

**REGULATION OF FATTY ACID SYNTHESIS PATHWAY IN
THE LACTATING RAT MAMMARY GLAND**

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**Submitted to the University of London for the Degree of
Doctor of Philosophy**

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London

May 1996

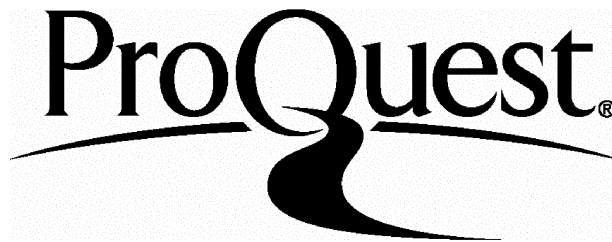
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ABSTRACT

The lactating rat mammary gland is highly active in synthesising fatty acids and isolated mammary acini were used to investigate the regulation and interdependence of specific regulatory steps in the pathway from glucose to fatty acids. Various putative effectors of lipogenesis were used to investigate the control and relative importance of these specific regulatory steps.

Acetoacetate, a potential physiological signal in starvation inhibited fatty acid synthesis (38%) but not acetyl-CoA carboxylase (ACC). Surprisingly, ACC was activated and this survived purification (V_{max} increased by 100%). ATP-citrate lyase (ATP-CL) and pyruvate dehydrogenase (PDH) were inhibited by 18% and 83%, respectively. However, neither of these are likely to account for the lipogenic inhibition, given the increase in citrate concentration caused by acetoacetate. Glucose uptake was reduced by 54% in the presence of acetoacetate, but this was not the result of a decrease in the rate of glucose transport which was unaffected. The data suggest that reduced glucose supply to the cells is the major factor in lipogenic inhibition, possibly via the consequent reduced production of NADPH for fatty acid synthesis, or glycerol-3-phosphate for esterification. Inhibition of glycolysis probably occurs as a result of phosphofructo-1-kinase (PFK-1) inhibition by the elevated citrate levels in combination with PDH inactivation.

Investigation of the profile of protein phosphatase activity in mammary gland showed the presence of okadaic acid-sensitive PP1 and PP2A. Okadaic acid is a potent cell-permeable inhibitor of PP1 and PP2A. It inhibited fatty acid synthesis (63%) without affecting the rate of glucose uptake by rat mammary acini. While GLUT 1 (the predominant glucose transporter in mammary acini) like GLUT 4 (the insulin stimulated glucose transporter) can be regulated by translocation to the plasma membrane, these studies confirm that phosphorylation is unlikely to be involved in this regulation. ACC was phosphorylated and inactivated (V_{max} decreased by 70%) in response to okadaic acid. This is an expected result of PP2A inhibition but a transient activation of AMP-PK was also observed. Time course studies showed that PDH inhibition in response to okadaic acid was a consequence of the inhibition of ACC, presumably via an increased acetyl CoA/CoA ratio that would activate PDH kinase.

The polyunsaturated fatty acid, eicosapentanoic acid (EPA), inhibited fatty acid synthesis in mammary acini in a dose-dependent manner. Maximal inhibition was achieved at 1mM EPA and at this concentration both PDH and ACC were inhibited. Linolenic and linoleic acids produced similar inhibitory effects with the degree of inhibition depending on the degree of unsaturation of the fatty acid. Clofibrate and gemfibrozil are lipid-lowering drugs with structures analogous to those of fatty acids. These drugs also inhibited fatty acid synthesis in acini (33% and 84%, respectively) via PDH and ACC inactivation. *In vivo* studies with gemfibrozil indicated that doses of this agent known to produce peroxisomal proliferation produced similar short term inhibition of fatty acid synthesis (68%), and cholesterol synthesis (81%) in rat liver *in vivo*. Surprisingly, fatty acid synthesis in white adipose tissue was increased by 71%.

To My Parents

ACKNOWLEDGEMENTS

I would like to take this opportunity to thank my supervisor Dr. M. R. Munday for his time and patience spent in the supervision of the project. I deeply appreciate his constant encouragement and guidance, and his reassuring manner has made working with him a great pleasure.

Many thanks to everyone in the Chemistry Department, in particular Mr M. Milic, Mr. P. Thomas, Miss C. Toomey, Miss C. Hemingway, Miss S. Cox and Mr. C. James for all their help and support.

Finally, I am also grateful to the London School of Pharmacy for a studentship and the Overseas Research Students Award Scheme for an ORS Award.

LIST OF ABBREVIATIONS

A ₂₈₀	Absorbance at 280nm
AABS	p-(p-aminophenylazo)-benzene sulphonic acid
AAT	Arylamine acetyltransferase
ACC	Acetyl-CoA carboxylase
ACK-2	Acetyl-CoA carboxylase kinase 2
ADP	Adenosine-5'-diphosphate
AMP	Adenosine-5'-monophosphate
AMP-PK	AMP-activated protein kinase
APAD	Acetylpyridine-adenine dinucleotide
ATP	Adenosine-5'-triphosphate
ATP-CL	ATP-citrate lyase
b.p.	Boiling point
BSA	Bovine serum albumin
cAMP	cyclic 3':5'-adenosine monophosphate
cAMP-PK	cyclic-AMP-dependent protein kinase
CoA	Coenzyme A
CS	Citrate synthase
DHA	Docosahexanoic acid
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EDTA	Ethylene-diamine-tetraacetic acid
EPA	Eicosapentanoic acid
F-6-P	Fructose-6-phosphate
F-1,6-BP	Fructose-1,6-bisphosphate
F-2,6-BP	Fructose-2,6-bisphosphate
FSBA	Fluorosulphonyl-benzoyl adenosine
GLUT	Glucose transporter
GPAT	sn-glycerol-3-phosphate acyltransferase
HDL	High density lipoprotein
HMG-CoA	hydroxymethyl glutaryl coenzyme A
H ₂ SO ₄	Sulphuric acid
IDL	Intermediate density lipoprotein
K _a	Activator constant
KH	Krebs Henseleit
K _i	Inhibitor dissociation constant
K _m	Michaelis constant
KOH	Potassium hydroxide
LDL	Low density lipoprotein
LPL	Lipoprotein Lipase
MgCl ₂	Magnesium chloride
MDH	Malate dehydrogenase

NAD(H)	Nicotinamide-adenine dinucleotide (reduced form)
NADP(H)	Nicotinamide-adenine dinucleotide phosphate (reduced form)
NaF	Sodium fluoride
NaHCO ₃	Sodium bicarbonate
NaN ₃	Sodium azide
NaOH	Sodium hydroxide
NaPP _i	Sodium pyrophosphate
PCA	Perchloric acid
PDH	Pyruvate dehydrogenase
PGI ₂	Prostaglandin 12
PFK	Phosphofructo-kinase
PMSF	Phenylmethylsulphonyl fluoride
PP	Protein phosphatase
PUFA	Polyunsaturated fatty acid
SBTI	Soya-bean trypsin inhibitor
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
S.E.M.	Standard error of the mean
TCA	Trichloroacetic acid
TEMED	Tetramethylethylenediamine
TLCK	N-p-tosyl-L-lysine chloromethyl ketone
TOFA	5-(Tetradecyloxy)-2-furoic acid
TPCK	N-tosyl-L-phenylalanine chloromethyl ketone
TxA ₂	Thromboxane A ₂
VLDL	Very low density lipoprotein
V _{max}	Maximum initial velocity of enzyme reaction at saturating substrate concentrations
v/v	Volume per volume
w/v	Weight per volume
w/w	Weight per weight

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CHAPTER 1

INTRODUCTION

1.1 Fatty Acid Synthesis In Mammals

The major sites of lipogenesis in most mammals are the liver and adipose tissues that synthesise fatty acids for secretion and for storage, respectively (Hardie, 1989). In the special case of lactating mammals, the mammary gland is extremely active in synthesising fatty acids for secretion into milk in the form of triglycerides, especially in mammals producing lipid-rich milk (Williamson, 1980). The common fatty acids that are found in mammals are monocarboxylic acids. The hydrocarbon chain usually has an even number of carbon atoms and may be unsaturated by the introduction of one or more double bonds. The most commonly occurring fatty acids in mammals are palmitate (C16:0) and oleate (C18:1).

1.2 Mammary Gland During Lactation

Rat milk has a relatively high fat content compared with other species (Williamson *et al*, 1984). The daily milk yield is approximately 40 ml at peak lactation and the lactating mammary gland synthesises fatty acid at a rate that

is 4-5 fold higher than in liver (per gram wet weight) and 15-fold higher than in mammary glands of virgin rats (Agius *et al*, 1979). This imposes a considerable demand for substrates on the lactating rat mammary gland. Approximately 50% of the fatty acids present in triglycerides are synthesised *de novo* from glucose in the mammary gland. The rest are derived from fatty acids mobilised from adipose tissue triglyceride stores by the action of hormone sensitive lipase and from hydrolysis of triglycerides in circulating VLDL and chylomicra by mammary gland lipoprotein lipase (LPL, EC 3.1.1.34; Baldwin and Yang, 1974). The relative contribution of these sources of fatty acids varies from species to species.

Lactation is characterised by physiological and metabolic changes in various tissues. Gross physiological changes that occur in lactation include increased cardiac output to the gland and increased weight of liver, intestine and mammary gland (Chatwin *et al*, 1969). At peak lactation, the gland receives 10% of the cardiac output compared to 0.6% in non-lactating rats (Williamson *et al*, 1984). The increase in blood flow to the gland ensures a higher rate of substrate delivery to meet the increased demand of the gland.

Integration of metabolism in various tissues, notably liver, adipose tissues and mammary gland occurs during lactation. In the liver of fed pregnant and lactating rats, the metabolism of fatty acids is directed primarily towards

synthesis of triglycerides (Williamson, 1980) which are then released into the bloodstream as VLDL. Upon breakdown of VLDL triglycerides by LPL, the fatty acids released are incorporated into milk lipids in the lactating mammary gland (Carrick and Kuhn, 1978). In adipose tissue, the rate of lipogenesis decreases dramatically at peak lactation (Robinson *et al*, 1978; Vernon and Flint, 1983; Williamson *et al*, 1986). LPL activity in the mammary gland increases, whilst the activity in adipose tissue decreases and remains low throughout lactation (Otway & Robinson, 1968; Hamosh *et al*, 1970; Flint *et al*, 1981). The changes in adipose tissue from its anabolic to catabolic role ensure plasma triglycerides and glucose are directed to the gland (Williamson, 1973).

1.3 The Mammary Gland

Before conception, rat mammary gland consists only of a system of short branching ducts lying at the inguinal fat depot with no true lobulo-alveolar secretory tissue (Knight, 1984). Throughout pregnancy and early lactation, cellular proliferation and development of mammary tissue occurs as levels of oestrogen and progesterone change (Cowie, 1971; Anderson, 1974). The progressive penetration and proliferation of the mammary gland ducts into the adipose tissue take place with the development of lobules which comprise tightly packed alveoli or acini and form the basic functional unit of the mammary gland. An acinus is an expanded sac of secretory cells well supplied by

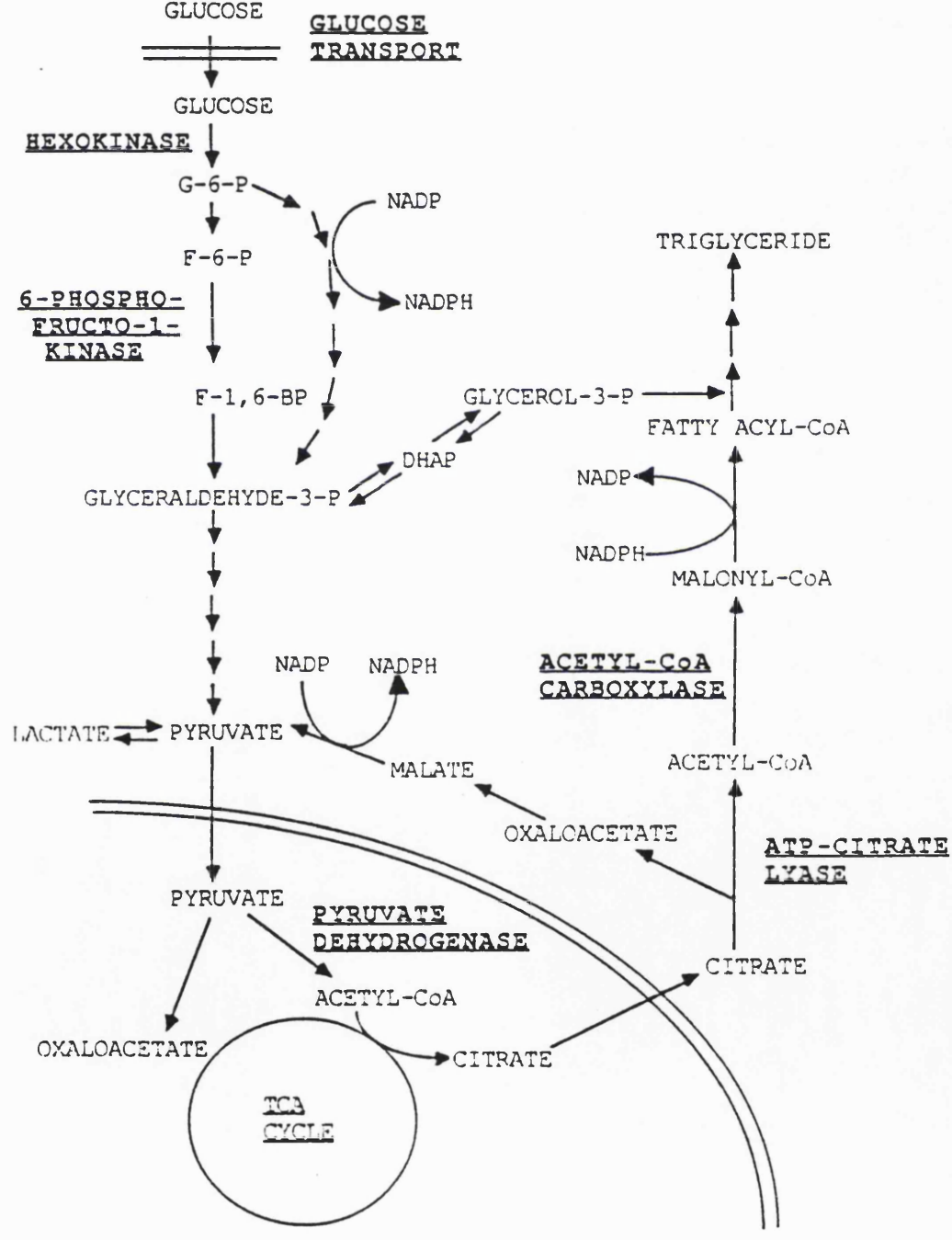
capillaries. It consists of a single layer of secretory parenchymal cells interspersed with adipocytes in a collagen matrix. *In vitro* treatment of minced mammary gland with collagenase leads to digestion of the collagen matrix and breaks up the lobules into individual acini which remain active (Katz *et al*, 1974).

Isolated acini have proved to be a good system for the *in vitro* studies of mammary gland metabolism (Katz *et al*, 1974). The preparation offers distinct advantages over the use of other *in vitro* systems such as tissue slices which contain adipocytes with different metabolic properties. This problem is eliminated in acinar preparation where minimal contamination by adipose cells present in the gland can be obtained (Katz *et al*, 1974). Isolated mammary acini retain the structural integrity of the intact gland. Metabolic changes that occur in the isolated cells reflect those of the gland *in vivo* under different physiological conditions (Robinson and Williamson, 1977a). Although isolated mammary acini are metabolically less active than the whole tissue, the preparation shows linearity and reproducibility of metabolic rates for at least 60 minutes. Such a preparation is therefore useful for investigating mammary gland metabolism, particularly biosynthesis of fatty acids and its hormonal regulation.

1.4 The Pathway of Fatty Acid Synthesis from Glucose in the Lactating Mammary Gland

Glucose is the major precursor for mammary gland lipogenesis in non-ruminant mammary tissues. Other potential lipogenic substrates include lactate and pyruvate (Robinson and Williamson, 1977a); acetate (Buckley and Williamson, 1977), ketone bodies (Hawkins and Williamson, 1972) and certain amino acids, in particular L-leucine (Vina & Williamson, 1981). However, the relative contributions of these substrates are small compared to glucose. At peak lactation, the uptake of glucose is high, approximately 30 mmol per day (Williamson, 1980). Of this, about 7 mmol (10-20%) is used for the synthesis of lactose, the major carbohydrate component of milk. A further 5-10% of glucose is oxidised for energy requirements via the tricarboxylic acid cycle and the remainder is used for *de novo* synthesis of fatty acids which takes place within the mammary cell (Katz *et al*, 1974; Robinson and Williamson, 1977a).

Figure 1.1 shows the pathway of fatty acid synthesis from glucose including the potential regulatory steps of glucose transport, hexokinase, phosphofructo-kinase, pyruvate dehydrogenase, ATP-citrate lyase and acetyl-CoA carboxylase. Fatty acids are synthesised by successive condensation of 2 carbon units derived from malonyl-CoA catalysed by the multifunctional enzyme fatty acid synthetase (Bloch and Vance, 1977). Rat mammary gland is unique



in that only the short and medium-chain fatty acids (C_8 - C_{14}) are synthesised (Grigor and Warren, 1980). This is due to the action of a tissue-specific medium-chain acylthioesterase which prematurely terminates chain elongation by cleaving the fatty acids from their thioester linkages with fatty acid synthetase (Knudson *et al*, 1976; Libertini and Smith, 1978). The enzyme is part of the fatty acid synthetase multifunctional enzyme (the seventh active site) which is not usually present in lipogenic tissues. The medium-chain fatty acids in milk fat are thought to be essential for maintaining the fluidity of milk (Chatterjee *et al*, 1979). Once the fatty acids are synthesised, they are esterified to form triglycerides, phospholipids and other lipids. In mammary tissue, the sn-glycerol-3-phosphate and monoglyceride pathways are operative in the synthesis of triglycerides. The mammary gland displays a characteristic stereospecific esterification of fatty acids to glycerol-3-phosphate in the synthesis of triglycerides which is not found in other lipogenic tissues (Chatterjee *et al*, 1979). The short and medium chain fatty acids are positioned almost exclusively at the sn-3 position of glycerol as a result of the specificity of acyltransferases.

The reducing power, NADPH required for fatty acid synthesis is generated mainly by the first two steps of the pentose phosphate pathway. The mammary gland of the lactating rat has a very high pentose phosphate pathway activity which is controlled by the cytosolic NADP : NADPH ratio (McLean, 1960). *In vitro* studies in isolated rat mammary acini showed that the pentose

phosphate pathway accounted for 25-50% of the glucose taken up by the acini and provided 70-100% of the NADPH required for fatty acid synthesis (Katz *et al*, 1974; Robinson and Williamson, 1977b). The activity of the pentose phosphate pathway is closely linked to fatty acid synthesis in the mammary gland. When the rates of fatty acid synthesis decrease, NADPH levels increase as a result of decreased utilisation. This leads to the inhibition of glucose-6-phosphate dehydrogenase and consequently decreases the flux of glucose into the pentose phosphate pathway (Glock and McLean, 1953).

1.5 Regulation of Fatty Acid Synthesis in the Mammary Gland of Lactating Rats

The high rate of lipogenesis of the lactating mammary gland imposes a high demand for the lipogenic substrate, glucose. Lipogenesis is therefore stringently regulated according to the nutritional and hormonal status of the animal (Williamson, 1980). This is to ensure that the maternal carbohydrate and fat reserve is not depleted when food availability is restricted such as in starvation.

1.5.1 Hormonal Regulation

Various hormones, notably prolactin and insulin are important in the

maintenance and regulation of fatty acid synthesis. The plasma level of prolactin released from the anterior pituitary gland increases with parturition. During lactation, milk removal from the gland by suckling of the young continues to stimulate prolactin secretion and a high rate of fatty acid synthesis is therefore maintained (Martyn and Hansen, 1980). Removal of pups for 24 hours from lactating rats at peak lactation resulted in a decrease in plasma prolactin by 78% (Agius *et al*, 1979). This is accompanied by decreased $^3\text{H}_2\text{O}$ incorporation into lipid in mammary gland by 95%. Conversely, removal of pups for 24 hours from lactating rats at peak lactation increased lipogenesis in liver and adipose tissue by 77% and 330%, respectively. Treatment of lactating rats with bromocryptine (Agius *et al*, 1979; Flint *et al*, 1981) decreased circulating levels of prolactin. This leads to inhibition of fatty acid synthesis in the mammary gland by 46% (Agius *et al*, 1979). Prolactin does not appear to play an important role in the short term regulation of fatty acid synthesis, as changes in its plasma concentration do not always parallel changes in fatty acid synthesis (Robinson *et al*, 1978b). For example, it has been shown that in starvation of lactating rats for 24 hours, plasma prolactin concentration is unchanged (Oller do Nascimento and Williamson, 1989).

The lactating rat mammary gland possesses a large number of insulin receptors (Flint, 1982) and is therefore highly insulin-sensitive and responsive (Jones *et al*, 1984a; 1984b; Burnol *et al*, 1987). A small physiological increase

in insulin concentration has been found to stimulate fatty acid synthesis, as demonstrated by its reactivation of lipogenesis in mammary glands of starved lactating rats (Agius *et al*, 1980) or in high fat feeding (Agius and Williamson, 1980). The number of insulin receptors on the lactating mammary epithelial cells increases during lactation (O'Keefe and Cuatrecasas, 1974; Flint, 1982) while the number of receptors on peripheral adipocytes is unchanged (Flint *et al*, 1979). This leads to the high rate of glucose utilisation and the redirection of substrates towards the lactating mammary gland (Williamson, 1980). A reverse relationship between plasma insulin concentration and weight of the lactating mammary gland has been demonstrated by Wilde and Kuhn (1979). This suggests that the gland is the major site of insulin extraction from the circulation.

Glucagon which acts by increasing the cAMP levels does not appear to play a role in the control of lipogenesis in the lactating mammary gland both *in vivo* (Bussmann *et al*, 1984) and *in vitro* in isolated mammary acini (Williamson *et al*, 1983; Robson *et al*, 1984). Mammary gland has no glucagon receptors (Robson *et al*, 1984) but β -adrenergic receptors (β_2 -subtype) are present (Clegg and Mullaney, 1985; Lavendero *et al*, 1985). A complete cAMP signalling system is present in the lactating mammary gland. The levels of cAMP decrease with the onset of lactation and increase at weaning (Sapag-Hagar and Greenbaum, 1973; Louis and Baldwin, 1975). However, the role of cAMP in mediating hormonal control of mammary gland metabolism is far from clear.

Adrenaline could potentially increase cAMP levels in the mammary gland but has no effect on mammary gland lipogenesis *in vivo* (Bussmann *et al*, 1984; Jones *et al*, 1984a) or *in vitro* in isolated mammary acini (Williamson *et al*, 1983; Robson *et al*, 1984). Munday and Williamson (1987) showed that adrenaline tends to increase lipogenesis in mammary acini *in vitro*. In mammary acini incubated with β -adrenergic agonists and phosphodiesterase inhibitors, the cAMP level is increased by 20-fold (Clegg and Mullaney, 1985) with no effect on fatty acid synthesis (Clegg *et al*, 1986).

Other hormones such as thyroid and corticosteroids do not play a role in the acute regulation of lipogenesis in the lactating mammary gland. They are involved in the development of mammary gland during pregnancy and early lactation, and in the initiation and maintenance of fatty acid synthesis (Topper and Freeman, 1980).

1.5.2 Diurnal Regulation

The lactating rats consume food continuously throughout the 24-hour cycle, with 35% of their daily food intake being consumed during the light period compared to virgin rats. The biosynthesis of fatty acids by the lactating mammary gland is extremely sensitive to the level of dietary intake. As with non-lactating rats, the rate of fatty acid synthesis in lactating rats exhibits a

marked diurnal variation which closely resembles the food intake of the animal, being highest at night and falls during the day when food consumption is low (Munday and Williamson, 1983).

1.5.3 Effect of Starvation

In response to 24 hour starvation, the rate of fatty acid synthesis in the lactating rat mammary gland decreased dramatically by 98%, as shown both *in vivo* (Robinson *et al*, 1978) and in isolated acini (Robinson and Williamson, 1977a). Similarly, short term starvation of lactating rats for 6 hours resulted in inhibition of fatty acid synthesis by 90% (Williamson *et al*, 1983; Jones *et al*, 1984a). This is accompanied by a 60% decrease in glucose utilisation, and lactate and pyruvate are released rather than being extracted by the gland (Hawkins and Williamson, 1972; Robinson and Williamson, 1977a). An increase in the plasma concentrations of ketone bodies has also been observed. These are potential physiological signals to the gland indicating decreased availability of glucose (Robinson and Williamson, 1977a; Hagopian *et al*, 1991). Starvation-induced inhibition of mammary gland lipogenesis is rapidly reversed by refeeding chow for 2 hours (Robinson *et al*, 1978; Robinson and Williamson, 1978b; Munday and Williamson, 1981; Williamson *et al*, 1983, Mercer and Williamson, 1986). Inhibition of fatty acid synthesis in starvation thus has a protective role in ensuring that glucose is used for the more immediate needs of

the mother than for the synthesis of milk lipids for the litters.

The plasma concentration of insulin has been implicated as an important signal in the regulation of lipogenesis in the starved-refed transition in the lactating mammary gland (Robinson *et al*, 1978; Munday and Williamson, 1981; Mercer and Williamson, 1986). In 24 hour-starved lactating rats, the plasma concentration of insulin decreased by approximately 50% (Robinson and Williamson, 1978b) and refeeding with chow diet for 2 hours restored the circulating insulin levels to control values (Robinson *et al*, 1978; Mercer and Williamson, 1986; Page and Kuhn, 1986). In addition, insulin administration with glucose partially restored fatty acid synthesis in the lactating mammary gland subjected to starvation for 24 hours (Munday and Williamson, 1981) and the hormone also reverses the acetoacetate inhibition of glucose utilisation and lipogenesis (Williamson *et al*, 1975; Robinson and Williamson, 1977c). The effects of chow refeeding was abolished by streptozotocin treatment of 24 hour-starved lactating rats (Robinson and Williamson, 1978b). Robinson *et al* (1978) also showed that acute insulin deficiency induced by 2 hour streptozotocin treatment caused marked inhibition of mammary lipogenesis *in vivo* by 80%, the effect of which persisted into the acini preparation. However, *in vitro*, it has proved impossible to demonstrate reactivation of fatty acid synthesis by insulin in acini from 24 hour starved lactating rats (Robinson and Williamson, 1977a). This could be due to the loss of insulin sensitivity of the cells.

1.5.4 High Fat Feeding

High fat diet fed to rats has been shown to influence the composition of milk lipids such that the fatty acid composition of the milk resembles that of the diet. The proportion of medium chain fatty acids in the milk is decreased but total milk fat content remains unchanged (Grigor and Warren, 1980). A high fat diet given throughout lactation inhibits the rate of lipogenesis in rat mammary gland (Agius *et al*, 1980; Munday and Williamson, 1987). In rats fed cafeteria diet (33% fat), a marked inhibition of fatty acid synthesis by 50% was observed (Agius *et al*, 1981a; Munday and Williamson, 1987). The *in vivo* effect of high fat diet is found to persist in isolated mammary acini *in vitro* and can be reversed by incubation of acini with insulin (Agius *et al*, 1980; Munday and Williamson, 1987). The rate of lipogenesis in the gland is also decreased by more than 80% in response to an intragastric load of triglycerides (Agius and Williamson, 1980). In this case, it was found that triglyceride with long chain fatty acids were more effective in inhibiting mammary gland lipogenesis (92%) than triglyceride with medium chain fatty acids (86%). Feeding rats a diet containing 20% coconut oil or peanut oil inhibited lipogenesis by 50 and 70%, respectively (Grigor and Warren, 1980). Therefore, it appears that increasing the availability of plasma fatty acids or triglycerides to the lactating mammary gland spares the use of lipogenic substrates by the gland which is an obvious physiological advantage.

1.6 Fatty Acid Synthesis in Liver

Liver is the major site of fatty acid synthesis in non-lactating mammals and birds. The major precursor for fatty acid synthesis in this tissue is lactate, derived mainly from metabolism of glucose in the intestinal mucosa. A small proportion of lactate is derived from erythrocytes and from anaerobic muscle metabolism. During starvation, the lactate concentration in the portal vein is 1-2 mM and this increases to 3-4 mM after a carbohydrate-rich diet (Hopkirk and Bloxham, 1977). Ketogenic amino acids which can be broken down to acetyl-CoA are also precursors for fatty acid synthesis, as has been shown in isolated hepatocytes (Clark *et al*, 1974). Glucose and glycogen are not utilised by liver for fatty acid synthesis (Geelen and Hindriks, 1984).

1.6.1 Hormonal Regulation of Hepatic Fatty Acid Synthesis

Insulin is involved in the regulation of liver lipogenesis *in vivo* (Stansbie *et al*, 1976). The stimulatory effect of insulin on rat hepatic lipogenesis *in vivo* can be observed after the oral administration of glucose which produces high plasma levels of insulin. Hepatic lipogenesis is inhibited in diabetic rats (Agius and Williamson, 1980). *In vitro*, fatty acid synthesis in isolated rat hepatocytes is stimulated by 40% in response to insulin (Holland and Hardie, 1985). Stimulation of hepatic lipogenesis by insulin is smaller in comparison to that

observed in isolated adipocytes where the hormone stimulates the rate of radioactive glucose incorporation into fatty acids by more than 25-fold (Haystead and Hardie, 1988).

The stimulatory effect of insulin on hepatic lipogenesis is antagonised by glucagon which inhibits fatty acid synthesis in isolated hepatocytes by 50% (Holland *et al*, 1984). *In vivo*, physiological glucagon concentrations inhibit hepatic lipogenesis by 60% (Cook *et al*, 1977) with an IC_{50} of approximately 10^{-9} M. Hardie *et al* (1984) also showed inhibition of lipogenesis by glucagon *in vivo* in rat liver. In isolated rat hepatocytes incubated with glucose, the effect of glucagon is thought to be mediated via inhibition of glycolysis which thus limits the supply of pyruvate and lactate for fatty acid synthesis (Beynen *et al*, 1979). This is reflected by a decrease in citrate concentration in the cells. The inhibitory effect of glucagon on fatty acid synthesis is partially reversed by the addition of pyruvate and/or lactate to the incubation media but the decrease in citrate levels is completely reversed (Watkins *et al*, 1977; McGarry *et al*, 1978). In the studies by Harris (1975), glucagon was also found to inhibit glycolysis and lipogenesis and this is accompanied by an increase in citrate levels. However, inhibition of glycolysis by glucagon is unlikely to be a significant factor in mediating the inhibitory effect of glucagon on lipogenesis *in vivo* because the major precursor for hepatic lipogenesis is probably the extrahepatic lactate (Hardie *et al*, 1984), and a major proportion of glucose absorbed by

intestinal mucosa is converted to lactate (Nicholls *et al*, 1983). Since glucagon and adrenaline are known to exert their effects via elevating intracellular cAMP concentrations, their inhibitory effect on lipogenesis is likely to be mediated via cAMP-dependent protein kinase.

1.7 Potential Regulatory Sites of Fatty Acid Synthesis Pathway

There are a number of potential rate-limiting steps in the pathway of lipogenesis from glucose. These include glucose transport, activities of hexokinase, phosphofructo-1-kinase, pyruvate dehydrogenase, ATP-citrate lyase and acetyl-CoA carboxylase (Williamson, 1980). In liver, since lactate is the major precursor of fatty acid synthesis, the potential regulatory steps are those catalysed by pyruvate dehydrogenase and acetyl-CoA carboxylase.

1.7.1 Glucose Transport

In lactating rat mammary gland, glucose transport increases sharply between parturition and the peak of lactation (Threadgold and Kuhn, 1984) with concomitant increases in the rates of glucose utilisation and lipogenesis. The extracellular glucose concentration is 5-6 mM (Wilde and Kuhn, 1979) and this decreases to less than 0.5 mM intracellularly, as measured in isolated mammary acini (Wilde and Kuhn, 1981). This large gradient of glucose concentration

across the plasma membrane suggests that uptake of glucose into the cell via a glucose transporter may play a potential regulatory role in the control of lipogenesis. Several lines of evidence suggest that glucose transport is the rate-limiting step in the overall metabolism of glucose (Threadgold *et al*, 1982; Threadgold and Kuhn, 1984). Studies by Threadgold and Kuhn (1984) showed that the rate of 2-deoxyglucose uptake closely match the overall glucose uptake by the lactating mammary gland *in vivo*. *In vitro*, the rate of uptake of 5 mM 2-deoxyglucose by the lactating rat mammary acini was similar to the rate of consumption of 5 mM glucose (Threadgold *et al*, 1982). Variations in the concentration of plasma glucose are therefore expected to affect the intracellular concentrations of glucose. This is shown by the linear increase in the rate of glucose consumption and lactose synthesis in isolated mammary acini in response to increasing concentrations of glucose (Wilde and Kuhn, 1981).

Glucose is a hydrophilic molecule that does not readily cross the plasma membrane. The transport of glucose into isolated mammary cells is a specific, saturable process (Threadgold *et al*, 1982). Molecular cloning showed that the cellular uptake of glucose is mediated by a family of highly homologous glucose transporters designated GLUT 1-7 based on the order in which they were isolated. Each glucose transporter has a characteristic tissue specific distribution (Table 1.1; for reviews see Bell *et al*, 1993; Gould and Holman, 1993). GLUT 4 is expressed in tissues such as muscle and adipose tissue which exhibit high

levels of insulin stimulated glucose transport and GLUT 1 transporters which have a much wider tissue distribution are expressed in a smaller amount (Zordano *et al*, 1989; Gould and Bell, 1990). In the basal state, GLUT 1 reside primarily in the plasma membrane whereas GLUT 4 are located almost exclusively in the intracellular compartment of the cell types in which they are expressed (James *et al*, 1987; Slot *et al*, 1991; Smith *et al*, 1991). Insulin is able to stimulate glucose transport by increasing the translocation of GLUT 4 from an intracellular pool to the plasma membrane (Wardzala *et al*, 1978; Cushman and Wardzala, 1980; Holman *et al*, 1990; Calderhead *et al*, 1990). The process is rapid, reversible and energy-dependent (Cushman and Wardzala, 1980) and an increase in the V_{\max} of glucose uptake in adipose tissue and muscle was observed in response to insulin (Taylor and Holman, 1981; Simpson and Cushman, 1986). In adipose tissue, the number of GLUT 4 transporters on the cell surface increases dramatically by 40-fold in response to insulin stimulation (Slot *et al*, 1991). This is antagonised by glucagon and β -agonists (Green, 1983; Smith *et al*, 1984) which increase cAMP levels without any corresponding changes in the number of glucose transporters in the plasma membrane (Simpson and Cushman, 1986). It has thus been suggested that insulin-stimulation of glucose transport activity is associated not only with translocation of transporters but also with activation of the intrinsic activity of GLUT 4 (Muhlbacher *et al*, 1988; Obermaier-Kusser *et al*, 1988; Tanti *et al*, 1989). In adipose tissue, GLUT 1 can be stimulated by insulin to translocate to the

plasma membrane but the intrinsic activity of GLUT 1 is not increased (Corvera *et al*, 1991).

Table 1.1 Major Sites of Expression and Function of Mammalian Facilitative Sugar Transporters

Modified from Bell *et al* (1993) and Gould and Holman (1993).

Isoforms	Major sites of expression and function
GLUT 1	Placenta; brain; blood-brain barrier, tissue culture cells; low levels in adipose and muscle tissue. Basal glucose uptake in many cells.
GLUT 2	Liver; pancreatic β -cells; kidney proximal tubule and small intestine. Transepithelial glucose and fructose transport.
GLUT 3	Brain; nerve; low levels in placenta, kidney, liver and human heart. Glucose transporter of neurones.
GLUT 4	Muscle; adipose tissue; heart. Mediates insulin-stimulated glucose transport.
GLUT 5	Small intestine; muscle tissue; low levels in adipose tissue and brain. Fructose transporter.
GLUT 6	Identified only in humans. Non-functional pseudogene.
GLUT 7	Liver. Microsomal glucose transporter.

Insulin-induced translocation of GLUT 4 to the cell surface is thought to be mediated by covalent modification of glucose transporters by phosphorylation (Haystead *et al*, 1989). Okadaic acid, a potent inhibitor of protein phosphatase 1 and 2A, stimulated glucose transport in isolated rat adipocytes suggesting the involvement of phosphorylation (Haystead *et al*, 1989). Studies by Kirsch *et al* (1985) showed that glucose transport activity in isolated fat cells is stimulated by phorbol esters which exert their effect via protein kinase C and this is

accompanied by translocation of GLUT 4 to the plasma membrane (Vogt *et al*, 1990). Further evidence of GLUT 4 phosphorylation comes from the studies of Kuroda *et al* (1987) who showed that insulin-stimulated glucose transport is inhibited by β -adrenergic agonists such as isoproterenol and cAMP derivatives. Since these compounds exert their effects by increasing the intracellular cAMP levels, this suggests that the inhibitory effects of these agents are likely to be mediated by cAMP-PK.

GLUT 4 is present at high levels in the mammary gland of virgin rats possibly due to the extensive presence of adipocytes at this state of mammary gland development (Knight, 1984). Throughout pregnancy, the proportion of fat cells in the mammary gland decreases and secretory cells form a major part of the mammary tissue as development proceeds. GLUT 1 is therefore expressed in mammary gland at high levels during lactation (Burnol *et al*, 1990) so that at peak lactation, the gland lacks the insulin-sensitive GLUT 4 transporter and contains only GLUT 1 (Corvera *et al*, 1989; Burnol *et al*, 1990; Madon *et al*, 1990).

Glucose uptake *in vivo* in rat mammary gland is known to be regulated by insulin (Hawkins and Williamson, 1972; Robinson and Williamson, 1977a; Threadgold and Kuhn, 1984; Page and Kuhn, 1986; Prosser, 1988). In starved rats, refeeding with chow diet restores glucose uptake by the gland and is

accompanied by a rise in plasma insulin (Munday and Williamson, 1981; Threadgold and Kuhn, 1984) which precedes the increase in glucose incorporation into either fatty acids or lactose (Bussman *et al*, 1984; Mercer and Williamson, 1986; Page and Kuhn, 1986). The decrease (90-95%) in the rate of glucose uptake in the mammary gland of lactating rats starved for 16 hours is readily reversed by refeeding the rat with chow for 2 hours or by administration of insulin (Threadgold and Kuhn, 1984). Inhibition of glucose uptake in starvation is associated with a decrease in the V_{\max} of glucose transport and the affinity of the glucose transporter for glucose remains unchanged (Threadgold and Kuhn, 1984). In lactating mouse mammary gland, the decrease and increase in glucose uptake in response to starvation and refeeding, respectively, is accompanied by parallel changes in transporter number at the mammary plasma membrane (Prosser, 1988). However, insulin has no effect on glucose transport in isolated mammary acini (Munday and Williamson, 1987) and mammary cells (Prosser and Topper, 1986) and Threadgold and Kuhn (1984) also showed that glucose transport by mammary acini is not affected by insulin or by previous starvation of the rat from which acini were prepared.

1.7.2 Hexokinase and 6-Phosphofructo-1-kinase

Hexokinase (EC 2.7.1.1) catalyses the formation of glucose-6-phosphate from glucose. In the mammary tissue, the enzyme exists in two isoforms, Type

I and Type II, with the K_m values for glucose of 4.5×10^{-5} M and 2.3×10^{-4} M, respectively (Walters and McLean, 1967). Type II isoform is the predominant enzyme present in mammary tissue (Walters and McLean, 1967). The activity of hexokinase in the lactating mammary gland increases at the onset of lactation but this is low compared with the activities of other enzymes (Gumaa *et al*, 1973). The activity of hexokinase is inhibited by physiological concentrations of glucose-6-phosphate with an apparent K_i of 0.16 mM (Grossbard and Schimke, 1966). *In vivo*, an inverse relationship between the concentration of glucose-6-phosphate and the rate of fatty acid synthesis in the lactating rat mammary gland has been observed (Jones *et al*, 1984a; Williamson *et al*, 1985).

The activity of hexokinase is closely linked to that of 6-phosphofructo-1-kinase (PFK-1, EC 2.7.1.11). PFK-1 is an important glycolytic enzyme that catalyses the formation of fructose-1,6-bisphosphate (F-1,6-BP) from fructose-6-phosphate. In starvation, an increase in the concentration of glucose-6-phosphate in the mammary gland as a result of lipogenic inhibition is associated with a decrease in the concentration of F-1,6-BP. The percentage of glucose accumulated as glucose-6-phosphate decreases from 40% in fed rats to 10% in 24 hour-starved rats, suggesting that hexokinase is inhibited (Jones *et al*, 1984a). Since glucose-6-phosphate is in equilibrium with fructose-6-phosphate, inhibition of PFK-1 leads to accumulation of these metabolites and subsequent inhibition of hexokinase. In the lactating rat mammary gland *in vivo*, the rate of

lipogenesis falls as the levels of glucose-6-phosphate rise (Jones *et al*, 1984a; Williamson *et al*, 1985). This is accompanied by a decrease in the concentration of F-1,6-BP, suggesting that PFK-1 is a primary regulatory enzyme in determining the flux through glycolysis.

Different isoenzyme forms of PFK-1 exist, all of which display similar kinetic properties (Dunaway, 1983). PFK-1 is activated by AMP and inhibited by ATP which lowers the affinity of the enzyme for fructose-6-phosphate. Therefore, when the ATP/AMP ratio is low, glycolysis is stimulated to ensure that the energy charge of the cell is restored. Citrate is a potent inhibitor of PFK-1 in rat liver (Randle *et al*, 1964) and in lactating rat mammary gland (Zammit, 1979). An increase in the citrate concentration signifies an over supply of biosynthetic precursor for lipogenesis and thus exerts negative feedback control on PFK-1. Fructose-2,6-bisphosphate (F-2,6-BP) is a potent stimulator of PFK-1 increasing the affinity of PFK-1 for fructose-6-phosphate, and decreasing the inhibitory effect of ATP (Pilkis *et al*, 1982). F-2,6-BP is synthesised by phosphofructo-2-kinase (PFK-2, EC 2.7.1.05) and degraded by fructose-2,6-bisphosphatase (F-2,6-BPase, EC 3.1.3.46). These enzyme activities are present within the same polypeptide chain (Pilkis *et al*, 1984).

PFK-1 purified from rat liver is phosphorylated by cAMP-PK *in vitro* and this has no effect on enzyme V_{\max} but increases its sensitivity to inhibition by

citrate (Sakakibara and Uyeda, 1983). Phosphorylation of purified liver PFK-2/F-2,6-BPase bifunctional enzyme on a single serine residue (Pilkis *et al*, 1983) by cAMP-PK *in vitro* inactivates PFK-2 and activates F-2,6-BPase (El-Maghrabi *et al*, 1981, 1982; Van Schaftingen *et al*, 1981, 1982). Therefore, changes in hepatic cAMP levels brought about by hormones such as glucagon, adrenaline and insulin are able to alter PFK-1 activity directly and indirectly via changes in the intracellular concentration of F-2,6-BP (Kuwajima and Uyeda, 1982). In contrast, rat muscle and bovine heart PFK-2/F-2,6-BPase is not phosphorylated and inactivated by cAMP-PK (Rider *et al*, 1985), and the enzyme from bovine heart contains 10 times less F-2,6-BPase activity (Rider and Hue, 1986). This has been shown to be due the presence of a different isozymic form of PFK-2/F-2,6-BPase in these tissues that lacks the phosphorylation site (Van Schaftingen and Hers, 1986). It is not known which isozymic form of PFK-2/F-2,6-BPase is present in the mammary tissue. However, since glycolytic flux does not change in response to cAMP, it seems likely that PFK-2/F-2,6-BPase in the mammary tissue resembles the muscle isoenzyme.

Between late pregnancy and peak lactation, the activity of PFK-1 and levels of F-2,6-BP increase by 4- and 3-fold respectively (Sochor *et al*, 1984). The increase in F-2,6-BP concentration is in part due to a decrease in citrate concentration and an increase in AMP levels during lactation which activates PFK-1 and PFK-2 (Hers *et al*, 1982). During lactation, the marked decrease in

the levels of cAMP in the mammary gland may also contribute to the rise in PFK-1 activity (Sochor *et al*, 1984).

Changes in the rate of lipogenesis are accompanied by reciprocal changes in the concentrations of glucose-6-phosphate, F-1,6-BP and citrate both in mammary tissue slices and intact mammary tissue (Williamson *et al*, 1975, 1980). Ward and Kuhn (1985) showed that the activity of mammary PFK-1 is highly dependent on the presence of F-2,6-BP, and observed small but significant changes in the concentration of F-2,6-BP during starvation and refeeding. However, the intracellular concentration of F-2,6-BP is found to approach saturation of the enzyme (Ward and Kuhn, 1985). Therefore, changes in the concentration of F-2,6-BP alone may not be sufficient to regulate the activity of PFK-1 during starvation and refeeding (Ward and Kuhn, 1985). In starvation, increased uptake of ketone body may lead to the increased concentrations of citrate in the mammary gland (Robinson and Williamson, 1977a; Jones *et al*, 1984a) that would produce PFK-1 inhibition and inhibition of glucose utilisation by the lactating mammary gland *in vivo* (Robinson and Williamson, 1977b) and *in vitro* (Williamson *et al*, 1975; Robinson and Williamson, 1977c).

1.7.3 Pyruvate Dehydrogenase

(a) Structure and Function

Pyruvate Dehydrogenase (PDH) is a multienzyme complex that catalyses the irreversible oxidative decarboxylation of pyruvate to acetyl-CoA in the mitochondria, in the presence of thiamine pyrophosphate, CoA and NAD⁺.

Mammalian PDH complex is a large multimeric enzyme complex in the mitochondrial matrix ($M_r > 7000$ kDa) organised around a core of dihydrolipoyl transacetylase (E2, EC 2.3.1.12) to which pyruvate decarboxylase (E1, EC 1.2.4.1) and dihydrolipoyl dehydrogenase (E3, EC 1.6.4.3) are bound noncovalently (Figure 1.2). E1, E2 and E3 are responsible for the catalytic activity of PDH. In bovine heart, the composition of the PDH complex is 30 E1 : 60 E2: 6 E3. E1 is composed of two non-identical subunits (α and β) and has a tetrameric structure whereas E3 is a homodimer which contains two molecules of FAD. Thiamine pyrophosphate acts as a cofactor and the binding site is located on the α -subunit of E1. The mammalian PDH complex also contains three to five molecules of PDH kinase and phosphatase which modulate the activity of PDH complex by reversible phosphorylation. The kinase is tightly bound to E2 and co-purified with the enzyme. PDH phosphatase is loosely bound to E2 in the presence of Ca²⁺ (Pettit *et al*, 1972). A recently identified subunit termed component X (De Marcucci and Lindsay, 1985) is tightly bound

(b) Regulation of Pyruvate Dehydrogenase

The acute regulation of PDH in response to hormonal and dietary changes is mediated by end product inhibition and reversible phosphorylation. The activity of the enzyme is inhibited allosterically by acetyl-CoA and NADH (Pettit *et al*, 1975; Kerbey *et al*, 1977). In starvation and alloxan-diabetes the activity of PDH complex in rat heart, liver and adipose tissue has been shown to be inhibited as a result of increased oxidation of fatty acids and ketone bodies which increased the mitochondrial ratios of [acetyl-CoA]/[CoA] and [NADH]/[NAD] (Garland and Randle, 1964a, 1964b; Wieland *et al*, 1972). The effect is rapidly reversed by 2-tetradecylglycidate, a fatty acid analogue which inhibits oxidation of long chain fatty acids (Caterson *et al*, 1982). However, administration of 2-tetradecylglycidate has no effect on the activity of PDH in heart, liver, kidney and adipose tissue of normal fed rats. This indicates that the effects of starvation and alloxan-diabetes to inhibit PDH in rat heart are mediated by oxidation of lipid fuels. End product inhibition may play a potential role in the regulation of PDH in lactating rat mammary gland subjected to starvation where an increase in ketone body utilisation is likely to increase the levels of acetyl-CoA. Direct allosteric regulation by end-products is believed to be quantitatively less important than regulation by reversible phosphorylation (Munday and Hardie, 1987a). PDH exists in an active, dephosphorylated and inactive, phosphorylated form. PDH kinase, an integral component of the

enzyme complex phosphorylates the α -subunit of E1 (Sugden and Randle, 1978; Hughes *et al*, 1980). Amino acid sequencing of the α -subunit purified from bovine heart or kidney suggests that there are multiple phosphorylation sites at three separate serine residues (Figure 1.3; Yeaman *et al*, 1978; Sugden *et al*, 1979). Phosphorylation at site 1 proceeds faster than sites 2 and 3 and it is the phosphorylation of site 1 that inactivates the PDH complex (Kerbey *et al*, 1979). Phosphorylation of sites 2 and 3, in addition to site 1 appears to inhibit the rate of reactivation of pig heart PDH by the phosphatase (Sugden *et al*, 1978). In contrast, studies by Teague *et al* (1979) revealed that phosphorylation of sites 1 and 2 inactivates PDH from bovine kidney and no change in the rate of reactivation was observed in response to phosphorylation of sites 2 and 3. The activity of PDH kinase is inhibited by pyruvate, CoA, NAD and ADP but stimulated by increased [acetyl-CoA]/[CoA], [NADH]/[NAD] and [ATP]/[ADP] ratios (for review see Randle, 1986). Phosphorylation of PDH complex is therefore promoted under conditions where end product inhibition occurs. The relative importance of direct allosteric end product inhibition is difficult to quantify because increased [acetyl-CoA]/[CoA] and [NADH]/[NAD] ratios also stimulate PDH kinase (Pettit *et al*, 1975). In rat heart and skeletal muscle, what was thought to be an additional regulatory mechanism of PDH kinase with a slower onset was reported by Hutson and Randle (1978). This involved a mitochondrial protein known as kinase activator protein (KAP), formed by cytoplasmic protein synthesis. Synthesis of KAP was reported in response to

α -subunit of E1. The activity of PDH phosphatase is inhibited by NADH (Pettit *et al*, 1975). In starvation and diabetes, the activity of phosphatase in bovine heart does not change but reactivation of PDH by the phosphatase may be decreased as a result of increased kinase activity (Sale and Randle, 1982).

In the rat mammary gland, the total activity of PDH increases by sevenfold from late pregnancy to peak lactation in parallel with the increased lipogenic capacity of the gland (Gumaa *et al*, 1973). There is a 3-fold increase in the proportion of PDH in its active dephosphorylated form (Coore and Field, 1974). Since formation of acetyl-CoA from glucose represents a net loss of carbohydrate from the body, regulation of the PDH step is essential in view of the high rate of glucose utilisation in the mammary gland of the lactating rat.

The regulation of PDH complex by reversible phosphorylation that has been demonstrated in other tissues also appears to be present in the rat mammary gland. Coore and Field (1974) showed that PDH from extracts of mammary tissue is inactivated by incubation with MgATP, the effect of which is reversed by the addition of Mg^{2+} and Ca^{2+} . Mammary gland PDH is phosphorylated and inactivated in response to starvation for 24 or 48 hours and the proportion of enzyme in its active form decreased by 71% and 77%, respectively (Kankel and Reinauer, 1976; Baxter and Coore, 1978). Inactivation of PDH is confirmed by the large increase in lactate and pyruvate production by mammary acini isolated from starved rats (Robinson and Williamson, 1977a).

The activity of PDH kinase is increased (Baxter and Coore, 1978) and that of PDH phosphatase is decreased (Baxter and Coore, 1979b) in response to starvation. The sensitivity of the kinase towards inhibition by pyruvate also appeared to be diminished in the mammary gland after 24 hour-starvation (Baxter and Coore, 1979b). Short term insulin deficiency induced by streptozotocin treatment has been shown to inactivate mammary gland PDH (Baxter *et al*, 1979). Inactivation of PDH in starved or streptozotocin-treated lactating rats is readily reversible by refeeding (Munday and Williamson, 1981) or by the administration of insulin and glucose *in vivo* (Baxter and Coore, 1978; Baxter *et al*, 1979). The effect of insulin is accompanied by a decrease in PDH kinase activity (Baxter and Coore, 1978) and reactivation of PDH phosphatase (Baxter and Coore, 1979a), and sensitivity of the kinase towards pyruvate is restored (Baxter *et al*, 1979). While dichloroacetate, a potent PDH kinase inhibitor, is able to reactivate PDH in mammary acini from 24 hour-starved rats (Munday and Williamson, 1981), insulin is not, suggesting that factors that mediate the effect of insulin *in vivo* are lost in the preparation of isolated acini.

Evidence so far suggests that regulation of PDH complex by reversible phosphorylation is similar in all mammalian tissues including the lactating rat mammary gland. The importance of PDH as a regulatory step in the pathway of lipogenesis in lactating rat mammary gland is emphasised by the parallel change in the phosphorylation state of PDH and the rate of fatty acid synthesis.

1.7.4 ATP-Citrate Lyase

(a) Structure and Function

ATP-Citrate lyase (ATP-CL, EC 4.1.3.8), also known as citrate-cleavage enzyme is a cytosolic enzyme and catalyses the following reaction :



Acetyl-CoA is produced in the mitochondria by the PDH reaction. Since the mitochondrial membrane is impermeable to acetyl-CoA, this lipogenic precursor leaves the mitochondria as citrate following condensation with oxaloacetate. In the cytoplasm, citrate is cleaved by the action of ATP-CL to regenerate acetyl-CoA and oxaloacetate. ATP-CL purified from rat mammary gland is a tetramer with subunit molecular weight of 116 kDa (Guy *et al*, 1981). During lactation, there is an 8-fold increase in ATP-CL activity in the rat mammary gland (Gumma *et al*, 1973; Martyn and Hansen, 1981). ATP-CL purified from rat liver is similar to that of the mammary gland in terms of subunit molecular weight and amino acid composition (Singh *et al*, 1976).

(b) Regulation of ATP-Citrate Lyase

The activity of ATP-CL is known to be dependent on the nutritional and hormonal status of the animal (Kornacker *et al*, 1965a; 1965b). The content of

ATP-CL in rat liver is decreased by starvation for 2 days and increased by refeeding a fat-free high carbohydrate diet to one third of the normal fed value (Kornacker *et al*, 1965a). These changes in enzyme activity correspond to changes in the mRNA content concentration of ATP-CL (Sul *et al*, 1984; Elshourbagy *et al*, 1990; Fukuda *et al*, 1992; Kim *et al*, 1992).

Regulation of ATP-CL by reversible phosphorylation was first suggested when highly purified enzyme from rat liver (Linn and Srere, 1979) and rat mammary gland (Guy *et al*, 1981) was found to contain 0.5 mol phosphate/mol subunit. The enzyme from rat mammary gland could be further phosphorylated *in vitro* by cAMP-PK (Guy *et al*, 1980), Ca^{2+} - and phospholipid-dependent protein kinase and Ca^{2+} - and calmodulin-dependent protein kinase (Hardie *et al*, 1986). Phosphorylation was shown to occur at a single serine residue of a tryptic peptide (Hardie *et al*, 1986).

Both insulin and glucagon (Alexander *et al*, 1979; Janski *et al*, 1979) were found to stimulate phosphorylation of ATP-CL in isolated rat hepatocytes although their effects on fatty acid synthesis are known to be counter-regulatory (Geelen *et al*, 1978). Both hormones increase phosphorylation of ATP-CL at the same serine residue phosphorylated by cAMP-PK. The level of phosphorylation by insulin and glucagon was only in the region of 0.1 - 0.15 mol phosphate/mol subunit (Alexander *et al*, 1982; Pierce *et al*, 1982) and is therefore unlikely to

be of any physiological significance. It has been shown that insulin treatment of rat adipocytes increased phosphorylation of one ATP-CL tryptic peptide (peptide A) and decreased phosphorylation of a second peptide (peptide B), with dephosphorylation of peptide B stimulating phosphorylation of peptide A (Pucci *et al*, 1983; Ramakrishna *et al*, 1989; 1990). It has been proposed that decreased phosphorylation of peptide B occurs via inactivation of a protein kinase by insulin. A protein kinase known as ATP-CL kinase and recently renamed as multifunctional protein kinase (MFPK) purified from rat liver specifically phosphorylates peptide B, and this leads to a decrease in phosphorylation in peptide A by cAMP-PK (Ramakrishna *et al*, 1989). The activity of MFPK is decreased in response to insulin, further suggesting that the effect of the hormone is mediated via MFPK (Ramakrishna and Benjamin, 1988). MFPK exhibits several properties that are similar to glycogen synthase kinase-3 and has now been found to be identical or highly related to glycogen synthase kinase-3 (Hughes *et al*, 1992).

Phosphorylation of ATP-CL purified from rat mammary gland (Janski *et al*, 1979; Guy *et al*, 1981) and rat liver (Ranganathan *et al*, 1982) has no effect on the catalytic activity of the enzyme although Houston and Nimmo (1985) reported a two-fold increase in the K_m for Mg/ATP upon phosphorylation of rat liver ATP-CL by cAMP-PK. This effect however, has no physiological significance as it is irreversible and the increase in K_m is still more than 10-fold

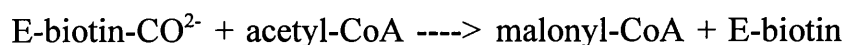
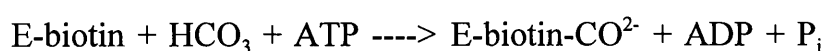
lower than the intracellular Mg/ATP concentrations.

The high amount of ATP-CL in the lactating mammary gland and the lack of physiological significance in its control by reversible phosphorylation suggest that ATP-CL is not involved in the regulation of mammary gland lipogenesis by reversible phosphorylation. The major regulation of the enzyme is probably achieved by altering the amount of the enzyme protein.

1.7.5 Acetyl-Coenzyme A Carboxylase

(a) Structure and Function

Acetyl-coenzyme A carboxylase (ACC, EC 6.4.1.2) is the rate limiting enzyme that catalyses the first committed step of fatty acid synthesis. Its activity was first described by Wakil (1958). ACC is one of the four known mammalian biotin-containing enzymes where biotin binds covalently to the enzyme as a prosthetic group to act as a mobile CO₂ carrier. ACC is the only cytosolic enzyme in this group, the others are mitochondrial enzymes (pyruvate carboxylase, propionyl-CoA carboxylase, 3-methyl crotonyl-CoA carboxylase). The overall carboxylation of acetyl-CoA by ACC to form malonyl-CoA can be partitioned into discrete half reactions (Moss and Lane, 1971):



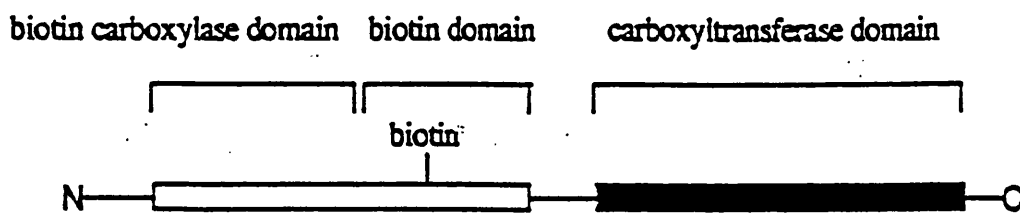
The first step involves the Mg/ATP dependent carboxylation of the biotinyl prosthetic group of the enzyme to form a 1'-N-carboxybiotinyl enzyme intermediate. In the second reaction, carboxyl transfer from the intermediate to acetyl-CoA gives rise to malonyl-CoA.

In *E.coli*, ACC can be resolved into three protein components (Guchait *et al*, 1974) : a biotin carboxylase, a carboxyl transferase and a carboxyl carrier protein. which is a non-enzymic protein contains the covalently bound biotin. Mammalian ACC is a single polypeptide and the proposed model of its domain structure (Figure 1.4) is based on homologies of the primary amino acid sequence with carbamoyl phosphate synthetase (catalyses ATP-dependent carboxylation reaction) and propionyl-CoA carboxylase, and the known location of the covalently bound biotin (Haase *et al*, 1982). The recent cloning and sequencing of cDNAs encoding rat and chicken ACC (Takai *et al*, 1988) suggest that ACC contains two active sites which have been combined by gene-fusion events. ACC can be cleaved by proteinases to yield two fragments of about 120 kDa (Wada and Tanabe, 1985). The site of cleavage has not been defined but presumably occurs between the putative carboxyl carrier and carboxyl transferase domains.

Mamalian ACC purified from rat liver, adipose tissue and mammary gland is a homodimer with a subunit molecular weight of 240 kDa as judged by

Figure 1.4 Model for the Domain Structure of Acetyl-CoA Carboxylase

Modified from Hardie (1989). The domain structure is based on the location of the covalently-bound biotin, and homologies with carbamoyl



SDS-PAGE and 265 kDa as calculated from the polypeptide chain predicted from the cDNA clone (Bianchi *et al*, 1990). An isozymic form of 280 kDa has been described in rat tissues by Bianchi *et al* (1990). This form is uniquely expressed in rat cardiac and skeletal muscle and has a higher K_m for citrate and K_m for acetyl-CoA than the 265 kDa form. Since rates of fatty acid synthesis in cardiac and skeletal muscle are low, the 280 kDa ACC may have an alternative role such as the formation of malonyl-CoA for the regulation of fatty acid oxidation or microsomal fatty acid elongation (Bianchi *et al*, 1990). The 265 kDa and 280 kDa forms of ACC are clearly the products of separate genes (Winz *et al*, 1994). The existence of a mitochondrial form of ACC which is

relatively inactive has also been reported (Allred and Roman-Lopez, 1988; Roman-Lopez *et al*, 1989). These authors suggested that the mitochondrial ACC represents a reservoir of enzyme which can be released into the cytosol and become activated under lipogenic conditions. Fasting shifted the subcellular distribution of ACC towards mitochondria and thus reduced the activity of the enzyme. Upon refeeding, the distribution shifted towards cytosol and was associated with increased ACC activity. The increase in the amount of inactive ACC associated with the mitochondria has also been observed in alloxan-diabetes (Roman-Lopez and Allred, 1987). However, Moir and Zammit (1990) observed no such changes in translocation of ACC from the mitochondria to the cytosol in rat liver upon refeeding starved rats.

(b) Regulation of Acetyl-CoA Carboxylase

(i) Long Term Control

ACC is a highly regulated enzyme, subjected to both long term and short term control. Long term control of ACC is achieved via changes in the mRNA levels and in the rate of protein synthesis and degradation (Majerus and Kilburn, 1969). The half-life of ACC was found to vary from 1-3 days according to metabolic status (Nakanishi and Numa, 1970). The rise in ACC concentration in liver upon refeeding after starvation can be prevented by treatment with puromycin, a protein synthesis inhibitor or actinomycin D, a transcription

inhibitor (Hicks *et al*, 1965). A fat-free diet induces synthesis of ACC and increases its activity in rat liver of 48 hour-starved animals due to simultaneous changes in the rate of degradation and synthesis of ACC (Nakanishi and Numa, 1970). Diet induced changes in the concentration of ACC in rat liver or epididymal fat pads also correlate positively with changes in the amount of ACC mRNA (Pape *et al*, 1988).

Rat mammary gland ACC content and activity increase after parturition as a result of increased synthesis of the enzyme (Mackall and Lane, 1977). This is accompanied by a 7-fold increase in the translatable ACC mRNA (Lopez-Cassilas *et al*, 1988). The mechanism controlling the expression of the ACC gene and the turnover of the enzyme has not been fully elucidated although anabolic hormones may play an important role. In the absence of either glucocorticoids, insulin or thyroxine, the increase in lipogenic capacity of liver upon refeeding of 36 hour-starved animals is greatly reduced (Wurdeman *et al*, 1978; Bouillon and Berdanier, 1980). Since the half-life of ACC is long (1-3 days), changes in enzyme protein concentration is unlikely to account for the short term hormonal control of ACC. However, 24 hour starvation has been shown to reduce the ACC content of the lactating mammary gland by 50% (Munday and Hardie, 1986).

(ii) Short Term Control

Allosteric Regulation

Short term regulation of ACC involves both allosteric and covalent modification of the enzyme. Citrate is an allosteric activator of all mammalian ACC (Moss and Lane, 1971). The concentration of citrate required for the half maximal activation of ACC (K_a) from mammary gland is approximately 2 mM (Munday *et al*, 1986) and the intracellular concentration varies between 0.2 to 0.5 mM (Williamson *et al*, 1975; Robinson and Williamson, 1977b), thus suggesting that citrate is an important potential physiological regulator of ACC. However, a close relationship between citrate concentration and fatty acid synthesis has not been demonstrated. Hagopian *et al* (1991) reported that starvation of lactating rats for 6 hours is accompanied by increased citrate concentrations and inhibition of ACC. Nishikori *et al* (1973) reported a rapid rise in citrate concentration, accompanied by increased ACC activity and rate of fatty acid synthesis in rat liver upon refeeding of starved animals. In rat epididymal fat pads, Halestrap and Denton (1974) reported increased ACC activity and rate of fatty acid synthesis in response to insulin with no corresponding changes in citrate concentration. In addition, adrenaline caused an increase in citrate concentration accompanied by inhibition of fatty acid synthesis (Denton and Halperin, 1968; Saggerson and Greenbaum, 1970).

Long chain fatty acyl-CoA esters are classical potent feedback inhibitors of ACC at micromolar concentrations (Bortz and Lynen, 1963; Lunzer *et al*, 1977; Ogiwara *et al*, 1978). Treatment of rat epididymal fat pads with insulin showed that an inverse relationship exists between the rate of fatty acid synthesis and intracellular levels of fatty acyl-CoA esters (Denton and Halperin, 1968; Saggerson and Greenbaum, 1970). *In vivo*, binding of fatty acyl-CoA esters to proteins and compartmentation within the cell (Lunzer *et al*, 1977) mean that the true intracellular concentrations are difficult to determine. As a result, the significance of the allosteric regulation of ACC *in vivo* by fatty acyl-CoA esters has yet to be confirmed.

Several other potential regulators of ACC have also been described. Guanine nucleotides have been shown to activate ACC in crude tissue extracts and the effect is lost during purification (Witters *et al*, 1981; Yeh *et al*, 1981; Beuchler and Gibson, 1984). In addition, polyphosphoinositides (Heger and Peter, 1977; Blyth and Kim, 1982), an autocrine factor identified as an oligosaccharide (Witters *et al*, 1988) and small molecular weight substances released from liver plasma membranes by insulin binding (Saltiel *et al*, 1983) have been suggested to modulate the activity of ACC. Recently, Moule *et al* (1992) showed that CoA inhibits ACC *in vitro* and Quayle *et al* (1993) have identified a small molecular weight protein regulator of ACC in rat liver. This regulator activated highly purified ACC by 2- to 3-fold at physiological citrate

concentrations and the effect is unlikely to be mediated by dephosphorylation or proteolysis of ACC. It is non-dialysable and can be inactivated by heating or by exposure to carboxypeptidase (Quayle *et al*, 1993). The physiological role of any of these molecules in regulating ACC is not established.

ACC exists in protomeric and polymeric forms with the polymeric form being more active. Studies by several authors (Hardie, 1980; Numa and Tanabe, 1984) have suggested that allosteric regulation of ACC by citrate and fatty acyl-CoA is associated with changes in the protomer : polymer ratio. The protomeric form of ACC is a homodimer (Gregolin *et al*, 1966a) which forms a filamentous polymer of up to 32 subunits in the presence of high concentrations of citrate (Gregolin *et al*, 1966b; Lane *et al*, 1974; Ahmad *et al*, 1978). Depolymerisation by agents such as malonyl-CoA which is also a potent product inhibitor of the enzyme (Moss and Lane, 1972a; 1972b) is associated with loss of catalytic activity of the enzyme. This is achieved via an increase in the carboxylated state of ACC (enzyme-biotin-CO²⁻) which has a greater tendency to depolymerise (Lane *et al*, 1974). Depolymerisation of ACC by Mg²⁺ is achieved by binding of Mg²⁺ with citrate, thus lowering the free citrate concentration (Beatty & Lane, 1983). Tanabe *et al* (1977) showed that ACC is less susceptible to proteolysis in the presence of citrate which may mean that it is protected by polymerisation. The polymers of ACC have been observed by electron microscopy, viscometry and ultracentrifugation of the purified enzyme and by gel filtration and sucrose

gradient velocity sedimentation of crude extracts (Ahmad *et al*, 1978). It was originally thought that the polymeric form of ACC was the active species of the enzyme but it was reported that activation of ACC by citrate precedes its polymerisation (Beatty and Lane, 1983).

Polymerisation of ACC may be of physiological significance *in vivo*. Whilst the polymeric form of ACC is resistant to inhibition by avidin, the protomeric form is avidin sensitive (Moss *et al*, 1972; Meredith *et al*, 1978). Binding of avidin to the biotinyl prosthetic group of the protomeric form leads to inactivation of the enzyme (Moss *et al*, 1972; Meredith and Lane, 1978). An increased in avidin sensitivity of hepatic ACC has been observed in chicks subjected to starvation for 6 hours or fed high fat diets (Ashcraft *et al*, 1980). Acute exposure of cultured chick hepatocytes to cAMP, where citrate concentrations were decreased by 90%, is associated with a reduction in the proportion of polymeric ACC (Meredith and Lane, 1978). Using digitonin treatment, which renders the plasma membrane permeable to cytoplasmic enzymes, Meredith and Lane (1978) have shown that inactivation of ACC in chicken hepatocytes treated with glucagon occurred as a result of depolymerisation of the enzyme. Halestrap and Denton (1974) observed an increase in the proportion of ACC in its polymeric form in rat adipose tissue treated with insulin. Similar observations was made by subsequent studies carried out by Borthwick *et al* (1987) who utilised rapid gel-permeation

chromatography (Superose 6) to separate the protomeric and polymeric form of ACC from rat adipocytes exposed to insulin. It was shown that ACC is highly polymerised in the presence of insulin.

Covalent Modification

Reversible phosphorylation as a mechanism of short term regulation of ACC was first suggested by Inoue and Lowenstein (1972) who found that ACC purified from rat liver contained covalently bound phosphate. The first evidence that the enzyme was regulated by reversible phosphorylation appeared when a partially purified preparation from rat liver was shown to be inactivated by incubation with MgATP, and the effect was reversed by treatment with a protein phosphatase preparation (Carlson and Kim, 1973). The enzyme purified from rat mammary gland was also later identified as a phosphoprotein (Ahmad *et al*, 1978; Hardie and Guy, 1980) that contains approximately 3 mol of alkali-labile phosphate/mol subunit (Hardie and Guy, 1980). The purified enzyme can be further phosphorylated *in vitro* by incubation with MgATP and the catalytic subunit of cAMP-PK (Hardie and Guy, 1980; Munday and Hardie, 1984) or a number of other protein kinases (Hardie, 1989, see section 1.8.).

Insulin has a stimulatory effect on fatty acid synthesis in isolated rat hepatocytes and adipocytes (Hardie *et al*, 1984). Since phosphorylation of ACC

is associated with a decrease in the activity of the enzyme, insulin would be expected to cause dephosphorylation and activation of ACC, and hence stimulate fatty acid synthesis. However, activation of ACC in response to insulin is found to be associated with increased phosphorylation within a tryptic peptide termed the "I" site (Brownsey and Denton, 1982). This was confirmed in isolated rat adipocytes (Haystead *et al*, 1988) and hepatocytes (Holland and Hardie, 1985). In contrast, activation of ACC in response to insulin with no accompanying increase in phosphorylation has also been reported (Witters *et al*, 1988). Brownsey *et al* (1981) reported that incubation of an adipocyte membrane fraction with partially purified adipocyte ACC produced a time- and MgATP-dependent activation of the enzyme. Phosphorylation of ACC was found to precede the activation. In cell free extracts of adipocytes incubated with insulin, ACC activity was increased. This led to the proposal that insulin caused the translocation from membrane to cytosol of a kinase which phosphorylates and activates ACC. In mammary cells, Munday *et al* (1986) reported that insulin caused ACC activation and this involved dephosphorylation of the inhibitory site of phosphorylation and in a Fao Reuber hepatoma cell line, Witters and co-workers (1988) reported an insulin-stimulated dephosphorylation of ACC. A small molecular weight molecule, phospho-oligosaccharide has been implicated in the effect of insulin (Saltiel *et al*, 1983; Haystead and Hardie, 1986; Witters *et al*, 1988). However, the exact mechanism by which this molecule could mediate the effect of insulin on phosphorylation of ACC is unknown.

Glucagon treatment of isolated hepatocytes inhibits fatty acid synthesis by 50% and this is accompanied by an increase in the phosphate content of ACC from 4.5 mol to 5.2 mol/mol subunit (Holland *et al*, 1984). The effect of glucagon is associated with decreased V_{\max} and increased K_a for citrate of ACC (Holland *et al*, 1984; 1985). In isolated rat adipocytes, phosphorylation and inactivation of ACC was stimulated by adrenaline (Brownsey *et al*, 1979). Phosphate content of ACC was increased from 3.3 mol to 4.7 mol/mol subunit (Holland *et al*, 1985). The effect of glucagon in hepatocytes and adrenaline in adipocytes is to increase the levels of cAMP implicating cAMP-PK in the phosphorylation of ACC. While cAMP-PK can phosphorylate ACC *in vitro*, the site at which the major increase in phosphate occurs (serine-79) and which appears to exert the major effect on ACC inactivation is in fact a substrate for AMP-activated protein kinase (AMP-PK) and not cAMP-PK (Sim and Hardie, 1988; Haystead *et al*, 1990; Munday *et al*, 1988; see section 1.8.6). The link between increased intracellular cAMP and serine-79 phosphorylation is not established. However, a second site of phosphorylation of ACC (serine-1200) that is phosphorylated by both cAMP-PK and AMP-PK *in vitro* is also phosphorylated in hepatocytes and adipocytes in response to glucagon and adrenaline respectively, and has also been shown to produce ACC inactivation (Ha *et al*, 1994) so that a physiological role for direct phosphorylation of ACC by cAMP-PK is not completely ruled out. Starvation is associated with inhibition of fatty acid synthesis in rat liver and the effect is reversed by refeeding for 2-4

hours (Holness *et al*, 1988; Holness and Sugden, 1990). While starvation is accompanied by decreased hepatic ACC concentration (Nakanishi and Numa, 1970; Munday *et al*, 1991), the fed to starved and starved to refeed transitions are paralleled by phosphorylation and inactivation, and dephosphorylation and activation of hepatic ACC, respectively (Jamil and Madsen, 1987; Thampy and Wakil, 1988; Munday *et al*, 1991).

In lactating rat mammary gland, starvation for 24 hours leads to inhibition of ACC and an increase in the alkali-labile phosphate content of approximately 1 mol/mol subunit (Munday and Hardie, 1986). This is accompanied by a large and significant decrease in the V_{\max} (73%) and an increase in K_a for citrate (75%). The inhibition of ACC in response to starvation could be reversed by PP2A purified from rabbit skeletal muscle *in vitro* (Munday and Hardie, 1986) or by refeeding 24 hour-starved lactating rats with chow for 2.5 hour (Munday and Hardie, 1986). The effect of refeeding appears to be mediated via increased arterial plasma insulin since this is restored to the control fed value upon refeeding of starved animals (Robinson *et al*, 1978), and streptozotocin treatment of starved lactating rats immediately before refeeding prevents the dephosphorylation and activation of ACC (Munday and Hardie, 1986). Short term starvation for 6 hours (Munday and Hardie, 1986) and acute insulin deficiency induced by streptozotocin treatment (McNeillie and Zammit, 1982; Munday and Williamson, 1982) do not significantly alter the activity and

phosphate content of mammary ACC although fatty acid synthesis is significantly inhibited. Inhibition of lipogenesis under these conditions appears to occur as a result of decreased substrate supply and inhibition of PDH (Hagopian *et al*, 1991). The decrease (60%) in the concentration of mammary ACC that occurred after 24 hour starvation of lactating rats is not restored by 2.5 hour refeeding (Munday and Williamson, 1982). This suggests that regulation of ACC by reversible phosphorylation is more important than changes in enzyme concentration in the acute control of mammary gland lipogenesis.

The inhibition of lipogenesis in lactating rat mammary gland in response to high fat (cafeteria) feeding (Munday and Hardie, 1987b) appears to result primarily via increased phosphorylation and inhibition of ACC (Munday and Hardie, 1987b; Munday and Williamson, 1987), and the effect is reversible by insulin in isolated acini *in vitro* (Munday and Hardie, 1987b). V_{\max} of ACC purified from insulin treated acini increased by 36%, and the phosphate content was decreased by 0.6 mol/mol subunit (Munday and Hardie, 1987b).

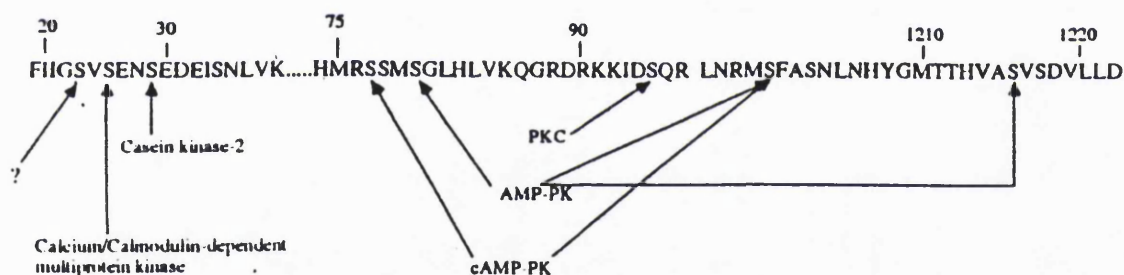
The regulation of ACC in rat liver, adipose tissue and lactating mammary gland by reversible phosphorylation is now firmly established and is probably the major mechanism of ACC regulation *in vivo*. Evidence from amino acid and cDNA sequencing have shown that there are up to nine serine residues of ACC which can be phosphorylated (Figure 1.5). Protein kinases that have so far been

shown to phosphorylate purified ACC *in vitro* are discussed below.

Figure 1.5 Map of the Phosphorylation Sites on Acetyl-CoA Carboxylase

The location of phosphorylation sites on ACC by various protein kinases is shown. The regions of sequence containing the phosphorylation sites have been illustrated in detail using the single letter amino acid code. The question mark indicates the serine residue found phosphorylated in intact adipocytes (Haystead *et al*, 1988) and the protein kinase that phosphorylates this site has not been identified. AMP-PK = AMP-activated protein kinase, cAMP-PK = cyclic AMP-dependent protein kinase and PKC = protein kinase C. Modified from Davies *et al* (1990).

Fig. 1.4. Map of the Phosphorylation Sites on Acetyl-CoA Carboxylase



1.8 Protein Kinases that Phosphorylate ACC

1.8.1 Casein Kinases-1 and 2

Casein kinases-1 and 2 have a widespread tissue distribution (Hathaway and Traugh, 1982) and have been partially purified and characterised from the lactating rat mammary gland (Munday and Hardie, 1984). Casein kinase-2

phosphorylates ACC at serine-29, which corresponds to a site phosphorylated in response to insulin in isolated adipocytes (Haystead *et al*, 1988). The casein kinase-1 phosphorylation site has not been identified. Phosphorylation of ACC by either kinase does not alter the activity or kinetic properties of ACC (Munday and Hardie, 1984; Hardie *et al*, 1986) so that their physiological significance is unclear.

1.8.2 Ca²⁺- and Calmodulin-dependent Multiprotein Kinase

Ca²⁺- and calmodulin-dependent multiprotein kinase has been purified and characterised in several different tissues (Cohen, 1988) including isolated lactating rat mammary acini (Brooks and Landts, 1985). The kinase phosphorylates ACC purified from rat mammary gland in a Ca²⁺- and calmodulin-dependent manner (Hardie *et al*, 1986) on serine-25 (Lopez-Casillas *et al*, 1988). The kinetic parameters of ACC remain unchanged as a result of this phosphorylation (Hardie *et al*, 1986).

1.8.3 Protein Kinase C

Protein kinase C phosphorylates ACC at ser-77 and ser-95 (Hardie *et al*, 1986), resulting in a modest decrease of V_{\max} (Haystead and Hardie, 1988; Lopez-Casillas *et al*, 1988). Phosphorylation is dependent on the presence of

Ca^{2+} and phospholipid and is reversed by protein phosphatase 2A. Since serine-77 is also phosphorylated by cAMP-PK (Munday *et al*, 1988), it is likely that the observed decrease in V_{\max} of ACC is the result of phosphorylation at this serine residue. Phorbol esters stimulate the activity of protein kinase C (Castagna *et al*, 1982). In adipocytes, lipogenesis is stimulated by treatment with phorbol ester (Van de Werve *et al*, 1985) but the activity of ACC is not stimulated (Haystead and Hardie, 1987). Therefore, the physiological significance of the phosphorylation of ACC by protein kinase C is unclear.

1.8.4 cAMP-dependent Protein Kinase

The catalytic subunit of cAMP-dependent protein kinase (cAMP-PK) purified from rabbit skeletal muscle or bovine heart was the first protein kinase shown to phosphorylate purified ACC (Hardie and Cohen, 1978; Munday *et al*, 1988). The kinase phosphorylates ACC at serine-77 and serine-1200, resulting in a decrease in the affinity of the enzyme for citrate (i.e. increase K_a) and a relatively small decrease (15%) of V_{\max} (Hardie and Guy, 1980; Munday *et al*, 1988). Phosphorylation is prevented by the heat stable protein inhibitor of cAMP-PK (Brownsey and Hardie, 1980) and is reversed by protein phosphatase 1, 2A or 2C (Ingebritsen *et al*, 1983). In contrast, Lent and Kim (1983) observed no phosphorylation and inactivation of hepatic ACC by cAMP-PK and have suggested that ACC is phosphorylated by a specific ACC kinase which is itself

phosphorylated and activated by cAMP-PK. In all physiological conditions, the small decrease in ACC V_{\max} achieved by cAMP-PK is insufficient to explain the phosphorylation and inactivation of ACC *in vivo*. For example glucagon in hepatocytes produces a 50-60% decrease in ACC V_{\max} (Holland *et al*, 1984) and 24 hour starvation of lactating rats produces a 75% decrease in the V_{\max} of ACC in mammary gland (Munday and Hardie, 1986). In rat liver, the major increase in cAMP-PK activity in response to starvation occurs after ACC inactivation (Munday *et al*, 1991) and in lactating rat mammary gland there is no increase in cAMP-PK activity in response to starvation (Clegg and Ottey, 1990). Pharmacological elevation of cAMP in isolated mammary acini has been shown to activate cAMP-PK and result in phosphorylation and activation of glycogen phosphorylase but have no effect on ACC (Clegg and Mullaney, 1985; Clegg and Ottey, 1990). This may be explained by the observation that the catalytic subunit of cAMP-PK in mammary tissue appears to be a distinct isozymic form with a poorer substrate specificity for ACC than the isoforms from skeletal muscle or heart (Takhar and Munday, 1992).

1.8.5 Acetyl-CoA Carboxylase Kinase-2

Acetyl-CoA carboxylase kinase-2 (ACK-2) which has been partially purified from lactating rat mammary gland was originally identified as a contaminant of ACC preparations from rabbit mammary gland (Munday and

Hardie, 1984). The kinase has been shown to phosphorylate ACC to bring about similar kinetic changes to those achieved by cAMP-PK (Munday and Hardie, 1984). Although ACK-2 has not been fully characterised, it can be distinguished from the catalytic subunit of cAMP-PK. ACK-2 has an apparent molecular weight on gel filtration of 76 kDa compared to 40 kDa for the catalytic subunit of cAMP-PK and is insensitive to the specific inhibitor of cAMP-PK (Munday and Hardie, 1984). Amino acid sequencing suggests that ACK-2 phosphorylates ACC at ser-1200 (Munday *et al*, 1988). However, the exact physiological role of ACK-2 remains unclear.

1.8.6 AMP-activated Protein Kinase

(a) Structure and Function

Studies on isolated rat hepatocytes by Holland *et al* (1984) showed ACC to have a low specific activity with a high phosphate content (4-5 mol phosphate/mol subunit) even in cells under basal conditions. This ACC could be reactivated with protein phosphatase with a large increase in V_{\max} and a decrease in K_a for citrate (Holland *et al*, 1984; Sim and Hardie, 1988). Since phosphorylation of ACC by cAMP-PK and ACK-2 only produce a moderate inhibition of V_{\max} , the presence of another ACC kinase in rat liver was suggested. Lent and Kim (1983) first described and characterised a rat liver kinase which produced a large decrease in the V_{\max} of ACC. The kinase activity

co-purified with ACC with a subunit molecular weight of 170 kDa. However, there was no evidence that the 170 kDa polypeptide correlated with the kinase activity. A protein kinase from rat liver was later purified which accounted for more than 90% of the cyclic nucleotide independent ACC kinase activity (Carling and Hardie, 1986; Carling *et al*, 1987). This kinase has been called the AMP-activated protein kinase (AMP-PK), as it was found to be stimulated by 5'AMP *in vitro*. A good correlation exists between inactivation of ACC and its phosphorylation by AMP-PK (Carling *et al*, 1989). Phosphorylation results in both an increase in K_a for citrate and a large decrease in V_{max} (80%) of ACC (Munday *et al*, 1988).

AMP-PK phosphorylates rat ACC at serine-79, serine-1200 and serine-1215 (Davies *et al*, 1990), as defined by amino acid sequencing (Munday *et al*, 1988). It is through phosphorylation of serine-79 that AMP-PK achieves its dramatic effect on V_{max} (80%). The phosphorylation of serine-77 explains the relatively diminished effect of cAMP-PK on V_{max} (Munday *et al*, 1988). The increased phosphorylation of serine-79 has been shown in isolated hepatocytes and adipocytes in response to glucagon and adrenaline, respectively (Sim and Hardie, 1988; Davies *et al*, 1990; Haystead *et al*, 1990) and serine-79 contains phosphate in rat liver *in vivo* (Davies *et al*, 1991). In none of these conditions did serine-77 contain phosphate even in intact cells when cAMP levels were increased. When the N-terminal segment of fully phosphorylated ACC (which

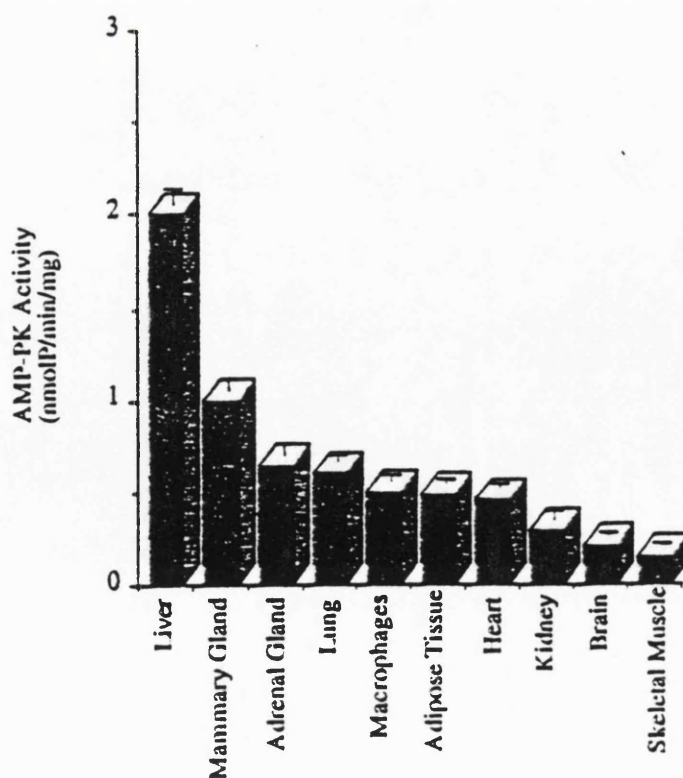
contains serine-79) was removed by limited proteolysis, enzyme activity was completely restored (Davies *et al*, 1990). This emphasized the importance of serine-79 and suggested that serine-1200 plays no part in regulating activity (serine-1215 is a minor site of phosphorylation). However, more recent site-directed mutations of ACC have suggested that phosphorylation of serine-1200 may play a part in ACC inactivation (Ha *et al*, 1994) and this site is phosphorylated by both AMP-PK and cAMP-PK and ACK-2 (Munday *et al*, 1988). Despite this recent observation, the overwhelming evidence is that AMP-PK and not cAMP-PK is the physiological ACC kinase.

AMP-PK is clearly involved in the general regulation of lipid metabolism. Its other major substrates currently identified are HMG-CoA reductase that regulates cholesterol biosynthesis and is phosphorylated and inactivated by AMP-PK (Carling *et al*, 1987) and hormone sensitive lipase that controls lipolysis where phosphorylation by AMP-PK blocks phosphorylation and activation by cAMP-PK (Garton *et al*, 1989). Tissue distribution studies show that AMP-PK is present in nine different rat tissues and in a mouse macrophage cell line (Figure 1.6; Davies *et al*, 1989). The highest activity of AMP-PK is found in those tissues most active in lipid biosynthesis (Figure 1.6). The ability of AMP-PK to inhibit lipid biosynthesis and its activation by 5'AMP has led to its proposed role as a mediator in the cellular stress response switching off biosynthetic pathways when ATP levels are depleted in response to fuel

to fuel limitation or anoxia (Corton *et al*, 1994). AMP-PK is a member of the yeast SNF1 protein kinase subfamily that includes protein kinases present in plants, nematodes and mammals (Hanks and Hunter, 1995). In rat liver it consists of a 63 kDa α -catalytic subunit that shares strong sequence homology with the catalytic domain of the yeast SNF1 kinase (Carling *et al*, 1994; Woods *et al*, 1994), a 38 kDa γ -subunit that is activatory and homologous to the SNF4p subunit and a 40 kDa β -subunit that is related to a family of transcription regulators (Stapleton *et al*, 1994). Recent evidence suggests the existence of isozymic forms of these subunits (Stapleton *et al*, 1996).

Figure 1.6 Tissue Distribution of AMP-activated Protein Kinase

The activity of AMP-PK in extracts prepared from various tissues is shown. The results are means for four separate measurements with S.E.M. indicated by the vertical bars. Modified from Davies *et al* (1990).



(b) Regulation of AMP-activated Protein Kinase

The activity of AMP-PK is stimulated 4- to 6-fold by 5'AMP which has a half maximal effect at 14 μ M that falls within the physiological range in rat liver (Carling *et al*, 1987). Adenosine inhibits AMP-PK with a half maximal effect at 200 μ M (Hardie, 1989). Other nucleotides (including ADP and cAMP) have no effect on AMP-PK activity but a number of synthetic analogues including ZMP (5 amino-4-imidazole carboxamide ribotide) produce stimulatory effects comparable to AMP (Sullivan *et al*, 1994). A large increase in AMP concentration is observed in rat hepatocytes during anoxia due to the action of adenylate cyclase which responds to a small drop in ATP levels (Vincent *et al*, 1982). Elevated levels of AMP in response to anoxia or fructose treatment stimulate AMP-PK *in vivo* or in isolated hepatocytes and result in phosphorylation and inactivation of the target enzymes ACC and HMG-CoA reductase (Ottey *et al*, 1989; Gillespie and Hardie, 1992). However, anoxia is not a physiological condition and hence, the relevance of this mechanism to hormonal regulation of lipid metabolism is unclear.

AMP-PK is also regulated by reversible phosphorylation (Carling *et al*, 1987). It was found that partially purified AMP-PK in its inactive form can be reactivated by the addition of MgATP. The reactivation appears to be due to a distinct "kinase-kinase", the activity of which can be stimulated by nanomolar

concentrations of palmitoyl-CoA (Carling *et al*, 1987) and by 5'AMP (Hawley *et al*, 1995). Phosphorylation of AMP-PK by kinase-kinase results in a more than 20-fold activation of the enzyme (Carling *et al*, 1987). There is thus an AMP-PK cascade that is sensitive to the energy charge of the cell and to produce inhibition (fatty-acyl-CoA) that can exert feedback control on fatty acid and cholesterol synthesis.

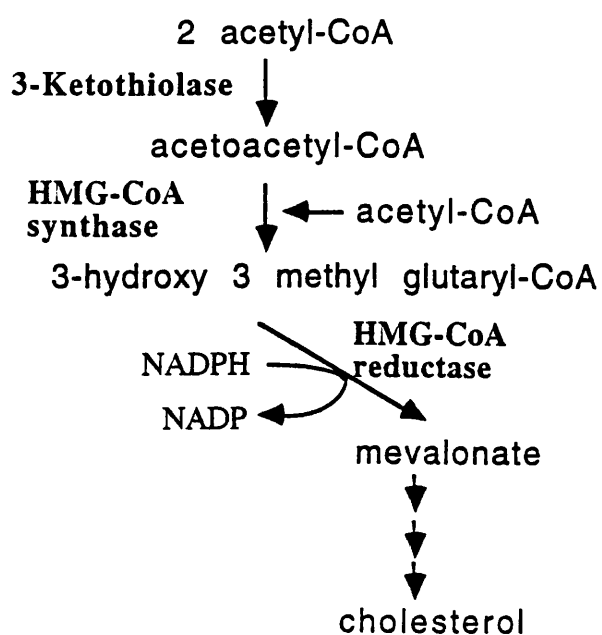
Activation of AMP-PK in response to starvation has been reported in the lactating rat mammary gland (Milic *et al*, 1992) and in rat liver (Munday *et al*, 1991). In the latter case, the increase in activity closely paralleled the decrease in plasma insulin concentration and insulin inhibition of AMP-PK has been reported in a Fao hepatoma cell line (Witters and Kemp, 1992).

1.9 3-Hydroxy-3-methylglutaryl-CoA Reductase and Cholesterol Synthesis

Cholesterol is an important component of all cellular membranes and is also a precursor for steroid hormones, vitamins and bile salts. The biosynthesis of cholesterol is a complex pathway of some 25 steps, some of which are summarised in Figure 1.7. Cholesterol synthesis utilises cytosolic acetyl-CoA as its precursor and NADPH as its reducing power. Lactate and pyruvate (3 mM) are thought to be the major substrates for mammalian hepatic cholesterol synthesis whilst glucose at physiological concentration (5-15 mM) provides only

6-12% of substrate for the process (Gibbons and Pullinger, 1979). Rat liver exhibits high rates of cholesterol synthesis whereas non-hepatic tissues other than intestine show rates that are less than 5% of those in the liver (Balasubramaniam *et al*, 1976). Hepatic cholesterol synthesis is known to undergo diurnal changes over a 24-hour period. This is related to the pattern of food intake of the animal, being minimal during the light phase and highest at the middle of the dark phase (Brown *et al*, 1973). Cholesterol synthesis in isolated rat hepatocytes is stimulated by insulin (Geelen and Gibson, 1975) and inhibited by glucagon (Beg *et al*, 1973) and cholesterol feeding (Shapiro and Rodwell, 1971). Such regulation is mediated by changes in the activity of HMG-CoA reductase.

Figure 1.7 Pathway of Cholesterol Synthesis



Mammalian HMG-CoA reductase is a transmembrane glycoprotein embedded in the endoplasmic reticulum with its active site orientated towards the cytosol where cholesterol synthesis occurs (Liscum *et al*, 1983; Brown and Simoni, 1984). Rat liver HMG-CoA reductase has a short half-life of approximately 3 hours (Edwards and Gould, 1972). The enzyme activity follows a diurnal rhythm, parallel to the nocturnal feeding behaviour of rats (Shapiro and Rodwell, 1969; Gibbons *et al*, 1983; Easom and Zammit, 1984). Diurnal variation in the activity of the enzyme is attributed to an increase in the rate of enzyme protein synthesis and a decrease in the rate of enzyme degradation (Higgins and Rudney, 1973). Changes in the membrane composition and fluidity in the vicinity of the enzyme also play a role in regulating the activity or degradation of HMG-CoA reductase (Brown and Goldstein, 1980).

HMG-CoA reductase is inactivated in a time-dependent manner after incubation of microsomal membranes with MgATP (Beg *et al*, 1978; Nordstrom *et al*, 1977) and this is reversed by incubation with a highly purified liver protein phosphatase (Ingebritsen *et al*, 1981). AMP-PK is the major reductase kinase in rat liver and it copurifies with ACC kinase activity during a six step, 4800-fold purification from rat liver and is inactivated with similar kinetics by treatment with fluorosulphonylbenzoyl adenosine (FSBA), a reactive ATP analogue (Carling *et al*, 1989). Phosphorylation of HMG-CoA reductase by AMP-PK occurs at a serine residue close to the C-terminal (serine-872 in the

human enzyme) and this leads to inactivation of HMG-CoA reductase *in vitro*. Clarke and Hardie (1990) showed that serine-871 can be phosphorylated in intact liver. Phosphorylation of HMG-CoA reductase is observed *in vivo* in rats fed cholesterol and *in vitro* in isolated rat hepatocytes incubated with cholesterol precursor mevalonic acid (Gibson and Parker, 1987) or fructose to raise AMP (Gillespie and Hardie, 1992).

In vitro, HMG-CoA reductase is also reported to be phosphorylated by protein kinase C (Beg *et al*, 1985) and Ca^{2+} - and calmodulin-dependent protein kinase (Beg *et al*, 1987). However, phosphorylation of HMG-CoA reductase by these kinases is unlikely to be of physiological importance since treatment of isolated rat hepatocytes with phorbol esters or Ca^{2+} ionophores which stimulate their activity does not lead to phosphorylation of HMG-CoA reductase (Zammit and Caldwell, 1991).

1.10 Protein Phosphatases

Extensive studies over the years have led to identification of a large number of serine/threonine-specific protein phosphatases (PP) and these are classified into two groups (for review, see Cohen, 1989). Type 1 dephosphorylate the β -subunit of phosphorylase kinase and are inhibited by nanomolar concentrations of heat and acid-stable proteins termed inhibitor-1 and

inhibitor-2. Type 2 dephosphorylate the α -subunit of phosphorylase kinase and are insensitive to inhibitor-1 and 2. Type 2 protein phosphatases are subclassified into PP2A, PP2B and PP2C, based on their requirements for divalent cations. PP2B and PP2C are active in the presence of Ca^{2+} and Mg^{2+} , respectively whilst PP2A is active in the absence of divalent cations.

Serine/threonine protein phosphatases have broad and overlapping specificities. They account for almost all protein phosphatase activity in tissue extracts toward a wide range of phosphoproteins that control glycogen metabolism, protein synthesis, fatty acid synthesis, cholesterol synthesis, glycolysis, gluconeogenesis, amino acid breakdown and various other processes. The recent discovery of okadaic acid, a potent tumour promoter has provided an improved procedure for the measurement of the activities and the relative proportion of serine/threonine protein phosphatases in tissue extracts (Cohen *et al*, 1989). Okadaic acid is a potent inhibitor of PP1 ($\text{IC}_{50} = 10 \text{ nM}$) and PP2A ($\text{IC}_{50} = 0.1 \text{ nM}$) but has no effect on PP2C or mitochondrial phosphatases and PP2B is relatively insensitive ($\text{IC}_{50} = 5 \text{ }\mu\text{M}$). Therefore measurement of PP1 and PP2A activities is achieved by utilising the differential inhibition of these enzymes by okadaic acid at different concentrations.

In vitro, PP2A has a high activity toward the regulatory enzymes of glycolysis, gluconeogenesis, fatty acid and cholesterol synthesis (Ingebritsen *et*

al, 1983). This suggests that it may also be the major protein phosphatase *in vivo*. In intact cells, treatment with okadaic acid stimulates hepatic gluconeogenesis and adipose tissue lipolysis and inhibits hepatic fatty acid synthesis (Haystead *et al*, 1989; Rutter *et al*, 1991).

In vivo, PP2A has been identified as the major phosphatase acting on ACC (Clarke *et al*, 1991). Assays in fractions of rat liver have demonstrated that PP2A (30%) and PP2C (66%) account for almost all of the phosphatase activity that dephosphorylates HMG-CoA reductase although treatment of hepatocytes with okadaic acid suggests that PP2A is the important activity (Clarke *et al*, 1991). PP2A (40%) and PP2C (60%) account for essentially all of the AMP-PK phosphatase activity in rat liver extracts. However, the lack of okadaic acid-sensitivity of AMP-PK phosphorylation in isolated hepatocytes suggests that PP2C may be responsible for AMP-PK dephosphorylation in the intact cell (Clarke *et al*, 1991).

1.11 Atherosclerosis

Atherosclerosis is the major cause of morbidity and mortality in the Western world. It is a disease characterised by gradual narrowing of the lumen of an artery as a result of focal areas of lipid deposition, smooth muscle cell proliferation, enhanced collagen formation and sometimes calcification that are

the features of atherosclerotic plaque formation. Epidemiological data provides convincing evidence of a direct relationship between increased plasma lipid concentrations and the development of atherosclerosis. Application of the British Hyperlipidaemic Association guidelines concerning the acceptable levels of plasma cholesterol and triglycerides would bring 25% of British adults into the hyperlipidaemic group (Tait and Shepherd, 1989). For many individuals hyperlipidaemia arises as a result of increased dietary fat consumption, but there are clearly genetic influences and a number of established clinical conditions whose hyperlipidaemia is purely a genetic defect in lipid metabolism eg Familial Hypercholesterolaemia (Brown and Goldstein, 1984; Tait and Shepherd, 1989).

Cholesterol, triglyceride, cholesterol ester and phospholipids are transported in the bloods as lipoproteins. The triglyceride-rich chylomicra carry dietary lipid from the intestine and very low density lipoproteins (VLDL) carry lipid secreted from the liver. Triglyceride is removed from these lipoproteins by the action of lipoprotein lipase in peripheral tissues and the resulting chylomicron remnants and intermediate density lipoprotein (IDL) are taken up by the liver. A certain proportion of IDL lose their apoprotein E constituent and become the cholesterol-rich low density lipoproteins (LDL). LDL have a long plasma half life and function to supply cholesterol to tissues but unfortunately are considerably atherogenic (Brown and Goldstein, 1984; Packard and Shepherd, 1987).

The first stage of atherosclerosis is the development of a fatty streak within the innermost layer of the artery wall, the intima. This is formed when LDL infiltrates a lesion in the endothelial cell layer that lines the intima of the artery. Here there is a tendency for LDL apoprotein B to become modified or oxidised. In response to the wound monocytes migrate into the intima of the artery where they take up the modified LDL and become lipid-laden macrophages or foam cells. As the foam cells within the fatty streak push up the endothelial cell layer, they may rupture the layer, exposing the smooth muscle cells. Platelets adhere to the smooth muscle cells where they become aggregated and platelet-derived growth factor is released. This stimulates smooth muscle cell proliferation and causes the cells to produce excess amount of fibrous connective tissue. A fibrous plaque is therefore a more advanced, complex, occlusive lesion consisting of a central necrotic core containing intracellular lipid within macrophages with a fibrous cap above it. A fibrous plaque may remain stable for many years. The degree of narrowing of the lumen depends on the amount of lipid deposited in the necrotic core of the plaque, the extent of smooth muscle cell proliferation and connective tissue formation. The final stage in atherosclerosis is the formation of a complex lesion, consisting of areas of haemorrhage, calcification, ulceration and thrombosis. Haemorrhage occurs when the fibrous plaque severely distorts the arterial-wall architecture, causing the small arterioles that supply the arterial wall to rupture and bleed. The fibrous plaque may crack, resulting in ulcers where a thrombogenic surface of smooth

muscle is exposed. Platelet aggregation stimulates formation of a thrombus or clot, which leads to arterial occlusion and may cause a heart attack or a stroke (Ross, 1993).

Non-pharmacological measures, ie diet and weight control are the first line of treatment of hyperlipidaemia. A weight reducing diet or diets low in dietary fat are recommended. Epidemiological evidence suggests that diets that are high in polyunsaturated fatty acids, such as those derived from marine fish oils which contain high levels of n-3 polyunsaturated fatty acids (eicosapentaenoic acid), may prevent or reduce hyperlipidaemia (Leaf and Weber, 1988). Drug intervention is initiated when dietary measures are inadequate or failed. Current popular drug therapy includes the use of bile acid binding resins eg cholestyramine that prevent bile acid reabsorption in the intestine. This reduces the hepatic cholesterol pool which is used for new bile acid synthesis. The liver increases its concentration of plasma membrane LDL-receptors to obtain cholesterol from circulating LDL and this results in a decrease in circulating LDL levels. Unfortunately the liver also increases its rate of *de novo* cholesterol synthesis such that HMG-CoA reductase inhibitors eg mevinollin, statins must be used as a supplementary drug treatment. Fibrates (eg clofibrate, gemfibrozil) are another popular treatment particularly for hypertriglyceridaemia and combined hyperlipidaemia as they reduce plasma levels of triglyceride and cholesterol via the activation of lipoprotein lipase in

the periphery, an increase in HDL levels and decreased production of VLDL (Brown and Goldstein, 1984; O'Connor *et al*, 1990).

1.12 Aims of The Present Studies

The *de novo* synthesis of fatty acids may not be a major direct contributor to the composition of hepatic VLDL (Gibbons, 1990). However, *de novo* synthesis will contribute to the intracellular pool of fatty acids and triglyceride and there are close correlations between the rates of hepatic fatty acid synthesis and cholesterol synthesis and the rate of VLDL secretion. An understanding of the regulation of the lipogenic pathway is of great importance to the understanding of substrate disposal and utilisation that has implications for a number of metabolic diseases including hyperlipidaemia, atherosclerosis and diabetes. The lactating rat mammary gland is extremely active in the synthesis of fatty acids and provides a good model for investigation of the importance and interaction between the different regulatory steps in the pathway and the signals that control them. The present study investigates the effects of physiological signals such as ketone bodies and polyunsaturated fatty acids, particularly given the therapeutic potential of the latter, and of the class of synthetic fibrate drugs that can be considered as fatty acid analogues. Pharmacological tools such as okadaic acid have been used to investigate the interaction of regulatory steps in the lipogenic pathway and their control by protein phosphorylation.

CHAPTER 2

MATERIALS AND METHODS

2.1 Animals

Rats of the Wistar strain were fed on standard chow diet (Special Diets Services Ltd, Kent, UK) and water *ad libitum* up to the time of killing. All animals were maintained on a 12-hour light/12-hour dark cycle (light from 7.00 a.m. to 7.00 p.m.). For mammary acini preparation, female rats with between 8 to 12 pups were used at the peak of lactation (10 to 16 days *post partum*). Animals were sacrificed with a non-recovery dose of intraperitoneal pentobarbitone injection (60 mg/kg body weight). Dissection was carried out as soon as deep anaesthesia was attained. For acini preparation, animals were killed between 10.00h to 10.30h and for *in vivo* studies, animals were killed 6 hours into the dark period (13.00h). Mammary tissue for freeze-clamping *in situ* was quickly and carefully freed the gland from the skin and abdominal wall. The tissue was then freeze-clamped and powdered under, and stored in, liquid nitrogen.

2.2 Radioactive Compounds

[γ - ^{32}P]ATP, [^{32}P]phosphate, [1- ^{14}C]-acetate (sodium salt), 2-deoxy-D-[1- ^3H] glucose, sn-[U- ^{14}C] glycerol-3-phosphate, sodium [^{14}C]-bicarbonate and $^3\text{H}_2\text{O}$ were obtained from ICN Flow, High Wycombe, U.K. or the Radiochemical Centre, Amersham, Bucks, U.K.

2.3 Chemicals

Acetate (Na^+ salt), acetoacetate (Li^+ salt), acetoacetyl-CoA, acetyl-CoA, ATP, bovine serum albumin (fatty acid free), CoA, 2-deoxy-D-glucose, sn-glycerol-3-phosphate, lactic acid, palmitoyl-CoA, phloretin, pyruvate, clofibrate, gemfibrozil, eicosapentanoic acid (EPA), linoleic acid and linolenic acid were obtained from Sigma Chemical Company, Poole, Dorset, U.K. Okadaic acid was obtained from Scientific Marketing Associates, Barnet, Herts. p-(p-aminophenylazo)-benzene sulphonic acid (AABS) was obtained from Pfaltz and Bauer, USA. All the other chemicals were of analytical or purer grade purchased from BDH Chemicals, Poole, Dorset, U.K. and Aldrich Fine Chemicals, Gillingham, Dorset, U.K.

2.4 Biochemicals

NAD⁺, NADH, NADP and all enzymes used for metabolite assays were obtained from Boehringer Corp., Mannheim, Germany. Benzamidine, dithiothreitol (DTT), phenylmethylsulphonyl fluoride (PMSF), soya-bean trypsin inhibitor (SBTI), N-p-tosyl-L-lysine chloromethyl ketone (TLCK), N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), collagenase, phosphorylase_b, phosphorylase kinase and pigeon liver acetone powder were obtained from Sigma Chemical Company, Poole, Dorset, U.K.

2.5 In Vitro Studies With Acini

2.5.1 Preparation of Mammary Acini

This method was based on the procedure described by Katz *et al* (1974) and Robinson and Williamson (1977a).

The inguinal mammary glands (5-8 g) were removed and minced finely with a scalpel blade on top of a petri dish. The subcutaneous fat and connective tissue were trimmed off during the process. The minced tissue was transferred to a 50 ml glass measuring cylinder and washed three times by decanting with warm Krebs Henseleit (KH) buffer containing 125 mM NaCl, 4.8 mM KCl, 25 mM NaHCO₃, 2.6 mM CaCl₂ and 5 mM glucose which was continuously gassed

with O₂/CO₂ (95% : 5%). The tissue was then transferred to a 250 ml plastic flask and was suspended in a total of 35 ml KH buffer containing 1 mg/ml collagenase. The suspension was shaken at 37°C in a Dubnoff-type shaker (180 strokes/min) with continuous gassing for 60-65 minutes. The acini were filtered through nylon gauze (mesh size 0.44 mm) and centrifuged at 400 rpm for 1 min. Hard spin was avoided as it caused aggregation of the acini. The precipitated acini were washed and centrifuged three times with 30 ml KH buffer. The weight of acini obtained was determined and this was typically between 3 to 5g. The acini were diluted with KH buffer to give approximately 50 mg wet weight of acini per ml.

2.5.2 Incubation of Acini

The acini (approximately 50 mg/ml) were incubated in a total volume of 4 ml KH buffer in 25 ml silicone-treated flasks with the additions of radio-labelled substrates and potential inhibitors or activators as indicated. The flasks were gassed with O₂/CO₂ (95% : 5%), sealed and incubated with gentle shaking (40 strokes/min) at 37°C in a Dubnoff-type shaker for 20, 40, 45 or 60 minutes as indicated.

For measurements of fatty acid synthesis or metabolite uptake and release, incubations were stopped with the addition of 0.4 ml of 20% perchloric acid.

The contents of the flasks were transferred into preweighed tubes and centrifuged at 4,000 rpm for 10 minutes. The supernatant was removed for metabolite determination (see section 2.7 for experimental details). Residual acini in the flask were removed with two 5 ml portions of 50% ethanol and added to the precipitated acini. Ethanol washings serve to remove electrolytes and soluble radioactive compounds from the acini (Katz *et al*, 1974). The tubes were centrifuged for a further 10 minutes and the supernatant decanted off. Lipid was then extracted from the acini by the addition of 10 ml chloroform : methanol (2 : 1) which was left overnight. The chloroform : methanol-acini suspensions were centrifuged at 4,000 rpm for 15 minutes and the supernatant removed and used for the determination of lipid biosynthesis (see Section 2.8 for experimental details). The acini pellet was dried down and the preweighed tubes were reweighed to calculate the dry defatted weight of acini in each incubation.

For measurements of enzyme activities, the acini at the end of the incubation were transferred into round bottom tubes and centrifuged at 4,000 rpm for 3 minutes. The supernatant was decanted off and the precipitated acini were frozen under liquid nitrogen and stored at -70°C until required for use.

2.6 In Vivo Studies

Male Wistar rats of between 220-250 g were fed chow diet and kept on a reverse cycle (light from 7.00 p.m. to 7.00 a.m.) for at least 7 days before being used. Rats were grouped and injected intraperitoneally with 250 mg/kg body weight of gemfibrozil in a total volume of 0.2 ml in DMSO or DMSO only (control) at 6 hours into the dark period (1.00 p.m.). Each group of rats were treated with gemfibrozil for 90 min, 150 min or 24 hours. Each animal was injected with 2 mCi of $^3\text{H}_2\text{O}$ (0.2 ml) intraperitoneally an hour before the animal was sacrificed. Liver and white adipose tissue were excised for determination of lipid synthesis (see Section 2.8.2).

2.7 Determination of Metabolites

Treatment of acinar incubations with 20% perchloric acid described in Section 2.5.2 resulted in an acidic supernatant. This was neutralized with 2 M KOH. The KClO_4 precipitate formed was removed by centrifugation for 15 minutes on a bench-top Biofuge (13,000 rpm). The concentrations of the following metabolites were then determined by enzymatic methods : glucose (Slein, 1963), pyruvate and lactate (Hohorst *et al*, 1959). The rates of metabolite accumulation or utilisation were expressed as $\mu\text{moles/min}$ per 100 mg defatted dry weight acini.

2.8 Measurement of Rate of Lipogenesis

2.8.1 Introduction

Rates of fatty acid synthesis were measured by the incorporation of tritium ($^3\text{H}_2\text{O}$) into fatty acids. Under different physiological conditions, various substrates including glucose, lactate, pyruvate and acetate are potential precursors for fatty acid synthesis. Measurement of the rate of fatty acid synthesis utilising ^{14}C as the tracer is unsatisfactory because this method measures only lipogenesis from the carbon precursor that is ^{14}C -labelled and true rates of lipogenesis are not obtained when a number of precursors are available. Tritium incorporation into fatty acids gives an absolute measurement of fatty acid synthesis regardless of the precursor because the tritium is incorporated in the reduction of the growing carbon chain of the fatty acid by NADPH and H^+ (Foster and Bloom, 1963).

2.8.2 Method

For *in vivo* determination of rates of lipogenesis, rats were injected with 5 mCi (0.2 ml) of $^3\text{H}_2\text{O}$ intraperitoneally. The incorporation of ^3H from $^3\text{H}_2\text{O}$ into fatty acids in liver and adipose tissue is linear for approximately 90 minutes (Hems *et al*, 1975; Lowenstein, 1971). Therefore, the animals were sacrificed 60 minutes after tritium injection which is within the linear time course of

tritium incorporation. Arterial blood was collected from the aorta into a heparinised syringe for the determination of specific radioactivity of plasma water. Duplicate samples of liver (approximately 2 g) and white adipose tissue (approximately 1 g) were removed, weighed and immersed in 5 ml of 30% KOH and 5 ml of absolute alcohol for saponification.

For determinations of lipogenesis in acini, the chloroform/methanol extract of lipid from precipitated acini was dried down under a stream of nitrogen at 50°C and the residual lipids were saponified by the addition of 3 ml of 30% KOH and 3 ml of absolute alcohol.

Saponification of lipids from tissue or acini samples was achieved by heating the KOH/ethanol solution at 80°C for 2 hours in a water bath. The tubes were then cooled. For the extraction of non-saponifiable lipids, the saponifiable mixture was extracted with three 8 ml portions of petroleum ether (b.p. 40-60°C). The pooled ether extracts were washed twice with 5 ml distilled water and transferred to scintillation vials and allowed to dry in the fume cupboard overnight. Scintillation fluid (10 ml) was added to each vial and the dpm of the tritiated non-saponifiable lipids was determined by scintillation counting. The remaining saponification mixture was acidified to pH 1 with 3 ml of 30% (v/v) H₂SO₄. The free fatty acids were then extracted with three 8 ml portions of petroleum ether (b.p. 40-60°C). The pooled extracts were washed with distilled

water and dried down as described above. The dpm of the tritiated free fatty acids were determined.

2.8.3 Determination of Specific Radioactivity of $^3\text{H}_2\text{O}$

Arterial blood samples collected from the animals used for *in vivo* studies were centrifuged at 13,000 rpm for 15 minutes. Supernatant (plasma) was diluted and dpm of triplicate aliquots (20 μl) of the plasma determined. For acini, triplicate aliquots of the incubation medium were diluted and counted. Specific radioactivity was calculated according to the following method :

Assuming that

1 ml plasma or medium = 1 ml H_2O = 1 g H_2O = 55.56 mmol H_2O

Therefore, the specific radioactivity of plasma or incubation medium :

$$\text{dpm}/\mu\text{mole } ^3\text{H}_2\text{O} = \frac{\text{dpm}}{55.56 \times 1000} \times \frac{1}{\text{dilution factor}}$$

The results of fatty acid synthesis (saponifiable) in the *in vitro* studies were expressed as $\mu\text{moles } ^3\text{H}_2\text{O}$ incorporated/min per 100 mg defatted dry weight acini. For *in vivo* studies, rates of fatty acid synthesis in liver and white adipose tissue (saponifiable and non-saponifiable) were expressed as $\mu\text{moles } ^3\text{H}_2\text{O}$ incorporated/hour per g wet weight.

2.9 Measurement of Glucose Transport

2.9.1 Introduction

Glucose transport in acini can be measured using 2-deoxy-[³H]-D-glucose, an analogue of D-glucose. 2-deoxy-D-glucose is transported into the cells and becomes phosphorylated in the same way as glucose. However, it cannot be metabolised further (Wick *et al*, 1957). A linear rate of uptake of this analogue has been measured in acini and shown to be competitively inhibited by D-glucose, indicating that both sugars enter the cells via the same stereospecific carrier (Threadgold *et al*, 1982). The K_m for 2-deoxy-D-glucose transport greatly exceeds its phosphorylation by hexokinase (Threadgold *et al*, 1982). Similar observations were made in experiments carried out with isolated adipocytes (Olefsky, 1975). Therefore, glucose transport under different physiological conditions can be determined by measuring the total uptake of radioactive 2-deoxy-D-glucose by the cells.

2.9.2 Method

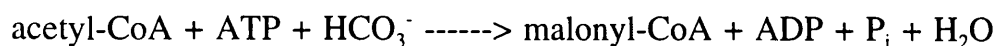
Acini (approximately 50 mg) were incubated at 37°C in a total volume of 4 ml KH buffer in which glucose was replaced with sodium L-lactate (4.7 mM) and sodium pyruvate (0.3 mM) as substrates. Incubations were carried out for 60 minutes in the presence and absence of potential effectors. At the end of

this period, 200 μ l of 100 mM 2-deoxy[3 H]-D-glucose (0.125 μ Ci/ μ mole) was added to each incubation to give a final concentration of 5 mM. Phloretin (1 mM), a potent inhibitor of glucose transport was added to some incubations to give a measure of non-specific 2-deoxy[3 H]-D-glucose trapping by the cells. Transport of the glucose analogue was arrested after 0, 20, 30, 40 and 60 seconds with the addition of 20 ml ice-cold 0.9% saline. The content of each flask was immediately filtered through a preweighed Millipore prefilter (Millipore AP 2002500) and washed with a further 40 ml of ice-cold saline. Filter papers were then dried, reweighed to calculate the dry weight of acini and placed in scintillation vials. 0.4 ml of 5% triton X-100 was added to the vials and left overnight to solubilise the cell membranes. Scintillation fluid was then added and radioactivity trapped within the cells was determined. Blank incubations carried out in the absence of acini were used for correction of the rate of glucose transport. The rate of glucose transport was expressed as nmol of 2-deoxy[3 H]-D-glucose taken up/mg dry weight acini over the incubation time course (0-60 seconds).

2.10 Measurement of Acetyl-CoA Carboxylase Activity

2.10.1 Introduction

Acetyl-CoA carboxylase (ACC) is the rate limiting enzyme catalysing the formation of malonyl-CoA from acetyl-CoA and bicarbonate :



The activity of ACC was measured by the incorporation of ^{14}C -bicarbonate into malonyl-CoA which is an acid-stable product. Activity in both crude homogenates and pure enzyme samples prepared from acini incubated under different conditions was determined.

2.10.2 Preparation of Crude Homogenates from Acini

Acini were incubated *in vitro* as described in Section 2.5.2. After harvesting the cells by centrifugation, the acini pellet was homogenised by hand in 10 volumes of homogenisation buffer containing 0.25 M mannitol, 100 mM Tris/HCl (pH 7.4 at 4°C), 2 mM EDTA, 50 mM NaF, 4 mM NaPP_i, 1 mM benzamidine, 2 µg/ml of SBTI, 1 mM DTT, 1 mM TLCK and 1 mM PMSF. The homogenate was centrifuged at 13,000 rpm for 60 seconds. Supernatant was removed carefully, avoiding the top lipid layer and used for enzyme activity measurement and protein concentration determination. The time interval between homogenisation and the start of the assay never exceeded 3 minutes.

2.10.3 Purification of Acetyl-CoA Carboxylase

Acini were homogenised as described in Section 2.10.2. The homogenate

was centrifuged at 16,000 g for 30 minutes at 4°C and the supernatant was filtered through glass wool to remove floating fat. Proteins, including ACC were precipitated by making the supernatant (post-mitochondrial fraction) 35% saturated with respect to ammonium sulphate. The mixture was kept at 4°C for 15 minutes and was then centrifuged at 12,000 g for 20 minutes. The resulting pellet was resolubilised in avidin-Sepharose column buffer containing 20 mM Tris/HCl (pH 7.5 at 4°C), 0.5 mM NaCl, 1 mM EDTA, 5 mM NaPP_i, 50 mM NaF, 10% glycerol, 0.02% NaN₃, 1 mM benzamidine, 2 µg/ml of SBTI, 1 mM DTT. The solution was centrifuged at 12,000 g for 20 minutes to remove insoluble material. The supernatant was filtered through glass wool and applied to a monomeric avidin-Sepharose affinity column (2 ml) prepared as described in Section 2.10.4. ACC binds to the column via the interaction of avidin bound to the column matrix with the biotin prosthetic group of the enzyme. The column was washed extensively with column buffer to remove the unbound proteins until the A₂₈₀ was below 0.05. Typically, 40 column volumes of buffer were required. ACC was eluted with 2 mM biotin in column buffer containing no glycerol as the latter interferes with the subsequent ACC assay. To ensure a sharp elution profile of ACC, half the column volume of elution buffer was allowed to flow into the column and the flow was then stopped. This allows time for biotin to exchange for ACC. After 30 minutes, the flow was continued and 0.5 ml fractions of eluate were collected. The fraction containing the highest amount of protein was diluted to 0.015 mg/ml with 0.1 M Tris/HCl (pH 7.4 at

4°C) and assayed immediately.

2.10.4 Preparation of Avidin-Sepharose Affinity Column

Sepharose CL-4B-200 gel (approximately 50 ml) was washed with one litre of ice cold water on a Buchner funnel under suction. The gel was then resuspended in 550 ml of 5 mM ice cold sodium phosphate buffer (coupling buffer) containing the mono and dihydrogen salts (pH 7.0 at 25°C). The suspension was adjusted to pH 11 with 2 M NaOH and transferred to a large beaker containing 15 g of cyanogen bromide which had been previously ground to a fine powder. The mixture was constantly stirred and the temperature was maintained at 0-2°C by the addition of ice and the pH maintained at 11 with the dropwise addition of 2 M NaOH. After 15-20 minutes, the mixture was filtered over ice on a Buchner funnel under suction. The gel was then washed immediately with 700 ml of 10 mM ice cold sodium phosphate buffer. The gel was finally resuspended in 125 ml of 10 mM sodium phosphate buffer containing 50 mg of avidin.

The suspension was allowed to mix end over end overnight at 4°C to maximise the cross-linking of avidin to Sepharose. The coupled gel was filtered under suction and resuspended in 200 ml of 1 M ethanolamine/HCl (pH 7.0) and was left at room temperature. Ethanolamine/HCl is used as a blocking agent to

block excess reactive groups that have not coupled to avidin. After 2-3 hours, the blocking buffer was suctioned off and the gel was rinsed with 3 M guanidine/HCl (pH 1.5, monomerisation buffer) and the gel was resuspended in the same buffer overnight at room temperature. This step monomerises the coupled avidin and decreases its binding capacity for ACC in order that ACC can be eluted from the column in subsequent purification.

The gel was packed into a column and washed with monomerisation buffer, followed by extensive washing with avidin-Sepharose column buffer containing 20 mM Tris/HCl (pH 7.5 at 4°C), 0.5 M NaCl, 1 mM EDTA, 5 mM NaPP_i, 50 mM NaF, 10% glycerol and 0.02% (w/v) NaN₃, until the A₂₈₀ measured against the column buffer was less than 0.05. The high affinity binding sites of the column were then blocked by washing with 5 volumes of column buffer containing 0.8 mM biotin. Prior to use, the column was washed alternately with 0.1 M glycine (pH 2) and column buffer. This was repeated three times before the column was extensively equilibrated with column buffer.

2.10.5 Assay of Acetyl-CoA Carboxylase Activity

The assay medium contained 100 mM Tris/HCl (pH 7.4 at 37°C), 2 mM MgCl₂, 4 mM ATP, 0.3 mM acetyl-CoA, 10 mg/ml BSA and 20 mM NaH¹⁴CO₃ (approximately 2000 cpm/nmol) in a total volume of 100 µl. Samples of crude

extract homogenate were assayed for initial activity at 0.5 mM (physiological concentration) and 10 mM citrate (saturating concentration) over 90 seconds unless indicated otherwise. Pure ACC was assayed over 4 minutes and the concentrations of citrate were varied from 0 to 20 mM. Citrate was added in the ratio of 1 : 1 with MgCl_2 . Incubations were started with the addition of ACC and terminated with 50 μl of 6% PCA which precipitated the proteins and released the unused $^{14}\text{CO}_2$ from the solution. The precipitated proteins were removed by centrifugation in a bench top microfuge at 13,000 rpm for 3 minutes. An aliquot (125 μl) of the supernatant was removed and dried down in a Gyrovap rotary vacuum evaporator (V A Howe & Co Ltd, London). The residue was dissolved in water (100 μl) followed by the addition of scintillation fluid (1 ml) and cpm were determined in a scintillation counter. Control incubations without the substrate acetyl-CoA was carried out concurrently to correct for non-specific $^{14}\text{CO}_2$ fixation.

2.10.6 Determination of Specific Radioactivity of $\text{NaH}^{14}\text{CO}_3$

An aliquot (10 μl) of stock $\text{NaH}^{14}\text{CO}_3$ (200 mM) was diluted 10-fold with water. 10 μl of the diluted solution was added to 1 ml of scintillation fluid (pH adjusted to 7.0 with 0.1 M NaOH) and cpm of the solution was determined by scintillation counting. The value represented the cpm of the 200 nmol $\text{NaH}^{14}\text{CO}_3$ in the 10 μl aliquot and from this, cpm/nmol of $\text{NaH}^{14}\text{CO}_3$ could be calculated

(approximately 2000 cpm/nmol). ACC activity was expressed as nmol of ^{14}C incorporated into acid stable product/min per mg of protein according to the following calculation :

$$\frac{\text{cpm of sample}}{\text{cpm/nmol of NaH}^{14}\text{CO}_3} \times \frac{150}{125} \times \frac{1}{\text{time of assay (minutes)}} \times \frac{1}{\text{mg of protein in assay}}$$

V_{\max} and K_a citrate (concentration of citrate producing half maximal activity) was calculated for pure enzyme using a computer programme fitting data to the equation :

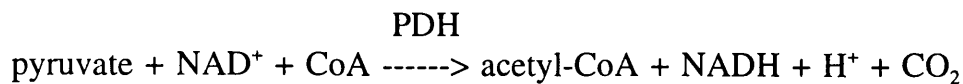
$$v = \frac{V_{\max}[c]h}{K_a + [c]h}$$

where v = initial rate
 h = Hill coefficient
 c = citrate concentration

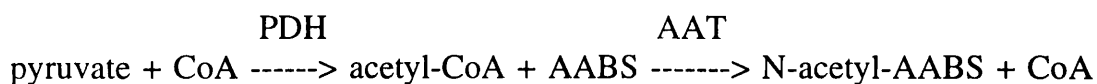
2.11 Measurement of Pyruvate Dehydrogenase Activity

2.11.1 Introduction

Pyruvate dehydrogenase (PDH) catalyses the irreversible oxidative decarboxylation of pyruvate to acetyl-CoA in the presence of thiamine pyrophosphate, CoA and NAD^+ :



PDH activity was measured in extracts of mitochondria spectrophotometrically by coupling to arylamine acetyltransferase (AAT) (Tabor *et al*, 1953) prepared as described in Section 2.11.4. The acetylation of p-(p-amino phenylazo) benzene sulphonic acid (AABS) by AAT using acetyl-CoA produced by PDH as substrate was monitored at 460 nm by measuring the disappearance of AABS (Jacobson, 1961; Caterson *et al*, 1982; Sugden and Holness, 1989) :



2.11.2 Preparation of Tissue Homogenates

PDH activity was assayed in crude homogenates prepared from acini. The frozen acini pellet prepared as described in Section 2.5.2 was freeze-thawed three times to facilitate rupturing of the mitochondrial membrane. The cells were then homogenised using a hand held glass teflon homogeniser in 10 volumes of homogenisation buffer containing 100 mM potassium phosphate (pH 7.4 at 4°C), 5 mM EDTA, 5 mM DTT and 10 mM pyruvate (freshly prepared). An aliquot of the homogenate was removed and mixed with rat blood serum in a ratio of 50 : 1. The serum serves to prevent proteolysis of PDH. The mixture was

centrifuged at 13,000 rpm for 20 seconds on a bench top microfuge and the supernatant was removed for assay immediately.

2.11.3 Assay of Pyruvate Dehydrogenase Activity

The assay was carried out in a total volume of 0.75 ml containing 100 mM Tris/HCl (pH 7.8 at 30°C), 1 mM MgCl₂, 0.5 mM EDTA, 5 mM 2-mercaptoethanol, 1 mM thiamine pyrophosphate, 0.5 mM NAD⁺, 0.1 mM CoA, 1 mM pyruvate and 15 µg of AABS. The reaction was initiated by the addition of 50 munits/ml of AAT and acetylation of AABS was monitored at 460 nm on a recording spectrophotometer. The linear rate of AABS acetylation was recorded for at least 10 minutes. The activity of PDH was calculated as follows :

$$\text{Activity} = \frac{A.V.}{e.d.v.t.} = \mu\text{mol/min/ml} = \text{Units/ml}$$

where A = change in absorbance

V = final assay volume (ml)

e = molar extinction coefficient (6.5)

d = lightpath (1 cm)

v = assay volume (ml)

t = assay time (min)

One unit of PDH activity converts 1 µmol of substrate into product per minute at 30°C and is expressed relative to the activity of citrate synthase (Section 2.12.4).

2.11.4 Preparation of Arylamine Acetyltransferase

This procedure was based on that described by Chou and Lipman (1952). Arylamine acetyltransferase (AAT, EC 2.3.1.5) was prepared from pigeon liver acetone powder. 10 g of acetone powder was homogenised for three minutes in 10 volumes of ice-cold water using a Polytron homogeniser fitted with a PT10 probe set at position six. The homogenate was centrifuged at 29,000 g for 15 minutes at 4°C. The supernatant was kept at approximately -10°C by standing the beaker in an ammonium chloride-ice mixture. The enzyme in the supernatant was fractionated by dropwise addition of ice-cold acetone with continuous stirring until a final concentration of 66% (w/v) acetone was achieved. The mixture was centrifuged for 15 minutes at 29,000 g at -10°C. AAT activity was recovered from the supernatant by three sequential precipitations with ice-cold acetone. Each addition of acetone was followed by centrifugation as above and the pellet retained. Pellets from each precipitation were resuspended in a small volume (0.5 ml) of 10 mM potassium phosphate buffer (pH 7.0 at 4°C) and assayed for AAT activity (section 2.11.5). Fractions containing less than 5 units/ml of activity were discarded. The enzyme was then stored in small aliquots at -20°C. Prior to use, the activity of AAT was diluted to 5 units/ml with 10 mM potassium phosphate buffer.

2.11.5 Assay of Arylamine Acetyltransferase

Arylamine Acetyltransferase (AAT) was assayed spectrophotometrically by monitoring the acetylation of p-(p-aminophenylazo)-benzene sulphonic acid (AABS) using acetyl-CoA in the presence of AAT. The change in extinction at 460 nm was followed as AABS was acetylated. The assay was performed at 30°C in a total volume of 0.75 ml containing 100 mM Tris/HCl (pH 7.8 at 30°C), 1 mM MgCl₂, 0.5 mM EDTA with 5 mM 2-mercaptoethanol, 15 µg AABS and 0.25 mM acetyl-CoA. The reaction was initiated by the addition of 5 µl AAT and monitored for at least 10 minutes. One unit of AAT activity is defined as the conversion of 1 µmol of substrate to product per minute at 30°C using the following formula:

$$\text{Activity} = \frac{A.V}{e.d.v.t} = \mu\text{mol/min/ml} = \text{Units /ml}$$

where A = change in absorbance

V = final assay volume (ml)

v = volume of AAT added (ml)

d = light path (= 1 cm)

t = assay time (minutes)

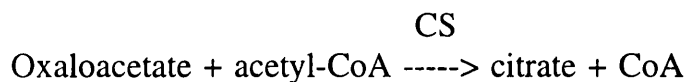
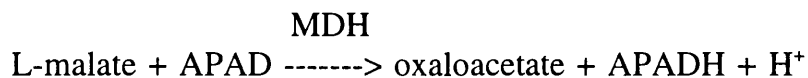
e = molar extinction coefficient (= 6.5)

2.12 Measurement of Citrate Synthase Activity

2.12.1 Introduction

Citrate synthase catalyses the condensation of oxaloacetate and acetyl-

CoA to form citrate. The assay consists of two reaction steps :



where APAD = acetylpyridine-adenine dinucleotide

MDH = malate dehydrogenase

CS = citrate synthase

The activity of citrate synthase was measured by monitoring the reduction of APAD (NAD⁺ analogue) and hence the utilisation of oxaloacetate by citrate synthase, spectrophotometrically at 365nm.

2.12.2 Preparation of Tissue Homogenates

Citrate synthase activity was measured in the same tissue homogenate as PDH. The supernatant prepared in Section 2.11.2 was diluted with homogenisation buffer to give a linear rate of citrate synthase activity.

2.12.3 Assay of Citrate Synthase

The procedure was based on that described by Stitt (1984). The assay was performed at 30°C in a final volume of 0.93 ml containing 80 mM triethanolamine (pH 8.5 at 37°C), 3 mM L-malate, 0.2 mM APAD, 13 units/ml malate dehydrogenase (MDH) and 0.18 mM acetyl-CoA. The assay was initiated

by the addition of acetyl-CoA. The linear rate of citrate synthase activity was monitored for at least 10 minutes on a recording spectrophotometer. The activity of citrate synthase was calculated as follows :

$$\text{Activity} = \frac{A.V}{e.d.v.t} \times \text{dilution factor of homogenate} = \mu\text{mol/min/ml} = \text{Units/ml}$$

where A = change in absorbance

V = final assay volume (ml)

e = molar extinction coefficient (0.911)

d = light path (1 cm)

v = sample volume (ml)

t = assay time (minutes)

A unit of citrate synthase activity converts 1 μmol of substrate into product per minute.

2.12.4 Expression of Pyruvate Dehydrogenase Activity

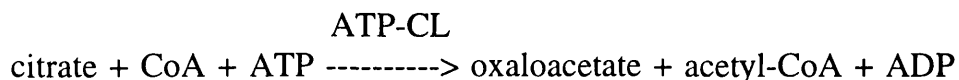
PDH is a mitochondrial enzyme and its activity is often expressed relative to that of citrate synthase. Citrate synthase is abundant in mitochondria and is a constitutive enzyme whose activity does not change in response to effects of hormones and other effectors. Activity of citrate synthase thus gives an indication of the efficiency of extraction of enzymes from mitochondria. The activity of PDH was expressed as a ratio of PDH : citrate synthase.

$$\frac{\text{PDH units/ml}}{\text{CS units/ml}} = \frac{\text{PDH}}{\text{CS}} \times 1000 = \text{mUnit PDH/Unit CS}$$

2.13 Measurement of ATP-Citrate Lyase Activity

2.13.1 Introduction

ATP-citrate lyase (ATP-CL), also known as citrate-cleavage enzyme, provides cytosolic acetyl-CoA for fatty acid synthesis from citrate produced in the mitochondria. The assay is based on the malate dehydrogenase-coupled procedure as described by Srere (1962).



The activity of ATP-CL was measured by following the amount of oxaloacetate formed via the oxidation of NADH in the presence of added malate dehydrogenase (MDH). The disappearance of NADH during the reaction was monitored spectrophotometrically at 340nm.

The enzyme activity was measured in crude homogenates prepared from acini and also for partially purified ATP-CL prepared from mammary tissue.

2.13.2 Preparation of Tissue Homogenates

Acini were homogenised in 10 volumes of homogenisation buffer containing 0.25 M mannitol, 100 mM Tris/HCl (pH 7.2 at 4°C), 1 mM EDTA, 50 mM NaF, 2 mM NaPP_i, 1 mM DTT, 1 mM benzamidine and 2 µg/ml SBTI.

The homogenate was centrifuged at 13,000 rpm for 60 seconds and the supernatant was divided into two portions. The first portion was used for measuring the activity of the enzyme directly, whilst the second portion was loaded onto a 2 ml Sephadex G-25 "spun column" (Section 2.13.4) and was centrifuged at 13,000 rpm for 1 minute. This procedure removed salts and low molecular weight molecules from crude homogenate preparations. The eluate from the column was collected into 50 mM NaF and 2 mM NaPP_i which were present at the bottom of the tube. This method ensured that while low molecular weight molecules were removed by the Sephadex gel filtration, the phosphatase inhibitors were replaced immediately prior to assay of ATP-CL activity.

2.13.3 Purification of ATP-Citrate Lyase

Lactating rat mammary tissue was homogenised in the homogenisation buffer described in Section 2.13.2. The homogenate was centrifuged at 16,000g for 30 minutes. The supernatant was then made 35% (w/v) saturated with ammonium sulphate by slow addition of the salt with gentle and continuous stirring at 4°C. The mixture was centrifuged at 12,000 g for 20 minutes. The supernatant was discarded and the ammonium sulphate pellet was resuspended in 20 mM KP_i buffer (pH 7.0 at 4°C, containing the mono and dihydrogen phosphate salts, 1 mM magnesium acetate, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM benzamidine, 2 µg/ml SBTI, 1 mM PMSF and 1 mM TLCK). This

was centrifuged at 12,000 g for 10 minutes to precipitate undissolved materials. The resulting supernatant was filtered through glass wool to remove floating fat, and loaded onto a DEAE ion-exchange column (10 ml) equilibrated with 20 mM KPi buffer. The unbound proteins were washed from the column with 20 mM KPi buffer until the A_{280} fell below 0.005. ATP-CL was then eluted from the column with 100 mM KPi buffer. Peak protein fractions were pooled and concentrated by vacuum dialysis at 4°C.

2.13.4 Preparation of Sephadex G-25 "Spun Column"

A "spun column" is a simple device to remove salts and low molecular weight compounds from crude homogenate preparations. Depending on the molecular size exclusion of Sephadex used, small molecules can be retained in the column effectively.

Sephadex G-25-150 was prepared by addition of water to the column material (1 g/ml water). This was left to stand at room temperature overnight. The material was then packed into a 2 ml disposable syringe and the column was placed in a round bottomed polypropylene centrifuge tube and centrifuged at 13,000 rpm for 3 minutes. The column was packed with more Sephadex and the process repeated until the packed bed volume of 2 ml was achieved. The column was then pre-equilibrated with homogenisation buffer as used for crude

extract preparation of ATP-CL. Prior to addition of sample, the column was centrifuged at 3,000 g for 3 minutes.

2.13.5 Assay of ATP-Citrate Lyase

The assay buffer contained 100 mM Tris/HCl (pH 7.0 at 37°C), 20 mM NaF, 0.4 mM NaPP_i, 20 mM citrate, 20 mM MgCl₂, 10 mM DTT, 1.2 units/ml MDH, 0.33 mM coenzyme A, 0.14 mM NADH in a total volume of 1 ml. The reaction was initiated with the addition of 5 mM ATP and the disappearance of NADH was monitored on a recording spectrophotometer for at least 10 minutes.

The activity of ATP-CL was calculated as follows :

$$\mu\text{moles of NADH utilised/min/mg protein} = \frac{A}{6.22} \times \frac{1}{C}$$

where A = change in absorbance per minute

C = protein concentration in assay (mg)

6.22 = molar extinction coefficient

The activity of ATP-CL was related to μmol of oxaloacetate produced and hence was expressed as $\mu\text{mol NADH utilised/min per mg protein}$.

2.14 Measurement of Glycerol-3-Phosphate Acyltransferase Activity

2.14.1 Introduction

Glycerol-3-phosphate acyltransferase (GPAT) catalyses the formation of 1-acyl glycerol-3-phosphate from glycerol-3-phosphate using fatty acyl-CoA as

the acyl donor. This is the first step of the sequential esterification of sn-glycerol-3-phosphate with three molecules of fatty acyl-CoA in the formation of triacylglycerol (Yamashita *et al*, 1972). The activity of this enzyme was assayed by the incorporation of radioactive glycerol-3-phosphate in the presence of palmitoyl-CoA into 1-acyl-glycerol-3-phosphate.

glycerol-3-phosphate + fatty acyl-CoA ----> 1-acyl-glycerol-3-phosphate

The activity of GPAT is measured at two different concentrations of bovine serum albumin : 6 mg/ml and 1.75 mg/ml (Bates and Saggerson, 1977; Sugden *et al*, 1980). GPAT utilises palmitoyl-CoA complexed to albumin. There are two isozymic forms of GPAT : a mitochondrial form located in the outer mitochondrial membrane which has a higher K_m for this complex, and a microsomal form which has a lower K_m for the complex. An albumin concentration of 6 mg/ml is therefore optimal for the mitochondrial enzyme and 1.75 mg/ml is optimal for the microsomal enzyme (Bates and Saggerson, 1979).

2.14.2 Preparation of Tissue Homogenates

Acini were homogenised by hand in 1 ml of 10 mM Tris/HCl (pH 7.4 at 4°C), 192 mM mannitol, 59 mM sucrose, 1 mM EDTA, 10 mM NaF, 1 mM DTT, 1 mM benzamidine and 2 µg/ml SBTI. The homogenate was centrifuged at 13,000 rpm for 30 seconds in a benchtop centrifuge and supernatant was

removed and diluted 1 in 5 with homogenisation buffer for assay. Protein concentration of the diluted enzyme was determined by the method of Bradford (1976) as described in Section 2.19.

2.14.3 Assay of Glycerol-3-phosphate Acyltransferase

The assay mixture (final volume 100 μ l) contained 120 mM KCl, 60 mM Tris/HCl (pH 7.3 at 37°C), 0.1 mg/ml DTT, 0.1 mg/ml palmitoyl-CoA, 6 mg/ml BSA, 10 mM NaF, 4 mM MgCl₂, 0.5 mM [U-¹⁴C]glycerol-3-phosphate (approximately 4000 cpm/nmol). The reaction was initiated with the addition of homogenate and was carried out at 37°C for 5 minutes. The reaction was stopped by the addition of 0.5 ml of water-saturated butanol and mixed thoroughly to ensure complete extraction of acylglycerols by the organic solvent. The mixture was then centrifuged at 13,000 rpm for 3 minutes to separate the lower aqueous and the upper organic layer with a layer of protein at the interface of the two. An aliquot (0.4 ml) of the upper butanol layer was removed and washed with 0.8 ml of butanol-saturated water. The mixture was centrifuged at 13,000 rpm for 3 minutes. An aliquot (0.3 ml) of the upper butanol layer was removed and added to 3 ml of scintillation fluid and its cpm was determined. GPAT activity was expressed as nmoles of glycerol-3-phosphate esterified/min per mg protein and was calculated as follows :

$$\begin{array}{ccccc}
 \text{cpm of sample} & & 1 & & 1 \\
 \hline
 & \times & \hline
 \text{cpm/nmol of} & \text{assay time} & \text{mg of protein} \\
 {}^{14}\text{C-glycerol-3-phosphate} & \text{(minute)} & \text{in assay}
 \end{array}$$

2.15 Measurement of AMP-activated Protein Kinase Activity

2.15.1 Introduction

The activity of AMP-activated protein kinase (AMP-PK) was assayed *in vitro* by measuring the incorporation of ^{32}P from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ into a synthetic peptide substrate, SAMS (His-Met-Arg-Ser-Ala-Met-**Ser**-Gly-Leu-His-Leu-Val-Lys-Arg-Arg). Amino acid sequencing studies revealed that AMP-PK phosphorylates ACC at serine-79 whereas cAMP-PK phosphorylates at serine-77 (Munday *et al*, 1988; Lopez-Cassilas *et al*, 1988). Synthesis of SAMS is based on the sequence of ACC around serine-79 (bold type). The replacement of serine-77 (the cAMP-PK phosphorylation site) with alanine has made the peptide a specific substrate for AMP-PK. The two arginine residues at the C-terminus facilitated removal of unreacted ATP as they increase the binding of the peptide to phosphocellulose paper (Davies *et al*, 1989).

2.15.2 Preparation of Tissue Homogenates

Homogenates from acini were prepared in the same way as that described for measurement of ACC activity using the same homogenisation buffer (Section

2.12.2). The homogenate was diluted 10-fold with 10 mg/ml of BSA and 5 μ l of this was removed for assay.

2.15.3 Assay of AMP-activated Protein Kinase

The assay medium contained 10 mM Hepes (pH 7.0 at 37°C), 0.2 mM EDTA, 50 mM NaF, 4 mM MgCl₂, 0.2 mM [γ -³²P]ATP (specific activity 4-6 x 10⁶ cpm/nmol) and 200 μ M SAMS synthetic peptide substrate (a generous gift from Dr D. Carling or was synthesised by Dr I. Toth at the School of Pharmacy).

The assay was initiated with the addition of MgATP and stopped after 2 and 4 minutes by the addition of 40 mM EDTA (final concentration 20 mM). A 30 μ l aliquot of the incubation mixture was spotted on to a 2 cm x 2 cm square of phosphocellulose paper (P81 Whatman) and dropped into 250 ml of 75 mM ortho-phosphoric acid. The squares were washed in three changes of acid solution with 5-10 minutes stirring between each changes. The paper squares were finally washed with water and transferred to scintillation vials containing 4 ml of scintillation fluid. The cpm of the bound ³²P peptide was determined by scintillation counting. Control incubations without the substrate SAMS were carried out concurrently to correct for non-specific ³²P binding and autophosphorylation of the kinase sample.

2.15.4 Determination of Specific Radioactivity of ^{32}P

An aliquot (5 μl) of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was diluted 200-fold with water. The concentration of ATP in the solution was determined spectrophotometrically at A_{260} against water and was calculated as follows :

$$\text{nmol/ml of } ^{32}\text{P} = \frac{A_{260} \text{ of diluted } ^{32}\text{P} \text{ solution}}{15 (= \text{extinction coefficient})} \times 200$$

The cpm of the same solution was determined by scintillation counting.

The specific radioactivity was calculated as follows :

$$\text{cpm/nmol of } ^{32}\text{P} = \frac{\text{cpm/ml of diluted } ^{32}\text{P} \text{ solution}}{\text{nmol/ml of diluted } ^{32}\text{P} \text{ solution}}$$

Activity of AMP-PK was expressed as nmol ^{32}P incorporated into SAMS peptide/min per mg protein :

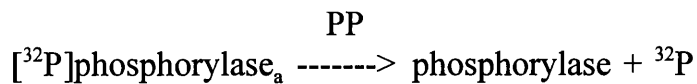
$$\frac{\text{cpm}}{\text{cpm/nmol of } ^{32}\text{P}} \times \frac{1}{\text{incubation time (minute)}} \times \frac{1}{\text{mg of protein in assay}}$$

2.16 Measurement of Protein Phosphatase 1 and 2A Activity

2.16.1 Introduction

Protein phosphatases (PP) 1 and 2A catalyse the dephosphorylation of serine and threonine residues of intracellular phosphoproteins, thus reversing the

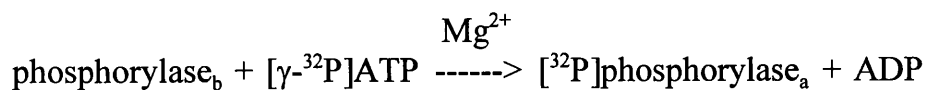
actions of protein kinases. The activities of PP1 and PP2A in cell extracts can be determined by measuring the release of acid-soluble ^{32}P -radioactivity from ^{32}P -labelled phosphorylase_a :



Quantitative analysis of PP1 and PP2A can be carried out by using okadaic acid, a potent inhibitor of PP1 and 2A (Chapter 4). PP1 and PP2A are inhibited by 5 μM okadaic acid. PP2A is the phosphorylase phosphatase activity which is inhibited by 2 nM okadaic acid. The activity of PP1 is therefore the proportion of phosphorylase phosphatase at 5 μM okadaic acid minus the activity at 2 nM okadaic acid.

2.16.2 Preparation of ^{32}P -Labelled Phosphorylase_a

Phosphorylase_a is a substrate for PP1 and PP2A prepared from phosphorylase_b (dephosphorylated form). Phosphorylation of phosphorylase_b by phosphorylase kinase occurs at a single serine residue (serine-14).



Phosphorylase_a is prepared by incubating 10 mg/ml of phosphorylase_b with phosphorylase kinase (2 units/ml) in a final volume of 1.2 ml in the presence of 50 mM Tris/HCl (pH 7.4 at 25°C), 0.1 mM EDTA, 1 mM DTT and

4 mM MgCl₂, 0.2 mM [γ -³²P]ATP (specific activity approximately 3 x 10⁵ cpm/nmol). The mixture was incubated for 4 hour at 37°C. The suspension was transferred to dialysis tubing and dialysed for 24 hour at 4°C against 50 mM Tris/HCl (pH 7.0 at 25°C), 0.1 mM EDTA and 1 mM DTT. Amberlite resins were added to aid the removal of residual radioactive ATP. The protein concentration of phosphorylase_a was adjusted to 2 mg/ml and the sample was stored in aliquots at 4°C until required for use.

2.16.3 Preparation of Rat Mammary Tissue Extracts

Powdered rat mammary gland was homogenised in 10 volumes of homogenisation buffer containing 0.25 M mannitol, 100 mM Tris/HCl (pH 7.4 at 4°C), 2 mM EDTA, 1 mM benzamidine, 2 µg/ml SBTI, 1 mM DTT, 1 mM TLCK and 1 mM PMSF. The homogenate was centrifuged at 13,000 rpm for 3 minutes. Supernatant was diluted 1 in 50 with homogenisation buffer and used for measurements of PP1 and PP2A activities.

2.16.4 Assay of Protein Phosphatase 1 and 2A

The assay was carried out in a total volume of 80 µl containing phosphorylase_a (2 mg/ml), okadaic acid (2 nM or 5 µM in 1% DMSO) or DMSO (1%). The reaction was stopped at 3 and 6 minutes by the addition of

200 μ l of 25% trichloroacetic acid (TCA) to a 35 μ l aliquot of the incubation mixture. The TCA precipitated assay mixture was kept in ice for 5 minutes. This was then centrifuged at 13,000 rpm for 5 minutes in a benchtop microfuge. 200 μ l of the supernatant were removed and scintillation fluid (1 ml) was added. The acid-soluble radioactivity was determined by scintillation counting.

The activity of PP1 and PP2A was expressed as nmoles phosphorylase_a dephosphorylated/min per mg protein and was calculated as follows :

$$\frac{\text{cpm of sample}}{\text{cpm/nmol of } ^{32}\text{P}} \times \frac{1}{\text{assay time (min)}} \times \frac{1}{\text{mg protein in assay}}$$

2.17 [³²P]Phosphate- Labelling of Mammary Acini

2.17.1 Method

Acini were prepared as described previously (Section 2.5.1). After harvesting, the acini were resuspended in KH buffer containing 0.2 mM phosphate to give 100 mg/ml acini and incubated at 37°C in a 250 ml polypropylene conical flask. [³²P]phosphate (100 μ Ci/ml) was added and cells were preincubated for 60 minutes under a stream of O₂/CO₂ (95 : 5). A 1 ml portion of the incubated acini suspension was removed and added into 55% PCA for determination of adenine nucleotides specific radioactivity. The cell suspension was then aliquotted out and transferred into 25 ml silicone-treated

flasks for treatment with various effectors as indicated. Cells were incubated for a further 45 minutes. The contents of the flask were then centrifuged to precipitate the acini and the pellets were used for ACC purification.

2.17.2 Determination of Incorporation of [³²P]-Phosphate into Acetyl-CoA Carboxylase

ACC was purified from [³²P]phosphate-labelled acini in the same way as described in Section 2.10.3). Following the loading of sample onto the avidin-Sepharose column (2 ml), the columns were washed with column buffer until the ³²P counts/min (Cerenkov) were less than 100 cpm/100 µl before elution of ACC with biotin as described in Section 2.10.3. The ³²P content of the fractions with the highest protein concentrations was determined by Cerenkov counting and the results expressed as cpm/mg protein. This gives an indication of the amount of radioactivity incorporated into ACC. Samples of homogenate, column breakthrough and purified ACC were retained and subjected to SDS-PAGE for further qualitative analysis (Section 2.18).

2.18 Polyacrylamide Gel Electrophoresis (PAGE)

2.18.1 Introduction

Electrophoresis is a powerful method for separating proteins and other

macromolecules. Polyacrylamide gel electrophoresis (PAGE) is perhaps the most commonly used method. The gels are readily formed from polymerisation of acrylamide, and their pore sizes can be controlled by choosing various concentrations of acrylamide and methylene bis-acrylamide (a cross-linking reagent) at the time of polymerisation. Electrophoresis in polyacrylamide gels in the presence of the anionic detergent, sodium dodecyl sulphate (SDS) is commonly used to separate protein subunits and the determinations of their molecular weights. In this case, the anionic detergent, SDS is used to disrupt all non-covalent interactions in native proteins and to equalise the negative charge on all proteins. Different proteins can be separated in an electric field purely on the basis of size and small molecules move through the gel more readily than larger molecules. The separated proteins can be visualised by Coomassie blue or silver staining depending on the amount of proteins present. Autoradiographs of radioactively labelled proteins can also be obtained.

2.18.2 Preparation of Protein Samples for SDS-PAGE

For qualitative analysis, the amount of protein in each sample was matched. Protein concentrations were measured by the method of Bradford (1976, Section 2.19). For purified ACC preparations, 5 μg of protein were used whilst 20 μg protein were used for samples of acini homogenates and avidin-Sepharose column breakthrough. Protein samples were precipitated according to

the method of Wessel and Flugge (1984). The protein solution was made up to 100 μ l with water. Methanol (400 μ l) was added, the mixture was well mixed and then centrifuged briefly (20 seconds) at 13,000 rpm. This was followed by the addition of chloroform (100 μ l) and water (400 μ l), with mixing and centrifugation after each addition of solvent. The top organic layer was removed and 100 μ l methanol was added. After mixing, the mixture was subjected to centrifugation at 13,000 rpm for 5 minutes. The supernatant was removed carefully and the remaining methanol was evaporated off. The precipitated proteins were solubilised in SDS sample buffer containing 62.5 mM Tris/HCl (pH 6.8 at 37°C), 2% SDS, 10% glycerol, 5% (v/v) 2-mercaptoethanol and 0.05 mg/ml bromophenol blue. The samples were boiled for 3 minutes and kept at -20°C until required for use.

2.18.3 Preparation of SDS-PAGE

The SDS Tris-glycine discontinuous buffer system (Laemmli, 1976) was used for the separation of ACC on SDS-PAGE. The running gel (5 ml) contained 7.5% acrylamide, 0.066% N'N'-methylenebisacrylamide, 0.1% SDS, 0.75 M Tris/HCl (pH 8.8 at 37°C). The stacking gel (5 ml) contained 3% acrylamide, 0.05% N'N'-methylenebisacrylamide, 0.1% SDS, and 68 mM Tris/HCl (pH 6.8 at 37°C). Both running gel and stacking gel were cross-linked with 0.05% (v/v) NN-N'N'-tetramethylethylenediamine (TEMED) and degassed

for 20 minutes. 80 μ l of 10% ammonium persulphate was added to each solution just before use.

Slab gels (8 cm x 10 cm) were cast using a standard casting kit. The running gel was loaded in between the two glass plates, which were previously cleaned and dried with ethanol, until it was approximately 6 cm in height. The top of the running gel was overlaid with a thin layer of isobutanol to prevent a meniscus from forming. Typically, the running gel polymerised after approximately 45 minutes at room temperature. The isobutanol was then rinsed off thoroughly with distilled water. The solution of stacking gel was loaded on top of the running gel until it was approximately 2 cm high. A 10-well comb was inserted to form sample wells. Any air bubbles introduced during the process of comb insertion were removed by moving the comb gently. The gel was allowed to set at room temperature (typically about 20 minutes). Prior to loading of protein samples, the comb was carefully removed and the wells were rinsed with reservoir buffer containing 25 mM Tris, 0.1% SDS, and the pH adjusted to 8.3 by the addition of glycine. This prevents polymerisation of acrylamide in the wells. The slab gel was then transferred to the apparatus where electrophoresis was to be carried out.

2.18.4 Separation of Proteins on SDS-PAGE

20 µl of protein samples prepared as described in Section 2.18.2 were loaded into the wells by means of fine disposable tips. Any unused wells were loaded with SDS sample buffer to ensure equal current distribution during the process of protein separation. A mixture of protein standards with known molecular weights (Sigma, Table 2.1) was run concurrently with the protein samples. Electrophoresis was performed at constant current of approximately 10 mA. When the samples had completely entered the stacking gel, the current was increased to 20-30 mA. Upon completion of protein separation (about 2 hours), gels were removed carefully from the glass plates and immersed into fixer solution which contained 50% methanol/10% acetic acid for 30 minutes. These were then stained for 30 minutes in 0.6% Coomassie Brilliant Blue R prepared in fixer. Excess stain was removed by washing the gels in several changes of destainer containing 10% methanol/10% acetic acid until protein bands could be visualised. Destaining was aided by the use of sponges which soaked up the dye. Gels were then air dried and gels that contained radiolabelled samples were subjected to autoradiography by exposing to X-ray film (Hyperfilm MR) in a Kodak X-Omatic cassette over 1 to 3 days at -70°C. The film was subsequently developed for 2 minutes with LX-24 developer and then fixed using FX-40 fixer.

Table 2.1 Molecular Weight of Protein Standards

<u>Proteins</u>	<u>Approximate Molecular Weight (Da)</u>
Myosin	205,000
B-Galactosidase	116,000
Phosphorylase B	97,000
Albumin (bovine plasma)	66,000
Albumin (egg)	45,000
Carbonic Anhydrase	29,000

2.19 Measurement of Protein Concentration

2.19.1 Introduction

Protein concentrations of crude homogenates prepared from acini or purified enzyme were determined by the method of Bradford (1976). The method is essentially based on the observation that Coomassie Brilliant Blue G-250 exists in two different colours, red and blue. The red form (470 nm) is converted to the blue form (595 nm) upon binding of Coomassie Blue dye to basic and aromatic amino acid residues of proteins.

Anion	----->	Neutral	----->	Cation
595 nm		650 nm		470 nm
blue		green		red

2.19.2 Method

Coomassie Brilliant Blue G-250 (30 mg) was dissolved in 100 ml of absolute ethanol. To this solution, 50 ml of concentrated phosphoric acid was added. The resulting solution was diluted to a final volume of 1 litre and was

filtered to remove undissolved dye.

To determine the protein concentration of unknown samples, a standard curve was constructed using the absorbance at A_{595} of bovine serum albumin (BSA) of known concentrations. The protein solutions containing 1 to 6 μg of BSA were diluted to 100 μl with distilled water in Eppendorf tubes. 1 ml of Coomassie Blue dye solution was added to each tube and the contents were mixed by vortexing. The absorbance at 595 nm was measured in a 1 ml plastic cuvette after 5 minutes against a reagent blank prepared from 100 μl of water and 1 ml of Coomassie Blue reagent. A standard curve was obtained by plotting the concentration of BSA against the corresponding absorbance. The slope of the standard curve was calculated.

For the determination of protein of unknown sample, the sample was diluted with water to ensure that the protein concentration falls within the concentration range of the BSA standard. 1 ml of Coomassie Blue reagent was added and the contents were mixed. The absorbance at 595 nm of the resultant protein-dye complex was measured. The protein concentration (mg/ml) of the unknown was calculated as follows :

$$\frac{A_{595} \text{ of unknown sample}}{\text{slope of standard curve}} \times \text{dilution factor of unknown sample}$$

CHAPTER 3

EFFECTS OF ACETOACETATE ON THE PATHWAY OF LIPOGENESIS IN ISOLATED MAMMARY ACINI

3.1 Introduction

Ketone bodies (acetoacetate and 3-hydroxybutyrate) are metabolic substrates of the brain and certain muscles and are also substrates of lipogenesis for neonatal brain, adipose tissue and the lactating mammary gland (Williamson, 1981; Zammit, 1981). Evidence from the activities of enzymes involved in the metabolism of ketone bodies suggests that the lactating rat mammary gland is a major potential site of ketone body utilisation (Page and Williamson, 1972). In addition, as shown by Kinsella (1970), 3-hydroxybutyrate can be metabolised for use in the synthesis of milk lipid constituents in cultured bovine mammary cells.

The major potential pathway of ketone body utilisation is in the mitochondria which provides acetyl-CoA for energy production or lipid synthesis. The enzyme 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30) which is located in the inner mitochondrial membrane catalyses oxidation of 3-hydroxybutyrate to acetoacetate. The reaction between acetoacetate and succinyl-

CoA, catalysed by 3-oxo-acid-CoA transferase (EC 2.8.3.5) leads to the formation of acetoacetyl-CoA and succinate. Acetoacetyl-CoA is cleaved to two molecules of acetyl-CoA by acetoacetyl-CoA thiolase (EC 2.3.1.9) which can either enter the tricarboxylic acid cycle for oxidation or be transported to the cytosol in the form of citrate for use in fatty acid synthesis. In the cytosolic pathway, acetoacetyl-CoA synthetase catalyses the conversion of acetoacetate to acetoacetyl-CoA. This is then cleaved by cytosolic acetoacetyl-CoA thiolase to form acetyl-CoA for lipid synthesis or forms hydroxymethylglutaryl-CoA for use in cholesterol synthesis. The cytosolic pathway of ketone body metabolism bypasses the need for citrate efflux from mitochondria to provide acetyl-CoA for lipid synthesis. Buckley and Williamson (1975) showed that acetoacetyl-CoA synthetase and acetoacetyl-CoA thiolase are present in the cytosol of the rat mammary gland. Therefore the cytosolic pathway, in addition to the mitochondrial, may be important in providing acetyl-CoA for the synthesis of lipids in the developing rat brain (Williamson and Buckley, 1973). Acetoacetyl-CoA generated in the cytoplasm can be converted into butyryl-CoA via 3-hydroxybutyryl CoA which is a better substrate for fatty acid synthesis than acetyl-CoA in rat mammary gland (Lin and Kumar, 1972).

The activities of enzymes of ketone body utilisation in the rat mammary gland increase rapidly from pregnancy to parturition and then decline on

removal of the litter (Page and Williamson, 1972; Buckley and Williamson, 1975). The high rate of ketone body uptake and utilisation by the lactating rat mammary gland is therefore associated with the high activities of the enzymes (Page and Williamson, 1972; Buckley and Williamson, 1975).

Lipogenesis in a tissue such as the lactating mammary gland is stringently regulated according to the nutritional and hormonal state of the animal. The lactating mammary gland utilises glucose at a high rate for use in the synthesis of fatty acids. This therefore represents a potential drain on the body glucose supplies. Starvation is characterised by alteration in tissue metabolism such that blood glucose concentration is maintained and carbohydrate is spared. Alternative fuels such as fatty acids and ketone bodies are used by most peripheral tissues in place of glucose.

In fed lactating rats, the concentrations of ketone bodies in the blood is similar to that of virgin rats which are reported to be 0.26 mM and 0.23 mM, respectively (Page and Williamson, 1972). However, it was reported that in the fed state, the actual contribution of ketone bodies to the total substrate uptake by the gland is less than 3% (Hawkins and Williamson, 1972). Therefore, ketone bodies do not seem to play an important role in the metabolism of the mammary gland in the fed state. During starvation of the rat, the concentrations of ketone

bodies increased to 2-3 mM (Page and Williamson, 1972). Uptake of ketone bodies by the starved lactating rat mammary gland has been shown to be concentration-dependent (Williamson and Hems, 1970). Studies by Hawkins and Williamson (1972) showed that the concentration of acetoacetate in the blood is increased by approximately 50% during starvation of the lactating rat for 16 hours. Acetoacetate causes increased lactate and pyruvate output and inhibition of glucose utilisation and glucose incorporation into lipid in lactating mammary gland slices (Hawkins and Williamson, 1972) and in isolated mammary acini (Robinson and Williamson, 1977c). In isolated mammary acini, the effect of acetoacetate is reversed upon addition of insulin (Williamson *et al*, 1975) or glycerol (Robinson and Williamson, 1977c). Similar effects of acetoacetate have also been observed *in vivo* where intravenous injection of acetoacetate into the fed lactating rat resulted in a decrease in the arterio-venous difference for glucose across the mammary gland (Robinson and Williamson, 1977b). In other rat tissues such as heart, diaphragm and skeletal muscle (Randle *et al*, 1964) and submaxillary gland (Thompson and Williamson, 1975), glucose utilisation has been shown to decrease in the presence of acetoacetate. Therefore, an increase in the concentration of ketone bodies could be the potential signal indicating decreased availability of carbohydrate and thus mediating metabolic changes in starvation (Hawkins and Williamson, 1972; Whitelaw and Williamson, 1977). A time-dependent increase in the plasma concentration of ketone bodies has

been observed during starvation of lactating rats (Hagopian *et al*, 1991). In this case, the arterial concentration of ketone bodies increased steadily over 24 hour starvation of the lactating rat and this was accompanied by a parallel decrease in the arterio-venous difference for glucose across the mammary gland (Figure 3.1).

So far, studies in isolated mammary acini suggest that glucose uptake and fatty acid synthesis are inhibited by acetoacetate. The aim of the present study was to identify the sites of action of acetoacetate in the pathway of lipogenesis in isolated lactating rat mammary acini.

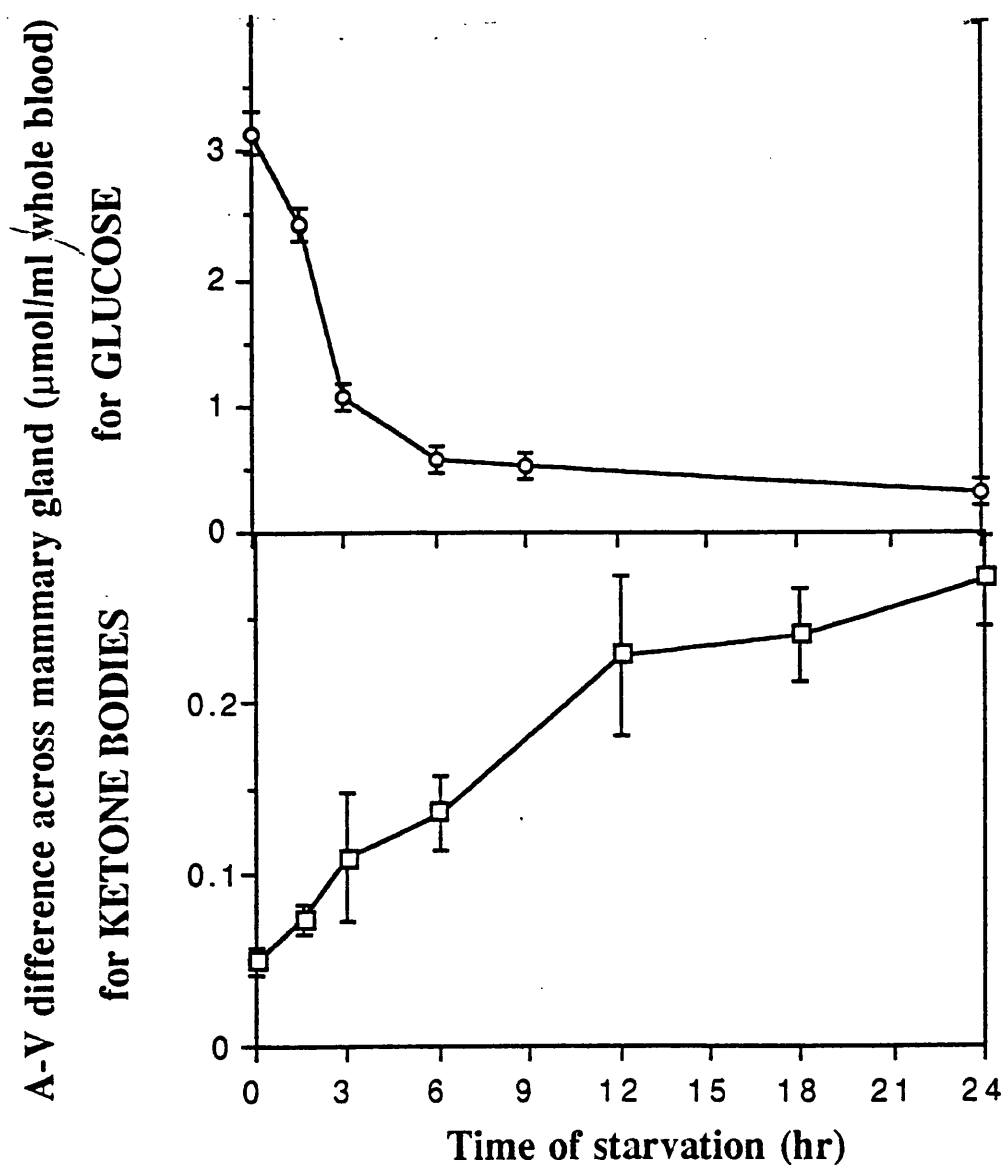
3.2 Effect of Acetoacetate on Fatty acid Synthesis

The rate of fatty acid synthesis in isolated control acini is in agreement with values reported previously for acini (Katz *et al*, 1974; Robinson and Williamson, 1977c). Incubation of isolated mammary acini with 2 mM acetoacetate leads to inhibition of fatty acid synthesis by 38% (Table 3.1). The extent of the inhibition was slightly lower than the 60% reported by Robinson and Williamson (1977c).

Figure 3.1 Arterio-venous Differences For Glucose and Ketone Bodies

Across Lactating Mammary Gland

The arterio-venous differences for glucose and ketone bodies were measured in lactating rat mammary gland over a time course of starvation. Results are expressed as $\mu\text{mol/ml}$ whole blood and are means for at least four observations at each time point with S.E.M. indicated by vertical bars. Modified from Hagopian *et al* (1991).



3.3 Effect of Acetoacetate on Glucose Utilisation

The rate of glucose uptake in the control cells is similar to that determined previously for acini (Katz *et al*, 1974; Robinson and Williamson, 1977c). Incubation of isolated mammary acini with 2 mM acetoacetate resulted in inhibition of glucose utilisation by 54% (Table 3.1). This is generally in agreement with the effects of acetoacetate on glucose utilisation in lactating rat mammary gland slices (Williamson *et al*, 1975) and in isolated mammary acini (Robinson and Williamson, 1977c) where glucose utilisation was inhibited by 30% and 75% respectively. Similar effect of acetoacetate on glucose uptake has also been observed *in vivo* in lactating rat mammary gland (Robinson and Williamson, 1977b), in rat heart and skeletal muscle (Randle *et al*, 1964). In fed lactating rats treated with an intravenous injection of acetoacetate, arteriovenous difference measurements across the mammary gland indicated that glucose uptake is decreased by 55% (Robinson and Williamson, 1977b).

The effect of acetoacetate on glucose utilisation in mammary gland slices (Williamson *et al*, 1975) and in isolated acini (Robinson and Williamson, 1977c) is reversible upon addition of insulin although the hormone has no direct stimulatory effect on glucose uptake in the absence of acetoacetate (Robinson and Williamson, 1977c). Glycerol (1 mM) has also been shown to relieve

**Table 3.1 Effects of Acetoacetate on Fatty Acid Synthesis and
Glucose Utilisation in Isolated Mammary Acini**

Acini were incubated in the presence and absence of 2 mM acetoacetate for 20, 40 and 60 min. The results are mean values \pm S.E.M. with the number of observations in parentheses and are expressed as $\mu\text{mol/min}$ per 100 mg defatted dry weight acini. Values that are significantly different by Student's t-test from the corresponding control values are shown : * $p < 0.005$; ** $p < 0.0005$.

	<u>Fatty acid synthesis</u>	<u>Glucose uptake</u>
	($\mu\text{mol } ^3\text{H}_2\text{O}$ incorp/ min/100 mg acini)	μmol glucose removed/ min/100 mg acini)
control	0.40 ± 0.03 (5)	1.32 ± 0.10 (4)
acetoacetate	0.25 ± 0.02 (5)*	0.61 ± 0.09 (4)*

inhibition of glucose utilisation by acini caused by acetoacetate, restoring it to 85% of the control rate (Robinson and Williamson, 1977c). However, in the absence of acetoacetate, glycerol decreased glucose uptake of acini by 60% (Robinson and Williamson, 1977c). The ability of insulin and glycerol to relieve inhibition of glucose utilisation caused by acetoacetate is thought to be mediated via an increase in the availability of glycerol-3-phosphate. These agents increase the levels of glycerol-3-phosphate in isolated acini by 70% and 9-fold, respectively (Robinson and Williamson, 1977c). Glucose is required to provide glycerol-3-phosphate for esterification of fatty acids *in vivo* (Baldwin and Yang, 1974). Inhibition of glucose utilisation by acetoacetate therefore resulted in a decreased concentration of glycerol-3-phosphate in acini (Robinson and Williamson, 1977c). Increase in the availability of glycerol-3-phosphate in response to insulin and glycerol may be able to promote esterification and hence relieve inhibition of lipogenesis in acini caused by acetoacetate (Robinson and Williamson, 1977c). Glycerol is a potential physiological substrate in the lactating mammary gland. The presence of an active glycerol kinase in the mammary gland has been shown by Baldwin and Milligan (1966). Metabolism of glycerol to glycerol-3-phosphate bypasses the phosphofructo-1-kinase (PFK-1) step in glycolysis. In addition, insulin and glycerol may relieve glucose utilisation by decreasing the cytoplasmic $[NAD^+]/[NADH]$ ratio. A good correlation has been found to exist between the concentration of glycerol-3-

phosphate and $[NAD^+]/[NADH]$ ratio in mammary gland (Robinson and Williamson, 1977c) as has been reported in adipose tissue (Saggerson and Greenbaum, 1970). The ratio of $[NAD^+]/[NADH]$ is increased in acini incubated with acetoacetate as indicated by the decrease in $[lactate]/[pyruvate]$ ratio (Robinson and Williamson, 1977c). The ability of glycerol to inhibit glucose utilisation in the absence of acetoacetate may also be related to the altered $[NAD^+]/[NADH]$ ratio.

In the presence of acetoacetate, inhibition of glucose utilisation by acini could occur either as a result of inhibition of fatty acid synthesis or inhibition of fatty acid synthesis may be due to lack of glucose uptake by the cells. In order to determine if inhibition of fatty acid synthesis is accounted for by decreased glucose uptake, the effect of acetoacetate on glucose transport in acini was measured.

3.4 Effect of Acetoacetate on Glucose Transport

Glucose transport is a major potential regulatory step in the pathway of lipogenesis (Williamson, 1980). Thus, in the lactating rat mammary gland which utilises glucose at a high rate, inhibition of glucose transport would have a profound effect on the rate of fatty acid synthesis. Glucose transport within the

mammary gland is known to be depressed during short term starvation (Threadgold and Kuhn, 1984). Therefore, the possibility that glucose transport into the cells is impaired by acetoacetate could explain the decreased glucose uptake.

Glucose transport in acini was measured as the rate of uptake of a radiolabelled glucose analogue, 2-deoxy-D-[1-³H]-glucose. This sugar molecule enters the cells and becomes phosphorylated by hexokinase in the same way as glucose but does not undergo further metabolism (Threadgold *et al*, 1982). The phosphorylated form of 2-deoxy-D-glucose is trapped within the cell and is therefore not subjected to regulatory influences of other steps in the pathway of glucose metabolism.

As reported by Threadgold *et al* (1982), the rate of 2-deoxy-D-[1-³H]-glucose uptake by acini was linear over the 60 second incubation (Figure 3.2). The rate of 2-deoxy-D-glucose uptake in the control acini was 3.72 ± 0.78 nmol/min/mg dry weight of acini. If dry weight of acini must comprise 25 - 50% protein, then the value obtained in the present study is close to the value reported by Threadgold *et al* (1982) of 16 nmol/min/mg protein.

In the presence of phloretin (1 mM), an inhibitor of glucose transport

across the cell membrane, glucose transport was abolished almost completely (Figure 3.2). This confirmed that our measurement of 2-deoxy-D-glucose is a reliable measure of glucose transport.

The presence of acetoacetate in the incubation media had no effect on the rate of 2-deoxy-[1-³H]-glucose uptake into the acini (Figure 3.2). This suggests that inhibition of glucose utilisation and fatty acid synthesis observed in isolated mammary acini could not be accounted for by inhibition of glucose transport. Inhibition of fatty acid synthesis may arise as a result of inhibition of later steps in the pathway of lipogenesis which leads to inhibition of glucose uptake by the cells, as postulated by Robinson and Williamson (1977c).

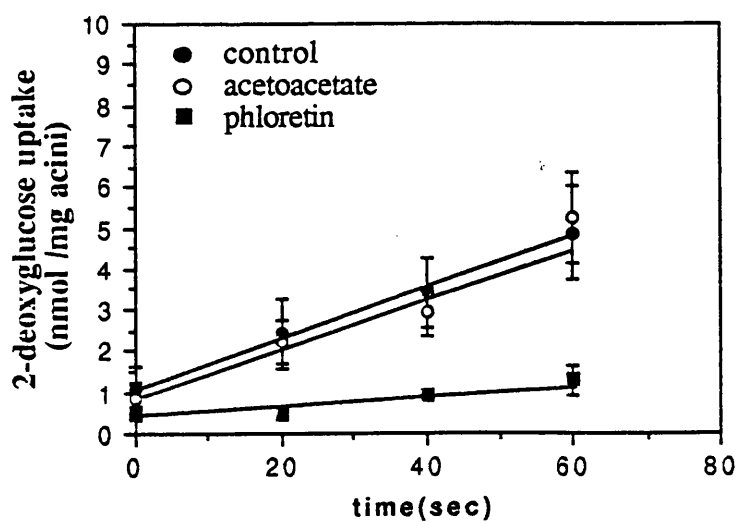
3.5 Effect of Acetoacetate on Pyruvate Dehydrogenase Activity

It was previously reported that in the presence of acetoacetate, an increased proportion of glucose utilised by isolated acini appeared as lactate and pyruvate (Robinson and Williamson, 1977c). Several studies have consistently taken the increased accumulation of lactate and pyruvate in isolated mammary acini *in vitro* as a reliable indication of PDH inactivation (Robinson and Williamson, 1977a; 1978b; Munday and Williamson, 1981). Since the activity of PDH is regulated by changes in the mitochondrial metabolite concentrations

Figure 3.2 Effect of Acetoacetate on 2-deoxy-D-[1-³H]glucose

Transport in Isolated Mammary Acini

For experimental details see Materials and Methods. Acini were preincubated for 30 min with pyruvate (0.3 mM) and lactate (4.7 mM) in the presence and absence of 2 mM acetoacetate. 2-deoxy-D-glucose was added to the final concentration of 5 mM and the uptake of 2-deoxy-D-glucose was measured over the initial 60 sec of the subsequent incubation. Mean values \pm S.E.M. at given time points are shown for acini from 5 separate rats.



of acetyl-CoA and NADH (Kerbey *et al*, 1976), it is possible that PDH activity may become inhibited due to increased ratios of [acetyl-CoA]/[CoA] and [NADH]/[NAD⁺] as a result of metabolism of acetoacetate to form acetyl-CoA.

As shown in Table 3.2, acetoacetate inhibits initial activity of PDH in isolated mammary acini by 83%. The effect of acetoacetate on PDH thus resembles that of short term starvation in which the activity of this enzyme measured in freeze-clamped mammary gland decreased sharply by 79% after 6 hours starvation with a concomitant increase in the plasma levels of acetoacetate (Hagopian *et al*, 1991).

The inhibition of PDH by acetoacetate is likely to occur as a result of increased phosphorylation of PDH. Changes in protein concentration of PDH do not occur readily in mammary gland in response to starvation, as indicated by the insignificant difference in the total activity of PDH in mammary glands from 24 hour-starved or fed lactating rats (Baxter and Coore, 1978). This is also unlikely to have changed in acini during the 60 minutes incubation with acetoacetate. In addition, the decrease in PDH activity was observed after a 150-fold dilution of the enzyme in the PDH assay which rules out allosteric inhibition of the enzyme. Increased phosphorylation of PDH could be due to enhanced PDH kinase or diminished PDH phosphatase activity. Activation of

PDH kinase could be brought about by increases in the concentrations of acetyl-CoA and NADH (Kerbey *et al*, 1976) as a result of oxidation of acetoacetate. Decreased activity of PDH phosphatase as a result of inhibition by NADH (Pettit *et al*, 1975) also leads to increased phosphorylation of PDH. Pyruvate is an inhibitor of PDH kinase (Randle, 1986). However, the sensitivity of PDH kinase to inhibition by pyruvate may be lost in acini, as occurred in mammary gland after 24 hour starvation (Baxter and Coore, 1978).

Study of perfused rat heart by Randle *et al* (1964) showed that glucose utilisation and PDH activity is inhibited in response to acetoacetate. However, addition of dichloroacetate, a pyruvate analogue which inhibits PDH kinase (Denton *et al*, 1975) and thus activates PDH, does not relieve inhibition of glucose uptake caused by acetoacetate in rat heart (Randle *et al*, 1964). Using dichloroacetate to activate PDH, Robinson and Williamson (1977c) have also shown that insulin restoration of decreased glucose uptake caused by acetoacetate is not mediated by PDH. This suggests that PDH inactivation may not be responsible for decreased glucose uptake caused by acetoacetate in rat heart and in isolated acini. The effects of dichloroacetate on the activity of PDH and glucose uptake by acini were not examined in the present study. Further experiments using dichloroacetate are therefore needed to determine the importance of PDH in mediating the inhibitory effect of acetoacetate on glucose

uptake by acini.

3.6 Effect of Acetoacetate on Acetyl-CoA Carboxylase Activity

In time courses of starvation of lactating rats, the slow inhibition of acetyl-CoA carboxylase (ACC) correlates more closely with the increase in concentration of ketone bodies in the blood (Hagopian *et al*, 1991). The rise in citrate concentration in acini in the presence of acetoacetate (Robinson and Williamson, 1977c) suggests that ACC might be inhibited.

The initial activity of ACC was measured at a physiological cell citrate concentration (0.5 mM) and at saturating citrate concentration (10 mM) in crude extracts of acini. In contrast to the inhibition of ACC by acetoacetate that one would expect, incubation of acini with 2 mM acetoacetate leads to activation of ACC over the 60 minutes incubation period (Table 3.2). The activity of ACC increased by approximately 50% above the control value and was not removed by desalting on a Sephadex G-25 "spun-column" (Table 3.3). In addition, inclusion of 2 mM acetoacetate to the assay mixture has no stimulatory effect on the activity of ACC (Table 3.3), suggesting that acetoacetate has no direct allosteric effect on the enzyme and that its presence in the assay mixture does not interfere with the enzyme assay.

Table 3.2 Effects of Acetoacetate on the Activities of Pyruvate Dehydrogenase and Acetyl-CoA Carboxylase in Isolated Mammary Acini

For measurements of PDH and ACC activity see Materials and Methods. Acini were incubated in the presence and absence of 2 mM acetoacetate for 60 min. The activity of PDH is expressed as munits PDH/unit citrate synthase activity and the activity of ACC is expressed as nmol $\text{NaH}^{14}\text{CO}_3$ incorporated/min per mg protein. The results are mean \pm S.E.M. for four observations. Values that are significantly different (Student's t-test) from the corresponding control values are shown : * $p < 0.001$; ** $p < 0.005$; *** $p < 0.0025$.

	<u>Activity of PDH</u>	<u>Activity of ACC</u>	
		<u>0.5 mM Citrate</u>	<u>10 mM Citrate</u>
Control	39.50 \pm 1.74	3.53 \pm 0.93	12.64 \pm 2.02
Acetoacetate	6.80 \pm 2.00 *	8.04 \pm 0.84 **	23.64 \pm 3.39 ***

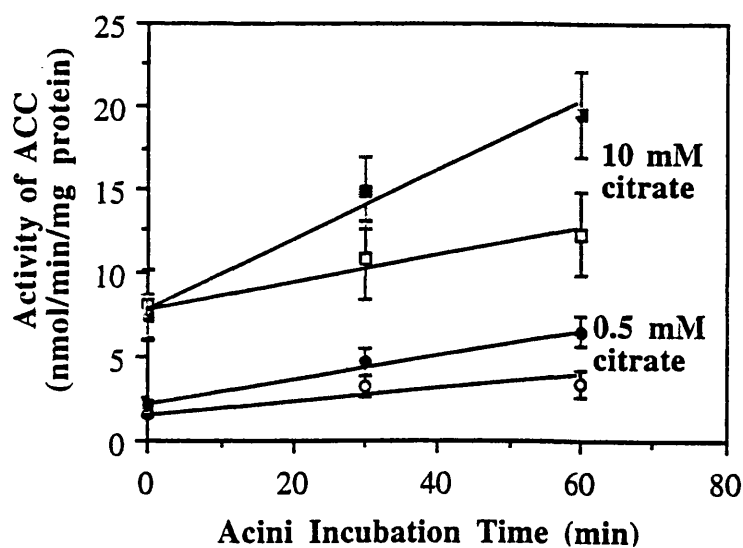
From Figure 3.3, it is clear that the activation of ACC by acetoacetate in acini is time-dependent. There is no significant activation of ACC by acetoacetate in comparison to the control values at 0 and 30 minutes incubation time points when the enzyme was assayed at 0.5 mM and 10 mM citrate. Activation of ACC was only apparent upon incubation of acini with acetoacetate for 60 minutes.

Activation of ACC by acetoacetate is also evident upon purification of the enzyme on avidin-Sepharose affinity column (Figure 3.4). This suggests that acetoacetate is unlikely to have an allosteric effect on ACC as this would not have survived purification. In the presence of acetoacetate, V_{\max} of the enzyme increased by 100% and K_a for citrate decreased by 18% in comparison to the control values (Table 3.5). Short term regulation of ACC could be mediated by allosteric regulation and/or by reversible phosphorylation of the enzyme (Chapter 1, Section 1.7.5). The results from the kinetic analysis suggest that acetoacetate may cause dephosphorylation and hence activation of ACC. In order to determine if acetoacetate decreased phosphorylation and thus increased activation of ACC, the phosphorylation state of ACC was measured in acini labelled with [^{32}P]phosphate.

Figure 3.3 Effect of Acetoacetate on the Activity of Acetyl-CoA

Carboxylase in Isolated Mammary Acini

For measurement of ACC activity in crude extracts of acini see Materials and Methods. Acini were incubated in the presence (close symbols) and absence (open symbols) of 2 mM acetoacetate for 20, 40 and 60 min. Results are expressed as nmol $\text{NaH}^{14}\text{CO}_3$ incorporated/min per mg protein and are means for at least four observations at each time point, with S.E.M. indicated by vertical bars.



**Table 3.3 Effects of Acetoacetate, Acetoacetyl-CoA and Desalting on
Sephadex G-25 Spun Column on the Activity of Acetyl-CoA
Carboxylase**

For experimental details see text. Activity of ACC in crude extracts of acini was measured at 10 mM citrate. Results are expressed as nmol $\text{NaH}^{14}\text{CO}_3$ incorporated/min per mg protein and are means \pm S.E.M. for four observations. Values that are significantly different from the corresponding control values by Student's t-test are shown : * $p < 0.05$.

	<u>ACC Activity</u>
(a) Control acini extract	10.5 \pm 1.3
(b) + 2 mM Acetoacetate added to assay	10.6 \pm 0.7
(c) + 2 mM Acetoacetyl-CoA added to assay	10.1 \pm 0.8
(d) Control acini extract desalted on G-25	9.9 \pm 0.6
(e) Acini incubated with 2 mM Acetoacetate extract desalted on G-25	19.6 \pm 2.5 *

Figure 3.4 Effect of Acetoacetate on the Activity of Acetyl-CoA Carboxylase Purified From Isolated Mammary Acini

For purification and assay of ACC see Materials and Methods. Acini were incubated in the presence and absence of 2 mM acetoacetate for 60 min. Results are expressed as Units/mg protein and are means for four observations with S.E.M. indicated by vertical bars.

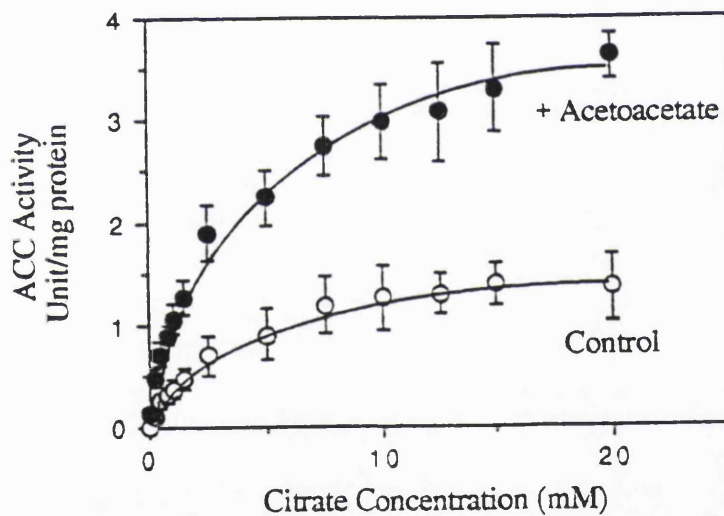


Table 3.4 Effects of Acetoacetate on the V_{\max} and K_a Citrate of Acetyl-CoA Carboxylase

V_{\max} (Unit/mg protein) and K_a citrate (mM) of ACC were determined as described in Materials and Methods. Each value is the mean for four observations and values that are significantly different from the corresponding control values by Student's t-test are shown : * $p < 0.05$.

	V_{\max}	K_a Citrate
Control	1.57 ± 0.07	1.91 ± 0.15
Acetoacetate	3.37 ± 0.70 *	1.57 ± 0.07 *

3.7 [^{32}P]-Labelling of Acini

Acini were labelled with [^{32}P]phosphate for 60 minutes and 2 mM acetoacetate was added and the incubation was allowed to proceed for a further 60 min. ACC was purified from the [^{32}P]-labelled cells on an avidin-Sepharose affinity column.

Figure 3.5 shows the Coomassie blue-stained gel and autoradiograph of crude extracts of acini labelled with [^{32}P]phosphate for 60 minutes. It is apparent that a number of phosphoproteins are radiolabelled, including ATP-CL (116 kDa) and ACC (Mr 240 kDa). Coomassie blue staining of the gel of the purified enzyme subjected to SDS-PAGE (Figure 3.6(A), lanes 3 and 5) revealed that it was homogeneous except that there was a minor contamination by a polypeptide of 147 kDa which is not ^{32}P -labelled (Figure 3.6(B), lanes 7 and 9). The 140 kDa fragment could be a proteolytic fragment of ACC or contamination by pyruvate carboxylase. Figure 3.6(B) shows an autoradiograph of purified ACC obtained after SDS-PAGE of the same amount of soluble proteins from control and acetoacetate-treated cells. Comparison of lanes 4 and 5 in Figure 3.5(B) show that the majority of ^{32}P -labelled proteins in the mammary acini do not change their phosphorylation status in response to acetoacetate with the exception of the 240 kDa band of ACC which appears to have less ^{32}P label in

acini treated with acetoacetate. This is more apparent following purification on avidin-Sepharose where comparison of lanes 7 and 9 in Figure 3.6(B) clearly show less ^{32}P in the ACC from acetoacetate-treated acini. Direct measurement of the radioactive content of the ACC eluted from the avidin-Sepharose column by scintillation counting confirmed this observation although not to the extent suggested by the autoradiograph. By scintillation counting the ^{32}P content appeared only to be approximately 10% lower in acetoacetate-treated acini.

The results suggest that phosphorylation of ACC might have decreased in response to acetoacetate, resulting in activation of the enzyme. Mild proteolysis of ACC is known to activate the enzyme (Guy and Hardie, 1981). However, there is no apparent change in the subunit size of ACC purified from acetoacetate-treated cells (Figure 3.6(A), lane 4) and there is no apparent appearance of low molecular weight fragments in the ^{32}P -labelled enzyme (Figure 3.6(B), lane 9). Therefore, activation of ACC in response to acetoacetate appears likely to be due either to its dephosphorylation (and the possibility that acetoacetate activates protein phosphatase 2A or inactivates AMP-activated protein kinase remains to be explored) or to an allosteric activator which binds so tightly to ACC that it survives purification.

Figure 3.5 Coomassie Blue-Stained Gel and Autoradiograph of Crude Extracts of Acini from [32 P]phosphate-Labelled Acini followed by Incubation with Acetoacetate

For experimental details see text. Acini were labelled with [32 P]phosphate for 60 min before addition of 2 mM acetoacetate. (A) Coomassie Blue-stained gel. Lanes are 1 : molecular weight marker; 2 : crude extract from control acini; 3 : crude extract from acetoacetate-treated acini. (B) Autoradiograph of Coomassie Blue-stained gel. Lanes are 4 : crude extract from control acini; 5 : crude extract from acetoacetate-treated acini.

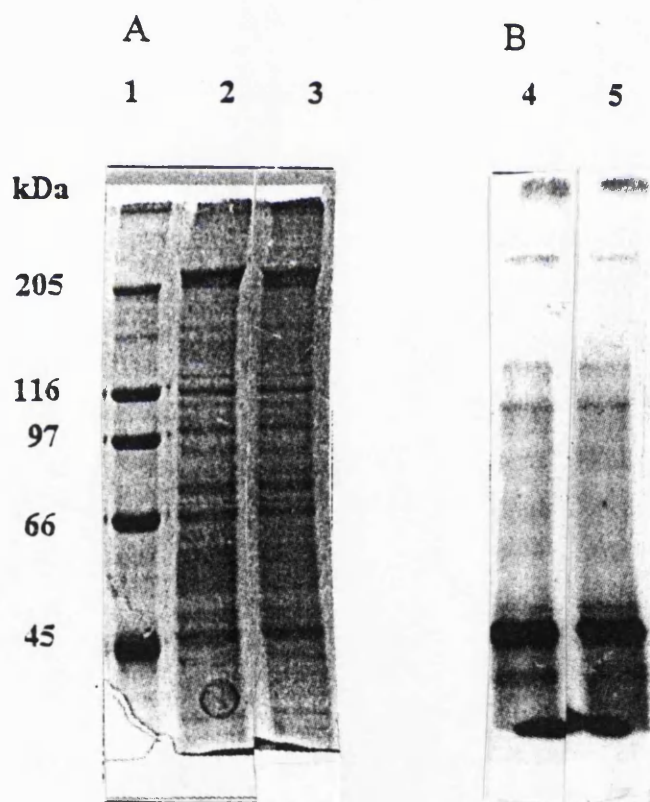
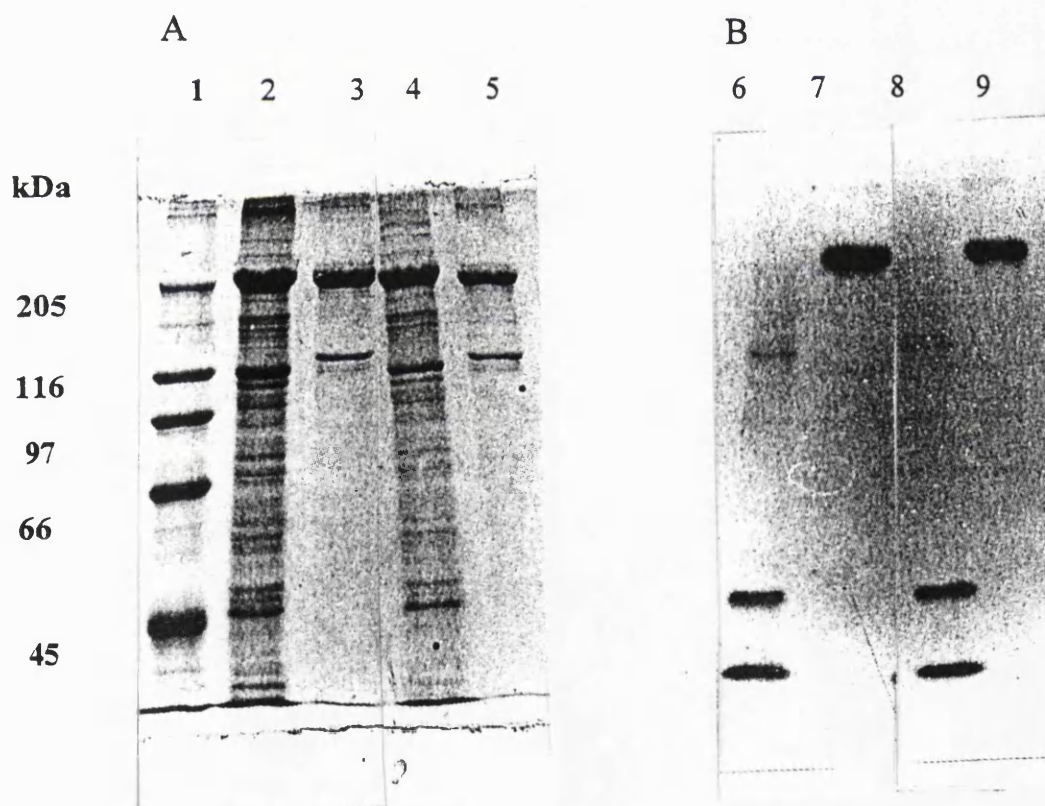


Figure 3.6 Coomassie Blue-Stained Gel and Autoradiograph of Breakthrough from Avidin-Sepharose Affinity Column and Purified Acetyl-CoA Carboxylase from [32 P]phosphate-Labelled Acini followed by Incubation with Acetoacetate

For experimental details see text. (A) Coomassie Blue-stained gel. Lanes are : 1 : molecular weight marker; 2 : column breakthrough from control acini; 3 : purified ACC from control acini; 4 : column breakthrough from acetoacetate-treated acini; 5 : purified ACC from acetoacetate-treated acini. (B) Autoradiograph of Coomassie Blue-stained gel. Lanes are : 6 : column breakthrough from control acini; 7 : purified ACC from control acini; 8 : column breakthrough from acetoacetate-treated acini; 9 : purified ACC from acetoacetate-treated acini.



3.8 Effect of Acetoacetyl-CoA on the Activity of Acetyl-CoA Carboxylase

Acetoacetate may be esterified in the cytoplasm of lactating rat mammary gland to form acetoacetyl-CoA via the action of acetoacetyl-CoA synthetase (Buckley and Williamson, 1975). In order to examine if acetoacetate exerts its activatory effect as acetoacetyl-CoA in a manner comparable to the allosteric inhibition of ACC by long chain fatty acyl-CoA (Halestrap and Denton, 1973), the effect of acetoacetyl-CoA on the activity of ACC was examined.

Addition of 2 mM acetoacetyl-CoA to the assay mixture had no stimulatory effect on ACC activity in crude extract of acini when measured at 10 mM citrate (Table 3.3). The concentration of acetoacetyl-CoA used in this case might not reflect the true physiological concentration in the cells as the extent of metabolism of acetoacetate is not known. However, it must be greater than the intracellular concentration since metabolism of acetoacetate decreases its concentration. The lack of effects of acetoacetyl-CoA on ACC activity suggests that other intrinsic factor(s) such as changes in the metabolite concentrations in the cells may be involved in stimulating ACC. Any such effector molecule(s) is likely to be tight-binding. The existence of small molecular weight molecules (besides citrate and fatty acyl-CoA esters) which are potential regulators of ACC have been described (Saltiel *et al*, 1983; Haystead

and Hardie, 1986). Recently, Quayle *et al* (1993) have identified an endogenous protein regulator from rat liver which activates ACC. The effect of this regulator on ACC is not mediated via dephosphorylation or proteolysis of the enzyme but was reported to involve direct interaction of the regulator with ACC. The identification of this regulator protein required further characterisation and until then, one may speculate that any allosteric activation of ACC by acetoacetate may in fact be mediated by a protein regulator as described by Quayle *et al* (1993).

3.9 Effect of Acetoacetate on ATP-Citrate Lyase Activity

ATP-CL catalyses the formation of cytosolic acetyl-CoA from citrate for use as the lipogenic precursor. To determine if the availability of acetyl-CoA was decreased by acetoacetate, the activity of ATP-CL was measured.

The activity of ATP-CL was measured in the crude extracts of acini. As shown in Table 3.5, the activity of ATP-CL was inhibited by 18% in the presence of acetoacetate and inhibition was not removed by desalting on a Sephadex G-25 "spun-column".

Figure 3.5A shows the Coomassie blue-stained SDS-PAGE gel of crude extracts prepared from [^{32}P]-labelled acini incubated in the presence and absence of acetoacetate. The autoradiograph (Figure 3.5(B), lanes 4 and 5) of the same gel gives no indication of changes in phosphorylation state of ATP-CL (Mr 116 kDa) in response to acetoacetate. ATP-CL is a phosphoprotein (Linn and Srere, 1979; Ramakrishna and Benjamin, 1979). However, there is no evidence that phosphorylation of ATP-CL changes its activity (Guy *et al*, 1981). In this case, inhibition of the enzyme by acetoacetate may have produced the changes in the concentration of citrate, which was increased by 37% in lactating rat mammary gland slices incubated with acetoacetate (Williamson *et al*, 1975).

3.10 Effect of Acetoacetyl-CoA on ATP-Citrate Lyase Activity

In order to determine if acetoacetyl-CoA is the allosteric effector responsible for decreasing the activity of ATP-CL, 2 mM acetoacetyl-CoA was added to crude extracts of acini prior to the assay. As seen in Table 3.6, The activity of ATP-CL was identical in the absence and presence of acetoacetyl-CoA. Similarly, no difference in activity was seen with the partially purified enzyme (Table 3.6). Thus, other contributing factors such as alterations in metabolite concentrations must be present in the cell in order for acetoacetate to exert its inhibitory effect on the enzyme.

**Table 3.5 Effect of Acetoacetate on the Activity of ATP-Citrate Lyase
in Isolated Mammary Acini**

Acini were incubated for 60 min in the presence and absence of 2 mM acetoacetate. ATP-CL activity was assayed in crude extract of acini before and after desalting on a Sephadex G-25 spun column. Results are expressed as μ moles NADH utilised/min per mg protein and are means \pm S.E.M. for five observations. Values that are significantly different (Student's t-test) from the corresponding control values are shown : * $p < 0.025$.

	ATP-CL Activity	
	<u>before desalting</u>	<u>after desalting</u>
Control	4.37 \pm 0.41	4.70 \pm 0.48
Acetoacetate	3.50 \pm 0.36 *	3.85 \pm 0.35 *

Table 3.6 Effect of Acetoacetyl-CoA on the Activity of ATP-Citrate Lyase

For experimental details see text. Results are expressed as $\mu\text{mole NADH}$ utilised/min per mg protein and are mean values \pm S.E.M. for three observations.

	ATP-CL Activity	
	<u>Crude extracts</u>	<u>Purified ATP-CL</u>
Control	4.85 \pm 0.19	2.45 \pm 0.20
Acetoacetyl-CoA	4.79 \pm 0.10	2.46 \pm 0.23

3.11 Effect of Acetoacetate On Glycerol-3-phosphate Acyltransferase

Activity

Glycerol-3-phosphate acyltransferase (GPAT) catalyses the esterification of fatty acids and glycerol to form triglycerides (Yamashita *et al*, 1972). GPAT exists in mitochondrial and microsomal isozymic forms. The activities of the mitochondrial and microsomal GPAT is measured at 6 mg/ml and 1.75 mg/ml BSA, respectively. The activity of GPAT is high in the lactating rat mammary gland which synthesises milk lipids at a high rate (Williamson, 1980) and this correlates with the rates of esterification of fatty acids (Zammit, 1984). The high activity of GPAT is to ensure high rates of triglyceride production as well as to ensure that the concentration of fatty acyl-CoA esters which are potent inhibitors of ACC remains low and that ACC activity is maintained. Inhibition of GPAT activity would therefore be expected to affect lipogenesis as the end product of lipogenesis i.e fatty acyl-CoA would accumulate and exert negative feedback control on ACC (Halestrap and Denton, 1973). The activity of mitochondrial GPAT is inhibited to 60% of the control value in adipose tissue of rats starved for 48 hours but the activity of the microsomal enzyme is unchanged (Bates and Saggerson, 1979). However, it is not known if the inhibition of the mitochondrial enzyme which occurred during starvation is mediated by acetoacetate. In the previous study carried out by Robinson and Williamson

(1978a), acetoacetate (2 mM) was shown to decrease esterification of oleate in isolated mammary acini by 60%. Therefore, it seems possible that acetoacetate may inhibit esterification of long chain fatty acyl-CoA esters. However, if this is the case, the accumulation of fatty acyl-CoA esters would lead to inhibition of ACC and one would not expect to see an increase in ACC activity in the presence of acetoacetate.

As shown in Table 3.7, the activity of GPAT in acini incubated with acetoacetate was not significantly different from that of the control. Therefore, acetoacetate has no effect on GPAT activity and inhibition of esterification of fatty acids is unlikely to be affected. Robinson and Williamson (1977c) showed that acetoacetate decreased the concentration of glycerol-3-phosphate in isolated mammary acini. Therefore, it is possible that acetoacetate may inhibit esterification by decreasing the supply of the glycerol-3-phosphate substrate or the activities of other enzymes in the esterification pathway.

Table 3.7 Effect of Acetoacetate on the Activity of Glycerol-3-phosphate Acyltransferase in Isolated Mammary Acini

For experimental details see text. Results are expressed as nmol of glycerol-3-phosphate esterified/min per mg protein and are means \pm S.E.M. for five observations.

	<u>GPAT Activity</u>
Control	9.51 \pm 1.69
Acetoacetate	8.94 \pm 1.25

3.12 Discussion

The results from the present study confirm the previous findings that acetoacetate inhibits glucose utilisation and fatty acid synthesis in isolated mammary acini (Table 3.1). These changes may be mediated by the increased formation of acetyl-CoA from acetoacetate which leads to a rise in citrate concentration (Williamson *et al*, 1975; Robinson and Williamson, 1977c). Citrate is a potent inhibitor of PFK-1 in mammary gland (Zammit, 1979). In starvation of lactating rats, the concentration of F-6-P in the mammary gland increased whereas that of F-1,6-BP decreased (Hagopian *et al*, 1991). Since conversion of F-6-P to F-1,6-BP is catalysed by PFK-1, an increase in the ratio of F-6-P : F-1,6-BP suggests that the activity of PFK-1 is likely to be inhibited by the rise in citrate concentration in starved lactating rats. The net result of this is decreased glycolytic flux which therefore limits the availability of glucose for fatty acid synthesis. It was postulated by Robinson and Williamson (1977c) that glycolysis at the step catalysed by PFK-1 is inhibited by acetoacetate. In isolated mammary acini incubated with acetoacetate, the citrate concentration is increased by 37% and this is accompanied by the accumulation of glucose-6-phosphate which was shown to increase by 60% (Williamson *et al*, 1975; Robinson and Williamson, 1977c). Inhibition of glycolysis by acetoacetate has been observed in mammary gland slices (Williamson *et al*, 1975) and in perfused rat heart

(Newsholme and Randle, 1964). The accumulation of glucose-6-phosphate which occurred as a result of inhibition of PFK-1 exerts negative feedback inhibition on hexokinase, and thus decreased glucose uptake. The net result is a decrease in the availability of glycerol-3-phosphate, and thus inhibition of fatty acid esterification. The activity of glucose-6-phosphate dehydrogenase is not stimulated by the increase in the concentration of glucose-6-phosphate. This presumably is a result of inhibition of glucose-6-phosphate dehydrogenase by high levels of NADPH (Sapag-Hagar *et al*, 1973) as fatty acid synthesis was inhibited. The pentose phosphate pathway is therefore unable to operate to full capacity. Williamson *et al* (1975) have observed that in the presence of acetoacetate, the activity of the pentose phosphate pathway is reduced by about 40% and that of glycolysis is reduced by 26% in mammary gland slices. The extent of inhibition of glycolysis is small in comparison to the decrease in glucose uptake. In the present study, glucose uptake by acini is inhibited by acetoacetate and this is not a result of inhibited glucose transport but more likely to occur as a result of secondary inhibition of hexokinase in response to primary inhibition of PFK-1. The free $[NAD^+]/[NADH]$ ratio in the cytosol is increased as a result of oxidation of acetoacetate. The altered redox state towards oxidation may contribute to the changes in overall mammary gland metabolism in response to acetoacetate.

The activity of PDH was inhibited in acini incubated with acetoacetate (Table 3.2). This is in keeping with the increase in the proportion of glucose utilised by acini that appeared as lactate and pyruvate (Robinson and Williamson, 1977c). However, dichloroacetate activation of PDH did not relieve inhibition of glucose uptake caused by acetoacetate in mammary acini (Robinson and Williamson, 1977c). This suggests that inhibition of PDH is not the primary cause of the decreased glucose uptake caused by acetoacetate. Inhibition of PDH is likely to occur as a result of metabolism of acetoacetate which leads to increased mitochondrial levels of acetyl-CoA and NADH. Activation of PDH kinase by these metabolites resulted in phosphorylation and inactivation of PDH.

Inhibition of PDH is not accompanied by a corresponding decrease in the activity of ACC. The unexpected stimulation of ACC by acetoacetate observed in the present study remains an open question. It appears that inactivation of ACC is not involved in the acute inhibition of fatty acid synthesis caused by acetoacetate. In short term starvation (6 hour) of lactating rats, the activity of ACC was not inhibited (Williamson *et al*, 1983; Munday and Hardie, 1986; Hagopian *et al*, 1991). It therefore appears that inactivation of ACC is not involved in the short term regulation of fatty acid synthesis during starvation. However, the activity of PDH is decreased by 79% in rats that have been starved for 6 hours (Hagopian *et al*, 1991). The importance of PDH rather than

ACC in the inhibition of fatty acid synthesis is likely to suggest the importance of carbohydrate precursor glucose, over lipid derived sources of acetyl-CoA in this tissue.

The results from ^{32}P -labelled acini and ACC purified to homogeneity suggest that ACC may become dephosphorylated in the presence of acetoacetate. The increase in the concentration of citrate in the cytosol in the presence of acetoacetate (Robinson and Williamson, 1977c) may promote polymerisation of ACC. The polymeric form of ACC is highly active and may show resistance towards phosphorylation by protein kinases. The exact mechanism of activation of ACC by acetoacetate is not known and may in fact be mediated by a protein regulator such as that described by Quayle *et al* (1993). Further elucidation of the mechanism involved in the activation of ACC in response to acetoacetate is required. In addition, measurement of the concentration of malonyl-CoA may give an indication of the true flux through the step catalysed by ACC.

The activity of ATP-CL is inhibited in response to acetoacetate. Although the extent of inhibition is small (18%), this may contribute to the overall inhibition of fatty acid synthesis in the isolated mammary acini. In starvation of the rat, inhibition of the activity of ATP-CL in liver occurred as a result of decreased enzyme concentration (Kornacker *et al*, 1965a and 1965b). However,

this is unlikely to have changed in the isolated mammary acini incubated with acetoacetate for 60 minutes.

Insulin and glycerol are able to relieve inhibition of glucose utilisation caused by acetoacetate in isolated mammary acini (Robinson and Williamson, 1977c). It was postulated by Robinson and Williamson (1977c) that insulin and glycerol increase the availability of glycerol-3-phosphate, thus encouraging esterification and activating ACC and fatty acid synthesis by decreasing the concentration of fatty acyl-CoA esters which are potent inhibitors of ACC (Halestrap and Denton, 1973). We have no evidence for fatty acyl-CoA mediated inhibition of ACC by acetoacetate although it is possible that such an allosteric inhibition would not survive our assay and purification procedures. One might speculate that the ACC activation we observe is a compensatory and antagonising reaction to such inhibition in the presence of plentiful substrate for the enzyme and feedforward activators such as citrate. It is clear from our results that any inhibition of esterification is not due to direct GPAT inhibition by acetoacetate but more likely due to the glycerol-3-phosphate deficiency.

In conclusion, the results from the isolated mammary acini suggest that the effects of acetoacetate are rather similar, but not identical to those seen in starvation. It appears that acetoacetate inhibits the activity of PDH and while in

starvation, inhibition of this enzyme is the major mechanism in mediating the inhibition of fatty acid synthesis. The inhibition of ATP-CL is small and it is hard to see how this could be the case under these conditions given the levels of citrate and acetyl-CoA that acetoacetate is likely to produce. ACC does not appear to be the direct target of acetoacetate in the short term inhibition of fatty acid synthesis despite the fact that it is the rate limiting enzyme in this biosynthetic pathway. One might speculate that it is increased citrate as a consequence of acetoacetate metabolism that is the key signal that via inhibition of PFK-1 inhibits glucose uptake, limits glycerol-3-phosphate production and the subsequent decrease in esterification allows fatty acyl-CoA to act as feedback inhibitors of fatty acid synthesis.

CHAPTER 4

REGULATION OF LIPOGENESIS BY OKADAIC ACID : AN INHIBITOR OF PROTEIN PHOSPHATASES 1 AND 2A

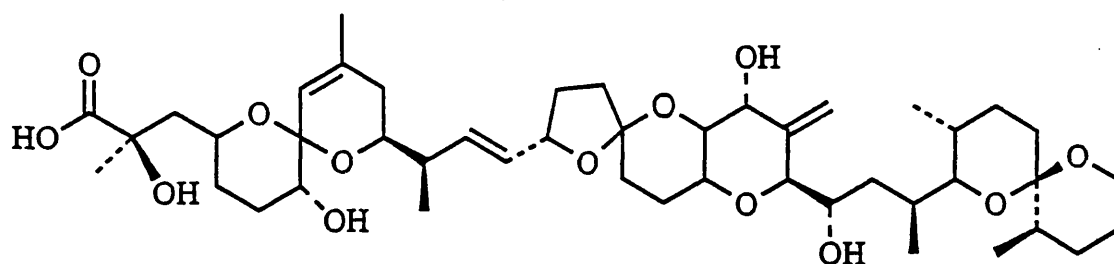
4.1 Introduction

Okadaic acid ($C_{44}H_{66}O_{13}$) is a polyether derivative of a C_{38} fatty acid with one carboxyl group and four hydroxyl groups (Figure 4.1). It is produced by marine dinoflagellates (phytoplankton or microalgae; Yasumoto *et al*, 1985) and can be ingested and accumulated in marine organisms such as sponges or shellfish as a result of filter feeding. The toxin is extracted from the black sponge *Halichondria okadaii* and *Halichondria melanodocia* (Tachibana *et al*, 1981) and is implicated as a causative agent of diarrhetic shellfish poisoning which has a worldwide occurrence and poses a threat to the shellfish industry.

Many of the physiological effects of okadaic acid may be attributed to the fact that it is a potent cell permeable inhibitor of protein phosphatases (Cohen *et al*, 1990). Studies of carcinogenesis have shown that okadaic acid is a potent, non-phorbol ester type, tumour promoter (Suganuma *et al*, 1988). Unlike other tumour promoters which bind to phorbol ester receptors and activate protein kinase C, okadaic acid exerts its effect by inhibiting protein phosphatases (Fujiki *et al*, 1986), thus allowing endogenous protein

phosphorylation to proceed unantagonised.

Figure 4.1 Structure of Okadaic Acid



There are two major types of serine/threonine phosphatase catalytic subunits in cytoplasm (Cohen, 1989) : Type 1 is able to dephosphorylate the β -subunit of phosphorylase kinase and is inhibited by thermostable protein inhibitors-1 and 2; and Type 2 which dephosphorylates the α -subunit of phosphorylase kinase and is insensitive to inhibitors-1 and 2. Type 2 comprises three distinct groups of enzymes, each of which has specific requirements for divalent cations. Type 2A is active in the absence of divalent cations whereas Type 2B and 2C require Ca^{2+} and Mg^{2+} respectively, for their activity. Protein phosphatase (PP) 1, 2A and 2C are the major protein phosphatases acting on a variety of phosphoproteins *in vivo* (Haystead *et al*, 1989; Cohen *et al*, 1989).

addition, PP2A has been shown to have particularly high activity towards the regulatory enzymes of glycolysis and fatty acid synthesis including PFK-1, PFK-2/F-6-BPase, ACC and ATP-CL (Ingebritsen *et al*, 1983).

Okadaic acid has an exceedingly high affinity for PP2A with an IC_{50} of 0.1 nM, whereas a higher concentration is required (IC_{50} 15-20 nM) to inhibit PP1 (Bialojan and Takai, 1988), despite the fact that there is a striking sequence homology (50%) between the catalytic subunits of these phosphatases (Berndt *et al*, 1987; Cohen and Cohen, 1989). The IC_{50} for PP2B is an order of magnitude higher whereas PP2C (Bialojan and Takai, 1988; Cohen *et al*, 1990) and mitochondrial phosphatases such as pyruvate dehydrogenase phosphatase (Haystead *et al*, 1989) are not affected. Kinetic studies (Takai *et al*, 1992) suggest that inhibition of PP2A by okadaic acid is a time-dependent process characteristic of a tightly binding inhibitor. The high affinity of okadaic acid for PP2A seems to be due to the very slow dissociation rather than fast binding of the toxin. Inhibition of PP1 and PP2A by okadaic acid is reversible as judged from its effects on myosin light chain phosphorylase (Bialojan and Takai, 1988). In the concentration range that is effective in inhibiting protein phosphatases, okadaic acid has no effect on protein kinases such as cAMP-PK, AMP-PK, casein kinases, Ca^{2+} -calmodulin-dependent protein kinases (Haystead *et al*, 1989) and protein kinase C (Suganuma *et al*, 1988). Recent studies by Haystead *et al* (1990b) suggest that okadaic acid can mimic the effects of insulin to

stimulate a number of cytoplasmic serine protein kinases including kinases acting on "S6 peptide" and myelin basic protein.

Okadaic acid has proved to be an extremely useful pharmacological tool for examining the importance of protein phosphorylation in cellular physiological events. Inhibition of PP1 and PP2A by okadaic acid allows the action of many intracellular protein kinases to proceed unchallenged. The resulting increase in phosphorylation of target proteins produces a diversity of physiological responses according to the cell types studied. Okadaic acid can therefore be used to identify physiological events that are regulated by reversible phosphorylation. Studies in guinea pig taenia coli suggest that okadaic acid enhanced the contraction of skinned smooth muscle fibres (Takai *et al*, 1987). Since smooth muscle contraction depends on the state of myosin phosphorylation, the effect of okadaic acid is therefore attributed to the inhibition of myosin light chain phosphatase activity as the toxin has no effect on myosin light chain kinase activity (Takai *et al*, 1987). In isolated cardiac myocytes, okadaic acid increased inward Ca^{2+} current and this has been taken as indication that the slow inward Ca^{2+} channel is activated by phosphorylation and inactivated by PP1 and PP2A (Hescheler *et al*, 1988). In Paramecium, PP1 is responsible for dephosphorylation and inactivation of the Ca^{2+} channel, this terminates backward swimming of this protozoan in response to adverse stimulations. Okadaic acid is able to bring about sustained backward swimming

in *Paramecium* by inhibiting PP1 (Proud, 1986; Cohen *et al*, 1990). In mouse skin treated with okadaic acid, the induction of tumours (Suganuma *et al*, 1988) is attributed to the fact that the toxin allows mitogenic and proliferative kinases such as protein kinase C to phosphorylate intracellular phosphoproteins without antagonism from PP1 and PP2A. Intubation of okadaic acid into the stomach of mice or rats caused diarrhoea with accumulation of a large volume of fluid in the stomach, small intestine and colon (Yasumoto *et al*, 1985). This effect of okadaic acid is thought to be mediated via unantagonised cAMP-PK phosphorylation of one or more proteins that control sodium secretion by intestinal cells.

It is now widely accepted that changes in the phosphorylation state of key regulatory enzymes are the major mechanism via which hormones control carbohydrate and lipid metabolism. In general, enzymes in biodegradative pathways are activated whereas those in biosynthetic pathways are inactivated by phosphorylation (Cohen, 1982). The activities of protein phosphatases and protein kinases are finely balanced according to the hormonal and nutritional status of the animal to ensure that the activity of regulatory enzymes and the flux in the metabolic pathways are finely tuned. Some of the confirmatory evidence for the involvement of reversible phosphorylation in the control of carbohydrate and lipid metabolism has come from the treatment of isolated adipocytes and hepatocytes with okadaic acid (Haystead *et al*, 1989; Rutter *et*

al, 1991). In isolated adipocytes and hepatocytes, fatty acid synthesis was inhibited by okadaic acid as a result of increased phosphorylation and inactivation of ACC (Haystead *et al*, 1989; Rutter *et al*, 1991) as PP2A was inhibited by the toxin. In addition, treatment of isolated hepatocytes with okadaic acid resulted in activation of gluconeogenesis with concomitant inhibition of glycolysis (Haystead *et al*, 1989). Inhibition of glycolysis must have occurred as a result of increased phosphorylation and inactivation of PFK-1 and PFK-2 as these are substrates for PP2A (Ingebritsen *et al*, 1983). Increased phosphorylation and activation of phosphorylase kinase and glycogen phosphorylase and inhibition of glycogen synthase by okadaic acid resulted in glycogen depletion (Haystead *et al*, 1989). This is consistent with PP1 being active against the enzymes of glycogen metabolism. In isolated adipocytes, okadaic acid increased phosphorylation and hence the activation of hormone sensitive lipase, resulting in a 2- to 3-fold increase in lipolysis (Rutter *et al*, 1991). These effects of okadaic acid have supported the view that PP1 and PP2A, rather than PP2C are the physiological phosphatases acting on enzymes in lipid and carbohydrate metabolism.

There is compelling evidence that fatty acid synthesis in the lactating mammary gland is subject to regulation by reversible phosphorylation of the key lipogenic enzymes. A number of potential rate limiting steps in the pathway from glucose to fatty acid may be regulated hormonally via reversible

phosphorylation. These include glucose transport into the cells and the steps catalysed by PDH and ACC. In particular, phosphorylation of ACC by AMP-PK has been shown to be of significant physiological importance in short term control of lipogenesis in mammary gland (Munday and Hardie, 1986; Hardie, 1989). The kinase phosphorylates ACC at serine-79, serine-1200 and serine-1215 with phosphorylation at serine-79 causing inactivation of the enzyme (Davies *et al*, 1989). ACC and AMP-PK are substrates of PP2A and PP2C, respectively (Ingebritsen *et al*, 1983; Haystead *et al*, 1990a). Inhibition of PP2A by okadaic acid is therefore expected to increase the phosphorylation of ACC by AMP-PK, thus inhibiting *de novo* fatty acid synthesis.

The aim of the present studies was to investigate the effects of reversible phosphorylation of lipogenic enzymes on the pathway of lipogenesis in isolated mammary acini and to examine the importance of the AMP-PK cascade and ACC inhibition in this tissue using okadaic acid as a specific inhibitor of PP1 and PP2A.

4.2 Identification of Protein Phosphatases in Lactating Rat Mammary Tissue Extracts

The discovery of okadaic acid and its specificity towards protein phosphatases has facilitated the studies of the activity of serine/threonine

phosphatases and the quantification of these enzymes in intact cells and cellular extracts. PP2A is taken as the activity which is inhibited by 2 nM okadaic acid. A higher concentration of okadaic acid (5 μ M) inhibits both PP1 and PP2A with the former being also sensitive to inhibitor-1 (Cohen *et al*, 1989). The activity of PP1 is therefore taken as the activity observed at 5 μ M okadaic acid subtracted from that measured at 2 nM okadaic acid. Using this differential inhibition of PP1 and PP2A by okadaic acid, the activities and the relative proportions of these enzymes in lactating rat mammary tissue extracts were measured using 32 P-labelled glycogen phosphorylase_a as substrate.

Table 4.1 shows the rate of dephosphorylation of 32 P-labelled phosphorylase_a in the presence and absence of okadaic acid. It is apparent that okadaic acid-sensitive protein phosphatase activity i.e PP1 and PP2A are present in the mammary tissue extracts. As shown in Figure 4.2, PP2A is the predominant phosphatase, contributing to 58% of the total glycogen phosphorylase phosphatase activity in mammary tissue extracts. PP1 contributed to 36% of the total activity with other phosphatases showing minimal activity, accounting for only 6% of the total activity of glycogen phosphorylase phosphatase. Assuming that the activity of other protein phosphatases is due to that of PP2C, the result is in agreement with the previous findings of the ratio of PP2A to PP2C of approximately 10 : 1 in rat liver and adipose tissue (Haystead *et al*, 1990a).

The findings that mammary tissue extracts have a high activity of PP2A is in agreement with the role of this phosphatase in the regulation of ACC *in vivo* (Ingebritsen *et al*, 1983; Haystead *et al*, 1990a).

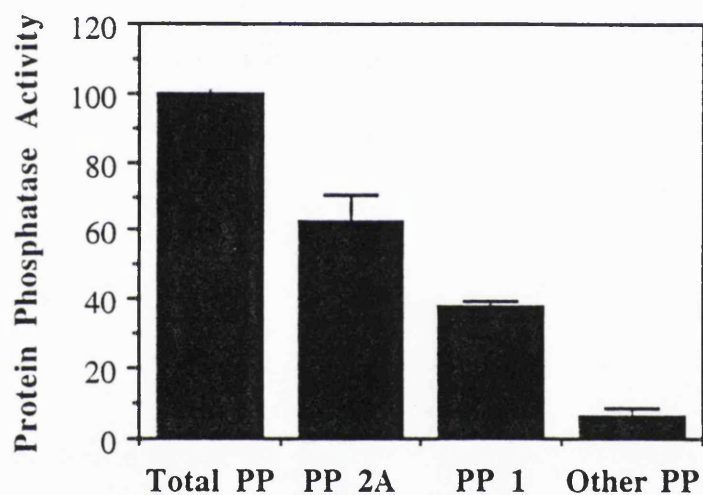
Table 4.1 Activities of Protein Phosphatases in Mammary Tissue Extracts

Experimental details are as described in text. The results are expressed as nmol ³²P released/min per mg protein and are mean values ± S.E.M for four separate observations.

<u>Protein</u>	<u>Rate of dephosphorylation</u>
<u>Phosphatase</u>	<u>of [³²P]-glycogen phosphorylase</u>
Total	396.0 ± 83.4
2A	244.1 ± 43.0
1	153.4 ± 38.5
Others	23.5 ± 5.8

Figure 4.2 Percentage of Protein Phosphatases in Mammary Tissue Extracts

Experimental details are as described in text. The protein phosphatase activities are expressed as a percentage of the total phosphatase activity in the tissue. Each value is the mean of four observations with S.E.M. indicated by vertical bars.



4.3 Effect of Okadaic Acid on Lipogenesis in Acini

As shown in Table 4.2, the rate of fatty acid synthesis in control acini is in agreement to those measured previously in acini (Katz *et al*, 1974). Incubation of isolated mammary acini with okadaic acid (1 μ M) significantly reduced the rate of $^3\text{H}_2\text{O}$ incorporation into fatty acids. The inhibition of fatty acid synthesis in okadaic acid-treated cells (63%) was highly significant. This is in agreement with the studies on isolated rat adipocytes and hepatocytes where the incorporation of ^{14}C -acetate into lipids is markedly inhibited by okadaic acid (Haystead *et al*, 1989). Inhibition of fatty acid synthesis in acini may have occurred as a result of lack of substrate availability i.e glucose, and/or inhibition of lipogenic enzymes. These parameters were measured and the results are discussed in the following sections.

4.4 Effect of Okadaic Acid on Glucose Utilisation

The rate of glucose uptake by isolated mammary acini in the presence of 1 μ M okadaic acid was not significantly different from that of the control (Table 4.2). The rate of glucose uptake by acini incubated in the absence of okadaic acid is in agreement to that reported previously (Katz *et al*, 1974). Since glucose utilisation by isolated mammary acini is not affected by okadaic acid, the inhibition of fatty acid synthesis could not therefore, be accounted for

by lack of substrate supply.

The lack of any effect of okadaic acid on glucose utilisation in acini is in marked contrast to the effect observed in isolated rat adipocytes and in skeletal muscle. Okadaic acid was found to mimic the stimulatory effect of insulin on glucose transport in isolated adipocytes (Haystead *et al*, 1989) and in skeletal muscle (Tanti *et al*, 1991). This has been taken as indication that stimulation of glucose transport activity by insulin in these tissues is mediated by an increase in phosphorylation of a protein on serine/threonine residues (Haystead *et al*, 1989). In adipose tissue, insulin stimulation of glucose transport involves translocation of the glucose transporter, GLUT 4 from an intracellular compartment to the plasma membrane (Wardzala *et al*, 1978; Cushman and Wardzala, 1980; Simpson and Cushman, 1986). This is accompanied by changes in the intrinsic activity of the glucose transporter (Muhlbacher *et al*, 1988; Obermaier-Kusser *et al*, 1988; Tanti *et al*, 1989). In contrast, GLUT 1 which is also expressed in insulin-sensitive tissues can be stimulated by insulin in adipose tissue to translocate to the plasma membrane without associated changes in the intrinsic activity of the transporter (Cushman and Wardzala, 1980; Corvera *et al*, 1991). There is evidence of increased phosphorylation of insulin-regulatable glucose transporter by agents such as β -adrenergic agonists and cAMP derivatives (James *et al*, 1989; Lawrence *et al*, 1990), suggesting that the inhibition of insulin-stimulated glucose transport activity by these agents is mediated

Table 4.2 Effects of Okadaic Acid on Rate of Fatty Acid Synthesis and Glucose Uptake in Isolated Mammary Acini

Acini were incubated in the presence and absence of 1 μ M okadaic acid and the incorporation of $^3\text{H}_2\text{O}$ into fatty acids, and glucose uptake were measured as described in the experimental section. Results are expressed as $\mu\text{mol}/\text{min}$ per 100 mg defatted dry weight acini and are means \pm S.E.M. with the number of observations in parenthesis. Values that are significantly different (by Student's t-test) from the corresponding control values are shown : ** $p < 0.005$.

<u>Treatment of Acini</u>	<u>Rate of</u> <u>Fatty Acid Synthesis</u>	<u>Rate of</u> <u>Glucose Uptake</u>
Control	0.52 ± 0.09 (3)	1.32 ± 0.10 (6)
Okadaic acid	0.19 ± 0.04 (3) **	1.10 ± 0.08 (6)

via an increase in the levels of cAMP. In addition, tumour promoters such as phorbol 12-myristate 13-acetate which acts via activation of protein kinase C has been shown to stimulate glucose transport activity in adipocytes (Kirsch *et al*, 1985).

In the presence of okadaic acid, stimulation of 2-deoxyglucose transport by insulin in isolated rat adipocytes is abolished completely as a result of inhibition of the insulin-induced translocation of GLUT 4 to the plasma membrane (Corvera *et al*, 1991). Stimulation of tyrosine autophosphorylation of its receptor by insulin was not inhibited by okadaic acid. It has been suggested that inhibition of insulin stimulated glucose transport by okadaic acid occurred at a step prior to recruitment of glucose transporter to the cell surface and a protein phosphatase may be involved (Corvera *et al*, 1991). The stimulatory effect of okadaic acid on glucose transport in adipocytes is small in comparison to the effect elicited by insulin (Lawrence *et al*, 1990; Corvera *et al*, 1991). In addition, the intrinsic activity of GLUT 4 in the plasma membrane of adipocytes treated with okadaic acid is found to be lower in comparison to the activity of GLUT 4 in insulin-treated cells. Studies by Reusch *et al* (1993) showed that phosphorylation of GLUT 4 had no effect on the translocation of the transporter to the plasma membrane. This suggests that phosphorylation of GLUT 4 is associated with intrinsic activation but not translocation of the transporter to the plasma membrane.

Although rat mammary gland is an insulin-responsive tissue, the lack of effect of okadaic acid in mimicking the effect of insulin to stimulate glucose transport could be explained by the findings of Burnol *et al* (1990). These authors showed that in mammary gland, the proportion of GLUT 4 declines progressively throughout pregnancy towards parturition but that of GLUT 1 increases. At peak lactation, GLUT 1 rather than GLUT 4 forms the predominant glucose transporter in this tissue (Figure 4.3). Studies in lactating mice showed that the activity of specific carrier of glucose is increased by 40-fold from the virgin to 10-day lactating state which is not mediated by translocation of glucose transporters to the plasma membrane (Prosser, 1988). Insulin is able to cause a small and acute effect on glucose uptake by the cells from pregnant mice but not in cells from fed lactating mice (Prosser, 1988). This suggests that there may be similar changes in the proportion of GLUT 1 and GLUT 4 in mice during the transition from pregnancy to lactation, as has been observed in rats by Burnol *et al* (1988).

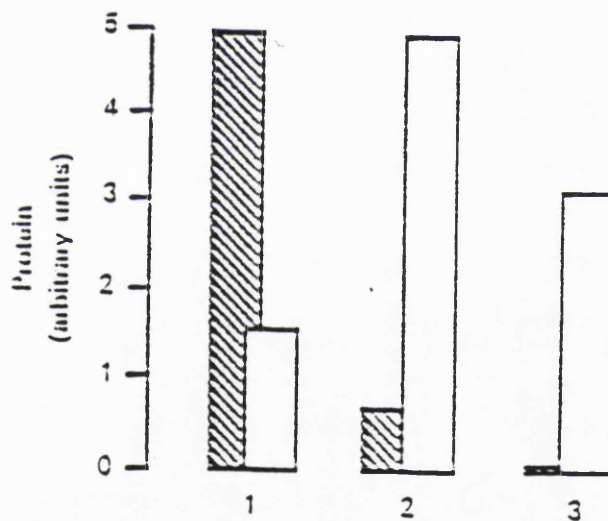
In mouse mammary epithelial cells isolated from lactating mice fasted for 16 hours, rate of glucose uptake was decreased by 3-fold. This is accompanied by parallel decreases in the V_{\max} of the transporter and the number of D-glucose-inhibitable cytochalasin-B binding sites is reduced by 70% (Prosser, 1988). Upon refeeding for 3 hours prior to isolation of the cells, the rate of glucose uptake and the number of glucose transporters in the plasma

membrane were restored (Prosser, 1988), suggesting recruitment of GLUT 1 to the plasma membrane. The decrease in the number of glucose transporters on the plasma membrane during starvation of the mice may have occurred either as a result of changes in the orientation of the transporter on the plasma membrane or translocation of the transporters to other membrane sites within the cells (Prosser, 1988). The effects of starvation and refeeding on the rate of glucose uptake observed in lactating mice (Prosser, 1988) has been shown in starved lactating rats (Threadgold and Kuhn, 1984). However, in contrast to the inhibition of glucose transport observed in mouse mammary cells (Prosser, 1988), starvation-induced inhibition of glucose transport is not apparent in acini isolated from starved lactating rats (Threadgold *et al*, 1982). This suggests that inhibition of glucose transporter activity associated with starvation of the animal was reversed during the isolation and subsequent incubation of the cells.

The lack of effect of okadaic acid on glucose utilisation in mammary acini observed in the present study suggests that GLUT 1 is not regulated by phosphorylation or at least not by phosphorylation that is reversible by the action of PP1 and PP2A.

Figure 4.3 Glucose Transporter Expression during Differentiation and Development of the Rat Mammary Gland

Quantification of Western blot analysis of GLUT 1 (unshaded area) and GLUT 4 (shaded area) during differentiation and development of rat mammary glands were from : Lane 1 : virgin rat; Lane 2 : 21-day pregnant rat; Lane 3 : 12-day lactating rat. Results are the means of two experiments. Modified from Burnol *et al* (1988).



4.5 Effect of Okadaic Acid on Acetyl-CoA Carboxylase Activity

ACC is a known substrate for PP2A (Ingebritsen *et al*, 1983). Inhibition of PP2A by okadaic acid is therefore expected to bring about hyperphosphorylation and inactivation of ACC.

Addition of okadaic acid to the incubation medium for 60 minutes strongly inhibited the activity of ACC in crude extracts of acini measured at citrate concentrations ranging from 0 to 20 mM in the presence of NaF and NaPP_i (Figure 4.4). Inactivation of ACC was rapid following addition of the toxin to the cells (Figure 4.9a). This is consistent with the acute effect of okadaic acid observed in several other studies using isolated rat hepatocytes and adipocytes. In isolated rat hepatocytes, the effect of okadaic acid on the activity of ACC was observed rapidly upon incubation of the cells with the toxin (Moore *et al*, 1991). Similarly, treatment of isolated hepatocytes with okadaic acid for 15 minutes resulted in a 2-3 fold increase in ³²P-labelling of cytosolic phosphoproteins, including ACC (Haystead *et al*, 1989). In isolated adipocytes, okadaic acid stimulated 2-deoxyglucose transport with a lag time of 5 minutes (Haystead *et al*, 1989; Lawrence *et al*, 1990) and the maximum rate of glucose uptake was attained after approximately 15 minutes (Lawrence *et al*, 1990). The acute effect of okadaic acid has also been illustrated by the studies of Rutter *et al* (1991) who showed that activity of ACC was inhibited in isolated rat

adipocytes and fat pads incubated with okadaic acid for 15 minutes.

The inhibition of ACC is in agreement with the results observed in rat epididymal fat pads and cells where the initial and total activity of ACC was shown to be inhibited by okadaic acid (Rutter *et al*, 1991). In this case, the initial activity of ACC in isolated adipocytes and fat pads was inhibited by 70% and 80% respectively by 1 μ M okadaic acid (Rutter *et al*, 1991). Haystead *et al* (1989) showed that dose dependent inhibition of ACC in hepatocytes correlated with phosphorylation of the enzyme. In the present study, the V_{\max} of ACC was decreased by 70% as a result of incubation of isolated mammary acini with okadaic acid (Table 4.3). The results thus confirm that inhibition of PP2A by okadaic acid resulted in phosphorylation and inactivation of ACC and consequently, inhibition of fatty acid synthesis.

Haystead *et al* (1989) showed that okadaic acid had no inhibitory effect when it was included in the assays of ACC. This suggests that the toxin has no direct allosteric effect on the enzyme. In order to confirm the changes in phosphorylation state of ACC in response to okadaic acid, acini were labelled with [32 P]phosphate.

Figure 4.4 Effect of Okadaic Acid on the Activity of Acetyl-CoA Carboxylase in Isolated Mammary Acini Extracts

Acini were incubated for 60 minutes in the presence and absence of 1 μ M okadaic acid. Activity of ACC in crude extracts of acini was measured as described in text. Results are expressed as nmol $\text{NaH}^{14}\text{CO}_3$ incorporated/min per mg protein and are means for 3 separate observations with S.E.M. indicated by vertical bars.

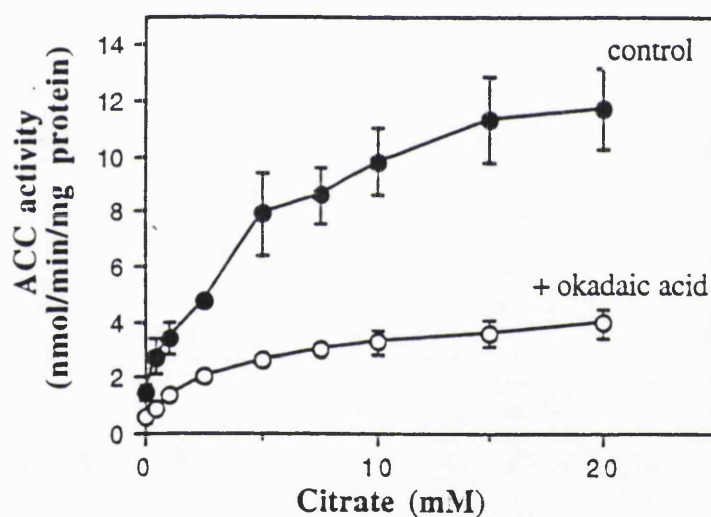


Table 4.3 Effects of Okadaic Acid on the V_{\max} and K_a Citrate of Acetyl-CoA Carboxylase Measured in Isolated Mammary Acini Extracts

For experimental details see text. Results are mean \pm S.E.M. for 3 separate observations. Values that are significantly different from the corresponding control values by Student's t-test are shown : * $P < 0.05$; ** $P < 0.005$.

	V_{\max}	K_a Citrate
	<u>(Unit/mg protein)</u>	<u>(mM)</u>
Control	14.03 \pm 2.11	2.75 \pm 0.13
Okadaic Acid	4.21 \pm 0.62 **	4.14 \pm 0.59 *

4.6 [³²P]-Labelling of Isolated Mammary Acini

Previous studies indicated that saturating concentrations of okadaic acid (1 μ M) increased the overall phosphorylation state of many phosphoproteins in isolated rat adipocytes and hepatocytes as a result of inhibition of PP1 and PP2A (Haystead *et al*, 1989, Rutter *et al*, 1991). When isolated mammary acini from lactating rats were incubated with [³²P]phosphate for 60 minutes followed by treatment with okadaic acid (1 μ M) for 45 minutes, a similar general increase in protein phosphorylation was observed. An autoradiogram of a Coomassie-stained gel of crude extracts of [³²P]-labelled acini subjected to SDS-PAGE (Figure 4.5B) showed that okadaic acid increased the amount of [³²P]phosphate incorporated into a number of phosphoproteins (lane 5). These included ATP-citrate lyase (116 kDa) and ACC (240 kDa). Upon purification of ACC on the avidin-Sepharose affinity column, it was found that in okadaic acid-treated cells, there was an increase of 32% in the amount of radioactivity in ACC (5981 cpm/mg protein) in comparison to the control (4335 cpm/mg protein). The autoradiogram of the purified enzyme (Figure 4.6B) showed that this occurs almost exclusively at 240 kDa which corresponds to the reported molecular weight of ACC (compare lanes 7 and 9). A minor band at 120 kDa which is not [³²P]-labelled is evident from the Coomassie-stained gel of the purified enzyme (Figure 4.6). This could be a proteolytic fragment of ACC or contamination by pyruvate carboxylase.

Figure 4.5 Coomassie Blue-Stained Gel and Autoradiograph of Crude Extracts of Acini from [32 P]phosphate-Labelled Acini followed by Incubation with Okadaic Acid

For experimental details see Materials and Methods. Acini were labelled with [32 P]phosphate for 60 min. before addition of 1 μ M okadaic acid. (A) Coomassie Blue-stained gel. Lanes are 1 : molecular weight marker; 2 : crude extract from control acini; 3 : crude extract from okadaic acid-treated acini. (B) Autoradiograph of Coomassie Blue stained gel. Lanes are 4 : crude extract from control acini; 5 : crude extract from okadaic acid-treated acini.

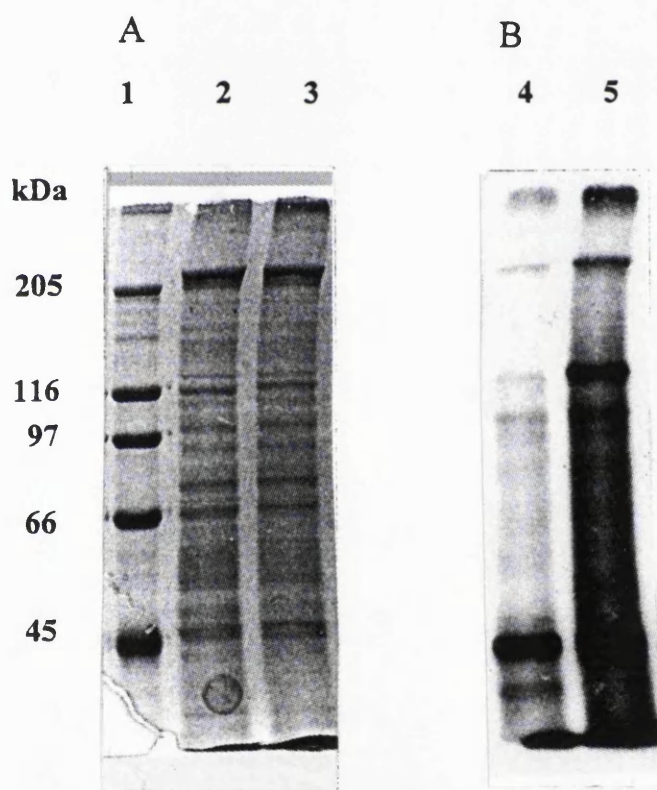
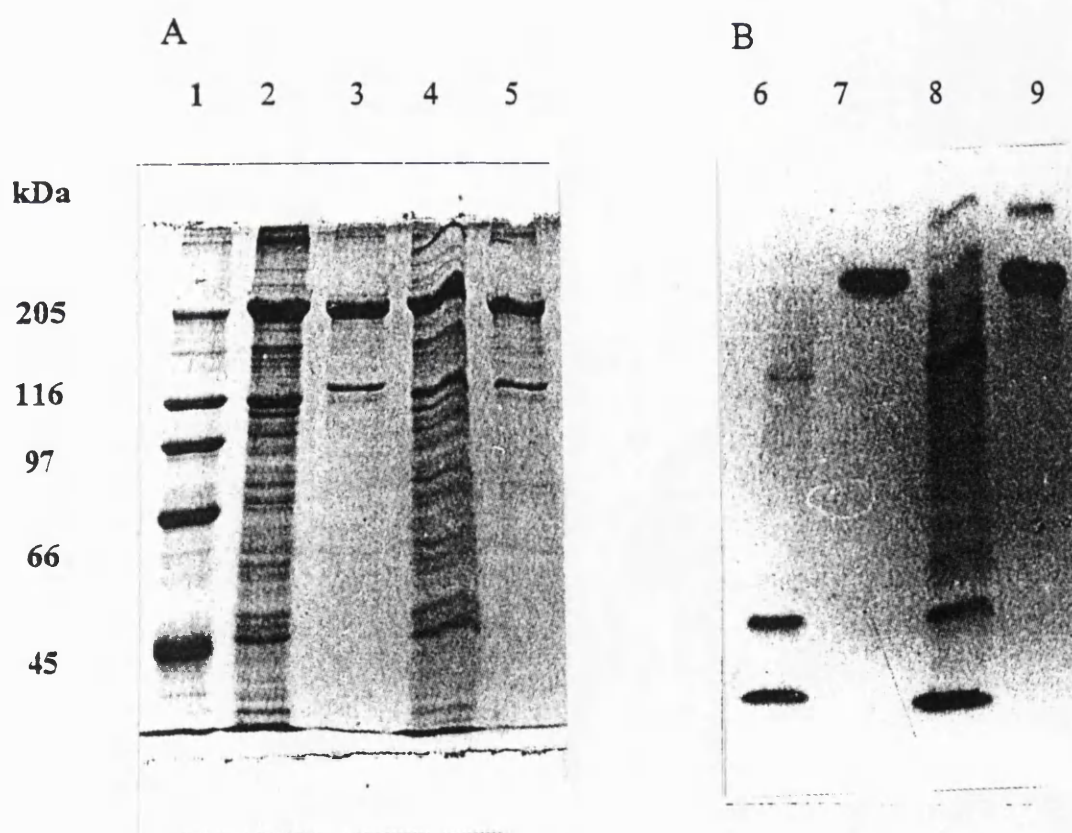


Figure 4.6 Coomassie Blue-Stained Gel and Autoradiograph of Breakthrough from Avidin-Sepharose Affinity Column and Purified Acetyl-CoA Carboxylase from [32 P]phosphate-Labelled Acini followed by Incubation with Okadaic Acid

For experimental details see Materials and Methods. (A) Coomassie Blue-stained gel. Lanes are : 1 : molecular weight markers; 2 : column breakthrough from control acini; 3 : purified ACC from control acini; 4 : column breakthrough from okadaic acid-treated acini; 5 : purified ACC from okadaic acid-treated acini. (B) Autoradiograph of Coomassie Blue-stained gel. Lanes are : 6 : column breakthrough from control acini; 7 : purified ACC from control acini; 8 : column breakthrough from okadaic acid-treated acini; 9 : purified ACC from okadaic acid-treated acini.



The results of the ^{32}P -labelling study of acini confirm that okadaic acid increased ^{32}P incorporation into ACC. Although the activity of purified ACC was not measured, inhibition of the enzyme activity in response to okadaic acid is highly significant in crude extracts of acini (Figure 4.5). This could only be accounted for by phosphorylation of ACC as a result of inhibition of PP2A by okadaic acid.

4.7 Effect of Okadaic Acid on AMP-activated Protein Kinase Activity

AMP-PK plays a central role in the protein kinase cascade that regulates fatty acid and cholesterol synthesis *in vivo*. Preliminary studies suggest that in acini incubated with 1 μM okadaic acid over a 60 minute period, there was an increase in the activity of AMP-PK in crude extracts of acini (Figure 4.7). The activity of AMP-PK showed a transient increase during the first 15 minute incubation period of acini with okadaic acid. This was followed by a decrease in the activity of the enzyme throughout the remaining incubation period. This correlated with the sharp decrease in the activity of ACC during the first 15 minute incubation with okadaic acid followed by a slower, steady decrease, measured in crude extracts of acini at 10 mM citrate (Figure 4.9a). This analysis of AMP-PK activity is however inconclusive, as linear phosphorylation of AMP-PK by $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ with time was not always reproducible. This was most likely to be due to the presence of inhibitory contaminants in the crude extracts

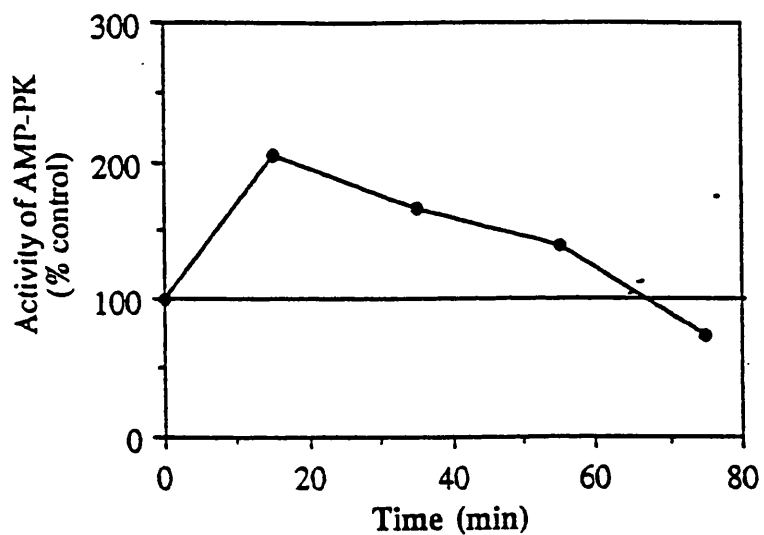
of acini. AMP-PK is a substrate of PP2C (Haystead *et al*, 1990a) which is insensitive towards inhibition by okadaic acid. If the activity of the kinase does indeed increase by treatment with okadaic acid, this may have occurred as a result of phosphorylation of another upstream component of the AMP-PK cascade, for example AMP-PK kinase (Carling *et al*, 1987). Further measurement using a reproducible assay method will be required to determine the activity of AMP-PK in response to okadaic acid.

4.8 Effect of Okadaic Acid on the Flux of Glucose Through the Lipogenic Pathway

To determine the flux of glucose through the lipogenic pathway in response to okadaic acid, the accumulation of lactate and pyruvate was determined in the presence and absence of 1 μ M okadaic acid. As shown in Figure 4.8a, there was a significant increase in the level of lactate in acini incubated with okadaic acid with a sharp rise of this metabolite occurring after 45 minute incubation. Accumulation of pyruvate in okadaic acid-treated acini was less significant (Figure 4.8b). Since accumulation of lactate and pyruvate has always been taken as a measure of the flux through the PDH step and hence the activation state of PDH (Robinson and Williamson, 1977a; 1978b; Munday and Williamson, 1981), the activity of this mitochondrial enzyme in response to okadaic acid was determined.

Figure 4.7 Effect of Okadaic Acid on the Activity of AMP-activated Protein Kinase in Isolated Mammary Acini

Activity of AMP-PK was measured as described in Materials and Methods. The results are expressed as a percentage of AMP-PK activity measured at 0 minute of incubation with 1 μ M okadaic acid for one experiment.

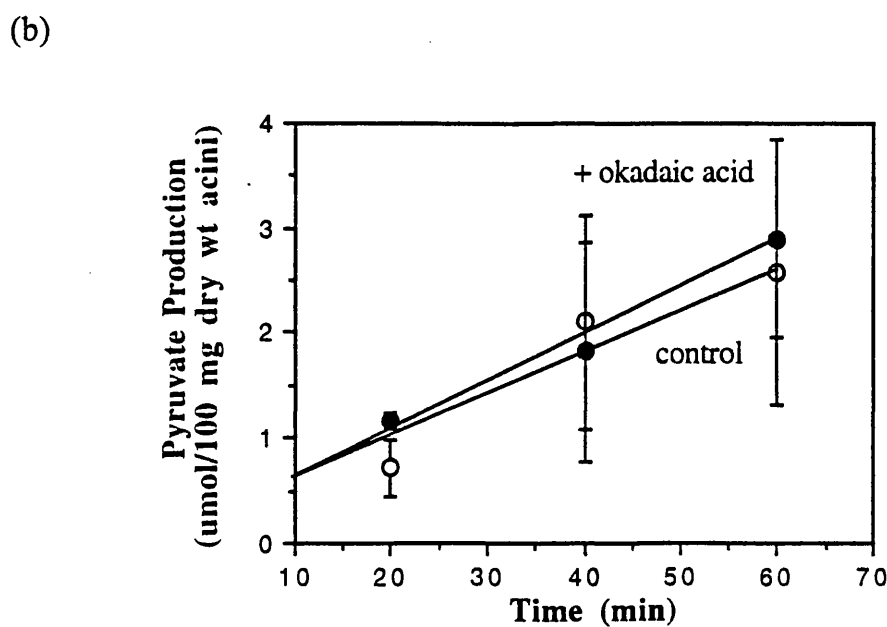
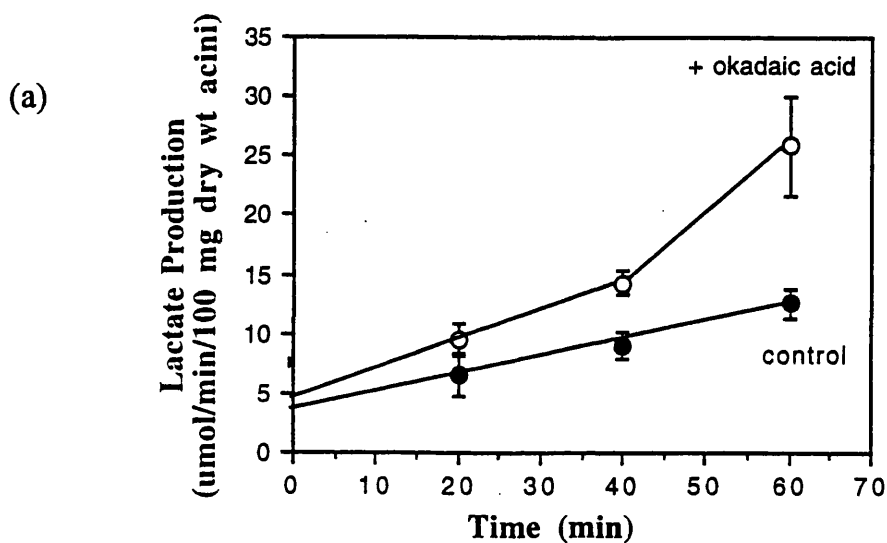


4.9 Effect of Okadaic Acid on Pyruvate Dehydrogenase Activity

Figure 4.9b shows the activity of PDH over a 75 minute incubation in the presence of 1 μ M okadaic acid. The activity of PDH remained constant during the first 15 minute incubation period of acini with okadaic acid and was not significantly different from that of the control. However, after 15 minutes, PDH activity began to decline steadily and maximum inhibition was observed after 45 minutes incubation (Figure 4.9b). This correlated with the sharp rise in the accumulation of lactate (Figure 4.8a). Since okadaic acid has no inhibitory effect on PDH phosphatase (Cohen *et al*, 1990), direct phosphorylation and inactivation of PDH in response to okadaic acid would not be expected. However, inhibition of PDH occurs as a consequence of activation of PDH kinase by changes in the ratio of metabolite concentrations. Increased ratios of [acetyl-CoA]/[CoA] and [NADH]/[NAD⁺] are known to stimulate the activity of PDH kinase (Randle, 1986). Increased [acetyl-CoA]/[CoA] ratio might occur in response to inhibition of ACC by okadaic acid. Careful comparison of the time courses of inactivation of PDH and ACC activities in response to okadaic acid suggests that inactivation of ACC precedes inactivation of PDH (Figure 4.9a and b). The effect of okadaic acid on the activity of PDH in acini is in contrast with the results reported for rat fat pads and cells where PDH activity was unaffected by okadaic acid (Rutter *et al*, 1991). This could be due to the fact that changes in the metabolite levels are not sufficient to activate PDH kinase

Figure 4.8 Effects of Okadaic Acid on Lactate and Pyruvate Accumulation in Isolated Mammary Acini

Acini were incubated in the presence and absence of 1 μ M okadaic acid. Results are expressed as μ mol/per 100 mg defatted dry weight acini and are mean values for at least four separate observations with S.E.M indicated by vertical bars.



in their studies. In this case, although okadaic acid has no inhibitory effect on PDH activity, it has been shown to inhibit insulin-stimulated PDH activity in rat epididymal fat pads and cells (Rutter *et al*, 1991). Insulin stimulates the flux from pyruvate to acetyl-CoA. However, since the activity of ACC was inhibited by okadaic acid, this allows sufficient accumulation of acetyl-CoA to activate PDH kinase, thus preventing stimulation of PDH by insulin.

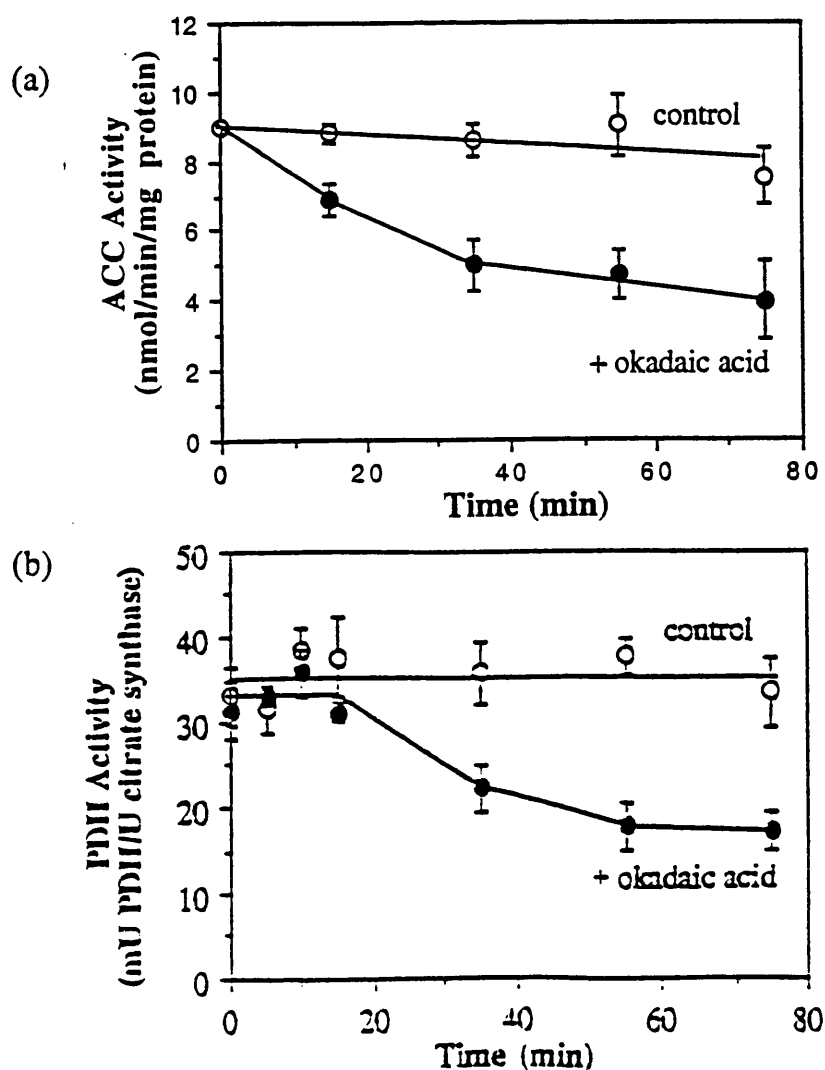
Inhibition of fatty acid synthesis in okadaic acid-treated cells is therefore a consequence of inactivation of ACC and PDH. We are able to demonstrate a continued relationship between ACC and PDH in mammary acini and that the activity of these two enzymes are closely linked. In this case, inactivation of ACC appears to induce PDH inactivation.

4.10 Discussion

The results from the present study indicated that reversible phosphorylation is an important short term mechanism in the regulation of lipogenesis in isolated lactating rat mammary acini. Using okadaic acid to inhibit PP1 and PP2A in isolated lactating rat mammary acini, we were able to show that ACC became phosphorylated and inactivated (Figure 4.4). This is most certainly the result of inhibited PP2A activity and also possibly as a result of increased activity of AMP-PK (Figure 4.7). This has been shown by the

Figure 4.9 Effects of Okadaic Acid on the Activities of Acetyl-CoA Carboxylase and Pyruvate Dehydrogenase in Isolated Mammary Acini

Acini were incubated in the presence and absence of 1 μM okadaic acid. Activities of ACC and PDH were measured as described in Materials and Methods. Results are expressed as nmol $\text{NaH}^{14}\text{CO}_3$ incorporated/min per mg protein for ACC and munits PDH/unit citrate synthase for PDH, and are means for three separate cell preparations with S.E.M. shown by vertical bars.



purification of ACC to homogeneity from [³²P]phosphate-labelled cells which revealed that ACC from okadaic acid-treated cells has a higher phosphate content (expressed as cpm/mg protein). Inhibition of PP1 and PP2A by okadaic acid also resulted in increased phosphorylation of ATP-CL (116 kDa) (Figure 4.5). However, phosphorylation of rat mammary gland ATP-CL is known to have no effect on the activity of this enzyme (Guy *et al*, 1981).

AMP-PK has been shown to be the physiological protein kinase of ACC (Hardie, 1989). Inhibition of PP2A by okadaic acid allows phosphorylation of ACC by AMP-PK to proceed unantagonised. Evidence suggests that AMP-PK is a substrate for PP2C (Haystead *et al*, 1989) and okadaic acid has been shown to have no effect on the activity of AMP-PK in hepatocytes (Moore *et al*, 1991), consistent with the lack of inhibitory effect of okadaic acid on PP2C. Although the results from the present study suggested that the activity of AMP-PK in okadaic acid-treated cells might be increased, further experiments will be necessary to confirm such changes.

In the present study, the inhibition of ACC by okadaic acid resulted in the accumulation of acetyl-CoA as the conversion of this metabolite to malonyl-CoA is reduced. The activity of PDH is allosterically inhibited by an increase in the levels of acetyl-CoA and since PDH phosphatase is insensitive to okadaic acid (Haystead *et al*, 1989), inhibition of PDH was therefore induced by

inactivation of ACC. The increase in the ratio of [acetyl-CoA]/[CoA] is able to stimulate PDH kinase which is an integral component of the PDH complex, resulting in increased phosphorylation and inactivation of PDH (Figure 4.9b). This was reflected by the accumulation of lactate in the cells (Figure 4.8a). A varied relationship is known to exist between the activity of PDH and ACC under different physiological conditions. In the lactating rats subjected to starvation for 6 hours, the activity of PDH in the freeze-clamped tissue was inhibited by 79% (Hagopian *et al*, 1991) whilst the activity of ACC was not rapidly inactivated and was refractory to inhibition with no significant change in expressed activity (Munday and Hardie, 1986; Williamson *et al*, 1983; Hagopian *et al*, 1991). Similarly, inhibition of PDH in isolated mammary acini incubated with 2 mM acetoacetate occurred without the corresponding changes in the activity of ACC (Chapter 3). In contrast, the results from the present study showed that PDH inactivation occurred as a result of inhibition of ACC. These observations are therefore indicative that different physiological signals are involved in the regulation of PDH and ACC. In short term starvation of the lactating rats (6 hours), low circulating levels of plasma insulin are probably the signal mediating the effects of starvation as the concentrations of this hormone fall by 48% during 6 hour starvation (Jones *et al*, 1984a). In the case of acetoacetate, metabolism of acetoacetate leads to an increase in the cytoplasmic pool of acetyl-CoA. The increased ratio of [acetyl-CoA]/[CoA] activated PDH kinase which in turn phosphorylates and inactivates PDH. Inhibition of PDH in

response to okadaic acid on the other hand, occurred as a result of inhibition of ACC which led to accumulation of acetyl-CoA.

Despite the significant inhibition of the activities of PDH and ACC by okadaic acid, no changes in the rate of glucose utilisation by acini were observed (Table 4.2). This suggests that the effect of the toxin is not mediated via inhibition of glucose transport. The lack of stimulatory or inhibitory effect of okadaic acid on glucose transport suggests that GLUT 1, unlike GLUT 4 in insulin-sensitive tissues, is not stimulated by phosphorylation or at least not by phosphorylation that is reversed by PP1 and PP2A. In adipocytes, stimulation of glucose transport by okadaic acid suggests that phosphorylation of serine/threonine residues is involved (Haystead *et al*, 1989). However, phosphorylation of GLUT 4 transporter by okadaic acid in adipose tissue is not associated with changes in the number of the transporters on the plasma membrane (Reusch *et al*, 1993). This suggests that phosphorylation of GLUT 4 in insulin-sensitive tissues is associated with intrinsic activation of the transporters.

Although we were able to show that PP2A is the predominant enzyme in mammary tissue, the current studies do not reveal that inhibition of PP2A is the sole reason for inhibition of fatty acid synthesis. This is because both PP1 and PP2A have intracellular concentrations of 0.1 - 1.0 μM which are inhibited

by similar levels of okadaic acid (Cohen *et al*, 1989).

In this study, we have utilised okadaic acid as an unique tool in quantifying protein phosphatases and for studying the role of reversible phosphorylation in the control of lipogenesis in mammary tissue. We have been able to conclude that fatty acid synthesis in the lactating rat mammary gland, as in liver and adipose tissue is controlled by reversible phosphorylation and that the AMP-PK cascade is functional and physiologically important.

CHAPTER 5

EFFECTS OF POLYUNSATURATED FATTY ACIDS ON LIPOGENESIS IN ISOLATED MAMMARY ACINI

5.1 Introduction

The low incidence of hyperlipidaemia and coronary heart disease in Greenland Eskimos consuming a diet rich in marine fish oils (Dyerberg *et al*, 1978) has stimulated interest in the investigation of potential protective factors present in the diet, namely the n-3 polyunsaturated fatty acids.

There are three major classes of unsaturated fatty acids: n-9 monounsaturated, n-6 polyunsaturated and n-3 polyunsaturated fatty acids (for review Berr *et al*, 1993). n-9 monounsaturated fatty acid such as oleic acid (C18:1) can be synthesised from saturated fatty acids. n-6 polyunsaturated fatty acids have two unsaturated carbon bonds of which the first double bond from the methyl end of the fatty acid is at the sixth carbon atom. n-6 polyunsaturated fatty acids are the principal polyunsaturated fatty acid from plant seeds. Since mammals lack the enzymes to introduce double bonds at carbon atoms beyond C-9 in the fatty acid chain, n-6 polyunsaturated fatty acids such as linoleic acid (C18:2) cannot be synthesised endogenously (Kelly, 1984). This fatty acid is

therefore an essential fatty acid which must be supplied in the diet. In addition, n-6 polyunsaturated fatty acids are important for the synthesis of a variety of other unsaturated fatty acids.

n-3 polyunsaturated fatty acids such as eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6) have their first double bond at the third carbon atom from the methyl end of the molecule. The parent fatty acid of EPA and DHA is α -linoleic acid (C18:3). This is an essential fatty acid present mainly in marine phytoplankton and in some plant seed oils (Figure 5.1). It is derived from elongation of linoleic acid which takes place in the chloroplast. Elongation and desaturation of dietary α -linoleic acid to form EPA and DHA can take place in the endoplasmic reticulum membrane in humans and animals. However, the process is slow and rather limited (Garg *et al*, 1988) and with ageing and possibly certain diseases, the endogenous synthesis of n-3 polyunsaturated fatty acids is abolished (Tinoco, 1982). Marine phytoplankton and zooplankton are rich sources of n-3 polyunsaturated fatty acids. The n-3 polyunsaturated fatty acids from these sources enter the food chain after consumption by fish. Therefore, marine fish oils are rich in n-3 polyunsaturated fatty acids (Feldt-Rasmussen *et al*, 1986).

Whilst fatty acids are synthesised in the cytosol, they are metabolised via the β -oxidation pathway which takes place in the mitochondria. The physical

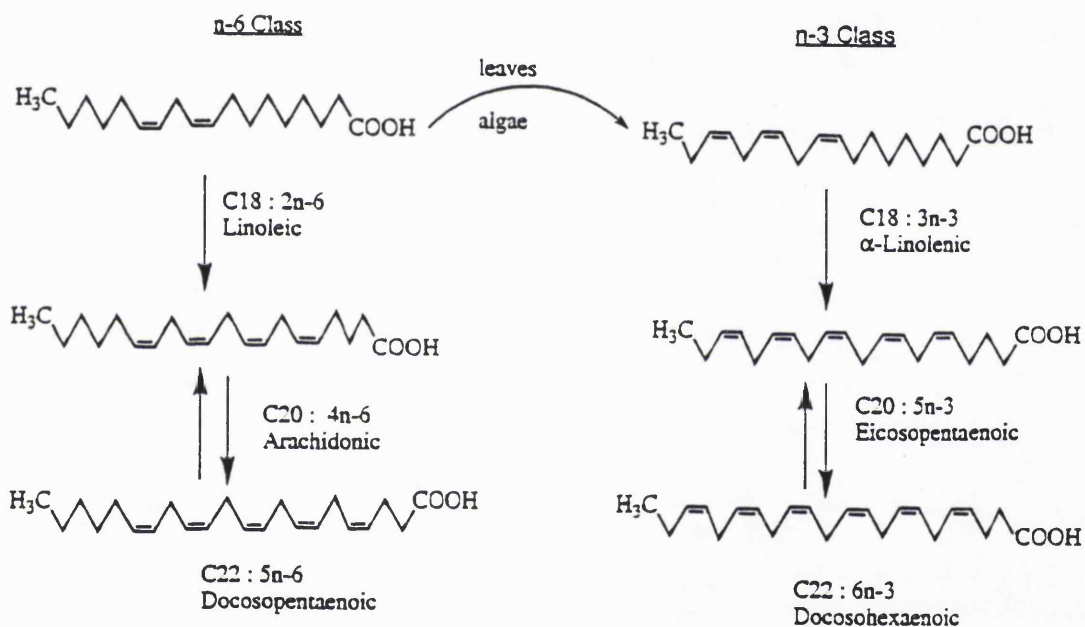
properties of fatty acids are dependent on their chain length and on their degree of unsaturation. Unsaturated fatty acids have a lower melting point than saturated fatty acids of the same length. Fatty acids with short chain length also have a lower melting point.

Fatty acids play an essential physiological role. They are structurally important in the formation of phospholipids and glycolipids present in cell membranes and are an important energy source in the form of triglycerides. In addition, fatty acid derivatives are important as hormones (eicosanoids) and intracellular messengers (eg diacylglycerol). Polyunsaturated fatty acid such as arachidonic acid (C20:4) is an important precursor for the synthesis of thromboxane and prostaglandins which are involved in a wide variety of physiological processes such as inflammation and thrombosis.

Extensive animal and clinical studies have now provided convincing evidence that n-3 polyunsaturated fatty acids may prevent or improve various pathological conditions, including atherosclerotic vascular disease (Kinsella *et al*, 1990) and hyperlipidaemia (Leaf and Weber, 1988). The public has been advised to increase their consumption of fish, particularly those with high n-3 fatty acid content such as mackerel and herring. Many individuals with raised triglyceride levels appear to respond to an increase proportion of dietary fat as polyunsaturated fat, particularly the n-3 series by displaying reduced blood

Figure 5.1 Relationship of n-6 and n-3 Polyunsaturated Fatty Acids

Modified from Leaf and Weber (1988). The transformation of n-6 linoleic acid by desaturation to form n-3 α -linolenic acid occurs only in leaves and algae and is indicated by the arching arrow. The formation of n-3 polyunsaturated acids from α -linolenic acid depicted by the arrows incorporates two or more reaction steps.



triglyceride levels.

The lipid lowering effects of marine fish oils have been illustrated by studies using MaxEPA. This is a natural triglyceride marine oil containing high levels of eicosapentaenoic acid (EPA, 18%) and docosahexaenoic acid (DHA,

12%). In a controlled double blind study carried out by Miller *et al* (1988) in hypertriglyceridaemic subjects, daily intake of MaxEPA (10 x 1 g capsules) was found to significantly decrease plasma triglyceride levels by 33% after one month. On the other hand, in subjects taking olive oil (placebo), no significant reduction in plasma triglyceride levels was observed. In the study of Simons *et al* (1985), dietary MaxEPA at 6 g/day (equivalent to 1.1 g EPA) given to hyperlipidaemic patients for 3 months was found to reduce plasma triglycerides by 33% in comparison to the placebo study which use olive oil that contains 78% oleic acid. VLDL cholesterol was decreased by 27% and HDL cholesterol was increased by 5%. In addition, the hypotriglyceridaemic effect of MaxEPA was found to be dose-related. This was shown in patients taking 16 g/day of MaxEPA (equivalent to 2.9 g EPA) where plasma triglycerides were reduced to 58%. Studies by Sanders *et al* (1985) showed that in hypertriglyceridaemic patients treated for 4 weeks with daily supplements of MaxEPA (15 g), a decrease of 26% in plasma triglyceride concentrations was observed.

Several other studies on the effects of polyunsaturated fatty acids on human subjects have also provided convincing evidence of the lipid-lowering effects of these fatty acids. In the studies of Schectman *et al* (1989), plasma triglyceride levels were significantly decreased by 33% in hypertriglyceridaemic patients receiving 4 g daily dose of n-3 polyunsaturated fatty acid of fish origin in comparison to safflower oil treatment. When fish oil at a daily dose of

7.5 g was given, a greater decrease (53%) in plasma triglyceride levels was observed. VLDL cholesterol levels were reduced by approximately 6% during fish oil treatment. In hyperlipidaemic subjects receiving 23% of their daily energy needs in the form of fish oil for over 2 weeks, VLDL triglyceride was found to be reduced to normal values (Nestel *et al*, 1984).

Studies by Harris *et al* (1983) showed that metabolic differences exist between dietary vegetable oils and fish oils. In their study carried out in normalipidaemic subjects, both salmon and safflower oils lowered plasma cholesterol by 14% but only salmon oil concomitantly decreased plasma triglyceride by 38%. The hypolipidaemic effects of dietary fish oils appear to be more potent in individuals with raised plasma lipid levels. Studies by Phillipson *et al* (1985) showed that dietary fish oils given to hyperlipidaemic subjects for 4 weeks lead to a decrease in plasma cholesterol, triglyceride and VLDL by 27%, 64% and 75%, respectively.

The long term lipid-lowering effects of polyunsaturated fatty acids have also been demonstrated in animal studies. In isolated hepatocytes prepared from rats fed a standard chow diet supplemented with 15% unsaturated fat in the form of corn oil for 7 days, hepatic lipogenesis and cholesterol synthesis were inhibited by 60% and 62%, respectively, in comparison to rats fed standard chow diet (Gibbons and Pullinger, 1986). In addition, VLDL triglyceride

secretion was reduced by 64% (Gibbons and Pullinger, 1987). In rats fed a standard chow diet supplemented with 8% (w/w) fish oil for 10 days, it was found that the rate of triglyceride secretion in perfused livers was reduced by about 50% in comparison to rats fed safflower oil (Topping *et al*, 1987).

Short term effects of n-3 polyunsaturated fatty acids on lipid secretion have also been studied. In isolated rabbit hepatocytes incubated with 800 μ M of EPA for 5 hours, VLDL secretion measured by [3 H]glycerol incorporation into VLDL triglyceride was decreased by over 50% (Benner *et al*, 1990). Willumsen *et al* (1993) showed that hepatic triglyceride was decreased by 50-60% after one day in rats fed EPA (1500 mg/kg body weight).

It is well documented that inhibition of fatty acid synthesis is a general consequence of adaptation to rats fed high fat diets. Studies by Grigor and Warren (1980) showed that in lactating rats fed a high fat diet of peanut oil (C18:1) and coconut oil (containing 45% lauric acid, C12:0), the rate of fatty acid synthesis in the mammary gland was reduced by 78% and 54% respectively in comparison to rats fed fat-free diets. Similar observations were found in the livers of rats fed peanut oil and coconut oil where the rate of fatty acid synthesis was decreased by 75% and 68%, respectively. Suppression of fatty acid synthesis is especially pronounced with n-3 polyunsaturated fatty acid in comparison to n-6 polyunsaturated fatty acids. As shown by the study of

Topping *et al* (1987), the rate of fatty acid synthesis was 37% lower in livers of rats fed marine fish oil (8% w/w) for 10 days than in rats fed safflower oil. The rate of hepatic lipogenesis was also found to decrease by 70% in response to intragastric intubation of EPA for 4 days whereas only a 35% decrease was observed in rats intubated with linoleic acid (Iritani *et al*, 1980).

In addition to the lipid-lowering effect, n-3 polyunsaturated fatty acids have a variety of other biological effects. These include their effects on platelet function and inflammation which contribute towards their anti-thrombotic effect. Arachidonic acid is the predominant polyunsaturated fatty acid found in phospholipids in cell membranes. It is released from phospholipids of platelets and endothelial cells for the synthesis of thromboxane and prostaglandin in response to various stimuli. Thromboxane and prostaglandin have antagonising effects, with thromboxane A₂ (TxA₂) being a potent vasoconstrictor and platelet aggregating factor and prostaglandin I₂ (PGI₂) being a potent vasodilator and anti-aggregatory factor. Dietary n-3 polyunsaturated acids such as EPA inhibit the synthesis of arachidonic acid from linoleic acid and compete with arachidonic acid for incorporation into cell membrane phospholipids (Goodnight *et al*, 1982). This leads to a decrease in plasma and cellular levels of arachidonic acid (Siess *et al*, 1980). In addition, EPA competes with arachidonic acid as the substrate for the cyclo-oxygenase enzymes, therefore inhibiting the production of TxA₂ by platelets and the small amounts of TxA₂ produced are

physiologically inactive (Needleman *et al*, 1979). In endothelial cells, the production of PGI₂ is not significantly inhibited. In addition, arachidonic acid is the precursor of the leukotriene-4 series that are chemo-attractants to monocytes and leucocytes but EPA produces the leukotriene-5 series which are less active and have antagonising effects to the 4 series. The net results of dietary n-3 polyunsaturated fatty acids are therefore to produce alternative signal molecules (eicosanoids, prostaglandins etc) with very little activity so that n-3 polyunsaturated fatty acids are in fact anti-inflammatory and anti-thrombotic.

Although the reduction of plasma lipid concentrations by dietary fish oil is a well-known effect, the exact mechanism of this effect is not fully understood. Several potential mechanisms of n-3 polyunsaturated fatty acids have been proposed including reduced hepatic lipogenesis (Iritani *et al*, 1980; Gibbons and Pullinger, 1986; Topping *et al*, 1987); decreased hepatic VLDL synthesis and secretion with a resultant decrease in LDL production (Philipson *et al*, 1985) and increase plasma clearance of VLDL (Harris *et al*, 1988). Since the lactating rat mammary gland exhibits an extremely high rate of fatty acid synthesis, acini isolated from this tissue is therefore an ideal preparation to be used for studying the effects of polyunsaturated fatty acids on the lipogenic pathway.

The aim of the present study was to examine the mechanism of action of

polyunsaturated fatty acids on the pathway of lipogenesis and to compare the effects of pure EPA with that of linolenic and linoleic acids.

5.2 Effect of Polyunsaturated Fatty Acids on Lipogenesis

Incubations of isolated mammary acini with EPA lead to inhibition of fatty acid synthesis as measured by the incorporation of $^3\text{H}_2\text{O}$ into fatty acids (Figure 5.2). This occurred in a dose-dependent manner and the rate of fatty acid synthesis was almost completely abolished (98%) by 1 mM EPA with an IC_{50} of approximately 0.8 mM.

Linolenic and linoleic acid at 1 mM concentrations also inhibited rates of fatty acid synthesis in acini by 43% and 38% respectively (Table 5.1). The inhibition of lipogenesis by linolenic and linoleic acid is consistent with that demonstrated previously by Abraham *et al* (1977) and Clarke *et al* (1976) in rat adipocytes and hepatocytes. In addition, it was found that feeding mice a fat-free diet supplemented with 2% linoleic acid inhibits hepatic fatty acid synthesis by 70% within 2 days (Allmann and Gibson, 1965). However, inclusion of palmitate or oleate in the diet had no such effect. *In vivo*, Clarke *et al* (1977) showed that feeding rats with a high carbohydrate, fat free diet supplemented with 3% linoleate and linolenate for 7 days leads to inhibition of hepatic fatty acid synthesis by 54% and 60% respectively. Studies by Gibbons and Pullinger

(1986) showed that in hepatocytes isolated from rats fed corn oil (15%), the rate of fatty acid synthesis is decreased by 60%. In the present study, the inhibition of lipogenesis by linolenic and linoleic acid is not as great as EPA at 1 mM concentration.

The inhibition of lipogenesis by EPA in acini is in agreement with observations in rat hepatocytes. In cultured rat hepatocytes incubated with 1 mM EPA, triglyceride secretion was inhibited by 60% (Nossen *et al*, 1986). In normolipidaemic rats fed EPA, the triglyceride-lowering effect occurred in a time-dependent manner over a 15 days period (Willumsen *et al*, 1993). In addition, Topping *et al* (1987) showed that feeding rats with diet supplemented with 8% (w/w) marine fish oil leads to inhibition of hepatic lipogenesis by 37% in comparison to rats fed safflower oil. An inhibitory effect of EPA on fatty acid synthesis has also been reported by Iritani *et al* (1980) in rats intubated with EPA for 4 days. In this case, hepatic lipogenesis was inhibited by 70%.

It is evident from the above findings that the substantial triglyceride-lowering effect of polyunsaturated fatty acids is brought about at least in part by inhibition of fatty acid synthesis. The magnitude is dose-related with n-3 polyunsaturated fatty acids being more effective than n-6 polyunsaturated fatty acids. The greater potency of EPA in inhibiting fatty acid synthesis is probably due to the fact that it is more unsaturated in comparison to linolenic acid, as

agreed by Yang and Williams (1978) who showed that C₂₀ unsaturated fatty acid is more potent than C₁₈ unsaturated fatty acid in inhibiting the incorporation of [1-¹⁴C]acetate or ³H₂O into fatty acids.

Acute (Agius and Williamson, 1980; Bussmann *et al*, 1984) and chronic (Agius *et al*, 1981a; Grigor and Warren, 1980; Munday and Williamson, 1987) feeding of a high fat diet is known to result in inhibition of fatty acid synthesis in lactating rat mammary gland. Studies by Agius *et al* (1980) have shown that in lactating rats fed cafeteria diet (consists of cheese crackers, chocolate chip cookies and potato chips) for a period of 10-14 days, a decrease in the rate of fatty acid synthesis by 61% in comparison to the rate of fatty acid synthesis of rats fed standard chow diet was observed. Similarly, studies by Munday and Williamson (1987) showed that in rats fed high fat cheese crackers throughout lactation, the *in vivo* rate of fatty acid synthesis is 50% lower relative to the control. The *in vivo* effect of high fat diet on fatty acid synthesis is found to persist in isolated acinar preparations where fatty acid synthesis was decreased by 40% (Munday and Williamson, 1987).

5.3 Effects of Polyunsaturated Fatty Acids on Glucose Utilisation

To determine if the observed inhibition of fatty acid synthesis could be due to decreased substrate availability, glucose uptake by isolated acini

Figure 5.2 Effect of EPA on the Rate of Fatty Acid Synthesis in Isolated Mammary Acini

Acini were incubated for 60 min in the presence and absence of EPA and the incorporation of $^3\text{H}_2\text{O}$ into fatty acids was measured as described in the experimental section. Results are expressed as $\mu\text{mol } ^3\text{H}_2\text{O}$ incorporated/min per 100 mg defatted dry weight acini and are means for 3 preparations with S.E.M. indicated by vertical bars.

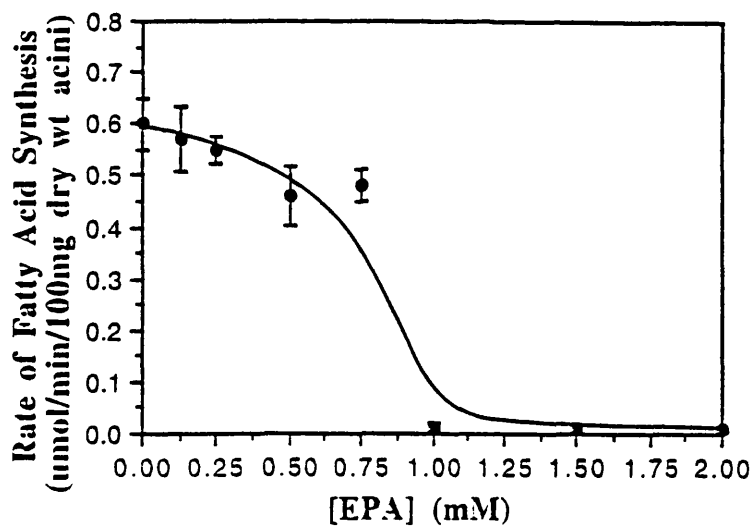


Table 5.1 Effects of PUFA on the Rate of Fatty Acid Synthesis in Isolated Mammary Acini

Acini were incubated for 60 min in the presence and absence of 1 mM EPA, linolenic and linoleic acid and the incorporation of $^3\text{H}_2\text{O}$ into fatty acids was measured as described in the experimental section. Results are expressed as $\mu\text{mol } ^3\text{H}_2\text{O}$ incorporated/min per 100 mg defatted dry weight acini and are means \pm S.E.M. for 4 preparations. Values that are significantly different (by Student's t-test) from control value are shown : * $P < 0.05$; ** $P < 0.005$.

	<u>Rate of Fatty Acid Synthesis</u>
Control	0.60 ± 0.05
EPA	0.02 ± 0.01 **
Linoleic Acid	0.41 ± 0.04 *
Linolenic Acid	0.34 ± 0.02 *

incubated with EPA, linoleic and linolenic acid was measured.

Over the range of EPA concentrations that inhibited fatty acid synthesis, no effect on glucose uptake by the cells was observed (Figure 5.3). Similarly, linoleic and linolenic acid at 1 mM concentration did not inhibit glucose utilisation by the acini (Table 5.2). Robinson and Williamson (1978a) showed that in isolated mammary acini, the monounsaturated fatty acid, oleate, has very little inhibitory effect on glucose utilisation by the cells and these results confirm that this is also true for polyunsaturated fatty acids.

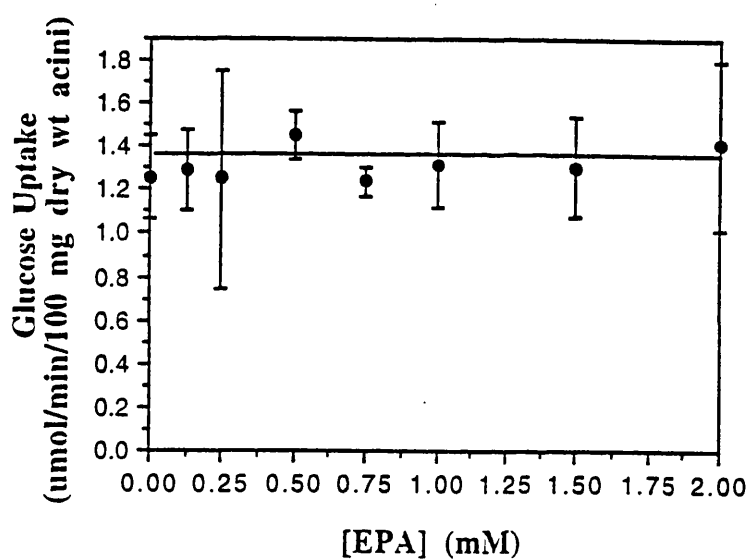
Since glucose utilisation by acini is not affected by any of the polyunsaturated fatty acids used in the study, inhibition of fatty acid synthesis is therefore not a consequence of lack of substrate supply.

5.4 Effects of Polyunsaturated Fatty Acids on Acetyl-CoA Carboxylase Activity

ACC is the rate-limiting step in the pathway of lipogenesis. Acute (Agius and Williamson, 1980) and chronic feeding of lactating rats on high fat diets (Agius *et al*, 1980; Grigor and Warren, 1980; Munday and Williamson, 1987) which results in inhibition of mammary lipogenesis is accompanied by inhibition of ACC (Munday and Williamson, 1987). This is reported to be due to increased

Figure 5.3 Effect of EPA on the Rate of Glucose Utilisation in Isolated Mammary Acini

Acini were incubated for 60 min in the presence and absence of EPA and glucose uptake was measured as described in the experimental section. Results are expressed as $\mu\text{mol}/\text{min}$ per 100 mg defatted dry weight acini and are means for 3 preparations with S.E.M. indicated by vertical bars.



**Table 5.2 Effects of PUFA on the Rate of Glucose Utilisation in
Isolated Mammary Acini**

Acini were incubated for 60 min in the presence and absence of 1 mM EPA, linoleic and linolenic acid and glucose uptake was measured as described in the experimental section. Results are expressed as $\mu\text{mol/min}$ per 100 mg defatted dry weight acini and are means \pm S.E.M. for 4 preparations.

	<u>Rate of Glucose Utilisation</u>
Control	1.26 ± 0.19
EPA	1.22 ± 0.11
Linoleic Acid	1.39 ± 0.17
Linolenic Acid	1.17 ± 0.25

phosphorylation of ACC (Munday and Hardie, 1987b). Purified mammary ACC from rats fed high fat diets showed a 64% decrease in V_{\max} with an increase in K_a for citrate of 36% and an increased phosphate content in comparison to chow fed rats (Munday and Hardie, 1987b). The effect of a high fat diet on lactating rat mammary gland can be relieved by insulin either administered *in vivo* to the rat (Agius *et al*, 1981) or added to the incubation medium of isolated acini *in vitro* (Munday and Williamson, 1987). The effect of insulin is accompanied by an increase in the activity of ACC to the control chow fed value (Munday and Williamson, 1987). ACC is therefore thought to be the site that mediates the inhibition of fatty acid synthesis in lactating rat mammary gland in response to high fat feeding.

In response to prolonged feeding of rats (10 days) a diet high in saturated fat (30% w/w beef tallow) or polyunsaturated fat diet (sunflower oil), Davies *et al* (1992) showed that the total activity of hepatic ACC was decreased by more than 90%. This has been shown to occur as a result of a decrease in the ACC mRNA. In addition, there was an increase in the phosphorylation state of ACC, reflected by a decrease in the expressed : total activity ratio in rats fed high fat diets (Davies *et al*, 1992). In the same study, it was also reported that high fat feeding of rats prevented the dephosphorylation of hepatic ACC that normally occurs during dark period when rats eat chow diet. This suggests that a possible mechanism of ACC inactivation in fat feeding is the reactivation of

dephosphorylated AMP-PK by kinase kinase which has been shown to be stimulated by palmitoyl-CoA (Carling *et al*, 1989). Alternately Davies *et al* (1992) suggest that lowered concentrations of ACC in the presence of unaltered AMP-PK activity result in increased phosphorylation of ACC.

Most of the studies on the effect of high fat diets observed so far have been carried out over long term periods. The present study investigated whether ACC inactivation was the cause of the observed short term inhibition of mammary lipogenesis by polyunsaturated fatty acids in isolated acini.

Figure 5.4 shows the activity of ACC measured in crude extracts prepared from acini incubated for 60 min with EPA over a range of concentrations. The activity of ACC measured at 10 mM citrate decreased steadily with increasing concentrations of EPA, reaching 50-60% inhibition at concentrations between 1-2 mM. Linolenic and linoleic acid also caused inhibition of ACC. At 1 mM concentration, the inhibition of ACC by EPA, linolenic and linoleic acid was 46%, 42% and 34%, respectively (Table 5.3). In addition, the inhibition of ACC by linolenic is significantly greater compared to the effect of linoleic acid (Table 5.3). This rapid inhibition of ACC in crude extracts prepared from acini incubated with polyunsaturated fatty acids for only 60 min is unlikely to have occurred as a result of decreased ACC concentration. Allosteric inhibition or ACC phosphorylation are likely mechanisms.

Figure 5.4 Effect of EPA on the Activity of Acetyl-CoA Carboxylase in Isolated Mammary Acini

Acini were incubated for 60 min in the presence and absence of EPA. Activity of ACC in crude extracts of acini was measured at 10 mM citrate as described in the experimental section. Results are expressed as nmol $\text{NaH}^{14}\text{CO}_3$ incorporated/min per mg protein and are means for 4 separate observations with S.E.M. indicated by vertical bars.

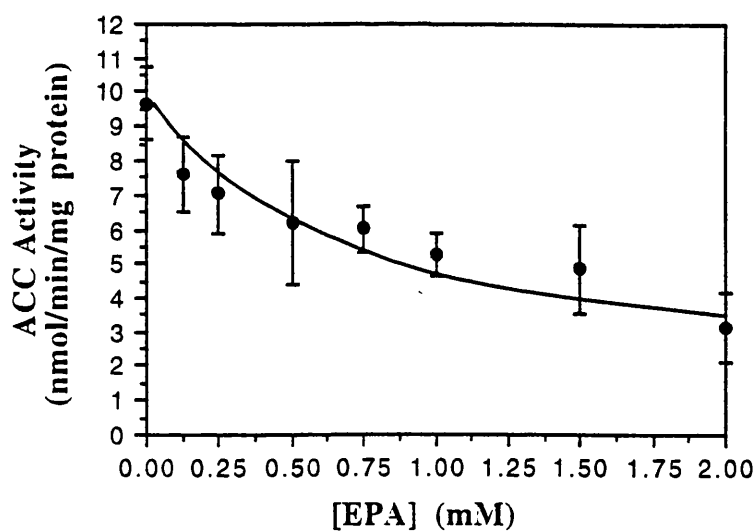


Table 5.3 Effects of PUFA on the Activity of Acetyl-CoA Carboxylase in Isolated Mammary Acini

Acini were incubated for 60 min in the presence and absence of 1 mM EPA, linoleic and linolenic acid. Activity of ACC in crude extracts of acini was measured at 10 mM citrate as described in the experimental section. Results are expressed as nmol $\text{NaH}^{14}\text{CO}_3$ incorporated/min per mg protein and are means \pm S.E.M. for 4 preparations. Values that are significantly different (by Student's t-test) from control value are shown : * $P < 0.05$; ** $P < 0.005$. The value for linolenic acid that is significantly different from linoleic acid (by Student's t-test) is shown : # $P < 0.01$.

	<u>Activity of ACC</u>
Control	9.68 ± 1.06
EPA	5.25 ± 0.66 *
Linoleic Acid	6.41 ± 0.21 **
Linolenic Acid	5.52 ± 0.36 * #

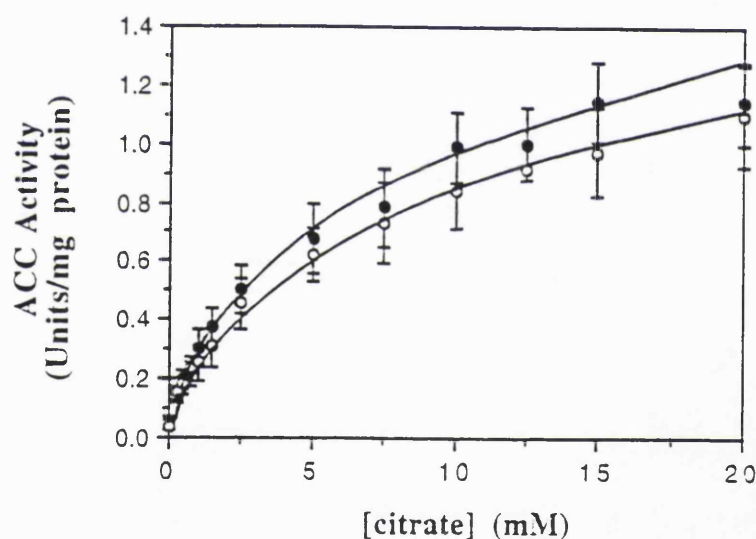
5.5 Effects of EPA on Purified Acetyl-CoA Carboxylase Activity

To determine if inhibition of ACC by EPA was due to phosphorylation of the enzyme, ACC was purified on an avidin-Sepharose column from extracts of isolated acini incubated with 1 mM EPA. The activity of ACC was measured at citrate concentrations ranging from 0 to 20 mM.

As shown in Figure 5.5, there was a small, but not statistically significant inhibition in the activity of purified ACC in EPA-treated cells. This was not comparable to measurements of ACC in crude extracts where the same concentration (1 mM) of this fatty acid inhibits the enzyme by 46% (Table 5.3). There was no significant difference between the K_a for citrate values of ACC from EPA-treated or control acini. However, a decrease of 20% in the V_{max} of ACC in acini incubated with 1 mM EPA was observed and was statistically significant (Table 5.4). Hence there is evidence to suggest that the inhibition of ACC by EPA is mediated by increases in its phosphorylation status. However, there is discrepancy between the level of ACC inactivation observed in crude extracts and following purification. The inhibition of ACC by PUFA is considerably less than the inhibition of the fatty acid synthesis, suggesting that PUFA may exert additional effects on other regulatory steps in the pathway of lipogenesis.

Figure 5.5 Effect of EPA on the Activity of Purified ACC from Isolated Mammary Acini

For purification and assay of ACC see Materials and Methods. Acini were incubated for 60 min in the presence (○) and absence (●) of 1 mM EPA. Results are expressed as Units/mg protein and are means for 3 observations with S.E.M. indicated by vertical bars.



**Table 5.4 Effect of EPA on the V_{\max} and K_a for Citrate of Purified
Acetyl-CoA Carboxylase**

V_{\max} and K_a citrate of ACC were determined as described in Materials and Methods. Each value is the mean \pm S.E.M. for 3 observations. Values that are significantly different (by Student's t-test) from the control value are shown : * $P < 0.01$.

	V_{\max} <u>(Unit/mg protein)</u>	K_a Citrate <u>(mM)</u>
Control	0.92 ± 0.14	1.82 ± 0.55
EPA	0.74 ± 0.14 *	1.33 ± 0.26

5.6 Effects of Polyunsaturated Fatty Acids On Lactate Accumulation

Incubations of acini with EPA lead to increased accumulation of lactate in a dose-dependent manner (Figure 5.6) which reached a maximum at concentrations of EPA of 1 mM and above. This response was a mirror image of the dose-dependent inhibition of fatty acid synthesis and ACC (Figure 5.2 and 5.4). Higher lactate production was also observed in acini incubated with 1 mM linolenic and linoleic acid (Table 5.5). The increase in lactate levels did not differ significantly between the three different polyunsaturated fatty acids and each produced a 90-120% increase in the rate of lactate accumulation.

It is interesting to note that in isolated mammary acini incubated with oleate, no change in lactate production was observed (Robinson and Williamson, 1978a). However, lactate production was increased when acini were incubated with medium and long chain triglycerides (Agius and Williamson, 1980). This suggests that even in the same tissue preparation, varying effects of fatty acids are possible depending on the nature of the fatty acid involved.

Increased accumulation of lactate in the presence of EPA, linolenic and linoleic acid suggests that the activity of PDH is likely to be decreased.

Figure 5.6 Effect of EPA on Lactate Accumulation in Isolated Mammary Acini

Acini were incubated for 60 min in the presence and absence of EPA. Results are expressed as $\mu\text{mol}/\text{min}$ per 100 mg defatted dry weight acini and are mean values for 3 separate observations with S.E.M. indicated by vertical bars.

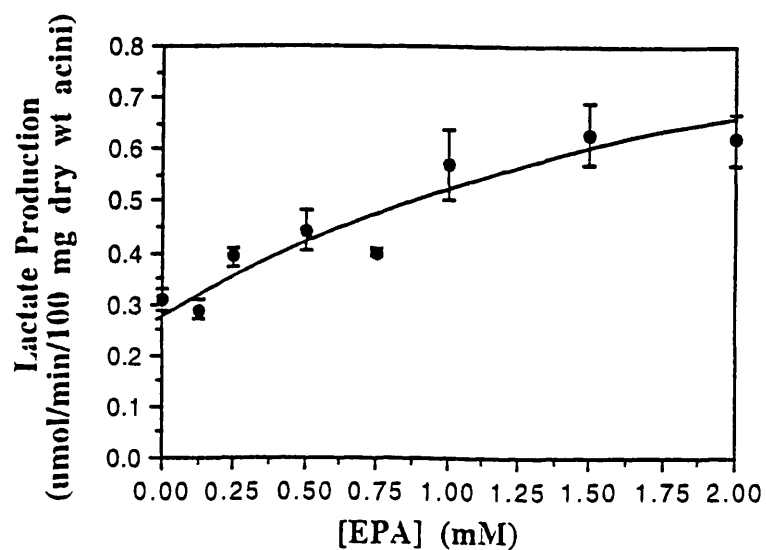


Table 5.5 Effects of PUFA on Lactate Accumulation in Isolated Mammary Acini

Acini were incubated in the presence and absence of 1 mM EPA, linoleic and linolenic acid. Results are expressed as $\mu\text{mol}/\text{min}$ per 100 mg defatted dry weight acini and are mean \pm S.E.M. for 3 preparations. Values that are significantly different (by Student's t-test) from the control value are shown : * $P < 0.05$; ** $P < 0.025$.

	<u>Rate of Lactate Accumulation</u>
Control	0.31 ± 0.02
EPA	0.57 ± 0.07 *
Linoleic Acid	0.67 ± 0.04 **
Linolenic Acid	0.72 ± 0.09 *

5.7 Effects of Polyunsaturated Fatty Acids on Pyruvate Dehydrogenase Activity

As shown in Table 5.6, EPA, linolenic and linoleic acid at 1 mM concentration significantly inhibit PDH activity in isolated mammary acini. It appeared that all three polyunsaturated fatty acids produced similar levels of PDH inhibition, with EPA, linolenic and linoleic acid causing 41%, 35% and 29% inhibition respectively. This is in keeping with the increases in lactate production that each caused.

Inhibition of PDH activity in liver in response to n-3 polyunsaturated fatty acids has been reported in the study carried out by Da Silva *et al* (1993). In their study, it was showed that dietary polyunsaturated fatty acid given to rats fed fat free diets for 2 weeks caused a marked inhibition of PDH activity. In this case, fish oil (10%) was found to inhibit PDH activity by 83% whereas inhibition by corn oil (10%) was only 49%. Total activity of hepatic PDH was decreased by 46% and 40% in rats fed diets supplemented with fish oil and corn oil, respectively.

Table 5.6 Effects of PUFA on the Activity of Pyruvate Dehydrogenase in Isolated Mammary Acini

Acini were incubated for 60 min in the presence and absence of 1 mM EPA, linoleic and linolenic acid. Results are expressed as munits PDH/unit citrate synthase and are mean \pm S.E.M. for 3 preparations. Values that are significantly different (by Student's t-test) from control value are shown :

** P < 0.005.

	<u>PDH Activity</u>
Control	45.4 \pm 2.5
EPA	20.7 \pm 2.1 **
Linoleic Acid	29.7 \pm 4.7 **
Linolenic Acid	28.0 \pm 3.0 **

5.8 Discussion

From the results of the present studies, it is evident that the polyunsaturated fatty acids EPA, linolenic and linoleic acid are inhibitors of fatty acid synthesis in lactating rat mammary acini. The n-3 polyunsaturated fatty acid EPA has the greatest effect in this respect. These findings are in agreement with the observed effects of n-3 polyunsaturated fatty acids reported in livers of rats fed fish oils (Yang and Williams, 1978; Iritani *et al*, 1980; Wong *et al*, 1984; Willumsen *et al*, 1993) and in isolated rat hepatocytes from rats fed unsaturated fats (Gibbons and Pullinger, 1986).

Inhibition of lipogenesis in response to polyunsaturated fatty acids clearly occurred as a result of inhibition of lipogenic enzymes. Earlier studies by Muto *et al* (1970) showed that intragastric intubation of polyunsaturated fatty acids (0.25% w/w) to rats for 3 days resulted in inhibition of hepatic activities of ACC, ATP-CL, glucose-6-dehydrogenase and malic enzyme. Iritani and co-workers (1980) also showed that the reduction of triglyceride production in rats intubated with marine fish oils for 4 days occurred as a result of a decrease in hepatic ACC, glucose-6-phosphate dehydrogenase and malic enzyme activities. Whilst EPA was able to cause inhibition of rat hepatic ATP-citrate lyase and ACC by 45-50%, palmitic acid exerted only a small suppressive effect (15%) upon these lipogenic enzymes over a 15 days treatment period (Willumsen *et al*,

1993). In rats fed a high polyunsaturated fat diet consisting of sunflower oil for 10 days, a decrease in the hepatic mRNA concentration of ACC was reported (Davies *et al*, 1992). These findings suggest that long term effects of n-3 polyunsaturated fatty acids on hepatic lipogenesis is accompanied by a decrease in the content of lipogenic enzymes.

Inhibition of lipogenic enzyme activities has also been reported in rats fed high fat diets. In acinar preparations isolated from rats fed a cafeteria diet (high fat cheese crackers) throughout lactation, inhibition of fatty acid synthesis (40%) was accompanied by a decrease in ACC activity by approximately 50% (Munday and Williamson, 1987). The effect of a high fat diet in lactating rat mammary gland can be relieved by insulin either administered *in vivo* to the rat (Agius *et al*, 1981a) or added to the incubation medium of isolated acini *in vitro* (Munday and Williamson, 1987). The effect of insulin is accompanied by an increase in the activity of ACC to the control chow fed value (Munday and Williamson, 1987). ACC has therefore, been implicated to be the site that mediates the inhibition of fatty acid synthesis in lactating rat mammary gland in the chronic response to high fat feeding.

Acute response of lactating mammary gland to dietary lipid has also been reported. Mercer and Williamson (1988) showed that oral administration of triglycerides (triolein) to starved/chow-refed lactating rats suppressed the

lipogenic switch-on in the mammary gland *in vivo* by 60%, between 60 and 90 minutes after the onset of refeeding. This is accompanied by decreased glucose uptake of approximately 40%.

In our studies in isolated mammary acini, the inhibition of ACC and PDH is clearly evident within 60 minutes of incubation of acini with polyunsaturated fatty acids. This suggests that the inhibition of these enzymes is unlikely to occur as a result of changes in the lipogenic enzyme concentrations. ACC is known to be regulated by allosteric effectors. Moss and Lane (1971) reported that ACC is activated by citrate and inhibited by long chain fatty acyl-CoA (Ogiwara *et al*, 1978). In addition, the enzyme may be phosphorylated and inactivated by AMP-PK, the central component of the kinase cascade that regulates the pathway of fatty acid and cholesterol synthesis. AMP-PK can be phosphorylated and activated by a distinct kinase kinase in a reaction stimulated by palmitoyl-CoA at nanomolar concentration (Carling *et al*, 1987). The effects of different classes of fatty acyl-CoA on kinase kinase have yet to be explored. It is likely that *in vivo*, polyunsaturated fatty acids are converted into their corresponding fatty-acyl-CoA esters. These could stimulate the activity of kinase kinase that in turn phosphorylates and activates AMP-PK. Studies by Flatmark *et al* (1988) showed that administration of n-3 fatty acids lead to a rapid rise in the levels of long chain fatty acyl-CoA. Inhibition of ACC by long chain fatty acids in perfused rat liver has been shown to occur as a result of an increase in

the levels of fatty acyl-CoA (Mayes and Topping, 1974) which could exert its effects on ACC allosterically or by stimulating the kinase kinase. However, the allosteric effect of long chain fatty acyl-CoA esters are likely to be lost as the effector molecule is extensively diluted in the enzyme assay, and upon purification of the enzyme. The lack of inhibitory effect of oleate in mammary acini (Robinson and Williamson, 1978a) and palmitate in adipocytes (Saggerson, 1976) on fatty acid synthesis may be attributed to the fact that the concentrations of their fatty acyl-CoA are not sufficiently high to inhibit ACC. As has been suggested by Lunzer *et al* (1977), lowering of fatty acyl-CoA levels could occur due to protein binding and compartmentation of fatty acyl-CoA within the cell. Since EPA is able to decrease the V_{\max} of purified ACC, the AMP-PK cascade may therefore, be the target for polyunsaturated fatty acids. As has been shown by the studies of Davies *et al* (1992) in rats fed sunflower oil diets for 10 days, a decrease in the initial : total activity ratio of hepatic ACC was observed. This is consistent with increased phosphorylation of the enzyme in response to high fat feeding.

In the present study, inhibition of PDH is likely to occur as a result of increased phosphorylation by PDH kinase. The kinase is activated by the increased ratio of [acetyl-CoA]/[CoA] which may be brought about by oxidation of polyunsaturated fatty acids and/or secondary to the inhibition of ACC. It has been shown in the time course experiment with okadaic acid (Chapter 4) that

inhibition of ACC by the toxin leads to accumulation of acetyl-CoA and there was a lag in the inhibition of PDH by okadaic acid. The results from the present study also suggest that the inhibition of PDH and ACC, the key regulatory enzymes in the lipogenic pathway, have an additive effect on the inhibition of fatty acid synthesis in response to polyunsaturated fatty acids.

In addition to the effects of polyunsaturated fatty acids on hepatic lipogenesis, the inhibitory effects of these fatty acids on cholesterol synthesis have also been illustrated in several studies. In rats fed 15% corn oil for 7 days, hepatic cholesterol synthesis was found to decrease by more than 60% (Gibbons and Pullinger, 1986). Inhibition of hepatic cholesterol synthesis by 28% was also observed in rats fed 20% EPA for 4 weeks (Ventura *et al*, 1989). Inhibition of cholesterol synthesis is likely to occur as a result of decreased HMG-CoA reductase activity. It has been shown by Field *et al* (1987) that in rabbits fed 10% n-3 polyunsaturated fatty acid in the form of fish oil for 14 days, the hepatic HMG-COA reductase activity was reduced by more than 75% compared to the control animals.

Decreased VLDL synthesis and secretion could also be brought about by the ability of fish oil to alter the hepatic partitioning of free fatty acids, channelling them away from triglyceride synthesis towards fatty acid oxidation, thus reducing triglyceride output (Wong *et al*, 1984; Aarsland *et al*, 1990). This

has been shown by the stimulation of mitochondrial fatty acid oxidation after one day of EPA feeding of rats with n-3 polyunsaturated fatty acids (Willumsen *et al*, 1993). The net result of this is a decrease in the availability of fatty acid for hepatic triglyceride synthesis. It appears that the greater inhibitory effect of n-3 fatty acids on lipogenesis could reside in the fact that stimulation of hepatic fatty acid oxidation by n-3 fatty acids is greater in comparison to the n-6 fatty acids (Yang and Williams, 1978). This is also shown by higher rates of fatty acid oxidation and ketogenesis manifested by rats fed MaxEPA in comparison to safflower oil-fed rats (Wong *et al*, 1984).

The other contributing factor towards the triglyceride-lowering effect of n-3 polyunsaturated fatty acids may be related to the fact that these fatty acids are poorer substrates for triglyceride synthesis and VLDL formation and secretion than are the more common fatty acids of the n-9 and n-6 series (Wong *et al*, 1984). This was supported by the observations made by Rustan *et al* (1988) and Zhang *et al* (1991). In their studies, they showed that utilisation of EPA for triglyceride and VLDL synthesis and secretion by perfused rat livers was lower when compared to oleic acid.

Since LDL are produced in the plasma during catabolism of VLDL, decreased VLDL production in response to fish oil could result in a decrease in plasma LDL. In chronic feeding of rats with 20% fish oil, plasma LDL

cholesterol was reduced by 38% but increased in rats fed 20% safflower and coconut oil (Ventura *et al*, 1989). The effect of fish oil was reported to be due to an increase in the hepatic LDL receptor activity where an increase of 105% was observed. Safflower oil also stimulated LDL receptor activity but to a much smaller extent (45%) and coconut oil had no significant effect on the receptor activity. Unlike fish oil however, the effect of safflower oil on the activity of the LDL receptor was offset by the increased production of LDL cholesterol. The effect of fish oil on LDL receptor activity could be the result of altered cell membrane fluidity occurring via incorporation of n-3 polyunsaturated fatty acids into phospholipid. As shown in isolated rat hepatocytes incubated with 800 μ M EPA for 8 hours, there was an increase of 91% in the amount of EPA incorporated into phospholipid (Benner *et al*, 1990). The fact that EPA is able to prevent the development of insulin resistance in rats fed a high fat diet (Storlien *et al*, 1987) may also be related to their ability to alter the membrane fluidity of the cell, thus affecting the binding of insulin and events subsequent to this.

In summary, the results from the present study indicates that the short term triglyceride-lowering effect of polyunsaturated fatty acids is in part mediated by inhibition of *de novo* fatty acid synthesis. The magnitude of lipogenesis inhibition appears to be dose-related. It is also evident that n-3 polyunsaturated fatty acid EPA and linolenic acid exhibit a greater effect in

inhibiting fatty acid synthesis in comparison to linoleic acid. While long term effects of PUFA may result in decreased ACC concentrations, the results from the present study have shown the short term effects of polyunsaturated fatty acids on PDH and ACC. This suggests phosphorylation of both enzymes and further elucidation of AMP-PK activity changes are required.

CHAPTER 6

EFFECTS OF THE LIPID LOWERING DRUGS CLOFIBRATE AND GEMFIBROZIL ON LIPOGENESIS

6.1 Introduction

Hyperlipidaemia is the major risk factor for atherosclerosis. The disease is characterised by elevated levels of plasma cholesterol and /or triglyceride. A plasma cholesterol level in excess of 220 mg/100 ml (5.7 mmol/l) and triglyceride level in excess of 140 mg/100 ml (4.9 mmol/l) are considered as abnormally high. There are two types of hyperlipidaemia. Primary, or inborn errors of hyperlipidaemia are associated with over-production of triglyceride- or cholesterol-rich lipoproteins, or disorders of lipoprotein metabolism. Secondary hyperlipidaemia is usually the result of cigarette smoking, excess alcohol consumption and complications of metabolic disorders such as hypertension and diabetes mellitus.

Several types of primary hyperlipidaemia have been defined (for reviews see Schaefer and Levy, 1985; Breslow, 1989). Familial hypercholesterolaemia is an autosomal dominant disorder with defective LDL receptors. The absence or deficiency of functional LDL receptors are associated with decreased LDL

catabolism and consequently elevated cholesterol levels in the plasma (Goldstein and Brown, 1983). In the heterozygous form, plasma LDL is twice the normal level and in the more severe homozygous form, plasma LDL is more than six times higher than normal. In many heterozygous individuals, an over-production of LDL which is thought to occur by an abnormal pathway not involving VLDL has also been observed. Plasma triglyceride and VLDL levels may be normal or mildly elevated and HDL-cholesterol tends to be low.

In familial hypertriglyceridaemia, there appears to be a defect leading to enhanced hepatic triglyceride synthesis with subsequent secretion of triglyceride-rich VLDL. Plasma cholesterol levels are modestly increased. LDL and HDL levels are low as a result of increased catabolism and decreased synthesis of apo-A1, respectively.

Familial combined hyperlipidaemia is an autosomal dominant trait characterised by elevated plasma levels of cholesterol and triglyceride, or of cholesterol or triglyceride alone. It is thought to occur as a result of over-production of VLDL with variable conversion of this lipoprotein to LDL. Concentrations of VLDL, LDL or of both lipoproteins are increased and HDL-cholesterol levels tend to be low.

Familial hyperchylomicronaemia is an autosomal recessive abnormality. It is associated with deficiency in the plasma lipoprotein lipase which therefore leads to incomplete catabolism of dietary triglyceride contained within chylomicrons. The disorder is characterised by high plasma triglyceride levels. There may be a moderate excess of VLDL and LDL and HDL levels are low.

In familial dysbetalipoproteinaemia, apoprotein E required for the binding of chylomicron remnants and LDL to hepatic receptors fails to bind to the receptors as a result of defect in the primary sequence of amino acids in ApoE. This leads to accumulation of remnant particles and consequently, elevated plasma levels of cholesterol and triglyceride. Plasma levels of LDL and HDL are low.

Various lines of evidence strongly suggests that treatment of hyperlipidaemia decreases or prevents atherosclerosis. Evidence from clinical trials showed that a decrease in plasma LDL-cholesterol can reduce the risk of atherosclerosis and hence coronary heart disease. The incidence of coronary heart disease in hyperlipidaemic patients could be reduced by 50% for a 35% decrease in plasma LDL-cholesterol (Lipid Research Clinics Program, 1984a, 1984b). Various treatments are available for treating hyperlipidaemias. Strict dietary and weight controls which aim to maintain a normal body weight and minimise

concentrations of lipids in plasma have been used as the first line treatment. A weight reducing diet or diets low in dietary fat are recommended. It is known that diets that are high in saturated fats increase plasma triglyceride and cholesterol levels whereas polyunsaturated fatty acids have the opposite effect and tend to reduce plasma lipid levels (Chapter 5). In patients where the hyperlipidaemic cause is secondary, patients are advised to minimise all risk factors that might potentiate the development of atherosclerosis. In patients with severe hyperlipidaemia, drug therapy is initiated if the dietary approach is inadequate. A few classes of lipid-lowering drugs are currently available. These include bile acid binding resins, nicotinic acid, statins and the fibric acid derivatives (for review see Illingworth, 1987; O'Connor *et al*, 1990).

Bile acid binding or anion-exchange resins such as cholestyramine (Questran) and colestipol (Colestid) decrease the plasma concentration of cholesterol by decreasing plasma LDL levels. The effect is achieved by binding of the resins to bile acids in the intestinal lumen, thus interrupting their enterohepatic circulation. Bile acids inhibit the microsomal hydroxylase that catalyses the conversion of cholesterol to bile acids. A decrease in bile acid pool size therefore leads to an increase in the hepatic synthesis of bile acids from cholesterol, the uptake of which from circulation is increased as LDL receptors are upregulated. The rate of LDL catabolism is therefore enhanced, resulting in

a lower plasma concentration of LDL cholesterol (Shepherd *et al*, 1980). Bile acid binding resins are effective in the heterozygous form of familial hypercholesterolaemia in which LDL receptors are deficient rather than absent. They are not recommended for the treatment of hypercholesterolaemia associated with elevated concentrations of triglyceride or VLDL since they can increase the plasma levels of VLDL.

Nicotinic acid (niacin) lowers both plasma cholesterol and triglyceride levels, but the predominant effect is on triglycerides where a 20-80% reduction may be seen depending on the initial concentration of VLDL (Carlson and Olsson, 1979). On the other hand, LDL-cholesterol is decreased by 10-15% (Kane *et al*, 1981). The primary effect of nicotinic acid appears to be the result of inhibition of hepatic secretion of VLDL (Grundy *et al*, 1981). This is achieved by inhibition of lipolysis in adipose tissue, thus reducing the availability of free fatty acids for hepatic triglyceride synthesis. Since LDL is derived from VLDL, a reduction in plasma LDL and hence cholesterol levels also occurs. Nicotinic acid is used in the treatment of hypercholesterolaemia and in combined hyperlipidaemia.

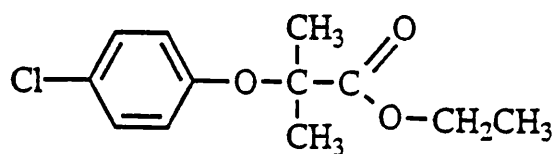
HMG-CoA reductase inhibitors such as compactin and mevinollin are fungal metabolites which exhibit hypocholesterolaemic properties. Statins such

as fluvastatin (Lescol), simvastatin (Zocor) and pravastatin (Lipostat) are newer synthetic drugs which lower plasma cholesterol. The drugs competitively inhibit cellular cholesterol production by inhibiting the rate limiting enzyme HMG-CoA reductase in the pathway of cholesterol synthesis. This leads to an increase in the expression of hepatic LDL receptors, thus reducing plasma levels of cholesterol. In heterozygous familial hypercholesterolaemia, the increase in the number of LDL receptors promotes LDL uptake from the bloodstream with a resultant 30-40% fall in LDL cholesterol. Statins also reduce plasma triglyceride and VLDL levels (Grundy, 1988), with 5-10% reduction being observed.

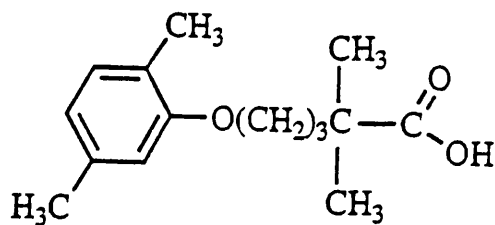
The fibric acid derivatives are effective triglyceride-lowering agents. Clofibrate (Atromid, Figure 6.1a), the first compound discovered in the 1960's is an ester derivative of phenoxy-butyric acid. Other fibric acid derivatives include gemfibrozil (Lopid, Figure 6.1b), bezafibrate (Bezalip, Bezalip Mono), fenofibrate (Lipantil) and ciprofibrate (Modalim). A common physical property of the fibrates is their hydrophobicity, being soluble only in lipid environments and organic solvents. The presence of a carboxylic acid functional group or group that can be readily oxidised to carboxylic acid is another characteristic of these molecules.

Figure 6.1 Structures of Clofibrate, Gemfibrozil and 5-(Tetradecyloxy)-2-Furoic Acid (TOFA)

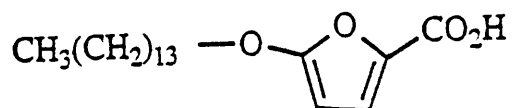
(a) Clofibrate



(b) Gemfibrozil



(c) TOFA



Clinical and animal studies have indicated that the fibrates are potent triglyceride-lowering agents and the drugs are used clinically in the treatment of familial hypertriglyceridaemia. In a study conducted over a 5 year period in hyperlipidaemic patients receiving treatment with clofibrate (1.8 g daily), plasma triglyceride concentration was reduced by 22% within 2-5 days after initiation of therapy and cholesterol was decreased by 6% (Coronary Heart Project, 1975). Newer derivatives of fibrate compounds such as gemfibrozil are more potent than clofibrate therapeutically (Todd and Ward, 1988). This is shown by the substantial decrease in plasma VLDL triglyceride by 54% and an increase in HDL cholesterol by 36% in chronic treatment (8 weeks) of hyperlipidaemic patients with a daily dosage of 1.2 g gemfibrozil (Saku *et al*, 1985). In the Helsinki Heart Study conducted over a 5 year period with hyperlipidaemic individuals, treatment with a twice daily dosage of 600 mg gemfibrozil induced a 54% decrease in triglyceride levels. HDL cholesterol levels were increased by 8%, and LDL-cholesterol and total cholesterol levels were decreased by approximately 8% (Frick *et al*, 1987). Studies with bezafibrate (200 mg three times daily) given to patients with familial dysbetalipoproteinaemia for 8 weeks resulted in a substantial fall in plasma triglyceride, cholesterol and VLDL cholesterol by 68%, 47% and 75%, respectively.

The fundamental mechanism underlying the lipid-lowering effects of the

fibrates is not entirely established although several mechanisms of action are evident:

- i) Increased plasma HDL levels (Frick *et al*, 1987)
- ii) Increased catabolism and plasma clearance of VLDL via stimulation of lipoprotein lipase activity (Shepherd *et al*, 1984). Extrahepatic lipoprotein lipase activity has been found to increase by 25% in hypertriglyceridaemic patients treated with gemfibrozil (1.2 g/day) for 8 weeks (Saku *et al*, 1985).
- iii) Reduced synthesis and secretion of VLDL triglycerides (Kesaniemi and Grundy, 1984). Incubation of cultured rat hepatocytes with 0.5 mM gemfibrozil for 20 minutes significantly decreased the incorporation of [1,3-¹⁴C]glycerol into cellular triglyceride by 30%, and VLDL triglyceride secretion was inhibited by 75% (Lamb *et al*, 1993). In Hep G2 cells incubated with fenofibrate (20 µg/ml) for 4 days, it was found that the incorporation of ¹⁴C-acetate into triglyceride was decreased by 30% and VLDL secretion was decreased by 40%. ApoB secretion, but not its synthesis was inhibited by both clofibrate and fenofibrate at 20 µg/ml (Hahn and Goldberg, 1992).

At least two mechanisms could explain the decreased synthesis and secretion of VLDL triglyceride in response to fibrates. These are the observed

increase in partitioning of intracellular fatty acids towards hepatic oxidation and away from esterification (Lazarow, 1977) and the observed inhibition of lipid biosynthesis.

6.1.1 Fibrates and Fatty Acid Oxidation

Animal studies have shown that fibrates are a novel class of chemical carcinogens, causing pronounced hepatic peroxisomal proliferation in rats and mice (Reddy *et al*, 1980; Milton *et al*, 1990). The acyl-CoA thioesters are thought to be the active species mediating the carcinogenic effect of the fibrates. As shown by the studies of Bronfman *et al* (1989), the acyl-CoA thioesters of clofibrate, bezafibrate and ciprofibrate were able to stimulate protein kinase C activity purified from rat brain and liver in a dose-dependent manner but the unesterified drugs were found to be ineffective. Recently, the *in vivo* formation of acyl-CoA esters of ciprofibroyl-CoA and clofibroyl-CoA have been reported in isolated rat hepatocytes (Bronfman *et al*, 1992). *In vivo*, the acyl-CoA thioesters are also the active species responsible for the lipid lowering effects of the drugs (Bronfman *et al*, 1986). Formation of fatty acyl-CoA thioesters is catalysed by a non-specific long chain fatty acyl-CoA synthetase (Amigo *et al*, 1992) localised in microsomes, in the outer mitochondrial membrane and in peroxisomes. The highest activity of this enzyme has been found in abdominal

fat and in liver (Amigo *et al*, 1992).

It was proposed that the effects of the fibrates on peroxisomal proliferation is brought about by binding of the fibroyl-CoA to one or more specific peroxisomal proliferator binding proteins or receptors. A cytosolic peroxisomal proliferator binding protein has been isolated from rat liver (Lalwani *et al*, 1983; 1987) although using ³H-ciprofibrate as labelled ligand, Milton *et al* (1988) found no evidence for the existence of specific receptors in rat liver.

The ability of the fibrates to induce peroxisomal proliferation is associated with hepatomegaly (Reddy *et al*, 1980); increased peroxisomal β -oxidation and induction of the activities of several peroxisomal enzymes (Lazarow and de Duve, 1976); induction of an isoenzyme of cytochrome P₄₅₀ which catalyses the ω -hydroxylation of fatty acids (Sharma *et al*, 1988; Milton *et al*, 1990); and increased mitochondrial oxidation and induction of carnitine acyltransferase 1 activity (Bhuiyan *et al*, 1988). Induction of peroxisomal and microsomal β -oxidation may enhance lipid catabolism and thus be related to the hypolipidaemic effects of the fibrates. Hepatic peroxisomes from rats fed clofibrate (5 g/kg chow) for 1 week showed an increase in peroxisomal fatty acyl-CoA oxidation of approximately 10-fold (Lazarow and de Duve, 1976).

Induction of peroxisomal enzyme activity was seen in cultured rat hepatocytes incubated with clofibrate and ciprofibrate where there was a concentration-dependent increase in fatty acyl-CoA oxidase with an IC_{50} of 0.028 mM and 0.82 mM, respectively (Kocarek and Feller, 1989).

A 3- to 4-fold induction of mitochondrial palmitate oxidation was reported in hepatocytes isolated from rats given subcutaneous clofibrate (200 mg/kg body weight) for 14 days (Mannaerts *et al*, 1979). The activity of hepatic mitochondrial carnitine acyltransferase 1 which catalyses the formation of acyl-carnitine was increased by more than 95% in rats fed 0.1% w/w ciprofibrate for 5 days (Bhuiyan *et al*, 1988). In rats fed clofibrate, bezafibrate, ciprofibrate and fenofibrate for 9-14 days, this activity was increased by more than 2-fold (Halvorsen, 1983). In addition, the concentration of hepatic carnitine is increased by approximately 2-fold in rats fed ciprofibrate for 5 days (Bhuiyan *et al*, 1988), and daily subcutaneous injection of rats with clofibrate (600 mg/kg body weight) for 5 days increases hepatic CoA levels by 3.2-fold (Voltti *et al*, 1979). These effects may contribute to increased rates of β -oxidation.

6.1.2 Fibrates and Lipid Biosynthesis

These is convincing evidence that at the very most, newly synthesised

fatty acids only contribute 20% towards the fatty acid content of VLDL triglyceride (Gibbon, 1990). *De novo* synthesis of cholesterol makes a larger contribution. However, the rate of VLDL secretion frequently correlates with the rate of lipogenesis (Gibbons, 1990).

Long term oral administration of fibrates to rats has been shown to inhibit fatty acid and cholesterol synthesis in liver slices or hepatocytes subsequently prepared from these animals (Panek *et al*, 1977; Haughom *et al*, 1992). This has been attributed to inhibition of acetyl-CoA carboxylase and HMG-CoA reductase that regulate fatty acid and cholesterol synthesis, respectively. These inhibitions are probably the result of decreases in enzyme concentration. However, despite the short term hormonal regulation of these enzymes, the speed of the fibrate inhibitions has not been investigated.

The fibrates share some structural features with 5-(tetradecyloxy)-2-Furoic Acid (TOFA, Figure 6.1c) which is also a potent hypolipidaemic agent though not used clinically. TOFA inhibited fatty acid and cholesterol synthesis in isolated rat hepatocytes (Ribereau-Gayon, 1976; Panek *et al*, 1977) and in rat liver *in vivo* (Kariya and Willie, 1978; Cook *et al*, 1978; McCune and Harris, 1979; McGarry and Foster, 1979). In isolated mammary acini incubated with 0.4 mM TOFA for 60 minutes, [6-¹⁴C]glucose incorporation into lipids was inhibited

by 81% (Robinson and Williamson, 1977a). The effects of TOFA were rapid (60 min) and given the structural similarities between TOFA and clofibrate and gemfibrozil (Figure 6.1), these compounds may have similar mechanisms of action.

The aim of the present study was to investigate short term effects of clofibrate and gemfibrozil on the regulatory steps on the pathway of fatty acid synthesis in isolated mammary acini. The lactating rat mammary gland has an extremely high rate of fatty acid synthesis and acini isolated from this tissue provide an ideal model for studying the effects of the fibrates on lipogenesis.

6.2 Effects of Clofibrate and Gemfibrozil on Lipogenesis in Isolated Mammary Acini

Clofibrate and gemfibrozil are potent inhibitors of fatty acid synthesis in isolated mammary acini as measured by the incorporation of $^3\text{H}_2\text{O}$ into fatty acids (Figure 6.2). Both compounds inhibit fatty acid synthesis in a dose-dependent manner with inhibition occurring at drug concentrations as low as 0.125 mM. In the presence of 1 mM clofibrate and gemfibrozil, the rates of fatty acid synthesis in acini were inhibited by 33% and 84%, respectively. Gemfibrozil is a more potent inhibitor of fatty acid synthesis in comparison to

clofibrate, with IC_{50} values of approximately 0.25 mM and 0.55 mM, respectively. The inhibitory effect of gemfibrozil on fatty acid synthesis in mammary acini was comparable to the observed effects of TOFA. Studies in isolated mammary acini showed that 0.4 mM TOFA inhibited fatty acid synthesis by 80% (Robinson and Williamson, 1977a) and in isolated hepatocytes incubated with 5 μ M TOFA, the rate of fatty acid synthesis was inhibited by 78% (McCune and Harris, 1979).

To examine if clofibrate and gemfibrozil inhibit fatty acid synthesis by decreasing glucose uptake into the cells, the effect of these drugs on glucose utilisation by isolated mammary acini was determined.

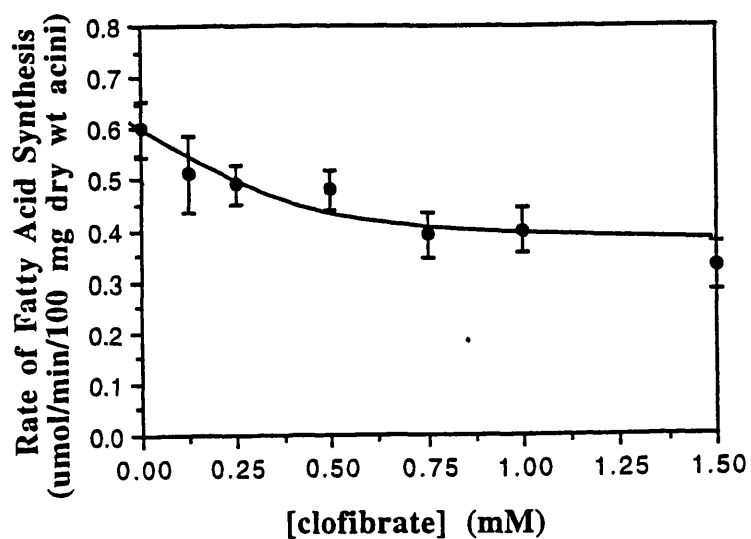
6.3 Effects of Clofibrate and Gemfibrozil on Glucose Utilisation in Isolated Mammary Acini

Glucose utilisation by acini is unaffected by the presence of clofibrate (Figure 6.3a) or gemfibrozil (Figure 6.3b) in the incubation media even at 1.5 mM which caused significant inhibition of fatty acid synthesis (Figure 6.2). These observations contrast with the effects of TOFA which inhibited glucose uptake by 57% in isolated mammary acini (Robinson and Williamson, 1977a) and 52% in isolated hepatocytes (McCune and Harris, 1979). The lack of effect

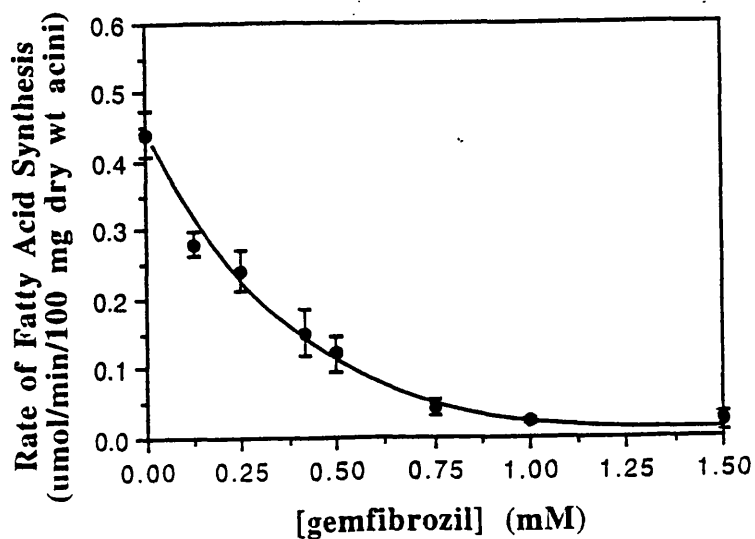
Figure 6.2 Effects of Clofibrate and Gemfibrozil on the Rates of Fatty Acid Synthesis in Isolated Mammary Acini

For experimental details see text. The results are expressed as $\mu\text{mol } ^3\text{H}$ incorporated/min per 100 mg dry defatted weight of acini. Each value is the mean of at least four separate observations with S.E.M. indicated by vertical bars.

(a) Clofibrate



(b) Gemfibrozil



of clofibrate and gemfibrozil on glucose uptake suggests that the inhibition of fatty acid synthesis in acini is not accounted for by decreased substrate (glucose) availability.

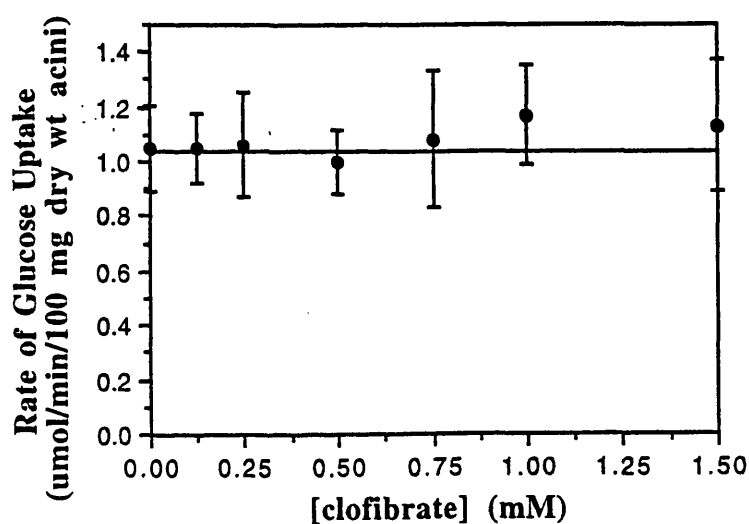
6.4 Effect of Clofibrate on [1-¹⁴C]Acetate Incorporation into Lipid in Isolated Mammary Acini

TOFA has been shown to inhibit translocation of citrate out of isolated mitochondria (Riberau-Gayon, 1976). Inhibition of citrate transport into the cytosol is associated with diminished production of acetyl-CoA, and hence a decrease in the availability of acetyl units for fatty acid synthesis. However, this effect was not observed by Panek *et al* (1977) who found that the cytoplasmic concentration of citrate in isolated hepatocytes was unchanged in the presence of 0.01 mM TOFA even when fatty acid synthesis was inhibited by 80%. McCune and Harris (1979) confirmed that the inhibition of fatty acid synthesis in isolated hepatocytes was not due to inhibition of citrate transport as TOFA increased the concentration of citrate in both mitochondria and cytosol. Robinson and Williamson (1977a) observed a significant increase (50%) in citrate concentration in the incubation medium of isolated mammary acini incubated with 0.4 mM TOFA. If inhibition of fatty acid synthesis by TOFA occurred as a result of inhibition of citrate translocation out of mitochondria,

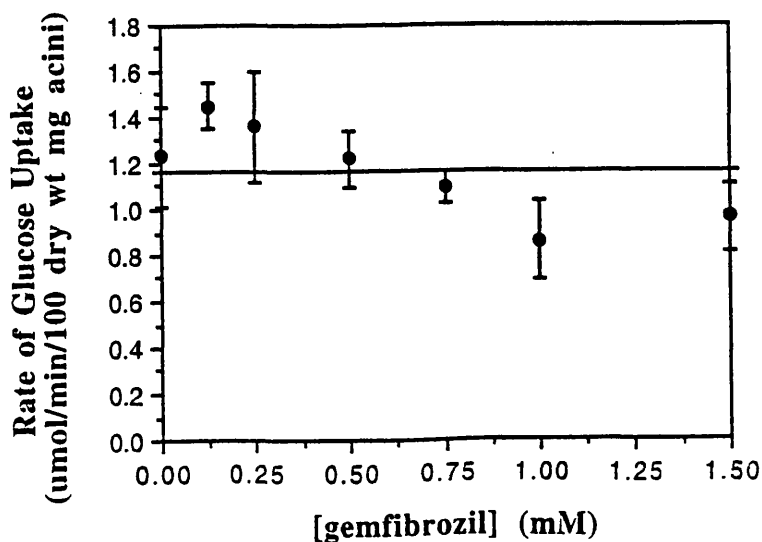
Figure 6.3 Effects of Clofibrate and Gemfibrozil on Glucose Utilisation in Isolated Mammary Acini

For experimental details see text. The results are expressed as μmol glucose utilised/min per 100 mg dry defatted weight of acini. Each value is the mean of at least four separate observations with S.E.M. indicated by vertical bars.

(a) Clofibrate



(b) Gemfibrozil



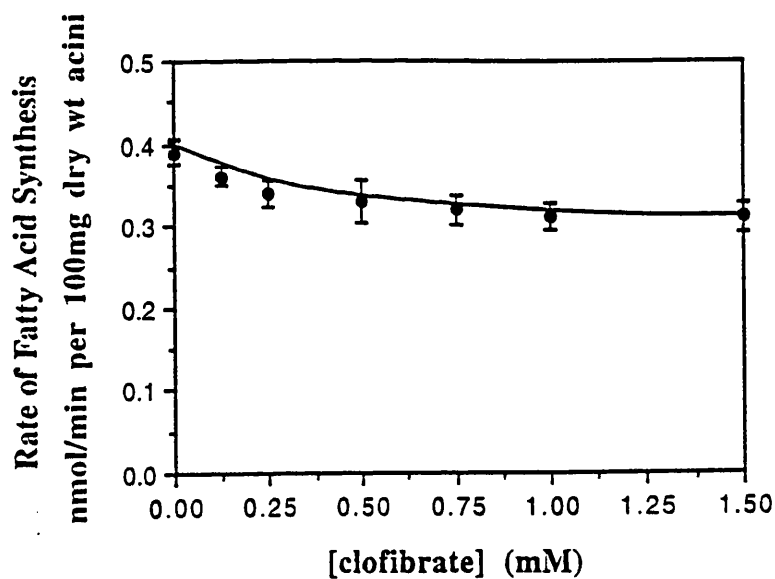
then citrate concentration would be expected to increase in the mitochondria.

To examine if inhibition of fatty acid synthesis by clofibrate occurred as a result of inhibition of translocation of citrate out of the mitochondria, the incorporation of [1-¹⁴C]acetate into fatty acids was measured in isolated mammary acini. Acetate is directly converted to acetyl-CoA by the action of acetyl-CoA synthetase (EC 6.2.1.1) in the cytoplasm (Barth *et al*, 1971). The reaction by-passes glycolysis and those steps involved in the transport of acetyl-CoA generated in the mitochondria, ie citrate synthase (EC 4.1.3.7), citrate transport out of mitochondria and ATP-CL.

The incorporation of [1-¹⁴C]acetate into fatty acids in isolated mammary acini showed a small but significant decrease when incubated with increasing concentrations of clofibrate (Figure 6.4). At 1 mM clofibrate, the rate of [1-¹⁴C]acetate incorporation into fatty acids was inhibited by 20% in comparison to the control value. This is smaller than the inhibition of tritium incorporation into fatty acids which was inhibited by 46% in the presence of 1 mM clofibrate (Figure 6.2a). Inhibition of [1-¹⁴C]acetate incorporation into fatty acids by clofibrate in acini is in keeping with the effect of TOFA (50 μM) in isolated hepatocytes where [1-¹⁴C]acetate incorporation is inhibited by 50% (Panek *et al*, 1977). Since the regulatory step in the conversion of acetate to fatty acids is via

Figure 6.4 Effect of Clofibrate on the Rate of Fatty Acid Synthesis from Acetate

For experimental details see Materials and Methods. The results are expressed as nmol [^{14}C]acetate incorporated/min per 100 mg dry defatted weight of acini and are means for six observations with S.E.M. indicated by vertical bars.



ACC, the results suggest that ACC may be the target of clofibrate in the inhibition of fatty acid synthesis. The effect of the fibrates on the activity of this enzyme was therefore determined.

6.5 Effects of Clofibrate and Gemfibrozil on Acetyl-CoA Carboxylase Activity in Isolated Mammary Acini

The dose-dependent effect of a 60 minute incubation of isolated mammary acini with increasing concentrations of clofibrate and gemfibrozil on the activity of ACC measured in crude homogenates is shown in Figure 6.5. ACC activity measured at 10 mM citrate decreased in a dose-related manner and the decrease in activity of the enzyme closely parallels the inhibition of fatty acid synthesis (Figure 6.2). The activity of ACC was inhibited by 24% in the presence of 1 mM clofibrate and 60% in the presence of gemfibrozil. This greater inhibitory effect of gemfibrozil on ACC correlates with that on the rate of fatty acid synthesis. TOFA has also been shown to inhibit ACC in a dose-dependent manner in isolated hepatocytes (McCune and Harris, 1979). Since ACC has a half-life of 1-3 days (Nakanishi and Numa, 1970), inhibition of the enzyme by clofibrate and gemfibrozil is unlikely to have occurred as a result of changes in the concentration of ACC. The effect of the fibrates on ACC is likely to be mediated via an allosteric effect or covalent modification of the enzyme.

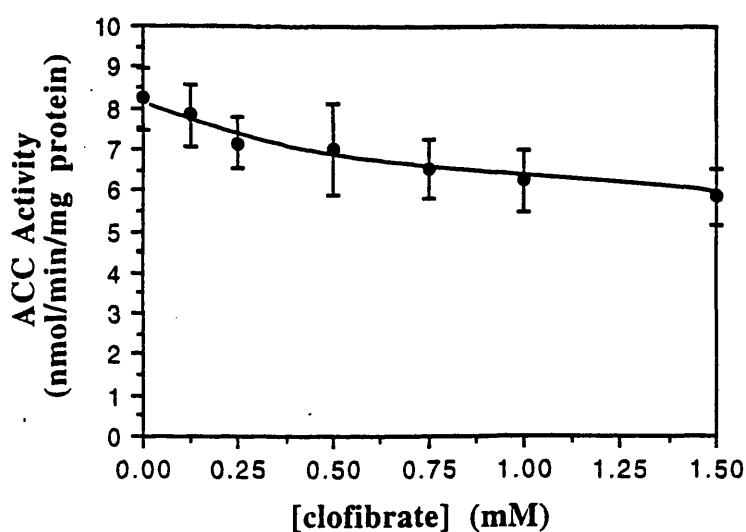
6.6 Effects of Clofibrate and Gemfibrozil on Purified Acetyl-CoA Carboxylase Activity In Isolated Mammary Acini

ACC was purified from acini incubated with 1 mM clofibrate and gemfibrozil using avidin-Sepharose chromatography. Inhibition of ACC activity by clofibrate and gemfibrozil was found to persist upon purification of the enzyme (Figure 6.6). Purified ACC from control acini showed citrate-dependent activity with approximately 20-fold activation from 0 to 20 mM citrate. Inhibition of the enzyme by clofibrate and gemfibrozil was evident at all citrate concentrations and kinetic analysis (Table 6.1) revealed that clofibrate and gemfibrozil decreased the V_{\max} of ACC by 11% and 30%, respectively. The concentration of citrate required for half-maximal activation of ACC (K_a citrate) was increased by 7% and 32% in the presence of clofibrate and gemfibrozil, respectively (Table 6.1). The inhibition of purified ACC by clofibrate is not statistically significant but the inhibition in response to gemfibrozil clearly is. This again, reflects the greater potency of gemfibrozil both as an inhibitor of ACC and lipogenesis and as a hypolipidaemic drug. Inhibition of purified ACC activity usually indicates increased phosphorylation of the enzyme. The known allosteric effectors of ACC such as CoA, fatty acyl-CoA and citrate cannot remain bound through the purification procedures, although it is possible that the fibrates are very tightly bound to the enzyme. In support of this possibility,

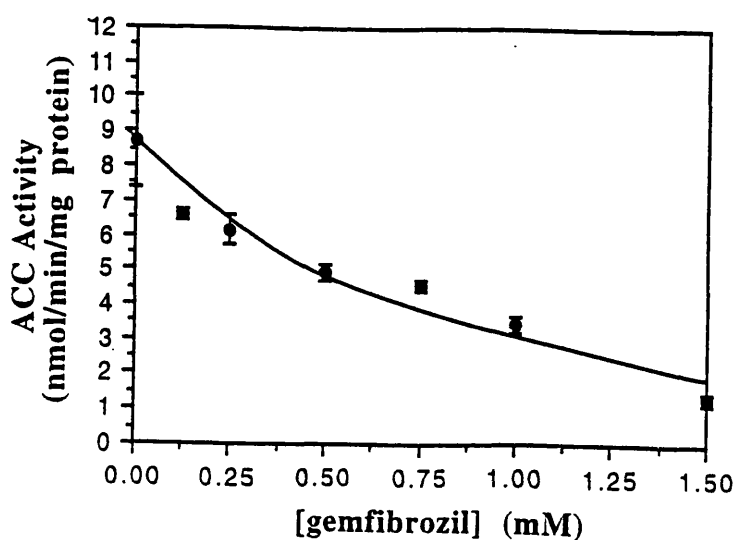
Figure 6.5 Effects of Clofibrate and Gemfibrozil on Acetyl-CoA Carboxylase Activity in Crude Extracts of Mammary Acini

For assay of ACC see Materials and Methods. Acini were incubated in increasing concentrations of (a) clofibrate and (b) gemfibrozil. Results are expressed as nmol $\text{NaH}^{14}\text{CO}_3$ incorporated/min per mg protein and are means for at least 4 observations, with S.E.M. indicated by vertical bars.

(a) Clofibrate



(b) Gemfibrozil



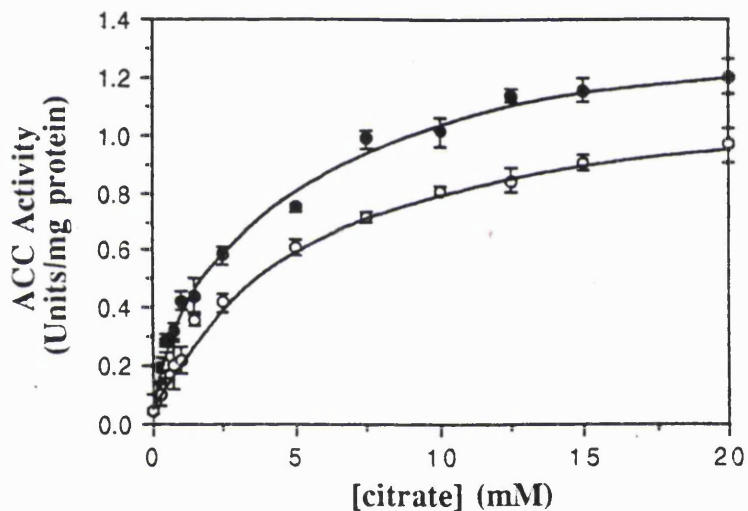
it is notable that the degree of ACC inhibition in response to 1 mM concentration of either fibrate following enzyme purification (Table 6.1) was considerably less than that measured in crude cell extracts (Figure 6.5).

Earlier studies showed that direct addition of clofibrate (1.25 mM) *in vitro* inhibited ACC purified from chicken livers by 50% (Maragoudakis, 1969). The inhibition was competitive for citrate and acetyl-CoA but non-competitive for ATP and HCO_3^- . Competitive inhibition occurs by either the drugs competing with the substrates for the same active site on ACC or by interfering with the activation of ACC by citrate (Maragoudakis, 1970). Studies by Sanchez *et al* (1993) also showed that addition of 1 mM clofibrate and gemfibrozil to the ACC assay mixture resulted in inhibition of rat liver ACC by 17% and 48%, respectively. This inhibition was dependent on the drug concentration. In contrast, no direct inhibition of ACC in crude homogenates of acini was observed in the present study when clofibrate and gemfibrozil were added at various concentrations to the assay mixture (Figure 6.7). The discrepancy between the results of the present study and that observed by the other authors is not known. It could be due to the source and/or the enzyme preparation. In the study of Sanchez *et al* (1993), the enzyme was prepared by ammonium sulphate precipitation (35%) of a cold-clamped rat liver homogenate and 250 μg of protein was used in the assay. In the present study however, crude homogenates

Figure 6.6 Effects of Clofibrate and Gemfibrozil on Purified Acetyl-CoA Carboxylase Activity

For purification and assay of ACC see Materials and Methods. Acini were incubated for 60 min with 1 mM of (a) clofibrate and (b) gemfibrozil. Results are expressed as Units/mg protein and are means for 4 observations, with S.E.M. indicated by vertical bars.

(a) Clofibrate



(b) Gemfibrozil

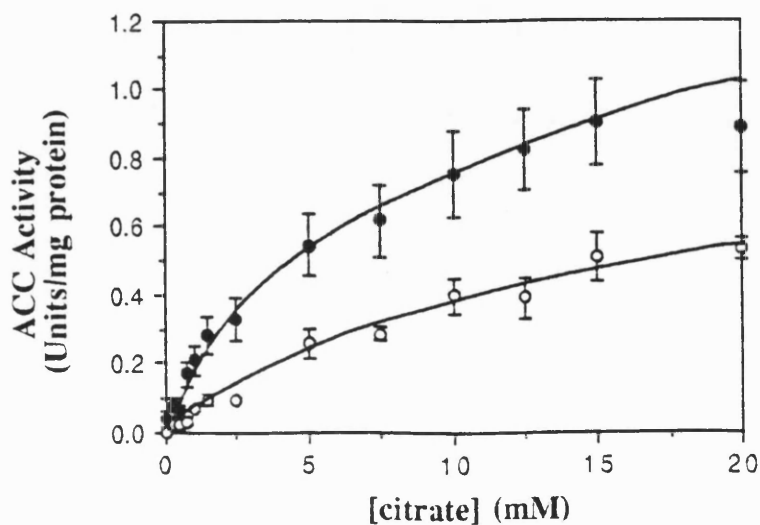


Table 6.1 Effects of Clofibrate and Gemfibrozil on the V_{\max} and K_a for Citrate of Purified Acetyl-CoA Carboxylase

V_{\max} and K_a citrate were determined as described in Materials and Methods. The results are mean values \pm S.E.M. for 4 observations. Values that are significantly different (by Student's t-test) from control values are shown :

* $P < 0.05$.

(a) Clofibrate

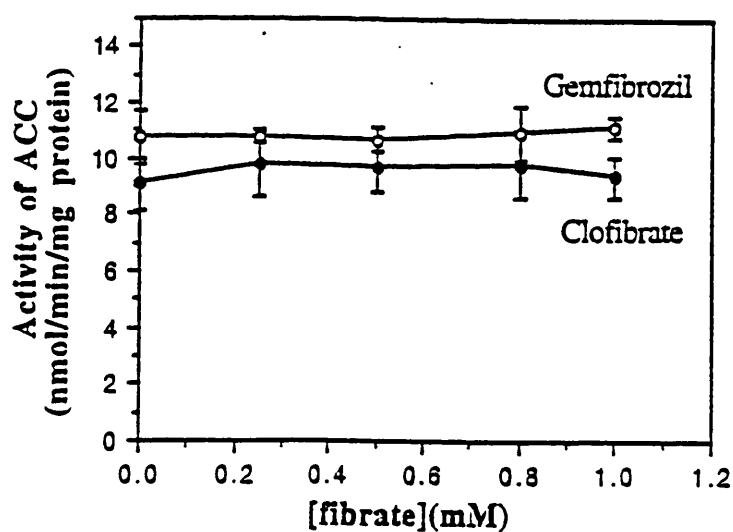
	V_{\max}	K_a citrate
Control	0.98 ± 0.04	1.25 ± 0.31
Clofibrate	0.87 ± 0.09	1.34 ± 0.17

(b) Gemfibrozil

	V_{\max}	K_a citrate
Control	0.79 ± 0.09	2.05 ± 0.26
Gemfibrozil	$0.55 \pm 0.04^*$	$3.05 \pm 0.79^*$

Figure 6.7 Direct Effects of Clofibrate and Gemfibrozil on Acetyl-CoA Carboxylase Activity

ACC activity in crude extracts of mammary acini was assayed as described in Materials and Methods in the presence of clofibrate and gemfibrozil added directly to the assay mixture at various concentrations. Results are expressed as nmol $\text{NaH}^{14}\text{CO}_3$ incorporated/min per mg protein and are means for at least 4 observations, with S.E.M. indicated by vertical bars.



of lactating rat mammary acini containing approximately 100 µg protein prepared were used in the assay.

McCune and Harris (1979) have reported that inhibition of fatty acid synthesis by TOFA in isolated rat hepatocytes is due to its conversion to the TOFyl-CoA ester which inhibits ACC. TOFyl-CoA was a potent inhibitor of partially purified rat liver ACC whereas TOFA of the same concentrations had no inhibitory effect. There is much evidence that the active species of the fibrates are their fatty acyl-CoA thioesters (see Section 6.1). However, there appears to be no correlation between the concentration of fibryl-CoA esters in isolated rat hepatocytes and their relative hypolipidaemic activity (Bronfman *et al*, 1992). These authors proposed that hydrophobicity of an individual fibrate may play a more important role in its action. The free intracellular concentrations of fibryl-CoA esters is difficult to determine. However, if the active species of the fibrates is indeed fibryl-CoA, inhibition of ACC may then be brought about by an allosteric inhibition analogous to that of fatty acyl-CoA esters which are known to be potent inhibitors of ACC (Ogiwara *et al*, 1978). Alternatively, fibryl-CoA may stimulate the activity of the AMP-PK-kinase that phosphorylates and activates AMP-PK in the kinase cascade that controls fatty acid and cholesterol synthesis. Activated AMP-PK in turn phosphorylates and inactivates ACC and fatty acid synthesis. The precedent for such a mechanism

is the stimulation of AMP-PK-kinase by palmitoyl-CoA (Carling *et al*, 1987).

6.7 [³²P]Phosphate-Labelling of Acini

The fact that inhibition of ACC by clofibrate and gemfibrozil has survived extensive dilution during the assay of crude extracts and the purification on avidin-Sepharose chromatography suggests that fibrates may stimulate phosphorylation and inactivation of ACC via AMP-PK. In order to determine if the phosphate content of ACC was increased in response to clofibrate and gemfibrozil, isolated acini were incubated with [³²P]phosphate followed by treatment with 1 mM clofibrate or gemfibrozil. ACC was purified from the ³²P-labelled cells by avidin-Sepharose chromatography and the incorporation of ³²P into ACC was determined as described in Chapter 2 (section 2.17).

Clofibrate and gemfibrozil increased the amount of radioactive phosphate incorporation into ACC (expressed as cpm/μg protein, Table 6.2) by 6% and 10% above the control value, respectively. SDS-PAGE and autoradiograph of the protein matched-samples of crude extracts and purified ACC from [³²P]-labelled acini incubated with clofibrate and gemfibrozil (Figure 6.8 and 6.9) does not reveal a significant increase. It is reported that ACC purified from rat

**Table 6.2 Effects of Clofibrate and Gemfibrozil on [³²P]phosphate
Incorporation into Acetyl-CoA Carboxylase**

Acini were incubated with ³²P for 60 min followed by incubation for a further 60 min in the presence or absence of 1 mM clofibrate or gemfibrozil. For experimental details see Materials and Methods. The ³²P content of the eluate with highest protein concentration from avidin-Sepharose chromatography were estimated by Cerenkov counting. The results are expressed as cpm/μg protein.

<u>Acini treatment</u>	<u>³²P content of ACC</u>
	<u>cpm/μg protein</u>
Control	4335.83
Clofibrate	4591.77
Gemfibrozil	4735.46

Figure 6.8 Coomassie Blue-Stained Gel and Autoradiograph of Crude Extracts of Acini from [32 P]phosphate-Labelled Acini followed by Incubation with Clofibrate and Gemfibrozil

For experimental details see Materials and Methods. Acini were labelled with [32 P]phosphate for 60 min before incubation for a further 60 min in the presence or absence of 1 mM clofibrate or 1 mM gemfibrozil. (A) Coomassie Blue-stained gel. Lanes are 1 : molecular weight markers; 2 : crude extract from control acini; 3 : crude extract from clofibrate-treated acini; 4 : crude extract from gemfibrozil-treated acini. (B) Autoradiograph of Coomassie Blue-stained gel. Lanes are 5: crude extract from control acini; 6 : crude extract from clofibrate-treated acini; 7 : crude extract from gemfibrozil-treated acini.

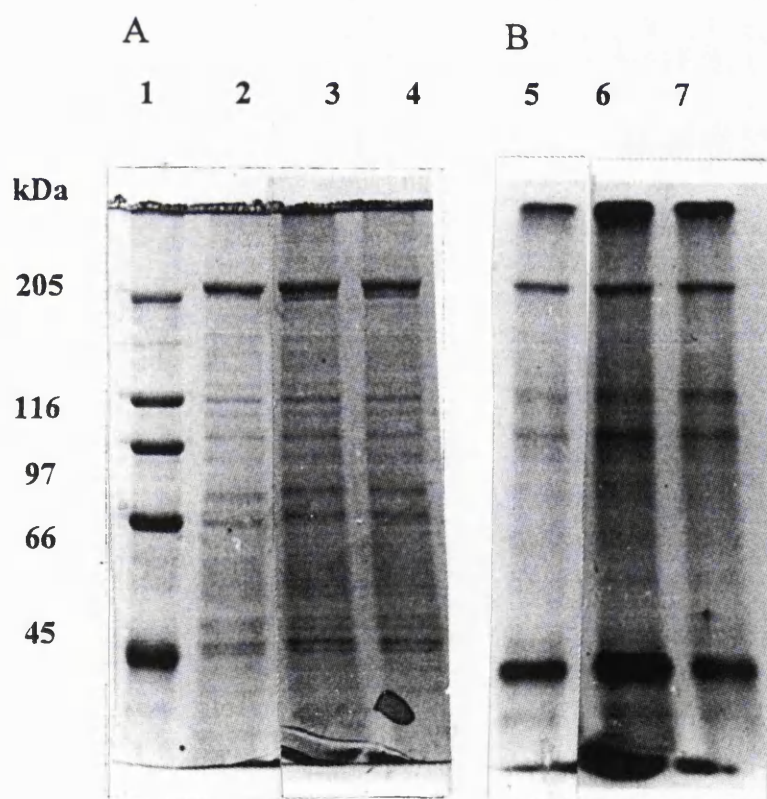
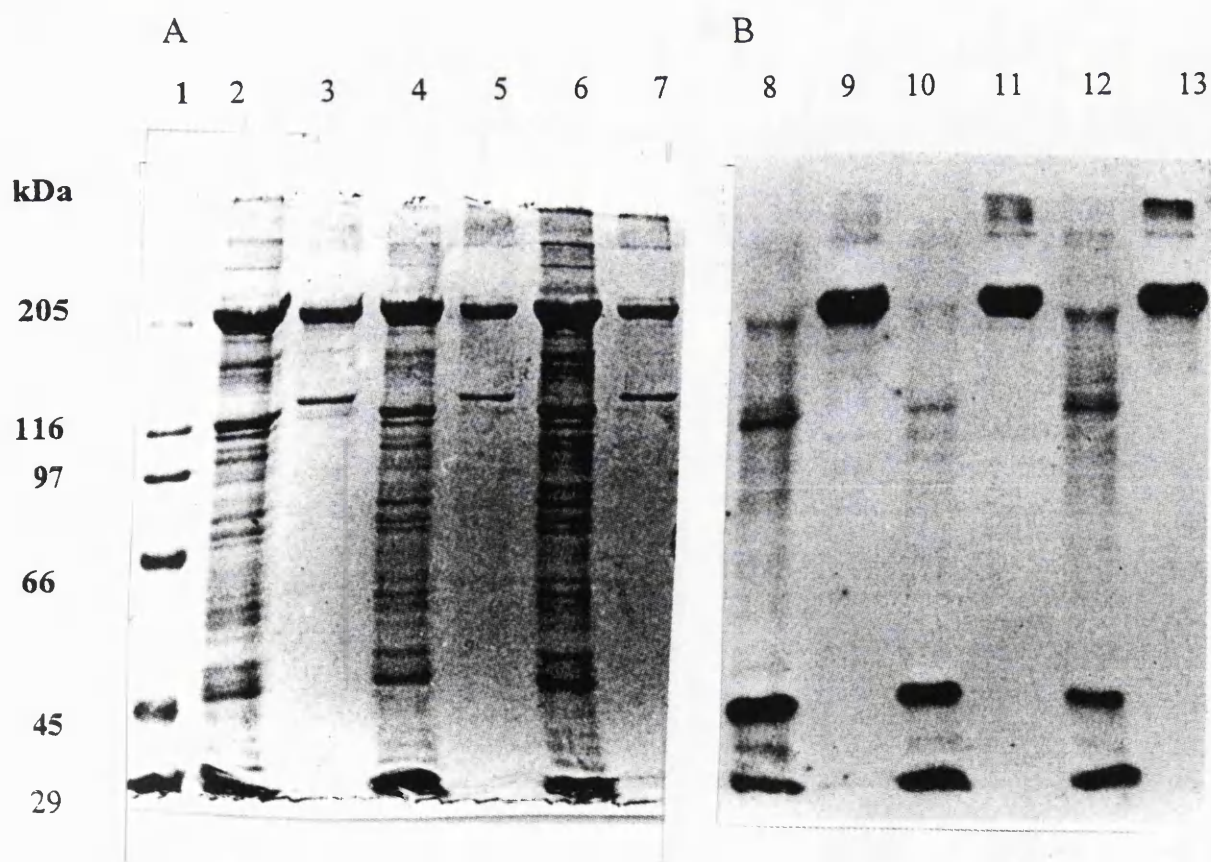


Figure 6.9 Coomassie Blue-Stained Gel and Autoradiograph of Breakthrough from Avidin-Sepharose Affinity Column and Purified Acetyl-CoA Carboxylase from [32 P]phosphate-Labelled Acini followed by Incubation with Clofibrate and Gemfibrozil

For experimental details see Materials and Methods. (A) Coomassie-stained gel. Lanes are 1 : molecular weight markers; 2 : column breakthrough from control acini; 3 : purified ACC from control acini; 4 : column breakthrough from clofibrate-treated acini; 5 : purified ACC from clofibrate-treated acini; 6 : column breakthrough from gemfibrozil-treated acini; 7 : purified ACC from gemfibrozil-treated acini. (B) Autoradiograph of Coomassie-stained gel. Lanes are 8: column breakthrough from control acini; 9 : purified ACC from control acini; 10 : column breakthrough from clofibrate-treated acini; 11 : purified ACC from clofibrate-treated acini; 12 : column breakthrough from gemfibrozil-treated acini; 13 : purified ACC from gemfibrozil-treated acini.



mammary gland contains 3-4 mol phosphate/mol (Hardie and Guy, 1980). A 10% increase in ^{32}P incorporation into purified ACC by 1 mM gemfibrozil would suggest an increase of phosphate content of 0.3-0.4 mol phosphate/mol. If this occurred exclusively in a single serine residue, for example serine-79, then it could account for the inactivation of ACC observed. The results from the present study are too preliminary for one to draw a conclusion as to whether phosphorylation plays a role in inhibiting ACC. Phosphorylation site analysis of the purified ^{32}P -labelled ACC would be necessary to see if phosphorylation of ACC in response to gemfibrozil has indeed occurred.

6.8 Effects of Clofibrate and Gemfibrozil on Lactate Production in Isolated Mammary Acini

Since the extent of inhibition of ACC by clofibrate and gemfibrozil is lower than the inhibition of fatty acid synthesis, one might expect inhibition of other steps in the pathway to contribute to the decrease in lipogenesis.

Figure 6.10 shows lactate production by mammary acini in response to clofibrate and gemfibrozil. The increase in accumulation of lactate in the incubation media occurred in a dose-dependent manner. At 1 mM clofibrate and gemfibrozil, lactate production in acini increased by 49% and 71%, respectively.

In acini incubated with clofibrate, increased lactate production reached a plateau at 1.5 mM whereas lactate production was still showing an increase at 1.5 mM gemfibrozil. This suggests that gemfibrozil may have a greater effect on the flux of glucose through the step catalysed by PDH. The effect of clofibrate and gemfibrozil on lactate production is in contrast to that of TOFA which was reported to cause a small but consistent decrease in lactate and pyruvate accumulation in isolated hepatocytes (Panek *et al*, 1977). This has been taken as an indication of an inhibitory effect of TOFA on glycolysis by these authors. However, Robinson and Williamson (1977a) observed no changes in lactate accumulation in mammary acini incubated with TOFA.

6.9 Effects of Clofibrate and Gemfibrozil on Pyruvate Dehydrogenase Activity in Isolated Mammary Acini

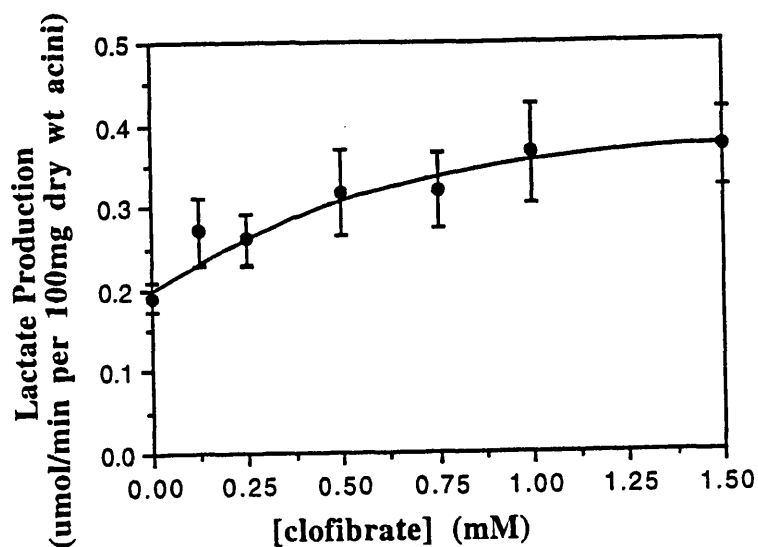
The activity of PDH in the presence of 1 mM clofibrate and gemfibrozil was reduced significantly by 33% and 30%, respectively (Table 6.3). Despite the significant difference in potency displayed by 1 mM clofibrate and gemfibrozil on fatty acid synthesis (Figure 6.2), ACC activity (Figure 6.4) and lactate production (Figure 6.10), both drugs inhibit PDH activity to a similar extent. The inhibition of PDH activity is likely to occur as a result of increased [acetyl-CoA]/[CoA] and/or [NADH]/[NAD] ratios which leads to activation of PDH

Figure 6.10 Effects of Clofibrate and Gemfibrozil on Lactate Production

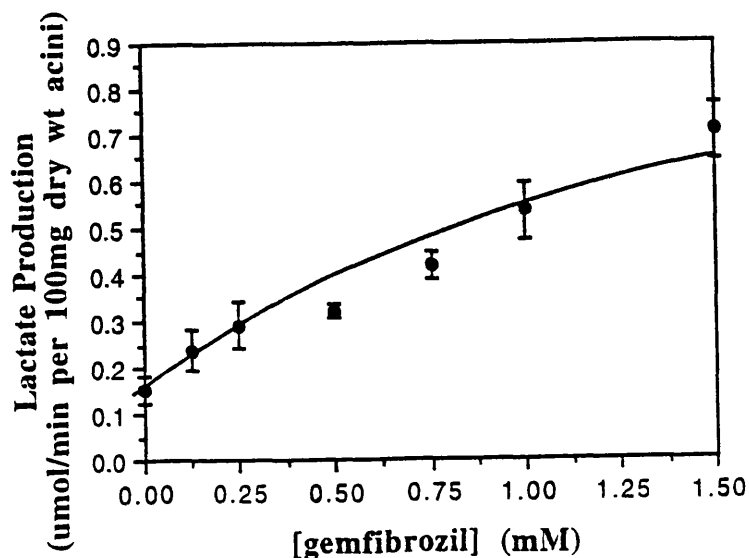
In Isolated Mammary Acini

For experimental details see Materials and Methods. Acini were incubated for 60 min with increasing concentrations of (a) clofibrate and (b) gemfibrozil. The results are expressed as $\mu\text{mol}/\text{min}$ per 100 mg dry defatted weight of acini and are means for at least 5 preparations with S.E.M. indicated by the vertical bars.

(a) Clofibrate



(b) Gemfibrozil



kinase. This in turn causes phosphorylation and inactivation of PDH. The increased [acetyl-CoA]/[CoA] may have occurred as a result of inactivation of ACC as found previously with okadaic acid (Chapter 4). Inhibition of PDH activity by clofibrate and gemfibrozil is likely to contribute to the decrease in fatty acid synthesis in acini as the flux through the PDH step is reduced.

6.10 *In Vivo* Studies of the Effects of Gemfibrozil on Lipid Biosynthesis

Short term inhibition of fatty acid synthesis by clofibrate and gemfibrozil has been clearly demonstrated in isolated rat mammary acini. It has been reported that hepatic VLDL and cholesterol synthesis are inhibited by long term administration of the fibrates (Kesaniemi and Grundy, 1984; Castillo *et al*, 1990). In view of the fact that the liver is the major tissue in which VLDL triglyceride and cholesterol synthesis takes place, the short term *in vivo* effects of gemfibrozil on the rate of fatty acid and cholesterol synthesis in rat liver were examined.

6.10.1 Hepatic Fatty Acid Synthesis

The administration of a single intraperitoneal dose of gemfibrozil (250 mg/kg) to normal fed rats at D6 in the diurnal cycle resulted in significant

Table 6.3 Effects of Clofibrate and Gemfibrozil on Pyruvate Dehydrogenase Activity

For measurement of PDH activity see Materials and Methods. Acini were incubated for 60 min with 1 mM (a) clofibrate and (b) gemfibrozil. Results are expressed as mUnits PDH/unit citrate synthase and are means \pm S.E.M. with the number of observations indicated in parentheses. Values that are significantly different (by Student's t-test) from the corresponding control values are shown : * $< P$ 0.05; ** $P < 0.005$.

(a) Clofibrate

	<u>PDH Activity</u>
Control	33.47 \pm 2.45 (6)
Clofibrate	22.63 \pm 2.76 (6) *

(b) Gemfibrozil

	<u>PDH Activity</u>
Control	38.03 \pm 2.42 (5)
Gemfibrozil	26.81 \pm 2.17 (5) **

inhibition of hepatic lipogenesis (Table 6.4). The rates of fatty acid synthesis in liver decreased by 54%, 68% and 69% relative to control animals after 90 min, 150 min and 24 hour following gemfibrozil administration, respectively. This was clearly a relatively rapid effect compared with long-term feeding of fibrates and is in keeping with the rapid inhibition of fatty acid synthesis by gemfibrozil in isolated mammary acini (Figure 6.2) and by TOFA in isolated hepatocytes (McCune and Harris, 1979). ACC is a likely target for gemfibrozil action in the liver. ACC is known to be inhibited *in vitro* by gemfibrozil (Maragoudakis and Hankin, 1971; Sanchez *et al*, 1993) and was inhibited in mammary acini in response to the drug (Figure 6.5). Furthermore ACC has been implicated as the site that mediates TOFA inhibition of lipogenesis in hepatocytes (McCune and Harris, 1979). The active intracellular compound is likely to be gemfibrozyl-CoA (see pp 255-256) and this could inhibit through mechanisms shown to exist for fatty acyl-CoA ie. allosteric inhibition (Ogiwara *et al*, 1978) or activation of the AMP-PK cascade resulting in ACC phosphorylation (Carling *et al*, 1987). A consequence of ACC inhibition would be a decrease in the concentration of malonyl-CoA formed from acetyl-CoA. Malonyl-CoA is a potent inhibitor of carnitine acyltransferase which is involved in the transportation of activated fatty acid into mitochondria. Lack of inhibition of this enzyme by malonyl-CoA leads to diversion of fatty acids towards β -oxidation and away from esterification. Increased activity of carnitine acyltransferase and β -oxidation of fatty acids in

response to the fibrates is well documented (Lazarow, 1977) and may therefore contribute to the hypolipidaemic effect of the drugs.

PDH inhibition accompanies ACC inhibition by gemfibrozil in isolated mammary acini (Figure 6.5, Table 6.3) although whether PDH inhibition was a consequence of ACC inhibition, as with okadaic acid treatment, was not tested. PDH does have a regulatory function in rat liver (Sugden *et al*, 1993) and could also be a gemfibrozil target.

6.10.2 Hepatic Cholesterol Synthesis

Cholesterol synthesis was inhibited by approximately 84% in rats treated with gemfibrozil for 90 min (Table 6.5). This inhibition did not increase at 150 min and in rats treated with gemfibrozil for 24 hours, the rate of cholesterol synthesis was found to return to the control value.

Inhibition of cholesterol synthesis is well documented in long term treatment of animals with clofibrate. Cohen *et al* (1974) showed that chronic treatment of rats with clofibrate inhibited cholesterol synthesis in rat liver by 25% and this was accompanied by inhibition of HMG-CoA reductase by 50%. In the studies of Castillo *et al* (1990), feeding clofibrate to chicks (3 mg/100 g

body weight) for 15 days resulted in more than 90% inhibition of hepatic HMG-CoA reductase. In addition, in rats treated with clofibrate for 7 days, rate of cholesterol synthesis was decreased by over 70% and this was accompanied by inhibition of HMG-CoA reductase activity by 50% (Haughom *et al*, 1992).

Despite the fact that fatty acid and cholesterol synthesis are related processes sharing a common precursor, their responses to gemfibrozil in rat liver were different. Both were rapidly inhibited within 90 min but the response of cholesterol synthesis was complete at 90 min whereas inhibition of fatty acid synthesis was slower and continued at 150 min (Table 6.5). This may reflect different sensitivities of ACC and HMG-CoA reductase to allosteric inhibition by gemfibrozyl-CoA or different rates of phosphorylation of ACC and HMG-CoA reductase by an activated AMP-PK. Furthermore, ACC turns over very slowly (half life 1-3 days) whereas HMG-CoA reductase turns over rapidly (half life 2-4 hours). Therefore the faster inhibition of cholesterol synthesis may reflect a combination of enzyme inactivation and decrease in enzyme concentration. Resynthesis of HMG-CoA reductase is the likely explanation for the restoral of cholesterol synthesis 24 hour after the single dose of gemfibrozil.

6.10.3 Fatty Acid Synthesis in White Adipose Tissue

In white adipose tissue, the rates of fatty acid synthesis were stimulated by 70% after administration of a single dose of gemfibrozil (250 mg/kg) for 90 and 150 minutes. The increase in the rate of fatty acid synthesis 24 hour after dosing was slightly lower, showing a 57% increase above the control value (Table 6.4).

White adipose tissue is an important site of lipogenesis in mammals. The effect of gemfibrozil on white adipose tissue lipogenesis raises the question as to the mechanism leading to lipogenic stimulation in this tissue. A possible explanation for the present findings is that inhibition of hepatic fatty acid and cholesterol synthesis by gemfibrozil leads to redistribution of lipogenic substrates towards adipose tissue. However, it is interesting that ACC and AMP-PK present in white adipose tissue (see Section 1.8.6) would appear not to be targets for gemfibrozil inhibition.

Table 6.4 Effects of Gemfibrozil on Hepatic and White Adipose Tissue Lipogenesis

For experimental details see the text. The results are mean values \pm S.E.M. with the numbers of observations in parentheses. The rates of (a) hepatic lipogenesis and (b) lipogenesis in white adipose tissue are expressed as $\mu\text{mol } ^3\text{H}_2\text{O}$ incorporated into saponified lipid/hour per g fresh wt. of tissue. Values that are significantly different (by Student's t-test) from the control values are shown : * $P < 0.001$; ** $P < 0.005$.

(a) Hepatic Lipogenesis

	<u>90 min</u>	<u>150 min</u>	<u>24 hour</u>
Control	13.44 \pm 1.26 (3)	13.24 \pm 1.05 (4)	15.22 \pm 0.75 (2)
Gemfibrozil	6.16 \pm 0.82 (5)**	4.23 \pm 0.76 (6)*	4.67 \pm 0.60 (4)*

(b) White Adipose Tissue Lipogenesis

	<u>90 min</u>	<u>150 min</u>	<u>24 hour</u>
Control	8.41 \pm 1.41 (3)	9.02 \pm 1.26 (4)	11.61 \pm 3.64 (2)
Gemfibrozil	33.81 \pm 2.71 (5)*	31.08 \pm 2.70 (6)*	27.16 \pm 3.88(4)*

Table 6.5 Effects of Gemfibrozil on Hepatic Cholesterol Synthesis

For experimental details see the text. The results are mean values \pm S.E.M. with the numbers of observations in parentheses. The rates of hepatic cholesterol synthesis are expressed as $\mu\text{mol } ^3\text{H}_2\text{O}$ incorporated into cholesterol/hour per g fresh wt. of tissue. Values that are significantly different (by Student's t-test) from the control values are shown : * $P < 0.001$.

Rate of Hepatic Cholesterol Synthesis

	<u>90 min</u>	<u>150 min</u>	<u>24 hour</u>
Control	7.93 \pm 1.08 (3)	7.60 \pm 1.34 (4)	7.38 \pm 1.65 (2)
Gemfibrozil	1.27 \pm 0.17 (5)*	1.39 \pm 0.06 (6)*	6.42 \pm 0.76 (4)

6.11 Conclusion

The results from this study have shown that gemfibrozil *in vivo* can produce a rapid inhibition of hepatic lipogenesis and cholesterol synthesis which may contribute to the decrease in VLDL production that this drug produces. The speed of action suggests short-term control of the regulatory steps in the pathways of lipid biosynthesis. In isolated mammary acini, both clofibrate and gemfibrozil inhibited fatty acid synthesis without affecting glucose uptake by the cells. Both agents inactivated ACC and PDH and this appeared to be mediated by phosphorylation of these enzymes. Activation of AMP-PK to phosphorylate and inactivate ACC, and activation of PDH kinase via an increased acetyl-CoA/CoA ratio, is a potential mechanism via which this could be achieved, and remains to be investigated.

A surprising observation was that gemfibrozil stimulated lipogenesis in white adipose tissue *in vivo*. This is possibly the result of an increase in circulating substrate concentrations resulting from the inhibition of hepatic lipid synthesis and further work will be necessary to confirm this action of gemfibrozil.

CHAPTER 7

GENERAL CONCLUSIONS

Acetoacetate is a potential physiological signal for the decrease in glucose uptake and fatty acid synthesis in lactating rat mammary gland during starvation. This study confirmed that glucose uptake of isolated mammary acini was inhibited by acetoacetate but has shown that this was not the result of an inhibition of glucose transport across the plasma membrane. PDH was inactivated by acetoacetate and this is likely to have occurred in response to an increased acetyl CoA/CoA ratio that would result from the metabolism of acetoacetate and which would activate PDH kinase. It is unlikely that PDH inhibition alone could account for the decreased glucose uptake. This is supported by the studies with okadaic acid-treated or gemfibrozil-treated acini in which PDH inhibition was not accompanied by any changes in glucose uptake. It seems likely from the observations of Williamson and coworkers that decreased glucose uptake in response to acetoacetate is primarily the result of PFK-1 inhibition by the elevated levels of citrate. The increased citrate concentration was not the result of ACC inactivation. On the contrary, ACC appears to be activated by acetoacetate via a mechanism that survives purification and suggests enzyme dephosphorylation. ATP-CL is a potential target whose inhibition would lead to citrate accumulation and ATP-CL was inhibited in response to acetoacetate but to an extent that was unlikely to account for the level of inhibition of fatty acid synthesis. Thus it can be speculated that the inhibition of fatty acid synthesis by acetoacetate is directly linked to the inhibition of glucose uptake, possibly via a reduced provision of NADPH for fatty acid synthesis or glycerol-3-phosphate for esterification.

Protein phosphatase activity (PP1 and PP2A) were shown to be present in the lactating rat mammary gland, and their importance to fatty acid synthesis was illustrated by the inhibition of fatty acid synthesis in isolated acini by okadaic acid. Glucose uptake was unaffected suggesting a lack of involvement of phosphorylation in the regulation of GLUT 1, the predominant glucose transporter in lactating mammary gland. Okadaic acid caused the phosphorylation and inactivation of ACC primarily as a result of PP2A inhibition but also via a transient activation of AMP-PK. This was an unexpected finding given that the protein phosphatase that is reported to act on AMP-PK (PP2C) is okadaic acid-insensitive. The explanation may be that there are components in the AMP-PK cascade preceeding AMP-PK that are regulated by okadaic acid-sensitive phosphorylation. The activity of PDH was also inhibited by okadaic acid despite the insensitivity of PDH phosphatase to the inhibitor. Time course studies showed that this occurred as a result of ACC inhibition presumably via stimulation of PDH kinase by the increased acetyl-CoA/CoA ratio caused by ACC inactivation. Previous studies have shown that during starvation, PDH is inactivated before ACC and is probably more important in the inhibition of lipogenesis. In the studies with okadaic acid we have shown that ACC inactivation is able to "drive" the inactivation of PDH.

Polyunsaturated fatty acids (PUFA) are known to have plasma lipid-lowering effects and have been reported to inhibit lipid biosynthesis in hepatocytes. In the present study EPA, linolenic acid and linoleic acid all inhibited fatty acid synthesis in isolated mammary acini. The effect was dose-dependent and also depended on the degree of unsaturation of the fatty acid. These effects were mediated, at least in part, by inactivation of ACC and PDH. Unlike acetoacetate, these PUFA did not inhibit glucose uptake by the acini. Two compounds that could be classed as synthetic fatty acid analogues are

clofibrate and gemfibrozil and both are used as hypotriglyceridaemic drugs. In isolated mammary acini both clofibrate and gemfibrozil had similar actions to the PUFA in that fatty acid synthesis, ACC and PDH were all inhibited. Furthermore, *in vivo* studies in rat liver showed that short term gemfibrozil treatment inhibited both fatty acid and cholesterol synthesis. The evidence from the effects in acini suggests that these hypolipidaemic agents inactivate ACC via an increase in phosphorylation. Thus the AMP-PK is a likely target for their action. Since the AMP-PK kinase is known to be stimulated by palmitoyl-CoA, the possibility that polyunsaturated fatty acyl-CoAs or fibryl-CoAs could have the same effect is worthy of further investigation.

7.1 Future Work

The present study has illustrated the regulation of lipogenesis in the lactating rat mammary gland by various putative effectors and the relationship between a number of regulatory steps. The use of okadaic acid has confirmed the role of reversible phosphorylation in short term control of mammary gland lipogenesis. It would be of great interest to investigate the role of AMP-PK kinase in this control. The possibility that polyunsaturated fatty acids, clofibrate and gemfibrozil stimulate AMP-PK kinase activity in a manner analogous to palmitoyl-CoA is worthy of investigation.

The studies in isolated hepatocytes carried out in the laboratory by Cheryl Hemingway have shown that ACC, HMG-CoA reductase and AMP-PK are targets of gemfibrozil. Further elucidation of the mechanism of action of gemfibrozil on the AMP-PK cascade and the effect of gemfibrozil on white adipose tissue lipogenesis will be important as this may have implications for the study of obesity and hyperlipidaemia.

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