

Malonyl-CoA Metabolism In Skeletal Muscle

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Abstract

Three skeletal muscle fibre types (type I, type IIa and type IIb) were assayed for the presence of the following enzymes ATP-citrate lyase, citrate-dependent acetyl-CoA carboxylase, fatty acid synthase and malonyl-CoA decarboxylase. All activities were present in each of the muscle fibre types studied. A cytosolic activity of malonyl-CoA decarboxylase would appear to offer a feasible route for the disposal of malonyl-CoA in skeletal muscle.

Rat soleus muscle strips were incubated with 5mM glucose followed by measurements of tissue contents of malonyl-CoA, long chain fatty acyl-CoA and carnitine esters. Alternatively muscle strips were incubated with 5mM glucose and 0.2mM palmitate followed by measurements of [^{14}C] palmitate CO_2 formation from exogenous palmitate or from fatty acids released from prelabelled glycerolipids.

Etomoxir at high concentrations (150 μM) significantly decreased the malonyl-CoA content by 50% and at low concentrations (50 & 100 μM) had no effect on malonyl-CoA. Etomoxir had no effect on the total long chain ester pool but significantly increased long chain acyl-CoA and decreased the ratio of acyl-carnitine/acyl-CoA suggesting that such changes could be diagnostic for inhibition of CPT 1.

Insulin and DCA (3mM) increased both malonyl-CoA and long chain fatty acyl-CoA content and decreased the ratio of fatty acylcarnitine/acyl-CoA and β -oxidation. Isoprenaline and palmitate (0.5mM) opposed the effects of insulin, by decreasing the contents of malonyl-CoA and long chain fatty acyl-CoA, increasing the ratio of fatty acylcarnitine/acyl-CoA and increasing β -oxidation. These findings are consistent with the notion that all these agents can cause acute regulation of CPT 1 in type I skeletal muscle.

In the presence of 5-amino-4-imidazolecarboxamide ribonucleoside (AICAR) (1mM) the malonyl-CoA content decreased by 65% and decreased the content of both long chain esters significantly, in particular long chain acyl-CoA.

Oxidation of ^{14}C -labelled exogenous and endogenous fatty acid was measured in soleus muscle strips incubated with insulin as mentioned above. Isoprenaline (10^{-7}M) increased both processes (28% and 103% respectively). Indicating that isoprenaline has a lipolytic effect. AICAR (1mM) increased oxidation of exogenous fatty acid by 102% but had no effect on endogenous oxidation. It is therefore concluded that AICAR causes a decrease in lipolysis in muscle. Similar experiments led to the suggestion that dichloroacetate (3mM) had a lipolytic effect in muscle.

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Contents	Page
Abstract	ii
Acknowledgements	iv
List Of Figures and Tables	xi
Abbreviations	xv
CHAPTER 1. Aims & Introduction	1
Aims	2
1.1 General Introduction	4
1.2 Structure of Skeletal Muscle	4
1.2.1 The Mechanism of Contraction	8
1.2.1.2 Excitation-Contraction Coupling	10
1.2.2 Types of Skeletal Muscle Fibres	11
1.3 Skeletal Muscle Energy Metabolism	15
1.3.1 Carbohydrate Metabolism	17
1.3.1.1 Glucose Transport	18
1.3.2 Glucose Phosphorylation	20
1.3.3 Phosphofructokinase	20
1.3.3.1 Fructose-2,6-bisphosphate	22
1.3.3.2 Regulation of PFK-2/FBPase	22
a) Hormones	22
b) Electrical Stimulation	23
1.3.3.3 Alternative Fuels	24
1.3.4 Pyruvate Dehydrogenase	25
1.3.4.1 Regulation of PDH Complex	25
a) PDH Kinase	25
b) PDH Phosphatase	26
1.3.4.2 Regulation of PDH Complex During Exercise	26
1.3.4.3 Starvation and Alternative Fuels	27
1.3.5 Glycogen Metabolism	28
1.3.5.1 Glycogen Synthesis	28

1.3.5.2 Regulation by Covalent Modification	29
1.3.5.3 Hormonal Regulation	30
a) Adrenaline	30
b) Insulin	31
c) Protein kinase B	32
1.3.6. Glycogen Phosphorylase	33
1.3.6.1 Hormonal Regulation	34
a) Adrenaline	34
b) Insulin	35
1.3.6.2 Glycogenolysis During Muscle Contractions	35
1.4 Lipid Fuels	38
1.4.1 Plasma Non-esterified Fatty Acids	38
1.4.2 Plasma Lipoprotein	40
1.4.3 Endogenous Triacylglycerols	42
1.4.4 Ketone Body Metabolism	43
1.5 Fatty Acid Oxidation	44
1.5.1 CPT 1 and CPT 2	45
a) CPT 2	45
b) CPT 1	45
1.5.2 Outline of The β -oxidation pathway	48
1.5.3 Regulation of Fatty Acid Oxidation	49
1.6 Acetyl-CoA Carboxylase	50
1.6.1 ACC 265 and ACC 280 Isoforms	51
1.6.2 Regulation of ACC 265	52
1.6.2.1 Short Term Regulation	52
a) Allosteric Regulation	52
b) Regulation By Phosphorylation/Dephosphorylation	52
1.6.2.2 Hormonal Regulation of ACC 265 Activity	55
a) Glucagon and Adrenaline	55
b) Insulin	56
1.6.2.3 Regulation Of ACC 265 In Response To Fasting/Refeeding	57
1.6.3 Regulation Of Muscle ACC 280	57

1.6.3.1 Regulation Of ACC 280 By Phosphorylation	57
1.6.3.2 Regulation Of Muscle ACC During Exercise	58
1.6.4. Regulation Of Muscle ACC In Response To Fasting/ Refeeding	61
1.6.5 Regulation Of Heart ACC 280	61
1.6.6 Human Isoforms Of ACC	62
1.7 Fuel Selection	63
1.7.1 PDH Complex	64
1.7.2 Malonyl-CoA/CPT 1	66
 CHAPTER 2. MATERIALS & METHODS	 71
2.1 Commercial Preparations	72
2.2 Laboratory Preparations	74
2.2.1 Palmitate Bound To The Albumin	74
2.2.1.1 Determination Of Palmitate Bound To Albumin	74
2.2.2 Purification Of Fatty Acid Synthase	75
2.3 Animals	76
2.4 The Soleus Strip Preparation	76
2.4.1 Isolation And Incubation Of Soleus Strips	77
2.5 Preparation Of Tissue Extracts For Metabolite Measurements	78
2.5.1 Preparation Of Acid Soluble Extract For Measuring Malonyl-CoA	78
2.5.2 Preparation Of Acid Insoluble Pellet For Measuring Long Chain acyl-CoA and acylcarnitine	79
2.6 Determination Of Metabolites	80
2.6.2 Long Chain Fatty Acyl-CoA (free coenzyme (CoASH))	83
2.6.3 Carnitine	84
2.6.3.1 Column Preparation	84
2.6.3.2 Carnitine Assay	84
2.6.3.3 Fractionation	86
2.6.4 HPLC Analysis Of Tissue Nucleotides	86
2.6.5 Lactate	87
2.7 Measurements Of Carbohydrate Metabolites In Isolated Soleus Strips	88
2.7.1 Formation of $^{14}\text{CO}_2$ From $[\text{U-}^{14}\text{C}]$ Glucose	88

2.7.2 Incorporation Of [$U^{14}C$] Glucose into Glycogen	89
2.8 Measurement Of Exogenous And Endogenous Palmitate Oxidation	89
2.8.1 Exogenous Palmitate Oxidation	89
2.8.2 Endogenous Palmitate Oxidation	90
2.8.3 Lipid Extraction And Fractionation	91
2.8.3.1 Lipid Extraction	91
2.8.3.2 Lipid Fractionation	92
2.8.3.3 Triacylglycerol	92
2.9 Preparation Of Subcellular Fractions	93
2.9.1 Muscle	93
2.9.2 Liver	96
2.10 Enzyme Assays	97
2.10.1 ATP-Citrate Lyase	97
2.10.2 Acetyl-CoA Carboxylase	97
2.10.3 Fatty Acid Synthase	98
2.10.3.1 Spectrophotometric Assay	98
2.10.3.2 Radiochemical Assay	98
2.10.4 Malonyl-CoA Decarboxylase	99
2.10.5 Citrate Synthase	100
2.10.6 Malonyl-CoA Deacylase	100
2.11 Measurement Of Protein Concentration	100
2.12 Scintillation Counting	101
2.13 Statistical Methods	101
CHAPTER 3. RESULTS & DISCUSSION	102
3.1 Enzymes Involved In The Synthesis Of Malonyl-CoA In Skeletal Muscle	103
3.1.1 ATP-Citrate Lyase	104
3.1.2 Acetyl-CoA Carboxylase	106
Conclusions	113
3.2 Enzymes Involved In The Disposal Of Malonyl-CoA In Skeletal Muscle	114
3.2.1 Fatty Acid Synthase	115
3.2.2 Malonyl-CoA Decarboxylase	120

Conclusions	127
3.3 Measurements Of Metabolite Intermediates In Incubated Soleus Muscle	128
Acute Regulation Of Fatty Acid Metabolite Levels In Isolated Soleus Muscles	129
3.3.1 Malonyl-CoA Content In Soleus Muscle	135
3.3.2 The Effects Of Insulin On Metabolite Levels	136
3.3.2.1 Malonyl-CoA	136
3.3.2.2 Long Chain Esters	138
3.3.3 The Effects Of Etomoxir On Metabolite Levels	144
3.3.3.1 Malonyl-CoA	145
3.3.3.2 Long Chain Esters	147
3.3.4 The Effects Of Dichloroacetate (DCA) On Metabolite Levels	152
3.3.4.1 Malonyl-CoA	153
3.3.4.2 Long Chain Esters	153
3.3.4.3 0.3mM DCA & 1mM DCA	155
Conclusions	159
3.3.5 The Effects Of Isoprenaline On Metabolite Levels	161
3.3.5.1 Malonyl-CoA	161
3.3.5.2 Long Chain Esters	163
3.3.6 The Effects Of Palmitate On Metabolite Levels	171
3.3.6.1 Malonyl-CoA	171
3.3.6.2 Long Chain Esters	171
3.3.7 The Effects Of AICAR On Metabolite Levels	174
3.3.7.1 Malonyl-CoA	177
3.3.7.2 Long Chain Esters	177
3.3.7.3 Nucleotide Analysis	186
Conclusions	188
3.3.8 Lactate Measurements	190
3.3.9 Overall Conclusion	192
CHAPTER 4. GENERAL DISCUSSION	196
4.1 General Discussion	197

CHAPTER 5.	FUTURE WORK	203
5.1 Suggestions For Future Work		204
CHAPTER 6.	REFERENCES	
6. References		205

A List Of Figures And Tables

Figures	Page
Fig 1.1 a Arrangement of skeletal muscle fibre filaments that produce the striated banding pattern	5
Fig 1.1 b Arrangement of thick and thin filaments in a single sarcomere	5
Fig 1.2 Arrangement of myosin stabilizing protein, titin and actin strengthening protein, nebulin	7
Fig 1.3 Chemical and mechanical changes during a cross bridge cycle	9
Fig 1.4 Pathways of glucose and glycogen metabolism	21
Fig 1.5 Features of the CPT enzyme in liver mitochondria	46
Fig 1.6 Shows the sites on liver ACC which are phosphorylated in response to different protein kinases	54
Fig 1.7 Summarises the events that occur in skeletal muscle during exercise	60
Fig 1.8 Proposed scheme for citrate as a link between the malonyl-CoA fuel sensing mechanism and glucose/fatty acid cycle	69
Fig 2.1 Standard curve for assay of small amounts of malonyl-CoA	81
Fig 2.2 Standard curve for carnitine determination	85
Fig 2.3 Muscles of the hindlimb, lateral view	94
Fig 2.4 Deeper muscles of the hindlimb, lateral view	95
Fig 3.1 Citrate activates ACC activity in muscle	107
Fig 3.2 Time course for ACC activity in different skeletal muscle fibre types	109
Fig 3.3 Time course for fatty acid synthase activity in muscles	118
Fig 3.4 Fatty acid synthase activity in muscles	119
Fig 3.5 Malonyl-CoA decarboxylase activity in cytosolic and mitochondrial fractions in muscles	122
Fig 3.6 Insulin increases glucose oxidation in muscle	131
Fig 3.7 Glycogen content in isolated soleus muscle strips	132

Fig 3.8 Palmitate oxidation in the presence and absence of insulin	134
Fig 3.9 Insulin increases malonyl-CoA content in soleus muscle	137
Fig 3.10 Insulin increases long chain acyl-CoA levels in soleus muscle	139
Fig 3.11 Insulin decreases long chain acylcarnitine levels in soleus muscle	140
Fig 3.12 Insulin decreases the ratio of long chain acylcarnitine/long chain acyl-CoA in soleus muscle	141
Fig 3.13 Insulin decreases total long chain esters in soleus muscle	143
Fig 3.14 150 μ M etomoxir significantly increases malonyl-CoA levels in soleus muscle	146
Fig 3.15 Etomoxir increases long chain acyl-CoA content in isolated soleus muscle	148
Fig 3.16 Etomoxir has no significant effect on long chain acylcarnitine levels in isolated soleus muscles	149
Fig 3.17 Etomoxir has no significant effect on total long chain esters in soleus muscle	150
Fig 3.18 Etomoxir decreases the ratio of long chain acylcarnitine/acyl-CoA in soleus muscle	151
Fig 3.19 DCA in the presence of glucose alone suppresses exogenous palmitate oxidation in isolated soleus muscle strips	156
Fig 3.20 Isoprenaline significantly decreases malonyl-CoA levels in soleus muscle	162
Fig 3.21 Isoprenaline in the presence of insulin lowers long chain acyl-CoA levels in soleus muscle	164
Fig 3.22 Isoprenaline in the presence of insulin increases long chain acylcarnitine levels in soleus muscle	165
Fig 3.23 Isoprenaline in the presence of insulin increases the combined level of long chain acyl-CoA & acylcarnitine esters	166
Fig 3.24 Isoprenaline in the presence of insulin increases the ratio of long chain acylcarnitine/long chain acyl-CoA	167
Fig 3.25 Isoprenaline in the presence of insulin stimulates exogenous [U- ¹⁴ C] palmitate oxidation in isolated soleus muscle strips	169
Fig 3.26 Proposed mechanism of action of AICAR in intact cells	175

Fig 3.27 Malonyl-CoA measurements in isolated soleus muscle in the presence of AICAR	178
Fig 3.28 Long chain acyl-CoA measurements in soleus muscle in the presence of AICAR	179
Fig 3.29 Long chain acylcarnitine measurements in the soleus muscle in the presence of AICAR	180
Fig 3.30 Measurements of total long chain esters in the presence of AICAR	181
Fig 3.31 The ratio of acylcarnitine/acyl-CoA in the presence of AICAR	182
Fig 3.32 AICAR in the presence of insulin increases exogenous [U- ¹⁴ C] palmitate oxidation in isolated soleus muscle strips	184
Fig 3.33 Analysis of nucleotides from isolated soleus muscle strips incubated with AICAR	187
Fig 3.34 Linear correlation between tissue contents of malonyl-CoA and long chain fatty acyl-CoA esters	194

Tables

Table 1.1 Summary of the properties of the different types of muscle fibres	16
Table 3.1 ATP-Citrate lyase activity in different skeletal muscle fibre types	105
Table 3.2 Acetyl-CoA carboxylase measurements in different muscle fibre types	111
Table 3.3 Fatty acid synthase activity in muscle	116
Table 3.4 Malonyl-CoA decarboxylase activity	124
Table 3.5 True malonyl-CoA decarboxylase activity	126
Table 3.6 Fatty acid metabolite measurements in the presence of DCA	154
Table 3.7 Measurements of exogenous and endogenous palmitate oxidation in soleus muscle (with DCA)	157
Table 3.8 Measurements of exogenous and endogenous palmitate oxidation in soleus muscle (with isoprenaline)	170
Table 3.9 Fatty acid metabolite measurements in the presence of palmitate	172
Table 3.10 Measurements of exogenous and endogenous palmitate oxidation	

in soleus muscle (with AICAR)	185
Table 3.11 Lactate Measurements in the soleus muscle	191

ABBREVIATIONS USED

ACC : acetyl-CoA Carboxylase

ACCK: acetyl-CoA carboxylase kinase 2

ACL: ATP-Citrate lyase

ADP: adenosine 5'-diphosphate

AICAR: 5-amino imidazole-4-carboxamide ribonucleoside

AMP: adenosine 5'-monophosphate

AMPK: AMP-activated protein kinase

ATP: adenosine 5'-triphosphate

BSA: Bovine Serum Albumin

Ca²⁺: calcium

CoASH: CoEnzyme A

CPT 1 and CPT 2 : the overt and latent forms respectively of mitochondrial carnitine palmitoyltransferase

cAMP: adenosine 5'-3'-cyclic monophosphate

CK2: casein kinase 2

CS: citrate synthase

DCA: dichloroacetic acid

EDL: extensor digitorum longus

FBPase-2: fructose-2,6- bisphosphatase

Fru-2,6-P₂: fructose-2,6- bisphosphatase

Fru-6-P: fructose-6-phosphate

GSK 3: glycogen synthase kinase 3

HSL: Hormone-sensitive-lipase

IMP: inosine 5'-monophosphate

Ins: Insulin

Iso: Isoprenaline

LPL: lipoprotein lipase

MDC: malonyl-CoA decarboxylase

NAD⁺: nicotinamide adenine dinucleotide

NADH: reduced nicotinamide adenine dinucleotide

NADPH: reduced nicotinamide adenine dinucleotide phosphate

NEFA: non-esterified fatty acids

pal: palmitate

PDH: pyruvate dehydrogenase

PDK 1: 3-phosphoinositide dependent protein kinase 1

PFK-1: 6-phosphofructo-1-kinase

PFK-2: 6-phosphofructo-2-kinase

Pi: inorganic phosphate

PI(4,5)P₂: Phosphatidylinositide 4,5 bisphosphate

PI(3,4,5)P₂: Phosphatidylinositide 3,4,5 triphosphate

PKA: protein kinase A

PKB: protein kinase B

PKC: protein kinase C

POCA: sodium 2-[5-(4-chlorophenyl)-pentyl]-oxirane-2-carboxylate

TAG: triacylglycerol

TDG-CoA: tetradecyl glycidyl CoA

ZMP: 5-aminoimidazole-4-carboxamide ribonucleoside monophosphate

ZTP: 5-aminoimidazole-4-carboxamide ribonucleoside triphosphate

*Dedicated to the most beautiful person in my life
my mum.*

Chapter One

Aims & Introduction

AIMS

Malonyl-CoA is an important intermediate in the synthesis and elongation of fatty acids (Cinti *et al*, 1992) and is present in significant quantities in lipogenic tissues (malonyl-CoA content in fed rat liver is 7-8 nmol/g wet wt.). Malonyl-CoA is formed in the cytosol via acetyl-CoA carboxylase (ACC) and used in the same compartment by the fatty acid synthetase complex (FAS). In the liver a regulatory role for malonyl-CoA has been widely recognised and studied (McGarry & Foster, 1980). The ability of malonyl-CoA to inhibit the overt form of carnitine palmitoyltransferase (CPT 1) in the mitochondria allows a reciprocal control of fatty acid synthesis and fatty acid oxidation to occur (McGarry & Foster, 1980). In the liver this has important bearing on the control of ketogenesis.

It was initially shown by Saggerson and Carpenter (1981) and then by Mills *et al*, (1983) that malonyl-CoA inhibits CPT 1 in mitochondria from non-lipogenic tissues (skeletal muscle and heart). Moreover, and intriguingly, the CPT 1 in these tissues is far more sensitive to malonyl-CoA than CPT 1 in liver. It was found that malonyl-CoA was present in skeletal muscle and heart at concentrations which were not very much different from those reported in liver (McGarry *et al*, 1983 and Awan & Saggerson, 1993) and if evenly distributed through the muscle cell it would lead to total inhibition of CPT 1 under all physiological conditions. Obviously this cannot be so since long chain fatty acids are readily used as metabolic fuels by oxidative muscles under many conditions. From this we derive the aims of this project.

- How is malonyl-CoA synthesized in muscle tissues ?

- How is it disposed of in these cell types (e.g. via FAS, or via the rather poorly characterized routes of malonyl-CoA decarboxylation to acetyl-CoA or deacylation to malonate) ?
- In the short term what effects would exposure to e.g. fatty acids, hormones (insulin), and various other effectors have on muscle malonyl-CoA levels ?

In answer to the first point, a muscle specific isoform of ACC has been characterized (Bianchi *et al*, 1990 and Trumble *et al*, 1995), but in this study measurements of ACC activity in the absence and presence of citrate in all three muscle types (type I, type IIa and type IIb) were made.

This investigation will provide an insight into the synthesis and the disposal of malonyl-CoA in muscle and its regulation in the short term.

1.1 GENERAL INTRODUCTION

Muscle cells have the ability to use chemical energy to produce force and movement which is limited in most other living cells.

On the basis of structure, contractile properties and control mechanisms 3 types of muscles are present in multicellular organisms 1) skeletal muscles, 2) smooth muscles and 3) cardiac muscle. Although there are significant differences in the form and function of these three types of muscle, the force generating mechanism is similar in all of them and only skeletal muscle will be discussed in great detail.

1.2 STRUCTURE OF SKELETAL MUSCLE

The cells of skeletal muscles are known as muscle fibres, which are formed during development by fusion of myoblasts. Individual cells surrounded by connective tissues are grouped together into bundles and are known as *FASICULI*, which are further grouped together and surrounded by a covering of connecting tissue the *epimysium*. The whole structure is the muscle and is attached to the bones by tendons at each end of the muscle. Each muscle fibre is composed of a series of light and dark bands orientated perpendicular to the long axis of the fibre. This pattern is due to the presence of cylindrical elements in the fibre cytoplasm known as myofibrils. Each myofibril is composed of thick and thin filaments arranged in a repeating pattern along the length of the myofibril. One unit of this repeating pattern is known as a sarcomere (Fig 1.1a). The filaments are composed entirely of the contractile proteins myosin (thick filaments), actin (thin filaments) and two other proteins troponin and tropomyosin (Fig 1.1a)

Fig 1.1

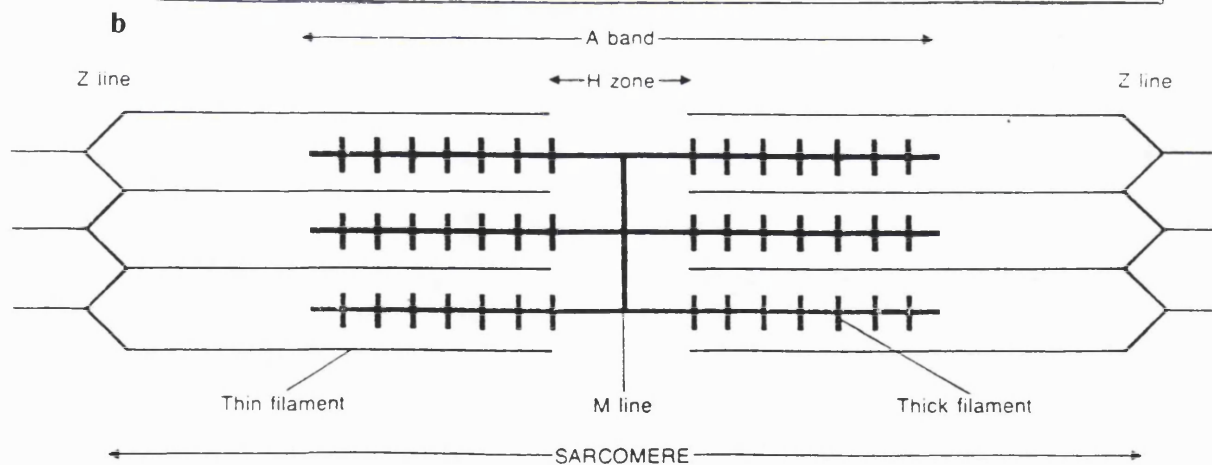
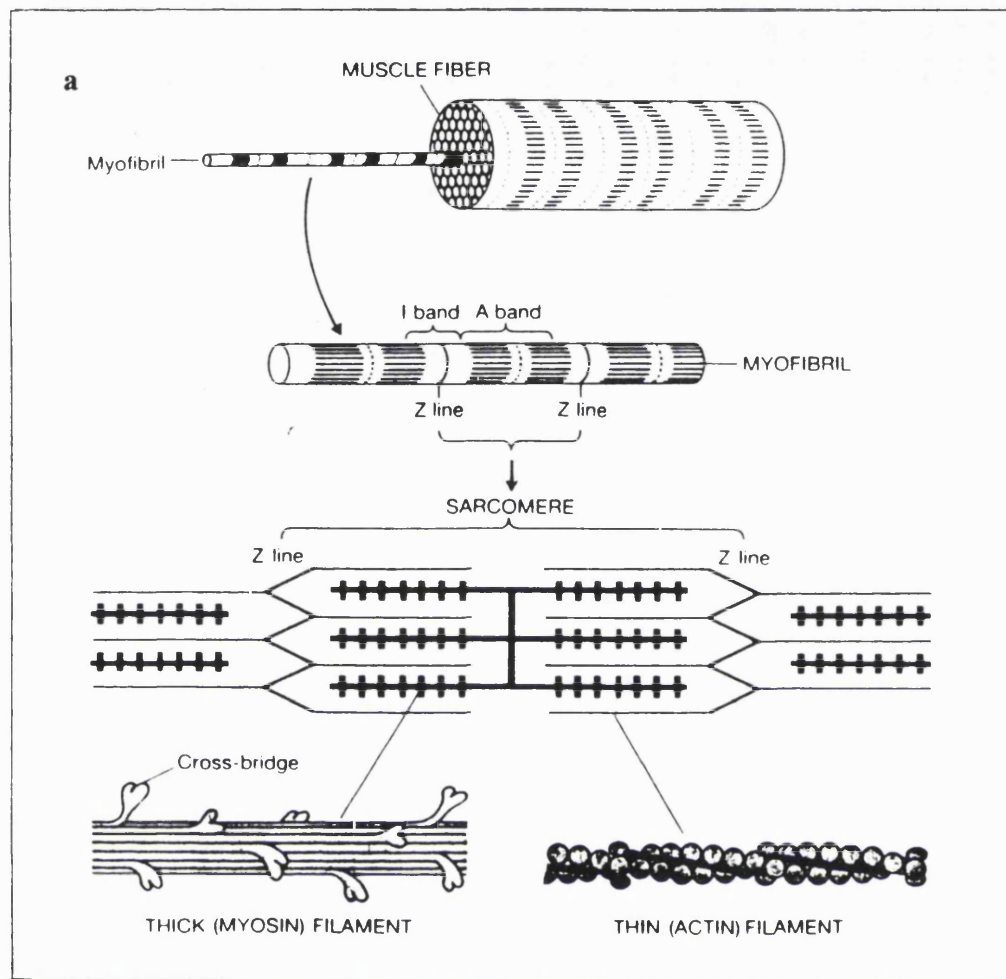


Fig 1.1 a Arrangement of skeletal muscle fibre filaments that produce the striated banding pattern

Fig 1.1 b Arrangement of thick & thin filaments in a single sarcomere

(Adapted from Human Physiology, (Vander, Sherman and Luciano))
(Fifth Edition)

The thick filaments are located in the middle of each sarcomere and their parallel arrangement produces the dark wide band, known as the A-band (Fig 1.1b). Whilst two sets of thin filaments are present one at each end of the sarcomere and one end of each thin filament is attached to a series of interconnecting proteins known as the Z-line, whereas the other end overlaps a portion of the thick filament (Fig 1.1a & b). The Z-line defines the limits of one sarcomere as shown in fig 1.1a. Portions of the thin filaments that do not overlap the thick filaments are present in the I-band. The thick and the thin filaments are not free floating, as the thin filaments are attached to the Z-line and the thick filaments are linked together by the M-line. The M-line correspond to the centre of the H-zone. The H-zone is found in the centre of the A-band and is the space between the ends of the two sets of thin filaments in each sarcomere (Fig 1.1b).

Myosin (thick) filaments are stabilised in the longitudinal axis by an array of thin filaments, composed of titin. Part of the titin strand is firmly attached to the myosin and the remainder extends through the I-band. Titin is thought to function as a longitudinal stabiliser for the myosin filament keeping it in the centre of the sarcomere during contraction and relaxation. Actin (thin) filaments contain two regulatory proteins troponin and tropomyosin and also a strengthening protein nebulin (fig 1.2).

Each thick filament is surrounded by a hexagonal array of six thin filaments and each thin filament is surrounded by a triangular arrangement of 3 thick filaments. Portions of myosin molecules extend from the surface of the thick filaments towards the thin filaments, forming cross bridges. During muscle contraction these cross bridges make contact with the thin filaments and exert force on them. These cross bridges are the force generating sites in muscle cells.

Fig 1.2

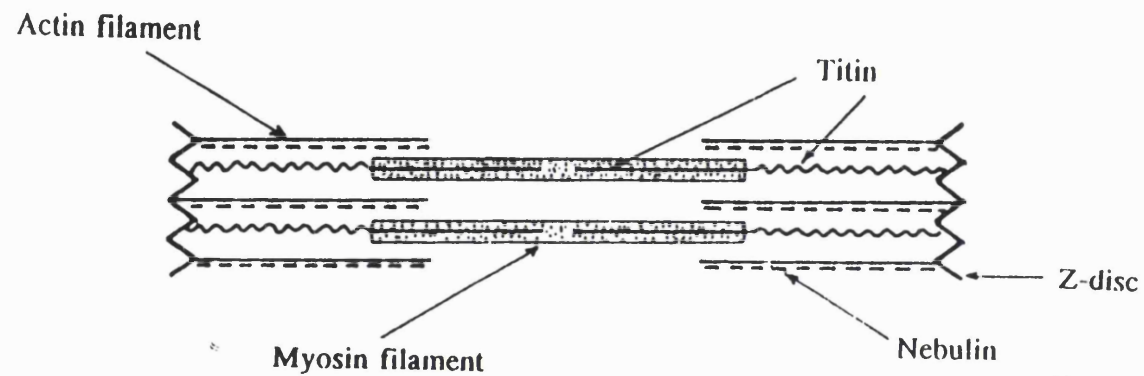


Fig 1.2 Arrangement of myosin stabilizing protein, titin and the actin strengthening protein, nebulin.

Adapted from Skeletal muscle: Form and function (McComas)

1.2.1 The Mechanism Of Contraction

When muscle fibres contract, cross bridges in the thick filaments bind to actin in the thin filaments, causing the thin filaments to move towards the centre of the sarcomere and as a result shortening of the muscle fibre occurs. There is no change in either the length of the thick or thin filaments, but the sarcomere is shortened. The sequence of events that occurs, between the time a cross bridge binds to a thin filament and the time it takes to repeat the process is known as a Cross-Bridge Cycle. The mechanism involved in a cross bridge cycle is as follows. Myosin has a globular head attached to the long tail of the myosin filament. Each globular head consists of a binding site for actin and an ATPase site. When a muscle fibre is stimulated to contract, the globular head of the myosin molecule binds to an actin molecule in the thin filament. This causes ADP and Pi to be released from myosin and results in the movement of the bound cross bridge (see fig 1.3). This linkage of myosin to actin must be broken in order to allow the cross bridge to reattach to a new actin molecule and repeat the cycle. ATP binds to myosin, breaking the link between actin and myosin. ATP bound to myosin is subsequently released, thereby the myosin can bind to a new actin filament and the cycle can be repeated as long as the fibre remains active (fig 1.3). ATP bound to myosin is necessary to free the myosin from the actin filament during the cross bridge cycle. Otherwise, in the absence of ATP the actin and myosin filaments remain locked together resulting in rigor mortis, stiffening of muscles which occurs several hours after death.

Actin filaments are associated with two regulatory proteins, tropomyosin and troponin. They inhibit the binding of cross bridges to actin during the resting state, thus the muscle is not in a continuous state of contractile activity.

Fig 1.3

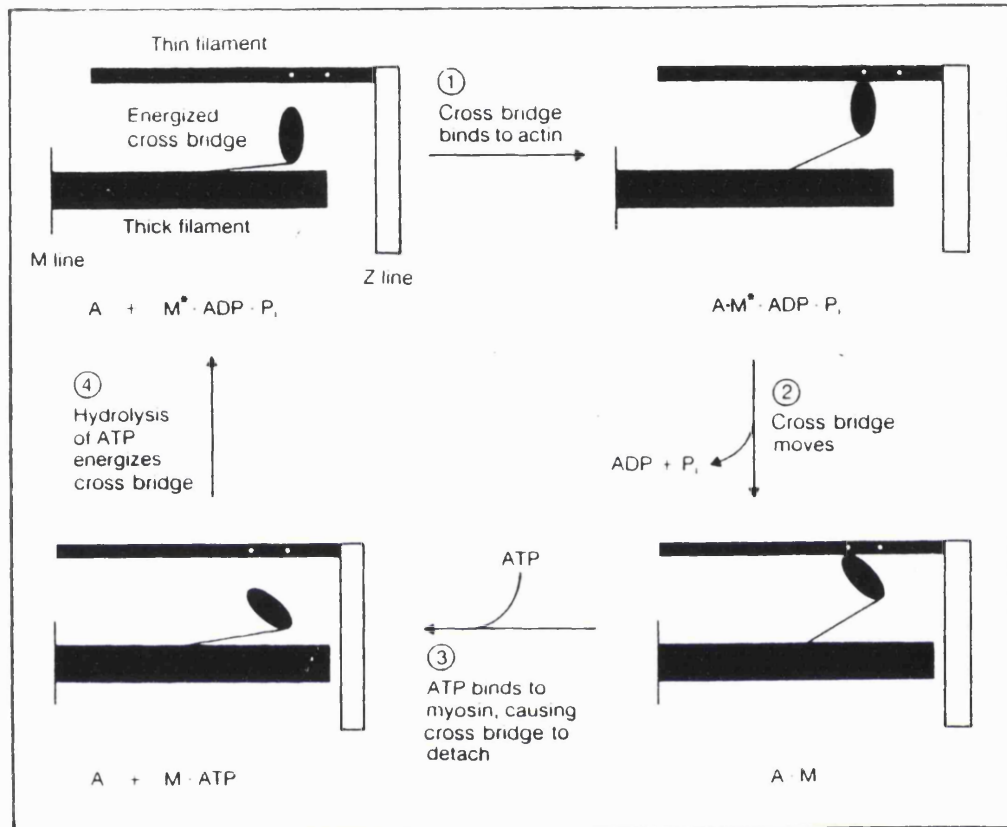


Fig 1.3 Chemical and mechanical changes during a cross bridge cycle. In a resting muscle fibre, contraction occurs with the binding of a cross bridge to actin in a thin filament

(Adpated from Human Physiology (Vander, Sherman and Luciano))

(Fifth Edition)

Tropomyosin, consists of a double helix , lying on the surface of the actin molecule and partially covers the myosin binding site on actin. Troponin is a complex of three polypeptides, one of which is attached to tropomyosin (troponin T). Troponin I binds to actin and prevents the actin from making contact with the myosin head. During muscle contraction, cytosolic Ca^{2+} rises, binding to specific sites on troponin, which then undergoes a conformational change moving the tropomyosin molecule away from actin. This exposes the sites on actin to which the cross bridges can attach and allows contraction to proceed. Conversely, removal of Ca^{2+} from the troponin, reverses the process and tropomyosin moves back in place, and this blocks the contact of actin with cross bridges.

1.2.1.1 Excitation-Contraction Coupling

In a resting muscle fibre, the concentration of free Ca^{2+} in the cytosol surrounding the thick and thin filaments is very low (10^{-7} M). As a result very few of the Ca^{2+} binding sites on troponin are occupied and thereby cross bridge activity is blocked by tropomyosin. Following an action potential, the cytosolic concentration of Ca^{2+} rises binding to troponin, relieving the inhibitory effects of tropomyosin on actin and cross bridges can bind to actin allowing contraction. This sequence of events is referred to as an excitation-contraction coupling.

This increase in Ca^{2+} concentration is from the sarcoplasmic reticulum within the muscle fibre. The sarcoplasmic reticulum in the skeletal muscle is the same as the endoplasmic reticulum in most other kinds of cells. Lateral sacs, located at each end of the reticulum are connected by a series of smaller tubular elements. These sacs store the Ca^{2+} that is released following membrane excitation. Transverse tubules (t-tubules) are found at the

junction of each A-I band, giving two tubular arrays per sarcomere and are like the plasma membrane in being able to conduct action potentials. As the action potential in a t-tubule passes the sarcoplasmic reticulum, it triggers the opening of calcium channels in the lateral sacs, and calcium diffuses into the cytosol. Muscle contractions are terminated, by the removal of calcium from the cytosol back into the sarcoplasmic reticulum. Pumping of calcium back into the sarcoplasmic reticulum is against a large concentration gradient and requires ATP to provide the necessary energy. The membranes of the lateral sacs contain Ca-ATPase transport proteins, that pump calcium ions from the cytosol back into the lumen of the reticulum. Once inside the sarcoplasmic reticulum, calcium ions are released from the pump and are taken up by calcium binding proteins.

1.2.2 TYPES OF SKELETAL MUSCLE FIBRES

Histochemical, biochemical and physiological studies of hindlimb skeletal muscle of guinea pig, rat and rabbit have shown the presence of three fibre types and each type has a distinctive combination of metabolic and mechanical characteristics (Edgerton & Simpson (1969), Barnard *et al* (1971), Ariano *et al* (1973), Dubowitz (1967) and Burke *et al*, (1971)). Skeletal muscle fibres are classified as slow-twitch oxidative (TYPE I), Fast-twitch oxidative-glycolytic (TYPE IIa) and fast-twitch glycolytic (TYPE IIb) based on 1) their twitch characteristics which correlates with the specific activity of myosin ATPase and 2) the major pathways used to form ATP by oxidative phosphorylation and glycolysis.

Myosin ATPase is the enzyme involved in hydrolyzing ATP during cross bridge movement. Both fast and slow fibres contain myosin ATPase but they differ in the

maximal rate at which they split ATP. This determines the rate at which actin and myosin filaments slide over each other and hence the speed of muscle shortening. From myosin ATPase staining and enzymatical studies (Engel 1962), it is possible to identify fast and slow twitch fibres. Type II fibres stain intensely for ATPase at pH 9.4 and also have the highest specific activity for myosin ATPase activity and thus are classified as fast fibres. Conversely, type I fibres stain poorly for myosin ATPase at pH 9.4 and have a lower activity for ATPase and are known as slow fibres. However, type IIa fibres have an intermediate myosin ATPase activity (between those observed in the type IIb muscle fibres and type I muscle fibres) and have an intermediate stain for myosin ATPase (Peter *et al*, 1972). By preincubating the type IIa muscle fibres at a different pH from type IIb fibres and then staining them for myosin ATPase at pH 9.4, it was possible to distinguish them from type IIb fibres (Brooke & Kaiser 1970, and Edgerton & Simpson, 1970). However, Close (1972) found that type IIa fibres have a high myosin ATPase activity and type IIb fibres have an intermediate activity for myosin ATPase. This is contradictory to what Peter *et al*, (1972) found (mentioned above). This difference could be due to variations in muscle preparations and experimental conditions.

In summary, type IIa and type IIb muscle fibres are fast contracting muscle fibres containing ATPase with a relatively high specific activity for myosin ATPase and type I muscle fibres are slow contracting fibres and contain myosin ATPase of a relatively low specific activity.

The other way of classifying skeletal muscle fibres is according to the type of enzymatic machinery available for synthesising ATP. By carrying out histochemical and enzymatical studies it is possible to assess the muscle's oxidative and glycolytic capacities to synthesize ATP (Peter *et al*, 1972, Barnard *et al*, 1971, Beaty *et al*, 1963 and Edgerton & Simpson 1969). Slow twitch oxidative fibres (type I) depend predominantly on

aerobic metabolism as they have an intermediate activity (between those observed in the fast twitch oxidative-glycolytic and fast twitch glycolytic fibres) for enzymes involved in aerobic metabolism e.g. succinate dehydrogenase. These fibres have numerous mitochondria, are abundant in myoglobin and have a high density of capillaries perfusing them allowing them to oxidise substrates from the blood. These fibres are resistant to fatigue due to a constant capillary flow which facilitates good diffusion of oxygen to the fibres resulting in rapid re-phosphorylation of ATP and creatine phosphate, thus providing energy for muscle contraction and a steady muscle performance (Hudlicka, 1975). These fibres have a low glycogen content and low phosphorylase and lactate dehydrogenase activity and rarely rely on anaerobic metabolism (Barnard *et al*, 1971).

Fast-twitch glycolytic muscles have a high glycogen concentration, high phosphorylase and lactate dehydrogenase activities, indicating that they rely mainly on anaerobic metabolism for production of ATP (Barnard *et al*, 1971). Their aerobic capacity is very low as they have minimal succinate dehydrogenase activity as well as a low myoglobin and mitochondrial content. Their role is to produce energy quickly, but as they depend upon stored substrates they cannot maintain this for a long period of time and thereby fatigue easily (Close, 1972 and Hudlicka, 1975). They are particularly important in the production of energy over short periods of time such as that required for sprinting.

Fast-twitch oxidative-glycolytic fibres have a powerful oxidative capacity for making ATP as they have the highest activity of succinate dehydrogenase as well as a high myoglobin concentration (Peter *et al*, 1971 and Edgerton & Simpson 1969). Also the capillary to fibres ratio in these fibres is high compared to fast twitch glycolytic fibres, thus a blood flow is present which is adequate for aerobic metabolism. However, they have the ability to produce ATP by glycolysis as they have a high glycogen content and a moderate lactate dehydrogenase activity. The high capacities for glycolytic,

glycogenolysis and oxidative phosphorylation together with their speed of contraction enables these fibres to be used during sustained contraction which requires a greater power output than the type I fibres are capable of and which could not be maintained by the easily fatiguable type IIb fibres.

The relationship between blood flow and oxygen consumption is quite different in fast and slow contracting muscles (cats) (Hudlicka, 1975). Slow contracting muscles (soleus), where both the activity of oxidative enzymes and the density of capillaries are high, have a resting flow several times higher than that of a fast contracting muscle (gastrocnemius). During increased contractions, oxygen consumption increases 7 fold in fast muscles with a concomitant increase in blood flow (2.5 fold). In contrast, in slow muscles (soleus) the blood flow hardly increases. The reason being, high capillary density and the relative consistency of capillary flow in slow muscles clearly facilitates good diffusion of oxygen to muscle fibres, and such a constant supply of oxygen ensures a very rapid rephosphorylation of ATP and creatine phosphate. This provides energy for muscle contraction and steady muscle performance without the development of fatigue. In contrast, an increased blood supply is essential for muscle performance in fast muscles, as they fatigue easily and are producing a greater amount of work than at rest thereby requiring an increased blood flow during contractions. It can be suggested that in fast muscles changes in muscle work and oxygen consumption are dependent on blood flow. However changes in oxygen consumption and muscle performance in slow muscles change independently of blood flow (Hudlicka, 1975).

Physical activity can alter the relative proportion of fibres within exercising muscles towards more oxidative muscle fibres (type I & Type IIa) and a decrease in type IIb fibres (Kriketos *et al*, 1995). Associated with this change in the relative proportion of muscle fibres during exercise, is an increase in activity of mitochondrial enzymes

associated with β -oxidation. It can be suggested that a shift in energy metabolism enhances the muscle's maximal work capacity, because the increase in mitochondrial enzyme activity causes an increase in the metabolism of fatty acids, thereby sparing glycogen stores (Kriketos *et al*, 1995).

Table 1.1 summarises the properties of the three types of muscle fibres. Soleus muscle is an example of type I muscle fibre and is composed of 89% type I muscle fibres. EDL is an example of type IIa muscle and is made up of approximately of equal proportions of type IIa and type IIb fibres. On the other hand the white gastrocnemius muscle is composed almost entirely of type IIb fibres (Kriketos *et al*, 1995).

1.3 SKELETAL MUSCLE ENERGY METABOLISM

Skeletal muscle in the resting state may be called upon to go into a state of contractile activity within seconds. ATP provides the source of energy and is synthesised either by anaerobic glycolysis of glucose (from stored glycogen or glucose taken up from blood) or from oxidation of pyruvate or free fatty acids (Holloszy & Booth, 1976). Also, ATP can be produced from creatine phosphate, by the action of creatine kinase. Creatine phosphate acts as a buffer store of ATP, as any fall in ATP concentration will lead to the formation of further ATP from ADP using creatine phosphate.

The oxidative enzymes are localised inside the mitochondria, whilst the enzymes of anaerobic glycolysis are present in the cytosol. Anaerobic glycolysis is relatively inefficient, but it has the advantage that it is unimpaired by the inadequacy in blood supply during sustained contractions. However, as the glycogen store is limited in muscle fibres, a continuously contracting muscle working anaerobically without fuel will soon

Table 1.1

**Summary Of The Properties Of The Different Types Of Muscle
Fibres**

<i>Property</i>	<i>Type I</i>	<i>Type IIa</i>	<i>Type IIb</i>
Rate of contraction	Slow	Fast	Fast
Myoglobin Content	High	High	Low
Mitochondrial Content	High	High	Low
Capillary Density	High	High	Low
Sarcoplasmic Reticulum	Low	High	High
Oxidative Metabolism	Good	Good	Poor
Anaerobic Capacity	Poor	Good	Excellent
Tendency To Fatigue	Low	Moderately low	High
Power Output	Low	Intermediate	High
Size	Small	Intermediate	Large

fatigue. In contrast, oxidative metabolism provides energy for an unlimited period of time as long as a sufficient blood supply is maintained. The different types of muscle fibres can to a certain extent shift between both metabolic pathways, but most fibres are specialized and differ in their content of enzymes and substrates for metabolism.

The synthesis of ATP occurs via a multi-enzyme process which occurs in the inner mitochondrial membrane and once synthesised it is transported to the cytosol where it is used by an ATP/ADP transport system in a 1:1 exchange with ADP. The ATP concentration in the three types of skeletal muscle fibres is not the same as they vary in the amount of mitochondria present and have differing capacities for oxidative and glycolytic metabolism. Skeletal muscle synthesises ATP using both stored fuel (glycogen and triacylglycerol) and substrates (glucose, fatty acid and ketone bodies) taken up from the blood. The fatty acids may be either plasma non esterified fatty acid (NEFA) or esterified fatty acids carried in the form of TAG in lipoproteins, hydrolyzed by lipoprotein lipase.

Skeletal muscles especially red muscle tend to oxidise fatty acids and ketone bodies rather than glucose under most conditions. This preference is further intensified by starvation and exercise.

The enzyme pathways that consume fats and glucose are highly regulated converting both fuels to acetyl-CoA, which is oxidised in the citric acid cycle producing CO₂ and H₂O with the consumption of oxygen and are discussed below.

1.3.1 CARBOHYDRATE METABOLISM

Skeletal muscle is the most important tissue for whole body glucose disposal in humans.

The main regulatory steps of glucose conversion to acetyl-CoA are discussed.

1.3.1.1 Glucose Transport

Glucose transport into the skeletal muscle occurs by facilitative diffusion mediated by the transport protein GLUT 4 and GLUT 1.

GLUT 4 and GLUT 1 transporter proteins belong to a family of glucose transporters (Gould & Holman, 1993), which are specific for D-glucose and are not coupled to any energy requiring components. In the absence of insulin, glucose uptake is the rate limiting step in glucose metabolism in skeletal muscle (Ren *et al*, 1993). Insulin stimulates glucose uptake by promoting translocation of GLUT 4 transporters to the plasma membrane (Holloszy *et al*, 1987, Wilson & Cushman 1994). Also, contraction mediates glucose uptake in muscles by stimulating translocation of GLUT 4 from an intracellular pool to the plasma membrane through a mechanism distinct from that of insulin (Lund *et al*, 1995). Evidence for contraction-stimulated translocation of GLUT 4 in skeletal muscle to occur through a mechanism distinct from that of insulin came from studies using wortmannin, a potent inhibitor of PI3-kinase. Wortmannin completely abolishes insulin stimulated glucose uptake in skeletal muscle, suggesting PI3-kinase is essential for the stimulation of glucose uptake by insulin (Lee *et al*, 1995). In contrast, wortmannin has no effect on contraction-stimulated glucose uptake (Lund *et al*, 1995 and Lee *et al*, 1995). This provides evidence that PI3-kinase is not involved in contraction-stimulated glucose uptake (Lee *et al*, 1995). However, other studies have found wortmannin to inhibit contraction stimulated glucose uptake in all three fibres, without affecting the muscle performance. The greatest effect was seen in the soleus muscle (Wojataszewski *et al*, 1996). This suggests that PI3-kinase may somehow be involved in contraction-stimulated glucose uptake in type I muscle fibres. An explanation for the discrepancy in the findings in the presence of wortmannin in

contracting-stimulated glucose uptake between the two groups is probably due to differences in the experimental design (Wojataszewski *et al*, 1996).

Excess glucocorticoid and free fatty acids decrease insulin stimulated glucose uptake in skeletal muscle (Dimitridiadis *et al*, 1997 and Jason *et al*, 1996). Exercise training leads to enhanced insulin stimulated glucose uptake in muscle. (Rodnick *et al*, 1990, Ivy *et al*, 1983, Leighton *et al*, 1989). Exercise increases the number of GLUT 4 transporters present at the plasma membrane (Slentz *et al*, 1992) and these have been shown not to originate from the insulin sensitive intracellular pool of GLUT 4 transporters. This suggests that the exercise sensitive pool is distinct from the intracellular insulin sensitive GLUT 4 transporter pool (Douen *et al*, 1990).

Skeletal muscle fibres also express GLUT 1 transporters at lower levels than those of GLUT 4. GLUT 1 is believed not to be involved in insulin stimulated glucose uptake (Mueckler, 1994). The expression of GLUT 4 in skeletal muscle fibre types correlates with the metabolic nature of the fibres rather than with their contractile properties. GLUT 4 is expressed more abundantly in oxidative muscles (Soleus) than in glycolytic muscles (gastrocnemius). This reflects oxidative muscles having a greater capacity for glucose uptake and a high degree of stimulation of glucose transport by insulin or by exercise (Marette *et al*, 1992 and Hocquette *et al*, 1994).

1.3.2 Glucose Phosphorylation

Glucose is phosphorylated to glucose-6-phosphate by hexokinase, this is the initial step in glycolysis converting glucose to pyruvate (see fig 1.4) and is virtually irreversible *in vivo*. Four isoforms of hexokinase are known to occur, hexokinase II & I are the predominant forms in skeletal muscle.

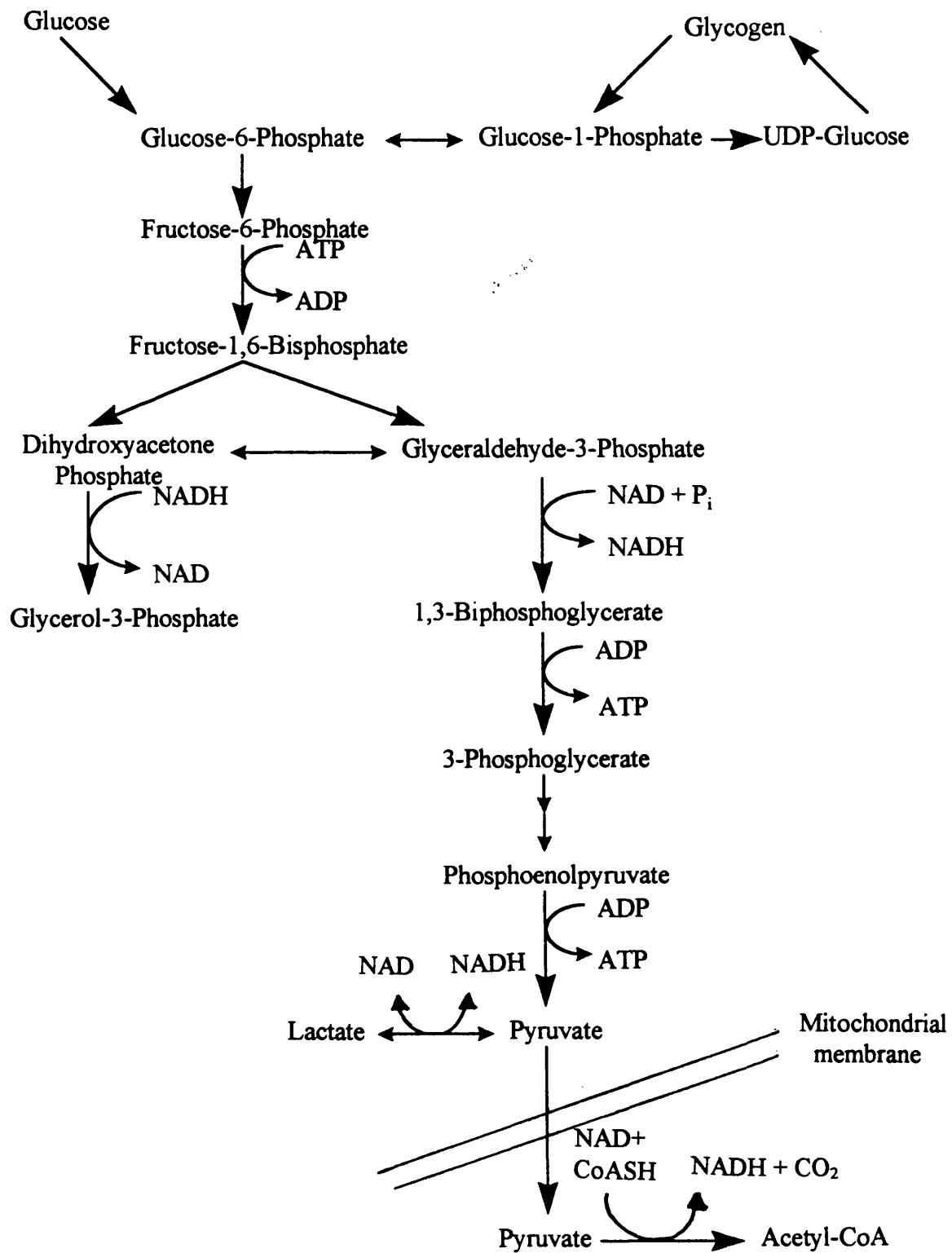
The hexokinase reaction is the rate limiting step in situations of high glucose transport. Glucose -6-phosphate, the product of the hexokinase reaction inhibits hexokinase and is thought to be the main regulator of hexokinase activity *in vivo*. Elevated levels of free fatty acids and ketone bodies indirectly inhibit hexokinase activity as a result of an accumulation of glucose-6-phosphate in heart and diaphragm muscle (Randle *et al*, 1963, 1964). It has been shown that increased free fatty acids (oleate) inhibit glucose phosphorylation in slow twitch muscles (type I muscle fibres) but not in fast twitch muscles (type IIb muscle fibres) (Nolte *et al*, 1994).

1.3.3 Phosphofructokinase

Phosphofructokinase (PFK-1) phosphorylates fructose-6-phosphate to fructose-1,6-bisphosphate and is the main site of regulation in the glycolytic pathway. PFK-1 is regulated by a number of substrates, reaction products and various cellular metabolites. Of these fructose-2,6-bisphosphate, fructose-1,6-bisphosphate, Pi, and AMP are efficient positive effectors while ATP, citrate and H⁺ ions are potent negative effectors (Thornheim & Lowenstein 1976, Uyeda, 1979, Kemp & Foe, 1983 and Spreit, 1991).

Figure 1.4

Pathways of Glucose and Glycogen Metabolism



1.3.3.1 Fructose-2,6-bisphosphate

Fructose-2,6-bisphosphate (Fru-2,6-P₂) is a potent activator of PFK-1 and was discovered through studies on the mechanism of glucagon action on liver glycolysis and gluconeogenesis (Van Schaftingen *et al*, 1980a, 1980b and Hers & Van Schaftingen, 1982). It is present in all mammalian tissues and acts in synergy with AMP in relieving the inhibition of PFK-1 by ATP and by citrate (Hue & Rider 1987 and Uyeda *et al*, 1982). Fru-2,6-P₂ is synthesised and degraded by the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-Bis-phosphatase (PFK-2/FBPase-2). Isoforms of PFK-2/FBPase-2 exist with differences in the relative proportion of the activities. In skeletal muscle the FBPase form is 5-10 times more active than PFK-2 and the bifunctional enzyme therefore is considered to be a phosphatase more than a kinase. In contrast the heart FBPase-2 activity is 80 times lower than PFK-2 activity and the enzyme is therefore more of a kinase than a phosphatase (Hue & Rider, 1987).

1.3.3.2 Regulation of PFK-2/FBPase

PFK-2/FBPase is inhibited by citrate, phosphoenolpyruvate and *sn*-glycerol 3-phosphate at physiological concentrations. Unlike the liver form, skeletal muscle PFK-2/FBPase is not phosphorylated by cAMP dependent protein kinase (Hue & Rider, 1987). However, PFK-2/FBPase activity in muscles is regulated by hormones and by *in vivo* conditions (e.g. exercise)

a)Hormones

Hormones such as adrenaline and insulin are known to increase Fru-2,6-P₂ in perfused hindlimb and thus stimulate glycolysis (Hue *et al*, 1982). This results most probably

from increased glucose transport and glycogenolysis, leading to the accumulation of glucose-6-phosphate and fructose-6-phosphate. By contrast, in the soleus muscle (red fibres) Fru-2,6-P₂ levels are not increased in the presence of adrenaline (Winder & Duan, 1992). This difference is probably due to metabolic differences between the two muscle preparation used, as the hindlimb contains a significant portion of white muscle fibres and the soleus is composed mainly of red fibres (section 1.2.2).

b) Electrical stimulation and Exercise

Electrical stimulation of the perfused hindlimb preparation increases glycolysis with a decrease in Fru-2,6-P₂ concentration. This increase in glycolysis may be due to the effects of changes in adenine nucleotide and phosphate levels and a fall in pH on PFK-1 (Hue *et al*, 1982).

In exercised fasted rats, increased levels of cAMP, Fru-2,6-P₂ and glucose-1,6-bisphosphate in white quadriceps muscles (type IIb muscle fibres) are associated with a rise in plasma adrenaline levels (Winder & Duan, 1992). This adrenaline-induced increase in Fru-2,6-P₂ is likely to be important in stimulating lactate production in type IIb muscle fibres during long term exercise when hepatic gluconeogenesis is the main source of glucose for type I muscle fibres (Winder *et al*, 1991 and Winder & Duan, 1992). Studies have shown a correlation between Fru-2,6-P₂ and muscle lactate production at various concentrations of adrenaline in perfused hindlimb. This confirms the earlier findings of Winder & Duan (1992), that the adrenaline-induced activation of glycolysis in type IIb muscle fibres is mediated by Fru-2,6-P₂ (Jones *et al*, 1994). However, adrenaline does not increase Fru-2,6-P₂ levels in red fibres as mentioned above (1.3.3.2a). This suggests the possibility that PFK-2 in red fibres may be similar to liver PFK-2 and is inactivated by cAMP dependent protein kinase (Winder & Duan, 1992).

The function and regulation of Fru-2,6-P₂ is well established in the liver as the level of Fru-2,6-P₂ determines which way glucose metabolism will go, either glycolysis or gluconeogenesis (Hue & Rider 1987). The role of Fru-2,6-P₂ in the stimulation of muscle glycolysis during exercise in oxidative muscles is unclear and it has been reported that Fru-2,6-P₂ does not contribute significantly to the control of glycolysis during intense muscular contractions (Wegener *et al*, 1990 and Krause & Wegener, 1996). It has been observed that during intense exercise Fru-2,6-P₂ levels are decreased. This makes it difficult to assign a regulatory role for Fru-2,6-P₂ during exercise. It has been suggested that oxidative muscles (working muscles) are capable of using substrates other than carbohydrates (fatty acids). Fru-2,6-P₂ could be involved in a mechanism to spare carbohydrate, as observed in the flight muscle of the locust. During sustained flight the locust muscle changes from using carbohydrate to lipid fuels as a main fuel and this is correlated with a decrease in Fru-2,6-P₂ (Wegener, *et al*, 1986). A similar mechanism could exist in mammalian working muscle during exercise, as Fru-2,6-P₂ in heart has been suggested to act as a glycolytic signal, switching on/off glycolysis in the absence and presence of alternative fuels, respectively (Hue & Rider, 1987).

1.3.3.3 Alternative Fuels

In the presence of alternative fuels such as ketone bodies, fatty acids and lactate, the rate of glycolysis decreases in skeletal muscle (Stanley & Connett, 1991). The effects are mediated by an increase in citrate concentration, inhibiting PFK-2 activity and Fru-2,6-P₂ synthesis. Inhibition of PFK-1, PFK-2 and Fru-2,6-P₂ synthesis, by citrate is considered to be of regulatory importance in down regulating glucose metabolism when fatty acid oxidation is increased *in vivo* (Rennie *et al*, 1976, Uyeda, 1979, Uyeda *et al*, 1982 and

James *et al*, 1985). This provides evidence for the involvement of this site in the glucose-fatty acid cycle (Randle *et al*, 1965).

1.3.4 Pyruvate Dehydrogenase

In skeletal muscle pyruvate can be converted to acetyl-CoA, lactate or alanine. Acetyl-CoA and CO₂ is formed by the oxidative decarboxylation of pyruvate, by the multienzyme pyruvate dehydrogenase complex (PDH). The complex is located within the mitochondria and comprises pyruvate dehydrogenase (E1), dihydrolipoamide acetyltransferase (E2) and dihydrolipoamide dehydrogenase (E3) which catalyses the oxidative decarboxylation of pyruvate and two regulatory enzymes, PDH Kinase and PDHP phosphatase with protein X linking E3 to the complex (Sugden & Holness, 1994).

1.3.4.1 Regulation Of PDH Complex

PDH complex activity is regulated by end product inhibition and by reversible phosphorylation at several sites. PDH kinase phosphorylates several sites on E1 and inactivates the complex. PDH phosphatase dephosphorylates and reactivates the complex (Randle *et al*, 1994).

a) PDH Kinase

PDH kinase is activated by increasing mitochondrial ratios of acetyl-CoA/ CoASH, NADH/NAD⁺ and ATP/ADP concentrations. The PDH kinase reaction is inhibited by pyruvate, thus acting as a feed forward activator for the PDH complex (Randle *et al*, 1994). However, during starvation cAMP and fatty acid levels rise and are responsible for an

increase in PDH kinase activity and/or abundance. Evidence for this came from culture experiments with the soleus muscle. (Stace *et al*, 1992).

b) PDH Phosphatase

Mg²⁺ is essential for PDH phosphatase activity and in the presence of Ca²⁺ (within the physiological range 0.1-10μM), activates it by influencing the binding of the phosphatase to E2 (Fuller & Randle, 1984 and Randle *et al*, 1994). During muscle contraction mitochondrial Ca²⁺ levels rise resulting in an increase in the activation of PDH phosphatase; thus more of the PDH complex is in the active form (Randle *et al*, 1994). PDH phosphatase activity can be inhibited by NADH (Randle *et al*, 1994). Insulin has been shown to activate PDH phosphatase in adipocytes, but no such effect has been observed in skeletal muscle (Randle *et al*, 1994).

1.3.4.2 Regulation Of PDH Complex During Exercise

During intense exercise, more of the PDH complex is in the active form throughout the exercise period despite an increased acetyl-CoA/CoASH ratio (Constantin-Teodosiu *et al*, 1992). This increase in acetyl-CoA does not inhibit the active form of PDH. The reason being, Ca²⁺ released from the sarcoplasmic reticulum as a result of muscle contraction activates PDH phosphatase and keeps the PDH complex in the active state. Also, formation of pyruvate is increased which inhibits PDH kinase, stimulating the transformation of PDH to the active form. This suggests that an increase in Ca²⁺ concentration and pyruvate during exercise overcomes the inhibitory effects of acetyl-CoA accumulation and results in an increase in the active portion of the PDH complex (Constantin-Teodosiu *et al*, 1991). It has been suggested that this increase in acetyl-CoA during exercise is accompanied by an increased store of acetyl-carnitine, which can

be utilised when the intensity of exercise is reduced and at rest (Constantin-Teodosiu *et al*, 1991 & 1992).

1.3.4.3 Starvation and Alternative Fuels

Starvation results in an increase in PDH kinase activity and a decrease in the activity of the PDH complex in skeletal muscle, heart and liver (Randle *et al*, 1994). Starvation in skeletal muscle leads to a decline in glucose utilisation and an increased PDH inactivation. This is brought about by an increase in PDH kinase activity due to a rise in mitochondrial NADH/NAD⁺ and acetyl-CoA/CoA ratios as a result of oxidation of lipid fuels (Fuller & Randle, 1984 and Holness *et al*, 1989).

In skeletal muscle, PDH inactivation is a relatively late event in starvation compared to the liver and heart (Randle, 1994) and complete reactivation of the PDH complex is not observed for at least 4-6 hours after re-feeding during which time hepatic glycogen is replenished (Sugden & Holness, 1989 and Sugden *et al*, 1993a). PDH reactivation is achieved more rapidly in Type II muscle e.g. gastrocnemius and extensor digitorum longus than in type I e.g. Soleus (oxidative muscles). This implies that in oxidative type I muscle fibres, suppression of PDH activity is greater than in type II muscle fibres. As type I muscle fibres are constantly working, their energy requirements are unlikely to be less during the starved to fed transition than in the fed state. The lower PDH activity in these muscles during the starved to fed transition than in the fed state suggests that ATP production is due to oxidation of non-carbohydrate fuels (fatty acids) (Sugden & Holness, 1989).

Associated with this decrease in PDH activity in oxidative muscles there is an increase in circulating concentration of NEFA, which is not observed if lipolysis is inhibited. This

provides evidence for the glucose/fatty acid cycle occurring via the activation of the PDH kinase in oxidative muscles (Holness *et al*, 1989).

In summary, suppression of glucose utilisation and of PDH activity in oxidative muscles is due to the preferential use of fatty acids as oxidative substrates during starvation, suggesting that the PDH complex is involved in the glucose/fatty acid cycle (Randle *et al*, 1994).

1.3.5 Glycogen Metabolism

Skeletal muscle glycogen plays a central role in the regulation of whole body carbohydrate metabolism and during exercise is an important source of energy. Glycogen synthesis and breakdown are two separate pathways regulated by contractile activity and hormones through covalent modification. Glycogen synthase is the enzyme involved in glycogen synthesis and glycogen phosphorylase is the enzyme involved in glycogen breakdown.

1.3.5.1 Glycogen Synthesis

Synthesis of glycogen from G-6-P is a three step reaction and the final step involving glycogen synthase is regulatory.

Glycogen synthase activity is controlled by multisite phosphorylation (Roach, 1991 and Cohen and Hardie, 1991) and by several allosteric effectors, of which G-6-P is the most important one. ATP, ADP and AMP result in inhibition of GS and this situation can be reversed by G-6-P.

1.3.5.2 Regulation by Covalent Modification

Phosphorylation of glycogen synthase results in a decrease in glycogen synthase activity. Nine serine residues are known to be phosphorylated *in vivo* (Cohen, 1986, Dent *et al*, 1990, Roach 1991 and Cohen & Hardie, 1991). Two of these serine residues are located in the N-terminus region of glycogen synthase (N7 + N10) and the remaining seven are present within the C-terminus region (C30, C34, C38, C42, C46, C87 and C100). Ser 10 in the N-terminus is phosphorylated by casein kinase 1, Ser 30-42 in the C-terminus is phosphorylated by glycogen synthase kinase 3 (GSK3), Ser 46 is phosphorylated by casein kinase 2 (CK2) and Ser 87 by protein kinase A (PKA). Ser 7 is phosphorylated by PKA and by other protein kinases *in vitro*, while Ser 100 is phosphorylated both by PKA and by calmodulin dependent multi protein kinase (Cohen & Hardie, 1991).

Phosphorylation of these different serine residues has distinct effects on the enzyme activity. Phosphorylation of the region C30-38 and combined phosphorylation of N7 and N10, has a considerable inactivating effect on glycogen synthase activity (Cohen & Hardie, 1991). In contrast, phosphorylation of C42, C46, C100 has no effect on glycogen synthase activity and phosphorylation of C87 has little or no effect on activity. However, phosphorylation of C46 by CK2 allows GSK3 to phosphorylate C42, which in turn acts as the recognition for the phosphorylation of C38, C34 and C30. It has been suggested that CK2 plays a role in the phosphorylation of glycogen synthase to form the recognition site for another protein kinase to phosphorylate a serine residue on glycogen synthase (Cohen & Hardie 1991).

Protein phosphatases 1 and 2A dephosphorylate and reactivate glycogen synthase. Protein phosphatase 1 dephosphorylates residues N7, N10 and the region C30-38.

G-6-P is able to reverse the effects of phosphorylation on glycogen synthase activity, and has been also suggested to be involved in the dephosphorylation of glycogen synthase by activating protein phosphatases **1** and 2A (Villar-Palasi, 1991).

1.3.5.3 Hormonal Regulation

Glycogen synthase activity is regulated by hormones such as adrenaline and insulin.

a) Adrenaline

Adrenaline increases the phosphorylation of every serine residue mentioned above except for Ser C42 and C46. However adrenaline only inhibits glycogen synthase activity by increased phosphorylation at Ser residues N7, N10 and the region C30-C38 (Cohen & Hardie, 1991).

A particular form of protein phosphatase 1, which is associated with glycogen particles dephosphorylates the sites which adrenaline phosphorylates and restores the activity. The catalytic subunit of protein phosphatase is bound to the glycogen binding (G) subunit of glycogen particles and is termed as **PP1^G**. The glycogen binding (G) subunit is phosphorylated by protein kinase A at two serine residues (sites 1 and 2) (Cohen & Hardie, 1991).

In the presence of adrenaline, cAMP levels are elevated and activate PKA, which phosphorylates site 2 on the G subunit. This results in the release of the catalytic subunit from the G subunit of protein phosphatase 1 and prevents protein phosphatase 1 from dephosphorylating glycogen synthase (Cohen & Hardie, 1991 and Wera & Hemmings, 1995). Also, PKA phosphorylates protein phosphatase inhibitor **1**, converting it to a potent inhibitor of the catalytic subunit of protein phosphatase 1 and suppressing further the ability of protein phosphatase 1 to dephosphorylate glycogen synthase (Cohen & Hardie, 1991).

In contrast insulin stimulates phosphorylation of site 1 through the MAP kinase pathway and rsk-2 resulting in activation of protein phosphatase 1 (Wera & Hemmings, 1995). Protein phosphatase 1 also catalyses the dephosphorylation and inactivation of phosphorylase kinase and glycogen phosphorylase. It has been suggested that inactivation of protein phosphatase 1 by PKA is likely to play an important part in the adrenergic stimulation of glycogenolysis.

b) Insulin

Insulin activates glycogen synthase by promoting dephosphorylation of the enzyme resulting in an increase in the -/+ G-6-P activity ratio and a decrease in the concentration of G-6-P needed to activate the enzyme (Lawrence, 1995 and Lawrence *et al*, 1997).

Initially it was thought that insulin exerts its effects by inhibiting PKA, but later it was shown that cAMP levels were unaffected by insulin (Craig & Larner, 1964 and Cohen *et al*, 1997). However, from tryptic peptide studies, it was found that insulin promoted the release of phosphates from the sites which GSK 3 phosphorylated (Ser 30-38). This led to the conclusion that insulin does not stimulate glycogen synthase activity by inhibiting PKA, but by inducing a decrease in GSK 3 activity or by activating protein phosphatase which dephosphorylates these sites. From the isolation of tryptic phosphopeptides and amino acid sequencing studies, it was established that insulin stimulated phosphorylation and inhibition of GSK 3 (Cross *et al*, 1994 & 1995 and Cohen *et al*, 1997). It was also shown in a skeletal muscle cell line (L6 myotubules) that insulin promoted phosphorylation of GSK 3 (Cross *et al*, 1995). Two insulin stimulated protein kinases were then identified which phosphorylated and inactivated both GSK3 isoforms at specific serine residues. One of them was MAP Kinase-activated protein kinase-I (MAPKAP-KI also known as p90^{rsk}) (Lawrence *et al*, 1997) and the other one was p70 S6 kinase, which phosphorylates ribosomal protein S6. However, from studies with

drugs i.e PD 98059 and rapamycin which inhibit raf activation of MAPKK and P70 S6 kinase, respectively, it was found that neither protein kinases were involved in the inhibition of GSK3 by insulin (Cohen *et al*, 1997). In contrast, wortmannin suppressed the inhibition of GSK3 by insulin, suggesting that inactivation was by a protein kinase whose activation by insulin is dependent on PI-3 Kinase activity (Cohen *et al*, 1997). This protein Kinase has been identified and has been termed as protein kinase B (PKB). The reason being it has a catalytic domain similar to PKA and PKC (Coffer & Woodgett, 1991).

In summary, PKB whose activity is increased by insulin in a wortmannin-sensitive fashion phosphorylates and inactivates GSK 3

c) Protein Kinase B (PKB)

PKB is activated by phosphorylation at Thr 308 and Ser 473 and is inactivated by protein phosphatase 2A (Cohen *et al*, 1997 and Alessi *et al*, 1996). From mutation studies, phosphorylation of both Thr 308 and Ser 473 is required for the full activation of PKB and phosphorylation of the two sites acts synergistically to produce a high level of PKB activity *in vivo* (Cohen *et al*, 1997). 3-phosphoinositide dependent protein kinase 1 (PDK1), has recently been identified and has been found to phosphorylate PKB at Thr 308 and increase its activity by 30 fold *in vitro* (Cohen *et al*, 1997 & Alessi *et al*, 1997). PDK1 is only active in the presence of PI(3,4,5)P₃ and PI(3,4,)P₂ and the concentration of PI(3,4,5)P₃ or PI(3,4,)P₂ required for half maximal activation of PDK *in vitro* is 1-2μM (Alessi *et al*, 1997). PDK 1 has a molecular mass of 67KDa and its catalytic domain is similar to PKA and PKB.

In conclusion, it is proposed that insulin activates glycogen synthase in skeletal muscle by the following mechanism. Insulin binds to its receptor leading to a cascade of events resulting in the activation of phosphatidylinositide (PtdIns) 3-kinase and formation of PI(3,4,5)P₃ and PI(3,4,)P₂ (by the conversion of PI(3,4,5)P₃ by a specific phosphatase). PI(3,4,5)P₃ and PI(3,4,)P₂ activates PDK-1 resulting in activation of PKB, which in turn inactivates GSK3 leading to increased dephosphorylation of glycogen synthase and an increase in glycogen synthesis (Cohen *et al*, 1997).

1.3.6 Glycogen Phosphorylase

Phosphorylase catalyses the rate limiting step in glycogenolysis. Phosphorylase exists in two forms, phosphorylase *b* (inactive) and phosphorylase *a* (active), the forms are interconvertible by phosphorylation and dephosphorylation.

Phosphorylase *b* is converted to the *a* form by phosphorylase kinase, which also exists in two forms. The less active dephosphorylated *b* form of phosphorylase kinase is converted to the active *a* form by protein kinase A in the presence of high levels of cAMP (Krebs, 1981 and Cohen, 1981). Calmodulin and troponin-C activate phosphorylase kinase, but the concentration of troponin-C required to activate phosphorylase kinase is greater than that required of calmodulin (Cohen, 1981). Phosphorylase kinase activity is dependent on Ca⁺. Phosphorylase kinase *a* is active at Ca⁺ concentrations found in resting muscles and phosphorylase kinase *b* is only active at Ca⁺ concentrations present in contracting muscles (Brostrom *et al*, 1971 and Cohen, 1981).

The phosphorylated form of phosphorylase is dephosphorylated to the inactive form by protein phosphatase 1 (Krebs, 1981 and Cohen, 1978 and 1981).

Finally, hypoxia in skeletal muscle does not increase the proportion of phosphorylase α , even though muscle glycogen content is decreased (Ren *et al*, 1992). It has been suggested that hypoxia causes an increase in P_i concentration and induces glycogenolysis in skeletal muscle without an increase in the proportion of phosphorylase α (Ren *et al*, 1992). In resting muscles, a significant proportion of phosphorylase is in the α active form, and one could assume that at rest phosphorylase α would be continuously breaking down glycogen. However this is not the case as it has been proposed that the limited availability of P_i prevents net glycogenolysis to occur in resting muscle despite a high proportion of phosphorylase being present in the active state (Ren *et al*, 1992)

1.3.6.1 Hormonal Regulation

a) Adrenaline

Adrenaline activates phosphorylase in muscles and stimulates glycogenolysis, whilst it has the opposite effect on glycogen synthase. This effect is explained by an increase in cAMP as a result of adrenaline. This increase in cAMP activates PKA, which phosphorylates and activates phosphorylase kinase, resulting in the phosphorylase b being converted to phosphorylase α (Stull and Mayer, 1971 and Coderre *et al*, 1991).

Adrenaline also stimulates the phosphorylation and inactivation of glycogen synthase as mentioned in section 1.3.5.3. It has been proposed that inactivation of glycogen synthase when glycogenolysis is stimulated probably limits the rate of futile cycling between glycogen and G-1-P.

Recent studies have shown that adrenaline-stimulated glycogenolysis is independent of the glycogen concentration (Jensen *et al*, 1997)

b) Insulin

Insulin has an inhibitory role on glycogen breakdown and a stimulatory effect on glycogen synthesis in fast-twitch glycolytic fibres (Challis *et al*, 1987). Insulin has been shown to promote dephosphorylation of phosphorylase in fast twitch glycolytic fibres *in vitro* (Zhang, *et al*, 1989). However, insulin had no effect on phosphorylase activity in diaphragm muscle, which is an example of an oxidative muscle. This lack of insulin effect in oxidative muscle fibres is probably due to differences of the metabolic properties in the muscles (section 1.2.2). Fast twitch glycolytic muscle fibres rely on anaerobic metabolism and the glycogen concentration is higher than that found in slow twitch oxidative fibres (Peter *et al*, 1972). Also fast twitch glycolytic fibres have several times more phosphorylase kinase and phosphorylase than slow twitch oxidative fibres, so that they are able to mobilize glycogen efficiently (Ritcher *et al*, 1982).

It has been suggested that insulin has a greater effect on muscle glycogenolysis than glycogen synthesis in fast glycolytic muscle fibres (Zhang *et al*, 1987), as insulin decreases the rate of glycogenolysis in fast glycolytic muscle fibres by 7 fold and increases glycogen synthesis by 3 fold (Challis *et al*, 1987).

1.3.6.2 Glycogenolysis during muscle contractions

In resting muscles, phosphorylase is completely in the inactive *b*-form. When muscles contract, Ca^{2+} released from the sarcoplasmic reticulum results in a rapid conversion of phosphorylase *b* to its active *a* form, catalysed by phosphorylase kinase, also there is allosteric activation of phosphorylase *b* by AMP and IMP (Ritcher *et al*, 1982 and

Aragon *et al*, 1980). It is generally believed that Ca^{2+} released from the sarcoplasmic reticulum during contraction accounts for exercise induced glycogenolysis in muscle by activating phosphorylase (Meinke & Edstrom, 1991). During intense exercise in fast glycolytic muscle fibres, adrenaline secretion is increased causing cAMP levels to rise, leading to the phosphorylation and activation of phosphorylase by PKA and stimulation of glycogenolysis. It has been suggested that adrenaline and muscle contraction jointly regulate muscle glycogenolysis during exercise depending on a number of factors such as the duration and intensity of exercise and muscle fibre type (Ritcher *et al*, 1982).

It has been shown that the concentration of glycogen is a major determinant of phosphorylase activity in contracting skeletal muscle (gastrocnemius/plantaris) (Hepel & Ritcher, 1992). The percentage of phosphorylase existing in the α form in the contracting skeletal muscle depends on the initial glycogen concentration. It has been observed that a high glycogen concentration is associated with a high phosphorylase α activity, while a low glycogen concentration is associated with a low phosphorylase α activity (Hespeel & Ritcher, 1992). In contrast, glycogenolysis has been shown to be independent of the glycogen concentration during exercise in fast twitch glycolytic muscle fibres (Coderre *et al*, 1991). Also from these studies it was concluded that muscle glycogenolysis was not related to the phosphorylation of glycogen phosphorylase during exercise. It has been suggested that glycogen phosphorylase activity during exercise is regulated by allosteric effectors i.e. AMP and IMP (Coderre *et al*, 1991). Evidence to support this has come from studies whereby elevated levels of muscle IMP and Pi during exercise activate phosphorylase b independently of its phosphorylation (Codrre *et al*, 1991). Recently, studies with the soleus muscle have suggested a mechanism to activate glycogenolysis and stimulate glycogen phosphorylase via activation of AMPK, as a result of an increase in AMP levels during muscle contractions (Young *et al*, 1996).

It should be taken into consideration that majority of these studies were carried out on fast twitch glycolytic muscle fibres and not on slow twitch oxidative fibres. Due to differences in the metabolic properties of the two muscle fibre types, different effects might be observed in slow twitch oxidative muscle in regards to glycogenolysis during muscle contractions.

During high intensity exercise, the energetic requirements of muscles are met mainly by the breakdown of glycogen. These glycogen stores are substantially repleted during the recovery period involving integrated regulation of the phosphorylation state of both glycogen phosphorylase and synthase in the muscle (Brau *et al*, 1997). At the onset of recovery, the glycogen synthase phosphorylation state is lower than at rest in red, white and mixed gastrocnemius but not in the soleus muscle where there is no glycogen mobilization, suggesting that a larger proportion of the enzyme is in the active dephosphorylated form. During the recovery period glycogen synthase phosphorylation increases progressively until it reaches the point where no further glycogen is being deposited (Brau *et al*, 1997). However, for optimal glycogen deposition to occur after exercise, it is not only necessary to activate glycogen synthase but the proportion of the active form of glycogen phosphorylase should decrease. It was observed that at the onset of recovery phosphorylase was in the dephosphorylated inactive form. Brau *et al*, (1997) suggested that this decrease in the phosphorylation of glycogen synthase and phosphorylase at the onset of recovery after a period of intense exercise is due to changes in the levels of glucose-6-phosphate, glycogen and H^+ levels and leads to repletion of glycogen content in muscle.

1.4 LIPID FUELS

Skeletal muscles are well capable of oxidizing fatty acids especially during periods of intense exercise and starvation. Fatty acids are obtained from both exogenous and endogenous sources. Exogenous fatty acids are provided in ^{the} form of circulating non-esterified fatty acids (NEFA) or in the form of triglyceride, and endogenous fatty acids are derived from lipolysis of triacylglycerol stores.

1.4.1 Plasma Non-Esterified Fatty Acids

In white adipose tissue, hormone sensitive lipase (HSL) is the main factor influencing the amount of plasma NEFA present. HSL catalyses the rate limiting step in adipose-tissue lipolysis and is under acute hormonal control (Yeaman *et al*, 1994). In the presence of lipolytic agents such as adrenaline, noradrenaline and glucagon it leads to an increase in the intracellular concentration of cAMP resulting in phosphorylation and activation of HSL by cAMP dependent protein kinase (Nilsson *et al*, 1980). Conversely, insulin the major antilipolytic agent, lowers the intracellular concentration of cAMP (Londos *et al*, 1988), and promotes dephosphorylation of HSL by increasing protein phosphatase activity (Stralfors and Honnor 1989).

AMP-activated protein kinase (AMPK) phosphorylates HSL at a site distinct from the site phosphorylated by cAMP dependent protein kinase. The basal site on HSL is phosphorylated by AMPK, preventing phosphorylation by cAMP dependent protein kinase at the regulatory site. By itself, phosphorylation of the basal site has no effect on the activity of the enzyme (Garton *et al*, 1989 and Garton and Yeaman, 1990).

AMPK is phosphorylated and activated by AMPK kinase, which itself also is activated by AMP (Garton *et al*, 1989 and Corton *et al*, 1995). The effect of AMPK on HSL is

considered to be antilipolytic as it inhibits isoprenaline-stimulated lipolysis in adipocytes (Sullivan *et al*, 1994). This effect was shown using AICAR, the cell permeable precursor of ZMP (see section 3.3.6). AICAR activates AMPK via ZMP, mimicking the activating effects of AMP on AMPK and AMPK Kinase. Adipocytes preincubated with AICAR showed a reduced response to the lipolytic agent isoprenaline, providing evidence that AMPK has an antilipolytic effect on HSL (Sullivan *et al*, 1994).

Low concentrations of long chain acyl-CoA esters stimulate AMPK kinase implying a possible feed back mechanism (Garton *et al*, 1989 and Carling *et al*, 1987). This suggests that fatty acids generated by lipolysis, which are converted to fatty acyl-CoA esters could activate AMPK, leading to phosphorylation of HSL at the basal site and antagonising HSL activation by cAMP dependent protein kinase (Garton *et al*, 1989). However, this effect has not been reproducible and it has been suggested that this effect was due to an artefact in the experimental procedure used (Hardie & Carling, 1997 quote unpublished results).

From immunological studies, evidence has been presented for the existence of HSL in heart and skeletal muscle (Holm *et al*, 1987).

Fatty acids liberated by the action of HSL bind to BSA and are transported to skeletal muscle via the blood stream. The fatty acids of most physiological importance are those containing 14 or more carbon atoms which are the preferred substrates for β -oxidation. In heart, it is thought that fatty acids are taken up by myocytes by a passive diffusion process or by a carrier mediated process (Van der Vusse, 1992). However, transport of fatty acids within the cytoplasm is thought to occur primarily by a tissue-specific fatty acid binding protein (FABP) directing the fatty acids to various metabolic pathways (Lopaschuk *et al*, 1994 and Faergeman & Knudsen, 1997).

1.4.2 Plasma Lipoprotein (Triacylglycerol)

Skeletal muscles are able to utilize circulating triglycerides. Chylomicrons and very low density lipoproteins (VLDL) particles (often known as triacylglycerol-rich lipoproteins) are involved in the delivery of exogenous triacylglycerol. They are composed of a hydrophobic lipid core (TAG and cholesterol esters) and a relatively hydrophilic outer surface consisting of phospholipids and unesterified cholesterol and various apoproteins.

Uptake of fatty acids from circulating chylomicrons and VLDL is dependent on lipoprotein lipase (LPL). LPL is synthesised in the parenchymal cells of extrahepatic tissues and anchored to the capillary wall by proteoglycan chains of heparan sulphate. LPL hydrolyses plasma triglycerides at the surface of the capillary and the fatty acids released are rapidly taken up by the tissue to be stored or oxidised. Muscle tissue stores relatively little lipid, yet uses NEFA for metabolic energy, thus LPL is important for supplying NEFA as a energy substrate for muscle.

LPL is present in all three skeletal muscle types with differing activities. Highest levels are found in slow twitch red fibres, with intermediate levels in the fast twitch red fibres and very low levels in fast twitch white fibres (Tan *et al*, 1977). This distribution is in keeping with the notion that red muscle fibres readily utilise fat as fuel, whereas white fibres mainly utilise carbohydrates. These differences in LPL activity between different types of skeletal muscle fibres are likely to result from differences in LPL gene transcription (Ong *et al*, 1994).

The expression of LPL in various tissues under different metabolic and hormonal influences is due to changes in LPL mRNA levels, translation and posttranslational processing. Regulation of LPL is more often than not inversely regulated and tightly co-ordinated between adipose tissue and muscle. Starvation results in a decrease in adipose

LPL activity which then increases in response to re-feeding. In contrast, during prolonged starvation LPL activity increases in skeletal muscle that have more red fibres (soleus) than in white fibres (gastrocnemius) (Ladu *et al*, 1991 and Ong *et al*, 1994). Recent studies have shown that a decrease in adipose LPL activity is associated with a decrease in plasma insulin concentration during prolonged starvation. However, upon re-feeding an increase in adipose LPL activity is accompanied by an increase in insulin concentration. This suggests that a striking correlation exists between the changes in adipose LPL activity and plasma insulin concentration during prolonged starvation and re-feeding (Sugden *et al*, 1993b). In contrast, skeletal muscle LPL activity increases in response to re-feeding, but no correlation is observed between skeletal muscle LPL activity and insulin concentration during starvation and re-feeding (Sugden *et al*, 1993b). In response to prolonged exercise LPL activity increases in slow-twitch red fibres and fast-twitch red fibres as fatty acids are a prime substrate for oxidation during exercise (Borensztajn 1975). This suggests that exercise results in an increased capacity by skeletal muscle to oxidise fatty acids. Also, uptake of plasma fatty acids by skeletal muscle is increased during exercise (Borensztajn 1975), thus the total oxidisable fatty acid pool is increased. Conversely, LPL activity in adipose decreases and in heart it does not seem to change in response to exercise (Borensztajn 1975, Ladu *et al*, 1991 and Ong *et al*, 1995). Changes in LPL activity during exercise are unaccompanied by changes in LPL mRNA, suggesting that these changes in LPL activity in adipose tissue, heart and skeletal muscle are post-translational (Ong *et al*, 1995).

1.4.3 Endogenous Triacylglycerols

Endogenous triacylglycerols located within the muscle fibres are also used for oxidative metabolism. Exercise has been shown to decrease the concentration of endogenous triacylglycerols (TAG) with a concomitant increase in plasma fatty acids. This suggests that endogenous TAG provides fuel for muscle contraction during exercise (Oscai *et al*, 1986, 1990 and Hurley *et al*, 1986).

It was originally proposed that LPL is involved in mobilizing endogenous TAG during exercise (Oscai *et al*, 1986). However Oscai *et al*, (1990) found that it was HSL that mobilizes intracellular TAG and not LPL.

Endogenous TAG is used as a substrate for energy production during electrical stimulation in isolated skeletal muscle preparations (Hopp & Palmer 1990a). The amount of endogenous TAG hydrolyzed is dependent on the frequency of the stimulation and the type of frequency (continuous or intermittent) (Hopp & Palmer 1990b). In the flexor digitorum brevis muscle it has been shown that when the frequency and the amount of muscle work are similar during continuous and intermittent stimulation. The extent of hydrolysis of triglycerides and free fatty acids are greater during continuous stimulation than during intermittent stimulation. However, as the frequency of the intermittent stimulation is increased, both triglycerides and exogenous fatty acids hydrolysis ~~are~~ increased and the amount of exogenous fatty acids that are esterified is also decreased (Hopp & Palmer 1990b). The reason being when the frequency of the intermittent stimulation is low, substrates other than lipids are providing a greater amount of energy for muscle contraction during the intermittent contraction than at continuous muscle contraction and hence a lower triglyceride hydrolysis during intermittent stimulation. However, when the frequency of the intermittent stimulation is

increased endogenous triglyceride content is reduced, as both triglycerides and fatty acids are providing energy to the contracting muscle (Hopp & Palmer, 1990b).

In conclusion the frequency of the stimulation and the type of electrical stimulation (continuous or intermittent) determines the amount of endogenous TAG that will be hydrolysed (Hopp & Palmer 1990b).

1.4.4 Ketone Body Metabolism

In liver, if fat oxidation predominates (e.g. during starvation, diabetes and fat feeding) the increased acetyl-CoA is used to synthesise ketone bodies (acetoacetate and β -hydroxybutyrate). Skeletal muscle, like heart, utilises ketone bodies. β -hydroxybutyrate is oxidised to acetoacetate by β -hydroxybutyrate dehydrogenase which is converted to acetoacetyl-CoA via a reaction with succinyl-CoA before being further metabolised. Ketone bodies are a major oxidative fuel for muscle during starvation and diabetes in resting muscle (Ruderman & Goodman, 1973). The rate at which ketone bodies are used in skeletal muscle is dependent on their concentration (Bates *et al*, 1968 and Ruderman & Goodman 1973). The activity of the enzymes involved in ketone utilisation have been compared in all three muscle types and slow twitch red muscle has the highest activity for all the enzymes involved in ketone utilisation (Maizels *et al* 1977 and Winder *et al*, 1974).

In the presence of acetoacetate, glucose uptake and glycolysis are decreased and glycogen synthesis is enhanced in slow red muscle (soleus) but no such effects are seen in fast red muscle (extensor digitorum longus) (Maziel *et al*, 1977). A possible explanation for this could be that fast red muscle comprises approximately equal amounts of red and

white fibres and so any changes in glucose metabolism could have been missed due to the presence of white fibres. Whether this is the sole reason has yet to be determined.

There is considerable difference in ketone body metabolism between physically trained and untrained individuals during exercise (Winder *et al*, 1973, 1974 and 1975). Physically trained individuals in contrast to untrained individuals have low blood ketone levels during and after exercise. Tolerance to an administered dose of ketones is greater in physically trained than in untrained individuals during and after exercise. This suggests that the levels of activity of the enzymes involved in ketone oxidation undergo an adaptive increase in muscle in response to exercise training and explains why physically trained individuals do not develop post-exercise ketosis (Winder *et al*, 1973, 1974 and 1975).

1.5 FATTY ACID OXIDATION

Long chain fatty acids can only be metabolised after they have been converted to their CoA thioesters by fatty acyl-CoA synthetase (FAS). Once activated, fatty acyl-CoAs can undergo β -oxidation in either the mitochondria or peroxisomes. Long chain acyl-CoAs are transported across the mitochondrial inner membrane to the matrix where β -oxidation occurs. Short chain fatty acids (C_{10}) directly enter the mitochondrial matrix whilst long chain acyl units are transported across by a system involving three carnitine dependent proteins. The first of these, CPT 1, is located on the outer mitochondrial membrane (Murthy and Pande, 1987a), and catalyses the formation of long chain acylcarnitine. Carnitine:acylcarnitine translocase transports acylcarnitine across the inner mitochondrial membrane in exchange for free carnitine. Finally the third enzyme CPT 2,

which is associated with the inner mitochondrial membrane (Murthy and Pande, 1987a & 1990) reforms acyl-CoA from acylcarnitine and mitochondrial CoASH (fig 1.5).

1.5.1 CPT 1 and CPT 2

It was initially thought that CPT 1 and CPT 2 were the same protein but from immunologically studies, cloning and expression of rat liver CPT 1 and CPT 2 cDNAs it was found that CPT 1 and CPT 2 are two distinct proteins (Kolodziej *et al*, 1992, Woeltje *et al*, 1987, 1990 and Esser *et al*, 1993).

a) CPT 2

CPT 2, which is malonyl-CoA insensitive and is easily solubilized by detergent in an active form, has been purified and characterised from rat liver mitochondria (Woeltje *et al*, 1987 and 1990). The full length cDNA clone predicts a protein consisting of 658 amino acids containing a 25 amino acid N-terminal leader sequence which is cleaved upon mitochondria import, yielding a protein of ~71kDa (Woeltje *et al*, 1990, Brown *et al*, 1991 and McGarry & Brown, 1997).

CPT 2 is expressed as the same protein throughout different rat tissues (Woeltje *et al*, 1990). CPT 2 has also been isolated from human liver and consists of 658 amino acids, but the human CPT 2 mRNA is larger than that of the rat CPT 2 (3Kb & 2.5Kb respectively) (McGarry & Brown, 1997).

b) CPT 1

Unlike CPT2, CPT1 is sensitive to inhibition by malonyl-CoA and is expressed as two isoforms, liver CPT (L-CPT 1) and muscle (M-CPT 1) (Kolodziej *et al*, 1992 McGarry 1995a and McGarry & Brown, 1997). CPT 1 retains the N-terminal peptide of the

Fig 1.5

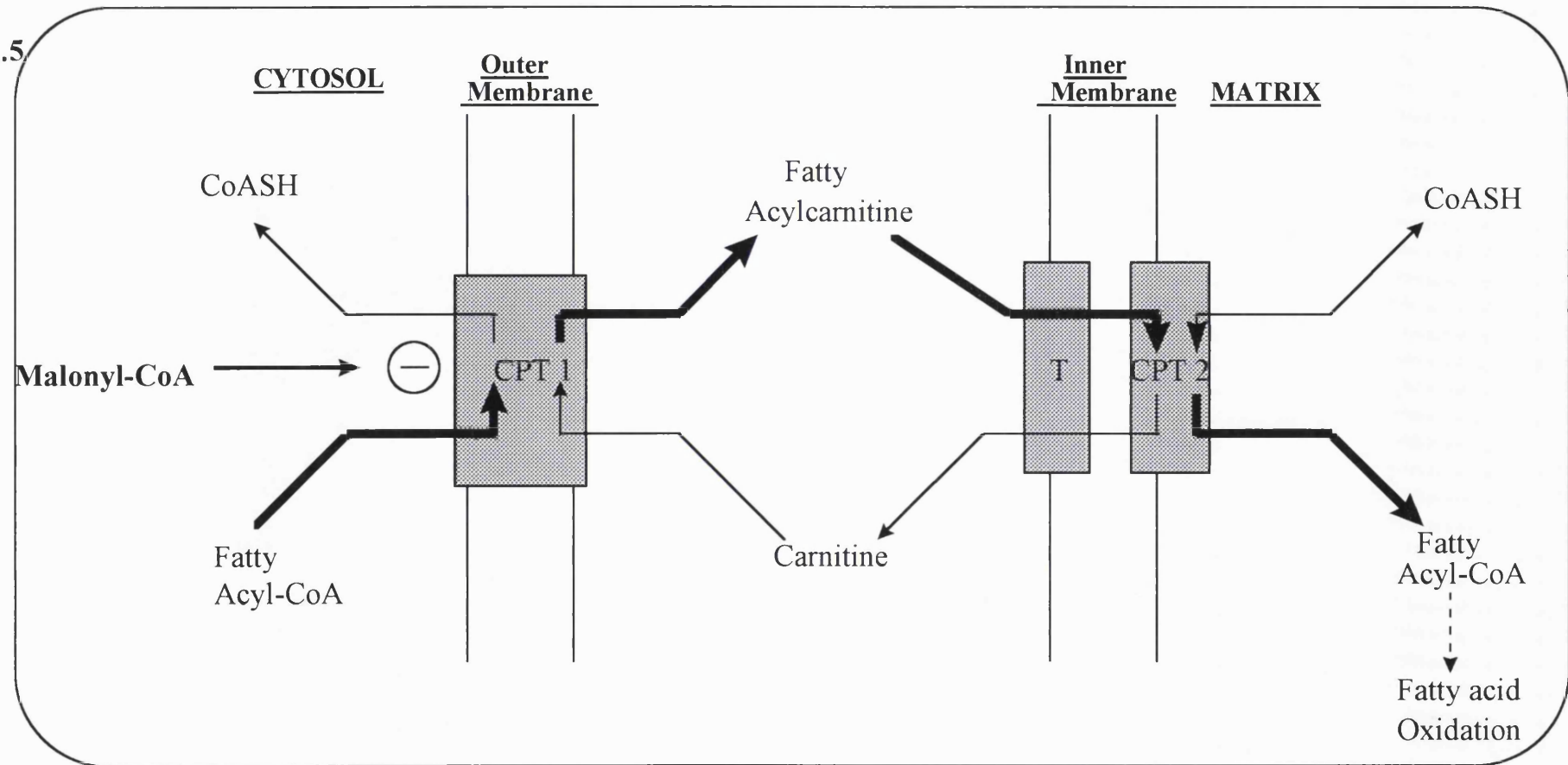


Fig 1.5 Features of the CPT enzyme in liver mitochondria. The malonyl-CoA-inhibitable CPT 1 that is associated with the outer membrane and a malonyl-CoA-insensitive CPT 2 associated with the inner membrane. Incoming fatty acyl-CoA thioesters are converted to acylcarnitine esters by CPT 1 followed by transport through the inner membrane by the acylcarnitine:carnitine translocase (T). Acyl-CoA thioesters are reformed by CPT 2 and are then oxidised (as described in section 1.5.1).

nascent protein during mitochondrial import (Kolodzetj *et al*, 1993). This supports the findings from Esser *et al*, (1993) that the two proteins are distinct.

L-CPT 1 has been characterised and isolated with great difficulty, as it is tightly associated to the outer membrane and when solubilized it loses its catalytic activity (McGarry *et al*, 1989, Murthy & Pande, 1987, 1990 and Woeltje *et al*, 1987). Expression of a full length cDNA clone encoding liver CPT 1 in COS cells increases CPT 1 activity by 10-20 fold, and this is inhibited by both malonyl-CoA and etomoxiryl-CoA (Esser *et al*, 1993 and McGarry 1995 a). This provided evidence that CPT 1 consists of a single polypeptide containing both the malonyl-CoA binding site and the catalytic site (Esser *et al*, 1993 and Brown *et al*, 1994).

L-CPT 1 is larger than CPT 2 as it has a mol wt of ~ 88 kDa and consists of 773 amino acids (Esser *et al*, 1993).

M-CPT 1 and L-CPT 1 are different proteins and evidence for this came from the following observations. Firstly, L-CPT 1 and M-CPT 1 display different kinetic properties and sensitivity to malonyl-CoA. The K_m for carnitine of the L-CPT 1 is lower than that of M-CPT 1 (~30 μ M and ~500 μ M respectively) and the IC_{50} values (IC_{50} value is the concentration required to inhibit 50% activity) for malonyl-CoA for the L-CPT 1 is higher than M-CPT 1 (~ 2.7 μ M and ~ 0.03 μ M respectively) (Saggerson & Carpenter, 1981 and McGarry *et al*, 1983). Secondly, L and M-CPT 1 have been predicted to contain 773 and 772 amino acids with molecular masses of 88kDa and 82kDa respectively on SDS PAGE (McGarry & Brown, 1997). The reason why they migrate so differently on the SDS PAGE is thought to be due to differences in their primary structure (McGarry & Brown, 1997).

The heart expresses both forms of CPT 1 (Weis *et al*, 1994 a & b, McGarry 1995a and 1995b) and it is interesting to note that the level of L-CPT 1 is relatively high in neonatal rat heart, contributing up to 25 % of total neonatal CPT 1 activity which then declines during development with a concomitant accumulation in tissue carnitine and increase in M-CPT 1 (Weis *et al*, 1994a & b and Brown *et al*, 1995). This implies that L-CPT 1 in neonatal rat hearts contributes significantly to the synthesis of fatty acylcarnitine despite the low levels of tissue carnitine (McGarry & Brown, 1997)

1.5.2 Outline Of The β -Oxidation Pathway

Carnitine : acylcarnitine translocase transports long chain acylcarnitine into the mitochondrial matrix in preference to free carnitine and short chain acylcarnitine (Murthy & Pande, 1987b). Once inside the mitochondrial matrix, long chain acylcarnitine is converted back to long chain acyl-CoA by CPT 2 and enters the β -oxidation pathway. Long chain acyl-CoAs are oxidised to acetyl-CoA via a series of four separate reactions.

- 1) Acyl-CoA is converted to trans- Δ^2 -enoyl-CoA by acyl-CoA dehydrogenase. Electrons from the dehydrogenase are transferred to the respiratory chain via electron transferring flavoprotein (ETF).
- 2) Trans- Δ^2 -enoyl-CoA is hydrated to L-3 hydroxy acyl-CoA by enoyl-CoA hydratase.
- 3) L-3-hydroxyacyl-CoA is converted to L-3-keto acyl-CoA by L-3 hydroxy-acyl-CoA dehydrogenase using NAD^+ as its coenzyme.
- 4) L-3-ketoacyl-CoA is cleaved by a thiolase, requiring a second CoASH yielding acetyl-CoA and a shorter acyl-CoA (2 carbons less than before) which undergoes another cycle of β -oxidation. The acetyl-CoA formed enters the citric acid cycle and is oxidised.

1.5.3 Regulation of Fatty acid oxidation

CPT 1 represents the main key regulatory point in the oxidation of fatty acids as it is uniquely inhibited by malonyl-CoA at low concentrations.

Initially shown by McGarry and Foster, malonyl-CoA inhibits CPT 1 in rat liver (McGarry and Foster, 1978 a). This lead to the conclusion that malonyl-CoA plays a key role in fatty acid oxidation and ketogenesis in lipogenic tissues (McGarry and Foster, 1980). Inhibition of CPT 1 by malonyl-CoA is not exclusive to lipogenic tissues but also occurs in non-lipogenic tissues (e.g. Heart and Skeletal muscle) (Saggerson and Carpenter, 1981). The malonyl-CoA contents in heart (4-5 nmol/g wet wt.) and skeletal muscle (1-2 nmol/g wet wt.) are much lower than in liver (7-8 nmol/g wet wt. of tissue) (McGarry *et al*, 1983). As mentioned in section 1.5.1, CPT 1 in these tissues is 50 times more sensitive to inhibition by malonyl-CoA than in the liver (Saggerson & Carpenter, 1981, McGarry *et al*, 1983 and section 1.5.1). This would suggest, that *in vivo* fatty acid oxidation should be completely inhibited in both heart and skeletal muscles. This clearly is not the case, suggesting that the majority of the tissue content of malonyl-CoA is not free or is inaccessible to CPT 1.

In heart, it has been proposed that malonyl-CoA is an important effector of CPT 1 and studies have shown an inverse correlation between the rates of fatty acid oxidation and malonyl-CoA levels, strongly supporting a regulatory role for malonyl-CoA. Evidence for this came from studies with perfused rat heart (Saddik *et al*, 1993) and cardiac myocytes (Awan & Saggerson, 1993). Substrates such as glucose and lactate in the presence of insulin increased malonyl-CoA levels with a concomitant decrease in palmitate oxidation and this effect was reversed in the presence of adrenaline (Awan & Saggerson, 1993). Saddik *et al* (1993) observed in the presence of dichloroacetate (a stimulator of

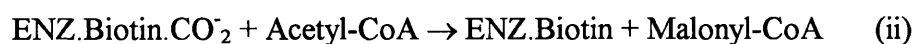
pyruvate dehydrogenase) that the tissue levels of acetyl-CoA and malonyl-CoA were raised with a simultaneous suppression of fatty acid oxidation. Saddik *et al* (1993) provided evidence that acetyl-CoA carboxylase, which synthesizes malonyl-CoA is an important regulator of fatty acid oxidation in heart and the availability of cytosolic acetyl-CoA is the main determinant of heart acetyl-CoA carboxylase activity. Further evidence to support this came from Kudo *et al* (1995), who demonstrated that high rates of fatty acid oxidation during reperfusion of ischemic hearts are associated with a decrease in malonyl-CoA levels resulting from a decrease in activity of acetyl-CoA carboxylase.

Malonyl-CoA in skeletal muscle probably exerts functional control over fatty acid oxidation. Malonyl-CoA levels fall in response to exercise and electrical stimulation accompanied by an increase in fatty acid oxidation (Winder *et al*, 1989 and Winder *et al*, 1996). However, studies on human skeletal muscle have shown no change in malonyl-CoA levels during exercise (Odland *et al*, 1996). Whether this represents a difference in species or muscle fibre type or a difference in experimental design is not known.

In conclusion, malonyl-CoA plays an important role in the regulation of fatty acid oxidation in heart and most probably in skeletal muscle.

1.6 ACETYL-CoA CARBOXYLASE

Acetyl-CoA carboxylase (ACC) (EC 6.4.1.2) catalyses the carboxylation of acetyl-CoA to malonyl-CoA and is the committed step in fatty acid synthesis. ACC is a biotin containing enzyme, catalysing two partial reactions :



In the first reaction a carboxybiotin intermediate is formed at the expense of ATP. The activated CO₂ is transferred to acetyl-CoA to form malonyl-CoA (reaction ii).

It consists of three essential catalytic components, biotin carboxylase (catalysing reaction (i)), a carboxyl transferase (catalysing reaction (ii)) and a carboxyl-carrier protein which covalently binds to biotin. In *Escherichia Coli*, the three components are encoded by separate polypeptides, whilst in eukarocytes they are encoded within a single multifunctional polypeptide (Lane *et al*, 1974 and Hardie, 1989).

1.6.1 ACC 265 and ACC 280 Isoforms

ACC has been purified and characterised from a variety of mammalian tissues (Nakanishi & Numa 1970, Inoue & Lowenstein, 1972, Hardie & Cohen, 1978 Bianchi *et al*, 1990 and Trumble *et al*, 1995) and two immunologically distinct isozymes of ACC have been identified (Thampy, 1989 and Bianchi *et al*, 1990).

The 265kDa form (ACC 265) is predominantly expressed in lipogenic tissues and the 280kDa form (ACC 280) is present in non-lipogenic tissues (heart and skeletal muscle) which exhibit high rates of fatty acid oxidation under certain circumstances (Bianchi *et al*, 1990). The 280kDa isoform is predominantly expressed in heart and skeletal muscle with small amounts of 265kDa isoform (Thampy 1989, and Bianchi *et al*, 1990). Recently Trumble *et al*, (1995) purified and characterised an ACC from skeletal muscle which was found to have a Mr of 272kDa. This isoform of ACC from skeletal muscle was found to contain minute amounts of the 265kDa form which was suggested to originate from adipocytes present in between the muscle fibres and not from the muscle itself (Trumble *et al*, 1995).

1.6.2 Regulation Of ACC 265

ACC 265 is subjected to several regulatory mechanisms in the short term (seconds - minutes) and long term (hours - days).

Long term control of ACC 265 in liver involves changes in protein synthesis and degradation (Nakanishi & Numa, 1970). Short term regulation includes allosteric regulation (by citrate and long chain fatty acyl-CoA) and covalent modification (phosphorylation/dephosphorylation) (Allred & Riley, 1997)

1.6.2.1 Short Term Regulation

a) Allosteric regulation

In the presence of citrate, liver ACC is polymerised into the active form (Thampy & Wakil, 1988). It is considered that citrate is a feed forward activator as it is a precursor for cytosolic acetyl-CoA (Lane *et al*, 1974). Palmitoyl-CoA inhibits ACC activity and appears to oppose the activation of ACC by citrate (Hardie, 1989). This inhibition is associated with the enzyme being converted to its depolymerised form. In addition CoASH at physiological concentrations has been shown to activate liver ACC (Yeh & Kim, 1980 and Yeh *et al*, 1981). By contrast, CoASH has been shown to be a potent inhibitor of ACC in rat epididymal tissue (Moule *et al*, 1992). Discrepancies between these two studies could be due to differences in the preparation of carboxylase from the two tissues.

b) Regulation by Phosphorylation/Dephosphorylation

Initial evidence that ACC could be regulated by phosphorylation came from observations that the purified enzyme from rat liver contained covalently bound phosphate (Inoue &

Lowenstein 1972). About the same time Carlson and Kim (1973) reported that crude preparations of liver ACC could be inactivated when incubated with MgATP in a time-dependent manner and this could be reversed in the presence of a protein phosphatase. It was eventually established that rat liver ACC is phosphorylated at multiple sites by a variety of protein kinases (Hardie, 1989). The sites of phosphorylation on ACC by various protein kinases have been extensively studied (Davies *et al*, 1989 and Hardie, 1989). Fig 1.6 shows the positions of all eight phosphorylation sites on ACC, six of these sites are located in the N-terminal region and two are located in the central region. Amongst the identified protein kinases, protein kinase A (PKA) and AMP-activated protein kinase (AMPK) can inactivate ACC *in vitro* (Hardie & Cohen 1978, Hardie & Guy 1980 and Munday *et al*, 1989). PKA causes a modest decrease in the V_{\max} and an increase in $A_{0.5}$ for citrate. By contrast AMPK results in a greater decrease in the V_{\max} and also an increased $A_{0.5}$ for citrate (Munday *et al*, 1988b). This suggests that the ACC sites that PKA and AMPK phosphorylate are distinct. The sites have been identified by amino acid sequencing. PKA phosphorylates Ser 77 and Ser1200 in rat liver ACC and phosphorylation of Ser 77 is responsible for the modest decline in V_{\max} . However, AMPK phosphorylates Ser 79, 1200, 1215 and phosphorylation at Ser 79 results in the dramatic decrease in V_{\max} (Munday *et al*, 1988a, 1988b and Davies *et al*, 1989). This has lead to the conclusion that AMPK is the principle kinase involved in the phosphorylation and inactivation of ACC *in vivo* and ACC is not a physiological substrate for protein kinase A (Davies *et al*, 1990). Further evidence for AMPK being the physiological inhibitor of ACC came from sequencing of phosphopeptides derived from ACC phosphorylation in isolated hepatocytes/adipocytes in the presence of glucagon or adrenaline (Sim & Hardie 1988 and section 1.6.2.2).

Fig 1.6

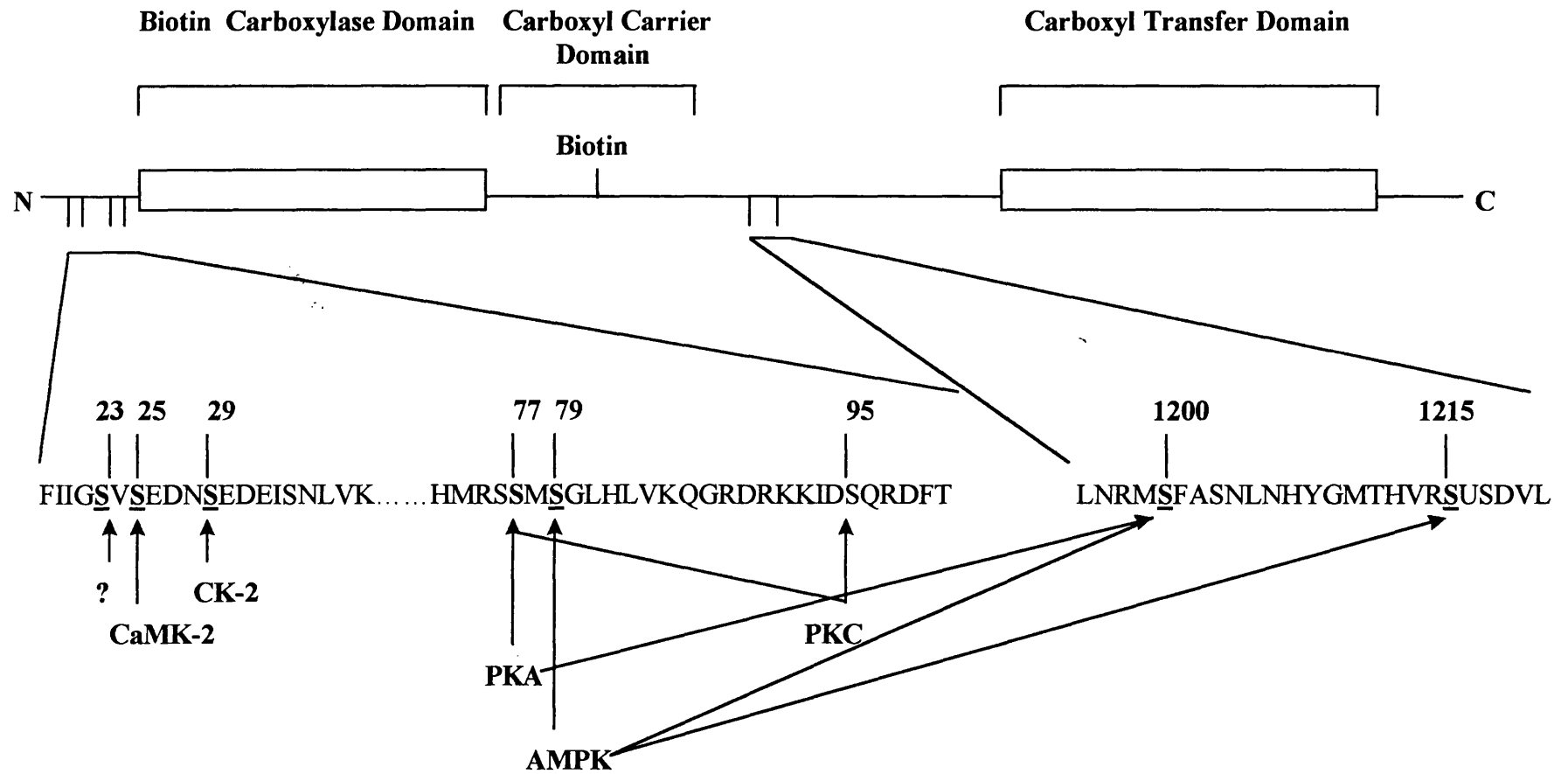


Fig 1.6 Shows the sites on liver ACC which are phosphorylated in response to different protein kinases. Of the eight phosphorylation sites only Ser 23, 25, 29, 79, 1200 & 1215 appear to be phosphorylated in intact cells. As of yet no kinases have been identified to phosphorylate Ser 23 *in vitro*. Phosphorylation of Ser 79 by AMPK appears to effect ACC activity *in vivo*, resulting inactivation of the enzyme *in vivo*.

In addition to PKA and AMPK, acetyl-CoA carboxylase kinase 2 (ACCK-2) a cAMP independent protein acetyl-CoA carboxylase kinase, has been purified from lactating mammary gland (Munday *et al*, 1988a). The effects of ACCK 2 on ACC are similar to the effects of protein kinase A on ACC, as it increases the $A_{0.5}$ for citrate and causes a modest decline in the V_{max} . It has been suggested that ACCK 2 and PKA probably phosphorylate the same site on ACC (Munday *et al*, 1988a). From amino acid sequencing, ACCK2 phosphorylates Ser 1200, which is also phosphorylated by PKA. (Munday *et al*, 1988a and Davies *et al*, 1989).

Recent evidence from mutagenesis studies have shown that phosphorylation by AMPK at Ser 79 and phosphorylation at Ser 1200 by protein kinase A are the critical phosphorylation sites for inactivation of liver ACC *in vitro* (Ha *et al*, 1994). This is contradictory to the previous findings of Davies *et al* (1990), that the effects of protein kinase A on ACC are mediated by phosphorylation of Ser 77 and not by Ser 1200.

In addition to AMPK, protein kinase A and ACCK2 which all phosphorylate and inactivate ACC, protein kinase C, casein I & II and calmodulin multi-protein kinase phosphorylate ACC. However, the physiological relevance of phosphorylation of ACC by these other protein kinases is not clear (Hardie, 1989).

1.6.2.2 Hormonal Regulation Of ACC 265 Activity

Hormones influence liver and adipose tissue ACC activity.

a) Glucagon and Adrenaline

Glucagon and adrenaline decrease enzyme activity with a concomitant increase in phosphorylation (Brownsey *et al*, 1979 and Mabrouk *et al*, 1990). Sim and Hardie (1988) showed that incubation of isolated adipocytes/hepatocytes in the presence of

glucagon or adrenaline resulted in phosphorylation of ACC at Ser 79 and Ser 1200 and no phosphorylation occurred at Ser 77 under these conditions. (Sim & Hardie 1988 and Haystead *et al*, 1990). This suggests that phosphorylation of ACC in response to hormones (glucagon & adrenaline) is due to AMPK and not due to PKA.

Adrenaline inactivates ACC by phosphorylating Ser 79 (inactivation by AMPK), but in the presence of an antagonist of binding of cAMP to the regulatory subunit of protein kinase A, the action of adrenaline is inhibited. This suggests that PKA does not directly phosphorylate ACC but is an essential component of the signal transduction pathway involving the AMPK (Haystead *et al*, 1990).

b) Insulin

Controversy surrounds the mechanism by which insulin stimulates liver or adipose ACC activity. It was originally proposed that insulin-mediated activation of ACC in adipocytes was by increased phosphorylation at a specific site, which was a substrate for casein kinase II (Brownsey *et al*, 1981, Brownsey & Denton 1982, Witters *et al*, 1983 and Haystead *et al*, 1988). Haystead & Hardie (1986) showed that insulin-induced phosphorylation of ACC is not related to enzyme activity, but the effects of insulin are mediated by an unidentified activator. Mabrouk's group proposed that insulin activates ACC by dephosphorylation via a phosphatase (Mabrouk *et al*, 1990). However, no further evidence has been presented to support Mabrouk's hypothesis. An alternative mechanism for the insulin induced activation of ACC has been suggested, that insulin acts via a protein phosphatase inhibiting AMPK (Witters & Kemp, 1992). If this is the case then both insulin and glucagon can respectively activate and inhibit ACC activity via AMP-activated protein kinase.

1.6.2.3 Regulation Of ACC 265 In Response To Fasting/Refeeding

Starvation or diabetes suppress enzyme activity and ACC gene expression. Conversely upon refeeding the enzyme activity is restored and synthesis of ACC is induced. Thampy & Wakil (1988 a & b) proposed that these changes in enzyme activity during feeding and fasting are due to phosphorylation and dephosphorylation with a concomitant depolymerisation and polymerisation of the protein. This suggests that the enzyme in the fed state exists in the active polymerised form and in the less active depolymerised form in the fasted state. The inactive form can be activated by citrate, overriding the effects of phosphorylation. This suggests that citrate plays a regulatory role under such conditions. The loss of activity of liver ACC during starvation is mediated by AMP-activated protein kinase (AMPK), phosphorylating Ser 79. Reactivation of ACC in response to refeeding results in a fall in AMPK activity accompanied by an increase in insulin (Munday *et al*, 1991).

1.6.3 Regulation Of Muscle ACC 280

Short term regulation of heart and skeletal muscle ACC 280 is relatively under-researched.

1.6.3.1 Regulation Of ACC 280 By Phosphorylation

Recently it has been shown that ACC activity in skeletal muscle is regulated by phosphorylation. Evidence for phosphorylation and inactivation of ACC came from *in vitro* studies of purified skeletal muscle ACC which was phosphorylated by liver AMPK (Winder and Hardie, 1996) Phosphorylation of ACC by AMPK results in a decrease in ACC V_{\max} and an increase in $|A_{0.5}$ of ACC for citrate activation. The findings from these

studies are similar to those in liver where AMPK phosphorylates and inactivates ACC (section 1.6.2.1 b)

Recently, protein kinase A (PKA) has shown to phosphorylate ACC from skeletal muscle with no significant effect on the V_{\max} or $K_{0.5}$ for citrate activation (Winder *et al*, 1997). Also, phosphorylation by PKA does not effect the phosphorylation of muscle ACC by AMPK. This suggests that AMPK is an important regulator of ACC activity in muscles and PKA has no functional effect on ACC activity (Winder *et al*, 1997). The serine sites which AMPK phosphorylates in rat skeletal muscle as yet have not been identified. However, both ACC 265 and 280 have been identified in human tissues (Abu-Elhegia *et al*, 1997, Ha *et al*, 1996 and Widmer *et al*, 1996) and the predicted amino acid sequences of human ACC 265 and ACC 280 have been compared providing an insight into the serine residues which might be phosphorylated in rat skeletal muscle ACC by AMPK. The potential phosphorylation sites in rat liver ACC 265, Ser 79 and Ser 77 (Ser 78 and Ser 80 in human ACC 265) are conserved in human ACC 280 as Ser 219 and Ser 221 (Abu-Elhegia *et al*, 1997). By contrast, Ser 1200 of rat liver ACC 265 (Ser 1201 in human ACC 265), the critical phosphorylation site for PKA is missing in human ACC 280 (Abu-Elhegia *et al*, 1997). It is suggested that rat skeletal muscle ACC could be phosphorylated at the same serine residues as those phosphorylated in human ACC 280 and is probably phosphorylated exclusively by AMPK.

1.6.3.2 Regulation Of Muscle ACC During Exercise

Considerable evidence has been provided showing that ACC activity decreases during exercise as a result of phosphorylation by AMPK. During exercise or electrical stimulation the AMPK activity increases with a parallel decrease in ACC activity and

malonyl-CoA content (Winder & Hardie, 1996 & Hutber *et al*, 1997). This decrease in malonyl-CoA has been considered to relieve CPT 1 inhibition and to serve as a signal for increasing fatty acid oxidation in muscle during prolonged exercise (Saha *et al*, 1995 & 1997).

Phosphorylation and inactivation of ACC by AMPK during exercise is due to a rise in Ca^{+} level and/or a rise in free 5'-AMP as a result of muscle contraction, leading to an activation of AMPK kinase which phosphorylates and activates AMPK or by direct allosteric activation of AMPK by 5'-AMP (see fig 1.7 Winder & Hardie, 1996 and Hutber *et al*, 1997). This activation can be reversed by the addition of phosphatases (phosphatase 2A), and complete reactivation of ACC in the presence of phosphatase requires glutamate and MgCl_2 (Vavvas *et al*, 1997). AMP, apart from activating AMPK has also been demonstrated to decrease the rate of inactivation of AMPK by protein phosphatases-2A and 2C (Davies *et al*, 1995).

It has been shown that the α 2 isoform of AMPK is involved in phosphorylating and inactivating ACC during muscle contraction and not the α 1 isoform (Vavvas *et al*, 1997). Finally, the existence of a dual mechanism for ACC regulation in muscle, has been suggested by Ruderman's group whereby the inhibition of ACC by phosphorylation (e.g. during contraction) overcomes the activating effects of citrate. As is discussed in section 1.7.2, in the presence of insulin and glucose malonyl-CoA content increases. This increase in malonyl-CoA content is not associated with a persistent increase in ACC activity but correlates with an increase in cytosolic citrate concentration (Section 1.7.2). Ruderman's group found that during muscle contraction, muscle malonyl-CoA content decreased which was associated with increased citrate and 5'AMP levels (Vavvas *et al*, 1997). This increase in 5'-AMP levels initiates the phosphorylation of ACC by AMPK

Fig 1.7

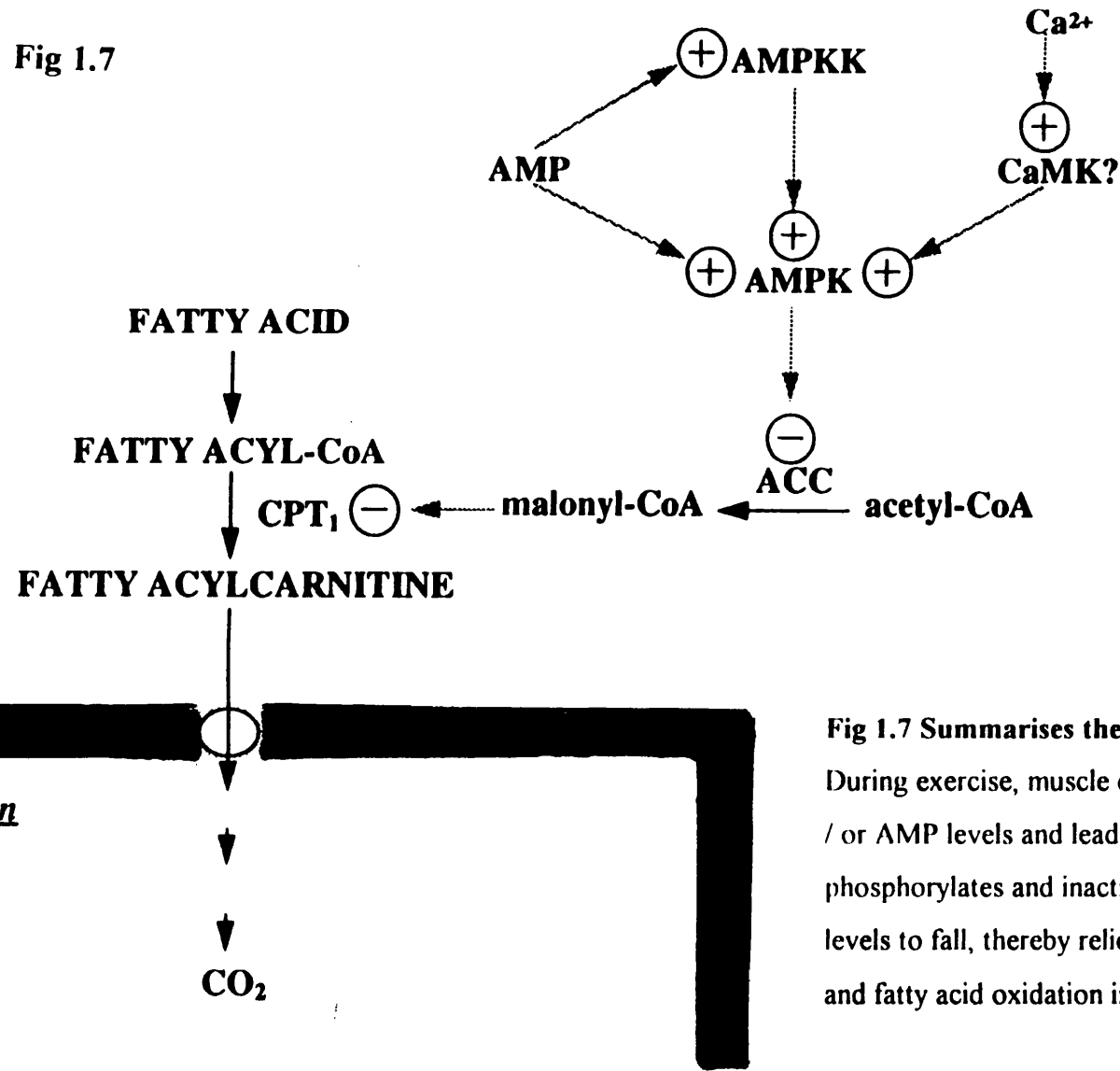


Fig 1.7 Summarises the events that occur in muscle during exercise
During exercise, muscle contraction increases which raises Ca^{2+} and / or AMP levels and leads to the activation of AMPK. Which in turn phosphorylates and inactivates ACC. This results in malonyl-CoA levels to fall, thereby relieving CPT 1 inhibition by malonyl-CoA and fatty acid oxidation increases

and overcomes the activating effects of citrate.. Also, citrate enhances the inhibition of PFK-1 by ATP an effect which is diminished by 5'AMP (section 1.3.3). Ruderman's group suggested that due to the dual effects of citrate and 5'AMP on ACC and PFK-1, citrate and 5'AMP complement each other in coordinating the regulation of these enzymes and also the use of glucose and fatty acids as fuels for muscle contraction (Vavvas *et al*, 1997).

1.6.4 Regulation Of Muscle ACC In Response To Fasting/Refeeding

Both heart and skeletal muscle ACC are unresponsive to nutritional manipulation (Bianchi *et al*, 1990). Winder *et al*, (1995) showed that skeletal muscle ACC V_{max} and the $A_{0.5}$ for citrate activation were not influenced by fasting/refeeding. From western blots it was also shown that the amount of ACC protein did not alter in response to fasting/refeeding. This suggests that ACC in heart and skeletal muscle may be controlled by a different mechanism from that observed in liver during fasting and refeeding (Bianchi *et al*, 1990 and Winder *et al*, 1995).

1.6.5 Regulation Of Heart ACC 280

Saddik *et al*, (1993) showed that in heart the concentration of acetyl-CoA is the main determinant in malonyl-CoA production as opposed to ACC activity *per se*, suggesting that ACC 280 is a substrate driven enzyme and is not influenced by phosphorylation or dephosphorylation as in the liver. However, it has been shown that purified rat liver AMPK phosphorylates and inactivates purified heart ACC 280 (Kudo *et al*, 1996). This suggests that heart ACC 280 is a physiological substrate for AMPK. Further evidence for this came from studies with reperfused ischemic hearts (Kudo *et al*, 1995). Kudo *et*

al, (1995) showed that 5'AMP levels increased at the end of ischemia and remained elevated throughout reperfusion. This was associated with a decrease in ACC activity during reperfusion as a result of accumulation of 5'AMP during ischemia, which in turn activates AMPK and subsequently phosphorylates ACC. Also, a negative correlation between AMPK and ACC was demonstrated in aerobic and reperfused ischemic heart, suggesting that AMPK is capable of phosphorylating heart ACC 280 (Kudo *et al*, 1996). In summary heart and skeletal muscle ACC is a physiological substrate of AMPK-like the liver isoform. During exercise muscle ACC is phosphorylated and inactivated by AMPK causing a fall in malonyl-CoA production and relieving inhibition of CPT I by malonyl-CoA thus allowing fatty acids to be oxidised and providing energy to the contracting muscle.

1.6.6 Human ACC Isoforms

In humans both isoforms of ACC are present in a variety of tissues. Human ACC 265 (HACC 265) and human ACC 275 (HACC 275 corresponds to rodent heart and skeletal muscle ACC 280 isoform) are expressed in adipose tissue, whilst the higher molecular mass form is expressed in heart and skeletal muscle. The cDNA's of HACC 265 and HACC 275 have been cloned and sequenced and are located on chromosome 17 and 12 respectively (Witters *et al*, 1994 and Widmer *et al*, 1996). HACC 265 and HACC 275 are more commonly known as ACC 1 and ACC 2, respectively (Abu-Elhegia *et al*, 1997 and Ha *et al*, 1996).

From comparing the predicted amino acid sequences at the N-terminus of human ACC 1 and human ACC 2, it was found that ACC 2 contains an extra 200 amino acid sequence at the N-terminus (Abu-Elhegia *et al*, 1997 and Ha *et al*, 1996). This unique sequence in

ACC 2 is thought to account for the difference in molecular mass between the two isoforms and has also been suggested to be involved in the function of ACC 2 in controlling fatty acid oxidation in skeletal muscle and heart (Ha *et al*, 1996). Ha *et al*, (1996) suggested that the first 25 amino acids of this unique sequence, which contains hydrophobic amino acids is responsible for membrane targeting of binding the ACC 2 to the outer membrane of the mitochondria i.e. near the malonyl-CoA binding site of CPT1.

1.7 FUEL SELECTION

Skeletal muscles derive energy from glucose and fatty acid oxidation. Fatty acids are the preferred source of energy during intense exercise and starvation.

Randle *et al* (1964) proposed that oxidation of fatty acids and ketones inhibits glucose oxidation in heart and diaphragm muscles, thus sparing carbohydrates (the glucose / fatty acid cycle). This was not demonstrated in resting skeletal muscles in the presence of increased fatty acids (Goodman *et al*, 1974). This finding led to the conclusion that this mechanism proposed by Randle, is confined to heart and diaphragm muscles and is not operative in skeletal muscles. However, other studies have shown that the glucose/fatty acid cycle occurs in skeletal muscles (Rennie *et al*, 1976). During exercise increased levels of fatty acids lead to the inhibition of carbohydrate utilisation in red muscle fibres (Rennie *et al*, 1976). It was also shown that in exercised muscle an increase in fatty acids caused a concomitant increase in citrate levels (Rennie *et al*, 1976). This suggests that like heart, increased fatty acids in muscles could indirectly inhibit glucose utilisation at the site of PFK-1 by citrate (Rennie *et al*, 1976 and Randle *et al*, 1964). These effects

of fatty acids were due to an increase in fatty acid oxidation (Neely and Morgan, 1974).

The reason why certain studies showed no effect with increased fatty acids was because the rat perfused hindquarter was being used. The rat hindquarter is composed of approximately 50% white fibres, and these fibres have a very low capacity to oxidise fatty acids in comparison to red fibres (section 1.2.2).

The glucose/fatty acid cycle is also operative in prolonged starvation, as a 40% decline in whole body glucose turnover in rats starved for 48 hours is due to the result of decreased glucose utilisation in muscles (oxidative muscles) which are capable of high rates of fatty acid oxidation (Issad *et al*, 1987). This decrease in glucose utilization is mediated by the inhibition of hexokinase, phosphofructokinase-1 and particularly the PDH complex (Randle, 1986). There is evidence to suggest that this fuel cross talk is not one sided, i.e. under appropriate conditions carbohydrate fuels can inhibit fatty acid oxidation. In the presence of glucose, or glucose and insulin, fatty acid oxidation is inhibited in human skeletal muscles thus reversing the glucose/fatty acid cycle (Sidossis & Wolfe, 1996 and Kelley *et al*, 1990). In addition, glucose, or glucose and lactate, inhibit fatty acid oxidation in perfused heart and in cardiac myocytes (Bielefeld *et al*, 1985 and Awan & Saggerson 1993). The mechanisms involved in reversing the glucose/fatty acid cycle have been of great interest and considerable evidence suggests that the malonyl-CoA/CPT 1 interaction seems to play an important role (Awan & Saggerson 1993, Kelley *et al*, 1990 and Saha *et al*, 1995).

1.7.1 PDH Complex

Increased fatty acids and ketones result in a decline in insulin dependent glucose uptake *in vitro* in diaphragm and heart (Randle, 1964). The mechanisms for this involves an

increase in the mitochondrial ratio of acetyl/CoA and NADH/ NAD⁺ activating PDH kinase and resulting in inhibition of the PDH complex, and at the same time indirectly inhibiting hexokinase and PFK-1 by accumulation of glucose-6-phosphate and citrate respectively (Randle 1986). This phenomenon is operative in skeletal muscles during the fed to starved transition (Issad *et al*, 1987 and Sugden & Holness, 1990a). This transition from fed to starved is characterised by a fundamental switch in fuel selection in oxidative muscles. The time course of the starvation-induced decrease in PDHa activity differs in individual tissues (Sugden & Holness, 1990a). Heart and liver PDH activity rapidly declined within 3-6 hours of starvation (Holness *et al*, 1989). In contrast, PDH inactivation in skeletal muscles occurred relatively later after food withdrawal (9-15 hr) associated with an increase in circulating fatty acid concentration (Sugden & Holness, 1990 a and Holness & Sugden, 1990). Both heart and skeletal muscle PDH inactivation can be reversed by inhibiting mitochondrial fatty acid oxidation (Holness & Sugden, 1989 and Holness *et al*, 1989). This suggests that the glucose fatty acid cycle is operative *in vivo* at the level of pyruvate oxidation in skeletal muscle in the resting state (Holness & Sugden, 1990 Sugden & Holness 1990a and Holness *et al*, 1989). In liver PDH activity is restored by administering insulin but not by inhibiting fatty acid oxidation, suggesting that PDH inactivation in liver is due to a decline in insulin concentration (Holness & Sugden 1989 and Sugden & Holness 1990 a & b). In oxidative muscles complete reactivation of PDH activity is not observed until after 6-8 hours of re-feeding and Fru-2,6-P₂ concentrations are lower than at the fed state, even though the supply of carbohydrates is increased and fatty acid and ketone body concentration have fallen (Sugden & Holness, 1989, and French *et al*, 1988). This suggests that oxidative muscles are able to supplement their oxidative requirements through the use of non-

carbohydrate substrates (endogenous and exogenous triacylglycerol and amino acids) (Sugden & Holness 1990a & 1989 and Holness *et al*, 1989).

1.7.2 Malonyl-CoA/CPT 1

In cardiac muscle it has been suggested in the presence of carbohydrate fuels that operation of the ACC/malonyl-CoA/CPT 1 interaction leads to inhibition of fatty acid oxidation, thus reversing the glucose/fatty acid cycle. Carbohydrate fuels such as lactate have been shown to inhibit palmitate oxidation accompanied by decreased levels of acylcarnitine (Bielefeld *et al*, 1985). This implies that inhibition of fatty acid oxidation by carbohydrate fuels involves the carnitine dependent reactions. Also, in perfused hearts DCA stimulates glucose oxidation with a concomitant suppression of palmitate oxidation (Saddik *et al*, 1993). This decrease in fatty acid oxidation was correlated with DCA-induced increases in acetyl-CoA and malonyl-CoA levels (Saddik *et al*, 1993). These changes can be reversed by removing glucose from the perfusate, resulting in a fall in acetyl-CoA and malonyl-CoA levels associated with increased fatty acid oxidation. The majority of the acetyl-CoA production in heart is intramitochondrial. It is therefore suggested by Saddik *et al*, (1993), that a rise in malonyl-CoA levels following DCA increases transport of acetyl-CoA units to the cytosol via a pathway involving carnitine acetyltransferase and a carnitine:acetylcarnitinetransferase translocase, thus providing the substrate for ACC. Evidence for the transport of acetyl-CoA from the mitochondria to the cytosol leading to a stimulation of ACC came from increasing intracellular carnitine levels. In the presence of high concentrations of carnitine, glucose oxidation is increased causing a decrease in fatty acid oxidation (Broderick *et al*, 1992). Carnitine stimulates the carnitine acetyltransferase transport system, increasing the transport of mitochondrial

acetyl units to the cytosol. This lowers the intramitochondrial levels of acetyl-CoA thus relieving PDH inhibition and stimulating glucose oxidation with a parallel decrease in fatty acid oxidation (Lopashuk & Gamble, 1994). This provides a mechanism whereby cytosolic acetyl-CoA generated from PDH, stimulates ACC activity and synthesis of malonyl-CoA, resulting in inhibition of fatty acid oxidation by malonyl-CoA at the site of CPT 1.

Another mechanism by which increased mitochondrial acetyl-CoA can inhibit fatty acid oxidation is by inhibiting 3-ketoacyl-CoA thiolase, but this does not account for the increase in malonyl-CoA levels observed in DCA perfused hearts (Saddik *et al*, 1993).

Recently it has been proposed that such an interplay between malonyl-CoA, acetyl-CoA and CPT 1 occurs in skeletal muscle leading to an inhibition of fatty acid oxidation. It has been well documented that malonyl-CoA concentration in rat skeletal muscle decreases in response to exercise (Winder *et al*, 1989 and Winder *et al*, 1990), starvation (McGarry *et al*, 1978), and increases on refeeding (McGarry *et al*, 1978). These changes are consistent with directional changes in fatty acid oxidation. The exact mechanisms whereby these factors alter malonyl-CoA concentrations is unclear. Ideally one could assume that these changes in malonyl-CoA levels and fatty acid oxidation activity could be controlled by the same mechanism as described by Saddik *et al* (1993), Lopashuk & Gamble (1994) and Lopashuk *et al* (1994) for heart. Recently it has been shown that increases in malonyl-CoA levels on the addition of glucose or glucose and insulin, are associated with a decrease in fatty acid oxidation in oxidative muscles (Soleus) (Duan & Winder 1993 and Vavvas *et al*, 1996). This increase in malonyl-CoA is not due to a persistent increase in ACC activity but is correlated with an increase in citrate concentration (Saha *et al*, 1997). This increase in malonyl-CoA content can be blocked by an inhibitor of ATP-citrate lyase, suggesting that the level of citrate the proposed

major precursor of the cytosolic acetyl-CoA is the main determinant of malonyl-CoA production in skeletal muscle (Vavvas *et al*, 1996). This suggests that under favourable conditions (e.g. glucose or insulin present) the citrate concentration is increased leading to an increase in cytosolic acetyl-CoA which stimulates ACC and malonyl-CoA production resulting in suppression of fatty acid oxidation. An increase in cytosolic acetyl-CoA under certain circumstances has been shown to be generated from substrates other than citrate such as acetyl-carnitine in the presence of acetoacetate (Saha *et al*, 1997 and Berger *et al*, 1976). Acetyl-carnitine levels in muscle have shown to increase in the presence of DCA (Constantin -Teodosiu *et al*, 1991).

As mentioned in section 1.6.3.2. exercise and contractions induced by electrical stimulation decrease malonyl-CoA levels in rat skeletal muscles, thus relieving inhibition of CPT 1 and allowing an increase in fatty acid oxidation during exercise (Winder *et al*, 1989 and Elayan & Winder 1991). This decrease in malonyl-CoA is associated with a decrease in ACC activity brought about by phosphorylation (Vavvas *et al*, 1996 and 1997). This suggests that fuel supply and energy expenditure alter malonyl-CoA levels in skeletal muscle by different mechanisms. From these findings Ruderman's group suggested that malonyl-CoA is a component of a fuel sensing mechanism and signalling pathway that responds acutely to changes in the fuel supply and energy expenditure of the muscle cell (Saha *et al*, 1995). A mechanism has been suggested whereby citrate links the malonyl-CoA fuel sensing mechanism to the glucose/fatty acid cycle (Saha *et al*, 1997). According to the proposed scheme an increase in the supply of glucose leads to an increase in cytosolic citrate resulting in an increased formation of malonyl-CoA by allosterically activating ACC or by increasing the concentration of its substrate cytosolic acetyl-CoA. This results in inhibition of fatty acid oxidation by malonyl-CoA as shown in fig 1.8. These findings from Ruderman's group provide a mechanism for inhibiting

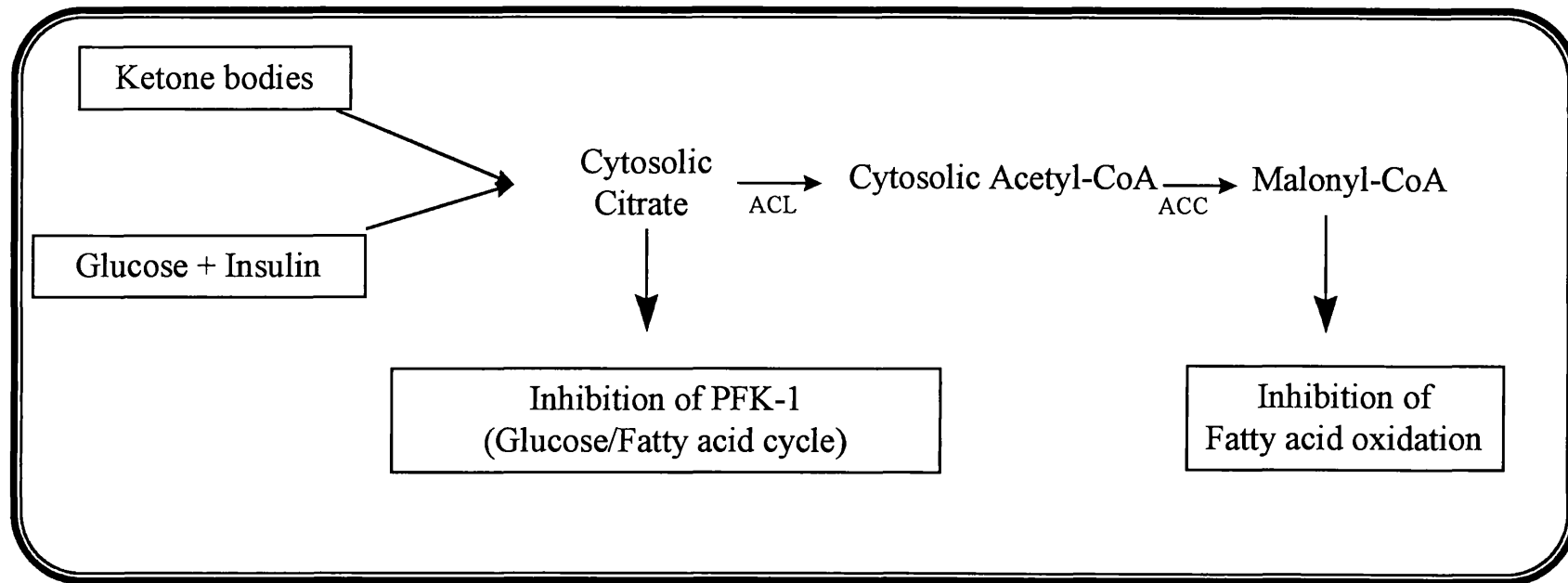
Fig 1.8

Fig 1.8 Proposed Scheme For Cytosolic Citrate as a link between the malonyl-CoA fuel sensing mechanism and the Glucose/fatty acid cycle (Adapted from Saha *et al*, 1997)

fatty acid oxidation by glucose and evidence for a citrate-dependent malonyl-CoA generating mechanism in skeletal muscle. The extent to which citrate provides acetyl-CoA and whether it acts as an allosteric activator of muscle ACC remains to be established.

Chapter Two

Materials & Methods

2.1 COMMERCIAL PREPARATIONS

All commonly used reagents were obtained from Sigma Chemical Co. Ltd Poole Dorset, UK and British Drug Houses Ltd, Poole, Dorset, UK In addition, the following were supplied by:

Sigma Chemical Co. Ltd,: L-Malic acid, Trizma-base, Tris-HCL, bis-Tris-propane, adenosine 5'-triphosphate (disodium) from Equine muscle, Bovine serum albumin (essentially fatty acid free, fraction V), benzethonium hydroxide (in methanol), Dowex 2 Chloride form (8% cross linkage-dry mesh 200-400), Hydrazine sulphate, Palmitic acid (sodium), Malonyl-CoA (Lithium), Acetyl-CoA (sodium), 5-amino-4-imidazolecarboxamide ribonucleoside (AICAR), NADPH (reduced form sodium salt),

L-isoproterenol, 5-amino-4-imidazolecarboxamide ribonucleotide (ZMP), Adenosine 5'-monophosphate (disodium), Adenosine 5'-diphosphate (disodium), Adenosine (disodium), L-isoproterenol (hydrochloride), Dichloroacetic acid, Acetyl-Phosphate (lithium phosphate) and Bicinchoninic acid protein assay kit.

Boehringer Mannheim: Malate dehydrogenase (from pig heart), Lactate dehydrogenase, Citrate synthase (from pig heart), Carnitine acetyl transferase (from breast muscle), Phosphotransacetylase (from *Bacillus stearothermophilis*), NAD (free acid), Coenzyme A (free acid), NADH (disodium), Insulin and Triglycerides fully enzymatic kit.

British Drug Houses Ltd: Dithiothreitol (Cleland's reagent)

May & Baker, Essex, U.K.: Citric acid (Trisodium)

Amersham International, Bucks., U.K.: Sodium [^{14}C] bicarbonate, [$1\text{-}^{14}\text{C}$] Acetyl-CoA, [^3H] Acetyl-CoA, [$2\text{-}^{14}\text{C}$] Malonyl-CoA and [$\text{U-}^{14}\text{C}$] Palmitate

BOC Ltd., London U.K.: Pressurised gasses ($\text{O}_2\text{:CO}_2$ 95% : 5%) and liquid nitrogen.

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

Alpha Laboratories Ltd., Eastleigh, Hants, U.K.: Wako NEFA C Test kit.

Spring & Wire Form., Reading Berkshire, U.K.: Stainless steel clips.

Etomoxir was a gift from Dr H. P. O. Wolf. (Byk Gulden Pharmazeytika., Konstanz, Germany.)

2.2 LABORATORY PREPARATIONS

2.2.1 Palmitate Bound To Albumin

The method of Evans & Muller (1963) was used to prepare palmitate bound to albumin. 250mg of sodium palmitate was added to 50ml of 15 % (w/v) BSA in 0.9% (w/v) NaCl and mixed. The mixture was repeatedly sonicated in a sonicating water bath until an even suspension was obtained. The suspension was incubated at 50°C for 20 minutes, cooled and left at 4°C overnight. The suspension was filtered through Whatman No.1 filter paper and centrifuged at 26000g_{av} for 30 minutes at 4°C to remove any remaining microcrystals of sodium palmitate. The supernatant was adjusted to pH 7.4 with NaOH and stored in aliquots at -20°C. The concentration of bound palmitate was determined using the WAKO NEFA C Kit as described below.

2.2.1.1 Determination Of Palmitate Bound To Albumin

The concentration of palmitate bound to albumin was determined using the WAKO NEFA C TEST KIT.

The main principles of this assay are as follows; free fatty acids are converted to acyl-CoA thioesters by acyl-CoA synthetase and are oxidised by acyl-CoA oxidase producing hydrogen peroxide as a by-product. Hydrogen peroxide in the presence of peroxidase forms a purple coloured adduct with a maximum absorbance at 550nm. The amount of NEFA present can be determined from the optical density at 550nm. The assay was carried out in duplicate in a final volume of 3ml. A series of tubes were labelled sample, standard and blank. To the appropriate tubes 50µl of bound

palmitate, fatty acid standard (oleic acid 1.0 nmol/L) provided in the kit or water was added.

1.0 ml of reagent A (50mM phosphate buffer (pH 6.9) containing 3 mM MgCl_2 , 0.3 U/ml acyl-Coenzyme A synthetase, 3U/ml ascorbate oxidase, 30 mg/ml ATP and 7 mg/ml CoASH) was added to each tube and incubated at 37°C for 10 min. 2.0 ml of reagent B (1.2 mM MEHA, 6.6 U/ml acyl-Coenzyme A Oxidase and 7.5 U/ml peroxidase) was then added and incubated as before. It was important that the ten minute incubations were accurately timed for the purpose of reproducibility. At the end of the incubation period, the tubes were allowed to equilibrate to room temperature and the absorbance was read at 550 nm against the blank.

2.2.2 Purification Of Fatty Acid Synthase

Fatty acid synthase was purified from rat livers by a procedure similar to that of Hsu *et al*, (1965). Livers were quickly removed, chopped and washed in ice cold homogenisation medium consisting of the following 10 mM Tris-HCl (pH 7.4), 225 mM mannitol, 75 mM sucrose, 0.5 M EDTA and 0.5 mM DTT. The livers were homogenised by 3-4 strokes of a motor driven teflon pestle (450 rev/min) in a glass potter elvehjem homogeniser (radial clearance 0.19mm).

The homogenate was centrifuged at 9200 g_{av} (Beckman Sorvall RC5-B centrifuge, SS-34 rotor) for 10 min at 0-4°C. The resulting supernatant was centrifuged at 100,000 g_{av} (Beckman L8 Ultracentrifuge, 75Ti rotor) for 1 hour at 0-4°C. Saturated ammonium sulphate was added slowly to the supernatant to 25 % saturation and stirred continuously for 15 min on ice. The suspension was centrifuged at 18,000 g_{av}

for 10 min and the pellet was discarded. The supernatant was brought to 40% saturation with saturated ammonium sulphate and centrifuged as above. The precipitate was dissolved in 20mM bis-Tris propane (pH 7.0) buffer containing 30mM NaCl, 3mM EDTA, 1mM DTT and then desalted by overnight dialysis with two changes of the same buffer. Finally the solution was applied to a Pharmacia f.p.l.c Hiload 16/10 Q Sepharose column previously equilibrated with 20mM bis-Tris propane buffer (pH7.0) containing 30mM NaCl, 3mM EDTA and 1mM DTT. The enzyme was eluted with a linear gradient of 0-0.6M NaCl in the same buffer over 2 column volumes. Most of the FAS activity was eluted between 0.2-0.25M NaCl with a specific activity > 100 nmol/min/mg of protein.

2.3 ANIMALS

Animals used throughout were male Sprague Dawley rats bred in the animal house at UCL.

They were maintained at 20-22°C, had constant access to drinking water and to Rat & Mouse Breeding Diet (Special Diet Services, Witham, Essex, UK). The light/dark cycle was 13h light and 11h dark, with light from 06:00 to 19:00h. Rats used for isolation of soleus strips were 4-6 weeks old (180-200g body wt.) and all other rats were 6-8 weeks (200-250g body wt.).

2.4 THE SOLEUS STRIP PREPARATION

The soleus muscle is commonly used for *in vitro* studies, as it can be easily dissected from the hindlimb with intact tendons and when incubated it possess the same shape

as *in vivo*, which is not the case for muscles such as the diaphragm or the epitrochlearis. The main problem with muscle incubation studies is the weight of the muscles. Muscles weighing more than 35 mg have problems of diffusion of substrates and this is the rate limiting factor in incubation studies. Crettaz *et al*, (1980) devised a method which overcame this diffusion problem. This was achieved by isolating thin strips of muscle (weighing 35mg) with tendons from the intact soleus.

2.4.1 Isolation And Incubation Of Soleus Strips

Soleus strips weighing approximately 35mg (2-3 strips per leg) were obtained by longitudinal dissection of the muscle, using the method of Crettaz *et al*, (1980). Rats were killed by cervical dislocation. The hindlimb was removed from the animal and fixed to the dissection board with the aid of needles. The gastrocnemius and the plantaris muscles were removed exposing the soleus muscle. The soleus muscle was dissected out, cleared of any fat surrounding it and fixed on to the dissection board (with needles). The intact muscle was divided into 3 strips with a scalpel. A thread was placed around the outer distal tendon and a knot was tied. The same was done at the outer proximal tendon. The muscle strip was cut below the knot at the distal tendon and cut above the knot at the proximal tendon. The muscle strip was attached to stainless steel clips (lightly stretched) and placed in pre-warmed (37°C) siliconized Erlenmeyer flasks containing 4ml Krebs Ringer-bicarbonate buffer (pH 7.4) with 20mg/ml BSA and 5mM glucose. The flasks were continuously gassed with O₂/CO₂, (95:5) and shaken in a water bath (75 strokes/min) for the preincubation period of 30min at 37°C. Another strip was prepared from the other leg.

In total four strips of muscle were obtained from each animal and this whole procedure took 7-8min. At the end of the preincubation period the muscles were either transferred to metabolic flasks (fitted with centre wells) as described in section 2.7.1 & 2.8.1 or to incubation flasks containing 4ml Krebs Ringer-bicarbonate buffer (pH 7.4) with 20mg/ml BSA, 5mM glucose plus the various additions. The flasks were left shaking for 60 min at 37°C and were gassed for the first 10 min of the incubation period. At the end of the incubation period the muscles were quickly removed from the flasks, either placed in liquid nitrogen and used to measure metabolites as described in section 2.5 or measured for glycogen as described in section 2.7.2.

Perchloric acid (25%) was added to the incubation medium and was neutralised by the addition of near saturated potassium bicarbonate (200µl). The neutralised medium was stored at -70°C and used for determination of lactate (2.6.4).

2.5 PREPARATION OF TISSUE EXTRACTS FOR METABOLITE MEASUREMENTS

2.5.1 Preparation Of Acid Soluble Extract For Measuring Malonyl-CoA

Muscle strips were isolated and incubated as described in section 2.4.1, at the end of the incubation period the strips were quickly removed from the stainless steel clips and placed in liquid nitrogen. Two frozen strips were ground to a fine powder in a mortar containing liquid nitrogen and then deproteinised by the addition of 6% perchloric acid [(w/v) 10µl/mg]. The frozen material was homogenised using an ultra

turrax tissue disintegrator until all the frozen material had melted. The contents of the homogeniser were centrifuged at $2000g_{av}$ for 10 min. After centrifugation the acid soluble extract was separated from the acid insoluble pellet. The pH of the acid soluble extract was adjusted to pH 6.0 by the addition of 5M potassium bicarbonate (30-50 μ l). The extract was centrifuged as before and the volume of the supernatant was recorded. Malonyl-CoA assays and nucleotide measurements were performed on these fresh extracts.

2.5.2 Preparation Of Acid Insoluble Pellet For Measuring Long Chain Fatty Acyl-CoA And Acylcarnitine

Long chain fatty acylcarnitine and long chain fatty acyl-CoA's are insoluble in dilute perchloric acid (Saggerson & Greenbaum 1970a) and can be separated from other acylcarnitines, free carnitines and CoA derivatives after acidic deproteinisation of the tissue. The acid-insoluble pellet was homogenized in 1.5ml KOH (0.2M) containing 10mM DTT. The suspension was incubated at 55⁰C for 2 hours, after which it was cooled on ice and 75 μ l of perchloric acid 60% (w/v) was added. The precipitate was removed by centrifugation ($2000g_{av}$) for 10 min. The supernatant was buffered by the addition of 25 μ l of near-saturated potassium dihydrogen phosphate and the pH was raised to 7.0 with KOH (10M and then 1M). The supernatant was centrifuged as above to remove the potassium perchlorate and the volume was recorded. Long chain fatty acyl-CoA and long chain acylcarnitine measurements were performed on these extracts as described in Section 2.6.2 and 2.6.3.

It should be noted that long chain fatty acyl-coA and long chain acyl carnitine were measured as CoASH and carnitine.

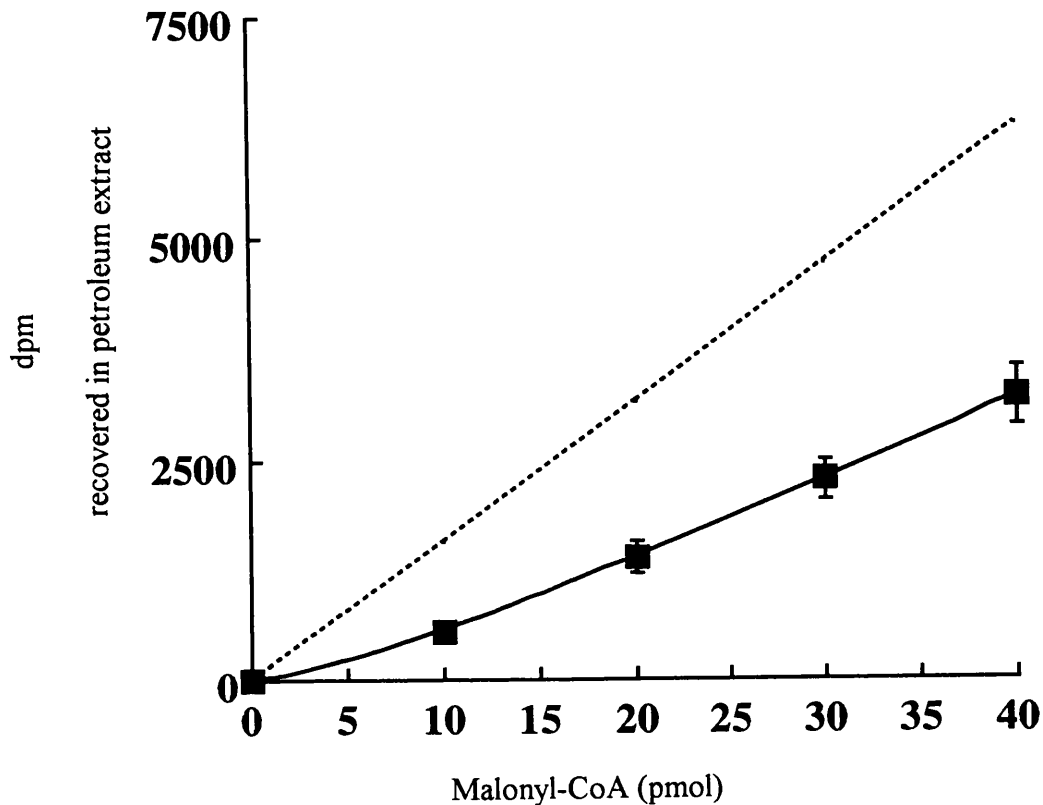
2.6 DETERMINATION OF METABOLITES

2.6.1 Malonyl-CoA

Malonyl-CoA was assayed radiochemically using the method of McGarry *et al* (1978 b) with slight modifications as explained below. The method measures the malonyl-CoA dependent incorporation of radioactive acetyl-CoA into palmitic acid, catalysed by fatty acid synthase in the presence of NADPH. McGarry *et al*, (1978 b) typically measured 100-200pmol amounts of malonyl-CoA in extracts from rat liver, where tissue malonyl-CoA content are relatively high and where quantity of tissue sample is generally not a problem. The assays of McGarry *et al*, (1978) were calibrated by the addition of malonyl-CoA as an internal standard (generally 200 pmol) in order to overcome the problem of dilution of [^3H] acetyl-CoA in the assay by unlabelled acetyl-CoA in the tissue extract. Under these conditions, the product formed was essentially all [^3H] palmitate and the product formation was linear with [malonyl-CoA]. However to reliably measure malonyl-CoA in small strips of soleus muscle (10-30 picomol in a typical assay) the assay was modified. This was because, the assay was non-linear at low concentrations of malonyl-CoA (fig 2.1) and this was initially shown by Singh *et al* (1984). This could be due to the binding of some malonyl-CoA by fatty acid synthase or as the ratio of acetyl-CoA/malonyl-CoA in the assay is relatively high, due to premature release from the fatty acid synthase complex of short or medium chain reaction products (Abdinejad *et al*, 1981) which are incompletely extracted into petroleum or are lost during drying down of the petroleum fraction. To minimize the problem of binding of malonyl-CoA the concentration of fatty acid

Fig 2.1

Standard Curve For Assay Of Small Amounts Of Malonyl-CoA



0.68 μ M [1- 14 C] acetyl-CoA (0.5 μ Ci/nmol) and standard quantities of malonyl-CoA were incubated with ImU of purified fatty acid synthase.

Incubations were for 2 hours and contained BSA, 0.1 % (w/v) as described in section 2.6.1.

The values are means \pm SEM from 24 different experiments. The dotted line shows the theoretical result if all the malonyl-CoA was converted into [3 H] palmitate.

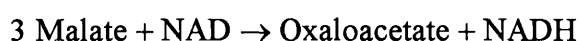
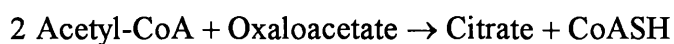
synthase was reduced to 1 m unit per assay and accordingly lengthened the incubation time to 2 h, but still observed sufficient lack of linearity to preclude the use of internal standards. However the use of internal standards was unnecessary in view of small amounts of tissue used. Assuming that 1g of skeletal muscle contains 0.65 g of intracellular water (Frayn, 1995), rat soleus muscle contains 2.7-5.2 nmol of acetyl-CoA per g wet. wt. (Spriet *et al*, 1992 and Dyck *et al*, 1996). Under our conditions, the concentration of acetyl-CoA was deliberately kept high (0.68 μ M), this would cause no more than a 5-9% dilution of the [3 H] acetyl-CoA in the malonyl-CoA assay. Accordingly, the assays of malonyl-CoA in muscle extracts were calibrated by reference to external standards which were always assayed in parallel on the same day.

The assay was carried out in duplicate in glass tubes. The assay mixture was made up to a final volume of 1ml containing 0.2M potassium phosphate buffer (pH 7.2) with 0.2mM EDTA, BSA 0.1% (w/v), 2.5mM DTT, 0.25mM NADPH, 0.68 μ M [3 H] acetyl-CoA (0.5 μ Ci/nmol), 150 μ l of muscle extracts and malonyl-CoA standards (10, 20, 30 & 40 picomol). The reaction was initiated by the addition of 1mU of purified fatty acid synthase. The tubes were vortexed and incubated for 2 hours at 37 $^{\circ}$ C. The reaction was terminated by the addition of 29 μ l of 60% (w/v) perchloric acid. 1ml of ethanol was added to each tube followed by 5ml of petroleum ether (b.p. 60-80 $^{\circ}$ C). Each tube was vortexed for 30 sec and centrifuged at 2000 g_{av} for 5-10min. 4ml of the petroleum ether phase was transferred to a second set of glass tubes containing 2ml of water. These tubes were vortexed and centrifuged as above. 3ml of the petroleum ether phase was transferred to glass tubes as above and the same procedure of vortexing and centrifuging was repeated. Finally 2ml of the petroleum ether phase

was transferred to scintillation vials and left overnight for the solvent to evaporate. 10ml of Ecoscint A was added to the scintillation vials before scintillation counting.

2.6.2 Long Chain Fatty Acyl-CoA (free Coenzyme (CoASH))

The recycling method of Allred & Guy (1969) was used to measure CoASH as shown in the reaction sequences below.



Overall Reaction



CoASH is recycled through a coupled enzyme system where the rate of formation of NADH is proportional to CoASH concentration when all the other substrates and enzymes are present in excess.

CoASH was assayed spectrophotometrically at 26°C in a final volume of 1ml containing 277mM Tris-HCl (pH 7.2), 55mM KCl, 10mM malate, 4.5mM acetyl-phosphate, 1mM DTT, 2mM NAD, 18.2µg citrate synthase 2units/ml, 7.5µg malate dehydrogenase 10units/ml, and tissue extract. The reaction was initiated with 17U of phosphotransacetylase and the rate of increase in absorbance at 340 nm was recorded against the reference cuvette containing 1ml of water. An internal standard (0.2nmol) of CoASH was added to the test cuvette and the further increase in absorbance was recorded. The background rate (tissue sample and CoASH standard omitted from the test cuvette) was measured for a period of 10 min and subtracted from the reaction rate observed. The amount of CoASH present in the muscle extract was calculated from measured rate before and after the addition of the internal CoASH standard.

2.6.3 Carnitine

Carnitine was assayed using the method of Cederblad & Lindstedt (1972). Radiolabelled acetylcarnitine is formed by incubating [^{14}C] acetyl-CoA with L-carnitine in the presence of carnitine acetyltransferase. The labelled acetylcarnitine is then separated from labelled acetyl-CoA by passing the reaction mixture through an anion exchange column. The column retains acetyl-CoA and the acetylcarnitine passes straight through.

2.6.3.1 Column Preparation

Dowex 2 (Chloride form, 8% cross linkage, dry mesh 200-400) was suspended as a stirred slurry in distilled water and poured into plastic columns. After settling for approximately 30 min the packed resin occupied approximately 2cm (length) x 0.8cm (diameter). Prior to the assays the water above the resin layer was reduced to approximately 2mm.

2.6.3.2 Carnitine Assay

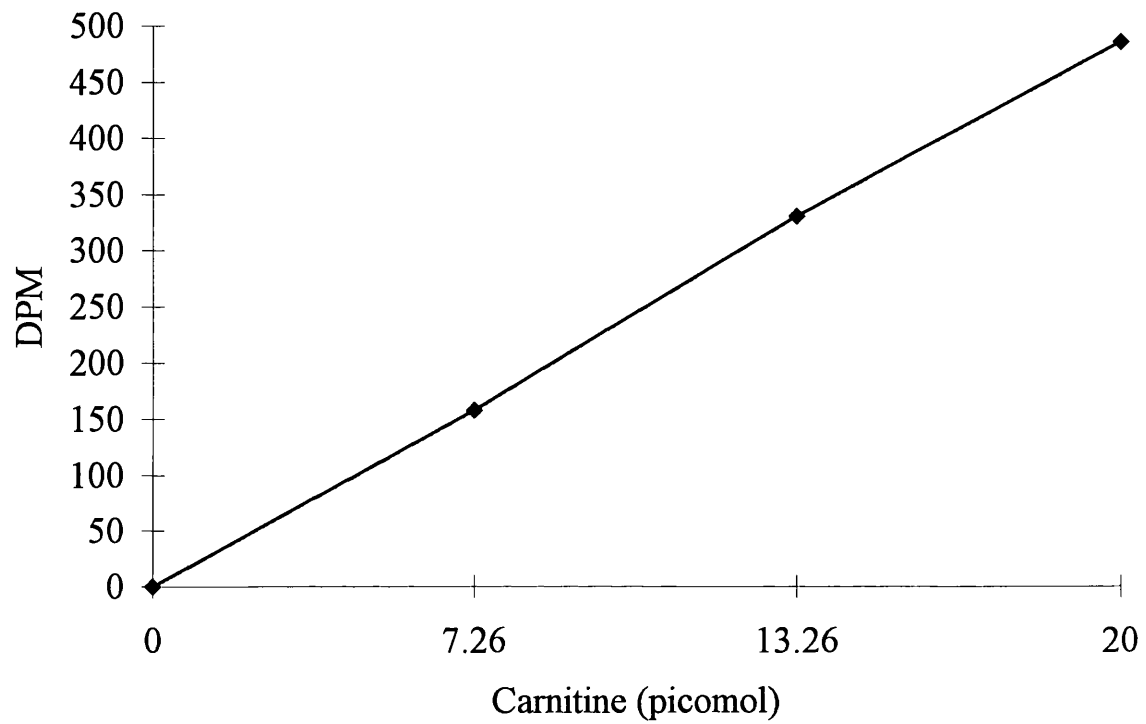
The following stock solutions were prepared and with the exception of the potassium phosphate buffer, the solutions were stored at -20°C .

- a) 1M potassium phosphate buffer (pH 6.5)
- b) 0.2mM acetyl-CoA
- c) [^{14}C] acetyl-CoA (diluted to $0.2\mu\text{Ci/ml}$)

Immediately before the start of the assay 1 volume of solution (a), 1 volume of solution (b) and 2 volumes of solution (c) were mixed. Incubations were carried out

Fig 2.2

Standard Curve For Carnitine Determination



The reaction was linear up to 20 picomol of L-carnitine. All assays of extracts were within the range of the standard curve. n = 1

in 1.5ml tubes containing 100 μ l of the mixture, 50 μ l of muscle extract as prepared in section 2.5.2 and carnitine standards (7.26, 13.26 and 20 picomol) (see figure 2.2 standard curve). The reaction was initiated by the addition of 100 μ g carnitine acetyltransferase (10U), and incubated at 37 $^{\circ}$ C for 30min to allow for the reaction to go to completion.

2.6.3.3 Fractionation

At the end of the incubation 0.5ml of distilled water was added to each tube and the total tube contents was then pipetted onto the Dowex column. This procedure was repeated once the water layer above the resin layer had reached approx 2mm. Columns were run into scintillation vials until the water layer was down to the top of the resin layer. 10ml of Ecoscint A was then added to each vial for scintillation counting.

2.6.4 HPLC Analysis Of Tissue Nucleotides

Samples for nucleotide determination were prepared as described in section 2.5.1 and then filtered through a 0.22 μ M millipore filter. HPLC analysis of soluble nucleotides was performed using a 15cm x 4mm column packed with APS-hyersil-5 (weak anion exchanger). A guard column 4cm x 4mm packed with the same material preceded the analytical column. The column was equilibrated at room temperature with 5mM KH₂PO₄, pH 3 (buffer A) at a flow rate of 1ml/min (1000 Bar pump pressure) with a linear gradient rising to 500mM KH₂PO₄, pH 3 (buffer B) over 30 min. The gradient profile used is shown on the next page.

Time (min)	% Buffer B (500mM KH ₂ PO ₄)
0	0
2	0
20	50
25	100
28	100
29	0
33	0
34	0

The buffers were filtered through a 0.22µM millipore filter and degassed under vacuum before use. 50µl of sample was applied to the column and the eluate was monitored at 259nm using a WATERS 486 tunable absorbance detector (626 pump) with a 11µl flow cell. The detector response was recorded and processed on Millenium 2010 software. 5-10 nmol of nucleotide standards were run under the same condition each time. AMP, ADP, ATP, and ZMP peaks were identified by comparison of their retention times with the known standards. The concentration of each nucleotide was determined from the area under its elution peak.

2.6.5 Lactate

Lactate released by muscle strips was measured enzymatically using the method of Hohorst (1965). Assays were carried out 26⁰C in a final volume of 3ml containing 0.2 M glycine buffer (pH 9.5), 0.5 M hydrazine sulphate, 2.7mM EDTA, 2.5mM NAD⁺ and 1.5ml of the neutralised incubation medium. The initial absorbance at 340nm was measured against a blank (sample omitted) and 100µg (10U) of lactate dehydrogenase was added to both cuvettes. The increase in absorbance was

measured and the concentration of lactate was calculated using the extinction coefficient $6.22\mu\text{mol}^{-1}\text{ml}$ for NADH.

2.7 MEASUREMENT OF CARBOHYDRATE METABOLITES IN ISOLATED SOLEUS STRIPS

2.7.1 Formation Of $^{14}\text{CO}_2$ From $[\text{U-}^{14}\text{C}]$ Glucose

The rate of glucose oxidation in isolated soleus strips was measured by the rate of $^{14}\text{CO}_2$ formation from $[\text{U-}^{14}\text{C}]$ glucose. Soleus strips were isolated and preincubated as described in section 2.4.1. At the end of the preincubation period the soleus strips were transferred to metabolic flasks fitted with centre wells, prewarmed to 37°C containing 4ml of Krebs Ringer-bicarbonate buffer (pH7.4), 5mM $[\text{U-}^{14}\text{C}]$ glucose ($1\mu\text{Ci/ flask}$) and 20mg/ml of BSA. The flasks were gassed for 5 min before being sealed and left shaking for 1 hour (75 strokes/min). The reactions were stopped by injecting 0.5ml of benzethonium hydroxide (1ml in methanol) into the centre well of each flask, followed by 0.2ml of 5M HCl into the outer compartment. The flasks were shaken for a further hour and the contents of the centre well were rinsed twice with 0.5ml methanol. The washes were added to 10ml Ecoscint A and counted. Duplicate blanks without muscle strips were run to correct for any ^{14}C substances made volatile during the incubation.

2.7.2 Incorporation Of [U-¹⁴C] Glucose Into Glycogen

Glycogen synthesis in isolated soleus strips was measured by the incorporation of [U-¹⁴C] glucose into glycogen using the method of Cuendet *et al*, (1976).

Muscle strips were incubated as described in section 2.4.1 in a final volume of 4ml Krebs Ringer-bicarbonate buffer (pH 7.4) containing 5mM [U-¹⁴C] glucose (1μCi/flask) and 20mg/ml BSA. At the end of the incubation the strips were removed and placed in glass tubes containing 0.5ml of 1M NaOH. After 60 min incubation at 70⁰C, the extracts were cooled down to room temperature and 10mg of carrier glycogen was added. 2ml of 66% ethanol was added to each tube and the glycogen was precipitated overnight at -20⁰C. The precipitate was centrifuged at 2000g_{av} for 10min and washed twice with 2ml of 66% ethanol. The precipitate was dissolved in 1ml of distilled water and 0.5 ml was added to 10ml of Optiphase for scintillation counting.

2.8 MEASUREMENT OF EXOGENOUS AND ENDOGENOUS PALMITATE OXIDATION

2.8.1 Exogenous Palmitate Oxidation

Oxidation of exogenous palmitate by muscle was measured by the formation of ¹⁴CO₂ from [U-¹⁴C] palmitate.

Soleus strips were isolated as described in section 2.4.1. The strips were preincubated in Erlenmeyer flasks in a final volume of 4ml Krebs Ringer-bicarbonate buffer (pH 7.4) containing 5mM glucose, 0.2mM palmitate bound to albumin and 20mg/ml BSA,

The muscles were then transferred to the outer compartments of metabolic flasks as described in section 2.7.1, containing 4ml of Krebs ringer bicarbonate buffer (pH 7.4) with 5mM glucose, 0.2mM [U-¹⁴C] palmitate bound to albumin (0.1μCi/μmol), 20 mg/ml BSA and ± additions. The flasks were gassed for 5 min before being sealed and were left shaking for 1 hour (75 strokes/min). The reactions were stopped by injecting 0.5ml of benzetonium hydroxide (1M in methanol) into the centre well of each flask and 0.32 ml of 60% (w/v) perchloric acid into the outer compartment of the flasks. The flasks were shaken for a further 60 min and the contents of the centre well were then removed and counted for ¹⁴CO₂ as described in section 2.7.1. Parallel blanks without any muscle strips were run to compensate for any [¹⁴C] labelled substances rendered volatile during the incubation.

The acidified medium in the outer compartment was neutralised by the addition of 0.32 ml of 1M triethanolamine and 0.28ml of saturated potassium phosphate. The medium was stored at -20°C and used for the determination of lactate.

2.8.2 Endogenous Palmitate Oxidation

Endogenous palmitate oxidation was measured in the muscle by the rate of formation of ¹⁴CO₂ from [U-¹⁴C] palmitate that had been incorporated into the tissue glycerides during a preincubation.

Muscle strips were isolated as described in section 2.4.1 and preincubated in prewarmed flasks (37°C) containing 4ml of Krebs Ringer bicarbonate buffer (pH 7.4) with 5mM glucose, 0.2mM [U-¹⁴C] palmitate bound to albumin (0.1μCi/μmol) and 20mg/ml BSA.

At the end of the preincubation period the muscle strips were washed in Krebs Ringer - bicarbonate buffer, and incubated as described in section 2.8.1, in a final volume of

4ml of Krebs Ringer-bicarbonate buffer (pH7.4) containing 5mM glucose, 0.2mM palmitate and 20mg/ml BSA.

The reaction was terminated as described in section 2.8.1 and the contents of the centre well were removed and counted for $^{14}\text{CO}_2$ production. The outer medium was neutralised as described in 2.8.1.

2.8.3 LIPID EXTRACTION AND FRACTIONATION

To determine the specific activity of labeled endogenous triglycerides that might act as precursors for endogenous fatty acid oxidation by the muscle strips the following procedure was carried out.

2.8.3.1 Lipid Extraction

Cellular lipids were extracted from muscles using the method of Folch *et al*, (1957) with modifications. Muscle strips were prepared as described in section 2.4.1 and were preincubated as described in section 2.8.2. At the end of the preincubation period the strips were removed, washed in Krebs Ringer-bicarbonate buffer and were homogenised in a hand held glass homogeniser (2-3 strokes) containing 20 volumes of 2:1 chloroform : methanol. The mixture was left for 90 min and sonicated every 10-15 min. The mixture was filtered through Whatman No 1 filter paper and 0.2 volumes of distilled water were added to the filtrate. The mixture was centrifuged for 5 min at $2000g_{av}$, the upper phase was removed and the lower chloroform phase was evaporated to dryness under a stream of O_2 -free N_2 . A small volume of chloroform (100 μl) was added to redissolve the residue of extracted lipids. A small aliquot of the chloroform extract was transferred to scintillation vials and further dried down. 10ml

of Ecosint A was added to the scintillation vial for counting total lipid content. A small, measured amount of the chloroform extract was used for lipid fractionation as described in section 2.8.3.2. The remainder of the chloroform extract was dried down and triacylglycerol content was determined as described in section 2.8.3.3.

2.8.3.2 Lipid Fractionation

Tri-, di- and monoacylglycerols, free fatty acids and phospholipids were separated as described by Crass *et al* (1970). A small, measured amount of the remaining chloroform extract was applied to a silica gel (SIL G) t.l.c. plate and developed in a solvent system of 90:20:3:2 (v/v/v/v) petroleum spirit (b.p. 40-60°C): diethylether: acetic acid: methanol, for 90 min. The t.l.c. plate was air dried and the lipid spots were visualised by staining with I₂ vapour. The spots were identified by comparing them to the standards applied to the t.l.c plate in parallel. The silica gel containing each spot was scraped from the plastic plate and added to 10ml of Ecosint A for scintillation counting.

2.8.3.3 Triacylglycerol

The TAG content of muscles was measured using the Boehringer Kit for TAG determination.

Briefly, TAG is hydrolysed to glycerol which is phosphorylated to glycerol phosphate, releasing ADP as a by-product. Phosphoenolpyruvate in the presence of ADP is dephosphorylated to pyruvate. Lactate is formed from pyruvate in the presence of

lactate dehydrogenase, resulting in oxidation of NADH which can be followed as a decrease in absorbance at 340nm.

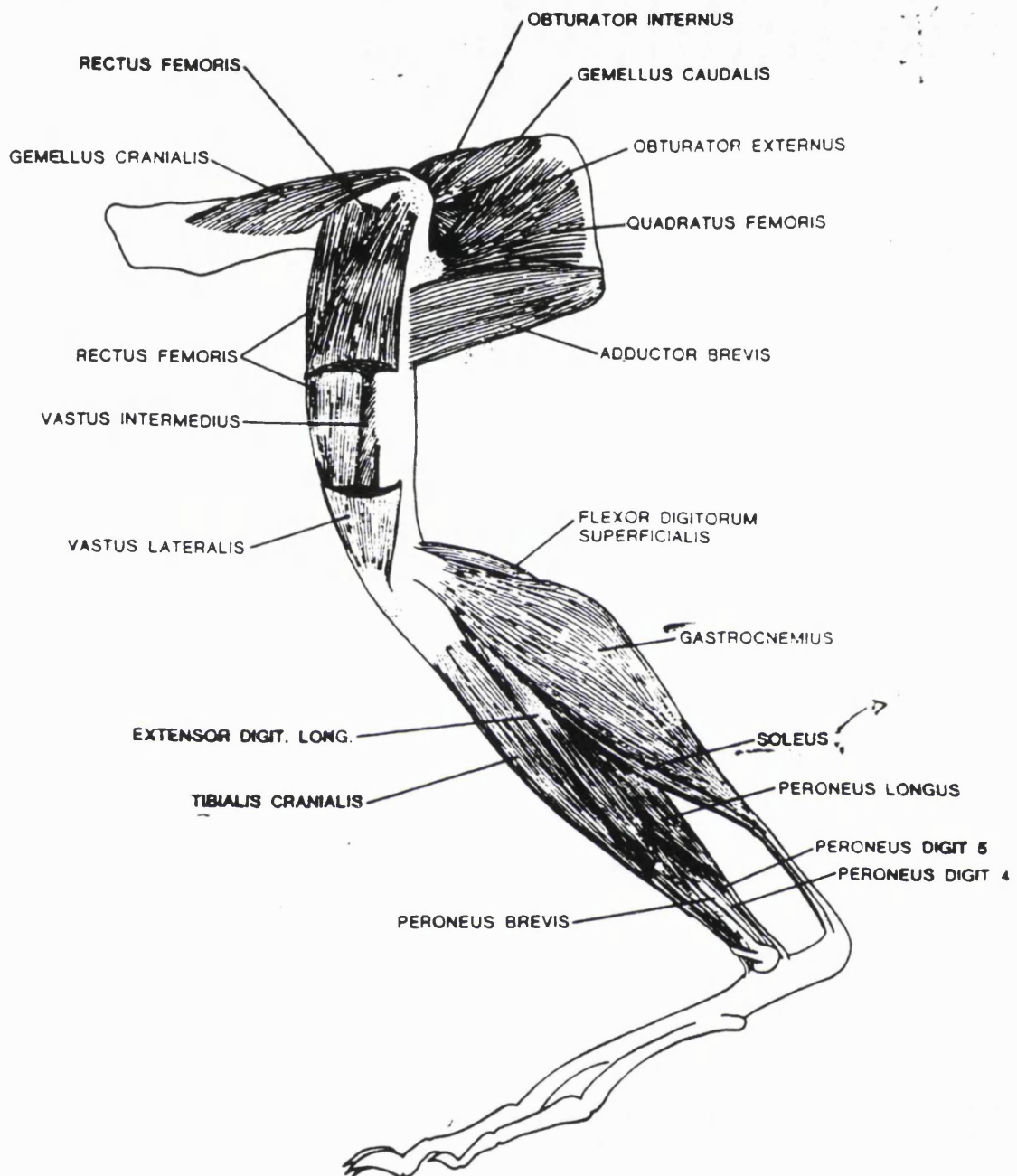
Lipids were extracted from muscle strips as described in section 2.8.3.1. and the final CH_3Cl phase was dried under a stream of O_2 -free N_2 . The residue was resuspended in 1.2ml of Reagent A (74mM Tris-citrate buffer (pH 8.2) containing 30.8mM MgCl_2 , 7.9mM sodium cholate, 2% polyethylene glycol, 0.05mM ATP, 0.3mM phosphoenolpyruvate, 0.25mM NADH, 4U/ml lipase and 0.5U/ml LDH) of the kit and incubated at 26°C for 10 min to hydrolyse TAG. 1ml of the solution was placed in plastic cuvettes and the absorbance was read at 340nm against 1ml of reagent A (blank). 10 μg of glycerol kinase was added and after 10 min the decrease in absorbance was read. The concentration of TAG was measured using the extinction co-efficient of $6.22\mu\text{mol}^{-1}\text{ ml}$ for NADH.

2.9 PREPARATION OF SUBCELLULAR FRACTIONS

2.9.1 Muscle

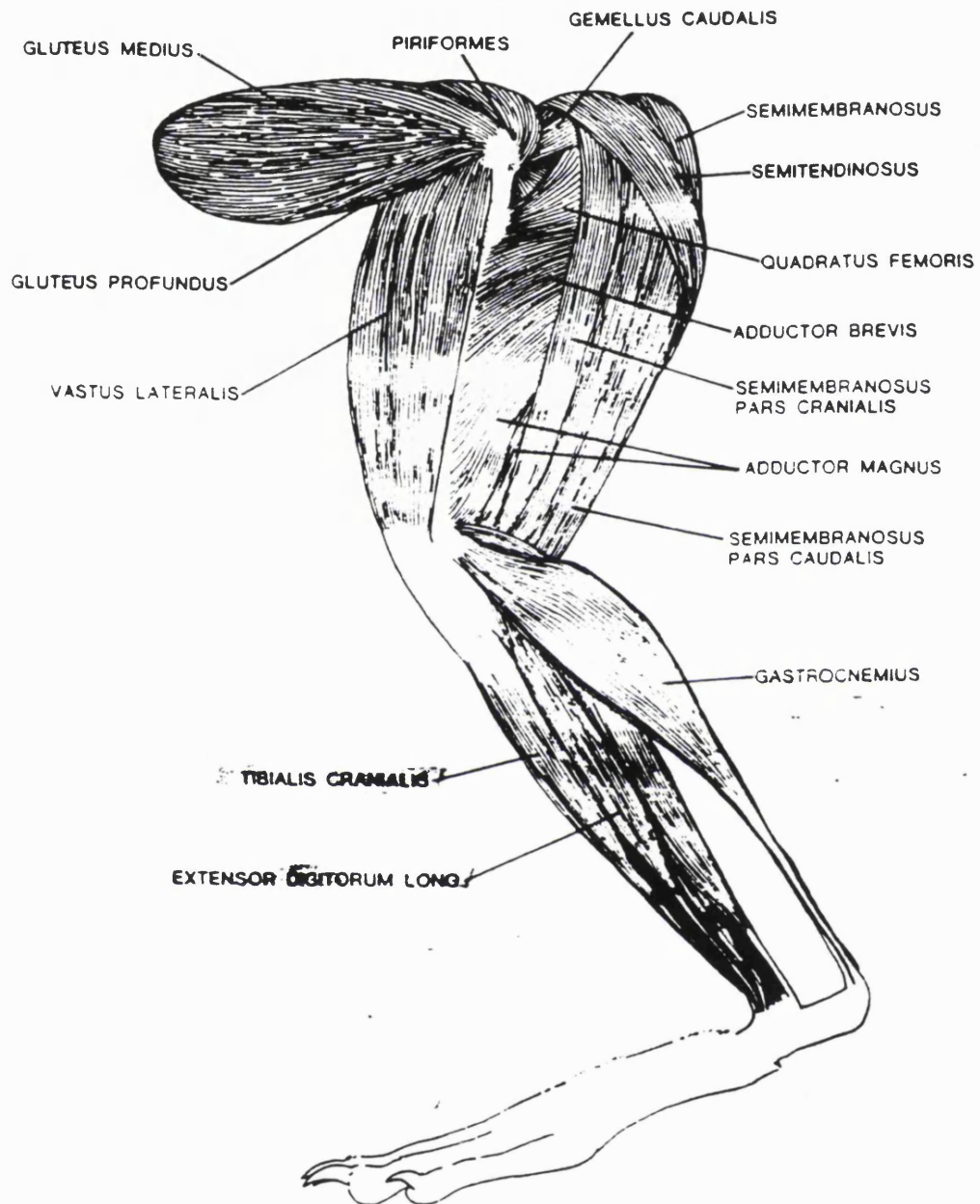
Fig 2.3 and 2.4 shows the location of the muscles used for the preparation of subcellular fractions. The following muscles were used the Soleus (type I), Gastrocnemius (type IIb) and EDL and Tibialis anterior (type IIa)

Rats were killed by cervical dislocation. Muscles were excised, blotted on filter paper, freed from fat and connective tissue and immersed in ice cold 0.15M KCl. The muscles were cut into small pieces and rinsed several times with 0.15M KCl. Cutting was continued until a fine mince was obtained. The minced tissue was rinsed several times with homogenisation medium consisting of 5mM TrisHCl (pH 7.4)



Adpated from the Laboratory Anatomy Series (White Rat)
(Odlaug, *et al*, 1988)

Fig 2.3 Muscles of the hindlimb, Lateral view



Adpated from the Laboratory Anatomy Series (White Rat)
(Odlaug, *et al*, 1988)

Fig 2.4 Deeper muscles of the Hindlimb, Lateral view

buffer containing 0.1M KCl, 5mM MgSO₄, 1mM EDTA and suspended in about 1 volume of the same medium (Ernster & Nordenbrand, 1964). The muscles were homogenised in 10 volumes of buffer using a relatively loose fitting all-glass Potter-Elvehjem (Teflon) homogeniser (radial clearance 0.1mm) for 1-2 min at 0-2°C. The homogenate was then centrifuged at 620g_{av} (Beckman Sorvall RC5-B, SS-34 rotor) for 10 min at 4°C. The supernatant was recentrifuged as above to remove residual myofibrils. The resulting supernatant was centrifuged at 1400g_{av} (Beckman Sorvall RC5-B, SS-34) for 10 min at 4°C. The mitochondrial pellet was resuspended in a small volume of homogenisation medium and centrifuged as above, this washing was repeated only once. The pellet suspended in 0.15M KCl and stored at -70°C.

The post-mitochondrial supernatant was centrifuged at 4°C for 1 hour at 105,000g_{av} (Beckman L8 Ultracentrifuge, 75Ti rotor) and the resulting supernatant (cytosolic fraction) was aliquoted out and stored at -70°C. The microsomal pellet was resuspended in a small volume of homogenising medium and stored at -70°C.

2.9.2 Liver

Rats were sacrificed as above and the liver was removed and placed in ice-cold isolation buffer (10mM Tris-HCl (pH 7.4) containing 0.25M sucrose, 1mM EDTA and 1mM DTT). The liver was minced and washed several times with isolation buffer. The liver was homogenised in 10 volumes of buffer using a Potter-Elvehjem homogeniser (radial clearance 0.19mm) fitted with a motor driven Teflon pestle. The homogenate was centrifuged at 620g_{av} (Beckman Sorvall RC-5B) to remove nuclear debris. The post nuclear supernatant was spun at 7250g_{av} for 10 min at 4°C to yield the mitochondrial pellet. The post mitochondrial supernatant was centrifuged at 4°C

for 1 hour at 105,000g_{av} (Beckman L8 Ultracentrifuge). The resulting supernatant was aliquoted out and stored at -70°C.

2.10 ENZYME ASSAYS

2.10.1 ATP-Citrate Lyase

ATP-Citrate Lyase (EC 4.1.3.8) was assayed spectrophotometrically at 26°C using the method of Denton & Martin (1970) in a 1ml final volume containing 5mM Triethanolamine buffer (pH 7.7), 7.5mM MgCl₂, 7mM ATP, 1mM trisodium citrate, 0.3mM Co-enzyme A, 0.3mM NADH, 1mM DTT, 10µg Malate Dehydrogenase (12U) and 100-200µl of tissue extract. The reaction was initiated by the addition of ATP. The change in absorbance was recorded at 340nm against a blank (minus ATP) on a Unicam SP-8-100 spectrophotometer. Enzyme activity was calculated from the extinction coefficient of 6.22µmol⁻¹ ml for NADH and expressed as nmol/min/g wet. wt.

2.10.2 Acetyl-CoA Carboxylase

Acetyl-CoA Carboxylase (EC 6.4.1.2) activity was measured radiochemically using the method of Holland *et al.*, (1984). The final assay mixture was made up to a final volume of 1ml containing 100mM Tris-HCl (pH 7.2), 2mM MgCl₂, 1.2mM DTT, 20mM NaH¹⁴CO₂ (0.1µCi/µmol), 0.3mM acetyl-CoA, BSA [0.1 % (w/v)] and 4mM ATP. The assays were performed in the presence and absence of 10mM trisodium citrate together with an equimolar concentration of MgCl₂ to compensate for the chelation of Mg²⁺ by citrate.

The reaction was initiated by the addition of 50 μ l of tissue extract and stopped after 20 minutes with 0.2ml HCl (6M) and placed on ice for 5 minutes. The samples were centrifuged for 5 minutes at 2000_{gav}, after which 0.8ml of the acid aqueous phase was evaporated to dryness in scintillation vials with the aid of a hairdryer. 10ml of Ecoscint A was added to each vial for scintillation counting.

2.10.3 Fatty Acid Synthase

2.10.3.1 Spectrophotometric Assay

Fatty acid synthase (EC 2.3.1.85) was assayed spectrophotometrically at 37⁰C using the method of Saggerson & Greenbaum (1970b) with modifications in a 1ml final volume containing 100mM KPO₄ buffer (pH 6.5), 2mM EDTA, 2mM DTT, 0.25mM NADPH, 0.03mM Acetyl-CoA and up to 100-200 μ l of tissue extract. The reaction was initiated by the addition of 0.05mM Malonyl-CoA and a fall in absorbance was measured against a blank (malonyl-CoA omitted) on a Unicam SP-8-100 spectrophotometer.

Enzyme activity was calculated from the extinction coefficient of 6.22 μ mol⁻¹. ml for NADPH and expressed as nmol/min/g wet. wt.

2.10.3.2 Radiochemical Assay

Fatty acid synthase (EC 2.3.1.85) was assayed radiochemically using the method of Carey & Dils (1975), by measuring the incorporation at 37⁰C of [2-¹⁴C] Malonyl-CoA (0.5 μ Ci/ μ mol) into synthesised fatty acids.

The final assay mixture was made up to a volume of 1ml containing 100 mM KPO₄ buffer (pH 6.5), 2 mM EDTA, 2 mM DTT, 0.25 mM NADPH, 0.03 mM Acetyl-CoA

and up to 100 μ l of tissue extract. The reaction was initiated by the addition of 0.05mM [2-¹⁴C] malonyl-CoA (0.025 μ Ci) and stopped after 30 min with 0.75 ml of alcoholic potassium hydroxide 15% (w/v). The glass tubes were transferred to a water bath at 65⁰C for 45 min after which they were put on ice for 5 mins. 1ml of cold 4M HCl was added to each tube to acidify the saponified fats and 3 extractions were made with 2.5 ml of petroleum spirit (b.p. 40-60⁰C). The extractions were pooled, transferred to scintillation vials and left overnight for the solvent to evaporate. 8mls of Ecoscint A was added to the scintillation vials for counting. True fatty acid synthase activity was calculated by subtracting the blanks (NADPH and Acetyl-CoA omitted) from experimental samples.

2.10.4 Malonyl-CoA Decarboxylase

This method measures the rate of formation of acetyl-CoA from malonyl-CoA by coupling it to citrate synthesis.

Malonyl-CoA Decarboxylase (E C 4.1.1.9) was assayed spectrophotometrically at 37⁰C using the method of Svoronos & Kumar (1988) in a 1ml final volume containing 0.1M Tris-HCl buffer (pH8.0), 0.5 mM DTT, 10 mM L-Malate, 0.5 mM NAD⁺, 1.7 Units of malate dehydrogenase and was incubated at 37⁰C for 7 min after which 1.7 Units of citrate synthase were added, mixed and left to stand for a minute. The reaction was initiated by the addition of 0.3mM malonyl-CoA and 100-200 μ l of tissue extract. An increase in absorbance at 340nm was observed after the first 30 seconds and enzyme activity was calculated from the extinction coefficient of 6.22 μ mol⁻¹. ml for NADH and expressed as nmol/min/g wet. wt.

2.10.5 Citrate Synthase

Citrate synthase was assayed spectrophotometrically at 25⁰C by the method of Shepherd and Garland (1969), in a 1ml final volume which contained the following 100mM Tris-HCl buffer (pH 8.0), 0.1% Triton X100, 0.1mM 5,5 Dithiobis (2 nitrobenzoic acid) 50μM Acetyl-CoA and 10-40μl of sample. The reaction was started by the addition of 50μM oxaloacetate and an increase in absorbance was measured at 412 nm against a blank (oxaloacetate omitted) on a Shimadzu UV-2101 PC Spectrophotometer. Enzyme activity was calculated from the extinction coefficient of 13.6μmol⁻¹. ml for DTNB and expressed as nmol/min/g wet. wt.

2.10.6 Malonyl-CoA Deacylase

Malonyl-CoA deacylase was assayed spectrophotometrically at 37⁰C in final volume of 1ml containing 50mM potassium phosphate buffer (pH 7.5), 20μM 4,4'-dithiopyridine and tissue extract. The reaction was initiated by the addition of 300μM malonyl-CoA and the increase in absorbance was measured at 324nm. Enzyme activity was calculated from the extinction coefficient of 19.44μmol⁻¹. ml for DTDP and expressed as nmol/min/g wet wt.

2.11 MEASUREMENT OF PROTEIN CONCENTRATION

Protein concentration of the mitochondrial and cytosolic fraction was determined using the Bicinchoninic acid protein assay kit obtained from Sigma.

The protein determination reagent was prepared by adding 1 part copper (II) sulphate pentahydrate 4 % solution to 50 parts bicinchoninic acid solution as provided in the kit.

1ml of the protein determination reagent was added to 50 μ l of sample and standards (fatty acid-poor albumin used as protein standard). The tubes were vortexed and incubated at 37⁰C for 30 min. The tubes were cooled to room temperature and the absorbance was read at 562nm on a Shimadzu UV-2101 PC Spectrophometer. The protein concentration of the samples were determined from the linear standard curve obtained.

2.12 SCINTILLATION COUNTING

The radioactivity in assays was measured on a Packard Tri-Carb 1900 CA analyser (Canberra Packard, Pangbourne, Berks., U.K.) which had been pre-programmed for counting ³H and ¹⁴C isotopes. The counter was equipped with a computer which stored the quench curves for ³H and ¹⁴C and enabled direct printing of radioactivity in disintegrations per minute. (DPM).

2.13 STATISTICAL METHODS

Statistical significance between different populations and within the same populations were evaluated by using Student's t-test respectively for paired or unpaired samples as appropriate. Values in Tables are given as means \pm S.E.M. The n values quoted throughout refer to the number of separate observations.

Chapter Three

Results & Discussion

3.1

Enzymes Involved In The Synthesis Of Malonyl-CoA In **Skeletal Muscle**

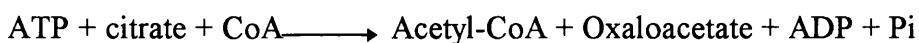
3.1 THE SYNTHESIS OF MALONYL-COA IN SKELETAL MUSCLE

Cytosolic enzymes involved in malonyl-CoA synthesis were measured in the following muscle types : Type I slow twitch red (e.g Soleus), Type IIa fast twitch red (e.g Extensor digitorum longus (EDL) & Tibialis anterior) and Type IIb fast twitch white (e.g. gastrocnemius). These muscles are located in the hindlimb of rats as shown in fig 2.3 & 2.4. Table 1.1 summarises the characteristics of the three types of skeletal muscle fibres.

Measurements of the enzymes were made in 100,000 x g supernatants from skeletal muscle homogenates and were compared between each skeletal muscle type.

3.1.1 ATP-Citrate Lyase

ATP-citrate lyase is the enzyme that catalyses the formation of acetyl-CoA from citrate in the cytosol. The reason being, acetyl-CoA is generated in the mitochondria by oxidative decarboxylation of pyruvate catalysed by PDH. Acetyl-CoA is a substrate for ACC the enzyme that synthesizes malonyl-CoA in the cytosol. Acetyl-CoA cannot directly enter the cytosol as the mitochondrial membrane is impermeable to it. Acetyl-CoA groups can enter the cytosol in the form of citrate via the tricarboxylate transport system. Citrate is converted back to acetyl-CoA by ATP-citrate lyase as shown in the reaction sequence shown below.



ATP-citrate lyase was measured spectrophotometrically in 100,000 x g supernatants. ATP-citrate lyase activity was found in all three skeletal muscle types (table 3.1). The

Table 3.1

**ATP-Citrate Lyase Activity In Different Skeletal Muscle
Fibre Types**

Muscle	Activity (nmol/min/g wet wt of muscle)
EDL (Type IIa)	2.7 ±0.82
Gastrocnemius (Type IIb)	9.2 ±2.2 ^B
Soleus (Type I)	3.1 ±0.5
T.anterior (Type IIa)	9.5 ±1.5 ^A

100,000 x g supernatant of skeletal muscle preparations were measured for ATP-Citrate Lyase activity at 26⁰C, as described in Materials and Methods (section 2.10.1.). The values are means ± SEM for 4 measurements.

^A indicate P<0.01 versus EDL and Soleus muscle.

^B indicate P<0.05 versus EDL and Soleus muscle.

tibialis anterior (type IIa) and gastrocnemius muscle (type IIb), were found to have the highest ATP-citrate lyase activities which were significantly different from those found in the soleus (type I) and EDL (type IIa) muscles.

Tibialis anterior and EDL muscles are both examples of a type IIa muscle and the ATP-citrate lyase activity in these two muscles were significantly different (table 3.1). The ATP-citrate lyase activity found in the EDL muscle was similar to that found in the soleus muscle, which is an example of a type I muscle.

3.1.2 Acetyl-CoA Carboxylase

Acetyl-CoA carboxylase (ACC) is the only enzyme known to be involved in the synthesis of malonyl-CoA in the cytosol.

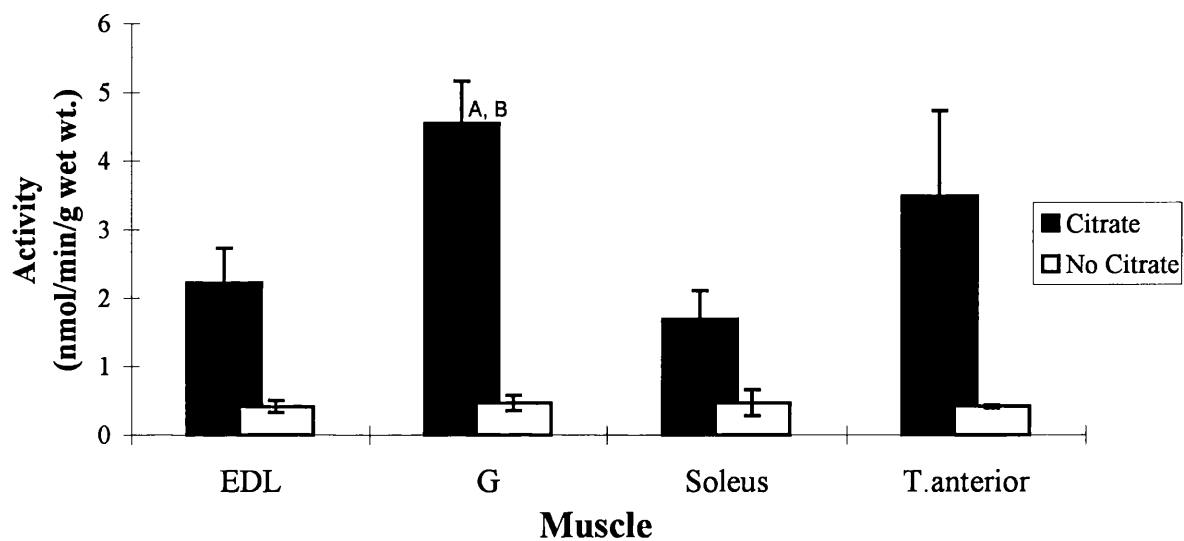
The total carboxylase activity was measured in all muscle types, by the incorporation of $\text{NaH}^{14}\text{CO}_3$ into acid-soluble products as described in section 1.10.2. These measurements will give an indication whether skeletal muscles synthesize malonyl-CoA.

In all the skeletal muscle types studied, $^{14}\text{CO}_2$ fixation dependent upon acetyl-CoA was observed in 100,000 x g supernatants.

ACC activity was measured in the presence and absence of citrate. Only small amounts of carboxylase activity was detected in the absence of citrate in all muscle types (fig 3.1). In the presence of citrate (10mM), ACC activity in all muscle types increased. This suggests that citrate activates ACC activity in muscles (fig 3.1). ACC activity in the presence of citrate, was found to be the greatest in the gastrocnemius muscle (type IIb) and the lowest activity was found in the soleus (type I) muscle. The ACC activity in the presence of citrate was compared between the two type IIa

Fig 3.1

Citrate Activates Acetyl-CoA Carboxylase Activity in Muscle



100,000 x g supernatant of skeletal muscle extracts were measured for ACC activity in the presence and absence of citrate (10mM) as described in Materials and Methods (section 2.10.2).

The values are means \pm SEM for 4 measurements.

^{A, B} indicate $P < 0.05$, < 0.01 versus EDL & Soleus muscle, respectively.

muscles (EDL and tibialis anterior) and the EDL muscle was found to have a lower ACC activity in the presence of citrate (fig 3.1).

Interestingly the soleus muscle and the EDL muscle had similar ACC activity in the presence of citrate and these two muscles also had similar activities of ATP-citrate lyase (table 3.1).

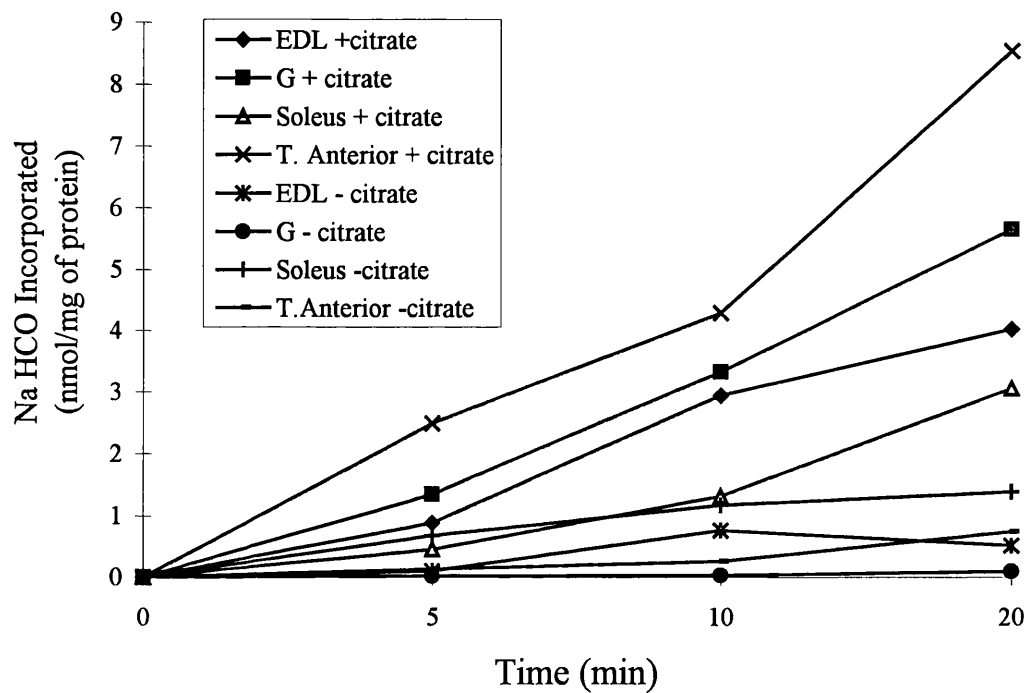
The ACC activity was linear for over 10 minutes for all the muscle types in the presence of citrate (fig 3.2).

Alternatively ACC activity was expressed as the *citrate-dependent carboxylase activity* as shown in Table 3.2 (page 111). The citrate dependent activity was calculated by subtracting the activity of ACC in the absence of citrate from that obtained in the presence of excess citrate. In all four muscles acetyl-CoA dependent bicarbonate fixation by the 100,000 g supernatant was shown to be highly citrate-dependent. This is consistent with the previous findings of ACC extracted from rat skeletal muscle (Trumble *et al*, 1995, Vavvas, *et al*, 1997, Winder & Hardie, 1996 and Hutber *et al*, 1997).

A small amount of citrate-independent carboxylase activity was present in all muscle fibre types and the activity was found to be similar in all four muscles. This citrate-independent carboxylase could be due to the mitochondrial propionyl-CoA carboxylase released from damaged mitochondria, into the 100,000 x g supernatant during subcellular fractionation preparation. Acetyl-CoA is known to be a substrate for propionyl-CoA carboxylase and unlike ACC is not activated by citrate (Martin & Vagelos, 1962 and Trumble *et al*, 1995). Therefore the citrate-independent carboxylase activity measured is a measure of propionyl-CoA carboxylase activity present in the cytosolic fraction as a result of mitochondria disruption.

Fig 3.2

**Time Course For ACC Activity In Different Skeletal
Muscle Fibre Types**



100,000 x g supernatants of skeletal muscle preparations were incubated with 20mM NaH¹⁴CO₃ in the presence and absence of citrate (10mM). The values are from a single experiment.

This citrate-dependent activity expressed in table 3.2, represents a minimal estimate (or under estimate) of the true ACC activity. This is because at the time of muscle dissection and extraction, ACC may have different degrees of phosphorylation in the different muscles and as protein phosphatase inhibitors were not included in the extraction buffer, this could result in affecting the *true* measurement of ACC activity. The citrate-dependent ACC values in table 3.2 are therefore minimum estimates although, since protein phosphatase inhibitors were not added in the extraction buffer there is a possibility that the most of the ACC activities measured largely reflect the dephosphorylated state.

When expressed as *per g of tissue*, the highest citrate-dependent carboxylase activity was found in the gastrocnemius muscle (type IIb) and the lowest citrate-dependent carboxylase activity was found in the soleus muscle (type I). An intermediate citrate-dependent carboxylase activity was found in the type IIa muscles (EDL and tibialis anterior). These differences in citrate-dependent carboxylase activity between the different types of muscles types largely disappeared when expressed relative to the soluble protein (Table 3.2). The reason being the amount of protein extracted from 1 g of soleus, EDL or tibialis anterior was quite similar, whilst approximately twice as much of protein was extracted from 1 g of gastrocnemius muscle. This is probably due to type IIb muscles (gastrocnemius) consisting of relatively large amounts of cytosolic (i.e glycolytic) enzymes.

From comparing the ratio of citrate-dependent carboxylase activity/citrate-independent carboxylase activity, it can be seen that the gastrocnemius and the tibialis anterior muscle has a higher ratio compared with the EDL and soleus muscles. This suggests that the ACC activity in type IIb muscle (gastrocnemius) and at least tibialis anterior is highly

Table 3.2

Acetyl-CoA Carboxylase Measurements in Different Muscle Fibre Types
(nmol/min/gram wet wt of muscle)

Muscle (n=4)	+ Citrate	- Citrate	Citrate-dependent activity (C _A)	Citrate dep/Citrate indep
EDL (Type IIa)	2.2 ±0.5	0.41 ±0.09	1.8 ±0.44 (296 ± 29)	4.4
Gastrocnemius (Type IIb)	4.5 ±0.6 ^{A, B}	0.46 ±0.11	4.0±0.60 ^C (260 ± 10)	8.7
Soleus (Type I)	1.6 ±0.4	0.46 ±0.19	1.2 ±0.25 (181 ± 42)	2.6
T. anterior (Type IIa)	3.4 ±1.2	0.41 ±0.02	2.9 ±1.11 (354 ± 61)	7.1

Carboxylase activity was measured at 37°C in 100,000g muscle supernatants as described in Materials & Methods (section 2.10.2) with and without citrate (10mM). Values are means ± S.E.M (n=4). The values in () are activities expressed as nmol/min/mg of protein.

The citrate-dependent activity (C_A) is a minimal estimate of the acetyl-CoA carboxylase activity present.

^{A, B} indicate P<0.05 <0.02 versus EDL & Soleus muscle, respectively.

^C indicate P< 0.05 versus EDL muscle.

citrate-dependent (irrespective of phosphorylation state) and citrate-independent carboxylase activity measured is most likely to be propionyl-CoA carboxylase.

Finally, it could be suggested that differences in citrate-dependent activity between the different muscle types may be due to absolute differences in ACC activity(i.e.

abundance of ACC protein)or differences in the phosphorylation states. The second possibility is difficult to evaluate as the muscle extracts were prepared in the absence of protein phosphatase inhibitors. However there is a good possibility that most of the ACC measured is in the dephosphorylated form.

It should be noted that ACC activity cannot be described as being more citrate dependent in some tissues, if one is to assume citrate independent activity is due solely to propionyl-CoA carboxylase. Both of the above explanations are possible, however experiments have not been carried out to explore which is true.

Conclusions

From these studies the following points emerge;

- ATP-citrate lyase activity was present in each of the muscle types studied and the activities within the different muscle types were in some cases significantly different.
- ACC activity was found to be present in each of the muscle types studied.
- In the presence of citrate, ACC activity was increased. The effect of citrate on ACC activity is more pronounced in the gastrocnemius (type IIb) and tibialis anterior (type IIa) muscles than in EDL (type IIa) or in soleus (type I) muscles.
- ACC activity in all four muscles was shown to be citrate-dependent which is consistent with recent studies of ACC extracted from rat skeletal muscle (Hutber *et al*, 1997, Vassa *et al*, 1997 and Winder *et al*, 1997).
- Differences in ATP-citrate lyase and ACC activity were shown to exist between the different types of muscles studied. These variations seen between the different types of muscles studied could be due to differences in the metabolic function of the muscles.
- This work clearly demonstrates that skeletal muscles are capable of synthesizing malonyl-CoA from acetyl-CoA by ACC and ATP-citrate lyase. Skeletal muscles are not considered to be lipogenic, but from these studies it suggests that skeletal muscles are able to produce malonyl-CoA.. It will be interesting to know the possible routes of disposal of malonyl-CoA in skeletal muscles, as discussed in the next section.

3.2

Enzymes Involved In The Disposal Of Malonyl-CoA In **Skeletal Muscle**

3.2 DISPOSAL OF MALONYL-CoA IN SKELETAL MUSCLE

Acetyl-CoA produced by ATP-Citrate lyase in the cytosol is carboxylated by ACC to malonyl-CoA. This provides a route for the synthesis of malonyl-CoA in skeletal muscle. The question that then arises is the route of disposal of malonyl-CoA. Several routes of disposal of malonyl-CoA in skeletal muscle are possible such as;

- a) Fatty acid synthesis - malonyl-CoA can be converted into long chain fatty acids by fatty acid synthase.
- b) Decarboxylation of malonyl-CoA to acetyl-CoA by malonyl-CoA decarboxylase
- c) Deacylation of malonyl-CoA to malonate by malonyl-CoA deacylase
- d) Use by the fatty acid elongation system

a-c of the above has been investigated in the three types of muscle and the results are discussed below.

3.2.1 Fatty Acid Synthase

Lipogenic tissues such as liver, adipose and mammary gland have a well-defined cytosolic fatty acid synthesis pathway which is able to utilise the malonyl-CoA produced in their cells (Wakil, 1961). The coenzyme NADPH and the substrate acetyl-CoA is essential for *de novo* fatty acid synthesis to occur in lipogenic tissues (Bressler & Wakil, 1961).

Fatty acid synthase activity can be assayed spectrophotometrically or radiochemically as described in the Materials and Methods (section 2.10.3). The radiochemical method is 10 times more sensitive in detecting fatty acid synthase activity than the spectrophotometric method. Using the spectrophotometric assay, fatty acid synthase activity was not detectable in 100,000 x g supernatants from skeletal muscle (table

Table 3.3**Fatty Acid Synthase Activity In Muscles****(pmol/min/g wet wt.)**

Muscle	Spectrophotometric Assay (n=2)	Radiochemical Assay (n=4)
EDL (type IIa)	n.d	434 ± 55
Gastrocnemius (type IIb)	n.d	421 ± 81
Soleus (type I)	n.d	309 ± 95
T.anterior (type IIa)	n.d	800 ± 25

100,000 x g supernatants from skeletal muscle were measured for fatty acid synthase activity using the spectrophotometric assay and the radiochemical assay, as described in the Materials and Methods (section 2.10.3).

The values are means ± SEM (n=4).

n.d = not detected

3.3). The reason being the lower detection limit for the spectrophotometric assay is approximately 100 pmol/min. In contrast, the detection limit for the sensitive radiochemical assay is approximately 2-3 pmol/min.

In the radiochemical assay, the incorporation of [2-¹⁴C] malonyl-CoA into petroleum spirit-soluble products, was demonstrated in the 100,000 x g supernatant from each of the muscle types (type I, type IIa and IIb). The activity was approximately linear for over 30 minutes for all the muscle types (fig 3.3).

Table 3.3 shows fatty acid synthase activity in different skeletal muscle fibre types. The highest activity was found in the Tibialis anterior muscle. The activity measured for the EDL, soleus and gastrocnemius muscle were similar.

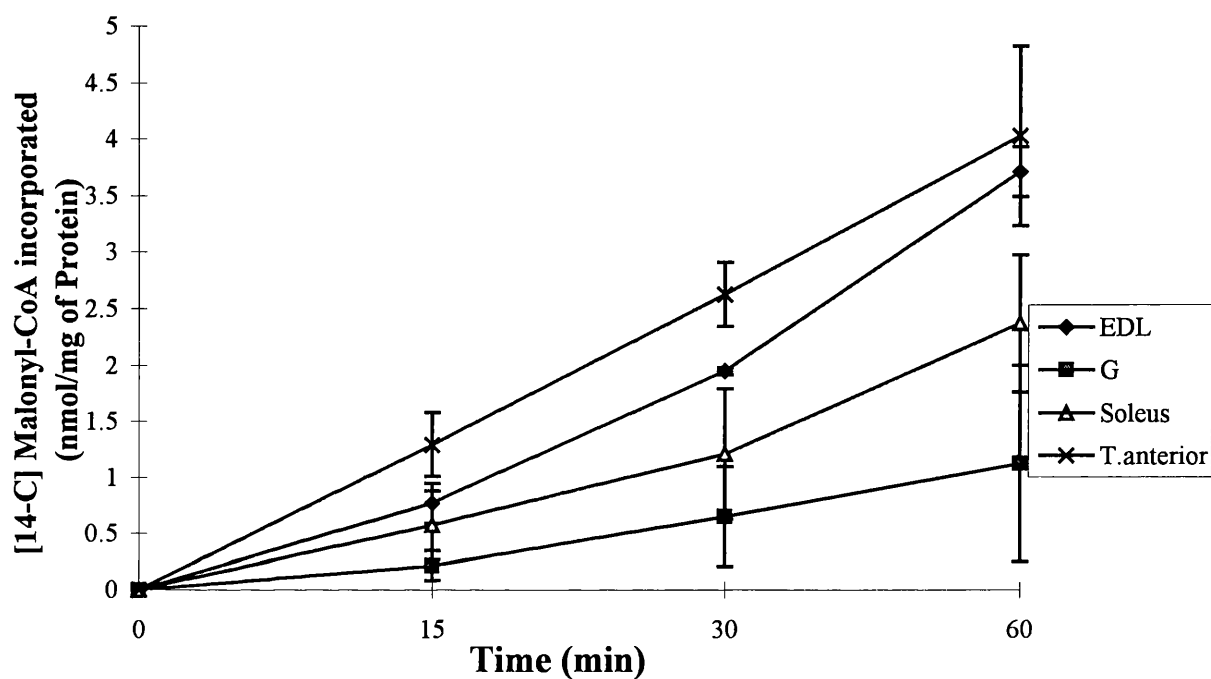
When expressed as *per mg of protein* (fig 3.4) the highest activity was found in extracts from the tibialis anterior muscle (type IIa) and the lowest activity was found in extracts from the gastrocnemius muscle (type IIb).

The fatty acid synthase activity in tibialis anterior extracts was found to be higher than that found in the EDL extracts, but the two activities are not significantly different from each other (fig 3.4).

Fatty acid synthase activities in type IIb and type I muscle fibres (gastrocnemius and soleus muscles) were found to be significantly lower than those activities found in type IIa muscles (tibialis anterior and EDL) (fig 3.4).

Fig 3.3

Time Course For Fatty Acid Synthase Activity In Muscles

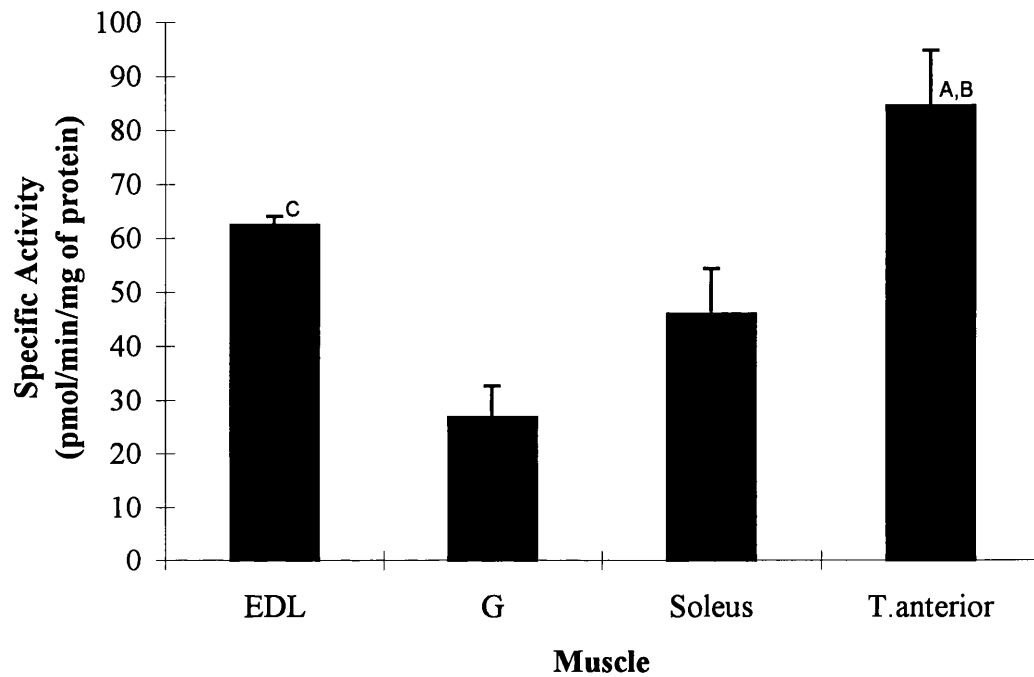


100,000 x g supernatants from skeletal muscle extracts were assayed using the radiochemical assay, as described in the Materials & Methods (section 2.10.3.2).

Values are expressed as a range of values for 2 experiments.

Fig 3.4

Fatty Acid Synthase Activity In Muscles



100,000 x g supernatant of skeletal muscle extracts were incubated with 50 μ M [2-¹⁴C] malonyl-CoA at 37°C for 30 min.

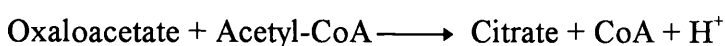
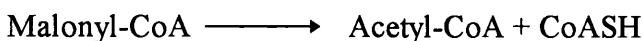
The results are expressed as means \pm SEM (n=4).

A, B indicate $P < 0.02, < 0.01$ versus soleus & gastrocnemius muscles, respectively.

C indicate $P < 0.01$ versus gastrocnemius muscle.

3.2.2 Malonyl-CoA Decarboxylase (MDC)

Malonyl-CoA decarboxylase is the enzyme involved in decarboxylation of malonyl-CoA to acetyl-CoA and is highly specific for malonyl-CoA. The assay reaction sequence is shown below.



(Kim & Kollatukudy, 1978 a & b)

Malonyl-CoA decarboxylase has been identified in a variety of tissues such as liver, heart, mammary gland and the uropygial gland in duck and goose (Scholte, 1973, Kim & Kolattukudy, 1978a, b & c and Buckner & Kolattukudy 1975). Malonyl-CoA decarboxylase is reported as being localised in the mitochondrial matrix of rat liver, guinea pig and rat heart (Scholte, 1969). In uropygial gland malonyl-CoA decarboxylase is located in the cytoplasm (Kim & Kolattukudy, 1978b).

The function of malonyl-CoA decarboxylase in mammalian tissue is obscure, and several roles for the enzyme in mammalian tissue have been suggested (Kim & Kolattukudy, 1978 c). Malonyl-CoA decarboxylase could be involved in regulating the chain length of fatty acids by controlling the [acetyl-CoA]:[malonyl-CoA] ratio in mammary gland. The mammary gland is known to generate large amounts of short chain fatty acids (< C₁₆), a high [acetyl-CoA]:[malonyl-CoA] ratio favours synthesis of short chain fatty acids. This function appears unlikely if decarboxylation of malonyl-CoA occurs only in the mitochondrial matrix and as yet a carrier system has

not been identified for transporting malonyl-CoA to the mitochondria from the cytoplasm (Kim & Kollatukudy, 1978a & c). However Kim & Kollatukudy, (1978a

& c) suggested that malonyl-CoA decarboxylase is involved in protecting mitochondrial enzymes (e.g. propionyl-CoA carboxylase) which are susceptible to inhibition by malonyl-CoA. This function seems highly unlikely, as mentioned above as yet no malonyl-CoA transport mechanism has been identified which transports malonyl-CoA to the mitochondria from the cytosol.

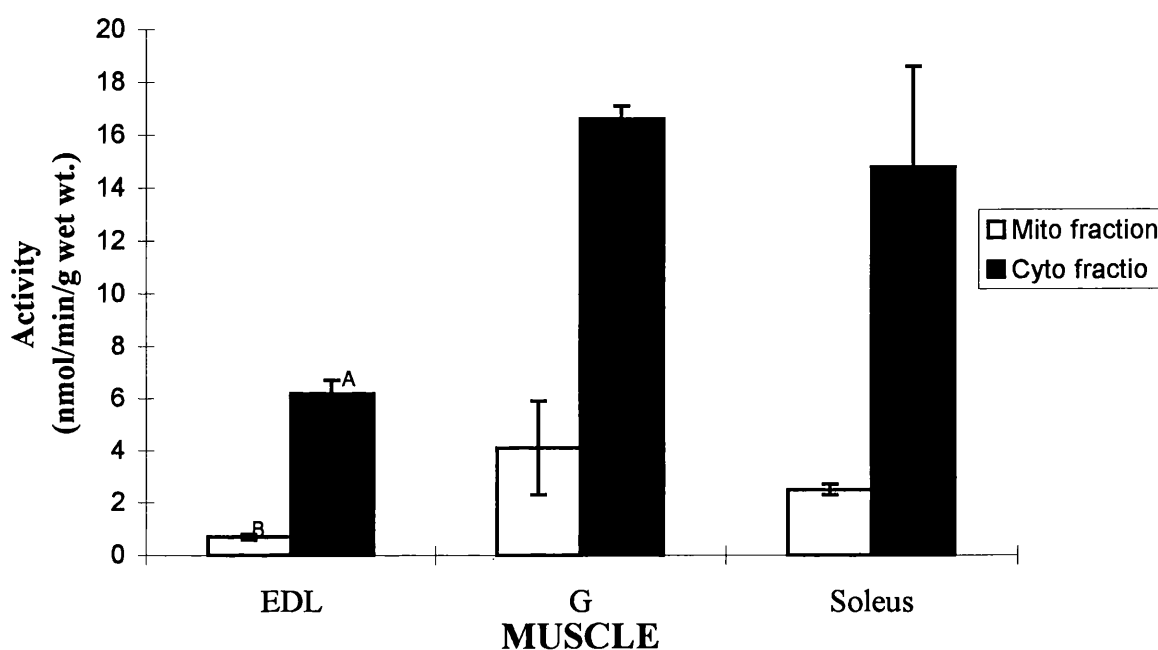
Uropygial gland synthesises large amounts of multi-methyl-branched fatty acids from methyl-malonyl-CoA. The fatty acid synthase in uropygial gland favours the production of unbranched fatty acids. However the natural occurring fatty acids present in uropygial glands are multi-methyl-branched fatty acids. Therefore, malonyl-CoA decarboxylase in the uropygial gland can ensure that methyl-malonyl-CoA is the only substrate available for fatty acid synthase to produce multi-methyl-branched fatty acids (Buckner & Kollatukudy, 1975).

Malonyl-CoA decarboxylase was assayed spectrophotomerically in both the mitochondrial and cytosolic fractions in all skeletal muscle types. Freeze thawed mitochondria prepared from skeletal muscle were assayed for malonyl-CoA decarboxylase in the presence of rotenone, an inhibitor of the NADH reductase. This was to ensure that the NADH formed in the assay was not oxidised back to NAD via the NADH Q reductase complex of the respiratory chain.

Malonyl-CoA decarboxylase activity was demonstrated in both the crude mitochondrial and the crude cytosolic fractions in all muscle types (Fig 3.5). The highest activity for malonyl-CoA decarboxylase in both the cytosolic and the mitochondrial fraction was found in extracts from the gastrocnemius muscle. The soleus muscle was found to exhibit an intermediate activity for malonyl-CoA

Fig 3.5

**Malonyl-CoA Decarboxylase Activity In Cytosolic
And Mitochondrial Fractions In Muscles**



Crude cytosolic and mitochondrial fractions of muscles were measured for malonyl-CoA decarboxylase activity, as described in the Materials and Methods (section 2.10.4).

Values are expressed as means \pm SEM. n = 4 for EDL muscle, n =3 for gastrocnemius and soleus muscle.

^{A, B} indicate $P < 0.001$ versus soleus muscle.

decarboxylase in both fractions. The lowest activity for malonyl-CoA decarboxylase in both fractions was found in the EDL muscle.

Considerable citrate synthase (CS) activity was found in the crude cytosolic fraction indicating extensive breakage of mitochondria during homogenisation of muscle samples (table 3.4). This complicates the estimation of any *true* cytosolic activity of MDC. However the activity ratio of MDC (cytosolic fraction)/CS (cytosolic fraction) is considerably higher than the activity ratio of MDC (mitochondria fraction)/CS (mitochondria fraction) in type IIa (EDL) and type I (soleus) muscles. This suggests the existence of some *true* cytosolic MDC activity in these muscles, despite the occurrence of extensive mitochondrial damage during homogenisation of tissue sample and subsequent release of CS. However the activity ratios of MDC/CS in both the crude cytosolic fraction and in the crude mitochondrial fraction in the type IIb (gastrocnemius) muscle were similar to each other (Table 3.4). It can be suggested that the MDC activity found in the cytosolic fraction in the gastrocnemius muscle is solely due to mitochondrial breakage during the preparation of mitochondria from the gastrocnemius muscle. An attempt was made to calculate the *true* distribution of MDC in the cytosolic and mitochondrial fraction based on the following relationships.

$$\frac{\text{Leaked MDC}}{\text{Leaked CS}} = \frac{\text{MDC activity measured in the mitochondrial fraction}}{\text{CS activity measured in the mitochondrial fraction}} \text{ -----1}$$

From 1

$$\text{Leaked MDC} = \text{Leaked CS} \times \frac{\text{measured mitochondrial MDC}}{\text{measured mitochondrial CS}} \text{ -----2}$$

From which *true* cytosolic and mitochondrial malonyl-CoA decarboxylase activity can be calculated using the equations on page 125.

Table 3.4

Malonyl-CoA Decarboxylase Activity
(nmol/min/g wet wt of muscle)

Muscle	Measured cytosolic MDC activity (A)	Measured mitochondrial MDC activity (B)	Measured CS (cytosolic) (C)	Measured CS (mitochondria) (D)	Ratio A/C (x1000)	Ratio B/D (x 1000)
EDL (type IIa)	6.2 ± 0.11^A	0.70 ± 0.11^A	963 ± 130	397 ± 60	6.43	1.76
Gastrocnemius (type IIb)	16.6 ± 0.5	4.1 ± 1.8	2138 ± 293	829 ± 198	7.76	4.95
Soleus (type I)	14.8 ± 3.8	2.5 ± 0.2	1126 ± 371	1310 ± 253	11.30	2.22

Malonyl-CoA decarboxylase activity was measured at 37°C in the crude cytosolic and the mitochondrial fractions as described in Materials & Methods (section 2.10.4). Values are means \pm SEM. n = 4 for EDL muscle. n = 3 for gastrocnemius and soleus muscle.

MDC is the measured malonyl-CoA decarboxylase activity. CS is the measured citrate synthase activity.

^A indicate P<0.001 versus soleus muscle.

$$\text{True cytosolic MDC} = \text{measured cytosolic MDC} - \text{leaked MDC} \quad \text{----- 3}$$

$$\text{True mito MDC} = \text{measured mitochondrial MDC} + \text{leaked MDC} \quad \text{----- 4}$$

Table 3.5 shows the calculated *true* MDC activity in both the cytosolic and mitochondrial fractions. The ratios of *true* mitochondrial MDC activity/*true* cytosolic malonyl-CoA decarboxylase activity were calculated as shown in table 3.5. The ratio varied appreciably between the different muscle types (Table 3.5). The gastrocnemius muscle had the highest activity ratio in comparison to the EDL and soleus muscle. This ratio suggests that a large proportion of MDC activity measured in the gastrocnemius muscle is from the mitochondria and a smaller proportion of mitochondrial MDC activity is present in the soleus and EDL muscle.

The *true* cytosolic MDC activity was compared against the fatty acid synthase activity (expressed as pmol/g wet wt.) (section 3.2.1). The *true* cytosolic activity in all muscle types studies was considerably greater than fatty acid synthase by factors of 10-, 17- and 38-fold in EDL, gastrocnemius and soleus muscles respectively. It can be suggested that MDC is a possible route for the disposal of malonyl-CoA that is formed by ACC in muscle cytosol.

3.2.3 Malonyl-CoA deacylase

Malonyl-CoA deacylase was assayed spectrophotometrically at 342nm in all skeletal muscle types. The activity measured was not consistent from one skeletal muscle subcellular fractionation preparation to the next. This made the data unreliable and so no valid results or conclusions could be derived.

Table 3.5

True Malonyl-CoA Decarboxylase Activity
(nmol/min/gram wet wt of muscle)

Muscle	n	<i>True</i> Mitochondrial activity (A)	True cytosolic activity (B)	Ratio A/B
EDL	4	2.4 ± 0.4	4.5 ± 0.5	0.53
Gastrocnemius	3	13.43 ± 2.3	7.3 ± 0.4	1.84
Soleus	3	5.6 ± 0.1	11.7 ± 3.7	0.48

True malonyl-CoA decarboxylase activity was calculated as described in section 3.2.3

Conclusion

On the basis of this study the following can be deduced regarding malonyl-CoA disposal in skeletal muscles.

- Firstly, there is evidence for a cytosolic MDC activity in the muscles types examined. Secondly, a very low activity of fatty acid synthase was detected in all muscle types studied. It is not known whether this fatty acid synthase activity originated entirely from skeletal muscle fibres or could have come from other cell types e.g. adipocytes. Whatever the source of the fatty acid synthase the data clearly shows that it is substantially lower than the calculated *true* cytosolic activity of MDC. Thus the majority of malonyl-CoA disposal in skeletal muscle is likely to be via this latter route.
- Apart from MDC being the main possible route of disposal of malonyl-CoA in muscle, MDC could be suggested to play an important role in oxidative muscles (soleus) during certain physiological conditions e.g. exercise, when fatty acids are the preferred source of fuel. CPT 1 and CPT 2 are involved in the transport of fatty acids to the mitochondrial matrix, where they undergo β -oxidation (section 1.5.1). CPT 1 activity in non-lipogenic tissues e.g. heart and skeletal muscle is inhibited by low concentrations of malonyl-CoA, thus suppressing fatty acid oxidation. During prolonged exercise, fatty acids are the preferred substrates providing energy to the continuously contracting muscle. Malonyl-CoA decarboxylase activity during exercise could enhance oxidation of fatty acids by converting malonyl-CoA (by decarboxylation) to acetyl-CoA, thus relieving inhibition of CPT 1. Further experiments are needed to investigate the full role and understand the regulation (in the short term) of cytosolic MDC in muscle.

3.3

Measurements Of Metabolite Intermediates In **Incubated Soleus Muscle**

3.3 ACUTE REGULATION OF FATTY ACID METABOLITE LEVELS IN

ISOLATED SOLEUS MUSCLES

In section 3.1. the potential for malonyl-CoA synthesis was demonstrated in skeletal muscles. Skeletal muscles are not considered to be lipogenic, thus the presence of malonyl-CoA in these tissues is intriguing.

Ruderman's group, proposed that malonyl-CoA is part of a fuel sensing mechanism and its level responds acutely to changes in fuel supply and energy expenditure in muscle (section 1.7.2). During intense exercise or starvation, fatty acids are the preferred substrates providing energy to the muscle and their oxidation is known to inhibit glucose oxidation (section 1.7.1). Suppression of glucose oxidation by fatty acids, is clearly not one sided, as under certain conditions carbohydrate fuels can suppress fatty acid oxidation in muscle (Kelley *et al*, 1990 and Sidossis & Wolfe, 1996). The mechanism by which carbohydrate-mediated suppression of fatty acid utilisation occurs in muscle is thought to involve the malonyl-CoA/CPT 1 interaction (section 1.7.2). According to Ruderman's group, in the presence of insulin and glucose, there is an increase in the formation of malonyl-CoA by insulin increasing the supply of cytosolic acetyl-CoA substrate for ACC (Saha *et al*, 1995 & 1997). Malonyl-CoA then inhibits CPT 1 and fatty acid oxidation is suppressed. Also similar findings have been found in perfused heart (Bielefeld *et al*, 1985 and Awan & Saggerson, 1993).

This section will provide an insight into the short term regulation of malonyl-CoA content and fatty acid oxidation in the soleus muscle (type I) under various test conditions.

Malonyl-CoA, long chain fatty acyl-CoA and long acylcarnitine measurements were made under different incubation additions. The various additions included insulin, etomoxir, dichloroacetate, palmitate, isoprenaline and AICAR.

In theory, any changes in the malonyl-CoA content of skeletal muscle due to the additions to the incubation medium should result in concomitant changes in the levels of long chain esters as a result of altered CPT 1 activity.

Malonyl-CoA measurements were used as an indication of ACC regulation in skeletal muscles. Similarly, measurements of long chain acyl-CoA and acyl-carnitine were used as an indication of CPT 1 activity.

Metabolite levels were measured in extracts from isolated incubated soleus muscle strips. The incubation was for 1 hour at 37°C with Krebs Ringer-bicarbonate buffer containing 5mM glucose and 2 % BSA as described in section 2.4.1.

The soleus muscle was used throughout for metabolite measurements as it is an easy muscle to dissect out from the rat hindlimb and several strips can be obtained from one leg. Also, it is comprised mainly of one type of muscle fibre (contains 89 % type I fibres) and is similar to the cardiac muscle as it undergoes oxidative metabolism (section 1.2.2).

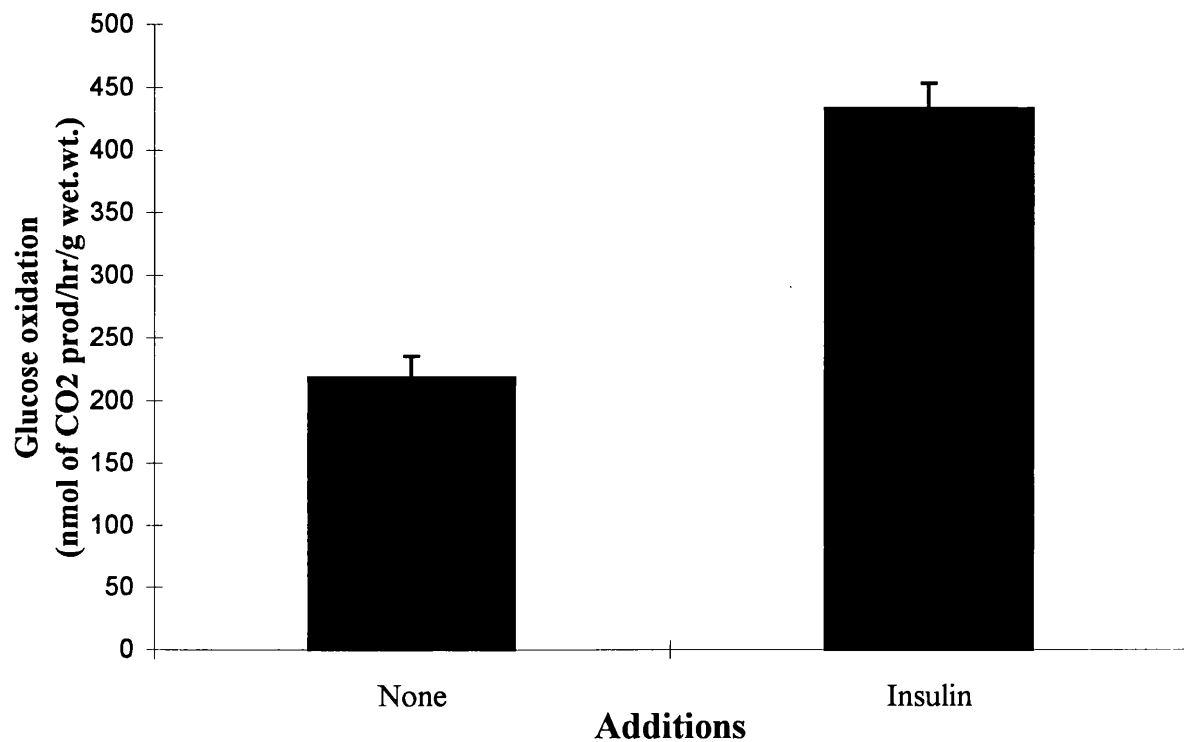
Prior to fatty acid metabolite measurements, glucose oxidation and the glycogen content of the soleus muscle were measured in the absence and presence of insulin (fig 3.6 & fig 3.7). This was to see that the incubation system used was viable.

Fig 3.6 shows that insulin increased glucose oxidation in soleus muscle by approximately 98 %. Also, glycogen synthesis was shown to occur during the incubation period (fig 3.7).

Under the various test conditions, fatty acid oxidation measurements (i.e. palmitate oxidation) were also made.

Fig 3.6

Insulin Increases Glucose Oxidation In Muscle



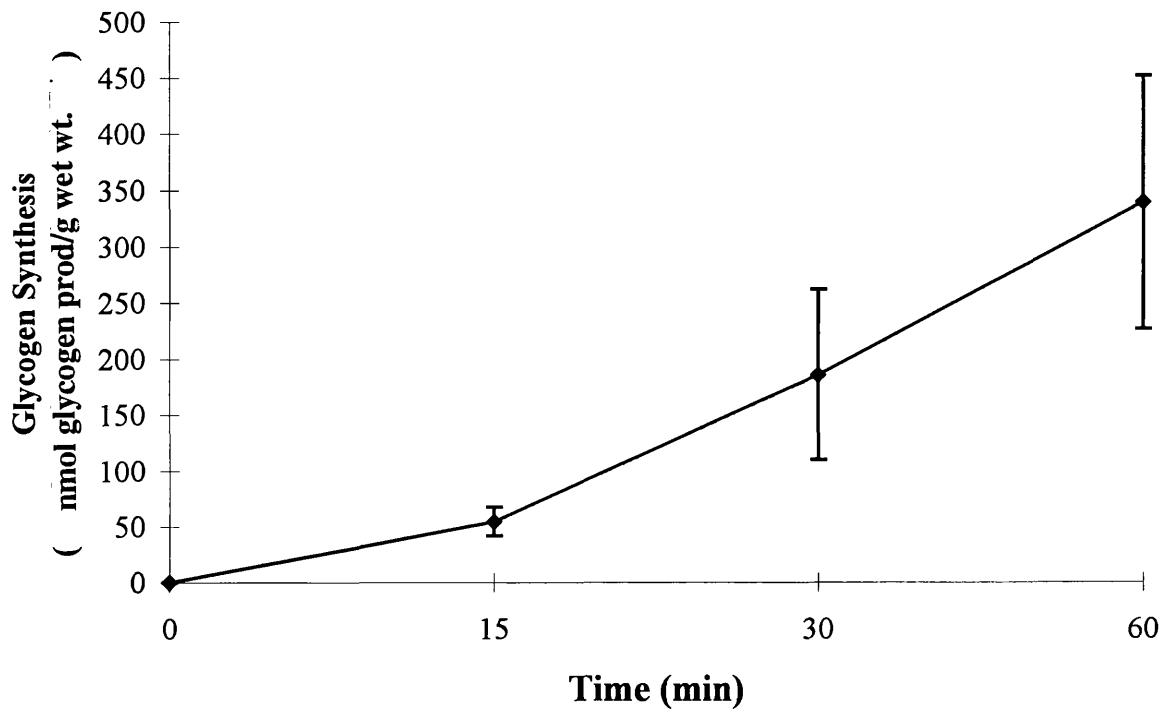
Isolated soleus muscle strips were incubated for 60 min as described in Materials and Methods (section 2.7.1) with 5mM glucose and fatty acid-poor albumin (20mg/ml).

The concentrations of other additions were : insulin 10^{-8} M.

Values are expressed as a range of values for 2 experiments.

Fig 3.7

Glycogen Content In Isolated Soleus Muscle Strips



Isolated soleus muscle strips were incubated with 5mM [U-¹⁴C] glucose (1 μ Ci/flask) and 2 % BSA as described in Materials and Methods (section 2.7.2).

Values are expressed as a range of values for 2 experiments.

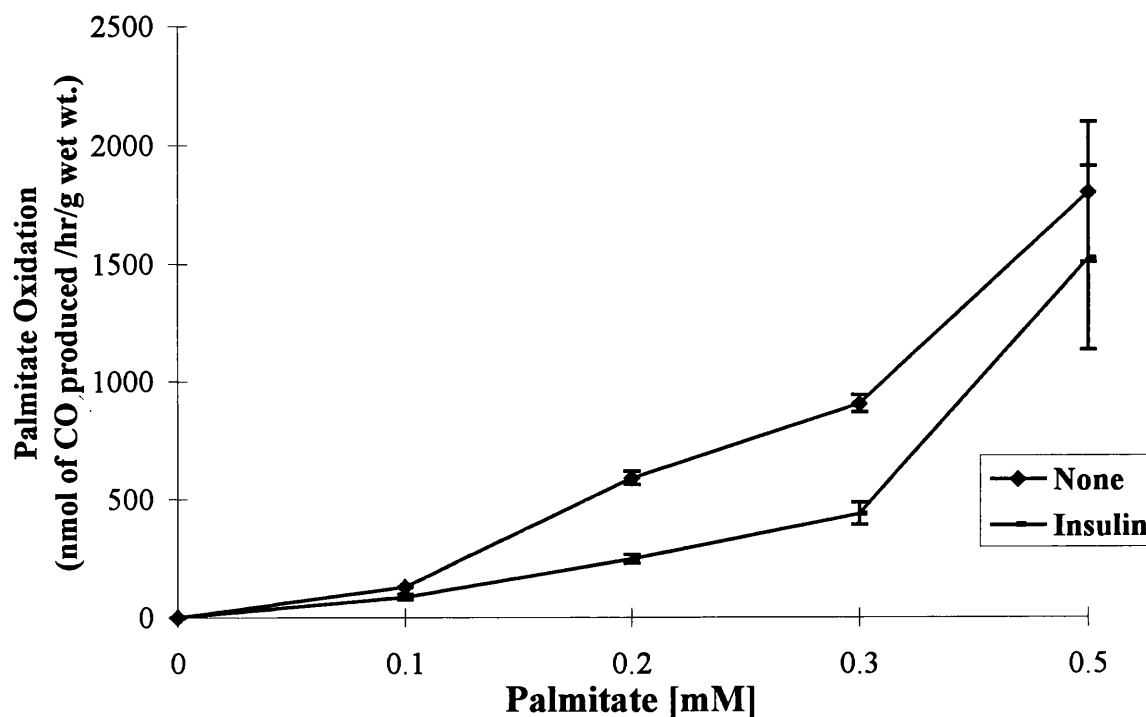
Initially isolated soleus muscle strips were incubated in Krebs Ringer bicarbonate buffer containing various concentrations of [U-¹⁴C] palmitate (0.1 μ Ci/ μ mol) in the presence and absence of insulin and ¹⁴CO₂ was collected as described in section 2.8 (Fig 3.8). Insulin was shown to have no effect at 0.1 mM palmitate but caused a reduction at 0.2 mM and 0.3mM palmitate. Insulin had the greatest effect at 0.2 mM palmitate as it decreased palmitate oxidation by 58 % (fig 3.8). However at 0.5mM palmitate, any effect of insulin was reversed and is discussed in section 3.3.6

By prelabelling muscle lipids during the preincubation phase, it was possible to obtain some index of the oxidation of fatty acids derived from endogenous sources. The content of triacylglycerol that was solvent extracted from muscle strips at the end of the preincubation with 0.2 mM palmitate (specific activity = 220 d.p.m. per nmol) was 272 ± 50 nmol/g wet wt. (n=8) and this had an overall specific activity of only 13 ± 2 d.p.m. per nmol of fatty acid. This value of 13 d.p.m. per nmol of fatty acid was likely to be an under estimate of the specific activity of the pool(s) of triacylglycerol that was/were turning over during the incubation phase and therefore the *absolute* rate of oxidation of endogenous fatty acids could not be determined. However, the paired changes in the ratio of d.p.m. in CO₂ derived from endogenous and exogenous sources are informative as discussed below.

The majority of experiments in this section were compared against glucose + insulin, as this condition is representative of the fed state.

Fig 3.8

**Palmitate Oxidation In The Presence And Absence
Of Insulin**



Isolated soleus muscle strips were incubated with 5mM glucose, 2% BSA and various concentrations of [U-¹⁴C] palmitate as described in the Materials and Methods (Section 2.8.1).

The concentration of insulin was 10⁻⁸M.

Values are expressed as a range of values for 2 experiments.

3.3.1 MALONYL-COA CONTENT OF SOLEUS MUSCLE

Measurements of malonyl-CoA from isolated soleus muscle incubated with glucose (5mM) only, were found to be 1.32 ± 0.15 nmol/g wet wt (n=14). This value is not that much different from that measured by Saha *et al*, (1995) in soleus muscles incubated with various concentrations of glucose only. The values lie in the range of 1.3 -2.4 nmol/g wet wt. (Saha *et al*, 1995 & 1997).

Malonyl-CoA has also been measured in gastrocnemius/plantaris muscles and the values reported lie in the range of 1.24 -1.66 nmol/g wet wt (Winder *et al*, 1989, Elayan & Winder, 1991 and Duan & Winder, 1993).

3.3.2 THE EFFECTS OF INSULIN ON METABOLITE LEVELS

3.3.2.1 Malonyl-CoA

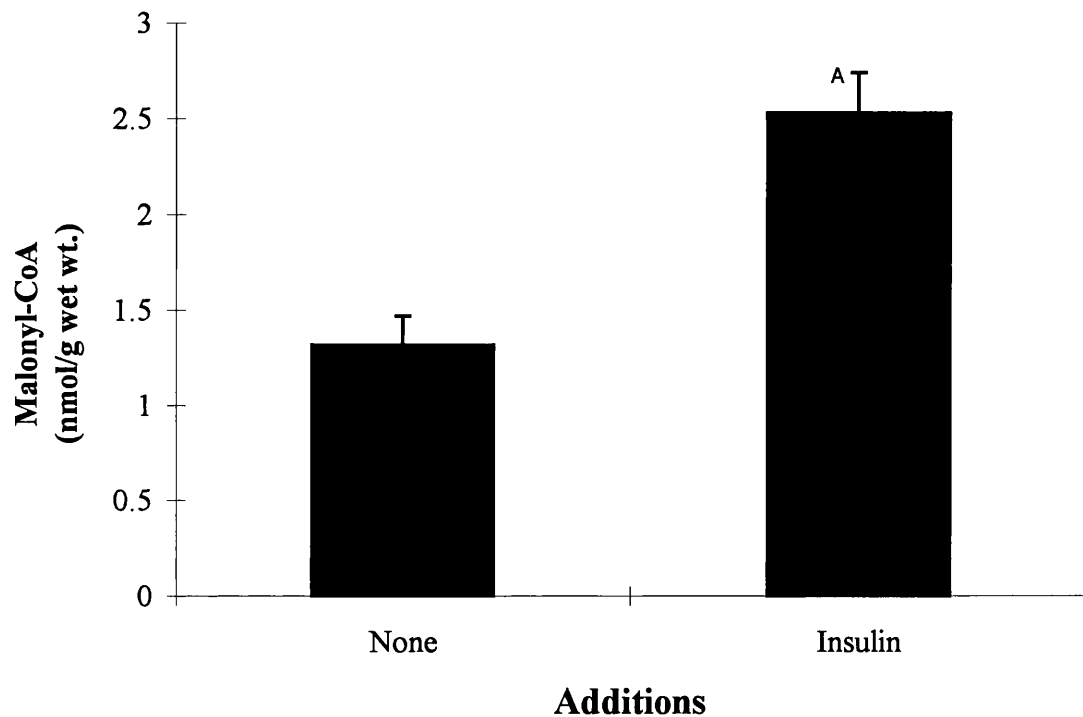
In the presence of glucose, insulin significantly increased the tissue content of malonyl-CoA by 92 % (fig 3.9)

These results are consistent with the findings from Ruderman's group (Saha *et al*, 1995 & 1997), that insulin increases malonyl-CoA content in the soleus muscle. Other studies have shown insulin to increase malonyl-CoA content in type IIb muscles (e.g. gastrocnemius muscle) (Duan and Winder, 1993).

The mechanism by which insulin acutely increases malonyl-CoA levels in skeletal muscle is not by increasing ACC activity (Saha *et al*, 1995), which is what occurs in the liver. However the exact mechanism of insulin activating liver ACC is unsure (section 1.6.3). Bianchi *et al*, (1990) showed muscle ACC to have a higher K_m for acetyl-CoA than liver ACC, suggesting that its activity might be regulated by substrate supply rather than by acute regulation by phosphorylation/dephosphorylation as in the liver. Recent work from Ruderman's group has shown that the insulin-induced increase in malonyl-CoA content in soleus muscle is accompanied by an increase in citrate concentration, a precursor of acetyl-CoA. An increase in citrate concentration could up-regulate malonyl-CoA synthesis, either by enhancing the supply of cytosolic acetyl-CoA or by allosterically activating ACC (Saha *et al*, 1995 and 1997). Studies with perfused gastrocnemius muscle have shown malonyl-CoA levels to rise in the presence of insulin without affecting citrate concentration (Duan & Winder, 1993 & Saha *et al*, 1995). A reason for this could be that the gastrocnemius muscle is comprised mainly of white fibres and these fibres are not as sensitive to the stimulatory effects of insulin as red fibres (soleus) (Saha

Fig 3.9

Insulin Increases Malonyl-CoA Content In Soleus Muscle



Isolated soleus muscles were incubated for 60 min as described in Material & Methods (Section 2.5) with 5mM glucose and 2% BSA.

The concentrations of other additions were: insulin, 10^{-8} M.

Results are expressed as mean \pm SEM.

All data for none and insulin were pooled (n= 14).

^A represents $P < 0.001$ versus None (unpaired).

et al, 1995 and Maizel *et al*, 1977). This lack of response to insulin in white fibres is probably due to the metabolic function of white fibres (Section 1.2.2. Marette *et al*, 1992 and Hocquette *et al*, 1995).

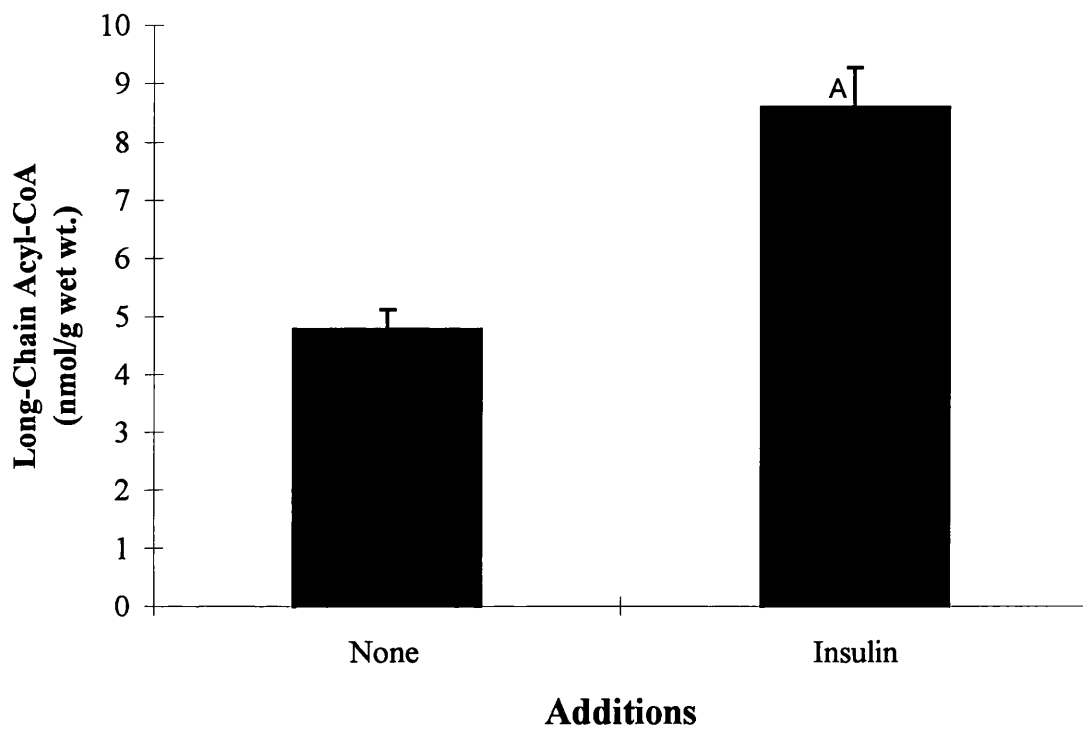
From these studies insulin has shown to significantly increase malonyl-CoA content in the soleus muscle. This increase is probably due to an increase in citrate concentration, resulting in an increase in supply of acetyl-CoA for ACC as suggested by Saha *et al*, (1995 and 1997)

3.3.2.2 Long Chain Esters

Insulin significantly increased long chain fatty acyl-CoA content by 79% (fig 3.10), and significantly decreased long chain acylcarnitine content by 55% (fig 3.11). This resulted in the ratio of long chain acylcarnitine : long chain fatty acyl-CoA being decreased significantly (fig 3.12). This increase in long chain acyl-CoA content and the decrease in long chain acylcarnitine content is consistent with those findings seen with etomoxir (section 3.3.2). From measuring long chain fatty acyl-CoA alone it does not tell us whether this increase in long chain fatty acyl-CoA in the presence of insulin is solely in the cytosol. Numerous studies in various tissues have attempted to quantify the intracellular distribution of long chain fatty acyl-CoA and as yet no conclusion has been reached in regards to the degree to which they are compartmented (i.e. the cytosol or the mitochondria) (Oram *et al*, 1975, Idell-Wenger *et al*, 1978, Deeney *et al*, 1992, Rasmusen *et al*, 1993 Kobayashi, *et al*, 1994 and Fabergemen & Knudsen, 1997). However studies with etomoxir (a pharmacological inhibitor of CPT 1) has provided evidence to suggest that this increase in long chain fatty acyl-CoA in the presence of insulin is *upstream* of CPT 1 i.e. in the cytosol or intermembrane space (section 3.3.3).

Fig 3.10

**Insulin Increases Long chain Acyl-CoA Levels In
Soleus Muscle**



Isolated soleus muscles were incubated for 60 min as described in the Material & Methods (Section 2.5) with 5mM glucose and 2% BSA.

The concentrations of other additions were: insulin, 10^{-8} M.

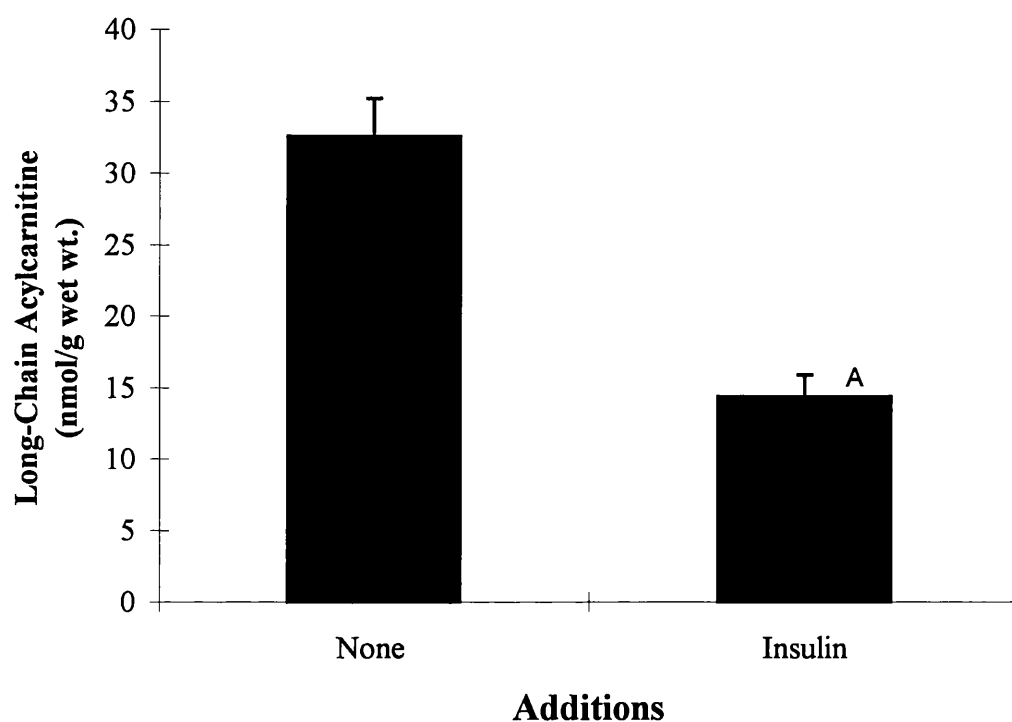
Results are expressed as mean \pm SEM.

All data for none and insulin were pooled (n= 13).

^A represents $P < 0.001$ versus None (unpaired).

Fig 3.11

Insulin Decreases Long Chain Acyl Carnitine Levels
In Soleus Muscle



Isolated soleus muscles were incubated for 60 min as described in the Material & Methods (Section 2.5) with 5mM glucose and 2% BSA.

The concentrations of other additions were: insulin, 10^{-8} M.

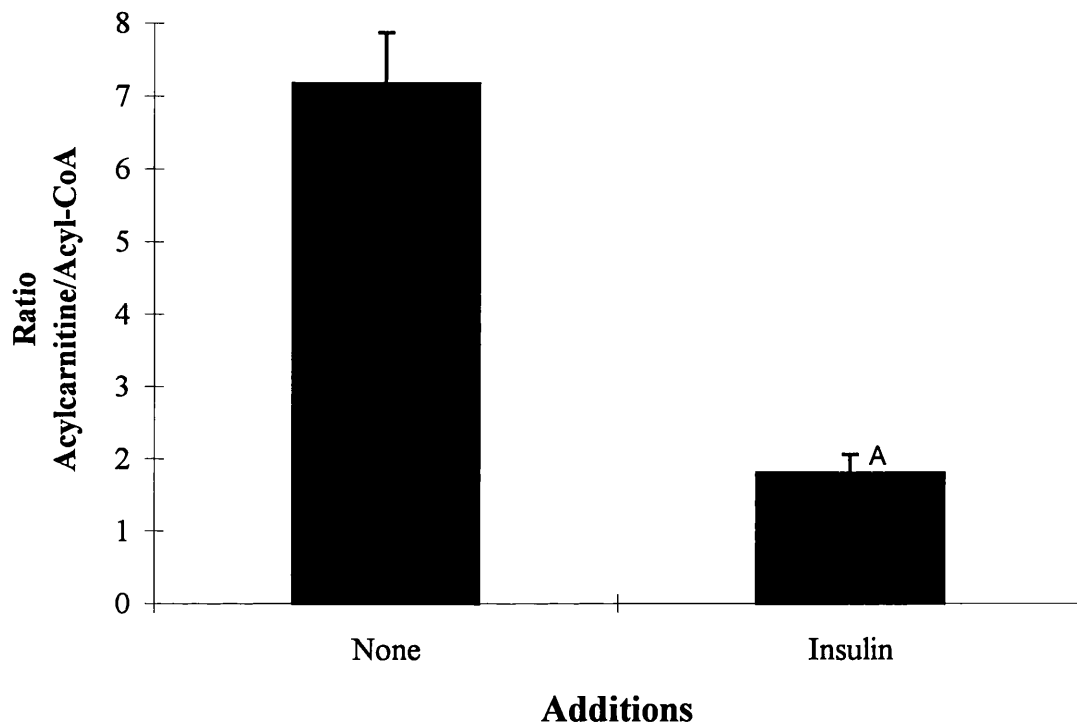
Results are expressed as means \pm SEM.

All data for none and insulin were pooled (n=12).

^A represents $P < 0.001$ versus None (unpaired).

Fig 3.12

**Insulin Decreases The Ratio Of Long Chain Acyl Carnitine
: Long Chain Acyl-CoA In Soleus Muscle**



Isolated soleus muscles were incubated for 60 min as described in the Material & Methods (Section 2.5) with 5mM glucose and 2% BSA.

The concentrations of other additions were: insulin, 10^{-8} M.

Results are expressed as mean \pm SEM.

All data for none and insulin were pooled (n=12 for insulin & n=13 for none).

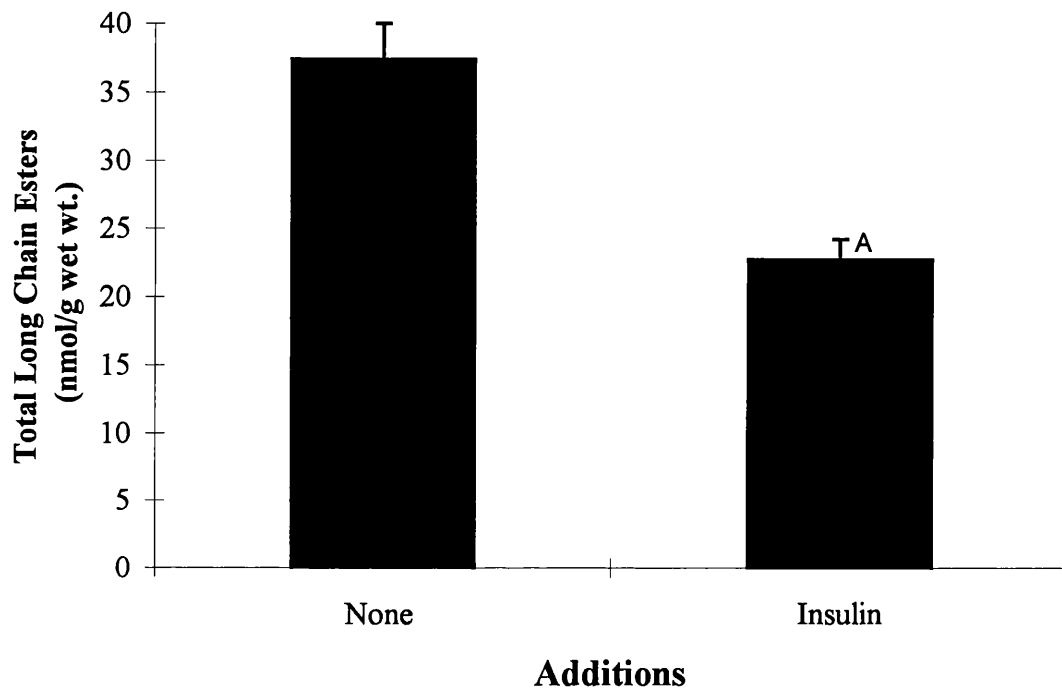
^A represents $P < 0.001$ versus None (unpaired).

Overall, insulin caused a small but significant fall in total long chain esters (fig 3.13). From fig 3.13, it can be suggested that insulin might be antilipolytic in soleus muscle. If this is the case then insulin will not increase the oxidation of endogenous fatty acids and therefore would decrease total (endogenous and exogenous) oxidation. Unfortunately there are no endogenous fatty acid oxidation measurements to support this claim.

Finally, it can be suggested that the changes in metabolite content seen in the presence of insulin were due to inhibition of CPT 1 probably by malonyl-CoA and this would not be inconsistent with the decrease in oxidation of palmitate seen with insulin as shown in figure 3.8.

Fig 3.13

Insulin Decreases Total Long Chain Esters in Soleus Muscle



Isolated soleus muscles were incubated for 60 min as described in the Material & Methods (Section 2.5) with 5mM glucose and 2% BSA. The concentrations of other additions were: insulin, 10^{-8} M.

Results are expressed as mean \pm SEM.

All data for none and insulin were pooled (n=12 for insulin & n=13 for none).

^A represents $P < 0.001$ versus None (unpaired).

3.3.3 THE EFFECTS OF ETOMOXIR ON METABOLITE LEVELS

The drug etomoxir belongs to a class of oxiraine carboxylic acid derivatives, which include tetradecylglycidate (TDGA) and 2-[5- (4-chlorophenyl)pentyl] oxiraine-2-carboxylate (POCA). They have the property of suppressing fatty acid oxidation as a result of inhibiting CPT 1 (Kiorpes *et al*, 1984 and Turnbull *et al*, 1984). These agents are converted intracellularly to their CoA esters which inactivate mitochondrial CPT 1.

Etomoxir has been shown to inhibit fatty acid oxidation in adipocytes and heart. In brown adipocytes, etomoxir (0.3mM) substantially decreased oxygen uptake and palmitate oxidation (Baht & Saggerson, 1984). In heart, etomoxir inhibits CPT 1 resulting in an accumulation of long chain fatty acyl-CoA and inhibits endogenous lipolysis (Hulsman *et al*, 1994).

It has been demonstrated that POCA, TDGA and oxfenicine, which inhibit fatty acid oxidation can protect the ischemic myocardium (Paulson *et al*, 1986 and Molaparas-Saless *et al*, 1987) due to a decrease in myocardial long chain acylcarnitine levels.

Lopaschuk's group demonstrated that etomoxir significantly improves myocardial function during reperfusion of perfused ischemic heart with palmitate. This effect was not associated to a decrease in myocardial long chain acyl carnitine levels but rather due to a switch from fatty acid oxidation to an increase in glucose utilisation, by the reperfused heart (Lopaschuk *et al*, 1988).

It has been suggested that etomoxir might be used in the treatment of diabetes since inhibition of fatty acid oxidation should overcome the fatty acid induced inhibition of glycolysis and increase glucose utilisation (Abdel-aleem *et al*, 1994, Nicholl *et al*, 1991 and Wall & Lopaschuk 1989).

Studies carried out so far with etomoxir have been involved with measuring fatty acid oxidation in heart under ischemic conditions. As yet no studies have been carried out

whereby fatty acid metabolites have been measured in the presence of etomoxir in skeletal muscle. As etomoxir is known to inhibit CPT 1 and suppress fatty acid oxidation, it was interesting to see the effects of etomoxir on fatty acid metabolite levels, in particularly long chain acyl-CoA levels in resting muscles.

Isolated soleus strips were incubated with various concentrations of extomoxir and fatty acid metabolites were measured as described in the Materials and Methods (section 2.4)

Incubations alone with etomoxir were compared against that with glucose only.

3.3.3.1 Malonyl-CoA

At 50 μ M and 100 μ M etomoxir, no significant effect was observed on malonyl-CoA content in soleus muscle (fig 3.14).

At higher concentrations of etomoxir (150 μ M), malonyl-CoA content in the soleus muscle was reduced significantly by 47 % (fig 3.14).

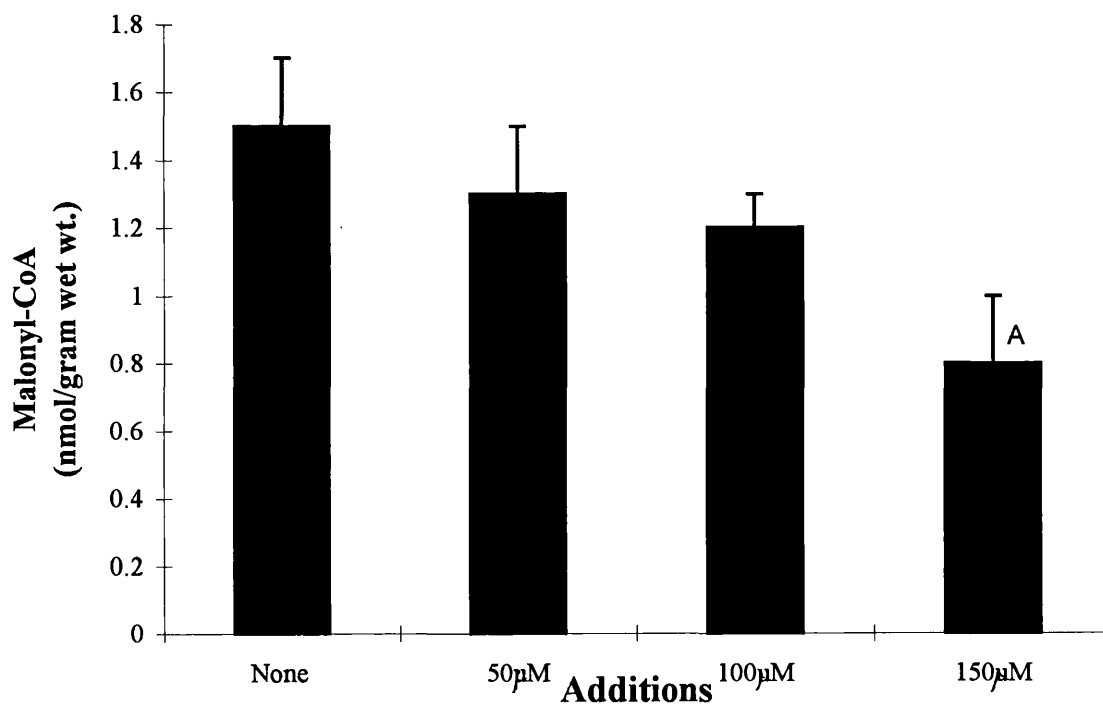
Long chain fatty acyl-CoAs are known to allosterically inhibit ACC activity (Hardie, 1989). It is possible that the fall in malonyl-CoA content in soleus muscle in the presence of 150 μ M etomoxir could be due to allosteric inhibition of ACC activity by long chain acyl-CoA, as a result of increased tissue content of long chain acyl CoA in the presence of 150 μ M etomoixr (fig 3.15).

This interpretation of the effect of etomoxir on malonyl-CoA content in soleus muscles at high concentrations has to be treated with caution. The reason being, at a high concentration of an inhibitor it will have deleterious metabolic effects on the tissue.

It should be noted that 50 μ M, 100 μ M and 150 μ M etomoxir are equally effective in raising fatty acyl-CoA but not in lowering malonyl-CoA. However it has been suggested above that fatty acyl-CoA inhibition of ACC may be the reason for the malonyl-CoA decrease observed at 150 μ M etomoxir.

Fig 3.14

150 μ M Etomoxir Significantly Decreases Malonyl-CoA Levels In Soleus Muscle



Isolated Soleus muscle strips were incubated for 60 min as described in the Materials and Methods (section 2.5) with 5mM glucose and fatty acid poor albumin (20mg/ml).

The concentrations of etomoxir added are stated in the figure.

Results are expressed as mean \pm SEM. (n = 5).

^D indicates P<0.005 versus none.

3.3.3.2 Long chain esters

Fig 3.15 shows that at all concentrations of etomoxir, long chain fatty acyl-CoA content was increased significantly and an insignificant decrease in long chain acylcarnitine content was observed in the soleus muscle (fig 3.16).

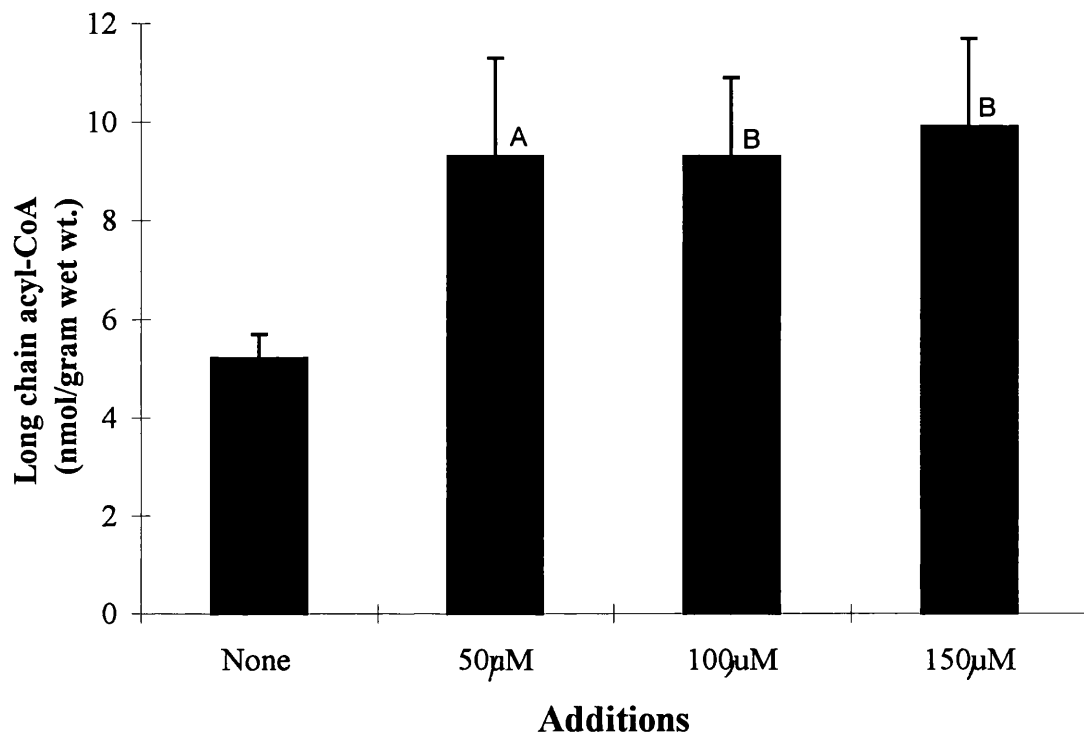
The total long chain esters did not change in the presence of etomoxir (fig 3.17).

However, the ratio of long chain acylcarnitine/long chain fatty acyl-CoA decreased significantly (fig 3.18). This decrease in the ratio is due to the increased tissue content in long chain fatty acyl-CoA as a result of etomoxir inhibiting CPT 1.

In conclusion, these results shows that etomoxir results in an increased tissue content of long chain fatty acyl-CoA with a concomitant decrease in the ratio of long chain acylcarnitine/long chain fatty acyl-CoA. This increase in long chain fatty acyl-CoA content in soleus muscle due to etomoxir is an important finding. An increase in long chain fatty acyl-coA as a result of inhibition of CPT 1 by etomoxir is probably upstream of CPT 1 i.e. cytosolic acyl-CoA. This suggests that the increase in long chain fatty acyl-CoA content observed in the presence of insulin might be cytosolic (section 3.3.2).

Fig 3.15

Etomoxir Increases Long Chain Acyl-CoA Content
In Isolated Soleus Muscle



Isolated Soleus muscle strips were incubated for 60 min as described in the Materials and Methods (section 2.5) with 5mM glucose and fatty acid poor albumin (20mg/ml).

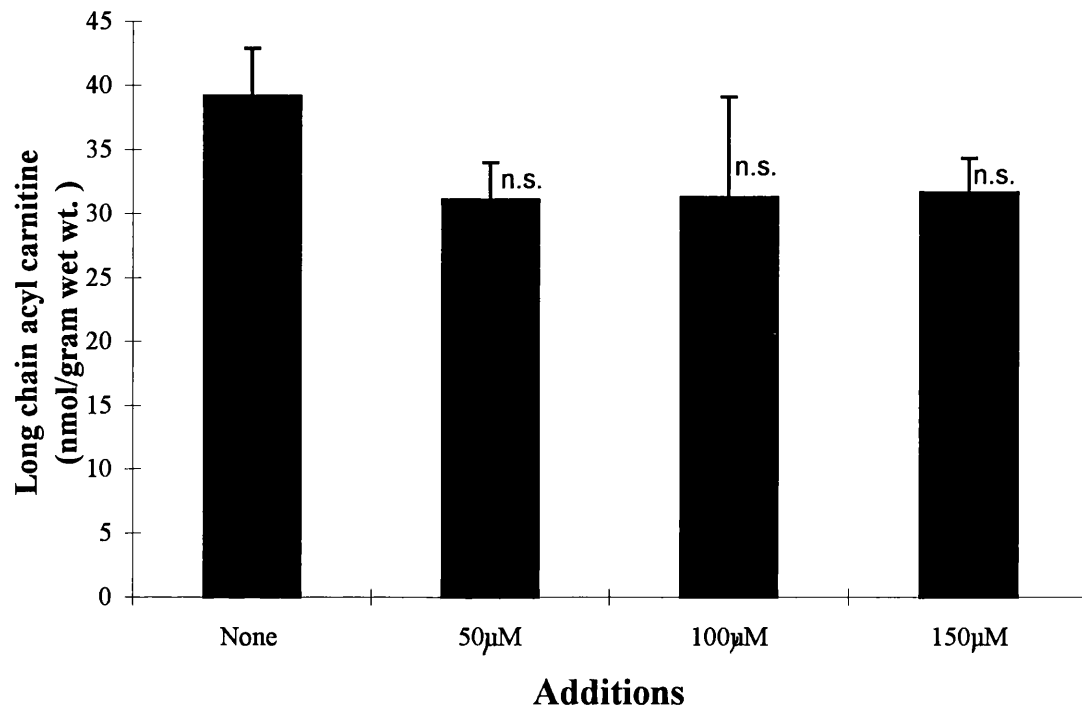
The concentrations of etomoxir added are stated in the figure.

Results are expressed as mean \pm SEM. (n = 5).

^{A, B} indicates $P < 0.025$, < 0.01 versus none.

Fig 3.16

**Etomoxir Has No Significant Effect On Long Chain
AcylCarnitine Levels In Isolated Soleus Muscle,**



Isolated Soleus muscle strips were incubated for 60 min as described in the Materials and Methods (section 2.5) with 5mM glucose and fatty acid poor albumin (20mg/ml).

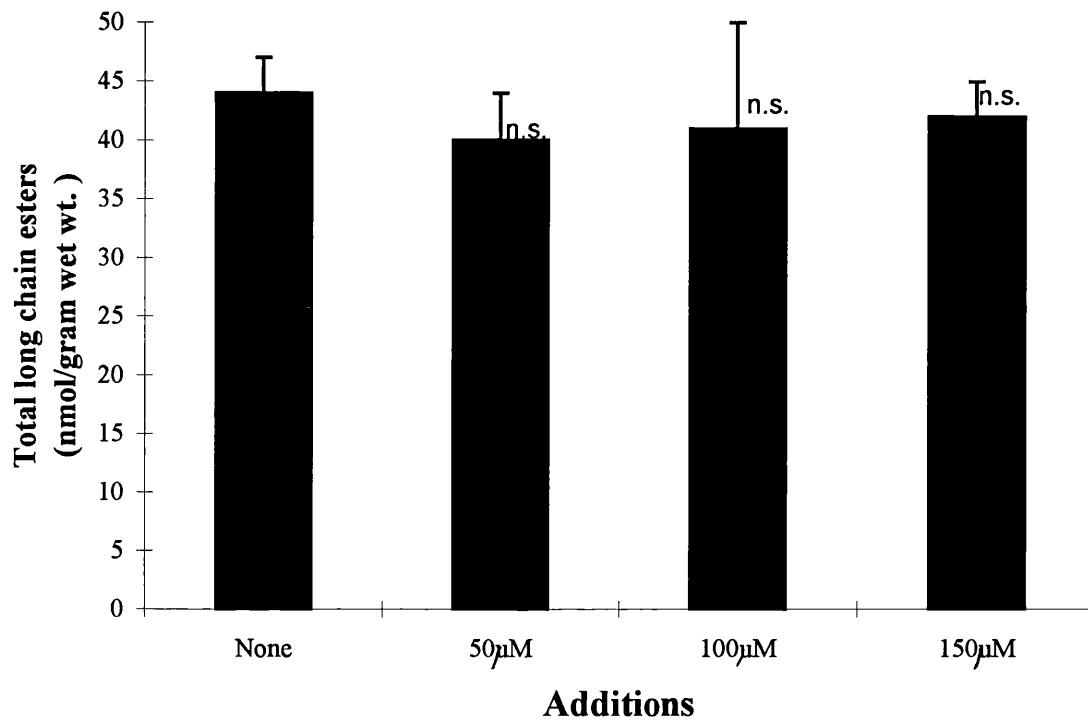
The concentrations of etomoxir added are stated in the figure.

Results are expressed as mean \pm SEM. (n = 5).

n.s indicates no significance against basal state.

Fig 3.17

Etomoxir Has No Significant Effect On Total Long Chain Esters In Soleus Muscle



Isolated Soleus muscle strips were incubated for 60 min as described in the materials and methods (section 2.5) with 5mM glucose and fatty acid poor albumin (20mg/ml).

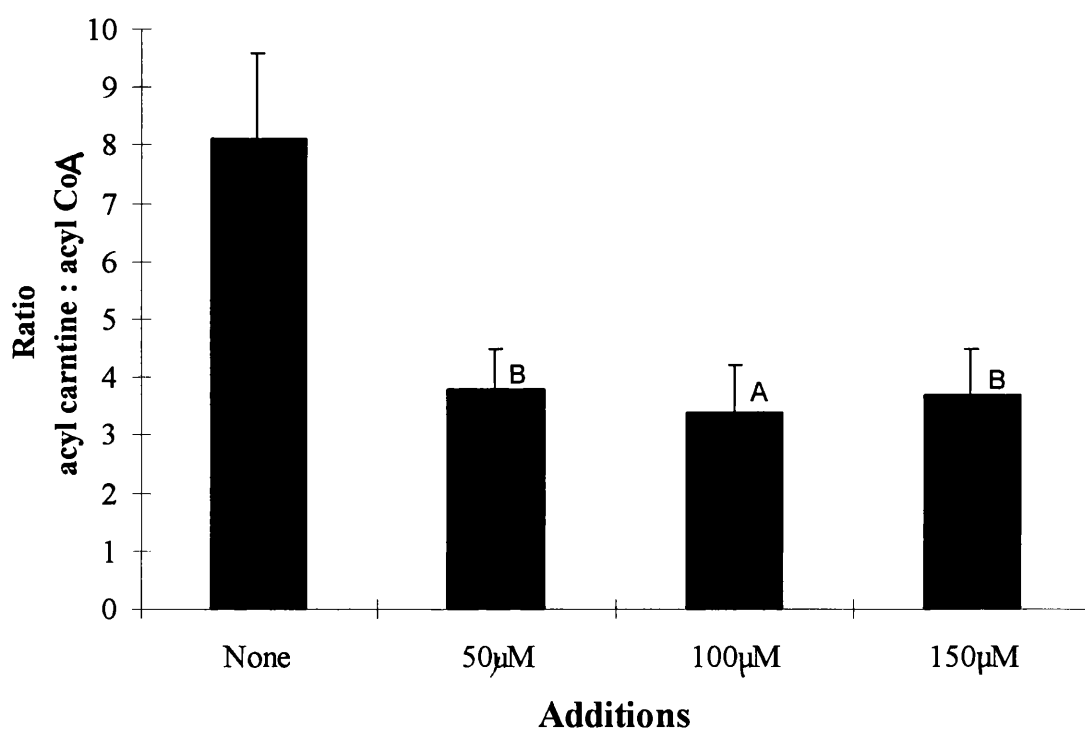
The concentrations of etomoxir added are stated in the figure.

Results are expressed as mean \pm SEM. (n = 5).

n.s indicates no significance against the basal state.

Fig 3.18

**Etomoxir Decreases The Ratio Of Long Chain Acyl
Carnitine : Acyl-CoA In Soleus Muscle**



Isolated Soleus muscle strips were incubated for 60 min as described in the materials and methods (section 2.5) with 5mM glucose and fatty acid poor albumin (20mg/ml).

The concentrations of etomoxir added are stated in the figure .

Results are expressed as mean \pm SEM. (n = 5).

^{A, B} indicates $P < 0.05$, < 0.025 versus the basal state.

3.3.4 THE EFFECTS OF DICHLOROACETATE (DCA) ON METABOLITE

LEVELS

In vitro studies with heart and diaphragm muscles show that DCA promotes glucose and pyruvate oxidation and inhibits the oxidation of long chain fatty acid, 3-hydroxybutyrate and lactate (Stackpole & Felts, 1970, McAllister *et al*, 1973 and Stackpole 1989). Initially it was thought that DCA may promote the oxidation of pyruvate by inhibiting fatty acid and ketone body oxidation (Stackpole & Felts, 1970). Later it was shown in perfused rat heart, isolated rat diaphragm and epididymal fat pads that DCA increases the proportion of active PDH by inhibiting the PDH kinase, resulting in increased hydrolysis of inactive PDH to active PDH (Whitehouse & Randle 1973 and Whitehouse *et al*, 1974).

McAllister *et al*, (1973) showed DCA to have other metabolic effects on rat heart and diaphragm. DCA in both tissues resulted in impaired oxidation of acetate, 3-hydroxybutyrate and palmitate, and a decrease in tissue citrate content associated with activation of phosphofructokinase and glycolysis. It was suggested that these effects occurred due to an activation of pyruvate oxidation by DCA. In rat epididymal fat pads DCA was shown to inhibit one or more reactions involved in the synthesis of fatty acids (Whitehouse *et al*, 1974). This suggests that DCA not only stimulates PDH activity but also has other metabolic effects.

More recently Saddik *et al*, (1993) showed that in perfused hearts, DCA (1mM) stimulated glucose oxidation, with a parallel decrease in fatty acid oxidation. As a result both acetyl-CoA and malonyl-CoA content increased in the perfused heart. It can be suggested that DCA increases the supply of acetyl-CoA for ACC. This provided evidence that ACC activity in heart could be determined by the supply of acetyl-CoA.

DCA was neutralised in NaOH before adding it to the incubation media. Various concentrations of DCA were used and the effects were compared against the basal state.

3.3.4.1 Malonyl-CoA

Table 3.6 shows that malonyl-CoA content in the soleus muscle was significantly increased by 3mM DCA.

DCA most probably increased malonyl-CoA content by stimulating PDH activity and increasing the rate of pyruvate oxidation. This could then lead to an increase in supply of acetyl-CoA for ACC and hence an increase in malonyl-CoA production.

In perfused heart, a 40% increase in malonyl-CoA level was seen in the presence of 1mM DCA (Saddik *et al*, 1993). However in the presence of 1mM DCA, it had no significant effect on malonyl-CoA content in the soleus muscle (table 3.6). Instead at 3mM DCA a 150 % increase in tissue malonyl-CoA content was observed.

It can be suggested that in the soleus muscle high concentrations of DCA are required to activate the PDH complex.

In essence the effects seen with 3mM DCA on malonyl-CoA content in muscle are similar to the effects seen with insulin (fig 3.9). An experiment was carried out to see whether the presence of insulin and DCA together caused any further increase in malonyl-CoA content. From table 3.6 it may be seen that insulin and DCA together had no further significant effect on any of the metabolite levels measured.

3.3.4.2 Long Chain Esters

In the presence of 3mM DCA, long chain fatty acyl-CoA content increased significantly (Table 3.6). The ratio of long chain acylcarnitine / long chain fatty acyl-CoA was decreased significantly (Table 3.6).

Table 3.6**Fatty Acid Metabolite Measurements In The Presence Of Dichloroacetate**

Addition to incubation	Malonyl-CoA (nmol/g wet wt.)	Long chain fatty acyl-CoA (nmol/g wet wt.) (A)	Long chain acylcarnitine (nmol/g wet wt.) (B)	Total long chain esters (A + B)	Ratio A/B
None	0.72 ± 0.14	4.8 ± 0.5	34.7 ± 3.4	39 ± 4	7.3 ± 0.2
0.3mM DCA	0.27 ± 0.05 ^B	5.0 ± 0.4	30.8 ± 7.2	36 ± 6	6.6 ± 1.8
1mM DCA	0.68 ± 0.12	5.1 ± 0.2	52.5 ± 2.6 ^C	58 ± 2 ^C	9.0 ± 0.6 ^B
3mM DCA	1.80 ± 0.18 ^C	7.9 ± 1.1 ^A	40.7 ± 4.2	49 ± 5	5.3 ± 0.5 ^B
Insulin	2.4 ± 0.3 ^{n.s}	9.2 ± 0.9 ^{n.s}	12.9 ± 1.2 ^{n.s}	22 ± 2 ^{n.s}	2.8 ± 0.1 ^{n.s}
Ins + DCA (3mM)	2.9 ± 0.2	8.3 ± 1.3	11.8 ± 2.7	19 ± 4	1.5 ± 0.2

Isolated soleus muscle strips were incubated for 60 min as described in the Materials & Methods (section 2.5), with 5mM glucose and fatty acid poor albumin (20mg/ml).

The concentration of dichloroacetate (DCA) added to the incubation media is mentioned in the table.

Results were expressed as means ± SEM. n = 4 for all measurements

^{A, B, C} indicate P<0.05, <0.025, <0.01 respectively versus incubations with no addition.

n.s. indicates not significant against insulin & DCA.

These effects seen are similar to those of insulin (fig 3.10 & fig 3.12).

This increase in long chain fatty acyl-CoA content seen at 3mM DCA was probably due to the increase in malonyl-CoA content. This would result in an increased inhibition of CPT1, causing an accumulation of long chain fatty acyl-CoA, as the pathway to the mitochondrial matrix is blocked.

Fatty acid oxidation (both exogenous and endogenous [^{14}C] palmitate) was measured in the soleus muscle with 3 mM DCA. Fig 3.19 and table 3.7 show that DCA suppressed both exogenous and endogenous palmitate oxidation by 68% and 35 % respectively. The fall in exogenous oxidation in the presence of DCA was greater than the fall in endogenous palmitate oxidation

A fall in the ratio for exogenous palmitate oxidation/endogenous palmitate oxidation was observed. This is interesting, as this suggests that DCA at 3 mM stimulates lipolysis, even though it also increases malonyl-CoA levels at this concentration.

This lipolytic effect with DCA in the soleus muscle was unexpected and maybe DCA has other metabolic effects that we are not aware of. This lipolytic effect could also have contributed to the increase in long chain acyl-CoA content observed in muscle at 3 mM DCA.

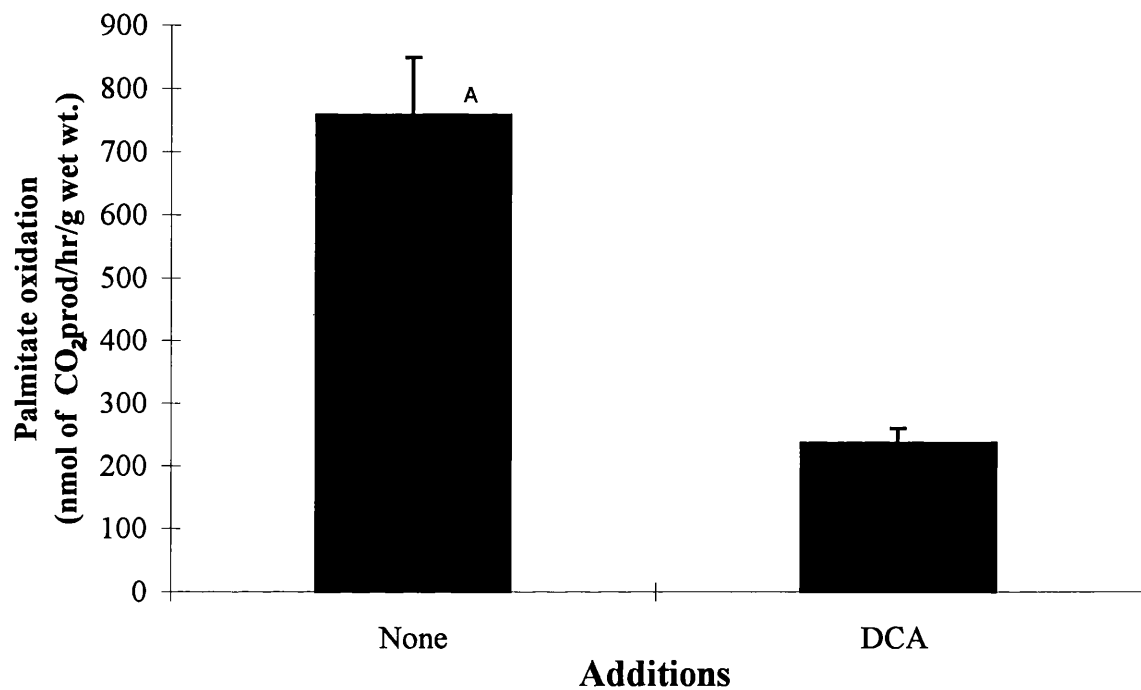
3.3.4.3 0.3 mM and 1 mM DCA

It is interesting to note that in the presence of 0.3 mM DCA a significant fall in malonyl-CoA content was observed (Table 3.6). 0.3 mM DCA had no significant effect on long chain esters.

By contrast 1mM DCA caused no change in malonyl-CoA content in soleus muscle (table 3.6). This coincides with no change in long chain fatty acyl-CoA content but a significant increase in long chain acylcarnitine content (table 3.6). This resulted in both

Fig 3.19

DCA In The Presence Of Glucose Alone Suppresses
Exogenous Palmitate Oxidation In Isolated Soleus Muscle
Strips



Incubation for 1 hour at 37⁰C and contained 2% BSA, 5mM glucose and 0.2mM [U-¹⁴C] palmitate as described in Materials & Methods (section 2.8.1). Other additions were DCA 3mM

Values are \pm SEM (n=5)

^A indicates P<0.005 versus DCA

Table 3.7

Measurements Of Exogenous And Endogenous Palmitate Oxidation In Soleus Muscle
(with DCA)

Addition to incubation	Exogenous oxidation (d.p.m. per g wet wt./h) (A)	Endogenous oxidation (d.p.m. per g wet wt./h) (B)	Ratio A/B	Exogenous oxidation (nmol CO ₂ per g wet wt./h)
None	13034 ± 1572	6837 ± 758	2.1 ± 0.4	760 ± 91
3mM Dichloroacetic Acid	4110 ± 416 ^A	4439 ± 630 ^A	1.0 ± 0.2 ^B	236 ± 23 ^A

Incubations were for 1 hour at 37°C and contained 2 % BSA, 5mM glucose and 0.2 mM [1-¹⁴C] palmitate oxidation, as described in the Materials and Methods (section 2.8.2).

Values are means ± SEM and n = 5 throughout.

^{A, B} indicate P<0.05, <0.005 for paired effects of Dichloroacetic acid.

the combined long chain esters and the ratio of long chain acyl carnitine/long chain acyl-CoA to be significantly increased (table 3.6).

Why is such an effect occurring at low concentrations of DCA in soleus muscle ?

It could be speculated that, at low concentrations, DCA has a lipolytic effect in muscle as 1mM DCA substantially increased total long chain esters, in particular long chain acyl-carnitine levels (table 3.6).

If 1mM & 0.3 mM DCA are causing a lipolytic effect in muscle then this could be the reason for observing a fall in malonyl-CoA levels at low concentrations of DCA (see section 3.3.6 for a possible mechanism for this observation).

In summary, the most important finding from this study was that 3 mM DCA, apart from increasing tissue malonyl-CoA content, seems to have a lipolytic effect in muscle.

At 3mM DCA the effects on metabolite levels are similar to the effects caused by insulin.

By contrast, at lower concentrations of DCA (1mM & 0.3 mM) the effects caused are suggested to be due to a lipolytic effect on muscle and are also similar to the effects caused by isoprenaline (see section 3.3.5)

CONCLUSIONS

- Insulin significantly increases malonyl-CoA content in the soleus muscle. This is probably due to an increase in the supply of acetyl-CoA for ACC, as a result of an increase in citrate concentration in the presence of insulin as suggested by Ruderman's group (Saha *et al*, 1995 and 1997).
- This increase in malonyl-CoA with insulin resulted in an increase in long chain fatty acyl-CoA content in muscle and experiments with etomoxir suggests that such an increase in long chain fatty acyl-CoA could be up-stream of CPT 1 i.e. in a cytosolic pool.
- This increase in malonyl-CoA content in the presence of insulin probably caused an increase in inhibition of CPT 1. Even though CPT 1 activity was not measured in the presence of insulin, the decrease in the ratio of long chain acylcarnitine/acyl-CoA and in [¹⁴C] palmitate oxidation suggests that insulin is able to down regulate the activity of CPT 1 and decrease fatty acid oxidation in muscle.
- Addition of Etomoxir in the incubation medium with isolated soleus muscle strips, inhibited CPT 1 as shown from the measurements of long chain esters (fig 3.15). These findings are consistent with those found by Lopashuk (1994), in reperfused ischemic hearts.
- At high concentrations of DCA (3mM), malonyl-CoA content in muscle was increased. The mechanism by which DCA causes this rise in malonyl-CoA content in muscle is probably by inhibiting the PDH kinase and resulting in an increase in the active proportion of PDH. This would result in an increase in the supply of acetyl-CoA for ACC and a rise in tissue malonyl-CoA content. This increase in malonyl-CoA content is associated with an increase in long chain fatty acyl-CoA content. This is

probably due to malonyl-CoA inhibiting CPT 1 and thereby blocking the pathway to the mitochondrial matrix where fatty acids undergo oxidation.

- 3mM DCA decreased both endogenous and exogenous [^{14}C] palmitate oxidation but the ratio for exogenous/endogenous oxidation fell. This suggests that DCA has a lipolytic effect in muscle, even though it increases tissue malonyl-CoA content. This lipolytic effect is not fully understood and requires further investigation.
- The findings from DCA at high concentrations are similar to those found with insulin. It can be suggested that DCA at high concentrations has an insulin-like effect, potentially since the effects of DCA and insulin were non-additive. This further supports the finding from Saha *et al*, (1997) that insulin increases malonyl-CoA content in soleus muscle by stimulating the conversion of carbohydrate precursors into citrate and acetyl-CoA.
- The findings from low concentrations of DCA are similar to what was found with isoprenaline (section 3.3.5). It can be proposed that DCA at low concentrations has isoprenaline-like effects (decreased malonyl-CoA levels and increased long chain fatty acylcarnitine, total long chain esters and ratio of acylcarnitine/acyl-CoA). The effects of isoprenaline are discussed in the next section

3.3.5 THE EFFECTS OF ISOPRENALINE ON METABOLITE LEVELS

Metabolite measurements were carried out using different concentrations of the β -adrenergic agonist isoprenaline in the presence of insulin. Therefore all measurements made were compared against insulin.

3.3.5.1 Malonyl-CoA

Fig 3.20 shows that isoprenaline at all tested concentrations, significantly reduced the malonyl-CoA content in muscle and the greatest effect (a 72 % decrease) was observed at 10 μ M isoprenaline. 0.1 μ M and 1 μ M isoprenaline caused a 50 % decline in malonyl-CoA content in soleus muscle.

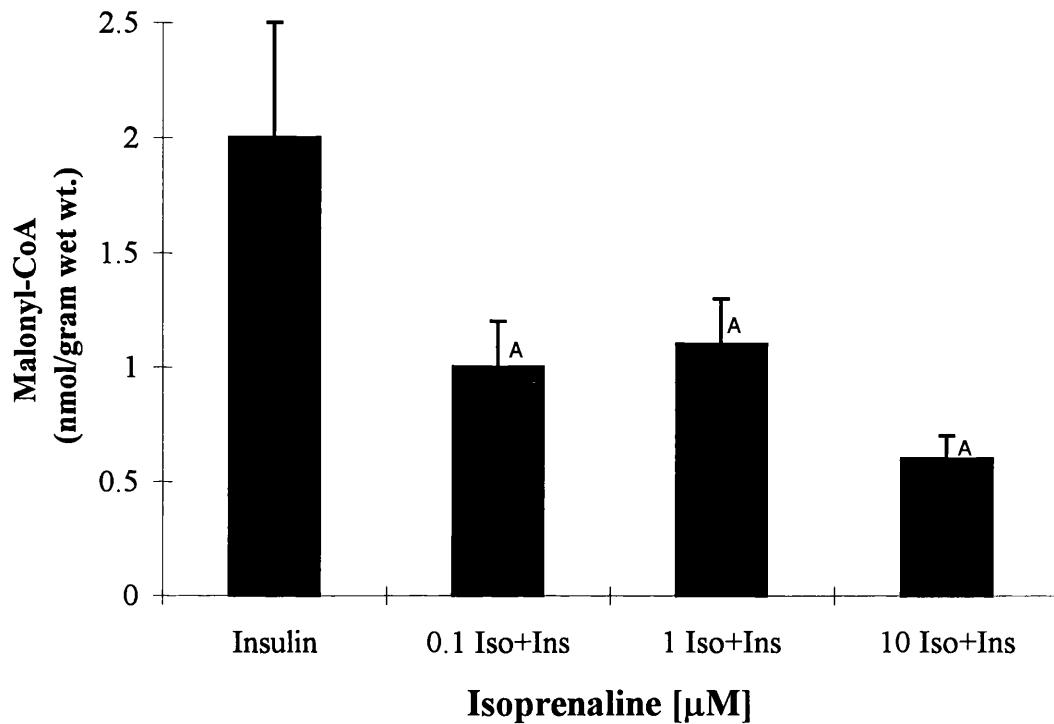
The data also strongly suggest that isoprenaline antagonises the effects of insulin.

A possible mechanism for isoprenaline causing this fall in malonyl-CoA content in muscle is suggested below.

It can be suggested that activated protein kinase A, as a result of increased cAMP levels due to isoprenaline, could phosphorylate and inactivate ACC activity. Winder *et al*, (1997) have shown ACC to be a substrate for protein kinase A. However protein kinase A-mediated phosphorylation has no effect on ACC activity. Possibly isoprenaline, acting via protein kinase A, regulates other enzymes involved in the synthesis and disposal of malonyl-CoA, e.g. malonyl-CoA decarboxylase and ATP-citrate lyase. Malonyl-CoA decarboxylase is the enzyme involved in the disposal of malonyl-CoA (section 3.3.2). Malonyl-CoA decarboxylase activity has been demonstrated in both the cytosolic and mitochondrial fraction of the soleus muscle. Possibly isoprenaline could activate cytosolic malonyl-CoA decarboxylase by protein kinase A or by some unidentified effector. This would result in decarboxylation of malonyl-CoA to acetyl-CoA, leading to

Fig 3.20

**Isoprenaline Significantly Decreases Malonyl-CoA
Levels In Soleus Muscle**



Isolated soleus muscles were incubated for 60 min as described in Materials & Methods (section 2.5.1) with 5mM glucose and 2% BSA. The concentration of insulin was 10^{-8} M and isoprenaline concentrations are stated in the figure.

Results are expressed as mean \pm SEM (n = 4).

^A indicates $P < 0.025$ versus insulin

a lowering of malonyl-CoA levels and relieving inhibition of CPT 1. This would allow fatty acids to be transported to the mitochondrial matrix where they undergo β -oxidation.

Possibly isoprenaline could suppress ATP-citrate lyase activity, by protein kinase A phosphorylating ATP-citrate lyase. This would result in a decrease in supply of cytosolic acetyl-CoA for ACC and subsequently a fall in malonyl-CoA production.

However the regulation of ATP-citrate lyase activity is not fully understood and the mechanism suggested above is just a speculation.

3.3.5.2 Long chain esters

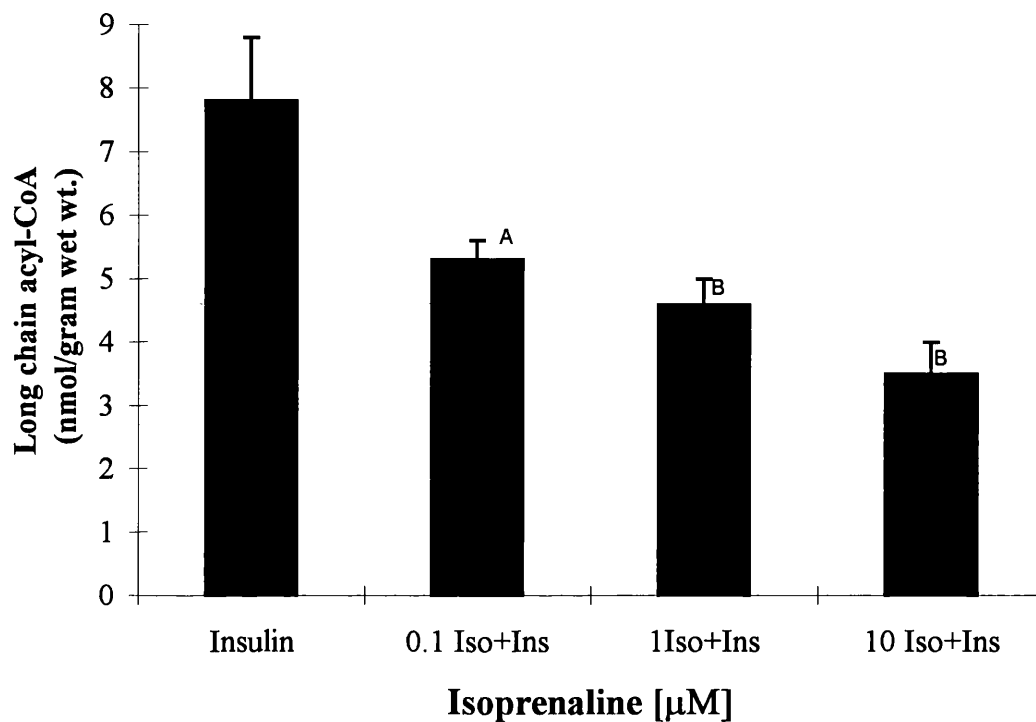
Isoprenaline at all concentrations, significantly decreased long chain fatty acyl-CoA content in soleus muscle (fig 3.21). 10 μ M isoprenaline had the greatest effect on long chain fatty acyl-CoA content, lowering it by 51%. 1 μ M and 10 μ M isoprenaline significantly increased both long chain acylcarnitine and combined total esters (fig 3.22 & fig 3.23). 0.1 μ M isoprenaline increased both long chain fatty acyl-CoA and long chain acylcarnitine, but the effect was insignificant (fig 3.21 & fig 3.22). Both 1 μ M and 10 μ M isoprenaline increased total long chain esters significantly, but 0.1 μ M isoprenaline did not alter the content of total long chain esters

A significant increase in the ratio of long chain acylcarnitine/long chain acyl-CoA was demonstrated at all concentrations of isoprenaline (fig 3.24).

This suggests that isoprenaline in the presence of insulin overcomes the effects of insulin and causes an increase in total long chain esters, especially long chain acylcarnitine levels. This could be attributed to a lipolytic effect of isoprenaline on muscle. Evidence for a lipolytic effect in the presence of isoprenaline came from fatty acid oxidation

Fig 3.21

Isoprenaline In The Presence Of Insulin Lowers Long Chain Acyl-CoA Levels In Soleus Muscle



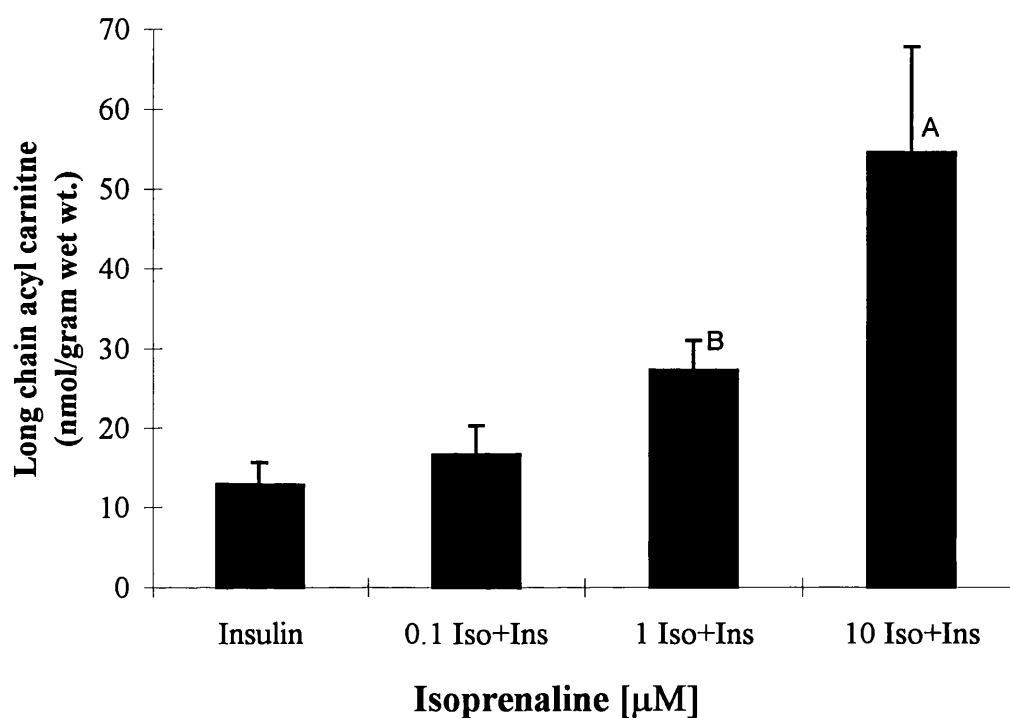
Isolated soleus muscles were incubated for 60 min as described in the Materials & Methods(section 2..5) with 5mM glucose and 2% BSA. The concentrations of insulin was 10^{-8} M and isoprenaline concentrations are stated in the figure.

Results are expressed as mean \pm SEM (n = 4).

^{A, B} indicates $P < 0.05 < 0.025$ versus insulin

Fig 3.22

**Isoprenaline In The Presence Of Insulin Increases
Long Chain Acyl Carnitine Levels In Soleus Muscle**



Isolated soleus muscles were incubated for 60 min as described in the Materials & Methods (section 2.5) with 5mM glucose and 2% BSA.

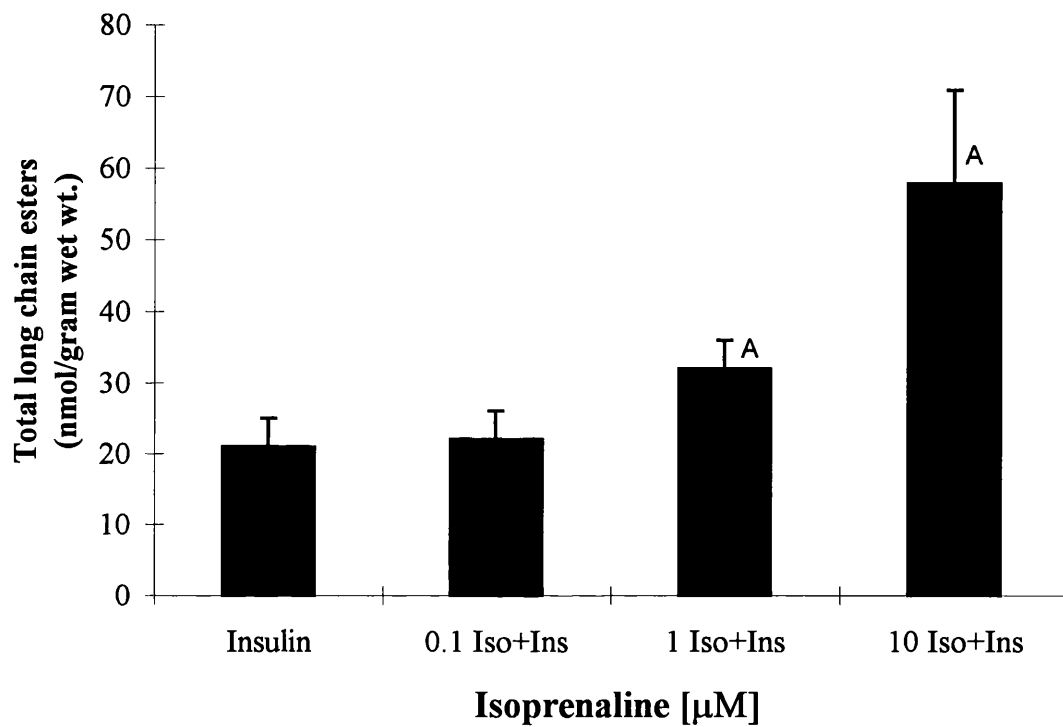
The concentration of insulin was 10^{-8} M and isoprenaline concentrations are stated in the figure.

Results are expressed as mean \pm SEM (n = 4).

^{A,B} indicates $P < 0.05$, < 0.025 versus insulin

Fig 3.23

**Isoprenaline In The Presence Of Insulin Increases
The Combined Level of Long Chain Acyl-CoA &
Carnitine Esters**



Isolated soleus muscles were incubated for 60 min as described in Materials & Methods (section 2.5) with 5mM glucose and 2% BSA.

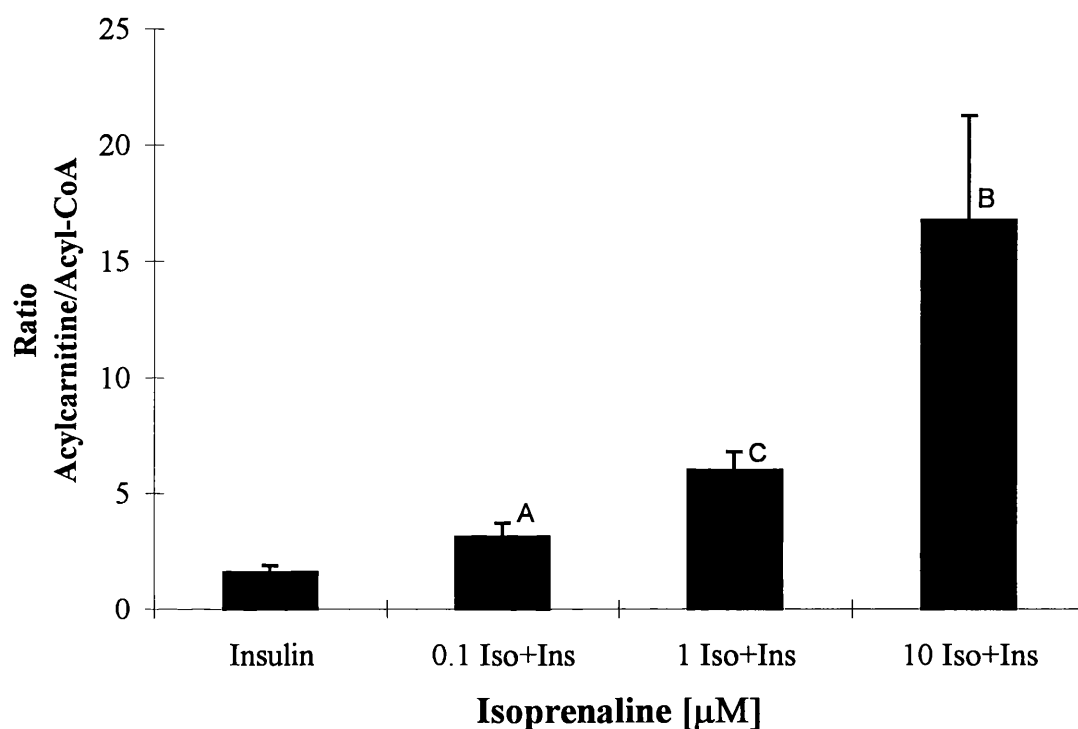
The concentration of insulin was 10^{-8} M and isoprenaline concentrations are stated in the figure.

Results are expressed as mean \pm SEM (n = 4).

^A indicates $P < 0.05$ versus insulin

Fig 3.24

**Isoprenaline In The Presence Of Insulin Increases
The Ratio Of Long Chain AcylCarnitine : Long
Chain Acyl-CoA**



Isolated soleus muscles were incubated for 60 min as described in the Materials & Methods (section 2.5)with 5mM glucose and 2% BSA.

The concentration of insulin was 10^{-8} M and isoprenaline concentrations are stated in the figure.

Results are expressed as mean \pm SEM (n = 4).

A,B,C indicates $P < 0.05$, < 0.025 , < 0.005 versus insulin

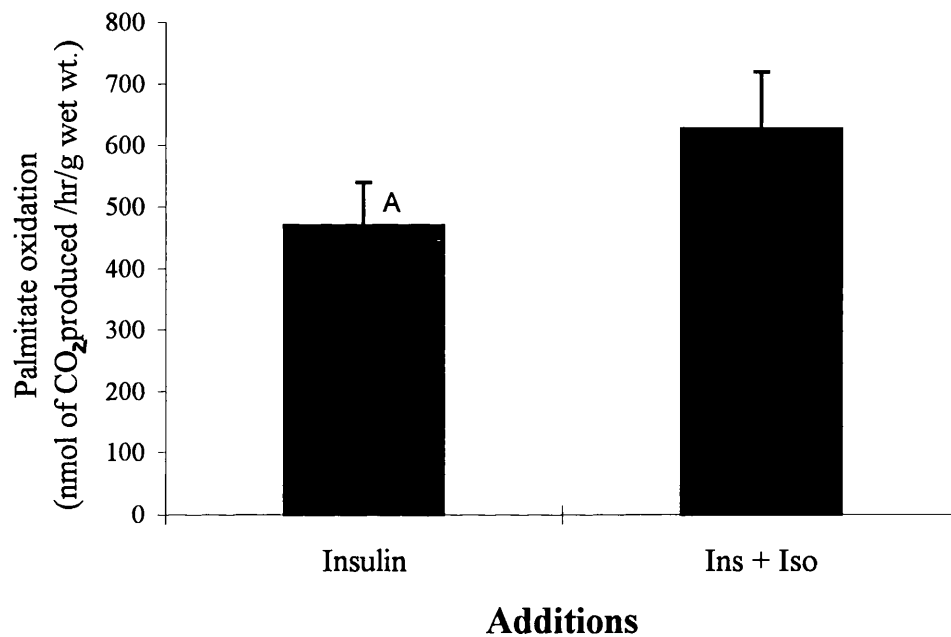
measurements. It was observed at the lowest concentration of isoprenaline (0.1 μ M) that both endogenous and exogenous [14 C] palmitate oxidation was increased (a 82 % and 22% increases respectively) (fig 3.25). This resulted in the ratio of exogenous/endogenous fatty acid oxidation to fall (table 3.8). This suggests that isoprenaline stimulates lipolysis in muscles. It can be suggested that isoprenaline stimulates lipolysis in muscle most probably by a cAMP-dependent mechanism involving HSL which is present in skeletal muscle (Oscai *et al*, 1990, Holm *et al*, 1987 and Holm *et al*, 1988). Isoprenaline has also been shown to stimulate lipolysis in adipocytes by elevating cAMP levels. This results in activation of PKA and in turn phosphorylates and activates HSL (Sullivan *et al*, 1995).

The increase in fatty acid oxidation with isoprenaline suggests that CPT 1 activity is enhanced and coincides with the increase in the ratio of acylcarnitine/acyl-CoA (fig 3.24 & fig 3.25).

Finally, it can be suggested that the decrease in malonyl-CoA content observed in the presence of isoprenaline might be due to phosphorylation and inactivation of muscle ACC by AMPK. As discussed further in section 3.3.6, this might occur if AMP is increased as a consequence of increased fatty acid/fatty acyl-CoA turnover.

Fig 3.25

**Isoprenaline In The Presence Of Insulin Stimulates
Exogenous[U-¹⁴C] Palmitate Oxidation In Isolated Soleus
Muscle Strips**



Incubations were for 1 hour at 37⁰C and contained 2% BSA, 5mM glucose and 0.2mM [U-¹⁴C] palmitate, as described in Materials & Methods (section 2.8.1).

Other additions were: insulin 10⁻⁸M and isoprenaline 0.1μM

Values are means ± SEM (n=5)

^A indicates P<0.025 versus Ins +Iso

Table 3.8

Measurements Of Exogenous And Endogenous Palmitate Oxidation In Soleus Muscle
(with Isoprenaline)

Addition to incubation	Exogenous oxidation (d.p.m. per g wet wt./h) (A)	Endogenous oxidation (d.p.m. per g wet wt./h) (B)	Ratio A/B	Exogenous oxidation (nmol CO ₂ per g wet wt./h)
Insulin	8737 ± 1128	2287 ± 450	4.5 ± 1.2	489 ± 63
Insulin + 0.1 µM Isoprenaline	11195 ± 1676 ^B	4662 ± 530 ^C	2.4 ± 0.2 ^A	627 ± 49 ^B

Incubations were for 1 hour at 37°C and contained 2 % BSA, 5mM glucose and 0.2 mM [1-¹⁴C] palmitate oxidation, as described in the Materials and Methods (section 2.8.2).

Values are means ± SEM and n = 5 throughout.

^{A, B, C} indicate P < 0.05, < 0.025 < 0.01 for paired effects of Isoprenaline.

3.3.6 THE EFFECTS OF PALMITATE ON METABOLITE LEVELS

0.5mM palmitate was added to incubation media containing insulin + glucose or glucose alone (none). Incubations with palmitate were compared against basal conditions (glucose only). Incubation with palmitate + insulin were compared against insulin alone.

3.3.6.1 Malonyl-CoA

Palmitate in the presence of insulin significantly decreased the malonyl-CoA content in soleus muscle by 58 % (table 3.9). This finding is consistent with that found in perfused heart (Awan & Saggerson, 1993).

Palmitate in the presence of insulin completely antagonised the effect of insulin on malonyl-CoA content, and is similar to the finding found with isoprenaline (section 3.3.5.1)

It is interesting to note that, palmitate alone had no significant effect on malonyl-CoA content in muscle.

The mechanism by which palmitate decreases malonyl-CoA levels is discussed in section 3.3.6.2.

3.3.6.2 Long Chain Esters

In principle, on addition of palmitate we would expect an increase in long chain fatty acyl-CoA as there is an increase in fatty acid being supplied to the muscle. It was observed however that long chain acyl-CoA levels significantly decreased in the presence of palmitate + insulin in comparison to insulin alone (Table 3.9). This contradicts what Awan & Saggerson (1993) found in perfused heart with palmitate (an increase in long chain acyl-CoA level). Both long chain acylcarnitine and the combined levels of long chain esters increased, but this effect was not significant (Table 3.9). However, the ratio

Table 3.9

Fatty Acid Metabolite Measurements In The Presence Of Palmitate

Addition to incubation	Malonyl-CoA (nmol/g wet wt.)	Long chain fatty acyl-CoA (nmol/g wet wt.) (A)	Long chain acylcarnitine (nmol/g wet wt.) (B)	Total long chain esters (A + B)	Ratio B/A
None	1.6 ± 0.2	4.3 ± 0.8	22.5 ± 1.7	27 ± 2	5.9 ± 1.3
Insulin	2.9 ± 0.3 ^C	8.6 ± 1.8 ^A	17.4 ± 3.2	26 ± 4	2.3 ± 0.7 ^B
Palmitate	1.4 ± 0.1	4.0 ± 0.7	37.9 ± 6.3	42 ± 5	11.3 ± 3.5
Ins +Pal	1.2 ± 0.1 ^F	3.3 ± 0.6 ^E	30.4 ± 7.2	34 ± 7	9.6 ± 2.4 ^D

Isolated soleus muscles were incubated for 60 min as described in the Materials & Methods (section 2.5), with 5mM glucose and 2% BSA.

The concentrations of the other additions were insulin 10⁻⁸M, 0.5mM palmitate.

Results are expressed as mean ± SEM n = 5 for malonyl-CoA and 4 for other measurements.

^{A, B, C} indicate P<0.05, <0.01, <0.005 respectively versus incubations with no addition. ^{D, E, F} indicate P<0.05, <0.025, <0.005 respectively versus incubations with insulin alone.

of long chain acylcarnitine/long chain acyl-CoA increased significantly in the presence of palmitate + insulin.

From these studies it is concluded that the effects of insulin on fatty acid metabolites are reversed in the presence of palmitate (section 3.3.2.1 & 3.3.2.2). This was also seen when the concentration of [^{14}C] palmitate was raised to 0.5 mM (fig 3.8). At 0.5 mM palmitate, it virtually abolished the effect of insulin to decrease fatty acid oxidation.

The effects of 0.5 mM palmitate (with insulin) were similar to those seen with isoprenaline. From the findings with isoprenaline and palmitate it can be suggested that an increased supply of long chain fatty acids from either exogenous/endogenous sources could result in increased throughput at the fatty acyl-CoA synthetase reaction with a concomitant formation of AMP. If the production of AMP is sufficient then it could activate AMPK (or AMPKK) leading to the phosphorylation and inactivation of ACC. This would result in a fall in tissue malonyl-CoA content as seen in the presence of palmitate or isoprenaline .

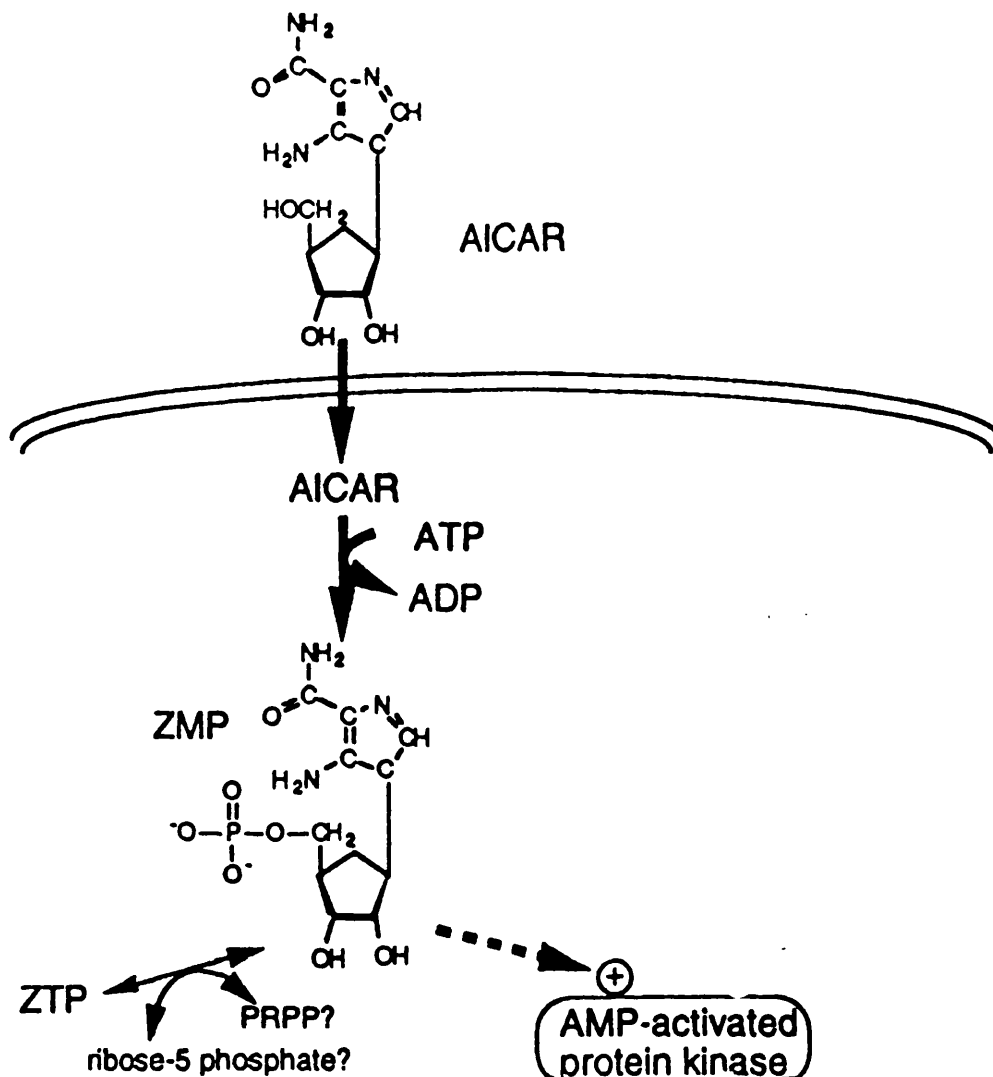
In summary, this study clearly shows that 0.5 mM palmitate, in the presence of insulin, overcomes the effects of insulin. This suggests that palmitate relieves inhibition of CPT 1 by malonyl-CoA, allowing activated long chain fatty acyl-CoA units to be transported across to the mitochondrial matrix, where they undergo β -oxidation.

3.3.7 THE EFFECTS OF AICAR ON METABOLITE LEVELS

AICAR is the cell permeable precursor of ZMP (5-aminoimidazole-4-carboxamide ribonucleoside monophosphate). ZMP is an intermediate of the *de novo* pathway of purine biosynthesis (Sabine *et al*, 1985a). AICAR has been shown to be taken up and metabolised to ZMP in a variety of mammalian tissues, including erythrocytes, CHO-K1 cells, fibroblasts heart and skeletal muscle (Sabina *et al*, 1985a & 1992). AICAR is transported across the plasma membrane, probably via the adenosine nucleoside transporter. Once inside the cell, it is phosphorylated presumably by adenosine kinase to ZMP see fig 3.26. ZMP inside the cell can be metabolised to IMP (inosine monophosphate) which in turn can undergo conversion into adenine and guanine nucleotides or degradation to terminal purine catabolites (hypoxanthine or uric acid). ZMP can also be phosphorylated to ZTP (fig 3.26) (Sabina *et al*, 1985 b)

AICAR, once converted to ZMP inside the cell, has been shown to affect a number of metabolic processes in adipocytes, hepatocytes and skeletal muscle. In isolated hepatocytes, ZMP was shown to inhibit gluconeogenesis and glycolysis (Vincent *et al*, 1991 & 1992). The effects of ZMP on gluconeogenesis are probably due to ZMP mimicking the effects of AMP on fructose-1-6-bisphosphatase (Vincent *et al*, 1991). ZMP also inactivates PFK-2, the enzyme that synthesises fructose-2-6-bisphosphate. This leads to a decrease in the concentration of fructose-2,6-bisphosphate, the main stimulator of PFK-1 (Vincent *et al*, 1992).

In adipocytes and hepatocytes, incubation with AICAR has led to an accumulation of ZMP, resulting in activation of AMPK which phosphorylates HSL at the basal site and inhibits lipolysis (Sullivan *et al*, 1994 and Corton *et al*, 1995). This suggests that AICAR



(Adapted from Sullivan *et al*, 1994)

Figure 3.26 Proposed mechanism of action of AICAR in intact cells

AICAR is transported across the plasma membrane (probably by adenosine transporter) and phosphorylated to ZMP in the cytoplasm. ZMP then activates AMPK as described in section 3.3.7. Phosphorylation of ZMP to ZTP may be by reversal of phosphoribosylpyrophosphate (PRPP) synthetase (Sabina *et al*, 1985a)

is an antilipolytic agent. Evidence for this came from adipocytes incubated with AICAR which had a reduced response to the lipolytic agent isoprenaline (Sullivan *et al*, 1994).

In adipocytes, AICAR also inhibits lipogenesis by ZMP activating AMPK and phosphorylating ACC. AICAR decreases the V_{\max} of ACC resulting in a 40% inhibition of ACC activity (Sullivan *et al*, 1994).

More recently, AICAR in soleus muscle has been demonstrated to activate glycogen phosphorylase and glycogenolysis by the activation of AMPK (Young *et al*, 1996).

The most likely mechanism by which AICAR exerts these metabolic effects is as follows: AICAR inside the cell results in an accumulation of ZMP. ZMP, by mimicking the effects of AMP, activates AMPK both by direct allosteric activation and by promoting increased phosphorylation by AMPK kinase (Carling *et al*, 1989 Weekes *et al*, 1994 Corton *et al*, 1995 and Sullivan *et al*, 1995). Hardie's group showed that even though ZMP mimics the effects of AMP on AMPK, it is a weaker activator than AMP (Corton *et al*, 1995).

The purpose of using AICAR in our studies was to see whether AICAR antagonised the effects of insulin similar to isoprenaline and palmitate.

Isolated soleus muscle strips were preincubated with AICAR (1mM) and then incubated with 1mM AICAR as described in the Materials and Methods (section 2.4.1).

1mM AICAR was used throughout, as this is the concentration of AICAR used in soleus muscle studies by Young *et al* (1996).

Incubations were carried out with AICAR alone and insulin +AICAR together. Experiments with AICAR alone were compared to the basal state (glucose only). Insulin + AICAR experiments were compared to insulin alone.

3.3.7.1 Malonyl-CoA

AICAR alone significantly reduced malonyl-CoA content in the soleus muscle, by 65 % (fig 3.27)

This suggests that AICAR is taken up into the muscle cell and is converted to ZMP which then activates AMPK resulting in phosphorylation and inactivation of ACC. Further evidence that AICAR is taken up by muscle and is converted to ZMP is shown in fig 3.33 (section 3.3.7.3)

In the presence of insulin, AICAR significantly decreased the malonyl-CoA content in the soleus muscle by approximately 64%, in comparison to insulin (fig 3.27).

This implies that AICAR in the presence of insulin appears to antagonise the effects of insulin, by decreasing the malonyl-CoA levels. The mechanism by which AICAR antagonises the insulin effect is probably by the mechanism suggested above.

3.3.7.2 Long Chain Esters

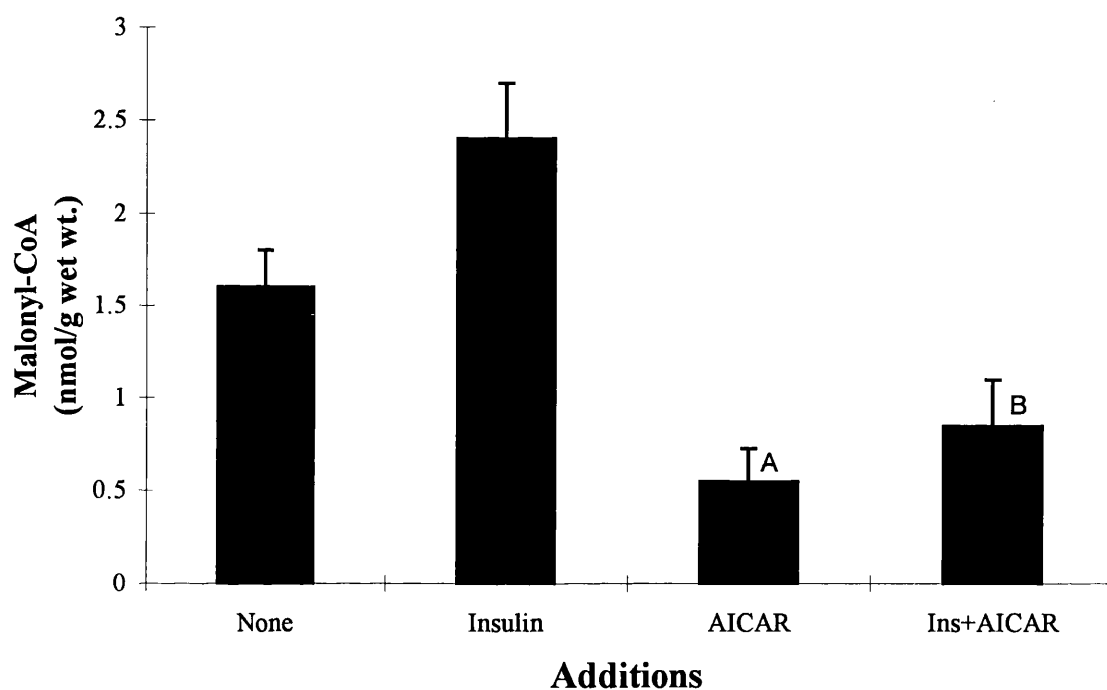
Long chain fatty acyl-CoA content was not altered by comparison with the basal state in the presence of AICAR alone (fig 3.28).

Long chain acylcarnitine content in soleus muscle was significantly reduced in the presence of AICAR alone (fig 3.29). The total long chain esters fell slightly in the presence of AICAR alone, but the effect was insignificant (fig 3.30). The ratio of long chain acylcarnitine/long chain acyl-CoA was significantly reduced by AICAR alone (fig 3.31).

AICAR, in the presence of insulin significantly decreased the long chain fatty acyl-CoA content in the soleus muscle by approximately 20% in comparison to insulin alone (fig 3.28). A small but insignificant fall in long chain acylcarnitine content also occurred in

Fig 3.27

**Malonyl-CoA Measurements In Isolated Soleus
Muscle In the Presence of AICAR**



Isolated Soleus strips were incubated for 60 min as described in the Materials & Methods (section 2.5), containing 5mM glucose and fatty acid poor albumin (20mg/ml).

The concentrations of additions were: insulin 10^{-8} M, AICAR 1mM.

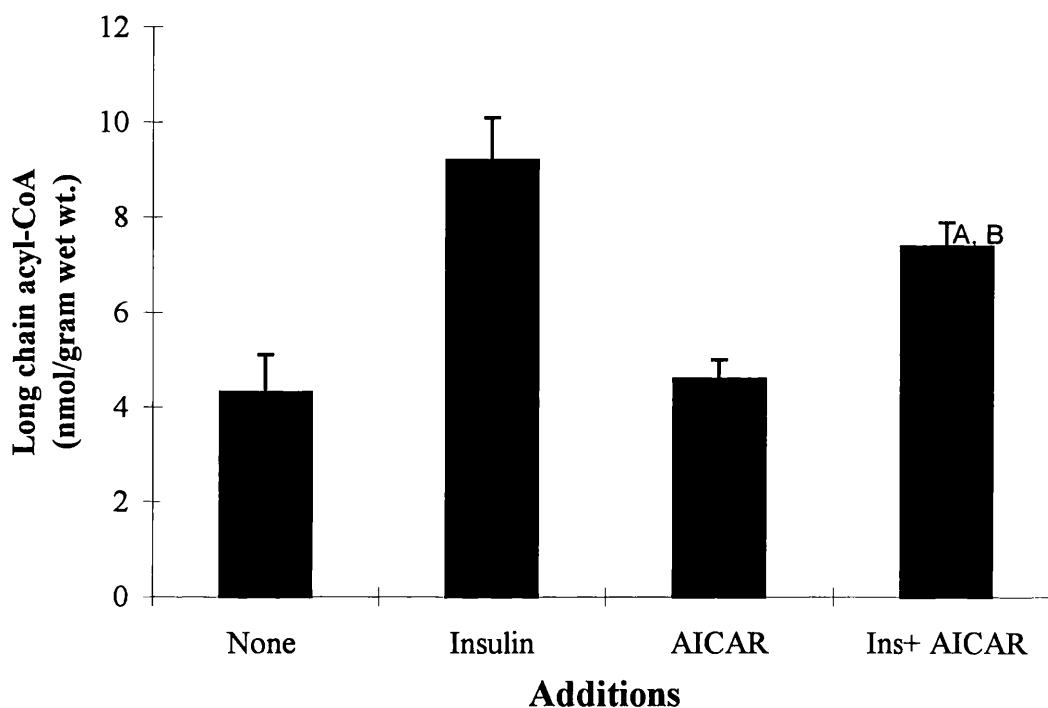
Results are expressed as means \pm SEM. n=5

^A, indicates $P < 0.01$ versus None (unpaired).

^B indicates $P < 0.0005$ versus insulin.

Fig 3.28

**Long Chain Acyl-CoA Measurements In Soleus
Muscle In The Presence Of AICAR**



Isolated Soleus strips were incubated for 60 min as described in the Materials & Methods (section 2.5), containing 5mM glucose and fatty acid poor albumin (20mg/ml).

The concentrations of additions were: insulin 10^{-8} , AICAR 1mM.

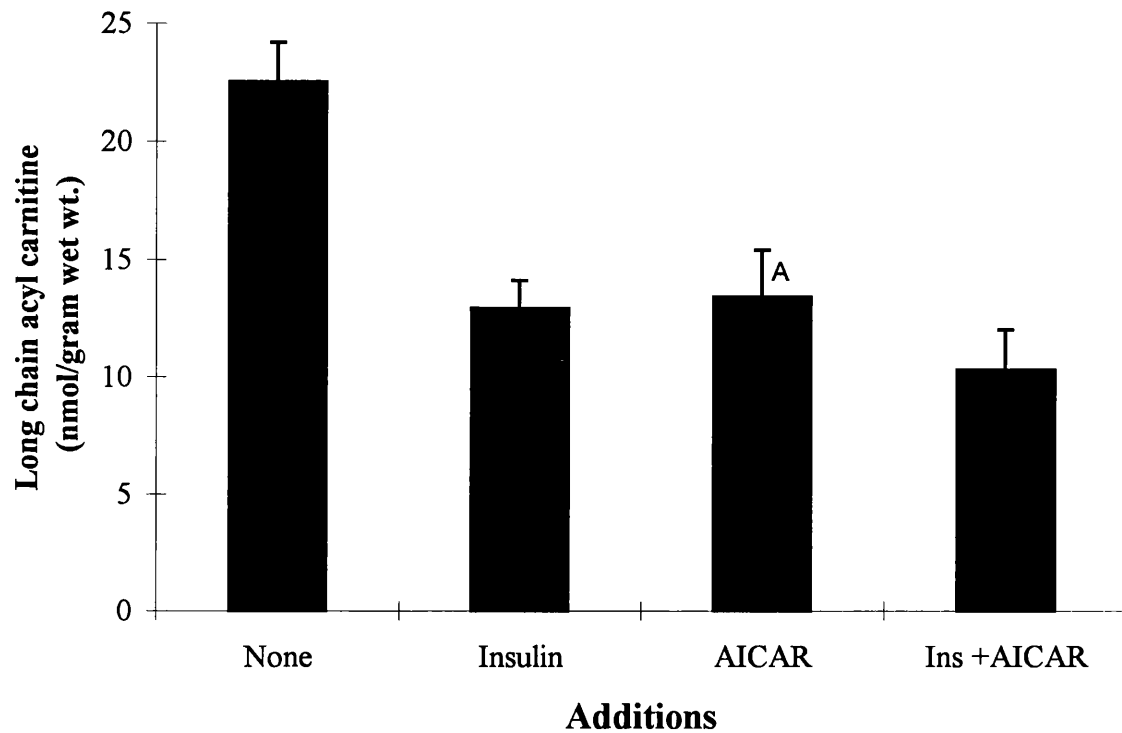
Results are expressed as means \pm SEM. n = 4

^A indicates $P < 0.025$ versus insulin.

^B indicates $P < 0.01$ versus AICAR alone.

Fig 3.29

Long Chain AcylCarnitine Measurements In The Soleus Muscle In The Presence Of AICAR



Isolated Soleus strips were incubated for 60 min as described in the Materials & Methods (section 2.5), containing 5mM glucose and fatty acid poor albumin (20mg/ml).

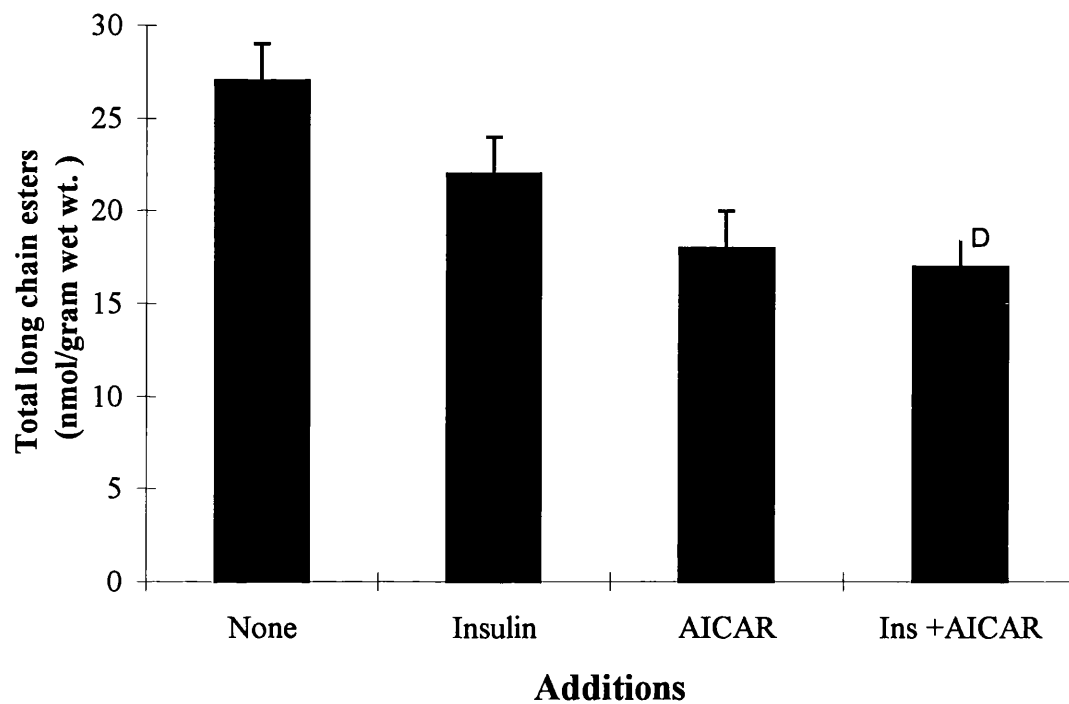
The concentrations of additions were: insulin 10^{-8} , AICAR 1mM.

Results are expressed as means \pm SEM. n = 4

^A indicates $P < 0.05$ versus none.

Fig 3.30

**Measurements Of Total Long Chain Esters In The
Presence Of AICAR**



Isolated Soleus strips were incubated for 60 min as described in the Materials & Methods (section 2.5), containing 5mM glucose and fatty acid poor albumin (20mg/ml).

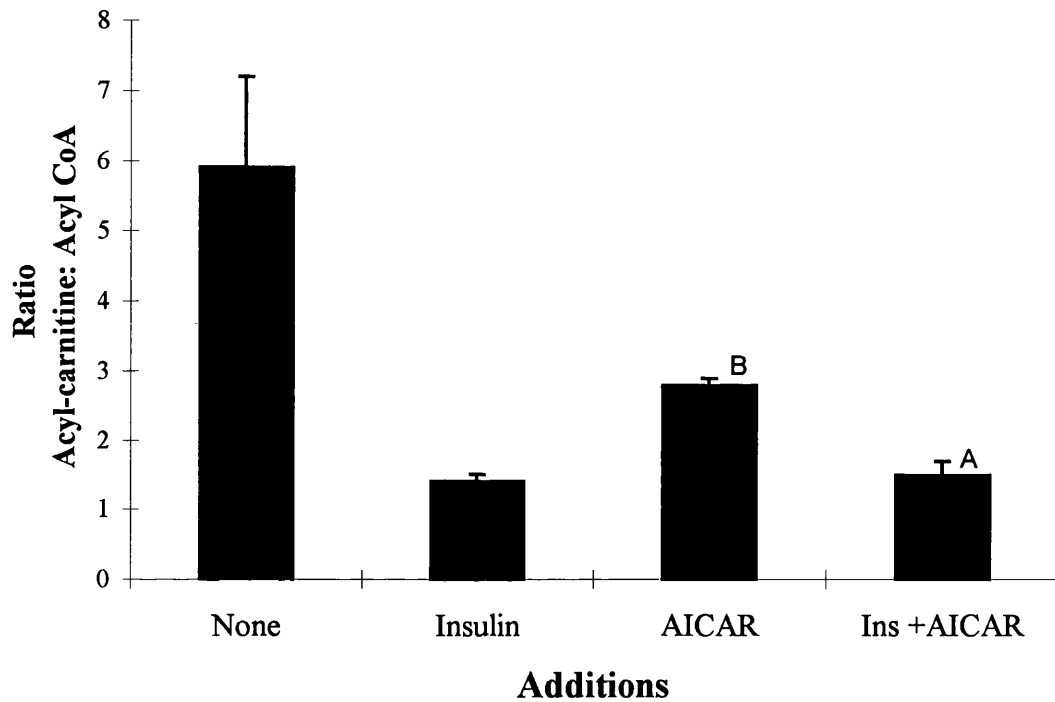
The concentrations of additions were: insulin 10^{-8} M, AICAR 1mM.

Results are expressed as means \pm SEM. n = 4

^D indicates $P < 0.05$ versus insulin.

Fig 3.31

**The Ratio Of Acyl-Carnitine : Acyl-CoA In The
Presence of AICAR**



Isolated Soleus strips were incubated for 60 min as described in the Materials & Methods (section 2.5), containing 5mM glucose and fatty acid poor albumin (20mg/ml).

The concentrations of additions were: insulin 10^{-8} M, AICAR 1mM.

Results are expressed as means \pm SEM. n = 4

^A indicates $P < 0.005$ versus AICAR alone.

^B indicates $P < 0.001$ versus none (unpaired)

the presence of AICAR + insulin (fig 3.29). The combined long chain esters were reduced significantly in the presence of AICAR + insulin (fig 3.30). However, AICAR + insulin did not alter the ratio of long chain acyl-carnitine : long chain acyl-CoA (fig 3.31).

AICAR in the presence of insulin was only slightly effective (20 %) in overcoming the increase in long chain acyl-CoA content seen with insulin. A significant 61 % insulin-dependent increase in long chain acyl-CoA was still seen, even in the presence of AICAR. This increase in long chain acyl-CoA content seen in the presence of insulin with AICAR, even when AICAR decreased malonyl-CoA, suggested that AICAR inhibits an alternative route of disposal of cytosolic fatty acyl-CoA, e.g. glycerolipid synthesis.

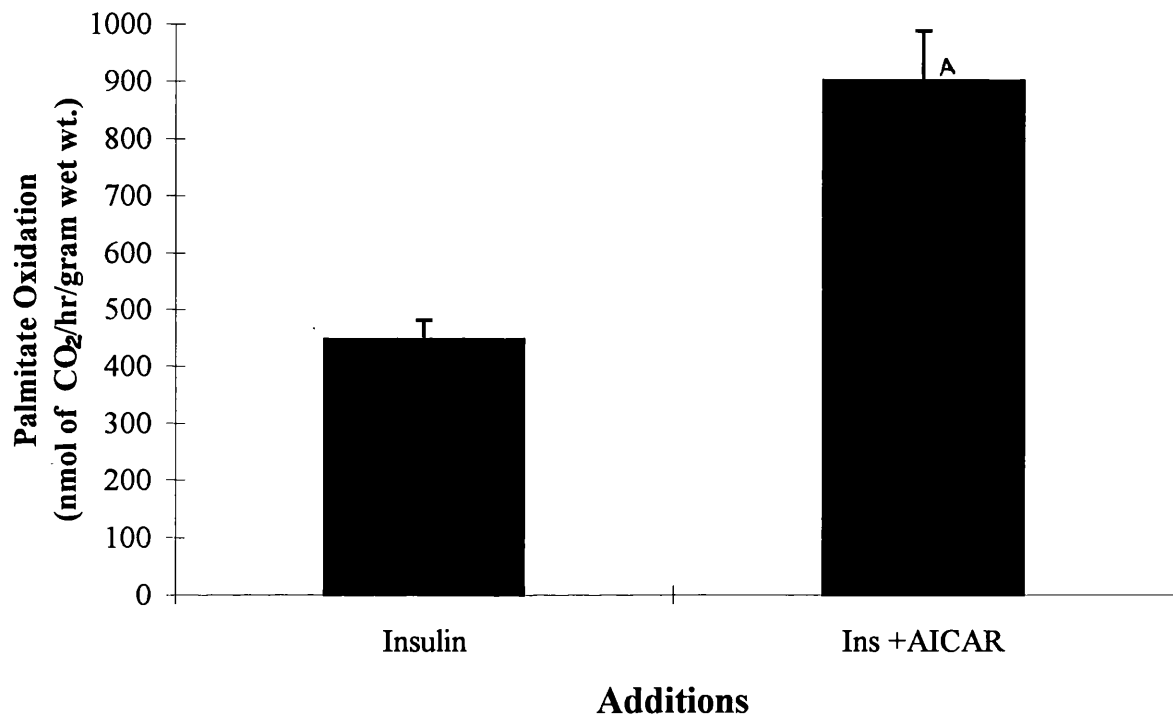
The decrease in total combined long chain esters with AICAR suggests that AICAR maybe having an antilipolytic effect on muscle. Fig 3.32 and table 3.10 shows that AICAR in the presence of insulin significantly increased exogenous palmitate oxidation by 102% (fig 3.32) but had no significant effect on endogenous palmitate oxidation (table 3.10). The ratio of exogenous palmitate oxidation : endogenous palmitate oxidation rose suggesting that AICAR decreased lipolysis in muscle.

In adipocytes AICAR acts as an antilipolytic agent, as adipocytes preincubated with AICAR have a reduced response to lipolytic agents (e.g. isoprenaline) (Corton *et al*, 1995). The antilipolytic effect of AICAR is presumably mediated via ZMP by activating AMPK and phosphorylating HSL. Phosphorylation of HSL opposes the phosphorylation by protein kinase A at the regulatory site and prevents the release of fatty acids by lipolysis.

Presumably the antilipolytic effect of AICAR in muscle is mediated by the same mechanism.

Fig 3.32

AICAR In The Presence Of Insulin Increases
Exogenous [U-¹⁴C] Palmitate Oxidation In Isolated
Muscle Strips



Incubations were for 1 hour at 37°C and contained 2% BSA, 5mM glucose and 0.2mM [U-¹⁴C] palmitate, as described in the Materials & Methods (section 2.8.1)

Other additions were insulin 10⁻⁸M and AICAR 1mM.

Values are means ± SEM (n=5).

^A indicates P<0.005 versus insulin

Table 3.10

Measurements Of Exogenous And Endogenous Palmitate Oxidation In Soleus Muscle
(with AICAR)

Addition to incubation	Exogenous oxidation (d.p.m. per g wet wt./h) (A)	Endogenous oxidation (d.p.m. per g wet wt./h) (B)	Ratio A/B	Exogenous oxidation (nmol CO ₂ per g wet wt./h)
Insulin	7759 ± 651	5601 ± 514	1.4 ± 0.2	447 ± 34
Insulin + 1mM AICAR	15675 ± 1611 ^A	5603 ± 1352	3.4 ± 0.7	903 ± 87 ^A

Incubations were for 1 hour at 37°C and contained 2 % BSA, 5mM glucose and 0.2 mM [1-¹⁴C] palmitate oxidation, as described in the Materials and Methods (section 2.8.2).

Values are means ± SEM and n = 5 throughout.

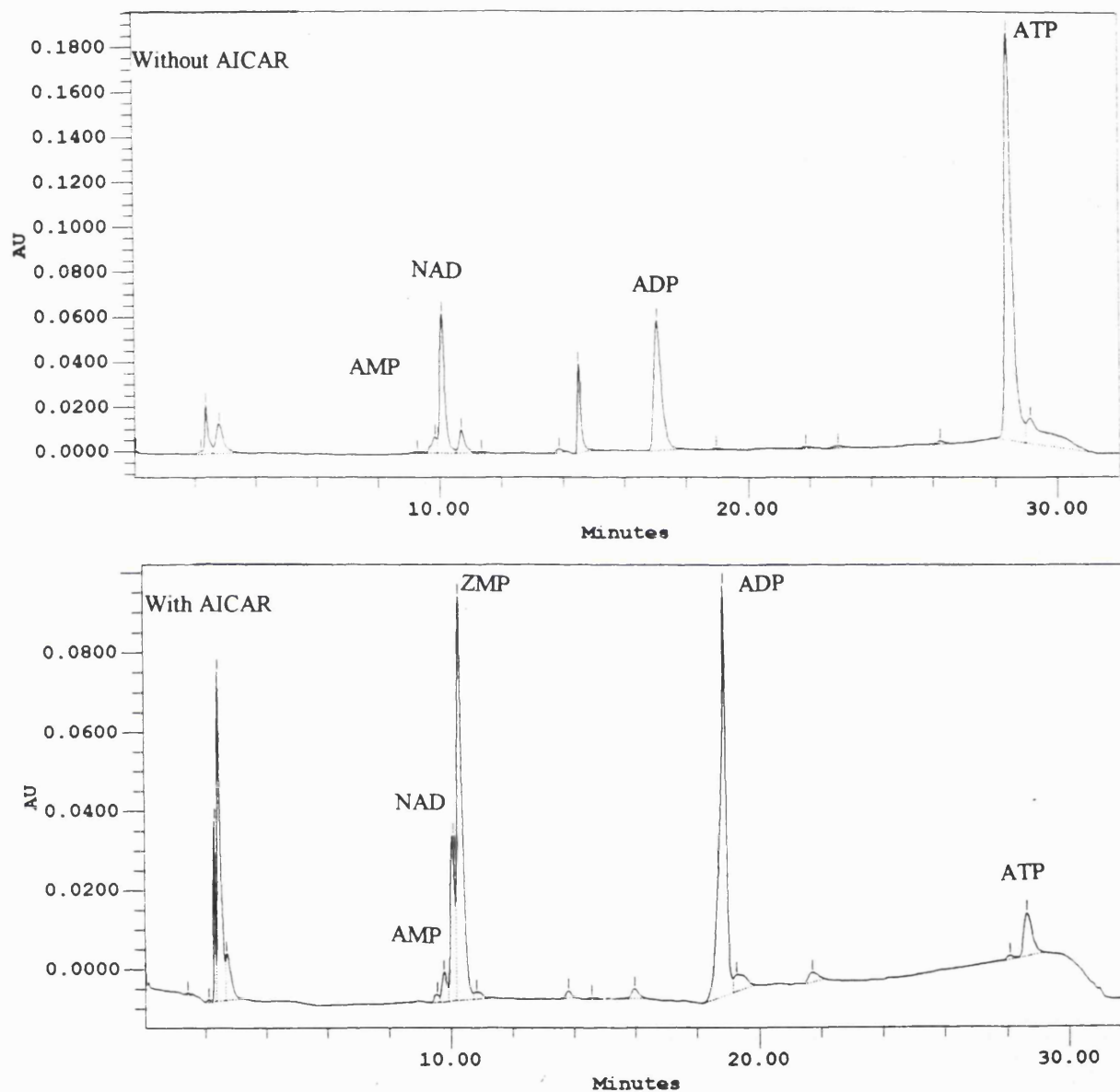
^A, indicate P<0.005 for paired effects of AICAR.

3.3.7.3 Nucleotide Analysis

Soleus muscle extracts incubated with/without AICAR were applied to an HPLC anion exchange column as described in the Materials and Methods (section 2.6.4). With AICAR a novel peak appeared with an elution time of approximately 10 min (fig 3.33). If a ZMP internal standard was added to a sample from tissue incubated with AICAR, it was seen that the ZMP comigrated with the novel peak (not shown). This confirms that AICAR was taken up by isolated soleus muscle strips resulting in an accumulation of ZMP. The amount of ZMP present in the muscle was calculated, and was found to be 428 nmol/g wet wt. of muscle (n=2). If we assume that muscle contains 650 mg H₂O/ g wet wt. of muscle, then the concentration of ZMP present in the pooled soleus strips is ~ 658 μM. This value is not much different from that calculated by Young *et al*, (1996) (460-720 μM).

Figure 3.33 Analysis of nucleotides from isolated soleus muscle strips

incubated with AICAR



Soleus muscle strips were incubated with/without AICAR for 60 min. Muscles were extracted in perchloric acid and analysed by ion-exchange HPLC (as described in section 2.6.4). Nucleotide peaks labelled (ADP, AMP, ATP, NAD and ZMP) were identified by spiking identical samples with internal standards of nucleotides (not shown).

Conclusions

- Palmitate, isoprenaline and AICAR were all shown to decrease malonyl-CoA levels in the soleus muscle.
- Several mechanisms have been suggested by which isoprenaline decreases malonyl-CoA levels in muscle. Firstly, isoprenaline lowers malonyl-CoA levels possibly by acting via protein kinase and activating malonyl-CoA decarboxylase. This would result in decarboxylation of malonyl-CoA to acetyl-CoA and hence a fall in malonyl-CoA levels. Secondly, isoprenaline was shown to have a lipolytic effect in muscle. This would result in an increase in long chain fatty acids. Prior to oxidation they are converted to long chain fatty acyl-CoA by fatty acyl-CoA synthetase with concomitant formation of AMP. Presumably AMP could then activate AMPK (or AMPK) and which in turn could phosphorylate and inactivate ACC and thereby cause a fall in malonyl-CoA levels. Hardie & Carling, (1997), recently suggested that under lipolytic conditions in adipocytes, AMP activates AMPK. It would be interesting to see whether this actually occurs in skeletal muscle under lipolytic conditions.
- Isoprenaline was shown to have a lipolytic effect on muscle and evidence for this came from the significantly increased endogenous fatty acid oxidation relative to exogenous fatty acid oxidation. The mechanism by which isoprenaline stimulates lipolysis in muscle is probably by isoprenaline elevating cAMP levels. cAMP probably activates PKA and in turn stimulates HSL and releases fatty acids.
- Muscle malonyl-CoA content falls in the presence of isoprenaline coinciding with increases in the ratio of long acylcarnitine/long chain acyl-CoA and of fatty acid oxidation. From this it can be suggested that isoprenaline enhances CPT 1 activity and fatty acid oxidation.

- Palmitate opposed the effects of insulin and the findings with palmitate are very similar with those found with isoprenaline.
- From the HPLC analyses it can be concluded that AICAR is taken up by the soleus muscle cells and is converted to ZMP, most probably by adenosine kinase.
- AICAR alone and AICAR in the presence of insulin, both appear to decrease malonyl-CoA content in the soleus muscle. The mechanism by which it exerts its effects is presumed to be via ZMP mimicking the effects of AMP to activate AMPK (or AMPK). Activated AMPK can then phosphorylate and inactivate ACC activity, resulting in a fall in malonyl-CoA production. In adipocytes it has been shown that AICAR inhibits ACC activity by 40% (Sullivan *et al*, 1994). From these studies we do not know the extent of AICAR on inhibition of ACC, but the results from this study suggests that ACC activity might be regulated by phosphorylation (via AMPK). *In vitro* studies have shown ACC to be regulated by phosphorylation. Winder *et al*, (1996) (section 1.6.3.1) showed that liver AMPK phosphorylates and inactivates purified skeletal muscle ACC resulting in a decrease in the V_{max} for ACC and an increase in $K_{0.5}$ for citrate. Winder has also showed that, AMPK levels are elevated during prolonged exercise resulting in inactivation of ACC by phosphorylation and a fall in malonyl-CoA level (Winder & Hardie, 1996). It can be proposed that AMPK plays an important role in the regulation of malonyl-CoA synthesis in muscle.
- AICAR decreased the content of long chain esters and significantly increased the oxidation of exogenous fatty acid oxidation relative to that of endogenous fatty acid oxidation. This suggests that AICAR has an antilipolytic effect in muscle.

3.3.8 LACTATE MEASUREMENTS

Under the various tests conditions the lactate content in muscle was measured as described in the Materials and Methods (section2.6.5)

Table 3.11 shows that under the different test conditions employed, the lactate content in the soleus muscle did not alter.

Table 3.11**Lactate Measurements In The Soleus Muscle**

Additions	Lactate ($\mu\text{mol/ hr/g wet. wt}$)
None	8.79 ± 1.89 (n = 4)
Insulin (10^{-8}M)	8.11 ± 0.86 (n = 4)
Etomoxir ($100\mu\text{M}$)	8.72 ± 0.92 (n = 5)
DCA 3mM	9.27 ± 2.0 (n = 5)
Isoprenaline ($0.1 \mu\text{M}$)	9.86 ± 1.1 (n = 4)
Palmitate (0.5 mM)	9.28 ± 1.0 (n = 4)
Palmitate + Insulin	9.87 ± 0.96 (n = 4)
AICAR + Insulin	9.60 ± 0.74 (n = 5)

Lactate was measured as described in the Materials and Methods (section 2.6.5)

The values are expressed as means \pm SEM.

3.3.9 OVERALL CONCLUSION

The work presented in this section clearly shows that insulin and DCA raise the malonyl-CoA content in muscle. This effect of insulin can be antagonised by palmitate, isoprenaline and AICAR.

The findings from the metabolite and fatty acid oxidation measurements under the different test conditions are summarised in the table below.

Test Conditions	Malonyl-CoA content	Fatty acyl-CoA content	Ratio Acylcarnitine/ acyl-CoA	Fatty acid oxidation (exogenous)
Insulin	Increased	Increased	Decreased	Decreased
DCA (3mM)	Increased	Increased	Decreased	Decreased
Pal + Ins	Decreased	Decreased	Increased	Increased
Iso + Ins	Decreased	Decreased	Increased	Increased

The table shows that under the conditions that increase malonyl-CoA content in muscles i.e insulin and DCA, an increase in long chain fatty acyl-CoA and a decrease in the ratio of acylcarnitine/ acyl-CoA and fatty acid oxidation is seen. By contrast the conditions that cause a decrease in a malonyl-CoA content, i.e. isoprenaline and palmitate, a decrease in long chain fatty acyl-CoA and an increase in the ratio of acylcarnitine/ acyl-CoA and fatty acid oxidation is observed.

This increase in malonyl-CoA (the physiological inhibitor of CPT 1) seen in the presence of insulin or DCA is likely to result in inhibition of CPT 1 activity. This presumably would lead to an accumulation of long chain fatty acyl-CoA (probably in the cytosol) as the pathway to the mitochondria is blocked and this will explain the fall in fatty acid oxidation. Conversely, a decrease in malonyl-CoA content, as seen with isoprenaline or palmitate, will result in relief of inhibition of CPT 1 by malonyl-CoA and result in CPT 1

increased activity. This would result in an increase in long chain fatty acyl unit transport to the mitochondrial matrix, and increased fatty acid oxidation.

Interestingly, from plotting the individual data of malonyl-CoA and long chain fatty acyl-CoA from all the experiments mentioned in section 3.3 (excluding AICAR) (fig 3.34), a highly significant positive correlation between the tissue contents of malonyl-CoA and long chain fatty acyl-CoA is seen.

Furthermore, the effects of insulin and DCA to raise malonyl-CoA and decrease fatty acid oxidation (presumably) are related. Similarly, the effects of isoprenaline and palmitate to decrease malonyl-CoA and increase fatty acid oxidation (presumably) are related. Therefore it can be suggested that malonyl-CoA in muscle plays a key regulatory role in fatty acid oxidation.

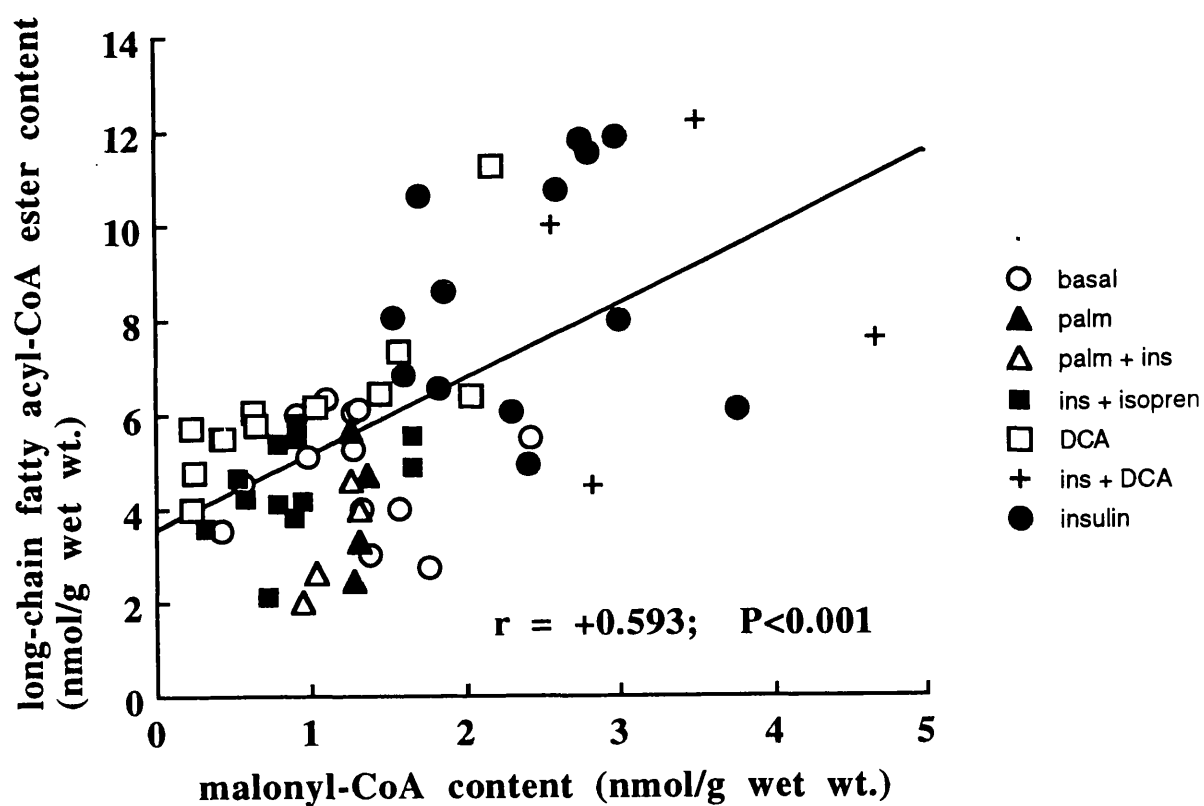
The mechanism by which insulin increases malonyl-CoA levels in soleus muscle is presumably by insulin increasing the cytosolic citrate concentration. This increase in citrate results either in increased allosteric activation of ACC or indirectly via an increase in the supply of acetyl-CoA for ACC, as proposed by Ruderman's group (Saha *et al*, 1995).

The effects of DCA were complex, as it elevated malonyl-CoA content at high concentrations but decreased it at low concentrations and also seemed to promote lipolysis. The increase in malonyl-CoA content observed at 3mM DCA is presumed to be due to DCA stimulating pyruvate oxidation. The findings suggest that DCA at high concentrations has an insulin-like effect and at low concentrations of DCA it has an isoprenaline-like effect.

It was proposed that decreases in malonyl-CoA content due to isoprenaline and palmitate are probably due to activation of AMPK (section 3.3.5 and 3.3.6) and subsequent

Fig 3.34

Linear Correlation Between Tissue Contents Of Malonyl-CoA and
Long Chain Fatty Acyl-CoA Esters



The figure shows individual values that were obtained in the experiments described in section 3.3 and are fitted to the regression line $y = 1.60x + 3.52$

inactivation of ACC. This requires further investigation. Also measurements of PKA and malonyl-CoA decarboxylase are required in the presence of isoprenaline.

An explanation was suggested in section 3.3.5. for the decrease in the long chain fatty acyl-CoA levels seen with palmitate or isoprenaline. It is suggested that a degree of flux control over fatty acid oxidation could be passed from CPT 1 to fatty acyl-CoA synthetase under these conditions. It is known that CPT 1 has a high flux control coefficient in liver mitochondria or in hepatocytes but as yet no comparable measurements in muscle have been made.

Interestingly, from the studies with AICAR, it is suggested that AMPK inhibits both ACC and lipolysis in muscle suggesting that AMPK probably plays an important role in regulating fatty acid metabolism in muscle.

It would be of interest to investigate whether AICAR and AMPK have an inhibitory role on glycerolipid synthesis in muscle as suggested in section 3.3.7.2. It has been shown in type IIa muscles that electrical stimulation results in both an increase in AMPK activity and also an inhibition of fatty acid esterification (Hutber *et al*, 1997, Vavvas *et al*, 1997 and Hopp & Palmer, 1990).

Malonyl-CoA in non-lipogenic tissues has been suggested to play an important role in the regulation of fatty acid oxidation (section 1.5.3, 1.6 and 1.7.2) and my studies seem to be consistent with this notion, although further studies are required. Therefore it is imperative that we further understand the regulation of malonyl-CoA synthesis and disposal and identify the regulatory mechanisms involved in all physiological conditions.

Chapter Four

General Discussion

4.1 GENERAL DISCUSSION

This work has provided evidence for the existence of enzymes capable of generating and disposing of malonyl-CoA in different skeletal muscle types (section 3.1 and 3.2).

ATP-citrate lyase activity was demonstrated in all the muscle types studied. This suggests that acetyl-CoA synthesised in the mitochondria can be transported to the cytosol in the form of citrate and converted back into acetyl-CoA by ATP-citrate lyase.

ACC activity was demonstrated in each of the muscle types studied and in the presence of citrate the total activity increased considerably (fig 3.1). The ACC activity was expressed as the citrate-dependent activity which is the minimal estimate of true ACC activity. This is because the mitochondrial enzyme propionyl-CoA carboxylase is probably present in the cytosol as a result of mitochondrial breakage during muscle preparation and the extent of phosphorylation of ACC is not known (section 3.1.2). Thus the activity was expressed as citrate-dependent activity. The citrate-dependent activity was greater in all muscle fibres than the citrate-independent activity. This suggests that ACC in muscle is dependent on citrate on for its activity and is consistent with the findings from Hutber *et al*, 1997, Vassa *et al*, 1997 and Winder *et al*, 1997.

Fatty acid synthase activity was demonstrated in each of the muscle fibres, using the more sensitive radiochemical method. Skeletal muscles are not considered to be lipogenic tissues, the activity detected could probably be due to adipocytes present within the muscle fibres. However further experiments are required to confirm this.

The cytosolic malonyl-CoA decarboxylase activity measured was approximately 15 fold greater than the fatty acid synthase activity measured. Therefore it can be suggested that decarboxylation of malonyl-CoA to acetyl-CoA is a possible route of disposal of

malonyl-CoA. Furthermore, the results clearly suggest a cytosolic malonyl-CoA decarboxylase activity

It is possible that malonyl-CoA decarboxylase (cytosolic) activity could be regulated by covalent modification (presumably by protein kinase A or AMPK), but further studies are required.

A functional role for the cytosolic malonyl-CoA decarboxylase can be suggested. It could be involved in increasing fatty acid oxidation by lowering malonyl-CoA levels during exercise. During exercise the demand for fuel is greater than at rest and is met by an increase in the oxidation of lipid fuels (i.e fatty acids) (section 1.7 Winder *et al*, 1989 and Elayan & Winder, 1991). Malonyl-CoA is a potent inhibitor of CPT 1, resulting in suppression of fatty acid oxidation. Presumably during exercise, cytosolic malonyl-CoA decarboxylase could decarboxylate malonyl-CoA to acetyl-CoA, relieving the inhibition of CPT 1 by malonyl-CoA resulting in an increase in fatty acid oxidation. This requires further investigation and is only a speculative function.

Even though malonyl-CoA decarboxylase activity was measurable in the mitochondria, the question that arises is what is its function in the mitochondria and is there evidence for a malonyl-CoA transport protein ?

Several roles for the mitochondrial malonyl-CoA decarboxylase have been suggested in liver and mammary gland (section 3.2.3). However the roles suggested seem unlikely as no malonyl-CoA transport protein as yet has been identified.

The enzyme activities in the EDL muscle (type IIa) were lower than those found in the tibialis anterior muscle, which is also a type IIa muscle. A reason for this difference in enzyme activities between the same type of muscle could be due to the relative proportion of the different muscle fibre types present. The EDL muscle is (approximately) made up of equal portions of type IIa and type I muscle fibres. The

enzyme activities measured in the EDL muscle are similar to those found in the soleus muscle (Type I). It can be suggested that most of the activity could have been contributed from the type I muscle fibres present within the EDL muscle. However I am not aware of the fibre composition of tibialis anterior muscle and thus I am not able to comment on which fibre type might be contributing to the enzyme activities measured.

Section 3.3. clearly showed that malonyl-CoA content in the soleus muscle can be acutely regulated by various effectors e.g. insulin, DCA, etomoxir, palmitate, AICAR and isoprenaline. Insulin increased malonyl-CoA content in the soleus muscle with a concomitant increase in long chain fatty acyl-CoA content, probably as a result of malonyl-CoA inhibiting CPT 1 (section 3.3.2). In contrast to these findings it was found by Awan & Saggerson (1993) and by Hamilton & Saggerson (unpublished) that insulin did not change the content of long chain fatty acyl-CoA in perfused heart or cardiac myocytes. This increase in long chain fatty acyl-CoA content was suggested to be in a cytosolic pool by analogy with studies with etomoxir. Significant evidence from Ruderman's group suggests that insulin increases muscle malonyl-CoA content not by increasing ACC activity *per se*, as proposed for liver ACC, but by increasing the concentration of citrate, the precursor of the cytosolic acetyl-CoA (section 1.6.4). This suggests that the activity of the two isoforms of ACC are regulated by different mechanisms in the presence of insulin (section 1.6.2.2 & 1.6.4).

The effects of insulin were opposed by palmitate and isoprenaline. They both decreased muscle malonyl-CoA content (section 3.3.5 & 3.3.6). The mechanism by which palmitate and isoprenaline lowered malonyl-coA content is suggested to be by the AMPK system (section 3.3.5 & 3.3.6), as a result of increased fatty acid supply. AMPK

could be activated as a consequence of increased fatty acid/ fatty acyl-CoA turnover in muscle.

This would result in a fall in ACC activity (as a result of phosphorylation) accompanied by a fall in muscle malonyl-CoA content. An alternative mechanism was suggested for the fall in muscle malonyl-CoA content in the presence of isoprenaline. Isoprenaline activates HSL via protein kinase A. It could be possible that protein kinase A, apart from activating HSL, could also activate malonyl-CoA decarboxylase. This would lead to a fall in muscle malonyl-CoA content.

In the presence of AICAR, malonyl-CoA content decreased (section 3.3.7). This fall in malonyl-CoA content is presumed to be due to inhibition of ACC activity in the presence of AICAR. (section 3.3.7). AICAR was shown to have an antilipolytic effect in soleus muscle. This suggests that AMPK might have a role in muscle fuel selection i.e. in regulating the extent to which fatty acids from endogenous stores are selected for β -oxidation as opposed to those provided from exogenous stores.

Hardie & Carling (1997) suggested that AMPK, the fuel gauge of mammalian cells, is able to switch both lipogenesis and lipolysis off (in lipogenic tissues) in times of environmental stress when ATP levels are depleted.

It has also been suggested by Hardie & Carling (1997) that AMPK is involved in switching fuel from carbohydrates to fats in muscles during intense exercise (Hardie & Carling, 1997). The supply of carbohydrates becomes limiting during intense exercise and cannot maintain the demand for ATP. This results in rises in the ratio of ADP/ATP and AMP/ATP, associated with the activation of AMPK by AMP. Activation of AMPK phosphorylates and inactivates ACC, causing malonyl-CoA levels to fall. This fall in

malonyl-CoA relieves the inhibition of CPT 1 and fatty acids become the main fuel for ATP production.

Studies by Hardie & Winder, (1995), Hutber *et al*, (1996) and Vassa *et al*, (1997 b) have all shown ACC activity to fall with a corresponding decline in malonyl-CoA levels, as result of increased phosphorylation by AMPK during exercise. It can be proposed that AMPK plays an important role in controlling muscle metabolism under certain physiological conditions.

These studies show that that malonyl-CoA content can be acutely regulated by insulin (increasing the supply of substrate of ACC) and by isoprenaline and by palmitate (presumably by phosphorylation of ACC by AMPK).

In conclusion these studies have shown that skeletal muscles are able to synthesize and dispose of malonyl-CoA and that the malonyl-CoA content in the soleus muscle can be acutely regulated. However these tissues are not lipogenic, what is the role of malonyl-CoA in these tissues ?

Ruderman's group suggested that malonyl-CoA with ACC is involved in a fuel sensing mechanism and signalling pathway that responds acutely to changes in the fuel supply and energy expenditure. It was suggested that ACC is the sensor, and alterations in its activity changes malonyl-CoA concentrations. Malonyl-CoA has been suggested to be the *signal* that influences whether long chain fatty acyl-CoAs are transferred into the mitochondria for oxidation (during electrically stimulation / exercise) or are kept in the cytosol, where they are converted to triglycerides or phospholipids (Saha *et al*, 1997). From the findings from this study it can be suggested that malonyl-CoA probably does act as a *signal* that determines whether long chain acyl-CoAs are kept in the cytosol or

are transported to the mitochondria to be oxidised, as suggested above (section 3.3.2, 3.3.5 & 6). Changes observed in the malonyl-CoA content under the various test conditions were presumed to be due to changes in ACC activity.

Furthermore the findings from this study suggest that malonyl-CoA and fatty acid oxidation are related (e.g. insulin & 3mM DCA increased malonyl-CoA and decreased fatty acid oxidation, isoprenaline and palmitate decreased malonyl-CoA content and increased fatty acid oxidation). It can be suggested that malonyl-CoA plays an important role in fatty acid oxidation in the soleus muscle linking the control of CPT 1 activity to ACC activity. However further studies are required to fully substantiate this.

To fully understand the role of malonyl-CoA in skeletal muscles, it is important to understand the regulation of malonyl-CoA synthesis in all muscle fibre types in all physiological conditions.

Chapter Five

Future Work

5 SUGGESTIONS FOR FUTURE WORK

- Elucidate the mechanisms involved in lowering malonyl-CoA content in muscles in the presence of palmitate and isoprenaline. Measuring AMPK activity, ACC activity and AMP levels in the presence of palmitate and isoprenaline. Also measure muscle protein kinase A activity and ATP-Citrate lyase activity in the presence of isoprenaline
- Characterise and purify cytosolic malonyl-CoA decarboxylase from muscle (soleus), Once purified, establish whether it is regulated by covalent modification (phosphorylation/dephosphorylation) or not.
- Identify whether a malonyl-CoA transport protein is present in muscles, transporting malonyl-CoA from the cytosol to mitochondria, where it is decarboxylated to acetyl-CoA.
- Measure malonyl-CoA levels in other muscle fibres (Type IIa and type IIb) and establish whether the malonyl-CoA content varies similarly in different muscle fibres. Also, study the short term regulation of malonyl-CoA levels in these different muscle fibres types and establish whether malonyl-CoA is regulated by the same mechanisms as in type I fibres.
- Finally, study long term regulation of malonyl-CoA content and ACC activity in muscles (starvation and exercise).

Chapter Six

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6 REFERENCES

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