

**METABOLIC ASPECTS  
OF THE  
HYPOXIC HEART**

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## **DECLARATION**

I declare that the work for this thesis was undertaken during my PhD studentship at The Cardiovascular Research Unit, Faculty of Medicine, University of Edinburgh and written up thereafter. I was the principal contributor to all sections except where indicated in the text.

Catherine A. Wardle.

## ABBREVIATIONS.

Acetyl CoA	Co-enzyme A ester of acetic acid
Acyl CoA	Co-enzyme A ester of long chain fatty acid
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
CK	Creatine phosphokinase
COMT	Catechol-O-methyl transferase
CP	Creatine phosphate
DHAP	Dihydroxyacetone phosphate
FABP	Fatty acid binding protein
GLC	Gas-liquid chromatography
$\alpha$ -GP	$\alpha$ -glycerophosphate
GTP	Guanine triphosphate
NAD <sup>+</sup>	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NADP <sup>+</sup>	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduce nicotinamide adenine dinucleotide phosphate
NEFA	Non-esterified fatty acid
Pi	Inorganic phosphate
PL	Phospholipid
RQ	Respiratory quotient
SAM	S-adenosyl-methionine
TAG	Triacylglycerol
UDP	Uridine diphosphate
UTP	Uridine triphosphate

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## **ABSTRACT**

Coronary heart disease is a major cause of death in Western society. Many studies have demonstrated the importance of lipid metabolism in the deterioration in cardiac function and the development of lethal arrhythmias during ischaemia.

It has been suggested that a TAG-NEFA (triacylglycerol-non-esterified fatty acid) "wasteful" cycle exists in the ischaemic heart and that increased operation of the cycle depletes ATP (adenosine triphosphate) supplies for the energy dependent ion pumps, disturbs membrane potentials and therefore causes arrhythmias. The aim of this thesis was to quantify energy wastage due to the operation of the TAG-NEFA cycle in the heart during oxygen deprivation. The hypoxic, perfused rat heart was used as the experimental model. Cannulation of the pulmonary artery allowed anaerobic sampling of coronary effluent for measurement of blood gases, and therefore calculation of oxygen uptake. Glycerol release was used as an index of TAG breakdown. Lactate production and tissue concentrations of ATP, CP (creatine phosphate), glycogen,  $\alpha$ -glycerophosphate, glycerol and TAG were measured. The rate of TAG-NEFA cycling could therefore be calculated.

The influence of severity of hypoxia on the rate of TAG-NEFA cycling was studied. A positive correlation between severity of hypoxia and energy utilization by the operation of the cycle was demonstrated. However, even during severe hypoxia (10%O<sub>2</sub>, 85%N<sub>2</sub>, 5%CO<sub>2</sub>), the proportion of the total ATP produced during hypoxia utilized in the operation of the cycle was small (less than 7%).

The influence of age, fasting (48h) and obesity on the operation of the cycle during normoxia and hypoxia was also studied. A five-fold difference in myocardial TAG concentration was found to be of little importance in determining the rate of the TAG-NEFA cycle. The rate of cycling was reduced in fasted rats.

The role of endogenous catecholamines in the metabolic changes induced by hypoxia

was investigated. Myocardial noradrenaline release could not be stimulated by oxygen deprivation. In contrast to isoprenaline-stimulated glycerol release, hypoxia stimulated glycerol release could not be inhibited by  $\beta$ -adrenoceptor blocking agents. Furthermore, depletion of endogenous catecholamines by pretreatment of rats with 6-hydroxydopamine caused only a 30% reduction in hypoxia-stimulated glycerol release.

The validity of the use of glycerol release as an index of myocardial lipolysis was questioned. Hypoxia-stimulated glycerol release was unaffected by the presence of 0.2% defatted albumin in the perfusate, suggesting that product inhibition caused by the presence of elevated tissue NEFA concentrations was not rate limiting. Nicotinic acid, a compound possessing antilipolytic properties in adipose tissue caused partial inhibition of isoprenaline-stimulated glycerol release. However, the drug had no effect on hypoxia-stimulated glycerol release in the heart. Labelling of myocardial TAG stores by infusion of radiolabelled glycerol and measurement of specific activity of glycerol released from the heart during normoxia, isoprenaline infusion and hypoxia inferred the presence of glycerokinase activity in the tissue and the existence of more than one TAG pool in the heart.

In conclusion: 1) The operation of the TAG-NEFA cycle during hypoxia does not appear to cause a major drain on high energy phosphate supplies in the heart. 2) The rate of cycling is independent of myocardial TAG concentrations. 3) Glycerokinase activity can be demonstrated in the rat heart. 4) Glycerol release during hypoxia is mediated by non-adrenergic mechanisms and cannot be assumed to be an accurate reflection of myocardial lipolysis.

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**CHAPTER 1**

**LITERATURE REVIEW**

### 1.1. Introduction.

Coronary heart disease is a major cause of death in Western society. A large proportion of deaths due to acute myocardial infarction happen within the first hour after the onset of symptoms (Kuller 1962, Armstrong et al. 1972).

Myocardial ischaemia is a common cause of arrhythmias, or disturbances in the normal electrical rhythm of the heart. Ischaemia can be defined as an imbalance between the supply of oxygenated blood and the oxygen requirements of the myocardium. Lethal arrhythmias are the most common cause of sudden cardiac death (Armstrong et al. 1972).

Myocardial ischaemia may be caused by coronary artery thrombus formation (Davies and Thomas 1985) or spasm (Olivia and Beckinbridge 1977) against a background of coronary heart disease. In situations such as these, the ensuing inadequate blood supply is inevitably accompanied by biochemical changes within the tissue. Many experimental studies have implicated biochemical changes within the heart in the development of ischaemic injury and arrhythmias.

The provision of energy for the maintenance of the function of the heart depends largely on oxidative metabolism, and during ischaemia there is a shift from  $\beta$ -oxidation of fatty acids to both aerobic and anaerobic glucose utilization. This is significant in that it is an oxygen sparing activity, as fatty acid oxidation requires more oxygen per mole of ATP produced than does glucose (P/O ratio 2.85 compared with 3.15 for glucose). Furthermore, oxidation of fatty acids in the heart requires even more oxygen than would be expected on the basis of the P/O ratio (Mjos 1971). The switch to increased glucose oxidation during ischaemia is therefore potentially beneficial, and increased fatty acid oxidation is likely to be deleterious to the already compromised myocardium.

This section reviews briefly some aspects of the metabolism of the heart during normoxia, hypoxia and ischaemia with particular emphasis on TAG and NEFA metabolism in the heart.

## 1.2. Substrate metabolism of the heart during normoxia.

It is not the intention to give a comprehensive review of the metabolism of the heart. For the sake of clarity a summary of the relevant aspects of the normal, ischaemic and hypoxic heart is given. The mammalian heart is in a constant state of mechanical and catabolic flux. The major purpose of the metabolism of the heart is to provide sufficient energy in the form of ATP to balance the requirements of contractile function. Calcium ions act as the physiological regulator of muscle contraction. Depolarization of the cell membrane activates an inward  $\text{Ca}^{++}$  current, which triggers release of  $\text{Ca}^{++}$  from the sarcoplasmic reticulum. The released  $\text{Ca}^{++}$  binds to troponin and causes conformational changes which are transmitted to tropomyosin and then to actin. Contractile force is thus generated, and ATP is concomitantly hydrolysed.

Bing (1954), using coronary sinus catheterization and comparing the composition of the arterial and coronary venous blood established that glucose, free fatty acids and lactate are the major substrates for energy metabolism of the heart. Uptake of tritiated palmitate was inhibited by 5.5mM lactate (Rose and Goresky 1977), indicating the preferential use of lactate by the heart. The myocardium is also able to use fuels such as pyruvate, acetate, ketone bodies and amino acids, although even when the supply of these substrates is raised, their relative contribution is too small for them to be major myocardial sources of energy. In addition, the heart may also use its internal stores of energy i.e. glycogen and lipid.

Substrate utilization in the heart is critically dependent on both substrate availability and hormonal status (Drake-Holland 1983). Though the amount of heat liberated per mole of fat oxidized (approximately 2.6 kcal) is more than twice that per mole of carbohydrate oxidized (approximately 0.7 Kcal). This is because more oxygen is required to oxidize a gram of fat than a gram of carbohydrate.

### 1.2.1. Glucose metabolism.

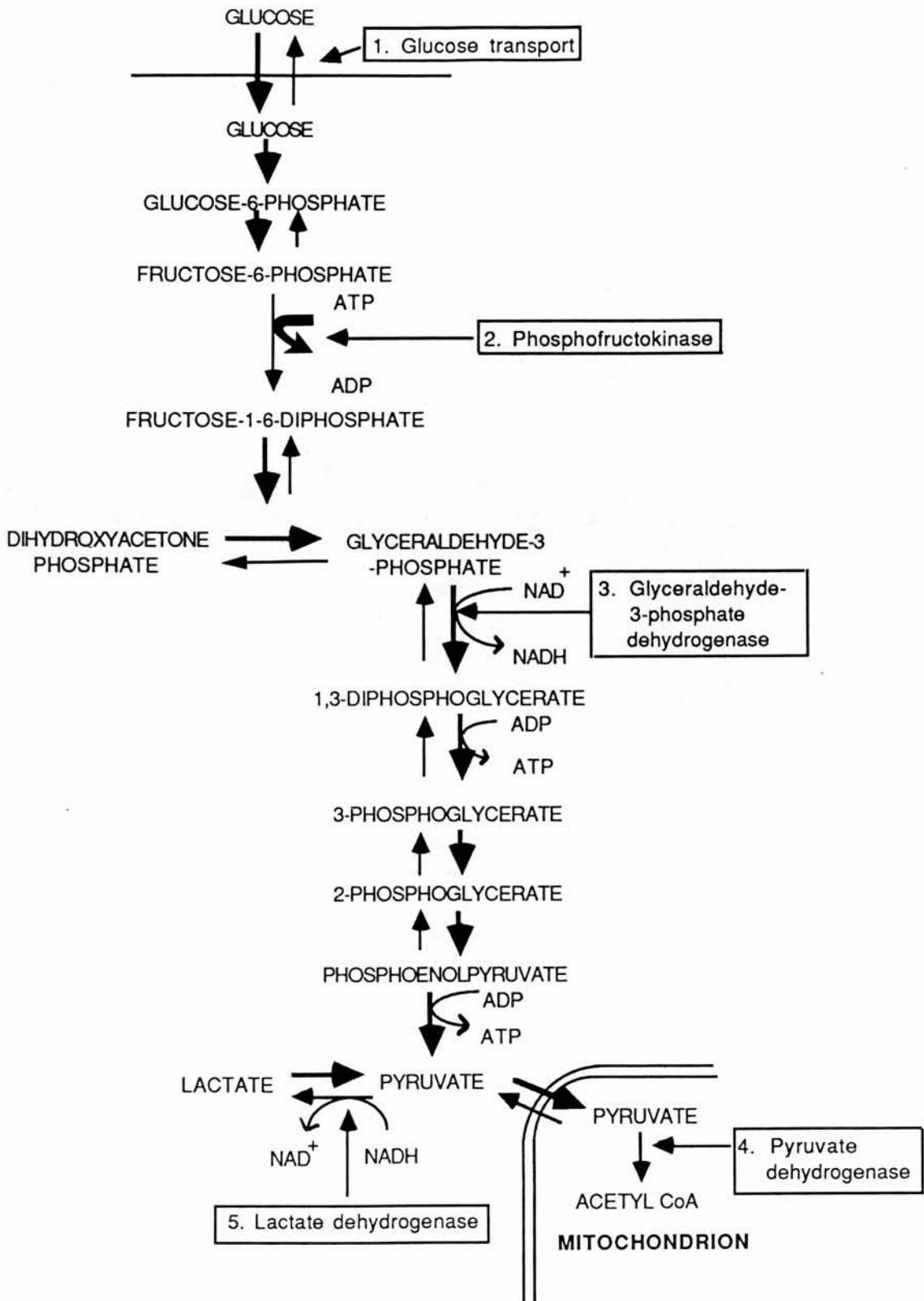
The metabolism of glucose in the heart has been extensively reviewed (Opie 1968). A brief summary is given here.

Uptake of glucose into the heart is controlled by a stereospecific glucose carrier which displays saturation kinetics (Morgan and Neely 1972). The carrier requires no energy for transport because the glucose concentration in the extracellular space is so much higher than in the cytosol (Opie 1984). Hormones such as insulin, adrenaline and cortisol increase transport of glucose into the heart (Morgan et al. 1961, Neely et al. 1967, Taegtmeier et al. 1980). In the normal myocardium, cardiac work, availability of substrates, plasma insulin and glucose concentration act in concert as the most important factors in regulating glucose uptake (Taegtmeier 1988). The amount of glucose taken up by the heart is very small compared with the plasma glucose concentration. Measurement of glucose uptake is therefore subject to large errors and its estimation is limited by the precision of the glucose assay being used. Samples must be analyzed numerous times in order to obtain accurate results.

During normal, oxidative metabolism, breakdown of glucose via glycolysis yields pyruvate which is then broken down aerobically in the citric acid cycle. Under normoxic conditions, the rate of glycolysis is limited by the high levels of citrate and ATP formed during oxidative metabolism. When fatty acids are oxidized, the glucose taken up by the heart is increasingly converted to glycogen. This is known as the "glucose sparing" effect of fatty acids. Conversion of 1 molecule of glucose to 2 molecules of pyruvate yields 2 molecules of ATP. The reactions involved in glycolysis and its regulation are summarized in Fig 1.1.

Phosphofructokinase1 (PFK1) is the most important regulatory enzyme in the control of glycolysis under normal conditions. ATP elicits allosteric control of PFK1 by binding to a highly specific regulatory site on the enzyme which is distinct from the catalytic

Figure 1.1. The glycolytic pathway.



site. The inhibitory action of ATP is reversed by AMP, and so the activity of the enzyme increases when the ATP/AMP ratio is lowered. As in the liver, fructose-2,6-bisphosphate, synthesized by phosphorylation of fructose-6-phosphate by PFK2 is a potent activator of PFK1, acting in a synergistic fashion with AMP to maintain PFK1 in its active form, and increasing the affinity of PFK1 for fructose-6-phosphate. However, unlike in the liver, PFK2 in the heart is not activated by cAMP-dependent protein kinase (Rider and Hue 1986). PFK1 is also allosterically regulated by citrate, an early intermediate in the citric acid cycle. A high level of citrate indicates that biosynthetic precursors such as  $\text{NAD}^+$  and ATP are abundant and so additional glucose should not be degraded for this purpose. The control of PFK1 by citrate plays a role in the control of selection of fuels by the myocardium by decreasing rates of glucose metabolism in the presence of NEFA, ketone bodies or pyruvate. Citrate inhibits PFK1 by enhancing the inhibitory effect of ATP.

The oxidation of NADH is facilitated by the transfer of electrons to dihydroxyacetone phosphate which is thereby reduced to  $\alpha$ -glycerophosphate by  $\alpha$ -glycerophosphate dehydrogenase, resulting in the formation of  $\text{NAD}^+$ .  $\alpha$ -glycerophosphate then diffuses through the outer mitochondrial membrane and is reoxidized by an  $\text{FAD}^+$ -linked  $\alpha$ -glycerophosphate dehydrogenase located on the external side of the inner mitochondrial membrane. The electrons are passed to coenzyme Q by a reductase complex located within the inner mitochondrial membrane. The dihydroxyacetone phosphate formed in this way is free to return to the cytoplasm and serve as an electron acceptor for the reoxidation of another molecule of NADH.

Another important regulatory enzyme of glycolysis is glyceraldehyde-3-phosphate dehydrogenase (Mochizuki and Neely 1979). This enzyme is sensitive to the  $\text{NADH}/\text{NAD}^+$  ratio in the cell, being activated by a decreased  $\text{NADH}/\text{NAD}^+$  ratio and vice versa.

Pyruvate kinase converts phosphoenolpyruvate to pyruvate, with the concomitant

production of ATP. This enzyme is subject to allosteric inhibition by ATP and activation by fructose-1,6-diphosphate and phosphoenolpyruvate.

Under normoxic conditions, most of the pyruvate formed during glycolysis is transported into the mitochondrial matrix by a specific carrier where it enters the citric acid cycle and is subsequently oxidized (Denton and Halestrap 1979).

In the mitochondria, pyruvate dehydrogenase catalyzes the conversion of pyruvate to acetyl coA, allowing entry into the citric acid cycle. The pyruvate dehydrogenase complex may exist in an active or inactive form and is covalently regulated by phosphorylation/dephosphorylation. Acetyl CoA and NADH, the products of the oxidation of pyruvate, inhibit the enzyme complex. These inhibitory effects are reversed by CoA and  $\text{NAD}^+$  respectively. Pyruvate dehydrogenase is also activated by AMP and inhibited by GTP.

Pyruvate may also be converted to lactate by the enzyme lactate dehydrogenase. Under normoxic conditions, the activity of this enzyme is limited by the low  $\text{NADH}/\text{NAD}^+$  ratio of the cell and entry of pyruvate into the citric acid cycle. However, some lactate is formed from pyruvate (Huckabee 1958).

### 1.2.2. Glycogen metabolism.

Excess glucose entering the myocyte is stored in the form of high molecular weight glycogen as large granules in the cytoplasm. This substrate store is in a constant state of turnover (Opie 1968). Glycogen metabolism is separated from glucose metabolism in that glucose-6-phosphate is formed in the first step in the breakdown of glucose, whereas glucose-1-phosphate is formed from glycogen. Glycogen and glucose breakdown converge in the formation of glucose-6-phosphate to glucose-1-phosphate. Two different pathways exist for glycogen synthesis and breakdown.

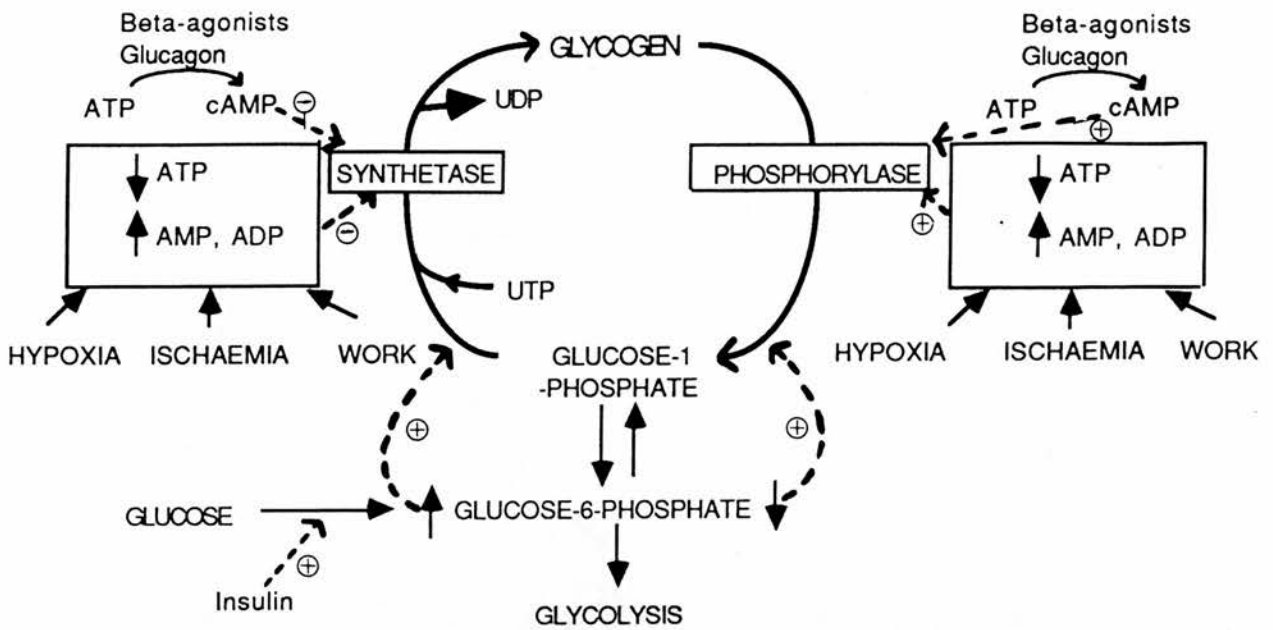
The key enzyme in glycogen synthesis is glycogen synthetase. This enzyme exists in

an inactive phosphorylated D form and an active dephosphorylated I form. Conversion of the active to the inactive form is ATP dependent and catalyzed by glycogen synthetase kinase. Glycogen synthetase kinase itself can be activated by cAMP and hence is under hormonal control. Inactive phosphorylated glycogen synthetase may become enzymatically active in its phosphorylated form in the presence of high intracellular concentrations of glucose-6-phosphate i.e. when glucose uptake into the cell is increased or when the glycolytic rate is decreased. Dephosphorylation of glycogen synthetase is controlled by glycogen synthetase phosphatase. This phosphatase is inhibited by glycogen. Thus when glycogen levels are high, glycogen synthetase will remain in its inactive (phosphorylated) form.

Glycogen degradation is catalyzed by glycogen phosphorylase. This enzyme may be present in an activated form as phosphorylase a and an inactivated form as phosphorylase b. The conversion of phosphorylase b to phosphorylase a is covalently regulated by phosphorylation by the action of phosphorylase kinase. Calcium ions are required for formation of phosphorylase a (Werth et al. 1982). Phosphorylase b is also active in the presence of high concentrations of AMP, which acts allosterically by binding to a binding site on the enzyme and hence altering its conformation. Glucose-6- phosphate also inhibits phosphorylase b, presumably by binding to another site.

Extracellular hormones such as adrenaline, noradrenaline and glucagon transform both glycogen synthetase and glycogen phosphorylase into a form which becomes less sensitive to intracellular metabolically regulated control mechanisms. Thus, activities of the enzymes involved in glycogen synthesis and breakdown are controlled in a concerted manner by hormonal as well as intracellular mechanisms.

**Figure 1.2. The regulation of glycogen metabolism.**



### 1.2.3. Non-esterified-fatty acid (NEFA) metabolism.

NEFA metabolism was originally thought to be unimportant in the heart as plasma levels are low in comparison with glucose and TAG. However, despite the low plasma concentrations of NEFA, their rate of turnover in the heart is high. Long chain non-esterified fatty acids (NEFA) are insoluble in water and are transported in the blood bound to albumin or in the form of triacylglycerol (TAG) complexed to hydrophilic lipoproteins (Frederickson and Gordon 1958). Short chain NEFA are water soluble and therefore may be transported in an unbound form in the blood.

Uptake of NEFA by the myocardium increases as the plasma concentration of NEFA, or more correctly, the NEFA:albumin molar ratio rises (Carlsten et al. 1961, Evans et al. 1963, Vik-Mo et al. 1979, Miller et al. 1976). Interaction of the NEFA-albumin complex with sites at the luminal membranes of the endothelial cells lining the myocardial capillaries has been proposed (Little et al. 1986). The nature of transport of NEFA from

the endothelial cells into the myocytes has not been completely elucidated. Simple diffusion due to the steep concentration gradient from plasma to sarcoplasm, or transendothelial and transinterstitial space transport, facilitated by fatty acid binding proteins (FABP) and albumin respectively have been put forward as possible mechanisms. The process of NEFA transport into the myocyte appears to be energy independent (Spector 1971).

Inside the myocytes FABP may be involved in the transport of fatty acids from the sarcolemma to the sites of conversion i.e. mitochondrial and sarcoplasmic reticulum (Fournier and Rahim 1985, Glatz and Veerkamp 1985). TAG must be hydrolysed to NEFA and mono- and/or diacylglycerol by lipoprotein lipase on the luminal side of the endothelium before the fatty acids can pass the endothelial barrier (Stam and Hulsmann 1985).

After being taken up by the myocardium, the first step in NEFA oxidation is the energy-dependent activation of NEFA to form acyl CoA. When the circulating NEFA concentrations are high, and NEFA extraction by the heart is also elevated, the rate of NEFA activation will also rise. However, as more acyl CoA is formed, the cytosolic free CoA concentration decreases, and activation of NEFA therefore slows down. Conversely, during increased heart work the rate of mitochondrial oxidation rises, tissue acyl CoA concentrations fall and NEFA activation is stimulated (Opie 1984).

Increased oxidation of NEFA results in a decrease in the rate of glycolysis due to increased ATP, NAD and citrate concentrations and therefore inhibition of the regulatory enzymes of glycolysis (Opie 1968, Neely and Morgan 1974).

The transport of long chain acyl CoA into the mitochondria is achieved by a shuttle mechanism in which acyl-carnitine, formed by the action of carnitine acyl transferase I, which can cross the mitochondrial membrane, is the intermediate. Carnitine-acylcarnitine translocase acts as a membrane carnitine exchange transporter.

Acylcarnitine is transported into the mitochondria, coupled with the transport out of one molecule of carnitine. Acylcarnitine then reacts with acyl CoA, catalyzed by carnitine acyltransferase II which is attached to the inside of the inner mitochondrial membrane. Acyl CoA is reformed in the mitochondrial matrix, with the subsequent liberation of carnitine. The stepwise degradation of acyl CoA to acetyl CoA via  $\beta$ -oxidation yields reduced flavoprotein and NADH. Acetyl CoA is further degraded by the enzymes of the Krebs cycle, also located in the mitochondrial matrix, leading to further formation of reduced coenzymes and 1 molecule of GTP. The reduced coenzymes are oxidized in the electron transfer chain with the concomitant formation of ATP. Alternatively, activated NEFA which is not oxidized may be ultimately stored as TAG or transferred to structural lipids.

#### 1.2.4. Triacylglycerol (TAG) metabolism.

TAG is stored in lysosomes and as lipid droplets in the sarcoplasm (Hulsmann and Stam 1979, Stam et al. 1980).

TAG is synthesized in the heart from acyl CoA and  $\alpha$ -glycerophosphate, the latter being derived from glycolysis or direct phosphorylation of glycerol by the action of glycerokinase. However, the activity of this enzyme is generally assumed to be negligible in the heart (Challoner and Steinberg 1965, Christian et al. 1968, Jesmok et al. 1977, Trach et al. 1984, Stam and Hulsmann 1981a).

The free hydroxy groups of  $\alpha$ -glycerophosphate are acylated with fatty acyl CoA by the action of glycerophosphate acyltransferase to form phosphatidic acid, an important intermediate in the synthesis of both TAG and phospholipids (phospholipid synthesis beyond the scope of this brief overview). Subsequent hydrolysis of the phosphate group at the 3 position by phosphatidate phosphatase yields diacylglycerol. The third free hydroxyl group then reacts with acyl CoA to form TAG. The release of fatty acids

stored in TAG is accelerated by the action of TAG lipases. As with glycogen, TAG stores are continually turning over. Separate pools of TAG with different turnover rates may exist in the heart (Stein and Stein 1968, De Groot et al. 1989). The regulation of TAG metabolism will be discussed in detail later (Section 1.4.2.).

### 1.2.5. High energy phosphate production and utilization.

Hydrolysis of ATP is the immediate source of energy for contraction, maintenance of ion gradients and other vital cellular functions. There is a delicate balance between synthesis and degradation of ATP.

Acetyl CoA entering the citric acid cycle produces  $\text{NADH} + \text{H}^+$ . Hydrogen atoms flow along the electron transfer chain where ADP is converted to ATP during oxidative phosphorylation. Once formed in the mitochondria, ATP is transported to the cytosol via an ATP translocase with counter exchange of ADP into the mitochondria. On entering the cytoplasm ATP may be utilized, resulting in ADP formation. The rates of synthesis and breakdown of ATP are therefore closely linked.

The rate at which the citric acid cycle operates is a major factor controlling the rate of production of ATP by the heart, because this cycle regulates the formation of  $\text{NADH} + \text{H}^+$ . Two dehydrogenases of the cycle, isocitrate dehydrogenase and  $\alpha$ -ketoglutarate dehydrogenase are sensitive to calcium.  $\alpha$ -Ketoglutarate dehydrogenase is also inhibited by succinyl CoA (Garland 1964). Pyruvate dehydrogenase is also activated by calcium ions (Denton and McCormack 1980, Coll et al. 1982).

Oxidation of NADH is coupled to phosphorylation of ADP in the mitochondria in such a way that 3 molecules of ATP are formed for each atom of oxygen utilized (P/O ratio of 3). Depending on the substrate oxidized, the exact P/O rates will vary from 2.85 to 3.15. The variations in the P/O ratio reflect the number of oxygen atoms of the substrates. Thus, glucose, already containing some oxygen atoms in its structure needs addition of

less oxygen added to produce H<sub>2</sub>O than does fatty acid.

Another important energy store in the heart is creatine phosphate (CP). This may act as a buffer store of high energy phosphate for ATP. A phosphate group may be transferred from creatine phosphate to ADP by the action of creatine kinase, resulting in formation of ATP. The equilibrium of this reaction favours formation of ATP by about 50 times (Opie 1984).

CP may also act as a shuttle in transporting high energy phosphate from the mitochondria to its site of utilization. Creatine phosphokinase converts newly synthesized ATP to CP in the mitochondria, liberating ADP which can be shuttled back to the mitochondrial matrix. CP then travels into the cytosol and is converted to ATP by creatine phosphokinase at or near the sites of ATP hydrolysis such as the contractile proteins and sarcolemma.

The concentration of CP in the heart is normally around 10 μmoles per gram wet weight. This is equivalent to twice the normal myocardial ATP concentration.

### 1.3. Comparison of hypoxia and ischaemia.

Hypoxia has been defined as a supply of oxygen insufficient to meet the oxygen requirements of the tissue whereas ischaemia is an imbalance between the supply of oxygenated blood and the oxygen requirements of the myocardium. The major difference between hypoxia and ischaemia is that coronary flow rate increases during hypoxia (although in some experimental models it may be controlled using a pump) whereas by definition ischaemia entails a decrease in flow rate.

Hypoxia may range from mild to severe. Complete lack of oxygen is termed anoxia. Complete abolition of blood flow to the myocardium is termed "global ischaemia". When ischaemia is confined to a region of the heart, but doesn't affect the whole heart, the term "regional ischaemia" is used. Many arrhythmias occur due to the onset of acute myocardial (regional) ischaemia (Reimer and Jennings 1986). Hypoxia is an important, perhaps the most important component of ischaemia.

Ischaemia results in a decreased supply of oxygen and nutrients to the tissue, and also decreased removal of waste products of metabolism. The distinction in myocardial metabolism between ischaemia and hypoxia is most dramatically demonstrated by contrasting the potential for energy production via anaerobic glycolysis in ischaemia and hypoxia (Neely et al. 1973). During ischaemia, glycolysis is initially stimulated due to the decreased energy status of the cell. However, the lactate concentration in the tissue rapidly increases and tissue acidosis ensues. This is accompanied by a marked slowing of anaerobic glycolysis (Jennings et al. 1981, Rovetto et al. 1973). Although glucose extraction is stimulated during ischaemia, glucose uptake is also governed by flow, and is thus limited.

The mechanism for the slowing of glycolysis is thought to be inhibition of glyceraldehyde-3-phosphate dehydrogenase by lactate and NADH and may also be due to inhibition of phosphofructokinase by lactate and hydrogen ions, and the declining

availability of pyruvate (Neely and Morgan 1974, Rovetto et al. 1975).

In contrast, during hypoxia, lactate accumulation and cellular acidosis are prevented by the maintained flow rate. Anaerobic glycolysis therefore proceeds at an accelerated rate so long as substrates remain available (Neely et al. 1975).

The effect of normoxia, hypoxia and ischaemia on glycolysis are shown in Figure 1.3. The major differences between hypoxia and ischaemia are shown in Table 1.1.

**Table 1.1.**

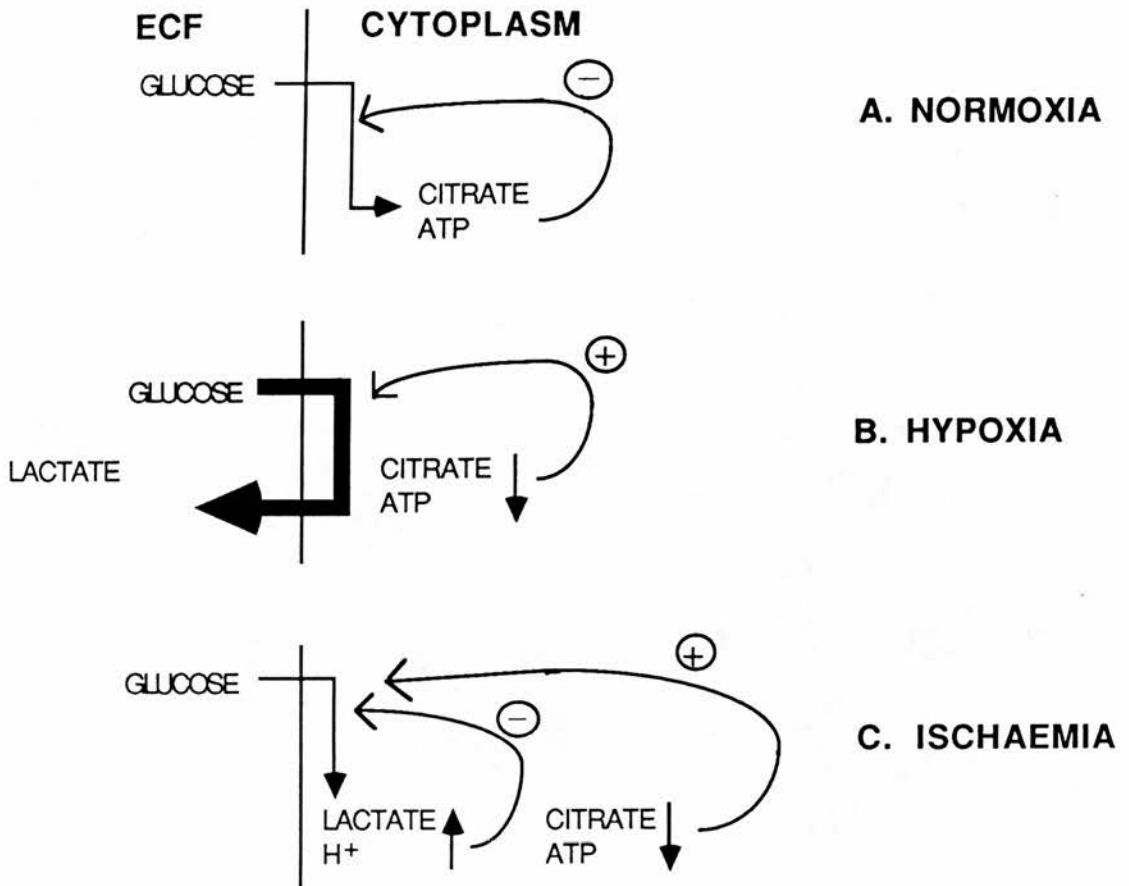
	HYPOXIA	ISCHAEMIA
Flow rate	↑	↓
Oxygen supply	↓	↓
Exogenous substrate supply	↑	↓
Removal of waste products	↑	↓

↓ = Decrease

↑ = Increase

In models of hypoxia in which coronary flow rate is controlled by means of a pump, substrate supply and removal of waste products are unchanged.

Figure 1.3. The control of glycolytic flux during normoxia, hypoxia and ischaemia.



Hypoxia is often used as an experimental model of myocardial ischaemia (section 1.6.). The following section gives a brief overview of the biochemical changes in the heart during hypoxia. Some aspects of metabolism have been studied in models of ischaemia only, and no data is available for the hypoxic heart. Although the effects of ischaemia cannot be directly extrapolated to hypoxia, the metabolic changes during early ischaemia are similar to those seen during hypoxia.

#### **1.4 Metabolism of the heart during hypoxia.**

At the onset of hypoxia, coronary flow is increased to compensate for the decreased oxygen supply to the tissue.

When oxygenation of the myocardium is compromised, the function of the electron transfer chain becomes limited due to the absence of oxygen as the ultimate acceptor of electrons. Electron transport is retarded, and formation of ATP by oxidative phosphorylation is inhibited (Scheuer 1967).

Reduced flavin and nicotinamide co-enzymes are not oxidized and hence accumulate in the mitochondria. The ensuing decrease in availability of  $\text{NAD}^+$  leads to inhibition of key enzymes in the citric acid cycle (isocitrate dehydrogenase and  $\alpha$ -ketoglutarate dehydrogenase) which require  $\text{NAD}^+$  as a cofactor. Conversion of pyruvate to acetyl CoA also requires  $\text{NAD}^+$  as a cosubstrate and is therefore inhibited when the oxygen supply is decreased. The pyruvate dehydrogenase complex is also inhibited via covalent modification by a cAMP-dependent protein kinase. Entry of acetyl CoA into the citric acid cycle is thus reduced. The regulatory enzymes of glycolysis are also affected. The relevant aspects of myocardial metabolism during hypoxia are discussed in the following section.

##### **1.4.1. High energy phosphate production and utilization during hypoxia.**

Myocardial ATP levels result from production and utilization buffered by a store of CP. As oxidative phosphorylation is inhibited during oxygen deprivation, the heart must rely on anaerobic glycolysis for ATP production. This process can only partly substitute for aerobic ATP generation. ATP is broken down to ADP and AMP and these metabolites increase in concentration when the oxygen supply is low. Enough ATP may be produced anaerobically to maintain some contractile function during hypoxia, providing that there is adequate substrate supply (Weissler et al. 1968). The high energy

phosphate stores in the heart could only support normal contractile activity for a few seconds without replenishment (Opie 1984). Decreased availability of oxygen is partly compensated for by increased coronary flow rate. Nevertheless, even under conditions of maximum stimulation, anaerobic glycolysis can produce no more than about 7% of the high energy phosphate needs of the working myocardium (Kobayashi and Neely 1979).

Creatine phosphate (CP) in the heart is continually turned over to form creatine, with the accompanying formation of ATP from ADP. When ATP formation is inhibited, ADP and AMP concentrations increase. ATP concentrations during hypoxia are initially maintained at the expense of CP. ATP is used directly in muscular contraction, causing isolated actomyosin threads to shorten (Opie 1984). It is logical that the concentration of ATP in the myocytes should be maintained at the expense of CP, with the CP pool suffering from more marked depletion than ATP. Hence, a small early fall of ATP precedes a large fall in CP (Feinstein 1962, Neely et al. 1973, Hearse 1979).

The rate of utilization of high energy phosphates is rapidly reduced during hypoxia because contractile function is suppressed. Nevertheless, electrical activity and minimal mechanical activity continue to utilize high energy phosphates; other reactions utilizing ATP include those involved in calcium sequestration by the sarcoplasmic reticulum, the sarcolemmal  $\text{Na}^+\text{-K}^+$  ATP ase and adenylate cyclase.

Compartmentalization of ATP between the mitochondria and the cytoplasm is well accepted. Subcompartments of ATP may also exist within the cytoplasm. It has been suggested that specific pools of ATP are used for contraction (Opie 1983). Decreased ATP and CP, and increased ADP concentrations are observed within 2 minutes of the onset of global ischaemia in the isolated, perfused rat heart. After 20 minutes, a further decrease in ATP concentration can be seen and ADP concentration returns to normal. At this point, AMP levels have increased five-fold. The effects of duration or severity of hypoxia rather than ischaemia on myocardial metabolism have not been studied.

#### 1.4.2. Glucose metabolism during hypoxia.

Glucose uptake by the heart is stimulated during hypoxia (Morgan et al 1961). Phosphofructokinase activity is also stimulated due to the increase in AMP concentration and concomitant decrease in ATP concentration in the tissue. In addition, the increased NADH/NAD<sup>+</sup> ratio inhibits glyceraldehyde-3-phosphate dehydrogenase activity and therefore reduces pyruvate formation. Glyceraldehyde- 3-phosphate dehydrogenase is also inhibited by decreased pH in the tissue (Rovetto et al. 1975), which is a consequence of hypoxia due to increased lactate production. Entry of pyruvate into the citric acid cycle is inhibited (Lemley and Meneely 1952) and conversion of pyruvate to lactate is stimulated by the increased NADH/NAD<sup>+</sup> ratio. Lactate formation serves to remove NADH and provide NAD<sup>+</sup> to maintain anaerobic glycolysis (Krasnow et al. 1962). In addition, the increased NADH/NAD<sup>+</sup> ratio favours formation of  $\alpha$ - glycerophosphate from dihydroxyacetone phosphate (DHAP), therefore providing a precursor for TAG synthesis.

The rate of anaerobic glycolysis is therefore stimulated during hypoxia but inhibition of oxidative metabolism results in increased lactate production and decreased oxidation of glucose.

#### 1.4.3. Glycogen metabolism during hypoxia.

During the first few seconds of hypoxia, glycogen phosphorylase activity is the rate limiting step for anaerobic energy production (Kubler and Spieckermann 1970).

However, there is a rapid increase in phosphorylase activity during the first 10 sec of ischaemia which is due to

- a) Activation of phosphorylase b by increased AMP and inorganic phosphate concentrations.
- b) Decreased inhibition of phosphorylase a because of decreased ATP and glucose-6-

phosphate concentrations (Opie 1976).

c) conversion of phosphoylease b to phosphorylase a (Dobson and Mayer 1973, Kubler and Spieckermann 1970). This is achieved through B-adrenergic stimulation of adenylate cyclase, resulting in cAMP production (Hough and Gevers 1975).

Glycogen synthetase activity may also increase due to cAMP-mediated activation of the kinase which activates glycogen synthetase (Huijing and Lerner 1966). Thus at the onset of hypoxia or ischaemia both glycogen synthesis and degradation may be activated (Williams and Mayer 1966, Opie 1970). As the duration of oxygen depletion increases, glycogen synthesis is limited by availability of high energy phosphates. Thus there is a shift towards increased glycogen degradation.

It has been suggested that myocardial glycogen stores may play an important role in the protection of the myocardium against arrhythmias (Russell et al. 1986). However, this function of glycogen is controversial and not well understood.

#### **1.4.4. Fatty acid metabolism during hypoxia.**

Oxidation of fatty acids is inhibited during hypoxia. It has been suggested that intermediates of fatty acid metabolism such as acyl CoA and acyl carnitine may accumulate in the tissue during oxygen deprivation and have deleterious effects on cellular function although this has only been demonstrated during ischaemia (Kurien and Oliver 1970, Opie 1970, Liedtke et al. 1978, Shug et al. 1978).

Studies looking at changes in NEFA have been hampered by technical difficulties in the measurement of tissue NEFA levels. Overestimation of NEFA concentrations has been attributed to methodological artefacts such as hydrolysis of tissue derived lipids during extraction and separation of lipids (Van der Vusse and Reneman 1984, Van der Vusse et al. 1985). The NEFA content of the heart represents less than 1% of the total amount of fatty acids (Van Bilsen et al. 1989). Hydrolysis of a small amount of either

phospholipids or TAG could thus lead to a large difference in NEFA levels.

*In vivo*, tissue NEFA concentrations may be raised during hypoxia or ischaemia because of increased extraction of NEFA from the plasma due to increased plasma levels (Riemersma 1979, Vik-Mo et al. 1979) and increased myocardial lipolysis. However, re-esterification of NEFA is also stimulated during hypoxia, removing some of the excess NEFA (Scheuer and Brachfield 1966, Jesmok et al. 1978, Shug et al. 1978).

Increased concentrations of arachidonic acid have been demonstrated in ischaemic myocardium in the early phase of injury (Chien et al. 1984, Prinzen et al. 1984). This fatty acid is a major component of phospholipids and its presence is thought to provide evidence for degradation of phospholipids. The tissue concentration of lysophospholipids also increases during ischaemia (Sobel et al. 1978, Corr et al. 1982). However, accumulation of lysophospholipids on a quantitative basis appears to be of minor importance (Shaikh and Downar 1981, Chien et al. 1984, Van Bilsen 1988) and becomes substantial only in necrotizing tissue after prolonged ischaemia (Steenbergen and Jennings 1984).

Elevated cellular NEFA concentrations may have deleterious effects on several cellular processes *in vitro*. These include effects on glycolytic enzymes (Lea and Weber 1968, Ramadoss et al. 1978), reduced activity of the sarcolemmal bound  $\text{Na}^+/\text{K}^+$  ATPase (Lamers and Hulsmann 1977) and disturbance of calcium homeostasis (Katz et al. 1979). However, accumulation of NEFA does not occur until one hour after the onset of ischaemia and infusion of NEFA into the blood during acute ischaemia failed to cause serious ventricular arrhythmias. NEFA accumulation is therefore an unlikely cause of arrhythmias during early ischaemia (Van der Vusse et al. 1987).

#### 1.4.5. Triacylglycerol metabolism during hypoxia

Myocardial lipolysis, mediated via a catecholamine-dependent mechanism is thought to be stimulated during oxygen deprivation (Crass and Pieper 1975, Christian et al. 1968, Jesmok et al. 1977). Anoxia-stimulated lipolysis, demonstrated by increased glycerol release, could not be shown in hearts from rats depleted of endogenous catecholamines by pretreatment with reserpine (Karwatowska-Krynska and Beresewicz 1983). However, re-esterification is also stimulated during hypoxia and this is facilitated by increased  $\alpha$ -glycerophosphate production due to increased glycolytic flux. Several investigators have reported an increase in the myocardial content of TAG in flow deprived tissues (Jesmok et al. 1978, Burton et al. 1986).

The onset of hypoxia evidently involves a complex and interacting series of biochemical changes. The changes during the first few minutes after the onset of hypoxia are compensatory and there is an increase in glycolytically produced ATP. This may minimize tissue damage. However, if hypoxia is prolonged, depletion of energy supplies may be so severe as to irreversibly damage myocardial function. This is demonstrated by amorphous matrix densities in the mitochondria and tiny breaks in the plasmalemma of the sarcolemma (Jennings and Reimer 1981, Reimer et al. 1983). Release and activation of lysosomal phospholipases or proteases is also apparent (Decker and Wildenthal 1978, Wattiaux and Wattiaux-De Connick 1984, Welman 1974).

## **1.5. Regulation of lipolysis in the heart.**

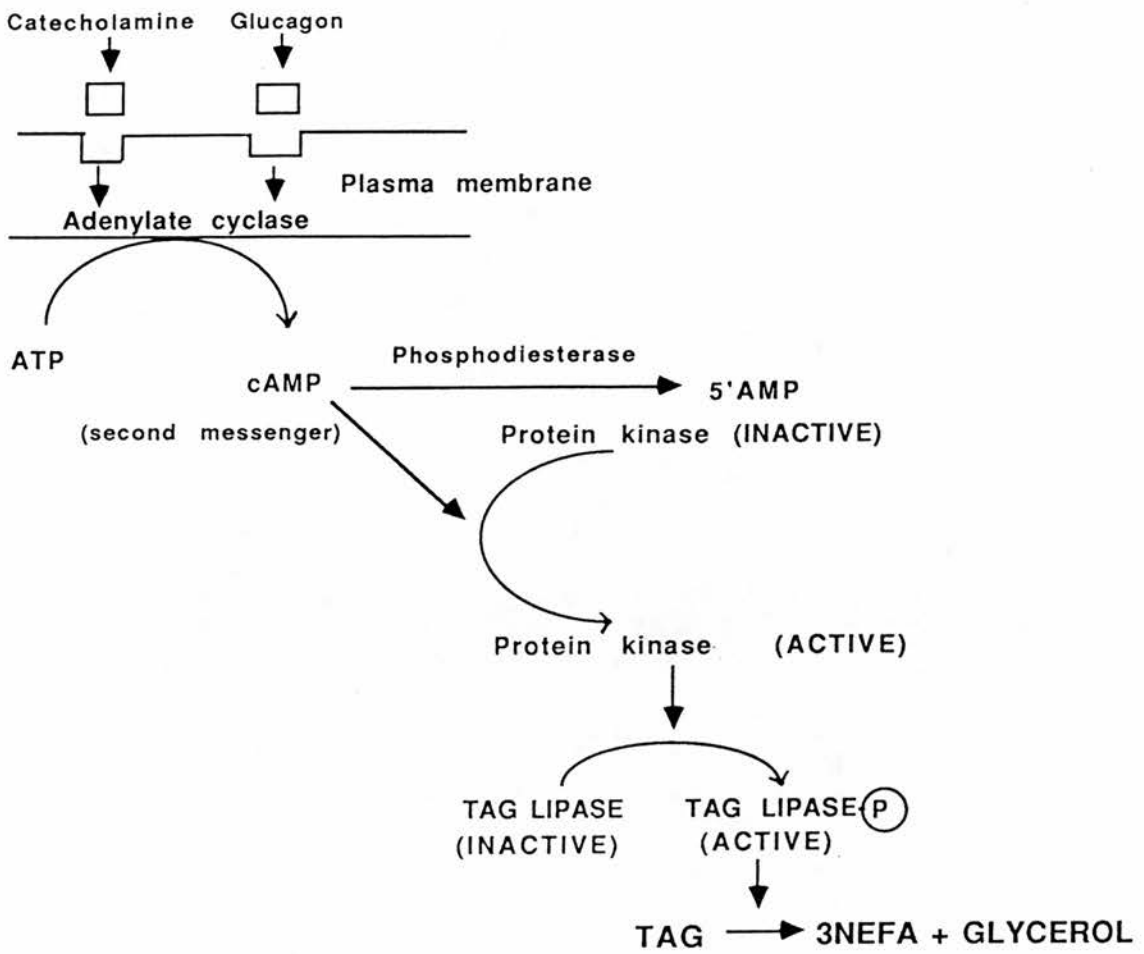
Although the process of endogenous lipolysis in the heart is a well known phenomenon, there is no consensus of opinion with regard to the mechanisms involved. The nature and localization of the metabolically active TAG pool (or pools) is unclear, and the enzymes involved in myocardial lipolysis under various conditions are unknown. A summary of present knowledge of the control of lipolysis is given in the following sections.

### **1.5.1. Hormone-stimulation of lipolysis.**

Each mole of TAG hydrolyzed in the heart gives rise to 3 moles of NEFA and 1 mole of glycerol. As glycerol is readily released into the coronary effluent, and myocardial glycerokinase activity is thought to be negligible, glycerol release has been well accepted as an index of TAG breakdown (Christian et al. 1968, Brownsey and Brunt 1977, Trach et al. 1984, De Groot et al. 1989). Catecholamines are well known to stimulate TAG hydrolysis in the heart as demonstrated both by increased rates of glycerol output (Williamson 1964, Challoner and Steinberg 1965, Christian et al. 1968, Brownsey and Brunt 1977, Hulsmann and Stam 1978) and decreased levels of TAG (Gartner and Vahoury 1973, Crass et al. 1975). In addition dibutyryl cAMP and theophylline have been shown to stimulate TAG breakdown (Crass et al. 1975).

Hormone-stimulated lipolysis in the heart is achieved via a  $\beta$ -receptor, cAMP, protein kinase mediated cascade (Christian et al. 1968; Fig 1.4).

Figure 1.4. Hormone-stimulation of lipolysis.



Isoprenaline-stimulated glycerol release may be blocked by the  $\beta$ -adrenoceptor antagonist propranolol, and a rise in cAMP levels was observed before the increase in glycerol release in isolated hearts perfused in the presence of the catecholamine analogue isoprenaline (Christian et al. 1968). In addition, increased glycerol release from hearts perfused with catecholamines was associated with an increased protein kinase activity ratio (Jesmok et al. 1977, Hron et al. 1976). This was shown to be dependent on a  $\text{Ca}^{++}$  concentration greater than 1.2mM (Hron et al. 1976). A protein kinase inhibitor was

found to block activation of a myocardial TAG lipase (Palmer et al. 1987). Also, blockade of noradrenaline reuptake in isolated perfused rat hearts by phenoxybenzamine increased TAG lipase activity (Hough and Gevers 1975), and depletion of endogenous catecholamines by pretreatment with reserpine resulted in inhibition of ischaemia induced increases in TAG lipase activity (Hough and Gevers 1975) and hypoxia-stimulated glycerol release (Karatowska-Krynska and Beresewicz 1983).

The effects of  $\alpha$ -adrenoceptor stimulation on myocardial lipolysis are not as well documented as  $\beta$ -adrenoceptor effects.  $\alpha_2$ -adrenoceptor stimulation is thought to inhibit lipolysis whereas  $\alpha_1$ -adrenoceptor stimulation results in the breakdown of phosphatidylinositol to form  $IP_3$  which stimulates release of  $Ca^{++}$  from the sarcoplasmic reticulum, and diacylglycerol which activates protein kinase C. The effects of increased intracellular  $Ca^{++}$  concentrations and protein kinase C activation on lipolysis in the heart do not appear to have been studied.

Holm et al. (1988) used an antibody raised against purified adipose tissue hormone-sensitive lipase to detect the enzyme in rat heart tissue. Hormone-sensitive lipase was partially purified from heart muscle and was found to be phosphorylated by cyclic AMP-dependent protein kinase. Phosphorylation of the purified enzyme resulted in increased enzyme activity (Small et al. 1989). Holm et al. (1988) determined the sequence of adipose tissue hormone sensitive lipase using cloned rat adipocyte complementary DNA. Messenger RNA species for the protein were found to be expressed in heart tissue.

### **1.5.2. Regulation of lipolysis by product inhibition.**

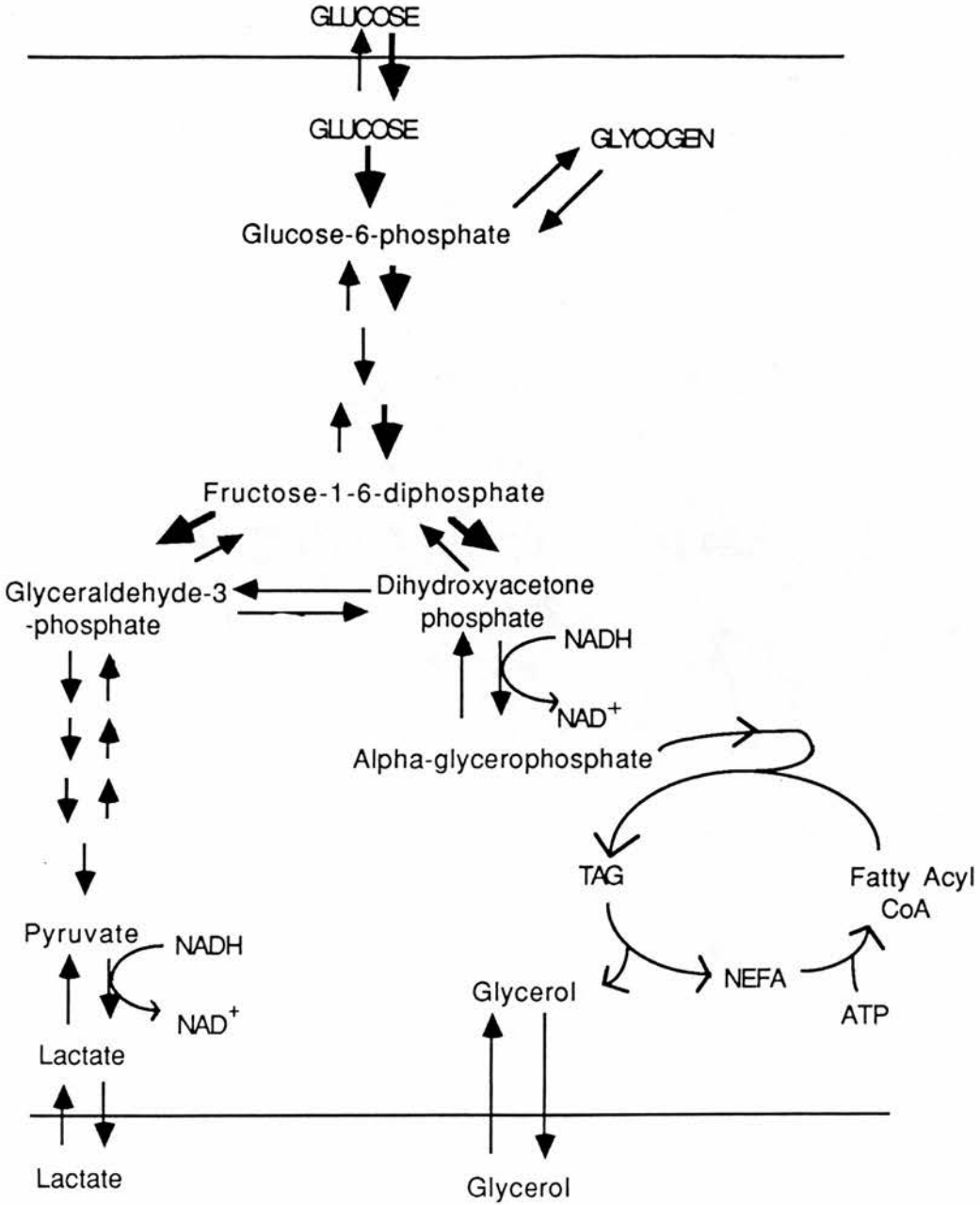
Under normoxic conditions myocardial lipolysis in the isolated, perfused rat heart was subject to product inhibition by increased exogenous concentrations of NEFA (0.25 mM to 0.6 mM; Crass III et al. 1975, Hron et al. 1978, Stam and Hulsmann 1981, Severson and Hurley 1982). Cardiac lipase activity in rat heart homogenates was inhibited in the

presence of palmitoyl CoA (100 $\mu$ M) and stimulated in the presence of albumin (3%). This was suggested to be via removal of NEFA (McDonough and Neely 1988). However, palmitoyl CoA is well known to possess detergent-like properties at concentrations much lower than 100  $\mu$ M and its effects at this concentration are therefore likely to be non specific.

### 1.5.3. The role of $\alpha$ -glycerophosphate in the control of lipolysis.

The supply of  $\alpha$ -glycerophosphate for TAG synthesis may also be an important factor in the control of lipolysis. This may be due to removal of product inhibition as a consequence of enhanced NEFA re-esterification (Schoonderwoerd et al. 1989). However, glycerol release from isolated, perfused rat hearts was increased in the presence of lactate (De Groot et al. 1989). This was attributed to stimulation of TAG turnover due to an increase in the NADH/NAD<sup>+</sup> ratio and hence increased  $\alpha$ -glycerophosphate production. The increased lipolysis in hearts perfused in the presence of lactate was accompanied by raised tissue NEFA levels compared with control hearts, ruling out the possibility that lipolysis was stimulated due to removal of product inhibition. Part of the myocardial TAG pool may have been converted into a separate pool which was more readily accessible to TAG lipase. TAG pools with different turnover rates have previously been reported (Stein and Stein 1968, Olson and Hoeschen 1967). The importance of  $\alpha$ -glycerophosphate production for TAG synthesis in rat heart homogenates has been demonstrated (Wood et al. 1972). cAMP protein-kinase stimulation of TAG lipase activity in rat heart homogenates was dependent on the presence of glycogen, probably for  $\alpha$ -glycerophosphate production (Schoonderwoerd et al. 1987). The involvement of  $\alpha$ -glycerophosphate in TAG metabolism is illustrated in Fig 1.5.

Figure 1.5. The relationship between glycolysis and lipolysis.



#### **1.5.4. Other factors involved in the regulation of lipolysis.**

Paracrine effects may also be present in the heart. Prostaglandins may influence lipolysis. Prostaglandin E<sub>1</sub> is known to be an inhibitor and the effects on myocardial lipolysis are dose-dependent (Riemersma 1979). Thromboxane and prostacyclin may be released from endothelial cells and subsequently influence metabolism of the myocytes. The influence of these substances on TAG metabolism in the heart does not appear to have been studied. Adenosine production in the heart results in inhibition of adenyl cyclase and thus decreased cAMP production and inhibition of hormone-stimulated lipolysis.

#### **1.5.5. TAG lipases of the myocardium.**

Several TAG lipases have been described in heart muscle.

a) Lipoprotein lipase - this enzyme is bound to the capillary endothelium and functions in the uptake of tissue NEFA by hydrolysis of the TAG component of circulating lipoproteins (Brockerhoff and Jensen 1974, Robinson 1970). Lipoprotein lipase may be released from its tissue sites by heparin. The enzyme has an alkaline pH optimum (8-8.5) and is inhibited by protamine sulphate and high NaCl concentrations (Twu et al 1975).

b) Neutral lipase - This enzyme has a pH optimum of 7.5 to 8.

c) Acid lipase - This enzyme is thought to originate from lysosomes (Wang et al. 1977, Hulsmann and Stam 1979) and has an acid pH optimum (4.5 to 5) (Severson 1979).

Besides TAG lipase activity, both microsomal DAG hydrolase and MAG hydrolase enzymes have been characterized (Stam et al. 1986). The activity of both these enzymes far exceeds that of TAG lipase, hence the latter enzyme is more important in the regulation of lipolysis.

### 1.5.6. Involvement of TAG lipases in hormone-stimulated lipolysis.

The identity of the lipase responsible for hormone-stimulated lipolysis in the heart has been controversial. Different studies have cited neutral lipase or lysosomal lipase as being responsible for basal and hormone-stimulated lipolysis in the heart.

Noradrenaline-stimulated lipolysis, demonstrated by glycerol release from the isolated, perfused rat heart was inhibited by the lysosomotropic agent chloroquine (Hulsmann and Stam 1978, Hulsmann et al. 1979), and lysosomal TAG levels decreased during perfusion (Stam et al. 1986). However, this decline represented only a small fraction (10%) of the total fall in TAG measured. Basal and glucagon-stimulated glycerol release were unaffected in isolated, perfused hearts from rats pretreated with cyclohexamide to deplete neutral TAG lipase activity (Stam et al. 1986). In addition, uptake and degradation of TAG by isolated lysosomes has been demonstrated (Schoonderwoerd et al. 1990). The lysosomotropic agent methylamine inhibited ischaemia-stimulated glycerol release in the isolated, perfused rat heart (Schoonderwoerd et al. 1990). However, this was in the presence of a relatively low  $\text{Ca}^{++}$  concentration (1.3mM) at which lipolysis has been shown to be inhibited (Hron et al. 1977). The effect of the drug was not examined in the presence of higher  $\text{Ca}^{++}$  concentrations.

Lysosomotropic agents were unable to inhibit basal or hormone stimulated glycerol release in isolated myocytes (Kryski et al. 1987) or the isolated, perfused rat heart (Severson et al. 1980, Rosen et al. 1981). Aupeple and Pinson (1982) suggested that lysosomal lipases may hydrolyse TAG only under conditions of lipid accumulation. However, even in myocytes isolated from diabetic rats in which TAG concentrations are raised, the lysosomotropic agent methylamine had no effect (Kryski et al. 1987). The reasons for the discrepancies between studies using lysosomotropic agents are not apparent, and other, possibly non-specific effects of the drugs should be taken into consideration. For example, chloroquine is an inhibitor of phospholipase  $\text{A}_2$  (Harris et al.

1988, Fazekas and Szekeres 1988).

The hormone-sensitive lipase purified from heart tissue by Small et al. (1989) had a neutral pH optimum. This enzyme exhibited identical properties to the hormone-sensitive lipase of adipose tissue. Furthermore, the enzyme was present in isolated myocytes, excluding the possibility that the lipase activity was derived from intramuscular adipocytes or other cell types. However, a lack of hormone-sensitivity of the neutral TAG lipase has been reported (Kawamura et al. 1981, Rosen et al. 1981, Stam and Hulsmann 1985).

More work is obviously needed to elucidate the key enzymes, and the mechanisms involved in the regulation of lipolysis.

### 1.6. The isolated Langendorff perfused hypoxic rat heart as an experimental model.

The rat is a useful animal model due to its small size, relatively low cost and availability. As it is commonly used as a model, results may be easily compared with those of other workers.

The isolated, perfused hypoxic rat heart provides a useful model for quantitative biochemical studies (De Leiris et al. 1984). The heart is isolated from the rest of the body and so is free of complicating factors such as hormonal and neural influences and cellular blood elements which may affect metabolism *in vivo*.

The acute consequences of hypoxia can be studied more easily in the isolated heart model. Both arterial and venous perfusate samples are readily accessible, allowing monitoring of substrate utilization and metabolite production. At any time necessary the heart may be freeze clamped, and concentrations of metabolites in the tissue measured. Non-invasive techniques such as nuclear magnetic resonance (NMR) may also be used to monitor the biochemical changes in the heart throughout an experiment. In addition, drugs may be easily infused into the heart during perfusion.

Hypoxia has some advantages over the regional ischaemic heart model for use in quantitative metabolic studies. When coronary effluent is collected from a regionally ischaemic heart, the sample consists of a mixture of perfusate from the normoxic tissue and perfusate from the ischaemic area and the relative amounts of these cannot easily be assessed. Also, the heart tissue is a mixture of normal and ischaemic myocardium. Although the normal and ischaemic tissue may be separated, this is technically difficult, particularly after freeze-clamping the tissue. The ischaemic area itself is not homogeneous. The main advantages of the use of ischaemia is that this model is closer to the situation in the human heart during acute myocardial infarction, and that arrhythmias may be monitored whereas arrhythmias do not occur during hypoxia.

Global low-flow ischaemia may be used. However, in this situation only small

volumes of coronary effluent may be collected, and maintenance of tissue temperature is difficult at low flow rates.

The isolated, perfused heart is a heterogeneous tissue and in addition to myocytes consists of other cell types such as endothelial cells, connective tissue and adipocytes. The contribution of the metabolism other cell types to the metabolism of the heart as a whole is not always clear. This may be a problem in measuring release of metabolites and hormones such as prostacyclin.

In most isolated heart models the perfusate does not contain haemoglobin as a means of oxygen transport. This requires that flow rates much higher than those *in vivo* are required, and perfusate of high oxygen tension must be used to maintain adequate oxygenation of the tissue. The use of high flow rates without replacing colloid content runs the risk of development of interstitial myocardial oedema.

Isolated myocytes may also be used as a model of hypoxia. The contribution of other cells is therefore removed, and diffusion of extracellular molecules such as oxygen is more efficient in isolated cells, removing the problem of unphysiological flow rates. However, isolation of the cells per se may change their properties. Separation of cells requires treatment with collagenase or hyaluronidase which may in itself affect cellular function. In addition, myocytes must be separated from each other at the intercalated discs (Yokoyama et al. 1961). This is usually accomplished by brief exposure to a calcium-free solution, which runs the risk of cells becoming calcium intolerant (Zimmerman et al. 1967, Ganote et al. 1981).

Obviously all experimental models have advantages and disadvantages. On the whole, the advantages of the hypoxic isolated, Langendorff perfused rat heart outweigh the disadvantages and it provides a useful model for the study of myocardial metabolism without the complicating factors which are present *in vivo*.

### 1.7. Myocardial lipid metabolism and arrhythmogenesis

The clinical observation that in patients admitted to hospital with acute myocardial infarction, incidental measurements of plasma NEFA concentrations are raised was made by Oliver et al. (1968). The rise in plasma NEFA concentrations during acute myocardial infarction has been attributed to increased sympathetic nervous system activity (Vetter et al. 1974, Christensen and Vidabaek 1974), stimulating lipolysis in adipose tissue. The increased noradrenaline secretion in dogs after coronary artery ligation was linked with, and put forward as, a possible cause of arrhythmias (Ceremuzynski et al. 1969).

In an alternative hypothesis, Kurien and Oliver (1970) suggested that acute lipid mobilization from adipose tissue may lead to accumulation of 'unbound' NEFA within the ischaemic myocardium where they may exert detergent effects on cell membranes causing cation loss and hence induce arrhythmias. Therefore, rather than being a "harmless" biochemical reflection of increased sympathetic nervous system activity, NEFA were thought to have a fundamental role in the genesis of arrhythmias.

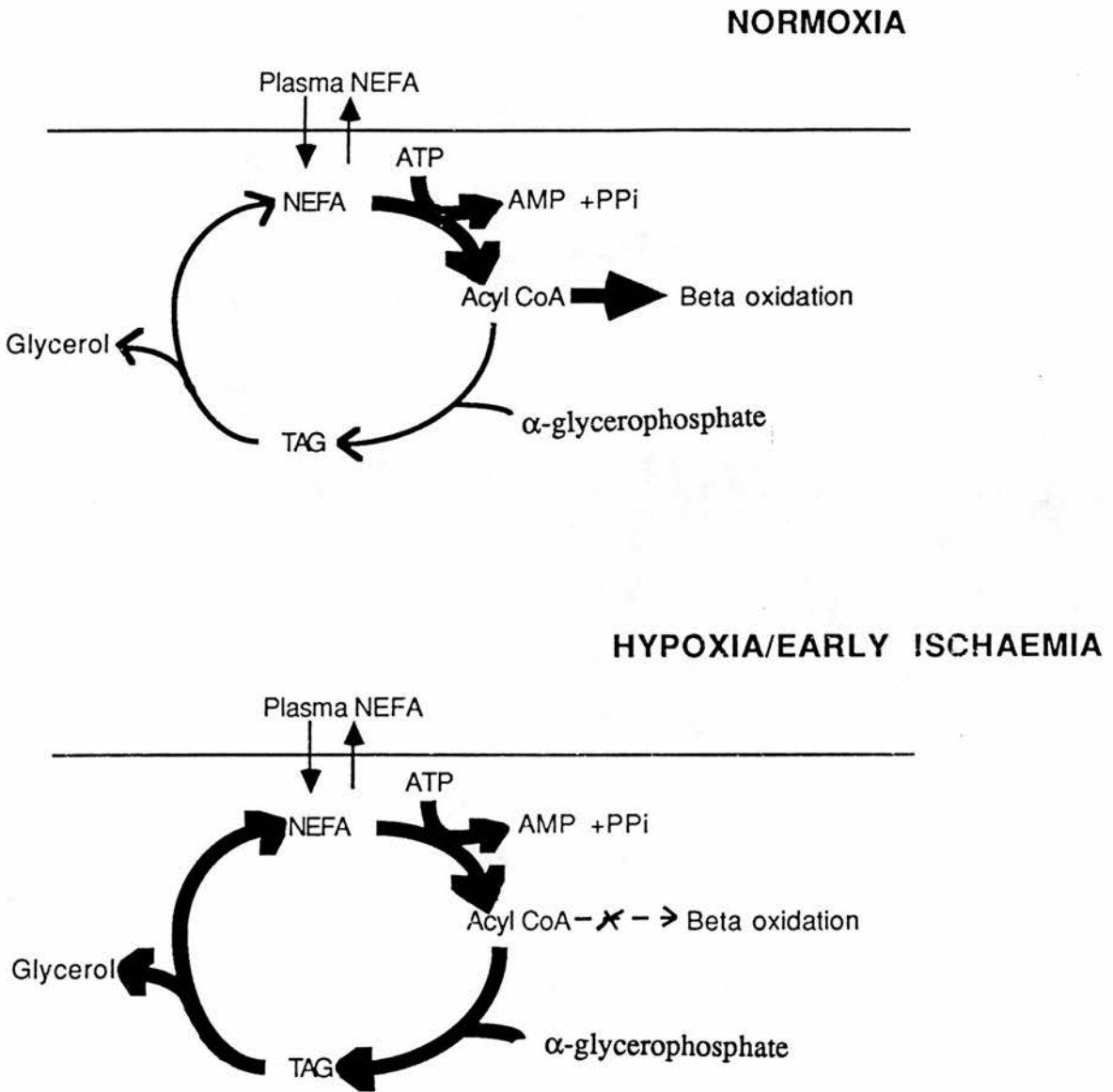
In support of this hypothesis, the incidence of arrhythmias during the early phase of acute myocardial infarction could be reduced by antilipolytic therapy independent of changes in plasma catecholamine concentrations (Rowe et al. 1975). Increased NEFA uptake by the heart in open-chest anaesthetized dogs could increase myocardial oxygen consumption, irrespective of effects on mechanical activity (Mjos 1971). In addition, inhibition of lipolysis by both antilipolytic agents and by increasing blood glucose levels was found to attenuate the increase in myocardial oxygen consumption induced by isoprenaline (Mjos 1976). Furthermore, inhibition of lipolysis by the nicotinic acid analogue  $\beta$ -pyridyl carbinol could prevent the increased S-T segment elevation caused by coronary occlusion in anaesthetized dogs (Kjekshus and Mjos 1973).

However, in subsequent studies using a similar model, infusion of NEFA salts bound to

albumin into the blood failed to precipitate ventricular fibrillation (Riemersma 1979).

This led to the suggestion that a TAG-NEFA "wasteful" cycle may operate in the ischaemic myocardium (Fig 1.6), and could be a stimulus for arrhythmias (Riemersma 1987). During early ischaemia, myocardial lipolysis is activated (Brownsey and Brunt 1977, Hough and Gevers 1975, Jesmok et al. 1978, Vik-Mo et al. 1979).  $\beta$ -oxidation is inhibited and NEFA re-esterification increases (Jesmok et al. 1978, Scheuer and Brachfield 1966, Shug et al. 1978). The operation of the cycle during acute myocardial ischaemia is therefore very likely. The TAG-NEFA cycle would be energy-requiring and may therefore be detrimental to the myocardium in consuming ATP supplies for the energy-dependent ion pumps. This may disturb membrane potentials and hence cause arrhythmias. If the rate limiting step of the cycle was lipolysis, infusion of NEFA may inhibit the cycle by exerting product inhibition on myocardial lipolysis, explaining the failure of infusion of NEFA to precipitate arrhythmias (Riemersma 1987).

Figure 1.6. The proposed TAG-NEFA cycle.



(Flux through the pathways is indicated by the width of the arrows.)

### 1.8. The TAG-NEFA cycle.

The operation of a TAG-NEFA cycle in adipose tissue is well established (Newsholme and Start 1974). The cycle is thought to be a means of improving the sensitivity of metabolic and endocrine control (Newsholme et al. 1984). It may also play a role in heat production during dietary-induced thermogenesis (White et al. 1984). The cycle has been shown to be sensitive to specific hormones. The cycling rate in adipose tissue was increased markedly by  $\beta$ -adrenergic agonists, and the effect was abolished by the  $\beta$ -blocker propranolol (Brooks et al. 1982). In addition, the rate of the TAG-NEFA cycle in adipose tissue *in vivo* was doubled by feeding (Brooks et al. 1983) and markedly increased by glucagon, ACTH and TSH (Brooks et al. 1982). There is strong evidence for the existence of a TAG-NEFA cycle in heart muscle during ischaemia or hypoxia (Vik-Mo and Mjos 1981, Vik-Mo 1983, Riemersma 1987). The use of *in vivo* models in these studies prevented quantification of ATP wastage and rates of TAG-NEFA cycling.

## **HYPOTHESIS.**

A TAG-NEFA "wasteful" cycle operates in the heart during oxygen deprivation. This leads to depletion of myocardial ATP supplies for the energy-dependent ion pumps, disturbance of membrane potentials and therefore the generation of arrhythmias.

## **CHAPTER 2**

### **METHODS**

## 2.1. Animal experiments.

### 2.1.1. Animal Housing Conditions

Rats were housed at constant temperature ( $21\pm 1^{\circ}\text{C}$ ), with a cycle of 12 h dark, 12 h artificial light. Free access to food (commercial laboratory chow; Bantin and Kingman) and water was given unless stated otherwise.

### 2.1.2. Langendorff perfusion

Rats were anaesthetised using an intraperitoneal injection of sodium pentobarbitone (60  $\mu\text{g/g}$  body weight). Heparin (0.25 units/g body weight) was injected via the femoral vein a minimum of 30 s prior to removing the heart to ensure adequate distribution throughout the blood pool. After abdominal section the chest cavity was opened by cutting through the diaphragm and ribs. The heart and lungs were rapidly excised and immediately placed in ice cold perfusate.

Table 2.1. Ionic Composition of Perfusate

ION	CONCENTRATION (mM)
$\text{K}^+$	5.6
$\text{Ca}^{2+}$	2.5
$\text{Na}^+$	141.0
$\text{Cl}^-$	126.0
$\text{Mg}^{2+}$	1.0
$\text{PO}_4^{3-}$	0.4
$\text{HCO}_3^-$	25.0

Bulk lung tissue was carefully removed and when no further contractions occurred, the heart was mounted on a perfusion cannula and secured using a 2/0 Mersilk thread. Hearts were perfused retrogradely, according to the Langendorff technique using a non-recirculating, double reservoir system (Fig 2.1). The perfusate was a modified Ringer Locke solution (Table 2.1), containing glucose (5.5 mM). This solution was freshly prepared using double distilled water before every experiment. The temperature was maintained at 37°C. The flow rate, controlled by a Harvard peristaltic pump was 10 ml/min. After a stabilization period of 20 min, the pulmonary artery was cannulated using Portex nonsterile polyethylene tubing (I.D. 0.86 mm) to enable anaerobic sampling of coronary effluent for calculation of myocardial oxygen uptake. Hearts were perfused for 30 min using 95%O<sub>2</sub>/5%CO<sub>2</sub> followed by a further 30 min perfusion as specified in the text for each experiment.

Samples were collected from the gassed perfusate in the reservoir, and the pulmonary artery. pH, pO<sub>2</sub>, and pCO<sub>2</sub> were measured using an Instrumentation Laboratories SYSTEM 1302 pH/Blood Gas Analyser. Throughout the perfusion period coronary effluent was collected at 5 or 10 min intervals. Samples were analyzed for glycerol and lactate either within 1h of collection or after storage at -40°C. At the end of the perfusion period, the hearts were rapidly cooled to the temperature of liquid nitrogen using a Wollenberger clamp, and then freeze dried for 48 hours (room temperature) using an Edwards Modulyo freeze drier.

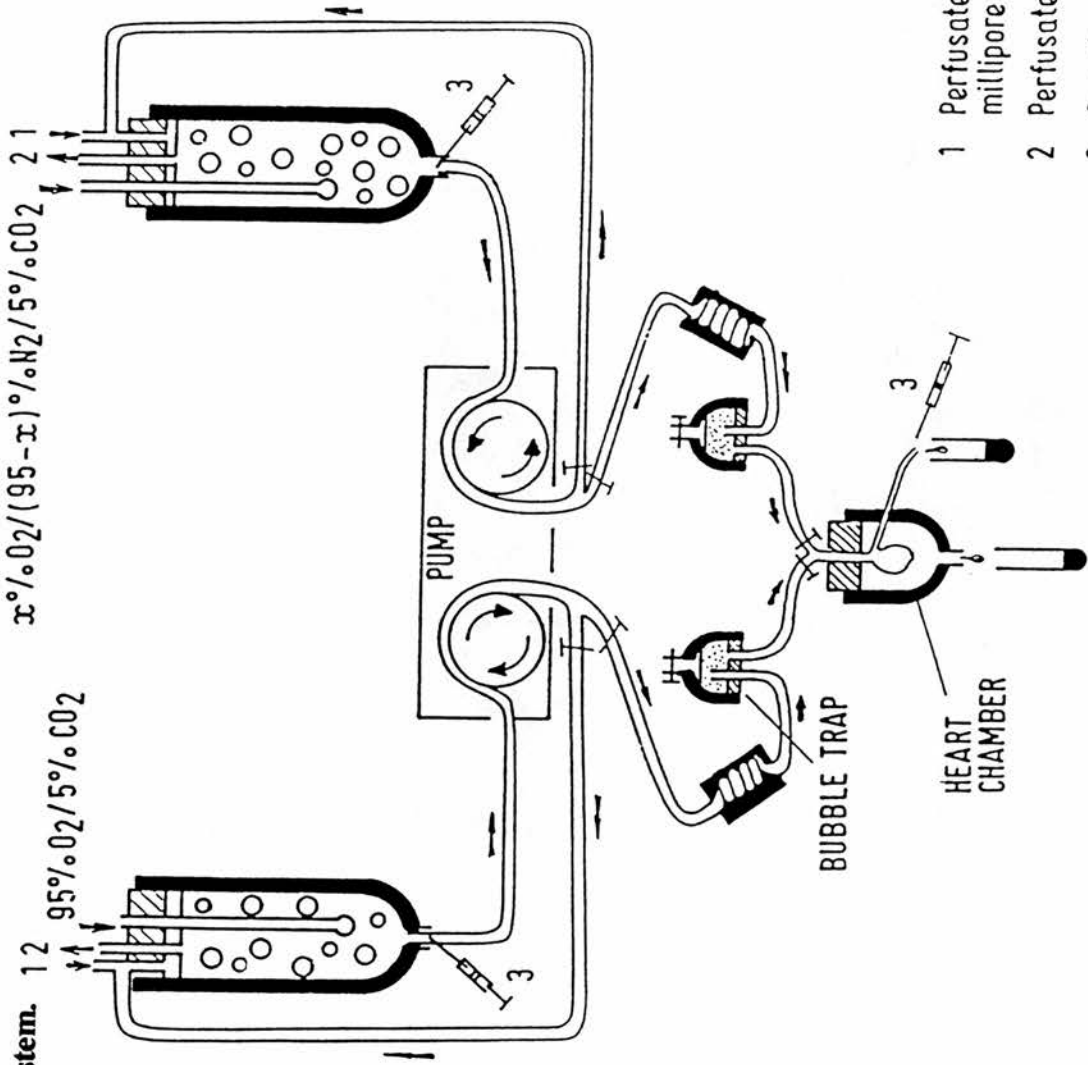


Figure 2.1.1. Perfusion system.

- 1 Perfusate pumped from reservoir, through millipore filter (type SM, pore size  $5\mu\text{M}$ )
- 2 Perfusate pumped back into reservoir
- 3 Sample collection points for pH  $\text{PO}_2$  +  $\text{PCO}_2$  analysis

### 2.1.3. Homogenization and extraction of metabolites

After freeze drying for 48 h, each heart was weighed. The atria and ventricles were separated and the ventricles pulverized using a B.Braun Mikro- dismembrator II (McKay and Lynn, Edinburgh).

Approximately 50 mg of powdered heart tissue was weighed and the exact weight noted. This tissue was then placed in a 5 ml plastic tube and 3 ml 0.6M perchloric acid was added.

The remainder of the powdered heart tissue was then weighed and stored at  $-40^{\circ}\text{C}$  before being analyzed for triacylglycerol.

Homogenate (200  $\mu\text{l}$ ) was removed, and added to 40  $\mu\text{l}$  potassium carbonate (molarity such that 200  $\mu\text{l}$  of perchloric acid was neutralized by 40  $\mu\text{l}$  potassium carbonate; referred to henceforth as matched potassium carbonate), mixed and then stored at  $-40^{\circ}\text{C}$  before being analysed for glycogen in duplicate.

The remaining homogenate was centrifuged (1200 g,  $4^{\circ}\text{C}$ , 20 min). A portion (500  $\mu\text{l}$ ) of the supernatant was pipetted into a clean 5 ml plastic tube. The pH was adjusted to 9.0-9.5 using 10 M KOH, the volume made up to 2.5 ml and the samples frozen at  $-40^{\circ}\text{C}$  before measurement of  $\alpha$ -glycerophosphate and glycerol concentrations.

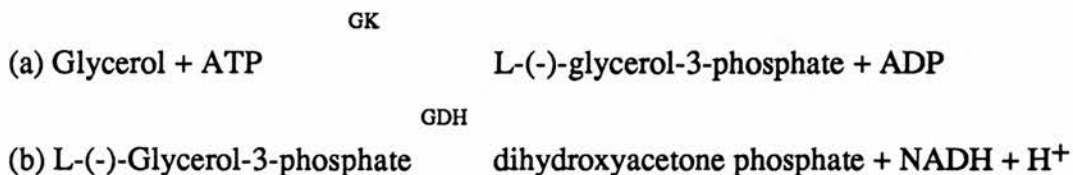
A further 2 ml of the supernatant was removed and added to matched potassium carbonate (400ul), then placed on ice for 30 min and centrifuged (1200 g,  $4^{\circ}\text{C}$ , 20 min). The supernatant was removed and analyzed for ATP and CP.

## 2.2. Biochemical analysis.

A Cobas Bio centrifugal analyser (Eisenweiner et al. 1980) was used for measurement of glycerol,  $\alpha$ -glycerophosphate, ATP, CP and glycogen concentrations.

### 2.2.1. Glycerol

Glycerol concentrations in perfusate and tissue samples were measured using an enzymic, fluorimetric method. The method was modified from that of Wieland (1974). Its principle is shown below.



GK = Glycerokinase

GDH = Glycerol-3-phosphate dehydrogenase

Glycerol is phosphorylated by ATP in the presence of glycerokinase to form  $\alpha$ -glycerophosphate.  $\alpha$ -glycerophosphate is then converted to dihydroxyacetone phosphate (DHAP) by glycerophosphate dehydrogenase leading to the formation of NADH.

Plotting of a calibration graph of the increase in fluorescence (excitation wavelength 339 nm, emission wavelength 450 nm) against glycerol standard concentrations allows determination of glycerol concentrations in samples.

The equilibrium of equation (b) lies far to the left. Therefore, to displace it in the desired direction the assay was carried out at pH 9.8 and the reaction product, DHAP was trapped with hydrazine. Samples were analyzed using a Cobas Bio centrifugal analyzer.

A "reagent mixture" was prepared as shown in Table 2.2.

**Table 2.2. Reagent mixture for glycerol analysis.**

Reagent	Concentration
Glycine/Hydrazine buffer	0.18 mM Glycine/0.52 M Hydrazine
NAD <sup>+</sup>	0.66 mM
ATP	1.64 mM
Glycerokinase*	1.12 U/ml

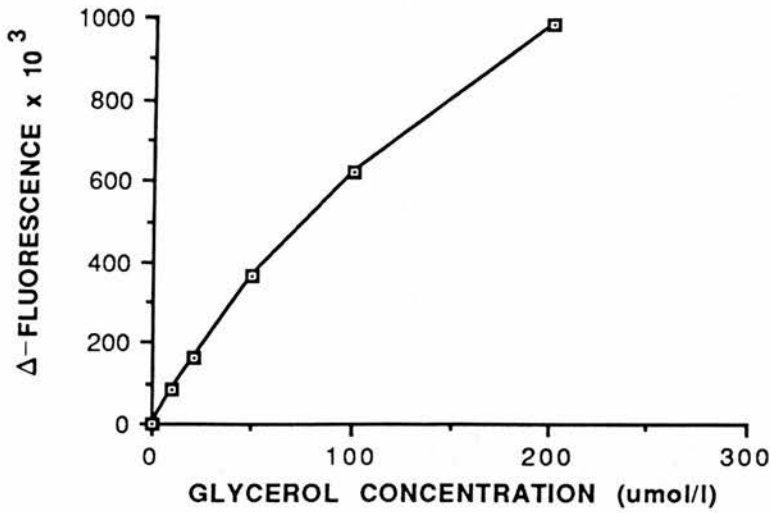
\* From *Candida mycoderma* (Boehringer Mannheim, 127 795)

A 10 min incubation period of samples with the reagent mixture at 25°C was required to ensure complete conversion of glycerol to  $\alpha$ -glycerophosphate before GDH (170 U/ml; from rabbit muscle, Boehringer Mannheim 127 752) was added to each cuvette.

**Standards:**

Glycerol standards were prepared by serial dilution of Precimat glycerol (Boehringer Mannheim) in double distilled water. Plotting of a standard curve revealed that the glycerol assay was non-linear (Fig 2.2). The DENS/Plot programme for the Cobas Bio allowed curve fitting of the fluorescence data of 6 standards, analyzed with each set of samples to a non-linear standard curve.

Figure 2.2. Calibration curve for glycerol assay.



Glycerol and  $\alpha$ -glycerophosphate were extracted from tissue samples as described in section 2.1.3. Analysis of tissue extracts using the glycerol method gave the sum of the glycerol and  $\alpha$ - glycerophosphate concentrations. Analysis for  $\alpha$ - glycerophosphate as described in section 2.2.2. and subsequent subtraction of the two results allowed calculation of the glycerol concentration in the tissue.

Coronary effluent  $\alpha$ -glycerophosphate concentrations were found to be below the detection limit of the assay (0.5  $\mu$ l) under both normoxic and hypoxic conditions. Perfusate samples were therefore analyzed for glycerol only.

The range of total glycerol and  $\alpha$ - glycerophosphate concentrations in tissue homogenates was found to be much higher than the range of glycerol concentrations found in perfusate samples (1-40  $\mu$ l in tissue vs 0-15  $\mu$ l in perfusate). Two different sets of standards were therefore used for tissue and perfusate samples. The standard concentrations used were 0-200  $\mu$ l for tissue samples and 0-20  $\mu$ l for perfusate samples.

The coefficient of variation of the method, calculated by repeated analysis of the 20  $\mu$ l standard throughout the studies was 4.7%. Recovery of 50 nmoles glycerol added to 50

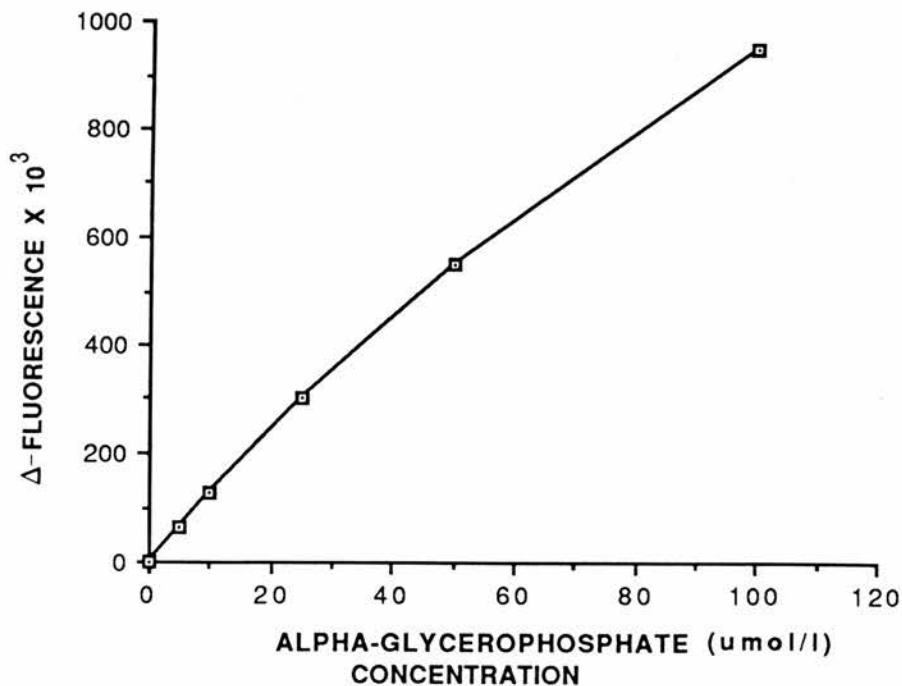
mg heart tissue before deproteinization was  $98.4 \pm 3.6\%$  (n=12).

### 2.2.2. L- $\alpha$ -glycerophosphate.

$\alpha$ -Glycerophosphate concentrations in heart tissue were measured using the same method as for glycerol (Section 2.2.1.), but glycerokinase was omitted from the reaction mixture.

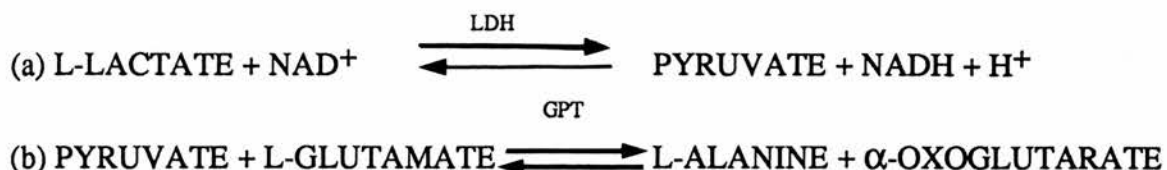
A series of standards was analyzed simultaneously with every set of samples. A typical calibration curve is shown in Fig 2.3. The coefficient of variation for the assay, determined by repeated analysis of the 50  $\mu$ l standard throughout the study was 2.04%. Recovery of 50 nmol  $\alpha$ -glycerophosphate added to 50 mg heart tissue before deproteinization was  $98.9 \pm 2.1\%$  (n=12).

Figure 2.3. Calibration curve for  $\alpha$ -glycerophosphate assay.



### 2.2.3. L-Lactate.

Lactate concentrations in coronary effluent were measured using a kit supplied by Boehringer Mannheim (Kit no. 256 773). The principle of the assay is shown below.



LDH = Lactate dehydrogenase

GPT = Glutamate-pyruvate transaminase.

The second reaction lies far to the right and thus drives the first reaction to completion by removal of pyruvate. NADH concentration, measured by the absorbance change at 340 nm (37°C), is proportional to the lactate concentration in the sample.

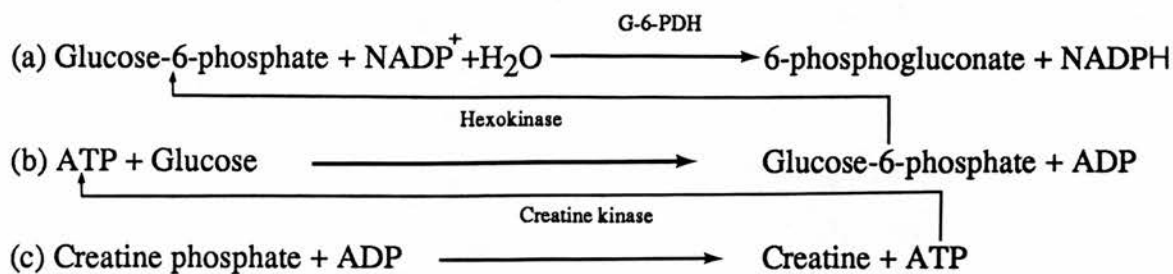
As the kit was designed for measurement of plasma lactate concentrations which are much higher than perfusate lactate concentrations, the NAD<sup>+</sup> concentration used for the assay was 60% lower than that stated in the kit. All other reagents were made up as described in the kit. A 1 mM standard solution of lactate was analyzed in triplicate with every set of samples.

Samples were analyzed using a Cobas Bio centrifugal analyzer. Precinorm S (diluted 1:3) was used as the quality control for the assay. The coefficient of variation of the lactate assay, calculated by repeated analysis of Precinorm S throughout the study was 5.4%.



#### 2.2.4. Adenosine triphosphate and Creatine phosphate

Tissue ATP and CP concentrations were determined by coupling the enzymic, UV methods of Lamprecht and Trautschold [1974] and Lamprecht et al. [1974] and adapting the methods for use on the Cobas Bio. The principle of the method is shown below.



G-6-PDH = Glucose-6-phosphate dehydrogenase

In stage 1, reactions (b) and (a) permit determination of sample ATP by measuring the increase in absorbance at 340 nm due to formation of NADPH.

CP levels are determined by reactions (c), (b) and (a).

Standards:

Stock ATP: 1 M in neutralized 0.6 M perchloric acid.

Stock CP: 1.5 M in neutralized 0.6 M perchloric acid.

**Table 2.3. Reagent mix for analysis of ATP and CP.**

Reagent	Final concentration (mM)
50 mM TEA HCl, pH=7.5-7.6	38.00
0.1 M MgCl <sub>2</sub> .H <sub>2</sub> O	6.66
0.5 M Glucose	50.00
63 mM NADP (Disodium salt)	0.33
ADP (Disodium salt)	0.15
Glucose-6-phosphate dehydrogenase	723 U/l

\*From yeast (Boehringer Mannheim, 127 655)

The reagent mix was prepared immediately before analysis.

The start reagent for the ATP assay was hexokinase 70 U/ml TEA HCl (From yeast, Boehringer Mannheim 127 809).

The use of a special mode on the Cobas Bio enabled consecutive measurement of ATP and CP. The start reagent for the CP assay was creatine kinase 4800 U/ml (From rabbit muscle, Boehringer Mannheim 126 969).

The inter-assay coefficients of variation for ATP and CP, calculated by repeated analysis of the 250 µl ATP/375 µl CP standard were 5.8% and 6.1% respectively.

### 2.2.5. Glycogen.

Tissue glycogen levels were determined using the UV- enzymic method of Keppeler and Decker [1974], adapted for the Cobas Bio centrifugal analyser. The principle of the method is as follows. Glycogen is converted to glucose by incubation with amyloglucosidase at pH 4.8. The glucose formed from this reaction is converted to glucose-6-phosphate by hexokinase in the presence of  $Mg^{2+}$  and ATP (pH=7.8). Glucose-6-phosphate is then converted to gluconate-6-phosphate in the presence of  $NADP^+$ , resulting in NADPH formation.

The amount of glucose liberated after the hydrolysis of glycogen is proportional to the NADPH production, measured using the absorbance change at 340 nm (37°C).

#### 2.2.5.(a) Preparation of standards

Glycogen (50 mg) (BDH Chemicals; from mammalian liver) was weighed out and placed in a 10 ml volumetric flask. Neutralized 0.6 M perchloric acid (5 ml) was added and the flask was placed in a water bath at 60°C for 10 min. The flask was removed from the water bath and the solution allowed to adjust to room temperature. The volume was then made up to 10 ml using neutralized 0.6 M perchloric acid.

A series of 5 standards (0.1 g/l to 0.5 g/l) were prepared. A portion (200  $\mu$ l) of each standard was pipetted in duplicate into 10 ml plastic tubes. Neutralized 0.6 M perchloric acid acted as a blank. Standards were treated in the same way as the samples throughout the analysis.

Tissue extracts were prepared as described in section 2.1.3.

Samples and standards were incubated with amyloglucosidase (1 mg/ml; from *Aspergillus niger*, Boehringer Mannheim 102 857) dissolved in acetate buffer (0.2 M, pH 4.8) for 2 h at 40°C.

After incubation, 0.6 M perchloric acid (0.5 ml) was added and the samples left on ice for 15 min. Matched potassium carbonate (0.1 ml) was then added and the samples left on ice for a further 30 min. The tubes were centrifuged (4°C, 15 min, 1200 g) and the supernatant removed to Cobas Bio cups for glucose analysis.

#### 2.2.5.(b) Glucose analysis.

Preciset-D-Glucose (Boehringer Mannheim) 0.505 mM was used as the standard for the glucose assay.

Precinorm S (Boehringer Mannheim) diluted 1:24 in neutralized 0.6M perchloric acid acted as a quality control for the assay.

Glucoquant Test Combination (Boehringer Mannheim) was used as the start reagent for the glucose assay.

The coefficient of variation of the assay, calculated by repeated analysis of the 0.1 g/l standard was 2.9%. Recovery of 0.1 mg glycogen glucose was  $90.7 \pm 5\%$  (n=20).

## 2.2.6. Triacylglycerol.

### 2.2.6.(a) Extraction and separation of lipids.

Freeze-dried heart tissue was pulverized as described in section 2.1.2. and each sample transferred to a quickfit tube (20 x 125 mm). Internal standard (0.2 mg triheptadecanoin in 1 ml chloroform) was added to each tube. A "blank" containing internal standard only was analysed simultaneously with each set of samples.

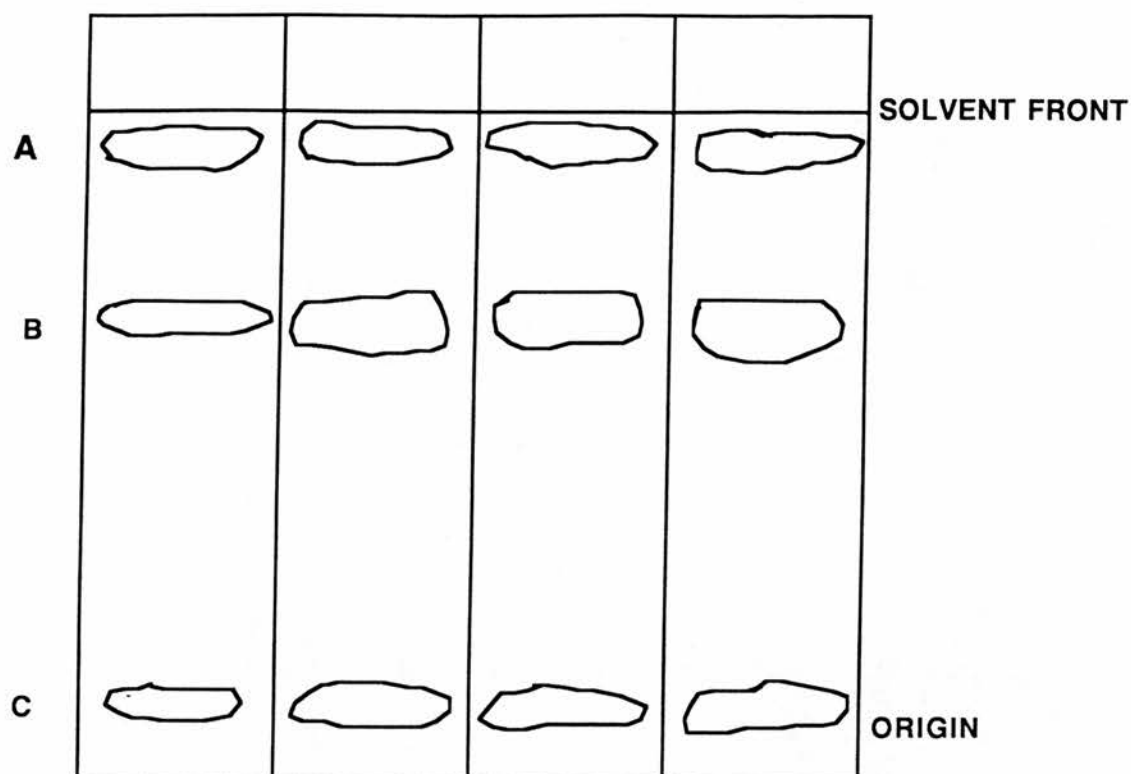
Lipids were extracted according to the method of Folch et al. (1957) using 29 ml chloroform: methanol (2:1 v/v).

The lipid extract was poured into a 1l Buchi flask and dried down under partial vacuum using a Buchi-R rotary evaporator over a water bath at 30°C. The lipids were redissolved in 6 ml chloroform:methanol (2:1 v/v) and poured into a quickfit tube. Samples were then dried down under vacuum and 1.0 ml chloroform:methanol (2:1 v/v) was added using a Hamilton syringe.

A portion of each extract (400 µl) was pipetted into a clean quickfit tube and applied as a 5 cm band on precoated silica gel 60 t.l.c. plates (Merck 5721; 20x20 cm). The remainder of the extract (600 µl) was stored at -40°C in case repeat analysis was required. Plates were developed in a tank containing hexane:diethylether:formic acid (80:20:2 by vol.) at room temperature for about 1 h or until the solvent front approached the top of the plate. Plates were then removed from the tank, air dried and sprayed with POPOP/PPO (6mg 1,4 bis [2-(5-phenyloxozolyl) benzene and 10 mg 2,5-Diphenyloxazole in 100 ml chloroform).

Bands were visualized under UV light at 365 nm. A typical separation is shown in Fig 2.4. The band, previously identified using known standards, was scraped from the plate and placed in a tube.

Figure 2.4. Separation of lipids.



**A=CHOLESTEROL ESTERS**

**B=TRIGLYCERIDES**

**C=PHOSPHOLIPIDS**

Base (0.5 M sodium methoxide) catalyzed transmethylation was carried out as described by Christie (1982). The methylesters were redissolved in 25  $\mu$ l chloroform and transferred to GLC vials flushed with nitrogen and stored at -20°C prior to analysis.

#### 2.2.6.(b) Gas Liquid Chromatography.

A Pye Unicam series 204 gas chromatograph fitted with a PU4700 autoinjector and linked to a Trilab Model II integrator (Trivector) was used. A glass column (1.5 m long, I.D. 2mm) packed with a stationary phase of 10% SP2330 on 100/120 mesh chromosorb WHW (Supelco) was used for all separations.

The following parameters were used:

Injector temperature	220°C
Detector temperature	300°C
Column temperature	180°C
Hydrogen gas flow	50 ml/min
Carrier gas flow	50 ml/min
Air flow	550 ml/min

Temperature programme:

Initial minutes	3
Rate °C/min	3
Final temperature	250°C
Final minutes	5

Solutions of methyl esters stored at -20<sup>o</sup> were allowed to equilibrate to room temperature before each sample (1 µl) was injected onto the column.

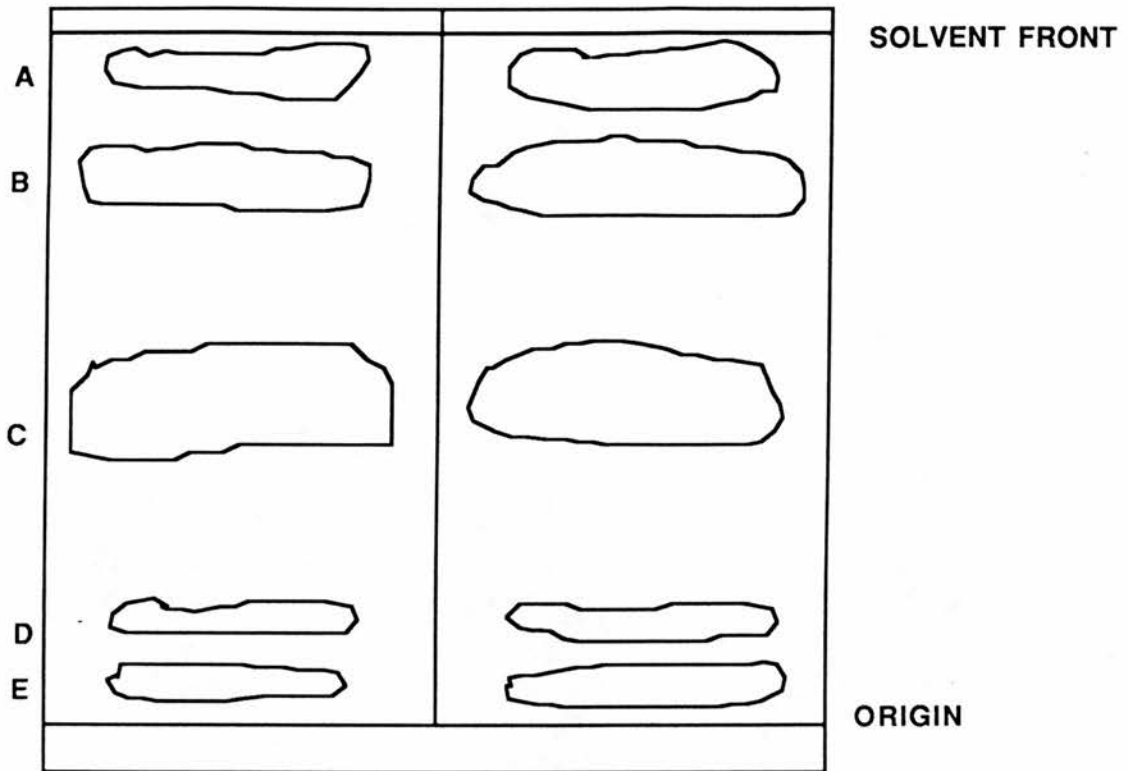
After visual inspection for correct separation of fatty acid peaks and baseline tracking, the samples were reanalysed where necessary.

### 2.2.7. Separation of Radioactive Phospholipids.

Phospholipids, extracted from heart tissue as described in section 2.2.6., were separated by t.l.c. using LK5 plates with a pre-adsorptive area (Whatman catalogue No. 4855 820 (20 x 20 cm). The plates were impregnated with 1.2% boric acid and reactivated at 100°C for 60 min (Fine and Sprecher 1982).

Lipid extract (500 µl) was spotted onto the plates. The plates were developed in chloroform:methanol:water:ammonia (120:75:6:2 by vol.) in a paperlined tank at room temperature. The plates were developed for about 1 h, air dried, and sprayed with dichlorofluorescein (0.1%). The phospholipid fractions were then visualized under UV light at 365 nm. A typical separation is shown in Fig 2.5. The position of each band had been previously identified using known standards. Each band was scraped off and placed in a liquid scintillation counting vial. Scintillation solution (10 ml) was added to each sample and samples were counted as described in section 2.2.8.

Figure 2.5. Separation of phospholipids.



A=CARDIOLIPIN

B=PHOSPHATIDYLETHANOLAMINE

C=PHOSPHATIDYLCHOLINE

D=PHOSPHATIDYLSERINE

E=PHOSPHATIDYLINOSITOL

### **2.2.8. Liquid scintillation counting.**

Samples were placed in Sarstedt scintillation counting vials (58 x 27 mm) and 10 ml of scintillation solution (Scintran, Cocktail T; BDH Chemicals 14509) was added. [<sup>3</sup>H] labelled toluene internal standard (Dupont NES-004 and NES-006 respectively) were added to each sample in order to account for quenching. Efficiency of [<sup>3</sup>H] counting was 28±2%.

#### **2.2.8.a) Fractionated lipid samples**

Fractionated lipid samples (Section 2.2.7.) were counted for 10 min.

#### **2.2.8.b) Perfusate samples**

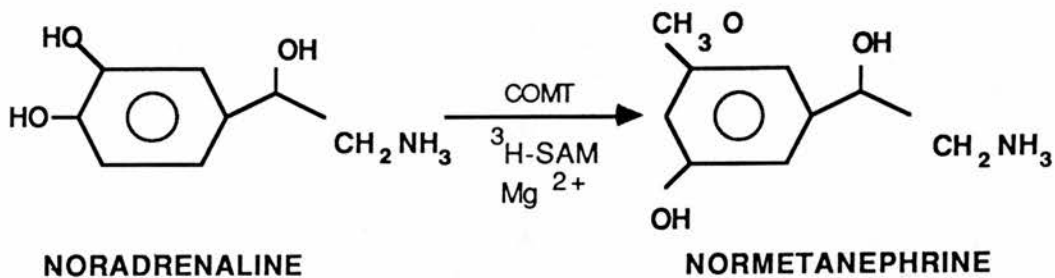
Each perfusate sample (1 ml) was counted for 20 min. A further portion of each sample (1 ml) was placed in a Sarstedt tube and [<sup>3</sup>H<sub>2</sub>O] evaporated by placing in a water bath at 70°C for 3 h. Each sample was then redissolved in cold perfusate (1 ml) and then counted for 20 min as above.

Unlabelled glycerol (30 µl; 100µM) was added to all samples prior to evaporation of water to prevent loss of [<sup>3</sup>H]-glycerol.

### 2.2.9. Measurement of Noradrenaline.

Noradrenaline concentrations in perfusate and tissue samples were measured radioenzymatically (DaPrada and Zurcher 1976).

Noradrenaline was converted to its 3-O-methylated derivative (normetanephrine) using catechol-O-methyl transferase (COMT) in the presence of the radioactive methyl donor  $^3\text{H}$ -methyl-5-adenosylmethionene ( $^3\text{H}$ -SAM) as shown below.



The product of the reaction (normetanephrine) was purified by selective ion pair extraction with tetraphenylborate, separated by t.l.c. and subsequently oxidized to vanillin. The final product was counted in a liquid scintillation counter. (LKB 1217 RACKBETA). All chemicals were from BDH and the standards from Sigma. The COMT (prepared from rat liver) was stable when stored at  $-20^\circ\text{C}$  for at least 6 months (Forfar 1985).

#### 2.2.9.a) Perfusate samples

Perfusate (500  $\mu\text{l}$ ) was stabilized with 0.6M perchloric acid (500  $\mu\text{l}$ ) plus EGTA (2 g/100 ml), and stored at  $-40^\circ\text{C}$  before analysis. The concentrations of the standards used were 1.2 and 12 pM.

#### **2.2.9.(b) Tissue samples.**

Freeze-dried hearts were pulverized as described in section 2.1.3. Heart tissue (approximately 10 mg) was weighed. The tissue was transferred to a Beckman tube and 1 ml 0.3M perchloric acid was added. The sample was mixed and then centrifuged (20000 g, 5 min) using a Hettich Mikroliter centrifuge (Arnold R Howell Ltd, London). The supernatant was stored at -40°C before analysis.

The coefficient of variation for the assay was 7% (Forfar 1985).

### **2.3. Expression of results and statistical analysis.**

Concentrations were expressed as amounts per gram wet weight of tissue throughout. A dry:wet weight ratio of 5:1 was used as the conversion factor where necessary. An unpaired Student t-test was used to indicate significant differences between groups. All results were presented as Mean $\pm$ SEM. As myocardial triglyceride values were not normally distributed, this data was presented as median (95% confidence intervals) and was normalised (log value) before statistical analysis. MINITAB statistics package (CLE.COM Ltd, Edgbaston) was used for all statistical analysis. The experiments were carried out using a rigorous protocol throughout, hence there were no missing data. The number of observations used in calculation of SEMs and for statistical analysis did not differ from those stated in the methods section of each chapter and were therefore not repeated in tables or figures.

#### 2.4. Calculation of RQ values

RQ values are a useful index in determining the main substrate being oxidized by a tissue. As both arterial and venous pH, PO<sub>2</sub> and PCO<sub>2</sub> could be measured using this model, in theory it should be possible to calculate RQ values for each experiment.

Values may be calculated as follows:

$$\text{Oxygen uptake} = [(PO_{2a} - PO_{2v}) \times a \times b] / 25.4 \text{ mM}$$

PO<sub>2a</sub> = Arterial PO<sub>2</sub>

PO<sub>2v</sub> = Venous PO<sub>2</sub>

a = Solubility of oxygen in buffer at 37°C (0.003ml O<sub>2</sub>/100ml buffer per mmHg)

b = Coronary flow rate (ml/min)

25.4 = Volume of ideal gas at 37°C

CO<sub>2</sub> production may be calculated by subtracting the sum of the concentrations of dissolved carbon dioxide and bicarbonate in the arterial perfusate from that in the venous perfusate:

$$H_2CO_3 = [PCO_2 \times c \times b] / 25.4$$

c = Solubility of CO<sub>2</sub> in buffer at 37°C (0.078 ml CO<sub>2</sub>/100 ml buffer per mmHg)

The bicarbonate concentration may be calculated using the Henderson-Hasselbach equation:

$$\text{pH} = \text{pK}' + \log ([\text{HCO}_3^-] / [\text{CO}_2])$$

$$\text{pK}' = 6.1$$

Therefore:

$$[\text{HCO}_3^-] = \text{H}_2\text{CO}_3 \times \text{antilog}(\text{pH} - 6.1) \text{ mM}$$

$$= (0.0348 \text{ PCO}_2) \times \text{antilog}(\text{pH} - 6.1) \text{ mM}$$

$$\text{Total CO}_2 = [\text{H}_2\text{CO}_3] + [\text{HCO}_3^-]$$

$$\text{CO}_2 \text{ production} = \text{Total CO}_{2v} - \text{Total CO}_{2a}$$

where a = arterial and v = venous.

Using data from a typical experiment:

$$\text{Oxygen uptake} = (14.9 \times 0.003 \times 10) / 25.4 \text{ mM}$$

$$= 0.02 \text{ mM}$$

$$[\text{H}_2\text{CO}_3]_a = (40 \times 0.078 \times 10 / 25.4)$$

$$= 1.22$$

$$[\text{HCO}_3^-]_a = 1.22 \times 19.5$$

$$= 23.95 \text{ mM}$$

$$\text{Total } [\text{CO}_2]_a = 23.95 + 1.22$$

$$= 25.17 \text{ mM}$$

$$[\text{H}_2\text{CO}_3]_v = (45.8 \times 0.078 \times 10) / 25.4$$

$$= 1.41 \text{ mM}$$

$$[\text{HCO}_3]_v = 1.41 \times 15.49$$

$$= 21.84 \text{ mM}$$

$$\text{Total } [\text{CO}_2]_v = 1.41 + 21.84$$

$$= 23.25 \text{ mM}$$

$$\text{CO production} = 25.17 - 23.25$$

$$= 1.92 \text{ mM}$$

$$\text{RQ} = 1.92/0.02$$

$$= 96$$

This value is obviously not a true RQ value. The error in calculating the value is presumably due to the effects of hypoxia. The increased production of hydrogen ions during hypoxia will neutralize bicarbonate ions in the perfusate leaving the heart. This will affect the  $\text{PCO}_2$  and pH. Variability of these parameters in turn will affect the calculated RQ value. It therefore appears that although RQ values may be calculated, the parameters measured in this study are not sufficiently reliable to provide a realistic estimation of this parameter.

**CHAPTER 3**

**THE EFFECTS OF  
INCREASING SEVERITY OF HYPOXIA  
ON MYOCARDIAL METABOLISM.**

### **3.1. Introduction.**

A substantial amount of evidence favours the likelihood of the operation of an energy wasting TAG- NEFA cycle during acute myocardial ischaemia (Section 1.7). However, little information is available in terms of the proportion of energy used by the cycle and its relative importance during oxygen deprivation.

The hypoxic isolated, perfused rat heart is a useful model for obtaining quantitative information about the biochemical changes taking place in the heart during oxygen deprivation (Section 1.6). The aim of this study was to use the model to examine the biochemical consequences of varying severities of hypoxia with particular regard to the operation of the TAG-NEFA wasteful cycle and its potential importance in the development of arrhythmias.

The use of the isolated, perfused rat heart inevitably entails a period of anoxia in the interval between removing the heart and commencing perfusion. This period obviously has biochemical consequences. If the effects of this period of anoxia are prolonged, they need to be considered when analyzing data. A preliminary study was therefore also carried out to look at the response of the heart to this period of anoxia and to try and determine the duration of its effects with a view to the design of subsequent experiments.

## 3.2. Methods.

### 3.2.1. Preliminary experiments

Hearts from male Sprague Dawley rats (body weight 400-450 g) were removed and perfused normoxically (95%O<sub>2</sub>/5%CO<sub>2</sub>) for 40min. Coronary effluent samples were collected at t=1, 2, 3, 4, 5, 6, 8, 10, 15, 20, 25, 30, 35 and 40 min and analyzed for glycerol and lactate.

### 3.2.2. The effects of increasing severity of hypoxia on myocardial metabolism.

Hearts from male Sprague Dawley rats (body weight 400-450g) were removed and perfused as described in section 2.1.2.

Four groups of rats were perfused normoxically (95%O<sub>2</sub>/5%CO<sub>2</sub>) for 30 min followed by a further 30 min period of hypoxic perfusion. The oxygen tension of the perfusate for each group (Mean±SD) is shown in Table 3.1.

Table 3.1. Oxygen tension of the perfusate with varying gas compositions.

Group	No. of hearts	Composition of gas	PO <sub>2</sub> (mmHg)
A	18	95% O <sub>2</sub> /5%CO <sub>2</sub>	600±6
B	16	30%O <sub>2</sub> /65%N <sub>2</sub> /5%CO <sub>2</sub>	220±6
C	7	20%O <sub>2</sub> /75%N <sub>2</sub> /5%CO <sub>2</sub>	133±8
D	8	10%O <sub>2</sub> /85%N <sub>2</sub> /5%CO <sub>2</sub>	58±8

For the sake of convenience, groups B, C and D will henceforth be referred to as mild, moderate and severe hypoxia groups respectively.

Coronary effluent samples were collected at 5 min intervals and analyzed for glycerol and lactate as described in sections 2.2.1 and 2.2.2 respectively. Oxygen uptake was monitored throughout the experiments. Tissue samples were analyzed for glycogen, ATP, CP,  $\alpha$ -glycerophosphate, glycerol and triacylglycerol (TAG) as described in Chapter 2.

Hearts were removed from a fifth group of rats, normoxically perfused for a few seconds to wash out blood and then freeze clamped. This group of hearts acted as "pre-perfusion" controls.

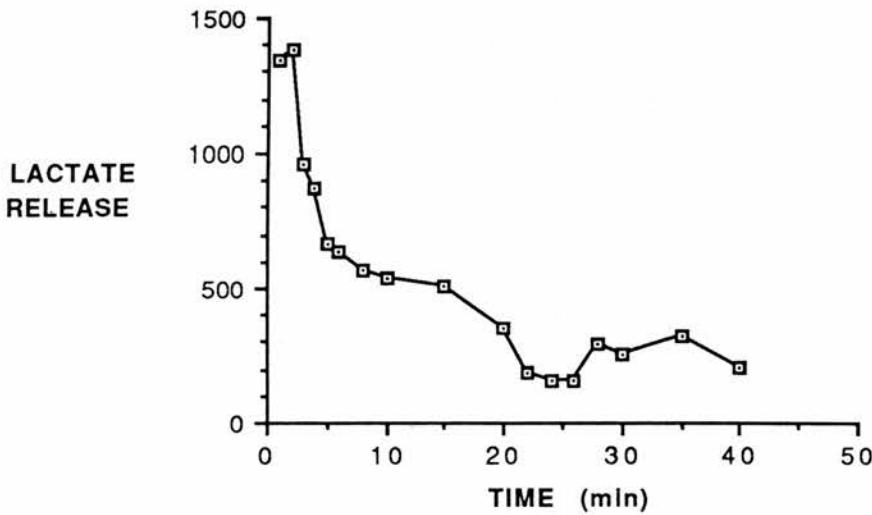
### 3.3. Results.

#### 3.3.1 Preliminary experiments.

Both lactate and glycerol release were elevated immediately after commencing perfusion. Efflux of both of these metabolites gradually decreased between  $t=1$  and  $t=20$  min, after which a low, stable rate of lactate and glycerol release continued for the remainder of the perfusion period (Figs 3.1 and 3.2 respectively).

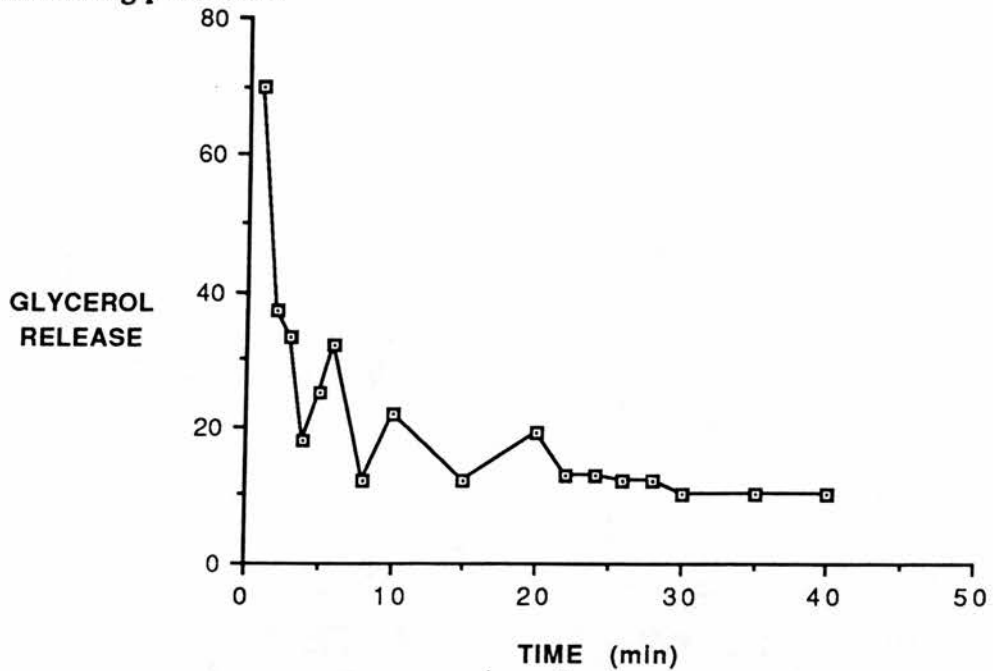
In the light of these results it was decided to include a preperfusion period of 20 min duration in subsequent experiments before collection of the first coronary effluent sample. Any hearts in which basal lactate release was greater than  $1.5 \mu\text{mol.g}^{-1}\text{wet wt.min}^{-1}$  were considered to be bad preparations and were therefore discounted from the results.

Figure 3.1. Lactate release from a typical heart during the first 40 min after commencing perfusion.



Lactate release is expressed as  $\text{nmol.g}^{-1}\text{min}^{-1}$ .

**Figure 3.2. Glycerol release from a typical heart during the first 40 min after commencing perfusion.**

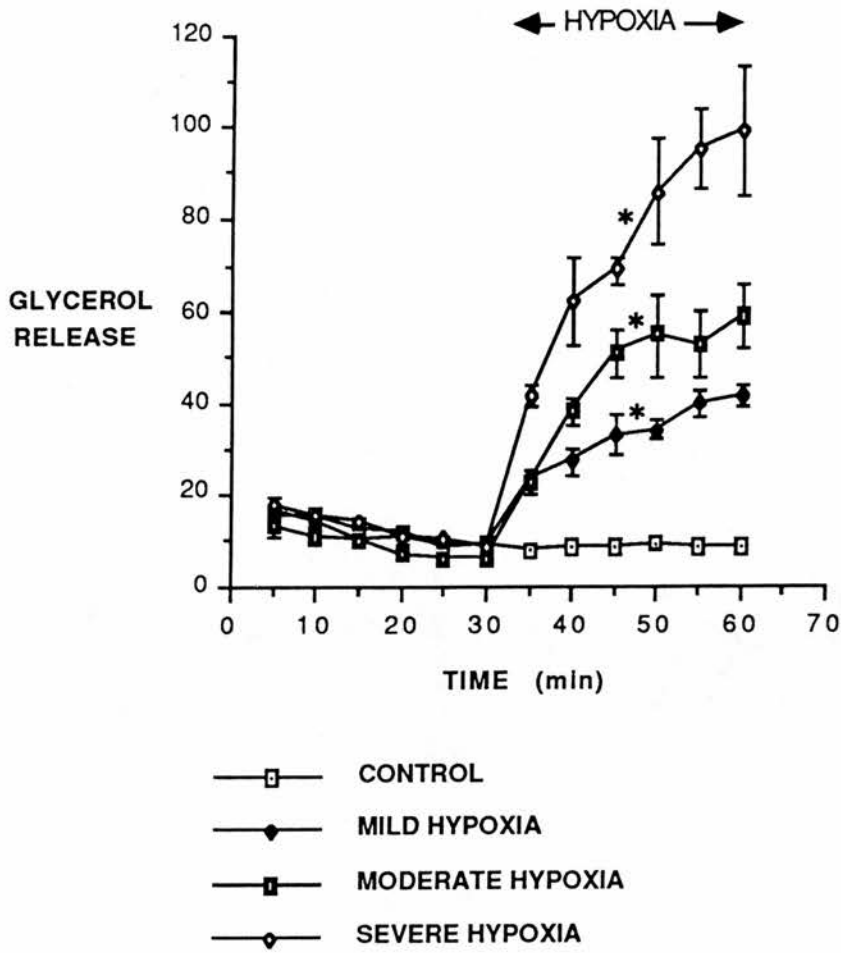


Glycerol release is expressed as  $\text{nmol.g}^{-1}\text{min}^{-1}$

### **3.3.2. Effects of increasing severity of hypoxia on myocardial metabolism.**

Glycerol and lactate efflux from the normoxically perfused hearts (control) remained constant throughout the 60min perfusion period. In contrast, hypoxia resulted in a significant increase in glycerol and lactate release (Figs 3.3 and 3.4 respectively). The total glycerol release between  $t=30$  and  $t=60\text{min}$ , calculated by measuring the area under the curve of glycerol release vs time for each group is shown in Fig 3.5.

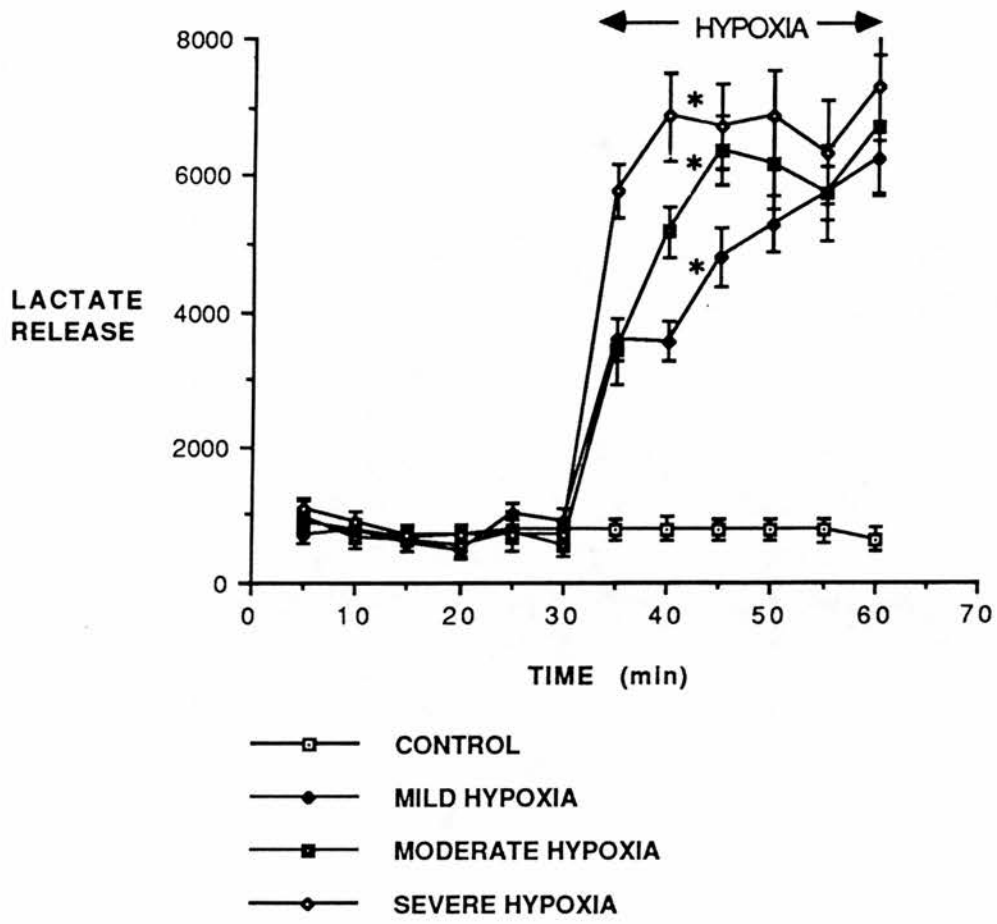
**Figure 3.3. The influence of severity of hypoxia on glycerol release from the isolated, perfused rat heart.**



Glycerol release is expressed as  $\text{nmol.g}^{-1} \text{ wet wt. min}^{-1}$  (Mean $\pm$ SEM).

\* $P < 0.01$  vs CONTROL (Analysis of variance).

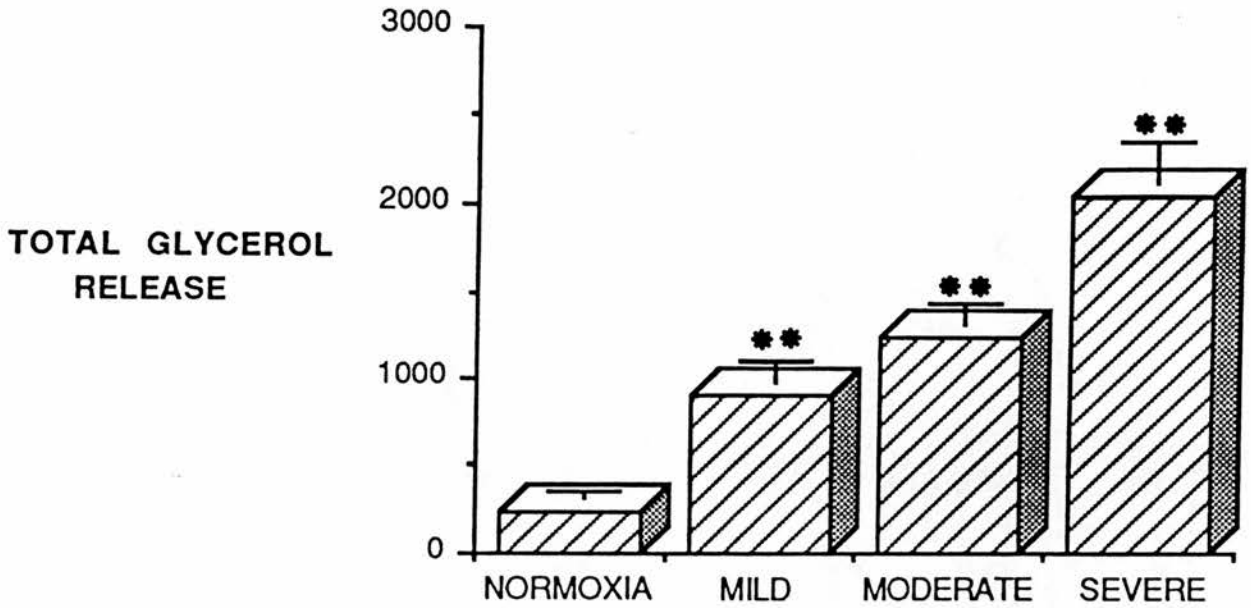
Figure 3.4. The influence of severity of hypoxia on lactate release from the isolated,perfused rat heart .



Lactate release is expressed as nmol.g<sup>-1</sup>min<sup>-1</sup> (Mean±SEM)

\* P<0.01 vs CONTROL (Analysis of variance)

Figure 3.5. The influence of severity of hypoxia on glycerol release from the isolated, perfused rat heart over a 30 min period.



Glycerol release is expressed as  $\text{nmol.g}^{-1}30\text{min}^{-1}$  (Mean $\pm$ SEM)

\*\*  $P < 0.01$  vs NORMOXIA (t-test).

Oxygen uptake had decreased significantly on collection of the first sample for blood gas analysis (t=35min) after the change to hypoxic perfusion. The mean oxygen uptake for each group is shown in Table 3.2. Oxygen uptake did not vary with time during the experimental period.

Table 3.2. The influence of severity of hypoxia on myocardial oxygen uptake.

Group	% OXYGEN	Oxygen Uptake (mmol.g <sup>-1</sup> min <sup>-1</sup> )
A	95 (Control)	6.35±0.2
B	30	2.38±0.05
C	20	1.45±0.03
D	10	0.20±0.04

Results are presented as Mean±SEM.

Measurements of tissue high energy phosphate levels revealed a significant decrease in ATP at each level of hypoxia in the experimental groups compared with the control group. ATP was significantly lower in the moderate hypoxia group than in the mild hypoxia group but there was no further decrease in ATP level when hearts were subjected to severe hypoxia.

After mild hypoxia no significant change in tissue CP concentration could be found when compared with the control group (2.28±0.04 μmol.g<sup>-1</sup>wet wt. mild hypoxia vs 2.56±0.02 control; Mean±SD). However, both the moderate hypoxia and severe hypoxia groups exhibited significantly lower CP levels than the control group. The CP concentration of the severe hypoxia group was significantly lower than that of the moderate hypoxia group (Fig 3.6). Tissue glycogen levels followed a similar pattern to CP levels (Fig 3.7).

Figure 3.6. The influence of severity of hypoxia on high energy phosphate concentrations in rat heart ventricle.

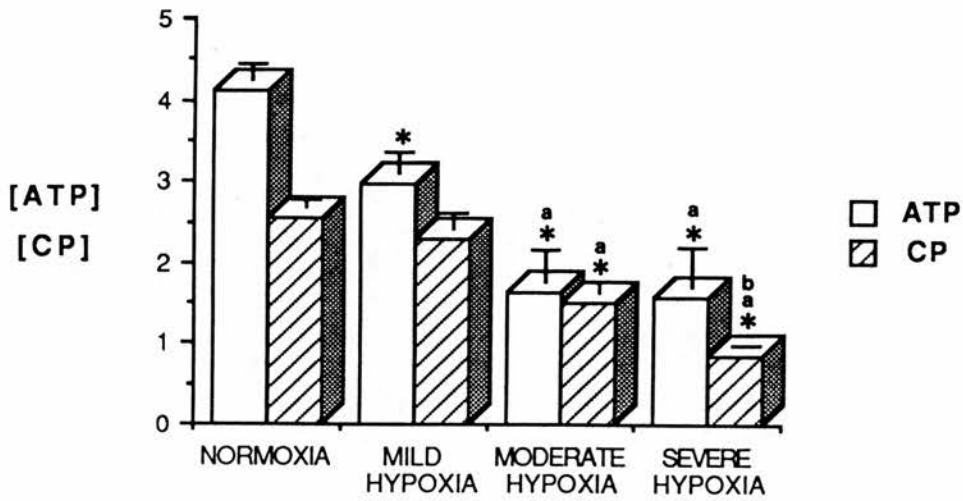
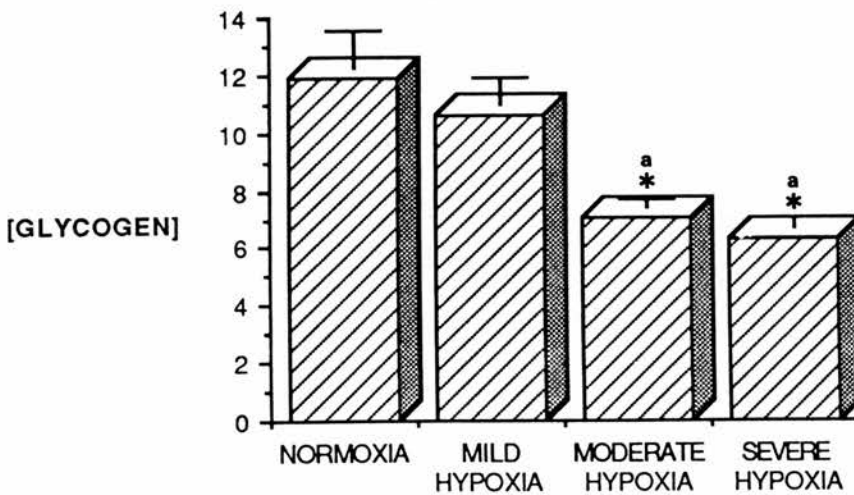


Figure 3.7. The influence of severity of hypoxia on glycogen concentrations in rat heart ventricle.



Metabolite concentrations are expressed as  $\mu\text{mol.g}^{-1}$  wet wt. (Mean $\pm$ SEM)

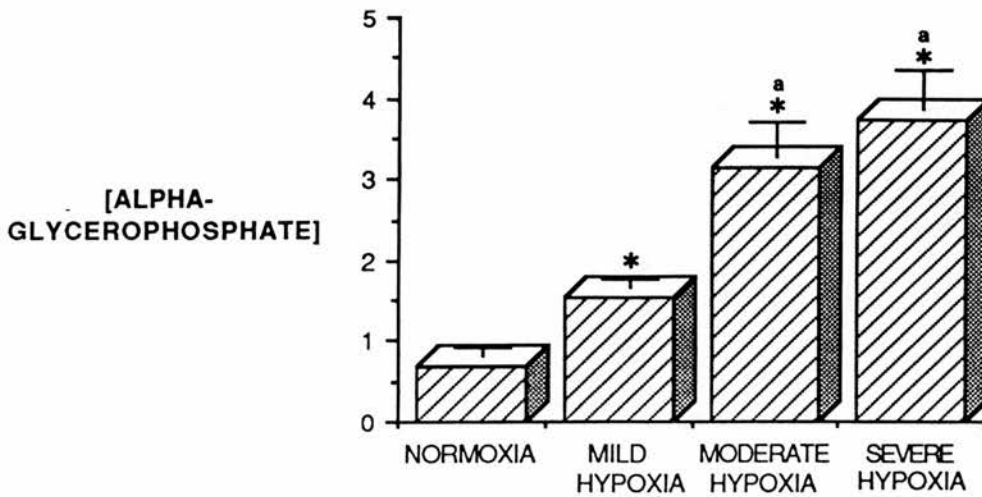
\* P<0.01 vs NORMOXIA

a P<0.01 vs MILD HYPOXIA

b P<0.01 vs MODERATE HYPOXIA

Hypoxia resulted in a significant increase in myocardial  $\alpha$ -glycerophosphate concentrations. The mean  $\alpha$ -glycerophosphate concentration in hearts subjected to moderate hypoxia group was significantly higher than in those subjected to mild hypoxia group, but there was no difference between the levels in the moderate hypoxia and severe hypoxia groups (Fig 3.8).

**Figure 3.8. The influence of severity of hypoxia on  $\alpha$ -glycerophosphate concentrations in rat heart ventricle.**



$\alpha$ -glycerophosphate concentrations are expressed as  $\mu\text{mol.g}^{-1}\text{wet wt.}$  (Mean $\pm$ SEM)

\*  $P < 0.01$  vs NORMOXIA (t-test)

<sup>a</sup>  $P < 0.01$  vs MILD HYPOXIA (t-test)

Myocardial TAG levels varied considerably *within* groups. As the results were not normally distributed, statistical analysis was performed using logarithms of the values. No significant difference in tissue TAG concentrations between groups was found (Table 3.3).

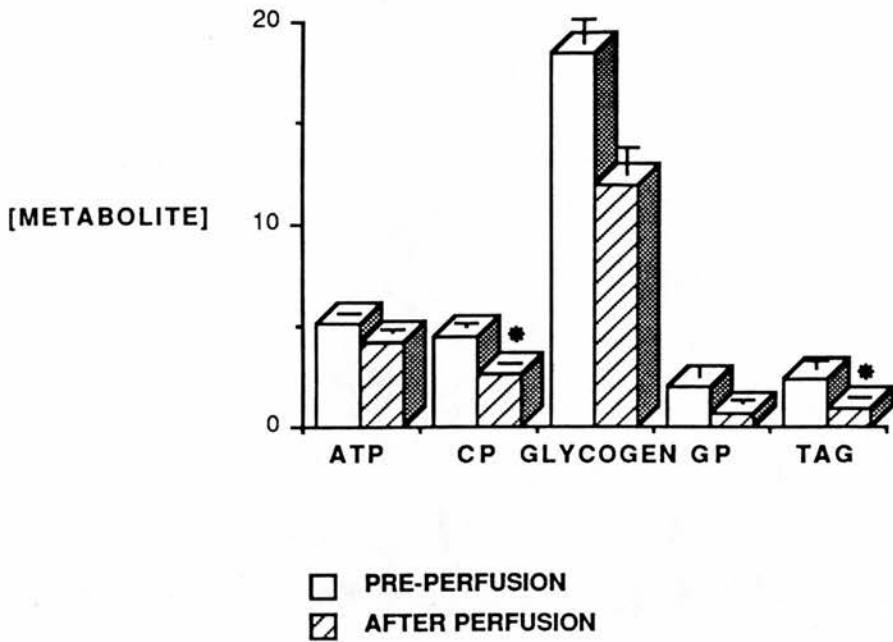
**Table 3.3. The influence of severity of hypoxia on myocardial TAG concentrations.**

<b>% OXYGEN</b>	<b>TAG concentration (<math>\mu\text{mol.g}^{-1}\text{wet wt.}</math>)</b>
95	0.5 (0.2, 1.9)
30	0.5 (0.2, 2.2)
20	0.5 (0.2, 2.0)
10	0.4 (0.1, 1.2)

Results are presented as Median (95% Confidence limits).

Biochemical analysis of the preperfusion control group of hearts and comparison with the normoxically perfused hearts (group A) revealed that a significant amount of TAG, and CP are utilized by the heart under normoxic conditions (Fig 3.9).

Figure 3.9. The influence of normoxic perfusion (60 min) on metabolite concentrations in rat heart ventricle.



Metabolite concentrations are expressed as  $\mu\text{mol.g}^{-1}$  wet wt. (Mean $\pm$ SEM)

\*  $P < 0.01$  vs PRE-PERFUSION (t-test)

### 3.4. Discussion.

The elevated lactate and glycerol release during the first few minutes of the perfusion period is presumably the result of a shift from aerobic to anaerobic respiration during the period of anoxia on removal of the heart. Lactate and glycerol production are thought to reflect anaerobic glycolysis and lipolysis respectively. Placing the heart in ice cold perfusate immediately after removal helps to minimize the effects of anoxia by "slowing down" the biochemical changes in the heart, inhibiting contraction and therefore preventing excessive loss of energy supplies and endogenous substrates. The high rates of lactate and glycerol release during the first few minutes of perfusion probably reflect the wash-out of lactate and glycerol accumulated during the anoxic period.

Endogenous triacylglycerol is utilised in the isolated, perfused rat heart under normoxic conditions despite the presence of 5.5 mM glucose as an exogenous substrate (Fig 3.9). This is in agreement with the findings of Neely et al.. (1970) and De Groot et al.. (1989). The decrease in CP observed during normoxic perfusion implies that oxygenation of the tissue was inadequate for the control group. It is well recognized that perfusion of isolated hearts may not provide the optimum requirements for the maintenance of aerobic metabolism.

The metabolic changes observed in the experimental groups are typical of the biochemical changes expected to occur during oxygen deprivation (Chapter 1).

When the oxygen supply to the heart is reduced the decreased capacity of the tissue for oxidative metabolism leads to inhibition of ATP production and accumulation of reduced flavin and nicotinamide co-enzymes in the mitochondria. The NADH/NAD<sup>+</sup> ratio subsequently increases and ADP and AMP levels increase. As a consequence of these changes, the rate of glycolysis increases and entry of pyruvate into the citric acid cycle is hindered. Hence lactate production by the tissue is increased.

Under mild hypoxic conditions the glycogen concentration in the heart did not decrease significantly compared with the control group of hearts. This may be a consequence of stimulation of both glycogen phosphorylase and glycogen synthetase during oxygen deprivation (Williams and Mayer 1966, Opie 1968). In contrast, tissue glycogen levels in the moderate and severe hypoxia groups were significantly lower than the control group, implying a shift towards increased glycogen breakdown and inhibition of glycogen synthesis during more severe hypoxia, possibly due to lack of availability of high energy phosphates for glycogen synthesis.

At low glycogen concentrations disruption of the glycogen particle and associated enzyme system may occur, thereby removing accessibility to phosphorylase (Entman et al. 1977). Alternatively, in the severe hypoxia group, the glycogen remaining in the heart may contain insufficient  $\alpha$ -1-4 linkages, preventing breakdown of glycogen by phosphorylase (Bailey et al. 1982).

As the amount of glucose taken up by the heart is small in comparison with the concentration in the perfusate, glucose uptake cannot be measured accurately owing to the large errors in attempting to evaluate such small changes in concentration. However, glucose uptake is well known to increase during oxygen deprivation (Morgan et al. 1961). Under mild hypoxic conditions, it may be that the stimulation of glucose uptake is sufficient to maintain the increase in glycolytic rate, whereas with more severe hypoxia although glucose transport into the heart is augmented, this may not be adequate in providing substrate for the increased glycolytic activity without depleting endogenous glycogen supplies (Morgan et al. 1959, Morgan et al. 1961). As neither glucose uptake nor the relative activities of glycogen synthetase nor glycogen phosphorylase were measured, this question cannot be answered conclusively.

After mild hypoxia, tissue ATP concentrations were depleted compared with the control group, but CP levels were unchanged. This was unexpected as CP is thought to

be depleted in preference ATP during oxygen deprivation (Neely et al.1973, Hearse 1979). In this case the increase in ATP production via anaerobic glycolysis may have been adequate to maintain levels at a sufficient level so that CP stores were not utilized. The fact that CP concentrations in the severe hypoxia group were significantly lower than in the moderate hypoxia group , whereas ATP concentrations were not different in the moderate and severe hypoxia groups is consistent with the generally held view that CP acts as an "emergency" store of high energy phosphate for use during severe energy depletion (Opie 1984). Hence, during more severe hypoxia, utilization of CP appears to preserve ATP. Glycolytically produced ATP may be preferentially available for maintenance of ionic gradients (Harris and Barry 1984, McDonald and MacLeod 1973, Mercer and Durham 1981). The loss of this source of ATP production could conceivably have lethal consequences.

The increase in glycerol release from the heart during hypoxia is commonly used as an index of myocardial lipolysis (Christian et al. 1968, Stam et al. 1979, Stam and Hulsman 1982, Severson et al. 1986, De Groot et al. 1989). Hypoxia is thought to stimulate release of endogenous catecholamines (Wollenberger and Shahab 1965, Karwatowska-Krynska and Beresewicz 1983) which activates a hormone sensitive TAG lipase in the heart by phosphorylation via a cAMP- protein kinase cascade system (Section 1.6.1).

If the glycerol released from the heart during hypoxia does reflect TAG breakdown, for every mole of glycerol leaving the heart, 3 of non-esterified fatty acid (NEFA) remain in the tissue. As no albumin was present in the perfusate, NEFA were unable to leave the heart. Furthermore, due to the decreased oxygen supply to the tissue, oxidation of NEFA is inhibited. NEFA are therefore likely to accumulate in the tissue. TAG concentrations in the heart were unchanged after hypoxia compared with normoxia despite the large increase in glycerol release implying that NEFA were re-esterified.

The within-group variation in TAG levels may reflect varying proportions of intermyocardial adipocytes in the hearts. Extraneous adipose tissue was removed before freeze-clamping hearts but no measure of the proportion of adipocytes within the heart was available. However, similar results have been obtained from experiments in which isolated myocytes were subjected to hypoxia (Larsen et al. 1989), suggesting that the presence of adipocytes within the heart does not significantly affect the conclusions drawn from these experiments.

Re-esterification of NEFA requires the presence of  $\alpha$ -glycerophosphate.  $\alpha$ -glycerophosphate levels increased as the severity of hypoxia increased. This was expected as the increased NADH/NAD<sup>+</sup> ratio during oxygen deprivation favours conversion of dihydroxyacetone phosphate to  $\alpha$ -glycerophosphate.  $\alpha$ -glycerophosphate may also be formed by phosphorylation of glycerol by the action of glycerokinase. The activity of this enzyme in rat heart is commonly dismissed as being negligible. However, the existence of the enzyme in rat heart has been demonstrated (Robinson and Newsholme 1967), its maximum activity being 10.8 nmol glycerol g<sup>-1</sup> fresh wt. min<sup>-1</sup>. This activity was measured in the presence of relatively high glycerol concentrations (50 $\mu$ M) and was diminished by AMP and ADP, both of which are likely to increase in concentration during hypoxia. However, as neither glycerokinase activity nor ADP and AMP concentrations were measured under the conditions used in this study, the possible contribution of glycerokinase activity to  $\alpha$ -glycerophosphate production should not be dismissed.

Given that 2 high energy phosphate groups are required for conversion of each NEFA to fatty acyl CoA prior to re-esterification, a total of 6 moles of ATP are required for each mole of TAG synthesized. Assuming that

- 1) 3 NEFA remain in the heart for every glycerol molecule released.
- 2) No NEFA oxidation takes place

3) The P/O ratio is 3,

the total aerobic and anaerobic ATP production may be calculated from the oxygen uptake and lactate release respectively and the proportion of ATP required to re-esterify the NEFA may be calculated from the glycerol release for each group of hearts. The potential proportion of energy used in TAG-NEFA cycling is shown in Table 3.4.

**Table 3.4. The influence of severity of hypoxia on energy utilized in the operation of the TAG-NEFA cycle.**

% OXYGEN	ATP production		ATP used for TAG-NEFA cycling	% ATP used
	Aerobic	Anaerobic		
30	428.4±7	137.1±22	5.38±0.4	0.95±0.03
20	261.1±3	165.2±30	7.50±0.4	1.76±0.03
10	36.0±7	196.9±30	12.20±0.6	5.24±0.03

ATP production is expressed as  $\mu\text{mol}\cdot\text{g}^{-1}\cdot 30\text{min}^{-1}$  (Mean±SEM).

Obviously these percentages are only approximations of the possible energy wastage. Some degree of NEFA oxidation cannot be ruled out, particularly in the mild hypoxia group. Unfortunately the relative contribution of the various substrates to the total ATP production could not be determined due to difficulty in calculating RQ values (section 2.4).

It is also possible that uncoupling of oxidative phosphorylation takes place during hypoxia. Conversion of NEFA to acyl CoA and a subsequent increase in size of the acyl CoA pool is also possible. High concentrations of acyl CoA may inhibit adenine nucleotide translocase, the enzyme responsible for transporting ATP from within the

mitochondria to the cytoplasm. (Ho and Pande 1974, Shug and Shrago 1973). This would mean that any ATP formed by oxidative phosphorylation would not be available for re-esterification of NEFA and for other energy-requiring processes in the myocyte.

Even if only glycolytically produced ATP is available for the re-esterification process, the loss of energy due to TAG-NEFA cycling during severe hypoxia amounts to less than 7% of the available ATP.

The proportion of energy wasted increases as the severity of hypoxia increases. During ischaemia when the blood supply to an area of tissue is prevented, an oxygen gradient exists across the tissue. A range of hypoxic states therefore exist - the outer edge of the ischaemic zone being mildly hypoxic and the centre of the ischaemic zone being severely hypoxic. It is therefore probable that TAG-NEFA cycling occurs at different rates across the ischaemic area. The heterogeneity of blood flow to an area of tissue is an important factor in the development of arrhythmias. Varying rates of wasteful cycling in an area of myocardium may therefore contribute to arrhythmogenesis.

In the severe hypoxia group in this study, glycolytically produced ATP accounted for as much as 85% of the total ATP produced, therefore making a highly significant contribution to myocardial energy production. Under ischaemic conditions glycolysis is inhibited and this source of ATP is therefore dramatically reduced. Hence these results cannot be directly extrapolated to ischaemia. In addition, the supply of oxygen and substrates remains constant during hypoxia whereas in ischaemia, oxygen and substrates are rapidly depleted and are only partly replaced. However, this study provides further evidence in favour of the operation of a TAG-NEFA cycle during myocardial hypoxia. The amount of energy consumed in the operation of the TAG-NEFA cycle during hypoxia does not appear to cause a significant drain of ATP supplies, suggesting that the cycle is not likely to be a major cause of arrhythmias. However, further experiments are needed to examine the effect of ischaemia on the operation of the TAG-NEFA cycle and

to study possible differences in rates of cycling in different parts of the cell. It is possible that the TAG-NEFA cycle plays an important role in protecting the hypoxic myocardium against the potentially deleterious effects of accumulation of fatty acids and fatty acyl CoA.

## **CHAPTER 4**

# **THE INFLUENCE OF MYOCARDIAL TRIACYLGLYCEROL LEVELS ON TAG-NEFA CYCLING DURING NORMOXIA AND HYPOXIA**

#### 4.1. Introduction.

Raised myocardial TAG levels may increase susceptibility of the heart to ischaemic or hypoxic damage. Endogenous catecholamines are thought to be released during hypoxia in the isolated, perfused heart (Wollenberger and Shahab 1965, Karwatowska-Krynska and Beresewicz 1983) and ischaemia (Dart et al. 1987, Schomig et al. 1987). Brownsey and Brunt (1977) increased TAG levels in rat hearts by feeding a high fat diet. These hearts were found to exhibit

1) An increased rise in resting tension and decline in heart rate during ischaemia compared with hearts from rats with normal myocardial TAG levels.

2) An increased incidence of arrhythmias and a greater tendency to deteriorate in mechanical performance in the presence of adrenaline.

Hearts from diabetic rats are more vulnerable to anoxia than normal rat hearts (Hearse et al. 1975). This increased susceptibility to injury may be due to the greater accumulation of endogenous TAG (Denton and Randle 1967). Isolated, perfused hearts from obese rats which also have raised TAG levels are more susceptible to arrhythmias than hearts from lean rats (Sargent 1990).

Trach (1986) suggested that TAG-NEFA cycling may become more important as a cause of arrhythmias when TAG levels in the heart are increased. In accordance with this view, basal and hormone-stimulated lipolysis are increased when tissue TAG levels are raised (Stam and Hulsmann 1978, Hulsmann et al. 1979).

Cardiac denervation is known to be protective against ischaemic arrhythmias (Ebert et al. 1970, Martin and Meesman 1985). In hearts from denervated rats, myocardial lipolysis, demonstrated by glycerol efflux, was not activated during ischaemia (Hough and Gevers 1975), supporting the hypothesis that TAG metabolism may be

involved in the development of arrhythmias.

Extraction of NEFA by the myocardium rises with the plasma NEFA/albumin ratio (Evans et al. 1963, Miller et al. 1976). Because the plasma albumin concentration is fairly constant, the rate of uptake is largely determined by the arterial concentration of NEFA (Carlsten et al. 1961, Vik-Mo et al. 1979). In ischaemic myocardium,  $\beta$ -oxidation is impaired, and increased NEFA extraction therefore results in accumulation of TAG in the tissue (Scheuer and Brachfield 1966, Opie et al. 1973, Jesmok et al. 1978).

Chronic ischaemia may thus lead to TAG accumulation (Brachfield 1969) and could in this way lead to a decrease in tolerance to ischaemic or catecholamine-induced stress.

The purpose of this study was to examine the effects of conditions associated with raised myocardial TAG levels, i.e. fasting (Wittels and Bressler 1964, Denton and Randle 1967) and obesity, on the rate of TAG-NEFA cycling during hypoxia, and also, as age is known to be a risk factor for increased vulnerability to hypoxia (Nakanishi et al. 1984), to compare the rates of TAG-NEFA cycling in hearts from rats of different ages.

## 4.2. Methods.

Hearts from three groups of male Sprague-Dawley rats and one group of inbred obese rats (ob/ob; supplied by The Rowett Research Institute, Aberdeen) were studied. The characteristics of each group of rats was as follows:

- A. Control (Body weight 450-500g; 18-20 weeks). Fed ad libitum; n=18 normoxia, n=16 hypoxia.
- B. Young (Body weight 280-320g; 12-14 weeks). Fed ad libitum; n=6 normoxia, n=6 hypoxia.
- C. Fasted (48 h; initial body weight 450-500g). Water ad libitum; n=6 normoxia, n=6 hypoxia.
- D. Obese (Body weight 500-550g; 18-20 weeks). Fed ad libitum; n=6 normoxia, n=9 hypoxia.

Rats were anaesthetized and the hearts removed and perfused. The protocol was as described in Section 2.1.2. All hearts were normoxically (95%O<sub>2</sub>/5%CO<sub>2</sub>) perfused for 30 min, after which hearts were subjected to either a further 30 min normoxia or 30 min hypoxia (30%O<sub>2</sub>/65%N<sub>2</sub>/5% CO<sub>2</sub>).

Coronary effluent was collected at 5 min intervals and analyzed for glycerol and lactate. Oxygen consumption was monitored throughout. After perfusion, hearts were freeze-clamped, freeze-dried and the tissue was analyzed for ATP, CP, glycogen,  $\alpha$ -glycerophosphate, glycerol and triacylglycerol as described in Chapter 2.

Additionally, hearts from control, young and fasted rats were freeze-clamped immediately after suspension and wash-out of blood (n=6 per group) to obtain comparison of metabolite concentrations between groups before perfusion. These hearts were also freeze-dried and analyzed as described above.

### 4.3 Results.

Body weights of rats fasted for 48 h decreased by  $49.5 \pm 4$  g (Mean  $\pm$  SEM;  $P < 0.05$ , t-test).

TAG levels in hearts from fasted rats were not significantly different than in hearts from fed (control) rats (Table 4.1). The only significant differences between hearts from control and young rats were in the tissue ATP and CP concentrations. Hearts from young rats had significantly lower ATP concentrations but significantly higher CP concentrations than the control group. However, the total high energy phosphate (ATP + CP) concentrations were not significantly different.

As reported by other workers, glycogen concentrations were significantly higher in hearts from fasted rats ( $28 \pm 0.6$  fasted vs  $18 \pm 0.3$   $\mu\text{mol}$  glucose equivalents  $\text{g}^{-1}$  wet wt fed). The amount of  $\alpha$ -glycerophosphate in hearts from fasted rats was significantly lower than in fed rats (Table 4.1).

**Table 4.1. Concentrations of metabolites in hearts from control, young and fasted rats before perfusion.**

METABOLITE	CONTROL	YOUNG	FASTED
ATP	5.10±0.05	4.63±0.11*	4.38±0.20
CP	4.49±0.10	4.92±0.12*	3.70±0.32
GLYCOGEN	18.40±1.20	18.27±0.78	27.56±1.37**
α-GP	2.02±0.45	1.72±0.45	0.05±0.02**
GLYCEROL	0.06±0.01	0.06±0.01	0.05±0.01
TAG	2.40±0.10	2.48±0.10	2.18±0.12

Metabolite concentrations are expressed as  $\mu\text{mol.g}^{-1}$  wet wt. (Mean±SEM).

Glycogen concentration is expressed as glucose equivalents.

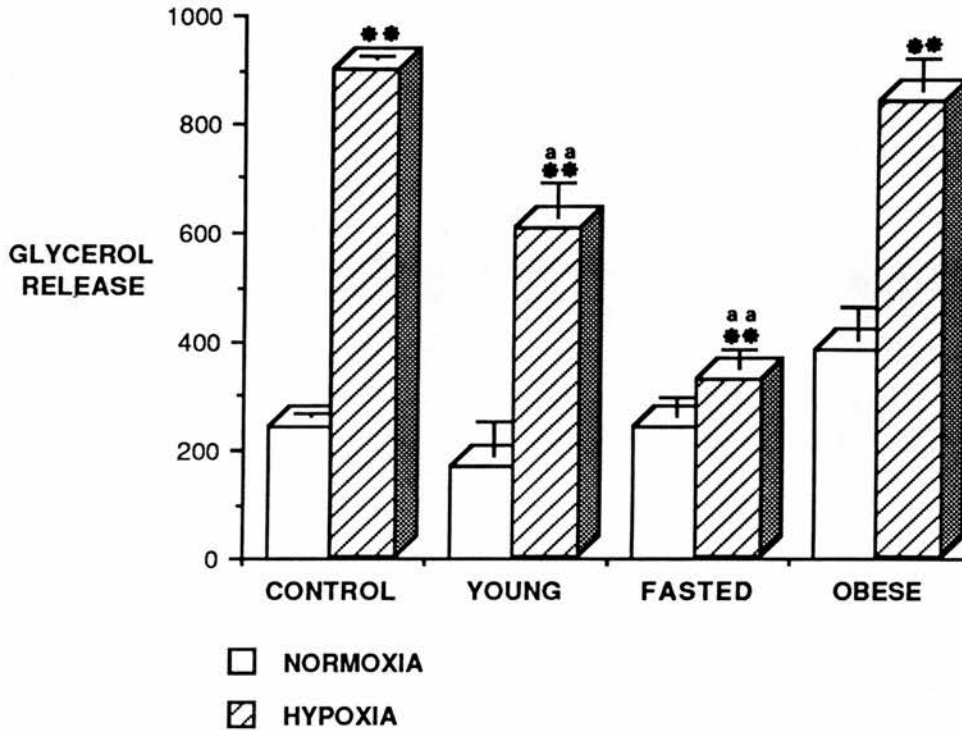
α-GP = α-glycerophosphate.

\* P<0.05 vs CONTROL

\*\* P<0.01 vs CONTROL

All groups of hearts released similar amounts of glycerol during normoxia (Fig 4.1). In all cases, hypoxia resulted in a significant increase in glycerol release. Hypoxia-stimulated glycerol efflux was significantly lower in hearts from young and fasted rats than in hearts from control (fed) rats.

Figure 4.1. The influence of age, fasting (48 h) and obesity on glycerol release from the isolated, perfused rat heart during normoxia and hypoxia.



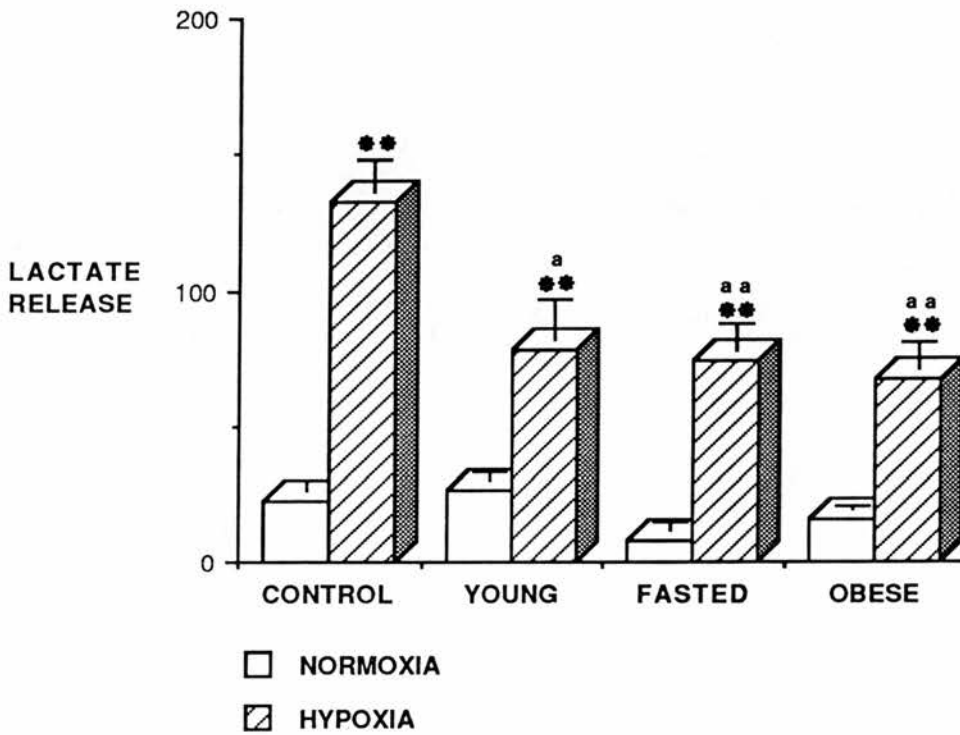
Glycerol release is expressed as  $\text{nmol.g}^{-1}\text{wet wt.per 30 min}$  (Mean $\pm$ SEM)

\*\*P<0.01 vs NORMOXIA (t-test)

aa P<0.01 vs CONTROL (t-test)

Lactate release from the normoxic heart was reduced in fasted and obese rats compared with hearts from fed, lean rats of similar age (control) (Fig 4.2). As expected, hypoxia resulted in a significant increase in lactate release from all groups of hearts. The amount of hypoxia-stimulated lactate release was similar in hearts from young, fasted and obese rats. This was significantly lower than lactate release from control hearts.

Figure 4.2. The influence of age, fasting (48 h) and obesity on lactate release from the isolated, perfused rat heart during normoxia and hypoxia.



Lactate release is expressed as  $\mu\text{mol.g}^{-1}$  wet wt. per 30 min (Mean $\pm$ SEM)

\*P<0.01 vs NORMOXIA (t-test)

<sup>a</sup>P<0.05 vs CONTROL (t-test)

<sup>a a</sup>P<0.01 vs CONTROL (t-test)

Oxygen uptake during normoxia was significantly lower in hearts from fasted rats than in hearts from fed rats. Hypoxia caused a significant decrease in oxygen uptake in all groups of hearts, none of the groups showing significant differences (Table 4.2.).

**Table 4.2. Oxygen uptake by hearts from control, young, fasted and obese rats during normoxia and hypoxia.**

Type of rat	Oxygen uptake ( $\mu\text{mol.g}^{-1}\text{min}^{-1}$ )	
	Normoxia	Hypoxia
Control	6.39 $\pm$ 0.2	2.38 $\pm$ 0.04**
Young	6.39 $\pm$ 0.2	2.34 $\pm$ 0.04**
Fasted	5.25 $\pm$ 0.2 <sup>aa</sup>	2.53 $\pm$ 0.05**
Obese	6.46 $\pm$ 0.1	2.38 $\pm$ 0.05**

Results are expressed as Mean $\pm$ SEM

\*\*P<0.01 vs Normoxia (t-test)

aa P<0.01 vs Control (fed) (t-test)

Each group showed a decrease in ATP and an increase in  $\alpha$ -glycerophosphate concentration in the heart after hypoxia. CP, glycogen and TAG concentrations were not significantly affected by hypoxia. Tissue ATP concentrations in hearts from young rats were significantly higher than in hearts from control rats after normoxic perfusion (Table 4.3). However, this difference was no longer observed after hypoxia. Other metabolite concentrations were not significantly different in hearts from control and young rats.

**Table 4.3. The influence of normoxic and hypoxic perfusion on metabolite concentrations in hearts from control and young rats.**

	CONTROL		YOUNG	
	Normoxia	Hypoxia	Normoxia	Hypoxia
ATP	4.13±0.2	2.99±0.2 <sup>aa</sup>	5.11±0.3*	3.32±0.3 <sup>aa</sup>
CP	2.50±0.1	2.28±0.2	3.26±0.4	2.99±0.3
GLYCOGEN	11.88±1.3	10.63±0.9	7.80±1.4	8.57±1.9
α-GP	0.68±0.1	1.52±0.1 <sup>aa</sup>	0.51±0.2	0.50±0.2 <sup>aa</sup>
GLYCEROL	0.32±0.1	0.00±0.0	0.45±0.2	1.08±0.1
TAG	0.50 (0.2,1.9)	0.50 (0.2,2.2)	0.40 (0.3,1.1)	0.60 (0.4,1.6)

Metabolite concentrations are expressed as  $\mu\text{mol.g}^{-1}$  wet wt. (Mean±SEM), except TAG concentrations which are expressed as Median (95% Confidence limits).

Glycogen concentration is expressed as glucose equivalents,  $\alpha\text{-GP} = \alpha$  glycerophosphate.

\*  $P < 0.05$  vs CONTROL (t-test)

<sup>aa</sup>  $P < 0.01$  vs Normoxia (t-test)

Although fasted rats exhibited elevated myocardial glycogen concentrations before perfusion, glycogen utilization rates were significantly higher in hearts from fasted rats after both normoxic and hypoxic perfusion than in hearts from fed rats, as demonstrated by the significantly lower glycogen concentrations in hearts from fasted rats after perfusion (Table 4.4). The  $\alpha$ -glycerophosphate concentration in fasted rat hearts was

significantly lower after normoxic perfusion but not different than in hearts from fed rats after hypoxic perfusion (Table 4.4).

**Table 4.4. The influence of normoxic and hypoxic perfusion on metabolite concentrations in hearts from control and fasted rats.**

	CONTROL		FASTED	
	Normoxia	Hypoxia	Normoxia	Hypoxia
ATP	4.13±0.2	2.99±0.2 <sup>aa</sup>	4.12±0.1	3.53±0.2 <sup>aa</sup>
CP	2.50±0.1	2.28±0.21	2.39±0.1	2.52±0.3
GLYCOGEN	11.88±0.3	10.63±0.93	4.71±0.4 <sup>**</sup>	5.08±0.5 <sup>**</sup>
α-GP	0.68±0.1	1.52±0.1 <sup>aa</sup>	0.15±0.2 <sup>**</sup>	0.58±0.05 <sup>aa</sup>
GLYCEROL	0.32±0.1	0.00±0.0	0.60±0.1	0.40±0.1
TAG	0.50 (0.2,1.9)	0.50 (0.2,2.2)	0.40 (0.3,1.4)	0.40 (0.3,1.3)

Metabolite concentrations are expressed as  $\mu\text{mol.g}^{-1}$  wet wt. (Mean±SEM), except TAG concentrations which are expressed as Median (95% Confidence limits).

Glycogen concentration is expressed as glucose equivalents.

\*\* P<0.01 vs CONTROL (t-test); aa P<0.01 vs Normoxia (t-test)

Hearts from obese rats exhibited elevated CP concentrations compared with control rat hearts after both normoxia and hypoxia (Table 4.5). The TAG concentrations in hearts from obese rats were significantly higher than in the control group after both normoxic and hypoxic perfusion. Glycogen concentrations were also raised in obese rat hearts

after normoxic perfusion but were not different than in hearts from the control group after hypoxia (Table 4.5).

**Table 4.5. The influence of normoxic and hypoxic perfusion on metabolite concentrations in hearts from control and fed rats.**

	CONTROL		OBESE	
	Normoxia	Hypoxia	Normoxia	Hypoxia
ATP	4.13±0.2	2.99±0.2	4.37±0.1	3.55±0.1
CP	2.56±0.1	2.28±0.2	3.35±0.2*	3.22±0.1*
GLYCOGEN	11.88±1.3	10.63±0.9	14.84±1.9*	12.56±1.1
α-GP	0.68±0.1	1.52±0.1	0.26±0.04	0.73±0.1
GLYCEROL	0.32±0.1	0.00±0.1	0.27±0.1	0.31±0.31
TAG	0.50 (0.2,1.9)	0.5 (0.2,2.2)	8.5 (6.5,12.4)**	9.0 (5.9,13.2)**

Metabolite concentrations are expressed as  $\mu\text{mol.g}^{-1}\text{wet wt.}$  (Mean±SEM), except TAG concentrations which are expressed as Median (95% Confidence limits) .

Glycogen concentration is expressed as glucose equivalents.

α-GP = α-glycerophosphate.

\* P<0.05 vs CONTROL (t-test).

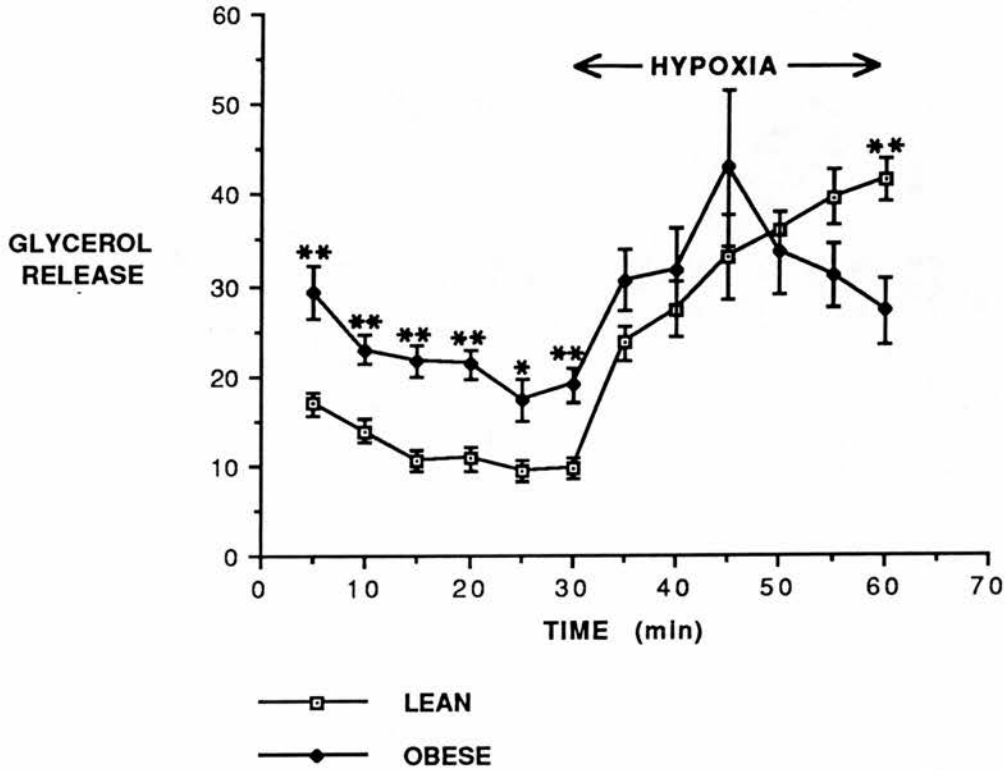
\*\* P<0.01 vs CONTROL (t-test).

aa P<0.01 vs Normoxia (t-test).

(For the sake of clarity the data from the control group is presented in Tables 4.3, 4.4 and 4.5).

The pattern of glycerol release from hearts from obese rats during hypoxia differed from that seen with hearts from the control group (Fig 4.3).

Figure 4.3. The influence of obesity on hypoxia-stimulated glycerol release from the isolated, perfused rat heart.



Glycerol release is expressed as  $\text{nmol.g}^{-1} \text{ wet wt.min}^{-1}$  (Mean  $\pm$  SEM).

\* $P < 0.05$  vs LEAN (t-test)

\*\* $P < 0.01$  vs LEAN (t-test)

Glycerol production from obese rat hearts was increased during the first 30 min (normoxic) perfusion, after which, in the normoxic group glycerol efflux decreased and

was not significantly different than in control rat hearts. When hypoxia was induced, glycerol efflux from hearts from obese rats gradually increased during the first 15 min, after which glycerol production began to decline. After 30 min hypoxia, the rate of glycerol release from control hearts was significantly higher than from obese rat hearts. Glycerol release from obese-rat hearts was more variable than from hearts in the control group (Standard deviation =  $13 \mu\text{mol}\cdot\text{g}^{-1}\cdot 30\text{min}^{-1}$  lean vs  $143 \mu\text{mol}\cdot\text{g}^{-1}\cdot 30\text{min}^{-1}$  obese).

Unlike glycerol, lactate production from hearts from obese rats showed a similar pattern to lactate production from hearts in the control group.

The amount of ATP utilized in TAG-NEFA cycling was calculated as described in Section 3.4. The results are as shown in Table 4.6.

**Table 4.6. ATP production, and utilization in TAG-NEFA cycling during hypoxia in hearts from different types of rat.**

Type of rat	ATP production		ATP used for TAG-NEFA cycling	% ATP used
	Aerobic	Anaerobic		
Control	428.4±7.2	137.1±21.6	5.38±0.4	0.95±0.03
Young	421.2±7.2	64.5±17.4	3.63±0.3	0.75±0.03
Fasted	455.4±9.0	85.2±15.6	1.96±0.2	0.36±0.03
Obese	428.4±9.0	67.0±14.9	5.04±0.6	1.02±0.03

ATP production and utilization is expressed as  $\mu\text{mol}\cdot\text{g}^{-1}\cdot 30\text{min}^{-1}$  (Mean±SEM).

#### 4.4. Discussion

The results from this study demonstrate that increased myocardial TAG concentrations in the obese rat do not have a significant effect on the total amount of glycerol released from the isolated, perfused rat heart during a 30 min period of hypoxia (30%O<sub>2</sub>/65%N<sub>2</sub>/5%CO<sub>2</sub>). However, the relationship of glycerol release with time was somewhat anomalous in this group. The proportion of total ATP production utilized in TAG-NEFA cycling was slightly increased in hearts with raised TAG concentrations i.e. hearts from obese rats, but this increase was associated with decreased anaerobic ATP production during hypoxia compared with hearts from lean rats, rather than increased lipolysis. Even when glycerol release from the obese rat hearts was at its maximum i.e. after 15 min hypoxia (Figure 4.3.), it can be calculated that only 1.5% of the ATP produced would be utilized for TAG-NEFA cycling. Furthermore, the increase in TAG-NEFA cycling in hearts from obese rats had no effect on high energy phosphate concentrations in the heart. On the contrary, CP levels were higher in hearts from obese rats than in hearts from lean rats after hypoxia.

Obviously, obesity is a pathological condition and results from these hearts may be complicated by factors such as changes in membrane structure and response to stimuli. However, as the hearts were removed from the animals and subjected to the same environment as the normal hearts, many of the complicating factors present *in vivo* are removed. The high variation in glycerol release from obese rat hearts during hypoxia compared with that in hearts from the control group may be due to variation in the number of adipocytes present in hearts from obese rats. It has been reported that few adipocytes are present in hearts from lean rats (Denton and Randle 1967).

Obesity is known to be accompanied by insulin resistance (Girolamo and Rudmen 1966). This would be expected to lead to decreased glucose uptake in hearts from obese

rats. Although insulin was not present in the perfusate during the experiment, the effects of insulin are known to remain for up to 30 min after commencing perfusion (Fisher and O'Brien 1972). After the preperfusion period, cannulation of the pulmonary artery takes approximately 5 min. Thus the first coronary effluent sample was collected after 30 min perfusion. Differences in metabolism due to the effects of endogenous insulin are therefore unlikely to be present during the experiment.

The lower lactate production and apparent decreased glycogen utilization during normoxia in hearts from obese rats compared with hearts from lean rats suggests decreased glycolytic flux in obese-rat hearts. This may be due to increased fatty acid oxidation, causing inhibition of phosphofructokinase by increased citrate and ATP production, and therefore a reduction in the rate of glycolysis. However, if glycerol production were to reflect TAG breakdown, and this could consequently be extrapolated to represent fatty acid oxidation during normoxia, no significant difference in fatty acid oxidation is apparent between hearts from lean and obese rats. Fatty acids may also be incorporated into the phospholipid pool, meaning that glycerol release would be an over-estimation of fatty acid oxidation. The amount of fatty acid oxidation could not be assessed due to the difficulty in obtaining accurate RQ values for this model as explained in Section 2.4.

The pattern of glycerol release from obese-rat hearts during hypoxia is unusual; in all other groups of hearts glycerol efflux showed no decline as the duration of hypoxia increased. Lipolysis in the heart has been related to mechanical activity (Stam and Hulsmann 1981). It is possible that the decline in glycerol efflux from hearts from obese rats during the later stages of hypoxia may reflect a concurrent decline in mechanical activity.

Lipolysis, assessed as glycerol release, is increased in hearts from rats in which myocardial TAG concentrations were raised by feeding on a high fat diet (Brownsey and

Brunt 1977, Stam and Hulsmann 1981a). In the obese rat, despite raised levels of TAG in the heart, the total amount of glycerol released was not different than that from lean-rat hearts. The existence of different TAG pools with varying turnover rates has been suggested (Stein and Stein 1968, Wood et al. 1972, De Groot et al. 1989). In the obese-rat heart, myocardial TAG stores may be less metabolically available, so that TAG accumulates in the heart but cannot be fully utilized for energy production.

Alternatively, if adipocytes react differently to hypoxia than do myocytes, a significant contribution of adipocytes in hearts from obese rats would explain the different metabolic profiles of the control and obese groups.

The effect of fasting on myocardial TAG concentration has been controversial. Several groups have reported increased TAG levels in rat hearts after fasting for 48 h (Wittels and Bressler 1964, Denton and Randle 1967, Lascano et al. 1981). All of these studies included MAG (monoacylglycerol) and DAG (diacylglycerol) in measurement of TAG concentrations and therefore probably overestimated TAG levels. However, concentrations of MAG and DAG in the heart are thought to account for less than 10% of the total glyceride concentration (Denton and Randle 1967). Their inclusion in TAG measurements is therefore unlikely to make a difference in the overall conclusion of an experiment, particularly as MAG and DAG were included in measurement of glyceride levels both before and after fasting. Other groups have reported decreased TAG levels in rat heart after fasting (Gorski and Kiryluk 1981, Arnall et al. 1988). This is surprising since myocardial TAG concentrations have been reported to be proportional to circulating NEFA concentrations (Paulson and Crass 1982) and plasma NEFA are known to be raised during fasting (Denton and Randle 1967). It should also be pointed out that due to the large variation of myocardial TAG levels within groups it is difficult to demonstrate a small difference between groups without the use of very large group sizes.

Sympathetic nervous system activity, demonstrated by noradrenaline turnover in the isolated, perfused rat heart, is suppressed during fasting, and this may be reversed by feeding (Landsberg and Young 1982). In addition, Brooks et al. (1983) demonstrated an increase in TAG-NEFA cycling in adipose tissue during feeding and attributed this to increased sympathetic nervous system activity and raised local concentrations of noradrenaline, due to the fact that feeding failed to increase the rate of cycling after injection of the  $\beta$ -blocker propranolol.

If the increase in glycerol and lactate release observed during hypoxia are the result of endogenous catecholamine release, diminished local concentrations of catecholamines in the heart during fasting could explain the decreased glycerol and lactate production from fasted-rat hearts during hypoxia compared with hearts from fed rats.

The age difference between the young and control rats used in this study was probably too small to show major differences in myocardial TAG concentrations. Hearts from rats younger than those used in this study would not be suitable for use in these experiments due to their small size and therefore difficulty in cannulation. Previous studies demonstrating differences in TAG metabolism have compared rats of 12 weeks with 1 year old rats (Reaven <sup>et al.</sup> 1978). The increased ATP concentrations in heart tissue from young rats after normoxia implies more efficient energy utilization in heart from young rats than in hearts from old rats. This could be explained by decreased ATP utilization for re-esterification of NEFA in hearts from young rats, as the decreased glycerol release during hypoxia implies a lower rate of TAG-NEFA cycling.

In summary, the changes in metabolism induced by age, fasting and obesity had little effect on the rate of TAG-NEFA cycling. The results suggest that the hypothesis that TAG-NEFA cycling becomes more important as a cause of energy wastage during oxygen deprivation when TAG levels are raised should be rejected. However, this study has compared the effects of metabolite concentrations only during normoxia and

relatively mild hypoxia. The influence of alterations in metabolite concentrations on the cycling rate during severe hypoxia and ischaemia remains to be studied.

If the responses observed in hearts from fasted rats are indeed the result of increased sympathetic nervous system activity, the findings of this study suggest that catecholamine release during hypoxia may be more important in determining biochemical changes during oxygen deprivation than levels of endogenous substrates. The involvement of endogenous catecholamines in the metabolic response to hypoxia is examined in the following chapter.

## **CHAPTER 5**

# **THE INVOLVEMENT OF ENDOGENOUS CATECHOLAMINE RELEASE IN HYPOXIA-STIMULATED GLYCEROL RELEASE FROM THE ISOLATED, PERFUSED RAT HEART DURING HYPOXIA.**

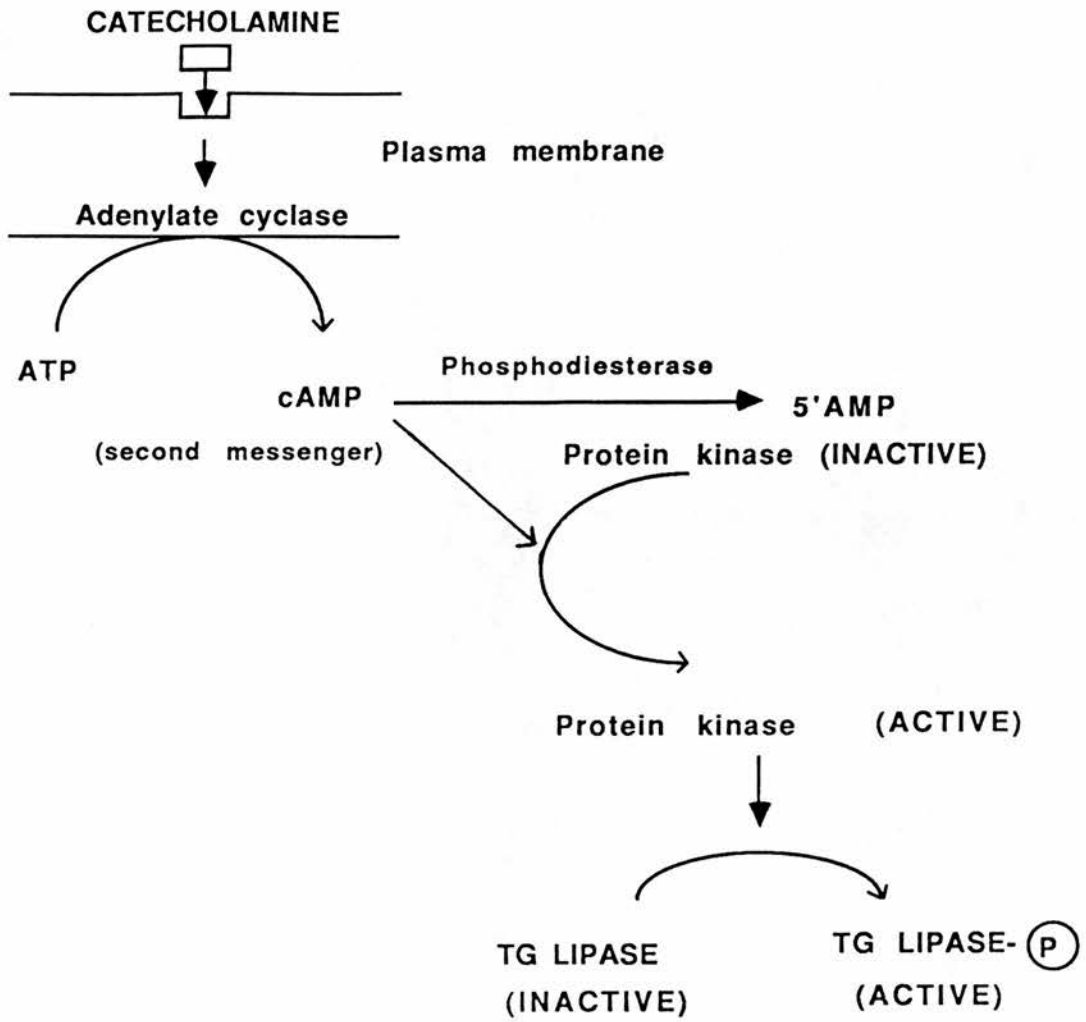
## 5.1. Introduction.

Alterations in myocardial metabolism are centrally involved in the development of arrhythmias. It is therefore important to understand the mechanisms controlling these changes during oxygen deprivation.

Hypoxia is thought to stimulate release of endogenous catecholamines in the heart (Wollenberger and Shahab 1965, Karwatowska-Krynska and Beresewicz 1983). These hormones bind to  $\beta$ -adrenoceptors on cell membranes and thus increase the activity of the enzyme adenylate cyclase. Adenylate cyclase catalyzes conversion of intracellular ATP to the second messenger cAMP. cAMP in turn activates protein kinases in the cytoplasm. These enzymes then phosphorylate other regulatory enzymes, either activating or deactivating them (Fig 5.1).

Catecholamine-mediated increases in cAMP concentrations in the myocardium have been linked to the occurrence of ventricular arrhythmias (Corr et al. 1978, Opie et al. 1979). Podzuweit et al. (1980) suggested that an increase in cAMP in the ischaemic heart may precede and cause ventricular fibrillation. In agreement with this theory, agents which increase intracellular levels of cAMP, such as inhibitors of phosphodiesterase (the enzyme which breaks down cAMP), are known to increase the severity of ischaemic arrhythmias (Kane et al. 1985). In the *in vitro*, coronary-artery-ligated rat heart, administration of the synthetic catecholamine analogue isoprenaline increased both cAMP levels and the incidence of ventricular fibrillation (Manning et al. 1985). Other groups have demonstrated a decreased incidence of arrhythmias in hearts depleted of endogenous catecholamines (Sethi et al. 1973, Sheridan et al. 1980). It is not known which of the biochemical changes induced by cAMP are arrhythmogenic.

Figure 6.2. The mechanism of action of  $\beta$ -adrenoceptor agonists.



Furthermore, the involvement of cAMP in the generation of arrhythmias has been questioned. Coronary occlusion-induced arrhythmias in the anaesthetized rat were not accompanied by temporally related increases in cAMP, although an unsustained increase in cAMP during early ischaemia was observed (Kane et al. 1985). Also, during global ischaemia in the rat heart, the activity of adenylate cyclase was significantly reduced compared with that in normal myocardium (Krause and England 1982, Reddy 1989). The role of catecholamine-mediated responses in the genesis of arrhythmias is therefore unclear.

A TAG lipase in the heart may be activated by  $\beta$ -adrenoceptor stimulation (Christian et al. 1968, Brownsey and Brunt 1977, Hough and Gevers 1975). Catecholamine-stimulated glycerol release from the isolated, perfused rat heart is preceded by an increase in cAMP (Christian et al. 1968). In addition, hormone-stimulated glycerol release in the heart is accompanied by an increase in the protein kinase ratio (Jesmok et al. 1977, Hron et al. 1977). Glycerol release was not increased during ischaemia in isolated, perfused hearts from animals denervated by pretreatment with reserpine (Hough and Gevers 1975) or hypoxia (Karwatowska-Krynska and Beresewicz 1983).

$\beta$  adrenoceptor blocking agents such as atenolol (a selective  $\beta_1$  receptor blocker) and timolol (a non-selective  $\beta$ -blocker) are well known to have antiarrhythmic properties (Campbell et al. 1982, Patterson et al. 1984, Sleight 1988). These drugs act as competitive antagonists by binding to  $\beta$  receptors and therefore preventing the binding of  $\beta$  receptor agonists such as the endogenous catecholamine noradrenaline or the synthetic catecholamine analogue isoprenaline.

The aim of this study was to assess the role and possible importance of endogenous

catecholamine release in the metabolic changes induced by hypoxia with the use of  $\beta$ -adrenoceptor agonists and antagonists.

## 5.2. Methods.

Hearts from male Sprague Dawley rats (body weight 400-450g) were removed and perfused as described in section 2.1.2.

The basic protocol consisted of a 60 min period of perfusion. An initial period (30 min) acted as a control for the second (experimental) period.

### 5.2.1. Noradrenaline release during normoxia and hypoxia.

The conditions used for the experiments were as shown in Table 5.1.

**Table 5.1. Experiment 1: Conditions used to study the role of noradrenaline release during normoxia and hypoxia on myocardial glycerol release.**

Group	No. of hearts	Conditions
A	6	95%O <sub>2</sub> /5%CO <sub>2</sub> (PO <sub>2</sub> =600±25 mmHg)
B	6	30%O <sub>2</sub> /65%N <sub>2</sub> /5%CO <sub>2</sub> (PO <sub>2</sub> =220±6 mmHg)
C	6	20%O <sub>2</sub> /75%N <sub>2</sub> /5%CO <sub>2</sub> (PO <sub>2</sub> =133±8 mmHg)
D	6	10%O <sub>2</sub> /85%N <sub>2</sub> /5%CO <sub>2</sub> (PO <sub>2</sub> =58±10 mmHg)

Coronary effluent was collected at 5 min intervals. Samples collected at t=5,15,25,35,45 and 55 min were analyzed for catecholamines. Samples for

catecholamine analysis were also collected from two hearts from each group for at 1 minute intervals from t=30 to t=40 min. Samples collected at t=10, 20, 30, 40, 50 and 60 min were analyzed for glycerol and lactate.

### 5.2.2. The influence of exogenous catecholamines and their antagonists on metabolic changes during normoxia and hypoxia.

The second series of experiments set out to examine the role of the  $\beta$ -adrenoceptor agonist isoprenaline and antagonists atenolol and timolol on myocardial glycerol release during normoxia and hypoxia. The conditions used are shown in Table 5.2.

**Table 5.2. Experiment 2: The conditions used to study the influence of exogenous catecholamines and their antagonists on metabolic changes during normoxia and hypoxia.**

Group	No. of hearts	Conditions
E	6	Normoxia + Isoprenaline
F	6	Normoxia + Isoprenaline + Atenolol
G	6	Hypoxia + Atenolol
H	8	Severe hypoxia
I	4	Severe hypoxia + Atenolol
J	6	Hypoxia + Isoprenaline
K	6	Hypoxia + Isoprenaline + Atenolol
L	3	Isoprenaline + Timolol
M	6	Hypoxia + Timolol

Normoxia = 95%O<sub>2</sub>/5%CO<sub>2</sub> (PO<sub>2</sub> 600±6 mmHg)

Hypoxia = 30%O<sub>2</sub>/65%N<sub>2</sub>/5%CO<sub>2</sub> (PO<sub>2</sub> 220±6)

Severe hypoxia = 10%O<sub>2</sub>/85%N<sub>2</sub>/5%CO<sub>2</sub> (PO<sub>2</sub> 58±8)

All drugs were infused proximal to the heart at a rate of 0.1ml/min. Atenolol and timolol were infused from t=0 to t=60 min; Isoprenaline was infused from t=31 to t=60 min.

Drug concentrations were as follows:

Isoprenaline 10 nM

Atenolol 10 μM

Timolol 10 μM

Coronary effluent was collected at 10 min intervals (from t=10 to t=60 min) and analyzed for glycerol and lactate. At t=60 min hearts were freeze-clamped, freeze-dried and the tissues analyzed for ATP, CP, glycogen, α-glycerophosphate, glycerol and TAG as described in Chapter 2.

### **5.2.3. Experiment 3: The effect of denervation on metabolic changes during normoxia and hypoxia.**

Rats received intraperitoneal injections of 6- hydroxydopamine-HBr (dissolved in sterile saline containing ascorbic acid (1%)) as follows:

Days 1 and 2 50 mg/kg

Days 8 and 9 100 mg/kg

The effects of chronic denervation on hypoxia- induced changes in metabolism was studied 10 days after the final dose was administered (Day 19).

Two sub groups were treated as follows:

Group I (n=6) - Hearts were removed, perfused for a few seconds in order to wash out

blood and then freeze-clamped. This group was used to examine the effects of pretreatment with the drug before perfusion.

Group II (n=6) - Hearts were removed, perfused normoxically for 30 min followed by 30 min hypoxia (30%O<sub>2</sub>/ 65%N<sub>2</sub>/5%CO<sub>2</sub>). Coronary effluent was collected at 10 min intervals from t=10 to t=60 min. At t=60 min hearts were freeze-clamped.

All hearts from 6-hydroxydopamine treated rats were freeze-dried and analyzed for catecholamines, ATP, CP, glycogen,  $\alpha$ -glycerophosphate, and TAG as described in Chapter 2.

Oxygen uptake was monitored throughout all experiments.

### 5.3 Results.

#### 5.3.1. Noradrenaline release during normoxia and hypoxia.

A period of 30 min hypoxia did not induce noradrenaline release from the isolated, perfused rat heart, irrespective of the severity of the hypoxic insult. On the other hand, hypoxia-stimulated glycerol and lactate release as described in Chapter 3 were evident (Glycerol release  $902 \pm 86 \text{ nmol.g}^{-1}$  per 30 min, lactate release  $144 \pm 14 \text{ } \mu\text{mol.g}^{-1}$  per 30 min; Mean  $\pm$  SEM,  $P < 0.01$  vs normoxia). There was no change in catecholamine release in any of the groups after induction of hypoxia (Table 5.3).

Table 5.3. Noradrenaline concentration in coronary effluent (nM) during normoxia and varying severities of hypoxia.

Group	A	B	C	D
% OXYGEN	95	30	20	10
No. of hearts	8	6	6	6
5 min	$0.53 \pm 0.2$	$0.29 \pm 0.1$	$0.20 \pm 0.03$	$0.14 \pm 0.1^a$
15 min	$0.40 \pm 0.1$	$0.31 \pm 0.05^{ac}$	$0.17 \pm 0.03^a$	$0.07 \pm 0.1^a$
25 min	$0.41 \pm 0.1$	$0.32 \pm 0.03^a$	$0.10 \pm 0.02^a$	$0.06 \pm 0.1^a$
		-----	HYPOXIA	-----
35 min	$0.39 \pm 0.1$	$0.28 \pm 0.03$	$0.14 \pm 0.04^{ab}$	$0.07 \pm 0.1^a$
45 min	$0.38 \pm 0.1$	$0.38 \pm 0.1$	$0.12 \pm 0.04^a$	$0.16 \pm 0.1$
55 min	$0.32 \pm 0.1$	$0.29 \pm 0.03$	$0.19 \pm 0.1$	$0.13 \pm 0.1$

a  $P < 0.05$  vs Group A; b  $P < 0.05$  vs Group B; c  $P < 0.05$  vs Group C  
Results are expressed as Mean  $\pm$  SEM.

Some differences in noradrenaline concentration in the coronary effluent were evident between groups during both normoxia and hypoxia. However, even in the groups in which noradrenaline release was raised compared with the other groups, the levels measured were much lower than the levels previously reported during anoxia (26 nM; Karwatowska-Krynska and Beresewicz 1983). Noradrenaline release from all groups of hearts remained constant during the first 10 min of hypoxia.

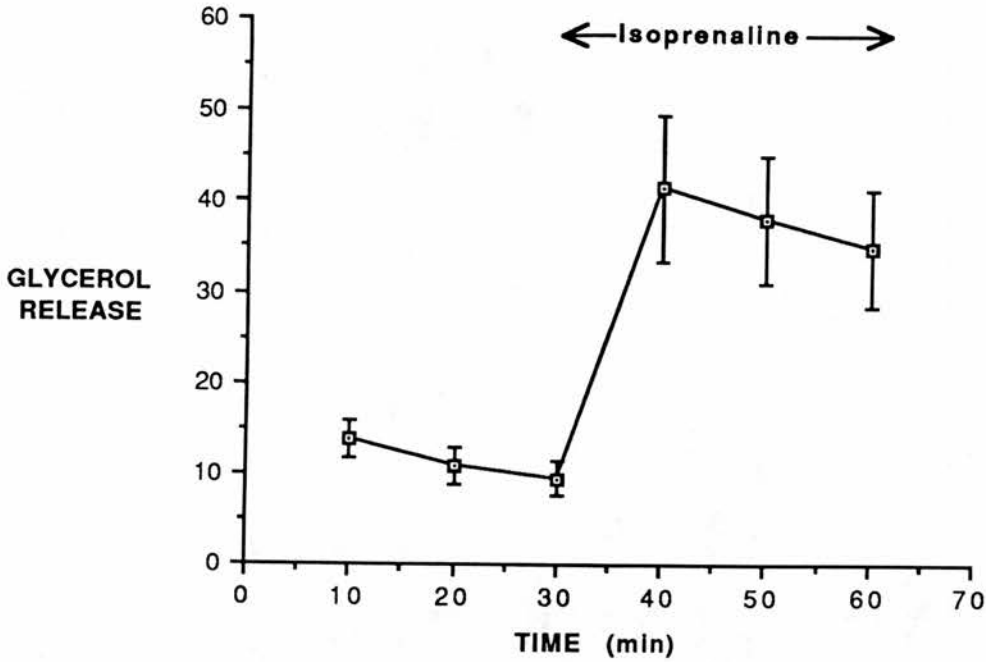
### **5.3.2. The influence of exogenous catecholamines and their antagonists on metabolic changes during normoxia and hypoxia.**

#### **5.3.2.(a) Isoprenaline**

Glycerol release was readily stimulated in the normoxic heart by infusion of isoprenaline (10nM) (Fig 5.2). The amount of glycerol released during a 30 min period of infusion of the drug ( $952 \pm 175$  nmol) was similar to the amount released during a 30 min period of hypoxia (30%O<sub>2</sub>/65%N<sub>2</sub>/5%CO<sub>2</sub>) ( $897 \pm 119$  nmol; Mean $\pm$ SEM).

Lactate production was also stimulated during isoprenaline infusion but no significant effect on oxygen uptake could be found (Table 5.4).

Figure 5.2. The influence of isoprenaline (10 nM) on glycerol release from the normoxic isolated, perfused rat heart.



Glycerol release is expressed as  $\text{nmol.g wet wt.}^{-1}\text{min}^{-1}$  (Mean $\pm$ SEM).

Tissue glycogen concentrations were significantly lower and CP concentrations significantly higher after isoprenaline infusion (Table 5.5). Tissue  $\alpha$ - glycerophosphate concentration was not affected by isoprenaline. Despite the increase in glycerol release during infusion of isoprenaline, myocardial TAG levels remained unchanged. Therefore, if glycerol release may be used as a measure of TAG breakdown, the results indicate that TAG-NEFA cycling is stimulated by exogenous catecholamines.

Table 5.4. The influence of exogenous catecholamines and their antagonists on glycerol ( $\text{nmol.g}^{-1}$  per 30 min) and lactate ( $\mu\text{mol.g}^{-1}$  per 30 min) release and oxygen uptake ( $\mu\text{mol.g}^{-1}\text{min}^{-1}$ ) during normoxia.

	Glycerol release	Lactate release	Oxygen uptake
Normoxic control	245±32	22±4.8	6.4±0.2
Isoprenaline	952±175**	196±26**	7.1±0.2
Isoprenaline + Atenolol	249±72 <sup>aa</sup>	101±6.8** <sup>aa</sup>	6.3±0.2
Isoprenaline + Timolol	194±46 <sup>aa</sup>	55±18 <sup>aa</sup>	4.4±1.4 <sup>aa</sup>

Results are expressed as Mean±SEM.

\*\* P<0.01 vs Normoxic control

<sup>aa</sup> P<0.01 vs Isoprenaline

### 5.3.2.(b) Isoprenaline and Atenolol.

As expected, isoprenaline-stimulated glycerol release was inhibited by the  $\beta$ -adrenoceptor blocking agent atenolol (Table 5.4). However, isoprenaline-stimulated lactate release was only partially blocked when atenolol was infused (Table 5.4). The decrease in tissue glycogen concentration observed after isoprenaline infusion was prevented by atenolol (Table 5.6).

### 5.3.2.(c) Hypoxia and Atenolol.

In contrast to the effect of atenolol on isoprenaline-induced glycerol release, the  $\beta$ -blocker failed to prevent hypoxia-stimulated glycerol release (Table 5.5). Hypoxia-stimulated lactate release was also unaffected. However, the drug prevented the hypoxia induced decrease in ATP and significantly attenuated the increase in tissue  $\alpha$ -glycerophosphate concentration observed after hypoxia (Table 5.6). Atenolol had no effect on oxygen uptake during hypoxia (Table 5.5).

**Table 5.5. The influence of exogenous catecholamines and their antagonists on glycerol ( $\text{nmol.g}^{-1}$  per 30 min) and lactate ( $\mu\text{mol.g}^{-1}$  per 30 min) release and oxygen uptake ( $\mu\text{mol.g}^{-1}\text{min}^{-1}$ ) during hypoxia.**

	Glycerol release	Lactate release	Oxygen uptake
Hypoxia	897 $\pm$ 119	133 $\pm$ 12	2.4 $\pm$ 0.04
Hypoxia + Atenolol	939 $\pm$ 105	162 $\pm$ 9	2.4 $\pm$ 0.05
Hypoxia + Timolol	778 $\pm$ 78	169 $\pm$ 27	2.4 $\pm$ 0.02
Hypoxia + Isoprenaline	1771 $\pm$ 307 <sup>b</sup>	217 $\pm$ 74	2.4 $\pm$ 0.1 <sup>aa</sup>
Hypoxia + Isoprenaline + Atenolol	984 $\pm$ 104	231 $\pm$ 21	2.4 $\pm$ 0.04
Severe hypoxia	2031 $\pm$ 245	179 $\pm$ 21	0.2 $\pm$ 0.05
Severe hypoxia + Atenolol	3163 $\pm$ 570	226 $\pm$ 38	0.4 $\pm$ 0.05

Results are expressed as Mean $\pm$ SEM.

aa P<0.01 vs Isoprenaline (Table 5.4)

b P<0.05 vs Hypoxic control

Glycerol and lactate release were significantly higher, and oxygen uptake significantly lower than the normoxic control values (Table 5.4;  $P < 0.01$ , not indicated).

#### 5.3.2.(d) Severe hypoxia and atenolol.

Glycerol and lactate release during severe hypoxia were also unchanged when atenolol was infused (Table 5.5). The decrease in ATP and CP observed during severe hypoxia was attenuated in the presence of atenolol. However, ATP and CP concentrations were lower than in normoxically perfused, untreated hearts. The increase in tissue  $\alpha$ -glycerophosphate concentration observed during severe hypoxia was prevented by atenolol (Table 5.6).

#### 5.3.2.(e) Hypoxia and Isoprenaline.

$\beta$ -adrenoceptors remained sensitive to isoprenaline during hypoxia, demonstrated by an increase in glycerol release from  $897 \pm 317$  nmol per 30 min (hypoxia) to  $1771 \pm 307$  nmol per 30 min (hypoxia and isoprenaline) ( $P < 0.05$ ; Table 5.5). ATP, glycogen and  $\alpha$ -glycerophosphate concentrations in the heart tissue were significantly lower than in normoxically perfused, untreated hearts. Glycogen and  $\alpha$ -glycerophosphate concentrations were significantly lower than after hypoxia alone but not significantly different than with isoprenaline alone (Table 5.6). Oxygen uptake was not significantly different than during hypoxia alone (Table 5.5).

Table 5.6. The influence of exogenous catecholamines and their antagonists on myocardial metabolite concentrations ( $\mu\text{mol}\cdot\text{g}^{-1}\text{wet wt.}$ ) during normoxia and hypoxia.

	ATP	CP	GLYCOGEN	$\alpha$ -GP	GLYCEROL	TAG
Normoxia	4.13 $\pm$ 0.2	2.56 $\pm$ 0.1	11.8 $\pm$ 1.3	0.68 $\pm$ 0.1	0.32 $\pm$ 0.1	0.50 (0.2,1.9)
Isoprenaline	3.61 $\pm$ 0.2	3.39 $\pm$ 0.3*	6.32 $\pm$ 0.8**	0.85 $\pm$ 0.2	0.46 $\pm$ 0.2	0.44 (0.2,1.2)
Isoprenaline + Atenolol	4.18 $\pm$ 0.2	3.02 $\pm$ 0.5	10.72 $\pm$ aa	0.39 $\pm$ 0.1	0.03 $\pm$ 0.02	0.38 (0.2,1.3)
Isoprenaline + Timolol	4.60 $\pm$ 0.2a	2.98 $\pm$ 0.2	19.22 $\pm$ 5.1	0.15 $\pm$ 0.03**a	0.45 $\pm$ 0.3	0.33 (0.2,1.3)
Hypoxia	2.99 $\pm$ 0.2**	2.28 $\pm$ 0.2	10.63 $\pm$ 0.9	1.52 $\pm$ 0.1**	0.00 $\pm$ 0.0	0.50 (0.2,2.2)
Hypoxia + Atenolol	3.75 $\pm$ 0.1bb	2.81 $\pm$ 0.2	9.93 $\pm$ 0.5	1.12 $\pm$ 0.1**b	0.02 $\pm$ 0.001	0.48 (0.3,1.7)
Hypoxia + Timolol	3.80 $\pm$ 0.1bb	2.84 $\pm$ b	14.99 $\pm$ 0.7bb	0.88 $\pm$ 0.1bb	0.23 $\pm$ 0.3	0.39 (0.3,1.6)
Hypoxia + Isoprenaline	2.85 $\pm$ 0.3**	2.92 $\pm$ 0.3	7.22 $\pm$ 0.4**bb	0.42 $\pm$ 0.005**bb	0.01 $\pm$ 0.001	0.69 (0.4,1.8)
Hypoxia + Isopr + Atenolol	3.49 $\pm$ 0.2**	3.02 $\pm$ 0.2	10.67 $\pm$ 0.5aadd	1.11 $\pm$ 0.1*bdd	0.14 $\pm$ 0.1	0.73 (0.3,1.9)
Severe hypoxia	1.58 $\pm$ 0.2**	0.84 $\pm$ 0.1**	6.29 $\pm$ 0.04**	3.73 $\pm$ 0.5	0.14 $\pm$ 0.1	0.40 (0.1,1.2)
Severe hypoxia + Atenolol	2.70 $\pm$ 0.03**	1.40 $\pm$ 0.1**b	7.24 $\pm$ 0.6**	1.69 $\pm$ 0.3	0.60 $\pm$ 0.05	0.41 (0.1,1.4)

Results are expressed as Mean  $\pm$ SEM, except TAG concentrations which are expressed as Median (95% Confidence limits).

\* P<0.054 vs Normoxia      b P<0.05 vs Hypoxia

\*\* P<0.01 vs Normoxia      bb P<0.01 vs Hypoxia

a P<0.05 vs Isoprenaline      c P<0.05 vs Severe hypoxia

aa P<0.01 vs Isoprenaline      dd P<0.01 vs Hypoxia + Isoprenaline

### **5.3.2.(f) Hypoxia, Isoprenaline and Atenolol.**

Atenolol prevented the isoprenaline-induced increase in glycerol release during hypoxia. When isoprenaline and atenolol were infused simultaneously during hypoxia, both glycogen and  $\alpha$ -glycerophosphate concentrations in the heart were significantly higher than when atenolol was omitted (Table 5.6). The tissue  $\alpha$ -glycerophosphate concentration was significantly lower than after hypoxia alone but significantly higher than in normoxically perfused, untreated hearts. The tissue ATP concentration was significantly lower than in normoxic, untreated hearts but not significantly different than with hypoxia alone or isoprenaline alone (Table 5.6). Atenolol had no effect on oxygen uptake in this group of hearts (Table 5.5).

### **5.3.2.(g) Isoprenaline and Timolol.**

The non-selective  $\beta$ -adrenoceptor blocking agent timolol inhibited isoprenaline-stimulated glycerol and lactate release (Table 5.4). ATP concentrations in heart tissue were significantly higher when timolol and isoprenaline were infused simultaneously than when isoprenaline was infused alone. However, ATP concentrations after timolol infusion were not significantly different than in normoxic, untreated hearts (Table 5.6). Tissue  $\alpha$ -glycerophosphate concentrations were significantly lower in the presence of timolol and isoprenaline compared with when isoprenaline was infused alone (Table 5.6). Oxygen uptake during isoprenaline and timolol infusion was significantly lower than during isoprenaline infusion alone, but not significantly different than in normoxic, untreated hearts (Table 5.4).

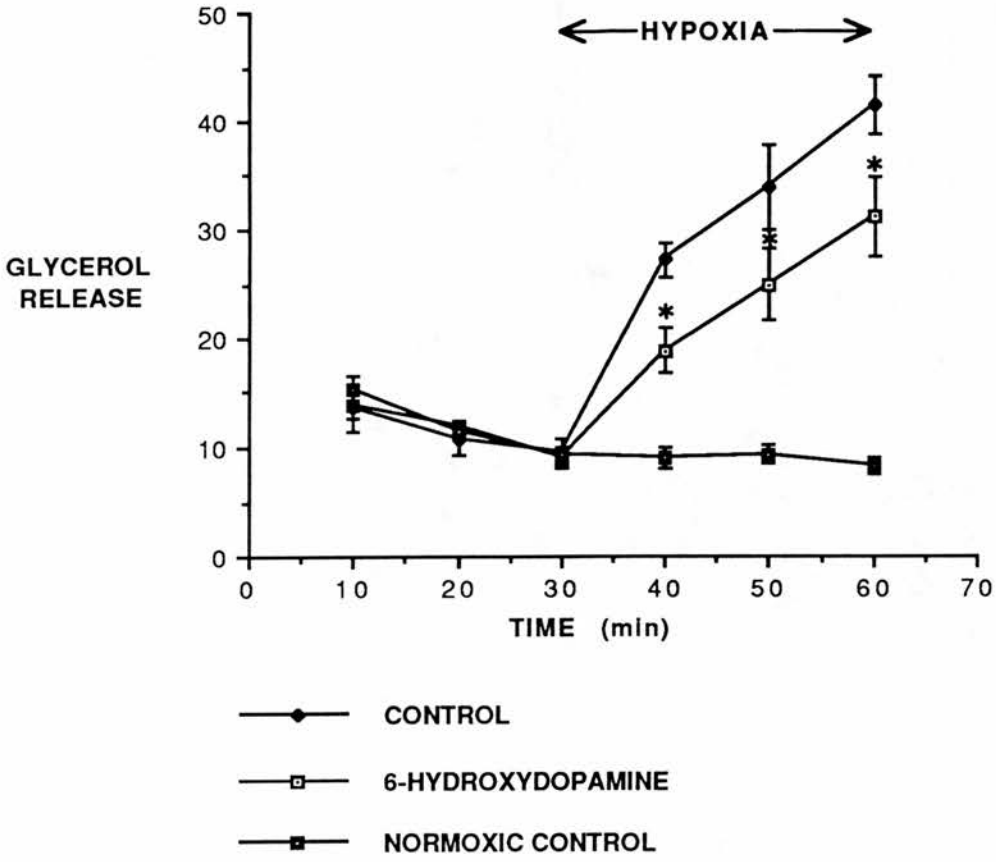
### **5.3.2.(h) Hypoxia and Timolol.**

Timolol had no effect on glycerol or lactate release during hypoxia (Table 5.5). CP and glycogen concentrations in heart tissue were significantly higher when timolol was infused during hypoxia. Glycogen concentrations were significantly higher than in normoxic, untreated hearts. Tissue  $\alpha$ - glycerophosphate concentrations were significantly lower when timolol was infused during hypoxia (Table 5.6).

### **5.3.3. The effect of denervation on metabolic changes during hypoxia.**

Glycerol (Fig 5.3) and lactate release were stimulated during hypoxia in hearts from 6-hydroxydopamine treated rats.

Figure 5.3. The influence of pretreatment with 6-hydroxydopamine on hypoxia-stimulated glycerol release from the isolated, perfused rat heart.



Glycerol release is expressed as nmol.g<sup>-1</sup>wet wt. min<sup>-1</sup> (Mean±SEM).

\* P<0.05 vs CONTROL

However, the total glycerol and lactate production was significantly lower than that from untreated rat-hearts (Table 5.7).

**Table 5.7. The influence of 6-hydroxydopamine pretreatment on glycerol and lactate release during hypoxia (30 min).**

	Glycerol release (nmol.g <sup>-1</sup> wet wt.)	Lactate release (μmol.g <sup>-1</sup> wet wt.)
Hypoxic control	896.9±129	132.5±7
6-Hydroxydopamine treated	628.9±72**	95.3±14**

Results are expressed as Mean±SEM.

\*\* P<0.01 vs Hypoxic control

ATP, CP and α-glycerophosphate concentrations in hearts from rats pretreated with 6-hydroxydopamine were significantly lower than in hearts from normal rats before perfusion (Table 5.8). Glycogen and TAG concentrations were not affected by treatment with 6-hydroxydopamine.

**Table 5.8. Effects of pretreatment with 6-hydroxydopamine on myocardial metabolite concentrations ( $\mu\text{mol.g}^{-1}\text{wet wt.}$ ) in the isolated, rat heart before perfusion.**

	Control	6-Hydroxydopamine treated
ATP	5.04 $\pm$ 0.1	4.84 $\pm$ 0.03**
CP	4.48 $\pm$ 0.1	3.66 $\pm$ 0.3*
Glycogen	18.40 $\pm$ 1.2	19.44 $\pm$ 1.2
$\alpha$ -Glycerophosphate	2.02 $\pm$ 0.5	0.8 $\pm$ 0.2*
TAG	1.81 (1.7,2.5)	2.52 (1.9,3.7)

Results are expressed as Mean $\pm$ SEM, except TAG concentrations which are expressed as Median (95% Confidence limits).

\* P<0.05 vs Control

\*\* P<0.01 vs Control

After 30 min hypoxia there was no difference in metabolite concentrations in hearts from 6- hydroxydopamine treated and untreated rats (Table 5.9).

**Table 5.9. The effects of pretreatment with 6-hydroxydopamine on myocardial metabolite concentrations ( $\mu\text{mol.g}^{-1}\text{wet wt.}$ ) in the isolated, perfused rat heart after 30 min normoxia and 30 min hypoxia.**

	Control	6-Hydroxydopamine treated
ATP	2.98 $\pm$ 0.4	3.58 $\pm$ 0.4 <sup>NS</sup>
CP	2.28 $\pm$ 0.3	2.68 $\pm$ 0.1 <sup>NS</sup>
Glycogen	10.62 $\pm$ 1.5	9.86 $\pm$ 0.7 <sup>NS</sup>
$\alpha$ -Glycerophosphate	1.52 $\pm$ 0.2	1.10 $\pm$ 0.2 <sup>NS</sup>
TAG	0.50 (0.2,2.2)	0.72 (0.2,2.2)

NS = Not significant.

Results are expressed as Mean $\pm$ SEM, except TAG concentrations which are expressed as Median (95% Confidence limits).

Oxygen uptake in hearts from treated rats was not significantly different than in hearts from untreated rats.

Myocardial noradrenaline concentrations after treatment with 6-hydroxydopamine were 0.02 $\pm$ 0.02 nmol.g<sup>-1</sup>wet wt. (Mean $\pm$ SEM). These levels are less than 1% of normal myocardial noradrenaline levels (~ 8 nmol.g<sup>-1</sup>wet wt; Abrahamsson et al. 1982).

#### 5.4. Discussion

Myocardial catecholamine release could not be demonstrated during hypoxia, even when the severity of hypoxia was increased. The effects of hypoxia on catecholamine release have not been studied previously. However, several groups have examined the effects of anoxia. The results presented here agree with those of Dart et al. (1987) and Schomig et al. (1987), but contradict those of Karwatowska-Krynska and Beresewicz (1983) who reported a significant increase in noradrenaline release, accompanied by a large increase in glycerol release from isolated, perfused rabbit hearts during the first 5 to 10 min of anoxia. It is possible that the discrepancies in results simply reflect species differences, but no systematic study of this appears to have been made. Another possibility is that the latter study used a constant pressure rather than a constant flow perfusion system. Consequently large increases in flow rates were recorded on induction of anoxia. Reuptake of endogenous catecholamines may therefore have been limited due to high flow rates and subsequent increased washout (Dart and Riemersma 1985). The large amount of glycerol produced by rabbit hearts during anoxia (95%N<sub>2</sub>/5%CO<sub>2</sub>) (~22000 nmol.g<sup>-1</sup>min<sup>-1</sup>; Karwatowska-Krynska and Beresewicz 1983) compared with that released from rat hearts during severe hypoxia (10%O<sub>2</sub>/85%N<sub>2</sub>/5%CO<sub>2</sub>) (99 nmol.g<sup>-1</sup>min<sup>-1</sup>) may reflect a higher proportion of adipocytes in rabbit heart than in rat heart, but data to confirm or deny this does not appear to be available. The pattern of glycerol release from the rabbit heart during anoxia was different from that observed in the hypoxic rat heart, in that glycerol efflux rapidly increased during the first five minutes of anoxia and then decreased, reaching levels not different than those observed in the normoxic heart after 30 min. In the rat heart, glycerol release continued to increase over a 30 min period of hypoxia (see Fig 5.3). Wollenberger and Shahab (1965) also reported anoxia-stimulated noradrenaline release from the rabbit heart. However, this was only recorded on reoxygenation of the

tissue. The report does not state whether a constant flow or constant pressure perfusion system was used.

The stimulation of lipolysis by exogenous catecholamines in the normoxically perfused heart, resulting in increased glycerol efflux has been well documented (Challoner and Steinberg 1965, Christian et al. 1968, Jesmok et al 1977, Stam and Hulsmann 1981a).

Infusion of isoprenaline (10nM) resulted in glycerol release similar to the amount released during hypoxia (30%O<sub>2</sub>/65%N<sub>2</sub>/59%CO<sub>2</sub>). As expected, isoprenaline-stimulated glycerol release was readily inhibited by the  $\beta_1$ -adrenoceptor antagonist atenolol. Isoprenaline-stimulated lactate release was only partially inhibited by atenolol, but almost completely inhibited by timolol, implying that both  $\beta_1$  and  $\beta_2$ -adrenoceptors may be involved in this process.

These observations are in sharp contrast to the failure of atenolol to block glycerol release during hypoxia, suggesting that hypoxia-stimulated glycerol efflux is not a result of endogenous catecholamine release. However, atenolol did reduce the hypoxia-stimulated decrease in ATP and prevented the decrease in tissue glycogen concentration observed during severe hypoxia, suggesting that some endogenous catecholamines are released and that their effects may be prevented by a  $\beta_1$ -adrenoceptor antagonist. The effects of the  $\beta$ -blocker on metabolism during hypoxia could also be a consequence of the infusion of the drug during the 30 min normoxic period prior to hypoxia, and hence inhibition of the actions of basal catecholamine release.

Isoprenaline-induced glycerol release was not significantly different during normoxia and hypoxia, suggesting that hypoxia neither impairs  $\beta$ -adrenoceptor function, nor affects the efficacy of the  $\beta$ -adrenoceptor blocking agents. Isoprenaline-stimulated glycerol release from isolated myocytes was attenuated during anoxia (95%N<sub>2</sub>/5%CO<sub>2</sub>) compared with during normoxia (Larsen et al 1989). This apparent disparity between results may reflect a difference between total oxygen deprivation (anoxia) and a

reduction in oxygen supply (hypoxia). Also, with the use of isolated myocytes, isolation of the cells per se may modify the response of receptors to anoxia. On the other hand, other cell types such as adipocytes are removed from isolated myocyte preparations and results therefore reflect purely myocyte metabolism. Thus it may be that the sensitivity of myocyte  $\beta$ -adrenoceptor function to oxygen deprivation in the isolated heart is masked by the response of adipocytes to isoprenaline. Adipocytes require much less energy than do myocytes due to their lack of mechanical activity and would therefore be expected to be less sensitive to hypoxia.

Atenolol is a selective  $\beta_1$ -adrenoceptor antagonist. The lipolytic effects of  $\beta$ -receptor stimulation are thought to be mediated via  $\beta_1$ -adrenoceptors (Arnold et al. 1966). Cardiac tissue has been reported to contain a significant proportion of  $\beta_2$ -adrenoceptors (Jones et al. 1989), although this varies between species (rat heart tissue contains mainly the  $\beta_1$  subtype). The effect of the non-selective  $\beta$ -adrenoceptor antagonist timolol was studied to eliminate the possibility that glycerol efflux during hypoxia was a result of  $\beta_2$ -adrenoceptor stimulation. The results suggest that hypoxia-stimulated glycerol release does not involve  $\beta_2$ -adrenoceptor stimulation.

Although the concentration of the  $\beta$ -adrenoceptor blocking agents used in this study was relatively high, it is possible that the action of endogenous catecholamines may not have been completely inhibited. 6-Hydroxydopamine causes selective degeneration of the terminal part of adrenergic neurons resulting in long-lasting depletion of endogenous catecholamines and functional sympathectomy (Haeusler <sup>et al.</sup> 1969). Pretreatment of rats with this drug should remove any catecholamine mediated responses observed during hypoxia.

Pretreatment of rats with 6-hydroxydopamine did not prevent stimulation of glycerol release during hypoxia. However, the total amount of glycerol released during the 30 min period of hypoxia was significantly lower than in hearts from untreated rats. Assuming that the decrease in hypoxia-stimulated glycerol release in pretreated rats represents

catecholamine-mediated glycerol release, it can be calculated that approximately 30% of the glycerol and 28% of the lactate released during hypoxia is the result of endogenous catecholamine release. This implies that infusion of  $\beta$ -adrenoceptor antagonists is inadequate in inhibiting endogenous catecholamine-mediated responses.

6-Hydroxydopamine pretreatment prevented the hypoxia-induced decrease in ATP, suggesting that the decline in ATP levels during hypoxia is catecholamine-mediated. However, the fact that high energy phosphate levels were lower in hearts from 6-hydroxydopamine pretreated rats than in hearts from control rats suggests that under normoxic conditions catecholamines may play a role in maintaining high energy phosphate levels in the heart. The increase in tissue CP concentration observed during isoprenaline infusion in the normoxic heart supports this argument.

Catecholamines are well known to cause an inotropic response which would be likely to increase energy utilization in the heart. The changes in metabolism observed during isoprenaline infusion may therefore be partly due to alterations in mechanical activity as well as biochemical changes. However, even if the heart rate is controlled, the work done by the heart cannot be easily monitored (Elzinga 1983). Also high energy phosphate levels are maintained with a 4 to 5 fold increase in heart rate (Kammermeir et al. 1990). Hypoxia-stimulated glycerol release has previously been attributed to the action of a hormone-sensitive lipase (Christian et al. 1968, Hough and Gevers 1975, Karwatowska-Krynska and Beresewicz 1983). These results demonstrate that the majority of the hypoxia-stimulated glycerol release was the result of non-adrenergic mechanisms. Non hormone-stimulated glycerol release must be the consequence either of activity of a lipase which is activated during hypoxia independent of endogenous catecholamines, or breakdown of  $\alpha$ -glycerophosphate or phospholipids rather than TAG.

The presence of both a lysosomal and a soluble fraction acid lipase with significant activity towards  $\alpha$ -glycerophosphate has been demonstrated in rat liver (Shibko and

Tappel 1962), and a similar acid phosphatase is known to be present in rat ventricle (Welman and Peters 1976). Activity of lysosomal enzymes increases during anoxia (Welman 1974). It is possible that increased acid phosphatase activity in the heart during hypoxia may lead to increased glycerol release via  $\alpha$ -glycerophosphate breakdown, although this needs to be further investigated.

Additionally, phospholipid breakdown may result in glycerol efflux during hypoxia. The content of myocardial phospholipids does not change significantly within 1 hour of ischaemia (Chien et al. 1984, Das et al. 1986, van der Vusse et al. 1987). However, a very small change in the phospholipid pool could conceivably result in a significant amount of glycerol release (van der Vusse et al. 1987). Since arachidonic acid is a fatty acid found almost entirely in phospholipid, increases in intracellular arachidonate levels are thought to provide evidence for increased phospholipase A<sub>2</sub> activity. The accumulation of unesterified arachidonate is minimal during 10-30 min of ischaemia in canine left ventricle and does not increase significantly until after 1-3 hours of ischaemia (Chien et al. 1984). However, deacylation and reacylation of phosphatidylcholine is thought to occur under normoxic conditions and during the early stages of myocardial ischaemia. This could lead to glycerol release from the heart. Changes in phospholipid metabolism during hypoxia have not been reported. However, addition of phospholipase C to isolated myocytes stimulates glycerol release during anoxia, but not during normoxia (Myrmel et al. 1989).

In conclusion, the results from this study show that approximately 30% of the glycerol release from the heart during hypoxia is due to myocardial lipolysis stimulated by endogenous catecholamines. More experiments are needed to determine the mechanisms involved in stimulating the remainder of the glycerol efflux during hypoxia and also the origin of the glycerol released from the heart during oxygen deprivation.

**CHAPTER 6**

**THE ORIGIN OF GLYCEROL RELEASE  
FROM THE ISOLATED, PERFUSED RAT HEART  
DURING HYPOXIA.**

## 6.1 Introduction.

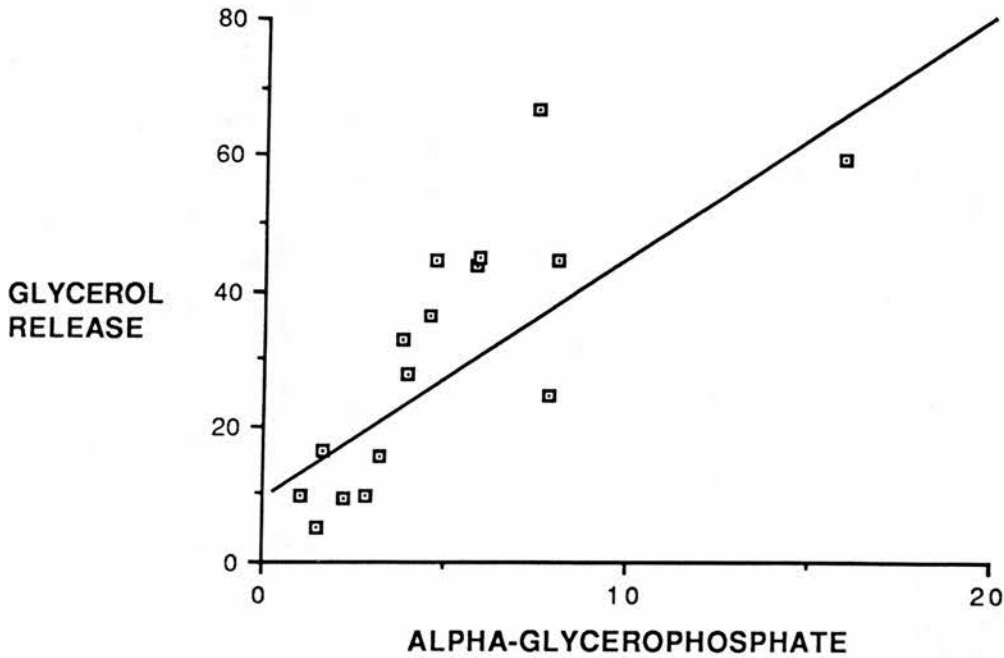
It is generally assumed that glycerol release during hypoxia is the result of hormone-stimulated lipolysis (Christian et al. 1968, Jesmok et al. 1977). A large proportion of glycerol released from the isolated, perfused rat heart during hypoxia is the result of non-adrenergic mechanisms). Hypoxia-stimulated glycerol release must therefore be either the result of a TAG lipase activated during hypoxia independent of catecholamines, or originate from a source other than TAG (ie. either  $\alpha$ -glycerophosphate or phospholipids). The common use of glycerol release as an index of lipolysis is thus brought into question (Chapter 5).

Breakdown of  $\alpha$ -glycerophosphate by acid phosphatase could lead to glycerol production. The possible contribution of acid phosphatase to glycerol release during hypoxia therefore needs to be investigated, particularly since there is a positive correlation between myocardial  $\alpha$ -glycerophosphate concentration and glycerol release over a range of conditions (Fig 6.1;  $r=0.65$ ).

Hormone-stimulated lipolysis in the isolated, perfused rat heart is thought to be sensitive to product inhibition by NEFA (Crass III et al. 1975, Stam and Hulsmann 1978, Severson and Hurley 1982). During hypoxia, if TAG breakdown is stimulated, intracellular NEFA are likely to increase in concentration in the buffer-perfused heart due to the decrease in oxidative metabolism, and therefore possibly exert feedback inhibition on lipolysis.

If albumin is present in the perfusate, NEFA are able to leave the heart and bind to fatty acid binding sites on the protein. Product inhibition may thus be removed and an increase in glycerol release would be expected (Fig 6.2). The positive correlation between myocardial  $\alpha$ -glycerophosphate concentration and glycerol release could also be accounted for by removal of product inhibition of lipolysis by  $\alpha$ -glycerophosphate in providing substrate for re-esterification of intracellular TAG.

Figure 6.1. The relationship between myocardial  $\alpha$ -glycerophosphate concentration and glycerol release.



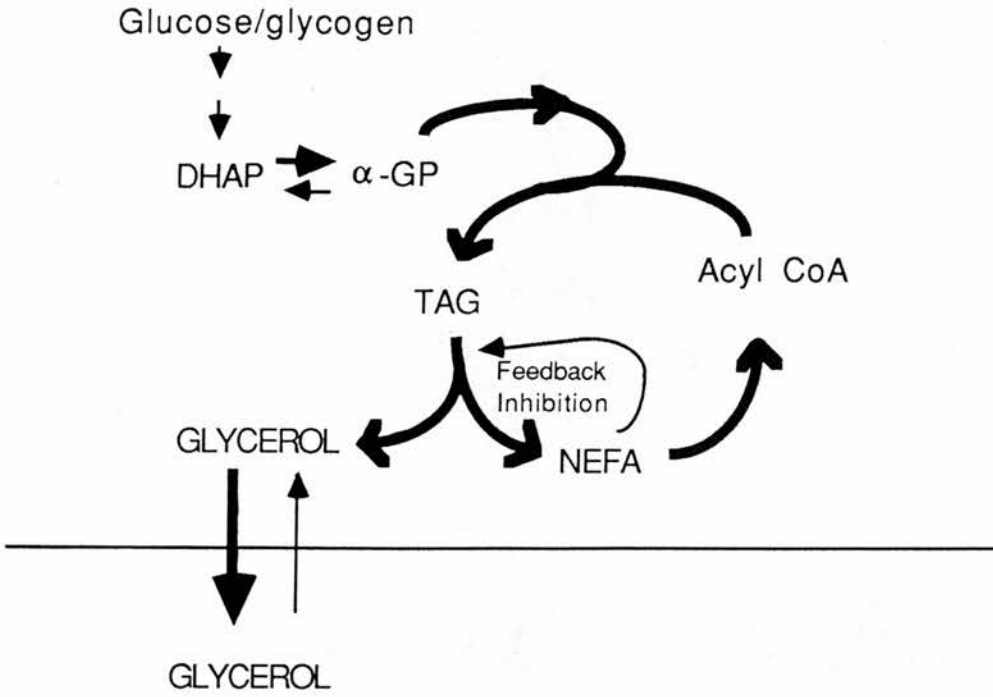
Glycerol release is expressed as  $\text{nmol.g}^{-1}\text{wet wt.min}^{-1}$

$\alpha$ -glycerophosphate concentration is expressed as  $\mu\text{mol.g}^{-1}\text{dry wt.}$

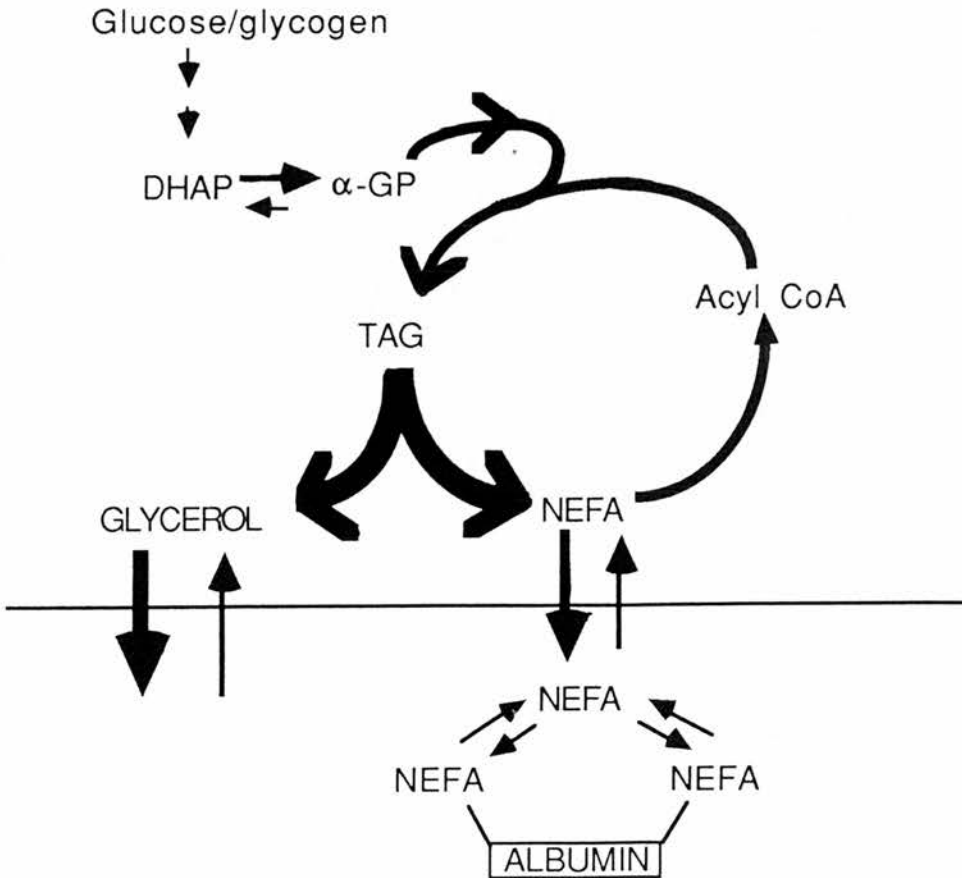
At the rate of glycerol release observed during severe hypoxia ( $\sim 90 \text{ nmol.g}^{-1}\text{min}^{-1}$ ; Chapter 3), assuming that each glycerol molecule released represents the hydrolysis of one TAG molecule,  $270 \text{ nmol.g}^{-1}\text{min}^{-1}$  NEFA would remain in the heart. Assuming a NEFA:albumin molar binding ratio of 2:1 (Goodman 1958), at a flow rate of 10 ml/min, an albumin concentration of 0.1% would be adequate to bind all NEFA leaving the tissue and entering the coronary effluent. The effects of an albumin concentration of 0.2% on hypoxia-stimulated glycerol release was therefore studied to ensure an excess of NEFA binding sites.

**Figure 6.2. The influence of extracellular albumin on myocardial lipolysis.**

1) Without albumin:

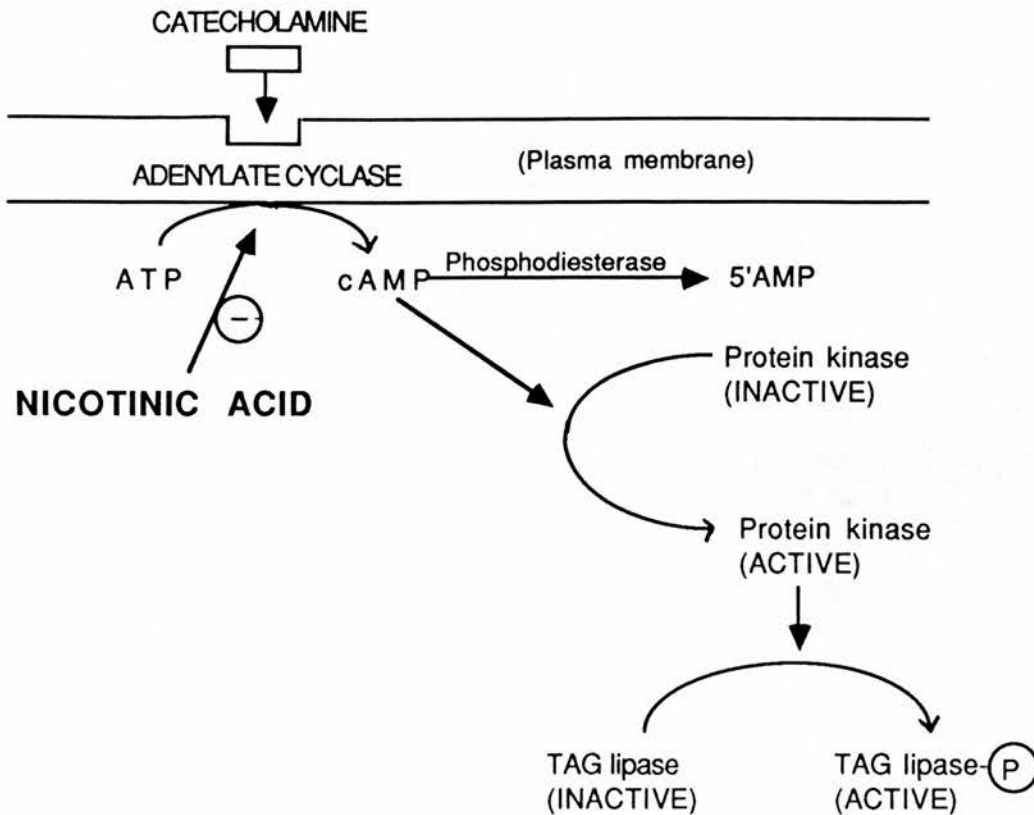


2) With albumin:



Nicotinic acid is well known to have antilipolytic properties in adipose tissue (Carlson and Oro 1962) and is thought to inhibit lipolysis by decreasing cAMP production (Butcher et al. 1968; Fig 6.3).

Figure 6.3. The mechanism of action of nicotinic acid.



The drug was found to inhibit isoprenaline-stimulated glycerol release in the isolated, perfused rat heart at concentrations ranging from 10 nM to 1mM (Christian et al 1968). However, at a concentration of 1 mM, it had no effect on cAMP release from the heart during isoprenaline infusion (O'Brien and Strange 1975). The effect of this drug on isoprenaline-stimulated and hypoxia-stimulated glycerol release was therefore examined to determine whether myocardial glycerol production is mediated by a mechanism similar to that in adipose tissue. It is generally assumed that glycerokinase activity in

the heart is negligible (Challoner and Steinberg 1965, Christian 1968, Jesmok et al. 1977, Trach et al. 1984, Stam and Hulsmann 1981a). However, the enzyme is known to exist in heart tissue (Robinson and Newsholme 1967), and glycerol utilization by the dog heart when arterial plasma glycerol concentrations are raised (ie.during infusion of isoprenaline) has been demonstrated (Vik-Mo et al. 1979). It should therefore be possible to label myocardial TAG by infusion of radiolabelled glycerol.

Lactate may be used preferentially as a substrate in the heart (Drake et al. 1980) and hence may attenuate fatty acid oxidation and also decrease oxidation of  $\alpha$ -glycerophosphate by increasing the NADH/NAD<sup>+</sup> ratio, promoting retention of radiolabelled glycerol in the TAG fraction. As it is generally assumed that the heart is unable to utilize glycerol, the mechanism of myocardial glycerol uptake and the effects of insulin on this process have never been studied. Insulin was found to increase incorporation of radiolabelled glycerol into myocardial TAG. If glycerol is taken up by the same carrier as glucose, the use of low perfusate glucose concentrations may facilitate uptake of glycerol. A second group of hearts was prelabelled using the perfusate composition allowing maximal incorporation of radiolabelled glycerol into TAG using an identical protocol.

Determination of the specific activity of glycerol released during normoxia, isoprenaline infusion and hypoxia and comparison with the specific activity of TAG should give an indication of the origin of glycerol released from the heart under each of these conditions.

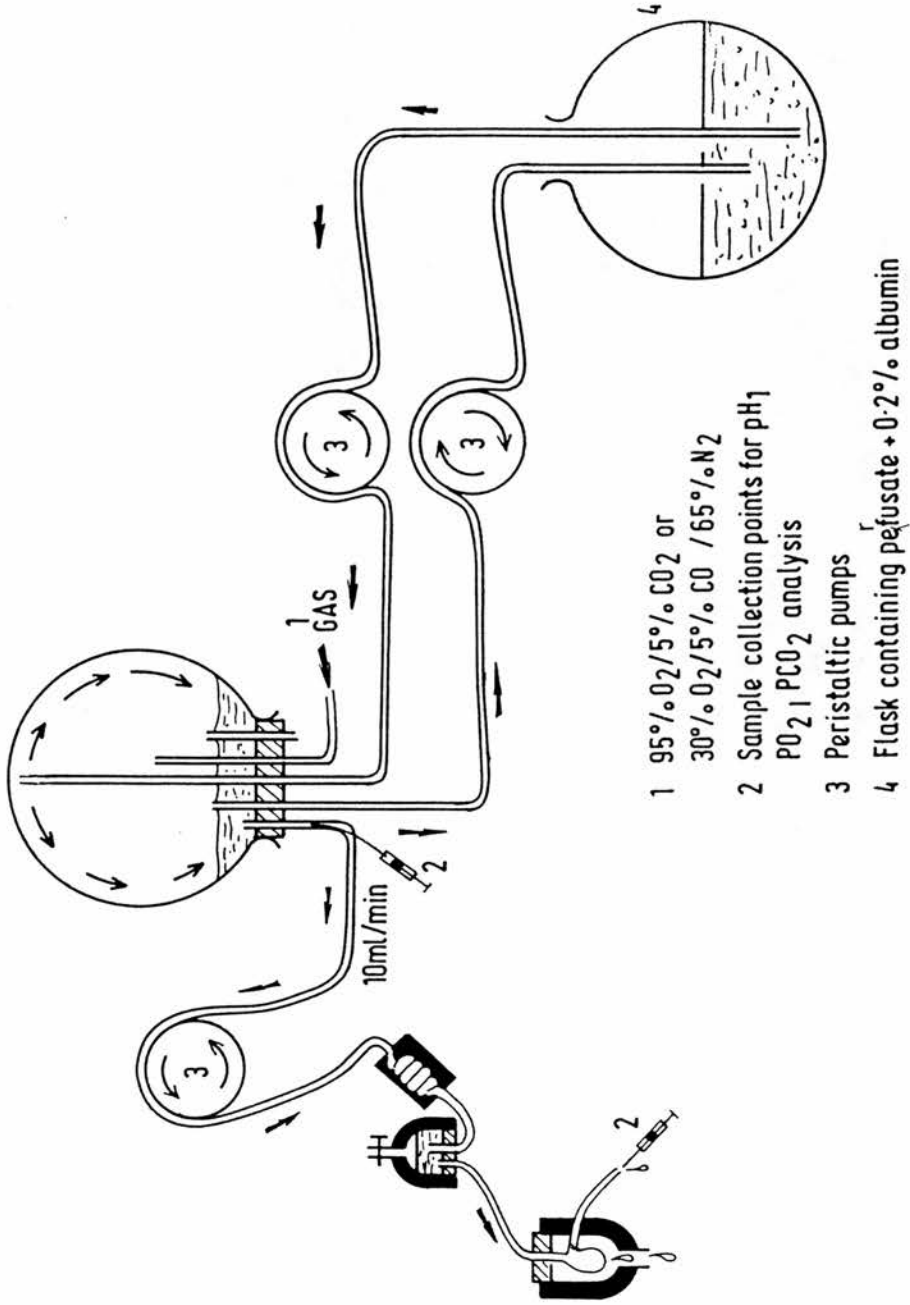
## 6.2 Methods.

### 6.2.1. The influence of albumin on hypoxia-stimulated glycerol release.

Perfusate containing albumin cannot be gassed in the usual way due to foaming of the solution. Surfactants are often used to prevent foaming but these compounds may affect membrane structure. The perfusion system illustrated in Figure 6.4 avoids the use of these compounds. Perfusate containing albumin is pumped by means of a peristaltic pump into an inverted round-bottomed flask (into which the appropriate gas mixture is also pumped), and allowed to cover the inside surface of the flask. A large surface area of the perfusate is thus exposed to the gas, allowing maximal equilibration of perfusate and oxygen (J Simpson, personal communication). The gassed perfusate is then pumped in the usual way to the heart using a non-recirculating perfusion system.

Male Sprague-Dawley rats ( $n=6$ ) were anaesthetized, the hearts removed and preperfused with albumin-free buffer for 20 min as described in section 2.1.2. Hearts were then perfused with perfusate containing 0.2% fatty-acid free bovine serum albumin (Sigma Chemical Company) using the perfusion system shown in Fig 6.4. Hearts were subjected to normoxia (95% $O_2$ /5% $CO_2$ ) (30 min), followed by hypoxia (30% $O_2$ /65% $N_2$ /5% $CO_2$ ) (30 min). Coronary effluent was collected at 10 min intervals. Samples were deproteinized as follows. A portion (1 ml) of each sample was removed and an equal volume of 0.6 M perchloric acid added. Matched potassium carbonate (200  $\mu$ l) was added and the samples left on ice for 30 min. Samples were then centrifuged (1500 g, 4°C, 20 min), after which the supernatant was analyzed for glycerol and lactate. Oxygen consumption was monitored throughout the experiments. After perfusion, hearts were freeze-clamped, freeze-dried and analyzed for ATP, CP, glycogen,  $\alpha$ -glycerophosphate, glycerol and TAG as described in Chapter 2.

Figure 6.4. Perfusion system for experiments using perfusate containing albumin.



### **6.2.2. The influence of nicotinic acid on glycerol release during isoprenaline infusion and hypoxia.**

Male Sprague-Dawley rats were anaesthetized, the hearts removed and perfused as described in Section 2.1.2. After the 20 min stabilization period, nicotinic acid (final concentration as specified for each experiment) was continuously infused (0.1 ml/min) proximal to the heart. After 30 min nicotinic acid infusion, both nicotinic acid and isoprenaline (10 nM) were infused simultaneously. Coronary effluent was collected at 10 min intervals and analyzed for glycerol.

A further group of hearts (n=6) was subjected to 30 min normoxia followed by 30 min hypoxia in the presence of the concentration of nicotinic acid conferring maximal inhibition of isoprenaline-stimulated glycerol release (100 $\mu$ M). Coronary effluent was collected at 10 min intervals and analyzed for glycerol and lactate. Oxygen consumption was monitored throughout. At the end of the experiment, hearts were freeze-clamped, freeze-dried and analyzed for ATP, CP, glycogen,  $\alpha$ -glycerophosphate, glycerol and TAG as described in Chapter 2 .

### **6.2.3. Radiolabelling of myocardial TAG by infusion of [2-<sup>3</sup>H]-glycerol.**

The first series of experiments served to identify the optimal conditions for labelling of the myocardial TAG pool. Male Sprague Dawley rats were anaesthetized, the hearts removed and perfused for a 20 min stabilization period as described in Section 2.1.2. Subsequently, [2-<sup>3</sup>H]-glycerol (Amersham 1Ci/mmol; 1mCi/ml) was infused (0.2 $\mu$ Ci/min) for 30 min. Infusion of radioactivity was then stopped and coronary effluent was collected at 2 min intervals and radioactivity measured as described in section 2.2.8. Hearts were then freeze-clamped, freeze-dried, the tissue lipids extracted and separated into fractions as described in section 2.2.7. The amount of [2-<sup>3</sup>H]-

glycerol in each lipid fraction and the amount of TAG were measured as described in sections 2.2.8. and 2.2.6. respectively. The specific activity of the TAG could therefore be calculated.

The conditions examined to determine the optimum requirements for incorporation of [2-<sup>3</sup>H]- glycerol into TAG were as follows:

1. 5.5 mM Glucose (n=3)
2. 5.5 mM Glucose and 5 mM Lactate (n=4)
3. 0.1 mM Glucose and Insulin\*(2munits/ml) (n=1)
4. 1.0 mM Glucose and Insulin (2munits/ml) (n=1)
5. 2.0 mM Glucose and Insulin (2munits/ml) (n=2)
6. 5.5 mM Glucose and Insulin (2munits/ml) (n=7)
7. 11 mM Glucose and Insulin (2munits/ml) (n=2)

\*Human velosulin, 100 units/ml, 10 ml, Wellcome.

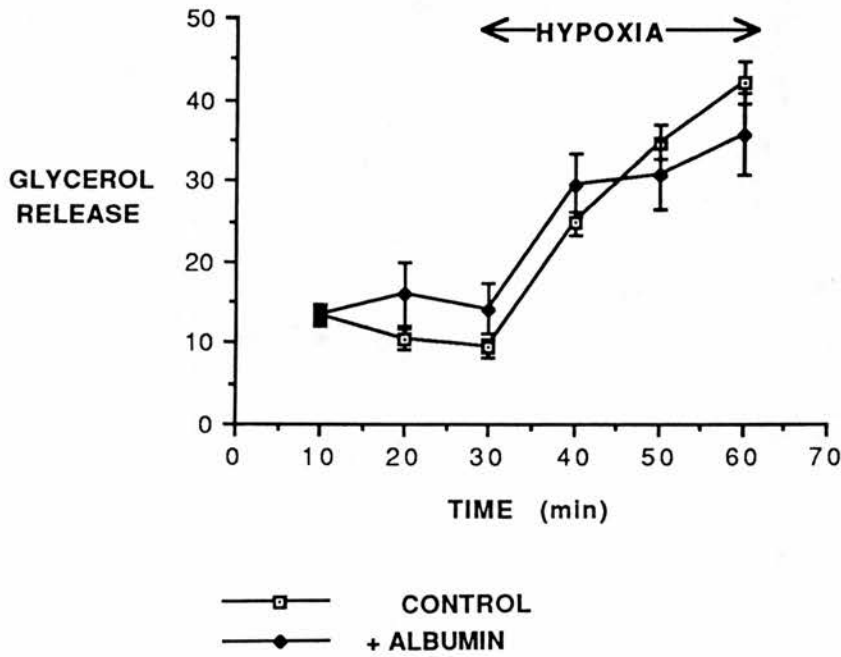
After infusing [2-<sup>3</sup>H]-glycerol for 30 min, and washing out radioactivity for 10 min, hearts were subjected to either normoxia (30 min, n=6), isoprenaline (10 nM) infusion (30 min, n=6) or hypoxia (30 min, n=6). Coronary effluent was collected at 5 min intervals from t=35 to 60 min and analyzed for glycerol and lactate. [2-<sup>3</sup>H]-glycerol and [2-<sup>3</sup>H<sub>2</sub>O] were measured as described in section 2.2.8.

At the end of the perfusion period, hearts were freeze-clamped, freeze-dried, lipids extracted and separated into fractions as described in section 2.2.7. [2-<sup>3</sup>H]-glycerol in each fraction was counted. The amount of TAG in each heart was measured as described in section 2.2.6. and the specific activity of TAG thus determined.

### 6.3. Results.

The presence of fatty acid free albumin (0.2%) in the perfusate had no effect on glycerol efflux during hypoxia (Fig 6.5).

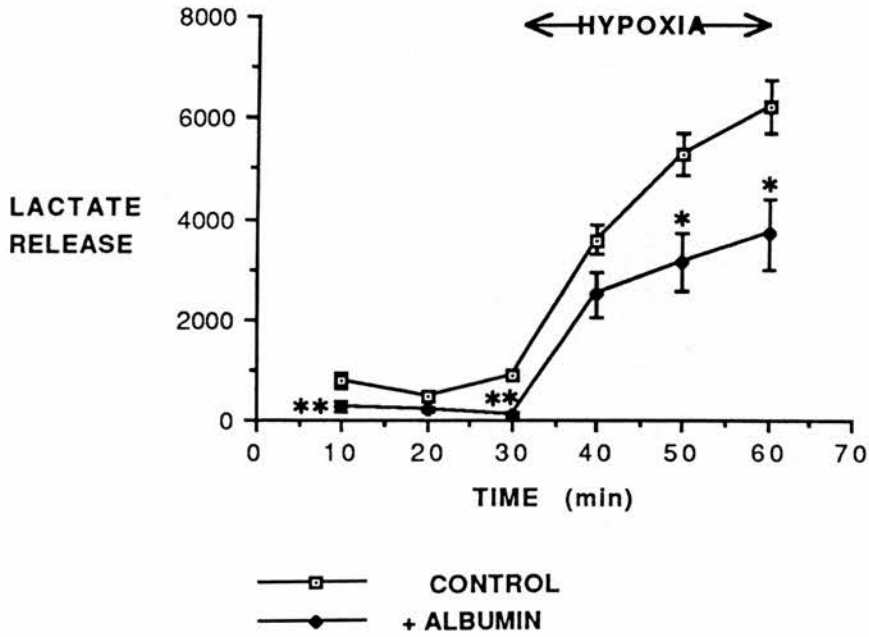
Figure 6.5. The influence of hypoxia on glycerol release in the absence and presence of fatty acid free albumin.



Glycerol release is expressed as  $\text{nmol.g}^{-1}\text{wet wt. min}^{-1}$  (Mean $\pm$ SEM)

In contrast, lactate production after the first 10 min of hypoxia was significantly lower when albumin was present (Fig 6.6). Total lactate production during the 30 min period of hypoxia was also significantly lower in the albumin group than in the control group ( $75 \pm 34$  with albumin vs  $133 \pm 12$   $\mu\text{mol}$  control;  $P < 0.05$ , t-test)

Figure 6.6. The influence of hypoxia on lactate release in the absence and presence of fatty acid free albumin.



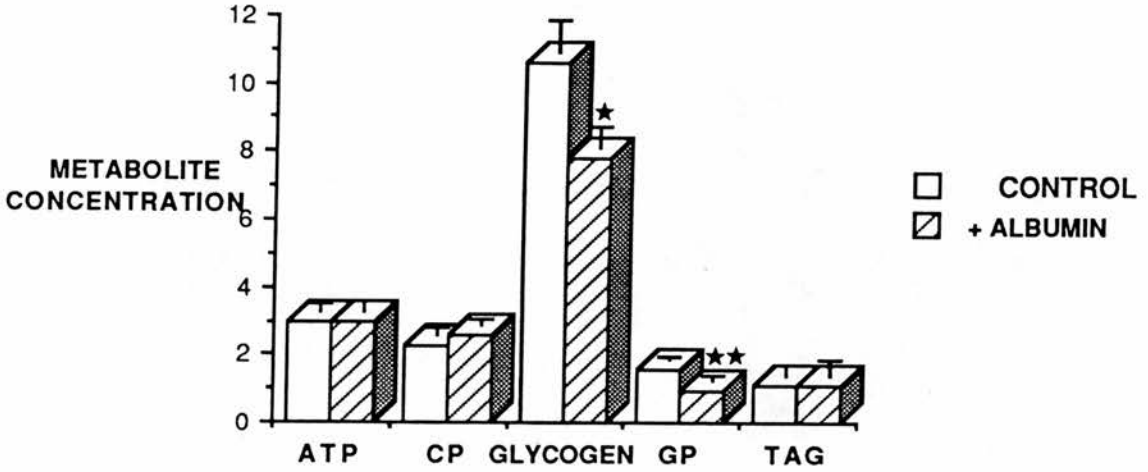
Lactate release is expressed as  $\text{nmol.g}^{-1}$  wet wt. (Mean  $\pm$  SEM)

\*  $P < 0.05$  vs CONTROL (t-test)

\*\* $P < 0.01$  vs CONTROL (t-test)

The presence of albumin in the perfusate had no effect on oxygen uptake ( $2.38 \pm 0.04$  control vs  $2.32 \pm 0.07 \mu\text{mol} \cdot \text{g}^{-1} \cdot \text{min}^{-1}$  with albumin). Myocardial glycogen and  $\alpha$ -glycerophosphate levels after hypoxic perfusion were significantly lower when albumin was present in the perfusate (Fig 6.7).

Figure 6.7. The influence of hypoxia on myocardial metabolite concentrations in the absence and presence of fatty acid free albumin (0.2%).



Metabolite concentrations are expressed as  $\mu\text{mol} \cdot \text{g}^{-1}$  wet wt. (Mean  $\pm$  SEM).

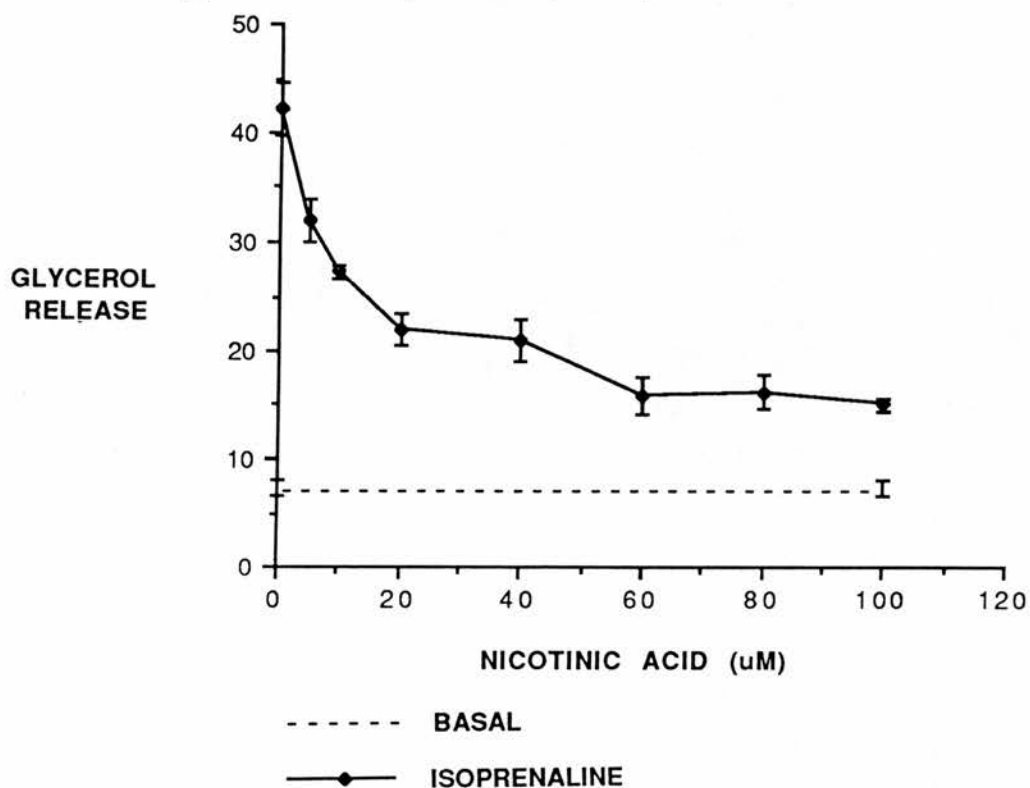
\*P < 0.05 vs CONTROL (t-test)

\*\*P < 0.01 vs CONTROL (t-test).

### 6.3.2. The influence of nicotinic acid on isoprenaline-stimulated and hypoxia-stimulated glycerol release.

Nicotinic acid inhibited isoprenaline-stimulated glycerol release in a dose dependent manner (Fig 6.8).

Figure 6.8. The influence of varying concentrations of nicotinic acid on glycerol release during infusion of isoprenaline (10 nM)



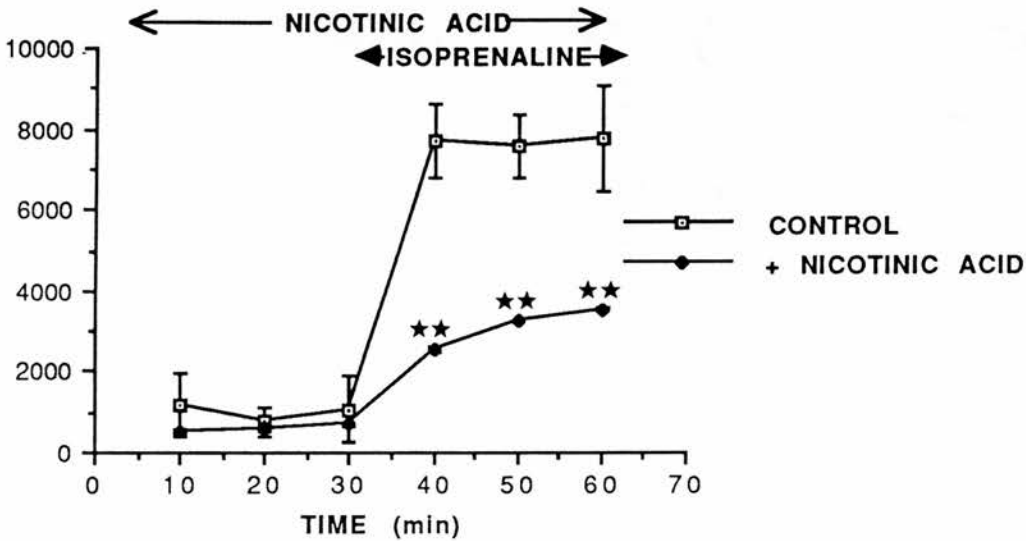
Glycerol release is expressed as  $\text{nmol.g}^{-1}\text{wet wt. min}^{-1}$  (Mean $\pm$ SEM)

BASAL (-----) = Glycerol release in the absence of isoprenaline.

None of the doses of nicotinic acid used fully inhibited isoprenaline-stimulated glycerol release. At a concentration of 60 $\mu$ M, nicotinic acid caused a 60% reduction in isoprenaline-stimulated glycerol release ( $P<0.01$ ). The inhibition was not complete when the concentration of nicotinic acid was increased to either 80  $\mu$ M or 100  $\mu$ M.

In addition to its inhibitory action on isoprenaline-stimulated glycerol release, nicotinic acid (100  $\mu$ M) significantly attenuated myocardial lactate production (Fig 6.9) and tissue  $\alpha$ -glycerophosphate concentration ( $0.85 \pm 0.1$  vs  $0.30 \pm 0.1 \mu.g^{-1}$  wet wt with nicotinic acid (100  $\mu$ M);  $P<0.05$ , t-test) after isoprenaline infusion.

Figure 6.9. The influence of isoprenaline (10 nM) on lactate release in the absence and presence of nicotinic acid (100  $\mu$ M).

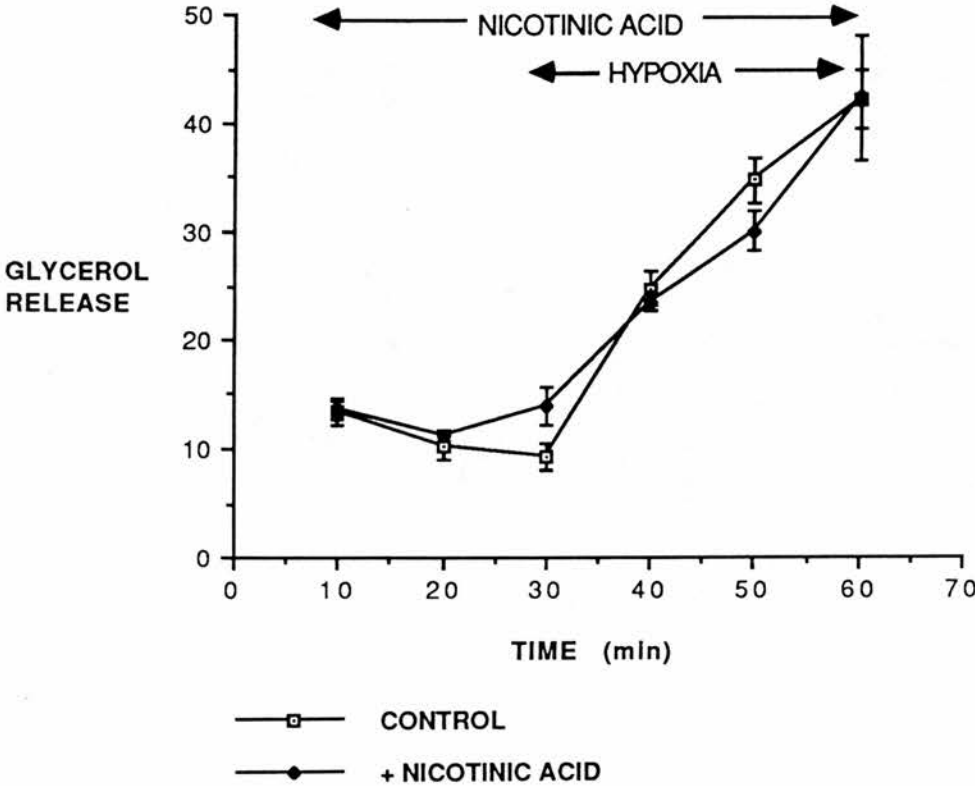


Lactate release is expressed as  $nmol.g^{-1}$  wet wt.  $min^{-1}$  (Mean $\pm$ SEM).

\*\*\*  $P<0.01$  vs CONTROL

The drug had no effect on oxygen uptake during isoprenaline infusion ( $7.1 \pm 0.2$  control vs  $7.0 \pm 0.5 \mu\text{g}^{-1}\text{min}^{-1}$  with nicotinic acid). In contrast to the effect of nicotinic acid on isoprenaline-stimulated glycerol release, the drug had no effect on hypoxia-stimulated glycerol release (Fig 6.10).

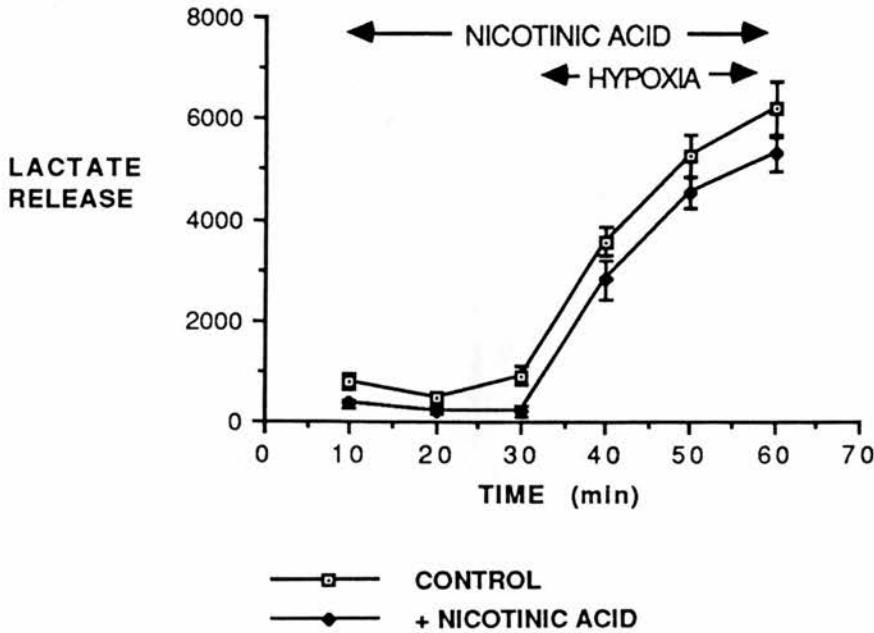
Figure 6.10. The influence of hypoxia on glycerol release in the absence and presence of nicotinic acid ( $100 \mu\text{M}$ ).



Glycerol release is expressed as  $\text{nmol.g}^{-1}\text{wet wt. min}^{-1}$  (Mean $\pm$ SEM)

The rate of lactate production during hypoxia was not significantly different in the absence or presence of nicotinic acid at t=40, 50 and 60 min (Fig 6.11).

Figure 6.11 The influence of hypoxia on lactate release in the absence and presence of nicotinic acid (100  $\mu$ M).

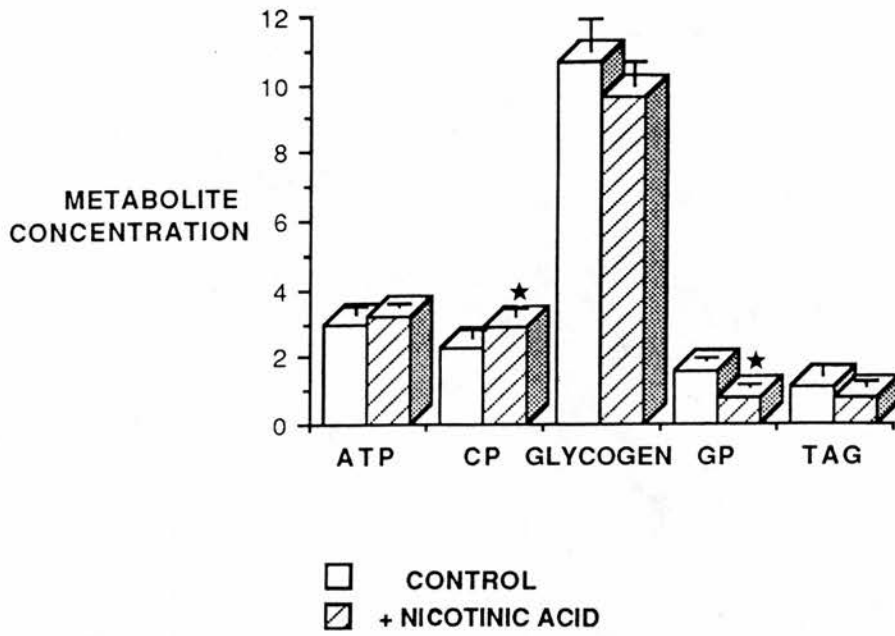


Lactate release is expressed as  $\text{nmol.g}^{-1}\text{wet wt. min}^{-1}$  (Mean $\pm$ SEM).

However, the total lactate release during the 30 min period of hypoxia was significantly lower in the presence of nicotinic acid ( $133\pm 12$  control vs  $101\pm 23 \mu\text{.g}^{-1}\text{min}^{-1}$  with nicotinic acid;  $P<0.05$ , t-test).

Tissue creatine phosphate levels were elevated and  $\alpha$ -glycerophosphate levels were reduced compared with the control group when nicotinic acid was infused during hypoxia (Fig 6.12).

**Figure 6.12 The influence of hypoxia on myocardial metabolite concentrations in the absence and presence of nicotinic acid (100  $\mu$ M).**



GP =  $\alpha$ -glycerophosphate

Metabolite concentrations are expressed as  $\mu\text{mol}\cdot\text{g}^{-1}\text{wet wt.}$  (Mean $\pm$ SEM).

\*P<0.01 vs CONTROL

### 6.3.3. Radiolabelling of myocardial TAG by infusion of [2-<sup>3</sup>H]-glycerol.

Incorporation of [2-<sup>3</sup>H] glycerol into the neutral lipid fraction of heart tissue under the various conditions varied (Table 6.1).

**Table 6.1. Specific activity of myocardial TAG under various experimental conditions.**

Conditions	Specific activity of neutral lipids (cpm.μmol <sup>-1</sup> )
5.5 mM Glucose	2940
5.5 mM Glucose +5 mM Lactate	6040
0.1 mM Glucose + Insulin	-
1.0 mM Glucose + Insulin	-
2.0 mM Glucose + Insulin	22785
11 mM Glucose + Insulin	24385
5.5 mM Glucose + Insulin	28420

(SEMs are not shown for this data due to the small numbers of observations).

Hearts perfused with 0.1 mM and 1.0 mM glucose as the exogenous substrate stopped beating before the end of the perfusion period and therefore could not be used for further studies. As the hearts perfused in the presence of 5.5 mM glucose and 0.2 mU/ml insulin showed the highest incorporation of [2-<sup>3</sup>H]-glycerol into the neutral lipid fraction, these conditions were used for subsequent experiments. Radiolabelled glycerol was readily washed out from the vascular space (Fig 6.13). Incorporation of labelled glycerol into other lipid fractions also varied (Table 6.2).

Figure 6.13 Washout of radiolabelled glycerol.

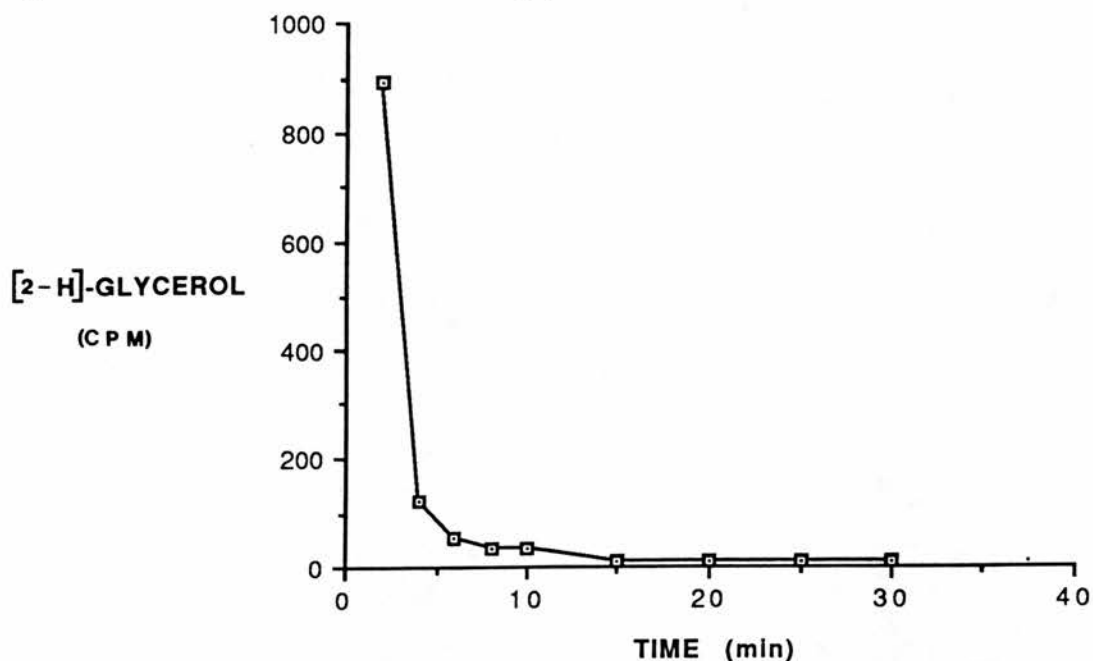
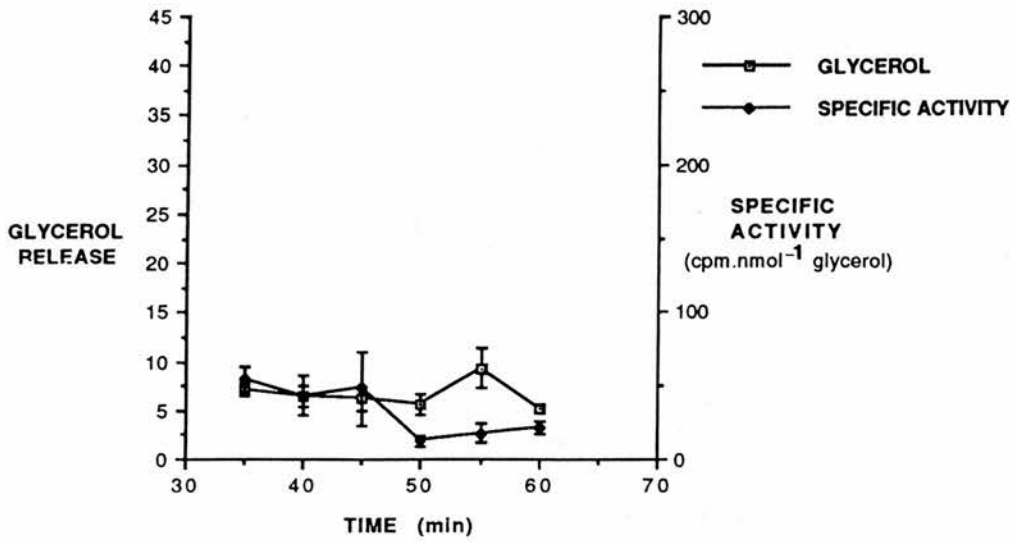


Table 6.2. Incorporation of [2-<sup>3</sup>H]-glycerol into lipid fractions of the heart.

Fraction	cpm.g <sup>-1</sup> wet wt. heart tissue
Neutral lipids	25840±8170
Cardiolipin	175±89
Phosphatidylethanolamine	2300±457
Phosphatidylcholine	5920±1455
Phosphatidylserine	217±138
Phosphatidylinositol	1965±1073

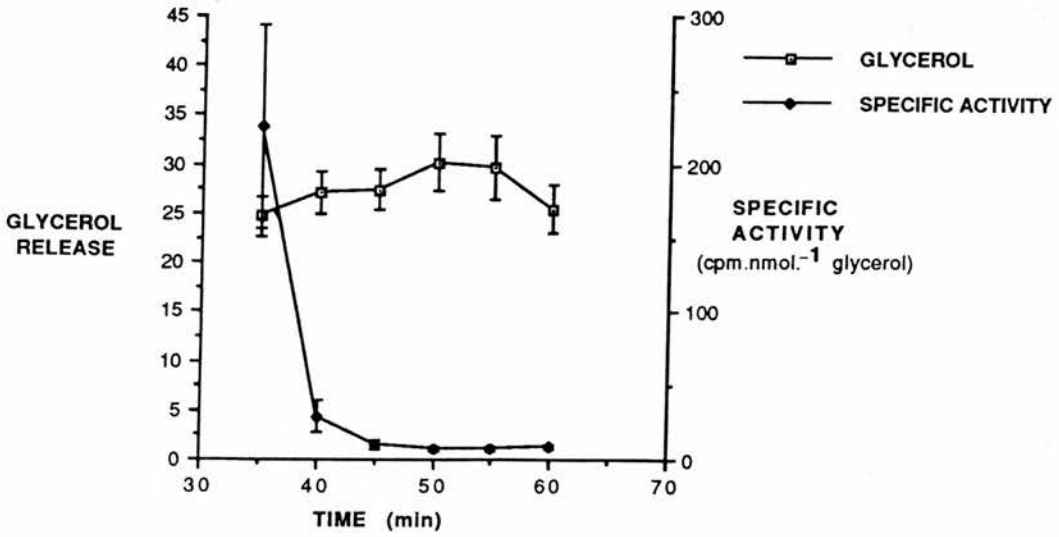
The specific activity of glycerol released during normoxia (Fig 6.14) was not significantly different than the specific activity of the TAG fraction in the heart at the end of the perfusion period (Table 6.3).

Figure 6.14. Specific activity of glycerol released during normoxia.



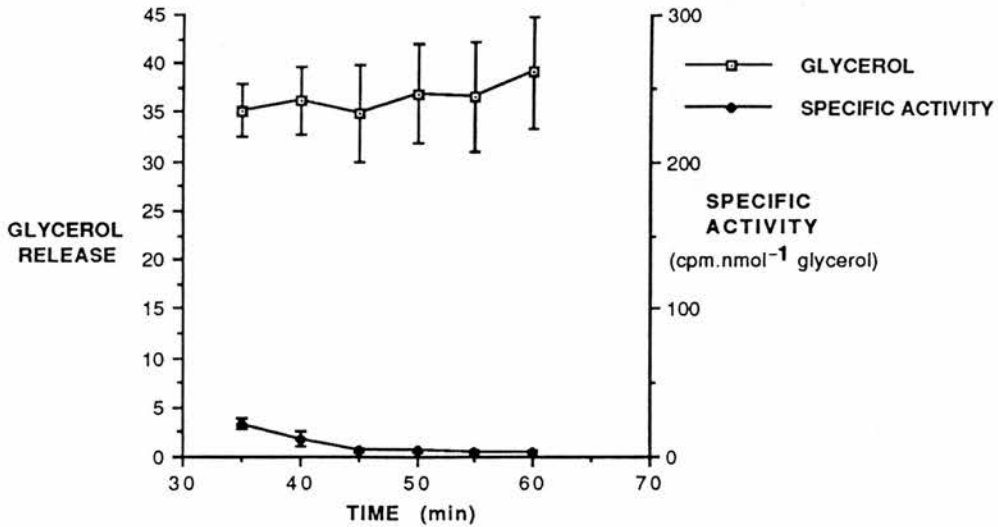
Although isoprenaline infusion caused a significant increase in glycerol release, after the first 5 min of infusion of the drug, the specific activity of the glycerol released (Fig 6.16) was significantly lower than the specific activity of the myocardial TAG (Table 6.3;  $P < 0.01$ ). It therefore appears that isoprenaline stimulates breakdown of a separate, unlabelled TAG pool.

**Figure 6.15. Specific activity of glycerol released during isoprenaline (10 nM) infusion.**



Hypoxia also caused a significant increase in myocardial glycerol release, but the specific activity of the glycerol in the coronary effluent (Fig 6.16) was significantly lower than the specific activity of the TAG in the heart (Table 6.3;  $p < 0.01$ ).

**Figure 6.16 Specific activity of glycerol released from the heart during hypoxia.**



TAG concentrations in the heart were not significantly different in any of the groups. However, the specific activity of TAG after isoprenaline infusion was significantly higher than after hypoxia (Table 6.3).

**Table 6.3. Specific activity of myocardial TAG after normoxia, hypoxia and isoprenaline infusion.**

	Normoxia	Isoprenaline	Hypoxia
[TAG]	0.97±0.2	0.71±0.1	0.73±0.1
Specific activity of TAG	39±7	33±3	23±2*

\*P<0.05 vs isoprenaline (t-test)

[TAG] is expressed as  $\mu\text{mol.g}^{-1}$  wet wt (Mean±SEM)

Specific activity of TAG is expressed as cpm  $\text{nmol}^{-1}$  TAG (Mean±SEM).

#### 6.4. Discussion.

The presence of fatty-acid free albumin in the perfusate had no effect on glycerol release during hypoxia. Thus if glycerol release was the result of TAG breakdown, either lipolysis was not affected by product inhibition, or there was no accumulation of NEFA within the tissue during hypoxia (tissue NEFA were not measured due to the well recognized technical difficulties; Van der Vusse and Reneman 1984, Van der Vusse et al. 1985). Previous reports of inhibition of lipolysis used exogenous fatty acids at concentrations of between 0.25 mM (Stam et al. 1979) and 0.6mM palmitate (Crass et al. 1975) to inhibit glucagon or adrenaline-stimulated lipolysis in the rat heart. During 30 min hypoxia, assuming that 3 moles of NEFA remain in the heart for every glycerol released, and none of these NEFA are re-esterified, the maximum NEFA concentration in the tissue would be around  $2.7\mu\text{M}$  (corresponding to a total glycerol release of  $\sim 900$  nmol). No effect of this concentration of NEFA on lipolysis has been reported and the effects of binding of intracellular NEFA to fatty acid binding proteins on lipolysis are unknown. However, reutilization of glycerol due to activity of glycerokinase would lead to underestimation of lipolysis when calculated using the amount of glycerol released from the heart. On the other hand, re-esterification would decrease the tissue NEFA even further. Thus the conclusion reached from the experiment with regard to the lack of effect of removal of NEFA by albumin is correct, even if reutilization did take place. During hypoxia, a large part of the glycerol release appears to be stimulated by a different mechanism than hormone-stimulated lipolysis (Chapter 5). Inhibition of hypoxia-stimulated lipolysis by NEFA has not been reported.

Glycogen levels were significantly lower in hearts perfused in the presence of albumin suggesting that removal of NEFA by albumin led to increased oxidation of glycogen. However, myocardial TAG concentrations were not reduced after perfusion with albumin compared with the control group (Fig 6.7), implying that a shift of NEFA from the

phospholipid to the TAG pool takes place. It is also possible that synthesis of NEFA is taking place.

Glycerol release during hypoxia was unaffected by nicotinic acid, providing further support for the hypothesis that a hormone-sensitive lipase is not activated during oxygen deprivation, as the drug acts by inhibiting cAMP production (Fig 6.3). However, it is not known whether metabolic changes in the tissue induced by hypoxia eg. decreased pH, would modify the activity of nicotinic acid.

The effects of nicotinic acid on isoprenaline-stimulated glycerol release conflict with the results of Christian et al. (1968) who reported decreasing inhibition of isoprenaline-stimulated glycerol release from the isolated, perfused rat heart when the nicotinic acid concentration was increased from 10 $\mu$ M to 100 $\mu$ M. These workers used a concentration of 30 nM isoprenaline (rather than 10 nM) to stimulate glycerol release. Additionally, the Ca<sup>++</sup> concentration used in this study was relatively low (1.22 mM). Hron et al. (1977) reported a lack of response to isoprenaline (1  $\mu$ M) at this Ca<sup>++</sup> concentration. Other differences were that the heart rate was controlled by electrical pacing, and that the rats were killed by decapitation rather than anaesthesia. It is not known whether these factors affect the response of the tissue to either isoprenaline or nicotinic acid. The effect of nicotinic acid on isoprenaline-stimulated glycerol release depends on both the concentrations of both nicotinic acid and isoprenaline and probably also on other variables.

It has been suggested that the rate of hormone-stimulated lipolysis may be determined by the availability of  $\alpha$ -glycerophosphate to remove product inhibition by re-esterifying NEFA (Schoonderwoerd et al. 1987). This is consistent with the finding that nicotinic acid caused a decrease in both myocardial  $\alpha$ -glycerophosphate concentrations and glycerol release during isoprenaline infusion. However, during hypoxia, nicotinic acid decreased myocardial  $\alpha$ - glycerophosphate concentrations but had no effect on glycerol

release. Thus, in this case, the relationship between tissue  $\alpha$ -glycerophosphate concentration and glycerol release is uncoupled.

Inhibitory effects of nicotinic acid on glycerol production in the isolated, perfused rat heart have been reported during low flow ischaemia (Trach et al. 1984) and stop flow ischaemia (van Bilsen et al. 1990) at concentrations of 1  $\mu$ M and 10  $\mu$ M respectively. However, in the latter study the decrease in tissue glycerol concentration brought about by the presence of the drug was not highly significant ( $0.05 < P < 0.1$ ). Adrenaline-stimulated glycerol release in the ischaemic, isolated, perfused rat heart was inhibited by nicotinic acid (5  $\mu$ M) (Brownsey and Brunt 1977), but the fact that the drug had no effect on basal ischaemic glycerol release was not emphasized.

The drug clearly has inhibitory properties with regard to hormone-stimulated lipolysis but its effects during hypoxia and ischaemia are controversial. Ischaemia (3 min) prevented the isoprenaline-induced increase in cAMP formation in the heart (Krause and England 1982). Thus it seems that the ability of nicotinic acid to inhibit ischaemia-induced glycerol release has been exaggerated.

Nicotinic acid had no effect on isoprenaline-stimulated increases in cAMP release from the heart (O'Brien and Strange 1975), suggesting that the drug is unlikely to inhibit activation of hormone-sensitive lipase in the myocyte. However, direct measurements of tissue cAMP levels have not been reported.

It is unclear whether nicotinic acid directly inhibits hormone-stimulated lipolysis in the heart or whether its inhibition of lipolysis is secondary to inhibition of glycolysis (i.e. via decreased production of  $\alpha$ -glycerophosphate)

. As nicotinic acid had no effect on hypoxia-stimulated glycerol release, breakdown of  $\alpha$ -glycerophosphate by acid phosphatase or hydrolysis of phospholipids cannot be ruled out as sources of glycerol.

The incorporation of [2-<sup>3</sup>H]-glycerol into TAG implies that glycerokinase activity

exists in the rat heart contrary to what is generally assumed. Incorporation of glycerol into TAG was stimulated in the presence of insulin. It is not known whether this hormone acts by stimulating glycerol uptake, glycerokinase activity, TAG synthesis or some other mechanism. Insulin inhibits lipolysis in adipose tissue (Jungas and Ball 1963) but was found to have no effect on isoprenaline-stimulated glycerol release from the isolated, perfused rat heart at a concentration of 100mU/ml (Christian et al. 1968). Even in the presence of insulin, less than 1% of the labelled glycerol infused into the heart was incorporated into TAG. Decreasing the glucose concentration in the perfusate did not increase incorporation of glycerol into TAG and concentrations of less than 2 mM glucose were detrimental to the heart. The presence of lactate in the perfusate increased incorporation of glycerol into TAG, possibly by decreasing dehydrogenation of  $\alpha$ -glycerophosphate due to an increased NADH/NAD<sup>+</sup> ratio.

The specific activity of the glycerol leaving the heart after five minutes of isoprenaline infusion was higher than would be expected from simple washout of radioactivity, suggesting that isoprenaline initially stimulates hydrolysis of radiolabelled TAG. This was followed by release of glycerol with specific activity lower than that of the myocardial TAG, implying that either more than one pool of TAG exists in the heart, or that the glycerol released after the first 10 minutes of isoprenaline infusion is the result of hydrolysis of newly formed TAG, from phospholipids or from  $\alpha$ -glycerophosphate. A possible role of  $\alpha$ -glycerophosphate formed during glycolysis in removing NEFA by re-esterification, and therefore stimulation of lipolysis is supported. Glycerol release from the isolated, perfused rat heart is stimulated in the presence of lactate which is likely to increase the NADH/NAD<sup>+</sup> ratio and subsequently the conversion of glyceraldehyde-3-phosphate via dihydroxyacetone phosphate to  $\alpha$ -glycerophosphate (De Groot et al. 1989). On the other hand, it seems unlikely that  $\alpha$ -glycerophosphate stimulates lipolysis by removing product inhibition (Schoonderwoerd et al. 1987) as glycerol release was not

stimulated when NEFA were removed by albumin. It therefore seems probable that NEFA are re-esterified with  $\alpha$ -glycerophosphate, forming a new, more metabolically active pool of TAG. TAG can be recovered in lipid droplets (Stein and Stein 1968) and in lysosomes (Stam et al. 1980) from the heart. The turnover of TAG stored in lipid droplets has been proposed to be lower than for TAG stored in intracellular organelles (Stein and Stein 1968). Transfer of TAG from a metabolically inert to a metabolically active pool may be important in regulation of myocardial TAG homeostasis (Schoonderwoerd et al. 1989).

Hypoxia-stimulated as well as isoprenaline-stimulated glycerol release appears to originate from either an unlabelled TAG pool, from phospholipids or from  $\alpha$ -glycerophosphate. Unfortunately this aspect could not be examined further. More complete labelling of TAG could be achieved by long term infusion of [2- $^3$ H] glycerol prior to the experiment. However, under these circumstances, it is likely that a significant amount of glycerol would also be incorporated into phospholipids. Labelling of this kind would therefore only be useful in determining whether glycerol was of lipid or of carbohydrate origin.

Obviously, much work is still needed to elucidate the mechanisms and controlling factors of myocardial TAG metabolism, particularly the changes in TAG metabolism during hypoxia and ischaemia. The evidence available so far suggests that glycolysis, TAG metabolism and phospholipid metabolism in the heart are closely linked and interdependent.

## **CHAPTER 7**

### **FINAL DISCUSSION**

### **7.1. The Model.**

The isolated perfused rat heart provides a useful model for quantitative metabolic studies. Arterial and venous perfusate samples are easily accessible, allowing direct measurements of metabolism to be made. The double-reservoir perfusion system allows the oxygen tension of the perfusate supplying the heart to be changed rapidly, enabling comparison of normoxic and hypoxic metabolism in the same heart. In addition, cannulation of the pulmonary artery allows anaerobic sampling of coronary effluent for blood gas analysis and therefore calculation of oxygen uptake.

A disadvantage of the model is that the cellular composition of the whole heart is heterogeneous. Measurements of metabolism therefore include adipose tissue, endothelial cells and smooth muscle cells as well as myocytes. Also, progressive changes in tissue metabolism during perfusion must be taken into account. For example, insulin is known to affect glucose uptake for up to 30 min after commencing perfusion (Fisher and O'Brien 1972). The biochemical changes induced by the period of anoxia between removal of the heart and commencing perfusion must be considered; a 20 min preperfusion period was required within each experiment to allow stabilization of the rates of lactate and glycerol release from the heart before collection of the first sample. Also, high flow rates are required to maintain adequate oxygenation of the tissue as haemoglobin is absent from the perfusate. Creatine phosphate concentration in the heart was found to decrease after normoxic perfusion, suggesting that even with high flow rates, oxygenation of the tissue was limited. Hence, this model is more useful for studying the effects of hypoxia rather than being used as a model of normoxic metabolism.

## 7.2. Metabolism in the Langendorff perfused rat heart during hypoxia.

Myocardial hypoxia was accompanied by decreased oxygen uptake, increased glycerol and lactate production, increased glycogen breakdown and decreased high energy phosphate levels. TAG concentrations in the heart were not altered.

Although glycerol release was stimulated during hypoxia, the TAG concentrations of normoxically and hypoxically perfused hearts were not different. This provides further evidence in favour of the operation of a TAG-NEFA cycle in the heart during oxygen deprivation. The TAG-NEFA cycle used only a small percentage (<7%) of the ATP produced in the heart even during severe hypoxia. Thus, if these results may be extrapolated to ischaemia, the operation of this cycle probably cannot create pathologically significant gradients in ATP. However, it should be pointed out that glycerokinase activity exists in the heart, signifying that measurement of myocardial glycerol release underestimates the amount of TAG hydrolysis. Hypoxia differs from ischaemia in many respects (Section 1.3), particularly in that products of metabolism such as glycerol and lactate cannot readily leave the tissue during ischaemia. Build-up of lactate causes tissue acidosis, which may in itself affect the metabolism of the heart. Caution must also be taken in extrapolating results directly from the *in vitro* situation where the heart is free from neural and hormonal influences to the *in vivo* situation.

More than one pool of TAG may exist in the heart (Stein and Stein 1968, Wood et al. 1972, De Groot et al. 1989) and previous studies have shown that ATP may be compartmentalized within the myocyte (Bricknell and Opie 1978). Glycolytically produced ATP has been specifically linked with the maintenance of cellular electrolyte gradients. It is therefore possible that localized TAG-NEFA cycling causing a significant drain on particular stores of high energy phosphate may be a stimulus for arrhythmias.

exhibiting a five-fold increase in TAG concentration compared with hearts from non-obese rats was only slightly higher than in hearts from the control (non-obese) group (Chapter 4), and the increase in the proportion of ATP utilized in the operation of the TAG-NEFA cycle was related to decreased anaerobic ATP production in hearts from obese rats, rather than increased lipolysis (Table 4.6). This finding is contrary to previous speculation that TAG levels in the heart may determine the rate of cycling, and that the cycle may become increasingly significant as a drain of ATP in the compromised heart (Trach et al 1984). Although basal glycerol release was elevated in hearts from obese rats, the response to hypoxia was unusual in that glycerol release was stimulated during the first 15 min of hypoxia but thenceforth declined (Fig 4.3). A higher proportion of adipocytes in hearts from obese rats may have been the reason for this, although unfortunately no data are available. The rate of cycling was lower in hearts from fasted rats than in hearts from fed rats, whereas the TAG levels in hearts from fed and fasted rats were not different. This provides further evidence that myocardial TAG concentration is not the major determinant for the rate of the TAG-NEFA cycle.

### **7.3. The mechanism and source of hypoxia-stimulated glycerol release.**

In contrast to isoprenaline-stimulated glycerol release, hypoxia-stimulated glycerol release could not be inhibited by the  $\beta$ -adrenoceptor blocking agents atenolol and timolol. However, pretreatment of rats with 6-hydroxydopamine to deplete stores of endogenous catecholamines (Hauesler et al. 1969), and subsequent perfusion of these hearts revealed that approximately 30% of hypoxia-stimulated glycerol release can be attributed to the action of endogenous catecholamines (Chapter 5). This is contrary to previous assumptions that hypoxia-stimulated glycerol efflux is entirely due to activation of a hormone-sensitive lipase in the heart by catecholamines (Hough and Gevers 1975, Karwartowska-Krynska and Beresewicz 1983).

Karwadowska-Krynska and Beresewicz 1983).

It is possible that the catecholamine-mediated glycerol release observed during hypoxia is due to the presence of adipocytes within the heart tissue and that glycerol release from the myocytes is stimulated by a different mechanism.  $\beta$ -adrenoceptor function in the heart did not appear to be affected by hypoxia, demonstrated by comparable amounts of glycerol release in response to exogenous isoprenaline during normoxia and hypoxia. In contrast to this, isoprenaline-stimulated glycerol release from isolated myocytes was attenuated during anoxia (Larsen et al. 1989). This may simply reflect a difference between anoxia and hypoxia, or alternatively could be interpreted as further evidence that changes in myocyte glycerol release from the isolated, perfused rat heart are masked by the response of adipocytes. However, this seems unlikely as in our studies nicotinic acid, a drug possessing antilipolytic properties in adipose tissue (Carlson and Oro 1966) was without effect on hypoxia-stimulated glycerol release in the heart (Fig 6.10).

As hypoxia-stimulated glycerol release from the heart is generally assumed to be mediated by adrenergic mechanisms, little attention has been paid to the role of myocardial lipases other than hormone-sensitive lipase. It may be that another TAG lipase, activated during hypoxia independent of catecholamines may play an important role in the metabolic changes induced by hypoxia.

Alternatively, a new suggestion which emerged from these studies is that hypoxia-stimulated glycerol release may be the result of breakdown of  $\alpha$ -glycerophosphate. Acid phosphatase activity is known to exist in the heart (Welman and Peters 1976), and direct breakdown of  $\alpha$ -glycerophosphate could therefore lead to glycerol release during hypoxia. However, the activity of this enzyme in the heart during hypoxia is unknown. Myocardial  $\alpha$ -glycerophosphate concentrations show a positive correlation with glycerol release from the isolated, perfused rat heart under a variety of conditions (Fig 6.1). It seems unlikely that  $\alpha$ -glycerophosphate production stimulates glycerol release by

presence of fatty acid free albumin in the perfusate had no effect on hypoxia-stimulated glycerol efflux (Fig 6.5). More probable is that re-esterification of NEFA with  $\alpha$ -glycerophosphate may lead to the formation of a more metabolically active TAG pool and thus stimulate glycerol release.

Another possible source of glycerol released from the heart during hypoxia is phospholipid. Addition of exogenous phospholipase C to energy-depleted isolated myocytes during anoxia caused an increase in glycerol release. This effect was absent in normal (non-energy depleted, normoxic) myocytes, (Myrmel et al. 1989). Endogenous phospholipase C activity in the heart during hypoxia has not been measured. It has been demonstrated several times that phospholipid concentrations in the heart are unaltered after 1h of ischaemia (Chien et al. 1984, Das et al. 1986, van der Vusse et al. 1987). However, a small change in the size of the phospholipid pool could lead to a significant amount of glycerol release. The phospholipid pool is continually turning over and the rate of turnover is thought to increase during ischaemia (van der Vusse et al. 1987).

Determination of the origin of glycerol released during hypoxia by labelling myocardial TAG via short-term infusion of [2-<sup>3</sup>H]-glycerol has proved to be complicated, probably due to the existence of more than one TAG pool. Long-term pre-labelling of TAG by administration of radiolabelled glycerol *in vivo* could be used to ensure labelling of all TAG pools. However, the label would also be incorporated into phospholipids, removing some of its specificity.

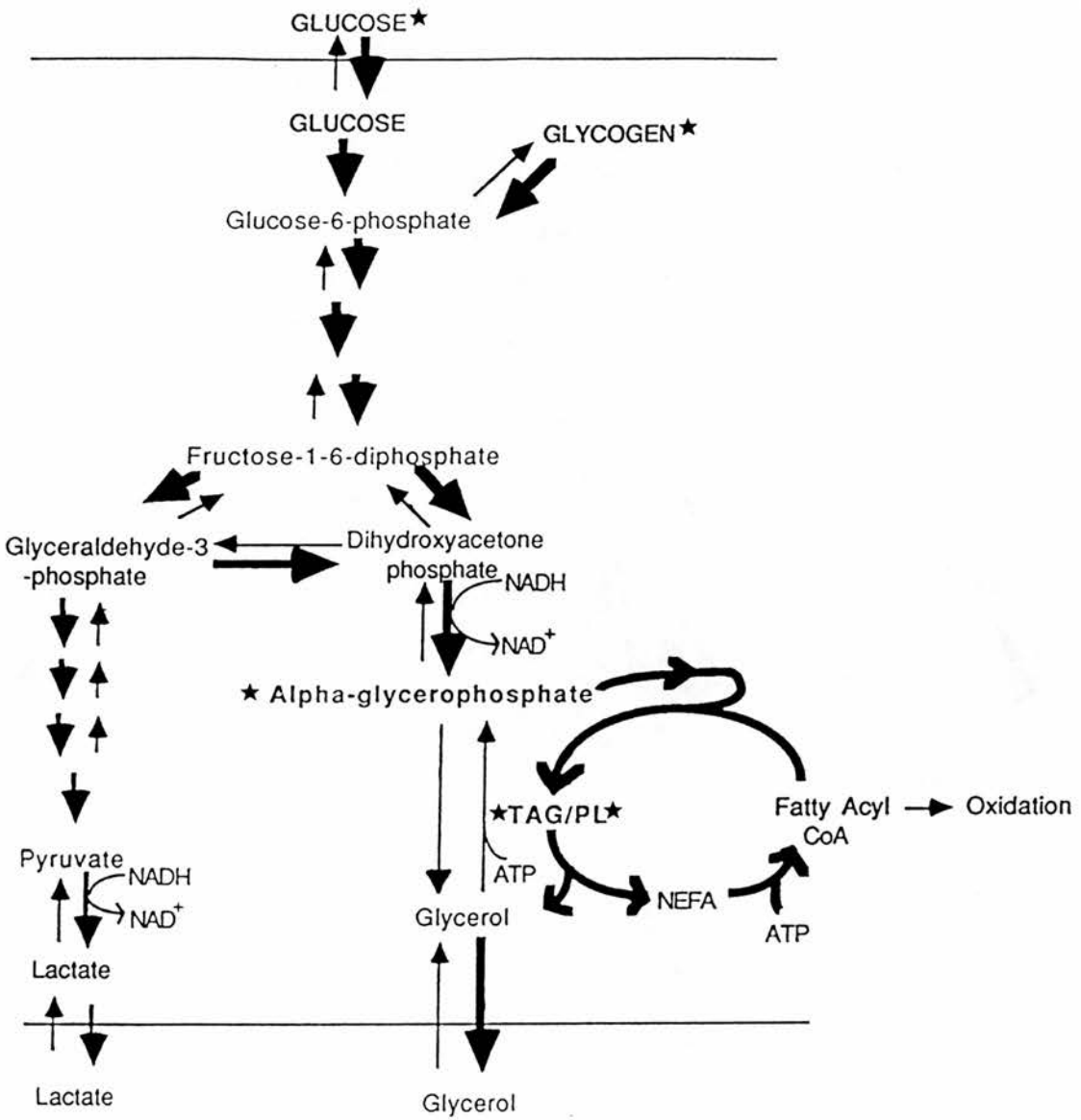
Perfusion with radiolabelled fatty acids bound to albumin is another possibility for measurement of the rate of lipolysis, although re-incorporation of NEFA into the TAG pool during TAG-NEFA cycling would make interpretation difficult as this would lead to underestimation of the rate of lipolysis. Again the label would be incorporated into phospholipids as well as TAG.

Selective labelling of  $\alpha$ -glycerophosphate by perfusion of hearts with radiolabelled

glucose would be complicated due to dilution of the label with glycogen-derived  $\alpha$ -glycerophosphate. Measurement of the relative activities of TAG lipases, phospholipases, acid phosphatase and glycerokinase during hypoxia would provide more information about potential sources of glycerol. However, it is customary to measure enzyme activities under optimal conditions, and inhibitory or stimulatory factors could be removed during the preparation of the hearts before measurements are made. This method could not be used for the study of enzyme activities during ischaemia as the reintroduction of oxygen causes as many biochemical changes as ischaemia itself. Whether similar problems could arise on re-oxygenation after hypoxia remains to be seen.

Clearly a combination of experiments are needed to gain accurate information about the origin of the glycerol released from the heart during hypoxia.

**Figure 7.1. Myocardial metabolism during hypoxia.**



★ Possible sources of glycerol  
 Flux through the pathways is indicated by the width of the arrows.

#### 7.4. Conclusion.

The major findings of this thesis are as follows:

- 1) The operation of the TAG-NEFA cycle in the isolated, perfused rat heart during hypoxia does not cause a major depletion of ATP supplies.
- 2) Myocardial TAG concentrations have little influence on the amount of energy utilized in the operation of the TAG-NEFA cycle during hypoxia.
- 3) Glycerol may be reutilized in the heart by the action of glycerokinase.
- 4) The majority of the glycerol release during hypoxia is mediated by non-adrenergic mechanisms and does not necessarily originate from TAG.

Thus, the results from this thesis suggest that rather than being detrimental to the heart, the TAG-NEFA cycle may play an important role in minimizing build up of NEFA concentrations during oxygen deprivation. Increased concentrations of NEFA and their metabolites may be harmful to the heart during oxygen deprivation (Kurien and Oliver 1970). Thus, contrary to its operation being harmful to the oxygen-deprived myocardium, the TAG-NEFA cycle may provide some protection against arrhythmias. Lipolysis in the heart during hypoxia is mediated by different mechanisms than in adipose tissue, and myocardial glycerol release should no longer be used as a measure of hypoxia-stimulated lipolysis. Glycolysis and lipolysis in the heart appear to be closely linked, glycolysis providing  $\alpha$ -glycerophosphate for re-esterification of NEFA. More work is needed to elucidate the mechanisms involved in stimulation of glycolysis and lipolysis and their interaction during hypoxia, and also to examine the extent and role of TAG-NEFA cycling *in vivo* when the heart is subjected to exogenous neural and hormonal influences.

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