a result of increased glucose oxidation but was via a direct inotropic effect of insulin.

6: DISCUSSION AND FUTURE WORK

6:1 Discussion

Aortic banding with a 0.7 mm OD needle produced a mild degree of myocardial hypertrophy (4-8 % increase in heart wt: tibia length ratio). Decreasing the size of the banding needle to 0.6 mm OD resulted in a greater degree of CH (10-25 % increase in heart wt: tibia length ratio). However, regardless of needle size, there was no evidence of functional impairment in hypertrophied hearts under any perfusion conditions. This implies that in this study a model of compensated hypertrophy was generated, which represents the early stages of CH.

When interpreting data from this model of CH for relevance to clinical studies, it was taken into account that it does not necessarily mimic a clinical condition in patients [Doggrell and Brown 1998]. CH produced from a short, sharp pressure overload as used in this study to model hypertrophy may differ slightly to that observed as a result of the more gradual process of essential hypertension in humans. However, numerous reviews have concluded at present that no one current model used in research mimics exactly all the symptoms of human heart disease due to the vast array of genetic and environmental factors [Smith and Nuttall 1985, Elsner and Riegger 1995, Doggrell and Brown 1998].

No differences were observed in myocardial glycogen content between control and hypertrophied hearts after any glycogen loading perfusion protocol. This suggests that there was no enhancement or deficit of glycogen storage in this model of CH. Glycogen content was observed to decrease from *in vivo* levels [Wolfe *et al.* 1993, Fraser *et al.* 1998] when hearts were perfused with only 5 mM glucose. This implies that myocardial glucose uptake may be limiting under these conditions as net glycogenolysis is required for energy provision. Indeed, in the perfused rat heart, glucose uptake has been reported to be linear over the range of 1.25 to 5 mM glucose, but not saturated until above concentrations of 10 mM [Opie *et al.* 1962]. Low glycogen levels have been observed in similar

perfusion studies when fatty acids and insulin were not present in the perfusate [Lagerstrom *et al.* 1988, Goodwin *et al.* 1995].

Myocardial glycogen content was maximised in the presence of glucose and physiological levels of insulin, with or without palmitate, but levels were lower than those reported *in vivo* [Wolfe *et al.* 1993, Fraser *et al.* 1998]. Further glycogen loading was not observed as insulin levels were increased up to pharmacological levels. This suggested that all the available space for intracellular glycogen deposition was occupied. However, further myocardial glycogen loading was observed when lactate was also present in the perfusate to levels comparable to those reported *in vivo* [Wolfe *et al.* 1993, Fraser *et al.* 1998]. The preferred oxidation of lactate (over glucose) in the heart re-directs extracellular glucose towards glycogen synthesis as a result of a greater inhibition of glycolysis than glucose uptake [Depre *et al.* 1993, Bolukoglu et al. 1996]. Therefore, as the available space for glycogen deposition was not limiting, it may be that insulin stimulation of glycogen deposition was not sufficient to maximally load the heart with glycogen.

The effects of insulin at pharmacological concentrations appeared to be more evident for glucose oxidation than for glycogen deposition. In the normal heart fatty acids are the dominant fuel, supplying 60 to 70 % of the energy requirements [Neely and Morgan 1974]. In contrast, in the presence of pharmacological levels of insulin the contribution of exogenous glucose to myocardial oxidative metabolism during normoxia was greater than 79 % in all cases, indicating a high level of activation of glycolysis through PDC. No differences were observed between control and hypertrophied hearts. The high level of insulin appears to have masked subtle differences in glucose oxidation between control and hypertrophied hearts as it has been previously reported in this model that glucose oxidation was increased in CH in the absence of insulin [Clarke 2001].

Physiological responses to ischaemic insults were similar in control and hypertrophied hearts, indicating that there was no increased susceptibility to ischaemic injury in the hypertrophied heart. During LFI slight myocardial function was still detectable but during GI function ceased within 7 minutes. Contracture was not observed in any hearts from either of the ischaemic conditions.

Alterations to glycogenolytic/glycolytic rates in CH have been implicated to increase the susceptibility of the hypertrophied heart to ischaemic injury [Bailey *et al.* 1982, Neely and Grotyohann 1984, Allard *et al.* 1994, Taegtmeyer 1994]. However, during all ischaemic perfusion protocols the amount of glycogen degradation and rate (phosphorylase *a* activity) were similar between control and hypertrophied hearts. The chief factor determining the degree of glycogen degradation was the severity in the reduction of flow. The presence of insulin preserved myocardial glycogen during LFI. This may be due to insulin increasing the rates of glycogen synthesis (from exogenous glucose) to close to the increased degradation rates during ischaemia, as no reductions in phosphorylase *a* activity were observed.

Increased glycogen degradation during ischaemia did not correlate with increased ischaemic injury. As contracture was not observed during GI, glycogen degradation for the anaerobic production of energy was favourable in the ischaemic heart. In support of this, myocardial contracture was observed in ischaemic hearts once glycogen stores were depleted [Steenbergen *et al.* 1990]. This study supports the theory that the ischaemic heart is better preserved if glycogenolysis is maintained during ischaemia [Bailey *et al.* 1982, Taegtmeyer *et al.* 1985, Goodwin and Taegtmeyer 1994, Cross *et al.* 1996].

Myocardial functional recovery on reperfusion was similar in control and hypertrophied hearts for all reperfusion protocols. Inosine release on reperfusion, a sensitive marker for ATP catabolism during ischaemia, was also

similar in control and hypertrophied hearts. This implies that in this model of CH there was not an increased vulnerability of the hypertrophied heart to ischaemic/reperfusion injury.

Work from this study has highlighted that the profile of substrate oxidation in CH may be normalised during reperfusion under physiological levels of substrates as no differences were observed in the profile of substrate use between control and hypertrophied hearts for any of the experimental protocols. However, this work reports the substrate utilisation averaged over the whole reperfusion period and does not rule out the possibility that immediate changes occur in the profile of substrate utilisation in CH on reperfusion. Due to the insensitivity of the ^{13}C-NMR technique for monitoring metabolism, any discrete changes occurring at reperfusion cannot be detected. Therefore it may be of importance for functional recovery post-ischaemia in CH to reveal the immediate changes likely to occur at the time of reperfusion.

In the absence of insulin, functional recovery during reperfusion of both control and hypertrophied hearts was poor. Recovery on reperfusion was markedly improved in the presence of insulin in both control and hypertrophied hearts. When insulin was present throughout the whole perfusion protocol, both control and hypertrophied hearts had a greater reliance on glucose oxidation during reperfusion compared to when insulin was there only at the time of reperfusion. Increased myocardial glucose oxidation has been reported to be of benefit for functional recovery during reperfusion [Lewandowski and White 1995, Doenst *et al.* 1999]. However, the presence of insulin prior to ischaemia was not a prerequisite to the beneficial effects of insulin on myocardial functional recovery post-ischaemia. Therefore, the improved recovery in both control and hypertrophied hearts during reperfusion in the presence of insulin was mainly due to a direct inotropic effect, and not the result of increased glucose oxidation. This is supported by a recent study in normal hearts that showed the inotropic

effects of insulin are immediate, but increases in the rates of glucose oxidation are more protracted [Doenst *et al.* 1999].

The effects of insulin at reperfusion may be an important aspect in the clinical treatment of ischaemic/reperfusion contractile dysfunction with glucose insulin and potassium (GIK therapy) [Sodi-Pallares *et al.* 1962, Gradinak *et al.* 1989, Fath-Ordoubadi *et al.* 1997]. GIK is proposed to be of benefit to the ischaemic/reperfused heart by manipulating energy metabolism and limiting calcium overload. The metabolic beneficial effects of GIK therapy could be due to any of the following; maintenance of basal ATP production during ischaemia, reductions in circulating fatty acid levels resulting in increased PDC activity, or increases in glucose uptake and oxidation [Stanley *et al.* 1997]. From the current study, which has shown that insulin has a direct inotropic effect on the post-ischaemic heart, it is fair to deduce that changes to glucose and fatty acid metabolism alone do not give rise to the beneficial effects of GIK therapy.

6:2 Future work

6:2:1 Model of hypertrophy

The model used in this study may represent early stages of CH. Therefore, a more severe model of hypertrophy may be required to emphasise the deleterious metabolic alterations observed in later stages of CH. It would therefore be interesting to assess glycolysis, glycogenolysis and oxidative substrate provision in hearts 9 weeks after intra-renal aortic constriction using a band of smaller diameter. Another contributing factor to the degree of observed CH may be the location of the band. In the model used in this study the ligature was tied further from the heart than in other studies where a clip/tie is placed on the ascending aorta. Greater degrees of hypertrophy have been reported when using a tighter band that is placed closer to the heart that may contribute to the metabolic

alterations observed in these studies [Schönekess *et al.* 1995, Allard *et al.* 1997, Wambolt *et al.* 1999].

Rather than using a tighter band, animals could be left for a longer period post surgery to determine if any detrimental changes occur chronically. In a study of CH, ascending aortic banding resulted in compensated left ventricular hypertrophy for several weeks [Feldman *et al.* 1993]. 20 weeks post surgery two further subgroups were clearly identified; rats showing no changes in LVDP and those with a reduction in LVDP, exhibiting signs of over heart failure [Feldman *et al.* 1993].

6:2:2 NMR studies of oxidative substrate selection

Work in this study has shown that the greatest myocardial glycogen loading occurs in this model of CH when there is a complex mixture of substrates (at physiological concentrations) and insulin present in the perfusate. Combining this glycogen loading protocol with a depletion/repletion protocol will allow the greatest proportion of glycogen to be labelled with ^{13}C under physiological conditions. The level of ^{13}C labelling obtained in glycogen may then be of sufficient quantity to follow its metabolism during ischaemia by NMR.

When hearts were perfused with a complex mixture of substrates, the relative proportions of substrate used in the TCA cycle included a large contribution from unlabelled sources. This was presumed to be mainly from lactate. By performing a parallel perfusion study to that used for REPLPI the relative contribution from lactate could be determined during reperfusion in hypertrophied hearts using NMR. Hearts could be perfused with U-^{13}C palmitate, 3-^{13}C lactate, 2-^{13}C pyruvate and unlabelled glucose to determine the relative contributions from lactate and pyruvate. Results from this experiment may highlight differences in the profile of substrate use in the hypertrophied heart during reperfusion. A similar set of experiments could be performed during

normoxia to give a better picture of the profile of substrate use in CH under normal conditions.

6:2:3 Ischaemic studies in cardiac hypertrophy

In low flow ischaemic studies it may be more appropriate to alter the coronary flow relative to the heart weight. This would allow control and hypertrophied hearts to experience the same degree of ischaemia, where ischaemia is expressed as flow (ml)/heart wet weight (g). To allow this, hearts could be weighed after trimming prior to hanging on the perfusion rig, to allow calculation of the required flow to use during ischaemia. It may also be more appropriate to the clinical situation if hearts were paced during the ischaemic episode. This would increase the metabolic stress on the heart and may help highlight any susceptibilities to injury in this model of CH during ischaemia.

Several studies have reported an enhanced glycolytic capacity in CH [Seymour *et al.* 1990, Allard *et al.* 1994] that may enhance susceptibility to ischaemic injury [Allard *et al.* 1994]. Other studies have shown beneficial/detrimental effects of glycogenolysis and glycolysis during ischaemia [Neely and Grotyohann 1984, Goodwin and Taegtmeyer 1994]. Depleting glycogen levels prior to an ischaemic episode has shown that minimising glycogen degradation during ischaemia is of benefit [Neely and Grotyohann 1984]. However, glycogen turnover was not taken into account. Significant rates of glycogen turnover have been observed under ischaemic conditions [Bolukoglu *et al.* 1996]. It was recently postulated that ATP derived from glycogenolysis may be used for different purposes to that from glycolysis under ischaemic conditions [King and Opie 1998]. Therefore, it would be of interest to investigate energy provision in CH under ischaemic conditions solely from glycolysis by halting glycogen degradation with the use of a glycogen phosphorylase inhibitor. An inhibitor of both the muscle and brain glycogen phosphorylase isoforms found in the heart has recently been reported [Treadway *et al.* 2001]. Similarly, using

phosphorylase inhibitors during total global ischaemia studies could define the role of glycogen in CH, as glycogenolysis is the only source of ATP under these conditions [Depre *et al.* 1998].

6:2:4 Pharmacological interventions to improve reperfusion recovery in CH

Several studies have recognised that increasing glucose oxidation during reperfusion increases recovery in the normal heart [Stacpoole 1989, M^{c}Veigh and Lopaschuk 1990, Fantini *et al.* 1994, Clarke *et al.* 1996]. Pharmacological interventions that increase glucose oxidation directly, by stimulation of PDC [Stacpoole 1989, M^{c}Veigh and Lopaschuk 1990], or indirectly, by suppressing fatty acid oxidation [Fantini *et al.* 1994, Clarke *et al.* 1996], result in improved recovery from ischaemia in normal hearts. However, the hypertrophied heart may be more susceptible to reperfusion injury than normal hearts, and as a result may benefit greater from pharmacological interventions. It has previously been reported that the percentage of PDC in the active form is reduced in cardiac hypertrophy [Seymour and Chatham 1997], which may limit the capacity for oxidative glucose use in the hypertrophied heart during reperfusion

Improved functional recovery has been observed in hypertrophied hearts when dichloroacetate (DCA) is administered at the time of reperfusion [Wambolt et al. 2000]. DCA inhibits pyruvate dehydrogenase kinase [Stacpoole 1989], which reduces phosphorylation of PDC, thereby increasing the activity of PDC. Improved recovery in the hypertrophied heart was accompanied by a marked increase in glucose oxidation [Wambolt et al. 2000]. Therefore, manipulation of energy metabolism to increase glucose oxidation may also be a useful approach to limit ischaemic/reperfusion injury in the hypertrophied heart, which could have relevance to the clinical situation. Further investigations are required to determine if alternative interventions such as GIK therapy and partial fatty acid oxidation inhibitors (ranolazine and trimetazidine) also exert beneficial effects in CH during ischaemia and reperfusion.

PUBLICATIONS

Abstracts

Richardson, S. and Seymour, A-M. L. Insulin resolves metabolic impairment in compensated hypertrophy. *J Mol Cell Cardiol* **34(6):** A53.

Richardson, S. and Seymour, A-M. L. Glycogen loading and ischemic injury in myocardial hypertrophy. *J Mol Cell Cardiol* **33(6):** A101.

Richardson, S. and Seymour, A-M. L. Insulin protects the hypertrophied heart against ischemia. *J Mol Cell Cardiol* **33(6):** A175.

Papers

Richardson, S. and Seymour, A-M. L. Improved functional recovery in the presence of insulin at reperfusion following global ischaemia in cardiac hypertrophy (Manuscript in preparation).

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