GLUCOSE VERSUS ACETATE AS SUBSTRATE DURING SUBTOTAL ISCHAEMIA

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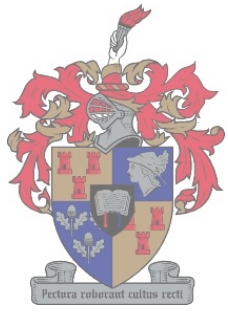

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Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Abstract

Background: The provision of glucose (Glu) is beneficial during ischaemia (Opie, 1970). Part of Glu protection may be due to Glu oxidation, specifically the Krebs Cycle and oxidative phosphorylation (Lopaschuk, 1998). Therefore, 30 mM acetate (Ac) may hypothetically provide protection similar to 10 mM Glu providing that there is residual oxygen available to allow functioning of the Krebs Cycle and oxidative phosphorylation. **Aim:** The aim of this study was to establish an optimal Ac concentration at which the consequences of ischaemia were minimal, to determine whether the protective effects of Glu and Ac is oxygen dependent, and to investigate whether equi-carbon concentrations of Ac and Glu would offer similar protection during low flow ischaemia. **Materials and Methods:** Isolated rat hearts (n = 6-8) were perfused (Langendorff, 95% 0_2, 5% $C0_2$) with 5 mM Ac for 30 minutes, followed by 2 ml/min low flow ischaemia with 1 mM or 5 mM or 10 mM or 30 mM Ac or 10 mM Glu. Additional hearts were subjected to anoxia during the same period. All hearts were reperfused for 30 minutes under the pre-ischaemic conditions. We measured the time to the onset of ischaemic contracture (TOIC), the percentage recovery of left ventricular developed pressure (LVDP), and tissue ATP, creatine phosphate, lactate, glycogen and cAMP content. **Results:** 5 mM and 10 mM Ac perfused hearts yielded optimal yet similar protection against the consequences of ischaemia. In addition, Ac (5 mM) and Glu (10 mM) treated hearts performed equally poorly in the absence of oxygen. This implies that the beneficial effects conferred by Glu is oxygen dependent, due possibly to the activity of the Krebs Cycle and oxidative phosphorylation. An equi-carbon concentration of Ac (30 mM) could not offer protection analogous to 10 mM Glu during oxygenated, low flow ischaemic conditions. Instead, 10 mM Glu provided optimal protection against

ischaemia while 30 mM Ac was associated with accelerated TOIC, reduced ATP and glycogen levels, elevated lactate and cAMP levels, and weakened function upon reperfusion. **Conclusion:** Glu provided maximal protection against the consequences of low flow ischaemia. However, this protection was dependent on the availability of oxygen, suggesting that oxidative phosphorylation and Krebs Cycle activity may contribute more to the protective effects of a particular substrate than previously anticipated.

Uittreksel

Agtergrond: Voorsiening van glukose (Glu) gedurende iskemie is voordelig (Opie, 1970). Glu beskerming mag gedeeltelik te danke wees aan Glu oksidasie, spesifiek oksidatiewe fosforilering en die Krebs siklus (Lopaschuk, 1998). Dus mag 30 mM asetaat (Ac) beskerming soortgelyk aan dié van 10 mM Glu verleen, indien daar genoeg suurstof beskikbaar is om funksionering van die Krebs Siklus en oksidatiewe fosforilering toe te laat. **Doel:** Die doel van hierdie studie was om 'n optimale Ac konsentrasie vas te stel waar die gevolge van iskemie minimaal is, om te bepaal of die beskermingseffekte van Glu en Ac suurstof afhanklik is, en om te ondersoek of ewe-koolstofkonsentrasies van Glu en Ac soortgelyke beskerming gedurende lae vloei iskemie sal bied. **Metodes:** Geïsoleerde rotharte (n = 6-8) is vir 30 minute met 5 mM Ac geperfuseer (Langendorff, 95 % O_2, 5 % CO_2), gevolg deur 2 ml/min lae vloei iskemie met óf 1mM, 5 mM, 10 mM, of 30 mM Ac, óf 10 mM Glu. Addisionele harte is tydens dieselfde periode aan anoksie blootgestel. Alle harte is vir 30 minute geherperfuseer onder die pre-iskemiese toestande. Ons het die tydsduur tot die begin van iskemiese kontraksie (TOIC), die persentasie wat die linkerventrikulêre ontwikkelde druk herstel (LVDP), en die weefsel ATP, kreatienfosfaat, laktaat, glikogeen en cAMP inhoud gemeet. **Resultate:** 5 mM en 10 mM Ac het optimale, maar soortgelyke beskerming teen die gevolge van iskemie gebied. Ac (5 mM) en Glu (10 mM) behandelde harte het, in die afwesigheid van suurstof, ewe swak resultate getoon. Dit impliseer dat die voordelige effekte verleen deur Glu suurstof afhanklik is en moontlik te danke is aan die aktiwiteit van die Krebs siklus en oksidatiewe fosforilering. Ewe-koolstofkonsentrasies van Ac (30 mM) kon nie soortgelyke beskerming as 10 mM Glu gedurende lae vloei iskemie bied nie. Terwyl Glu optimale beskerming teen iskemie gebied het, is Ac geassosieer met vervroegde TOIC,

verlaagde ATP- en glikogeenvlakke, verhoogde laktaat- en cAMP-vlakke en swak herstel van funksie tydens herperfusie. **Gevolgtrekking:** Glu het maksimale beskerming teen die gevolge van lae vloei iskemie gebied. Díe beskerming is egter suurstof afhanklik wat aandui dat oxidatiewe fosforilasie en die Krebs Siklus 'n groter bydra het tot die beskermende effekte van 'n spesifieke substraat as wat voorheen verwag is.

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Research Output

Results of this study have been presented at the following meetings:

- **16th World Congress of the International Society for Heart Research (1998), May 27-31, Rhodes Island, Greece.** Van Rooyen J, Segadavan A, McCarthy J, and Podzuweit T: Superiority of glycolysis as a source of ATP during low flow ischaemia.
- **26th Annual Congress of the Physiological Society of Southern Africa (1998), September 20–23, Rustenberg**. Segadavan, A., Podzuweit, T., Thomas, S., van Rooyen, J.: Glucose versus acetate as substrate during subtotal ischaemia.

Published proceedings of the International Society for Heart Research Meeting

- **Van Rooyen J, Segadavan A, McCarthy J, Podzuweit T** Superiority of glycolysis as a source of ATP during low flow ischaemia. **Journal of Molecular and Cellular Cardiology**, 1998:30:629

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Abbreviations

Ac	acetate
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
Ca^{++}	calcium ion
cAMP	cyclic adenosine monophosphate
CF	coronary flow
CHD	coronary heart disease
CP	creatine phosphate
DAG	diacylglycerol
ECG	electrocardiogram
ETOH	ethanol
FA	fatty acids
FAD(H_2)	flavin adenine dinucleotide (reduced)
G3PDH	glyceraldehyde-3-phosphate dehydrogenase
GIK	glucose-insulin-potassium
Glu	glucose
H^+	proton
HK	hexokinase

HR	heart rate
IC	ischaemic contracture
IHD	ischaemic heart disease
IP_3	inositol triphosphate
K^+	potassium ion
K_{ATP}	ATP sensitive potassium channel
LVDP	left ventricular developed pressure
Mg^{++}	magnesium ion
Na^+	sodium ion
Na^+/ Ca^{++}	sodium-calcium exchanger
Na^+/ K^+ATPase	ATP dependent sodium-potassium pump
Na^+/H^+	sodium-proton exchanger
NAD(H)	nicotinamide adenine dinucleotide (reduced)
PCA	perchloric acid
PDH	pyruvate dehydrogenase complex
PFK	phosphofructokinase
RMP	resting membrane potential
SR	sarcoplasmic reticulum
TOIC	time to the onset of ischaemic contracture

Chapter 1

INTRODUCTION

Since the introduction of the Glucose Hypothesis by Opie (1970), it is generally accepted that glucose has a protective effect on the ischaemic myocardium (Opie,1970; Apstein et al., 1983; Opie, 1989; Owen et al., 1990; Oliver and Opie, 1994)). Possible beneficial actions of glucose include decreased incidence of ischaemic contracture (Owen et al., 1990), reperfusion induced arrhythymias (Bernier and Hearse, 1988), and stunning (Ferrari et al., 1983). These protective effects may be mediated by increased production of glycolytic adenosine triphosphate (ATP) (Owen et al., 1990) which may, in turn, improve ion homeostasis through better control of the sarcoplasmic reticulum (SR) calcium pump, as well as other membrane pumps. If glycolytic ATP is responsible for the beneficial actions of glucose, glucose protection may be independent of oxygen availability since glycolysis does not require oxygen. The protection offered by glucose during low flow ischaemia should then be similar during anoxia.

Alternatively, the protection conferred by glucose on the ischaemic heart may not be due solely to glycolysis (Lopaschuck, 1988). Under normoxic conditions, glucose yields more ATP through the Krebs Cycle and oxidative phosphorylation combined (34 ATP), than during glycolysis (2 ATP). Therefore, part of glucose protection may be due to increased ATP production by way of the former processes. If this is true, acetate, (a two carbon molecule) which is incorporated into the Krebs Cycle as acetyl coenzyme A (CoA), may offer protection similar to glucose (a six carbon molecule). This is provided

that the same number of carbon atoms are oxidised to carbon dioxide (CO_2) and there is sufficient oxygen to allow functioning of the Krebs Cycle and oxidative phosphorylation.

Therefore, the aim of this study was:

a) To establish an optimal acetate concentration which best protects the isolated rat heart against the consequences of low flow ischaemia

b) To determine whether the availability of oxygen during low flow ischaemia influences the protective effect of glucose and acetate

c) To ascertain whether equi-carbon concentrations of acetate and glucose offers similar protection during subtotal ischaemia.

Chapter 2

LITERATURE REVIEW

2.1 Ischaemia

The word ischaemia is derived from the Greek words, "τσχειυ" meaning "to restrain" and "αιμα" meaning "blood." There has however been much debate concerning the actual definition of myocardial ischaemia (Hearse, 1994). According to Opie (1987), it is simply defined as "a reversible oxygen deficit in the heart due to insufficient perfusion of the coronary arteries." Factors causing this myocardial oxygen imbalance include those which raise the *oxygen demand* such as tachycardia, wall stress, contractility, and metabolically driven "oxygen wastage". In contrast, coronary artery narrowing due to atheroma or spasm or both would decrease the *oxygen supply* (Opie, 1989). Ischaemic heart disease (IHD), coronary heart disease (CHD) and atherosclerosis all refer to clinical syndromes that may reduce blood flow to a region of the heart, thus disturbing the oxygen balance in the myocardium (Glass, 1977 p. 2; Katz, 1977 p. 419; Sharkey, 1979 p. 180). If prolonged, ischaemia may develop into myocardial infarction, that is, necrosis (cell death) of the myocardium.

Various experimental models exist for the investigation of myocardial ischaemia. These include regional ischaemia which is the result of coronary artery occlusion. In surgical ischaemia on the other hand, the entire heart is subjected to global ischaemia. During low flow ischaemia the coronary flow has simply been reduced, whereas in total ischaemia the coronary flow has been completely restricted. In practise however,

coronary flow is seldom totally arrested due to the presence of collateral flow in the ischaemic area. Growth of these collaterals is encouraged by the oxygen deficient heart in order to compensate for the oxygen imbalance. They occur on the epicardial surface, and are found in patients with IHD (Opie, 1987). The presence of collateral flow can prolong the time to necrosis. This, however depends on the degree of collateral blood flow present. The guinea pig, for example, has excellent collaterals, whereas rats have none (Opie, 1987). Therefore it is believed that extensive pre-existing collateral circulation can protect the ischaemic zone (Schaper, 1984).

2.2 Myocardial metabolism

The metabolism of the ischaemic myocardium is characterised by an ATP shortage as well as inhibition of glycolytic enzymes by the accumulation of lactate. This contributes further to the ATP deficiency (Opie,1988). Other detrimental changes include inadequate functioning of membrane pumps (Opie, 1990), potassium (K^+) loss in the ischaemic zone (Opie, 1970; Weiss and Lamp, 1987), sarcolemmal damage, intracellular acidosis (Opie, 1975; Williamson et al., 1976), accumulation of cyclic adenosine monophosphate (cAMP) (Wollenberger et al., 1969), and deficient Ca^{++} homeostasis (Henry et al., 1977). All of these factors contribute to ventricular arrhythmias and an eventual decline in myocardial contractility (i.e. pump failure). This may lead to acute heart failure (sudden death) where death occurs soon after coronary flow is reduced. Alternatively, the loss of functional myocardial tissue may initially be tolerated by the rest of the heart with death occurring after a prolonged period. This is termed as chronic heart failure (Katz, 1977 p.421).

The early consequences of ischaemia are potentially reversible as there is no detectable membrane damage associated with the initial K^+ loss (Opie, 1987). Late ischaemic K^+ loss is due to severe ATP depletion and inhibition of the sodium pump (Opie, 1989). Four main factors indicate irreversible ischaemic damage: 1) critical ATP loss 2) membrane damage 3) mechanical effects such as cell swelling and membrane rupture as a result of elevated osmotic pressure and finally 4) calcium overload (Opie, 1984).

2.3 Energy Bearing Substrates

Cardiac muscle utilises various metabolic fuels including fatty acids, glucose, lactate and ketone bodies (Opie, 1968; Neely et al., 1974). The rate of utilisation of these substrates depends on:

a) the substrate concentration in the plasma

b) the availability of alternate competitive substrates

c) oxygen delivery to the myocardium

d) mechanical activity of the heart and

e) plasma levels of regulating hormones (Neely et al., 1972).

The myocardium depends on aerobic metabolism. The preferred substrate is fatty acids which fulfil 60%-90% of myocardial energy requirements (Braunwald, 1992 p.1183). These fatty acids are metabolised through ß-oxidation and the Krebs/ Tricarboxylic Acid Cycle (Neely et al., 1972). Glycolysis and glucose oxidation are inhibited by high levels of fatty acids (Neely and Morgan, 1974) as well as the metabolism of ketone bodies and lactate (Opie, 1990).

During ischaemia there is a shift from aerobic to anaerobic metabolism (Oliver & Opie, 1994). An oxygen deficiency prevents the oxidation of non-glucose substrates such as free fatty acids (FFA) and lactate. Glucose and glycogen now become the primary substrates while glycolysis and glucose oxidation are enhanced (Opie, 1990). Therefore the provision of appropriate energy-giving substrates, such as glucose, may protect against ischaemic injury (Oliver & Opie, 1994).

2.3.1 Glucose

As postulated by Opie (1970), glucose may be good for the survival of the ischaemic myocardium. Indices of protection include a reduction of reperfusion-induced arrhythmias (Bernier and Hearse, 1988), the attenuation of stunning (Ferrari et al., 1983), and a decrease in the occurrence of ischaemic contracture (Owen et al., 1990). The mechanism underlying the protection conferred may be attributed to increased production of glycolytic ATP (Owen et al., 1990) and the resultant improvement in cytosolic Ca^{++} homeostasis (Opie, 1989), the inhibition of the ATP-sensitive K^{+} channels (K_{ATP}) (Weiss & Lamp, 1987), and lowering of cAMP levels in the ischaemic zone (Podzuweit et al., 1993). Furthermore, De Leiris et al. (1975) found increased enzyme release in hearts perfused with FFA in contrast to lower enzyme release in glucose perfused hearts.

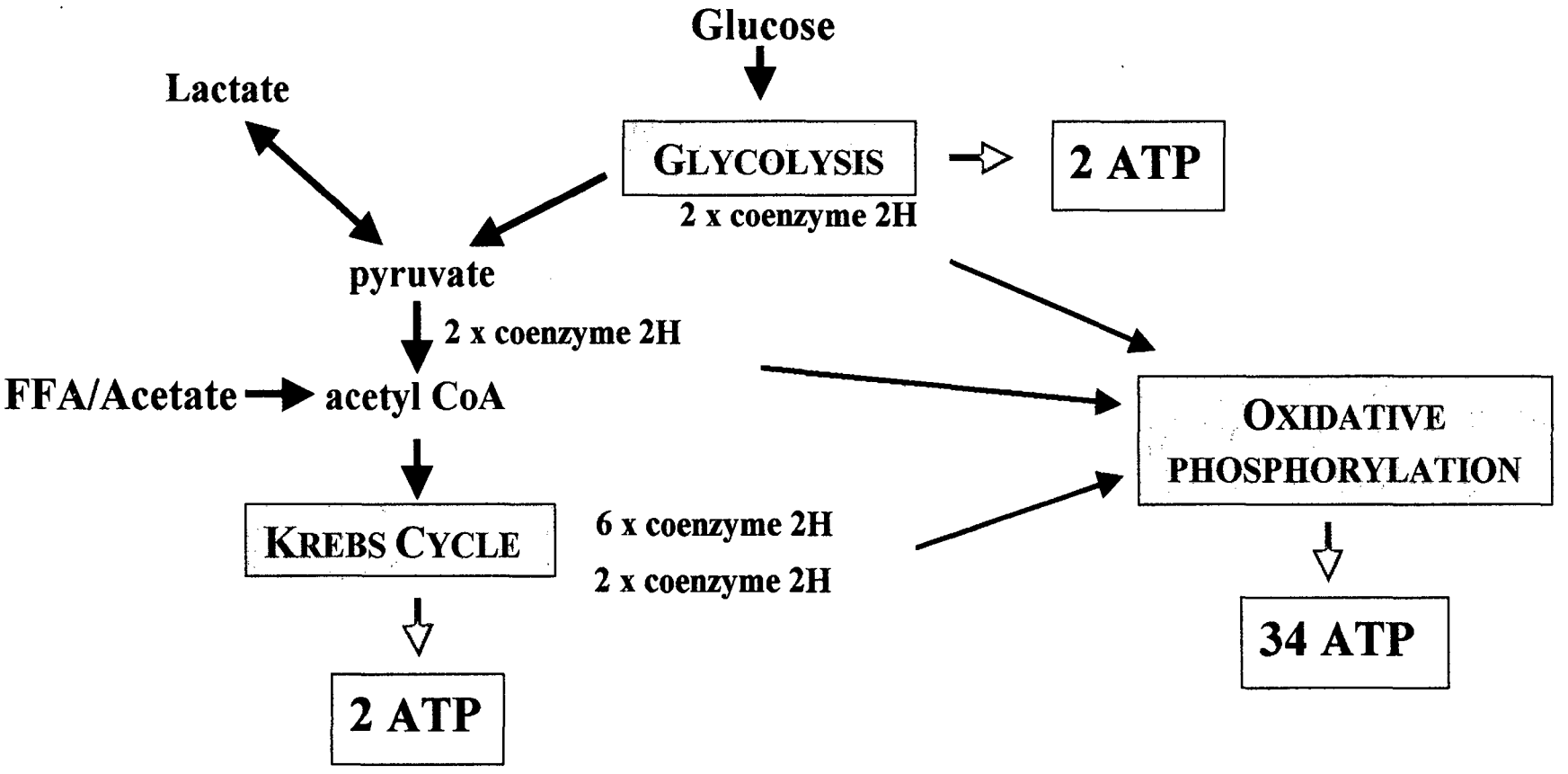


Figure 2.1. Energy Producing Pathways. Complete oxidation of glucose to CO_2 yields more ATP through oxidative phosphorylation than via glycolysis and the Krebs Cycle. Glucose oxidation through glycolysis yields 2 pyruvate, 2 ATP molecules and 2 coenzyme 2H. Pyruvate is converted to acetyl CoA and in the process another 2 coenzyme 2H is produced. Acetyl CoA is incorporated into the Krebs Cycle which then yields 2 ATP and 8 coenzyme 2H. Coenzyme 2H renders a total of 34 ATP via oxidative phosphorylation. Acetate and FFA are introduced into this system as acetyl CoA.

Normally, fatty acids are preferentially oxidised in the perfused rat heart (Shipp et al., 1961). However, an oxygen deficiency prevents oxidation of these non-glucose substrates. Instead glucose uptake and glycolysis is enhanced (Opie, 1990) and glucose becomes the main source of energy.

To maximise glucose uptake into the cardiocytes there must be adequate glucose delivery by the coronary blood flow and efficient transport of glucose from the blood to the interstitial space and finally into the cytosol (Achs and Garfinkel, 1977). Anoxia, where the oxygen tension is reduced, encourages glucose transport into the cell (Morgan et al., 1961). Similarly, moderate ischaemia, during which oxygen deprivation is due to limited coronary flow, also enhances glucose uptake (Rovetto et al., 1973). During severe ischaemia, where the coronary flow is drastically decreased, glucose uptake is reduced since its delivery to the myocardium is restricted (Achs and Garfinkel, 1977; Rovetto et al., 1973). Besides the coronary flow rate, α- and ß-adrenergic receptors may also play a role in the control of glucose uptake through a Ca^{++} dependent control mechanism (Clark and Patten, 1984).

While anoxia accelerates glucose transport, the rate of anaerobic glycolysis is enhanced by reduced inhibition of glycolytic enzymes. Free fatty acids and high energy phosphate compounds within the cell are responsible for this enzymatic inhibition (Opie, 1975). In moderate ischaemia, the majority of glucose enters the Krebs Cycle to produce energy via oxidative metabolism. Severe ischaemia limits flux through the Krebs Cycle and lactic acid accumulates within the myocardium (Rovetto et al., 1973). Together with protons, lactate inhibits glycolytic enzymes such as phosphofructokinase (PFK),

hexokinase (HK), phosphorylase kinase C, and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) (Figure 2.2) (Neely et al., 1975). Thus during ischaemia, glycolytic flux is regulated by both the rate of glucose delivery and the severity of enzymatic inhibition. As the coronary flow is reduced, the influence of metabolic inhibition becomes increasingly important with metabolite accumulation limiting glycolytic flux at zero coronary flow (King et al., 1995). This may explain why glycolytic flux is enhanced during mild ischaemia, and inhibited during severe ischaemia (Opie, 1990).

Ischaemic myocardial metabolism may be improved by the promotion of glycolytic flux (Opie, 1989). Glycolysis can be enhanced by administration of glucose, insulin and potassium (GIK) (Broderick et al., 1992). The administration of insulin accelerates glucose uptake (Morgan et al., 1961; Van Rooyen et al., 1998), while the provision of glucose increases the anaerobic glycolysis rate, lowers circulating FFA levels (Grayson and Irvine, 1968), and alters the extracellular volume (Opie, 1970). However, while glucose uptake and enzymatic regulation serve to influence glycolysis, the actual rate of ATP production may determine the degree of protection offered by glucose.

Inhibition of glycolytic flux decreases the amount of ATP available which is required to control ion gradients (Opie, 1988). The uptake of Ca^{++} into the SR occurs by means of an ATP-dependent pump. Glycolytic ATP maintains this pump and when glycolysis is suppressed, Ca^{++} may accumulate intracellularly leading to the development of ischaemic contracture, a form of ischaemic injury (Opie, 1987). Therefore glycolysis has a protective effect in the ischaemic and reperfusion period via better control of Ca^{++}

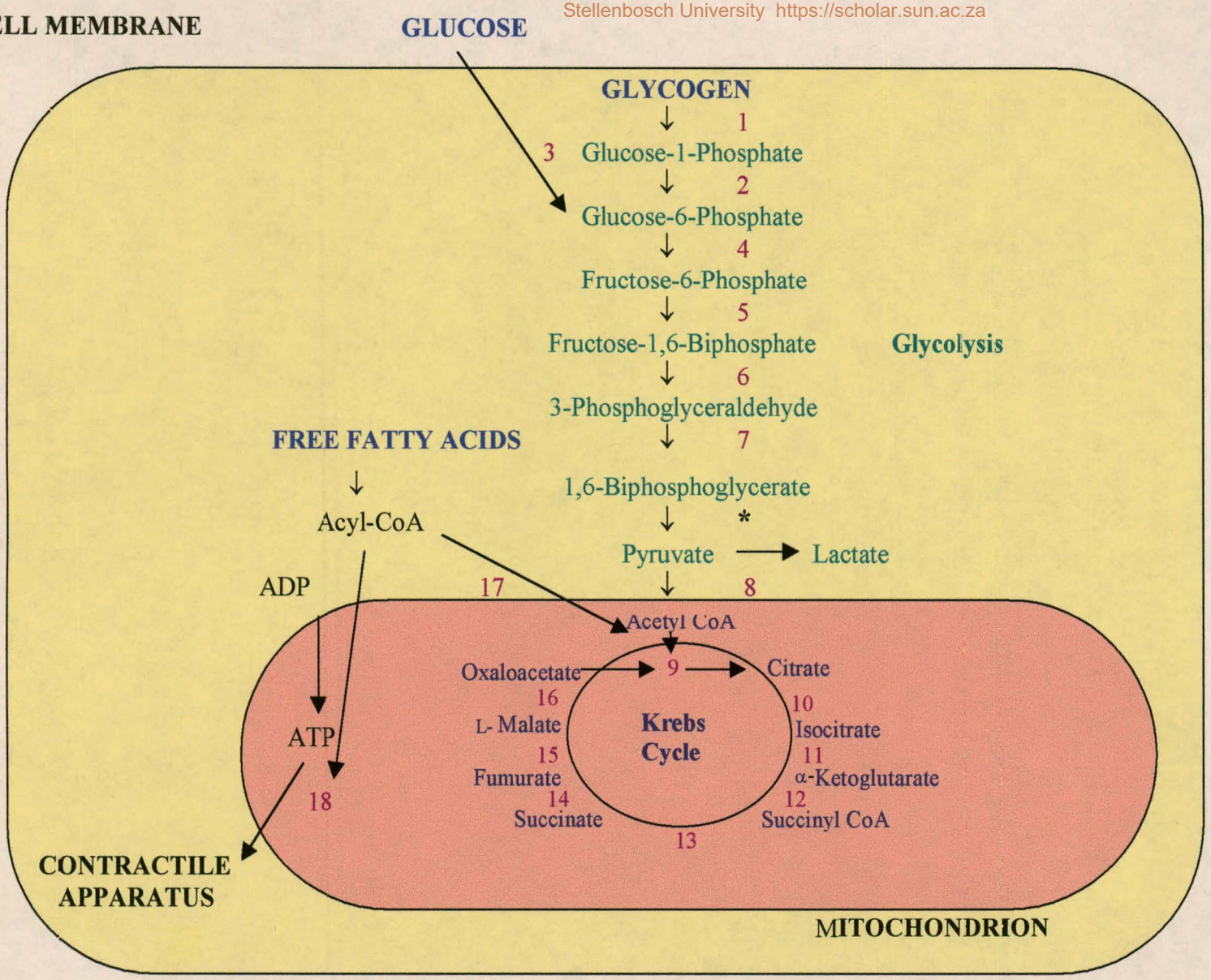
homeostasis (Owen et al., 1990). Glycolysis may, through the production of ATP, also play a role in the movement of K^+ across the cell membrane. ATP is required to prevent opening of the ATP-sensitive potassium (K_{ATP}) channels and ensure functioning of the Na^+/K^+ ATPase. Inhibition of glycolysis and ATP synthesis may lead to K^+ loss. Weiss and Lamp (1987) showed similar findings when glycolysis was limited by the absence of glucose in the perfusate. They found a marked K^+ loss from the cell as a result of reduced inhibition of the ATP-sensitive K^+ channels when glucose was replaced by pyruvate or acetate as myocardial substrates.

Despite evidence for a protective role of glucose, the effect of glycogen during ischaemia is controversial (Neely and Grotyohann 1984; Goodwin and Taegtmeyer, 1994). Glycogenolysis is both a source of glycolytic ATP as well as lactate and protons. Glycolytic ATP maintains ion gradients (Opie, 1988), while lactate and protons contribute to ischaemic injury by inhibiting glycolysis and inducing acidosis (Neely et al., 1973). Thus the effects of glycogen during ischaemia may depend on whether its beneficial qualities outweigh the detrimental consequences (Cross et al., 1996). Using a total, global ischaemic model, Neely and co-workers (1984) found that high pre-ischaemic glycogen levels had a deleterious effect on functional recovery. However this may have been due to reduced washout of lactate and protons which then accumulate and inhibit glycolysis. In contrast, Cross et al. (1996) observed that high pre-ischaemic glycogen levels in a low flow model delayed the onset of ischaemic contracture, but was also associated with poor recoveries. If however these hearts were reperfused prior to the onset of IC, recoveries improved to 89%. This led to the proposal that in the absence of glucose during ischaemia, depletion of glycogen would lead to reduced

glycolytic activity and subsequent accumulation of protons. On reperfusion, this build-up of protons increases sodium (Na^+)/hydrogen (H^+) exchange which provokes poor recoveries of function. Thus the effect of high tissue glycogen during ischaemia may depend on the degree of ischaemic coronary flow which ensures washout of harmful metabolites as well as the extent of ischaemic glycogen depletion.

2.3.2 Fatty Acids (FA)

Fatty acids (FA) are the preferred metabolic fuel in the perfused rat heart (Shipp et al., 1961). Intracellular FA must be transported into the mitochondria before it can be metabolised. This process involves three stages. In the first stage the FA undergoes esterification to yield fatty acyl-CoA. The acyl group is then transferred to the carrier molecule, carnitine, and transported across the mitochondrial membrane as fatty acyl carnitine. Once in the mitochondrion, the acyl group is transferred to intramitochondrial CoA. The acyl CoA then undergoes β-oxidation where acetyl CoA units are removed from the fatty acid and finally incorporated into the Krebs Cycle (Neely and Morgan, 1974). Acetate, a fatty acid homologue without detergent properties (Bricknell and Opie, 1978) is a two carbon compound which does not undergo β-oxidation. Instead, it supplies acetyl-CoA directly to the Krebs Cycle (Mathews and van Holde, 1990 p.491-493). This increased production of acetyl CoA accompanying fatty acid oxidation inhibits pyruvate dehydrogenase complex (Figure 2.2), thus limiting carbohydrate entry into the Krebs Cycle (Braunwald, 1992 p.1183).



1. phosphorylase kinase
2. phosphoglucomutase
3. hexokinase
4. phosphohexoisomerase
5. phosphofructokinase
6. aldolase
7. glyceraldehyde-3-phosphate dehydogenase
8. pyruvate dehydrogenase complex
9. citrate synthase
10. aconitase
11. isocitrate dehydrogenase
12. α-keto glutarate dehydrgenase complex
13. succinyl CoA synthetase
14. succinate dehydrogenase
15. fumarase
16. malate dehydrogenase
17. carnitine acyltransferase
18. adenine nucleotide translocasetranslocase

Figure 2.2 Glycolysis and the Krebs Cycle. * denotes where the glycolytic pathway has been condensed. (Adapted from Braunwald, 1992 p.1183)

Increased catecholamine stimulation experienced during ischaemia, contributes to the intracellular accumulation of FA via the breakdown of triglycerides (Opie, 1968). These high levels of FA are detrimental to the ischaemic myocardium (Opie, 1975). Oxidation of FA during ischaemia is impeded by the O_2 shortage (Neely and Morgan, 1974), and if ß-oxidation is inhibited, unoxidised products such as acyl CoA and acyl carnitine accumulate intracellularly. These products may have deleterious effects in the myocardium.

Mechanisms involved in fatty acid toxicity include acylcarnitine induced inhibition of the SR Ca^{++} pump, inhibition of the sarcolemmal Na^{+}/Ca^{++} exchanger and Na^{+}-K^{+} pump, as well as activation of the Ca^{++} channels. These actions could promote intracellular Ca^{++} overload (Oliver & Opie, 1994). Furthermore, accumulation of acyl-CoA inhibits ATP transfer from the mitochondria to the cytoplasm (De Leiris et al., 1975) (Figure 2.2). ATP produced in the mitochondrion is therefore inaccesible to the contractile apparatus and the sarcolemmal membrane pumps. Besides hindering ATP production (Opie, 1975), accumulation of tissue FA contributes to K^{+} loss by opening K_{ATP} channels (Kim and Duff, 1990), depresses contractility (Davies et al., 1992) and are arrhythmogenic in isolated rat hearts (Makiguchi et al., 1991). Since an intracellular ATP deficiency promotes the release of enzymes (Wilkinson and Robinson, 1974), increased provision of FA could indirectly accelerate enzyme release from infarcting tissue. The magnitude of enzyme release has been associated with the ultimate extent of heart cell necrosis (De Leiris et al., 1975).

2.4 Messengers during ischaemia

The function of all cells is controlled by signalling systems which translate external information into internal signals (Berridge and Irvine, 1984). The first step in this pathway is the binding of the chemical messenger to the receptor. The myocardium possesses α- and β-adrenergic receptors, each activating different signal transduction pathways. The β-adrenergic system activates adenylate cyclase to generate cAMP as second messenger (Sutherland et al., 1968; Schomig et al., 1990 p.396). The α-adrenergic system generates inositol trisphophate and diacylglycerol as second messengers. These signalling systems regulate intracellular Ca^{++}, which coincidentally rises during ischaemia, therefore implicating the involvement of these systems in ischaemic injury.

2.4.1 Inositol 1, 4, 5-trisphosphate (IP_3)

When stimulated, the α-adrenergic receptor couples to a G-protein (Piper, p.396 1990; Vander et al., 1990 p.160). The G-protein has α-, β- and ϑ-subunits. Once coupled to the receptor, the α-subunit separates from the βϑ - subunit and appears to activate phospholipase C (Berridge, 1984). Phospholipase C catalyses the breakdown of phosphatidylinositol 4,5-biphosphate (PIP_2), an inositol lipid located on the inner leaflet of the plasma membrane (Berridge and Irvine, 1984). As a result two second messengers, diacylglycerol (DAG) and inositol 1,4,5 - trisphosphate (IP_3) are formed (Berridge, 1984). These messengers are produced rapidly, they function in a synergistic manner and act at very low concentrations. Levels of these signals depend on their rate of formation and degradation. Inositol trisphosphate is degraded through its conversion to IP_2 by inositol triphosphatase while DAG is either phosphorylated to phosphatidic acid

by diacyl glycerol kinase, or deacylated to monoacylglycerol by diacyl glycerol lipase (Berridge, 1984).

DAG and IP_3 function as second messengers to activate two independent, but parallel signalling pathways. DAG remains in the membrane and activates protein kinase C (Berridge, 1984). Protein kinase C may act on numerous proteins such as the Ca^{++} ATPase, Na^+-K^+ ATPase, Na^+/H^+ exchanger, the glucose transporter, troponin T and I, glycogen phosphorylase kinase and phosphofructokinase. Protein kinase C has also been implicated in the regulation of contraction by phosphorylation of substrates and modulation of Ca^{++} and other ion levels with inotropic and chronotropic effects.

In contrast, IP_3 mobilises Ca^{++} from intracellular stores, particularly the SR (Berridge & Irvine, 1984). The Ca^{++} releasing property of IP_3 was first demonstrated in a preparation of rat pancreatic acinar cells (Streb et al., 1983). This observation has been verified in a number of cell types including cardiac microsomes (Hirata et al., 1984). It is not certain how IP_3 increases internal Ca^{++}. It occurs either by acting on internal receptors to stimulate release, or inhibiting uptake mechanisms responsible for the removal of intracellular Ca^{++} into the SR (Berridge, 1984). IP_3 levels decrease during ischaemia, but rise rapidly during reperfusion and may play a role in the harmful accumulation of Ca^{++}.

2.4.2 Cyclic Adenosine 3,5 – monophosphate (cAMP)

The β-adrenergic system activates adenylate cyclase to generate cAMP as a second messenger (Sutherland et al., 1968; Schomig et al., 1990). G-proteins also play a role in

the formation of cAMP. If stimulated, the G-protein (G_s) couples adenylate cyclase to β_1 and β_2 adrenoreceptors as well as other stimulatory receptors such as histamine and prostacyclin receptors. If inhibited, the protein (G_i) couples the cyclase to inhibitory receptors such as A_1 adenosine receptors and M_2 cholinergic receptors (Opie, 1992). Increased cAMP levels occur due to greater formation from ATP (Wollenberger et al., 1969), or reduced removal/hydrolysis to 5-AMP by phosphodiesterase (PDE), or a combination of the two (Sutherland et al., 1968). According to Podzuweit et al. (1996), the accumulation of cAMP in the ischaemic heart is partly due to metabolic activation of adenylyl cyclase and or inhibition of phosphodiesterase activity.

Cyclic AMP plays a role in the myocardium's positive inotropic response to catecholamines (Sutherland et al., 1968). It activates protein kinase A which then phosphorylates various proteins such as the sarcolemmal Ca^{++}-channels and phospholamban. Phosphorylation of the voltage-gated Ca^{++}-channels in the cell membrane opens these channels. The resultant influx of Ca^{++} triggers additional Ca^{++} release from the SR via a process known as calcium induced calcium release. This increased cytosolic Ca^{++} level ultimately contributes to a stronger contraction. Phosphorylation of phospholamban, a regulatory protein on the SR, leads to enhanced activity of the Ca^{++} -ATPase which is responsible for transporting Ca^{++} back to the SR. (Katz, 1988). Therefore cAMP also ensures that Ca^{++} is returned to the SR more rapidly thereby shortening the duration of the myocardial contraction.

The rise of cAMP in the ischaemic zone has an arrhythmogenic effect (Podzuweit et al., 1976). The cAMP hypothesis postulates that cAMP plays an important role in the

mediation of adrenergic effects on the heart's vulnerability to fibrillation. Elevated cAMP levels increases intracellular Ca^{++} which then acts as a third messenger (Podzuweit et al.,1976) and promotes delayed after depolarisations (Coetzee et al., 1987). It has been proposed that β-blocking agents (e.g. propranolol) can reverse the arrhythmogenic effects by inhibiting the binding of catecholamines to the β-receptors (Katz, 1977). Beta- blockade would limit an increase in cAMP levels (Wollenberger et al., 1969), reduce cytosolic Ca^{++} overload and help prevent ventricular fibrillation in the early stages of acute myocardial infarction (Lubbe et al., 1992).

Evidence exists against the theory of the relation between tissue cyclic AMP levels and ventricular fibrillation. Metoprolol, a ß-adrenergic blocker, failed to reduce the cAMP levels in the ischaemic myocardium (Muller et al., 1986), while forskolin induced increases in cAMP levels in isolated rat hearts which were not accompanied by ventricular fibrillation (Manning et al., 1985). Muller et al.(1986) tested the relationship between the effects of ß-blockade on ventricular fibrillation and tissue metabolic changes in the pig model. They found an initial rise and subsequent fall in cAMP levels and suggested that elevated cAMP could only be partially blocked by ß-blockade possibly due to the inhibition of phosphodiesterase by intracellular acidosis. The subsequent reduction in cAMP may be due to downgrading or "switching off" of the ß-receptors or links in the receptor-cyclase-cAMP chain.

High cAMP levels are also associated with the regulation of energy generating pathways. In cardiac muscle, glycogen turnover may be controlled by intracellular levels of cAMP (Neely and Morgan, 1974). It catalyses the phosphorylation of phosphorylase *b* to *a*,

thereby facilitating the conversion of glycogen to glucose-1-phosphate. Under energy-deprived conditions, 5'-AMP may accumulate and also stimulate glycogenolysis by activating phosphorylase *b*. cAMP inhibits glycogen synthesis (Mathews and van Holde, 1990 p.464) and enhances glycolysis by stimulating PFK activity (Katz, 1977 p.421). Together with adenosine, cAMP also inhibits fatty acid metabolism in oxygen deficient hearts. This may have an energy sparing effect by limiting the undesirable accumulation of long chain acyl-CoA derivatives (Neely and Morgan, 1974).

2.5 Metabolic consequences of myocardial ischaemia

2.5.1 Adenosine triphosphate (ATP)

ATP is essential for most cellular processes. It is a source of energy for muscle contraction and the specific transport of substances across the cell membrane (Mathews and Von Holde 1990 p.83). It plays an integral role in the maintenance of ATP-dependent pumps such as the K^+- channel and the Ca^{++}- channel and is utilised to form cAMP. Furthermore, ATP may act as a vasodilator, either directly or by conversion to adenosine (Kirsch et al., 1990; Ferrari and Opie, 1992). Thus, the lack of energy during an oxygen deficiency may be the main cause of all functional and structural changes (Spiekermann, 1990).

The initial reduction in work output during ischaemia occurs without any change in cellular ATP (Hoerter et al., 1988). Only after the myocardial O_2 pressure falls to low critical values, does breakdown of high energy phosphates occur. This initially includes mainly creatine phosphate (CP) and a little ATP. Anaerobic glycolysis is now stimulated

(Spiekermann, 1990) by the products of ATP degradation, ADP and Pi (Rovetto et al., 1973). ATP usually inhibits the activity of citrate synthase, phosphorylase and PFK (Figure 2.2). Therefore an ATP shortage, as well as increased formation of intracellular activators such as AMP, serve to enhance glycolytic flux (Neely & Morgan, 1974).

Mild ischaemia accelerates glycolytic flux leading to increased ATP production (Ferrari and Opie, 1992). During severe ischaemia however, carbohydrate flux through the Krebs Cycle is inhibited by the oxygen shortage and results in lactate accumulation (Rovetto et al., 1973). The extent of lactate accumulation depends on the severity of coronary flow restriction and thus washout of metabolites. Together with protons, lactate hinders glycolysis by inhibiting glycolytic enzymes such as PFK, HK, and G3PDH (Neely et al., 1975). Furthermore, lactate formation results in acyl CoA accumulation which in turn inhibits adenine nucleotide translocase. The latter is responsible for transporting ADP from the cytoplasm to the mitochondrion and ATP in the opposite direction (Rovetto et al., 1975). As a result, there may be insufficient ADP in the mitochondrion for ATP synthesis as well as insufficient ATP in the cytoplasm for cellular processes. These actions contribute to an ATP shortage.

ATP helps maintain ion concentrations and pumps such as the K_{ATP}, the SR Ca^{++} pump and the sarcolemmal Ca^{++} channel amongst others. An ATP shortage would therefore result in opening of the K_{ATP} channels causing K^{+} loss from the cell (Noma, 1983). Since ATP is needed for Ca^{++} uptake by the SR and Ca^{++} extrusion by the sarcolemma in order to maintain a relaxed state (Opie, 1990), an ATP deficiency would lead to intracellular Ca^{++} accumulation which may induce ischaemic contracture (Koretsune and Marban,

1990; Ventura-Clapier and Veksler, 1994). An ATP shortage also promotes enzyme release (Wilkinson and Robinson, 1974) while ATP degradation may liberate protons which in turn causes acidosis (Opie, 1989), both of which contribute further to necrosis of the ischaemic tissue.

The breakdown of ATP to AMP and ADP during ischaemia may be potentially beneficial. Protective effects include mediating vasodilation by means of the formation of adenosine from AMP (Kirsch et al, 1990), reducing heart rate and contractility by opening the K_{ATP} and inhibiting the Ca^{++} current (Isenberg and Belardinelli, 1984; Opie, 1992).

Gross ATP content may however not be the most critical determinant of ischaemic injury. Neely et al. (1973) found that the ATP content of ischaemic tissue had decreased by only 25 % by the time ventricular pressure started to decrease. They suggested that the ventricular failure may be due to compartmentalisation of available ATP in an inaccessible pool rather than a net shortage of ATP. By the time ischaemia had become irreversible, the ATP content had been further reduced by 70%. Since non-ischaemic tissue functioning at low ATP levels is not irreversibly impaired, they proposed that the ATP depletion is indicative, rather than a cause of irreversible ischaemic tissue damage. Alternatively, the turnover rate of high-energy phosphates may be more important in predicting the survival of the ischaemic myocardium (Vanoverschelde et al., 1994). However, this may be limited by the loss of tissue nucleosides (Mustafa et al., 1975). During net ATP hydrolysis, there is a loss of purine bases from oxygen deficient cells. This limits the restoration of ATP during reperfusion (Reibel and Rovetto,1978). If

however residual glycolysis is maintained, some ATP may be resynthesized thereby preventing the complete degradation of ATP and subsequent purine loss (Podzuweit et al., 1998). This supports the proposal that the extent to which ATP returns to control levels following a period of ischaemia may depend on how long ATP is maintained at a low level (Reibel and Rovetto,1978).

2.5.2 Lactate

Under aerobic conditions, the myocardium extracts lactate from arterial blood to serve as a metabolic substrate (Braunwald 1992 p. 1183). In mild ischaemia, glucose and glycogen is converted to pyruvate where some is oxidised and some forms lactate. These are features of mixed aerobic and anaerobic metabolism. During severe ischaemia however, reduced oxygen availability prevents pyruvate flux through the Krebs Cycle. Lactate extraction is therefore reduced and replaced by net lactate production. Restriction of Krebs Cycle activity also results in acetyl CoA accumulation (Ferrari and Opie, 1992) which in turn inhibits the pyruvate dehydrogenase complex. The outcome is a greater conversion of pyruvate to lactate (Neely and Morgan, 1974). Severely restricted coronary flow where washout of metabolites is reduced may also contribute to lactate build up.

Deleterious effects of lactate accumulation include the inhibition of glycolysis at various steps. It inhibits the G3PDH reaction (Figure 2.2) responsible for converting 3-phosphoglyceraldehyde to 1.3-biphosphoglyceraldehyde (Williamson et al., 1976). High intracellular lactate levels also inhibit HK, PFK, phosphorylase kinase (which activates phosphorylase b to the a form) and carnitine acyl tranferase, responsible for the transfer

of acyl CoA from the cytoplasm to the mitochondria (Rovetto et al., 1973). The resultant build up of acyl CoA suppresses the activity of adenine nucleotide translocase, thereby inhibiting the exchange of ADP and ATP between the cytoplasm and mitochondria (De Leiris et al., 1975). According to Neely and Grotyohann (1984), high ischaemic lactate levels have also been inversely related to recovery of myocardial function during reperfusion. Thus, it appears that the glycolytic rate could be enhanced (Neely and Morgan, 1974) and reperfusion recoveries improved if washout of lactate and protons is accelerated.

It has been reported that high levels of tissue lactate may contribute to intracellular acidosis (Brachfeld, 1969). More recently Dennis et al. (1991) suggested that protons during ischaemia is more likely to be a result of reduced mitochondrial ATP production in relation to glycolytic ATP synthesis. Nevertheless, acidosis could also possibly increase intracellular Ca^{++} and aid the development of ischaemic contracture (Allen, 1988). It inhibits glycolytic flux and the malate-aspartate cycle, responsible for the transport of oxaloacetic acid from the mitochondria to the cytoplasm (Williamson et al., 1976). In addition, it is suggested that a low intracellular pH may contribute to ventricular failure. Accumulation of intracellular H^{+} may interfere with the uptake and release of Ca^{++} from the SR (Nakamura and Schwartz, 1972), or may compete with Ca^{++} for receptors on the troponin molecules, thus impairing the actin-myosin interaction (Braunwald, 1992).

2.5.3 Ionic changes

a) Calcium (Ca^{++})

Calcium plays an important role in the regulation of the energy metabolism of cardiac muscle. Disturbances in the amount and distribution of intracellular calcium affects the energetics of myocardial cells (Henry et al., 1977) whilst Ca^{++} "out of control" is said to be harmful to cardiocytes (Poole-Wilson et al., 1984).

Cell membranes are permeable to Ca^{++} and since the ratio of the extracellular to intracellular free Ca^{++} is of the order of 10 000, the low intracellular Ca^{++} ion concentrations ($\approx 10^{-7}$ M) in myocardial cells must be maintained by active processes (Henry et al., 1977). Under normal physiological conditions, the myocardial cell has a complex system to control the transsarcolemmal Ca^{++} fluxes. This includes the Ca^{++} ATPase pumps, the sodium potassium (Na^{+}-K^{+}) ATPase pumps, the Na^{+}/Ca^{++} exchanger, the Na^{+}/H^{+} exchanger, and the L-type Ca^{++} channel. If these processes are disrupted, Ca^{++} accumulates within the cell (Henry et al., 1977) with detrimental effects for the survival of the myocardium.

Intracellular Ca^{++} concentrations increase through ß-adrenergic cAMP mediated influx via the slow Ca^{++} channels (Podzuweit et al., 1978) and by α-adrenergic IP_3 mediated effects on the SR (Schomig et al., 1990). Ca^{++} entry via the slow channels increase intracellular Ca^{++} levels further by inducing Ca^{++} release from the SR. Catecholamine ß - adrenergic stimulation also enhances Ca^{++} uptake by the Ca^{++} pump into the SR which leads to a greater rate of relaxation, termed the lusitropic effect (Katz, 1988). Ca^{++}

regulates cellular processes through the phosphorylation of various proteins and is therefore generally regarded as a third messenger, with catecholamines as first and cAMP / IP_3 as second. Taking into account the various intervening processes including activation of protein kinases, Ca^{++} may sometimes be considered as the fifth or sixth messenger (Opie, 1992).

According to Bricknell and Opie (1978), the early rise in cytosolic Ca^{++} may be from internal sources such as the SR, while the late rise is due to influx through the damaged sarcolemma (Opie,1987). Raised intracellular Ca^{++} levels may be the result of increased catecholamine secretion or reduced ATP stores. An ATP shortage hinders the Na^{+}-K^{+} pump activity which in turn increases the intracellular Na^{+} concentration. To rectify this, activity of the Na^{+}-Ca^{++} exchanger is enhanced resulting in greater Ca^{++} influx. An ATP deficit also inhibits the SR Ca^{++} pump with reduced Ca^{++} uptake into the SR and egress from the cells (Sedlis et al., 1983).

Deleterious effects of raised intracellular Ca^{++} levels includes activation of Ca^{++}-dependent ATPases which encourages ATP degradation (Burton et al., 1981), activation of Ca^{++} dependant lipases and proteases (Burton et al., 1981) leading to sarcolemmal phospholipid degradation, and release of arachidonic acid (Murphy et al, 1985). It also promotes glycogen degradation by enhancing the conversion of phosphorylase b to the a form (Neely & Morgan, 1974), where the latter is responsible for converting glycogen to glucose-1-phosphate. Ca^{++} loading of the mitochondria contributes further to the ATP shortage by impairing ATP synthesis (Jacobus et al., 1975). Moreover, elevated cytosolic Ca^{++} precipitates arrhythmias by promoting delayed after-depolarisations

(Coetzee et al., 1987). Thus, excess intracellular free Ca^{++} not only enhances ATP utilisation and decreases ATP production, but also causes membrane damage with Ca^{++} overloading, and arrhythmias (Burton et al., 1981).

b) Sodium (Na^+)

Maintenance of the resting membrane potential (RMP) is crucial in cell viability since the Na^+ and Ca^{++} channels fail to open when the cell is completely depolarised. The Na^+-K^+ pump is one of the basic mechanisms in maintaining the potassium (K^+) gradient and resting membrane potential across the sarcolemma and responds to ATP in the micromolar range (Niki et al., 1989).

Variations in intracellular Na^+ levels occur mainly through the Na^+-K^+ pump and Na^+/ Ca^{++} exchangers which are reversible depending on concentration gradients of these ions. The ATP dependent Na^+-K^+ pump is usually responsible for the transport of K^{+-} into the cell and Na^+ in the opposite direction in order to maintain the RMP. Failure of this pump during ischaemia due to energy depletion results in raised intracellular Na^+ levels and lower K^+ levels. This increased cytosolic Na^+ levels now reduces the Na^+ electrochemical gradient to such an extent that it can no longer drive Ca^{++} extrusion via the Na^+/Ca^{++} exchange. As a result, the exchange reverses and Na^+ extrusion is coupled to Ca^{++} ingress (Sedlis et al., 1983). A low intracellular pH may also increase the cytosolic Na^+ concentration through the Na^+/H^+ exchanger. This would then also contribute to subsequent Ca^{++} accumulation via the Na^+/Ca^{++} exchanger (Aronson, 1985). Thus cytosolic Na^+ imbalance precedes the cytosolic Ca^{++} rise (Allshire and Cobboid, 1990) with developed tension being strongly dependent on Na^+. Additional

deleterious effects of Na^+ accumulation may include increased osmolarity, cell swelling, disruption of the RMP and generation of abnormal action potentials.

c) Potassium (K^+)

The separation of ions across the semi-permeable sarcolemma is responsible for the resting membrane potential. Potassium has a higher intracellular concentration and as a result tends to move down the concentration gradient to the extracellular space. It plays a role in the maintenance of the RMP by creating a positive charge outside the cell During the action potential, K^+ efflux is retarded and contributes to the depolarisation of the sarcolemma. Opening of the K^+ channels permits ions to leave the cell thereby aiding repolarisation (Katz 1977 p.245).

Rapid K^+ loss is characteristic of the early phase of ischaemia but is not associated with detectable membrane damage. Late ischaemic K^+ loss is due to severe ATP depletion and inhibition of the Na^+-K^+ pump which transports K^+ back into the cell (Opie, 1987; 1989). There are various hypotheses for the mechanism of K^+ loss. Firstly, production of negatively charged lactate and inorganic phosphates occur due to anaerobic glycolysis and CP/ATP breakdown respectively. The positively charged K^+ accompanies the negatively charged ions when leaving the cell in order to balance the charges (Kleber, 1984). K^+ loss may also occur via inhibition of the Na^+-K^+ pump (Sedlis et al., 1983), and through opening of the ATP sensitive K^+ channels (Noma, 1983) as a result of an ATP deficiency.

ATP-sensitive potassium channels (K_{ATP}) exist in numerous tissues including skeletal muscle, smooth muscle, the brain (Spruce et al., 1985) and cardiac myocytes (Noma, 1983). Channel closure is caused by the association of one ATP molecule with the intracellular ATP receptor in the channel (Nichols et al., 1991). Adenosine diphosphate (ADP) has less effect than ATP in this aspect, whereas AMP has no effect on the K_{ATP} (Noma, 1983). Other intracellular ligands known to open the K_{ATP} are nucleoside diphosphates, protons and lactate (Findlay, 1994). Under normal conditions the channel is closed by intracellular ATP where ATP hydrolysis is compensated for by ATP production, and levels of other channel effective metabolites would be low. Ischaemia is characterised by an ATP deficiency and increased levels of ADP, protons and lactate. As a result, the K_{ATP} opens and affects myocardial contractility by lowering the RMP (Hollard et al., 1977) and reducing the action potential duration. Furthermore, extracellular K^+ is high in the ischaemic zone whereas it is lower (less elevated or normal) in the non-ischaemic zone. The current resulting from this K^+ gradient is known as depolarisation-induced automaticity (Mugelli et al., 1983).

Openers of K_{ATP} include cromakalim, pinacidil, isimakalim, levcromakalim and nicorandil (Cavero and Premmereur, 1994; Grover, 1994) while sulphonylurea drugs inhibit the opening of K_{ATP} (Findlay, 1994). Most studies indicate that K_{ATP} openers protect mechanical functioning of the heart, but are proarrhythmic while K_{ATP} blockers are anti-arrhythmic but are also associated with a greater loss of mechanical function (Siegl, 1994). However the notion that openers of the K_{ATP} channels are proarrhythmic and blockers are anti-arrhythmic is controversial as is whether K_{ATP} blockers or openers are of potential benefit in IHD (Cavero and Premmereur, 1994; Grover, 1994; Siegl, 1994).

2.6 Functional consequences of myocardial ischaemia

2.6.1 Ischaemic contracture

Ischaemic contracture (IC), defined as a 5% rise in diastolic tension (Owen et al., 1990), is one of the earliest consequences of myocardial ischaemia. Within 10 seconds of the beginning of ischaemia, the systolic pressure is visibly depressed and ischaemic contracture develops once the systolic pressure is either greatly reduced or already abolished (Ventura-Clapier and Veksler, 1994). The clinical equivalent of IC is known as the "stone heart" and is recognised as a complication of cardiopulmonary bypass (Cooley et al., 1972) and a marker for the onset of irreversible injury during coronary artery occlusion (Baroldi et al., 1974).

Hypothetically, IC may develop as a consequence of a raised cytosolic Ca^{++} concentration (Bricknell and Opie, 1978) which causes active cycling crossbridges (Ventura-Clapier and Veksler, 1994). Thus the elevation of cytosolic Ca^{++} precedes the rise in resting tension (IC) during ischaemia (Kihara et al., 1989). Yet data indicate that reduced ATP availability correlates much better with the occurrence of IC than changes in internal Ca^{++} (Koretsune and Marban, 1990; Owen et al., 1990). An ATP deficiency prevents restoration of resting cytosolic Ca^{++} levels and inhibits dissociation of the actin-myosin cross-bridges (Grossman and Barry, 1980). However, the exact relationship between ATP depletion and IC is not fully understood. It has also been suggested that phosphocreatine depletion and Mg^{++}-ADP accumulation may contribute to the development of contracture in the ischaemic myocardium (Ventura-Clapier and Veksler, 1994).

The development of IC is analogous with severe microscopic damage (Apstein et al., 1983) and massive cell disruption (Garcia-Dorado et al., 1992). It may reduce the coronary flow even more via mechanical compression of the coronary bloodvessels (Humphrey et al., 1980). This would aggravate ischaemic injury by reducing washout of potentially toxic metabolites such as lactate or H^+ (Neely & Groyohann, 1984). Ischaemic contracture contributes further to the progression of necrosis by raising the mechanical stress on adjacent myocytes through the intercalated discs (Garcia-Dorado et al., 1992).

Different ischaemic substrates may influence the occurrence of IC. No incidence of IC was found during the ischaemic period in glucose perfused hearts. Yet when glycolysis was inhibited with iodoacetate/ deoxyglucose, or hearts were perfused with non-glucose substrates such as fatty acids, IC developed quickly (Bricknell and Opie, 1978; Van Rooyen et al., 1996). This supports the hypothesis that a certain rate of glycolytic ATP synthesis from glucose is needed for protection against ischaemic contracture in globally underperfused isolated rat hearts (Owen et al., 1990). This may also explain why the protective effect of glucose on IC is lost during severe ischaemia when glucose delivery is limited (Rovetto et al., 1973). Furthermore, ATP production via glycogenolysis may be less effective than glycolysis from glucose in maintaining Ca^{++} homeostasis across the sarcolemma. Some glycogen breakdown, is however required during the early stages of low flow ischaemia to delay the onset of IC (King et al., 1995).

2.6.2 Arrhythmias

Ventricular fibrillation is the most common cause of sudden cardiac death (Opie, 1987). A number of consequences of ischaemia have been shown to be potentially arrhythmogenic:

1) increased adrenergic activity (Harris et al., 1954, Podzuweit et al., 1976)

2) localised K^+ loss (Harris et al., 1954)

3) insufficient glycolytic ATP (Russel and Oliver, 1979)

4) elevated lactic acid levels (Wissner, 1974)

5) accumulation of fatty acids and acyl CoA (Kurien and Oliver, 1970) and

6) myocardial oxygen gradients during regional ischaemia that may cause inhomogeneous metabolic and electrical activity (Beck, 1958).

Catecholamine stimulation increases the heart's vulnerability to ventricular fibrillation (Hoffman et al., 1955). Administration of norepinephrine during ischaemia has been shown to elicit tachycardia or fibrillation in a number of animal models such as the isolated rat heart (Lubbe et al., 1978), the dog (Opie et al., 1980), and the pig (Podzuweit, 1980). Similarly, when sympathetic activity is inhibited by either surgical denervation (Ebert et al., 1970), depletion of myocardial epinephrine stores (Meesman, 1980) or blockade of the receptors (Sehti et al., 1973), there is protection against ventricular fibrillation.

Catecholamines induce arrhythmias by raising cAMP and Ca^{++} levels (Opie, 1987). Both cAMP (Wojtczak, 1982) and Ca^{++} (De Mello, 1982) delay intercellular conduction as they augment the intercellular resistance. Elevation of cAMP levels in the ischaemic myocardium is found to precede ventricular fibrillation (Podzuweit et al., 1978; Opie et al., 1979). In the isolated heart, epinephrine as well as dibutyryl cyclic 3'-5'-AMP (a form of cAMP thought to penetrate the cell membrane) enhanced ventricular vulnerability to fibrillation (Lubbe et al., 1976; Lubbe et al., 1978). Furthermore, agents such as propranolol (Lubbe et al., 1981) and adenosine (Lubbe et al., 1983) reduces myocardial cAMP levels in the ischaemic zone and protects against the occurrence of ventricular fibrillation.

Hypokalaemia also plays an important role in provoking ventricular arrhythmias (Johansson and Dziamski, 1984). Increased extracellular K^+ levels in the ischaemic zone slows conduction which in turn predisposes the heart to re-entrant arrhythmias (Janse et al., 1987). Therefore, agents which diminish K^+ loss also lessen ventricular fibrillation as observed in the pig by Muller et al. (1986). Due to the loss of cellular K^+, extracellular K^+ levels are higher in the ischaemic zone and less elevated or normal in the non-ischaemic area. This concentration gradient may inspire a phenomenon known as depolarisation-induced automaticity (Mugelli et al., 1983) which is also influenced by changes in the external Ca^{++} concentration (Katzung, 1975). Hence, Ca^{++} antagonists, which inhibit the Ca^{++} current, may also relieve arrhythmias by lowering the K^+ loss and reducing the depolarisation-induced current (Johnson et al., 1991).

2.6.3 Membrane damage

Proposed mechanisms for early membrane damage include:

- Unoxidised fatty acid metabolites (e.g. acyl CoA and acylcarnitine) accumulate within the cell and contribute to ischaemic damage via their detergent effect on the cell membrane (Kurien & Oliver, 1970) and inhibition of glycolysis (Liedtke, 1981).
- The accumulation of Ca^{++} within the cell partly activates phospholipases. These enzymes are responsible for the degradation of membrane lipids.
- Oxygen free radicals contribute to membrane damage via the formation of lipid peroxide. They are derived in part from neutrophils (Lucchesi et al., 1995) and are formed during the ischaemic and reperfusion phases.

As ischaemia progresses, the membrane undergoes more destruction. This leads to a greater release of intracellular enzymes and ultimately cell death (De Leiris et al., 1975).

2.6.4 Coronary flow

A close relationship exists between coronary flow, myocardial oxygen consumption and contractile performance of the heart where changes in coronary flow translate into changes in contractile performance (Vatner, 1980). Therefore, coronary flow rate during ischaemia is an important determinant of the degree of recovery upon reperfusion (King et al., 1995) and the rate and extent of cell death (Schaper et al., 1987). It is argued then that ischaemia, where the coronary flow is restricted, is more detrimental to cellular function and metabolism than anoxia, where the coronary flow is maintained at normal rates (Rovetto et al., 1973).

The harmful effects precipitated by a restriction of coronary flow is due to the lack of washout of the interstitial space, as well as reduced delivery of oxygen and metabolic substrates to the tissue. The time to the onset of cell death may even be directly proportional to the restriction of flow (Neely et al., 1975). King et al. (1995) found that the severity of the reduction in coronary flow plays a critical role in the protective effects of glucose. In the globally ischaemic isolated rat heart, a coronary flow reduction of 30% caused an increase in glucose uptake. A further 15 % reduction had less of an effect, but when coronary flow fell to 4-10 %, glucose uptake was inhibited (Rovetto et al., 1973). Neely et al. (1975), on the other hand, found that a 50 % reduction in coronary flow in the isolated working rat heart resulted in ventricular failure within eight minutes of the decrease in coronary flow. Ischaemic flow rates less than 2 ml/ minute resulted in glycolytic inhibition. Alternatively, coronary flow rates upon *reperfusion* may determine the rate and extent of cell death (King et al., 1995). Although the myocardium is reperfused, the coronary flow may still be restricted by mechanical compression of the coronary arteries by ischaemic contracture. This suppresses reperfusion coronary flow (Humphrey et al., 1980) with subsequent accumulation of deleterious products.

It is important to note that in experimental models, isolated rat hearts are perfused with a Krebs-Henseleit bicarbonate buffer. This crystalline solution has a lower oxygen carrying capacity than blood so the coronary arterioles dilate maximally due to local control mechanisms. As a result, the coronary flow is higher than the in vivo rates and range between 10-15 ml/ minute (Neely et al., 1975). The ischaemic coronary flow is artificially fixed in order to prevent a contracture-induced reduction in flow (Humphrey et al., 1980; King et al., 1995).

2.6.5 Developed pressure

There are numerous abnormalities of diastolic and systolic function during severe ischaemia which ultimately affects the developed pressure. These include a reduced relaxation rate, ischaemic contracture (Apstein et al., 1983) and systolic failure (Miyazaki et al., 1990). Systolic pressure decreases within seconds of the beginning of ischaemia (Ventura-Clapier and Veksler, 1994) which is followed by an increase in diastolic tension or ischaemic contracture. According to Ventura-Clapier and Veksler (1994), rigor tension (ischaemic contracture) or stiffness under these circumstances, may appear as a "switch off" of energy expenditure for contraction. Besides IC, additional factors which have negative effects on developed pressure include high ischaemic tissue lactate levels (Neely and Grotyohann, 1984), glycolytic inhibition (Kusoka and Marban, 1994), inhibition of oxidative phosphorylation (Weiss and Hiltbrand, 1985), and increased fatty acids. Glucose, on the other hand, has been shown to aid recovery of developed pressure (Coleman et al., 1989; King et al., 1995) following an ischaemic period.

2.7. Reperfusion

Clinical manifestations of myocardial ischaemia such as myocardial infarction or ventricular fibrillation, depends on metabolic changes that may be modified by reperfusion (Opie, 1987). Reperfusion improves the myocardial metabolism via increased washout of harmful metabolic products (Neely and Grotyohann, 1984) and providing oxygen and nutrients to the ischaemic myocardium. However, it may also cause damage which is referred to as reperfusion injury. This is characterised by a spectrum of events which include stunning, reperfusion arrhythmias, microvascular

injury, and accelerated or reperfusion-induced cell necrosis (Opie, 1989). The mechanism of reperfusion injury appears to be metabolic in origin, related to either free radicals or excess accumulation of cytosolic Ca^{++} (Opie, 1987). It is suggested that reperfusion mediated events are largely a consequence of injury during ischaemia. Prevention of these ischaemic-specific events can effectively preclude reperfusion induced injury (Vanoverschelde et al., 1994).

Reperfusion stunning refers to depressed or delayed myocardial mechanical function which occurs when ischaemic tissue is reperfused. The mechanical function should nevertheless return to expected levels with continued reperfusion (Braunwald and Kloner, 1982). The two main hypotheses for the cause of stunning are cytosolic Ca^{++} overload (Du Toit and Opie, 1992) and the formation of oxygen free radicals (Bolli, 1990). Oxygen free radicals, derived partly from neutrophils, may play a role in stunning by reducing the response of the organelles to Ca^{++} (Engler and Corell, 1987). When these free radicals are diminished by scavengers such as superoxide dismutase, improved recovery from ischaemia is found (Ambrosio et al., 1987).

Elevated Ca^{++} levels during reperfusion has been implicated as the mediator of postischaemic contractile dysfunction (Tani and Neely, 1989). The use of Ca^{++} channel blockers or modulation of flux via the SR during early reperfusion has a protective effect and lessens stunning (Du Toit and Opie, 1992). Lazduski et al. (1985) proposed that rapid washout of extracellular H^+ during reperfusion may create an intracellular-to-extracellular H^+ gradient. Via a cascade of events, which involve the Na^+/H^+ and Na^+/Ca^{++} exchangers, this H^+ gradient is ultimately responsible for raising intracellular

Ca^{++} levels. According to Jeremy et al. (1992) the promotion of glucose utilisation may reduce stunning by increasing glycolytic ATP production which controls cytolsolic Ca^{++} levels.

No-reflow is the phenomenon which occurs when removal of the coronary occlusion does not lead to the restoration of the coronary flow (Bernier et al., 1986). There are two explanations for this phenomenon. Either the microvascular damage caused endothelial cell edema, or IC may have prevented normal flow by squeezing the coronary arteries (Opie 1991). The consequences of microvascular damage include reduced formation of vasodilatory substances such as nitric oxide, and formation of vasoconstrictory endothelin. Endothelial damage may also counteract factors which inhibit platelet plugging and neutrophil adherence (Forman et al., 1989).

Arrhythmias are an abnormal heart rhythm and is classified as ventricular arrhythmias or supraventricular arrhythmias. This includes tachycardias and bradyarrhythmias (Opie, 1991). The mechanisms which may underly ventricular arrhythmias include automaticity (Hauswirth et al., 1969; Mugelli et al., 1983), reentry circuits (Janse et al., 1987), and after-depolarisations. Reperfusion arrhythmias may also be due to excess cycling of intracellular Ca^{++}(Coetzee et al., 1987), and the formation of free radicals. Free radicals can induce electrophysiological changes such as membrane depolarisation (Coetzee et al., 1990) and when present in high concentrations, can modify the gating properties of the SR so that excess Ca^{++} is released (Cumming et al., 1990). This Ca^{++} overload triggers reperfusion arrhythmias (Opie, 1987) by promoting delayed after depolarisations (Coetzee et al., 1987) and provoking inward depolarising currents (Johnson et al., 1991).

The nature of the reperfusion solution may affect the incidence of reperfusion arrhythmias. The inhibitory effect of glucose on reperfusion ventricular arrhythmias is established (Manning and Hearse, 1984; Bernier and Hearse, 1988; Coleman et al., 1989). Recent findings include acidic reperfusion as having an inhibitory effect as well. Avkiran and Ibuki (1992) found that early acidic reperfusion significantly suppresses reperfusion induced ventricular fibrillation. The protection afforded was maximal at pH 6,6 and the duration of acidic reperfusion needed to be at least two minutes. The protective mechanism of transient acidic reperfusion may involve pH-mediated inhibition of Na^+ influx and perhaps enhanced recovery of the Na^+-K^+ ATPase (Avkiran et al., 1996).

Chapter 3

MATERIALS AND METHODS

3.1 Animals

Male Sprague-Dawley and Wistar rats were used. Each weighed between 260g-380g. The rats had free access to food (standard lab chow) and water. They were maintained in animal quarters at a constant temperature (22°C) and humidity (40%) with a twelve hour artificial light cycle.

3.2 Study design

The rats were anaesthetised with diethyl ether vapour (B and M Scientific). Once asleep, they were injected with 200U sodium heparin (Novo Nordisk) into the femoral vein. The heart was excised quickly and placed in ice cold Krebs-Henseleit solution. The isolated rat heart was then mounted on a cannula via the aorta and perfused within 60 seconds of the excision. The heart was subsequently freed of excess non-cardiac tissue. All hearts were perfused in accordance with the Langendorff technique with an oxygenated Krebs-Henseleit bicarbonate buffer initially containing 5 mM sodium acetate as a substrate. The purpose of the acetate was to reduce pre-ischaemic tissue glycogen levels (Neely et al., 1970) and thus preclude its effects on performance during ischaemia. This initial perfusion period lasted 30 minutes, the purpose of which was to stabilise the heart and washout any blood and enzymes (De Leiris et al., 1975). A Gilson Minipuls 3 pump kept the meniscus of the buffer at a constant level so as to maintain a perfusion pressure of 100 cm H_2O. The myocardial temperature of 37°C was maintained throughout the

experiment by means of a waterbath (CND Scientific) which circulated warm water through the water jacketed glass reservoirs. A thermistor probe was inserted into the right coronary sinus to monitor the myocardial temperature. Coronary flow rates varied between 10 - 15 ml/min. during normal perfusion of 100 cm H_2O.

3.3 Experimental protocols

The protocol consisted of three sequential periods lasting 30 minutes each (Figure 3.1):

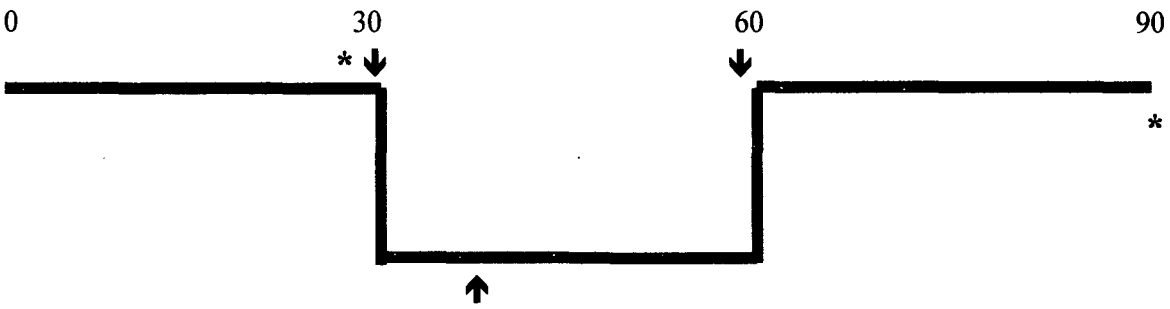
- 0 - 30 min.: During the pre-ischaemic phase, hearts were perfused under standard conditions at a pressure of 100 cm H_20 (coronary flow: 10-15 ml/min.) with 5 mM sodium acetate (Ac) as a substrate.
- 30 - 60 min.: During the ischaemic phase hearts were subjected to global low flow ischaemia. A Gilson Minipuls 2 pump regulated the coronary flow at a rate of 2 ml/min. which was equivalent to a 80-87% reduction in flow rate, i.e. 13%-20% of the pre-ischaemic values. This ischaemic coronary flow rate was higher than what is usually employed in order to lessen the accumulation of harmful metabolites and ensure residual oxygen delivery. The ischaemic perfusate contained one of the following substrates: 10 mM Glu, 1 mM Ac, 5 mM Ac, 10 mM Ac, or 30 mM Ac. Additional hearts were also subjected to an ischaemic period without residual oxygen (ischaemia-anoxia). The perfusate, containing either 5 mM Ac or 10 mM Glu, was aerated with nitrogen gas during ischaemia-anoxia instead of the oxygen-carbon dioxide mixture used in the previous experiments.
- 60 - 90 min.: During the reperfusion phase hearts were perfused under standard conditions once again where the perfusion pressure was 100 cm H_20 and the coronary flow rates varied from 10-15 ml/min.

3.4 Tissue Samples

For the purpose of biochemical analysis, a number of hearts were freeze clamped with Wollenburger metal tongs which was precooled in liquid nitrogen. The first group (n = 6) was freeze clamped at the end of the pre-ischaemic phase (30 minutes). Additional hearts were freeze clamped at the onset of IC (± 33 minutes), and at the end of the ischaemic phase (60 minutes). The exact time period between the beginning of ischaemia and the onset of IC varied depending on the substrate used during ischaemia. The ventricular tissue was then stored in a -80 °C freezer until analysed.

3.5 Measured variables

The variation in systolic and diastolic pressure was monitored through the insertion of a compliant balloon into the left ventricle. The balloon was made of plasticised PVC ("Glad Wrap") and inflated with distilled water. Any alteration of the balloon's volume, caused by the contraction of the heart against the balloon, was converted to a pressure reading by a pressure transducer (Washington PT 400 S/N 173). This was recorded on a Washington 400 MD 2C oscillograph. The balloon's volume was maintained throughout the experiment. The onset of IC, as indicated by a 5 % increase in diastolic pressure above baseline, was followed by a continued rise in diastolic pressure. After the initial rise, a peak was reached and ensued by a gradual fall in diastolic pressure. This sigmoidal graph is characteristic of IC. The time lapse from the start of ischaemia to the onset of IC was measured.



CORONARY FLOW:	10-17 ml/min	2 ml/min	10-17 ml/min	
SUBSTRATE:	5 mM Ac	1 mM Ac or 5 mM Ac or 10 mM Ac or 30 mM Ac	5 mM Ac	**Aim 1:** To establish an optimal Ac concentration which best protects the heart during subtotal ischaemia
	5 mM Ac	5 mM Ac or 10 mM Glu	5 mM Ac	**Aim 2:** To determine the effect of O_2 availability during ischaemia on the beneficial effects of Glu and Ac
	5 mM Ac	30 mM Ac or 10 mM glucose	5 mM Ac	**Aim 3:** To determine whether equi-carbon concentrations of Ac and Glu offer similar protection during subtotal ischaemia

Figure 3.1 Study Design. (↓ indicates the points at which hearts were freeze clamped; * indicates pressure readings used to determine % recovery of left ventricular developed pressure (LVDP); Ac=acetate, Glu=glucose)

Coronary flow (ml/min.) was measured periodically by collecting the volume of the heart's effluent in one minute. The coronary flow rate, heart rate (indicated by the ECG), and temperature was noted after five minutes of perfusion and every ten minutes thereafter.

The pH and pO_2 of the perfusate was determined with a blood-gas analyser using samples taken from both the low flow and normal flow systems.

Functional recovery, as indicated by the developed pressure at the end of the reperfusion period, was expressed as a percentage of the pre-ischaemic developed pressure after the initial 30 minutes of perfusion.

3.6 Exclusion criteria

Hearts with coronary flow rates outside the range of 10 - 17 ml/min., and heart rates outside the range of 200-380 beats/min were excluded. The temperature was maintained within one degree more or less than 37 °C.

3.7 Perfusion solutions

The perfusion solution, a crystalloid Krebs-Henseleit bicarbonate buffer, contained 118.5 mM NaCl, 25.0 mM $NaHCO_3$, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4.7H_2O$ and 1.3 mM $CaCl_2.2H_20$ (Merck). The solution was aerated with 95 % O_2 ; 5 % CO_2 for fifteen minutes prior to the experiment as well as throughout the protocol. The substrates used during ischaemia were 10 mM glucose (Merck) and 1 mM or 5 mM or

10 mM or 30 mM sodium acetate (Sigma) while 5 mM sodium acetate was also used during the pre-ischaemic and reperfusion phases (Figure 3.1). Solutions were filtered with a Milli-Q-System obtained from Millipore (cat. no. HVLPO4700). Buffers were prepared and stored in a fridge on the previous day and filtered through 0.45 μm millipore filters just prior to usage.

A sample of the perfusate was taken prior to the experiment to determine pH and PO_2. The low flow system had a pH =7,45 and PO_2 =64.4 kPa. The perfusate in the high flow system had a pH =7,45 and PO_2 =63.0 kPa..

3.8 Biochemical analysis

The ventricular tissue was freeze-dried (Christ Alpha 1-4 freeze-dryer) and teased in order to obtain a homogenous sample. The tissue was then extracted with either KOH (3.8.1) to determine its glycogen levels, or with perchloric acid (3.8.2), to determine the ATP, creatine phosphate, lactate and cAMP levels. All metabolite concentrations were assayed enzymatically with the use of a spectrophotometer or radioimmunoassay kit.

3.8.1 Glycogen extraction

1. Add ± 10 mg of the freeze-dried tissue to Eppendorf tubes. (Note the weight of the tissue.)
2. Add 0.2 ml 40 % cold KOH

3. Heat to 95 °C on Eppendorf thermostat for 30 minutes, shaking at 10 minute intervals to ensure that the tissue is broken up. Place a weight on the lids to prevent them from popping open. Allow to cool.
4. Add 0.8 ml 100% ethanol (ETOH). Glycogen precipitates overnight in the fridge.
5. Spin for 3 minutes (Eppendorf centrifuge) at 4 °C at 14000 rpm.
6. Aspirate supernatant, wash pellet at least twice with cold 100% ETOH. Aspirate supernatant again.
7. Add 1 ml acetate buffer with 1mg amyloglucosidase (7.3 U/ mg) to the pellet.

   acetate buffer: 0.2 M (16.4g) sodium acetate + 4.8 ml 96% acetic acid
8. Shake pellet well and then heat for 2 hrs at 37 °C on the Eppendorf thermostat to hydrolyse the glycogen to glucose. Shake pellets intermittently.
9. Add 75 ml PCA (70%)
10. Spin for 3 minutes (Eppendorf centrifuge) at 14000 rpm
11. Remove 1 ml clear supernatant/acid extract and place in clean Eppendorf tubes. Add 350 µl 2M NaOH for neutralisation.

Use this neutralised sample to determine the tissue glucose levels by means of a standard assay.

Calculations:

Calculate the dilution factor (F).

$$F = \frac{\text{vol. PCA} \times \text{total volume (ml)}}{\text{dry heart weight (g)}} \qquad \text{where total volume} = \frac{\text{total neutralised vol.}}{\text{vol. of acid extract}}$$

3.8.2 Tissue extraction with perchloric acid (PCA)

1. Add 9-10 mg teased freeze-dried tissue to Eppendorf tubes. (Note the weight of the tissue.)
2. Add cold PCA (5%) to tissue (50 µl PCA/ mg dry tissue) and homogenise (Ultrasonic Disintegrator, Branson) sample immediately while keeping tube on ice. (Note the amount of PCA used.)
3. Spin homogenised samples in the centrifuge (Eppendorf centrifuge) for 3 minutes at 14000 rpm.
4. Add the supernatant to a clean Eppendorf tube. (Note the exact volume of supernatant used.)
5. Add 5 µl universal indicator and neutralise the acid extract with 5N KOH to pH 6.5-7.5 (sample appears yellow-green in colour). (Note the amount of KOH used to neutralise the sample)
6. Spin (Eppendorf centrifuge) samples for 3 minutes at 14000 rpm.
7. Decant the supernatant into clean Eppendorfs and store at –196 °C in liquid nitrogen

Use this neutralised sample to determine the tissue ATP, CP, lactate and cAMP levels by means of a standard assay.

Calculations:

Calculate the dilution factor (F).

$$F = \frac{\text{vol. PCA} \times \text{total volume (ml)}}{\text{dry heart weight (g)}}$$

total volume = vol. acid extract/supernatant used + vol. of KOH used to neutralise the extract + 5 µl universal indicator

3.8.3 Assays

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

Measurement of ATP and CP is dependent upon the following enzymatic reactions which result in the formation of NADPH. The variation in absorbance is measured with a spectrophotometer at 340 nm as $NADP^+$ is reduced to form NADPH. The increase in absorbance is directly proportional to the creatine kinase activity.

Creatine kinase (CK)

$$CP + ADP \longrightarrow ATP + creatine$$

Hexokinase (HK)

$$ATP + glucose \longrightarrow ADP + G6P$$

glucose 6-phosphate dehydrogenase (G6PDH)

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

Cocktail		**Standard**	
Reagent	**Vol/cuvette**	**Reagent**	**Vol**
Glucose (10mM)	100 µl	ATP (10 mM)	100 µl
NADP (10 mM)	50 µl	(50 nmol/ 50 µl)	
$MgCl_2$ (1 M)	20µl	CP (10 mM)	100 µl
G6PDH (1 U/1.5 µl)	1.5µl	H_20	800 µl
Tris (0.5 M; pH 7.5)	400 µl		
H_20	379 µl		
Total	950.5 µl	Total	1000 µl

Assay:

ATP

1. Add 50 µl neutralised sample to 950 µl cocktail
2. Shake sample and wait 10 minutes
3. Use a spectrophotometer to measure the baseline absorbance (E1) at 340 nm.
4. Add 10 µl hexokinase (3 U/ 10 µl)
5. Wait 30 minutes and then measure absorbance again (E2)

CP

6. Add 50 µl ADP (10 mM) and wait 10 minutes
7. Measure the baseline absorbance (E1)
8. Add 10 µl creatine kinase (2 mg/ 500 µl ≅ 2 U/ 10 µl)
9. Wait 30 minutes and then measure the absorbance again (E2)

b) L-Lactate

The measurement of lactate is dependant upon the following enzymatic reaction:

Lactate dehydrogenase (LDH)

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

Cocktail		**Standard**	
Reagent	**vol/ cuvette**	**Reagent**	**vol**
NaOH (2 M)	350 µl	Lactate standard (1 M/ L)	20 µl
Hydrazine buffer	350 µl	(100 nmol/ 50 µl)	
NAD (10 mM)	100µl	H_20	980 µl
H_20	150 µl		
Total	950 µl	Total	1000 µl

Hydrazine buffer: 26.02 g Hydrazinesulphur (1M) + 30.03 g Glycine (2 M) + 0.370 g EDTA (5 mM) + water to final volume of 200 ml

Assay:

1. Add 50 µl neutralised sample to 950 µl cocktail
2. Shake sample and wait 10-15 minutes
3. Use a spectrophotometer to measure the baseline absorbance (E1) at 340 nm.
4. Add 10 µl LDH (25 µg/ 10 µl)
5. Wait 30 minutes and then measure the absorbance again (E2)

c) Glucose

The measurement of glucose is dependant upon the following enzymatic reactions:

Hexokinase (HK)

$$\text{Glucose} + \text{ATP} \longrightarrow \text{Glucose 6-phosphate} + \text{ADP}$$

Glucose 6-phosphate dehydrogenase (G-6-PDH)

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

Cocktail		**Standard**	
Reagent	**vol/ cuvette**	**Reagent**	**vol**
ATP (10 mM)	100 µl	Glucose (10 mM)	100 µl
NADP (10 mM)	100 µl	(50 nmol/ 50 µl)	
$MgCl_2$ (1 M)	20µl	H_20	900 µl
G-6-PDH (1U/1.5 µl)	1.5 µl		
Tris (0.5 M; pH 7.5)	400 µl		
H_20	329 µl		
Total	950.5 µl	Total	1000 µl

Assay:

1. Add 50 µl neutralised sample (from glycogen extraction) to 950 µl cocktail
2. Shake sample and wait 10 minutes
3. Use a spectrophotometer to measure the baseline absorbance (E1) at 340 nm.
4. Add 10 µl hexokinase (1.5 µl enzyme + 8.5 µl buffer = 1U/ 10 µl)
5. Wait 30 minutes and then measure the absorbance again (E2)

Calculations to determine the ATP, CP, lactate and glucose concentrations

$\Delta E = E2-E1$

$$\text{Concentration} = \frac{\Delta E \times F3 \times \text{cuvette vol.}}{E\ (6.3) \times \text{sample vol.} \times d\ (=1)}$$

d) cAMP

A kit (Amersham, England) was used to determine tissue cAMP content. The assay is based on the competition between unlabelled cAMP and a fixed quantity of tritium labelled cAMP for binding to a protein. This protein has a high specificity and affinity for cAMP. The amount of labelled cAMP-protein complex formed is inversely related to the amount of unlabelled cAMP present in the sample. Therefore by measuring the protein-bound radioactivity, the amount of unlabelled cAMP in the sample may be calculated. The protein bound cAMP is separated from the unbound nucleotide through the absorption of the free nucleotide onto coated charcoal. Through centrifugation, a supernatant is obtained and removed for liquid scintillation counting. A standard curve is used to determine the concentration of unlabelled cAMP in the sample.

3.9 Statistical analysis

A software package, GraphPAD Instat™ was used to conduct the statistical analysis. All data was expressed as means ± SEM. Each experimental group consisted of 6-8 hearts. Results were compared using ANOVA two-way analysis of variance followed by the students t-test with $p \leq 0.05$ considered as significant.

Chapter 4

RESULTS

4.1 Aim 1: To establish an optimal acetate concentration which best protects the heart during subtotal ischaemia

4.1.1 Heart rate (Table 4.1)

After 25 minutes of pre-ischaemic perfusion, the heart rate (HR) in all groups ranged from 250-283 beats/min with no significant variation amongst the groups. After 5 minutes of ischaemia the HR in all groups, except 5 mM Ac, decreased significantly. The greatest decline ($p \leq 0.001$) was noted in the 1 mM Ac hearts where the HR deteriorated to only 18.8±0.7 beats/min and remained low throughout ischaemia. With initial reperfusion, heart rates in 5 mM and 10 mM Ac treated hearts had recovered to 265.8±10.3 and 242.5±9.0 beats/min respectively. On the other hand, 1 mM Ac and 30 mM Ac yielded significantly weaker recoveries of 60.0±0.0 and 170.3±52.1 respectively. After 25 minutes of reperfusion, the HR in all groups except 1 mM Ac had recovered to at least 210.8±43.0 beats/min. (84 % of the last pre-ischaemic recording) while 1 mM Ac hearts had recovered to only 160.0±80.0 beats/min. There was however no significant difference in HR amongst the groups at this point.

Table 4.1 Heart Rate (beats/min) at different stages during the experiment where various ischaemic substrates were administered. Ac = acetate, Glu = glucose.

* p≤0.05 vs 1 mM Ac at the same time interval; # p≤0.05 vs HR at 25 min in the same group.

	Time	1 mM Ac	5 mM Ac	10 mM Ac	30 mM Ac	10 mM Glu
Pre -	5	342.5±15.2	334.0±11.8	315.0±16.9	372.5±27.8	260.0±11.4
ischaemic	15	290.0±3.2	272.5±17.5	287.5±31.8	292.5±26.5	247.5±5.1
Phase	25	252.5±19.1	270.7±13.8	282.5±17.1	250.0±26.5	257.5±10.5
Ischaemic	35	18.8±0.7#	192.0±36.6*	145.0±19.2#	106.7±24.4#	135.0±21.2
Phase	45	17.4±1.1	188.3±26.1	125.0±28.6	57.5±13.6	127.5±22.8
	55	18.0±0.0	118.6±20.4	120.0±31.7	60.0±12.8	162.5±18.5
Reperfusion	65	60.0±0.0	265.8±10.3*	242.5±9.0*	170.3±52.1	235.0±6.3
Phase	75	60.0±0.0	270.8±13.0	285.0±27.7	174.7±53.7	257.5±7.2
	85	160.0±80.0♣	272.5±14.6	277.5±23.8	210.8±43.0	247.0±6.4

♣ The HR could only be determined for 2 of the 6 hearts at this particular time interval, hence the large SEM. The rest of the hearts fibrillated at this point.

4.1.2 Coronary Flow (Table 4.2)

Pre-ischaemic coronary flow (CF) was similar in all groups and ranged between 12.4-15.6 ml/min. During ischaemia, the CF was fixed at 2 ml/min. Upon reperfusion the CF returned to pre-ischaemic values and after 25 minutes of reperfusion, the CF in all groups except 1 mM Ac had recovered to at least 11.0±0.9 ml/min. (equivalent to a minimum of 85% of the CF prior to ischaemia). There were no significant differences in CF at the end of the reperfusion period.

Table 4.2 Coronary flow (ml/min) at different stages during the experiment where various ischaemic substrates were administered. Ac = acetate, Glu = glucose.

	Time	1 mM Ac	5 mM Ac	10 mM Ac	30 mM Ac	10 mM Glu
Pre -	5	15.6±0.5	14.3±1.2	15.9±0.6	17.0±0.5	16.1±0.5
ischaemic	15	13.3±1.0	13.6±1.1	14.0±1.2	14.3±0.7	14.4±±0.8
Phase	25	12.4±1.2	13.8±1.4	13.2±1.1	13.0±0.8	13.5±0.5
Ischaemic	35	2.02±1.7	2.0±2.6	2.0±2.6	2.0±1.7	2.1±2.6
Phase	45	2.02±3.1	2.1±5.0	2.0±0.0	2.0±0.0	2.0±2.1
	55	2.05±2.2	2.0±2.6	2.1±2.2	2.0±1.7	2.1±3.1
Reperfusion	65	10.1±1.1	12.2±1.3	12.0±1.0	9.6±0.7	13.2±0.8
Phase	75	9.43±1.2	13.4±1.7	13.2±0.8	10.6±0.9	12.0±0.7
	85	9.1±1.3	13.6±1.7	13.4±1.0	11.0±0.9	11.6±0.5

4.1.3 Time to the Onset of Ischaemic Contracture (TOIC) (Figure 4.1)

Ischaemic contracture was delayed in the 10 mM Ac hearts (202.0±0.04 s) while 30 mM acetate accelerated the time to onset of IC (65.0±9.8 s). However, there was no significant difference between 5 mM and 10 mM Ac hearts and similarly between 1 mM and 30 mM Ac.

4.1.4 Percentage recovery of Left Ventricular Developed Pressure (LVDP) (Figure 4.2)

The percentage recovery of LVDP was greatest in 5 mM Ac hearts (53.5±2.74 %) with 1 mM Ac producing the weakest recoveries (5.0±2.9 %) ($p \leq 0.001$ vs 5 mM, 10 mM and 30 mM Ac). Despite yielding the best recoveries, there was no significant difference between 5 mM and 10 mM Ac.

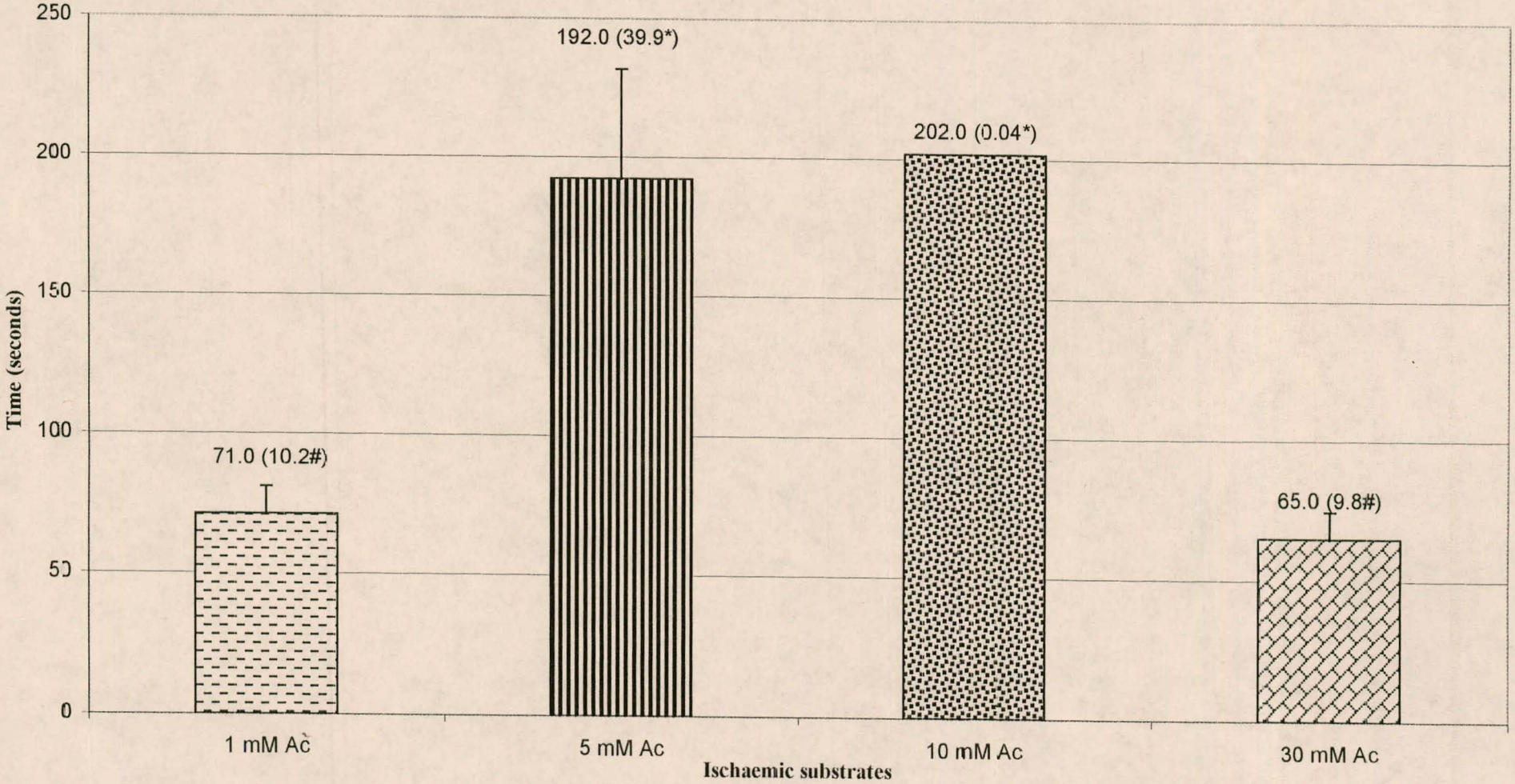


Figure 4.1 Time to onset of ischaemic contracture (TOIC) in acetate (Ac) perfused hearts.
*p<0.05 vs 1 mM Ac; # p<0.05 vs 5 mM Ac

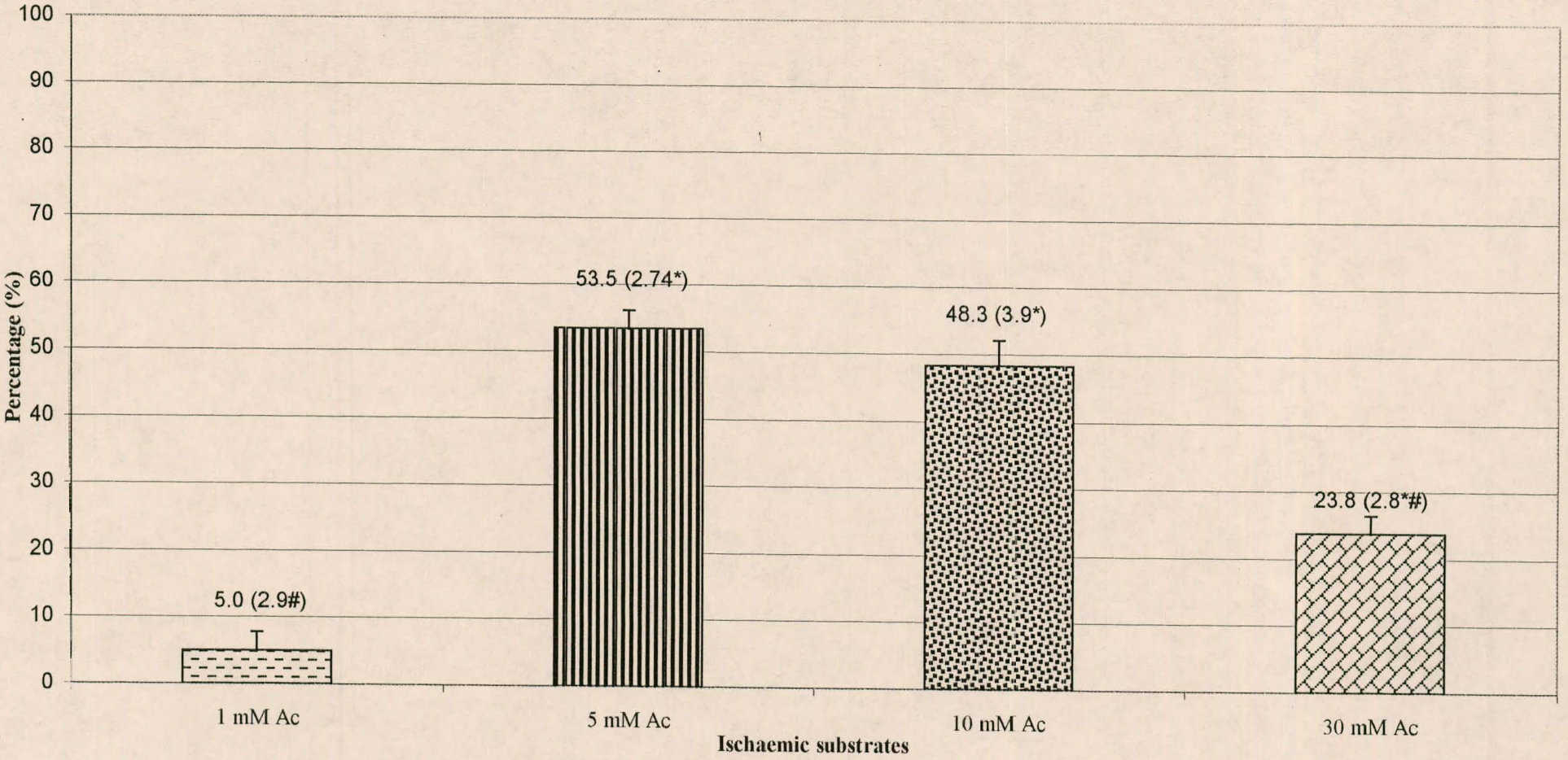


Figure 4.2 Percentage recovery of left ventricular developed pressure (LVDP) in acetate (Ac) perfused hearts. *$p<0.05$ vs 1 mM Ac; # $p<0.05$ vs 5 mM Ac

4.1.5 Tissue ATP content. (Table 4.3 and Figure 4.3)

Tissue ATP levels in control hearts, which were freeze clamped after 30 minutes of pre-ischaemic perfusion, was 22.2±0.5 umol/g d.w. Tissue ATP content decreased ($p \leq 0.05$) at the onset of IC in all Ac treated hearts and declined even further after 30 minutes of ischaemia with values ranging from 9.5±0.9 umol/g d.w (30 mM Ac) to 12.4±1.0 umol/g d.w. (5 mM Ac). There was however no difference in ATP content amongst any of the groups at either the onset of IC or end of ischaemic period.

4.1.6 Tissue CP content. (Table 4.3 and Figure 4.4)

The tissue CP content prior to ischaemia was 39.56±0.42 umol/g d.w.. At the onset of IC it had decreased in all groups to values that ranged from 16.38±1.85 umol/g d.w (1 mM Ac) to 20.91±1.16 umol/g d.w. (10 mMAc). By the end of the ischaemic period, the tissue CP levels diminished even further in the 10 mM Ac hearts. There were no differences in tissue CP content amongst the Ac hearts at the various intervals.

4.1.7 Tissue Lactate content. (Table 4.3 and Figure 4.5)

Tissue lactate level in control hearts measured 1.5±0.4 umol/g d.w. Lactate levels had increased ($p \leq 0.05$) in all hearts at onset of IC, with the highest levels observed in the hearts treated with 30 mM Ac (7.0±0.9 umol/g d.w) and lowest in 1mM Ac (3.28±0.37 umol/g d.w) hearts. Tissue lactate levels in 1mM Ac treated hearts were significantly lower than that of 30 mM Ac at the onset of IC. After 30 minutes of ischaemia the tissue lactate content of hearts perfused with 10 mM and 30 mM Ac increased even further. Differences within the groups was again only significant between 1 mM Ac and 30 mM Ac ($p \leq 0.01$) at the end of the ischaemic period.

4.1.8 Tissue Glycogen content. (Table 4.3 and Figure 4.6)

Glycogenolysis, as indicated by reduced tissue glycogen levels, had increased in all Ac hearts both at the onset of IC and after 30 minutes of ischaemia. The smallest decrease ($p \leq 0.05$) in tissue glycogen at the end of the ischaemic period was observed in 5 mM Ac (12.6±3.4 umol/g d.w) with the largest decline ($p \leq 0.0005$) noted in 1mM (7.66±0.55 umol/g d.w) and 10 mM Ac (4.8±1.2 umol/g d.w.). There were, however, no significant differences in tissue glycogen content amongst the Ac hearts.

4.1.9 Tissue cAMP content. (Table 4.3 and Figure 4.7)

Compared to cAMP levels prior to ischaemia, there was an increase in tissue cAMP at the onset of IC in all groups ($p \leq 0.01$). The greatest increase was noted in hearts perfused with 30 mM Ac (4.1±0.3 nmol/g d.w) ($p \leq 0.0005$) and was associated with a shorter time to the onset of IC. cAMP levels at the end of the ischaemic period was not different to the control values. There was no significant variation in cAMP amongst the Ac hearts at either the onset of IC or after the 30 minutes of ischaemia.

Table 4.3 Tissue ATP, CP, lactate, glycogen, and cAMP content determined prior to ischaemia (30 min), at the onset of ischaemic contracture (OIC) and at the end of the ischaemic period (60 min). Ac = acetate; Glu = glucose

	Substrate	ATP (umol/g d.w.)	CP (umol/g d.w.)	Lactate (umol/g d.w.)	Glycogen (umol/g d.w.)	cAMP (nmol/g d.w.)
30 min	**(control)**	22.2±0.5	39.56±0.42	1.5±0.4	62.5±10.6	2.3±0.1
OIC	**1 mM Ac**	16.11±0.8#	16.38±1.85#	3.28±0.37**	30.19±5.95*	3.48±0.14#
	5 mM Ac	17.5±1.2**	20.02±1.07#	4.8±0.4#	27.4±5.4*	3.8±0.2#
	10 mM Ac	17.9±0.9**	20.91±1.16#	5.6±1.1**	27.6±4.7*	3.9±0.3**
	30 mM Ac	18.5±0.3#	19.58±1.67#	7.0±0.9#	33.7±5.3*	4.1±0.3#
	10 mM Glu	21.6±0.5	18.68±1.96#	11.8±1.7#	42.6±9.0	3.0±0.2**
60 min	**1 mM Ac**	12.21±0.6#	17.79±1.21#	3.56±0.24**	7.66±0.55#	2.33±0.27
	5 mM Ac	12.4±1.0#	18.81±1.44#	11.5±4.0*	12.6±3.4*	2.2±0.2
	10 mM Ac	10.0±1.2*	12.73±2.35#	13.8±2.8#	4.9±1.2#	2.4±0.1
	30 mM Ac	9.5±0.9#	17.13±1.07#	23.2±2.7#	16.0±5.4**	2.7±0.3
	10 mM Glu	19.0±1.5	19.33±0.64#	12.18±1.3**	43.0±4.5	2.4±0.1

*$p \leq 0.05$ vs control (30 min); **$p \leq 0.01$ vs control (30 min); #$p \leq 0.0005$ vs control (30 min)

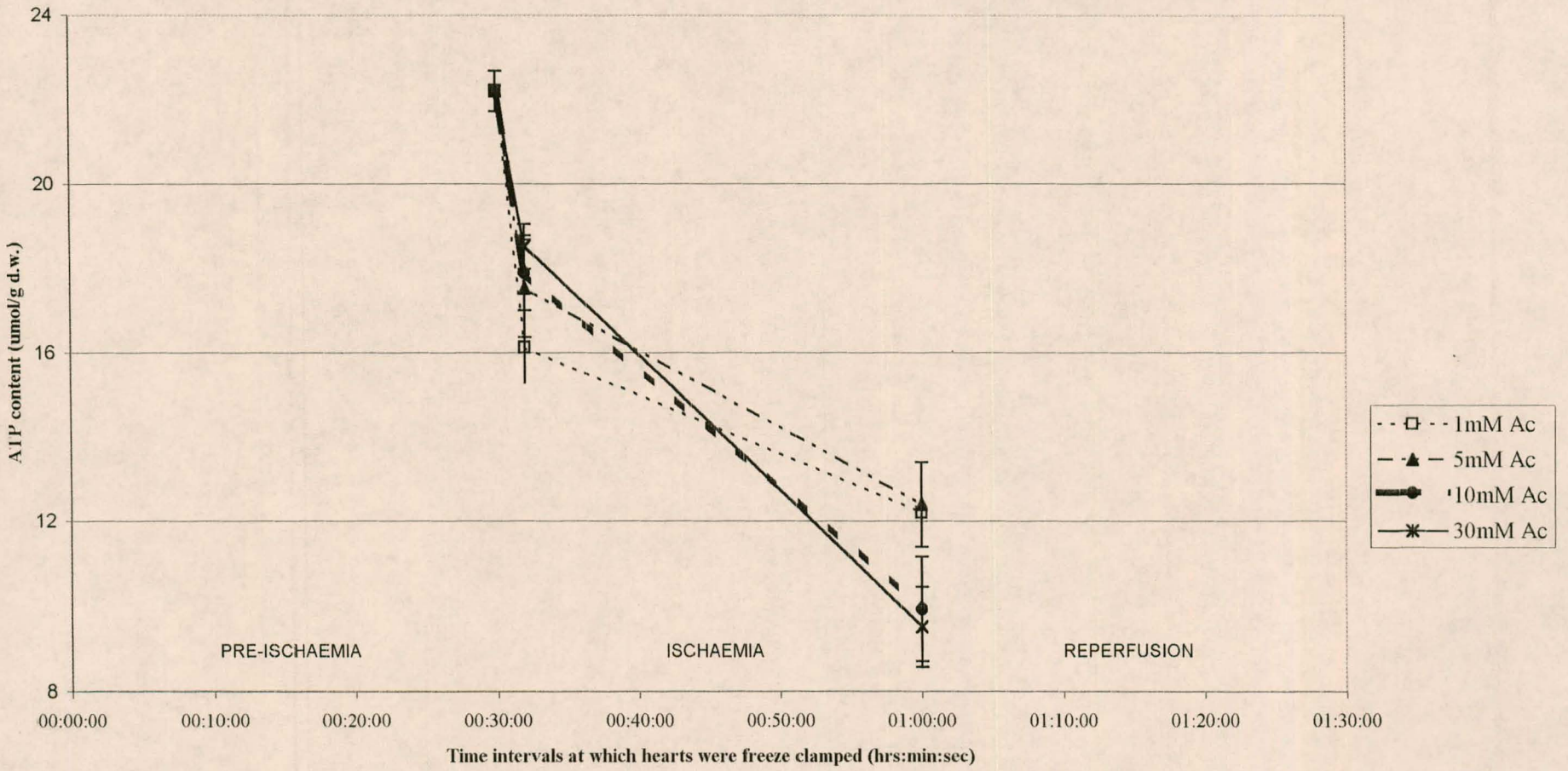


Figure 4.3 Tissue ATP content in acetate (Ac) perfused hearts

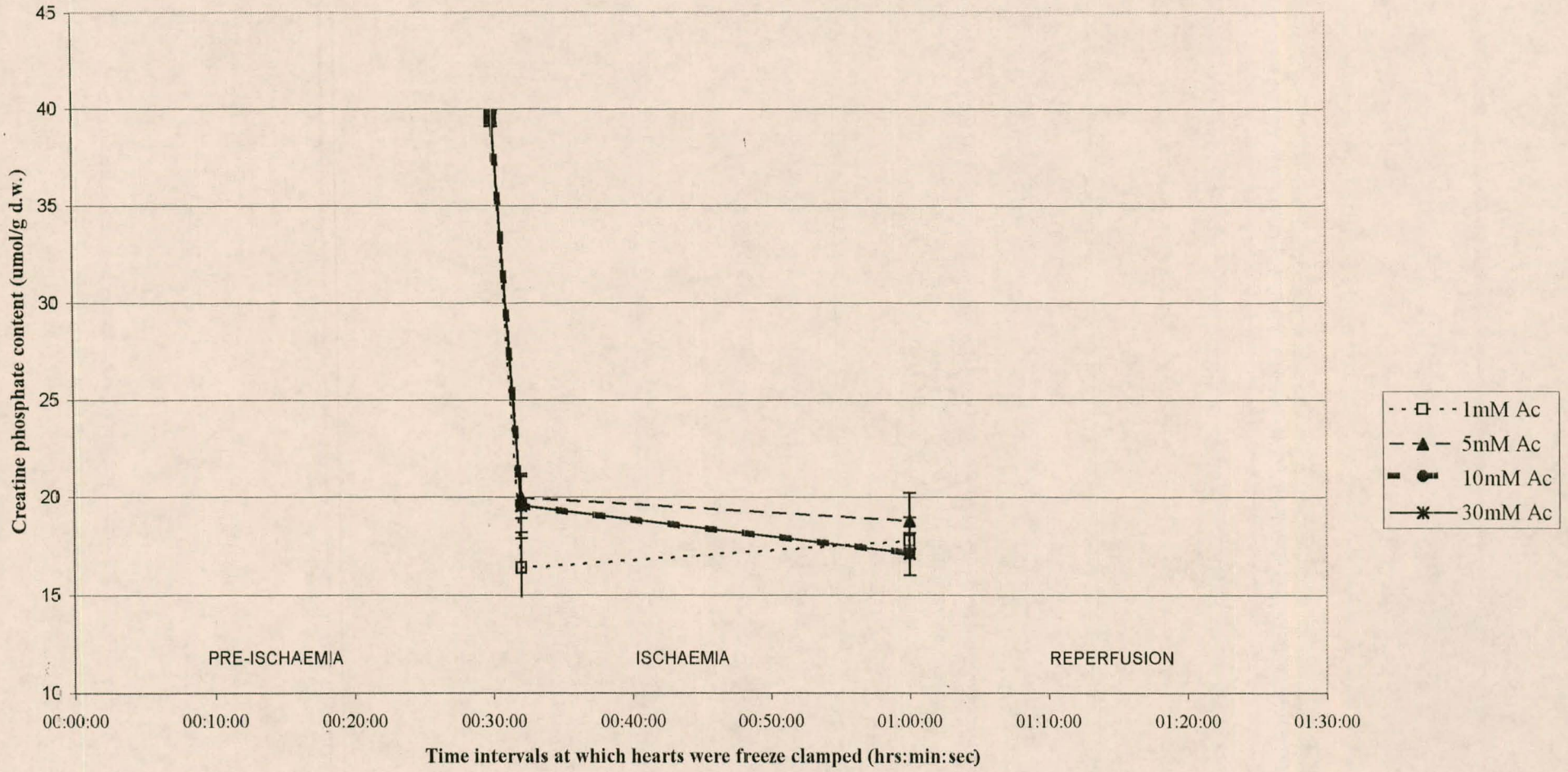


Figure 4.4 Tissue creatine phosphate content in acetate (Ac) perfused hearts

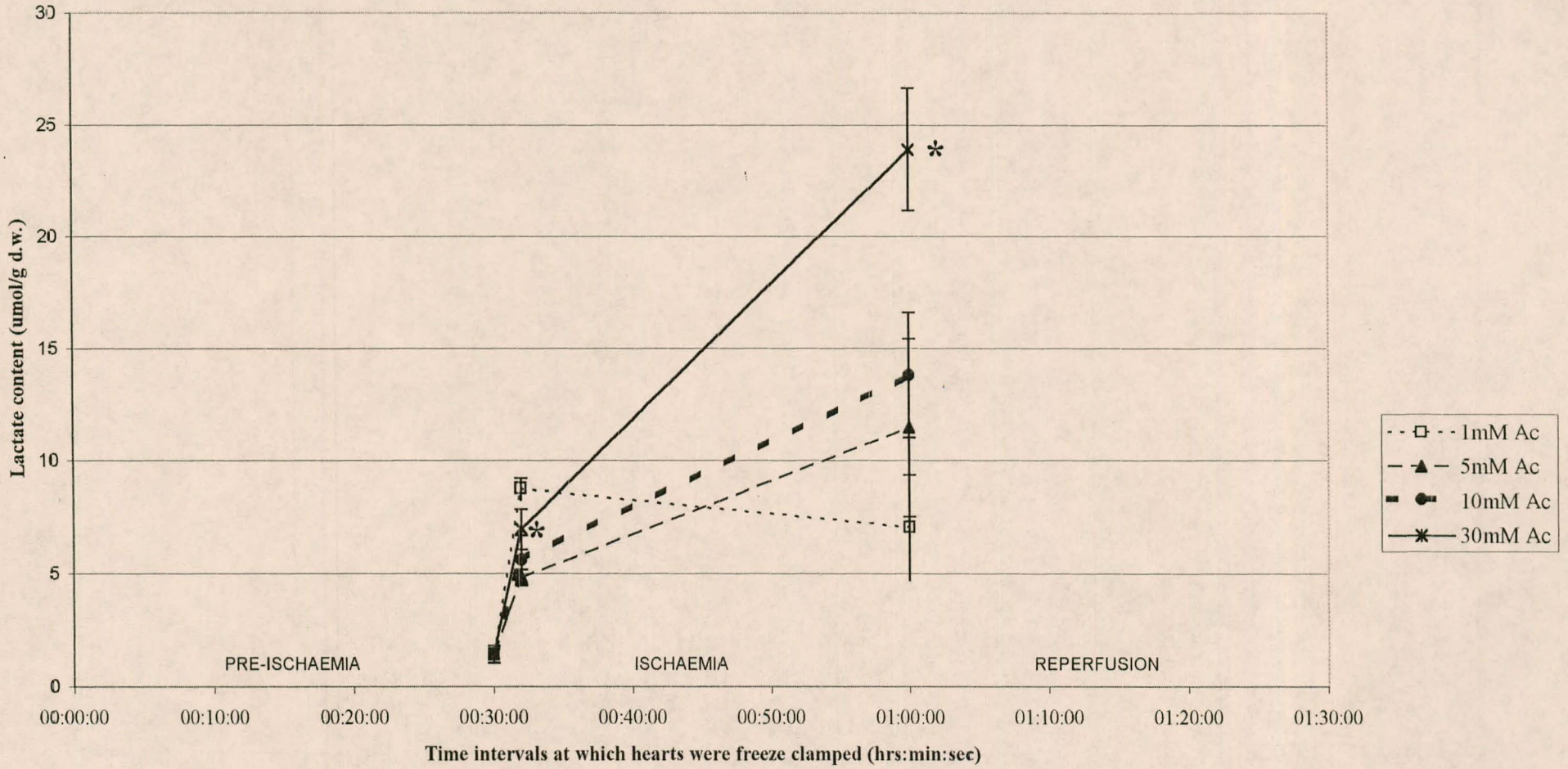


Figure 4.5 Tissue lactate content in acetate (Ac) perfused hearts. *$p<0.05$ vs 1mM Ac

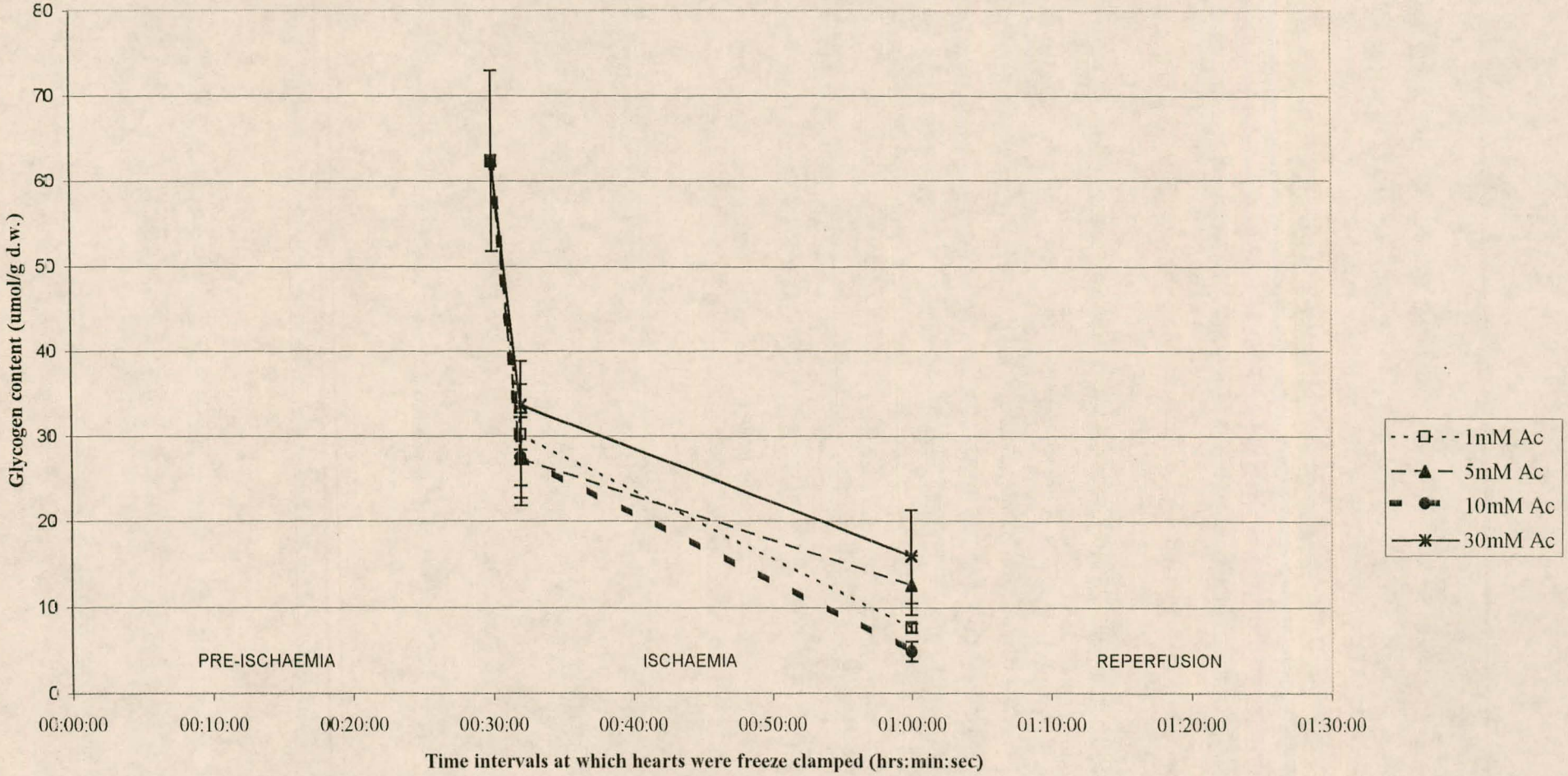


Figure 4.6 Tissue glycogen content in acetate (Ac) perfused hearts

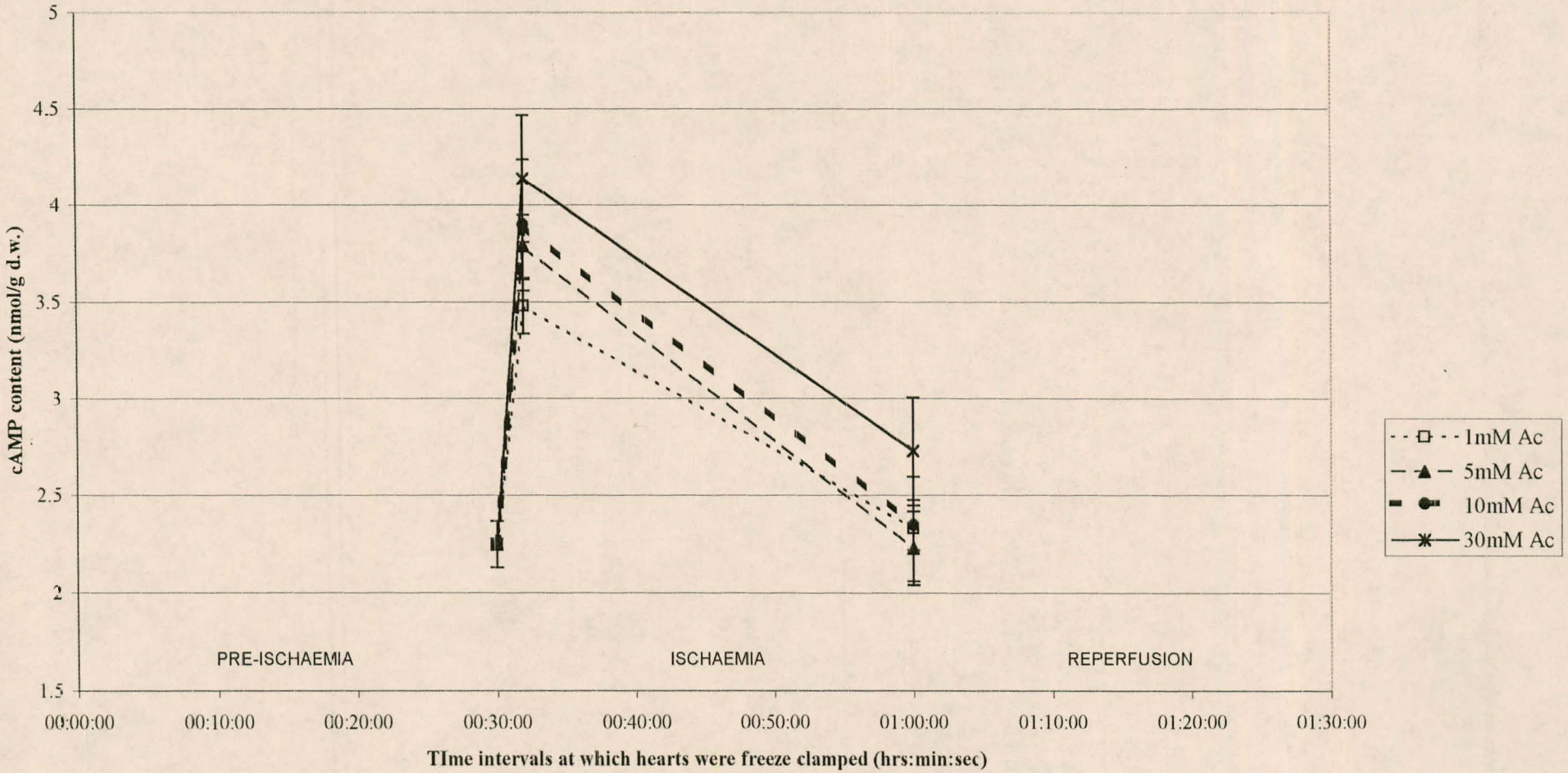


Figure 4.7 Tissue cAMP content in acetate (Ac) perfused hearts

4.2 Aim 2: To determine the effect of oxygen availability during ischaemia on the beneficial effect of glucose and acetate

4.2.1 Heart Rate (Table 4.4)

Initial HR in 5 mM Ac and 10 mM Glu hearts were 323 beats/min and 262 beats/min respectively with no significant difference in the pre-ischaemic-anoxic HR between the two groups. However, after 5 minutes of ischaemia-anoxia, the HR in both groups decreased significantly ($p \leq 0.001$) to 90.8±90.8 (5 mM Ac) and 114.0±25.4 beats/min. (10 mM Glu). They remained low throughout the ischaemic-anoxic period and recovered marginally during the reperfusion phase. After 25 minutes of reperfusion, the Ac hearts had recovered to 21.3±4.4 beats/min (individual values: */ 18/ */ */ 16/ 30 beats/min.; * indicates that the hearts fibrillated so the HR could not be measured) while Glu treated hearts recovered to 22.0±7.9 beats/min (14/ */ 130/ */ */ * beats/min). There was however no significant difference in the HR between the groups at this point due to the large difference in SEM.

4.2.2 Coronary Flow (Table 4.5)

There was no significant difference in the pre-ischaemic-anoxic CF between the two groups. During the ischaemic-anoxic period the CF was regulated at 2 ml/min. The recovery of CF upon reperfusion was equally poor ($p \leq 0.001$) in both the Ac and Glu treated hearts. By the end of the experiment, CF in the Ac perfused hearts had only recovered to 6.0±1.5 ml/min. with Glu recovering to 4.0±1.4 ml/min.

Table 4.4 Heart Rate (beats/min) at different stages during the experiment in ischaemic-anoxic hearts. Ac = acetate, Glu = glucose.

	Time (min)	5 mM Ac	10 mM Glu
Pre - ischaemic Phase	5	322.5±12.1	307.5±16.2
	15	287.5±17.1	276.7±30.9
	25	275.0±13.8	262.5±18.9
Ischaemic-anoxic Phase	35	90.8±90.8	114.0±25.4
	45	*	37.5±7.5
	55	*	53.0±26.1
Reperfusion Phase	65	23.5±7.6	46.0±37.0
	75	26.0±2.4	18.0±0.0
	85	21.3±4.4	22.0±7.9

*HR could not be determined as there was no visible developed pressure or ECG recording after approximately 5 minutes of ischaemia-anoxia.

Table 4.5 Coronary flow (ml/min) at different stages during the experiment in ischaemic-anoxic hearts. Ac = acetate, Glu = glucose.

	Time (min)	5 mM Ac	10 mM Glu
Pre - ischaemic Phase	5	17.2±0.4	15.5±1.2
	15	16.7±0.7	14.5±1.3
	25	15.1±0.5	13.9±0.7
Ischaemic-anoxic Phase	35	2.0±3.4	1.9±0.1
	45	2.0±3.1	2.0±0.02
	55	2.0±2.1	2.0±0.0
Reperfusion Phase	65	5.0±1.1	2.3±0.8
	75	5.5±1.3	3.0±0.9
	85	6.0±1.5	4.0±1.4

4.2.3 Time to the onset of ischaemic contracture (TOIC) (Figure 4.8)

There was no significant difference in the TOIC between the Ac (63.0±6.01 s) and Glu (64.0±9.5 s) treated hearts.

4.2.4 Percentage recovery of left ventricular developed pressure (LVDP) (Figure 4.9)

There was no significant difference in the percentage recovery of LVDP between the Ac (0.5±0.5) and Glu (2.0±2.0) treated hearts. One out of six hearts recovered in each group which accounts for the unusual SEM.

4.2.5 Tissue ATP content (Table 4.6 and Figure 4.10)

Prior to ischaemia-anoxia tissue ATP levels measured 22.2±0.5 umol/g d.w. By the onset of IC it had declined ($p \leq 0.001$) to 14.7±1.0 (Ac) and 13.9±0.8 umol/g d.w. (Glu) and continued to decrease throughout the ischaemic-anoxic period. There was no difference in ATP content between the two groups at the onset of IC or after 30 minutes of ischaemia-anoxia.

In comparison with the ischaemic groups, the ischaemic-anoxic hearts showed a sharper decline in tissue ATP content at the onset of IC. By the end of the ischaemic period the ATP levels in ischaemic-anoxic hearts were significantly lower than that of the ischaemic, particularly the Glu treated hearts.

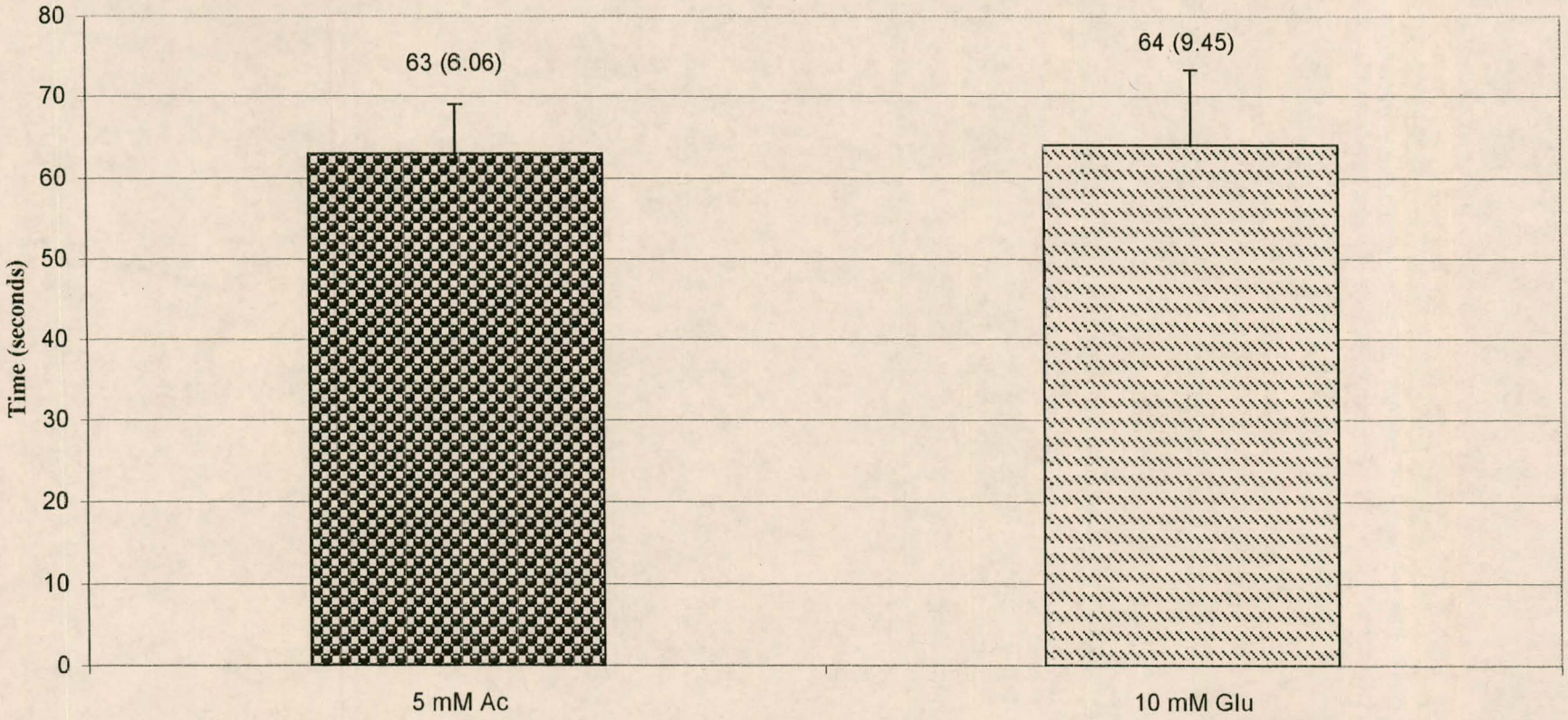


Figure 4.8 Time to onset of ischaemic contracture (TOIC) in ischaemic-anoxic hearts perfused with either 5 mM acetate (Ac) or 10 mM glucose (Glu).

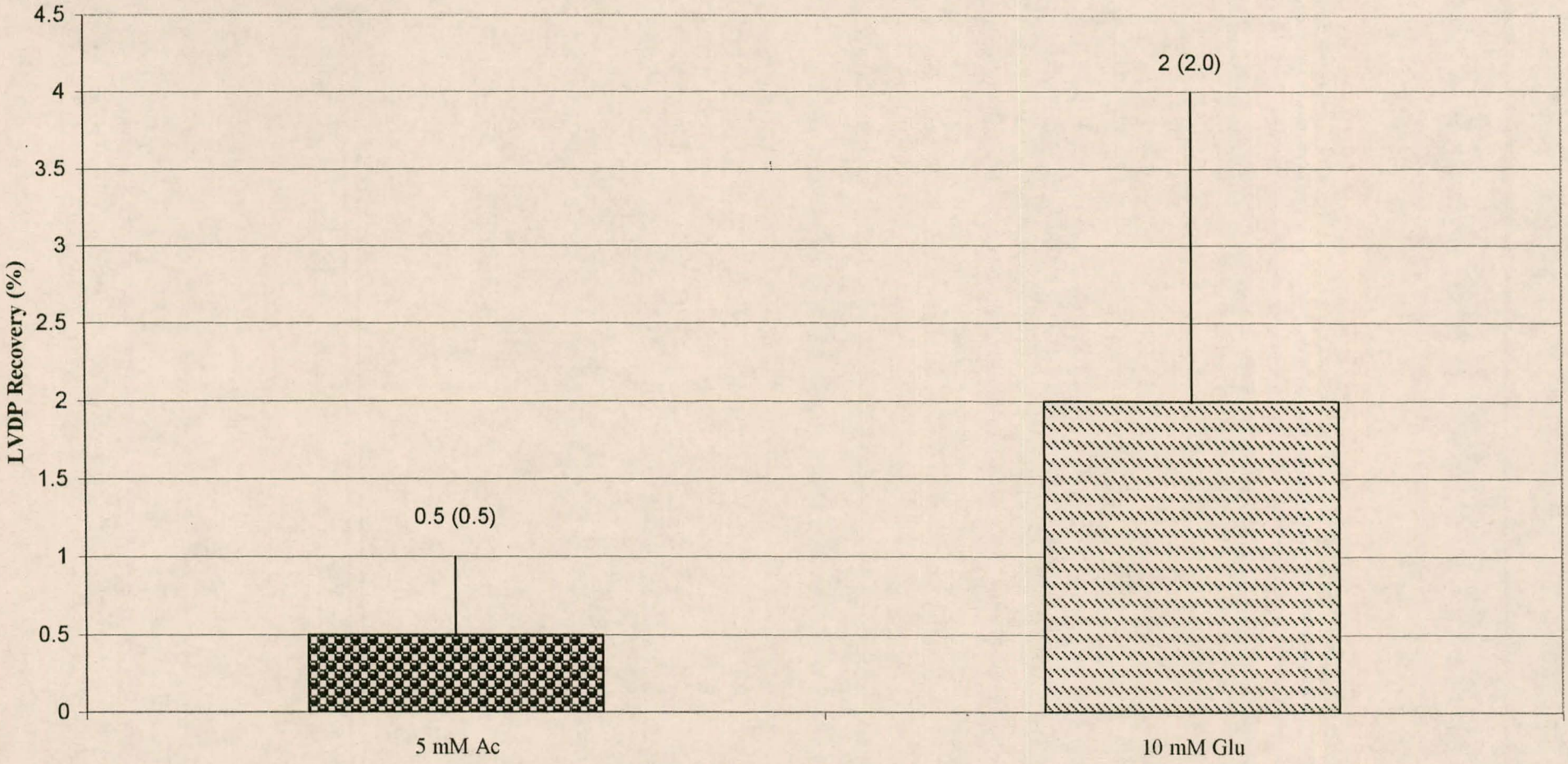


Figure 4.9 Percentage recovery of left ventricular developed pressure (LVDP) in ischaemic-anoxic hearts perfused with either 5 mM acetate (Ac) or 10 mM glucose (Glu).

4.2.6 Tissue CP content (Table 4.6 and Figure 4.11)

Pre-ischaemic tissue CP levels measured 39.6 ± 0.4 umol/g d.w. During ischaemia-anoxia tissue CP decreased ($p \leq 0.01$) to 10.4±1.5 umol/g d.w. (Ac) and 11.6±0.9 umol/g d.w. (Glu) as measured at the onset of IC. There was no difference between the Ac and Glu hearts at this point. After 30 minutes of ischaemia-anoxia, CP levels in Ac hearts (1.3±0.7 umol/g d.w.) were significantly lower than in the Glu treated hearts. Furthermore, anoxia increased CP break down as shown by the lower CP levels in comparison with the groups subjected to ischaemia during the same period.

4.2.7 Tissue Lactate content (Table 4.6 and Figure 4.12)

The tissue lactate level prior to ischaemia was 1.5±0.4 umol/g d.w. An increase ($p \leq 0.001$) was observed at the onset of IC in both the Ac (16.1±2.5 umol/g d.w.) and Glu (14.8±1.3 umol/g d.w.) hearts. The apparent slight reduction in lactate noted at the end of the ischaemic-anoxic period was not significant. There was also no significant difference in tissue lactate between the Ac and Glu hearts at both the onset of IC and at the end of ischaemia-anoxia. The increase in tissue lactate content during this period was also greater in ischaemic-anoxic hearts than ischaemic groups.

4.2.8 Tissue Glycogen content (Table 4.6 and Figure 4.13)

Compared to the pre-ischaemic levels (62.4 ± 10.6 umol/g d.w.), tissue glycogen content had decreased significantly at the onset of IC in both the Ac (30.6±3.3 umol/g d.w.) and Glu (17.3±1.5 umol/g d.w.) perfused hearts. The glycogen levels had diminished even further by the end of the ischaemic-anoxic period (Ac 6.5±1.4 umol/g d.w.; Glu 6.7±1.8 umol/g d.w.). There was however no differences in tissue glycogen content between the

two groups at the onset of IC or after 30 minutes of ischaemia. While ischaemic 5 mM Ac treated hearts showed glycogen decreases similar to ischaemic-anoxic Glu and Ac perfused hearts, ischaemic Glu hearts lessened the fall in tissue glycogen content significantly.

4.2.9 **Tissue cAMP content** (Table 4.6 and Figure 4.14)

In comparison with tissue cAMP content prior to ischaemia-anoxia (2.3±0.1 nmol/g d.w.), tissue cAMP content increased ($p \leq 0.001$) at the onset of IC in the Ac (4.4±0.3 nmol/g d.w.) and Glu (3.8±0.1 nmol/g d.w.) treated hearts. By the end of the ischaemic-anoxic period, these levels had decreased significantly to 1.4±0.3 nmol/g d.w. (Ac) and 2.3±0.3 nmol/g d.w. (Glu). However, there was no significant difference in tissue cAMP content between the two groups at the onset of IC or after 30 minutes of ischaemia-anoxia.

Ischaemia-anoxia increased the tissue cAMP levels at the onset of IC in Glu treated hearts in comparison with hearts subjected to ischaemia. In this respect, ischaemic-anoxic glucose treated hearts performed just as poorly as those perfused with 5 mM Ac subjected to anoxia and or ischaemia. By the end of the ischaemic-anoxic period however, ischaemic-anoxic 5 mM Ac hearts had significantly lower cAMP levels than the other groups.

Table 4.6 Tissue ATP, CP, lactate, glycogen, and cAMP levels in ischaemic-anoxic hearts determined prior to ischaemia (30 min), at the onset of ischaemic contracture (OIC) and at the end of the ischaemic period (60 min). Ac = acetate; Glu = glucose

	Substrate	ATP (umol/gd.w.)	CP (umol/gd.w.)	Lactate (umol/g .w.)	Glycogen (umol/gd.w.)	cAMP (nmol/gd.w.)
30 min	**(control)**	22.2±0.5	39.6±0.4	1.5±0.4	62.4±10.6	2.3±0.1
OIC	**5 mM Ac**	14.7±1.0**	10.4±1.5*	16.1±2.5**	30.6±3.3*	4.4±0.3**
	10 mM Glu	13.9±0.8**	11.6±0.9*	14.8±1.3**	17.3±1.5**	3.8±0.1**
60 min	**5 mM Ac**	7.2±0.6**	1.3±0.7**	14.8±7.8	6.5±1.4**	1.4±0.3
	10 mM Glu	9.3±1.9**	6.6±1.7**	14.6±3.7	6.7±1.8**	2.3±0.3

*p≤0.05 vs control (30 min); **p≤0.001 vs control (30 min)

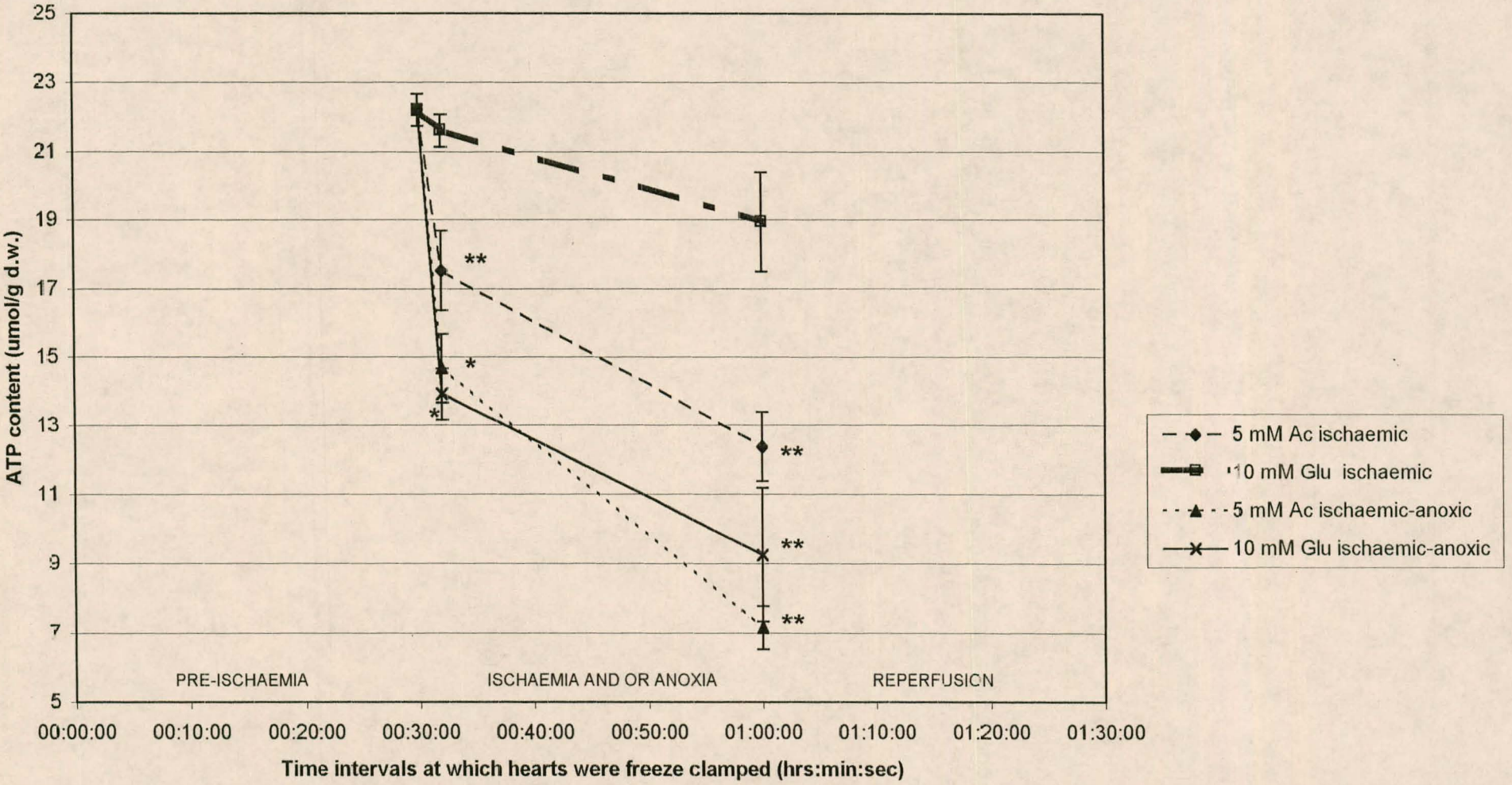


Figure 4.10 Tissue ATP content in ischaemic and ischaemic-anoxic hearts perfused with either 5 mM acetate (Ac) or 10 mM glucose (Glu). *p<0.05; **p< 0.001 vs 10 mM Glu ischaemic

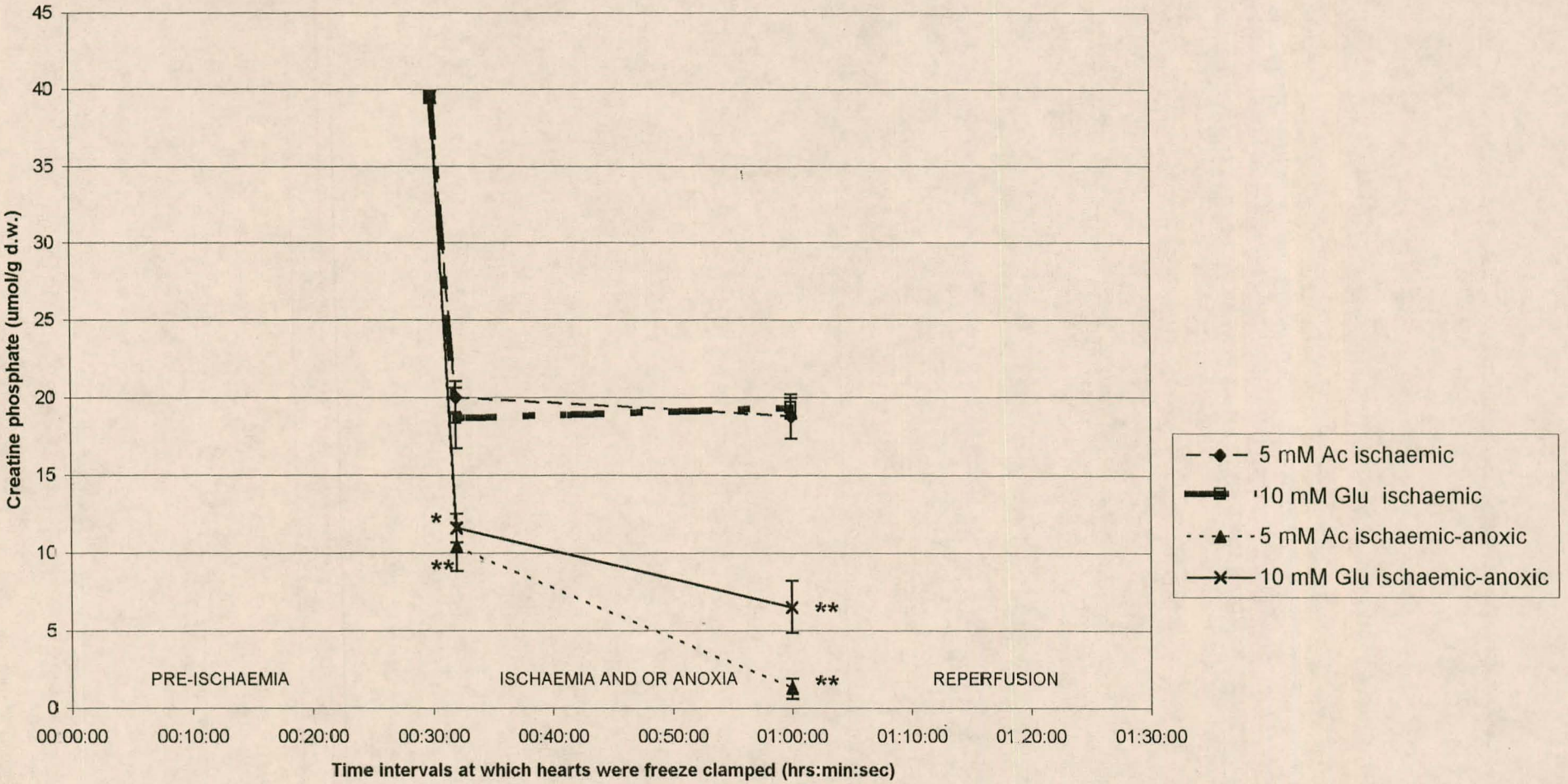


Figure 4.11 Tissue creatine phosphate content in ischaemic and ischaemic-anoxic hearts perfused with either 5 mM acetate (Ac) or 10 mM glucose (Glu). *p<0.05; **p< 0.001 vs 10 mM Glu and 30 mM Ac ischaemic

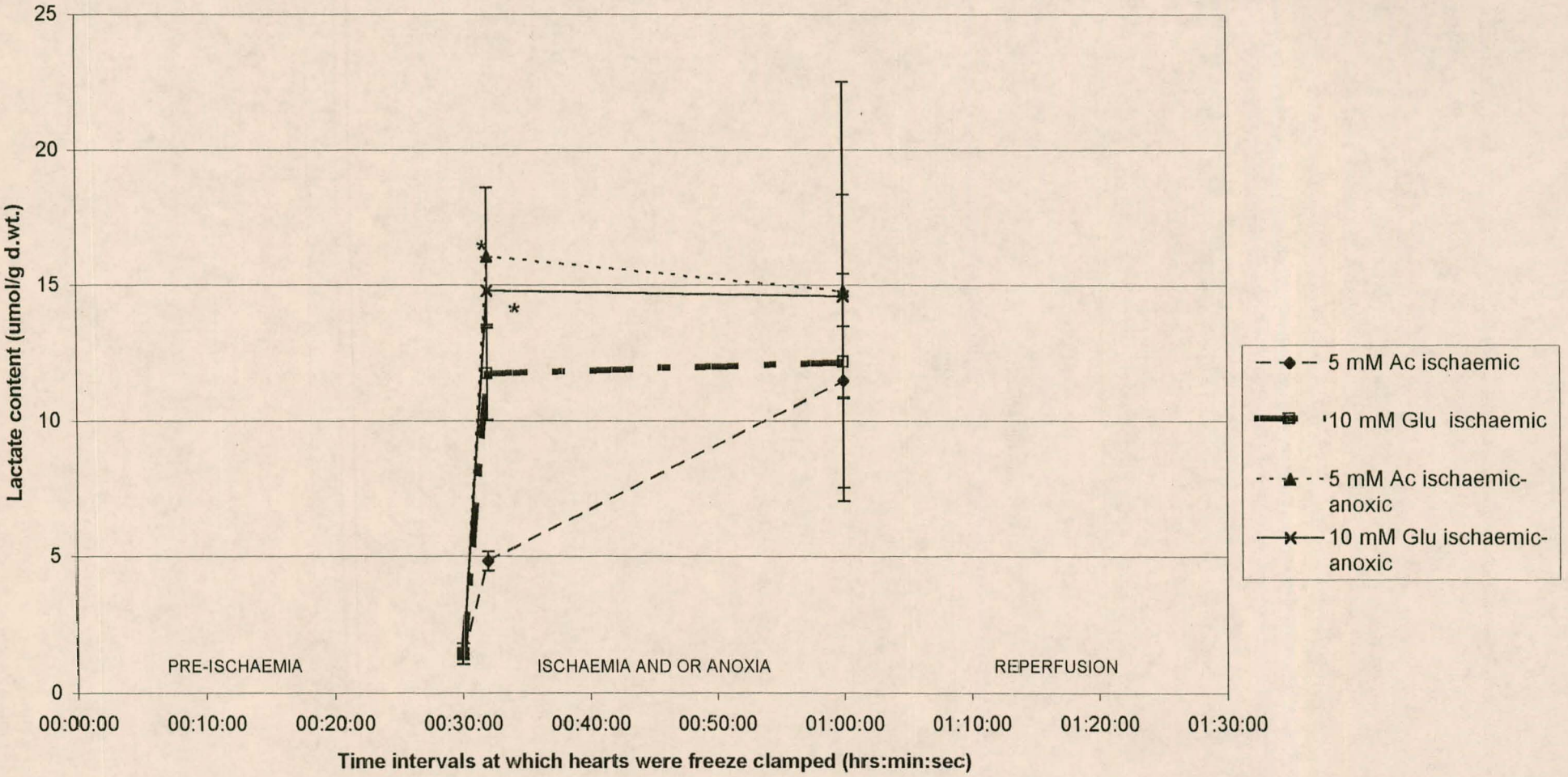


Figure 4.12 Tissue lactate content in ischaemic and ischaemic-anoxic hearts perfused with either 5 mM acetate (Ac) or 10 mM glucose (Glu). *$p<0.01$ vs 5 mM Ac ischaemic

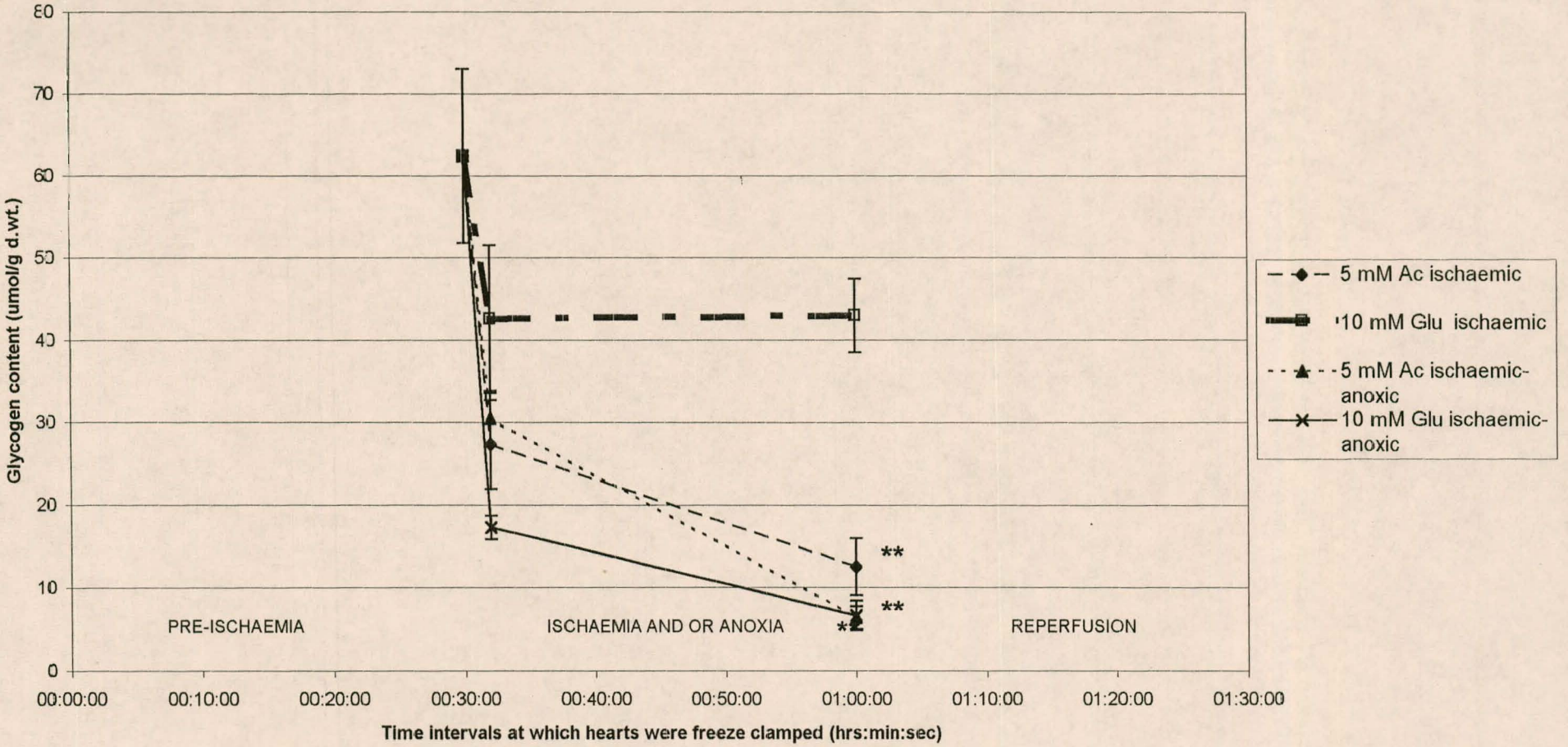


Figure 4.13 Glycogen content in ischaemic and ischaemic-anoxic hearts perfused with either 5 mM acetate (Ac) or 10 mM glucose (Glu). **p<0.001 vs 10 mM Glu ischaemic

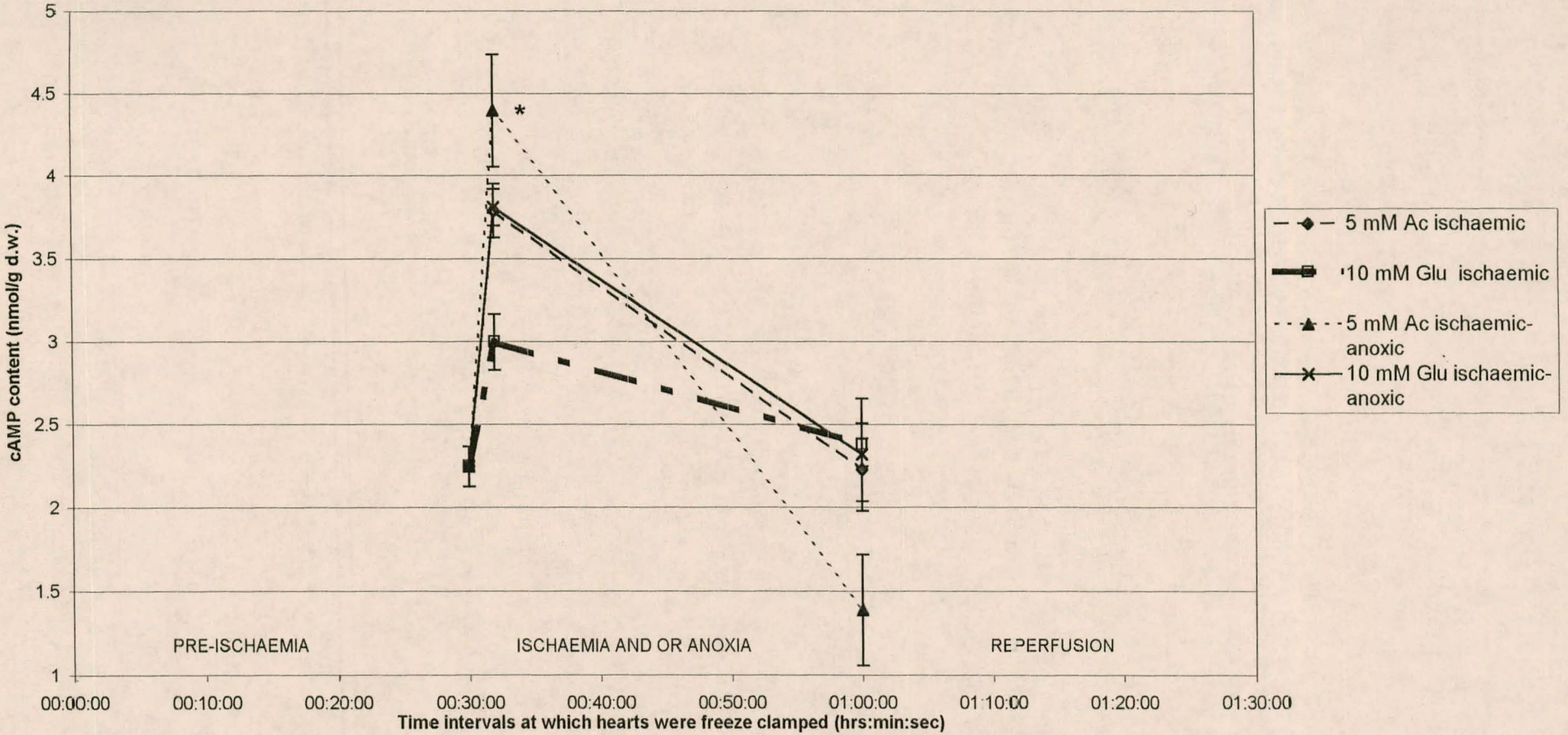


Figure 4.14 Tissue cAMP content in ischaemic and ischaemic-anoxic hearts perfused with either 5 mM acetate (Ac) or 10 mM glucose (Glu). *$p<0.01$ vs 10 mM Glu ischaemic

4.3 Aim 3: To ascertain whether equi-carbon concentrations of glucose and acetate offer similar protection during subtotal ischaemia

4.3.1 Heart Rate (Table 4.1; p. 52)

The pre-ischaemic HR in the two groups was similar, but after 5 minutes of ischaemia the HR had declined to 106±24.4 beats/min (Ac) and 135.0±21.2 beats/min (Glu). By the end of the ischaemic period the HR in Ac treated hearts had declined even more to 60.0±12.8 beats/min while than the Glu perfused hearts had HR of 162.5±18.5 beats/min.. However, after five minutes of reperfusion, the Ac HR recovered to 170.0±52.1 beats/min which was not significantly different to that of Glu treated hearts at 235.0±6.3 beats/min.

4.3.2 Coronary Flow (Table 4.2; p. 53)

Variations in coronary flow followed a trend similar to that of the HR. Pre-ischaemic CF rates were similar, while ischaemic rates were regulated at 2ml/min. Recovery of coronary flow was initially delayed in Ac hearts (9.6±0.7 ml/min) in comparison ($p \leq 0.01$) with that of Glu (13.2±0.8) which may indicate some degree of stunning in the Ac hearts. There were no significant differences in CF after 90 minutes of perfusion.

4.3.3 Time to Onset of Ischaemic Contracture (TOIC) (Table 4.7)

Glucose abolished IC while Ac accelerated the onset to IC (65 ± 9.8 s).

4.3.4 Percentage recovery of Left Ventricular Developed Pressure (LVDP) (Table 4.7)

With 30 mM Ac as substrate, the recovery of LVDP was 23.8±2.8 % whereas hearts perfused with 10 mM glucose showed improved recoveries of 79.8±4.1 % ($p \leq 0.0005$).

Table 4.7 Time to the onset of ischaemic contracture (TOIC) and the percentage recovery of left ventricular developed pressure (LVDP) in hearts perfused with either acetate (Ac) or glucose (Glu)

	30 mM Ac	**10 mM Glu**
TOIC (seconds)	65.0 ± 9.8	None
% Recovery LVDP	23.8 ± 2.8	79.8 ± 4.1

4.3.5 Tissue ATP content (Table 4.3 and Figure 4.15)

Tissue ATP levels declined during ischaemia in the Ac hearts only. The difference in ATP between Glu and Ac was already noticeable at the onset of IC ($p \leq 0.0005$) while levels in Glu hearts (19.0±1.5 umol/g d.w.) was double that of Ac (9.5±0.9 umol/g d.w.) perfused hearts after 30 minutes of ischaemia ($p \leq 0.0005$).

4.3.6 Tissue CP content (Table 4.3 and Figure 4.16)

Creatine phosphate also decreased during ischaemia, but there were no significant differences between the Ac and Glu hearts.

4.3.7 Tissue Lactate content (Table 4.3 and Figure 4.17)

Ac treated hearts showed more of an increase in lactate content than glucose hearts at the onset of IC ($p \leq 0.05$). By the end of the ischaemic period, lactate levels in Ac hearts (23.2±2.7 umol/g d.w.) exceeded that of Glu hearts (12.18±1.3 umol/g d.w.)($p \leq 0.005$).

4.3.8 Tissue Glycogen content (Table 4.3 and Figure 4.28)

Glu had a glycogen sparing effect while glycogen mobilisation was increased in Ac hearts. The decrease in tissue glycogen at the onset of IC was similar in both groups. However Glu abated a further reduction in glycogen (43.0±4.5 umol/g d.w.) while Ac had the opposite effects (16.0±5.4 umol/g d.w.) ($p \leq 0.005$).

4.3.9 Tissue cAMP content (Table 4.3 and Figure 4.19)

The increase in cAMP observed at the onset of IC was greater in Ac (4.1±0.3 nmol/g d.w.) hearts compared to Glu (3.0±0.2 nmol/g d.w.) treated hearts ($p \leq 0.05$). Tissue cAMP levels had declined similarly in both groups by the end of the ischaemic period.

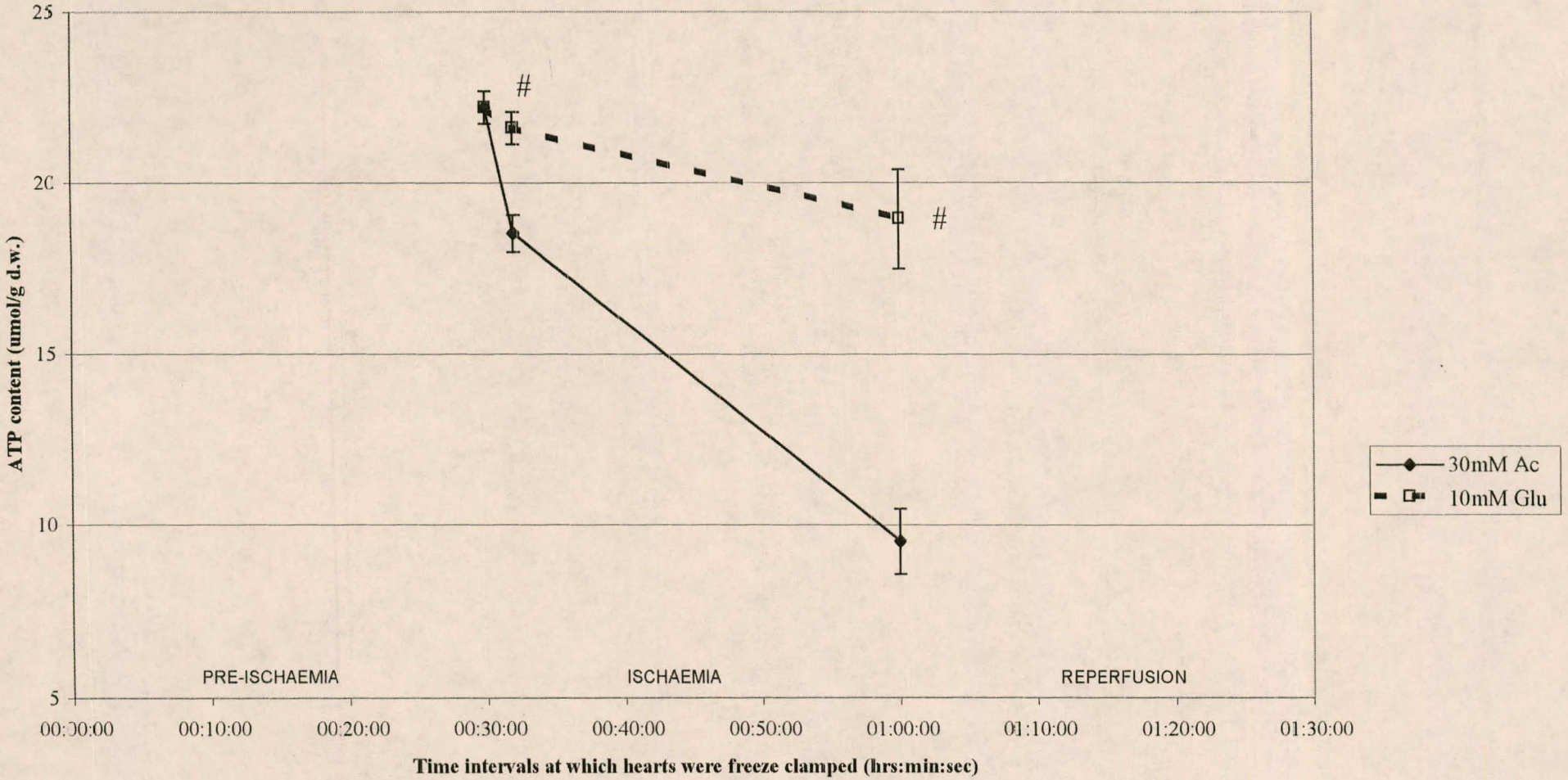


Figure 4.15 Tissue ATP content in hearts perfused with either 30 mM acetate (Ac) or 10 mM glucose (Glu). #p<0.0005 vs 30 mM Ac

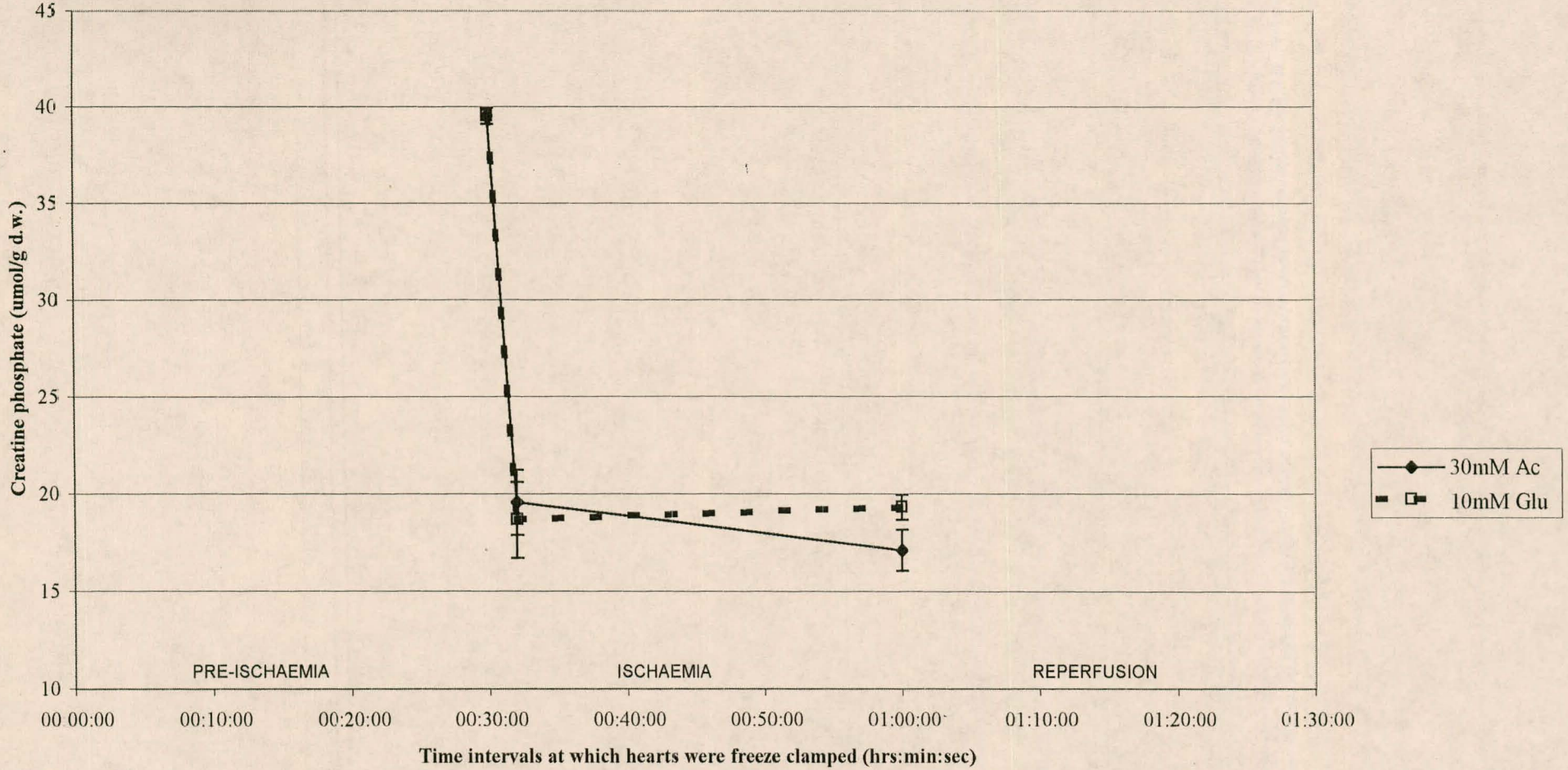


Figure 4.16 Tissue creatine phosphate content in hearts perfused with either 30 mM acetate (Ac) or 10 mM glucose (Glu)

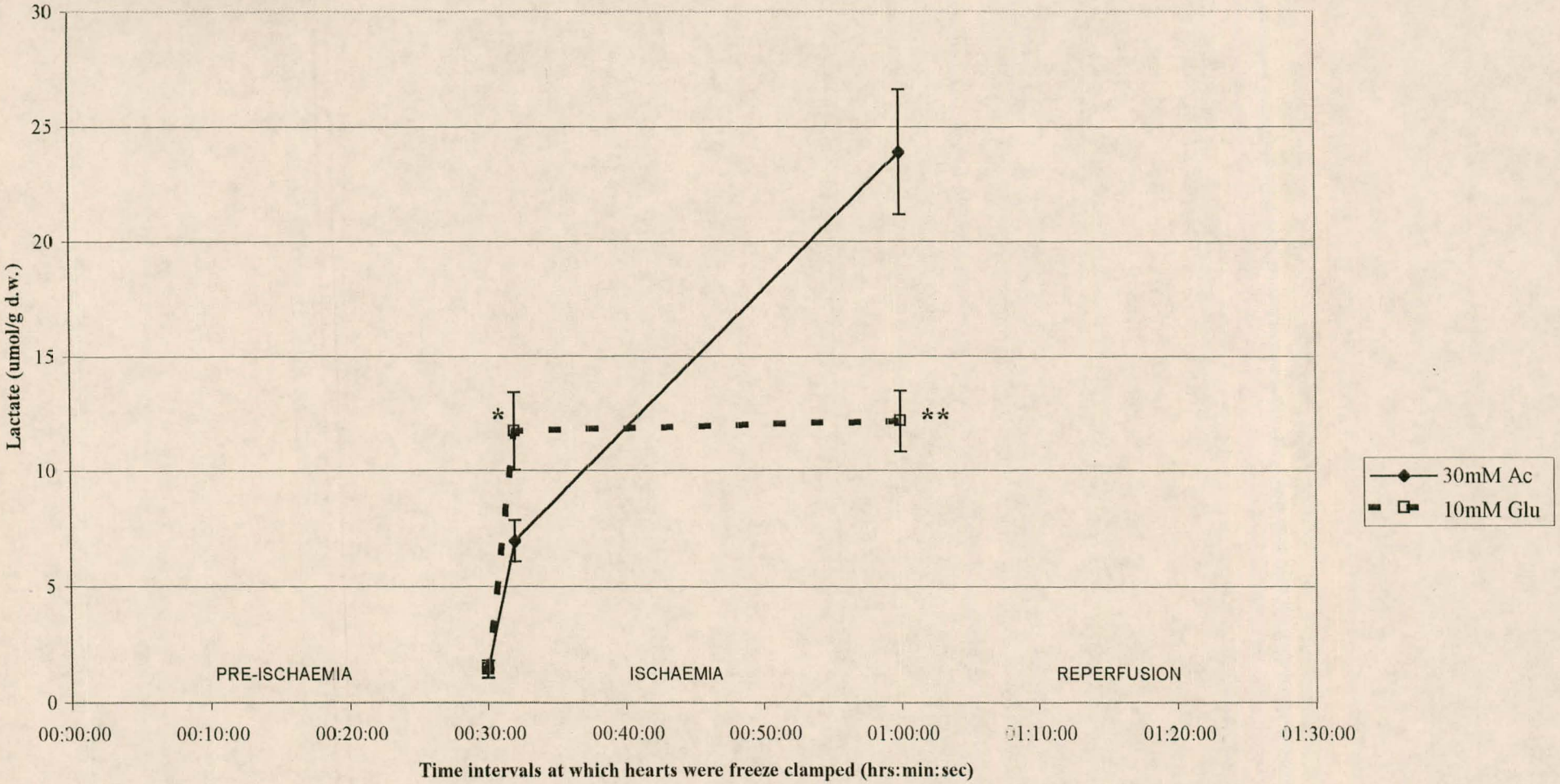


Figure 4.17 Tissue lactate content in hearts perfused with either 30 mM acetate (Ac) or 10 mM glucose (Glu). *p<0.05; **p<0.005 vs 30 mM Ac

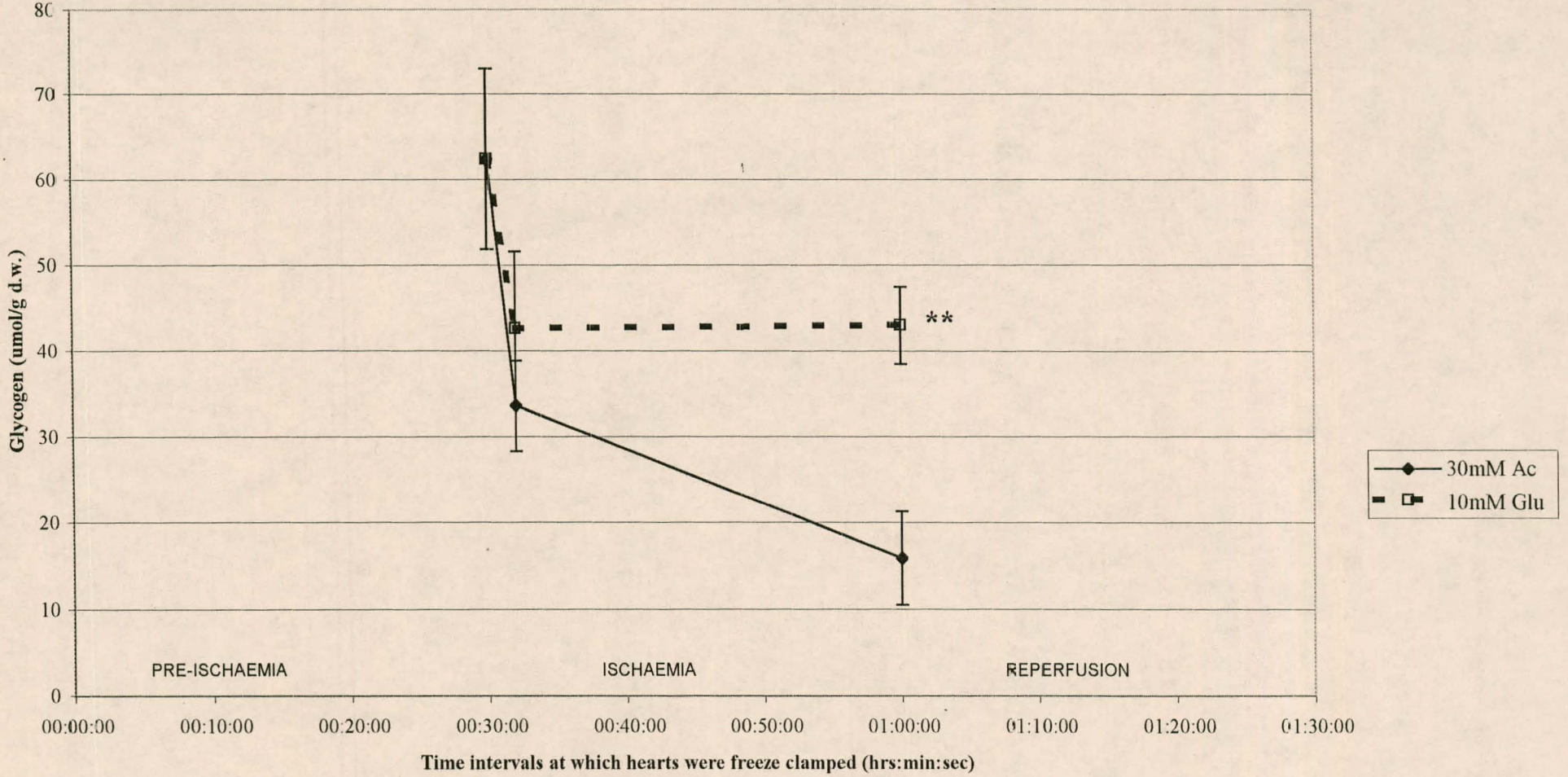


Figure 4.18 Tissue glycogen content in hearts perfused with either 30 mM acetate (Ac) or 10 mM glucose (Glu). **p<0.005 vs 30 mM Ac

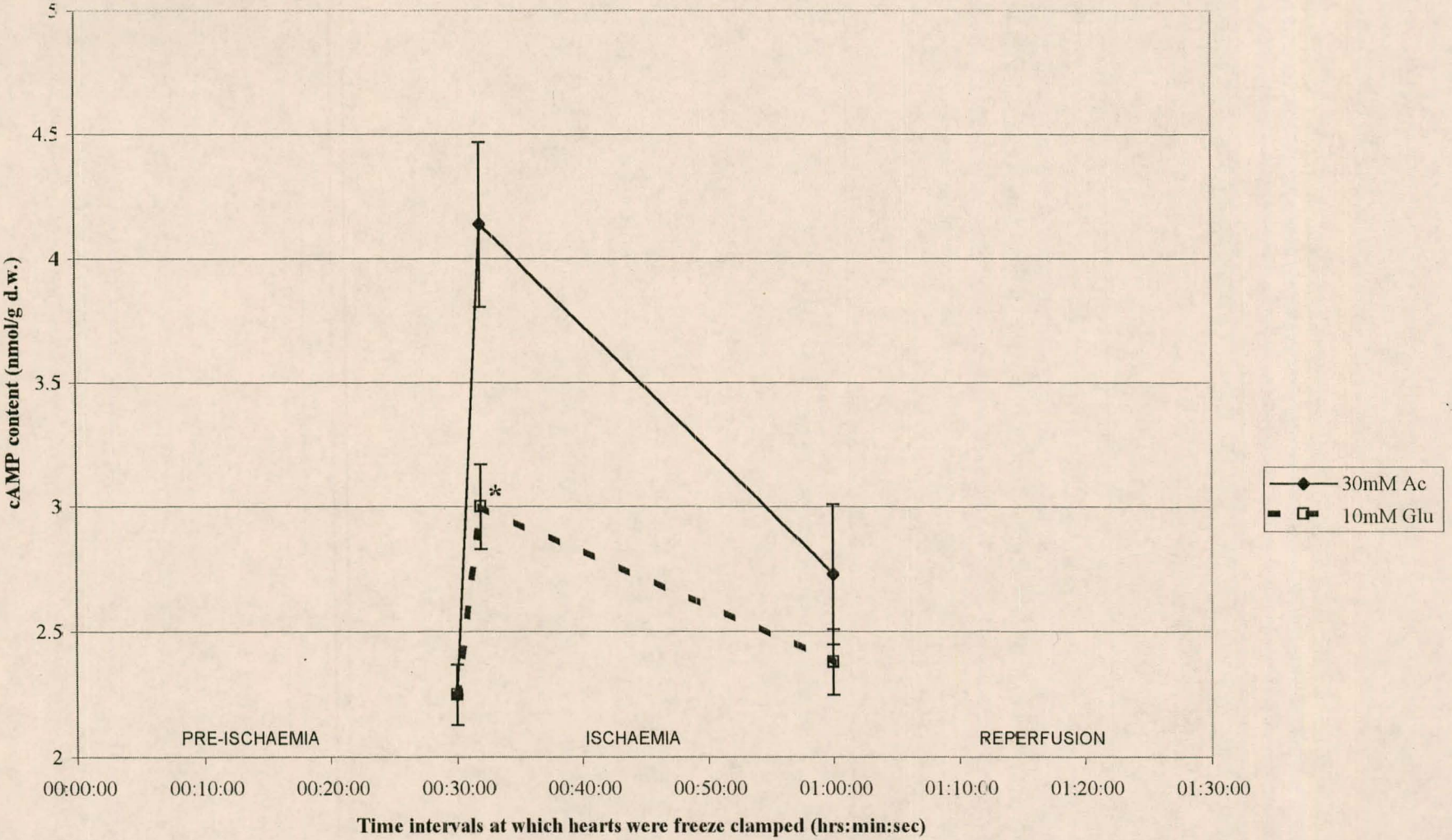


Figure 4.19 Tissue cAMP content in hearts perfused with either 30 mM acetate (Ac) or 10 mM glucose (Glu). * $p<0.05$ vs 30 mM Ac

Chapter 5

DISCUSSION

5.1 Acetate dose response

The rate of utilisation of any substrate depends on, amongst others, the substrate concentration and oxygen delivery to the myocardium (Neely et al., 1972). Previous studies involving acetate as a substrate utilised concentrations varying from 3,6 to 10 mM (Williamson, 1964; Bricknell and Opie, 1978; Owen and Opie, 1990). This study implies that the optimum concentration for the provision of acetate to the isolated perfused rat heart during subtotal ischaemia appears to be 5 mM and 10 mM when the ischaemic coronary flow was regulated at 2 ml/min. These concentrations had fewer deleterious effects indicated by improved reperfusion recovery and delayed TOIC.

The biochemical data showed no significant differences amongst the groups. The TOIC and percentage recovery of LVDP were therefore the only criteria which gave conclusive evidence as to the benefit or harm of a particular acetate concentration.

5.1.1 Ischaemic contracture and percentage recovery of LVDP

In agreement with Ventura-Clapier and Veksler (1994), the systolic pressure decreased visibly within seconds of the onset of ischaemia. Ischaemic contracture developed in under four minutes in all acetate perfused hearts. The difference in TOIC between the 5 mM and 10 mM acetate treated hearts was insignificant, implying that this may be the

optimal range of acetate concentration to use as an ischaemic substrate. Furthermore, hearts with the earliest TOIC displayed the poorest recovery of LVDP. These results support the findings of Apstein et al. (1983) who found low reperfusion recoveries following increased diastolic pressure during the ischaemic phase in isolated perfused rabbit hearts. Since acetate was the sole exogenous substrate, recoveries of 53.5±2.74 % (5 mM Ac) and 48.3±3.9 % (10 mM Ac), despite being low, indicate residual Krebs Cycle activity in maintaining reperfusion function.

Another factor which may have affected the TOIC is the Na^+ concentration of the ischaemic perfusate. The acetate was derived from s*odium* acetate, therefore the incremental increases in the acetate concentration of the ischaemic perfusate was possibly accompanied by similar increases in the Na^+ concentration.

Increased intracellular Na^+ levels may reduce the Na^+ electrochemical gradient across the sarcolemma and lead to reversal of the Na^+ /Ca^+ exchanger. Na^+ extrusion from the cell is thus coupled to Ca^{++} ingress resulting in a rise in cytosolic Ca^{++} levels (Sedlis et al., 1983). Cytosolic Ca^{++} accumulation has been implicated as a cause of IC (Bricknell and Opie, 1978; Kihara et al., 1989; Ventura-Clapier and Veksler, 1994). Therefore, if the increases in the acetate concentration is related to increased Na^+ levels in the perfusate, hearts perfused with higher concentrations of acetate should hypothetically develop IC sooner. Although the present study showed that hearts perfused with a high concentration (30 mM) of acetate had the shortest TOIC, their was no trend showing a relationship between increased acetate concentrations and a shorter TOIC. Additional

deleterious effects of Na^+ accumulation include increased osmolarity, disruption of the RMP and the generation of abnormal action potentials.

We cannot however confirm any changes in the Na^+ levels since we did not measure the electrolyte concentrations of the perfusate.

5.1.2 Interaction of biochemical and functional parameters

a) High Energy Phosphates

Our data indicate a significant decrease in tissue ATP at the onset of IC in all acetate treated hearts. However, in support of Owen et al. (1990), the current study showed that the initial breakdown of CP was greater than that of ATP. This suggests that the decrease in tissue ATP during ischaemia was buffered, but could not be prevented by CP (Spiekermann, 1990).

Factors that may have contributed to the decline in ATP during ischaemia include those which decrease ATP production and increase ATP utilization. ATP production is limited by, amongst others, reduced oxygen availibility and as well as increased lactate levels (Rovetto et al., 1973; Neely et al., 1975). Lactate not only inhibits glycolysis, but also leads to acyl CoA accumulation. This in turn prevents transport of ADP from the cytoplasm to the mitochondrion. As a result, there may be insufficient ADP in the mitochondrion for ATP synthesis (Rovetto et al., 1975). Enhanced ATP utilization may be stimulated by increased activity of ATP dependent pumps. As previously mentioned, incremental increases in the acetate concentration could have been accompanied by

similar increases in the Na^+ content of the ischaemic perfusate. The resultant rise in the cytosolic Na^+ levels would have induced greater activity of the ATP dependent Na^+/K^+ pumps thereby contributing to the decline in ATP levels during ischaemia.

Hypothetically, IC is a consequence of reduced ATP levels and or raised cytosolic Ca^{++} levels (Koretsune and Marban, 1990; Owen et al., 1990; Ventura- Clapier and Veksler, 1994). In the current study however, tissue ATP content in the various groups did not correlate well with the TOIC. Therefore, in keeping with the findings of Owen et al. (1990), the present study could not find a clear relationship between tissue ATP levels and the onset of IC. In this respect, Bricknell and Opie (1978) and Vanoverschelde et al. (1994) found that the actual rate of ATP synthesis, specifically glycolytic ATP (Owen et al., 1990) may be more important than gross tissue ATP content. Furthermore, in support of Neely and Grotyohann (1984), this study could not show a correlation between tissue ATP and recovery of function during reperfusion either.

Alternatively, ATP *availability* may influence the incidence of IC. Increased provision of acetate may have resulted in elevated acyl CoA production. This in turn inhibits adenine nucleotide translocase which is responsible for the transfer of ATP from the mitochondrion to the cytoplasm (Rovetto et al., 1975). Therefore the amount of ATP available in the cytoplasm may have been reduced. If diminished ATP availability predisposes IC, the incremental increase in acetate concentrations should correspond with a decrease in TOIC. Our data however does not reflect this trend.

b) Lactate

The raised tissue lactate content during ischaemia corresponded with the increase in acetate concentration. Differences in tissue lactate between 1mM and 30 mM acetate hearts could be due to either the degree of glycogenolysis or inhibition of carbohydrate flux through the Krebs Cycle. The latter may be the result of increased acetyl CoA formation which inhibits PDH and results in a greater conversion of pyruvate to lactate (Neely and Morgan, 1974; Ferrari and Opie, 1992). There were no significant differences in tissue glycogen content between the groups, therefore implying that 1 mM acetate exercised less inhibition on carbohydrate flux and resulted in lower tissue lactate content. Although PDH activity and tissue acetyl CoA content was not measured, our data may suggest that 30 mM acetate was associated with increased lactate levels, possibly due to PDH inhibition.

Accumulation of tissue lactate is also believed to be detrimental to ventricular functional recovery (Neely and Grotyohann, 1984) by inhibiting glycolysis (Williamson et al., 1976), and leading to intracellular acidosis (Brachfeld, 1969). In contrast, the current study found that the 1 mM acetate group, with the lowest lactate content, was associated with the poorest recovery. The opposing findings may be due to differences in experimental design. The current study used a low flow ischaemic model whereas Neely and Grotyohann (1984) used a no-flow ischaemic model where lactate accumulation was a result of reduced metabolite washout.

c) Glycogen

Tissue glycogen content decreased similarly in all acetate treated hearts. According to King et al. (1995), glycogen is capable of delaying the onset of IC, yet in the current study, IC developed within 4 minutes in all groups. Furthermore, 5 mM and 10 mM acetate delayed the TOIC significantly longer than 1 mM and 30 mM acetate despite the fact that all acetate treated hearts had similar tissue glycogen levels at the onset of IC. Instead our data support the proposal by Owen et al. (1996) that glycolytic ATP synthesis from glucose is needed for protection against IC.

The decreased ischaemic glycogen content may have contributed to the poor recovery of LVDP in the acetate perfused hearts. Pre-ischaemic perfusion with 5 mM acetate is known to reduce the tissue glycogen content, provoking speedier glycogen depletion during ischaemia. According to Cross et al. (1996) glycogen depletion would lead to reduced glycolytic activity and subsequent accumulation of protons. Upon reperfusion, this proton accumulation increases Na^+/H^+ exchange which provokes poor recoveries. However, no correlation between the glycogen content and percentage recovery of LVDP amongst all groups could be found in the present study.

d) cAMP

Elevated cAMP levels were observed at the onset of IC, suggesting that it plays a role in ischaemic contracture. Mechanisms for the increase in cAMP could have been due to either metabolic activation of adenylyl cyclase or inhibition of phosphodiesterase (Podzuweit et al., 1996). The decrease in cAMP levels observed at the end of ischaemia may have been the result of downgrading of the β-receptors (Muller et al., 1987).

Cyclic AMP, together with adenosine, inhibits fatty acid metabolism in oxygen deficient hearts (Neely and Morgan, 1974). This may lead to an ATP shortage which contributes to the development of IC (Koretsune and Marban, 1990; Owen et al., 1990; Opie, 1990). Alternatively, cAMP could be more closely associated to the occurrence of IC by increasing intracellular Ca^{++} levels (Podzuweit et al., 1976; Ventura-Clapier and Veksler, 1994) through phosphorylation of the Ca^{++} channels and subsequent Ca^{++} stimulated Ca^{++} release. This cannot however be confirmed as the Ca^{++} levels was not measured in the current study.

5.2 Effect of oxygen availability on beneficial effect of glucose and acetate

The current study showed that, in the presence of oxygen, 10 mM glucose protected the ischaemic heart by abolishing IC and improving recovery of function upon reperfusion. In contrast, 5 mM Ac accelerated the TOIC and yielded weaker reperfusion recoveries. However, in the absence of oxygen during ischaemia, both glucose and acetate treated hearts developed IC soon after initiation of ischaemia and displayed equally poor reperfusion recoveries. These results imply that the protective effects of glucose is oxygen dependent and the Krebs Cycle may play a role in these effects.

5.2.1 Interaction between ischaemic contracture and biochemical parameters

Ischaemic contracture, supposedly a result of deficient ATP and or Ca^{++} overload (Bricknell and Opie, 1978; Grossman and Barry, 1980; Korestsune and marban, 1990; Owen et al., 1990), developed soon after the beginning of ischaemia in both the ischaemic-anoxic glucose and acetate treated hearts. The early development of IC in

ischaemic-anoxic acetate perfused hearts was expected since acetate metabolism, involving β-oxidation and oxidative phosphorylation, is oxygen dependent. Acetate treated hearts would then have had to rely solely on glycogen as a source for ATP synthesis. The decrease in tissue glycogen content during ischaemia supports this theory. However, Owen et al. (1990) speculated that ATP production from glycogen is less capable than glycolysis from glucose in maintaining Ca^{++} homeostasis across the sarcolemma, assuming that inadequate Ca^{++} homeostasis is linked to IC. And since the TOIC was accelerated in acetate treated hearts, the current study found, contrary to King et al. (1995), that glycogen was ineffective in delaying the onset of IC during ischaemia-anoxia.

Assuming that glycolytic ATP production from glucose maintains Ca^{++} homeostasis (Owen et al., 1990) and elevated cytosolic Ca^{++} causes IC (Grossman and Barry, 1980; Ventura-Clapier and Veksler, 1994), ATP synthesis via anaerobic glycolysis should hypothetically prevent IC. However, the current study showed that TOIC was accelerated in glucose perfused ischaemic-anoxic hearts. A possible explanation for the development of IC in these hearts could be that anaerobic glycolysis may only play a minor role in preventing IC. The beneficial effects of glucose may either be directly dependant on oxygen through oxidative phosphorylation, or indirectly through the incorporation of pyruvate into the Krebs Cycle. Anoxia inhibits pyruvate flux through the Krebs Cycle which is then converted to lactate (Neely and Morgan, 1974). When lactate accumulates, glycolysis is inhibited (Neely et al., 1975; Williamson et al., 1976). The resultant ATP shortage would hinder Ca^{++} homeostasis and predispose intracellular Ca^{++} overload accompanied by IC (Kusuoka and Marban, 1994). This would account for

lower ATP levels and development of IC as noted in both acetate and glucose perfused anoxic hearts. Thus, the protection conferred by glucose, in the presence of O_2, against the development of IC may be attributed to either ATP production via oxidative processes or by reducing lactate accumulation which inhibits glycolytic flux.

Similar increases in tissue cAMP in the glucose and acetate ischaemic-anoxic hearts corresponded with the onset of IC. However, cAMP levels in Glu hearts subjected to ischaemia-anoxia were significantly lower compared to hearts perfused with a buffer aerated with 95%O_2 ; 5%CO_2. Therefore, if elevated cAMP levels increase cytosolic Ca^{++} which in turn causes IC (Grossman and Barry, 1980; Ventura-Clapier, 1994), IC should have developed in glucose hearts perfused with oxygenated buffer during ischaemia. Instead, glucose abolished IC in the presence of O_2 while TOIC was accelerated when O_2 was omitted from the perfusate despite significant differences in cAMP content. In accordance with Koretsune and Marban (1990) our results suggest that an ATP deficit correlates much better with the occurrence of IC. Alternatively, cAMP fluctuations may not necessarily determine the occurrence of IC. According to Owen and Opie (1978) hypoxic damage or ATP depletion would result in the ingress of Ca^{++} and have the same effects as cAMP accumulation.

5.2.2 Percentage Recovery of Left Ventricular Developed Pressure

Ischaemia-anoxia impaired the recovery of LVDP similarly in both the glucose and acetate treated hearts. These poor recoveries may be attributed to various factors. Neely et al. (1975) suggested that inhibition of O_2 dependent energy generating pathways increased lactate induced glycolytic inhibition with subsequent ATP shortage. According

to Spiekermann (1990), this lack of energy may be the main cause of functional impairment. In support of Neely et al. (1975), the present study showed lower tissue ATP levels corresponding with higher tissue lactate levels in all ischaemic-anoxic hearts compared to ischaemic hearts. This increased ischaemic lactate levels may have contributed to the low recovery of function during reperfusion (Neely and Grotyohann, 1984). In keeping with the findings of King et al. (1995) the current study also showed poor recovery of reperfusion coronary flow in all ischaemic-anoxic hearts. This may have been due to mechanical compression of coronary arteries when IC developed (Humphrey et al., 1980). As a result there may have been insufficient washout of metabolic products which contributed to tissue damage (Neely et al., 1973) and poor reperfusion recovery.

According to Neely and Grotyohann (1984) glycogen breakdown also affects the extent of reperfusion recovery by contributing to tissue damage through metabolite accumulation. The present study showed that ischaemia-anoxia stimulated glycogenolysis, as reflected by similar decreases in ischaemic tissue glycogen content in all groups (Neely and Morgan, 1974). However, Neely and Grotyohann (1984) used a total, global ischaemic model whereas a low-flow (2ml/min.) model was used in the current study. Therefore the detrimental effects of glycogen breakdown in their study may be attributed to reduced washout of metabolic products which is not applicable to the current study.

5.3 Equi-carbon concentrations of glucose and acetate as ischaemic substrates

Despite the presence of residual oxygen, 30 mM acetate could not offer protection equivalent to that of 10 mM glucose during low flow ischaemia. These results support Bricknell and Opie (1978) who also found that glucose abolished IC while acetate accelerated the onset of IC. In addition, acetate worsened LVDP which, assuming that acetate is a fatty acid, is in keeping with Coleman et al. (1989) who found that fatty acids have negative effects on developed pressure.

It is accepted that glucose may protect the ischaemic myocardium (Opie, 1970; Owen et al., 1990). Part of glucose protection may be due to glucose oxidation, specifically oxidative phosphorylation and the Krebs Cycle (Lopaschuk, 1998). In this respect, an equi-carbon concentration of acetate (30 mM), which is incorporated into the Krebs Cycle as acetyl-CoA, may offer protection similar to glucose (10 mM).

According to Bricknell et al. (1981) glycolytic ATP is needed for protection against IC. This implies that acetate treated hearts would have had to rely on glycogenolysis to prevent IC. Since IC developed in acetate treated hearts but not in glucose perfused hearts, these results support the proposal that ATP production from glycogen may be less effective than that from glucose in preventing IC (Owen et al., 1990). A similar trend was found by Oliver and Opie (1994) who proposed that Glu protected the ischaemic myocardium, possibly through better control of Ca^{++} homeostasis, while acetate had the opposite effects, assuming that IC is linked to inadequate Ca^{++} homeostasis.

5.3.1 Tissue ATP content

These results show that the decrease in tissue ATP during ischaemia was buffered by the provision of glucose but not by acetate. The onset of IC in acetate hearts was followed by a rapid decrease in tissue ATP content as found by Vanoverschelde et al. (1994) and Cross et al. (1996). This may have been due to inhibition of flux through the Krebs Cycle by enhanced production of acetyl CoA (Braunwald, 1992), which accompanies increased provision of acetate. Not only would this hinder ATP production but also increase conversion of pyruvate to lactate in acetate treated hearts (Neely and Morgan, 1974). Acetate, as a fatty acid, would also inhibit glycolysis (Shipp et al., 1961), thus further promoting an ATP deficiency. The decline in ATP content in acetate treated hearts may also be attributed to increased activity of the ATP dependent sodium-potassium pump.

5.3.2 Tissue Lactate content

Tissue lactate levels at the onset of IC were higher in glucose hearts, possibly due to accelerated glycolytic flux. However, it had not increased further by the end of ischaemia. This may indicate maintenance of Krebs Cycle activity, therefore preventing lactate accumulation. In contrast, lactate levels in acetate hearts were elevated at the end of the ischaemic period. This may have been due to increased formation of acetyl-CoA which inhibits PDH, thereby encouraging lactate formation (Neely and Morgan, 1974; Braunwald, 1992).

Increased tissue lactate levels may have contributed to the early development of IC and poor reperfusion recovery in acetate treated hearts. Ischaemic contracture is supposedly

a consequence of reduced ATP availability (Koretsune and Marban, 1990; Owen et al., 1990). Ischaemic lactate production has been said to inhibit glycolysis (Neely et al., 1975) and thus contribute indirectly to an ATP deficiency. This ATP deficiency may then encourage the development of IC by preventing restoration of resting Ca^{++} levels and inhibiting dissociation of the actin-myosin cross-bridges (Grossman and Barry, 1980). In addition, Ca^{++} uptake into the SR is controlled by an ATP-dependent pump which is maintained by glycolytic ATP (Opie, 1987). Therefore, when glycolysis is inhibited, Ca^{++} may accumulate and lead to the development of IC. Furthermore, lactate is believed to contribute to intracellular acidosis (Rovetto et al., 1973), which in turn inhibits glycolysis (Williamson et al., 1976) and thus aids the development of IC (Allen, 1988). However, this argument is questionable since Dennis et al. (1991) has shown that lactate is an unlikely source of protons. High ischaemic tissue lactate has also been associated with poor reperfusion ventricular function recovery in a total global ischaemic model (Neely and Grotyohann,1984). This may partially account for poorer reperfusion recoveries in Ac treated hearts compared to Glu hearts, a trend which was also found by Coleman et al. (1989).

5.3.3 Tissue cAMP content

Since IC was not observed in the glucose hearts, higher cAMP levels at the onset of IC in acetate hearts may implicate its involvement in the generation of IC. Elevated cAMP reportedly increased intracellular Ca^{++} (Podzuweit et al., 1976) which may cause IC (Ventura-Clapier and Veksler, 1994). High cAMP levels may also inhibit fatty acid metabolism (Neely and Morgan, 1974) which necessitates increased glycogen utilisation (Neely et al., 1970). A similar trend was found in this study where lower tissue glycogen

content was associated with raised cAMP levels at the onset of IC. The reduced tissue glycogen content may be due to increased phosphorylation of phosphorylase b to a by cAMP, resulting in the breakdown of glycogen to glucose-1-phosphate (Neely and Morgan, 1974).

5.3.4 Tissue Glycogen content

Glycogen utilisation increased during ischaemia in hearts perfused with acetate. Neely et al. (1970) found similar results in hearts perfused with fatty acids, while Cross et al. (1996) observed that provision of glucose throughout ischaemia depleted glycogen minimally. Similarly, the present study showed that provision of exogenous glucose buffered a decrease in tissue glycogen. Neely and Grotyohann (1984) found that anaerobic glycolysis from glycogen leads to intracellular accumulation of lactate and protons in the total global ischaemic model. The resultant acidosis could increase intracellular Ca^{++} and aid the development of IC (Allen, 1988). This would explain why glycogen break down was higher in acetate hearts which developed IC as opposed to glucose treated hearts where there was no incidence of IC (Owen et al., 1990). However, we cannot confirm that glycogenolysis predisposed acidosis or increased intracellular Ca^{++}.

It is evident that 30 mM acetate could not offer protection equivalent to that of 10 mM glucose during low flow ischaemia. Possible reasons could be that glycolytic ATP is chiefly responsible for the protection conferred by glucose. ATP derived from oxidative phosphorylation and the Krebs Cycle may only play a minor role in this respect. Acetate perfused hearts would, as expected, perform poorly since it is believed that glycolytic

ATP from glucose is more effective than that from glycogen in protecting the ischaemic heart (Owen et al., 1990). Alternatively, insufficient oxygen supply to allow functioning of oxidative phosphorylation and prevent conversion of pyruvate to lactate may have contributed to the poor results. This would leave glycolysis as the main source of ATP. Acetate perfused hearts would then have had to rely on glycogen as a source of energy. This may explain the lower ATP and glycogen levels noted in acetate treated hearts.

The acetate which was used as a substrate in the ischaemic perfusate was derived from *sodium* acetate. The solution used to perfuse the acetate treated hearts would then have had a higher sodium concentration than the glucose treated hearts. Since increased sodium levels have detrimental effects such as increased osmolarity, cell swelling, disruption of the RMP, generation of abnormal action potentials, increased ATP utilization and raised cytosolic calcium levels, it may account for the poor performance of the acetate treated hearts.

Another plausible explanation is that despite providing the hearts with equi-carbon concentrations of glucose and acetate, the ATP production from these substrates is quite different. Theoretically, oxidation of 10 mM glucose would yield more ATP than 30 mM acetate. Acetate is a two carbon substrate which does not undergo ß-oxidation but provides acetyl-CoA directly to the Krebs Cycle. However, ß-oxidation appears to be an important source of NADH and $FADH_2$ which is oxidised to form ATP via oxidative phosphorylation. Perhaps the metabolism of a longer chain fatty acid, involving ß-oxidation, may yield more favourable results.

Since acetate does not contribute to anaplerotic pathways, a reduced oxaloacetate level may have also contributed to the dismal performance of acetate treated hearts. Lower oxaloacetate concentrations would prevent the incorporation of acetyl CoA into the Krebs Cycle and as a result, limit ATP production. Glucose on the other hand, contributes to the anaplerotic pathways through the conversion of pyruvate to oxaloacetate and malate. Acetate treated hearts would have had to rely on glycogen as a source for pyruvate production. The synthesis of oxaloacetate from pyruvate is ATP dependent, therefore the low ATP levels noted in acetate treated hearts would have inhibited this pathway and ultimately leading to a further decrease in ATP levels. We cannot however be sure that the oxaloacetate content was the limiting factor as we did not measure its concentration.

Our aim was to provide the hearts with equi-carbon concentrations of acetate and glucose in the form of 30 mM acetate and 10 mM glucose. However, 2-C atoms of the glucose 6-C are exhaled as CO_2 when pyruvate is converted to acetyl CoA. Only 4-C atoms are therefore burned up during the Krebs Cycle, so it may be argued that 20 mM acetate is actually equi-carbon to 10 mM glucose. Since the acetate dose response showed that 5 mM and 10 mM acetate were equally protective during ischaemia, perhaps 20 mM acetate would have exerted beneficial effects as well.

Finally, the high concentration of acetate may have simply been too toxic. Mechanisms underlying fatty acid toxicity include inhibition of glycolysis, preventing pyruvate flux through the Krebs Cycle and accelerated enzyme release (Wilkinson and Robinson, 1974). In addition, fatty acids promote Ca^{++} overload via inhibition of the SR Ca^{++}

pump, the sarcolemmal Na^+/Ca^{++} exchanger and Na^+-K^+ pump, as well as activation of the Ca^{++} channels (Oliver & Opie, 1994). FFA are also directly arrhythmogenic in the isolated rat heart (Makiguchi et al., 1991). Therefore the deleterious effects of 30 mM acetate outweighed any beneficial effects of an exogenous substrate during subtotal ischaemia.

Chapter 6

CONCLUSION

The optimal acetate concentration which best protected the isolated rat heart against the consequences of low flow ischaemia was both 5 mM and 10 mM acetate. These concentrations yielded improved, yet similar, function upon reperfusion and delayed the onset of IC equally. Hearts perfused with 1mM and 30 mM acetate consistently displayed poor recoveries and accelerated TOIC.

This study showed that the protection offered by glucose to the ischaemic myocardium is oxygen dependent. Therefore, the beneficial effects of glucose may be partly attributed to the maintenance of Krebs Cycle activity and oxidative phosphorylation. However, the deleterious effects of ischaemia-anoxia on glycolysis may have also contributed to the poor performance of glucose treated anoxic hearts.

Equi-carbon concentrations of acetate and glucose could not offer similar protection during subtotal ischaemia. Acetate (30 mM) treated hearts accelerated the time to the onset of IC and worsened the percentage recovery of LVDP while glucose (10 mM) abolished IC and improved reperfusion recovery. Acetate hearts displayed increased tissue cAMP and lactate levels accompanied by lower ATP and glycogen content during ischaemia. Thus 10 mM glucose protected maximally against the consequences of ischaemia in the presence of oxygen. This may argue for additional protection via oxidative phosphorylation and the Krebs Cycle.

Reservations to this study

1. In order to obtain a more comprehensive image of metabolic changes during ischaemia, additional tissue samples should be taken at shorter intervals for biochemical analysis.

2. Measuring biochemical changes during the reperfusion period could help to substantiate LVDP data.

3. The oxaloacetate concentration is a crucial factor in determining the incorporation of acetate into the Krebs cycle. It would therefore be advisable to measure tissue oxaloacetate content to determine acetate flux through the Krebs Cycle.

4. The acetate, which was used as a substrate in the ischaemic perfusate, was derived from *sodium* acetate. Solutions containing acetate would then have had higher sodium levels than those without acetate. Similarly, incremental increases in the acetate concentration would also have resulted in increased sodium levels. To maintain a balance in the sodium content of the perfusion solutions, sodium should be omitted from any buffers to which sodium acetate is to be added. Measuring the electrolyte concentations of all perfusion solutions would also ensure that the substrate is the only variable component in the solution.

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