Malonyl-CoA Metabolism in Cardiac Myocytes

by

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Abstract

Subcellular fractions of rat heart homogenates were prepared and the pathways of malonyl-CoA utilisation studied. The presence of fatty acyl-CoA elongation (FACE) activity was not detected in any subcellular fraction but an appreciable amount of malonyl-CoA decarboxylase activity was. Approximately 70% of the decarboxylase activity was localised in the mitochondrial fraction. Intact mitochondrial studies suggested the presence of malonyl-CoA decarboxylase activity overt to the inner mitochondrial membrane.

Oxygen electrode studies showed that 5mM acetyl-carnitine (in the presence of 3mM malate) was a good respiratory substrate for intact rat heart mitochondria, while 1mM malonyl-CoA was not.

Cardiac myocytes were used to study levels of malonyl-CoA under various physiological conditions. In the presence of 5mM glucose, malonyl-CoA content was measured at 6.5-7.5 nmol/g dry wt in triacylglycerol (TAG)-loaded and non-TAG-loaded myocytes. 10nM insulin increased this by 25% in non-TAG-loaded myocytes but not in TAG-loaded myocytes. 0.5mM palmitate lowered malonyl-CoA levels by 35% and 39% in non-TAG-loaded and TAG-loaded myocytes respectively, while increasing glucose concentration (0-5mM), resulted in a parallel increase in malonyl-CoA in both.

Adrenaline (0-2μM), isoprenaline (0-1μM) and phenylephrine (0-1μM) had no significant effect on the level of malonyl-CoA in insulin treated, non-TAG-loaded myocytes.

Long-chain ester levels were measured in TAG-loaded and non-TAG-loaded myocytes. 0.5mM palmitate increased long-chain acyl-carnitine levels by 23% in TAG-loaded only, while 10nM insulin decreased levels by ~14% in both. Increasing glucose concentration (0-5mM) increased long-chain acyl-CoA and long-chain acylcarnitine levels by 27% and 46% respectively in non-TAG-loaded myocytes. Levels of long-chain esters were measured in freeze-clamped non-working perfused rat hearts and were 2-2.7 fold lower than in myocytes.

The rate of malonyl-CoA synthesis was estimated in glucose depleted myocytes. Re-addition of 5mM glucose increased malonyl-CoA levels from 3.0 to 5.0 nmol/g dry wt. within 12 min, with a half time of ~1.8 min.

5μM isoprenaline increased lipolysis in non-TAG-loaded myocytes by 94%. 0.5mM palmitate decreased lipolysis by 54% and 37% in TAG-loaded and non-TAG-loaded myocytes respectively.

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Abbreviations

ACBP	Acyl-CoA binding protein
ACC	Acetyl-CoA carboxylase
ACTH	Adrenocorticotropic hormone
AICAR	5-aminoimadazole-4-carboxamide ribonucleoside
ADP	Adenosine 5'-diphosphate
AMP	Adenosine 5'-monophosphate
AMPK	AMP activated protein kinase
AMPK kinase	AMP activated protein kinase kinase
ATP	Adenosine 5'-triphosphate
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CAMK	Ca^{2+}/calmodulin dependent protein kinase
cAMP	Cyclic adenosine monophosphate
CAT	Carnitine acetyltransferase
CK	Casein kinase
CoA	Coenzyme A
CoASH	Coenzyme A (reduced form)
CPT	Carnitine palmitoyltransferase
CS	Citrate synthase
DCA	Dichloroacetate
DHAP	Dihydroxyacetone phosphate
DNA	Deoxyribonucleic acid
DTNB	5-5'dithiobis(2-)nitrobenzoic acid
DTT	Dithiothreitol
ETF	Electron transferring flavoprotein
ETF:QO	Electron transferring flavoprotein ubiquinone oxidoreductase
FA	Fatty acid
FABP	Fatty acid binding protein

FACE	Fatty acid chain elongation
FAS	Fatty acid synthase
FBPase	Fructose bisphosphatase
FPLC	Fast pressure liquid chromatography
Fru-6-P	Fructose-6-phosphate
Fru-1,6-P_2	Fructose-1,6-bisphosphate
Fru-2,6-P_2	Fructose-2,6-bisphosphate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Glu-6-P	Glucose-6-phosphate
GPAT	Glycerolphosphate acyltransferase
GS	Glycogen synthase
GSK	Glycogen synthase kinase
HDL	High density lipoprotein
HK	Hexokinase
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
HPLC	High performance liquid chromatography
HSL	Hormone sensitive lipase
KHB	Krebs-Henseleit bicarbonate
LDH	Lactate dehydrogenase
LPL	Lipoprotein lipase
MDH	Malate dehydrogenase
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NEFA	Non-esterified fatty acid
PAP	Phosphatidic acid phosphohydrolase
PDH	Pyruvate dehydrogenase
PDK	Phosphoinositide dependent protein kinase
PFK	Phosphofructokinase

P_i	Inorganic phosphate
PK	Pyruvate kinase
PKA	Protein kinase A
PKC	Protein kinase C
PtdIns	Phosphatidylinositide
RNA	Ribonucleic acid
SR	Sarcoplasmic Reticulum
TAG	Triacylglycerol
UDP	Uridine 5'-diphosphate
VLDL	Very low density lipoprotein

Chapter 1 - Introduction

1.1 General Introduction

The heart can utilise a variety of carbohydrate and lipid fuels to provide energy for contractile function. The rate of oxidation of these fuels is demand led, in that an increase in work rate and adenosine triphosphate (ATP) demand, increases oxidative phosphorylation and citric acid cycle activity. Under normal aerobic conditions the heart takes its energy from substrates in the bloodstream so that endogenous energy stores of triacylglycerol (TAG) and glycogen, are preserved. Nutritional and pathological conditions can dramatically alter the supply of lipids and/or carbohydrates to the heart and the rate of their oxidation.

Oxidation of fatty acids in mitochondria provides 60-70% of the energy requirements of the myocardium. It has been well established that fatty acids inhibit the utilisation of carbohydrates in the heart via a series of regulatory mechanisms known as the glucose/fatty acid cycle. Much less is known about mechanisms by which fatty acid oxidation is inhibited by carbohydrates. It has become increasingly clear that myocardial malonyl-CoA (a precursor of fatty acid synthesis) plays an important role in regulation of fatty acid oxidation and fuel selection via regulation of long-chain fatty acid transport into the mitochondria. High levels of fatty acids are known to be detrimental to the ischaemic myocardium and during reperfusion. Therefore, strict control of fuel selection in the heart is essential for efficient and continued contractile function.

1.1.1 Structure and Function of the Heart

The heart is the central organ that by rhythmic contraction, pumps blood continuously through the blood vessels to convey nutrient and other materials to and from the various parts of the organism. The mammalian heart consists of four chambers: right atrium, left atrium, right ventricle and left ventricle, with the two sides of the heart being separated by an internal septum. The direction of the flow of blood is largely determined by the presence of valves at the entry to and exit from the ventricles.

Heart muscle cells have properties somewhere in between those of skeletal and smooth muscle. They are small, striated and branching cells with a single nucleus. Each cell is connected to its neighbour by intercalated discs, within which are "gap junctions". These gap junctions allow the passage of electrical currents and small molecules between adjacent cells and are controlled by the metabolic state of the cells. The myocardium consists of three types of muscle fibre. Muscle cells found in the sinus node and the atrioventricular node are often smaller than other myocardial cells, are only weakly contractile, are autorhythmic, and exhibit very slow conduction between cells. The largest myocardial cells, found in the ventricular endocardium, are also weakly contractile, but are specialised for fast conduction and constitute the system for spreading the excitation over the heart. The intermediate-sized myocardial cells are strongly contractile and constitute the bulk of the heart.

In summary, the heart is a muscular pump whose continuous rhythmical contraction is essential for the maintenance of life.

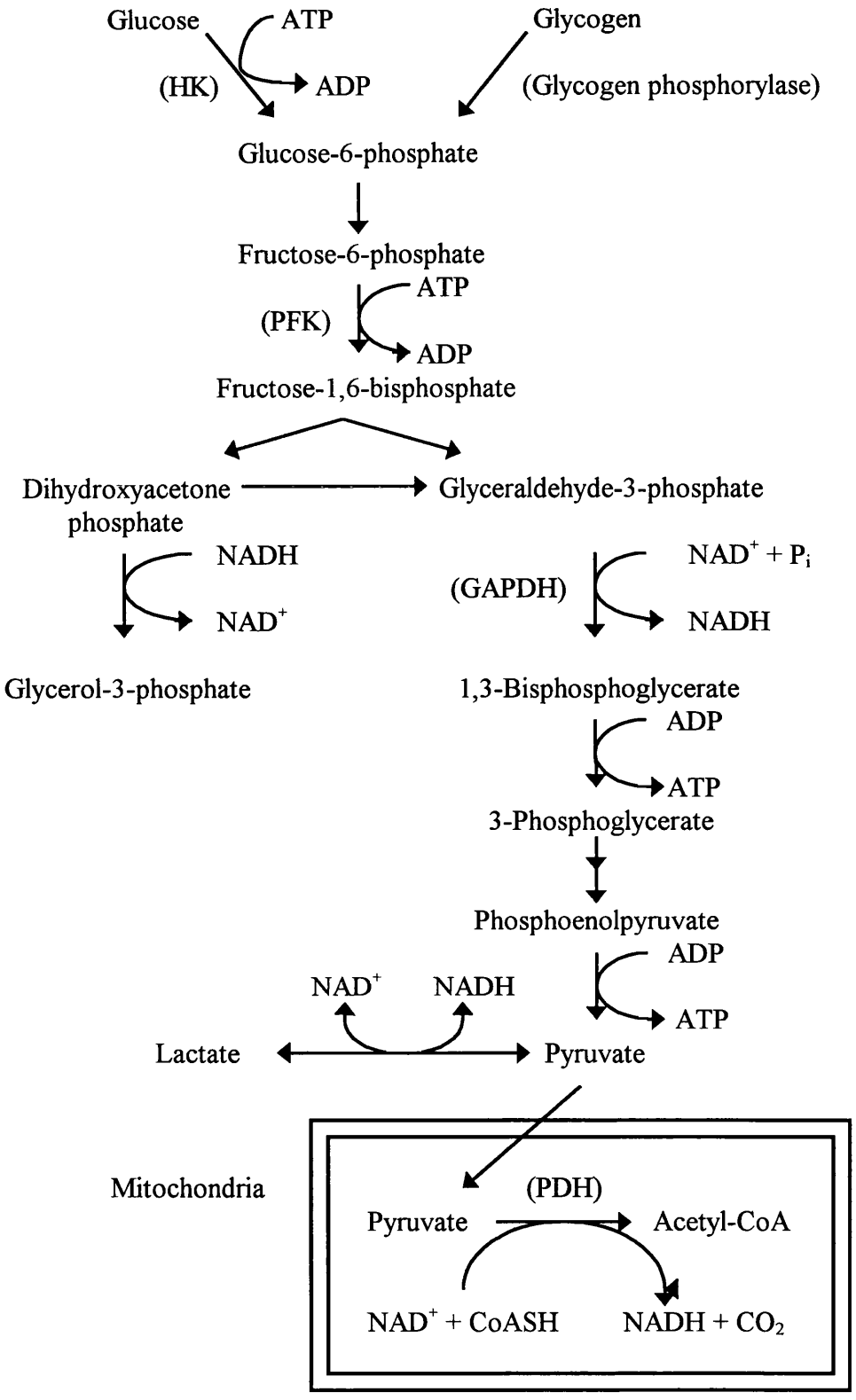
1.2 Cardiac Energy Metabolism

The heart can produce energy from a wide range of substrates including dietary fatty acids (FA) and carbohydrates but also from various molecules produced during their metabolism, such as lactate, pyruvate and ketone bodies. Ultimately all fuels are broken down to acetyl-CoA and CoA which is oxidised within mitochondria by the enzymes of the citric acid cycle to produce reduced coenzymes. These reduced coenzymes along with those produced from glycolysis and fatty acid oxidation interact with the electron transport chain to produce ATP. ATP synthesised within the mitochondria is transported to the cytosol by the adenine nucleotide translocase in a one to one exchange with adenosine diphosphate (ADP). The cardiac muscle shifts continuously from one fuel source to another depending on substrate availability, workload and pathophysiological state.

1.2.1 Carbohydrate Fuels

Carbohydrates are not the preferred myocardial fuel under normal conditions, but do contribute significantly to the total energy requirements of the heart. Their contribution can both increase and decrease under certain physiological and pathological conditions, such as carbohydrate feeding, exercise (increased workload), starvation, diabetes, hypoxia and ischaemia. Utilisation of glucose and glycogen by heart muscle is regulated at a number of key steps in their conversion to acetyl-CoA which are outlined below. The pathways of glucose metabolism in heart are shown in figure 1.1.

Figure 1.1 Pathways of glucose metabolism in the heart



Abbreviations: HK, hexokinase; PFK, phosphofructokinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PDH pyruvate dehydrogenase.

1.2.1.1 Glucose Transport

Transport of glucose across the plasma membrane is mediated by a family of glucose transporters (Gould & Holman, 1993) which are specific for D-glucose and do not require energy. The transport of glucose occurs down a concentration gradient as the concentration of glucose in the extracellular fluid is much higher than in the cytosol. At normal cardiac workload, circulating insulin regulates the rate of glucose extraction by cardiomyocytes (Taegtmeyer, 1985). Increased workload increases glucose uptake in an insulin independent manner, the mechanism of which is incompletely understood. In the absence of insulin, glucose transport is the rate-limiting step in the utilisation of exogenous glucose in normoxic perfused hearts (Kobayashi & Neely, 1979). In the presence of insulin, glucose transport is no longer rate-limiting and imposes little restraint on glucose uptake and utilisation. Insulin markedly increases the rate of glucose transport and is thought to act by stimulating the recruitment of GLUT 4 glucose transporters from intracellular vesicles to their site of action at the sarcolemma. A number of other agents are also known to increase the rate of glucose transport in heart muscle, including growth hormone(s), adrenaline, anoxia and increased ventricular pressure development (Neely & Morgan, 1974). On the other hand, oxidation of fatty acids, ketone bodies and pyruvate have all been shown to inhibit glucose transport (Randle & Tubbs, 1979).

1.2.1.2 Glucose Phosphorylation

The first step of glycolysis in mammalian cells, including heart cells, is the phosphorylation of glucose by ATP to produce glucose-6-phosphate (Glu-6-P) (fig 1.1). This reaction is catalysed by hexokinase (HK) and is essentially irreversible

under physiological conditions. Glucose-6-phosphatase, the enzyme that catalyses the reverse reaction in liver is not present in heart muscle. HK, like all other kinases requires Mg^{2+} or another divalent metal ion such as Mn^{2+} for activity. HK exists in 4 isoforms, types I, II and III are present in heart muscle, with type II accounting for more than 50% of total activity. The activity of this isoform is markedly reduced in hearts from diabetic rats (Katzen *et al*, 1970). In the presence of insulin, when the rate of glucose transport is high, phosphorylation of glucose by HK may become the rate-limiting step in glucose utilisation. Regulation of HK *in vivo*, is mainly mediated by changes in the levels of its product Glu-6-P (Neely & Morgan, 1974; Randle & Tubbs, 1979) which acts as a feedback inhibitor. Hexokinase is also inhibited by the oxidation of fatty acids and ketone bodies (Randle & Tubbs, 1979) the mechanism of which is discussed later. Glu-6-P is converted to fructose-6-phosphate (Fru-6-P) by the enzyme phosphoglucoisomerase. This reaction is not an important rate-controlling step in glycolysis.

1.2.1.3 Phosphofructokinase

Phosphofructokinase-1 (PFK-1) catalyses the phosphorylation of Fru-6-P by ATP (requires Mg^{2+}) to produce fructose-1,6-bisphosphate (Fru-1,6-P_2). PFK-1 is a multimodulated enzyme and is thought to be one of the most important regulatory sites in the glycolytic pathway. Allosteric control of PFK-1 is extensive and produces large changes in catalytic activity. PFK-1 is inhibited by ATP, creatine phosphate, citrate and protons (H^+), and is stimulated by adenosine monophosphate (AMP), ADP, inorganic phosphate (P_i) and by its product Fru-1,6-P_2. The activators of PFK-1 are particularly important as their intracellular concentrations rise in heart muscle in

response to anoxia and ischaemia. Increased PFK-1 activity causes a drop in Glu-6-P, relieving its inhibition of HK driving glycolysis forward. PFK-1 activity is greatly affected by intracellular pH, especially over the physiological range pH 6.8 to 7.3. Studies using purified heart enzyme show a sigmoid relationship between enzyme activity and concentration of Fru-6-P at pH 6.9, and a marked sensitivity to allosteric regulation. At pH 8.2, PFK-1 exhibits Michaelis-Menten kinetics, has increased activity, and is not subject to regulation (Trivedi & Danforth, 1966; Mansour, 1972a). The product of the PFK-1 reaction, Fru-1,6-P_2, is a potent activator of the enzyme. PFK-1 has two binding sites for Fru-1,6-P_2, a low affinity site, and a high affinity site (Setlow & Mansour, 1972). Binding to the low affinity site probably accounts for allosteric regulation at pH 6.9, while binding to the high affinity site affects catalytic activity even at pH 8.2. Adrenaline increases PFK-1 activity in muscle (Mansour, 1972b) which may be the result of more extensive binding of effectors, especially Fru-1,6-P_2. Removal of Fru-1,6-P_2 by addition of fructose bisphosphatase or aldolase inhibits PFK-1 activity (El-Badry *et al*, 1973). Adrenaline also stimulates PFK-1 activity in rat heart by Ca^{2+}-dependent α- and β-adrenergic mechanisms (Clark & Patten, 1984).

A small decrease in ATP level results in a marked increase in PFK-1 activity due to an amplification mechanism involving adenylate kinase (2 ADP $\leftrightarrow$ ATP + AMP) (Newsholme, 1971). The equilibrium constant of adenylate kinase is 0.44, thus a 15% reduction in ATP levels produces a 5 fold increase in AMP level.

In vitro studies of cardiac PFK-1 have shown it to be a substrate for cyclic AMP-dependent- (PKA) and Ca^{2+}/calmodulin-dependent (CAMK) protein kinases

(Mahrenholz *et al*, 1991). Phosphorylation results in an increase in the sensitivity of PFK-1 to inhibition by ATP as well as an increase in its K_m for Fru-6-P. These effects are minor compared to allosteric effects and it is not known whether phosphorylation of cardiac PFK-1 has a regulatory role *in vivo*.

Glycolysis is inhibited by the oxidation of fatty acids or ketone bodies in what is known as the glucose/fatty acid cycle (see section 1.3). Oxidation of fatty acids or ketone bodies increases the citrate content of perfused rat hearts (Opie, 1991). Therefore, the glucose-sparing effect of these alternative oxidizable fuels results in part, from a citrate-mediated inhibition of PFK-1 (Garland *et al*, 1963). The increase in citrate content is accompanied by a fall in the concentration of fructose-2,6-bisphosphate (Fru-2,6-P_2) (Hue *et al*, 1988), a potent activator of PFK-1 (see section 1.2.1.3.1). This drop in Fru-2,6-P_2 is also mediated by citrate as it is a potent inhibitor of 6-phosphofructo-2-kinase (PFK-2), the enzyme responsible for its synthesis. Therefore, increased citrate levels can maintain fatty acid and ketone body oxidation via inhibition of glycolysis at the level of both PFK-1 and PFK-2. Lactate, which accumulates during exercise inhibits both glycolysis and fatty acid oxidation in the heart (Drake *et al*, 1980). The inhibition of glycolysis by lactate is mainly exerted by a citrate-mediated inhibition of PFK-1 and PFK-2 (Depre *et al*, 1993b) resulting in a decrease in Fru-2,6-P_2. Perfusion of hearts with insulin in the presence of glucose also produces an increase in citrate levels (Opie, 1991), yet under these conditions glycolysis is stimulated. This would suggest that there are other mechanisms involved in the regulation of glycolytic flux in the heart.

1.2.1.3.1 Fructose-2,6-bisphosphate Regulation of PFK-1

Fru-2,6-P_2 is present in all mammalian tissues and is an important activator of PFK-1. It was discovered by Van Schaftingen and co-workers while studying the mechanism of glucagon action on glycolysis and gluconeogenesis in liver (Van Shaftingen *et al*, 1980a, 1980b). To date, all PFK-1 isoenzymes studied are sensitive to Fru-2,6-P_2 (Van Schaftingen, 1987). At physiological concentrations of substrates and effectors, heart PFK-1 is almost completely inactive unless physiological concentrations (μM) of Fru-2,6-P_2 relieve the inhibition by ATP and citrate (Hue & Rider, 1987; Lawson & Uyeda, 1987).

Fru-2,6-P_2 is synthesised from Fru-6-P and ATP (requires Mg^{2+}) by the bi-functional 6-phosphofructo-2-kinase (PFK-2)/fructose-2,6-bisphosphatase (FBPase-2) (Van Schaftingen, 1987; Hue & Rider, 1987; Pilkis *et al*, 1988). The FBPase-2 activity of the enzyme is responsible for the degradation of Fru-2,6-P_2, hence the concentration of Fru-2,6-P_2 is dependent upon the two activities of this single enzyme. In the heart, two isoforms of PFK-2/FBPase-2 (a 58,000 M_r- and 54,000 M_r-form) have been described, which originate from alternative splicing of the same primary transcript (Rider *et al*, 1992). Other tissue specific isoenzymes exist (Rousseau & Hue, 1993) but are not discussed in detail. Heart PFK-2/FBPase-2 is interesting in that the PFK-2 activity is 80 times greater than the FBPase-2 activity, compared to the liver enzyme where PFK-2 activity is only 4 times greater (Rider & Hue, 1986). PFK-2 activity is inhibited by citrate and *sn*-glycerol-3-phosphate (Hue & Rider, 1987), and stimulated by insulin. Insulin acts by increasing the V_{max} (Rider & Hue, 1984), possibly via the phosphatidylinositol 3-kinase pathway (Hue *et al*, 1995). Heart PFK-2/FBPase-2 is a

substrate of both PKA and CAMK. Phosphorylation activates PFK-2 by decreasing its K_m for Fru-6-P but has no effect on FBPase-2 activity (Rider *et al*, 1992). *In vitro* phosphorylation of purified bovine heart PFK-2/FBPase-2 by PKA and CAMK results in the same activation of the enzyme (Rider *et al*, 1992). Both kinases probably phosphorylate the same sites (Depre *et al*, 1993a). Preparations of PFK-2/FBPase-2 purified from hearts submitted to high workload showed similar changes in PFK-2 activity (Depre *et al*, 1993). These changes in PFK-2 activity *in vivo* are likely to be due to phosphorylation by CAMK rather than PKA, as concentrations of cAMP are unaltered under these conditions (Hue *et al*, 1994).

Conditions which reduce glycolysis such as oxidation of fatty acids, ketone bodies or lactate are citrate mediated and are accompanied by a decrease in Fru-2,6-P_2 levels (Hue & Rider, 1987; Hue *et al*, 1995), whereas stimulation of glycolysis by glucose, insulin, adrenaline, or increased workload is paralleled by an increase in the Fru-2,6-P_2 content of isolated perfused hearts (Hue & Rider, 1987; Lawson & Uyeda, 1987; Depre *et al*, 1993a). As well as their direct effects on PFK-1 and PFK-2, insulin and adrenaline both stimulate glycolysis by increasing Fru-6-P levels (by stimulation of glucose transport and glycogenolysis respectively). Fru-2,6-P_2 can therefore be thought of as an intracellular signal that controls glycolysis in various tissues under normoxic conditions (Hue & Rider, 1987).

1.2.1.4 Glyceraldehyde-3-phosphate Dehydrogenase

The next step in the glycolytic pathway is catalysed by aldolase and is the conversion of fructose-1,6-bisphosphate into the two triose phosphates, dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (glyceraldehyde-3-P).

Glyceraldehyde-3-P is on the direct pathway of glycolysis, whereas DHAP is not (fig 1.1), but can be converted to glyceraldehyde-3-P in a reaction catalysed by triose phosphate isomerase. Thus two molecules of glyceraldehyde-3-P are formed from one molecule of fructose-1,6-bisphosphate. DHAP can also be converted to glycerol-3-phosphate (glycerol-3-P) by glycerol-3-P dehydrogenase (glycerol-3-PDH) for use in TAG synthesis. Neither the aldolase or triose phosphate isomerase reactions are considered important regulatory steps in the heart.

Glyceraldehyde-3-P is oxidised to 1,3-bisphosphoglycerate by the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and requires NAD^+ as a coenzyme. This is the first step where a high energy phosphate compound (acyl phosphate) is generated. GAPDH is inhibited by its products, 1,3-bisphosphoglycerate and NADH, and by lactate (Mochizuki & Neely, 1979) and ATP (Meriwether & Park, 1971). Activation of PFK-1 leads to an accumulation of Fru-1,6-P_2 and, under certain circumstances, to restriction of glycolytic rate further down the pathway. The control of glycolytic rate is shifted from PFK-1 to GAPDH in hearts perfused under anoxic or ischaemic conditions resulting in accumulation of Fru-1,6-P_2 and triose phosphate. Under ischaemic or anoxic conditions reoxidation of NADH to NAD^+ is impaired. The resulting increase in NADH inhibits GAPDH and stimulates the conversion of DHAP to glycerol-3-P by glycerol-3-PDH, thus promoting TAG synthesis.

1,3-bisphosphoglycerate is used to generate the first molecule of ATP from glycolysis by transfer of the phosphoryl group from the acyl phosphate of 1,3-bisphosphoglycerate to ADP, a reaction catalysed by phosphoglycerate kinase. The products of this reaction are ATP and 3-phosphoglycerate. 3-phosphoglycerate is

converted to 2-phosphoglycerate and then to phosphoenolpyruvate by the enzymes phosphoglyceromutase and enolase respectively. These three intermediate reactions have not been implicated as regulatory reactions.

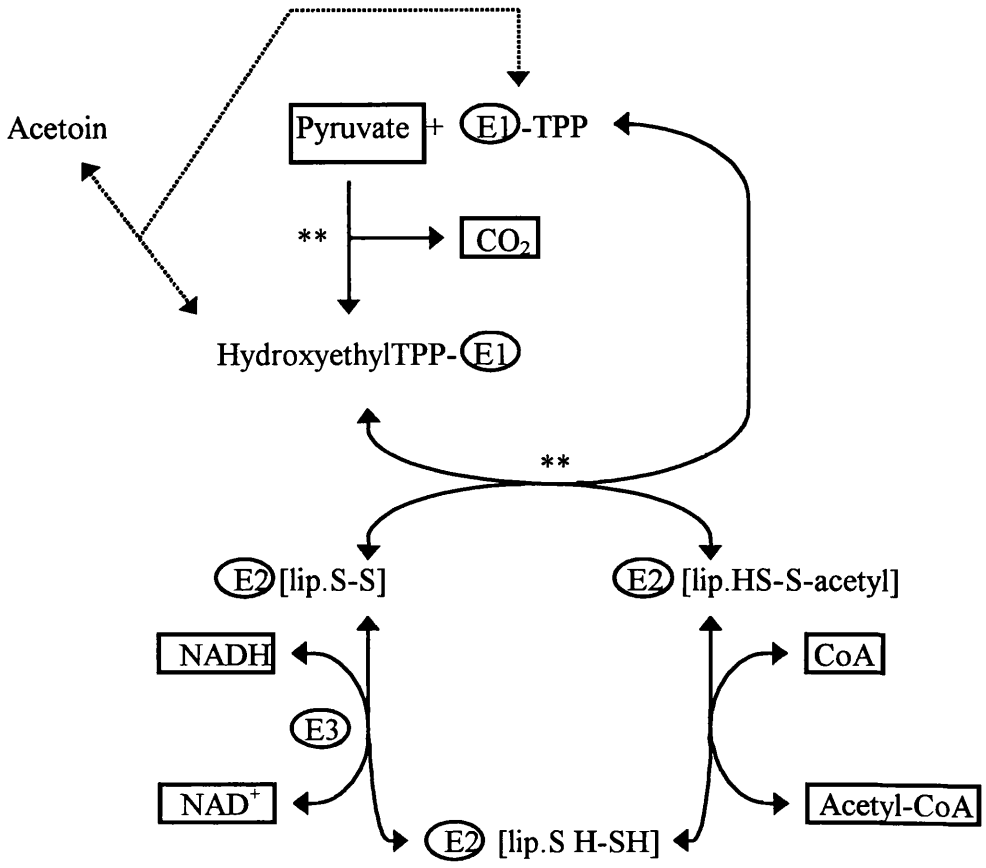
1.2.1.5 Pyruvate Kinase

The next step in the glycolytic pathway that has been suggested to be a regulatory site is pyruvate kinase (PK) which catalyses the reaction of phosphoenolpyruvate with ADP to yield pyruvate and ATP. The heart isoform of PK is inhibited by ATP via competition at the ADP site. Inhibition leads to an accumulation of metabolites preceding this step, including 1,3-bisphosphoglycerate which is a potent inhibitor of GAPDH. The majority of pyruvate produced from glycolysis in heart muscle is converted to acetyl-CoA and lactate, with a small amount being converted to alanine. Conversion of pyruvate to lactate as opposed to acetyl-CoA depends upon the oxidation state of the cell, the levels of NADH and acetyl-CoA, and the activity of pyruvate dehydrogenase (PDH).

1.2.1.6 Pyruvate Dehydrogenase

The pyruvate dehydrogenase complex catalyses the reaction of pyruvate with CoASH and NAD^+ to form acetyl-CoA, NADH and CO_2 in the presence of thiamine pyrophosphate (TPP) and Mg^{2+}. PDH is a mitochondrial multienzyme complex containing multiple copies of three component enzymes (E1, E2 and E3) and a binding protein (protein X) which binds E3 to the complex. The three enzymes pyruvate dehydrogenase (E1), dihydrolipoamide acetyltransferase (E2) and dihydrolipoamide dehydrogenase (E3) catalyse the reactions shown in figure 1.2.

Figure 1.2 Primary reactions of the PDH complex in heart



Abbreviations: Lip, lipoate; TPP, thiamin pyrophosphate. Enzymes are circled; substrates and products are boxed. ** Reactions inhibited by phosphorylation of E1α. Taken from Randle *et al*, 1994.

The decarboxylation reaction catalysed by E1 is essentially irreversible, conferring irreversibility on the holocomplex reaction, while all the other reactions are freely reversible. Interactions of the three enzymes are affected by lipoate bound covalently to the mobile arm of E2 which visits the active site of the three enzymes sequentially (Reed, 1969) and transfers acetyl (to CoASH) and $2e^-$ via the flavoprotein E3 to NAD^+.

The PDH complex is regulated by end-product inhibition and by reversible phosphorylation. Reversible phosphorylation is the major mechanism regulating the PDH complex, though end-product inhibition may be important in rapid adjustments in flux (Randle *et al*, 1994a). Inhibition by acetyl-CoA (competes with CoASH), and NADH (competes with NAD^+) results in reversal of reactions involving E2 and E3 leading to the formation of hydroxyethyl-TPP and acetoin, which prevent the decarboxylation of pyruvate. In the absence of CoASH and NAD^+, the reactions of E2 and E3 are blocked and pyruvate is decarboxylated producing hydroxyethyl-TPP and acetoin as side products. Acetoin inhibits the PDH complex by competing with pyruvate (fig 1.2).

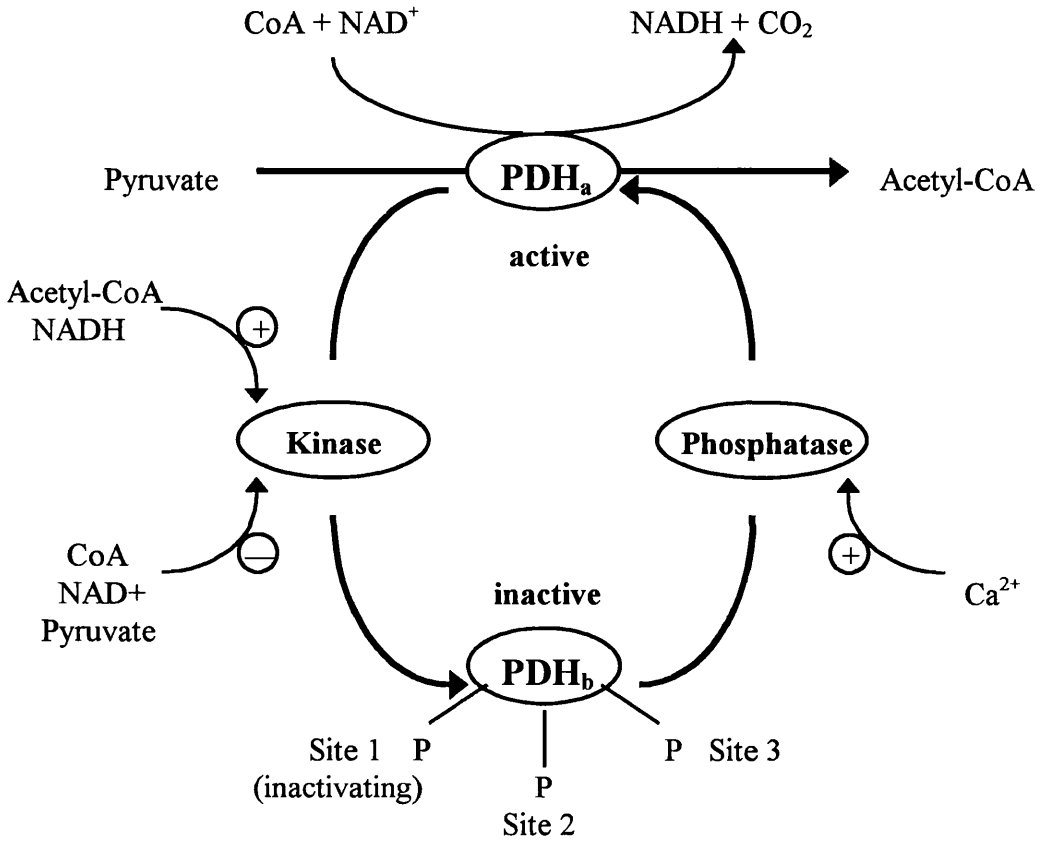
When purified, the PDH complex contains an intrinsic PDH kinase which catalyses the phosphorylation and inactivation of the PDH complex. There are no known activators of the phosphorylated inactive complex (PDH_b) (Randle *et al*, 1994a). A PDH phosphatase, which separates from the complex during purification is responsible for the dephosphorylation and reactivation of the complex (PDH_a). PDH kinase phosphorylates three serine residues in the α-chain of the E1 component of the PDH complex resulting in inactivation of the two reactions catalysed by E1, and of the

holocomplex reaction (fig 1.2); the other reactions of the holocomplex are unaffected. Phosphorylation of site 1 on the α-chain of E1 is >90% responsible for the inactivation of the PDH complex in mitochondria. Phosphorylation of sites 2 and 3 inhibits the dephosphorylation of site 1 by PDH phosphatase therefore retarding reactivation (Randle *et al*, 1994a).

The total amount of PDH complex (PDH_a + PDH_b) is constant under most physiological and pathological conditions, but the percent in active (PDH_a) and inactive (PDH_b) form can vary greatly under various conditions. In the resting state, ~20% of the PDH complex is in the active form. Starvation, high fat/low carbohydrate diet and insulin dependent diabetes all decrease the percent of PDH_a, while stimulation of glycolysis, e.g. with exercise can increase the percent of PDH_a to as much as 90% (Opie, 1991).

The activity of PDH kinase is stimulated in the short term by increasing mitochondrial ratios of acetyl-CoA/CoA, NADH/NAD^+ and ATP/ADP. The activating effects of acetyl-CoA and NADH involve reductive acetylation or reduction of lipoate in the complex and are reversed by CoASH and NAD^+ (Randle *et al*, 1994a); ADP competitively inhibits the substrate ATP. PDH kinase is also inhibited by pyruvate and inhibition is enhanced by increasing ADP concentration. Figure 1.3 shows the overall reaction catalysed by the PDH complex along with the regulation of the enzymes catalysing the interconversion of PDH_a and PDH_b. Longer-term regulation of PDH kinase activity (i.e. due to starvation), is mediated by increased plasma non-esterified fatty acids (NEFA) in conjunction with increased cyclic-AMP (cAMP) concentrations and results in an increase in the specific activity of PDH kinase (Randle *et al*, 1994b).

Figure 1.3 Regulation of pyruvate dehydrogenase (PDH) complex activity and the enzymes catalysing the interconversion between active and inactive forms



Abbreviations: P, Phosphate. Taken from Sugden & Holness, 1994.

Myocytes incubated with agents that increase cAMP levels (glucagon or dibutytyl-cAMP or 8-bromo-cAMP), or with fatty acids (n-octanoate or albumin-bound palmitate) show a 2-3 fold increase in PDH kinase activity (Randle *et al*, 1994a).

Increases in the percentage of PDH complex in the active form are achieved through the dephosphorylating activity of PDH phosphatase. PDH phosphatase requires Mg^{2+} for its activity (Hucho, *et al*, 1972; Randle *et al*, 1974) and is activated by physiological concentrations of Ca^{2+} (0.1-10 μM) (Denton *et al*, 1972; Randle *et al*, 1974). Increasing Ca^{2+} concentration in perfused hearts (McCormack *et al*, 1990) and isolated mitochondria (Midgley *et al*, 1987) increases the percentage of PDH in the active form due to dephosphorylation by the activated PDH phosphatase. Ca^{2+} may exert its effect by enhancing the binding of the phosphatase to E2 of the PDH complex. PDH phosphatase may be inhibited by NADH (reversed by NAD^+) (Randle *et al*, 1994a).

Perfusion of hearts with adrenaline results in an increase in the percentage of PDH in the active form. Muscle contraction also causes an increase in the amount of PDH_a. The major factor responsible for these increases in PDH_a is activation of PDH phosphatase due to an increase in cytosolic (and therefore mitochondrial) Ca^{2+} (McCormack *et al*, 1990; Randle *et al*, 1994a). Increased muscle contraction also decreases mitochondrial acetyl-CoA/CoA and $NADH/NAD^+$ ratios thus inhibiting PDH kinase and relieving its inhibition of the PDH complex. Perfusion of rat hearts with fatty acids or ketone bodies causes a 70% decrease in the proportion of PDH in the active form (Randle *et al*, 1994a). The role of the PDH complex in the glucose/fatty acid cycle is discussed in section 1.3.1.

1.2.1.7 Glycogen

Glucose is taken up from the circulation and stored in the heart as glycogen, which can later be mobilised and used as a glycolytic fuel. Glycogen metabolism has been extensively studied in skeletal muscle, while much less is known about its metabolism and physiological function in cardiac muscle. Glycogen is present in the cytosol in the form of granules with diameters ranging from 100 to 400 Å. These granules contain the enzymes that catalyse the synthesis and degradation of glycogen, but are not a multienzyme complex like PDH, as the bound enzymes are not present in defined ratios. Glycogen granules also contain some of the enzymes which regulate these processes. The synthesis and degradation of glycogen occur by different reaction pathways which are regulated reciprocally by a complicated series of control mechanisms. Glu-6-P is incorporated into glycogen in a series of reactions outlined below:

1) Gluc-6-P $\rightarrow$ glucose-1-phosphate (phosphoglucomutase)
2) Glucose-1-phosphate + UTP $\rightarrow$ UDP-glucose + PP_i (UDP-glucose pyrophosphorylase)
3) $PP_i + H_2O \rightarrow 2\ P_i$
4) UDP-Glucose + $glycogen_n \rightarrow glycogen_{n+1}$ + UDP (glycogen synthase)
5) UDP + ATP $\rightarrow$ UTP + ADP (nucleoside diphosphokinase)

Reaction 4 is the major regulatory step of glycogen synthesis and is catalysed by glycogen synthase (GS), an enzyme that is subject to both hormonal and non-

hormonal control (Stalmans *et al*, 1987). GS activity is regulated by reversible phosphorylation between a phosphorylated inactive form GS_b and a dephosphorylated active form GS_a. GS_a has an increased affinity for UDP-glucose (such that it is active in the presence or absence of Glu-6-P), and is only slightly inhibited by ATP and P_i. In contrast GS_b is essentially inactive in the absence of Glu-6-P and its activation by Glu-6-P is inhibited by ATP and P_i. The majority of knowledge of GS subunit structure, kinetic properties and phosphorylation-dephosphorylation interplay between the different protein kinases and phosphatases involved in the regulation of the enzyme stem from studies on rabbit skeletal muscle GS.

The complete amino acid sequence of rabbit skeletal muscle GS is known (Zhang *et al*, 1989) and at least 11 different phosphorylation sites have been identified that can be phosphorylated *in vitro* by PKA and various cAMP-independent protein kinases. *In vivo*, rabbit skeletal muscle GS is known to be phosphorylated at nine serine residues by a minimum of six protein kinases, including GS kinase-3 (GSK_3), PKA, casein kinase-1, -2 (CK1, CK2) and Ca^{2+}/calmodulin-dependent protein kinase-2 (CAMK2) (Poulter *et al*, 1988; Nakielny *et al*, 1991). Phosphorylation of four residues, sites 2a, 2b, 3a and 3b, leads to a decrease in activity, but the effects are cumulative so that almost complete inhibition occurs when all four sites are phosphorylated (Nakielny *et al*, 1991; Skurat & Roach, 1995). Larner and co-workers demonstrated that insulin stimulates glycogen synthesis in skeletal muscle by promoting dephosphorylation of GS (Craig & Larner, 1964). Therefore, insulin must exert this effect via inhibition of a protein kinase and/or activation of a protein phosphatase. There is evidence to support the hypothesis that activation of skeletal

muscle GS in response to insulin involves inhibition of GSK_3, a kinase that phosphorylates sites 3a, 3b, and 3c (Cohen, 1986; Roach, 1991). The ability of insulin to stimulate skeletal muscle GS occurs via an insulin-stimulated protein kinase cascade which is triggered by the activation of phosphatidylinositide (PtdIns) 3-kinase. The first enzyme in the cascade is termed 3-phosphoinositide-dependent protein kinase (PDK1), because it is only active in the presence of PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2. PDK1 activates protein kinase B (PKB), which in turn inactivates GSK_3, leading to the dephosphorylation and activation of GS and hence to an increase in glycogen synthesis (Cohen *et al*, 1997).

Cardiac muscle glycogen metabolism is quite different from that of skeletal muscle and the hormonal regulation of heart GS is not yet fully understood. Rat heart GS, like skeletal muscle GS, appears to be heavily phosphorylated on multiple sites *in vivo* (Ramachandran *et al*, 1983; Grekinis *et al*, 1995). Studies using purified rat heart GS preparations have shown that it can be phosphorylated by PKA, GSK_3, CAMK2, PKC, phosphorylase kinase and CK2 (although CK2 does not inactivate GS). As in skeletal muscle, inactivation appears to be correlated with the phosphorylation state of the enzyme. Studies using isolated heart preparations have shown that insulin activation of heart GS is independent of either Ca^{2+} or cAMP, whereas β-adrenergic agonists and glucagon act via cAMP, while α-agonists most likely act via Ca^{2+} translocation (Ramachandran *et al*, 1982, 1983). Therefore, it appears that the phosphorylation and inactivation of heart GS *in vivo*, may be catalysed by at least three types of protein kinase: (1) cAMP-dependent, (2) Ca^{2+}-dependent, (3) cAMP/Ca^{2+}-independent. Although the complete amino acid sequence of heart GS

has not been determined recent studies indicate that the structure and enzymatic properties of the heart enzyme are very similar to those of the skeletal muscle enzyme (Grekinis *et al*, 1995).

Changes in GS activity produced by phosphorylation can be reversed by the removal of phosphoryl groups. Dephosphorylation (and activation) of GS is catalysed mainly by the glycogen-bound form of protein phosphatase 1 ($PP1_G$), which is capable of dephosphorylating all of the sites in GS. The catalytic action of $PP1_G$ is blocked by the phosphorylated form of protein phosphatase inhibitor-1 (PPI-1) which has been shown to be hormonally regulated in myocytes by PKA (Gupta *et al*, 1996).

It is likely that differences in the synthesis of heart and skeletal muscle glycogen are determined by the interplay of the kinases and phosphatases which could lead to a difference in the phosphorylation state, and thus the activity state, of GS in the two tissues.

Glycogen is phosphorolytically cleaved by the enzyme glycogen phosphorylase to yield glucose-1-phosphate (Glu-1-P), which is converted to Glu-6-P by phosphoglucomutase. This is energetically advantageous because the released sugar is already phosphorylated. Hydrolytic cleavage of glycogen to glucose would mean it would have to be phosphorylated at the expense of an ATP molecule in order to enter the glycolytic pathway. Like GS, glycogen phosphorylase is regulated by reversible phosphorylation except that phosphorylation produces activation of the enzyme, not inactivation. Glycogen phosphorylase is activated by a specific phosphorylase kinase, which itself is activated through phosphorylation by PKA. Again the changes in activity due to phosphorylation are reversed by $PP1_G$. Hence both glycogen synthesis

and degradation are regulated reciprocally such that a rise in cellular cAMP levels, caused by β-adrenergic stimulation, will initiate a cascade of events leading to the activation of glycogen phosphorylase and inactivation of GS, while a fall in cAMP levels will have the opposite effect.

Increased cardiac workload and anoxia stimulate glycogenolysis due to a drop in ATP and a rise in AMP and P_i. The resulting depletion of glycogen stores is responsible for stimulating the resynthesis of glycogen following increased cardiac work or ischaemia (Opie, 1991). Most mammalian cells store some glycogen which acts as a buffer against short-term alterations in blood glucose. In heart, this system also provides protection against other factors that lead to ATP depletion such as hypoxia, ischaemia and increased workload and ensures efficient generation of ATP from a limited supply of endogenous substrate (Goodwin *et al*, 1996).

1.2.2 Lipid Fuels

Although the heart can utilise a variety of substrates to fulfill its high energy demands, fatty acids are preferred under normal conditions providing 60–70% of the hearts energy requirements (Neely & Morgan, 1974). The main proportion of fatty acids delivered to the heart are as non-esterified fatty acids (NEFA) bound to albumin (Evans, 1964). An additional source of fatty acids are those released from TAG-containing particles (lipoproteins), such as chylomicrons and very-low-density lipoproteins (VLDL), after hydrolysis by lipoprotein lipase (LPL), located on the luminal surface of the endothelial cell membrane (Stam & Hulsmann, 1985). Fatty acids can also be derived by lipolysis of endogenous TAG stores and under certain conditions, endogenous phospholipids. To date the relative proportion of fatty acids

derived from these sources is unclear. The majority of fatty acids are oxidised in the mitochondria to produce energy for electromechanical activity and other ATP-requiring processes (Neely & Morgan, 1974). A small proportion of fatty acids are esterified and stored in pools of TAG and phospholipids. Normally, tissue levels of non-esterified fatty acids is very low (Van der Vusse & Reneman, 1983)

1.2.2.1 Extracellular Sources of Fatty Acids

Adipose tissue TAG is quantitatively the most important source of stored energy in mammals. Hydrolysis of this TAG is under acute neural and hormonal control (Yeaman *et al*, 1994) with the release of energy regulated to meet the constantly changing energy demand of the animal. The enzyme responsible for the hydrolysis of adipose TAG is hormone-sensitive lipase (HSL). HSL catalyzes the rate-limiting step in the lipolysis of TAG, releasing glycerol and fatty acids which may then be oxidised by other tissues to provide energy (Nilsson *et al*, 1980). Regulation of lipolysis is mediated via reversible phosphorylation of HSL. Lipolytic agents such as adrenaline, noradrenaline, corticotropin and glucagon activate the TAG-hydrolase activity by increasing the intracellular cAMP concentration, resulting in activation of cyclic-AMP-dependent protein kinase (PKA). This leads to phosphorylation and activation of HSL (Nilsson *et al*, 1980). Conversely insulin, the major anti-lipolytic hormone, decreases phosphorylation (and activity) of HSL, in part by lowering intracellular cAMP (Londos *et al*, 1985) by activation of a low K_m cAMP phosphodiesterase (Elks *et al*, 1983). There is evidence that insulin can exert a cAMP-independent anti-lipolytic effect, the mechanism of which has been proposed as via activation of protein phosphatases (Stralfors & Honnor, 1989).

HSL is activated by phosphorylation at a single site by PKA *in vitro* (Garton *et al*, 1988). This site (site 1) has been termed the regulatory site and is located at position 563 of the rat HSL polypeptide. A second site (site 2) phosphorylated in the absence of lipolytic stimuli, has been termed the basal site and is located at position 565. Peptide mapping studies have shown that these two sites are mutually exclusive, i.e. phosphorylation of site 1 blocks subsequent phosphorylation of site 2 and *vice versa* (Garton & Yeaman, 1990). Site 2 can be phosphorylated *in vitro*, by a number of protein kinases including AMP-activated protein kinase (AMPK), CAMK and glycogen synthase kinase-4 (GSK_4) (Garton *et al*, 1989). Phosphorylation at site 2 has no direct effect on the activity of HSL, but renders site 1 inaccessible to phosphorylation by PKA and hence HSL cannot be activated. Of the above mentioned protein kinases, AMPK is thought most likely to be responsible for phosphorylation of the basal site of HSL *in vivo*, since it is known to regulate two other key enzymes in lipid metabolism. Acetyl-CoA carboxylase (ACC) and 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, the key regulatory enzymes in fatty acid and sterol synthesis respectively, are both phosphorylated and inactivated by AMPK *in vivo* (Carling *et al*, 1987b; Hardie *et al*, 1989). Also, activation of AMPK decreases isoprenaline-stimulated lipolysis in adipocytes, suggesting an important anti-lipolytic role in regulating HSL activation *in vivo* (Sullivan *et al* 1994). AMPK can be phosphorylated and activated by an AMP-activated protein kinase kinase (AMPK kinase), which is itself activated by AMP (Weekes *et al*, 1994) (see section 1.3.5). It has been reported that low concentrations of palmitoyl-CoA can stimulate reactivation of AMPK by endogenous AMPK kinase (Carling *et al*, 1987b; Weekes *et al*, 1996),

providing a possible feedback regulation of lipolysis via phosphorylation of the basal site of HSL, preventing activation of the lipase. However, this result was obtained using a crude system and has not been reproduced in a more purified reconstituted system (Weeks *et al*, 1996).

1.2.2.1.1 Plasma Lipoproteins

Exogenous TAG is also supplied to the heart via the circulation. Hydrophobic TAG molecules form the core of lipoprotein particles which are coated by a hydrophilic layer consisting of phospholipids, cholesterol, and apoproteins (Dolphin, 1985). Depending on their size, chemical composition and site of formation, these particles are called very low density lipoprotein (VLDL) or chylomicrons. VLDL are synthesised by parenchymal liver cells and secreted into the hepatic vein (Spector, 1984), whereas chylomicrons are synthesised by enterocytes in the small intestine and enter the circulation via the thoracic duct. Although endothelial cells are capable of internalising intact lipoprotein particles (Cryer, 1989), and studies conducted using isolated rat hearts have suggested that some circulating TAG can be extracted without chemical modification (Scheuer & Olson, 1967), it is generally accepted that the majority of fatty acids are extracted by cardiac tissue after hydrolysis of the TAG core of lipoprotein particles on the lumenal side of the endothelium (Cryer, 1981, 1989). The amount of fatty esters present in lipoproteins is dependent on the nutritional state of the animal. While *in vitro* studies indicate that fatty acyl moieties present in lipoproteins can serve as the major source of cardiac energy (Delcher *et al*, 1965), the actual contribution of fatty acids from circulating lipoproteins to overall fatty acid uptake by the heart in situ is thought to be small to moderate (Carlson *et al*, 1970).

In the heart, hydrolysis of the TAG core of lipoproteins occurs at the luminal surface of the coronary vascular endothelium by the actions of lipoprotein lipase (LPL). Lipoproteins bind transiently to endothelial sites where their TAG are acted upon by LPL. The fatty acid products of LPL hydrolysis are taken up by the endothelial cells and myocytes and move down a concentration gradient created by their use for catabolic and anabolic processes (Smith & Scow, 1979), or reesterification within cells (Scow & Blanchette-Mackie, 1985). LPL also exhibits phospholipase A_1 activity, exerted by breaking down the outer phospholipid layer of lipoproteins to reveal the TAG core for hydrolysis (Groot *et al*, 1979). For optimal activity LPL requires a neutral to alkaline pH and the presence of activator apoprotein CII embedded in the outer phospholipid layer of VLDL and chylomicrons (Cryer, 1989).

The gene expressing LPL is located in myocytes where the enzyme is synthesised (Camps *et al*, 1990). After protein synthesis and post-transcriptional processing the enzyme is secreted into the interstitial space and transported to the endothelial surface, where it is functionally active. LPL is a 55 kDa glycoprotein, anchored to the lumenal membrane of the endothelial cell by chains of heparin sulphate proteoglycans in the glycocalyx of the plasma membrane (Cryer, 1989). LPL is continuously synthesised, secreted, bound, and released from the endothelial surface. The half-life of LPL at the luminal surface of the endothelium is normally in the order of 90 min (Stam *et al*, 1984), although Bagby (1983) have calculated the half life to be just 20 min. This short half life suggests that continuous release of the enzyme, to its site of action, is an important regulator of the actual activity of the enzyme. The expression of LPL at the luminal surface may be controlled at a number of stages, including control of the rate

of synthesis of the proenzyme, its chemical modification and activation in the myocyte, enzyme storage, transport and degradation in the parenchymal cell, release of the enzyme into the interstitial space, transfer of the enzyme to the site of action at endothelial cell, attachment to and release from the luminal surface, and interaction of the enzyme with its substrate (Cryer, 1989). In the fed state ~78% of overall myocardial LPL is localised in the myocytes, ~4% is localised in the extracellular space, and ~18% is localised in the capillary endothelium (Blanchette-Mackie *et al*, 1989). Within the myocyte LPL is located primarily in the sarcoplasmic reticulum (SR), Golgi sacs, transport vesicles, and at the cell periphery, while extracellular LPL is located near the opening of secretory vesicles and in the space between the myocytes and capillary endothelium.

In heart and adipose tissue, LPL activity is very much dependent on the nutritional status of the animal. In the fed state, when fatty acids are being stored, LPL activity is high in adipose tissue, and low in heart; whilst in the starved animal, when fatty acids are mobilised for oxidation, LPL activity is low in adipose tissue and high in heart (Hollenberg, 1959; Borensztajn *et al*, 1970). These long-term changes in LPL activity are under hormonal control, with insulin being one hormone directly involved in LPL regulation. While adipose tissue and heart seem to show reciprocal changes in LPL activities, it remains to be established whether one or more hormones can change the activity of LPL in opposite directions in different tissues. It is more likely that the hormone(s) which modulate adipose tissue LPL activity differ from those which affect LPL activity in heart. Insulin plays an important role in regulating adipose tissue LPL activity *in vivo*, but appears to have little effect on cardiac LPL activity (Borensztajn

et al, 1972). Perfusion of hearts with adrenaline or glucagon increases the functional activity of LPL on the luminal side of the endothelium, with a concomitant decrease in LPL activity in the tissue compartment of the heart (Stam & Hulsmann, 1984). This results from stimulation of LPL transport from the intracellular compartment to the vascular endothelial site of action, and may be mediated by cAMP induced stimulation of the microtubular system (Stam & Hulsmann, 1984; Cryer, 1989). Long-term regulation of cardiac LPL activity involves changes in enzyme quantity and/or activity. Stimulatory effects on overall cardiac LPL activity after treatment with adrenocorticotropic hormone (ACTH) and thyroxine (T_4) (Stam *et al*, 1984), are probably the result of either increased rates of enzyme synthesis or decreased rates of enzyme degradation. Cardiac LPL activity is also increased by fat feeding (Jensen *et al*, 1975; Hulsmann *et al*, 1979), most likely mediated by glucocorticoids (Pedersen *et al*, 1981), fasting (Borensztajn *et al*, 1970) and diabetes, and decreased by carbohydrate feeding (Cryer, 1981; Stam *et al*, 1984). Although insulin has almost no effect on overall LPL activity in rat heart (Borensztajn *et al*, 1975), insulin may be required in glucocorticoid-mediated effects on cardiac LPL activity (Friedman *et al*, 1980).

1.2.2.2 Fatty Acid Uptake & Intracellular Transport

Fatty acids released from adipocyte TAG, are bound to albumin in blood plasma, increasing their solubility several fold. Normal arterial fatty acid levels range between 0.2 and 1 mmol/l plasma, but can increase dramatically (up to 2 mmol/l plasma) under certain conditions such as starvation, diabetes and myocardial infarction. The plasma concentration of albumin is ~ 0.6 mmoles/l plasma, with each molecule of protein

containing 6-8 fatty acid binding sites (Spector *et al*, 1971). Therefore, plasma albumin can easily accommodate the fatty acids present in the blood with <0.1% of fatty acid molecules present in an unbound form (Spector, 1971). The amount of fatty acids extracted by myocytes is determined by the arterial concentration of fatty acids, the workload of the heart, and the presence of competing substrates (Nikkila, 1971; Drake *et al*, 1980).

While the transport of fatty acids between organs is carried out by plasma albumin or as TAG contained within lipoproteins, the intracellular transport of long-chain fatty acids as well as the modulation of free cytoplasmic fatty acid concentrations, is thought to occur primarily by fatty-acid binding proteins (FABP) (Bassingthwaighte *et al*, 1989b; Glatz & Van der Vusse, 1989). FABPs belong to a family of low molecular weight (14-15 kDa) cytosolic proteins which have a high affinity for long-chain fatty acids (Bass, 1988; Veerkamp *et al*, 1991) and are found in a wide variety of mammalian tissues (Sweetser *et al*, 1987). Heart tissue contains appreciable amounts of FABP and are thought to play a crucial role in the transport of fatty acids from the cellular membrane to the intracellular site of oxidation.

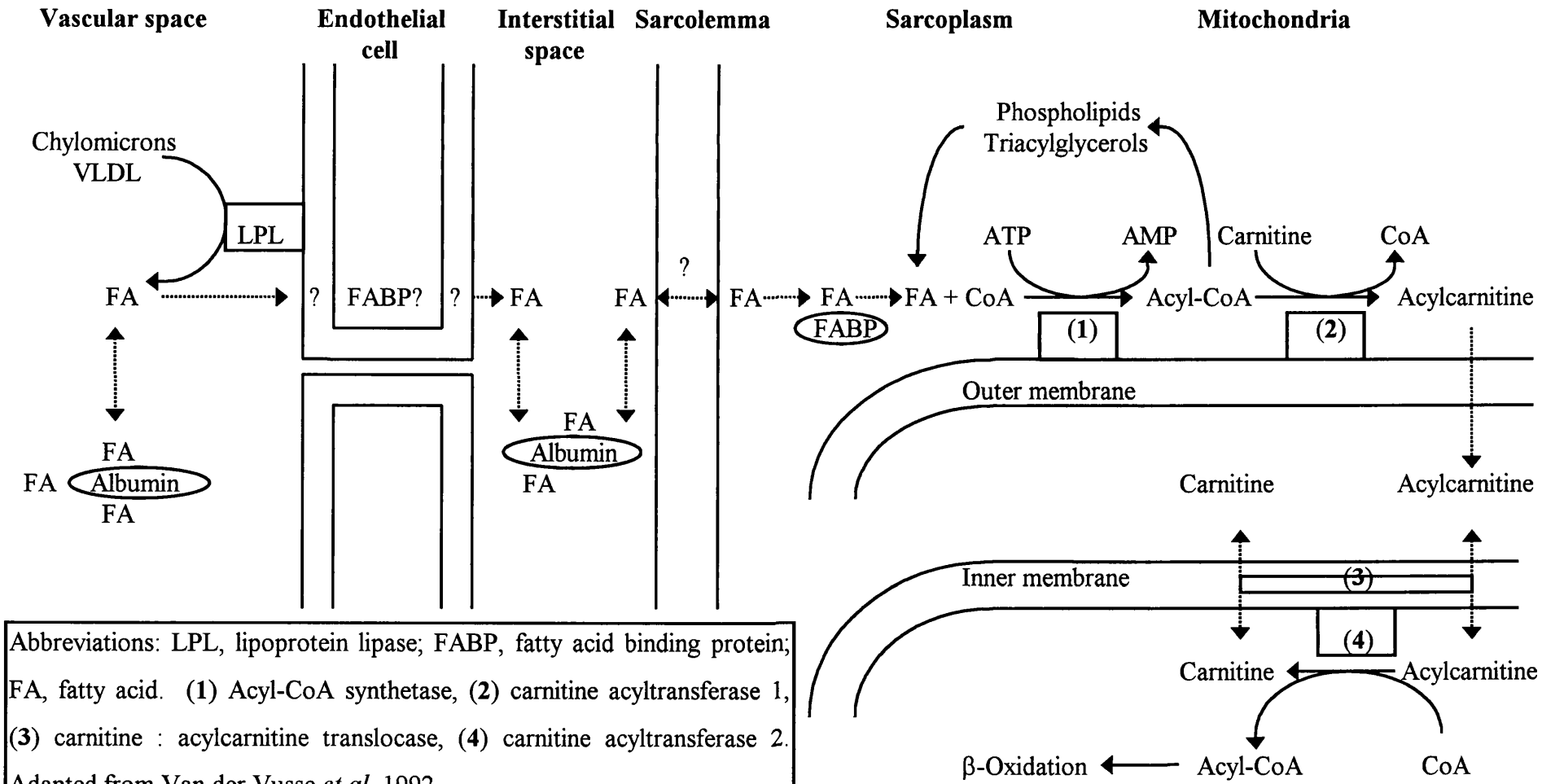
Fatty acid extraction by the heart is rapid and highly efficient, and occurs at the luminal surface of the endothelium. The exact mechanism of fatty acid transport from the vascular compartment to the site of oxidation in cardiomyocytes is still a controversial subject (Bassingthwaighte *et al*, 1989a). It is thought that following their release from albumin, fatty acids are translocated across the luminal cell membrane of the endothelial cell and diffuse through the endothelial cytoplasm, possibly facilitated by cytoplasmic FABP ($FABP_c$), and are translocated through the

abluminal cell membrane into the interstitial space. The fatty acids diffuse through the interstitial space between the endothelial and parenchymal cells of the heart (probably mediated by albumin), and cross the sarcolemma, passively by diffusion along a concentration gradient, or possibly facilitated by plasma membrane FABP ($FABP_{pm}$). Finally after translocation across the sarcolemma, the fatty acids may bind to $FABP_c$ to facilitate cytoplasmic transport to reach the mitochondrial outer membrane (fig 1.4).

Fatty acid oxidizing capacities of several tissues have been shown to vary in parallel with their FABP content (Glatz *et al*, 1989; Veerkamp & Van Moerkerk, 1993). During postnatal development there is a marked increase in FABP content up to 70 days, with a concomitant rise in fatty acid oxidation capacity of the heart (Veerkamp & Van Moerkerk, 1993). Apart from fatty acid uptake, FABP may influence other mechanisms of intracellular fatty acid disposal. Facilitation of substrate supply or product removal by $FABP_c$ stimulates the activity of several membrane bound enzymes of lipid metabolism *in vivo* (Fournier *et al*, 1983; Bass, 1988). $FABP_c$ also has the ability to sequester long-chain fatty acids preventing them from inhibiting enzymes such as Na^+ -K^+ -ATPase and mitochondrial adenine nucleotide translocase (Bass, 1988). The reputed protective effect of $FABP_c$ may be of great importance in the heart during ischaemia when there is an accumulation of fatty acids (Van der Vusse *et al*, 1982; Glatz *et al*, 1988; Srimani *et al*, 1990) which are potentially harmful to the myocardium.

Fatty acids derived from exogenous TAG sources are preferentially oxidised, with only a small amount being re-esterified to TAG, while the opposite is true of exogenous NEFA taken up from blood plasma (Tamboli *et al*, 1983). This would suggest two

Figure 1.4 Schematic representation of fatty acid uptake, transport and activation in the heart



Abbreviations: LPL, lipoprotein lipase; FABP, fatty acid binding protein; FA, fatty acid. **(1)** Acyl-CoA synthetase, **(2)** carnitine acyltransferase 1, **(3)** carnitine : acylcarnitine translocase, **(4)** carnitine acyltransferase 2. Adapted from Van der Vusse *et al*, 1992.

different pathways for the uptake of NEFA and TAG-derived fatty acids. It has been suggested that fatty acids derived by LPL hydrolysis of TAG enter an interfacial membrane continuum which delivers them to the mitochondria for oxidation (Scow & Blanchette-Mackie, 1992), thus providing a pathway for the preferential oxidation of TAG-derived fatty acids.

1.2.2.3 Endogenous Triacylglycerol

Acyl groups present on long-chain acyl-CoA can either be targeted for oxidation in mitochondria or for incorporation into myocardial lipid stores. Phospholipids and TAG are the main forms of fatty acyl moieties present in the heart. Phospholipids are essential constituents of the sarcolemma and intracellular membranes and account for ~85% of the total endogenous pool of fatty acids (Van der Vusse, 1992). Perfusion of isolated hearts under various conditions has shown that phospholipids do not significantly contribute fatty acids for oxidation (Olson & Hoeschen, 1967; Crass *et al*, 1971; Saddik & Lopaschuk, 1991). Storage of fatty acids in endogenous myocardial TAG pools provides the heart with an important source of fatty acids for oxidative metabolism (Saddik & lopaschuk, 1991). Cardiac myocytes store TAG as membrane-bound lipid particles, as free-floating cytoplasmic droplets or in lysosome-like particles and all or some of this TAG is in a dynamic state of continuous synthesis and degradation (De Groot *et al*, 1989). Similar particles have been isolated from bovine heart and have been shown to posses FAS, acyltransferase (Christiansen, 1975) and lipase (Schousboe *et al*, 1973) activities. Therefore, TAG synthesis, storage and mobilisation can all occur within these individual, mobile particles making them a versatile unit of for lipid utilisation.

The concentration of exogenous fatty acids plays an important role in the regulation of endogenous TAG lipolysis (Crass, 1972). Increasing the concentration of exogenous fatty acids in isolated perfused hearts, inhibits lipolysis of endogenous TAG and stimulates TAG synthesis, possibly via stimulation of phosphatidic acid phosphohydrolase (PAP) (Schoonderwoerd *et al*, 1989a). Endogenous TAG fatty acids are used in preference to glucose. In the absence of exogenous fatty acids in excess of 50% of the hearts energy requirements are met by utilisation of endogenous TAG reserves (Saddik & Lopaschuk, 1991). In the presence of physiological levels of exogenous fatty acids (0.4mM palmitate), endogenous TAG fatty acids provide in excess of 40% of overall ATP production (Saddik & Lopaschuk, 1991). Even in the presence of high concentrations of exogenous fatty acids (1.2mM palmitate), endogenous TAG fatty acids can account for 11% of total myocardial ATP production, with no change in size of the endogenous TAG pool. The fact that the TAG pool does not alter in size suggests that it is a rapidly turning over metabolic intermediate (Saddik & Lopaschuk, 1991).

Cardiac content of TAG is increased by isoproterenol (Jodalen *et al*, 1982) and in type I diabetes (Denton & Randle, 1967) implying an upregulation of myocardial TAG synthesis. Myocardial ischaemia has also been shown to increase incorporation of fatty acids into TAG (Trach *et al*, 1986). At present an integrated metabolic control system for cardiac TAG synthesis/mobilisation has yet to be described. Two enzymes of the TAG synthesis pathway, glycerolphosphate acyltransferase (GPAT) and PAP, have been particularly documented within the context of liver or adipose tissue (Tijburg *et al*, 1989), while less is known about the enzymology and regulation of the

two enzyme activities in heart. However, a recent study by Swanton & Saggerson (1997), has described the properties and subcellular distribution of PAP and GPAT activities in cardiac myocytes and rat heart ventricle muscle. GPAT activity isolated from the sarcoplasmic reticulum (SR) fraction of ventricle muscle, was found to have a significantly higher K_m for glycerol-3-P (4.06mM) than that of the GPAT from adipose tissue microsomes (0.97mM) (Swanton & Saggerson, 1997). Glycerol-3-P levels in incubated cardiac myocytes appear higher than in perfused hearts and can rise from ~1mM under normoxic conditions to as much as 6mM during prolonged hypoxia (Myrmel *et al*, 1991, 1992). It has been suggested that the high K_m for glycerol-3-P of this GPAT found in the SR of cardiac myocytes, means that TAG synthesis can vary quite widely with changes in the tissue level of glycerol-3-P (provided fatty acid supply is maintained) (Swanton & Saggerson, 1997).

1.2.2.4 Ketone Bodies

Under normal conditions acetyl-CoA formed from fatty acid oxidation enters the citric acid cycle for the production of ATP. However, under conditions where fatty acid oxidation predominates (e.g. during starvation, diabetes or fat feeding), acetyl-CoA in the liver is utilised in formation of the ketone bodies β-hydroxybutyrate and acetoacetate. The reason for this is that entry of acetyl-CoA into the citric acid cycle depends on the availability of oxaloacetate for the formation of citrate. Under conditions of fasting and diabetes, oxaloacetate is diverted to gluconeogenesis and hence is unavailable for condensation with acetyl-CoA which is thus unable to enter the citric acid cycle. Also, in ketotic states such as starvation and diabetes, a fall in the absolute level of circulating insulin, coupled with elevation of the glucagon/insulin

ratio stimulates the mobilisation of free fatty acids from peripheral fat depots as well as shifting the balance of fatty acid traffic away from esterification and towards the oxidative pathways (McGarry & Brown, 1997). Carbon flow through glycolysis and ACC is reduced, the malonyl-CoA level falls, and fatty acid synthesis ceases. This fall in malonyl-CoA also causes CPT 1 to be derepressed, such that incoming free fatty acids readily undergo β-oxidation resulting in accelerated ketone body production (McGarry & Foster, 1980; McGarry *et al*, 1989). Ketone bodies are formed in the liver and are readily utilised by most tissues except the liver (due to low levels of 3-oxoacid CoA transferase needed to activate acetoacetate). The heart efficiently uses circulating ketone bodies by first activating them to acetoacetyl-CoA in a reaction with succinyl-CoA (from the citric acid cycle) catalysed by a specific 3-oxoacid CoA transferase. Acetoacetyl-CoA is then cleaved by a thiolase to yield two molecules of acetyl-CoA which can enter the citric acid cycle. The rate at which ketone bodies are taken up and oxidised is limited only by their availability (Opie, 1969; Robinson & Williamson, 1980). Perfusion of isolated hearts with the ketone bodies β-hydroxybutyrate and acetoacetate, inhibits the oxidation of both octanoate (carnitine independent) and oleate (carnitine dependent) fatty acids (Forsey *et al*, 1987), suggesting inhibition at the level of β-oxidation *per se*. In isolated cardiomyocytes Chen *et al* (1984), found contrasting results in that β-hydroxybutyrate inhibits the carnitine dependent oxidation of palmitate, but has only minor effects on the carnitine independent oxidation of octanoate, suggesting inhibition at the level of fatty acid transport into mitochondria by the CPT system. This difference is probably due to differences in the metabolism of isolated perfused hearts compared to isolated

cardiomyocytes. There are a number of possible mechanisms by which ketone bodies inhibit fatty acid oxidation: (1) Activation of acetoacetate by the 3-oxoacid CoA transferase could reduce the levels of CoASH thus inhibiting octanoate activation. (2) Accumulation of acetoacetyl-CoA could inhibit β-oxidation directly as it is a potent inhibitor of acyl-CoA dehydrogenase (McKean *et al*, 1979). (3) An increase in acetyl-CoA and decrease in CoASH due to increased oxidation of ketone bodies, could inhibit β-oxidation at the level of 3-ketoacyl-CoA thiolase which is very sensitive to regulation by the acetyl-CoA/CoA ratio (Olowe & Schulz, 1980). In addition to a decrease in fatty acid uptake, the presence of acetoacetate in a perfusate containing palmitate, increases the incorporation of palmitate into endogenous TAG in isolated perfused rat heart by approximately fivefold (Olson, 1962). Ketone bodies are therefore able to divert fatty acids away from oxidation and towards esterification as TAG, helping to explain the increase in myocardial TAG found in experimental diabetes (Murthy & Shipp, 1977).

1.2.3 Fatty Acid Oxidation

The enzymes of mitochondrial β-oxidation all act on CoA esters, so a preliminary to β-oxidation is the ATP-dependent formation of fatty acyl-CoA esters, catalysed by fatty acyl-CoA synthetase (FAS). The heart contains a number of FAS activities with differing chain-length specificities and subcellular locations (Aas, 1971). Long-chain FAS in the heart is primarily located on the outer side of the mitochondrial outer membrane (Norman *et al*, 1983), but some may be localised at the sarcoplasmic reticulum (SR). Activation of long-chain fatty acids to their CoA esters requires energy derived from the hydrolysis of ATP. This reaction is controlled by the

cytoplasmic acyl-CoA/CoASH ratio and the total concentration of CoASH (Idell-Wenger & Neely, 1978) and is inhibited by its products, P_i and AMP (Groot *et al*, 1976). Fatty acids from both endogenous and exogenous sources must first be activated prior to β-oxidation. The newly formed long-chain acyl-CoA is relatively insoluble in water and is probably bound to $FABP_c$, lipid membranes and/or acyl-CoA binding protein (ACBP). ACBP is a widely distributed cytosolic protein of molecular mass 10 kDa that has been found in all eukaryotes tested. Activities of both aqueous cytoplasmic (i.e., acetyl-CoA carboxylase) and mitochondrial enzymes (i.e., adenine nucleotide translocase) are inhibited by long-chain acyl-CoA esters (Rasmussen *et al*, 1993). Binding of long-chain acyl-CoA esters to ACBP may act to relieve inhibition upon these enzymes and may also function in the site-specific transport of acyl-CoA within the aqueous cytoplasm. In addition ACBP readily protects acyl-CoA against hydrolysis by microsomal hydrolases and stimulates mitochondrial long-chain acyl-CoA synthetase (Rasmussen *et al*, 1993; Færgeman & Knudsen, 1997).

1.2.3.1 The Carnitine Palmitoyltransferase System

Short-chain acyl-CoA ($<C_{10}$) can directly enter the mitochondrial matrix where the enzymes of the β-oxidation pathway are located, while long-chain acyl-CoA needs the action of a specific carnitine-dependent transport system to overcome the permeability barrier of the inner mitochondrial membrane to acyl-CoA esters. This system consists of three enzymes. (1) Carnitine palmitoyltransferase (CPT) 1, located in the outer mitochondrial membrane (Murthy and Pande, 1987), catalyses the conversion of acyl-CoA to acylcarnitine. (2) Carnitine:acylcarnitine translocase transports the acylcarnitine across the inner mitochondrial membrane in a 1:1 exchange with

carnitine. The mechanism of the carnitine:acylcarnitine translocase appears to be of the "ping-pong" type, which makes it unique among all the mitochondrial metabolite transporters (Palmieri *et al*, 1996). (3) CPT 2, located on the matrix side of the inner mitochondrial membrane, catalyses the regeneration of acyl-CoA from acylcarnitine and mitochondrial matrix CoASH (Bieber, 1988). Although the CPT system for entry of acyl moieties into the mitochondria has been known since the 1960s, until recent years the enzymology of the system has been controversial. Recent cloning and expression of rat liver CPT 1 and 2 cDNAs has provided strong evidence that they are distinct proteins (Brown *et al*, 1994; Johnson *et al*, 1995; McGarry & Brown, 1997) and not a single polypeptide as once thought.

CPT 1 is a key enzyme in the regulation of fatty acid oxidation (see section 1.2.3.3) and is regulated mainly by the simple molecule, malonyl-CoA. Malonyl-CoA, the product of acetyl-CoA carboxylase (ACC), is a key regulatory step in fatty acid synthesis, and a potent inhibitor of CPT 1 acting at a site distinct from the catalytic site (Murthy and Pande, 1987, 1988; Woeltje *et al*, 1990a, 1990b). CPT 1 has been shown to exist in at least two isoforms designated as liver-type (L-CPT 1) and muscle type (M-CPT 1), based upon the tissues originally identified as expressing these isoforms (Weis *et al*, 1994a, 1994b). However, northern blot analysis and [^{3}H] etomoxir-CoA labeling of CPT 1 in mitochondria from a number of tissues has shown expression of both isoforms to be widespread.

L- and M-CPT 1 have been shown to migrate on SDS/polyacrylamide gels with apparent monomeric sizes of ~88 and 82 kDa respectively (Woeltje *et al*, 1990; Weis *et al*, 1994b). However, recent isolation of L- and M-CPT 1 cDNAs predicted

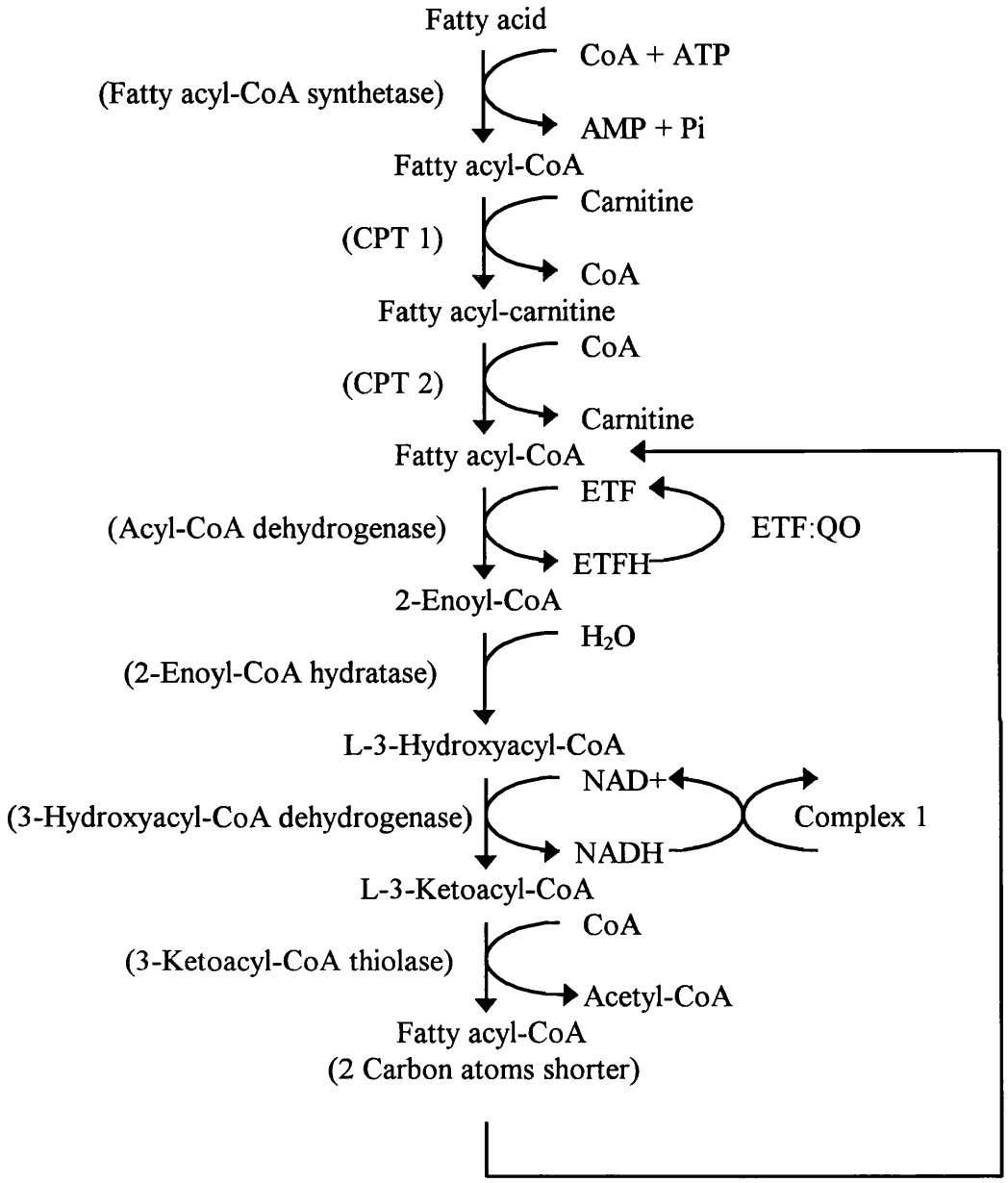
proteins of 773 (88150 Da) and 772 (88227 Da) amino acids respectively (Esser *et al*, 1993, 1996; McGarry & Brown, 1997). The fact that *in vitro* transcribed and translated cDNAs (Esser *et al*, 1996) also migrate differently on SDS/polyacrylamide gels, infers that their different electrophoretic mobilities is an intrinsic property of their primary structures, as opposed to selective post-translational modification. L- and M-CPT 1 differ in their kinetic characteristics (Km for carnitine, ~30 and 500μM, respectively and the concentration of malonyl-CoA required to inhibit CPT 1 activity by 50% (IC_{50}), ~2.7 and 0.03μM, respectively) (McGarry *et al*, 1983). Adult rat heart mitochondria expresses both the M- and L-type enzymes (Weis *et al*, 1994a, 1994b) in a 98:2 ratio of M- to L-CPT 1 resulting in a CPT 1 activity that has kinetic characteristics in-between those of liver and skeletal muscle (K_m for carnitine ~200μM, IC_{50} for malonyl-CoA ~0.1μM). The level of heart L-CPT 1 in the late fetal/neonatal rat is particularly high (Brown *et al*, 1995). Use of the inhibitor, 2-[6-(2,4-dinitrophenoxy)hexyl]oxirane-2-carboxylic acid (DNP-Et) (specific for L-CPT 1) has shown that in the neonatal heart, L-CPT 1 contributes ~25% to total CPT 1 activity (in terms of V_{max}). This is important as myocardial carnitine content is very low at birth and L-CPT with its lower K_m for carnitine (~30μM) provides 60% of total cardiac fatty acid oxidation in the newborn rat. L-CPT 1 contribution decreases simultaneously with the accumulation of carnitine in the growing heart (with a concomitant increase in expression of the M isoform) to reach its adult level of 2-3% (Brown *et al*, 1995).

1.2.3.2 The β-oxidation Pathway

Once the acyl moieties are transferred into the mitochondrial matrix and converted back to long-chain acyl-CoA, they enter the β-oxidation pathway (fig 1.5). There are multiple enzymes for each of the constituent steps of the pathway, which vary in their chain-length specificity. The first (rate limiting) step involves the conversion of acyl-CoA to 2-enoyl-CoA by the action of acyl-CoA dehydrogenase. Electrons from the dehydrogenase are transferred via electron transferring flavoprotein (ETF), to ETF:ubiquinone oxidoreductase (ETF:QO) and thence to the mitochondrial respiratory chain at the level of ubiquinone. The second step catalyses the addition of water to 2-enoyl-CoA by the enzyme enoyl-CoA hydratase to form L-3-hydroxyacyl-CoA. In the third step, L-3-hydroxyacyl-CoA dehydrogenation is catalysed by the enzyme L-3-hydroxyacyl-CoA dehydrogenase to produce L-3-ketoacyl-CoA. In this step, NAD^+ is involved in the dehydrogenation as the coenzyme and electrons are transferred via NADH dehydrogenase to the respiratory chain at the level of NADH-Q reductase. In the final step of the pathway, L-3-ketoacyl-CoA is cleaved at the 2,3 position, by 3-ketoacyl-CoA thiolase, which requires CoASH to yield acetyl-CoA and a shortened acyl-CoA derivative. This shorter acyl-CoA molecule feeds back to the first step in the pathway and passes through the β-oxidation pathway again. Acetyl-CoA produced from each cycle of β-oxidation is now available to the citric acid cycle where it is catabolised to CO_2.

The mitochondrial carnitine:acylcarnitine translocase is thought to have an upper limit with regard to acyl group length and it is thought that oversized molecules are initially

Figure 1.5 Pathway of mitochondrial β-oxidation in heart



Abbreviations: CPT, carnitine palmitoyl transferase; ETF, electron transfer flavoprotein; ETF:QO, ETF:ubiquinone oxidoreductase; ETFH, reduced ETF. Adapted from Eaton *et al*, 1996.

shortened in chain length by undergoing partial oxidation in peroxisomes, which contain a separate β-oxidation pathway. These chain-shortened fatty acyl groups are transferred from CoASH to carnitine by the action of peroxisomal carnitine medium/long-chain acyltransferases and, subsequently, migrate to the carnitine translocase for mitochondrial import (Cronin, 1997). Liver peroxisomes have been shown to contain malonyl-CoA-sensitive (strongly associated with the peroxisomal membrane) and -insensitive (located in the peroxisomal matrix) carnitine medium/long-chain acyltransferases (Singh *et al*, 1996). It has been established that the malonyl-CoA insensitive CPT activity of peroxisomes, previously referred to as carnitine octanoyltransferase (COT), differs in monomeric size, primary structure and immunologically from the internal CPT proteins of mitochondria and microsomes (Ramsay, 1988; McGarry & Brown, 1997). While the role of the CPT system in mitochondria is well understood, its function in other organelles, such as peroxisomes and microsomes, is still unclear. While it is generally accepted that isolated peroxisomes do not require carnitine for the oxidation of fatty acids (Kunau *et al*, 1995), it is possible that these structures are less permeable to CoA esters, *in situ*, and therefore require a CPT shuttle for the inward transport of fatty acyl moieties. A CPT system may also be needed for the export of medium chain acyl groups, which are the major products of peroxisomal fatty acid oxidation in the liver (Kunau *et al*, 1995). Peroxisomal β-oxidation occurs similar to that of mitochondria, except that electrons are transferred directly to molecular oxygen to yield H_2O_2, which is broken down by catalase. Peroxisomal β-oxidation is virtually inactive towards short-chain (C_6 and C_4) fatty acids and so these intermediates must be further oxidised in the mitochondria

(Lazarow, 1978; Mannaerts, 1982). In rat hearts the relative contribution of peroxisomes to the total oxidative capacity is 10-30% for common fatty acids, but for fatty acids of chain length greater than 22 carbon atoms, can reach 45% (Norseth & Thomassen, 1983; Veerkamp & Van Moerkerk, 1986; Reubsaet *et al*, 1989). Fatty acids can undergo alpha and omega fatty acid oxidation but the presence of these pathways in heart has yet to be established.

1.2.3.3 Regulation of Fatty Acid Oxidation

The rate of fatty acid oxidation is controlled by a number of factors including supply of fatty acids by the blood, levels of high energy phosphates, redox state of the mitochondria, energy demand of the tissue and availability of CoASH in the mitochondrial compartment and cytoplasmic space (Oram *et al* 1973; Moore, 1985). Fatty acid oxidation may be regulated at a number of key steps. Transport of long-chain acyl-CoA across the mitochondrial inner membrane by the CPT system is likely to be the primary site of regulation (Bielefeld *et al*, 1985; Latipaa *et al*, 1989), although modulation of 3-ketoacyl-CoA thiolase, which catalyses one of the steps in the β-oxidation pathway, may also be a site of regulation as it is highly sensitive to inhibition by acetyl-CoA produced from the PDH reaction (Olowe & Schulz, 1980).

1.2.3.3.1 Carnitine Palmitoyltransferase 1

While liver CPT 1 increases in activity and decreases its sensitivity to inhibition by malonyl-CoA during fasting (Cook, 1984) and in the diabetic state (Cook & Gamble, 1987), neither of these changes seem to occur in heart CPT 1 (Cook, 1984; Paulson *et al*, 1984; Cook & Lappi, 1992). This suggests an important difference in the regulation of hepatic and myocardial fatty acid oxidation at the level of CPT 1. While

there is no evidence at present that cardiac CPT 1 is regulated by a phosphorylation-dephosphorylation mechanism, several studies by Guzmán and co-workers using digitonin-permeabilized hepatocytes, led to the suggestion that phosphorylation-dephosphorylation might be involved in the short-term control of hepatocyte CPT 1 activity (reviewed by Zammit, 1994). However, further research has shown that the increase in CPT 1 activity observed in okadaic acid treated hepatocytes was not due to direct phosphorylation of CPT 1, but may involve interactions between the mitochondrial outer membrane and extra-mitochondrial, non-diffusible cell components (e.g. cytoskeletal elements) (Guzmán *et al*, 1994). Okadaic acid stimulation of hepatic CPT 1 has been shown to be dependent on the permanent activation of Ca^{2+}/calmodulin-dependent protein kinase-2 (CAMK2) by autophosphorylation, i.e. okadaic acid inhibits the phosphatases involved in the dephosphorylation (deactivation) of autophosphorylated CAMK2 (Valasco *et al*, 1996, 1997). The mechanism by which CAMK2 activates hepatic CPT 1 is not known, but recent studies have indicated that cytoskeletal components may be involved (Valesco *et al*, 1997).

The heart is not regarded to be a lipogenic (nor ketogenic) tissue and it is therefore surprising to find that the heart isoform of CPT 1 is as much as 50 times more sensitive to inhibition by malonyl-CoA than the liver enzyme (Saggerson & Carpenter, 1981; Bird & Saggerson 1984; Edwards *et al*, 1985). Given that heart malonyl-CoA levels have been shown to fluctuate between 3 and 10μM under various physiological conditions (McGarry *et al*, 1983; Awan & Saggerson, 1993; Lopaschuk *et al*, 1994b), fatty acid oxidation would be almost completely inhibited due to the very high

sensitivity to inhibition by malonyl-CoA (IC_{50}~0.1μM) of heart CPT 1. Presumably, most of the cellular malonyl-CoA is unavailable for interaction with CPT 1. There are several mechanisms which may account for this. (1) Binding of malonyl-CoA to cellular membranes or to some as yet unidentified element. It has been suggested that the condensing enzyme of the fatty acid chain elongation (FACE) system may provide a binding/sequestration sink for malonyl-CoA (Awan & Saggerson, 1993), but this has yet to be studied in detail (see section 1.2.3.3.4). (2) The presence of a small amount of L-CPT 1, whose IC_{50} is 100-fold higher than that of M-CPT 1 (McGarry *et al*, 1983), may allow fatty acid oxidation to occur at a basal level when malonyl-CoA concentrations are high (Weis *et al*, 1994a; Brown *et al*, 1995). (3) Compartmentation of malonyl-CoA within the cell would prevent it interacting with CPT 1. (4) Malonyl-CoA may bind to a low affinity malonyl-CoA/long-chain acyl-CoA binding site on CPT 1 distinct from the inhibitory site. Binding of malonyl-CoA at this site would result in incomplete inhibition of CPT 1 at infinitely high malonyl-CoA concentrations (McMillan *et al*, 1994), allowing fatty acid oxidation to proceed when malonyl-CoA levels are high.

1.2.3.3.2 Acetyl-CoA Carboxylase

Malonyl-CoA is synthesised in the cytoplasm from acetyl-CoA by the enzyme acetyl-CoA carboxylase (ACC), and to a lesser extent in the mitochondria by propionyl-CoA carboxylase. Like all carboxylases, ACC is a biotin containing enzyme which catalyses the carboxylation of acetyl-CoA to form malonyl-CoA (Kim *et al*, 1989), and is the first committed step in the pathway of fatty acid biosynthesis. Two immunologically distinct isoforms of ACC have been identified (Thampy, 1989; Bianchi *et al*, 1990); a

265 kDa isoform, which predominates in lipogenic tissues such as liver and white adipose tissue, and a 280 kDa isoform which predominates in tissues with a high fatty acid oxidative capacity, such as heart and skeletal muscle (Bianchi *et al*, 1990). In the heart, ACC-280 is the predominant isoform expressed, although ACC-265 is also present, but to a much lesser extent (Saddik *et al*, 1993). The opposite is true in the liver where ACC-265 is the predominant isoform but the tissue does contain a small amount of ACC-280 (Bianchi *et al*, 1990).

Regulation of ACC-265 in tissues such as liver and adipose tissue has been well characterised and has been shown to operate over two different time frames. A short term regulation (minutes) involving changes in covalent phosphorylation, allosteric regulation and polymerisation (Hardie *et al*, 1989), and a long-term (hours-days) regulation (Pape *et al*, 1988), involving changes in enzyme mass in response to hormonal and nutritional status. The long-term regulation of ACC-265 involves the tissue-specific use of two distinct ACC gene promoters (PI and PII), and changes in the rate of transcription, synthesis and degradation of ACC (Lopez-Cassilas & Kim, 1989; Luo *et al*, 1989). In the short term, citrate promotes polymerisation of ACC-265 increasing its activity, whilst long-chain acyl-CoA is a potent inhibitor (Moule *et al*, 1992). Although several kinases such as protein kinase C (PKC) and casein kinase-2 (CK2) can phosphorylate ACC-265 at multiple phosphorylation sites *in vitro* (Kim *et al*, 1989), PKA and AMPK appear to be primarily responsible for phosphorylation and inactivation of ACC-265 in the intact cell (Kim *et al*, 1989; Hardie, 1989; Ha *et al*, 1994). PKA phosphorylates ACC-265 at Ser-77 and Ser-1200 *in vitro*. Phosphorylation of the former residue results in inhibition of ACC-265 due to a 33%

decrease in V_{max} and a 2-fold increase in the K_a for citrate (the allosteric activator) (Hardie & Guy, 1980; Davies *et al*, 1990). AMPK phosphorylates ACC-265 at Ser-79, Ser-1200 and Ser-1215 *in vitro*. Phosphorylation of Ser-79 results in a 75% decrease in V_{max} and a 5-fold increase in K_a for citrate, while phosphorylation of Ser-1200 and Ser-1215 has no apparent affect on activity (Davies *et al*, 1990). Glucagon and adrenaline stimulate phosphorylation and inhibition of ACC in isolated hepatocytes and adipocytes, by 50% and 65% respectively. It was thought that inhibition of ACC-265 and hence fatty acid oxidation by these cAMP-elevating hormones, resulted from a stimulation of PKA mediated phosphorylation of ACC-265. However, Hardie and co-workers found that the serine residues of ACC-265 which were phosphorylated in isolated adipocytes and hepatocytes in response to cAMP-elevating hormones, were Ser-79 and Ser-1200 and but there was no phosphorylation of Ser-77 under any conditions used (Sim and Hardie, 1988; Haystead *et al*, 1990). This would suggest that the phosphorylation of ACC-265 in response to hormones which increase cAMP is due to the action of AMPK and not PKA. Hence AMPK appears to be the important inhibitory regulator of ACC in the liver and adipose tissue. Even though PKA phosphorylates ACC *in vitro*, this phosphorylation does not reach significant levels *in vivo*. There are two possible reasons for this: (1) Phosphorylation of Ser-79 by AMPK prevents phosphorylation of Ser-77 by PKA. (2) ACC is a relatively poor substrate for PKA. PKA may still be involved in the phosphorylation of ACC by inhibiting dephosphorylation by protein phosphatases.

As stated earlier, ACC is present in tissues which have a very low capacity for fatty acid synthesis and high capacity for fatty acid oxidation, such as heart and skeletal

muscle (Bianchi *et al*, 1990). ACC is believed to play a role in the control of fatty acid oxidation based on the following observations. (1) CPT 1, an essential component of fatty acid oxidation, is extremely sensitive to inhibition by malonyl-CoA (Cook, 1984; McGarry *et al*, 1989) which is generated by ACC. (2) Muscle tissues are non-lipogenic and yet contain a large amount of ACC. (3) Physiological conditions that cause a decrease in ACC activity and malonyl-CoA levels are accompanied by increased rates of fatty acid oxidation in liver (McGarry *et al*, 1978) and heart (Saddik *et al*, 1993; Lopaschuk *et al*, 1994b; Kudo *et al*, 1995). These observations also suggests that regulation of fatty acid oxidation is the primary role of ACC in non-lipogenic tissues (Bianchi *et al*, 1990; Saddik *et al*, 1993).

Considerably less is known about the regulation of the heart/muscle isoform of ACC (ACC-280) than the liver isoform (ACC-265). Heart ACC-280 has a very low affinity (K_m~100μM) for its substrate acetyl-CoA (Bianchi *et al*, 1990; Saddik *et al*, 1993). Since normal cytosolic acetyl-CoA levels are well below the K_m of ACC-280, ranging between 10 and 50μM, it would suggest that heart ACC-280 is sensitive to regulation by cytosolic acetyl-CoA levels (Saddik *et al*, 1993). It has been suggested that the acute regulation of ACC-280 activity differs from ACC-265, in that it may be regulated primarily by acetyl-CoA supply as opposed to regulation by phosphorylation or by the allosteric activator, citrate (Saddik *et al*, 1993). However, it has been shown that ACC activity in rat hearts can be stimulated 2-fold by citrate (Kudo *et al*, 1995) and that cardiac ACC is activated by a purified liver protein phosphatase resulting in a decrease in citrate dependence (Thampy, 1989). Therefore, ACC-280 would appear to be regulated by reversible phosphorylation similar to ACC-265 in liver. AMPK

activity increases in perfused working rat hearts with a concomitant decrease in ACC activity (Kudo *et al*, 1995). High rates of fatty acid oxidation observed in reperfused ischaemic hearts is due to a decrease in malonyl-CoA levels resulting from a decrease in ACC activity (Kudo *et al*, 1995, 1996). It is thought that an increase in AMPK activity is primarily responsible for the phosphorylation and inactivation of ACC during ischaemia and reperfusion due to an increase in AMP which activates AMPK both allosterically and via an upstream AMPK kinase (see section 1.3.5).

Recently it has been shown that rat and human skeletal muscle contain a distinct 272-275 kDa isoform of ACC (Trumble *et al*, 1995; Witters *et al*, 1994), as well as a minor amount of ACC-265. At present there is no compelling evidence (apart from cross reactivity with specific antibodies raised against unique regions of the liver ACC-280) suggesting that skeletal muscle ACC-272-275 is the same protein as heart or liver ACC-280. Whether the 270-280 isoforms of ACC present in liver, heart and skeletal muscle all represent the same gene product has yet to be determined. Both ACC-265 and -280 have been cloned and sequenced from human liver (Abu-Elheiga *et al*, 1997; Ha *et al*, 1996). Ha *et al* (1996), have reported the complete amino acid sequence of an isoform of ACC with molecular mass 275 kDa, which is primarily expressed in heart and skeletal muscle. This isoform contains an amino acid sequence at the N-terminus of about 200 amino acids long which may be related to the role of ACC-275 in controlling CPT 1 activity and mitochondrial fatty acid oxidation (Ha *et al*, 1996). It has been suggested that this region may be required for targeting and/or anchoring to the mitochondrial outer membrane in such a way as to control the malonyl-CoA regulatory site of CPT 1, which faces the cytosolic side of the outer mitochondrial

membrane. Phosphorylation-dephosphorylation of the mito-sequence (the first 25 amino acids at the N terminus of ACC-275) at serine and threonine residues could control ACC-275 binding and/or anchoring to the outer mitochondrial membrane by altering the hydrophobic interactions of ACC with the lipid membrane. Such interactions could increase the local concentration of a metabolite as much as 1000-fold (Attardi & Schatz, 1988).

In vitro studies of purified skeletal muscle ACC have found it can be phosphorylated by AMPK isolated from rat liver. Phosphorylation results in a decrease in V_{max} and an increase in the K_a for citrate (Winder & Hardie, 1996). Malonyl-CoA decreases in rat skeletal muscle during exercise and in response to electrical stimulation (Winder *et al*, 1990; Duan & Winder, 1992), thus relieving its inhibition of CPT 1 and allowing fatty acid oxidation to proceed at a higher rate. This drop in malonyl-CoA is accompanied by an increase in AMPK activity which is responsible for phosphorylation and inactivation of ACC (Winder & Hardie, 1996). AMPK is sensitive to changes in AMP, ADP and ATP levels, yet they appear unchanged during exercise. However, it is possible that changes in these adenine nucleotides do occur during exercise, but that they return to resting levels before the muscle can be removed and frozen for analysis. It is also possible that the increase in sarcoplasmic free calcium concentration caused by muscle contraction, may activate the putative AMPK kinase (Winder & Hardie, 1996).

1.2.3.3.3 Malonyl-CoA

It is thought that changes in absolute malonyl-CoA levels may be primarily responsible for control of fatty acid oxidation in the heart via inhibition of CPT 1 (Lopaschuk *et*

al, 1994a; Lopaschuk & Gamble, 1994). A number of studies have demonstrated that the rate of fatty acid oxidation exhibits a strong inverse correlation with malonyl-CoA concentration in perfused hearts and isolated cardiac myocytes (Awan & Saggerson, 1993; Saddik *et al*, 1993). Stimulation of glucose oxidation in perfused hearts by addition of dichloroacetate (DCA), results in an increase in malonyl-CoA levels, with a concomitant decrease in myocardial fatty acid oxidation (Saddik *et al*, 1993); whereas perfusion of hearts in the absence of glucose, such that all ATP production comes from fatty acid oxidation, results in a decrease in malonyl-CoA to almost undetectable levels (Saddik *et al*, 1993). Adrenaline decreases malonyl-CoA levels in perfused hearts and increases palmitate oxidation in isolated myocytes, whilst insulin has the opposite effect. Addition of exogenous palmitate to perfused hearts results in a decrease in their malonyl-CoA content (Awan & Saggerson, 1993). Lopaschuk *et al* (1994b) showed that maturation of fatty acid oxidation in hearts from new born rabbits is paralleled by a decrease in levels of malonyl-CoA, while Kudo *et al* (1995, 1996) found that during reperfusion of ischaemic hearts, the increase in fatty acid oxidation observed is coupled with a dramatic drop in malonyl-CoA levels, providing further evidence that malonyl-CoA levels determine myocardial fatty acid oxidation.

1.2.3.3.4 Malonyl-CoA Decarboxylase

The fact that myocardial malonyl-CoA levels can change dramatically yet transiently under the conditions described above suggests there must be a mechanism by which malonyl-CoA can be degraded. One possible mechanism is decarboxylation of malonyl-CoA by the enzyme malonyl-CoA decarboxylase. Malonyl-CoA decarboxylase has been observed in both plants and animals (Hayaishi, 1955; Hatch &

Stumpf, 1962) and has been purified and sequenced from the uropygial gland of the goose (Jang *et al*, 1989). It is located exclusively in the mitochondria of most tissues (Kim *et al*, 1979a, 1979b; Kim & Kolattukudy, 1978) except in specialised organs such as the mammalian Harderian gland and the uropygial glands of birds. In these organs malonyl-CoA decarboxylase accumulates in the cytoplasm and plays an important role in the generation of multimethyl-branched fatty acids by assuring that methylmalonyl-CoA (and not malonyl-CoA) is the only chain elongation substrate available for FAS (Buckner and Kolattukudy, 1975a, 1975b, 1976). In the liver malonyl-CoA decarboxylase is located exclusively in the mitochondrial matrix, where it is thought to protect key enzymes such as pyruvate carboxylase (Scrutton & Utter, 1967) and methylmalonyl-CoA mutase (Babior, 1973) from inhibition by malonyl-CoA generated by the mitochondrial matrix enzyme propionyl-CoA carboxylase. However, since rat heart contains little if any FAS or pyruvate carboxylase, whereas malonyl-CoA decarboxylase activity is quite high (about one third that of rat liver mitochondria), it seems likely that there is another metabolic role for the enzyme in heart. For cytoplasmic malonyl-CoA to be degraded by malonyl-CoA decarboxylase in the heart, it must be readily available to the enzyme. As yet the presence of malonyl-CoA decarboxylase external to the inner mitochondrial membrane in heart has not been established nor has a mechanism by which malonyl-CoA can be transported into the mitochondrial matrix for degradation by the decarboxylase. Whether or not malonyl-CoA decarboxylase can regulate cytoplasmic malonyl-CoA is still a point of debate.

1.2.3.3.5 Fatty Acid Chain Elongation

The fatty acid composition of various mammalian tissues differs slightly from that provided by diet. Fatty acyl groups present in mammalian tissues and subcellular membranes are derived from three different processes. (1) Diet, which provides the bulk of fatty acids present in phospholipids, TAG, and cholesterol esters. (2) *De novo* fatty acid synthesis, the main product of which is palmitic acid. (3) Modification of fatty acids derived from (1) and (2) by enzymes present in the endoplasmic reticulum of the cell, which catalyse a series of saturation and chain elongation reactions to yield the required acyl (enoyl) group (Cinti *et al*, 1992). The enzymes of the fatty acid chain elongation (FACE) system have been extensively studied in liver and brain but are also found in a number of other tissues including kidney, small intestine, adrenal cortex, testis, lung, retina, placenta and aorta (Cinti *et al*, 1992). Fatty acid elongation is thought to occur primarily in the endoplasmic reticulum of mammalian cells but there are reports of elongation processes in mitochondria and peroxisomes. The main reactions of the microsomal FACE system using a 14-carbon chain length substrate (myristoyl-CoA) are outlined below:

1) Myristoyl-CoA + malonyl-CoA $\rightarrow$ β-ketopalmitoyl-CoA + CO_2 + CoASH

2) β-Ketopalmitoyl-CoA +NADPH + H^+ $\rightarrow$ β-hydroxypalmitoyl-CoA +$NADP^+$

3) β-Hydroxypalmitoyl-CoA $\rightarrow$ *trans*-2,3-hexadecenoyl-CoA + H_2O

4) *Trans*-2.3-hexadecenoyl-CoA + NADPH + H^+ $\rightarrow$ palmitoyl-CoA + $NADP^+$

The microsomal fatty acid chain elongation reaction begins with the condensation of an activated fatty acid, such as myristoyl-CoA with malonyl-CoA. The rate limiting step in the total elongation reaction is this first step catalysed by the condensing enzyme (Nugteren, 1965; Bernert & Sprecher, 1977). Numerous studies have shown that the 2-carbon elongation unit is provided by malonyl-CoA and cannot be replaced by acetyl-CoA (Nugteren, 1965; Podack *et al*, 1974; Keyes & Cinti, 1980). In contrast, the mitochondrial elongation system appears to use acetyl-CoA instead of malonyl-CoA. In the reductive steps 2 and 4, both NADH and NADPH appear to be able to be used, although when added in rate limiting quantities, NADPH is preferred over NADH (Nugteren, 1965).

Very little is known about the FACE system in cardiac tissue, especially in cardiac myocytes. Incubation of cardiac myocytes with radiolabelled 18:2, 18:3, 20:4, or 20:5 fatty acids showed no formation of elongation/desaturation products (Hagve & Sprecher, 1989), whereas a crude particulate fraction of rat cardiac myocytes has been shown to incorporate [2-^{14}C]malonyl-CoA into petroleum-soluble products (Awan & Saggerson, 1993). The fact that the content of C_{18} fatty acids in heart relative to C_{16} fatty acids is nearly twice that found in plasma (Cinti *et al*, 1992), suggests that heart cells either selectively take up C_{18} fatty acids from the circulation, or that fatty acid elongation occurs within the heart cells (at least up to C_{18}).

Saggerson's group have recently suggested that provided the initial condensing enzyme of the FACE system is adequately abundant then it may provide a high-abundance low-affinity binding/sequestration "sink" for myocardial malonyl-CoA, thus reducing the amount of free malonyl-CoA available for interaction with CPT 1.

1.3 Cardiac Fuel Selection

It is well established that long-chain fatty acids represent the major substrate for the normoxic heart (Neely & Morgan, 1974). Other important sources of energy are the oxidation of glucose, lactate, pyruvate, ketone bodies and to a lesser extent, ATP production from glycolysis. The contribution of these pathways to the overall ATP production can vary dramatically and is mainly dependent on substrate availability and cardiac workload.

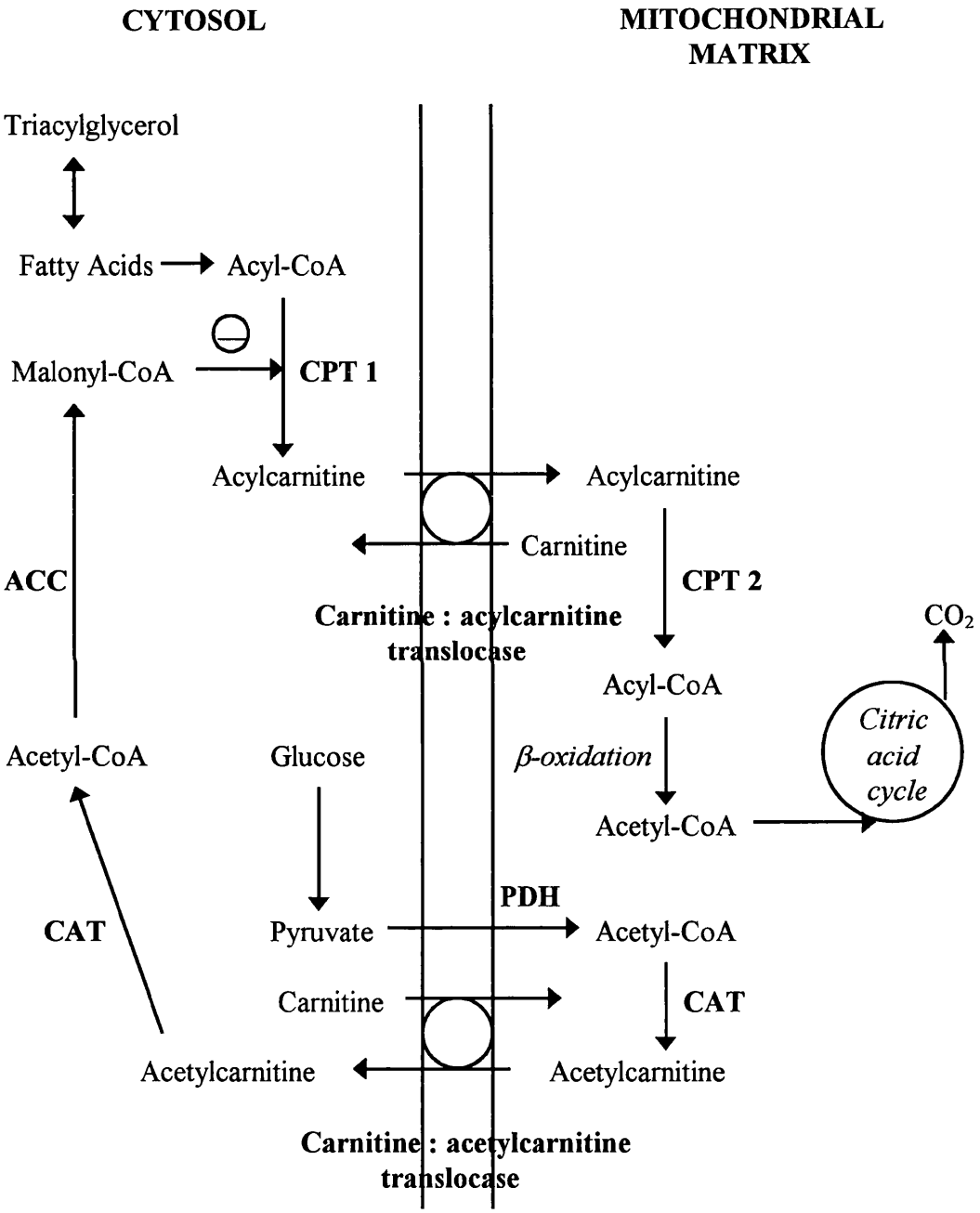
Blood levels of fatty acids, glucose and lactate are approximately 0.2-0.5, 5.0, and 1.0 mmol/l plasma respectively. During a single capillary passage, ~50% of both fatty acids and lactate are extracted compared to only a small proportion of glucose (Van der Vusse *et al*, 1982). Under normal aerobic conditions the heart takes its energy from substrates in the bloodstream so that endogenous energy stores, TAG and glycogen, are preserved. Moreover, a hierarchy exists among the oxidizable substrates. Glucose is not a preferred substrate for the heart and glycolysis is inhibited by fatty acids and ketone bodies in what is known as the glucose/fatty acid cycle. While the mechanisms by which fatty acids inhibit carbohydrate oxidation have been well established (Randle *et al*, 1963; Neely & Morgan, 1974; Hue *et al*, 1995), much less is known about how fatty acid oxidation is regulated by carbohydrates in the heart (Abdel-aleem *et al*, 1996). Under normal aerobic conditions, fatty acids (endogenous or exogenous) are preferred over glucose as a substrate for energy production in the heart. In the absence of exogenous fatty acids, glucose contributes ~50% of the total cardiac ATP production while fatty acids still contribute ~40% due to mobilisation of

endogenous TAG stores within the heart (Lopaschuk & Gamble, 1994). In the presence of high levels of exogenous fatty acids (1.2mM palmitate) the contribution of glucose to total cardiac ATP production falls to below 5% and that of fatty acids increases to ~90% (~80% from exogenous and ~10% from endogenous sources) (Lopaschuk & Gamble, 1994). This parallel decrease in glucose oxidation with increasing fatty acid oxidation is due to inhibition of glucose transport, HK, PFK-1 and a marked inhibition of PDH activity (Kerby *et al*, 1976; Randle, 1986; Patel & Roche, 1990) (see section 1.3.1). Perfusion of isolated hearts with the fatty acid oleate, causes a decrease in glucose oxidation and an increase in lactate production, suggesting that inhibition of glucose oxidation is much greater than the inhibition of glucose removal. Thus oleate diverts glucose from oxidation to glycolysis (Taegtmeyer *et al*, 1980). The effect of physiological concentrations of insulin to activate glucose transport in heart muscle is inhibited by fatty acids and ketone bodies. Addition of high levels of insulin can overcome the inhibition of glucose transport but not that of intracellular glucose metabolism. Direct inhibitory effects of fatty acids on glycolytic enzymes have been reported (Lea & Weber, 1968; Ramadoss *et al*, 1976), but it is unlikely that cytoplasmic fatty acid concentration is sufficiently high to exert a direct negative affect on the glycolytic pathway under physiological conditions (Van der Vusse *et al*, 1982).

The relationship between carbohydrate and fatty acid oxidation is not all one sided. Increased carbohydrate oxidation causes a concomitant decrease in fatty acid oxidation (Saddik *et al*, 1993). Fatty acid oxidation is inhibited by the presence of glucose or glucose plus lactate in perfused hearts (Forsey *et al*, 1987) and isolated

cardiac myocytes (Chen *et al*, 1984; Awan & Saggerson, 1993). Glucose also suppresses the extraction of oleate by perfused working hearts (Taegtmeyer *et al*, 1980). Recent evidence suggests that the depression of fatty acid oxidation induced by increasing carbohydrate oxidation is mediated through an increase in malonyl-CoA concentration in the heart (Awan & Saggerson, 1993; Saddik *et al*, 1993; Lopaschuk & Gamble, 1994). Stimulation of PDH activity (i.e. glucose oxidation) with agents such as DCA (Stacpoole, 1989), results in an increase in malonyl-CoA levels, with a parallel decrease in fatty acid oxidation in isolated perfused hearts (Saddik *et al*, 1993). This decrease in fatty acid oxidation can potentially be explained by an increase in intramitochondrial acetyl-CoA, derived from PDH, directly inhibiting β-oxidation at the level of 3-ketoacyl-CoA thiolase (Schulz, 1994; Abdel-aleem *et al*, 1996), but this does not explain the increase in malonyl-CoA observed with DCA treatment. Lopaschuk & Gamble (1994) have proposed a model to explain the effect of DCA on glucose/fatty acid oxidation. Excess acetyl groups produced by the PDH complex are transferred to acetylcarnitine via carnitine acetyltransferase (CAT) and exported to the cytosol by carnitine:acetylcarnitine translocase in a 1:1 exchange with carnitine (Lysiak *et al*, 1986), thus increasing the concentration of cytoplasmic acetyl-CoA available for ACC-280. This in turn would stimulate production of cytosolic malonyl-CoA, which would inhibit CPT 1 and the oxidation of long-chain fatty acids (fig 1.6). Further support for this hypothesis comes from the observation that the majority of the acetyl-CoA generated from carbohydrate by PDH is readily accessible to CAT, whereas acetyl-CoA produced from β-oxidation is more readily available to the citric acid cycle (Lysiak *et al*, 1988). This hypothesis can also be used to explain

Figure 1.6 Proposed mechanism by which glucose oxidation regulates fatty acid oxidation



Abbreviations: ACC, acetyl-CoA carboxylase; CPT, carnitine palmitoyltransferase; CAT, carnitine acetyltransferase; PDH, pyruvate dehydrogenase. Adapted from Lopaschuk & Gamble, 1994.

how changes in cardiac workload regulate fatty acid oxidation. When cardiac workload decreases, acetyl-CoA accumulates in the mitochondria due to reduced demand by the citric acid cycle, resulting in export of acetyl-CoA to the cytosol. Increased cytosolic acetyl-CoA stimulates synthesis of malonyl-CoA by ACC-280, inhibiting CPT 1 and decreasing fatty acid oxidation. On the other hand, when cardiac workload increases, the increased demand of the citric acid cycle means intramitochondrial acetyl-CoA is unavailable for export and synthesis of malonyl-CoA. The decrease in cytosolic malonyl-CoA relieves the inhibition of CPT 1 and increases the rate of fatty acid oxidation to meet the increased energy demand of the heart.

A recent study has shown that the oxidation of both palmitate (CPT dependent) and octanoate (CPT independent) by isolated cardiac myocytes is decreased by DCA to a similar extent (although not significantly) (Abdel-aleem *et al*, 1996). This suggests that the decrease in fatty acid oxidation by DCA treatment is regulated directly by acetyl-CoA produced by the PDH complex, via inhibition of 3-ketoacyl-CoA thiolase, and not by regulation of cytosolic malonyl-CoA and inhibition of CPT 1 as proposed by Lopaschuk & Gamble (1994). The observation that DCA also inhibits the oxidation of 3-hydroxybutyrate (which is independent of the CPT system), in cardiac tissues (McAllister *et al*, 1973), provides further evidence that inhibition occurs at the level of β-oxidation (3-ketoacyl-CoA thiolase) and not CPT 1.

1.3.1 Hexokinase, Phosphofructokinase-1 & Pyruvate Dehydrogenase

The activity of the key glycolytic enzymes HK, PFK-1 and PDH are all inhibited in perfused rat hearts by the oxidation of short- and long-chain fatty acids, lipoprotein fatty acids, and ketone bodies (Williamson, 1964; Randle *et al*, 1966; Ontko &

Randle, 1967). Extraction and subsequent oxidation of fatty acids and ketone bodies in perfused hearts causes an increase in citrate levels as well as a decrease in Fru-2,6-P_2 levels, the combined effect of which is a decrease in PFK-1 activity resulting in accumulation of Glu-6-P which inhibits both HK and glucose transport. Citrate release from cardiac mitochondria is minimal and unlikely to have an effect on PFK-1 activity *in vivo*, although it has been shown to modulate PFK-1 activity *in vitro* (Sluse *et al*, 1971). It is not only fatty acids and ketone bodies which inhibit glycolysis at the level of PFK. Oxidation of lactate is also known to inhibit glycolysis via inhibition of PFK-1 and PFK-2 (Hue *et al*, 1995). PFK-1 is also modulated by cytoplasmic ATP levels in that high concentrations of ATP decrease PFK-1 activity (Neely & Morgan, 1974).

PDH is also important in regulation of glucose metabolism by fatty acids (Randle *et al*, 1994a). Perfusion of hearts in the absence of fatty acids results in the rate of glycolysis being twice as high as the rate of glucose oxidation (Saddik & Lopaschuk, 1991). Addition of fatty acids results in a marked decrease in glucose oxidation with only a small decrease in glycolytic rate (Saddik & Lopaschuk, 1991). This would suggest that inhibition of glucose oxidation by fatty acids occurs more at the level of PDH than at the level of PFK-1. The substantial rise in intramitochondrial acetyl-CoA/CoA and $NADH/NAD^+$ ratios induced by oxidation of fatty acids and/or ketone bodies, decreases the amount of PDH in the active form (due to activation of PDH kinase) resulting in a decrease in glucose oxidation (Weis *et al*, 1989; Saddik & Lopaschuk, 1991). Increasing workload of isolated perfused hearts causes a decrease in intramitochondrial $NADH/NAD^+$ and acetyl-CoA/CoA with a concomitant increase

in fatty acid oxidation (Neely *et al*, 1969; Neely & Morgan, 1974). Decreased intramitochondrial NADH/NAD^+ and acetyl-CoA/CoA also stimulates glycolysis via an increase in the amount of PDH in the active form (Opie, 1991).

1.3.2 Lactate & Pyruvate

Metabolic intermediates such as lactate and pyruvate are also able to regulate glucose/fatty acid oxidation and influence fuel selection in the heart. Lactate is capable of competing successfully with fatty acids for mitochondrial oxidation (Spitzer, 1974; Drake *et al*, 1985). Normal plasma lactate concentration is ~0.5-1.0 mmol/l, which when extracted and oxidised is not sufficient to produce all the energy required for normal heart function. However, during physical exercise extracellular lactate levels can rise to ~5 mmol/l. At this concentration lactate has been shown to account for >85% of the energy produced by substrate oxidation in anaesthetised animals (Spitzer, 1974; Drake *et al*, 1985). Lactate has been shown to inhibit fatty acid oxidation in perfused rat hearts (McDonough *et al*, 1982; Bielefeld *et al*, 1985) and in isolated cardiac myocytes (Montini *et al*, 1981; Awan & Saggerson, 1993). Extraction of fatty acids is inhibited in canine hearts infused with lactate (Spitzer, 1974) or when lactate levels are raised due to endotoxic or hemorrhagic shock (Scott *et al*, 1972; Spitzer & Spitzer, 1972). Lactate is thought to exert its inhibition of fatty acid oxidation via inhibition of CPT 1. It has been hypothesised that lactate stimulates ACC, possibly by altering ACC phosphorylation (Louis & Witters, 1992), resulting in an increase in malonyl-CoA which inhibits CPT 1, thus preventing the transport of long-chain fatty acids into the mitochondrial matrix for oxidation (Van der Vusse *et al*, 1992). This hypothesis is supported by the fact that perfusion of hearts with high

concentrations of lactate decreases palmitate oxidation but does not alter the oxidation of octanoate, a medium-chain fatty acid which does not require carnitine for its transport into the mitochondrial matrix (Bielefeld *et al*, 1985; Forsey *et al*, 1987). Perfusion of isolated hearts with lactate also causes a decrease in acylcarnitine levels (Bielefeld *et al*, 1985) further supporting this hypothesis.

Pyruvate is usually present in minor quantities in the blood, but is also able to compete with fatty acids for oxidative degradation in cardiac muscle (Forsey *et al*, 1987). Fatty acid oxidation is inhibited by increased supply of pyruvate. The mechanism of this inhibition is thought to be at the level of intramitochondrial CoASH as it is used in the conversion of pyruvate to acetyl-CoA by PDH.

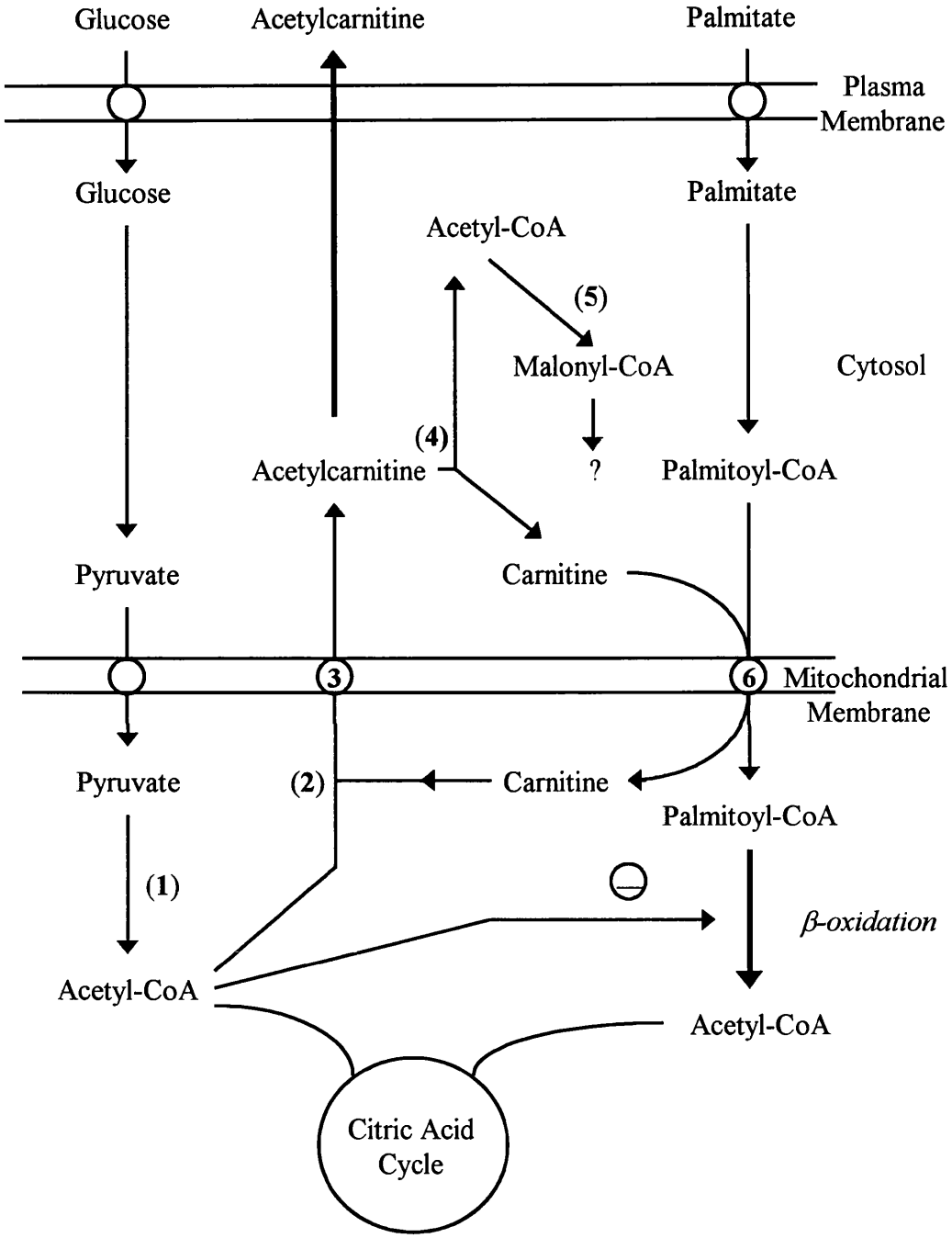
1.3.3 L-Carnitine

L-carnitine is an essential cofactor in fatty acid transport into mitochondria, but is also important in regulating intramitochondrial acetyl-CoA levels. Studies using isolated mitochondria have demonstrated that L-carnitine lowers the intramitochondrial acetyl-CoA level due to stimulation of CAT (Pearson & Tubbs 1967; Lysiak *et al*, 1988) and export of acetylcarnitine from the mitochondria. The resulting decrease in intramitochondrial acetyl-CoA would be expected to stimulate PDH activity leading to an increase in glucose oxidation. In support of this, Lopaschuk and co-workers have demonstrated that increasing myocardial carnitine content in isolated hearts perfused with fatty acids, results in an increase in glucose oxidation with a parallel decrease in fatty acid oxidation (Broderick *et al*, 1992; Lopaschuk & Gamble, 1994). A decrease in intramitochondrial acetyl-CoA would normally be expected to stimulate fatty acid oxidation by relieving inhibition of 3-ketoacyl-CoA thiolase in the mitochondrial

matrix. However, fatty acid oxidation is decreased suggesting that inhibition occurs at a different site of action. Carnitine stimulated export of acetyl-CoA from the mitochondria (via CAT and carnitine:acetylcarnitine translocase), would also be expected to increase cytosolic acetyl-CoA, stimulating ACC-280 (fig 1.6). Therefore, inhibition of fatty acid oxidation caused by increasing carnitine content of perfused hearts, is thought to occur via inhibition of CPT 1 by malonyl-CoA (Lopaschuk & Gamble, 1994).

In a recent study by Abdel-aleem *et al* (1996), incubation of isolated cardiac myocytes with L-carnitine significantly increased the oxidation of palmitate with little effect on glucose oxidation. This result opposes the Lopaschuk model and suggests that regulation of fatty acid oxidation by L-carnitine is not mediated by CPT 1, but rather through the β-oxidation system. The proposed mechanism is that L-carnitine stimulates the efflux rates of acetyl-CoA generated from pyruvate by the PDH complex. Acetyl-CoA is converted into acetylcarnitine by CAT and transported across the mitochondrial membrane into the cytosol by carnitine:acetylcarnitine translocase (fig 1.7). The majority of acetylcarnitine is released into the external medium, while any that is not transported is converted back to acetyl-CoA and free carnitine in the cytosol by CAT. The decrease in intramitochondrial acetyl-CoA relieves inhibition of 3-ketoacyl-CoA thiolase, thus increasing the rate of fatty acid oxidation (Abdel-aleem *et al*, 1996). It is also possible that L-carnitine may increase palmitate oxidation by increasing mitochondrial uptake of palmitate, especially in isolated myocytes where the isolation procedure may deplete endogenous carnitine.

Figure 1.7 Proposed mechanism for the regulation of glucose and fatty acid oxidation in isolated myocytes



Abbreviations: (**1**) Pyruvate dehydrogenase complex; (**2**) carnitine acetyltransferase; (**3**) carnitine : acetylcarnitine translocase; (**4**) cytosolic carnitine acetyltransferase; (**5**) acetyl-CoA carboxylase; (**6**) carnitine palmitoyltransferase 1, carnitine : acylcarnitine translocase and carnitine palmitoyltransferase 2. Adapted from Abdel-aleem *et al*, 1996.

However, Awan & Saggerson (1993), demonstrated that incubation of isolated myocytes with 1mM L-carnitine does not enhance the rate of oxidation of palmitate, suggesting that the procedure used for isolation of cardiac myocytes does not deplete carnitine to any great extent. Further evidence for the regulation of fatty acid oxidation at the level of β-oxidation comes from studies on isolated rat heart mitochondria by Wang *et al* (1991), who demonstrated that L-carnitine stimulates the oxidation of palmitoylcarnitine, whose oxidation does not require the actions of the carnitine dependent CPT system.

1.3.4 Calcium

The mechanisms by which Ca^{2+} alters myocardial contractile function have been extensively studied, whilst much less is known about the role of Ca^{2+} in regulating ATP production. It is well known that increasing extracellular [Ca^{2+}] increases cytoplasmic [Ca^{2+}] which can stimulate both glycolysis and glycogenolysis by stimulation of PFK and activation of phosphorylase *b* kinase (Werth *et al*, 1982; Taegtmeyer 1985; Leite *et al*, 1988). The inner mitochondrial membrane contains a number of Ca^{2+} transport systems that are thought to be regulators of Ca^{2+} dependent enzymes involved in mitochondrial energy metabolism (Denton *et al*, 1972, 1978; McCormack *et al*, 1990). The tight control of intramitochondrial Ca^{2+} and its close coupling to cytoplasmic Ca^{2+}, led McCormack *et al* (1990) to suggest a mechanism whereby mitochondrial oxidative metabolism can be stimulated to meet the increased demands for ATP that are invariably associated with the stimulation of the processes promoted by increases in cytosolic Ca^{2+}. This mechanism also offers a possible

explanation as to how increased mitochondrial ATP production can occur under such conditions, without any increases or decreases in the ADP/ATP ratio.

An increase in intramitochondrial Ca^{2+} can occur secondary to either an increase in cytoplasmic Ca^{2+} or in response to adrenergic stimulation, such as with adrenaline (Halestrap, 1987). The increase in intramitochondrial Ca^{2+} can activate or regulate key enzymes such as the PDH complex (presumably by activation of the pyruvate dehydrogenase phosphatase) (McCormack & Denton, 1984, 1989), and the citric acid cycle enzymes isocitrate dehydrogenase and oxoglutarate dehydrogenase (McCormack *et al*, 1990). It has been suggested that Ca^{2+} dependent stimulation of the citric acid cycle can increase the rate of fatty acid oxidation (McCormack *et al*, 1990). Increasing mitochondrial Ca^{2+} increases the affinity of both isocitrate and oxoglutarate dehydrogenase for their substrates and hence has the potential to increase citric acid cycle activity and flux through β-oxidation. In rat liver mitochondria an increase in $[Ca^{2+}]$ can result in an increase in matrix volume. A rise in pyrophosphate (PP_i) may be responsible, at least in part, for this increase in matrix volume (Davidson & Halestrap 1988, 1990; Halestrap & Davidson 1990), which is responsible for a number of important changes in intramitochondrial metabolism, including activation of the respiratory chain, fatty acid oxidation and glutaminase. It remains to be substantiated whether this Ca^{2+}-induced increase in mitochondrial matrix volume can occur in cardiomyocytes in which mitochondria are tightly packed in with the array of contracting proteins (Lopaschuk *et al*, 1994a). Indeed, Griffiths & Halestrap (1993) have shown that at physiological Mg^{2+} concentrations, heart mitochondria do not

show significant regulation of their matrix volume by physiological concentrations of Ca^{2+}, nor is an effect of Ca^{2+} on mitochondrial PP_i seen in the perfused heart.

In the presence of palmitate (1.2mM), both glycolytic and glucose oxidation rates are decreased dramatically. The decrease seen in glycolysis is due to citrate inhibition of PFK (Taegtmeyer, 1985), whereas the decrease in PDH complex activity is probably due to an increase in the intramitochondrial acetyl-CoA/CoA ratio (Kobayashi & Neely, 1979). When Ca^{2+} is increased (from 1.25 to 2.5mM) in the presence of 1.2mM palmitate, there is a concurrent increase in glycolytic and glucose oxidation rates (64% and 270% increase respectively), but not palmitate oxidation (Schonekess *et al*, 1995). Interestingly these effects of Ca^{2+} are not seen in the absence of fatty acids, where the PDH complex is already primarily in the active dephosphorylated form. This suggests that Ca^{2+} can stimulate both PFK and PDH complex activity when fatty acid metabolites are acting to inhibit these enzymes. It is unlikely that the effects of increasing perfusate [Ca^{2+}] on glucose metabolism are secondary to changes in myocardial workload as very little further change in contractile function occurs above 1.25mM Ca^{2+} (Schonekess *et al*, 1995).

1.3.5 AMP-Activated Protein Kinase

AMPK is an important enzyme in cardiac fuel selection as it is known to be responsible for the phosphorylation and regulation of a number of key enzymes in lipid metabolism, including HMG-CoA reductase, ACC and HSL. AMPK was the second protein kinase cascade to be discovered and has been extensively studied in recent years. AMPK is allosterically activated up to 5-fold by physiological concentrations of AMP. At high (mM) concentrations, ATP inhibits AMPK by antagonising activation

by AMP (Corton *et al*, 1995). It was once thought that AMPK could be stimulated by ADP and possibly cAMP but studies with purified enzyme have shown it to be highly specific for AMP (Ferrer *et al*, 1985; Carling *et al*, 1989). The AMP analogues adenosine 5'-thiomonophosphate and adenosine 5'-phosphoramidate both activate AMPK more potently than AMP, while the AMP analogue 5-aminoimidazole-4-carboxamide ribonucleoside monophosphate (ZMP) is less potent but still activates AMPK to the same extent as AMP (Corton *et al*, 1995; Henin *et al*, 1996). Evidence suggests that AMP also promotes phosphorylation and activation of the kinase via an upstream AMPK kinase. There are two mechanisms for this effect: (1) AMP binds to the allosteric site of AMPK changing its conformation making it a better substrate for AMPK kinase. (2) AMP directly activates the upstream AMPK kinase (Hawley *et al*, 1995). Surprisingly, AMP also affects dephosphorylation of AMPK by binding to phosphorylated AMPK, making it a poor substrate for protein phosphatases (Davies *et al*, 1995; Henin *et al*, 1996). The result of these mechanisms is a highly sensitive system which can dramatically increase AMPK activity in response to a small increase in AMP concentration.

Hardie & Carling (1997), proposed that the AMPK cascade acts as a "fuel gauge" or perhaps a "low-fuel warning system", being switched on by depletion of ATP, therefore protecting cells against nutritional and environmental stress. Fatty acid oxidation is regulated by AMPK at the level of CPT 1, which is inhibited by the product of ACC, malonyl-CoA. In the resting state, the energy requirements of the heart are met by oxidation of fatty acids and glucose. These conditions produce a high ATP/AMP ratio which means that AMPK is in its inactive form, ACC is in its active

dephosphorylated form, and malonyl-CoA levels are relatively high, inhibiting CPT 1, and decreasing the rate of fatty acid oxidation. When the heart is put under "stress" such as increased workload, ATP is depleted (AMP levels rise) with consequent activation of AMPK, phosphorylation and inactivation of ACC and a drop in malonyl-CoA, therefore removing its inhibition on CPT 1 and increasing the rate of fatty acid oxidation. The overall result of this mechanism is to replenish ATP levels in the cell to normal. AMPK therefore appears to be involved in fuel selection, switching from carbohydrate to fat when required. In perfused rat hearts, ischaemia produces a large increase in AMP levels, a 2-3-fold activation of AMPK, a reasonable inactivation of ACC, and a 2-fold decrease in malonyl-CoA (Kudo *et al*, 1995). During reperfusion AMPK activity remains high, further depressing ACC and decreasing malonyl-CoA levels dramatically. This suggests that AMPK is at least partly responsible for the high rates of fatty acid oxidation seen during reperfusion (Hardie & Carling 1997) which are potentially damaging to the myocardium. It is not known why AMPK remains activated during reperfusion as AMP levels return to normal, but the continued activity is due to sustained phosphorylation of the enzyme.

It has recently been demonstrated that ZMP is capable of mimicking the effect of AMP on allosteric activation of rat liver AMPK (Sullivan *et al*, 1994). Moreover, administration of the dephosphorylated form 5-aminoimadazole-4-carboxamide ribonucleoside (AICAR) to intact cells causes ZMP to accumulate inside the cell (Sabina *et al*, 1985). Unlike existing methods for activating AMPK in intact cells (e.g. fructose, heat shock or arsenite), AICAR does not perturb the cellular contents of ATP, ADP or AMP (Corton *et al*, 1995) and is therefore a more specific method for

activating AMPK in intact cells. When incubated with intact cells, AICAR is taken up via a nucleoside transporter and phosphorylated to the monophosphate form, ZMP, by adenosine kinase (Sabina *et al*, 1985; Vincent *et al*, 1996). ZMP is a normal intermediate in purine-nucleotide biosynthesis, but in many cells the rate of formation of ZMP from AICAR is fast compared to its rate of further metabolism, and ZMP accumulates to millimolar concentrations. AICAR has been used to show that activation of AMPK inhibits fatty acid sterol synthesis in hepatocytes (Vincent *et al*, 1991; Corton *et al*, 1995) and lipolysis in adipocytes (Sullivan *et al*, 1994; Corton *et al*, 1995) and in theory could be used to test the effect of AMPK activation on any cellular process. However, in cardiac myocytes, ZMP does not accumulate due to the low activity of adenosine kinase in these cells (Javaux *et al*, 1995). Hence AICAR cannot be used to test the effect of AMPK activation in cardiac myocytes. A negative result with AICAR, in the absence of measurements of ZMP and/or AMPK, cannot therefore be taken as evidence that AMPK is not involved in the system (Hardie & Carling, 1997).

1.4 Pathological Alterations in Cardiac Energy Metabolism

1.4.1 Ischaemia and Reperfusion

Ischaemia is characterised by reduced coronary flow resulting in an inadequate supply of oxygen and substrates to and removal of waste products (CO_2, H^+ and lactate) from the affected tissue. Anoxia (no oxygen) and hypoxia (low oxygen) differ from ischaemia in that coronary flow is maintained. During ischaemia, profound alterations in cardiac lipid and carbohydrate metabolism occur. Carbohydrate metabolism shifts from oxidative degradation to anaerobic production of lactate, whereas fatty acid oxidation is depressed (Opie, 1968; Lerch *et al*, 1985). During ischaemia the reduced supply of oxygen to the mitochondria decreases the flux of electrons through the mitochondrial respiratory chain, resulting in increased $NADH/NAD^+$ ratios in the mitochondrial matrix and cytosol (Liedtke, 1981). The increased $NADH/NAD^+$ ratio in the mitochondria inhibits the oxygen dependent β-oxidation of fatty acids leading to accumulation of intermediates of this pathway. The accumulation of fatty acids and their carnitine and CoA esters in ischaemic cardiac tissue is accompanied by an increase in levels of plasma fatty acids (Van der Vusse *et al*, 1992). It is generally accepted that fatty acids and fatty-acyl esters become harmful when their extracellular or intracellular concentrations reach critical levels (Katz & Messineo, 1981; Corr *et al*, 1984; Liedtke, 1988). High concentrations of fatty acyl moieties give rise to the formation of micelles, which can interfere with membrane function and ultimately lead to membrane instability and disruption due to their detergent effects (Katz & Messineo, 1981). High levels of fatty acids and acyl-CoA inhibit a number of

membrane bound enzymes including the sarcolemmal Na^+/K^+ ATPase and mitochondrial adenine nucleotide translocase.

The increase in myocardial fatty acid content is thought to be supplied mainly by lipolysis of endogenous TAG (Schoonderwoerd *et al*, 1989b). Myocardial lipolysis has been shown to be accelerated during ischaemia possibly due to increased release of endogenous noradrenaline (Opie, 1976; Heathers *et al*, 1985). The markedly elevated content of free arachidonic acid, amongst other fatty acids, in ischaemic cardiac tissue suggests increased degradation or reduced reutilisation of phospholipids during ischaemia (Van der Vusse, 1982; Van Bilsen *et al*, 1989) which may also add to the increase in myocardial fatty acid content.

Inhibition of β-oxidation due to the raised tissue $NADH/NAD^+$ ratio leads to an increase in fatty acids available for reesterification (Whitmer *et al* 1977; Saddik & Lopaschuk, 1992). TAG synthesis is indeed seen to increase significantly following reperfusion and may be important in preventing fatty acid concentrations from reaching concentrations which are deleterious to myocardial function (Piper *et al*, 1983). A futile cycle of TAG hydrolysis and synthesis has been shown to occur in ischaemic hearts (Trach *et al*, 1986; Van Bilsen *et al*, 1989; Saddik & Loppaschuk, 1992a) and isolated myocytes (Myrmel *et al*, 1991, 1992). As TAG turnover consumes ATP, it has been suggested that this process is detrimental to the myocardium in which ATP supply is already low (Van Bilsen, *et al*, 1989). However, it has been calculated that the energy loss associated with the re-esterification cycle is only 2.5-4.4% of the total ATP production in ischaemic rat hearts (Trach *et al*, 1986; Schoonderwoerd *et al*, 1989b). Hence, it has been suggested that enhanced TAG

turnover may act to protect the ischaemic myocardium by reducing tissue fatty acid and fatty acyl-CoA levels by increasing their esterification to endogenous TAG (Trach *et al*, 1986; Schoonderwoerd *et al*, 1989b).

In the early stages of ischaemia, both the uptake of exogenous glucose and intracellular glycogen degradation are increased to provide glucose moieties for anaerobic glycolysis. Glycolytic flux is increased through allosteric activation of PFK-1 due to high intracellular levels of ADP, AMP and P_i and low levels of ATP. Prolonged severe ischaemia results in an increase in NADH, H^+ and lactate levels which inhibit glucose utilisation via inhibition of glyceraldehyde-3-phosphate and PFK-1. Increased levels of fatty acids and their metabolites in ischaemic-reperfused hearts inhibit glucose metabolism by mechanisms described by the glucose/fatty acid cycle (see section 1.3). Previous studies have demonstrated that glucose oxidation is very low in hearts reperfused in the presence of high concentrations of fatty acids (Lopaschuk *et al*, 1990; McVeigh & Lopaschuk, 1990; Lopaschuk & Saddik, 1992). This over reliance on fatty acid oxidation following reperfusion is thought to occur at the level of CPT 1. Inactivation of ACC by AMPK results in a decrease in malonyl-CoA production, relieving its inhibition of CPT 1 (Kudo *et al*, 1995). It has been suggested that inhibition of myocardial glucose utilisation is involved in the mechanisms by which fatty acids exert their deleterious effects on the myocardium (Opie, 1975; Lopaschuk *et al*, 1988, 1990). High plasma fatty acid concentrations increase the severity of ischaemic damage and can lead to a depression of mechanical function following reperfusion (Liedtke *et al*, 1978). Stimulation of glucose oxidation during reperfusion has been shown to significantly improve the degree of functional

recovery, suggesting that a potential therapeutic aim in myocardial ischaemia would be to overcome fatty acid inhibition of glucose oxidation, as opposed to directly inhibiting fatty acid oxidation at the level of CPT 1 (Lopaschuk & Saddik, 1992).

Reperfusion of previously ischaemic myocardium does not result in a return to normal metabolism. Fatty acid content continues to rise despite the fact that fatty acid oxidation rates are the same or higher in the reperfused ischaemic heart (Liedtke *et al*, 1988; Lopaschuk *et al*, 1990; Saddik & Lopaschuk, 1992). Although G3P levels fall upon reperfusion of previously ischaemic hearts, TAG synthesis appears to be enhanced compared to normal aerobic hearts (Lopaschuk *et al*, 1990; Saddik & Lopaschuk, 1992). As TAG-fatty acid cycling is thought to cease upon reperfusion (Van Bilsen *et al*, 1989), an increase in the TAG pool is seen as a result of the enhanced TAG synthesis (Lopaschuk *et al*, 1990; Saddik & Lopaschuk, 1992).

1.4.2 Myocardial Hypertrophy

Myocardial hypertrophy is well established as a risk factor in congestive heart failure, sudden death and myocardial infarction (Frohlich *et al*, 1992). Hypertrophied myocardium has a depressed pre- and post-ischaemic function (Hearse *et al*, 1978; Anderson *et al*, 1990). Myocardial hypertrophy is described as an increase in ventricular mass due to an increase in cell size (not cell number), and may be induced under conditions of pressure or volume overload. Hypertrophied heart exhibits a decrease in fatty acid oxidation thought to occur due to a decreased carnitine content (due to poor carnitine uptake) inhibiting the translocation of long-chain fatty acids into the mitochondria. Studies of hypertrophied rat heart have shown that the rate of fatty acid oxidation is depressed by low levels of fatty acids (Schonekess *et al*, 1993;

Lopaschuk *et al*, 1994a). Under these conditions, the percentage of total ATP production from fatty acid oxidation is decreased from 69% in normal hearts, to 55% in hypertrophied hearts (Lopaschuk *et al*, 1994a). The reduced total ATP production is partly offset by an increase in glycolytic rate (Allard *et al*, 1994). Increased supply of exogenous fatty acids appears to overcome the decrease in CPT 1 activity that occurs due to the decrease in carnitine levels, indicating that the enzymes involved in β-oxidation are not altered in the hypertrophied heart and that decreased carnitine content may only be limiting when fatty acid concentration is low (Lopaschuk *et al*, 1994a).

1.4.3 Diabetes

Hearts from diabetic animals are similar to hypertrophic hearts in that they also have a reduced carnitine content (Smith *et al*, 1978). However, this decrease in carnitine content does not depress fatty acid oxidation in the diabetic heart. In fact, the diabetic heart relies almost entirely on fatty acid oxidation to meet its ATP requirements (Wall & Lopaschuk, 1989). This increased reliance on fatty acid oxidation is primarily due to increased levels of circulating fatty acids and TAG content, as well as a decrease in glycolysis and glucose oxidation. Although the diabetic heart is more dependent on fatty acid oxidation, it is unclear if actual β-oxidation rates are altered in these hearts. The rate of exogenous fatty acid oxidation has been shown to be depressed or unchanged in perfused diabetic hearts, especially if the lower heart work observed is accounted for (Kreisberg, 1966; Lopaschuk & Tsang, 1987).

The primary effect of diabetes on cardiac glucose metabolism is decreased glucose uptake across the sarcolemma due to lack of insulin (Morgan *et al*, 1961). The

increase in circulating fatty acids also inhibits glycolysis and glucose oxidation by the mechanisms of the glucose/fatty acid cycle (see section 1.3). Increased citrate concentrations inhibit PFK-1, with glycogenolysis and glucose uptake being subsequently decreased due to accumulation of Glu-6-P. Pyruvate oxidation is also decreased in diabetes due to inhibition of the PDH complex by fatty acids (Garland *et al*, 1964). The role of fatty acid metabolism in decreasing glucose oxidation rates in diabetics has led to the development of a number of hypoglycaemic drugs such as tolbutamide, POCA and Etomoxir, which act by blocking the key step in long-chain fatty acid oxidation, CPT 1 (Stephens *et al*, 1985; Cook, 1987).

The diabetic heart also has an elevated TAG content (Denton & Randle, 1967). This is probably due to an increase in long-chain fatty acyl-CoA levels resulting from an increase in exogenous fatty acids and increased CoASH synthesis within the myocyte (Lopaschuk *et al*, 1986; Murthy & Pande, 1987). The cytosolic carnitine:CoA ratio has been shown to be important in determining the fate of fatty acids in several tissues. In the normal heart, where the major fate of fatty acids is oxidation, cytosolic CoASH levels are low. Whereas in the diabetic heart, increased cytosolic CoASH and decreased carnitine levels push fatty acids towards TAG synthesis and away from fatty acid oxidation (Lopaschuk *et al*, 1986). The diabetic heart also possesses an increased capacity for synthesis of TAG (Murthy & Shipp, 1977), as well as an increase in the activities of key enzymes in TAG synthesis, phosphatidic acid phosphohydrolase (PAP) and diacylglycerol acyltransferase (DGAT) (Schoonderwoerd *et al*, 1990a).

It has been suggested that high levels of circulating fatty acids may decrease the rate of TAG lipolysis which in combination with the increased TAG synthesis would

contribute to the accumulation of TAG in the diabetic heart. This is supported by the fact that perfusion of diabetic hearts with a high concentration of exogenous fatty acids, results in a lower rate of TAG hydrolysis than in normal hearts perfused with a low concentration of fatty acids (Paulson & Crass, 1982). Therefore, low rates of glucose oxidation in diabetic hearts, without a concomitant increase in fatty acid oxidation (endogenous or exogenous), may lead to a decreased ability of the heart to maintain metabolic demand under certain conditions. In contrast it has recently been shown that diabetic myocytes incubated in the presence of 1.2mM oleate have a higher rate of lipolysis than normal myocytes incubated without exogenous fatty acids (Larsen & Severseen, 1990). The increased TAG pool is important to the diabetic heart as a source of endogenous fatty acids which can be rapidly utilised if circulating fatty acid levels decrease. It is also important as an assured supply of fatty acids for β-oxidation and ATP production to replace that normally derived from glycolysis and glucose oxidation.

Alterations in lipid and carbohydrate metabolism that occur as a result of diabetes can have detrimental effects on the heart. Fatty acids are important in mediating myocardial ischaemic injury in the diabetic rat. Even in the absence of diabetes, free fatty acids accelerate mechanical failure of ischaemic hearts (Lopaschuk & Spafford, 1989). Myocardial long-chain acylcarnitine and long-chain acyl-CoA levels accumulate during ischaemia and their accumulation is potentiated in hearts of diabetic animals (Garland & Randle, 1964; Feuvray *et al*, 1979). These amphiphilic intermediates are known to interfere with a number of cellular processes and membrane-bound enzymes (see section 1.3.1).

1.5 Project Objectives

Due to the high sensitivity of the heart isoform of CPT 1 to inhibition by malonyl-CoA (50 times higher than the liver isoform), malonyl-CoA concentrations in fed rats are sufficiently high to effect almost complete inhibition of cardiac CPT 1 unless malonyl-CoA is bound, compartmentalised or sequestered in some way. Numerous studies have measured malonyl-CoA in whole heart but there are other cell types present in the whole organ which may interfere with these measurements. Therefore, it is important to make measurements using isolated cardiac myocytes for comparison. High levels of fatty acids are detrimental to the ischaemic myocardium and during reperfusion. Regulation of fatty acid oxidation by malonyl-CoA inhibition of CPT 1 may be important in these conditions. This is highlighted by the fact that some drugs which appear to alleviate ischaemic heart damage have similar effects to malonyl-CoA. It is therefore important to learn more about the routes of malonyl-CoA synthesis and disposal and its role in the fuel selection of the heart..

The aims of the project were:

1) Make measurements of malonyl-CoA under various physiological conditions in viable, isolated cardiac myocytes, for comparison with measurements previously made in whole heart.

2) To investigate the routes of malonyl-CoA synthesis and disposal in order to gain a greater insight into the regulation of its levels in cardiac tissue.

3) Examine the hypothesis that cytosolic malonyl-CoA may be bound, compartmentalised or sequestered in some way (possibly by the FACE system), therefore preventing it from interacting with CPT 1.

Chapter 2 - Materials and Methods

2.1 Commercial Preparations

Commonly used reagents were obtained from Sigma Chemical Co. Ltd., Poole, Dorset, U.K. In addition, the following were supplied by:

Sigma Chemical Co. Ltd., Poole, Dorset, U.K.: L-adrenaline (bitartarate), L-isoprenaline (hydrochloride), L-phenylephrine (hydrochloride), halothane (1-chloro-2-bromo-1, 1, 1-trifluoroethane), adenosine 5'-triphosphate (ATP), adenosine diphosphate (ADP), ascorbic acid, 5-5'dithiobis(2-)nitrobenzoic acid (DTNB), sodium palmitate, fatty acid poor bovine serum albumin fraction V (BSA), Dowex 2 chloride (8% cross-linkage, dry mesh 200-400), malonyl-CoA (lithium), bicinchoninic acid protein (BCA) assay kit.

Boehringer Mannheim, Lewes, Sussex, U.K.: cytochrome c, palmitoyl-CoA, insulin, nicotinamide adenine dinucleotide (NAD), reduced nicotinamide adenine dinucleotide (NADH), reduced nicotinamide adenine dinucleotide phosphate (NADPH), lactate dehydrogenase, pyruvate kinase, glycerokinase, phosphoenolpyruvate, malate dehydrogenase, citrate synthase,

Amersham International, Bucks., U.K.: [^{3}H] acetyl-CoA, [1-^{14}C] acetyl-CoA, [2-^{14}C] malonyl-CoA

British Drug Houses, Poole Dorset. U.K.: Dithiothreitol (DTT).

BOC Ltd., London., U.K: Pressurized gasses (O_2 : CO_2 95% : 5%), liquid nitrogen

Alpha Laboratories Ltd., 40 Pelham Drive, Eastleigh, Hants. U.K.: Wako NEFA C test kit

National Diagnostics, Manville, New Jersey, U.S.A.: Ecoscint A

Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A.: collagenase type 2 (from *Clostridium hystolyticum*).

2.2 Laboratory Preparations

2.2.1 Palmitate Bound to Albumin

Palmitate was bound to albumin using the method of Evans & Mueller (1963). 250mg sodium palmitate was added to 50ml of 15% (w/v) BSA in a 0.9% (w/v) NaCl solution. The mixture was repeatedly sonicated in a sonicating waterbath. The resulting suspension was incubated for 20 min at 50^0C before leaving to cool overnight at 4^0C. The suspension was filtered through Whatman No.1 filter paper and the filtrate centrifuged at 0-4^0C for 30 min at 26,000g_{av} to remove any microcrystals of sodium palmitate. The supernatant was adjusted to pH 7.4 with NaOH (1M) and stored in aliquots at -20^0C. The concentration of palmitate bound to albumin was measured using the Wako NEFA C kit as described in section 2.8.5.

2.2.2 Purification of Fatty Acid Synthase

Fatty acid synthase (FAS) was partially purified from rat liver using a method similar to that used by Hsu *et al* (1965), for the purification of FAS from pigeon liver. Rats were killed by a blow to the head followed by cervical dislocation. Livers were removed and promptly washed and chopped in homogenization buffer (approx. 5ml/g tissue) containing 225mM mannitol, 75mM sucrose, 10mM Tris-HCl, 0.05mM EDTA, 0.5mM DTT, adjusted to pH 7.4. The livers were homogenised using a Potter-Elvehjem homogeniser fitted with a motor-driven Teflon pestle (450 rev/min)(radial clearance

0.19mm). The homogenate was centrifuged at 0-4^{0}C for 10 min at 600g_{av} in a SS-34 rotor (r_{av}=10.8cm) of a Sorvall RC5-B Superspeed centrifuge and the resulting supernatant centrifuged at 0-4^{0}C for 10 min at 9,200g_{av}. The resulting mitochondrial supernatant was then centrifuged at 0-4^{0}C for 1 hr at 100,000g_{av} in a 75Ti rotor (r_{av} = 4.5 cm) of a high speed L-7 Beckman Ultracentrifuge. All pellets from centrifugation steps were discarded leaving just the high speed supernatant. Ice-cold saturated ammonium sulphate was added slowly to the supernatant until 25% saturation was reached. The mixture was kept stirring on ice for 30 min, and then centrifuged at 0-4^{0}C for 10 min at 18,000g_{av}. The pellet was discarded and the supernatant brought to 40% saturation with further ice-cold ammonium sulphate and centrifuged as before. The pellet was collected and dissolved in a minimum volume of buffer containing 20mM bis-Tris propane (pH 7.0), 30mM NaCl, 3mM EDTA, and 1mM DTT, and then desalted overnight by dialysis (at 0-4^{0}C) with two changes of buffer (100 x sample volume each). The sample was then applied to a Pharmacia FPLC HiLoad 16/10 Q sepharose column previously equilibrated with 20mM bis-Tris propane buffer (pH 7.0) containing 30mM NaCl, 3mM EDTA and 1mM DTT. The enzyme was eluted with a linear gradient of 0-0.6M NaCl dissolved in the same buffer over 20 column volumes. 10ml fractions were collected and most of the FAS activity was found to elute between 0.18-0.23M NaCl. Fractions containing the bulk of the activity were pooled, assayed for activity and stored in aliquots at -70^{0}C, with the addition of 10% (v/v) glycerol to aid storage. Aliquots of FAS were assayed once a month to check activity was stable on storage.

2.3 Animals

The animals used in these experiments were male Sprague-Dawley rats bred in the animal colony at University College London. Rats selected for experimentation were 250-300g body weight (6-8 weeks old). They were maintained at 20-22^{0}C and had constant access to drinking water and Rat and Mouse No. 3 Breeding Diet (Special Diet Services, Witham, Essex, U.K.). This contained 21% protein, 4% fat and 39% starches and sugars (by weight). The light/dark cycle was 13 hr/11 hr, with light from 06:00 to 19:00 hr.

2.4 Isolation of Cardiac Myocytes

2.4.1 Non-Triacylglycerol (TAG)-Loaded Myocytes

Myocytes were isolated using a slight modifications of the method described by Fuller *et al* (1990). Hearts were removed from halothane anaesthetized rats and immediately placed in ice-cold Krebs-Henseleit bicarbonate (KHB) medium (25mM $NaHCO_3$, 118.5mM NaCl, 4.7mM KCl, 1.2mM $MgSO_4$, 1.2mM KH_2PO_4, 10mM glucose) containing 2.5mM $CaCl_2$. Hearts were washed twice, with gentle squeezing to expel the blood from inside the heart and trimmed of adhering adipose and connective tissue before being cannulated. Hearts were perfused through the aorta with oxygenated, calcium-free KHB (at 37^{0}C, under 80cm hydrostatic pressure) until clear of blood (bloody perfusate was discarded). After 4 min recirculating perfusion, collagenase (1mg/ml), $CaCl_2$ (50μM) and BSA (1mg/ml) were added to the perfusate and the perfusion continued for a further 4-5 min or until white patches due to ischaemia, appeared on the ventricles. Hearts were removed

from the cannula and the atria discarded. The remaining ventricles were cut into a star shape to increase surface area, and placed in a 25ml conical flask with 3.3ml KHB containing collagenase (1mg/ml), $CaCl_2$ (50μM) and BSA (20mg/ml). Flasks were sealed and shaken (150 strokes/min) with constant gassing (95%O_2 : 5%CO_2) in a Gallenkamp shaking waterbath for 7 min at 37^{0}C. Myocytes released by collagenase action were isolated by filtering through nylon gauze (250μm). The filtrate was made up to 10 ml with oxygenated KHB containing 20mg/ml BSA, 50μM $CaCl_2$ and cells allowed to settle under gravity. The remaining ventricular tissue was re-incubated for 6 min in KHB containing collagenase (1mg/ml), $CaCl_2$ (50μM), and BSA (20mg/ml) as before and cells collected in a separate tube. This procedure was repeated up to 3 times or until all the ventricular tissue had been digested. The cell pellets were pooled and washed six times by gentle resuspension in 10ml KHB containing 20mg/ml BSA and gradually increasing concentrations of calcium (50μM, 100μM, 200μM, 400μM, 800μM and 1.3mM) with 6 min incubations (37^{0}C and gassing) between washes to allow viable cells to settle whilst non-viable cells floated and were discarded in subsequent washes. The final cell pellet was resuspended in oxygenated KHB containing 1.3mM $CaCl_2$, 20mg/ml BSA and 5mM glucose at a dilution appropriate for the experiment being done.

All glassware was silicone treated prior to use. BSA was dialyzed for 48 hr against distilled water and stored in aliquots at -20^{0}C.

2.4.2 Triacylglycerol (TAG)-Loaded Myocytes

Myocytes were isolated as described above except that 0.5mM palmitate bound to albumin (NEFA) was included in the collagenase digestion flasks and the first four of the

five washes so that myocytes were able to maintain their endogenous TAG pool during the isolation.

2.4.3 Glucose Depleted Myocytes

Myocytes were isolated as described as in section 2.4.1 except that all KHB perfusion and collagenase digestion media contained 5mM glucose instead of 10mM. Washes 4, 5 and 6 were devoid of glucose and the final incubation medium also contained no glucose thus producing myocytes with depleted glucose.

2.4.4 Myocyte Isolation from Two Hearts Simultaneously

In order to make certain metabolic measurements it was necessary to perfuse two hearts simultaneously to obtain enough myocytes. This was achieved by setting up two perfusion apparatus independently of each other and starting two isolation procedures together. The same KHB isolation media was used for both procedures and the heart material from the two was combined at the collagenase flask stage. Both hearts were placed in a single 50ml conical flask with 6.6ml KHB containing collagenase (1mg/ml), $CaCl_2$ (50μM) and BSA (20mg/ml) and shaken as normal for 7 min. The remainder of the procedure was carried out as described in section 2.4.1.

2.4.5 Assessment of Myocyte Viability

Myocyte preparations containing >70% viable cells were used for metabolic studies. Viability was assessed by staining with 0.4% (w/v) trypan blue in 0.9% (w/v) NaCl. Viable cells excluded trypan blue and were of a typical rod shape with visible cross-striations. Non viable cells did not exclude trypan blue and were rounded with no distinct ultrastructure. Each preparation was assessed for viability before use and those of <70% viability were discarded.

2.5 Measurement of Myocyte Dry Weight

Quadruplicate aliquots of resuspended myocytes and resuspension buffer were placed in separate pre-weighed 10ml glass beakers and heated at 70^0C until no further decrease in weight was observed (normally 24 hr). The beakers were then re-weighed and the dry weight of myocytes calculated by subtracting the dry weight of the suspension medium.

2.6 Preparation of Myocyte Extracts

2.6.1 For Metabolite Measurements

Myocytes were incubated under conditions described in individual experiments in plastic scintillation vials and reactions stopped by the addition ice-cold perchloric acid (final concentration of 6% [w/w]), and immediately placed on ice for 30 min. The acid-soluble extract was separated from the acid-insoluble pellet by centrifuging at 0-4^0C for 10 min at $2000g_{av}$ in a MSE benchtop centrifuge.

2.6.2 Acid-soluble Extract

The acid-soluble extract was buffered by addition of 5M bis-Tris propane (pH 6.0) (0.1ml/ml) and its pH raised to 6.0 with KOH (10M and then 1M). The tubes were placed on ice for 30 min to allow all the potassium perchlorate to precipitate. The tubes were centrifuged at 0-4^0C for 10 min at $2000g_{av}$ in a MSE benchtop centrifuge and the supernatant collected and the volume recorded. Assays for the metabolites malonyl-CoA

and glycerol were usually carried out the same day but supernatants were occasionally stored at -70^{0}C overnight.

2.6.3 Acid-insoluble Pellet

Both long-chain acylcarnitine and long-chain acyl-CoA are insoluble in dilute perchloric acid (Saggerson and Greenbaum 1970) and can therefore be separated from free CoASH and carnitine as well as from other CoASH and carnitine derivatives by acid precipitation of cells or tissue. 1.5ml 0.2M KOH containing 10mM DTT was added to the acid-insoluble pellet which was then homogenised with an Ultra-Turrax homogeniser at 0-4^{0}C. The tubes were incubated for 2 hr at 55^{0}C releasing free CoASH and free carnitine from their long-chain acyl derivatives. The suspension was cooled on ice and 75µl of 60% (w/w) perchloric acid was added whilst vortexing and the resulting protein precipitate removed by centrifugation at 0-4^{0}C for 10 min at 2000g_{av} in a MSE benchtop centrifuge. The supernatant was buffered by the addition of near saturated KH_2PO_4 and the pH adjusted to 7.0 with KOH (10M and then 1M) and left on ice for 30 min. The potassium perchlorate precipitate was removed by centrifugation at 0-4^{0}C for 10 min at 2000g_{av} in a MSE benchtop centrifuge and the supernatant collected and the volume recorded. Supernatants were stored at -70^{0}C and assays for free CoASH and free carnitine were carried out within a few days of storage.

2.7 Heart Perfusion

Hearts were excised from halothane-anaesthetized rats and placed in ice-cold KHB (25mM $NaHCO_3$, 118mM NaCl, 4.7mM KCl, 1.2mM KH_2PO_4, 1.2mM $MgSO_4$, 5mM

glucose) containing 0.1% BSA (w/v). Hearts were trimmed of fatty and connective tissue, cannulated and perfused with oxygenated KHB containing 2% BSA (w/v) and 1.3mM $CaCl_2$ by the Langendorff technique (Langendorff, 1895) using the method of Mowbray & Ottaway (1973), until clear of blood. After 45 min of recirculating perfusion, the heart was removed from the cannula and immediately crushed between two stainless steel plates cooled to -70^{0}C and ground to a fine powder under liquid nitrogen using a mortar and pestle. The powder was subsequently extracted with ice-cold perchloric acid 6% (w/w)(5ml per gram fresh tissue) and homogenised with an Ultra-Turrax homogeniser at 0^{0}C. The resulting homogenate was centrifuged at 0-4^{0}C for 10 min at 2000g_{av} in a MSE benchtop centrifuge. After centrifugation the acid-soluble extract was separated from the acid-insoluble pellet as described above and metabolites measured as described in section 2.8.

2.8 Determination of Metabolites

2.8.1 Malonyl-CoA

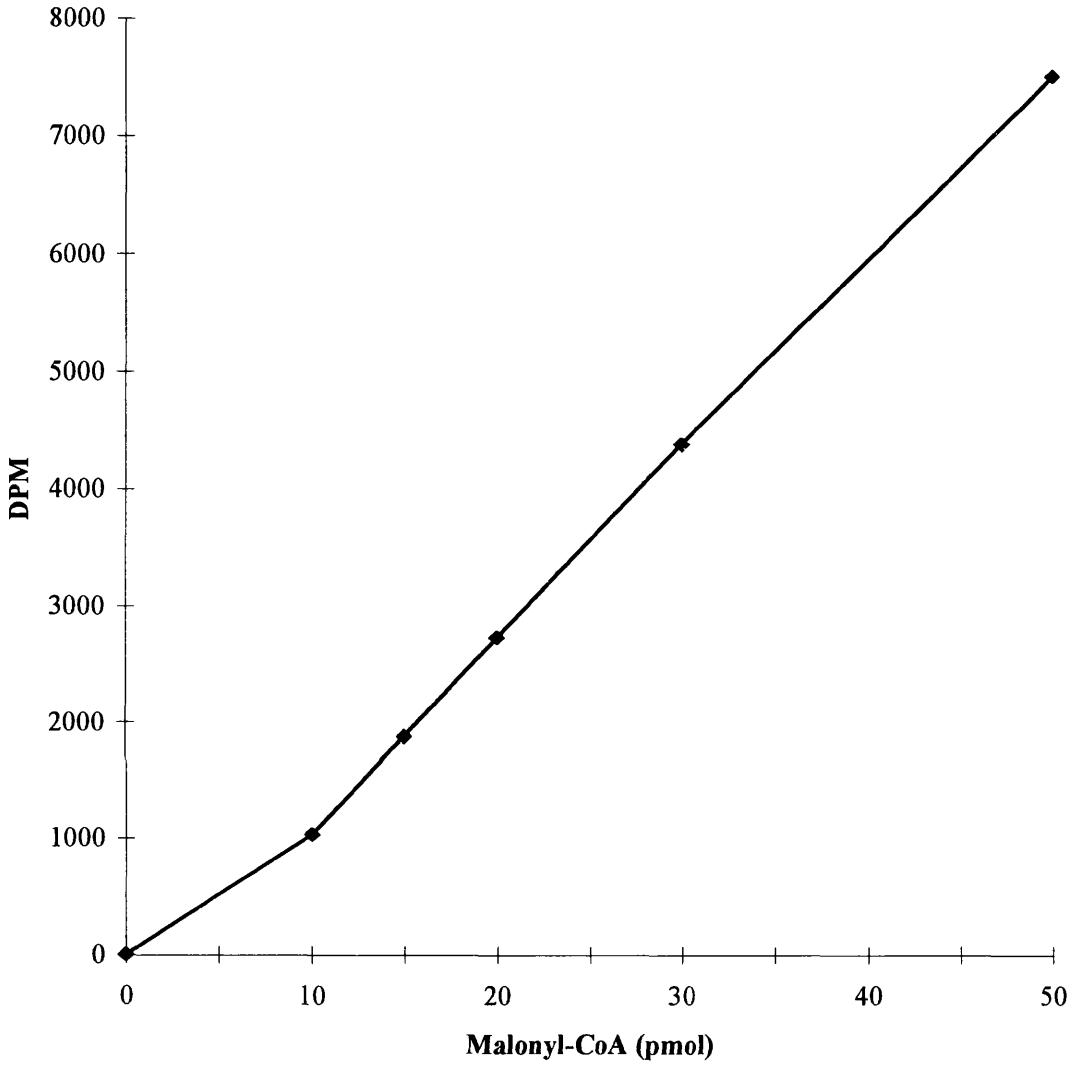
Malonyl-CoA was assayed by the method of McGarry *et al* (1978) with slight modifications (see section 3.1.2). Assays were carried out in duplicate in glass test tubes. The reaction was assayed in a 1ml final volume containing 0.2M KH_2PO_4 (pH 7.0), 2mM EDTA, BSA 0.1% (w/v), 2.5mM DTT, 0.25mM NADPH, 1.0μM [^{3}H] acetyl-CoA (0.5μCi/nmol) and appropriate quantities of heart extract or malonyl-CoA standard. The reaction was initiated by the addition of 1mU of purified FAS (see section 2.2.2) and

incubated at 37^{0}C for 90 min. The reaction was terminated by the addition of 29µl of 60% (w/w) perchloric acid. 1ml of ethanol was added followed by 5ml petroleum ether (BP 60-80^{0}C). Each tube was vortex mixed for a minimum of 20 sec and centrifuged at room temperature for 2 min at 2,000g_{av} in a MSE benchtop centrifuge to get better separation between the organic and aqueous phases. 4ml of the organic solvent phase was transferred to 2ml of distilled water. After vortexing and centrifugation as above, 3ml of the organic phase was transferred to 2ml of distilled water. After vortexing and centrifugation as above, 2ml of the organic phase was transferred to glass scintillation vials and placed in a fume cupboard to evaporate to dryness. Once dry, 2ml petroleum spirit was added and the vials vortexed to resuspend any fatty acids adhering to the bottom of the glass vial. 10ml Ecoscint A was added for scintillation counting. 20µl and 40µl samples of reaction mixture were also counted such that total reaction counts and theoretical values could be calculated. Blanks containing no malonyl-CoA or heart extract and a set of standards (0-50pmol) were included with each set of assays. Sample malonyl-CoA levels were determined by comparison with the standard curve (Fig 2.1).

2.8.1.1 Malonyl-CoA Standards.

Standard quantities of malonyl-CoA (0-50pmol), were assayed in duplicate. Standards were incubated for 90 min at 37^{0}C with 1.0µM [^{3}H] acetyl-CoA (0.5µCi/nmol) and 1mU of purified FAS (see section 2.2.2). The malonyl-CoA standards were measured as in the assay above.

Figure 2.1 Malonyl-CoA standard curve



Shows a sample standard curve for the determination of malonyl-CoA. Assays were carried out in a final volume of 1ml as described in section 2.8.1.

2.8.2 Glycerol

Glycerol released from myocytes was measured spectrophotometrically by the method of Garland & Randle (1962). Assays were carried out in duplicate at 26^0C in a final volume of 3ml containing 50mM bis-Tris propane (pH 7.6), 5mM $MgCl_2$, 5mM KCl, 0.3mM ATP, 0.4mM phosphoenolpyruvate, 0.1mM NADH, 20μg lactate dehydrogenase, 20μg pyruvate kinase and 1.5ml of myocyte extract (as prepared in section 2.5.1) The initial absorbance was read at 340nm using a Unicam SP8-100 spectrophotometer against a blank containing water in place of sample and the reaction initiated by addition of 10μg glycerokinase. The resulting decrease in absorbance (approx. 15 min after start) was measured and used to calculate glycerol concentration using the extinction coefficient of 6.22$\mu mol^{-1}ml$ for NADH. Glycerol contained within myocytes at the start of the incubation was also measured to allow calculation of glycerol output during the incubation.

2.8.3 Free Coenzyme A (CoASH).

CoASH was measured by the method of Allred and Guy (1969). Assays were carried out in duplicate at 26^0C in a final volume of 1ml containing: 200mM Tris-HCl buffer (pH 7.2), 55mM KCl, 10mM L-malate, 4.5mM acetyl phosphate, 7.5ug malate dehydrogenase (9U), 18.2ug citrate synthase (2U), 2mM NAD, 1mM DTT and sample (prepared as in section 2.6.3) (max. vol. 0.4ml). The reaction was started by the addition of 17U of phosphotransacetylase and the rate of increase in absorbance measured at 340nm against a blank containing water on a Unicam SP8-100 spectrophotometer. An internal standard of 0.1nmol CoASH was then added to the test cuvette and the increase in absorbance at 340nm recorded. The background rate (sample and CoASH standard omitted from the

test cuvette) was measured and subtracted from the reaction rates. CoASH levels were calculated using the following equation:

0.1 nmol x sample rate / (sample + standard rate) - (sample rate)

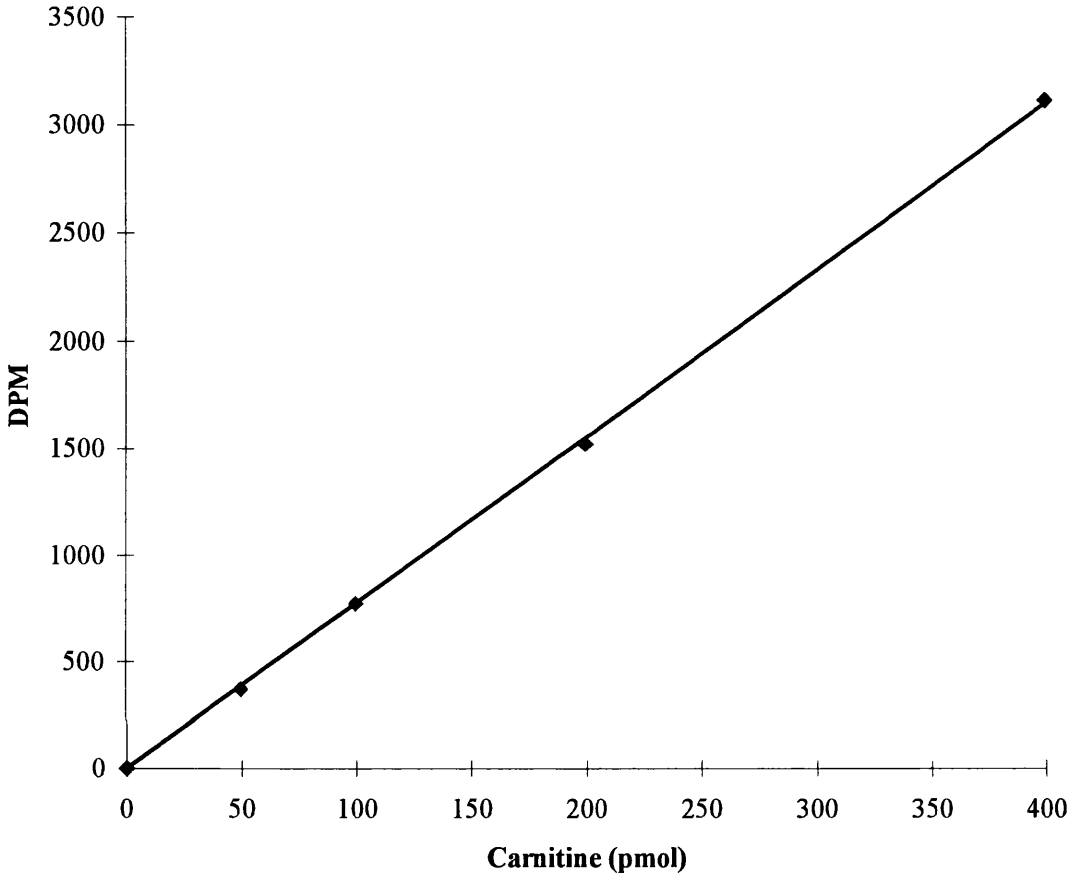
2.8.4 Free Carnitine

Free carnitine was measured using a slight modification of the method described by Cederblad and Linstedt (1972). Assays were carried out in duplicate in a 0.2ml reaction volume containing 125mM KH_2PO_4, 25μM [1-^{14}C] Acetyl-CoA (0.004uCi/nmol) and 100μl of tissue extract. The reaction was initiated by the addition of 1U carnitine acetyltransferase and tubes incubated at 37^{0}C for 30 min to allow the reaction to reach completion. 0.5ml distilled water was added to each tube, vortex mixed, and the contents loaded onto individual Dowex columns (see below). Columns were allowed to drip directly into glass scintillation vials until all the sample had entered the resin upon which the sample tubes were rinsed with a further 0.5ml distilled water which was loaded onto the columns. The columns continued to drip into scintillation vials until all the sample had eluted. Ecoscint A was added to the vials for scintillation counting. A set of l-carnitine standards (0-400pmol) were included with each set of assays and carnitine levels calculated by comparison with the linear standard curve (Fig 2.2).

2.8.4.1 Dowex Column Preparation

Dowex 2 (chloride form, 8% crosslinkage, dry mesh 200-400) was thoroughly washed and suspended in distilled water to form a slurry which was poured into 10ml plastic columns. The resin was allowed to settle and occupied approximately 3cm (length) by 0.8cm (diameter) in each column. The resin was kept wet until just before use when the

Figure 2.2 Carnitine standard curve



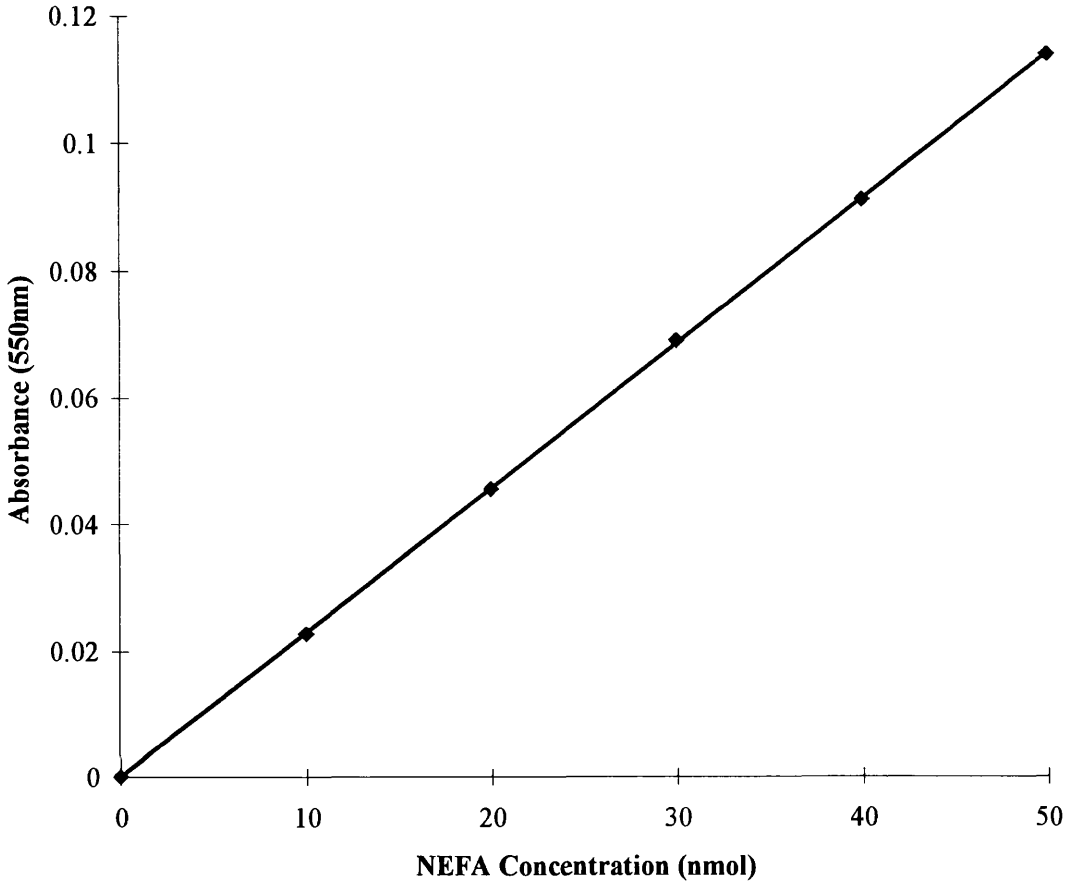
Shows a sample standard curve for the determination of carnitine. Assay were carried out in a final volume of 200µl as described in section 2.8.4.

water above the resin was reduced to a few mm and the samples loaded. Column resin was only used once and was discarded after use.

2.8.5 Non-Esterified Fatty Acids (NEFA)

The concentration of palmitate bound to albumin was determined using the Wako NEFA C test kit. In summary, acyl-CoA synthetase in the presence of ATP, Mg^{2+} and CoASH converts non-esterified fatty acids to acyl-CoA thioesters. The acyl-CoA is then oxidised by acyl-CoA oxidase to produce hydrogen peroxide. Hydrogen peroxide in the presence of added peroxidase allows the oxidative condensation of 3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline (MEHA) with 4-aminoantipyrine to form a purple coloured adduct with absorption maximum at 550nm which allows the NEFA concentration to be determined. The assay was carried out in duplicate in a reaction volume of 3ml. Fatty acid standards were set up with 0-50nmol oleic acid in 2% BSA (provided in the kit). 50µl samples were placed in plastic test tubes and 1ml of reagent A (50mM phosphate buffer (pH 6.9) containing 3mM $MgCl_2$, 5.44mM ATP, 0.3mg/ml 4-aminoantipyrine, 0.3U/ml acyl-CoA synthetase, 3U/ml ascorbate oxidase, 0.7mg/ml CoASH) added to each tube and incubated at 37^0C for 10 min. 2ml reagent B (1.2mM MEHA, 6.6U/ml acyl-CoA oxidase, 7.5U/ml peroxidase) was then added to each tube and incubated for a further 10 min at 37^0C. Tubes were allowed to cool to room temperature and the absorbance measured at 550nm using a Shimadzu UV-2101 PC spectrophotometer against a blank (water replacing sample). The NEFA concentration was calculated by comparison with the linear standard curve (Fig. 2.3)

Figure 2.3 NEFA C standard curve



Shows a sample standard curve for the determination of non-esterified fatty acids. Assays were carried out in a final volume of 3ml as described in section 2.8.5.

2.9 Tissue Preparations

2.9.1 Preparation of Liver Subcellular Fractions

Rats were killed by a blow to the head followed by cervical dislocation and livers removed, washed and chopped with scissors in ice-cold isolation buffer containing 0.25M sucrose, 20mM Tris-HCl, 2mM EGTA, 0.01% (v/v) saturated PMSF, adjusted to pH 7.4. The livers were homogenised in 10 volumes of the same buffer using a Potter-Elvehjem homogeniser fitted with a motor-driven Teflon pestle (radial clearance 0.19mm) (450 rev/min). The homogenate was centrifuged at 0-4^{0}C for 10 min at 600g_{av} in a SS-34 rotor (r_{av}=10.8cm) of a Sorvall RC5-B Superspeed centrifuge to yield a nuclear pellet. The resulting supernatant was centrifuged at 0-4^{0}C for 10 min at 9,200g_{av} to yield a mitochondrial pellet. The post-mitochondrial supernatant was centrifuged at 0-4^{0}C for 65 min at 100,000g_{av} in a 50.2Ti rotor (r_{av}=4.5cm) of a high speed L-8 Beckman Ultracentrifuge to yield a microsomal pellet and high speed supernatant (cytosolic fraction). All pellets were resuspended in a small amount of homogenization buffer and stored at -70^{0}C in aliquots along with the cytosolic fraction.

2.9.2 Preparation of Heart Subcellular fractions

Fractions of heart mitochondria, sarcoplasmic reticulum (SR), and cytosol were prepared as described by Wientzek and Katz (1991). Rats were sacrificed as above and the hearts rapidly removed and placed in ice-cold buffer A containing 10mM $NaHCO_3$ (pH 7.4) and 4mM DTT. The hearts were minced with scissors and washed in 10 volumes of the same buffer. The hearts were then homogenised on ice in a further 10 volumes of buffer using three 10 sec bursts of a Polytron homogeniser. The homogenate was then centrifuged at

0-4^{0}C for 15 min at 330g_{av} in a SS-34 rotor of a Sorval RC-5B Superspeed centrifuge. The resulting nuclear pellet was resuspended in a small volume of buffer A and stored in aliquots at -70^{0}C whilst the supernatant was centrifuged at 0-4^{0}C for 15 min at 5,000g_{av} to yield a crude mitochondrial pellet. The mitochondrial pellet was resuspended in a small volume of buffer A and stored in aliquots at -70^{0}C and the post-mitochondrial supernatant was centrifuged at 0-4^{0}C for a further 30 min at 23,000g_{av} to yield the SR pellet and crude cytosol. The SR pellet was resuspended in buffer B containing 39mM histidine-HCl buffer (pH 7.0), 0.6M KCl and 4mM DTT and centrifuged at 0-4^{0}C for 30 min at 23,000g_{av} to yield SR membranes free of myofibrils. These SR membranes were resuspended in a small volume of buffer C containing 0.1M Tris-HCl buffer (pH 7.2), 0.25M Sucrose, 0.3M KCl and stored in aliquots at -70^{0}C. The crude cytosol was centrifuged at 0-4^{0}C for 60 min at 100,000g_{av} in a 50.2Ti rotor (r_{av}=4.5cm) of a high speed L-8 Beckman Ultracentrifuge to yield a small SR fragment pellet and the pure cytosolic fraction which was stored in aliquots at -70^{0}C.

2.9.3 Isolation of Intact Rat Heart Mitochondria

Intact rat heart mitochondria were isolated from rat hearts by the following method. Rats were sacrificed as above and the hearts quickly removed and immediately placed in ice-cold MST buffer containing 8.25mM Tris-HCl buffer (pH 7.2), 0.21M mannitol, 70mM sucrose. The hearts were chopped with scissors and washed three times to remove any blood. The hearts were homogenised for a few seconds only using a Polytron homogeniser and then centrifuged at 0-4^{0}C for 5 min at 300g_{av} in a SS-34 rotor of a Sorvall RC-5B Superspeed centrifuge to remove any large debris and the nuclear fraction. The supernatant was then centrifuged at 0-4^{0}C for 8 min at 3,000g_{av} to pellet

the mitochondria. The supernatant was discarded and the pellet resuspended using a hand held Potter-Elvehjem homogeniser in MSTEB buffer containing Tris-HCl buffer (pH 7.2), 0.21M mannitol, 70mM sucrose, 1mM EGTA and 0.5mg/ml BSA. This was then centrifuged again at 0-4^{0}C at 3,000g_{av} for 8 min and this wash procedure repeated a further two times. The final pellet was resuspended in a small volume (usually 1ml) of MSTEB buffer and the protein content determined.

2.10 Oxygen Electrode

A Rank oxygen electrode (Berkshain., Cambridge, U.K.) was used to measure the rate of oxygen consumption of rat heart mitochondria prepared as above. Reactions were carried out in duplicate at 37^{0}C in a 3ml reaction volume containing 10mM Tris-HCl buffer (pH 7.3), 120mM KCl, 0.5mM EGTA, 1mM KH_2PO_4, BSA (1.3mg/ml), 1mM ADP and 0.5-0.8mg/ml mitochondrial protein. The zero oxygen concentration of the reaction medium was determined with sodium dithionite and the chamber thoroughly washed with H_2O before beginning experiments. Additions of 3mM malate, 3mM pyruvate, 1mM malonyl-CoA, 1mM carnitine and 5mM acetyl-L-carnitine were made singularly and in combination and changes in oxygen tension recorded on a Pharmacia LKB-REC 2 dual pen chart recorder. Oxygen consumption was calculated given that at 37^{0}C 0.02386 volumes of O_2 dissolve in 1 volume of buffer when air is 20.95% O_2. Therefore O_2 saturation of buffer at 37^{0}C is 0.22mM and 3ml buffer contains 0.66 μmoles O_2. Oxygen consumption was expressed as nmol/min/mg mitochondrial protein.

2.11 Attempted Purification of the Fatty Acid Chain Elongation (FACE) System from Rat Liver Microsomes

Purification of the fatty acid chain elongation (FACE) system was attempted from liver microsome fractions previously isolated as described in section 2.9.1. Solublizations were carried out in duplicate at 0-4^{0}C in a 1ml final volume containing 10mM KH_2PO_4 buffer (pH 7.2), 10% glycerol (v/v), 5μg/ml pepstatin, 5μg/ml bestatin, 5μg/ml leupeptin, 0.01% (v/v) saturated PMSF, 5-10mg/ml microsomal protein and varying concentrations of detergent at and above their critical micelle concentration (detergents were diluted in reaction buffer). The reaction was kept on ice with regular vortexing for 1 hr. The mixture was then transferred to TLA-100.3 ultracentrifuge tube and centrifuged at 0-4^{0}C for 1 hr 30 min at 200,000g_{av} in a TLA-100.3 rotor of a Beckman TL-100 ultracentrifuge. The supernatants were transferred to fresh tubes and assayed for FACE activity spectrophotometrically as described in section 2.12.2.2. The pellets were resuspended in a small volume of reaction buffer and assayed for FACE activity.

The four detergents cholate, deoxycholate, triton-X 100 and Tween 20 were used to try and solublize the FACE system from liver microsomes.

2.12 Enzyme Assays

2.12.1 Fatty Acid Synthase (FAS)

Fatty acid synthase was assayed spectrophotometrically at 26^0C by a method based on that of Saggerson & Greenbaum, (1970). Assays were carried out in duplicate in a 1ml reaction containing 100mM KH_2PO_4 buffer (pH 6.6), 2mM EDTA, 2mM DTT, 0.25mM NADPH, 0.03mM acetyl-CoA and 100µl of tissue extract. The reaction was initiated by the addition of 0.05mM malonyl-CoA and the decrease in absorbance measured at 340nm against a reference cuvette (H_20 replacing malonyl-CoA) on a Unicam SP8-100 spectrophotometer. FAS activity was calculated using the extinction coefficient for NADPH of 6.22µmol^{-1} ml and was expressed as nmol/min/mg protein

2.12.2 Fatty Acid Chain Elongation (FACE)

2.12.2.1 Radiochemical Assay

Fatty acid chain elongation activity was measured at 37^0C, as described by Prasad and Cinti (1986). Reactions were carried out in duplicate in a 1 ml final volume containing 0.1M Tris-HCl buffer (pH 7.4), 0.4mM [2-^{14}C]malonyl-CoA (0.23µCi/µmol), BSA 0.4% (w/v) and 30µM palmitoyl-CoA. The reaction was initiated by the addition of an appropriate volume of tissue extract and stopped after 30 min by the addition of 0.75ml 15% (w/v) KOH in methanol, followed by saponification at 65^0C for 45 min. After acidification with 1ml cold HCl (4M), the reaction products were extracted three times with 2.5ml of petroleum ether (BP 40-60^0C). On each extraction the sample mixture was vortexed for a minimum of 20 sec and then centrifuging at room temperature for 2 minutes at 2,000g_{av} in a MSE benchtop centrifuge to obtain a good separation of the

organic and aqueous phases before transferring the organic solvent layer into scintillation vials. Samples were left to evaporate to dryness in a fume cupboard and 8ml of Ecoscint A was added for scintillation counting. 20µl and 40µl samples of reaction mixture were also counted so that total reaction counts could be calculated.. FACE activity was calculated from the total counts and was expressed as nmol/min/mg protein

2.12.2.2 Spectrophotometric Assay

Activity of the condensing enzyme (the rate limiting step) of the FACE system was measured using the method of Nagi *et al* (1989). Reactions were carried out in duplicate in a 1ml reaction volume containing 0.1M Tris-HCl buffer (pH 7.4), 40µM palmitoyl-CoA, 40µM BSA, and 250µg/ml microsomal protein. A baseline was recorded using a Unicam SP8-100 spectrophotometer at 37^0C by scanning from 400-290nm against an identical reference cuvette. The reaction was initiated by the addition of 60µM malonyl-CoA (final concentration) to the sample cuvette and an equal volume of water to the reference cuvette. The reaction was monitored by repeated scanning every 2 min and the change in absorbance at 303nm, due to the formation of an enolate-BSA complex, calculated. Any change in absorbance at 350nm was subtracted from that at 303nm.

The condensation activity was calculated using the extinction coefficient for the enolate complex of $29mM^{-1}cm^{-1}$ as determined by Nagi *et al* (1989) and expressed as nmol/min/mg protein.

2.12.3 Malonyl-CoA Decarboxylase

Decarboxylase activity was measured using the method of Kim and Kolattukudy (1978). Reactions were carried out in duplicate a 1ml reaction volume containing 0.1M Tris-HCl buffer (pH 8.0), 0.5mM DTT, 10mM L-malate, 0.5mM NAD^+ and 8.5U malate

dehydrogenase. The reaction mixture was incubated at 37^{0}C for 7 min so that the malate dehydrogenase equilibrium could be established. 4.25U of citrate synthase was added to the cuvette and incubated for a further 1 min for equilibrium to be reached between the two enzymes. 100µl sample and 5µM rotenone was added and the reaction started with 60µl of 5mM malonyl-CoA. The increase in absorbance at 340nm due to the reduction of NAD^+ to NADH, was measured using a Unicam SP8-100 spectrophotometer at 37^{0}C against a reference cuvette (H_2O replacing malonyl-CoA). The change in absorbance was recorded after the first 30 sec. Malonyl-CoA decarboxylase activity was calculated using the extinction coefficient for NADH of 6.22µm^{-1}ml.

2.12.4 Citrate Synthase

Citrate synthase activity was measured by the method of Shepherd and Garland (1969). Reactions were carried out in duplicate and in a 1 ml reaction volume containing 100mM Tris-HCl buffer (pH 8.0), 0.1%, 0.1mM DTNB, 50µM acetyl-CoA and 40µl sample. The reaction was initiated by the addition of 50µM oxaloacetate to the sample cuvette. The reaction of free CoASH with DTNB causes the release of a thiolate anion which has an absorption maximum at 412nm. The increase in absorbance at 412nm, was measured for 60 sec against a reference cuvette (H_2O replacing oxaloacetate). Absorbance increase was measured on a Shimadzu UV-2101 PC spectrophotometer at 26^{0}C and the enzyme activities were calculated using the extinction coefficient for the thiolate anion of 13.6µmol^{-1}ml.

2.12.5 Lactate Dehydrogenase

Lactate dehydrogenase (LDH) was measured spectrophotometrically using the method of Saggerson (1974). Reactions were carried out in duplicate in a 1 ml reaction volume

containing 65mM Tris-HCl buffer (pH 7.4), 0.3mM NADH and 20µl sample. The reaction was started by the addition of 1.3mM pyruvate to the sample cuvette. The drop in absorbance due to the oxidation of NADH to NAD^+ was measured against a reference cuvette (H_2O replacing pyruvate). The drop in absorbance at 340nm was measured using a Shimadzu UV-2101 PC spectrophotometer at 26^0C for 10 mins and enzyme activities were calculated using the extinction coefficient for NADH of 6.22µmol^{-1}ml.

2.12.6 Malate Dehydrogenase

Malate dehydrogenase was measured in duplicate in a 1ml reaction volume containing 1M Tris-HCl buffer (pH 8.0),0.5mM NADH, 5µM rotenone and 20µl sample. The reaction was initiated by the addition of 5mM oxaloacetate to the sample cuvette. The decrease in absorbance at 340nm due to the oxidation of NADH to NAD^+ was measured on a Shimadzu UV-2101 spectrophotometer at 26^0C against the reference cuvette (H_2O replacing oxaloacetate) for 10 mins. Malate dehydrogenase activity was calculated using the extinction coefficient for NADH of 6.22µm^{-1}ml.

2.13 Protein Determination.

Protein concentrations of homogenates and subcellular fractions were measured using a bicinchoninic acid (BCA) assay kit supplied by Sigma Chemical Co. The BCA solution is a modification of the reagent originally proposed by Smith *et al* (1985). Assays were carried out in duplicate in a reaction volume of 1.05ml. 50µl samples were placed in plastic test tubes and 1ml protein determination reagent (prepared by adding 1 part copper (II) sulphate pentahydrate 4% to 50 parts BCA solution) added. Tubes were

vortex mixed and incubated at 37^0C for 30 min. Tubes were allowed to cool to room temperature before the absorbance at 562nm was determined using a Shimadzu UV-2101 spectrophotometer against a reference cuvette (H_2O replacing sample). The protein concentration was calculated by comparison with the linear standard curve constructed using 0-100μg BSA (Fig. 2.4).

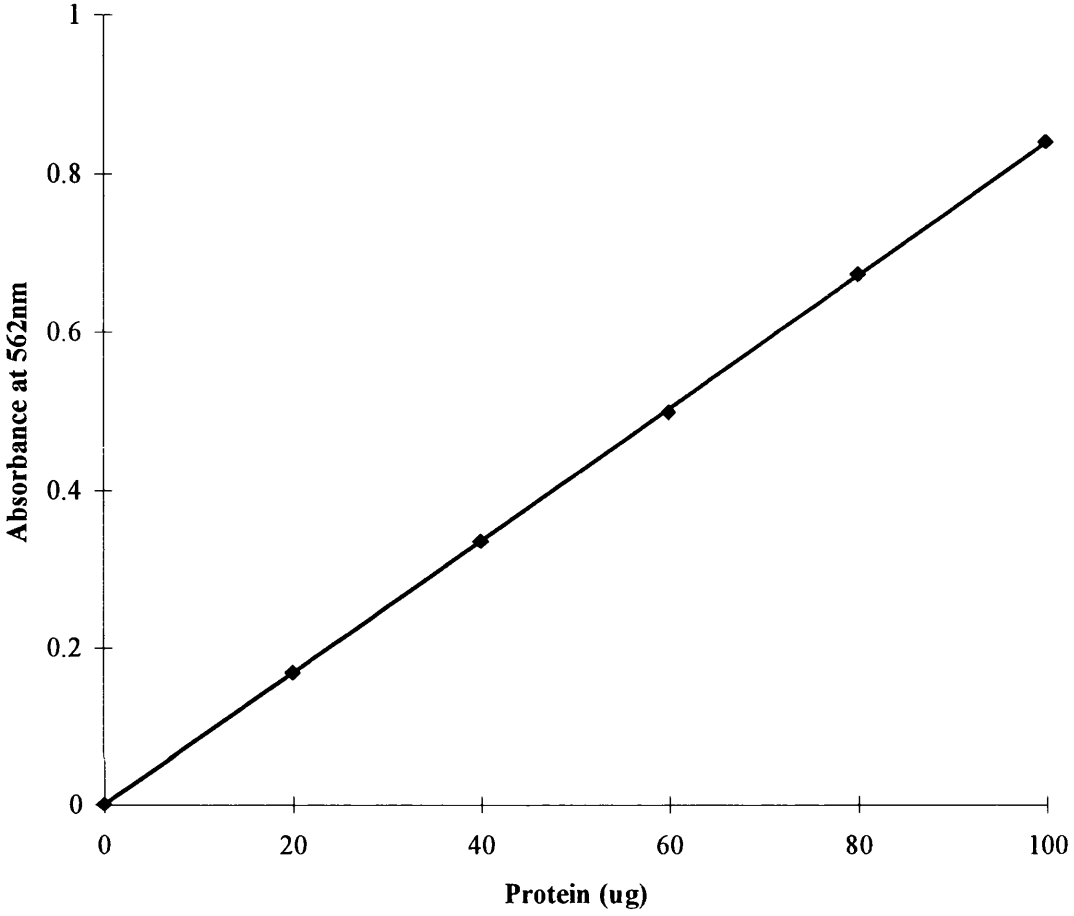
2.14 Scintillation Counting

Radioactive products from radiochemical assays were measured on a Packard Tri-Carb 1900CA analyzer (Canberra Packard, Pangbourne, Berks., U.K.) which had been pre-programmed for counting ^{14}C and ^{3}H isotopes. The counter was equipped with a computer which stored the quench curves for ^{14}C and ^{3}H and enabled direct printing of radioactivity in disintergrations per minute (dpm).

2.15 Statistical Analysis

Values are presented as means ± SEM. The n values quoted refer to the number of separate preparations. The statistical significance between different preparations and within the same preparations was assessed using the Student's t-test for unpaired or paired samples as appropriate.

Figure 2.4 Protein standard curve



Shows a sample standard curve for the determination of protein using the BCA protein assay kit. Assays were carried out in a final volume of 1.05ml as described in section 2.13.

Chapter 3 - Results

3.1 Development of Conditions for Measuring Malonyl-CoA in Cardiac Myocytes

Malonyl-CoA has been measured in a number of tissues including heart (McGarry *et al*, 1983) and is an important regulator of mitochondrial fatty acid oxidation through its inhibitory action on CPT 1. Tissue concentrations of malonyl-CoA are low (in the region of 2 to 4 nmol/g wet weight tissue), but it is not clear to what extent these measurements are representative of cardiac myocytes, rather than the non-myocyte cells of the heart.

Uncontaminated, viable cardiac myocytes must therefore be isolated and a highly sensitive malonyl-CoA assay devised, to accurately measure malonyl-CoA in these cells.

3.1.1 Isolation of Cardiac Myocytes

Several methods over the years have been developed for the isolation of cardiac myocytes. The majority of successful methods, involve perfusion and shaking with digestive enzymes, such as collagenase. The inability of isolated myocytes to maintain structural and metabolic integrity in the presence of physiological concentrations of Ca^{2+} (Farmer *et al*, 1977; Vahouny *et al*, 1979) has been a major problem in the isolation of viable myocytes. This problem was overcome by slowly reintroducing Ca^{2+} to the myocytes in a series of washes. Isolation of cardiac myocytes by perfusion and shaking with Krebs-Henseleit bicarbonate (KHB) medium containing collagenase followed by sequential washing in KHB medium containing gradually increasing $[Ca^{2+}]$, produces a high yield of myocytes with good viability which are Ca^{2+} tolerant (see section 2.4). As well as being judged

viable on morphological criteria, myocytes isolated using this method by previous co-workers of the Saggerson group have shown them to retain normal metabolic characteristics upon isolation (Awan & Saggerson, 1993; Swanton PhD Thesis, 1996).

In order to obtain sufficient cardiac myocytes for the measurement of malonyl-CoA, it was sometimes necessary to pool myocytes isolated from two rat hearts which were removed and perfused simultaneously (see section 2.4.4). This enabled a number of incubations to be carried out using the same batch of cells. Using this method it was possible to isolate a good quantity (3-4 cm^3 packed cells) of >70% viable myocytes. Myocyte viability was determined as described in section 2.4.3.

A number of adjustments were made to the isolation procedure depending on the incubations and experiments to be carried out. TAG-loaded (0.5mM palmitate included in isolation medium and washes), non-TAG-loaded (no palmitate in isolation medium and washes) and glucose depleted (low/no glucose in isolation medium and washes) myocytes were all isolated with >70% viability (see sections 2.4.1-2.4.3). The isolation procedure and incubation conditions are described for each experiment and stated in figure legends where appropriate.

3.1.2 The Malonyl-CoA Assay

McGarry *et al* (1978), developed an assay system for measuring malonyl-CoA, which measures the malonyl-CoA-dependent incorporation of radiolabeled acetyl-CoA into palmitic acid catalysed by fatty acid synthase (FAS) in the presence of excess NADPH. Reaction products are extracted into petroleum spirit and radioactivity measured. This method was found to be unreliable when levels of malonyl-CoA were low (i.e. in cardiac myocytes), due to the non-linearity of the assay and possible dilution of radiolabeled acetyl-CoA by endogenous acetyl-CoA.

Smith *et al* (1984), have shown that using labeled butyryl-CoA as a primer for fatty acid synthesis in place of labeled acetyl-CoA, improves both the linearity and sensitivity of the assay. Butyryl-CoA is the preferred primer for fatty acid synthesis when equal concentrations of butyryl-CoA and acetyl-CoA are used. Unfortunately, labeled butyryl-CoA is not yet commercially available and its synthesis is a complicated procedure and so could not be used.

The non-linearity of the malonyl-CoA standard curve can be explained, in part, by the fact that FAS converts significant amounts of acetyl-CoA into short-chain fatty acids (e.g. butyric acid) as well as long-chain fatty acids (Abdinejad *et al*, 1981). The radioactivity incorporated into short-chain fatty acids is not detected in the assay as some short-chain fatty acids are not extracted into petroleum spirit, while others are evaporated during drying of the extracts. A small amount of malonyl-CoA may remain as an enzyme substrate complex and will not be extracted into petroleum spirit for scintillation counting. The amount of uncompleted fatty acid chains increases with enzyme concentration, and their relative contribution to the total radioactivity is large when the malonyl-CoA:acetyl-CoA ratio is low (Singh *et al*, 1984), which is the case in cardiac myocytes. It was therefore necessary to further develop the method of McGarry *et al* (1978), in order to accurately measure the very low concentrations of malonyl-CoA present in cardiac myocytes.

3.1.2.1 Development of the Malonyl-CoA Assay

A number of factors within the malonyl-CoA assay were altered to improve the sensitivity and linearity of the assay; these included the quantity of FAS added to each reaction (decreased from 6mU to 1mU), the reaction time (increased from 45 min to 90 min) and the concentration of non-labeled acetyl-CoA (increased from 0.68μM (0.5μCi/nmol) to 1.0μM (0.5μCi/nmol)).

Figure 3.1 shows standard curves determined using both the original and improved method, as well as a theoretical standard curve. The theoretical standard curve is calculated assuming that there is a 7:1 ratio of acetyl-CoA:malonyl-CoA incorporated into petroleum spirit soluble products by FAS. This also assumes that all of the malonyl-CoA in the sample is incorporated into palmitate and that palmitate is the only product of the FAS reaction.

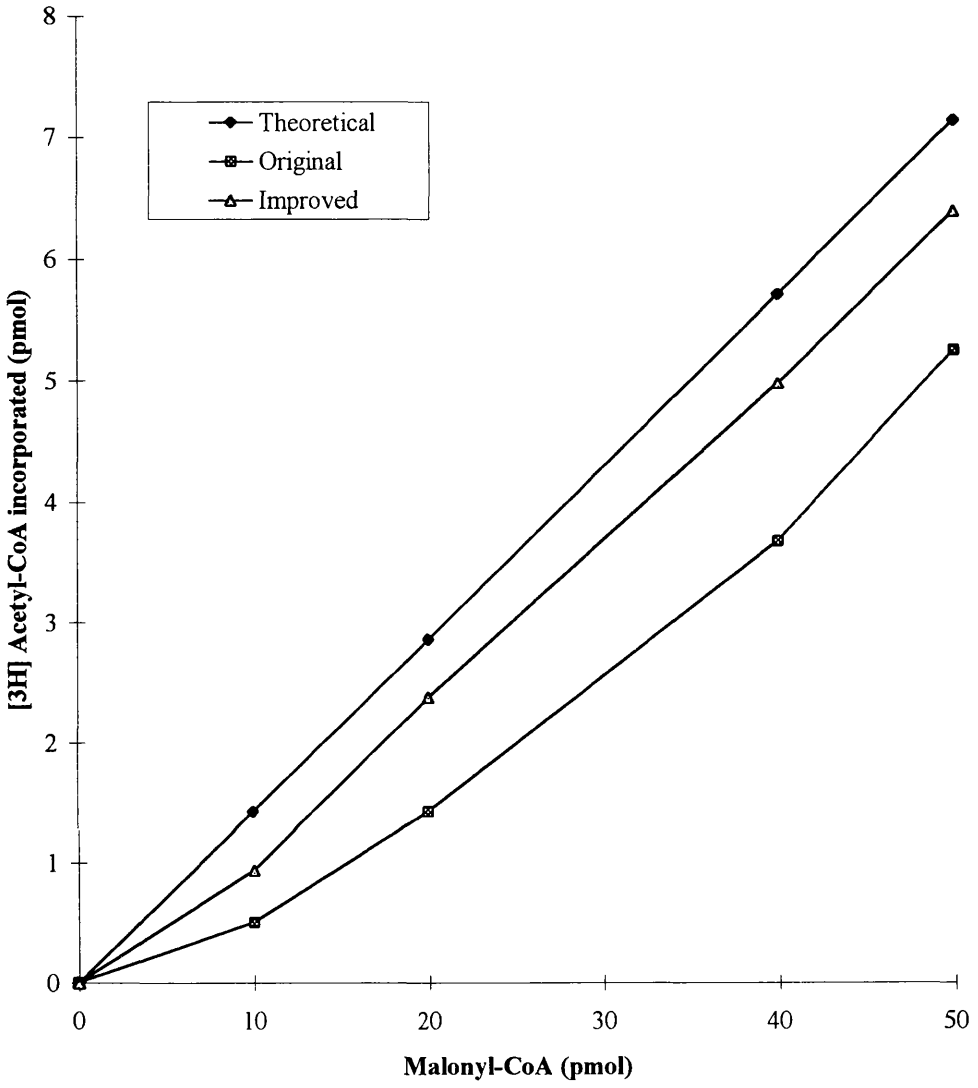
Decreasing the concentration of FAS, increasing the concentration of acetyl-CoA and increasing the reaction time significantly improved both the sensitivity and linearity of the malonyl-CoA assay, producing a standard curve which was almost linear and close to the theoretical one (fig 3.1).

Decreasing the amount of FAS decreases the amount of uncompleted and short-chain fatty acids formed, while increasing the reaction time allows the reaction to go to completion with the lower concentration of FAS. Decreasing the amount of FAS also reduces the amount of partially synthesised fatty acids which are bound to the enzyme and are not extracted into petroleum spirit.

Dilution of radiolabeled acetyl-CoA by endogenous acetyl-CoA is another factor contributing to the non-linearity of the assay, especially at low concentrations of malonyl-CoA. Endogenous acetyl-CoA within the sample reduces the specific radioactivity of labeled acetyl-CoA in the assay, thus reducing its sensitivity. This results in a reduced recovery of radioactivity in the petroleum spirit extracts and values which are lower than they should theoretically be.

The acetyl-CoA content of isolated hearts from adult fed rats, as determined by Allred & Guy (1969), is ~38 nmoles/g wet weight tissue. A typical quantity of myocytes used in incubations was 10mg (wet weight), which therefore contains ~0.38 nmoles endogenous acetyl-CoA. Myocyte incubations were carried out in a

Figure 3.1 Development of malonyl-CoA standard curve



Malonyl-CoA standard curves were determined as described in section 2.8.1. Typical standard curves are shown using either the original method with 6mU FAS, 0.68μM acetyl-CoA (0.5μCi/nmol) and a 45 min reaction time, or the improved method with 1mU FAS, 1.0μM acetyl-CoA (0.5μCi/nmol) and a 90 min reaction time. Theoretical values are calculated assuming a 7:1 ratio of acetyl-CoA:malonyl-CoA incorporated into petroleum spirit-soluble products by FAS.

final volume of 1ml, such that the concentration of endogenous acetyl-CoA in the incubation would be ~0.38μM. Incubations were stopped by the addition of perchloric acid (6% (w/w) final concentration) and 350μl of the 1ml aqueous sample assayed for malonyl-CoA. Malonyl-CoA is assayed in a 1ml reaction volume which is a 2.86 x dilution of the sample. Hence the concentration of endogenous acetyl-CoA added to the assay as a result of the myocytes would be ~0.133μM. In the original assay, the concentration of acetyl-CoA in the reaction is 0.68μM (0.5μCi/nmol). An additional 0.133μM acetyl-CoA from the sample would dilute the specific radioactivity by ~20%. Whereas in the improved assay, where the concentration of acetyl-CoA is 1.0μM (0.5μCi/nmol), the addition of 0.133μM acetyl-CoA would dilute it by only ~13%.

Increasing the concentration of acetyl-CoA in the assay from 0.68μM (0.5μCi/nmol) to 1.0μM (0.5μCi/nmol) improved the sensitivity of the malonyl-CoA assay, especially at low concentrations of malonyl-CoA, and produced a curve which was more linear and was closer to the theoretical one (fig 3.1). It would be unwise to increase the concentration of acetyl-CoA in the assay any further as the relative contribution of uncompleted fatty acid chains is large when the malonyl-CoA:acetyl-CoA ratio is low (Singh *et al*, 1984).

Addition of an internal malonyl-CoA standard, as suggested by McGarry *et al* (1978), to compensate for any acetyl-CoA or other acyl-CoAs acting as primers for FAS, is only possible if the standard curve is linear. Although the improved standard curve is more linear than the original, it is still not completely linear and hence an internal standard cannot be used.

As a result of all these factors the improved malonyl-CoA assay with 1mU FAS, 1.0μM acetyl-CoA (0.5μCi/nmol) and a 90 min reaction time was used for all determinations of malonyl-CoA in this study and a standard curve was performed in parallel with each assay.

3.1.3 Purification of FAS

The FAS used in the malonyl-CoA assay was purified from rat liver cytosol as described in section 2.2.2. FAS was purified by a series of steps: (1) Subcellular fractionation of cytosol from rat liver homogenate. (2) Ammonium sulphate precipitation (25-40% cut). (3) Ion exchange (Q sepharose) chromatography using FPLC.

Ammonium sulphate precipitation was carried out on ice at 4^0C. A 25-40% ammonium sulphate cut of rat liver cytosol resulted in a 4.2 fold increase in FAS specific activity with a yield of 71% (table 3.1). Ammonium sulphate was removed from the sample after precipitation by overnight dialysis before FAS activity was determined and before any further purification was carried out.

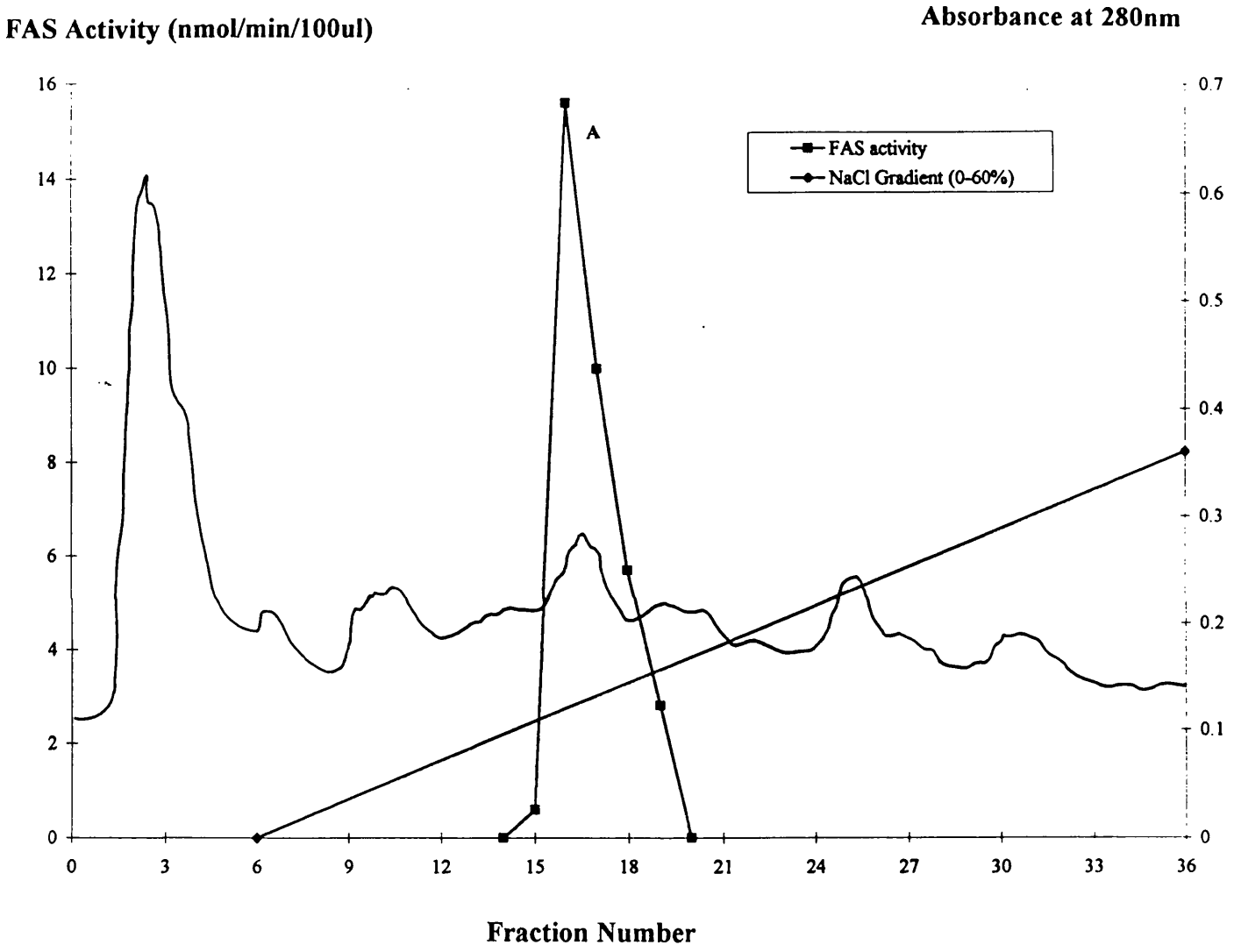
The dialyzed ammonium sulphate precipitate was loaded, via a 30ml super loop, on to a previously equilibrated Pharmacia FPLC HiLoad 16/10 Q sepharose column. Figure 3.2 shows an FPLC trace from a typical FPLC purification procedure. The majority of FAS activity eluted between 0.18M and 0.23M NaCl and corresponds to the large peak of protein (marked A) which eluted from the column (fig 3.2). 10ml fractions were collected and those containing the bulk of the activity were pooled and stored at -70^0C. Mono Q ion exchange chromatography resulted in a 4.7 fold increase in FAS specific activity and a yield of just under 100%. It is not unusual to get a yield of more than 100% as very often the purification procedure

Table 3.1 Fatty acid synthase purification

Purification Step	Total Protein (mg)	Total Activity (nmol/min)	Specific Activity (nmol/min/mg)	Percentage Yield (%)	Purification Factor
Fractionated Cytosol	1374.3	14614.8	10.6	-	-
Ammonium Sulphate 25-40% Cut	231.8	10392.5	44.8	71.1	4.2
Mono Q Ion-Exchange	49.5	10372.8	209.6	71.0	19.7

Fatty acid synthase was purified from rat liver cytosol as described in section 2.2.2. FAS activity was assayed at 26^0C as described in section 2.12.1. Protein concentration was measured as described in section 2.13. Values are from a representative purification procedure.

Figure 3.2 Purification of rat liver FAS using FPLC mono Q ion exchange chromatography



The partially purified FAS sample recovered from subcellular fractionation and ammonium sulphate precipitation was further purified using a Pharmacia FPLC HiLoad 16/10 Q sepharose column as described in section 2.2.2. The sample was desalted prior to loading by overnight dialysis in 20mM bis-Tris propane buffer (pH 7.0) containing 30mM NaCl, 3mM EDTA and 1mM DTT and eluted with a linear gradient of 0-0.6M NaCl over 20 column volumes.

removes inhibitors of the enzyme which results in an increase in enzyme activity even though some enzyme is lost during the procedure.

FAS activity was regularly checked in frozen aliquots. It was found that storage of FAS in normal storage buffer for 2 months, resulted in a 28% decrease in activity. Addition of 10% glycerol (final concentration) to the storage buffer reduced the loss of activity to 9% in 2 months. When FAS activity in frozen aliquots had decreased by 15% a new batch of FAS was prepared and the old one discarded.

Table 3.1 shows the techniques used to purify FAS as well as the specific activity, total activity, percentage yield and purification factor at each step for a typical FAS purification from rat liver cytosol. This typical FAS purification procedure resulted in a total purification factor of 19.7 which is similar to that obtained by Hsu *et al* (1965), who achieved a purification factor of 21 upon purification of FAS from pigeon liver cytosol. The 71% yield of FAS was higher than that obtained by Hsu *et al* (1965), who obtained a yield of only 24%. Further purification was not thought necessary due to the high specific activity of FAS purified (~210 nmol/min/mg protein). This high specific activity meant that a very small volume of FAS was added to reactions for the malonyl-CoA assay.

3.1.4 Conclusions

An isolation procedure which produces a good yield of uncontaminated, viable cardiac myocytes is essential for any biochemical investigation of myocardium at the cellular level. The isolation procedure used in this study has been used successfully by previous members of the Saggerson laboratory for a number of years and has been shown to produce cardiac myocytes which are viable both morphologically and biochemically (Swanton, PhD thesis 1996).

The further development of the malonyl-CoA assay has meant that accurate measurements of malonyl-CoA can be made in cardiac myocytes where cell mass and malonyl-CoA concentration are low. The combination of a good myocyte isolation procedure (section 3.1.1) with reliable metabolite assays, allows reliable measurements of malonyl-CoA and other metabolites to be made in cardiac myocytes.

3.2 Synthesis of Malonyl-CoA in Cardiac Myocytes

Malonyl-CoA is synthesised in the cytosol from acetyl-CoA by the enzyme acetyl-CoA carboxylase (ACC) or, to a lesser extent, by the action of propionyl-CoA carboxylase in the mitochondria. In lipogenic tissues such as liver and adipose tissue, the ACC-dependent production of malonyl-CoA is important as the first committed step of fatty acid synthesis. However, an immunologically distinct isoform of ACC (ACC-280) has been identified in tissues such as heart and skeletal muscle, which are not considered to be lipogenic. The presence of ACC-280 in the heart, and the fact that malonyl-CoA is a potent inhibitor of mitochondrial CPT 1 has lead to the suggestion that the primary role of ACC in heart, is regulation of mitochondrial fatty acid oxidation (Bianchi *et al*, 1990; Saddik *et al*, 1993).

Cardiac malonyl-CoA levels are known to increase dramatically and transiently under various conditions such as dichloroacetate (DCA) -treatment (Saddik *et al*, 1993), and glucose treatment (Louis & Witters, 1992). To date, no studies have investigated the rate of ACC-dependent malonyl-CoA synthesis in cardiac myocytes.

3.2.1 Effect of Glucose Concentration on Malonyl-CoA Levels in Non-TAG-loaded Myocytes

Non-TAG-loaded myocytes were incubated at 37^0C for 60 min in Krebs-Henseleit bicarbonate (KHB) medium containing 2% BSA and a range of glucose concentrations (0, 1, 2.5 and 5mM glucose).

As shown in figure 3.3, incubation of myocytes with increasing concentrations of glucose resulted in an almost linear increase in malonyl-CoA level. The malonyl-

CoA content at each glucose concentration was significantly higher than at the previous glucose concentration (fig 3.3).

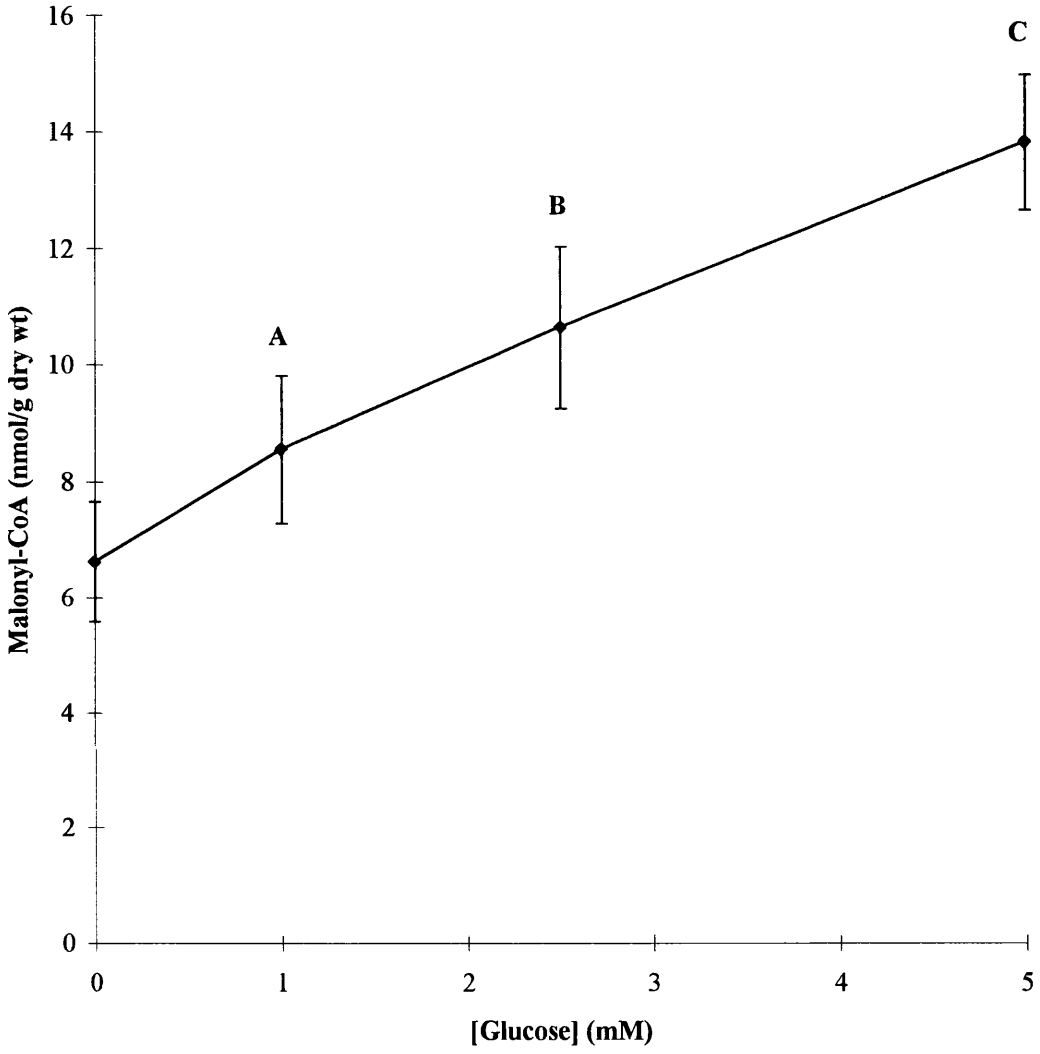
It should be noted that non-TAG-loaded myocytes are normally isolated in buffers containing 10mM glucose. For this experiment the last wash did not contain any glucose so as to significantly dilute the glucose in the incubation medium and myocytes prior to incubation. As a result, the 0mM glucose incubation contained a small, but unknown amount of glucose as it was impossible to remove all glucose from the incubation medium and myocytes prior to incubation. It was expected that in the absence of glucose, malonyl-CoA synthesis, and hence malonyl-CoA level, would be considerably reduced. The presence of this unknown concentration of glucose in the incubation medium is most likely responsible for the surprisingly high level of malonyl-CoA measured in the 0mM glucose incubation. This additional glucose may also result in the level of malonyl-CoA at other glucose concentrations, being higher than they would have been had this extra glucose been absent.

The isolation of glucose depleted myocytes would remove this problem and allow more accurate measurements of glucose induced malonyl-CoA levels to be made. It would also allow greater insight into the glucose induced regulation of ACC and the rate of malonyl-CoA synthesis upon glucose repletion (see section 3.2.2). The mechanism by which glucose acutely regulates ACC, and malonyl-CoA levels in cardiac myocytes is discussed in the section 3.2.2. below.

3.2.2 Glucose Regulation of Malonyl-CoA in Cardiac Myocytes

Glucose depleted myocytes were isolated as described in section 2.4.3. The isolation procedure differs from normal in that isolation buffers contain a reduced concentration of glucose (5mM instead of 10mM), and glucose is absent from the

Figure 3.3 Effect of glucose concentration on malonyl-CoA levels in non-TAG-loaded myocytes



Non-TAG-loaded myocytes were incubated at 37^{0}C for 60 min with KHB medium containing 2% BSA and indicated concentrations of glucose. Values are expressed as means ± SEM of 5 independent preparations. **A**, represents $p<0.005$ versus 0mM glucose. **B**, represents $p<0.01$ versus 1mM glucose. **C**, represents $p<0.01$ versus 2.5mM glucose (paired t-test).

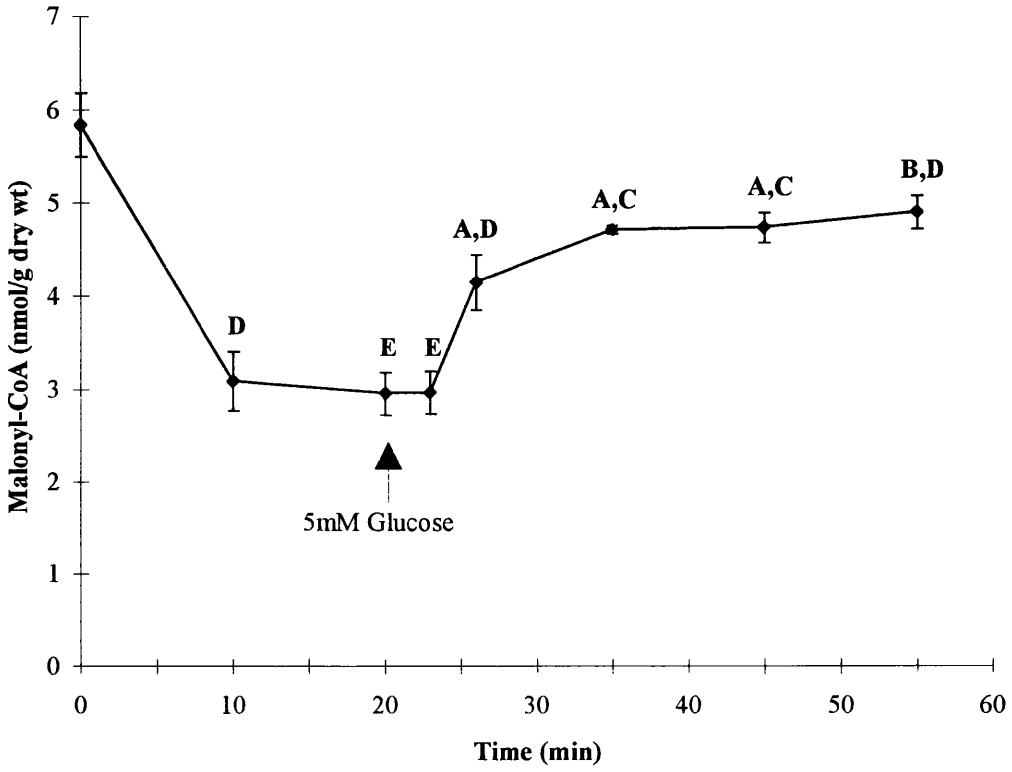
final three washes and incubation buffer. The result of this procedure is the isolation of myocytes which are depleted of glucose and presumably have some depletion of glycogen stores. Even in the absence of exogenous fatty acids, the energy demands of glucose depleted myocytes are entirely met by the oxidation of fatty acids,. This is due to the mobilisation of fatty acids from endogenous TAG stores present within the heart (Saddik & Lopaschuk, 1991). It is expected that malonyl-CoA levels would be low under such conditions so as to allow fatty acid entry into mitochondria and β-oxidation to proceed freely.

Glucose depleted myocytes were incubated at 37^0C with gentle shaking for 20 min in gassed KHB medium containing no glucose and 2% BSA. After 20 min incubation, glucose was rapidly reintroduced (5mM final concentration) and cells incubated for a further 35 min. Reactions were stopped by the addition of ice cold perchloric acid (6% (w/w) final concentration) at 0, 10, 20, 23, 25, 35, 45 and 55 min time points. Malonyl-CoA levels were determined as described in section 2.8.1 and values expressed as nmol/g dry weight of cells.

3.2.2.1 Effect of Glucose Removal on Malonyl-CoA Levels in Cardiac Myocytes

Incubation of myocytes for 10 min in glucose-free medium significantly reduced malonyl-CoA level by ~46% compared to time 0 min (fig 3.4). It is important to note that myocytes had been in a low/no glucose medium for up to 20 min prior to the start of the incubation. As a result, malonyl-CoA level would presumably have already started to decrease prior to the start of the incubation. Therefore, the percentage decrease in malonyl-CoA due to the removal of glucose was probably significantly higher than ~46%. The rate of malonyl-CoA disposal from myocytes was found to be relatively slow, decreasing from 5.8 nmol/g dry wt. to 3.1 nmol/g

Figure 3.4 Rate of formation of malonyl-CoA on re-addition of 5mM glucose to glucose depleted myocytes



Glucose depleted myocytes were incubated at 37^{0}C for 20 min with KHB medium containing no glucose and 2% BSA, and subsequently incubated for 35 min with KHB medium containing 5mM glucose and 2% BSA. Reactions were stopped at time points indicated by the addition of perchloric acid (6% final concentration). Values are means ± SEM of 5 independent preparations. **A**, **B** indicate $p<0.005$, <0.0005 respectively versus 23 min incubation. **C**, **D**, **E** indicate $p<0.025$, <0.005, <0.0005 respectively versus 0 min incubation (paired t-test).

dry wt. in the first 10 min of incubation. However, since glucose was not removed from the cells instantly, but was removed gradually by consecutive washes in glucose free KHB medium prior to incubation, it was not possible to determine the exact rate of malonyl-CoA disposal from cardiac myocytes from this experiment. Between 10 and 20 min, malonyl-CoA level did not fall any further and remained at a steady basal level of ~3.0 nmol/g dry wt. (fig 3.4).

The decrease in malonyl-CoA upon removal of glucose, represents either an increased malonyl-CoA utilisation/degradation, possibly by malonyl-CoA decarboxylase, or a decrease in ACC activity, which could be due to several effects, including reduced substrate availability for ACC and/or changes in ACC phosphorylation state.

It has been suggested that the cytosolic acetyl-CoA/CoA ratio is an important determinant of ACC activity in the heart (Saddik *et al*, 1993). In the absence of glucose, acetyl-CoA production via the PDH complex will be vastly reduced and the intramitochondrial acetyl-CoA/CoA ratio will be low. As a result, the cytosolic acetyl-CoA/CoA ratio will also be low since intramitochondrial acetyl-CoA transport to the cytosol, via the actions of carnitine acetyltransferase (CAT) and carnitine:acetylcarnitine translocase, will be reduced. This low level of cytosolic acetyl-CoA, combined with the fact that heart ACC has a very low affinity for acetyl-CoA (Bianchi *et al*, 1990; Saddik *et al*, 1993), means ACC activity is greatly reduced. The resulting decrease in malonyl-CoA level will reduce the inhibition on CPT 1 in the mitochondria and stimulate fatty acid oxidation to meet the energy demands of the heart. The decrease in the acetyl-CoA/CoA ratio also releases the suppression of 3-ketoacyl-CoA thiolase which is very sensitive to inhibition by acetyl-CoA (Olowe & Schulz, 1980), further stimulating fatty acid

oxidation. It has been proposed that acetyl-CoA from β-oxidation is directed mainly into the citric acid cycle as opposed to being available for transport to the cytosol (Abdel-aleem *et al*, 1996). This would keep the acetyl-CoA/CoA ratio low and prevent acetyl-CoA from reacting with ACC, thus keeping malonyl-CoA level low and fatty acid oxidation rates high. The fact that malonyl-CoA level did not fall to undetectable levels in the absence of glucose suggests that either some glycogen is still present and is being metabolised to provide acetyl-CoA for ACC or that some acetyl-CoA from β-oxidation is available to ACC. However, Lopaschuk & Gamble (1994) demonstrated that perfusion of hearts in the absence of glucose resulted in a diminution of malonyl-CoA to almost undetectable levels, suggesting that no acetyl-CoA from β-oxidation is available to ACC. Marked decreases in malonyl-CoA concentrations have been observed in hearts during exercise (Thampy, 1989; Bianchi *et al*, 1990). The increase in acetyl-CoA demand by the citric acid cycle during exercise, is thought to reduce the availability of acetyl-CoA for transport to the cytosol and reaction with ACC (Lopaschuk & Gamble, 1994). This further supports the hypothesis that the acetyl-CoA/CoA ratio and acetyl-CoA availability play a major role in regulation of ACC activity, malonyl-CoA level and fuel selection in the heart.

The major regulatory protein kinase active on ACC is AMPK (Carling *et al*, 1987, 1989; Davies *et al*, 1989). AMPK is stimulated dramatically by an increase in AMP level. During isolation and subsequent incubation in substrate free medium, glucose depleted myocytes may become slightly fuel starved, causing a rise in AMP level (Louis & Witters, 1992). This rise in AMP would stimulate AMPK, resulting in phosphorylation and inactivation of ACC (see section 1.2.3.3.2). The resulting decrease in malonyl-CoA level wouldfavourthe oxidation of fatty acids to maintain

cellular ATP levels. AMPK is thought to play an important role in the regulation of ACC phosphorylation and fuel selection in the heart, and is itself, ultimately regulated by fuel availability and rates of oxidation.

3.2.2.2 Effect of Re-addition of Glucose on Malonyl-CoA Levels in Cardiac Myocytes

Re-addition of glucose (5mM final concentration) increased malonyl-CoA content rapidly and significantly by ~61% from a basal level of ~3.0 nmol/g dry wt. to a plateau of ~4.8 nmol/g dry wt. within 15 min (fig 3.4). There was an initial lag of approximately 2 min before malonyl-CoA levels were seen to rise. This lag period is likely due to the time taken for glucose to be taken up by myocytes and for glucose oxidation to become stimulated. The intracellular disposition of glucose transporters upon isolation may also influence the rate at which glucose can be transported into the myocyte.

Upon re-addition of glucose malonyl-CoA reached half its maximum level ($K_{0.5}$) in approximately 1.8 min, indicating acute regulation of ACC and implies a direct effect of glucose on ACC.

The mechanism by which glucose stimulates the ACC dependent synthesis of malonyl-CoA in heart is complex. The observed rise in malonyl-CoA upon re-addition of glucose may be the result of increased substrate flux through ACC, allosteric activation of ACC by citrate, glucose-induced changes in ACC phosphorylation and/or changes in malonyl-CoA utilisation/degradation, possibly by malonyl-CoA decarboxylase.

The mechanism by which re-addition of glucose increases substrate flux through ACC is thought to be via increased transport of acetyl-CoA, produced by the PDH complex within the mitochondria, to the cytosol. Stimulation of PDH activity

upon re-addition of glucose results in an increase in intramitochondrial acetyl-CoA. Studies with isolated rat heart mitochondria have suggested that acetyl-CoA generated from carbohydrate is accessible to CAT, whereas that produced by β-oxidation is more readily available to the citric acid cycle (Lysiak *et al*, 1988; Abdel-aleem *et al*, 1996). It has been proposed that as intramitochondrial acetyl-CoA level increases, acetyl-CoA is transported to the cytosol via the actions of CAT and carnitine:acetylcarnitine translocase (Lopaschuk & Gamble, 1994). The resulting increase in cytosolic acetyl-CoA enhances ACC activity, increasing the level of malonyl-CoA and inhibiting fatty acid oxidation at the level of CPT 1. Evidence to support this proposed mechanism comes from the fact that treatment of hearts with DCA (inhibits PDH kinase) has been shown to be associated with an increase in malonyl-CoA level, accompanied by a reduction in myocardial fatty acid oxidation (Saddik *et al*, 1993).

Glucose and other metabolic substrates have been shown to acutely regulate ACC activity and phosphorylation state in intact hepatoma and islet cells (Louis & Witters, 1992). Since these changes in ACC activity are acute (occurring within minutes), they are distinct from the long term activation of ACC resulting from glucose induced changes in enzyme mass previously reported (Giffhorn & Katz, 1984; Spence *et al*, 1985). Glucose induced changes in ACC phosphorylation may involve changes in the activities of protein kinases or phosphatases active on ACC, or conformational changes in ACC due to allosteric interactions with metabolites. It has been suggested that AMPK plays a major role in the glucose stimulated increase in ACC activity and malonyl-CoA level (Louis & Witters, 1992). As stated earlier, isolation and incubation of myocytes in substrate free medium may cause a rise in the level of AMP resulting in activation of AMPK. Glucose

repletion would at least partially restore the depleted adenylate charge, lowering AMP levels. This decrease in AMP levels would result in a decrease in AMPK activity, reducing ACC phosphorylation, thereby increasing ACC activity. This hypothesized role for AMPK in this mechanism of ACC activation has been shown to be supported by the nature of the kinetic changes observed in AMPK activation of ACC (Louis & Witters, 1992).

The malonyl-CoA level remained at ~4.8 nmol/g dry wt. for the final 20 min of the incubation. This was significantly lower than malonyl-CoA level at the start of the experiment. Myocyte viability (based on morphological appearance) was assessed at the end of the experiment and was found not to be significantly less than at the start (data not shown). Therefore, a reduction in morphologically viable myocytes is not responsible for the decrease in malonyl-CoA levels observed from time 0 to 55 min. One possible explanation for this drop in malonyl-CoA content is that deprivation of glucose for up to 43 min (20 min during isolation and 23 min incubation) may cause metabolic changes in myocytes which are permanent, or which take longer than 35 min to revert to normal. For example, the time taken for AMPK to become inactivated and ACC to become dephosphorylated on addition of glucose may be greater than 35 min. In the absence of glucose myocytes rely entirely on the oxidation of fatty acids. It is possible that re-addition of glucose does not return the balance of fatty acid and glucose oxidation to normal in cells which have been so severely glucose depleted. Given that depletion of glycogen stores is responsible for stimulating the resynthesis of glycogen, it seems reasonable that upon re-addition of glucose a proportion of glucose will be diverted towards glycogen synthesis to re-establish the buffer that glycogen provides, thus reducing glycolysis and acetyl-CoA available for ACC. In order to

determine whether the effect of glucose depletion on malonyl-CoA levels in myocytes is permanent or not, the level of malonyl-CoA upon re-addition of glucose needs to be followed for longer than 35 min.

As stated earlier, malonyl-CoA levels could be increased due to decreased utilisation/degradation. As yet, very little is known about the mechanism(s) by which malonyl-CoA is decreased in the heart, although decarboxylation to acetyl-CoA by the mitochondrial enzyme, malonyl-CoA decarboxylase, has been suggested as a possibility (Awan & Saggerson, 1993). As to whether the removal and/or re-addition of glucose has any effect on any such mechanism is not known, although malonyl-CoA decarboxylase is known to be inhibited by acetyl-CoA (Kim & Kolattukudy, 1978). Therefore, an increase in intramitochondrial acetyl-CoA, as seen on re-addition of glucose, would inhibit malonyl-CoA decarboxylase allowing malonyl-CoA levels to rise. It is important to note that the changes in malonyl-CoA observed were rapid which has not been examined before.

In summary, when glucose is available and efficiently transported into myocytes, the activation of ACC promotes storage of glucose carbon as fatty acid and glycogen, while simultaneously inhibiting the mitochondrial entry and oxidation of fatty acids through allosteric inhibition of CPT 1 by the product of ACC, malonyl-CoA (McGarry & Foster, 1980). When glucose is unavailable, the acetyl-CoA supply to ACC, and hence ACC activity, is reduced due to the fact that acetyl-CoA produced from β-oxidation is thought to be directed into the citric acid cycle and not transported to the cytosol for synthesis of malonyl-CoA (Lysiak *et al*, 1988; Abdel-aleem *et al*, 1996). Thus, malonyl-CoA level falls allowing fatty acid oxidation to proceed at an increased rate to compensate for the lack of glucose. The rapid switching between fatty acids and glucose as fuels is important to the

heart especially during exercise. Rapid alterations in the level of malonyl-CoA provides a suitable mechanism of fuel selection. As well as regulation of malonyl-CoA synthesis, it is important to remember that regulation of malonyl-CoA disposal may also be of importance in the control of fuel selection of the heart.

3.2.3 Conclusions

When glucose and exogenous fatty acids are unavailable, lipolysis of endogenous TAG is responsible for maintaining the energy demands of the myocyte. Under these conditions it is essential that fatty acid transport into mitochondria for oxidation be uninhibited. Therefore, malonyl-CoA levels need to be low under these conditions so that mitochondrial CPT 1 is uninhibited and fatty acid oxidation is able to proceed.

Removal of glucose from cardiac myocytes decreases malonyl-CoA level dramatically. This decrease in cardiac malonyl-CoA level is due to reduced ACC activity and possibly increased malonyl-CoA disposal. ACC activity is strongly dependent on substrate supply due to the fact that the heart isoform of ACC has a low affinity for its substrate, acetyl-CoA. Removal of glucose reduces PDH activity which in turn reduces the supply of acetyl-CoA to ACC in the cytosol. Acetyl-CoA produced by the PDH complex is thought to be transported from the mitochondria to the cytosol by the actions of CAT and carnitine:acetylcarnitine translocase. Whereas, acetyl-CoA produced from β-oxidation is thought to be directed into the citric acid cycle, thereby preventing acetyl-CoA transport to the cytosol for synthesis of malonyl-CoA.

AMPK is responsible for the phosphorylation and inactivation of ACC. In the absence of glucose and exogenous fatty acid, glucose depleted myocytes would likely become fuel starved, resulting in a rise in AMP level, activation of AMPK,

and inactivation of ACC. The combination of reduced substrate supply and inhibition due to phosphorylation produces a dramatic decrease in cardiac malonyl-CoA level.

Re-addition of glucose rapidly increases malonyl-CoA level within a few minutes. This is thought to be due, in part, to increased substrate supply to ACC due to increased acetyl-CoA production (by the PDH complex) and transport to the cytosol via CAT and carnitine:acetylcarnitine translocase. Re-addition of glucose also reduces AMP levels such that AMPK is no longer activated and ACC becomes dephosphorylated and re-activated. Malonyl-CoA level did not return to normal within 35 minutes of re-addition of glucose which might indicate that the level of AMP, and hence AMPK activity, are not completely returned to normal after such a period of fuel starvation.

Malonyl-CoA disposal, possibly by the mitochondrial enzyme, malonyl-CoA decarboxylase, may also have an effect on the rate of glucose stimulated malonyl-CoA synthesis, as well as the rate of malonyl-CoA disposal in the absence of glucose. Malonyl-CoA decarboxylase is capable of degrading malonyl-CoA, but little is known about its regulation or its role in regulating myocardial malonyl-CoA level. The fact that the rate of malonyl-CoA re-synthesis is faster than the rate of disposal, might suggest that the level of malonyl-CoA is regulated primarily by changes in its rate of synthesis.

3.3 Disposal of Malonyl-CoA in Cardiac Tissue

In lipogenic tissues such as liver, adipose tissue and mammary gland, cytosolic malonyl-CoA is utilised for the synthesis of fatty acids. Heart muscle is not regarded as a lipogenic tissue. However, an isoform of ACC that is distinct from the liver enzyme has been isolated from rat heart (Thampy, 1989; Bianchi *et al*, 1990) and malonyl-CoA has been detected in rat heart tissue (McGarry *et al*, 1983). As well as a distinct isoform of ACC, cardiac tissue also possesses a distinct isoform of mitochondrial CPT 1 which is highly sensitive to inhibition by malonyl-CoA (as much as 50 times more sensitive than the liver isoform). Heart malonyl-CoA levels have been shown to range from approximately 3 to 10μM under various physiological conditions (McGarry *et al*, 1983; Awan & Saggerson, 1993; Lopaschuk *et al*, 1994b). These are at least one order of magnitude in excess of those that inhibit heart mitochondrial CPT 1 (Saggerson & Carpenter, 1981; Edwards *et al*, 1985; Saggerson, 1986), which would result in cardiac fatty acid oxidation being almost completely inhibited. It is clear that CPT 1 is not inhibited to this extent under normal conditions, as β-oxidation of fatty acids in mitochondria provides 60-70% of the energy requirements of the myocardium. Presumably, most cellular malonyl-CoA in heart is unavailable for interaction with CPT 1 and must therefore be bound to cell components other than CPT 1 or be compartmentalised within the cell, away from CPT 1.

3.3.1 The Fatty Acid chain Elongation (FACE) System

It has been suggested that the condensing enzyme of the fatty acid chain elongation (FACE) system, may provide a high-abundance, low affinity "sink" for the

binding/sequestration of malonyl-CoA. This could act to bring the free levels of malonyl-CoA in the cell down to those that would not totally inhibit CPT 1 under all conceivable conditions (Awan & Saggerson, 1993). A considerable quantity of low affinity (K_D~30μM) binding sites for malonyl-CoA have been reported in crude mitochondrial fractions from rat heart (Bird & Saggerson, 1984), which may represent the condensing enzyme of the FACE system (Awan & Saggerson, 1993). The FACE system has been extensively studied in both liver and brain, and is present in a variety of other tissues (see section 1.2.3.3.5). Fatty acid elongation occurs primarily in the endoplasmic reticulum of these tissues by the actions of 4 enzymes, a condensing enzyme, a β-ketoreductase, a dehydrase and an enoyl reductase. If present in rat heart, it would be reasonable to expect the enzymes of the FACE system to be localised in the sarcoplasmic reticulum (SR).

3.3.2 Comparison of FACE Activities in Subcellular Fractions of Rat Heart, Liver and Brain Using Radiochemical and Spectrophotometric Assays

The rate limiting reaction of fatty acid chain elongation (i.e. the condensation reaction) was assayed radiochemically and spectrophotometrically as described in sections 2.12.2.1 and 2.12.2.2 respectively, in subcellular fractions of rat heart. FACE activity was also assayed in liver and brain microsomes for comparison. Table 3.2 shows the measurements of FACE activity obtained using both assay methods for the various tissue subcellular fractions.

3.3.2.1 FACE Spectrophotometric Assay

As shown in table 3.2, no FACE activity was detected in any subcellular fraction of rat heart when measured spectrophotometrically. Significant FACE activity was demonstrated in rat liver and brain microsomes (0.44 ± 0.01 & 0.43 nmol/min/mg respectively). These values of malonyl-CoA decarboxylase activity

Table 3.2 Fatty acyl-CoA elongation (FACE) activity in subcellular fractions of liver, brain and heart, measured using spectrophotometric and radiochemical assays

Tissue Fraction	Spectrophotometric Assay (nmol/min/mg) (mitochondrial protein)	Radiochemical Assay (nmol/min/mg) (mitochondrial protein)	Revised Radiochemical Assay (nmol/min/mg) (mitochondrial protein)
Heart Sarcoplasmic Reticulum	n.d.	0.52 ± 0.05 (n=5)	n.d.
Heart Mitochondria	n.d.	0.33 ± 0.06 (n=5)	n.d.
Heart Cytosol	n.d.	0.31 ± 0.10 (n=5)	n.d.
Liver Microsomes	0.44 ± 0.01 (n=4)	0.58 ± 0.02 (n=4)	0.39 ± 0.09 (n=4)
Brain Microsomes	0.43 (n=1)	0.83 (n=1)	0.36 ± 0.12 (n=3)

Fatty acyl-CoA elongation activity in subcellular fractions of heart, liver and brain were determined by radiochemical and spectrophotometric assays (at 37^0C) as described in sections 2.12.2.1 and 2.12.2.2 respectively. Values are expressed as means ± SEM from 4-5 separate preparations except for brain microsomes where n=1. n.d. indicates that FACE activity was not detected.

for rat liver and brain microsomes are comparable to those previously reported by Cinti *et al* (1992) (0.44 and 0.33 nmol/min/mg respectively).

3.3.2.2 FACE Radiochemical Assay

A significant amount of FACE activity was demonstrated in all subcellular fractions of rat heart when measured using the radiochemical assay (table 3.2). The greatest specific activity of FACE (0.52 ± 0.05 nmol/min/mg) was measured in the SR fraction. This is where it was expected that the enzymes of the FACE system would be located if present in heart. However, appreciable FACE activity was also demonstrated in the mitochondrial and cytosolic fractions of heart (0.33 ± 0.06 and 0.31 ± 0.10 nmol/min/mg respectively).

The presence of FACE activity in the mitochondrial fraction could be explained by the fact that sarcoplasmic reticulum can become entangled around mitochondria during fractionation therefore contaminating the mitochondrial fraction with SR. However, the subcellular fractionation procedure used to isolate subcellular fractions of rat heart was designed to minimize contamination of mitochondria with SR. A small amount of contamination between fractions is unavoidable, particularly as heart tissue is very tough and needs thorough homogenization before fractionating. However, this would not account for the significant amount of FACE activity measured in the mitochondrial fraction. Contamination of the cytosolic fraction with SR, or any membrane particles, is extremely low and would certainly not account for the appreciable FACE activity detected in the cytosolic fraction when measured radiochemically. These results suggest that the radiochemical assay is not specifically measuring FACE activity, but is measuring some other enzyme activity as well, or instead of, FACE activity.

3.3.2.3 Revision of FACE Radiochemical Assay

On close examination of the radiochemical assay it became apparent that the presence of the mitochondrial matrix enzyme, malonyl-CoA decarboxylase, within samples, is capable of interfering with the FACE assay. Malonyl-CoA decarboxylase interferes with the FACE assay by converting [2-^{14}C]malonyl-CoA to [^{14}C]acetyl-CoA, which upon saponification is converted into [^{14}C]acetic acid. [^{14}C]Acetic acid is petroleum spirit extractable and therefore contributes to the radioactivity measured upon scintillation counting giving a false measurement of FACE activity. This problem was solved by thoroughly drying the petroleum spirit extracts before scintillation counting, thus evaporating any [^{14}C]acetic acid present in the sample and leaving only the radiolabelled true elongation products behind for scintillation counting. When the revised radiochemical assay was used and petroleum spirit extracts were thoroughly dried, no FACE activity was detected in any subcellular fraction of rat heart (table 3.2).

Significant FACE activity was detected in liver and brain microsomes (0.58 ± 0.02 and 0.83 nmol/min/mg respectively) when measured using the original radiochemical assay (table 3.2). These values are somewhat higher than those previously reported by Cinti *et al* (1992), (0.44 and 0.33 nmol/min/mg respectively) and than those obtained using the spectrophotometric assay (0.44 and 0.43 nmol/min/mg respectively). Again, this was thought to be due to the presence of malonyl-CoA decarboxylase in the samples giving an overestimate of FACE activity. When measured using the revised radiochemical assay, the FACE activity detected in liver and brain microsomes was 0.39 ± 0.09 and 0.36 ± 0.12 respectively. These values are much more comparable to those obtained using the spectrophotometric assay and to those obtained by Cinti *et al* (1992).

3.3.3 Malonyl-CoA Decarboxylase can be Measured Using the Non-Revised FACE Radiochemical Assay

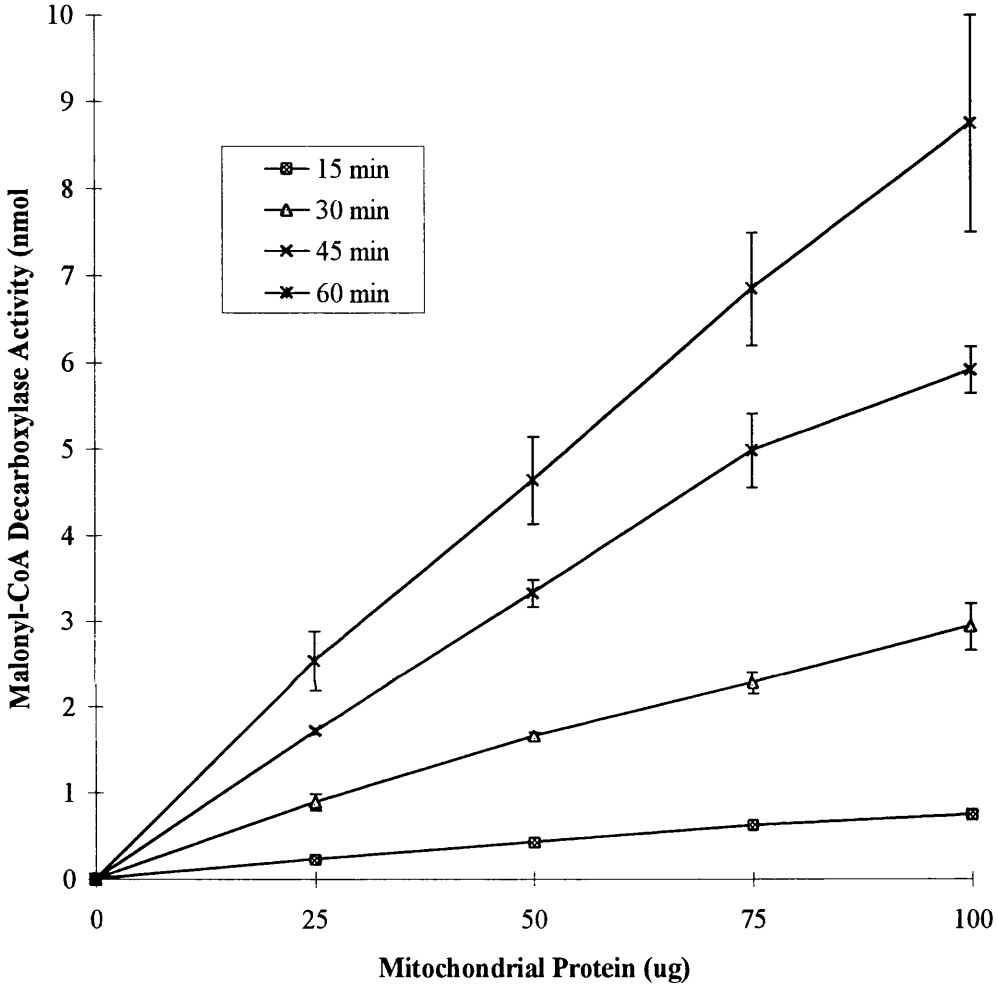
Figure 3.5 demonstrates that the non-revised FACE radiochemical assay can measure malonyl-CoA decarboxylase in freeze/thawed rat heart mitochondria. A reaction time of 60 min produced an almost linear curve with varying samples of 0-100μg mitochondrial protein. However, the large error bars indicate that this is not a reliable assay for measuring malonyl-CoA decarboxylase but certainly demonstrates the ability of the radiochemical FACE assay to detect malonyl-CoA decarboxylase if the petroleum spirit extracts are not evaporated prior to scintillation counting.

3.3.4 Attempted Purification of Rat Liver FACE

It was expected that an appreciable quantity of FACE activity would be detected in subcellular fractions of rat heart. However, as shown by the above results, FACE activity was not detected in any subcellular fraction of heart. Originally the purification of FACE was attempted in both rat liver microsomes and rat heart mitochondria (thought to contain the bulk of the FACE activity) using the radiochemical assay to detect FACE activity. Once the problem with the radiochemical assay was discovered, the spectrophotometric assay was used for all subsequent assays and it became apparent that rat heart did not contain any detectable FACE activity.

Purification of FACE from rat liver microsomes was attempted as described in section 2.11 using the four detergents, cholate, deoxycholate, triton-X 100 and Tween 20 at varying concentrations, at and around their critical micelle concentration.

Figure 3.5 Malonyl-CoA decarboxylase measured using the non-revised FACE radiochemical assay



Freeze thawed rat heart mitochondria were assayed using the non-revised FACE radiochemical assay. Sample size (0-100μg) and reaction time (0-60 min) were varied to determine an optimum assay procedure. Petroleum spirit extracts were not dried before scintillation counting. Values are expressed as means ± SEM of 3 independent preparations.

Under no conditions was FACE successfully removed from the microsomal membranes in an active state. Increasing detergent concentrations resulted in an increase in protein concentration in the supernatant due to solublization of microsomal proteins. This was accompanied by a decrease in FACE activity in the microsomal membrane pellet due either to solublization of FACE from the microsomal membrane or inactivation of FACE by the detergent. Unfortunately no FACE activity was detected in the supernatant at any concentration of detergent which resulted in solublization of microsomal protein. Therefore, it is unclear if the enzymes of the FACE system are solublized from the microsomal membrane and inactivated by the detergent or not solublized from the membrane and inactivated by the detergent. The spectrophotometric assay used to detect FACE activity measures the rate limiting condensation reaction. Therefore, it is the sensitivity of the condensing enzyme of the FACE system to inactivation by detergents which makes purification of FACE extremely difficult. Several groups have attempted to purify FACE from rat liver membranes but have been unsuccessful due to its sensitivity to inactivation by detergent.

3.3.5 Conclusions

Contamination of subcellular fractions of rat heart, brain and liver with the mitochondrial matrix enzyme malonyl-CoA decarboxylase, interferes with the radiochemical FACE assay. Evaporation of petroleum spirit extracts before scintillation counting prevents false measurements of FACE due to the activity of malonyl-CoA decarboxylase in samples.

FACE activity was undetectable in any subcellular fraction of rat heart using either the spectrophotometric or revised radiochemical assay. Therefore, it appears that the enzymes of the FACE system are not present in heart, or if they are, enzyme

levels are lower than the detection range of the assay. In either case it is highly unlikely that the condensing enzyme of the FACE system can act as a high abundance low affinity sink for malonyl-CoA. Hence the question still remains as to how CPT 1 activity is maintained when the cellular concentration of malonyl-CoA is such that it should be totally inhibited.

It has been suggested that the cytosolic enzyme, fatty acid synthase, may provide a possible route for the disposal of malonyl-CoA. However, Awan & Saggerson (1993), have shown there to be no "true" fatty acid synthase detectable in 100,000 g_{av} supernatants from isolated myocytes.

Zammit's group have begun research into the possibility of a malonyl-CoA binding protein which could act to lower the free concentration of malonyl-CoA in the heart. As yet no data has been published to support this hypothesis, but it is still the most plausible hypothesis to explain this intriguing question.

3.3.6 Malonyl-CoA Decarboxylase

The fact that acute interventions such as substrate deprivation or insulin treatment can cause dramatic yet transient changes in myocardial malonyl-CoA levels (Saddik *et al*, 1993), suggests there must be a mechanism by which malonyl-CoA can be degraded in the heart. One possibility is decarboxylation by malonyl-CoA decarboxylase, a mitochondrial enzyme which is known to be active in a number of tissues including heart (Kim & Kolattukudy, 1978).

Malonyl-CoA decarboxylase catalyses the decarboxylation of malonyl-CoA to form acetyl-CoA and has been identified in a number of tissues including liver, kidney, heart, mammary gland and the uropygial gland of birds (Kim & Kolattukudy, 1978). In the uropygial gland of birds, large amounts of malonyl-CoA decarboxylase in the cytosol ensures that methylmalonyl-CoA and not

malonyl-CoA, is the only chain elongation substrate available to cytosolic FAS for the formation of multimethyl branched fatty acids (Buckner & Kolattukudy, 1975a; 1975b; 1976). In most mammalian tissues malonyl-CoA decarboxylase is localised in the mitochondrial matrix and protects key mitochondrial enzymes such as, methylmalonyl-CoA mutase and pyruvate carboxylase from inhibition by malonyl-CoA (Kim & Kolattukudy, 1978; Jang *et al*, 1989). In the mitochondria, malonyl-CoA is generated by propionyl-CoA carboxylase. Since mitochondria can neither metabolize malonyl-CoA nor transport it to the cytosol, only decarboxylation prevents accumulation of inhibitory levels of malonyl-CoA in the mitochondria (Kim & Kolattukudy, 1978).

Rat heart contains an appreciable quantity of malonyl-CoA decarboxylase activity (about one third that of rat liver) (Scholte, 1969), which is more than adequate to decarboxylate the amount of malonyl-CoA which might be generated in the mitochondria by propionyl-CoA carboxylase, therefore suggesting another role for this enzyme in the heart.

The precise intracellular localisation of malonyl-CoA decarboxylase in heart has yet to be determined although it is generally accepted to be mitochondrial. Should a proportion of this mitochondrial malonyl-CoA decarboxylase be located external to the inner mitochondrial membrane, then it would capable of decarboxylating and regulating cytosolic malonyl-CoA. The possibility of a truly cytosolic malonyl-CoA decarboxylase in heart has also not been ruled out as a possible mechanism for malonyl-CoA degradation. Hence, changes in malonyl-CoA decarboxylase activity may be partly responsible for changes in cardiac malonyl-CoA concentrations. Little is known about the regulation of malonyl-CoA decarboxylase *in vivo*. The purified enzyme is known to be severely inhibited by

thiol-directed agents such as *p*-hydroxymercuribenzoate and *N*-ethylameimide, and is also inhibited by the CoA esters, acetyl-CoA, propionyl-CoA and methylmalonyl-CoA (Kim & Kolattukudy, 1978).

3.3.6.1 Intracellular Localisation of Malonyl-CoA Decarboxylase in Rat Heart

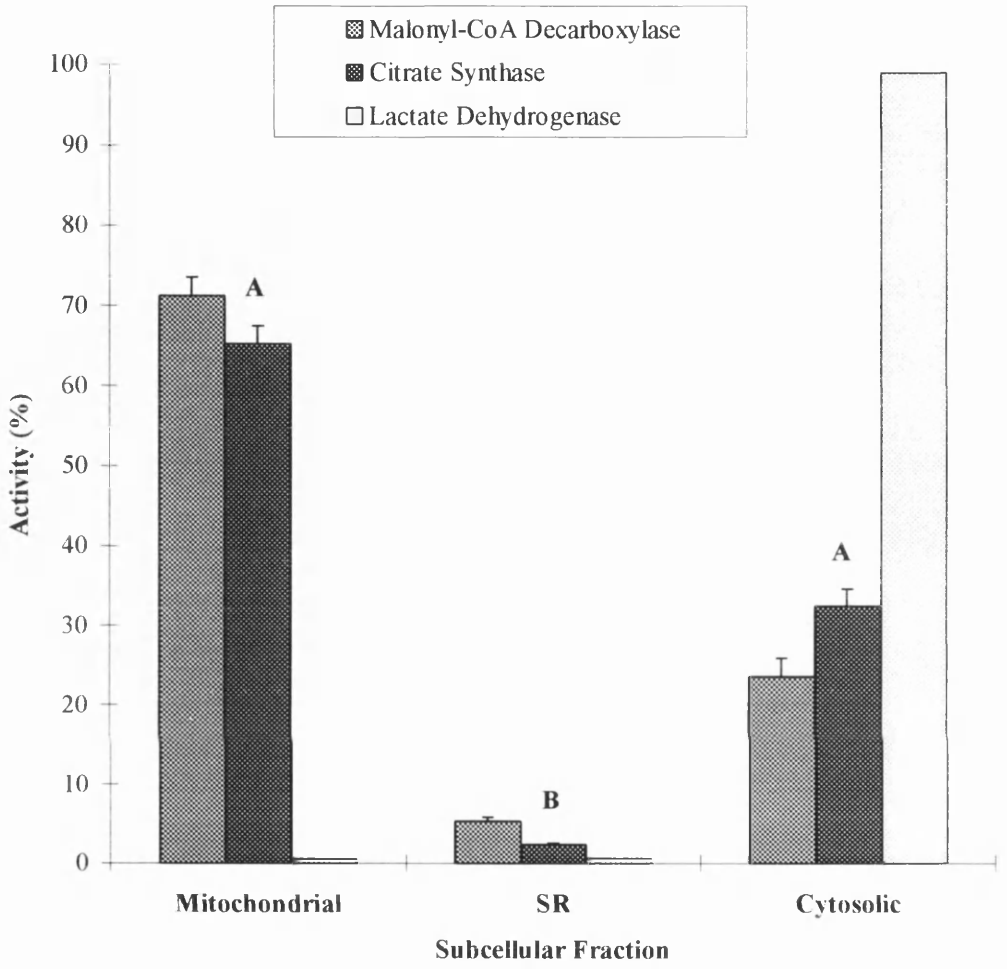
The exact intracellular localisation of malonyl-CoA decarboxylase in heart is unclear. Previous reports have demonstrated that hepatic malonyl-CoA decarboxylase is localised exclusively in the mitochondrial matrix enzyme (Scholte, 1969).

The distribution of citrate synthase (mitochondrial matrix enzyme), lactate dehydrogenase (LDH) (cytosolic enzyme) and malonyl-CoA decarboxylase activities were determined in subcellular fractions of rat heart, in an attempt to further elucidate the intracellular localisation of malonyl-CoA decarboxylase.

Figure 3.6 shows the percentage activities of citrate synthase, LDH and malonyl-CoA decarboxylase in subcellular fractions of rat heart. As expected for the cytosolic enzyme LDH, ~99% of LDH activity was detected in the cytosolic fraction with less than 1% being located in the mitochondrial and SR fractions. This demonstrates that there is practically no contamination of the mitochondrial and SR fractions with cytosolic protein.

As shown in figure 3.6, ~32% of the mitochondrial matrix enzyme, citrate synthase, was detected in the cytosolic fraction, indicating that a significant quantity of mitochondria were broken during subcellular fractionation resulting in leakage of citrate synthase into the cytosol. The percentage of citrate synthase recovered in the cytosol is a representation of the percentage of mitochondria disrupted during isolation. Heart tissue is very tough and the technique used to

Figure 3.6 Intracellular localisation of malonyl-CoA decarboxylase, citrate synthase and lactate dehydrogenase in rat heart



Subcellular fractions of rat heart were prepared as described in section 2.9.2. Samples were subjected to 10 x 5 sec bursts of sonication at 4^0C prior to being assayed. Enzyme activities were measured as described in sections 2.12.3, 2.12.4 and 2.12.5. Percentage of total activity was calculated using the sum of mitochondrial, sarcoplasmic reticulum (SR) and cytosolic activity as the total enzyme activity. Values are means ± SEM of 4 independent preparations. **A**, **B** indicate $p<0.05$ and <0.005 respectively versus malonyl-CoA decarboxylase (paired t-test).

homogenize it is quite severe. The disruption of ~32% of heart mitochondria during isolation is low for subcellular fractionation of heart, but would be unacceptably high for fractionation of rat liver which is more easily homogenized.

A very small percentage (~2%) of citrate synthase activity was measured in the SR fraction while the remaining ~65% was measured in the mitochondrial fraction (fig 3.6) where the majority of activity should be located. The ~2% citrate synthase activity measured in the SR is probably the result of it being captured in SR vesicles which re-form after the SR is disrupted. The citrate synthase within these vesicles is released, and therefore detected, when samples are sonicated prior to being assayed.

As shown in figure 3.6, ~71% of malonyl-CoA decarboxylase activity was measured in the mitochondrial fraction, while ~5% was measured in the SR fraction and ~24% was measured in the cytosolic fraction. This distribution of malonyl-CoA decarboxylase follows that of citrate synthase very closely, although not exactly, suggesting it to be a mitochondrial enzyme. However, this does not prove that malonyl-CoA decarboxylase is exclusively localised in the mitochondrial matrix. A slightly greater (but statistically significant) percentage of malonyl-CoA decarboxylase was recovered in the mitochondrial and SR fractions compared with citrate synthase. This suggests that malonyl-CoA decarboxylase may be slightly "sticky", resulting in it adhering to SR membranes when it is released from broken mitochondria upon fractionation. Another possible explanation is that a proportion of malonyl-CoA decarboxylase is loosely bound to the inner mitochondrial membrane. Scholte (1969), has demonstrated that the enzymes present in rat liver mitochondria stripped of their outer membrane, differ with respect to the tightness with which they are bound to the inner membrane in the presence of digitonin. In

descending order come cytochrome c oxidase, aspartate aminotransferase, malate dehydrogenase (MDH), malonyl-CoA decarboxylase = glutamate dehydrogenase, and propionyl-CoA carboxylase.

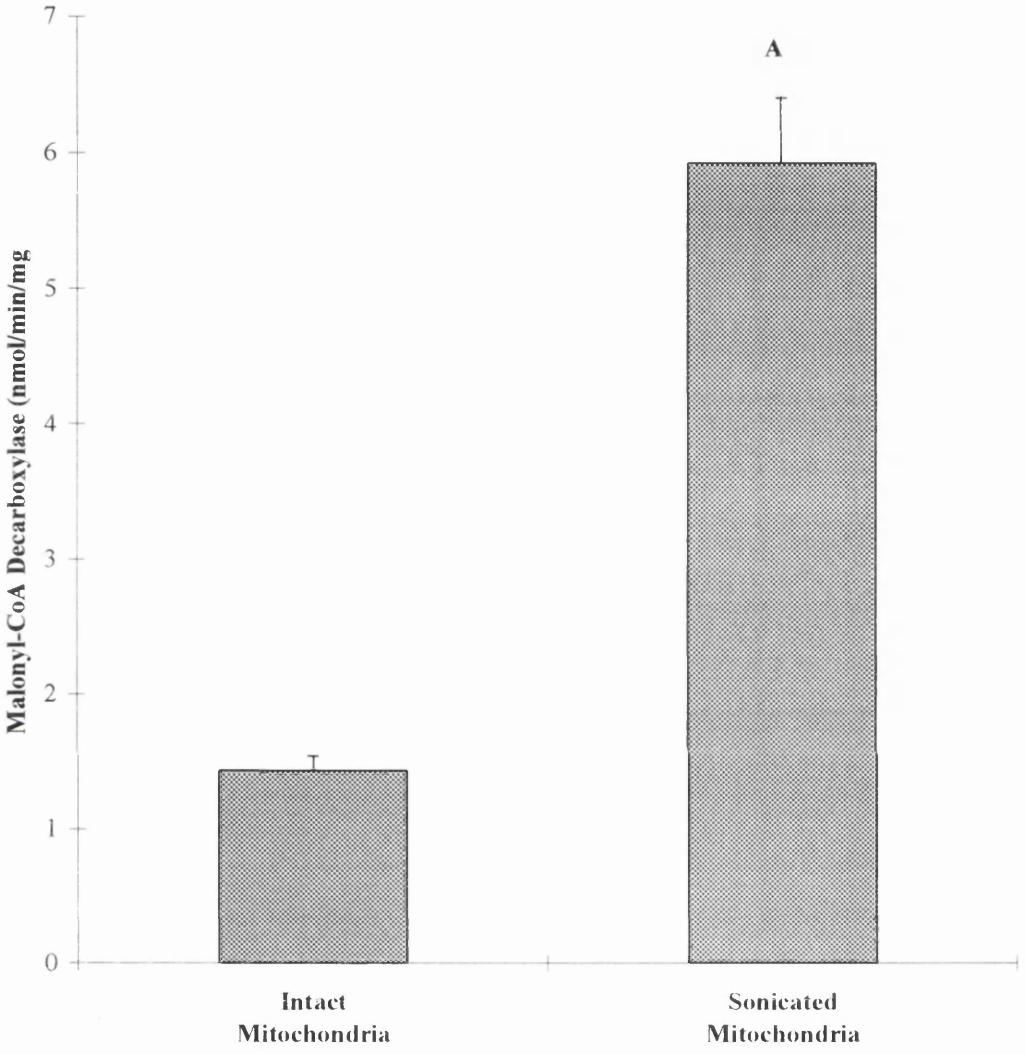
The percentage of malonyl-CoA decarboxylase activity recovered in the cytosolic fraction is slightly less (although statistically significant) than that of citrate synthase (fig 3.6). This demonstrates that there is no truly cytosolic malonyl-CoA decarboxylase in rat heart which could act to alter cytosolic malonyl-CoA levels.

3.3.6.2 Effect of Sonication on Malonyl-CoA Decarboxylase Activity in Intact Rat Heart Mitochondria

Intact rat heart mitochondria were isolated as described in section 2.9.3 and subjected to 10 x 5 sec bursts of sonication using a probe sonicator. Malonyl-CoA decarboxylase activity was assayed as described in section 2.12.3 before and after sonication.

As shown in figure 3.7, sonication of intact rat heart mitochondria significantly increased malonyl-CoA decarboxylase activity by a factor of 4.14 from 1.43 ± 0.11 to 5.93 ± 0.48 nmol/min/mg mitochondrial protein. This indicates that malonyl-CoA decarboxylase activity in intact rat heart mitochondria is latent, in that it is greatly increased by sonication. These values are similar to those measured in rat liver mitochondria by Scholte (1969), who obtained values before and after sonication of 1.28 and 9.7 mU/mg respectively (stimulation factor 7.5). The latency of malonyl-CoA decarboxylase in rat heart mitochondria suggest it to be localised mainly within the matrix or loosely bound to the matrix side of the inner mitochondrial membrane. The small amount of malonyl-CoA decarboxylase activity detected when intact mitochondria were assayed before sonication is most likely due to a small amount of enzyme leakage from damaged or broken

Figure 3.7 Effect of sonication on malonyl-CoA decarboxylase activity in rat heart mitochondria



Intact rat heart mitochondria were isolated as described in section 2.9.3 and subjected to 10 x 5 sec bursts of sonication at 4^0C. Malonyl-CoA decarboxylase activity was measured as described in section 2.12.3, before and after sonication. Values are expressed as means ± SEM of 7 independent preparations. **A** indicates $p<0.0005$ versus intact mitochondria (paired t-test).

mitochondria, but may also represent the presence of some malonyl-CoA decarboxylase activity external to the inner mitochondrial membrane.

3.3.6.3 Percentage Malonyl-CoA Decarboxylase, Citrate Synthase and Malate Dehydrogenase Activity Overt to the Mitochondrial Inner Membrane

Intact rat heart mitochondria were isolated and subjected to 10 x 5 sec bursts of sonication. Malonyl-CoA decarboxylase, citrate synthase and MDH activities were measured before and after sonication. The percentage activity overt to the mitochondrial inner membrane was calculated using the equation below:

% Overt Activity = [Intact activity / Sonicated activity] x 100

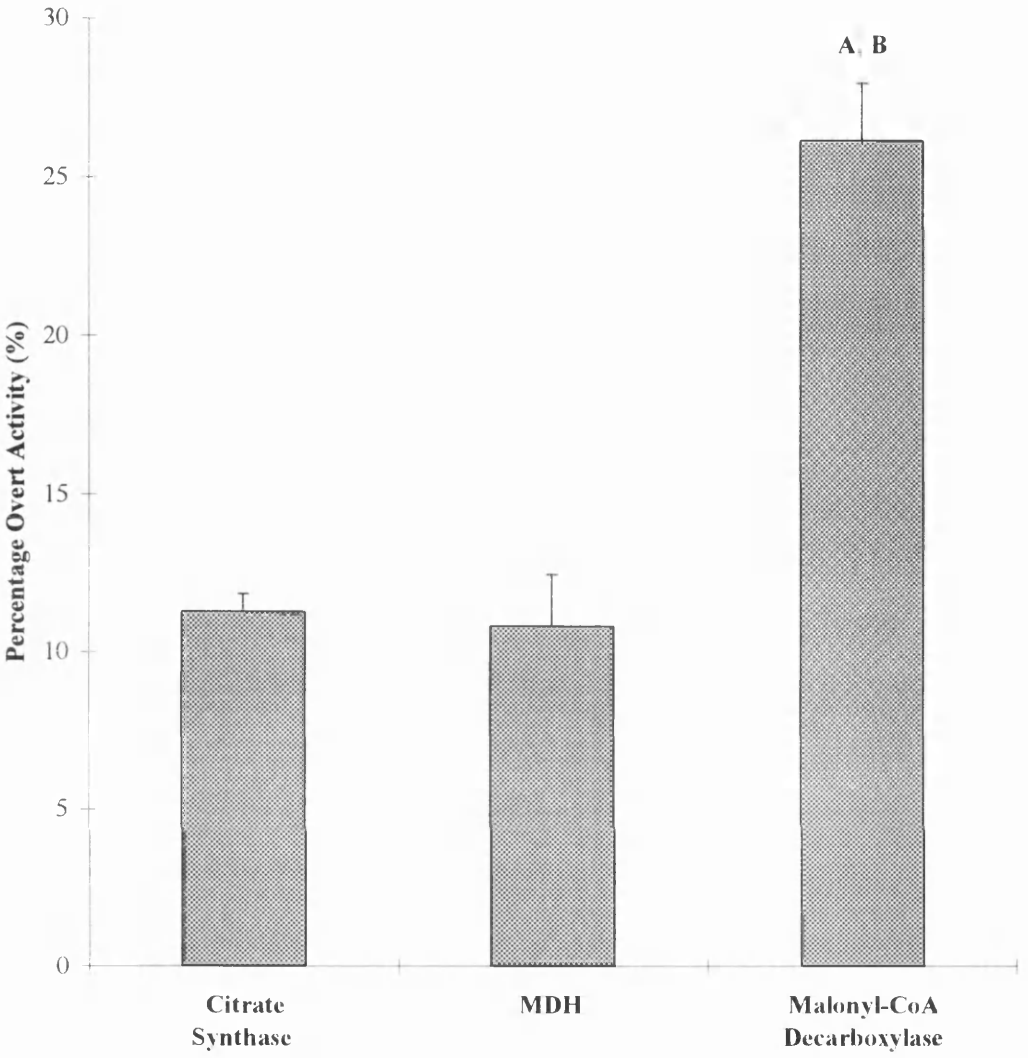
where intact activity = true overt activity + activity due to leakage

and sonicated activity = total activity.

As shown by figure 3.8, the percentage overt activity of the mitochondrial matrix enzymes citrate synthase and MDH, were 11.3 ± 0.6% and 10.8 ± 1.6% respectively. This overt activity is possibly due to the leakage of these mitochondrial matrix enzymes from damaged mitochondria and if malonyl-CoA decarboxylase were exclusively localised in the mitochondrial matrix, its percentage overt activity would be the same as that of citrate synthase and MDH. However, the percentage overt activity of malonyl-CoA decarboxylase was approximately 2.4 times greater than that of citrate synthase and MDH (fig 3.8). This might suggest the presence of a small amount of malonyl-CoA decarboxylase activity which is truly overt.

In principle, MDH and citrate synthase could be assayed in "intact" mitochondria if some mitochondria had burst after their isolation and leaked enzymes into the surrounding medium. It is possible that malonyl-CoA decarboxylase leaks out more readily than MDH and citrate synthase, but is unlikely. It is also possible that

Figure 3.8 Percentage enzyme activities overt to mitochondrial matrix



Intact rat heart mitochondria were isolated as described in section 2.9.3 and subjected to 10 x 5 sec bursts of sonication at 4^0C. Enzyme activities were measured before and after sonication. Percentage activity overt to mitochondrial inner membrane was calculated as intact/sonicated activity x 100. Values are expressed as means ± SEM of 2-7 independent preparations (malonyl-CoA decarboxylase and citrate synthase n=7, MDH n=2). **A**, **B**, indicate $p<0.001$ and <0.0005 respectively versus MDH (unpaired t-test) and citrate synthase (paired t-test) respectively.

some mitochondria may have become slightly degraded so that they admitted small molecules (such as the substrates for MDH, citrate synthase or malonyl-CoA decarboxylase) without the enzymes themselves being released. If this was the case then malonyl-CoA decarboxylase would have to be more readily accessed by its substrate in these permeable mitochondria than MDH or citrate synthase. One cannot rule out these possibilities in addition to the possibility that some of the mitochondrial malonyl-CoA decarboxylase is truly overt.

3.3.7 Effect of Various Substrates on the Oxygen Consumption of Intact Rat Heart Mitochondria

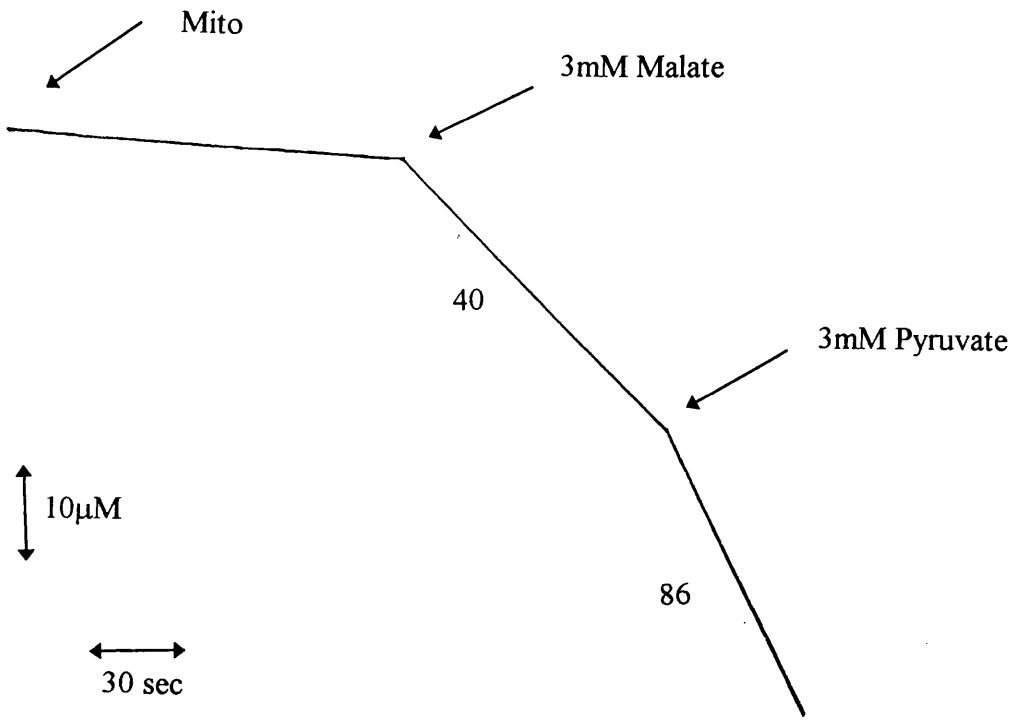
In theory, the existence of malonyl-CoA decarboxylase on the cytosolic face of the inner mitochondrial membrane should allow cytosolic malonyl-CoA to be decarboxylated to acetyl-CoA outside the mitochondria. There are a number of possible fates for acetyl-CoA produced in this way. One possibility is that it is re-carboxylated to malonyl-CoA by ACC in the cytosol, thus creating a futile cycle. If malonyl-CoA were being constantly synthesised and degraded in this way, it would provide a mechanism by which the level of malonyl-CoA could be rapidly altered, by changes in either ACC or malonyl-CoA decarboxylase activity.

Another possibility is that acetyl-CoA produced from decarboxylation of malonyl-CoA is transported into the mitochondria, by the actions of CAT and carnitine:acetylcarnitine translocase, for oxidation by the citric acid cycle. This theory would imply that acetyl-CoA produced by malonyl-CoA decarboxylase at the inner mitochondrial membrane would be in closer proximity to CAT than to ACC. If this hypothesis is correct then mitochondria would be able to utilise malonyl-CoA as a substrate for respiration. It is also possible that malonyl-CoA may be taken up by the mitochondria by an, as yet unknown, transport system.

Once inside the mitochondria, malonyl-CoA would be rapidly decarboxylated by malonyl-CoA decarboxylase to produce acetyl-CoA. This in turn would be oxidised by the enzymes of the citric acid cycle. In either case, an increase in oxygen consumption by intact mitochondria, upon addition of malonyl-CoA, would demonstrate its transport into, and oxidation by, the mitochondria via one of these suggested routes.

Intact rat heart mitochondria were isolated as described in section 2.9.3 and the rate of oxygen consumption measured using an oxygen electrode as described in section 2.10. All incubations were carried out in a 3ml reaction volume at 37^0C in incubation medium containing 10mM Tris-HCl buffer (pH 7.3), 120mM KCl, 0.5mM EGTA, 1mM KH_2PO_4, 1mM ADP and 1.3mg/ml BSA. The presence of ADP in the incubation medium is essential for oxidative metabolism to occur. The oxygen consumption due to mitochondria in incubation medium alone was found to be negligible (fig 3.9), and so the rates of oxygen consumption measured are actually increases in the rate of oxygen consumption due to the substrate included. Figure 3.9 shows a typical oxygen electrode trace obtained upon addition of 3mM malate and subsequent addition of 3mM pyruvate to isolated intact rat heart mitochondria. The average rate of oxygen consumption from 4 such experiments was calculated to be 88 ± 2 nmol/min/mg mitochondrial protein (table 3.3). Malate is required by the mitochondria in order to maintain levels of citric acid cycle intermediates. Pyruvate is permeable to the inner mitochondrial membrane and hence enters the mitochondria where it reacts with the PDH complex to form acetyl-CoA. The acetyl-CoA produced by the PDH complex in turn enters the citric acid cycle where it is oxidised resulting in an increase in oxygen

Figure 3.9 A typical trace from an oxygen electrode experiment



Intact rat heart mitochondria were isolated as described in section 2.9.3 and incubated at 37^0C in incubation medium containing 10mM Tris-HCl buffer (pH 7.3), 120mM KCl, 0.5mM EGTA, 1mM KH_2PO_4, 1mM ADP and 1.3mg/ml BSA with additions of 3mM malate and 3mM pyruvate. The oxygen consumption was measured using an oxygen electrode as described in section 2.10. Numerals indicate oxygen consumption in nmol/min/mg protein.

Table 3.3 Effect of various substrates on the oxygen consumption rate of isolated rat heart mitochondria

Additions to Isolated Mitochondria	Oxygen Consumption (nmol/min/mg) (mitochondrial protein)
3mM Malate + 3mM Pyruvate	88.0 ± 2.4
3mM Malate	45.7 ± 1.8
3mM Pyruvate	30.5 ± 1.1
3mM Malate + 5mM Acetyl-L-Carnitine	84.2 ± 2.0
5mM Acetyl-L-Carnitine	14.5 ± 1.9

Intact rat heart mitochondria isolated as described in section 2.9.3 were incubated for 5 min at 37^0C in incubation medium containing 1mM ADP, BSA (1.3mg/ml) and other additions indicated. Mitochondrial oxygen consumption was measured at 37^0C using an oxygen electrode as described in section 2.10. Values are expressed as means ± SEM of 4 independent preparations.

consumption, as measured by the oxygen electrode. This increase in oxygen consumption in the presence of malate and pyruvate compared with mitochondria alone, indicates that the mitochondria are in state 3 respiration.

Addition of malate and pyruvate individually resulted in oxygen consumptions of 46 ± 2 nmol/min/mg and 31 ± 1 nmol/min/mg respectively (table 3.3). The increase in oxygen consumption as a result of malate addition is likely due to the presence of a small amount of endogenous oxidisable substrate, such as pyruvate or acetyl-CoA within the mitochondria. The increase in oxygen consumption as a result of pyruvate addition is likely due to the presence of low levels of citric acid cycle intermediates present in the isolated mitochondria. However, these intermediates became depleted within a few minutes if malate was not present, resulting in a steady decrease in oxygen consumption until no oxygen decrease was observed (data not shown).

Addition of 5mM acetyl-L-carnitine in combination with 3mM malate resulted in an oxygen consumption of 84 ± 2 nmol/min/mg (table 3.3), which was comparable to that observed with pyruvate and malate (table 3.3). This indicates that acetyl-L-carnitine is transported into the mitochondrial matrix by carnitine:acetylcarnitine translocase. Once inside, it is converted to acetyl-CoA by CAT, located on the matrix face of the inner mitochondrial membrane, and subsequently oxidised by the enzymes of the citric acid cycle. The fact that addition of acetyl-L-carnitine on its own results in a small oxygen consumption (15 ± 2 nmol/min/mg) compared with pyruvate on its own (31 ± 1) is probably due to the fact that acetyl-L-carnitine requires a transport mechanism to enter the mitochondrial matrix whereas pyruvate does not. In the presence of malate, the transport of acetyl-L-carnitine into the

mitochondria does not appear to be rate limiting. This might suggest that replenishing citric acid cycle intermediates by the addition of malate stimulates the flux of acetyl-L-carnitine into the matrix for oxidation, thus removing any restraint that transport puts on the oxidation of acetyl-L-carnitine. The fact that in the presence of malate, both pyruvate and acetyl-L-carnitine produce almost identical rates of oxygen consumption, also suggests that transport of acetyl-L-carnitine does not limit its rate of oxidation and that the rate of oxidation by the citric acid cycle with these substrates is at a maximum.

Given that malate is essential for oxidation by the citric acid cycle to occur freely, it was included in all subsequent experiments. Table 3.4 shows the increase in oxygen consumption measured upon addition of various substrates to mitochondria which have already had malate added to them. Addition of pyruvate results in an increases in the oxygen consumption by the mitochondria of 42.3 $\pm$ 0.3 nmol/min/mg. As expected, addition of acetyl-L-carnitine results in a comparable increase in oxygen consumption (39 $\pm$ 2 nmol/min/mg) to that of pyruvate. No significant increase in oxygen consumption was detected upon addition of 1mM malonyl-CoA in the presence of 1mM L-carnitine (table 3.4). This indicates that malonyl-CoA is not being transported into the mitochondria for oxidation by an as yet unknown transport mechanism. However, due to the absence of overt CAT in an intact mitochondria preparation it is not known whether malonyl-CoA is being decarboxylated to acetyl-CoA by overt malonyl-CoA decarboxylase for transport into the mitochondria.

The fact that malonyl-CoA is not utilised by mitochondria as a substrate for oxidation does not rule out the possibility that malonyl-CoA decarboxylase on the

Table 3.4 Effect of various substrates in addition to 3mM malate, on the increase in oxygen consumption rate of isolated rat heart mitochondria

Addition to Isolated Mitochondria in Addition to 3mM Malate	Increase in Oxygen Consumption (nmol/min/mg)
3mM Pyruvate	42.3 ± 0.3
5mM Acetyl-L-Carnitine	38.5 ± 1.7
1mM Malonyl-CoA + 1mM Carnitine	-0.03 ± 0.03

Intact rat heart mitochondria isolated as described in section 2.9.3 were incubated for 5 min at 37^{0}C in incubation medium containing 3mM malate, 1mM ADP, BSA (1.3mg/ml) and other additions indicated. Mitochondrial oxygen consumption was measured at 37^{0}C using an oxygen electrode as described in section 2.10. Values are expressed as means ± SEM of 4 independent preparations.

cytosolic face of the mitochondrial inner membrane is capable of regulating cytosolic malonyl-CoA levels.

3.3.8 Conclusions

Disposal of cardiac malonyl-CoA is essential in order that malonyl-CoA levels be acutely regulated. Malonyl-CoA decarboxylase has been shown to be a mitochondrial enzyme in rat heart with the majority of activity lying internal to the inner mitochondrial membrane. However, this data suggests that a proportion of the total mitochondrial malonyl-CoA decarboxylase activity may be localised overt to the inner mitochondrial membrane where, in theory, it would be capable of decarboxylating cytosolic malonyl-CoA. The data clearly shows that malonyl-CoA is not a respiratory substrate i.e. it is unlikely to be transported in and then decarboxylated by intra-mitochondrial malonyl-CoA decarboxylase. The inability of malonyl-CoA to be transported into mitochondria for oxidation, via an unknown transport mechanism does not rule out malonyl-CoA decarboxylase as a route of malonyl-CoA disposal.

Malonyl-CoA decarboxylase still provides a possible mechanism for the disposal of malonyl-CoA in the heart. Little is known about the regulation of malonyl-CoA decarboxylase activity and its role (if any) in regulation of cardiac malonyl-CoA levels. Inhibition of malonyl-CoA decarboxylase by acetyl-CoA, in combination with increased ACC activity, may provide a possible mechanism by which increased glucose utilisation could inhibit fatty acid oxidation via increased malonyl-CoA level. Further studies of malonyl-CoA decarboxylase are required if its role in regulating cardiac malonyl-CoA levels is to be fully understood.

3.4 Acute Regulation of Fatty Acid Metabolite Levels in Cardiac Myocytes

Metabolic mechanisms whereby the oxidation of fatty acids predominates over that of carbohydrate fuels (glucose/fatty acid cycle) have been well documented (Randle *et al*,1963; Neely & Morgan, 1974; Hue *et al*, 1995), while much less is known about how fatty acid oxidation is regulated by carbohydrates in the heart. An important site of regulation in the heart is thought to be the interaction of malonyl-CoA, produced predominantly by a 280 kDa isoform of ACC, with mitochondrial CPT 1. A close negative correlation has been observed to occur between malonyl-CoA concentration and the rate of fatty acid oxidation in perfused hearts and isolated cardiac myocytes (Awan & Saggerson, 1993; Saddik *et al*, 1993), providing further evidence for the role of malonyl-CoA in cardiac fuel selection. Regulation at this, and other sites, maintains an optimal balance between fatty acid and carbohydrate oxidation in the heart.

The liver isoform of ACC (ACC-265) has been extensively studied while considerably less is known about the heart/muscle isoform (ACC-280). Liver ACC is subject to complex acute regulation based on polymerization/depolymerization and protein phosphorylation/dephosphorylation. Conditions such as carbohydrate feeding or insulin administration lead to a more active polymerized form, whereas glucagon and adrenaline have the opposite effect (Hardie *et al*, 1989; Haystead *et al*, 1990; Davies *et al*, 1992; Witters & Kemp, 1992). Heart ACC has been shown to be activated by citrate, inhibited by palmitate and activated with a phosphoprotein phosphatase preparation (Thampy, 1989). However, Saddik *et al*

(1993), have suggested that acetyl-CoA supply is a key determinant of heart ACC activity. Heart ACC also appears to be regulated differently from the liver isoform in that, unlike the liver enzyme, abundance of ACC in the heart is not affected by fasting and re-feeding (Bianchi *et al*, 1990). Awan & Saggerson (1993), have provided evidence that the concentration of malonyl-CoA in heart is under acute regulation by hormones such as insulin and adrenaline, but that this may be via an indirect mechanism.

This section provides further insight into the regulatory mechanisms involved in cardiac fuel selection and the acute regulation of ACC (and malonyl-CoA) due to substrate and hormonal influences.

3.4.1 Effect of Insulin and Palmitate on Fatty Acid Metabolite Levels in Cardiac Myocytes

Malonyl-CoA content of cardiac myocytes was measured as described in section 2.8.1, under various physiological conditions to compare with measurements previously made in whole heart. Myocyte malonyl-CoA levels are an indication, at least in part, of cardiac ACC activity and can be used to gain an insight into ACC regulation under various physiological conditions.

Long-chain acyl-CoA and long-chain acylcarnitine were measured as described in sections 2.8.3 and 2.8.4 respectively, to illustrate changes in mitochondrial CPT 1 activity. Hence, changes in malonyl-CoA concentration, and therefore CPT 1 activity, should be followed by concomitant changes in long-chain acyl-CoA and long-chain acylcarnitine levels and particularly in the long-chain acyl-CoA:long-chain acylcarnitine ratio.

Malonyl-CoA, long-chain acyl-CoA and long-chain acylcarnitine measurements were made in cardiac myocytes incubated at 37^0C for 60 min with KHB buffer

containing 5mM glucose, 2% BSA and various additions including, 0.5mM palmitate (NEFA), 10nm insulin and both 0.5mM palmitate and 10nm insulin together. Reactions were stopped by the addition of perchloric acid (6% (w/w) final concentration), metabolites extracted, and assays carried out as described in material and methods.

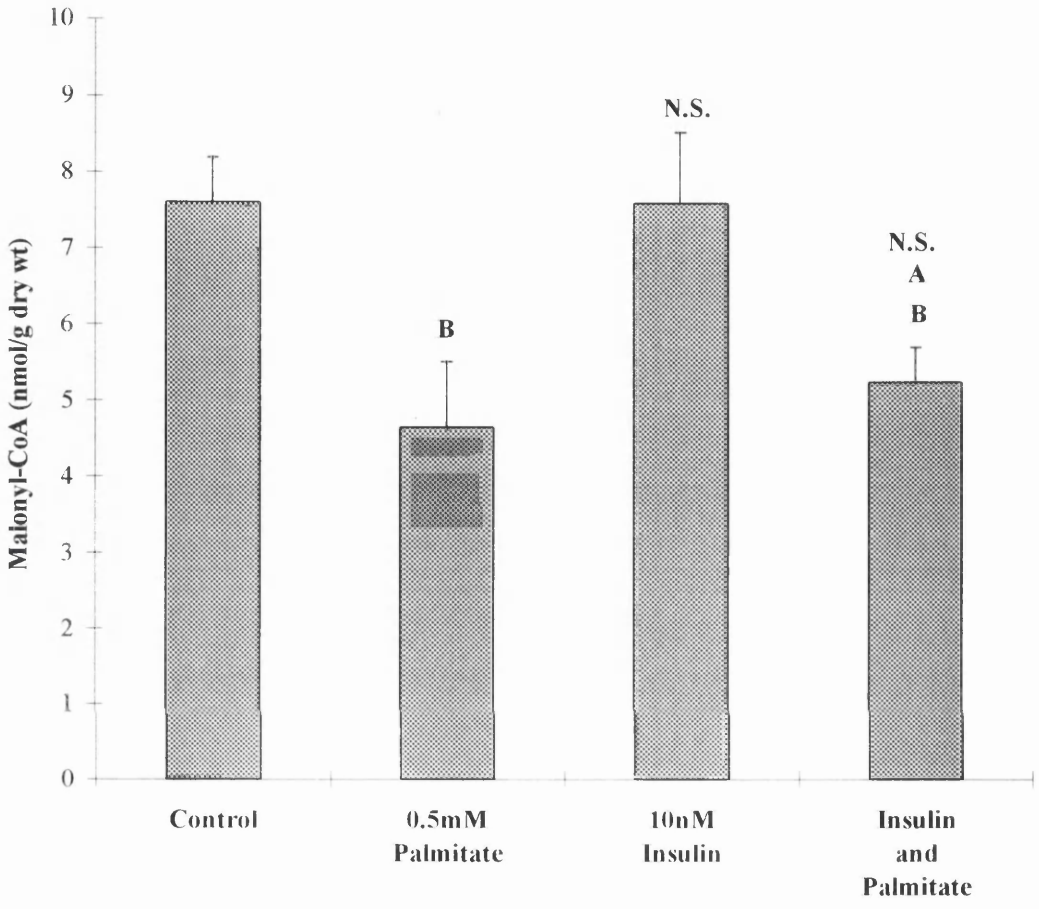
Experiments were carried out using TAG-loaded myocytes (isolated in the presence of 0.5mM palmitate). The presence of 0.5mM palmitate during isolation provides a more physiological environment for the myocytes during isolation as well as maintaining the level of endogenous TAG pools. Experiments were also carried out using non-TAG-loaded myocytes (isolated in the absence of palmitate).

3.4.1.1 Effect of Insulin and Palmitate on Malonyl-CoA Levels in TAG-Loaded Myocytes

Palmitate significantly decreased malonyl-CoA level by ~39% in TAG-loaded myocytes (fig 3.10). This decrease in malonyl-CoA is comparable to that observed in perfused hearts from fed rats (Awan & Saggerson, 1993). The mechanism by which palmitate decreased malonyl-CoA levels is not fully understood.

Thampy (1989), has shown that ACC purified from heart is allosterically inhibited by palmitoyl-CoA. However, due to the abundance of cytosolic fatty acid/acyl-CoA binding proteins, the free concentration of palmitoyl-CoA in the cell is probably orders of magnitude lower and too low to inhibit ACC directly. One possible mechanism for the palmitate induced decrease in malonyl-CoA is through AMPK mediated phosphorylation of ACC. Exogenous palmitate must be "activated" to its CoA-thioester before it can be metabolised further by the cell. This ATP-dependent reaction is catalysed by FAS and produces 1 mol of AMP per mol fatty acid activated. Therefore, addition of exogenous palmitate will increase

Figure 3.10 Effect of insulin and palmitate on malonyl-CoA levels in TAG-loaded myocytes



TAG-loaded myocytes were incubated at 37^{0}C for 60 min with KHB medium containing 5mM glucose and 2% BSA, in the presence or absence of the indicated concentrations of insulin and palmitate. Values are means ± SEM of 5 independent preparations. **A** indicates $p<0.01$ for the effect of insulin and palmitate versus insulin. **B** indicates $p<0.005$ versus control. **N.S.** indicates no significant difference for the effect of insulin versus control and the effect of insulin and palmitate versus palmitate (paired t-test).

substrate flux through FAS, possibly resulting in an increase in AMP levels. This in turn could activate AMPK (directly and via activation of the upstream AMPK kinase) leading to the phosphorylation and inactivation of ACC. The resulting decrease in malonyl-CoA level would relieve malonyl-CoA inhibition of CPT 1 and allow fatty acid oxidation to proceed at an increased rate.

Activation of AMPK by the upstream AMPK kinase is also thought to be stimulated by long-chain fatty acyl-CoA esters (Carling *et al*, 1987b; Hardie, 1989) which would further increase the effect of exogenous palmitate on AMPK activity and ACC phosphorylation. However, no significant increase in long-chain fatty acyl-CoA esters was observed on addition of palmitate.

It should be noted, that TAG-loaded myocytes used in control incubations (and all other incubations) were isolated in the presence of 0.5mM palmitate. Even though palmitate was greatly diluted by washing myocytes in palmitate free KHB medium prior to incubation, it was not possible to remove all palmitate from the cells. Therefore, the presence of a small, unknown quantity of palmitate within the incubation, may have resulted in the malonyl-CoA content of these cells being lower than would have been seen had all exogenous palmitate been removed. As a result, the effect of palmitate on malonyl-CoA levels may actually be greater than was observed. However, the effect of palmitate on malonyl-CoA level in non-TAG-loaded myocytes (fig 3.14) was very similar to that in TAG-loaded myocytes and no significant difference was observed between malonyl-CoA levels in control incubations of TAG-loaded and non-TAG-loaded cells (fig 3.18). This would suggest that low levels of exogenous palmitate have little effect on malonyl-CoA levels, and/or that incubation of myocytes for 60 min in the absence of exogenous

palmitate is sufficient to reverse any decrease in malonyl-CoA content that may have resulted from being isolated in the presence of 0.5mM palmitate.

Insulin (in the presence of 5mM glucose) had no significant effect on malonyl-CoA level in TAG-loaded myocytes (fig 3.10). This differs from observations previously made in isolated perfused hearts from fed rats, in which insulin increased malonyl-CoA levels dramatically (Awan & Saggerson, 1993). It has been suggested that insulin exerts its effect on malonyl-CoA levels in perfused rat hearts via acute regulation of ACC.

It is possible that this lack of response to insulin may be secondary to insulin's ability to stimulate glucose transport in isolated myocytes, which in turn, may depend on the activity and intracellular disposition of glucose transporters at the time of isolation. The effect of glucose and insulin on malonyl-CoA levels and the difference between TAG-loaded and non-TAG-loaded myocytes is studied in more detail in section 3.4.5.

Palmitate and insulin (in the presence of 5mM glucose) together, decreased malonyl-CoA level by ~31% compared to control incubations (fig 3.10). Hence, insulin was unable to overcome the effects of palmitate as no significant difference in malonyl-CoA level was observed between incubations containing palmitate alone and those containing both palmitate and insulin. It is not surprising that insulin was unable to overcome the effects of palmitate in TAG-loaded myocytes since insulin alone had no significant effect on malonyl-CoA level on its own.

3.4.1.2 Effect of Insulin and Palmitate on Long-chain Acyl-CoA and Long-chain Acylcarnitine Levels in TAG-loaded Myocytes

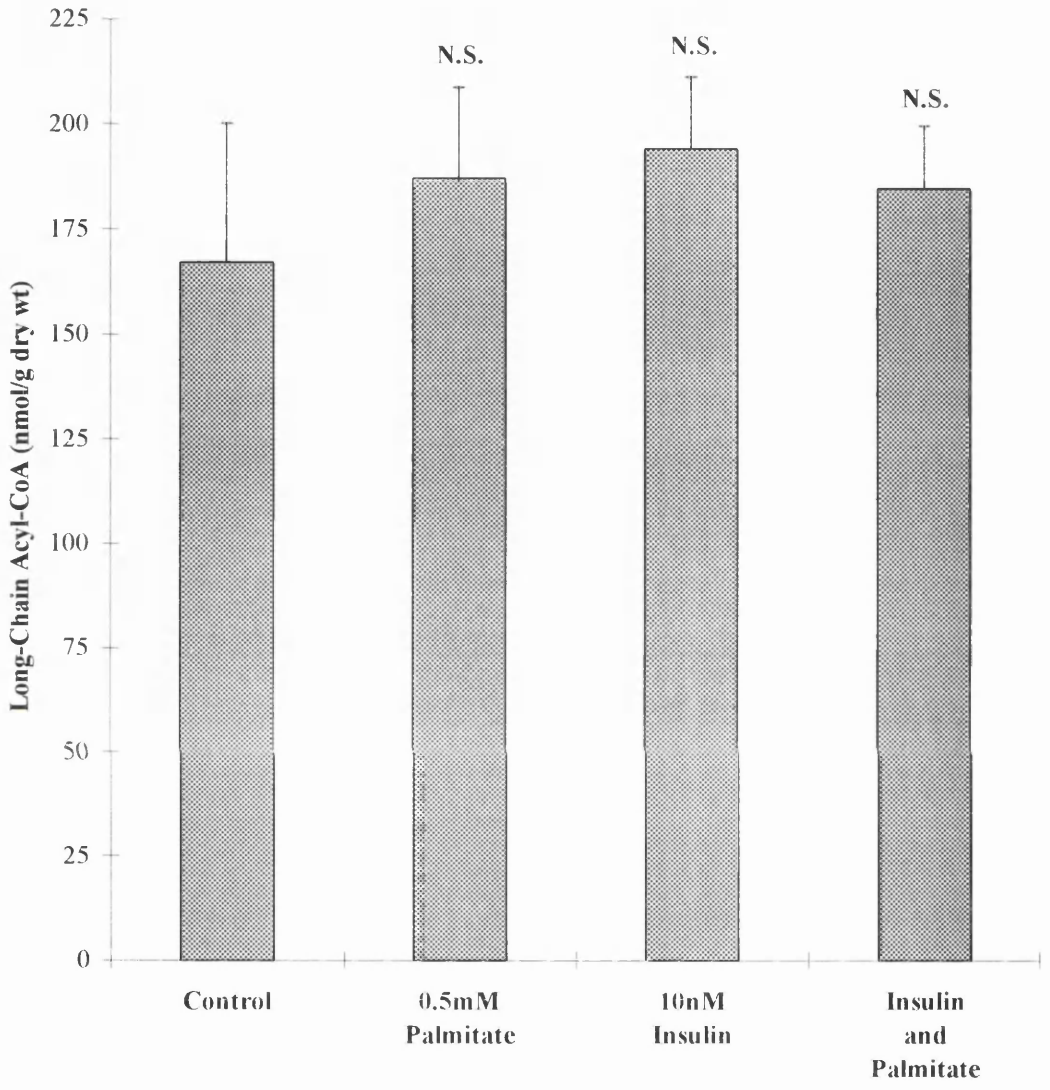
In theory, changes in myocyte malonyl-CoA levels should produce changes in mitochondrial CPT 1 activity, due to the fact that the heart isoform of CPT 1 is

highly sensitive to inhibition by malonyl-CoA. A decrease in malonyl-CoA level would decrease inhibition of CPT 1, increasing the rate of conversion of long-chain acyl-CoA to long-chain acylcarnitine for transport into the mitochondria, which would result in a drop in the long-chain acyl-CoA:long-chain acylcarnitine ratio. Conversely, an increase in malonyl-CoA level would further inhibit CPT 1, blocking the entry of long-chain acyl-CoA into mitochondria resulting in an increase in the long-chain acyl-CoA:long-chain acylcarnitine ratio.

Palmitate very slightly increased (although not significantly) long-chain acyl-CoA level in TAG-loaded myocytes (fig 3.11). Addition of palmitate might be expected to increase levels of long-chain acyl-CoA as exogenous palmitate is activated to palmitoyl-CoA by FAS. However, palmitate decreased malonyl-CoA levels by ~39% in TAG-loaded myocytes (fig 3.10). In theory, this decrease in malonyl-CoA will reduce the inhibition of CPT 1, resulting in an increase in long-chain acyl-CoA conversion to long-chain acylcarnitine for transport into mitochondria and an increase in fatty acid oxidation rate. Therefore, the increase in long-chain acyl-CoA due to activation of exogenous palmitate to palmitoyl-CoA within the myocyte may be cancelled out by stimulation of its conversion to long-chain acylcarnitine by CPT 1 and subsequent oxidation in the mitochondria. Hence the long-chain acyl-CoA level remains approximately constant on addition of palmitate.

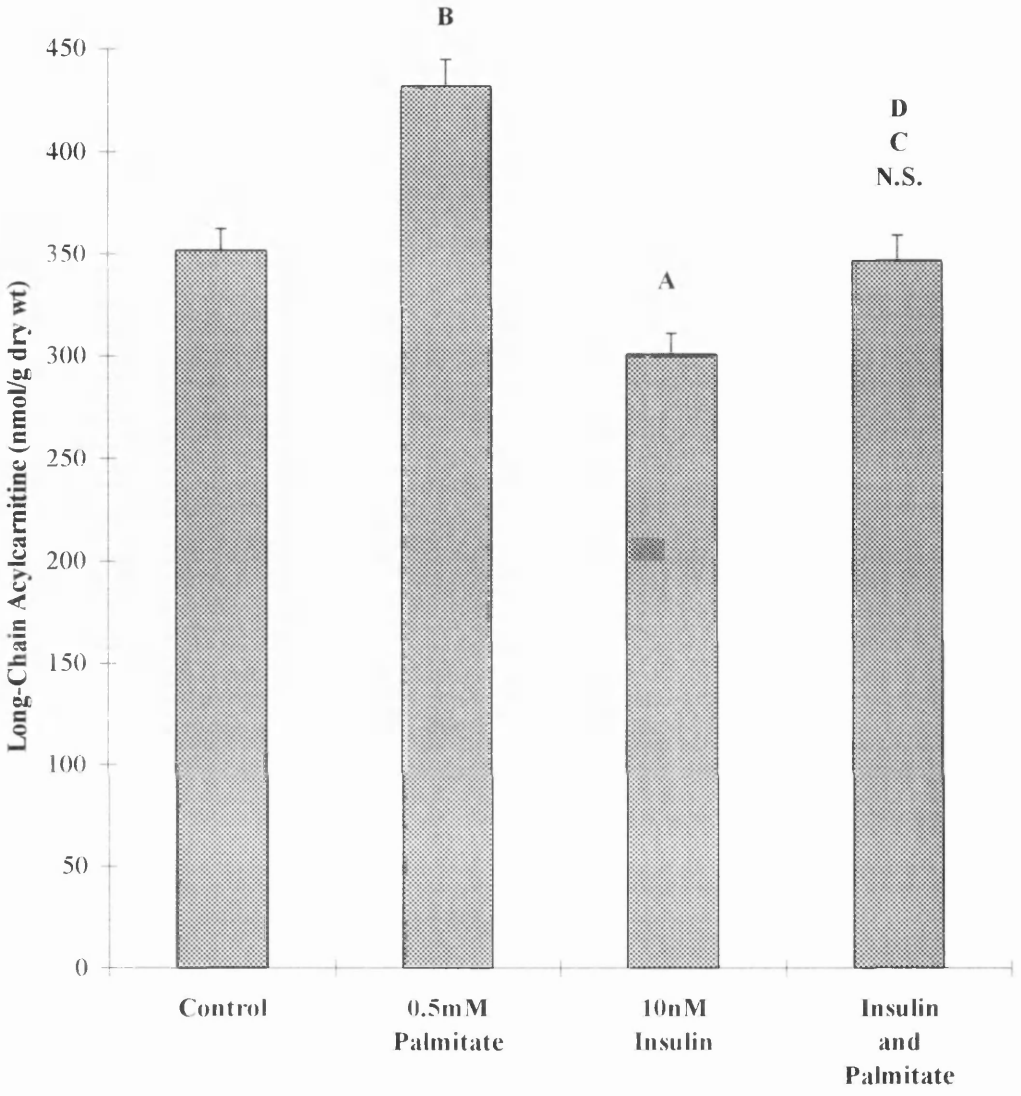
Palmitate significantly increased long-chain acylcarnitine level by ~23% (fig 3.12). This result gives further support to the proposed mechanism above, whereby reduced inhibition of CPT 1 due to decreased malonyl-CoA level, increases the conversion of long-chain acyl-CoA to long-chain acylcarnitine for transport into the mitochondria.

Figure 3.11 Effect of insulin and palmitate on long-chain acyl-CoA levels in TAG-loaded myocytes



TAG-loaded myocytes were incubated at 37^{0}C for 60 min with KHB medium containing 5mM glucose and 2% BSA, in the presence or absence of the indicated concentrations of insulin and palmitate. Values are means ± SEM of 4 independent preparations. **N.S.** indicates no significant difference versus control and for the effect of insulin and palmitate versus insulin or versus palmitate (paired t-test)

Figure 3.12 Effect of insulin and palmitate on long-chain acylcarnitine levels in TAG-loaded myocytes



TAG-loaded myocytes were incubated at 37^{0}C for 60 min with KHB medium containing 5mM glucose and 2% BSA, in the presence or absence of the indicated concentrations of insulin and palmitate. Values are means ± SEM of 5 independent preparations. **A**, **B**, **N.S.** indicate $p<0.01$, <0.005 and no significant difference respectively versus control. **C** indicates $p<0.025$ for the effect of insulin and palmitate versus insulin. **D** indicates $p<0.001$ for the effect of insulin and palmitate versus palmitate (paired t-test).

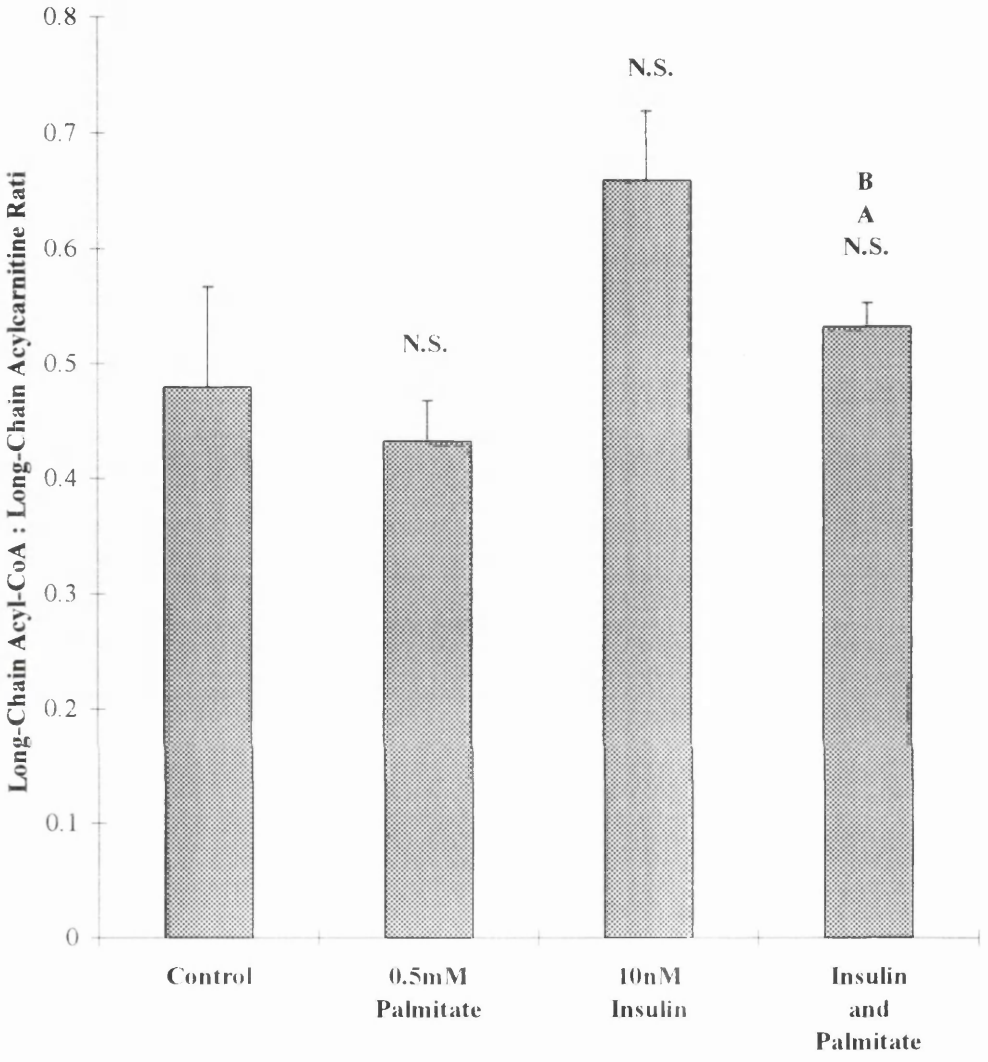
Palmitate slightly reduced (although not significantly) the long-chain acyl-CoA:long-chain acylcarnitine ratio by ~10% (fig 3.13). This indicates a small overall increase in CPT 1 activity, presumably as a result of reduced malonyl-CoA inhibition. If the primary role of ACC in heart is as suggested, to regulate fatty acid oxidation via malonyl-CoA inhibition of CPT 1, one might expect to see a more dramatic change in CPT 1 activity with such a significant decrease in malonyl-CoA level. Perhaps measurements of long-chain esters are not as good an indication of CPT 1 activity in isolated myocytes as first thought, or perhaps other factors are influencing the levels of long-chain esters in isolated TAG-loaded myocytes.

Insulin had no significant effect on the level of long-chain acyl-CoA (fig 3.11). This was not unexpected since insulin had no effect on malonyl-CoA level either.

Insulin caused a small, but significant decrease in long-chain acylcarnitine level of ~15% (fig 3.12). Since insulin had no effect on malonyl-CoA level, it is surprising to see a decrease in long-chain acylcarnitine level. This might suggest that insulin is inhibiting CPT 1 via a mechanism which does not involve changes in ACC activity and malonyl-CoA levels, although this would also result in an increase in the level of long-chain acyl-CoA, which was not observed.

In the liver, conditions which decrease both levels of malonyl-CoA as well as the malonyl-CoA sensitivity of CPT 1 include fasting and diabetes (Cook, 1984; McGarry *et al*, 1983). The malonyl-CoA sensitivity of cardiac CPT 1 in response to fasting is widely debated (Paulson *et al*, 1984; Fiol *et al*, 1987; Cook & Lappi, 1992) despite the fact that malonyl-CoA concentrations decrease in fasted hearts (McGarry *et al*, 1983). Liver CPT 1 is sensitive to hormonal regulation by insulin, so that enzyme activity is decreased and the sensitivity to inhibition by

Figure 3.13 Effect of insulin and palmitate on the long-chain acyl-CoA:long-chain acylcarnitine ratio in TAG-loaded myocytes



TAG-loaded myocytes were incubated at 37^{0}C for 60 min with KHB medium containing 5mM glucose and 2% BSA, in the presence or absence of the indicated concentrations of insulin and palmitate. Values are means ± SEM of 4 independent preparations. **N.S.** indicates no significant difference versus control. **A** indicates $p<0.05$ for the effect of insulin and palmitate versus insulin. **B** indicates $p<0.01$ for the effect of insulin and palmitate versus palmitate (paired t-test).

malonyl-CoA is increased (Cook & Gamble, 1987). The effect of insulin on heart CPT 1 activity is controversial and as yet, no consistent effect of hormonal levels of insulin have been reported for CPT 1 in the heart (Fiol *et al*, 1987; Cook & Lappi, 1992). However, the effects of fasting, diabetes and insulin on liver CTP 1 sensitivity are not acute effects but are quite long-term. Therefore, it is unlikely that the effect of insulin on the level long-chain acylcarnitine is due to changes in sensitivity of CPT 1 to malonyl-CoA.

Insulin increased (although not significantly) the long-chain acyl-CoA:long-chain acylcarnitine ratio by ~37% (fig 3.13). This is not expected since insulin had no significant effect on malonyl-CoA level.

Incubation of TAG-loaded myocytes with palmitate and insulin together had no significant effect on long-chain acyl-CoA levels (fig 3.11), long-chain acylcarnitine levels (fig 3.12), or the long-chain acyl-CoA:long-chain acylcarnitine ratio (fig 3.13) despite a significant decrease in the level of malonyl-CoA (fig 3.10). In the presence of insulin and palmitate, the long-chain acylcarnitine level was significantly lower (~20% lower) and the long-chain acyl-CoA:long-chain acylcarnitine ratio was significantly higher (23% higher) compared to myocytes incubated with palmitate alone, despite the fact that malonyl-CoA levels were comparable. This also suggests that insulin is inhibiting CPT 1 activity via a mechanism which is independent of changes in ACC activity and malonyl-CoA levels, i.e. through increased sensitivity of CPT 1 to inhibition by malonyl-CoA. A closer study into the effects of insulin on CPT 1 activity in TAG-loaded myocytes is required before any definite conclusions can be made.

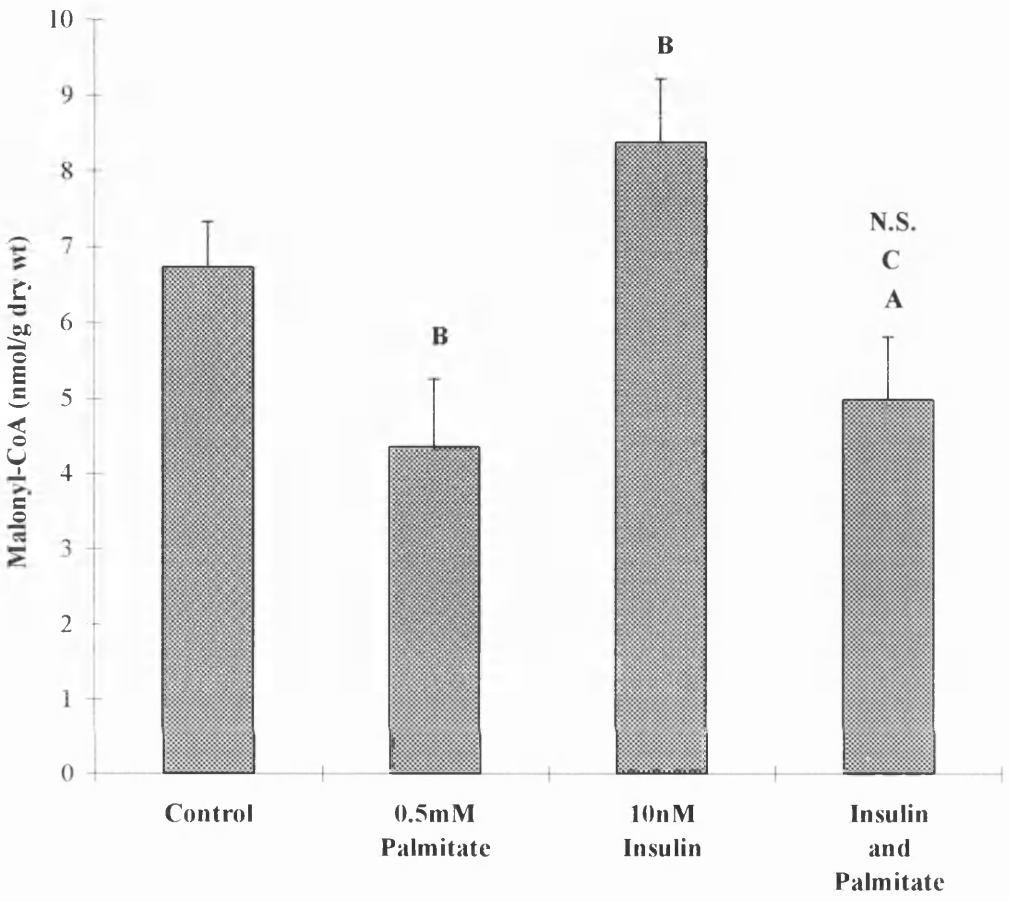
3.4.1.3 Effect of Insulin and Palmitate on Malonyl-CoA Levels in Non-TAG-loaded Myocytes

Palmitate significantly decreased malonyl-CoA levels by ~35% in non-TAG-loaded myocytes (fig 3.14), which is comparable to that seen in TAG-loaded myocytes (fig 3.10) and to that previously observed in perfused hearts from fed rats (Awan & Saggerson, 1993). The mechanism by which palmitate reduces malonyl-CoA levels in non-TAG-loaded myocytes is very likely the same as that in TAG-loaded myocytes (see section 3.4.1.1).

In contrast to TAG-loaded myocytes, insulin (in the presence of 5mM glucose) significantly increased malonyl-CoA levels by ~25% in non-TAG-loaded myocytes (fig 3.14). Awan & Saggerson (1993), observed a 41% increase in malonyl-CoA content of isolated rat hearts perfused with 8nM insulin. The mechanism by which insulin acutely increases malonyl-CoA levels in the heart is thought to be primarily via an increase in acetyl-CoA supply from the PDH complex as opposed to activation of ACC as in the liver. However, it has been suggested that cAMP elevating hormones increase phosphorylation at AMPK sites on ACC. The mechanism for this is unclear, although it is likely to be mediated by inhibition of protein phosphatase activity. Insulin decreases cAMP levels and appears to promote dephosphorylation of ACC, at least in mammary cells and Fao hepatoma cells. Again the mechanism is unclear but may involve activation of protein phosphatases (Hardie, 1989).

Much less is known about the regulation of the heart isoform of ACC (ACC-280) compared to the liver isoform (ACC-265). Liver ACC-265 is regulated by phosphorylation/dephosphorylation and by the allosteric activator, citrate. This differs from heart ACC-280 which is thought to be primarily regulated by acetyl-

Figure 3.14 Effect of insulin and palmitate on malonyl-CoA levels in non-TAG-loaded myocytes



Non-TAG-loaded myocytes were incubated at 37^{0}C for 60 min with KHB medium containing 5mM glucose and 2% BSA, in the presence or absence of the indicated concentrations of insulin and palmitate. Values are means ± SEM of 6 independent preparations. **A**, **B** indicate $p<0.05$, <0.01 respectively versus control. **C** indicates $p<0.005$ for the effect of insulin and palmitate versus insulin. **N.S.** indicates no significant difference for the effect of insulin and palmitate versus palmitate (paired t-test).

CoA supply (Saddik *et al*, 1993). Heart ACC-280 has a low affinity (K_m~100μM) for its substrate acetyl-CoA (Bianchi *et al*, 1990; Saddik *et al*, 1993) which supports the hypothesis that it is regulated primarily by acetyl-CoA supply. It has been suggested that the acetyl-CoA generated from carbohydrate is available for transport to the cytosol via the actions of CAT and carnitine:acetylcarnitine translocase, whereas acetyl-CoA produced by β-oxidation is more readily available to the citric acid cycle (Lysiak *et al*, 1986). Hence, increased acetyl-CoA production by the PDH complex in response to insulin, stimulates the transport of acetyl-CoA to the cytosol. The resulting increase in cytosolic acetyl-CoA provides substrate for ACC and thereby increases cytosolic malonyl-CoA level, which in turn inhibits fatty acid oxidation at the level of CPT 1. This hypothesis is supported by the fact that stimulation of glucose oxidation with DCA (inhibits PDH kinase) has been shown to decrease fatty acid oxidation and is closely correlated by an increase in cardiac malonyl-CoA level. This increase in malonyl-CoA level was also closely correlated with a DCA induced increase in cardiac acetyl-CoA level, suggesting that an increase in acetyl-CoA supply from the PDH complex stimulates ACC activity (Saddik *et al*, 1993).

Incubation of myocytes with insulin and palmitate resulted in a decrease in malonyl-CoA level of ~26% (fig 3.14) compared to control incubations. Insulin did not overcome the effects of palmitate as no significant difference in malonyl-CoA level was observed between incubations containing palmitate alone and those containing both palmitate and insulin. Incubation of myocytes in the presence of insulin and palmitate decreased malonyl-CoA level by ~40% compared with insulin alone. Awan & Saggerson (1993), demonstrated a similar response in isolated perfused rat hearts, whereby perfusion with palmitate and insulin resulted in a

~53% decrease in malonyl-CoA compared with insulin alone. The fact that insulin did not overcome the effects of palmitate might suggest that AMPK activation by increased AMP levels (and AMPK kinase) is greater than insulin's ability to inactivate AMPK by activating protein phosphatases.

3.4.1.4 Effect of Insulin and Palmitate on Long-chain Acyl-CoA and Long-chain Acylcarnitine Levels in Non-TAG-loaded Myocytes

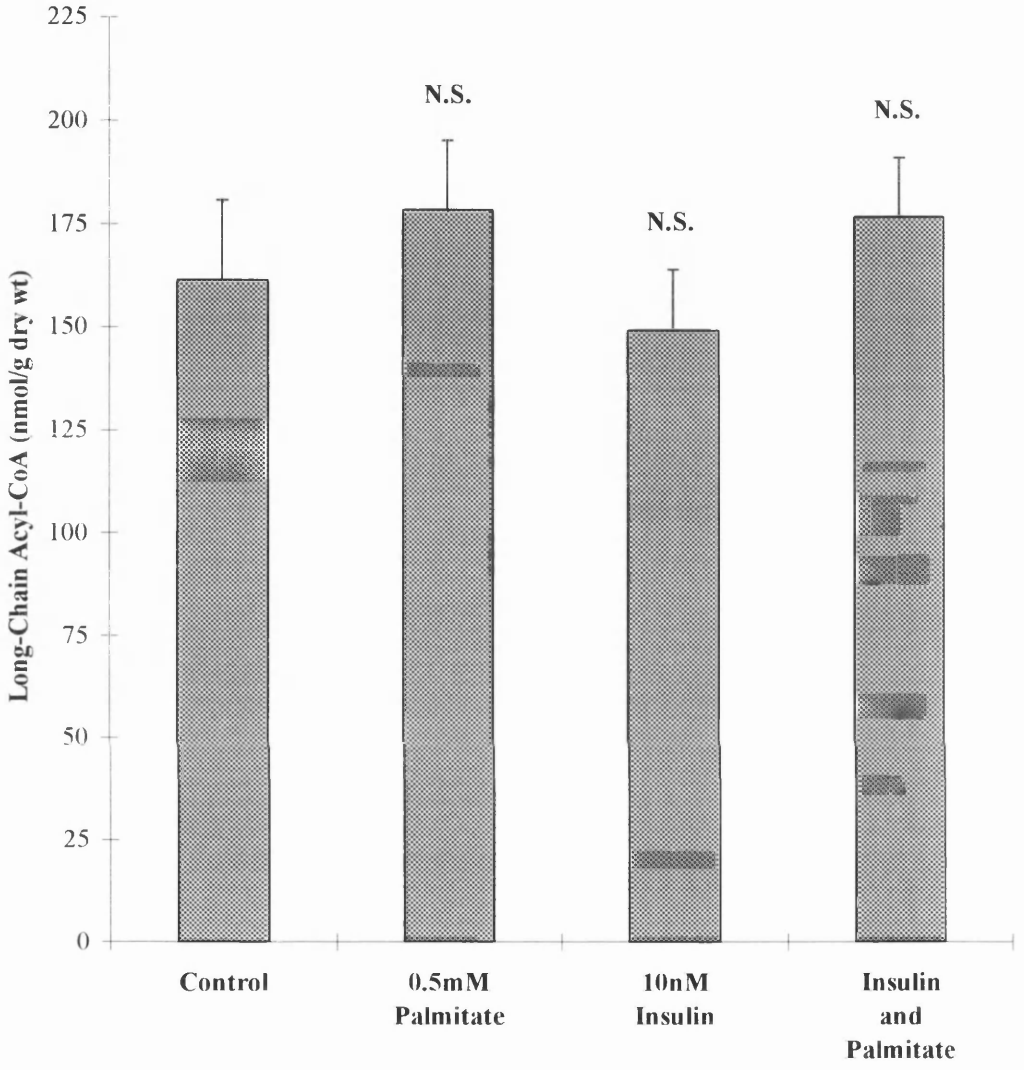
Palmitate had no significant effect on long-chain acyl-CoA level in non-TAG-loaded myocytes (fig 3.15) despite a ~35% decrease in malonyl-CoA level. This is most likely due to the same reason that no change in the level of long-chain acyl-CoA was observed on addition of palmitate to TAG-loaded myocytes (see section 3.4.1.2).

Palmitate slightly increased (although not significantly) long-chain acylcarnitine level (fig 3.16). An increase in long-chain acylcarnitine level would be expected since malonyl-CoA was decreased by ~35%, thus relieving its inhibition on CPT 1. The fact that a significant increase in long-chain acylcarnitine level was not observed suggests that in non-TAG-loaded myocytes, a proportion of exogenous fatty acids might be used in the repletion of endogenous TAG pools as opposed to being transported into the mitochondria by CPT 1.

Palmitate had no significant effect on the long-chain acyl-CoA:long-chain acylcarnitine ratio (fig 3.17), indicating little or no change in mitochondrial CPT 1 activity. Again, this is unexpected since malonyl-CoA level was significantly reduced which should decrease the long-chain acyl-CoA:long-chain acylcarnitine ratio.

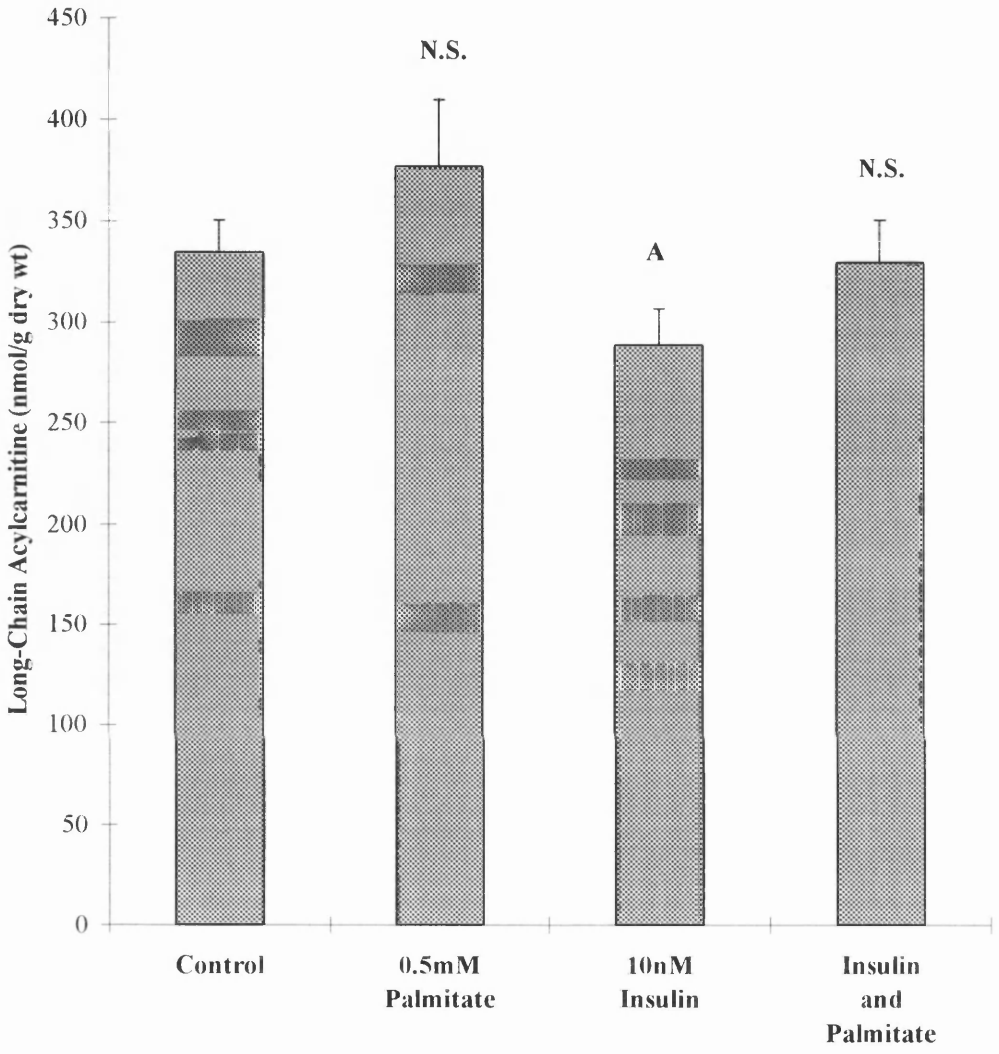
Insulin (in the presence of 5mM glucose) had no significant effect on long-chain acyl-CoA level (fig 3.15) despite a ~25% increase in malonyl-CoA level (fig 3.14).

Figure 3.15 Effect of insulin and palmitate on long-chain acyl-CoA levels in non-TAG-loaded myocytes



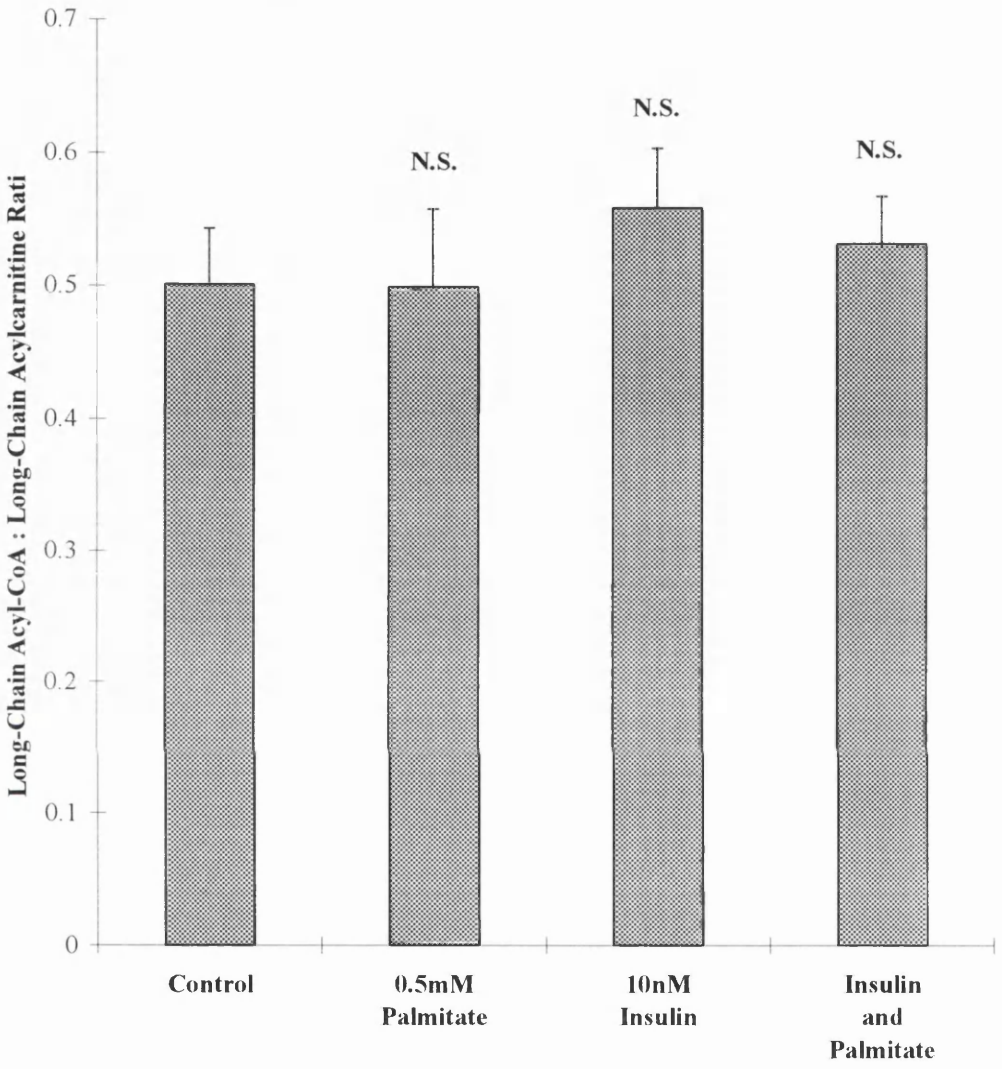
Non-TAG-loaded myocytes were incubated at 37^{0}C for 60 min with KHB medium containing 5mM glucose and 2% BSA, in the presence or absence of the indicated concentrations of insulin and palmitate. Values are means ± SEM of 4 independent preparations. **N.S.** indicates no significant difference versus control and for the effect of insulin and palmitate versus insulin or versus palmitate (paired t-test).

Figure 3.16 Effect of insulin and palmitate on long-chain acylcarnitine levels in non-TAG-loaded myocytes



Non-TAG-loaded myocytes were incubated at 37^{0}C for 60 min with KHB medium containing 5mM glucose and 2% BSA, in the presence or absence of the indicated concentrations of insulin and palmitate. Values are means ± SEM of 6 independent preparations. **A** indicates $p<0.025$ versus control. **N.S.** indicates no significant difference versus control and for the effect of insulin and palmitate versus insulin or versus palmitate (paired t-test).

Figure 3.17 Effect of insulin and palmitate on the long-chain acyl-CoA:long-chain acylcarnitine ratio in non-TAG-loaded myocytes



Non-TAG-loaded myocytes were incubated at 37^{0}C for 60 min with KHB medium containing 5mM glucose and 2% BSA, in the presence or absence of the indicated concentrations of insulin and palmitate. Values are expressed as means ± SEM of 4 independent preparations. **N.S.** indicates no significant difference versus control and for the effect of insulin and palmitate versus insulin or versus palmitate (paired t-test).

It is becoming increasingly clear that long-chain acyl-CoA measurements do not correspond with expected changes in CPT 1 activity, especially in non-TAG-loaded myocytes.

Insulin significantly decreased long-chain acylcarnitine levels by ~14% (fig 3.16). This may be the result of increased inhibition of CPT 1 due to increased malonyl-CoA levels, an increase in the sensitivity of CPT 1 to inhibition by malonyl-CoA, or both.

Insulin had no effect on the long-chain acyl-CoA:long-chain acylcarnitine ratio (fig 3.17). Given that insulin increased the level of malonyl-CoA by ~25%, one might have expected an increase in the long-chain acyl-CoA:long-chain acylcarnitine ratio, especially if as suggested, insulin increases the sensitivity of CPT 1 to inhibition by malonyl-CoA.

Incubation with insulin and palmitate had no significant effect on long-chain ester levels (fig 3.15, 3.16) or their ratio (fig 3.17) despite a 26% decrease in malonyl-CoA levels (fig 3.14).

The above results suggest that either changes in malonyl-CoA levels have little effect on CPT 1 activity in non-TAG-loaded myocytes, which is unlikely, or that changes in long-chain esters are not representative of CPT 1 activity. This is in contrast to results observed in soleus muscle where appreciable changes in the ratio of long-chain acyl-CoA:long-chain acylcarnitine are seen (Alam & Saggerson, 1998). The validity of long-chain ester measurements as an indicator of CPT 1 activity in cardiac myocytes is discussed further in section 3.4.3.

3.4.2 Effect of TAG-loading on Fatty Acid Metabolites in Cardiac Myocytes

No significant difference in malonyl-CoA content was observed between TAG-loaded and non-TAG-loaded myocytes which had been incubated for 60 min in

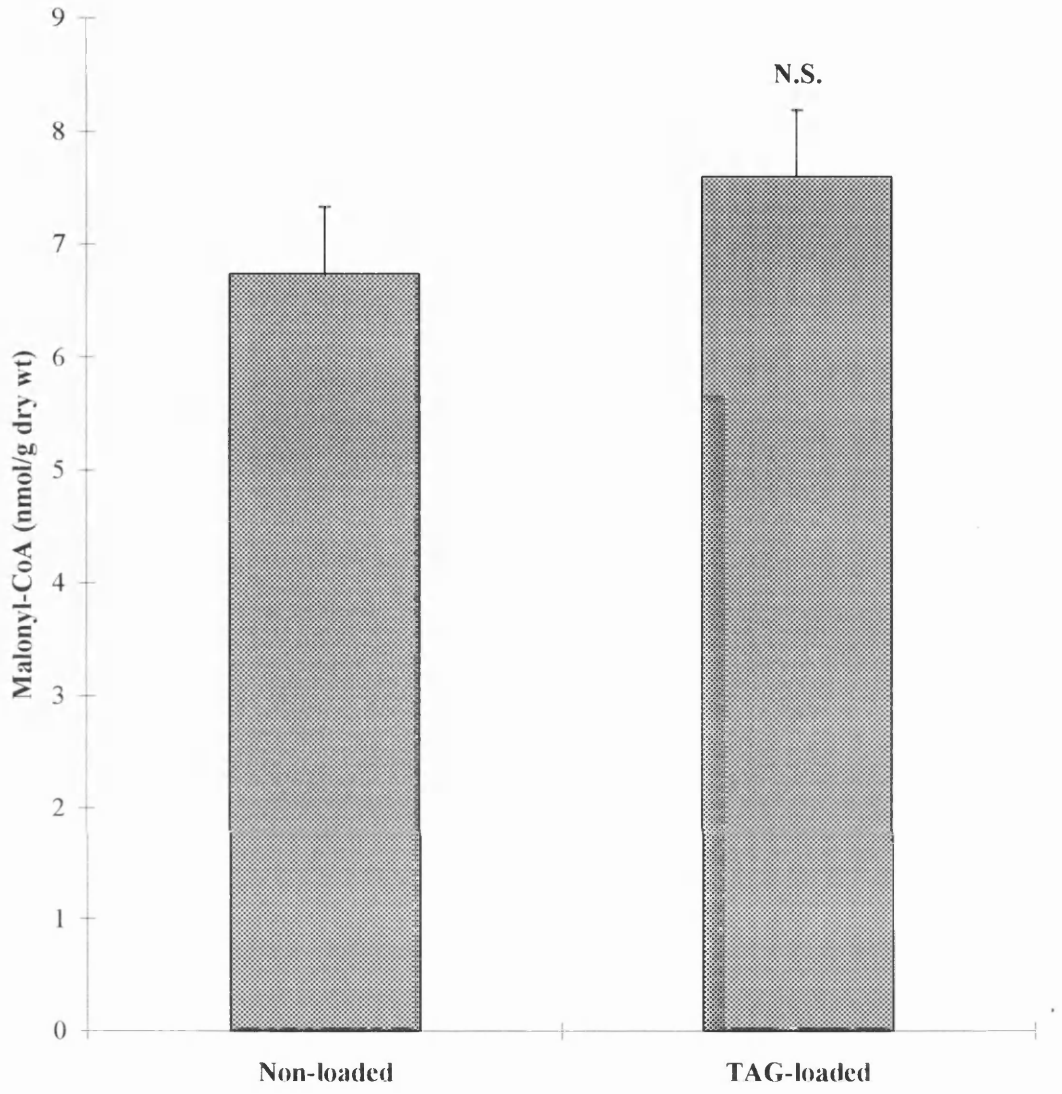
KHB medium containing 5mM glucose and 2% BSA (fig 3.18). Also, no significant difference in long-chain acyl-CoA levels (fig 3.19), long-chain acylcarnitine levels (fig 3.20) or the long-chain acyl-CoA:long-chain acylcarnitine ratio (fig 3.21) was observed between TAG-loaded and non-TAG-loaded myocytes incubated under the same conditions.

The fact that fatty acid metabolite levels are comparable between TAG-loaded and non-TAG-loaded myocytes after a 60 min incubation in the absence of exogenous fatty acids, would suggest that any alterations in fatty acid metabolite levels which occur during the two isolation procedures are not permanent and are reversed within 60 min. This means that comparisons of absolute levels of fatty acid metabolites can be made between TAG-loaded and non-TAG-loaded myocytes. It also means that any differences in fatty acid metabolite levels in response to various stimuli are the result of differences in the metabolism of TAG-loaded or non-TAG-loaded myocytes as opposed to differences in the basal levels of fatty acid metabolites.

3.4.3 Long-chain Acyl-CoA and Long-chain Acylcarnitine Levels in Myocytes and Perfused Rat Hearts

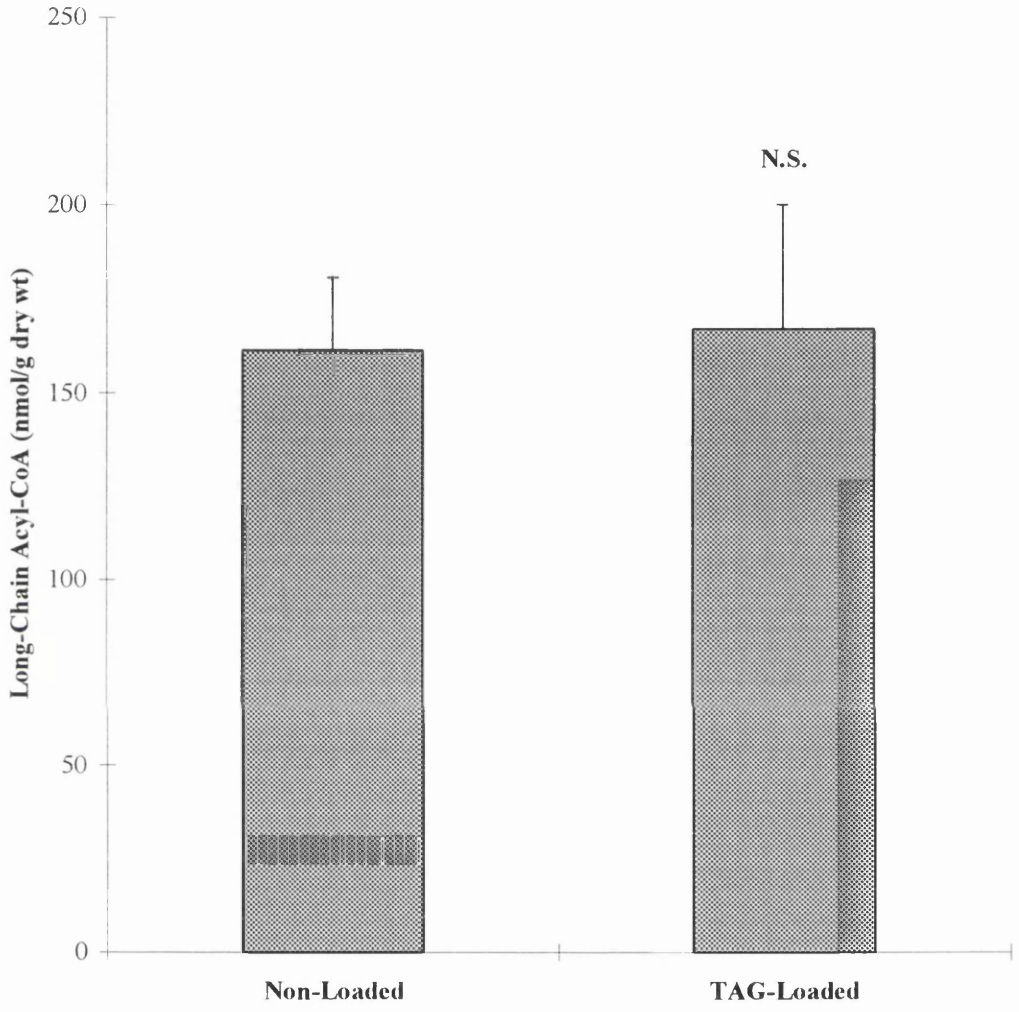
Non-TAG-loaded myocytes were isolated as described in section 2.4.1 and incubated for 45 min at 37^0C in KHB medium containing 5mM glucose and 2% BSA. Isolated rat hearts were perfused under identical conditions as described in section 2.7. Myocyte incubations were stopped by the addition of perchloric acid (6% (w/w) final concentration), while heart perfusions were stopped by freeze clamping and crushing under liquid nitrogen. Long-chain esters were extracted as described in section 2.6.

Figure 3.18 Effect of TAG-loading on malonyl-CoA levels in cardiac myocytes



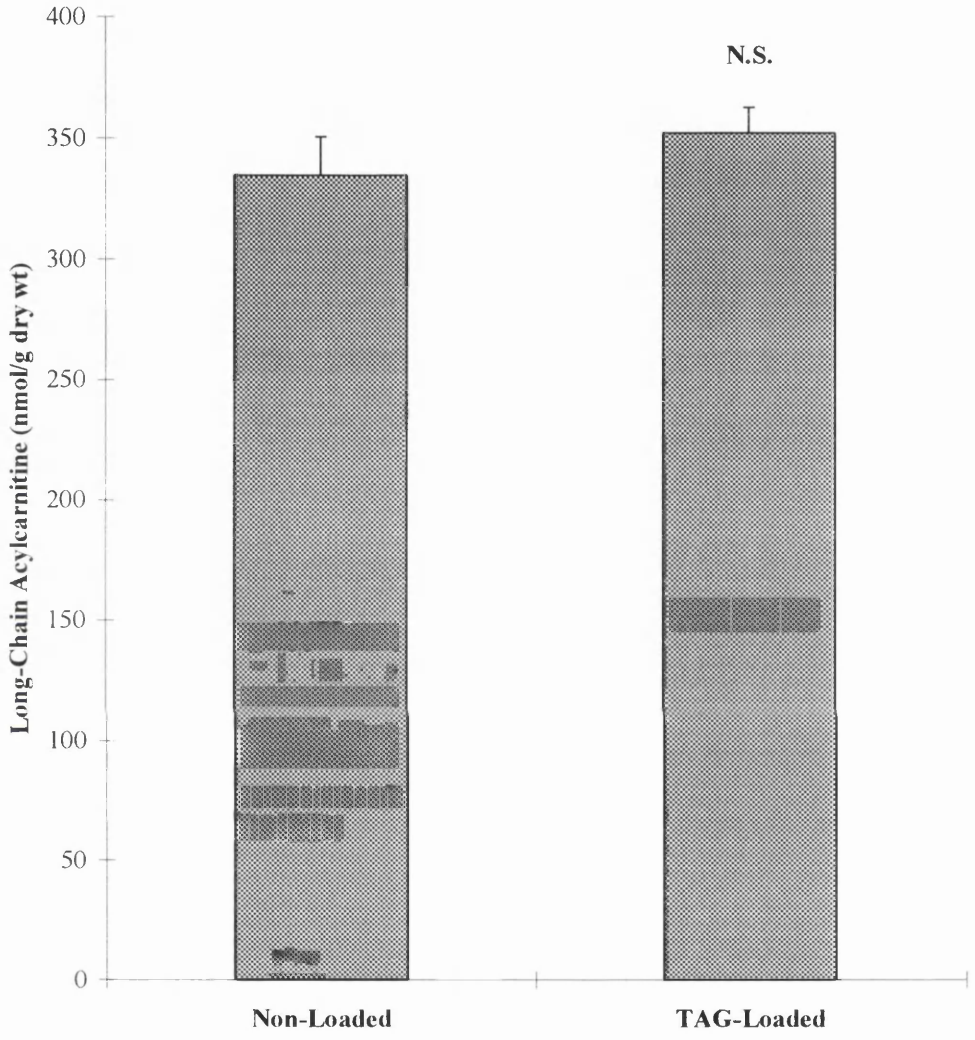
TAG-loaded and non-TAG-loaded myocytes were incubated at 37^0C for 60 min in KHB medium containing 5mM glucose and 2% BSA. Values are means ± SEM of 5-6 independent preparations (TAG-loaded n=5, non-TAG-loaded n=6). **N.S.** indicates no significant difference between TAG-loaded and non-TAG-loaded myocytes (unpaired t-test).

Figure 3.19 Effect of TAG-loading on long-chain acyl-CoA levels in cardiac myocytes



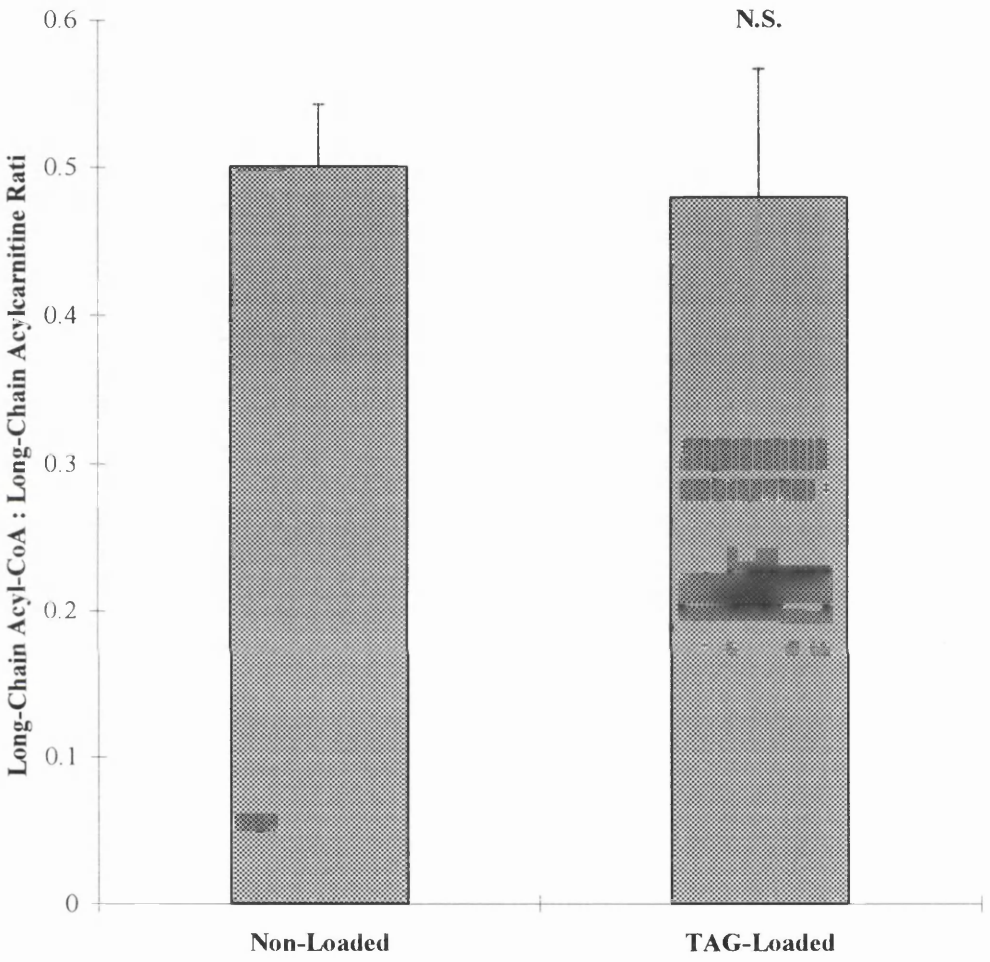
TAG-loaded and non-TAG-loaded myocytes were incubated at 37^0C for 60 min with KHB medium containing 5mM glucose and 2% BSA. Values are expressed as means ± SEM of 4 independent preparations. **N.S.** indicates no significant difference versus non-TAG-loaded (unpaired t-test).

Figure 3.20 Effect of TAG-loading on long-chain acylcarnitine levels in cardiac myocytes



TAG-loaded and non-TAG-loaded myocytes were incubated at 37^0C for 60 min with KHB medium containing 5mM glucose and 2% BSA. Values are expressed as means ± SEM of 4 independent preparations. **N.S.** indicates no significant difference versus non-TAG-loaded (unpaired t-test).

Figure 3.21 Effect of TAG-loading on the long-chain acyl-CoA : long-chain acylcarnitine ratio in cardiac myocytes



TAG-loaded and non-TAG-loaded myocytes were incubated at 37^{0}C for 60 min with KHB medium containing 5mM glucose and 2% BSA. Values are expressed as means ± SEM of 4 independent preparations. **N.S.** indicates no significant difference versus non-TAG loaded (unpaired t-test).

Table 3.5 shows the long-chain acyl-CoA and long-chain acylcarnitine levels as well as the long-chain acyl-CoA:long-chain acylcarnitine ratio for both myocytes and whole heart. The level of long-chain acyl-CoA and long-chain acylcarnitine measured in perfused rat heart was 29 ± 5 and 81 ± 12 nmol/g wet weight respectively. These values are approximately twice as high as those previously observed in isolated perfused hearts by Awan & Saggerson (1993).

The level of long-chain acyl-CoA and long-chain acylcarnitine measured in isolated non-TAG-loaded myocytes was 161 ± 19 and 335 ± 16 nmol/g dry weight respectively. Under the assumption that the myocardium is ~50% water (for which there is some evidence) these values must be halved in order to compare with those made in perfused heart. Hence, the level of long-chain acyl-CoA and long-chain acylcarnitine in non-TAG-loaded myocytes was 81 ± 10 and 167 ± 8 respectively, which is approximately 2-2.5 fold higher than measurements made in perfused heart. The reason for these quite large differences is not clear but is obviously the result of isolating myocytes from the whole heart.

Isolated myocytes are considerably more dilute compared to myocytes within a whole heart where they are tightly packed together to form the whole organ. The large dilution effect on isolation is one possible cause of the high levels of long-chain esters measured within isolated myocytes. The presence of non-myocyte cells within the whole heart may also influence the level of long-chain esters.

Tissue content of long-chain acyl-CoA and long-chain acylcarnitine can be dramatically altered by ischaemia. The effect of ischaemia on the tissue content of acyl-CoA and acylcarnitine is species-dependent and is influenced by the presence of extracellular substrates during the low-flow ischaemia. In isolated rat hearts, perfused with a fatty acid containing medium, the acylcarnitine and acyl-CoA

Table 3.5 Long-chain acyl-CoA and long-chain acylcarnitine concentrations in whole heart and cardiac myocytes

Tissue	Long-Chain Acyl-CoA	Long-Chain Acylcarnitine	Long-Chain Acyl-CoA:Long-Chain Acylcarnitine Ratio
Myocytes	161 ± 19 (n=4)	335 ± 16 (n=6)	0.5 ± 0.04 (n=4)
Whole Heart	29 ± 5 (n=6) [B]	81 ± 12 (n=6) [B]	0.36 ± 0.008 (n=6) [A]

Non-TAG-Loaded myocytes were incubated at 37^{0}C for 45 min with KHB medium containing 5mM glucose and 2% BSA. Isolated rat hearts were perfused at 37^{0}C for 45 min with KHB medium containing 5mM glucose and 2% BSA as described in section 2.7. After 45 min hearts were removed from perfusion apparatus and immediately freeze clamped in liquid nitrogen. Long-chain acyl-CoA's and long-chain acyl-carnitines were extracted and measured as described in section 2.8.3 and 2.8.4. Myocyte values are nmol/g dry weight. Perfused heart values are nmol/g wet weight. Values are means ± SEM of 4-6 independent preparations. [A, B] indicates $p<0.02$ and <0.001 respectively versus myocytes (unpaired t-test).

content has been shown to increase by a factor of 5.5 and 2 respectively during low-flow ischaemia (Neely & Feuvray, 1981). In low-flow ischaemic, glucose-perfused rat hearts, a moderate accumulation of acylcarnitine was observed, whereas acyl-CoA levels were increased to the same extent as palmitate-perfused rat hearts (Neely & Feuvray, 1981). Accumulation of long-chain acyl-CoA and long-chain acylcarnitine is a rapid process occurring within 5 min after the onset of low-flow ischaemia (Whitmer *et al*, 1978).

It is possible that during the isolation procedure, cardiac myocytes may have experienced a short period which is comparable to low-flow ischaemia in perfused hearts. This most likely occurred when myocytes were left to settle after they had been digested away from the heart. During this part of the isolation procedure, although the KHB medium is gassed, myocytes are not agitated for up to 21 min and may become ischaemic due to the lack of flow around the cells. Therefore, the high levels of long-chain acyl-CoA and long-chain acylcarnitine measured in isolated myocytes could be due to them experiencing some degree of ischaemia during the isolation procedure. If this hypothesis is correct then it must take a long time for the long-chain ester levels to return to original levels. As a result, measurements of these long-chain esters in myocytes are probably not as informative as they might have been. This may also explain why expected changes in long-chain acyl-CoA and long-chain acylcarnitine levels due to changes in malonyl-CoA levels, were not always observed.

The ratio of these long-chain esters was significantly higher in isolated non-TAG-loaded myocytes than in perfused hearts (table 3.5). A higher long-chain acyl-CoA:long-chain acylcarnitine ratio indicates that transport of long-chain acyl-CoA into mitochondria, and hence fatty acid oxidation, is lower in isolated myocytes

than in perfused hearts. This difference may be the result of myocytes experiencing some degree of ischaemia during isolation, since during ischaemia fatty acid oxidation has been shown to be depressed (Opie, 1968; Lerch *et al*, 1985). It could also reflect the lower energy demand of a quiescent myocyte compared with a beating heart.

3.4.4 Conclusions

Addition of exogenous fatty acids such as palmitate inhibits ACC activity in both TAG-loaded and non-TAG-loaded myocytes. The mechanism for this inhibition is not thought to be by direct allosteric regulation (due to the abundance of fatty acid binding proteins) but by phosphorylation by AMPK. AMPK is activated due to a rise in AMP levels as exogenous fatty acids (palmitate) are activated by FAS, requiring ATP. Acetyl-CoA produced from β-oxidation of fatty acids is not thought to be transported to the cytosol, but is directed into the citric acid cycle. Therefore, increased fatty acid oxidation will not increase ACC activity via increased acetyl-CoA supply. It seems logical that exogenous fatty acids would decrease malonyl-CoA levels such that fatty acid transport into mitochondria is uninhibited and β-oxidation of fatty acids be responsible for a greater proportion of the ATP production in the myocyte.

Insulin increased malonyl-CoA content in non-TAG-loaded myocytes but not in TAG-loaded myocytes. The lack of insulin response in TAG-loaded myocytes is discussed in section 3.4.6. The mechanism by which insulin increases malonyl-CoA levels in non-TAG-loaded myocytes is thought not to be via an increase in ACC intrinsic activity but by an increase in acetyl-CoA supply from the PDH complex. Acetyl-CoA from the oxidation of carbohydrate is thought to be readily

transported to the cytosol by CAT and carnitine:acetylcarnitine translocase for reaction with and stimulation of ACC.

Insulin did not overcome the effect of palmitate to decrease malonyl-CoA levels. If palmitate is indeed decreasing malonyl-CoA level via an increase in AMP, AMPK activity and hence phosphorylation and inactivation of ACC, then an increase in acetyl-CoA supply to ACC from the PDH complex due to insulin would be of little consequence.

One must not forget the possible importance of malonyl-CoA disposal as a means of regulating malonyl-CoA levels. Changes in malonyl-CoA disposal in combination with alterations in ACC activity could provide a mechanism for rapidly changing malonyl-CoA levels. Malonyl-CoA decarboxylase may be one such route of malonyl-CoA disposal. The fact that malonyl-CoA decarboxylase is inhibited by acetyl-CoA may provide the link between ACC activity and malonyl-CoA disposal.

Measurements of long-chain esters as an indication of CPT 1 activity in isolated myocytes are not reliable due to the fact that they appear significantly increased in isolated myocytes compared with perfused hearts. This might be due to myocytes being exposed to a short period of ischaemia which is known to cause accumulation of long-chain esters. Therefore, although these measurements do in some cases support the hypothesis that changes in malonyl-CoA levels are paralleled by changes in CPT 1 activity, in other cases they do not.

3.4.5 Effect of Glucose Concentration and Insulin on Fatty Acid Metabolite Levels in Cardiac Myocytes

Insulin was previously shown to have no significant effect on malonyl-CoA level in TAG-loaded myocytes (fig 3.10). In the intact heart, substrate (glucose)

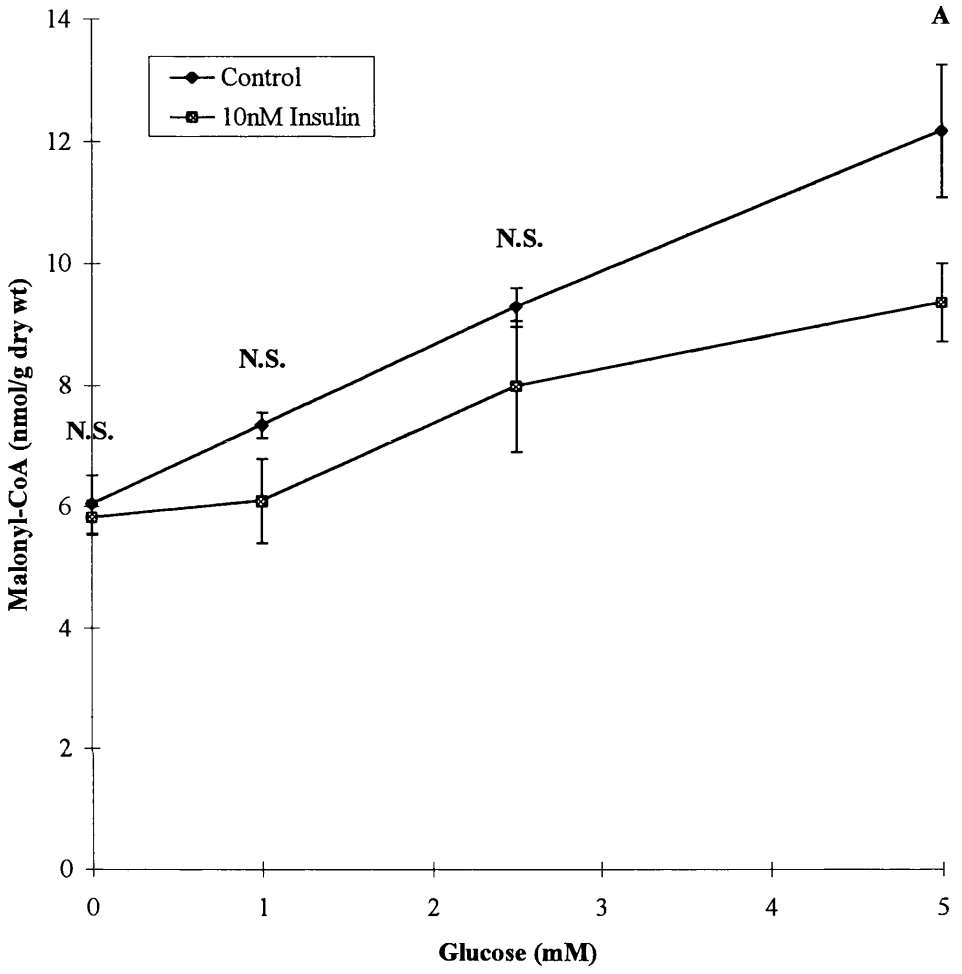
availability is dependent upon the coronary circulation. The presence of non-myocyte cells within the whole heart may also affect the availability of glucose to the myocytes. In isolated myocytes, the heart cells are in direct contact with the incubation medium and so there is no restraint on substrate availability. As a result, it was thought that the concentration of glucose present in myocyte incubations was such that insulin may be unable to stimulate glucose utilisation any further. If this were true, then insulin should exert an effect when the concentration of glucose in myocyte incubations is lowered. This hypothesis was tested by incubating myocytes in a range of glucose concentrations (0-5mM) in the presence or absence of insulin (10nM).

3.4.5.1 Effect of Glucose Concentration and Insulin on Malonyl-CoA Levels in TAG-loaded and Non-TAG-loaded Myocytes

As shown in figure 3.22, increasing glucose concentration resulted in a parallel increase in malonyl-CoA levels in TAG-loaded myocytes. The increase in malonyl-CoA from 0-1.0mM glucose was not significant, while the increase from 1.0-2.5mM ($p<0.025$) and from 2.5-5.0mM glucose ($p<0.05$) were significant (paired t-test). The mechanism by which glucose regulates malonyl-CoA levels in myocytes is discussed in detail in section 3.2.2 and is thought to be via an increase in acetyl-CoA supply to ACC.

In the presence of insulin, increasing glucose concentration also resulted in an increase in malonyl-CoA levels in TAG-loaded myocytes. Again the increase in malonyl-CoA from 0-1.0mM glucose was not significant, while the increase from 1.0-2.5mM ($p<0.05$) and 2.5-5.0mM glucose ($p<0.05$) were significant (using paired t-test). However, incubation with insulin did not result in an increase

Figure 3.22 Effect of glucose concentration and insulin on malonyl-CoA levels in TAG-loaded myocytes



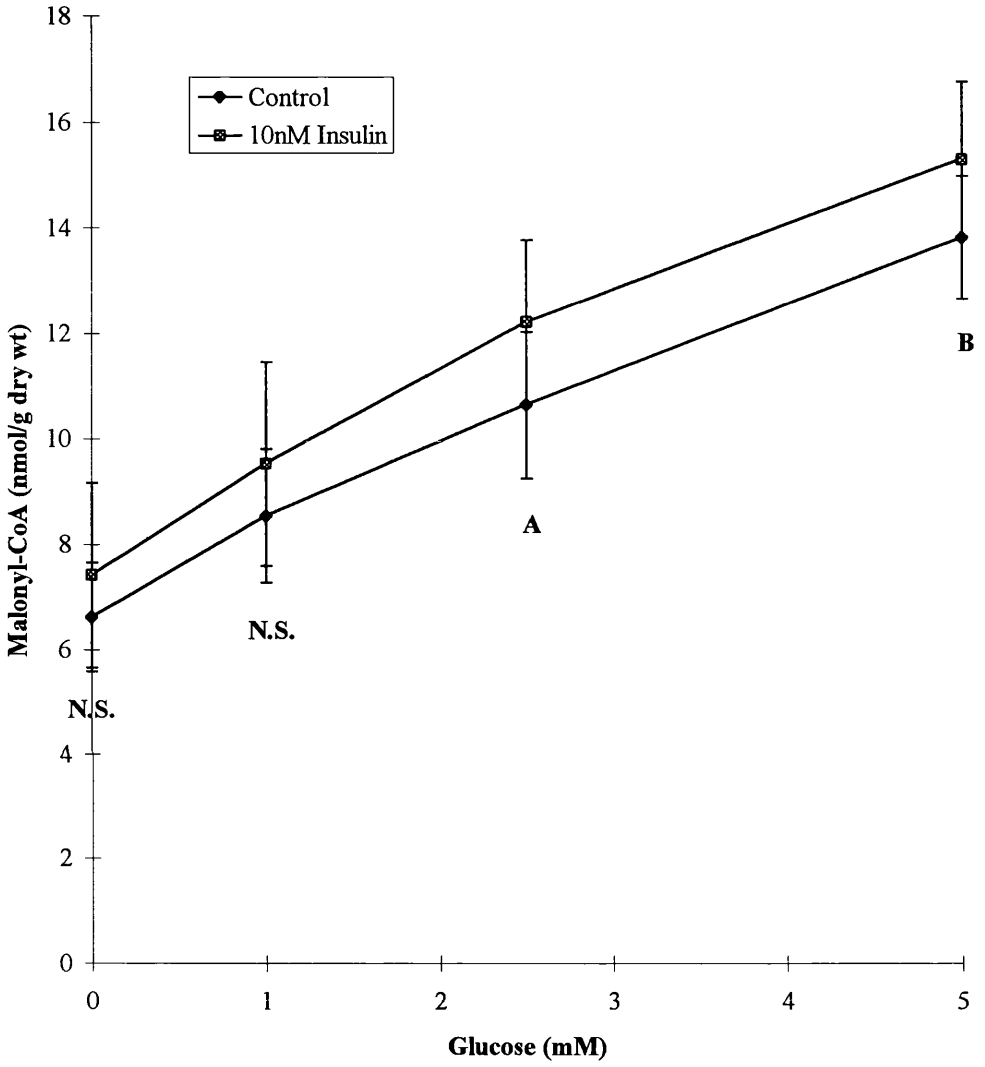
TAG-loaded myocytes were incubated at 37^{0}C for 60 min with KHB medium containing 2% BSA and indicated concentration of glucose, in the presence or absence of 10nM insulin. Values are means ± SEM of 4 independent preparations. **A**, **N.S.**, represent $p<0.05$ and no significant difference respectively versus insulin (paired t-test).

malonyl-CoA levels at any tested concentration of glucose. In fact, malonyl-CoA levels tended to be lower in the presence of insulin, especially at a glucose concentration of 5mM where they were significantly lower (fig 3.22). The reason for this decrease in malonyl-CoA upon incubation with insulin is unclear. However, it disproves the hypothesis that insulin is unable to exert an effect on malonyl-CoA level in TAG-loaded myocytes because the glucose concentration in the incubation medium is such that no further stimulation is possible. Therefore, this lack of insulin response must be the result of TAG-loading.

Increasing glucose concentration in non-TAG-loaded myocytes, both in the presence or absence of insulin, resulted in a parallel increase in malonyl-CoA levels (fig 3.23). In the absence of insulin each increase in glucose concentration resulted in a significant increase in malonyl-CoA (0-1.0mM $p<0.005$, 1.0-2.5mM $p<0.01$, 2.5-5.0mM $p<0.01$) (paired t-test). In the presence of insulin each increase in glucose also resulted in a significant increase in malonyl-CoA (0-1.0 $p<0.025$, 1.0-2.5 $p<0.005$, 2.5-5.0 $p<0.005$) (paired t-test). However, in contrast to TAG-loaded myocytes, insulin significantly increased malonyl-CoA levels at glucose concentrations of 2.5mM and 5.0mM glucose by ~16% and ~30% respectively (fig 3.23). The mechanism by which insulin increases malonyl-CoA levels in non-TAG-loaded myocytes is discussed in section 3.4.1.3 and is thought to involve an increase in acetyl-CoA supply to ACC as opposed to an increase in ACC activity.

It should be noted that both TAG-loaded and non-TAG-loaded myocytes are isolated in buffers containing 10mM glucose. Glucose was absent from the final wash of the isolation procedure in an attempt to remove all glucose prior to incubation. However, it is not possible to remove all glucose from the myocytes in this way. As a result, a small, unknown amount of extra glucose is present in these

Figure 3.23 Effect of glucose concentration and insulin on malonyl-CoA levels in non-TAG-loaded myocytes



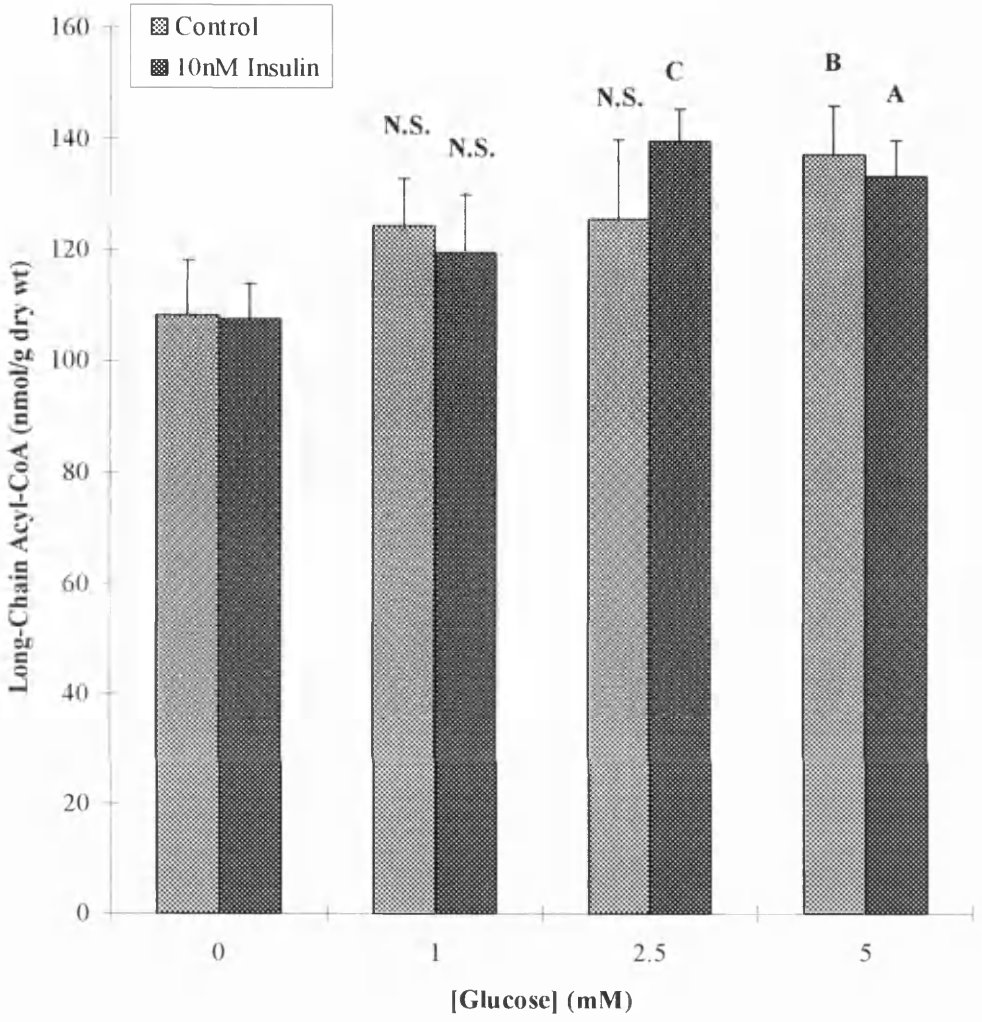
Non-TAG-loaded myocytes were incubated at 37^{0}C for 60 min with KHB medium containing 2% BSA and indicated concentration of glucose, in the presence or absence of 10nM insulin. Values are means ± SEM of 5 independent preparations. **A**, **B**, **N.S.**, represent $p<0.05$, <0.025 and no significant difference respectively versus insulin (paired t-test).

incubations. This might help to explain why the levels of malonyl-CoA in both TAG-loaded and non-TAG-loaded myocytes, at a glucose concentration of 5mM, are significantly higher than was observed in sections 3.4.1.1 and 3.4.1.3 respectively. For the same reason, the 0mM glucose incubation will actually contain a small unknown amount of glucose which will result in the level of malonyl-CoA being higher than expected. This problem of glucose removal can be solved by glucose depleting myocytes prior to incubation, as was done in section 3.2.2. Despite this, it can still be concluded that the lack of response to insulin by TAG-loaded myocytes is not due to the relatively high concentration of glucose present in the incubation medium and must therefore be a direct result of TAG-loading. This effect of TAG-loading is discussed below in section 3.4.5.3.

3.4.5.2 Effect of Glucose Concentration and Insulin on Long-chain Acyl-CoA and Long-chain Acylcarnitine Levels in Non-TAG-loaded Myocytes

Unfortunately, measurements of long-chain esters were not made in TAG-loaded myocytes to compare with those made in non-TAG-loaded myocytes. Measurements of long-chain esters were made to give an indication of CPT 1 activity, although as previously discussed in section 3.4.3, these measurements are not thought to be a good indication of CPT 1 activity in isolated cardiac myocytes. In the presence or absence of insulin, increasing glucose concentration resulted in a parallel increase in long-chain acyl-CoA level which was significant at a glucose concentration of 5mM (fig 3.24). Insulin did not result in an increase in long-chain acyl-CoA level at any concentration of glucose (fig 3.24), despite the fact that malonyl-CoA level was significantly higher than in the absence of insulin (fig 3.23). Given that malonyl-CoA level more than doubled (in the presence or absence of insulin) from 0mM to 5mM glucose, the resulting increase in long-chain acyl-CoA

Figure 3.24 Effect of glucose concentration and insulin on long-chain acyl-CoA levels in non-TAG-loaded myocytes



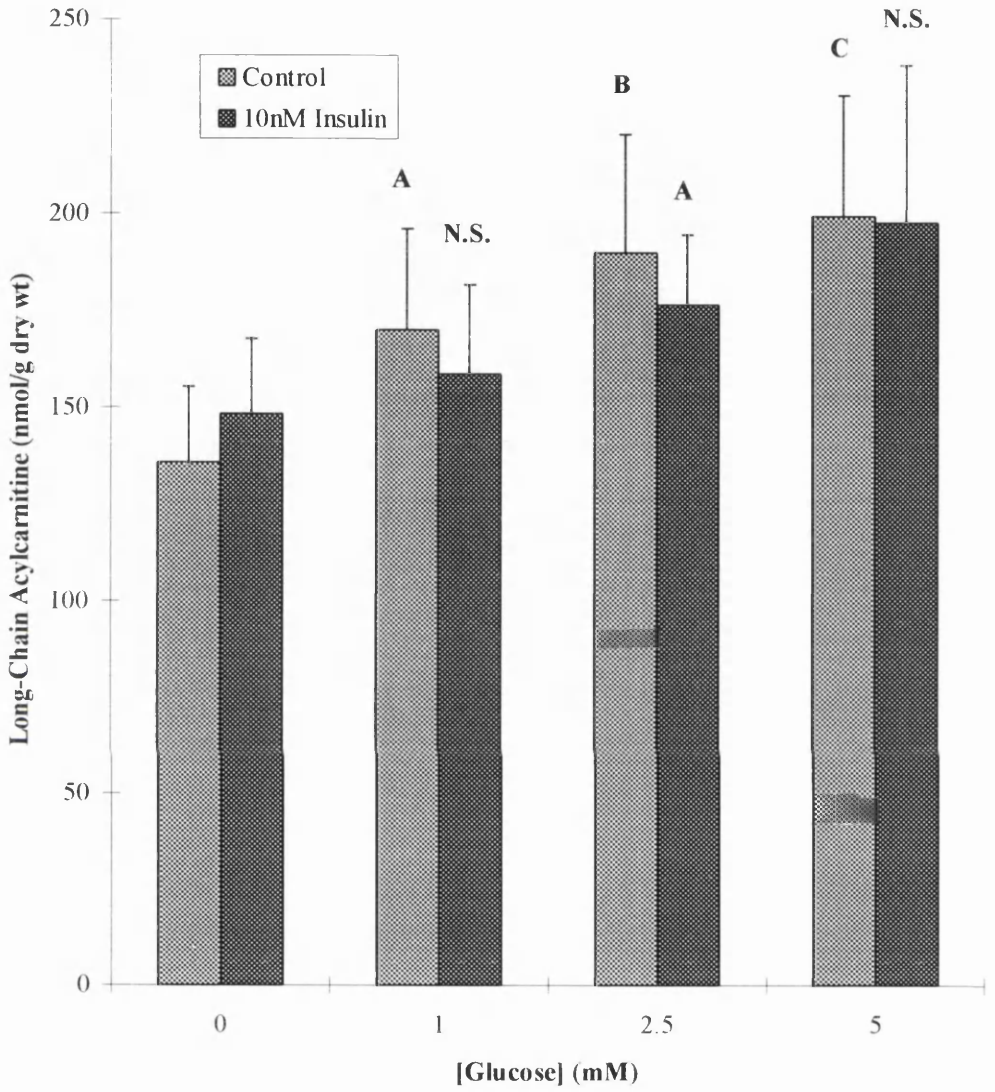
Non-TAG-loaded myocytes were incubated at 37^{0}C for 60 min with KHB medium containing 2% BSA and indicated concentrations of glucose, in the presence or absence of 10nM insulin. Values are means ± SEM of 4 independent preparations. **A**, **B**, **C**, and **N.S.** represent $p<0.05$, <0.025, <0.01 and no significant difference respectively versus control (paired t-test).

is surprisingly low. This might suggest that a considerable increase in malonyl-CoA is required to have a significant effect on long-chain acyl-CoA level. However, this is unlikely since the heart isoform of CPT 1 is highly sensitive to inhibition by malonyl-CoA and should be dramatically inhibited by such a large increase in malonyl-CoA.

Long-chain acylcarnitine levels also increased in parallel with glucose concentration both in the presence or absence of insulin. This increase in long-chain acylcarnitine level compared with 0mM glucose incubation was significant at concentrations of 2.5mM and 5mM glucose (fig 3.25). This was very unexpected since malonyl-CoA level was shown to increase with glucose concentration (fig 3.23). An increase in malonyl-CoA level should inhibit CPT 1 resulting in a decrease in long-chain acylcarnitine. Insulin had no significant effect on long-chain acylcarnitine level at any concentration of glucose (fig 3.25), even though a significant increase in malonyl-CoA level was observed in the presence of insulin. The reason for this increase in long-chain acylcarnitine levels is unclear but would appear to be a result of increasing glucose concentration as opposed to changes in malonyl-CoA levels and CPT 1 activity.

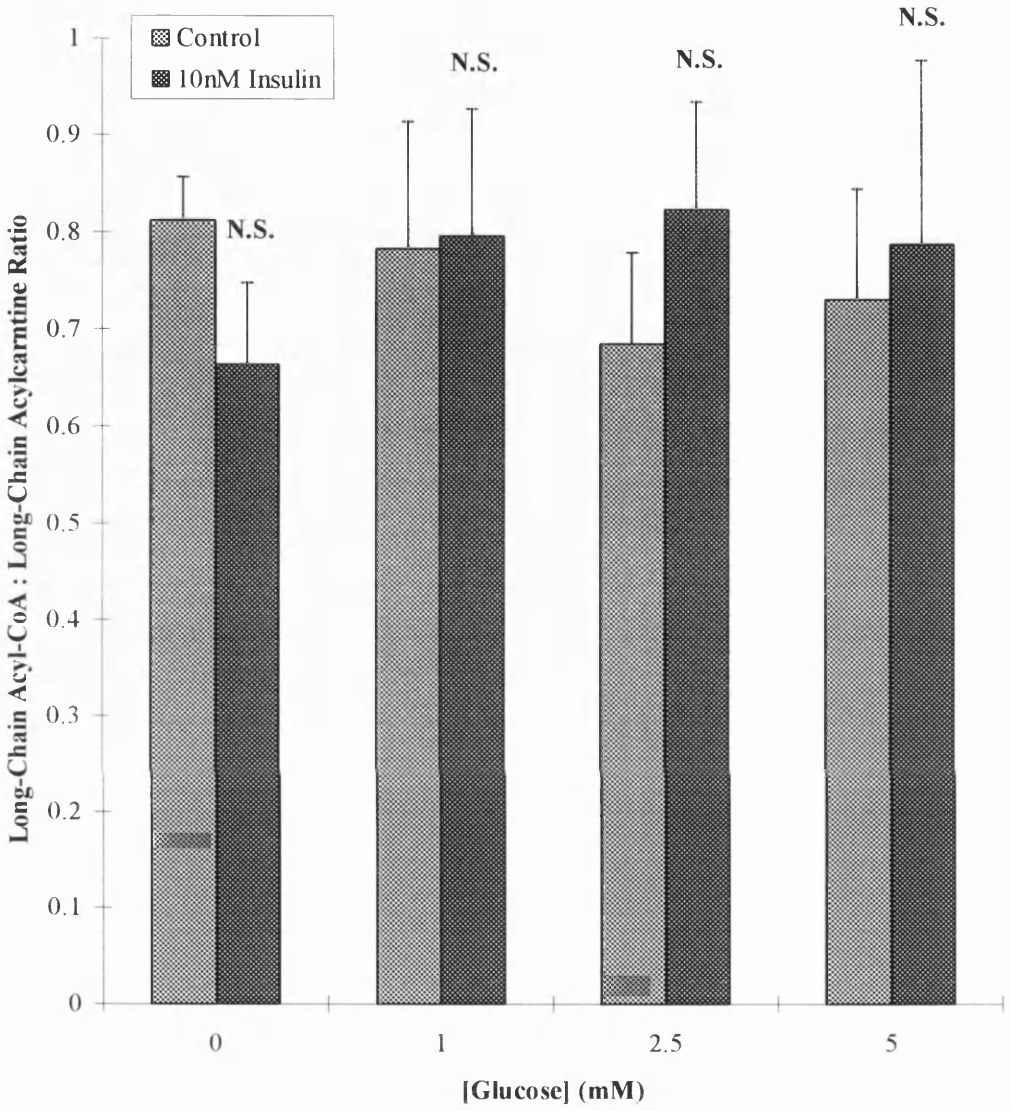
Increasing glucose concentration in the presence or absence of insulin had no significant effect on the long-chain acyl-CoA:long-chain acylcarnitine ratio (fig 3.26). This would suggest that there is no change in CPT 1 activity in spite of the marked increase in malonyl-CoA level. It seems more likely that other factors are affecting the levels of these long-chain esters making them unreliable as an indicator of CPT 1 activity (see section 3.4.3).

Figure 3.25 Effect of glucose concentration and insulin on long-chain acylcarnitine levels in non-TAG-loaded myocytes



Non-TAG-loaded myocytes were incubated at 37^{0}C for 60 min with KHB medium containing 2% BSA and indicated concentrations of glucose, in the presence or absence of 10nM insulin. Values are means ± SEM of 4 independent preparations. **A**, **B**, **C**, and **N.S.** represent $p<0.05$, <0.025, <0.01 and no significant difference respectively versus control (paired t-test).

Figure 3.26 Effect of glucose concentration and insulin on the long-chain acyl-CoA:long-chain acylcarnitine ratio in non-TAG-loaded myocytes



Non-TAG-loaded myocytes were incubated at 37^{0}C for 60 min with KHB medium containing 2% BSA and indicated concentrations of glucose, in the presence or absence of 10nM insulin. Values are means ± SEM of 4 independent preparations. **N.S.** indicates no significant difference versus control (paired t-test).

3.4.6 Effect of TAG-loading on Malonyl-CoA Levels in Response to Glucose Concentration and Insulin

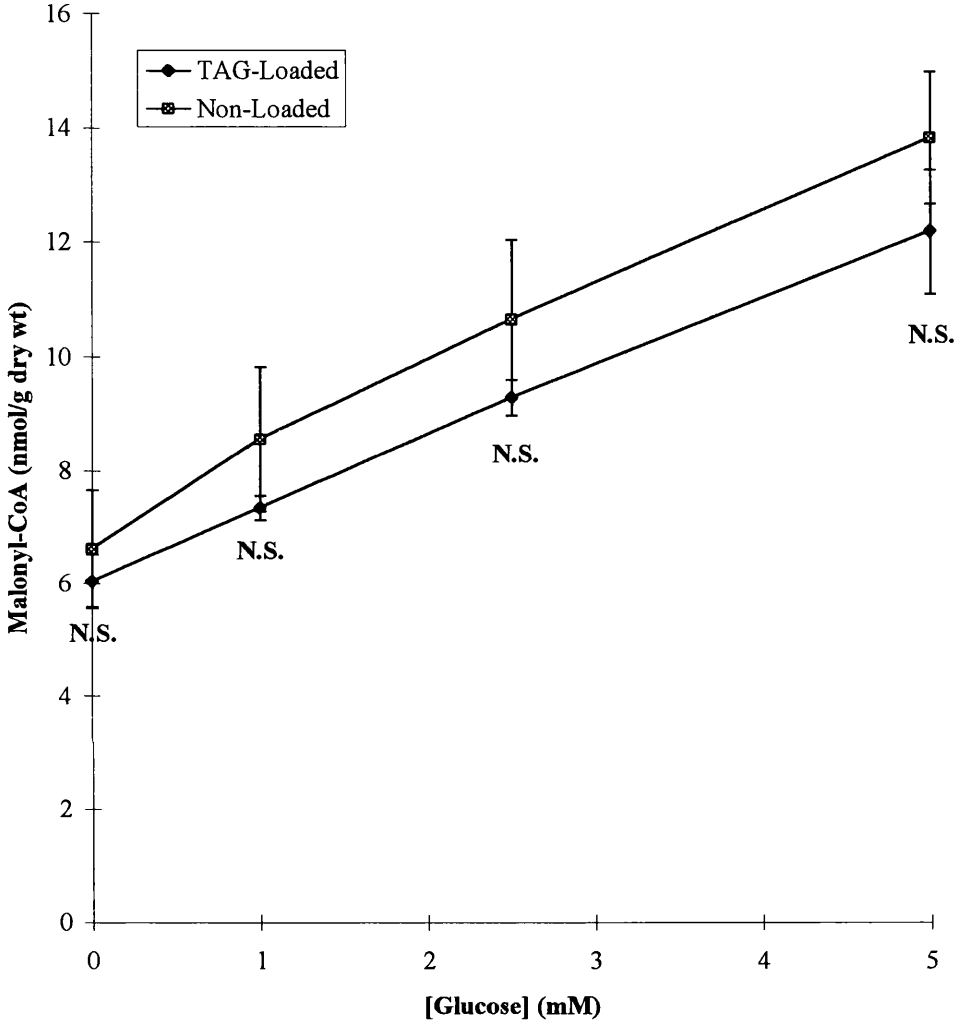
As previously discussed, in the absence of insulin, the level of malonyl-CoA in both TAG-loaded and non-TAG-loaded myocytes increased linearly with increasing glucose concentration (fig 3.27). No significant difference in malonyl-CoA level was observed at any concentration of glucose, between TAG-loaded and non-loaded myocytes (fig 3.27). This suggests that TAG-loading does not influence the glucose induced increase in malonyl-CoA level.

In the presence of insulin, the level of malonyl-CoA in non-TAG-loaded myocytes was higher than in TAG-loaded myocytes at all concentrations of glucose and significantly higher at concentrations of 2.5mM and 5mM glucose (fig 3.28). In fact, in the presence of insulin and 5mM glucose, the level of malonyl-CoA in non-TAG-loaded myocytes was ~64% higher than in TAG-loaded myocytes (fig 3.28). The fact that TAG-loading has no effect on the glucose induced increase in malonyl-CoA but abolishes the effect of insulin to increase malonyl-CoA might suggest that the effects of insulin and glucose on malonyl-CoA are independent.

TAG-loading does not alter the glucose induced increase in malonyl-CoA and therefore is unlikely to interfere with either glucose transport, PDH activity, the transport of acetyl-CoA from the mitochondria to the cytosol or ACC activity. Therefore, it suggests that TAG-loading interferes with the insulin response at the level of either the insulin receptor or some part of the insulin signaling pathway. Further work is required to elucidate the exact point of the pathway which is affected and how.

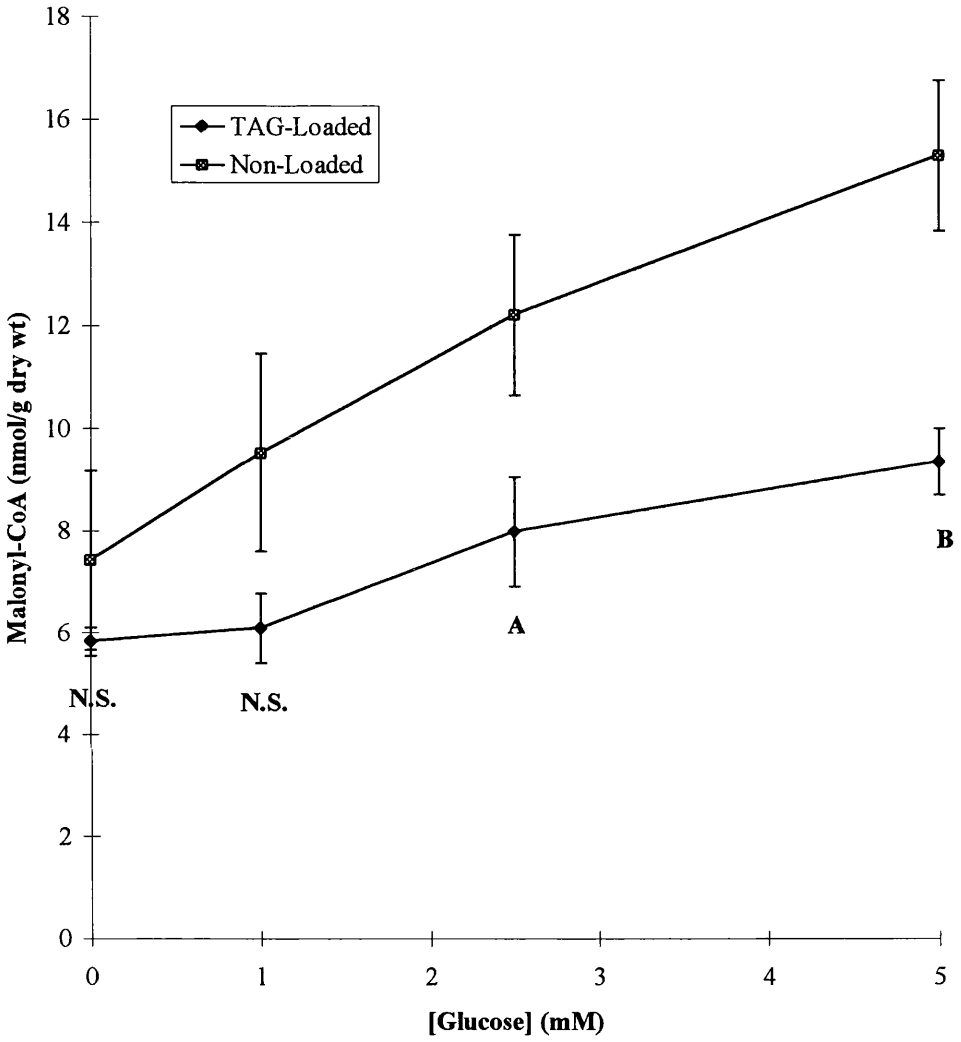
Awan & Saggerson (1993), demonstrated that insulin (in the presence of glucose) significantly increases malonyl-CoA content in perfused rat hearts. It is possible

Figure 3.27 Effect of TAG-loading and glucose concentration on malonyl-CoA levels in cardiac myocytes



TAG-loaded and non-TAG-loaded myocytes were incubated at 37^{0}C for 60 min with KHB medium containing 2% BSA and indicated concentrations of glucose. Values are means ± SEM of 4-5 preparations (TAG-loaded n=4, non-TAG-loaded n=5). **N.S.** indicates no significant difference versus non-TAG-loaded myocytes (unpaired t-test).

Figure 3.28 Effect of TAG-loading and glucose concentration on malonyl-CoA levels in insulin treated cardiac myocytes



TAG-loaded and non-TAG-loaded myocytes were incubated at 37^{0}C for 60 min with KHB medium containing 10nM insulin, 2% BSA and indicated concentrations of glucose. Values are means ± SEM of 4-5 independent preparations (TAG-loaded n=4, non-TAG-loaded n=5). **A**, **B** and **N.S.** indicate $p<0.05$, <0.01 and no significant difference versus non-TAG-loaded myocytes (unpaired t-test).

that the perfused heart system used in their experiments would be TAG-depleted after 45 min of perfusion. This may be why an effect of insulin was observed in these perfused hearts.

TAG-loading has been shown to have other affects on myocyte metabolism. TAG-loading significantly effects the rate of lipolysis in cardiac myocytes in the presence of exogenous fatty acids (section 3.5.3). The effect of adrenaline on fatty acid oxidation is also altered by TAG-loading. Adrenaline has been shown to increase fatty acid oxidation in non-TAG-loaded myocytes (Awan & Saggerson, 1993) but has been shown to have the opposite effect in TAG-loaded myocytes (Swanton & Saggerson, 1997b).

3.4.7 Effect of AICAR on Malonyl-CoA Levels in TAG-loaded Myocytes

As previously suggested, palmitate may exert its effect on malonyl-CoA levels in cardiac myocytes indirectly via phosphorylation and inactivation of ACC by AMPK (see section 3.4.1). This hypothesis was tested with the use of 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), a cell permeable precursor of 5-aminoimidazole-4-carboxamide ribonucleoside monophosphate (ZMP). ZMP is an AMP analogue known to activate AMPK and AMPK kinase to a similar degree as AMP.

AICAR has been shown to be taken up (via a nucleoside transporter) and metabolised to ZMP by a number of mammalian tissues including hepatocytes (Vincent *et al*, 1991; Corton *et al*, 1995), adipocytes (Sullivan *et al*, 1994; Corton *et al*, 1995), human erythrocytes (Zimmerman & Deeprose, 1978), fibroblasts (Thomas *et al*, 1981), skeletal muscle and heart (Sabina *et al*, 1982). Once synthesised within the cell, ZMP can be further phosphorylated to ZTP or

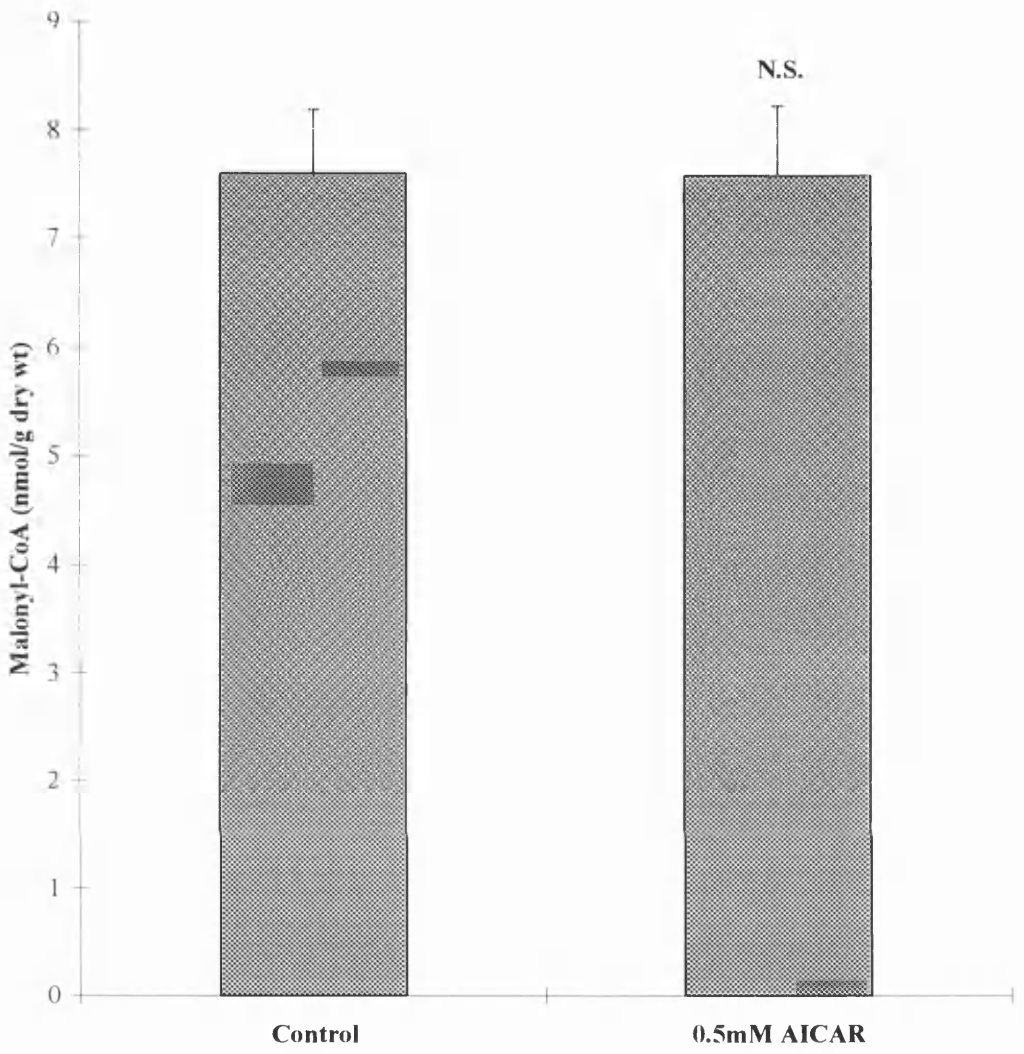
metabolised to inosine monophosphate (IMP), which can be further metabolised to adenine and guanine or degraded to hypoxanthine or uric acid.

TAG-loaded myocytes were incubated for 60 min in KHB medium containing 5mM glucose, 2% BSA, in the presence or absence of 0.5mM AICAR.

AICAR had no significant effect on malonyl-CoA levels in TAG-loaded myocytes at the end of the 60 min incubation (fig 3.29). In the process of determining the effect of AICAR in myocytes, both on malonyl-CoA levels and rates of lipolysis (see section 3.5.4), Hardie & Carling (1997), published a paper which explained that while in some cells ZMP accumulates to millimolar concentrations, in others, ZMP levels do not accumulate at all due to low adenosine kinase activity. Myocytes are one of those cell types which does not accumulate ZMP due to low adenosine kinase activity and hence AICAR has little or no effect in isolated cardiac myocytes. Without making measurements of ZMP and/or AMPK activity, this negative result is not proof that AMPK is not involved in the phosphorylation and inactivation of ACC.

Recent work by Alam & Saggerson (1998), has shown that AICAR is taken up and metabolised to ZMP in isolated soleus muscle strips. The accumulation of ZMP within soleus muscle strips was shown using HPLC chromatography. Incubation of soleus muscle strips with 1mM AICAR significantly decreased malonyl-CoA levels by ~65%, presumably as a result of AMPK activation and subsequent ACC phosphorylation and inactivation. In the presence of insulin, AICAR was shown to antagonize the effects of insulin by significantly decreasing the malonyl-CoA content of soleus muscle strips (Alam & Saggerson, 1998). From these results it seems likely that the heart/muscle isoform of ACC (ACC-280) is regulated by

Figure 3.29 Effect of 0.5mM AICAR on malonyl-CoA in TAG-loaded myocytes



TAG-loaded myocytes were incubated at 37^{0}C for 60 min with KHB medium containing 5mM glucose and 2% BSA, in the presence or absence of 0.5mM AICAR. Values are expressed as means ± SEM of 4-6 independent preparations (control n=6, AICAR n=4). **N.S.** indicates no significant difference versus control (unpaired t-test).

AMPK resulting in changes in malonyl-CoA content and concomitant changes in fatty acid oxidation.

3.4.8 Effect of Adrenaline, Isoprenaline, and Phenylephrine on Malonyl-CoA Levels in Insulin-Treated, Non-TAG-loaded Myocytes

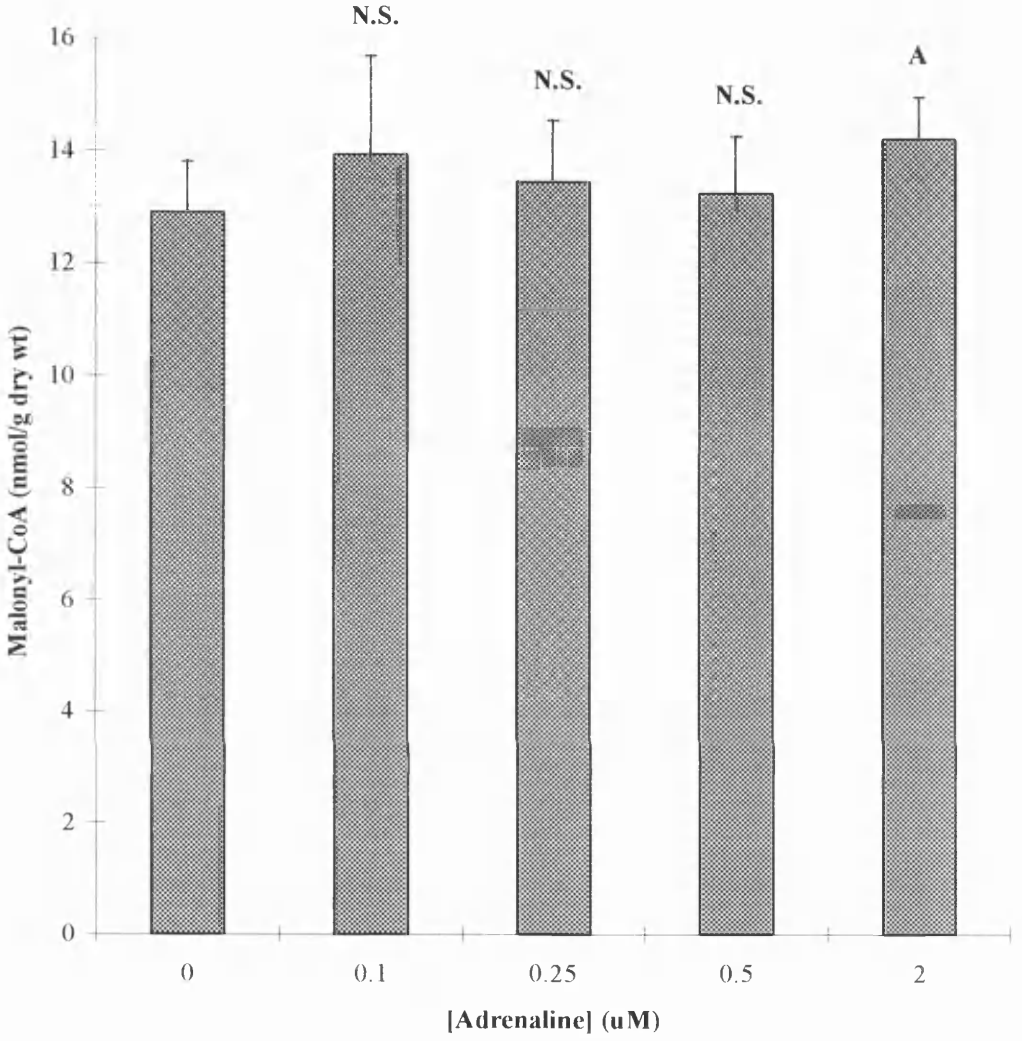
Isolated non-TAG-loaded myocytes were incubated for 60 min at 37^0C in KHB medium containing 5mM glucose, 2% BSA, 10nM insulin and a range of concentrations of adrenaline (0-2μM), isoprenaline (0-1μM), and phenylephrine (0-1μM).

Non-TAG-loaded myocytes were used in these experiments because they had previously been shown to produce a significant increase in malonyl-CoA content in response to insulin. Indeed, incubation of non-TAG-loaded myocytes with insulin alone resulted in a malonyl-CoA content of between 12-13.5 nmol/g dry weight (figs 3.30, 3.31, 3.32). This is significantly higher than the malonyl-CoA levels previously determined in non-TAG-loaded myocytes incubated in the absence of insulin (see section 3.4.1.3), and indicates a good response to insulin by these myocytes.

Adrenaline had no significant effect on malonyl-CoA levels at concentrations of 0, 0.1, 0.25 and 0.5μM (fig 3.30). At a concentration of 2.0μM, adrenaline increased malonyl-CoA levels very slightly (but significantly) (fig 3.30). This result differs from that previously observed in perfused hearts from fed rats, whereby adrenaline (5μM), in the presence of insulin, decreased malonyl-CoA levels by 47% (Awan & Saggerson, 1993).

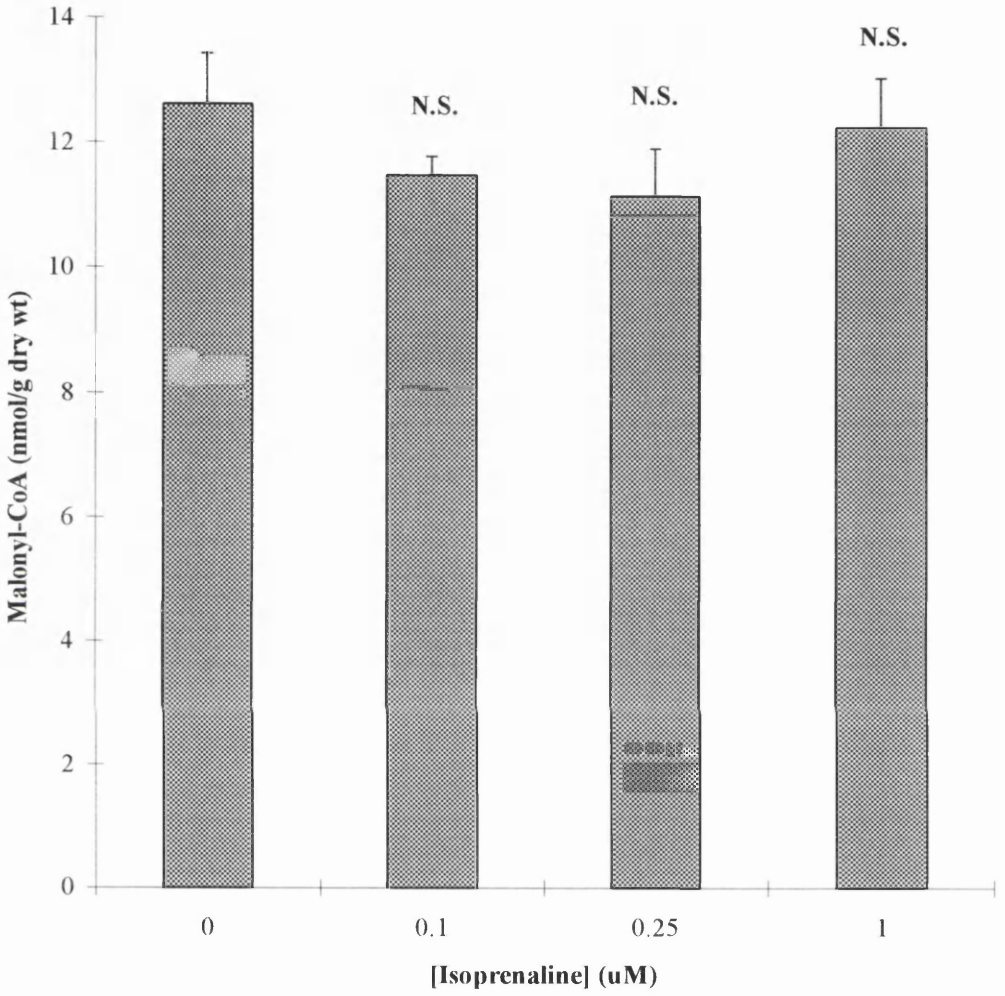
Adrenaline increases cAMP levels which activates PKA. Although ACC-280 has been shown to be a substrate for PKA (Winder *et al*, 1997), PKA-mediated

Figure 3.30 Effect of adrenaline concentration on malonyl-CoA levels in insulin -treated, non-TAG-loaded myocytes



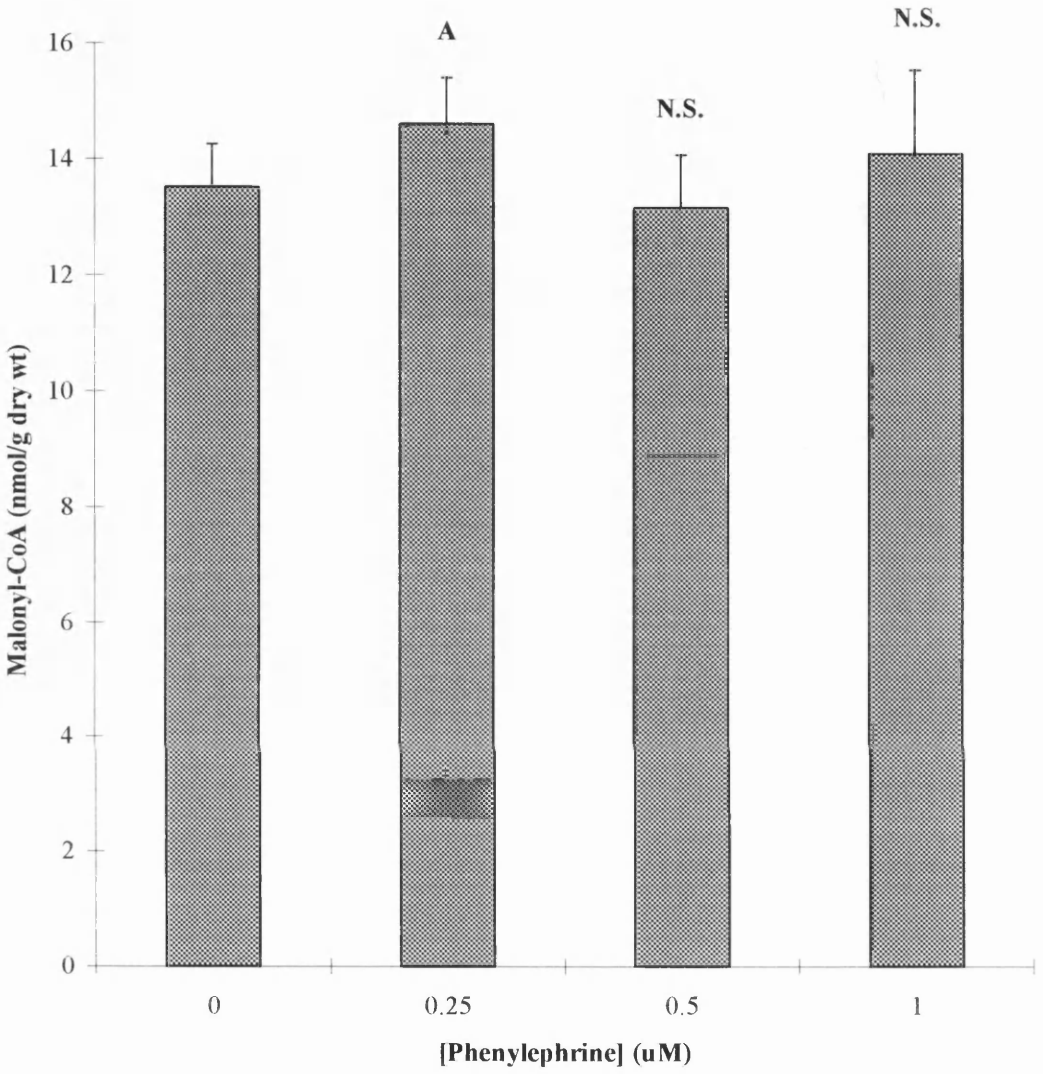
Non-TAG-loaded myocytes were incubated at 37^{0}C for 60 min with KHB medium containing 5mM glucose, 2% BSA, 10nM insulin and indicated concentration of adrenaline. Values are means ± SEM of 6 independent preparations. **A**, **N.S.** represent $p<0.005$ and no significant difference respectively versus 0μM adrenaline (paired t-test).

Figure 3.31 Effect of isoprenaline concentration on malonyl-CoA levels in insulin -treated non-TAG-loaded myocytes



Non-TAG-loaded myocytes were incubated at 37^{0}C for 60 min with KHB medium containing 5mM glucose, 2% BSA, 10nM insulin and indicated concentration of isoprenaline. Values are means ± SEM of 5 independent preparations. **N.S.** represents no significant difference versus 0μM isoprenaline (paired t-test).

Figure 3.32 Effect of phenylephrine concentration on malonyl-CoA levels in insulin -treated, non-TAG-loaded myocytes



Non-TAG-loaded myocytes were incubated at 37^{0}C for 60 min with KHB medium containing 5mM glucose, 2% BSA, 10nM insulin and indicated concentration of adrenaline. Values are means ± SEM of 4 independent preparations. **A**, **N.S.** represent $p<0.05$ and no significant difference respectively versus 0μM phenylephrine (paired t-test).

phosphorylation has no effect on ACC activity. It has been suggested that hormones which increase cAMP levels, such as adrenaline, increase phosphorylation at AMPK sites on ACC, possibly via inhibition of protein phosphatase activity through phosphorylation of protein phosphatase inhibitor-1 by PKA (Hardie, 1989). Insulin decreases cAMP levels and therefore will oppose the effects of adrenaline on AMPK activation possibly by an increase in phosphatase activity.

Insulin is thought to increase malonyl-CoA levels in non-TAG-loaded myocytes primarily by increasing acetyl-CoA supply to ACC via activation of glucose transport and PDH activity. It was previously demonstrated that palmitate overcame the stimulatory effect of insulin on malonyl-CoA levels in non-TAG-loaded myocytes by inhibiting ACC activity (see section 3.4.1.3). The fact that adrenaline was unable to overcome the effect of insulin indicates that it does not act via the same mechanism i.e. phosphorylation and inactivation of ACC by AMPK. The difference in results obtained in non-TAG-loaded myocytes compared with perfused hearts from fed rats (Awan & Saggerson, 1993) may be due to lower endogenous TAG levels in non-TAG-loaded myocytes such that adrenaline has less of a lipolytic effect. However, the TAG content of perfused hearts was not determined and so may have been comparable with that of non-TAG-loaded myocytes.

Given that adrenaline had no significant effect on the insulin stimulated increase in malonyl-CoA, it was not surprising that the β-receptor agonist isoprenaline had no significant effect on malonyl-CoA levels at all concentrations tested (fig 3.31), or that the α_1-adrenergic receptor agonist phenylephrine had no significant effect on malonyl-CoA levels at concentrations of 0.5 and 1.0μM, although a small but

significant increase was seen at a concentration of 0.25μM (fig 3.32). These results suggest that the effects of adrenaline, isoprenaline and phenylephrine are insufficient to overcome the effect of insulin on malonyl-CoA levels in non-TAG-loaded myocytes.

3.5 Lipolysis in Cardiac Myocytes

Fatty acids are the preferred substrate for oxidative metabolism in the heart (Neely & Morgan, 1974). In addition to exogenous fatty acids from circulating lipoproteins and non-esterified fatty acids from adipose tissue, the heart contains a considerable pool of endogenous TAG. This endogenous TAG may be present in cardioadipocytes or as free-floating cytosolic droplets, membrane bound particles and lipid-filled vacuoles within myocardial cells.

The rate of oxidation of endogenous TAG is at least partly dependent on its rate of lipolysis and must be tightly regulated to prevent lipolysis of endogenous TAG when fatty acid utilisation is low. The identity of the lipase(s) primarily responsible for mobilisation of endogenous myocardial TAG is a matter of some controversy. There are at least three lipases present within the myocardium capable of TAG lipolysis. (1) LPL, present both intracellularly and on the vascular endothelium. (2) A neutral intracellular lipase which is distinct from LPL. (3) An acid lipase present in lysosomes. The fact that LPL activity is high and that it is synthesised within the myocyte before being transported to the vascular endothelium, makes the study of cardiac lipases difficult. Isolated myocytes (which contain little extracellular LPL) and hearts perfused with heparin (which removes LPL from the vascular endothelium), both contain neutral (pH 7-7.5) and acid (pH 4-4.5) TAG lipase activities (Severson, 1979; Ramirez *et al*, 1985). Removal of intracellular LPL using heparin sepharose-affinity chromatography reveals a neutral lipase which is immunologically distinct from LPL (Ramirez *et al*, 1985). There is now considerable evidence to suggest that this neutral lipase is actually HSL (Heathers

et al, 1985; Schoonderwoerd *et al*, 1987; Small *et al*, 1989; Kraemer *et al*, 1993). The relative contribution of HSL, LPL and lysosomal acid lipase to endogenous cardiac TAG lipolysis is unknown. Cardiac lipolysis is acutely regulated by catecholamines suggesting that HSL is responsible for the hormone-stimulated lipolysis of endogenous TAG, while lysosomal acid lipase may be primarily responsible for basal levels of lipolysis (Schoonderwoerd *et al*, 1990b).

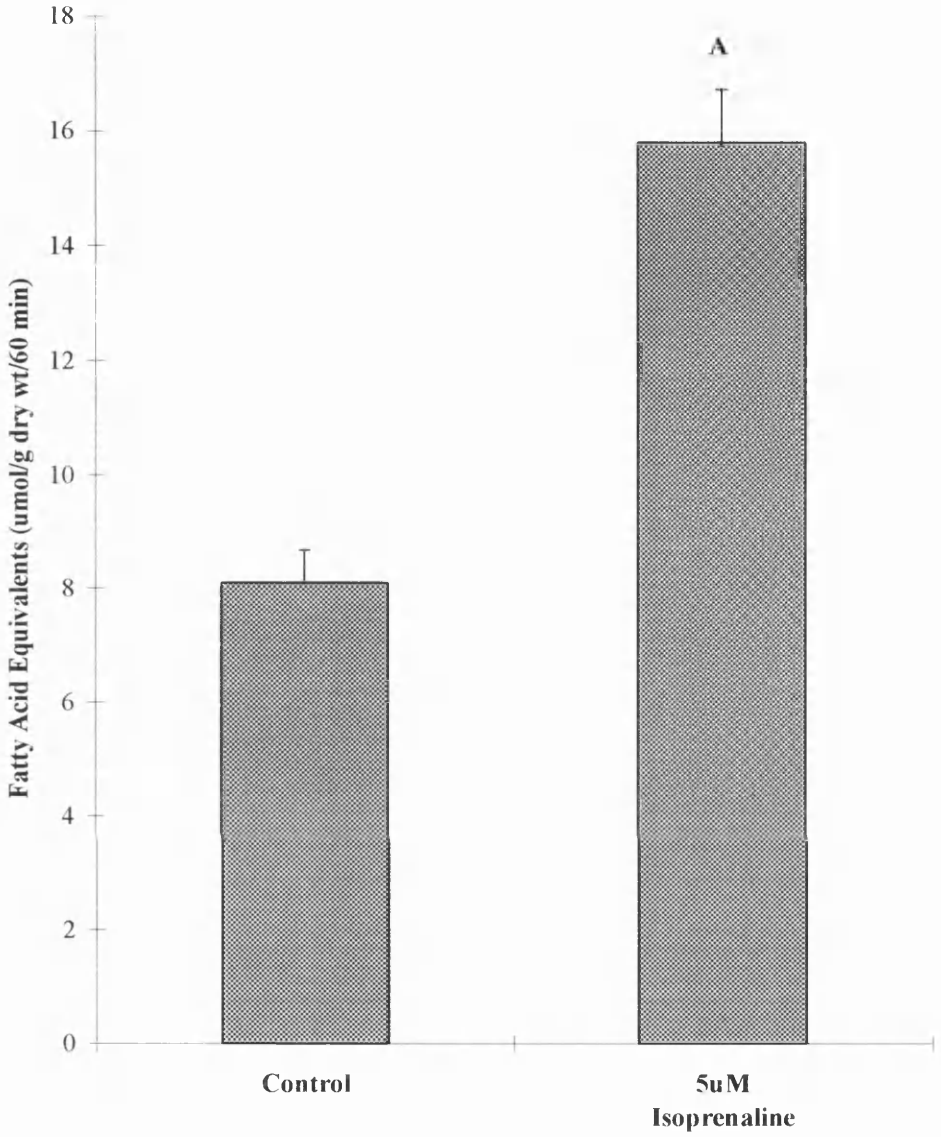
The rate of endogenous lipolysis was determined following the incubation of isolated cardiac myocytes by measuring glycerol release into the incubation medium. The use of isolated myocytes is beneficial in that the possible contribution of other cell types, such as cardioadipocytes, is removed. Also, isolated myocytes are quiescent and therefore, unlike perfused hearts, results obtained using isolated myocytes are not complicated by the fact that cardiac contractility and mechanical performance are important determinants of the utilisation of myocardial TAG (Crass, 1977).

Non-TAG-loaded myocytes were incubated for 60 min at 37^0C in KHB medium containing 5mM glucose, 2% BSA in the presence or absence various additions including 5μM isoprenaline and a range of concentrations of palmitate. In all experiments the rate of lipolysis was measured by the output of glycerol into the incubation medium of the cells as described in section 2.8.2 and is expressed in fatty acid equivalents (μmoles/g dry wt. 60 min^{-1}).

3.5.1 Effect of Isoprenaline on Lipolysis in non-TAG-loaded myocytes

Isoprenaline significantly increased lipolysis by ~94% in non-TAG-loaded myocytes (fig 3.33). This is comparable to the isoprenaline induced stimulation of lipolysis observed in non-TAG-loaded myocytes by Larsen & Severson (1990).

Figure 3.33 Effect of isoprenaline on lipolysis in non-TAG-loaded myocytes



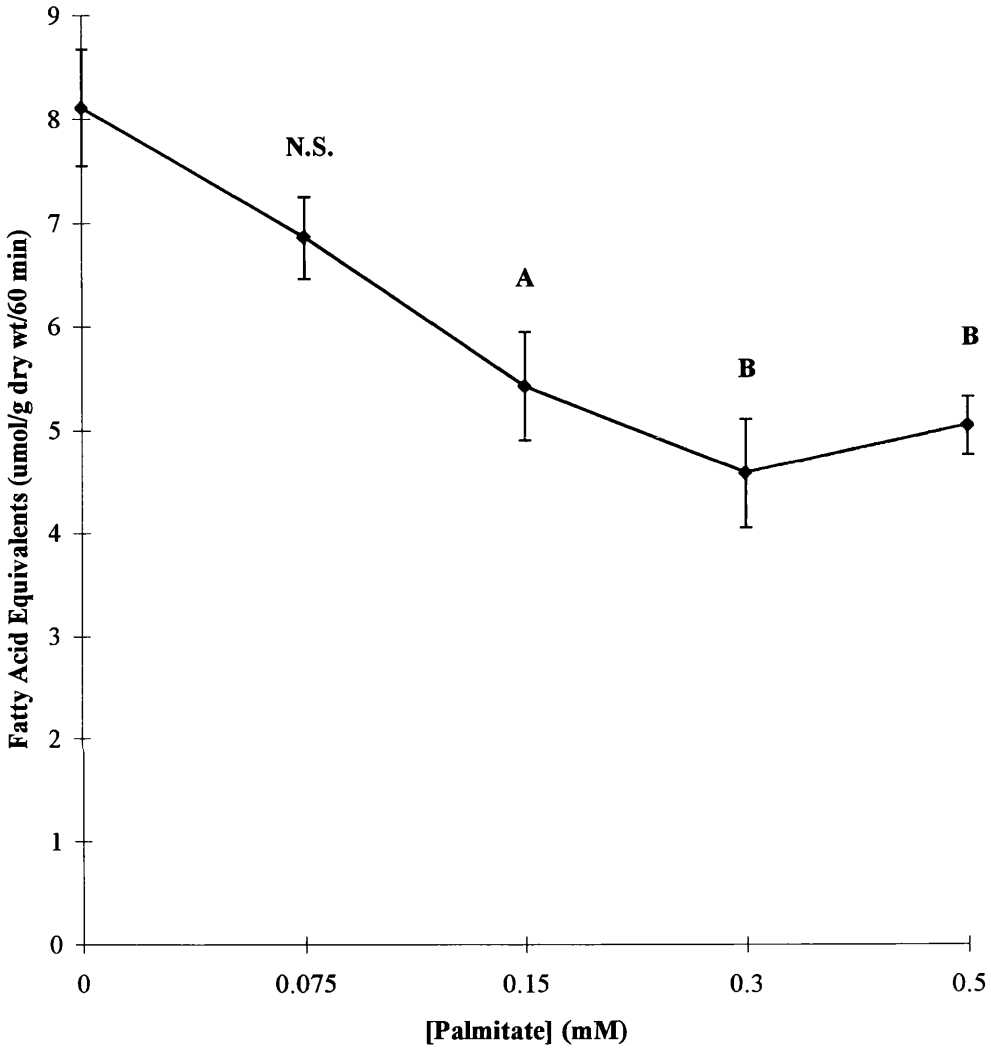
Non-TAG-loaded myocytes were incubated at 37^0C for 60 min with KHB medium containing 5mM glucose and 2% BSA, in the presence or absence of 5μM isoprenaline. Values are means ± SEM of 4 independent preparations. **A** indicates $p<0.005$ versus control (paired t-test).

Hormone sensitive lipase (HSL) is thought to be the lipase responsible for mobilisation of TAG stores in myocytes. As described in section 1.2.2.1, regulation of lipolysis is mediated via reversible phosphorylation of HSL at two sites, a regulatory site (site 1) and a basal site (site 2). In adipose tissue, lipolytic agents such as adrenaline, noradrenaline, and isoprenaline activate the TAG-hydrolase activity by increasing the intracellular cAMP concentration, resulting in activation of PKA. Phosphorylation of site 1 by PKA is responsible for activation of HSL. Phosphorylation of site 1 blocks the subsequent phosphorylation of site 2 and *vice versa* (Garton & Yeaman, 1990). Site 2 can be phosphorylated *in vitro*, by a number of protein kinases including AMPK (Garton *et al*, 1989). Phosphorylation at site 2 has no direct effect on the activity of HSL, but renders site 1 inaccessible to phosphorylation by PKA and hence HSL cannot be activated. Previously in section 3.4.8, isoprenaline was shown to have no significant effect on malonyl-CoA level in insulin-treated non-TAG-loaded myocytes. The fact that isoprenaline had a significant effect on lipolysis in non-TAG-loaded cells indicates that these cells are capable of responding to this agent.

3.5.2 Effect of Palmitate Concentration on Lipolysis in Non-TAG-loaded Myocytes

As shown in figure 3.34, the presence of exogenous palmitate significantly reduced the rate of lipolysis in non-TAG-loaded myocytes in a dose-dependent manner between 0-0.3mM palmitate. Addition of 0.3mM palmitate inhibited lipolysis by ~43%. No further inhibition of lipolysis was observed at concentrations of palmitate above 0.3mM (fig 3.34). Larsen & Severson (1990), showed that in non-TAG-loaded myocytes the basal rate of lipolysis was unaffected by exogenous oleate (0-1.2mM). However, when the lipolytic rate was elevated by the presence

Figure 3.34 Effect of palmitate concentration on lipolysis in non-TAG-loaded myocytes



Non-TAG-loaded myocytes were incubated at 37^{0}C for 60 min with KHB medium containing 5mM glucose, 2% BSA and indicated concentrations of palmitate. Values are means ± SEM of 6 independent preparations. **A**, **B**, **N.S.**, indicate $p<0.05$, 0.01 and no significant difference versus 0mM palmitate (paired t-test).

of isoprenaline (10μM), glycerol output was reduced by 30% in the presence of oleate (1.2mM).

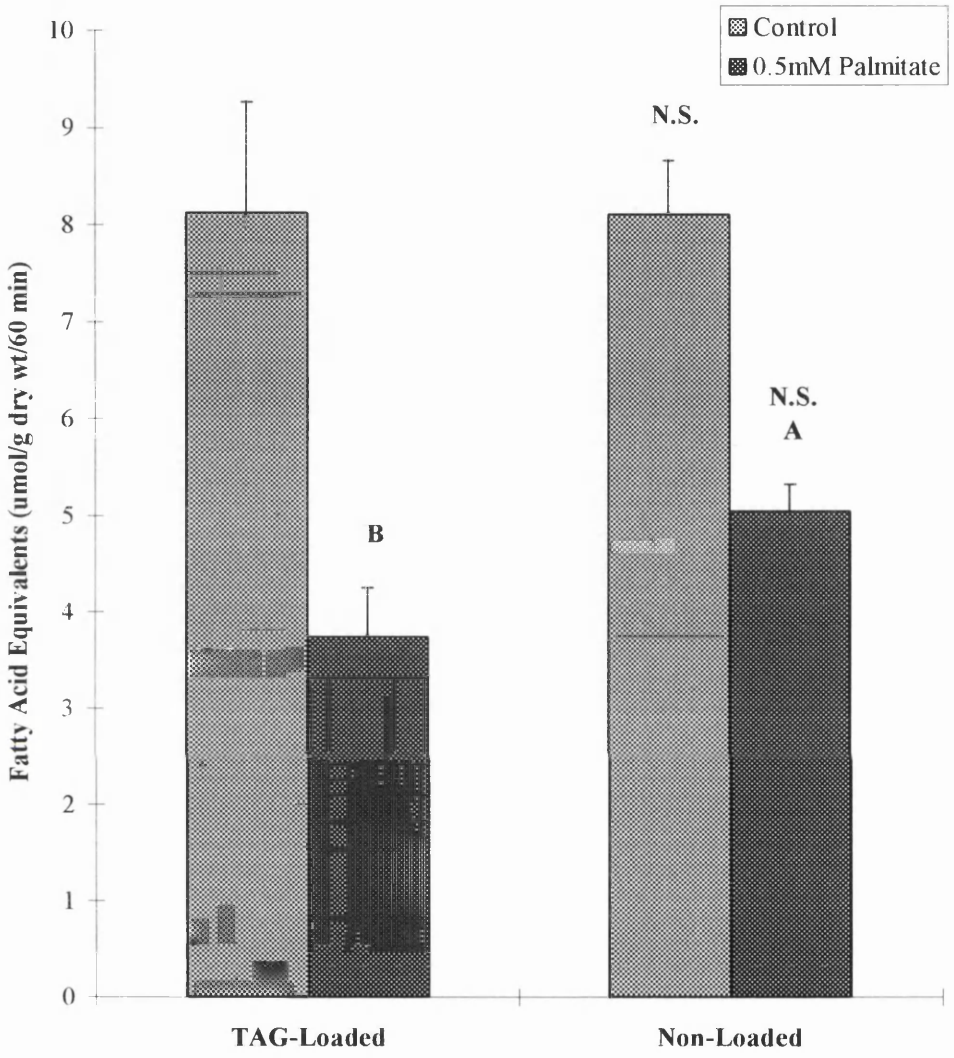
It has been suggested that this effect of exogenous fatty acids is due to feedback inhibition on the TAG lipase (HSL). However, significant inhibition of lipolysis was observed at fatty acid:albumin ratios which were such that the concentrations of free oleate would be very low (1-2μM). This would suggest another mechanism for the inhibition of lipolysis by exogenous fatty acids.

AMPK has been suggested to be involved in the regulation of fatty acid oxidation (Kudo *et al*, 1996). It is also possible that AMPK plays a role in regulation of myocardial lipolysis via phosphorylation of the regulatory site (site 2) of HSL. As described previously in section 3.4.1.1, exogenous fatty acids must be "activated" to their CoA-thioester before further metabolism can occur. This reaction, catalysed by FAS, results in the formation of 1mol AMP per mol fatty acid activated. Therefore, utilisation of exogenous palmitate will increase AMP levels which in turn will activate AMPK (directly and via an upstream AMPK kinase). Once activated, AMPK will phosphorylate site 2 of HSL, thereby preventing phosphorylation and activation of HSL at site 1 and thereby inhibit lipolysis. This proposed mechanism could help explain the decrease in lipolysis observed in response to such low concentrations of exogenous palmitate. It seems likely that AMPK may play a role in cardiac lipolysis as it is known to regulate several other key enzymes in lipid metabolism.

3.5.3 Effect of TAG-loading on the Inhibition of Lipolysis by Palmitate

The effect of exogenous palmitate on lipolysis was examined in both TAG-loaded and non-TAG-loaded myocytes. As shown in figure 3.35, there is no significant difference in basal lipolytic rates between myocytes prepared in the presence of

Figure 3.35 Effect of 0.5mM palmitate on lipolysis in TAG-loaded and non-TAG -loaded myocytes



TAG-loaded and non-TAG-loaded myocytes were incubated at 37^0C for 60 min with KHB medium containing 5mM glucose and 2% BSA, in the presence or absence of 0.5mM palmitate. Values are means ± SEM of 3-6 preparations (TAG-loaded control n=6, palmitate n=3; non-TAG-loaded control n=4, palmitate n=4). **A** indicates $p<0.01$ versus control (paired t-test). **B** indicates $p<0.001$ versus control (unpaired t-test). **N.S.** indicates no significant difference between TAG-loaded and non-TAG-loaded (unpaired t-test).

0.5mM palmitate (TAG-loaded) and myocytes prepared in the absence of palmitate (non-TAG-loaded). This differs from previous observations whereby myocytes prepared in the presence of oleate were shown to have lipolytic rates approximately two times higher than non-TAG-loaded myocytes (Larsen & Severson, 1990). This discrepancy in results may reflect differences in the initial TAG content of myocytes isolated in the presence of palmitate as opposed to oleate. Differences in the isolation procedures and the length of time myocytes were exposed to exogenous fatty acids during isolation may be of importance.

Several groups have demonstrated a relationship between the initial TAG content and glycerol output in perfused hearts (Rösen *et al*, 1981) and isolated myocytes (Kenno & Severson, 1985). When the initial TAG content of myocytes is increased by the presence of fatty acids in the isolation medium, rates of endogenous lipolysis are elevated in subsequent incubations (Kenno & Severson, 1985; Kryski *et al*, 1987). Unfortunately, measurements of initial TAG content in TAG-loaded and non-TAG-loaded myocytes were not made in these experiments, and so the extent to which the initial TAG content was increased by isolation in the presence of palmitate is unknown. However, Swanton & Saggerson (1997b), used the same isolation procedure and showed that the TAG-content of myocytes loaded with palmitate was 60μmol fatty acid equivalents per g dry wt. compared with only 6-8μmol in myocytes isolated in the absence of palmitate.

Kenno & Severson (1985), showed that preparation of myocytes in the presence of palmitate increased the initial TAG content from a control content of 12.1 nmol/10^6 cells to 139 nmol/10^6 cells. Whereas preparation in the presence of oleate increased initial TAG content to 189nmol/10^6 cells (Kryski *et al*, 1987). Hence, isolation in the presence of palmitate does increase the initial TAG content

but not as much as oleate. This might help to explain why no significant difference in basal rates of lipolysis was observed between TAG-loaded and non-TAG-loaded myocytes (fig 3.35).

As shown in figure 3.35, no significant difference in lipolytic rate was observed between TAG-loaded and non-TAG-loaded myocytes incubated in the presence of palmitate. However, in TAG-loaded myocytes, palmitate significantly decreased lipolysis by ~54% compared with a decrease of only ~37% in non-TAG-loaded myocytes. This indicates a greater inhibition of lipolysis by exogenous fatty acid in TAG-loaded myocytes compared to non-TAG-loaded myocytes and is probably the result of an increase in the initial TAG content of TAG-loaded myocytes. This is comparable to results obtained by Larsen & Severson (1990), who showed that incubation of TAG-loaded myocytes with 1.2mM oleate significantly inhibited basal lipolysis by 40% and isoprenaline stimulated lipolysis by 53%. While in non-TAG-loaded myocytes, incubation with 1.2mM oleate did not significantly inhibit basal lipolysis, and inhibited isoprenaline stimulated lipolysis by only 30%.

These results suggest that the availability of endogenous TAG substrate is an important regulator of endogenous rates of lipolysis.

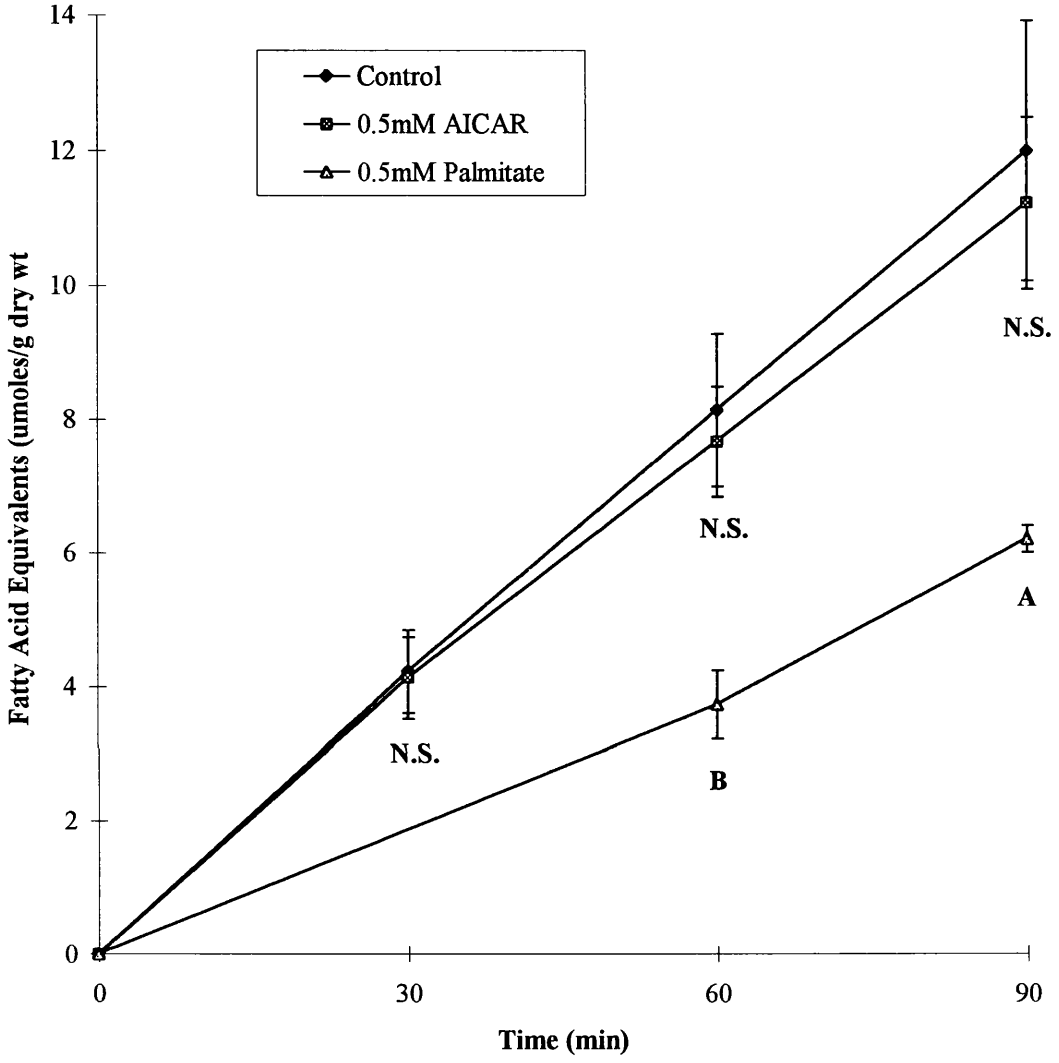
3.5.4 Effect of AICAR on Lipolysis in TAG-Loaded Myocytes

As was previously discussed in section 3.5.2, AMPK may play an important role in lipolysis via phosphorylation of the regulatory site of HSL. AICAR, a cell permeable precursor of ZMP (an AMP analogue) was used to test the possible role of AMPK in regulation of HSL and TAG lipolysis in cardiac myocytes in the same way as it was used to test the role of AMPK in the palmitate induced inhibition of ACC (see section 3.4.7).

Myocytes were incubated for 30, 60 and 90 min at 37^0C in KHB medium containing 5mM glucose, 2% BSA in the presence or absence of either 0.5mM palmitate (bound to albumin) or 0.5mM AICAR.

As shown in figure 3.36, the basal rate of lipolysis in TAG-loaded myocytes incubated in the absence of exogenous fatty acid is linear with time. As previously demonstrated, palmitate significantly reduced the rate of lipolysis in TAG-loaded myocytes by approximately 50% (fig 3.36). However incubation with 0.5mM AICAR had no significant effect on the rate of lipolysis in TAG-loaded myocytes (fig 3.36). This is due to the fact that ZMP levels do not accumulate in cardiac myocytes due to low adenylate kinase activity (Hardie & Carling, 1997) and hence AMPK and AMPK kinase are not activated. Without making measurements of ZMP and/or AMPK activity, this negative result does not mean that AMPK is not involved in the phosphorylation and inactivation of ACC or HSL.

Figure 3.36 Effect of palmitate and AICAR on lipolysis in TAG-loaded myocytes



TAG-loaded myocytes were incubated at 37^{0}C for 30, 60 and 90 min with KHB medium containing 5mM glucose and 2% BSA, in the presence or absence of indicated concentrations of palmitate and AICAR. Values are expressed as means ± SEM of 3-6 independent preparations (control & AICAR n=6, palmitate n=3). **A**, **B**, **N.S.** indicate $p<0.05$, <0.001 and no significant difference respectively versus control (paired t-test).

Chapter 4 - General Discussion

The development of the raddicoc:hemical malonyl-CoA assay for the accurate measurement of malonyl-CoA vwas an essential part of this study. This was mainly due to the very small quantity c obf myocytes used in experiments as well as the low concentration of malonyl-CoA . within these cells. Without this development no statistically significant changes in malonyl-CoA would have been detected.

4.1 General Discussion

The work within this thesis has helped to gain a greater understanding of the synthesis and disposal of malonyl-CoA in cardiac myocytes.

Malonyl-CoA is synthesised in heart by a tissue specific isoform of ACC (ACC-280). Conditions for measuring malonyl-CoA in cardiac myocytes were developed so that accurate measurements could be made (chapter 3.1). Malonyl-CoA content in isolated cardiac myocytes was found to be comparable to that previously measured in perfused heart by McGarry's group and Saggerson's group.

Preparation of glucose-depleted myocytes enabled the rate of glucose dependent malonyl-CoA synthesis to be studied for the first time. Removal of glucose from myocytes resulted in a dramatic decrease in malonyl-CoA level. After a short lag period (2 min), re-addition of glucose resulted in a rapid increase in malonyl-CoA level ($T_{0.5}$ of ~1.8 min). It was assumed that changes in malonyl-CoA level represented changes in ACC activity. The rapid synthesis of malonyl-CoA on re-addition of glucose indicates acute regulation of ACC activity and implies a direct effect of glucose on ACC in cardiac myocytes.

The malonyl-CoA content of both TAG-loaded and non-TAG-loaded cardiac myocytes was shown to increase in parallel with increasing glucose concentration. This increase in malonyl-CoA level is thought to be due to an increase in acetyl-CoA supply to ACC in the cytosol. Insulin was shown to have no significant effect on the level of malonyl-CoA in TAG-loaded myocytes but significantly increased it in non-TAG-loaded myocytes. Further work is required to elucidate the

mechanism by which TAG-loading interferes with the effect of insulin to increase malonyl-CoA level in cardiac myocytes.

The heart is not regarded to be a lipogenic tissue yet contains an isoform of CPT 1 which is as much as 50 times more sensitive to inhibition by malonyl-CoA than the liver enzyme. Heart malonyl-CoA levels have previously been shown to range between 3 and 10μM under various physiological conditions. At these levels of malonyl-CoA, fatty acid oxidation would be almost completely inhibited due to the very high sensitivity of heart CPT 1 to inhibition by malonyl-CoA (IC_{50}~0.1μM). It was proposed that the condensing enzyme of the fatty acid chain elongation (FACE) system may provide a binding/sequestration sink for malonyl-CoA.

The presence of the mitochondrial enzyme, malonyl-CoA decarboxylase in subcellular fractions of rat heart was found to interfere with the radiochemical assay for measuring FACE activity. Upon revision of the radiochemical assay, as well as the use of a spectrophotometric assay, no FACE activity was detected in any subcellular fraction of rat heart. Therefore, the condensing enzyme of FACE is unlikely to provide a high abundance, low affinity, binding/sequestration "sink" for malonyl-CoA. The question still remains as to how fatty acid oxidation can proceed, when in theory, CPT 1 will be almost completely inhibited at normal levels of malonyl-CoA. The most likely hypothesis is that malonyl-CoA is bound to a malonyl-CoA binding protein. Zammit's group are presently working on this hypothesis, but as yet, no data has been published to provide evidence of a malonyl-CoA binding protein in heart.

The fact that myocardial malonyl-CoA levels can change dramatically yet transiently under various conditions suggests there must be a mechanism by which malonyl-CoA can be degraded in the heart. One possible mechanism is

decarboxylation by the enzyme malonyl-CoA decarboxylase. A significant quantity of malonyl-CoA decarboxylase was detected in subcellular fractions of rat heart and was shown to closely follow the subcellular distribution of citrate synthase. This would suggest that the majority of malonyl-CoA decarboxylase in heart is localised in the mitochondrial matrix. Several suggestions for the role of malonyl-CoA decarboxylase in mitochondria have been made but it is still an area of debate. Studies using intact rat heart mitochondria showed that malonyl-CoA decarboxylase activity was approximately 2.5 fold higher than both citrate synthase or MDH when expressed as a percentage activity overt to the mitochondrial matrix. This might suggest the presence of a malonyl-CoA decarboxylase activity which is overt to the inner mitochondrial membrane which might be capable of decarboxylating cytosolic malonyl-CoA. This would provide a possible mechanism for the regulation of cytosolic malonyl-CoA and hence fatty acid oxidation.

Little is know about the regulation of malonyl-CoA decarboxylase activity, although it is known to be allosterically inhibited by acetyl-CoA. Further work is required into the regulation of malonyl-CoA decarboxylase before its role in the regulation of cardiac malonyl-CoA can be elucidated.

Malonyl-CoA was shown not to be a respiratory substrate for intact rat heart mitochondria. Therefore, it is unlikely that malonyl-CoA is transported in and decarboxylated by intra-mitochondrial malonyl-CoA decarboxylase. Due to the lack of overt CAT activity in an intact mitochondria preparation, it is not clear whether malonyl-CoA may be decarboxylated to acetyl-CoA by the suggested overt malonyl-CoA decarboxylase and subsequently transported into the mitochondria, via the actions of CAT and carnitine:acetylcarnitine translocase for

oxidation in the matrix. Again, further investigation into this proposed mechanism of malonyl-CoA disposal is required.

It was clearly demonstrated that the level of malonyl-CoA in isolated cardiac myocytes can be acutely regulated by palmitate and insulin. Palmitate significantly decreased malonyl-CoA content in both TAG-loaded and non-TAG-loaded cardiac myocytes. The mechanism by which palmitate decreases malonyl-CoA is suggested to be by the AMPK system as conversion of exogenous fatty acid to fatty acyl-CoA by FAS, produces AMP. Hence when the level of exogenous fatty acids is high, AMPK is activated, ACC is inhibited and fatty acid oxidation is uninhibited due to the fall in malonyl-CoA level. Unfortunately, incubation of myocytes with AICAR does not result in accumulation of ZMP due to low levels of adenylate kinase and so the role of AMPK could not be confirmed in this way.

Insulin increased malonyl-CoA content in non-TAG-loaded myocytes but had no significant effect in TAG-loaded myocytes. Insulin is thought to increase the level of malonyl-CoA in non-TAG-loaded myocytes primarily via an increase in acetyl-CoA supply from the PDH complex as opposed to activation of ACC as in the liver. However, the fact that increasing glucose concentration resulted in a parallel increase in malonyl-CoA in both TAG-loaded and non-TAG-loaded myocytes, suggests that the effects of glucose and insulin on malonyl-CoA levels are distinct. It would also suggest that TAG-loading interferes with the effect of insulin at some point in the insulin signaling pathway.

The levels of long-chain esters were found to be 2-2.5 fold higher in non-TAG-loaded myocytes than in isolated perfused rat hearts. It is possible that this difference is due to myocytes experiencing a short period of ischaemia during isolation. On the other hand, it may be due to the dilution effect upon isolation of

myocytes. Long-chain ester measurements were originally made in isolated myocytes to give an indication of CPT 1 activitty. These high levels of long-chain esters measured in myocytes, may help explaim why expected changes in CPT 1 activity, and hence long-chain esters, due to chanɡes in malonyl-CoA level, were not always seen. In contrast Alam & Saggerson (1998), showed insulin increased malonyl-CoA content in soleus muscle with a concomitant increase in long-chain acyl-CoA content, presumably as a result of mallonyl-CoA inhibition of CPT 1.

Isoprenaline significantly increased the rate of lipolysis in non-TAG-loaded myocytes. Palmitate significantly decreased the rate of lipolysis in cardiac myocytes. The effect of exogenous fatty acids on lipolysis is not thought to be via feedback inhibition of HSL but possibly via the AMPK system. Unfortunately, this could not be tested with AICAR for the reason explained previously. The effect of palmitate to inhibit lipolysis was significantly greater in TAG-loaded myocytes than in non-TAG-loaded myocytes. Several groups have demonstrated a relationship between the initial TAG content and glycerol output in isolated myocytes, and that lipolysis in TAG-loaded myocytes is inhibited to a greater degree by exogenous fatty acids than non-TAG-loaded myocytes. Measurements of initial TAG-content in TAG-loaded and non-loaded myocytes would be useful to gain a greater insight into the effects of TAG-loading on lipolysis.

Chapter 5 - Bibliography

Aas, M. (1971) Biochim. Biophys. Acta. **231**, 32-47

Abdinejad, A., Fisher, A. M. & Kumar, S. (1981) Arch. Biochem. Biophys. **208**, 135-145

Abu-Elheiga, L., Almarza-Ortega, D. B., Baldini, A. & Wakil, S. J. (1997) J. Biol. Chem. **272**, 10669-10677

Alam, N. & Saggerson, E. D. (1998) Biochem. J. **334**, 233-241

Allard, M. F., Schonekess, B. O., Henning, S. L., English, D. R. & Lopaschuk, G. D. (1994) Am. J. Physiol. **267**, 2, H742-50

Allred, J. B. & Guy, D. G. (1969) Anal. Biochem. **29**, 293-299

Anderson, P. G., Allard, M. F., Thomas, G. D., Bishop, S. P. & Diegerness, S. B. (1990) Circ. Res. **67**, 948-959

Attardi, G. & Schatz, G. (1988) Annu. Rev. Cell. Biol. **4**, 289-333

Babior, B. M. (1973) J. Biol. Chem. **248**, 1445-1450

Bagby, G. J. (1983) Biochim. Biophys. Acta. **753**, 47-52

Bass, N. M. (1988) Int. Rev. Cytol. **111**, 143-184

Bassingthwaighte, J. B., Noodleman, L., Van der Vusse, G. & Glatz, J. F. C. (1989a) Mol. Cell. Biochem. **88**, 45-49

Bassingthwaighte, J. B., Noodleman, L., Van der Vusse, G. & Glatz, J. F. C. (1989b) Mol. Cell. Biochem. **88**, 51-88

Bernert, J. T. & Sprecher, H. (1977) J. Biol. Chem. **252**, 6736-6744

Bianchi, A., Evans, J. L., Iverson, A. J., Nordlund, A. C., Watts, T. D. & Witters, L. A. (1990) J. Biol. Chem. **265**, 1502-1509

Bieber, L. L. (1988) Ann. Rev. Biochem. **57**, 261-283

Bielefeld, D. R., Vary, T. C. & Neely, J. R. (1985) J. Mol. Cell. Cardiol. **17**, 619-625

Bird, M. I. & Saggerson, E. D. (1984) Biochem. J. **222**, 639-647

Blanchette-Mackie, E. J., Masuno, H., Dwyer, N. K., Olivecrona, T. & Scow, R. O. (1989) Am. J. Physiol. **256**, 19, E818-E828

Borensztajn, J., Otway, S. & Robinson, D. S. (1970) J. Lipid. Res. **11**, 102-110

Borensztajn, J., Samols, D. R. & Rubenstein, A. H. (1972) Am. J. Physiol. **223**, 6, 1271-1275

Broderick, T. L., Quinney, H. A. & Lopaschuk, G. D. (1992) J. Biol. Chem. **267**, 3758-3763

Brown, N. F., Esser, V., Foster. D. W. & McGarry, J. D. (1994) J. Biol. Chem. **269**, 26438-26442

Brown, N. F., Weis, B. C., Husti, J. E., Foster, D. W. & McGarry, J. D. (1995) J. Biol. Chem. **270**, 8952-8957

Buckner, J. S. & Kolattukudy, P. E. (1975a) Biochemistry, N.Y. **14**, 1768-1773

Buckner, J. S. & Kolattukudy, P. E. (1975b) Biochemistry, N.Y. **14**, 1774-1782

Buckner, J. S. & Kolattukudy, P. E. (1976) Biochemistry, N.Y. **177**, 539-551

Camps, L., Reina, M., Lobera, M., Vilard, S. & Olivecrona, T. (1990) Am. J. Physiol. **258**, 27, C673-C681

Carling, D., Clarke, P. R., Zammint, V. A. & Hardie, D. G. (1989) Eur. J. Biochem. **186**, 129-136

Carling, D., Zammit, V. A. & Hardie, D. G. (1987a) FEBS Lett. **223**, 217-222

Carling, D., Zammit, V. A. & Hardie, D. G. (1987b) FEBS Lett. **353**, 33-36

Carlson, L. A., Kayser, L. & Lassers, B. W. (1970) J. Mol. Cell. Cardiol. **1**, 467-475

Cederblad, M. & Lindstedt, S. (1972) Clin. Chim. Acta. **37**, 235-243

Chen, V., Wagner, G. & Spitzer, J. J. (1984) Horm. Metab. Res. **16**, 243-247

Christiansen, K. (1975) Biochim. Biophys. Acta. **380**, 390-402

Cinti, D. L., Cook, L., Nagi, M. N. & Suneja, S. K. (1992) Prog. Lipid Res. **31**, 1-51

Clark, M. G. & Patten, G. S. (1984) J. Biol. Chem. **259**, 15204-15211

Cohen, P. (1986) The Enzymes. **17**, 461-497

Cohen, P., Alessi, D. R. & Cross, D. A. E. (1997) FEBS lett. **410**, 3-10

Cook, G. A. (1984) J. Biol. Chem. **259**, 12030-12033

Cook, G. A. (1987) J. Biol. Chem. **262**, 4968-4972

Cook, G. A. & Gamble, M. S. (1987) J. Biol. Chem. **262**, 2050-2055

Cook, G. A. & Lappi, M. D. (1992) Mol. Cell. Biochem. **116**, 39-45

Corr, P. B., Gross, R. W. & Sobel, B. E. (1984) Circ. Res. **55**, 135-154

Corton, J. M., Gillespie, J. G., Hawley, S. A. & Hardie, D. G. (1995) Eur. J. Biochem. **229**, 558-565

Craig, J. W. & Larner, J. (1964) Nature Lond. **202**, 971-973

Crass III, M. F., (1972) Biochim. Biophys. Acta. **280**, 71-81

Crass III, M. F., (1977) Fed. Proc. Fed. Am. Soc. Exp. Biol. **36**, 1995-1998

Crass III, M. F., McCaskill, E. S., Shipp, J. C. & Mundy, V. K. (1971) Am. J. Physiol. **220**, 428-435

Cronin, C. N. (1997) Eur. J. Biochem. **247**, 1029-1037

Cryer, A. (1981) Int. J. Biochem. **13**, 525-541

Cryer, A. (1989) Mol. Cell. Biol. **88**, 7-15

Davidson, A. M. & Halestrap, A. P. (1988) Biochem. J. **254**, 379-384

Davidson, A. M. & Halestrap, A. P. (1990) Biochem. J. **268**, 147-152

Davies, S. P., Carling, D. & Hardie, D. G. (1989) Eur. J. Biochem. **186**, 123-128

Davies, S. P., Carling, D., Munday, M. R. & Hardie, D. G. (1992) Eur. J. Biochem. **203**, 615-623

Davies, S. P., Helps, N. R., Cohen, P. T. W. & Hardie, D. G. (1995) FEBS Lett. **377**, 421-425

Davies, S. P., Sim, A. T. R. & Hardie, D. G. (1990) Eur. J. Biochem. **187**, 183-190

De Groot, M. J. M., Willemsen, P. H. M., Coumans, W. A., Van Bilsen, M. & Van der Vusse, G. J. (1989) Biochim. Biophys. Acta. **1006**, 111-115

Delcher, H. K., Fried, M. & Shipp, J. C. (1965) Biochim. Biophys. Acta. **106**, 10-18

Denton, R. M. & Randle, P. J. (1967) Biochem. J. **104**, 416-422

Denton, R. M., Randle, P. J. & Martin, B. R. (1972) Biochem. J. **128**, 161-163

Denton, R. M., Richards, D. A. & Chin, J. G. (1978) Biochem. J. **176**, 894-906

Depre, C., Rider, M. H., Veitch, K. & Hue, L. (1993a) J. Biol. Chem. **268**, 13274-13279

Depre, C., Veitch, K. & Hue, L. (1993b) Acta Cardiologica. **48**, 147-164

Drake, A. J., Haines, J. R. & Noble, M. I. M. (1980) Cardiovasc. Res. **14**, 65-72, 1980

Dolphin, P. J. (1985) Can. J. Biochem. Cell. Biol. **63**, 850-869

Duan, C. & Winder, W. W. (1992) J. Appl. Physiol. **72**, 901-904

Edwards, M. R., Bird, M. I. & Saggerson, E. D. (1985) Biochem. J. **230**, 169-179

El-Badry, A. M., Ontani, A. & Mansour, T. E. (1973) J. Biol. Chem. **248**, 557-563

Elks, M. L., Manganiello, V. C. & Vaughan, M. (1983) J. Biol. Chem. **258**, 8582-8587

Esser, V., Britton, C. H., Weis, B. C., Foster, D. W. & McGarry, J. D. (1993) J. Biol. Chem. **268**, 5817-5822

Esser, V., Brown, N. F., Cowan, A. T., Foster, D. W. & McGarry, J. D. (1996) J. Biol. Chem. **271**, 6972-6977

Evans, J. R. (1964) Can. J. Biochem. **42**, 955-969

Evans, W. H. & Mueller, P. S. (1963) J. Lip. Res. **4**, 39-45

Færgeman, N. J. & Knudsen, J. (1997) Biochem. J. **323**, 1-12

Ferrer, A., Caelles, C., Massot, N. & Hegardt, F. G. (1985) Biochem. Biophys. Res. Commun. **132**, 497-504

Feuvray, D., Idell-Wenger, J. A. & Neely, J. R. (1979) Circ. Res. **44**, 322-329

Forsey, R. G. P., Reid, K. & Brosnan, J. T. (1987) Can. J. Physiol. Pharmacol. **65**, 401-406

Fournier, N. C., Zuker, M., Williams, R. E. & Smith, I. C. P. (1983) Biochemistry. **22**, 1863-1872

Friedman, G., Stein, O. & Stein, Y. (1980) Atherosclerosis. **36**, 289-298

Frohlich, E. D., Chobanian, A. V. & Devereux, R. B. (1992) N. Engl. J. Med. **327** (14), 998-1008

Fuller, S. J., Gaitanaki, C. J. & Sugden, P. H. (1990) Biochem. J. **266**, 727-736

Garland, P. B., Newsholme, E. A. & Randle, P. J. (1964) Biochem. J. **93**, 665-678

Garland, P. B. & Randle, P. J. (1962) Nature. **196**, 987-988

Garland, P. B. & Randle, P. J. (1964) Biochem. J. **93**, 678-687

Garland, P. B., Randle, P. J. & Newsholme, E. A. (1963) Nature Lond. **200**, 169-170

Garton, A. J., Campbell, D. G., Carling, D., Hardie, D. G., Colbran, R. J. & Yeaman, S. J. (1989) Eur. J. Biochem. **179**, 249-254

Garton, A. J., Campbell, D. G., Cohen, P. & Yeaman, S. J. (1988) FEBS Lett. **229**, 68-72

Garton, A. J. & Yeaman, S. J. (1990) Eur. J. Biochem. **191**, 245-250

Giffhorn, S. & Katz, N. R. (1984) Biochem. J. **221**, 343-350

Glatz, J. F. C., Baerwaldt, C. C. F., Veerkamp, J. H. & Kempen, H. J. M. (1984) J. Biol. Chem. **25**, 4295-4300

Glatz, J. F.C. & Van der Vusse, G. J. (1989) Mol. Cell. Biochem. **88**, 37-44

Glatz, J. F.C. & Van der Vusse, G. J. (1990) Mol. Cell. Biochem. **251**, 237-251

Glatz, J. F.C. & Van der Vusse, G. J. & Veerkamp, J. H. (1988) News. Physiol. Sci. **3**, 41-43

Goodwin, G. W., Ahmad, F. & Taegtmeyer, H. (1996) J. Clin. Invest. **97**, 1409-1416

Gould, G. W. & Holman, G. D. (1993) Biochem. J. **295**, 329-341

Grekinis, D., Reimann, E. M. & Schlender, K. K. (1995) Int. J. Biochem. Cell Biol. **27**, 565-573

Griffiths, E. J. & Halestrap, A. P. (1993) Biochem. J. **290**, 489-495

Groot, P. H. E., Oerlemans, M. C. & Scheek, L. M. (1979) Biochim. Biophys. Acta. **530**, 91-98

Groot, P. H. E., Scholte, H. R. & Hulsman, W. C. (1976) Adv. Lipid Res. **14**, 75-126

Gupta, R. C., Neuman, J., Watanabe, A. M., Lesch, M. & Sabbah, H. N. (1996) Am. J. Phsiol. Heart and Circulatory Physiology. **39**, H1159-H1164

Guzmán, M., Kolodziej, M. P., Caldwell, A., Costorphine, C. G. & Zammit, V. A. (1994) Biochem. J. **300**, 693-699

Ha, J., Lee, J.-K., Kim, K.-S., Witters, L. A. & Kim, K.-H. (1996) Proc. Natl. Acad. Sci. USA. **93**, 11466-11470

Hagve, T., Sprecher, H. (1989) Biochim. Biophys. Acta. **1001**, 338-344

Halestrap, A. P. (1987) Biochem. J. **244**, 159-164

Halestrap, A. P. & Davidson, A. M. (1990) Biochem. J. **268**, 153-160

Harano, Y., Kashiwagi, A., Kojima, H., Suzuki, M., Hashimoto, T. & Shigeta, Y. (1985) FEBS Lett. **188**, 267-272

Hardie, D. G. (1995) J. Biol. Chem. **270**, 27186-27191

Hardie, D. G. (1989) Prog. Lipid Res. **28**, 117-146

Hardie, D. G., Carling, D. & Sim, A. T. R. (1989) Trends Biochem. Sci. **14**, 20-23

Hardie, D. G. & Guy, P. S. (1980) Eur. J. Biochem. **140**, 325-333

Hatch, M. D. & Stumpf, P. K. (1962) Plant Physiol. **37**, 121-126

Hawley, S. A., Selbert, M. A., Goldstein, E. G., Edelman, A. M., Carling, D. &

Hayaishi, O. (1955) J. Biol. Chem. **215**, 125-136

Haystead, T. A. J., Moore, F., Cohen, P. & Hardie, D. G. (1990) Eur. J. Biochem. **187**, 199-205.

Hearse, D. J., Stewart, D. A. & Green, D. G. (1978) Eur. J. Cardiol. 7, 437-450

Heathers, G. P., Al-Muhtaseb, N. & Brunt, R. V. (1985) J. Mol. Cell. Cardiol. **17**, 785-796

Henin, N., Vincent, M. F. & Van den Berghe, G. (1996) Biochim. Biophys. Acta. **1290**, 197-203

Hollenberg, C. H. (1959) Am. J. Physiol. **197**, 667-670

Hsu, R. Y., Wasson, G. & Porter, J. W. (1965) J. Biol. Chem. **240**, 10, 3736-3746

Hucho, F., Randall, D. D., Roche, T. E., Burgett, M. W., Pelley, J. W. & Reed, L. J. (1972) Arch. Biochem. Biophys. **151**, 328-340

Hue, L., Dupre, C., Lefebvre, V., Rider, M. H., Veitch, K. (1995) Biochem. Soc. Trans. **23**, 311-315

Hue, L., Maisin, L. & Rider, M. H. (1988) Biochem. J. **251**, 541-545

Hue, L. & Rider, M. H. (1987) Biochem. J. **245**, 313-324

Hulsmann, W. C., Geelhoed-Mieras, M. M., Jansen, H. & Houtsmuller, U. M. T. (1979) Biochim. Biophys. Acta. **572**, 183-187

Jang, S. H., Cheesbrough, T. M. & Kolattukudy, P. E. (1989) J. Biol. Chem. **264**, 6, 3500-3505

Jansen, H., Hulsmann, W. C., Van Zuylen-Van Wiggen, A., Struyk, C. B. & Houtsmuller, U. M. T. (1975) Biochem. Biophys. Res. Commun. **64**, 747-751

Javaux, F., Vincent, M. F., Wagner, D. R. & Van den Berghe, G. (1995) Biochem. J. **305**, 913-919

Jodalen, H., Lie, R. & Rotevatn, S. (1982) Res. Exp. Med. **181**, 239-244

Johnson, T. M., Mann, W. R., Dragland, C. J., Anderson, R. C., Nemecek, G. M. & Bell, P. A. (1995) Biochem. J. **309**,689-693

Katz, A. M. & Messineo, F. C. (1981) Circ. Res. **48**, 1-16

Katzen, H. M., Soderman, D. D. & Wiley, C. E. (1970) J. Biol. Chem. **245**, 4081-4096

Kenno, K. A. & Severson, D. L. (1985) Am. J. Physiol. **249**, H1024-H1030

Kerby, A. L., Randle, P. J., Cooper, R. H., Whitehouse, S., Pask, H. T. & Denton, R. M. (1976) Biochem. J. **154**, 327-348

Keyes, S. R. & Cinti, D. L. (1980) J. Biol. Chem. **255**, 11357-11364

Kim, K., Lopez-Casillas, F., Bai, D. H., Luo, X. & Pape, M. E. (1989) FASEB J. **3**, 2250-2256

Kim, Y. S. & Kolattukudy, P. E. (1978) Arch. Biochem. Biophys. **190**, 234-246

Kim, Y. S., Kolattukudy, P. E. & Boos, A. (1979a) Comp. Biochem. Physiol. **B62**, 443-447

Kim, Y. S., Kolattukudy, P. E. & Boos, A. (1979b) Int. J. Biochem. **10**, 551-555

Kobayashi, K. & Neely, J. R. (1979) Circ. Res. **44**, 166-175

Kraemer, F. B., Patel, S., Saedi, M. S. & Sztalryd, C. (1993) J. Lip. Res. **34**, 663-671

Kreisberg, R. A. (1966) Am. J. Physiol. **210**, 2, 379-384

Kryski, A. J., Larsen, T. S., Ramirez, I. & Severson, D. L. (1987) Can. J. Physiol. Pharmacol. **65**, 226-229

Kudo, N., Barr, A. J., Barr, R. L., Desai, S. & Lopaschuk, G. D. (1995) **270**, 29, 17513-17520

Kudo, N., Gillespie, J. G., Kung, L., Witters, L. A., Schultz, R., Clanachan, A. S. & Lopaschuk, G. D. (1996) Biochim. Biophys. Acta. **1301**, 67-75

Kunau, W. H., Dommes, V. & Schulz, H. (1995) Prog. Lipid Res. **34**, 267-342

Langendorff, O. (1895) Pflugers Archiv fur die gesamte Physiologie des Menschen und der Tiere. **61**, 251-332

Larsen, T. S. & Severson, D. L. (1990) Can. J. Physiol. Pharmacol. **68**, 1177-1182

Latipaa, P. M. (1989) J. Mol. Cell. Cardiol. **21**, 765-771

Lawson, J. W. R. & Uyeda, K. (1987) J. Biol. Chem. **262**, 3165-3173

Lazarow, P. B. (1978) J. Biol. Chem. **253**, 1522-1528

Lea, M. A. & Weber, G. (1968) J. Biol. Chem. **243**, 1096-1102

Leite, A., Abrahao, J. N., Leyton, J. F., Crivellaro, O. & El Dorry, H. A. (1988) J. Biol. Chem. **263**, 17527-17533

Lerch, R. A., Bergman, S. R., Ambos, H. D., Welch, M. J., Ter-Pogossian, M. M. & Sobel, B. E. (1985) Circulation. **65**, 731-738

Liedtke, A. J. (1981) Prog. Cardiovasc. Dis. **23**, 321-336

Liedtke, A. J. (1988) J. Mol. Cell. Cardiol. **20**, Suppl. **II**, 65-74

Liedtke, A. J., Demainson, L., Eggleston, A. M., Cohen, L. M. & Nellis, S. H. (1988) Circ. Res. **62**, 535-542

Liedtke, A. J., Nellis, S. H. & Neely, J. R. (1978) Circ. Res. **43**, 652-661

Londos, C., Honnor, R. C. & Dhillon, G. S. (1985) J. Biol. Chem. **260**, 15139-15145

Lopaschuk, G. D., Belke, D. D., Gamble, J., Toshiyuki, I. & Schonekess, B. O. (1994a) Biochim. Biophys. Acta. **1213**, 263-276

Lopaschuk, G. D. & Gamble, J. (1994) Can. J. Physiol. Pharmacol. **72**, 1101-1109

Lopaschuk, G. D., Hansen, C. A. & Neely, J. R. (1986) Am. J. Physiol. **250**, H351-H359

Lopaschuk, G. D. & Spafford, M. A. (1989) Circ. Res. **65**, 378-387

Lopaschuk, G. D., Spafford, M. A, Davies, N. J. & Wall, S. R. (1990) Circ. Res. **66**, 546-553

Lopaschuk, G. D. & Tsang, H. (1987) Circ. Res. **61**, 853-858

Lopaschuk, G. D., Wall, S. R., Olley, P. M. & Davies, N. J. (1988) Circ. Res. **63**, 1036-1043

Lopaschuk, G. D., Witters, L. A., Itoi, T., Barr, R. & Barr, A. (1994b) J. Biol. Chem. **269**, 25871-25878

Louis, N. A. & Witters, L. A. (1992) J. Biol. Chem. **267**, 2287-2293

Lysiak, W., Lilly, K., Dilisa, F., Toth, P. P. & Bieber, L. L. (1988) J. Biol. Chem. **263**, 1511-1516

Lysiak, W., Toth, P. P., Suelter, C. H. & Bieber, L. L. (1986) J. Biol. Chem. **261**, 13698-13703

Mahrenholz, A. M., Lan, L. & Mansor, T. E. (1991) Biochem. Biophys. Res. Commun. **174**, 1255-1259

Mannaerts, G. P. & Debeer, L. (1982) Ann. NY. Acad. Sci. **386**, 30-39

Mansour, T. E. (1972a) Curr. Top. Cell Regul. **5**, 1-46

Mansour, T. E. (1972b) J. Biol. Chem. **247**

McAllister, A., Allison, S. P. & Randle, P. J. (1973) Biochem. J. **134**, 1067-1081

McCormack, J. G. & Denton, R. M. (1984) Biochem. J. **218**, 235-247

McCormack, J. G. & Denton, R. M. (1989) Mol. Cell. Biochem. **89**, 121-125

McCormack, J. D., Halestrap, A. P. & Denton, R. M. (1990) Physiol. Rev. **70**, 391-425

McDonough, K. H., Burns, A. H. & Spitzer, J. J. (1982) J. Mol. Cell. Cardiol. **14**, 49

McGarry, J. D. & Brown, N. F. (1997) Eur. J. Biochem. **244**, 1-14

McGarry, J. D. & Foster, D. W. (1980) Annu. Rev. Biochem. **49**, 395-420

McGarry, J. D., Mills, S.E., Long, C. S. & Foster, D. W. (1983) Biochem. J. **214**, 21-28

McGarry, J. D., Stark, M. J. & Foster, D. W. (1978) J. Biol. Chem. **253**, 22, 8291-8293

McGarry, J. D. Woeltje, K. F., Kuwajima, M. & Foster, D. W. (1989) Diabetes Metab. Rev. **5**, 271-284

McKean, M. C., Frerman, F. E. & Mielke, D. M. (1979) J. Biol. Chem. **254**, 2730-2735

McVeigh, J. J. & Lopaschuk, G. D. (1990) Am. J. Physiol. **259**, H1070-H1085

Meriwether, F. S. H.& Park, J. H. (1971) J. Biol. Chem. **246**, 5433-5441

Midgley, P. J. W., Rutter, G. A., Thomas, A. P. & Denton, R. M. (1987) Biochem. J. **241**, 371-377

Mochizuki, S. & Neely, J. R. (1979) J. Mol. Cell. Cardiol. **11**, 221-236

Montini, J., Bagby, G. J. & Spitzer, J. J. (1981) J. Mol. Cell. Cardiol. **13**, 903-911

Moore, K. H. (1985) Mol. Physiol. **8**, 549-563

Morgan, H. E., Cadenas, E., Regen, D. M. & Park, C. R. (1961) J. Biol. Chem. **236**, 262-268

Moule, S. K., Edgell, N. J., Borthwick, C. & Denton, R. M. (1992) Biochem. J. **283**, 35-38

Mowbray, J. & Ottaway, J. H. (1973) Eur. J. Biochem. **36**, 362-368

Murthy, M. S. R. & Pande, S. V. (1987) Proc. Natl. Acad. Sci. USA. **84**, 378-382

Murthy, V. K. & Shipp, J. C. (1977) Diabetes. **26**, 222-229

Myrmel, T., Forsdahl, K. & Larsen, T. S. (1992) J. Mol. Cell. Cardiol. **24**, 855-868

Myrmel, T., Forsdahl, K., Sager, G. & Larsen, T. S. (1991) J. Mol. Cell. Cardiol. **23**, 207-215

Nagi, M. N., Cook, L., Sunjea, S. K., Osei, P. & Cinti, D. L. (1989) Anal. Biochem. **179**, 251-261

Nakielny, S., Campbell, D. G. & Cohen, P. (1991) Eur. J. Biochem. **199**, 713-722

Neely, J. R., Bowman, R. H. & Morgan, H. E. (1969) Am. J. Physiol. **216**, 804-811

Neely, J. R. & Feuvray, D. (1981) Am. J. Pathol. **102**, 282-291

Neely, J. R. & Morgan, H. E. (1974) Ann. Rev. Physiol. **36**, 413-459

Newsholme, E. A. (1971) Cardiology. **56**, 22-34

Nikkila, E. A. (1971) Prog. Biochem. Pharmacol. **6**, 102-129

Nilsson, N. O., Stralfors, P., Fredrikson, G. & Belfrage, P. (1980) FEBS Lett. **111**, 125-130

Norman, P. T., Norseth, J. & Flatmark, T. (1983) Biochim. Biophys. Acta. **752**, 474-481

Norseth, J. & Thomassen, M. S. (1983) Biochim. Biophys. Acta. **751**, 312-320

Nugteren, D. H. (1965) Biochim. Biophys. Acta. **106**, 280-290

Olowe, Y. & Schulz, H. (1980) Eur. J. Biochem. **109**, 425-429

Olson, R. E. (1962) Nature. **195**, 597-599

Olson, R. E. & Hoeschen, R. J. (1967) Biochem. J. **103**, 791-801

Ontko, J. A. & Randle, P. J. (1967) Biochem. J. **104**, 43c-44c

Opie, L. H. (1968) Am. Heart J. **76**, 685-698

Opie, L. H. (1969) Am. Heart J. **77**, 100-122 & 383-410

Opie, L. H. (1975) Am. J. Cardiol. **36**, 938-953

Opie, L. H. (1976) Circ. Res. **38**, Suppl. 1, 52-68

Opie, L. H. (1991) in The Heart: Physiology and Metabolism (Opie, L. H., ed), pp 208-395, Raven Press, New York

Oram, J. F., Bennetch, S. L. & Neely, J. R. (1973) J. Biol. Chem. **248**, 5299-5309

Palmieri, F., Bisaccia, F., Capobianco, L., Dolce, V., Fiermonte, G., Iacobazzi, V., Indiveri, C. & Palmieri, L. (1996) Biochim. Biophys. Acta. **1275**, 127-132

Pape, M. E., Lopez-Casillas, F. & Kim, K. H. (1988) Arch. Biochem. Biophys. **267**, 104-109

Patel, M. S. & Roche, T. E. (1990) FASEB J. **4**, 3224-3233

Paulson, D. J. & Crass III, M. F. (1982) Am. J. Physiol. **242**, H1084-H1094

Paulson, D. J., Ward, K. M. & Shug, A. L. (1984) FEBS Lett. **176**, 381-384

Pearson, D. J. & Tubbs, P. K. (1967) Biochem. J. **105**, 953-963

Pedersen, M. E., Wolf, L. E. & Schotz, M. C. (1981) Biochim. Biophys. Acta. **666**, 191-197

Pilkis, S. J., El Maghrabi, M. R. & Claus, T. H. (1988) Annu. Rev. Biochem. **57**, 755-783

Piper, H. M., Seze, O., Schwartz, P., Huetter, J. F. & Spieckmann, P. G. (1983) **732**, 193-203

Podack, E. R., Lakomek, M., Saathoff, G. & Seubert, W. (1974) Eur. J. Biochem. **45**, 13-23

Poulter, L., Ang, S. G., Gibson, B. W., Williams, D. H., Holmes, C. F. B., Caudwell, F. B., Pitcher, J. & Cohen, P. (1988) Eur. J. Biochem. **175**, 497-510

Prasad, M. R. & Cinti, D. L. (1986) Biochim. Biophys. Acta. 248, 479-488

Ramachandran, C., Angelos, K. L. & Walsh, D. A. (1982) J. Biol. Chem. **257**, 1448-1457

Ramachandran, C., Angelos, K. L. & Walsh, D. A. (1983) J. Biol. Chem. **258**, 13377-13383

Ramadoss, C. S., Russel, R. O., Rogers, W. J., Mantle, J. A. & McDaniel, H. G. (1976) J. Biol. Chem. **251**, 98-107

Ramirez, I., Kryski, A. J., Ben-Zeev, O., Schotz, M. C. & Severson, D. L. (1985) Biochem. J. **232**,229-236

Ramsay, R. R. (1988) Biochem. J. **249**, 239-245

Randle, P. J. (1986) Biochem. Soc. Trans. **14**, 799-806

Randle, P. J., Denton, R. M., Pask, H. T. & Severson, D. L. (1974) Biochem. Soc. Symp. **39**, 75-88

Randle, P. J., Garland, P. B., Hales, C. N., Newsholme, E. A. (1963) Lancet **i**, 785-794

Randle, P. J., Garland, P. B., Hales, C. N., Newsholme, E. A., Denton, R. M. & Pogson, C. I. (1966) Recent Prog. Horm. Res. **22**, 1-44

Randle, P. J., Preistman, D. A., Mistry, S. C. & Halsall, A. (1994a) J. Cell. Biochem. **55S**, 1-11

Randle, P. J., Preistman, D. A., Mistry, S. C. & Halsall, A. (1994b) Diabetologia. **37**, 2, S155-S161

Randle, P. J. & Tubbs, P. K. (1979) in The Handbook of Physiology: The Cardiovascular System I, pp805-844, American Physiological Society, Bethseda, Maryland

Rasmussen, J. T., Rosendal, J. & Knudsen, J. (1993) Bioche,. J. **292**, 907-913

Reed, L. J. (1969) Curr. Top. Cell. Regul. **1**, 233-241

Reubsaet, F. A. G., Veerkamp, J. H., Trijbels, J. M. F. & Monnens, L. A. H. (1989) Lipids. **24**, 945-950

Rider, M. H. & Hue, L. (1984) FEBS Lett. **176**, 484-488

Rider, M. H. & Hue, L. (1986) Biochem. J. **240**, 57-61

Rider, M. H., Vandame, J., Lebeau, E., Vertommen, D., Vidal, H., Rousseau, G. G., Vandekerckhove, J. & Hue, L. (1992) Biochem. J. **285**, 405-411

Roach, P. J. (1991) FASEB J. **4**, 2961-2968

Robinson, A. M. & Williamson, D. H. (1980) Physiol. Rev. **60**, 143-187

Rousseau, G. G. & Hue, L. (1993) Prog. Nucleic Acids Res. Mol. Biol. **45**, 99-127

Sabina, R. L., Kernstine, K. H. Boyd, R. L., Holmes, E. W. & Swain, J. L. (1982) J. Biol. Chem. **257**, 10178-10183

Sabina, R. L., Patterson, D. & Holmes, E. W. (1985) J. Biol. Chem. **260**, 6107-6114

Saddik, M., Gamble, J., Witters, L. A. & Lopaschuk, G. D. (1993) J. Biol. Chem. **268**, 25836-25845

Saddik, M. & Lopaschuk, G. D. (1991) J. Biol. Chem. **266**, 8162-8170

Saddik, M. & Lopaschuk, G. D. (1992) J. Biol. Chem. **267**, 3825-3831

Saggerson, E. D. (1974) Biochem. J. **140**, 211-224

Saggerson, E. D. & Carpenter, C. A. (1981) FEBS lett. **129**, 229-232

Saggerson, E. D. & Greenbaum, A. L. (1970) Biochem. J. **119**, 193-219

Scheuer, J. & Olson, R. E. (1967) Am. J. Physiol. **212**, 301-307

Scholte, H. R. (1969) Biochim. Biophys. Acta. **178**, 137-144

Schonekess, B. O., Allard, M. F. & Lopaschuk, G. D. (1993) Eur. Heart. J. **14** (Abstract Suppl.), P1245

Schonekess, B. O., Brindley, P. G. & Lopaschuk, G. D. (1995) Can. J. Physiol. Pharmacol. **73**, 1632-1640

Schoonderwoerd, K., Broekhoven-Shokker, S., Hulsmann, W. C. & Stam, H. (1987) Basic. Res. Cardiol. **82**, Suppl. 1, 29-35

Schoonderwoerd, K., Broekhoven-Shokker, S., Hulsmann, W. C. & Stam, H. (1989b) Basic. Res. Cardiol. **84**, 165-173

Schoonderwoerd, K., Broekhoven-Shokker, S., Hulsmann, W. C. & Stam, H. (1990a) Biochem. J. **268**, 487-492

Schoonderwoerd, K., Broekhoven-Shokker, S., Hulsmann, W. C. & Stam, H. (1990b) Basic Res. Cardiol. **85**, 153-163

Schoonderwoerd, K., Van der Kraaij, T., Hulsmann, W. C. & Stam, W. (1989a) Mol. Cell. Biochem. **88**, 129-137

Schousboe, I., Bartels, P. & Jensen, P. K. (1973) FEBS Lett. **35**, 279-283

Schulz, H. (1991) Biochim. Biophys. Acta. **1081**, 109-120

Schulz, H. (1994) J. Nutr. **124**, 165-171

Scow, R. O. & Blanchette-Mackie, E. J. (1985) Prog. Lipid. Res. **24**, 197-241

Scrutton, M. C. & Utter, M. F. (1967) J. Biol. Chem. **242**, 1723-1753

Setlow, B. & Mansour, T. E. (1972) Biochemistry. **11**, 1478-1486

Severson, D. L. (1979) J. Mol. Cell. Cardiol. **11**,569-583

Shepherd, D. & Garland, P. B. (1969) Biochem. J. **114**, 597-610

Sim, A. T. R. & Hardie, D. G. (1988) FEBS Lett. **233**, 294-298

Singh, B., Stakkestad, J. A., Bremer, J. & Borrebaek, B. (1983) Anal. Biochem. **138**, 107-111

Skurat, A. V. & Roach, P. J. (1995) J. Biol. Chem. **270**, 12491-12497

Sluse, F. E., Meyer, A. J. & Tager, J. M. (1971) FEBS Lett. **18**, 149-153

Small, C. A., Garton, A. J. & Yeaman, S. J. (1989) Biochem. J. **258**, 67-72

Smith, C. M., Cano, M. L. & Potyraj, J. (1978) J. Nutr. **108**, 854-862

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. & Klenk, D. C. (1985) Anal. Biochem. **150**, 76-85

Smith, L. C. & Scow, R. O. (1979) Prog. Biochem. Pharmacol. **15**, 109-138

Spector, A. A. (1971) Prog. Biochem. Pharmacol. **6**, 130-176

Spector, A. A. (1984) Clin, Physiol. Biochem. **2**, 123-134

Spector, A. A., Fletcher, J. E. & Ashbrook, J. D. (1971) Biochemistry. **10**, 3229-3232

Spence, J. T., Koudelka, A. P. & Tseng-Crank, J. C. L. (1985) Biochem. J. **227**, 939-947

Spitzer, J. J. (1974) Am. J. Physiol. **226**, 213-217

Srimani, B. N., Engelman, R. M., Jones, R. & Das, D. K. (1990) Circ. Res. **66**, 1535-1543

Stacpoole, P. W. (1989) Metabolism. **38**, 11, 1124-1144

Stalmans, W., Bollen, M. & Mvumbi, L. (1987) Diab. Metab. Rev. **3**, 127-161

Stam, H. & Hulsmann, W. C. (1984) Biochim. Biophys. Acta. **794**, 72-82

Stam, H., Schoonderwoerd, K., Breeman, W. & Hulsmann, W. C. (1984) Horm. Metab. Res. **16**, 293-297

Stephens, T. W., Higgins, A. J., Cook, G. A. & Harris, R. A. (1985) Biochem. J. **227**, 651-660

Stralfors, P. & Honnor, R. C. (1989) Eur. J. Biochem. **182**, 379-385

Stam, H. & Hulsmann, W. C. (1985) Eur. Heart. J. **6**, 158-167

Sullivan, J. E., Brocklehurst, K. J., Marley, A. E., Carey, F., Carling, D. & Beri, R. K. (1994) FEBS Lett. **223**, 217-222

Sullivan, J. E., Carey, F., Carling, D. & Beri, R. K. (1994) Biochem. Biophys. Res. Commun. **200**, 1551-1556

Swanton, E. M. S. (1996) PhD Thesis, University College London, University of London

Swanton, E. M. S. & Saggerson, E. D. (1997a) Biochim. Biophys. Acta. **1346**, 93-102

Swanton, E. M. S. & Saggerson, E. D. (1997b) Biochem. J. **238**, 913-922

Sweetser, D. A., Heuckeroth, R. O. & Gordon, J. I. (1987) Ann. Rev. Nutr. **7**, 337-359

Taegtmeyer, H. (1985) Circulation. **72**, suppl. IV. 1-8

Taegtmeyer, H., Hems, R. & Krebs, H. A. (1980) Biochem. J. **186**, 701-711

Thampy, K. G. (1989) J. Biol. Chem. **264**, 17631-17634

Thomas, C. B., Meade, J. C. & Holmes, E. W. (1981) J. Cell. Physiol. **107**, 335-344

Tijburg, L. B., Geelen, M. J. & Van Golde, L. M. (1989) Biochim. Biophys. Acta. **1004**, 1-19

Tolbert, N. E. (1981) Annu. Rev. Biochem. **50**, 133-157

Trach, V., Buschmans-Denkel, E. & Schaper, W. (1986) Basic Res. Cardiol. **81**, 454-464

Trivedi, B. & Danforth, W. H. (1966) J. Biol. Chem. **241**, 4110-4114

Trumble, G. E., Smith, M. A. & Winder, W. W. (1995) Eur. J. Biochem. **231**, 192-198

Uziel, G., Garaveaglia, B. & DiDonato, S. (1988) Muscle Nerve. **11**, 720-724

Valesco, G., Guzmán, M., Zammit, V. A. & Geelen, M. J. H. (1997) Biochem. J. **321**, 211-216

Valesco, G., Sánchez, C., Geelen, M. J. H. & Guzmán, M. (1996) Biochem. Biophys. Res. Comm. **224**, 754-759

Van Bilsen, M., Van der Vusse, G. J., Willemsen, P. H. M., Coumans, W. A., Roeman, T. H. M. Reneman, R. S. (1989) Circ. Res. **64**, 304-314

Van der Vusse, G. J., Prinzen, F. W., Van Bilsen, M., Engels, W. & Reneman, R. S. (1987) Basic Res. Cardiol. **82**, suppl. 1, 157-167

Van der Vusse, G. J. & Reneman, R. S. (1983) **16**, 215-237

Van der Vusse, G. J., Roemen, T. H. M., Prinzen, F. W., Coumans, W. A. & Reneman, R. S. (1982) Circ. Res. **50**, 538-546

Van Schaftingen, E. (1987) Adv. Enzymol. **59**, 315-395

Van Shaftingen, E., Hue, L. & Hers, H. G. (1980a) Biochem. J. **192**, 887-895

Van Shaftingen, E., Hue, L. & Hers, H. G. (1980b) Biochem. J. **192**, 897-901

Veerkamp, J. H., Peeters, R. A. & Maatman, R. G. H. J. (1991) Biochim. Biophys. Acta. **1081**, 1-24

Veerkamp, J. H. & Van Moerkerk, H. T. B. (1986) Biochim. Biophys. Acta. **875**, 301-310

Veerkamp, J. H. & Van Moerkerk, H. T. B. (1993) Mol. Cell. Biochem. **123**, 101-106

Vincent, M. F., Bontemps, F. & Van den Berghe, G. (1996) Biochem. Pharmacol. **52**, 999-1006

Vincent, M. F., Marangos, P. J., Gruber, H. E. & Van den Berghe, G. (1991) Diabetes **40**, 1259-1266

Wall, S. R. & Lopaschuk, G. D. (1989) Biochim. Biophys. Acta. **1006**, 97-103

Wang, H-Y, Baxter Jr, C. F. & Schulz, H. (1991) Arch. Biochem. Biophys. **289**, 274-280

Weekes, J., Hawley, S. A., Corton, J., Shugar, D. & Hardie, D. G. (1994) Eur. J. Biochem. **219**, 751-757

Weis, B.C., Cowan, A. T., Brown, N., Foster, D. W. & McGarry, J. D. (1994a) J. Biol. Chem. **269**, 26443-26448

Weis, B. C., Esser, V., Foster, D. W. & McGarry, J. D. (1994b) J. Biol. Chem. **269**, 18712-18715

Weis, R. G., Chacko, V. P. and Gerstenblith, G. J. (1989) Mol. Cell. Cardiol. **21**, 469-478

Werth, D. K., Hathaway, D. R. & Watanabe, A. M. (1982) Circ. Res. **51**, 448-456

Whitmer, J. T., Idell-Wenger, J. A., Rovetto, M. J. & Neely, J. R. (1978) J. Biol. Chem. **253**, 4305-4309

Williamson, J. R. (1964) Biochem. J. **93**, 97-106

Winder, W. W., Arogyasami, J., Elayan, I. M. & Cartmill, D. (1990) Am. J. Physiol. **259** (Endocrinol. Metab. 22) E266-E271

Winder, W. W. & Hardie, D. G. (1996) Am. J. Physiol. **270**, (Endocrinol. Metab. 33) E299-E304

Winder, W. W., MacLean, P. S., Lucas, J. C., Fernley, J. E. & Trumble, G. E. (1995) J. Appl. Physiol. **78**, 578-582

Winder, W. W., Wilson, H. A., Hardy, D. G., Rasmussen, B. B., Hutber, C. A., Call, G. B., Clayton, L. M., Connley, S. Y. & Zhau, B. (1997) J. Appl. Physiol. **82**, 1, 2219-2295

Witters, L. A. & Kemp, B. E. (1992) J. Biol. Chem. **267**, 2864-2867

Witters, L. A., Widmer, J., King, A. N., Fassihi, K. & Kuhajda, F. (1994) Int. J. Biochem. **26**, 589-594

Woeltje, K. F., Esser, V., Weis, B., Cox, W. F., Schroeder, J. G., Liao, S. T., Foster, D. W. & McGarry, J. D. (1990a) J. Biol. Chem. **265**,18, 10714-10719

Woeltje, K. F., Esser, V., Weis, B., Sen, A., Cox, W. F., McPhaul, M. J., Slaughter, C. A., Foster, D. W. & McGarry, J. D. (1990b) J. Biol. Chem. **265**,18, 10720-10725

Yeaman, S. J., Smith, G. M., Jepson, C. A., Wood, S. L. & Emmison, N. (1994) Adv. Enz. Reg. **34**, 355-370

Zammit, V. A. (1994) Diabetes Rev. **2**, 132-155

Zhang, W., Browner, M. F., Fletterick, R. J. DePaoli-Roach, A. A. & Roach, P. J. (1989) FASEB J. **3**, 2532-2536

Zimmerman, T. P. & Deeprose, R. D. (1978) Biochem. Pharmacol. **27**, 709-716