THE UNIVERSITY OF HULL

Biochemical Adaptations in Cardiac Hypertrophy

being a Thesis submitted for the degree of
Doctor of Philosophy in the University of Hull

by

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Abstract

Cardiac hypertrophy is the adaptive response of the heart to chronic overload. The metabolic adaptations that occur during hypertrophy are initially beneficial, but can ultimately deteriorate into heart failure. The mechanisms underlying this are unknown. Evidence of impaired energy reserve, which may be caused by changes in the profile of substrate use, has been implicated in the transition of compensatory hypertrophy to heart failure. The work of this thesis characterises the alterations in substrate utilisation that occur in the heart, secondary to pressure-overload induced cardiac hypertrophy, the their implications on heart function.

Pressure-overload hypertrophy was induced surgically in male Sprague-Dawley rats by inter-renal ligation. ^{13}C-NMR spectroscopy was performed on extracts from hypertrophied and control hearts perfused with ^{13}C-labelled substrate mixtures to determine the profile of substrate utilisation. Nine weeks pressure-overload achieved a moderate hypertrophy, evidenced by a 10-15% increase in heart mass to tibia length. The hypertrophied hearts showed an increased reliance on glucose and endogenous substrate contribution to TCA cycle oxidation for the production of ATP (15.0% versus 11.0%) compared to control hearts.

Prolonged fifteen weeks pressure-overload resulted in further metabolic changes including impaired long-chain fatty acid oxidation and the accumulation of long-chain acylcarnitines. Alteration in substrate utilisation preceded any change in heart function and is strong evidence to suggest that impaired substrate delivery at the level of the mitochondria in cardiac hypertrophy plays an important role in the development of heart failure and is not a secondary phenomenon. At high workloads both hypertrophied and control hearts, showed similar profiles of substrate use, with glucose being the predominant substrate utilised for TCA cycle oxidation. At high workloads, hypertrophied hearts initially exhibited significantly higher mechanical function, but was not sustained, suggesting that physiological changes were becoming detrimental. This study highlights that sequential metabolic adaptations occur during the development of hypertrophy and precede any functional abnormality, providing potential prognostic markers.

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Declaration

All the work described in this thesis was carried out in the Department of Biological Sciences, University of Hull, between October 1996 to October 1999, under the supervision Dr Anne-Marie Seymour. Except where stated it is the original work of the author. The views expressed in this thesis are those of the author and not necessarily those of the University.

Samantha Jayne Clarke

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Abbreviations

4-AA	4-Aminoantipyrine
ΔA	Absorbance change
ACE	Angiotensin converting enzyme
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ANF	Atrial natriuretic factor
Ang	Angiotensin
ANOVA	One-way analysis of variance
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
cAMP	Cyclic adenosine monophosphate
carnitine	β-hydroxy-trimethylammonium butyrate
CK	Creatine kinase
CH_3CN	Acetonitrile
CHF	Chronic heart failure
CoA	Coenzyme A
CPT1	Carnitine palmitoyl transferase 1
CPT2	Carnitine palmitoyl transferase 2
CS	Citrate synthase
D_2O	Deuterium oxide
DAP	Dihydroxyacatone phosphate
DCA	Dichloroacetate
DEAE	Diethylaminoethyl
DP	Diastolic pressure
DTT	Dithiothreitol
E-C	Excitation-contraction
EDV	End-diastolic volume
EDP	End-diastolic pressure
EDTA	Ethylenediaminetetraacetic acid
Etomoxir	Ethyl-2-[6-{4-chlorophenoxy}hexyl]oxirane-2-carboxylate
FA	Fatty acid
Fc	Fractional enrichment of acetyl CoA
FFA	Free fatty acid
FGF	Fibroblast growth factor
G-1-P	Glucose-1-phosphate
G-1-PDH	Glucose-1-phosphate dehydrogenase
G-6-P	Glucose-6-phosphate
G-6-PDH	Glucose-6-phosphate dehydrogenase
GIDH	Glutamate dehydrogenase
GK	Glycerol kinase
GLUT1	Glucose transporter 1

HBr	Hydrogen bromide
HCl	Hydrochloric acid
HEPES	N-2-Hydroxyethylpiperazine N'-2-Ethanesulphonic acid
HF	Heart failure
HK	Hexokinase
HR	Heart rate
HT	Hypertrophy
INT	2-(p-iodophenyl)-3-p-nitrophenyl-5-phenyltetrazolium
INTH	Formazan
KF	Potassium fluoride
KH_2PO_4	Potassium dihydrogen phosphate
KOH	Potassium hydroxide
LDH	Lactate dehydrogenase
LV	Left ventricle
LVDP	Left ventricular developed pressure
LVH	Left ventricular hypertrophy
MeOH	Methanol
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnessium sulphate
MHC	Myosin heavy chain
mRNA	Messenger ribonucleic acid
N_2	Nitrogen
Na_2CO_3	Sodium carbonate
NaCl	Sodium chloride
NAD+	Nicotinamide adenine dinucleotide (oxidised form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADP	Nicotinamde adenine dinucleotide phosphate (oxidised form)
NADPH	Nicotinamde adenine dinucleotide phosphate (reduced form)
NaOH	Sodium hydroxide
NEFA	Non-esterified fatty acids
NH_3	Nitrite
NMR	Nuclear magnetic resonance
OGDH	Oxoglutarate dehydrogenase
PCA	Perchloric acid
PCr	Phosphocreatine
PDH	Pyruvate dehydrogenase
PDHa	Pyruvate dehydrogenase (active form)
PDHt	Pyruvate dehydrogenase (total activity)
PEG	Polyethylene glycol
PFK	Phosphofructokinase
Pi	Inorganic phosphate
RAAS	Renin-angiotensin-aldosterone system
RNA	Ribonucleic acid
RPP	Rate pressure product
rRNA	Ribosomal ribonucleic acid

SHR	Spontaneously hypertensive rat
SP	Systolic pressure
TBH	Tribromohydroxybenzoic acid
TCA	Tricarboxylic acid
TG	Triglyceride
TPP	Thiamine pyrophosphate
TRIS	Tris [Hydroxymethyl] aminomethane
y	Relative rate of anaplerosis

Chapter one:

Introduction

1.1. Physiology of the heart.

1.1.1. Anatomy of the heart.

The heart is a muscular pump organ located in the thoracic cavity, enclosed in a fibrous sac, the pericardium (reviewed by Vander *et al* 1990). Its primary function is to supply oxygen and nutrients to the body, essential for life. The walls of the heart are composed primarily of cardiac muscle (myocardium). The human heart is divided longitudinally into right and left halves, each consisting of two chambers, an atrium and a ventricle (Figure 1.1).

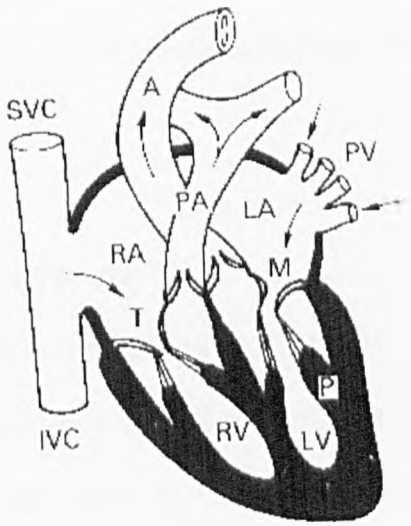

Figure 1.1. Schematic cross-section through the heart. (RA, LA, right and left atrium; RV, LV, right and left ventricle; T, M, tricuspid and mitral valves; P, papillary muscle with chordae tendineae; A, aorta; PA, PV, pulmonary artery and veins; SVC, IVC, superior and inferior venae cavae (Levick 1995).

1.1.2. Cardiac contraction cycle.

The atria and ventricles contract in sequence, in a cycle of pressure and volume changes (reviewed by Vander *et al* 1990, Levick 1995). The cardiac cycle has four phases. In the filling phase the heart is in diastole (relaxation), the arterial outlet valves (aortic and pulmonary) are closed and the atrioventricular inlet

valves (tricuspid and mitral) are open, allowing rapid, passive filling of the ventricles. This is augmented by atrial systole (contraction) towards the end of the phase. During the isovolumetric contraction phase, ventricular systole raises the ventricular pressure, closing the atrioventricular valves. Ventricular pressure then rises rapidly, but is quickly terminated by opening of the arterial outlet valves and the onset of the ejection phase, in which two-thirds of the ventricular volume is ejected. As ejection diminishes, ventricular pressure falls until the outlet valves are closed by a small back-flow of blood. In the isovolumetric relaxation phase the ventricular pressure falls rapidly below the arterial pressure, at which point the atrioventricular valves open and rapid filling begins again.

1.1.3. Ultrastructure of myocyte.

The myocyte is branched and attached to adjacent cells end-to-end (reviews by Harrington and Rodgers 1984 and Zak and Galhotra 1983). The junction between cells (intercalated disc) contains two specialised junctions, namely desmosomes and gap junctions. Desmosomes hold adjacent cells together, whereas the gap junctions are thought to be electrically conductive regions through which ionic currents pass (Levick 1995, Langer 1978). The myocyte is packed with longitudinal bundles of myofibrils. Each myofibril is composed of sarcomeres, the basic contractile unit, aligned across the cell, giving a striated appearance. A sarcomere is defined as the region between two Z lines and contains thick and thin filaments (Figure 1.2). The thick filaments lie parallel in a central region of the sarcomere (A band). The thin filaments lie in the Z line and form the I band. The cell also contains non-contractile cytoskeletal

filaments (connectin or tinin) which contribute to its stiffness.

At a molecular level cardiac muscle consists of the proteins myosin, actin, tropomyosin and troponin complex (I, C, T), of which myosin light and heavy chains make up the thick filaments, and actin, tropomyosin and troponin the thin filaments (reviews by Harrington and Rodgers 1984 and Zak and Galhotra 1983). Myosin is a large hexameric protein consisting of two heavy chain subunits, two essential myosin light chain subunits and two regulatory light chain subunits. The actin complex is composed of a double helix of actin monomers. The tropomyosin dimer molecule lies in the groove of this double helix. At the end of each tropomyosin molecule lies the troponin complex, consisting of troponin T which binds tropomyosin, troponin I which is the inhibitory troponin and troponin C which binds calcium.

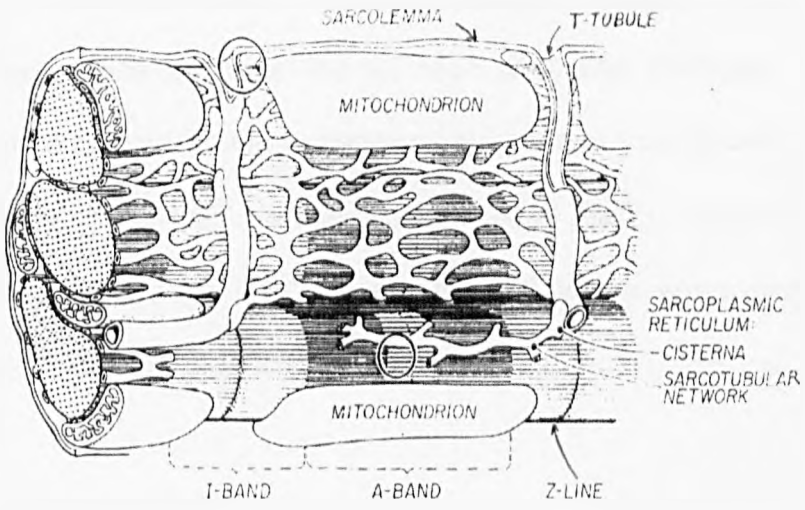

Figure 1.2. Schematic diagram of a normal cardiac myofibril.

The surface membrane (sarcolemma) is invaginated into a series of transverse tubules (T-tubules), which run into the cell interior (Vander *et al* 1990). The T-tubules transmit the electrical stimulus rapidly into the cell interior. A second

closed series of tubules, the sarcoplasmic reticulum (SR), contains the store of Ca^{2+} ions.

1.1.4. Mechanism of contraction and excitation-contraction coupling.

The depolarisation (excitation) of the plasma membrane of cardiac muscle cells triggers their contraction (Noble 1983). The gap junctions between connecting myocardial cells allow the action potential to spread from cell to cell. The mechanism coupling excitation and contraction is an increase in cytosolic Ca^{2+} concentration. During each action potential, a small amount of Ca^{2+} enters the cell. This initial Ca^{2+} influx, triggers the release of large amounts of Ca^{2+} from the SR by way of Ca^{2+}-specific release channels (ryanodine receptor)(Fabiato 1983, Valdivia *et al* 1995). This combines with the regulator protein, troponin, to remove tropomyosin inhibition of cross-bridge formation between actin and myosin head, which contains the ATPase (Zak and Galhotra 1983). (Figure 1.2). The force of contraction in cardiac myocytes is modulated by variations in the magnitude of the Ca^{2+} transient (Alpert *et al* 1992). Relaxation occurs by the subsequent extrusion of Ca^{2+} into the SR by an adenosine triphosphate (ATP)-dependent calcium pump.

1.1.5. Cardiac energetics.

Heart muscle uses adenosine triphosphate (ATP) as energy for muscle contraction, through the actin-myosin cross-bridge fibre site (Harrington and Rodgers 1984). ATP has to be continuously re-synthesised from its products, adenine diphosphate (ADP) and inorganic phosphate (Pi), in order to sustain its work. The greater the 'cardiac output', the higher the rate of ATP turnover. The

heart therefore requires the ability to adapt to changes in energy demand by altering the flux of metabolic pathways, to maintain a state of energy homeostasis (Taegtmeyer 1994).

1.2. Cardiac metabolism.

Cardiac function is dependent upon cardiac metabolism. ATP energy is generated in the myocardium primarily by oxidative phosphorylation and to a much lesser extent by anaerobic glycolysis. The heart is richly endowed with mitochondria, the cell organelles that contain the enzymes of oxidative metabolism and thus meets most of its energy needs by the latter (Taegtmeyer 1994). A close relationship exists between mitochondrial density, heart rate and total body oxygen consumption (Neely *et al* 1967, La Noue and Schoolwerth 1979). As ATP production is tightly coupled to ATP use, therefore substrate oxidation is tightly coupled to cardiac work (Taegtmeyer 1994, Drake-Holland 1983).

1.3. Major energy providing substrates.

The heart can use a variety of substrates for the production of ATP, namely fatty acids (non-esterified) derived from lipids, carbohydrates (glucose and lactate) and to a lesser extent ketone bodies and amino acids (Saddik and Lopaschuk *et al* 1991, van der Vusse and de Groot 1992, Allard *et al* 1994), see Figure 1.3. Under certain circumstances endogenous energy stores of glycogen and lipids can be utilised. The heart can also use its reserves of high-energy phosphate bonds in the form of phosphocreatine (PCr). Phosphoryl

transfer from PCr to ADP via the creatine kinase reaction can maintain ATP levels, acting as an energy reservoir (Ingwall *et al* 1990).

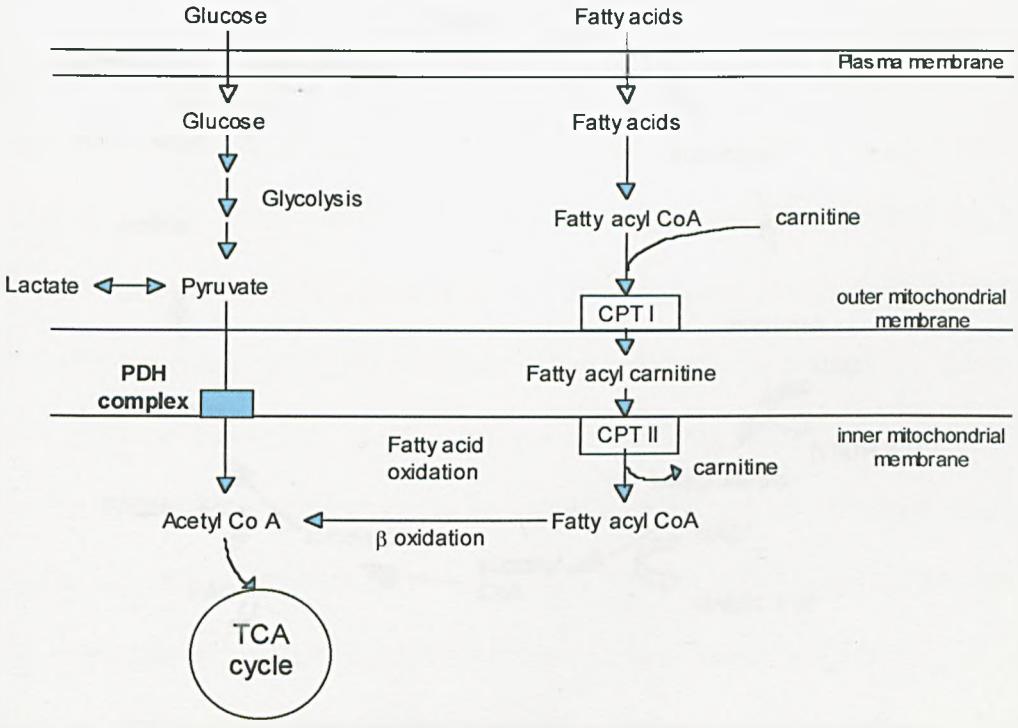

Figure 1.3. Major metabolic pathways of the heart.

In both fatty acid and carbohydrate metabolism there is a common intermediate produced, acetyl-CoA, which in turn enters the TCA cycle (Drake-Holland 1983, Heinman and Balaban 1992). Metabolism of acetyl-CoA via oxidative phosphorylation leads to the reduction of NAD and generation of reducing equivalents (NADH). These reducing equivalents transfer electrons through the electron transfer chain coupled to ATP synthetase resulting in the production of ATP (Figure 1.4). Inside the myocardial cell, ATP hydrolysis enhances flux through the tricarboxylic acid (TCA) cycle by increasing [ADP] and [NADH] oxidation (Taegtmeyer 1994, Drake-Holland 1983).

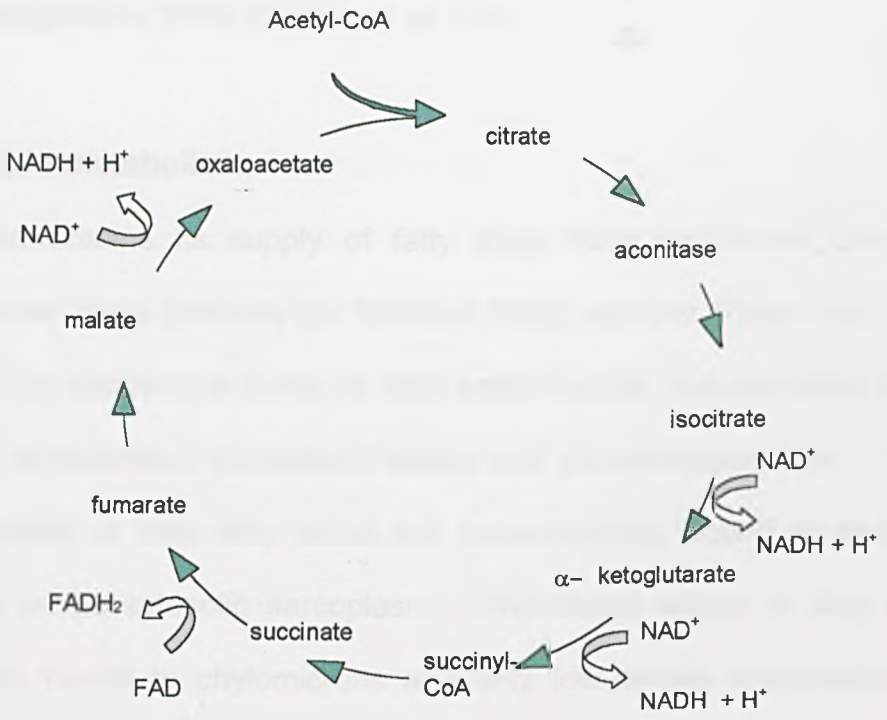

Figure 1.4. TCA cycle showing the formation of reducing equivalents.

Oxidative phosphorylation is thought to be controlled by NADH-linked dehydrogenases (Heinman and Balaban 1992) and mitochondrial NADH concentration (From *et al* 1990, Sholtz *et al* 1995), and to a much lesser extent ADP concentration (From *et al* 1990). In addition Ca^{2+} ions could provide a link between ATP use and production by the TCA cycle (McCormack and Denton 1989, Denton and McCormack 1980). Ca^{2+} ions can stimulate oxidative phosphorylation through their influence on intramitochondrial Ca^{2+}-sensitive dehydrogenases (namely isocitrate dehydrogenase and oxoglutarate dehydrogenase) (McCormack and Denton 1989, Hansford 1991). In the presence of adequate substrate supply, the maximal rate of substrate oxidation

is determined by the capacity of the 2-oxoglutarate DH reaction in the TCA cycle (Taegtmeyer 1994, Cooney *et al* 1981).

1.3.1. Lipid metabolism.

The heart obtains its supply of fatty acids from exogenous circulating or endogenous lipids (reviews by Tahiliani 1992, van der Vusse and Reneman 1983). The exogenous forms of fatty acids include non-esterified fatty acids (NEFA), triglycerides, cholesterol esters and phosphoglycerides. Circulating non-esterified or free fatty acids are predominantly bound to proteins and localised in the cytosolic sarcoplasm. Cholesterol esters of fatty acids are essentially bound to chylomicrons and very low-density lipoproteins (VLDL), whereas circulating phospholipids are bound to high-density lipoproteins (HDL) (reviewed by Pownall and Gotto 1992). Triglycerides can be hydrolysed outside the cell by lipoprotein lipase (LPL) associated with the vascular endothelium.

1.3.1.1. Endogenous source.

Triacylglycerol is thought to be the main endogenous lipid store, present in the lysosome and as lipid droplets in the sarcoplasm (Stam *et al* 1980). Release of fatty acids stored in triacylglycerol is accelerated by the action of triacylglycerol lipase. A variety of lipolytic enzymes are involved in the degradation of myocardial triacylglycerol (Severson 1979). These include LPL, a neutral triglyceride lipase and an acid lysosomal lipase. In addition di- and monoglyceride lipases are also present in the myocardial cells and are responsible for complete degradation of triglycerides (Stam *et al* 1986). Both catecholamines and glucagon are proposed to stimulate myocardial lipolysis

(Severson 1979), mediated by adenylate cyclase, cyclic AMP and protein kinase A (Keely *et al* 1975).

1.3.1.2. Exogenous free fatty acids.

Fatty acids are the major source of energy in the heart (Neely and Morgan 1974, Lopaschuk 1997). Fatty acids represent a substantial fuel for respiration in heart muscle because oxidation of long chain fatty acids (LCFA) can release 6 times as much energy as the oxidation of an equal mass of glucose. The main source of fatty acids for the heart is in the form of free fatty acids (predominantly oleic acid and palmitic acid) bound to albumin. Initial uptake of fatty acids is by energy-independent reversible binding of fatty acids to the cell membrane (Spector and Steinberg 1965). Translocation across the cell membrane may be diffusion- and carrier-mediated (Paris *et al* 1978, Samuel *et al* 1976). Inside the aqueous cytoplasm, fatty acids bind to heart-type binding proteins (FABPs) and are activated to long-chain acyl-CoA by acyl-CoA synthetase (van der Vusse and Reneman 1983, Lopaschuk 1997).

The acyl groups are transferred into the mitochondria by a complex of three enzymes involving carnitine palmitoyltransferase I (CPTI), carnitine: acylcarnitine translocase, and carnitine palmitoyltransferase II (CPTII) (Eaton *et al* 1996, Lopaschuk 1997, McGarry and Brown 1997). Inside the mitochondrial matrix long-chain acyl-CoA passes through a β-oxidation enzyme system (or spiral) to produce acetyl-CoA. The acetyl-CoA produced enters the TCA cycle leading to the liberation of $2CO_2$, 3NADH and $1FADH_2$. The NADH and $FADH_2$ are oxidised via the electron transport chain, ultimately reducing molecular O_2 to

H_2O. The H^+ gradient generated by the electron transport chain drives the synthesis of ATP from ADP and Pi.

1.3.2. Carbohydrate metabolism.

The carbohydrates of major importance for cardiac energy metabolism under physiological conditions are glucose and lactate as exogenous substrates and glycogen as endogenous substrate (Drake-Holland 1983). Two energy-yielding stages of glucose metabolism exist, the glycolytic pathway leading to pyruvate and lactate, and oxidation through the TCA cycle and the respiratory chain.

1.3.2.1 Glucose.

Glucose has been shown to be an important substrate for the production of ATP specifically for membrane ion channels and pumps (Balaban and Bader 1984, Weiss and Lamp 1989), as well as having an anaplerotic role by replenishing TCA cycle intermediates (reviewed by Taegtmeyer 1994). In normal heart muscle, the availability of alternative substrates as well as plasma glucose and insulin concentration can act as the most important factors regulating glucose uptake.

1.3.2.1.1. Glucose uptake.

Specific glucose transporter proteins, GLUT-1 and GLUT-4 mediate the majority of glucose uptake by the heart muscle. The GLUT-1 isoform is involved in glucose transport in the basal metabolic state, whereas the GLUT-4 isoform is insulin sensitive and increases glucose uptake during insulin stimulation (Slot *et al* 1991). GLUT1 and GLUT4 have different affinities and maximum rates of

activity and aggregate in distinctly separate areas of tissues in their resting states. GLUT1 is readily available at the sarcolemma and although the translocation of GLUT1 can be weakly stimulated by insulin, its activity is generally regarded as insulin-independent. GLUT4 is sequestered in intracellular vesicles near the trans-Golgi region (Slot *et al* 1991). GLUT4 moves to the sarcolemma (Watanabe *et al* 1984) with insulin (James *et al* 1988, Zaninetti *et al* 1988), contraction (James *et al* 1985), ischaemia (Liedtke 1981, Opie and Camici 1992) or hypoxia (Eblenkamp *et al* 1996).

1.3.2.1.2. **Glucose metabolism.**

Glucose is catabolised via the glycolytic pathway before entering the TCA cycle. Phosphorylation of glucose by hexokinase becomes rate limiting for glycolysis at high rates of glucose transport (Manchester *et al* 1994). Once inside the cell and phosphorylated, glucose enters the glycolytic pathway.

Glycolysis is regulated by phosphofructokinase (PFK) activity modulated by levels of ATP present in the cytosol (Newsholme and Leach 1983). PFK is stimulated by ADP and Pi and inhibited by ATP. In the absence of oxygen, resulting in a decline in cytosolic ATP concentration, glycolysis is stimulated (Bailey *et al* 1981). However even working at maximal efficiency it is estimated that glycolysis could only supply 20% of the ATP necessary for normal cell function (Newsholme and Leach 1983). Under severe ischaemic conditions, glycolysis is inhibited due to the accumulation of protons and their action on PFK (Taegtmeyer 1994, Garlick *et al* 1979).

The common metabolic product of glucose, glycogen and lactate is pyruvate (Taegtmeyer 1994). The final step in aerobic glycolysis is the conversion of pyruvate to acetyl-CoA, which then enters the TCA cycle. Pyruvate provides both acetyl-CoA and oxaloacetate for the TCA cycle and is a critical intermediate when workload and turnover of the TCA cycle increase. Pyruvate crosses the inner mitochondrial membrane and is oxidised to acetyl-CoA in a reaction mediated by the multi-enzyme complex PDH. The oxidative decarboxylation of pyruvate by PDH thus plays a central role in the regulation of substrate supply to the heart, linking glycolysis and oxidative phosphorylation.

1.3.2.1.3. Pyruvate dehydrogenase enzyme complex.

PDH plays a crucial role in the oxidation of glucose, converting pyruvate into acetyl-CoA for further oxidation via the TCA cycle to CO_2 (Wieland *et al* 1971, Olson *et al* 1983, Latipaa *et al* 1985). The PDH complex contains three component enzymes: pyruvate decarboxylase, dihydrolipoate acetyltransferase, dihydrolipoyl dehydrogenase (Kerbey *et al* 1976). PDH activity is regulated by a phosphorylation / dephosphorylation cycle (Wieland and Siess 1970, Kerbey *et al* 1976), where phosphorylation by PDH kinase leads to inactivation of the complex and dephosphorylation by PDH phosphatase results in activation to the active form of PDH (PDHa), see Figure 1.5.

The PDH complex and its associated kinase and phosphatase are not influenced by cyclic AMP or cyclic AMP-dependent kinase (Kerbey *et al* 1976). The kinase and phosphatase reactions may be influenced by mitochondrial [Ca^{2+}]; Ca^{2+} and Mg^{2+} are required for the phosphatase reaction, but inhibit the kinase reaction (McCormack and Denton 1989). In addition PDH activity can be

modulated directly by the acetyl-CoA/CoA ratio, the ATP/ADP ratio and the $NADH/NAD^+$ ratio and by pyruvate concentration (Kerbey *et al* 1976).

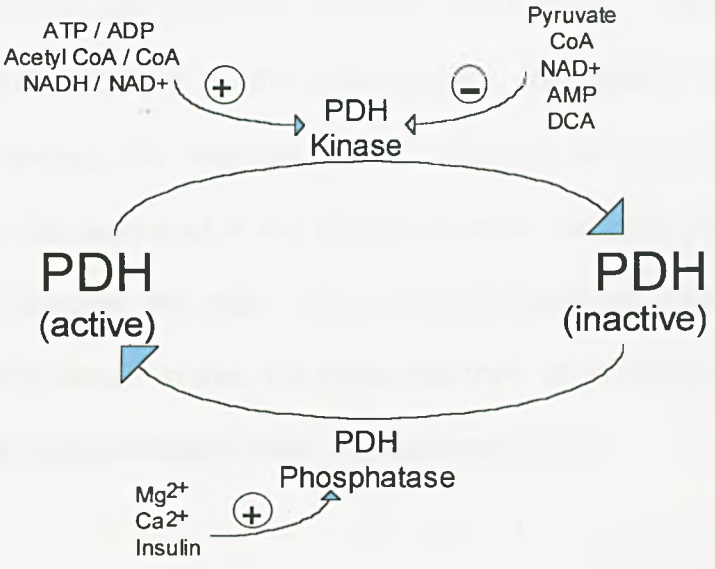

Figure 1.5. PDH enzyme regulation.

1.3.2.1.4. Insulin.

Insulin stimulation promotes glucose transport by enhancing GLUT4 response at the levels of transcription and translation. Reduced levels of GLUT4 mRNA have been reported in insulin-deficient muscle and in the streptozotocin injected rat, a model of Type 1 diabetes (Gerrits *et al* 1993). Insulin causes a rapid increase in glucose uptake in the heart (Morgan *et al* 1961). Specifically the addition of insulin to isolated rat ventricular cardiomyocytes increases GLUT4 mRNA by 200% (Petersen *et al* 1995), suggesting that insulin signalling is involved in pre-translational events in the nucleus.

1.3.2.2. Lactate.

The heart is considered both a lactate producer and consumer, in which studies have demonstrated a simultaneous extraction and release of lactate during

normal conditions in animals and humans (Guth *et al* 1990, Gertz *et al* 1981, Wisneski *et al* 1985). Lactate oxidation is dependent on the activity of lactate dehydrogenase (LDH) and pyruvate dehydrogenase (PDH). The hydrogen ion, pyruvate and NADH formed by the LDH reaction are rapidly removed in the aerobic heart, forcing the reaction in the direction of pyruvate formation. Lactate will only be produced if the oxygen supply becomes limited and the pyruvate levels become too high. It is thought lactate is used to preserve glycogen and triglyceride stores for circumstances of inadequate exogenous substrate supply (Drake-Holland 1983, Taegtmeyer 1994).

1.2.2.3. Glycogen.

Carbohydrates are stored in myocardial tissue as high molecular mass molecules known as glycogen, consisting of D-glucose units (van der Vusse and Reneman 1983). The D-glucose molecules are coupled by α(1-4) linkages and the glucose chains formed are highly branched by α(1-6) linkages. Glycogen is usually present in the form of granules, localised in the interstices surrounding the mitochondria, the inter-fibrillar sarcoplasm, the perinuclear areas and areas around the sub-sarcolemmal cisternae. Glycogen is catabolised to G-6-P by glycogen phosphorylase. This step can be accelerated by activation of phosphorylase, which exists in two forms. The b form is dependent on G-6-P, ATP and ADP concentration for its activity, whereas the a form is more active as a result of hormonal stimulated phosphorylation, by the thyroid hormones, glucagon and catecholamines, mediated through Ca^{2+} and cAMP (Drake-Holland and Noble 1983).

1.4 Substrate competition.

The reciprocal relationship between the rates of oxidation of glucose and fatty acids by muscle, such as in the heart, has been explained by a concept known as the glucose-fatty acid cycle, put forward by Randle as early as 1963 (reviewed by Newsholme and Leach 1983). Under conditions when the glycogen store in the liver is depleted, fatty acids are mobilised from adipose tissue. The resulting elevated concentration of fatty acids in the blood stream increases the rate of fatty acid oxidation in muscle, which in turn decreases the rate of glucose oxidation (Newsholme 1976). Conversely when glycogen levels are restored in liver, the rate of fatty acid release by adipose tissue is reduced, decreasing their rate of oxidation, so that the rate of glucose utilisation by the muscle increases.

Blood glucose concentration remains relatively constant despite a range of physiological states (Newsholme 1976), such that blood glucose concentration per se is unlikely to be quantitatively important in regulating the rate of fatty acid mobilisation. Instead blood ketone body concentrations, 3-hydroxybutyrate in particular, are thought to be important in reducing the blood concentration of fatty acids, as a glucose-fatty acid-ketone body cycle (reviewed by Newsholme and Leach 1983). A high concentration of ketone body could exert its effect by either reducing the rate of lipolysis in adipose tissue, increasing the sensitivity of adipose tissue to the effect of insulin, or by stimulating insulin secretion (McGarry 1998).

1.5. Cardiac hypertrophy.

1.5.1. Phenotype.

Hypertrophy is the response of the heart to chronic haemodynamic overload (Capasso *et al* 1990, Chien *et al* 1991, Komuro and Yazaki 1993, Shapiro and Sugden 1996, Sugden and Fuller 1997). It is characterised by an increase in myocyte size and an increase in protein content per cell (Swynghedauw *et al* 1990). Adult cardiac myocytes are highly specialised, terminally differentiated cells which are unable to divide. In pressure-overload (concentric) hypertrophy, the ventricular wall is grossly thickened in the absence of chamber enlargement (Smith and Bishop 1985, Campbell *et al* 1989). The increase in heart muscle mass following increased workload is the adaptive mechanism to normalise ventricular wall stress ('compensated' state). However in the longer term, ejection fraction may decrease and heart failure may ensue ('decompensated' state) (Sakata *et al* 1998). The events leading to cardiac hypertrophy can be divided into; an extracellular stimulus, intracellular signal transduction, and activation of early gene products, which allow alterations of the phenotype.

1.5.2. Hypertrophic stimulus.

Many factors can trigger hypertrophy, chief amongst them is mechanical stretch (Sadoshima *et al* 1992, Komuro *et al* 1995). Mechanical stretch of the myocytes has been associated with increased protein synthesis and a rise in both messenger and ribosomal RNA levels (Swynghedauw *et al* 1990), as well as activating multiple signalling transduction pathways (Sadoshima and Izumo 1993). In addition to mechanical effects such as stretch stimuli, several other factors may play a role in initiating cardiac hypertrophy (reviewed by Sugden

and Clerk 1998, Cooper 1997, Swynghedauw *et al* 1990). Angiotensin II is a prime candidate (Sadoshima and Izumo 1993, Pennica *et al* 1995) alongside α1-adrenergic agonists (Simpson *et al* 1991). Growth factors, such as fibroblast growth factors (Parker *et al* 1990), endothelin-1 (Arai *et al* 1995), insulin-like growth factor I (Parker *et al* 1990), cardiotrophin-1 (Wollert *et al* 1996) and phorbol esters (Henrich and Simpson 1988), can also induce hypertrophy in cardiac myocytes. In addition thyroid hormones can stimulate growth of heart chambers (Strauer and Scherpe 1975, Morgan 1989). More recently calcineurin (a calcium-calmodulin-dependent phosphatase) has been implicated (Ding *et al* 1999, Lim *et al* 2000). Calcineurin activation, regulated by increased basal calcium levels has been hypothesised as a primary pathway for the induction of hypertrophy (Molkentin *et al* 1998) and creation of cardiac dilation (Sussman *et al* 1999).

1.5.3. Signal transduction.

Signal transduction mechanisms in cardiac hypertrophy, covers a wide area of investigation, much of which is beyond the scope of this study. Briefly the three main categories of hypertrophic stimuli can act on the cell as shown simplistically in Figure 1.6. (reviewed in depth by Francis and Carlyle 1993, Hefti *et al* 1997, Glennon *et al* 1995, Sugden and Clerk 1998, Sadoshima and Izumo 1997, Schaub *et al* 1997). Firstly, growth factors bind to receptors, which have a tyrosine kinase activity (RPTKs), initiating a signalling cascade including the oncoproteins Ras and Raf-1 (Thorburn *et al* 1993, Schaap *et al* 1993). Secondly, angiotensin II, endothelin-1 and α-adrenergic agonists all bind to specific G protein coupled receptors (possibly G_i/G_o or G_q/G_{11}), resulting in

activation of the phospatidylinositol pathway and protein kinase C (Ito *et al* 1991, Sadoshima and Izumo 1993, Knowlton *et al* 1993). Thirdly, stretching of myocytes stimulates, unidentified mechanoreceptors, which may lead to increased intracellular calcium (Sadoshima *et al* 1992), and may exert indirect hypertrophic effects via paracrine mechanisms (Sadoshima *et al* 1992). The three major pathways are thought to converge on the mitogen-activated protein (MAP) cascade (Sadoshima *et al* 1992), to amplify the signals.

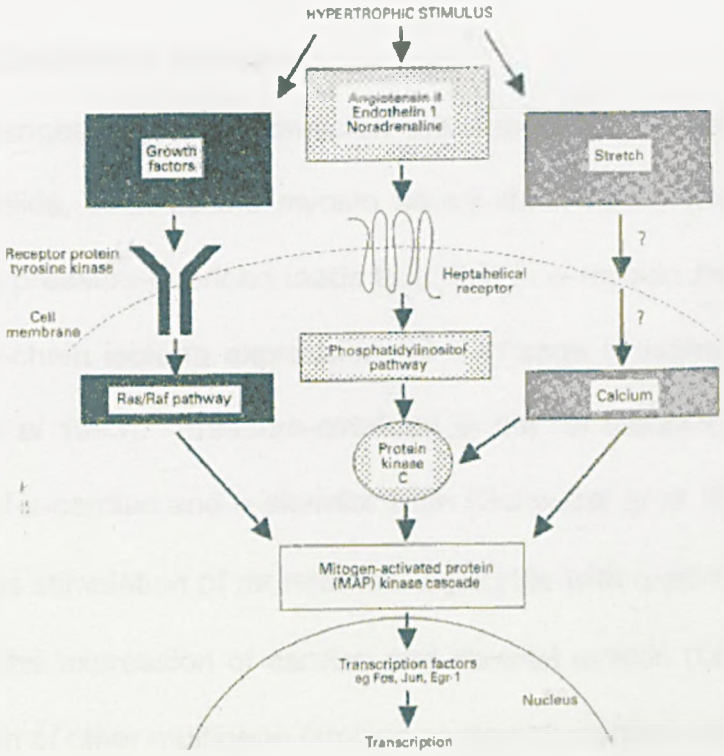

Figure 1.6. Potential signal transduction pathways leading to cardiac hypertrophy (Glennon *et al* 1995).

1.5.4. Gene expression in cardiac hypertrophy.

The changes in gene expression of non-dividing myocytes with chronic haemodynamic overload are qualitative (phenotypic conversions characterised

by isoform switches) and quantitative (characterised by modulation of individual gene expressions) (reviews by Boeheler and Schwartz 1992, Bugaisky *et al* 1992). One major feature of switches in gene expression during hypertrophy is the recapitulation of a 'foetal' gene programme, with re-expression of atrial natriuretic factor (ANF) in the ventricular myocyte (Calderone *et al* 1995). This feature is lost during normal post-natal development and can be used as a marker of ventricular hypertrophy (Zhu *et al* 1996).

1.5.4.1. Qualitative changes.

Qualitative changes in gene expression are due to differential expression of multigene families, such as the myosin heavy-chain family (Deschamps *et al* 1991). In rats, pressure-overload leads to a shift in α-myosin heavy-chain to β-myosin heavy-chain isoform expression (or a change in isomyosin V1 to V3) (van Bilsen *et al* 1993). Pressure-overload in the rat induces changes in the expressions of α-cardiac and α-skeletal actin (Schwartz *et al* 1986, Izumo *et al* 1988), whereas stimulation of rat neonatal myocytes with α-adrenergic agonists also leads to the expression of cardiac and skeletal α-actin (Long *et al* 1989). The expression of other multigene families controlling cardiac contraction is also modified in cardiac hypertrophy. Ventricular myosin light-chains–1 and –2 appear in pressure-overloaded human atria (Cummins 1982). The mRNA encoding the β-isoform of tropomyosin, is transitorily activated at the beginning of pressure-overload hypertrophy (Izumo *et al* 1988).

Changes in the expression in isoenzymes of intermediary metabolism have also been reported, such as increases in foetal type isoenzymes (BB + MB) of creatine kinase and the M-LDH isoform of lactate dehydrogenase

(Ingwall *et al* 1985). In addition an increase in RNA encoding the α3-isoform of the Na^+/K^+-ATPase membrane protein was found in rat heart hypertrophy (Sainte Beuve *et al* 1992).

1.5.4.2. Quantitative changes.

Cardiac hypertrophy growth differs from normal post-natal growth (Glennon *et al* 1995). A main feature of myocyte hypertrophy is an increased protein content per cell. At the molecular level this may result from a selective up- and down-regulation of the expression of distinct genes (Glennon *et al* 1995, Chien *et al* 1991). On exposure of myocytes to hypertrophic stimulation, immediate early genes encoding transcription factors or proto-oncogenes (c-jun, c-fos, c-myc) are rapidly and transiently up-regulated (Chien *et al* 1991, Komuro *et al* 1988, Izumo *et al* 1988).

Genes involved in tissue response to stress are also selectively up-regulated after pressure-overload, such as the heat-shock proteins HSP70, HSP68 and HSP58 (Delacyre *et al* 1988). Accumulation of mRNAs encoding transforming growth factor β1 (TGFβ1), insulin-like growth factor-I and early growth response factor-1 (Erg-1) are also found in experimental hypertrophy (Chien *et al* 1991, Schneider and Parker 1990).

In contrast, failure to up-regulate the expression of individual genes can lead to a relative decrease of the level of gene products in the hypertrophied heart. With pressure-overload, there is neither an induction of a new isoform nor an apparent activation of the gene encoding Ca^{2+}-ATPase of the SR (SERCA2a) (Boheler and Schwartz 1992). Other non-activated genes include

the Na^+ channel and the β_1-adrenergic and muscarinic receptors (Chien *et al* 1991).

1.5.5. Functional consequences at the level of protein expression.

In addition to transcriptional regulation, control is also exerted at the level of protein accumulation. Thus hypertrophic stimuli cause changes in gene expression and increase the overall rate of protein synthesis translation (Sugden and Fuller 1991). It is probable that changes in protein isoform expression may result in altered contractile characteristics and intracellular calcium handling in the hypertrophied heart as part of the consequences of these gene changes.

A switch in isoforms of α-MHC to β-MHC, results in a slower rate of ATP cycling and a lower velocity of contraction in the hypertrophied fibre (Boeheler and Schwartz 1992). Re-expression of the α- skeletal muscle actin isoform is a potential regulatory mechanism of protein interactions in the actomyosin complex in response to the development of systolic wall stress (Izumo *et al* 1987). In addition, ANF, a peptide hormone, plays a significant role in regulating blood pressure (Nakao *et al* 1986, Sugawara *et al* 1988). Its re-introduction (Hosoda *et al* 1991, Takahashi *et al* 1992) may contribute to a reduction in pre- and after-load, thus normalising pump function (Boeheler and Schwartz 1992), whereas alterations in the expression of heat shock proteins and / or antioxidant enzymes such as catalase are thought to provide protection against ischaemic injury (Yellon *et al* 1993, Black and Lucchesi 1993, Marber *et al* 1995).

Cardiac dysfunction in hypertrophy and failure is associated with abnormal handling of intracellular calcium (Dhalla *et al* 1991, Perreault *et al* 1990). The troponin complex plays an integral role in regulating muscle contraction by controlling the interaction of thick and thin filaments in response to alterations in intracellular Ca^{2+} concentrations (Winegrad 1984). Changes in the calcium binding characteristics of troponin C can be modulated through troponin I and T (Parmecek and Leiden 1991). Expression of slow skeletal troponin I in the developing heart is associated with a relative insensitivity of myofilament to Ca^{2+} activation to acidic pH range (Solaro *et al* 1988), whereas developmental changes in isoform expression of troponin T are associated with alterations in troponin C Ca^{2+} binding (McAuliffe *et al* 1990). Altered troponin I isoform expression is not a feature of hypertrophy in the rat (Cumming *et al* 1995) or the human heart at end-stage heart failure (Sasse *et al* 1993). However troponin T isoform expression changes have been reported in heart failure in humans (Sasse *et al* 1993, Anderson *et al* 1995) and in pressure-overload hypertrophy models of the guinea-pig (Gulati *et al* 1994), the rabbit (Chen *et al* 1997) but not in the rat (Cumming *et al* 1995).

Alterations in proteins associated with the uptake and release mechanisms of calcium-handling, have been observed in experimental models of pressure overload hypertrophy including decreased activity of the sarcoplasmic reticular Ca^{2+}-ATPase (De La Bastie *et al* 1990, Boateng *et al* 1997, Calderone *et al* 1995) and human heart failure (Arai *et al* 1993). The SR-Ca^{2+}-ATPase is the major protein responsible for the sequestration of cytosolic calcium. This loss of activity is thought to reduce the sequestration of Ca^{2+} during relaxation and decrease the amount of calcium available for muscle

contraction. The SR-Ca^{2+}-ATPase activity is mediated by phospholamban phosphorylation. Phospholamban expression is also depressed after pressure-overload (Arai *et al* 1993, Nagai *et al* 1989, Feldman *et al* 1991). As a result calcium transient times are prolonged with cardiac hypertrophy, which may contribute to the generation of arrhythmias and enhanced potential for sudden death (Thandroyen *et al* 1991).

In addition, the calcium-release channels (dihydropyridine and ryanodine receptors)(Arai *et al* 1993, Takahashi *et al* 1992, Brillantes *et al* 1992), the Na^+ / Ca^{2+} exchanger, (responsible for calcium extrusion) (Hanf *et al* 1988) and the Na^+ / K^+ ATPase, (responsible for Na^+-K^+ homeostasis)(Charlemagne *et al* 1995) have also been shown to alter in experimental hypertrophy and heart failure. These protein changes may also contribute to the prolongation of calcium transient times in cardiac hypertrophy.

1.5.6. Non-myocyte changes.

The myocardium is composed of cardiac myocytes and interstitial cells (including vascular endothelial cells, vascular smooth muscle cells, fibroblasts, macrophages and mast cells) (Weber *et al* 1991). The major macromolecules of the extracellular matrix connecting these cells are secreted by fibroblasts (Lin and Bissell 1993, Brilla *et al* 1995). These macromolecules include fibrous proteins (structural collagens and elastin) and cell adhesive or anti-adhesive molecules such as fibronectin, vitronectin, laminin and proteoglycans (Linn and Bissell 1993).

1.5.6.1. Reduced coronary reserve.

A lack of adaptation of the coronary circulation during left ventricular hypertrophy may be an important factor contributing to the fall in coronary reserve, increased coronary vascular resistance and under-perfusion of the subendocardium (Tomanek 1990). This inappropriate adaptation of the coronary vasculature may ultimately contribute to the development of ischaemia, arrhythmia and heart failure. Capillary volume density and intercapillary distance may be equally important (Tomanek *et al* 1991).

1.5.6.2. Fibrosis and collagen turnover.

Cardiac hypertrophy is not only due to an enlargement of myocyte size. Perivascular fibrosis of intramyocardial coronary arteries and an interstitial fibrosis can often accompany myocyte hypertrophy (Zhu *et al* 1996). Three patterns of fibrosis have been described in left ventricular hypertrophy: reactive fibrosis (growth of collagen in parallel with myocyte enlargement), excessive fibrosis (thick collagen fibres around the myocyte) and necrotic fibrosis (restorative fibrosis with tissue necrosis in the centre) (Cody *et al* 1986, Weber *et al* 1988, Weber *et al* 1989).

The interstitial collagens are major constituents of the cardiac extracellular matrix and play a crucial role in maintaining the structural and functional integrity of the heart (Weber *et al* 1989, Weber *et al* 1995). Collagen turnover in the heart is dynamic and under stringent regulation (Laurent 1987, Bishop *et al* 1994, Weber *et al* 1995). Pressure-overload cardiac hypertrophy is accompanied by an increased accumulation of fibrillar collagens in the myocardium (Weber *et al* 1988). Chapman *et al* (1990) characterised this at

both the protein and mRNA levels. It was demonstrated that mRNA levels for types I and III collagens increased in the rat myocardium during the first week after abdominal aortic constriction and can be followed by fibroblast proliferation (Lindy *et al* 1972, Morkin and Ashford 1968). Villarreal and Dillman (1992) showed similar transient increase in left ventricular collagen mRNA levels immediately after thoracic aortic banding. This accumulation of collagen might increase myocardial stiffness and ultimately lead to ventricular dysfunction (Jalil *et al* 1989, Brilla *et al* 1991).

1.5.6.3. Collagen network remodelling and diastolic stiffness.

Structural remodelling of the myocardial collagen matrix and a disproportionate accumulation of fibrous tissue, particularly in the extracellular space, is thought to contribute to the progression of LV dysfunction (Weber *et al* 1993, Jalil *et al* 1989) and eventual cardiac failure (Eghbali and Weber 1990). Addition of a stiff element, fibrillar collagen, increases myocardial diastolic stiffness and predisposes the heart cells to abnormal electrical dispersion.

In normal myocardium it is thought that elastic elements are aligned both in parallel and in series with a contractile element and is a major determinant of passive stiffness (Fry *et al* 1964). The intact myocardium requires a structural protein network to provide myocyte to myocyte connections and a three-dimensional support structure with its own important elastic properties. This support network enables the myocytes to relax or to be stretched (Weber *et al* 1993). Perimysial strands of collagen connect adjacent muscle bundles preventing muscle bundle slippage or misalignment while facilitating force transmission (Factor and Robinson 1988), whereas, coiled perimysial fibres

store energy during myocyte contraction, which assists in subsequent re-lengthening of the myocytes and relaxation of the ventricle (Factor *et al* 1988).

Even a small increase in the concentration of inelastic collagen has been shown to add substantially to myocardial stiffness (Weber *et al* 1993, Doering *et al* 1988). Factors that contribute to the functional consequences of fibrillar collagen are distribution, configuration and alignment with respect to the myocytes (Weber *et al* 1983). Collagen cross-linking is thought an important factor that could be altered in adaptations of the heart to pressure or volume overload (Brilla *et al* 1991, Anversa and Capasso 1991). Although the mechanism is not clear, the renin-angiotensin-aldosterone system (RAAS), both circulating and / or local systems, are thought to be involved (Wilke *et al* 1996, Brilla *et al* 1995, Danser 1996). These mechanisms are known to promote an excessive accumulation of collagen types I and II within the extracellular matrix of the myocardium leading to diastolic and systolic dysfunction and ultimately to congestive heart failure (Wilke *et al* 1996, Brilla *et al* 1995). In addition noradrenaline is also known to stimulate collagen synthesis and the proliferation of cultured fibroblasts (Boucek and Noble 1973).

1.5.6.4. Renin-angiotensin-aldosterone system (RAAS).

Studies suggest that the effector hormones, Ang II and aldosterone, of the RAAS are important regulators in the structural remodelling of the myocardial collagen matrix (Brilla *et al* 1995). In cultured adult cardiac fibroblasts, Ang II and aldosterone stimulate collagen synthesis, while angiotensin II inhibits matrix metalloproteinase I activity, a key enzyme for interstitial collagen degradation in the myocardium (Mohabir *et al* 1994, Zhu *et al* 1996). In addition Ang II in

culture can stimulate collagen synthesis in vascular smooth muscle cells (Katto *et al* 1991) and increase fibroblast proliferation and growth of myocytes (Morgan and Baker 1991).

The inactive decapeptide, Ang I is cleaved from the prohormone angiotensinogen by the action of the protease renin (reviewed by Dzau and Pratt 1992). Ang II is cleaved from Ang I by the action of the dipeptidyl carboxyl zinc metallopeptidase angiotensin converting enzyme (ACE) (Figure 1.7). Ang II acts as a potent vasoconstrictor playing a critical role in the regulation of vascular resistance and intravascular volume. It also increases aldosterone secretion, enhances central sympathetic outflow and catecholamine release from the adrenal medulla and promotes the release of vasopressin. Virtually all of the physiological effects of Ang II are mediated by the AT1 receptor (Matssusaka and Ichikawa 1997). It has been shown that there is an increased ACE activity and mRNA expression in rats with pressure-overload hypertrophy, in association with a higher Ang I to Ang II conversion in isolated perfused hypertrophied hearts (Eberli *et al* 1992).

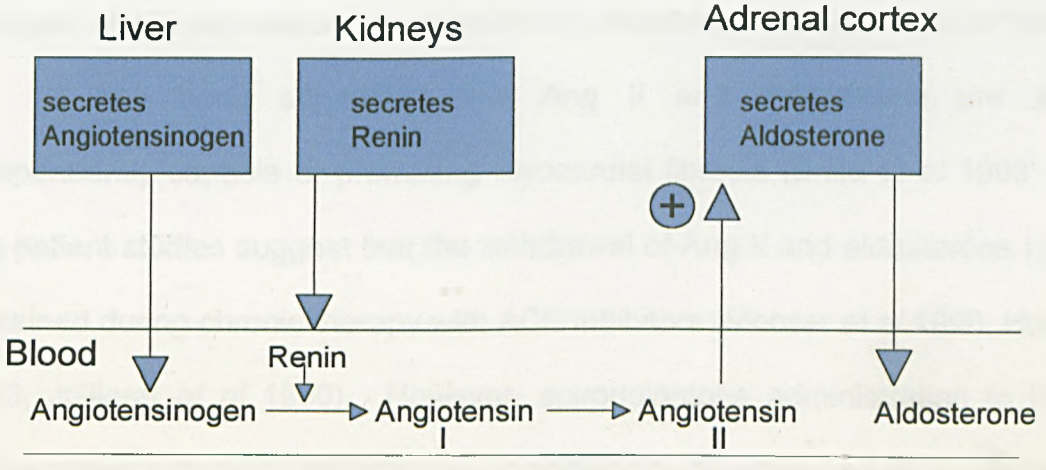

Figure 1.7. Renin-angiotensin-aldosterone interactions.

1.5.6.5. Regression with ACE inhibitors.

Further evidence for the involvement of the RAAS system in cardiac fibrosis is found in pharmacological studies. Treatment of cardiac hypertrophy with both ACE inhibitors and AT1 receptor agonists is associated with a regression of hypertrophy and fibrosis in spontaneously hypertensive rats (SHR) and animal model of renovascular hypertension as well as in hypertensive patients (Kim *et al* 1995, Panizo *et al* 1995, Nagasawa *et al* 1995, Vacher *et al* 1995, Bruckschlegel *et al* 1995, Sen *et al* 1980). Regression of both cardiac hypertrophy and fibrosis was found in SHRs treated with quinapril (ACE inhibitor) (Panizo *et al* 1995), whereas lisinopril (ACE inhibitor) treatment also reduced collagen accumulation (Brilla *et al* 1991). In the renovascular hypertensive rat model, ramipril treatment completed inhibited fibrosis without lowering the blood pressure (Nagasawa *et al* 1995). This suggested that the signalling mechanisms for cardiac fibrosis may be independent of blood pressure. In addition, administration of Enalapril, another ACE inhibitor, to cardiomyopathic Syrian hamsters (a model of cardiomyopathy and heart failure) also revealed that collagen concentration, the collagen type III:I ratio and type III collagen mRNA expression was significantly decreased (Masutomo *et al* 1996).

It has been suggested that Ang II and aldosterone are each independently capable of promoting myocardial fibrosis (Brilla *et al* 1993). In fact patient studies suggest that the withdrawal of Ang II and aldosterone is not sustained during chronic therapy with ACE inhibitors (Mooser *et al* 1990, Husain 1993, Juillerat *et al* 1990). However, spironolactone administration to heart failure patients to block aldosterone, in addition to diuretics and ACE inhibition, can positively improve heart rate variability and reduce myocardial collagen

turnover (MacFadyen *et al* 1997) and may provide a more beneficial therapeutic intervention.

1.5.7. Metabolic adaptations during hypertrophy.

1.5.7.1. Glucose use and glycolysis.

Increased glycolytic capacity (Seymour *et al* 1990) and glucose transport (Bhutta *et al* 1994) has been demonstrated in experimental cardiac hypertrophy *in vitro* and *in vivo* (Zhang *et al* 1995). It is proposed that myocardial substrate switches away from fatty acid oxidation towards glucose metabolism in hypertrophy (Allard *et al* 1994, Taegtmeyer and Overturf 1988, Christie and Rogers 1994, Massie *et al* 1995)(Figure 1.8). This is consistent with the theory of energy conservation in the hypertrophied heart, in part because the use of glucose yields 10% more ATP per mole of oxygen consumed than the utilisation of fatty acids (Taegtmeyer and Overturf 1988, Collins-Nakai *et al* 1994). It is also indicative of a regression to the foetal phenotype, in which glucose is the more predominant substrate available (Lopaschuk *et al* 1994). Accumulation of the foetal-type (BB+MB) creatine kinase isoenzymes have also been found in hypertrophied adult myocardium (Ingwall 1984).

1.5.7.2. Fatty acid oxidation.

In parallel with the changes in glucose use, there is a marked reduction in the oxidation of long-chain fatty acids such as palmitate in the hypertrophied myocardium (Chiekh *et al* 1994, Reibel *et al* 1983, El-Alaoui-Talibi *et al* 1992). This decrease in fatty acid oxidation is thought to occur due to a reduced myocardial carnitine content (Chiekh *et al* 1994, Reibel *et al* 1983, El-Alaoui-

Talibi *et al* 1992). In the presence of normal blood plasma carnitine levels, a decreased myocyte carnitine content may be produced due to impaired carnitine uptake (DiMauro *et al* 1980). Carnitine is an essential component for the transport of activated long-chain fatty acids (acyl-CoA) from the cytosol to the mitochondrion. It is thought that the reduction in carnitine content in the hypertrophied heart may be rate limiting in the translocation of activated long-chain fatty acyl groups into the mitochondria and therefore depress the rate of long-chain fatty acid oxidation.

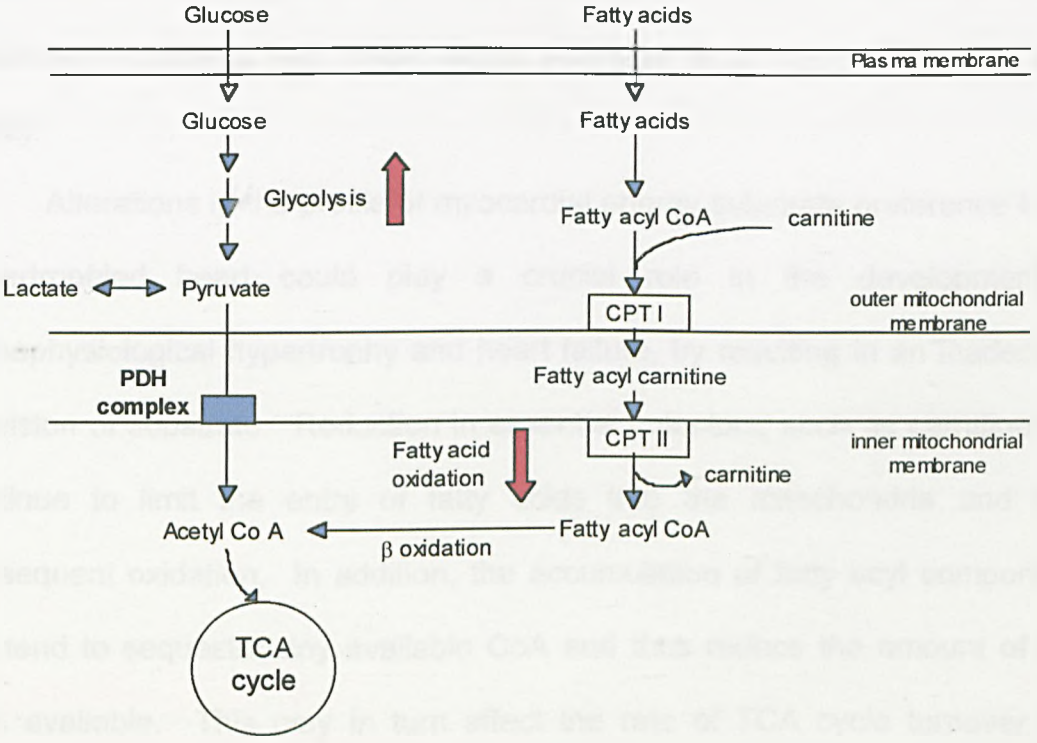

Figure 1.8. Metabolic adaptations in cardiac hypertrophy.

1.5.7.3. Metabolic role in cardiac dysfunction and heart failure.

Cardiac hypertrophy has been shown to be associated with impaired contractile function and an enhanced susceptibility to ischaemic injury in studies of both animal models and humans (Anderson *et al* 1990, Schaper *et al* 1978, Gaasch

et al 1990). The mechanisms underlying these effects are not clearly understood, but one hypothesis proposes that the hypertrophied heart is in an energy deprived state due to an imbalance between energy production and utilisation (Katz 1990, Ingwall 1993). Evidence that the hypertrophied failing heart is in an energy-deprived state arises from studies of myocardial energy metabolism in animal models of cardiac hypertrophy and failure, showing reduced levels of phosphocreatine alongside impaired cardiac performance (Seymour *et al* 1990, Field *et al* 1994, Raine *et al* 1993, Osbakken *et al* 1993, Buscer *et al* 1990, Ingwall *et al* 1990, Massie *et al* 1995). This has also been observed in patients with heart failure (Conway *et al* 1991, Neubauer *et al* 1992).

Alterations in the profile of myocardial energy substrate preference in the hypertrophied heart could play a crucial role in the development of pathcphysiological hypertrophy and heart failure, by resulting in an inadequate provision of substrate. Reduction in essential cofactors, such as carnitine, will continue to limit the entry of fatty acids into the mitochondria and their subsequent oxidation. In addition, the accumulation of fatty acyl components will tend to sequester any available CoA and thus reduce the amount of free CoA available. This may in turn affect the rate of TCA cycle turnover and contractile function (Russell and Taegtmeyer 1992). Alterations in the amount of available CoA will also have consequences for the oxidation of glucose (Timmons *et al* 1996). A decrease in available CoA will increase the acetyl-CoA to CoA ratio, an important factor in the regulation of pyruvate dehydrogenase, the key enzymatic step in the oxidative use of glucose. In consequence, despite an increased reliance on glucose metabolism, the hypertrophied heart

may have a limited capacity to use both glucose and fatty acids and become energy depleted and unable to function.

1.6. Heart failure.

Heart failure is generally characterised in terms of altered pump function and abnormal circulatory dynamics (Katz 1990). Heart failure can simply be defined as the inability of the heart to meet the haemodynamic demands of the body. This cardiac output deficiency is characterised by systolic and diastolic abnormalities (Alpert *et al* 1995), in which the velocity of contraction and rate of pressure development as well as the rate of relaxation are decreased in heart failure. Systolic dysfunction is characterised by a decreased left ventricular ejection fraction and an increase in systolic and end-diastolic volume. Diastolic dysfunction is characterised by an increased left ventricular end-diastolic pressure in association with normal end-diastolic volume, normal / reduced systolic volume and a preserved ejection fraction (Lorell 1991, Lorell *et al* 1990).

1.6.1. Functional changes.

Ventricular hypertrophy initially develops as a beneficial response to the increased pressure overload, termed the 'compensatory phase' when no signs of haemodynamic failure are present. In order to cope with an increase in pressure or volume overload, the heart can alter its mechanical performance initially, by modifications of the excitation-contraction-relaxation coupling (such as length of fibre, adrenergic stimulation and frequency of stimulation), (Pouleur 1990). In this phase the muscle function (defined by the velocity of shortening

for a given load) is normal and pump fuction (the ejection volume) is increased by ventricular dilation (Crozatier 1990). During the creation of the overload, the ventricle adapts by using its 'pre-load reserve' (where the sarcomeres are stretched to reach their maximum length). In the longer term structural alterations occur. Muscle function remains normal and pump function is augmented by the dilation of the ventricle but not by further lengthening of the sarcomeres, which are already maximal. This produces a misalignment of the z-bands, with a slippage of myofibrils and the creation of new sarcomeres. Therefore during hypertrophy the preload remains maximal and the ventricular function is increased (Levick 1995).

Myocardial failure appears when there is a mismatch between the afterload and preload reserve (Ross 1976), known as 'decompensatory cardiac hypertrophy. In this case chronic mechanical overloading of the heart regularly becomes associated with a progressive decline in myocardial contractility, accompanied by myocardial diastolic stiffness.

At the molecular level, the alterations in myocardial performance that lead to failure are directly dependent upon the characteristics of the myosin-actin cross-bridge cycle in conjunction with the degree and rate of myocardial activation and inactivation (calcium cycling) (Huxley 1957, Lymm and Taylor 1971). Specifically the calcium cycling proteins SR Ca^{2+}-ATPase, phospholamban and the ryanodine receptor are depressed in failing hearts (Nagai *et al* 1989, Arai *et al* 1991, Mercadier *et al* 1990, Feldman *et al* 1991). Depressed contractile protein ATPase activity is also associated with a decrease in the velocity of shortening (Alpert *et al* 1992) and thought to contribute to failure.

1.6.2. Adaptations to reduced cardiac output.

Clinical manifestations reflect inappropriate circulatory responses to the chronic low output (Zelis *et al* 1988). In acute heart failure, blood pressure is generally maintained by vasoconstriction and later by salt and water retention (Morse and Bing 1961), both mediated by neurohumoral stimulation. Increased levels of circulating aldosterone augment distal renal tubular resorption of sodium and water, leading to an expansion of intravascular volume. This is a short-term compensation for decreased cardiac output. However, this fluid and sodium retention produces volume overload leading to deterioration of haemodynamic conditions. Subsequently the cardiac output decreases further, producing a drop in renal blood flow, which continues to induce the RAAS (Zannad 1995). Ultimately left ventricular dysfunction, by increasing left atrial pressure, can cause dyspnea, pulmonary congestion and pulmonary oedema (Katz 1992). In addition aldosterone enhances urinary potassium and magnesium excretion. The consequence of these electrolyte disturbances can include myocardial electrical instability and myocyte death (Zannad 1995).

1.7. Nuclear magnetic resonance (NMR) spectroscopy.

Magnetic resonance spectroscopy is a non-invasive technique that uses a powerful external magnetic field and radio-frequency pulses to detect the magnetic resonance signal of nuclei, which possess magnetic moment (Gadian 1982). Several atomic nuclei are biologically suitable for magnetic resonance spectroscopy, these include hydrogen (^{1}H), carbon (^{13}C), sodium (^{23}Na), phosphorus (^{31}P), nitrogen (^{15}N), oxygen (^{17}O) and potassium (^{39}K).

1.7.1. Carbon-13 NMR Spectroscopy.

Carbon-13 is a stable isotope, with a natural abundance of only 1.1%, so it can be used as an isotope tracer in perfusion studies. ^{13}C NMR spectroscopy and ^{13}C-labelled substrates are becoming increasingly used to study cardiac metabolism (Bailey *et al* 1981, Chatham and Forder 1993, Chatham and Glickson 1992, Neurohr *et al* 1983, Weiss *et al* 1989). If hearts are perfused with ^{13}C-labelled substrates, the various intermediates of metabolism become labelled with ^{13}C. ^{13}C-NMR spectroscopy provides a continuous, non-destructive method of quantifying these ^{13}C-labelled metabolic intermediates, if present at sufficient concentrations.

^{13}C-NMR spectroscopy can be used to study glucose uptake in intact hearts. The use of ^{13}C-glucose allows one to follow the metabolic fate of glucose, including incorporation of ^{13}C into the glycogen pool (Garlick and Prichard 1993, Shulman *et al* 1990). The adaptation of isotopomer analysis of ^{13}C natural abundance or labelled compounds permits the analysis of flux through specific pathways, particularly the TCA cycle and glycogen turnover (Lewandowski 1992, Malloy *et al* 1987, Weiss *et al* 1989, 1992, Laughlin *et al* 1992).

The approach is particularly useful in measurement of absolute TCA cycle flux. The main parameter measured being incorporation of ^{13}C into the glutamate pool. Pre-steady-state incorporation of ^{13}C into the tissue glutamate pool has been shown to correlate with oxidative flux through the TCA cycle (Veerkamp *et al* 1986, Lopaschuk *et al* 1986, Chance *et al* 1983, Weiss *et al* 1992, Malloy *et al* 1987, Robitaille *et al* 1993, Cohen *et al* 1994, Chatham *et al* 1995, Yu *et al* 1995, Lewandowski 1992, Sherry *et al* 1988, 1985, 1992). The

investigators have focussed on the substrate preference of the heart and quantitative metabolic flux through the oxidative and anaplerotic routes of the TCA cycle, using several specifically ^{13}C-enriched substrates. Other studies have concentrated on measurements of relative TCA cycle flux based on the steady-state distribution of label in glutamate (Lewandowski 1992, Malloy *et al* 1987, Malloy *et al* 1990, Weiss *et al* 1989). Using this technique it is possible to investigate substrate selection and measure the contribution of each substrate to the energy provision of the perfused heart.

^{13}C-NMR spectroscopy has also been successfully used to investigate metabolic compartmentation in the perfused heart (Chatham and Forder 1996, Yu *et al* 1995). ^{13}C-glucose NMR spectroscopy has also been used to measure glucose metabolism in various pathological states such as acute ischaemic hibernation (McNulty *et al* 1996) and diabetes (Chatham and Forder 1993, 1997).

Many advantages are associated with ^{13}C-NMR spectroscopy (Malloy *et al* 1993, Jeffrey *et al* 1991). Firstly ^{13}C-NMR spectroscopy allows direct measurement of flux through various pathways of energy metabolism. Secondly the contribution of individual energy substrates to TCA cycle activity can be measured, allowing substrate competition to be assessed by a single carbon-13 experiment. In addition the use of ^{13}C-labelled energy substrates is amenable to in vivo studies (Robitaille *et al* 1993).

1.8. Objectives.

It is clear that hypertrophy of the heart leads to both biochemical and functional abnormalities that contribute to the deterioration of the heart. However the

relationship between these two elements remains undetermined. The objective of this study was to investigate the relative contribution of different substrates towards energy provision in the hypertrophied heart. Also to determine the relationship between these changes and the abnormalities in cardiac contractile function that develop in hypertrophy and lead to heart failure. These studies aimed to clarify the biochemical mechanisms involved in the development of heart failure and provide new rationales to improve the prognosis of patients with left ventricular hypertrophy and heart failure. The aims were:

- To test the hypothesis that the pressure-overload hypertrophied heart is in an energy-deprived state due to inadequate provision of substrate.
- To determine the sequence of metabolic adaptations that occur during the progression of hypertrophy.
- To test whether glucose oxidation in the hypertrophied heart is inhibited at the level of pyruvate dehydrogenase as a result of reductions in cellular co-factors, such as carnitine and the amount of free CoA available.
- To investigate whether a limitation of energy in the hypertrophied myocardium results in impaired cardiac function and is the underlying cause of the increased susceptibility of the heart to heart failure.

Chapter two:

Materials and Methods.

2.1. Materials.

Biochemicals, insulin, Chelex, and diagnostic kits for glucose, triglycerides, lactate and β-hydroxybutyrate were obtained from Sigma Chemical Company Ltd, Dorset. Diagnostic kits for free fatty acids, glutamate, glutamine, and enzymes were obtained from Boehringer Mannheim Biochemical Ltd, Sussex. Intergen Company, New York, USA, supplied fatty acid free bovine serum albumin. ^{13}C Labelled isotopes ([1-^{13}C]-D-glucose, [1-^{13}C]-hexanoate and [U-$^{13}C16$]-palmitic acid), were supplied by Cambridge Isotope Laboratories, Andover, USA. Deuterium oxide was obtained from Goss Scientific Instruments Ltd, UK. All other chemicals were obtained from BDH and were of analaR reagent grade. Thiovet (sodium thiopentone) was obtained from C-Vet Veterinary Products, Leyland; Ketaset (ketamine) from Willows Francis Veterinary, West Sussex; Antisedan (Atipamezole hydrochloride) was obtained from Pfizer Ltd, Kent; Dormitor (medetomidine hydrochloride) from SmithKline Beecham Animal Health, Surrey and Amfipen (Ampicillin) from Intervet UK LTD, Cambridge. Water was of 18MΩ quality in all experiments.

2.2. Model of cardiac hypertrophy.

Male Sprague-Dawley rats (Charles River Inc. Kent) were operated on and cared for in accordance with the Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act 1986. Pressure overload cardiac hypertrophy was induced surgically in male Sprague-Dawley rats, (weighing 220-250g), by constriction of the abdominal aorta based on the method of Boateng *et al* (1997).

Food intake was restricted for 12 hours prior to surgery. Animals were anaesthetised with an intraperitoneal injection containing a mixture of ketamine/ medetomidine/ sterile water (ratio 2.4:1:9). A laparotomy was performed and the abdominal descending aorta was exposed between the right and left renal arteries. The aorta was then partially ligated (Figure 2.1), using 3mm braided silk suture (Ethicon, Johnson and Johnson International Manufacturers, Brussels, Belgium) and 0.7mm OD blunted Yale spinal needle (Becton Dickinson, New Jersey, USA), Boateng *et al* (1997). The needle was subsequently removed and the left kidney observed to ensure that blood supply had been fully restored. 4 mls of sterile saline was administered directly into the abdominal cavity, to allow for any loss of fluid.

The abdominal cavity was closed using catgut (Chromic suture 3/0, Ethicon) and the cutis layer using a monofilament polyamide suture thread (Ethicon). Animals received intra-peritoneal injections of antibiotic (Amphipen 30 mg/Kg body mass) and an anaesthetic reversal agent (Antesedan 1.4 mg/Kg body mass). Sham operated control animals were subjected to the same procedure, but no aortic constriction was performed. Animals were allowed to recover, housed in groups of three or four and maintained for nine or fifteen weeks post surgery with *ad libitum* access to food and water.

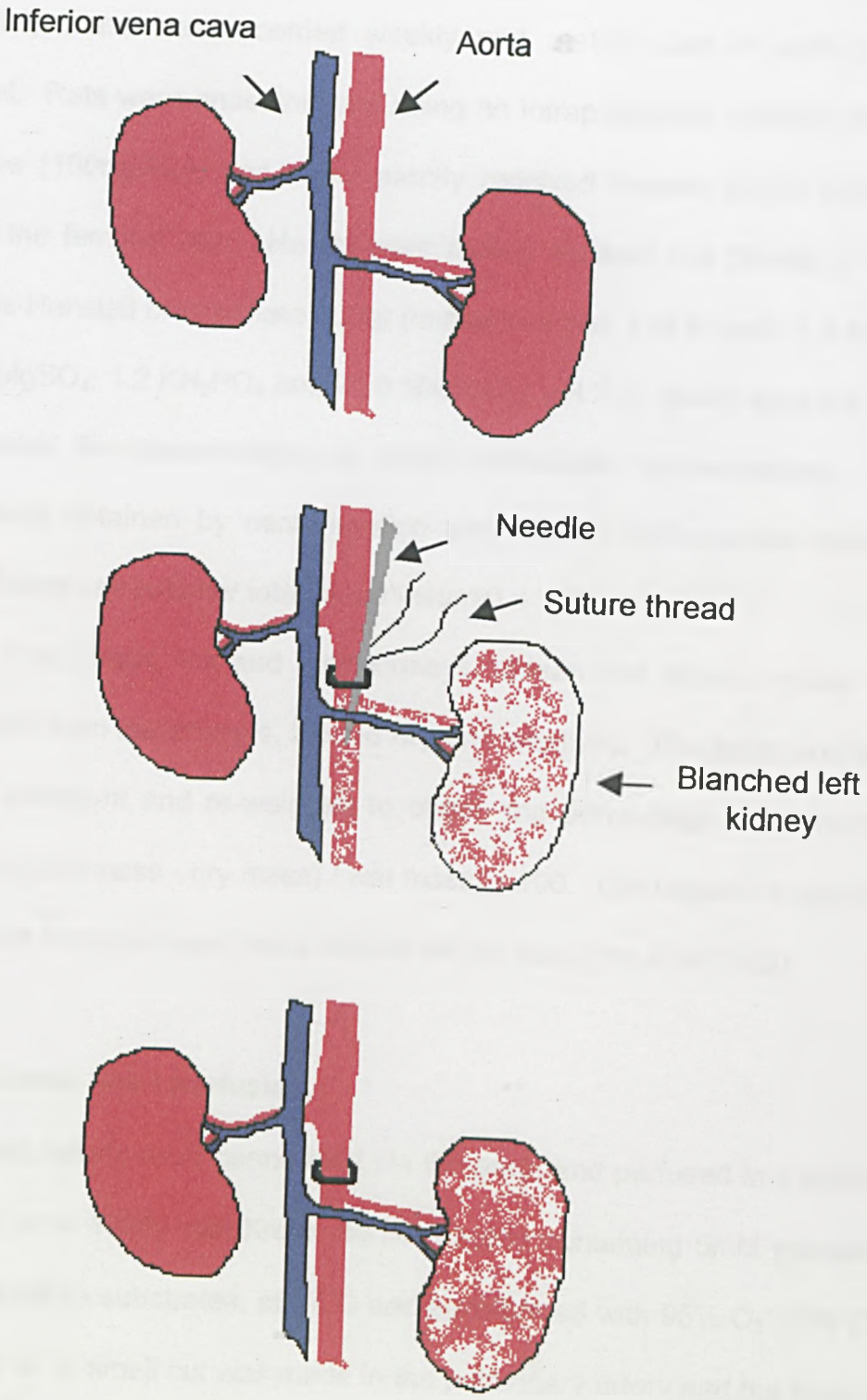

Figure 2.1. Surgical procedure.

2.2.2. Tissue collection and morphology.

Animal body mass was recorded weekly and at the start of each perfusion experiment. Rats were anaesthetised using an intraperitoneal injection of sodium thiopentone (100mg/Kg), and subsequently received heparin (1000 IU/Kg body mass) via the femoral vein. Hearts were rapidly excised and placed in ice cold (4°C) Krebs-Hensleit bicarbonate buffer (containing mM: 118.5 NaCl, 4.8 KCl, 1.25 $CaCl_2$ 1.2 $MgSO_4$, 1.2 KH_2PO_4 and 25.0 $NaHCO_3$), pH 7.4. Blood samples (10mls) were collected for determination of serum metabolite concentrations. Serum fractions were obtained by centrifugation and packed cell volumes determined using (red blood cell volume/ total blood volume) x 100.

The liver, lungs, left and right kidneys, spleen and adrenal gland organs were removed from the animals, blotted dry and weighed. The lungs and kidneys were dried overnight and re-weighed to obtain the percentage water contained, calculated by (wet mass - dry mass) / wet mass) x 100. The degree of hypertrophy was evaluated from the heart mass to tibial length ratio (Yin *et al* 1982).

2.3. Isovolumic heart perfusion.

Once removed, hearts were cannulated via the aorta and perfused in a retrograde mode (Ogino *et al* 1996) with Krebs-Hensleit buffer containing 5mM glucose and 0.3mM fatty acid as substrates, at 37°C and equilibrated with 95% O_2 / 5% CO_2 to maintain pH 7.4. A small cut was made in the pulmonary artery and the heart apex punctured with a needle to facilitate Thebesian drainage. Hearts supplied with glucose and hexanoate as substrates were perfused through constant pressure of

85cm H_2O (63.5mmHg) using a Langendorff rig. Where palmitate was used as a substrate, hearts were perfused with 3% albumin under constant flow mode using an oxygenator apparatus (P. Brookes, Scientific Glassblowers, Witney)(Gamcsik *et al* 1996, Figure 2.2). Hearts were perfused at a flow rate equivalent to 85cm H_2O in the Langendorff mode, using a continuous calibrated pump (MHRE/22 Mk.3 Flow Inducer, Watson-Marlow).

2.3.1. Physiological measurements.

Left ventricular function was monitored using a balloon catheter made of cling film, inserted into the left ventricle (Figure 2.3(a), Ogino *et al* 1996). The balloon was connected via a fluid filled line to a physiological pressure transducer (SensoNor), linked to a MacLab recording system (AD instruments). The balloon volume was inflated using a 2ml micrometer syringe, to obtain an end-diastolic pressure between 5-10 mmHg (Ogino *et al* 1996). Figure 2.3(b) shows a typical functional trace obtained during a perfusion experiment.

Systolic and diastolic pressures and heart rates were continuously recorded. Contractile function was assessed by calculating the left ventricular developed pressure (LVDP) (systolic pressure - diastolic pressure) and rate pressure product (RPP) (heart rate x LVDP), from these measurements, averaged over the whole perfusion length. Hearts with less than 20 x 10^3 mmHg/min were excluded from the studies. Coronary flow was monitored periodically throughout the perfusions and remained consistently within the range of 13-15 mls per minute.

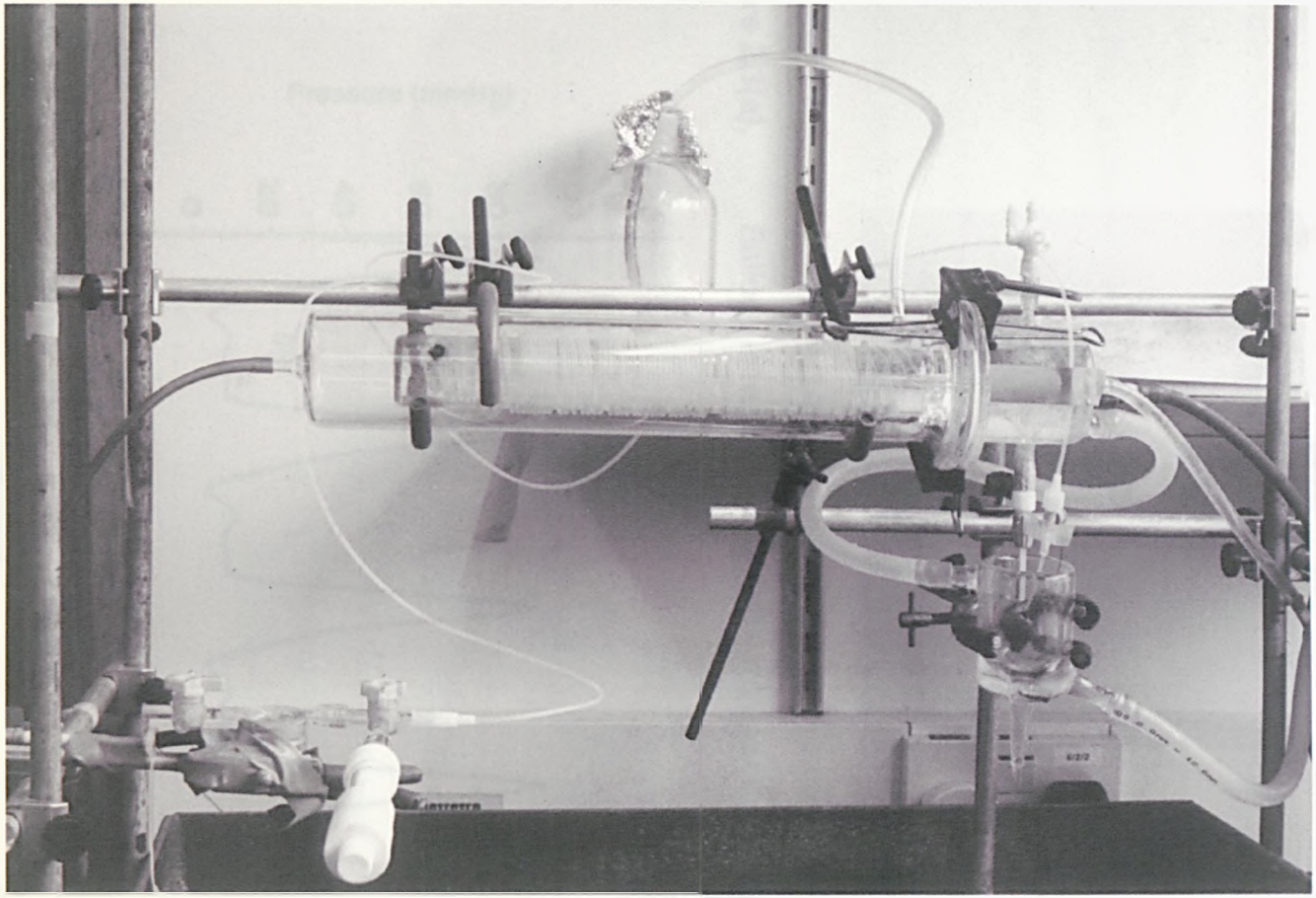

Figure 2.2. Oxygenator perfusion apparatus.

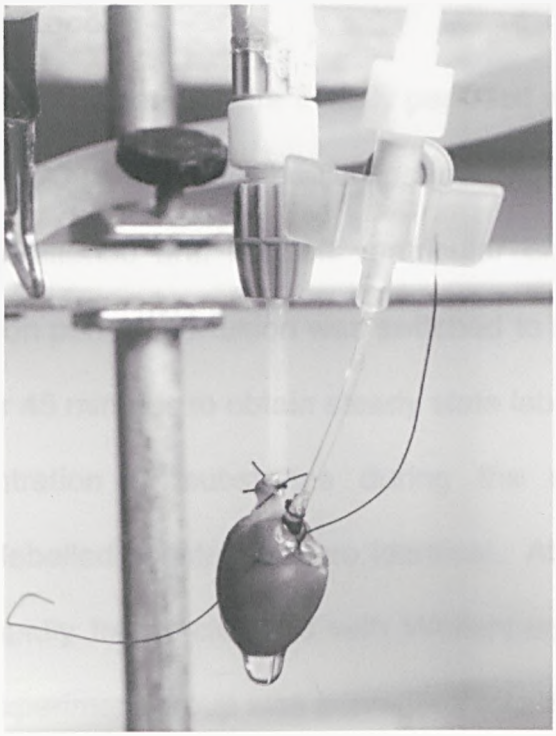

Figure 2.3(a). Balloon inserted into the left ventricle of a cannulated heart.

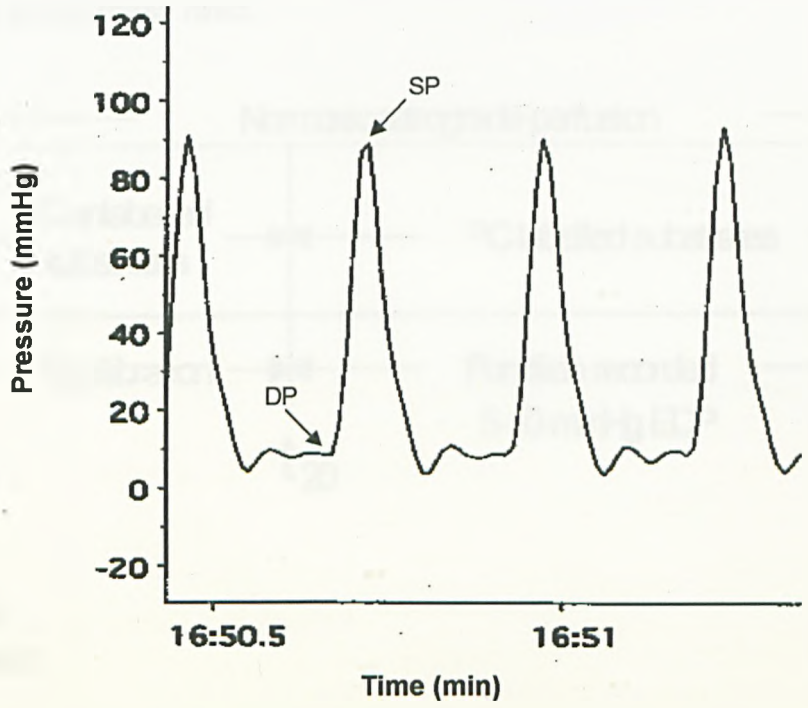

Figure 2.3(b). A typical example of a functional trace.

2.3.2. Perfusion protocol.

Hypertrophied and control hearts were initially perfused with buffer containing only ^{12}C substrates, for a 20 minute equilibration period (Figure 2.4). During this time the balloon was positioned and the left ventricular end-diastolic pressure set. After the equilibration period, perfusion was switched to the ^{13}C labelled substrate mixture for a further 45 minutes to obtain steady state labelling.

The concentration of substrates during the equilibration period and perfusion with ^{13}C-labelled substrates were identical. At the end of the perfusion the hearts were rapidly freeze-clamped with Wollenberger tongs. Frozen heart tissue from each experiment group was pulverised into a fine powder under liquid nitrogen using a mortar and pestle, and stored in liquid nitrogen for further analysis. A small portion of this ground tissue was used for determination of the wet mass to dry mass ratio.

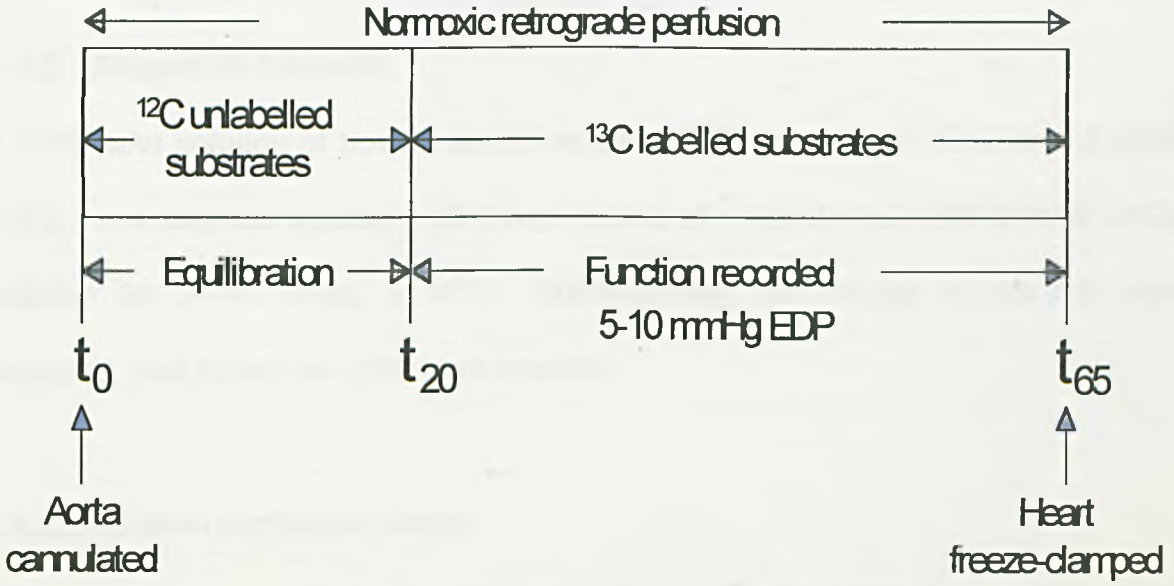

Figure 2.4. Schematic diagram of perfusion protocol.

2.4. Preparation of bovine serum albumin (BSA) containing buffer.

Hearts were perfused with Krebs-Hensleit bicarbonate buffer containing 3% bovine serum albumin (fatty acid free), which had been dialysed against 2.5mM $CaCl_2$ and 118mM NaCl to remove any small molecular mass impurities and saturate the calcium binding sites.

2.4.1. Preparation of dialysis tubing.

Dialysis tubing (10-20cm in length) with a molecular mass cut off of 12-14,000 Daltons, (Medicell) was boiled for 10 minutes in a large volume of 2% (w/v) Na_2CO_3 and 1mM EDTA (pH 8.0). The tubing was rinsed thoroughly and boiled again for 10 minutes in 1mM EDTA (pH 8.0). The tubing was then allowed to cool, and rinsed prior to cold storage. The tubing was handled with gloves to avoid contamination and rinsed thoroughly before use.

2.4.2. Dialysis of Albumin.

A 30% (w/v) solution of bovine serum albumin (BSA) in 118mM NaCl and 2.5mM $CaCl_2$, was dialysed against a 20 times volume of 118mM NaCl and 2.5mM $CaCl_2$ solution for 24-48 hours, at 4°C. Subsequently the volume of albumin was recorded, and frozen at -20°C until required.

2.4.3. Albumin perfusion buffer.

Albumin containing buffer was made up freshly on the day perfusion by diluting the 30% BSA 10 fold with distilled water to give a final concentration of 3% BSA buffer

(w/v). The other components of Krebs-Hensleit bicarbonate buffer and any substrates were dissolved in this buffer (with appropriate corrections for $CaCl_2$ (2.5mM) and NaCl (118mM). The palmitate (either ^{13}C labelled or unlabelled) was first dissolved in hot distilled water before adding to the BSA buffer. The BSA buffer was filtered through 5μm and 0.45μm low protein binding filters (AP and AW type filters, Millipore) prior to use. The concentration of Ca^{2+} and Na^{+} electrolytes (mean +/- SEM) within the BSA buffer were determined using a blood gas analyser (ABL5 Radiometer Medical A/S, Copenhagen, Denmark) given in Table 2.1.

Treatment Groups	pH	K^{+} mmol/l	Na^{+} mmol/l	Ca^{2+} mmol/l	Cl^{-} mmol/l
Control (n=8)	7.05 (0.10)	5.6 (0.05)	134.7 (0.92)	1.21 (0.05)	110.8 (0.92)
Hypertrophy (n=9)	7.04 (0.13)	5.6 (0.05)	134.9 (0.88)	1.16 (0.08)	110.7 (0.88)
Control + Insulin (n=4)	7.26 (0.27)	5.6 (0.05)	134.3 (1.29)	1.14 (0.11)	110.7 (1.17)
Hypertrophy + Insulin (n=4)	7.47 (0.24)	5.6 (0.05)	134.4 (1.53)	1.06 (0.11)	111.3 (1.25)

Table 2.1. Electrolyte ion concentrations in albumin containing buffers used in Chapter 3.

2.5. Preparation of sodium salt of palmitic acid from free acid.

[U-^{13}C]-palmitic acid was converted to [U-^{13}C]-sodium palmitate using the method

of Wolfe *et al* (1980). 30.9mM NaOH was made up in 80% methanol and 0.3 mmoles of palmitic acid was dissolved in 25mls of hexane in a round-bottomed flask, and 10mls of the NaOH/MeOH solution was added to the hexane/palmitic acid solution. This mixture was briefly heated to 60°C in a water bath, mixed and evaporated to dryness with N_2 for several hours. The resulting white solid sodium salt was stored desiccated at –20°C until required.

2.6. Tissue metabolite concentrations.

2.6.1. Perchloric Acid Extraction.

Frozen ground cardiac tissue was extracted with 6% perchloric acid (PCA) (Seymour *et al* 1990). To a known mass of tissue was added ice-cold 6% perchloric acid in a ratio of 5:1 (1ml of 6% PCA to 200mg of tissue), mixed and left on ice for 10 minutes. The resultant mixture was centrifuged at 4°C for 10 minutes at 600g, to remove the precipitated protein. A known volume of supernatant was neutralised using 6M KOH (approximately 50-100μl), at 4°C. All volumes were accurately recorded. Subsequently, the sample was centrifuged again and the resulting supernatant decanted and stored at -70°C, for further analysis. Aliquots were assayed for glutamate, glutamine, PCr and ATP concentrations. For NMR extractions, the resulting supernatant was lyophilised and stored desiccated at -20°C until reconstitution.

2.6.2. Glutamate and Glutamine assay.

The concentrations of glutamate and glutamine in PCA heart extracts were

determined using a diagnostic kit from Boehringer Mannheim (Cat. No. 139 092). Absorbance was measured at 492nm, 25°C.

$$\text{L-Glutamate} + NAD^+ + H_2O \xrightarrow{\text{GIDH}} \text{oxoglutarate} + NADH + NH_4^+$$

$$NADH + INT + H^+ \xrightarrow{\text{Diaphorase}} NAD^+ + \text{formazan}$$

$$\text{L-Glutamine} + H_2O \xrightarrow{\text{Glutaminase}} \text{L-glutamate} + NH_3$$

2.6.3. Phosphocreatine and ATP assay.

Phosphocreatine and ATP concentrations were assayed spectrophotometrically using a modified method of Bergmeyer (1974), at 340nm and 25°C (Table 2.2).

$$PCr + ADP \xrightarrow{CK} \text{Creatine} + ATP$$

$$ATP + \text{glucose} \xrightarrow{HK} \text{G-6-P} + ADP$$

$$\text{G-6-P} + NADP^+ + H_2O \xrightarrow{\text{G6PDH}} \text{6-Phosphogluconic acid} + NADPH + H^+$$

The initial absorbance (A1) was read prior to the addition of HK. After 45 minutes incubation at 30°C absorbance (A2) was measured from which ATP concentrations were determined. Absorbance (A3) was read following 45 minutes at 30°C after the addition of CK, from which PCr concentrations were determined. Concentrations of ATP and PCr were calculated using the extinction coefficient 6.22 for NADP at 340nm. Standard curves for ATP and PCr are given in Figures 2.5 and 2.6.

Pipette into cuvette	Volume (ml)	Concentration in assay mixture
Reagent mixture	0.95	17.5mM TRIS buffer (pH 7.5) 0.035% NADP.$3H_2O$ 288mM $MgCl_2.6H_2O$ 1mM Glucose G-6-PDH (2 μg/ml ~ 240 mU/ml)
Sample	0.05	
Hexokinase	0.005	3.3 μg/ml ~ 470 mU/ml
ADP	0.005	17.3mM ADP
Creatine kinase	0.005	17 μg/ml ~ 306 mU/ml

Table 2.2. Reagents in cuvettes for PCr and ATP assay.

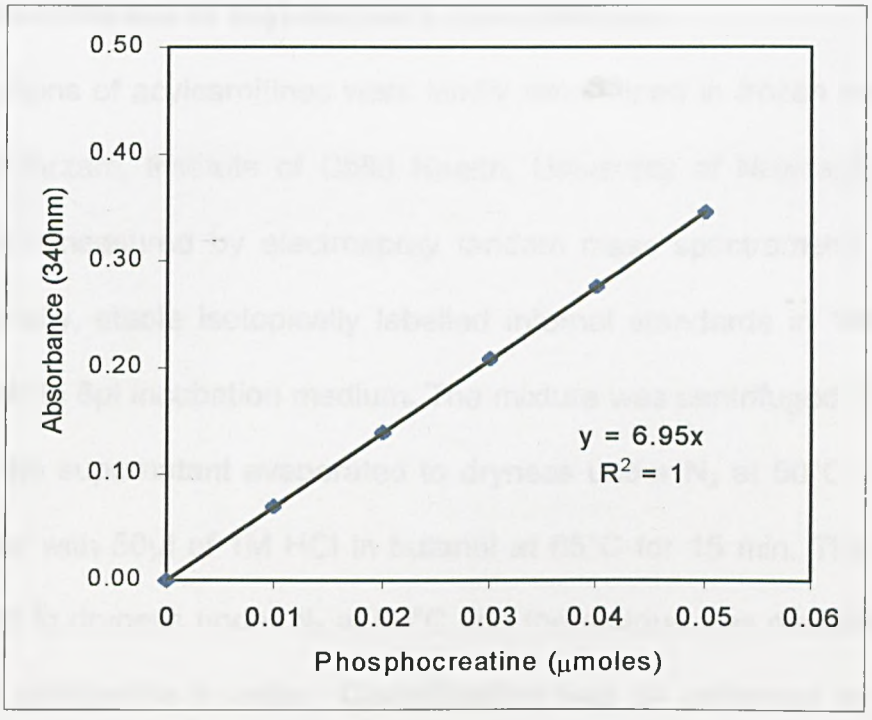

Figure 2.5. Standard curve for PCr assay.

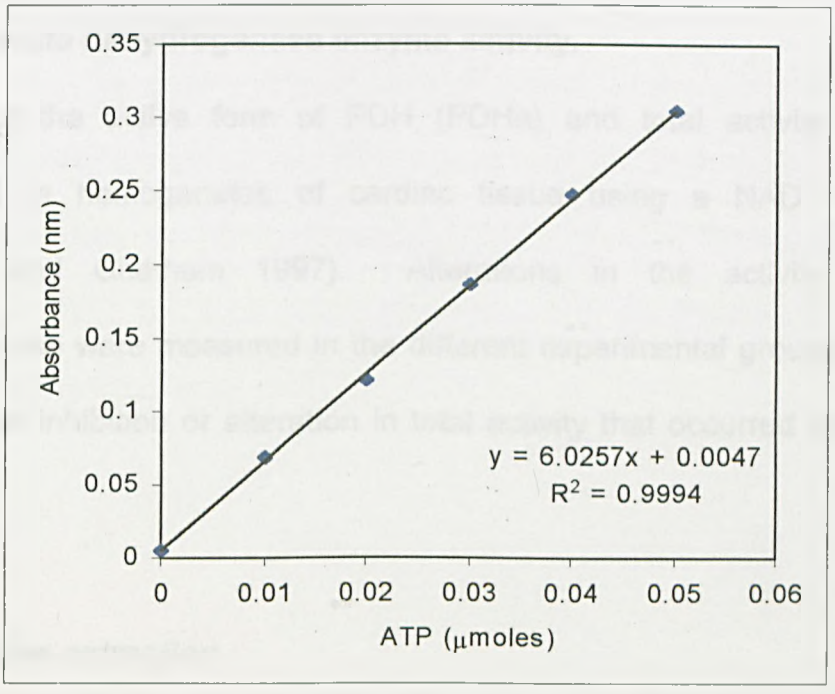

Figure 2.6. Standard curve for ATP assay.

2.6.4. Determination of acyl-carnitine concentration.

Concentrations of acylcarnitines were kindly determined in frozen heart tissue by Dr M Pourfarzam, Institute of Child Health, University of Newcastle. Carnitine esters were measured by electrospray tandem mass spectrometry (Eaton *et al* 1996). Briefly, stable isotopically labelled internal standards in 100µl methanol were added to 5µl incubation medium. The mixture was centrifuged (11500 g for 2 min) and the supernatant evaporated to dryness under N_2 at 50°C. The residue was treated with 50µl of 1M HCl in butanol at 65°C for 15 min. The mixture was evaporated to dryness under N_2 at 60°C and the residue was dissolved in 50µl of 60% (v/v) acetonitrile in water. Quantification was by reference to the internal standards.

2.7. Pyruvate dehydrogenase enzyme activity.

Activities of the active form of PDH (PDHa) and total activity (PDHt) were determined in homogenates of cardiac tissue using a NAD^+ based assay (Seymour and Chatham 1997). Alterations in the activity of pyruvate dehydrogenase were measured in the different experimental groups to determine the extent of inhibition or alteration in total activity that occurred due to differing substrates.

2.7.1 Enzyme extraction

Active form of PDH:

The active fraction of PDH (PDHa) was extracted under conditions where both

PDH phosphatase and PDH kinase were inhibited (Seymour and Chatham 1997), (Table 2.3).

Pipette into eppendorff	Volume ratio	Concentration in active PDH extraction buffer
Extraction buffer : tissue	5 : 1	25mM HEPES 25mM KH_2PO_4 25mM KF 1mM Dichloroacetate 3mM EDTA 1mM ADP 1mM Dithiothreitol 0.05 mM Leupeptin 1% Triton X-100 pH 7.0

Table 2.3. Reagents in active PDH extraction buffer.

Total form of PDH:

Total PDH enzyme activity (PDHt) was extracted under conditions where PDH phosphatase was stimulated and PDH kinase was inhibited, based on the method of Paxton and Sievert (1991) (Table 2.4). The homogenisation procedures for extraction of PDHa and PDHt were identical, using the method of Seymour and Chatham (1997), see Figure 2.7.

Pipette into eppendorff	Volume ratio	Concentration in total PDH extraction buffer
Extraction buffer : tissue	5 : 1	75mM HEPES 5mM Dichloroacetate 5mM $MgCl_2$ 1mM ADP 1mM Dithiothreitol 0.05 mM Leupeptin 1% Triton X-100 pH 7.0

Table 2.4. Reagents in total PDH extraction buffer.

2.7.2. PDH assay.

PDH activity was assayed spectrophotometrically at 340nm and 30°C, using the method of Seymour and Chatham (1997). 25-50μl of PDHt or 50-200μl of PDHa extract was added to 0.955 ml of reaction buffer, pH 7.2 (Table 2.5). The reaction rate was followed over a 2 minute time period using a 4 cell Spectronic PU8720 UV/VIS scanning spectrophotometer (Spectronic, Leeds), with a reaction rate kinetics program to give absorbance/minute (ΔA/min).

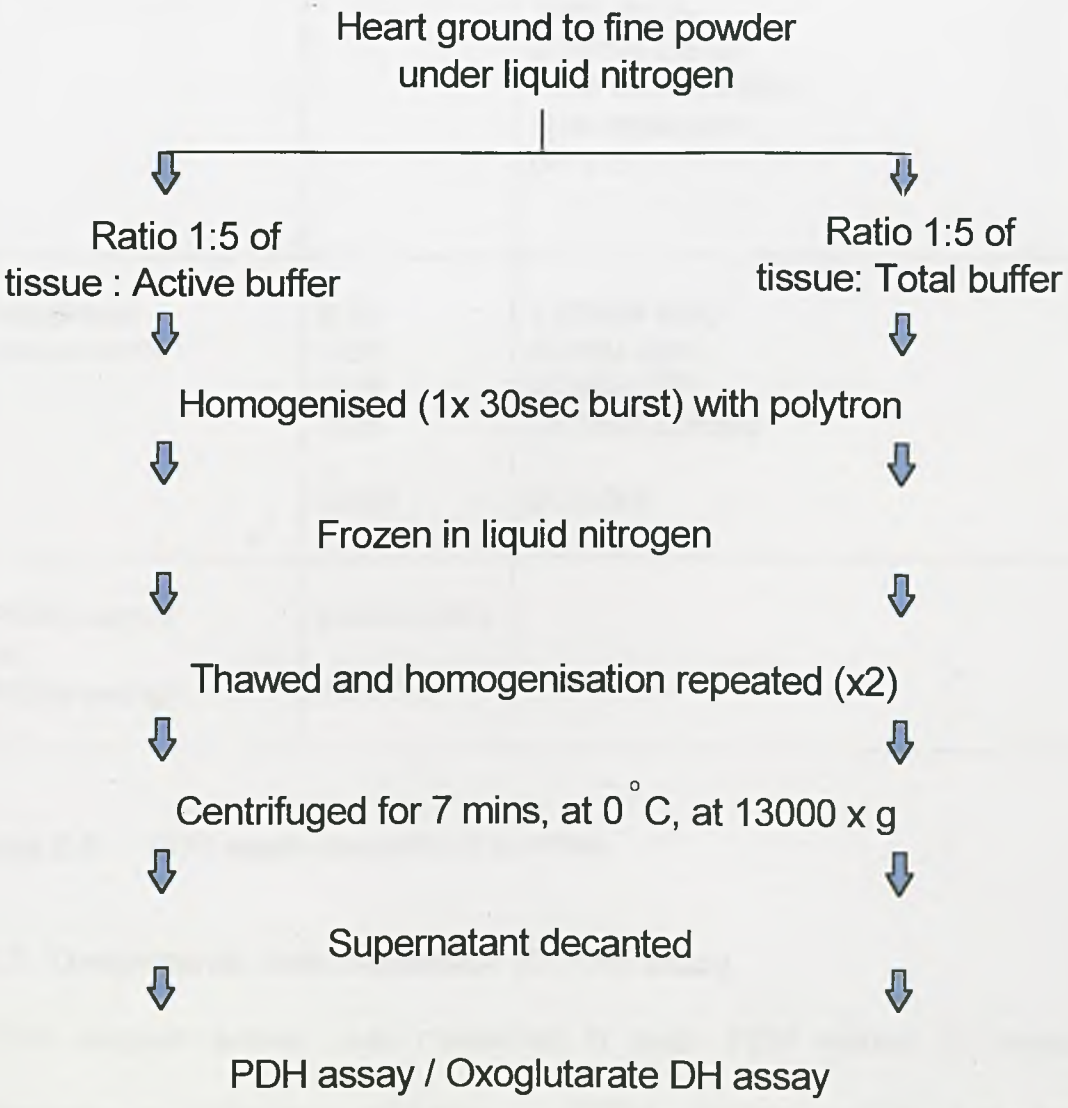

Figure 2.7. PDH extraction summary.

The rate of NADH production was determined over the first 30 seconds.

Pipette into eppendorff	Volume (ml)	Concentration in PDH reaction buffer
Reaction buffer	0.75	50mM HEPES 1mM $MgCl_2$ 0.08mM EGTA 1mM Dithiothreitol 4μM Rotenone pH 7.2
Additional components	0.05 0.05 0.05 0.05 0.005	1.67mM NAD 0.1mM CoA 0.2mM TPP 16.7mM Lactate 2U LDH
PDHt extract or PDHa extract	0.025-0.050 0.05-0.2	

Table 2.5. PDH assay reagents in cuvette.

2.7.3. Oxoglutarate dehydrogenase (OGDH) assay.

OGDH enzyme activity was measured in each PDH extract to correct for differences in extraction efficiency. OGDH enzyme activity was assayed spectrophotometrically at 340nm, at 30°C, using the method of Hansford (1991). 20-50μl of the active and total PDH extracts were added to 1.0 ml of reaction buffer (Table 2.6). The reaction was monitored over 1-2 minutes and the rate determined over the first 30 seconds.

$$\text{2-Oxoglutarate} + NAD^+ \xrightarrow{\text{OGDH}} \text{succinate} + NADH + H^+$$

Pipette into eppendorff	Volume (ml)	Concentration in OGDH reaction buffer
Reaction buffer	0.75	50mM KH_2PO_4 10mM $MgSO_4$ 5mM $MgCl_2$ 1mM EDTA 1mM Dithiothreitol 2μM Rotenone pH 7.2
Additional components	0.05 0.05 0.05 0.05 0.05	2mM NAD 0.15mM CoA 0.2mM TPP 10mM 2-oxoglutarate 2mM ADP
PDHt / PDHa extract	0.02-0.05	

Table 2.6. OGDH assay reagents in cuvette.

2.7.4. PDH enzyme results.

Calculations of enzyme activity were expressed as μmoles/min/gramme wet mass of tissue, using the following equation:

$$\frac{OD/min}{\text{Extinction Coefficient}} \times \frac{\text{Total assay volume (μl)}}{\text{Volume of extract (μl)}} \times 5 \times \text{Dilution factor of extract}$$

= μmoles/ min/ g. wet mass of tissue

The proportion of PDH in active form was expressed relative to the proportion of

PDH total, corrected for extraction efficiency:

$$\frac{(\text{PDHa / OGDHa or CSa})}{(\text{PDHt / OGDHt or CSt})} \times 100 = \%\ \text{PDHa}$$

2.7.5. Citrate synthase assay.

Citrate synthase activity, a marker of mitochondrial density (Smith *et al* 1990), was determined in each PDH extract. Citrate synthase was assayed using the method of Morgan-Hughes *et al* (1977). The enzyme activity was followed spectrophotometrically at 412nm, 30°C, by the conversion of 5,5'-Dithio-bis-2-nitro benzoic acid to thionitrobenzoic acid over 2 minutes (Table 2.7). Enzyme activity was expressed as μmoles/min/gram wet mass of tissue, and calculated as shown above.

$$\text{Oxaloacetate} + \text{Acetyl CoA} + H_2O \xrightarrow{\text{CS}} \text{Citrate} + \text{HS-CoA} + H^+$$

Pipette into eppendorff	Volume (ml)	Concentration in CS reaction buffer
Reaction buffer	0.99	50mM Tris 0.2mM 5,5'dithio-bis-2-nitrobenzoic acid 0.1mM Acetyl CoA 0.5mM Oxaloacetate 0.05% (v/v) Triton X-100 pH 8.1
PDHt / PDHa extract	0.01	

Table 2.7. Citrate synthase reaction reagents.

2.8. Serum metabolite concentrations.

Blood samples were centrifuged at 600g for 10 minutes at 4°C. Concentrations of glucose, fatty acids, triglycerides, ketone bodies and lactate were determined using spectrophotometric assay kits from Sigma Chemical Company or Boehringer Mannheim. Mrs Kath Bulmer kindly carried out these assays. Calculations for these assays are given in Appendix 1.2.

2.8.1. Glucose assay.

Glucose concentration was assayed using a diagnostic kit from Sigma Chemical Company (Cat No. 510-A) based on the following coupled enzymatic reactions.

$$\text{Glucose} + 2H_2O + O_2 \xrightarrow{\text{Glucose oxidase}} \text{Gluconic acid} + 2H_2O_2$$

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

(colourless) (brown)

Absorbances were determined at 450nm within 30 minutes of completion of the reaction.

2.8.2. Free fatty acid assay (Half micro test).

Free (non-esterified) fatty acids in serum were assayed at 540nm using a diagnostic enzymatic assay kit from Boehringer Mannheim (Cat. No. 1383 175). The principle of the assay relied on a coupled enzymatic assay system.

$$\text{FFA} + \text{CoA} + \text{ATP} \xrightarrow{\text{Acyl CS}} \text{Acyl-CoA} + \text{AMP} + \text{pyrophosphate}$$

$$\text{Acyl-CoA} + O_2 \xrightarrow{\text{ACOD}} \text{Enoyl-CoA} + H_2O_2$$

$$H_2O_2 + \text{4-AA} + \text{TBH} \xrightarrow{\text{POD}} \text{Red dye} + 2H_2O + \text{HBr}$$

2.8.3. Triglyceride assay.

Triglyceride concentration in serum was determined using an enzymatic assay kit from Sigma Chemical Company (Cat. No. 336-10) based on the reactions given below. The absorbance at 500nm was directly proportional to the triglyceride concentration in the sample.

$$\text{Triglycerides} \xrightarrow{\text{Lipoprotein lipase}} \text{Glycerol} + \text{Fatty acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{DAP} + \text{NADH}$$

$$\text{G-1-P} + \text{NAD} \xrightarrow{\text{G-1-PDH}} \text{DAP} + \text{NADH}$$

$$\text{NADH} + \text{INT} \xrightarrow{\text{Diaphorase}} \text{Formazan} + \text{NAD}$$

2.8.4. β - Hydroxybutyrate (β-HBA) assay.

β- hydroxybutyrate concentration was measured in serum samples using an enzymatic diagnostic kit from Sigma Chemical Company (Cat. No. 310-A).

$$\beta\text{-hydroxybutyrate} + \text{NAD} \xrightarrow{\beta\text{-HBDH}} \text{Acetoacetate} + \text{NADH}$$

2.8.5. Lactate assay.

Serum lactate concentrations were determined spectrophotometrically at 340nm, by the generation of NADH, using an enzymatic diagnostic assay kit from Sigma Chemical Company (Cat. No. 826-A).

$$\text{Lactic acid} + \text{NAD} \xrightarrow{\text{LDH}} \text{Pyruvic acid} + \text{NADH}$$

2.9. Nuclear magnetic resonance spectroscopy.

2.9.1. NMR sample preparation.

Lyophilised extracts of cardiac tissue were reconstituted by dissolving in a known volume of 50mM KH_2PO_4/D_2O (pH 7.4) buffer (1ml). A small spatula of Chelex-100 was added and mixed well, to remove paramagnetic metal ion contamination (Seymour *et al* 1990). The sample was filtered using a 22μM filter (Millipore) into a 5mm NMR tube.

2.9.2. Carbon-13 NMR spectroscopy.

Samples were analysed by high resolution carbon-13 NMR spectroscopy using a JEOL JNM-LA400 spectrometer interfaced with a 9.4 Tesla vertical bore superconducting magnet and a Stacman auto sample changer. Proton-decoupled carbon-13 spectra were acquired at 101MHz in a 1H broad band decoupling coil. Spectra were proton decoupled using a WALTZ decoupling sequence (bi-level decoupling mode) using the parameters described in Table 2.8. The temperature was maintained at approximately 25°C by using an in-built thermocouple within the

NMR probe.

NMR sampling parameters	Settings
IrrMode	bi-level decoupling (BCM)
Sampling points	64K
No. of scans	10000
No. of dummy scans	2
Pulse delay (sec)	3.0
Obs freq (MHz)	100.40
Irr freq (MHz)	399.65
Solvent	D_2O
Temperature (°C)	25.5

Table 2.8. ^{13}C NMR settings for sample analysis.

2.9.3. TCA cycle labelling patterns.

Oxidative metabolism was studied by ^{13}C NMR in hearts supplied with ^{13}C enriched substrates, following the incorporation of the ^{13}C label into glutamate. Analysis of the TCA cycle is based on the fact that glutamate is in fast exchange with α-ketoglutarate (Malloy *et al* 1990, Weiss *et al* 1992). TCA cycle intermediates are too low in concentration to be measured using ^{13}C NMR.

2.9.3.1. [1-^{13}C]-glucose and [6-^{13}C]-hexanoate labelled substrates.

^{13}C label from either [1-^{13}C]-glucose or [6-^{13}C]-hexanoate enters the TCA cycle via the methyl carbon of acetyl CoA (C-2). A schematic profile of TCA labelling with ^{13}C is represented in Figure 2.8. The carbon atom number given is used to identify the site of enrichment within the intermediates. Following the condensation of labelled acetyl CoA the ^{13}C label is transferred to the C-4 position of citrate (blue coloured carbon atoms). The labelled carbon is then metabolised

into the C-4 positions of α-ketoglutarate and glutamate. In the subsequent steps of the TCA cycle, the C-4 label becomes incorporated into the C-2 or C-3 positions of succinate and fumarate due to symmetry and possible scrambling in these intermediate pools. In one complete turn of the TCA cycle, oxaloacetate becomes labelled in either the C-2 or C-3 carbon positions. Following condensation with another acetyl CoA molecule, (labelled or unlabelled), in the second turn of the cycle, the original ^{13}C label arises in the C-3 or C-2 position of citrate and ultimately in the C-3 or C-2 position of glutamate (red coloured carbon atoms). Additional ^{13}C label entering the second turn of the TCA cycle will result in labelling of both the C-4 and C-3 carbons or C4 and C2 positions of glutamate. ^{13}C label therefore initially appears in the C-4 position of glutamate and transfers to the C-3, C-2 and C-1 carbons of glutamate during continuing turns of the TCA cycle.

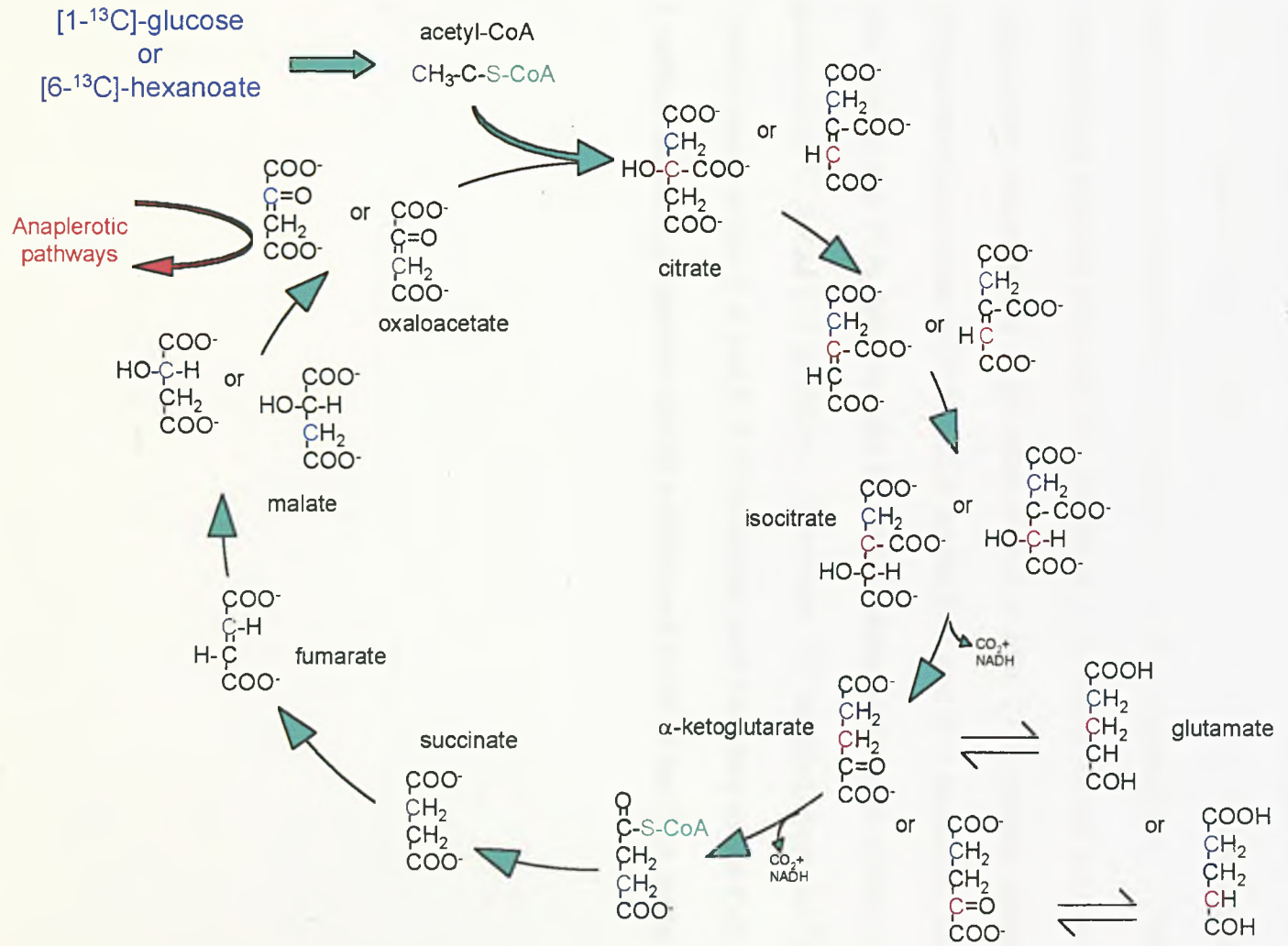

Figure 2.8. TCA cycle labelling from [1-^{13}C]-glucose and [6-^{13}C]-hexanoate labelled substrates.

2.9.3.2. [U-^{13}C]-palmitate labelled substrate.

[U-^{13}C]-palmitate is uniformally labelled, unlike the previous substrates. When metabolised by the heart each resulting acetyl-CoA molecule becomes ^{13}C labelled in both carbon atoms (Figure 2.9). During initial labelling of the TCA cycle these ^{13}C labelled carbon atoms appear in the C-4 and C-5 positions of citrate following the condensation of acetyl-CoA and oxaloacetate. They are then metabolised into the C-4 and C-5 positions of α-ketoglutarate and glutamate. At subsequent steps in the TCA cycle, the C-4 and C-5 labelled carbons become incorporated into either, C-1 and C-2, or the C-3 and C-4 positions. Completion of one turn of the TCA cycle results in oxaloacetate labelled in either, C-1 and C-2 positions or C-3 and C-4 positions. Therefore ^{13}C labelling from [U-^{13}C]-palmitate initially arises in the C-4 and C-5 of glutamate and transfers to the C-3, C-2 and C-1 carbon atoms of glutamate during subsequent turns of the TCA cycle.

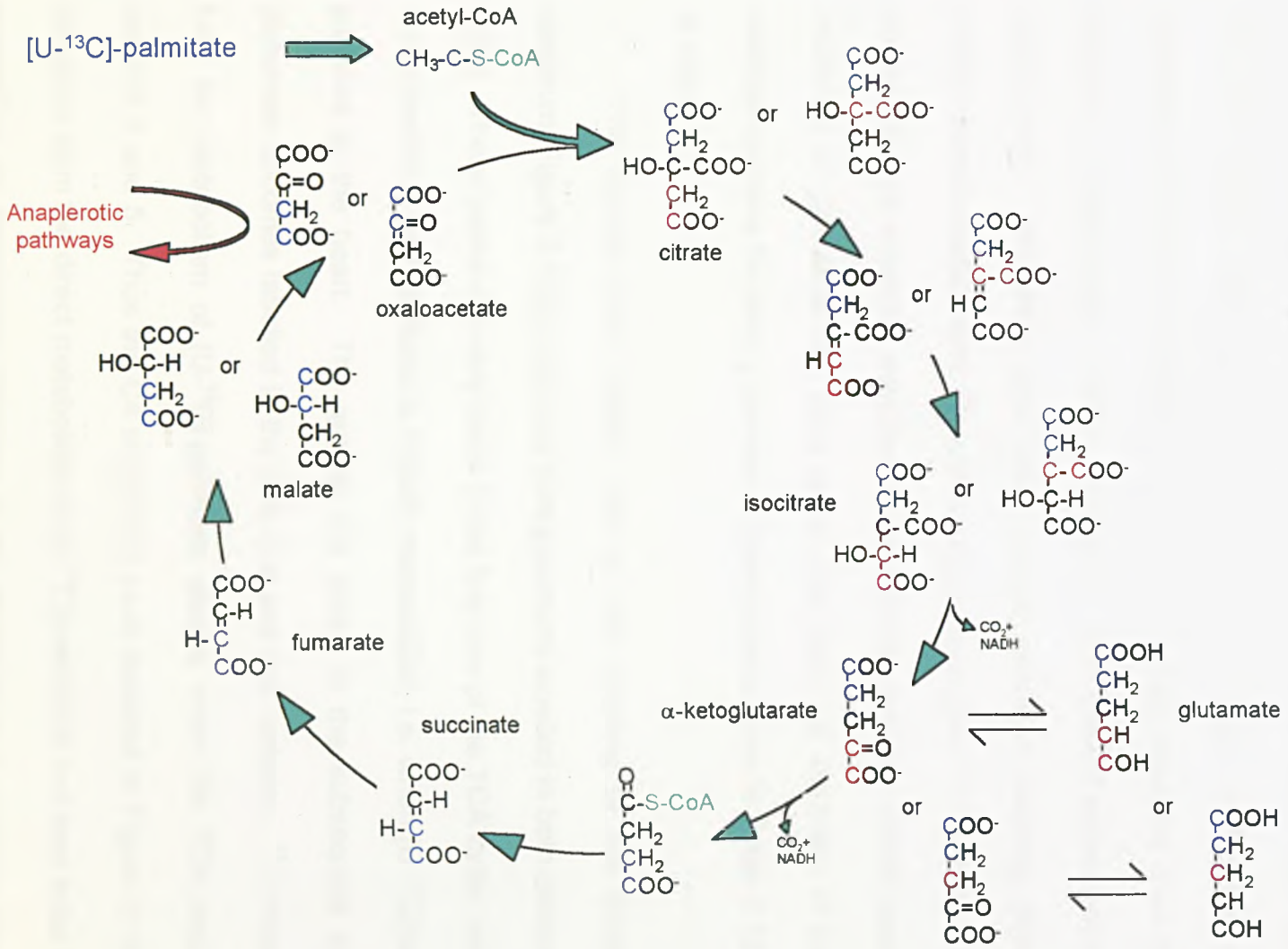

Figure 2.9. TCA cycle labelling from [U-^{13}C]-palmitate labelled substrate.

2.9.4. NMR spectra analysis.

2.9.4.1. Data processing.

The ^{13}C resonance patterns of glutamate derived from the oxidation of ^{13}C labelled substrates are used to monitor substrate metabolism and TCA cycle activity. Simulated proton-decoupled ^{13}C NMR spectra of each of the five carbons of glutamate (C1-C5) are shown in Figures 2.10(a-e), obtained from the Rogers Magnetic Resonance Center website (http://www2.swmed.edu/rogersmr2/carbon.html). Multiplets arise from carbon spin-spin coupling from adjacent carbon atoms labelled with ^{13}C within proton decoupled ^{13}C spectra. Depending on the labelled substrate supplied, its metabolism and the carbon examined, the multiplets of glutamate can have up to nine lines. A summary of the possible multiplet patterns for each glutamate carbon atom is given in Table 2.10 (Weiss *et al* 1992).

The doublet (D45) peaks, due to J45 coupling in the glutamate C-4 spectrum, Figure 2.10(a) originate from glutamate labelled in both carbons 4 and 5 alone. These peaks can only occur in the first turn of the TCA cycle, when acetyl-CoA labelled in both carbons is initially metabolised, i.e. when [U-^{13}C]-palmitate is supplied to the heart. The quartet (Q) arises in the subsequent turns when glutamate becomes labelled in the C-3, C-4 and C-5 carbons. ^{13}C labelled atoms from the metabolism of [U-^{13}C]-palmitate always enter the TCA cycle in both carbons 4 and 5. Thus the C4 singlet (S) peak depicted in Figure 2.10(a) would not occur from the direct metabolism of [U-^{13}C]-palmitate, but may arise from label scrambling via anaplerotic pathways or the metabolism of naturally abundant ^{13}C

substrates. The glutamate C-3 ^{13}C NMR spectrum, Figure 2.10(b), has multiplets labelled as a singlet (S), doublet (D) or triplet (T). The spin-spin couplings between C2-C3 (J23) and C3-C4 (J34) are equivalent giving rise to one doublet. The triplet (T) occurs due to a doublet of doublets originating from labelling in the C2, C3 and C4 carbons, however the two inner resonance peaks overlap to produce a 1:2:1 peak resonance ratio. An example of a proton-decoupled ^{13}C NMR spectrum of C-2 glutamate is shown in Figure 2.10(c). Again each multiplet is labelled as a singlet (S), a doublet with relevant coupling (D) or a quartet (Q). The proton-decoupled ^{13}C NMR spectra of C-1 and C-5 glutamate appear in close proximity in the spectral region, Figure 2.10(d) and (e). Considering C-1, the singlet (C1S) is composed of isotopomers in which the C1 is labelled, the C-2 is unlabelled and the C-3, C-4 and C-5 may or may not be labelled. The doublet (C1D) results from isotopomers in which both C-1 and C-2 are labelled and C-3, C-4 and C-5 may or may not be labelled. The singlet (S) in C-5 glutamate, Figure 2.10(e), originates from glutamate labelled in C-5 only. This C-5 singlet (S) would not occur from the direct metabolism of [U-^{13}C]-palmitate, in the same way as the singlet (S) in C-4 glutamate, due to the simultaneous labelling of carbon atoms 4 and 5.

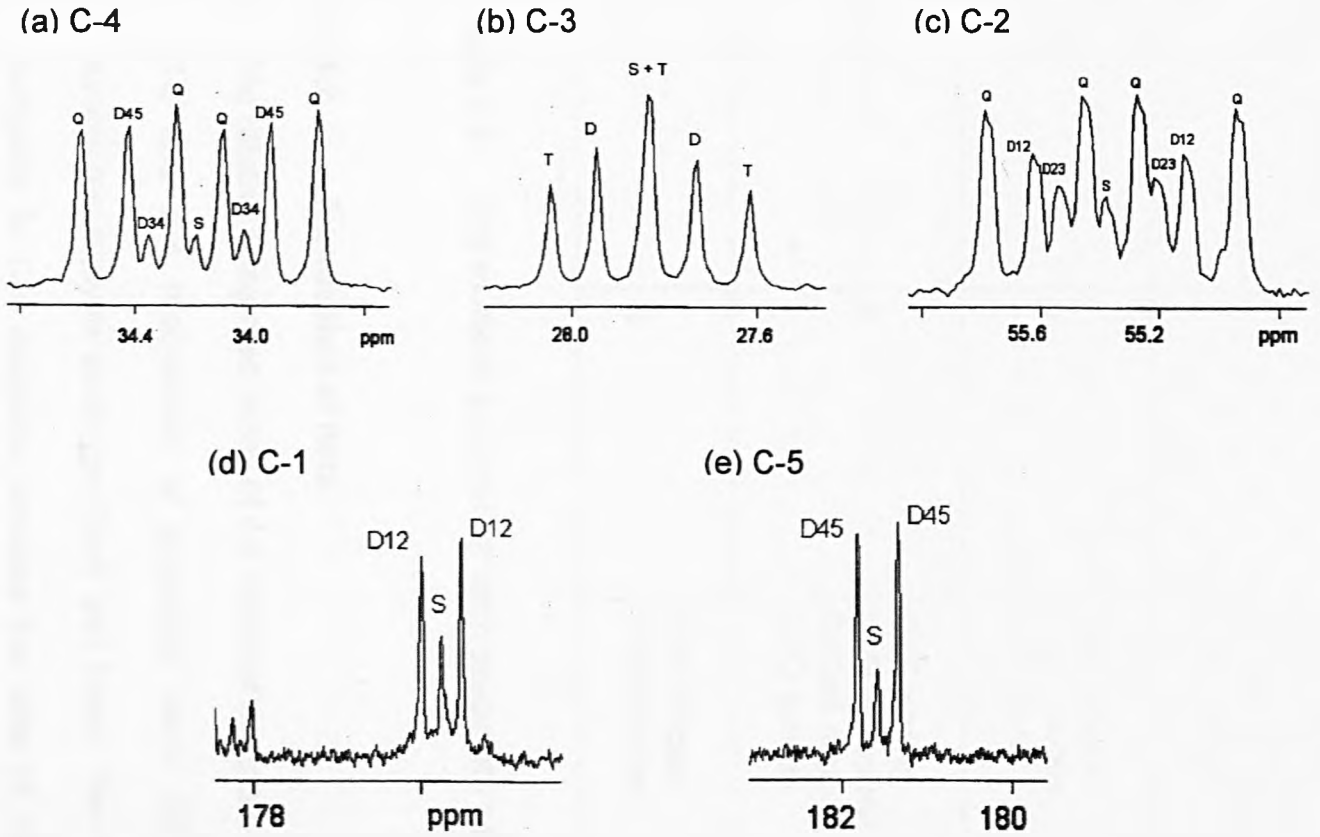

Figure 2.10. Typical examples of proton-decoupled ^{13}C-NMR spectra of glutamate molecules following perfusion with [U-^{13}C]-palmitate. (a) Gutamate C-4 spectra showing a singlet (S), doublets (D34) and (D45), and quartet (Q). (b) Glutamate C-3 ^{13}C NMR spectrum example with multiplets labelled as a singlet (S), doublet (D) or triplet (T). (c) Glutamate C-2 ^{13}C NMR spectrum example with multiplets labelled as a singlet (S), a doublet with relevant coupling (D) or a quartet (Q). Carbon multiplets of both Glutamate C-1 (d) and Glutamate C-5 (e) are labelled as a singlet (S) and a doublet with relevant coupling (D), (http://www2.swmed.edu/rogersmr2/carbon.html).

Carbon atom	Possible number of lines	Multiplets
1	3	C1S singlet C1D doublet
2	9	C2S singlet C2D12 doublet C2D23 doublet C2Q quartet
3	5	C3S singlet C3D doublet C3T triplet
4	9	C4S singlet C4D34 doublet C4D45 doublet C4Q quartet
5	3	C5S singlet C5D doublet

Table 2.9. The multiplet patterns for each glutamate carbon atom.

2.9.4.2. Expression of data.

1. The relative integrated areas of the multiplet components in each of the C-2, C-3 and C-4 resonances of glutamate were determined using UNIX compatible analysis packages Stork and Swan. The ratio of integrated C-3 multiplets to C-4 mutiplets provides the ratio of carbon atom fractional enrichment between the two different carbon atoms.
2. The peaks heights of each multiplet were measured and expressed as a percentage of the total peak heights for each C-2, C-3 and C-4 carbon atom

in the spectrum, to give their fractional intensity values. The total possible fractional intensity within any carbon atom of glutamate molecule is 1.0.

2.9.4.3. Analysis using TCAcalc™.

The TCAcalc™ program of Malloy *et al* (1990), performs an isotopomer analysis to estimate relative pathway fluxes at metabolic steady state and can be used to determine the contribution of different substrates both labelled and unlabelled towards acetyl-CoA production and relative flux through the TCA cycle of competing substrates. It employs algebraic equations that describe the ^{13}C NMR spectrum in terms of metabolic (pathway fluxes) and experimental (substrate labelling patterns and enrichment) parameters. These parameters are estimated by non-linear least-squares analysis of the multiplet information determined from the NMR spectral measurements (Malloy *et al* 1990). Isotopomer analysis using the TCAcalc™ program requires the following data:

1. fractional enrichment values of each multiplet of C-2, C-3, and C-4 of glutamate, as in previous section,
2. the ratio of C-3 to C-4 resonance area,
3. the relative rate of anaplerosis giving rise to oxaloacetate, (y), and
4. the fractional enrichment of acetyl CoA, (Fc).

Anaplerosis refers to the reactions that allow the entry of carbon into the TCA cycle intermediate pools other than via citrate synthase. The anaplerotic pathways are included in the TCAcalc™ program by considering the reactions that feed directly into the oxaloacetate pool. However the contribution of anaplerosis to TCA cycle labelling is assumed to be very low in the experiments performed throughout this thesis. The set of parameters defining

the fractional isotopomer enrichments in the acetyl-CoA pool include (Malloy *et al* 1990):

Fc0 - Fraction of acetyl-CoA that is unlabelled,

Fc1 - Fraction of acetyl-CoA labelled at the carbonyl carbon (C-1),

Fc2 - Fraction of acetyl-CoA labelled at the methyl carbon (C-2),

Fc3 - Fraction of acetyl-CoA labelled at both carbons,

in which:- $Fc0 + Fc1 + Fc2 + Fc3 = 1$

Fc2 corresponds to labelling of the carbon 2 of acetyl CoA, found in hearts perfused with labelled glucose, hexanoate, while Fc3 correponds to labelling of the carbons C1 and C2 of acetyl CoA, found in hearts perfused with labelled palmitate. The TCAcalc program uses the following ratios to calculate a value for the relative fractional enrichment through the pathway specified:

Glutamate C3/ C4 ratio = $1/(2y + 1)$, and

Glutamate C4D/ total C4 ratio = $Fc/(2y + 1)$

As shown in Figure 2.8 [1-^{13}C]-glucose substrate when metabolised by the heart provides two acetyl CoA molecules (one labelled and one unlabelled), which can enter the TCA cycle, thus the fractional enrichment value obtained from the TCAcalc™ program corresponds to half of the original glucose molecule utilised. In contrast, the substrate [6-^{13}C]-hexanoate provides three acetyl CoA molecules into the TCA cycle, only one of which is labelled, thus the fractional enrichment value obtained from the TCAcalc program corresponds to one third of the original hexanoate molecule utilised. The fractional enrichment value in the TCAcalc program for acetyl CoA obtained from [U-^{13}C]-palmitate directly reflects the ratio of palmitate molecules utilised because both carbons of acetyl CoA entering the TCA cycle are labelled.

2.9.4.4. Model assumptions.

The following assumptions are used in the TCAcalc™ model [Rogers Magnetic Resonance Center website (http://www2.swmed.edu/rogersmr2/ carbon.html), (Malloy *et al* 1990)]:

1. The metabolic reactions are at steady-state (Katz 1985).
2. The enrichment of substrates supplying the acetyl-CoA pools and the anaplerotic substrates are constant.
3. ^{13}C occurring from natural abundance does not influence the ^{13}C NMR spectrum.
4. ^{13}C entry into the intermediate pools as a result of carboxylation reactions is negligible (Katz 1985).
5. Glutamate and α-ketoglutarate are in fast exchange with each other, relative to the TCA cycle flux (Randle *et al* 1970, Kelly *et al* 1979, Malloy *et al* 1990).
6. The pathway fluxes have values relative to TCA turnover, which is considered to be 1.0 (Malloy *et al* 1990).

2.10. Statistical analysis.

All calculated results were expressed as mean +/- SEM (for n>4). Significance of the results was analysed using Students t-test for unpaired comparisons of two groups. One-way analysis of variance (ANOVA) was performed to assess difference in parameters involving more than two experimental groups. Significant differences were then subjected to post-hoc analysis using the Newman-Keuls method. A probability less than 5% was considered as statistical significance.

Chapter 3:

Relative contribution of substrates to TCA cycle oxidation in compensated cardiac hypertrophy.

3.1. Introduction.

3.1.1. Energy status of the hypertrophied heart.

A continuous energy supply is critical in maintaining heart function. A delicate balance is thought to exist between its energy provision and its utilisation. This is especially true for the hypertrophied heart, in which energy demands are further increased (Meerson *et al* 1972). Alterations in energy status, characterised by a depletion of high-energy phosphate reserves, have been observed in many models of cardiac hypertrophy (Bhutta *et al* 1995, Massie *et al* 1995, Seymour *et al* 1990, Lortet *et al* 1995, Zhang *et al* 1993).

3.1.2. Glycolysis and glucose oxidation.

There is as yet no clear understanding of pressure or volume overload-induced changes in myocardial energy metabolism and its effects on heart function. Hypertrophied hearts resulting from pressure or volume overload exhibit alterations in their profile of substrate oxidation. Previous studies have shown that the hypertrophied heart exhibits an increase in its capacity for glucose metabolism (Seymour *et al* 1990, Taegtmeyer and Overturf 1988, Zhang *et al* 1995, Massie *et al* 1995), assumed from its increased cellular uptake. Supporting evidence for increased glucose uptake has been measured by the cellular accumulation of 2-deoxyglucose-6-phosphate (2DGP), a glucose analogue, using ^{31}P-NMR spectroscopy during infusion of 2-deoxyglucose (2DG) in hearts. Zhang *et al* (1995) demonstrated increased accumulation of 2DGP in pressure overloaded canine left ventricles, and that the accumulation positively correlated with the degree of hypertrophy obtained, being most marked in the subendocardium. This is further supported by the observation

that glucose uptake is greater in hypertensive rat hearts (Leipala *et al* 1989) compared to control hearts. Glucose oxidation measured directly using U-^{14}C-glucose was also found to be moderately elevated in spontaneously hypertensive rat (SHR) hearts (Christe and Rodgers 1994).

Alterations in gene expression are thought to occur in the heart in response to a chronic increase in haemodynamic load, as in cardiac hypertrophy. This can be considered as the re-expression of a 'foetal gene program' (van Bilsen and Chien 1993). In the rat, pressure-overload induced altered gene expression leads to a hypertrophic phenotype affecting myocyte contractility. This is characterised by a reduction in Ca^{2+}-ATPase activity (Mercadier *et al* 1990, Nagai *et al* 1987), a shift from αMHC to βMHC (Nagai *et al* 1987) and expression of MLC-2 (Cummins 1982) and skeletal α-actin (Bishopric *et al* 1992). Other changes in gene expression are associated with substrate use such as glucose transporters for substrate uptake (Depre *et al* 1998) and carnitine palmitoyltransferase I for long-chain fatty acid substrate oxidation (Depre *et al* (1998). Increased glucose oxidation is a metabolic adaptation thought to arise due to the re-expression of foetal genes, characteristic of cardiac hypertrophy at a molecular level (Depre *et al* 1998). Gene expression measured by real-time quantitative (RT-PCR) in hypertrophied rat heart was associated with a 40% decrease of mRNA coding for the adult isoform of GLUT-4, with no change in GLUT-1 the isoform predominantly found in the foetal heart (Depre *et al* 1998).

The foetal heart has a greater dependence on glucose metabolism for provision of energy than the normal adult heart. Glucose oxidation is a more efficient process in terms of the amount of ATP derived per atom of oxygen

consumed compared to fatty acid oxidation (3.2 ATP per atom of oxygen versus 2.8, Collins-Nakai *et al* 1994), such that glucose yields 10% more ATP per mole of oxygen consumed compared to fatty acids. Therefore a shift towards increased utilisation of glucose would be consistent with the proposal that the hypertrophied heart switches (from fatty acid oxidation) towards a more efficient system (glucose metabolism).

Changes have also been noted in relation to glycolysis in cardiac hypertrophy. Hypertrophied rat hearts resulting from hyperthyroidism have been shown to exhibit increases in both glycolytic and glycogenolyic fluxes (Seymour *et al* 1990), as measured by significantly elevated levels of G-6-P and F-6-P and a 40% increase in PFK enzyme activity. These results are taken as evidence for a switch in substrate preference away from fatty acids. Interestingly an increase in glycolytic capacity accompanies chronic pressure overload even in the absence of left ventricular hypertrophy (Taegtmeyer and Overturf 1988). Investigators have reported that the concentrations of several glycolytic enzymes are increased in hypertrophied myocardium (Bishop and Altschuld 1970). Fox and Reed (1969) showed that lactate dehydrogenase isozymes from canine hearts with right ventricular hypertrophy shifted towards anaerobic muscle type isoforms and away from the aerobic heart isoforms, without any alteration in total LDH activity. Such an isoform shift is thought to favour the production of lactate and is further evidence for re-expression of a foetal phenotype. However the metabolism of glucose to lactate is much less efficient. The regulation of LDH isoform synthesis is still unknown but may occur as a result of hypoxia or an alteration in substrate availability.

3.1.3. Fatty acid oxidation.

It is well established that long-chain fatty acids are the preferred substrate metabolised by well-oxygenated normal myocardium, with the normal heart obtaining up to 70% of its metabolic energy from this substrate (Neely and Morgan 1974, Saddik and Lopaschuk 1991, Schonekess *et al* 1997). However alongside enhanced glycolysis and glucose utilisation, investigators have shown evidence of a decrease in fatty acid oxidation in both pressure and volume overload hypertrophy models (Wittels *et al* 1968, El Alaoui-Talibi *et al* 1992).

In guinea pig hearts, chronic volume overload resulted in a reduction in the rate of long-chain fatty acid metabolism in the rat heart, measured by $^{14}CO_2$ production from [U-^{14}C] palmitate. This was paralleled by a decrease in the O_2 consumption rate. The SHR heart model has also been found to exhibit suppressed fatty acid oxidation when perfused with 9,10-^{3}H-palmitate (Christe and Rodgers 1994). Highly impaired fatty acid oxidation has also been shown to exist in patients with idiopathic hypertrophic cardiomyopathy (HCM), using radionuclide imaging of iodine-123-BMIPP metabolism, a beta-methyl-branched-fatty acid (Nakata *et al* 1996). This metabolic alteration occurred despite a lack of myocardial dysfunction or a perfusion abnormality. However the pathophysiological implications of reduced fatty acid metabolism might underlie the development of heart failure.

There are a number of possible mechanisms by which a decrease in long-chain fatty acid oxidation may be explained. Firstly a reduction in the total tissue carnitine level may lead to a decrease in carnitine mitochondrial transport. Carnitine is an essential co-factor in the transport of activated long-

chain fatty acids (acyl CoA) from the cytosol to the mitochondria. Altered carnitine metabolism has been observed in volume- (Cheikh *et al* 1994) and pressure-overload (Reibel *et al* 1983) hypertrophy models. Secondly, down regulation of the carnitine palmitoyltransferase 1 (CPTI) enzyme, which catalyses the transfer of acyl groups from CoA to carnitine may also contribute. This is a key regulatory step in the transport of fatty acids across the mitochondrial membrane. In support of this Depre *et al* (1998) measured a 40% decrease in the mRNA coding for the adult muscle isoform of CPTI in hypertrophied rat heart, but found no change in the level of foetal isoform of CPTI (liver CPTI). A decrease in either or both of these could result in a reduction of long-chain fatty acid oxidation. Further evidence exists in the fact that short-chain fatty acid oxidation is not found to be impaired in models of cardiac hypertrophy, in contrast to that seen in long-chain fatty acid oxidation (El Alaoui-Talibi *et al* 1989, 1992). Short-chain fatty acid oxidation does not require carnitine or CPTI for transport across the mitochondrial membrane.

Some hypoglycaemic agents such as methyl palmoxirate, oxfenicine, and etomoxir, not only reduce fatty acid utilisation but also cause cardiac hypertrophy in normal rats (reviews by Rupp and Jacob 1992, Bressler and Goldman 1993), suggesting that substrate utilisation is involved in the development of cardiac hypertrophy (versus being a consequence). Etomoxir in particular inhibits the mitochondrial carnitine palmitoyltransferase 1, thus reducing long-chain fatty acid uptake of the mitochondria. Rupp and Jacob discovered that etomoxir induced allometric growth of right and left ventricles both in normal and overloaded rat hearts, whereas aortic stenosis induced left ventricular hypertrophy alone. This suggests that the growth signals in over-

loaded hearts and hearts with a reduced fatty acid utilisation differ.

However the mechanisms underlying the effects of etomoxir are not clear. A study by Lesniak *et al* (1995) on adult rat cardiomyocytes in culture appears to conflict with these findings. Although etomoxir inhibited the oxidation of palmitate and stimulated the oxidation of exogenous glucose in adult cardiomyocytes, it caused no direct hypertrophic effect, measured by the amount of ANF production. This suggests the effects of etomoxir *in vivo* might not be due to its metabolic action at the myocyte level.

3.1.4. Aims.

Left ventricular hypertrophy is a progressive disease (Anversa and Capasso 1991). Previous work on cardiac hypertrophy described above indicates that hypertrophied hearts from pressure- or volume-overload exhibit many alterations in their substrate oxidation profile and energy status. However the exact sequence and time scale of these metabolic adaptations still needs to be clarified. Despite the extensive research on energy metabolism during cardiac hypertrophy to date, a number of questions still remain unanswered.

- Do changes in glucose and fatty acid metabolism occur simultaneously during cardiac hypertrophy?
- What consequences do these substrate alterations have on heart function?
- What role does insulin play in determining substrate selection during cardiac hypertrophy and at what stage does insulin resistance develop?

This study proposes that alterations in substrate availability play an important role in the adaptive process of left ventricular hypertrophy and its progression to heart failure. By identifying the major regulating sites of these substrate

adaptations it may be possible to identify which patients exhibit a predisposition to heart failure. This will allow successful early diagnosis of patients, the stage of disease progression and provide insight into potential therapeutic targets in the prevention of heart failure.

3.2. Methods.

Chapter 2 describes in detail the methods employed in this study. The animal model in this chapter was nine weeks pressure overload cardiac hypertrophy, induced surgically by abdominal aortic constriction in male Sprague-Dawley rats. Sham operated rats without aortic constriction acted as the control group (Chapter 2, section 2.2).

The profile of substrate utilisation was investigated in isolated perfused hypertrophy and control hearts as described in Chapter 2, section 2.3. Three different substrate mixtures were used:

- 5mM glucose and 0.3mM [6-^{13}C]-hexanoate, or
  5mM [1-^{13}C]-glucose and 0.3mM unlabelled hexanoate.
- 5mM glucose and 0.3mM [U-^{13}C]-palmitate, or
  5mM [1-^{13}C]-glucose and 0.3mM unlabelled palmitate
- 5mM glucose, 0.3mM [U-^{13}C]-palmitate and 100μU/ml insulin, or
  5mM [1-^{13}C]-glucose, 0.3mM unlabelled palmitate and 100μU/ml insulin.

Hearts were supplied with the short chain fatty acid, hexanoate using Langendorff perfusion rig, whereas palmitate was introduced to the hearts in the form of palmitate bound to 3% albumin using an oxygenator perfusion apparatus (Chapter 2, section 2.3). Perfusion pressure and coronary flow were maintained consistent between the perfusion methods. All metabolite and ion concentrations were kept at physiological concentrations to mimic the *in vivo* situation.

Preliminary long chain fatty acid perfusions were performed using 100% [U-^{13}C]-palmitate. Due to the excellent signal to noise ratio obtained, the continuing perfusions were performed using 50% [U-^{13}C]-palmitate and 50%

unlabelled palmitate. This enabled the economy of ^{13}C label, therefore reducing the cost of each experiment.

Results were expressed as mean +/- SEM (to 2 d.p). Significance of the results was analysed using Students t-test for unpaired comparisons of two groups. One-way analysis of variance (ANOVA) was performed to assess difference in parameters involving more than two experimental groups. Probability values less than 5% were considered statistically significant.

3.3. Results.

3.3.1. Characterisation of hypertrophy.

3.3.1.1a. Morphology of hypertrophy.

Table 3.1(a) shows the morphology of nine week hypertrophied and control rats. The results show that aortic constriction of the abdominal aorta led to a significant increase in heart mass (11.6%, $p<0.001$), with no significant difference in body mass or tibia length, consistent with previous observations (Boateng *et al* 1997, Ogawa *et al* 1996, Seymour and Chatham 1997). Aortic constriction also caused a significant decrease (11.5%, $p<0.0001$) in left and increase (9.9%, $p<0.002$) in right kidney mass with respect to the relevant control kidneys. This resulted in a significant mass difference between the right and left hypertrophied kidneys (25%, $p<0.0001$).

Treatment Groups	Body Mass (g)	Heart Mass (g)	Tibia Length (cm)	Left Kidney Mass (g)	Right Kidney Mass (g)
Control (n=30)	581.67 (10.11)	2.51 (0.06)	4.57 (0.02)	1.82 (0.03)	1.82 (0.03)
Hypertrophy (n=33)	580.25 (8.29)	2.80* (0.06)	4.56 (0.02)	1.61* (0.03)	2.00*+ (0.04)

Table 3.1(a). Morphology in rats following nine weeks aortic constriction, mean +/- SEM. (* Significant difference compared to control group, + significant difference compared to left kidney).

3.3.1.1b. Morphology of heart failure.

Table 3.1(b) shows the morphology of the hypertrophied and control rats used in this study in relation to heart failure. There were no significant differences in

the lung wet to dry mass ratios, liver or adrenal wet mass between control and hypertrophied rats. Aortic constriction for nine weeks did however result in a significant increase in wet spleen mass ($p<0.05$). In addition, no significant differences were observed in the wet to dry mass ratios of the heart, left and right kidneys, adrenal gland or spleen, (see Appendix Table 2.1 for values).

Treatment Groups	Lung Wet / Dry Ratio	Liver Mass (g)	Adrenal Mass (g)	Spleen Mass (g)
Control (n=30)	3.81 (0.08)	19.91 (0.54)	0.03 (0.00)	0.90 (0.02)
Hypertrophy (n=33)	3.84 (0.07)	19.77 (0.48)	0.03 (0.00)	0.97* (0.03)

Table 3.1(b). Heart failure morphology in rats following nine weeks aortic constriction.

3.3.1.2. Indices of Hypertrophy.

The indices used to determine the degree of hypertrophy obtained are shown in Table 3.1(c). Aortic constriction resulted in a significant increase (12.3%, $p<0.001$) in heart mass to body mass ratio. The heart mass to tibia length was significantly increased by 10.9% ($p<0.001$) in the hypertrophied group, whereas the right to left kidney ratio was significantly increased by 25.0% ($p<0.001$). This represents a moderate compensated hypertrophy model. Animals with a right to left kidney ratio greater than 3:1 were excluded from this study. Figure 3.1 shows transverse cross sections of left ventricular myocardium from control (a) and hypertrophied (b) rat heart, to show the difference in myocyte size and structural arrangement of the heart muscle. The myocytes from a

hypertrophied heart are of greater diameter and area than in the control heart. The hypertrophied myocytes have a greater intercellular distance in which they are situated further apart from neighbouring myocytes compared to myocytes in the control heart.

Treatment Groups	Heart Mass / Body Mass ($x10^{-3}$)	Heart Mass / Tibia Length (g/cm)	Right / Left Kidney Mass Ratio
Control (n=30)	4.32 (0.09)	0.55 (0.01)	1.00 (0.01)
Hypertrophy (n=33)	4.85 (0.11)*	0.61 (0.01)*	1.25 (0.02)*

Table 3.1(c). Indices of hypertrophy.

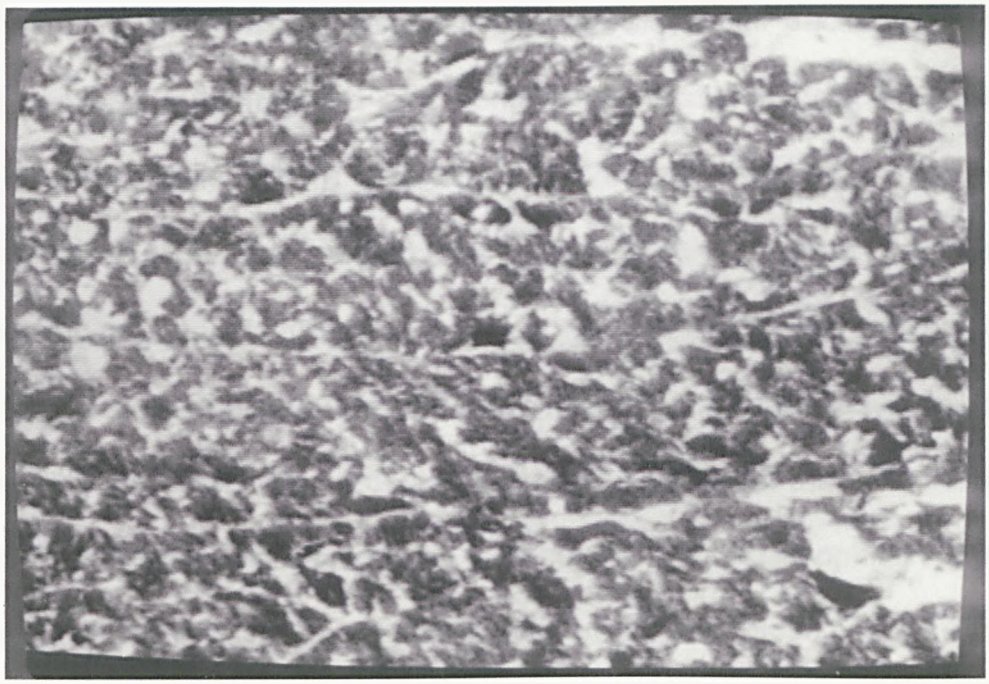

Figure 3.1(a). Transverse cross-section of control myocardium (x 25 magnification) stained with Eosin.

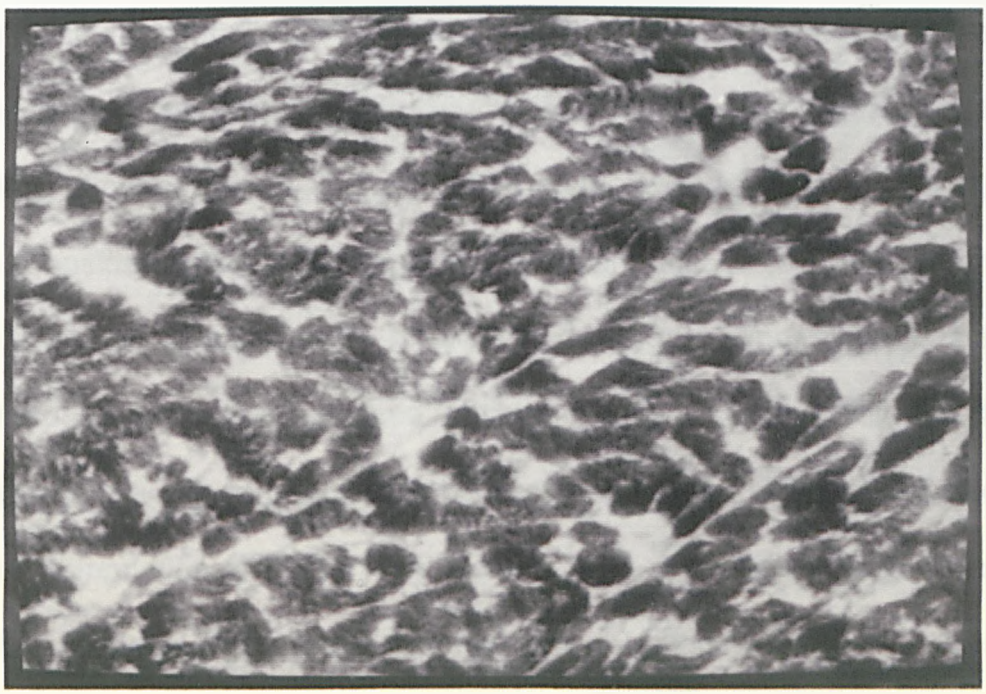

Figure 3.1(b). Transverse cross-section of nine week hypertrophied myocardium (x 25 magnification) stained with Eosin. (Photographs kindly provided by K. Linehan).

3.3.1.3. Blood Characteristics.

No significant difference was seen in the red blood cell packed cell volume ratios between hypertrophied and control rats (Table 3.1(d)), indicating no evidence of anaemia. There were also no significant differences in the plasma concentrations of the metabolites triglyceride, lactate, glucose, β-hydroxybutyrate and fatty acids, between hypertrophied and control groups. This observation highlights the fact that there were no changes in substrates delivered to the heart cells preceding sacrifice. The higher than physiologically expected glucose levels, recorded in both control and hypertrophied rat plasma was most likely caused by haemolysation of the red blood cells during the centrifugation procedure. Heparin administered to the animal prior to sacrifice to prevent blood clots in the myocardium, may have been the cause of the higher than physiologically expected fatty acid concentrations measured in both control and hypertrophied rat plasma. Heparin augments the breakdown of triglycerides to free fatty acids in the blood. The resulting increase in plasma fatty acid concentration is then combated by increased fatty acid uptake by the cells. Considering the gas values, no significant differences were observed in pH, dissolved carbon dioxide or dissolved oxygen concentrations between hypertrophied and control plasma samples (Table 3.1(e)). Oddly the plasma samples showed signs of acidosis, characterised by a low pH, possibly due to impaired circulation at the time of sacrifice as a result of excess anaesthetic. Similarly analyses of plasma electrolyte concentrations revealed no significant differences between hypertrophied and control plasma.

Treatment Groups	RBC	TG mmol/l	Lactate mmol/l	Glucose mmol/l	HBA mmol/l	FA mmol/l
Control (n=4)	0.73 (0.02)	1.09 (0.33)	2.44 (0.14)	8.88 (0.48)	0.05 (0.02)	2.44 (0.36)
Hypertrophy (n=6)	0.71 (0.03)	1.74 (0.28)	4.16 (1.21)	9.78 (0.49)	0.10 (0.05)	2.90 (0.51)

Table 3.1(d). Plasma metabolite concentrations after nine weeks aortic constriction.

Treatment Groups	pH	pCO_2 mmHg	pO_2 mmHg	ctHb g/dl	Hctc %	HCO^{3-} mmol/l	K^+ mmol/l	Na^+ mmol/l	Ca^{2+} mmol/l	Cl^- mmol/l
Control (n=6)	6.7 (0.1)	37.7 (8.5)	113.3 (8.1)	0.0 (0.0)	1.0 (0.0)	30.5 (0.2)	4.7 (0.3)	139.2 (0.8)	1.4 (0.0)	95.0 (0.7)
Hypertrophy (n=7)	6.8 (0.1)	29.3 (5.2)	131.5 (6.6)	0.1 (0.0)	1.0 (0.0)	30.6 (0.8)	4.8 (0.2)	138.1 (1.3)	1.3 (0.1)	94.3 (0.8)

Table 3.1(e). Plasma gas values after nine weeks aortic constriction.

3.3.2. Substrate selection in hypertrophy

3.3.2.1. Glucose and short chain fatty acid hexanoate as substrates.

3.3.2.1a. Metabolic analysis.

Figure 3.2 shows a typical example of a ^{13}C NMR spectrum (0-120 ppm range) obtained from a hypertrophied heart perfused with [6-^{13}C]-hexanoate and unlabelled glucose. The expanded regions (20-60 ppm) clearly show the isotopomer splitting patterns of C3 and C4 glutamate. There was little difference between ^{13}C labelling of the hypertrophied and control hearts. A typical ^{13}C NMR spectrum from a hypertrophied heart perfused with [1-^{13}C]-glucose and unlabelled hexanoate is shown in Figure 3.3. It is clear that the signal to noise ratio is poorer when the ^{13}C label is supplied as [1-^{13}C]-glucose compared to [6-^{13}C]-hexanoate (Figure 3.2).

Using the TCAcalc™ program, the relative proportions of labelled substrates utilised to TCA cycle oxidation were determined in each heart (Figure 3.4). In both the hypertrophied and control hearts, the short chain fatty acid, hexanoate contributes approximately 100% of the total substrate oxidised in the TCA cycle. Poor incorporation of ^{13}C label from [1-^{13}C]-glucose meant that the proportion of glucose oxidised was indeterminable in both groups. Myocardial glutamate levels were determined in the perfused hearts to ensure that no absolute changes in TCA intermediate concentrations had occurred. No differences were measured in total glutamate or glutamine concentrations (μmoles/g. wet mass) between the hypertrophied and control hearts (7.01 +/- 0.19, n=6 versus 6.86 +/- 0.08, n=6 and 2.62 +/- 0.10, n=6 versus 2.29 +/- 0.10, n=6 respectively).

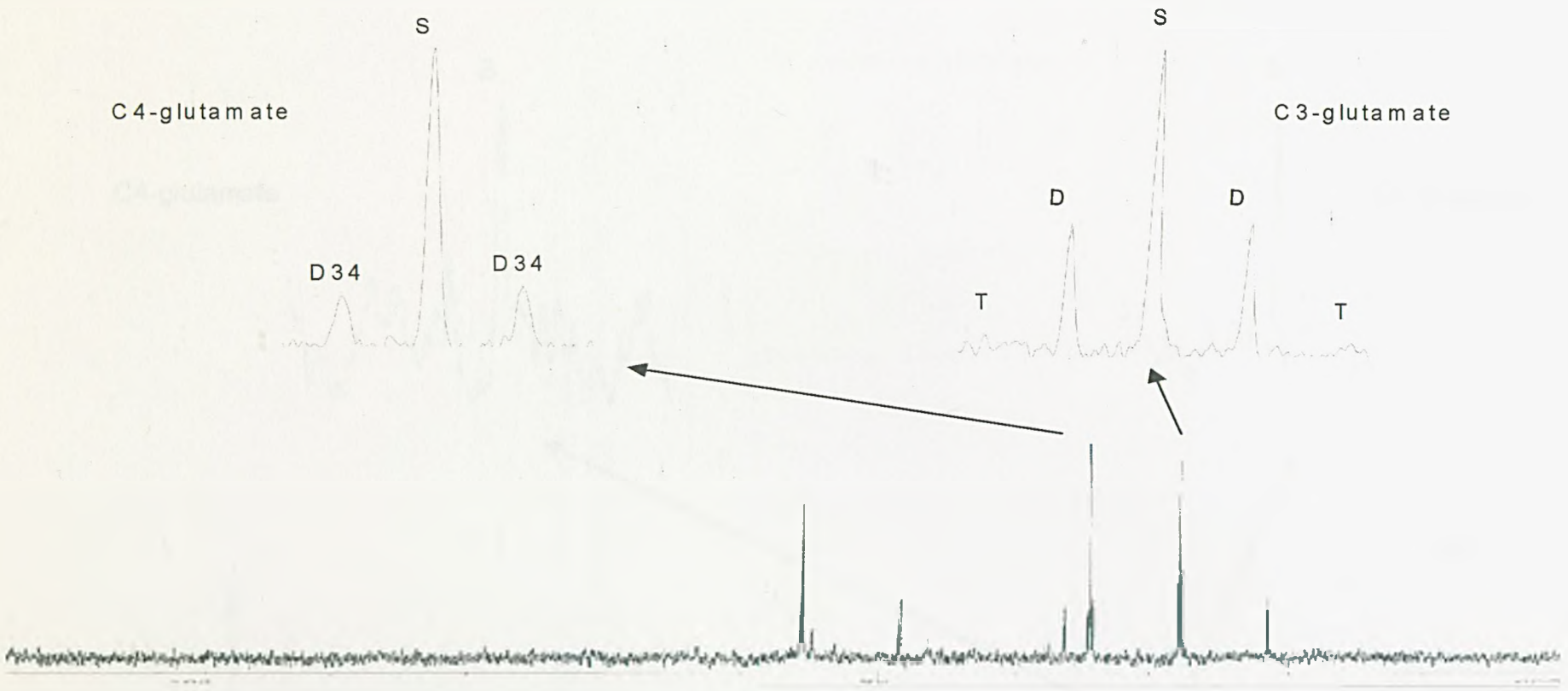

Figure 3.2. Typical example of a ^{13}C NMR spectrum obtained from a hypertrophied heart perfused with [6-^{13}C]-hexanoate and unlabelled glucose. The C3 expanded region consists of a central singlet (C3S) peak corresponding to labelling of carbon 3 alone, a doublet (C3D) corresponding to labelling of the carbons 3 and 4 and a triplet (C3T) corresponding to labelling of carbons 2, 3 and 4 simultaneously (Jeffrey *et al* 1991). The C4 isotopomer pattern arises from a singlet peak (C4S), corresponding to labelling of carbon 4 alone and a doublet (C4D34) corresponding to labelling of the carbons 3 and 4.

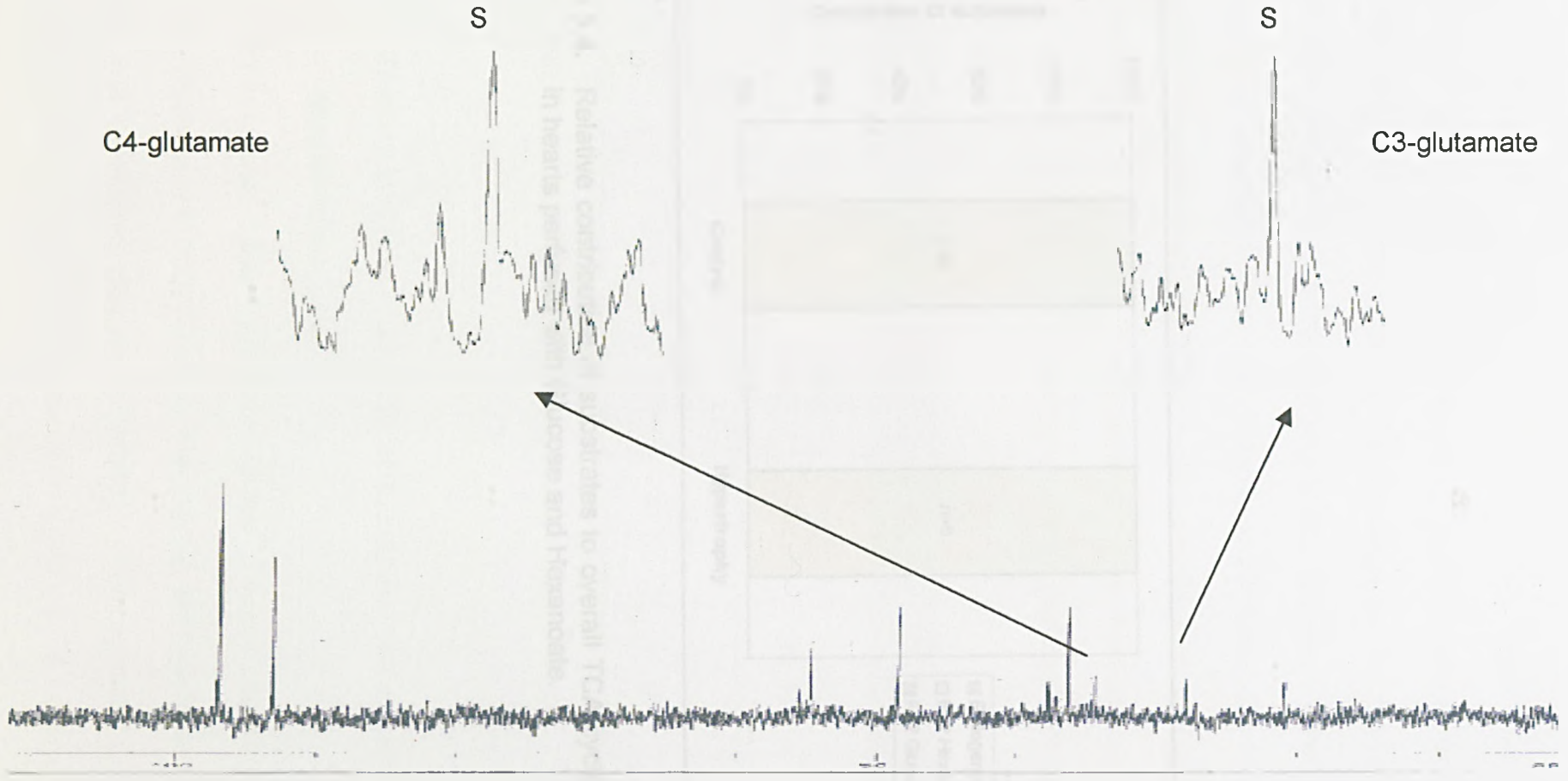

Figure 3.3. A typical ^{13}C NMR spectrum from a hypertrophied heart perfused with [1-^{13}C]-glucose and unlabelled hexanoate.

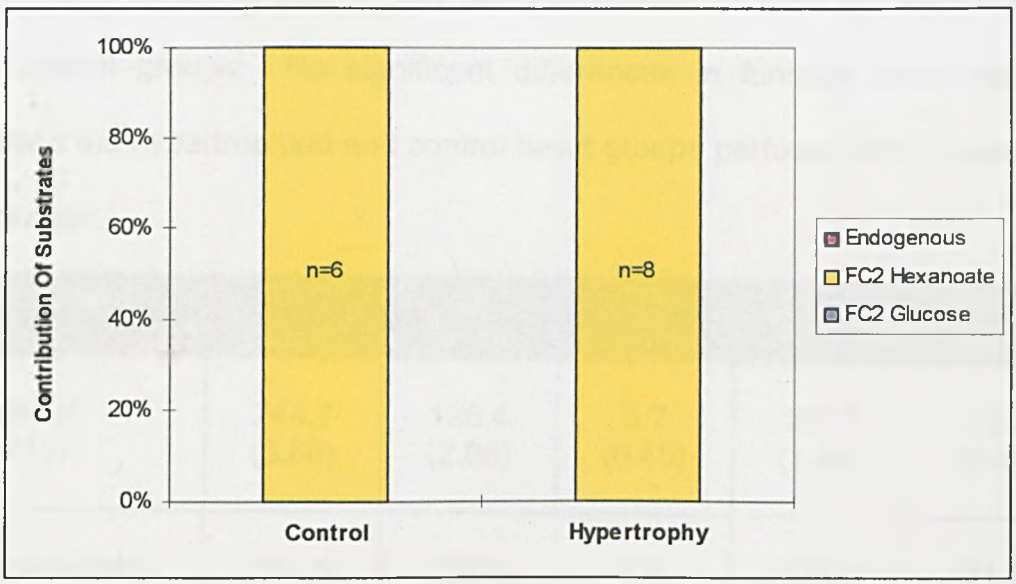

Figure 3.4. Relative contribution of substrates to overall TCA cycle oxidation in hearts perfused with Glucose and Hexanoate.

3.3.2.1b. Functional data.

Table 3.2(a) shows the functional characteristics (mean +/- SEM) for hypertrophied and control hearts perfused with glucose (5mM) and the short chain fatty acid, hexanoate (0.3mM). ^{13}C labelling did not significantly alter heart function within the hypertrophied and control groups whether supplied in the glucose or hexanoate substrate. Therefore the functional data from the consecutive labelling experiments were combined for both the hypertrophied and control groups. No significant differences in function were observed between the hypertrophied and control heart groups perfused with glucose and hexanoate.

Treatment Groups	HR (bpm)	SP (mmHg)	DP (mmHg)	LVDP (mmHg)	RPP ($X10^3$)
Control (n=10)	244.7 (3.60)	126.4 (2.06)	5.7 (0.45)	120.7 (1.94)	29.4 (0.45)
Hypertrophy (n=12)	254.0 (3.61)	132.4 (1.20)	6.4 (0.11)	126.0 (1.18)	31.7 (0.39)

Table 3.2(a). Function in Glucose & Hexanoate substrate groups.

3.3.2.2. Glucose and long chain fatty acid, palmitate as substrates.

3.3.2.2a. Metabolic analysis.

The spectra in Figures 3.5 and 3.6 show typical ^{13}C spectra obtained from nine week hypertrophied and control hearts respectively, perfused with ^{13}C labelled palmitate and unlabelled glucose substrates. The expanded C3 and C4 glutamate resonances can be seen for each spectrum. A small central peak

was occasionally present in the centre of the C4 glutamate resonance pattern. This corresponded to approximately 2-4% of the total label incorporation and was only clearly detectable when 50% [U-^{13}C]-palmitate was supplied. There are two possible explanations for this peak, one being due to the natural abundance of ^{13}C glutamate and the second being due to 2-4% Fc2 labelling entering the cycle via anaplerotic pathways. The splitting pattern for C3 glutamate resonances using labelled palmitate occurs in the same way as described for the C3 resonances using [6-^{13}C]-hexanoate.

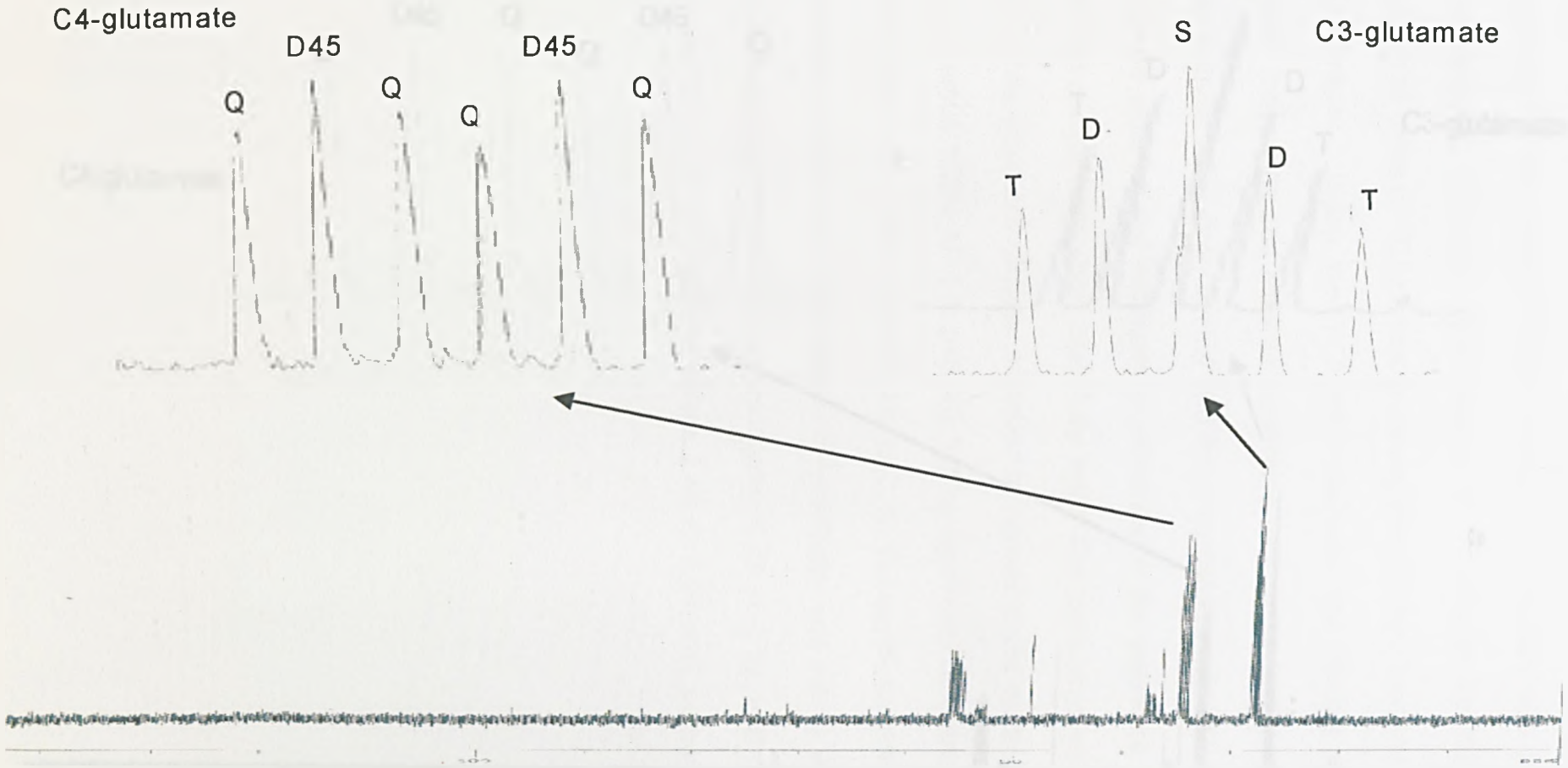

Figure 3.5. Typical ^{13}C spectra obtained from a nine week hypertrophied heart perfused with [U-^{13}C]-palmitate and unlabelled glucose substrates. The doublet peaks (C4D45), occur due to this labelling of the C4 and C5 carbons of glutamate. In the C4 NMR resonances, the quartet peaks (C4Q), arise from the simultaneous labelling of the C3, C4 and C5 carbons of glutamate.

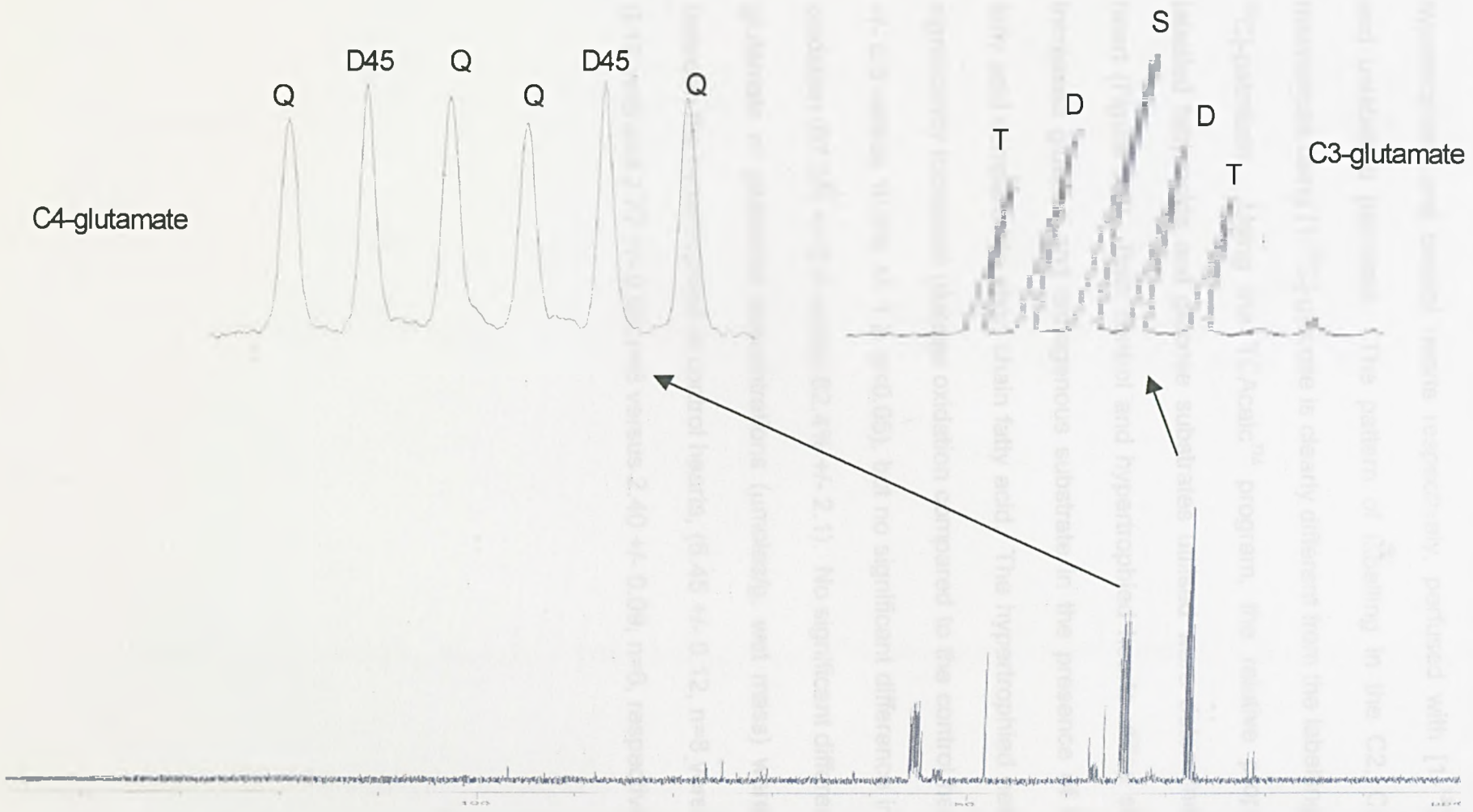

Figure 3.6. Typical ^{13}C spectra obtained from a nine week control heart perfused with [U-^{13}C]-palmitate and unlabelled glucose substrate.

The spectra in Figures 3.7 and 3.8 show ^{13}C spectral examples of hypertrophied and control hearts respectively, perfused with [1-^{13}C]-glucose and unlabelled palmitate. The pattern of labelling in the C2, C3 and C4 resonances using [1-^{13}C]-glucose is clearly different from the labelling using [U-^{13}C]-palmitate. Using the TCAcalc^{TM} program, the relative proportions of labelled fatty acids and glucose substrates utilised were determined in each heart (Figure 3.9). Both control and hypertrophied hearts utilise significantly increased glucose and endogenous substrate in the presence of long chain fatty acid compared to short chain fatty acid. The hypertrophied heart exhibits significantly increased glucose oxidation compared to the control heart (18.4% +/- 0.8 versus 10.5% +/- 1.3, $p<0.05$), but no significant difference in palmitate oxidation (67.3% +/- 2.4 versus 62.4% +/- 2.1). No significant differences in the glutamate or glutamine concentrations (μmoles/g. wet mass) were observed between the hypertrophied or control hearts, (6.45 +/- 0.12, n=8 versus 6.07 +/- 0.17, n=6 and 2.77 +/- 0.08, n=8 versus 2.40 +/- 0.09, n=6, respectively).

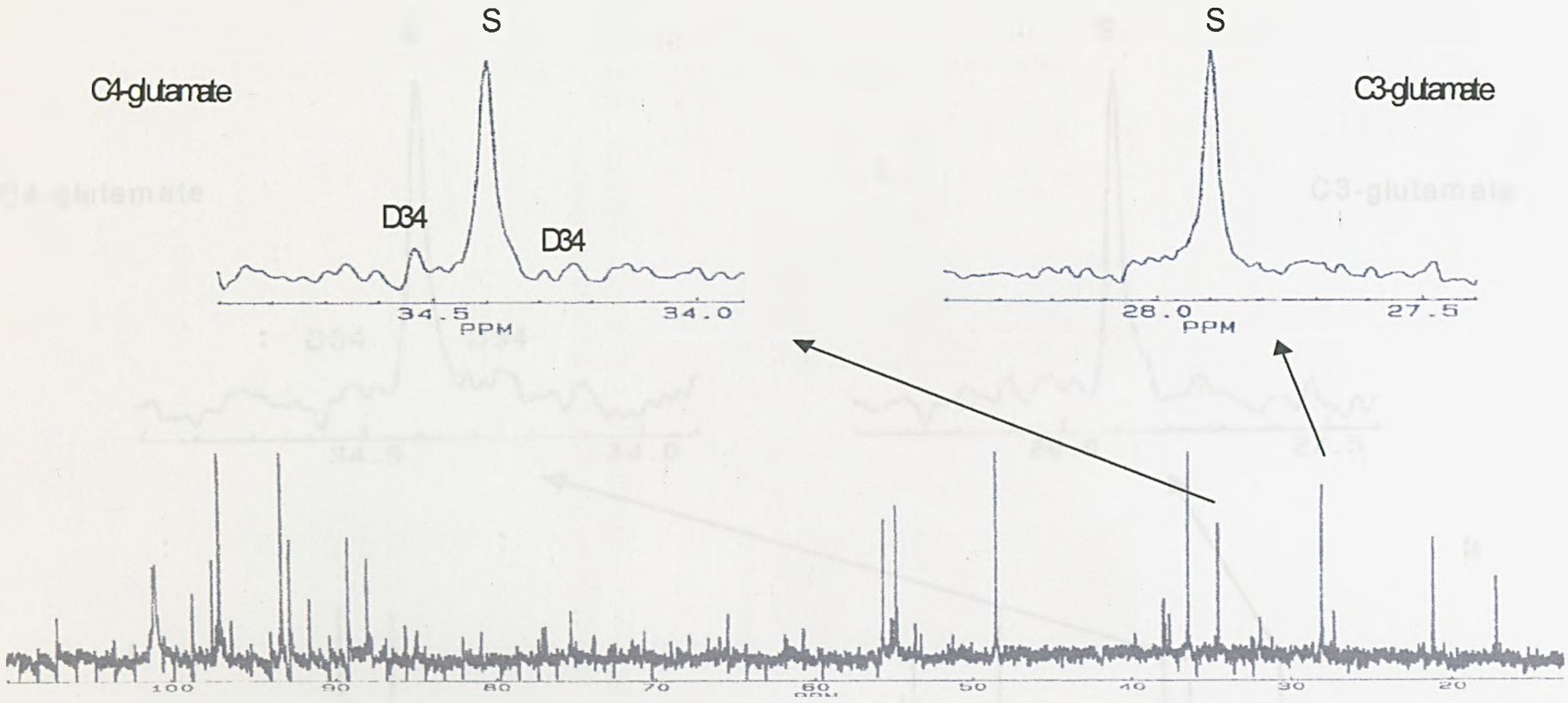

Figure 3.7. Typical ^{13}C NMR spectra of a nine week hypertrophied heart perfused with [1-^{13}C]-glucose and unlabelled palmitate. The expanded regions show the isotopomer patterns of the C3 and C4 glutamate resonances. The glutamate resonances consist of a C4 singlet peak (C4S) due to labelling of the C4 carbon alone and a doublet (C4D34) from labelling of the C3 and C4 carbons. The isotopomer pattern for C3 glutamate resonances using labelled glucose occurs as described previously in Figure 3.2.

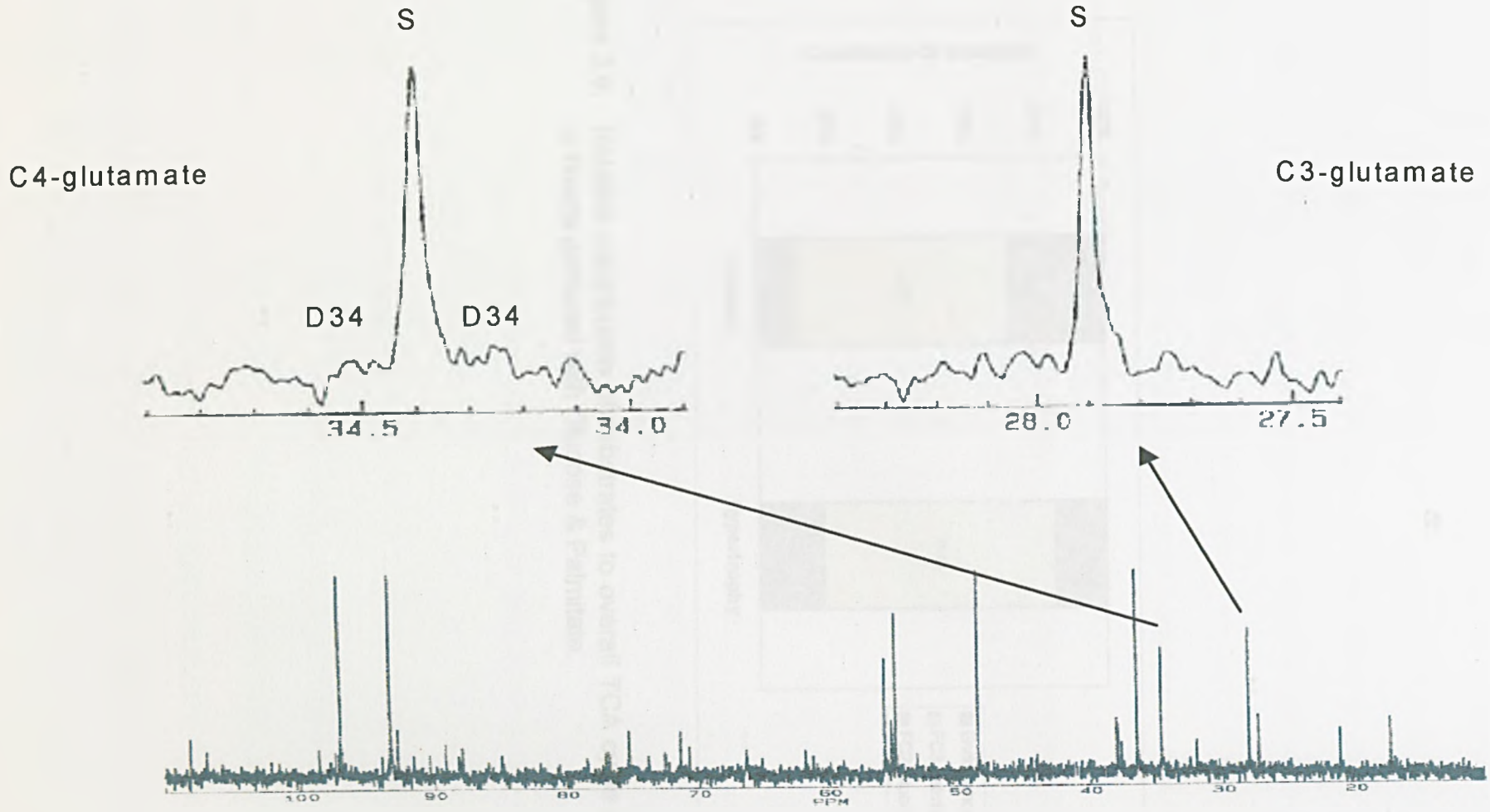

Figure 3.8. Typical ^{13}C NMR spectra of a nine week control heart perfused with [1-^{13}C]-glucose and unlabelled palmitate.

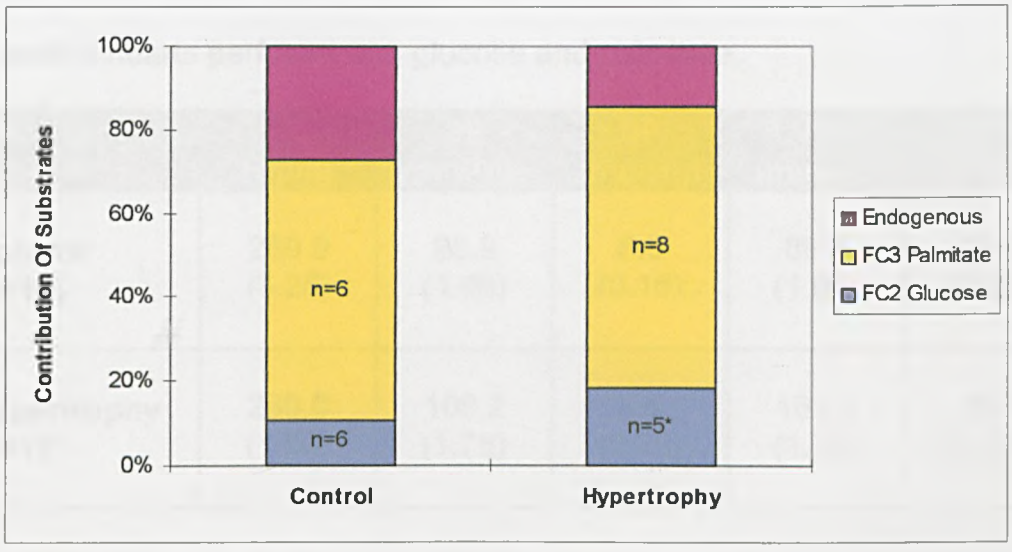

Figure 3.9. Relative contribution of substrates to overall TCA cycle oxidation in hearts perfused with Glucose & Palmitate.

3.3.2.2b. Functional data.

Table 3.2(b) shows a summary of the functional characteristics for hypertrophied and control hearts perfused with glucose and the long chain fatty acid, palmitate. Again the ^{13}C label had no adverse effect on heart function, and thus the functional data from consecutive labelling experiments were combined for hypertrophied and control groups. No significant differences were measured in any of the functional parameters between the hypertrophied and control hearts perfused with glucose and palmitate.

Treatment Groups	HR (bpm)	SP (mmHg)	DP (mmHg)	LVDP (mmHg)	RPP ($X10^3$)
Control (n=10)	269.9 (3.28)	95.9 (1.06)	6.6 (0.16)	89.3 (1.04)	23.9 (0.29)
Hypertrophy (n=12)	260.0 (1.50)	108.2 (1.75)	6.6 (0.20)	101.5 (1.72)	26.1 (0.31)

Table 3.2(b). Function in Glucose & Palmitate substrate groups.

3.3.2.3. Glucose and long chain fatty acid palmitate in the presence of insulin.

3.3.2.3a. Metabolic analyses.

Figures 3.10 and 3.11 show ^{13}C NMR spectra of hypertrophied and control hearts respectively, perfused with [1-^{13}C]-glucose, unlabelled palmitate and insulin. The incorporation of glucose label is greatly increased in the presence of insulin, highlighted by the increased signal to noise ratio. The isotopomer patterns of the C3 and C4 glutamate resonances from hearts perfused with

glucose, palmitate and insulin are identical to those in hearts perfused with glucose and palmitate in the absence of insulin (Figures 3.7 and 3.8), only the peak height proportions alter.

Comparisons within the substrate group show that although insulin induces an increase in the amount of glucose utilised, in both hypertrophied and control hearts (36.0% +/- 4.2 versus 50.7% +/- 5.0 respectively), the effect is attenuated in the hypertrophied hearts (Figure 3.12). Interestingly this increase in glucose oxidation is compensated by a decrease in fatty acid oxidation. In the presence of insulin the hypertrophied heart shows a significant reduction in the oxidation of palmitate compared to control hearts (47.2% +/- 2.2 versus 59.1% +/- 2.5, $p<0.05$), replaced by a greater contribution of endogenous substrate use. The total glutamate concentration (μmoles/g. wet mass) was not significantly different between hypertrophied and control hearts (6.03 +/- 0.29, n=5 versus 6.18 +/- 0.25, n=7). This was also the case for total glutamine concentrations (μmoles/g. wet mass) between hypertrophied and control hearts (2.60 +/- 0.07, n=5 versus 2.42 +/- 0.06, n=7).

Comparison between substrate groups showed that both control and hypertrophied heart groups perfused with glucose and hexanoate had significantly increased percentage fatty acid oxidation compared to those perfused with both glucose and palmitate and glucose, palmitate and insulin ($p<0.001$). Comparing palmitate oxidation values between control groups showed no significant difference between the presence or absence of insulin, whereas hypertrophied hearts perfused with insulin showed significantly reduced palmitate oxidation compared to hypertrophied hearts perfused with palmitate without the presence of insulin ($p<0.05$). There was also a significant

increase in glucose oxidation in hearts perfused with glucose, palmitate and insulin compared to control ($p<0.01$) and hypertrophied ($p<0.05$) heart groups perfused with glucose and palmitate without insulin, whereas, total glutamate and glutamine concentrations were not significantly different, when substrate groups were compared.

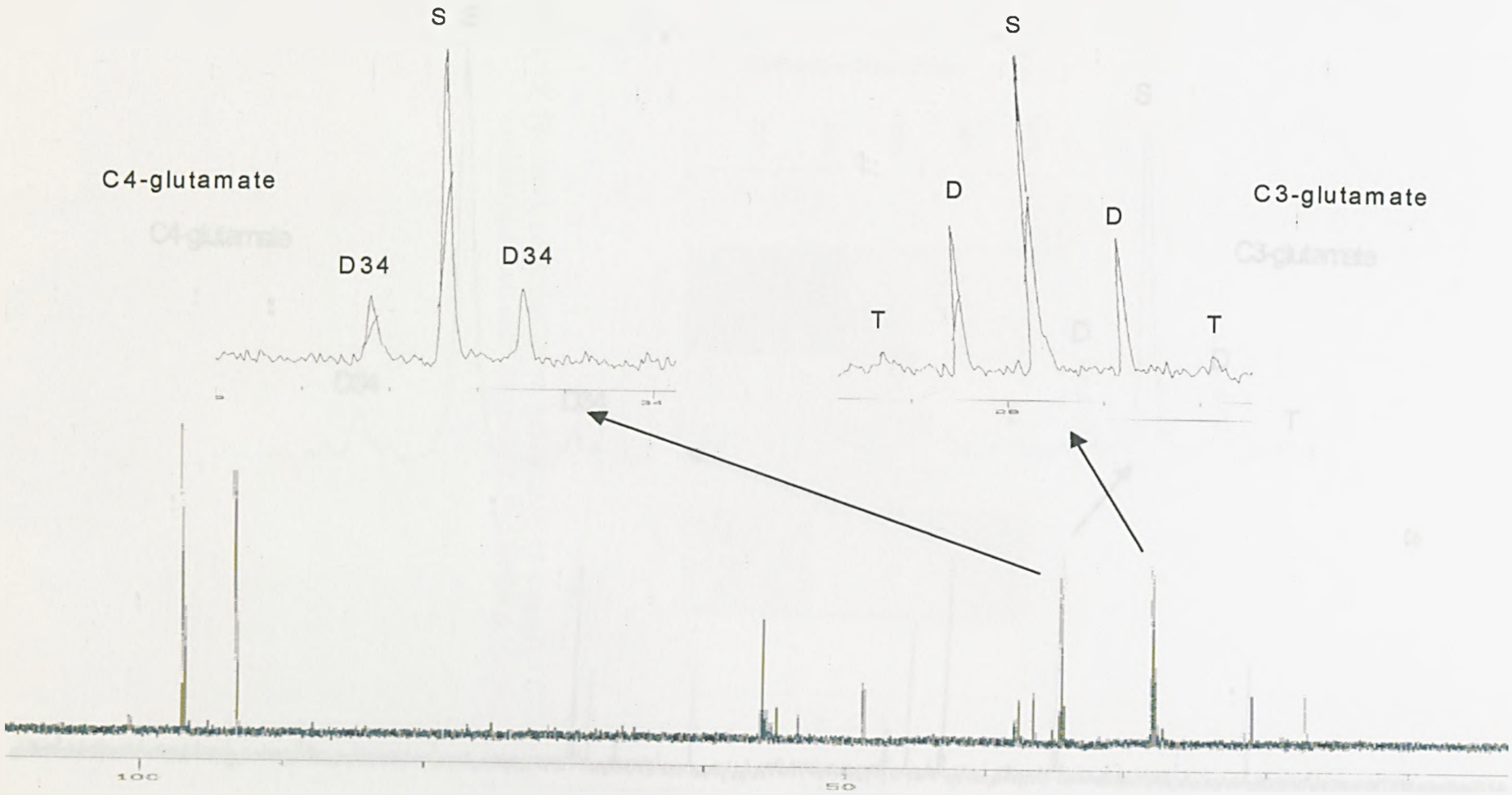

Figure 3.10. Typical ^{13}C NMR spectra of a nine week hypertrophied heart perfused with [1-^{13}C]-glucose and unlabelled palmitate in the presence of insulin.

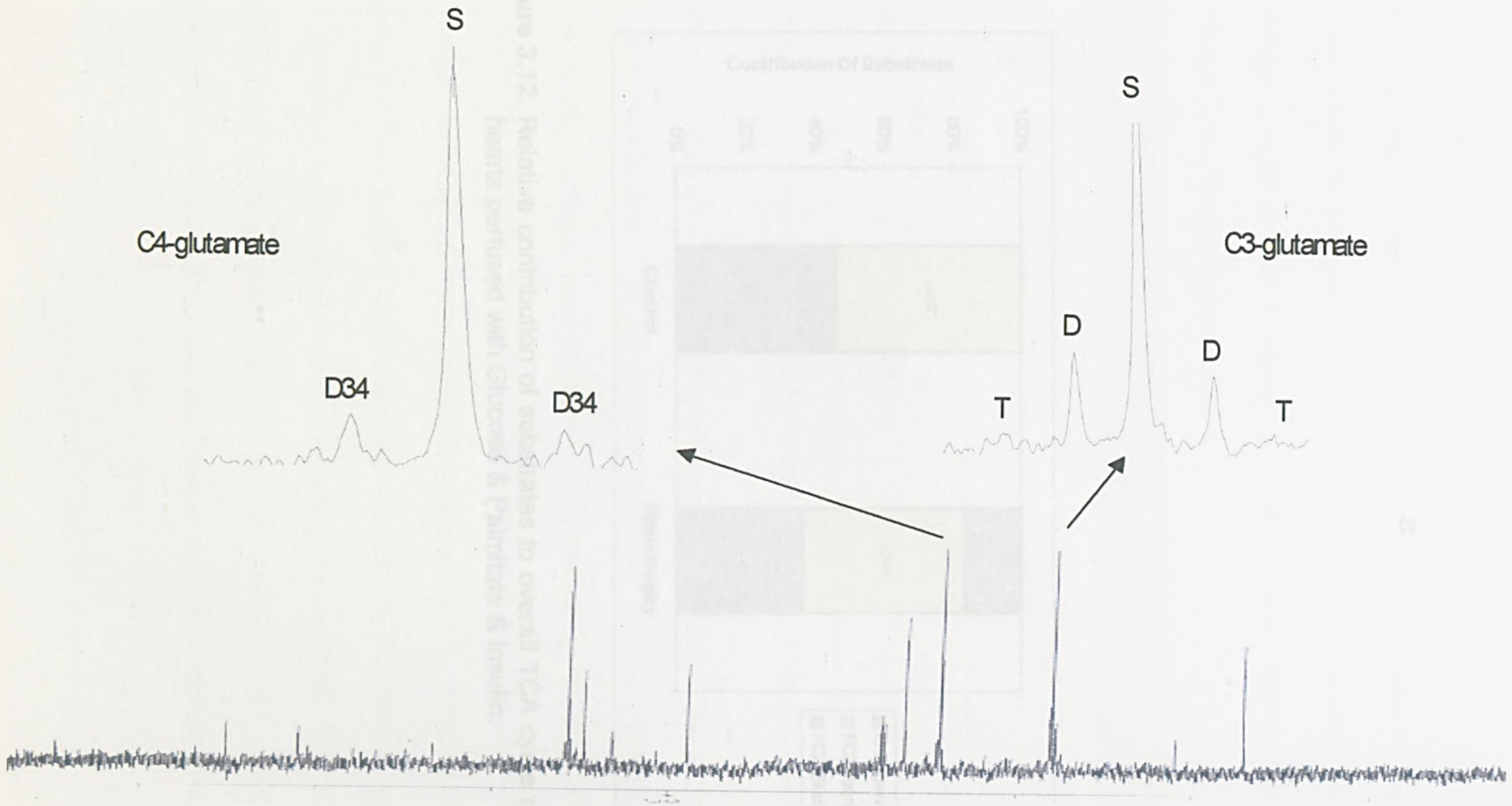

Figure 3.11. Typical ^{13}C NMR spectra of a nine week control heart perfused with [1-^{13}C]-glucose and unlabelled palmitate in the presence of insulin.

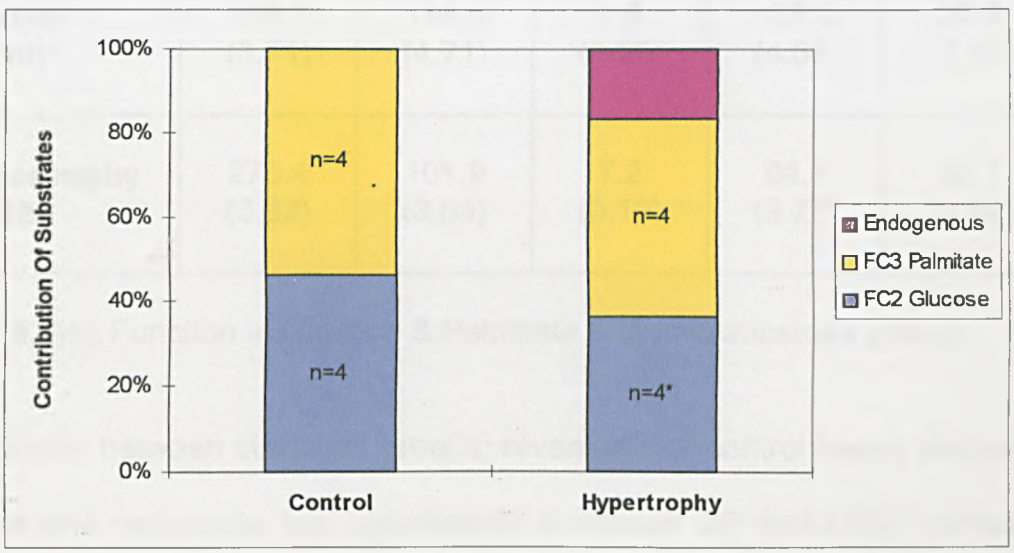

Figure 3.12. Relative contribution of substrates to overall TCA cycle oxidation in hearts perfused with Glucose & Palmitate & Insulin.

3.3.2.3b. Functional data.

Table 3.2(c) shows a summary of the functional characteristics for hypertrophied and control hearts perfused with glucose, fatty acids and insulin. No significant differences in any of the functional parameters were observed between the hypertrophied and control hearts.

Treatment Groups	HR (bpm)	SP (mmHg)	DP (mmHg)	LVDP (mmHg)	RPP ($X10^3$)
Control (n=10)	257.7 (3.71)	114.8 (4.71)	7.8 (0.25)	107.0 (4.61)	27.5 (1.19)
Hypertrophy (n=12)	275.4 (3.92)	101.9 (3.64)	7.2 (0.19)	94.7 (3.77)	25.7 (0.81)

Table 3.2(c). Function in Glucose & Palmitate & Insulin substrate groups.

Comparison between substrate groups, revealed that control hearts perfused with glucose and hexanoate had significantly increased SP and LVDP compared to control hearts perfused with glucose and palmitate ($p<0.05$). Control hearts perfused in the presence of insulin showed comparable functional characteristics with those perfused with any substrate composition. Hypertrophied hearts perfused with glucose and hexanoate had significantly increased SP, LVDP and RPP compared to the hypertrophied hearts perfused with both glucose and palmitate and glucose, palmitate and insulin ($p<0.05$). No other significant differences were found in functional parameters between substrate groups.

3.4. Discussion.

3.4.1. Model of hypertrophy.

After nine weeks of abdominal aortic constriction, a moderate degree of cardiac hypertrophy was achieved, as indicated by the 10.9% increase in heart mass to tibia length ratio (Table 3.1(c)). This index of hypertrophy is much lower than the 20-25% increase previously reported with this model (Seymour and Chatham 1997, Boateng *et al* 1997). The hearts in these previous studies were weighed immediately after excision and had not been subjected to perfusion unlike the hearts in this study. Oedema caused by the crystalloid perfusion may be one reason for a difference between the two studies, causing a high heart mass to tibia length ratio in control hearts and masking the real extent of hypertrophy.

However parallel histological studies have shown a greater than 30% increase in myocyte size (Linehan *et al* 2001). This increase in cell size correlates well with the changes seen in the kidney ratios (Table 3.1(a)). The 25% difference in right to left kidney ratio may therefore be a more accurate determinant of cardiac hypertrophy for this study. This model of hypertrophy showed no evidence of pulmonary hypertension as determined by the wet to dry ratios of the lungs or hepatic congestion from examination of liver wet mass (Table 3.1(b)). Plasma metabolite concentrations were not different between control and hypertrophied hearts (Table 3.1(c)). It was therefore valid to use physiological concentrations of glucose and fatty acids during the heart perfusion experiments. It can be concluded that nine weeks of aortic constriction created a model of compensated hypertrophy, allowing early metabolic adaptations to be investigated.

One interesting observation was the resulting 8% hypertrophy of the spleen (Table 3.1(b)). Splenic hypertrophy in this hypertrophy model may be due the effects of a number of stimulatory growth factors. *In vitro* studies have shown that pressure-overload hypertrophy can be mimicked by the administration of various stimuli (Morgan and Baker 1991). These can include adrenergic agonists (Zierhut and Zimmer 1989, Simpson *et al* 1991, Ikeda *et al* 1991), endothelin-1 (Shubeita *et al* 1990), angiotensin II (Baker and Aceto 1990), FGF and TGFβ (Parker *et al* 1990, Schneider *et al* 1990) and cardiotrophin-1 (Pennica *et al* 1995). Cardiotrophin-1 (CT-1) is a recently discovered cytokine thought to be a possible autocrine factor that mediates hypertrophy. CT-1 has the ability to induce cardiac hypertrophy in mice *in vivo* (Jin *et al* 1996) and *in vitro* (Pennica *et al* 1995) and CT-1 mRNA expression is reportedly elevated in SHR rats (Ishikawa *et al* 1996). CT-1 is not singly specific to the heart and it can be expressed in other tissues. It has been shown to stimulate the growth of other organs in mice such as the spleen, liver, and kidney (Jin *et al* 1996). No organs measured besides the heart and spleen were found to hypertrophy in the aortic-banded rat model used in this study. It is therefore unlikely that a cytokine such as CT-1 would only affect the heart and spleen organs, unless factors such as species differences and severity of the haemodynamic stress are involved. CT-1 is unlikely to be the only growth factor involved in the cardiac hypertrophic response and further investigation would be required to determine the distinct cause of this splenic phenomenon.

3.4.2. Substrate selection in cardiac hypertrophy.

3.4.2.1. Glucose and short chain fatty acid metabolism.

3.4.2.1.1. ^{13}C NMR data.

The ^{13}C NMR data obtained here and the determination of the relative contributions of different substrates revealed that both the hypertrophied and control hearts utilised hexanoate almost exclusively, despite the presence of physiological concentrations of glucose (Figure 3.4). This finding is consistent with that of Weiss *et al* (1989) who demonstrated that hexanoate can inhibit glucose oxidation in control hearts at the PDH enzyme step. Chatham and Forder (1997) also obtained similar results.

The ^{13}C NMR spectra from the labelled hexanoate perfused heart extracts exhibited good signal to noise ratios and clearly defined glutamate isotopomer splitting patterns (Figure 3.2). In contrast the ^{13}C NMR spectra obtained from perfusions with [1-^{13}C]-glucose as substrate showed almost no label incorporation (Figure 3.3), and its relative percentage utilisation was non-determinable using the TCAcalc™ program. These studies support the observation that in normal heart muscle, fatty acids are the preferred substrates for oxidation in order to meet energy demands (reviews by Neely and Morgan 1974, and Lopaschuk *et al* 1994). As hexanoate is directly transported into the mitochondria and circumvents the carnitine-acylcarnitine step (Webster *et al* 1964) there are no problems with substrate delivery. The fact that short chain fatty acid oxidation in the hypertrophied heart remained unaffected during pressure-overload reveals that there can be no defects in the β-oxidation of short-chain fatty acids or respiratory

chain function per se.

In support of these findings El Alaoui-Talibi *et al* (1989, 1992) demonstrated that octanoate oxidation and $^{14}CO_2$ production remained well preserved in volume-overloaded hypertrophied hearts, despite a reduction in tissue carnitine concentration. Similarly, a study by Cheikh *et al* (1994) on the respiratory chain found no limitations in mitochondrial respiration when the octanoate substrate was supplied to volume-overloaded rat hearts.

^{13}C NMR studies and glutamate isotopomer distribution analyses have been used to study heart substrate metabolism in the control (Bailey *et al* 1981, Chance *et al* 1983, Sherry *et al* 1985, Malloy *et al* 1990) and diabetic heart (Chatham and Forder 1993 and 1997) for many years. ^{13}C isotopomer analysis enables determination of endogenous and exogenous substrate contribution to TCA cycle oxidation and substrate competition to be assessed in a single experiment (Malloy *et al* 1993). It also has the advantage that it does not require the contributions from the fatty acid to be completely oxidised. The TCAcalc™ program relies on two major assumptions (Malloy *et al* 1990). Firstly that cardiac glutamate concentration does not change. In the present study total glutamate concentration in the heart extracts did not alter between hypertrophied and control hearts fulfilling this condition. The second assumption is that glutamate is in direct rapid equilibrium with α-ketoglutarate via transaminase reactions involving glutamate dehydrogenase. Furthermore, glutamate dehydrogenase activity is low in the heart and concerns have been voiced as to whether cardiac glutamate dehydrogenase is in equilibrium with its reactants (Opie 1984). Unfortunately

NMR spectroscopy on heart extracts alone cannot provide information concerning the magnitude of flux through the TCA cycle. A dynamic timecourse and mathematical modelling of ^{13}C incorporation into C3 or C2 compared to C4 glutamate of perfused hearts are required to determine the TCA cycle flux (Malloy *et al* 1993, Weiss *et al* 1992).

3.4.2.1.2. Heart function with glucose and short chain fatty acid metabolism.

The functional performance of pressure-overloaded hearts perfused with 0.3mM hexanoate and 5mM glucose, as competing substrates did not differ significantly from that of control hearts (Table 3.2). This was reflected in all parameters measured. The maintenance of mechanical function in hypertrophied hearts highlights the adequate provision of substrate. The mechanical activity in this study supports the ^{13}C NMR data, providing no evidence of limited substrate availability when short-chain fatty acid hexanoate and glucose are supplied. This study therefore proves that the use of short chain fatty acid, hexanoate did not alter the contractility or energy turnover of hypertrophied hearts. In support of this El Alaoui-Talibi *et al* (1989 and 1992) found that the mechanical performance of hypertrophied hearts, as measured by cardiac output ($ml.min^{-1}.gdw^{-1}$) and pressure work ($bpm.Torr.10^{-3}$) was comparable to control hearts when 2.4mM octanoate was used as the major exogenous substrate.

Oxygen consumption data was not obtained for this study but data was

measured by El Alaoui-Talibi *et al* (1992), who have shown that hypertrophied hearts utilising octanoate as the main exogenous substrate showed O_2 consumption rates ($\mu mol.min^{-1}.g.dry\ wt^{-1}$) comparable to control hearts perfused with the same substrate.

3.4.2.2. Glucose and long-chain fatty acid metabolism.

3.4.2.2.1. ^{13}C NMR analysis.

Control and hypertrophied hearts perfused with this substrate mixture showed a very different profile of substrate use compared to hearts perfused with glucose and short chain fatty acid substrates. In the presence of the long-chain fatty acid, palmitate, there was oxidation of glucose and palmitate, in both control and hypertrophied hearts, seen clearly in the NMR spectra (Figures 3.7 and 3.8), sufficient for accurate determination of their relative contributions (Figure 3.9). This demonstrates that the metabolic relationship of glucose oxidation in the presence of long chain fatty acid differs to that in the presence of short chain fatty acid.

3.4.2.2.2. Glucose metabolism.

Hypertrophied hearts supplied with glucose and palmitate as oxidisable substrates, utilised significantly 75% more glucose compared to control hearts (18.4 versus 10.5% respectively; Figure 3.9). Increased glucose utilisation as a myocardial substrate in this pressure-overload hypertrophy model supports the findings of other studies, in which increased capacity for glucose metabolism has

been reported in different models of hypertrophy (Seymour *et al* 1990, Taegtmeyer and Overturf 1988, Yonekura *et al* 1985, Leipala *et al* 1989). Glucose oxidation in both hypertrophied and control hearts is characterised by the incorporation of ^{13}C-label from [1-^{13}C]-glucose to the C2, C3 and C4 carbons of glutamate. This is the first study to show that the relative percentage of glucose oxidised in the TCA cycle is increased during pressure-overload hypertrophy using ^{13}C NMR and is in agreement with studies using ^{14}C substrates (Allard *et al* 1994).

The potential mechanisms resulting in increased glucose utilisation need to be considered. Increased glucose oxidation in the hypertrophied hearts may be due to increased glucose transport, an elevation of glycolysis, increased glucose flux into the TCA cycle or some combination of the above. Increased glucose metabolism during hypertrophy may arise as an early adaptation in cardiac hypertrophy. Clear evidence exists for the re-expression of foetal genes such as those encoding glucose transporters GLUT-I and -4 (Depre *et al* 1998). The foetal heart has a greater dependence on glucose metabolism for energy provision compared to the normal heart (Lopaschuk *et al* 1992, 1994). Glucose oxidation can be considered more efficient yielding 36 ATP molecules per mole of glucose (Taegtmeyer 1994). In the hypertrophied myocardium, limited oxygen delivery to the myocyte could result from reduced capillary density and increased oxygen diffusion distances, associated with increased muscle mass of hypertrophied hearts (Katz 1990). As a consequence the enhanced glucose utilisation seen here during cardiac hypertrophy may be related to a conservation of energy stores in the presence of reduced oxygen availability. This study would support the

proposal of a shift towards glucose oxidation.

In addition the increase in glucose uptake witnessed in hypertrophied hearts could result from chronically increased sympathetic activation (i.e. norepinephrine) during the pressure overload exposure *in vivo*. α-Adrenergic receptor activation is known to mediate translocation of the glucose transporter to the sarcolemma (Rattigan *et al* 1986), thus aiding glucose uptake into the heart cells by increasing the availability of glucose transporters. This activation may also help explain the reported increase in glycolysis (Seymour *et al* 1990) in hypertrophy, where PFK a major rate limiting enzyme in the glycolytic pathway is activated by β– and α–adrenergic receptor stimulation (Patten *et al* 1982).

3.4.2.2.3. Fatty acid oxidation.

^{13}C NMR spectra from hearts perfused with uniformly labelled palmitate gave very good signal to noise ratios. Subsequently perfusions were continued using 50% of the fatty acid labelled with ^{13}C. No inaccuracy was incurred by switching to this method because the percentage of fatty acid contribution calculated was comparable between the two methods. This study indicates that under aerobic conditions, long-chain fatty acid is the preferred myocardial substrate in both hypertrophied and control rat hearts (67.3% versus 62.4% respectively; Figure 3.9). This is consistent with early heart studies showing that the oxidation of long-chain fatty acids can provide up to 70% of metabolic energy of well oxygenated myocardium (Neely and Morgan 1974, Weiss *et al* 1976).

The proportion of long chain fatty acid utilisation in nine week hypertrophied

hearts did not differ from that of the control hearts. In contrast abnormalities of fatty acid metabolism have been demonstrated in other hypertrophied heart studies. Cardiac failure secondary to chronic pressure overload has been shown by Wittels *et al* (1968) to result in defective long chain fatty acid metabolism in guinea pig hearts, whereas chronic volume overload induced a reduction of long chain fatty acid metabolism in the rat heart (El Alaoui-Talibi *et al* 1992). Hypertensive Dahl rat hearts have also shown reduced long chain fatty acid uptake compared to normal hearts (Yonekura *et al* 1985). However in the present hypertrophied hearts, normal fatty acid oxidation was accompanied by an increase in glucose oxidation. The major proposal is that during the development of cardiac hypertrophy there is a shift from fatty acid to glucose utilisation. The present study provides evidence against alterations in fatty acid and glucose oxidation occurring simultaneously in pressure-overload cardiac hypertrophy. Instead, it identifies sequential changes in the substrate profile during the development of cardiac hypertrophy. Certainly in this mild model of compensated hypertrophy, the changes in glucose appear to precede any changes in fatty acid use. In addition this is the first study that identifies the fact that glucose changes precede fatty acid alterations and highlights the possible role of metabolism in the progression of disease.

A decrease in long chain fatty acid use has generally been attributed to a decrease in tissue carnitine levels. High levels of myocardial carnitine are essential for the β-oxidation of long chain fatty acids (Borrebaek *et al* 1976, Bremer 1962). Carnitine ensures transmembrane fluxes of long chain fatty acyls

from the cytosol to the mitochondria, in which acyl transferases play a key role (Pande and Parvin 1976, Idell-Wenger *et al* 1982). It is proposed that decreased tissue carnitine levels may compromise free fatty acid oxidation and lead to an accumulation of triacylglycerols in the heart (Wittels *et al* 1968, Reibel *et al* 1983, Bowe *et al* 1984). This is possibly due to conditions of low cytosolic carnitine to CoA ratios limiting fatty acid activation and long-chain fatty acyls translocation from the cytosol to mitochondria.

Myocardial carnitine concentration was found to decrease by 30% in the present nine week pressure-overload model (K. Hornby, Personal communication 1999), consistent with other studies on myocardial hypertrophy in guinea-pigs (Wittels *et al* 1968), rabbits (Revis and Cameron 1979), rats (Reibel *et al* 1983, Bowe *et al* 1984, El Alaoui-Talibi *et al* 1992) and Syrian hamsters with cardiomyopathy (Whitmer *et al* 1986). Myocardial carnitine levels are maintained normally by passive diffusion and carrier-mediated transport from the blood (Bohmer *et al* 1977, Bahl *et al* 1981, Vary *et al* 1982). Such alterations in tissue carnitine content could result from a decrease in carrier-mediated transport and/or changes in carnitine efflux from the heart. El Alaoui-Talibi *et al* (1989) found such depressed total and carrier-mediated transport in volume-overload hypertrophy alongside a decreased rate of palmitate oxidation and overall contribution of long-chain fatty acids to overall energy turnover.

Although there was an approximate 30% decrease in total carnitine levels associated with this pressure-overload model, it did not have any effect on fatty acid oxidation. One reason may be that cytosolic carnitine content was decreased

but mitochondrial content was unchanged. El Alaoui-Talibi *et al* (1992) reported palmitoylcarnitine translocase proceeded normally in volume-overload hypertrophy despite a 30% decrease in carnitine levels, suggesting the limitation of palmitate oxidation was not at the level of carnitine-acylcarnitine translocase. It may well be that carnitine depletion must be very severe to have an impact (i.e. that the Km for CPT is very low)(Bohmer *et al* 1977, Bahl *et al* 1981).

The fact that nine weeks pressure-overload hypertrophy, in this study did not result in any alteration of the percentage long-chain fatty acid contribution to TCA cycle oxidation compared to control hearts, suggests the hypertrophied hearts contained adequate carnitine-acyl-CoA transferase and carnitine acylcarnitine translocase enzyme activities (Bremer 1983). Unlimited carnitine-acyl-CoA transferase and carnitine-acylcarnitine translocase activities are required to promote high rates of cytosolic long-chain acyls translocation into the mitochondria for oxidation (Bremer 1983), accompanied by free carnitine recycling back to the cytosol (Oram *et al* 1973).

Surprisingly, when 50% labelled fatty acid substrate was supplied to the hearts it revealed a small singlet peak in the centre of the C4 glutamate isotopomer spectra. This occurred in both the control and hypertrophied hearts (Figures 3.5 and 3.6). It can be attributed to ^{13}C labelling of the C4 carbon of glutamate alone, which cannot possibly be accounted for by the initial metabolism of uniformly labelled palmitate, which always labels the C4 and C5 carbons of glutamate simultaneously. Measurement of this peak shows that it represents a consistent 2-4% of the total fatty acid contribution to the TCA cycle. There are at

least two likely explanations for the occurrence of this singlet peak. The first explanation being due to ^{13}C natural abundance of C4 glutamate, in which case natural abundance labelling would also appear in the other carbons of glutamate to the same extent. This singlet peak can be simulated in the TCAsim™ computer package by inserting low values of Fc2 substrate label alongside Fc3 labelling. Another explanation can therefore be considered in which ^{13}C labelled TCA cycle intermediates leave the TCA cycle and rejoin with scrambled labelling, resulting from anaplerotic reactions. This would involve malic enzyme, although malic enzyme is thought to have very low activity in the heart. Unfortunately the TCA calculation package used in this work, to determine the relative contribution of substrate oxidation in the TCA cycle, is not able to accurately determine the involvement of anaplerosis.

3.4.2.2.4. Glycolysis and endogenous substrate use.

^{13}C label from [1-^{13}C]-glucose is also incorporated into lactate and glycogen as seen in Figures 3.7 and 3.8. This indicates that other pathways of glucose metabolism are effective in hypertrophy (glycolysis and glycogen synthesis). It also supports earlier studies which have reported increases in the concentrations of glycolytic enzymes (Bishop and Altschuld 1970, Taegtmeyer and Overturf 1988), glycolytic flux (Seymour *et al* 1990) and decreased glycogen levels (Lortet *et al* 1995) in hypertrophied myocardium

The use of endogenous substrates, such as glycogen, was determined in this study by subtracting the relative percentage glucose and fatty acid

contributions from 100% total TCA cycle substrate use (14.3% versus 27.1% for hypertrophied and control hearts respectively, Figure 3.9). A decreased use of endogenous substrate use balanced the increased reliance on glucose in the hypertrophied hearts. This would suggest that the hypertrophied hearts rely more heavily on the supply of exogenous substrates rather than intracellular stores.

One explanation why this study did not show increased endogenous use compared to other studies could be that competing substrates were supplied i.e. fatty acids were present in the perfusion medium. The study by Seymour *et al* (1990) only included glucose as the exogenous substrate, whereas Lortet *et al* (1995) reported lower glycogen contents in T3-induced hypertrophied hearts, perfused with a glucose-pyruvate substrate mixture. It appears that the choice of substrate(s) supplied is an important consideration when interpreting relative substrate use in the hypertrophied heart. Another reason for discrepancies between the studies could lie in the severity of the hypertrophy model being examined. Lortet *et al* (1995) was examining hearts that had hypertrophied by 50% compared to the 10% hypertrophy achieved in this study.

3.4.2.2.5. Heart function with glucose and long chain fatty acids.

No significant differences were observed in any functional parameters between hypertrophied and control hearts supplied with glucose and palmitate as substrates, despite the observed changes in their percentage substrate use (Table 3.2(b)). This indicates that the hypertrophied hearts in this study were not substrate or oxygen limited.

However comparing different substrates groups hearts supplied with palmitate did not perform as well as those supplied with hexanoate. Hearts perfused with glucose and hexanoate showed markedly higher SP and LVDP than hearts perfused with glucose and palmitate (124.4 versus 95.9 mmHg and 120.7 versus 89.3 mmHg). A similar trend was observed in the hypertrophied hearts. These differences may indicate that substrate availability is a key determinant of function.

The metabolism of glucose and palmitate requires specific transport processes and activation to enter the mitochondria for the final steps leading to the formation of acetyl-CoA. Hexanoate requires no specific transporter or activation, is able to circumvent the carnitine-acylcarnitine translocase system, and thus can directly enter β-oxidation leading to the production of acetyl-CoA (Fritz 1963). Therefore hexanoate can be utilised more readily than palmitate. The effect of hexanoate on contractile activity was similar here to the response of the isolated perfused hypertrophic hearts to short chain fatty acids (Cheikh *et al* 1994). A significant improvement in cardiac function was reported in hypertrophied hearts perfused with glucose plus octanoate compared with hearts perfused with glucose plus palmitate. Cheikh *et al* (1994) suggested that in the presence of palmitate there was a substrate limitation of the respiratory chain that was removed in the presence of octanoate, resulting in an increase in cardiac function.

3.4.2.3. The effect of insulin on glucose and long chain fatty acid metabolism.

3.4.2.3.1. ^{13}C NMR analysis.

Substrate selection in the heart can be influenced not only by workload or oxidative status, but also by extrinsic factors such as insulin (reviewed by Taegtmeyer 1994). The aim of this study was to determine whether insulin modified the profile of substrate selection seen previously.

3.4.2.3.2. Effect of insulin on glucose use.

The presence of a physiological concentration of insulin significantly increased the proportion of exogenous glucose oxidised, in both hypertrophied and control hearts, compared to the insulin-free perfusions (36.0% and 50.7% versus 18.4 and 10.5% respectively; Figures 3.9 and 3.12). The increased availability of glucose due to the action of insulin (Taegtmeyer 1994) is likely to enhance relative glucose oxidation in both the hypertrophied and control hearts. The relative increase in glucose oxidation produced by insulin was slightly attenuated in the hypertrophied hearts compared the effect on the control hearts (36.0% versus 50.7%), but was not significant. Therefore there was no evidence of insulin resistance at this level of hypertrophy.

In contrast to this study Christe and Rodgers (1994) measured elevated glucose oxidation directly in SHR using U-^{14}C-glucose oxidation, in the presence of palmitate and insulin. Massie *et al* (1995) also reported differences in exogenous glucose metabolism in left ventricular hypertrophy in pigs. In which the

proportion of glucose oxidised was increased without an increase in the total amount of glucose uptake, providing direct evidence of increased glucose oxidation *in vivo*.

The discrepancy between these studies and the present study in terms of glucose use could be due to the severity of the hypertrophy studied. Certainly Christe and Rodgers (1994) examined glucose oxidation in SHR hearts, a genetic model of severe hypertension and showed 32% hypertrophy (increased heart/body mass ratio). The work presented here is thought to correspond to very early changes in the development of compensated hypertrophy. Other groups may be investigating substrate changes associated with the transition from compensated to de-compensated hypertrophy.

In most *in vitro* heart perfusion studies the amounts of substrates used to study substrate preference in the heart are often not consistent with each other or indeed at physiological levels (Allard *et al* 1994, Cheikh *et al* 1994). Measurement of energy metabolism *in vitro* should be performed in hearts perfused with relevant concentrations of both carbohydrates and fatty acids (reviewed by Lopaschuk 1997). Secondly some studies include insulin while others do not consider its action on substrate selection, where it is an essential component. The present study did include a physiological concentration of insulin. The effect that insulin has on PDH enzyme is less clear and will be examined in Chapter 4.

3.4.2.3.3. Effect of insulin on long-chain fatty acid use.

In the control hearts the relative proportion of fatty acid oxidised was not

significantly different to control hearts without insulin (59.1% versus 62.4%, Figures 3.9 and 3.12). Instead the increased proportion of glucose oxidation replaced endogenous substrate use in the control hearts. Therefore in control hearts, glucose and insulin at physiological concentrations did not alter the relative contribution of fatty acid oxidation to ATP production, whereby fatty acid still remained the primary substrate utilised by the control heart.

In contrast to glucose utilisation, significant differences were apparent in the proportion of fatty acid use in the hypertrophied hearts. In the presence of glucose and insulin, the relative proportion of fatty acid was decreased significantly in the hypertrophied heart group compared to the absence of insulin (47.2% versus 67.3%, Figures 3.9 and 3.12). This is consistent with other investigators where reduced levels of fatty acid oxidation has been reported in hypertrophied-failing guinea pig hearts (Wittels and Spann 1968) and hypertrophied rat hearts (Allard *et al* 1994, Christian *et al* 1998). The decrease in fatty acid contribution in the hypertrophied hearts in this study was partially compensated for by an increased contribution of endogenous substrate use. This is consistent with the study by Allard *et al* (1994) who found the decrease in ATP production from fatty acid oxidation in hypertrophied hearts was partially compensated for by an increased contribution of glycolysis to ATP production.

Sidossis *et al* (1996) investigated glucose and insulin regulation of fatty acid oxidation in humans using a hyperinsulinemic-hyperglycemic clamp. The proposed mechanism of glucose/insulin inhibition of fatty acid oxidation was at the site of CPTI by increased malonyl-CoA concentrations. The oxidation of

octanoate, a short chain fatty acid whose oxidation is independent of the CPTI site was not inhibited. The reduction of relative long-chain fatty acid oxidation in the hypertrophied hearts appears to be secondary to enhanced glucose and endogenous substrate use, (possibly enhanced glycolysis and glycolytic capacity), because a reduction in fatty acid oxidation was not found in the absence of insulin. The reduction in fatty acid oxidation could be the result of feedback inhibition of CPTI, whereby, increased metabolism of endogenous substrates, such as glycogen or lactate, could result in increased availability of pyruvate. In the presence of insulin, decreased levels of cAMP coupled to increased pyruvate availability, results in increased formation of malonyl-CoA, a potent inhibitor of CPTI (McGarry *et al* 1977). Thus if cytosolic malonyl-CoA increases, long chain fatty acid uptake will be inhibited and fatty acid oxidation will decrease (McGarry *et al* 1977).

Incorporation of ^{13}C from enriched hexanoate into the TCA derived glutamate amino acid proved that citrate synthase, the TCA cycle enzymes, and the amino-transaminases were not appreciably inhibited by the presence of hexanoate. Instead glucose metabolism is inhibited by hexanoate by the inhibition of pyruvate dehydrogenase. It appeared from the Weiss study that short chain fatty acid inhibition of glucose oxidation, at the level of PDH, was greater than the positive effects of insulin on glucose oxidation. This suggested that insulin would have little effect on glucose oxidation with short chain fatty acid present as the competing substrate, which does not require the CPTI site.

Allard *et al* (1994) proposed that the decrease in fatty acid oxidation in

hypertrophied hearts may be a consequence of the decrease in carnitine levels, found in many models of hypertrophy (El Alaoui-Talibi *et al* 1992, Reibel *et al* 1983, Allard *et al* 1994), including this model. Carnitine is essential for the translocation of activated long-chain fatty acids across the mitochondrial inner membrane to the site of β-oxidation (Pande and Parvin 1976). Without sufficient carnitine the enzymes CPTI and CPTII would be unable to convert the activated long-chain fatty acids into carnitine esters for transfer to CoA within the mitochondrial matrix. The inner mitochondrial matrix would remain impermeable to the activated fatty acids and thus long-chain fatty acid would be subsequently inhibited (Fritz 1963). A reduction in tissue carnitine levels cannot simply account for the reduction in fatty acid oxidation in this study because fatty acid oxidation was not reduced in the hypertrophied hearts in the absence of insulin with reduced carnitine concentrations.

3.4.2.3.4. Effect of insulin on glycolysis and endogenous substrate use.

Control hearts only utilised exogenous substrates in the presence of insulin, where the contribution of endogenous substrate use was negligible in the control hearts perfused in the presence of insulin. In contrast the hypertrophied hearts still retained considerable reliance on endogenous substrate use, when insulin was present (16.8%, Figure 3.12).

Exogenous substrate delivery may be reduced in hypertrophied hearts compared to control hearts due to alterations in the myocardial vasculature. Clinical and laboratory studies suggest that left ventricular hypertrophy is

associated with abnormal regulation of the coronary circulation, namely a decrease in the coronary vasodilatory reserve, and changes in regional myocardial blood flow (Karam *et al* 1990, Marcus *et al* 1982, Nakano *et al* 1989, Wexler *et al* 1988). During hypertrophic growth the myocardial capillaries fail to increase in proportion to the wall thickening, alongside an increased vascular permeability (Koyanagi *et al* 1982). These alterations may contribute to a greater dependence on endogenous substrate use during hypertrophy.

Potential sources of endogenous substrates are lactate, glycogen and to a lesser extent triglycerides or proteins. It is beyond the scope of this study to determine exactly which endogenous substrate(s) are likely to be significant, however some speculation is possible. Studies demonstrate that there is a simultaneous extraction and release of lactate during non-ischaemic, normoxic conditions in animals and humans (Guth *et al* 1990, Gertz *et al* 1981, Wisneski *et al* 1985). In support of this Allard *et al* (1994) found that that the hypertrophied heart oxidised substantial quantities of lactate. Therefore lactate may contribute to the endogenous substrate supply in the hypertrophied hearts, although Massie *et al* (1995) reported that the amount of lactate release and proportion of lactate derived from exogenous glucose did not alter significantly in left ventricular hypertrophy.

Glycogen could be a principal source of unlabeled substrate. Increased glycolytic activity and enhanced glucose uptake have previously been observed in animal models of left ventricular hypertrophy (Bishop and Altschuld 1970, Yonekura *et al* 1985, Seymour *et al* 1990, Leipala *et al* 1989, Smith *et al* 1990,

Zhang *et al* 1995). An increased glycolytic capacity can also accompany chronic pressure-overload even in the absence of left ventricular hypertrophy (Taegtmeyer and Overturf 1988). Allard *et al* (1994) found that the contribution of glycolysis to ATP production was significantly higher in hypertrophied rat hearts compared to control hearts. Alongside this finding Allard *et al* (1994) reported that the acceleration of glycolysis in moderate pressure overloaded hearts was not followed by a stimulation of glucose oxidation, whereby the contribution of glucose and lactate oxidation to ATP production in the hypertrophied heart did not differ from control hearts.

3.4.2.3.5. Effect of insulin on heart function.

Hypertrophied hearts perfused with glucose and palmitate in the presence of insulin did not show any differences in heart function. This is in contrast to Zhang *et al* (1995) and Massie *et al* (1995) who both showed that hypertrophied hearts exhibited significantly increased left ventricular systolic pressure. Although there are no significant differences between the functional characteristics of the hypertrophied and control hearts, perfused with glucose and palmitate with insulin, the same cannot be said for the comparison between substrate groups. Insulin inclusion in the perfusion buffer improved heart function in control hearts utilising palmitate and glucose. Making the heart function parameters comparable to control hearts perfused with hexanoate and glucose alone. This highlights the possible positive effect insulin and increased glucose use can exert on heart function. However, insulin did not show the same positive effects on heart function

in the hypertrophied hearts perfused with palmitate and glucose, in hypertrophied hearts, insulin does not improve heart function. Instead it is comparable to hypertrophied hearts perfused with glucose and palmitate without insulin, but still significantly lower than hypertrophied hearts perfused with glucose and hexanoate. This is likely to be due to the fact that the hypertrophied heart is more reliant on endogenous substrate use compared to the control heart in the presence of insulin. Alternatively the lack of improved function in the hypertrophied hearts may be a consequence of alterations in the contractile apparatus of the hypertrophied hearts or calcium handling within the myocardium that may adversely affect function.

3.4.3. Summary.

This series of studies has established the following alterations in substrate selection that are associated with compensated cardiac hypertrophy:

- Increased glucose oxidation during hypertrophy, in the absence of insulin, precedes a decrease in fatty acid oxidation reported by other investigators.
- No evidence of insulin resistance occurred in moderate cardiac hypertrophy.
- Evidence of impaired substrate accessibility / delivery exists in hypertrophy in the presence of insulin. Endogenous substrate use increases and fatty acid oxidation decreases during cardiac hypertrophy in the presence of insulin, which may indicate limited availability of CoA.

The exact mechanisms responsible for these changes need further evaluation, particularly at the subcellular level and are addressed in Chapter 4.

Chapter 4:

Involvement of pyruvate dehydrogenase in substrate use during compensated cardiac hypertrophy

4.1 Introduction.

A delicate balance exists between substrate oxidation and energy utilisation in the heart, which requires precise regulation. Metabolic regulation has to be flexible because substrate availability is not constant, even in normal tissue. Altering the supply of one particular energy substrate has considerable impact on the flux through other pathways (Saddik *et al* 1991).

4.1.1. Glucose metabolism.

4.1.1.1. Pyruvate dehydrogenase.

Research suggests in both tissue extractions (Wieland *et al* 1971, Olson *et al* 1983, Latipaa *et al* 1985) and isolated beating hearts (Weiss *et al* 1989) that a critical enzyme involved in the regulation of glucose metabolism by fatty acid oxidation is the pyruvate dehydrogenase (PDH) enzyme. The PDH enzyme is a large multi-enzyme complex that provides the link between glycolysis and the TCA cycle.

4.1.1.2. Regulation of PDH activity.

The PDH enzyme complex exists in an active (de-phosphorylated) and inactive (phosphorylated) form. Inter-conversion between these two forms via a reversible phosphorylation / dephosphorylation cycle, catalysed by a phosphatase and kinase, respectively, provides a major inhibitory mechanism of this complex (Linn *et al* 1969, Wieland and Siess 1970, Kerbey *et al* 1976). The complex is inactivated when a specific serine residue of the PDH enzyme is phosphorylated

by PDH kinase dependent on ATP (Kerbey *et al* 1976). The enzyme is activated when the phosphoryl group is hydrolysed by a Mg^{2+} / Ca^{2+} dependent PDH phosphatase (Linn *et al* 1969, Denton *et al* 1972).

Various metabolic effectors modulate the activities of these inter-conversion enzymes, which in turn regulate the percentage of PDH complex in the active form. PDH kinase activity, thus phosphorylation, is enhanced by high ratios of ATP / ADP, acetyl-CoA / CoA and NADH / NAD^+ (Kerbey *et al* 1976). PDH kinase activity can be decreased by increased sensitivity to pyruvate inhibition (Cooper *et al* 1974, Kerbey *et al* 1976, Carter and Coore 1995), and inhibited by high concentrations of Mg^{2+} and Ca^{2+} (Cooper *et al* 1974). An additional factor in PDH regulation can be increased transcription of PDH kinase (Kerbey *et al* 1976).

A second regulatory mechanism involves feedback inhibition. The products, namely acetyl-CoA and NADH, act as competitive inhibitors of the PDH reaction, whose inhibitory effects are reversed by CoA and NAD^+ (Garland and Randle 1964). PDH activity is also regulated by nucleotides, where PDH activity is inhibited by GTP and activated by AMP. Thus the activity of the complex is reduced when there is abundant free energy.

4.1.1.3. Influence of calcium on PDH activity.

Under normal physiological conditions, the primary function of the Ca^{2+}-transport system of the mitochondrial inner membrane in mammalian tissues, may be to relay changes in cytoplasmic Ca^{2+} concentrations into the mitochondrial matrix and thus control the concentration of Ca^{2+} in this compartment (McCormack and

Denton 1989). Alternatively its function may be to set the extra-mitochondrial Ca^{2+} concentration (Akerman and Nicholls 1983). Either way Ca^{2+} exerts a messenger role to ensure that ATP synthesis is increased to meet enhanced cellular demand (Denton and McCormack 1985). Ca^{2+} activates PDH indirectly by causing increases in the amount of active, non-phosphorylated PDH, through activation of PDH phosphatase (Denton *et al* 1972). In addition, Ca^{2+} may also activate PDH through inhibition of PDH kinase (Cooper *et al* 1974).

4.1.1.4. Influence of insulin on PDH activity.

The heart is responsive to insulin *in vivo* (James *et al* 1985), where studies indicate that glucose uptake in the rat heart *in vivo* is sensitive to stimulation by insulin in the physiological range (i.e. up to 150μU/ml). However Awan and Saggerson (1993) have observed that insulin has a variable and inconsistent effect to suppress fatty acid oxidation. Bryson *et al* (1995) also found differential tissue responses to short term changes in insulin levels and conclude that local intracellular fatty acid metabolism is important in the regulation of intracellular glucose oxidation. The mechanism by which insulin suppresses fatty acid oxidation is still being elucidated but is likely to involve PDH activity, in that PDH activity can be altered by relatively short term increases in plasma insulin (Bryson *et al* 1995). In support of this, Sugden *et al* (1997) showed that insulin suppressed PDH kinase activity in control cardiac myocytes cultured with fatty acid and dibutyryl cAMP, which would result in a higher percentage of PDH in the active form.

Many hormones that activate energy-requiring processes such as contraction by causing increases in the cytoplasmic concentration of Ca^{2+} also stimulate oxidative metabolism (Denton and McCormack 1985). This is thought to occur due to the activation of three key Ca^{2+}-sensitive intra-mitochondrial dehydrogenases (PDH, NAD^{+}-isocitrate and 2-oxoglutarate dehydrogenase) as the result of accompanying increases in the concentration of Ca^{2+} in the mitochondrial matrix (Denton and McCormack 1985). Therefore hormones such as insulin, play an important role in the regulation of metabolism by triggering cascades of modification reactions that lead to highly amplified changes in metabolism. The 'extrinsic' regulation of PDH by Ca^{2+} is thought to be distinct from 'intrinsic' regulation by local metabolite end products concentration ratios (Denton and McCormack 1980). By using Ca^{2+} in this way, insulin could override intrinsic mechanisms, whereby ATP synthesis could be stimulated without the need for large changes in NAD+/NADH or ADP/ATP concentration ratios (Denton and McCormack 1980).

The short-term metabolic effects of insulin are reported to involve at least three distinct signalling pathways (reviewed by Moule and Denton 1997). The first leading to increases in glucose transport and the activation of enzymes such as glycogen synthase and acetyl-CoA carboxylase, which may involve phosphatidylinositol 3-kinase and protein kinase B. The second leading to the effects of insulin on protein synthesis, which may involve phosphatidylinositol 3-kinase and p70 S6 kinase. The third is thought to lead to the activation of PDH, which uniquely does not require activation of phosphatidylinositol 3-kinase.

4.1.2. Interaction of fatty acids with glucose metabolism.

4.1.2.1. Glucose-fatty acid cycle.

The reciprocal relationship between the rates of oxidation of glucose and fatty acids by muscle has been explained by a concept known as the glucose-fatty acid cycle (Randle 1963). Studies on isolated *in vitro* muscle preparations have shown that fatty acid oxidation decreases the rate of glucose utilisation via effects on glucose transport, hexokinase, 6-phosphofructokinase and pyruvate dehydrogenase. As the flux through glycolysis is decreased, the concentrations of the pathway reaction products (namely intracellular glucose, fructose-6-phosphate and pyruvate) are increased. Increases in the concentrations of the inhibitors described above have been demonstrated during fatty acid oxidation in muscle. Support for the fact that β-oxidation is necessary for fatty acid suppression of glucose oxidation arise from the observations that inhibitors of CPTI, such as tetradecylglycidate and etomoxir can overcome this suppression of glucose oxidation and PDH activity (Lopaschuk *et al* 1990, Caterson *et al* 1982).

4.1.2.2. Fatty acid metabolism.

It has been suggested that the availability and oxidation of free fatty acids play an important role in the regulation of PDH activity in the heart (Chicco *et al* 1991). In support of this administration of a high fat diet in rats leads to a stable increase in cardiac PDH kinase activity (Orfali *et al* 1995, Sugden *et al* 1997). Increased PDH kinase activity (Marchington *et al* 1990) and desensitisation of PDH kinase to

inhibition by pyruvate (Priestman *et al* 1996) has also been observed in cultured cardiac myocytes from rats fed a high fat diet. Fatty acids and ketone bodies have also been shown to decrease the percentage of PDH in the active form in rat heart extracts (Olson *et al* 1983, Latipaa *et al* 1985). Wieland (1983) reported an inverse correlation between plasma free fatty acid levels and PDH in the active form in normal rats. Perfusion of various even-numbered fatty acids or ketone bodies in the perfused rat heart results in equal degrees of inhibition of PDH flux, although with varying states of inactivation of the PDH complex (Olson *et al* 1983). In addition, Weiss *et al* (1989) devised a non-invasive quantification method of the TCA cycle flux in intact cardiac tissue using ^{13}C NMR, to measure PDH activity indirectly via the flux of label enriched substrates into the TCA cycle. It was demonstrated that the mechanism of fatty acid inhibition of the PDH flux was by reversible inactivation of the PDH complex.

The proposed factors that contribute to the inter-conversion of the active and inactive forms of PDH include the CoA / acetyl-CoA, NAD^+ / NADH and ADP / ATP ratios (Kerbey *et al* 1976). Decreased levels of CoA / acetyl-CoA and NAD/ NADH were measured in hearts metabolising fatty acids (Garland and Randle 1964), while Weiss *et al* (1989) calculated lower ADP levels in glucose and short chain fatty acid perfused hearts compared with hearts perfused with glucose alone. Latipaa *et al* (1985) found that PDH inter-conversion over-rides feedback inhibition as a regulatory mechanism under most conditions, due to amplification effect by the PDH phosphatase and kinase.

4.1.2.3. Regulation of long-chain fatty acid metabolism by glucose.

Under appropriate circumstances carbohydrate fuels can decrease fatty acid utilisation by heart cells. Glucose can suppress the removal of oleate by the working perfused heart (Taegtmeyer 1994). In addition elevation *in vivo* of blood lactate decreases fatty acid extraction and oxidation by the heart (Spitzer 1974), the perfused heart (Bielefeld *et al* 1985) and isolated myocytes (Montini *et al* 1981).

4.1.2.3.1. Carnitine palmitoyltransferase I (CPTI).

Carnitine palmitoyltransferase I (CPTI), situated on the outer mitochondrial membrane catalyses the first step in the mitochondrial oxidation of long chain fatty acids (McGarry *et al* 1989) and exists as two isoforms in rat heart mitochondria (Weis *et al* 1994). These two isoforms, L-CPTI and M-CPTI indicating their association with liver and muscle respectively (Weis *et al* 1994), differ not only in monomeric size but also in their kinetic characteristics (Km) for carnitine and malonyl-CoA (McGarry *et al* 1983). The L-CPTI isoform is thought to contribute significantly in the regulation of fatty acid oxidation in the new-born heart, when carnitine levels are low, with the expression pattern switching to the mainly to the M-CPTI isoform during adult life, when carnitine levels are more abundant (Brown *et al* 1995).

CPTI can be feedback inhibited by malonyl-CoA, the primary step in fatty acid synthesis, in the heart. CPTI exerts major control over fatty acid transport and thus the oxidation of long chain fatty acid by its inhibitability by malonyl-CoA,

the product of the acetyl-CoA carboxylase reaction (McGarry *et al* 1989). This malonyl-CoA / CPTI interaction is considered an important component in the regulation of fatty acid and carbohydrate oxidation in the heart (Saddik *et al* 1993).

4.1.2.3.2. Acetyl-CoA carboxylase.

Acetyl-CoA carboxylase (ACC) is a biotin-containing enzyme which catalyses the carboxylation of acetyl-CoA to form malonyl-CoA, a potent inhibitor of CPTI (Mabrouk *et al* 1990, McGarry *et al* 1989). Since CPTI is a key regulatory enzyme in the mitochondrial uptake of fatty acids, ACC has also been suggested to play an important role in regulating fatty acid oxidation (Bianchi *et al* 1990). In the heart the supply of malonyl-CoA, as opposed to the sensitivity of CPTI to malonyl-CoA inhibition, may be the key factor regulating myocardial fatty acid oxidation (Cook and Lappi 1992). In support of this, Saddik *et al* (1993) demonstrated a strong correlation between increased acetyl-CoA production from PDH activation and an increase in myocardial malonyl-CoA production. This increase in malonyl-CoA production also correlated with a reduction in overall fatty acid oxidation rates, suggesting that ACC is an important regulator of fatty acid oxidation in the heart. The hypothesis being that acetyl-CoA derived from PDH is transported from the mitochondria to the cytosol, to act as a substrate for ACC, resulting in an increase in malonyl-CoA production and decreasing fatty acid oxidation. The acetyl-CoA produced is thought to be transported via a carnitine acetyl-transferase and carnitine acetyltranslocase pathway active in the heart, that transports acetyl groups from mitochondrial acetyl-CoA to cytosolic CoA (Lysiak *et al* 1988). In

support of this hypothesis Lysiak *et al* (1988) observed that most of the acetyl-CoA generated from pyruvate by PDH is readily accessible to carnitine acetyltransferase, whereas acetyl-CoA generated from β-oxidation is more available to the TCA cycle.

4.1.2.3.3 Long-chain fatty acyl-CoA esters and acylcarnitines.

Long-chain acyl-CoA esters are important intermediates in fatty acid degradation and lipid biosynthesis. Attention is now being focussed on these amphipathic molecules as important physiological regulators of cellular systems and functions, including ion channels, ion pumps, translocators, enzymes, membrane fusion and gene regulation (reviewed by Faergeman and Knudsen 1997). With regard to their effects on energy metabolism, acyl-CoA esters have been shown to be potent inhibitors of adenine nucleotide translocase (ANT). ANT catalyses the exchange of ADP and ATP across the mitochondrial inner membrane, and is generally accepted as a major rate-limiting step in energy metabolism (Heldt and Klingenberg 1968). Therefore inhibition by acyl-CoA esters would be expected to decrease ATP synthesis, resulting in adverse effects on muscle contraction and electrical conduction.

The β-oxidation of fatty acids is inhibited mainly by feedback inhibition. When flux through the TCA cycle is limited, the acetyl-CoA level increases and inhibits β-ketothiolase (reviewed by Faergeman and Knudsen 1997). An accumulation of fatty acyl-CoA intermediates would inhibit the first steps of the pathway catalysed by the chain-length specific acyl-CoA dehydrogenases,

ultimately resulting in inhibition of fatty acid oxidation.

The most prominent intracellular fatty-acyl derivatives are the acylcarnitine esters, by action of long-chain carnitine acyltransferase enzymes present in the outer membrane of the mitochondria (reviewed by Brady *et al* 1993). There is now evidence for a direct link between the long-chain acyl-CoA pool and the long-chain acylcarnitine pool (Arduini *et al* 1996), whereby the acylcarnitine pool may act as a buffer for activated acyl chains *in vivo*, when there is a free flow of acyl-chains between the acyl-CoA and the acylcarnitine pools.

Regarding glucose oxidation, the enzyme glucose-6-phosphatase can be inhibited by acyl-CoA esters (reviewed by Faergeman and Knudsen 1997), implicating that acyl-CoA esters are important in controlling the efflux of glucose. In addition, it has been shown that an increase in mitochondrial acyl-CoA, such as palmitoyl-CoA, inhibits the activity of pyruvate dehydrogenase enzyme (reviewed by Faergeman and Knudsen 1997), suggesting that long-chain acyl-CoA is a common regulator of this enzyme. Such an increase in the acyl-CoA / CoA ratio can rise in the mitochondrial matrix during the starvation state, resulting in an inhibition of PDH and a switch to β-oxidation for energy production.

4.1.3. Role of pyruvate dehydrogenase in cardiac hypertrophy.

Reduction in the proportion of fatty acid oxidation in the hypertrophied myocardium (El Alaoui-Talibi *et al* 1992, Cheikh *et al* 1994) is thought to occur in part as a result of a decrease in the myocardial tissue carnitine content (Cheikh *et al* 1994, Riebel *et al* 1983). Such reductions in carnitine concentrations have been

measured in the model of hypertrophy used in this thesis (Chapter 3). Carnitine is an essential co-factor for the transport of activated long chain fatty acids from the cytosol to the mitochondrion, thus any reductions in this co-factor will limit entry of fatty acids into the mitochondria and their subsequent oxidation. In addition Juvenile Visceral Steatosis (JVS) mice, a model of systemic carnitine deficiency (Kuwajima *et al* 1991), exhibit progressive cardiac hypertrophy and systemic circulatory impairment (Narama *et al* 1997). Alongside carnitine deficiency JVS mice show a disordered expression of CPTI (Uenaka *et al* 1996) and CPTII (Hotta *et al* 1996). Further to this, studies have shown that inhibitors of CPTI, an essential step in fatty acid oxidation, results in cardiac hypertrophy in rodents (Higgins *et al* 1985, Lee *et al* 1985, Rupp *et al* 1972). The effects of one such inhibitor, etomoxir, have been attributed to the accumulation of long chain acylcoenzyme A (Severson and Hurley 1982, Hulsmann *et al* 1994). Thus, the accumulation of fatty acyl components due to reduced fatty acid oxidation is likely to sequester any available CoA. The resulting decreased levels of CoA are thought to affect the rate of TCA cycle turnover and contractile function (Russell and Taegtmeyer 1992). Perhaps more importantly alterations in the availability of CoA could affect glucose oxidation at the level of PDH, in which an increased acetyl-CoA to CoA ratio could lead to a decreased PDH activity.

Pyruvate dehydrogenase enzyme activity and its regulation due to the phosphorylation / dephosphorylation state, have been studied in several models of heart disease, although there is still speculation as to the importance of PDH enzyme activity involvement. Di Lisa and co-workers (1993) reported a lower

fraction of PDH in the active form in glucose-perfused isolated hearts of two myopathic strains of Syrian hamsters, alongside a decrease in contractile performance. This correlated with a lower mitochondrial calcium concentration when the isolated myocytes were electrically stimulated, which is likely to result in a lower TCA cycle activity in response to increased workload. Indeed even in mild compensated hypertrophy there has been shown a concomitant decrease in the fraction of PDH in the active form (Seymour and Chatham 1997), likely to create a decrease in the flux of glucose to acetyl-CoA. These studies indicate that myocardial substrate availability to the mitochondria may be impaired at the level of PDH, and may ultimately contribute to energy depletion and heart failure. It appears that understanding how the production of ATP is regulated in the hypertrophied heart, at the level of PDH, is critical to understanding how the alterations in energy metabolism may contribute to the severity of cardiac disease.

4.1.4. PDH as a potential therapeutic target in heart disease.

Understanding of the complex regulation and interaction of the metabolic pathways in heart disease remains incomplete. Pharmacological agents being introduced to treat patients with heart disease have functions specifically aimed at modifying energy metabolism in the heart. Situations in which maintaining glucose oxidation, through the activation of PDH, is critical in maintaining heart function are during the recovery periods following haemorragic shock (Granot and Steiner 1985, Kline *et al* 1997), ischaemia (McVeigh and Lopaschuk 1990, Lopaschuk *et al* 1993), and cardioplegic arrest for transplantation (Smolenski *et al*

1999).

The activity of PDH is tightly coupled to the work demand of the heart, as well as the myocardial redox potential (Kobayashi and Neely 1983). Conditions such as ischaemia and haemorragic shock, can decrease the heart workload and produce intracellular conditions that may increase the action of PDH kinase, which phosphorylates and inactivates the PDH complex (Kobayashi and Neely 1983, Behal *et al* 1993). As a result the net conversion of pyruvate and lactate to acetyl-CoA maybe become limited by shock conditions (Kerbey *et al* 1985). This situation is particularly detrimental to the heart subjected to haemorragic shock which becomes dependent upon lactate for up to 90% of its total oxidisable carbon source (Spitzer and Spitzer 1972, Weiner and Spitzer 1994). The activation of enzymatic pathways leading to net carbohydrate oxidation, such as the PDH reaction, during or following shock conditions, can sustain the heart with an adequate flow of oxidisable substrates through the TCA cycle to regenerate its high energy phosphate levels.

The pharmacological agent administered to increase PDH activity in the aforementioned studies was Dichloroacetate (DCA). DCA is a non-metabolised analogue of pyruvate (Whitehouse *et al* 1974), which activates PDH by inhibiting PDH kinase, preventing the conversion of the active form of PDH to the phosphorylated inactive form (Stacpoole 1989, Stacpoole and Greene 1992). Stimulation of PDH with DCA occurs in most tissues, particularly the myocardium (Stacpool 1989, McAllister *et al* 1973) and leads to increased glucose utilisation, glycolysis and utilisation of lactate for aerobic respiration. Myocardial

consumption of free fatty acids is simultaneously inhibited with the overall effect of a change of substrate utilisation from predominantly non-esterified fatty acids to glucose and lactate (Stacpoole 1989, McAllister *et al* 1973, Latipaa *et al* 1985, Weiss *et al* 1989, Saddik *et al* 1993).

During early reperfusion of the post-ischaemic myocardium, the PDH enzyme is largely in the phosphorylated inactive state (Patel and Olson 1984). This is likely due to the elevated levels of free fatty acids resulting from ischaemia that decrease the percentage of PDH that is in the active form (Behal *et al* 1993) and limit the recovery of hearts during reperfusion following ischaemia (McVeigh and Lopaschuk 1990). High levels of circulating fatty acids are known potential inhibitors of glucose oxidation (Lopaschuk *et al* 1990), therefore fatty acids are thought to exert their detrimental effects by inhibiting glucose oxidation rates post-ischaemia. Administration of DCA does not improve heart function during moderate ischaemia (Mazer *et al* 1995, Stanley *et al* 1996), but improves heart function during reperfusion following ischaemia (McVeigh and Lopaschuk 1990, Lopaschuk *et al* 1993, Lewandowski and White 1995) and in humans with congestive heart failure (Bersin and Wolfe 1994), by restoring glucose utilisation. This observation is supported by the beneficial effect of etomoxir, an inhibitor of CPTI activity (Lopaschuk *et al* 1990). Decreasing fatty acid uptake into the mitochondria during ischaemic reperfusion using Etomoxir improves heart function and increases glucose oxidation simultaneously.

The exact mechanism behind the beneficial effect of increased flux through PDH on cardiac performance following ischaemia is still largely unknown.

However the effect is thought directly related to the extent of carbohydrate oxidation within the mitochondria than to associate increases in glycolysis and glycolytic energy production (McVeigh and Lopaschuk 1990, Lopaschuk *et al* 1993). Other investigators cite the possibility that substrate-induced activation of PDH may account for the beneficial effect of high pyruvate concentrations with supplemental glucose in the reperfusion media (Bunger *et al* 1983). Alternatively that a potential inotropic action of pyruvate metabolism enhances contractile function of reperfused myocardium through elevations in energetic potential, evidenced by high-energy phosphate levels (Bunger *et al* 1983). These observations, linked to the evidence that increased flux through the PDH complex can improve the functional recovery of the heart following ischaemia, suggest that agents such as DCA may have a future therapeutic use in the correction of substrate metabolism during cardiac hypertrophy.

4.1.5. Aims.

The first aim of this chapter was to investigate the level of pyruvate dehydrogenase enzyme activity in control hearts under conditions of competing substrates. Dichloroacetate was the agent used to determine the plasticity of this enzyme, in an attempt to modulate PDH activity by inhibition of PDH kinase, and subsequently increase glucose utilisation. It was necessary to investigate whether DCA and/or the increase in PDH enzyme activity would be beneficial or detrimental to heart function before the PDH complex can be considered as a therapeutic target.

The second section of this chapter deals with the role of PDH enzyme activity in the progression of cardiac hypertrophy. The establishment of alterations in energy substrate use during compensatory cardiac hypertrophy highlighted in this model led to the requirement of further investigation into characterisation of the metabolic state at the sub-cellular level. Thus determining how these alterations may have been regulated, it was important to determine the regulatory role of PDH enzyme activity during substrate utilisation in compensated

4.2 Methods

4.2.1 Activation of cardiac pyruvate dehydrogenase.

Modulation of pyruvate dehydrogenase activity and its effects on heart function were determined in isolated perfused hearts, from eight week old control Sprague-Dawley rats. The Krebs-Hensleit perfusion buffer contained one of the following substrate mixtures:

- 5mM glucose
- 5mM glucose and 1mM hexanoate
- 5mM glucose, 1mM hexanoate and 1mM DCA
- 5mM glucose, 1mM hexanoate and 2mM DCA
- 5mM glucose, 1mM hexanoate and 5mM DCA

Hearts were perfused initially for a 15 minutes equilibration period to establish a diastolic pressure setting of 5-10 mmHg. Function was recorded for a further 45 minutes perfusion. The hearts were freeze-clamped at the end of each experiment, weighed and stored in liquid nitrogen before enzyme extraction. Activities of the active form of pyruvate dehydrogenase (PDHa) and total enzyme activity (PDHt) were determined in homogenates of cardiac tissue using a NAD^+ based spectrophotometric assay (Chapter 2, section 2.2.7). PDH activities were calculated by determining the absolute values (μmol/min/gramme wet mass) for the active extracts PDHa and OGDHa, and the total extracts PDHt and OGDHt. Relative activities were obtained by normalising for OGDH activity to give % [(PDHa/OGDHa)/(PDHt/OGDHt)] to allow for extraction efficiency and subdivided into substrate groups.

4.2.2. Hypertrophied heart study.

The effect of differing substrates on PDH enzyme activity was investigated in nine week hypertrophied and control rat hearts. Frozen heart tissue for this study was obtained from the isolated perfused heart experiments in Chapter 3. The competing substrate groups examined were:

- 5mM glucose and 0.3mM hexanoate,
- 5mM glucose and 0.3mM palmitate,
- 5mM glucose, 0.3mM palmitate and 100μU/ml insulin.

In this study 0.3mM hexanoate was perfused in parallel with the 0.3mM palmitate concentration. The equilibration period was extended to 20 minutes compared to the previous 15 minutes (section 4.2.1). The activities of PDH in both the active and the total extracts were normalised for extraction efficiency by comparing to the citrate synthase enzyme activity. PCr and ATP concentrations (μmoles/gramme wet mass) were determined in tissue PCA extracts from the hypertrophied and control rat hearts perfused with each substrate mixture (Chapter 2, section 2.6.3). Frozen tissue samples from hypertrophied and control hearts, perfused with glucose and palmitate were sent to Newcastle Institute of Child Health, for analysis of acylcarnitine concentration (nmol/gramme wet mass), using the method described in Chapter 2, section 2.6.4.

4.3. Results.

4.3.1. Control heart pyruvate dehydrogenase study.

4.3.1.1. Morphology.

A summary of the morphology of control rats used in this study is given in Appendix Table 2.2. The body mass and heart mass of the control rats from the substrate groups glucose, hexanoate and 2mM DCA and glucose, hexanoate and 5mM DCA were significantly smaller than those used in the other substrate groups ($p<0.05$). However, more importantly the ratio of heart mass to body mass was not significantly different between any of the substrate groups. There were also no significant differences in serum fatty acids, triglycerides, glucose, lactate and ketone body concentrations between any of the substrate groups. The comparable RBC packed cell volume ratios, confirm the absence of anaemia as anticipated. This data is presented in Appendix Table 2.3.

4.3.1.2. Control heart function.

Contractile function in control hearts perfused with differing substrates is presented in Table 4.1(a). Heart rate was comparable between all substrate groups. Diastolic pressure was set in each heart to be within a range of 5-10mmHg, therefore no significant differences were observed in diastolic pressure between the substrate groups. Hearts perfused with glucose, hexanoate and 2mM DCA showed significantly higher systolic pressure compared to hearts from other substrate groups ($p<0.05$), with the exception of the 5mM DCA group. 2mM DCA significantly increased the LVDP compared to all other substrate groups ($p<0.05$),

and increased the RPP significantly compared to hearts perfused without DCA present ($p<0.05$).

Treatment Groups	HR (bpm)	SP (mmHg)	DP (mmHg)	LVDP (mmHg)	RPP ($X10^3$)
Glu (n=7)	247.1 (9.11)	100.6 (4.28)	7.5 (0.42)	93.1 (3.96)	23.7 (1.83)
Glu+Hex (n=7)	261.3 (5.73)	94.2 (1.60)	7.3 (0.30)	86.9 (1.55)	22.7 (0.65)
Glu+Hex+1mM DCA (n=5)	295.6 (9.95)	92.9 (2.00)	5.3 (0.23)	87.8 (1.81)	25.8 (1.47)
Glu+Hex+2mM DCA (n=6)	279.1 (2.14)	132.5~ (3.02)	7.0 (0.44)	125.5* (2.75)	35.1# (0.82)
Glu+Hex+5mM DCA (n=6)	264.9 (6.67)	113.1 (4.24)	8.0 (0.47)	105.1 (3.87)	27.8 (1.15)

Table 4.1(a). Function in control hearts with varying substrates, (mean +/- SEM). (* Significant difference compared to all substrate groups, ~ significant difference compared to Glu, Glu+Hex, Glu+Hex+1mM DCA, # significant difference compared to Glu and Glu+Hex).

4.3.1.3. PDH enzyme activity.

Activities of PDHa, OGDHa, PDHt and OGDHt in heart extracts for each substrate group are shown in Table 4.1(b). Only hearts perfused with 2mM DCA showed comparable PDHa activity compared to glucose alone, whereas all substrate groups showed comparable PDHt activities compared to glucose alone. Hearts perfused with 2 and 5 mM DCA exhibited significantly higher OGDH enzyme

activities in both active and total extracts, compared to the other substrate groups ($p<0.05$). This indicated differences in extraction efficiency between the substrate groups. The variation in OGDH enzyme activity was a great cause for concern. To overcome this problem in future experiments PDH enzyme activities were normalised for extraction efficiency by comparing to citrate synthase enzyme activity. The extractions were however consistent between the glucose, glucose and hexanoate and glucose, hexanoate and 1mM DCA substrate groups and between the glucose, hexanoate and 2mM and 5mM substrate groups.

The PDHa/OGDHa ratio of hearts perfused with glucose and hexanoate and glucose and hexanoate with 1 and 2mM DCA are significantly lower than the ratio for hearts perfused with glucose alone ($p<0.05$). Conversely, 5mM DCA increased the PDHa/OGDHa ratio compared to the glucose only group ($p<0.05$). Concerning the PDHt/OGDHt ratios only glucose, hexanoate and 1mM DCA showed a significantly lower ratio compared to the glucose only group ($p<0.05$). This again resulted from an alteration in extraction efficiency.

Treatment Groups	PDHa	OGDHa	PDHa/ OGDHa	PDHt	OGDHt	PDHt/ OGDHt
Glu (n=7)	0.84 (0.11)	1.39 (0.05)	0.60 (0.06)	1.73 (0.18)	1.16 (0.05)	1.44 (0.08)
Glu+Hex (n=7)	0.17* (0.02)	1.48 (0.08)	0.11* (0.01)	1.25 (0.10)	1.21 (0.08)	1.05 (0.06)
Glu+Hex+ 1mM DCA (n=5)	0.23* (0.02)	1.59 (0.07)	0.14* (0.01)	1.31 (0.08)	1.54 (0.02)	0.86* (0.05)
Glu+Hex+ 2mM DCA (n=6)	0.82 (0.05)	2.57* (0.05)	0.32* (0.01)	2.29 (0.13)	2.19* (0.13)	1.07 (0.03)
Glu+Hex+ 5mM DCA (n=6)	2.19* (0.11)	2.44* (0.05)	0.89* (0.03)	2.62 (0.11)	2.06* (0.05)	1.27 (0.03)

Table 4.1(b). Enzyme activities (μmoles/min/gramme wet mass) in hearts perfused with varying substrate mixtures, (mean +/- SEM). * Significant difference compared to glucose alone.

Figure 4.1 shows the percentage PDH in the active form relative to the total, corrected for extraction efficiency, for each substrate group. Hearts perfused with the substrates glucose and hexanoate and glucose, hexanoate and 1mM DCA, show significantly decreased percent PDH active compared to hearts perfused with glucose alone ($p<0.05$). The presence of 2mM DCA increased the percent PDH active enzyme values comparable to hearts perfused with glucose alone.

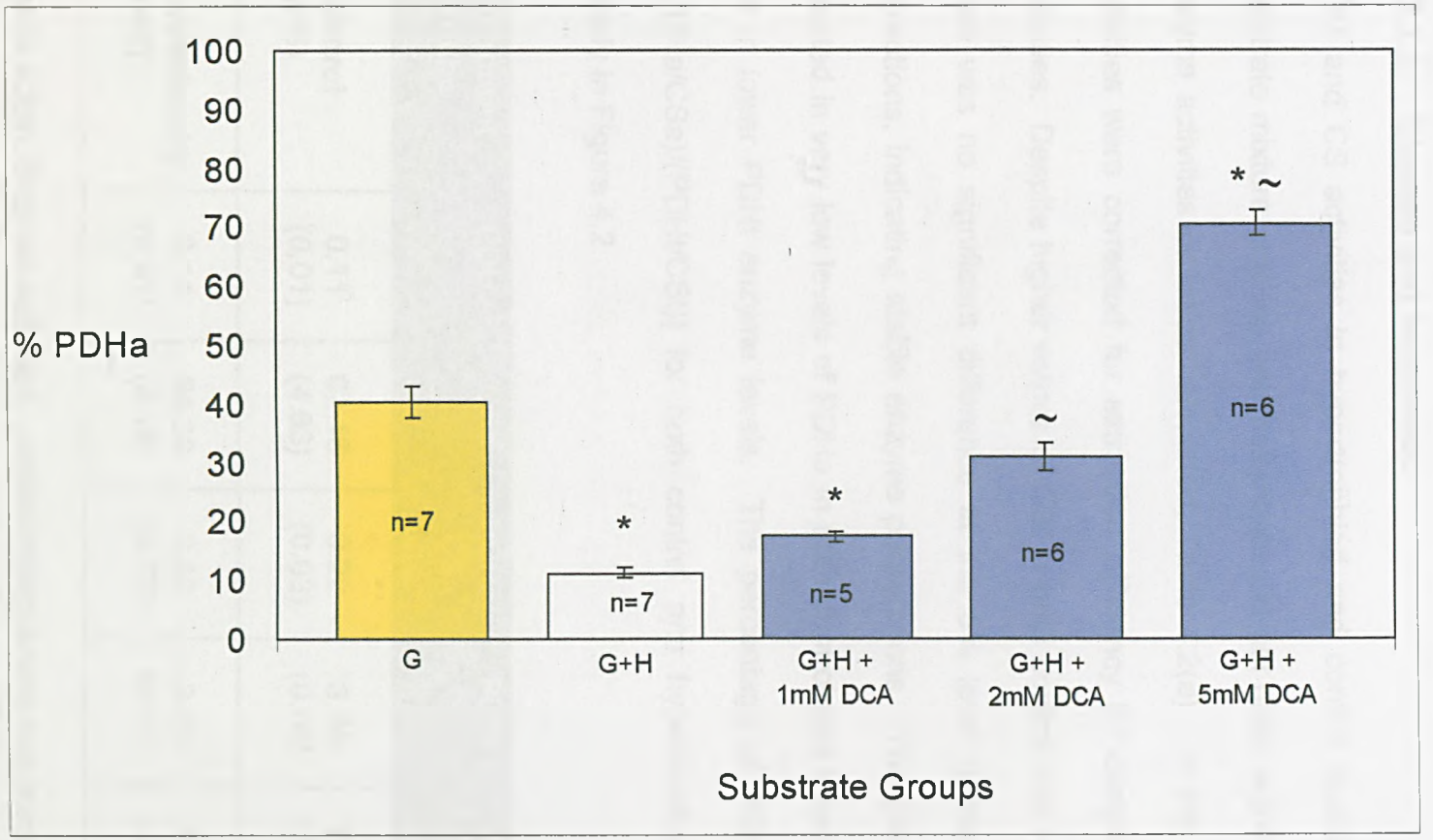

Figure 4.1. Percentage PDH enzyme in the active form (mean +/- SEM) in control hearts perfused with varying substrate mixtures. (* significant difference, $p<0.05$, compared to glucose substrate alone. ~ significant difference, $p<0.05$, compared to glucose and hexanoate as substrates).

4.3.2. Hypertrophied heart study.

4.3.2.1. Glucose and hexanoate.

PDH and CS activities in hypertrophied and control hearts perfused with the substrate mixture glucose and hexanoate did not differ significantly in any of the enzyme activities or ratios calculated, Table 4.2(a). In this study PDH enzyme activities were corrected for extraction efficiency by comparing to CS enzyme activities. Despite higher values of CSt in both control and hypertrophied hearts, there was no significant difference at the 5% level between active and total extractions, indicating stable enzyme preparations. The presence of hexanoate resulted in very low levels of PDHa in both control and hypertrophied hearts, but not in lower PDHt enzyme levels. The percentage of PDH in the active form [(PDHa/CSa)/(PDHt/CSt)] for both control and hypertrophied hearts is shown clearly in Figure 4.2.

Treatment Groups	PDHa	CSa	PDHa/ CSa ($x10^{-2}$)	PDHt	CSt	PDHt/ CSt ($x10^{-2}$)
Control (n=6)	0.11 (0.01)	62.16 (4.63)	0.22 (0.03)	3.74 (0.04)	75.55 (3.31)	5.28 (0.36)
Hypertrophy (n=7)	0.11 (0.01)	64.39 (2.14)	0.18 (0.02)	3.93 (0.12)	79.14 (1.14)	4.97 (0.15)

Table 4.2(a). Enzyme activities, μmoles/min/gramme wet mass in hearts perfused with Glucose & Hexanoate (mean +/- SEM).

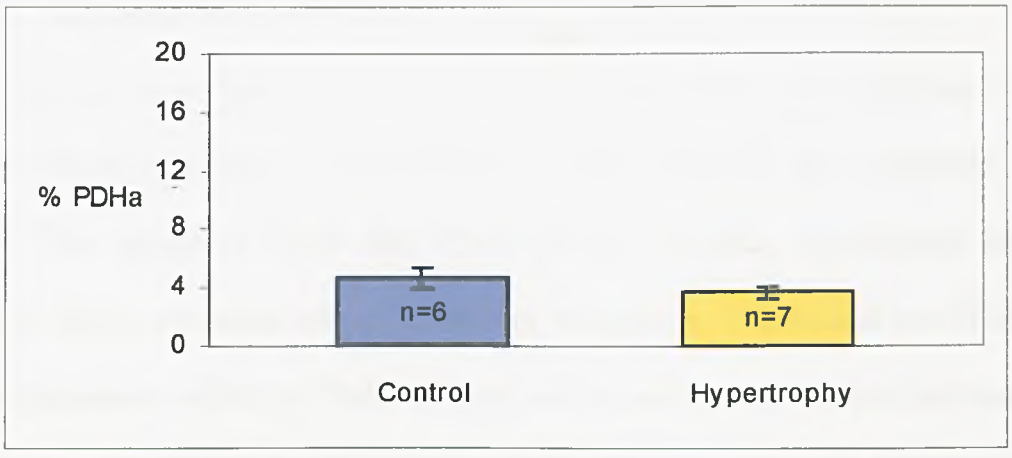

Figure 4.2. Percentage PDH enzyme in the active form (mean +/- SEM) in nine week hypertrophy and control hearts perfused with Glucose & Hexanoate.

Table 4.2(b) shows the concentrations of ATP and PCr, in nine week hypertrophied and control hearts, perfused with glucose and hexanoate. No significant difference was seen in the levels of ATP, PCr or the PCr/ATP ratio, a measure of energy status, between the two groups.

Treatment Groups	ATP	PCr	PCr/ ATP
Control (n=3)	8.49 (2.42)	16.70 (5.76)	1.89 (0.12)
Hypertrophy (n=5)	12.07 (0.64)	19.65 (0.57)	1.66 (0.06)

Table 4.2(b). High-energy phosphate concentrations, μmoles/gramme wet mass (mean +/- SEM) in hearts perfused with Glucose & Hexanoate.

4.3.2.2. Glucose and palmitate.

There were no significant differences in any of the PDH or CS activities between control and hypertrophied hearts perfused with glucose and palmitate, Table 4.2(c). The values of PDHt and PDHt/CSt do not differ significantly from the values in hearts perfused with glucose and hexanoate. PDHa and the PDHa/CSa values increased slightly in both control and hypertrophied hearts perfused with palmitate as opposed to hexanoate as the fatty acid source, although not significant at the 5% level. The percentage of PDH in the active form is again very low in the presence of fatty acid, in this experiment palmitate, for both control and hypertrophied hearts, as shown in Figure 4.3. There is again no significant difference in PDHa/CSa between the two treatment groups.

Treatment Groups	PDHa	CSa	PDHa/ CSa ($x10^{-2}$)	PDHt	CSt	PDHt/ CSt ($x10^{-2}$)
Control (n=7)	0.18 (0.02)	61.44 (1.48)	0.29 (0.03)	3.39 (0.09)	64.72 (1.97)	5.31 (0.12)
Hypertrophy (n=8)	0.16 (0.01)	60.61 (2.18)	0.27 (0.02)	3.58 (0.15)	63.64 (2.38)	5.63 (0.11)

Table 4.2(c). Enzyme activities, μmoles/min/gramme wet mass (mean +/- SEM) in hearts perfused with Glucose & Palmitate.

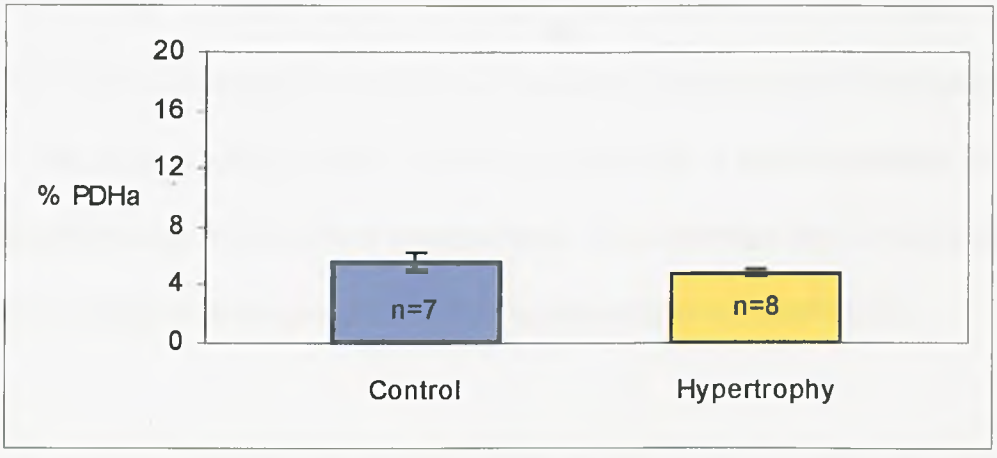

Figure 4.3. Percentage PDH enzyme in the active form (mean +/- SEM) in hearts perfused with Glucose & Palmitate after nine weeks hypertrophy.

Hypertrophied hearts perfused with glucose and palmitate contain similar concentrations of ATP and PCr compared to their respective control group. This results in a comparable PCr/ATP ratio, shown in Table 4.2(d). The levels of ATP, PCr and the PCr/ATP ratio in both control and hypertrophied hearts perfused with palmitate were not significantly different to those in hearts perfused with hexanoate.

Treatment Groups	ATP	PCr	PCr/ ATP
Control (n=4)	8.50 (0.36)	14.73 (0.32)	1.78 (0.10)
Hypertrophy (n=4)	10.19 (0.43)	15.50 (1.19)	1.57 (0.14)

Table 4.2(d). High-energy phosphate concentrations, μmoles/gramme wet mass (mean +/- SEM) in hearts perfused with Glucose & Palmitate.

The concentrations of myocardial acylcarnitine molecules (nmoles/ gramme wet

mass) are shown in Table 4.2(e). No significant differences were seen in any of the acylcarnitine molecule concentrations between hypertrophied and control heart tissue. The hypertrophied hearts do show a trend for a slight increase in the C5-OH (pentylhydroxyacylcarnitine) acylcarnitine concentration and a slight decrease in the DC4 (butyl-dicarboxyacylcarnitine) acylcarnitine concentration.

Chain Length		Control (n=3)	Hypertrophy (n=3)
Short Chain	C2	117.75 (35.52)	150.85 (77.74)
	C3	0.73 (0.20)	0.60 (0.12)
	C4	0.37 (0.02)	0.42 (0.06)
	C5	0.21 (0.02)	0.18 (0.01)
	C4-OH	0.26 (0.02)	0.26 (0.02)
	C6	0.71 (0.13)	0.66 (0.08)
	C5-OH	0.45 (0.00)	0.61 (0.06)
	C8	0.18 (0.03)	0.17 (0.02)
Medium Chain	DC3	0.15 (0.02)	0.14 (0.03)
	C10	0.22 (0.04)	0.23 (0.04)
	DC4	1.34 (0.14)	0.98 (0.05)
	DC5	0.20 (0.02)	0.16 (0.01)
	C12:1	0.09 (0.01)	0.06 (0.03)
	C12	0.68 (0.15)	0.57 (0.08)
	C12-OH	0.37 (0.02)	0.34 (0.02)
	C14:2	0.10 (0.01)	0.08 (0.02)
	C14:1	0.36 (0.03)	0.32 (0.05)
	C14	2.49 (0.79)	2.27 (0.42)
	C14:2-OH	0.10 (0.02)	0.11 (0.03)
	C14:1-OH	0.14 (0.03)	0.43 (0.27)
	C14-OH	0.73 (0.06)	0.85 (0.09)
Long chain	C16:1	0.83 (0.09)	0.89 (0.11)
	C16	6.18 (2.84)	6.34 (1.74)
	C16:1-OH	0.20 (0.03)	0.34 (0.12)
	C16:OH	1.46 (0.13)	3.11 (1.43)
	C18:2	0.45 (0.07)	0.70 (0.17)
	C18:1	0.68 (0.12)	1.16 (0.38)
	C18	1.09 (0.22)	1.34 (0.35)
	C18:2-OH	0.12 (0.03)	0.16 (0.02)
	C18:1-OH	0.20 (0.03)	0.25 (0.01)
	C18-OH	0.23 (0.04)	0.35 (0.11)
	C20:4	0.60 (0.05)	0.60 (0.10)

Table 4.2(e). Acylcarnitine concentration in nmoles/gramme wet mass (mean +/- SEM) in hearts perfused with Glucose & Palmitate. The C number represents the chain length of the molecule, while the OH signifies the presence of a hydroxyacylcarnitine. The DC notation indicates dicarboxyacylcarnitines.

4.3.2.3. Glucose, palmitate and insulin.

Table 4.2(e) shows the enzyme activities for hypertrophied and control hearts perfused with glucose and palmitate in the presence of insulin. No significant differences were observed in any of the enzyme activities or ratios between the two groups. The presence of insulin significantly increased the activity of PDHa in both hypertrophied and control hearts compared to those perfused without insulin ($p<0.05$). This significant increase is also reflected in the percentage of PDH in the active form [(PDHa/CSa)]/[(PDHt/CSt)] for both hypertrophied and control hearts as shown in Figure 4.4, (12.1 +/- 0.5 versus 4.8 +/- 0.3 and 10.6 +/- 0.8 versus 5.6 +/- 0.7 respectively, $p<0.05$).

Treatment Groups	PDHa	CSa	PDHa/ CSa ($x10^{-2}$)	PDHt	CSt	PDHt/ CSt ($x10^{-2}$)
Control (n=6)	0.37 (0.03)	72.67 (1.31)	0.50 (0.04)	3.83 (0.10)	80.11 (1.79)	4.80 (0.13)
Hypertrophy (n=8)	0.49 (0.02)	77.91 (1.70)	0.64 (0.03)	4.26 (0.13)	81.10 (2.07)	5.29 (0.14)

Table 4.2(e). Enzyme activities, μmoles/min/gramme wet mass (mean +/- SEM) in hearts perfused with Glucose & Palmitate & Insulin.

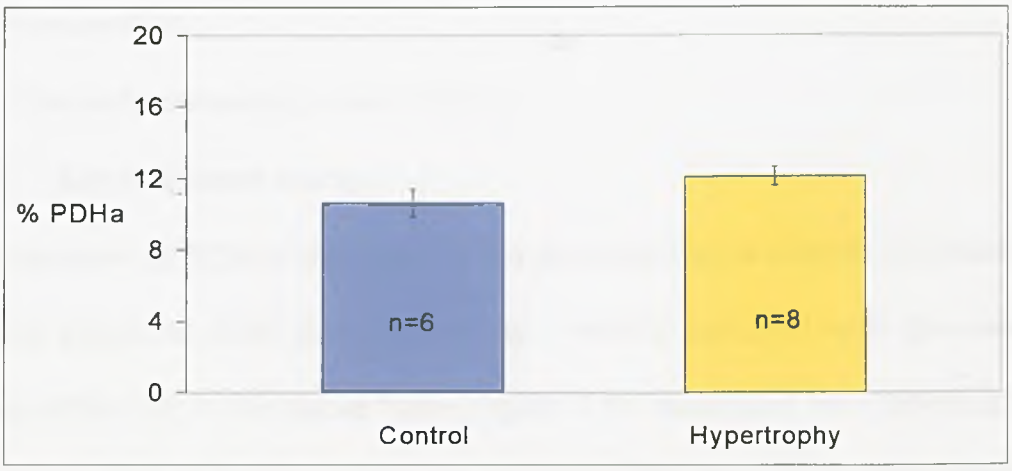

Figure 4.4. Percentage PDH enzyme in the active form (mean +/- SEM) in hearts perfused with Glucose & Palmitate & Insulin after nine weeks hypertrophy.

The concentrations of ATP and PCr do not differ between control and hypertrophied hearts perfused with the substrate mixture glucose and palmitate in the presence of insulin (Table 4.2(f)). The levels of ATP, PCr and the PCr/ATP ratio in both control and hypertrophied hearts perfused with palmitate in the presence of insulin were not significantly different to those in hearts perfused with palmitate without insulin or hexanoate.

Treatment Groups	ATP	PCr	PCr/ ATP
Control (n=4)	8.85 (0.34)	14.50 (0.61)	1.65 (0.09)
Hypertrophy (n=5)	10.60 (0.57)	15.56 (0.81)	1.52 (0.11)

Table 4.2(f). High-energy phosphate concentrations, μmoles/gramme wet mass (mean +/- SEM) in hearts perfused with Glucose & Palmitate & Insulin.

4.4. Discussion.

4.4.1. Pyruvate dehydrogenase activity.

4.4.1.1. Control heart study.

The proportion of PDH in the active form revealed that substrate provision has a profound effect on PDH enzyme activity. Hearts perfused with glucose alone showed 40% PDH in the active form (Figure 4.1), consistent with previous reports using fatty acids or ketone bodies as sustrates (Wieland *et al* 1971, Olson *et al* 1983, Dennis *et al* 1979, Latipaa *et al* 1985). Inclusion of hexanoate as a competing substrate decreased the PDHa enzyme activity to a level of 11%, a level comparable to that found by Latipaa *et al* (1985). Latipaa *et al* (1985) reported correlation between tissue PDH in the active form and PDH flux. As demonstrated in Chapter 3, control hearts perfused with glucose in the presence of hexanoate, exhibit depressed glucose utilisation, relying almost entirely on hexanoate for substrate oxidation. Work from other groups (Neely and Morgan 1974, Weiss *et al* 1989) supports the observation that glucose oxidation by the heart is markedly depressed in the presence of fatty acids.

Hexanoate oxidation in the present study may decrease PDH enzyme activity by either or both of two mechanisms, (i) feedback inhibition or (ii) enzyme interconversion. PDH kinase activity is enhanced by the ATP/ADP ratio (Kerbey *et al* 1976). Weiss *et al* (1989) measured lower ADP levels likely to increase the proportion of PDH in the inactive form. In support of these findings Kerbey *et al* (1976) also found that perfusion of heart with fatty acids and ketone bodies decreased the proportion of active PDH, and that the ATP concentrations of the

hearts and PDH activities were inversely correlated.

When comparing the two regulatory mechanisms of PDH activity, Latipaa *et al* (1985) found covalent inter-conversion overrides the feedback regulation (product inhibition) of active PDH under most conditions, due to the amplification effect by the PDH phosphatase and kinase. They also discovered that the acetyl-CoA/CoA ratio exhibited a higher controlling power on the PDH inter-conversions than the $NADH/NAD^+$ ratio. In this study, hexanoate may thus control PDH via phosphorylation / dephosphorylation.

Fatty acid inhibition of glucose utilisation in hearts can be reversed by DCA (Weiss *et al* 1989). DCA is a non-metabolised analogue of pyruvate (Whitehouse *et al* 1974) and is thought to increase the proportion of PDH in the active form in rat hearts by inhibiting PDH kinase, while not being associated with increased amounts of total PDH enzyme (Kerbey *et al* 1976, Whitehouse *et al* 1974). McVeigh and Lopaschuk (1990) found that lower concentrations of DCA (0.1 and 0.5 mM DCA) did not result in a significant increase in the steady-state oxidation rates of glucose perfused in the presence of high palmitate, whereas 1mM DCA increased glucose oxidation without affecting heart rate or function. 1mM DCA in the present study increased the proportion of PDH in the active form compared to hearts perfused with glucose and hexanoate (16.3% versus 10.5%), although not significantly. In the study by Weiss *et al* (1989), 1mM DCA restored carbon flux from glucose through the PDH reaction in the presence of hexanoate. This led to the conclusion that the primary mechanism and contribution to TCA energy production in the isolated heart was by reversible inactivation of the PDH enzyme.

Carter and Coore (1995) studied the effect of DCA on PDH kinase activity and found that both 1 and 10mM DCA concentration had an inhibitory effect on PDH kinase. The present study supports these findings in which no differences in PDH total were measured.

Stimulation of PDH creates a greater clearance of pyruvate from the glycolytic pathway, accelerating the formation of acetyl-CoA from pyruvate into the TCA cycle. This increases the contribution of glucose to the production of ATP, while simultaneously inhibiting the consumption of fatty acids; the overall change in substrate utilisation being from predominantly fatty acids to glucose and lactate (Stacpoole 1989, McAllister *et al* 1973, Latipaa *et al* 1985, Weiss *et al* 1989, McVeigh and Lopaschuk 1990, Saddik *et al* 1993). Thus it appears that DCA can almost completely reverse the inhibitory effects of fatty acids on glucose oxidation.

In isolated working rat hearts perfused with glucose, fatty acids and DCA, Saddik *et al* (1993) found that tissue levels of acetyl-CoA and malonyl-CoA were significantly higher, in the presence of DCA. They proposed that an increase in acetyl-CoA production by the stimulation of PDH by DCA could directly inhibit the β-oxidation of long-chain fatty acid via an inhibition of ketoacyl-CoA thiolase. As hexanoate is not influenced by CPT1 activity, increased glucose oxidation by PDH stimulation is likely to affect fatty acid oxidation at the level of β-oxidation and not uptake by the mitochondria. Other effects of DCA on metabolism have been noted including the production of glycerol-3-phosphate (Chacko and Weiss 1993). The proposed mechanism is that DCA activates PFK (Stacpoole 1989, Stacpoole and Greene 1992) to cause an accumulation of dihydroxyacetone phosphate and

glyceraldehyde–3-phosphate. Dihydroxyacetone phosphate can then be reduced to glycerol-3-phosphate by glycerol 3-phosphate dehydrogenase.

When 2mM DCA was included in the competing substrate mixture PDH enzyme activity further increased to 30%, a level comparable to the control level achieved with glucose alone. Latipaa *et al* (1985) found a positive linear relationship between the fraction of PDH existing in the active form and the pyruvate oxidation rate until active PDH increased to 48%, at which point the TCA cycle was saturated. When 5mM DCA was used, a concentration equivalent to total PDH activity in other studies, the proportion of PDH in the active fraction reached a value of 70%. This suggests that 100% PDH activity may be unobtainable in an isolated heart preparation when more than one substrate is available for utilisation, unless potentially detrimentally high levels of DCA are provided. This may be explained by the observation of Latipaa *et al* (1985), in which activation of PDH beyond 48% did not further increase the metabolic flux through PDH into the TCA cycle. More importantly, even if higher levels of PDH in the active form are achieved, there is no further improvement in functional performance of the heart. Thus substrate delivery through PDH is optimal between 2 and 5mM DCA concentration and at 5mM DCA the TCA cycle has become saturated.

Considering the technical aspects of the study, active (PDHa) and total (PDHt) pyruvate dehydrogenase enzyme activities were measured in separate extracts of myocardial tissue. One potential disadvantage of this assay technique is that differences in extraction efficiency could affect the PDHa/PDHt ratio. PDH

enzyme activities of both active and total extracts were therefore normalised for enzyme extraction efficiency by using the activities of oxoglutarate dehydrogenase (OGDH)(see Table 4.1(b) for individual values)(based on Seymour and Chatham 1997). The enzyme OGDH was chosen because as a membrane multi-subunit enzyme complex it is very similar to PDH. The maximal velocity of OGDH is considered reliable due to the fact that it is not regulated by phosphorylation / dephosphorylation (Hansford *et al* 1990). Thus equal extraction efficiency was expected of both enzymes from the inner mitochondrial membrane, with the advantage that OGDH activity was unlikely to be affected by the different extraction buffers (Seymour and Chatham 1997).

The activity of OGDH in the control rat groups in this study, lay in the range of 1-3 μmoles/gram wet mass of cardiac tissue. These values are approximately 50% of the levels seen previously in control rat hearts (Seymour and Chatham 1997), using an identical enzyme assay protocol, based on a method by Hansford (1991). Enzyme activities measured in this study were obtained from isolated perfused hearts compared to the study by Seymour and Chatham (1997), where the hearts were frozen at the time of sacrifice. However, OGDH values in the present study do compare with absolute control OGDH levels measured in isolated hamster hearts perfused with glucose (Di Lisa *et al* 1993). The low OGDH enzyme activity in this study is unlikely to be due to the presence of hexanoate because similar activities were observed with glucose alone as substrate. The explanation is more likely to be due to the difference in the extraction efficiency of the individual performing the assay. The fact that there is no difference in OGDH

values between the active and total extractions for each substrate group allows the valid calculation of percentage fraction of PDH in the active form normalised for extraction efficiency.

Interestingly high levels of DCA (2 and 5 mM concentration) significantly increased the OGDH enzyme activity in active and total extractions compared to the other substrate groups. This is in parallel to the positive effect DCA exhibits on PDH active and total enzyme activity. However Di Lisa *et al* (1993) did not witness this phenomenon when glucose and 5mM DCA were included in the perfusion buffer. The fact that the only difference between the normalised PDHt/OGDHt values lay between the glucose only and the glucose, hexanoate and 1mM DCA substrate groups suggests that the OGDH and PDH enzyme increases are not caused by the presence of DCA, but is again due to extraction efficiency.

4.4.1.2. Hypertrophied hearts perfused with glucose and short-chain fatty acid.

Hypertrophied and control hearts perfused with glucose and hexanoate as competing substrates, both contained very low levels of PDH enzyme in the active form (Figure 4.2). This is consistent with the findings in the previous chapter (section 3.3.2.1). In addition, short-chain fatty acid oxidation is not found to be impaired in models of cardiac hypertrophy (El Alaoui-Talibi *et al* 1989, 1992). This supports the idea that short chain fatty acid utilisation, in this case hexanoate can inhibit glucose oxidation at the level of the PDH enzyme in hypertrophied as well

as control hearts (Olson *et al* 1983, Weiss *et al* 1989, Latipaa *et al* 1985). The most important finding of this experiment is that the PDH enzyme data are consistent with the ^{13}C NMR study of relative substrate utilisation. Inhibition of glucose oxidation and utilisation of hexanoate as evidenced by the ^{13}C NMR data (section 3.3.2.1) and the corresponding low PDHa levels revealed that even low levels of short chain fatty acid are sufficient to inhibit glucose use at the PDH reaction.

The mechanisms by which hexanoate utilisation in the present study inhibits the PDH complex in hypertrophied and control hearts are similar to that described in the control hearts (section 4.4.1.1), despite the fact that the hexanoate concentration supplied here was 0.3mM compared to 1mM previously.

The proportion of PDH in the active form was lower in the hypertrophied hearts (3.7%) compared to the control hearts (4.6%), but was not significantly different. The fact that both the hypertrophied and control hearts utilised hexanoate almost exclusively indicates that PDH was inhibited to the same extent in both groups. At this stage of hypertrophy there is no evidence to suggest that metabolic changes in substrate utilisation occur when glucose and hexanoate are the substrates supplied. In contrast, Seymour and Chatham (1997) found a significant reduction in PDHa in an identical nine week pressure-overload cardiac hypertrophy model, in which PDHa levels were reduced from 61% to 36% during hypertrophy. Concerning the decrease in PDHa during hypertrophy, there could be several reasons for this discrepancy, found within the same animal model.

Firstly PDH activity was measured in hypertrophied hearts isolated and

perfused with glucose and hexanoate in the present study, whereas Seymour and Chatham (1997) measured PDH activity in hearts frozen at the time of sacrifice. Secondly the hearts in the present study exhibited a less severe degree of hypertrophy compared to a 25% increase in heart mass to body mass ratio observed by Seymour and Chatham (1997). In a more severe model of hypertrophy, Di Lisa (1993) reported a lower proportion of PDH in the active form in hearts from hypertrophic cardiomyopathic Syrian hamsters. These animals progress to heart failure beyond the age of nine months. Thus the severity of hypertrophy achieved is likely to be an important factor greatly influencing the activity of PDHa.

Considering the technical aspects of the assay the levels of PDHa measured here may be low compared to other groups due to extraction efficiency. The sensitivity of the assay at these low levels of activity could be masking any differences between the control and hypertrophied groups. The previous DCA and control PDH enzyme experiment clearly demonstrates that the assay can measure precisely high levels of PDHa, but some accuracy may be lost in measuring very low levels of PDHa. The active and total extract PDH enzyme activities in this experimental section were normalised to citrate synthase enzyme activities. This method of calculation was consistently used in future PDH assays. Citrate synthase enzyme measurements were found to be more reproducible within heart groups compared to the OGDH enzyme measurements in the control heart study (section 4.3.1.3.). Normalising PDH activity in this manner alleviated any concerns over OGDH extraction with the added advantage of providing an

in Table 4.2(a). No differences were seen between hypertrophied and control values of PDHa, CSa, PDHt and CSt, indicating that neither total enzyme content, mitochondrial density or extraction efficiency was altered between control and hypertrophied hearts.

4.4.1.3. Hypertrophied hearts perfused with glucose and long-chain fatty acid.

The major finding in this study of substrate selection during hypertrophy is that %PDHa activity per se does not accurately reflect the relative amount of glucose substrate used by the hearts. The ^{13}C NMR data in Chapter 3 (section 3.3.2.2a.) clearly demonstrated a significant increased reliance on glucose oxidation at the expense of endogenous substrate use, when hypertrophied hearts were supplied with glucose and long chain fatty acid compared to control hearts. However the proportions of PDH in the active form did not significantly alter between nine week hypertrophied and control hearts (4.8% versus 5.6% respectively, Figure 4.3). Thus PDH activity does not accurately reflect the flux through this reaction. It is also worth noting that the total PDH enzyme expression does not change between the groups. Any alteration in PDH activity is therefore due to the phosphorylation / dephosphorylation state of the PDH complex or regulating molecules.

Glucose is a major source of pyruvate, which itself is an inhibitor of PDH kinase activity (Carter and Coore 1995). Thus hearts utilising relative more glucose as its major TCA cycle oxidative substrate, in this case the hypertrophied hearts, as evidenced from the ^{13}C NMR data, would be expected to have a

decreased PDH kinase activity. This would result in a greater proportion of PDH in the active form. Although PDHa did not differ significantly between the hypertrophied and control hearts, care has to be taken in the interpretation of this PDH enzyme activity data. The PDH activity *in vitro* does not equal flux. The 5-6% PDHa in hypertrophied hearts may be sufficient to allow the increased flux. Therefore these PDH experiments may support the observations of increased glucose uptake (Seymour *et al* 1990) and enhanced rates of glycolysis and glycolytic enzyme activities (Allard *et al* 1994, Bishop and Altschuld 1970, Leipala *et al* 1989, Smith *et al* 1990, Taegtmeyer and Overturf 1988) seen during hypertrophy.

Comparison between substrate mixtures reveals that the utilisation of fatty acid has the ability to inhibit %PDHa to similar extents regardless of chain length. The %PDHa values of hypertrophied and control hearts perfused with glucose and palmitate were comparable to those perfused with glucose and hexanoate. Adaptations in PDH phosphatase and PDH kinase reported in cardiac hypertrophy may act as potential modulators of flux through the PDH enzyme complex (Orfali *et al* 1995, Lydell *et al* 1999). There exist conflicting reports regarding PDH kinase. Orfali *et al* (1995) observed an increase in PDH kinase activity following triiodothyronine (T3) treatment, an agent known to cause cardiac hypertrophy. Lydell *et al* (1999) however did not observe any changes in either PDH activity or PDH kinase activity during pressure overload hypertrophy versus the model in this present study. PDH kinase levels and activity were not measured here, so conclusions as to their influence on these PDH measurements cannot be made.

4.4.1.4. Effect of insulin on PDH activity in hypertrophied hearts.

The proportion of PDH in the active form is regulated not only by substrate availability but also by the hormonal status of the animal (Randle 1988). Cardiac PDH activity increases both *in vitro* and *in vivo* when exposed to insulin. A wide variation in the proportion of PDH in the active form has been measured in control conditions (ranging from 8-90%) (Kobayashi and Neely 1983, Hansford *et al* 1990, Behal *et al* 1993, Seymour and Chatham 1997). This may result from differences in hormonal and nutritional state of the heart at the time of sacrifice, the method of sacrifice, and perfusion. The model here contained physiological substrate concentrations and a high physiological insulin level representing a fed state. Inclusion of insulin in the perfusion buffer at a concentration of 100μU/ml significantly increased the level of PDH in the active form, (Figure 5.3). The enzyme activities were approximately doubled in both control and hypertrophied hearts, to values 10.6% and 12.1% respectively. These increases in %PDHa are consistent with the ^{13}C-NMR study (section 3.3.2.3a).

The similarity in PDHa percentage between control and hypertrophied heart groups suggests that no insulin resistance existed with acute administration, at this stage of compensated hypertrophy. In contrast, in a chronic situation, Orfali *et al* (1995) showed that cardiac hypertrophy following T3 treatment, resulted in an increase in PDH kinase activity, and is consistent with the observations of a decrease in PDHa in the pressure-overload rat model (Seymour and Chatham 1997). Orfali *et al* (1995) reported a direct involvement of insulin alongside T3 in

the regulation of cardiac PDH activity via stable changes in the activity of PDH kinase. A lower proportion of PDH in the active form has also been reported in the hearts of cardiomyopathic hamsters (Di Lisa *et al* 1993), but no change in PDH effector concentrations (pyruvate, CoASH, acetyl-CoA and NAD^+). Here the alterations of PDH activity were associated with alterations in calcium handling, in which the rise of mitochondrial [Ca^{2+}] was less following electrical or adrenergic stimulation, accounting for a decrease in PDH phosphatase activity and less active form of PDH (McCormack and Denton 1989). Studies measuring the effect of insulin on PDH enzyme activity have been conducted on rat models of high fat feeding (Bryson *et al* 1995) and starvation (Sugden *et al* 1997), in which marked decreases in cardiac PDH activation have been found. There is speculation concerning the exact mechanism by which insulin alters the phosphorylation state and increases PDH enzyme activity.

By increasing the PDH activity, and subsequently glucose oxidation, insulin can influence long chain fatty acid oxidation. An increase in acetyl-CoA production from PDH may directly inhibit β-oxidation of fatty acid via an inhibition of ketoacyl-CoA thiolase (Saddik *et al* 1993). The observation that fatty acid oxidation is decreased in hypertrophied hearts in the presence of insulin, suggests that flux through PDH may be increased in the hypertrophied hearts, irrespective of the %PDHa values. In support of this the major fate of additional glucose taken up in response to insulin in the control heart is not found to be glycogen (Holness and Sugden 1991). The effect of glucose oxidation decreasing fatty acid oxidation

through insulin has also been attributed to an increase in cardiac malonyl-CoA concentrations and inhibition of CPTI (Awan and Saggerson 1993). CPTI is highly sensitive to inhibition by malonyl-CoA (McGarry *et al* 1983). Malonyl-CoA levels are therefore likely to play an important regulatory role in controlling fuel selection by the heart. In a study on rats hearts perfused with 5mM glucose, malonyl-CoA levels were acutely raised by insulin (Awan and Saggerson 1993). However Awan and Saggerson (1993) have observed that insulin has a variable and inconsistent effect on suppressing palmitate oxidation. They suggest that acute regulation of long-chain fatty acid oxidation by insulin is not a major feature of cardiac metabolism.

4.4.2. Heart function in DCA study on control hearts.

The work performed by the heart is a key determinant of carbon substrate oxidation rates, therefore the mechanical function of control hearts perfused with differing substrates was continuously monitored throughout the perfusion periods. Control rat hearts perfused with glucose in the presence of hexanoate did not exhibit any alterations in functional parameters, compared to hearts perfused with glucose alone (Table 4.1(a)). Thus inhibition of glucose oxidation by hexanoate in control hearts in the present study did not adversely affect heart function, despite the fact that more oxygen is required per molecule of ATP produced for fatty acid oxidation compared to glucose oxidation (Liedtke *et al* 1978, Kahles *et al* 1982). Although it is worth considering that hexanoate, a short chain fatty acid, has easier access to the mitochondria compared to long chain fatty acids; hexanoate

circumvents the carnitine-acylcarnitine translocase transport and is activated in the matrix of the mitochondria by a butryl-CoA synthetase (Webster *et al* 1964).

Improved contractile performance was obtained in hearts perfused with glucose, hexanoate and 2mM DCA only. The heightened control heart function following perfusion with 2mM DCA was determined by increases in systolic pressure, LVDP and RPP (Table 4.1(a)). The mechanism by which DCA can affect heart function is still speculative. It has been suggested that DCA may have a positive inotropic effect on the heart (Weiss *et al* 1989), although this is unlikely to account for the improved heart function in this study. Alternatively the increase in mechanical function with 2mM DCA is more likely to be explained by altered substrate oxidation, whereby stimulation of PDH by DCA has been shown to significant increase in glycolysis alongside glucose oxidation (McAllister *et al* 1973). Non-esterified fatty acids are a relatively inefficient fuel source, compared to glucose and lactate (Kahles *et al* 1982). Thus switching myocardial substrate utilisation from hexanoate to glucose or endogenous lactate could improve the oxygen consumption efficiency of the heart (McAllister *et al* 1973, Stacpoole *et al* 1988).

It is interesting to note that 5mM DCA did not further improve heart function. This suggests that 2mM DCA is the optimum concentration required to improve substrate supply in the control heart. In support of this finding Lewandowski and White (1995) observed no difference in function between control heart and 5mM DCA-treated hearts during normal perfusion with pyruvate as the sole carbon based substrate.

4.4.3. Potential therapeutic use of DCA

The present study clearly demonstrated that PDH enzyme activity is effectively modulated, using DCA, thus overcoming the inhibition of glucose utilisation even by short chain fatty acid. The findings revealed that the addition of varying concentrations of DCA increased the PDH enzyme activity in a dose response relationship. The study also highlighted the relationship between substrate use via PDH activation and its effect on heart function.

Beneficial effects of DCA administration have been confirmed in situations where substrate provision is thought to be impaired, such as experimental infarction models (McVeigh and Lopaschuk 1990, Lopaschuk *et al* 1993, Lewandowski *et al* 1995) and under some clinical conditions in patients with congestive heart failure (Bersin *et al* 1994). The beneficial effect of increasing flux through PDH has generally been attributed to the increase in carbohydrate oxidation rather than associated to increases in glycolysis and glycolytic energy production (McVeigh and Lopaschuk 1990, Lopaschuk *et al* 1993). Decreased expression of GLUT4 (Depre *et al* 1998) and possibly HK can occur in hypertrophy, which may limit the availability of glucose. In addition during the development of cardiac hypertrophy cellular conditions may arise that increase the action of PDH kinase (Seymour and Chatham 1997). It would be paradoxical for the heart to become dependent upon carbohydrate for energy production, with concomitant inactivation of a key enzyme responsible for carbohydrate oxidation. Therefore pharmacological activation of PDH could maintain adequate substrate provision during cardiac hypertrophy, and warrants further investigation.

PDH enzyme activities in this study were determined in enzyme preparations from tissue homogenates. One limitation is whether changes in enzyme activities from homogenates accurately reflect the situation *in vivo*. Even if DCA stimulates PDH, increasing glucose oxidation, substrate entry of the cell may in turn become the rate-limiting step. Therefore, further studies are necessary to determine whether DCA has clinical potential to regulate substrate metabolism during the development of cardiac hypertrophy.

4.4.4. Energetic status in hypertrophied hearts.

The heart requires an adequate flow of oxidisable carbon through the TCA cycle to regenerate high-energy phosphate stores. ATP and PCr metabolite concentrations and the ratio PCr/ATP (phosphorylation potential), provide important information about the energy status of the myocardium. Absolute myocardial high-energy phosphate ATP and PCr levels were comparable in extracts from control and nine week hypertrophied hearts perfused with glucose and hexanoate (Table 4.2(b)). Subsequently the PCr/ATP ratio was also unaltered following nine weeks hypertrophy under the conditions of these experiments (1.9 +/- 0.1 versus 1.7 +/- 0.1 for control and hypertrophied hearts). This indicates that there was adequate substrate provision for energy production in both control and nine week hypertrophied hearts under these perfusion conditions. These ratios are consistent with PCr/ATP ratios reported in control hearts by other investigators using ^{31}P NMR spectra analysis (Chatham and Forder 1993), but differ compared to other hypertrophy studies, in which a depletion of high-energy phosphate

reserves, have been observed in many models of cardiac hypertrophy (Bhutta *et al* 1995, Massie *et al* 1995, Seymour *et al* 1990, Lortet *et al* 1995, Zhang *et al* 1993).

Static levels of ATP and PCr measured in heart extracts, provide an indication of energy reserves but do not provide information concerning the rates of ATP production or utilisation. A change in the rate of ATP utilisation would not be detectable or accompanied by a change in the level of ATP and PCr levels if rates of ATP production changed in parallel with rates of utilisation. Rates of ATP production by the heart depend on ATP utilisation (Neely and Morgan 1974). Therefore it is unlikely given that cardiac function was similar between the two heart groups (Chapter 3, section 3.3.2.1b), that the associated ATP turnover rates differed between the control and hypertrophied hearts in this study.

The concentrations of ATP and PCr did not alter significantly between the nine week hypertrophied and control hearts perfused with glucose and palmitate as substrates (Table 4.2(d)). This led to comparable PCr/ATP ratios, indicating that that there was no limitation of energy production in nine week hypertrophied hearts perfused with glucose and palmitate as substrates. There is as yet no clear understanding of pressure- or volume-overload induced changes in myocardial energy metabolism and its effects on heart function. There are also limitations in the measurements of high-energy phosphate levels in extracts compared to in situ measurements by ^{31}P-NMR, given that PCr is a labile compound. However in support of these findings Seppet *et al* (1985) demonstrated that even with a decrease in PCr concentration, no direct effect on energy metabolism and short-term regulation of mitochondrial activity by ADP and Pi could be found. It was

concluded that PCr was not involved in the adaptation of the energy-producing pathway to chronic sustained workload.

The ATP, PCr and PCr/ATP ratios were unchanged between the nine week hypertrophied and control hearts perfused with glucose, palmitate and insulin (Table 4.2(f)). Therefore despite the alterations in relative substrate use between, the hypertrophied hearts showed no limitation in energy reserves. However care has to be taken when interpreting high-energy phosphate obtained from freeze-clamped extracts. High-energy phosphates are compartmentalised at varied concentrations within different heart cells (namely myocytes, smooth muscle cells, endothelial cells, fibroblasts, conductive cells and blood). Greater than 85% is located within the myocytes (reviewed by Harmsen and Seymour 1988), implying that changes in ATP and PCr concentrations greater than 15% should reflect differences within the myocyte alone. However in conditions such as hypertrophy, the proportions of cell types within the heart regions (subepi- versus subendocardial) may alter, which could give rise to non-uniform high-energy phosphate distribution. Marked differences have also been shown in high-energy phosphate concentrations in the cytosol and mitochondrion of control hearts. ATP and PCr are considered to be primarily cytosolic, whilst free ADP levels are higher in the mitochondria (Asimakis and Sordahl 1981). It is impossible to determine if these levels change during hypertrophy using tissue extractions in this study.

The positive effect insulin exerted on mechanical function in the glucose and palmitate perfused hearts compared to those without insulin was not reflected in either the PCr and ATP levels or the PCr/ATP ratios. All high-energy phosphate

parameters were comparable between all substrate groups and their varied function performances. ATP concentration in the myocardium remains markedly constant and independent of workload (Beyerbacht *et al* 1996). However, ATP utilisation may well be altered in relation to the changes in substrate utilisation profiles seen in hypertrophy. ATP produced by membrane-bound glycolytic enzymes and/or glycogen is used predominantly by ionic pumps (reviewed by Harmsen and Seymour 1988). Since hypertrophied hearts utilised significantly more endogenous substrate compared to the control hearts in the presence of insulin, the sites of ATP use may also have been altered accordingly, but is beyond the scope of this study.

4.4.5. Myocardial acylcarnitine content.

The most abundant acylcarnitine concentration in both hypertrophied and control hearts was that of 2 carbons in length. Control and hypertrophied hearts showed comparable levels of short-, medium- and long-chain acylcarnitines concentrations (Table 4.2(e)). This was despite the 30% decrease in tissue carnitine levels measured in hypertrophied hearts of this model (Hornby 1999, personal communication). Long-chain acylcarnitines formed by CPTI are preferentially transported into mitochondria. This is because the affinity of carnitine-acylcarnitine translocase for extra mitochondrial acylcarnitine is higher than for external carnitine and short-chain acylcarnitine (Parvin and Pande 1976, Idell-Wenger *et al* 1982). The translocase activity is determined by free carnitine concentrations in the mitochondrial matrix (Pande and Parvin 1976).

A decrease in carnitine content in the hypertrophied heart (El Alaoui-Talibi *et al* 1992, Reibel *et al* 1983) leads to a decrease in long-chain fatty acid oxidation (El Alaoui-Talibi *et al* 1992). However due to the fact that the Km for CPTI is low (Bohmer *et al* 1977, Bahl *et al* 1981) suggests that the concentration of carnitine in the model of hypertrophy used here is adequate to maintain long-chain fatty acid oxidation. In addition only total carnitine concentration was measured in the present study and may not reflect the mitochondrial concentration of carnitine.

4.4.6. Summary.

The following conclusions can be drawn from this study:

- The proportion of PDH in the active form can be effectively modulated in control hearts, perfused with the substrates, glucose and hexanoate, in the presence of varying concentrations of DCA.
- PDH in the active form was not altered during compensated hypertrophy, in hearts perfused with any of the substrate mixtures.
- %PDHa per se does not reflect the alterations in substrate use observed in Chapter 3.
- High-energy phosphate status did not alter during cardiac hypertrophy, indicating the hypertrophied hearts were not energy limited.
- Acylcarnitine levels had not increased during this stage of hypertrophy despite reported decreases in tissue carnitine concentrations.

Chapter 5:

Prolonged pressure-overload cardiac hypertrophy in the rat.

5.1. Introduction.

Left ventricular hypertrophy has been recognised as a risk factor for sudden death, myocardial infarction and congestive heart failure (Kannel *et al* 1983, Lenfant 1994). The cardiac response to a prolonged pressure-overload is an initial adaptive ventricular hypertrophy that can progress towards maladaptive heart failure (Anversa and Capasso 1991). Mechanisms responsible for the transition from compensated to decompensated hypertrophy are still poorly understood. One hypothesis is that the transition occurs due to a metabolic defect (Katz 1990), in which impairment of the energy producing system of the myocyte already present in the compensated stage could underlie the progression to failure.

5.1.1. Heart Failure.

5.1.1.1. Metabolic alterations of the failing heart.

Myocardial energy metabolism is markedly altered in both experimental and clinical studies of heart failure resulting in a decrease in the myocardial energy reserve (Seymour *et al* 1990, Ingwall 1993, Tian *et al* 1996). Ingwall *et al* (1990) found low PCr concentrations in an animal model of the failing heart using ^{31}P-NMR. Rajagopalan *et al* (1987) and Conway *et al* (1991) have both observed a reduced PCr/ATP ratio at rest in failing human myocardium, while Neubauer *et al* (1992) and Luyten *et al* (1991) reported a decrease in patients with dilated cardiomyopathy. Conflicting results in patient studies (Schaefer *et al* 1990, Auffermann *et al* 1991) however suggest that decreased myocardial PCr content is present only in patients with severe cardiac dysfunction (reviewed by Ingwall and

Weiss 1993).

Reduction in the concentration of PCr is thought to occur due to a depletion of intracellular creatine. Some reports have shown that severe creatine depletion in normal rats following chronic feeding of β-guanidinoproprionate (β-GP), results in impaired cardiac performance (Kapelko *et al* 1989, Zweier *et al* 1991). Depletion of the creatine pool resulting in lower PCr concentration has been reported in animal models of hypertrophy (Wexler *et al* 1988, Zhang *et al* 1993) and in the human heart (Conway *et al* 1991).

In the heart phosphoryl transfer from phosphocreatine to ADP via the creatine kinase (CK) reaction can maintain ATP levels, acting as an energy reservoir. In addition to high-energy phosphate concentration, the activity of key CK has been reported to decrease, possibly due to changes in isoenzyme distribution (Stephens *et al* 1979, Bittl *et al* 1987, Smith *et al* 1990), or altered CK kinetics (Osbakken *et al* 1992). Whether changes in myocardial enzyme kinetics and high-energy phosphate concentration may contribute to diastolic dysfunction and development of heart failure, if the overload is maintained for prolonged periods, needs further investigation. The findings so far in the present study have shown that the concentration of high-energy phosphates does not alter in nine week compensated pressure-overload hypertrophied hearts (Chapter 4).

5.1.1.2. Detrimental consequences of altered substrate utilisation in cardiac hypertrophy.

Increased mechanical stress induces adaptation by isoform switching in the

energy providing and consuming pathways (Depre *et al* 1998). This allows for a rapid adaptation of myocardial energetics to meet the changes in energy demands. The alterations in substrate utilisation identified in Chapter 3, namely the increased reliance on glucose oxidation for ATP production, may begin as beneficial adaptations to increased workload demands during the development of cardiac hypertrophy. However there are many detrimental consequences associated with these changes that may limit generation of ATP ultimately in the hypertrophied heart and lead to energy depletion in the longer term.

5.1.1.3. Limitations in substrate availability.

Reductions in myocardial carnitine co-factor amounts, noted by other researchers (Chiekh *et al* 1994, Reibel *et al* 1983), limit the entry of fatty acids into the mitochondria and inhibit fatty acid oxidation. A decrease in total tissue carnitine concentration has been observed in the present hypertrophied heart model following nine weeks overload (Horby 1999, Personal communication). Inhibition of fatty acid oxidation in the heart is thought to result in an accumulation of acyl-CoA molecules (Russell and Taegtmeyer 1991, 1992) which may affect the rate of TCA cycle turnover and contractile function. Accumulations of fatty acyl components, such as acyl-CoA molecules, may in turn sequester any CoA molecules and thus reduce the amount of free CoA available. This would result in an increase of the acetyl-CoA to CoA ratio, an important regulator of pyruvate dehydrogenase. Pyruvate dehydrogenase itself is a key enzyme in the oxidation of glucose, therefore an alteration in the amount of free CoA could have

detrimental effects on glucose oxidation. In addition decreases in carnitine concentration, itself an important regulator of glucose oxidation may contribute to the alteration of the acetyl-CoA to CoA ratio. The ultimate consequence may be that the heart is unable to utilise either fatty acids or glucose as substrates.

5.1.1.4. Long-term effects of reduced fatty acid utilisation.

Studies have investigated the role of fatty acids in the contribution to changes in cardiac hypertrophy / cardiomyopathy, where inborn errors of fatty acid metabolism are associated with cardiomyopathy (Strauss *et al* 1995). Administrations of CPTI inhibitors, which subsequently inhibit fatty acid oxidation, have been shown to evoke cardiac hypertrophy (Kusaka *et al* 1995, Bressler and Goldman 1993, Vetter and Rupp 1994). Fatty acids per se may act as secondary messengers (Van Bilsen and van der Vusse 1995), in which they can modulate the activity of various cardiac voltage-gated ion-channels (e.g. K^+-, L-type Ca^{2+}- and Na^+-channel). Perhaps more importantly exposure of cardiac myocytes to saturated fatty acids (C16:0 or C18:0) has been shown to result in cell death as evidenced by the release of intracellular proteins like LDH (Van Bilsen *et al* 1997).

5.1.1.5. Mitochondrial dysfunction.

The Syrian cardiomyopathic hamster, develops a hereditary myocardiopathy terminating in congestive heart failure and has been used to study the sequence of events leading to heart failure (Gertz 1972, Hunter *et al*1984, Ottenweller *et al* 1987). Some authors demonstrate that the pathological events leading to heart

failure are associated with mitochondrial dysfunction (Schwartz *et al* 1968, Nascimben *et al* 1995). Others show *in vitro* oxidative phosphorylation may be normal if the mitochondria are adequately supplied with substrates (Wrogemann *et al* 1972, Wrogemann and Nylen 1978), which further supports the hypothesis that insufficient substrate delivery to the mitochondria underlies the progression to heart failure.

5.1.1.6. Insulin resistance.

Coupled with observed changes in energy metabolism, both animal (Paternostro *et al* 1995, Katz *et al* 1995) and human studies (Paternostro *et al* 1999) suggest that left ventricular hypertrophy is also associated with insulin resistance. In one study involving the spontaneously hypertensive rat (SHR), a common model of hypertension, insulin resistance was demonstrated by a reduced uptake of 2DG6P in isolated rat hearts in the presence of insulin together with reduced mRNA levels of the insulin-responsive glucose transporter GLUT4 (Paternostro *et al* 1995). Alterations in glucose transporters, namely a decrease in the GLUT 4/ GLUT 1 ratio have been implicated as a possible cause of insulin resistance and decreased glucose uptake (reviewed by Gould and Holman 1993). In support of this pressure-overload cardiac hypertrophy in the rat has been shown to exhibit such a decreased ratio of GLUT 4 to GLUT 1 mRNA expression (Weinberg *et al* 1995, Depre *et al* 1998). With GLUT 4 being an insulin-regulated glucose transporter, pressure-overload hypertrophy can render the heart insulin-resistant for glucose substrate supply.

Insulin bound to cell membrane receptors requires energy-dependent internalisation (Im *et al* 1985). Depletion of ATP in normal isolated myocytes resulted in decreased insulin uptake and degradation. This process may be compromised in energy deficient hypertrophied heart cells. A limitation of glucose uptake due to insulin resistance during hypertrophy would exert a negative effect on glucose oxidation at the level of PDH enzyme, in myocardium that may have already come to rely heavily on glucose for its energy provision. Further limiting the availability of this glucose substrate may eventually contribute to the deterioration of heart function. In support of this Katz *et al* (1995) demonstrated that mice deficient in GLUT4 exhibited decreased longevity associated with cardiac hypertrophy and post-prandial hyperinsulinaemia, indicating possible insulin resistance. This raises the possibility that alterations in substrate utilisation such as decreased glucose metabolism may itself contribute to cardiac disease rather than arise purely as a consequence.

5.1.2. Decompensated hypertrophy.

Events leading to cardiac hypertrophy firstly involve an increase in systolic stress due to pressure-overload and secondly an increase in diastolic stress due to loss of myocardium and impaired calcium handling due to a decrease in the expression the Ca^{2+}-ATPase of the SR (Levitsky *et al* 1991). This loss of myocardium can result from myocardial ischaemia leading to necrotic cell death. Therefore cardiac hypertrophy is thought to be a prognostic factor in the development of cardiac failure (Levy 1990). Pathological changes are characterised by a switch towards

the re-expression of foetal isoform of functionally important proteins such as β-myosin heavy chains, α-skeletal actin, α-smooth muscle actin (reviewed by Schwartz *et al* 1986) and the BB-isoform of cytosolic creatine kinase (Smith *et al* 1990). Another phenotypic change in the pathological hypertrophy is the induction of the synthesis and secretion of atrial natriuretic factor (ANF) Takahashi *et al* 1992).

5.1.2.1. Susceptibility of hypertrophied heart to ischaemia.

At a physiological level the hypertrophied heart increases in both myocyte and whole organ size, increasing the intercapillary distance and potentially limiting the supply of oxygen and substrate supply. Myocardial ischaemia is characterised by insufficient delivery of oxygen and substrates to the myocardium to meet its energy needs. When oxygen delivery is inadequate for oxidative needs, fatty acid oxidation decreases and anaerobic glycolysis increases, resulting in insufficient energy transduction, accumulation of lactate and hydrogen ions, and a decrease in contractile function (Opie 1984). During myocardial ischaemia ATP is broken down to its purine constituents which can then be released into the coronary circulation and lost from the myocardial cell (Smolenski *et al* 1994). Release of purines from the myocardium can result in a substantial depletion of the intracellular adenine nucleotide pool, thus rendering the heart more vulnerable to reperfusion injury should it occur (Bailey *et al* 1981). The deleterious effects of myocardial ischaemia are thus due to inadequate ATP production and local tissue acidosis.

Impaired fatty acid metabolism during myocardial ischaemia has been associated with many of the detrimental electrophysiological, biochemical and mechanical effects that lead to accelerated tissue damage (Katz and Messineo 1981, Corr *et al* 1984). Ischaemic inhibition of fatty acid oxidation can lead to an abnormal accumulation of lipid metabolites such as acyl-CoAs and long-chain acylcarnitines (DaTorre *et al* 1991). These molecules are thought to contribute to a loss of membrane integrity (Piper and Das 1987), targeting of ion channels (Corr *et al* 1987) and decreased mechanical function (Corr *et al* 1984).

High levels of fatty acids and fatty acyl metabolites can also lead to inhibition of both glycolytic flux and glucose oxidation (Neely and Morgan 1974, Randle 1981, Lopaschuk *et al* 1994). Increased free fatty acid availability with the build up of acetyl-CoA and NADH will decrease PDH in its active form and lower glucose oxidation (Randle 1981). Investigations have suggested that increased carbohydrate utilisation may protect the heart during ischaemia (Runnman *et al* 1990), possibly by improving myocardial efficiency and decreasing acidosis. Thus reducing ability of the ischaemic heart to utilise glucose could result in severe tissue damage, by contributing to a decrease in the efficiency of energy coupling, lactate accumulation and the resultant potential for H^+, Na^+ and Ca^{2+} accumulation and overload as proposed by Lopaschuk *et al* (1994).

5.1.2.2. Apoptosis (programmed cell death) and necrosis.

The myocardium can also lose cells through apoptosis or necrosis. Mitochondria

appear to control the switch between apoptosis and necrosis (reviewed by Cai and Jones 1999). Apoptosis differs from necrosis in that it occurs by activating an energy-requiring molecular suicide program, while necrosis is generally regarded as an unregulated phenomenon (Wu *et al* 1997). Inappropriate initiation of apoptotic cell death may play an integral part in the pathogenesis of cardiac dysfunction related to ischaemia, reperfusion or failure. There is increasing evidence to suggest that apoptotic cells are a clear feature of heart failure in various animal models, in ischaemia / reperfusion-treated hearts, as well as in human end-stage failing hearts (reviewed by MacLellan and Schneider 1997). Programmed cell death may serve as one of the underlying mechanisms for the transition from hypertrophy to decompensated heart failure. The implication of p38 in apopotosis of cardiomyocytes provides a potential signalling pathway for such an apoptotic response (Wang *et al* 1998). Wang *et al* (1998) reported that activation of p38 MAP kinase activities during hypertrophy, and the opposing effects of hypertrophy and cell death mediated by members of the p38 MAP kinase family suggests a potential role of the p38 pathway in the onset of hypertrophy and heart failure. In addition early heart failure is characterised by elevated plasma atrial natriuretic peptide levels (Wei *et al* 1993).

5.1.2.2.1. Mitochondrial involvement in apoptosis.

An early response to apoptotic stimuli is the release of cytochrome c from the mitochondrial inter-membrane space, leading to caspase activation and the proteolytic cleavage of target proteins responsible for the rearrangements of the

cytosol, nucleus and plasma membrane that are characteristic of apoptosis (reviews by Halestrap 1999, Crompton 1999). There is a family of proteins associated with or recruited to the mitochondria that can either, inhibit cytochrome c release and protect from apoptosis (e.g. Bcl-2 and Bcl-X_L), or enhance the process and stimulate apoptosis (e.g. BAX, BAD and BID) (Li and Yuan 1999, Korsmeyer 1999). There is still controversy however over the molecular mechanism(s) of cytochrome c release (Halestrap 1999, Crompton 1999). One possibility is by transient opening of the MPTP, leading to swelling of the mitochondria sufficient to disrupt the outer membrane and release cytochrome c, but still maintaining sufficient ATP synthesis for apoptosis (Leist *et al* 1997).

5.1.5. Aims.

Cardiac hypertrophy is a short-term adaptive response to cope with increased pressure overload. Chapters 3 and 4 have helped to establish some of the early beneficial metabolic changes associated with moderate pressure-overload created in a rat model using aortic constriction. At the nine week stage of the cardiac hypertrophy pressure-overload model there is no evidence to suggest that the metabolic changes in substrate utilisation are causally related to the development of heart failure or cardiac dysfunction. However continuation of pressure-overload in this model may create further metabolic adaptations that ultimately have detrimental consequences on heart function. This chapter proposes that the metabolic alterations that occur in prolonged pressure-overload hypertrophy may play a significant role in the development of decompensated hypertrophy and

heart failure.

Identification of distinct metabolic changes that are detrimental in cardiac hypertrophy could be vital in early detection and prevention of heart failure clinically. The aim of this chapter was to address the following questions:

- Does prolonged pressure-overload result in further alterations in substrate metabolism?
- Does the heart exhibit sufficient ATP production to maintain normal mechanical function during prolonged hypertrophy?

5.2. Methods.

5.2.1. Fifteen week aortic banding.

Pressure overload was induced surgically in male Sprague-Dawley rats as previously described (Chapter 2, section 2.2). Animals were sacrificed fifteen weeks post surgery. The resulting extent of hypertrophy and anatomical changes were determined as in Chapter 3 (section 3.3.1). The profile of substrate utilisation at fifteen weeks hypertrophy was determined in isolated perfused hypertrophied and control rat hearts following 45 minutes perfusion with:

- 5mM [1-^{13}C]-glucose and 0.3mM unlabelled palmitate.
- 5mM unlabelled glucose and 0.3mM [U-^{13}C]-palmitate,
- 5mM [1-^{13}C]-glucose, unlabelled palmitate and 100μU/ml insulin,
- 5mM unlabelled glucose, 0.3mM [U-^{13}C]-palmitate and 100μU/ml insulin.

Heart function was recorded and determined as previously described (Chapter 2, section 2.3.1) in parallel with substrate perfusion. At the end of perfusion the hearts were freeze-clamped and PDH activity was determined as described in Chapter 2 (section 2.7). Acylcarnitine concentration was measured as described in Chapter 2 (section 2.6.4). PCr and ATP concentration was determined in frozen heart extracts using the assay described in Chapter 2 (section 2.6.3).

5.3. Results.

5.3.1. Characterisation of fifteen week hypertrophy.

5.3.1.1a. Morphology of hypertrophy.

Morphology relating to the degree of cardiac hypertrophy is presented in Table 5.1(a). Constriction of the abdominal aorta for fifteen weeks led to a significant 15.3% increase in heart mass ($p<0.02$), with no significant difference in body mass or tibia length compared to the control group. Aortic constriction also caused a significant 19.9% decrease ($p<0.01$) in left and 31.3% increase ($p<0.01$) in right kidney mass with respect to the control group.

Treatment Groups	Body Mass (g)	Heart Mass (g)	Tibia Length (cm)	Left Kidney Mass (g)	Right Kidney Mass (g)
Control (n=13)	617.50 (26.35)	2.55 (0.07)	4.70 (0.03)	1.91 (0.07)	1.92 (0.08)
Hypertrophy (n=12)	646.67 (23.20)	2.94* (0.11)	4.76 (0.05)	1.53* (0.10)	2.52*+ (0.16)

Table 5.1(a). Morphology in rats following fifteen weeks aortic constriction, mean +/- SEM. (* significant difference from control group, + significantly different compared to the left kidney).

Comparison between the nine week (Table 3.1a) and fifteen week hypertrophied groups revealed that the tibia continued to increase in length in both fifteen week control (4.57 versus 4.70, $p<0.01$) and hypertrophied groups (4.56 versus 4.76, $p<0.01$), indicative of continuing growth. The fifteen week hypertrophied group also showed a significant increase in body mass (580.25 +/- 8.29 versus 646.67

+/- 23.20, $p<0.02$). More importantly the extra six weeks of aortic constriction did not exacerbate the degree of hypertrophy.

5.3.1.1b. Morphology of heart failure.

Indices of heart failure were measured (Table 5.1(b)). No significant differences were observed in the lung wet to dry mass ratios, liver or adrenal wet masses between fifteen week control and hypertrophied rats, providing no evidence of heart failure in the fifteen week model. As with the nine week model, no significant differences were observed in the wet to dry mass ratios of any organs (see Appendix Table 3.1 for values). The lung wet to dry mass ratio, liver and adrenal mass did not differ between the nine and fifteen week models.

Treatment Groups	Lung Wet / Dry Ratio	Liver Mass (g)	Adrenal Mass (g)	Spleen Mass (g)
Control (n=13)	3.86 (0.04)	20.81 (1.25)	0.03 (0.00)	0.87 (0.04)
Hypertrophy (n=12)	3.84 (0.05)	20.88 (0.79)	0.03 (0.00)	1.01* (0.04)

Table 5.1(b). Absolute morphology in rats following fifteen weeks aortic constriction, mean +/- SEM. (* significantly different from control group).

5.3.1.2. Indices of Hypertrophy.

The heart mass to tibia length ratio was significantly increased (14.8%, $p<0.01$) in the hypertrophied group, whereas the right to left kidney ratio was significantly increased by 72.3% ($p<0.002$), relative to the control group. Fifteen weeks aortic

constriction resulted in an 8.8% increase in heart mass to body mass ratio (Table 5.1(c)) but did not reach 5% level of significance. Comparison between the nine and fifteen week models revealed that only the right to left kidney ratio increased significantly (1.25 versus 1.74, $p<0.02$).

Treatment Groups	Heart Mass / Body Mass ($x10^{-3}$)	Heart Mass / Tibia Length (g/cm)	Right / Left Kidney Mass Ratio
Control (n=13)	4.19 (0.15)	0.54 (0.01)	1.01 (0.01)
Hypertrophy (n=12)	4.56 (0.12)	0.62* (0.02)	1.74* (0.17)

Table 5.1(c). Indices of hypertrophy, mean +/- SEM. (* significantly different from the control group).

5.3.1.3. Blood Characteristics.

No significant differences were observed in either plasma metabolite concentrations or electrolytes between hypertrophied and control groups Table (5.1(d)). Equally none of the blood parameters changed significantly between the nine and fifteen week control and hypertrophy groups.

Treatment Groups	pH	pCO_2 mmHg	pO2 mmHg	RBC Ratio	HCO^{3-} mmol/l	K^{+} mmol/l	Na^{+} mmol/l	Ca^{2+} mmol/l	Cl^{-} mmol/l	Glucose mmol/l	Lactate mmol/l
Control (n=9)	7.2 (0.2)	23.2 (6.8)	134.4 (8.4)	0.8 (0.0)	29.8 (1.6)	4.5 (0.2)	136.9 (1.0)	1.3 (0.1)	94.2 (0.6)	9.2 (0.3)	2.7 (0.5)
Hypertrophy (n=6)	6.9 (0.2)	28.6 (6.9)	131.95 (10.1)	0.8 (0.0)	31.2 (0.5)	4.3 (0.2)	137.0 (1.5)	1.3 (0.1)	94.2 (1.28)	9.9 (0.6)	2.1 (0.2)

Table 5.1(d). Plasma characteristics after fifteen weeks aortic constriction, mean +/- SEM.

5.3.2. Substrate selection in fifteen week hypertrophied hearts.

5.3.2.1. Profile of substrate selection in hearts perfused with glucose and palmitate.

A summary of the relative contribution of glucose, palmitate and endogenous substrates to overall TCA cycle oxidation in fifteen week hypertrophied and control hearts is shown in Figure 5.1. In this preliminary study the fifteen week hypertrophied hearts showed increased utilisation of glucose, 15.0% versus 11.3 +/- 1.3% in control hearts, although this is not statistically significant due to the small numbers in each group. The individual values are 14, 10 and 10% for glucose use in control hearts and 10 and 20% for hypertrophied hearts. There is a more marked reduction in palmitate use in the fifteen week hypertrophied hearts compared to the control hearts, (63.57% +/- 2.3 versus 74.84% +/- 3.4, p=0.59), although not significant due to small sample size. The individual values are 74.8, 73.8 and 81.2% fatty acid use in control hearts and 58.9, 66.1 and 65.8% in hypertrophied hearts. This preliminary study revealed that the fifteen week hypertrophied hearts relied more heavily on endogenous substrates compared to the control hearts.

Fifteen week hypertrophied and control hearts perfused with glucose and palmitate showed a very similar profile to the nine week hypertrophied hearts perfused with the same substrate mixture. Although the relative contributions of glucose and palmitate oxidised do not alter significantly between the nine and fifteen week control groups (Table 5.2(a)), there is a small increase in endogenous

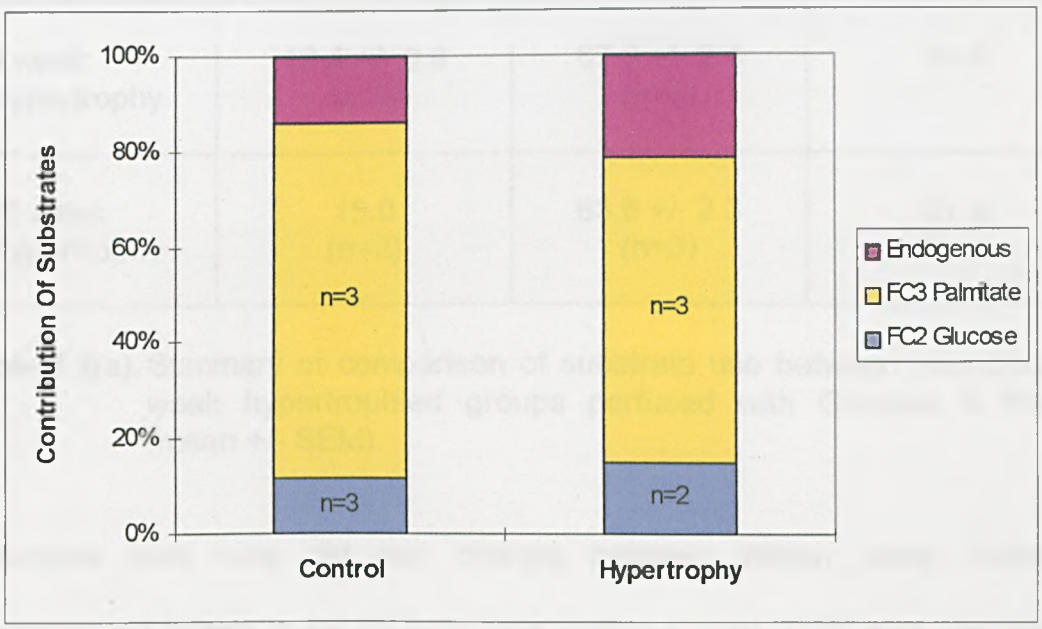

Figure 5.1. Substrate selection in hearts perfused with Glucose & Palmitate after fifteen weeks hypertrophy.

substrate use when comparing fifteen and nine week hypertrophied hearts (14.3% to 21.4%).

Treatment Groups	% Glucose use	% Fatty acid use	% Endogenous use
9 week Hypertrophy	18.4 +/- 0.8 (n=5)	67.3 +/- 2.4 (n=8)	14.3
15 week Hypertrophy	15.0 (n=2)	63.6 +/- 2.3 (n=3)	21.4

Table 5.2(a). Summary of comparison of substrate use between nine and fifteen week hypertrophied groups perfused with Glucose & Palmitate, (mean +/- SEM).

Glutamate pool size did not change between fifteen week control and hypertrophied hearts (6.65 +/- 0.20, n=5 versus 6.39+/- 0.22, n=5, μmoles/g. wet mass). This is also true of the glutamine concentrations in the fifteen week control and hypertrophied heart tissue (2.57 +/- 0.19, n=5 versus 2.86 +/- 0.24, n=5, μmoles/g. wet mass) which do not differ significantly. Therefore alterations in substrate utilisation measured using the ^{13}C NMR and TCA calculation method do not occur as a result of changes in glutamate or glutamine pool size.

5.3.2.2. Function in glucose and palmitate perfused hearts.

No significant difference was observed in heart rate, systolic pressure, diastolic pressure, left ventricular developed pressure or rate pressure product between the hypertrophied and control heart groups perfused with glucose and palmitate

following fifteen weeks aortic constriction (Table 5.2(b)).

However a comparison of function between the fifteen and nine week control and hypertrophied heart groups did reveal differences. Both groups showed significantly reduced heart rate (238.21 +/- 2.18 versus 269.89 +/- 3.28 for controls, 236.28 +/- 3.01 versus 260.02 +/- 1.50 for hypertrophied hearts, $p<0.05$), but only control hearts showed a decreased rate pressure product (21.01 +/- 0.35 versus 23.94 +/- 3.21, $p<0.05$) compared to nine week control hearts.

Treatment Groups	HR (bpm)	SP (mmHg)	DP (mmHg)	LVDP (mmHg)	RPP ($x10^3$)
Control (n=6)	238.21 (2.18)	96.47 (1.79)	8.02 (0.26)	88.45 (1.77)	21.01 (0.35)
Hypertrophy (n=5)	236.28 (3.01)	106.06 (2.34)	7.79 (0.32)	98.57 (2.14)	23.29 (0.58)

Table 5.2(b). Function in Glucose & Palmitate substrate groups after fifteen weeks hypertrophy (mean +/- SEM).

5.3.2.3. PDH enzyme activity in fifteen week control and hypertrophied hearts.

No significant differences were observed in enzyme activities between the control and hypertrophied hearts (Table 5.2(c)). There was no significant difference in percentage PDH enzyme in the active form between the hypertrophied and control heart groups (7.76% +/- 0.76 versus 6.63 % +/- 0.73 respectively, Figure 5.2).

Treatment Groups	PDHa	CSa	PDHa/ CSa	PDHt	CSt	PDHt/ CSt
Control (n=5)	0.24 (0.03)	70.69 (2.61)	0.003 (0.000)	3.58 (0.13)	71.45 (2.15)	0.050 (0.001)
Hypertrophy (n=5)	0.34 (0.04)	74.85 (3.84)	0.005 (0.001)	4.49 (0.09)	77.04 (3.38)	0.060 (0.003)

Table 5.2(c). Enzyme activities (mean +/- SEM) in hearts perfused with Glucose & Palmitate after fifteen weeks hypertrophy.

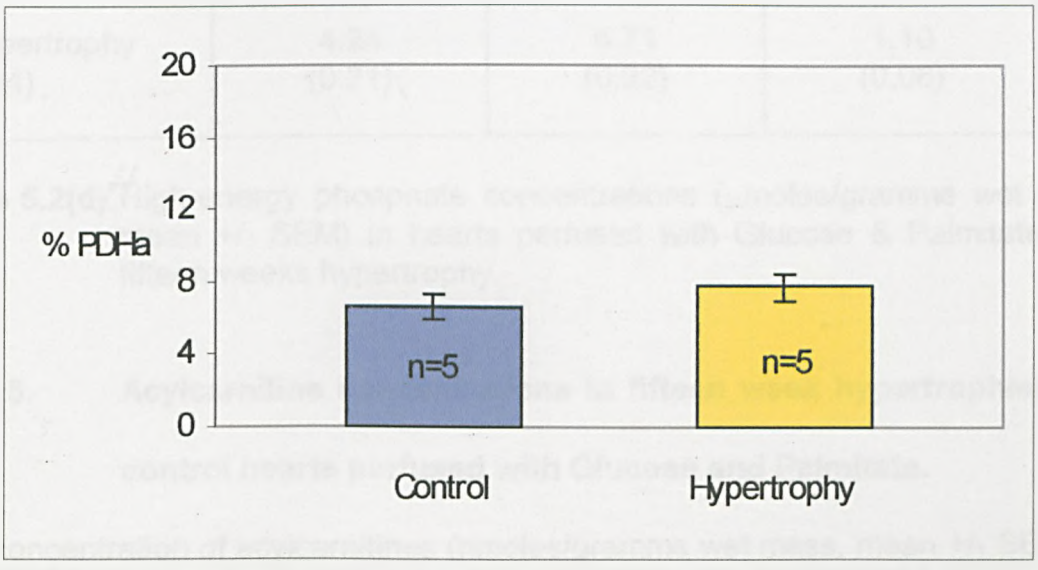

Figure 5.2. Percentage PDHa in hearts perfused with Glucose & Palmitate after fifteen weeks hypertrophy (mean +/- SEM).

A comparison between the nine and fifteen week model showed that fifteen week hypertrophied hearts had a significantly increased proportion of PDHa (0.34 +/- 0.04 versus 0.16 +/- 0.01, $p<0.05$) and PDHt (4.49 +/- 0.09 versus 3.58 +/- 0.15, $p<0.05$). Yet no difference was observed in either enzyme activity when normalised for CS activity and is therefore likely to reflect differences in extraction efficiency.

5.3.2.4. High energy phosphate status of hearts perfused with Glucose and Palmitate.

The concentration of ATP and PCr and the PCr/ATP ratio were not significantly different between control and hypertrophied groups at fifteen weeks (Table 5.2(d)).

Treatment Groups	ATP	PCr	PCr/ ATP
Control (n=6)	4.90 (0.12)	5.80 (0.38)	1.26 (0.04)
Hypertrophy (n=4)	4.24 (0.21)	4.71 (0.23)	1.10 (0.06)

Table 5.2(d). High-energy phosphate concentrations (μmoles/gramme wet mass, mean +/- SEM) in hearts perfused with Glucose & Palmitate after fifteen weeks hypertrophy.

5.3.2.5. Acylcarnitine concentrations in fifteen week hypertrophied and control hearts perfused with Glucose and Palmitate.

The concentration of acylcarnitines (nmoles/gramme wet mass, mean +/- SEM) for fifteen week hypertrophied and control hearts perfused with the substrates glucose and palmitate are shown in Table 5.2(e). The fifteen week hypertrophied hearts contained significant increased concentrations of acylcarnitines with the carbon chain length ten and eighteen compared to the control hearts ($p<0.05$).

Chain Length		Control (n=3)	Hypertrophy (n=5)
Short Chain	C2	97.33 (24.59)	84.83 (11.27)
	C3	0.36 (0.12)	0.27 (0.01)#
	C4	0.47 (0.12)	0.39 (0.01)
	C5	0.26 (0.05)	0.18 (0.01)
	C4-OH	0.30 (0.05)	0.23 (0.01)
	C6	0.33 (0.06)#	0.50 (0.07)
	C5-OH	0.75 (0.09)	0.69 (0.05)
	C8	0.14 (0.02)	0.36 (0.06)
Medium Chain	DC3	0.25 (0.06)	0.23 (0.02)
	C10	0.21 (0.02)	0.61 (0.07)*
	DC4	2.72 (0.46)#	2.33 (0.27)
	DC5	0.05 (0.03)#	0.09 (0.02)#
	C12:1	0.16 (0.01)#	0.34 (0.04)#
	C12	0.83 (0.02)	2.25 (0.29)
	C12-OH	0.17 (0.01)#	0.44 (0.08)
	C14:2	0.22 (0.01)#	0.52 (0.10)
	C14:1	0.64 (0.06)#	1.50 (0.22)
	C14	3.18 (0.15)	8.34 (1.13)
	C14:2-OH	0.09 (0.04)	0.27 (0.08)
	C14:1-OH	0.79 (0.31)	1.79 (0.49)
	C14-OH	0.31 (0.06)#	0.64 (0.11)
Long chain	C16:1	1.92 (0.22)#	3.47 (0.37)#
	C16	15.76 (1.20)#	38.55 (5.21)#
	C16:1-OH	0.53 (0.15)	1.15 (0.25)
	C16:OH	4.06 (1.25)	10.78 (3.08)
	C18:2	4.66 (0.55)#	5.53 (0.62)#
	C18:1	5.98 (0.68)#	7.05 (0.88)#
	C18	1.63 (0.13)	4.28 (0.54)*#
	C18:2-OH	0.31 (0.02)#	0.48 (0.05)#
	C18:1-OH	0.35 (0.07)	0.66 (0.12)
	C18-OH	0.10 (0.04)	0.50 (0.10)
	C20:4	0.56 (0.02)	1.05 (0.13)

Table 5.2(e). Acylcarnitine concentration in nmoles/gramme wet mass (mean +/- SEM) in hearts perfused with Glucose & Palmitate after fifteen weeks hypertrophy. (* significant difference ($p<0.05$) compared to the control hearts, # significantly different ($p<0.05$) from the nine week groups).

Fifteen week hypertrophied and control hearts showed significantly increased concentrations of many acylcarnitines in comparison to their respective nine week groups ($p<0.05$), as signified by the # symbol. Most of the increases occurred in the medium and long chain acylcarnitines.

5.3.3. Effect of insulin on substrate selection in fifteen week hypertrophied hearts.

5.3.3.1. Profile of substrate selection.

Insulin increased glucose utilisation, in both fifteen week hypertrophied (32 and 34%, mean 33.0%, n=2) and control hearts (51.0, 42.0 and 30.0%, mean 41.0 +/- 6.09, n=3, $p<0.05$) as shown in Figure 5.3. In the presence of insulin the proportion of palmitate use did not significantly alter between the fifteen week hypertrophied or control hearts. However the hypertrophied hearts showed an increased contribution from endogenous substrates compared to the control hearts.

Comparison between substrate groups revealed that the presence of insulin resulted in a significant 22% decrease in palmitate use in control hearts (52.5 +/- 4.5 versus 74.8 +/- 3.4, $p<0.05$), but not in hypertrophied hearts (46.8 +/- 6.5 versus 63.6 +/- 2.3, $p=0.08$). Glutamate pool size was unchanged between fifteeen week control and hypertrophied hearts with insulin (5.90 +/- 0.6, n=5 versus 6.20 +/- 0.2, n=5, μmoles/g. wet mass). Glutamine pool sizes in the fifteen week control and hypertrophied hearts perfused with insulin were also comparable (2.62 +/- 0.2, n=5 versus 2.95 +/- 0.1, n=5, μmoles/g. wet mass).

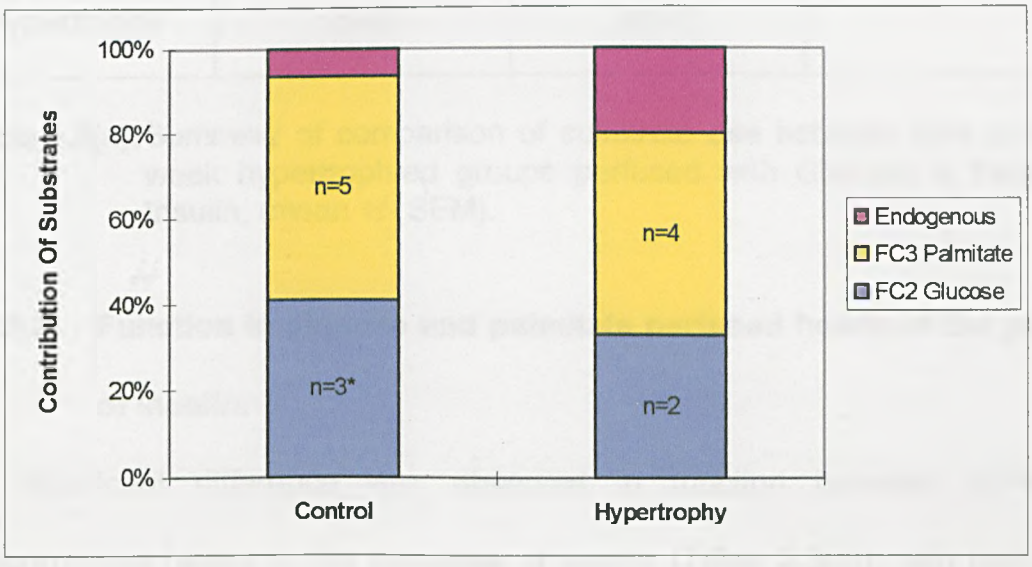

Figure 5.3. Substrate selection in hearts perfused with Glucose & Palmitate & Insulin after fifteen weeks hypertrophy. (* significantly different compared to absence of insulin)

Extending the duration of hypertrophy (Table 5.3(a)) did not modify the profile of substrate oxidation.

Treatment Groups	% Glucose use	% Fatty acid use	% Endogenous use
9 week Hypertrophy	36.0 +/- 4.2 (n=4)	47.2 +/- 2.2 (n=4)	16.8
15 week Hypertrophy	33.0 (n=2)	46.8 +/- 6.5 (n=4)	20.2

Table 5.3(a). Summary of comparison of substrate use between nine and fifteen week hypertrophied groups perfused with Glucose & Palmitate & Insulin, (mean +/- SEM).

5.3.3.2. Function in glucose and palmitate perfused hearts in the presence of insulin.

No significant difference was observed in function between control and hypertrophied hearts in the presence of insulin (Table 5.3(a)), with both groups showing comparable function to that seen in the nine week model.

Treatment Groups	HR (bpm)	SP (mmHg)	DP (mmHg)	LVDP (mmHg)	RPP ($x10^3$)
Control (n=10)	267.66 (8.24)	100.80 (3.72)	7.60 (0.24)	92.97 (3.72)	24.47 (0.86)
Hypertrophy (n=12)	265.58 (5.22)	105.53 (3.64)	7.65 (0.24)	97.88 (3.62)	25.83 (0.98)

Table 5.3(b). Function in Glucose & Palmitate & Insulin substrate groups after fifteen weeks hypertrophy (mean +/- SEM).

5.3.3.3. PDH enzyme activity in fifteen week control and hypertrophied hearts perfused with Glucose, Palmitate and Insulin.

No significant difference was observed in any of the individual PDH and CS enzyme activities or their ratios, between the control and hypertrophied heart groups (Table 5.3(b)). Although the percentage active PDH, [(PDHa/CSa)/(PDHt/CSt)] value is higher in the hypertrophied heart group, it was not significantly different from the control group (Figure 5.4).

Insulin significantly increased the percentage of PDH in the active form in hypertrophied hearts (13.57 +/- 0.95 versus 7.76 +/-0.76, $p<0.05$) and in control hearts (11.90 +/-0.84 versus 6.61 +/- 0.74, $p<0.05$). Comparison between the nine and fifteen week models perfused with insulin revealed no differences in enzyme activities between either the control groups or hypertrophied groups.

Treatment Groups	PDHa	CSa	PDHa/ CSa	PDHt	CSt	PDHt/ CSt
Control (n=6)	0.36 (0.01)	69.85 (1.00)	0.005 (0.000)	3.76 (0.13)	83.31 (1.97)	0.045 (0.002)
Hypertrophy (n=6)	0.48 (0.03)	77.53 (1.60)	0.006 (0.000)	4.03 (0.15)	85.57 (1.64)	0.047 (0.002)

Table 5.3(c). Enzyme activities (μmoles/min/gramme wet mass, mean +/- SEM) in hearts perfused with Glucose & Palmitate & Insulin after fifteen weeks hypertrophy.

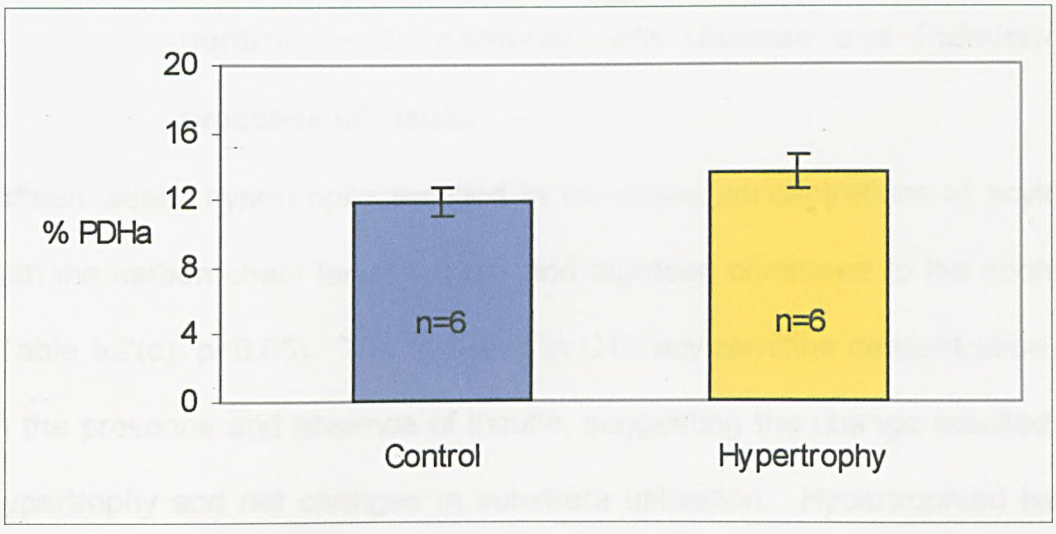

Figure 5.4. Percentage PDH enzyme in the active form in hearts perfused with Glucose & Palmitate & Insulin after fifteen weeks hypertrophy.

5.3.3.4. High energy phosphate status of hearts perfused with Glucose and Palmitate in the presence of insulin.

The cardiac concentrations of ATP and PCr did not alter significantly between the control and hypertrophied hearts perfused with glucose, palmitate and insulin (Table 5.3(d)).

Treatment Groups	ATP	PCr	PCr/ ATP
Control (n=7)	4.41 (0.10)	6.16 (0.16)	1.40 (0.03)
Hypertrophy (n=5)	4.63 (0.07)	6.20 (0.17)	1.35 (0.07)

Table 5.3(d). High-energy phosphate concentrations (μmoles/gramme wet mass) in hearts perfused with Glucose & Palmitate & Insulin after fifteen weeks hypertrophy.

5.3.3.5. Acylcarnitine concentrations in fifteen week hypertrophied and control hearts perfused with Glucose and Palmitate in the presence of insulin.

Fifteen weeks hypertrophy resulted in increased concentrations of acylcarnitines with the carbon chain lengths three and eighteen compared to the control hearts (Table 5.2(d), $p<0.05$). The increase in C18 acylcarnitine concentration occurred in the presence and absence of insulin, suggesting the change resulted from the hypertrophy and not changes in substrate utilisation. Hypertrophied hearts also showed a significant increase in C4-OH acylcarnitine concentration (0.36 +/-0.02 versus 0.23 +/-0.01, $p<0.05$) in the presence of insulin. This increase may result from extended periods of hypertrophy.

Chain Length		Control (n=4)	Hypertrophy (n=4)
Short Chain	C2	88.18 (34.01)	170.61 (23.89)
	C3	0.19 (0.01)	0.45 (0.03)*
	C4	0.53 (0.09)	0.55 (0.04)
	C5	0.19 (0.02)	0.18 (0.02)
	C4-OH	0.33 (0.08)	0.36 (0.02)
	C6	0.70 (0.21)	0.81 (0.08)
	C5-OH	0.97 (0.16)	0.89 (0.03)
	C8	0.26 (0.07)	0.26 (0.02)
Medium Chain	DC3	0.23 (0.02)	0.32 (0.02)
	C10	0.71 (0.29)	0.46 (0.01)
	DC4	3.74 (0.42)	4.07 (0.58)
	DC5	0.11 (0.04)	0.15 (0.02)
	C12:1	0.42 (0.16)	0.23 (0.02)
	C12	3.14 (1.43)	1.35 (0.06)
	C12-OH	0.68 (0.34)	0.36 (0.05)
	C14:2	0.57 (0.24)	0.31 (0.02)
	C14:1	1.26 (0.39)	0.94 (0.07)
	C14	4.25 (0.97)	5.42 (0.28)
	C14:2-OH	0.27 (0.10)	0.27 (0.04)
	C14:1-OH	0.80 (0.19)	1.13 (0.27)
	C14-OH	0.78 (0.24)	0.68 (0.07)
Long chain	C16:1	2.23 (0.36)	2.66 (0.21)
	C16	17.97 (2.67)	24.53 (1.65)
	C16:1-OH	0.57 (0.10)	0.70 (0.12)
	C16:OH	5.27 (1.68)	7.27 (1.52)
	C18:2	3.27 (0.61)	4.07 (0.54)
	C18:1	3.91 (0.64)	5.34 (0.62)
	C18	1.86 (0.26)	3.07 (0.13)*
	C18:2-OH	0.33 (0.08)	0.39 (0.03)
	C18:1-OH	0.53 (0.10)	0.55 (0.06)
	C18-OH	0.31 (0.06)	0.50 (0.08)
	C20:4	0.69 (0.09)	0.86 (0.05)

Table 5.3(d). Acylcarnitine concentration in nmoles/gramme wet mass (+/-SEM) in hearts perfused with Glucose & Palmitate & Insulin after fifteen weeks hypertrophy. (* significant difference ($p<0.05$) compared to the fifteen week control hearts).

5.4. Discussion.

5.4.1. Substrate use in prolonged pressure-overload hypertrophy.

5.4.1.1. Glucose utilisation.

Analysis of preliminary ^{13}C NMR spectra obtained from hearts perfused with [1-^{13}C]-glucose and palmitate revealed that fifteen week hypertrophied hearts utilised more glucose than the control hearts (15.0 versus 11.3% respectively, Figure 5.1). The substrate profile for glucose use in fifteen week hypertrophied hearts was similar to that obtained in the nine week hypertrophied hearts. This indicates a continued greater dependence on glucose oxidation for the provision of ATP, in the hypertrophied hearts. The present study compares well with those showing increased rates of glucose utilisation in hypertensive hearts (Christe and Rodgers 1994, Yonekura *et al* 1985) and in left ventricular hypertrophy (Kagaya *et al* 1990. Massie *et al* 1995), alongside an enhanced capacity for glycolysis (Seymour *et al* 1990).

Increased reliance on glucose utilisation in the hypertrophied heart could result from increased glucose uptake, as shown in SHR hearts (Yonekura *et al* 1985, Leipala *et al* 1989) and in pressure-overloaded dog hearts using 2DGP accumulation (Zhang *et al* 1995). Alternatively it could result in response to re-expression of a foetal phenotype, in which the concentration of several glycolytic enzymes are reportedly increased in the hypertrophied myocardium (Bishop and Altschuld 1970, Taegtmeyer and Overturf 1988). Indeed an increase in glycolytic capacity has been shown to accompany chronic pressure-overload even in the absence of left ventricular hypertrophy (Taegtmeyer and Overturf 1988).

The trend of increased reliance on relative glucose utilisation during fifteen weeks hypertrophy was not as great as that seen in the nine week hypertrophied model. Glucose transport across the sarcolemma in myocytes is mediated by specific glucose transporters GLUT 1 and GLUT 4 (Kraegen *et al* 1993). The GLUT 1 isoform is normally located in the sarcolemmal membrane and thought to be involved in glucose transport in the basal metabolic state (Slot *et al* 1991). However there is no evidence to suggest impaired GLUT-1 at this stage of hypertrophy. In support of this a study in the hypertensive rat heart reported no changes in GLUT-1 mRNA content (Paternostro *et al* 1995).

5.4.1.2. Insulin sensitivity.

Insulin increased the proportion of glucose utilisation in both fifteen week hypertrophied and control hearts, in this preliminary study, giving no indication of insulin resistance. Following insulin stimulation, translocation of GLUT-4 from an intracellular pool to the sarcolemmal membrane (Slot *et al* 1991, Barnard 1992) accelerates glucose transport. The major fate of the additional glucose taken up in response to insulin in the heart is glucose oxidation, rather than glycogen synthesis (Holness and Sugden 1991).

Insulin resistance has been shown to be a feature of the hypertrophied heart in both patients (Paternostro *et al* 1999) as well as animal models (Verma *et al* 1999, Paternostro *et al* 1995, Katz *et al* 1995), in which the response of glucose uptake to insulin in the hypertrophied human heart is reduced (Paternostro *et al* 1999). It has been explained in part by altered expression of glucose transporters,

in which decreases of the Glut 4 / Glut 1 ratio have been measured (Paternostro *et al* 1995, Weinberg *et al* 1995, Depre *et al* 1998). Sun *et al* (1994) have shown that myocardial ischaemia also causes translocation of GLUT-4 to the plasma membrane of cardiac myocytes leading to increased glucose uptake. Therefore a down-regulation of GLUT-4 expression as reported in some hypertrophied studies could limit glucose availability under conditions of hypoxia and contribute to myocardial dysfunction.

Extending the duration of pressure-overload hypertrophy for a further six weeks in the present model did not modify the profile of substrate oxidation in relation to insulin sensitivity. The similarities of relative glucose utilisation between the hypertrophied and control hearts in the present study suggest that there is no limit in the delivery of glucose and no insulin resistance. Thus insulin resistance may be a feature of decompensated hypertrophy and heart failure and not compensated hypertrophy. Assumptions concerning glucose transport itself study in regards to the rate of uptake or ratio of GLUT 4 / GLUT 1 cannot be made in the present study.

5.4.1.3. Fatty acid utilisation.

Interestingly the fifteen week hypertrophied hearts showed a reduction in palmitate use compared to control hearts, in this prelininary study (63.6% versus 74.8% respectively, Figure 5.1), although not significant due to small sample numbers. This study suggests that the profile of substrate utilisation in prolonged pressure-overload hypertrophy switches away from fatty acid utilisation towards increased

reliance on glucose and endogenous use. Experimental studies on the heart in the compensated phase of hypertrophy have also shown a reduction in the oxidation of long chain fatty acids in hearts (Cheikh *et al* 1994) and quiescent hypertrophied myocytes (Torielli *et al* 1995). Other studies on hypertrophied rat hearts have shown that the depressed rate of fatty acid oxidation (Schonekess *et al* 1995) is partially offset by increased glycolytic rates (Allard *et al* 1994). In addition switches in myocardial substrate utilisation towards glucose and away from fatty acids has been demonstrated in hypertensive rats (Christie and Rodgers 1994, From *et al* 1990). The presence of insulin did not further modify the proportion of palmitate use in hypertrophy, but resulted in a 22% decrease in the control hearts. The lack of effect of insulin in the hypertrophied hearts could be due to the fact that the contribution of palmitate to TCA cycle ATP production was already lowered in the hypertrophied hearts.

A trend for a continuing decrease in long-chain fatty acid use, as pressure-overload hypertrophy progresses is highlighted when palmitate utilisation is compared between the fifteen and nine week models (Table 5.2(a)). The fact that no decrease in fatty acid oxidation was observed in the nine week model but is more apparent in the fifteen week model indicates that alterations in fatty acid use occur at a late stage in the development of hypertrophy. This provides evidence that sequential metabolic adaptations arise during cardiac hypertrophy. Decreased fatty acid oxidation may therefore play a key role in the transition from compensated hypertrophy to decompensated hypertrophy and heart failure, where abnormalities in fatty acid metabolism have been demonstrated in more severe

models of hypertrophy. Cardiac failure secondary to chronic pressure-overload has shown to result in defective long-chain fatty acid metabolism in guinea pig hearts (Wittels and Spann 1968), while chronic volume-overload induces a reduction in long-chain fatty acid metabolic capacity in the rat heart (El-Alaoui-Talibi 1992).

In support of this, evidence is emerging that during the development of heart failure, the proportion of myocardial energy derived from fatty acid oxidation decreases. Sack *et al* (1996) has identified a possible gene regulatory pathway involved in this metabolic switch, in which fatty acid enzyme expression is down regulated at the mRNA levels in failing left ventricle. Examination of the enzymes catalysing the first (medium-chain acyl-CoA dehydrogenase, MCAD) and third (long-chain acyl-CoA dehydrogenase, LCAD) steps in the fatty acid oxidation cycle of β-oxidation of fatty acids, showed that MCAD mRNA levels were down-regulated in both pressure-overload hypertrophy and heart failure in rats (Sack *et al* 1996). However the enzyme activities and protein levels did not decrease significantly until heart failure occurred. This may account for why other investigators have reported that the activity of β-oxidation enzymes is variably affected (Smith *et al* 1990, Taegtmeyer and Overturf 1988).

Alternatively the contribution of palmitate to TCA cycle oxidation in the fifteen week hypertrophied hearts may continue to decrease due to a depletion of carnitine, an essential co-factor for long-chain fatty acid oxidation. An alteration in fatty acid oxidative capacity of the heart has often been associated with a decrease in tissue carnitine content (Sievers *et al* 1983, Reibel *et al* 1983, El

Alaoui-Talibi *et al* 1992). Tissue carnitine levels were measurably decreased in the present nine week hypertrophied heart model (Hornby 1999, personal communication). In a rat model of pressure-overload hypertrophy a deficiency of myocardial carnitine content was directly correlated to the extent of cardiac hypertrophy (Yang *et al* 1992). Although tissue carnitine levels were not measured in the fifteen week hypertrophied hearts, if tissue carnitine levels continued to decline throughout the prolonged more severe hypertrophy it could explain why fatty acid oxidation has decreased even further. However decreased fatty acid oxidation, in the presence of depressed carnitine content, has been shown to be corrected when fatty acid levels are elevated (reviewed by Lopaschuk *et al* 1994). They suggest that increased supply of exogenous fatty acids may overcome the decrease in CPTI activity occurring secondary to the decrease in carnitine levels. It has thus been argued that the enzymes involved in β-oxidation are not altered in the hypertrophied heart and that the decreased carnitine content may only play a role in decreasing fatty acid oxidation to overall ATP production, at low concentrations. The JVS mouse model of carnitine deficiency is associated with mitochondrial abnormalities in muscle (Miyagawa *et al* 1995) and a disordered expression of CPTI (Uenaka *et al* 1996) and CPTII (Hotta *et al* 1996).

5.4.1.4. Endogenous substrate use.

Fifteen week hypertrophied hearts showed a dramatic increase in endogenous substrate utilisation compared to control heart in both the presence and absence of insulin (20.2% versus 6.48%, Figure 5.3 and 21.4% versus 13.9%, Figure 5.1

respectively). Increased endogenous substrate contribution was also apparent at fifteen week compared to the nine week hypertrophied hearts endogenous use (21.4% versus 14.3%, Table 5.2(a); 20.2 versus 16.8%, Table 5.3(a) for insulin). Therefore during the progression of hypertrophy the hearts relies more heavily on endogenous substrate metabolism to compensate for any decrease in relative fatty acid use.

A likely endogenous substrate contributing to this increase is the utilisation of glycogen. Previous investigators report that the concentrations of several glycolytic enzymes are increased in the hypertrophied myocardium (Wittels and Spann 1968, Bishop and Altschuld 1970). PFK a major rate limiting enzyme in the glycolytic pathway is also activated by β- and α-adrenergic receptor stimulation (Patten *et al* 1982). An increase in anaerobic production of energy would tend to suggest reduced oxygen availability and possible involvement of hypoxia and ischaemia, in which the myocyte is attempting to maintain ATP levels. Studies have shown that during the development of heart failure, the heart also reverts to reliance on glycolysis as the primary pathway for energy production due to a re-expression of foetal energy metabolism (Taegtmeyer 1994, Wittels and Span 1968, Bishop and Altschuld 1970). This substrate switch from fatty acid utilisation to glycolysis has also been observed in the SHR heart (Christe and Rodgers 1994) and patients with idiopathic dilated cardiomyopathy (Feinendegen *et al* 1995). The present study however is only relevant to the oxidative use of glycogen and not the anaerobic use.

5.4.1.5. PDH activity.

Examination of the percentage of PDH enzyme in the active form revealed comparable levels between the fifteen week hypertrophied hearts and control hearts (7.8% versus 6.6%, Figure 5.2). The presence of insulin significantly increased the proportion of PDH in the active form in both hypertrophied and control hearts, although no difference was seen within the substrate group (13.57 versus 11.9, Figure 5.4). Thus presence of long-chain fatty acid inhibited the PDH enzyme activity in the fifteen week model as in the nine week model, in which the products of fatty acid β-oxidation (acetyl-CoA and NADH) would inhibit the PDH complex (Neely and Morgan 1974, Randle 1981). Prolonging the pressure-overload study did not however change the proportions of PDHa in either the hypertrophied or control hearts. This does not necessarily mean that flux through the PDH enzyme does not alter however, as explained in Chapter 4. The fact that the levels of %PDHa do not appreciably alter after fifteen weeks of hypertrophy indicates that there is no major ischemic injury. In ischaemia elevated levels of free fatty acids have been shown to reduce the percentage of PDH in the active form (Behal *et al* 1993). However these measurements were made on homogenised extracts of whole heart, therefore small areas of ischaemia may be present, but at a level not yet detectable by the assay technique.

Decreased flux through PDH enzyme, secondary to a decrease in fatty acid oxidation and more severe depletion of carnitine tissue levels. If carnitine levels continue to decrease as hypertrophy progresses, the entry of fatty acids into the mitochondria for oxidation will be inhibited. This would result in an accumulation

of fatty acyl molecules and sequestration of available CoA. A decrease in available CoA increases the acetyl-CoA to CoA ratio, which is an important regulator of PDH activity and subsequently glucose oxidation. In support of this marked decreases in the proportion of PDH in the active form have been reported in compensated hypertrophied hearts (Seymour and Chatham 1997).

5.4.2. Morphological indicators of heart failure

5.4.2.1. Model of hypertrophy after fifteen weeks aortic constriction.

Prolonged abdominal aortic constriction for fifteen weeks resulted in a significant 15% increase in heart mass to tibia mass ratio compared to control rats (Table 5.1(c)). Although this index of hypertrophy has slightly increased in comparison to the nine week model, it still represents a moderate degree of cardiac hypertrophy. However when right to left kidney ratios are considered, a more severe pressure overload was achieved by fifteen weeks aortic constriction compared to that at nine weeks, where the right to left kidney ratio differed by 50% in the fifteen week model compared to a 25% difference at nine weeks. Body mass and tibia length continued to increase in both the fifteen week hypertrophied and control rats compared to the nine week model, indicating that prolonged hypertrophy did not exert any detrimental effects on animal growth.

Despite achievement of increased pressure-overload in the fifteen week model no evidence was found to suggest pulmonary hypertension or hepatic congestion had occurred (Table 5.1(b)). Analysis of the blood characteristics revealed no differences between the fifteen week hypertrophied and control rats.

Likewise the blood characteristics did not alter between the nine and fifteen week models (Table 5.1(d)). Thus prolonged aortic constriction for fifteen weeks resulted in a moderately compensated non-failing hypertrophied heart, but slightly more severe than the nine week model. The fifteen week hypertrophied hearts therefore presented a valid model for the examination of further metabolic alterations that may result during prolonged pressure-overload.

5.4.2.2. Mechanical function.

The alterations in substrate use described during fifteen week hypertrophy and control hearts were not reflected in the mechanical function. Functional parameters did not differ between the fifteen week hypertrophied and control hearts (Table 5.2(b)). Equally the presence of insulin did not reveal any alterations in functional parameters (Table 5.3(b)). This indicates that neither substrate or oxygen delivery was limited at this level of hypertrophy to a great enough extent to impair contractility. In contrast the capacity of the heart to respond to a hemodynamic stress was found to be impaired in a rat pressure-overload model exhibiting reduced carnitine content (Micheletti *et al* 1994).

Comparison between the nine and fifteen week models did reveal differences. Both the fifteen week hypertrophied and control hearts exhibited significantly reduced heart rates compared to their respective nine week counterparts. This suggests an ageing factor is likely to be the cause. However, no different in function was observed between nine week and fifteen week hypertrophied and control hearts when insulin was supplied in the perfusion

media. This would suggest that improving glucose substrate use during ageing helps maintain normal heart function since glucose oxidation provides more ATP per molecule of oxygen consumed than other substrates (Taegtmeyer 1994).

Considering that alterations in substrate use have been observed but that heart function is not compromised at this level of hypertrophy suggests that a decrease in fatty acid oxidation is a primary event in the transition from compensated hypertrophy to heart failure, rather than a secondary phenomenon.

5.4.3. Metabolic basis of heart failure.

5.4.3.2. High-energy phosphates.

No differences were observed in the high-energy metabolite ATP and PCr concentrations or the PCr/ATP ratios between the fifteen week hypertrophied and control hearts (Table 5.2(d)). This was regardless of whether insulin was present of not in the perfusion medium (Table 5.3(d)). This implies that there is sufficient substrate availablity and no ischaemia at fifteen weeks. Unfortunately these parameters were determined from PCA extracts that had been frozen and stored at –70°C, unlike the analysis from non-frozen PCA extracts in the nine week study, therefore no direct comparison of high-energy phosphate levels can be made between the two models. Low PCr levels have been reported in moderate pressure-overload hypertrophy in the rat (Bhutta *et al* 1995), porcine (Massie *et al* 1995) and canine model (Zhang *et al* 1993), using ^{31}P NMR detection, resulting in decreased PCr/ATP ratios. This has generally been attributed to depleted creatine transport and handling in animal models of cardiac hypertrophy (Seppet

et al 1985) and SHR hearts (Ingwall *et al* 1990) and diseased human myocardium (Ingwall *et al* 1985). Uptake of creatine by the heart under normal physiological conditions is by active Na^+-linked transport (Loike *et al* 1986).

The importance of PCr and creatine in cardiac energy transduction itself has come under scrutiny. Mekhfi *et al* (1990) have shown that creatine depletion in the rat was associated with a change in the isozyme expression of myosin V_1 to the slower V_2 and V_3 forms, global cardiac hypertrophy and a slowing of cross-bridge cycling. It was thought the heart was demonstrating adaptations to improve the economy of cardiac contraction. However in contrast Moerland *et al* (1990) found no change in ventricular myosin expression in mice fed on β-guanidinopropionic acid, a competitive inhibitor of creatine transport.

Much controversy exists concerning energy status during the various stages of hypertrophy. Some investigators report that myocardial high-energy phosphate levels (PCr and ATP) are decreased in the initial stages of hypertrophy (Meerson *et al* 1972, Shimamoto *et al* 1982, Peyton *et al* 1982), return to normal levels during stable hypertrophy and again decrease during the progression to congestive heart failure, whereas, there are some investigators (Cooper *et al* 1973, Buser *et al* 1990, Wexler *et al* 1988, Stephens *et al* 1979, Bittl *et al* 1987) who report no change during any stage of hypertrophy. The present study would support the latter observations, in which no changes have been observed at either the nine or fifteen week stages of hypertrophy.

5.4.3.3. Acylcarnitine content.

Analysis of acylcarnitine tissue concentrations revealed that acylcarnitines of carbon lengths ten and eighteen had significantly increased by fifteen weeks of hypertrophy (Table 5.2(e)). Likewise in the presence of insulin the fifteen week hypertrophied hearts exhibited enhanced levels of acylcarnitines three and eighteen carbons in length (Table 5.3(e)). An increase in acylcarnitine concentration is indicative of impaired fatty acid oxidation and supports the preliminary ^{13}C NMR data showing a reduction in the proprtion of fatty acid oxidised during fifteen weeks hypertrophy. Acyl-CoAs and acylcarnitine levels have been shown to increase proportionally in both ishaemic and hypoxic rat hearts (Whitmer *et al* 1978). Increases in long-chain acylcarnitine concentration are attributed to the inhibition of CPTII (Hulsmann *et al* 1991, Chiodi *et al* 1992). The accumulation of acylcarnitines and the trend of a reduction in the proportion of fatty acid utilised, suggest that during fifteen weeks hypertrophy, the hearts are beginning to show alterations in carnitine transport and CPTII isoform expression.

An accumulation of acylcarnitines and reduced fatty acid oxidation may eventually produce substrate limitation at the level of the mitochondria and lead to the development of decompensated hypertrophy. Elevated acylcarnitines may eventually sequester available CoA and alter the ratio of acetyl-CoA to CoA ratio. This would have a negative effect on PDH activation. In fitting with the glucose-fatty acid cycle (Randle *et al* 1963), inhibition of PDH results in increased citrate concentrations, which lead to inhibition of PFK and the accumulation of G-6-P. Subsequently increased G-6-P levels would inhibit hexokinase and decrease

glucose uptake by the heart cell.

5.4.5. Summary.

- This preliminary study indicates that sequential metabolic alterations arise during the development of pressure-overload hypertrophy.
- Preliminary data suggests that during prolonged pressure-overload, hypertrophied hearts exhibit a switch in substrate preference as evidenced by a trend for reduced fatty acid oxidation alongside enhanced reliance on glucose and endogenous substrates.
- Modifications of substrate utilisation in cardiac hypertrophy arise independently of changes in heart function, indicating that reduced fatty acid oxidation may be a contributory factor in the transition of compensated hypertrophy to heart failure.

Chapter 6:

Substrate utilisation in pressure-overload cardiac hypertrophy at increased workload.

6.1. Introduction.

Cardiac hypertrophy is associated with impaired contractile function and an enhanced susceptibility to ischaemic injury (Schaper *et al* 1978, Gaasch *et al* 1990, Anderson *et al* 1990). Chapter 5 highlighted a number of basal metabolic alterations that arise during prolonged pressure-overload hypertrophy. Myocardial relaxation and ventricular filling require adequate energy supplies, while the integrity of the tissue itself has to be maintained. Many investigators have implicated alterations in energy metabolism in contributing to the depression in contractile function (Bishop and Altschuld 1970, Ingwall 1984, Cunningham *et al* 1990, El Alaoui-Talibi 1992, Allard *et al* 1994, Taegtmeyer and Overturf 1988). This may help explain why diastolic properties of the heart can be altered in a number of cardiac disorders including left ventricular hypertrophy, where abnormal left ventricular relaxation and filling may precede the onset of decreased heart function (reviewed by Smith and Zile 1992). In support of this Jacob *et al* (1983) reports that only hypertrophied hearts with sustained haemodynamic load are prone to fail.

6.1.1. Bioenergetics during hypertrophy and heart failure.

The bioenergetics of several rodent models of congestive heart failure has been studied. These include the cardiomyopathic Syrian hamster (Wikman-Coffelt *et al* 1986, Nascimben *et al* 1995), the spontaneous hypertensive and heart failure rat (SHHF) (O'Donnell *et al* 1998, Sack and Kelly 1998) and the spontaneously hypertensive rat (Pfeffer *et al* 1982). In the cardiomyopathic Syrian hamster

(Markiewicz *et al* 1986) and SHHF rat (O'Donnell *et al* 1998), the failing hearts have been found to contain reduced PCr and increased in Pi concentrations. However ATP levels in many animal models of congestive heart failure have been reported as unchanged or slightly depressed (Scheuer 1993, O'Donnell *et al* 1998, Pool *et al* 1967, Coleman *et al* 1971, Wexler *et al* 1988).

6.1.2. Reduced oxygen delivery in cardiac hypertrophy.

Myocardial blood flow in the hypertrophied heart has been shown to be generally normal in the basal state and rises considerably with increased cardiac workload (Bache *et al* 1981, Dellsperger and Marcus 1990). It is thought that a combination of factors regulates coronary flow (Haddy and Scott 1975). Included is adenosine, a potent vasodilator, in which the production of adenosine by the myocardial cell appears to parallel oxygen consumption (Berne 1980). Oxygen itself is also thought to be a mediator of coronary flow (Laird 1983). However tissue oxygen tension is not uniform even in normal myocardium (Shubert *et al* 1978). In addition reduced capillary density and increased oxygen diffusion distances are associated with the increase in muscle mass of hypertrophied hearts (Anversa *et al* 1990), thought to indicate a predisposition to ischaemia (Bache *et al* 1981). Oxygen requirements per gram myocardium themselves undergo an increase during exercise in the pressure-overloaded heart than in normal heart (Bache and Dai 1990). However, controversy still exists concerning whether impaired coronary reserve associated with hypertrophy can be equated to myocardial ischaemia.

6.1.3. Diastolic dysfunction in cardiac hypertrophy.

Left ventricular hypertrophy development in response to pressure-overload is thought to be a beneficial adaptation in order to normalise peak systolic wall stress (Shapiro and McKenna 1984). Pressure-overload myocardial hypertrophy is however associated with changes in myocardial contractility that includes diastolic dysfunction and impairment of relaxation (Capasso *et al* 1981, Taegtmeyer and Overturf 1988, Micheletti *et al* 1994). This increased duration of contraction and relaxation is paralled by a shift in myosin isoenzymes from a faster (V_1) to a slower (V_3) enzymatic form (Cappelli *et al* 1989, Mercardier *et al* 1981). However the mechanisms responsible for these changes in contractile function are not yet fully understood. These changes may occur to either complement or counteract the changes in Ca^{2+} uptake by the sarcoplasmic reticulum.

6.1.3.1. Altered calcium homeostasis in cardiac hypertrophy.

The functional properties of the sarcoplasmic reticulum (SR) are central to the excitation-contraction coupling of cardiac muscle. In the heart the SR plays a crucial role in the regulation of intracellular Ca^{2+} through its ability to sequester calcium. The entry of extracellular Ca^{2+} via Ca^{2+} channels is necessary to trigger Ca^{2+} release from the SR, leading to tension development by the myocardium, whereas Ca^{2+} uptake by the SR results in muscle relaxation (Fabiato 1983). The important proteins in the SR membrane, regulating Ca^{2+} uptake are the Na+/Ca^{2+} exchanger, Ca^{2+}-ATPase and PLB. Relaxation is achieved through the combined effects of sarcolemmal extrusion via the Na^{+}/Ca^{2+} exchange (Struder *et al* 1994)

and SR sequestration via the SR Ca^{2+}-ATPase (Boheler 1992); the function of which is regulated by the SR spanning protein phospolamban and its phosphorylation state (James *et al* 1989, Kiss *et al* 1995). In cardiac hypertrophy and failure, in both human and animal models, decreased SR Ca^{2+}-ATPase mRNA and protein levels have been observed (Boateng *et al* 1997, de la Bastie 1990, Mercadier *et al* 1990, Feldman *et al* 1991). Similarly decreased PLB mRNA expression levels have been shown in pressure-overloaded and failing rabbit hearts (Feldman *et al* 1993) and in end-stage human heart failure (Mercadier *et al* 1990, Feldman *et al* 1991).

A reduction in Ca^{2+} transport by the SR has been reported in experimental models of cardiac hypertrophy induced by mechanical overload and the transport capacity is further decreased in the failing heart (Suko *et al* 1970, Ito *et al* 1974, Dhalla *et al* 1978, Lamers and Stinis 1979 de la Bastie *et al* 1990). Alterations in SR function have also been reported in human heart failure (Hasenfuss *et al* 1994, Movsesian *et al* 1994, Schwinger *et al* 1995).

It is possible that changes in calcium handling in the hypertrophied heart and their detrimental effects on mechanical function eventually contribute to the deterioration in heart function during decompensated hypertrophy and heart failure. In support of this the prolongation of the time course of contraction and relaxation in pressure-overloaded hypertrophied myocardium correlates with a similar prolongation of the Ca^{2+} transient (Bentivegna *et al* 1991). However the relationship may not be quite that simple, in which Limas *et al* (1980), reported increased rather than decreased Ca^{2+} transport in mild pressure-overload

hypertrophy.

6.1.3.2. Diastolic stiffness and collagen deposition in hypertrophy.

Cardiac dysfunction may be caused by the development of fibrosis, alterations of the structure and composition of the extracellular matrix and by degenerative changes in myocytes (Anversa *et al* 1986, 1990, Capasso *et al* 1990, Olivetti *et al* 1991, Weber *et al* 1993). Previous studies of hypertrophy have demonstrated that it is not merely the accumulation of collagen but also the configuration, location and alignment of fibrillar collagen with respect to cardiac muscle that can determine myocardial stiffness (Weber *et al* 1993). Bing *et al* (1978) found that inhibition of collagen cross-linking prevented the abnormal rise in resting tension observed in isolated cardiac muscle strips from hypertrophied left ventricle. This illustrates that structure and contractile function of the heart cannot be dissociated. It reinforces the importance of correlating results from functional and metabolic studies with work investigating collagen orientation in cardiac hypertrophy.

6.1.3.3. Myocardial damage resulting from hypoxia / ischaemia.

The incidence of sudden death after myocardial infarction is significantly higher in patients with hypertension and chronic left ventricular hypertrophy (Pye and Cobbe 1992, Ghali *et al* 1991). Many studies have shown that hypertrophied hearts have an enhanced susceptibility to ischemic and hypoxic contractile dysfunction, in which impaired diastolic relaxation is prominent (Lorell *et al* 1986, 1990, Wexler *et al* 1988, Cunningham *et al* 1990, Hearse *et al* 1978, Snoeckx *et al*

1990, Anderson *et al* 1990, Buser *et al* 1990, Gaasch *et al* 1990). One hypothesis is that enhanced conversion of angiotensin I to angiotensin II in rats with pressure-overload hypertrophy can contribute to the increased sensitivity of hypertrophied hearts to diastolic dysfunction during low-flow ischaemia (Eberli *et al* 1992). There is evidence for an intrinsic tissue renin-angiotensin system in the heart that may play a role in modulating cardiac hypertrophy (Wright *et al* 1983, Rogers *et al* 1986, Dzau 1988, Jin *et al* 1988). In fact increased angiotensin converting enzyme activity and mRNA expression in rats with pressure-overload hypertrophy has been observed alongside higher angiotensin I to angiotensin II conversion (Shunkert *et al* 1990). This increased angiotensin II activation has been shown to result in severe but reversible impairment of diastolic function in the hypertrophied heart (Schunkert *et al* 1990). Ischaemia leads to cellular damage linked to energy depletion and the accumulation of toxic metabolites (Neely *et al* 1973, Peuhkurinen *et al* 1983, Neely and Grotyohann 1984, Jennings and Steenburgen 1985).

Increased risk to ischaemia may be due in part to derrangements in cardiac electrical activity, as a result of hypertrophy, such as prolongation of the action potential (Hart 1994) and altered intrinsic electophysiological response to metabolic stress (Baghdady and Nichols 1994). Prolonged action potentials shorten more than normal during global ischaemia (Kohya *et al* 1988). When intracellular concentrations of ATP decrease, cardiac ATP-sensitive K^+ channels are activated and pass current (Kakei *et al* 1985), particularly during myocardial ischaemia and infarction (Weiss and Lamp 1989). Glycolysis is the major energy-

producing pathway during cardiac ischaemia. Weiss and Lamp reported that ATP generated by glycolysis preferentially inhibits K^+-ATP channels in cardiac myocytes when intracellular ATP consumption is increased. In support of this Yuan *et al* (1997) discovered that hypertrophy increases the maximal activity K^+-ATP channel activity in the absence of ATP and the effectiveness of the hypertrophied ventricular cells to inhibit K^+-ATP channel activity by glycolytically produced ATP is decreased.

Tissue oedema is considered one of the main factors contributing to irreversible cardiac injury during ischaemia. During ischaemia, the net influx of sodium results in higher than physiological concentrations without the efflux of potassium. This non-equilibrium causes water to enter the cells. Microscopic studies have shown up to 20% oedema in heart slices after a normothermic ischaemic insult (Vogel *et al* 1986). The development of oedema has been attributed to intracellular organelles such as the mitochondria. Accompanied by the disturbance of electrolyte homeostasis (Macknight and Leaf 1977), such as the accumulation of calcium ions, swelling and de-energisation of the mitochondria and release of intracellular enzymes (reflecting the breakdown of membrane integrity) have been implicated in irreversible ischemic damage of the cell.

6.1.3.4. Apoptosis-induced myocardial remodelling.

Loss of cardiomyocytes during myocardial hypertrophy can lead to progressive dysfunction in hearts with chronic haemodynamic overload (Narula *et al* 1996, Olivetti *et al* 1997). Cardiomyocyte loss most likely occurs sequentially in

association with cell degeneration and atrophy throughout the course of myocardial remodelling in the hypertrophied hearts. However little is known about the mechanisms involved. In hypertrophied myocardium showing no evidence of inflammatory response or cell necrosis, apoptosis has been implicated as a possible cause (MacLellan and Schneider 1997, Yamamoto *et al* 2000).

Various triggers have been suggested to induce apoptosis in myocardial hypertrophy and heart failure (Teiger *et al* 1996, James *et al* 1996, James 1997) including hypoxia (Tanaka *et al* 1994), ischaemia (Gottlieb *et al* 1994), norepinephrine (Communal *et al* 1998) and heat shock proteins (Benjamin and McMillan 1998). Overstretching of cardiac myocytes itself may also act as a stimulus to induce apoptosis as well as structural rearrangements of the myocardium (Cheng *et al* 1995). A model by Wang *et al* (1998) proposed that haemodynamic stress, as a result of mechanical overload or chronic ischaemia, can activate the p38 MAP kinase activities, which subsequently contribute to the hypertrophic response in the initial compensatory phase. As the stress persists the balance between hypertrophic and apoptotic signalling may be disrupted and result in cardiomyocytes losing their cellular viability and structural integrity and enter the cell death pathway. Loss of contractile function and viable cells would eventually place more stress on the surviving myocytes and initiate the irreversible deterioration of cardiac function ultimately resulting in heart failure.

6.1.3.5. Effect of ageing on heart function.

The heart can demonstrate deterioration in contractile function due to age-

associated changes in structure and metabolism that may predispose the aged cardiovascular system to cardiac dysfunction and disease. Studies have shown that elastic fibres are significantly modified in senescence (de Carvalho Filho *et al* 1996). Myocyte volume is also increased in aged hearts and aged hearts exhibit impaired calcium handling (Wei 1984, Carre *et al* 1993). Additional changes have been observed in the amount and isoenzyme distribution of contractile proteins including myosin (Bhatnagar *et al* 1984) and actin (Carrier *et al* 1992), suggesting aged myocardium has altered ATP utilisation required for its contraction and relaxation.

Regarding age-related changes in ATP synthesis and utilisation it has been suggested that the capacity for oxidative phosphorylation declines with age (Atlante *et al* 1998, Nakara *et al* 1998, Clandinin *et al* 1983), associated with increased mutations of mitochondrial DNA in heart tissue (Wallace 1996, Ozawa *et al* 1990). Observations of reduced cardiac ANT activity (Kim *et al* 1988) and alterations in the lipid membrane of cardiac mitochondria from aged rats (Kim *et al* 1988) suggest a decreased respiratory activity and decrease in ATP synthesis. Energy reserves of the heart may also be altered with ageing signified by studies showing that CK activity is reduced (Saupe *et al* 2000), which may render the heart more susceptible to dysfunction, if associated with conditions such as cardiac hypertrophy. In contrast to this although the senescent heart has been shown to contain lower baseline PCr and creatine levels, the PCr/creatine ratio and ATP content was found to be normal (Bak *et al* 1998), suggesting a balance between ATP synthesis and utilisation. However during hypoxia the aged heart

CK failed to use existing PCr stores to maintain ATP levels (Bak *et al* 1998). This is thought to be due to partial reversible inhibition of the CK enzyme (Bittl *et al* 1987) and suggests the aged heart was better able to preserve its ATP content during acute hypoxic stress by other mechanisms.

6.1.4. Myocardial substrate use during hypertrophy.

A key factor controlling flux through metabolic pathways is the work being performed by the heart (Lopaschuk 1997). Rates of ATP production by the heart depend on ATP utilisation rates (Neely and Morgan 1974). One hypothesis proposed for impaired contractile function during cardiac hypertrophy is the existence of an energy deprived state resulting from an imbalance between energy production and utilisation (Katz 1990, Ingwall 1993). The functional demand of the heart is reflected in the dense vascularisation of the myocardium and a very high reliance on oxidative metabolism, as indicated by the abundance of mitochondria, occupying close to 30% of the myocardial cell volume (Williams 1983). However in compensated hypertrophy the ratios of mitochondria to myofibril have been shown to be reduced possibly leading to inadequate energy production. (Anversa *et al* 1986).

During hypoxia mitochondrial acetyl units are transferred to carnitine and the resulting acetylcarnitine is rapidly transported back to the cytosol where it contributes to feedback inhibition of long-chain fatty acid activation. The adjustments in myocardial carbohydrate metabolism have also been reported. Lactate production by the hypertrophied heart has been shown to increase during

hypoxia (Anderson *et al* 1990) and ischaemia (Bladergroen *et al* 1990), indicative of enhanced glycolytic capacity. In addition glycogen is thought to contribute to the energy production of hearts exposed to increased workloads (Neely *et al* 1967), or hypoxic and ischaemic conditions (Neely and Morgan 1974). Adjustment of substrate utilisation during the adaptive phase of left ventricular hypertrophy, coupled to elevated workloads, super-imposed on anatomically restricted oxygen delivery (Anversa *et al* 1990), may play a crucial role in the development of pathophysiological hypertrophy and heart failure.

6.1.5. Effect of high work on substrate selection in hypertrophy.

In the intact heart, it has been suggested that under the conditions of high workload, when fatty acids constitute the major source of metabolic energy, the overall energy turnover is determined by the rate of carnitine-acylcarnitine exchange (Neely and Morgan 1974) and not the rate of mitochondrial oxidation. Allard *et al* (1994) found that the extra ATP required for mechanical function during high workload in hypertrophied hearts, was obtained primarily from an increase in glucose and lactate oxidation. The contribution of palmitate oxidation to overall energy production was not found to be depressed, whereas Christe and Rodgers (1994) found that glucose oxidation was enhanced and fatty acid oxidation was suppressed in SHR especially during elevated workloads, thought to be related to a conservation of energy stores in the presence of reduced oxygen availability. The favouring of glucose utilisation may maintain ATP synthesis during moderately reduced oxygen delivery, particularly at elevated workloads

(Alpert *et al* 1992), to offset the increased ratio of oxygen consumption calculated wall stress at a given workload (Brooks *et al* 1993).

6.1.6. Aims.

This Chapter proposes that the metabolic adaptations that had occurred following fifteen weeks aortic banding may become detrimental to the heart during an acute increased demand in workload. The workload of the heart can be increased by a number of factors including a change in preload, pressure, inotropy, heart rate or a combination of such. In the present study increase in work was achieved by raising the aortic pressure to enhance ventricular pressure development. Lactate metabolite release in the effluent flow-through collected, was taken as evidence of ischaemia, at intervals throughout the work regime. The following questions were asked:

- In light of the substrate alterations during prolonged pressure-overload, can hypertrophied hearts generate sufficient ATP energy to sustain contractile function when increased work demands are imposed?
- Alternatively do physiological changes associated with hypertrophy combined with altered substrate use cause the development of ischaemia or contractile dysfunction?

6.2. Methods.

6.2.1. Fifteen week aortic banding.

Pressure overload was induced surgically in male Sprague-Dawley rats as previously described (Chapter 2, section 2.2). The rats were sacrificed fifteen weeks post surgery (as in Chapter 5, section 5.2.1). The profile of substrate utilisation at fifteen weeks hypertrophy was determined in isolated perfused hypertrophied and control rat hearts using the following substrate mixtures:

- 5mM [1-^{13}C]-glucose, unlabelled palmitate and 100μU/ml insulin,
- 5mM glucose, 0.3mM [U-^{13}C]-palmitate and 100μU/ml insulin.

6.2.2. Increased workload.

Hearts were perfused initially for 20 minutes at low perfusion pressure (80mmHg). After this equilibration period the workload imposed on the hearts was increased by approximately 70% to a high perfusion pressure (135mmHg).

6.2.3. Lactate release.

Effluent samples were collected at specific time intervals (0, 30 and 45 minutes) during low and high workload perfusion and stored at –20°C until analysis. The concentration of lactate release and pH was measured in the effluent samples, to indicate whether the hearts experienced ischaemia or hypoxic damage during the increase in work demands.

6.3. Results.

6.3.1. Model of hypertrophy.

The morphology relating to hypertrophy and heart failure for the rats in this study can be found in Appendix Table 4.1 and 4.2 respectively. Aortic banding for fifteen weeks resulted in a 13.7% increase in heart mass compared to the control group ($p<0.01$). This level of hypertrophy was comparable to the 15.7% previously seen following fifteen weeks aortic banding (Chapter 5, section 5.3.1.1a).

As in the previous Chapter no evidence of heart failure was found at this level of hypertrophy (Appendix Table 4.2). Although smaller animals were used in this study the 11.9% increase in heart mass/tibia length ($p<0.05$) achieved in the hypertrophied group (Table 6.1), was comparable to the 14.8% increase found in Chapter 5 (section 5.3.1.2).

Treatment Groups	Heart Mass / Body Mass ($x10^{-3}$)	Heart Mass / Tibia Length (g/cm)	Right / Left Kidney Mass Ratio
Control (n=8)	4.70 (0.09)	0.59 (0.01)	1.01 (0.01)
Hypertrophy (n=9)	5.42* (0.04)	0.66* (0.00)	1.29* (0.02)

Table 6.1. Indices of hypertrophy. (* significantly different from the control group).

Blood characteristics for the hypertrophied and control groups used in this study can be seen in Appendix Table 4.4. No significant differences were observed between these two groups. Neither was there any difference in comparison with

the fifteen week hypertrophied and control groups from Chapter 5.

6.3.2. Substrate selection in fifteen week hypertrophied hearts following increased work demands.

6.3.2.1. Profile of substrate selection.

Figure 6.1. shows a summary of the relative contribution of glucose, palmitate and endogenous substrates to overall TCA cycle oxidation in fifteen week hypertrophied and control hearts subjected to increased work demands. No significant differences in substrate use were observed between fifteen week hypertrophied and control hearts at high workload (71.9 +/- 6.1% versus 69.3 +/- 7.1% for glucose and 35.3 +/- 1.8% versus 36.8 +/- 3.3% for palmitate, respectively). In both hypertrophied and control hearts the relative proportions of glucose and palmitate substrate use together accounted for approximately 100% total substrate use, therefore endogenous substrate contribution was negligible.

Analysis between groups revealed that increased workload significantly enhanced the proportion of glucose use in both hypertrophied and control hearts compared to those at low workload in Chapter 5 (71.9% versus 33.0%, $p<0.01$ and 69.3% versus 41.0%, $p<0.05$, respectively, Table 6.2(a)). High work demands significantly reduced the relative proportion of palmitate use in control hearts (36.8 versus 52.5%, $p<0.05$), but not in hypertrophied hearts (35.3 versus 46.8%, $p=0.09$). Glutamate measured as μmoles/gramme wet mass of tissue does not alter significantly between fifteeen week control and hypertrophied hearts during high workload (5.15 +/- 0.11 versus 4.69 +/- 0.06 respectively). This is also true of

the glutamine concentrations in the fifteen week control and hypertrophied heart tissue (2.43 +/- 0.11 versus 2.25 +/- 0.06) which does not differ significantly.

Treatment Groups	% Glucose use	% Fatty acid use	% Endogenous use
15 week Hypertrophy at low work	33.0 (n=2)	46.8 +/- 6.5 (n=4)	20.2
15 week Hypertrophy at high work	71.9 +/- 6.1* (n=4)	35.3 +/- 1.8 (n=5)	n.d

Table 6.2(a). Summary of comparison of substrate use in fifteen week hypertrophied hearts at low and high workload (mean +/- SEM). (* significant difference compared to low workload).

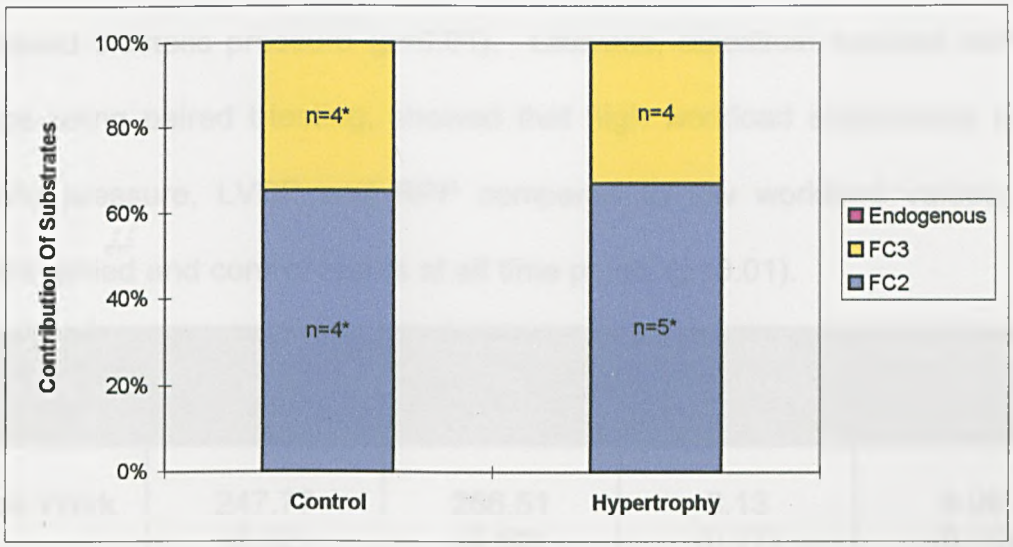

Figure 6.1. Substrate selection in hearts perfused with Glucose & Palmitate & Insulin after fifteen weeks hypertrophy following increased work demands. (* significant difference compared to low workload).

6.3.2.2. Function in glucose and palmitate perfused hearts during low and high work demands.

Heart rate did not differ significantly between hypertrophied and control hearts at low or high workloads and did not change due to workload increase (Table 6.2(b)). Diastolic pressure did not alter significantly between control and hypertrophied hearts at either workload, however paired t-tests within both control and hypertrophied heart groups revealed that high work demands significantly increased diastolic pressure ($p<0.01$). Likewise, statistical analysis within heart groups using paired t-testing, showed that high workload significantly increased systolic pressure, LVDP and RPP compared to low workload values, in both hypertrophied and control hearts at all time points ($p<0.01$).

Treatment Groups	Control HR (n=8)	Hypertrophy HR (n=9)	Control DP (n=8)	Hypertrophy DP (n=9)
Low Work	247.77 (3.32)	266.51 (2.59)	8.13 (0.17)	8.08 (0.16)
High Work	255.79 (2.07)	263.30 (4.02)	14.27* (0.66)	14.10* (0.65)

Table 6.2(b). Heart rate (bpm) and diastolic pressure (mmHg) in control and hypertrophied hearts during low and high workloads (mean +/- SEM). (* significant difference compared to low workload).

Alterations in SP between groups during the workload study are shown in Figure 6.2. Both heart groups start at 85mmHg. At the start of high work the hypertrophied hearts show significantly 23% increased SP compared to control

hearts (154mmHg versus 125mmHg, $p<0.05$), but eventually equilibrated at a non-significant 10% increase at the end of perfusion (190mmHg versus 170mmHg). These changes are reflected in LVDP (Figure 6.3). Control hearts began at 80mmHg, increased to 110mmHg at the start of high work and finally equilibrated to 155mmHg at the end of the perfusion period. Fifteen week hypertrophied hearts started perfusion at 80mmHg, increased to significantly 150mmHg ($p<0.05$) at high workload and equilibrated to 170mmHg (ns) at the end of perfusion. The RPP values follow a similar pattern (Figure 6.4). Thus hypertrophied hearts showed significantly augmented SP, LVDP and RPP between the time points 19 to 30 minutes ($p<0.05$), but all ceased to be significant after 40 minutes of perfusion.

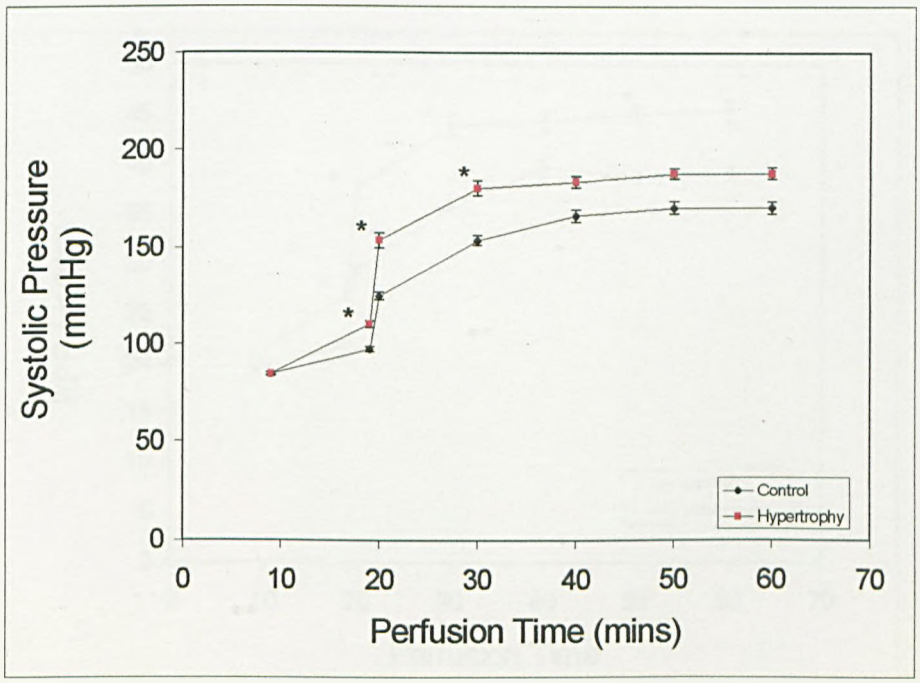

Figure 6.2. Systolic pressure values in fifteen week hypertrophied and control hearts during a workload study. (mean +/- SEM). (* significant difference from control hearts).

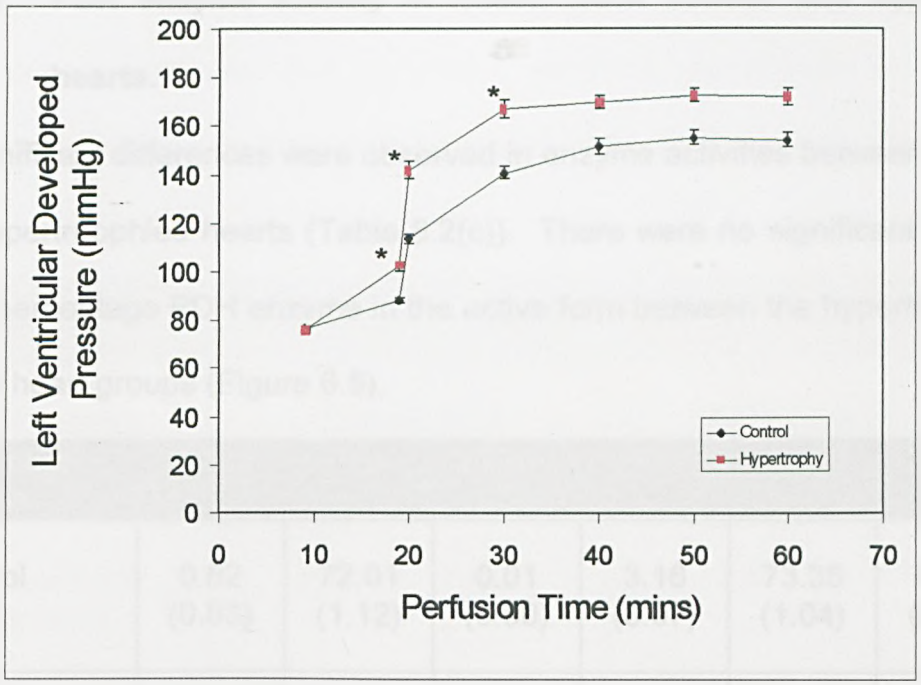

Figure 6.3. Left ventricular developed pressure measurements in fifteen week hypertrophied and control hearts during a workload study. (mean +/- SEM). (* significant difference from control hearts).

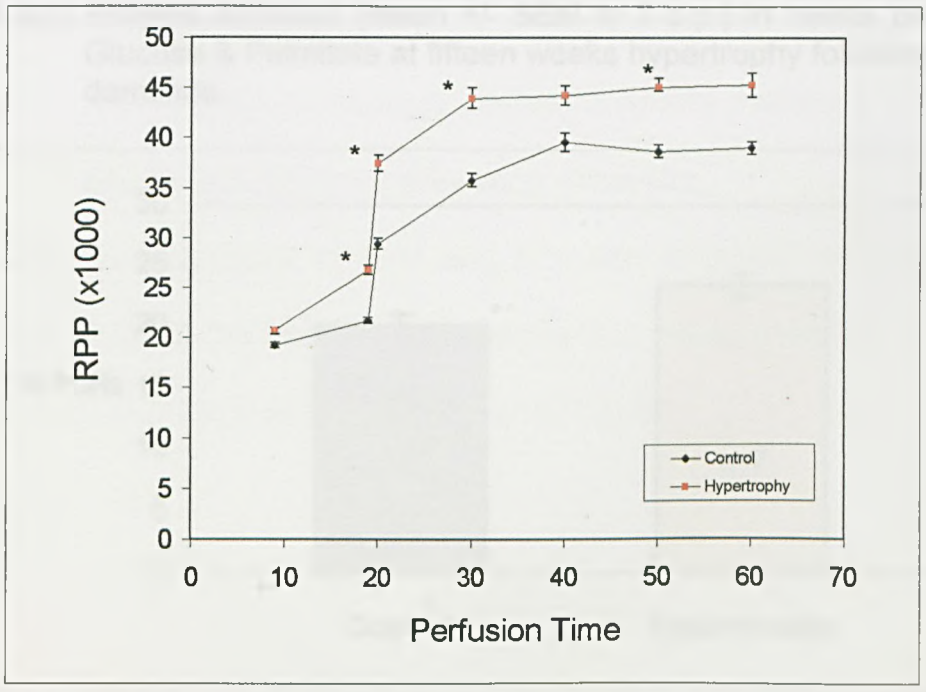

Figure 6.4. Rate pressure product values for fifteen week hypertrophied and control hearts during a high workload study (mean +/- SEM). (* significant difference from control hearts).

6.3.2.3. PDH enzyme activity in fifteen week control and hypertrophied hearts.

No significant differences were observed in enzyme activities between the control and hypertroophied hearts (Table 6.2(c)). There were no significant differences in the percentage PDH enzyme in the active form between the hypertrophied and control heart groups (Figure 6.5).

Treatment Groups	PDHa	CSa	PDHa/ CSa	PDHt	CSt	PDHt/ CSt
Control (n=7)	0.62 (0.03)	72.01 (1.12)	0.01 (0.00)	3.16 (0.07)	73.35 (1.04)	0.04 (0.00)
Hypertrophy (n=7)	0.68 (0.04)	72.79 (0.95)	0.01 (0.00)	3.08 (0.10)	73.50 (1.28)	0.06 (0.00)

Table 6.2(c). Enzyme activities (mean +/- SEM to 2 d.p.) in hearts perfused with Glucose & Palmitate at fifteen weeks hypertrophy following high work demands.

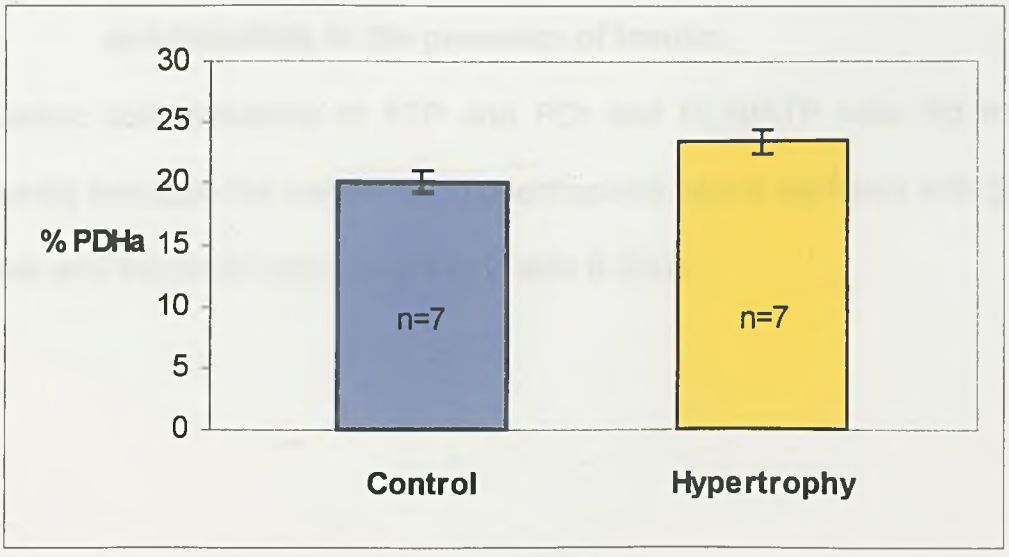

Figure 6.5. Percentage PDH enzyme in the active form in hearts perfused with Glucose and Palmitate at fifteen weeks hypertrophy following high work demands (mean +/- SEM).

Comparison between the fifteen week hypertrophied and control hearts at low and high workloads revealed that enhanced workload significantly increased the percentage of PDHa in both hypertrophied and control hearts (Table 6.2(d), $p<0.05$).

Treatment Groups	15 week Control	15 week Hypertrophy
% PDH activity at low work	7.76 +/- 0.76 (n=5)	6.63 +/- 0.73 (n=5)
% PDH activity at high work	20.04 +/- 1.02* (n=7)	23.39 +/- 1.57* (n=7)

Table 6.2(d). Summary of comparison of PDH enzyme activity in fifteen week hypertrophied and control hearts at low and high workloads (mean +/- SEM).

6.3.2.4. High-energy phosphate status of hearts perfused with Glucose and Palmitate in the presence of insulin.

The cardiac concentrations of ATP and PCr and PCR/ATP ratio did not alter significantly between the control and hypertrophied hearts perfused with glucose, palmitate and insulin at high workloads (Table 6.2(e)).

Treatment Groups	ATP	PCr	PCr/ ATP
Control (n=6)	6.23 (0.10)	6.64 (0.08)	1.07 (0.02)
Hypertrophy (n=6)	5.88 (0.14)	5.99 (0.13)	1.03 (0.03)

Table 6.2(e). High-energy phosphate concentrations (μmoles/gramme wet mass, mean +/- SEM) in hearts perfused with Glucose & Palmitate & Insulin during fifteen weeks hypertrophy at high workload.

6.3.2.5. Effluent metabolite analysis.

Analyses of the effluent samples showed no significant differences in pH or lactate release between the fifteen week hypertrophied and control hearts obtained at various time points throughout perfusion (Appendix Table 4.5).

6.4. Discussion.

6.4.1. Substrate utilisation in hypertrophied hearts subjected to increased work demands.

6.4.1.1. Glucose utilisation.

Work demands play a major role in controlling the flux through various metabolic pathways (Lopaschuk 1997). As rates of ATP utilisation increases the heart has to meet these demand by adjusting its ATP production (Neely and Morgan 1974). The profile of substrate oxidation in hearts at high workload was very different from that seen at low workload in both groups but particularly in the hypertrophied hearts (Table 6.2(a)). Under conditions of high workload glucose became the predominant substrate for TCA cycle oxidation, in both the hypertrophied and control hearts (Figure 6.2.). The production of ATP derived from glucose oxidation was enhanced more than two fold in both groups, when a 70% increase in perfusion pressure was imposed upon the hearts. This finding is supported by Allard *et al* (1994) who investigated the contribution of oxidative metabolism and glycolysis to ATP production in the hypertrophied heart at high workloads. Allard *et al* (1994) determined that the extra ATP production required for mechanical function was obtained primarily from enhanced oxidation rates of glucose and lactate, in both hypertrophied and control hearts.

The increase in glucose use in both the hypertrophied and control hearts is likely to result from increased glucose uptake. Regulation of glucose transport activity in insulin-sensitive tissues such as the heart is achieved by translocation of the GLUT-4 glucose transporter between the inactive intracellular location and

the active sarcolemmal location (Holman and Cushman 1994). Translocation of GLUT-4 to the sarcolemma of cardiomyocytes is induced by workload (Wheeler *et al* 1994).

Early studies in humans during exercise reported that the contribution of exogenous glucose to myocardial oxidative metabolism was decreased or unchanged during exercise (Kaijser *et al* 1972, Lassers *et al* 1971, Keul 1971), as determined by arterial-coronary sinus measurements. This method of calculation reflects the net balance and could under-estimate the actual amount of substrate being utilised by the myocardium. However Wisneski *et al* (1985) found that enhanced glycolysis occuring during exercise resulted in an increase in exogenous glucose oxidation, with minimal or no change in the arterial-coronary sinus glucose difference. In contrast experiments using isolated perfused hearts have demonstrated enhanced glycolysis during conditions of increased myocardial work (Opie *et al* 1971, Marshall *et al* 1981). In support of this Gertz *et al* (1988) found increased myocardial oxidation of exogenous glucose and lactate in humans during moderate intensity exercise, with over 50% calculated to be oxidised (Gertz *et al* 1988).

6.4.1.2. Fatty acid utilisation.

Chapters 3 and 5 established that free fatty acids are the major source for myocardial oxidative metabolism in control and hypertrophied hearts in the basal work state, confirming the observations seen by others (Opie 1968, Neely *et al* 1974, Liedtke *et al* 1981). Although at low workload the fifteen week

hypertrophied hearts exhibited impaired fatty acid oxidation, compared to the control hearts. During high workload however the increase in glucose oxidation was associated with no change in palmitate use in the hypertrophied hearts and a decrease in the palmitate use in control hearts (Figure 6.3). This resulted in a similar profile of substrate use for both groups, with the impaired fatty acid use in the hypertrophied hearts no longer being apparent. Thus the greater energy requirements associated with mild increases in cardiac work were met preferentially by the utilisation of carbohydrates rather than fatty acids in both the hypertrophied and control hearts.

Allard *et al* (1994) reported that the contribution of palmitate to overall ATP production decreased in hypertrophied and control hearts, to comparable levels. A preferential switch to glucose use compared to palmitate oxidation has also been demonstrated in normal hearts exposed to epinephrine (Collins-Nakai *et al* 1994) and increased concentrations of calcium. In contrast Christe and Rodgers (1994) reported a difference in the coupling of workload to myocardial fuel oxidation between SHR and normotensive hearts, in which fatty acid oxidation was suppressed in the SHR heart alongside markedly elevated glucose oxidation, regardless of workload. The exact mechanism of suppressed fatty acid oxidation in SHR was not known (Christe and Rodgers 1994). It was thought to be associated with altered coupling of workload to triglyceride turnover, enzymes of the β-oxidation spiral, regulation of the CPT enzyme complex, or in FAD- and NAD-linked long-chain acyl CoA dehydrogenases. The differences may be due to the fact that SHR is a more severe genetic model.

Myocardial extractions of various substrates used for energy production has been correlated with circulating levels of these substrates in animal and human studies (Lassers *et al* 1971, Gertz *et al* 1980). This does not appear to be the case in isolated perfused hearts during elevations in workload and ATP demands, where palmitate use was decreased at high workload but the concentration supplied was identical to that at low workload. Fatty acid oxidation was most likely inhibited at the CPTI activity, by malonyl-CoA. Increased glucose metabolism could increase malonyl-CoA production by enhancing the supply of acetyl-CoA, to acetyl-CoA carboxylase. Studies suggest that when glucose oxidation increases the acetyl groups can be translocated from acetyl-CoA within the mitochondrial matrix to acetyl-CoA in the cytoplasm (reviewed by Lopaschuk *et al* 1994). In support of this stimulation of glucose oxidation has been shown to increase malonyl-CoA in the rat heart, which correlated with a decrease in long-chain fatty acid oxidation (Saddik *et al* 1993). In the hypertrophied hearts other mechanisms inhibiting fatty acid use may still be present, such as reduced carnitine levels. The pre-existence of reduced fatty acid use at low workloads observed in Chapter 5 may account for why no further decrease was observed.

In contrast to the present study, the rate of total fatty acid oxidation has been reported to increase by contractile stimulation in isolated working hearts (Goodwin *et al* 1998). It is proposed that increments in AMP during increased work are insufficient to promote the phosphorylation of acetyl-CoA carboxylase by AMP-stimulated protein kinase and thus fatty acid oxidation is augmented due to

the degradation of malonyl-CoA by malonyl-CoA decarboxylase (Goodwin and Taegtmeyer 1999).

6.4.1.3. Endogenous substrate use.

Endogenous substrates did not contribute to TCA cycle oxidation in either hypertrophied or control hearts during high workload. The metabolism of both exogenous glucose and endogenous substrates has been suggested to increase during exercise. Animal studies investigating glycogen levels have shown that myocardial glycogen content decreases with moderate to heavy intensity exercise (Judd and Poland 1972, Goldfarb *et al* 1986). This study has only determined the contribution of endogenous substrate stores to oxidative metabolism and no assumptions can be made concerning the anaerobic use of endogenous substrate.

6.4.1.4. PDH activity.

Accompanying the enhanced utilisation of glucose, under conditions of high workload, the proportions of PDH in the active form increased significantly in both the hypertrophied and control hearts, compared to those observed at low workloads (Table 6.2(c)). This finding correlates well with studies by Kobayashi and Neely (1983) and Pearce *et al* (1980) who have shown that the proportion of PDH in the active form was dependent on cardiac work in isolated perfused control hearts. Interestingly the levels of PDHa achieved in both hypertrophied and control hearts did not reach maximal levels, in relation to that achieved using DCA

activation in Chapter 4, despite the increased work performed.

PDH activity is regulated by two inter-dependent mechanisms. The first is by inter-conversion of the enzyme between active (non-phosphorylated) and inactive (phosphorylated) forms (Wieland *et al* 1971). This is catalysed by an ATP-dependent kinase and a Mg^{2+} and Ca^{2+} activated phosphatase (Denton *et al* 1976). The kinase is activated by increases in the ratios of acetyl-CoA/CoA, $NADH/NAD^+$ and ATP/ADP. The second mechanism is by competitive feedback inhibition due to the reaction products NADH and acetyl-CoA (Bremer 1962). With the increase in workload imposed and a greater demand for acetyl-CoA ensuing, the intra-mitochondrial ratios of acetyl-CoA/CoA and $NADH/NAD^+$ probably decreased, which removed any fatty acid inhibition of PDH. It has been proposed that different mechanisms are involved in controlling PDH activity depending on the workload imposed. It was determined that at low work PDH inter-conversion occurs by a rise in the $NADH/NAD^+$ ratio and flux is limited by the amount of active enzyme (Pearce *et al* 1980). In contrast at high workloads PDH flux can be restricted by product inhibition by acetyl-CoA (Pearce *et al* 1980).

Calcium activates PDH indirectly by causing increases in the amount of active non-phosphorylated PDH through activation of PDH phosphatase (Denton *et al* 1976). The role of the calcium mechanism ensures that ATP synthesis is increased to meet the enhanced demand for ATP, during contraction without the need to decrease cellular ATP concentrations (Denton and McCormack 1980, McCormack and Denton 1989). The calcium handling system in this model of hypertrophy has been shown to be impaired (Boateng *et al* 1997), even at the nine

week stage. It was expected that these inappropriate calcium-handling conditions would have adverse effects on calcium-sensitive metabolic enzymes such as PDH, in the hypertrophied hearts, particularly at high workloads. This was not shown to be the case in the present study.

In addition Kobayashi and Neely (1983) propose that pyruvate availability may contribute towards limiting the flux through PDH at high workloads in hearts. It was shown in hearts perfused with glucose and insulin that the glycolytic rate increased in proportion to developed ventricular pressure until a saturation point was reached, when lactate production declined, despite a linear increase in oxygen consumption. The pyruvate production by glycolysis became rate-limiting the demand of the TCA cycle for acetyl-CoA and the contribution of endogenous fatty acid to overall respiration rate increased. In the present study no evidence was found to suggest that endogenous substrates contributed to TCA cycle oxidation. Assumptions concerning the rates of substrate oxidation cannot be made in this study; however Pearce *et al* (1980) found that the rates of glucose oxidation were similar in control heart perfused under high and low workloads.

6.4.2. Heart function.

Studies have shown that hypertrophied hearts exhibit impaired contractile function (Schaper *et al* 1978, Gaasch *et al* 1990, Anderson *et al* 1990). The hypothesis that this may occur due to an imbalance between energy production and utilisation (Katz 1990, Ingwall 1993) was tested in this chapter, by imposing greater work demands on the hypertrophied hearts.

Initially systolic pressure was significantly enhanced in the hypertrophied hearts with comparable diastolic pressure at high workload compared to the control hearts. This resulted in augmented LVDP and RPP. Zhang et al (1995) and Allard et al (1994) have also observed enhanced systolic pressure in hypertrophied hearts. However in the present study after ten minutes of high workload the improved mechanical performance ceased to be significantly higher that that of the control hearts.

End-diastolic pressures increased comparably in both hypertrophied and control hearts during the elevated work period, indicating no evidence of abnormal relaxation in the hypertrophied hearts. In contrast Zhang *et al* (1995) has observed significantly greater end-diastolic pressures in hypertrophied hearts. Additionally, at no point during the perfusion periods did the hypertrophied hearts show arrhythmias or contractile dysfunction, indicating that the fifteen week hypertrophied hearts were compensated and not failing hearts. This contrasts to work on more severe models in which impaired performance was observed in the glucose-perfused myopathic heart (Di Lisa *et al* 1993). Myocyte hypertrophy improves contractility by increasing the total mass of myocardial tissue available for contraction (Krayenbeuhl *et al* 1983, Frohlich 1983). It also reduces wall stress by increasing the wall thickness of a dilated chamber (Strauer 1979).

One question raised is why do the hypertrophied hearts not sustain this increased performance? There could be several reasons but it can only be speculated on at this stage. Firstly impaired myocardial metabolism has been found in patients (Kobayashi *et al* 1995, Nakata *et al* 1996) and animals (Kalpelko

et al 1989, Whitmer 1986) with hypertrophic cardiomyopathy. Popovich *et al* (1995) discovered nuclear and cellular myocyte hypertrophy in patient myocardium was associated with lower creatine-stimulated mitochondrial respiration rate. The depressed mitochondrial function was thought to arise from altered expression of the CK enzyme (Khuchua *et al* 1992) and SR Pi accumulation (Veksler and Ventura-Clapier 1994), in which resulting insufficient ATP energy at the myofibrils caused impaired diastolic relaxation. In contrast to these studies the pressure-overload hypertrophied heart is a less severe model and did not show abnormal diastolic pressure development. Assumptions concerning mitochondrial respiration rates cannot be made in this work. However examination of the substrate profiles and proportion of PDH in the active form suggests substrate limitation at the level of the mitochondria is not the underlying cause.

Secondly altered calcium homeostasis could be the underlying reason. Abnormal calcium handling is thought to be responsible at least in part for the impaired diastolic and systolic function in the failing heart (Gwathmey *et al* 1987). The pressure-overload hypertrophied heart has also been associated with a decreased expression of calcium handling proteins, SR Ca^{2+}-ATPase and PLB in this model (Boateng *et al* 1997), and in the failing heart alongside a decreased affinity of the SR Ca^{2+}-ATPase for Ca^{2+} (Kiss *et al* 1995). In the myopathic heart impaired work performance was linked to a less active PDH, correlated to a lower $[Ca^{2+}]$ mitochondrial value, when the myocytes were electrically stimulated, which was ultimately thought to decrease TCA cycle flux. The smaller rise in $[Ca^{2+}]m$ transients were attributed to lower systolic transients in the cytosol ($[Ca^{2+}]c$). The

proportion of PDH in the active form was not found to be lower in the present study, so is unlikely to be a contributory factor.

Thirdly hypertrophied myocardium exhibits limited coronary flow reserve and leads to exercise-induced myocardial ischaemia (Wolfe *et al* 1990). Heterogeneously reduced fatty acid use due to exercise-induced ischaemia was observed in the hypertensive heart (Wolfe *et al* 1990). During ischaemia, the heart resorts to anaerobic glycolysis for energy production (Vary *et al* 1981). Zhao *et al* (1995) found that lactate was derived exclusively from exogenous glucose during low pressure-low flow ischaemia, whereas glycogen was mobilised only during a more severe global ischaemic insult. Unfortunately only extracts could be analysed in the present study which may have masked small areas of disturbed energy metabolism or ischaemic injury which may have occurred. It can only be speculated on whether the function in the hypertrophied hearts would continue to decline if perfused for longer.

6.4.3. High-energy phosphate status.

Impaired high-energy transfer via the creatine phosphokinase system has been correlated with poor functional reserve (Nascimben *et al* 1995) and in some forms of heart failure there are declines in ATP and total adenine nucleotides (Pool *et al* 1967). However the extent to which such changes are associated with the transition from compensatory hypertrophy to decompensated hypertrophy and failure is still speculative. In this study the concentrations of ATP and PCr did not differ between the hypertrophied and control hearts at high workload (Table 6.2(e)). Subsequently the ATP/PCr ratio did not altered between the two groups, indicating no evidence of substrate limitation or impaired coronary reserve. This finding contrasts to work by other researchers. Diminshed high-energy phosphate levels have been demonstrated in other experimental models of cardiac hypertrophy (Kapelko *et al* 1991, Zhang *et al* 1993). In addition the contents of PCr and creatine have been reported in animal models of hypertrophied-failing hearts (Neubauer *et al* 1989, Nascimben *et al* 1995) and the myocardium of patients with dilated cardiomyopathy (Conway *et al* 1991, Hardy *et al* 1991), as well as decreased CK activity (Nascimben *et al* 1996).

In terms of heart function, no impairment was found in the hypertrophied hearts compared to control hearts during high workload, which is consistent with the high-energy phosphate levels. This indicates that the hypertrophied hearts in the present study were non-failing. Zhang *et al* (1995) found that despite the loss of high-energy phosphates, the hypertrophied hearts were able to sustain increased pressure-overload imposed by aortic banding without evidence of

cardiac failure, concluding that the size of the high-energy phosphate pool may not be critical to contractile performance. However in more severe models such as the failing Syrian hamster (Markiewicz *et al* 1987) and SHF rat heart (O'Donnell *et al* 1995), the PCr was found to decrease while Pi increased, when the hearts were stimulated to higher workloads. The decline in PCr/ATP ratio was thought to be indicative of the transition to heart failure. In addition Conway *et al* (1998) has shown deteriorating myocardial function in patients is associated with lower PCr/ATP levels, possibly due to inefficient creatine kinase activity (Ingwall *et al* 1990). The PCr/ATP results in the present study indicate that even at high workload the hypertrophied hearts are non-failing.

The PCr / ATP ratio is a sensitive marker of ischaemia (Schaefer *et al* 1989, 1990). Severe myocardial ischaemia causes a depletion of PCr and ATP, with a concommitant increase in Pi, particularly in patients with ischaemic heart disease (Bottomley *et al* 1987). The hypertrophied hearts in the present study showed no evidence of ischaemia, as evidenced by the PCr/ATP levels compared to control hearts. In support of this, Massie *et al* (1995), could find no evidence of impaired coronary reserve or ischaemia, in pigs with left ventricular hypertrophy, in which the PCr / ATP ratio did not alter in response to a three fold increase in oxygen consumption. However measurements of intracellular phosphate compounds in intact hearts by ^{31}P-NMR have revealed that the ADP and the PCr/ATP ratio do not change over a variety of myocardial oxygen consumption (Heinman and Balaban 1990). The acceptor (respiratory) control ratio and ATP/O ratio are thought to be better estimators of mitochondrial metabolism.

6.4.4. Lactate release.

Hypertrophied hearts are reportedly more susceptible to ischaemic injury (Schaper *et al* 1978, Gaasch *et al* 1990, Anderson *et al* 1990). It has been shown that coronary flow may not be homogeneous in the hypertrophied heart, in which a decrease in perfusion creates regional disturbances or heterogeneity of myocardial metabolism (Gertz *et al* 1981). Gertz *et al* (1981) found heterogeneity of lactate metabolism at rest in patients with ischaemic heart disease, where the severity of heterogeneity was related to the severity of the coronary artery disease. Heterogeneity of myocardial blood flow has also been demonstrated in animal preparations (Cobb *et al* 1974, Falsetti *et al* 1975). In addition differences in oxygen tension in various layers of left ventricle have been reported in open-chest canine preparations (Kirk and Honig 1964). If the supply of oxygen is insufficient, pyruvate is converted to lactate. If lactate was present it may have inhibited the lactate/H^+ transport causing increased acidification of the myocyte, that competes with intracellular Ca^{2+} at the level of the myofibrillar contraction and cause a decrease in force production (Liu *et al* 1993). However the effluent lactate concentrations from both the hypertrophied and control hearts during low and high workload were minimal (Appendix Table 4.5). This is evidence to suggest that oxygen supply was not limited and ischaemia did not occur throughout the entire work period. In addition the pH of the effluent samples was not found to decrease, in either the hypertrophied or control hearts (Appendix Table 4.5), indicating that no acidosis had occurred throughout the high work conditions.

Ischaemia and hypoxia have been shown to induce translocation of GLUT-4 to the sarcolemma of myoctes (Sun *et al* 1994, Wheeler 1988), and glucose uptake and translocation of GLUT-4 is stimulated during low-flow ischaemia in dogs *in vivo* (Young *et al* 1997). In addition the contribution of glucose to energy metabolism is increased during ischaemia (Liedtke *et al* 1988, Lopaschuk *et al* 1990). This is unlikely to be the cause of increased glucose use in the present study because no evidence of ischaemia was found. Thus the enhanced contribution of glucose metabolism to TCA cycle oxidation in both the hypertrophied and control hearts can be attributed to increased work demands and not ischaemia.

6.4.5. Summary.

- Abnormalities in the relative proportions of substrate utilisation found in hypertrophied hearts during low work are normalised during high work conditions.
- Glucose is the predominant substrate oxidised for ATP production in both hypertrophied and control hearts during high workload.
- Hypertrophied hearts exhibited enhanced mechanical function at high perfusion pressure, but were unable to sustain this significance over time.
- No evidence of impaired energy provision, ischaemia or contractile dysfunction was observed in hypertrophied hearts subjected to increased workloads.

Chapter 7:

Discussion.

7.1 Conclusions.

The work of this thesis has highlighted important sequential changes in substrate use that occur during the development of pressure-overload left ventricular hypertrophy. Initially the hypertrophied heart exhibited an increased reliance on glucose oxidation, with no change in long-chain fatty acid use. As the disease progressed the hypertrophied heart showed a trend for impaired fatty acid utilisation and an accumulation of long-chain acylcarnitine molecules. Ultimately myocardial substrate selection switched from fatty acid oxidation towards increased reliance on glucose and endogenous substrate metabolism in hypertrophy. Alteration in substrate utilisation preceded any change in heart function and is strong evidence to suggest that impaired substrate delivery at the level of the mitochondria in cardiac hypertrophy plays an important role in the development of heart failure and is not a secondary phenomenon.

7.2. Potential detrimental effects of reduced fatty acid metabolism.

Accumulation of acylcarnitines may eventually become detrimental to the hypertrophied heart. Long-chain acylcarnitines have been shown to cause ventricular arrhythmias and cardiac dysfunction during myocardial ischaemia (Corr *et al* 1989). Studies on pressure-overload hypertrophied hearts have shown greater susceptibility exists to ischaemic injury (Eberli *et al* 1992, Schonekess *et al* 1996) and ischaemic ATP breakdown (Harnsen *et al* 1994). In hypertensive patients, left ventricular hypertrophy is associated with an increased risk for morbidity and mortality, with most of the complications resulting from myocardial

ischaemia (Bache *et al* 1988, Dellsperger and Marcus 1990).

In addition lipid droplets have been found in the myocardium of humans with dilated cardiomyopathy (Schaper *et al* 1991). Exposure of cardiac myocytes to saturated fatty acids (C16:0 or C18:0) results in cell death as evidenced by the release of intracellular proteins such as LDH (Van Bilsen *et al* 1997). Non-esterified long-chain fatty acids also have the ability to uncouple oxidative phosphorylation (Skulachev 1991) and have been implicated in inducing the mitochondrial permeability transition pore (MPTP) in tissues such as liver (Catisti and Vercesi 1999).

Maintenance of a permeability barrier by the mitochondrial inner membrane is essential for ATP synthesis by oxidative phosphorylation and for the compartmentalisation of cellular metabolism. However a non-specific increase in the permeability of this membrane can occur when mitochondrial matrix [Ca^{2+}] is greatly increased, especially when coupled to oxidative stress and adenine nucleotide depletion, resulting in uncoupling and swelling of the mitochondria (reviews by Crompton 1999, Halestrap 1999). The conditions that occur in ischaemic damaged tissues, namely a decrease in ATP and loss of ionic homeostasis, are the exact conditions that can cause this phenomenon. Hypertrophied hearts may be even more prone to this phenomenon due to reports of impaired calcium handling (Levitsky *et al* 1991, Boateng *et al* 1997).

Once the MPTP is open, mitochondria actively hydrolyse ATP and without adequate ATP, cellular damage can occur. This inevitably leads to cell death by necrosis (Halestrap 1999, Halestrap *et al* 1998). Alternatively low-dose oxidants,

which occur following ischaemia, can trigger the MPTP, release both cytochrome c (Cai *et al* 1999) and apoptosis-inducing factor (AIF)(Susin *et al* 1996) and activate apoptosis. Cell loss due to necrosis or apoptosis in the hypertrophied heart would then place greater strain on the residual myocardium and ultimately lead to heart failure.

7.3. Future work.

7.3.1. ^{13}C Nuclear Magnetic Resonance.

This work has established biochemical changes, useful as prognostic markers to detect the early stages of hypertrophy. This could improve both diagnosis and management of cardiac hypertrophy in patients before it progresses to heart failure. The use of ^{13}C labelled energy substrates would be amenable to *in vivo* studies. This study points out that infused ^{13}C-labelled glucose in patients would give a limited success, whereas very low concentrations of ^{13}C-labelled fatty acid would be more easily visible using the NMR techniques.

Establishment of the relationship between glucose and fatty acid utilisation changes during the development of hypertrophy has provided a basis for the investigation into more complex substrate mixtures. This has already been successfully achieved in diabetic hearts (Chatham *et al* 1997). Hearts can be perfused with uniformly labelled [U-^{13}C]-palmitate together with [3-^{13}C]-lactate, in the presence of unlabelled glucose. The only proviso is that each label produces an individual labelling pattern when entering the TCA cycle. In this case [U-^{13}C]-palmitate would label C1 and C2 of acetyl-CoA, whereas [3-^{13}C]-lactate would only

label C2 of acetyl-CoA.

The measurement of metabolic flux through biochemical pathways during cardiac hypertrophy would also be useful experiments to perform. These experiments would require oxygen consumption and the rate of ^{13}C-label incorporation (Weiss *et al* 1992, Malloy *et al* 1990). Ideally these non-steady state experiments would compliment the steady-state isotopomer analysis carried out on the same experimental tissue. In addition a new ^{13}C method of quantitating local myocardial oxygen consumption has been developed, to help determine the profiles of aerobic energy metabolism (van Beek *et al* in press), which could be successfully applied to future cardiac hypertrophy experiments.

7.3.2. Therapeutic intervention.

For future therapeutic treatment of hypertrophied heart patients, several questions need consideration. Would reversing the metabolic changes that occur in cardiac hypertrophy and improving energy substrate supply in the absence of hypertrophy reversal, be adequate to prevent the transition from stable hypertrophy to heart failure? Or would hypertrophy need to be reversed alongside the improvement of substrate provision? It may be worth exploiting the beneficial adaptations such as increased glucose use, whilst aiming to prevent those that are detrimental such as reduced fatty acid oxidation and the build up of acylcarnitines. In this way it may be possible to halt the progression of hypertrophy in terms of loss of cells through apoptosis and so prevent the overload on the remaining myocardium. Two potential therapeutic agents, DCA and propionyl-L-carnitine have been chosen for

discussion due to their experimental success on improving substrate utilisation and function in myocardial ischaemia to date.

7.3.2.1. DCA treatment.

During ischaemia anaerobic glycolysis increases, resulting in insufficient energy transduction, local accumulation of lactate and hydrogen ions and a decrease in function (Opie 1984). However during early reperfusion of the post-ischaemic elevated levels of free fatty acids are thought to decrease the percentage of PDH in the active form (Behal *et al* 1983), limiting glucose oxidation and the functional recovery of the hearts (McVeigh and Lopaschuk 1990). Thus the deleterious effects of myocardial ischaemia are thought to be due to inadequate ATP production and local tissue acidosis. Administration of DCA improves heart function during reperfusion following ischaemia in animal models (McVeigh and Lopaschuk 1990, Lewandowski and White 1995) and in humans with congestive heart failure (Bersin and Wolfe 1994), by restoring glucose oxidation through PDH enzyme activation (McAllister *et al* 1973, Bunger *et al* 1983).

In the hypertrophied heart, besides the increased susceptibility to ischaemic injury, the accumulation of long-chain fatty acyl molecules resulting from decreased long-chain fatty acid use may sequester any available CoA. Reduction of free CoA, may in turn increase the acetyl-CoA to CoA ratio, an important regulator of pyruvate dehydrogenase activity. Thus the hypertrophied heart may exhibit a limited capacity to utilise both glucose and fatty acids and become energy depleted. DCA administration may have a therapeutic use in

hypertrophied hearts, by ensuring flux through the PDH enzyme and restoring glucose substrate oxidation. Questions that still need to be addressed include, would increasing glucose and glycogen usage in severe hypertrophy by the supply of agents such as DCA be beneficial in the long term? Or would it be beneficial in the short term, ultimately resulting in the depletion of essential endogenous substrate stores?

7.3.2.2. Propinyl-L-carnitine treatment.

Studies administering propionyl-L-carnitine chronically to diabetic animals (Broderick *et al* 1996) have shown that impaired myocardial function can be overcome with a concomitant improvement in substrate oxidation. In addition long-term administration of propionyl-L-carnitine in volume- and pressure-overloaded rat models increased both glucose and long chain fatty acid oxidation rates in the perfused hearts, resulting in improved functional recovery from ischaemia (El Alaoui-Talibi *et al* 1997, Schonekess *et al* 1995). Torelli *et al* (1994) found that isolated myocytes from pressure-overloaded rats exhibited stimulated palmitate oxidation in the presence of propionyl-L-carnitine. In contrast Schonekess *et al* (1995) found that propionyl-L-carnitine improved hypertrophied heart function in the rat, without reversing the depressed contribution of fatty acid oxidation to ATP production.

The mechanisms behind the improved substrate oxidation remain controversial (Broderick *et al* 1996, Russell *et al* 1995, Tassani *et al* 1994), but is likely due to an anaplerotic effect. One hypothesis is that propionyl-L-carnitine

contributes to the replenishment of succinate lost from the cell during ischaemia and that improved post-ischaemic function may be due in part to substrate-level phosphorylation during ischaemia via the succinyl-CoA synthase reaction (Russell *et al* 1995). Tassani *et al* (1994) showed that propionyl-L-carnitine stimulates PDH activity in rat heart alongside stimulating the oxidation of the resulting acetyl-CoA. In addition the conversion of long-chain acyl-CoA esters to their carnitine derivatives may reduce their mitochondrial toxicity (Russell *et al* 1995). The relationship between functional improvement and biochemical normalisation in hypertrophied hearts following therapeutic intervention with propionyl-L-carnitine has yet to be clearly defined.

7.3.2.3. Regression of hypertrophy by ACE inhibition.

In humans, treatment with ACE inhibitors is associated with an improvement of diastolic function (Schneeweiss *et al* 1990) and is considered an effective method of inducing the regression of cardiac hypertrophy (Rockman *et al* 1994). The use of Ramipril, one such inhibitor of the angiotensin converting enzyme, has been shown to decrease the extent of left ventricular hypertrophy (Linz *et al* 1992) and reduce fibrosis (Brilla *et al* 1991) in experimental models. It is still undetermined whether this regression simply causes a reversal of the changes induced by pressure-overload hypertrophy or whether heart size is reduced without a parallel decrease in workload.

Studies using the aortic banding model have shown that a sub-antihypertensive dose of Ramipril was accompanied by partial normalisation of

energy metabolism (Bhutta *et al* 1994). Phosphocreatine levels were restored alongside a reduction in the uptake of glucose. In the same model the increase in diastolic stiffness was markedly attenuated. These results lead to the question of what effect Ramipril administration would have on substrate selection during the regression of hypertrophy? Would correction of the renin angiotensin system and reversal of fibrosis during hypertrophy also correct the changes in substrate utilisation? One study by Gohlke and Unger (1995) suggests that chronic ACE inhibitor treatment can improve cardiac function and metabolism in SHRs, independently of antihypertensive and antihypertrophic drug actions. This was evidenced by increase tissue concentrations of glycogen, ATP and CK in the myocardium.

ACE inhibitor treatment may be beneficial in preventing many other detrimental conditions associated with hypertrophy and heart failure. Increased occurrence of apoptosis in chronically pressure-overload SHR myocardium has been observed during the transition from stable hypertrophy to heart failure (Li *et al* 1997). There is the possibility that the renin-angiotensin system may mediate myocyte apoptosis. In a study of SHR failing hearts treated with Captopril, the number of apoptotic cells was not found to differ from that in SHR non-failing hearts (Li *et al* 1997). Therefore ACE inhibition may help prevent unnecessary cell loss during hypertrophy. Clinical studies have demonstrated the presence of insulin resistance and hyperinsulinemia in hypertensive patients (Paternostro *et al* 1999). The exact mechanism by which this insulin resistance occurs is not fully understood, but one study has suggested that Ang II plays a role in the

pathogenesis of insulin resistance in hypertension (Rao 1994). In support of this observations that ACE inhibitors can successfully improve insulin sensitivity have been reported (Berne *et al* 1991).

On a function level ACE inhibitor treatment of SHRs can significantly reduce the incidence of arrhythmias and increased heart rate (Chevalier *et al* 1995). ACE inhibition is thought to reduce arrhythmias not only due to the reduction of fibrosis, but also by preventing Ang II effects. Ang II has been reported to increase cytosolic calcium concentration (Allen *et al* 1988), which may mediate cardiac arrhythmias. In support of this Boateng *et al* (1998) showed that a sub-antihypertensive dose of Ramipril, normalised Ca^{2+}-ATPase expression and function following cardiac hypertrophy, independent of blood pressure changes in the rat. Normalisation was associated with inhibition of cardiac ACE activity.

ACE inhibition may also help protect against ischaemic injury. Cargnoni *et al* (1994) found a cardioprotective effect from ischaemia using Quinaprilat, independent from haemodynamic changes or direct reduction of oxygen free-radical toxicity, thought to be related to a reduction in the release of noradrenaline, maintenance of high energy phosphates and membrane integrity. Moreover chronic ACE inhibitor treatment at sub-antihypertensive doses has been shown to induce myocardial capillary growth and improve cardiac metabolism in SHRs (Unger *et al* 1992, Gohlke *et al* 1993). Thus further investigation is needed into the reversal of cardiac hypertrophy using ACE inhibitors, particularly with respect to normalising substrate utilisation and protection against the progression to heart failure.

Publications

Abstracts

XVII European section of the International Society for Heart Research 1997:

SJ Clarke, K Bulmer, JC Chatham, A-M L Seymour. Modulation of substrate selection by pyruvate dehydrogenase. JMol.Cell.Cardiol; 29, 5: A85, 1997.

XXI American section of the International Society for Heart Research 1999:

SJ Clarke, A-ML Seymour. Effects of insulin on fuel selection in cardiac hypertrophy. JMol.Cell.Cardiol; 31,5:H4, 1999.

Papers

Clarke SJ, Seymour A-M L. Substrate competition in cardiac hypertrophy (in preparation for Am J Physiol).

Clarke SJ, Seymour A-M L. Effects of high workload on substrate selection in prolonged cardiac hypertrophy (in preparation).

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Appendix:

Appendix 1.1. Calculations of metabolite concentrations.

Calculation of tissue dilution to correct for μmoles / g wet mass of tissue:

metabolite concentration (μmoles/ml) x $\frac{\text{total neutralising volume}}{\text{known volume of PCA}}$ x $\frac{1}{\text{g dry tissue in known volume}}$

Glutamate and Glutamine assay.

Glutamate calculation:

The concentration of glutamate was calculated as follows:

$$C = \frac{\Delta A \text{ sample}}{\Delta A \text{ standard}} \times c \text{ standard (mM)}$$

Glutamine calculation:

L-glutamine concentration (mM) was detemined using the following equation:

$$C = \frac{V \times MW}{\varepsilon \times d \times v \times 1000} \times \Delta A$$

where: V= final volume (ml)
MW = molecular mass of substance assayed (g/L)
ε = extinction coefficient of formazan at 492nm (19.9 mmol/L x l xcm^{-1})
d = light path (cm)
v = sample volume (ml)

Glucose concentration was calculated as follows:

Serum glucose (mg/dL) = $\frac{\text{A test}}{\text{A standard}}$ x 100 x dilution factor

Glucose concentration (mM) = mg/dL x 0.056.

Calculation of Free Fatty Acid Concentration:

$$C = \frac{V}{\varepsilon \times d \times v} = \frac{1.15 \times \Delta A}{19.3 \times 1 \times 0.05}$$

where: V = final volume (ml)
v = sample volume (ml)
d = light path (cm)
ε = absorption coefficient of the dyestuff at 546nm.

Calculation of Triglyceride concentration:

$$\text{Triglycerides (mg/dL)} = \frac{\text{A test - A blank}}{\text{A standard - A blank}} \times \text{Concentration of standard} \times \text{Dilution factor}$$

Triglyceride concentration (mM) = mg/dL x 0.0113.

Calculation of β - Hydroxybutyrate (β-HBA) concentration:

A blank = Final A blank - Initial A blank
A standard = Final A standard - Initial A standard
A sample = Final A sample - Initial A sample

$$\text{Serum β-HBA (mg/dL)} = \frac{\Delta A \times V \times MW \times 100}{\varepsilon \times v \times 1000}$$

where: V = total reaction volume (ml)
MW = molecular mass of β-HBA
100 = conversion of mg/ml to mg/dL
ε = millimolar absoptivity of NADH at 340nm
v = sample volume
1000 = conversion of μg to mg

β-HBA concentration (mM) = mg/dL x 0.096.

Calculation of lactate concentration:

Serum lactate concentration (mmol/L) was calculated as follows:

$$C\ (\text{mmol/L}) = \frac{\Delta A\ 340 \times V}{\varepsilon \times v \times d}$$

where: ΔA 340 = final maximum absorbance at 340nm
V = reaction volume (ml)
ε = millimolar absorptivity of NADH at 340nm
v = volume (ml) of sample in cuvette
d = lightpath (cm).

Appendix 2.1. **Morphology of nine week hypertrophied and control hearts.**

Treatment Groups	Heart	Left Kidney	Right Kidney	Adrenal Gland	Spleen
Control (n=30)	6.24 (0.26)	3.74 (0.04)	3.70 (0.05)	2.76 (0.05)	3.82 (0.02)
Hypertrophy (n=33)	5.86 (0.22)	3.72 (0.06)	3.75 (0.05)	2.73 (0.04)	3.86 (0.01)

Appendix Table 2.1. Wet to dry mass ratios in nine week hypertrophied and control rats (means +/- SEM).

Appendix 2.2. Morphology of control DCA heart study.

Treatment Groups	Body Mass (g)	Heart Mass (g)	Heart Mass/ Body Mass
Glu (n=7)	349.17 (16.44)	1.96 (0.07)	0.01 (0.00)
Glu+Hex (n=7)	356.20 (18.45)	1.87 (0.09)	0.01 (0.00)
Glu+Hex+1mM DCA (n=5)	360.20 (20.16)	2.09 (0.11)	0.01 (0.00)
Glu+Hex+2mM DCA (n=6)	282.35* (3.99)	1.67* (0.04)	0.01 (0.00)
Glu+Hex+5mM DCA (n=6)	255.88* (8.92)	1.61* (0.03)	0.01 (0.00)

Appendix Table 2.2. Morphology of control groups (means +/- SEM). * Indicates significant difference compared to other group means, $p<0.05$).

Treatment Groups	RBC	TG mmol/l	Lactate mmol/l	Glucose mmol/l	HBA mmol/l	FA mmol/l
Glu (n=7)	0.63 (0.01)	1.40 (0.26)	7.25 (1.53)	14.09 (1.72)	0.14 (0.07)	1.54 (0.29)
Glu+Hex (n=7)	0.67 (0.02)	1.01 (0.09)	5.63 (0.79)	12.04 (0.67)	0.17 (0.06)	1.55 (0.32)
Glu+Hex+1mM DCA (n=5)	0.62 (0.03)	0.81 (0.23)	7.25 (1.98)	13.60 (1.78)	0.25 (0.09)	1.04 (0.38)
Glu+Hex+2mM DCA (n=6)	0.67 (0.02)	1.22 (0.20)	4.59 (1.49)	11.50 (1.07)	0.06 (0.04)	1.67 (0.32)
Glu+Hex+5mM DCA (n=6)	0.67 (0.02)	0.94 (0.08)	9.81 (2.30)	15.25 (2.08)	0.09 (0.04)	0.78 (0.32)

Appendix Table 2.3. Plasma characteristics of control groups (means +/- SEM).

Appendix 3.1. **Morphology of fifteen week hypertrophied and control hearts.**

Treatment Groups	Heart	Left Kidney	Right Kidney	Adrenal Gland	Spleen
Control (n=13)	5.73 (0.06)	3.78 (0.03)	3.77 (0.03)	2.55 (0.02)	3.75 (0.01)
Hypertrophy (n=12)	5.75 (0.05)	3.78 (0.05)	3.60 (0.06)	2.55 (0.03)	3.68 (0.01)

Appendix Table 3.1. Wet to dry mass ratios in fifteen week hypertrophied and control rats (means +/- SEM).

Appendix 4.1. **Morphology of rats in high workload study.**

Treatment Groups	Body Mass (g)	Heart Mass (g)	Tibia Length (cm)	Left Kidney Mass (g)	Right Kidney Mass (g)
Control (n=8)	585.63 (14.65)	2.70 (0.03)	4.59 (0.01)	1.80 (0.02)	1.82 (0.03)
Hypertrophy (n=9)	567.22 (4.65)	3.07* (0.02)	4.65 (0.01)	1.56* (0.02)	2.00^{+} (0.03)

Appendix Table 4.1. Morphology following fifteen weeks aortic constriction for use in a high workload study. (* significantly different from the control, $^{+}$ significantly different compared to the left kidney).

Treatment Groups	Lung Wet / Dry Ratio	Liver Mass (g)	Adrenal Mass (g)	Spleen Mass (g)
Control (n=8)	3.95 (0.01)	18.84 (0.46)	0.03 (0.00)	0.74 (0.05)
Hypertrophy (n=9)	3.84 (0.02)	18.62 (0.23)	0.03 (0.00)	0.84 (0.03)

Appendix Table 4.2. Morphology relating to heart failure following fifteen weeks aortic constriction for use in a high workload study.

Treatment Groups	Heart	Left Kidney	Right Kidney	Adrenal Gland	Spleen
Control (n=8)	7.62 (0.15)	4.01 (0.02)	3.98 (0.02)	2.64 (0.14)	3.83 (0.01)
Hypertrophy (n=9)	7.95 (0.26)	3.67 (0.09)	4.43 (0.18)	2.49 (0.07)	3.79 (0.03)

Appendix Table 4.3. Wet to dry mass ratios in fifteen week hypertrophied and control rats in the high workload study (means +/- SEM).

Treatment Groups	pH	pCO_2 mmHg	pO_2 mmHg	RBC Ratio	K^+ mmol/l	Na^+ mmol/l	Ca^{2+} mmol/l	Cl mmol/l	Glucose mmol/l	Lactate mmol/l
Control (n=3)	7.8 (0.05)	21.9 (2.4)	174.3 (2.1)	0.7 (0.03)	4.6 (0.2)	140.3 (0.3)	0.8 (0.04)	98.0 (0.5)	10.8 (0.04)	3.6 (0.5)
Hypertrophy (n=3)	7.8 (0.01)	20.9 (0.7)	174.8 (5.1)	0.6 (0.03)	5.1 (0.1)	138.7 (0.3)	0.9 (0.01)	98.3 (1.2)	11.0 (0.70)	3.4 (0.4)

Appendix Table 4.4. Plasma characteristics after 15 weeks aortic constriction used in the high workload study (mean +/- SEM).

Group	Perfusion Phase	pH	K^+ mmol/l	Na^+ mmol/l	Ca^{2+} mmol/l	Cl^- mmol/l	Glucose mmol/l	Lactate mmol/l
Control (n=4)	**Beginning**	7.63 (0.03)	6.05 (0.05)	145.5 (1.16)	0.94 (0.02)	121.67 (0.97)	5.12 (0.04)	0.08 (0.01)
	Low work	7.64 (0.02)	6.11 (0.07)	147.1 (1.89)	0.95 (0.01)	123.29 (1.84)	5.19 (0.07)	0.06 (0.01)
	High work	7.57 (0.01)	6.00 (0.04)	143.8 (0.94)	0.97 (0.00)	118.75 (0.95)	5.01 (0.04)	0.23 (0.02)
	End	7.57 (0.01)	6.00 (0.05)	145.0 (1.44)	0.98 (0.00)	119.50 (1.40)	5.13 (0.04)	0.18 (0.04)
HT (n=4)	**Beginning**	7.60 (0.02)	5.92 (0.04)	141.8 (0.94)	0.94 (0.01)	117.60 (1.08)	4.96 (0.05)	0.16 (0.02)
	Low work	7.61 (0.02)	6.17 (0.06)	147.7 (1.43)	0.96 (0.01)	123.14 (1.42)	5.09 (0.07)	0.07 (0.02)
	High work	7.58 (0.02)	6.06 (0.08)	146.38 (1.99)	0.94 (0.00)	122.63 (2.36)	5.01 (0.05)	0.19 (0.02)
	End	7.55 (0.04)	6.03 (0.12)	145.0 (3.31)	0.98 (0.01)	120.75 (3.15)	5.03 (0.14)	0.30 (0.09)

Appendix Table 4.5. Effluent metabolite concentrations for fifteen week hypertrophied and control hearts in the high workload study (mean +/- SEM).

Appendix 5. Statistical Limitations

Appendix 5.1. Low sample populations

Several experiments presented in this thesis contained low sample populations. This was particularly evident in the ^{13}C NMR substrate utilisation studies in Chapter 5, rendering it impossible to calculate the statistical significance for these heart groups. Therefore the majority of the subtrate utilisation work in Chapter 5 represents a preliminary study and care has to be taken when drawing any conclusions from the results.

Ideally all experiments would have contained sample populations of eight or more, however this was not always possible for a number of reasons. The animals were subjected to strict exclusion criteria and were removed from studies if they exhibited necrosed kidneys as a result of surgery or under-perfusion during the Langendorf heart preparations as evidenced by low function and poor incorporation of ^{13}C labelling in the heart extracts. If an animal was excluded from one category it was subsequently removed from all experimental analyses. Although adequate sample sizes were obtained for the morphology sections of Chapter 5, the fifteen week hypertrophied and control hearts had to be further divided into perfusion experiments containing substrates labelled with either ^{13}C-glucose or ^{13}C-palmitate, each with and without the presence of insulin. This ultimately reduced the numbers in each substrate group, below the level acceptable for statistical analysis.

Futhermore, the work in this was thesis restricted to a three year time scale. When this was coupled to the fifteen week period required for the model of

prolonged hypertrophy to develop and time necessary to perform the experiments and analyse the results by NMR, it meant there was less opportunity to increase the sample sizes for the substrate utilisation experiments in Chapter 5.

Appendix 5.2. Statistical significance testing.

Comparison of the significant difference between two group sample means was assessed using the Student t-test. The major assumption using this test was that the population from which the sample was drawn was a normal population. The reason being that a number of natural phenomena are normally distributed. However this assumption is less reliable if the sample size is small, which was the case for a number of studies in this thesis. In experiments where the sample populations were small it may have been more appropriate to use non-parametric tests, such as the sign test. Non-parametric tests have the advantage that they do not require many assumptions and are best employed in testing situations in which the normality assumption is not satisfied and the sample size is not large. The sign test in particular only has to statisfy the assumptions that the population being sampled is a continuous population and that the population has a median.