THE INFLUENCE OF DIETARY FATTY ACIDS ON CARDIAC FUNCTION

A thesis submitted for the degree of

DOCTOR OF PHILOSOPY

by

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I'd give all wealth that years have piled,

The slow result of life's decay,

To be once more a little child

For one bright summer-day.

Rev. Charles Lutwidge Dodgson

to the child who always asks questions

to the power of a child's dream

CONTENTS

ABBREVIATIONS

$\delta P/\delta t$	Pressure-Time Integral
μmol	micro moles
Σ	sum of
AA	arachidonic acid
ADP	adenosine diphosphate
AL	afterload
ATP	adenosine triphosphate
bpm	beats per minute
C	control group
CCCP	carbonyl cyanide-*m*-chlorophenyl hydrazone
CHD	ischaemic coronary heart disease
CPP	coronary perfusion pressure
DHA	docosahexaenoic acid
DMA	dimethyl acetate
dw	dry weight
E-C	excitation-contraction
ECG	electrocardiogram
EPA	eicosapentaenoic acid
FO	polyunsaturated fish oil
g	gram
Hz	hertz
J	joules
K-H	Krebs-Henseleit solution
Kg	kilogram

LA	linoleic acid
LV_{fp}	left ventricular filling pressure
M	molar
min	minutes
mL	millilitres
mmHg	millimetres of mercury
mmol	millimoles
ms	milliseconds
MVO_2	myocardial oxygen consumption
NAD^+	nicotinamide adenine dinucleotide (oxidised)
NADH	nicotinamide adenine dinucleotide (reduced)
P_i	inorganic orthophosphate
PDH	pyruvate dehydrogenase
PL	preload
PO_2	oxygen partial pressure
PTI	Pressure-Time Integral
PUFA	polyunsaturated fatty acids
RBC	red blood cell perfusate
REF	reference diet
rpm	revolutions per minute
RR	ruthenium red
RY	ryanodine
SAT	saturated sheep fat supplemented diet
SCD	sudden cardiac death
SD	standard deviation
sec	seconds
U	units
VFT	ventricular fibrillation threshold
VPB	ventricular premature beats
VT	ventricular tachycardia
w/w	weight per weight

ABSTRACT

The aim was to study the direct effects of dietary fatty acids on myocardial function in rats. In particular, the effects of dietary fat intake on cardiac function stressed with myocardial ischaemia and reperfusion were investigated. Although dietary fat can influence the development of atherosclerosis, thrombosis, cardiac ischaemia and myocardial infarction, little is known of any direct effects by dietary fat on cardiac performance. It has also been clearly demonstrated that the fatty acid profile of myocardial phospholipids are predominantly dependent on the qualitative properties of dietary lipid intake. Such alterations in the cellular lipid environment may be associated with a direct dietary fatty acid influence on cardiac function.

Hooded-Wistar rats (4months old) were placed into 3 dietary groups: REF, a reference base diet or a 12% (w/w) addition of either saturated fatty acid rich sheep fat (SAT) or fish oil rich in polyunsaturated marine n-3 fatty acids (FO). Animals were maintained on the diets for a minimum of four months prior to experimental use. In order to directly study cardiac function and precisely control electrolyte and metabolic substrate availability, neural and humoral factors, preload, workload, humidity and temperature, the isolated working heart method was selected. To overcome limitations which reduce the suitability of most isolated working heart models of global ischaemia for the study of the progression of ischaemic injury, a new model of low flow global ischaemia was developed. This method did not cause total cessation of ventricular function or coronary flow and thus permitted investigation of ischaemic processes as they occurred, by simultaneous

measurement of ventricular function, oxygen uptake and metabolite release in venous outflow. This new model utilised a novel placement of two valves to permit coronary perfusion pressure reductions with maintained afterload, to provide a greater ischaemic insult yet allowing simultaneous functional and metabolic evaluation. In addition, a buffer with washed porcine erythrocytes at 40% haematocrit in a modified Krebs-Henseleit/dextran solution was utilised for improved oxygen delivery, viscosity, colloid osmotic pressure (to reduce oedema) and improved mechanical performance on which to impose the ischaemic insult.

Under control conditions, compared to REF hearts, SAT hearts demonstrated an elevated MVO_2 with no performance dividend while FO hearts had reduced MVO_2 with no performance deficit. The higher oxygen delivery in SAT hearts was achieved by intrinsically raised coronary flow. Ischaemic production of lactate, cellular efflux of K^+, creatine kinase, development of venous acidosis and increased arrhythmia vulnerability were enhanced in SAT hearts and reduced in FO hearts. Better post-ischaemic recovery of functional performance was evident in reperfused FO than in SAT hearts. A paradoxical increase in MVO_2 (despite reduced coronary flow, contractility and external work) was observed during ischaemia and reperfusion in all groups except SAT hearts. However, MVO_2 remained higher in SAT hearts during ischaemia and reperfusion compared to REF and FO hearts. The dietary differences in MVO_2 were still evident following equalisation of coronary flow with hydralazine but were abolished in K^+ arrested hearts. Maintenance of a constant diet-related MVO_2 differential

despite work related increases and during contractile inhibition by ryanodine suggest an activation-dependent mechanism not linked to contraction. Rather the abolition of the high MVO_2 in SAT hearts by ruthenium red indicates a role of mitochondrial Ca++.

This thesis study has demonstrated the advantages of utilising an isolated working heart method which uses an erythrocyte buffer that allows oxygenation in the physiological range and a method of global ischaemia that is more appropriate for the study of the progression of ischaemic injury. The new model enhanced the capacity to control and directly monitor experimental ischaemic events in progress in a manner that may be more physiologically relevant than previous models and permitted observations that would have not been possible by alternative methods. Although definitive identification of the link between myocardial membrane fatty acid composition and intracellular functional changes was not provided by this study, the results confirm and provide a possible basis for the widely reported antiarrhythmic or proarrhythmic actions of fish oil or fatty acids respectively.

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of this thesis. All experimental work was conducted solely by myself with the exclusion of the fatty acid analyses of diet and myocardial tissue samples as specified in the "Acknowledgements". I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying in accordance with the copyright laws.

Salvatore Pepe

Date: 20/11/91

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I. GENERAL INTRODUCTION

I.1. Ischaemic Coronary Heart Disease

Ischaemic coronary heart disease (CHD) is the major cause of mortality in the economically advanced nations of Western Society. As a result of this widespread condition, not only is there the premature loss to the individual, but also to the nation. In Australia, it is estimated that CHD is responsible for the loss of 2000 million dollars per year to the economy (**Leeder,1988**). It has been of great interest to observe that since 1967 there has been a small (3-5% per annum), but steady fall in mortality due to CHD (acute myocardial infarction and sudden cardiac death) (**Leeder & Wilson 1987;Jackson & Beaglehole,1987; Hobbs,*et al.*,1984; Thompson,*et al.*, 1988; Hetzel,*et al.*,1989**). The enormous improvement in medical diagnosis and treatment, with the advent of superior resuscitation and support equipment, new pharmacological agents, angioplasty and coronary artery by-pass operations, does not account for the large fall in first incidence sudden cardiac death. Indeed the decline of CHD in Australia, U.S.A, Canada, Belgium, Norway, Finland and New Zealand over the last 20 years may be related to increased health awareness/education regarding physical exercise and dietary habit. The fall in CHD may be associated with the increased consumption of polyunsaturated cooking oil/margarine, chicken and fish, as well as the reduced consumption of eggs, butter, sheep (cooking) fat and cigarettes (**Thompson,*et al.*,1988**). Despite this decline, according to the most recent Australian Government record of mortality in Australia, heart disease-related disorders accounted for 32.4% of all deaths in Australia, making this the

leading cause of death in 1989. Coronary heart disease was the cause of death in 81.1% of the people who died of heart related disorders, or in 26.3% of all deaths (**Australian Bureau of Statistics,1990**). The magnitude of this wastage of human life necessitates better management of cardiac health which must include fundamental research into the underlying influences in the mechanisms of CHD.

I.2. Defining Myocardial Ischaemia

Clinically, CHD is manifested as angina pectoris, congestive heart failure, cardiac arrhythmias and sudden cardiac death. Sudden cardiac death occurs almost always following acute cardiac arrhythmias, specifically, ventricular fibrillation, which leads to an abrupt loss of effective circulation and thus consciousness. This syndrome can occur unexpectedly and rapidly, within one hour of onset of acute symptoms, in an individual with or without pre-diagnosed heart disease (**Myerburg & Castellanos,1988**). Pathological conditions which underlie CHD may include varying combinations of: atherosclerosis of the coronary arteries, vasospastic myocardial ischaemia, and thrombosis. Ischaemia has traditionally been described as the reduction in coronary artery blood flow resultant from the lumenal narrowing of coronary blood vessels due to the build up of lipids, cholesterol and atherosclerotic plaques on the vessel walls thus restricting the delivery of oxygen and metabolic substrates to myocardial tissues, (including the removal of myocardial metabolites). The most severe condition would be complete occlusion. If coronary artery occlusion persists, irreversible injury of

ischaemic cardiac cells occurs leading to myocardial infarction (**Jennings & Reimer,1991**).

Within the definition of myocardial ischaemia, coronary artery blood flow and oxygen delivery must be considered in concert with the energy demands of the myocardium. In healthy humans, increased physical activity requires increased energy availability and thus more oxygen is made available because of increased coronary artery blood flow. Humans with atherosclerosis will not have the same coronary "reserve" and may not be able to meet the energy requirements of exercise or emotional stress. This can result in anginal pain or further consequences even though no ischaemia existed at rest. Non-exertional angina indicates a greater severity of ischaemia. Vasospastic angina may occur even at rest where coronary vasospasm can induce ischaemia (**Nakamura,1985**).

I.3. Consequences Of Ischaemia

Myocardial ischaemia starts a cascade of biochemical alterations that eventually lead to cellular insult and finally irreversible myocardial injury and necrosis (myocardial infarction). The primary consequence of ischaemia is an imbalance between myocardial demand for oxygen and oxygen supply via coronary artery blood flow. This state of oxygen and metabolic substrates deficit is closely followed by a build up of heat and metabolites such as lactate, carbon dioxide, and protons which can eventually contribute to toxic acidosis (**Considine,1985**).

Oxidative phosphorylation in myocardial mitochondria produces the high energy phosphate, adenosine triphosphate (ATP). This process is the only one that can meet all of the myocardium's needs. The major substrate for the production of energy in cardiac muscle are the fatty acids. Indeed, up to 90% of oxygen consumption is utilised in the oxidation of fatty acids (**Whitmer,*et al.*,1978**). The unavailability of oxygen due to ischaemia halts aerobic oxidation of fatty acids, glucose and lactate in the Krebs' cycle. Anaerobic glycolysis commences but yields only 2 moles of ATP per mole of glucose, which is insufficient for energy demands and leads to a rapid impairment of cardiac contractility. Due to restricted blood flow, the accumulation of end products of anaerobic glycolysis, (nicotinamide-adenine dinucleotide, hydrogen ion, and lactate), attenuate further anaerobic glycolysis (**Kobayshi & Neely, 1979;Neely & Feuvray,1981**).

Isochronously there is a decreased availabilty of glutamate, aspartate and other amino acid precursers of Krebs' cycle intermediates due to altered amino acid metabolism. This prevents the formation of ATP by an alternative source and it has been proposed that it is a contributing factor that limits the resumption of oxidative metabolism during reperfusion (**Penney & Cascarno 1970; Bittl & Shine,1983; Mudge,*et al.*,1976**).

With the reduced presence of ATP there is an inhibition of ATP-dependant sodium potassium ATPase pumps which regulate cell volume. This produces alterations to the flux and distribution of monovalent ions. Cellular water, sodium and chloride increase while potassium and magnesium ions are lost from the cell (**Buja,*et al.*,1983**). In addition to the onset of oedema,

increased cell membrane permeability to extracellular calcium raises the concentration of intracellular calcium. ATP-dependant calcium pumping into the sarcoplasmic reticulum ceases and can lead to mitochondrial damage due to calcium overload as ATP exhaustion occurs later in the mitochondria **(Nayler,1981a; Jennings & Reimer,1983)**.

The above scenario (by no means thorough), leads to plasma membrane damage. Membrane integrity is lost with the increase in membrane fluidity and permeability, such that intracellular enzymes are released from the cell (including creatine kinase, lactate dehydrogenase & malate dehydrogenase, **Hearse & Humphrey,1975**). Associated with these events, electrical instability ensues with the spontaneous depolarisation of myocytes, giving rise to ectopic ventricular contractions and the possibility of ventricular fibrillation **(Nayler,1981b)**. Increased extracellular $[K^+]$ at the onset of ischaemia has been associated with reduced conduction, altered refractoriness and arrhythmias **(Kleber,1987)**. Considerable evidence also implicates the involvement of intracellular Ca^{++} overload and hence can even occur prior to significant cell damage **(Tani'90)**.

I.4. Coronary Risk Factors For Sudden Cardiac Death (SCD)

Of age, heredity, gender and race, only age and gender have been shown to contribute most to the risk of SCD **(Myerburg & Castellanos,1988)**. It has been reported that 76% of CHD deaths in the 20 to 39 year age group occur suddenly and without notice **(Kuller,*et al.*,1966)**. In the 45 to 54 year age group 62% of CHD deaths were sudden and this proportion decreased to 58%

in the 55 to 64 age group and 42% in the 65 to 74 year age group although the absolute incidence continues to increase with age. **(Doyle,*et al.*1976; Kannel & Thomas,1982).**

The lowest incidence of SCD has been observed in women and it has been proposed to be due to the low incidence of atherosclerosis in premenopausal women **(Shatzkin,*et al.*,1984a;1984b)**. Even with the low SCD incidence women are prone to similar CHD risk factors as men. In a 20 year study the male:female ratio of SCD incidence has been shown to peak at 6.75:1 in the 55 to 64 year age group and this then falls to 2.17:1 in the 65 to 74 year age group **(Doyle,*et al.*1976;Kannel & Thomas,1982)**.

Most risk factors are related to lifestyle. Cigarette smoking and obesity are closely associated with the incidence of CHD and SCD **(Kannel & Thomas,1982)**. Although it has been suggested that there is a relationship between low levels of exercise and increased CHD death **(Paffenbarger,*et al.*,1977)**, a high incidence of SCD has been reported to occur at high levels of exercise **(Kannel & Thomas,1982)**. In addition, a significant association between psychosocial stress due to isolation, high levels of life-stress and SCD has been observed **(Rahe,*et al.*,1974;Friedman & Rosenman,1959)**. It has also been reported that increased cigarette smoking, alcohol consumption, obesity and reduced regular exercise occurred with greater frequency in persons with low levels of education **(Lambert,*et al.*,1982)**. Indeed, in a study of female sudden death victims, a history of psychiatric treatment, cigarette smoking, greater alcohol consumption, and lower levels of education

were more prevalent than in age-related controls from the same neighbourhood (**Talbott,*et al.*,1977**).

Although age, systolic blood pressure, heart rate, ECG abnormalities, serum cholesterol and triglycerides, relative body weight, cigarette consumption, and lung vital capacity are recognised risk factors for CHD, no relationship or particular risk factor emerges as a predictor of SCD. It is recognised though, that hypertension is a highly significant risk factor for SCD (**Doyle,*et al.*,1976;Kannell,*et al.*,1975**). However, in general, no specific indicator is currently known to predict the onset of SCD.

Sudden cardiac death by definition precludes its victims from prophylactic antiarrhythmic drug therapy as most of them would have exhibited no significant prior symptom of cardiovascular disorder (**Lown,-1980; Buxton,1986; Keefe,*et al.*,1987**). By reducing the incidence of CHD the proportion of people predisposed to SCD incidence would be expected to decrease also. It is of great importance to establish an alternative to *apost-eriori* medical treatment, one that prevents or limits the extent of CHD, reducing its expense to medical and human resources. The findings discussed below and in the following chapters indicate that the type of dietary fat intake may provide a means of limiting the incidence or severity of CHD in part by altering cardiac performance during increased cardiac stress, post-ischaemic recovery and myocardial vulnerability and sensitivity to SCD trigger events. This thesis has focused on the type of dietary lipid as a potential modulator of CHD incidence, post-ischaemic survival in CHD dysfunction and vulnerability to SCD, by investigating dietary fatty acid influences on basic

mechanisms and parameters of cardiac physiology that are independent of effects on coronary vasculature.

I.5. Dietary Fatty Acids

Epidemiological studies of Eskimo and Japanese fishing communities have indicated that there may be an association between increased dietary intake of long chain omega-3 (n-3) polyunsaturated fatty acids (PUFAs) and CHD (**Dyerberg,1986;Bang,*et al.*,1976;Goodnight,*et al.*,1982**). As a result of such observations and previous experimental studies, research interest has developed the idea that increasing PUFAs and decreasing saturated fatty acids in the diet may reduce the incidence of CHD predominantly by lowering plasma triglyceride and cholesterol levels, lowering systemic blood pressure, reducing the incidence of thrombosis and limiting the development of atherosclerosis (**Leaf & Weber,1988;Harris,1989;Kinsella,*et al.*,1990; Knapp,1990**). Most research effort has thus been expended investigating the effect of dietary fatty acids on artherosclerosis and thrombosis with little regard for possible direct effects on myocardial performance and metabolism.

However, it has been well demonstrated (**Charnock,*et al.*,1985;1986; Abeywardena,*et al.*,1986;1987**) that the composition of myocardial phospholipid fatty acids can be altered by modification of dietary fat intake. Cardiac function may be affected as a result of change to the composition of myocardial membrane fatty acids that influence calcium fluxes, membrane enzyme and receptor systems and eicosanoid synthesis (**Egwin & Kummerow, 1972;Farias,*et al.*,1975;Spector & Yorek,1985;Lands,1979;Willis,**

1981). Although altered fatty acid composition of myocardial membranes are proposed to influence these cellular systems, to date most of these findings have been observed in isolation and as yet little is known of the role of dietary fatty acids in the relationship between membrane composition and whole heart function. PUFA dietary supplements of linoleic acid (LLA; n-6, vegetable origin), eicosapentaenoic acid and docoshexaenoic acid (EPA & DHA respectively; n-3, marine origin), have been reported to reduce thrombogenesis (**Goodnight,*et al.*,1982**) as well as decrease the susceptibility to arrhythmias induced by catecholamines (**McLennan,*et al.*,1987**) or coronary artery ligation (**McLennan,*et al.*,1985**), *in vitro* and *in vivo*. Saturated animal fats have been reported to be thrombogenic and arrhythmogenic in these animal models. Considering that nonesterified fatty acids are the preferred energy substrate of the healthy myocardium, it is feasible that dietary differences may directly influence energy requirements, oxygen utilisation and the production of metabolites. Dietary fat type may more (or less) readily facilitate the events, or modify the time course or magnitude of ischaemia.

I.6. Fatty Acids In Myocardial Ischaemia

In the ischaemic myocardium, fatty acids, fatty acid esters, triglycerides, acyl coenzymeA and acyl carnitine accumulate causing the inhibition of a variety of enzymes and processes (**Corr,*et al.*,1981**). It has been proposed that fatty acid esters, such as lysophospholipids, act as detergents and can destroy membranes (**Kinnaird,*et al.*,1988**). Another event of ischaemia is the activation of polymorphonuclear leukocytes. These leukocytes contain lipoxygenase

enzymes which metabolise the membrane fatty acid arachidonic acid (AA) to produce substances which are xenobiotic to the healthy myocardium (**Mullane, *et al.*,1987**).

Membrane fatty acids may act as precursers of eicosanoids. In the presence of lipoxygenase,the fatty acids AA, EPA and DHA contribute to the synthesis of the leukotrienes and other lipoxygenase products, while in the presence of cyclo-oxygenase they contribute to the production of prostaglandins, prostacyclins and thromboxane (**Willis,1981**). Notably, many of these eicosanoids are either vasoactive and/or influence platelet aggregation, thus it follows that they may affect cardiac function. Both EPA and DHA interfere with eicosanoid synthesis to produce the benefits described by a mechanism(s) that is not fully elucidated (**Cashman,1985**). The metabolism of membrane fatty acids also serves as a source of oxygen-derived cytotoxic free radicals. Although many organisms have peroxidase, catalase and superoxide dismutase enzyme systems that normally catalyse oxygen-derived free radicals to form water, their efficacy is limited during the course of ischaemic injury. The increase of destructive oxygen metabolites can influence fatty acid metabolism and membrane integrity and myocardial viability (**Simpson,*et al.*,1987**).

I.7. Models of Myocardial Ischaemia

Investigations into myocardial ischaemia have in the past been conducted by various methods (see **Ross,1972; Neely & Rovetto,1975; DeLeiris,*et al.*,1984;Manning,*et al.*,1980; Foëx,1988**):

i) Whole animal studies involve surgical intervention whereby the coronary arteries are ligated (regional ischaemia) in a manner which can also allow reperfusion (**Bajusz & Jasmin,1964**). For experiments with awake animals the animal must be allowed to recover first and trained for the experimental protocol while in other studies the experiment is conducted under anaesthetic. The precise measurement of coronary flow, myocardial oxygen utilisation and metabolic end products in venous outflow is difficult with this approach unless large animals such as dogs, sheep, and pigs are used. This approach usually limits animal numbers, as such experiments are expensive in terms of maintenance, materials, time and experiment personnel. This method is most successfully implemented in the study of regional ischaemia-induced arrhythmias.

ii) Isolated tissue studies include experiments that utilise portions of atria, ventricles, or papillary muscles from animals that may/may not have been excised from hearts that previously experienced coronary ligation. These have been used successfully in studies of electrolyte and/or pharmacological action where muscular contraction is the measurable end point. However this approach usually investigates post-ischaemic/post-infarction performance or hypoxia rather than ischaemia.

iii) Isolated myocyte culture experiments are particularly effective at a molecular and cellular level of investigation, but limited with direct studies of cardiac function during ischaemia.

iv) Isolated heart preparations are used to investigate metabolism and haemodynamic ventricular function because these methods offer numerous

advantages over whole animal preparations, including the exclusion of exogenous neural and humoral influence on cardiac function. In particular, it is possible to precisely control coronary perfusion pressure, perfusate concentrations of substrates, salts, hormones, metabolites, pH, and PO_2. This model includes the ability to measure physiological parameters of function such as coronary flow, contractility and myocardial oxygen consumption and venous effluent can be assayed for substrate utilisation and release.

It is disturbing to see studies of myocardial ischaemia that test the potency of various pharmacological products to reduce the extent of injury induced in Langendorff and working heart models of global ischaemia without careful consideration of physiological oxygenation (utilisable oxygen), maintained workloads (oxygen demand) and coronary perfusion pressures. The consequences of using inadequate techniques are perilous and expensive in the long term. It is clear that these commonly used methods are inadequate if the ultimate aim is to relate functional and metabolic parameters of the isolated heart under conditions with or without ischaemia to the *in vivo* situation.

A suitable model according to Fallen, *et al.*, **(1967)**, should at least satisfy the following criteria:

i) The oxygen supply must meet the energy demands of the working heart.

ii) The preparation must be stable for the entire experimental period. This means no change in the ventricular pressures should occur under control conditions.

iii) All physical variables known to influence cardiac function should be easily controlled and measured (e.g. coronary flow, coronary perfusion pressure, systolic pressure, diastolic pressure, oncotic pressure, oxygen tension of media, heart rate, stroke volume, pH and temperature).

iv) The arterial concentration of perfusion fluid should be constant and the rate of substrate utilisation easily measured.

v) The apparatus should be simple in design and easy to assemble and clean. Such a model may have widespread use throughout a number of disciplines investigating cardiac function and heart disease.

The following chapter considers previous methods and their limitations and presents a new model of global ischaemia that is tested against a previous best model, and in addition, the use of a red blood cell perfusate is compared to the traditional Krebs-Henseleit solution.

I.8. Thesis Aims

The aim of this doctoral study at its commencement in late March 1988 was to investigate the influence of dietary saturated animal fat and dietary fish oil on cardiac function and myocardial ischaemia. Specifically, the aim was to test the hypothesis that prior feeding of a diet high in omega n-3 polyunsaturated fatty acids (deep sea fish oil origin) to laboratory animals may provide a degree of protection during ischaemia and permit improved post-ischaemic recovery in the isolated perfused working heart, devoid of neural

and humoral influence. In addition, prior feeding of a diet high in saturated fatty acids (peri-renal sheep fat), may worsen the detrimental effects of ischaemia as well as retard and attenuate post-ischaemic recovery in isolated working hearts. The previous findings of McLennan *et al.*, **(1985-90)** from whole animal and isolated papillary muscle experiments provide the basis for the above hypotheses. Although the influence of dietary fat type on some basic physiological parameters including ischaemic and reperfusion arrhythmias have been identified by these workers, the basis is unclear. Thus, study at the isolated heart level (without neural and humoral influence) is necessary in order to directly measure basic cardiac function and test the above hypotheses. Such an approach could permit a closer investigation of the events during the progression of ischaemic injury and the underlying mechanisms by which it occurs. Prior to achieving this objective it was perceived that a new model of global ischaemia in the isolated working heart had to be developed which superceded the critical limitations of previous models by being inexpensive, not oxygen limited and allowing controllable, graded ischaemia while maintaining a normal work load. The principal aim of this thesis was to implement such a model of global ischaemia to investigate whether dietary fat type can influence the cardiac responses to ischaemia.

The work of this thesis has focused firstly, in Chapter II, on the development of a new experimental model and secondly, in Chapter III, on the use of the new method in order to obtain information regarding the influence of dietary lipids on cardiac physiology and metabolism that could not be observed by alternative techniques (Chapter III). In Chapter IV the influence

of dietary fats on the vulnerability of the erythrocyte-perfused isolated working heart to cardiac arrhythmias induced by ischaemia, reperfusion or programmed ventricular stimulation is assessed. In Chapter V the role of coronary flow differences in the influence of dietary fatty acids on myocardial performance is investigated utilising the vasodilator hydralazine in order to standardise coronary flow rates between dietary regimes. Chapter VI involves the study of intracellular calcium handling and metabolism in the possible mechanism underlying both the paradoxical increase in oxygen consumption during and after ischaemia and the effects of dietary lipids on cardiac function and metabolism. In Chapter VII the influence of diet on physiological response to a range of altered preloads and coronary perfusion pressures (degrees of ischaemia) is examined in the presently developed isolated working heart. The final experimental chapter, Chapter VIII, studies the effectiveness of different levels of n-3 fatty acids to produce the physiological effects observed in Chapter III. In Chapter IX the findings of this project are discussed, their significance is interpreted and the direction and mode of future investigation is proposed.

II. GENERAL METHODS

The Maintained Afterload Model of Global Ischaemia In The Erythrocyte Perfused Isolated Working Rat Heart

II. 1. Introduction

Myocardial ischaemia has been studied under a great number of conditions but to date, simple methods utilising small animals have often been lacking in their capacity to simultaneously impose ischaemia upon a continuously functioning heart and to provide a means of following the ischaemic event while in progress. Isolated heart preparations are used to investigate metabolism and haemodynamic ventricular function because these methods offer numerous advantages over whole animal preparations, including the exclusion of exogenous neural and humoral influence on cardiac function. In particular, it is possible to precisely control coronary perfusion pressure, workload, heart rate, perfusate concentrations of substrates, salts, hormones, metabolites, pH, and PO_2. Together these variables influence cardiac output and myocardial oxygen consumption.

The Langendorff isolated heart preparation **(Langendorff,1895)** uses an oxygenated electrolyte solution such as Krebs-Henseleit buffer **(Krebs & Henseleit,1932)** for retrograde perfusion through the aorta to supply the coronary arteries. As the left ventricle conducts no volume-work it has a low metabolic demand and oxygen consumption compared to isolated working hearts with the same coronary flow rate **(Neely,*et al.*,1967)**. Ventricular work and cardiac output cannot be evaluated and restriction of coronary flow and/or

oxygen supply in this "low demand" situation provides a less than satisfactory model of *in vivo* ischaemia. Adaptations to insert into the left ventricle a small fluid filled balloon that is attached to a pressure transducer, permit pressure development to be measured (isovolumic pressure work model (**Fallen,*et al.*, 1967;Opie,1967**)). This has been popular with researchers because of the ease of using this model. However the model is quite invasive for rat or other small hearts as it can involve the cutting, tearing, and/or piercing of atria, mitral valves, papillary muscles and the apex of the left ventricle either unintentionally or to accommodate ventricular balloons (**Kligfield,*et al.*,1976; Miller,*et al.*1987;Serur,*et al.*,1976;Vogel,*et al.*,1980**).

Neely, *et al.*, (**1967**), developed the isolated working rat heart preparation in order to permit the measure of aortic output, coronary flow, external work and ventricular pressure development. These parameters, plus oxygen and substrate uptake, provide valuable information regarding metabolic status and performance of isolated hearts that can, with qualification, be extrapolated to the heart *in vivo*. Only the "*working*" heart preparation has this capacity. Hearts perfused in modified Langendorff fashion can only develop "*tension*". The working heart method offers immediately visible evidence of alterations in myocardial function as measured by changes in pressure development and aortic output. Perfusate is supplied to the left atrium at a specific preload pressure and passes into the left ventricle during diastole. The left ventricle contracts in systole, ejecting the perfusate via the aorta against a fixed hydrostatic pressure head or afterload. During diastole, the afterload of the pressure head closes the aortic valve and causes perfusate to enter the

coronary arteries at a perfusion pressure that is determined by the afterload. This method has been extremely valuable for the study of myocardial function and metabolism.

Studies of the effect of ischaemia in the working heart have encountered a number of difficulties. Regional ischaemia, similar to that caused by coronary vasospasm or atherosclerosis and thrombosis, can be induced in the working heart preparations by ligating (reversibly) a coronary artery **(Bajusz & Jasmin,1964)**. A disadvantage of using regional ischaemia is that accumulated metabolites cannot be collected from the region during ischaemia. Assessment of the metabolic and functional state of the discrete ischaemic region is particularly difficult in small hearts. Coronary ligation experiments though have been valuable for the study of ischaemia-induced arrhythmias **(Bernier,*et al.*,1989)**, long term regional ischaemia and infarction **(Bester,*et al.*,1972)**, or for investigation of the effects of reperfusion **(Bolli,1990;Greenfield,*et al.*, 1988;Jennings & Reimer,1983)**. Global ischaemia, on the other hand, will affect the entire ventricular myocardium, thus assessment of whole heart function and metabolism is truly representative of the ischaemic region **(Manning,*et al.*,1980)**.

In order to induce global ischemia, Neely, *et al.*, **(1973)** modified their original working heart preparation, by adding a one-way ball valve in the aortic cannula, close to the coronary orifices. This means that during systole, ejection of the left ventricle contents can occur against the hydrostatic pressure head, but diastolic back pressure would close the ball valve, prevent coronary perfusion and produce ischemia. Cardiac output was severely reduced within

30 sec of ischemia and ventricular failure occurred within 5 to 10 min. Apart from the rapidity of heart failure, the disadvantage with this model is that the degree of ischaemia produced cannot be adjusted. In subsequent studies these workers used forced coronary perfusion, utilising a mechanical pump connected to the aortic cannula, in order to control the coronary flow rate following ventricular failure early in the ischaemic period (**Neely,*et al.*,1975**). Figure II. 1A depicts the normal working heart while the method described above and two other commonly utilized methods (described below) are depicted in Figure II. 1; B, C and D. Panels E and F in Figure II. 1 represent the principle of the new ischaemic method developed in the present study.

In an alternative method (Figure II. 1C), investigators induce brief global ischemia in the working heart, (10-60 min depending on species), by clamping the aorta. This leads to total cessation of coronary flow and cardiac function. During the remaining period of ischemia these hearts do not conduct "work". Upon unclamping the aorta to allow coronary reperfusion, the heart often will not restart in working mode. The majority of studies have required a period of perfusion in Langendorff mode prior to returning to working heart mode (**Greenfield,*et al.*,1988;Humphrey,*et al.*,1985;Snoeckx,*et al.*,1986**). It has often been necessary to maintain post-ischaemic Langendorff perfusion for 10-20 min during which time the heart does no work (low metabolic demand) and thus the study of immediate post-ischaemic function is not possible. While this method has become the method of choice for studying the post-ischaemic ventricular mechanical dysfunction that occurs even in the absence of irreversible myocardial damage (the "stunned myocardium" (**Bolli,**

1990;Braunwald & Kloner,1982)), it is of little value for the study of ischemia.

The limitation of this and other models of ischemia that rapidly curtail ventricular function and cardiac output is that the events *during* the progression of ischemia cannot be closely observed, only reperfusion injury and post-ischaemic myocardial performance can be studied. Indeed, during the ischaemic period such hearts are no longer "working", thus the requirement for oxygen and energy are less than may be expected during an ischaemic episode *in situ*.

One model that does permit ventricular ejection throughout the ischaemic period involves lowering the aortic hydrostatic pressure head to decrease coronary perfusion pressure **(Haneda,*et al.*,1986;Ichihara,*et al.*, 1980;1981;1983)**. However in doing so, the afterload is also reduced. This means that less work is done to eject ventricular contents and less energy is required by the myocardium. Thus, although coronary perfusion is reduced, so are the metabolic requirements of the heart and the consequences of ischemia will be less than expected if the heart was attempting to work as it does in the control period.

Other working heart models utilise hypoxia rather than ischemia by reducing the oxygen content of the perfusate **(Whitmer,*et al.*,1978)**. Although the workload is maintained, coronary flow is not reduced (and may increase), and the build up of toxic metabolites that may play a major role in the ischaemic response cannot occur. The use of hypoxia has been most useful in pharmacological and electrophysiological experiments that utilise isolated

myocytes, ventricular strips, atria, or papillary muscles in small volume organ baths (**Foëx,1988**). Ischemia will occur if oxygen and other substrates are limited in supply to the myocardium so that the metabolic demand cannot be met, or the cardiac metabolic demand increases at a rate and magnitude that cannot be supported by a restricted supply of substrates. This premise, along with the rare clinical occurrence of hypoxia compared to the incidence of ischemia related cardiac injury, limits the value of hypoxic models.

A further common disadvantage of all of the above-mentioned methods is the use of low viscosity oxygenated physiological electrolyte solution as the perfusate. Although these solutions can be highly saturated with oxygen, the oxygen carrying capacity is well below the physiological level (**Bergman,*et al.*,1979;Gaudel,*et al.*,1985**). This can lead to relative hypoxia even under control conditions, and to compensate there is an abnormally high degree of coronary vasodilation and increased coronary flow. When coronary flow decreases in response to low flow global ischemia, oxygen availability declines, altering metabolism and myocardial function at levels of coronary flow that are high compared to *in vivo* (**DeLeiris,*et al.*,1984**). The contrast in oxygen availability between normoperfusion and ischaemia is thus reduced and ischaemic autoregulatory mechanisms may be already partially activated in the coronary vasculature prior to the intended ischemia. A number of workers have expressed concern and provided some evidence in support of deficient oxygenation of the myocardium by erythrocyte-free buffers (**Duvelleroy,*et al.*,1976;Gaudel,*et al.*,1982;1985;Gibbs,*et al.*,1980**). In addition, as perfusion time increases, these solutions produce oedema and associated ionic

imbalances because they have an inadequate colloid osmotic pressure (**Buja,*et al.*,1983;Cobbe & Poole-Wilson,1980;Fallen,*et al.*,1967;Nakamura,1985; Neely & Feuvray,1981**). It is possible to eliminate these problems with the use of blood to increase the oxygen carrying capacity. Early attempts to perfuse rat hearts with blood experienced limited success, with poor performance and rapid heart failure due to the quality of the blood; protein denaturation, fat embolism, blood coagulation, haemolysis, and inadequate filtration (**Duvelleroy,*et al.*,1976;Gamble,*et al.*,1970;Gaudel,*et al.*,1985;Neely & Rovetto,1975**). The use of a live blood donor animal has the advantages of physiological oxygenation and metabolite clearance but contains the complication of physiologically active humoral substances, and anaesthetic agents. The approach also overcomes problems of blood coagulation and filtration however it is expensive, laborious, and especially with animals having a small blood volume, difficult (**Gamble,*et al.*,1970;Miller,*et al.*, 1987;Satoh,*et al.*,1986;Tosaki,*et al.*1988;Vogel & Lucchesi,1980;Wetstein, *et al.*1984**). These difficulties have caused most researchers to avoid the use of blood perfusates, but at great cost to the efficacy of their model, and relevance of their data to the *in vivo* state. However a small number of researchers have turned to the use of washed erythrocytes resuspended in a solution of physiological salts, substrates, and agents which increase colloid osmotic pressure (**Bergman,*et al.*,1979;Duvelleroy,*et al.*,1976;Gaudel,*et al.*, 1985;Ichihara,*et al.*1981;Isoyama,*et al.*,1987;Oye,*et al.*,1964**). Platelets, plasma lipids, white cells and other humoral substances are removed by centrifugation washing of erythrocytes, thus preventing the blood coagulation

and other difficulties encountered with whole blood perfusion. Continuous artificial oxygenation of the perfusate can occur by gas exchange across a large surface area without bubbling the buffer **(Duvelleroy,*et al.*,1976)**. With careful filtration and a well considered heart perfusion apparatus design, red blood cell fragment presence due to abrasive interactions with perfusion pumps and glassware can be minimised. Such a preparation is an inexpensive and easily prepared perfusate that is valuable to heart function experiments in providing data which is relevant *in vivo*.

The aim of this study was to develop an inexpensive, erythrocyte perfused, working heart model of controllable graded ischemia without altering work load and preload. One that allows the study of myocardial function and metabolism *during* an ischaemic event.

II. 2. Methods

II. 2. a) Perfusion media

The Krebs-Henseleit solution (K-H), pH 7.4, consisted of the following final concentrations (in mM): NaCl, 118; KCl, 3; $MgSO_4$, 1.164; KH_2PO_4, 1.18; $NaHCO_3$, 24.88; $CaCl_2$, 1.4; glucose, 11.1. This buffer was filtered, oxygenated with a humidified gas mixture of 95% O_2 and 5% CO_2 and maintained at 37°C. Pig blood (Metropolitan Meat Co., Mt. Barker, South Australia) was collected into a solution (100ml/l of blood) consisting of sodium citrate 38% w/v and 10 times concentrated K-H, without $CaCl_2$ at pH 7.4. After gentle stirring it was carried to the laboratory on ice. The blood was filtered through sheets of fine cotton poplin, and stored in 500ml polyethylene containers at 4°C. Although the blood was viable for perfusion for up to 2 weeks, (without the addition of neomycin sulphate and adenine), all blood was used within 5 days of storage. On the day of use the blood was centrifuged with a Beckman J14 rotor at 4°C, 2500 r.p.m., in a Beckman J2-21 centrifuge for 15 min. The supernatant and buffy coat were removed by suction and the erythrocytes were resuspended to the original volume in ice cold 0.9% saline. The erythrocytes were washed in saline 3 times for 15 min. at 2500 r.p.m., 4°C. Prior to the 3rd wash the red blood cell suspension was again filtered through cotton poplin sheets. The washed red blood cells were resuspended to a haematocrit of 40% in an oxygenated (95% O_2, 5% CO_2) solution that consisted of the following (final concentrations in mM): NaCl,118; KCl,3; $MgSO_4$,1.164; KH_2PO_4, 1.18; $NaHCO_3$,24.88; glucose,11.1; $CaCl_2$,1.4 and Dextran (70000 m.w.), 15g/l. The KCl and $CaCl_2$ concentrations were used

according to the findings of Curtis **(1989)** and Yamamoto, ***et al.*, (1984)**. High molecular weight dextran (available as Macrodex solution, Pharmacia, Uppsala, Sweden) was selected to increase the colloid osmotic pressure of the perfusate **(Duvelleroy,*et al.*,1976)**. This red blood cell buffer (RBC) was continuously oxygenated as it slowly passed over the walls of a glass bubble column as a thin film against the flow of humidified 95% O_2, 5% CO_2 gas (Figure II.2).

II. 2. b) Working Heart Preparation

Male Hooded Wistar rats weighing 350-500 g were fasted overnight before use. Rats received 1000 I.U. of heparin i.p. 1 hour before they were sacrificed by cervical dislocation. The thoracic cavity was rapidly opened by lateral incisions along the rib cage and transversely across the top of the abdominal cavity and the wall of the anterior chest was folded back to reveal the cavity. The pericardium and associated tissues were gently pulled away from the heart with fingers moistened with isotonic saline solution and the heart was picked up and rapidly excised with a single cut allowing sufficient length to remain to the blood vessels arising from the heart. The heart was placed in a beaker of ice cold 0.9% sodium chloride. Within a few seconds the contractions were arrested. Each heart was transferred to a petri dish containing ice cold saline. Excess pericardium and aortic tissue were quickly trimmed and using fine tipped forceps the aorta was slipped a few mm onto the perfusion cannula and secured with ligature. Perfusion of the coronary vessels was commenced immediately in Langendorff mode, with Krebs-

Henseleit buffer (37°C) at a pressure of 75 mmHg. This allowed the heart to resume spontaneous rhythm, equilibrate the buffer substrate concentrations with the interstitial fluid and remove blood from the coronaries. During this period (10 min) the left atrium and the pulmonary artery were cannulated, platinum electrocardiogram recording electrodes were attached to the ventricular apex and the portion of the aorta above the ligature. For paced hearts (Narco SI-10 stimulator, 300 b.p.m., 2 ms duration at twice the threshold voltage) stainless steel electrodes were attached to the right atrium. All hearts were placed in a glass water jacket chamber to maintain the myocardium at 37°C and at constant high humidity. The entire perfusion apparatus was contained in an air conditioned perspex cabinet maintained at 37°C.

II. 2. c) Experimental design

The purpose of this study was to compare the performance of working hearts perfused in the mode of Neely *et al.*, (1967), with either Krebs-Henseleit solution (K-H), or the erythrocyte perfusate (RBC) described above, and to compare two methods of low flow global ischemia that utilise these buffers. The method of reduced afterload (RA) ischemia,(**Haneda,*et al.*1986;Ichihara,*et al.*,1980;1981;1983**), was selected for comparison to the newly developed "micro-valve" method of maintained afterload (MA) ischemia, (described below), because both permit ventricular ejection, aortic output and collection of venous effluent throughout the ischaemic period.

Hearts were allocated into four experimental groups:

i) K-H perfused working heart with RA ischaemia.

ii) K-H perfused working heart with MA ischaemia.

iii) RBC perfused working heart with RA ischaemia.

iv) RBC perfused working heart with MA ischaemia.

The working heart perfusion mode, was initiated by opening the tube that supplied perfusate to the left atrium from the atrial overflow bubble trap (Figure II. 2) and the Langendorff aortic inflow tract was switched off to allow systolic outflow through the aorta. The perfusate was supplied to the left atrium at a preload pressure of 10 mmHg via the pulmonary vein. Left ventricular contraction in systole, ejected the perfusate via the aorta against a fixed hydrostatic pressure head (75 mmHg).

In the maintained afterload (MA) model a stainless steel one-way microvalve (Nupro) was placed between the aorta and an elasticity chamber. During diastole, afterload back pressure kept the microvalve (valve 1) closed, preventing coronary perfusion at the afterload pressure (Figure II.1.,2). Between the aorta and the first one-way microvalve an inlet from a second one-way microvalve placed in reverse and therefore kept closed by systolic pressure allowed perfusate to flow from a jacketed reservoir to the coronary blood vessels during diastole. Under control conditions, while the afterload (valve 1) and the coronary reservoir perfusion (valve 2) remained equal at 75 mmHg, the valves were without influence and a normal working heart configuration was in effect. Low flow global ischemia was induced by quickly lowering the coronary perfusion reservoir to provide a reduced coronary

perfusion pressure of 35 mmHg while maintaining the afterload at 75 mmHg (MA).

In the reduced afterload (RA) model hearts were perfused in working heart mode under the same control conditions, with a preload of 10 mmHg and afterload of 75 mmHg. Low flow global ischemia was induced by reducing the afterload from 75 mmHg to 35 mmHg, thus reducing coronary perfusion pressure to 35 mmHg.

Both groups were subdivided such that hearts were either perfused with Krebs-Henseleit throughout or with erythrocyte buffer when they were switched from Langendorff to working heart mode. All hearts were subjected to 20 min ischemia after a 20 min control working heart period. Reperfusion was attained by either raising the coronary perfusion reservoir (MA) or the afterload height (RA). Arterial perfusate was sampled from the atrial reservoir and venous coronary effluent was sampled from the pulmonary artery cannula in order to measure PO_2, PCO_2 and pH with an IL 813 blood gas analyser (Instrumentation Laboratories). Coronary flow was periodically measured by timed collection of effluent from the pulmonary artery cannula and the right atrium. Coronary effluent was not recirculated. Aortic output pumped to the afterload height was periodically measured with a calibrated tube and recirculated. The RBC perfusate was continuously filtered by a series of in-line poplin fabric filters placed after a Gilson Minipuls 2 pump. The perfusate was replenished between each perfusion in order to maintain an arterial pH of 7.4 and to prevent the possible build up of any red cell fragments and haemolysis products from the RBC perfusate, that may have been produced by abrasion

in glassware or pump tubing, and were too small to be removed by filtration. Aortic pressure was measured with a Statham P23Db pressure transducer (Gould, Oxnard,CA), and with the electrocardiogram, was traced onto chart paper by a Grass Instrument Co. 79D polygraph. At the end of each perfusion, hearts were dissected separating the atria, right and left ventricles, blotted and weighed. Tissues were then dried in an oven at 100°C until a constant dry weight was determined.

II. 2. d) Calculations

Cardiac output was calculated as the sum of coronary flow and aortic output. External cardiac work was calculated as the product of mean aortic pressure, cardiac output per gram dry weight, and the coefficient 1.333222 x 10^{-4}, which expresses work in J/min (**Duvelleroy,*et al.*,1976**). The pressure-time integral was determined from the product of average aortic systolic pressure (mmHg), ejection time (sec) and heart rate (b.p.m.) (**Neely,*et al.*,1967**). The content of oxygen dissolved in solution was calculated as the Bunsen Solubility Coefficent (α) x PO_2 in mL/mL solution (α=0.0237 for blood; α=0.0240 for Krebs solution). The combined oxygen was calculated as the haemoglobin concentration (g/mL) x % saturation x haemoglobin oxygen content (mL/g) and expressed as mL O_2/mL perfusate. For porcine erythrocytes haemoglobin concentration a 40% haematocrit was calculated to be 0.14g/mL and haemoglobin oxygen content as 1.38mL/g. The % saturation was determined as $(PO_2/P_{50})^n/1+(PO_2/P_{50})^n$x 100 (for porcine erythrocytes: P_{50}=31; n=2.59; Bohr Effect=-0.42). For % Saturation below 100% the P_{50}

was corrected for the change in pH and the new P_{50} was substituted in the % Saturation calculation (Correction=P_{50} at pH7.4 x log(Bohr Effect x change in pH)). The oxygen content of perfusate was calculated as the sum of oxygen combined with erythrocytes and oxygen dissolved in solution. Myocardial oxygen extraction was calculated from the arterial-venous oxygen content difference and expressed as a percentage of arterial oxygen content. Myocardial oxygen consumption (mmol/min/g) was calculated as the product of the arterial-venous O_2 content difference and coronary flow per gram dry weight (**Gamble,*et al.*,1970**).

II. 2. e) Statistical Analysis

Results are expressed as the arithmetic mean $\pm$ standard deviation. Ten hearts were used in each of the experimental groups. To test for a significant effect of RBC and K-H in the working heart under control conditions parametric t-tests were calculated. The presence of a significant effect of perfusate type and mode of global ischemia was tested by a 2-way analysis of variance for each parameter investigated. Individual comparisons between groups were made with *Scheffe's post hoc test* in order to minimise the possibility of Type I errors. A significant probability was considered at the level of $P<0.05$ or less.

II. 3. Results

II. 3. a) Performance of Unpaced Hearts

Hearts were not weighed prior to their cannulation and perfusion in order to minimise the period of non-experimental ischaemia that occurred between the moment of heart removal from the rats until reperfusion. Figure II. 3 shows the left ventricle wet and dry weights in each group measured at the conclusion of each perfusion. Left ventricle wet weights following K-H perfusion were significantly higher (+31.2 %) than those hearts perfused with RBC perfusate irrespective of the apparatus configuration. To standardise flow rates and other measures, all calculations and units are expressed as g dry weight.

Table II. 1 presents the characteristics of the K-H and RBC perfusates in the working rat heart in our hands. No significant differences in arterial pH and PCO_2 were measured between K-H and RBC perfusates. Although K-H buffer had an arterial PO_2 nearly three times that of the RBC perfusate, it had only 1/15th the arterial oxygen content. The PO_2 measured in coronary effluent from K-H perfused hearts was 68% higher than from RBC perfused hearts. The highest percentage oxygen extraction was measured in K-H perfusion (76%), compared to RBC perfusion (15%) of hearts, yet more than three times as much oxygen was taken up by RBC perfused hearts in absolute terms.

Coronary flow rates in all experimental groups are presented in Figure II.4. Under control working heart conditions, (CPP=75 mmHg), the coronary flow was significantly greater in K-H perfused hearts than in RBC perfused

hearts. No significant difference in coronary flow was observed between RA and MA perfusion configurations. Reduction of coronary perfusion pressure to induce ischaemia decreased coronary flow significantly in all groups (measured after 5 min). The largest decrease was observed in the coronary flow of RBC perfused hearts. Notably, by 20 min of ischaemia coronary flow had increased to return towards pre-ischaemic coronary flow rates in all groups. The greatest degree of coronary artery hyperaemia was seen after 20 min of maintained afterload ischaemia. In addition to this, Figure II. 5 shows that control perfusion with K-H buffer resulted in a lower aortic output compared to RBC buffer. During RA ischaemia, hearts had a higher aortic output than MA ischaemic hearts. Compared to control perfusion, aortic output measured during ischaemia decreased to negligible levels in K-H perfused hearts and very low levels in RBC perfused hearts.

Myocardial contractility under control conditions was significantly greater in RBC perfused hearts as indicated by the pressure time integral (PTI) standardised for heart rate, (Figure II. 6). During ischaemia PTI decreased in all groups, but this occurred least in K-H perfused hearts where the PTI was already low during control perfusion. The reduction in PTI during ischaemia was greater with the MA than the RA configuration. External work was maintained relatively equal across all groups under control conditions (Figure II.7). During the ischaemic period, external work conducted by the hearts decreased by 79-94%. The largest reductions in work were observed during MA ischaemia. Measurement of perfusate pH revealed a significant increase in

venous hydrogen ion content during MA ischaemia that was not observed with RA ischaemia (Figure II. 8).

Under control conditions myocardial oxygen consumption was significantly greater in RBC perfused hearts (Figure II. 9). Oxygen consumption during ischaemia was paradoxically increased in the RBC perfused hearts but not significantly in the K-H perfused hearts. During reduced afterload ischaemia a higher rate of oxygen consumption was apparent compared to hearts treated to maintained afterload ischaemia with RBC buffer.

Heart rates differed significantly between the groups presented in Figure II. 10. Under control conditions the heart rate in RBC perfused hearts was significantly lower than in K-H perfused hearts. While ischaemia reduced the heart rate across the groups, during perfusion with RBC buffer, significantly lower heart rates were measured with the induction of maintained afterload ischaemia.

II. 3. b) Correction for Heart Rate: Paced Hearts

In order to circumvent the confounding effect of the differing heart rates in RA and MA ischaemia with RBC perfusate a further series of experiments were conducted using paced hearts. Hearts were paced at 300 bpm throughout the perfusion period and were also subjected to reperfusion following 20 min of ischaemia. With pacing, cardiac output was higher than in the unpaced hearts under control conditions. The presence of microvalves had no effect on coronary flow rates measured in paced hearts under control perfusion (Figure II. 11). No significant coronary flow rate difference was

observed between RA and MA ischaemia, however, coronary flow was considerably lower than in unpaced hearts at 20 min ischaemia (Figure II. 4). Pacing hearts at 300 bpm prevented the hyperaemic increase in coronary flow following 20 min of ischaemia that was previously observed in unpaced hearts which slowed during ischaemia. There was no significant difference in aortic output between the groups in paced RBC perfused working hearts (Figure II. 12). Reducing the coronary perfusion pressure to 35 mmHg revealed a significantly lower aortic output during maintained afterload ischaemia compared to reduced afterload ischaemia. Reperfusion of hearts was conducted by returning coronary perfusion pressure to 75 mmHg. After 5 min reperfusion aortic output reached 80% and 56% recovery of pre-ischaemic levels following reduced afterload ischaemia and maintained afterload ischemia, respectively.

Left ventricular external work decreased significantly during reduced and maintained afterload ischaemia, compared to control perfusion (Figure II. 13). Upon reperfusion, hearts treated to maintained afterload ischaemia generated less external work (55% of control) than those subjected to reduced afterload ischaemia (76% of control). While the pressure-time integral was reduced following both reduced afterload and maintained afterload ischaemia, the latter tended to have a lower PTI. Indeed, following reperfusion, MA ischemia produced a poorer level of recovery of the pressure-time integral (67% of control) (Figure II. 14). A significant fall in venous pH occurred during ischaemia, (Figure II. 15). Hearts subjected to maintained afterload ischaemia had a significantly lower pH compared to RA ischaemia.

In these paced heart experiments the control oxygen consumption did not differ significantly between the groups. Oxygen consumption paradoxically increased in both reduced and maintained afterload ischaemia, but the largest increase in oxygen consumption occurred in maintained afterload ischaemia (Figure II. 16). When reperfusion was permitted after 20 min of ischaemia, myocardial oxygen consumption remained at an increased rate in both groups. The group subjected to maintained afterload ischaemia still had a significantly higher rate of oxygen consumption. This increased consumption during ischaemia was related to a greater increase in oxygen extraction in the group with maintained afterload, and this was even more marked upon reperfusion (Figure II. 17).

Table II. 1 Effect of RBC and K-H perfusates in isolated working hearts at 10 mmHg preload, 75 mmHg workload. K-H =Krebs Henseleit perfusate; RBC = red blood cell suspension in modified KH buffer. * =$p<0.05$, significant difference, K-H v RBC, Student's t-test, n=20. See Methods for details.

	Perfusate	
	K-H	RBC
Haematocrit %	0	40
ARTERIAL MEASURES:		
Haemoglobin sat. %	-	99.7 ± 0.07
PO_2 mmHg	443.0 ± 21.0	*159.5 ± 10.0
O_2 Content mL O_2/mL	0.012 ± 0.002	*0.188 ± 0.001
PCO_2 mmHg	36.9 ± 2.8	36.8 ± 5.3
pH	7.433 ± 0.022	7.395 ± 0.014
VENOUS MEASURES:		
Haemoglobin sat. %	-	72.5 ± 4.2
PO_2 mmHg	87.2 ± 11.0	51.9 ± 4.0
O_2 Content mL O_2/mL	0.003 ± 0.001	*0.159 ± 0.007
PCO_2 mmHg	36.25 ± 3.1	*45.20 ± 5.7
pH	7.302 ± 0.009	7.309 ± 0.015
ARTERIAL-VENOUS:		
O_2 Difference mL O_2/mL	0.009 ± 0.002	*0.029 ± 0.02
O_2 Extraction %	76.86 ± 6.05	*15.44 ± 0.09

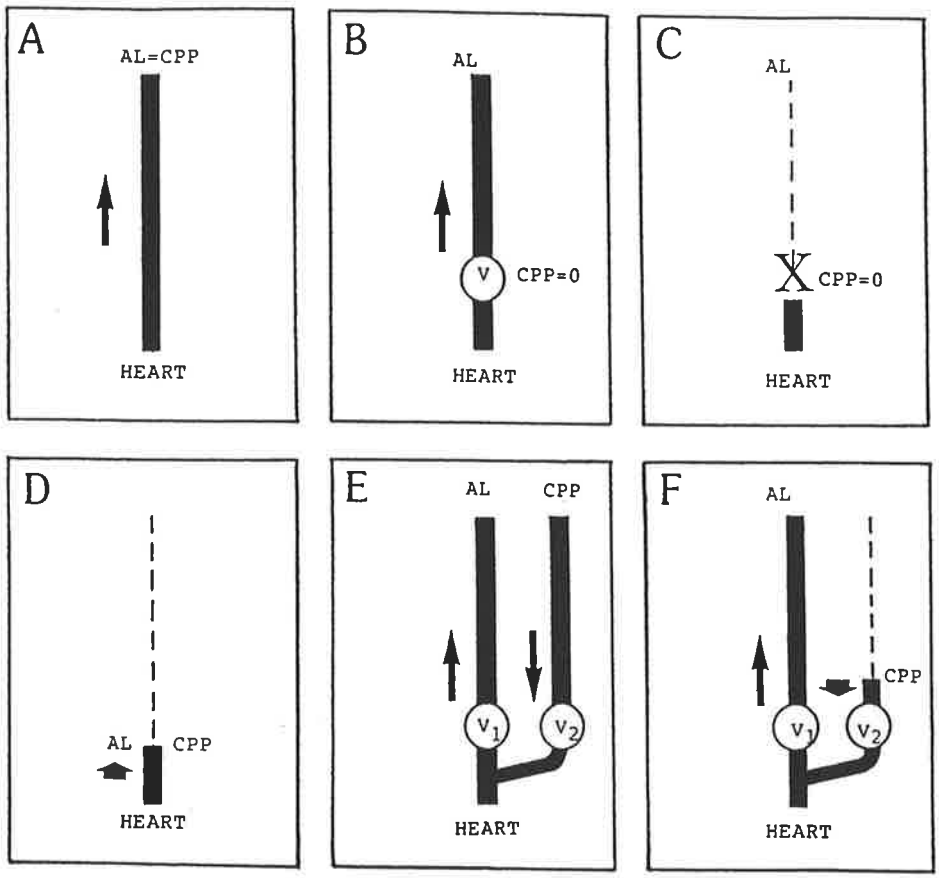

Figure II. 1 The schematic representation of 3 models of global ischemia.

AL=afterload; CPP=coronary perfusion pressure; H=isolated working heart; V=one-way valve.

A. During normal working heart perfusion the heart performs work against an AL that also determines the CPP.

B. Placing a one-way valve in the aortic cannula prevents coronary perfusion during diastole without changing the AL. Rapid ventricular failure prevents assessment of cardiac function during the progression of ischemia.

C. Clamping the aorta prevents coronary perfusion. This is usually conducted with either the reduction of preload or clamping of the left atrial cannula. Rapid ventricular failure occurs.

D. Reducing AL results in a reduction in CPP. The heart has a low aortic output but maintains ventricular ejection. Due to the reduced AL, the heart workload is low and thus has a reduced metabolic demand compared to panel F. (Reduced Afterload model of ischemia (RA)).

E. CPP and AL are maintained at an equivalent level during normal working heart. V_1 opens in systole while V_2 (placed in reverse) opens in diastole. (While AL=CPP this configuration is equivalent to panel A.).

F. CPP can be reduced while maintaining AL. In diastole V_1 closes preventing coronary perfusion, however V_2 opens to allow a perfusate to enter the coronary arteries at a reduced CPP. Thus ischemia can be induced at a maintained workload (Maintained Afterload model of ischemia (MA)).

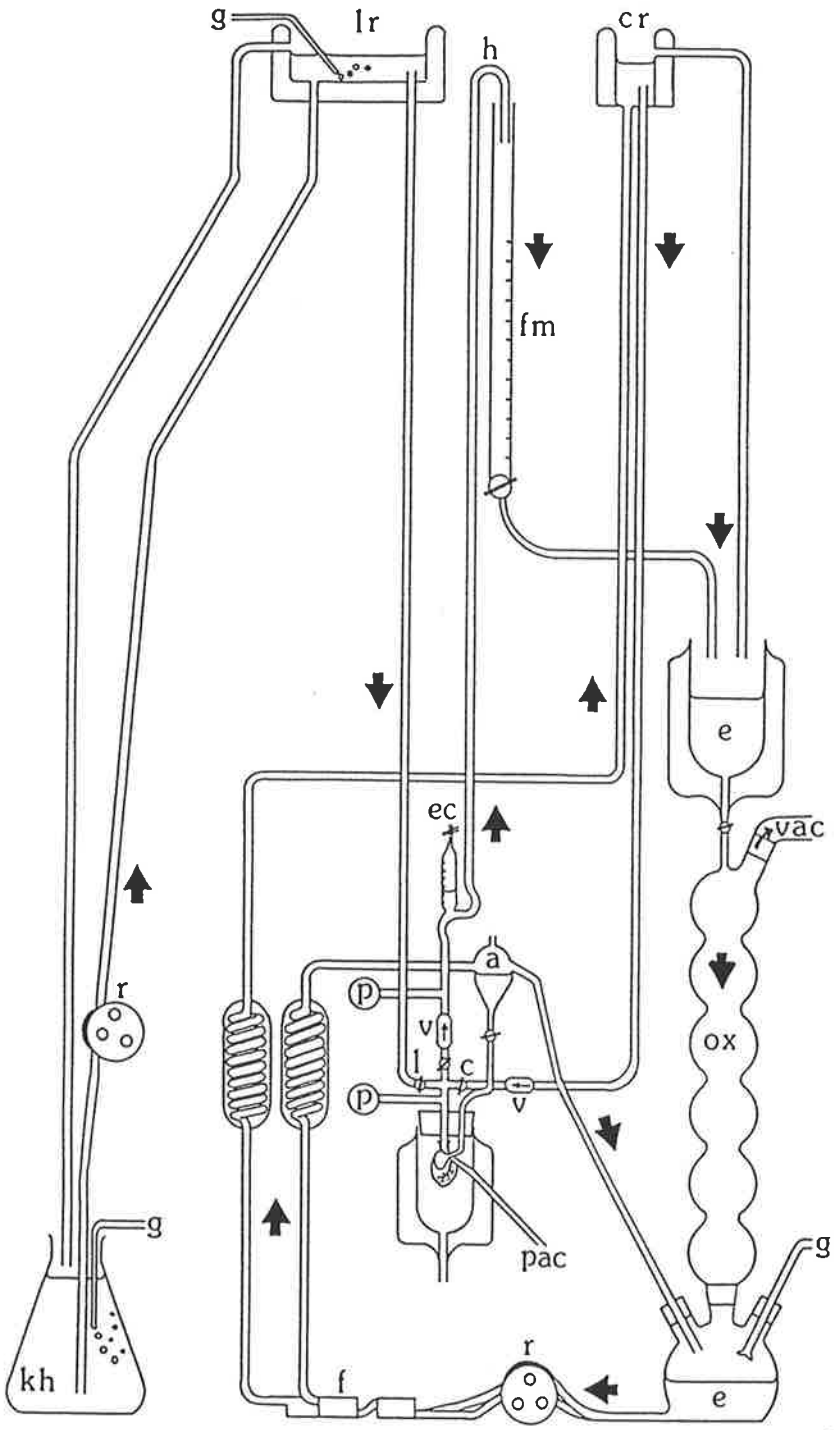

Figure II. 2 Scheme of the modified working heart preparation and Langendorff perfusion circuit.

Langendorff perfusions with Krebs-Henseleit buffer (K-H) for 10 min at a coronary perfusion pressure (CPP) of 75mmHg while cannulation of pulmonary arteries (pac) and electrode placement occurred. Working heart perfusion started by closing tap l and opening tap c and the atrial perfusion reservoir and bubble trap, a. For maintained afterload ischemia experiments 2 stainless steel one-way microvalves were placed as indicated by v (direction of flow is indicated by arrows). Ischemia was induced by reducing cr, the coronary perfusion reservoir, from a CPP of 75mmHg to 35mmHg. For reduced afterload ischemia experiments both valves were absent, and ischemia was induced by lowering h, the height of the afterload from a CPP of 75mmHg to 35mmHg. All glassware was jacketed and the entire apparatus is housed in an airconditioned perspex cabinet. The complete system is kept at 37°C.

ec= elasticity chamber, e= RBC perfusate (for K-H perfusions e= K-H), f= filters, fm= aortic flowmeter, g= 95% O_2, 5% CO_2, ox= RBC oxygenater, p= pressure transducer, r= roller pump, vac= low vacuum pressure. See Methods for more detail.

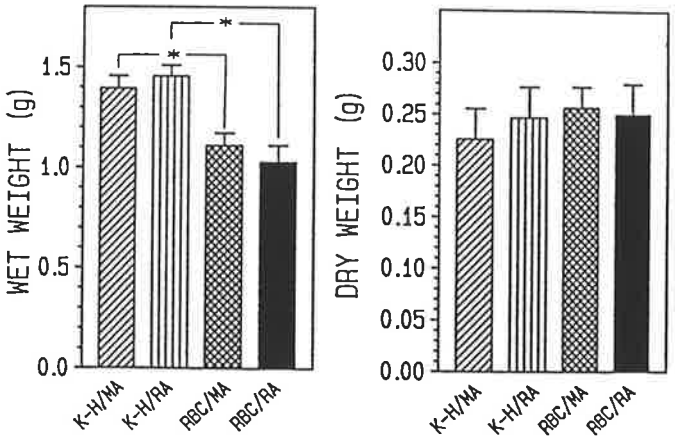

Figure II. 3 Effect of Krebs-Henseleit solution (K-H) or red blood cell suspension in modified K-H (RBC) perfusion of control working hearts (coronary perfusion pressure (CPP) =75 mmHg) and during maintained afterload ischemia (MA) or reduced afterload ischemia (RA) configuration (CPP =35 mmHg) on ventricular wet and dry weight. See Methods for details. Values are expressed as mean ± S.D., n=10, * =p<0.01 significant difference, Scheffe's comparison.

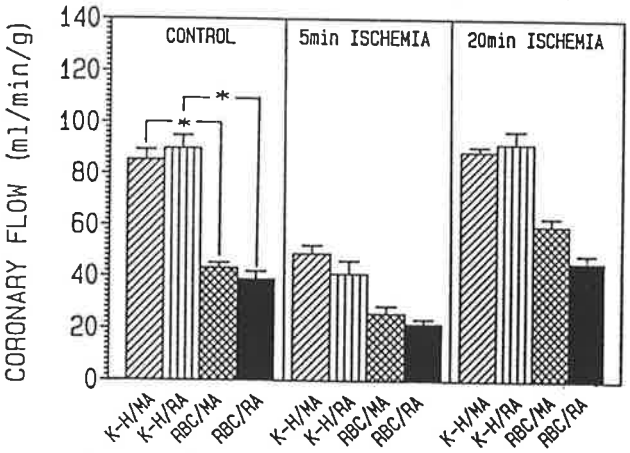

Figure II. 4 Effect of K-H or RBC perfusion of control working hearts and during MA or RA configuration ischemia on coronary flow. Values are expressed as mean ± S.D., n=10, * =p<0.01, significant difference, Scheffe's comparison. See Figure 3 legend and Methods for details.

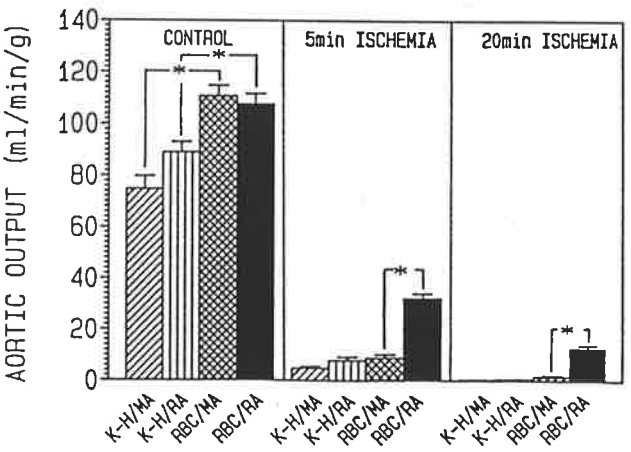

Figure II. 5 Effect of K-H or RBC perfusion of control working hearts and during MA or RA configuration ischemia on aortic output. Values are expressed as mean ± S.D., n=10, * =p<0.01, significant difference, Scheffe's comparison. See Figure 3 legend and Methods for details.

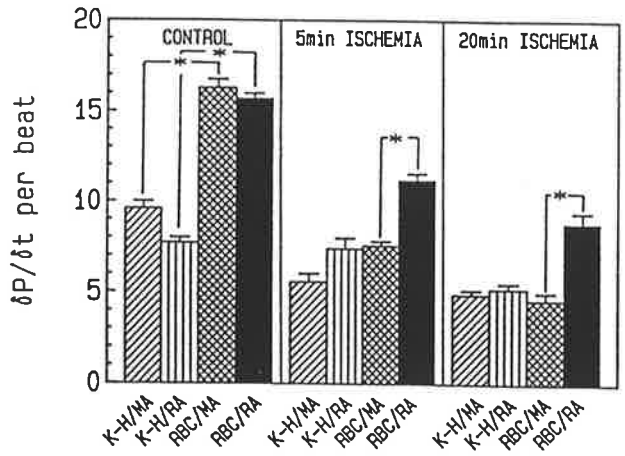

Figure II. 6 Effect of K-H or RBC perfusion of control working hearts and during MA or RA configuration ischemia on the pressure-time integral (dP/dt) per beat. Values are expressed as mean ± S.D., n=10, * =p<0.01, significant difference, Scheffe's comparison. See Figure 3 legend and Methods for details.

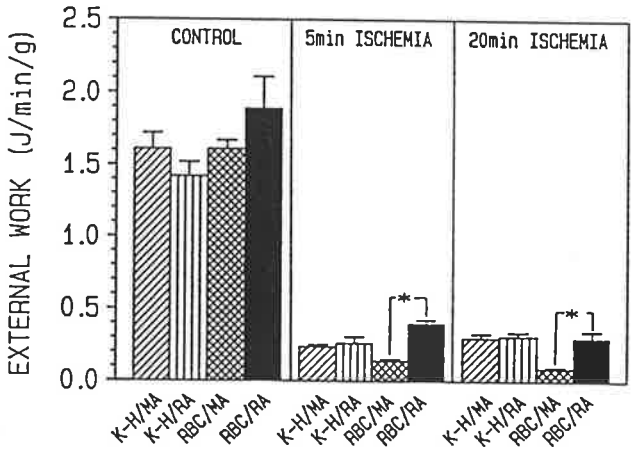

Figure II. 7 Effect of K-H or RBC perfusion of control working hearts and during MA or RA configuration ischemia on external work. Values are expressed as mean ± S.D., n=10, *= p<0.01, significant difference, Scheffe's comparison. See Figure 3 legend and Methods for details.

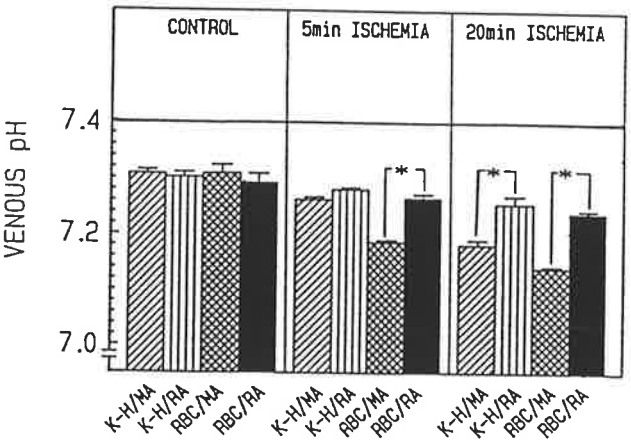

Figure II. 8 Effect of K-H or RBC perfusion of control working hearts and during MA or RA configuration ischemia on venous pH. Values are expressed as mean ± S.D., n=10, *= p<0.01, significant difference, Scheffe's comparison. See Figure 3 legend and Methods for details.

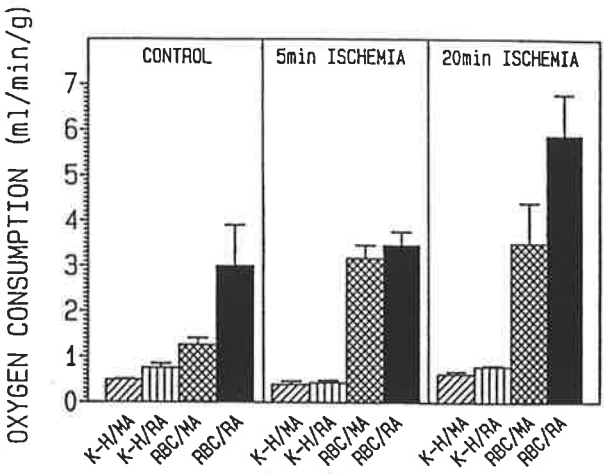

Figure II. 9 Effect of K-H or RBC perfusion of control working hearts and during MA or RA configuration ischemia on oxygen consumption. Values are expressed as mean ± S.D., n=10, * =p<0.01, significant difference, Scheffe's comparison. See Figure 3 legend and Methods for details.

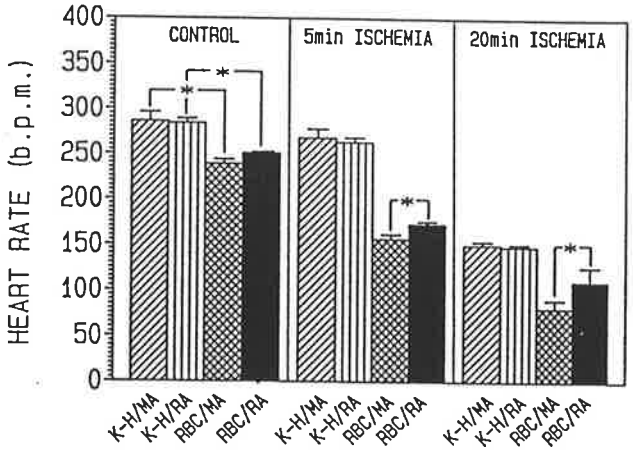

Figure II. 10 Effect of K-H or RBC perfusion of control working hearts and during MA or RA configuration on heart rate. Values are expressed as mean ± S.D., n=10, * =p<0.01, significant difference, Scheffe's comparison.
See Figure 3 legend and Methods for details.

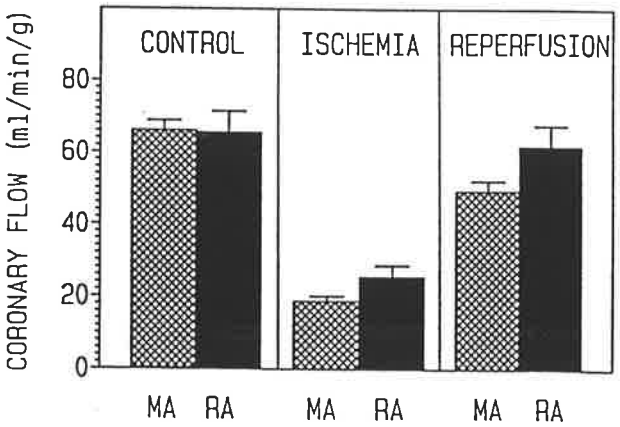

Figure II. 11 Paced heart experiments: The effect of MA or RA in RBC perfused hearts (paced at 300 bpm) on coronary flow during ischemia (CPP=35mmHg)(at 15 min) and reperfusion (CPP=75mmHg)(at 5 min). Values are expressed as mean ± S.D., n=10, * =p<0.01, significant difference, Scheffe's comparison. See Methods for details.

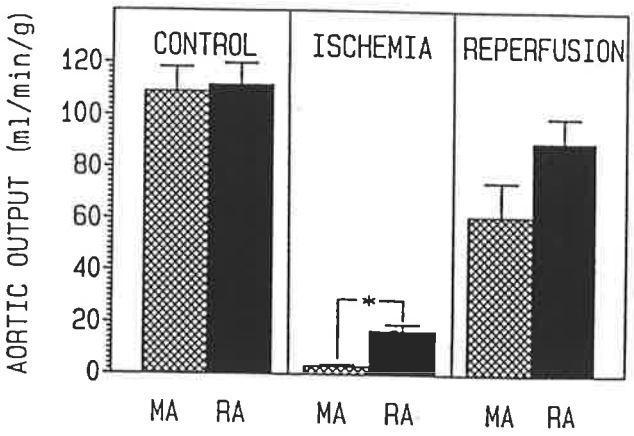

Figure II. 12 Paced heart experiments: The effect of MA or RA in RBC perfused hearts on aortic output during ischemia and reperfusion. Values are expressed as mean ± S.D., n=10, * =p<0.01, significant difference, Scheffe's comparison. See Figure 11 and Methods for details.

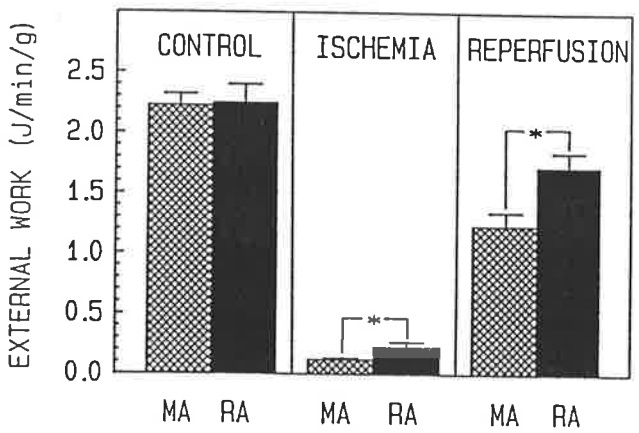

Figure II. 13 Paced heart experiments: The effect of MA or RA in RBC perfused hearts on external work during ischemia and reperfusion. Values are expressed as mean ± S.D., n=10, * =p<0.01, significant difference, Scheffe's comparison. See Figure 11 and Methods for details.

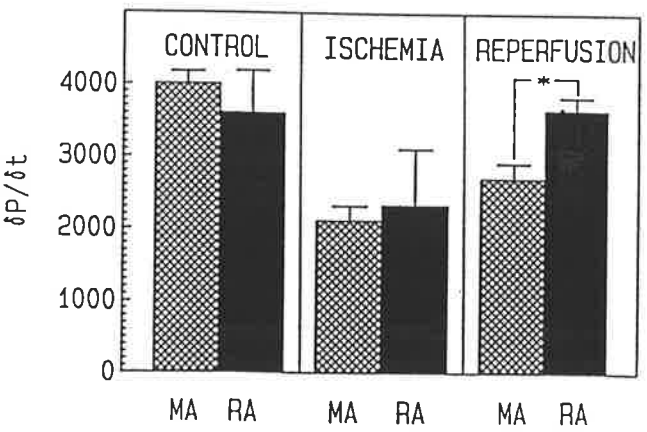

Figure II. 14 Paced heart experiments: The effect of MA or RA in RBC perfused hearts on the pressure-time integral (dP/dt) during ischemia and reperfusion. Values are expressed as mean ± S.D., n=10, * =p<0.01, significant difference, Scheffe's comparison. See Figure 11 and Methods for details.

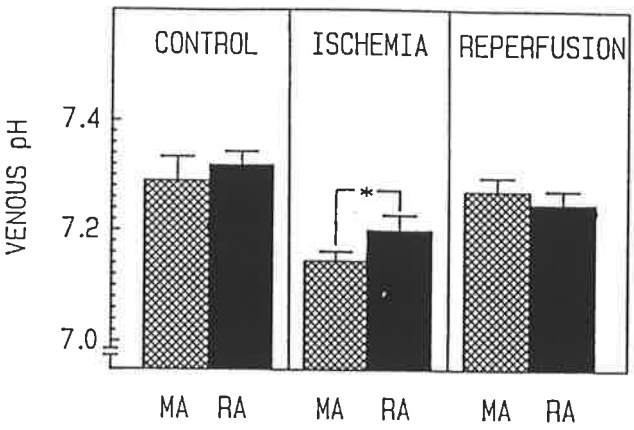

Figure II. 15 Paced heart experiments: The effect of MA or RA in RBC perfused hearts on venous pH during ischemia and reperfusion. Values are expressed as mean ± S.D., n=10, * =p<0.01, significant difference, Scheffe's comparison. See Figure 11 and Methods for details.

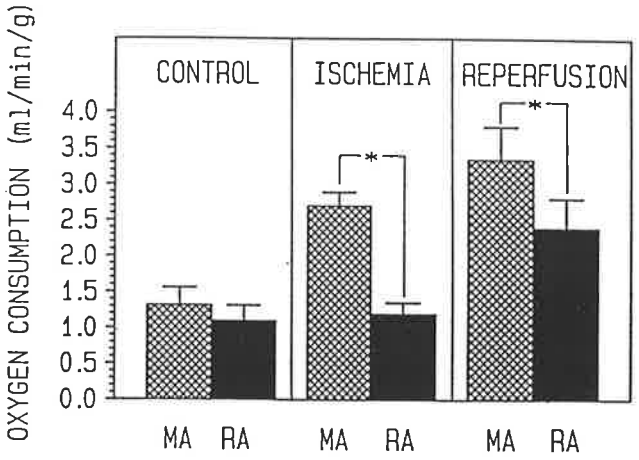

Figure II. 16 Paced heart experiments: The effect of MA or RA in RBC perfused hearts on oxygen consumption during ischemia and reperfusion. Values are expressed as mean ± S.D., n=10, * =p<0.01, significant difference, Scheffe's comparison. See Figure 11 and Methods for details.

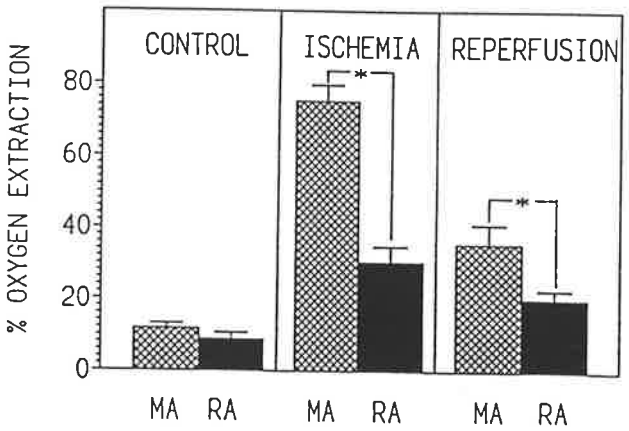

Figure II. 17 Paced heart experiments: The effect of MA or RA in RBC perfused hearts on the percentage of oxygen extraction during ischemia and reperfusion. Values are expressed as mean ± S.D., n=10, * =p<0.01, significant difference, Scheffe's comparison. See Figure 11 and Methods for details.

II. 4. Discussion

The isolated working heart preparation, has been perfused with a washed red blood cell, dextran and physiological salt solution (40% haematocrit), in order to investigate cardiac performance and oxygen metabolism with *in vivo-like* oxygen delivery. Wet weight tissue analysis showed that use of this RBC buffer in working heart perfusion limited oedema compared to perfusion with K-H solution. The water content in K-H perfused hearts was more than 30% greater (by weight) than in RBC perfused hearts. This finding supports the observation of others that the development of oedema in isolated hearts is greater when using a physiological salt solution (**Bergman,*et al.*,1976;Buja,*et al.*,1983;Cobbe & Poole-Wilson,1980;Greenfield,*et al.*,1988;Nakamura, 1985;Neely & Feuvray,1981**). As seen in Table 1, K-H arterial PO_2 was over three times that of RBC arterial PO_2, yet the oxygen content in arterial K-H solution was less than 7% of the arterial RBC perfusate. Even at the high PO_2 of >400 mmHg, very little oxygen is available to sustain function. An oxygen extraction of 76% of the oxygen content in K-H perfused working hearts compared to 15% in RBC perfused hearts, demonstrates the limited capacity of K-H solution to deliver oxygen to the working myocardium.

As there is so little oxygen remaining in the venous coronary effluent in K-H compared to perfusion with RBC buffer during working heart performance, little scope exists for increased oxygen extraction if the heart is stressed with ischaemia and/or increased work (Figure II. 9).

Perfusion with K-H solution produced significantly higher coronary flow rates compared to RBC perfusate. This increased flow rate is likely to be due to

coronary vasodilation in response to a limited oxygen supply and lower viscosity in contrast to the RBC buffer.

In RBC perfused hearts a large reduction in coronary flow occurred during the early phase of ischaemia, followed eventually by an increase in coronary flow due to compensatory coronary vasodilation and/or increased time in diastole due to slowed heart rate. Both the coronary flow reduction and subsequent compensation were greater in hearts with maintained afterload ischaemia. Along with a significantly greater decrease in aortic output during MA ischaemia, this indicated that a more severe ischaemia was possible with a maintained afterload. With K-H perfusion, similar trends in cardiac output components were observed throughout the perfusion period. However, the coronary hyperaemia that occurred towards the end of the ischaemic period returned coronary flow in K-H perfused hearts to preischaemic flow rates, unlike the RBC perfused hearts which overcompensated. This may indicate that K-H perfused hearts are close to their maximum coronary flow limits prior to ischaemia, leaving little room for further vasodilation. The lack of compensatory flow increase during ischaemia in paced hearts suggests that it is essentially due to a longer time in diastole during which coronary arteries are not impeded by contracted myocardium.

Hearts perfused with RBC beat more slowly particularly during ischaemia. This heart rate reduction may be related not only to the greater viscosity of RBC perfusate, but may reflect an increase in ventricular filling time of all ischaemic hearts in an attempt to maintain cardiac output. This compensation is a protective mechanism by which the heart can reduce the

level of work and thus myocardial oxygen demand (**Bernier,*et al.*,1989; Hearse & Humphrey,1975;Tosaki,*et al.*,1988**). This is supported by the observation that MA hearts in which functional decline was most marked, also had the slowest heart rate. The sino-atrial node may have been sensitive to the production of H^+ or other metabolite produced during ischaemia. By pacing hearts during RBC perfusion, the complication of spontaneous heart rate was removed. Indeed, pacing the hearts clarified a number of observations made in unpaced heart experiments, (such as the coronary hyperaemia seen at 20 min of ischaemia). The significantly reduced cardiac output, contractility (PTI), external work, pH and significantly higher oxygen consumption during maintained afterload ischaemia indicates that this model of global ischaemia is functionally more severe.

It has been shown that myocardial energy production and mechanical work closely parallels oxygen consumption in normal working hearts (**Neely,*et al.*,1967;Sarnoff,*et al.*1958;Taeghtmeyer,*et al.*,1980;Whalen,1961**). If cardiac output, external work and the pressure-time integral are reduced during ischaemia, it would be expected that oxygen consumption should decrease. Instead we see this anomalous increase in oxygen consumption, that has previously been reported by a small number of researchers, (**Krause,*et al.*,1986;Krukenkamp,*et al.*,1986;Stahl,*et al.*,1988;Laster,*et al.*,1989**). Although the underlying mechanism remains elusive, evidence supports the decoupling of oxygen consumption from myocardial work processes following short term ischaemia as hearts are failing at this time. It has been proposed that this increased oxygen consumption may be due to an increased energy

requirement to regulate intracellular calcium (**Hoerter,*et al.*,1986**). Lakatta, *et al.*, (**1985**), have shown increased spontaneous calcium release from sarcoplasmic reticulum and asynchrony of contractile elements following ischaemic injury. It has also been proposed that sarcoplasmic reticulum calcium uptake may be impaired, thus increased energy could be required for alternative mechanisms to remove cytosolic calcium in compensation (**Laster,*et al.*,1989**).

The large increase in oxygen consumption that we have measured in ischaemic RBC-perfused hearts is not as evident in K-H perfusion perhaps because of the restricted oxygen availability in the perfusate oxygen extraction. The RBC-perfused unpaced hearts subjected to reduced afterload ischaemia consumed more oxygen than those subjected to maintained afterload ischaemia. This was associated with a faster heart rate. When heart rates in both groups were normalised by atrial pacing at 300 bpm the opposite result was produced. Here the greater oxygen consumption was seen during and following maintained afterload ischaemia. As an increased heart rate (by pacing) decreases the diastolic period, less time is available for coronary flow. Thus the higher coronary flow in unpaced hearts is not apparent with pacing and results in reduced oxygen delivery and total oxygen consumption. In paced hearts coronary flow in ischaemia was very low with a high heart rate. Thus increased demand exists with a reduced substrate supply, enhancing the severity of ischaemia. A greater oxygen demand would therefore be evident with a greater severity of ischaemia. Indeed oxygen consumption was highest

in paced RBC perfused hearts during maintained afterload ischaemia and reperfusion.

II. 5. Conclusion

Perfusion of isolated working hearts with a buffer containing erythrocytes increased the oxygen delivery to the myocardium and resulted in measures of cardiac output, myocardial oxygen consumption and external work that resemble the respective measures *in vivo* (**Duvelleroy,*et al.*,1976**). Myocardial contractility was improved and a much smaller proportion of the cardiac output was required by the heart as coronary flow. The findings of this present study therefore indicate the value of using a perfusate physiologically relevant *in vivo*. The erythrocyte-perfused hearts were plainly in a more healthy state on which to impose an ischaemic insult.

By inducing global myocardial ischaemia in the isolated working heart we were able to measure the reduced mechanical performance and metabolic dysfunction that correspond directly to the ischaemic ventricles. The new model permitted global ischaemia by reducing coronary perfusion pressure without reducing the afterload and thus the workload. Of importance, significant ischaemia occurred while still providing low coronary flow sufficient to prevent immediate ventricular failure. This feature permits continued ventricular ejection, and allows measures of cardiac output, and most importantly venous perfusate to be taken during the progression of ischaemia and immediately upon reperfusion. This facility was available through the reduced afterload technique, but the consequences of ischaemia

(reduced mechanical function, reduced heart rate and acidosis) were more severe and occurred more rapidly when the heart was forced to work against a maintained afterload. Indeed, a poorer recovery of aortic output and contractility measured 5 min after reperfusion from maintained afterload ischaemia supports this. It is apparent from this study that when the afterload is reduced the progression of ischaemia is retarded and its full effects may be attenuated. The erythrocyte perfusate provided optimum oxygen delivery on which to impose ischaemia and by maintaining afterload the impact of ischaemia was not blunted by the method of ischaemia itself. It should by now be apparent to the investigator of cardiac function that the isolated heart perfused in Langendorff manner has limited application and should by superseded by the "blood" perfused isolated working heart. In the following chapters the influence of dietary fatty acids is investigated using the new isolated working heart maintained afterload method of global ischaemia.

III. EFFECT OF DIETARY FATTY ACID SUPPLEMENTS ON CARDIAC PERFORMANCE: ISCHAEMIC INJURY & POST-ISCHAEMIC RECOVERY

III. 1. Introduction

Since Hunter (**1779**) first conducted an autopsy on a patient who suffered from angina pectoris and described that the coronary arteries were "ossified", medical interest has been concentrated on atherosclerosis and associated ischaemia, thrombosis, and cardiac arrhythmias. It was Sinclair (**1956**) who first proposed that atherosclerosis was related to a reduced intake of essential fatty acids or inappropriate ratio of saturated to unsaturated fatty acids. Indeed the severity of experimental atherosclerosis has been shown to be dependent on type rather than amount of dietary fat intake (**Mahley,1982**). With the realisation that the type and quantity of dietary fat may influence the incidence and/or severity of atherosclerosis and CHD, efforts in dietary fat intake modification have concentrated on minimising the atherogenic profile of lipoproteins, triglycerides, plasma cholesterol and preventing coronary thrombosis due to blood clotting. This approach has been adopted in both clinical (human) and experimental (animal model) studies (**Harris,1989; Kinsella,*et al.*,1990;Knapp,1990;Budowski,1988**). Although this approach has been valuable, increased consideration and research into the underlying additional mechanisms of dietary fatty acid influence on cardiac function *per se* has been comparatively limited. It is recognised that atherosclerosis

contributes to myocardial infarction and heart failure or fatal cardiac arrhythmias by causing ischaemia. Diets excessively high in saturated fat may be atherogenic while polyunsaturated fatty acids can protect against the development of atherosclerosis **(Dyerberg,1986;Goodnight,*et al.*,1982)**. This has led researchers to consider atherosclerosis to be almost the sole factor involved with an increased susceptibility to arrhythmias. The validity of this assumption must be questioned if we consider the finding that as many as 1 in 5 cardiac sudden deaths occur in the absence of significant atherosclerosis **(Keefe,1987)**, and arrhythmias fatal or otherwise do not occur in all atherosclerotic patients. To date, none of the recognised risk factors for CHD, including cholesterol, can be used to discriminate potential SCD victims **(Myerburg & Castellanos,1989)**.

It has been shown that the rat is highly resistant to atherosclerosis following diets rich in saturated fats unless treated with grossly exaggerated cholesterol loading and amino acid supplementation **(Naka,*et al.*,1989)**. Dietary supplementation with saturated fat or polyunsaturated n-3 fish oil, or n-6 sunflower seed oil alone for up to 15 months in rats produces no significant alterations to their vasculature. Yet polyunsaturated oil supplementation significantly lowered plasma cholesterol and triglyceride levels while saturated fat supplementation significantly raised them **(Turner,*et al.*,1990)**. These findings indicate that if dietary fat can influence cardiac function in the rat, it must act independently of atherosclerosis. It is this particular aspect, the direct influence of altered type of dietary fat on myocardial function that will be briefly reviewed here and forms the basis of this dissertation.

It has been demonstrated (**Charnock,*et al.*,1985;1986**) that the composition of myocardial phospholipid fatty acids and triglycerides can be altered by modification of dietary fat intake. It has been proposed that cardiac function can be affected as a result of myocardial membrane fatty acid alteration by an influence upon a wide variety of cellular and membrane functions such as: ion efflux and influx (including calcium), respiratory electron transport, carrier-mediated transport, membrane-bound enzyme activity, receptor systems and eicosanoid synthesis. An effect in any one of these systems could have extensive impact on cardiac function and metabolism under physiological and pathophysiological conditions.

In the late 1970's it was observed that dietary supplementation of rats with linoleic acid (sunflower seed oil origin, 18:2, n-6 polyunsaturated fatty acid: See Table III. 2. for a nomenclature guide) increased the contractility and left ventricular work of heart muscle (papillary muscle and isolated perfused hearts) and increased coronary flow without an increase in oxygen consumption in Langendorff perfused hearts but had no effect in isolated working hearts compared to saturated fat (lard) fed rats (**DeDeckere,*et al.*,1979;1980**). The susceptibility to cardiac arrhythmias (**Hoffman,1982**) and experimentally induced myocardial infarction (**Lepran,1981**) was shown to decrease with similarly increased linoleic acid feeding. More recently (**Charnock,*et al.*1985;McLennan,*et al.*,1987a;1987b**) it was observed that a decreased positive inotropic response to increased extracellular [Ca^{++}] and a decreased incidence of isoprenaline-induced arrhythmias occurred in cardiac papillary muscles and atria from rats and primates fed sunflower seed oil or

tuna fish oil compared to a saturated sheep fat or standard reference diet. McLennan, *et al.*, **(1985;1988)** utilised coronary artery ligation in an *in vivo* model of cardiac arrhythmias and myocardial infarction to demonstrate an increased susceptibility to arrhythmias and infarct size in rats fed a diet supplemented with saturated sheep fat compared to isocaloric tuna fish oil, sunflower seed oil or a standard reference diet. The most striking finding from these results was the distinct reduction in the incidence of ventricular tachycardia and fibrillation during coronary occlusion and upon reperfusion in fish oil fed animals. These landmark findings indicated that dietary supplementation with n-3 marine source polyunsaturated fatty acids could provide protection against ischaemic and reperfusion induced arrhythmias following the metabolic stress of regional cardiac ischaemia. The results of recent studies conducted by Hock, *et al.* **(1987;1989;1990)**, using Menhaden (n-3 fatty acid rich) fish oil have confirmed the findings of the McLennan research group.

A small number of conflicting studies have been reported that have not observed the fatty acid supplementation effects discussed above, however, these studies had limitations in experimental design which included insufficient length of feeding (1 week only), essential fatty acid deficiency and abnormally high levels of starch and sucrose **(Chardigny,*et al.*,1988;1991)**. In dietary fat experiments in all areas of physiological interest, aside from failure to analyse all diets (including commercially purchased) and tissues for fatty acid profiles, there have been significant differences in the dietary models, energy values, diet storage and antioxidant levels, period of experimental diet feeding, animal

species, animal model and age of subjects at the time of experimentation **(Kwei & Bjeldanes,1989;Johnston & Fritsche,1989)**. In many studies not all of the above variables have been considered or discussed clearly and comprehensively. Such inconsistencies between experimental groups are serious oversights which compromise the data and prevent confident interpretation of results.

Standard commercially prepared unrefined diets are often used as "control diet" against which comparisons of experimental diets are made. The composition of commercial diets change from time to time without notice according to the availability and price of various ingredients, thus the ratios of saturated to unsaturated fatty acids may change considerably. Levels of essential vitamins and minerals may also fluctuate such that particular animal species may suffer deficiencies **(Johnston & Fritsche,1989)**. Comparisons against such "control" diets should be made with care. Nevertheless, it is valuable to have a reference point in order to judge the effects of experimental diets. If two experimental diets are compared to each other alone there can be no assessment of whether one has a positive effect or the other a negative effect, or indeed whether both induce a change from "normal" in the same direction but to different degrees. Having a *priori* reference diet and two experimental diets allows the effects of both diets to be considered independently of each other. It allows the evaluation of a dietary supplemented animal against the "normal" colony animal.

Although changes to myocardial membrane composition can occur within a few days of dietary modification, physiological changes were

significantly evident only with longer periods of feeding (**Charnock,*et al.*,1985**). Age has been implicated as a risk factor for fatal cardiac arrhythmias in humans (**Myerberg & Castellanos,1988**). McLennan *et al.*,(1989) observed that the severity of ischaemia induced arrhythmias increased with age. The influence of diet increased with age also. In recognition that increased myocardial vulnerability to arrhythmia trigger mechanisms occur with aging the use of mature, adult animals may be more appropriate than weanlings in models of arrhythmias and cardiac function in general.

To date most observations have been made in the whole animal. The experiments described in this section were designed to investigate the influence of dietary polyunsaturated fish oil and saturated sheep fat on normal heart function and responses to ischaemia under conditions which could accurately be controlled and the metabolic and physiological consequences measured. The isolated working heart is appropriate for such an investigation, particularly because it is free of potentially confounding neural and humoral influences. The dietary design implemented by the current study involved supplementation of a commercially available base diet (constituents known) with either saturated sheep (peri-renal fat) (12% w/w) or 12% (w/w) polyunsaturated refined fish oil containing high levels of n-3 eicosapentaenoic and docosahexaenoic acid (similar to MaxEPA). The two supplements were prepared ensuring that no essential fatty acid deficiencies could occur, that fat provided 35% of total energy and thus were isocaloric. While supplementation strategy normally leads to a "dilution" of components, analysis of the prepared diets indicated that protein, vitamins, minerals and fatty acids were present in

sufficient quantities. The aim of the present chapter was the assessment of the influence of diet on a wide cross-section of measures of cardiac function and metabolism under control and ischaemic conditions.

III. 2. Methods

III. 2. a) Diets

The following three diets were prepared: i) Reference diet (REF), ii) Saturated fat (SAT) and iii) Polyunsaturated fish oil (FO). The reference diet consisted of standard commercially available unrefined "Joint Stock" rat pellets (Milling Industries, Australia) composed of the following (expressed as % w/w): moisture, 11.6; protein, 20.5; fat, 7.6; crude fibre, 5·3. The fat in the dry mix base diet was derived from wheat, oats, soya bean meal, lucerne meal, meat meal, and fish meal. The Joint Stock REF diet was prepared by the manufacturer by blending all of the ingredients as a dry mix and spraying with beef tallow at the time of extrusion pelleting. This diet included the following vitamins, minerals and trace elements (mg/kg): thiamine (vitamin B1), 54.0; riboflavin, 10.0; pyridoxine, 12.0; pantothenate, 20.0; niacin, 18.0; folate, 2.0; biotin, 0.11; retinol (vitamin A), 12.0; α-tocopherol (vitamin E), 28.0; vitamin K, 8.3; vitamin B12, 0.12; manganese, 140.0; molybdenum, 1.0; iodine, 0.6; cobalt, 0.42; zinc, 84.0; copper, 17.0; iron, 280.0; lead, 0.60; cadmium, 0.16; selenium, 0.52; and expressed in g/kg: calcium, 7.45; magnesium, 1.68; sodium, 2.83; potassium, 6.68; phosphorus, 5·99.

For the preparation of the lipid-supplemented diets the "Joint Stock" diet was obtained in dry mix form without added tallow. This base diet had

a fat content of 3.7% by weight. The SAT diet was prepared by adding locally available sheep peri-renal fat (12% w/w), which is rich in saturated fatty acids (stereate 18:0 & palmitate 16:0), to the dry mix. The FO diet was prepared by adding (12% w/w) fish oil rich in the polyunsaturated fatty acids; eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6)(Shaklee, San Francisco, U.S.A.). The fish oil also included 1.0% of the *in vivo* antioxidant vitamin E (by volume of oil), 70 IU/g vitamin A, and 40 IU/g vitamin D. The peroxide value of the fish oil did not exceed 10 meq/kg. All diets contained 0.05% butylated hydroxytoluene to prevent peroxidation of fats during storage. Vitamin E was added to the SAT diet to the same level as the FO diet. Following repelleting and drying all diets were stored at 0-4°C prior to use and new diet was prepared every 14 days. Thus fat-supplemented diets contained 15.3% total fat and had an energy value of 18.5-19.3 kJ/g according to combustion calorimetry assay, while REF diet (7.6% fat) contained 16.7 kJ/g. The following table shows the fatty acid profile of each diet analysed by gas liquid chromatography (5710A Hewlett Packard, U.S.A) according to the method of Bligh & Dwyer **(1959)**, using SP2310 column packing material (55% cyanopropyl chromosorb WAW 100/120 (Supelco,Bellefont, U.S.A.)).

Table III.1 Fatty acid composition of the experimental diets.

The fatty acids are expressed as a % of total fat content. Values below the level of detection are represented by - . The nomenclature used for fatty acid identification is C:d, n-x, where C is chain length of the fatty acid, :d is the number of double bonds and n-x is the number of carbon atoms from the last double bond to the methyl end of the molecule. Dimethylacetal (DMA) is derived from plasmalogen methylation indicative of ether linkages more common in phosphatidyl-ethanolamine (measurable in SAT diet at physiologically insignificant levels).

FATTY ACIDS	REF	SAT	FO
14:0	1.094	2.663	6.061
16:0 DMA	-	0.282	-
16:0	19.381	22.825	10.501
16:1, n-7	1.894	1.600	9.839
18:0 DMA	-	0.161	-
18:0	7.614	28.498	4.579
18:1, n-9	20.713	27.109	11.372
18:2, n-6	33.855	6.699	5.640
18:3, n-3	3.345	1.524	1.200
20:0	0.210	0.488	3.778
20:1	0.471	0.462	1.488
20:3	-	-	0.151
20:4, n-6	0.620	0.091	0.960
20:5, n-3	2.235	0.358	24.283
22:5, n-3	-	0.129	1.523
22:6, n-3	6.173	0.764	11.840
24:0	0.761	-	0.378
% TOTAL	98.366	93.653	93.593
Σ SAT.	29.06	54.92	25.30
Σ POLYUNSAT.	46.23	9.57	45.45
P/S	1.59	0.17	1.80
Σ n-9	20.71	27.57	12.86
Σ n-6	34.48	6.79	6.60
Σ n-3	11.753	2.78	38.85
% TOTAL FAT	7.6	15.3	15.3

III. 2. b) Animals

Weight matched (450-540 g when 8 months old), male, Hooded-Wistar rats (4 months old) were allocated to one of the three dietary groups REF, SAT, FO, (n=10 per group). The rats were housed 5 per cage at 23°C with constant 55% humidity on a 12h light/dark cycle. Although the food and water was available *ad libitum*, the animals consumed a consistent amount of food daily (approximately 20 g/rat). While SF and FO diets were isocaloric, the energy values of the fat-supplemented groups were slightly higher than REF. However, the average daily caloric intakes were similar between the 3 groups (approximately 400 kJ/day), because SF and FO groups consumed 1-3 g/day less than the REF group. This follows the observation that laboratory animals usually adjust their food intake to maintain constant energy consumption **(Johnston & Fritsche,1989)**. However, during the first week of feeding FO, rats ate very little probably due to the strong fish oil odour. In order to avoid the possibility of fat peroxidation, leftover food was discarded daily prior to refilling feed containers. Food was replenished daily in the late afternoon to minimise the period between exposure to room temperature,light and air and its consumption. The amount of food placed in each cage was sufficient such that there was always at least 1-5 g of leftovers minimising wastage. The rats were maintained on their respective dietary regimes for four months.

III. 2. c) Perfusion Protocol

Rats were fasted overnight prior to each experimental day. The animals were sacrificed and the hearts removed and prepared for working heart perfusion

(maintained afterload ischaemia configuration) with the erythrocyte buffer (40% haematocrit) as described in chapter II. Following 10 min in Langendorff perfusion mode during which pulmonary artery cannulation, pacing electrode placement, and blood washout took place, hearts were switched to working heart mode (Coronary perfusion pressure, CPP=75mmHg, preload= 10mmHg, workload =75mmHg, paced heart rate =300 bpm). Hearts were perfused in this manner for 10 min to stabilise. At the end of this period measures were taken (every 5 min until the end of the experiment) of cardiac output, aortic pressure, arterial and venous blood gas content, and pH as well as collection of coronary effluent samples for later biochemical analyses. Arterial and coronary venous samples following oxygen tension and pH readings were spun to separate red blood cells from the perfusate using a small bench top centrifuge (Corning). The supernatant was frozen in liquid nitrogen and stored at -60°C. Ischaemia was induced by lowering the coronary perfusion reservoir such that the coronary perfusion pressure was 35mmHg. After 15 min, reperfusion was initiated by raising the same reservoir back to the original height. Following each perfusion hearts were quickly removed from the apparatus, blotted dry and weighed whole and with atria & ventricles dissected. A small piece of tissue was cut from hearts and dried for dry weight calculations. The ventricles were frozen in liquid nitrogen and stored at -60°C until fatty acid analysis could be performed.

Notably, the ischaemic period used was 15 min rather than the 20 min used in Chapter II. The reason for this change was to establish uniformity with a large number of other researchers who have induced 15 min ischaemia

in the isolated rat heart for reperfusion studies. In order to be confident that 15 min ischaemia was sufficient to produce ischaemic injury, in a pilot study, a few hearts (reference rat diet) were prepared for electron microscopy. Samples of ventricle from ischaemic and normal hearts were fixed in 3% formaldehyde, 3% glutaraldehyde, polyvinylpyrrolidone (40 000 mw) and 0.1% osmium tetroxide in 0.1 M phosphate buffer at pH 7.4, dehydrated, and embedded in propylene oxide and epoxy resin. Sections (0.5μm) were observed under a JEOL 100S transmission electron microscope. Sections that had been treated to 15 min ischaemia demonstrated membrane disruption and mitochondrial swelling, indicating that myocardial injury had occurred. The use of this time period was appealing because the visible damage was not extensive or severe enough to completely exclude the study of post-ischaemic recovery.

III. 2. d) Creatine Kinase Analysis

Arterial and venous samples were allowed to reach room temperature, and then diluted 1/40 in warmed (37°C) creatine kinase N-acetyl-L-cysteine reagent (CK-NAC diagnostic test kit, Behring Dignostics,Inc.,U.S.A.). The reagent was designed to allow the assay of creatine kinase activity in the catalysis of ATP and creatine from the substrates ADP and creatine phosphate according to the method of Szasz **(1979)**. The sample was placed in a quartz cuvette of a Gilford M250 Spectrophotometer (Gilford Instruments, U.S.A.), and the reaction was allowed to proceed over a few minutes at 340 nm wavelength, 1 cm light path and 37°C. Creatine kinase was quantified

according to the rate of change of absorbance. Results were expressed in U/L and in U/L/min when adjusted according to coronary flow rates.

III. 2. e) Lactate Release Analysis

Lactate concentrations in collected samples were analysed by Cobass Bio (Hoffman-La Roche, Basle, Switzerland) at 340 nm wavelength. Samples (5μL) were incubated at 37°with 8μL of L^+ lactate dehydrogenase (3.2 mol/L in ammonium sulphate solution, pH 7, Boehringer Mannheim, U.S.A.) and with 150μL of reagent (4 mg/mL of ß-nicotinamide adenine dinucleotide (Sigma Chemical Co.,U.S.A.) in 0.5 M glycine buffer with 0.4 M hydrazine hydrate (BDH,) at pH 9). Results were expressed in mmol/L or mmol/L/min when adjusted according to coronary flow rates.

III. 2. f) K^+ Flamephotometry

All samples were analysed in duplicate for their extracellular K^+ concentrations with an IL143 Flamephotometer (Instrumentation Laboratories, U.S.A.) calibrated with 5 mmol/L K^+. Results were expressed in mmol/L.

III. 2. g) Data Handling & Statistical Analysis

All calculations of cardiac output, coronary flow, external work pressure-time integral, oxygen extraction and oxygen consumption were conducted as described in chapter II. All results were expressed as mean $\pm$ SD. For each parameter the effect of dietary treatment was tested by Analysis of Variance

and between individual comparisons, Scheffe's *post hoc F*-test. The level of significance was considered at $P<0.05$ or less.

III. 3. Results

III. 3. a) Animal Growth & Tissue Composition

Following four months of dietary treatment, body weights obtained at the time of sacrifice did not differ significantly between the three dietary groups (Figure III.1). Similarly no dietary effect was observed on ventricular dry weight (Figure III.2). The fatty acid profile of the 3 diets (Table III.1) showed that FO contained approximately 45% polyunsaturated fatty acids (25% EPA & 12% DHA) and SAT had 10% (<0.5% EPA). The SAT diet contained 55% saturated fatty acids while FO had 25%. The REF diet contained 30% saturated fatty acids and 46% polyunsaturated fatty acids of which 8% were long chain n-3 fatty acids, EPA and DHA. This diet of course had only half the total fat content of the supplemented diets. The total phospholipid fraction fatty acid profiles in Table III.2 show that FO treated hearts incorporated a significantly larger proportion of EPA and DHA (20:5 & 22:6 respectively) into the myocardial membranes compared to the other diets. A significantly larger incorporation of arachidonic acid (AA, 20:4), linoleic acid (LA, 18:2) occurred in SF compared to FO.

The percentage of the sum total of saturated fatty acids (33-35%) and the sum total of polyunsaturated fatty acids (55-57%) incorporated into myocardial membranes did not differ between the diets and thus neither did the P/S ratio. However, the ratio Σ n-6:Σ n-3 was significantly decreased in

FO treated hearts due to the higher percentage of n-3 fatty acid membrane incorporation. The ratio of Σ n-6 to Σ n-3 ratio for the dietary groups were 2.3 REF, 1.6 SAT, and 0.76 FO.

Table III. 2. The effect of lipid supplementation on the fatty acid components of the total phospholipids of rat ventricle. Values are expressed as mean ±SD. See Table III. 1. for nomenclature and other details.

FATTY ACIDS	REF	SAT	FO
14:0	0.103 ± 0.039	0.114 ± 0.040	0.153 ± 0.035
16:0 DMA	0.489 ± 0.119	0.434 ± 0.151	0.413 ± 0.089
16:0	11.239 ± 0.619	9.909 ± 1.087	9.429 ± 0.960
16:1, n-7	0.560 ± 0.131	0.451 ± 0.191	0.791 ± 0.097
18:0 DMA	0.409 ± 0.134	0.666 ± 0.181	0.378 ± 0.035
18:0	21.261 ± 1.454	23.294 ± 0.749	24.231 ± 0.670
18:1, n-9	7.612 ± 1.401	7.665 ± 0.763	6.360 ± 0.348
18:2, n-6	21.634 ± 2.197	13.860 ± 1.686	10.205 ± 0.825
18:3, n-3	0.127 ± 0.119	0.090 ± 0.020	0.132 ± 0.033
20:0	0.053 ± 0.007	0.042 ± 0.020	0.048 ± 0.013
20:1	0.288 ± 0.078	0.167 ± 0.050	0.400 ± 0.045
20:2	0.148 ± 0.009	0.110 ± 0.008	0.090 ± 0.008
20:4, n-6	17.675 ± 1.619	20.337 ± 1.193	14.134 ± 0.988
20:5, n-3	0.325 ± 0.137	0.286 ± 0.111	3.127 ± 0.397
22:4, n-6	0.368 ± 0.070	0.274 ± 0.348	-
22:5, n-3	1.416 ± 0.241	1.840 ± 0.182	1.836 ± 0.113
22:6, n-3	15.375 ± 1.391	19.464 ± 0.813	27.225 ± 1.727
24:0	0.324 ± 0.062	0.297 ± 0.074	0.230 ± 0.016
% TOTAL	99.330 ± 0.155	99.076 ± 0.337	99.207 ± 0.131
Σ SAT.	33.88	34.76	34.88
Σ UNSAT.	57.04	55.97	56.97
P/S	1.68	1.61	1.63
Σ n-9	7.90	7.83	6.76
Σ n-6	39.80	34.58	24.65
Σ n-3	17.24	21.39	32.32

III. 3. b) Cardiac Output

During control perfusion (CPP=75 mmHg) there was no difference in cardiac output between the diets (Figure III.3). For control working heart perfusion SAT hearts had a significantly increased coronary flow (Figure III.4) and decreased aortic output (Figure III.5) compared to the other diets. With the induction of ischaemia, coronary flow and aortic output decreased to very low levels. At 15 min ischaemia the greatest reduction in coronary flow was measured in the SAT group with an 80% drop, while REF coronary flow fell by 67% and FO by 70%. However, ischaemic coronary flow measures did not differ significantly between the dietary groups. Following 5 min reperfusion the best recovery was observed in FO hearts. The FO hearts had the highest aortic output, reaching 85% of pre-ischaemic levels, REF attained 63% of original levels and SAT the lowest aortic output with only 53% recovery.

III. 3. c) Ventricular Function

Myocardial contractility as indicated by the pressure-time integral (Figure III.6), was significantly lower in SAT hearts throughout the control, ischaemic and reperfusion periods compared to REF. In contrast, contractility was significantly greater in FO hearts through all perfusion phases. Ischaemia produced 43-47% reductions in contractility in all dietary groups compared with respective controls. Myocardial external work (Figure III.7) was maintained at the same level with all diets during control perfusion and decreased greatly during ischaemia in all groups. Following reperfusion, SAT hearts made the poorest return to pre-ischaemic external work levels while the best recovery was observed in FO hearts.

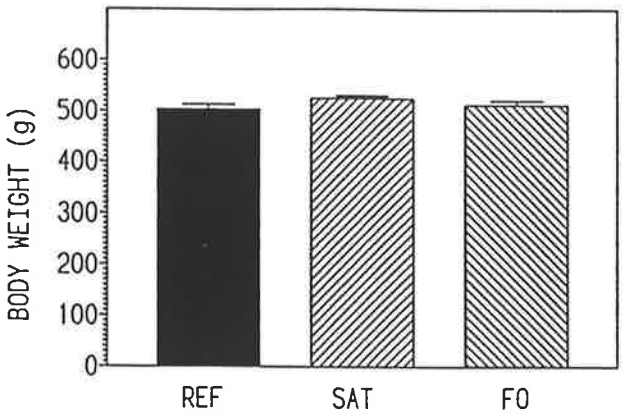

Figure III. 1. No significant effect of dietary fat supplement on body weight. Measurements were determined after overnight fasting prior to preparation of working heart perfusions (n=10 per group, mean±SEM). REF=Reference diet; SAT=Saturated fat rich diet; FO=Polyunsaturated rich fish oil diet. See Methods for details.

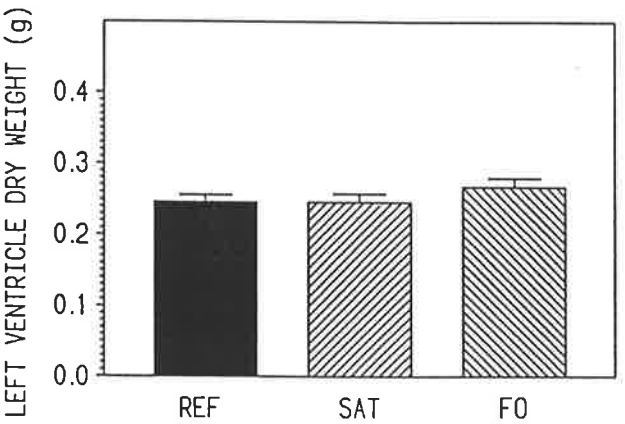

Figure III. 2. No significant effect of dietary fat supplement on left ventricle dry weight (n=10 per group, mean±SEM). REF=Reference diet; SAT=Saturated fat rich diet; FO=Polyunsaturated rich fish oil diet. See Methods for details.

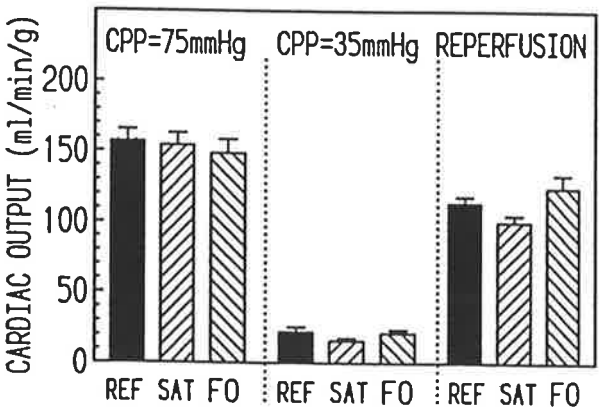

Figure III. 3. The effect of dietary fat supplementation on cardiac output during control working heart perfusion, ischaemia and reperfusion. Values are expressed as mean±SEM, g dry weight, n=10 per group. REF=Reference diet; SAT=Saturated fat rich diet; FO=Polyunsaturated rich fish oil diet; CPP=coronary perfusion pressure (control=75mmHg); 15min ischaemia= CPP=35mmHg. See Methods for details.

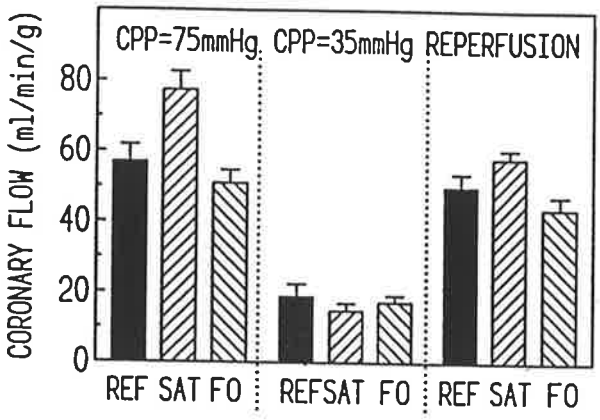

Figure III. 4. The effect of dietary fat supplementation on coronary flow during control working heart perfusion, ischaemia and reperfusion. Values are expressed as mean±SEM, g dry weight, n=10 per group. REF=Reference diet; SAT=Saturated fat rich diet; FO=Polyunsaturated rich fish oil diet; CPP=coronary perfusion pressure (control=75mmHg); 15min ischaemia= CPP=35mmHg. See Methods for details.

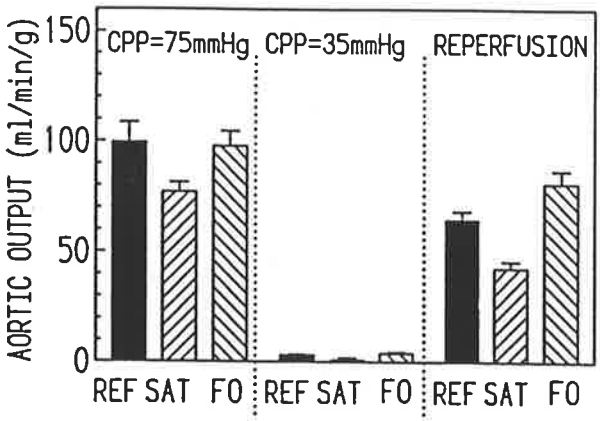

Figure III. 5. The effect of dietary fat supplementation on aortic output during control working heart perfusion, ischaemia and reperfusion. Values are expressed as mean±SEM, g dry weight, n=10 per group. REF=Reference diet; SAT=Saturated fat rich diet; FO=Polyunsaturated rich fish oil diet; CPP=coronary perfusion pressure (control=75mmHg); 15min ischaemia= CPP=35mmHg. See Methods for details.

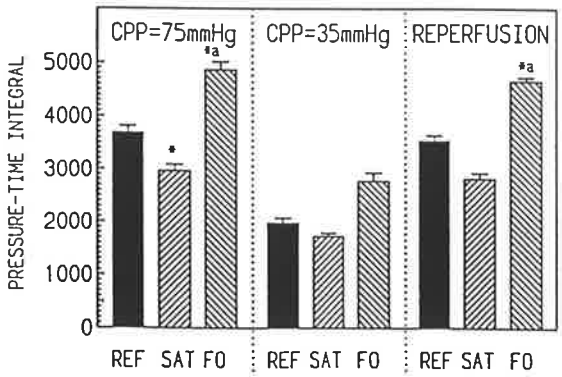

Figure III. 6. The effect of dietary fat supplementation on the pressure-time integral ($\delta P/\delta t$) during control working heart perfusion, ischaemia and reperfusion. Values are expressed as mean±SEM; n=10 per group; *=$p<0.05$, significantly different compared to REF; a=$p<0.05$, significantly different compared to SAT; Scheffe's comparison. REF=Reference diet; SAT=Saturated fat rich diet; FO=Polyunsaturated rich fish oil diet; CPP=coronary perfusion pressure (control=75mmHg); 15min ischaemia= CPP=35mmHg. See Methods for details.

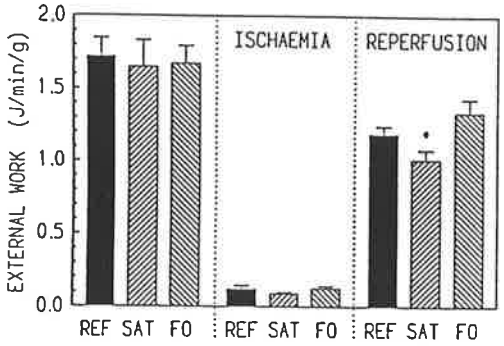

Figure III. 7. The effect of dietary fat supplementation on left ventricular external work during control working heart perfusion, ischaemia and reperfusion. Values are expressed as mean±SEM; g dry weight; n=10 per group; *=p<0.05, significantly different compared to REF; Scheffe's comparison. REF=Reference diet; SAT=Saturated fat rich diet; FO=Polyunsaturated rich fish oil diet; CPP=coronary perfusion pressure (control=75mmHg); 15min ischaemia= CPP=35mmHg. See Methods for details.

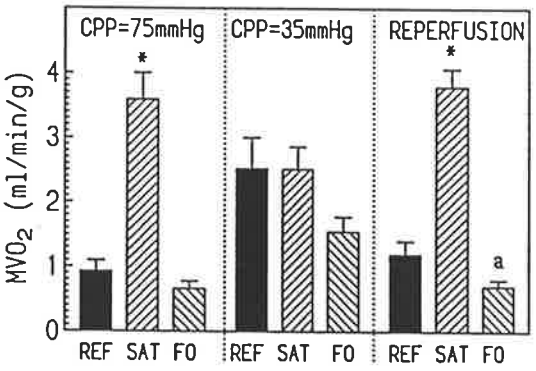

Figure III. 8. The effect of dietary fat supplementation on myocardial oxygen consumption during control working heart perfusion, ischaemia and reperfusion. Values are expressed as mean±SEM; g dry weight; n=10 per group; *=p<0.05, significantly different compared to REF; a=p<0.05, significantly different compared to SAT; Scheffe's comparison. REF=Reference diet; SAT=Saturated fat rich diet; FO=Polyunsaturated rich fish oil diet; CPP=coronary perfusion pressure (control=75mmHg); 15min ischaemia= CPP=35mmHg. See Methods for details.

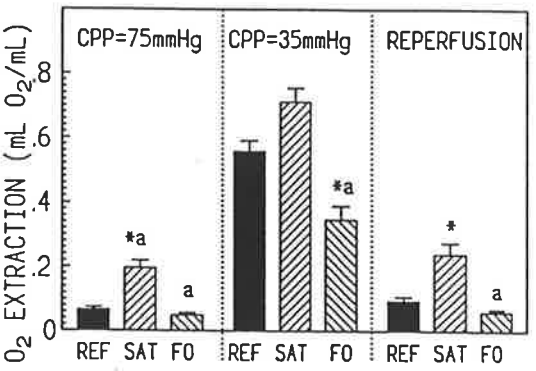

Figure III. 9. The effect of dietary fat supplementation on myocardial oxygen extraction during control working heart perfusion, ischaemia and reperfusion. Values are expressed as mean±SEM; n=10 per group; *=p<0.05, significantly different compared to REF; a=p<0.05, significantly different compared to SAT; Scheffe's comparison. REF=Reference diet; SAT=Saturated fat rich diet; FO=Polyunsaturated rich fish oil diet; CPP=coronary perfusion pressure (control=75mmHg); 15min ischaemia= CPP=35mmHg. See Methods for details.

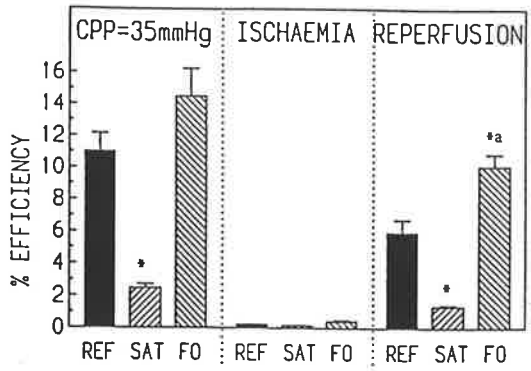

Figure III. 10. The effect of dietary fat supplementation on myocardial energy utilisation % efficiency during control working heart perfusion, ischaemia and reperfusion. Values are expressed as mean±SEM; n=10 per group; *=p<0.05, significantly different compared to REF; a=p<0.05, significantly different compared to SAT; Scheffe's comparison. REF=Reference diet; SAT=Saturated fat rich diet; FO=Polyunsaturated rich fish oil diet; CPP=coronary perfusion pressure (control=75mmHg); 15min ischaemia= CPP=35mmHg. See Methods for details.

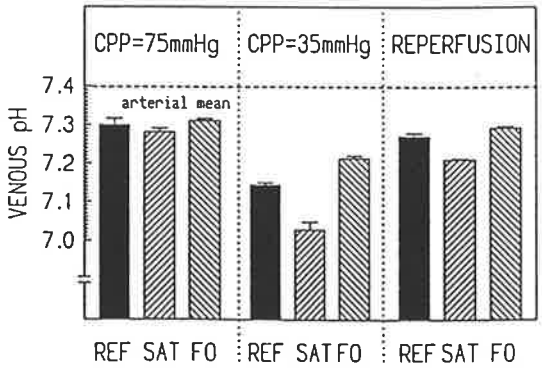

Figure III. 11. The effect of dietary fat supplementation on venous pH during control working heart perfusion, ischaemia and reperfusion. Values are expressed as mean±SEM; n=10 per group; *=p<0.05, significantly different compared to REF; Scheffe's comparison. REF=Reference diet; SAT=Saturated fat rich diet; FO=Polyunsaturated rich fish oil diet; CPP=coronary perfusion pressure (control=75mmHg); 15min ischaemia= CPP=35mmHg. See Methods for details.

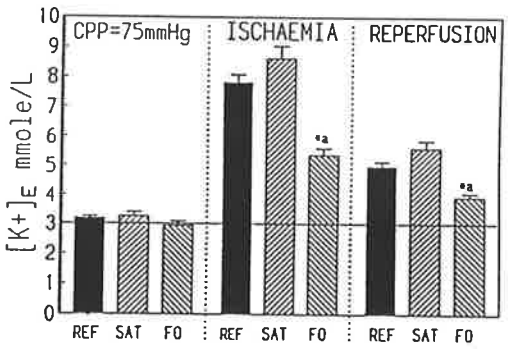

Figure III. 12. The effect of dietary fat supplementation on coronary effluent extracellular [K+] during control working heart perfusion, ischaemia and reperfusion. Values are expressed as mean±SEM; n=10 per group; *=p<0.05, significantly different compared to REF; a=p<0.05, significantly different compared to SAT; Scheffe's comparison. REF=Reference diet; SAT=Saturated fat rich diet; FO=Polyunsaturated rich fish oil diet; CPP=coronary perfusion pressure (control=75mmHg); 15min ischaemia= CPP=35mmHg. See Methods for details.

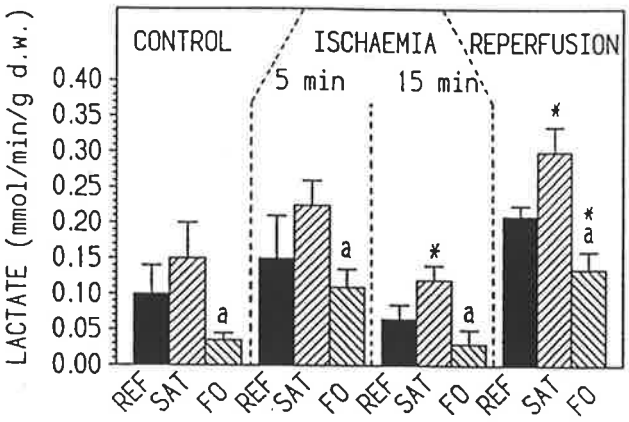

Figure III. 13. The effect of dietary fat supplementation on myocardial lactate release during control working heart perfusion, ischaemia and reperfusion. Values are expressed as mean±SD; n=10 per group; *=p<0.05, significantly different compared to REF; a=p<0.05, significantly different compared to SAT; Scheffe's comparison. REF=Reference diet; SAT=Saturated fat rich diet; FO=Polyunsaturated rich fish oil diet; CPP=coronary perfusion pressure (control=75mmHg); 15min ischaemia= CPP=35mmHg. See Methods for details.

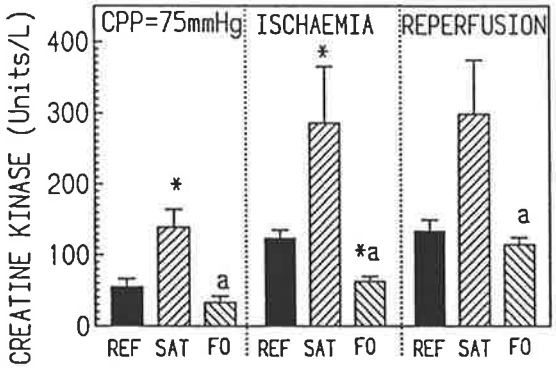

Figure III. 14. The effect of dietary fat supplementation on myocardial creatine phosphokinase release during control working heart perfusion, ischaemia and reperfusion. Values are expressed as mean±SEM; n=10 per group; *=p<0.05, significantly different compared to REF; a=p<0.05, significantly different compared to SAT; Scheffe's comparison. REF=Reference diet; SAT=Saturated fat rich diet; FO=Polyunsaturated rich fish oil diet; CPP=coronary perfusion pressure (control=75mmHg); 15min ischaemia= CPP=35mmHg. See Methods for details.

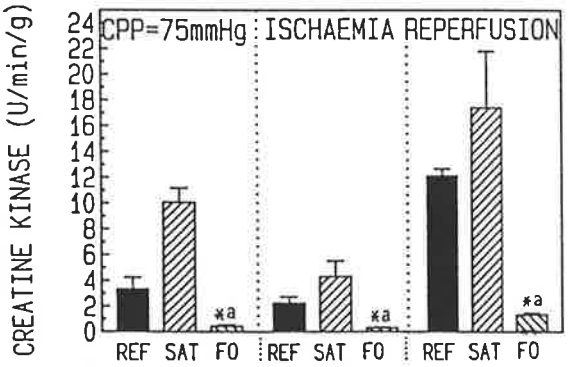

Figure III. 15. The effect of dietary fat supplementation on myocardial creatine phosphokinase release (corrected for coronary flow per g dry weight) during control working heart perfusion, ischaemia and reperfusion. Values are expressed as mean±SEM; n=10 per group; *=p<0.05, significantly different compared to REF; a=p<0.05, significantly different compared to SAT; Scheffe's comparison. REF=Reference diet; SAT=Saturated fat rich diet; FO=Polyunsaturated rich fish oil diet; CPP=coronary perfusion pressure (control=75mmHg); 15min ischaemia= CPP=35mmHg. See Methods for details.

III. 3. d) Myocardial Oxygen Consumption

Figure III.8 illustrates the effect of diet on myocardial oxygen consumption (MVO_2). Of particular note, under control working heart perfusion SAT hearts consumed significantly more oxygen than the other diets. The effect of 15 min ischaemia was to increase MVO_2 in all groups except SAT where MVO_2 fell from its already elevated level. On reperfusion MVO_2 was reduced towards control levels for REF and FO but in REF hearts MVO_2 still remained slightly elevated compared to pre-ischaemic levels. In contrast the MVO_2 of SAT hearts returned to the high levels measured during control perfusion. If we consider oxygen extraction levels corrected for coronary flow rates (Figure III.9), oxygen extraction was significantly greater in SAT hearts than the other groups during control perfusion. Oxygen extraction increased in all diets, including SAT, when coronary flow was limited during ischaemia. In reperfusion oxygen extraction returned to pre-ischaemic levels in all groups. Figure III.10 demonstrates the very low energy utilisation efficiency (ratio of work output energy/energy input) of SAT hearts. In reperfusion the efficiency of SAT remained the lowest while FO hearts displayed a significantly greater energy efficiency compared to both REF and SAT hearts.

III. 3. e) Metabolism

While venous pH was slightly lower in SAT than REF and FO, it remained relatively similar across the diets during control perfusion (Figure III. 11). Acidosis occurred in all diets during ischaemia, especially in SAT hearts. The FO group experienced a significantly smaller fall and SAT a greater fall in

venous pH than REF hearts. After 5 min reperfusion the venous pH had returned to pre-ischaemic levels in REF and FO but was only partially restored in the SAT group.

The extracellular K^+ concentration in venous coronary effluent increased during ischaemia in all dietary groups from the control level of approximately 3mmol/L. At 15 min this concentration was almost trebled in REF and SAT hearts but was significantly lower being only doubled in FO hearts (Figure III. 12). The concentration of K^+ decreased upon reperfusion in both REF and SAT dietary groups but was still very high compared to the respective control concentration. In comparison, FO hearts had significantly lower $[K^+]$ levels also during reperfusion and these had almost returned to pre-ischaemic levels.

The amounts of lactic acid released into coronary effluent during control working heart perfusion were significantly lower in FO hearts than REF and SAT hearts (Figure III. 13). Coronary effluent lactate levels rose in all groups during the first 5 min of ischaemia. Lactic acid production during ischaemia was significantly higher in SAT hearts and lower in FO hearts. After 5 min reperfusion, lactate levels were significantly elevated above control concentrations. While SAT concentrations of lactate were the highest, FO lactate concentrations were significantly lower.

III. 3. f) Creatine Phosphokinase

The concentration of creatine kinase released into coronary effluent was significantly higher in SAT and lower in FO hearts during normal coronary

perfusion (Figure III.14). Creatine kinase concentration was increased in ischaemia and reperfusion, remaining highest in SAT and lowest in FO hearts. When creatine kinase release was corrected according to the respective coronary flow rates and dry weights (Figure III.15), the relationship between diets observed in Figure III.14 during control working heart perfusion was still present. The release from hearts was negligible in control conditions, however, significantly increased concentrations of creatine kinase were released by SAT and to a lesser extent in FO and REF hearts. These levels did not increase during reperfusion, indeed they were attenuated slightly in all groups. The largest amount of creatine kinase release was from SAT hearts during reperfusion.

III. 4. Discussion

This study using the erythrocyte perfused isolated working rat heart was conducted under control perfusion conditions and with maintained afterload low flow global ischaemia. This model has permitted an examination of the direct consequences of increased dietary polyunsaturated fish oil or saturated sheep fat consumption on myocardial function and metabolism without the complication of neural and humoral influences present in whole animal coronary artery occlusion studies (**McLennan,*et al.*,1985-1990**). For the first time it has been shown that following qualitative modification of dietary fatty acids, the changes to the biochemical composition of cardiac membrane phospholipids which ensue, are also associated with altered working heart metabolism, mechanical function, haemodynamic performance and suscep-

tibility to ischaemic injury, thus extending the previous findings of McLennan, *et al.*, (1985-1990).

Under control conditions there was no difference between the dietary groups in the production of external work or cardiac output. However, myocardial oxygen consumption was more than 3 times higher in SAT hearts than REF group hearts. This was achieved by a 35% increase in coronary flow and an increase in the percentage extraction of oxygen from 8% in REF to 24% in SAT hearts. This increase in MVO_2 was reflected in a markedly decreased energy utilisation efficiency and, although cardiac output and external work were maintained, contractility (as indicated by $\delta P/\delta t$) was significantly depressed. The reduction in contractility and significant release of creatine kinase in SAT hearts suggests some functional deficit. Creatine phosphokinase released from the myocardium is used clinically as an indicator of myocardial cell membrane damage (**Lott,*et al.*,1980;1984;Lee,*et al*,1987; Wu,*et al.*,1987**). This measurable creatine kinase in control perfused hearts may be related to the process of heart removal from the whole animal, but it is unclear as to the mechanism responsible for the greater levels of creatine kinase in control perfused REF and SAT hearts than FO hearts since all hearts were subject to the same conditions. Nevertheless external work and cardiac output were maintained while venous pH, and extracellular K^+ release were not significantly different under control conditions. It is of particular interest to note that FO hearts had, associated with increased aortic output and contractility, the lowest levels of creatine phosphokinase release and lactic acid production during control working heart perfusion. In addition FO hearts

possessed a distinctly higher energy utilisation ratio than SAT and to a lesser extent REF hearts. This indicates that FO hearts, having an altered metabolic and functional capacity may be less susceptible than REF and SAT hearts to the potentially detrimental aspects of increased work or ischaemic stress.

The greater coronary flow in control perfused SAT hearts may be a direct effect of the diet on coronary vasculature or may be partly related to compensatory vasodilation due to increased metabolic requirements (**Mohrman & Feigl,1978;Braunwald,*et al.*,1968;Drake-Holland, *et al.*,1984**). The very poor utilisation efficiency observed in SAT hearts and the augmented lactate production indicate metabolic changes which suggest that the vasodilation was compensatory, although increased flow can induce increased MVO_2 (**Gregg,*et al.*,1967**).

It was observed in the previous chapter that during ischaemia and reperfusion, oxygen consumption was paradoxically increased even though myocardial contractility and external work were reduced significantly. The few researchers that have addressed this increased oxygen consumption have proposed that it may be due to an increased energy requirement to regulate intracellular calcium (**Hoerter,*et al.*,1986;Laster,*et al.*,1989**), which is raised during ischaemia (**Nayler,1981**). The sarcoplasmic reticulum ability to deal with the increased Ca^{++} entry may be impaired. Indeed, increased spontaneous calcium release from sarcoplasmic reticulum and impaired contractility following ischaemic injury has been observed (**Lakatta,*et al.*,1985**). In the present study when coronary perfusion pressure was decreased coronary flow fell to a common low level in all groups inducing a low flow global

ischaemia. In the face of the greatly decreased coronary flow myocardial oxygen consumption was paradoxically increased in REF and to a lesser extent FO hearts, but not in SAT. This was achieved by greatly increased oxygen extraction. The percentage of oxygen extraction was 70% in REF hearts, 45% in FO hearts. During restricted coronary flow oxygen delivery in SAT hearts was insufficient to support additional increases in MVO_2 despite 88% oxygen extraction. However, SAT heart MVO_2 during ischaemia was still significantly higher than FO hearts with paradoxically increased MVO_2.

The other consequences of ischaemia documented in Chapter II were observations of venous acidosis, high extracellular coronary effluent K^+ concentrations and release of creatine kinase, all of which were augmented by the SAT diet during ischaemia and following reperfusion. This suggests that greater ischaemic and reperfusion injury occurred in this group. These markers of ischaemic injury were significantly reduced in the FO group during ischaemia and reperfusion. Thus this supports evidence from whole animal coronary occlusion studies that FO has had a protective influence against ischaemic injury while SAT has enhanced the severity and extent of ischaemia induced myocardial injury (**McLennan,*et al.*,1988;1989**). Many of the dietary differences became greater or were only manifest during ischaemia. If the maintained afterload global ischaemia and the erythrocyte perfusate, had not been utilised, the above differences may not have been clearly evident. If we consider the low oxygen carrying capacity of physiological salt perfusates it is very likely that the ischaemic changes in venous pH, extracellular K^+, creatine phosphokinase, and lactate would have occurred to some extent prior

to reduced coronary perfusion pressure, thus attenuating the contrasting differences between control perfusion and ischaemia.

The physiological effects of ischaemia were quite severe in all diets and when coronary flow was restored, further consequences of ischaemia became evident. By 5 min reperfusion, although less than complete, the greatest recovery in all parameters of mechanical and metabolic function, including oxygen energy utilisation efficiency was observed in FO hearts and the least in SAT hearts. Creatine kinase was washed out in large quantities from the coronary effluent of SAT hearts in reperfusion, but only in minimal amounts from REF and FO hearts. Whether this increased release of creatine kinase was related directly to ischaemia or reperfusion cell damage is unclear as this was not specifically investigated. The presence of extracellular creatine kinase during ischaemia indicates that some ischaemic cell damage had commenced prior to reperfusion in these hearts.

It is unlikely that the effects of the SAT diet in the present study could have been manifested as increased MVO_2 had the study been conducted in isolated working hearts with erythrocyte-free perfusion media. This is because physiological salt perfused hearts have little capacity to increase oxygen extraction and increase coronary flow. The results of Chapter II suggest this and this has very recently been recorded in some detail by Olders, *et al.*, **(1990)**. Erythrocyte perfused working hearts responded to increased preload by an increase in myocardial oxygen consumption and cardiac output, including coronary flow, but Tyrode's-perfused hearts were incapable of altering coronary flow or MVO_2. It has been shown by Gregg and coworkers

(1967) that increases in coronary perfusion pressure and thus coronary flow, result in increased MVO_2. Therefore it is important to determine the relationship between coronary flow and oxygen consumption in SAT hearts. Is the increased coronary flow the result of increased oxygen demand or *vice versa*? Are other factors responsible for these abnormal measures in SAT hearts? These possible relationships are investigated in Chapter V. Alternatively there may be dietary lipid related differences in basal metabolism (See Chapter VI, potassium arrest experiment) that may influence or underlie the increased MVO_2 in SAT hearts. Or considering the apparent relationship between altered energy consumption and calcium overload in ischaemia, it is possible that altered calcium metabolism underlies the dietary influenced alterations to cardiac function and performance, even under control conditions. Such an investigation, utilising selective drugs such as ryanodine and ruthenium red to target intracellular calcium handling sites may provide insight into both ischaemia and dietary-induced differences (See Chapter VI).

The early findings of DeDeckere & Ten Hoor (1979) showed that contractility and work capacity were 10-20% higher in hearts from animals on a polyunsaturated fatty acid (linoleic acid) enriched dietary regime and dietary saturated hydrogenated coconut oil influenced an increased coronary flow. These differences were marginal and related to slightly higher spontaneous heart rates in polyunsaturated dietary fatty acid treated hearts and lower heart rates in the saturated fat treated hearts (not statistically significant). However, the failure of this research group to observe significant dietary fat induced differences in a number of parameters, including myocardial oxygen

consumption could be due to a number of factors. Firstly, experimental diets were fed for only four weeks and to young rats which may be an insufficient feeding period for many dietary induced physiological alterations to become apparent (**McLennan,*et al.*,*1989***). Secondly, the dietary groups compared did not vary by a large margin in dietary lipid composition. Thirdly, the Langendorff perfusion method was preferred to the isolated working heart in most experiments and the influence of ischaemia was not investigated in these studies. Finally, all perfusions were conducted with saline or Krebs-Henseleit solution (**DeDeckere & Ten Hoor,1979;1980**) and even increased preload could not induce changes in coronary flow in these experiments (**DeDeckere, 1981**). In general, few experimental studies have investigated dietary lipid influences on cardiac function. A few of these have reported negative results, but closer examination of their data indicates that the results are confounded due to inadequate attention to dietary lipid composition, storage and handling of feed, length of feeding, age of animals, limitations in experimental models, and limitations in experiment design and execution (**Chardigny,*et al.*,1988;-1991;Johnston & Fritsche,1989**).

The dietary induced functional differences evident from this current study are supported by whole animal experiments using similar dietary regimes. Radionuclide angiography using *in vivo* labelled erythrocytes with ^{99m}Tc in marmosets (small primates) demonstrated that the pressure-rate product, an indirect indicator of myocardial oxygen consumption *in vivo* was increased following saturated fat supplementation. Contractility, as measured by the left ventricular ejection fraction, increased significantly following a

polyunsaturated lipid dietary regime compared with saturated fat supplementation (**Charnock,*et al.*,1987**). In addition to this, dietary polyunsaturates, in particular fish oil supplementation have been shown to reduce and saturated fat increase the susceptibility to ischaemia and reperfusion-induced ventricular arrhythmias in rats (**McLennan,*et al.*,1988**). In the experimental study described in this chapter although ventricular arrhythmias were monitored they have been addressed at length in the following chapter (IV). Indeed in the erythrocyte perfused isolated working heart model the dietary influence on the distribution of ischaemia and reperfusion induced arrhythmias are similar to the findings of McLennan and coworkers. The ischaemia-induced increase in extracellular K^+ concentrations may be related to altered electrical potentials that influence changes to the electrocardiogram and the onset of arrhythmogenesis and the dietary differences in the ischaemic levels of this ion may vary with the extent of the dietary influence on arrhythmias (See following chapter).

It is apparent that dietary supplementation with polyunsaturated fish oil improved ventricular performance in terms of contractility and energy utilisation efficiency. This became more apparent with ischaemia as fish oil supplementation provided protective properties to the myocardium making it less susceptible to ischaemic injury and permitting better post-ischaemic recovery. Saturated sheep fat supplementation made the hearts more vulnerable to ischaemic injury and less capable of adequate post-ischaemic recovery. Of particular interest was the abnormal increase of oxygen consumption during ischaemia and reperfusion, particularly in REF hearts, the

abnormal MVO_2 and coronary flow of SAT hearts under control conditions and the lower MVO_2 of FO hearts. These observations provided the impetus of the ensuing investigations (Chapters V,VI,VII).

IV. THE INFLUENCE OF DIETARY FAT SUPPLEMENTATION ON CARDIAC ARRHYTHMIA INCIDENCE AND VULNERABILITY

IV. 1. Introduction

Following the development of methods permitting the study of the electrocardiogram (ECG), early research found atrial fibrillation of greatest clinical interest despite evidence associating myocardial ischaemia, infarction and ventricular fibrillation. Due to the common observation of atrial fibrillation clinically, it was incorrectly assumed that ventricular fibrillation (VF) occurred rarely and was of minor importance (**Garrey,1924**). Such a misconception arose because most often VF, if sustained, would lead to immediate death without recognition. Thus early studies of mechanisms influencing ventricular arrhythmias were not differentiated from general cardiac arrhythmia studies. However, Wiggers,*et al.*(**1940**), showed that the ventricular fibrillation threshold (VFT) could be reduced following coronary artery occlusion. They postulated that such a reduction may induce ectopic beats that would generate VF. Only in recent decades, through the clinical practice of continuous ECG monitoring of patients with ischaemic heart disease has the direct involvement of ventricular fibrillation been documented as an important factor in sudden cardiac death (**Lown,1979;Nikolic,*et al.*, 1982;Roelandt,*et al.*,1984**).

With the occurrence of cardiac arrhythmias, the elongation of the S-T segment of the ECG has been observed in humans. Arrhythmias have been monitored during or at maximal ischaemia-induced S-T elevation and at reperfusion following the return to pre-ischaemic S-T segment length levels (**Araki,*et al.*,1983;Previtali,*et al.*,1983**). Manning & Hearse (**1984**) observed that susceptibility to arrhythmias was greatest during early ischaemia (within 7 min from the onset of coronary occlusion ischaemia in an *in vivo* rat model) and then gradually decreased. The peak susceptibility was found to occur later in dogs (40 min) possibly due to the increased presence of collateral coronary blood vessels in the dog. Despite observing arrhythmias during ischaemia the greatest interest has been in those ensuing reperfusion. Perhaps this is due to the high incidence of arrhythmias after cardiac surgery, thrombolysis or possible influence of vasospasm relief on sudden cardiac death (**Tzivoni,*et al.*,1983**). Carbonin,*et al.*(**1980**), found that susceptibility to reperfusion arrhythmias was reliant on the severity of the ischaemic insult. Indeed, Curtis and Hearse (**1989**) demonstrated that VF incidence had a sigmoidal relationship to the size of the ischaemic zone following coronary artery ligation in the rat. While the majority of studies investigating experimentally induced arrhythmias have utilised left descending coronary artery ligation models, Curtis and Hearse (**1989**) observed reperfusion VF in 100% of globally ischaemic isolated hearts and suggested that arrhythmogenesis was not contingent on the interface between ischaemic and normoxic tissue but rather on the state of the reperfused tissue.

The occurrence of cardiac arrhythmias whether fatal or not is conditionally influenced by the state of the ischaemia-modified myocardium and the subsequent interaction of factors such as impaired left ventricular function, the sympathetic nervous system, electrolyte imbalance, drug side-effects, increased heart rate, increased physical and emotional stress, which all modulate ventricular premature depolarisations that may trigger arrhythmias (**Lown & Wolf,1971**). Clinically, approximately 75% of sudden cardiac deaths occur following a previous myocardial infarction that had often gone undetected by the subject or physician (**Bigger,*et al.*,1977**). Thus sudden cardiac death is dependent not only on the occurrence of a trigger event but also on the predisposition of the myocardium to that trigger event and ventricular fibrillation. This means that those with identifiable myocardial infarction or other cardiac disorder that produces ventricular dysfunction have a very high risk of succumbing to fatal arrhythmias. Where treatment with antiarrhythmic drugs has been fortuitously possible the general reduction of cardiac arrhythmias has not been highly successful in preventing sudden fatal arrhythmias, even with the superior drugs currently available (**Echt,*et al.*, 1983**). Thus, considering these difficulties, it would be rational and discerning to select a prophylactic approach of reducing myocardial vulnerability to arrhythmias, one that is not contingent upon detection and diagnosis of risk factors or pathophysiology in potential victims of cardiac disorder. Such an approach could effectively be fulfilled by dietary intervention and modification of dietary lipid intake, as it has already been effective in averting the incidence of atherosclerosis (**Goodnight,*et al.*,1982;Kroumhout,*et al.*,1985**).

The recent studies of McLennan,*et al.*(**1985-1990**) have provided robust evidence supporting the correlation between dietary lipid type and susceptibility to arrhythmias in a number of *in vivo* and *in vitro* rat and primate models. The incidence of isoprenaline induced dysrhythmias in rat and marmoset (primate) isolated papillary muscles is greater following feeding with saturated sheep fat and reduced by dietary supplementation with polyunsaturated fatty acid rich fish oil or sunflower seed oil (**McLennan,*et al.*,1986;1987**). The same authors utilised coronary artery ligation in an *in vivo* model of sudden cardiac death to demonstrate an increased susceptibility to arrhythmias such as VF in rats fed a diet supplemented with saturated fat and reduced susceptibility with dietary polyunsaturated fatty acid supplementation. Of greatest importance was the distinct reduction in the incidence of ventricular fibrillation during coronary occlusion and upon reperfusion in the fish oil groups. The protective benefit of a fish oil dietary regime was evident both in aged animals and after cross-over from long term consumption of increased dietary saturated fat. This latter finding indicates that it would be of value to explore the possibility of altering the composition of dietary fat intake in middle-aged to elderly humans with high saturated fat consumption in order to reduce the prevalence of sudden cardiac death in this high risk group.

In the Chapter III of this thesis it was reported that saturated fat (SAT) supplementation influenced a greater release of extracellular [K^+] into coronary effluent during global ischaemia and reperfusion compared to polyunsaturated fish oil (FO). Indeed FO provided substantial protection against this effect of ischaemic insult. This release cannot be considered to be

related solely, if at all, to reduced membrane integrity because such damage does not occur immediately upon the onset of ischaemia. Harris,*et al.*(**1954**), first observed that the onset of arrhythmias following coronary artery occlusion in the canine heart corresponded with increased [K^+] in venous coronary effluent. Since then many have provided evidence to support this concept of [K^+] imbalance and arrhythmogenesis (**Regan,*et al.*,1967;Fisch,*et al.*,1973;Jennings,*et al.*,1957;Thomas,*et al.*,1970**). Closer investigation has revealed that within the first minutes of ischaemia there is a net loss of potassium from myocardial cells to the extracellular fluid (**Kleber,1984**). Associated temporally with this is the shortening of myocardial cell action potentials. Opie,*et al.*(**1979**) proposed that myocardial potassium loss exerts a role in reducing the membrane resting potential and thus increasing the possibility of arrhythmogenesis. The diminished conduction and altered refractoriness traits of ischaemic myocardium are influenced by the build up of extracellular K^+ to increase the likelihood of arrhythmias (**Kleber,1987**).

As discussed above, dietary fatty acid influences on arrhythmias have previously been observed in isolated cardiac muscle following excess catecholamine stimulation and following ischaemia or reperfusion in the whole animal. The arrhythmogenic influence of ischaemia, reperfusion and dietary lipid modulation of these events in the whole animal may be dependent on neural or humoral control or the properties of the myocardium *per se*. The erythrocyte perfused isolated working heart technique permits the study of the direct effects on the heart with the exclusion of humoral and neural influence. The increased extracellular K^+ release in ischaemia and reperfusion of SAT

hearts compared to FO hearts (Chapter III) may predispose these hearts to arrhythmias in isolation even in the absence of excess catecholamines. As arrhythmias tend to be intermittent and most often not clinically evident, clinical assessment of myocardial vulnerability to arrhythmia and effectiveness of antiarrhythmic therapy has recently utilised programmed electrical stimulation technique (**Axelrod,*et al.*,1975;Richards,*et al.*,1983;Gomes,*et al.*,1984;Brugada,*et al.*,1984,Hessen,*et al.*,1990**). The technique is also used to predict increased arrhythmia vulnerability. By triggering an electrical pulse at an appropriate stimulus level during the ventricular vulnerable period of the cardiac cycle, ventricular fibrillation can be initiated (**Shumway,*et al.*,1957**). The threshold current required to induce fibrillation has been used as a relative indicator of myocardial arrhythmia vulnerability. This approach arose from the finding that ventricular fibrillation ensued when early ventricular premature beats interupted the T-wave in patients with acute myocardial infarction (**Lown & Wolf,1971**).

In this chapter the influence of dietary fats are examined on both spontaneous arrhythmia generation during ischaemia and reperfusion (Experiment A) and vulnerability to electrically induced arrhythmias during control working heart perfusion and ischaemia (Experiment B).

IV. 2. Methods

IV. 2. a) Animals

Sixty male Hooded-Wistar rats (4 months old) were divided into two experimental then three dietary groups REF, SAT, FO (n=10 per group).

Diets were prepared, stored and fed to rats in the manner described in Chapter III. The rats were housed 5 per cage at 23°C with constant 55% humidity on a 12h light/dark cycle. Although the food and water was available *ad libitum*, the animals were fasted overnight prior to experimental usage. The animals were kept on their respective diets for four months.

IV. 2. b) Experiment A. Perfusion Protocol

In this experiment hearts were perfused as described in the previous chapter and the incidence of ischaemia-induced arrhythmias was assessed. Isolated hearts were prepared for working heart perfusion (maintained afterload ischaemia configuration) with the erythrocyte buffer (40% haematocrit) as described in Chapter II. During Langendorff perfusion mode the pulmonary artery was cannulated, pacing electrodes were attached to the right atrium and the coronary vessels were washed out. Hearts were switched to erythrocyte perfused working heart mode (CPP=75mmHg, preload=10mmHg, workload =75mmHg), as described earlier. All hearts were paced at 300 bpm, with square wave pulses of 2ms duration at twice the threshold current with 2 Grass subdermal platinum alloy needle electrodes and a Narco Biosystems stimulator. The hearts were atrially paced in order to preclude the influence of heart rate on arrhythmia vulnerability as well as to ensure that normal driving of the ventricles via the atrio-ventricular node so that the direction of current flow was normal. The hearts were perfused in working heart mode for 10 min to stabilise. At the end of this period measures were taken (every 5 min until the end of the experiment) of cardiac output, aortic pressure, pH,

arterial and venous blood gas content. Ischaemia was induced by lowering the coronary perfusion reservoir such that the coronary perfusion pressure was 35mmHg. After 15 min, reperfusion was initiated by raising the same reservoir back to 75 mmHg. The electrocardiogram (ECG) was recorded continuously with two Grass platinum alloy subdermal recording electrodes placed at the surface of the ventricular apex and the aortic stump and attached to a Grass preamplifier and EKG signal unit in a Grass polygraph (Grass, Quincy, M.A., U.S.A.).

IV. 2. c) Experiment B. Programmed Electrical Stimulation Protocol

In this experiment the vulnerability to ventricular fibrillation was examined. Programmed electrical stimulation involved using square wave pulses (2 ms duration) at a stimulation intensity of twice the mid-diastolic excitation threshold. A Grass S8800 digital stimulator sent pulses through to a Grass SIU5 stimulus isolation unit then a Grass CCU1 constant current unit and finally to subdermal needle platinum alloy electrodes (Grass Instruments, Quincy, MA) attached to the ventricular apex (cathode) and just below the left anterior atrio-ventricular border (anode). Working hearts were permitted to maintain their spontaneous heart rate except just prior to a programmed stimulation event heart rate was driven with a constant ventricular pacing train of 10 pulses (S_1) at 200 ms intervals and then a single extra stimulus (S_2) was introduced after a S_1/S_2 coupling interval which was decreased from 140ms until refractoriness occurred within the effective refractory period limits. The intensity of the extra stimulus was increased in the vulnerable period by

raising current in 2.5-5.0 mA steps until ventricular arrhythmias were induced or maximal current (43mA) had been delivered. This programme of stimulation was conducted under control working heart perfusion, and at the end of 15 minutes of global ischaemia.

IV. 2. d) Ventricular Arrhythmia Assessment

The following definitions were used in the assessment and scoring of arrhythmias according to the "Lambeth Convention" (**Walker,*et al.*,1988**).

i) Ventricular premature beats (VPB): discrete QRS complexes that are premature in relation to the P wave. Bigeminy is scored as a variant of this.

ii) Salvos: 2 or 3 ventricular premature beats were not considered to be ventricular tachycardia but termed salvos.

iii) Ventricular tachycardia (VT): 4 or more consecutive ventricular premature beats of similar morphology were deemed to be tachycardia.

iv) Ventricular Fibrillation (VF): This was defined as a signal from which QRS deflections are no longer distinguishable from others and no sinus rythm can be accurately measured (distinct from the flat signal of asystole where no sinus rhythm is evident).

IV. 2. e) Statistical Analysis

All results were expressed as mean $\pm$ SD (except data in Figure IV. 2). For each parameter the effect of dietary treatment was tested by Analysis of Variance and between individual comparisons, Scheffe's *post hoc F*-test. The level of significance was considered at $P < 0.05$ or less.

IV. 3. Results

Examples of arrhythmias and ventricular fibrillation threshold measurement are illustrated in Figure IV. 1. During the low flow ischaemic period of Experiment A. only infrequent ventricular premature beats (VPB) were observed, most of which occurred between 10 and 15 min ischaemia. Figure IV. 2. shows that although even they were infrequent, a significantly higher number of VPBs during ischaemia were measured in the SAT group while the lowest number were seen in FO hearts. Upon reperfusion, arrhythmias were more evident and ranged from VPB to VF. The distribution of reperfusion arrhythmias is presented in Figures IV. 3.a) and b) ($n=10$ per group). The incidence of ventricular tachycardia was significantly reduced in the FO dietary group ($<15\%$) compared to both SAT and REF (60-65%), which did not differ (Figure IV. 3.). Ventricular fibrillation incidence was at approximately 60% for the REF group while in SAT hearts VF incidence rose to 80%. Reperfusion VF was not observed in any FO dietary supplemented hearts (Figure IV. 3.). In reperfusion, the ventricular fibrillation episodes were sustained (>30s VF) in 5 out of 8 SAT and 3 out of 6 REF hearts.

Experiment B. was aimed at examining ventricular fibrillation threshold vulnerability in dietary groups challenged with programmed electrical stimulation. The spontaneous beat rates of control perfused isolated hearts (mean $\pm$SD) were: REF, 278 $\pm$14; SAT, 306 $\pm$12; FO, 248 $\pm$11 beats per min. SAT heart rate was significantly elevated compared to REF ($p=0.04$, $n=10$) and FO ($p=0.016$, $n=10$), while FO had a significantly lower heart rate compared to REF ($p=0.034$) and SAT. After 15 min of low

flow ischaemia the mean (±SD) spontaneous heart rates had fallen significantly to: REF, 124 ±11; SAT, 132 ±15; FO, 170 ±14. The heart rates in the REF group were significantly lower than those of SAT (p=0.04) and FO hearts (p=0.038).

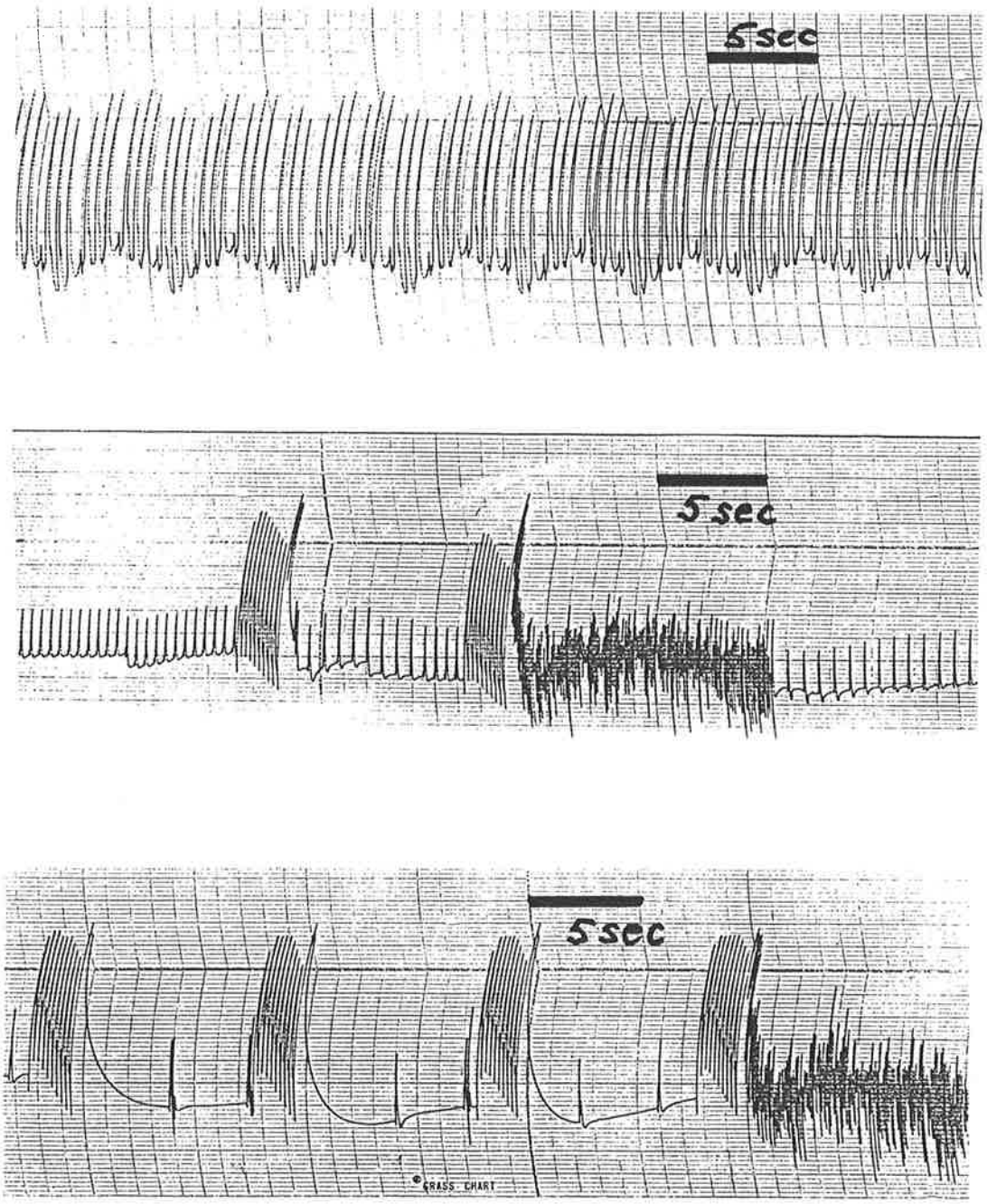

Figure IV. 1. Examples of spontaneous and triggered arrhythmias. Top panel indicates displays spontaneous arrhythmias in a SAT heart at 15min ischaemia. Middle panel illustrates the programmed VF stimulation during control perfusion and shows spontaneous defibrillation in a SAT heart. Bottom panel shows programmed stimulation during ischaemia (at 15min) in a SAT heart which failed to spontaneously defibrillate. See Methods and Results for details.

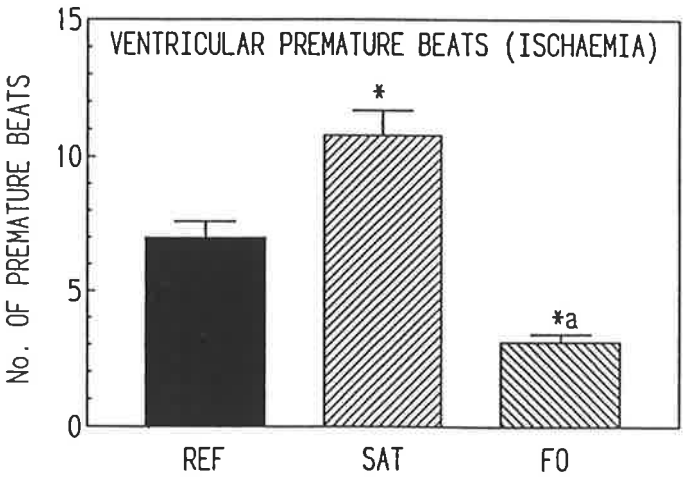

Figure IV. 2. The effect of dietary fat supplementation on the number of ventricular premature beats during ischaemia. Values are expressed as mean±SEM; n=10 per group; *=p<0.05, significantly different compared to REF; a=p<0.05, significantly different compared to SAT; Scheffe's comparison. REF=Reference diet; SAT=Saturated fat rich diet; FO=Polyunsaturated rich fish oil diet; 15min ischaemia= CPP=35mmHg. See Methods for details.

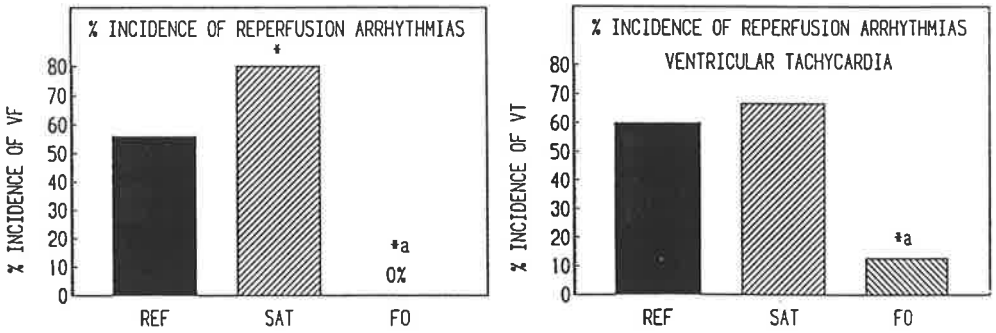

Figure IV. 3. The effect of dietary fat supplementation on the % incidence of reperfusion arrhythmias (ventricular tachycardia and ventricular fibrillation). Results are expressed as median values; n=10 per group; *=p<0.05, significantly different compared to REF; a=p<0.05, significantly different compared to SAT; Scheffe's comparison. REF=Reference diet; SAT=Saturated fat rich diet; FO=Polyunsaturated rich fish oil diet. See Methods for details.

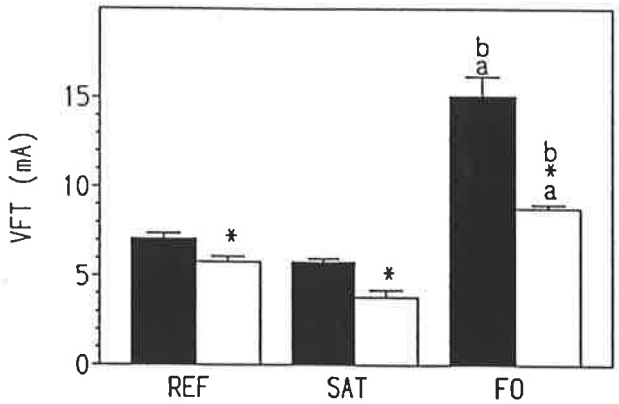

Figure IV. 4. The effect of dietary fat supplementation on ventricular fibrillation threshold (VFT) during control working heart perfusion and ischaemia. Values are expressed as mean±SD; n=10 per group; *=p<0.05, significantly different compared to working heart control; a= significantly different compared to REF; b=p<0.05, significantly different compared to SAT; Scheffe's comparison. REF=Reference diet; SAT=Saturated fat rich diet; FO=Polyunsaturated rich fish oil diet. Solid bars =control working heart perfusion; open bars =15min ischaemia. See Methods for details.

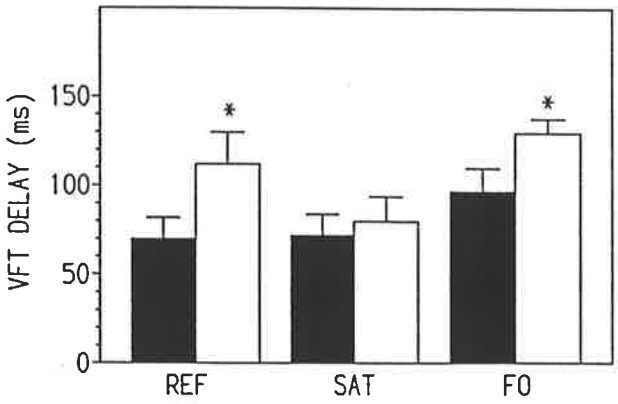

Figure IV. 5. The effect of dietary fat supplementation on the refractory period during control working heart perfusion and ischaemia. Values are expressed as mean±SD; n=10 per group; *=p<0.05, significantly different compared to working heart control; Scheffe's comparison. REF=Reference diet; SAT=Saturated fat rich diet; FO=Polyunsaturated rich fish oil diet. Solid bars =control working heart perfusion; open bars =15min ischaemia. See Methods for details.

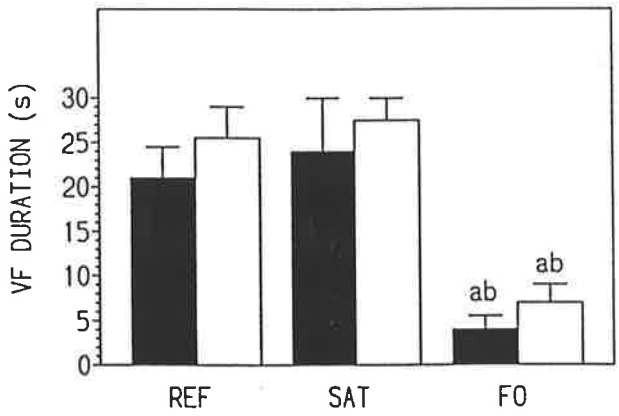

Figure IV. 6. The effect of dietary fat supplementation on ventricular fibrillation duration during control working heart perfusion and ischaemia. Values are expressed as mean±SD; n=10 per group; a= significantly different compared to REF; b=p<0.05, significantly different compared to SAT; Scheffe's comparison. REF=Reference diet; SAT=Saturated fat rich diet; FO=Polyunsaturated rich fish oil diet. Solid bars =control working heart perfusion; open bars =15min ischaemia. See Methods for details.

Figure IV. 1. provides example of ischaemia-induced S-T segment elevation and show the induction of VF by programmed stimulation. The spontaneous defibrillation common to rat hearts is also illustrated. It was observed that the threshold current in all dietary groups decreased during ischaemia (Figure IV. 4). In control working heart perfusion SAT and REF hearts required a smaller current to induce fibrillation compared to FO hearts. This dietary relationship was maintained following 15 min ischaemia despite a reduction in the current required to induce VF in all groups. Indeed SAT hearts at 15 min ischaemia required very little stimulation to invoke fibrillation (Figure IV. 4.). The refractory period increased slightly in all groups with ischaemia however both during control and ischaemic perfusion FO hearts had a significantly longer refractory period compared to SAT and REF (Figure IV. 5.). The duration of ventricular fibrillation episodes is presented in Figure IV. 6. Sustained ventricular fibrillation (VF duration >30s) occurred in 4 out of 10 SAT group hearts during the control period and no sustained VF was observed in REF or FO hearts. However, only 2 out of 10 SAT hearts and 2 out of 10 REF hearts had a VF duration of less than 5s. In contrast 8 out of 10 FO hearts had a VF duration of less than 5 seconds. Ischaemia slightly raised the mean duration of non-sustained VF from control perfusion levels in all dietary groups. The longest mean duration of VF was observed in REF and SAT groups while FO hearts quickly defibrillated spontaneously giving a significantly lower VF duration even during ischaemic insult. In a few hearts from REF and SAT groups following programmed stimulation at 15 min ischaemia sinus rhythm did not resume. Attempts were made to defibrillate the

hearts by over drive pacing after 45 s VF but were unsuccessful. For statistical evaluation these few hearts were assigned a VF duration equal to the longest duration (30 s) of non-sustained VF. Notably sustained VF was not observed in any FO heart, but 4 out of 10 SAT hearts and 3 out of 10 REF group hearts had VF sustained for more than 30 s at 15 min of global ischaemia. Even in ischaemia VF episodes lasted for less than 5 s in 8 out of 10 FO hearts. In comparison, 1 REF heart and no SAT hearts had any such brief VF episodes.

IV. 4. Discussion

This study has for the first time demonstrated that a relationship exists between the increased consumption of dietary polyunsaturated fatty acids and a reduction of myocardial vulnerability to arrhythmogenesis in the globally ischaemic isolated heart. These findings support the previous whole animal coronary artery occlusion studies, but free of potentially confounding neural, humoral and variable blood pressure influences (**McLennan,*et al.*,1989**). Hearts from the FO group were less susceptible to arrhythmias during ischaemia and reperfusion in comparison to SAT and REF hearts. The complete absence of spontaneous ventricular fibrillation in reperfused FO hearts distinctly demonstrated the protective attributes of FO (Figure IV. 4). It was evident that SAT diet treatment was pro-arrhythmogenic because of increased arrhythmias measured in this group compared to REF and FO hearts as has been observed by others (**McLennan,*et al.*,1987;1989**). In the previous chapter it was observed that the concentration of potassium in coronary

effluent increased substantially during ischaemia and particularly in SAT hearts, but to a lesser extent in FO hearts. As discussed earlier, the increased extracellular accumulation of K^+ occurs with the onset of ischaemia and abnormally high K^+ levels are associated with reduced conduction, altered refractoriness and arrhythmogenesis (**Kleber,1987**). Whether the dietary effect on ischaemic extracellular [K^+] accumulation is directly linked to specific mechanisms responsible for the dietary lipid induced differences observed or is merely related to altered affectors is unclear. However, a number of mechanisms of potassium handling have been proposed to be altered by ischaemia (**Wilde,*et al.*,1990**):

i) Na^+/K^+ pump inhibition could influence reduced inward transport and increased efflux of K^+;

ii) Increased efflux of K^+ could follow intracellular anions (e.g. lactate) generated by abnormal metabolism;

iii) Modified cellular and intracellular volume could increase K^+ efflux with transmembrane osmotic movement secondary to the production of osmotically active metabolites.

iv) It has been demonstrated that cardiac ATP-regulated K^+ channels mediate increased K^+ efflux during ischaemia and hypoxia and this release is an important contributor to arrhythmogenesis (**Kantor,*et al.*,1990;Smallwood,*et al.*,1990;Noma,1983**). Glibenclamide, a drug with selectivity for the ATP-dependent K^+ channel, attenuates K^+ release during ischaemia and has been shown to abolish ventricular fibrillation during ischaemia but

have no effect on lactate, phosphocreatine and ATP content or glucose utilisation (**Kantor,*et al.*,1990**).

The supportive evidence to the above ischaemia-altered K^+ mechanisms requires extension and consolidation. It is however feasible on the basis of the present study, that ischaemic influences on potassium ion movement may also be altered by dietary lipid modulation.

The programmed electrical ventricular stimulation proved to be a useful and convenient way of testing susceptibility to arrhythmias in the isolated erythrocyte perfused working heart preparation, particularly in a dietary lipid supplemented heart model previously shown to be free of vascular disease (**Turner,*et al.*,1990**). Despite use of this testing method in the evaluation of antiarrhythmic drug efficacy in experimental animals and in the clinical assessment of human myocardial vulnerability to arrhythmias, this is the first study to use programmed stimulation in isolated rat heart preparations modified by dietary fatty acid supplementation. Increased dietary fish oil consumption served to increase the threshold current required to induce ventricular fibrillation during control working heart perfusion whereas it was more easy to invoke ventricular fibrillation in SAT hearts with the programmed electrical stimulation paradigm utilised.

It was observed that with ischaemia, VFT was reduced across all dietary groups, supporting the previous findings that ischaemic and hypoxic insult of the myocardium lowers the ventricular fibrillation threshold (**Wiggers,*et al.*,1940;Shumway,*et al.*,1957;Han,1969;Murnaghan,1975; Lubbe,*et al.*,1975**). Even with the reduction of VFT due to myocardial ischaemia in FO

hearts, it remained significantly higher than that recorded in SAT hearts during control working heart perfusion. Myerburg, *et al.*, **(1984)** recognised that increased myocardial substrate vulnerability is a critical factor that predisposes a subject to sudden cardiac death in conjunction with a trigger event and/or factors which modulate the severity of the trigger or the sensitivity of the myocardium to the effect of the trigger event. Since myocardial vulnerability to arrhythmias was altered by dietary lipid supplementation under both the control perfusion and ischaemic conditions of this present study and in the face of trigger events that were constant across dietary groups, it is feasible that dietary fatty acids could be used as a prophylactic to modulate the cardiac response to a range of proarrhythmic factors that would normally initiate fatal ventricular arrhythmias.

It is interesting to note that spontaneous heart rates differed between the dietary groups. While the mean heart rate was elevated by increased dietary saturated fatty acid supplementation, dietary polyunsaturated fish oil supplementation lowered it in comparison to the reference diet during control perfusion. It was shown in Chapter II that ischaemia induced a reduction in heart rate. Although heart rate decreased in all dietary groups during ischaemia the least reduction occurred in FO treated hearts and the largest fall in heart rate occurred in SAT hearts. It has been observed clinically **(Adgey,1982)** in a study of 48 patients who experienced VF during very early stages of myocardial infarction that prior to an episode of VF heart rate rose significantly. This suggests that high heart rates may be conducive to arrhythmogenesis during the early phases of coronary ischaemia in humans. Chadda,

et al.,(1974), demonstrated that during experimental coronary occlusion in dogs ventricular premature depolarisations and ventricular fibrillation occurred at low heart rates (60-90 bpm) and at high rates (180-200 bpm) but not at intermediate rates (90-180 bpm). Notably, the SAT heart rates were quite low by 15 min ischaemia when the highest incidence of VF was observed and the heart rate of FO hearts although lowered during ischaemia were at comparatively intermediate levels. Indeed early re-entrant arrhythmias have been shown to be less prevalent during bradycardia and the reduced heart rates were associated with reduced myocardial fibre damage (**Kaplinsky,*et al.*, 1972;Scherlag,*et al.*,1976**) but this heart rate reduction led to increased non-re-entry-related arrhythmias during long term ischaemia. It has also been found that heart rates increased to very high levels exacerbate arrhythmias, ischaemic zone size and increased severity of ischaemic cell injury in acute coronary occlusion ischaemia (**Kaplinsky,*et al.*,1972;Shell & Sobel,1973**). The advantages of bradycardia in ischaemia could be to reduce myocardial oxygen requirements. In the programmed stimulation model utilised in Experiment B and the paced working heart model of Experiment A, the heart rate was controlled in all the groups during the initiation of arrhythmias to eliminate heart rate influence of ischaemia. The dietary differences in the spontaneous heart rates measured in the latter experiment indicate that SAT hearts may indeed be predisposed to arrhythmogenesis while FO hearts may have a greater capacity to optimise heart rate to a non-arrhythmogenic level. Furthermore, in the previous chapter it was apparent that even after normalisation of heart rate, the SAT hearts had higher oxygen requirements. At

spontaneous rates, this differential may be even greater. Myocardial oxygen demand may in itself be an important determinant of substrate vulnerability.

A determinant of whether ventricular fibrillation has a fatal outcome is its duration and whether it is sustained. Figure IV. 6. showed that the fish oil supplemented group was able to limit the duration of ventricular fibrillation (<4-6 s). The duration of VF was almost negligible compared to the comparatively very long duration of VF in SAT hearts. The effect of ischaemia was to slightly increase the duration of ventricular fibrillation in all the dietary groups. Even with this influence FO hearts had a significantly shorter mean fibrillation episode compared to SAT and REF hearts. The results suggest that the n-3 polyunsaturated fatty acids of fish oils exert a protective influence on the electrophysiological properties of the myocardium which support the continuance of instability and ventricular fibrillation.

Human epidemiological studies have linked low mortality from cardiovascular disease to the increased consumption of fish or fish oils (**Kroumhout,*et al.*,1985;Leaf & Weber,1988;Harris,1989**). Burr,*et al.*(**1990**), observed that increased consumption of fish reduced mortality over two years in patients with a previous myocardial infarction. It did not reduce the incidence of heart attack, only the incidence of death. Since post-infarction patients are at very high risk of VF and consequently sudden cardiac death, it is possible in the light of the present study that mortality was reduced by decreasing the proportion of events which progressed to fatal arrhythmias. The reduction of fat intake alone had no protective effect in these subjects. This is similar to the comparison between SAT and REF in which fat intake was

reduced from 15.3% to 7.6% (w/w) but the types of fatty acids were changed very little and the incidence of ventricular fibrillation was not very different. Thus it would seem most worthwhile to conduct clinical trials designed to specifically investigate arrhythmia incidence and sudden cardiac death to further evaluate the efficacy of increased n-3 polyunsaturated fatty acid consumption as a prophylactic against myocardial arrhythmia vulnerability.

The current study clearly demonstrated that dietary lipid modulation altered arrhythmic vulnerability to several triggers under conditions of strictly controlled pressure and arterial ionic concentrations. It most likely resides in directly altering the properties of the myocardium.

V. THE INFLUENCE OF CORONARY FLOW ON MYOCARDIAL OXYGEN CONSUMPTION

V. 1. Introduction

The notion that cardiac autoregulation of coronary blood flow is influenced by the augmentation of coronary vasodilator metabolites proportional to the rate of energy consumption is based on the observation that coronary blood flow covaries closely with myocardial oxygen consumption (**Khouri,*et al.*,1967**). Such a mechanism is likely to explain the greater coronary flow in SAT hearts during control working heart perfusion compared to FO and REF (Chapter III). On the other hand it has been shown that increasing coronary perfusion pressure and flow results in increased contractile force, external work and oxygen consumption (**Gregg,1963;Gregg,*et al.*,1965**), the increased myocardial oxygen consumption in association with greatly reduced contractility and external work during ischaemia observed in Chapter II and III must be considered paradoxical. In order to investigate this closer it was important to first establish whether the high cardiac oxygen consumption in SAT hearts is due to high coronary flow. An increase in coronary flow would usually be expected to produce increases in contractility and external work, however, this was not seen in SAT hearts. Nevertheless, if cardiac MVO_2 is directly related to coronary flow alone, then equalising the coronary flow in all hearts should abolish the dietary induced differences in myocardial oxygen consumption.

The classical vasodilator hydralazine was selected in an attempt to match FO and REF coronary flow rates with the high coronary flow rates seen

in SAT hearts in normal working heart perfusion (Chapter III) by producing maximal coronary vasodilation in all groups. This maximal vasodilation would also make it possible to observe whether dietary fatty acid supplementation restricted maximal coronary flow and to observe any changes in coronary vascular resistance with changes in type of fat diet or ischaemia.

V. 2. Methods

V. 2. a) Animals and Dietary Supplementation

Thirty male Hooded-Wistar rats (4 months old) were weight matched (400-500 g at end of experiment) and placed in the three dietary groups REF, SAT, FO, (n=10 per group). The diets were prepared, stored and presented to the rats as described in Chapter III. The animals were kept on their allocated diet and housed under the conditions described in Chapter III. until they were approximately 8 months old (four months on experimental diet) and then were fasted overnight prior to each experimental day.

V. 2. b) Perfusion Protocol

Isolated hearts were prepared for working heart perfusion (maintained afterload ischaemia configuration) with the erythrocyte buffer (40% haemato-crit) and perfused and monitored as described in Chapter II. Hearts were perfused in Langendorff mode for 10 min while the pulmonary artery was cannulated, pacing electrodes were positioned, and coronary blood was flushed out. Working heart perfusion was commenced (coronary perfusion pressure, preload and afterload set at 75, 10 and 75 mmHg respectively) and the hearts

were paced at 300 bpm (5Hz, 2 ms duration at twice the threshold current) with 2 platinum alloy subdermal needle electrodes placed on the right atrium (Grass Instruments, Quincy, MA, U.S.A.).

Following 5 min working heart perfusion, allowing hearts to equilibrate and stabilise, measures of cardiac output, aortic pressure, arterial and venous blood gas content, and pH were taken every 5 min until the end of the experiment. Coronary effluent samples were also collected for later biochemical analyses. Following oxygen tension and pH readings erythrocytes from arterial and coronary venous samples were removed by centrifugation with a small Corning bench top centrifuge. The sample supernatant was frozen in liquid nitrogen and stored at -60°C. Following assessment of control working heart performance a second identical (parallel) perfusion circuit was opened and the former circuit closed. The perfusate in the second circuit was identical to the erythrocyte buffer previously used except it contained 10^{-4}M hydralazine (1-hydrazinophthalazine, Sigma Chemical Co., U.S.A.). After 5 min of hydralazine treatment all measures were repeated then working hearts were made ischaemic by lowering the coronary perfusion pressure to 35mmHg. After 15 min, reperfusion was initiated by returning coronary perfusion pressure to 75mmHg. Hearts continued to receive hydralazine throughout ischaemia and reperfusion. Following each perfusion, hearts were quickly removed from the apparatus, blotted dry and weighed whole and with atria & ventricles dissected then prepared for dry weight determination.

V. 2. c) Vasodilator Treatment

In order to determine the effective dose for maximal coronary vasodilation a preliminary experiment was conducted using 10^{-8}M-10^{-3}M hydralazine in paced erythrocyte buffer perfused isolated working hearts (n=2 per dose) from rats that were not on experimental diets (standard reference commercial diet). A sigmoidal curve log-dose dependent effect was observed. Maximal coronary flow occurred following administration of 10^{-4}M hydralazine, and thus this dose was selected for use (Figure V. 1). No significant influence on myocardial oxygen consumption was observed at this dose although a very small negative inotropic effect was observed involving a slight decline in the PTI and systolic pressure (<5mmHg).

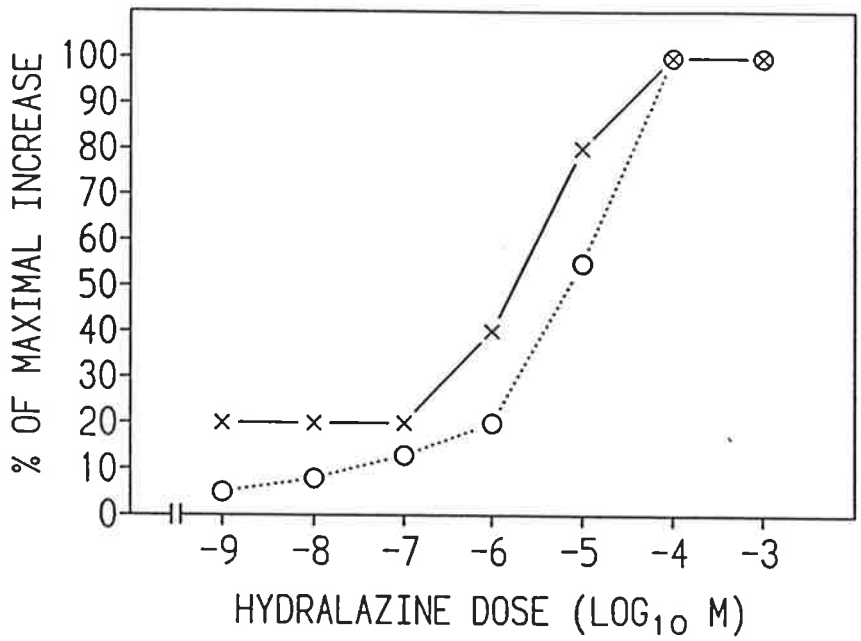

Figure V.1. The log dose-response of hydralazine (M) on coronary flow (open circles) and aortic output (crosses) in isolated working hearts (reference diet). CPP=75mmHg; Preload=10mmHg; Workload=75mmHg. Results are expressed as % of maximal increase (median values; n=2).

V. 2. d) Analysis of Coronary Effluent Contents

Arterial and venous samples were assayed for creatine kinase and lactic acid concentrations as described in Chapter III. Creatine kinase was quantified according to the rate of change of absorbance. Results were expressed in U/min/g dry weight adjusted according to coronary flow rates. Lactic acid concentrations were expressed in μmol/min/g dry weight when adjusted according to coronary flow rates. For potassium ion concentration determination all samples were diluted in 1.5 mmol/L Caesium diluent and analysed in duplicate for extracellular K^+ concentrations with an automated IL943 Flamephotometer (Instrumentation Laboratories, U.S.A.). Results were expressed in mmol/L.

V. 2. e) Data Handling and Statistical Analysis

Myocardial left ventricular external work, the pressure-time integral, perfusate oxygen content, oxygen extraction, myocardial oxygen consumption and energy utilisation efficiency percentage were calculated as described in Chapter II. All results were expressed as mean $\pm$ SD. For each parameter the effect of dietary treatment was tested by Analysis of Variance and between individual comparisons with Scheffe's *post hoc* *F*-test. The level of significance was considered at $P < 0.05$ or less.

V. 3. Results

V. 3. a) Vasodilator Influence on Coronary Flow & Cardiac Output

The administration of 10^{-4}M hydralazine increased the coronary flow rates of REF and FO hearts during control working heart perfusion but did not cause any increase in the coronary flow of SAT hearts. In the presence of hydralazine there was no significant difference in coronary flow rate between the 3 diets (Table V.1.a)). Hydralazine had no significant effect on coronary flow after 15 min ischaemia in any dietary group compared with ischaemic perfusion without hydralazine. Upon reperfusion, coronary flow in FO hearts was significantly increased relative to the reperfusion period in non-hydralazine treated FO hearts. Thus upon reperfusion coronary flow was not significantly different between FO and SAT hearts. The aortic output did not differ significantly between the dietary groups during control perfusion, ischaemia and reperfusion following hydralazine, despite the trend for a smaller aortic output in SAT hearts in all perfusion phases (Table V. 1 b)). Hydralazine attenuated the significant differences in cardiac output between the dietary groups in the control, ischaemia and reperfusion phases of the experiment (Table V. 1.c)).

V. 3. b) Vasodilator Influence on Ventricular Performance

Hydralazine had a negative inotropic effect in all dietary groups during control perfusion. Despite a similar cardiac output between the dietary groups the pressure-time integral was significantly higher in FO hearts than in REF and was significantly lower in SAT hearts compared to REF (Table V. 2. a)).

While contractility during ischaemia tended to differ slightly between the 3 groups, $\delta P/\delta t$ in all groups was depressed to the same very low level observed in the absence of hydralazine during ischaemia. Contractility returned to near control levels ensuing reperfusion in hydralazine treated hearts but remained lower than in hearts reperfused in the absence of hydralazine.

Left ventricular external work during control perfusion with or without hydralazine treatment did not differ significantly between the 3 dietary treatment groups (Table V. 2. b)). However, with hydralazine treatment the FO hearts tended to produce more external work, and SAT hearts less external work than the REF group. The level of left ventricular external work fell to very low levels in all groups during ischaemia. Post-ischaemic external work was slightly but not significantly higher after treating the 3 groups with hydralazine. The best return towards pre-ischaemic external work levels at 5 min reperfusion was observed in FO hearts compared to SAT and REF hearts.

V. 3. c) Vasodilator Influence on Oxygen Utilisation

The percentage of oxygen extracted was significantly lower in SAT hearts treated with hydralazine than without hydralazine during control perfusion. Hydralazine did not significantly alter oxygen extraction in REF or FO hearts under these conditions. With or without hydralazine treatment the highest percentage of oxygen extracted was observed in SAT hearts and the least oxygen extraction in FO hearts (Table V. 3. a)). Ischaemia induced an increase in oxygen extraction in all hearts but hydralazine treatment attenuated the extent of the increased oxygen extraction evident in REF and SAT hearts

with the absence of hydralazine. The highest oxygen extraction was measured in ischaemic SAT hearts and it was significantly lower in ischaemic FO hearts regardless of hydralazine presence. Ensuing reperfusion (5 min post-ischaemia) oxygen extraction fell in all 3 dietary groups yet remained significantly elevated compared to their respective control group. In the presence of hydralazine the extent of return to pre-ischaemic oxygen extraction levels in the 3 groups was much less than in hearts perfused without hydralazine.

Myocardial oxygen consumption (MVO_2) was significantly reduced by hydralazine treatment in SAT hearts but significantly increased in FO hearts during control working hearts perfusion (Table V. 3.b)). Coronary flow rates were equalised after hydralazine treatment but SAT hearts still had a significantly higher MVO_2 compared to REF, while FO had the lowest oxygen consumption. Ischaemia induced an increase in MVO_2 in all dietary groups in hydralazine treated hearts. During ischaemia in the absence of hydralazine MVO_2 increased in the REF and FO hearts but fell in the SAT hearts. The MVO_2 during ischaemia in each dietary group with hydralazine was not different to the MVO_2 of ischaemic non-hydralazine treated hearts. Oxygen consumption returned to control levels in hydralazine-free hearts but remained significantly elevated during reperfusion with hydralazine. SAT hearts had significantly higher MVO_2 than FO and REF with or without hydralazine. The post-ischaemic MVO_2 in FO hearts was significantly lower than in REF hearts in the absence of hydralazine.

While hydralazine produced a slight attenuation in the ratio of energy utilisation to oxygen consumption (% efficiency of energy utilisation) in FO and REF hearts, an increased efficiency was observed in SAT hearts during control perfusion (Table V. 3.c)). Nevertheless SAT hearts had a significantly lower percentage energy utilisation efficiency compared to FO and REF hearts. Energy utilisation efficiency was extremely low during ischaemia in all hearts with or without hydralazine. Post-ischaemic recovery of energy utilisation efficiency was diminished by hydralazine. Efficiency was significantly less in SAT hearts compared to REF and FO. The highest efficiency during reperfusion was measured in FO dietary fatty acid supplemented hearts.

V. 3. d) Vasodilator Influence on Ischaemic Injury

Creatine kinase release was lower in SAT hearts treated with hydralazine than in SAT hearts perfused in control conditions in the absence of hydralazine, but this was still significantly greater than hydralazine treated FO and REF hearts (Table V. 4. d)). FO hearts released the least CPK with hydralazine treatment under control perfusion conditions. Hydralazine did not induce any alteration in the rate of creatine kinase release into the coronary effluent from FO and REF hearts during control perfusion. During ischaemia and reperfusion the creatine kinase release was not significantly different in the presence of hydralazine. With or without hydralazine, SAT hearts exhibited a significantly higher creatine kinase release compared to FO in ischaemia and reperfusion.

Significantly lower venous pH in coronary effluent from SAT hearts compared to FO hearts was observed with and without the presence of

hydralazine during ischaemia (Table V. 4. a)). No significant post-ischaemic pH differences were evident between the 3 groups with hydralazine present.

The extracellular K^+ concentration was increased in ischaemia and returned towards control levels in reperfusion. During ischaemia, venous K^+ concentration was significantly higher in SAT than in FO hearts. There were no significant differences between hearts treated or not treated with hydralazine in any perfusion period (Table V. 4. b)).

Hydralazine treatment increased lactic acid release into the coronary effluent of REF and SAT hearts (Table V. 4. c)). In control working heart perfusion SAT hearts released significantly higher levels of lactate compared to REF and FO, while FO hearts produced the lowest release regardless of hydralazine presence. Following 15 min ischaemia in the presence of hydralazine lactic acid release was significantly lower compared to non-hydralazine treated ischaemic hearts. The lactate levels in SAT and REF hearts remained significantly higher compared to FO dietary fatty acid supplemented hearts. Except for FO hearts, in which lactate remained very low throughout, lactate release increased in reperfusion with or without hydralazine.

Table V.1. a) Coronary Flow (mL/min/g d.w.)

DIET	REF	SAT	FO
CONTROL			
HYDRALAZINE	66.9 ± 12.0	71.2 ± 9.8	73.5 ± 9.9§
NO HYDRALAZINE	51.1 ± 10.1*	77.19 ± 5.2	50.9 ± 3.7*
ISCHAEMIA 15min			
HYDRALAZINE	20.07 ± 4.8	15.91 ± 1.6	16.14 ± 2.8
NO HYDRALAZINE	18.76 ± 3.7	14.85 ± 2.1	17.26 ± 1.9
REPERFUSION 5min			
HYDRALAZINE	35.9 ± 9.3§	58.73 ± 6.2	62.9 ± 7.3§
NO HYDRALAZINE	49.9 ± 3.8	58.17 ± 2.4	43.9 ± 3.4*

*=significantly different compared to SAT; §=significantly different compared to no hydralazine; n=10, mean ± SD. See methods for details.

Table V.1. b) Aortic Output (mL/min/g d.w.)

DIET	REF	SAT	FO
CONTROL			
HYDRALAZINE	97.1 ± 14.0	80.6 ± 13.2	89.9 ± 17.5
NO HYDRALAZINE	115.2 ±17.5*	77.2 ± 4.3	97.7 ± 6.9*
ISCHAEMIA 15min			
HYDRALAZINE	4.5 ± 2.6	1.5 ± 0.9	5.0 ± 1.3*
NO HYDRALAZINE	3.1 ± 0.6*	0.9 ± 0.1	4.0 ± 0.6*
REPERFUSION 5min			
HYDRALAZINE	80.7 ± 14.4	53.4 ± 12.2	75.0 ± 13.2
NO HYDRALAZINE	64.5 ± 3.9*	42.6 ± 3.1	81.0 ± 5.8*

*=significantly different compared to SAT; §=significantly different compared to no hydralazine; n=10, mean ± SD. See methods for details.

Table V.1. c) Cardiac Output (mL/min/g d.w.)

DIET	REF	SAT	FO
CONTROL			
HYDRALAZINE	163.9 ± 21.1	151.8 ± 21.7	163.5 ±26.5
NO HYDRALAZINE	166.3 ± 23.7	154.4 ± 8.6	148.6 ± 9.9
ISCHAEMIA 15min			
HYDRALAZINE	24.6 ± 7.0	17.4 ± 2.2	21.2 ± 3.1
NO HYDRALAZINE	21.8 ± 3.7	15.7 ± 2.1	21.3 ± 2.4*
REPERFUSION 5min			
HYDRALAZINE	116.5 ± 21.7	112.1 ± 39.6	137.9 ±19.7
NO HYDRALAZINE	114.4 ± 4.4*	100.8 ± 4.9	125.0 ±9.0*

*=significantly different compared to SAT; §=significantly different compared to no hydralazine; n=10, mean ± SD. See methods for details.

Table V.2. a) Pressure-Time Integral

DIET	REF	SAT	FO
CONTROL			
HYDRALAZINE	2232 ± 485§	2179 ± 237§	3844±388*§
NO HYDRALAZINE	3868 ± 402*	2970 ± 380	4868±471*
ISCHAEMIA 15min			
HYDRALAZINE	1908 ± 343	1573 ± 390	2390 ± 418*
NO HYDRALAZINE	1979 ± 277	1720 ± 202	2774 ± 510*
REPERFUSION 5min			
HYDRALAZINE	2147 ± 420	2114 ± 161§	3751±374*§
NO HYDRALAZINE	3546 ± 329	2823 ± 341	4664 ±222*

*=significantly different compared to SAT; §=significantly different compared to no hydralazine; n=10, mean ± SD. See methods for details.

Table V.2. b) External Work (J/min/g d.w.)

DIET	REF	SAT	FO
CONTROL			
HYDRALAZINE	2.016 ± 0.310	1.851 ± 0.330	2.221 ± 0.423
NO HYDRALAZINE	1.836 ± 0.380	1.650 ± 0.182	1.674 ± 0.122
ISCHAEMIA 15min			
HYDRALAZINE	0.158 ± 0.065	0.092 ± 0.011	0.154 ± 0.041
NO HYDRALAZINE	0.120 ± 0.025	0.087 ± 0.012	0.124 ± 0.017
REPERFUSION 5min			
HYDRALAZINE	1.225 ± 0.262	1.168 ± 0.447	1.740 ± 0.317
NO HYDRALAZINE	1.189 ± 0.054*	1.013 ± 0.068	1.339 ± 0.095*

*=significantly different compared to SAT; §=significantly different compared to no hydralazine; n=10, mean ± SD. See methods for details.

Table V.3. a) % Oxygen Extraction

DIET	REF	SAT	FO
CONTROL			
HYDRALAZINE	9.32 ± 1.89	14.03 ± 2.83§	7.02 ± 3.36*
NO HYDRALAZINE	9.95 ± 2.33*	24.20 ± 6.72	5.91 ± 2.51*
ISCHAEMIA 15min			
HYDRALAZINE	52.99 ± 7.32*§	78.18 ± 3.59	48.40 ± 4.9*
NO HYDRALAZINE	70.71 ± 3.36*	88.35 ± 7.73	48.25 ± 2.4*
REPERFUSION 5min			
HYDRALAZINE	41.63 ± 6.48§	54.39 ± 5.01§	28.14 ± 3.58*§
NO HYDRALAZINE	12.04 ± 0.47*	34.03 ± 2.09	7.85 ± 0.52*

*=significantly different compared to SAT; §=significantly different compared to no hydralazine; n=10, mean ± SD. See methods for details.

Table V.3. b) Myocardial Oxygen Consumption (mL/min/g d.w.)

DIET	REF	SAT	FO
CONTROL			
HYDRALAZINE	1.182 ± 0.279	1.885 ± 0.364§	1.023 ±0.224*§
NO HYDRALAZINE	0.955 ± 0.451*	3.589 ± 0.410	0.353 ± 0.114*
ISCHAEMIA 15min			
HYDRALAZINE	2.011 ± 0.516	2.356 ± 0.167	1.475 ± 0.169*
NO HYDRALAZINE	2.523 ± 0.479	2.515 ± 0.350	1.547 ± 0.231*
REPERFUSION 5min			
HYDRALAZINE	2.904 ± 1.100*§	6.176 ± 2.497	3.293 ± 0.52*§
NO HYDRALAZINE	1.192 ± 0.213*	3.796 ± 0.277	0.710 ± 0.145*

*=significantly different compared to SAT; §=significantly different compared to no hydralazine; n=10, mean ± SD. See methods for details.

Table V.3. c) % Energy Utilisation Efficiency

DIET	REF	SAT	FO
CONTROL			
HYDRALAZINE	8.80 ± 1.88	5.09 ± 1.48§	11.08 ± 2.28
NO HYDRALAZINE	11.03 ± 1.41*	2.46 ± 0.26	14.53 ± 1.75*
ISCHAEMIA 15min			
HYDRALAZINE	0.38 ± 0.08§	0.19 ± 0.02	0.52 ± 0.09
NO HYDRALAZINE	0.24 ± 0.02*	0.17 ± 0.01	0.44 ± 0.07*
REPERFUSION 5min			
HYDRALAZINE	2.23 ± 0.49§	0.95 ± 0.10§	2.68 ± 0.54§
NO HYDRALAZINE	5.95 ± 0.79*	1.36 ± 0.09	10.11 ± 0.78*

*=significantly different compared to SAT; §=significantly different compared to no hydralazine; n=10, mean ± SD. See methods for details.

Table V.4. a) Venous pH

DIET	REF	SAT	FO
CONTROL			
HYDRALAZINE	7.294 ± 0.012	7.287 ± 0.004	7.308 ± 0.007*
NO HYDRALAZINE	7.300 ± 0.017	7.282 ± 0.010	7.311 ± 0.006*
ISCHAEMIA 15min			
HYDRALAZINE	7.151 ± 0.039	7.085 ± 0.053	7.173 ± 0.031
NO HYDRALAZINE	7.144 ± 0.006*	7.028 ± 0.020	7.213 ± 0.007*
REPERFUSION 5min			
HYDRALAZINE	7.146 ± 0.048§	7.203 ± 0.034	7.234 ± 0.022§
NO HYDRALAZINE	7.270 ± 0.009*	7.209 ± 0.003	7.292 ± 0.005*

*=significantly different compared to SAT; §=significantly different compared to no hydralazine; n=10, mean ± SD. See methods for details.

Table V.4. b) Extracellular [K^+] (mmol/L)

DIET	REF	SAT	FO
CONTROL			
HYDRALAZINE	3.340 ± 0.237	3.178 ± 0.206	3.190 ± 0.252
NO HYDRALAZINE	3.170 ± 0.209	3.266 ± 0.472	2.943 ± 0.399
ISCHAEMIA 15min			
HYDRALAZINE	6.395 ± 0.306*	7.217 ± 0.332	5.009 ± 0.368*
NO HYDRALAZINE	7.729 ± 0.819	8.630 ± 1.319	5.181 ± 0.698*
REPERFUSION 5min			
HYDRALAZINE	4.019 ± 0.347	4.503 ± 0.296	3.660 ± 0.236*
NO HYDRALAZINE	4.963 ± 0.444	5.603 ± 0.788	3.780 ± 0.595*

*=significantly different compared to SAT; §=significantly different compared to no hydralazine; n=10, mean ± SD. See methods for details.

Table V.4. c) Lactate Production (μmol/min/g d.w.)

DIET	REF	SAT	FO
CONTROL			
HYDRALAZINE	155.32 ± 54.35§	241.10 ± 60.56	0.027 ± 0.01*
NO HYDRALAZINE	60.78 ± 27.95*	149.08 ± 37.35	0.048 ± 0.02*
ISCHAEMIA 15min			
HYDRALAZINE	81.65 ± 33.40§	98.34 ± 32.81§	0.24 ± 0.07*§
NO HYDRALAZINE	159.35 ± 39.25*	263.63 ± 37.58	0.10 ± 0.04*
REPERFUSION 5min			
HYDRALAZINE	113.68 ± 49.3*§	261.29 ± 20.11§	0.04 ± 0.01*§
NO HYDRALAZINE	272.73 ± 33.21*	382.41 ± 29.37	0.08 ± 0.02*

*=significantly different compared to SAT; §=significantly different compared to no hydralazine; n=10, mean ± SD. See methods for details.

Table V.4. d) Creatine Phosphokinase Release (U/min/g d.w.)

DIET	REF	SAT	FO
CONTROL			
HYDRALAZINE	4.763 ± 0.950*	7.333 ± 0.960§	0.497 ± 0.121*
NO HYDRALAZINE	3.315 ± 0.930*	10.059 ± 1.113	0.430 ± 0.108*
ISCHAEMIA 15min			
HYDRALAZINE	2.573 ± 0.396	3.952 ± 0.407	0.224 ± 0.027
NO HYDRALAZINE	2.229 ± 0.495	4.316 ± 1.220	0.294 ± 0.048
REPERFUSION 5min			
HYDRALAZINE	8.226 ± 1.434	11.938 ± 3.242	1.206 ± 0.103*
NO HYDRALAZINE	12.118 ± 5.331	17.394 ± 4.400	1.331 ± 0.133*

*=significantly different compared to SAT; §=significantly different compared to no hydralazine; n=10, mean ± SD. See methods for details.

V. 4. Discussion

The erythrocyte perfused isolated working rat heart and the maintained afterload model of global ischaemia have been utilised to investigate the relationship between the increased coronary flow rates of SAT dietary lipid supplemented hearts, the increased myocardial oxygen consumption in these hearts and the dietary influenced differences in cardiac performance, cardiac metabolism and susceptibility to ischaemic injury. The principal aim of this experiment was to determine whether the very high coronary flow rate observed in SAT hearts during control working heart perfusion was directly due to the abnormally high MVO_2 or *vice versa*. Hydralazine, a classical vasodilator, was used to increase the coronary flow in FO and REF hearts to the high levels of SAT hearts in order to make coronary flow a constant factor in myocardial oxygen metabolism. The previously reported vasodilatory properties of hydralazine and the slight negative inotropy at supratherapeutic doses (**Zacest,*et al.*,1972;Spokas & Wang,1980;Rendig,*et al.*,1988**), were confirmed in this study. However, the high coronary flow in SAT hearts could not be further increased.

Although hydralazine produced coronary flow equalisation and some attenuation of the dietary differences in contractility, ventricular performance in FO hearts, especially remained enhanced compared to SAT hearts. Certainly, extracellular creatine kinase release, a clinical indice of myocardial cell membrane ischaemic injury (**Lott,*et al.*,1984;Lee,*et al.*,1987;Wu,*et al.*, 1987**), was significantly higher in SAT hearts indicating that SAT hearts were more susceptible to such injury than FO hearts even after coronary flow equalisation with hydralazine. Indeed a degree of protection was evident in FO

hearts because creatine kinase release did not increase in this dietary group following ischaemia and reperfusion. Associated with this finding was the observation that acidosis, lactate release, and extracellular K^+ concentration were significantly increased in SAT hearts compared to FO hearts also after hydralazine-induced coronary flow equalisation. These variables also indicated that FO dietary supplementation provided a protective influence against ischaemic and reperfusion injury. This property, first reported in Chapter III does not seem to have been impeded by hydralazine.

Despite the equivalent coronary flow rates induced by hydralazine during control perfusion, dietary influenced disparities in oxygen metabolism were still present and measurable. Saturated fat supplemented hearts treated with hydralazine still had greater oxygen extraction and oxygen consumption compared to FO during control perfusion although the differences were somewhat attenuated. This attenuation was achieved by two opposite actions. Firstly, MVO_2 was increased in REF and FO hearts by hydralazine. Secondly, the abnormally high MVO_2 in SAT hearts was unexpectedly reduced compared to perfusion in the absence of hydralazine even though coronary flow in these hearts was not significantly altered by hydralazine. The former effect can be explained primarily by an increase in oxygen delivery rather than an increase in oxygen extraction. This implies that it was related to the hydralazine-induced increased coronary flow, confirming the ability of increased coronary flow to increase MVO_2 **(Gregg,1963;Khouri,*et al.*,1967;Braunwald,*et al.*, 1968;Mohran & Feigl,1978)**. Nevertheless FO hearts had the lowest oxygen extraction and consumption rates under all control conditions. The high percentage oxygen extraction and MVO_2 in SAT hearts during control

perfusion as well as their elevated coronary flow rates suggests that the altered oxygen metabolism is not totally dependent upon nor directly related to a higher coronary flow. It has been demonstrated (at least in canine models, **Khouri,*et al.*,1965;Restorff,*et al.*,1977**) that at times of increased myocardial oxygen demand (i.e. exercise), the requirements are met not only by increased coronary flow but also increased oxygen extraction. It is likely that other factors are also responsible for the abnormally high MVO_2 in SAT hearts. The concurrent reduction in oxygen consumption which occurred in SAT hearts under the influence of hydralazine may be related to the mode of hydralazine action. Some understanding of the pharmacology of hydralazine may even give a clue to the mechanism of SAT-induced elevation in MVO_2.

Although hydralazine has been in wide use for the treatment of hypertension and congestive heart failure for over 40 years the exact mechanisms of hydralazine function directly on the myocardium are still unknown (**Zacest & Reece,1984**). It has been shown that the vasodilatory effects of hydralazine occur in arterial but not venous smooth muscle *in vitro* (**Pang & Sutter,1980;Worcel,*et al.*,1980**). Some evidence supports hydralazine as an inhibitor of dopamine-ß-hydroxylase which thus restricts the production of noradrenaline (**Liu,*et al.*,1974;Songkittiguna,*et al.*,1980**). Others have proposed that hydralazine may act on smooth muscle purine sensitive receptors to oppose the contractile actions of noradrenaline in the rat tail artery preparation (**Worcel,*et al.*,1980**). McLean, *et al.*,(**1978**) provided evidence to suggest that supratherapeutical concentrations of hydralazine ($> 10^{-4}$M) prevent Ca^{++} influx into aortic smooth muscle cells during contraction thus permitting vasodilation. Greenberg (**1980**), proposed that hydrala-

zine reduced the availability of calcium ions to the smooth muscle contractile system and thus suppressed excitation-contraction mechanisms. This proposal of reduced Ca^{++} availability to the excitation-contraction system of smooth muscle has received further indirect support (**Morita,*et al.*,1988**). Other data suggests that hydralazine inhibits calcium ions from being released at binding sites within intracellular storage localities (**Lipe & Mould,1981**). This conclusion stemmed from experiments in which human arterial smooth muscle preparations were perfused in calcium-free media and the contractile response to noradrenaline, serotonin, 80mmol/L KCl and $BaCl_2$ was observed. Hydralazine reduced, by varying degrees, the contractile response of all of these agonists except $BaCl_2$. The mechanism of hydralazine action on smooth muscle could not be receptor mediated because it non-competitively antagonised all of the contractile agonists used, particularly KCl which produces smooth muscle contraction probably by direct smooth muscle cell depolarisation. The efficacy of hydralazine to antagonise these smooth muscle contractile agents was high when agonist and tissue sensitivity reduced in the absence of extracellular calcium ions. The observation that hydralazine could not prevent barium ions from crossing the cell membrane to produce smooth muscle vasoconstriction (barium substitution for calcium ions), strongly supports the proposal of these authors that hydralazine does not act by inhibiting the influx of external calcium but rather interferes with the intracellular calcium handling involved with the mechanisms of contraction. The finding that hydralazine depressed contractility in all dietary groups, even in the face of increased coronary flow (FO and REF hearts), could be explained if hydralazine can also influence myofibril contraction by similar mechanisms. A negative

inotropic effect on cardiac muscle has previously been observed in papillary muscle preparations treated with 10^{-4}M hydralazine (**Rendig,*et al.*,1988**). If hydralazine acts by restricting calcium fluxes in cardiac cells, then its ability to reduce MVO_2 in SAT hearts alone without significant changes to coronary flow suggests that the abnormally high MVO_2 in SAT hearts may be explained by altered calcium handling. A link between excess intracellular calcium concentrations, mitochondrial energy metabolism and mitochondrial oxygen consumption has been reported (**McCormack & Denton,1984;Unitt,*et al.*, 1989;Grover,*et al.*,1990**).

In light of the current observations, the null hypothesis; "that the elevated MVO_2 in SAT hearts is due to their high coronary flow rates", must be rejected because when hydralazine equalised coronary flow between the groups, the MVO_2 was still greater in SAT than FO hearts, despite some increase in the MVO_2 of REF and FO hearts and slight reduction in the MVO_2 of SAT hearts. The increase in MVO_2 by hydralazine in REF and FO hearts was most likely due to the above mechanism but they failed to achieve the very high levels seen in untreated SAT hearts. On the other hand, the possible effects of hydralazine on calcium-related mechanisms and its ability to reduce oxygen consumption in SAT hearts gives a clue to the origin of the abnormally high MVO_2 and is worthy of further investigation with highly specific drugs which can influence intracellular Ca^{++} movement. Such a study is pursued in Chapter VI.

VI. THE INFLUENCE OF CALCIUM IN DIETARY LIPID AND ISCHAEMIA INDUCED CHANGES IN OXYGEN METABOLISM AND VENTRICULAR PERFORMANCE.

VI. 1. Introduction

By using the new model of global ischaemia with maintained afterload and preload in the erythrocyte perfused isolated working rat heart described in Chapter II it was possible to follow changes in myocardial oxygen demand during ischaemia. The result was a paradoxical increase in MVO_2 (dependant of course on the continued provision of some coronary flow). Most importantly, this represented an increase in oxygen demand not associated with contraction, which was markedly depressed at the time.

In Chapter III it was observed that dietary saturated fat was also associated with increased MVO_2 without any external work dividend, even under control conditions. Such was the elevation in MVO_2 under control conditions that coronary flow and oxygen delivery was insufficient to support any further paradoxical increase in MVO_2 during ischaemia. Contrast this to a diet containing fish oil which reduced the MVO_2 without any performance deficit under control conditions and depressed the paradoxical ischaemic increase in MVO_2. None of these observations have been reported before. However, some indirect evidence of a Ca^{++}-related mechanism described in the previous chapter together with a number of isolated observations from other studies when drawn together give some insight into the possible mechanisms. It is feasible that similar mechanisms may be responsible for both the ischaemic and dietary saturated fat effects.

The increase in MVO_2 in ischaemia is paradoxical because reduced coronary flow, contractility and external work would normally be associated with a proportionate reduction in MVO_2 (**Gregg,1963;Khouri,*et al.*,1967; Braunwald,*et al.*1968;Weber & Janicki,1977;Mohran & Feigl,1978; Suga,1979;Suga,*et al.*,1987**). Indeed, it cannot occur (despite increased demand) when coronary flow is stopped rather than reduced. However, paradoxical increases in MVO_2 have been observed after reperfusion of an ischaemic heart or heart region suggesting that extreme limitations in oxygen delivery with total ischaemia and/or methodological limitations preventing measurement during ischaemia may have been responsible for the absence of reports of increased oxygen demand during ischaemia.

In the mid 1970's it was found that even short periods of ischaemia were followed by an interval of impaired mechanical function (**Hendryckx,*et al.*,1975;Apstein,*et al.*,1976**). It was proposed that this delayed contractile recovery occurred as a result of abnormalities in energy metabolism, which may be the cause of the reduced high energy phosphates and adenine nucleotides observed in the post-ischaemic myocardium (**Reibel & Rovetto, 1972;Sharma,*et al.*,1975;Kannengeisser,*et al.*,1979;DeBoer,*et al.*,1980; Rhiemer,*et al.*,1981;Takeo,*et al.*,1988**).

Observations of abnormally increased MVO_2 have been limited mainly to the reperfusion period (**Krukenkamp,*et al.*,1985;1986;Stahl,*et al.*,1988; Dean,*et al.*,1990;Bavaria,*et al.*,1990**). However, Weiss (**1980**) used the anaesthetised open chest dog preparation to investigate coronary artery ligation effects on MVO_2, **during** regional ischaemia. In this study microspectrophotometric methods were used to determine regional oxygen extraction while

radioisotope labelled microspheres were used to determine regional coronary blood flow. Despite increased oxygen extraction, MVO_2 was reported to be reduced in the occluded zone. This result may be related to total restriction of blood flow within the occluded site.

Kannengeiser, *et al.* **(1979)**, found that in isolated working rat hearts, reperfusion after 15 min coronary ligation resulted in rapid restoration oxygen uptake to pre-ischaemic levels even though contractility, external work and energy utilisation efficiency was reduced. Krukenkamp, *et al.* **(1985;1986)**, induced cardiac arrest with cold cardioplegic K^+ solution in the anaesthetised open chest dog preparation. Following 2 hours ischaemia the aorta was unclamped to allow normothermic reperfusion and resumption of ventricular systolic pressure development. Post-ischaemic MVO_2 was significantly increased but mechanical efficiency reduced compared to controls. This effect was not evident in empty, beating hearts. These findings were supported by Stahl, *et al.* **(1988)**, using regional ischaemia in the anaesthetised open chest dog. Oxygen extraction and consumption in the region previously made ischaemic was increased significantly during reperfusion of these hearts. These researchers proposed that the reduced contractility and mechanical work was due to inefficient energy transfer into myofibrillar contraction and/or increased energy consumption for non-contractile activities. Laster, *et al.* **(1989)**, observed that the abnormally high post-ischaemic MVO_2 in isolated rabbit hearts was not due to differences in basal MVO_2 following high [K+] arrest, but proposed that it was related to a defect in either electromechanical coupling or contraction processes. These findings were also supported by Bavaria, *et al.* **(1990)**, following global cardiac ischaemia in sheep and by Dean, *et*

al. (1990), using coronary occlusion in dogs. The latter workers also calculated mitochondrial respiratory rates and oxidative phosphorylation capacity, *in vitro*, from post-ischaemic tissue, but these were not significantly different from non-ischaemic controls. These results also indicated that the paradoxically high post-ischaemic MVO_2 may be mostly related to processes other than contraction. On the basis of the above studies, the mechanisms possibly responsible for the paradoxically high MVO_2 (in the face of ischaemia-induced reductions in mechanical performance and energy utilisation efficiency), may be related to defects in processes involving energy production, energy transport and/or energy utilisation. While not dependent on contraction, they do appear to be related to activation.

In addition to energy metabolism abnormalities in the ischaemic and reperfused myocardium, altered sarcolemmal and intracellular calcium handling have been observed. Immediately upon reperfusion and reoxygenation, increased levels of calcium accumulate into the cytosol and mitochondria of cardiac cells (**Nayler,1981;Ferrari,*et al.*,1982;Shen & Jennings,-1972**). Numerous studies have implicated calcium in ischaemia/reperfusion related cellular injury (**Cheung,*et al.*,1986;Poole-Wilson,*et al.*,1984**). This calcium increase has been shown to inhibit ATP synthesis (**Nayler,*et al.*,1980;Williams,*et al.*,1982**). The activation of phospholipases or calcium dependent ATPases by calcium influx may be involved in cell organelle damage observed in reperfusion injury (**Nayler,*et al.*,1981**). In experiments where isolated hearts are perfused with solutions low in extracellular calcium or that have been treated with pharmacological agents that restrict calcium entry into myocytes, diminished ischaemia/reperfusion cell injury has been ob-

served (**Cheung,*et al.*,1986;Poole-Wilson,*et al.*,1984;Ferrari,*et al.*,1988**). Abnormally high intracellular Ca^{++} levels may cause mitochondrial damage such that futile mitochondrial cycling of calcium and inefficient energy utilisation may occur (**Thomas,*et al.*,1988**).

Considerable evidence from rat heart studies indicates that hormones or positive inotropic drugs which stimulate energy-requiring cardiac contraction, produce enhanced ATP utilisation and increase the cytosolic calcium concentration. They also increase intra-mitochondrial calcium concentrations to activate pyruvate dehydrogenase, NAD^{+}-isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase and thus stimulate oxidative phosphorylation. These key enzymes permit the production of NADH for increased oxidative metabolism and still maintain high ATP/ADP ratios. (**Denton&McCormack,1985;McCormack&Denton,1986;Hansford,1985;-Unitt, *et al.*,1989**). These workers even use measures of pyruvate dehydrogenase activity as an indicator of intra-mitochondrial and cytosolic Ca^{++} levels. To date no studies have examined the effect of ischaemia/reperfusion induced increases in calcium on these three enzymes, the related oxidative metabolism and MVO_2. However, Ruthenium Red (RR) a potent inhibitor of calcium ion uptake into isolated mitochondria (**Moore,1971;Vasington,*et al.*,1972**), has been shown to inhibit the increase of activated mitochondrial pyruvate dehydrogenase in response to increased extra-mitochondrial calcium or positive inotropic agents in isolated perfused hearts (**Denton,*et al.*,1980;-McCormack & England,1983**). Infusion of RR during myocardial ischaemia also improved post-ischaemic mitochondrial function, limited intracellular calcium increases and produced significant increases in ADP induced

respiration and ATP production (**Peng,*et al.*,1980;Smith,,1980;Thomas & Reed,1988**). In addition, RR perfusion in isolated hearts improved post-ischaemic contractile function, limited ischaemic cellular injury and improved the efficiency of oxygen utilisation without an increase in oxygen consumption (**Grover,*et al.*,1990;Park,*et al.*,1990**).

Cardiac work may be increased by simply raising the extracellular concentration of calcium and thus raising cytosolic calcium (**Nayler,1966**). In a study by Ito, *et al.*, (**1985**) using an anaesthetised open chest canine preparation, intracoronary calcium infusion increased regional contractile function that had been diminished by regional ischaemia. This suggested that defective excitation-contraction coupling and altered calcium fluxes were implicated in the post-ischaemic dysfunction. Positive inotropic agents can also increase sarcoplasmic reticulum calcium loading, but continued calcium accumulation into cells past an optimal level can reduce contractility and is associated with arrhythmogenesis(**Vassalle,*etal.*,1962;Ferrier,1976;Capogrossi,*etal.*,1988**). Although cardiac systole occurs by action potential driven calcium oscillation, independent, asynchronous and spontaneous calcium oscillations also occur at single or multiple locii of sarcoplasmic reticulum (**Hansford & Lakatta,1987;Cappogrossi,*et al.*,1984;1985;Fabiato & Fabiato,1972;1975;1983;-Kort & Lakatta,1984**). These are attributed to calcium-dependent calcium release because ryanodine, an inhibitor of sarcoplasmic reticulum calcium release (**Sutko,*et al.*,1985;Hansford & Lakatta,1987**), suppressed their frequency and amplitude (**Lakatta,*et al.*,1985**). It has been observed that following global ischaemia and reperfusion in the presence of ryanodine, myocardial mechanical dysfunction and reperfusion arrhythmias are suppressed

through preserved sarcoplasmic reticulum function (**Thandroyen,*et al.*,1988;-Limbruno,*et al.*,1989**).

It was the aim of this chapter to investigate the effects of ruthenium red and ryanodine in the erythrocyte perfused isolated working heart using the maintained afterload model of low flow global ischaemia, in order to test the possibility that ischaemia-altered intracellular calcium handling influences not only cardiac mechanical performance but also myocardial oxygen consumption and energy utilisation efficiency. Few researchers have investigated this aspect in whole working hearts. In particular, it was aimed to test the hypothesis that the functional and metabolic differences observed between REF, FO and SAT groups in the previous experiments are related to distinct differences in active metabolism specifically related to differences in intracellular calcium handling. The possibility that alterations in basal rather than active metabolism may contribute to dietary or ischaemic differences in MVO_2 was also investigated in the high $[K^+]$ arrested heart.

VI. 2. Methods

VI. 2. a) Animals & Diets

In this series of experiments a total of 129 male Hooded-Wistar rats (4 months old) were placed in the three dietary groups REF, SAT, FO. Thirty rats were allocated to Experiment A, 75 rats to Experiment B (See below) and 21 rats kept on reference diet, were used to study ryanodine dose response effects in erythrocyte perfused isolated working hearts. The diets were prepared, stored and presented to the rats as described in Chapter III. The animals were kept on their allocated diet and housed under the conditions described in Chapter

III until they were at least 8 months old (body mass =400-500 g). They were fasted overnight prior to each experimental day.

VI. 2. b) Isolated Working Heart Preparation & Perfusion Protocol

Isolated hearts were prepared for working heart perfusion (maintained afterload ischaemia configuration) with the erythrocyte buffer (40% haematocrit) and perfused and monitored as described in Chapter II. Hearts were perfused in Langendorff mode for 10 min while the pulmonary artery was cannulated, pacing and ECG recording electrodes were positioned, and coronary vessel blood was flushed out. Erythrocyte perfused working heart mode was commenced (coronary perfusion pressure, preload and afterload set at 75, 10 and 75 mmHg respectively) with right atrial pacing (300 bpm) as previously described. Subsequent to 5 min working heart perfusion, when hearts had equilibrated and were stabilised, measures of cardiac output, aortic pressure, arterial and venous blood gas content, and pH were taken every 5 min for the entire perfusion. After the equilibration of working hearts ischaemia was induced and maintained for 15 min by reducing coronary perfusion pressure to 35mmHg then reperfused at a coronary perfusion pressure of 75 mmHg. Following oxygen tension and pH readings in arterial and coronary venous samples, erythrocytes were removed by centrifugation with a small Corning bench top centrifuge. The sample supernatant was frozen in liquid nitrogen and stored at -60°C for later biochemical analyses.

VI. 2. c) EXPERIMENT A: Determination of Basal Metabolism: K^+ Arrest

Thirty animals, treated to either REF, SAT, or FO dietary supplementation were divided into 2 groups. In the first group, REF, SAT, FO hearts (n=5), were subjected to the perfusion phases described above except that following 5 min reperfusion, contraction was arrested by infusing erythrocyte perfusate (40% haematocrit) containing 30 mM KCl through the aorta into the coronary arteries. A peristaltic pump (Cole Parmer, U.S.A) infused the high KCl perfusate at a rate which maintained coronary flow at 20 mL/min. The second group of REF, SAT, and FO isolated working hearts (n=5), was not subjected to ischaemia, but these hearts were arrested by an infusion of 30mM KCl at the time point corresponding to KCl infusion in the first group. Arterial and venous PO_2 measures were taken during high $[K^+]$ -induced cardiac arrest in order to calculate basal oxygen metabolism.

VI. 2. d) EXPERIMENT B: The Modification of Extracellular $[Ca^{++}]$

In this experiment, following the control working heart period the perfusion circuit was switched to a parallel circuit which contained the same erythrocyte buffer but with the addition of either 3.18 μM ruthenium red (RR; Sigma Chemical Co.,U.S.A.), or 10^{-9}M ryanodine (RY; Agri Systems, U.S.A.), 6mM Ca^{++} (final concentration),or 3.18μM RR+6mM Ca^{++},or 10^{-9}M RY + 6mM Ca^{++} (for all treatments, n=5 per dietary group). The RR concentration used was based on the findings of McCormack & England (**1983**) that showed this was the minimum dose capable of blocking increases in active, non-phosphorylated pyruvate dehydrogenase (PDH_a), caused by positive inotropic

agents or by raised extracellular [Ca^{++}], while minimising the negative inotropic effect in isolated rat heart.

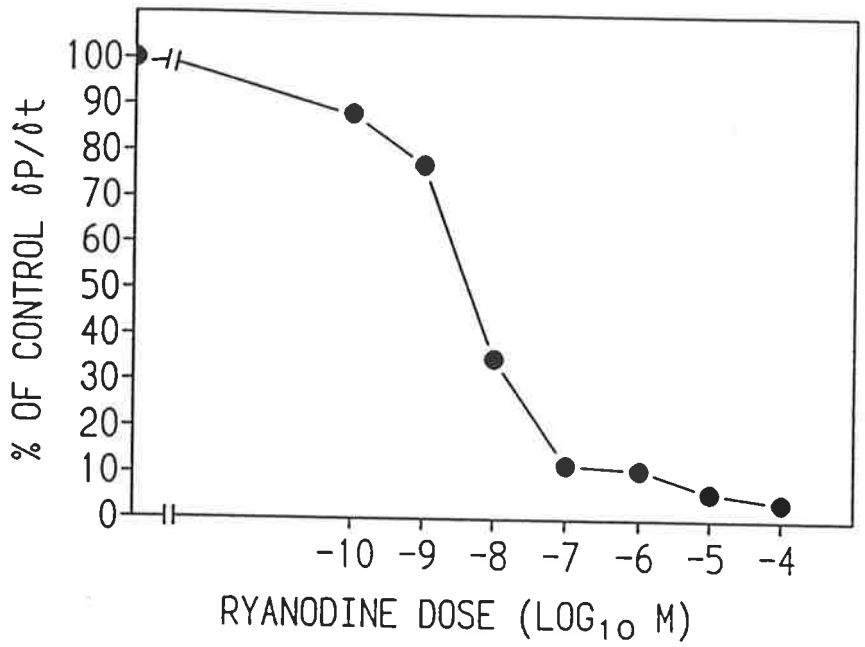

Figure VI.i. The log dose-response effect of ryanodine on the pressure-time integral ($\delta P/\delta t$) after 10min in isolated working heart mode (Reference diet). Values are expressed as % of control $\delta P/\delta t$ (median values, n=3).

Instead of using a positive inotropic adrenergic agonist to return contractility to control levels during RR perfusion, the effects of increased cAMP and phosphorylase kinase activation (independent of Ca^{++}) could be avoided by raising extracellular [Ca^{++}] from 1.4mM (control buffer) to 6mM (**Cohen,-1978**). The RY concentration used was selected following a dose response study (10^{-10} M-10^{-4} M RY, n=3 per dose) with a separate group of 21 REF isolated erythrocyte perfused working hearts. The ryanodine dose selected was that which reduced working heart contractility (Figure VI.i.) and aortic output by no more than 50% (to avoid ventricular failure), but with 6mM Ca^{++} contractility could be restored to match control hearts. Ryanodine began to

reduce contractility within a few minutes of perfusion and at concentrations greater than 10^{-8}M led to ventricular failure by 10 to 15 min depending on the dose. Washout perfusion with ryanodine-free medium failed to reverse the effects of ryanodine. Hearts were perfused according to the protocol described in section VI.2.b) with each drug treatment.

VI. 2. e) Analysis of Coronary Effluent Contents

Arterial and venous samples were assayed for creatine kinase, lactic acid and K^+ concentrations as described in Chapter III. Creatine kinase was quantified according to the rate of change of absorbance. Results were expressed in U/min/g dry weight adjusted according to coronary flow rates. Lactic acid concentrations were expressed in μmol/min/g dry weight when adjusted according to coronary flow rates. For potassium ion concentration determination all samples were analysed as described in Chapter V and results were expressed in mmol/L.

V. 2. f) Data Handling and Statistical Analysis

Myocardial left ventricular external work, the pressure-time integral, perfusate oxygen content, oxygen extraction, myocardial oxygen consumption and energy utilisation efficiency percentage were calculated as described in Chapter II. Ventricular arrhythmias were assessed according to the Lambeth Convention (**Walker,*et al.*,1988**) as described in Chapter IV. All results were expressed as mean $\pm$ SD. For each parameter, the effect of dietary treatment was tested by Analysis of Variance and between individual comparisons with Scheffe's *post hoc F*-test. The level of significance was considered at $P < 0.05$.

VI. 3. Results

VI. 3. EXPERIMENT A: Basal Oxygen Metabolism

Myocardial oxygen consumption following reperfusion, just prior to high [K^+] arrest, was significantly increased in REF and SAT but not in FO hearts compared to their time matched controls (ii. vs i., Table VI.1). MVO_2 was significantly higher in SAT hearts and significantly lower in FO hearts compared to REF hearts in either control conditions or following ischaemia-reperfusion. Infusion with 30mM KCl solution completely arrested contraction in working hearts within 30s. The coronary flow rate was maintained by pump at 20 mL/min during arrest in those hearts. Oxygen consumption decreased in all hearts during potassium arrest. Although [K^+] hearts subjected to ischaemia and reperfusion had slightly higher MVO_2 than their respective [K^+] arrested controls this marginal difference was not statistically significant (iv vs iii, Table VI.1). Basal MVO_2 in K^+ arrested REF, SAT and FO hearts was reduced to approximately 17%, 13% and 20% respectively, of the total MVO_2 in either control or reperfused non-arrested working hearts. There were no significant differences in basal MVO_2 between dietary groups.

VI. 3. EXPERIMENT B: The Modification of Extracellular [Ca^{++}]

VI. 3. B: a) Pressure-Time Integral

The pressure-time integral (PTI) was increased by Ca^{++} 6mM in REF (5%), SAT (9%) and FO hearts (12%) in the control perfusion period (Table VI.11.a.). In comparison, the PTI was reduced by RR (Table VI.3.a) and RY (Table VI.7.a) under control conditions in REF (8% & 20%), SAT (8% &

13%) and FO hearts (12% & 16%). No significant dietary differences in contractility were observed under the influence of these two agents. The combination of 6mM Ca^{++} with either RR (Table VI.15.a) or RY (Table VI.19.a) prevented the reductions in contractility seen with these agents alone in all dietary groups. Contractility was greatest in FO hearts and lowest in SAT hearts during pre- and post-ischaemic periods with 6mM Ca^{++} alone but these differences were not significant with RR+Ca^{++}. During ischaemia and reperfusion the PTI was similar in all dietary groups and between each treatment, although contractility tended to be lowest in the presence of RY in all dietary groups.

VI. 3. B: b) Myocardial External Work

Perfusion with 6 mM calcium resulted in significantly increased ventricular external work in all dietary groups compared to 1.4 mM Ca^{++} controls (Figure VI.2). The greatest external work with 6 mM Ca^{++} was observed in FO hearts and the smallest was in SAT during all perfusion phases. Ryanodine reduced myocardial external work by 48%, 38% and 52% in REF, SAT and FO respectively (Table VI.7.). Myocardial external work was also depressed with RR in REF, SAT and FO by 39%, 24% and 54% respectively. Both RR and RY abolished the dietary differences in external work under control conditions and following ischaemia and reperfusion.

The addition of 6mM Ca^{++} prevented the reductions in external work recorded with RR (Table VI.15) or RY (Table VI.19) alone with 1.4mM Ca^{++} during pre- and post-ischaemic perfusion. Ryanodine but not

ruthenium red prevented the increase in external work seen with elevated Ca^{++} alone. External work decreased to a common low level during ischaemia in all dietary groups irrespective of treatment but tended to be lowest with RR or RY alone.

VI. 3. B: c) Cardiac Output

Perfusion with 6mM calcium increased aortic output in REF, SAT and FO hearts by 31%, 30% and 21% respectively (Table VI.10.b). These effects were reflected in an increased cardiac output from REF, SAT and FO hearts by 27%, 26% and 23% respectively (Table VI.10.c). With RR (Table VI.2.c) and RY (Table VI.6.c), cardiac output decreased by 29% & 38%, 25% & 40%, 46% & 37% in REF, SAT and FO control working hearts respectively such that there were no significant differences between dietary groups. Similarly, aortic output was reduced in all groups (Tables VI.2.b.-6.b). The depression in cardiac output produced by either RR or RY was prevented in the presence of 6mM Ca^{++} (Tables VI 14.c. & 18.c), and RY but not RR prevented the Ca^{++}-induced increase in cardiac output. Although no dietary differences were observed during ischaemia, post-ischaemic cardiac output in FO hearts was greater than in SAT hearts for all treatments.

VI. 3. B:d) Coronary Flow

Coronary flow was reduced in the presence of RR and RY in REF (36% & 46%), SAT (46% and 46%) and FO hearts (29% and 19%) respectively such that all coronary flow differences were abolished by both agents (Tables

VI.2.a. & 6.a). By contrast, with 6mM Ca^{++}, coronary flow increased in all groups compared to controls, but remained significantly higher in SAT hearts (Table VI.10.a). This increase in coronary flow with 6mM Ca^{++} alone was prevented by RY (Table VI.18.a) whereas RR attenuated this coronary flow increase in REF & SAT but not in FO hearts such that significant dietary differences were abolished (Table VI.14.a). The coronary flow during ischaemia was similar in all dietary groups with all treatments and returned towards pre-ischaemic levels in reperfusion.

VI. 3. B:e) % Oxygen Extraction

The percentage oxygen extracted was significantly reduced by 30% in REF and 16% in SAT, but not in FO hearts during working heart perfusion with RR (Table VI.4.). No significant differences in oxygen extraction were observed between the dietary groups in any perfusion phases with RR although it intended to be higher in the SAT group throughout. Oxygen extraction rose during ischaemia in all groups and returned close to pre-ischaemic levels following reperfusion.

Ryanodine reduced the percentage of oxygen extraction in REF and SAT hearts, but not FO hearts (Table VI.8.a). Despite the reduction, SAT hearts still had the highest % of oxygen extraction compared to REF and FO hearts. During ischaemia, oxygen extraction rose significantly in REF and SAT, but only slightly in FO hearts (not significant).

When extracellular calcium concentration was increased to 6mM, oxygen extraction (Table VI.12.a), was increased significantly in all dietary

groups. The highest percentage of oxygen extracted was in SAT hearts and the least in FO hearts. During ischaemia, oxygen extraction increased further to very high levels in all groups without changing the existing dietary differences. Following reperfusion the percentage of oxygen extraction declined only slightly in all 3 groups and remained higher than in control. The highest extraction occurring in SAT and the lowest in FO hearts.

In the presence of RR + 6mM Ca^{++} the percentage oxygen extraction rose in REF and FO hearts but fell slightly (not statistically significant) in SAT hearts (Table VI.16.a.). During ischaemia the % oxygen extraction rose significantly to levels higher than with RR alone and then fell during reperfusion in all groups. In all perfusion periods with RR + 6mM Ca^{++}, FO hearts had significantly lower % oxygen extraction compared to SAT and REF and no significant difference was observed in oxygen extraction between REF and SAT hearts.

The percentage oxygen extraction was increased in REF and FO hearts during control perfusion with RY + Ca^{++}, but not in SAT hearts. During ischaemia, oxygen extraction increased significantly by 60%, 75% and 80% in REF, SAT and FO hearts respectively (Table VI.20.a)). While oxygen extraction did not differ between SAT and REF during ischaemia, these diets had significantly higher % oxygen extraction compared to FO hearts.

VI. 3. B: f) Myocardial Oxygen Consumption

Myocardial oxygen consumption (MVO_2) was significantly higher in SAT hearts (5.7 $\pm$ 0.73,n=25) than in REF hearts (3.5 $\pm$ 0.56,n=25). FO

hearts had significantly lower MVO_2 (2.0 ± 0.32,n=25) under control conditions. When extracellular calcium concentration was increased to 6mM, MVO_2 was increased significantly in all dietary groups. The MVO_2 was highest in SAT hearts and the lowest in FO hearts (Table VI.12.b., Fig.VI.1).

Ruthenium red markedly reduced the MVO_2 in REF and SAT but the already low MVO_2 in FO hearts was unchanged (Table VI.4.b., Figure VI.1). In the presence of RR there were no significant differences in MVO_2 between dietary groups. Ryanodine had no significant effect on control MVO_2 in any dietary group (Table VI.8.b). The combination of RR+Ca^{++} prevented the fall in MVO_2 seen in REF and SAT hearts with RR alone and induced a significant increase in MVO_2 in FO hearts (Table VI.16.b.). Ryanodine prevented the 6mM Ca^{++}-induced increase in MVO_2 in all dietary groups and there was no significant change from control perfusion conditions with this combination (Table VI.20.b).

During ischaemia or reperfusion in the presence of RR, the MVO_2 did not change from the pre-ischaemic levels in any dietary groups (Table VI.4.b). With RY (Table VI.8.b), MVO_2 increased in ischaemia and rose further in reperfusion in all dietary groups. With Ca^{++} 6mM alone (Table VI.12.b) or in combination with RR (Table VI.16.b), the MVO_2 fell during ischaemia in all dietary groups and in reperfusion rose to pre-ischaemic levels in SAT hearts, rose marginally in REF hearts but remained unchanged in FO hearts. With RY+Ca^{++} (Table VI.20.b) the MVO_2 rose significantly during ischaemia in REF and FO hearts but only slightly in SAT hearts and rose further in all dietary groups during reperfusion.

VI. 3. B: g) % Efficiency

The ratio of oxygen-derived energy utilisation to work conducted was expressed as % efficiency. There was a trend for the percentage efficiency of energy utilisation to be increased in REF and SAT and reduced with FO in the presence of RR (Table VI.4.c.). The main effect of RR was to abolish any dietary differences in % efficiency. During perfusion with RY (Table VI.8.c), 6mM Ca^{++} (Table VI.12.c), RR+Ca^{++} (Table VI.16.c) and RY+Ca^{++} (Table VI.20.c), the energy utilisation efficiency in all dietary groups decreased compared to the respective treatment free controls. The lowest level of efficiency was observed in SAT hearts and the highest in FO hearts for all perfusion periods.

VI. 3. B: h) Metabolites & Enzyme Content of Coronary Effluent

No significant differences in venous pH and extracellular [K^+] (Table VI.5.), were observed between the 3 dietary groups in the presence of RR. However, lactate and creatine kinase release was significantly higher in SAT hearts compared to FO with RR present. Following perfusion with RY or RY + Ca^{++}, dietary differences were still present in venous pH. The SAT hearts had significantly lower pH compared to REF and FO (Table VI.9.a. & 21.a.). During ischaemia, venous pH was slightly lower again in all the groups but SAT hearts had significantly greater acidosis. Following reperfusion venous pH was significantly lower in SAT hearts compared to REF and FO.

Acidosis was augmented in SAT hearts compared to the other treatments during working heart perfusion with 6mM Ca^{++} (Table VI.13.a).

During ischaemia and reperfusion, venous pH was decreased in all groups compared to control perfusion and the lowest pH was observed in SAT hearts and the highest in FO. With RR + Ca^{++}, ischaemic acidosis was attenuated and venous pH did not differ significantly between the dietary groups (Table VI.17.a).

During control working heart perfusion with RY or RR there was no significant difference in coronary effluent potassium concentration, lactate and creatine kinase between the dietary groups (Table VI.5 & 9.). However, higher amounts of lactate and creatine kinase were observed during ischaemia and reperfusion in SAT hearts compared to REF and FO (no significant difference).

No significant difference in the concentration of extracellular K^+ was observed between the dietary groups during control working heart perfusion with 6mM Ca^{++} (Table VI.13.b). During ischaemia and reperfusion the potassium concentration in coronary effluent was significantly elevated in SAT hearts compared to REF and FO hearts. In FO hearts extracellular [K^+] during reperfusion with 6mM Ca^{++} returned closer to preischaemic levels compared to REF and SAT. The concentration of lactic acid in coronary effluent tended to increase in all dietary groups perfused with 6mM Ca^{++}, but this rise was significant only in FO hearts (Table VI.13.c). The SAT hearts released significantly more lactate and creatine kinase compared to REF and FO hearts (Table VI.13.d). The dietary differences were maintained throughout ischaemia and reperfusion even though lactate and creatine kinase

concentrations were increased in reperfusion compared to preischaemic perfusion with 6mM Ca^{++}.

Extracellular $[K^+]$ increased in the presence of RR + 6mM Ca^{++} in all dietary groups (Table VI.17.b). During ischaemia extracellular $[K^+]$ rose further in all groups with the highest concentration being observed in SAT hearts. No significant difference in coronary effluent potassium concentration was observed during reperfusion compared to pre-ischaemic hearts of any dietary group.

During RR + 6mM Ca^{++} working heart perfusion lactate and creatine kinase release was decreased slightly in SAT but increased in REF and FO hearts (Table VI.17.c,d). Following a slight reduction in the amount of extracellular lactate and creatine kinase during ischaemia, these were increased during reperfusion in all dietary groups. Through each period SAT hearts had significantly greater release of lactate and creatine kinase than FO hearts.

Extracellular $[K^+]$ increased significantly in SAT hearts but only slightly in FO hearts with RY + Ca^{++} (Table VI.21.b). During ischaemia extracellular $[K^+]$ increased in all groups but was significantly higher in SAT than FO hearts. This dietary difference was maintained during reperfusion, but FO extracellular $[K^+]$ returned closely to pre-ischaemic levels while in SAT hearts this was delayed. A similar dietary relationship was observed in the amount of lactate in coronary effluent (Table VI.21.c).

Compared to non-treated controls, lactate increased in REF, SAT and FO working hearts perfused with RY + Ca^{++} by 13%, 10% and 18% respectively and this release increased further with ischaemia and reperfusion (Table

VI.21.c). Creatine kinase release followed the same dietary propensity as extracellular lactate (Table VI.21.d)). The highest levels of lactate and creatine kinase were collected from SAT hearts and the lowest from FO hearts in all perfusion periods with RY + Ca^{++}.

VI. 3. B: i) Effect of Ca^{++} on Arrhythmogenesis

Although this study was not specifically designed to observe cardiac arrhythmias (group sizes may be too small), Table VI.22. presents the assessment of this activity during ischaemia and upon reperfusion. Relatively few spontaneous arrhythmias were observed during ischaemia, most of which occurred between 10 and 15 min ischaemia. These arrhythmias were not severe, as only ventricular premature beats (VPB's) were observed. Nevertheless, perfusion with 6mM Ca^{++} resulted in increased VPB's in all dietary groups, compared to observations of spontaneous arrhythmias in Chapter IV, experiment A (Table VI.22.a). The highest number occurring in SAT hearts and the least in FO hearts. In the presence of RY + Ca^{++} significantly less VPB's were observed than with 6mM Ca^{++} alone and the dietary differences were not significant. In the presence of RR + Ca^{++}, the frequency of VPB's was significantly less than perfusion with Ca^{++} alone. The SAT and REF hearts still exhibited more frequent VPB's compared to RY + Ca^{++} and significantly more VPB's were observed in SAT hearts than in FO hearts.

The percentage incidence of ventricular tachycardia (VT) in reperfusion was significantly increased in the presence of 6mM Ca^{++} summed across all

dietary groups (Table VI.22.b). The percentage incidence of ventricular fibrillation (VF) during reperfusion with 6mM Ca^{++} was also significantly increased across all groups compared to controls (ChapterIV, experiment A) such that 5/5 SAT hearts, 4/5 REF hearts and 1/5 FO hearts experienced VF (Table VI.22.c). The incidence of VF in FO hearts was significantly lower than SAT in the presence of 6mM Ca^{++}. Reperfusion arrhythmias were inhibited by RY or RR with VT observed only in 1 of 5 (20%) REF and SAT hearts with RR. The elevated incidence of VT and VF with 6mM Ca^{++} alone was abolished by RY but not by RR (Tables VI.22.b.& c.iv & v).

VI. 4. Discussion

The first aim of this section of the study was to test whether the abnormally high MVO_2 in SAT hearts was related to increased basal oxygen consumption and to observe if basal oxygen metabolism is altered by ischaemia. This was measured by arresting the hearts with 30mM K^+. The very high extracellular K^+ concentration reduces the resting cell membrane potential of the myocytes such that the action potentials driving contraction cease. Once contraction has ceased, active metabolism ceases while a degree of basal energy consumption continues to sustain energy dependant ion pumps for sodium and calcium homeostasis **(Gibbs & Chapman,1979)** and also for the transport of amino acids, for synthesis and degradation of proteins and other macromolecules **(Millward,,*et al.*,1975;Gibbs,1978;Shreiber,*et al.*,1977;Burns & Reddy,1978)**. Although there was a trend for basal MVO_2 to be higher in SAT hearts than FO hearts and basal MVO_2 to be slightly

higher in reperfusion after ischaemia, no significant effect of dietary lipid regime or of ischaemia and reperfusion was observed on basal oxygen metabolism. This is the first study to investigate basal oxygen consumption in isolated hearts from dietary lipid supplemented rats. However, regarding the post-ischaemic effect, these findings are in agreement with those of Laster and coworkers (**1989**) using isolated rabbit hearts and Dean, *et al.*, (**1990**) using anaesthetised open chest dogs. This is also supported by other mitochondrial isolation studies and P^{-31} NMR spectroscopy (**Edoute,*et al.*,1983;Sako,*et al.*,1987**). Together these results indicate that the paradoxically high post-ischaemic MVO_2 or the elevated MVO_2 after SAT feeding are not related to electrogenic uncoupling within mitochondria (basal metabolism) but may be mostly related to altered cost of increasing and decreasing intracellular $[Ca^{++}]$ with each contraction (activation metabolism) or may involve electromechanical uncoupling or defective contractile mechanisms (contraction metabolism).

This study subsequently investigated the possible involvement of altered intracellular Ca^{++} handling in both the abnormally high MVO_2 in SAT hearts and the paradoxical increase in MVO_2 that occurs during ischaemia and reperfusion (Chapter III). Thus increased extracellular $[Ca^{++}]$ was utilised to elevate intracellular $[Ca^{++}]$ while RR and RY were employed to selectively investigate the role of mitochondria and SR in modulating intracellular Ca^{++}.

When extracellular Ca^{++} was increased from 1.4 to 6 mM, cardiac performance significantly increased in all dietary groups. FO hearts maintained the highest cardiac output, contractility, external work and the percentage energy utilisation efficiency and SAT hearts the lowest. Con-

versley, SAT hearts maintained very high oxygen extraction and MVO_2 irrespective of Ca^{++} concentration. Coronary flow was increased in all groups and SAT hearts sustained the highest while FO had the lowest flow rate. Although not directly measured, but on the basis of the above performance and previous studies **(Nayler,1969;1981;1983;Ito,*et al.*,1985;Lamers,*et al.*,1984)**, it is assumed that perfusion with 6mM Ca^{++} resulted in increased cytosolic $[Ca^{++}]$, which was then available for sequestration to specific intracellular sites. The increased MVO_2 was only partly due to calcium-induced increased contractility and external work in hearts prior to ischaemia because energy utilisation efficiency decreased.

During ischaemia and reperfusion, the increase of extracellular $[Ca^{++}]$ and thus intracellular $[Ca^{++}]$ also augmented acidosis and as well as $[K^+]$, creatine kinase and lactate release, of which the greatest magnitude occurred in SAT hearts and the least in FO hearts. In addition, this was associated with increased ischaemic and reperfusion arrhythmias, which occurred most frequently in SAT hearts and the least in FO hearts. Thus, increased calcium contributed to the detriment of cardiac performance and metabolism in ischaemia and reperfusion. The involvement of intracellular Ca^{++} overload in the pernicious effects of ischaemia and reperfusion is well documented **(Grinnwald & Nayler,1981;Nayler,*et al.*1983;Sharma,*et al.*,1983;Cheung,*et al.*,1986;Murphy,*et al.*,1987;Steenburgen,*et al.*,1987;Thomas,*et al.*,1988)** and supported by the present findings.

It is noteworthy to consider the possibility that reduced venous pH may be partly responsible for the post-ischaemic reduction in contractility.

Intracellular acidosis is reported to contribute to ischaemia induced negative inotropy (**Smith,1926;Cingolani,*et al.*,1970;Wang & Katz,1965;Pannier & Leusen,1968;Ellis & Thomas,1976**). Evidence exists for myocardial acidosis to diminish calcium delivery to the myofilaments by altering Na^+/H^+ exchange and thus Na^+/Ca^{++} exchange (**Eisener & Lederer,1985;Van - Heel,*et al.*, 1986**), or by altering mitochondrial release of Ca^{++} and displacement of Ca^{++} by H^+ in the cytoplasm (**Williams & Fry,1979;- Crompton,1985;Denton & McCormack,1985**). In addition, acidosis may inhibit myofibrillar responsiveness to calcium by decreasing contractile protein sensitivity to Ca^{++} (**Marban & Kasuoka,1987;Kentish & Nayler,1978;- 1979;Blanchard & Solaro,1984;Donaldson,1978;Fabiato & Fabiato,1978;- Solaro,*et al.*,1988**). Thus it is possible that in SAT hearts the even greater reduction of contractility in ischaemia and depressed return in reperfusion compared to REF and FO, may be due to increased ischaemic alterations of pH and calcium fluxes by the aforementioned mechanisms. The situation using the low flow model of ischaemia is different from total ischaemia in which contractile failure is due to cessation of intracellular Ca^{++} release secondary to action potential failure (**Stern,*et al.*,1988**).

Ryanodine and ruthenium red produced contrasting effects in the isolated blood perfused working heart. Both the elevated MVO_2 of SAT hearts during control perfusion and the paradoxical increase in MVO_2 that was observed during ischaemia and reperfusion were abolished by RR. Ryanodine had no effect on MVO_2. In contrast, RR & RY had equal depressant effects on external work in all dietary groups, which were greatest in FO and least

in SAT hearts. In the presence of RR or RY there were no dietary differences in external work. Consequently RY greatly reduced the % efficiency of energy utilisation in all dietary groups while RR reduced efficiency in FO (to a lesser degree) but increased the % efficiency in the REF and SAT groups. With RR, the coronary flow rates of SAT hearts (controls) were also reduced and other functional differences, present in control perfusion were no longer observed between the 3 dietary groups. The effects of RR are in contrast to the effects of hydralazine (which induces coronary vasodilation via putative calcium mechanisms in smooth muscle). In Chapter V, when hydralazine equalised coronary flow between the dietary groups by coronary vasodilation, neither the significantly greater MVO_2 in SAT hearts, nor the paradoxical ischaemia-induced increase in MVO_2 was abolished.

Ruthenium red is a large molecular weight inorganic polycationic polysaccharide dye used as a glycocalyx stain in electron microscopy and it is argued by some that it cannot enter intact cells. Nevertheless sufficient evidence both direct and indirect has been presented to support an intracellular action. It has been reported to enter intact cardiac cells of hearts perfused for 15 min with 2.5 to 5μM RR (**Forbes & Sperelakis,1979;Gupta,*et al.*,1988**). The findings of the present study that RR decreased myocardial performance in cardiac output, contractility, external work and also decreased MVO_2, creatine kinase and lactate release were in agreement with previous reports (**Smith,1980; Ferrari,*et al.*,1982;McCormack & England,1983;Busselen, 1985;Unitt,*et al.*,1988;Moreno-Sanchez & Hansford,1988;Park,*et al.*,1990; Grover,*et al.*,1990**).

Increased extracellular [Ca^{++}] resulted in increased MVO_2, and RR reduced MVO_2 in control perfusion of working hearts. Indeed, when both RR and 6mM Ca^{++} were administered the negative inotropic effect of RR was reversed and slight positive inotropy was observed (less than 6mM Ca^{++} alone). The increased contractility, cardiac output and external work observed in all groups was related to the increased Ca^{++}. However, dietary differences in MVO2 were abolished as had been seen with RR alone. Although notably the % oxygen extraction, lactate production, creatine kinase and K^+ release was lower in FO hearts than REF and SAT. This indicates that although RR prevented the increased MVO_2, increased extracellular Ca^{++} still permitted some detrimental aspects of ischaemia to occur albeit limited in comparison to the absence of RR at either high or low extracellular Ca^{++} levels.

The work of Grover and coworkers (**1990**), showed that 3 μM RR could reduce MVO_2 before and after 30 min global ischaemia in isolated rat hearts and improve the efficiency of oxygen utilisation. In the present study, with RR the dietary differences in venous pH, extracellular [K^+], and creatine kinase release were also abolished and were altered by a lesser extent than with perfusion with 6mM Ca^{++}. Along with the findings of other workers this indicates a role of increased calcium in the alteration of energy metabolism and utilisation as well as the detrimental consequences of ischaemia, especially in the unique case of SAT hearts.

It has been observed that the abnormally high intracellular Ca^{++} levels that accumulate as a consequence of ischaemia may damage mitochondria causing futile calcium cycling and inefficient energy utilisation (**Thomas,*et***

al.,**1988**). The production of intracellular H_2O_2 from accumulated hypoxanthine by xanthine oxidase in reperfusion can induce Ca^{++} efflux that commences Ca^{++} reuptake thus causing futile Ca^{++} cycling across inner mitochondrial membranes and producing increases in oxygen consumption (**Vlessis & Mela-Riker,1989**).

Ruthenium red may exert some of its effects, such as reduced MVO_2 in the myocardium, by limiting the accumulation of calcium into intracellular compartments. Ruthenium red has been shown to act *in vitro* by selectively inhibiting mitochondrial uptake of calcium (**Moore,1971;Vasington,1972;-Gupta,*et al.*,1989**). Peng, *et al.*, (**1977**) in an anaesthetised whole pig preparation infused RR during regional myocardial ischaemia preventing post-ischaemic intracellular calcium increases and produced significant increases in ADP induced respiration and ATP production in mitochondria isolated from these hearts compared to controls. Indeed, Cheung, *et al.*, (**1986**) and Murphy, *et al.*, (**1987**) have observed increased mitochondrial uptake of calcium after reperfusion of ischaemic hearts, while others observed rapid accumulation of calcium into mitochondria to competitively inhibit ATP production as well as mitochondrial lesions (**Ferrari,*et al.*,1982;Vercesi,*et al.*,1978;Renlund,*et al.*,1984**).

Although it has been demonstrated that the predominant action of RR is mitochondrial, evidence exists for some action on the sarcoplasmic reticulum (SR). Calcium release from SR has been shown to be inhibited by RR in an *in vitro* study with SR vesicles (**Chamberlain,*et al.*,1984**). However, Gupta and associates (**1989**) found that RR inhibited calcium

movement across mitochondrial membranes at a concentration 10 times less than that required to inhibit calcium movement across sarcolemmal or sarcoplasmic reticulum membranes. It was observed that 1 μM RR failed to prevent calcium accumulation in SR following ischaemia but did inhibit calcium overload in mitochondria indicating that at such a concentration RR acted selectively at mitochondrial sites (**Ferrari,*et al.*,1982**). Stone and coworkers (**1989**) showed that high concentrations of RR inhibit uptake of ^{45}Ca in reoxygenated myocytes and perfused isolated hearts during and after 40 min of hypoxia and resulted in reduced creatine kinase release and cell damage suggesting a sarcolemmal site of action also. Smith (**1980**), using an anaesthetised open chest dog preparation found that when RR was administered at reperfusion, post-ischaemic contractile function was improved and reperfusion cell damage was reduced. However, when the calcium channel antagonist verapamil was administered under the same conditions it failed to reduce reperfusion injury. Thus the evidence to date predominantly supports an intracellular and particularly a mitochondrial site of action for RR.

Ryanodine on the other hand has a selective effect at the sarcoplasmic reticulum to inhibit Ca^{++} release (**Hansford & Lakatta,1987;Wier,*et al.*, 1985;Sutko,*et al.*,1985;Fabiato,1985**). Therefore the ability of RR but not RY to abolish dietary and ischaemic differences in MVO_2 implicates altered mitochondrial calcium handling as the basis for both effects. As mentioned earlier, RY depressed contraction and thus greatly reduced the % efficiency of mechanical energy utilisation in all dietary groups. In FO, with low MVO_2, the effect of RR is chiefly on contractile mechanisms thus reducing the %

efficiency. In SAT (and also REF) with high MVO_2 the RR effect is mainly on oxygen consumption thus increasing the % efficiency.

It has been demonstrated that hormones or positive inotropic drugs which stimulate energy requiring cardiac contraction, produced increases in cytosolic calcium concentration and enhanced ATP utilisation (**Williamson, 1975;Randle & Tubbs,1979**). In addition it has been observed (**Denton,*et al.*, 1980;McCormack & Denton,1988**) that such calcium movement into the cytosol caused intramitochondrial calcium concentrations to indirectly activate pyruvate dehydrogenase (PDH), NAD^+-isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase and thus stimulate oxidative phosphorylation. These key enzymes assist NADH production for increased oxidative metabolism and still maintain high ATP/ADP ratio. In an elegant study by Wolska & Lewartowski (**1991**), carbonyl cyanide *m*-chlorophenyl-hydrazone, CCCP (100μM), activated Ca^{++} release from mitochondria in perfused isolated guinea pig hearts which were at rest or which were electrically stimulated. Prior to CCCP treatment these tissues were equilibrated with ^{45}Ca, thus permitting the measurement of stimulated calcium release. These workers demonstrated that hearts stimulated with increased contraction rates had increased mitochondrial calcium content compared to rested hearts.

The collective work of Denton and McCormack (**1985**) and Hansford (**1985**) suggests that calcium may perform a crucial role in a feed-back system that links energy consumption to energy production. No studies have yet examined the effect of ischaemia and/or reperfusion induced increases in calcium on these three enzymes, the related oxidative metabolism or MVO_2.

However, by inhibiting calcium uptake into mitochondria, RR has been shown to inhibit the increase of activated mitochondrial PDH in response to increased extra-mitochondrial calcium or positive inotropic agents in isolated perfused hearts **(Denton,*et al.*,1980;McCormack & England'83)**.

Bunger and coworkers **(1983;1984)** found that by raising extracellular Ca^{++} they could match noradrenalin stimulated increases in activated pyruvate dehydrogenase in isolated perfused hearts. Associated with the increased PDH was increased MVO_2. In a study where K^+ or veratridine stimulation of isolated myocytes was used to increase cytoplasmic calcium concentration, an increase in oxygen uptake ensued **(Sanchez & Hansford,1988)**. Oxygen uptake increased with the elevation of extracellular $[Ca^{++}]$ or pH, while RR-induced inhibition of mitochondrial dehydrogenases and thus respiration, was associated with reduced oxygen uptake. Unitt, *et al.***(1988)**, showed that the oxygen uptake and lactate production in isolated perfused rat hearts was increased following stimulation of hearts with isoprenaline (and thus increased intracellular calcium). Infusion of 3.4μM RR had no effect on peak contractile force but did reduce isoprenaline stimulated increases of MVO_2 and lactate. These findings are supported by the present study.

The present study reported the effect of calcium on ischaemia and reperfusion induced cardiac arrhythmias. As most of the arrhythmias during ischaemia occurred between 10 and 15 min ischaemia, it is of interest to note a report that showed cytosolic $[Ca^{++}]$ to be increased within 10 min of global ischaemia in isolated rat hearts **(Steenburger,*et al.*,1987)**. In the present study, increased extracellular $[Ca^{++}]$ led to an increased incidence of

ischaemia and reperfusion arrhythmias. It has been observed that action potential-independent, Ca^{++}-dependent, asynchronous spontaneous calcium release from SR occurs in cardiac preparations and can be abolished by RY (**Bloom,*et al.*,1974;Capogrossi,*et al.*,1984;1985;Fabiato & Fabiato,1972;1975;1978;1985;Kass,*et al.*,1982;Kort & Lakatta,1984a;1984b;Cannell,*et al.*,1985;Lakatta,*et al.*,1985**). These spontaneous Ca^{++} oscillations from SR have been shown to increase during ischaemia-induced calcium overload and it has been proposed that they may play a role in arrhythmogenesis (**Thandroyen,*et al.*,1988**). This is supported by the observation that RY can inhibit the incidence of ischaemic and reperfusion arrhythmias (**Hajdu & Leonard,-1961;Kahn,*et al.*,1964;Thandroyen,*et al.*,1988**).

Ryanodine completely abolished reperfusion arrhythmias even in the presence of high Ca^{++} and reduced ischaemic arrhythmias and the proarrhythmic effect of high Ca^{++} in ischaemia.In contrast, while RR was quite effective at inhibiting arrhythmias with a low Ca^{++} perfusate it was unable to overcome the proarrhythmic effect of high extracellular Ca^{++}. This result indicates that RR may have had only a limited effect on the sarcoplasmic reticulum.

It has been proposed that the plant alkaloid ryanodine binds to the spanning protein complex linked to the sarcoplasmic reticulum calcium-release channel, locking it in the open state (**Lai,*et al.*,1987;1988;Inuie,*et al.*,1987;-Lederer,*et al.*,1989**). Ryanodine specifically inhibits Ca^{++} release from SR by promoting its leakage from release sites into the cytoplasm where it is then pumped out of the myocyte. This continues until SR Ca^{++} stores are impoverished and further Ca^{++} release from the SR is inhibited limiting Ca^{++}

release in subsequent excitations (**Hansford & Lakatta,1987;Wier,*et al.*, 1985;Sutko,*et al.*,1985;Fabiato,1985**). Ryanodine has been of great value in the study of the excitation-contraction (E-C) coupling in the myocardium because it has been shown not to interfere with other factors involved in E-C coupling such as mitochondrial Ca^{++} uptake; sarcolemmal Ca^{++} transport by Ca^{++}-ATPase or Na^{+}-Ca^{++} exchanger; cAMP; Ca^{++} binding to cardiac troponin; and passive sarcolemmal Ca^{++} leak (**Sutko,*et al.*,1985;Hansford & Lakatta,1987**).

It has been repeatedly demonstrated that ryanodine has negative inotropic qualities at nanomolar concentrations in myocardial preparations (**Nayler,1962;Jenden & Fairhurst,1969;Sutko,*et al.*,1985**). At such low concentrations this negative inotropy may be due to reduced Ca^{++} release from the SR while at high concentrations it is due to reduced Ca^{++} accretion in the SR (**Su,1988**). These findings are supported by the results of the current study where RY reduced myocardial performance in all dietary groups. However, unlike RR, RY did not abolish the abnormally high MVO_2 of control perfused SAT hearts, nor was the abnormally high MVO_2 in ischaemic or post-ischaemic hearts abolished. Indeed, RY had no significant effect in altering the dietary group differences in venous pH, lactate production, creatine kinase release, and only slightly attenuated the extracellular $[K^{+}]$ of SAT hearts. When RY was perfused together with 6mM Ca^{++} the negative inotropic effects of RY and the positive inotropic effects of 6mM Ca^{++} were cancelled out, but RY had no effect on the dietary related differences in MVO_2 and oxygen extraction either alone or together with 6mM Ca^{++}. Ryanodine was

also able to prevent the increase in MVO_2 associated with increased Ca^{++} suggesting a contraction-dependent mechanism. On the other hand neither the ischaemia nor dietary induced increases in MVO_2 were inhibited by RY.

The consensus of published evidence supports an SR site of action for ryanodine and a selective action of ruthenium red at mitochondria. Many studies have associated ischaemia/reperfusion with increased cytosolic calcium and increased mitochondrial and SR calcium. In turn the McCormack, Denton and Hansford research groups have demonstrated that procedures which elevate cytosolic Ca^{++} lead to increased mitochondrial calcium which stimulates oxygen consumption, probably by stimulating pyruvate dehydrogenase or other calcium activated activity.

Other studies as well as the present have demonstrated a paradoxical increase in MVO_2 that is not related to contraction or to basal metabolism. Some suggest excess mitochondrial Ca^{++} can increase MVO_2 by futile cycling across mitochondrial membranes (**Thomas,*et al.*,1988**) or that ischaemic damage of mitochondria promotes Ca^{++} loss and oxygen consuming futile re-uptake/ loss cycling (**Vlessis & Mela-Riker,1989**).

These individual observations have not previously been linked. However, in light of the current observations it appears likely that both the paradoxical increase in MVO_2 and the SAT-induced increase in MVO_2 are due to abnormal Ca^{++}-dependent oxygen consumption in the mitochondria. On the other hand, it is likely that the arrhythmias that occur in ischaemia and upon reperfusion may be dependent on excess Ca^{++} in the SR. This has not been

confirmed by direct Ca^{++} measurements nor has the cause of the postulated elevated cytosolic Ca^{++} in SAT hearts been identified.

It is apparent that the results of the experiments performed for this thesis indicate that intracellular calcium may rise during ischaemia as well as reperfusion to induce altered Ca^{++}-dependent oxygen demand or consumption. However, some reports suggest that calcium uptake does not occur until reperfusion of ischaemic myocardium. Tani & Neely **(1990)**, reported no alteration in $^{45}Ca^{++}$ uptake in Langendorff perfused hearts made globally ischaemic for 30 min despite large increases in intracellular Na^{+} concentration. Upon reperfusion a large and rapid uptake of $^{45}Ca^{++}$ was observed. Similarly, Panagiotopoulos, *et al.***(1990)**, observed no change in atomic absorption-determined intracellular Ca^{++} content in Langendorff perfused hearts made globally ischaemic for 60 min. However, in reperfused heart tissue and in mitochondria from reperfused hearts calcium content was significantly increased.

The novel findings of altered Ca^{++}-dependent oxygen consumption obtained in this thesis suggestive of increased cytosolic calcium levels in the myocardium during ischaemia may be explained by a few main possibilities. Firstly, there may be a redistribution of calcium between intracellular compartments without an overall increase in total intracellular calcium. This may occur in response to increased cytosolic-free calcium because of reduced compartmental Ca^{++} uptake during ischaemia **(Steenburgen,*et al.*,1987)** due to acidosis-dependant reduced myofibrillar responsiveness to calcium via altered Na^{+}/H^{+} exchange and Na^{+}/K^{+} ATPase inactivation **(Lazdunski,*et***

al.,1985;Neubauer,*et al.*,1987;Huang & Askari,1984). In addition, this may also be due to increased [P*i*] and acidosis-dependent abatement in SR Ca^{++} accumulation (Kimurn,*et al.*,1990;Koretsune & Marban,1990;Fabiato & Fabiato,1978), possibly allowing calcium redistribution to mitochondria. Increased intracellular [Ca^{++}] can occur due to increased uptake or reduced removal. As Ca^{++} removal can be energy dependent this may be depressed during ischaemia. Indeed, it has been observed that Ca^{++}ATPase activity is reduced in sarcolemmal vesicles from ischaemic myocardium (Dhalla,*et al.*,1988).

This thesis did not utilise total global ischaemia as others have (Tani & Neely,1990;Panagiotopoulos,*et al.*,1990). Complete cessation of coronary flow may have limited availability of external calcium to permit increased calcium uptake. Indeed, Nayler, *et al.*(1988), have reported increases in tissue concentrations of calcium during hypoxia where coronary flow was maintained relatively high. When buffer concentrations of calcium were reduced from 1.3mM to 0.1mM Ca^{++} no increase in tissue calcium content was observed over the same period of hypoxia. In addition, in most models of ischaemia total cessation of coronary flow is also associated with total arrest of contractile function. Although Tani & Neely (1990) electrically stimulated contractions in their preparation they ceased doing so within 5min of a 30min ischaemic period, possibly corresponding with the time of total ventricular failure. This means that cross-bridge activity and SR utilisation of calcium would have also ceased during this period and was only restored during reperfusion where demands for calcium-dependent contractile function and

mitochondrial energy production resumed. Most important, however, is probably the failure of action potentials in anoxia and the concomitant voltage-dependent Ca^{++} entry (**Stern,*et al.*,1988**). In the unique model of ischaemia developed for this thesis, low flow global ischaemia may have provided sufficient external calcium and oxygen to permit observations of MVO_2 increases, continued contraction and the production of external work to a limited extent during ischaemia. Certainly, contraction and therefore action potentials did not cease, thus leaving an avenue for beat to beat Ca^{++} entry in this model.

In summary, the results presented in this chapter indicate that basal metabolism was not altered by dietary lipid supplementation and thus was not related to the dietary differences in MVO_2 during control perfusion. In addition, basal metabolism was not altered during ischaemia or reperfusion and therefore did not influence the paradoxical increase in MVO_2. The increased MVO_2 induced by SAT dietary supplementation was prevented by ruthenium red but not by ryanodine. The 6mM Ca^{++}-induced increase in MVO_2 occurred such that dietary differences were maintained, however this MVO_2 rise was prevented by ryanodine but not ruthenium red. The ischaemia-induced paradoxical increase in MVO_2 was prevented by ruthenium red but not by ryanodine. These results indicate that the 6mM Ca^{++}-induced increase in MVO_2 may be largely related to contraction (possibly by activation of actomyosin ATPase) or SR-Ca^{++} release/reuptake related (Ca^{++} ATPase). However, the increased MVO_2 induced by SAT or ischaemia appear to be

related to mitochondrial uptake of Ca^{++} but are dependent upon beat to beat activation.

It is clear that these issues may be resolved with the execution of experiments that involve the direct measurement of Ca^{++} content in the cytosol and in all subcellular compartments during a time course of ischaemia without reperfusion.

Table VI. 1. The influence of dietary lipid supplementation and ischaemia-reperfusion on basal oxygen consumption.

PROTOCOL	REF	SAT	FO
(i)	3.245 ± 0.25	5.181 ± 0.31*	1.808 ± 0.28*
(ii)	3.915 ± 0.26§	5.924 ± 0.33*§	2.185 ± 0.25*
(iii)	0.572 ± 0.18	0.683 ± 0.19	0.359 ± 0.13
(iv)	0.668 ± 0.21	0.775 ± 0.22	0.445 ± 0.19

Heart contraction was arrested with infusion of 30mM KCl solution. Oxygen consumption values for (i)-(iv) are presented as mL/min/g dry weight.

(i)= control hearts (not subjected to ischaemia and reperfusion), perfusion time matched with (ii).

(ii)= hearts subjected to 15 min ischaemia and 5 min reperfusion.

(iii)= KCl arrested control hearts, perfusion time matched with (iv).

(iv)= KCl arrested reperfused hearts. See methods for further details.

*=significant difference compared to REF, §= significant difference compared to the respective control (i), Scheffe's test, $p<0.05$, $n=5$ for all means ± SD.

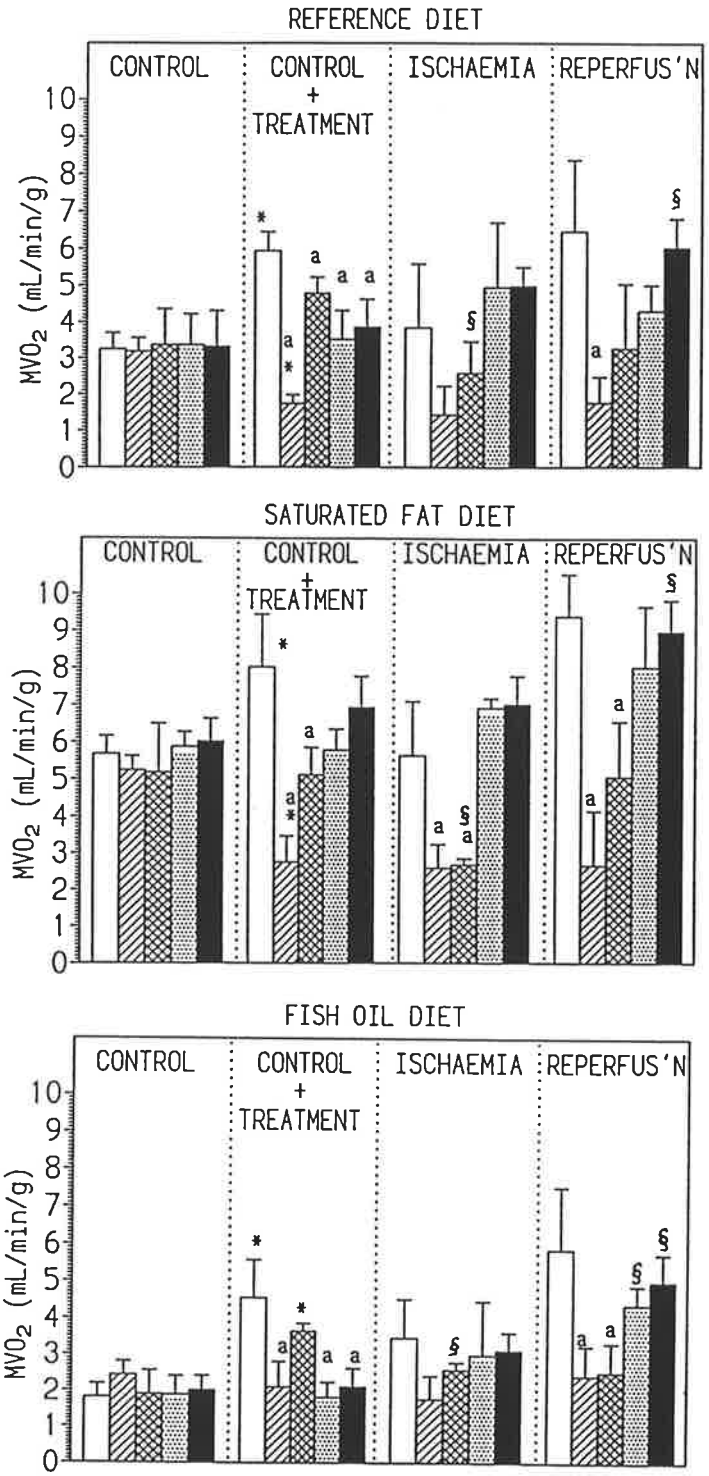

Figure VI.1. The effect of extracellular calcium modification in the three dietary groups on myocardial oxygen consumption (MVO_2,mL/min/g dry weight). Open bars= perfusate Ca^{++} increased from 1.4mM to 6mM. Diagonal bars= 3.4μM ruthenium red+1.4mM Ca^{++}. Criss-cross bars =3.4μM ruthenium red+6mM Ca^{++}. Speckled bars =10^{-9}M ryanodine+1.4mM Ca^{++}. Solid bars =10^{-9}M ryanodine+6mM Ca^{++}. *=significant difference compared to respective control, §=significant difference compared to respective control + treatment, a=significant difference compared to 6mM Ca^{++}, $p<0.05$, n=5 for each mean ±SD, Scheffe's test. See Methods & Results for details.

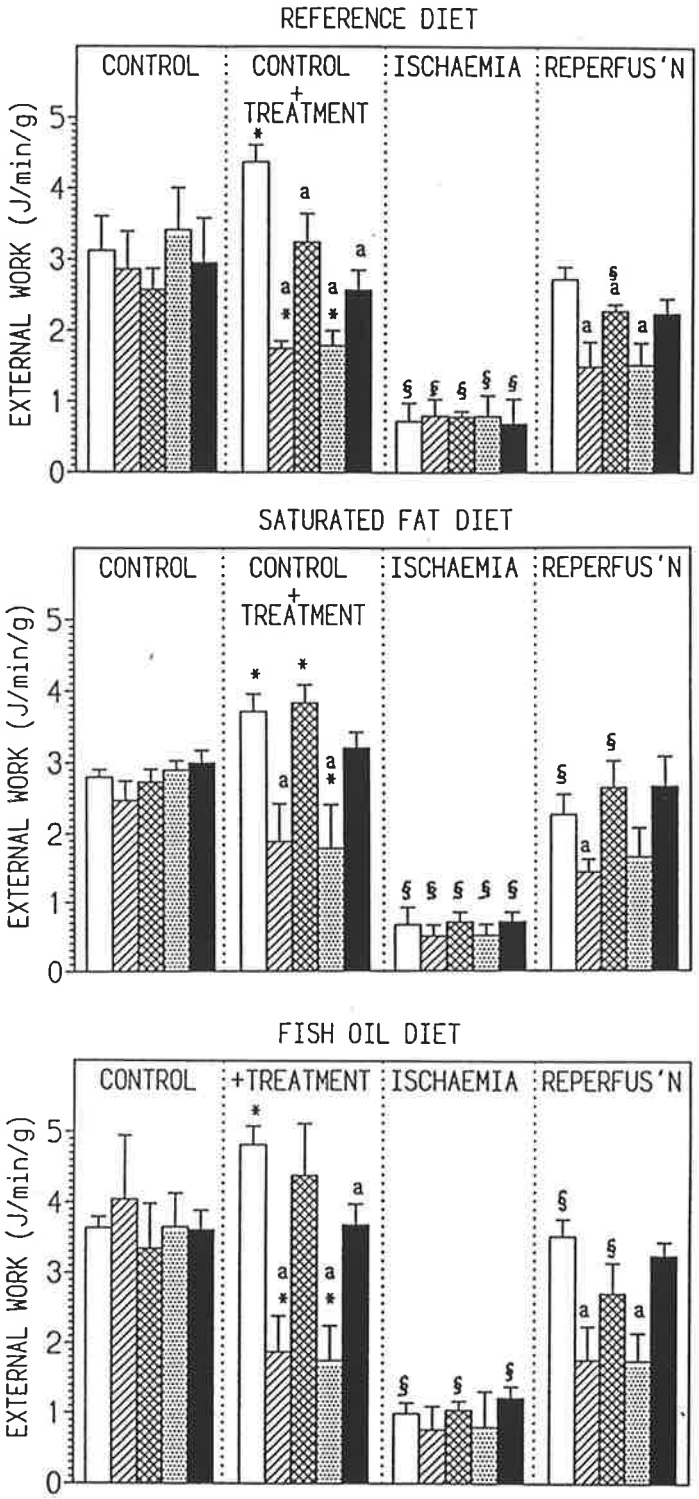

Figure VI.2. The effect of extracellular calcium modification in the three dietary groups on external work (J/min/g dry weight). Open bars= perfusate Ca^{++} increased from 1.4mM to 6mM. Diagonal bars= 3.4μM ruthenium red+1.4mM Ca^{++}. Criss-cross bars =3.4μM ruthenium red+6mM Ca^{++}. Speckled bars =10^{-9}M ryanodine+1.4mM Ca^{++}. Solid bars =10^{-9}M ryanodine+6mM Ca^{++}. *=significant difference compared to respective control, §=significant difference compared to respective control + treatment, a=significant difference compared to 6mM Ca^{++}, $p<0.05$, n=5 for each mean ±SD, Scheffe's test. See Methods & Results for details.

Table VI.2. Ruthenium Red (3.4μM) Treatment

a) Coronary flow mL/min/g

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	44.77 ± 2.57	28.71 ± 2.80	18.91 ± 1.24	24.96 ± 6.30
SAT	57.33 ± 4.17*	31.22 ± 8.45	20.75 ± 9.26	29.91 ± 4.81
FO	43.93 ± 3.50	31.29 ± 9.24	21.50 ± 9.01	30.07 ± 8.93

b) Aortic Output mL/min/g

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	148.90 ± 15.30	105.23 ± 5.20§	75.66 ± 6.23	93.42 ± 17.63
SAT	126.57 ± 19.96	107.75 ± 25.50	35.27 ± 6.70	81.36 ± 9.17
FO	210.09 ± 37.20*	113.66 ± 41.10§	56.32 ± 11.20	105.02 ± 36.20

c) Cardiac Output mL/min/g

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	193.69 ± 17.70	133.94 ± 3.29§	88.10 ± 8.08	118.38 ± 22.75
SAT	183.90 ± 19.94	138.96 ± 32.20	56.02 ± 14.60	111.27 ± 11.73
FO	254.02 ± 42.95	144.95 ± 42.90§	77.82 ± 20.20	135.10 ± 37.96

Table VI.3. Ruthenium Red (3.4μM) Treatment

a) Pressure-Time Integral

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	2055.8 ± 264	1885.2 ± 329	1246.5 ± 276	1638.8 ± 108
SAT	2082.6 ± 315	1913.9 ± 517	1343.4 ± 451	2101.8 ± 450
FO	2139.6 ± 362	1872.0 ± 180	1095.6 ± 258	1829.0 ± 152

b) External Work J/min/g

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	2.861 ± 0.533	1.751 ± 0.102§	0.805 ± 0.232	1.499 ± 0.347
SAT	2.469 ± 0.277	1.881 ± 0.538	0.502 ± 0.159	1.435 ± 0.184
FO	4.043 ± 0.900*	1.877 ± 0.512§	0.759 ± 0.331	1.759 ± 0.479

*=significant difference compared to REF, §= significant difference compared to the respective control, Scheffe's test, $p<0.05$, $n=5$ for all means ± SD. See methods for protocol details.

Table VI.4. Ruthenium Red (3.4μM) Treatment

a) % Oxygen Extraction

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	44.5 ± 2.6	31.4 ± 2.3	59.2 ± 11.5	36.1 ± 8.5
SAT	53.1 ± 4.7*	44.5 ± 8.9	67.5 ± 8.4	46.6 ± 6.3
FO	29.3 ± 3.0*	34.6 ± 4.7	44.0 ± 8.9	41.9 ± 2.1

b) Myocardial Oxygen Consumption (MVO_2) mL/min/g

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	3.816 ± 0.37	1.739 ± 0.24§	1.440 ± 0.78	1.756 ± 0.72
SAT	5.831 ± 0.62*	2.759 ± 1.11§	2.595 ± 0.12	2.669 ± 0.47
FO	2.430 ± 0.38*	2.098 ± 0.71	1.756 ± 0.65	2.417 ± 0.82

c) % Efficiency of Oxygen Utilisation

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	3.755 ± 0.67	5.057 ± 0.42§	3.412 ± 1.85	4.692 ± 1.45
SAT	2.133 ± 0.37	3.675 ± 0.96§	0.980 ± 1.02	2.724 ± 0.46
FO	8.760 ± 2.54*	4.884 ± 1.87	2.120 ± 0.22	3.823 ± 1.45

Table VI.5. Ruthenium Red (3.5μM) Treatment

a) Venous pH

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	7.321 ± 0.026	7.300 ± 0.037	7.202 ± 0.304	7.232 ± 0.077
SAT	7.300 ± 0.059	7.252 ± 0.018	7.145 ± 0.106	7.160 ± 0.048
FO	7.320 ± 0.008	7.272 ± 0.023	7.238 ± 0.015	7.272 ± 0.023

b) Extracellular K^+ mM/L

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	3.210 ± 0.241	3.240 ± 0.305	4.640 ± 0.471	4.542 ± 0.392
SAT	3.260 ± 0.454	3.160 ± 0.411	5.230 ± 0.958	5.115 ± 0.893
FO	3.194 ± 0.317	3.310 ± 0.362	3.750 ± 0.255	3.950 ± 0.325

c) Extracellular Lactate μmol/min/g

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	80.495 ± 22.5	95.631 ± 28.5	75.192 ± 30.7	98.727 ± 39.6
SAT	165.327 ± 18.7*	149.421 ± 22.3	96.352 ± 26.4	168.292 ± 48.2
FO	20.532 ± 2.9*	75.424 ± 13.6§	65.321 ± 20.9	79.618 ± 22.4

d) Creatine Phosphokinase Release U/min/g

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	4.592 ± 0.92	6.090 ± 0.78	3.251 ± 0.29	7.251 ± 0.63
SAT	12.393 ± 0.98*	8.950 ± 0.42§	3.952 ± 0.36	8.722 ± 0.93
FO	1.988 ± 0.17*	5.070 ± 0.45§	2.903 ± 0.35	6.672 ± 0.63

*=significant difference compared to REF, §= significant difference compared to the respective control, Scheffe's test, $p<0.05$, $n=5$ for all means ± SD. See methods for protocol details.

Table VI.6. Ryanodine (10^{-9}M) Treatment

a) Coronary flow mL/min/g

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	46.35 ± 8.6	30.87 ± 1.9§	17.52 ± 1.4	29.58 ± 2.3
SAT	60.28 ± 3.2*	32.71 ± 9.5§	19.95 ± 10.9	27.95 ± 8.9
FO	48.85 ± 3.5	39.60 ± 4.3§	22.95 ± 8.6	42.50 ± 7.6

b) Aortic Output mL/min/g

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	163.62 ± 12.5	100.50 ± 5.9§	73.67 ± 5.4	85.73 ± 6.7
SAT	126.70 ± 10.6*	80.68 ± 19.5§	32.22 ± 11.6	75.83 ± 20.2
FO	190.40 ± 13.6*	110.40 ± 40.4§	60.45 ± 15.4	100.5 ± 42.6

c) Cardiac Output mL/min/g

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	209.97 ± 17.8	131.37 ± 7.8§	91.19 ± 8.4	115.31 ± 11.4
SAT	186.98 ± 14.6	113.40 ± 29.6§	53.17 ± 15.4	103.80 ± 22.4
FO	239.27 ± 25.8	151.00 ± 44.7§	83.40 ± 14.8	143.00 ± 32.9

Table VI.7. Ryanodine (10^{-9}M) Treatment

a) Pressure-Time Integral

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	2230.0 ± 281	1782.3 ± 130	1195.8 ± 362	1497.6 ± 382
SAT	2080.4 ± 102	1823.8 ± 153	1311.9 ± 329	1662.4 ± 289
FO	2160.0 ± 150	1822.0 ± 146	1050.6 ± 304	1803.0 ± 260

b) External Work J/min/g

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	3.420 ± 0.588	1.792 ± 0.214	0.798 ± 0.295	1.522 ± 0.310
SAT	2.892 ± 0.132	1.782 ± 0.624	0.510 ± 0.162	1.650 ± 0.421
FO	3.647 ± 0.480	1.753 ± 0.493	0.795 ± 0.511	1.749 ± 0.396

*=significant difference compared to REF, §= significant difference compared to the respective control, Scheffe's test, $p<0.05$, $n=5$ for all means ± SD. See methods for protocol details.

Table VI.8. Ryanodine (10^{-9}M) Treatment

a) % Oxygen Extraction

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	44.44 ± 2.32	32.03 ± 4.50§	60.80 ± 10.60	43.58 ± 11.60
SAT	52.58 ± 5.35*	42.68 ± 3.99	65.78 ± 7.78	45.38 ± 5.80
FO	29.25 ± 2.86*	34.46 ± 4.66	43.78 ± 9.10	41.66 ± 3.18

b) Myocardial Oxygen Consumption (MVO_2) mL/min/g

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	3.362 ± 0.853	3.518 ± 0.795	4.953 ± 1.75	4.295 ± 0.704
SAT	5.876 ± 0.399*	5.792 ± 0.562	6.925 ± 0.261	8.021 ± 1.647
FO	1.876 ± 0.531*	1.804 ± 0.426	2.985 ± 1.481	4.362 ± 0.514

c) % Efficiency of Oxygen Utilisation

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	4.953 ± 1.203	2.900 ± 2.43	1.154 ± 0.99	2.260 ± 0.89
SAT	2.490 ± 0.283*	1.502 ± 0.98§	0.420 ± 0.83	1.065 ± 0.54
FO	10.027 ± 2.930*	3.84 ± 3.14§	1.110 ± 0.85	2.130 ± 0.66

Table VI.9. Ryanodine (10^{-9}M) Treatment

a) Venous pH

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	7.322 ± 0.034	7.302 ± 0.034	7.285 ± 0.021	7.299 ± 0.024
SAT	7.304 ± 0.032	7.249 ± 0.013	7.195 ± 0.015	7.205 ± 0.011
FO	7.321 ± 0.042	7.280 ± 0.051	7.225 ± 0.064	7.294 ± 0.058

b) Extracellular K^+ mM/L

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	3.275 ± 0.211	3.291 ± 0.207	4.622 ± 0.431	4.311 ± 0.298
SAT	3.459 ± 0.316	3.298 ± 0.195	6.012 ± 0.951	5.803 ± 0.876
FO	3.356 ± 0.241	3.317 ± 0.245	4.962 ± 0.215	4.498 ± 0.797

c) Extracellular Lactate μmol/min/g

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	82.732 ± 27.6	75.739 ± 30.5	68.592 ± 24.4	70.214 ± 28.9
SAT	177.341 ± 36.5*	143.724 ± 68.7	111.467 ± 47.3	163.620 ± 79.2
FO	24.621 ± 10.3*	21.775 ± 14.9	25.243 ± 20.9	30.972 ± 22.3

d) Creatine Phosphokinase Release U/min/g

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	4.923 ± 0.832	5.022 ± 0.762	6.521 ± 0.792	7.022 ± 0.855
SAT	10.299 ± 0.951*	8.362 ± 1.524	8.921 ± 4.595	8.567 ± 0.743
FO	1.746 ± 0.294*	4.895 ± 0.392§	6.753 ± 3.211	6.953 ± 1.214

*=significant difference compared to REF, §= significant difference compared to the respective control, Scheffe's test, $p<0.05$, $n=5$ for all means ± SD. See methods for protocol details.

Table VI.10. Increased Extracellular Calcium (6mM) Treatment

a) Coronary flow mL/min/g

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	47.27 ± 3.75	54.58 ± 2.53§	17.69 ± 9.01	47.16 ± 14.80
SAT	58.15 ± 2.74*	67.43 ± 2.07*§	31.52 ± 7.93	48.14 ± 4.32
FO	41.65 ± 4.92	51.22 ± 2.83§	25.39 ± 6.04	47.21 ± 4.02

b) Aortic Output mL/min/g

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	160.31 ± 15.92	209.60 ± 15.50§	44.16 ± 6.22	177.90 ± 49.20
SAT	128.68 ± 6.58*	167.05 ± 21.34§	56.56 ± 3.41	124.65 ± 18.90
FO	195.55 ± 12.82*	236.52 ± 10.87§	65.25 ± 9.02	200.36 ± 26.75

c) Cardiac Output mL/min/g

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	207.58 ± 17.73	264.18 ± 16.21§	61.81 ± 14.79	225.10 ± 57.00
SAT	186.83 ± 5.34	234.48 ± 23.10§	88.08 ± 8.19	175.24 ± 18.90
FO	237.21 ± 34.14	292.53 ± 25.99§	90.65 ± 5.71	247.57 ± 27.80

Table VI.11. Increased Extracellular Calcium (6mM) Treatment

a) Pressure-Time Integral

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	2183.8 ± 59.9	2298.6 ± 30.9§	1427.2 ± 226.2	1957.1 ± 292.7
SAT	2029.3 ± 53.8	2204.2 ± 38.2§	1137.6 ± 244.3	1783.2 ± 222.0
FO	2161.8 ± 58.8	2427.2 ± 15.9§	1306.2 ± 192.6	2300.0 ± 266.0

b) External Work J/min/g

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	3.122 ± 0.487	4.377 ± 0.238§	0.727 ± 0.258	2.730 ± 0.173
SAT	2.798 ± 0.110	3.709 ± 0.244§	0.668 ± 0.255	2.260 ± 0.288
FO	3.634 ± 0.159	4.816 ± 0.262§	0.991 ± 0.149	3.522 ± 0.245

*=significant difference compared to REF, §= significant difference compared to the respective control, Scheffe's test, $p<0.05$, $n=5$ for all means ± SD. See methods for protocol details.

Table VI.12. Increased Extracellular Calcium (6mM) Treatment

a) % Oxygen Extraction

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	36.13 ± 6.28	57.07 ± 2.62§	82.01 ± 2.75	80.53 ± 1.58
SAT	50.79 ± 2.09*	62.30 ± 3.38§	94.71 ± 1.59	88.95 ± 3.67
FO	22.51 ± 4.71*	42.93 ± 7.85*§	71.58 ± 6.37	64.4 ± 14.14

b) Myocardial Oxygen Consumption (MVO_2) mL/min/g

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	3.249 ± 0.449	5.937 ± 0.512§	3.844 ± 1.750	6.461 ± 1.921
SAT	5.681 ± 0.480*	8.024 ± 1.417*§	5.641 ± 1.458	9.401 ± 1.137
FO	1.808 ± 0.383*	4.553 ± 1.020§	3.463 ± 1.054	5.859 ± 1.673

c) % Efficiency of Oxygen Utilisation

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	4.893 ± 1.167	3.652 ± 0.556§	1.439 ± 0.424	2.233 ± 0.558
SAT	2.470 ± 0.261*	2.398 ± 0.800§	0.609 ± 0.212	1.377 ± 0.269
FO	10.360 ± 2.570*	5.479 ± 0.430§	1.522 ± 0.430	3.133 ± 0.716

Table VI.13. Increased Extracellular Calcium (6mM) Treatment

a) Venous pH

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	7.310 ± 0.054	7.276 ± 0.025§	6.975 ± 0.120	7.078 ± 0.061
SAT	7.324 ± 0.036	7.200 ± 0.059§	6.888 ± 0.092	7.067 ± 0.067
FO	7.323 ± 0.039	7.292 ± 0.066§	7.074 ± 0.104	7.107 ± 0.065

b) Extracellular K^+ mM/L

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	3.522 ± 0.314	4.192 ± 0.113§	8.769 ± 0.204	6.257 ± 0.472
SAT	3.414 ± 0.216	4.975 ± 0.204§	9.895 ± 0.217	8.734 ± 0.301
FO	3.291 ± 0.254	3.925 ± 0.211§	5.953 ± 0.542	4.562 ± 0.211

c) Extracellular Lactate μmol/min/g

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	62.75 ± 25.3	98.43 ± 16.9	195.7 ± 39.9	297.30 ± 28.9
SAT	143.13 ± 27.5*	180.95 ± 31.8*	269.8 ± 35.3	399.70 ± 31.7
FO	1.13 ± 0.13*	29.00 ± 5.7*§	35.9 ± 3.1	37.92 ± 2.9

d) Creatine Phosphokinase Release U/min/g

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	3.227 ± 0.673	4.954 ± 0.291§	3.792 ± 0.253	17.433 ± 0.895
SAT	9.975 ± 0.765*	12.637 ± 0.952*§	4.983 ± 0.299	21.631 ± 1.253
FO	0.632 ± 0.137*	2.051 ± 0.399*§	1.739 ± 0.175	3.052 ± 0.956

*=significant difference compared to REF, §= significant difference compared to the respective control, Scheffe's test, $p<0.05$, $n=5$ for all means ± SD. See methods for protocol details.

Table VI.14. Ruthenium Red (3.4μM) + 6mM Calcium Treatment

a) Coronary flow mL/min/g

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	46.92 ± 8.93	50.31 ± 5.93	17.25 ± 3.43	40.40 ± 11.4
SAT	55.59 ± 6.06	59.25 ± 5.85	17.77 ± 0.82	52.02 ± 9.08
FO	47.58 ± 7.25	63.21 ± 9.84	24.66 ± 3.09	55.80 ± 6.90

b) Aortic Output mL/min/g

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	157.4 ± 4.99	174.06 ± 8.51§	62.70 ± 10.85	120.40 ± 15.90
SAT	138.6 ± 12.49	179.13 ± 15.40§	64.94 ± 7.94	145.52 ± 22.12
FO	185.0 ± 16.29*	198.49 ± 30.52	61.87 ± 7.37	203.60 ± 23.03

c) Cardiac Output mL/min/g

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	204.3 ± 6.01	224.1 ± 12.20	79.11 ± 14.15	160.8 ± 26.2
SAT	194.2 ± 17.73	238.4 ± 19.30	82.71 ± 8.86	197.5 ± 28.24
FO	226.3 ± 15.80	261.7 ± 39.98	86.71 ± 8.86	259.4 ± 28.99

Table VI.15. Ruthenuium Red (3.4μM) + Calcium (6mM) Treatment

a) Pressure-Time Integral

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	2010.3 ± 283.5	2419.5 ± 305.1	1215.0 ± 187.1	1737.2 ± 234.0
SAT	2311.2 ± 387.2	2598.6 ± 788.0	1122.7 ± 233.2	2197.6 ± 444.3
FO	2065.3 ± 68.1	2245.6 ± 395.6	1688.4 ± 331.2	2165.4 ± 333.3

b) External Work J/min/g

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	2.572 ± 0.299	3.247 ± 0.402	0.790 ± 0.075	2.280 ± 0.096
SAT	2.725 ± 0.183	3.826 ± 0.255	0.703 ± 0.147	2.640 ± 0.375
FO	3.341 ± 0.642	4.385 ± 0.731	1.036 ± 0.128	2.710 ± 0.431

*=significant difference compared to REF, §= significant difference compared to the respective control, Scheffe's test, $p<0.05$, $n=5$ for all means ± SD. See methods for protocol details.

Table VI.16. Ruthenium Red (3.4μM) + Calcium (6mM) Treatment

a) % Oxygen Extraction

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	37.17 ± 7.85	50.79 ± 7.33§	82.11 ± 3.68	68.06 ± 12.56
SAT	48.69 ± 8.38	45.55 ± 6.81	80.00 ± 6.32	51.58 ± 13.16
FO	20.42 ± 2.62*	30.89 ± 4.19*§	55.26 ± 5.79	36.84 ± 5.69

b) Myocardial Oxygen Consumption (MVO_2) mL/min/g

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	3.369 ± 0.984	4.782 ± 0.447	2.585 ± 0.880	3.264 ± 1.770
SAT	5.182 ± 1.319	5.121 ± 0.733	2.686 ± 0.174	5.068 ± 1.489
FO	1.890 ± 0.673*	3.642 ± 0.214§	2.582 ± 0.203	2.513 ± 0.802

c) % Efficiency of Oxygen Utilisation

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	4.031 ± 1.15	3.424 ± 0.68	1.828 ± 0.84	2.380 ± 0.92
SAT	2.753 ± 0.67	3.803 ± 0.76§	1.305 ± 0.26	2.705 ± 0.58
FO	9.275 ± 2.14*	5.981 ± 0.86§	2.016 ± 0.36	5.537 ± 0.90

Table VI.17. Ruthenium Red (3.4μM) + Calcium (6mM) Treatment

a) Venous pH

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	7.302 ± 0.051	7.245 ± 0.028	7.075 ± 0.099	7.114 ± 0.036
SAT	7.261 ± 0.053	7.227 ± 0.056	7.022 ± 0.180	7.127 ± 0.003
FO	7.324 ± 0.044	7.242 ± 0.098	7.095 ± 0.064	7.137 ± 0.037

b) Extracellular K^+ mM/L

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	3.195 ± 0.213	3.603 ± 0.225	4.080 ± 0.344	3.695 ± 0.415
SAT	3.721 ± 0.257	3.995 ± 0.310	5.250 ± 0.932	5.055 ± 0.863
FO	3.021 ± 0.211	3.176 ± 0.242	3.930 ± 0.275	3.256 ± 0.213

c) Extracellular Lactate μmol/min/g

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	66.38 ± 14.7	90.05 ± 20.4	60.24 ± 12.7	110.20 ± 18.5
SAT	150.91 ± 27.4 *	125.31 ± 22.6	91.02 ± 24.5	187.30 ± 29.6
FO	18.63 ± 3.5*	30.12 ± 1.95§	50.58 ± 13.9	34.95 ± 3.21

d) Creatine Phosphokinase Release U/min/g

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	3.952 ± 0.65	4.251 ± 0.29	2.912 ± 0.28	10.01 ± 0.23
SAT	9.875 ± 0.25*	7.348 ± 0.28§	4.232 ± 1.11	11.56 ± 0.29
FO	1.138 ± 0.19*	1.356 ± 0.11	1.102 ± 0.202	6.987 ± 0.16

*=significant difference compared to REF, §= significant difference compared to the respective control, Scheffe's test, $p<0.05$, $n=5$ for all means ± SD. See methods for protocol details.

Table VI.18. Ryanodine (10^{-9}M) + 6mM Calcium Treatment

a) Coronary flow mL/min/g

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	45.82 ± 4.95	49.82 ± 6.34	18.95 ± 7.24	48.65 ± 6.75
SAT	63.53 ± 4.86*	62.41 ± 6.78	21.75 ± 0.92	59.75 ± 7.38
FO	50.65 ± 6.89	49.80 ± 8.34	25.80 ± 9.91	52.73 ± 7.54

b) Aortic Output mL/min/g

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	150.38 ± 7.93	140.20 ± 3.49	60.15 ± 9.85	120.4 ± 10.8
SAT	129.33 ± 16.20	143.60 ± 21.30	51.35 ± 10.24	115.4 ± 10.2
FO	201.66 ± 12.60*	205.60 ± 10.63*	60.35 ± 10.75	200.2 ± 16.9

c) Cardiac Output mL/min/g

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	196.2 ± 12.4	190.0 ± 10.4	79.1 ± 14.7	169.1 ± 14.8
SAT	192.8 ± 18.9	206.0 ± 23.4	73.1 ± 13.6	175.2 ± 14.8
FO	252.3 ± 19.0*	255.4 ± 15.8*	86.2 ± 12.85	252.8 ± 15.7

Table VI.19. Ryanodine (10^{-9}M) + Calcium (6mM) Treatment

a) Pressure-Time Integral

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	2084.8 ± 263.2	2003.7 ± 263.2	1231.0 ± 288.4	1760.6 ± 250.3
SAT	2098.8 ± 160.3	2391.2 ± 432.1	1100.6 ± 316.4	2117.5 ± 329.6
FO	2156.0 ± 60.5	2200.0 ± 53.7	1695.0 ± 140.8	2310.0 ± 43.7

b) External Work J/min/g

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	2.952 ± 0.632	2.576 ± 0.283	0.692 ± 0.352	2.240 ± 0.210
SAT	2.994 ± 0.175	3.202 ± 0.214	0.710 ± 0.138	2.657 ± 0.421
FO	3.605 ± 0.280	3.683 ± 0.299	1.215 ± 0.159	3.245 ± 0.193

*=significant difference compared to REF, §= significant difference compared to the respective control, Scheffe's test, $p<0.05$, $n=5$ for all means ± SD. See methods for protocol details.

Table VI.20. Ryanodine (10^{-9}M) + Calcium (6mM) Treatment

a) % Oxygen Extraction

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	37.41 ± 7.83	50.74 ± 7.75	82.06 ± 3.87	68.15 ± 12.55
SAT	48.43 ± 8.18	45.51 ± 6.67	79.96 ± 6.39	51.48 ± 12.80
FO	20.68 ± 2.57*	30.71 ± 4.00*§	55.41 ± 5.73	37.09 ± 5.56

b) Myocardial Oxygen Consumption (MVO_2) mL/min/g

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	3.323 ± 0.982	3.862 ± 0.765	4.980 ± 0.523	6.015 ± 0.782
SAT	6.012 ± 0.631	6.933 ± 0.838	7.014 ± 0.765	8.980 ± 0.850
FO	2.005 ± 0.404*	2.097 ± 0.522*	3.118 ± 0.494	4.986 ± 0.744

c) % Efficiency of Oxygen Utilisation

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	4.08 ± 1.29	3.70 ± 0.08	0.91 ± 0.64	2.33 ± 0.10
SAT	2.61 ± 0.27	1.94 ± 0.17	0.44 ± 0.18	1.57 ± 0.25
FO	9.29 ± 2.18	8.65 ± 3.49	1.61 ± 0.35	4.97 ± 3.19

Table VI.21. Ryanodine (10^{-9}M) + Calcium (6mM) Treatment

a) Venous pH

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	7.318 ± 0.029	7.306 ± 0.018	7.221 ± 0.013	7.285 ± 0.017
SAT	7.289 ± 0.047	7.218 ± 0.063	7.103 ± 0.042	7.149 ± 0.071
FO	7.330 ± 0.038	7.318 ± 0.015	7.205 ± 0.019	7.220 ± 0.011

b) Extracellular K^+ mM/L

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	3.295 ± 0.224	3.767 ± 0.298§	7.327 ± 0.411	5.192 ± 0.210
SAT	3.292 ± 0.210	3.958 ± 0.302§	7.835 ± 0.422	7.091 ± 0.306
FO	3.172 ± 0.213	3.336 ± 0.318	5.122 ± 0.387	4.216 ± 0.231

c) Extracellular Lactate μmol/min/g

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	93.25 ± 29.5	105.63 ± 28.3	165.22 ± 37.7	229.42 ± 26.2
SAT	164.33 ± 42.8	181.95 ± 48.3	228.42 ± 52.4	269.38 ± 28.6
FO	25.72 ± 7.3*	30.43 ± 5.4*	36.79 ± 4.14	39.95 ± 3.3

d) Creatine Phosphokinase Release U/min/g

DIET	CONTROL (C)	C+TREATMENT	ISCHAEMIA	REPERFUSION
REF	5.008 ± 0.652	5.136 ± 0.692	6.313 ± 0.311	7.951 ± 0.348
SAT	10.913 ± 0.891*	11.725 ± 0.729*	9.986 ± 0.672	13.663 ± 0.582
FO	1.670 ± 0.214*	3.056 ± 0.264*§	2.572 ± 0.295	4.992 ± 0.273

*=significant difference compared to REF, §= significant difference compared to the respective control, Scheffe's test, $p<0.05$, $n=5$ for all means ± SD. See methods for protocol details.

Table VI. 22.

The Incidence of Arrhythmias after Ca^{++} 6mM, RY and RR Treatments

a) The Number of VPBs During Ischaemia (n=5, mean±SD)

DIET	(i)	(ii)	(iii)	(iv)	(v)	(vi)
REF	7.0 ± 0.78æ	49.8 ± 10.56	5.0 ± 0.24	4.0 ± 0.78	12.0 ± 4.82æ¶	19.2 ± 1.64æ¶
SAT	10.8 ± 0.85*	66.3 ± 11.28*	6.0 ± 0.48	7.0 ± 1.55	18.6 ± 4.75	30.8 ± 2.02*
FO	3.2 ± 0.50*§	24.6 ± 8.45*§	5.0 ± 0.62	5.0 ± 1.28	12.8 ± 4.50	11.4 ± 1.42*§

b) The % Incidence of Reperfusion Arrhythmias (VT) (n=5)

DIET	(i)	(ii)	(iii)	(iv)	(v)	(vi)
REF	60 (n=10)	100æ	0	20	0¶	40
SAT	66 (n=9)	100	0	20	0	60
FO	12 *§ (n=9)	40	0	0	0	20

c) The % Incidence of Reperfusion Arrhythmias (VF) (n=5)

DIET	(i) (n=10)	(ii)	(iii)	(iv)	(v)	(vi)
REF	60	80	0	0	0	40
SAT	80*	100	0	0	0	60
FO	0*§	20	0	0	0	0

(i) Control, taken from Chapter IV, Expt.A
(ii) Ca^{++} 6mM,
(iii) RY 10^{-9}M,
(iv) RR 3.4μM,
(v) RY+Ca^{++} 6mM,
(vi)RR+Ca^{++} 6mM.

*=Sig. different from REF $p<0.05$; §=Sig. different from SAT $p<0.05$;
æ=Sig. treatment effect compared with control 1.4mM Ca^{++} (i);
¶=Sig treatment effect compared with 6mM Ca^{++} alone (ii).
See methods for details.

VII. EVALUATION OF FUNCTIONAL PERFORMANCE EFFICIENCY BY MYOCARDIAL LOAD TESTING: CORONARY PERFUSION PRESSURE & PRELOAD ALTERATIONS WITH MAINTAINED AFTERLOAD

VII. 1. Introduction

The maintenance of cardiac output is dependent on four major determinants of cardiac function: heart rate, preload, afterload and myocardial contractility. In the preceding chapter, and as reported by other researchers, it was evident that free calcium, particularly intracellular [Ca^{++}] can determine the velocity and force of cardiac contraction. It is well established that force and velocity are inversely related such that with no load, force is negligible and velocity maximal. Conversely, with isometric contraction no external myofibril shortening occurs resulting in maximal force and negligible velocity. Contractility, the condition of cardiac muscle determined by a change in developed force at a given resting fibre length is dependent on the level of preload and afterload (**Sarnoff,1955;Karliner,*et al.*,1971**).

The afterload (AL) is the tension to be developed by the cardiac muscle during the systolic ejection period and consists of forces that resist myocardial fibre shortening such that when AL is increased fibre shortening is decreased by inverse proportions (**Sarnoff,1955;Sonneblick,*et al.*,1969**). In the isolated working heart the height of the perfusate column that ventricular output is pumped over and the hydrostatic properties of the column weight determine the AL. This AL pressure will usually also act as the coronary perfusion

pressure (CPP). However, as discussed in Chapter II, CPP can be set independently from the AL pressure to simulate coronary artery disease in the unique isolated heart perfusion model developed for this thesis.

The preload (PL) on a heart is the load exacted on cardiac muscle fibrils and the resultant sarcomere length before contraction (**Abbot & Mommaerts,*et al.*,1954;Gaasch & Zile,1984**). Frank (**1895**) and Starling (**1914**) demonstrated that with increasing left ventricular filling pressure cardiac output increases until further excessive increases in filling pressure produce a decline in cardiac output. Sonnenblick (**1974**) later observed that the maximal cardiac output corresponded to the optimal stretch of the sarcomeres and further stretch above this results in the gradual detriment of actin and myosin filament contraction. The left ventricular filling pressure can serve as a measure of PL but at higher filling pressures (above optimal sarcomere length) the volume-pressure relationship is increasingly non-linear and thus inaccurately predicts PL (**Levine & Gaasch,1978**). It has also been reported that increased PL can result in increased myocardial oxygen demand and MVO_2 because PL-related increased wall stress may be sustained by greater force development (**Neely,*et al.*,1967;Weber & Janicki,1977; Krukenkramp,*et al.*,1987;Rooke & Feigl,1982;Scholz,*et al.*,1990**).

Clinical observations of a number of syndromes indicate that even in the absence of abnormal myocardial function, failure to support the cardiac output requirements of corporal metabolism can occur when the heart is suddenly burdened with ventricular overload or impeded filling (**Braunwald, 1980**). Following the emergence of such defects, compensatory

mechanisms attempt the maintenance of cardiac output by increased PL, increased release of catecholamines for raised contractility and/or myocardial hypertrophy but these mechanisms are limited and ultimately fail (**Braunwald, 1974**). Thus it is only after provocation that the properties underlying the myocardial condition may become evident. In clinical studies diagnostic assessment is sometimes only possible following stimulation of specific mechanisms in organ function, for instance; exercise ECG or angiography, programmed electrical stimulation of arrhythmias, velocity assessment in nerve conduction and kidney or liver function tests. This approach is also of great value in experimental studies (**Doring,1989**). Indeed, it was evident from the previous chapters that many of the dietary lipid induced differences were precipitated only following ischaemia or programmed electrical stimulation.

In this thesis the spontaneous heart rate was observed to alter with ischaemia, reperfusion or dietary lipid modulation. Heart rate, AL and PL are all important determinants of ventricular performance and were hence held constant to evaluate the influence of dietary fats on cardiac contractility and other measures of ventricular performance and metabolism. The aim of this chapter was to further evaluate the influence of dietary lipids under a range of stresses. Firstly, the influence of increasing PL was investigated in the isolated working heart model developed in Chapter II. This stimulus was used to observe whether dietary lipid-related differences in ventricular performance and metabolism are maintained or possibly augmented under these stress conditions. Secondly, the effect of low flow global ischaemia was investigated

under high PL conditions. Thirdly, the mechanical and metabolic function and the influence of dietary fats was evaluated under varying lesser degrees of ischaemia other than the 35mmHg CPP used extensively through this study.

VII. 2. Methods

VII. 2. a) Animals & Diets

A total of 60 male Hooded-Wistar rats were allocated to the dietary groups REF, SAT and FO when they were 4 months old. The experimental diets were prepared, stored and presented to the animals as described in Chapter III for a further 4 months. Animals were fasted overnight prior to each experimental day (body mass = 400-500g).

VII. 2. b) Isolated Working Heart Preparation & Perfusion Protocol

Isolated working hearts perfused with 40% haematocrit erythrocyte buffer were prepared for perfusion in control and maintained afterload global ischaemia modes as described in Chapter II. During initial Langendorff perfusion (< 10 min) coronary blood was flushed out, the pulmonary artery was cannulated, pacing and ECG electrodes were positioned. Following 5 min control erythrocyte perfusion, equilibrated and stabilised atrially paced (300 bpm) working hearts (CPP, preload and afterload set at 75, 10 & 75 mmHg respectively), were monitored for functional performance and arterio-venous PO_2 and pH differences as in Chapter II. Coronary effluent samples, taken every 5 min were stored as supernatants at -60°C for later biochemical analyses.

VII. 2. c) Experimental Design

The animals were allocated into 2 experimental groups, A with 15 and B with 45 subjects. Experiment A involved REF, SAT and FO hearts (n=5). Subsequent to control perfusion at 10mmHg preload each heart was perfused with a constant afterload and CPP, but preload was altered by adjusting the left atrial buffer reservoir to produce a left ventricular filling pressure (LV_{fp}) of 5, 10, 15 or 20mmHg. The preload was first lowered to 5mmHg, then raised by increments of 5 mmHg. At each point, hearts were allowed to equilibrate for 5 min prior to arterio-venous sampling and measurement of cardiac performance. After assessment at the LV_{fp} of 20 mmHg, ischaemia was induced by lowering CPP to 35 mmHg. After 15 min ischaemia hearts were reperfused with CPP returned to 75 mmHg. The LV_{fp} was maintained at 20mmHg throughout the ischaemia and reperfusion periods.

Throughout experiment B preload in REF, SAT and FO hearts (n=15) was maintained at 10mmHg and afterload at 75mmHg but the degree of ischaemia induced for 15min was varied when CPP was decreased from 75 mmHg to either 65, 55 or 45mmHg in each dietary group (n=5 per CPP).

VII. 2. d) Analysis of Coronary Effluent Contents

Arterial and venous samples were assayed for creatine kinase, lactic acid and K^+ concentrations as described in Chapter III. Creatine kinase was quantified according to the rate of change of absorbance. Results were expressed in U/min/g dry weight adjusted according to coronary flow rates. Lactic acid concentrations were expressed in μmol/min/g dry weight when adjusted

according to coronary flow rates. For determination of potassium ion concentration all samples were analysed as described in Chapter V and results were expressed in mmol/L.

VII. 2. e) Data Handling and Statistical Analysis

Myocardial left ventricular external work, the pressure-time integral, perfusate oxygen content, oxygen extraction, myocardial oxygen consumption and percentage energy utilisation efficiency were calculated as described in Chapter II. All results were expressed as mean $\pm$ SD. For each parameter, the effect of dietary treatment was tested by Analysis of Variance and between individual comparisons with Scheffe's *post hoc F*-test. The level of significance was considered at $P < 0.05$ or less.

VII. 3. Results

VII. 3. a) External Work

The relationship between left ventricular filling pressure (LV_{fp}) and myocardial left ventricular external work is presented in Figure VII.1. as a curvi-linear association. Varying the preload over the range 5-15mmHg resulted in increased external work, however, with a further increase to 20mmHg LV_{fp} work levels declined in all dietary groups. At low preload, external work was also low and no dietary differences were evident. Between 10 and 20 mmHg the external work curve was shifted upwards in FO hearts and downwards in SAT hearts in comparison to the REF external work curve.

VII. 3. b) Contractility

The pressure-time integral relationship increased with the rise in LV_{fp} from 5mmHg but tended to fall slightly at 20mmHg LV_{fp} in all three dietary groups (Figure VII.2). At 5mmHg LV_{fp} no significant difference was observed between REF and FO hearts, in contrast, SAT hearts had slightly lower contractility. The three curves were distinctly separated when preload was increased further. Lower contractility was observed in SAT hearts and higher contractility in FO hearts compared to REF, particularly at 15 and 20mmHg LV_{fp}.

VII. 3. c) Myocardial Oxygen Consumption

In all dietary groups MVO_2 increased progressively with the elevation of preload from 5 to 20mmHg LV_{fp}. The relationship between MVO_2 was linear between 5 and 20 mmHg LV_{fp} and parallel between the dietary groups. A significantly lower MVO_2 was observed in FO hearts compared to REF hearts across the range of LV_{fp}. In contrast, the MVO_2 in SAT hearts was significantly greater than both REF and FO at all preloads investigated. At 5mmHg LV_{fp} SAT MVO_2 was approximately 7 fold greater than FO and by 20mmHg LV_{fp} this was 2.5 fold greater than FO hearts. The absolute difference of approximately 4.5 ml/min/g between the dietary groups was maintained at all preloads.

VII. 3. d) Energy Utilisation Efficiency

The relationship between the % mechanical efficiency and preload is shown in Figure VII.4. In all groups the % efficiency rose with the increase in LV_{fp} from 5 to 15 mmHg but was reduced at 20mmHg. The % efficiency of SAT hearts was lower than REF and FO hearts for the entire preload pressure range. In comparison, the % efficiency was markedly increased in FO hearts, yet despite this it was sharply decreased at 20mmHg LV_{fp}.

VII. 3. e) Cardiac Output

It can be observed in Figure VII.5. that as LV_{fp} was increased over the range 5 to 15mmHg, the cardiac output increased. At 5 and 10mmHg LV_{fp} no significant dietary differences were observed. When LV_{fp} was increased to 15mmHg cardiac output was significantly greater in FO than in REF and SAT hearts. With a further preload increase to 20mmHg LV_{fp}, cardiac output increased further in FO hearts but declined in REF and SAT hearts such that FO hearts had significantly greater cardiac output than REF and SAT hearts.

VII. 3. f) Lactic Acid Production & Venous pH

It was apparent that between 5 and 20mmHg, the increase in preload pressure influenced an increase in coronary effluent lactate content. At 10, 15 and 20 mmHg LV_{fp} SAT hearts produced significantly more lactate than REF hearts while in contrast FO hearts produced significantly less lactate for the respective LV_{fp}.

A similar trend was observed in the venous pH presented in Table VII.1. As LV_{fp} increased from 5 to 20 mmHg venous pH decreased in all

groups but to a greater extent in SAT hearts. However, in REF and FO hearts, venous pH rose significantly at 20mmHg compared to 10mmHg LV_{fp}. Significant dietary differences in venous pH were observed at each preload pressure such that the highest venous pH values (i.e. those closes to arterial levels) occurred in FO hearts and the lowest values were in SAT hearts.

VII. 3. g) The Effect of Ischaemia with High Preload Stress

When isolated working heart perfusion was maintained with a high preload of 20mmHg LV_{fp}, as presented above, most of the cardiac performance parameters (except MVO_2) were either slightly raised or close to those observed at 10mmHg LV_{fp} because they were on the descending limb of the cardiac function curves at this preload. After 15 min low flow global ischaemia (CPP=35mmHg) with high preload (Table VII.2.b), coronary flow was not significantly different from that obtained after 15 min ischaemia with 10mmHg LV_{fp} for each respective dietary group (See Figures III.5.-III.15.).

However, cardiac output and external work were greater during ischaemia with 20mmHg LV_{fp} compared to 10mmHg preload. In FO hearts during ischaemia, MVO_2 was higher compared to 10mmHg LV_{fp} ischaemia. Other parameters were not markedly different between 10 and 20mmHg LV_{fp} during ischaemia despite distinct dietary group differences. In exception, with 20mmHg LV_{fp}, creatine kinase and particularly lactate production were greater in all dietary groups compared to 10mmHg. The largest increases were observed in REF and SAT hearts.

At 5 min reperfusion with 20mmHg LV_{fp}, a close return to preischaemic cardiac output was observed: 92%, 81%, 95% in REF SAT and FO hearts respectively (Table VII.2.c). Contractility was significantly higher in FO and significantly lower in SAT hearts compared to REF hearts. However, external work was reduced in REF and SAT by 17% and 11% respectively compared to preischaemic performance.

In particular in reperfusion, MVO_2 was elevated above preischaemic levels in all dietary groups and was greatest in SAT hearts and the least in FO hearts. This observation was reflected in the significantly lower % mechanical energy utilisation efficiency in SAT and significantly higher % efficiency in FO compared to REF hearts. In addition, although SAT hearts had significantly higher reperfusion levels of extracellular $[K^+]$, lactate production, and creatine kinase release and FO hearts significantly lower levels compared to REF hearts, only FO hearts did not have significantly increased coronary effluent amounts of K^+, lactate and creatine kinase compared to preischaemia at 20mmHg LV_{fp}.

VII.3.h) The Effect of Coronary Perfusion Pressure Reductions

When coronary perfusion pressure (CPP) was reduced from 75mmHg to either 65, 55, or 45mmHg with preload maintained at 10mmHg and afterload at 75mmHg (Tables VII.4.-9.), coronary flow, cardiac output, PTI, external work, MVO_2, and % efficiency were reduced in a curvi-linear manner (Figures VII.7.-11.). Coronary flow was reduced linearly from 75mmHg to 55mmHg in all dietary groups but at 45mmHg the coronary flow rates fell to levels similar to 35mmHg causing the curve to flatten out (Figure VII.7.).

Conversely, the % oxygen extraction rose with each reduction in CPP in all dietary groups (Figure VII.12.). There was a parallel relationship between the dietary groups with the reduction of coronary flow such that SAT hearts had the highest % extraction while FO had the lowest. Myocardial oxygen consumption (Figure VII.10.) decreased with the decline in CPP by virtue of the reduced coronary flow rate (Figure VII.7).

The highest values of cardiac output, PTI, external work and % mechanical efficiency were observed in the FO dietary group while the lowest values were observed in SAT hearts. However, SAT hearts exhibited not only the highest levels of MVO_2 and % oxygen extraction, but also the highest levels of lactate production, extracellular [K^+] and creatine kinase release compared to FO hearts for each CPP (Tables VII.7.-9.).

The measures of cardiac function at 5 min following reperfusion of hearts from reduced CPP are presented in Tables VII.4.c.-6.c. The closest returns to pre-ischaemic cardiac performance were observed following a CPP of 65mmHg and to a lesser extent 55mmHg, where cardiac performance recovered to within 5-20% of pre-ischaemic levels. However, MVO_2, % oxygen extraction, lactate production and creatine kinase release were raised, particularly in SAT and to a lesser extent REF hearts upon reperfusion.

Cardiac performance was most affected following reperfusion after a CPP of 45mmHg for 15 min. Cardiac output was reduced by 26%, 30%, and 20% in REF, SAT and FO hearts respectively at 5 min reperfusion. Similarly, the lowest values for the pressure-time integral, external work, venous pH and % mechanical efficiency were observed in SAT hearts compared to FO hearts.

In contrast, the highest % oxygen extraction, MVO_2, lactate production, $[K^+]$ and creatine kinase release was observed in SAT hearts with reperfusion.

VII. 4. Discussion

In these experiments, preload was controlled by altering the height of the atrial perfusate reservoir of the perfusion apparatus to modify left atrium filling pressure and thus left ventricular filling pressure (LV_{fp}), in order to generate cardiac function curves in the isolated erythrocyte perfused working heart. Dietary lipid-related differences were observed across the range of filling pressures in most measures. As the LV_{fp} was increased, external work, cardiac output, contractility, % mechanical energy utilisation efficiency and MVO_2 also increased. At high LV_{fp} (20mmHg) these parameters declined except for MVO_2 and lactate production. These findings closely follow the Frank-Starling relationship between ventricular performance and preload **(Frank,1895;Starling,1914;Patterson,*et al.*,1914)** according to which ventricular function increases with increased stretch (diastolic volume) but beyond a certain point (15-20mmHg) the relationship changes and function declines. These findings are supported by previous observations **(Neely,*et al.*,1967;Sonnenblick,1974;Weber & Janicki,1977;Levine & Gaasch,1978;-Suga,1977;1979;Rooke &Feigl,1982;Krukenkramp,*et al.*,1987;Scholz,*et al.*,1990)**.

The effects of dietary polyunsaturated fish oil and saturated sheep fat on the cardiac function curves were observed for the first time in this study. The cardiac function curves illustrated that the external work, contractility,

cardiac output and % efficiency performance were significantly reduced in SAT hearts compared to REF and FO across a range of preloads. The significantly higher MVO_2, lactate production and lower venous pH in SAT hearts compared to REF and FO hearts was augmented further with higher LV_{fp}. When ischaemia was induced with high preload (LV_{fp}=20mmHg) the cardiac output was better able to be maintained because of the Frank-Starling effect of increased preload but coronary flow was still at very low levels. The metabolic consequences of the greater work production in the face of limited oxygen supply were more severe with increased lactate, $[K^+]$, and creatine phosphokinase release. The detrimental consequences of ischaemia were greatest in SAT hearts. This was most striking in the measures of lactate production and creatine kinase release which increased not only in comparison to REF and FO but also to SAT hearts made ischaemic at lower LV_{fp} (10mmHg). Together this indicated that SAT hearts were more prone to reduced performance efficiency with increased preload. Thus, as the heart is progressively stimulated to do more work its requirements for oxygen also increase putting it under even greater stress if ischaemia is imposed against this background.

In contrast, FO hearts maintained their elevated performance despite high preload. This was manifest as greater external work, contractility, cardiac output and % mechanical efficiency but lower lactate production and MVO_2 compared to REF and SAT hearts. Indeed, even at the highest preload the FO function curves were less inclined to decline (or "flatten") than the REF and SAT heart function curves. During ischaemia and reperfusion with

high preload, FO hearts had significantly greater cardiac output, work, contractility and mechanical efficiency with comparatively lower MVO_2, lactate production, acidosis, creatine kinase release and coronary effluent $[K^+]$ content compared to REF and SAT hearts.

In the experiments where different degrees of reduced CPP were imposed but with maintained afterload (75mmHg) and preload (10mmHg), the left ventricular function declined proportionally to the reduction in CPP. The relationship between the coronary flow, pressure-time integral and MVO_2 over the range of 75-35 mmHg CPP indicated that cardiac performance in these parameters occurred as a "reverse effect" of Gregg's Phenomenon (**Gregg,-1963**). While the reduction in MVO_2 with CPP reduction may have been primarily related to the reduced coronary flow and oxygen delivery, it may have also been related to reduced oxygen demand due to the proportionate decline in external work, contractility and cardiac output (**Abel & Reis,1970**). These observations have previously been reported (**Suga,*et al.*,1988;Goto,*et al.*,1991**).

The reductions in these functional parameters may be consequentially related (via Frank-Starling mechanisms) to reduced distensibility of the myocardium, particularly around the coronary blood vessels, thus altering cardiac compliance by changing the relationship between LV_{fp} and myocardial fibre length (**Belloni & Sparks,1977;Gaasch & Bernard,1977**).

Suga, *et al.*(**1988**), reported that CPP reduction resulted in reduced MVO_2 for basal and excitation-contraction metabolism. It has also been proposed that reduced MVO_2, contractility and external work following CPP

reduction may be related to reduced Ca^{++} supply to myofibrils (**Allen & Orchard,1983**), intracellular acidosis (**Fabiato & Fabiato,1978**), intracellular accumulation of P_i (**Allen,*et al.*,1985**), reduced availability of ATP and creatine phosphate (**Gibbs,1985**), and decreased myofibrillar sensitivity to calcium (**Allen & Orchard,1983**). The significantly higher MVO_2 in SAT hearts across the range of coronary perfusion pressures compared with REF and FO may be related to intrinsically higher intracellular [Ca^{++}] levels (Chapter VI) despite reduced Ca^{++} delivery to the myofibrils. However, the highest level of performance was seen in FO hearts where cardiac output, contractility and external work was greater than in SAT hearts for each CPP reduction. The dietary differences were reduced with each step down until little difference was observed between FO and SAT at 45mmHg, but only at 35mmHg CPP were there no significant dietary differences in cardiac output and external work.

Dietary differences in contractility tended to increase with the decline in CPP. This was related to the greater rate of reduction in contractility of SAT hearts than in FO hearts as the CPP declined. Notably, contractility in REF hearts tended to resemble that of FO (although reduced compared to FO) at a higher CPP but with further CPP reduction and thus increased severity of ischaemia, the contractility tended to approach that of SAT hearts. This indicates that a more curvi-linear relationship between CPP and $\delta P/\delta t$ was present in REF hearts than SAT and FO hearts.

The % efficiency was greater in FO hearts because of their lower MVO_2 to higher external work ratio compared with SAT and REF hearts.

Despite reductions in MVO_2 during the reduction of CPP the % oxygen extraction was increased in all hearts, with the greatest % extraction being in SAT hearts and the lowest in FO hearts. Elevations in lactate production and creatine kinase release were observed particularly at 45 and 35mmHg CPP indicating the presence of greater ischaemic stress.

The CPP of 35mmHg was utilised in Chapter II because from preliminary experiments it was deemed to be the lowest CPP that permitted low flow global ischaemia while allowing sufficient coronary flow to occur so that coronary flow rates, venous PO_2, venous pH and concentrations of coronary effluent constituents to be determined. Coronary perfusion at pressures below this led to cardiac failure resembling that of total "stop flow" global ischaemia (**Neely,*et al.*,1973;Bolli,1990**) and prevented any assessment during ischaemia. The findings of this chapter taken with those of Chapter III suggest that FO hearts may be able sustain function on a lower CPP or for a longer period than those hearts from SAT animals. Moreover, it is apparent that SAT hearts may be more vulnerable to even a small degree of ischaemia compared with FO. It may be expected that the response equivalent to that seen with angina of effort would be earlier in onset with the SAT diet.

When hearts were perfused at a CPP of 65mmHg, performance was reduced only slightly, particularly on reperfusion. At 55mmHg CPP the slight detriment was augmented in most functional parameters with dietary differences also increasing, but only at 45mmHg were distinct reductions in post ischaemic recovery observed. At this level of ischaemia dietary

differences are evident with FO hearts having the greatest recovery of pre-ischaemic cardiac output, contractility and external work compared with SAT.

Although the paradoxically high post-ischaemic level of MVO_2 **(Krukenkamp,*et al.*,1985;1986;Stahl,*et al.*,1988;Dean,*et al.*,1990;Bavaria, *et al.*,1990)**, becomes most evident after reperfusion from 45mmHg CPP, (next to 35mmHg CPP, Chapter III), this could be observed to some extent at 65 and 55mmHg, particularly in SAT hearts that had the highest post-ischaemic MVO_2 rates compared to REF and FO hearts. In addition, creatine kinase release and lactate production increased further after reperfusion from all CPP levels, the greatest of which occurred in SAT hearts after 45mmHg CPP (next to 35mmHg). Thus from these experiments it is clear that while 45mmHg would have provided similar details of dietary related differences in cardiac performance and metabolism, the extent of ischaemic injury or myocardial stunning would not have been as great as with a CPP of 35mmHg.

Dietary lipid modulation was clearly capable of influencing ventricular performance over a range of conditions and with increased preload and ischaemic stress these underlying dietary lipid induced differences were augmented. To date this has not been clearly demonstrated by others. DeDeckere & Ten Hoor **(1979;1980)**, reported higher cardiac external work curves for hearts from sunflower seed oil fed rats compared to the external work produced by hearts from lard fed animals in response to increasing LV_{fp}. These results were observed particularly when animals were fed for periods longer than 1 or 4 weeks. However, they failed to report extensive dietary differences in a number of other performance parameters in these experiments.

The conditions used in those experiments varied in a number of ways from the present study. They used weanling rats fed for short periods only, they used a 70mmHg afterload, they did not use erythrocyte buffer, and the heart rate was permitted to vary spontaneously providing a confounding effect. The effects of ischaemia were not evaluated at all.

It is clear from the experiments conducted for this thesis to date that SAT hearts have altered intrinsic properties distinct from REF and FO that are predominantly evident when subjected to a variety of stresses. The limited capacity of SAT to respond to elevated preload or ischaemic stress is in contrast to FO hearts that deal with this comparatively more efficiently and with maintained performance. The abnormally high MVO_2 of SAT hearts compared to REF and FO during control perfusion was still evident, even when MVO_2 rose in all dietary groups in response to increased preload. Indeed, the influence of diet on MVO_2 was much greater than the influence of altered preload. This was exacerbated by the high stressor of increased preload and ischaemia-reperfusion.

This study approach has indicated that provocation with detrimental stimuli can provide valuable information not always clearly apparent under steady-state control conditions. Indeed, this is similar to the *in vivo* occurrences of high load or pathological stress on a heart with apparently normal cardiac performance. It has been observed clinically that preload pressure indices increased in patients with either mitral insufficiency, decompensated aortic insufficiency or dilated cardiomyopathy and markedly decreased with aortic stenosis as a compensatatory measure (**Razzolini,*et al.*,1988**). In hearts

with underlying dysfunction (but unprecipitated), upon a sudden increase in preload, afterload or increased myocardial oxygen and nutrient demand the apparently "normal" cardiac performance (possible because of compensatory mechanisms already in operation) does not alter rapidly or sufficiently enough to meet the new metabolic needs, causing compensatory mechanisms to be insufficient, eventually leading to cardiac failure. The pathological restriction of coronary blood flow in angina may be sufficient to maintain the metabolic needs of the myocardium while the patient remains at rest or conducts low levels of exercise. However, the coronary flow reserve may be limited at very high levels of exercise and the myocardial oxygen and nutrient demand increases above supply thus causing myocardial ischaemia. Treadmill exercise is utilised clinically to gradually raise physical exercise to high levels in patients to a point where the ECG can be monitored for ischaemia induced alterations or arrhythmias, particularly in the case of patients with suspected previous myocardial infarction but with no currently evident signs (**Chirife & Spodick,1972;Haissly,*et al.*,1974;Kerber,*et al.*,1975;Spodick & Lance,-1976;Shepherd,1987;Hasegawa,1989;1990**). It is by similar means that such provocation has permitted us to observe major dietary differences in response to altered preload, ischaemic severity and reperfusion.

Despite marked increments in MVO_2 with increasing preload, the dietary differentials remained almost constant highlighting the conclusion from the previous chapter that the abnormally high MVO_2 in SAT hearts was not directly related to contraction.

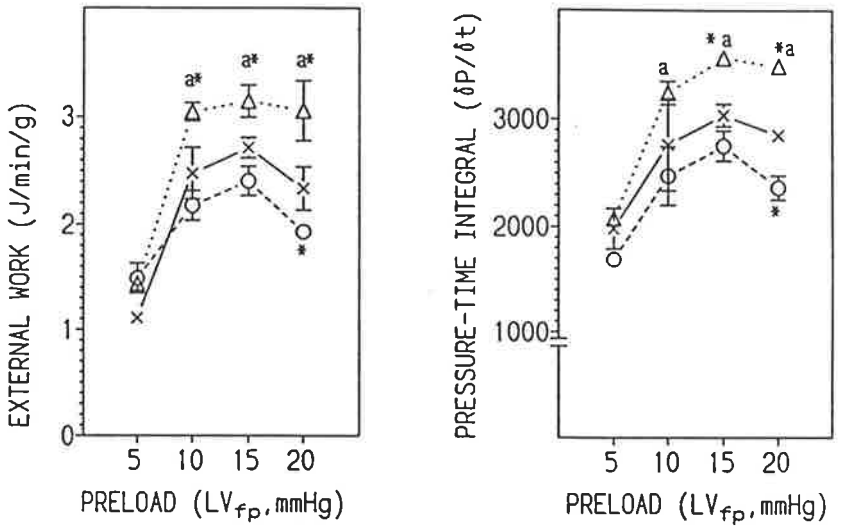

Figure VII.1. The relationship between left ventricular filling pressure and external work. Values are expressed as mean ±SD (g dry weight), n=5, *=p<0.05, significantly different compared to REF; a=p<0.05, significantly different to SAT, Scheffe's comparison. Cross=REF, circle=SAT, triangle=FO. See Methods for details.

Figure VII.2. The relationship between left ventricular filling pressure and the pressure-time integral. Values are expressed as mean ±SD (g dry weight), n=5, *=p<0.05, significantly different compared to REF; a=p<0.05, significantly different to SAT, Scheffe's comparison. Cross=REF, circle=SAT, triangle=FO. See Methods for details.

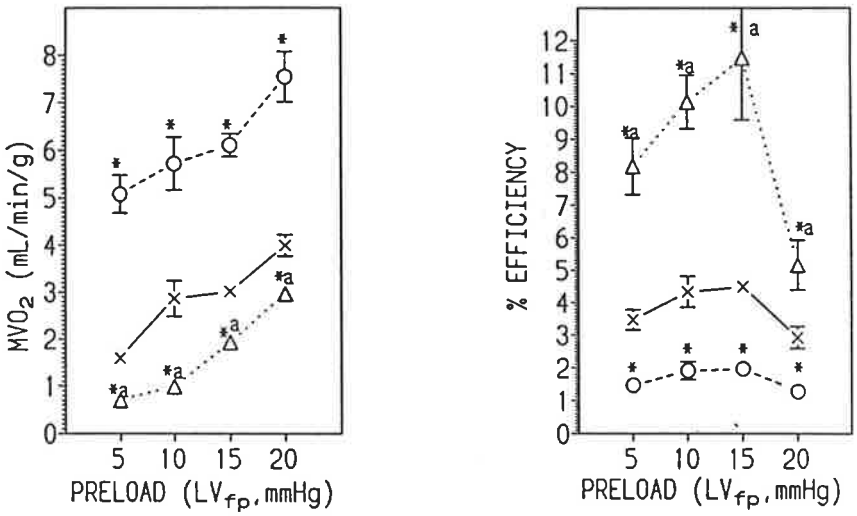

Figure VII.3. The relationship between left ventricular filling pressure and myocardial oxygen consumption. Values are expressed as mean ±SD (g dry weight), n=5, *=p<0.05, significantly different compared to REF; a=p<0.05, significantly different to SAT, Scheffe's comparison. Cross=REF, circle=SAT, triangle=FO. See Methods for details.

Figure VII.4. The relationship between left ventricular filling pressure and % energy utilisation efficiency. Values are expressed as mean ±SD, n=5, *=p<0.05, significantly different compared to REF; a=p<0.05, significantly different to SAT, Scheffe's comparison. Cross=REF, circle=SAT, triangle=FO. See Methods for details.

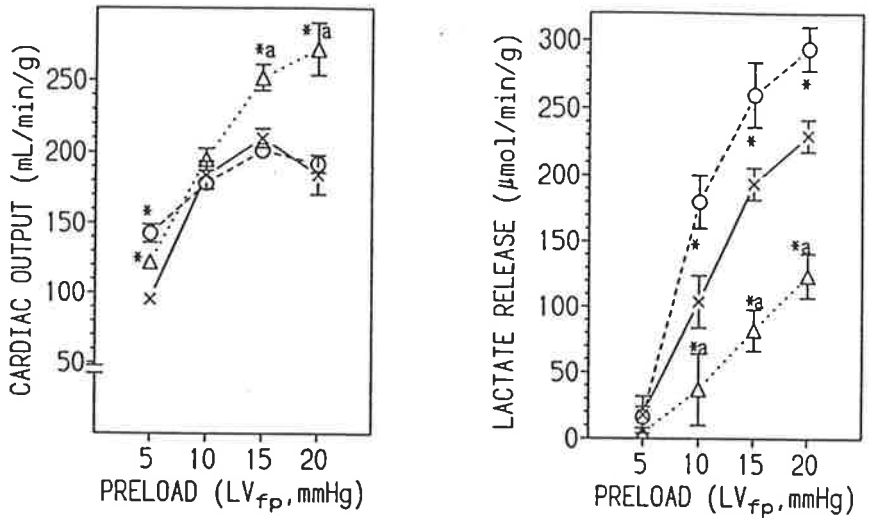

Figure VII.5. The relationship between left ventricular filling pressure and cardiac output. Values are expressed as mean ±SD (g dry weight), n=5, *=p<0.05, significantly different compared to REF; a=p<0.05, significantly different to SAT, Scheffe's comparison. Cross=REF, circle=SAT, triangle=FO. See Methods for details.

Figure VII.6. The relationship between left ventricular filling pressure and lactic acid release. Values are expressed as mean ±SD (g dry weight), n=5, *=p<0.05, significantly different compared to REF; a=p<0.05, significantly different to SAT, Scheffe's comparison. Cross=REF, circle=SAT, triangle=FO. See Methods for details.

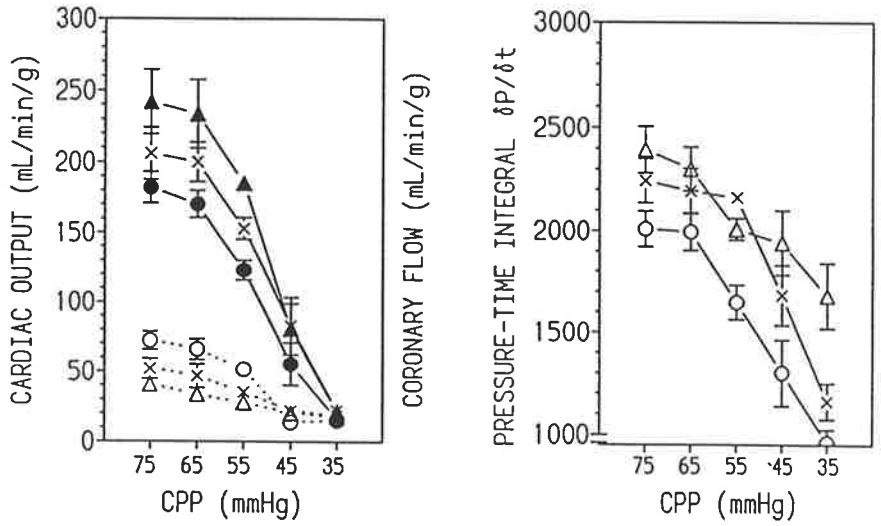

Figure VII.7. The effect of coronary perfusion pressure reductions on cardiac output. Values are expressed as mean ±SD (g dry weight), n=5, *=p<0.05, significantly different compared to REF; a=p<0.05, significantly different to SAT, Scheffe's comparison. Cross=REF, circle=SAT, triangle=FO, solid symbols=cardiac output, open symbols=coronary flow. See Methods for details.

Figure VII.8. The effect of coronary perfusion pressure reductions on the pressure-time integral. Values are expressed as mean ±SD, n=5, *=p<0.05, significantly different compared to REF; a=p<0.05, significantly different to SAT, Scheffe's comparison. Cross=REF, circle=SAT, triangle=FO. See Methods for details.

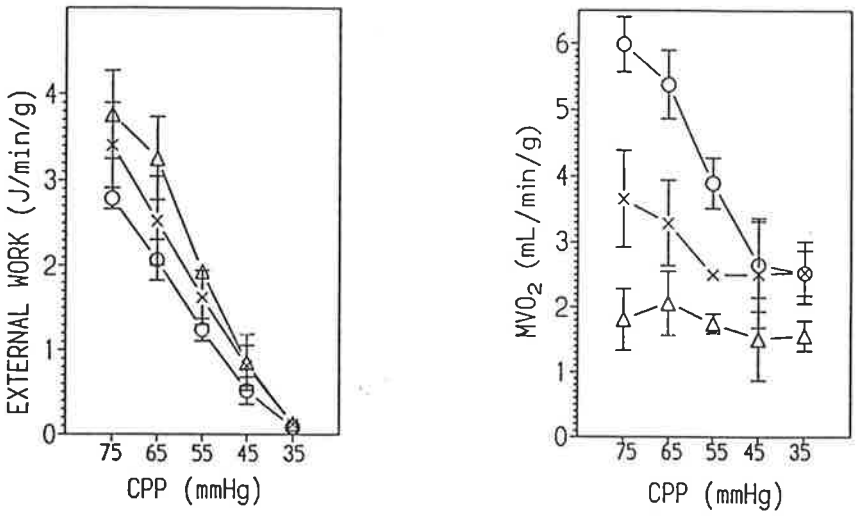

Figure VII.9. The effect of coronary perfusion pressure reductions on external work. Values are expressed as mean ±SD (g dry weight), n=5. Cross=REF, circle=SAT, triangle=FO. See Methods for details.

Figure VII.10. The effect of coronary perfusion pressure reductions on myocardial oxygen consumption. Values are expressed as mean ±SD (g dry weight), n=5, *=p<0.05, significantly different compared to REF; a=p<0.05, significantly different to SAT, Scheffe's comparison. Cross=REF, circle=SAT, triangle=FO. See Methods for details.

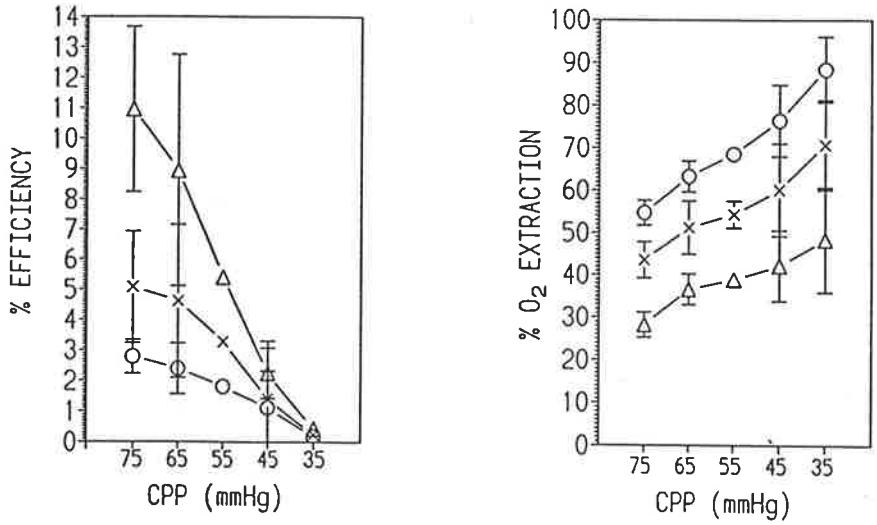

Figure VII.11. The effect of coronary perfusion pressure reductions on % energy utilisation efficiency. Values are expressed as mean ±SD, n=5, *=p<0.05, significantly different compared to REF; a=p<0.05, significantly different to SAT, Scheffe's comparison. Cross=REF, circle=SAT, triangle=FO. See Methods for details.

Figure VII.12. The effect of coronary perfusion pressure reductions on % oxygen extraction. Values are expressed as mean ±SD, n=5, *=p<0.05, significantly different compared to REF; a=p<0.05, significantly different to SAT, Scheffe's comparison. Cross=REF, circle=SAT, triangle=FO. See Methods for details.

Table VII.1. The Influence of Preload Alteration on Ventricular Function

a) Preload=5mmHg

DIET	(i)	(ii)	(iii)	(iv)	(v)	(vi)	(vii)	(viii)
REF	95.40 ±4.8	29.62 ±2.70	1980.0 ±191	1.109 ±0.041	28.36 ±0.73	1.599 ±0.169	3.48 ±0.314	7.325 ±0.002
SAT	142.61 ±6.6*	87.09 ±1.08*	1683.0 +44	1.488 ±0.142*	30.70 ±2.32	5.083 ±0.400*	1.47 ±0.170*	7.289 ±0.004*
FO	122.05 ±4.3*§	20.83 ±2.11§	2075.3 ±65§	1.437 ±0.063*	17.92* ±1.20§	0.710* ±0.066§	8.19 * ±0.858§	7.362* ±0.002§

b) Preload=10mmHg

DIET	(i)	(ii)	(iii)	(iv)	(v)	(vi)	(vii)	(viii)
REF	183.70 ±9.75	45.24 ±4.4	2764.5 ±429	2.48 ±0.24	33.58 ±4.57	2.864 ±0.380	4.340 ±0.48	7.314 ±0.009
SAT	178.50 ±4.82	90.19 ±3.6*	2474.0 ±273	2.18 ±0.14	33.34 ±2.46	5.721 ±0.554*	1.916 ±0.27*	7.291 ±0.005*
FO	195.24 ±7.70	31.01 ±2.0*§	3244.0 ±106*§	3.05 ±0.08*§	16.78 ±0.70*§	0.992 * ±0.101§	10.140 ±0.82*§	7.320 ±0.003

c) Preload=15mmHg

DIET	(i)	(ii)	(iii)	(iv)	(v)	(vi)	(vii)	(viii)
REF	209.7 ±6.58	58.41 ±3.26	3039.7 ±104	2.72 ±0.096	27.27 ±1.6	3.017 ±0.14	4.50 ±0.11	7.256 ±0.002
SAT	201.3 ±2.83	56.36 ±3.53	2756.0 ±139	2.41 ±0.137	57.26 ±4.29*	6.106 ±0.24*	1.97 ±0.17*	7.247 ±0.004
FO	251.9 ±9.04*§	44.16 ±2.15*§	3564.0 ±69*§	3.15 ±0.148*§	22.93 ±0.76*§	1.928 ±0.03*§	11.48 ±1.89*§	7.305* ±0.002§

d) Preload=20mmHg

DIET	(i)	(ii)	(iii)	(iv)	(v)	(vi)	(vii)	(viii)
REF	184.4 ±13.8	53.36 ±3.98	2853.0 ±30	2.339 ±0.201	39.44 ±0.80	3.993 ±0.228	2.927 ±0.34	7.290 ±0.002
SAT	191.8 ±6.1	72.09 ±0.89*	2367.0 ±112*	1.933 ±0.064*	55.11 ±3.63*	7.543 ±0.530*	1.280 ±0.07*	7.242 ±0.002*
FO	271.6 ±18.1*§	38.42 ±2.01*§	3485.4 ±47*§	3.060 ±0.274*§	40.55 ±1.91§	2.962 ±0.166*§	5.172 ±0.77*§	7.312 * ±0.002§

(i) Cardiac Output (mL/min/g d.w.); (ii) Coronary Flow (mL/min/g d.w.)

(iii) Pressure-Time Integral ($\delta P/\delta t$); (iv) External Work (J/min/g d.w.)

(v) % Oxygen Extraction; (vi) Myocardial Oxygen Consumption (mL/min/g d.w.)

(vii) % Efficiency of Energy Utilisation; (viii) Venous pH

*=Significantly different from REF $p<0.05$; §=Significantly different from SAT $p<0.05$; See Methods for details.

Table VII.2.

The Effect of Ischaemia and Reperfusion With High Preload

a) Control Working Heart Perfusion (Preload=20mmHg)

DIET	(i)	(ii)	(iii)	(iv)	(v)	(vi)	(vii)	(viii)
REF	184.4 ±13.8	53.36 ±3.98	2853.0 ± 30	2.339 ±0.201	39.44 ±0.80	3.993 ±0.228	2.927 ±0.34	7.290 ±0.002
SAT	191.8 ±6.1	72.09 ±0.89*	2367.0 ±112*	1.933 ±0.064	55.11 ±3.63*	7.543 ±0.530*	1.280 ±0.07*	7.242 ±0.002*
FO	271.6 ±18.1*§	38.42 ±2.01*§	3485.4 ±47*§	3.060 * ±0.274§	40.55 ±1.91§	2.962 * ±0.166§	5.172 ±0.77*§	7.312 * ±0.002§

b) Ischaemia (15min) (Preload=20mmHg)

DIET	(i)	(ii)	(iii)	(iv)	(v)	(vi)	(vii)	(viii)
REF	99.05 ±5.08	18.90 ±1.56	1606.0 ±36.7	0.647 ±0.420	70.51 ±3.4	2.519 ±0.09	1.28 ±0.10	7.188 ±0.003
SAT	88.07 ±5.90	16.43 ±1.68	1107.0 ±75.8*	0.390 ±0.033	91.27 ±1.2*	2.828 ±0.31	0.69 ±0.05*	7.083 ±0.006*
FO	115.87 ±5.35	20.25 ±0.72	1897.0 ±62.5*§	0.677 ±0.014	58.71 ±1.8*§	2.256 ±0.13*§	1.50 ±0.12§	7.218 * ±0.002§

c) Reperfusion (Preload=20mmHg)

DIET	(i)	(ii)	(iii)	(iv)	(v)	(vi)	(vii)	(viii)
REF	170.56 ± 7.06	42.93 ±5.01	2346.9 ± 48	1.952 ±0.11	62.20 ±3.2	5.043 ±0.42	1.944 ±0.27	7.193 ±0.002
SAT	156.04 ±10.22	59.10 ±2.75*	1891.3 ±115*	1.729 ±0.11	83.78 ±3.2*	9.376 ±0.76*	0.921 ±0.05*	7.145 ±0.002*
FO	256.36* ±10.70§	29.65 ±1.40§	3549.0 ± 67*§	3.135 ±0.10*§	58.09 ±1.5§	3.278 ±0.19*§	4.780 ±0.36*§	7.292 * ±0.003§

(i) Cardiac Output (mL/min/g d.w.); (ii) Coronary Flow (mL/min/g d.w.)

(iii) Pressure-Time Integral ($\delta P/\delta t$); (iv) External Work (J/min/g d.w.)

(v) % Oxygen Extraction; (vi) Myocardial Oxygen Consumption (mL/min/g d.w.)

(vii) % Efficiency of Energy Utilisation; (viii) Venous pH

*=Significantly different from REF $p<0.05$; §=Significantly different from SAT $p<0.05$.

See Methods for details.

Table VII.3.

Influence of Ischaemia & Reperfusion on High Preload (20mmHg)

a) Extracellular K^+ mM/L

DIET	HIGH PRELOAD	ISCHAEMIA	REPERFUSION
REF	5.28 ± 0.31	8.95 ± 0.42	6.05 ± 0.30
SAT	6.72 ± 0.52*	10.46 ± 0.34*	8.23 ± 0.42*
FO	4.56 ± 0.37§	5.99 ± 0.24*§	4.29 ± 0.39*§

b) Extracellular Lactate μmol/min/g

DIET	HIGH PRELOAD	ISCHAEMIA	REPERFUSION
REF	232.18 ± 22.30	320.20 ± 28.68	369.40 ±33.76
SAT	294.36 ± 19.75*	386.50 ± 32.24	420.30 ±44.72
FO	123.95 ± 21.23*§	129.87 ± 39.42*§	149.50 ±38.62*§

c) Creatine Phosphokinase Release U/min/g

DIET	HIGH PRELOAD	ISCHAEMIA	REPERFUSION
REF	5.78 ± 0.56	3.53 ± 0.20	18.95 ± 0.83
SAT	15.22 ± 0.96*	8.47 ± 0.28*	24.33 ± 0.75*
FO	3.93 ± 0.21*§	1.84 ± 0.17*§	5.21 ± 0.71*§

*=significant difference compared to REF, §= significant difference compared to the respective control, Scheffe's test, $p<0.05$, $n=5$ for all means ± SD. See Methods for protocol details.

Table VII.4. The Effect of Ischaemia (15min)(CPP=65mmHg) & Reperfusion on Erythrocyte Perfused Isolated Working Heart Function With Maintained Afterload (75mmHg) & Preload (10mmHg)

a) Control Working Heart Perfusion

DIET	(i)	(ii)	(iii)	(iv)	(v)	(vi)	(vii)	(viii)
REF	205.92 ±18.3	52.26 ±7.21	2243 ±107	3.403 ±0.49	43.48 ±4.28	3.307 ±0.732	5.08 ±1.84	7.322 ±0.001
SAT	181.78 ±11.2	72.34 ±6.54	2008 ± 87*	2.785 ±0.12	54.67 ±5.83*	5.978 ±0.415*	2.78 ±0.56*	7.309 ±0.004*
FO	242.25 ±22.5	40.72 ±2.95	2392 ±112§	3.759 ±0.51§	28.20 ±2.97*§	1.802 ±0.475*§	10.96 ±2.72*§	7.341 * ±0.003§

b) Ischaemia (15 min)

DIET	(i)	(ii)	(iii)	(iv)	(v)	(vi)	(vii)	(viii)
REF	200.31 ±14.2	46.76 ±8.42	2193 ±110	2.526 ±0.52	51.16 ±6.34	3.291 ±0.651	4.62 ±2.53	7.310 ±0.002
SAT	170.63 ± 9.7*	65.82 ±7.49*	1994 ± 92	2.066 ±0.24	63.25 ±6.98	5.382 ±0.515*	2.40 ±0.84	7.290 ±0.003
FO	233.52 ±24.2§	33.42 ±1.83§	2300 ±106§	3.253 ±0.48§	36.53 ±3.67§	2.061 ±0.482§	8.94 ±3.82*§	7.335 ±0.002*§

c) Reperfusion (5 min)

DIET	(i)	(ii)	(iii)	(iv)	(v)	(vi)	(vii)	(viii)
REF	209.31 ±15.25	49.86 ±6.8	2231 ± 89	3.428 ±0.62	52.92 ±4.83	3.861 ±0.811	4.160 ±2.71	7.316 ±0.003
SAT	183.29 ±12.63	68.28 ±3.9*	2022 ±115	2.801 ±0.27	64.34 ±6.99	6.913 ±0.603*	2.013 ±0.62	7.300 ±0.004*
FO	244.37 ±26.71§	48.72 ±4.2§	2406 ±122§	3.742 ±0.44§	39.22 ±3.64*§	2.264 ±0.493*§	9.521 ±3.45§	7.351 * ±0.002§

(i) Cardiac Output (mL/min/g d.w.); (ii) Coronary Flow (mL/min/g d.w.)

(iii) Pressure-Time Integral ($\delta P/\delta t$); (iv) External Work (J/min/g d.w.)

(v) % Oxygen Extraction; (vi) Myocardial Oxygen Consumption (mL/min/g d.w.)

(vii) % Efficiency of Energy Utilisation; (viii) Venous pH

*=Significantly different from REF $p<0.05$; §=Significantly different from SAT $p<0.05$. See Methods for details.

Table VII.5. The Effect of Ischaemia (15min)(CPP=55mmHg) & Reperfusion on Erythrocyte Perfused Isolated Working Heart Function With Maintained Afterload (75mmHg) & Preload (10mmHg)

a) Control Working Heart Perfusion

DIET	(i)	(ii)	(iii)	(iv)	(v)	(vi)	(vii)	(viii)
REF	204.3 ±13.8	52.45 ±4.52	2253 ± 96	2.991 ±0.602	45.75 ±4.67	3.798 ±0.324	5.617 ±1.840	7.326 ±0.002
SAT	186.7 ± 8.3	72.34 ±3.84*	2012 ±119	2.681 ±0.578	52.51 ±4.94*	5.924 ±0.537*	2.754 ±0.513*	7.298 ±0.002*
FO	258.5 ±11.2§	39.45 ±2.47*§	2405 ±105§	3.982 ±0.748	28.98 ±3.47*§	2.495 ±0.236*§	10.274 ±2.043*§	7.367 ±0.002*§

b) Ischaemia (15 min)

DIET	(i)	(ii)	(iii)	(iv)	(v)	(vi)	(vii)	(viii)
REF	152.5 ±7.8	35.13 ±1.33	2162 ±38.6	1.620 ±0.321	54.24 ±3.2	2.499 ±0.11	3.288 ±0.11	7.215 ±0.003
SAT	122.7 ±6.9*	51.38 ±2.14*	1647 ±85.5*	1.239 ±0.132	68.52 ±1.1*	3.898 ±0.38*	1.810 ±0.08*	7.176 ±0.004*
FO	184.8 ±5.35*§	27.51 ±0.92*§	2007 ±52.6*§	1.927 ±0.084§	38.78 ±1.4*§	1.736 ±0.15*§	5.412 ±0.10*§	7.268 * ±0.006§

c) Reperfusion (5 min)

DIET	(i)	(ii)	(iii)	(iv)	(v)	(vi)	(vii)	(viii)
REF	163.8 ±11.9	48.22 ±7.2	2088 ±113	1.995 ±0.492	49.28 ±2.5	3.992 ±0.13	1.452 ±0.14	7.287 ±0.004
SAT	148.4 ±10.3	68.37 ±6.6*	1794 ±128	1.828 ±0.623	60.37 ±3.7*	6.895 ±0.16*	0.964 ±0.09	7.226* ±0.007
FO	239.5 ±12.6*§	39.12 ±2.86§	2291 ±103§	3.596 * ±0.716§	31.54 ±3.9*§	2.891 ±0.12*§	2.493 ±0.25§*	7.333*§ ±0.004

(i) Cardiac Output (mL/min/g d.w.); (ii) Coronary Flow (mL/min/g d.w.)

(iii) Pressure-Time Integral ($\delta P/\delta t$); (iv) External Work (J/min/g d.w.)

(v) % Oxygen Extraction; (vi) Myocardial Oxygen Consumption (mL/min/g d.w.)

(vii) % Efficiency of Energy Utilisation; (viii) Venous pH

*=Significantly different from REF $p<0.05$; §=Significantly different from SAT $p<0.05$. See Methods for details.

Table VII.6. The Effect of Ischaemia (15min)(CPP=45mmHg) & Reperfusion on Erythrocyte Perfused Isolated Working Heart Function With Maintained Afterload (75mmHg) & Preload (10mmHg)

a) Control Working Heart Perfusion

DIET	(i)	(ii)	(iii)	(iv)	(v)	(vi)	(vii)	(viii)
REF	195.9 ±26.3	53.74 ±3.67	2095 ±160	2.972 ±0.594	46.45 ±4.8	3.882 ±0.392	5.26 ±2.18	7.324 ±0.004
SAT	184.0 ±20.7	68.95 ±7.56*	2008 ±110	2.605 ±0.673	55.34 ±5.2	5.916 ±0.593*	2.63 ±0.42	7.299 ±0.003*
FO	260.4 ±29.6*§	42.39 ±3.15*§	2243 ± 91	4.006 ±0.850	29.36 ±4.7*§	2.482 * ±0.510§	10.07 ±3.75§	7.356 * ±0.002§

b) Ischaemia (15 min)

DIET	(i)	(ii)	(iii)	(iv)	(v)	(vi)	(vii)	(viii)
REF	82.47 ±20.6	22.03 ±4.7	1682 ±148	0.813 ±0.241	60.13 ±10.9	2.504 ±0.82	1.412 ±1.90	7.209 ±0.005
SAT	55.18 ±15.2	14.05 ±3.2	1300 ±162*	0.516 ±0.163	76.42 ± 9.3	2.651 ±0.72	1.124 ±1.20	7.152 ±0.006*
FO	80.26 ±18.3	20.61 ±2.4§	1940 ±158§	0.852 ±0.333	42.15 ± 8.4§	1.505 ±0.64	2.26 ±0.83§	7.259 * ±0.008§

c) Reperfusion (5 min)

DIET	(i)	(ii)	(iii)	(iv)	(v)	(vi)	(vii)	(viii)
REF	145.63 ±24.6	48.12 ±5.1	1992 ±167	1.827 ±0.205	49.78 ±4.63	4.216 ±0.44	1.041 ±0.429	7.272 ±0.011
SAT	130.12 ±21.6	51.92 ±8.8	1793 ±148	1.779 ±0.512	58.85 ±9.52	7.085 ±0.67*	0.380 ±0.625	7.208 ±0.013
FO	210.36 ±31.4*§	39.47 ±4.2§	2198 ±110§	3.716 * ±0.437§	34.89 ±9.25§	2.873 ±0.42*§	1.192 ±0.578	7.299 ±0.009§

(i) Cardiac Output (mL/min/g d.w.); (ii) Coronary Flow (mL/min/g d.w.)

(iii) Pressure-Time Integral ($\delta P/\delta t$); (iv) External Work (J/min/g d.w.)

(v) % Oxygen Extraction; (vi) Myocardial Oxygen Consumption (J/min/g d.w.)

(vii) % Efficiency of Energy Utilisation; (viii) Venous pH

*=Significantly different from REF $p<0.05$; §=Significantly different from SAT $p<0.05$. See Methods for details.

Table VII.7.

Influence of Ischaemia (15min)(CPP=65mmHg) on Coronary Effluent Concentrations of K^+, Lactate and Creatine Phosphokinase

a) Extracellular K^+ mM/L

DIET	CONTROL	ISCHAEMIA	REPERFUSION
REF	3.156 ± 0.232	3.324 ± 0.247	3.272 ± 0.282
SAT	3.521 ± 0.356	3.682 ± 0.363	3.650 ± 0.379
FO	3.208 ± 0.211	3.369 ± 0.298	3.259 ± 0.262

b) Extracellular Lactate μmol/min/g

DIET	CONTROL	ISCHAEMIA	REPERFUSION
REF	76.52 ± 29.4	82.19 ± 32.5	90.22 ± 30.2
SAT	165.29 ± 32.2*	175.94 ± 36.5*	182.64 ± 34.2*
FO	20.48 ± 8.3*§	24.28 ± 7.2*§	29.73 ± 8.5*§

c) Creatine Phosphokinase Release U/min/g

DIET	CONTROL	ISCHAEMIA	REPERFUSION
REF	5.438 ± 0.784	5.450 ± 0.575	5.562 ± 0.611
SAT	9.826 ± 0.837*	10.025 ± 0.758*	10.673 ± 0.795*
FO	1.809 ± 0.324*§	1.815 ± 0.319*§	1.992 ±0.328*§

*=significant difference compared to REF, §= significant difference compared to the respective control, Scheffe's test, $p<0.05$, $n=5$ for all means ± SD. See Methods for protocol details.

Table VII.8.

Influence of Ischaemia (15min)(CPP=55mmHg) on Coronary Effluent Concentrations of K^+, Lactate and Creatine Phosphokinase

a) Extracellular K^+ mM/L

DIET	CONTROL	ISCHAEMIA	REPERFUSION
REF	3.172 ± 0.245	3.552 ± 0.269	3.426 ± 0.218
SAT	3.606 ± 0.298	4.372 ± 0.412	3.958 ± 0.321
FO	3.214 ± 0.194	3.767 ± 0.209	3.249 ± 0.225

b) Extracellular Lactate μmol/min/g

DIET	CONTROL	ISCHAEMIA	REPERFUSION
REF	70.69 ± 22.7	98.26 ± 37.6	123.62 ± 34.5
SAT	171.35 ± 38.4*	196.83 ± 31.9*	210.25 ± 41.4*
FO	22.95 ± 10.3*§	29.42 ± 4.9*§	35.08 ± 10.7*§

c) Creatine Phosphokinase Release U/min/g

DIET	CONTROL	ISCHAEMIA	REPERFUSION
REF	5.392 ± 0.652	5.436 ± 0.721	6.214 ± 0.811
SAT	9.958 ± 0.704*	9.982 ± 0.824*	11.050 ± 0.653*
FO	1.893 ± 0.385&§	1.856 ± 0.324*§	2.795 ± 0.632*§

*=significant difference compared to REF, §= significant difference compared to the respective control, Scheffe's test, $p<0.05$, $n=5$ for all means ± SD. See Methods for protocol details.

Table VII.9.

Influence of Ischaemia (15min)(CPP=45mmHg) on Coronary Effluent Concentrations of K^+, Lactate and Creatine Phosphokinase

a) Extracellular K^+ mM/L

DIET	CONTROL	ISCHAEMIA	REPERFUSION
REF	3.312 ± 0.258	4.592 ± 0.287	4.106 ± 0.307
SAT	3.722 ± 0.319	4.818 ± 0.366	4.351 ± 0.414
FO	3.342 ± 0.208	4.270 ± 0.197	3.620 ± 0.211

b) Extracellular Lactate μmol/min/g

DIET	CONTROL	ISCHAEMIA	REPERFUSION
REF	78.25 ± 26.43	95.93 ± 33.42	190.34 ± 39.62
SAT	185.21 ± 32.49*	228.34 ± 46.73*	355.27 ± 37.76*
FO	24.36 ± 8.76*§	33.65 ± 6.93*§	39.75 ±12.13*§

c) Creatine Phosphokinase Release U/min/g

DIET	CONTROL	ISCHAEMIA	REPERFUSION
REF	3.958 ± 0.80	3.324 ± 0.42	10.42 ± 2.07
SAT	8.65 ± 0.94*	6.822 ± 0.29*	16.51 ± 2.31*
FO	2.22 ± 0.20*§	2.318 ± 0.17*§	3.60 ± 0.45*§

*=significant difference compared to REF, §= significant difference compared to the respective control, Scheffe's test, $p<0.05$, $n=5$ for all means ± SD. See Methods for protocol details.

VIII. DOSE EFFECT RELATIONSHIP BETWEEN DIETARY POLYUNSATURATED FISH OIL, CARDIAC FUNCTION AND METABOLISM.

VIII. 1. Introduction

Polyunsaturated fatty acids are important structural and functional components of cell membranes that regulate membrane properties such as fluidity and permeability, the function of membrane bound enzymes, and ion transport systems **(Egwin & Kummerow,1972;Farias,*et al.*,1975;Spector & Yorek, 1985)**. Despite early evidence of a potential role of direct myocardial action, the predominance of research interest has been concentrated on the clinical role of omega-3 polyunsaturated fatty acids to: lower plasma cholesterol levels; lower systemic blood pressure; reduce the incidence of the low density plasma lipo-proteins associated with atherosclerotic plaque development; and regulate the functions of platelets, monocytes, macrophages, arterial and endothelial cells to reduce the incidence of atherosclerosis and thrombosis **(Harris,1989;Kinsella,*et al.*,1990;Knapp,1990)**.

The dearth of investigation into the direct influence of omega-3 polyunsaturated fish oils on cardiac function makes the findings reported in the earlier chapters of this thesis noteworthy and unique. In particular, it was observed that FO hearts were afforded protection against ischaemic and reperfusion injury, but had enhanced ventricular contractility and cardiac output while limiting MVO_2, particularly the paradoxical increase in MVO_2 during ischaemia and reperfusion. McLennan, *et al.* **(1989)**, demonstrated that

the arrhythmogenic effect of SAT diet consumption in rats could be reversed by dietary change-over to a n-3 polyunsaturated fatty acid rich diet. Reversal could also be achieved with a diet rich in n-6 polyunsaturates but to a lesser extent. In an identical dietary regime Abeywardena, *et al.*(**1987**), observed that the myocardial phospholipid fatty acid profile formed by long term saturated fat supplementation could be reversed following dietary cross-over to polyunsaturated-supplemented diets. The facile reversal of the adverse cardiac effects of long term feeding of high saturated fat diet suggests that alteration of the type of dietary fat consumption is worthy even after prolonged consumption of a diet associated with a high risk of ischaemic heart disease.

The studies described previously in this thesis have clearly demonstrated that a diet in which the predominant source of fat was fish oil can produce a different physiological and metabolic profile in the isolated working heart to that achieved with a principally SAT diet. Little is known about whether similar effects can be achieved with lesser proportions of fish oil fatty acids, especially in conjunction with a predominantly saturated fat diet. Therefore, the aim of the experiment presented in this chapter was to investigate the effect of replacing saturated fat supplementation with various increments of n-3 polyunsaturated fatty acid rich fish oil on ventricular performance and metabolism in the erythrocyte perfused isolated working hearts, particularly during ischaemia and reperfusion. This part of the study additionally utilised a shorter period of fish oil feeding and a prefeeding of a SAT diet to all animals.

VIII. 2. Methods

VIII. 2. a) Animals & Diets

A total of 38 male Hooded-Wistar rats (5 months old) were commenced on a SAT diet regime for 6 weeks. The animals were then divided into 4 groups, in which they were crossed-over to a diet supplemented with 12% fat consisting of: (i) 12% SAT (n=8); (ii) 9% SAT, 3% FO (n=10), (iii) 6% SAT, 6% FO (n=10); or (iv) 12 %FO (n=10) for a further 6 weeks.

Although at the time of experimental use the animals were approximately the same age as in the earlier experiments of this thesis, this experiment was designed to implement FO dietary supplementation for 6 weeks only because of an extreme shortage of Shaklee EPA fish oil. The diets were formulated to have the fatty acid compositions described in Table VIII.1. These diets were prepared, stored and presented to the rats as described in Chapter III. Diets (ii) and (iii) differed from (i) and (iv) only in the proportion of saturated fat and fish oil in the supplement. The animals were housed under the conditions described in Chapter III. The body mass at the time of experimental use ranged between 450 and 500g. They were fasted overnight prior to each experimental day.

VIII. 2. b) Isolated Working Heart Preparation & Perfusion Protocol

Isolated hearts were prepared for working heart perfusion (maintained afterload ischaemia configuration) with the erythrocyte buffer (40% haematocrit) and perfused and monitored as described in Chapter II. Erythrocyte perfused working heart mode was commenced (coronary perfusion pressure,

preload and afterload set at 75, 10 and 75 mmHg respectively) with right atrial pacing (300 bpm) as previously described. Subsequent to 5 min working heart perfusion, when hearts had equilibrated and were stabilised, measures of cardiac output, aortic pressure, arterial and venous blood gas content, and pH were taken every 5 min for the entire perfusion. After the equilibration of working hearts, ischaemia was induced and maintained for 15 min by reducing coronary perfusion pressure to 35mmHg then reperfused at a coronary perfusion pressure of 75 mmHg. Following oxygen tension and pH readings in arterial and coronary venous samples, erythrocytes were removed by centrifugation with a Corning bench top centrifuge. The sample supernatant was frozen in liquid nitrogen and stored at -60°C for later analyses.

VIII. 2. c) Analysis of Coronary Effluent Contents

Arterial and venous samples were assayed for creatine kinase, lactic acid and K^+ concentrations as described in Chapter III. Creatine kinase was quantified according to the rate of change of absorbance per minute. Results were expressed in U/min/g dry weight adjusted according to coronary flow rates. Lactic acid concentrations were expressed in μmol/min/g dry weight when adjusted according to coronary flow rates. For determination of potassium ion concentration all samples were analysed as described in Chapter V and results were expressed in mmol/L.

Table VIII.1. The Calculated Fatty Acid Composition of Rat Diets

DIET	0% FO	3% F0	6% FO	12% FO
% TOTAL FAT	15.52	15.52	15.52	15.52
% FAT ADDED	12.00	12.00	12.00	12.00
Σ SATURATES	53.15	46.50	39.85	26.55
Σ MONOUNSAT-URATES	28.13	27.24	26.35	24.57
Σ POLYUNSAT-URATES	12.88	21.42	29.96	47.05
18:2 n-6	9.16	9.04	8.93	8.69
18:3 n-3	1.68	1.66	1.64	1.60
20:4 n-6	0.14	0.37	0.60	1.06
20:5 n-3	0.50	5.99	11.48	22.46
22:4 n-6	0.00	0.19	0.39	0.77
22:5 n-3	0.00	0.41	0.81	1.62
22:6 n-3	1.41	3.71	6.01	10.61
Σ n-6	9.29	9.66	10.03	10.76
Σ n-3	3.58	11.76	19.93	36.29
P/S RATIO	0.24	0.46	0.75	1.77
n-6/n-3	2.59	0.82	0.50	0.30

Calculated composition of diets supplemented with 12% fat (i) 12% SAT,0% FO (ii) 9% SAT,3% FO (iii) 6% SAT,6% FO (iv) 0% SAT,12 %FO. See text above for additional detail and Chapter III.2. for details of SAT and FO diets and fatty acid nomenclature.

VIII. 2. d) Data Handling and Statistical Analysis

Myocardial left ventricular external work, the pressure-time integral, perfusate oxygen content, oxygen extraction, myocardial oxygen consumption and percentage energy utilisation efficiency were calculated as described in Chapter II. All results were expressed as mean ± SD. For each parameter,

the effect of dietary treatment was tested by Analysis of Variance and between individual comparisons with Scheffe's *post hoc* *F*-test. The level of significance was considered at $P < 0.05$ or less.

VIII. 3. Results

VIII. 3. a) % Oxygen Extraction

Increasing the percentage of fish oil in the diet (0,3,6,12%) resulted in a proportional reduction of the % oxygen extraction in working hearts under control conditions (Table VIII.1. & Figure VIII.1.). It was significantly lower in 6% and 12% FO hearts compared to SAT hearts. During low flow ischaemia the % oxygen extraction significantly rose in all dietary groups reaching almost 80% in SAT hearts. The rise in % oxygen extraction during ischaemia was inversely proportional to the level of fish oil supplementation and it was significantly lower than SAT with 3,6 and 12% FO. During reperfusion the % oxygen extraction was reduced slightly from ischaemic levels in all dietary groups and the inverse relationship between the % oxygen extraction and % dietary FO remained unchanged. The % oxygen extraction was significantly lower in the 6% and 12% dietary FO hearts than in SAT hearts.

VIII. 3. b) Myocardial Oxygen Consumption

The increase in the percentage of fish oil supplementation produced a similar proportionate decrease in MVO_2 of control perfused hearts as was observed with % oxygen extraction (Table VIII.2. & Figure VIII.2.). Compared to SAT

hearts, MVO_2 was significantly reduced at 3%FO as well as 6%FO and 12%FO. During ischaemia MVO_2 rose significantly in 3,6 and 12%FO hearts but in SAT hearts MVO_2 decreased by approximately 50%. There was no significant difference in MVO_2 during ischaemia between SAT and 3% or 6% FO hearts while MVO_2 was significantly lower in 12% FO hearts. Following reperfusion MVO_2 rose significantly in SAT and 3% FO hearts. The greatest increase in MVO_2 occurred in SAT hearts. Post-ischaemic MVO_2 was elevated in all dietary groups compared to their respective control values.

VIII. 3. c) Myocardial External Work

During control working heart perfusion external work was elevated with each increment in % dietary FO compared to SAT (Figure VIII.3.). In control hearts the 6% and 12%FO performed significantly more external work than the SAT hearts. Following 15 min ischaemia a marked reduction in external work was observed in all dietary groups, but remained significantly higher in 12%FO hearts compared to SAT hearts. At 5 min reperfusion external work returned towards control in all dietary groups. The trend towards external work being proportional to % dietary FO was maintained in reperfusion, and in post-ischaemic 12%FO hearts it was significantly greater than in SAT hearts.

VIII. 3. d) Cardiac Output

During control working heart perfusion cardiac output was proportional to the %FO in the diet (Figure VIII.4). It was significantly greater at 6% and 12%FO compared to SAT hearts. Cardiac output decreased markedly during ischaemia in all dietary groups by approximately 80% and remained significantly higher in 6% and 12%FO hearts compared to SAT hearts. Following 5min reperfusion cardiac output recovered towards control levels in all dietary groups. Post-ischaemic cardiac output was significantly greater in 12%FO hearts compared to SAT hearts.

VIII. 3. e) Coronary Flow

The control working heart coronary flow rates were significantly lower in 3%FO hearts than SAT hearts (Figure VIII.4.). Small further reductions were seen in 6%FO and 12%FO hearts, with the lowest coronary flow having occurred in 12%FO compared to SAT hearts. During ischaemia there were no significant differences in coronary flow between the dietary groups. During reperfusion the coronary flow rates returned towards control levels but remained significantly depressed in SAT hearts.

VIII. 3. f) Pressure-Time Integral

Contractility, as determined by the pressure-time integral was significantly higher in 3%, 6% and 12%FO than in SAT hearts (Figure VIII.5.). There was a trend for contractility to increase with each increment in %FO. The dietary relationship to contractility was maintained throughout ischaemia and

reperfusion. During ischaemia contractility decreased significantly by approximately 50% in all groups. Upon reperfusion, close recovery to within 10% of pre-ischaemic contractility was observed in all dietary groups.

VIII. 3. g) % Efficiency

The energy efficiency ratio in control perfused hearts was significantly increased with each %FO increment from the very low % efficiency in SAT hearts (Figure VIII.6.). During ischaemia % efficiency decreased to very low levels in all dietary groups. By 5 min of reperfusion the % efficiency ratio had risen significantly from ischaemic levels in all dietary groups. This increase was proportional to the % dietary FO. However, the % efficiency post-ischaemia was significantly lower in all dietary groups than their respective control measure.

VIII. 3. h) Metabolites & Enzyme Content of Coronary Effluent

Table VIII.8. presents the values of venous pH during the control, ischaemia and reperfusion periods of each dietary group. Throughout each experimental period SAT hearts had significantly lower venous pH compared to 3%, 6% and 12%FO whereas little difference in venous pH was observed between each increment of %FO. The venous pH fell during ischaemia in all groups and returned towards control on reperfusion. The extent of acidosis was significantly greater in SAT hearts than the other dietary groups.

During control working heart perfusion venous [K^+] did not differ significantly either between the dietary groups or from arterial [K^+] (Table

VIII.9.). During ischaemia venous [K^+] rose significantly in all groups. However, the greatest increase occurred in SAT hearts and the magnitude of this arterio-venous difference declined with each increment in %FO. After reperfusion, venous [K^+] decreased in all groups but this remained highest in SAT and lowest in 12%FO hearts where the closest return to pre-ischaemic extracellular [K^+] occurred. No significant difference was observed in extracellular [K^+] between SAT and 3%FO hearts nor between 6%FO and 12%FO hearts after 5 min reperfusion.

The production of lactic acid was significantly elevated through all perfusion phases in SAT hearts compared to all other groups (Table VIII.10.). It increased significantly in all groups during ischaemia where the greatest rise in lactate production occurred in SAT hearts. This was markedly less in 3%FO hearts compared to SAT and a further decline occurred with the increase in FO to 6% or 12%. Extracellular lactate levels were augmented further after reperfusion such that the highest lactate output occurred in SAT hearts and the least in 12% FO.

Creatine phosphokinase release was significantly increased in SAT hearts (approximately 10 fold) compared to the other groups during control perfusion (Table VIII.11). No significant difference was observed between 3%, 6% and 12%FO hearts. During ischaemia, creatine kinase output increased in 3%, 6% and 12%FO hearts and decreased in SAT hearts such that no dietary differences were observed. However, reperfusion caused a significant increase in creatine kinase output in SAT hearts to nearly double control levels. Creatine kinase release also increased further in reperfusion in

3% and 6% FO hearts but not in 12% FO hearts. All FO hearts had lower creatine kinase release during reperfusion than the SAT hearts.

Table VIII.2.

The Effect of Dietary Saturated Fat Supplement

Following Replacement with Fish Oil: Venous pH

DIET	CONTROL	ISCHAEMIA	REPER'N
SAT	7.284 ±0.004	7.083 ±0.033	7.213±0.004
3% FO	7.318 ±0.006*	7.205 ±0.007*	7.257±0.008*
6% FO	7.322 ±0.005*	7.232 ±0.018*	7.300±0.005*
12% FO	7.319 ±0.004*	7.217 ±0.005*	7.293±0.004*

Table VIII.3.

The Effect of Dietary Saturated Fat Supplement

Following Replacement with Fish Oil: Extracellular K^+ (mM/L)

DIET	CONTROL	ISCHAEMIA	REPER'N
SAT	3.32 ± 0.28	9.79 ± 1.25	5.92 ± 0.69
3% FO	3.11 ± 0.21	6.02 ± 0.32*	5.49 ± 0.30
6% FO	3.03 ± 0.13	5.29 ± 0.36*	4.06 ± 0.28*
12% FO	3.05 ± 0.26	4.94 ± 0.53*	3.70 ± 0.35*

*=significant difference compared to SAT, $p<0.05$, n=10 (except SAT, n=8), mean ± SD. REPER'N = reperfusion. See Methods for more details.

Table VIII.4.

The Effect of Dietary Saturated Fat Supplement

Following Replacement with Fish Oil: Extracellular Lactate (μmol/min/g)

DIET	CONTROL	ISCHAEMIA	REPER'N
SAT	156.1 ± 42.7	243.3 ± 36.73	374.3 ± 27.7
3% FO	34.56 ± 3.27*	43.95 ± 2.98*	70.92 ±4.85*
6% FO	17.53 ± 2.96*	29.23 ± 3.85*	57.58 ±4.26*
12% FO	20.32 ± 3.40*	28.73 ± 4.19*	39.58 ±5.24*

Table VIII.3.

The Effect of Dietary Saturated Fat Supplement

Following Replacement with Fish Oil: Creatine Phosphokinase (U/min/g)

DIET	CONTROL	ISCHAEMIA	REPER'N
SAT	9.858 ± 1.652	4.857 ± 1.493	16.47 ±3.95
3% FO	0.992 ±0.224*	5.799 ± 2.175	9.659 ±2.44
6% FO	0.953 ±0.196*	5.682 ± 2.093	8.732 ±3.47*
12% FO	1.026 ±0.268*	5.048 ± 2.175	5.388 ±1.94*

*=significant difference compared to SAT, $p<0.05$, $n=10$ (except SAT, $n=8$), mean ± SD. REPER'N = reperfusion. See Methods for more details.

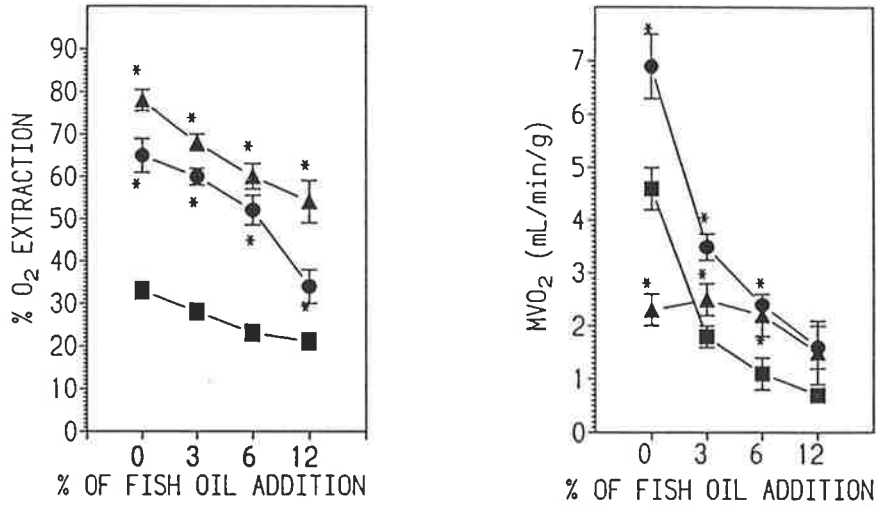

Figure VIII.1. The effect of different levels of fish oil dietary supplementation on % oxygen extraction. Values are expressed as mean ±SD, n=10, *=p<0.05, significantly different compared to control working heart perfusion. Square=control perfusion (CPP=75mmHg), triangle=ischaemia (CPP=35mmHg,15min), circle=reperfusion. 0% FO addition=SAT. See Methods for details.

Figure VIII.2. The effect of different levels of fish oil dietary supplementation on myocardial oxygen consumption. Values are expressed as mean ±SD, n=10, *=p<0.05, significantly different compared to control working heart perfusion. Square=control perfusion (CPP=75mmHg), triangle=ischaemia (CPP=35mmHg,15min), circle=reperfusion. 0% FO addition=SAT. See Methods for details.

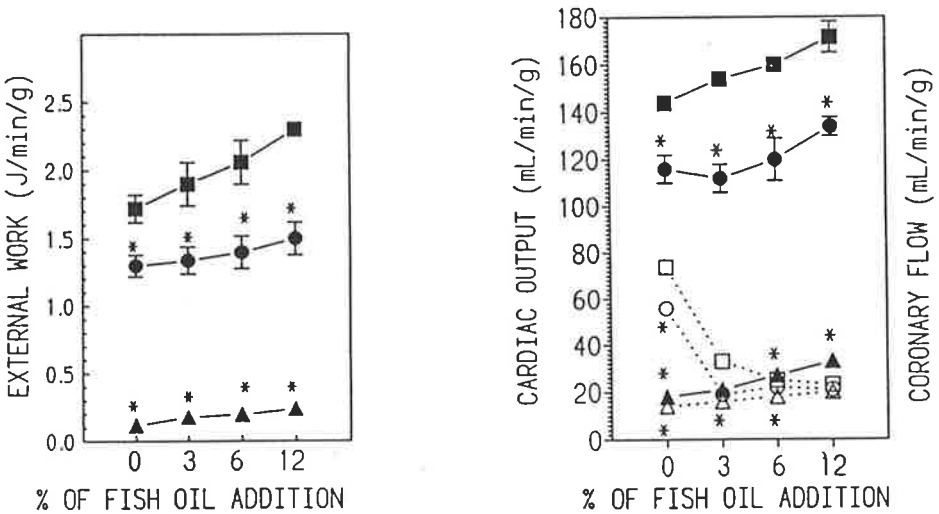

Figure VIII.3. The effect of different levels of fish oil dietary supplementation on myocardial external work. Values are expressed as mean ±SD, n=10, *=p<0.05, significantly different compared to control working heart perfusion. Square=control perfusion (CPP=75mmHg), triangle=ischaemia (CPP=35mmHg,15min), circle=reperfusion. 0% FO addition=SAT. See Methods for details.

Figure VIII.4. The effect of different levels of fish oil dietary supplementation on cardiac output. Values are expressed as mean ±SD, n=10, *=p<0.05, significantly different compared to control working heart perfusion. Square=control perfusion (CPP=75mmHg), triangle=ischaemia (CPP=35mmHg,15min), circle=reperfusion. Open symbols=coronary flow. 0% FO addition=SAT. See Methods for details.

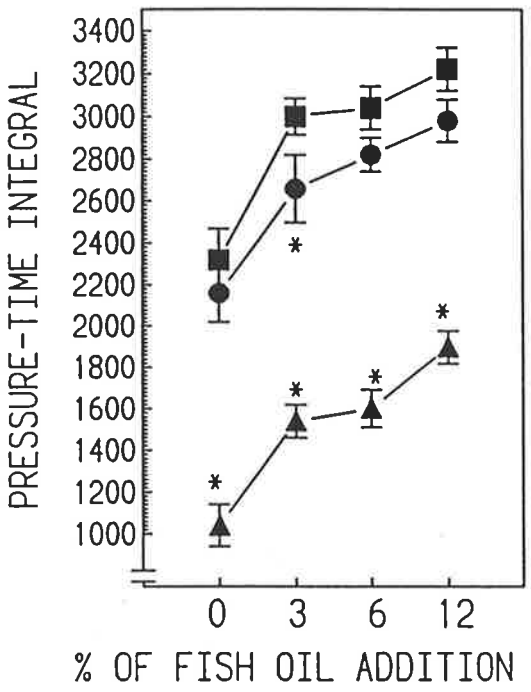

Figure VIII.5. The effect of different levels of fish oil dietary supplementation on the pressure-time integral. Values are expressed as mean ±SD, n=10, *=p<0.05, significantly different compared to control working heart perfusion. Square=control perfusion (CPP=75mmHg), triangle=ischaemia (CPP=35mmHg,15min), circle=reperfusion. 0% FO addition=SAT. See Methods for details.

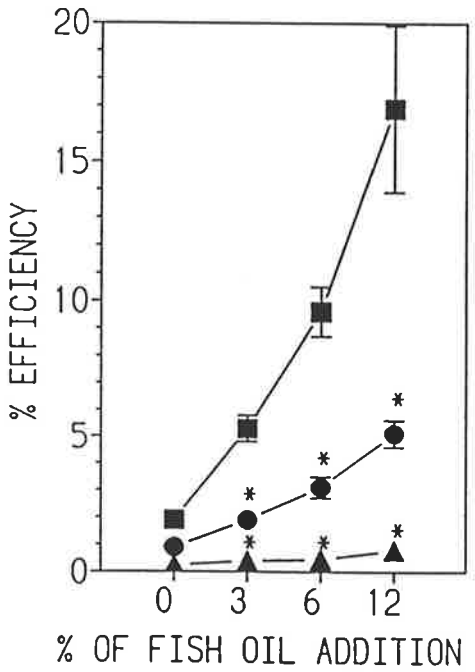

Figure VIII.6. The effect of different levels of fish oil dietary supplementation on % efficiency of energy utilisation. Values are expressed as mean ±SD, n=10, *=p<0.05, significantly different compared to control working heart perfusion. Square=control perfusion (CPP=75mmHg), triangle=ischaemia (CPP=35mmHg,15min), circle=reperfusion. 0% FO addition=SAT. See Methods for details.

VIII. 4. Discussion

This study has demonstrated that as little as six weeks feeding of dietary polyunsaturated fish oil can produce the effects of limiting MVO_2 without functional detriment even if the animals have been prefed a saturated fat supplemented diet. During control working heart perfusion, cardiac output, contractility, external work and the % mechanical efficiency of 12%FO hearts was significantly greater than in SAT hearts. In addition, coronary flow, % oxygen extraction and myocardial oxygen consumption were significantly lower in 12%FO than in SAT hearts. These results are similar to those reported after 16 weeks feeding to the same final age (Chapter III). These findings are complementary to and extend those of McLennan, *et al.*(1989) to the erythrocyte perfused isolated working heart model, thus gaining insight into the effect of dietary cross-over on ventricular performance and metabolism. Notably, this was a relatively short feeding period with younger rats, compared to 9 months feeding of SAT followed by 9 months feeding of fish oil supplemented diet in the study by McLennan, *et al.*(1989).

Ventricular performance was augmented in a dose-related manner with the concomitant increase in the proportion of FO and decrease in the proportion of SAT in the diet. In addition, the MVO_2 during control perfusion, ischaemia and reperfusion was reduced from that evident with 12% SAT with each reduced proportion of SAT and increased proportion of FO. Since the total amount of dietary fat supplementation was maintained at 12% as in previous experiments, each increase in dietary n-3 fatty acids was achieved by a concomitant reduction in saturated fatty acids. Thus, it is

possible that the cardiac effects observed with increased % dietary FO could be due merely to a reduced % of dietary SAT. Indeed, it was observed in Chapter IV and in studies by McLennan, *et al.*(**1985-1990**) that saturated fat-rich dietary supplementation may be pro-arrhythmogenic. The reduction in the % dietary SAT may in itself contribute to the changes in ventricular performance and oxygen metabolism rather than, or in addition to increased dietary FO.

Sargent and Riemersma (**1990**) observed in Langendorff perfused isolated hearts from a rat strain having high arrhythmia incidence that ventricular fibrillation was inversely related to linoleic acid and positively related to the saturated fatty acids stearate and palmitate in adipose tissue. In an earlier study these authors (**Riemersma & Sargent,1989**), observed non-significant reductions (18%) of ventricular fibrillation following coronary ligation in rats fed low levels of saturated and n-3 polyunsaturated fatty acids. They proposed that the effects of polyunsaturates in general on the incidence of sudden cardiac death may be due to reduced saturated fat intake. It has also been observed (**Hornstra,1989;Elvevoll,*et al.*,1990**) that the Greenland Eskimo diet associated with reduced risk of mortality from ischaemic heart disease (**Bang,*et al.*,1980**) contains approximately 50% less saturated fatty acids than the European diet. However, Hornstra (**1989**), reported that in a rat model of arterial thrombosis the anti-thrombotic effect of polyunsaturated n-3 fatty acids of marine origin were more effective against the pro-thrombotic effect of saturated fatty acids than that of n-3 and n-6 polyunsaturated fatty acids of terrestrial origin. This author proposed that the benefits of dietary

marine polyunsaturates could be increased by concomitant reduction in the intake of dietary saturated fats.

In this thesis it was observed that the REF diet, lower in total fat (7.6% total) and particularly low in saturated fatty acids compared to SAT and FO diets, did not influence ventricular function or metabolism similar to FO diet. Indeed, REF hearts tended to function similar to SAT hearts for a number of parameters but to a lesser extent. McLennan, *et al.*(**1985-1990**), have observed similar findings with a different reference diet (4% total fat). In addition, these workers observed that 12% dietary supplementation with sunflower seed oil rich in n-6 polyunsaturates was not as consistently anti-arrhythmic as 12% dietary fish oil supplementation, yet the saturated fat proportion was displaced less by the latter diet. In very recent experiments this research group (**Charnock,*et al.*,*1*991;1992), blended saturated fat (6%) with fish oil (6%) to produce their 12% dietary fat supplement. It was observed that the incidence of arrhythmic activity was significantly reduced with the supplement of saturated fat/fish oil blend compared with a blend of saturated fat with n-6 polyunsaturated fatty acid rich sunflower seed oil, even though total saturated fat content and the ratio of polyunsaturates to un-saturates were similar. Thus, these results in combination, do not permit us to accept the possibility that reductions in dietary saturated fat are totally responsible for the improved cardiac output, contractility and % energy efficiency, rather than the increase in % dietary fish oil.

Nor is it likely that the proportion of polyunsaturate to saturate, the P:S ratio of the diet, provides a simple explanation. For it is only with the

12% FO diet that the P:S ratio exceeds that of the REF diet used in the previous chapters (and then only slightly). In this present study it was observed that: a significant reduction in extracellular [K^+] during ischaemia; a marked reduction in extracellular lactate in all perfusion phases; and a significant reduction in control and reperfusion creatine kinase release was observed with as little as 3% FO (with 9% SAT) compared to 12% SAT. Indeed, with these and other parameters many marked effects were observed with 3% while with further increments of %FO the benefits produced were often only marginally greater. It is therefore likely that the cardiac mechanical and metabolic alterations resulting from the replacement of SAT diet with FO is more related to the presence of dietary n-3 polyunsaturates of marine origin rather than the reduction of saturated fatty acids. However, in these experiments, the confounding nature of the alteration to the 2 dietary variables SAT and FO which could not be avoided without altering another variable, means that the reduction in SAT proportions can not be totally excluded as a contributing cause of the beneficial attributes of fish oil supplementation.

Burr, *et al.*(**1989**), conducted a randomised, controlled dietary intervention clinical trial for the secondary prevention of myocardial infarction in over 2000 men for 2 years. It was found that advice to generally reduce dietary fat intake was no more effective than no dietary fat advice for the prevention of subsequent myocardial reinfarction (non-fatal) or ischaemic heart disease fatality. However, a group given advice to add a modest amount of fish (200-400g/week) to their diets experienced fewer fatalities despite having

no reduction in the incidence of ischaemic events compared to those not given this advice.

The efficacy of lower level fish oil supplementation (even with 9%SAT), to alter cardiac mechanical function and metabolism following a diet rich in saturated fats as seen in this chapter indicates the benefits of dietary change, even after only 6 weeks. Moreover these results suggest that the effects of high dose n-3 fatty acid supplementation on heart function described throughout this thesis and in other studies (**McLennanetal'85-90**) can be attained with low (clinically achievable) doses, much as has been observed in some human studies (**Burr,*et al.*,1989;Kromhout,*et al.*,*1985***).

IX. GENERAL DISCUSSION & CONCLUSIONS

IX. 1. Introduction

At the outset of this study it was evident that ischaemic coronary heart heart disease (CHD) was and still remains the major cause of death in Australia and the western nations of North America and Europe. This is closely related to atherosclerosis and/or thrombosis that leads to coronary artery narrowing and eventual coronary occlusion. The altered interaction between plasma triglycerides, lipoproteins, platelets, monocytes, and arterial endothelium and smooth muscle antecedes the formation of lipid and cholesterol deposits, foam cells, plaques and blood clots in atherosclerosis and thrombogenesis (**Leaf & Weber,1988;Harris,1989;Knapp,1990**). Although a number of risk factors for CHD such as age, heredity, gender, psychosocial stress, cigarette smoking, obesity, exercise, heart rate, ECG abnormalities, systolic blood pressure, serum cholesterol and triglycerides are recognised, little is known to predict the onset of sudden cardiac death (SCD) which can afflict a large proportion of those with high CHD risk (**Myerburg & Castellanos,1988;Leeder & Wilson,1988**). In recent years epidemiological consideration of the positive association between fish consumption and reduced incidence of atherosclerosis and CHD mortality has led to a renewed interest in the n-3 polyunsaturated fatty acids, particularly those predominant in fish of marine origin (**Dyerburg & Jorgensen,1982;Kagawa,*et al.*,1982; Goodnight,*et al.*,1982**). In contrast, the contribution of dietary saturated fatty acids and cholesterol to facilitate atherogenesis has been recognised

(Steinberg,1988;Bonanome & Grundy,1988). It is thus feasible that a reduced incidence of CHD may occur by nutritional means. In the long term, energy and funds spent on providing nutritional advice to entire nations of people may be more successful and less expensive compared to the identification, treatment and intensive care of high risk individuals. Nutritional behaviour modification for the prevention or decline in CHD, if effective, would indirectly lead to a reduced incidence of SCD.

The role of dietary fat influence and metabolism on myocardial function has undergone limited examination, particularly in relation to pathophysiological stress. Even in a 1991 review, the influence of dietary factors on cardiovascular responses to stress received limited consideration **(Herd,1991)**. Indeed, in this review the only dietary factor discussed was the effect of sodium consumption. However, while the majority of research effort has concentrated on atherosclerosis and thrombosis, the investigation of potential direct effects on myocardial function by dietary fatty acids has been limited. This is despite a number of discrete findings that modification of dietary fat type may change the myocardial cell membrane phospholipid fatty acid profile and cell function. Altered myocardial membrane fatty acid composition has been shown to influence membrane fluidity, ion fluxes, membrane enzyme and receptor systems and eicosanoid metabolism. Most often these *in vitro* observations cannot be directly associated with physiological outcomes, being conducted with purified enzyme or membrane systems devoid of *in vivo* modulators. Importantly, such changes to the dietary fatty acid profile have been shown to directly influence myocardial vulnerability to

arrhythmic stimuli in whole animal models without atherosclerotic pathology. The details of these previous findings were discussed in Chapter I, III and IV.

The aim of this thesis, in the face of these previous findings, was to closely investigate the direct effects of dietary saturated animal fat and polyunsaturated fish oil on cardiac function and metabolism, particularly during the stress of ischaemia, reperfusion, increased preload, and programmed ventricular electrical stimulation of arrhythmias. For even with long-term dietary supplementation there have been no reports of outward signs of cardiac or any other disease. Significant differences in cardiac performance and metabolism have been observed between the dietary groups in this study. They were subsequently examined more closely by the modulation of intracellular calcium handling and metabolism as a potential mechanism underlying differences. The results described in this thesis were obtained using a unique model of maintained afterload global ischaemia in the erythrocyte perfused isolated working rat heart, developed especially for this study in order to:

(a) eliminate the neural and humoral limitations of whole animal models;

(b) overcome physiological oxygen utilisation and colloid osmotic pressure restrictions in Krebs-Henseleit or Tyrode's solution perfused isolated hearts and **(c)** permit precise control over temperature, humidity, preload and graded ischaemia while maintaining a normal workload. It is likely that many of the principal findings of this thesis would not have been possible with alternative models. These findings are summarised and discussed below.

IX. 2. The Erythrocyte Perfused Isolated Working Rat Heart

In Chapter II the performance of erythrocyte (RBC) perfused (40% haematocrit) working hearts was compared to that of Krebs-Henseleit solution (K-H) perfused hearts. The K-H perfused hearts were found to incur significantly greater oedema than RBC perfused hearts that contained dextran for the same period of perfusion and ischaemia. Despite K-H buffer containing an arterial PO_2 more than three times that of RBC it had only 1/15th the arterial content. This was reflected in the observation that K-H perfused hearts extracted over 75% of available oxygen in contrast to 15% in RBC perfused working hearts, however, in absolute terms more than three times as much oxygen was extracted by RBC perfused hearts. In addition, during control perfusion K-H hearts had significantly greater coronary flow (above physiological levels) and heart rates but significantly lower aortic output, contractility and MVO_2. The measures of MVO_2, cardiac output and external work in these experiments utilising perfusate with physiologically relevant oxygen delivery and viscosity were similar to those measured *in vivo* (**Duvelleroy,*et al.*,1976**).

Oxygen consumption during ischaemia was paradoxically increased (despite reduced external work and contractility) in RBC but not significantly in K-H perfused hearts. These results along with the higher coronary flow during control perfusion and the limited coronary hyperaemia towards the end of the ischaemic period in K-H hearts indicated that K-H perfused hearts had restricted oxygen availability during control perfusion and less scope for adaptive changes during ischaemia compared to RBC perfused hearts.

The autoregulatory capacity of erythrocyte perfused isolated hearts was most elegantly illustrated in a recent study by Olders, *et al.*(**1990**). They clearly demonstrated that erythrocyte perfused hearts had the capacity to improve their oxygen supply during hypoxia by vasodilation to increase coronary flow. In contrast, Tyrode perfused hearts having a limited oxygen supply already were unable to increase coronary flow further in compensation as it was already close to maximal. The importance of a capacity for such adaptive changes became most apparent in the evaluation of dietary effects in the present study.

A similar finding was also observed by Van Beek (**1989**) in the isolated rabbit heart. Indeed Bergman, *et al.*(**1979**), also observed similar results but found that when haematocrit was raised from 25% to 40%, oxygen uptake increased by 60% without an increase in coronary flow indicating that to some extent oxygen limitation may have been present even at 25% haematocrit. The physiological and metabolic function of hearts in the study of Olders and coworkers (**1990**) which used a 25% haematocrit were slightly depressed compared with the present study with 40% haematocrit. Although there is some disagreement about the oxygen delivery adequacy of electrolyte buffers (**Opie,1984**), the general conclusion supported by this study is that the isolated working heart with a relevant workload is at least borderline hypoxic (**Duvelleroy,*et al.*,1976;Gaudel,*et al.*,1982;1985;Gibbs,1980;Figulla,*et al.*,1983;Robiolio,*et al.*,1989;Olders,*et al.*,1990;Bergman,*et al.*,1979;VanBeek,1989**).

These experiments also confirmed the intuitive expectation that RBC perfused working hearts made globally ischaemic with the maintained afterload

configuration would produce a more severe ischaemia compared to the reduced afterload ischaemia configuration when normalised for heart rate. Indeed, the hearts in the reduced afterload ischaemia configuration group had significantly lower oxygen extraction and consumption, greater contractility and aortic output and higher venous pH during ischaemia. The paradoxically increased post-ischaemic oxygen consumption was less evident in reduced afterload hearts compared to maintained afterload hearts and functional recovery was better. It is apparent that the reduced afterload configuration provides a degree of protection against the stress of ischaemia.

The post-ischaemic MVO_2 (RBC perfusion) was considered paradoxical because it was significantly but unexpectedly increased above control levels even though mechanical performance had not fully recovered. Although a small number of researchers have also observed this finding in reperfused hearts (**Krause,*et al.*,1986;Krukenkamp,*et al.*,1986;Stahl,*et al.*,1988; Laster,*et al.*,1989**), few have considered this closely for possible underlying mechanisms. Moreover, these experiments showed that this phenomenon is not limited to reperfusion but can also be observed in response to low flow global ischaemia in physiologically sound hearts that are capable of adaptive changes in oxygen extraction.

The use of a low flow global model additionally ensured that ventricular function did not cease immediately upon induction of ischaemia thus permitting continued assessment during the development of ischaemic consequences. Manning & Hearse (**1980**) have proposed that a low flow global ischaemia model may have greater physiological relevance over total zero flow

ischaemia because coronary flow has not been found to completely cease even in severely ischaemic tissue. This model may resemble angina or silent ischaemia where ischaemia can occur with maintained (or increased) workload. Together these critical model attributes have enable the successful observation of changes in cardiac function and metabolism following alterations in dietary fat type, ischaemia, reperfusion and preload, especially the paradoxical increase in MVO_2 during ischaemia and reperfusion.

IX. 3. Dietary Fatty Acid Influence on Cardiac Function

The results of this thesis have shown that altering the dietary fatty acid profile lead to changes in the composition of the cardiac membrane phospholipids which were associated with altered haemodynamic performance, metabolism and susceptibility to the stresses of ischaemia, increased preload, electrical stimulation of arrhythmias and reperfusion in these electrically paced working hearts. Despite cardiac output and external work being similar in SAT, REF and FO hearts during control perfusion, SAT hearts had significantly increased MVO_2 achieved by increasing both coronary flow and the percentage oxygen extraction. The high MVO_2 resulted in reduced energy utilisation efficiency and occurred despite lower contractility in SAT hearts compared to REF and FO hearts. Even during control perfusion significant creatine phosphokinase release into coronary effluent (a clinical marker of myocardial cell injury), was observed in SAT hearts. In contrast, FO hearts during control perfusion had a low MVO_2, significantly greater contractility, the lowest creatine kinase and lactate output and a higher energy utilisation efficiency. Therefore while

outwardly giving the appearance of healthy function, numerous signs suggested that SAT hearts may have been at increased risk to marginal stresses. Indeed, under control perfusion conditions, programmed electrical stimulation with even a small current could induce fibrillation in SAT and REF hearts whereas a significantly higher current was required in FO hearts. Notably, the foregoing observations were made with the hearts paced at a constant rate. Under control conditions spontaneous heart beat rates were significantly higher in SAT (approx. 300 bpm) compared to FO hearts (approx. 250 bpm). Differences in MVO_2 and biochemical markers may have been even more marked at these disparate spontaneous heart rates. It has been demonstrated that significantly increased heart rates aggravate cardiac ischaemia and worsen occlusion-induced arrhythmias (**Scherlag,*et al.*,1970;Kent,*et al.*,1973**) while reduced heart rate may provide protection against reperfusion induced arrhythmias (**Tosaki,*et al.*,1988**). This effect is possibly related to increased time in systole thus reducing coronary perfusion and augmenting the degree of ischaemia as well as increasing energy requirements at higher work rates (See Chapter II,IV).

Despite reductions in coronary flow and contractile function to similar low levels, the greatest differences between the dietary groups were observed in the metabolic parameters during ischaemia and reperfusion. Extracellular $[K^+]$, creatine kinase release, lactate production and venous acidosis were significantly greater in SAT hearts than FO hearts during ischaemia and reperfusion. Following reperfusion the coronary effluent content of these returned close to control levels in FO hearts, to a lesser extent in REF and

least in SAT hearts. Functional recovery was also better in FO hearts. Notably, MVO_2 paradoxically rose in FO and REF hearts during ischaemia because of the capacity to increase oxygen extraction. Although oxygen extraction during ischaemia rose also in SAT hearts it was insufficient to overcome the reduction in coronary flow. Despite the reduced MVO_2 in ischaemic SAT hearts, this was still significantly greater than the MVO_2 of ischaemic REF and FO hearts. After reperfusion, MVO_2 was increased in SAT hearts and remained elevated above control levels in REF and FO hearts. The responses of erythrocyte perfused SAT hearts to ischaemia were similar to those observed with erythrocyte-free buffer perfusion. Complete autoregulatory changes were not possible. This was further emphasised by the inability of hydralazine to induce further increases in coronary flow in SAT hearts. **It is most unlikely that the dietary and ischaemic differences in MVO_2 would have been observed with a low oxygen carrying perfusate.**

Coincident with the large differences in venous blood chemistry was reduced vulnerability of FO hearts to programmed electrical stimulation in ischaemia and to spontaneous arrhythmia generation, particularly ventricular fibrillation in reperfusion. No spontaneous ventricular fibrillation was observed in any FO heart. In contrast to the protective attributes of FO, dietary SAT treatment was pro-arrhythmogenic.

Functional and metabolic differences between the dietary groups were investigated further by studying the effect of altered preload on cardiac function. Although classical Frank-Starling curves for cardiac output, contractility and external work were observed, SAT hearts exhibited less

improvement in contractility and external work in response to increases in preload than did FO hearts. The decline in contractility, external work and cardiac output at high preload was less evident in FO hearts and cardiac output even increased further. As preload was increased MVO_2 also increased in all dietary groups. However, the dietary differences were maintained across the preload range.

The effect of ischaemia with high preload further illustrated the greater vulnerability of SAT hearts to stress. The combination of limited oxygen delivery with high workload amplified the metabolic consequences to increase lactate production, $[K^+]$ and creatine kinase release in SAT hearts. On the otherhand FO hearts maintained a higher performance even with the extra stress of increased preload during ischaemia. In particular, FO hearts had the capacity for greater external work, contractility, cardiac output, and energy utilisation efficiency with comparatively lower MVO_2, acidosis, and coronary effluent lactate, $[K^+]$ and creatine kinase compared to REF and SAT hearts.

When graded ischaemia was imposed in erythrocyte perfused isolated working hearts with maintained afterload and preload, the coronary flow, contractility and MVO_2 were altered over the range of decreasing coronary perfusion pressure in the manner of a "reverse" Gregg's Phenomenon **(Gregg,1967)**. The MVO_2 proportionally declined with reduced oxygen delivery and perhaps as a consequence of reduced oxygen demand following the proportionate decline in contractility, external work and cardiac output. Although MVO_2 and the % oxygen extraction remained significantly higher in SAT hearts compared to REF and FO hearts, FO hearts maintained higher

contractility, external work, cardiac output and % energy utilisation efficiency. Lactate production and creatine kinase release were significantly increased with increasing ischaemic stress, particularly in SAT hearts. Yet again, it was evident that SAT hearts were more vulnerable to ischaemia than FO hearts.

Thus it was demonstrated in this thesis that alteration of the type of dietary lipid intake could influence ventricular performance and metabolism and the underlying differences were augmented and more distinctly characterised with increased preload and ischaemic stress as well as electrical ventricular stimulation. These findings have not been clearly demonstrated by others perhaps because of a number of critical factors. Although DeDeckere & Ten Hoor **(1979;1980)** showed that with increased preload the external work curves of hearts isolated from polyunsaturated sunflower seed oil fed rats were significantly higher than lard fed animals, further extensive differences were not reported. These workers did not provide oxygenation with an erythrocyte buffer, used a low afterload, short feeding periods and very young rats as subjects. In addition, ischaemic stress was not investigated and heart rate varied spontaneously to confound the results. Chardigny, *et al.*(**1988**), and Chardigny & Moreau (**1991**), also observed limited dietary fat influence on cardiac performance. In their 1988 study, using isolated working hearts perfused with a low glucose (5.5mM) Krebs-Henseleit solution, a saturated fatty acid rich diet significantly increased heart rate and reduced cardiac output compared to sunflower seed oil. In addition arrhythmias invoked by coronary ligation were predominant in the saturated diet group but

not the polyunsaturated group. In contrast to fish oil in the present study, coronary flow and oxygen consumption was significantly raised in the polyunsaturated diet group. It is difficult to assess whether the differences attributable to the acute (7 day) ingestion of an essential fatty acid deficient diet (their saturated fat diet) are comparable to manipulations within a sufficient range of essential dietary fatty acids. The 1991 report by the same group using the same model showed that fish oil diet did not differ substantially from control or saturated fat dietary groups. They concluded that n-3 polyunsaturated fatty acids must exert their reported protective effects because of their anti-atheroslerotic, anti-thrombotic and hypotriglyceridemic attributes rather than any direct cardiac effects. However, considering all the evidence including this thesis, their results (or lack thereof) are more likely to be due to limitations in the experimental model, dietary model and feeding period, increasing the likelihood that the earlier findings related to essential fatty acid deficiency.

Attribution of the current results to direct myocardial effects is supported by the failure of even long term feeding of the saturated diet used herein to produce atherosclerosis, fat deposition or any other vascular pathology in the rat that could differentiate it from fish oil or other diets. **(Turner,*et al.*,1990)**.

Another very recent study by Demaison and Grynberg **(1991)**, compared diets containing 10% sunflower seed oil (18:2, n-6) or 10% linseed oil (18:3, n-3) as the sole lipid source fed to weanling rats for 8 weeks. The hearts were isolated and perfused by Krebs-Henseleit solution in working heart

mode. Although heart rate and 1-^{14}C palmitate oxidation were significantly lower in the n-3 rich dietary group, cardiac output and work were not affected. These workers did not investigate myocardial oxygen consumption, but claimed that Krebs-Henseleit solution provided adequate oxygen to the working heart. Although their description of the methods implemented is very brief and vague it is likely that the experiment was limited by the experimental model and design outlined previously. These workers did not stress their working heart preparation with either increased preload, workload, heart rate, electrical ventricular stimulation or ischaemia thus limiting the chances of precipitating or augmenting functional differences.

It is evident that the electrically paced, erythrocyte perfused isolated working heart model with optimal oxygen and nutrient delivery, temperature, humidity including maintained workload and preload without neural or humoral influence was a critical foundation for this thesis. Equally important, was the dietary model, age and species of animal used. The ability to produce fundamental cardiac membrane and cardiac function differences in the absence of atherosclerosis or other vascular pathology was also critical for demonstrating a direct influence of dietary fat on cardiac performance. It was also observed that cardiac stress imbalanced the complex auto-compensatory mechanisms operative in the maintenance of demand-determined heart performance and metabolism thus producing a distinct performance reduction. Under these conditions the dietary fat influence on cardiac function was characterised and was most distinct. It was apparent that myocardial oxygen demand may have been an important contributor to substrate arrhythmia

vulnerability as the high oxygen demand of SAT hearts was associated with increased arrhythmias. Indeed, Poole-Wilson (**1990**) proposed, on the basis of a number of clinical trial studies, that the oxygen consumption at the onset of ischaemia may be a determinant contributing to the size of myocardial infarct. Thus, SAT hearts may be subject to greater infarction following long term ischaemia because of their higher oxygen requirements than FO hearts. This remains to be investigated.

IX. 4. Study Of Mechanisms Underlying Altered Oxygen Metabolism

The paradoxical effects of dietary lipids and ischaemia/reperfusion on myocardial oxygen consumption became the chief focus of this study. A possible difference in basal requirements independent of activation or contraction was discounted when no significant dietary difference in the basal MVO_2 of control and post-ischaemic hearts was observed after KCl arrest. Nor was there a significant effect of ischaemia on basal oxygen metabolism despite the paradoxical increase in post-ischaemic MVO_2.

The possibility that the greater coronary flow could be directly responsible for the greater MVO_2 in SAT hearts was also discounted when maximum vasodilation with hydralazine failed to abolish the differences. However, hydralazine did attenuate the MVO_2 difference between SAT and the other dietary groups. The MVO_2 was raised in REF and FO hearts primarily by raising coronary flow and thus increased oxygen delivery. In addition, hydralazine had a confounding effect of markedly reducing MVO_2 in SAT hearts without significant coronary flow alteration. Although the

molecular mode of hydralazine action in the heart is currently not clearly defined, it is possible that hydralazine influenced intracellular calcium fluxes in myocardial cells (**McLean,*et al.*,1978;Lipe & Mould,1981;Morita,*et al.*,1988;Rendig,*et al.*, 1988**), thereby depressing activational oxygen requirements. Despite this MVO_2 attenuation in SAT hearts, acidosis, lactate release and extracellular [K^+] were significantly augmented even with hydralazine-induced coronary flow equalisation between the dietary groups. The increased coronary flow was a response to, rather than a cause of, the high MVO_2 SAT hearts.

Intracellular acidosis during ischaemia may stimulate intracellular calcium content by altering Na^+/H^+ and Na^+/Ca^{++} exchange; mitochondrial release of Ca^{++} and its displacement by H^+ in the cytoplasm; and inhibit myofibrillar responsiveness to calcium. Intracellular calcium overload has been observed as a consequence of ischaemia and reperfusion (See Chapter VI). This suggested that altered intracellular calcium handling may be related to either or both the abnormal MVO_2 of SAT hearts and the paradoxical increase in MVO_2 during and after ischaemia in all dietary groups.

Ryanodine (RY) and ruthenium red (RR) were used as tools to modify the calcium handling of sarcoplasmic reticulum and mitochondria respectively (See Chapter VI). Both the paradoxical increase in MVO_2 during ischaemia and reperfusion in addition to the abnormally high MVO_2 in SAT hearts were abolished by RR, a putative inhibitor of calcium uptake into mitochondria (**Moore,1971;Vasington,1972;Gupta,*et al.*,1989**). Even the augmentation of the intrinsically high MVO_2 of SAT hearts by elevated extracellular Ca^{++} was

prevented by RR. These effects were not due to depression of contractile function because they were not shared by RY, a proposed inhibitor of SR calcium release (**Hansford & Lakatta,1987; Fabiato,1985**). Ruthenium red was effective in inhibiting arrhythmias in the presence of low but not high extracellular calcium perfusion. However, RY completely abolished the high incidence of arrhythmias in SAT hearts during ischaemia and reperfusion even in the presence of high extracellular calcium. The findings of this extensive portion of the study suggest that the paradoxical ischaemia-induced increase in MVO_2 and the dietary-SAT induced abnormal MVO_2 may both be related to excess uptake of calcium into mitochondria. In addition, the ability of RY to abolish the heightened arrhythmia vulnerability in SAT hearts points to calcium overload in SR (**Thandroyen,*et al.*,1988; Kort & Lakatta,1984a;-1984b;Lakatta,*et al.*,1985**). Together the results suggest that SAT hearts may have elevated intracellular calcium which gets sequestered into SR and mitochondria to produce the observed effects. The confounding reduction in SAT MVO_2 by hydralazine could have been due to a calcium antagonist action that has been reported in vascular smooth muscle.

IX. 5. Potential Mechanisms of Altered Calcium Metabolism

The results presented in Chapter VI, together, indicated that the ischaemia-induced paradoxical increase in MVO_2 and the SAT-induced increase in MVO_2 may be related to altered mitochondrial uptake of Ca^{++} whereas the 6mM Ca^{++}-induced increase in MVO_2 may be mainly related to contraction (possibly by activation of actomyosin ATPase) or is possibly SR-Ca^{++}

release/reuptake related (Ca^{++} ATPase). The thesis results suggested that calcium may be a mediator of dietary altered cardiac and metabolic function. Although not the subject of direct investigation in this study the following subsections will consider the relationship between dietary fatty acids, eicosanoids, free radicals and myocardial ischaemia that may provide a basis for the altered calcium metabolism observed in the findings of this thesis.

IX. 5. a) Dietary Fatty Acid Effect on Eicosanoids

All eicosanoids (prostaglandins, thromboxanes, and leukotrienes) have polyunsaturated fatty acid precursors. In example, arachidonic acid (AA), the principal prostaglandin precursor, can be obtained directly from the diet but mainly comes from desaturation and chain elongation of linoleic acid (**Hassam & Crawford,1976**). Arachidonate is predominantly cell membrane bound in phospholipids and intracellular free fatty acids are present in very low concentrations (**Vogt,1978**). Intracellular accumulation of AA has been shown to be increased even by brief ischaemia (**Hsueh & Needleman,1978**). The activation of phospholipase A_2 to cleave the ß position of the phospholipid to liberate increased levels of esterified AA has been shown to be under the regulatory control of a number of hormones including the glucocorticoids and has been shown to be increased by ischaemia (**Berger,*et al.*,1976;Kraemer,*et al.*, 1976;Blackwell,*et al.*,1980**). Activation of phospholipases and the production of eicosanoids is also associated with the ischaemic process that may lead to loss of cell viability and the release of cytosolic enzymes such as creatine kinase and lactate dehydrogenase (**Jackson,*et al.*,1987**). Notably,

increased intracellular calcium (as observed in reperfusion calcium overload) has been proposed to increase phospholipase A_2 activity (**Karmazyn,1986a**). This of course raises the question of whether dietary lipids modulate calcium entry which in-turn facilitates eicosanoid production, or differential eicosanoid production modulates calcium entry producing a feedback loop.

The cyclooxygenase and lipoxygenase metabolites of AA and other polyunsaturates have been associated with thrombosis, myocardial ischaemia and infarction and possess a wide range of pharmacological properties. For extensive reviews see **Ogletree,1987;Karmazyn,1989;Weber,1987; Simmett & Peskar,1986;Leaf & Weber,1988;Mehta & Nichols,1990;Fauler & Frolich,1989**). Cyclooxygenase products such as the prostaglandins or thromboxane posses varying degrees of vasoactive or platelet aggregatory activity. A principal mediator of vascular thrombosis, thromboxane A_2, has been found to be a potent vasoconstrictor, platelet aggregator and proarrhythmic (**Lefer,1985;Ogletree,1987;Parratt,*et al.*,1987**). Prostacyclin (prostaglandin I_2), mainly produced by vascular endothelium, has been shown to be a potent vasodilator, inhibitor of platelet aggregation and to possess antiarrhythmic properties (**Araki & Lefer,1980;Parratt,*et al.*,1987**). Both appear to be produced by myocardium and the balance changed in favour of thromboxane by saturated fat treatment and in favour of prostacyclin by fish oil treatment in a dietary model similar to this thesis (**Abeywardena,*et al.*,1986;1987**).

A number of events in myocardial infarction resemble those of acute inflammation, e.g. platelet, leukocyte, macrophage and phagocyte infiltration of cells (**Davies,*et al.*,1981**). These have been also associated with non-

necrotic myocardial tissue damage and functional detriment (**Rheimer & Jennings,1979**). It has been observed that aspirin and other nonsteroidal anti-inflammatory drugs can inhibit cyclooxygenase function (**Abramson,*et al.*,1985**). Lipoxygenase, present in ischaemia activated polymorphonuclear leukocytes or neutrophils convert AA to hydroxy fatty acids and leukotrienes which have also been purported to exert negative inotropic effects and coronary constriction in isolated hearts (**Letts & Pipier,1982;Hattori & Levi,1984;Lefer & Yanagisawa,1986**). It is has been proposed that leukotrienes may reduce transsarcolemmal calcium influx (**Hattori & Levi, 1984**). See Fauler & Frolich (**1989**) for a review. It is not known whether these substances can be produced by myocardium and they may not be a factor in the observations made using the *in vitro* system of this study.

Dusting and coworkers (**1979**) proposed that an unfavourable balance between prostaglandin I_2/thromboxane A_2 contributed to the development of atherosclerosis and CHD. Atherosclerosis in clinical and animal experiments has been associated with reduced prostacyclin and elevated thromboxane A_2 thus the pro-thrombotic, vasoconstrictor and pro-arrhythmic attributes of the latter detrimentally influence cardiac function (**Bunting,*et al.*,1983;Simmet & Peskar,1986;Lefer,1985;Ogletree,1987;Parrat,*et al.*,1987**). Although some report that thromboxane B_2 (the highly stable thromboxane A_2 hydrolysis product) is not biologically active it has also been reported by others to have a positive inotropic effect in isolated rat heart that is non-competitively antagonised by propranolol. In addition, thromboxane B_2 was also found to inhibit Na^+/K^+-ATPase (**Pascual,*et al.*,1988**). It has also been suggested that

increased prostaglandin $F_{1\alpha}$ and thromboxane B_2 contribute to reperfusion injury (**Karmazyn,1986;Farber & Gross,1990**). Karmazyn & Neely (**1989**), observed in isolated working hearts that exogenous prostacyclin reduced post-ischaemic recovery of aortic output and increased the extent of ischaemia-induced lactate production. These effects of prostacyclin were prevented with verapamil treatment by blocking slow calcium channel activity. Okada (**1991**), observed that the production of a number of prostaglandins (E_2, $F_{1\alpha}$) during hypoxia were associated with the coronary vasodilation in perfused isolated rat hearts.

The protective effects of fish oils, rich in eicosapentaenoic acid (EPA, 20:5, n-3) may be related to the production of eicosanoids with reduced toxic action. It has been observed that EPA is a substrate for cyclooxygenase production of thromboxane A_3 which possesses little biological activity (**Fisher & Weber,1984**). The production of this metabolite in the place of thromboxane A_2 is considered beneficial and preferable (**Lorenz,1983**). Similarly, lipoxygenase facilitated conversion of EPA to various leukotrienes that do not produce deleterious cardiac effects is also considered attractive (**Strasser,*et al.*,1985;Lee,*et al.*,1984**). It has been proposed that dietary supplementation with saturated fatty acids alters the balance of myocardial eicosanoid production in favour of thromboxane while fish oil promotes a balance in favour of prostacyclin (**Abeywardena,*et al.*,1991a;1991b**). These authors suggest dietary fish oil supplementation may not only replace arachidonate with n-3 fatty acids as a substrate but may specifically inhibit the thromboxane A_2 synthetase complex.

The defective oxidative phosphorylation of mitochondria isolated from reperfused hearts has been associated with intracellular calcium overload, including mitochondrial calcium accumulation. Treatment with prostaglandin synthesis inhibitors increases post-ischaemic ventricular function during reperfusion (**Karmazyn,1986;Moffat,*et al.*,1985**). It has been suggested by these studies that endogenous prostaglandins may participate in reperfusion-related mitochondrial dysfunction. Indeed, McLennan and coworkers (**1987**) observed that papillary muscles isolated from tuna fish oil supplemented rats were less sensitive to the positive inotropic response of calcium and less prone to tachyarrhythmias under isoprenaline treatment compared to papillary muscle preparations from reference diet or saturated fat diet supplemented rats. Treatment with indomethacin reduced the extent of the observed effects in the dietary saturated fat and reference groups but had no effect in the fish oil treated group. Indomethacin at the concentration used in this experiment was proposed to inhibit cyclooxygenase activity but may have been sufficient to directly influence calcium transport (**Flower,1972;Northover,1972;Burch,*et al.*,1983**). Prostaglandins have been shown to augment myocardial mitochondrial respiration but only in association with elevated calcium concentrations (**Karamzyn,1986**). Kirtland & Baum (**1972**) have suggested that prostaglandin E_1 increases calcium binding to the inner mitochondrial membrane as would a calcium ionophore. Presumably this may be possible because of the highly lipophilic nature of this prostaglandin (**Carston & Miller,1977**). Although the mechanism remains undefined, prostaglandins

were clearly able to alter cardiac mitochondrial respiration by facilitating calcium-related changes to respiration.

The extent and nature of nonvascular eicosanoid production and function in the isolated working heart and *in vivo* remains to be defined. It is evident that eicosanoid metabolism by virtue of its relationship to fatty acid metabolism could play a critical role in cardiac function during dietary fat modulation and during ischaemia/reperfusion. A number of such aspects just discussed may have contributed to the results observed in this thesis.

IX. 5. b) Direct Intracellular Fatty Acid Interactions

The predominance of research interest in fatty acids has been focused on their role as metabolic substrate or in anabolism. However, it has been recognised that fatty acids are not only important structural components but also regulate membrane fluidity and permeability, ion transport systems and the function of membrane bound enzymes (**Egwin & Kammerow,1972;Farias,*et al.*,1975;-Spector & Yorek,1985**). Below, considerable evidence from diffuse studies have been brought together in order to elucidate potential mechanisms of dietary fatty acid influence in this thesis. It has been suggested that amphipathic metabolites such as the lysophospholipids or acyl carnitine (**Katz & Meissineo,1981;Corr,*et al.*,1984**) participate in the membrane dysfunction of myocardial ischaemia. These have been shown to accumulate during ischaemia (**Corr,*et al.*,1982;Steenburger & Jennings,1984**) and be involved in the precipitation of cardiac arrhythmias (**Corr,*et al.*,1981;Man & Choy, 1982;**

Kinnaird,*et al.*,1988;Man,1988). How they may be differentially modulated by dietary fatty acid types is less clear.

Although free fatty acids have been proposed to be arrhythmogenic **(Opie & Lubbe,1975;Corr,*et al.*,1984)**, it is unlikely that these could have been present in significant quantities at the commencement of perfusion with washed erythrocyte buffer in isolated working hearts from fasted rats utilised in this thesis. It has however, been observed that phospholipase A_2 can facilitate the generation of free lysophospholipids and fatty acids within 10 min of ischaemia **(Corr,*et al.*,1982;Lenzen,*et al.*,1989;Rustenbeck & Lenzen,1989)**. Notably, it has also been observed *in vitro* that cis-unsaturated oleic, linoleic, linolenic and arachidonic acids dose-dependently inhibited myocardial phospholipase A_2 lipid catalysis whereas palmitic acid was non-inhibitory **(Franson,*et al.*,1989)**. The reduction of pH to 4.5 (as in ischaemia) reversed the effect of the unsaturated fatty acids on phospholipase A_2.

Opie **(1988)**, proposed that high levels of free fatty acids may be mildly arrhythmic but may also be "sensitising" agents and markers of enhanced catecholamine activity, increased coronary venous hyperkalaemia and intracellular enzyme loss, in addition to reduced mechanical efficiency of work. At very high concentrations free fatty acids can impair left ventricular function and augment myocardial oxygen consumption **(Simonsen & Kjekshus,1978)**. Perhaps such levels could be achieved locally under physiological conditions set in the current study. While such concentrations posses detergent-like membrane destructive actions, lower concentrations of free fatty acids and lysophospholipids exert complex affects related to their hydrophobic

and hydrophilic moieties. Murnaghan (**1981**), demonstrated that hypoxia-reduced ventricular arrhythmia threshold in Langendorff perfused rabbit heart was potentiated by perfusion with saturated and monounsaturated fatty acids. This effect was antagonised by the polyunsaturated fatty acids, linolenic (18:3) and linoleic acids (18:2). These results have similarities with those observed following programmed electrical stimulation in ischaemic SAT and FO hearts of Chapter IV.

Lysophosphatidyl choline (**Karli,*et al.*,1979**), acyl carnitine (**Adams,*et al.*,1979a;1979b**) and some fatty acids (**Lamers & Hulsmann,1977**) have been shown to inhibit Na^+/K^+ ATPase. However, Kim & Duff (**1990**) proposed that release of $[K^+]$ in ischaemia and reperfusion (as observed in this thesis) is partly related to the actions of free unsaturated arachidonic or linoleic acids and lysophospholipids but not saturated fatty acids to rapidly inhibited K^+ channel activity. Indeed, this may explain the increase in arrhythmias during 10-15 min ischaemia observed in this thesis, and in particular may contribute to increased arrhythmias in SAT hearts and a reduced incidence in FO hearts.

Philipson (**1984**) found that anionic and cationic amphiphiles stimulate and inhibit Na^+/Ca^{++} exchange respectively. These effects were correlated with cardiac contractility (**Philipson,*et al.*,1985;Burt,*et al.*,1984**). Philipson and coworkers (**1985**) found Na^+/Ca^{++} exchange to be more potently stimulated in cardiac sarcolemmal vesicles by unsaturated fatty acids than saturated fatty acids by relatively low concentrations. These authors proposed that bilayer hydrophobic region perturbations and increased anionic surface

charge along with fatty acid accumulation during ischaemia may alter sarcolemmal Ca^{++} transport.

The Ca^{++}/Mg^{++}-ATPase in cardiac SR has been shown to be modulated by fatty acid unsaturation and chain length, phospholipid composition and cholesterol (**Messineo,*et al.*,1980;Katz & Messineo,1981;1982;-Swanson,*et al.*,1989**). Ambudkar & coworkers (**1988**) observed that lysphopholipids inhibited Ca^{++}/Mg^{++}-ATPase activity and decreased calcium uptake in cardiac SR. However, others have also shown that dietary fish oil intake in rats resulted in significantly higher levels of docosahexaenoic and eicosapentaenoic acids (22:6 (n-3), 20:5 (n-3)) in cardiac SR phospholipids and associated with this was a lower relative activity of Ca^{++}/Mg^{++}-ATPase, a lower initial rate of calcium transport and increased calcium uptake in SR compared to dietary corn or olive oil (**Swanson,*et al.*,1989**).

Rustenbeck & Lenzen (**1989**) showed that arachidonate and lysophosphatidylcholine could each reduce the mitochondrial membrane potential and stimulate mitochondrial calcium release in liver cells. Calcium uptake in mitochondria isolated from liver can also be inhibited by a range of cis-unsaturated fatty acids (including the marine n-3 polyunsaturated fatty acids) and a range of lysophospholipids (**Lenzen,*et al.*,1989a;1989b**). Saturated fatty acids and trans-polyunsaturated fatty acids were relatively inactive. The effect of inhibition increased with the number of double bonds (unsaturation) and decreased with chain length, thus γ-linolenic acid was optimal while docosahexaenoic acid was comparatively less active. In contrast, Chow & Jondal (**1990**) demonstrated in an *in vitro* preparation of leukemic T cells that

polyunsaturated n-3 fatty acids induced rapid increases of cytosolic calcium that was not mitochondrial in origin and was not due to calcium intracellular influx but rather from endoplasmic reticulum via a mechanism independent of inositol lipid hydrolysis. The extent of calcium release was positively correlated to the degree of unsaturation and chain length.

While the majority of these observations have been made in highly purified subcellular systems and the exact nature of these mechanisms is yet to be clearly defined *in situ* of physiologically relevant cardiac preparations, it is clear that the intracellular actions of fatty acids could act to modulate cardiac function in a number of ways. Thus the dietary fatty acids used in the experiments of this thesis may also exert direct influence to alter intracellular handling of calcium, particularly during ischaemia and reperfusion.

IX. 5. c) Potential Role of Free Radical Metabolism

The superoxide free radicals produced by the incomplete reduction of oxygen can lead to the production of hydroxyl or other secondary free radicals. Free radicals are highly reactive species with unpaired outer shell electrons. Nearly all parts of the cell are subject to the highly destructive free radical reactions (**Ambrosio & Chiarello,1988**). These free radicals are produced *in vivo* and under normal conditions superoxide dismutase, catalase and glutathione peroxidase related reactions are capable of metabolising them to non-destructive forms. These protective enzymes may be functionally impaired by myocardial ischaemia (**McCord,1985**). The reperfusion injury that paradoxically occurs with the reintroduction of oxygen to the ischaemic heart has

been proposed to be mediated by increased free radical activity but some evidence suggests that this commences even during ischaemia (**Bolli,*et al.*,1988;1989;Kako,*et al.*,1987**). A number of studies have attributed reperfusion induced arrhythmias to free radical mediated injury because treatment with antioxidants or free radical "scavenging" enzymes have limited the extent of these arrhythmias in a number of ischaemic models and animal species (**Manning,*et al.*,1984;Bernier,*et al.*,1986;Chambers,*et al.*,1985; Woodward & Zacharia,1985**). Numerous studies have used this approach to demonstrate reduced infarct size and cellular necrosis following ischaemia and much of this effect has been associated with the mechanisms active during the inflammation process (**Jolly,*et al.*,1984;Ambrosio,*et al.*,1986;Zeier,*et al.*,1987;Simpson,*et al.*,1987;Mullane,*et al.*,1984;Romson,*et al.*,1983**).

It has been proposed that free radical injury may contribute to the reduced mechanical efficiency and increased oxidative metabolism characteristic of post-ischaemic hearts (**Bolli,*et al.*,1989**). In this thesis and other studies evidence suggests that polyunsaturates may be protective against ischaemia as observed by reduced extent of injury, improved cardiac output and post-ischaemic recovery, improved energy efficiency and reduced ischaemia/reperfusion arrhythmias. It is therefore confounding to find that susceptibility to free radical damage increases with increased unsaturation. Lipid peroxides and hydroperoxides are formed from oxygen radical reaction with polyunsaturates incorporated in membranes (**Kramer,*et al.*,1984**). These products have been found to be increased during reperfusion (**Romaschin,*et al.*,1987**). Hydroperoxides have been indirectly found to stimulate intracellular calcium overload

in normoperfused papillary muscles and myocytes and this could be prevented by ryanodine pretreatment (**Hayashi,*et al.*,1989;Nakaya,*et al.*,1987**). Part of this effect has been attributed to sarcolemmal calcium pump depression that has been observed following treatment of sarcolemmal vesicles with xanthine and xanthine oxidase or with hydrogen peroxide and catalase. Superoxide dismutase and mannitol were found to prevent calcium pump depression in this preparation (**Kaneko,*et al.*,1989**).

It has been observed that mitochondria possess high concentrations of polyunsaturated fatty acids predominantly incorporated as phosphatidylcholine and ethanolamine (approx.80% of total phospholipid). Cardiolipin, specific to mitochondria (approx. 18% of total phospholipid), consists of 90% un-saturated fatty acids (**Daum,1985**). Thus, lipid peroxidation in mitochondria has been considered by many as a major target for free radical generation both from *in vitro* and *in vivo* experiments (**Guarnieri,*et al.*,1983;Schlafer,*et al.*,1982;Ambrosio,*et al.*,1987a;1987b**). However, mitochondrial membrane fatty acid composition was not measured in this thesis and therefore whether the mitochondrial phospholipid composition was subject to dietary fat alteration is not known in this study. It is likely that the type and amount of specific fatty acids are regulated by structural and functional requirement limitations specific to the phospholipids of organelle membranes because it has been shown that alteration of dietary lipid type did not alter the distribution of phospholipids (**Charnock,*et al.*,1991**). Vlessis & Mela-Riker (**1989**) indirectly demonstrated hydrogen peroxide induced calcium efflux in preloaded liver mitochondria following xanthine and xanthine oxidase

reactions (which accumulate during ischaemia). This calcium efflux was proposed to stimulate calcium reuptake (separate to the efflux system) in mitochondria to initiate a futile calcium cycle that markedly increased mitochondrial oxygen consumption. This calcium efflux effect could be specifically inhibited by catalase and also dose dependently by exogenous ATP. The ATP inhibition of calcium efflux could be diminished by ruthenium red. Elevated H^+ and free fatty acids have been found to promote similar calcium efflux and it was suggested that these conditions (as in ischaemia) enhanced susceptibility to hydrogen peroxide induced calcium efflux. It is highly feasible that similar processes would be operative in ischaemic myocardium.

Although these findings may provide a basis for the paradoxically increased oxygen consumption during ischaemia and reperfusion it is difficult to find a potential basis for the high MVO_2 of SAT hearts and low MVO_2 of polyunsaturated hearts. If oxygen free radicals are involved in the development and progression of calcium overload because of their detection during reperfusion reoxygenation, the very high oxygen consumption in SAT hearts would be expected to increase the amount of oxygen potentially available for free radical generation. Conversely, FO hearts would generate fewer free radicals because of the lower oxygen metabolism. It is apparent that few studies have directly investigated the role of saturated fatty acids and the majority of reports assume that free radical generation detected in their experimental preparation is directly related to polyunsaturated metabolism. Few studies have addressed the possibilities of species-related differences in

free radical generation. Most of the evidence regarding free radical metabolism has been gleaned through *in vitro* preparations that may have limited physiological significance. In addition, past methods only demonstrated indirect detection by a number of reactive indicator solutions. The move to use magnetic electron spin resonance (ESR) for specific detection of unpaired electrons is a recent step forwards. Oxygen derived free radical production bursts measured in the coronary effluent of ischaemic myocardium by ESR are very short lived in concentrations less than 1μM and total tissue content is also very low. The role of free radicals in the development of calcium overload is questionable and remains to be elucidated (**Rao,*et al.*,1983;Garlick,*et al.*, 1987; Zweier,1988;Tani,1990**).

If long term saturated fatty acid intake has been associated with the development of atherosclerosis in humans it is likely that increased polymorphonuclear leukocytes and other blood cells involved with atherosclerosis, inflammatory response and myocardial infarction would be activated. These cells would be converting arachidonate (n-6 polyunsaturate more prevalent in SAT rather than FO hearts in this study) via lipoxygenase to the leukotrienes. Importantly, n-3 polyunsaturates (EPA, DHA) have been shown to competitively inhibit lipoxygenase and cyclooxygenase (**German,*et al.*,1987;88;-Lokesh,*et al.*,1988**). The leukocytes and other "white" blood cells have been demonstrated to be potent generators of superoxide free radicals (**Kinsella,*et al.*,1990;Leaf & Weber,1988**). Thus, by such mechanisms, saturated fatty acid rich diet could promote increased free radicals and decreased free radical generation by polyunsaturates. The experimental preparation used in this thesis

was devoid of all blood constituents except erythrocytes thus free radical generation was not possible by these means. These findings indicate that closer investigation of n-6 fatty acid metabolism in myocardial function is pressing. In addition, the specific role of n-3 polyunsaturates of marine origin in the generation of free radical damage is yet to be defined.

IX. 6. Future Directions

It is evident that the isolated working heart model of ischaemia developed for this thesis may have wide spread applications to many areas of physiological, pharmacological and biochemical cardiac research. In this thesis alone, many avenues of research remain unexplored. In particular, intracellular calcium content of the various dietary groups investigated in this thesis needs to be directly measured at the mitochondrial and SR level following isolation from control, ischaemic and reperfused isolated hearts in order to define possible dietary lipid and ischaemia induced alterations to intracellular calcium. Functional differences in calcium mediated respiration may be measured by utilising the methods of McCormack and associates **(1984-1990)** which permit assay of calcium-activated pyruvate dehydrogenase as well as other enzymes of oxidative metabolism. In addition to this, the role of eicosanoids could be studied by measuring their presence in coronary effluent and myocardial tissue with and without various inhibitors at a number of rate limiting steps of eicosanoid synthesis during ischaemia and reperfusion. This would extend the observations of Abeywardena, *et al.***(1987-1991)** made *in vitro* under highly controlled conditions. The role of free fatty acid infusion on the synthesis of

eicosanoids and/or free radicals could also be investigated in the erythrocyte perfused working heart. With all of these studies the critical role of calcium should be addressed. Finally, it is not possible to differentiate the effects of dietary fish oil n-3 fatty acids from polyunsaturates in general from the results presented here. This needs to be evaluated. However, on the basis of many different studies it is possible that n-3 and n-6 polyunsaturates may act quite differently.

The requirement of utilising an experimental model that is physiologically relevant *in vivo* is critical in order to assess pathological alteration to biological systems. It is clear that *in vivo*, complex multivariate factors are involved in cardiac function and the interaction between these is interdependent and simultaneous. This complexity makes it difficult to accurately control under experimental conditions and to assess experimental anomolies as error, artifact or related to some as yet unknown variable. Such complexity may well explain the difficulties involved with clinical trials that do not always support data obtained in other species. The erythrocyte perfused heart used in this thesis draws closer the gaps between Langendorff perfused hearts and *in vivo* cardiac function, permitting increased control of functional variables.

There is little published work with which to compare this study. It has demonstrated basic dietary-induced changes in oxygen requirements and progressed some little way towards the identification of intracellular mechanisms responsible. Although the study does not provide a definitive identification of the link between myocardial membrane fatty acid composition and intracellular functional changes, it does provide confirmation and a

possible basis for the widely reported antiarrhythmic or proarrhythmic actions of fish oil or saturated fatty acids respectively. Moreover the thesis poses the question as to whether diet can influence cardiac function in apparently healthy individuals, particularly under stress and is suggestive of potential roles in reducing the risk of sudden cardiac death in the prevention and therapy of heart failure. However, the complex nature of fatty acid metabolism and influence on myocardial function and pathogenesis warrants extreme caution in the application of our current knowledge to humans.

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