ASPECTS OF THE REGULATION OF LIPID METABOLISM IN THE MAMMARY GLAND AND ADIPOSE TISSUE OF THE RAT.

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TABLE OF CONTENTS

TABLE OF CONTENTS (CONTD.)

TABLE OF CONTENTS (CONTD.)

TABLE OF CONTENTS (CONTD.)

TABLE OF CONTENTS (CONTD.)

TABLE OF CONTENTS (CONTD.)

SUMMARY

The effects of insulin, glucagon and other agents on rates of lipogenesis and activity of acetyl-CoA carboxylase were measured in rat mammary glands during lactation *in vitro*. As a comparison to this, the effect of glucagon on rates of lipolysis in rat adipose tissue *in vitro* was investigated at different stages of the reproductive cycle.

The rate of lipogenesis in acini isolated from mammary glands of mid-lactating rats was studied by measuring the rate of incorporation of ^{3}H from $^{3}H_2O$ into total lipid and fatty acids, with glucose as a substrate. Glucagon did not affect the rate of lipogenesis in rat mammary glands *in vitro* in the presence or absence of agents which modulate its effects on adipocytes. Glucagon did not antagonize the maximal stimulatory effect of insulin, nor did it alter the insulin dose-response curve. Theophylline, at concentrations up to 20mM, was a potent inhibitor of lipogenesis in acini. Glucagon did not augment the degree of inhibition of lipogenesis induced by 5mM-theophylline. The results suggest that the mammary gland acini do not respond to glucagon *in vitro*. In agreement with these observations, only a minimal degree of specific binding of ^{125}I-labelled glucagon could be detected in acini which bound insulin normally. This difference in responsiveness of mammary and adipose cell preparations

in vitro to glucagon suggests that the two tissues may be differentially responsive to changes in the circulating insulin/glucagon concentration ratio *in vivo*.

Other agents, adenosine and adenosine deaminase were found to have no effect on lipogenesis in mammary gland acini. Relationships between the cyclic AMP content and the rate of lipogenesis in acini prepared from lactating rat mammary tissue were investigated by exposing them to theophylline which increases their cyclic AMP content in the presence or absence of insulin. The dose-dependent inhibition of lipogenesis by theophylline in acini isolated from fed rats was highly correlated with the induced increases in acinar cyclic AMP content. Cyclic AMP of acini from 24h-starved lactating rats was more sensitive in its response to theophylline than cyclic AMP in acini from fed animals.

The activity of acetyl-CoA carboxylase in mammary acini was studied under the same conditions as were used for the lipogenesis experiments. Insulin and theophylline were found to have no effect on the activity of acetyl-CoA carboxylase which was in contrast to their effects on rates of lipogenesis. Glucagon, adenosine and adenosine deaminase had no effect, which supported earlier findings.

The dose-response curves for the effect of insulin on lipogenesis in rat mammary gland acini were obtained for preparations from lactating animals in different physiological and pathological conditions. A much closer correlation between changes in acinar rates of lipogenesis *in vitro* and mammary lipogenesis *in vivo* induced by different physiological states was observed than in previous studies. Acini from diabetic and starved rats had the lowest rates of lipogenesis. Cafeteria-feeding did not affect either the basal or maximally insulin-stimulated rates. Acini from streptozotocin-diabetic rats were most sensitive and responsive to insulin, whereas those from early-lactating rats were least responsive. In addition, lipogenesis in acini from early lactating rats and cafeteria-fed rats were least sensitive to insulin. Starvation (24h) did not affect insulin sensitivity but, after 2h refeeding, insulin sensitivity was significantly decreased compared to that in acini from normal, fed mid-lactating rats. The results indicated that in conditions with low-circulating insulin and low rates of lipogenesis, relative to fed animals, the response to insulin was greater and the concentration of insulin required for the half-maximal response was smaller i.e. increased sensitivity.

A study of rates of lipolysis in adipocytes from rats during pregnancy and lactation was undertaken. Incubations were performed in the presence or absence of

sub-maximally effective concentrations of adenosine deaminase and in the presence of several concentrations of glucagon (10^{-11} to 10^{-5}M). Both the maximal response to glucagon and the concentration of the hormone required to elicit half-maximal response (EC_{50}) were markedly altered during pregnancy and lactation compared to those cells from weight-matched virgin rats. The effects of 24h-starvation on these parameters was also altered in pregnant and lactating animals. In the fed state, the highest glucagon-stimulated rates of lipolysis were obtained in adipocytes from mid-lactating rats whereas in the starved state the highest rates were obtained in cells from pregnant and mid-lactating rats. Adipocytes from fed or starved late-pregnant animals had EC_{50} values for glucagon that were several-fold lower than those from animals in any of the other physiological states studied. By contrast, adipocytes from early lactating rats were least responsive and least sensitive to glucagon. These changes in sensitivity and response of lipolytic rate to glucagon in isolated adipocytes correlated with the extent of triacylglycerol mobilization from adipose tissue <u>in vivo</u> as evidenced from changes in mean cell volume of adipocytes obtained from animals in different stages of the reproductive cycle.

ABBREVIATIONS

ACTH	Adrenocorticotropin hormone
ADP	Adenosine 5'-diphosphate
AMP	Adenosine 5'-monophosphate
ATP	Adenosine 5'-triphosphate
BSA	Bovine serum albumin
CoA	Coenzyme A
DAG	Diacylglycerol
DTT	Dithiothreitol
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetra-acetate
EGTA	Ethylene glycol-bis-(2-aminoethyl)-tetra-acetate
GMP	Guanosine 5'-monophosphate
GDP	Guanosine 5'-diphosphate
GTP	Guanosine 5'-triphosphate
GTPase	Guanosine 5'-triphosphatase
Hepes	4-(2-hydroxylethyl)-1-piperizine-ethane-sulphonic acid
IP_3	Inositol 1,4,5-trisphosphate
MCR	Mean cell rate
MCV	Mean cell volume
MOPS	4-morpholinepropanesulphonic acid
NADH	Nicotinamide adenine dinucleotide, reduced
$NADP^+$	Nicotinamide adenine dinucleotide phosphate, oxidised
NEFA	Non-esterified fatty acids

PEP	Phosphoenolpyruvate
Pi	Phosphate
PI	Phosphatidylinositol
PIP_2	Phosphatidylinositol 4,5-bisphosphate
POS	Phospho-oligosaccharide
PPi	Pyrophosphate
Tris	Tris(Hydroxy-methyl)-aminomethane
VLDL	Very low density lipoprotein

LIST OF FIGURES

LIST OF TABLES

PUBLICATIONS

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CHAPTER 1

INTRODUCTION

1.1 LACTATION

1.1.1 Physiological Changes

Pregnancy and lactation are physiological conditions in which the mother must radically re-align her metabolism in order to meet the additional metabolic demands made by the offspring. Milk production by the mammary gland is the additional metabolic system competing for available nutrients with other metabolic processes during lactation.

Various physiological changes occur in the mother that are geared towards promoting an adequate supply of substrates to meet the high metabolic demands of the mammary gland. In the rat, at peak lactation the food intake is two to three-fold higher than in the unmated animal (Fell *et al.*, 1963; Ota & Yokoyama, 1967; Kumaresan & Turner, 1968; Cripps & Williams, 1975) and the cardiac output is increased up to two-fold (Chatwin *et al.*, 1969). In addition to the mammary gland, various organs hypertrophy, in particular the liver and alimentary tract, and the blood flow to these organs is also increased (Fell *et al.*, 1963; Chatwin *et al.*, 1969; Craft, 1970; Smith, 1975; Grigor *et al.*, 1982). In spite of the increased food intake, lactation is generally associated with net depletion of adipose tissue reserves, in contrast to the increased accumulation

of lipid observed during pregnancy (Spray, 1950; Beaton *et al.*, 1954; Knopp *et al.*, 1970; Bershtein & Aleksandrov, 1977; Flint *et al.*, 1979; Sinnett-Smith *et al.*, 1980; Steingrimsdottir *et al.*, 1980a; Naismith *et al.*, 1982; Oller do Nascimento & Williamson, 1986). This is a major adaptation in order to spare substrates such as glucose for the mammary gland and to meet the high demand for fatty acids required for milk production during lactation.

Biochemical changes, e.g. in the plasma concentration of several hormones, accompany these physiological adaptations and different tissues respond to these endocrine alterations in different ways that are discussed later (Vernon, 1989). The result of these concerted changes is that during lactation lipid reserves in adipose tissue are utilised to meet the increasing demands of the biosynthesis of milk lipids by the lactating mammary gland. Milk fatty acid synthesis in the mammary gland is further supplemented by other substrates available in the circulation derived endogenously and from the diet (Williamson, 1973; Vernon & Flint, 1983). Hence there is a fundamental re-direction of lipid metabolism during lactation, the mammary gland becoming a major site both of lipogenesis *de novo* and of the uptake and utilisation of plasma triacylglycerol fatty acids from lipoproteins (Williamson, 1986).

1.1.2 Fate of Dietary Components

In fed lactating rats the main substrates taken up by the mammary gland are glucose and triacylglycerol-fatty acids, the triacylglycerol being available in very low density lipoproteins (VLDL) and chylomicrons. Several studies have shown that plasma glucose is the precursor for the synthesis of lactose (Wilde and Kuhn, 1979) and for a large proportion of milk lipids (O'Hea & Leveille, 1969; Kuhn, 1978). However the substrate requirements for the lactating ruminant mammary gland are different because of their distinctive diet and digestive physiology. Glucose, in lactating ruminants, is not as important a precursor of milk lipids in the mammary gland as it is in lactating rats, with acetate and, to a lesser extent, β-hydroxybutyrate being the main precursors of milk lipids (Balmain et al., 1952; Linzell et al., 1972; Bickerstaffe et al., 1974; Moore & Christie, 1979). Glucose, due to its limited availability in the ruminant, is primarily a precursor for lactose synthesis, the glycerol moiety of milk lipids and the generation of reducing equivalents for lipogenesis (Hardwick et al., 1963; Bickerstaffe et al., 1974).

A number of potential rate-limiting steps in the pathway from glucose to fatty acids have been

identified, in particular the activity of acetyl-CoA carboxylase and the rate of glucose transport across the plasma membrane. Acetyl-CoA carboxylase (EC 6.4.1.2) catalyses the first step committed to fatty acid synthesis and is generally believed to be an important regulatory enzyme in this pathway in the mammary gland (Williamson, 1980; Munday & Williamson, 1981; Clegg 1988). From parturition to peak lactation, an increase in the activity of acetyl-CoA carboxylase in the rat mammary gland parallels the increased rates of fatty acid synthesis (Mackall & Lane, 1977; Agius & Williamson, 1980; Martyn & Hansen, 1981). At the onset of lactation, the amount of acetyl-CoA carboxylase in the mammary gland increases and is accompanied by an increase in the proportion of acetyl-CoA carboxylase in the active state relative to non-lactating rats (Mackall & Lane, 1977; McNeillie & Zammit, 1982). Acetyl-CoA carboxylase is an enzyme subject to reversible phosphorylation/dephosphorylation mediating activation and inhibition of the enzyme by various hormones, but no change in the phosphorylation state is apparent at the different stages of lactation once lactation is established (McNeillie & Zammit, 1982). The ratio of 'initial' acetyl-CoA carboxylase activity (measured in crude extracts with no preincubation) to 'total' acetyl-CoA carboxylase activity (measured after preincubation with Mg^{2+} and citrate) is used as an

index of the state activation of acetyl-CoA carboxylase. The ratio of initial to total acetyl - CoA carboxylase is a measure of the degree of phosphorylation of the enzyme.

Glucose transport, a carrier facilitated process, in the mammary gland has been shown to control the overall rate of glucose utilisation in the tissue and to change in parallel with lipogenesis in mammary acini and *in vivo* (Threadgold *et al.*, 1982; Threadgold & Kuhn, 1984). The mechanism of regulation of glucose transport in the rat and mouse mammary gland may be due to either phosphorylation affecting the activity of the transporter or to changes in the number of functional transporter molecules or the orientation/conformation of the transporter within the plasma membrane (Prosser, 1988).

Because of the importance of glucose and triacylglycerol for the synthesis of milk constituents in the rat mammary gland, the following discussion will concentrate on these metabolites, and especially on the role of glucose in lipogenesis.

1.1.3 Glucose and Fatty Acid Synthesis

1.1.3.1 Regulation of Glucose Homeostasis

Insulin and glucagon are released directly into the portal vein from the β and α cells of the endocrine pancreas, respectively. In the liver, glucagon stimulates rates of glycogenolysis and gluconeogenesis, while insulin causes increases in the rates of glycogenesis and lipogenesis. The liver is the major organ for the uptake of insulin and glucagon in the non-lactating rat and also an important site of their degradation, which is important not only for the elimination of the hormones from the bloodstream, but also for determining the insulin/glucagon concentration ratio between portal and peripheral blood (Lefebvre & Luyckx, 1979; Parman, 1979; Jungermann, 1983). Variation of the peripheral insulin/glucagon concentration ratio can occur as the result of changes in several factors; pancreatic hormone secretion, change of the hepatic hormone extraction or extrahepatic hormone removal (Balks & Jungermann, 1984). A high peripheral insulin/glucagon concentration ratio is considered to indicate a high plasma glucose 'status', a low ratio reflecting low glucose 'status'. Insulin and 'counter-regulatory' hormones (e.g. glucagon, adrenaline, vasopressin) affect glucose homeostasis, with insulin stimulating

glucose uptake and utilisation by many peripheral tissues. During lactation, the mammary gland has a prominent role in determining the circulatory insulin concentration, being an important site for the uptake of insulin (Jones et al., 1984a). The mammary gland also becomes the major glucose-utilising tissue in the body, with lipogenesis being quantitatively the most important pathway in terms of glucose utilisation in the gland (Williamson, 1980). However, fatty acid biosynthesis in the lactating mammary gland must be able to respond rapidly to changes in the availability of glucose in the circulation to maintain the carbohydrate balance within the mother. Hence, the rate of mammary gland lipogenesis is closely regulated according to the dietary status of the rat and is sensitive both to the amount of food eaten and the composition of the diet.

1.1.3.1.1 Diurnal Variations

During lactation, there is a diurnal variation in the rate of food intake of rats which, although generally similar to that of virgin rats, shows some differences. Mid-lactating rats consume a higher proportion (35%) of their diet during the light period (Tachi et al., 1981; Munday & Williamson, 1983) than do virgin rats (14%) (Kimura et al.,

1970; Bruckdorfer et al., 1974; Munday & Williamson, 1983). In lactating animals, the peak rate of lipogenesis in the liver occurs around mid-dark, decreasing to a lower rate around mid-light as observed in non-lactating rats; although the diurnal variations in hepatic lipogenesis are not very marked (Kimura et al., 1970; Munday & Williamson, 1983). Hepatic glycogen stores are replenished when food intake is still high and hepatic lipogenesis is declining. The decline in glycogen content parallels the decline in food intake. This indicates that when food intake is relatively decreased, hepatic glycogen plays an important role in supplying the demand for carbohydrate i.e. as a precursor for lipogenesis (Nowell, 1970; Munday & Williamson, 1983). Hence it is not surprising that there is no observed diurnal variation (Carrick & Kuhn, 1978) in the arterial concentration of glucose (4-5mM) (Robinson et al., 1978; Burnol et al., 1983) in the lactating rat. A diurnal variation in the arterial concentration of insulin (20-40mU/l, 1-2ng/ml) has been observed in the lactating rat with minimum values occurring during daylight hours corresponding to lowest dietary intake (Carrick & Kuhn, 1978; Munday & Williamson, 1983). A similar pattern has been observed for the non-lactating female rat and the male rat (Kaul & Berdanier, 1972; Bellinger et al., 1975; Balks & Jungermann, 1984). No such measurement of glucagon concentration has

been made in the lactating rat, and studies with male rats indicate that glucagon is not subject to diurnal changes (Balks & Jungermann, 1984). If similar diurnal relationships between insulin and glucagon exist in lactating rats, then the apparently small diurnal variation of insulin observed (a decline of 50% occurring around midday) (Carrick & Kuhn, 1978; Munday & Williamson, 1983) would be more significant when considered as the insulin/glucagon concentration ratio, and would provide a great amplitude of variation in the signal bringing about the necessary metabolic changes in response to the varying food intake.

A decline in food intake e.g. in the post-absorptive period of feeding, leads to a lowering of the peripheral insulin/glucagon ratio and to glucose sparing by peripheral tissues sensitive to insulin, such as mammary gland, in favour of essential tissues not sensitive to insulin e.g. brain, in the rat (Robinson et al., 1978; Burnol et al., 1983). Hence lipogenesis in the mammary gland at peak lactation also parallels hepatic lipogenesis, and peaks just before maximum food intake (Munday & Williamson, 1983). Variations in mammary gland lipogenesis were much more marked than those observed for hepatic lipogenesis, implying that the mammary gland is very sensitive to the metabolic status of the animal (Munday & Williamson, 1983).

However, diurnal changes in lactose synthesis do not coincide with the peripheral plasma insulin changes, despite the close correlation between lactose synthesis and food intake. This implies that although lactose synthesis is very sensitive to the nutritional status of the mother, insulin does not appear to be the dominant hormone controlling this process (Carrick & Kuhn, 1978). More recent evidence suggests the involvement of a 'novel' gastrointestinal peptide (not a known gut peptide) which works synergistically with insulin, in the regulation of glucose uptake in the mammary gland of the lactating rat (Page, 1989).

1.1.3.2 Glucose Utilisation

The glucose turnover rate is enhanced by 80% in mid-lactating rats relative to virgin or non-lactating rats. This is due to increased glucose production by the liver, increased glucose absorption from the gut and glucose utilisation. An increase in glucose utilisation is observed at peak lactation in the rat and ruminants (Dils & Parker, 1982). In the rat this occurs at low blood glucose and plasma insulin concentrations in the peripheral circulation (glucose: virgin = 6-7mM, lactating = 4-5mM (Robinson et al., 1978; Burnol et al., 1983); insulin: virgin = 50μU/ml or 2.1ng/ml, lactating = 16-20μU/ml or 1ng/ml; (Robinson et al., 1978; Flint

et al., 1979; Burnol et al., 1983). Glucose utilisation depends both on the blood glucose concentration and on the ability of tissues to absorb glucose (a parameter that is itself partly dependent on insulin concentration). The glucose metabolic clearance rate (MCR) is an index of the capability of tissues to utilise glucose independent of blood glucose concentration (Cherrington et al., 1978). The MCR is increased two fold at peak lactation, the rise being mainly due to the active mammary gland. The enhanced rate of glucose utilisation of the mammary gland represents 45% of the total glucose utilisation by the lactating rat (Davis & Mepham, 1974; Robinson & Williamson, 1977a; Jones & Parker, 1978; Burnol et al., 1983). Studies in the cow and goat show that glucose uptake by the mammary gland represents 60% of the total glucose turnover rate (Dils & Parker, 1982). The use of the euglycaemic clamp technique (Burnol et al., 1983, 1986b, 1987, 1988) has demonstrated an increased insulin responsiveness of MCR during lactation which confirms that glucose metabolism in the mammary gland is affected by insulin in vivo. Measurement of the rate of lipogenesis in vivo gave direct evidence for the insulin responsiveness of the mammary gland (Burnol et al., 1983, 1986b, 1987, 1988). However, despite this evidence of increased glucose utilisation by the mammary gland under the influence of insulin in vivo, the effects of insulin

on glucose metabolism *in vitro* in the mammary gland were debated until relatively recently. These studies performed *in vitro* on mammary slices or isolated acini of rat mammary gland (Martin & Baldwin, 1971b; Robinson & Williamson, 1977a) suggested that glucose uptake by the mammary gland was not influenced by insulin, whereas insulin stimulated the incorporation of glucose into fatty acids (Martin & Baldwin, 1971a; Robinson & Williamson, 1977a). As plasma insulin is lower in lactating rats than in virgin or non-lactating rats, it is necessary to reconcile the apparent paradox of low plasma insulin being correlated with a high rate of glucose metabolism in the mammary gland (discussed later in section 1.1.5). Consequently, certain dietary and physiological manipulations have assisted in elucidating this further.

1.1.3.3 Short-Term Starvation

The response of mammary gland lipogenesis to starvation is rapid, since 6h starvation results in 88% inhibition of lipogenesis (Williamson *et al.*, 1983; Jones *et al.*, 1984b). Withdrawal of food for 24h, results in a 98% inhibition of mammary gland lipogenesis which is accompanied by a reduction of acetyl-CoA carboxylase activity and a 90-95% inhibition in the rate of glucose transport *in vivo*, which is rapidly reversed by refeeding for 2h

(Robinson et al., 1978; Robinson & Williamson, 1978; Agius & Williamson, 1980; Munday & Williamson, 1981, 1982; McNeillie & Zammit, 1982; Williamson et al., 1983; Bussmann et al., 1984; Jones et al., 1984b; Jones & Williamson, 1984; Threadgold & Kuhn, 1984; Mercer & Williamson, 1986; Munday & Hardie, 1986a). A change in insulin concentration accompanies these states, and induction of short-term insulin-defiency by administration of streptozotocin to fed lactating rats results in substantial inhibition of lipogenesis and acetyl-CoA carboxylase activity in the mammary gland (Robinson & Williamson, 1977a; Robinson et al., 1978; Munday & Williamson, 1981, 1982; McNeillie & Zammit, 1982). Administration of streptozotocin to 24h-starved rats prior to refeeding with chow, prevents activation of mammary gland lipogenesis (Robinson et al., 1978; Munday & Williamson, 1981). This suggests that insulin may be a necessary hormone signal during refeeding. Administration of insulin and glucose to 24h-starved rats restores glucose transport and the activation state of acetyl-CoA carboxylase in vivo (Munday & Williamson, 1981; Threadgold & Kuhn, 1984), but only partially reverses the effects of starvation on lipogenesis in vivo, with none of these effects being observed in vitro (Robinson & Williamson, 1977a; Munday & Williamson, 1981). Insulin infusion in the absence of refeeding fails to reactivate lipogenesis fully in the gland of 24h-starved rats

(Jones *et al.*, 1984b). These observations imply that some other systemic factor(s) e.g. a gastrointestinal peptide, and some other inhibitory factor, may be responsible for the changes observed *in vivo* (Page, 1989).

More recent experiments with 18h starved/30 min. refed lactating rats show that on refeeding, there is a peak in the circulating insulin concentration, followed by recovery of glucose uptake and, after a time lag, by an increase in the lipogenic activity in the mammary gland (Bussmann *et al.*, 1984; Mercer & Williamson, 1986; Page & Kuhn, 1986). The presence of a retardant of carbohydrate digestion, or refeeding with oral triglyceride, or limited food availability on refeeding, either suppresses lipogenesis (i.e. in the former two cases) or prevents a prolonged increase (in the latter case) in lipogenesis unlike that seen if the animals are fed *ad libitum* after starvation (Bussmann *et al*, 1984; Mercer & Williamson, 1987, 1988; Page, 1989). This implies that the rat mammary gland in the lactating rat is highly attuned to the availability of dietary carbohydrate and probably the gastrointestinal peptide or other as yet unknown modulating factors (Mercer & Williamson, 1987, 1988; Page, 1989). Continued hepatic gluconeogenesis during refeeding is also required to maintain stimulation of mammary gland lipogenesis (Williamson

et al., 1985). Supression of insulin secretion after the initial surge of insulin abolishes the activation of lipogenesis, suggesting that the insulin-sensitivity of the gland may be acutely enhanced over this period of refeeding (Mercer & Williamson, 1986; Page & Kuhn, 1986).

1.1.4 Triacylglycerols and Fatty Acid Synthesis

The products of triacylglycerol digestion in the rat are mainly monoacylglycerols and long chain fatty acids, which are re-esterified and incorporated into chylomicrons in the intestinal epithelium, to be made available in the peripheral venous blood supply. Another important source of fatty acids are endogenous triacylglycerols secreted from the liver as VLDL. Lipoprotein triacylglycerol can be taken up by any tissue, following hydrolysis by lipoprotein lipase (EC 3.1.1.34) (Clegg, 1988). The resultant fatty acids predominantly enter the cell, whereas the glycerol is removed by the circulation and metabolised by the liver and kidney.

In the fed non-lactating rat, chylomicron- and VLDL-triacylglycerol is removed from the circulation in part by adipose tissue for storage and is also an important site of lipogenesis from glucose (Williamson, 1980). During lactation the rate of lipogenesis in adipose tissue is considerably

decreased (Smith, 1973; Robinson et al., 1978; Flint et al., 1979). Also the activity of lipoprotein lipase decreases rapidly at parturition and remains low throughout lactation (Otway & Robinson, 1968; Hamosh et al., 1970; Zinder et al., 1974; Oller do Nascimento & Williamson, 1986). Concomitantly the rate of lipolysis in adipose tissue from lactating rats increases and adipose tissue becomes more sensitive to lipolytic stimuli, such as glucagon (Smith & Walsh, 1976; Zammit, 1988). The net result of these changes is that, in lactation, less glucose and triacylglycerol are removed by adipose tissue and more non-esterified fatty acids (NEFA) and glycerol are released into the bloodstream from adipose tissue.

There are reciprocal changes in the activity of lipoprotein lipase in mammary gland to those of adipose tissue; the lipoprotein lipase activity in mammary gland increases rapidly at parturition and remains high throughout lactation (Zinder et al., 1974). In this way, the mammary gland becomes the predominant site of utilisation of fatty acids from VLDL. The tissue uses these in addition to the NEFA made available from adipose tissue to supplement the supply of fatty acids required from milk secretion (Hawkins & Williamson, 1972). The net result of these changes, is the direction of triacylglycerol

to the mammary gland and away from the adipose tissue.

Although the ruminant generally derives much less triacylglycerol from its diet than the rat, nevertheless it too depends on its supply of fatty acids from adipose tissue and from its diet for its source of long-chain fatty acids for the synthesis of milk lipids (Tulloh, 1966; Schoefl & French, 1968; West et al., 1972; Annison, 1974; Bickerstaffe et al., 1974; Smith & Baldwin, 1974; Chilliard et al., 1977, 1978; Moore & Christie, 1979; Vernon et al., 1981).

1.1.4.1 High Fat Diets

During lactation, in rats and mice, the proportion of fat in the diet does not affect the fat content of milk, but the fat composition of the milk resembles that of the diet when this is rich in fat (Garton, 1963; Coniglio & Bridges, 1966; Farid et al., 1978; Grigor & Warren, 1980). High-fat or 'cafeteria' diets (chow plus palatable high energy food) given throughout lactation decrease lipogenesis in the mammary gland in vivo in both mice (Romsos et al., 1978) and rats (Agius et al., 1980; Grigor & Warren, 1980, Munday & Williamson, 1987) and in vitro (Coniglio & Bridges, 1966; Agius et al., 1980; Bussmann et al., 1984). Decreased

rates of glucose utilisation and acetyl-CoA carboxylase activity *in vitro* are observed with the high fat diets, which correlate with the inhibition of lipogenesis *in vivo* (Agius *et al.*, 1980; Munday & Hardie, 1986b; Munday & Williamson, 1987). The addition of insulin, *in vivo* and *in vitro*, restores lipogenesis, the removal of glucose and the activity of acetyl-CoA carboxylase to normal values i.e. those fed chow only (Agius *et al.*, 1980, 1981; Munday & Hardie, 1986b; Munday & Williamson, 1987).

An intragastric load of medium or long-chain triacylglycerols inhibits lipogenesis in the lactating rat mammary gland *in vivo*, long-chain triacylglycerols having the greater effect (Agius & Williamson, 1980). This inhibition of rat mammary gland lipogenesis is reversed by insulin administration *in vivo* (Agius *et al.*, 1981). However glucose utilisation *in vitro* by mammary gland acini from triacylglycerol-fed rats was normal (Agius & Williamson, 1980), in contrast to that observed for rats fed a 'cafeteria' diet. In ruminants, different long-chain fatty acids produced different effects on lipogenesis i.e. some stimulated, some inhibited and others had no effect, and these fatty acids also promoted incorporation of specific fatty acids into triacylglycerols in the mammary gland e.g. palmitate stimulates lipogenesis

and promotes incorporation of butyrate into milk lipids (Hansen & Knudsen, 1987a,b).

Short-term starvation in lactating rats resulted in a decrease of triglyceride uptake by mammary gland; refeeding (2h) ad libitum restored triglyceride uptake in this tissue (Oller do Nascimento & Williamson, 1988). Insulin-defiency prior refeeding did not prevent the restoration of lipid accumulation and lipoprotein lipase activity to fed values in the mammary gland. This implied that lipid accumulation in mammary gland may not be affected by changes in plasma insulin concentration and is less sensitive to starvation than is lipogenesis or lactose synthesis. The activity of lipoprotein lipase in the mammary gland may also be unresponsive to insulin, or only responsive to much lower concentrations of insulin than are required for lipoprotein lipase in adipose tissue, thereby providing a different regulatory mechanism of this enzyme in the two tissues during lactation (Oller do Nascimento & Williamson, 1988). These adaptations have the advantage that milk lipid content can still be maintained for a short while after withdrawal of food.

1.1.5 Hormonal Regulation of Mammary Gland and Adipose Tissue Metabolism

The regulation of mammary gland and adipose tissue metabolism during lactation can be ascribed to long-term and short-term effects of hormones. Chronic regulation of the metabolism of these two tissues involves the long-term effects of hormones in the development and maintenance of lactation. These hormones are involved in the induction of enzyme synthesis and are responsible for changes in concentrations of these enzymes (total activity) at the onset of lactation. Acute regulation of the mammary gland involves the short-term effects of hormones which bring about changes in the activity of pre-existing enzymes in response to e.g. the nutritional state of the animal or to short-term pathological conditions. Changes in the activity of enzymes are brought about by a variety of effects; covalent modification e.g. phosphorylation and dephosphorylation (Munday & Hardie, 1987), binding of an effector molecule(s), or association / dissociation of enzyme subunits.

1.1.5.1 Effects of Prolactin

Studies with a variety of species have shown that the onset of lactogenesis is dependent on the rise in serum prolactin and the fall in serum

progesterone, in conjunction with a requirement for glucocorticoids (Baldwin & Yang, 1974; Kuhn, 1977; Cowie *et al.*, 1980). There are species differences in the minimum requirement to restore or to maintain lactation in hypophysectomised animals and in culture systems. These experiments, *in vivo* and *in vitro* with mice and rats, have generally concluded that prolactin, cortisol and insulin are all important in the induction of enzyme synthesis in the mammary gland at the onset of lactation (Baldwin & Martin, 1968; Hallowes *et al.*, 1973; Baldwin & Yang, 1974; Topper & Oka, 1974; Topper & Freeman, 1980). In ruminants, a high prolactin concentration is less important once lactation is established (Forsyth, 1986). Instead growth hormone has a greater role to play in ruminants, yet growth hormone is not essential for lactation in rodents (Forsyth, 1986; Madon *et al.*, 1986).

Prolactin is also associated with the longer-term control of lipogenic enzyme concentrations in the lactating rat mammary gland (Field & Coore, 1976; McNeillie & Zammit, 1982). The rat mammary gland is known to possess receptors for prolactin, so enabling prolactin to exert a direct effect (Flint *et al.*, 1981). However, adipose tissue does not have such receptors and therefore it has been assumed that prolactin exerts an effect indirectly (Flint *et al.*, 1981; Vernon & Flint, 1983).

Conditions in which the circulating concentration of prolactin has been decreased, such as administration of bromocryptine, an inhibitor of prolactin secretion, for 48h, or removal of pups (24h or 48h) result in a decrease of lipogenesis and activity of lipoprotein lipase in the mammary gland and an increase in lipogenesis and lipoprotein lipase in adipose tissue, which suggests that prolactin is responsible for the changes observed (Robinson & Williamson, 1977a; Agius et al., 1979; Flint et al., 1981; Oller do Nascimento et al., 1989). Administration of prolactin reverses the effects of prolactin-defiency, but it cannot reverse the effects of pup removal for 24h or 48h (Agius et al., 1981; Flint et al., 1981). The effects of prolactin-deficiency or weaning can be reproduced under conditions where there is no change in plasma prolactin i.e. complete sealing of teats (24h) with continued suckling, but there is an increase in plasma insulin (Oller do Nascimento et al., 1989). A parallelism between plasma insulin, lipoprotein lipase activity and lipid accumulation in adipose tissue has been drawn from the above and other experiments, which suggests that plasma insulin is more important than prolactin in regulating lipid deposition in adipose tissue during lactation (Oller do Nascimento et al., 1989).

1.1.5.2 Insulin Receptors

The numbers of insulin receptors on tissue cells change during pregnancy and lactation relative to those observed in tissues of virgin animals. Both adipocyte and hepatocyte insulin receptor numbers increase during pregnancy but decline at parturition, and during lactation the number of insulin receptors is similar to those of virgin rats (Flint et al., 1979; Flint, 1980). Conversely, the number of insulin receptors on epithelial cells of the mammary gland are increased during lactation (Flint, 1982). The sensitivity of the liver to insulin is unchanged in the lactating rat, which would agree with the unchanged affinities and numbers of hepatic receptors observed relative to virgin rats (Burnol et al., 1983). Similarly the increase in insulin receptor affinities and numbers for the mammary gland are as expected for the increased sensitivity of the mammary gland during lactation (Burnol et al., 1983, 1986b, 1987; Jones et al., 1984a). The fact that there is no change in receptor number implies that adipose tissue may be insulin resistant and/or that some other factor is responsible for the decreased lipogenesis observed. A post-receptor defect has been indicated, with more recent experiments showing that it is a failure of the signal-transduction system in the plasma membrane at steps subsequent to insulin binding to

its receptor i.e. a failure to release the insulin 'mediator' (Walker & Flint, 1983; Vernon & Flint, 1983; Kilgour & Vernon, 1987, 1988). This could account for the decreased ability of adipose tissue to respond to insulin during lactation.

1.1.5.3 Regulation of Adipose Tissue

In adipose tissue the relative concentrations and activities of enzymes associated with lipogenesis are low and hence the tissue does not have the potential to respond to such stimulation by insulin as demonstrated by the inability of insulin to stimulate fatty acid synthesis in adipose tissue *in vivo* (Smith, 1973; Robinson *et al.*, 1978; Agius *et al.*, 1979; Flint *et al.*, 1979; Sinnett-Smith *et al.*, 1980; Burnol *et al.*, 1983, 1986a,b, 1987; Jones *et al*, 1984a). In addition, there is evidence that adipose tissue may also have decreased sensitivity to insulin and increased sensitivity to glucagon (Burnol *et al.*, 1983, 1986a,b, 1987; Kilgour & Vernon, 1988; Zammit, 1988).

1.1.5.3.1 Lipolysis and Effects of Adenosine in Adipose Tissue

Lipolysis is the predominant pathway in adipose tissue during lactation and is favoured by a low insulin/glucagon ratio; glucagon stimulating

lipolysis, insulin inhibiting lipolysis. The combined effects of a low insulin/glucagon ratio, the decreased responsiveness of rat adipose tissue to insulin, and increased sensitivity of lipolysis to glucagon during lactation should favour high rates of triglyceride mobilisation required at peak lactation (Zammit, 1988). However the response of adipose tissue to the lipolytic effects of catecholamines is unchanged during lactation (Aitchison et al., 1982; Vernon et al., 1983).

The response of lipolysis in adipose tissue is affected by adenosine in vitro and in vivo during lactation. Adenosine has wide ranging effects on several aspects of cell metabolism that can be observed both when released by adipose tissue in vivo (Fredholm & Sollevi, 1981) and its accumulation in the incubation medium with isolated cell preparations in vitro (Schwabe et al., 1973). It is an important effector of adenylate cyclase activity and a modulator of the effects of insulin and glucagon on tissues that are responsive to these hormones (Fernandez & Saggerson, 1978; Lambert & Jacquemin, 1983).

Two types of adenosine receptor have been described. A_1 sites mediate inhibition of adenylate cyclase in all cases and the A_2-receptor in most tissues, mediates activation of adenylate cyclase (Fain &

Malbon, 1979; Fain, 1980; Londos et al., 1983). However, the rat adipocyte does present a paradox with regard to the effects of adenosine, in that both occupancy of both the A_1 and A_2 sites depresses cyclic AMP accumulation in the intact cell, which in turn inhibits lipolysis (Fain, 1973; Fain et al., 1973; Trost & Stock, 1977).

Adipocytes isolated from lactating rats have an increased ability to respond to adenosine, but the sensitivity to adenosine is unchanged compared to pregnant or virgin rats (Vernon et al., 1983, 1987; Zammit, 1988). 24h starvation has no effect on rates of lipolysis during lactation, but an increase in sensitivity is observed. Administration of growth hormone can diminish the response to adenosine, but is not thought to be important in regulating adenosine concentration in vivo during lactation. The factor(s) responsible for these changes is unknown, prolactin having no effect on the response of adipocytes to adenosine (Vernon et al., 1987).

1.1.5.4 Differential Regulation of Mammary and Adipose Tissue

In adipose tissue, insulin stimulates and glucagon inhibits key steps in the pathway of lipogenesis from glucose in isolated adipocytes in vitro (Zammit

& Corstorphine, 1982b; Green 1983). The relationships between the effects of glucagon and insulin on adipocytes suggest that lipogenesis in adipose tissue *in vivo* is very sensitive to changes in the circulatory insulin/glucagon ratio (Zammit & Corstorphine, 1982b). By analogy, the same effects of these hormones might be expected to be observed in the mammary gland. The low insulin/glucagon ratio in rats at peak lactation (half that of virgins) (Robinson *et al.*, 1978), would tend to favour the counter-regulatory actions of glucagon versus the effects of insulin on a tissue. However, if a tissue is insensitive to the effects of glucagon and is acutely regulated by changes in circulatory insulin, rather than by the insulin/glucagon ratio, a mechanism would thereby exist enabling the two tissues to be differentially responsive to the circulatory insulin concentration. The rat mammary gland is one such tissue, lipogenesis not being inhibited by glucagon *in vivo* or in isolated acini *in vitro* (Williamson *et al.*, 1983; Bussmann *et al.*, 1984; Jones *et al.*, 1984b). Hence the increased sensitivity of the mammary gland to insulin, in conjunction with a lack of response to glucagon would compensate for the low insulin/glucagon ratio that favours inhibition of lipogenesis in other tissues.

1.2 HORMONE ACTION

In this section the action of insulin and glucagon, important regulators of mammary gland and adipose tissue lipogenesis, are discussed. Discussion has been limited to these two hormones because the practical work reported in the following chapters was concerned exclusively with their action on adipose and mammary metabolism.

1.2.1 Glucagon

Glucagon is a polypeptide hormone containing 29 amino acid residues, which is released from the pancreas in response to dietary stimuli. Glucagon binds to a specific receptor which is an intrinsic glycoprotein located in the plasma membrane. A heterogeneous population of glucagon receptors expressing high- and low-affinities is known to exist (Rodbell et al., 1971a,b,c; Sonne et al., 1978), which is analogous to adrenaline acting through α_1- and β-adrenergic receptors (Lefkowitz et al., 1983). It has been suggested that, at least in the liver, the two classes of receptors act via different second messenger systems : high affinity sub-type via inositol phosphate, low affinity sub-type via cyclic AMP (Wakelam et al., 1986).

Sub-maximal concentrations of glucagon added to adipocytes, resulting in low receptor occupancies, stimulate adenylate cyclase in a dose-dependent manner, maximal activation of adenylate cyclase occurring at full occupancy of sites (Sonne & Gliemann, 1977). This mode of action is known to occur via the low affinity sites (Wakelam et al., 1986).

1.2.1.1 Adenylate Cyclase

Adenylate cyclase, a plasma membrane enzyme located on the internal surface, catalyses the formation of cyclic AMP and PP_I from ATP, and is part of a multi-enzyme complex. The enzyme system, which is located in the cell membrane, is composed of at least three classes of components (see Figure 1.1). The glucagon receptor (R) is functionally distinct from adenylate cyclase and only couples to it in the presence of glucagon (Houslay et al., 1977). At the inner face of the membrane are the catalytic unit (C) and the nucleotide regulatory components (G) of adenylate cyclase. The catalytic unit carries out the conversion of ATP into cyclic AMP. Each G-component contains site(s) for binding GTP and is responsible for mediating the effects of GTP and the hormones on the activity of C-component. Two types of GTP-binding proteins linked to adenylate cyclase have been distinguished functionally. One mediates

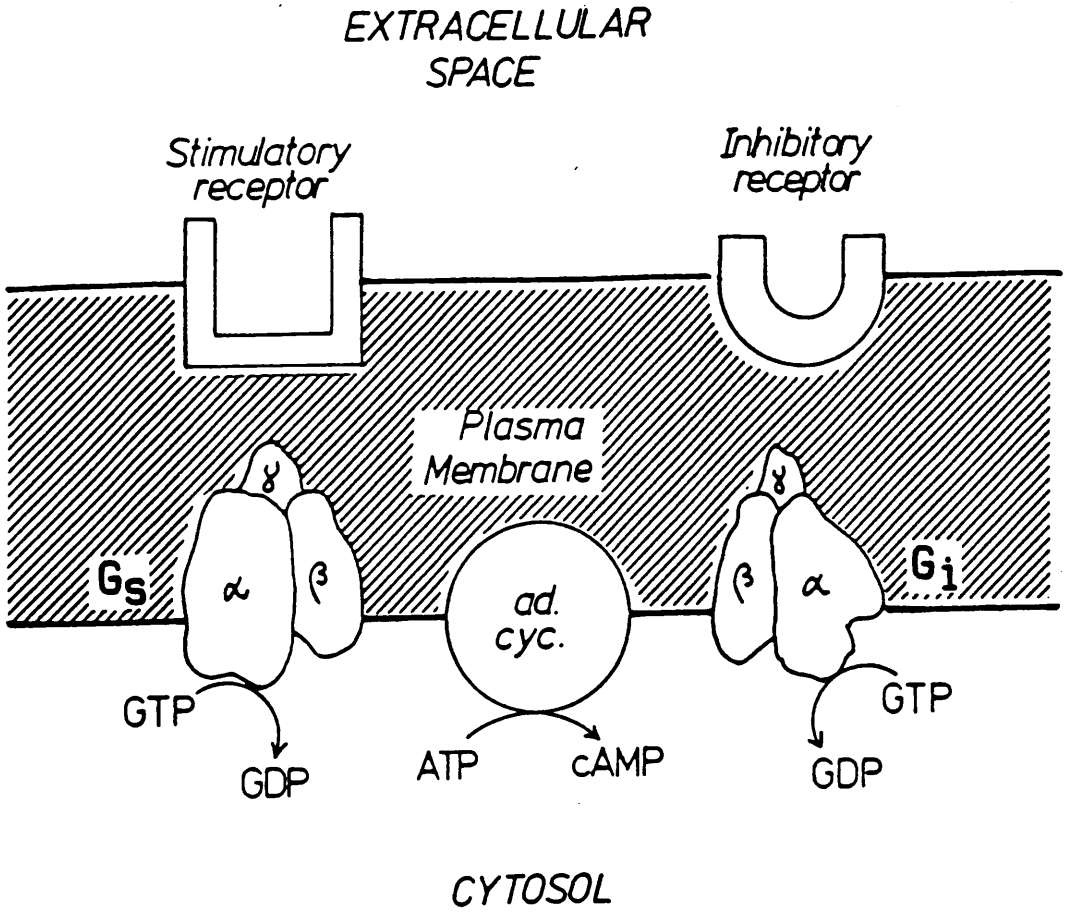


Fig. 1.1 Diagrammatic representation of the adenylate cyclase system in mammalian cells.

stimulation (termed G_s), the other inhibition (G_i) of the adenylate cyclase activity (Ross & Gilmann, 1980; Rodbell, 1980). The overall activity of adenylate cyclase is regulated by the relative activities of G_s and G_i, which in turn reflect the hormone occupancy of the relevant receptors.

Stimulation of adenylate cyclase activity requires the stimulatory hormone, e.g. glucagon, low concentrations of GTP (10^{-7}M) and an intact adenylate cyclase complex. The binding of GTP both decreases the affinity of hormones for their receptor and synergistically amplifies the hormonal stimulation of activity. A GTPase associated with the catalytic subunit (GC complex) breaks down GTP to GDP and Pi and returns the nucleotide-receptor complex (GR) back to its uncoupled state (Cooper, 1983; Birnbaumer et al., 1985)

1.2.1.2 Cyclic AMP as a Second Messenger

Cyclic AMP is formed from ATP by the action of adenylate cyclase. It acts as a second messenger for glucagon by binding to an enzyme, a cyclic AMP-dependent protein kinase. The cyclic AMP-dependent protein kinase consists of regulatory (R) and catalytic (C) subunits (Krebs & Beavo, 1979, Pall, 1981) forming a tetrametric structure. Each R-subunit monomer has two binding sites for cyclic

AMP as well as a site for binding C-subunits (Corbin et al., 1978; Weber & Hilz, 1979; Builder et al., 1980). The binding of cyclic AMP causes conformational changes in the R-subunit that results in its dissociation from and consequent activation of the catalytic subunit. This active protein catalyses the transfer of the ɣ-phosphate (phosphoryl-) group from ATP to esterifiable (-hydroxyl) groups on amino acid residues (serine, threonine) in proteins. The resulting covalent modifications of the protein (i.e. phosphorylation), are known to change their biological activity e.g. the activation or deactivation of several hormonally regulated enzymes.

Termination of the cyclic AMP signal within cells is effected by the hydrolysis of the messenger molecule to 5'AMP by specific cyclic AMP-phosphodiesterases, which are present in multiple forms in most tissues (Thompson & Strada, 1978; Beavo et al., 1982; Houslay et al., 1983; Houslay, 1986b). Two distinct types, which seem the most important in regulating cyclic AMP levels, at least in the liver, are a 'dense-vesicle' phosphodiesterase and a plasma-membrane bound phosphodiesterase (52kDa protein) (Marchmont et al., 1981; Heyworth et al., 1983b; Pyne et al., 1987, 1989). The 'dense-vesicle' phosphodiesterase is activated by both glucagon and insulin (Loten et al., 1978, 1980;

Heyworth et al., 1983b; Houslay et al., 1983). The mechanism whereby glucagon activates this enzyme is distinct from that employed by insulin (as yet unidentified) and is mediated by cyclic AMP (Heyworth et al., 1983b; Wilson et al., 1983). Insulin is known to activate the plasma-membrane bound phosphodiesterase by direct tyrosine phosphorylation in the intact cell. This phosphorylation, in turn, converts the enzyme into a substrate for cyclic AMP-dependent protein kinase (Pyne et al, 1989). However, glucagon has no effect on the plasma-membrane bound enzyme, though there is evidence that pretreatment of isolated cells with glucagon prevents the activation of the enzyme by insulin (Heyworth et al., 1983b; Wilson et al., 1983). A third species of phosphodiesterase, less well-defined, has been identified (Heyworth et al., 1984). It may be the so-called cyclic GMP-activated cyclic AMP phosphodiesterase, which can be activated by an insulin-mediator preparation (Pyne & Houslay, 1988).

Another mechanism of terminating the cyclic AMP signal is the inhibition of cyclic AMP-dependent protein kinase. This has been demonstrated in hepatocytes where insulin decreases the affinity of the protein kinase for cyclic AMP, but does not alter the concentration of cyclic AMP (Gabbay & Lardy, 1984, 1987).

A long-loop feedback system involving mechanisms which produce desensitisation and/or down-regulation of hormone receptors has been described (Gavin et al., 1974). The actions of these systems results in diminished cyclic AMP accumulation after chronic exposure to hormones, and refractoriness to further hormonal stimulation.

1.2.1.3 Calcium Ions and Phospholipid Metabolism as Second Messengers

The effects elicited by glucagon binding to the high-affinity receptor sites are thought to be mediated by an elevation of intracellular calcium ion levels, thus facilitating activation of calcium-dependent protein kinases (Sistaire et al., 1985; Mauget & Claret, 1986). A mechanism whereby glucagon might exert such an effect has been proposed (Wakelam et al., 1986). There are documented examples of hormones e.g. vasopressin, adrenaline via α_1 receptors, binding to surface receptors in a variety of tissues, which trigger the cleavage, by a phospholipase C type hydrolytic enzyme, of phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). A G protein (G_p), which may be $p21^{RAS}$, is thought to link the receptor binding to the activation of this phospholipase C (Gomperts, 1983; Berridge & Irvine, 1984; Farese et al., 1985;

Wakelam et al., 1986). IP_3 can release Ca^{2+} from intracellular stores causing a transient increase in cytosolic calcium ion levels (Hendrickson & Reinertsen, 1971; Buckley & Hawthorne, 1972; Jones & Michell, 1975, 1978; Putney et al., 1980; Prpic et al., 1982; Berridge, 1983; Berridge et al., 1984; Berridge & Irvine, 1984; Farese, 1984). Glucagon, at physiological concentrations, has been found to cause the breakdown of inositol phospholipids and the production of inositol phosphates (Wakelam et al., 1986). This effect is considerably smaller than the stimulation of inositol phospholipid metabolism elicited by vasopressin in hepatocytes (Creba et al., 1983; Thomas et al., 1984). Although it is known that α_1 adrenoreceptors in liver seem to produce a disproportionately large physiological response in relation to their rather small stimulation of inositol phospholipid metabolism (Downes & Michell, 1985). These effects of glucagon are in contrast with earlier findings in rat liver that glucagon does not have any effect on Ca^{2+}-independent PIP_2 hydrolysis or Ca^{2+}-dependent phosphatidylinositol (PI) hydrolysis (Bell & Majerus, 1980). At present, these experiments with glucagon have been done using only liver cells, so it is uncertain whether it also exerts this action on extrahepatic tissues. Insulin can stimulate production of DAG, but not stimulate IP_3 metabolism in adipocytes and myocytes (Farese, 1984; Farese et

al., 1985). The role of DAG generated by insulin is unknown, although a possible role for DAG as a second messenger has been postulated, as it is known to activate a protein kinase C that can phosphorylate specific proteins (Takai et al., 1979; Nishizuka, 1983; Berridge et al., 1984). However a role for DAG with respect to this in conjunction with glucagon action has not been identified.

There have also been suggestions that glucagon may be a precursor of at least one biologically active peptide (Mallat et al., 1987). Experiments have indicated that this peptide has no effect on adenylate cyclase, but it can inhibit both the Ca^{2+}-activated and Mg^{2+}-dependent ATPase activity and Ca^{2+} transport in liver plasma membranes. Further studies are needed in this area to ascertain the full implications of such peptide in the actions of glucagon.

1.2.2 Insulin

Insulin is a small polypeptide protein made up of chains (designated A and B) which are held together by disulphide bridges. The insulin receptor is a glycoprotein and an integral component of the plasma membrane, with the carbohydrate portion of the receptor located on the external surface of the cell (Jacobs et al., 1980a,b). It consists of a

heterotetramer with two α-subunits and two β-subunits linked covalently by disulphide bonds.

Insulin can elicit a maximum biological response with only a small number of receptors occupied, the additional 97-99% of receptors are termed spare receptors (Kono & Barham, 1971; Megyesi et al., 1975). These are not inactive receptors, as other biological responses in these and other cells may require greater levels of receptor occupancy (Kono & Barham, 1973). Receptor occupancy by insulin obviously contrasts with that observed for glucagon, which does not involve spare receptors.

The mechanism of insulin action is only partly understood. At one end of the signalling chain, the structure of the insulin receptor is known in detail, and at the other end, insulin controls cellular metabolism by regulating the phosphorylation of serine and threonine residues in key target enzymes. The molecular events linking the occupied receptor to changes in target enzyme phosphorylation remain obscure. Several theories concerning the mechanism whereby insulin-receptor mediated activity is elicited, are :

(a) by a membrane-bound insulin-dependent tyrosine kinase;
(b) by an inositol phosphate mediator;

(c) by a insulin-specific guanine nucleotide regulatory protein (G_{INS}) activated protein kinase.

1.2.2.1 Tyrosine Kinase Activity

Receptors for insulin express tyrosine kinase activity, acting upon target proteins, the best characterised of which is its own β-chain, in lymphocytes and hepatoma cells (Kasuga et al., 1982; Petruzelli et al., 1982, 1984; Van Obberghen et al., 1983). The insulin receptor can phosphorylate tyrosine residues of exogenous substrates, e.g. histone, casein, and is cyclic AMP-independent (Petruzelli et al., 1982; Kasuga et al., 1983; Stadtmauer & Rosen, 1986). The phosphorylation of the 52kDa plasma membrane phosphodiesterase by insulin in isolated rat hepatocytes is the first real evidence of the receptor tyrosyl kinase activating directly an enzyme known to be insulin-sensitive (Pyne et al., 1989). Whether the receptor autophosphorylation might be coupled to the activation or inactivation of specific protein kinases remains to be elucidated. One possibility is that the interaction of insulin with its receptor stimulates the generation of low molecular weight 'mediators' which serve as intermediates in the signalling process (see section 1.2.2.2). Other possibilities of action are to modify the activity

of a membrane protease which in turn generates a second messenger; or to initiate a cascade of phosphorylation and dephosphorylation reactions that are responsible for the effects of insulin (Kasuga et al., 1982)

1.2.2.2 'Mediator' as Second Messenger

Originally, these 'mediators' were thought to be peptides or complex molecules composed of peptide, sugar and lipids (Seals & Czech, 1980, 1981; Larner et al., 1981; Saltiel et al., 1981; Kiechle et al., 1981). More recent evidence indicates that these putative 'mediators' of insulin action are phospho-oligosaccharides (POS) composed of phosphate, inositol, glucosamine and galactose (Mato et al., 1987a,b; Saltiel et al., 1987) Phosphodiesteratic hydrolysis of a phosphatidylinositol glycan (PI-glycan) by a phosphatidylinositol (PI)-specific phospholipase C generates POS, the polar head group, and DAG (Saltiel & Cuatrecasas, 1986; Saltiel et al., 1986; Mato et al., 1987a, 1989). The PI-glycan is located at the outer surface of a variety of cells (adipocytes, hepatocytes) and POS, once released enters the cells by an unknown mechanism (Mato et al., 1989). Differences in the structure of POS have been detected in different cells or tissues (Alemany et al., 1987). PI-glycan is also very similar to a PI-glycan involved in regulating

the attachment of proteins to cell membranes (Low et al., 1986; Low, 1987), but the PI-glycan identified by Mato et al., (1987a,b) is not thought to have this role as it does not contain amino acids or ethanolamine residues to anchor the protein to the glycolipid (Mato et al., 1989).

POS is able to mimic many actions of insulin; it can modulate the phosphorylation state of key target proteins mediating insulin action (Mato et al., 1989), and is thought to mediate cyclic AMP-dependent and cyclic AMP-independent effects of insulin (Alemany et al., 1987). Evidence to support this theory has been demonstrated in cell free systems and intact cells. POS can affect the activity of many enzymes in a manner similar to insulin, such as high-affinity cyclic AMP phosphodiesterase and pyruvate dehydrogenase in adipocytes (Saltiel et al., 1986; Saltiel, 1987), adenylate cyclase (Saltiel, 1987), hormone-sensitive lipase (Alemany et al., 1987), and cyclic AMP-dependent protein kinase (Villalba et al., 1988). POS also has been demonstrated to have insulin-like effects on intact cells e.g. in adipocytes on lipolysis and lipogenesis (Kelly et al.,1987; Saltiel & Sorbara-Cazan, 1987), and in hepatocytes on cyclic AMP levels and lipogenesis (Alvarez et al., 1987; Mato et al., 1989). However, the 'mediator' is not able to stimulate glucose

transport (Kelly et al., 1987). However the DAG (distinct from that generated by IP_3 metabolism) generated in conjunction with PDS, is thought to possibly regulate glucose transport (Kelly et al., 1987; Saltiel & Sorbara-Cazan, 1987), and protein kinase C activity (Blumberg, 1980) or an analogous protein kinase.

As to what links the action of insulin binding to its receptor and the activation of the PI-specific phospholipase C is not certain. However, by analogy to G_p, a specific G protein that links the insulin receptor to its phospholipase has been postulated (Houslay et al., 1986).

1.2.2.3 Guanine Nucleotide Regulatory Protein (G_{INS}) as Second Messenger

The insulin receptor is known to interact with, and activate, a specific GTP-binding protein called G_{INS}, similar to 'G_s' and 'G_i' that act on adenylate cyclase shown diagrammatically in Fig. 1.1 (Heyworth et al., 1983a,b; Heyworth & Houslay, 1983b; Houslay & Heyworth, 1983; Houslay, 1985, 1986a,b). As insulin inhibits adenylate cyclase, it was expected that it might act via G_i. However, recent evidence has shown that insulin does not mediate its action on adenylate cyclase through G_i (Gawler et al., 1988). G_{INS} is thought to have properties that bear

comparison with the G_I identified as mediating inhibitory effects of adenylate cyclase, but to be a distinct species from G_I (Heyworth & Houslay, 1983b). It has been proposed that the effect of insulin on liver plasma-membrane cyclic AMP phosphodiesterase and adenylate cyclase is mediated by G_{INS} (Heyworth et al., 1983a,b; Heyworth & Houslay, 1983b; Houslay & Heyworth, 1983).

1.3 KEY ENZYMES OF LIPOGENESIS & LIPOLYSIS

Acetyl-CoA carboxylase and hormone-sensitive lipase play key roles in the regulation of fatty acid synthesis and lipolysis, respectively. The way in which the activity of these enzymes is regulated, with particular reference to insulin and glucagon, is discussed.

1.3.1 Acetyl-CoA Carboxylase

1.3.1.1 Alteration in the Concentration of the Enzyme in Tissues

Alterations in the concentration of acetyl-CoA carboxylase in tissues (Allman et al., 1965; Muto & Gibson, 1970) are generally a result of long-term changes in the physiological states of the animal such as lactation or weaning. A reduction of the enzyme content being accompanied by decreased total

activity of the enzyme, an increase resulting in increased total activity (McNeillie & Zammit, 1982; Sinnett-Smith et al., 1982).

1.3.1.2 The Actions of Effector Molecules

Effector molecules are known to regulate allosterically acetyl-CoA carboxylase (Miller & Levy, 1969; Denton et al., 1977; Yeh et al., 1981); citrate increases the activity of acetyl-CoA carboxylase and long-chain acyl-CoA esters inhibit the enzyme (Halestrap & Denton, 1974; Lane et al., 1979). The protomeric form of acetyl-CoA carboxylase is a homodimer which in the presence of high concentrations of citrate forms polymers (Gregolin et al., 1966; Hardie, 1980; Numa & Tanabe, 1984; Brownsey & Denton, 1985). The role of polymerisation and citrate has been explored in adipose tissue and mammary tissue (Borthwick et al., 1987). In the mammary gland, citrate is needed to promote polymerisation which supported earlier findings (Halestrap & Denton, 1974). The phosphorylation of acetyl-CoA carboxylase by cyclic AMP-dependent protein kinase results in a decreased ability to polymerise in the presence of citrate, though some polymerisation does occur (Lee & Kim, 1978; Lent et al., 1978; Swenson & Porter, 1985). Exposure of adipose tissue to insulin in the absence of citrate resulted in increased polymerisation, as

suggested by Halestrap & Denton, (1974), implying that the presence of the effector molecule is not required since effects persisted after purification, but that the covalent modification e.g. phosphorylation, is the most likely cause (Borthwick et al., 1987).

1.3.1.3 Phosphorylation

Changes in the activity and the degree of phosphorylation of the enzyme in isolated rat liver cells and adipocytes have been demonstrated after the exposure of these preparations to various hormones (Halestrap & Denton, 1974; Geelen et al., 1978; Brownsey et al., 1979; Witters et al., 1979; Witters, 1981; Brownsey & Denton, 1982; Holland et al., 1984, 1985). Acetyl-CoA carboxylase is phosphorylated by a variety of protein kinases, including cyclic AMP- and Ca^{2+}/calmodulin-dependent protein kinase, protein kinase C, casein kinase-2 and AMP-activated protein kinase (Hardie & Guy, 1980; Hardie et al., 1986; Haystead et al., 1988; Haystead & Hardie, 1988; Sim & Hardie, 1988). Acetyl-CoA carboxylase from the rat has now been completely sequenced (Lopez-Casillas et al, 1988) and 7 major regulatory phosphorylation sites have been identified (Haystead et al., 1988; Munday et al., 1988a). However, the actions of protein kinase C and Ca^{2+}/calmodulin-dependent protein kinase are

not thought to be important in the regulation of acetyl-CoA carboxylase *in vivo* (Hardie *et al*, 1986; Haystead *et al*., 1988; Haystead & Hardie, 1988). Acetyl-CoA carboxylase purified from the rat mammary gland (Hardie & Guy, 1980; Munday & Hardie, 1984), liver (Tipper & Witters, 1982) or adipose tissue (Brownsey *et al*., 1981) is reversibly phosphorylated by cyclic AMP-dependent protein kinase, and until recently this phosphorylation and inactivation was thought to occur in hepatocytes and adipocytes in response to cyclic AMP-elevating hormones (Witters *et al*., 1979; Brownsey & Hardie, 1980; Holland *et al*., 1984, 1985). However, more recent studies have shown that the increased phosphorylation in response to glucagon elevation of cyclic AMP causing inactivation of acetyl-CoA carboxylase in hepatocytes, occurs at the sites phosphorylated by AMP-activated protein kinase, not at sites phosphorylated by cyclic AMP-dependent protein kinase (Sim & Hardie, 1988). This finding suggests that acetyl-CoA carboxylase may not be a substrate for cyclic AMP-dependent protein kinase in intact cells (Sim & Hardie, 1988; Hardie *et al*., 1989). Acetyl-CoA carboxylase cannot be phosphorylated by both cyclic AMP-dependent protein kinase and AMP-activated protein kinase in the same molecule (Munday *et al*., 1988b). Prior phosphorylation of acetyl-CoA carboxylase with cyclic AMP-dependent protein kinase prevents the inactivation and

phosphorylation normally produced by AMP-activated protein kinase (Munday et al., 1988b). The physiological significance of the phosphorylation by cyclic AMP-dependent protein kinase in hepatocytes is unclear in view of the evidence discussed above.

In adipocytes and hepatocytes, insulin stimulates phosphorylation of acetyl-CoA carboxylase at site(s) distinct from those phosphorylated by cyclic AMP-dependent protein kinase (Brownsey & Denton, 1982; Witters et al., 1983; Holland & Hardie, 1985; Haystead & Hardie, 1986a), although phosphorylation produces no change in activity (Witters et al., 1983; Holland & Hardie, 1985; Haystead & Hardie 1986b). However purification of the enzyme on avidin-sepharose could result in the loss of the effects of insulin on isolated hepatocytes and adipocytes due to the poor binding of the polymerised form of the enzyme (Witters et al., 1983, Holland & Hardie, 1985; Borthwick et al., 1987). It has also been suggested that the activity alterations measurable in crude extracts of adipocytes after insulin treatment result from the interaction with acetyl-CoA carboxylase of a tightly bound allosteric mediator (Haystead & Hardie, 1986b). Recent analysis of the site(s) phosphorylated on acetyl-CoA carboxylase in response to insulin in isolated adipocytes have shown then to correspond to the sites phosphorylated by casein

kinase-2 (Haystead et al., 1988). However it is not clear as to whether insulin activates either casein kinase-2 or a protein kinase with the same specificity as casein kinase-2 (Sommercorn et al., 1987a; Haystead et al., 1988). Phosphorylation of acetyl-CoA carboxylase by casein kinase-2 has been shown to promote dephosphorylation at sites phosphorylated by cyclic AMP-dependent protein kinase, thus resulting in a secondary effect which would lead to the activation of the enzyme (Sommercorn et al., 1987b; Witters et al., 1988). Increased phosphorylation and inactivation of acetyl-CoA carboxylase has been demonstrated in mammary gland extracts from 24h starved rats and insulin-deficient rats (Munday & Hardie, 1986a). A novel protein kinase has been described (Carling & Hardie, 1986) that may be responsible for phosphorylation of acetyl-CoA carboxylase; the activity of this kinase is stimulated by AMP and appears to have properties similar to AMP-activated protein kinase (Carling et al., 1987). This mammary gland kinase may mediate the effects of 24h-starvation or high-fat feeding upon acetyl-CoA carboxylase in the lactating rat mammary gland.

1.3.1.4 Dephosphorylation

Dephosphorylation of acetyl-CoA carboxylase occurs by the hydrolytic cleavage of phosphoryl residues

under the direction of several protein phosphatases differing in their specificities. The most significant phosphatases for which acetyl-CoA carboxylase is a substrate are protein phosphatases-1, 2A and 2C (Ingebritsen et al., 1983). All three have been shown to reverse the phosphorylation induced by cyclic AMP-dependent protein kinase and lead to the activation of acetyl-CoA carboxylase in rat liver, rat mammary gland and rabbit mammary gland (Hardie & Guy, 1980; McNeillie et al., 1981; Tippers & Witters, 1982; Ingebritsen & Cohen, 1983; Munday & Hardie, 1984). The exposure of acetyl-CoA carboxylase, which has previously been activated or inhibited, and phosphorylated by a hormone, to an antagonistic hormone, not only results in the phosphorylation of its site, but also results in making the antagonistic site more susceptible to phosphatase action and thereby becoming dephosphorylated.

Insulin has been shown to stimulate protein phosphatase activity in cultured mouse cells and that this activity could be attributed to a type-1 phosphatase (Chan et al., 1988). It has also been shown in hepatoma cells that insulin can activate acetyl-CoA carboxylase by either inhibiting protein kinase activity or by stimulating the activation of protein phosphatases that control the

phosphorylation state of the enzyme (Witters et al., 1988).

In mammary tissue, there is evidence that insulin action may be acting through an entirely different mechanism to that of phosphorylation of acetyl-CoA carboxylase, i.e. a dephosphorylation of acetyl-CoA carboxylase (Munday & Hardie, 1986a; Sommercorn et al., 1987b). Refeeding for 2.5h reverses the effects of 24h starvation and is accompanied by dephosphorylation (Munday & Hardie, 1986a). The effect of refeeding can be mimicked by the action of protein phosphatase 2A in vitro, which would seem to indicate that insulin is probably responsible for the reversal of phosphorylation related to the inhibition of acetyl-CoA carboxylase (Munday & Hardie, 1986a). Dephosphorylation and an increase in activity of acetyl-CoA carboxylase in liver in vivo has been demonstrated in rats subjected to hyperglycaemia; an increase in insulin accompanying the hyperglycaemia being responsible for the effects on acetyl-CoA carboxylase (Jamil & Madsen, 1987). From the foregoing it is evident that the way in which insulin modulates acetyl-CoA carboxylase activity is still unclear.

1.3.2 Hormone-Sensitive Lipase

Hormone-sensitive lipase is the rate-limiting step in the hydrolysis of triacylglycerols and catalyses the breakdown of stored triacylglyceride first to DAG and then to monoacyl-glyceride and free fatty acids. Lipolytic hormones, e.g. catecholamines, glucagon and ACTH, stimulate and insulin inhibits the activity of hormone-sensitive lipase (Belfrage et al., 1980; Belfrage et al., 1983a,b). Hormone-sensitive lipase activity is controlled by reversible phosphorylation/dephosphorylation (Steinberg & Huttunen, 1972; Steinberg et al., 1975; Steinberg, 1976; Severson, 1979). Phosphorylation by cyclic AMP-dependent protein kinase has been demonstrated in adipocytes (Belfrage et al., 1977, 1978), which closely parallels the activation of the enzyme (Fredrikson et al., 1981), the rate of phosphorylation being comparable with that found in intact cells (Belfrage et al., 1983a). Phosphorylation by cyclic AMP protein kinase occurs exclusively on a serine residue at one site of the two phosphorylation sites on the enzyme (Fredrikson et al., 1981; Belfrage et al., 1983b; Stralfors & Belfrage, 1983; Garton et al., 1988; Olsson & Belfrage, 1988). This site is termed the 'regulatory' site as its phosphorylation mediates the change (inhibition) in activity. This 'regulatory' site is phosphorylated in intact

adipocytes in response to noradrenaline and other lipolytic hormones (Nilsson *et al.*, 1980; Stralfors & Belfrage, 1983; Stralfors *et al.*, 1984) A second 'basal' site appears to be insensitive to short-term modulation (Stralfors *et al.*, 1984; Garton *et al.*, 1988). Phosphorylation of the 'basal' site by either AMP-activated hormone or Ca^{2+}- and calmodulin-dependent protein kinase has no direct effect on the activity of the enzyme, but it does greatly reduce the extent of phosphorylation and activation achieved subsequently by cyclic AMP-dependent protein kinase (Garton *et al.*, 1989). This suggests that 'basal' site phosphorylation may have an antilipolytic role *in vivo* and act as a feedback system (Hardie *et al.*, 1989).

Dephosphorylation of hormone-induced phosphorylation can be brought about by incubation of adipocytes with insulin. It is accompanied by a decrease in hormone-sensitive lipase activity (Nilsson *et al.*, 1980; Nilsson, 1981). Insulin has no effect on basal hormone-sensitive lipase phosphatase activity, but incubation of adipocytes with insulin before exposure of cells to a lipolytic hormone prevents the increase in phosphorylation of hormone-sensitive lipase (Nilsson, 1981; Stralfors *et al.*, 1984). It is known that hormone-sensitive lipase in cyclic AMP treated cells is rapidly dephosphorylated in parallel with the deactivation by a protein

phosphatase from rat adipose tissue (Belfrage et al., 1983a,b; Olsson et al., 1984; Olsson & Belfrage, 1987, 1988). Hence dephosphorylation in vivo could be a combination of inhibition of cyclic AMP-mediated phosphorylation and the promotion of dephosphorylation by the activation of protein phosphatases (Nilsson et al., 1980; Nilsson, 1981; Stralfors et al., 1984). Recent work examining the mode of insulin regulation of lipolysis in rat adipocytes, indicates that insulin affects the state of phosphorylation of hormone-sensitive lipase via a cyclic AMP-dependent pathway, i.e. lowering the cyclic AMP concentration and through a cyclic AMP-independent pathway involving the activation of protein phosphatase activity, which together results in dephosphorylating both the regulatory and basal phosphorylation sites of hormone-sensitive lipase (Stralfors & Honnor, 1989).

CHAPTER 2

MATERIALS AND METHODS

2.1 RATS

Rats of the Albino Wistar strain, obtained from A Tuck & Son, Battlebridge, Essex, UK, were used for all experiments. The rats were fed ad libitum on Oxoid Breeding Diet (4% fat, 23% protein by energy) (Oxoid Limited, Basingstoke, Hants, UK) and had access to water at all times. They were housed at a temperature of 20°C and on a 12 hour light:12 hour dark cycle, the light period beginning at 08:00 hours.

For experiments described in Chapter 3 (Study of the regulation of mammary gland lipogenesis to insulin and glucagon during lactation), conditions were as follows.

The rats were delivered to the animal house at 8 to 9 weeks of age (body weight 180-200g) and were kept for at least 10-20 days before mating for use during lactation. At birth the litters were limited to 8 pups. The dams weighed about 270g at 10-12 days lactation. Lactating rats used within 2 days post-partum are referred to as early-lactating rats and those used between 10 and 12 days post-partum as mid-lactating rats.

For experiments described in Chapter 4 (Study of the regulation of adipose tissue lipolysis to glucagon during pregnancy and lactation), conditions were as follows.

The rats were bought in at 7 to 8 weeks of age (body weight 160-180g) and were kept for 2 days before mating for use during pregnancy and lactation, the time of mating being determined by the appearance of vaginal plugs. The pregnant and lactating rats were of approximately the same age (11-12 weeks) as the virgin rats in the same experiments. Virgin rats weighed 240-260g. Pregnancy lasted about 21 days, parturition occurring on day 22. Rats used on days 19-20 of pregnancy are referred to as late pregnant rats and weighed 350-355g. At birth the litters were limited to 8 pups. Lactating rats that were used 1 day post-partum are referred to as early-lactating rats, (240-275g). Rats used between 10 and 12 days post-partum are referred to as mid-lactating rats and weighed 270-320g.

All experiments were started between 09:00 hours and 10:00 hours.

2.2 FEEDING

Starved rats were deprived of chow for the 24 hour period immediately preceding their experimental use.

Animals referred to as starved-refed were starved for 24 hours, then refed chow for 2 hours before the experiment.

2.2.1 High-Energy Diets

Palatable high-energy diets have been used in previous studies to induce hyperphagia and obesity in rats (Sclafini & Springer, 1976; Agius et al., 1980, 1981; Rolls et al., 1980). In this study, to investigate the effects of alterations in energy intake on lactating rats, a high-energy 'cafeteria' diet was used. This consisted of cheese cheddars and chocolate chip biscuits, and potato crisps. These foods contain proportionally more fat (25-38% by energy) and less protein (5-11% by energy) (e.g. Rolls et al., 1980) than rat breeding diet chow. The rats also had chow available ad libitum at all times. This diet was fed ad libitum to lactating rats for 7 days i.e. from day 3 to day 10 of lactation.

2.3 INSULIN DEFIENCY

Insulin deficiency was induced by injection of streptozotocin (Schein et al., 1971). For long-term insulin deficiency in lactating rats, a single intraperitoneal injection of streptozotocin (50-100mg/kg body weight, delivered in 0.5ml of a

0.01M citrate buffer, pH 4.5, (referred to later as 50-100% streptozotocin doses)) was given three days after parturition and the animals were killed seven days later. A 5% glucose solution was substituted for water for the first 24 hours after the injection (Nosadini et al., 1982).

2.3.1 Determination of Blood Glucose Concentration

This determination was made the day before the experiment. The rat was maintained under light halothane anaesthesia and a sample of blood was taken from the tail vein. An equal volume of perchloric acid (3% w/v) was added to a volume of blood, mixed and centrifuged at 1000 x g for 10 minutes. The supernatant was removed and centrifuged again at 10,000 x g for 10 minutes in an Eppendorf 4312 centrifuge. The supernatant was diluted 100 fold and neutralised with triethanolamine hydrochloride/potassium hydroxide (0.5M/2M) to pH 7. The sample was centrifuged again at 10,000 x g for 10 minutes to remove precipitated potassium perchlorate. The sample was assayed for glucose by the method of Bergmeyer et al., (1974) using a Gilford 250 spectrophotometer.

2.4 MATERIALS

Fatty acid-poor albumin, adenosine, aprotinin, bacitracin, antipain, leupeptin, pepstatin, crystalline bovine insulin (24 U/mg), Ficoll 400, theophylline, pentobarbitone, adenosine trisphosphate (sodium salt), triethanolamine-hydrochloride, tris(hydroxymethyl)-aminomethane, L-malic acid, $NADP^+$, DNA (Calf Thymus, Type I), phospho(enol) pyruvate (trimonocyclohexylammonium salt), Activated charcoal, Fraction V BSA, cyclic AMP (sodium salt), dithiothreitol, acetyl-CoA, mops (4-morpholinepropanesulphonic acid), adenosine deaminase (EC 3.5.4.17), hexokinase (EC 2.7.1.1), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), pyruvate kinase (EC 2.7.1.40), L-lactate dehydrogenase (EC 1.1.1.27), glycerokinase (EC 2.7.1.30), protein kinase (3', 5'-cyclic AMP dependent) (EC 2.7.1.37) were obtained from Sigma Chemical Company Limited (Poole, Dorset, UK).

Streptozotocin was purchased from Upjohn Company (Kalamazoo,M.I., USA).

Collagenase (EC 3.4.24.3) was type CLS III from Worthington (Lorne Diagnostics Ltd., Unit 11, Cratfield Road, Moreton Hall Industrial Estate, Bury

St. Edmunds, Suffolk, IP32 7DF, UK) used for experiments in Chapter 3.

Collagenase (EC 3.4.24.3) from Clostridium histolyticum used for experiments in Chapters 3 and 4 and NADH were from The Boehringer Corporation (London) Limited (Lewes, East Sussex, UK).

Crystalline pig glucagon was a gift from Lilley Research Laboratories (Indianapolis, IN, USA).

The tritiated water ($^{3}H_{2}O$) (specific radioactivity 5Ci/ml), cyclic (8-^{3}H) AMP (specific radioactivity 28Ci/mmol), and $KH^{14}CO_{3}$ (specific radioactivity 0.5Ci/mol) were from Amersham International plc (Aylesbury, Bucks, UK).

Compound Hoeschst H33258 (Bis-benzinoid) was purchased from Fluka A G (Buchs, Switzerland).

All other organic and inorganic chemicals were obtained from BDH Chemicals Limited (Poole, Dorset, UK).

2.5 MEASUREMENT OF LIPOGENESIS IN MAMMARY ACINI AND ADIPOCYTES

2.5.1 Preparation of Rat Mammary Acini and Adipocytes (Chapter 3)

Rats were anaesthetised with pentobarbitone (60mg/kg body weight) at 09:30 hours; subsequent procedures up to the conclusion of the acini preparation were carried out in a warm room at 37°C. The inguinal/abdominal mammary glands were rapidly dissected and placed in oxygenated (O_2/CO_2,19:1) Krebs-Henseleit (1932) bicarbonate buffered saline (120mM NaCl; 4.7mM KCl; 1.3mM $CaCl_2.6H_2O$; 1.2mM KH_2PO_4; 1.2mM $MgSO_4.7H_2O$; 24mM $NaHCO_3$) containing 5mM glucose (referred to below as 'saline'). The tissue was minced with scissors and finely chopped with razor blades. The resulting minced tissue was rinsed twice in 5 volumes of 'saline' and resuspended in 30ml of 'saline' which contained 2% fatty acid-poor albumin, 5% dialysed Ficoll and 0.1% collagenase. The suspension was placed in a 250ml plastic conical flask, which was incubated with shaking (180 strokes/minute) for 60 minutes. The flask was gassed throughout with O_2/CO_2. The resulting digested tissue was strained through a nylon mesh (approximately 0.15mm mesh size) and the acini (not retained by the mesh) were washed 3 times and separated from adipocytes and cell-fragments by

light centrifugation (400 x g, 15 seconds) from a medium containing 2% Ficoll in 'saline'. Finally the acini were resuspended in 6.5ml of 'saline' containing 2% Ficoll and 4% fatty acid-poor albumin.

Adipocytes from parametrial fat pad were prepared by a modification of the method of Rodbell (1964) using the same composition of the media as for acini except that Ficoll was omitted and 4% fatty acid-poor albumin was used in the digestion, washes and final suspension. The adipocytes were resuspended finally in 3.0ml of suspension medium.

2.5.2 Incubations of Acini and Adipocytes

Portions (1ml) of the final cell suspension of acini were dispersed into each of six 25ml plastic conical flasks, which contained 4ml of the same incubation medium, either without or with addition of hormones and/or effectors. The flasks were gassed with O_2/CO_2 (19:1), stoppered and incubated, with shaking (180 strokes/minute) at 37°C for 15 minutes, after which time 1mCi of ^3H_2O (in 100µl) was added to each flask and incubations allowed to proceed for a further 45 minutes. Rates of ^3H incorporation into lipid by acini were determined to be linear over this time interval. At the end of the incubations the acini were separated by light centrifugation (400 x g, 15 seconds), the supernatant was discarded

and the acini were homogenised in 6ml chloroform/methanol (2:1,v/v) with a Polytron tissue homogeniser (Kinematica, Basle, Switzerland). Portions (1ml) of the final cell suspension of adipocytes were incubated as for mammary acini except that the incorporation of $^{3}H_{2}O$ was performed over 15 minutes after prior exposure to hormones for 5 minutes. This was necessary to ensure linearity of ^{3}H incorporation with time. At the end of the incubations the adipocytes were separated by light centrifugation (400 x g, 15 seconds).

2.5.3 Extraction of Acini and Quantification of Lipid Synthesis

Following the first extraction of acini with chloroform/methanol, after separation of acini and solvent by light centrifugation (400 x g, 15 seconds) the solid matter was re-extracted with another 6ml of the same solvent mixture. To the combined extracts (12ml) were added 3ml of 0.88% KCl and 0.1ml of 6M HCl The mixture was shaken and spun (400 x g, 5 minutes) to obtain two distinct phases. The upper phase was discarded and the lower phase was rinsed twice with 5ml of methanol/0.88% KCl/chloroform (48:47:3 by volume). Finally the lower phase was filtered (Whatman No 1 filter paper) and evaporated to dryness in a rotary evaporator. When required a few drops of ethanol were added to

ensure removal of traces of water. The dry residue was redissolved in 6ml of choroform/methanol (9:1, v/v), transferred to a scintillation vial and evaporated to dryness under a stream of nitrogen. The residue was redissolved in 2ml of toluene. A portion of this toluene solution (0.95ml) was taken for measurement of ^{3}H radioactivity in the total lipid fraction. Another sample was taken for extraction of fatty acids as follows.

To 0.95ml of the toluene solution was added 2ml of methanolic H_2SO_4 (one volume of concentrated H_2SO_4 in 100 volume of methanol). The mixture was heated at 50°C overnight in a stoppered glass tube. It was subsequently cooled and extracted sequentially with 4ml of n-heptane and 4ml of water. The upper phase was separated and retained. The lower phase was washed again with 4ml of n-heptane. The combined upper (organic) phases were washed with 3ml of water and the radioactivity remaining in the washed upper phase was measured to obtain a value for the incorporation of ^{3}H into fatty acids. Radioactivity in a sample of the combined lower phase and washings was measured to obtain a value for the incorporation of ^{3}H into acylglycerol glycerol. The scintillation cocktail that was used for radioactive counting in total lipid and fatty acid fractions was a toluene scintillator (supplied by Packard), whereas that used with other samples was an aqueous-compatible

formulation (Packard Scintillator 299). The specific radioactivity of $^{3}H_{2}O$, in the acini- or adipocyte-free incubation medium (recovered from the centrifugation step by which the cells were isolated prior to lipid extraction), was determined by counting aliquots of this media. The radioactivity in the lipid extracts was expressed as umol $^{3}H_{2}O$-equivalents/minute/100mg defatted dry weight. Defatted dry weight was obtained from the remaining solid matter after extraction for lipid. This procedure was considerably more involved and lengthy than those used by other workers (see, e.g. Robinson & Williamson, 1977b). However, it was designed specifically for studying lipogenesis in mammary tissue, since it prevents the loss of short-chain fatty acids as their volatile methyl esters during subsequent extraction of the acini. Short- and medium-chain fatty acids form a substantial proportion of those synthesised by mammary tissue (Strong & Dils, 1972).

2.5.4 Quantification of Lipogenesis by Adipocytes

This was performed as described by Stansbie et al., (1976). After separation from their incubation medium by light centrifugation, 3ml of 30% (w/v) KOH was added to the adipocytes and the mixture was heated on a hot block at 70°C for 30 minutes. Following saponification, the lipid was esterified

by heating for a further two hours after adding 3ml of 95% (v/v) ethanol. After cooling, samples were acidified with 3ml 6M-H_2SO_4 and the lipid was extracted with two 10ml volumes of petroleum ether (bp 40-60°C). The petroleum ether extracts of the saponified lipid were combined and washed three times with 10ml volumes of distilled water to remove contamination by ^3H_2O and other water-soluble tritiated substances, and they were evaporated to dryness. The dry residue was dissolved in 10ml of Scintillator 299 (Packard Instruments) and the radioactivity determined. The specific activity of ^3H_2O in the incubation medium, obtained as an infranatant after centrifugal floatation of the adipocytes, was determined. The results were expressed as μmol ^3H_2O incorporated into lipid/minute per mUnit of adipocyte $NADP^+$-malate dehydrogenase activity. This was assayed at 30°C in the infranatant of a sample of adipocytes disrupted by vortex-mixing in a glass tube, using a coupled spectrophotometric assay as described previously (Zammit & Corstorphine, 1982b).

2.6 SPECIFIC BINDING OF ^{125}I-LABELLED INSULIN AND ^{125}I-LABELLED GLUCAGON TO RAT MAMMARY ACINI

Acini were prepared as previously described in 2.5.1. Specific binding and degradation of ^{125}I-labelled insulin (specific radioactivity 50-

70μCi/μg) were determined in the presence of bacitracin (600 Units/ml) as described by Flint (1982), except that the pH was 7.6 and the time of incubation was 24 hours. Identical protocols were followed to determine the binding and degradation of ^{125}I-labelled glucagon (2.5ng/ml). Three ^{125}I-labelled glucagon preparations, with differing specific activities, were used as follows.

Experiment 1	52μCi/μg
Experiment 2	346μCi/μg
Experiment 3	295μCi/μg

Those used in experiments 1 and 2 were prepared from native glucagon by the chloroamine-T method, whereas that used in experiment 3 was a monoiodinated commercial product (NEN Chemicals, Dreiech, Germany). Control experiments, used to correct for non-specific binding, were performed either by shortening the duration of the incubation (one hour, 18°C) or by diminishing the extent of glucagon degradation (by using aprotinin, 2.5 Units/ml; pepstatin, 0.5mM; and antipain, 0.5mM, singly or in various combinations).

The measurements of insulin- and glucagon-binding in these experiments were performed by Dr. D.J. Flint.

2.7 DETERMINATION OF CYCLIC AMP LEVELS IN RAT MAMMARY ACINI

2.7.1 Preparation and Incubation of Acini

Acini were prepared essentially as described before, except that the acini were finally resuspended in 7.5ml of 'saline' containing 2% Ficoll and 4% fatty acid-poor albumin.

Portions (1ml) of the final suspension medium of acini were dispensed into each of five 25ml plastic conical flasks, which contained 6ml of the same incubation medium, either without or with addition of varying concentrations of theophylline. The flasks were gassed with O_2/CO_2, stoppered and incubated at 37°C with shaking (160 strokes/minute). For the zero time point, the cells were added to the incubation medium, mixed, and a 1ml sample was immediately removed for assay of cyclic AMP. For later time points, samples (1ml) were removed at 3, 15, 30, 45 and 60 minutes. The incubations were stopped by adding the sample to ice-cold perchloric acid (final concentration 5%). They were then mixed and rapidly frozen in liquid nitrogen. Such samples could then be stored for up to four weeks without any decrease in cyclic AMP levels.

2.7.2 Determination of Cyclic AMP Concentration

The cyclic AMP assay is based on the competition between unlabelled cyclic AMP and a fixed amount of the tritium labelled compound for binding to a sub-stoichiometric quantity of a protein which has a high affinity and specificity for cyclic AMP (Method of Gilman, 1970 as modified by Tovey *et al.*, 1974). When equilibrium has been reached between cyclic AMP binding protein and the cyclic AMP present, measurement of the protein-bound radioactivity enables the amount of the unlabelled cyclic AMP in a sample to be calculated.

A standard curve relating known concentrations of cyclic AMP based on the above principle was prepared. The cyclic AMP concentration of unknowns could be determined with reference to the calibration curve. The standards were prepared by diluting a stock cyclic AMP solution with assay buffer (50mM-Tris-HCL, 4mM-EDTA, pH 7.0) to the appropriate final concentrations (0-160 pmol/ml) on the day of the assay. The standards were treated with perchloric acid in the same way as were the unknown samples and also subsequently treated in exactly the same way as unknown samples.

All assay procedures were carried out in duplicate at 0°C. To aliquots (50µl) of unknown sample or

standard cyclic AMP solutions were added 50µl (0.025µCi; 0.89pmol) of [^{3}H]-cyclic AMP in assay buffer, 80µl of assay buffer and 20µl of binding protein solution (0.4mg cyclic AMP dependent-protein kinase/ml assay buffer containing 5mg/ml BSA). Control or 'charcoal blank' tubes contained 20µl of assay buffer instead of binding protein solution. Each tube was mixed for five seconds and left to stand on ice for at least two hours in order to allow equilibrium binding of cyclic AMP in samples containing up to 8pmol of cyclic nucleotide. Then to each tube was added 100µl charcoal suspension (2.6mg activated charcoal/ml assay buffer containing 2mg/ml BSA). This was mixed and left on ice for 1.5 minutes, then spun at 10,000 x g for two minutes in an Eppendorf centrifuge. Unbound cyclic AMP was adsorbed onto the charcoal at this step. The fraction of [^{3}H]-cyclic AMP that was bound to the binding protein remained non-sedimentable and was quantified by withdrawing a 200µl sample of the supernatant and adding it to 10ml of Scintillator 299, prior to the determination of radioactivity in a scintillation counter. Results were expressed as pmol cyclic AMP/g wet weight of acini.

2.8 MEASUREMENT OF ACETYL-COA CARBOXYLASE ACTIVITY IN MAMMARY ACINI

The preparation and incubation of mammary acini was as described in section 2.5.1 with the exception that an aliquot (100µl) of non-radioactive water was used. At the end of the incubations, the acini were separated by light centrifugation (400 x g, 15 seconds) and frozen in liquid nitrogen. They were subsequently stored frozen at -30°C.

2.8.1 Preparation of Homogenates

The frozen acinar pellets were weighed, powdered with a spatula cooled in liquid nitrogen and homogenised (two periods of 10 seconds) with a polytron tissue homogeniser in 1.5ml of ice-cold extraction medium containing 0.25M-sucrose, 20mM-Tris, 20mM-Mops, 1mM-dithiothreitol, 2mM-EDTA and 2mM-EGTA, pH 7.4 at 4°C. Fatty acid-poor albumin (10mg/ml) was added to the homogenate, which was immediately centrifuged at 10,000 x g for 60 seconds in an Eppendorf 5412 centrifuge.

2.8.2 Assay of Acetyl-CoA Carboxylase Activity

Acetyl-CoA carboxylase activity was assayed at 30°C as described by Brownsey et al., (1979). The assay medium contained 100mM-Tris/acetate, 0.5mM-EDTA,

10mM-magnesium acetate, 1mM-dithiothreitol, 150μM-acetyl-CoA, 5mM-ATP, 20mM-magnesium citrate, 15mM $KH^{14}CO_3$ and 10mg of fatty acid-poor albumin/ml in a total volume of 0.45ml contained in stoppered plastic tubes. The final pH was 7.4. The sample was incubated at 30°C for one minute before assaying in order to heat the sample to the same temperature as that of the assay mix. The reaction was initiated by addition of 20μl of sample either (i) immediately or (ii) after pre-incubation for 15 minutes in the presence of 20mM-potassium citrate. They were terminated at either (a) 60 seconds and 120 seconds for condition (i) or (b) 30 seconds and 60 seconds for condition (ii) by addition of 0.1ml of 6M-HCl. Results determined in (i) represent the Initial activity (I) of acetyl-CoA carboxylase. Results determined in (ii) represent the citrate activity (C) of acetyl-CoA carboxylase. For controls, conditions were as for (i) and (ii), except that acid was added before the addition of the sample.

2.9 MEASUREMENT OF GLYCEROL RELEASE BY ADIPOCYTES

2.9.1 Preparation of Adipocytes from Parametrial Fat

Rats were anaesthetized with pentobarbitone (60mg/Kg body weight) at 09:30 hours, all subsequent procedures were carried out in a warm room at 37°C.

The parametrial fat pads from one rat were rapidly dissected from the urinogenital tract, then rinsed in oxygenated (O_2/CO_2, 19:1) Krebs-Henseleit (1932) bicarbonate-buffered saline (120mM NaCl; 4.7mM KCl; 1.3mM $CaCl_2.6H_2O$; 1.2mM KH_2PO_4; 1.2mM $MgSO_4.7H_2O$; 24mM $NaHCO_3$) containing 5mM-glucose (referred to below as 'saline') and finely minced. Adipocytes were prepared using the technique of Rodbell (1964) as follows. The minced adipose tissue was divided between three flasks; each flask contained 5ml of 'saline' which contained 4% fatty acid-poor albumin and 3% collagenase. The suspensions were incubated with shaking (160 strokes/minute) for 60 minutes. The resulting digested tissue was strained and the adipocytes were washed twice by centrifugal floatation from the same basal medium (without collagenase) and finally resuspended in a total of 16ml.

2.9.2 Incubations of Adipocytes

Portions (1ml, containing approximately 10^5 cells) of the cell suspension were dispensed into each of fourteen 25ml plastic conical flasks, which contained 4ml of the same basal medium, either without or with addition of hormones and/or effectors. The flasks were gassed with O_2/CO_2 (19:1), stoppered and incubated at 37°C in a shaking water bath (160 strokes / minute) for 50 minutes.

Rates of glycerol release by adipocytes were determined to be linear over this time interval. At the end of the incubations the adipocytes were separated from the medium by light centrifugation. To 4.5ml of the medium was added 0.45ml 45% (w/v) ice-cold perchloric acid. The acidified sample was spun at 1000 x g for 10 minutes. The supernatant was removed and to it was added 0.25ml triethanolamine-HCl (1M), followed by a saturated solution of K_2CO_3 until neutrality was achieved (using universal indicator). The resulting extract was centrifuged again at 1000 x g for 10 minutes. The supernatant was retained and kept on ice for the assay of glycerol.

2.9.3 Glycerol Assay

Glycerol was assayed spectrophotometrically at 340nm using a Gilford 250 spectrophotometer by the method of Garland & Randle (1962). The assay medium in 2.0ml contained 0.1mM-triethanolamine-HCl/6mM-magnesium sulphate/2mM-potassium hydroxide, pH 7.6, 3mM-NADH, 75mM-ATP, pH 7.0, 35mM-PEP, 20mU-pyruvate kinase, 200mU-L-lactic dehydrogenase and sample from which a base line was obtained. The reaction was started with the addition of glycerokinase (2mU) and allowed to go to completion. The difference between the two steady states gave a measure of the amount of glycerol in the sample. Glycerol release from

adipocytes was expressed as μmol/hour/10^5 cells or μmol/hour/10 mg DNA.

2.10 MISCELLANEOUS PROCEDURES

2.10.1 Cell Number Determinations

Adipocyte size was determined as described by Vernon (1977). A sample of the cell suspension was placed in a haemocytometer which was mounted on the stage of a projecting microscope. The diameter of 100 cells was measured and from this the adipocyte volumes and mean volume were calculated. A dry weight determination was made on a 200μl sample of the final suspension of cells by drying to constant weight in an oven at 60°C. From these determinations it was possible to calculate the number of cells per 1ml of cell suspension by making the assumption that 1ml adipocyte volume accounted for a weight of 0.91g.

2.10.2 DNA Determination

An aliquot (1ml) of adipocyte suspension was disrupted by vortex-mixing in a glass tube. The suspension was left to stand at room temperature for five minutes, then at 4°C for 15 minutes. The infranatant was removed and an equal volume of ice-cold ether was added. The tubes were mixed

thoroughly and left to stand for five minutes. This was followed by slow centrifugation. The ether layer was removed as completely as possible, and any remaining ether was evaporated using a stream of air. The samples were then diluted 20-fold with a medium (A) containing 0.01mM-$NaPO_4$ and 2.0M-NaCl; pH 7.4.

The DNA content of the samples was measured by monitoring enhancement of fluorescence that occurred on addition of bisbenzimidazole (Hoechst 33258) to the DNA-containing sample (Labarca & Paigen, 1980). Stock solutions of DNA (200µg/ml) were prepared in 2mM EDTA and stored at -80°C. DNA standards were prepared by diluting stock DNA solution with phosphate-saline buffer to the appropriate final concentration (0-20µg/ml) on the day of the assay. A stock solution of fluor (H33258) (200µg/ml) was prepared and stored at 4°C. On the day of the assay, this stock solution was diluted with medium A to a final concentration of 2µg/ml.

To duplicate aliquots (500µl) of unknown sample or standard DNA solutions were added 500µl fluor. For 'unknown sample blanks' 500µl of medium A was added instead of fluor to aliquots of unknown samples. Each tube was mixed and left to stand at room temperature for at least one hour, before reading fluorescence at an excitation wavelength 355nm and

an emission wavelength 445nm using a Baird Atomic Fluorimeter.

CHAPTER 3

STUDY OF THE REGULATION OF MAMMARY GLAND LIPOGENESIS BY INSULIN AND GLUCAGON DURING LACTATION

The aims of this study were two fold :

(a) To determine the way in which mammary gland lipogenesis is regulated; the effects of glucagon and other agents (which are known to affect lipid metabolism in adipose tissue) on the rates of lipogenesis and the activity of acetyl-CoA carboxylase in acini isolated from the mammary gland of fed mid-lactating rats were studied.

(b) To determine the effect of insulin on the rates of lipogenesis in acini isolated from the mammary glands of rats at different stages of lactation, and in acini isolated from mid-lactating rats that were subjected to various physiological (and pathological) conditions. Starvation, diabetes and cafeteria-feeding were studied. From these data of the dose-response characteristics of different acini with respect to insulin, the sensitivity to insulin of mammary lipogenesis under different conditions could be determined.

3.1 STUDY OF THE RATES OF LIPOGENESIS AND THE ACTIVITY OF ACETYL-COA CARBOXYLASE IN ACINI ISOLATED FROM THE MAMMARY GLANDS OF FED MID-LACTATING RATS

3.1.1 Lipogenesis

The absolute rates of ^3H incorporation into fatty acids by acini in the absence of any additions were similar to the rates of fatty acid synthesis reported previously by other workers (Robinson & Williamson, 1977c; Munday & Williamson, 1981). Experiments in which the incorporation of ^3H_2O into lipid was measured as a function of time demonstrated that rates of lipogenesis were linear between 5 and 60 minutes. Subsequently rates were measured routinely between 15 and 60 minutes, the initial 15 minute period being used to ensure the attainment of the full response to the effectors added. The incorporation of label into acylglycerol glycerol is about 20% of that into fatty acids under all conditions studied.

3.1.1.1 Effects of Glucagon

Incubation of acini with glucagon had no effect on the rates of synthesis of total lipid or fatty acids (Table 3.1). The concentration of glucagon used in the present study was about 100-fold higher than those found to give a maximal inhibitory response on

	Incorporation of ^3H from ^3H_2O (μmol/min per 100mg defatted dry wt.)		
	Total lipid	Fatty acid	Acylglcerol glycerol
Additions			
None (11)	0.543±0.023	0.429±0.026	0.113±0.007
Glucagon (11)	0.561±0.027	0.432±0.033	0.129±0.017
Insulin (11)	0.812±0.042*	0.668±0.046*	0.146±0.011
Glucagon plus insulin (3)	0.907±0.110*	0.774±0.160†	0.133±0.015

Table 3.1. Effects of glucagon plus insulin on the rates of incorporation of ^3H from ^3H_2O into total lipid, fatty acid and acylglycerol glycerol in acini from mammary glands of mid-lactating rats.

Acini were incubated as described in the Materials and Methods chapter for 15 min either in the absence of any additions or in the presence of glucagon (2μg/ml) or insulin (1μg/ml) or a combination of the two hormones. After 15 min, ^3H_2O (100μl, containing 1mCi) was added and the incubations were allowed to proceed for a further 45 min. The acini were separated from the medium by centrifugation and extracted as described in the Materials and Methods chapter. Values are means ± S.E.M. for determinations on separate preparations of acini (numbers shown in parentheses). Levels of statistical significance (paired t test) for the differences in rates obtained under different conditions from those obtained in the absence of additions are denoted by *$P<0.01$, and †$P<0.05$.

acetyl-CoA carboxylase activity in adipocytes (Zammit & Corstorphine, 1982b). This high concentration was used to ensure that degradation of the hormone by the acini did not significantly deplete the incubations of glucagon. The complete lack of effect of glucagon on the rate of lipogenesis in mammary acini was in contrast with the inhibition of lipogenesis observed in adipocytes (Table 3.2) and in agreement with results of two determinations reported by Williamson et al., (1983) since these experiments were performed. More recent work involving the infusion of glucagon into fed lactating rats, at a rate sufficient to cause a 5-6 fold increase in the plasma concentration of the hormone (Soman & Felig, 1978), indicated that no change in the rates of lipogenesis in the liver, mammary gland or adipose tissue could be elicited (Jones et al., 1984a), although adipose tissue rates tended to be lower.

To further investigate these results, the specific binding of ^{125}I-labelled insulin and ^{125}I-labelled glucagon to rat mammary acini were studied. These results (Table 3.3) showed that the specific binding and degradation of ^{125}I-labelled insulin were similar to those reported by Flint (1982) for mammary acini of 10 day lactating rats. However, significant specific binding of ^{125}I-glucagon to preparations of acini could not be detected, suggesting that, unlike

Table 3.2. Effects of insulin and glucagon on the rate of ^3H incorporation from ^3H_2O into fatty acids by adipocytes from parametrial adipose tissue.

Adipocytes were prepared and incubated in a manner identical with that described for mammary acini (see Materials and Methods chapter), except that Ficoll was omitted from the media. Adipocytes were incubated for 5 min in the absence or presence of insulin or glucagon, after which 1mCi of ^3H_2O was added (100µl) and the rate of lipogenesis measured after a further 15 min of incubation as described by Stansbie et al. (1976). Values are means S.E.M. for three separate determinations and are expressed as ^3H incorporation into fatty acids per munit of $NADP^+$–malate dehydrogenase activity at 30°C in the infranatant of a sample of adipocytes disrupted by vortex–mixing in a glass tube. Values significantly different from controls ($P<0.01$) are indicated by *.

Additions	Incorporation of ^3H from ^3H_2O (µmol/min per munit of malate dehydrogenase activity)
None	13.3±0.3
Insulin (1µg/ml)	17.5±0.3*
Glucagon (2µg/ml)	9.0±0.2*

Table 3.3. Specific binding of ^{125}I-labelled insulin, but not of ^{125}I-labelled glucagon, to rat mammary acini. Specific binding and degradation of ^{125}I-labelled insulin (sp. radioactivity 50–70 μCi/μg) were determined in the presence of bacitracin (600units/ml) exactly as described by Flint (1982), except that the pH was 7.6 and the time of incubation was 24h. the results obtained were similar those reported by Flint (1982) for mammary acini of 10-day lactating rats. Identical protocols were followed to determine the binding and degradation of ^{125}I-labelled glucagon (2.5ng/ml). The results of three separate experiments, each done with a single preparation of acini, are given. Three ^{125}I-labelled glucagon preparations, with different specific radioactivities, were used as follows : Expt.1, 52μCi/μg; Expt. 2, 346μCi/μg; Expt. 3, 295μCi/μg. Those used in Expts. 1 and 2 were prepared from native glucagon by the chloromine-T method, whereas that used in Expt. 3 was a monoiodinated commercial product (NEN Chemicals, Dreieich, Germany). Control experiments (not shown) demonstrated that neither by shortening the duration of the incubation (1h, 18°C) nor by diminsihing the extent of glucagon degradation (by using aprotinin, 2.5units/ml; pepstatin, 0.5mM; and antipain, 0.5mM, singly or in various combinations) was it possible to increase the measurable specific glucagon binding : indeed, both modifications resulted in decreases in binding.

	^{125}I-labelled insulin		^{125}I-labelled glucagon	
Expt. no.	Specific binding (c.p.m./100μg of DNA)	Degradation (%)	Specific binding (c.p.m./100μg of DNA)	Degradation (%)
1	3571	5	164	60
2	7239	10	559	42
3	5601	5	432	33

that in adipose tissue, mammary tissue lipogenesis is not likely to be sensitive to circulatory glucagon *in vivo*.

3.1.1.2 Effects of Insulin

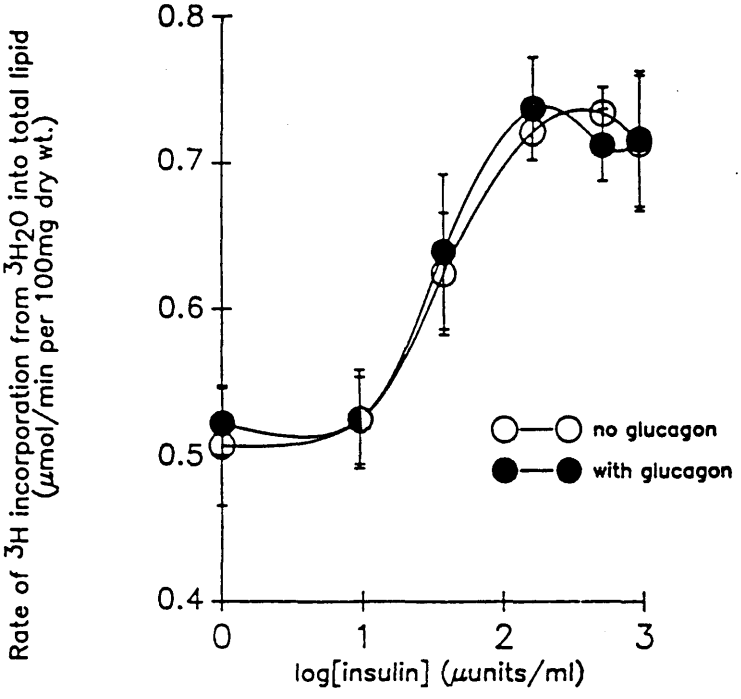
Inclusion of insulin in the incubation medium resulted in a marked stimulation (50-60%) of lipogenesis in mammary acini (Table 3.1). This stimulation was much larger than that observed previously (Robinson & Williamson, 1977a; Agius & Williamson, 1980; Williamson *et al.*, 1983) for acini prepared from lactating rats fed on a comparable diet, although a similar degree of stimulation by insulin was reported by the same workers when they used acini isolated from rats fed on a high-energy diet (Agius & Williamson, 1980). The reason(s) for these differences are unclear, though Williamson *et al.*, (1983) have proposed that the low percentage stimulation by insulin obtained by them may be due to lack of another effector(s) such as the gastrointestinal peptide, that acts synergistically with insulin *in vivo* (Page, 1989). Rates of lipogenesis *in vivo* (Robinson *et al.*, 1978; Agius & Williamson, 1980) are much higher compared to those reported *in vitro* (Robinson & Williamson, 1977a; this thesis), but there is a parallelism between *in vivo* and *in vitro*. Incubation of acini with both insulin and glucagon in the incubation medium did

not have any significant effect on the rate of lipogenesis in acini, compared with the rates obtained when insulin alone was added, contrary to the antagonistic effects of the two hormones in adipocytes (Zammit & Corstorphine, 1982b; Green, 1983). Moreover, glucagon did not affect the sensitivity to insulin of lipogenesis by acini, as judged by its lack of effect on the dose-response curve for insulin (Fig. 3.1).

3.1.1.3 Effects of Phosphodiesterase Inhibitor

Theophylline is known to augment an increase in the concentration of cyclic AMP by inhibiting cyclic AMP phosphodiesterase and/or as antagonist of the adenosine A_1-site in tissues in which the inhibitory A_1-site predominates. In experiments with adipocytes, incubation with theophylline results in inhibition of lipogenesis (Trost & Stock, 1979). Therefore, in order to test whether glucagon might have an inhibitory effect on lipogenesis in mammary acini by augmenting the potential effects of theophylline, experiments were performed in which either glucagon or insulin was added to the incubation medium simultaneously with theophylline (5mM). Theophylline at this concentration inhibited the rate of lipogenesis by about 15% (Table 3.4, experimental series I). Glucagon did not augment this effect. However, insulin still had a

Fig3.1 Dose–response curve for the effect of insulin on the rate of lipogenesis by mammary–gland acini in the absence or presence of glucagon.



Acini were incubated as described in Materials and Methods chapter for 15min. in the presence of either glucagon (2μg/ml) or insulin (1μg/ml). After 15 min., ^3H_2O (100μl, containing 1mCi) was added and the incubations were allowed to proceed for a further 45 min.. The acini were separated from the medium by centrifugation and extracted as described in the Materials and Methods chapter. Values are means ± S.E.M. for three separate determinations.

Table 3.4. Effects of glucagon and insulin on the rates of incorporation of ^3H from ^3H_2O into total lipid, fatty acid and acylglycerol glycerol in the presence of theophylline or adenosine deaminase in acini from mammary glands of lactating rats.

Acini were incubated for 15 min in the presence of hormones and/or effectors (see Table 3.1); ^3H_2O was added and incubations were terminated after a further 45 min.. Values are means ± S.E.M. for two separate series of experiments (I and II; n = 4 and 3 respectively). See Table 3.1 for statistical treatment of data (*P<0.01, †P<0.05).

	Incorporation of ^3H from ^3H_2O (μmol/min per 100mg defatted dry weight)					
	Total Lipid		Fatty acid		Acylglycerol glycerol	
Series of Experiments...	I	II	I	II	I	II
Additions						
None	0.64±0.15	0.52±0.03	0.51±0.14	0.40±0.03	0.14±0.01	0.12±0.02
Glucagon (2μg/ml)	0.65±0.10	0.56±0.05	0.47±0.10	0.41±0.12	0.18±0.03	0.15±0.05
Insulin (1μg/ml)	1.03±0.31*	0.79±0.05*	0.72±0.18*	0.62±0.06*	0.17±0.01	0.18±0.02
Theophylline (5mM)	0.56±0.13†	–	0.45±0.11†	–	0.12±0.06	–
Theophylline + glucagon	0.55±0.14†	–	0.42±0.14†	–	0.13±0.01	–
Theophylline + insulin	0.81±0.20	–	0.63±0.20†	–	0.18±0.04	–
Adenosine deaminase (1 unit/ml)	–	0.55±0.06	–	0.42±0.06	–	0.13±0.01
Adenosine deaminase + glucagon	–	0.55±0.06	–	0.41±0.05	–	0.14±0.02
Adenosine (50μM)	–	0.56±0.08	–	0.41±0.06	–	0.15±0.04

stimulatory effect on lipogenic rates in the presence of theophylline (Table 3.4).

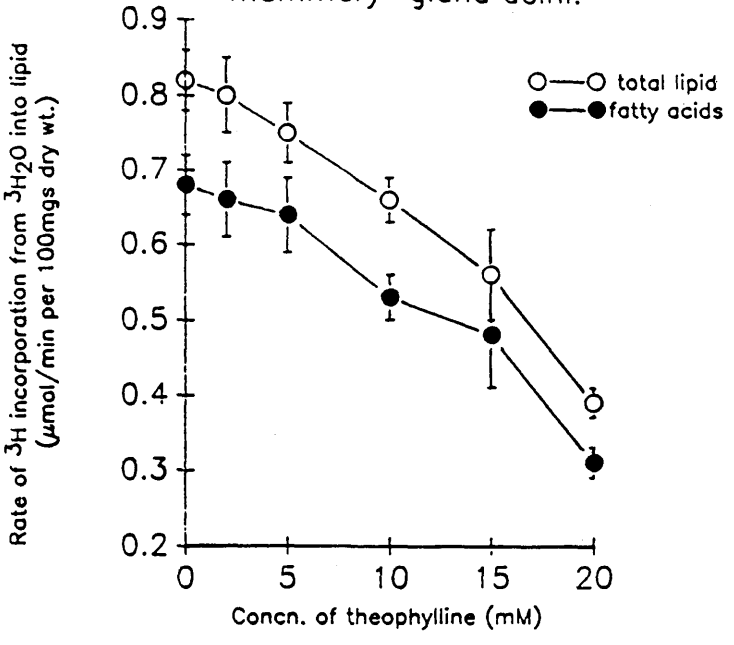
3.1.1.4 Effects of Adenosine

A similar series of experiments was performed with adenosine and adenosine deaminase, which, in adipose tissue, induce lipogenic and antilipogenic effects respectively (Trost & Stock, 1979; Honnor & Saggerson, 1980; Vernon et al., 1983, 1987; Zammit, 1988). Neither adenosine nor adenosine deaminase affected the lipogenic rates of mammary acini (Table 3.4, series II). Glucagon added in combination with adenosine deaminase also did not result in any inhibition of the rate of lipogenesis (Table 3.4). Both adenosine and adenosine deaminase were added to acini at concentrations in excess of those required to elicit their respective responses in adipose tissue. However, the apparent unresponsiveness of acini to these agents has to be interpreted with caution, since the amounts of cell protein in these incubations were much higher than those involved in adipocyte incubations (e.g. Zammit & Corstorphine, 1982b). Therefore rates of production and/or destruction of adenosine in incubations of acini could be much higher, thus tending to mask the effects of exogenously added adenosine or adenosine deaminase.

3.1.1.5 Effects of Cyclic AMP

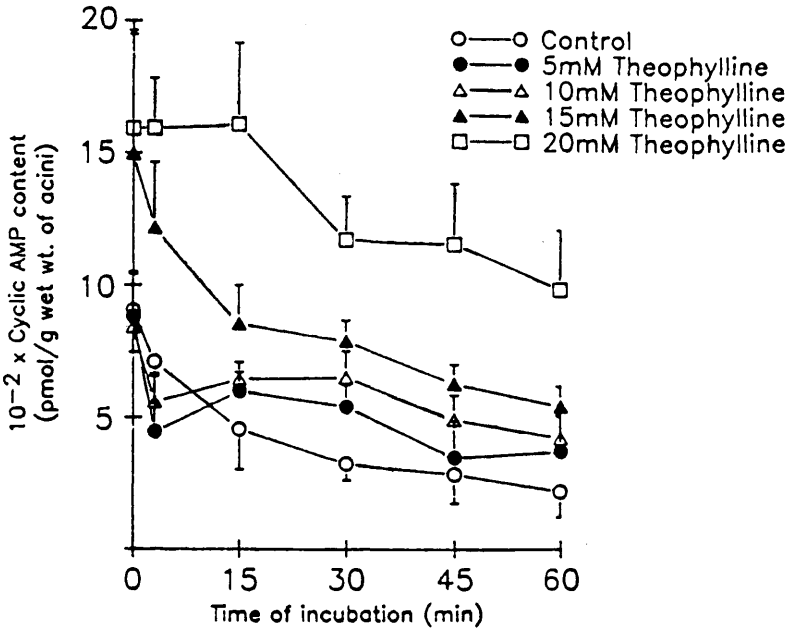
The observation that theophylline, when added to the incubation medium at a concentration of 5mM, gave a modest but significant inhibition of lipogenesis in acini prompted us to study the dependence of the extent of inhibition on increasing theophylline concentrations (Fig. 3.2). The results suggest that theophylline, in the concentration range normally used in studies on isolated adipocytes to increase intracellular concentrations of cyclic AMP and stimulate lipolysis (Mosinger & Vaughan, 1967; Steinfelder & Joost, 1982), is a potent inhibitor of lipogenesis in mammary acini. Direct measurements of cyclic AMP concentration in mammary acini showed that the inhibitory effects observed with theophylline on lipogenic rates were accompanied by a rise in the concentration of the cyclic nucleotide (Fig. 3.3). The concentration of cyclic AMP in mammary acini from fed rats was increased 2-3 fold over controls in the presence of 20mM theophylline. However, at the lowest concentration tested (5mM), theophylline was without statistically significant effect on cyclic AMP content of acini (Fig. 3.3). The concentration of cyclic AMP in freshly prepared acini diluted into incubation medium in the absence of theophylline was similar to that reported previously for milk-free rat mammary tissue (Sapag-Hagar & Greenbaum, 1974).

Fig3.2 Effects of theophylline on the rate of incorporation of ^{3}H from $^{3}H_2O$ into total lipid and fatty acids by rat mammary gland acini.



Acini were incubated with the appropriate concentration of theophylline for 15 min., and the rate of incorporation of ^{3}H was measured over the following 15 min. after addition of 1mCi of $^{3}H_2O$.

Fig3.3 Theophylline–induced increase in intracellular cyclic AMP content of mammary acini: effect of duration of incubation.



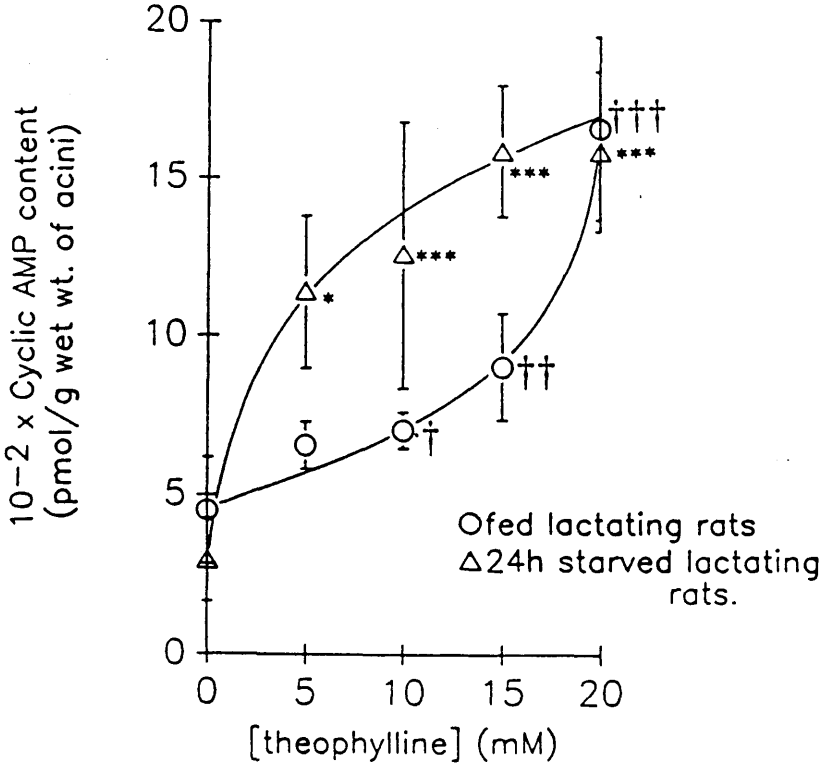
Acini from fed lactating rats were incubated at 37°C for the times indicated, in the absence of theophylline (O) or in the presence of theophylline at a concentration of 5mM (●), 10mM (△), 15mM (▲) or 20mM (□), before samples were rapidly quenched in $HClO_4$ and their content of cyclic AMP was measured. Bars indicate S.E.M. values around the means plotted, which are from five separate preparations incubated at each theophylline concenatration.

The sensitivity of the response of intracellular cyclic AMP to theophylline was increased in mammary acini isolated from lactating rats that had been starved for 24h (Fig. 3.4). Comparison of values measured in the presence of theophylline to those measured for the corresponding group in the absence of theophylline (tested by Student's t test for paired samples) indicated that for fed rats, 10mM-, 15mM- and 20mM-theophylline values, and for starved rats, 5mM-, 10mM-, 15mM- and 20mM-theophylline values, were significantly different (Fig. 3.4). Group mean values were significantly different (Student's t test, $P<0.05$) between acini from fed and starved rats at 5mM-, 10mM- and 15mM-theophylline.

There was a positive correlation (coefficient 0.9705) between the percentage inhibition of lipogenesis and the concentration of cyclic AMP in mammary acini measured at the end of the incubation, i.e. after 60 minutes (Fig. 3.5). This observation suggested that the inhibition of lipogenesis by theophylline was related to a rise of the cyclic AMP concentration, but does not necessarily indicate a causal relationship between the two parameters.

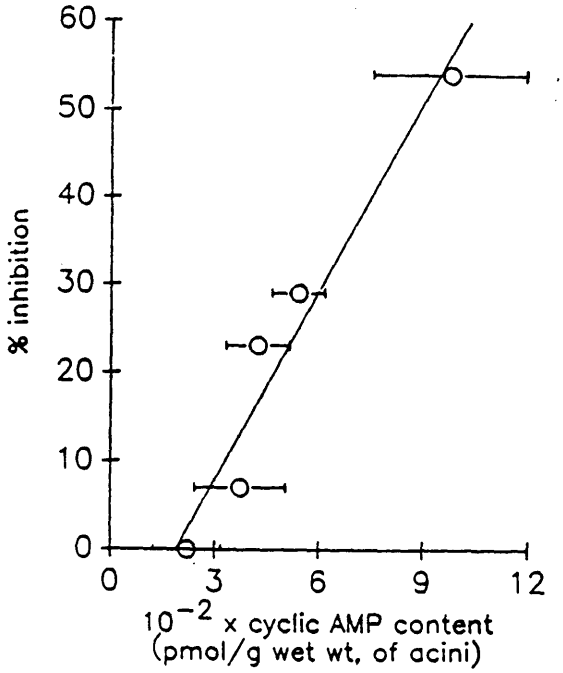
The cyclic AMP concentrations in acini discussed above were those measured after 60 minutes of incubation at 37°C. Measurements were also made at

Fig3.4 Dose–dependent increase in intracellular cyclic AMP content of mammary acini treated with theophylline.



Acini prepared from either fed (O) or 24h-starved lactating rats (△), were incubated at the indicated concentrations of theophylline and sampled as in Fig. 3.3. The mean cyclic AMP concentrations after incubation of acini (five separate preparations from rats in each state) for 15 min. are plotted; bars indicate S.E.M. Values significantly different from those measured for the corresponding group in the absence of theophylline (tested by Student's t test for paired samples) are indicated as follows : †, *, $P<0.05$; ††, **, $P<0.01$; †††, ***, $P<0.001$. Group mean values were significantly different (Student's t test, $P<0.05$) between acini from fed and starved rats at 5mM-, 10mM- and 15mM-theophylline.

Fig3.5 Correlation between intracellular cyclic AMP content and inhibition of lipogenesis provoked by treatment of mammary acini with theophylline.



Values for inhibition of lipogenesis are calculated from Fig. 3.3 on the effect of theophylline on the rate of lipogenesis in rat mammary acini; in that work, lipogenesis was measured as an average rate between 15 and 60 min. of incubation. Linear-regression analysis by the least squares method gave the line shown, with a correlation coefficient of 0.9705.

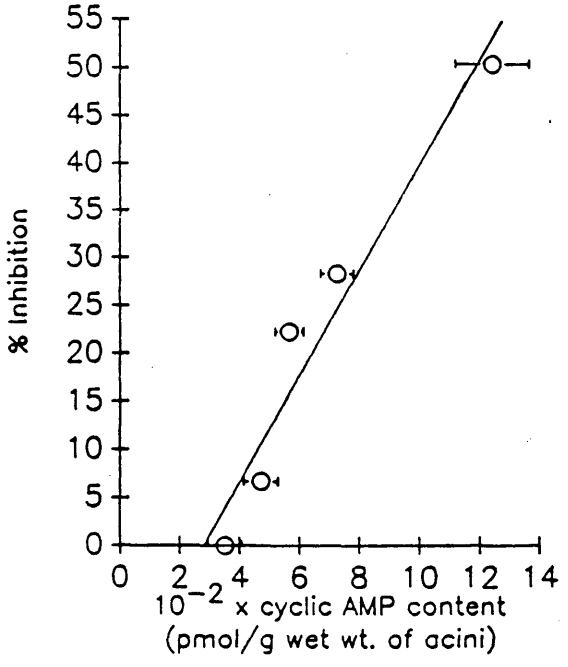
other time-points during incubations of acini with or without theophylline. Cyclic AMP concentrations were not constant during 60 minutes of incubation but decreased. The rate of this decrease was rapid during the initial phase of the incubation (0-5 minutes) except at the highest concentration of theophylline tested (20mM), and then much slower between 15 and 60 minutes (Fig. 3.3). At all concentrations of theophylline tested and also in its absence, the cyclic AMP content was significantly lower at 60 minutes than at time zero. However, using Student's t-test for paired samples, it was ascertained that there was a significant decrease in cyclic AMP between 15 minutes and 60 minutes of incubation in the presence of 5mM ($p<0.05$) and 20mM ($p<0.01$) theophylline, but not in its absence.

These values, at any given concentration of theophylline, could justifiably be pooled all together at times between 15 and 60 minutes for the plot of theophylline concentration and percentage inhibition of lipogenesis, since lipogenesis was measured by $^{3}H_{2}O$ incubation throughout the same interval. Qualitatively, similar results were obtained to those given in Figs. 3.4.and 3.5 respectively. In the case of the latter plot, the correlation coefficient actually increased slightly

to 0.9747 when the data were handled in this way (Fig. 3.6).

The reasons for the observed biphasic pattern of decline in cyclic AMP concentration are not clear. It is possible that the initially high levels of cyclic AMP in freshly prepared acini bring about an activation of phosphodiesterase (as found in liver cells treated so as to increase intracellular cyclic AMP (Houslay et al., 1983a)), this giving a rapid breakdown of the cyclic nucleotide over the first 15 minutes which slows down as the cyclic AMP concentration reaches a new near-steady state. Since theophylline (a methylxanthine) is an inhibitor of phosphodiesterase (as well as an antagonist of the adenosine A_1 site) theophylline (20mM) would therefore be expected to attenuate the decrease in cyclic AMP observed in its absence as seen in Fig. 3.3. If the effects of theophylline on acinar lipogenesis are mediated through this effect, the persistence of the lipogenic effect of insulin (Table 3.4) under these conditions would be compatible with the known ability of insulin to reverse modest increases in intracellular concentrations of cyclic AMP in isolated adipocytes (Manganiello et al., 1971) and hepatocytes (Pilkis et al., 1975). However no comparable effect was found to accompany insulin treatment of mammary acini in the presence of a isobutyl methylxanthine,

Fig3.6. Correlation between intracellular cyclic AMP content and inhibition of lipogenesis provoked by treatment of mammary acini with theophylline.



Values for inhibition of lipogenesis are calculated from Fig. 3.3 on the effect of theophylline on the rate of lipogenesis in rat mammary acini; in that work, lipogenesis was measured as an average rate between 15 and 60 min. of incubation. Cyclic AMP content at corresponding theophylline concentrations (0, 5, 10, 15 and 20mM) are therefore averages of values recorded from five separate preparations from fed lactating rats at 15, 30, 45 and 60 min. of incubation. Linear-regression analysis by the least squares method gave the line shown, with a correlation coefficient of 0.9747.

isoprenaline (Clegg & Mullaney, 1985). This and other results suggest that the rate of lipogenesis and the cyclic AMP content in mammary acini can vary independently of one another. Indeed, there are reports of antagonism by insulin of the effects of adrenaline which is not accompanied by any change in cyclic AMP concentration (e.g. in muscle) (Walkenbach et al., 1980). In addition, the necessity for insulin-mediated depression of cyclic AMP concentration in the mechanism of insulin action has been disputed (Gabbay & Lardy, 1984, 1986). However there is evidence that insulin can reverse cyclic AMP accumulation in adipocytes up to a certain percentage stimulation of lipolysis, whereas at greater levels of stimulation another mechanism of antagonism occurs (as yet unidentified) (Beebe et al., 1985; Honnor et al., 1985; Londos et al., 1985).

3.1.2 Acetyl-CoA Carboxylase

3.1.2.1 Effects of Insulin

Insulin had no effect on acetyl-CoA carboxylase activity in mammary acini (Table 3.5), unlike the effect of insulin stimulation of lipogenesis in acini (Table 3.1). These results are contradictory to the many reports of insulin activation of acetyl-CoA carboxylase in many tissues, including the

Table 3.5 Effect of insulin and theophylline on acetyl–CoA carboxylase in isolated mammary acini.

Acini were incubated at 37°C for 45 mins in the presence or absence of insulin or theophylline as indicated. Acetyl–CoA carboxylase was measured in crude extracts of acini and in purified preparations of the enzyme as described in the Materials & Methods chapter. The activity of the crude extracts was measured as an 'initial' activity in the absence of added citrate. Values given are means ± S.E.M.; the number of determinations are shown in parentheses. Statistical testing by student's t test for paired samples revealed no significant differences between values from control and treated acini.

	μmol/min/100mg defatted dry weight
Control (no additions)	1.306 ± 0.15 (5)
Insulin (1.68mU/ml)	1.197 ± 0.10 (4)
Theophylline (20mM)	1.211 ± 0.11 (5)

mammary gland *in vitro* and *in vivo* (Halestrap & Denton, 1973, 1974; McCormack & Denton, 1977; Witters *et al.*, 1979; Munday & Williamson, 1982; Buechler *et al.*, 1984; Clegg & Calvert, 1988). The lack of effect of insulin on acetyl-CoA carboxylase observed above could be accounted for by the possibility that the enzyme in isolated acini may already be in a fully dephosphorylated state, at least with respect to the 'insulin site(s)'. An alternative hypothesis is that the effect of insulin on lipogenesis in mammary acini may not be the result of action on acetyl-CoA carboxylase.

3.1.2.2 Effects of Theophylline

Acini were exposed to 20mM theophylline for 60 minutes, and acetyl-CoA carboxylase activity was measured. No effect of theophylline was observed (Table 3.5) compared to the 60% decrease observed for lipogenesis (Fig. 3.2). As suggested previously for insulin, the effect of theophylline on lipogenesis may not be mediated through acetyl-CoA carboxylase, despite the observation that theophylline does increase the cyclic AMP concentration in mammary acini. Although this could be expected to trigger the mammary gland protein kinase thought to have properties very similar to AMP-activated protein kinase found in hepatocytes and adipocytes (Carling & Hardie, 1986; Carling *et*

al., 1987; Sim & Hardie, 1988). It is, however, possible to envisage circumstances under which this alone would not lead to an increase in the steady-state phosphorylation of acetyl-CoA carboxylase. The possibility exists that this protein kinase may be inactive in mammary acinar preparations.

Data presented in Table 3.6 are part of a preliminary investigation into the activity of acetyl-CoA carboxylase in mammary acini. Due to the nature of the results, no further work was done in this area, and hence as they stand they should be taken as indicative of the possible lack of effect of adenosine and adenosine deaminase on acetyl-CoA carboxylase, which supports earlier findings using these effectors on lipogenesis in acini (Table 3.4).

3.1.2.3 Effects of Glucagon

Glucagon had no effect on the activity of acetyl-CoA carboxylase in acini (Table 3.6) which supports findings presented earlier (Table 3.1) on the lack of effect of glucagon on lipogenesis in acini. By analogy with the accepted mode of action of glucagon, if the hormone were to exert an effect on acetyl-CoA carboxylase, it might be through the mammary gland protein kinase. This is through the same mechanism described for theophylline and therefore it is possible to envisage similar

Table 3.6 Effect of various agents on acetyl–CoA carboxylase in isolated mammary acini.

Acini were incubated at 37°C for 45 mins in the presence or absence of various agents as indicated. Acetyl–CoA carboxylase was measured in crude extracts of acini and in purified preparations of the enzyme as described in the Materials & Methods chapter. The activity of the crude extracts was measured as an 'initial' activity in the absence of added citrate. Values given are means ± S.E.M.; the number of determinations are shown in parentheses. Statistical testing by student's t test for paired samples revealed no significant differences between values from control and treated acini.

	μmol/min/100mg defatted dry weight
Control (no additions)	1.306 ± 0.15 (5)
Adenosine deaminase	1.160 ± 0.14 (5)
Glucagon (2μg/ml)	1.440 ± 0.13 (5)
Theophylline (5mM)	1.256 ± 0.10 (5)
Theophylline (20mM)	0.872 ± 0.08 (3)
+ insulin (1.68mU/ml)	
Adenosine (50μM)	1.027 ± 0.07 (4)

conditions occurring for glucagon as discussed for theophylline.

At low concentrations (5mM), theophylline did not appear to have an effect on the activity of acetyl-CoA carboxylase (Table 3.6). Addition of insulin at the same time as 20mM theophylline had no significant effect either. This result was perhaps surprising in view of earlier reports (Table 3.4) of the ability of insulin to reverse the effect of theophylline on lipogenesis.

Overall, acetyl-CoA carboxylase activity did not respond under any conditions, and only the effects of glucagon and adenosine correlated with results obtained with lipogenesis (section 3.1.1).

3.2 STUDY OF THE RATES OF LIPOGENESIS IN ACINI ISOLATED FROM THE MAMMARY GLANDS OF RATS AT DIFFERENT STAGES OF LACTATION AND IN ACINI ISOLATED FROM MID-LACTATING RATS THAT WERE SUBJECTED TO VARIOUS PHYSIOLOGICAL (AND PATHOLOGICAL) CONDITIONS

A variety of physiological and pathological conditions were studied in the lactating rat. A summary of the main observations to arise from the insulin dose-response experiments follows, with the results being expressed relative to those for the fed mid-lactating rat mammary acini.

(i) 24h starvation and long-term diabetes both resulted in a large decrease in lipogenesis, but long-term diabetes resulted in an increase in sensitivity to insulin, whereas 24h starvation had no effect on the sensitivity to insulin.

(ii) Acini from early lactating rats had a low rate of lipogenesis and decreased sensitivity to insulin.

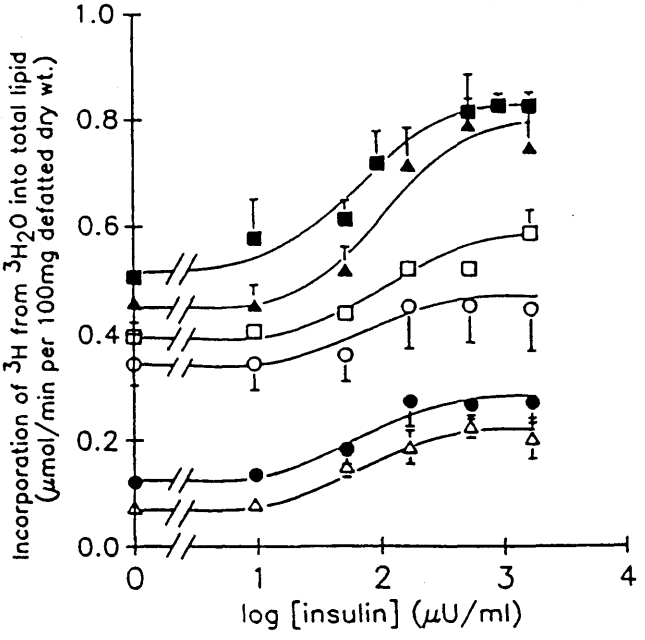
(iii) 2h refeeding of 24h starved rats resulted in a partial reversal of the rate of lipogenesis from the starved value to the fed value, and was accompanied by a decreased sensitivity to insulin relative to acini from the 24h starved and fed mid-lactating rats.

(iv) Acini from Cafeteria-fed mid-lactating rats showed no significant change in the rates of lipogenesis, though the sensitivity was decreased.

3.2.1 Effects of Different Stages of Lactation

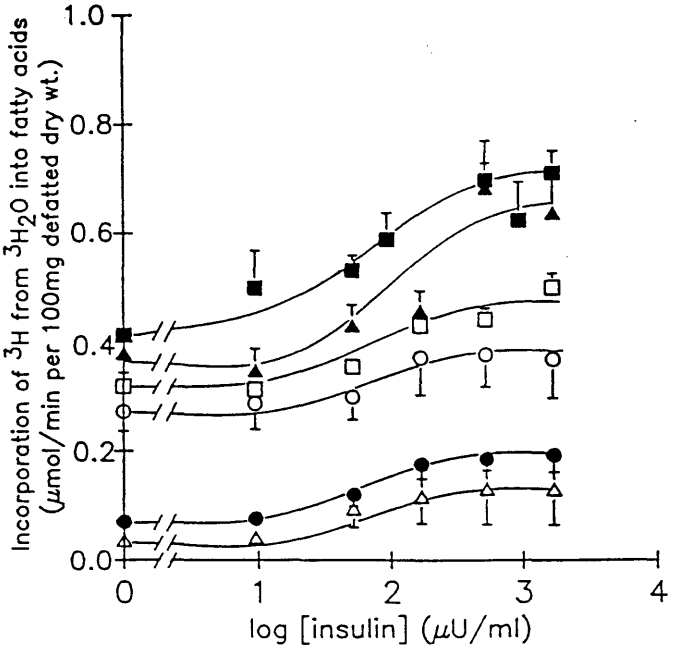
Incubation of acini, from fed mid-lactating rats, with insulin resulted in the stimulation of lipogenesis (Figs. 3.7 & 3.8). The maximal stimulation observed (about 50-60%) was obtained at concentrations of insulin above 168μU/ml (Figs. 3.7

Fig3.7. Dose–response curves for the rate of incorporation of ^{3}H from $^{3}H_2O$ into the total lipid by acini from mammary glands of lactating rats under different physiological states.



Acini were prepared from mammary glands of normal, fed early-lactating rats (□) or from mid-lactating rats that were fed a normal (■) or cafeteria diet (▲) or that were made diabetic (△), or starved for 24h (●) or starved and refed (2h) (○). See Materials and Methods chapter for experimental treatments. The values shown are means (±S.E.M.) for the number of determinations given for the corresponding data in Table 3.7.

Fig3.8. Dose–response curves for the rate of incorporation of ^{3}H from $^{3}H_2O$ into fatty acids by acini from mammary glands of lactating rats under different physiological states.



Acini were prepared from mammary glands of normal, fed early-lactating rats (□) or from mid-lactating rats that were fed a normal (■) or cafeteria diet (▲) or that were made diabetic (△), or starved for 24h (●) or starved and refed (2h) (○). See Materials and Methods chapter for experimental treatments. The values shown are means (±S.E.M.) for the number of determinations given for the corresponding data in Table 3.7.

& 3.8). The concentration required to exert half the maximal effect was approximately 59μU/ml for both total lipid synthesis and fatty acid synthesis (Table 3.7). This concentration is of the same order as that found in the plasma of the mid-lactating rat (19.7μU/ml; 1.3ng/ml and 16μU/ml respectively) (Robinson et al., 1978; Flint et al., 1979; Burnol et al., 1983).

The rate of lipogenesis observed for early lactating rats (two days post-partum) was about 80% of that observed for mid-lactating rats (Figs. 3.7 & 3.8). This was a higher percentage than would have been expected from the much lower concentrations of lipogenic enzymes reported previously for the stage of lactation in vivo at this time (Sinnett-Smith et al., 1979; Martyn & Hansen, 1980; McNeillie & Zammit, 1982). However, the overall response of lipogenesis to insulin for early lactating rats was decreased by about 25-30% (Fig. 3.9) relative to that for mid-lactating rats and the sensitivity of the tissue to insulin, as judged by the concentration of insulin required to elicit half-maximal stimulation was greater (approximately 95μU/ml) (Table 3.7) than observed for mid-lactating rats. This would seem to indicate that acini from these rats were markedly less sensitive to insulin. This observation is to be expected in view of the lower number of insulin receptors present at this

Table 3.7 Rates of ^3H incorporation from ^3H_2O into fatty acids and total lipid in mammary gland acini, from lactating rats in different physiological states, measured in the absence (basal) and presence of optimal insulin concentration (1.7mU/ml).

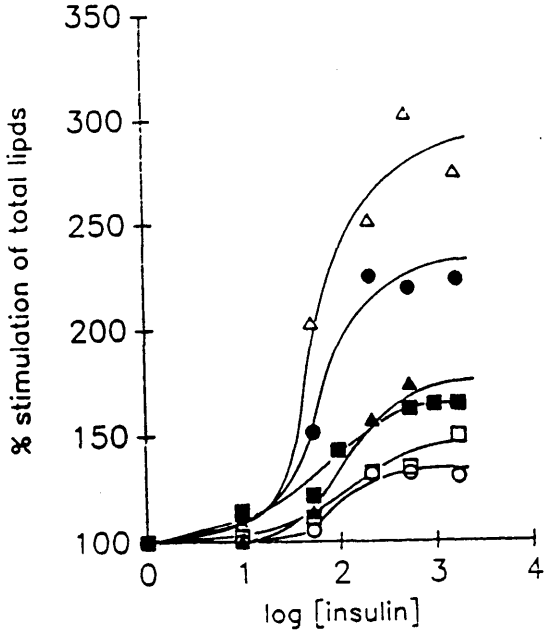
Acini were incubated at 37°C for 45 min in the presence of insulin (1.7mU/ml) or in the absence of insulin as described in the Materials & Methods chapter. The concentration of insulin required to give half-maximal activation of lipogenesis (EC_{50}) is also given for each physiological state. Values are means (± S.E.M.) for the number of separate determinations shown in parentheses. Levels of statistical significance of differences (t test) are indicated by superscripts.

Animal	State	Rate of incorporation of ^3H from ^3H_2O (μmol/min per 100mg defatted dry wt)				EC_{50} (μU/ml)
		Fatty acids		Total lipid		
		Basal	Insulin-stimulated	Basal	Insulin-stimulated	
Early-lactating	Fed (3)	0.32 ± 0.03^{c}	0.50 ± 0.40^{c}	0.40 ± 0.03^{c}	0.59 ± 0.04^{c}	94.9 ± 15.2^{a}
Mid-lactating	Fed (7)	0.41 ± 0.01	0.67 ± 0.04	0.51 ± 0.02	0.83 ± 0.03	59.2 ± 9.1
Mid-lactating	24h-starved (4)	0.07 ± 0.01^{c}	0.19 ± 0.03^{c}	0.12 ± 0.01^{c}	0.27 ± 0.04^{c}	67.8 ± 10.5
Mid-lactating	24h-starved 2h-refed (4)	0.27 ± 0.04^{b}	0.38 ± 0.08^{c}	0.34 ± 0.04^{b}	0.45 ± 0.08^{c}	87.0 ± 7.2^{a}
Mid-lactating	Cafeteria fed (3)	0.38 ± 0.04	0.63 ± 0.09	0.46 ± 0.04	0.75 ± 0.07	103.5 ± 9.6^{b}
Mid-lactating	Diabetic	0.04 (2)	0.13 (2)	0.07 ± 0.01^{c} (3)	0.20 ± 0.04^{c} (3)	38.1 ± 7.3^{c} (3)

^{a}, $P<0.10$; ^{b}, $P<0.01$; ^{c}, $P<0.001$ vs mid-lactating, fed

Fig.3.9.

Percentage stimulation by insulin of the rate of incorporation of ^{3}H from $^{3}H_2O$ into total lipids by acini from mammary glands of lactating rats under different physiological states.



The results from Fig. 3.8 were pooled together and expressed as percentage increases, relative to the control (no insulin) quoted as 100%, for each condition studied - normal, fed early-lactating rats (□), mid-lactating rats that were fed a normal (■) or cafeteria diet (▲) or diabetic rats (△), or rats starved for 24h (●) or starved and refed (2h) (○).

stage of lactation compared to mid-lactation, though the affinity of the receptors is unchanged (Flint, 1982).

3.2.2 Effects of Starvation

Starvation (24h) in mid-lactating rats resulted in a marked decrease in basal lipogenesis (about 80-90%) (Figs. 3.7 & 3.8) in mammary acini as has been observed previously *in vivo* and *in vitro* (Robinson & Williamson, 1977a; Robinson *et al.*, 1978; Munday & Williamson, 1981). Although addition of insulin to acini from starved rats did stimulate lipogenesis, it did not restore it to the rate of lipogenesis observed in acini from fed lactating rats. However, the overall percentage stimulation by insulin was much greater (approximately 100%, Fig. 3.9) than that observed for the fed rat, although the half-maximal stimulation (EC_{50}) was achieved at a similar concentration of insulin (approximately 68μU/ml) (Table 3.7) as for fed rats, indicating that the starved rat is much more responsive, but not any more sensitive to insulin compared to the fed rat at the same stage of lactation.

The lack of any change in sensitivity to insulin of the rate of lipogenesis in mammary acini from 24h starved rats could be indicative of the fact that the decrease in the rates of lipogenesis observed is

not entirely due to the decreased insulin/glucagon concentration ratio at this time, but that some other factor(s) may be having an effect. Similarly, the fact that the rate of lipogenesis in acini from 24h starved rats is not restored to the level of lipogenesis observed for fed rats by insulin supports the possibility that some other factor e.g. gastrointestinal peptide, which would work synergistically with insulin, is required to elicit the full effect (Williamson et al., 1975; Mercer & Williamson, 1987; Page, 1989).

3.2.3 Effects of Starvation and Glucagon

Since the effect of insulin on starved lactating rats was markedly different from that observed for fed rats, it was necessary to test whether starvation might alter sensitivity to glucagon in the mammary gland. However, the incubation of acini from starved (24h) mid-lactating rats with glucagon had no effect on the rates of synthesis of total lipids or fatty acids (Table 3.8). Nor did glucagon alter the rates of lipogenesis in the presence of insulin (Table 3.8). Hence it would seem that rat mammary acini are insensitive to glucagon irrespective of the physiological state of the donor animal.

Additions		Incorporation of ^3H from ^3H_2O (μmol/min per 100mg defatted dry wt.) Total lipid	Fatty acid	Acylglycerol glycerol
None (4)		0.171±0.044	0.111±0.034	0.060±0.011
Glucagon (4)		0.167±0.034	0.110±0.029	0.057±0.005
Insulin (4)	528μU/ml	0.266±0.054	0.199±0.047	0.065±0.009
	53μU/ml	0.200±0.055	0.142±0.045	0.058±0.010
Glucagon plus insulin (4)	528μU/ml	0.265±0.048	0.196±0.042	0.069±0.010
	53μU/ml	0.187±0.038	0.135±0.033	0.053±0.007

Table 3.8. Effects of glucagon plus insulin on the rates of incorporation of ^3H from ^3H_2O into total lipid, fatty acid and acylglycerol glycerol in acini from mammary glands of 24h-starved mid-lactating rats.

Acini were incubated as described in the Materials and Methods chapter for 15min either in the absence of any additions or in the presence of glucagon (2μg/ml) or insulin (53μU/ml or 528μU/ml) or a combination of the two hormones. After 15 min, ^3H_2O (100μl, containing 1mCi) was added and the incubations were allowed to proceed for a further 45 min. The acini were separated from the medium by centrifugation and extracted as described in the Materials & Methods chapter. Values are means ± S.E.M. for determinations on separate preparations of acini (numbers shown in parentheses).

3.2.4 Effects of Refeeding after Starvation

Refeeding of starved mid-lactating rats for 2h resulted in a complete restoration of lipogenesis *in vivo* to that of the normally fed animal (Robinson *et al.*, 1978; Robinson & Williamson, 1978; Munday & Williamson, 1981; Mercer & Williamson, 1986). The changes in sensitivity of lipogenesis to insulin that accompany such a transition were studied. As the results in figs. 3.7 and 3.8 indicate, there was only a partial reversal by refeeding of the inhibition of lipogenesis indicated in acini isolated from starved-refed rats such that the rate of lipogenesis was increased to about 50% of that found for fed mid-lactating rats (Fig . 3.9). The sensitivity of lipogenesis to insulin in these rats *in vitro* was significantly increased to that for fed mid-lactating rats (EC_{50} values: 87μU/ml for starved-refed, 59μU/ml for fed) (Table 3.7), indicating that the sensitivity and the responsiveness of mammary tissue to insulin is impaired in the starved-refed lactating rat. The rapid reversal by refeeding of the metabolic effects of starvation on mammary tissue *in vivo* is thought to be indicative of enzyme activation rather than induction of enzymes (Robinson & Williamson, 1977a). It is possible that in the mammary acini preparations used in the experiments in Table 3.7, activation of a particular enzyme is not occurring

to allow the full expression of refeeding. Acetyl-CoA carboxylase has previously been mentioned as being activated by refeeding, but as previous experiments in Tables 3.5 & 3.6 have shown, this enzyme does not appear to be very responsive, nor to have an effect on lipogenesis under these conditions studied. This may be a reason why the results presented here do not agree entirely with earlier reports (Robinson et al., 1978; Robinson & Williamson, 1978; Munday & Williamson, 1981; Mercer & Williamson, 1986).

3.2.5 Effects of Cafeteria Diet

Decreased rates of lipogenesis occur in vivo in the mammary gland of mid-lactating rats fed from parturition on a cafeteria diet (high fat) in addition to chow (Agius et al., 1980; Grigor & Warren, 1980; Rolls et al., 1980; Munday & Williamson, 1987). The basal and maximal rates of lipogenesis observed in the present study for acini from mid-lactating rats fed a cafeteria diet (plus chow) from day 3 of lactation were unchanged compared to those of mid-lactating rats fed chow alone (Figs. 3.7 & 3.8). However, the EC_{50} value for the cafeteria-fed rat (approximately 104μU/ml) was noticeably higher than the EC_{50} value for the mid-lactating rat (approximately 59μU/ml),

indicating that the sensitivity of lipogenesis in the cafeteria rat is reduced.

3.2.6 Effects of Long-term Insulin Defiency

Long-term insulin defiency resulting in the development of severe hyperglycaemia (28mM blood glucose as opposed to approximately 5mM blood glucose in non-diabetic rats) induced in lactating rats at day 3 of lactation by intraperitoneal injection of streptozotocin (100mg/Kg body weight) resulted in a decreased rate of lipogenesis in acini subsequently isolated from these rats. Short- and long-term diabetes also results in diminished rates of mammary lipogenesis *in vivo* (Walters & McLean, 1968; Baldwin & Yang, 1974; Robinson & Williamson, 1977a; Robinson *et al.*, 1978; Cowie *et al.*, 1980). These observations confirm that mammary tissue is an insulin-responsive tissue both with respect to long and short-term mechanisms of the hormone (Field & Coore, 1976; McNeillie & Zammit, 1982; Jones *et al.*,1984a,b). Treatment of acini from long-term diabetic rats with insulin resulted in much larger fractional stimulation of lipogenesis (approx. 200%) (Fig. 3.9) than it caused in those from animals in other physiological states. However, as in the case of starvation, insulin did not restore the rate of lipogenesis in acini from long-term diabetic rats to that observed in acini from fed lactating rats.

This lack of response *in vitro* contrasts with that observed when insulin is administered to short-term streptozotocin (3h) rats *in vivo*; such treatment completely restores the rate of lipogenesis (Bussmann *et al.*, 1984). The reason for these differences, between short-term and long-term diabetes in the rat, is that in long-term diabetic rats the total enzyme concentrations and activity of enzymes associated with lipogenesis are greatly reduced. Whereas in the short-term diabetic rat only the activities of the lipogenic enzymes are affected and hence insulin can quite easily reverse these effects. The sensitivity to insulin of acini from long-term diabetic rats was markedly increased relative to that for acini from normal lactating rats. Thus the EC_{50} (approximately 38μU/ml) for diabetic rats was in keeping with the increased sensitivity expected under these conditions compared to fed lactating rats. The observed increased sensitivity to insulin could be the result of an increase in the number of insulin receptors and/or affinity of insulin receptors which might compensate for the very low circulating insulin concentration.

In order to study the effect of varying degrees of insulin-deficiency *in vivo* on the insulin-responsiveness of acini *in vitro*, the effect of injecting different doses of streptozotocin into mid-lactating rats on acinar lipogenesis was

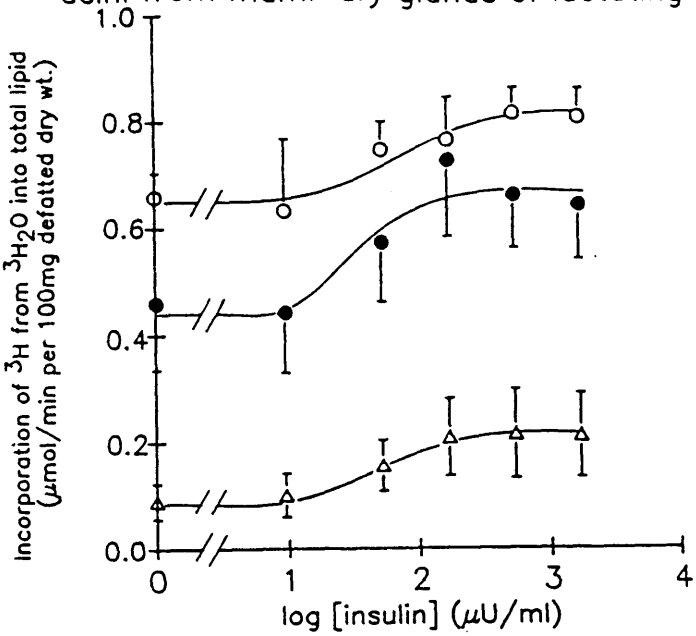
investigated. Doses of either 50mg (Group A) or 80mg (Group B) streptozotocin/Kg body weight were administered intraperitonealy on day 3 of lactation, and acini were isolated from these rats on day 10. The basal rate of lipogenesis observed in acini isolated from both groups of rats were similar to each other (Figs. 3.10 & 3.11) and to the rates observed for acini isolated from normal fed lactating rats. However, the acini isolated from Group B rats were more sensitive to insulin than those isolated from normal rats, but were less sensitive than acini isolated from rats treated with a still higher dose of streptozotocin (100mg/Kg body weight) (Table 3.9). The implications of such results are not clear. Possibly the doses of streptozotocin in Group B rats are sufficient to affect the sensitivity of acini to insulin by altering either the number or affinity of the insulin receptors on mammary acini, but not enough to impair the rate of lipogenesis in any way (Table 3.9). The moderate degree of hypoinsulinaemia achieved by Group A rats did not result in any significant increase in sensitivity of the rate of lipogenesis in acini isolated from these animals relative to that observed for acini from normal rats. Rates of lipogenesis intermediate between those observed for 80% (ie 80% of maximally effective dose) streptozotocin-treated rats, or fed rats, and 100% streptozotocin-treated rats were very

Table 3.9 Rates of ^3H incorporation from ^3H_2O into fatty acids and total lipid in mammary gland acini, from lactating rats in different diabetic states, measured in the absence (basal) and presence of optimal insulin concentration (1.7mU/ml).

Acini were incubated at 37°C for 45 min in the presence of insulin (1.7mU/ml) or in the absence of insulin as described in the Materials & Methods chapter. The concentration of insulin required to give half-maximal activation of lipogenesis (EC_{50}) is also given for each physiological state. Values are means (± S.E.M.) for the number of separate determinations shown in parentheses. Levels of statistical significance of differences (t test) are indicated by superscripts.

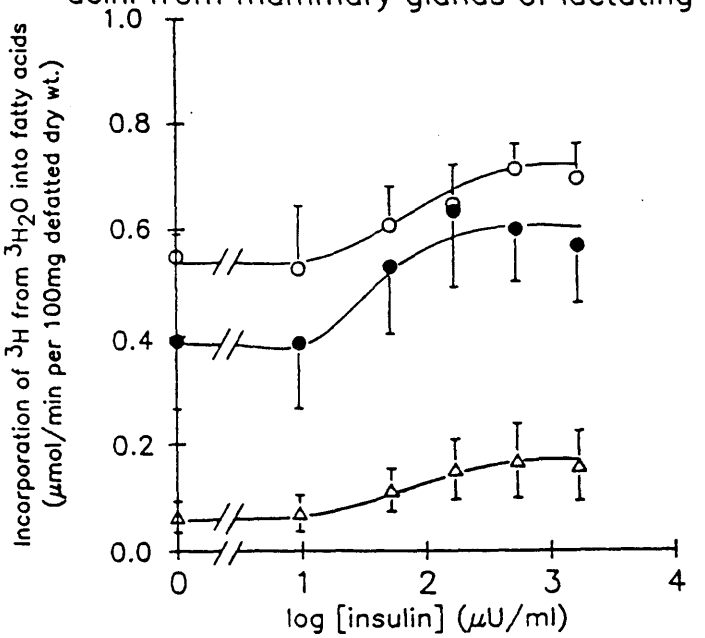
Streptozotocin concentration	Glucose concentration (mM)	Rate of incorporation of ^3H from ^3H_2O (μmol/min per 100mg defatted dry wt) Basal		Insulin-stimulated		EC_{50} (μU/ml)
		Total Lipid	Fatty Acid	Total Lipid	Fatty Acid	
Control	4–5 *	0.498±0.03	0.398±0.02	0.746±0.04	0.636±0.04	87.0± 7.2
50% (3) (50mg/Kg body wt.)	14±8	0.659±0.05	0.549±0.04	0.820±0.05	0.717±0.05	47.7±26
80% (3) (80mg/Kg body wt.)	21±4	0.459±0.12	0.391±0.13	0.665±0.10	0.601±0.10	49.4±20
100% (6) (100mg/Kg body wt.)	28±2.1	0.090±0.03	0.064±0.03	0.211±0.08	0.158±0.07	39.5±9.6

Fig3.10. Effects of different concentrations of streptozotocin on the insulin stimulated incorporation of ^{3}H from $^{3}H_2O$ into total lipid by acini from mammary glands of lactating rats.



Acini were prepared from mammary glands of fed, mid-lactating rats that were made diabetic by different doses of streptozotocin - 50mg/Kg (O), 80mg/Kg (●) or 100mg/Kg (△). See Materials and Methods chapter for experimental treatments. The values shown are means (±S.E.M.) for the number of determinations given in Table 3.9.

Fig3.11 . Effects of different concentrations of streptozotocin on the insulin stimulated incorporation of ^{3}H from $^{3}H_2O$ into fatty acids by acini from mammary glands of lactating rats.



Acini were prepared from mammary glands of fed, mid-lactating rats that were made diabetic by different doses of streptozotocin - 50mg/Kg (O), 80mg/Kg (●) or 100mg/Kg (△). See Materials and Methods chapter for experimental treatments. The values shown are means (±S.E.M.) for the number of determinations given in Table 3.9.

difficult to achieve experimentally by manipulation of the streptozotocin dose. Attempts to obtain these reproducibly were not successful.

CHAPTER 4

STUDY OF THE REGULATION OF ADIPOSE TISSUE LIPOLYSIS BY GLUCAGON DURING PREGNANCY AND LACTATION

During lactation, the mammary gland is the major site of lipogenesis despite the low absolute concentration of plasma insulin and of a much lower insulin/glucagon concentration ratio compared to unmated animals. One of the reasons for this may be the insensitivity of the mammary gland to glucagon (Chapter 3). In the non-lactating and especially the pregnant rat the major lipogenic tissue is adipose tissue. In the fed state, the high insulin/glucagon concentration ratio favours a high rate of lipogenesis. However, in the presence of a low insulin/glucagon concentration ratio (e.g. starvation) adipose tissue is able to readily become lipolytic. It supplies non-esterified fatty acids to other tissues primarily as substrates for energy formation. During lactation, the adipose tissue has a very low rate of lipogenesis and a high rate of lipolysis, favoured by the low insulin/glucagon concentration ratio and other hormonal changes including elevated prolactin and glucocorticoid concentration (Baldwin & Yang, 1974; Cowie et al., 1980). The rates of lipogenesis in adipose tissue are low during lactation. Thus direct comparisons to the mammary gland of the sensitivity of this process in this tissue to insulin and glucagon during lactation was not possible because of the practical difficulties in measuring such low rates. Hence it was decided to examine the sensitivity of glucagon to lipolysis in adipocytes isolated from

animals at different stages of reproduction. In addition the effects of starvation on this parameter were also studied.

In the present study, the range of concentrations of glucagon required to stimulate lipolysis (see below) were similar to, but slightly higher than, those required to affect the activity (Zammit & Corstorphine, 1982b) and the state of phosphorylation (Holland et al., 1985) of acetyl-CoA carboxylase. However previous experiments in vitro with adipose tissue have required higher concentrations than the range of circulatory glucagon concentrations in vivo to elicit a relatively modest enhancement of the lipolytic rate (Vaughan, 1960; Hagen, 1961). A possible reason for this requirement for slightly higher concentrations may be the longer incubation periods involved in the present study to enable a measurable accumulation of glycerol in the medium.

4.1 EFFECT OF ADENOSINE DEAMINASE ON THE RATE OF LIPOLYSIS

Adenosine is released by adipocytes into the incubation medium in vitro (Schwabe et al., 1973) and from adipose tissue in vivo (Fredholm & Sollevi, 1981). Low concentrations of adenosine are known to inhibit the lipolytic effect of glucagon (Vaughan,

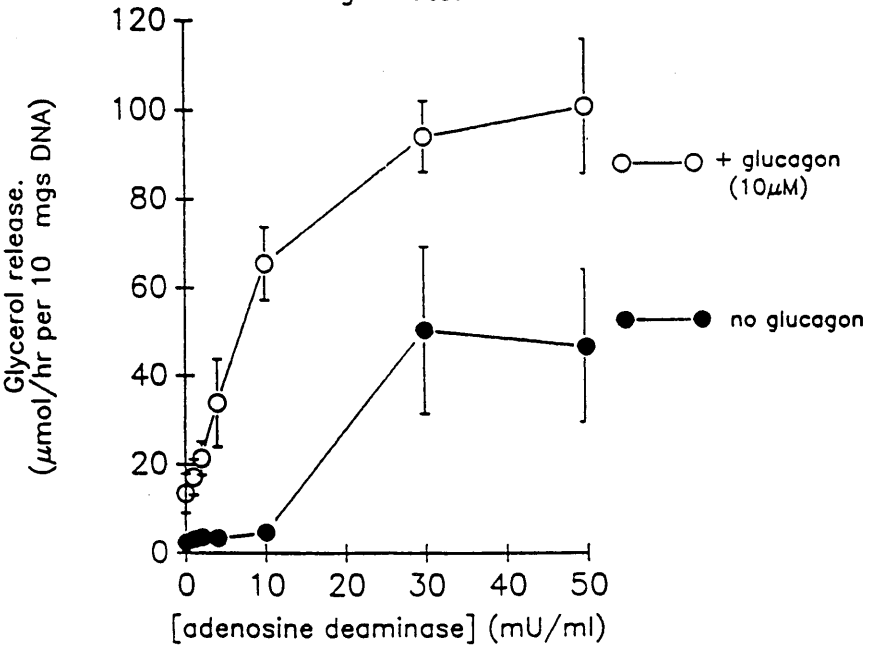
1960; Hagen, 1961; Blecher et al., 1969; Fernandez & Saggerson, 1978; Saggerson, 1986; Vernon et al., 1983, 1987; Zammit, 1988) through the adenosine-mediated inhibition of adenylate cyclase. The response of adipocytes to hormones that activate this enzyme is modified depending on the dose-response relationship between the particular metabolic activity affected and the concentration of adenosine in the medium. Inclusion of adenosine deaminase in the incubation medium of adipocytes lowers the concentration of adenosine hence relieving the inhibition of lipolysis (Schwabe et al., 1975; Fain, 1977), which can lead to enhanced responsiveness of triacylglycerol hydrolysis to glucagon in adipocytes from male rats (Honnor & Saggerson, 1980; Honnor et al., 1985; Saggerson, 1986), to glucagon in female lactating rats (Zammit, 1988) and to noradrenaline in female lactating rats (Aitchison et al., 1982; Vernon et al., 1987). So in this study, it was decided that the effect of glucagon on glycerol release in adipocytes from female rats during pregnancy and lactation would be measured in the absence and presence of adenosine deaminase.

In order to determine what concentration of adenosine deaminase was required to observe effects on lipolysis, isolated adipocytes from fed virgin and fed mid-lactating rats were incubated with

increasing concentrations of adenosine deaminase in the absence and presence of glucagon (10^{-5}M). Virgin and lactating rats were chosen because preliminary experiments had indicated that these had the lowest and highest rates of lipolysis respectively. The adenosine deaminase dose-response curve in the absence of glucagon (Figures 4.1 & 4.2) showed that adenosine deaminase itself, presumably by removing adenosine, increased basal lipolysis (see above). Moreover, the use of a high concentration (50mU/ml) of adenosine deaminase tended to mask the effect of increasing concentrations of glucagon on lipolysis (Figure 4.3).

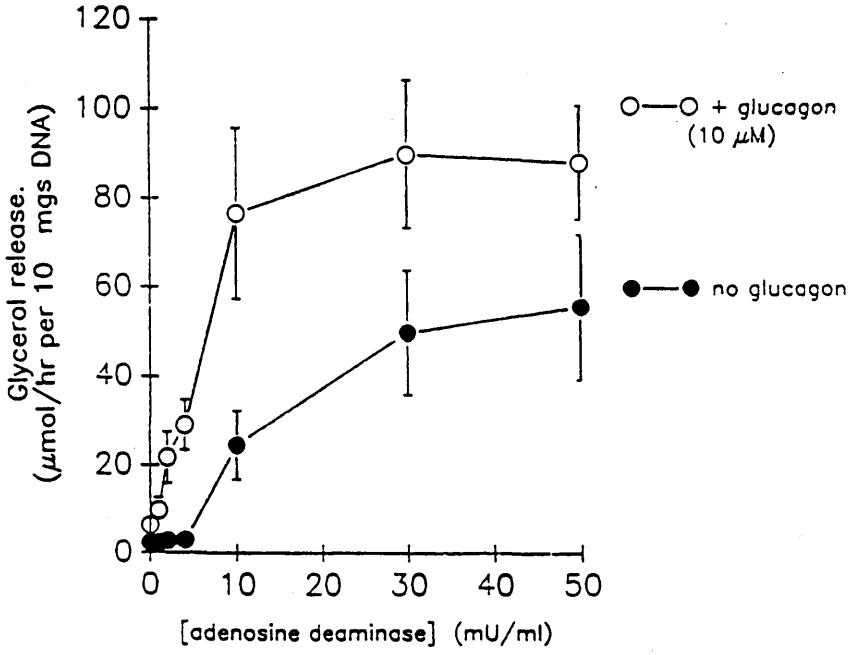
A comparison of the rates of lipolysis obtained with and without glucagon at each concentration of adenosine deaminase used, revealed a greater enhancement of lipolysis in response to glucagon at low concentrations of adenosine deaminase compared to high concentrations of adenosine deaminase (Figures 4.1 & 4.2). Consequently, a concentration of 4mU/ml adenosine deaminase was used because it had a minimal effect on basal lipolysis, yet gave a greater amplitude of response of lipolysis to glucagon. The use of the same concentration of adenosine deaminase for experiments on adipocytes from starved and diabetic male rats in the presence of glucagon was chosen for similar reasons by Honnor

Fig.4.1. Dose response curves for the effect of adenosine deaminase on the rate of lipolysis in adipocytes from fed virgin rats.



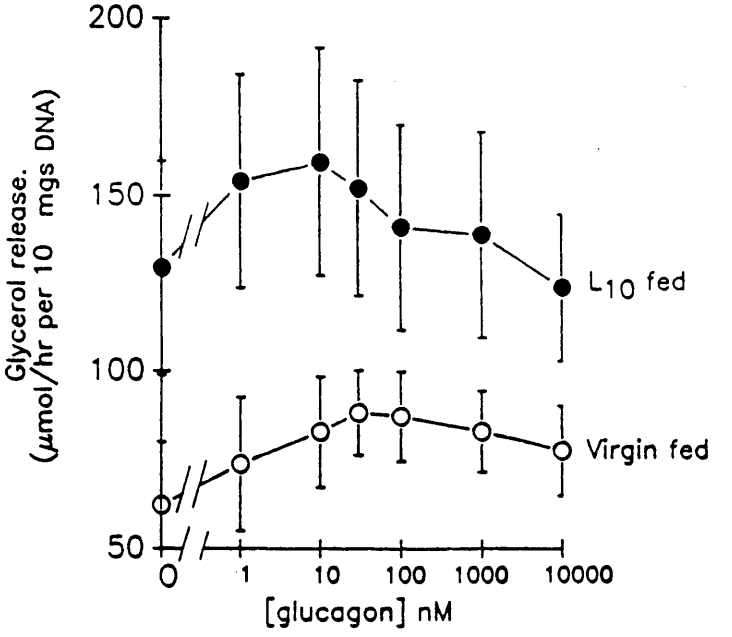
Rates of glycerol release were measured as described in the Materials and Methods chapter for cells incubated either in the absence (●) or presence (○) of maximally effective concentrations of glucagon (10^{-5}M). Values are means ± S.E.M. from 3 separate determinations.

Fig.4.2. Dose response curves for the effect of adenosine deaminase on the rate of lipolysis in adipocytes from fed mid-lactating rats.



Rates of glycerol release were measured as described in the Materials and Methods chapter for cells incubated either in the absence (●) or presence (O) of maximally effective concentrations of glucagon (10^{-5}M). Values are means ± S.E.M. from 3 separate determinations.

Fig.4.3. Dose–response curves for the actions of glucagon on the rate of lipolysis in adipocytes isolated from virgin fed or L_{10} fed rats in the presence of 50 mU/ml adenosine deaminase.



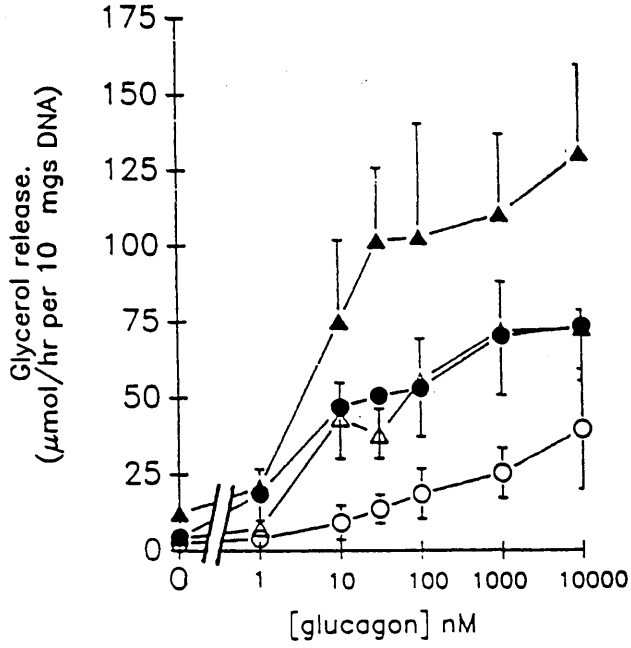
Rates of glycerol release were measured as described in the Materials and Methods chapter for cells incubated in the presence of 50mU/ml adenosine deaminase. Values are means ± S.E.M. from 3 separate determinations.

& Saggerson, (1980). Therefore results of the present study and those of previous studies are directly comparable. However, it is appreciated that working at this concentration of adenosine deaminase, the rates of lipolysis obtained may be very sensitive to small differences in the amount of enzyme used in individual experiments. Consequently variability in rates was observed between different series of experiments although within individual series reproducibility was good. Any variability in absolute rates could not, of course, affect the EC_{50} values which are expressed relative to the maximal rates observed within the same experiment.

4.2 BASAL AND MAXIMALLY GLUCAGON-STIMULATED RATES OF LIPOLYSIS

The basal rate of glycerol release (ie no glucagon and no adenosine deaminase) was similar for adipocytes from virgin, late pregnant (P_{20}), early- (L_1), mid- (L_{10}) and late- (L_{20}) lactating rats whether fed or starved (Figure 4.4, Table 4.1). The presence of adenosine deaminase in the medium caused either no change or an increase in the basal rates (Figure 4.4, Table 4.1). The increase in the basal rates for starved pregnant and fed mid-lactating rats seem to indicate that lipolysis is more sensitive to the presence of adenosine at this time; although the variation of the basal rate of the fed

Fig.4.4. Dose–response curves with respect to glucagon for the rate of lipolysis in adipocytes isolated from 20–day pregnant rats.



Rates of glycerol release were measured as described in the Materials and Methods chapter for cells isolated from fed (○ , ●) or 24h-starved (△ , ▲) animals. Incubations were performed in the presence (filled symbols) or absence (open symbols) of adenosine deaminase (4mU/ml). Values are means ± S.E.M. for 4 or 5 separate determinations.

Table 4.1 Rates of lipolysis in adipocytes isolated from female rats in different stages of the reproductive cycle.

Measurements were performed as described in the Materials & Methods chapter in the presence or absence of adenosine deaminase (4mU/ml) and glucagon 10^{-5}M). Values are means (±S.E.M.) for the number of determinations shown in parentheses. Values that are statistically significantly different ($P<0.05$) from their respective counterparts in the absence (*) or presence (†) of glucagon are indicated.

	Rates of lipolysis (µmol glycerol released/h per 10mgs DNA)			
	No glucagon		Plus 10^{-5} glucagon	
	No additions	Adenosine deaminase added	No additions	Adenosine deaminase added
Virgin				
Fed	4.3±1.3 (5)	4.9±1.5 (4)	12.2±4.1 (5)	35.5± 8.7 (5)†
Starved	4.5±0.6 (3)	5.6±1.4 (4)	20.2±7.4 (3)	107.3±29.4 (4)†
Pregnant				
Fed	2.5±0.4 (4)	4.5±1.2 (5)	30.5±7.9 (4)	73.2±18.0 (5)
Starved	4.1±0.4 (4)	12.3±1.6 (5)*	72.4±6.2 (4)	130.5±20.9 (5)
Early lactating				
fed	4.6±2.3 (4)	6.7±1.6 (5)	21.9±3.3 (5)	59.1± 6.9 (5)†
Starved	3.8±0.6 (4)	4.7±1.1 (5)	28.6±5.6 (4)	62.5±10.7 (5)†
Mid–lactating				
fed	3.5±0.3 (5)	16.1±5.1 (3)*	27.1± 9.7 (5)	136.7±17.9 (5)†
Starved	6.6±0.9 (4)	8.6±1.5 (5)	71.5±32.3 (4)	178.1±55.2 (5)
Late–lactating				
fed	3.7±0.8 (5)	7.0±3.5 (3)	30.0±4.5 (3)	97.5±14.9 (3)†

mid-lactating rats is very great compared to other basal values.

The addition of glucagon in the absence of adenosine deaminase to incubations of adipocytes from animals in all the physiological conditions studied resulted in a marked stimulation of glycerol release (Figure 4.4, Table 4.1). In the fed state, the rate of lipolysis in adipocytes increased gradually from a low value observed in virgin rats to higher values observed in pregnancy and lactation. Although, a slightly lower value was obtained in adipocytes at one day post-partum compared to adipocytes from other stages of lactation (Table 4.1). 24h-starvation resulted in an even greater increase in the response of the cells to glucagon (approximately two fold) except during early lactation when there was no significant change (Table 4.1).

The presence of adenosine deaminase in addition to glucagon in the incubation medium resulted in a further stimulation of lipolysis for animals in all conditions (Figure 4.4, Table 4.1), following the trend observed in the absence of adenosine deaminase (Table 4.1). However, as in the absence of adenosine deaminase, there was no significant difference between the rates in adipocytes of fed or starved early lactators. There was a three-fold increase in maximal response of starved virgin rats

relative to fed virgin rats; smaller starvation-induced increases were observed for pregnant and mid-lactating rats (Table 4.1). This relatively small increase was due primarily to a much greater effect of the inclusion of adenosine deaminase on the lipolytic rate in adipocytes from these fed animals (five-fold stimulation) than in those from starved animals. Therefore even in the presence of maximally effective concentrations of glucagon the enhanced response of adipocytes from fed lactating rats to partial removal of adenosine was apparent (see above).

4.3 CHANGES IN SENSITIVITY OF LIPOLYTIC RATE OF GLUCAGON CONCENTRATION

Dose-response curves for the rate of lipolysis at increasing concentrations of glucagon were obtained for adipocytes from rats in the various physiological conditions studied. Experiments were performed in the absence or presence of adenosine deaminase (4mU/ml, see above). Maximal stimulation of lipolysis by glucagon was always obtained at 10^{-7}-10^{-5}M glucagon. A representative set of dose-response curves is shown in Figure 4.4. The concentration of glucagon required to elicit half this maximal response (EC_{50}) was used as an index of the sensitivity of the cells to glucagon. In the absence of adenosine deaminase the EC_{50} for glucagon

was highest in the cells from virgin rats and lowest in the cells from pregnant rats, with early lactating and mid-lactating rat cells displaying EC_{50} values of intermediate values. This trend was observed however in cells from fed and starved animals (Table 4.2). However, the absolute value of EC_{50} for starved rats was much lower than corresponding values for fed rats, indicating increased sensitivity under these conditions.

The addition of adenosine deaminase to the medium of cells from fed and starved animals resulted in lower EC_{50} values in comparison to those for adipocytes in the absence of adenosine deaminase with one exception. This was afforded by starved early-lactating rats that had the same EC_{50} whether in the presence or absence of adenosine deaminase. However, there was no change in the sensitivity of cells from starved rats in comparison to cells from fed rats in the presence of adenosine deaminase, except for cells from starved early-lactating rats that showed an increased EC_{50} value (Table 4.2).

Thus it can concluded that generally the presence of adenosine led to a greater increase in sensitivity to glucagon for fed animals (40-fold) than for starved animals (4 to 10-fold). This would seem to indicate that starved rats are less sensitive to the removal of adenosine than were fed animals, with

Table 4.2 Changes in sensitivity (EC_{50}) to glucagon of the rates of lipolysis in adipocytes from female rats in different stages of the reproductive cycle.

Measurements were performed as described in the Materials & Methods chapter in the presence or absence of adenosine deaminase (4mU/ml) at different concentrations of glucagon. Values are means (±S.E.M.) for the number of determinations on separate adipocyte preparations shown in parentheses. Values that are statistically significantly different ($P<0.05$) between the fed and starved states in the presence (*) or absence (+) of adenosine deaminase are indicated.

	Glucagon EC_{50} (nM)			
	No additions		Adenosine deaminase added (4mU/ml)	
	FED	STARVED	FED	STARVED
Virgin	640.0±166 (4)	146.7±36.7 (3)	16.8±2.4 (4)	31.3±14.5 (3)
Pregnant	96.0±40.8 (4)	35.2± 7.5 (4)	2.6±0.3 (4)	4.3± 0.4 (3)
Early–Lactating	160.0±11.2 (5)	52.0±14.8 (4)	15.0±4.8 (4)	54.3±10.7 (4)*
Mid–Lactating	126.7±34.7 (5)	93.3±28.4 (4)	10.8±0.8 (4)	18.0± 8.7 (4)
Late–Lactating	29.7± 9.6 (3)		10.9±3.3 (3)	

starved early-lactating rats being particularly insensitive to its removal.

4.4 ADIPOCYTE VOLUME DURING PREGNANCY AND LACTATION

The adipocyte mean cell volume (MCV) of fed rats fell significantly from 434±72pl at 20 days of pregnancy to 131±30pl at 20 days lactation (Figure 4.5). The values obtained for rat adipocyte MCV are in agreement with those previously reported (Aitchison et al., 1982) except for the value of virgin rats. The reason for this difference is not known. The effects of 24h starvation on parametrial adipocyte volume in virgin, pregnant and lactating rats are shown in Figure 4.5. Mean cell volume was decreased by starvation in all groups of animals, although statistical significance was achieved only in the case of adipocytes from 20-day pregnant animals ($p<0.05$ starved versus fed; Student's t-test) which had undergone a net lipid mobilisation equivalent to 125pl per adipocyte (difference between fed and starved mean cell volume) after 24h starvation. In marked contrast, starvation-induced lipid mobilisation from adipocytes of early lactation animals was minimal (13pl per adipocyte) although net lipid mobilisation tended to rise in mid-lactating animals (30pl/cell). Starvation of weight-matched virgin animals resulted in a volume loss of 36pl per adipocyte. This pattern of lipid

Fig.4.5. Effects of pregnancy and lactation on the mean adipocyte volume in parametrial adipose tissue of fed and starved rats.

Adipocyte cells were obtained and measured as described in Materials and Methods chapter. Mean adipocyte cell volume was measured for parametrial adipose tissue from virgin rats (V), late pregnant rats (20 days pregnant), early-lactating rats (1 day lactating), mid-lactating rats (10 days lactating) and late-lactating (20 days lactating) in fed or starved animals. Values are means ± S.E.M. for the number of determinations shown in parentheses.

mobilisation after starvation of animals at different stages of pregnancy and lactation is broadly consistent with that of the changes in sensitivities to glucagon and the absolute rates of lipolysis *in vitro* of adipocytes for corresponding categories of starved animals (see Table 4.2). Conversely, there appears to be little correspondence between glucagon sensitivities of adipocytes from fed animals throughout pregnancy and lactation and the decline in adipocyte volume *in vivo* accompanying their starvation for 24h.

4.5 DISCUSSION

There are not many reports to which the results presented in this chapter can be compared to directly. Previous work measuring rates of lipolysis in pregnant and lactating rats, compared to virgin rats, have been either performed in adipose tissue slices (Knopp *et al.*, 1970; Smith & Walsh, 1976; Farid *et al.*, 1978; Gillon, 1981), or else in adipocytes during various stages of pregnancy and lactation, but using different lipolytic hormones eg noradrenaline, adrenaline, corticotrophin, to enhance lipolysis (Aitchison *et al.*, 1982; Vernon *et al.*, 1983, 1987). A subsequent study, prompted by the experiments reported here (Zammit, 1988), does provide a more direct comparison to the work reported above. However, the

concentration of adenosine deaminase (1 Unit/ml) used by Zammit (1988) is much greater, resulting in an insignificant amount of adenosine being present in the incubation medium, and the concentration of glucagon (25nM) used to elicit a maximal response of lipolysis in the presence of 1U/ml adenosine deaminase is much lower (Zammit, 1988) compared to the concentrations of glucagon used in Figure 4.4, Table 4.1. Also the rates of lipolysis in response to glucagon in adipocytes from starved rats could only be measured in the presence of a maximally anti-lipolytic dose of insulin (Zammit, 1988). The results expressed in Table 4.1 agree with the results of Zammit (1988) in that adipocytes from mid-lactating rats in both studies have the highest maximal rates of lipolysis in the presence of glucagon in fed and 24h-starved rats, but the rates of lipolysis for the other conditions studied were different. The sensitivity of lipolysis to glucagon (Table 4.2) in adipocytes from rats at different stages of the reproductive cycle, whether fed or starved, are of a different order of magnitude to those expressed by Zammit (1988), and indicate that in the fed and starved state that late-pregnant animals are the most sensitive to glucagon (Table 4.2) in contrast to late-pregnant animals being the least sensitive to glucagon in the fed animal (Zammit, 1988). The reasons for these differences between the two studies is most likely to be due to

the differing amounts of adenosine deaminase used, and therefore the amount of adenosine present within the incubation medium.

The rates of lipolysis obtained for experiments presented in this chapter are generally in agreement with what one might expect. Namely, that the rates of lipolysis are generally increased during late pregnancy and lactation compared to the virgin state. One apparent anomaly would appear to be early-lactating rats, which had low absolute rates of lipolysis and low sensitivity to glucagon. These properties, if reflected *in vivo* would result in minimal rates of triacylglycerol mobilisation from adipose tissue of early-lactating animals both in the fed and in the 24h-starved states. These observations can be rationalised in terms of the low requirement for exogenous substrates by the mammary gland at a time when lactation is not yet established.

In mid-lactating rats both the absolute rates of lipolysis and the sensitivity to glucagon are much higher. These observations agree with the generally accepted view that preformed fatty acids released from adipose tissue supplement *de novo* synthesis of fatty acids in the mammary gland (see Zammit, 1985). This suggests that glucagon may have an important role in effecting the direction of substrates

towards the gland during lactation. The lipolytic rates obtained for adipocytes from pregnant animals were most interesting and they demonstrate the potential role of glucagon at this time. In these cells half-maximal rates of lipolysis were obtained at glucagon concentrations that were of an order of magnitude lower than those required by cells from animals in other physiological conditions studied. These observations suggest that during starvation adipose tissue of pregnant rats is likely to respond by mobilising triacylglycerols at a greater rate than adipose tissue of rats at other stages of the reproductive cycle. Changes in the rates of lipolysis in response to starvation *in vivo* are regulated by a variety of factors, not least by changes in the molar concentration ratio of glucagon (lipolytic) to insulin (antilipolytic). The present observations suggest that changes in EC_{50} values for glucagon may form a significant part of the adaptive increase in adipose tissue lipolysis that occurs during starvation especially in late pregnant animals. Such an enhanced response could account for the increased susceptibility of late pregnant animals to the development of ketonaemia, because the increased release of fatty acids from adipose tissue would furnish additional substrate for hepatic ketogenesis, while concomitant changes in the liver would facilitate their use for oxidation (Zammit, 1980, 1981; Zammit & Corstorphine, 1982a).

The accumulation of adenosine in the medium of adipocytes seemed to have a greater effect on adipocytes from fed rats than starved rats for all physiological conditions studied, with exception of early-lactating rats. In vivo, adenosine acts as a vasodilator in adipose tissue (Sollevi & Fredholm, 1981), thereby affecting the rate of fatty acid release into the blood stream (Scow, 1965), yet at the same time, adenosine is also rapidly metabolised and removed by the blood (Fredholm & Sollevi, 1981). Therefore the build up of adenosine observed in vitro would not occur to the same extent in vivo. The conditions created for the experiments presented in this chapter i.e. the removal of some, but not all adenosine by adenosine deaminase, may well approximate to conditions in vivo. The reasons for the differences between adipocytes from fed and starved rats is not clear. It is possible that there is an increased rate of adenosine production in fed rats compared to starved rats, or fed rats may not be able to metabolise adenosine as well as starved rats due to an increase in the sensitivity of fed rats to adenosine, or a combination of these effects may occur. It has been suggested (Vernon et al., 1983) that adenosine has a role regulating the rate of lipolysis to ensure that the stores of triacylglycerol do not become depleted too rapidly, especially during lactation when lipid reserves are low compared to pregnancy. Adenosine may have a

similar role to play for fed and starved rats; a greater rate of lipolysis being required during starvation to meet the needs of the rat at that time than in the fed animal.

CHAPTER 5

GENERAL DISCUSSION

The significance of the findings for the regulation of substrate utilization for lipogenesis in mammary gland and adipose tissues during pregnancy and lactation are discussed in this chapter.

The major finding of the results presented in chapter 3 was that glucagon had an effect on adipose tissue but not on mammary gland during lactation. Glucagon did not inhibit lipogenesis in rat mammary acini, nor did glucagon reverse the stimulatory effect of insulin on lipogenesis in the rat mammary acini. The lack of effect of glucagon on mammary gland was primarily due to the fact that there were no receptors for glucagon on the rat mammary gland. The significance of this finding is that the mammary gland will only be responsive to the concentration of plasma insulin, not the insulin/glucagon concentration ratio. Whereas adipose tissue is dependent on the insulin/glucagon concentration ratio as it is responsive to both insulin and glucagon. This also means that the actions of insulin in the mammary gland cannot be antagonised by glucagon, nor can glucagon enhance the effects of other inhibitory hormones. Hence the difference in responsiveness of mammary and adipose cell preparations <u>in vitro</u> to glucagon suggest that the two tissues may be differentially responsive to changes in the circulating insulin/glucagon concentration ratio <u>in vivo</u> during lactation.

Insulin had a stimulatory effect on lipogenesis in adipose tissue and mammary gland, but at different times during the reproductive cycle. During pregnancy, adipose tissue lipogenesis is increased relative to lipogenesis in the tissue in the virgin rat (Vernon & Flint, 1983). This is due to a high insulin/glucagon concentration ratio (Saudek et al., 1975) and increased insulin receptor numbers (Flint et al., 1979). At the onset of lactation, the rate of lipogenesis observed in adipose tissue decreases to a very low level and the concentration of lipogenic enzymes in adipose tissue is greatly reduced (Smith, 1973; Robinson et al., 1978; Agius et al., 1979; Flint et al., 1979; Sinnett-Smith et al., 1980; Burnol et al., 1983, 1986a,b, 1987; Jones et al, 1984a). The insulin/glucagon concentration ratio drops to a level lower than that observed in virgin rats (Robinson et al., 1978; Flint et al, 1979), which in itself is unfavourable for lipogenesis in the adipose tissue. This is also accompanied by a decrease in the number of insulin receptors on adipose tissue to a level similar to that observed in the virgin rat, but lower than the level observed in the pregnant rat (Flint et al., 1979; Flint, 1980). This implies that despite the normal level of insulin receptors relative to the virgin rat, adipose tissue must also have a reduced sensitivity to insulin (Kilgour & Vernon, 1988; Zammit, 1988). The mammary gland on the other hand has a high rate of lipogenesis at this time despite the low insulin/glucagon ratio (Robinson et al., 1978). The number of insulin

receptors on the mammary gland increase from late pregnancy (day 20) to mid-lactation (day 10), with the greatest increase occurring between late lactation and early lactation (day 2), but with no change in affinity of the receptors at this time (Flint, 1982). However, by mid-lactation the sensitivity of the receptors has also increased (Flint, 1982). The increased numbers and affinity of the insulin receptors on the mammary gland ensure that the stimulatory effects of insulin on the mammary gland metabolism are promoted despite the low plasma insulin concentration.

In the virgin rat, adipose tissue is capable of lipogenesis and lipolysis depending on the nutritional and physiological status of the animal and the insulin/glucagon concentration ratio. Insulin and glucagon act antagonistically to each other in adipose tissue. Lipogenesis is stimulated by insulin and inhibited by glucagon, hence very small changes in the plasma insulin/glucagon concentration can determine the rate of lipogenesis in adipose tissue. Lipolysis is regulated in a similar way and is just as sensitive to changes in the insulin/glucagon ratio, except that insulin inhibits lipolysis and glucagon stimulates lipolysis. So that during pregnancy when the insulin/glucagon ratio is high and adipose tissue has increased sensitivity to insulin, high rates of lipogenesis are observed, but the rates of lipolysis are low because of the high levels of insulin and its

inhibitory effects on lipolysis (Knopp et al., 1973; Flint et al., 1979; Naismith et al., 1982). Conversely, during lactation when the insulin/glucagon concentration ratio is low, and adipose tissue has decreased sensitivity to insulin, rates of lipolysis are high partly due to the levels of glucagon in the plasma, but also due to the low levels of insulin (Vernon et al, 1983, 1987; Kilgour & Vernon, 1988; Zammit, 1988).

The results in chapter 4 demonstrated the sensitivity of lipolysis in adipose tissue to glucagon through pregnancy and lactation. Lipolysis in adipocytes from late pregnant rats reflected the changes starting to occur in the mother in preparation for parturition and the onset of lactation. There was a switch from low rates of lipolysis to high rates of lipolysis which was also accompanied by an increased sensitivity to glucagon relative to that observed in the virgin rat. During early lactation, when lactation is not yet fully established, lipolysis rates dropped to a low level which was accompanied by a decrease in sensitivity to glucagon as there is not the demand from the mammary gland for triacylglycerols at this stage of lactation. However as lactation progressed, the rates of lipolysis and the sensitivity of adipose tissue to glucagon increased as the demand for preformed fatty acids increased. The changes in response of adipose tissue lipolysis to glucagon appeared to facilitate the mobilization of fatty acids from adipose tissue either for the energy demands

of the mother (especially in pregnancy) and/or for the substrate demands of the mammary gland when lactation was fully established. By contrast, during early lactation adipose tissue became refractory to glucagon action. It is suggested that this represents part of a general adaptation that enables the sparing of stored triacylglycerol in spite of the large decrease in insulin concentrations from the high levels observed during pregnancy (Saudek et al., 1975) to the low concentrations that occur during early lactation (Robinson et al., 1978; Flint et al., 1979). The decreased effectiveness of glucagon on adipose tissue would counteract the diminution of the antilipolytic effects of insulin.

The sensitivity to insulin of lipogenesis in the mammary gland varied throughout lactation with low rates of lipogenesis and sensitivities being observed during early lactation, increasing to high rates of lipogenesis and sensitivity at mid-lactation as might be expected as lactation progressed and the demand for milk increased (Robinson et al., 1978). Nutritional effects on the rates of lipogenesis during lactation were investigated. A high energy diet (cafeteria diet) did not result in decreased rates of lipogenesis as reported before in this tissue (Agius et al., 1980; Rolls et al., 1980), but maintained the rates of lipogenesis observed in the acini from rats fed a normal diet (chow only). However acini from rats fed a cafeteria diet did have decreased sensitivity to insulin. 24h starvation resulted in

decreased rates of lipogenesis and an increased sensitivity to insulin indicating that starvation conserves energy at this time, but also primes the tissue to be responsive to increases in insulin that would occur on refeeding. As might be expected, refeeding from starvation does indeed increase the rate of lipogenesis, but not to the levels observed prior to starvation implying that some other factor is required to permit the normal levels of lipogenesis to be met. Diabetic rats had very low rates of lipogenesis as a result of the very low plasma insulin concentration similar to the effect observed for starved animals. However, acini from these rats were most sensitive and responsive to insulin. Although, the addition of insulin did not return the rates of lipogenesis to the level observed in fed rats as mammary gland acini from diabetic rats do not have the capability to achieve such rates due to low levels of lipogenic enzymes present at this time.

The various changes in enzyme activities in adipose tissue and mammary gland, and their differing sensitivities of each tissue to insulin and glucagon, explain how these two tissues work together in a complementary way. Thus, when demand for milk is high e.g. during mid-lactation, the elevated rates of lipogenesis in the mammary gland can be maintained, with adipose tissue lipolysis supplementing <u>de novo</u> fatty acid synthesis. Conversely, when demand for mammary gland fatty acids is low e.g. early lactation, lower rates of

mammary gland lipogenesis and adipose tissue lipolysis are observed.

The points raised in the discussion suggest that there are important physiological implications for the reciprocal regulation of the rates of lipogenesis in adipose tissue and mammary gland in the lactating rat. For a tissue that is sensitive to the insulin/glucagon ratio (such as adipose tissue) the hormone-sensitive steps of the lipogenic pathway will be relatively inhibited, whereas in a tissue that is not sensitive to glucagon (mammary gland) the activity of the lipogenic pathway would not be markedly affected. Hence the differential responsiveness of the two tissues to circulatory insulin during lactation would be achieved.

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