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

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Abstract of Thesis

The mammary gland of the lactating rat uses 30 mmol of glucose a day. 70% of this is used for the synthesis of fatty acids which is therefore stringently regulated. This thesis describes investigations designed to elucidate some of the mechanisms of this regulation.

Acetyl-CoA carboxylase (ACC) and pyruvate dehydrogenase (PDH) are key regulatory enzymes in the pathway from glucose to fatty acid. Pyruvate dehydrogenase catalyses the oxidative decarboxylation of pyruvate to acetyl-CoA and ACC catalyses the committed step in the synthesis of fatty acids from acetyl-CoA. In lactating rat mammary gland the activities of both enzymes are profoundly inhibited by starvation and rapidly re-activated (within 3 hours) upon refeeding. Unexpectedly during these dietary manipulations the rate of fatty acid synthesis can be more closely correlated with PDH than ACC even though the latter is considered to be the major rate determining enzyme.

Perfusion of the mammary gland *in situ* was performed using known inhibitors of fatty acid synthesis in the perfusate. Palmitic acid inhibited PDH but not ACC; acetoacetate had no effect on either enzyme.

In vitro experiments suggest differential effects of palmitate (16:0) and oleate (18:1), palmitate being a less potent inhibitor of fatty acid synthesis and ACC than oleate.

ACC in lactating rat mammary gland is phosphorylated and inactivated in response to 24hr starvation. One possible candidate for such phosphorylation is cAMP-dependent protein kinase. This had been purified from lactating mammary gland and its unusual tissue specific properties are described and discussed in relation to the results of in vivo experiments showing that in mammary gland the activity of this kinase does not correlate with the rate of fatty acid synthesis or ACC activity.

The partial purification of a kinase capable of phosphorylating and inhibiting ACC in a manner identical to that which occurs during starvation in vivo is described. Its characteristics, regulation and physiological significance are discussed.

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ABBREVIATIONS

Apart from those listed below, the abbreviations used throughout this thesis follow the recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature, as detailed in the Biochemical Journal (225, pp 1-26, 1985).

AABS	p-(p-aminophenylazo)-benzene sulphonic acid.
AAT	arylamine acetyl transferase
ACC	Acetyl-CoA carboxylase
APAD	acetylpyridine-adenine dinucloetide
AMP-PK	AMP-activated protein kinase
A-V	arterio-venous
BSA	bovine serum albumin
BZ	benzamidine
cAMP-PK	cAMP-dependent protein kinase
CNBr	cyanogen bromide
CS	citrate synthase
DTT	dithiothreitol
F-1,6-P_2	fructose-1,6-bisphosphate
F-2,6-P_2	fructose-2,6-bisphosphate
FSBA	fluorosulphonyl-benzoyl adeonsine
FPLC	fast protein liquid chromatography
G-1-P	glucose-1-phosphate
G-6-P	glucose-6-phosphate
HPLC	high pressure liquid chromatography
IBMX	3-isobutyl-1-methyl xanthine
MG	mammary gland
PDH	pyruvate dehydrogenase
PEG	poly(ethylene) glycol
PFK-1	6-phosphofructo-1-kinase
PFK-2	6-phosphofructo-2-kinase
PMSF	phenylmethylsulphonyl fluoride
SBTI	soya bean trypsin inhibitor
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis

TLCK	Nα-tosyl-L-phenylalanine chloromethyl ketone

Note: the dimensions of chromatography columns are quoted as bed height x internal diameter.

FIGURES

TABLES

CHAPTER 1

INTRODUCTION

1.1. BIOSYNTHESIS OF FATTY ACIDS IN MAMMALS

The common fatty acids that occur in mammals are monocarboxylic acids with a long unbranched hydrocarbon chain with a terminal carboxyl group. The chain usually has an even number of carbon atoms and may be unsaturated by the introduction of one or more double bonds. The most commonly occurring fatty acids in animal tissues are palmitate (16:0) and oleate (18:1).

It was originally believed that fatty acid biosynthesis was a simple reversal of the degradative pathway. However the discovery of malonyl-CoA as an intermediate during studies of fatty acid synthesis (Wakil 1958) generated research which elucidated the pathway. ß-oxidation of fatty acids occurs in the mitochondria and peroxisomes whereas biosynthesis occurs almost exclusively in the cytosol, elongation beyond sixteen carbons and also desaturation are accomplished by microsomal enzyme systems.

The committed step for fatty acid synthesis is the production of malonyl-CoA by the carboxylation of acetyl-CoA. This reaction is catalysed by the biotin containing enzyme acetyl-CoA carboxylase. The elongation process from acetyl-CoA, malonyl-CoA and NADPH is catalysed by the multienzyme complex fatty acid synthase. The mammary gland is unique in that it contains medium chain acylthioesterase (Smith and Abraham 1975, Libertini and Smith 1978) which causes early chain termination and thus changes the pattern of the fatty acid synthetase product from C-16 fatty acid to a mixture of C-8 to C-12 fatty acids. Cow milk contains C-4 and C-6 fatty acids which are the products of rumen

fermentation.

Prolactin is an absolute requirement for the induction of medium chain thioesterase in pregnant animals although sometimes insulin and corticosterone are also required. (Wang et al 1972, Forsyth et al 1972). The physiological requirements for the high concentration of medium chain fatty acids in milk (Table 1) are unclear. There is evidence that neonates utilize medium chain triglycerides better than long chain triglycerides (Odle et al 1989) and also that medium chain fatty acids have a sparing effect on critical fuels such as glycogen and protein in the neonate (Benevenga et al 1989). It has also been shown in mature animals and humans that ingestion of medium chain triglycerides produce marked postprandial thermogenesis when compared to the effects of long chain triglyceride (Baba et al 1982, Seaton et al 1986, Hill et al 1990). This effect if it occurs in neonates may indicate thermoregulation as an additional property of the medium chain fatty acids of milk fat.

1.2 LIPID CONTENT OF MILK

Rat milk is relatively rich in fat containing 10.3% (w/v) of fat in comparison to humans or cows which contain 3.8% (w/v) and 3.7% (w/v) respectively (Davies et al 1983). Triacylglycerols constitute 87.5% of rat milk fat. The rest is composed mostly of diacyglycerols and free fatty acids (Table 2). Approximately 50% of rat milk fatty acids are synthesised *de novo* the rest are derived from the diet, adipose tissue lipolysis and hepatic lipogenesis (Hawkins and Williamson 1972). These extra-mammary sources also provide most of the long chain fatty acids present in milk and the very short chain ones in ruminant milk (see above).

16 mg of cholesterol are secreted in rat milk per day (Clarenburg and Chaikoff 1966). Cholesterol synthesis is low in the rat mammary gland even though HMG-CoA reductase,

Table 1 FATTY ACID COMPOSITION OF THE TRIGLYCERIDE FRACTION OF RAT, HUMAN AND COW MILK

Fatty Acid	Rat	Human	Cow
4:0	-	-	3.3
6:0	-	-	1.6
8:0	1.1	-	1.3
10:0	7.0	1.3	3.0
12:0	7.5	3.1	3.1
14:0	8.2	5.1	9.5
15:0	-	0.4	0.6
16:0	22.6	20.2	26.3
16:1	1.9	5.7	2.3
17:0	0.3	-	0.5
18:0	6.5	5.9	14.6
18:1	26.7	46.4	29.8
18:2	16.3	13.0	2.4
18:3	0.8	1.4	0.8

Fatty acids denoted by number of carbon atoms: number of double bonds.
Values are expressed as weight per cent of total fatty acids (Davies et al 1983)

Table 2 THE MAIN LIPID CLASSES OF RAT MILK COMPARED WITH HUMANS AND COWS

Lipid Class	Rat	Human	Cows
Triacylglycerols	87.5	98.2	97.5
Diacylglycerols	2.9	0.7	0.36
Monoacylglycerols	0.4	trace	0.03
Cholesterol esters	-	trace	trace
Cholesterol	1.6	0.25	0.31
Free fatty acids	3.1	0.40	0.27
Phospholipids	0.7	0.20	0.60

Values expressed as weight per cent of total lipids (From Davies et al 1983).

the rate limiting enzyme for cholesterol synthesis, is abundant in the tissue (Gibbons et al 1983). A maximum of 30-40% of the cholesterol secreted into rat milk is synthesized within the mammary gland, and when rats are maintained on normal, low fat, high carbohydrate chow about 11% is derived from the diet, the rest is synthesized in the liver (Gibbons et al 1983).

1.3 REGULATION OF FATTY ACID SYNTHESIS

At peak lactation (10-15 days post partum) the mammary gland of the rat utilises 30 mmol of glucose per day (Williamson and Robinson 1977) of which about 7 mmol is used for lactose synthesis (Carrick and Kuhn, 1978) another 10% is oxidised completely (Katz and Wals, 1972); the rest is used for lipogenesis, primarily for fatty acid and triglyceride synthesis (Williamson 1980). The daily turnover of glucose by the mammary gland of the lactating rat is approximately the same as the daily whole body turnover of a male rat of similar size and rates of lipogenesis per gram wet weight of tissue are five times higher than in the liver of virgin lactating rats (Robinson et al 1978b). The metabolism of the mammary gland is therefore stringently regulated not only to ensure sufficient supplies of substrates for lactogenesis when the diet is adequate but to modify milk synthesis to ensure efficient use of available substrate, for example a high fat diet fed for 7 days depresses mammary lipogenesis by up to 80% and the rat milk composition reflects that of the diet (Grigor and Warren 1980). There is also a decrease in the glucose metabolic clearance rate in lactating rats fed a high fat diet. It is suggested that this is a consequence of the reduced glucose requirements of the mammary gland (Burnol et al 1986). These changes are probably due to inhibition of specific lipogenic enzymes such as ACC (Munday and Hardie, 1986b) and glucose-6-phosphate dehydrogenase (Young et al 1990), as well as suppression of expression of tissue lipogenic enzymes as has been shown for fatty acid synthetase in adipose tissue

and liver (Shillabeer et al 1990).

Lipogenesis in the rat mammary gland falls by 87% after 6 hours starvation (Williamson et al 1983). This decrease is mediated by decreased glycolytic flux and/or decreased oxidation of pyruvate thus limiting the supply of acetyl-CoA for fatty acid synthesis, glycerophosphate for acylglyceride synthesis and the glucose-6-phosphate needed to generate NADPH (via the pentose phosphate pathway) that is necessary for fatty acid synthesis. When the animals are starved for more than 6 hours inhibition of ACC also has a role to play in keeping the rate of lipogenesis depressed (Munday and Williamson 1982).

1.3.1 HORMONAL REGULATION OF LIPOGENESIS

Lipogenesis, but not lactose synthesis (the other major glucose utilizing pathway in the lactating mammary gland), is sensitive to insulin (Bussmann et al 1984). Short term diabetes reduces lipogenesis (Robinson and Williamson 1977a; Robinson et al 1978b) and insulin is able to restore lipogenesis to normal in situations where the rate is depressed (Munday and Williamson 1981; Freed et al 1988). The number of insulin receptors on lactating mammary epithelial cells rises during lactation (O'Keefe and Cuatrecasas 1974, mice; Flint 1982, rats) while the numbers of receptors on peripheral adipocytes is unchanged (Flint et al 1979). Glucose is therefore diverted to the mammary gland.

In ruminant mammary tissue the utilisation of glucose for either lipogenesis or lactose synthesis is not regulated by insulin (Campbell et al 1987). Furthermore insulin binding by bovine mammary gland falls from early to mid lactation when milk yield is highest and begins to rise at late lactation when milk yield is declining (Campbell et al 1987). Acetate is the primary fatty acid precursor in ruminant mammary gland and its uptake for lipogenesis is not regulated by insulin (Laarveld et al 1985). The

contrasting roles of insulin in the mammary gland of ruminant and mono-gastric animals reflects the relative importance of glucose as a lipogenic precursor between the two.

Rat mammary gland has no glucagon receptors (Robson et al 1984) so this hormone cannot antagonise insulin effects as it does in liver. The mammary gland does respond to ß-adrenergic agonists with elevated cAMP levels (Clegg and Mullaney 1985) but in contrast to liver, lipogenesis is not affected (Clegg et al 1986). The role of cAMP and cAMP dependent protein kinase is discussed in section 1.5 and the introduction to Chapter 3.

Prolactin appears to have a limited role in the short term regulation of mammary lipogenesis, changes in its plasma concentration do not always parallel lipogenesis (Robinson et al 1978b) though deficiency causes a decrease in lipogenesis (Agius et al 1979) and inhibition of pyruvate dehydrogenase (Field and Coore 1976). Prolactin appears to be involved in the regulation of protein synthesis (Williamson et al 1984) and undoubtedly regulates gene expression (Vonderhaar 1987). There is evidence that it mediates the insulin resistance of adipose tissue which occurs during lactation (Ros et al 1990).

Other hormonal signals eg thyroxine and corticosteroids play no role in the acute regulation of lipogenesis; they are involved in the initiation and maintenance of lactogenesis and also mammary gland development during pregnancy and early lactation (Topper and Freeman 1980).

1.4 POTENTIAL SITES OF REGULATION OF FATTY ACID SYNTHESIS

Studies on glucose transport (Threadgold and Kuhn 1984) and also measurement of the relative activities of glycolytic and lipogenic enzymes (Gumaa et al 1973) indicate that there are five potential control points in the pathway from glucose to fatty acid which are important

for the acute regulation of fatty acid synthesis. These are (1) glucose transport, (2) hexokinase, (3) phosphofructokinase-1, (4) pyruvate dehydrogenase and (5) acetyl-CoA carboxylase (Fig. 1.1).

1.4.1 GLUCOSE TRANSPORT

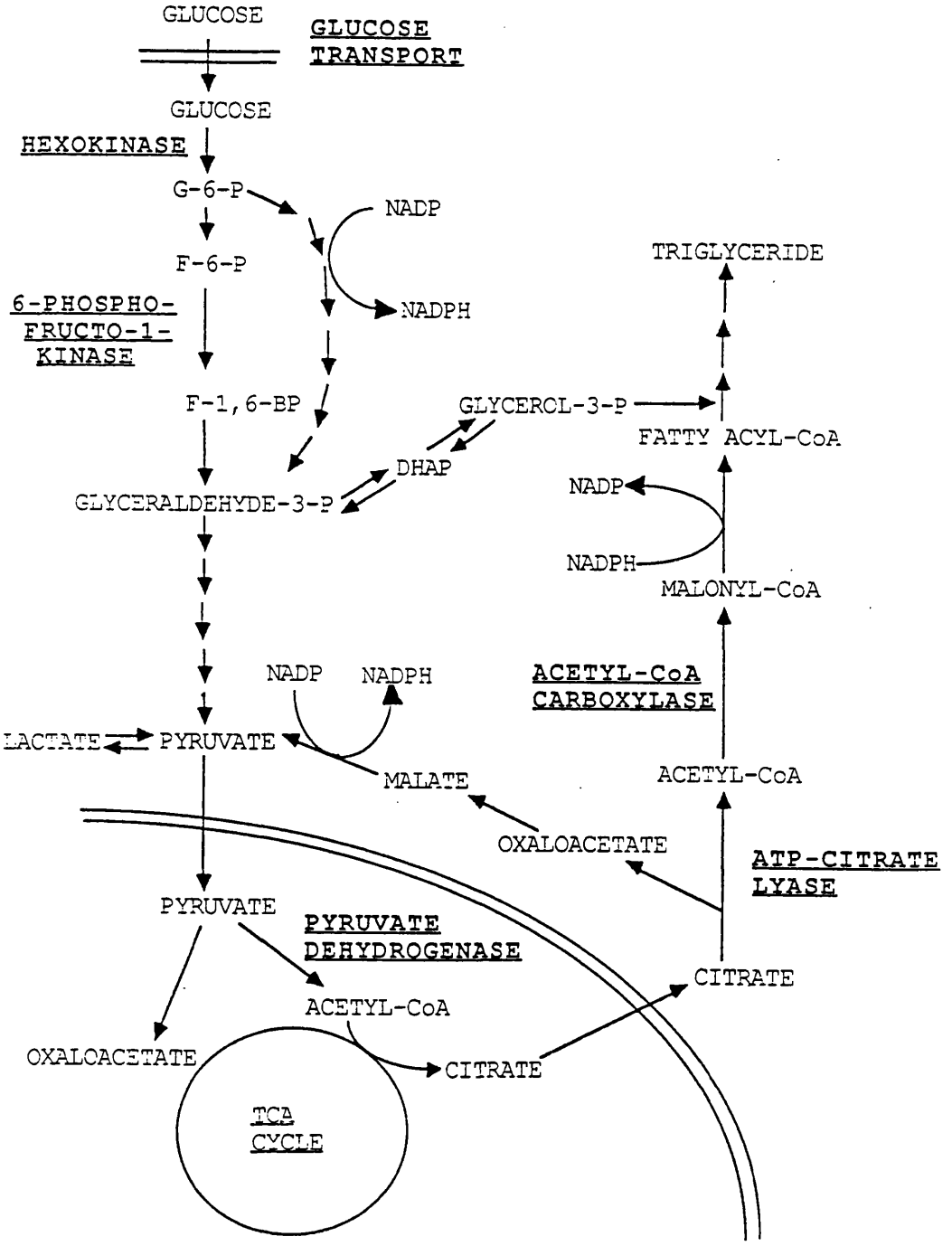
In adipose tissue glucose transport and lipogenesis are stimulated by insulin. The hormone binds to its receptor and by an unknown mechanism causes the number of functional glucose transporters on the plasma membrane to rise (Simpson and Cushman 1986). The identity of the putative second messenger(s) responsible for this and other insulin effects is unknown and the subject of much debate (Houslay et al 1986; Espinal 1987). In contrast, insulin stimulates lipogenesis in the normal fed rat lactating mammary gland (Jones et al 1984a; Bussmann et al 1984) and in mammary acini from rats fed a high fat diet (Munday and Williamson 1987) but has no effect on glucose transport except when transport is already depressed by starvation (Threadgold and Kuhn 1984; Prosser 1988). In confirmation of these functional studies two papers have reported that mammary tissue lacks the insulin regulated glucose transporter (GLUT-4) and contains predominantly the GLUT-1 transporter (Madon et al 1990; Burnol et al 1990). This transporter has recently been reported to translocate to the plasma membrane of fat cells in response to insulin (Vogt et al 1991). This would explain the recruitment of transporters to the plasma membrane of starved lactating mouse mammary epithelial cells in response to re-feeding (Prosser 1988).

However, there is little to support glucose transport in this tissue as limiting for glucose uptake or utilization as stated by Threadgold and Kuhn (1984). Furthermore the intracellular concentrations and ratio of glucose/glucose-6-phosphate rise during starvation (Jones et al 1984a) when lipogenesis is decreased by 80%. This would have been expected to fall if the entry of glucose was the primary regulatory step for glucose utilization.

Figure 1.1 THE PATHWAY FOR FATTY ACID SYNTHESIS FROM GLUCOSE IN THE MAMMARY GLAND OF THE RAT

(From Munday and Hardie 1987).

In ruminants acetate (from rumen fermentation) is the major precursor for fatty acid synthesis, and ATP citrate lyase activity is very low.



Notwithstanding this, the maximum rate of glucose transport does reflect the glycolytic capacity of the lactating mammary gland. The basal rate of glucose transport in murine mammary epithelial cells increased nearly forty fold from virgin to 10 days lactation (Prosser and Topper 1986). Similarly large increases in glucose transport also occur in rat mammary tissue between parturition and the peak of lactation (Threadgold and Kuhn 1984) when glucose utilisation, for lactose and fatty acid synthesis, is also rising rapidly.

1.4.2 HEXOKINASE AND PHOSPHOFRUCTOKINASE-1

Hexokinase [EC 2.7.1.1] catalyses the synthesis of glucose-6-phosphate (G-6-P) from glucose. It is inhibited by its reaction product with an apparent Ki of 0.16 mM (Grossbard and Schimke 1966) and inhibition is non-competitive with respect to its substrate glucose. This means that hexokinase activity can decrease even in the presence of elevated intracellular glucose levels and also allows for the co-ordinate regulation of hexokinase and phosphofructokinase-1, [EC 2.7.1.11] (PFK-1); the next regulatory enzyme in the glycolytic pathway. PFK-1 catalyses the synthesis of fructose-1,6-bisphosphate (F-1,6-P_2) from fructose-6-phosphate (F-6-P). The latter is in equilibrium with G-6-P therefore inhibition of PFK-1 (and the concomitant rise in F-6-P) necessarily increases the concentration of G-6-P and thus may also bring about the inhibition of hexokinase.

Decreased glucose utilisation is associated with elevated G-6-P levels in mammary tissue (Williamson et al 1987; Robinson and Williamson 1977c) and lipogenesis correlates inversely with G-6-P concentrations; thus indicating that the inhibition of glycolysis is distal to hexokinase. In contrast inhibition of lipogenesis in mammary tissue is associated with a decrease in F-1,6-P_2 levels (Mercer and Williamson 1987), thus identifying PFK-1 as a primary regulatory enzyme of glycolysis. Mammary

PFK-1 (as in other tissues studied) is subject to allosteric control by ATP and citrate (Zammit 1979). These effectors produce inhibition of PFK-1 by decreasing the affinity of the enzyme for its substrate F-6-P, furthermore citrate augments the inhibitory effects of ATP (Zammit 1979). The most important activators of PFK-1 are AMP and fructose-2,6-bisphosphate (F-2,6-P_2) which act in synergy. F-2,6-P_2 is synthesized and degraded by the bifunctional enzyme phosphofructokinase-2/fructose 2,6-bisphosphatase [EC 2.7.1.05/EC 3.1.3.46] (PFK-2/F-2,6-Pase). The kinase catalyses the transfer of phosphate from ATP to the C-2 hydroxyl of F-6-P and the phosphatase catalyses hydrolysis of F-2,6-P_2 to F-6-P. Until recently F-2,6-P_2 was the most potent known activator of PFK-1. At micromolar levels it is able to relieve the inhibition of PFK-1 by physiological levels of ATP, and except during anoxia, the rate of glycolysis generally follows the level of F-2,6-P_2 (Hue and Rider 1987; Pilkis et al 1987; Hue et al 1988). A new activator of PFK-1, ribose 1,5-bisphosphate (Ribose-1,5-P_2) has been identified in rat brain (Ogushi et al 1990). This effector is synthesised in the first few seconds of ischaemia and like F-2,6-P_2 activates PFK-1 at micromolar levels. Whether this activator is responsible for the rapid glycolysis seen in other anoxic tissues (Hue and Rider 1987) has yet to be determined.

The levels of F-2,6-P_2 and the activity of PFK-1 rise 3 fold and 4 fold respectively between late pregnancy and peak lactation (Socher et al 1984). Furthermore, the levels of AMP, a positive effector of PFK-1 and PFK-2, rises while citrate (an inhibitor) falls. These changes, together with the observations that mammary F-2,6-P_2 decreased by 40% after an overnight fast (Ward and Kuhn 1985) and was doubled by a hyperinsulinaemic clamp (Burnol et al 1988) indicate that F-2,6-P_2 is an important regulator of glycolytic flux in the lactating gland.

Both PFK-1 and PFK-2/F-2,6-Pase from liver are subject to <u>in vitro</u> phosphorylation by cAMP-PK (Foe and Kemp 1982;

Sakakibara and Uyeda 1983). Phosphorylated PFK-1 from muscle and liver is more sensitive to allosteric inhibition by ATP and citrate and less sensitive to activation by AMP or F-2,6-P_2. In contrast PFK-1 from heart is activated when phosphorylated by an as yet unidentified kinase (Pilkis et al 1987). Similarly phosphorylation of PFK-2 has opposite effects according to the tissue from which it is isolated. In bovine heart PFK-2 activity is increased by phosphorylation but in liver phosphorylation inhibits the kinase activity (Sakata and Uyeda 1990). These authors show that the difference may be due to the different location of the phosphorylation sites between the heart and liver isoenzymes. The nature of the PFK-1 and PFK-2/F-2,6-Pase enzymes in mammary gland has not been studied. Their regulation by phosphorylation would be an attractive mechanism working in synergy with the known metabolic effectors; especially when changes in the levels of effectors do not seem to be sufficient to account for the observed changes in glycolysis (Ward and Kuhn 1985).

1.4.3. PYRUVATE DEHYDROGENASE (PDH)

The 'total' activity of PDH in the mammary gland of the lactating rat increases seven-to-ten fold from mid pregnancy to mid lactation (Gumaa et al 1973, Coore and Field 1974) since, fatty acids are synthesized from acetyl-CoA derived mainly from glucose this is in keeping with the increased lipogenic capacity of the tissue during this transition.

The PDH complex is located in the mitochondrial matrix space. It is a multienzyme complex which catalyses the oxidative decarboxylation of pyruvate to acetyl-CoA via a co-ordinated series of reactions. The overall reaction is essentially irreversible and regulates the supply of acetyl-CoA which is available for oxidation or fatty acid synthesis.

The PDH reaction is catalysed sequentially by three components of the complex (Fig. 1.2). Pyruvate decarboxylase (E_1), [EC 1.2.4.1]; lipoate acetyltransferase (E_2), [EC 2.3.1.12] and dihydrolipoyl dehydrogenase (E_3), [EC 1.6.4.3]. The reactions catalysed are described below and illustrated schematically in Fig. 1.2

$\underline{E}_1$ $CH_3CO.COOH + E_1\,[TPP] \rightarrow E_1[CH_3CHOH - TPP] + CO_2$
(pyruvate)

$E_1[CH_3CHOH\text{-}TPP] + E_2[lip\text{-}S_2] \rightarrow E_1[TPP] + E_2[CH_3CO\text{-}S\text{-}lip\text{-}SH]$
$\underline{E}_2$ oxidised acetylated
$E_2[CH_3O\text{-}S\text{-}lip\text{-}SH] + CoA\text{-}SH \rightarrow E_2[lip\text{-}(SH)_2] + CH_3CO\text{-}SCoA$
reduced (acetyl-CoA)

$E_2[lip\text{-}(SH)_2] + E_3[FAD] \rightarrow E_2[lip\text{-}S_2] + E_3\,[FADH_2]$
$\underline{E}_3$ reduced oxidised
$E_3[FADH_2] + NAD^+ \rightarrow E_3[FAD] + NADH + H^+$

TPP = thiamine pyrophosphate; lip = lipoyl moiety.
Sum :-
$CH_3CO.COOH + NAD^+ + CoA\text{-}SH \rightarrow CH_3CO\text{-}SCoA + NADH + H^+ + CO_2$
pyruvate acetyl-CoA

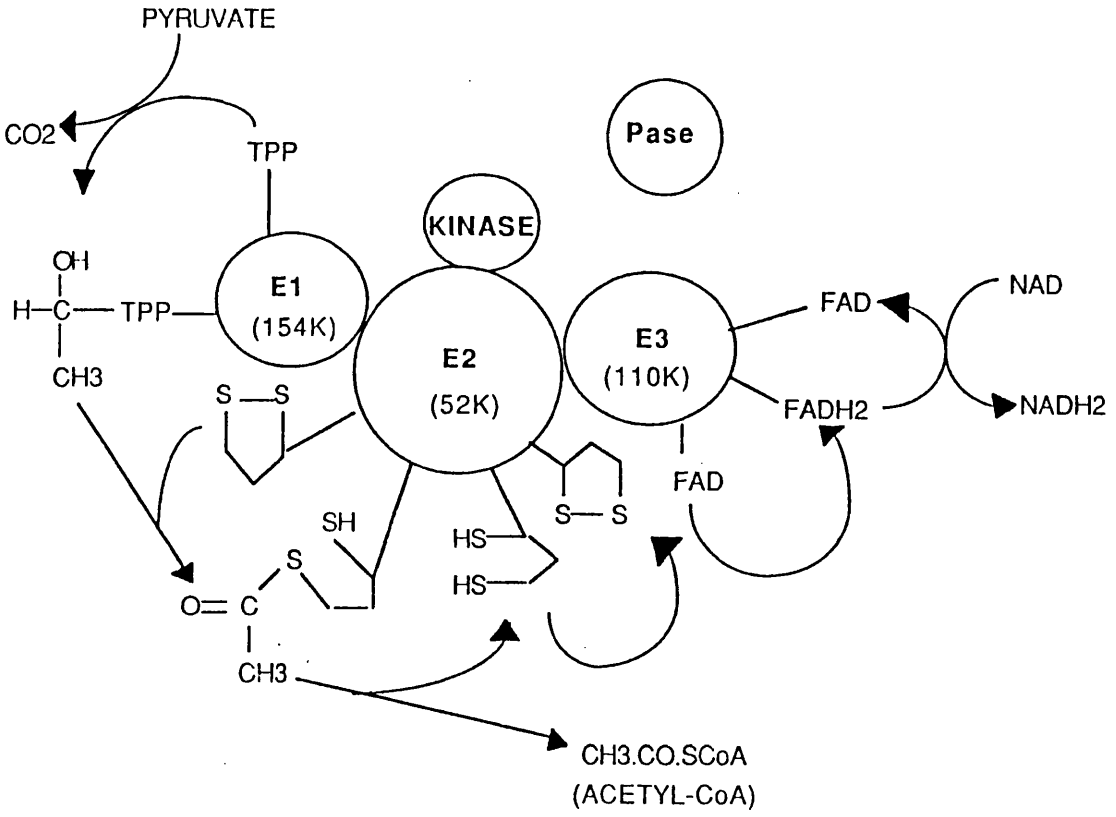
The structure of the PDH complex from mammary gland has not been studied but thus far it has shown only small variations in different tissues. Mammalian PDH complex is organised as a core of 60 E_2 subunits each with two covalently attached lipoyl moieties. This core is linked via non-covalent interactions to 20 - 30 E_1 subunits and 6 E_3 subunits. E_1 is a tetramer with a structure of $\alpha_2\beta_2$ and E_3 is a homodimer containing 2 molecules of FAD. A further component of the mammalian PDH complex, termed component X, has been identified (De Marcucci and Lindsay 1985). It binds tightly to the E_2 core and is thought to be involved with the transfer of reducing equivalents from the lipoyl moiety of the E_2 component to the E_3 component prior to their transfer to NAD^+ (Gopalakrishnan et al 1989). Three

Figure 1.2 SCHEMATIC REPRESENTATION OF THE PYRUVATE DEHYDROGENASE COMPLEX

(From Munday and Hardie 1987).

The steps catalysed by each subunit are described in the text.

E1 - pyruvate decarboxylase
E2 - dihydrolipoyl transacetylase
E3 - dihydrolipoyl dehydrogenase



to five molecules of PDH kinase and phosphatase are associated with the complex. The kinase is tightly bound to E_2 and co-purifies with the PDH complex. The phosphatase only binds to E_2 in the presence of Ca^{++} (Pettit et al 1972). The PDH complex is inhibited when phosphorylated by its intrinsic kinase. Phosphorylation occurs on specific serine residues in the α subunit of the pyruvate decarboxylase (E_1) (Barrera et al 1972; Sugden and Randle 1978).

The PDH complex is also inhibited by its reaction products acetyl-CoA and NADH (Pettit et al 1975, Kerbey et al 1976, 1977). It is suggested that the high ratios of acetyl-CoA/CoA and NADH/NAD^+ inhibit by lowering the number of lipoyl groups in the oxidised form (E_2[lip-S_2]) which in turn leads to a reduction of E_1[TPP] thus limiting the rate of pyruvate decarboxylation (Denton et al 1975). End product inhibition may play a role in the acute regulation of PDH in lactating mammary tissue but is believed to be quantitatively less important than regulation by reversible phosphorylation (Munday and Hardie 1987). Moreover the relative importance of direct end product inhibition is difficult to quantify because elevated NADH/NAD^+ and acetyl-CoA/CoA ratios stimulate PDH-kinase (Pettit et al 1975). A second regulator of PDH kinase activity was identified in 1982 in rat heart and termed kinase/activator (KAP) because it could be either additional free PDH kinase and/or an activator of PDH kinase. This regulatory mechanism is slower in onset and requires new protein synthesis (Kerbey and Randle 1982). The synthesis of KAP increases in response to starvation and diabetes (Kerbey and Randle 1981, 1982; Kerbey et al 1984). It has recently been reported that KAP is indeed a free form of PDH kinase (Mistry et al 1991; Jones and Yeaman 1991). These authors suggest that the appearance of free PDH-kinase in the mitochondria of starved or diabetic animals is due to the tissue synthesising more kinase than is able to bind to the E_2 subunits (Jones and Yeaman 1991) or alternatively that KAP is merely kinase which has dissociated from the

PDH complex during tissue preparation.

The importance of covalent modification to the regulation of PDH activity, and hence lipogensis, is illustrated by the three-fold increase in the proportion of active complex which occurs from parturition to peak lactation (Coore and Field 1974). This change is superimposed on a seven-fold increase in the total amount of PDH over the same period. Thus the mammary gland has the potential for a twenty-one-fold increase in the rate of acetyl-CoA synthesis.

Lipogenesis and PDH activity in the mammary gland falls when lactating rats are made insulin deficient (Field and Coore 1976) or starved (Kankel and Reinauer 1976; Baxter and Coore 1978) and is rapidly restored by insulin treatment (Field and Coore 1976) or refeeding (Munday and Williamson 1981). These changes are mediated by reversible phosphorylation of PDH. The proportion of mammary PDH in its active (dephosphorylated) form falls by more than 70% after 24 hours starvation (Kankel and Reinauer 1976; Baxter and Coore 1978) Furthermore starvation and insulin deprivation both correlate with an increase in PDH-kinase activity (Baxter and Coore 1978) and a reduction in PDH phosphatase activity (Baxter and Coore 1979a).

The precise mechanism whereby physiological effectors such as insulin and free fatty acids cause changes in PDH activity has not been elucidated. It has been reported (Kiechle et al 1980, 1981; Saltiel 1981) that insulin stimulates the production of an 'enzyme modulator' from plasma membranes which is able to activate PDH activity. Saltiel (1987) claims this is through the activation of PDH phosphatase as the effect was not seen in the presence of phosphatase inhibitor (100 mM NaF). It has been shown that a high fat diet decreases the activity of PDH in liver (Weiland et al 1972) and in white adipose tissue (Stansbie et al 1976). Laker and Mayes (1984) have also observed inhibition of PDH in livers perfused with elevated levels

of free fatty acids. Stansbie et al (1976) measured no change in PDH phosphatase activity in high fat fed animals, suggesting that PDH-kinase is activated in conditions where plasma free fatty acids are elevated. The precise mediator is unknown however.

1.4.4 ACETYL-CoA CARBOXYLASE

Structure and Function

Acetyl-CoA carboxylase [EC 6.4.1.2], (ACC) is a cytosolic biotinyl enzyme whose activity was first described by Wakil in 1958. ACC catalyses the committed step in the synthesis of fatty acids from acetyl-CoA namely the carboxylation of acetyl-CoA to form malonyl-CoA.

The overall reaction catalysed by ACC is:

$$\text{acetyl-CoA} + \text{ATP} + HCO_3^- \xrightarrow{ACC} \text{malonyl CoA} + \text{ADP} + \text{Pi}$$

The reaction occurs in two stages and the catalytic sites for each half reaction occur on the same polypeptide chain (Lane et al 1974). The protomeric form of the enzyme is a homodimer (Gregolin et al 1966) and contains one mole of biotin per subunit. In lactating mammary gland each subunit has a M_r of 240 kDa as judged by SDS-polyacrylamide gel electrophoresis and 265 kDa as calculated from the polypeptide chain predicted from the cDNA clone (Bianchi et al 1990) An isozymic form of ACC of 280 kDa has been described in rat tissues by Bianchi et al (1990). This form is uniquely expressed in heart and skeletal muscle and co-expressed with the previously known form in liver and mammary gland. The 280 kDa ACC has a higher Ka for citrate and Km for acetyl-CoA than the 265 kDa form, and it is suggested that the novel isoform of ACC is able to promote fatty acid oxidation.

LONG TERM REGULATION

For many years it has been known that the activity of ACC is regulated in the long term by varying the quantity of enzyme in the cell. Hicks et al (1965) showed that the rise in ACC concentration that occurs in liver upon refeeding after starvation can be prevented by treatment with puromycin, a protein synthesis inhibitor or actinomycin D, a transcription inhibitor. Nakanishi and Numa (1970) showed that the amount of immunotitratable ACC in rat liver is more than halved by a 48 hour fast and increased nearly 3-fold above controls when the animals were refed a fat free diet. This was due to simultaneous changes in the rate of degradation and synthesis of ACC. (Table 3.)

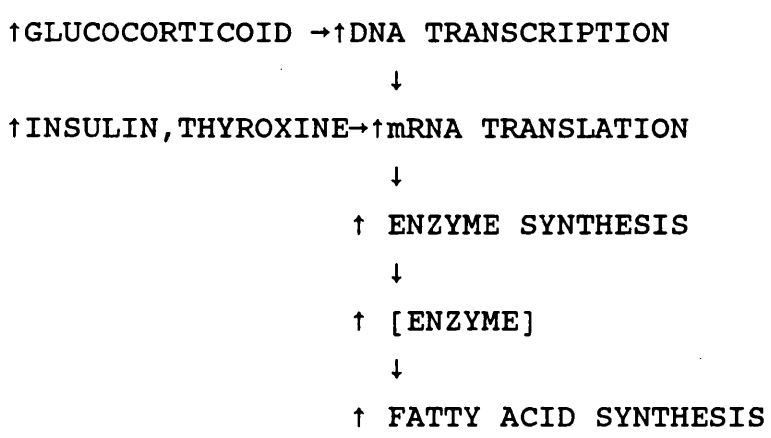
Table 3 EFFECT OF STARVATION AND REFEEDING ON THE TURNOVER OF ACC IN RAT LIVER

	TOTAL ACC (arbitary units)	$t_{½}$ for degradation(hours)	Relative rate of synthesis.
CONTROL	1.0	59	1.0
48 hour fast	0.5	31	0.5
48 hour fast) 72 hour refed) fat free diet)	2.7	55	4.0

Degradation and synthesis were estimated by ^{3}H-leucine incorporation. ACC was measured by immunotitration (from Nakanishi and Numa, 1970).

After parturition rat mammary ACC content and activity increases as a result of increased synthesis of the enzyme (Mackall and Lane, 1976), this is accompanied by a seven-fold elevation in translatable ACC mRNA (Lopez-Cassillas 1987). Similarly, diet-induced changes in the concentration of ACC in liver or epididymal fat pads correlate positively with changes in the amount of ACC mRNA (Pape et al 1988).

The mechanism controlling the gene expression of ACC and also the turnover of the enzyme have not been fully elucidated though anabolic hormones have a role to play:



The absence of either glucocorticoids, insulin or thyroxine significantly reduces the increase in lipogenic capacity that occurs in liver upon refeeding after a fast of more than 36 hours (Wurdeman et al 1978, Bouillon and Berdanier 1980). Furthermore, transcription and translation of the lipogenic enzyme glucose-6-phosphate dehydrogenase is increased by these hormones (Fritz et al 1986, Kletzien et al 1985, Fritz and Kletzien 1987). It is highly probable that other lipogenic enzymes including ACC are influenced similarly.

SHORT TERM REGULATION

Allosteric Effectors

When rats are fasted for less than 24 hours or subjected to acute insulin deficiency the decrease in the activity of ACC is greater than explained by enzyme turnover. The activity of ACC can be altered *in vitro* allosterically by citrate (Martin and Vagelos 1962, Moss and Lane 1971) and fatty acyl-CoA thioesters (Bortz and Lynen 1963; Lunzer et al 1977; Ogiwara et al 1978) which activate and inhibit the enzyme respectively. *In vitro* inhibition of ACC by fatty acyl-CoA esters is competitive with citrate (Goodridge 1972; Halestrap and Denton 1974;

Ogiwara et al 1978) and acetyl-CoA (Bortz and Lynen 1963). This evidence would suggest a plausible mechanism for the regulation of ACC is the forward activation by citrate (the immediate precursor for acetyl-CoA) antagonised by end product inhibition by fatty acyl-CoA thioesters.

In vivo there is some evidence to support this. The concentration of citrate required for the half maximal activation (Ka citrate) of ACC from mammary gland is approximately 2 mM (Munday and Hardie 1884, 1986) and the intracellular concentration varies between 0.2 to 0.5 mM (Williamson et al 1975, Robinson and Williamson 1977b) making it a potential physiological regulator. However, there is no conclusive evidence showing a close correlation between fatty acid synthesis and citrate levels. Nishikori et al (1973) reported a rapid rise in citrate and fatty acid synthesis in the liver of starved and re-fed rats. To contrast , in rat epididymal fat pads stimulation of fatty acid synthesis by insulin was associated with an unchanged or decreased tissue concentration of citrate whereas adrenaline caused an increase in citrate concentrations along with a decrease in fatty acid synthesis. (Denton and Halperin 1968, Saggerson and Greenbaum 1970, Halestrap and Denton 1974). By using fluoroacetate which is metabolised to fluorocitrate a potent inhibitor of aconitase it is possible to raise citrate levels ten-fold. When this was done using epididymal fat pads no marked increase in the initial activity of ACC was observed (Brownsey et al 1977).

An inverse relationship between the rate of fatty acid synthesis and intracellular levels of fatty acyl-CoA esters has been demonstrated in rat epididymal fat pads treated with insulin (Denton and Halperin 1968; Saggerson and Greenbaum 1970) also in the liver of starved rats refed a fat free diet (Nishikori et al 1973). Jacobs and Majerus (1973) using skin fibroblasts found no such relationship. The role of fatty acyl-CoA esters in vivo will be extremely difficult to resolve largely because the free intracellular

levels are difficult to measure. They are modulated by fatty acid binding proteins (Lunzer et al 1977) and also compartmentalization within the cell. It is estimated that a total fatty acid concentration of 50 - 150 μM is reduced to a free concentration of 1.5 - 15 μM. (Brownsey and Denton 1987, Lunzer et al 1977) but the Ki of palmitoyl-CoA for ACC has been reported as <10 nM (Ogiwara et al 1978, Nikawa et al 1979). This would mean that free fatty acids are always at inhibitory levels, and thus lessens the likelihood that they are physiological mediators.

Several other potential regulators of ACC have been described. These include GTP and other guanine nucleotides (Witters et al 1981, Beuchler and Gibson 1984), polyphosphoinositides especially phosphatidyl inositol 4,5-bisphosphate (Heger and Peter 1977, Blyth and Kim 1982), an autocrine factor tentatively identified as an oligosaccharide (Witters et al 1988), small molecular weight substances released from liver plasma membranes by insulin binding (Saltiel et al 1983) and ADP-ribosylation (Witters and McDermott 1986). The physiological relevance of these potential regulators is still unclear.

ACC polymerises to form linear helices of up to 30 dimers in length (Ahmed et al 1978). Citrate induces this polymerisation <u>in vitro</u> and fatty acyl-CoA esters cause disaggregation. The filamentous form of ACC is more active than the protomeric dimer but activation of the ACC subunits precedes their polymerization in the presence of citrate (Beaty and Lane 1983). There is evidence that the polymerization of ACC occurs when it is activated irrespective of the effector agent. Thus ACC activation by Coenzyme-A (Yeh et al 1981) or limited trypsinolysis (Iritani et al 1969) both induce polymerisation. The possible importance of polymerisation <u>in vivo</u> has been considered by Borthwick et al (1987) who have reported that ACC from insulin treated tissue which has elevated ACC activity is more highly polymerised in the absence of citrate than that from tissue treated with the ß-agonist

isoprenaline which inhibits lipogenesis. Similarly Ashcraft et al (1980) have reported that fat feeding or starvation, both of which decrease lipogenesis, cause a decrease in the amount of polymeric ACC in chicken liver. Thus indicating that the polymerisation of ACC may be of significance in vivo. Tanake et al (1977) have reported that ACC is less susceptible to proteolysis in the presence of citrate when it is in the polymeric form.

Allred and co-workers have published work describing the regulation of ACC by the translocation of an inactive form of the enzyme from the mitochondrial outer membrane to the cytosol where upon the enzyme becomes active (Allred and Roman-Lopez 1988). The proportion of active cytosolic to inactive mitochondrial ACC is decreased by fasting and increased by refeeding (Allred et al 1985; Roman-Lopez et al 1989). These workers also show short term insulin deficiency increases the amount of inactive ACC associated with the mitochondria. (Roman-Lopez and Allred 1987) thus providing a mechanism for the decreased lipogenesis seen in diabetic animals It has also been shown that the increased ACC activity characteristic of genetically obese Zucker rats is not due to a greater amount of enzyme but is the result of a greater proportion of ACC being in the active cytosolic form (Allred et al 1989). All this work has been done in rat liver but a similar mechanism if it occurs in the mammary gland would allow rapid control of ACC activity. It must be noted however that Moir and Zammit (1990) have reported in direct contrast that the hepatic mitochondrial ACC pool does not replenish the cytosolic ACC upon refeeding of starved rats. There are no reports addressing possible interactions between enzyme translocation, and the allosteric and covalent modification mechanisms for controlling ACC activity.

COVALENT MODIFICATION

The regulation of ACC by phosphorylation and dephosphorylation was first confirmed by Kim and co-workers

(Carlson and Kim 1974, Lee and Kim 1977) in rat liver and was reported in rat mammary gland by Hardie and Guy (1980). These early reports showed that phosphorylation and dephosphorylation of ACC was probably a mode of acute regulation in vivo. The dephosphorylation of ACC decreased the Ka for citrate from 2.4 mM to 0.2 mM and elevated Vmax (Carlson and Kim 1974). The latter authors also showed that the dephosphorylated enzyme was less susceptible to inhibition by palmitoyl-CoA.

In vitro ACC can be phosphorylated by a number of protein kinases (Table 4). The first characterised kinase shown to phosphorylate purified ACC and change its activity was cAMP dependent protein kinase (cAMP-PK) (Hardie and Cohen 1978). Phosphorylation by the catalytic subunit of cAMP-PK causes a modest decrease in Vmax and a doubling in the Ka for citrate (Hardie and Guy 1980, Munday et al 1988a). Similar changes in the kinetic parameters are seen when ACC is phosphorylated by acetyl-CoA carboxylase-2 (ACK2) purified from lactating rat mammary gland (Munday and Hardie 1986, Table 3). cAMP-PK and ACK2 both generate the same major ^{32}P-labelled peptides as identified by double trypsin/chymotrypsin digestion followed by separation on reversed phase HPLC (Munday et al 1988b). The maximum effects of phosphorylation by these two kinases are seen at subsaturating levels of citrate (0.1 -1.0 mM) (Hardie and Guy 1980) which corresponds to the physiological levels of citrate in liver (Nishikori et al 1973) and hepatocytes (Holland et al 1984) as well as mammary gland (Munday and Hardie 1984, 1986).

It was noted that ACC purified from rabbit mammary gland (Hardie and Cohen 1979, Hardie and Guy 1980) or hepatocytes (Holland et al 1984, Sim and Hardie 1988) in the presence of phosphatase inhibitors often had a high phosphate content (>5 moles phosphate/mole enzyme) and a low specific activity at saturating citrate concentrations. The activity in mammary gland could be doubled by dephosphorylation with protein phosphatase I (Hardie and

Table 4 KINASES WHICH PHOSPHORYLATE ACC IN VITRO

Protein Kinase	Effect on ACC activity	Reference
1. cAMP-PK	Vmax↓ Ka↑	Hardie & Cohen, 1978; Hardie & Guy, 1980.
2. ACK-2	Vmax↓ Ka↑	Hardie & Cohen, 1978; Munday & Hardie, 1984.
3. ACC-Kinase	Vmax↓	Shiao et al, 1981.
4. ACC-Kinase	Vmax↓ Ka↑	Lent & Kim, 1982; Jamil &b Madsen, 1987a.
5. AMP-PK	Vmax↓ Ka↑	Carling & Hardie, 1986, Munday et al 1988b.
6. Ca^{++} Calmodulin-dependent	no effect	Hardie et al, 1986
7. Ca^{++} Phospholipid-dependent	Vmax↓ Ka↑	Hardie et al, 1986.
8. Casein Kinase I	no effect	Munday & Hardie, 1984.
9. Casein Kinase II	no effect	Munday & Hardie, 1984.
10. Multifunctional Kinase	Vmax↑	Ramakrishna & Benjamin 1985

Guy 1980) and increased more than ten-fold in hepatocytes by treatment with the catalytic subunit of protein phosphatase 2A (Holland et al 1984). Subsequent phosphorylation of the activated ACC with cAMP-PK could not reduce the Vmax of ACC to original values (Hardie and Guy 1980). These observations indicated the presence of an endogenous kinase capable of profoundly inhibiting ACC. In vitro cAMP-PK produced a 13% and ACK2 a 20% fall in the Vmax of purified ACC (Munday et al 1988a). However in vivo ACC attains specific activities markedly lower than phosphorylation by either cAMP-PK or ACK2 can explain (Munday and Hardie 1986). It was shown that 24 hour starvation decreased the Vmax of ACC purified from lactating mammary gland by 73%. This was associated with an increase in the alkali-labile phosphate of approximately 1 mole Pi/mole enzyme subunit.

Novel kinases which inactivate ACC have been reported by Shiao et al (1981), Lent and Kim (1982) and Carling and Hardie (1986). The kinase described by Shiao et al (1981) has an apparent MW_r of 160 kDa and autophosphorylates to a small extent; incorporating 0.1 to 0.2 moles of phosphate per mole. The kinase incorporates 2 to 2.5 moles of phosphate into ACC which correlates with a rapid decrease in the activity of the enzyme. The ACC kinase described by Lent and Kim has an apparent MW_r of 170 kDa as determined by SDS-PAGE and was associated with ACC during purification. The incorporation of one mole of phosphate into ACC caused complete inactivation of ACC when measured at 2 mM-citrate; its effects on the Vmax of the enzyme were not stated. This ACC-kinase was activated by Coenzyme-A (Lent and Kim 1982, 1983a) and by incubation with cAMP-PK (Lent and Kim 1983b). The latter workers were unable to phosphorylate ACC directly with cAMP-PK. They therefore have proposed that cAMP-PK inactivated ACC by means of a bicyclic cascade. In their model cAMP-PK phosphorylated and activated the ACC-kinase which in turn phosphorylated and inactivated ACC. This kinase has been studied further by Jamil and Madsen (1987). They have reported that the

kinase produced an 82% fall in the activity of ACC (measured at 10 mM-citrate) with the concomitant incorporation of 0.45 mol phosphate/mol ACC and an increase from 1.4 mM to 2.1 mM in the apparent Ka for citrate. Jamil and Madsen (1987) identified through the use of two dimensional thin layer chromatography two peptides in ACC tryptic digests whose phosphorylation correlated with the loss of activity of ACC. The AMP-activated protein kinase from rat liver (AMP-PK) described by Carling and Hardie (1986, 1989) has several properties in common with that purified by Lent and Kim. The AMP-PK is also associated with ACC during the early stage of purification (Carling and Hardie 1986) and its phosphorylation produces an 80% fall in the Vmax of ACC as well as an increase in the Ka for citrate (Table 5). The AMP-PK accounts for more than 90% of the cAMP independent kinase activity against ACC in the liver of male rats. It is activated four to six fold by 5'AMP with a half maximal effect at approximately 1.4 μM. (Carling et al 1989). AMP-PK is itself regulated by phosphorylation in a bicyclic cascade (Carling et al 1987) but the kinase activating AMP-PK is unidentified, it is not cAMP-PK (Davies et al 1989). The AMP-PK phosphorylates and inactivates hydroxymethylglutaryl-CoA reductase the rate limiting enzyme for cholesterol synthesis. A model for the co-ordinate regulation of fatty acid synthesis and cholesterol synthesis has therefore been proposed (Carling et al 1987). The channelling of acetyl-CoA, the precursor for both pathways, would therefore be diverted from anabolic to catabolic pathways during, for example, starvation. AMP-PK phosphorylates hormone sensitive lipase (HSL) (Garton et al 1989). This has no intrinsic effect on the latter's activity but it prevents subsequent activation of HSL by cAMP-PK. AMP-PK therefore has the potential to exert antilipolytic effects in adipose tissue. AMP-PK phosphorylates glycogen synthase at a site (N7) which is known to cause inactivation (Carling and Hardie 1989). Herein lies a potential mechanism for the inhibition of glycogen synthesis in exercising muscle when intracellular

Table 5 KINASES WHICH CHANGE THE ACTIVITY OF ACC

Kinase	Phosphate incorporated mol/mol ACC	% change Vmax	Ka Citrate
cAMP-PK[1]	1.49	13↓	234↑
ACK-2[2]	0.89	20↓	155↑
'ACC-Kinase'[3]	0.45	82↓	66↑
AMP-PK[4]	1.77	81↓	280↑
'ACC Kinase'[5]	0.9	(80↓ activity at 2mM citrate)	

1 from bovine heart (Munday et al 1988a)

2 from lactating rat mammary gland (Munday et al 1988a)

3 from rat liver (Jamil and Madsen 1987)

4 from rat liver (Munday et al 1988a)

5 from rat liver (Lent and Kim 1982)

AMP levels are elevated. However the concentration of AMP-PK is low in muscle (Davies et al 1989) and so it may not be the physiological mediator. AMP-PK is reported to be widespread in mammalian tissues and especially active in those with high lipid and cholesterol metabolism (Davies et al 1989).

The rate of activation of AMP-PK by AMP-PK kinase in vitro is markedly enhanced by the presence of nanomolar amounts of palmitoyl-CoA. (Carling et al 1988) If this occurs in vivo it would allow for the inhibition of fatty acid synthesis by direct feedback inhibition of ACC allosterically and indirect inhibition by stimulating the phosphorylation of ACC by AMP-PK.

The multifunctional kinase described by Ramakrishna and Benjamin (1985) is unusual in that phosphorylation increases the Vmax of ACC. This observation is of interest because the activation of ACC by insulin is generally associated with increased phosphate content. Unfortunately no further reports clarifying the effects of the multifunctional kinase on ACC have been published. The other kinases in Table 4 have no significant effect on the activity of ACC and so their phosphorylation of ACC is generally believed to be gratuitous.

THE PHYSIOLOGICAL SIGNIFICANCE OF THE PHOSPHORYLATION SITES ON ACC

The sites phosphorylated on ACC in vitro and in intact hepatocytes and adipocytes have been identified in the Hardie laboratory (Haystead and Hardie 1988, Haystead et al 1988, Munday et al 1988a, Sim and Hardie 1988). These are shown in Table 6 with the appropriate effectors involved. Six of the eight phosphorylation sites occur in a cluster in the first 100 amino acids at the amino terminus, the remaining sites are ser-1200 and 1215 in the rat liver sequence numbered from the N-terminus (Lopez-Cassillas et al 1988). Only phosphorylation of serines 77 and 79

Table 6 SITES PHOSPHORYLATED ON ACC

Serine residue	Site	Peptide sequence	Protein kinases
1200	1	Arg-Met-Ser(P)[1]Phe	1-cAMP-PK, AMP-PK, ACK-2
77,79	2,3	Ser-Ser(P)[2]-Met-Ser(P)[3]-Gly-Leu	2-cAMP-PK and PKC 3-AMP-PK
23,25, 26,	4,5,6	Phe-Ileu-Ileu-Gly-Ser(P)[4]-Val-Ser(P)[5]-Glu-Asp-Asn-Ser(P)[6]-Glu	4 - unknown effector 5-calmodulin PK 6-casein Kinase II -insulin
95	7	Lys-Ileu-Asp-Ser(P)[7]-Gln-Arg	7 - PKC but not altered when cells treated with phorbol ester or insulin
1215	8	Val-Ala-Ser(P)[8]-Val Ser-Asp-Val-Leu	8-AMP-PK

directly correlate with the inactivation of ACC (Munday et al 1988a) and removal of these sites by limited trypsinisation completely restores the activity even though serines 1200 and 1215 remain fully phosphorylated (Davies et al 1990). Moreover only the modification of serine 79, which is phosphorylated exclusively by AMP-PK, produces the large decrease in the Vmax of ACC that occurs in vivo. Phosphorylation of serine 77 (i.e. by cAMP-PK) produces only small effects on the maximal activity of ACC.

Experiments using isolated adipocytes treated with phorbol ester show that ACC is not a physiological substrate for protein kinase C. (Haystead and Hardie 1988). There is also uncertainty about the role of cAMP-PK in the regulation of ACC activity in vivo. Challenge of adipocytes and hepatocytes with glucagon or adrenaline causes a decrease in fatty acid synthesis concomitant with the phosphorylation and inactivation of ACC. (Witters et al 1979, 1983, Holland et al 1984, 1985). These authors attributed the phosphorylation of ACC to cAMP-PK because both glucagon and adrenaline stimulate cAMP production and they both cause an increase in the phosphorylation of the T1 peptide which is phosphorylated by cAMP-PK in vitro. The T1 peptide contains serine 77 and 79. However, sequencing of the relevant ACC phosphopeptides showed that all the phosphate was always on serine 79, the AMP-PK site. (Sim and Hardie 1988) The role of cAMP-PK in vivo, if any, must therefore be permissive. It has been proposed (Hardie 1989) that glucagon and ß adrenergic agonists cause the activation of cAMP-PK which then inhibits ACC phosphatase thus allowing the apparent activity of AMP-PK to increase which is observed as an increase in the phosphate on serine-79. However, more recently protein phosphatase 2A has been identified as the physiological ACC phosphatase and as yet there is no evidence for regulation of this phosphatase by cAMP-PK (Clark et al 1991)

The effect of insulin treatment on ACC in isolated adipose cells is paradoxical. ACC activity is increased

but the enzyme is also more phosphorylated. This was first observed by Brownsey and Denton (1982) who reported the increased phosphate in a specific tryptic peptide (the 'I site') and has been confirmed by several others (eg Witters et al 1983, Holland and Hardie 1985, Haystead and Hardie 1988). The function of this phosphorylation is unknown as the activation of ACC observed in extracts from insulin treated cells (Halestrap and Denton 1973) does not survive purification by avidin-Sepharose affinity chromatography although the phosphorylation is preserved. Furthermore dephosphorylation of ACC in crude extracts from insulin-treated cells with exogenously added protein phosphatase 2A did not reverse the activation of ACC (Haystead and Hardie 1986). Quantitation of sites modified in the presence of insulin reveal that the I site described by Brownsey and Denton (1982) is minor, the primary effect of insulin is the phosphorylation of serine-29, the same site phosphorylated by casein-kinase II (Haystead et al 1988). Phosphorylation at this site has no effect on ACC activity. It has been proposed that the phosphorylation of serine-29 enhances the dephosphorylation of sites affecting the activity of ACC (Sommercorn et al 1987). This could therefore be a mechanism for the increase of ACC activity. Witters et al (1988) reported that a 30 minute exposure of Fao hepatoma cells to insulin produced an activation of ACC as expected but there was also dephosphorylation of the enzyme. These workers suggest that insulin acts by inhibiting ACC kinases and suggest that their use of digitonin lysis of the cells allowed more rapid 'quenching' of cell metabolism thus allowing an insulin stimulated dephosphorylation to be observed for the first time.

Several authors have proposed that the rapid effects of insulin on ACC are mediated by a small molecular weight molecule because they do not survive purification (Haystead and Hardie 1986, Saltiel et al 1983, Witters et al 1988). The latter two reports have identified the molecule as an oligosaccharide. The mechanism by which the effector functions, and its physiological role are not clear

however. It may simply be an insulinomimetic substance rather than a real mediator of insulin action.

1.5 cAMP-DEPENDENT PROTEIN KINASE

cAMP dependent protein kinase [EC2.7.1.37] (cAMP-PK) is a ubiquitous eukaryotic enzyme and is the primary if not the only intracellular receptor for the secondary messenger cAMP. The kinase exists as an inactive tetramer consisting of two regulatory subunits and two catalytic subunits (R_2C_2) (Carlson et al 1979). The holoenzyme is activated by elevated levels of intracellular cAMP. The cyclic nucleotide binds to the regulatory subunits and decreases their affinity for the catalytic subunits by approximately four orders of magnitude. The latter dissociate and are thus activated.

$$\underset{\text{inactive}}{R_2C_2} \xrightarrow{cAMP} R_2\,(cAMP)_4 + \underset{\text{active}}{2\ C}$$

The Km for activation by cAMP is approximately 2×10^{-8} M (Corbin et al 1975).

In most tissues cAMP-PK occurs in two major isoforms as judged by salt gradient elution from anion exchange columns (Corbin et al 1975). The isoforms are termed type I and type II (Corbin et al 1975; Flockhart et al 1982) according to their order of elution from DEAE - cellulose and differ in the structure of the regulatory subunit.

The R subunit of the type I enzyme has a molecular weight of 49,000 as judged by SDS polyacrylamide gel electrophoresis. Two forms of the type II R subunit have been reported: a 54,000 M_r species found in most tissues (Takio et al 1984) and a 51,000 M_r species found in brain, adrenal tissue and granulosa cells (Weldon et al 1985; Jahnsen et al 1985). Type II regulatory subunit readily undergoes autophosphorylation in the holoenzyme form. Two moles of phosphate per mole of RII subunit can be incorporated by the catalytic subunit. Phosphorylated RII

subunit has a lower rate of reassociation with the catalytic subunit (Rosen et al 1975; Rangel-Aldao and Rosen 1976, 1977). The physiological significance of this is unclear however. The type I isoenzyme has a high affinity binding site for MgATP which is distinct from the substrate site (Hoffman et al 1975). Ringheim and Taylor (1990) have shown that the binding of MgATP there prevents dissociation of the holoenzyme at physiological salt concentrations (see also Corbin et al 1975). Without MgATP, significant activation could occur in the absence of cAMP due to salt induced dissociation of the complex. Ringheim and Taylor also suggest that MgATP accelerates the reassociation of the holoenzyme thus facilitating termination of the activation process when cAMP is removed.

It has been generally assumed that C subunits from different tissues and different holoenzymes are identical (Bechtel et al 1977; Krebs and Beavo 1979; Flockhart and Corbin 1982; Nairn et al 1985) largely because C-subunits from all sources studied have had the same molecular weight around 40,000 (Carlson et al 1979) and similar tryptic peptide maps (Zoller et al 1979). However two forms of the C subunit termed C_A and C_B have been separated chromatographically using cation exchange (Kinzel et al 1987), and two genes coding for the C subunit have been identified by Uhler et al (1986 a,b) and have been designated Cα and Cß. Both are found in all tissues but Cß is particularly abundant in brain tissue. Other evidence for heterogeneity of the catalytic subunit has also been published by Peters et al (1977) where three isoforms were identified according to their isoelectric points and more recently by Van-Patten et al (1986,1988) where the formation of two major and one minor complex between the C-subunit and its specific inhibitor has been attributed to multiple forms of the catalytic subunit. The latter workers have correlated the two major complexes with the two forms of the C-subunit described by Kinzel et al (1987). A third form of C-subunit found specifically in testicular tissue has been isolated. This isoform has

been termed Cγ, (Beebe et al 1990).

Different physiological roles for the isoenzymes type I and II have not been firmly established, however evidence for selective hormonal activation has been published by Livesey et al (1982, 1984) where the hormone calcitonin was shown to selectively activate isoenzyme type II in two strains of human breast cancer cells. It has also been reported that prostaglandin E_2 stimulated isoenzyme type I in malignant rat osteoblasts but in contrast stimulated isoenzyme type II in normal osteoblasts (Livesey et al 1982). Other workers report specific down regulation of the amount of rat liver type I isoenzyme in response to starvation or a low protein diet, (Chan et al 1979; Ekanger et al 1988) others however find no change (McClung and Kletzien 1981). No significant differences in the activity of the C-subunit isoforms α and ß has been reported. They have a 90% sequence homology and bind to either type I or II regulatory subunits. They also have similar kinetic properties (Kinzel et al 1987; Olsen and Uhler 1989). Cγ however has only 83% amino acid homology with Cα and 79% homology with Cß. This together with its tissue specific expression suggests that Cγ may be functionally distinct from the other C-subunits characterised thus far.

As mentioned earlier (Section 1.3) mammary gland is different from liver and adipose tissue in that it does not respond to ß-adrenergic stimulation with a decrease in lipogenesis (Williamson et al 1983; Robson et al 1984; Clegg and Mullaney 1985). The increased intracellular levels of cAMP do not correlate with increased phosphorylation of ACC (Clegg et al 1986, 1987) thus questioning the role of cAMP in the lactating mammary gland.

The reasons for the refractory nature of mammary gland to adrenergic stimulation is unknown. It is possible that the lactating mammary gland has, like the testis mentioned

above, an unusual form of cAMP-PK. It is also possible that the unusual environment of the mammary gland confers specific properties upon the cAMP within it. Gabbay and Lardy (1987) have reported that in rat hepatocytes insulin can decrease the affinity of cAMP-PK for cAMP, so the Ka is increased but the Vmax remains unchanged. They suggest that insulin exerts these effects by decreasing the binding of cAMP to the site 2 cyclic nucleotide binding site on the regulatory subunit. It is possible that similar regulatory mechanisms occur in the mammary gland considering the high insulin sensitivity of the tissue (Burnol et al 1986). Compartmentalization of the cAMP-PK has been proposed as a possible regulatory mechanism for cAMP-PK (Dreyfuss et al 1978). In human erythrocytes isoenzyme type I is predominantly membrane bound while type II is cytoplasmic. It is proposed that membrane bound R subunits can affect the substrates phosphorylated by the C-subunits by positioning the holoenzyme in closer proximity to certain substrates. However these authors find no difference in the catalytic properties of the cytosolic and membrane bound cAMP-PK so such compartmentalization may not confer significant regulatory advantage in the mammary gland. It is possible that the major role of cAMP-PK during lactation is the regulation of the transcription and/or translation of genes as occurs in the liver (Quinn and Granner 1990).

1.6 AIMS OF THE PRESENT WORK

The aim of the present work was to gain a better understanding of the molecular mechanisms controlling the synthesis of fat in the lactating mammary gland with particular reference to the regulation of acetyl-CoA carboxylase by phosphorylation/dephosphorylation. To this end the protein kinases that phosphorylate and inactivate ACC were investigated. The effects of fasting on mammary metabolism as well as the role of some putative regulators of fatty acid synthesis were studied.

CHAPTER 2

MATERIALS AND METHODS

2.1 ANIMALS

Wistar rats were fed a standard chow diet (Special Diets Services Ltd, Kent, UK) and water ad libitum unless otherwise indicated. The animals were kept in windowless rooms, lit 08.00h to 20.00h; dark 20.00h to 08.00h. The female rats were mated at 130-150g body weight and used at peak lactation (10-15 days post partum). Animals with 8-12 pups were used.

Experimental procedures were started between 09.30h and 10.00h and unless otherwise stated the rat was anaesthetized with a non-recovery dose of pentobarbital (60mg/kg body weight) administered intraperitoneally. Dissection was commenced once deep anaesthesia was attained. Mammary tissue for freeze clamping was quickly but carefully freed from the skin and abdominal wall without cutting the blood vessels. The gland was thus sampled with the circulation intact.

2.2 CHEMICALS

2.2.1 Biochemicals

All reagents were of analytical grade or purer. Enzymes, co-enzymes, NAD^+, NADH and fatty acid-free bovine serum albumin (BSA) were obtained from Boehringer Mannheim (BCL), Lewes, Sussex. p-(p-aminophenylazo)-benzene sulphonic acid (AABS) was purchased from Pfaltz and Bauer, (USA). Other reagents were obtained from BDH Chemicals, Poole, Dorset,UK., Sigma Chemical Company Ltd., Poole, Dorset, UK. or Aldrich Fine Chemicals, Gillingham, Dorset, UK. Where the supplier and type of product is critical the product number will be given.

2.2.2 Radiochemicals

[γ -^{32}P] ATP, [^{14}C]-bicarbonate, and tritiated water were both obtained from ICN Radiochemicals, California, USA or Amersham International, Bucks, UK. [^{14}C]-Fluorosulphonyl-benzoyl adenosine ([^{14}C]-FSBA) was from New England Nuclear, Boston, USA.

2.2.3 Pharmacological Chemicals

Sodium pentobarbitol and (-)isoprenaline were obtained from Sigma Chemicals, Poole, Dorset, UK. Insulin (pig) was from Boots Co., Nottingham, UK.

2.2.4 Peptide Substrates and Inhibitors

cAMP-dependent protein kinase substrate Kemptide (K1127) and protein kinase inhibitor both native protein (P8140) and synthetic peptide (P3294) were obtained from Sigma Chemical Co. Poole, Dorset, UK.

Peptide substrates based on ACC phosphorylation sites and specific for AMP activated kinase and cAMP-dependent protein kinase were a generous gift from Dr D. Carling.

2.2.5 Protease Inhibitors

The following protease inhibitors were used routinely for all protein purifications.

Trypsin and Trypsin like proteases:

- Benzamidine (BZ)
- Soyabean trypsin inhibitor (SBTI)
- Nα-p-tosyl-L-lysine chloromethyl ketone (TLCK)

Chymotrypsin:

- N-tosyl-L-Phenylalanine chloromethyl ketone (TPCK)

Serine Proteases: (eg: elastase, trypsin, chymotrypsin)

- Phenylmethylsulphonyl Fluoride (PMSF)

Metalloproteinases:

- EDTA and/or EGTA

The protease inhibitors were added to buffers as indicated.

2.3 PREPARATION OF AFFINITY CHROMATOGRAPHY MEDIA

Buffers required for cyanogen bromide activation of Sepharose and coupling of ligand to gel are listed below.

Coupling Buffer

10 mM-Na phosphate buffer - prepared by mixing appropriate quantities of the mono and dihydrogen sodium salt to give pH 7.0 at 25°C.

Blocking Buffer

1 mM-Ethanolamine adjusted to pH 7.0 with HCl.

Monomerisation Buffer

3 M-Guanidine in 0.2 M-KCl adjusted to pH 1.5 with 0.2 M-HCl.

Column Buffer

20 mM-Tris/HCl pH 7.5 at 4°c
0.5 M-NaCl
1 mM-EDTA
5 mM-$NaPP_i$
50 mM-NaF
10% (w/v)-glycerol
0.02% (w/v)-NaN_3

Glycine Buffer

0.1 M-Glycine adjusted at 25°C to pH 2.0 with HCl.

METHOD

Approximately 50 ml of Sepharose CL-4B-200 gel was washed with one litre of water under suction. The gel was resuspended in 550 ml 5 mM-phosphate buffer (COUPLING BUFFER diluted 1:1 with water), and the pH adjusted to pH 11.0 with 2 M-NaOH.

A fume cupboard was used for the following operations involving cyanogen bromide.

The gel suspension was then mixed with 15 g of finely ground cyanogen bromide in a large beaker. The reaction started immediately with the evolution of heat and protons. The temperature was maintained at 2.0°C by adding chipped ice, and the pH maintained at 11.0 by the dropwise addition of 2 M-NaOH. After approximately twenty minutes the mixture was filtered over ice with suction, then washed with 700 ml COUPLING BUFFER with suction. The gel was resuspended in 125 ml COUPLING BUFFER containing 0.4 mg/ml avidin or 5 mg/ml histone IIAS, (Sigma Chemicals).

The gel suspension was transferred to a screw top jar and mixed overnight (15 hours at 4°C) on an 'end-over-end' mixer to maximise coupling of ligand to the activated Sepharose. The coupled gel was filtered under suction and resuspended in 200 ml BLOCKING BUFFER for two to three hours at room temperature. This blocked uncoupled cyanogen bromide bridges.

2.3.1 For Avidin-Sepharose only

The gel was suctioned free of blocking buffer and rinsed with MONOMERISATION BUFFER and the gel was resuspended in 200 ml monomerisation buffer and left at room temperature overnight. This step monomerises the coupled avidin and so decreases its binding capacity for acetyl CoA carboxylase.

The gel was poured into a column and washed through with guanidine/HCl buffer. The column was washed with five volumes of COLUMN BUFFER until the A_{280} against column buffer was ≤ 0.05. The column was washed with five column volumes of 0.8 mM-biotin in COLUMN BUFFER and prepared for use by washing with three volumes of COLUMN BUFFER followed by three volumes of GLYCINE BUFFER. This was repeated twice more and the column finally equilibrated with column buffer. This treatment ensured that high affinity avidin sites which are capable of irreversibly binding ACC were blocked as the glycine buffer does not remove biotin from them.

2.3.2 Histone - Sepharose

The coupled gel was suctioned free of coupling buffer then resuspended in 200 ml AMP-PK buffer (see section 4.1) with 0.2% (w/v)-NaN_3 and stored at 4°C. Before use the gel was packed into a column and washed with 5 column volumes of 1M-NaCl in 100 mM-Tris/HCl pH 7.0 followed by equilibration in AMP-PK buffer.

2.4 TREATMENT OF FATTY ACID FREE BOVINE SERUM PRIOR TO USE IN BIOCHEMICAL ASSAYS

Fatty acid free BSA (BCL 774111) was dissolved in 0.9% (w/v) KCl at 0.5g per ml then dialysed over 24 hours at 4°C, first against 10 volumes of 0.9% KCl with two changes followed by dialysis against 10 volumes of water with three changes. The solution was then lyophilised and stored at

4°C. This procedure ensured the removal of low molecular weight contaminants (eg citrate).

2.5 PREPARATION OF ALBUMIN BOUND FATTY ACID FOR MAMMARY GLAND PERFUSIONS AND ACINI INCUBATIONS.

(from Evans and Mueller, 1963)

The following procedure was conducted at 60°C using a 50 ml round bottom flask. 80 μmoles of palmitic or oleic acid were heated to 60°C in a water bath and a minimum volume of absolute ethanol added to dissolve the fatty acid. 85 μmoles of solid K_2CO_3 was added followed by 1.0 ml of water heated to 60°C. The ethanol was blown off using a stream of nitrogen. The fatty acid potassium salt was mixed with 8 ml of 17% BSA at 50°C. The albumin-bound fatty acid solution was made up to 10 ml with water and stored in 2.5 ml aliquots at -20°C.

2.6 PURIFICATION OF ACETYL-CoA CARBOXYLASE (ACC) (after Tipper and Witters 1982)

Buffered solutions

ACC HOMOGENISATION BUFFER

0.25 M-sucrose
2 mM-EDTA
50 mM-NaF
72 mM-Tris/HCl pH 8.5 at 4°C
5 mM-NaPPi
0.1% (v/v)-2-Mercaptoethanol
or 2 mM-DTT

When dephosphorylated protein was required the phosphatase inhibitors NaF and NaPPi were omitted.

Protease Inhibitor cocktail for Homogenisation

2 mM-BZ
8 μg/ml-SBTI
0.05 mM-PMSF
0.1 mM-TPCK
0.2 mM-TLCK

AVIDIN SEPHAROSE BUFFER
(prepared as large stock, 5l)
20 mM-Tris/HCl pH 7.4 at 4°C
0.5 M-NaCl
1 mM-EDTA
5 mM-NaPPi
50 mM-NaF
0.2% (w/v)-NaN_3
10% (w/v)-glycerol
just prior to use 2 mM-DTT or 0.1% (v/v)-2-Mercaptoethanol was added.

Protease Inhibitor cocktail for Chromatography of ACC
2 mM-BZ
8 μg/ml-SBTI
0.2 mM-TLCK

2.6.1 PREPARATIVE PURIFICATION OF ACETYL-CoA CARBOXYLASE (ACC)

Female Wistar Rats at peak lactation (10 - 15 days post partum) were killed by stunning and cervical dislocation or anaesthetized by intraperitoneal injection of sodium pentobarbital at 60 mg/kg body weight. The inguinal and thoracic mammary glands were dissected out, weighed and placed in ice cold homogenisation buffer. The tissue was rinsed in the same buffer and then chopped finely using scissors.

All subsequent procedures were performed at 4°C. The chopped mammary gland was homogenised in homogenisation

buffer (50ml buffer per 10g tissue) using a Philips domestic blender at full speed in three thirty second bursts. The homogenate was transferred to 250 ml centrifuge tubes and centrifuged at 30,000g for 20 minutes.

The supernatant was filtered through glass wool to remove any floating fat, its volume measured, and then taken to 35% saturated ammonium sulphate by the gradual addition with stirring of either a neutral solution of 90% saturated ammonium sulphate (prepared at room temperature; 100% saturation taken to be 4.1 M) or the solid ammonium sulphate. When the solid ammonium sulphate was used the pH of the homogenate was checked after addition to ensure it was not below pH 8.0, and adjusted with 1 M-NaOH if necessary. The ammonium sulphate solution was stirred for twenty minutes then transferred to centrifuge tubes and centrifuged at 30,000g for 20 minutes.
The supernatant was discarded and the pellet frozen at -20°C if not to be used immediately.

The ammonium sulphate pellet was resuspended in a minimum volume of avidin Sepharose buffer containing protease inhibitors (2 mM-BZ; 4 μg/ml-SBTI; 0.2 mM-PMSF; 0.2 mM-TLCK and 2 mM-DTT or 15 mM-2-mercaptoethanol). This was typically one fifth of the volume of the initial supernatant. The resuspension was clarified by centrifuging at 30,000g for 20 minutes at 4°C followed by filtering through glass wool.

The solubilized pellet was applied to a 50 ml (6 cm x 1.6 cm) avidin Sepharose affinity column previously equilibrated with avidin Sepharose buffer and washed with the avidin Sepharose buffer until no protein was detectable at 280 nm. Acetyl-CoA carboxylase was eluted with 0.5 mg/ml d-biotin in avidin Sepharose buffer and 5.0 ml fractions were collected. The high protein fractions as measured by A_{280} absorbance or Bradford protein assay were pooled and concentrated by ultrafiltration using a YN membrane with a 100,000 Da cut off (Amicon Ltd) or the

addition of a neutral 90% saturated ammonium sulphate solution to a final fractional saturation of 50%.

After concentration the ACC was dialysed into 'ACC storage buffer' (50 mM-Hepes/NaOH pH 7.0 at 4°C, 1.0 mM-EDTA, 1.0 mM-EGTA, 50 mM-NaF, 50 mM-NaCl, 10% (w/v) glycerol, 2 mM-DTT) and stored frozen at -20°C.

2.6.2 ANALYTICAL PURIFICATION OF ACETYL-CoA CARBOXYLASE (ACC)

Mammary gland was freeze clamped and the tissue pulverized under liquid nitrogen. 1.0-1.2 g of the tissue powder was homogenised in 10 volumes of ACC HOMOGENISATION BUFFER using a hand held glass/teflon homogenizer. The homogenate was centrifuged at 50,000 g for 20 min at 4°C. The supernatant was filtered through glass wool and then taken to 40% saturated ammonium sulphate by the gradual addition of the solid salt, (0.243 g/ml). The suspension was kept on ice for 5 min then centrifuged at 4°C for 10 min at 50,000 g. The supernatant was discarded and the pellet snap frozen in liquid nitrogen before storage at -70°C if not to be used immediately.

The ammonium sulphate pellet was resuspended in 5 ml of avidin Sepharose buffer. The resuspension was applied directly to a 2 ml avidin Sepharose column (2.5 cm x 0.5 cm) previously equilibrated with avidin Sepharose buffer, glycerol and sodium azide are excluded to avoid any inhibition of ACC activity. The column was washed with 40 ml of the same buffer then the protein eluted with 2 mM-biotin in glycerol and sodium azide free avidin Sepharose buffer. 0.5 ml fractions were collected and the fraction containing the highest protein concentration (usually 60 - 100 μg/ml) as assessed by the Coomassie blue protein assay (section 2.20) was taken for immediate assay of ACC activity (Section 2.14.2). The fraction was diluted to 15 μg/ml of ACC using 100 mM-Tris/HCl, pH 7.4 at 37°C and containing 1% BSA as at this ACC concentration the assay

was linear with respect to time and protein added.

2.7 PURIFICATION OF ARYLAMINE ACETYLTRANSFERASE (for PDH assay) (from Chou and Lipman 1952)

Arylamine acetyltransferase (AAT, EC 2.3.1.5) was prepared from pigeon liver acetone powder (Sigma). 10 g of acetone powder was homogenised for three minutes in ten volumes of ice cold water using a Polytron homogeniser fitted with a PT10 probe set at position six. The homogenate was centrifuged at 290,000 g for fifteen minutes at 4°C. The supernatant was cooled to -10°C by packing the beaker in an NH_4Cl salt-ice mixture. 0.66 volumes of ice cold acetone was added dropwise with slow but continuous stirring. The mixture was centrifuged at -10°C and 26,000g for fifteen minutes. AAT activity was recovered from the supernatant by three sequential precipitations with 25 ml of ice cold acetone added dropwise. After each addition the mixture was centrifuged as above and the pellet retained. 50 ml of acetone was added as a final step. The pellets from each precipitation were resuspended in approximately 0.5 ml of 10 mM-potassium phosphate buffer (pH 7.0) then assayed for AAT activity. Fractions containing less than 5 units per ml were discarded. The enzyme was stored at -20°C in 30 µl aliquots and diluted prior to use with 10 mM-phosphate buffer (pH 7.0) to give 5 units per ml. (Units defined below)

2.7.1 Assay of Arylamine Acetyltransferase

AAT was assayed spectrophotometrically at 460 nm by monitoring the acetylation of p-(p-aminophenylazo)-benzene sulphonic acid (AABS) by acetyl-CoA. (Jacobson 1961). The assay was conducted at 30°C in a volume of 0.75 ml containing 100 mM-Tris HCl pH 7.8 , 1 mM-$MgCl_2$, 0.5 mM-EDTA, with 5 mM-2-mercaptoethanol, 15 µg-AABS and 10 mM-acetyl CoA and initiated by the addition of 5 µl of the AAT resuspension. 1.0 unit of activity is defined as the conversion of 1 µmol of substrate to product per minute at

30°C.

2.8 PREPARATIVE SEPARATION OF cAMP-PK HOLOENZYMES TYPE I AND II FROM RAT LIVER

Buffer A for homogenisation and chromatography

10 mM-Tris/HCl pH 7.4 at 4°C
1 mM-EDTA
15 mM-2-mercaptoethanol

Protease Inhibitor cocktail

1 mM-BZ
0.1 mM-PMSF
4 μg/ml-SBTI

Method

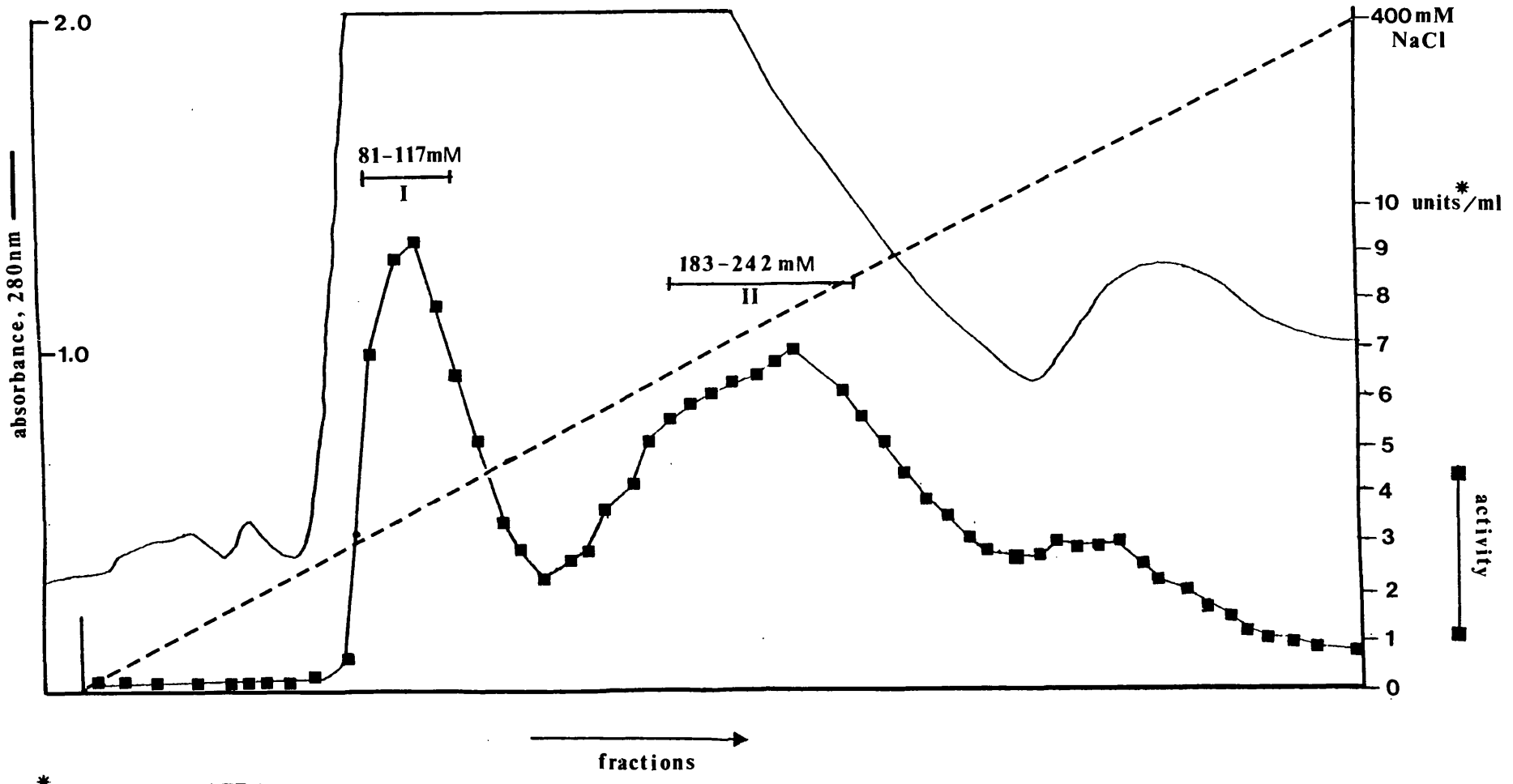
4 male rats (body wt 160-180 g) were killed by stunning and cervical dislocation. Their livers were rapidly excised and chopped finely with scissors in ice cold buffer A. The tissue (approx. 50 g) was rinsed with buffer A through a wide mesh nylon strainer and then homogenised in two batches in 10 volumes (v/w) of fresh buffer A for 45 seconds using a Polytron fitted with a PT10 probe set at number 6. The homogenate was centrifuged at 26,000g for 30 minutes. The supernatant was decanted through glass wool and applied directly to a DE-52 cellulose (Whatman) column (23 x 2 cm) previously equilibrated with buffer A. The column was washed until the A_{280} versus buffer was zero. The kinase activity was eluted with a NaCl gradient in buffer A (300 ml) from 0 to 0.4 M flowing at 1.6 ml/min. Two peaks of kinase activity were resolved, eluting at approximately 100 mM and 200 mM-NaCl respectively. These were pooled as indicated in Fig. 2.1 and dialysed overnight at 4°C versus twenty-five volumes of buffer A.

Figure 2.1 SEPARATION OF cAMP-PK ISOENZYMES I AND II FROM RAT LIVER BY ANION EXCHANGE

Experimental details are described in text. The DE-52 cellulose column was developed using a linear gradient of 0-400 mM-NaCl in 300 ml of buffer. 5ml fractions were collected at 1.6 ml/min. cAMP-PK activity was assayed as described in Materials and Methods in the presence of cAMP using histone-IIA as a substrate (see section 2.13).

SEPARATION OF cAMP-PK ISOENZYMES I AND II FROM RAT LIVER BY ANION EXCHANGE

Figure 2.1



*(unit= 1nmol ATP/min)

The dialysed fractions were then applied to two small (18 ml bed volume) DEAE-52 columns pre-equilibrated with buffer A in order to concentrate them. After washing the columns with buffer A the protein was eluted batchwise with 150 mM-NaCl in buffer A for isoenzyme type I and 400 mM-NaCl in buffer A for isoenzyme type II. The peaks of activity were pooled separately and dialysed for five hours into one litre of buffer A prepared with 50% (w/v) glycerol in order to concentrate them further and also to stabilise the protein. Each isoenzyme was aliquoted into 1.0 ml fractions and stored at -20°C.

2.9 ANALYTICAL SEPARATION OF cAMP-PK HOLOENZYMES TYPE I AND TYPE II FROM LACTATING RAT MAMMARY GLAND (From Livesey 1982)

Buffer B for homogenisation and chromatography

10 mM-MES/NaOH pH 6.8 at 4°C
0.2 mM-EDTA
0.1 mM-DTT

Protease Inhibitor cocktail

1 mM-BZ
0.1 mM-PMSF
4 μg/ml-SBTI

For homogenisation only

0.5 mM-3-isobutyl-1-methylxanthine (IBMX)

Method

Freeze clamped inguinal mammary glands from lactating female rats (see section 2.1) were pulverised under liquid nitrogen using a pestle and mortar. 0.3 to 0.5 g of tissue powder was placed in ice cold, preweighed tubes and the final weight noted. After weighing, these

fractions were immediately capped and stored in liquid nitrogen or -70°C until required.

All the following procedures were performed on ice or at 4°C. Ten volumes of buffer B plus 0.5 mM-IBMX and protease inhibitors were added directly to the frozen powder and homogenised for ten seconds using a Polytron fitted with a PT10 probe and set on number four (18-20,000 rpm). 500 μl of the tissue extract was taken and frozen at -20°C for later determination of DNA and protein. The rest of the homogenate was centrifuged at 20,000 g at 4°C for 20 minutes.

The supernatant was filtered through glass wool, diluted 1/10 with buffer B and then 1 ml applied to a 1 ml DE-52 column previously equilibrated with buffer B. This was washed through with 10 ml of buffer B. Isoenzyme I was eluted with 5 ml of 150 mM-NaCl in buffer B, followed by 5 ml of 400 mM-NaCl in buffer B to elute isoenzyme II.

The eluates and washings were assayed for CAMP-PK activity without further dilution as previous experiments had shown that activity was linear in the conditions used.

Batch elution of the isoenzymes was used for speed of separation. Gradient elution from DEAE cellulose (Whatman) showed that 150-mM NaCl and 400 mM-NaCl were required to separate the two isoenzymes in mammary gland. (Fig. 3.2).

2.10 ASSAY FOR 5' AMP-ACTIVATED PROTEIN KINASE (AMP-PK) ACTIVITY

AMP-PK activity was assayed at 30°C in a volume of 25 μl containing 20 mM-Tris HCl (pH 6.8), 0.5 mM-EDTA, o.5 mM-EGTA, 5 mM-DTT and 10% (w/v)-glycerol with $MgCl_2$ and [γ-^{32}P] ATP (specific activity 0.1 - 0.2 μCi/nmol) added to a final concentration of 4 mM and 0.2 mM respectively. 5' AMP (0-4 mM) was added to some experiments to show activation but

was not used routinely.

For many experiments purified ACC (0.6 mg/ml) or Histone type IIS (1.0 mg/ml) were used as substrate. For some experiments a specific peptide substrate (see section 2.2.4) was employed at a concentration of 200 μM. Experiments were initiated by the addition of ATP/Mg. When histone or peptide were used as substrate the assay was stopped by pipetting 20 μl of the reaction mixture onto squares of phosphocellulose paper (P81 Whatman) which were immediately dropped into a stirred beaker of 75 mM-orthophosphoric acid. The squares were washed in three changes of acid solution before counting on a phosphorus -32 programme in aqueous compatible scintillant. Some assays were stopped by the addition of 40 mM-EDTA to a final concentration of 20 mM to chelate the magnesium ions. The solution was then pippetted onto phosphocellulose paper, washed and counted as described above. For ACC the assays were stopped with 1 ml of 25% trichloroacetic acid (TCA) and then 30 μl of 1% BSA added to assist precipitation of substrate protein. The assay tubes were centrifuged at 5,000 g for 5 min, the supernatant removed and the pellet washed three times with 1 ml of 25% TCA. The pellet was then counted by Cerenkov counting.

Assays were performed with quantities of kinase which produced linear rates of phosphorylation with respect to both time and protein added.

2.11 ASSAY OF AMP-KINASE KINASE.

This activity could only be measured indirectly by observing the increased activity of AMP-PK after it had been incubated in phosphorylating conditions.

AMP-PK purified to the DEAE stage was incubated at 30°C in AMP-PK buffer (see section 4.1) containing 0.2 mM-ATP, 4 mM-$MgCl_2$ and 2 units/μl of cAMP-PK inhibitor. After incubation the reaction was stopped by the addition of 50

mM-EDTA to a final concentration of 12.5 mM. These 'pre-incubations' were passed down 1 ml desalting columns (prepared as described in the following section) to remove unreacted ATP/Mg and the excess EDTA. The protein was then assayed for AMP-PK kinase activity as described in section 2.10.

This technique was used to reduce the problem of the very high reaction blanks that occurred if preincubation was performed with radioactive ATP followed by the direct addition of exogenous substrate.

2.12 'Spun-column' chromatography
(Maniatis et al 1982)

A 1 ml disposable syringe was plugged with siliconised glass wool and filled with Sephadex G-25-150, equilibrated in AMP-PK buffer. The syringe was placed in a polypropylene centrifuge tube and centrifuged at 150 g for 3 minutes. More Sephadex was added and the process repeated until the packed bed volume was 1.0 ml. Before use 2 x 60 μl of a 5 mg/ml solution of BSA was spun through the columns before re-equilibration in pyrophosphate free AMP-PK buffer. This procedure minimized non specific protein binding to the Sephadex.

The reaction mixture was applied to the column in a volume of 60 μl. The cup of a microcentrifuge tube was placed in bottom of the centrifuge tube under the end of the syringe. Centrifugation was performed as before; the unincorporated ATP/Mg remained in the syringe and the phosphorylated protein was collected in the tube.

2.13 ASSAY OF cAMP-DEPENDENT PROTEIN KINASE (cAMP-PK)

(from Roskoski 1983)

cAMP-PK activity was assayed at 30°C with Kemptide (130 μM) as a substrate in a volume of 60 μl containing 13 mM - MOPS KOH, pH 7.0, 10 mM-Mg Acetate, 0.07 mM-[γ^{32}P] ATP (Specific activity 0.2 μCi/nmol) and 2.5 mM-DTT, 0.7 μM-cAMP or 0.6 mg/ml specific protein inhibitor of cAMP-PK (Sigma P8140) were included as necessary to determine 'total' cAMP-PK activity and non cAMP-dependent kinase activity, respectively. The reaction was initiated by the addition of 10 μl of sample (diluted as necessary to ensure linearity over the assay time) and terminated by pipetting 30 μl onto phosphocellulose squares (Whatman P81) and immersing these in 75 mM-orthophosphoric acid. The squares were washed three times in 75 mM-orthophosphoric acid transferred to vials and counted using a phosphorus-32 programme in an aqueous compatible scintillant.

The assay was performed in duplicate as follows:

1	2	3
± Kemptide	± Kemptide	± Kemptide
- cAMP	+ cAMP	- cAMP
- Kinase inhibitor	- Kinase inhibitor	+ Kinase inhibitor

In all groups phosphorylation in the absence of Kemptide was subtracted as this represents phosphorylation of endogenous substrates. cGMP dependent kinase can be activated by cAMP, however activity that was cAMP activated but kinase inhibitor resistant was not seen in mammary tissue under the conditions used therefore the control assay: ± Kemptide/+cAMP/+kinase inhibitor was not routinely performed.

Group 1 - measures 'basal' kinase activity due to free catalytic subunit in the sample.
Group 2 - measures 'total' kinase activity due to dissociation of holoenzyme by cAMP.
Group 3 - measures cAMP independent kinase activity.

Thus 1 - 3 = basal cAMP dependent kinase activity.

2 - 3 = total cAMP dependent kinase activity.

'Total' activity was measured at 0.7 μM cAMP as this concentration was found to produce maximal stimulation. See Fig. 3.1 in Chapter 3.

2.13.1 ACTIVITY RATIO OF cAMP DEPENDENT-PROTEIN KINASE

The activity ratio of cAMP-PK is defined as

$$\frac{\text{BASAL activity}}{\text{TOTAL activity}}$$

and was first introduced by Corbin (1983).

The activity ratio of cAMP-PK was adopted as a method of determining changes in cellular cAMP levels because it is extremely difficult to measure small changes of intracellular cAMP in mammary gland due to the relatively high concentration of the nucleotides in milk, approximately 2.5 μM (Sapag-Hagar and Greenbaum, 1974).

2.14 ASSAY OF ACETYL-CoA CARBOXYLASE (ACC)

Principle of Assay

ACC (EC 6.4.1.3) catalyses the synthesis of malonyl-CoA from acetyl-CoA and bicarbonate. The overall reaction can be summarised by the following half reactions.

1) $ATP{:}Mg^{2+} + HCO_3^- + \text{E-biotin} \rightarrow \text{E-biotin-}CO_2 + ADP + Pi$

2) $\text{E-biotin-}CO_2^- + \text{acetyl-CoA} \rightarrow \text{malonyl-CoA} + \text{E-biotin}$

The activity of ACC is quantified by measuring the incorporation of ^{14}C-bicarbonate into malonyl-CoA in usual practice this is determined as acid stable ^{14}C. In mammary gland, fixed ^{14}C not derived from the ACC catalysed reaction is negligible.

2.14.1 Assay of ACC in Crude Homogenates

Approximately 300 mg of powdered freeze clamped mammary gland was homogenised using a Polytron PT10 probe in Tris-MOPS buffer (20 mM-Tris, 20 mM-MOPS, 0.25 M-sucrose, 2 mM-EGTA, 1 mM-DTT, pH 7.4 at 4°C) in the ratio 1:4 w/v. After homogenisation BSA was added to give a concentration of 10 mg/ml. 100 μl of homogenate was taken for DNA measurement. The rest was centrifuged for one minute at 8,500g in a benchtop Eppendorf centrifuge. The supernatant was diluted 2/5 with Tris-MOPS buffer before assay for enzyme activity.

The reaction mixture consisted of 125 mM-Tris Acetate, pH 7.4 at 30°C with 0.625 mM-EDTA, 12.5 mM-Mg Acetate, 7.5 mM-ATP, 0.15 mM-acetyl-CoA, 1 mM-DTT, 1% BSA, 15 mM-$KH^{14}CO_3$ with specific activity 75 μCi/ml and where stated 20 mM citrate/magnesium in a volume of 200 μl. This was warmed to 30°C for approximately three minutes, 50 μl of the supernatant was then added to start the reaction. The assay was stopped after 30 seconds by the addition of 100 μl of 6 M hydrochloric acid. 300 μl was transferred to glass vials and dried under a stream of heated air (approximately 70°C). The residue was dissolved in 250 μl of water and the radioactivity counted for ten minutes per vial in 10 ml of aqueous compatible scintillant.

Expression of Results

"Initial" activity was measured as described at 0 mM-citrate/magnesium or 20 mM-citrate/magnesium.

"Total" activity was measured at 20 mM-citrate/magnesium by allowing the ACC to be dephosphorylated by endogenous phosphatases. This was achieved by incubating the sample for twenty minutes in the reaction mixture at 30°C in the absence of acetyl CoA, ATP or bicarbonate. These three were then added, the reaction timed from the addition of bicarbonate and stopped after

thirty seconds. All results were expressed as μmol of carbon-14 incorporated into acid stable product per min per μg of DNA.

2.14.2 Assay of Purified ACC
(Holland et al 1984)

ACC purified by avidin affinity chromatography was diluted to between 15 and 20 μg/ml with 0.5 M-Tris HCl (pH 7.4 at 37°C) containing 1 mM-DTT and 2% BSA. Activity of ACC was measured at 37°C in a total volume of 100 μl containing 100 mM-Tris HCl (pH 7.4 at 37°C), 0.1 mM-DTT, 0.3 mM-acetyl-CoA, 2% BSA, 4 mM-ATP, 2 mM-magnesium chloride, 2 mM-$NaH^{14}CO_3$ and citrate/$MgCl_2$ ranging in concentration from 0 to 15 mM. The assay was started by the addition of 10 μl of ACC solution (to give a final ACC concentration of 1.5 - 2.0 μg/ml). After four minutes incubation at 37°C the reaction was stopped by the addition of 50 μl of 5% (v/v) perchloric acid.

The stopped assays were centrifuged to clarify the solution. 125 μl of the supernatant was transferred to clean 'Eppendorf' tubes and then dried down in a rotary vacuum evaporator (Gyrovap, V A Howe & Co. Ltd. London) set at 40°C. The residue was resuspended in 100 μl of water and then 1.0 ml of aqueous compatible scintillant. The tubes were left to stand for at least twelve hours before being counted for ten minutes on a carbon-14 programme. Results were expressed as μmol of carbon-14 incorporated into acid stable product per min per mg protein.

Vmax, Ka for citrate and the Hill co-efficient (h) were determined using computational fitting of the initial rates (v) at each citrate concentration (c) to the following equation:

$$v = \frac{Vmax[C]^h}{Ka+[C]^h}$$

2.15 PYRUVATE DEHYDROGENASE ASSAY

Principles of Assay

PDH activity was assayed using a system coupled to arylamine acetyl transferase (AAT) (Tabor et al 1953). The acetylation of p-(p-amino phenylazo) benzene sulphonic acid (AABS) by acetyl-CoA was followed spectrophotometrically at 460 nm (Jacobson 1961).

(1) Pyruvate + NAD^+ + CoA $\xrightarrow{PDH}$ Acetyl-CoA + NADH + H^+ + CO_2

(2) Acetyl CoA + AABS $\xrightarrow{AAT}$ N-acetyl - AABS + CoA

Preparation of mammary tissue for measurement of pyruvate dehydrogenase activity.

Homogenising buffer (pH 7.0 at 4°C.)

- 100 mM-potassium phosphate
- 5 mM-EDTA
- 5 mM-DTT
- 10 mM-pyruvate (added just before use)

Approximately 100 mg of powdered freeze clamped mammary tissue was homogenised in 10 volumes of homogenisation buffer using a 5 ml hand held glass teflon homogeniser. 20 μl of rat blood serum was mixed with 1.0 ml of extract to prevent proteolysis then centrifuged for 20 seconds in a benchtop microfuge (>10,000g). The supernatant was transferred to a clean tube placed in ice and used immediately.

METHOD (Coore et al, 1971)

Pyruvate dehydrogenase activity was measured in crude homogenates at 30°C in a final volume of 0.75 ml containing 100 mM-Tris HCl, 1 mM-$MgCl_2$, 0.5 mM-EDTA at pH 7.8 and in addition 5mM-2-mercaptoethanol, 1mM-thiamine pyrophosphate, 0.5 mM-NAD^+, 0.1 mM CoA, 1 mM-pyruvate and 15 μg AABS. The reaction was started by the addition of

50 munits (10 μl) of arylamine acetyltransferase and the increase in absorbance at 460 nm was followed for at least ten minutes.

Expression of PDH activity

The activity of PDH has been expressed versus gram wet weight of tissue and also as a ratio versus citrate synthase activity. Citrate synthase is an abundant mitochondrial enzyme whose activity is not acutely regulated. Its activity can therefore be a useful standard against which other mitochondrial enzymes can be expressed, as there will be automatic compensation for varying mitochondrial extraction between experiments.

2.16 CITRATE SYNTHASE ASSAY

Principle of Assay

Citrate synthase (EC 4.1.3.7) catalyses the condensation of acetyl-CoA with oxaloacetate to form citrate. Its activity is measured using a coupled system where the rate of oxaloacetate disappearance and thus the reduction of the NAD^+ analogue acetylpyridine-adenine dinucleotide (APAD) is monitored spectrophotometrically at 365 nm.

(1) L- Malate + $APAD^+ \xrightarrow{MDH}$ oxaloacetate + APADH + H^+
(2) Oxaloacetate + acetyl CoA $\xrightarrow{CS}$ citrate + CoA

MDH = malate dehydrogenase (EC 1.1.1.37)
CS = citrate synthase

METHOD

Citrate synthase activity was measured immediately after PDH using the same tissue extract, diluted as necessary.

The assay was conducted in a final volume of 930 μl

according to the method of Stitt (1984).

Calculation of results for PDH and Citrate Synthase

Activity for both enzymes is expressed as μmol of substrate converted into product per min per gram wet weight of tissue at 30°C.

2.17 MEASUREMENT OF FATTY ACID SYNTHESIS

(from Jungas 1968)

Principle of Assay

Fatty acid synthesis was measured by the incorporation of tritium (from tritiated water) into fatty acids during the reduction steps of the biosynthetic pathway (Fig 2.2). This is considered the best method because incorporation of hydrogen (from water) and hence radioactivity is proportional to the rate of fatty acid synthesis irrespective of the original carbon precursor.

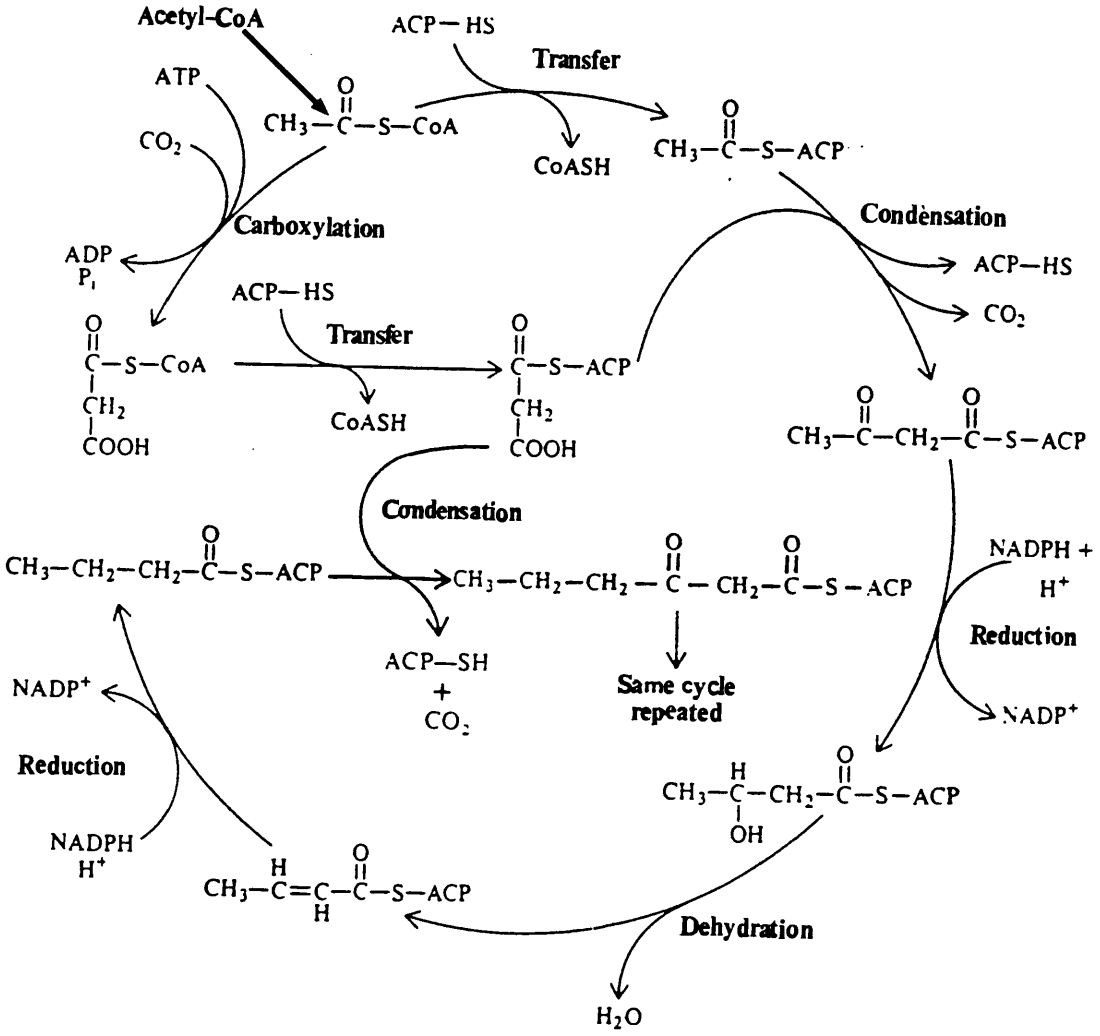
2.17.1 FATTY ACID SYNTHESIS DETERMINATION IN MAMMARY ACINI

Approximately 150 mg mammary acini were prepared and incubated in INCUBATION BUFFER (see section 2.15) containing 1 mM-palmitate or oleate, 4% BSA and 0.1 mU/ml insulin (see section 2.24). The acini were preincubated for ten minutes at 37°C in a shaking water bath with a constant flow of O_2:CO_2 (95:5).

100 μl of tritiated water (equivalent to 1.0 mCi) was then added and the acini incubated for 60 minutes. The acini were then transferred to pre-weighed glass tubes. The tubes were spun at 800g for 2 minutes in an MSE bench top centrifuge. The supernatant was decanted and an aliquot kept for calculating the specific radioactivity of the tritiated water.

Figure 2.2 MAMMALIAN FATTY ACID SYNTHESIS

Tritium (from [^{3}H]-H_2O) is incorporated into the fatty acid at each reduction step.



The acini pellet was homogenised in 6.0 ml of chloroform:methanol (2:1 v/v) using a Polytron homogeniser set at maximum and fitted with a PT10 probe. The tubes were centrifuged as above and the solvent fraction kept. The pellet was extracted a second time and the extracts pooled. 3.0 ml of 0.88% KCl and 0.1 ml 6 M-HCl were added to the 12 ml pool and shaken vigorously. The tubes were centrifuged at 800g for 3 minutes to separate the phases. The lower phase was taken and washed twice with 5.0 ml of methanol:0.88% KCl: chloroform (48:47:3). The lower phase was filtered through Whatman No. 1 filter paper pre-wetted with methanol:chloroform (2:1) into scintillation vials. The filtrate was evaporated to dryness under nitrogen at 35° - 40°C. 200 μl of absolute ethanol was then added to each vial and re-evaporated to ensure total dryness before the radioactivity was measured.

The tubes containing the pellets were dried overnight at 110°C and reweighed to calculate the weight of defatted acini. The specific radioactivity of the supernatant from each incubation was also measured by adding 100 μl to 10 ml of aqueous compatible scintillant and counting on a tritium channel.

Results were expressed as μmol of tritiated water incorporated per min per 100 mg defatted dry weight and calculated as follows:

$$\frac{\text{dpm of extracted lipid}}{\text{specific activity of (dpm/}\mu\text{mol } H_2O)} \times \frac{1}{\text{incubation time (min)}} \times \frac{100}{\text{defatted dry wt of acini (mg)}}$$

= μmol ^3H incorporated/min/100 mg defatted dry weight
the second and third terms define the results in terms of time and quantity of tissue respectively. The first term therefore gives the amount of radioactivity incorporated per flask.

For calculation of specific radioactivity of the

medium it was assumed that the sum of the solvated volumes of the constituent solutions was negligible thus:-

1 ml medium = 1 ml H_2O with the assumption that 1 ml of water was equal to 55.56 mmol.

This method measures total lipid synthesis rather than fatty acid synthesis per se. However cholesterol synthesis in the lactating mammary gland is negligible (Gibbons et al 1983). Some tritium will be incorporated into glycerol when dihydroxyacetone phosphate is reduced to make glycerol-3-phosphate and also a small amount into the phospholipid headgroups. However the proportions of 'fixed' tritium that will be due to these factors is very small in comparison with that incorporated into fatty acids (Chapter 1, Table 2). The rates of tritium incorporation can therefore be regarded as an accurate reflection of fatty acid synthesis.

2.17.2 ASSAY OF FATTY ACID SYNTHESIS IN VIVO

Rates of fatty acid synthesis *in vivo* were measured by the incorporation of ^3H_2O into fatty acid exactly as described in Robinson et al (1978) with the exception that the period of measurement was for 30 min. The rates expressed for each time point of starvation (Chapter 5) represent the rate measured over a period of 30 min directly preceeding the time quoted.

2.18 ASSAY OF FREE FATTY ACIDS

An *in vitro* colourimetric test from Wako Chemicals, West Germany was used: Code No: 994-7504.

Principle of assay:

The test measures free fatty acids (NEFA) indirectly using a coupled assay system. In the presence of added acyl-CoA synthetase (ACS) and coenzyme A (CoA) fatty acids

form acyl-CoA. This is then oxidised by added acyl-CoA oxidase (ACOD). In the presence of peroxidase (POD) and acceptor dyes, the hydrogen peroxide thus produced causes the formation of a coloured adduct which is measured colorimetrically at 550 nm.

(1) NEFA + ATP + CoA $\xrightarrow{ACS}$ Acyl-CoA + AMP + PPi
(2) Acyl-CoA + O_2 $\xrightarrow{ACOD}$ 2,3-trans-enoyl CoA + H_2O_2
(3) $2H_2O_2$ + dye substrates $\xrightarrow{POD}$ Purple adduct

METHOD

A standard curve of 0-2 milliequivalents (meq) of free fatty acids was constructed and the assays performed according to the instructions and using the reagents supplied with the kit. No preparation of the sample was required beyond removal of red blood cells.

2.19 ASSAY OF DNA IN CRUDE HOMOGENATES (from Labarca and Paigen, 1980)

Principle of Assay

The DNA content of tissue homogenates was measured via the enhancement of fluorescence seen when the fluorochrome bisbenzimide (Hoechst H33258) binds to DNA. RNA does not interfere with the assay.

METHOD

Salmon sperm DNA (Sigma D1626) was used as a standard. The DNA was stored at -20°C as a stock solution of 200 μg/ml in 2 mM-EDTA. A standard curve from 0-6 μg DNA in a final assay volume of 3 ml was constructed. The tissue homogenates and salmon DNA were diluted in assay buffer (2M-NaCl, 50 mM-NaH_2PO_4,pH 7.4) containing 1 μg/ml of fluorochrome so that the fluorometer readings fell within 0-2 μg/ml of DNA. At lower dilutions turbidity of the homogenate samples hindered the attainment of stable

readings. In mammary tissue final dilutions in the range 1:3000 to 1:6000 (i.e. tissue homogenised in 10 vols w/v and then the homogenate diluted 1:300 or 1:600 in assay buffer) proved suitable.

A Perkin-Elmer 3000 spectrofluorometer was used with excitation wavelength set at 355 nm and emission wavelength set at 445 nm. Fluorescence was expressed in arbitary units.

2.20 ASSAY OF PROTEIN

Protein was measured:

(I) at 595 nm using a Coomassie Blue dye binding assay with BSA (BDH, 6935390A) as a standard (Bradford 1976)
or (II) directly by absorbance at 280 nm.

Principle of Assay

The assay is based upon the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. The dye interacts principally with basic and aromatic amino acids.

Anion		Neutral		Cation
595 nm	⇌	650 nm	⇌	470 nm
(blue)		(green)		(red)

2.21 METABOLITE ASSAYS

2.21.1 BLOOD METABOLITES

Venous blood was collected from the superficial epigastric vein (see Fig. 2.3) and arterial blood was collected from the aorta into heparinized syringes. The whole blood concentrations of glucose, and lactate, pyruvate, acetoacetate and D-3-hydroxybutyrate were assayed as described by Robinson and Williamson (1977a). In

experiments with perfused tissue, afferent perfusate (i.e flowing into the catheterized epigastric artery) and efferent perfusate (i.e. flowing from the catheterized superficial epigastric vein) were treated as equivalent to arterial and venous blood. Thus arterio-venous differences across the mammary tissue of 'normal' and perfused rats was calculated by subtraction in the same manner.

2.21.2 TISSUE METABOLITES

Freeze clamped mammary tissue was powdered under liquid nitrogen and 200 mg of frozen powder homogenized in 4 ml of 6% (w/v) $HClO_4$. The homogenate was centrifuged at 30,000g for 20 min to clarify it and the supernatant neutralised using 20% (w/v) KOH added dropwise and with universal indicator also present to indicate when neutrality had been achieved. The following metabolites were assayed in the tissue: citrate (Mollering, 1985) glucose-6-phosphate (Michal 1984a), fructose-1,6-bisphosphate (Michal 1984b), glucose, pyruvate, L-lactate, acetoacetate and D-3-hydroxybutyrate (Robinson and Williamson 1977a).

2.22 PERFUSION OF MAMMARY TISSUE

The perfusion of inguinal mammary glands was conducted exactly as described in Clegg and Calvert (1988) except that human erythrocytes replaced goat cells for some of the experiments. This modification was made because human red cells are smaller and so less capillary blockage occurs during perfusions.

Additions of insulin, palmitate, acetoacetate or isoprenaline were made to the bulk perfusate as detailed in the relevant results sections.

Typically perfusions were performed for 100 minutes before freeze clamping of the mammary gland.

2.23 SURGICAL PREPARATION FOR ANOXIC MAMMARY GLANDS

Lactating rats weighing 200-300g and 10-14 days post partum were anaesthetized with pentobarbital (40 mg/kg body weight) administered intraperitoneally.

An incision was made along the midline from the tail to sternum. The skin was then cut across down the sides in line with the diaphragm. The left inguinal mammary gland was freed from the abdominal wall and the vessels tied with suture ligatures as illustrated in Fig. 2.3. Experimental anoxia was timed from the ligation of the superficial epigastric vessel. After 12 minutes the anoxic mammary gland was freeze clamped and the tissue stored at -70°C. The right inguinal mammary gland was freeze clamped as a paired control during the timed anoxia of the left gland.

2.24 PREPARATION OF ACINI FROM MAMMARY TISSUE OF LACTATING RAT

(from Katz et al. 1974)

Before anaesthesia of the rat and dissection of the mammary tissue the following solutions were made:

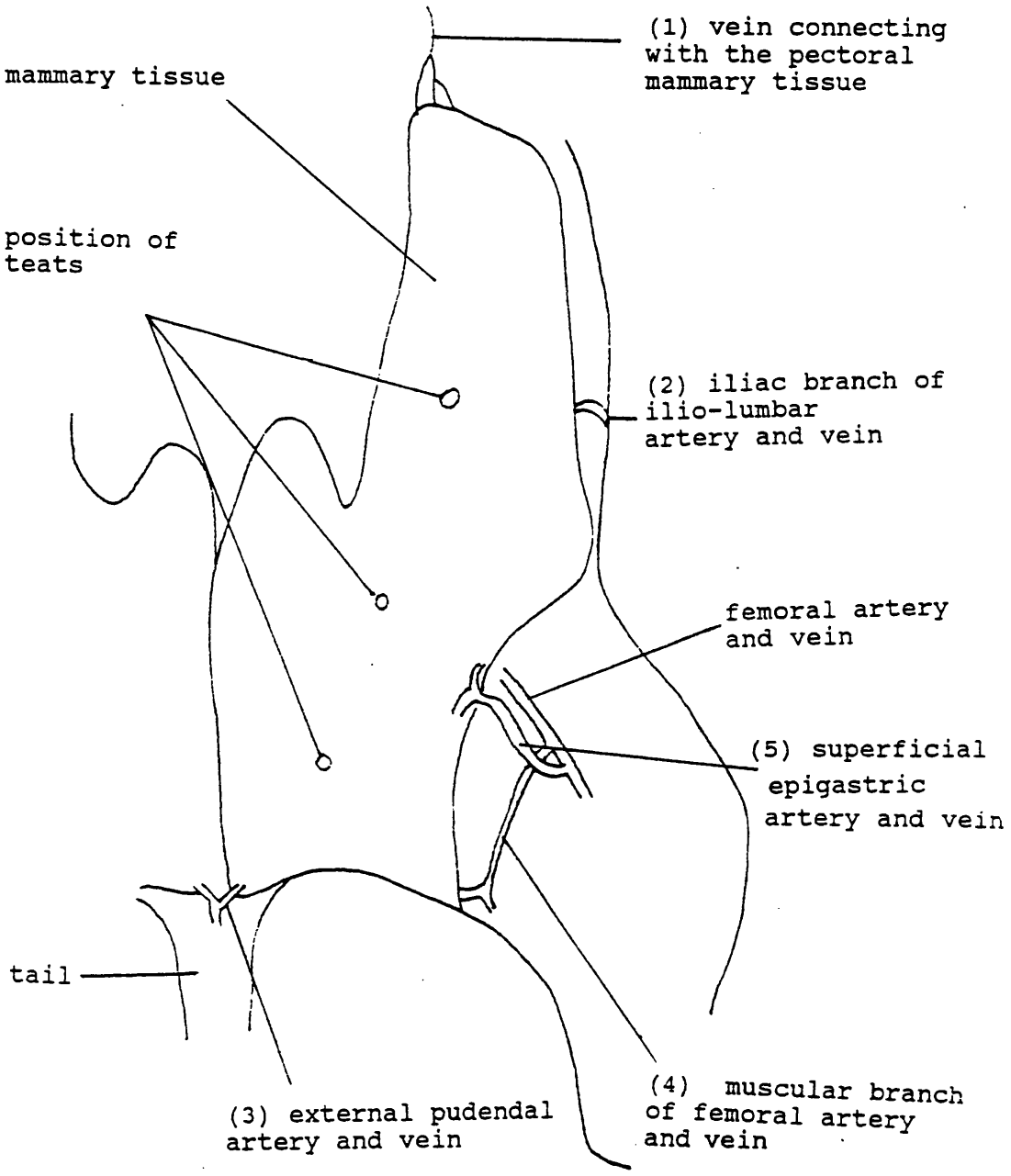
KREBS-GLUCOSE BUFFER

(KRB-gluc) - prepared by mixing the following solutions (all w/v) in the stated ratios:

	ml
NaCl (4.5% soln)	102.0
KCl (5.75% soln)	4.0
$CaCl_2.6H_2O$ (12% soln)	1.5
$MgSO_4.7H_2o$ (19.5% soln)	1.0
KH_2PO_4 (10.55% soln)	1.0

40 ml of the mixture was diluted to 200 ml with water; 30 ml of 155 mM $NaHCO_3$ added and then 0.214g glucose. The KRB-gluc buffer (final concentration: 125 mM-NaCl; 5 mM-KCl; 1.3 mM-$CaCl_26.H_2O$; 1.3 mM-$MgSO_4.7\ H_2O$; 1.2 mM-KH_2PO_4; 20 mM-$NaHCO_3$, 5 mM-glucose) was gassed for 15 minutes with

Fig. 2.3 LEFT INGUINAL MAMMARY GLAND OF THE RAT-SHOWING VESSELS SERVING THE TISSUE AND THE ORDER IN WHICH THEY ARE LIGATED TO INDUCE EXPERIMENTAL ANOXIA



O_2:CO_2 (95:5). The vessel was then tightly stoppered and placed in a 37°C shaking water bath.

COLLAGENASE DIGESTION BUFFER:

2% (w/v) BSA (BCL fraction V, fatty acid free, 774111 treated as in section 2.4), 30 mg collagenase (Worthington, type CLS lll 102 u/mg. 5% (w/v) Ficoll-400, (Sigma, F4375) in KRB-gluc buffer; 30 ml prepared.

WASH BUFFER

2% (w/v) Ficoll-400 in KRB-gluc buffer; 50 ml prepared.

RESUSPENSION BUFFER

2% (w/v) Ficoll-400, 4% (w/v) BSA in KRB-gluc buffer; 25 ml prepared.

INCUBATION BUFFER

2% (w/v) Ficoll-400, 2-3% (w/v) BSA in KRB-gluc; 30 ml prepared.

All mixtures were prepared in polypropylene 250 ml conical flasks and the air space above was gassed with O_2:CO_2 (95:5) before being stoppered tightly and placed in a 37°C shaking water bath.

METHOD

All procedures were performed in a 'hot room' or water bath at 37°C.

The rat was anaesthetized using pentobarbital (60 mg/kg body weight) administered intraperitoneally. The inguinal mammary tissue was dissected out rapidly then placed in 30 ml of KRB-gluc; chopped coarsely with scissors, drained and then rinsed with a further 20 ml of KRB-gluc. The tissue was drained again then chopped very finely using a razor blade mincer. The tissue was added to

the warmed and oxygenated collagenase digestion buffer, the air space gassed and then the flask replaced in the 37°C shaking water bath set at 180 strokes/min. The tissue was digested for 60 minutes with a vigorous swirl every 20 minutes to facilitate the process.

When the digestion was complete the flask's contents were strained through a nylon sieve (approximately 150 μ mesh). The filtrate was transferred to a 50 ml plastic centrifuge tube and centrifuged at 400g for 15 seconds in an MSE bench centrifuge. The fat cake and supernatant were removed and the sides of the tube wiped clean.

The acini pellet was washed by gentle resuspension in 15 ml of wash buffer using a wide tipped 5 ml plastic pipette. The cells were then centrifuged for 15 seconds at 400g. The cells were washed twice more. The final pellet was resuspended in 7.5 ml of resuspension medium and kept at 37°C until used.

2.24.1 WET WEIGHT DETERMINATION OF ACINI

200 μl of well mixed acini preparation was pippetted into 2 pre-weighed microtubes. The tubes were spun at 8000g for 2 minutes. The supernatant was removed and the walls of the tube blotted dry. The tubes were re-weighed and the difference taken as the wet weight per 200 μl, The yield of acini was normally between 100 and 200 mg/ml.

The wet weight of acini in the stock suspension prepared as described above was used to calculate the amount of tissue present in the subsequent incubations. Dilution of the stock acini preparation was such that this was generally between 20 mg and 40 mg per ml.

2.24.2 INCUBATIONS OF ACINI WITH PALMITATE OR OLEATE FOR ASSAY OF ACC, PDH and CS.

The incubations were performed in 25 ml polycarbonate

flasks and timed from the addition of the acini. The incubations (6 ml) consisted of INCUBATION BUFFER containing 0.1 munits per ml of insulin, 4% BSA, 120 - 140 mg acini, and 1 mM fatty acid complexed with albumin (section 2.5). Control incubations contained the same amount of BSA but no fatty acid.

The acini were added last to the incubation mixture, the flask gassed with O_2:CO_2 (95:5) and then incubated at 37°C in a water bath shaking at 180 strokes/min. After 15 minutes the flasks were removed and 4ml transferred to a polypropylene tube for ACC assay and 2 ml transferred to another for assay of PDH and CS.

The tubes were spun at 400 g for 15 seconds. The supernatant was poured off and the pellet frozen in liquid nitrogen. The pellets were stored at -70°C and assayed within two days.

2.25 POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE) OF PROTEINS

2.25.1 Denaturing PAGE

The sodium dodecyl sulphate (SDS) Tris-glycine discontinuous buffer system as described by Laemmli, (1970) was employed. The gels were run overnight at 10°C using constant current; approximately 10 mA per slab gel of 15 x 15 x 0.2 cm.

The protein solutions were solubilised by boiling for 3 minutes in 62.5 mM-Tris HCl (pH 6.8) with 2% SDS, 10% glycerol, 5% (v/v) 2-mercaptoethanol and 0.05 mg/ml of bromophenol blue as a marker.

2.25.2 Non-denaturing PAGE

The Laemmli system (Laemmli 1970) was used but with SDS omitted from all buffers. The gels were run at 4°C over three hours.

2.26 PROTEIN DETECTION AFTER PAGE

2.26.1 Coomassie blue dye stain

When electrophoresis was complete the proteins were fixed by soaking the gels for at least one hour in a 50% methanol, 10% acetic acid solution (fixer). They were then stained by soaking the gels for 10-20 minutes in 0.6% serva blue R prepared in fixer. The protein bands were visualised by washing the gel in several changes of destainer (10% acetic acid, 10% methanol) until the background had cleared.

2.26.2 Silver stain

Gels were stained with silver according to the method described by Morrissey, 1981.

Gels were preserved by drying onto cellophane.

2.26.3 Autoradiograph of radioactive proteins.

Autoradiography of samples separated by PAGE after labelling with [^{14}C]-FSBA was carried out using Kodak X-omat film exposed in a Kodak X-omatic cassette at -70°C. The film was developed for 2 min with LX-24 developer and fixed using FX-40 fixer.

2.27 ANALYSIS OF ADENINE NUCLEOTIDES BY REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

2.27.1 Tissue Preparation

Freeze clamped tissue was powdered in liquid nitrogen using a pestle and mortar. The tissue was deproteinised by homogenisation in 3.25 volumes (ml/g tissue) of 6% (w/v) perchloric acid. Homogenisation was performed using a Polytron homogeniser fitted with a PT10 probe and set at number 4. The extracts were clarified by centrifugation

at 2500g for 10 mins at 4°C. The supernatants were neutralised and freed of perchlorate by extraction with 1.1 volumes of a 50:50 mixture of tri-n-octylamine and 1,1,2-trichlorotrifluoroethane according to the method of Khym, (1975).

2.27.2 Measurement of ATP

The ATP concentration of neutralised tissue extracts was measured using a luminometric assay system supplied by LKB Ltd (Bromma, Sweden), based on the luciferin/luciferase reaction where the intensity of luminescence is proportional to the concentration of ATP.

2.27.3 HPLC Separation of Nucleotides (from Willis et al 1986).

50 μl of the neutralised extract (2.27.1) was sufficient to obtain nucleotide profiles by reversed phase chromatography using a polymeric column (150 mm x 4.6 mm, 300Å, 8μ particle size Polymer Labs RP-S). The solvents used for the gradient elution were 1% (v/v) methanol in 83.3 mM triethylammonium phosphate, pH 6.0 (solvent A) and 20% (v/v) methanol in 83.3 mM triethylammonium phosphate pH 6.0 (solvent B). Adenine nucleotide peaks were assigned by comparison with retention times of authentic standards.

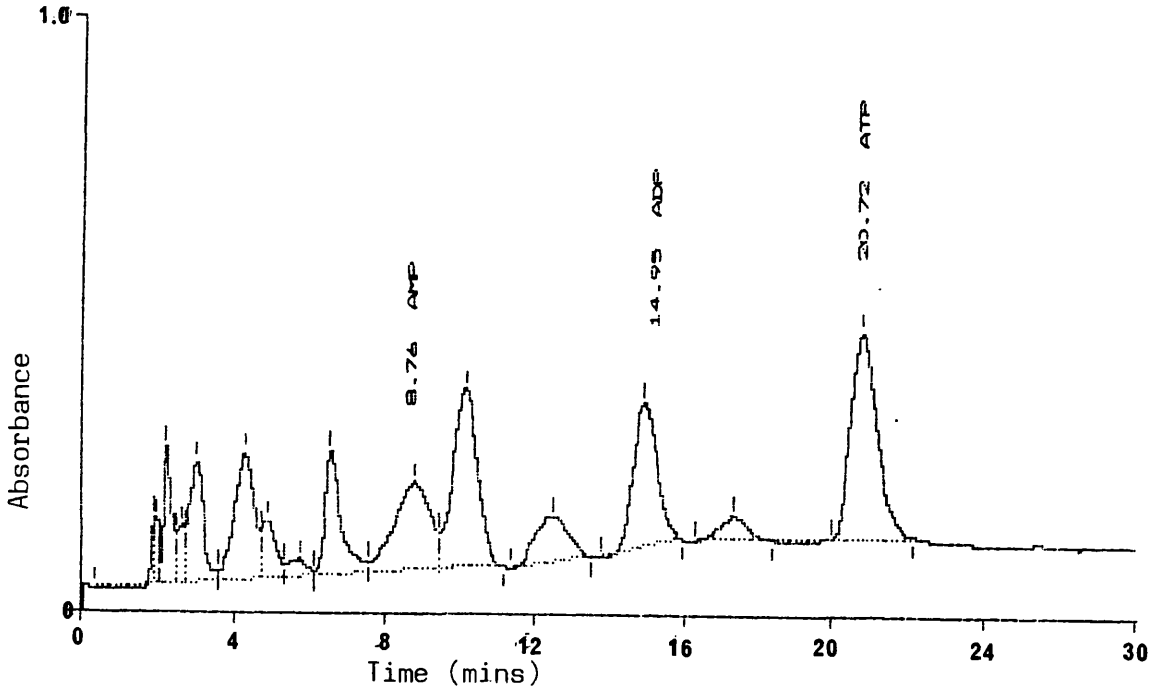
The elution programme employed was as follows: 0-3.0 min, 100% A : 0% B; 3.0 - 22.0 min, 0% B - 45%B; 22.0 min - 32 - min, 45% B - 100% B; 32.1 min - 34 min, 0% A : 100% B. The flow rate was 1 ml per minute.
Absorbance was monitored at 254 nm.

2.27.4 Calculation of Adenine Nucleotide Concentrations

ATP was measured luminometrically before chromatography of the sample. This value was assigned to the peak of absorbance corresponding to ATP. Quantitation

Figure 2.4 SEPARATION OF ADENINE NUCLEOTIDES BY REVERSED PHASE HPLC

Neutralized H ClO_4 extracts of mammary tissue were prepared as described in Materials and Methods. The trace is a representative profile of the adenine nucleotides present in normal mammary tissue from lactating rat. 50 μl of extract was loaded and chromatographed as described in text.



of the other adenine nucleotides was achieved by comparison of their integrated peak areas with that for ATP using the following values of relative absorbance at 254 nm: ATP, 1.0; ADP, 1.0; AMP, 0.994. (Dawson et al., 1986) All values are expressed as μmol/g wet weight.

A typical profile from lactating mammary tissue extract is shown (Fig. 2.4).

2.28 COVALENT MODIFICATION OF AMP-PK BY [^{14}C-FLUOROSULPHONYL-BENZOYLADENOSINE ([^{14}C]-FSBA)

AMP-PK preparations were labelled with [^{14}C]-FSBA at 30°C in a 100 μl incubation mixture containing: [^{14}C]-FSBA (0.039 mM, 5.1 mCi/nmol) with kinase preparation in 20 mM-Tris HCl pH 7.4; 0.5 mM-EDTA; 0.5 mM-EGTA; 5 mM-NaPPi; 5 mM-DTT; 10% (w/v)-glycerol; and 0.02% (w/v)-Brij 35. In order to distinguish proteins with AMP-binding sites 0.4 mM-AMP was included in some incubations. The incubations were terminated after 60 mins by the addition of 25 μl of SDS sample solubilizer to give a final concentration of 62.5 mM-Tris HCl (pH 6.8 at 25°C); 10% (w/v)-glycerol; 2% (w/v)-SDS; 5% (v/v)-2-mercaptoethanol, and boiled for 3 min before loading into a 7% - 10% gradient resolving gel with a 5% stacking gel. The gel was electrophoresed as described in section 2.25.1, dried onto cellophane and then autoradiographed as described in section 2.26.3.

2.29 ANALYSIS OF PHOSPHORYLATION SITES ON [^{32}P]-LABELLED ACETYL CoA CARBOXYLASE.

Analysis of phosphorylation sites was performed by reverse phase HPLC using a Beckman-Altex system.

^{32}P-labelled acetyl-CoA carboxylase was precipitated in a microfuge tube (to 45% fractional saturation) using 90% saturated (4.1 M) and neutralised ammonium sulphate solution with 400 μg of BSA as a carrier. After centrifugation (5 mins, >10,000 g) the pellet was rinsed

twice with 1 ml of 45% saturated ammonium sulphate. The pellets were digested by resuspension in 300 µl of 50 mM-Hepes/NaOH, pH 7.0 with the addition of trypsin and/or chymotrypsin the latter TPCK treated (Worthington Diagnostic Systems Inc., USA) in the ratio 50 mg ACC to 1 mg of protease.

After incubation for 16 hours at 37°C the digestion was stopped with 100% (w/v) trichloroacetic acid (TCA) to give a final concentration of 10%. Undigested material was removed by centrifugation and the supernatant extracted three times with 0.8 ml water saturated diethyl ether to remove all traces of TCA.

The digests were separated at 1 ml/min on a Beckman Ultrasphere ODS column (250 x 4.6 mm) using linear gradients of 0-40% acetonitrile in aqueous 0.1% trifluoroacetic acid. Radioactivity was analyzed continuously by Cerenkov counting.

2.30 ASSAY OF ALKALI-LABILE PHOSPHATE IN ACC

Principle of assay

The measurement of phosphate is based upon the quantitative formation of phosphomolybdate which produces a stable blue colour with a peak of absorbance at approximately 820 nm (Bartlett 1959) when heated in reducing conditions.

Method

All solutions were prepared using double distilled and deionised water and all glassware was washed with phosphate free chromic acid and double distilled and deionised water before use.

Approximately 0.1 nmol of ACC (0.24 mg) was precipitated in a microfuge tube by 1 ml of 25% (w/v) of

trichloroacetic acid (TCA). The protein was pelleted by centrifugation at >10,000g for 3 minutes, then washed three times with 25% (w/v) TCA to remove non covalently bound phosphate. The pellet was incubated at 37°C for 16-20 hours with 150 μl of 1M-NaOH to liberate covalently bound phosphate. 50 μl of 100% (w/v) TCA was added to acidify the mixture which was then kept on ice for 10 min. After centrifugation the 200 μl of supernatant was transferred to a clean tube and 300 μl of phosphomolybdate reagent (6 vol 0.42% (w/v) ammonium molybdate in 0.5 M-H_2SO_4 + 1 vol freshly prepared 10% (w/v) ascorbic acid) added and the reaction incubated at 45°C for 20 min. A standard curve between 0 and 5 nmoles phosphate was incubated simultaneously. It was prepared using KH_2PO_4 in 25% (w/v) TCA. The phosphate content of the incubations was measured by absorbance at 820 nm.

CHAPTER 3

3.1 cAMP-PK ACTIVITY IN MAMMARY TISSUE OF THE LACTATING RAT

It is well established that in adipose tissue and liver as well as isolated cells from these tissues, lipogenesis is inhibited by ß-adrenergic agonists and the hormone glucagon. (Holland et al 1983, Robson et al 1984, Stark and Keller 1987).
These effectors all cause an increase in intracellular cAMP and thus cAMP-PK activity by the stimulation of adenylate cyclase.

Acini prepared from lactating rat mammary tissue do not respond to adrenaline, glucagon or adrenergic agonists with a decrease in lipogenesis (Robson et al 1984, Williamson et al 1983, Clegg and Mullaney 1985). The tissue has no glucagon receptors (Robson et al 1984) but it does possess $ß_2$-adrenergic receptors which are competently coupled to adenylate cyclase (Clegg and Mullaney 1986). However it required the treatment of mammary acini with ß-agonists simultaneously with phosphodiesterase inhibitors to produce significant increases in intra-cellular cAMP levels, (Clegg and Mullaney 1985). However even when intracellular cAMP concentrations were elevated to twenty-fold above normal no decrease in lipogenesis, nor in the activity or phosphate content of acetyl-CoA carboxylase was observed (Clegg et al 1986,1987).

The poor response of cAMP levels in mammary acini to adrenergic agents (Williamson et al 1983, Clegg and Mullaney 1985) can be explained by the high activity of cyclic nucleotide phosphodiesterases in mammary tissue; they have a total activity three orders of magnitude in excess of that of adenylate cyclase (Clegg and Mullaney 1984). Thus it may be the case that intracellular cAMP changes mediated by adrenergic agents are effectively nullified except when phosphodiesterase inhibitors are also

present.

The two major isoenzymes of cAMP-PK are present in the lactating mammary gland of the rat; 34±5% of total activity occurs as isoenzyme type I (See section 3.7).

Thus the role of cAMP-PK in mammary tissue is not clear. Indeed its activity rises rapidly in pregnancy and falls at parturition (Majumder and Turkington 1971 a,b) in parallel with changes in adenylate cyclase activity and cAMP levels (Sapag-Hagar and Greenbaum 1973,1974) as well as ß-receptor number (Marchetti et al 1990).

This chapter describes experiments designed to clarify the role of cAMP-PK in vivo during dietary manipulation and also shows that the mammary enzyme has several properties unique to the tissue.

3.2 DEPENDENCE OF cAMP-PK ACTIVITY ON cAMP

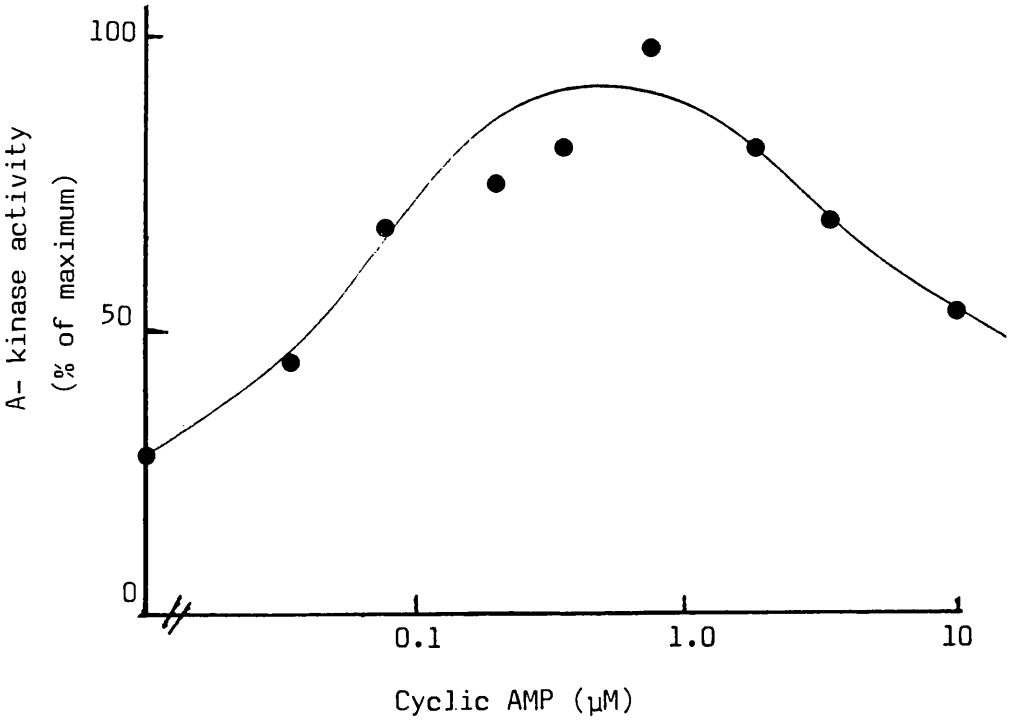
In order to achieve linearity in the assay of cAMP-PK activity over at least 15 minutes with respect to both time and quantity of sample it was necessary to dilute the mammary tissue homogenate to a final concentration equivalent to 1 part tissue by weight to 1000 parts of buffer by volume. 1.0 ml tissue extract thus diluted was derived from tissue containing 3-6 μg of DNA. In order to stabilize the catalytic subunit the buffer used for dilution contained 10 mg BSA/ml.

The basal activity of cAMP-PK was stimulated by cAMP up to a final concentration of 0.6 μM. It was then progressively inhibited by higher concentrations (Fig 3.1): only 50% of maximum activity being measured at 10 μM cAMP. Inhibition of cAMP-PK activity by high concentrations of cAMP is commonly seen but more than 100 μM is usually required.

A decrease in cAMP-PK activity in response to elevated cAMP has been observed *in vivo* and in isolated acini (See section 3.6). It has also proved difficult to purify catalytic subunit from mammary gland by affinity elution using cAMP because the activity was rapidly lost. These observations suggest that the mammary gland has a mechanism whereby free catalytic subunit is rapidly inactivated when cAMP levels are elevated above basal conditions.

Figure 3.1 cAMP DEPENDENT PROTEIN KINASE IN MAMMARY TISSUE-DEPENDENCE ON cAMP

Mammary tissue homogenate was diluted 1:1000 (w/v) with buffer containing 10 mg/ml BSA and assayed at concentrations of cAMP between zero and 10 μM. 100% activity in the experiment illustrated is equivalent to 36.6 nmol phosphate transferred to Kemptide/min/mg DNA.



3.3 POST EXTRACTION ACTIVATION AND INACTIVATION OF cAMP-PK

The very high affinity of the regulatory subunits for cAMP mean that the dissociation and activity of the kinase is directly related to the concentration of cAMP. However measurement of activity ratio can only be taken as an indication of changes of intracellular cAMP and protein kinase activation if steps are taken to ensure that the tissue homogenisation procedure does not artefactually increase or decrease the degree of holoenzyme dissociation. Corbin et al (1975) have shown that 150 mM-NaCl is sufficient to cause significant dissociation of isoenzyme type I. However, dissociated type II isoenzyme will reassociate in low ionic strength buffer. Workers have sought to avoid dissociation or reassociation of isoenzyme type I by extraction of tissues in zero NaCl buffers containing phosphodiesterase inhibitors. For assay of isoenzyme II reassociation was prevented by extraction in 0.5 M-NaCl. Intermediate conditions; 0.15 M-NaCl and phosphodiesterase inhibitors; were used when both isoenzymes were being assayed.

Palmer et al (1980) and more recently Murray et al (1990) have shown that these precautions are not necessarily sufficient to prevent artefactual activity ratio measurement. It is necessary to determine this for each tissue and experimental protocol.

3.4 DETERMINATION OF CONDITIONS NECESSARY TO PREVENT POST EXTRACTION ACTIVATION OR INACTIVATION OF TYPE I AND II cAMP-PK ISOENZYMES IN THE LACTATING RAT MAMMARY GLAND.

Animals were treated, and tissue dissected and homogenised as described for analytical extraction of cAMP-PK (see Materials and Methods section 2.9). The experiments were performed as shown in Table 1 with salt concentrations, and the addition of partially purified isoenzymes I and II, as described.

Holoenzymes type I and II were purified from rat liver as described in Materials and Methods and when used were added to the mammary tissue prior to homogenisation such that the total kinase activity assayed in the presence of 0.7 μM cAMP was doubled.

The measured activation state of the exogenous enzyme was calculated by subtracting the activity contributed by the endogenous protein kinase (this was determined from a simultaneous assay containing mammary extract alone). The predicted activity ratio was that of the exogenous holoenzyme assayed alone at the NaCl concentration indicated.

The results (Table 1) show that the extraction of mammary tissue in homogenisation buffer containing 0.5 mM IBMX but no added NaCl gave the best correlation between predicted and actual activity ratios. This procedure was therefore used for all extractions where cAMP-PK activity was to be measured. The higher activity ratio observed at 150 mM and 400 mM NaCl were due to the inhibition of total activity by NaCl.

Table 1

Effects of extraction procedure on observed activity ratios of cAMP dependent protein kinase isoenzymes.

NaCl in homogenisation buffer	Additions to mammary tissue	Observed activity ratio	Predicted activity ratio
o mM	None	.11	
	Type I	.08	.09
	Type II	.15	.14
150 mM	None	.14	
	Type I	.20	.07
	Type II	.14	.09
400 mM	None	.15	
	Type I	.14	.09
	Type II	.16	.10

3.5 EFFECT OF FASTING AND SUBSEQUENT REFEEDING ON THE ACTIVITY OF cAMP-PK AND ACC IN MAMMARY TISSUE OF THE LACTATING RAT.

The activity ratio of cAMP-PK in extracts of freeze clamped mammary tissue was 0.118 and did not change significantly when the animals were starved for 24 hours before sampling, when lipogenesis is inhibited by 98% (Robinson et al 1978) or from those that had been starved for 24 hours then re-fed for 2 hours, when lipogenesis has almost returned to control levels. (Table 2)

In contrast there is a 50% fall in the Vmax of ACC after 24 hour starvation (Table 3). This is accompanied by an increase of 1.5 moles of alkali labile phosphate from 5.9 to 7.4 moles per mole ACC. Upon refeeding the activity of ACC is restored to approximately 80% of control Vmax values within 2 hours. These changes in ACC activity occur although there is no change in the activity of cAMP-PK (see Table 2). This observation provides no support for the hypothesis that cAMP-PK is involved in the control of lipogenesis and ACC activity in the lactating mammary gland of the rat during starvation and refeeding.

3.6 EFFECT OF ISOPRENALINE ON cAMP-PK ACTIVITY IN MAMMARY TISSUE OF LACTATING RAT

Treatment of the rats with isoprenaline (0.5 mg/kg body weight, delivered intraperitoneally) 30 minutes before sampling of mammary tissue produced a four fold increase in the activity ratio of cAMP-PK (Table 4). A similar effect was produced in isolated acini incubated with 1 μM isoprenaline and 1 mM Ro7-2956 for 15 minutes (Table 4). This result demonstrates directly that treatment known to increase intracellular cAMP does indeed cause an increase in cAMP-PK activity whether in intact animals or isolated acini and indicates that the resistance of ACC to phosphorylation in conditions where cAMP is elevated (Clegg et al 1986, 1987) is not because cAMP-PK is not being

Table 2 Activity of cAMP-PK in mammary tissue extracts from control, 24hr starved and 24hr starved - 2hr refed lactating rats.

Activity = ^{32}P transferred from γ-^{32}P-ATP to Kemptide/μg DNA/min

	BASAL ACTIVITY (no cAMP)	TOTAL ACTIVITY (0.7 uM cAMP)	ACTIVITY RATIO BASAL:TOTAL
ad libitum fed n=7	2.36 ± 0.55	19.93 ± 3.50	0.118
24hr starved n=7	1.71 ± 0.39	18.10 ± 3.49	0.094
2hr ad libitum refed n=7	2.04 ± 0.04	23.92 ± 3.90	0.085

For experimental details see text

Results are mean values ± SEM; n = number of observations

Table 3 Activity of purified ACC from mammary tissue of control, 24 hr starved and 24hr starved - 2hr refed lactating rats.

(1.0 unit activity = μmol/min)

	Vmax (Units/mg ACC)	Ka Citrate (mM)	Hill coefficient
ad libitum fed n=7	3.48 ± 0.23	2.35 ± 0.16	0.99 ± 0.04
24hr starved n=10	1.67 ± 0.13***	3.89 ± 0.45**	1.10 ± 0.03
2hr ad libitum refed n=3	2.71 ± 0.50^{++}	3.00 ± 0.22^{+}	0.90 ± 0.08

For experimental details see text.

Results are mean values ± SEM; n=number of observations.

Values that are significantly different from those of ad libitum fed rats (as assessed by Student's t-test) are indicated by

$^{***}P<0.0001$, $^{**}P<0.025$

Values for refed animals that are significantly different (as assesed by Student't t-test) are indicated by $^{++}P<0.025$, $^{+}P<0.05$

Table 4 ACTIVITY OF cAMP-PK IN MAMMARY TISSUE AND ISOLATED MAMMARY ACINI TREATED WITH ISOPRENALINE

(Activity = ^{32}P transferred/min/μg DNA)

Treatment	BASAL ACTIVITY (no cAMP)	TOTAL ACTIVITY (with cAMP)	ACTIVITY RATIO (BASAL:TOTAL)
ACINI n=9 none	1.28±0.23	11.23±1.02	0.114±0.030
ACINI n=9 Isoprenaline + Ro7-2956	2.43±0.46*	4.89±0.69***	0.471±0.050***
TISSUE n=9 none	2.18±1.44	33.84±5.22	0.071 ±0.024
TISSUE n=8 Isoprenaline	4.89±1.43+	21.10±1.53***	0.280±0.067***

For experimental details see text.

Results are mean values ± SEM; n = number of observations. Values that are significantly difference from relevant controls (as assessed by Student's t-test) are indicated by *P< 0.05; **P< 0.025; ***P<0.001

+ One way analysis of variance, by using $\log_{10}$ transformed to normalize the data, demonstrated this value to be significantly different (P<0.025) from a pooled population of untreated acini and tissue.

activated. The break in the usual cascade of events is clearly distal to this. Recent evidence (Clegg and Ottey 1990) has shown that in isolated acini this increase in activity of cAMP-PK results in the phosphorylation and activation of phosphorylase. However no significant effect on the activity of ACC is seen in acini treated with isoprenaline (Clegg and Ottey, 1990; Clegg et al 1986; 1987, Table 5). This is further evidence that different mechanisms for the acute regulation of ACC must be present in the mammary gland.

The increased activity ratio of cAMP-PK in mammary tissue produced by isoprenaline was the result of both an increase in the basal activity as well as a substantial (37%) decrease in the total cAMP stimulated activity (Table 4). A similar loss of total activity was also seen in isolated acini incubated in the presence of 1 μM isoprenaline and 1 mM Ro7-2956 (Table 4) and could be correlated with a loss of immunotitratable catalytic subunit from the cytosol (Clegg and Ottey, 1990). The mean content of cytosolic catalytic subunit in extracts from isoprenaline treated acini extracts, as a percentage of the paired control value, was $54\pm7\%$ (mean ± SEM, n = 5) and the corresponding value for cAMP-PK activity in the same extracts was $64\pm6\%$ (mean ± SEM, n = 5). Keely et al (1975) have also reported the translocation of C-subunit from the cytosol to particulate fractions in rat hearts exposed to elevated cAMP levels but are non-commital with regard to its physiological significance.

Alhanaty and co-workers (1979,1981, 1985) have shown the presence in intestinal brush border membranes of a specific protease for the C-subunit of cAMP-PK which rapidly degrades the native subunit (40,000 M_r) to an inactive 34,000 M_r fragment. A similar protease if present in mammary tissue might explain the loss of kinase activity observed in isoprenaline treated tissue and acini. However, this does not appear to be true in mammary tissue as the loss of total activity measurable is due to a

Table 5 ACTIVITY OF ACC IN MAMMARY ACINI TREATED WITH ISOPRENALINE

(μmol/min/mg DNA)

	INITIAL ACT. 0mM Citrate	INITIAL ACT. 20mM Citrate	TOTAL ACT. 20mM Citrate	ACT. RATIO INIT:TOTAL
CONTROL n=7	0.092±0.018	0.157±0.029	0.273±0.046	0.56±0.02
ISOPRENALINE + Ro7-2956 n=7	0.095±0.017	0.165±0.026	0.263±0.033	0.62±0.02

Extracts were prepared from acini treated as described in Table 4 and assayed for ACC activity by the incorporation of $H^{14}CO_3$ into acid stable soluble products, as described in Materials and Methods.

Results are mean ± SEM for n observations.

quantitative loss of immunoprecipitable catalytic subunit from the cytosol by binding to sedimentable protein. (R A Clegg unpublished results). A similar loss of C-subunit triggered by agents which activate cAMP-PK has been observed in porcine epithelial cells (LLC-PK_1) by Hemmings (1986). He suggests proteolysis is responsible but shows no accumulation of proteolytic products. It is therefore possible that a similar phenomenon is occurring in this system.

The decrease in the cytosolic C-subunit which occurs when the cell is exposed to very high levels of cAMP may be a regulatory mechanism serving a 'protective' function thus preventing over stimulation of cAMP-PK sensitive pathways. Alternatively the translocation of the C-subunit to membranes may be the method whereby the effects of cAMP elevating effectors are relayed to the nucleus. cAMP-PK has been implicated in mammary development during pregnancy and the oestrus cycle (Sapag-Hagar and Greenbaum 1974; Marchetti et al 1990; Marchetti and Labrie 1990). The differential expression and subcellular location of cAMP-PK subunits has clearly been shown to be important in the regulation of cell differentiation and development (Pariset et al 1989).

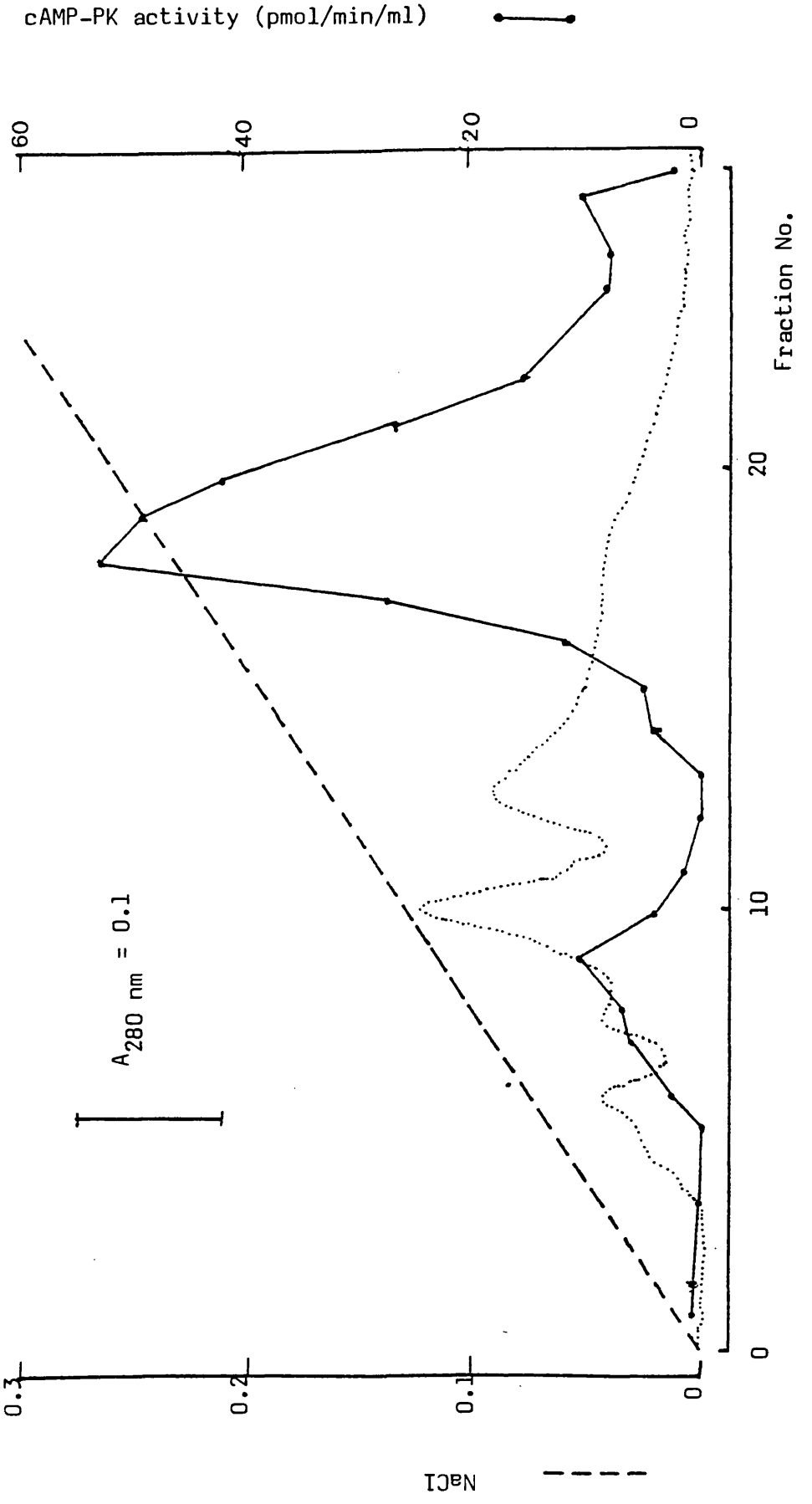
3.7 DISTRIBUTION OF ISOENZYMES TYPE I AND II OF cAMP-PK IN LACTATING MAMMARY GLAND.

Separation of cAMP-PK by DE-52 cellulose anion exchange shows that mammary tissue contains both isoenzymes type I and II. Type I eluted at approximately 110 mM-NaCl and type II at 230 mM-NaCl. The reported NaCl concentrations at which isoenzymes type I and II elute varies slightly between authors and is usually due to varying buffer compositions but Corbin et al (1975) report that isoenzyme type II from rat adipose tissue and rat heart do not show a peak of activity at exactly the same position when eluted from DEAE cellulose under identical conditions. Thus indicating multiple forms of type II

Figure 3.2 SEPARATION OF cAMP-PK ISOENZYMES BY ION-EXCHANGE CHROMATOGRAPHY ON DEAE-CELLULOSE

A 1 ml portion of a 100,000g supernatant fraction (total cAMP-PK activity of 1.42 nmol/min/ml) prepared from mammary tissue homogenate was loaded on to a column (140 mm x 8 mm) of DEAE-cellulose equilibrated with 10 mM-MES/NaOH, 0.2 mM-EDTA, 0.1 mM -DTT, pH 6.8. After washing with this buffer until no further protein was eluted the column was developed with a linear gradient of NaCl (0-0.4 M) in the same buffer at 3 ml/min. Recovery of activity in the separation illustrated (representative of six experiments) was 78%.

Figure 3.2 Chromatographic Separation of Isoenzymes I and II of cAMP-PK from Rat Lactating Mammary Gland



regulatory subunit; possibly due to varying degrees of autophosphorylation (Fig. 3.2). Type II is the predominant isoenzyme in mammary gland comprising approximately 70% of total cAMP-PK activity recovered from ad libitum fed rats. This proportion did not change significantly when cAMP-PK was isolated from rats starved for 24 hours or those starved for 24 hours and then refed for 2 hours (Table 6).

Hence heterologous activation as seen in other tissues (Livesey et al 1982, 1984; Ekanger et al 1988) does not occur in lactating mammary gland during short term starvation or refeeding of the animal. Thus the constancy of activity ratio in mammary extracts from rats treated as described was not due to a differential inactivation of one isoenzyme together with activation of the other thereby producing no overall change in cAMP-kinase activity.

Treatment of normally fed animals with the ß agonist isoprenaline caused co-ordinate activation of both isoènzymes I and II (Table 6) The higher cAMP-PK activity observed (Table 4) was due to dissociation of both isoenzymes to a similar extent (Table 6).

Table 6 Recovery and Distribution of Total cAMP-PK Activity from DE-52 ion exchange chromatography

TREATMENT	ISOENZYME I (% total activity)	ISOENZYME II (% total activity)	II/I	Recovery of activity
FED n=5	16.80 ± 1.82	53.20 ± 5.51	3.17	70%
24h Starved n=5	16.22 ± 2.18	56.22 ± 5.48	3.47	72%
3hr Refed n=5	15.96 ± 2.94	50.20 ± 7.14	3.15	66%
Isoprenaline n=5	16.75 ± 2.1	60.00 ± 7.78	3.58	77%

The isoenzymes were separated by batch elution and assayed as described in Materials and Methods. The amount of each isoenzyme is quoted as a percentage of the total activity applied to the columns.
Values are means ± SEM of n observations.

3.8 STUDIES ON THE CATALYTIC SUBUNIT OF cAMP-PK PURIFIED FROM MAMMARY GLAND.

The refractory nature of the cAMP cascade in the mammary gland particularly with respect to the phosphorylation of ACC suggests that the kinase may have unusual properties conferred upon it either by the special environment of actively secreting mammary epithelial cells or even some inherent property of the kinase itself. Workers who have shown phosphorylation and inhibition of ACC by cAMP-PK (Hardie and Guy 1980, Munday et al 1984, 1988 a,b.) have all used cAMP catalytic subunit purified from rabbit skeletal muscle or bovine heart. This was due to evidence indicating that the catalytic subunit is an invariant enzyme. This is explained in detail in the introduction.

The purification and partial characterisation of the catalytic subunit from lactating mammary gland described in this chapter suggests that this may not be true.

3.9 PURIFICATION OF THE CATALYTIC SUBUNIT OF cAMP-PK FROM RAT LACTATING MAMMARY GLAND

3.9.1 SEPARATION ON PHOSPHOCELLULOSE

The inguinal and thoracic mammary tissue from rats at peak lactation, killed by stunning and cervical dislocation, were dissected out and homogenised in five volumes of HOMOGENISATION BUFFER (Materials and Methods section 2.6) containing 1 mM-BZ, 4 μg/ml-SBTI and 0.1 mM-PMSF. The homogenate was centrifuged at 100,000 g for 60 minutes and the supernatant stirred with approximately 150 ml (packed volume) of freshly prepared phosphocellulose (type P11, Whatman) for twenty minutes at 4°C.

The phosphocellulose was washed with a total of 750 ml of phosphocellulose COLUMN BUFFER (60 mM-Tris/HCl, 150 mM-NaF, 1 mM-EDTA, 1 mM-EGTA, 10% (w/v) glycerol, pH 7.5 at

Figure 3.3 SEPARATION OF C-SUBUNIT ON PHOSPHOCELLULOSE

Tissue extract and the column were prepared as described in the text. The column was washed with 100 mM-NaCl in COLUMN BUFFER. The bulk of the kinase activity was eluted by the addition of 300 mM-NaCl in COLUMN BUFFER at the point indicated. Protein was monitored by absorbance at 280 nm. Protein kinase activity was assayed at 30°C in a volume of 25 μl in 10 mM-glycerophosphate, pH 7.5 at 25°C, 0.4 mM-EDTA, 0.1 mM-EGTA, 0.2 mM [γ^{32}P]-ATP (0.05μCi/nmol), 5 mM-$MgCl_2$, +/- kinase inhibitor. Histone IIA at 2.2 mg/ml was the phosphate acceptor. After a 60 minute incubation 20ul was transferred to 2 x 2 cm squares of phosphocellulose paper and washed three times in tap water (mineral content ~50 mM) followed by a final wash in acetone and drying before counting in non-aqueous scintillant using a phosphorus 32-programme.

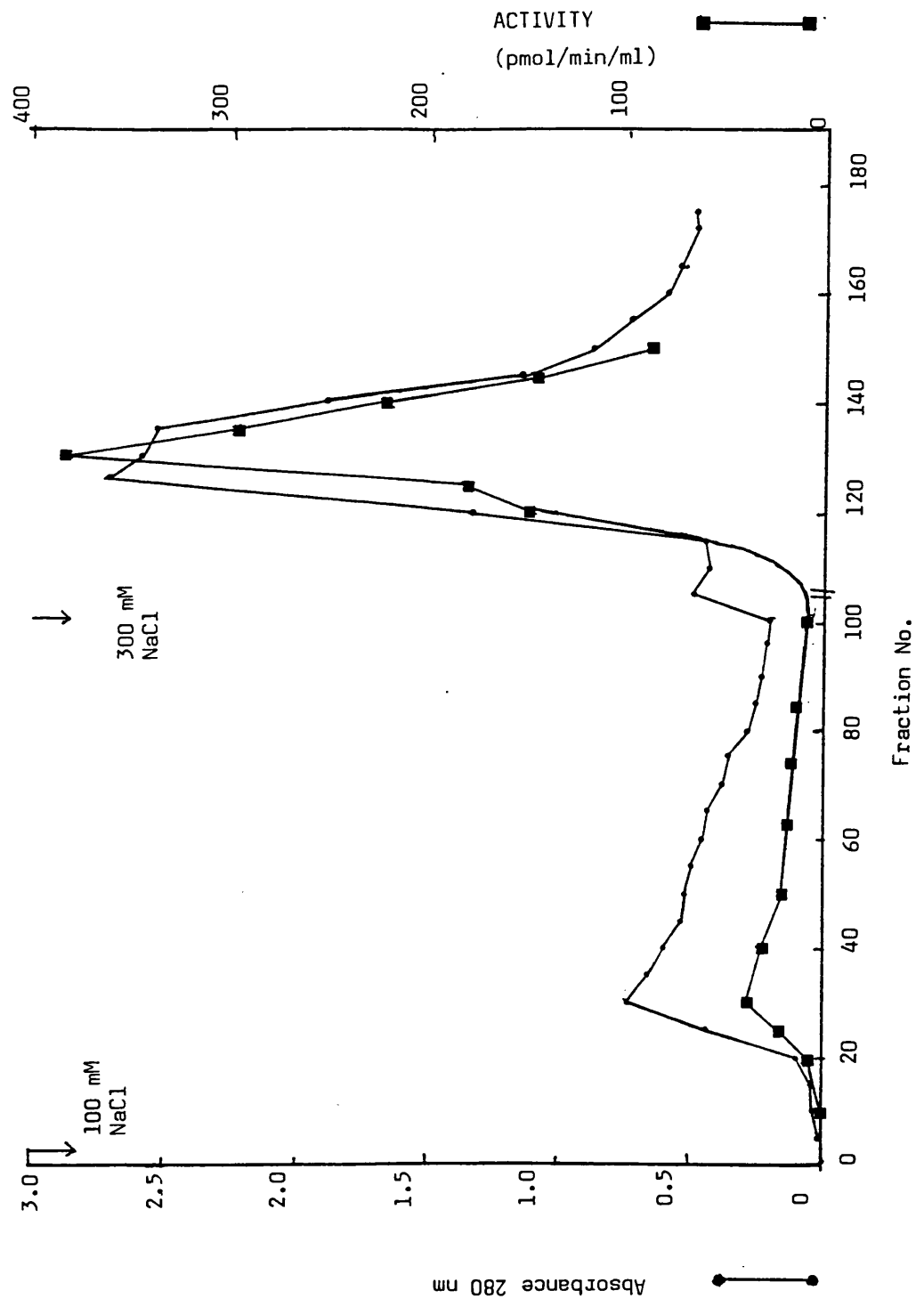


Figure 3.3 Purification of the Catalytic Subunit of cAMP-PK on Phosphocellulose

4°C, with protease inhibitor cocktail as above) using a Buchner funnel and gentle suction. The matrix was packed into a column with 4 cm diameter and washed with COLUMN BUFFER containing 100 mM-NaCl until no protein was detectable at 280 nm. A small amount of kinase activity was eluted by the 100 mM NaCl. This was not pooled for further purification.

The catalytic subunit of cAMP-PK was eluted batchwise by 300 mM-NaCl in COLUMN BUFFER (Fig. 3.3). Fractions with the greatest kinase activity against Histone IIA were pooled and concentrated by vacuum dialysis prior to dialysis into 45 mM-phosphate buffer pH 7.0 at 4°C, with 10% (w/v) glycerol, 1 mM-EDTA and 0.1 mM-EGTA.

Phosphocellulose is a combined ion exchange/affinity absorbent. The charged phosphate groups can function as weak cation exchangers and also as pseudosubstrates for proteins which are able to interact with phosphate esters. The catalytic subunit being positive at pH 7.5 will therefore be able to bind to phosphocellulose through either of these modes. The elution of kinase activity by 100 mM-NaCl was probably due to a small amount of kinase interacting with the matrix differently from the bulk. It is likely that the activity eluting at 100 mM NaCl was that bound ionically because proteins bound through affinity interactions normally require higher salt concentrations for elution.

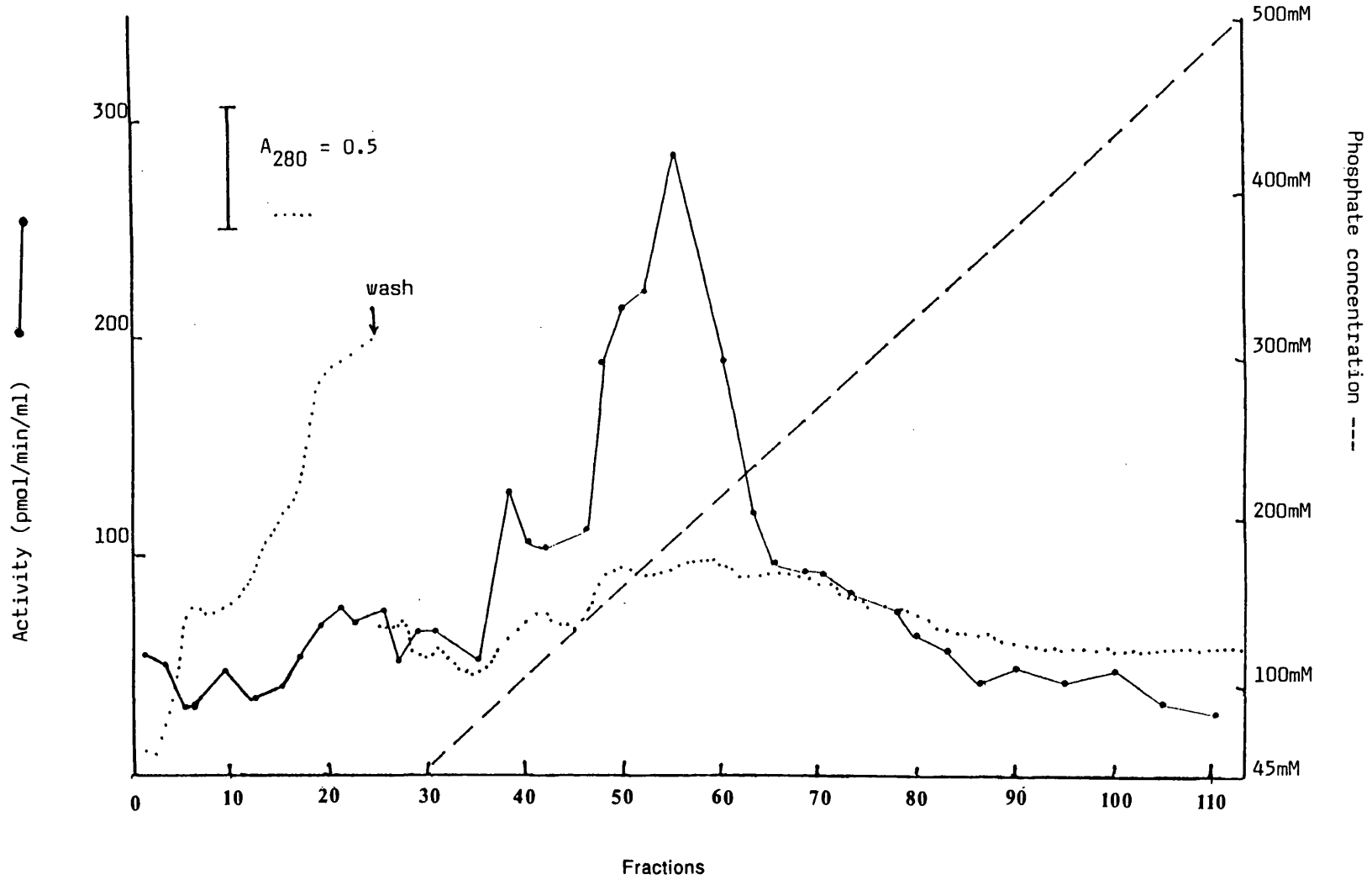
3.9.2 SEPARATION ON HYDROXYAPATITE

The dialysed protein solution was applied to a hydroxyapatite column equilibrated with 45 mM-phosphate buffer, pH 7.0 and washed until the A_{280} was zero. The large amount of protein that did not bind to the column and thus came through in the wash produced a significant purification even before the gradient was applied. A gradient of 45 mM to 500 mM-phosphate was used. The kinase eluted with a peak of activity at approximately 180

Figure 3.4 SEPARATION OF C-SUBUNIT ON HYDROXYAPATITE

The column was developed with a linear gradient from 45-500 mM-phosphate. Protein was monitored by absorbance at 280 nm. Kinase activity was assayed as described in Figure 3.3. The fractions with highest activity (48-62) were pooled and concentrated before dialysis into COLUMN BUFFER containing 200 mM-NaCl.

Figure 3.4 Separation of Catalytic Subunit of cAMP-PK on Hydroxyapatite



mM-phosphate (Fig. 3.4). The peak fractions (48-62) were pooled and concentrated by vacuum dialysis, then dialysed into COLUMN BUFFER containing 200 mM-NaCl.

The catalytic subunit is basic at neutral pH so it can bind to hydroxyapatite through the phosphate sites with hydroxyapatite acting as a cation exchanger or affinity matrix. The situation is similar to that for phosphocellulose and may explain the appearance of a minor peak of activity prior to the major one. It is likely that the two peaks of activity represent the catalytic subunit binding in the two possible modes.

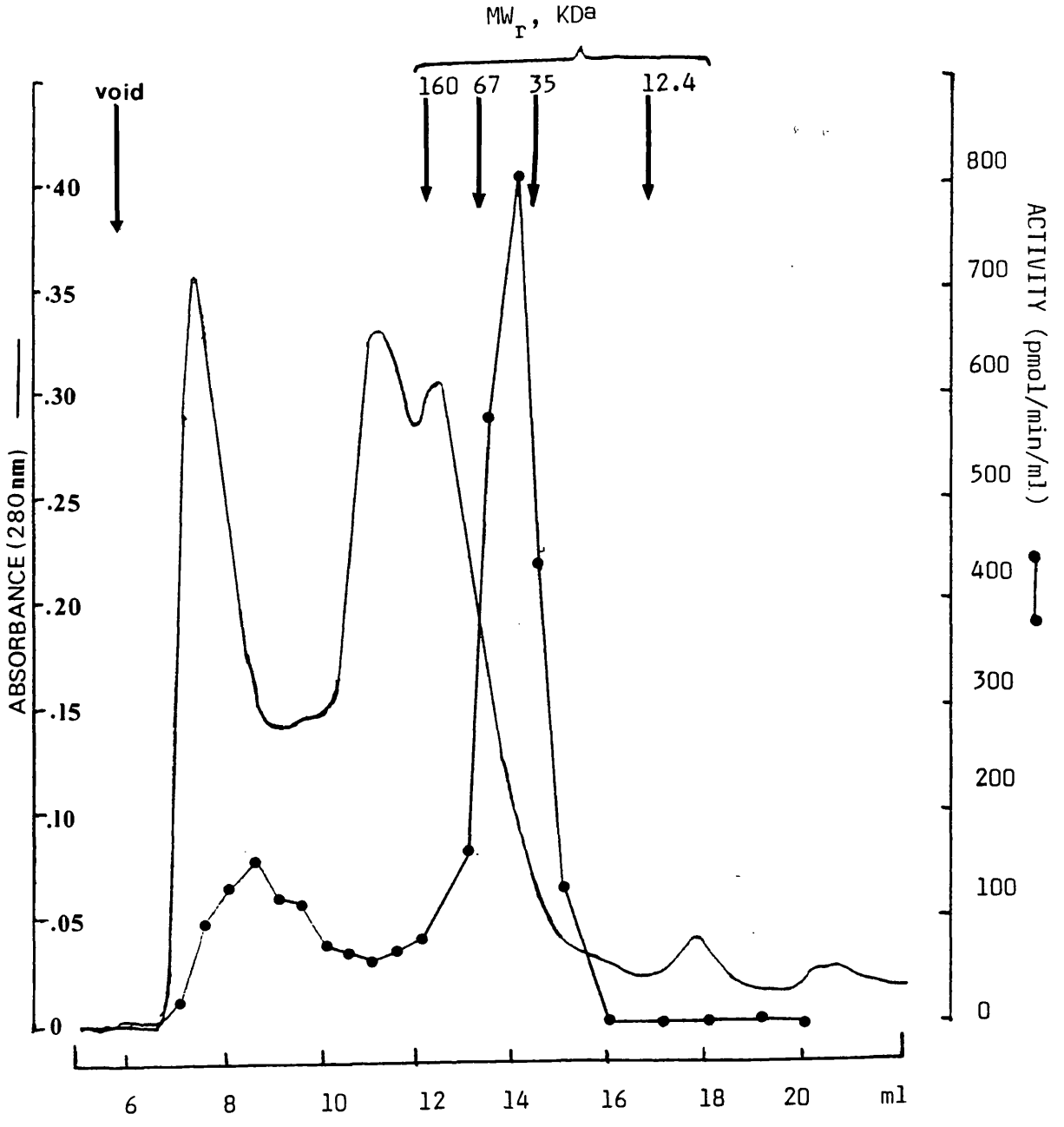
The two forms of catalytic subunit isolated on cation exchange columns by Kinzel et al (1987) separate with only a 10 mM change in salt concentration. It is therefore unlikely that the two peaks shown in Fig. 3.4 which are separated by nearly 100 mM-phosphate correspond to the isoforms he describes.

3.9.3 SEPARATION BY GEL FILTRATION

The preparation was further purified on a Superose 12 gel filtration column (Pharmacia) (Fig. 3.5). This proved to be a very good step as most of the protein loaded eluted before the kinase activity. A small amount of activity appeared in the void volume. A sharp peak of activity was resolved at 14 ml which is equivalent to 47,000 MWr. The MWr of cAMP-PK catalytic subunit is generally quoted as 40,000 as judged by SDS gel electrophoresis. Such accuracy is not possible with Superose 12 gel filtration. The difference of 7000 is within the range of accuracy possible with Superose 12 as the range of the column is <1000 to approximately 160,000 over 10 ml, i.e. 15,000 MW_r per ml. The peak activity fractions were pooled and concentrated by vacuum dialysis before dialysis into Hepes buffer (50 mM-Hepes/NaOH, pH 7.4, 1 mM-EDTA, 1 mM-EGTA, 50 mM-NaF, 50 mM-NaCl, 2 mM-DTT, 30% (w/v) glycerol). The kinase preparation was stored at 4°C.

Figure 3.5 PURIFICATION OF C-SUBUNIT BY MOLECULAR WEIGHT

The concentrated peak fractions from hydroxyapatite were dialysed into COLUMN BUFFER containing 200 mM-NaCl and then chromatographed on a Superose-12 column equilibrated with the same. The column was developed at 0.5 ml/min. The protein was monitored continuously at 280 nm and 0.5 ml fractions collected. Kinase activity was monitored by phosphorylation of histone IIA as described in Fig. 3.3 The elution volumes of molecular weight standards are indicated by the arrows at the top of the figure.



3.10 SUBSTRATE SPECIFICITY OF cAMP-PK FROM LACTATING RAT MAMMARY GLAND AND RAT HEART

Table 7 shows that the substrate specificities of the cAMP-PK catalytic subunit from mammary gland and heart are not identical. The table shows that ACC is not a good substrate for cAMP-PK from rat heart however more than 1.0 mole of phosphate per mole of ACC can be transferred by the kinase during a 60 minute incubation. In contrast cAMP-PK from mammary gland phosphorylates ACC to a negligible extent.

These results show that the catalytic subunit of cAMP-PK is not a functionally invariant enzyme.

The inability of cAMP-PK from mammary gland to phosphorylate ACC yet retain activity against other substrates which is comparable to that achieved by the heart enzyme provides a clear and complete explanation for the results of several authors showing that elevation of cAMP-PK activity in mammary tissue has no direct effect on lipogenesis or the phosphorylation state of ACC in the gland.

Table 8 shows assays that were performed using the holoenzyme for both mammary gland and heart in the presence of 10 μM cAMP. cAMP-PK from mammary tissue is only stable in the undissociated form and so the heart enzyme was also used as holoenzyme so that assays could be directly comparable. The results show that bovine heart cAMP-PK phosphorylates the synthetic peptide 'SSMS' nearly as well as it phosphorylates Kemptide. The mammary gland cAMP-PK also phosphorylates SSMS though it is a poorer substrate than Kemptide.

Table 7 Substrate specificity of cAMP-dependent protein kinase from lactating rat mammary gland and rat heart.

The values show initial velocities measured at the indicated substrate concentrations and are expressed relative to histone.

Substrate	Substrate concentration(mg/ml)	Relative rate for cAMP-PK Mammary gland	Heart
Histone	0.8	100	100
Acetyl-CoA Carboxylase	0.48	<1	17
ATP-citrate lyase	0.24	50	7
Glycogen synthase	0.17	130	110
Phosphorylase kinase	0.67	310	240
Casein	2.00	53	26
Kemptide	0.133	2850	1100

Table 8 Phosphorylation of synthetic peptide substrates by cAMP-PK from bovine heart and rat mammary gland.

Substrate	Substrate concentration μM	Bovine heart cAMP-PK	Mammary Gland cAMP-PK
Kemptide	170	100	100
SSMS	100	70	12

Peptide 'SSMS' is the name given for the synthetic peptide corresponding to residues 73 to 85 inclusive on ACC; it has two extra arginines on the C terminus.
[His-Met-Arg-Ser-Ser*-Met-Ser-Gly-Leu-His-Leu-Val-Lys-Arg-Arg](Carling et al 1989). * denotes the serine phosphorylated by cAMP-PK. The results are expressed as initial rates of phosphorylation relative to that for Kemptide. For heart and mammary enzyme 100% represented the same kinase activity to within ± 5%.

The results in Table 8 are surprising when compared to those in Table 7 where ACC is shown to be a poor substrate for both cAMP-PKs. They indicate that the secondary and/or tertiary structure of ACC must play an important role in determining substrate recognition by cAMP-PK. ACC holoenzyme is a poor substrate for cAMP-PK but when the

phosphorylation site was presented as a short primary sequence of fifteen residues (the 'SSMS' peptide) it proved an 800 fold better substrate for heart cAMP-PK. Similarly a 3000 fold difference in the rate of phosphorylation between ACC holoenzyme and Kemptide was reduced to an eight fold different between the phosphorylation of 'SSMS' and Kemptide for mammary gland cAMP-PK.

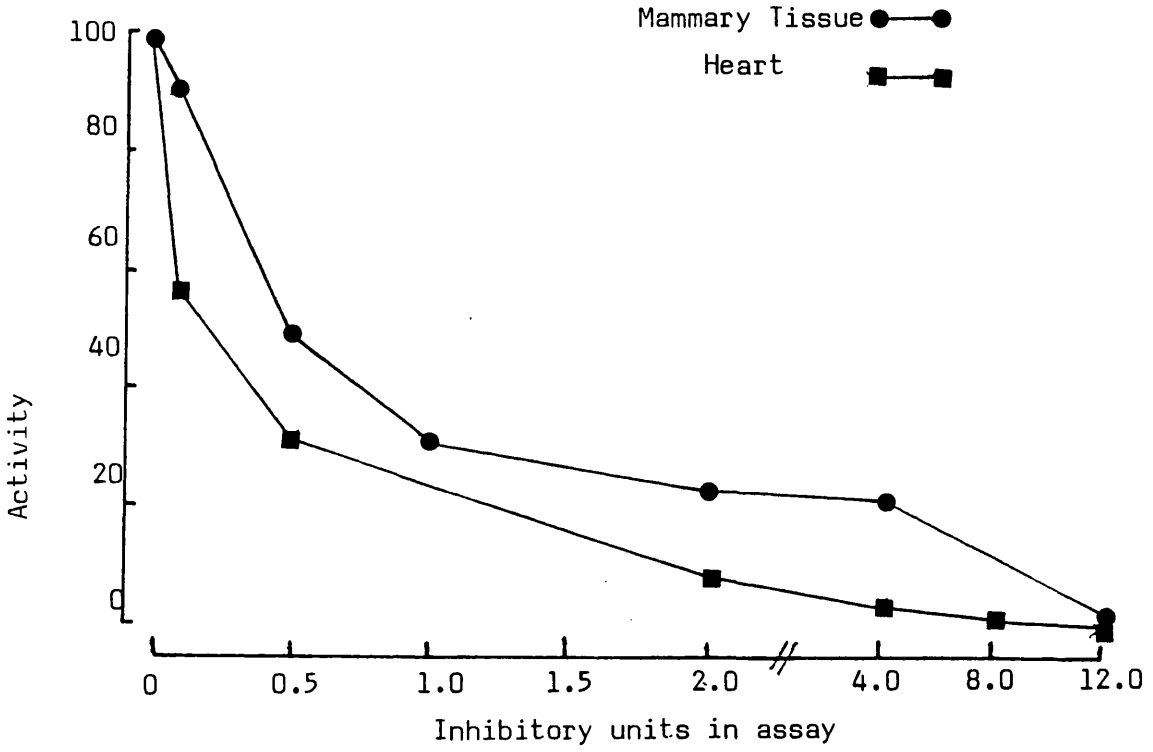
Heart cAMP-PK phosphorylated 'SSMS' six times better than the mammary enzyme indicating that fundamentally the difference in the rates of phosphorylation of native ACC was due to the varying specificity with which the two catalytic subunits recognised the primary sequence. The heart catalytic subunit appears to have less stringent requirements for phosphorylation sites than the mammary enzyme. It phosphorylates the sequence His-Met-Arg-Ser-Ser*-Met-Ser ('SSMS') with 70% of the efficiency with which it phosphorylates Leu-Arg-Arg-Ala-Ser*-Leu-Gly (Kemptide) whereas the mammary kinase phosphorylates 'SSMS' at only 12% of the rate with which it phosphorylates Kemptide. Perhaps mammary gland cAMP-PK is more stringent in its requirement for two basic amino acids prior to the target serine (i.e. Lys/Arg-Arg-X-Ser) as described by Cohen (1985).

3.11 SENSITIVITY OF cAMP-PK CATALYTIC SUBUNIT FROM RAT MAMMARY GLAND AND RAT HEART TO THE SPECIFIC PEPTIDE INHIBITOR OF cAMP-PK.

When a similar total activity of cAMP-PK activity from both mammary gland and heart was assayed in the presence of the specific peptide inhibitor of the catalytic subunit it was found that the mammary gland exhibited 'resistance' to inhibition. This 20 amino acid synthetic peptide is identical to the cAMP-PK binding site on the native protein inhibitor of cAMP-PK known as Walsh inhibitor. The Ki for the catalytic subunit was approximately 0.5 versus 0.2 inhibitory units for the heart enzyme (Fig. 3.6). The reasons for this are unclear, it is possible that the

Figure 3.6 INHIBITION OF CATALYTIC SUBUNIT FROM MAMMARY GLAND TISSUE AND HEART BY THE SPECIFIC PEPTIDE INHIBITOR OF cAMP-PK

The initial activity of the catalytic subunits was assayed as described in Materials and Methods with Kemptide as the phosphate acceptor and is expressed as a % of the activity in the absence of peptide inhibitor. Inhibitory units are defined by Sigma Chemicals as the inhibition of the transfer of 1.0 pmol of phosphate from [γ^{32}P]ATP to hydrolysed and partially dephosphorylated casein per minute at pH 6.5 at 30°C.



mammary catalytic subunit is structurally different from that in the heart. This is likely considering its different substrate specificity.

The mammary free catalytic subunit unlike the heart enzyme is highly unstable which greatly hindered its purification. It is therefore possible that the higher Ki observed is due to some of the inhibitor binding to already inactive catalytic subunit present in the preparation.

3.12 SUMMARY AND CONCLUSIONS

1. The activity of cAMP-PK in mammary gland does not change upon 24 hour starvation or 2 hour refeeding of standard chow. It bears no correlation with the rate of lipogenesis or activity of ACC *in vivo* even when the activity of cAMP-PK is elevated. These results show by direct assay of cAMP-PK activity that cAMP-PK is not involved directly in the regulation of ACC and lipogenesis in mammary tissue.

2. Selective activation of isoenzymes type I and II does not occur in mammary tissue during starvation, refeeding or isoprenaline treatment unlike in liver (Chan et al, 1979, Ekanger et al, 1988, Livesey 1982), breast cancer cells (Livesey 1984) osteoblasts or parotid salivary gland (Schwoch 1987).

3. Isoprenaline treatment increased the activity ratio of cAMP-PK in both tissue and isolated acini. A substantial depression of 'total' activity as well as an increase in basal activity was observed. The decrease in total activity was due to loss of catalytic subunit through binding to sedimentable proteins not proteolysis as occurs in intestinal brush border membranes (Alhanaty et al 1979, 1981, 1985).

4. The cAMP-PK from rat mammary gland has a different substrate specificity profile to that from bovine heart.

Most significantly ACC does not seem to be a substrate for the mammary enzyme. This explains why *in vivo* even sustained elevation of cAMP-PK activity produces no change in the activity of ACC in the mammary gland. However cAMP-PK purified from mammary tissue does phosphorylate Kemptide and also the synthetic peptide 'SSMS' corresponding to the known cAMP-PK phosphorylation site on ACC. The result shows that the secondary and perhaps tertiary structure of ACC are important for the substrate recognition mechanism of cAMP-PK. The primary sequence of ACC also plays a part in making it an especially poor substrate for mammary cAMP-PK. The heart enzyme phosphorylates 'SSMS' six times more rapidly than the mammary enzyme, and nearly as well as it phosphorylates Kemptide. This suggests that the heart catalytic subunit has less stringent requirements than the mammary catalytic subunit for the recognition of phosphorylation sites.

5. Mammary gland catalytic subunit has a lower affinity than the heart enzyme for the specific peptide inhibitor of cAMP-PK. This may be due to the subunit having a different structure or the presence of inactive catalytic subunit in the preparation.

CHAPTER 4

AMP-ACTIVATED PROTEIN KINASE (AMP-PK) IN RAT LACTATING MAMMARY GLAND

It is well established that ACC is regulated by reversible phosphorylation and that phosphorylation by particular kinases is associated with a decline in activity. (Hardie and Guy 1980, Tipper and Witters 1982, Munday and Hardie 1984, Carling et al 1989)

Studies show that 24 hour starvation of lactating rats causes a concomitant increase in the phosphate content of ACC with a decrease in its activity (Munday and Hardie 1986). Until recently the only kinases shown to affect the activity of ACC in lactating mammary gland were (bovine heart) cAMP-PK (Hardie and Guy 1980) and acetyl-CoA carboxylase kinase-2, ACK2 (Munday and Hardie 1984). *In vitro* phosphorylation of ACC by these kinases did not, however, produce changes in the kinetic parameters of ACC that matched those seen in mammary tissue from 24 hour starved rats. Furthermore the activity of cAMP-PK (as discussed in Chapter 3) does not change significantly during starvation and effectors which elevate intracellular cAMP do not inhibit fatty acid synthesis or ACC in rat lactating mammary gland (Williamson et al 1983) or in isolated mammary cells.

The existence in lactating mammary tissue of a cAMP independent kinase capable of phosphorylating and inhibiting ACC was first reported by McNeillie et al (1981) though a cAMP independent protein kinase which inactivated ACC had been first reported in liver in 1973 by Carlson and Kim. Most researchers have, as described in Chapter 1, investigated hepatic ACC kinases. The most recent major advance in these studies has been the purification and characterisation of 5'AMP-activated protein kinase, AMP-PK from rat liver (Carling et al 1989). Phosphorylation *in*

vitro of ACC by this kinase produces an 81% decrease in the Vmax of ACC purified from rat mammary gland (Munday et al 1988a). This kinase if present in the rat mammary gland could easily explain the large (≥50%) decrease in the Vmax of ACC caused by 24 hour starvation. (Munday and Hardie 1986, Chapter 3, Table 3).

This chapter describes the purification of AMP-PK from lactating mammary gland, and also looks at the physiological role of the kinase in the tissue.

4.1. PURIFICATION OF AMP-ACTIVATED PROTEIN KINASE (AMP-PK)

AMP-PK Buffer used for all steps after homogenisation:

20 mM-Tris HCl pH 7.2 at 4°C
0.5 mM-EDTA
0.5 mM-EGTA
5 mM-NaPPi
10% (w/v)-glycerol
5 mM-DTT
0.02%-Brij 35

Protease inhibitor cocktail used unless otherwise stated:

1.0 mM-BZ
4 μg/ml-SBTI
0.1 mM-TLCK

4.1.1 Preparation of Tissue

Rats at peak of lactation (10-15 days post partum) were killed by stunning and cervical dislocation or intraperitoneal injection of sodium pentobarbitone (60mg/kg body weight). The mammary glands were dissected out, flash frozen in liquid nitrogen then ground to a fine powder under liquid nitrogen using a pestle and mortar. The tissue was stored in liquid nitrogen or -70°C until used. The tissue was homogenised in 10 volumes of HOMOGENISATION BUFFER (Section 2.6) in three thirty second

bursts using a domestic blender. The homogenate was taken to 2.5% w/v PEG 6000 by the slow addition, with mixing, of 0.0533 vols of a 50% (w/v) solution of PEG 6000.

The 2.5% PEG 6000 protein suspension was stirred slowly for 20 min at 4°C and then centrifuged at 20,000 g for 20 minutes (Beckman JA21 centrifuge, JA14 rotor). The supernatant was filtered through glass wool to remove floating fat and 0.0747 volumes of 50% PEG 6000 were added slowly with mixing to give a final concentration of 6% (w/v) PEG 6000. The protein/PEG suspension was mixed and centrifuged as described above. The 6% PEG supernatant was discarded and the pellet used immediately or frozen at -20°C until required. Up to this stage the purification is essentially the same as that for ACC. AMP-PK was sought in this fraction because Carling and Hardie (1986) reported that liver AMP-PK was associated with ACC at the earlier stages of purification as is the ACC-Kinase described by Lent and Kim (1982). It was estimated that 96% of the AMP-PK activity in the crude homogenate prepared from lactating rat mammary gland was precipitated by the PEG-6000. (Table 1).

4.1.2 SEPARATION BY ANION EXCHANGE CHROMATOGRAPHY

Anion rather than cation exchange was tried because the liver AMP-PK had been shown to bind to anion exchange columns at pH 7.4 (Carling and Hardie 1986). The DEAE column was used prior to the Mono-Q because the 6% PEG resuspension contained too much protein to be chromatographed on the Mono-Q 5/5 column (which has a maximum capacity of 50 mg protein) in a reasonable number of runs.

The 6% PEG pellet was resuspended in AMP-PK buffer (1/5 of the volume of the 2.5% supernatant) and then applied to a DEAE anion exchange column. (Fig. 4.1) The column was washed with 50 mM-NaCl in the same buffer. Preliminary experiments showed that 50 mM-NaCl removed

Figure 4.1 DEAE ANION EXCHANGE

The pellet was resuspended in one fifth of the volume of the 2.5% PEG-6000 supernatant using AMP-PK buffer with 4 μl/ml SBTI, 0.02 mM-TLCK and 0.2 mM-BZ and then centrifuged at 20,000g for 20 minutes at 4°C. The clarified resuspension was filtered through glass wool and then applied to a DEAE-Sepharose column 50 ml, 10 x 2.5 cm, previously equilibrated with AMP-PK buffer. Loading, washing and elution were performed at 1.5 ml/min.

The column was then washed until the absorbance at 280 nm was zero versus AMP-PK buffer then washed further with AMP-PK buffer plus 50 mM-NaCl. When no further protein was leaving the column, AMP-PK was eluted with 200 mM-NaCl in AMP-PK buffer. Eluate from the second batch elution showed kinase activity versus ACC. The peak of activity, typically 20-30 ml, from the 200 mM-NaCl elution was pooled and dialysed into 100 volumes of NaCl-free AMP-PK buffer overnight.

Protein was measured in each fraction by absorbance at 280 nm. Kinase activity was measured using 5 μl of eluate in a 25 μl assay with ACC (0.48 mg/ml) or Histone IIAS (2 mg/ml) as substrate. Assays were conducted at 30°C in AMP-PK buffer lacking NaPPi but with 50 mM-NaF, 0.2 mM [γ^{32}P]-ATP (0.1 μCi/nmol) and 4 mM-$MgCl_2$. The assays were stopped by spotting 20 μl of the incubation onto phosphocellulose paper squares when histone was substrate or by the addition of 1 ml of 25% (w/v) trichloroacetic acid when ACC was substrate.

Figure 4.1

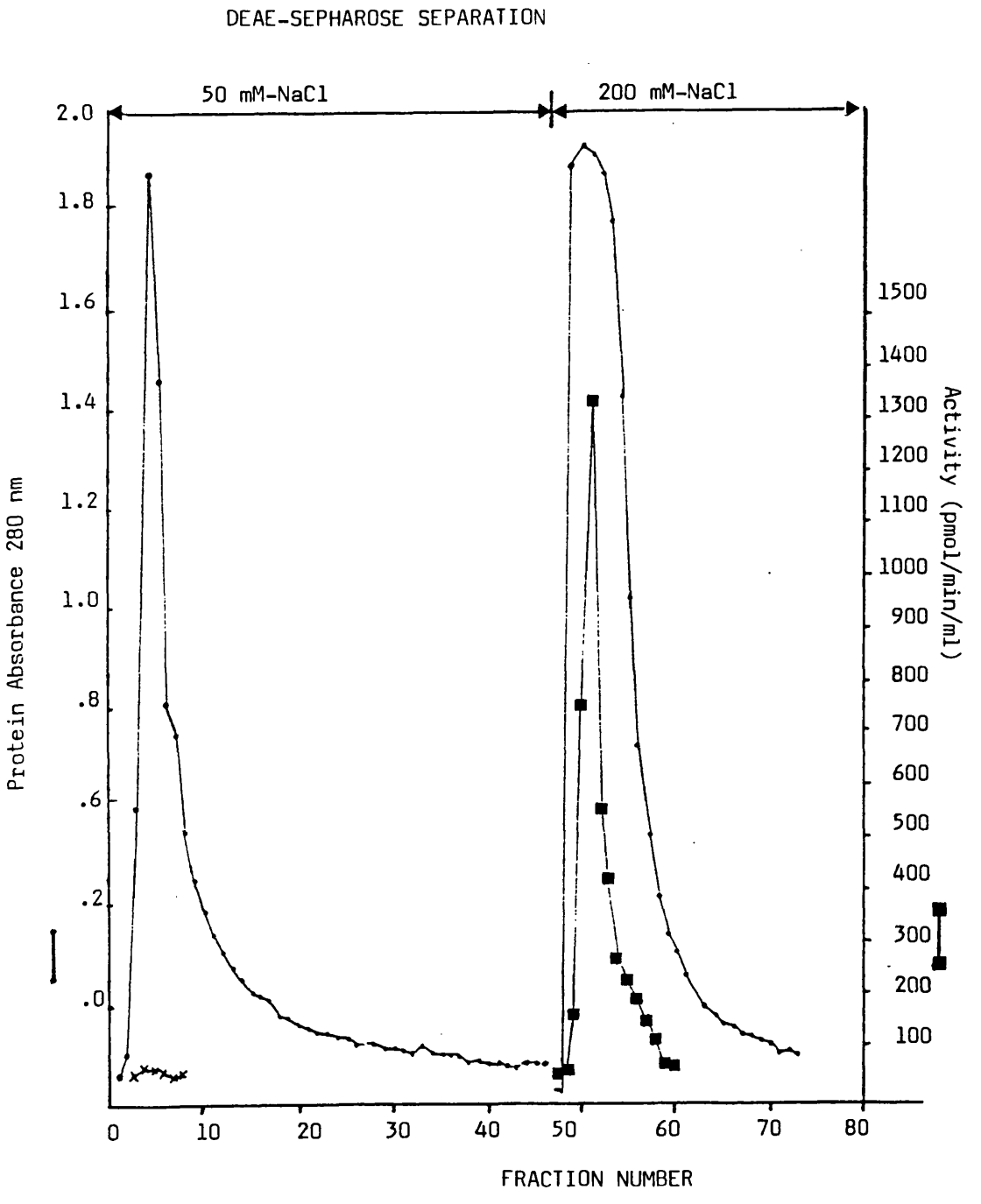

DEAE-SEPHAROSE SEPARATION
50 mM-NaCl
200 mM-NaCl
Protein Absorbance 280 nm
Activity (pmol/min/ml)
FRACTION NUMBER
2.0
1.8
1.6
1.4
1.2
1.0
.8
.6
.2
.0
1500
1400
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
10
20
30
40
50
60
70
80

Figure 4.2 FPLC-MONO-Q ANION EXCHANGE

The dialysed DEAE eluate was centrifuged at 50,000 g for 20 mins at 4°C. The supernatant was filtered using a 0.2 μ nitrocellulose filter (Millipore Inc) just prior to loading it onto a Mono-Q 5/5 anion exchange column (Pharmacia LKB).

After loading, the column was washed with 10 ml of AMP-PK buffer. The column was then developed with a linear gradient of zero to 400 mM-NaCl in AMP-PK buffer over 20 ml. Activity eluted between 200 and 250 mM-NaCl. Protein was monitored continuously by absorbance at 280 nm using a flow through UV meter (Pharmacia). Kinase activity was monitored as described in Figure 4.1.

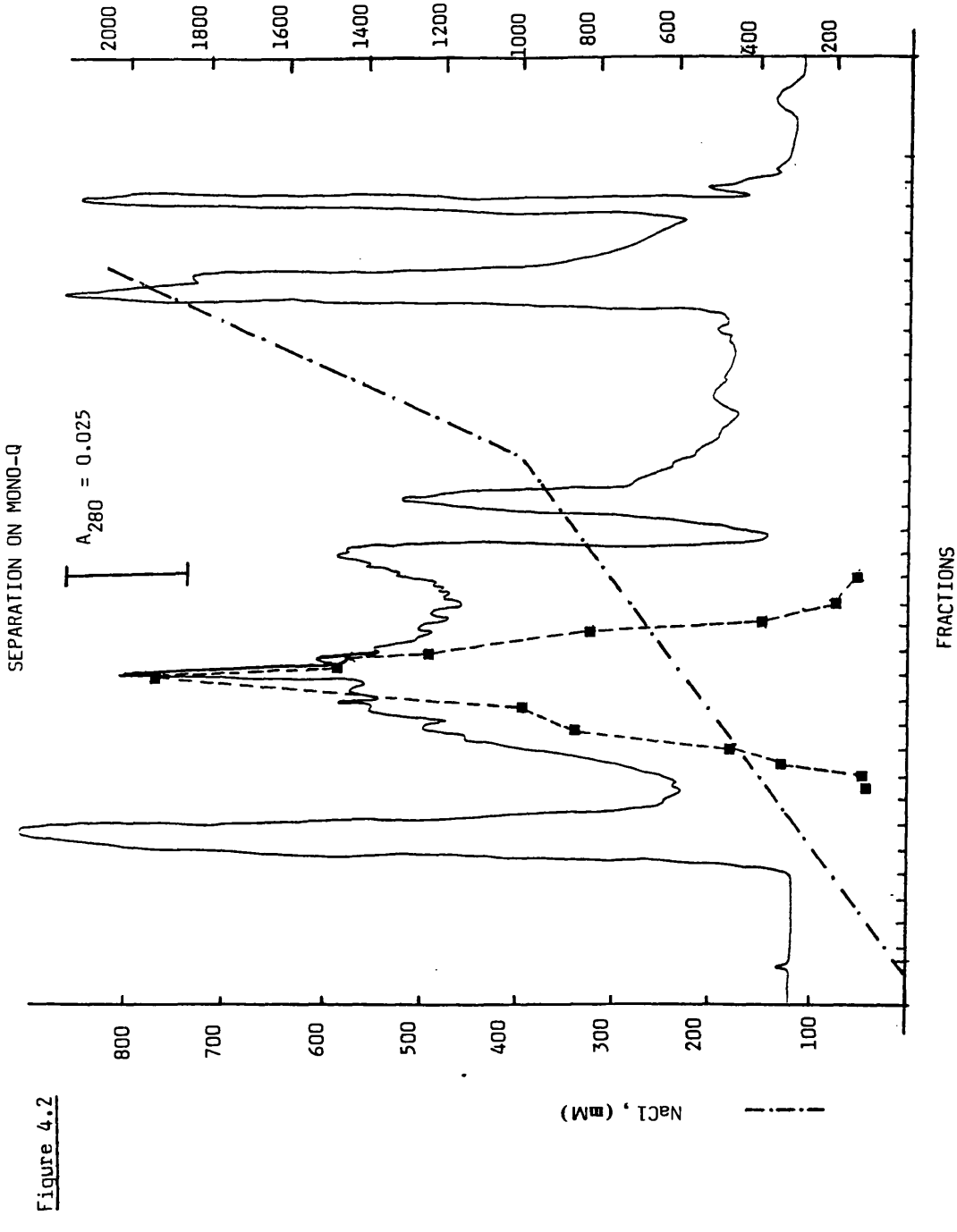

SEPARATION ON MONO-Q
A_{280} = 0.025
NaCl , (mM)
FRACTIONS
2000
1800
1600
1400
1200
1000
800
600
400
200
800
700
600
500
400
300
200
100

Figure 4.2

significant amounts of protein but no AMP-PK activity was eluted, and so this step was added to improve purification. AMP-PK activity was eluted in the 200 mM-NaCl step elution. The peak of kinase activity from the batch elution was pooled and dialysed into 100 volumes NaCl free AMP-PK buffer. The dialysed DEAE elute was applied to a Mono-Q anion exchange column (Fig. 4.2) and developed using a 0-400 mM-NaCl linear gradient in the AMP-PK buffer. The peak of kinase activity eluted from Mono-Q at approximately 230 mM-NaCl. AMP-PK activity eluted at a higher salt concentration from Mono-Q than DEAE because of tighter binding to the matrix. Table 1 shows that there was significant loss of kinase activity at the DEAE and Mono-Q anion exchange steps. This can be attributed to the loss of AMP-PK activity during dialysis. Approximately 50% of activity was lost after 36 hours at 4°C and 75% after approximately 60 hours at 4°C, but loss of activity was negligible when the kinase solution was stored at -20°C. It is reasonable to assume that activity was being lost continuously once the kinase was eluted from the DEAE column. The peak of protein from the 200 mM-NaCl batch elution was therefore usually pooled and dialysed into salt free AMP-PK buffer without assay of the individual fractions. Also the time that the dialysed DEAE eluate was at room temperature during the use of the Mono-Q column was kept to a minimum (less than 2 hours).

4.1.3 SEPARATION USING AFFINITY CHROMATOGRAPHY

After dialysis into salt free buffer the pool of high activity eluate from Mono-Q was loaded onto blue agarose which has the blue dye Cibacron F3G-A covalently attached to the matrix. Cibacron F3G-A has a three dimensional structure similar to that of purine nucleotides. Thus it was expected to show affinity for AMP-PK which has sites for both ATP and AMP. Figure 4.3 shows that blue agarose was indeed a good purification step as 73% of the recovered activity eluted with 1.0 M-NaCl but with only 16% of the protein.

Most proteins requiring adenylyl-containing substances and also some non-enzyme proteins eg. albumin can bind to blue agarose and these will compete with AMP-PK. As AMP-PK is a small percentage of the total protein in the Mono-Q eluate, its binding was maximised by ensuring that the protein concentration of the solution being loaded was more than 1 mg/ml and also by loading it slowly; typically at 0.25 ml/min. These precautions ensured that greater than 90% of AMP-PK activity was retained on the column. The kinase activity was recovered by batch elutions between 0.5M and 1.0M-NaCl (Fig. 4.3). As mentioned earlier 73% of the AMP-PK activity was recovered in the 1M-NaCl batch elution. A small amount of kinase activity eluted with 0.5M-NaCl This was probably due to a proportion of the kinase binding less specifically and less tightly to the matrix. Batch elution was employed because the protein concentration of the fractions was higher than when a gradient was used and so better recovery of kinase activity was achieved. Thus some purification was sacrificed to preserve the activity as from this stage of the purification the kinase became highly labile in dilute solution. The high affinity activity fractions from the blue agarose affinity chromatography were concentrated and stabilised by dialysis into AMP-PK buffer containing 50% glycerol.

Further purification was achieved by affinity chromatography on histone Sepharose (Fig. 4.4) This column separated AMP-PK through interaction of the protein substrate binding site with the histone rather than through the adenyl nucleotide binding sites as occurs with the blue agarose. No measurable protein was present in the breakthrough from the column, or in the NaCl free wash. Typically 90% of recovered activity was present in the 0.25M to 0.5M-NaCl cut (See Fig. 4.4) together with approximately 50% of the protein. 1M-NaCl did not remove further protein from the gel. Inspection of Table 1 and the amount of protein recovered suggests that this should have given a two fold purification however an apparent reduction in specific activity was measured. This

Figure 4.3 SEPARATION ON BLUE AGAROSE

The blue agarose gel (Sigma) was swollen in AMP-PK buffer containing 1.0 mg/ml BSA to block the very high affinity protein binding sites. A 25 ml column (12 x 1.0 cm) was packed and washed extensively with 1M-NaCl in 100 mM-Tris HCl (pH 7.4 at 25°C) then equilibrated with AMP-PK buffer. The dialysed peak fractions from the Mono-Q separation were applied to the blue agarose and the gel washed with AMP-PK buffer until absorbance at 280 nm was zero. Protein was eluted batchwise in two steps using the same buffer containing 0.5M and 1.0M-NaCl respectively. Protein concentration and kinase activity were assayed as described in Fig. 4.1.

The peak fractions from the 1.0M-NaCl elution were pooled and dialysed overnight into twenty volumes of AMP-PK buffer containing 50% (w/v) glycerol. Dialysis into 50% glycerol was used for concentration and stabilisation of the kinase activity.

Figure 4.3 SEPARATION ON BLUE AGAROSE

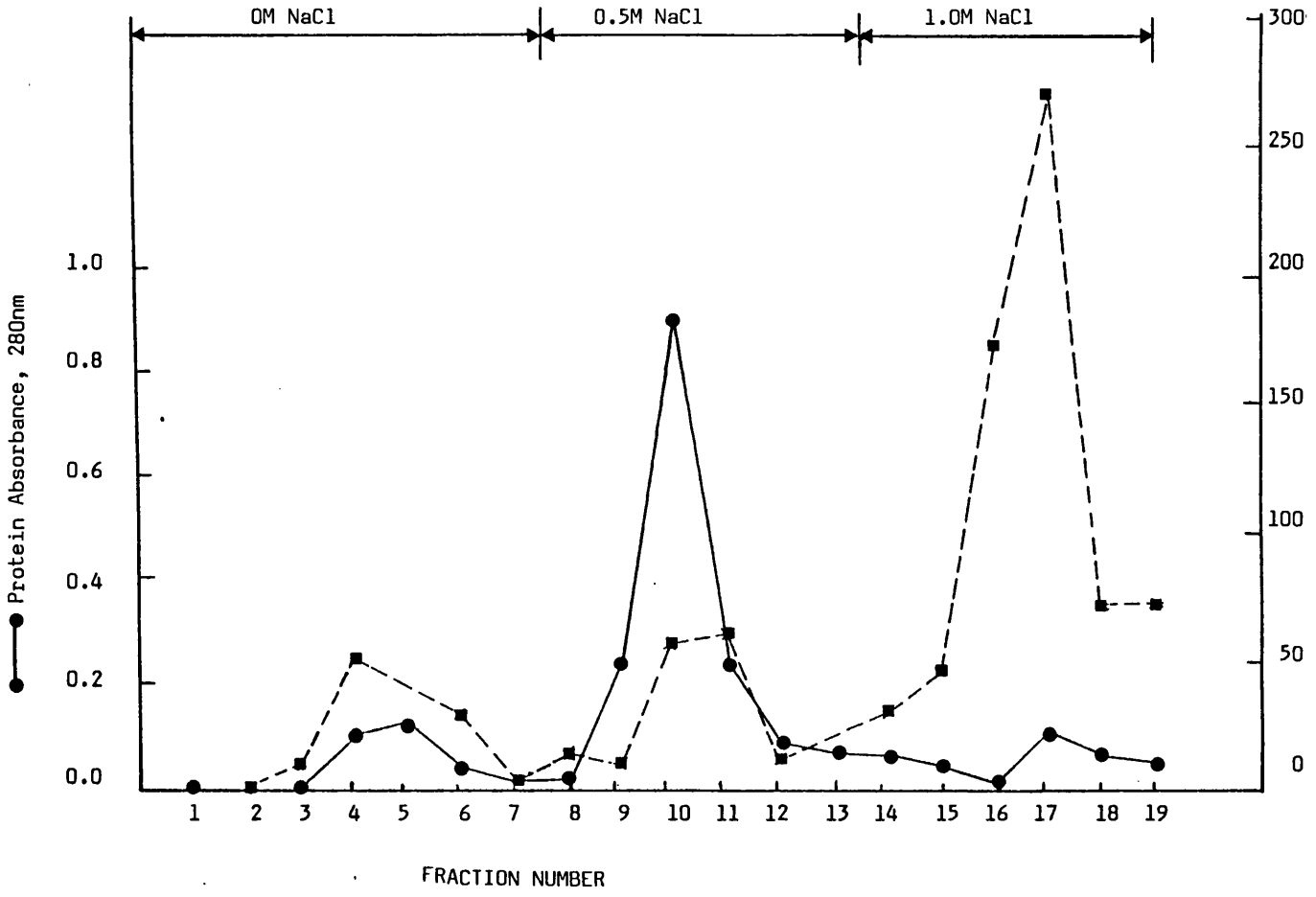


Figure 4.4 SEPARATION ON HISTONE SEPHAROSE

Histone-Sepharose affinity gel was prepared as described in Materials and Methods and a 10 ml (6 x 0.7 cm) column packed. The gel was equilibrated with AMP-PK buffer and the pooled and dialysed activity from the blue agarose step loaded. The gel was washed with AMP-PK buffer until absorbance at 280 nm was zero. Protein was eluted from the column batchwise in two steps using 0.25M and 0.5M-NaCl. Protein and kinase activity were assayed as described in Fig. 4.1.

No measurable protein was present in the breakthrough from the column or in the salt free wash. Typically 90% of recovered activity was present in the 0.5M-NaCl eluate together with the rest of the protein. 1M-NaCl did not remove further protein from the gel.

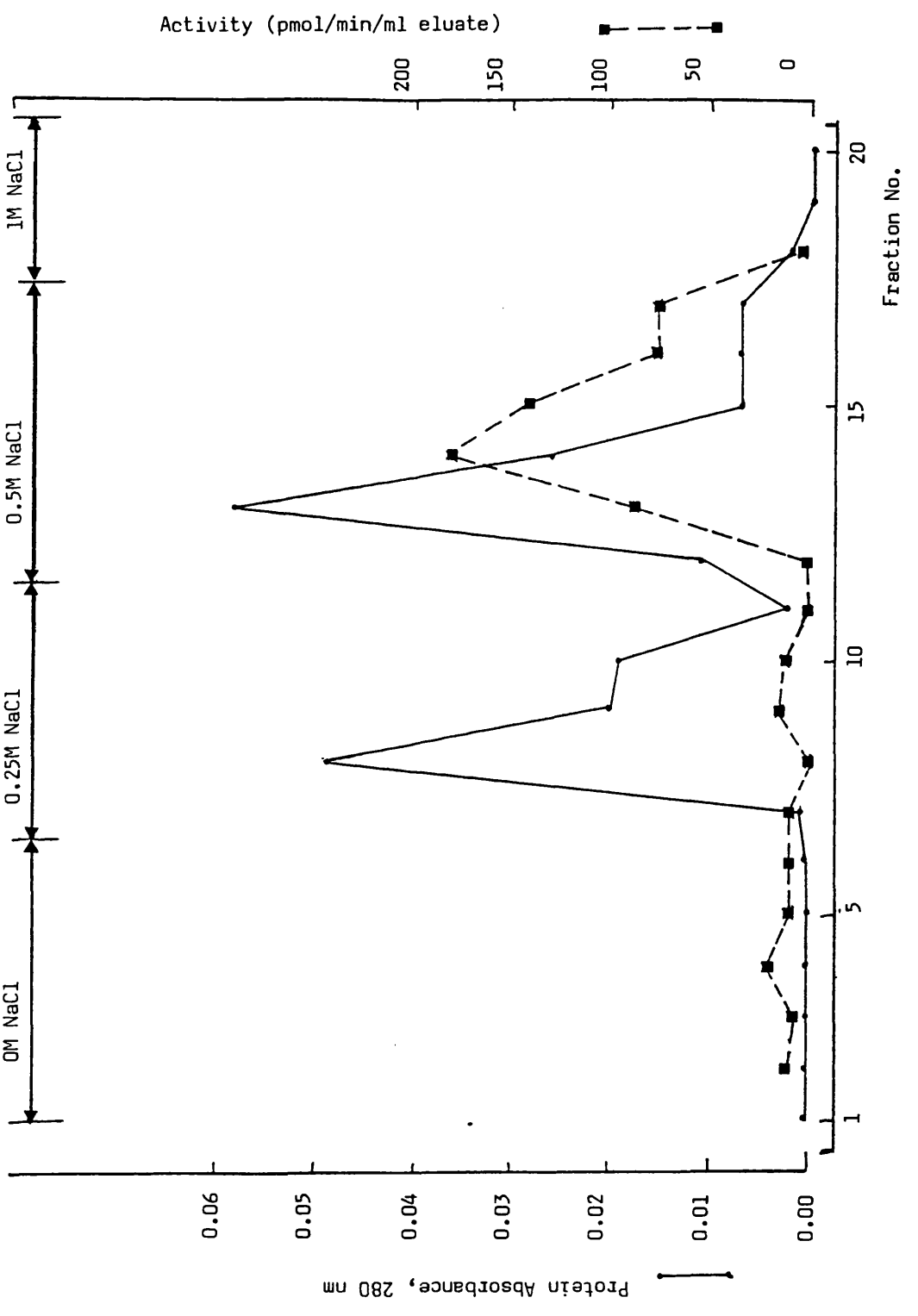


Figure 4.4 Separation on Histone Separation

reduction was due to loss of kinase activity as it emerged in dilute solution from the column and the delay while the fractons were assayed. As with the blue agarose step batch elution was used because the method was faster than gradient elution and so more kinase activity was recovered. The fractions with highest activity were pooled then concentrated to 0.5 ml first by dialysis against dry PEG-20,000 and finally by ultrafiltration using Centricon apparatus with a 30,000Da cut off.

Phosphocellulose and ATP agarose columns were also tested as affinity purification steps. Both columns bind proteins which catalyse ATP dependent reactions. However neither proved successful with AMP-PK, due to poor binding of the kinase to the matrix. It is likely that the pyrophosphate ions present in the buffer interfered with the binding of the kinase through competition between the free pyrophosphate and the immobilised ligand for the ATP site. The phosphatase inhibitor was necessary however to maintain AMP-PK activity and so these column were not used. The success of the blue agarose steps (with the precautions mentioned) would suggest that the AMP rather than the ATP site may be the important factor in the binding of the kinase to this matrix.

4.1.4 SEPARATION ON THE BASIS OF MOLECULAR WEIGHT

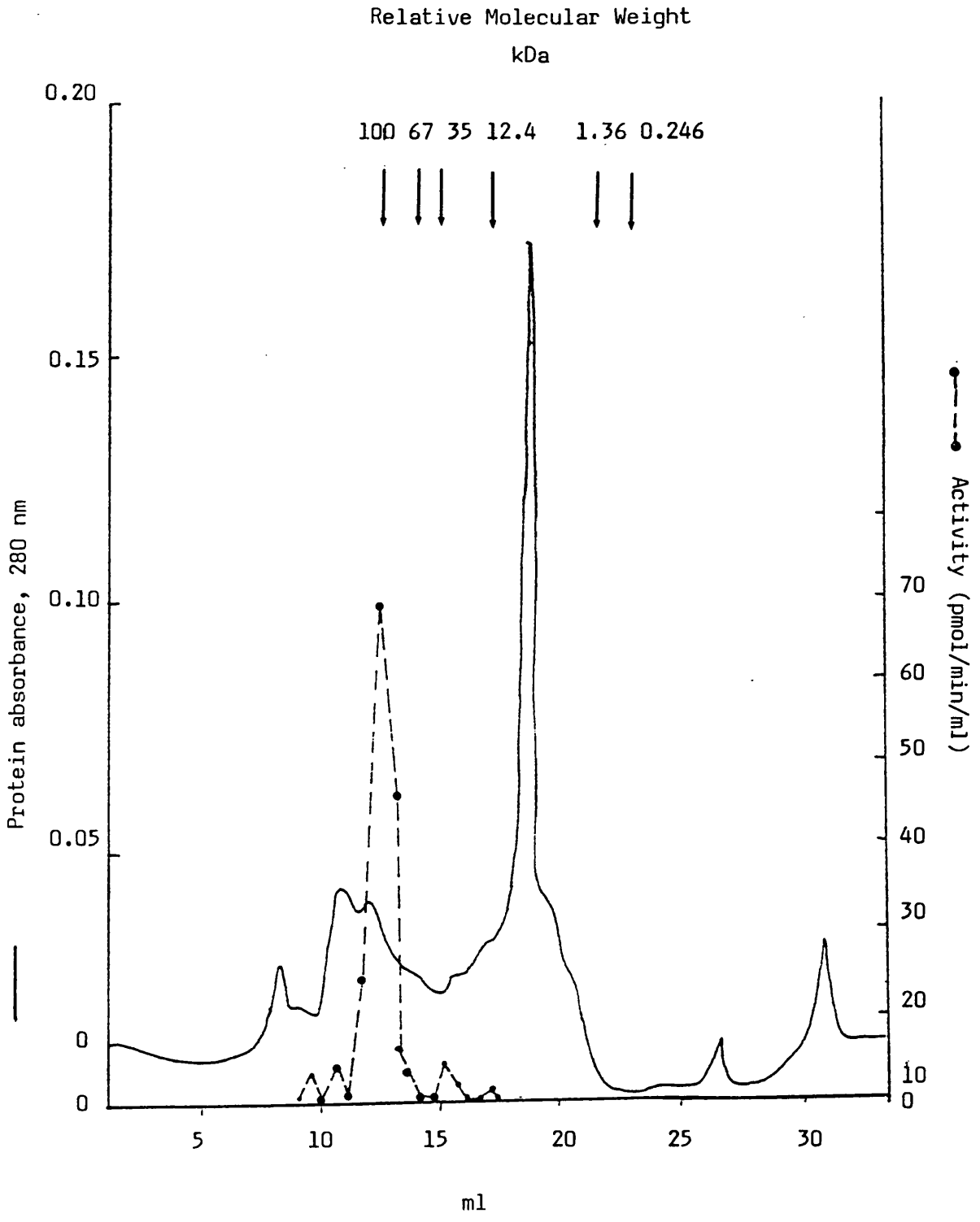
The kinase preparation was finally separated on the basis of molecular weight using a Superose-12 'FPLC' gel filtration column (Fig. 4.5) which has a range of approximately 150,000Da over 10 ml. AMP-PK activity was separated in AMP-PK buffer containing 0.5M-NaCl to minimise non specific protein-protein interactions since kinase activity was detected in the void volume when lower salt concentrations were used. AMP-PK resolved at 12 ml which was estimated to be approximately 160,000Da as determined by calibration with protein standards supplied by Pharmacia. This molecular weight is slightly larger than the 100 $\pm$ 30 kDa quoted by Carling et al (1989) for the

Figure 4.5 SEPARATION BY SUPEROSE-12 GEL FILTRATION

The concentrated histone eluate was applied to a Superose-12 gel filtration column (Pharmacia) and run in 0.5M-NaCl AMP-PK buffer at 0.5 ml/min.

The column was calibrated using standards supplied by Pharmacia and the kinase estimated to have a relative molecular weight of approximately 160,000. Protein was monitored continuously at 280 nm and kinase measured as described in Fig. 4.1.

Figure 4.5


Relative Molecular Weight
kDa
100 67 35 12.4 1.36 0.246
0.20
0.15
0.10
0.05
0
0
Protein absorbance, 280 nm
70
60
50
40
30
20
10
0
Activity (pmol/min/ml)
5
10
15
20
25
30
ml

liver AMP-PK. The peak of activity from gel filtration was pooled and concentrated by ultrafiltration using Centricon apparatus with a 30,000Da cut off and stored at -20°C.

Gel filtration using a conventional gel filtration column (packed with 123 ml Sepharose S-200 (Pharmacia) and with the dimensions 61 x 1.6 cm) was not satisfactory as a purification step because of the slow flow rates required to give good resolution and also the great dilution of protein activity that occured. The peak of kinase activity corresponded to a relative molecular weight of approximately 160,000 in concordance with the result from the Superose-12 column.

Purification by molecular weight was also attempted using non-denaturing polyacrylamide gel electrophoresis, but the recovery of kinase activity from the gel slices was very poor and so this method was not developed.

The purification would normally take 3-4 days with overnight dialysis. The kinase was relatively stable if the protein concentration was high (>3 mg/ml), stored at -20° and not subjected to repeated freeze-thawing. The concentrated Mono-Q or blue agarose eluates were used for most experiments because of their relative stability. The purification method, for reasons of economy, was developed using Histone IIAS in the presence of the specific inhibitor of cAMP-PK, as a substrate in the AMP-PK assay. However, all stages have been performed using the specific peptide substrate for AMP-PK (Davies et al 1989, refer to section 4.2.4 for details of peptide) and these are presented in the Purification Table.

4.1.5 PURIFICATION TABLE FOR AMP-PK

Table 1 shows a typical purification of the AMP-PK from the inguinal mammary tissue of nine rats at peak

Table 1

PURIFICATION TABLE FOR AMP-PK

Fraction	Total Protein mg	Total Activity nmol	Specific Activity nmol/mg	Yield %	Purification
Homogenate	9149	431	0.05	-	1
2.5% PEG	6082	481	0.08	100	1.7
6% PEG	1348	461	0.34	96	7.2
DEAE	116	150	1.29	31	27
Mono Q	15.16	24	1.59	5.0	34
Blue Agarose	2.28	10	4.56	2.2	97
Histone Sepharose	0.452	1.3	2.79	0.25	59
Superose 12	0.054	0.78	14.50	0.16	309

The table shows a typical purification profile using the inguinal mammary tissue from nine rats as starting material. The low apparent purification is due to the instability of the kinase. Thus loss of activity during purification caused the specific activity to be lower than could be expected given that separation of contaminating proteins from the kinase had clearly been achieved.

Protein was measured by the Coomassie dye binding method using BSA as standard (Bradford 1976). The specific AMP-PK peptide substrate 'SAMS' was used. Activity was measured as described in Materials and Methods in 25 μl containing 100 μM 'SAMS'; 0.2 mM-ATP and 4 mM-$MgCl_2$ in AMP-PK buffer lacking NaPPi but with 50 mM-NaF. All activities were linear in their phosphorylation of SAMS with respect to time and concentration of protein added.

lactation. The table shows that a 309 fold purification was achieved. This is a lower figure than the purification profiles would indicate. As mentioned earlier this was largely due to apparent purification as determined by separation of the AMP-PK from other proteins being offset by greater losses in total activity recovered. The specific activity therefore did not increase as expected. This was seen at the Mono-Q step and also at the histone step. With both of these columns attempts were made to minimise the losses of activity rather than abandon the steps because they were good purification steps with respect to protein. Figure 4.6 shows this is especially true for the histone Sepharose step.

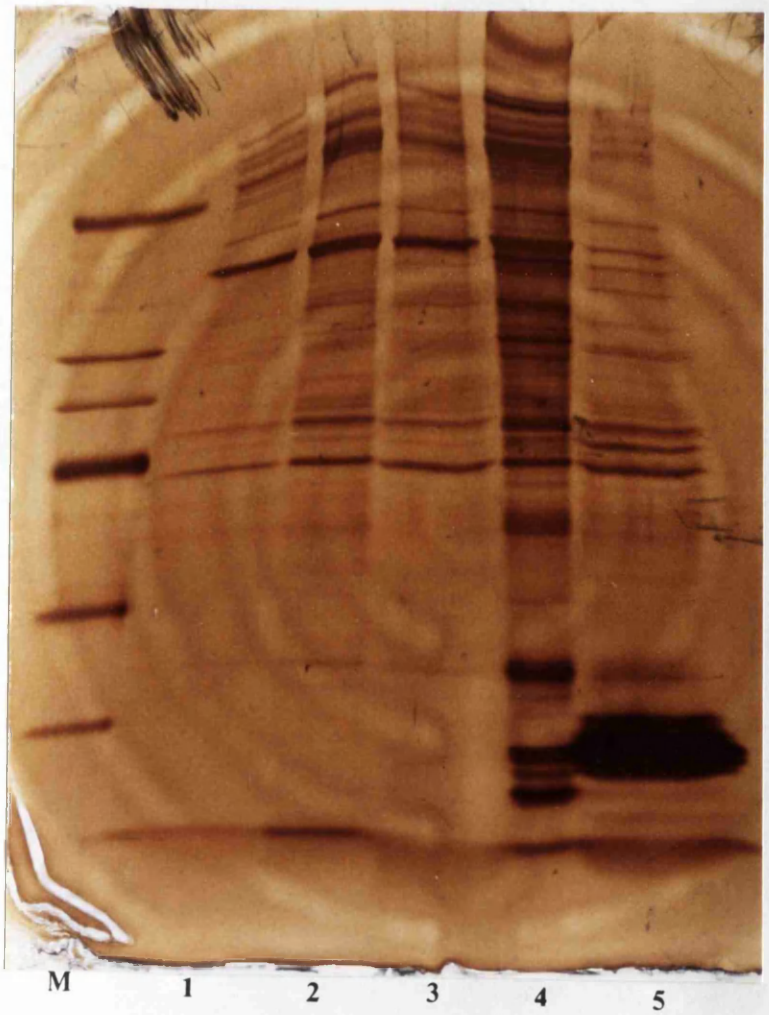
Figure 4.6 shows a silver stained polyacrylamide gel of the most purified fractions of AMP-PK (Lanes 1-3) and of a partially pure gel-filtered sample (Lanes 4 and 5). This gel shows that even the most pure sample of AMP-PK was not homogeneous and consisted of some 20-30 different proteins. However, a number of protein bands were noticeably enriched by the histone-Sepharose purification step compared with the gel filtration. On the basis that the AMP-PK subunit from rat liver has a M_r of 63,000 Da and runs as a dimer on superose 12 gel filtration with a M_r of 100,000 ± 30 Da (Carling et al 1989), one could speculate that a reasonable candidate for the AMP-PK from mammary gland is the protein indicated in Figure 4.6 at $M_r \approx 80,000$ Da which could have run as the dimer of $M_r \approx 160,000$ Da on superose 12 gel filtration shown in Figure 4.5

However, from the data available in Figure 4.6 one cannot rule out the possibility that the prominent band than ran just below the 55 KDa marker represents the AMP-PK from mammary gland that is directly comparable in molecular weight to the 63 KDa liver enzyme. Furthermore, the prominent band at approx. 160,000 Da may equally represent mammary gland AMP-PK which ran as a single subunit on superose 12. Labelling of AMP-PK with the radio-active analogue of ATP, ^{14}C-FSBA, has attempted to resolve this

Figure 4.6 DETAIL OF THE PURIFICATION ON HISTONE SEPHAROSE AND ON SEPHAROSE S-200

The figure shows a 5% - 15% linear gradient polyacrylamide slab gel stained with silver in which the fractions from Histone Sepharose showing peak AMP-PK activity have been electrophoresed. The fractions showing the most AMP-PK activity from a Sepharose-200 gel filtration are also shown.

Lanes 1,2 and 3 show concentrated Histone-Sepharose eluates: 1 - immediately preceding the peak; 2 - immediately following the peak and 3 - the fraction containing the peak of AMP-PK activity. Lane 4 shows the breakthrough from the S-200 gel filtration column and lane 5 a concentrated pool of the resolved proteins from the same column showing the peak of AMP-PK activity.



question (see Fig. 4.17a).

4.2 THE PHOSPHORYLATION OF ACC BY AMP-PK

4.2.1 THE REVERSIBLE PHOSPHORYLATION AND INACTIVATION OF ACC

In order to promote a particular kinase as a candidate for the physiological regulation of ACC it must be established that the kinase phosphorylates ACC reversibly and that the change in covalent modification causes concurrent inactivation and reactivation of the ACC of a similar magnitude to that seen *in vivo*. These criteria are satisfied by AMP-PK which reversibly phosphorylates and inactivates ACC. (Fig. 4.7)

The activity of ACC was determined at 0.5 mM citrate, (the physiological concentration), in parallel experiments containing non-radioactive ATP. After five minutes incubation with AMP-PK 0.55 moles of phosphate were incorporated into ACC and the activity had fallen by 70%. A further mole of phosphate was incorporated over the next 55 minutes but this only produced a further 20% fall in activity. At 60 min phosphorylation was stopped by the addition of EDTA to 15 mM and dephosphorylation started by the addition of protein phosphatase-2A to 5 u/ml. This caused the loss of 0.85 moles of phosphate within five minutes and was accompanied by the regaining of 55% of ACC activity. A further 0.3 moles of phosphate were removed after 60 minutes and 100% of activity was regained.

4.2.2 EFFECT OF AMP-PK ON THE KINETIC PARAMETERS OF ACC

Table 2 shows the effect on the kinetic parameters of ACC when phosphorylated by cAMP-PK, acetyl-CoA carboxylase kinase 2 (ACK2) or AMP-PK; the three kinases known to alter the activity of ACC. Neither cAMP-PK nor ACK2 caused large decreases in the Vmax of ACC (13% and 20% respectively). Their primary effect was to decrease the sensitivity of ACC

Figure 4.7 THE REVERSIBLE PHOSPHORYLATION AND INACTIVATION OF ACC BY AMP-PK

The figure shows the results of one typical experiment. Phosphorylation and activity are expressed as a percentage of the control. The control was ACC incubated with ATP but no AMP-PK.

Phosphorylation of ACC was conducted as described in Materials and Methods. Separate phosphorylations were performed simultaneously using non-radioactive ATP and [γ^{32}P]ATP together with appropriate controls to monitor endogenous phosphorylation in the ACC and AMP-PK preparations. Aliquots from the non-radioactive phosphorylation were removed at the times indicated, diluted appropriately and the ACC activity measured immediately at 0.5 mM citrate as described in Materials and Methods for pure ACC. At the same time points aliquots from the radioactive phosphorylation were stopped with 10% trichloroacetic acid and processed as described in Materials and Methods to measure the amount of radioactive phosphate incorporated. The arrow indicates the point at which EDTA and protein phosphatase-2A were added to respectively stop the phosphorylation reaction and initiate the dephosphorylation. Experimental details are described in the text.

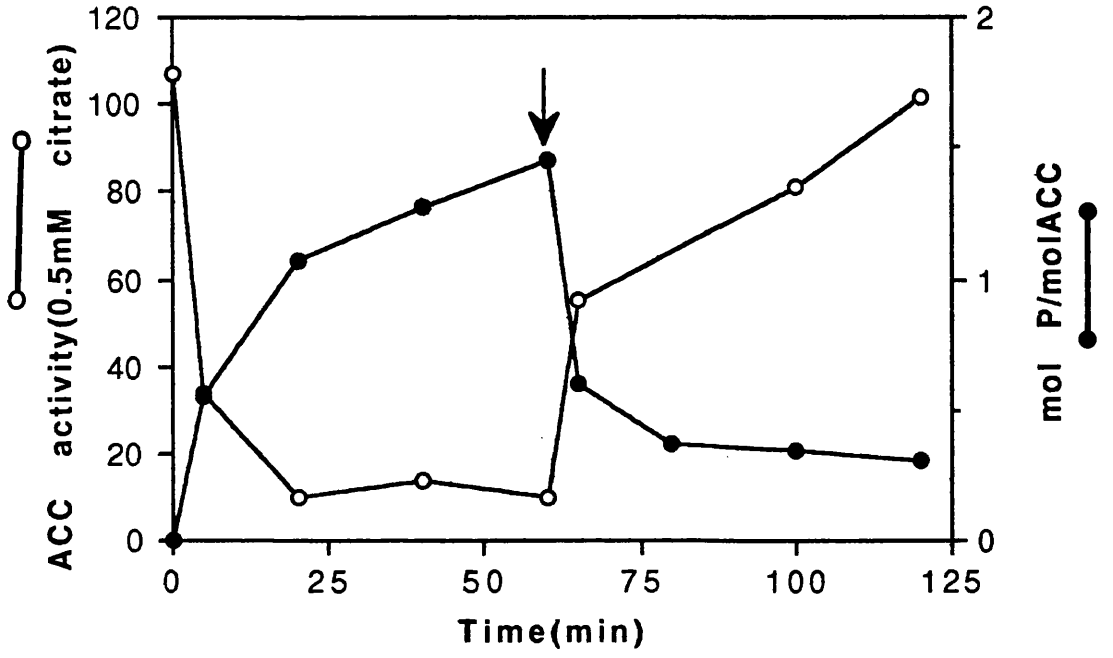


Table 2 KINETIC PARAMETERS OF PURIFIED MAMMARY ACC FOLLOWING PHOSPHORYLATION BY PROTEIN KINASES IN VITRO

KINASE	PHOSPHATE INC. mol/mol ACC)	Vmax (Units/mg)	A0.5 CITRATE (mM)
None	0.03	3.42	4.14
AMP-PK	1.92	0.48(86%↓)	7.79(88%↑)
cAMP-PK	1.49	2.97(13%↓)	9.69(134%↑)
ACK2	0.89	2.74(20%↓)	6.40(55%↑)

Units of ACC activity = μmol $H^{14}CO_3$ transferred/min

A0.5 citrate = concentration of citrate producing half maximal activity of ACC.

AMP-PK and ACK2 were purified from rat mammary tissue.

cAMP-PK catalytic subunit was purified from bovine heart.

ACC was incubated with each kinase for 60 minutes at 30°C.

for its activator citrate. Thus neither cAMP-PK nor ACK2 could be responsible for the large decrease in the Vmax of ACC produced by 24 hour starvation. (Chapter 3, Table 3). Moreover the effects of cAMP-PK shown in Table 2 are greater than would be expected in vivo because as described in Chapter 3 cAMP-PK purified from lactating mammary gland barely phosphorylates ACC. AMP-PK however produced an 86% fall in the Vmax of ACC an effect of the same order as the 73% decrease in Vmax reported in 24 hour starved rats by Munday and Hardie (1986), and described in Chapter 3, Table 3.

AMP-PK is the only kinase identified in the lactating mammary gland which is able to mimic the effects of starvation on ACC. Its kinetic effects on ACC at saturating citrate concentrations and its activation by 5'AMP (See section 4.4 of this chapter) indicate that this is a similar activity as that first described in rat liver by Carling and Hardie (1986) and Carling et al (1987). It has been shown that phosphorylation of ACC by liver AMP-PK produced an 80% decrease in the Vmax of ACC (Munday et al 1988a) which is similar to the 86% inhibition observed with mammary AMP-PK (Table 2). The liver AMP-PK causes a greater increase in the $A_{0.5}$ for citrate (2.8-fold) versus the approximately 88% increase produced by mammary AMP-PK. Both kinases incorporated similar amounts of phosphate (1.77 mol/mol ACC for liver AMP-PK versus 1.92 mol/mol ACC for mammary gland AMP-PK) so the reason for the difference is unclear and may be due to differences in the AMP-PK as isolated from the two sources.

Starvation of lactating rats for 24 hours produced only a 60% increase in the Ka for citrate (Chapter 3, Table 3) so the primary importance of Vmax changes over Ka is confirmed in vivo during starvation where the inhibition of ACC is correlated with higher phosphorylation (Section 3.5).

4.2.3 SITES OF PHOSPHORYLATION ON ACC BY AMP-PK

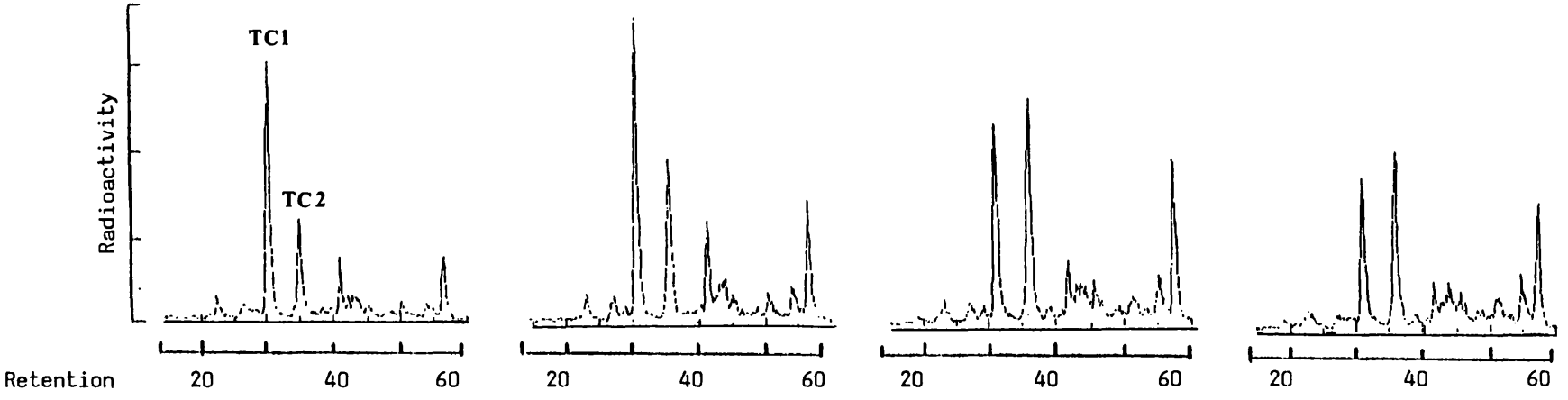
Figure 4.8 shows a time course of phosphorylation of ACC by AMP-PK similar to that shown in Fig. 4.7, but peptide analysis was performed on the ACC at each time point in order to identify the sites of action of AMP-PK.

When ACC previously phosphorylated with [γ ^{32}P]-ATP using AMP-PK is digested by simultaneous incubation with trypsin and chymotrypsin three major phosphopeptides can be separated by reversed phase HPLC. They are termed TC1, TC2 and TC3 according to their order of elution from the column. The amino acid sequence of TC1 (^{76}Ser-Ser-Met-Ser-Gly-Leu^{81}) and TC2 (^{1198}Arg-Met-Ser-Phe^{1201}) has been established by Munday et al (1988a) and corresponds to residues 76 to 81 and 1198 to 1201 respectively on native ACC (Lopez-Casillas et al 1988). Figure 4.8 shows that AMP-PK phosphorylated TC1 most rapidly and that the proportion of radioactivity in TC1 correlated with the decrease in the Vmax of ACC. The proportion of phosphorylation of TC2 only increased significantly after 20 minutes when the Vmax of ACC was already inhibited by 80%. TC3 was phosphorylated least rapidly and its rate of phosphorylation bore no relation to the kinetic changes of ACC. It is therefore probably only a gratuitous phosphorylation. These results explain the rapid decline of ACC activity illustrated in Fig. 4.7. It is clear that most of the phosphate incorporated into ACC in the first five minutes of the incubation must have been in the sequence corresponding to peptide TC1 as this was when the greatest inhibition of ACC Vmax occurred. The function of the phosphorylation in TC2 is unclear as it is secondary to TC1 and it does not seem to have any effect on the Vmax of ACC.

4.2.4 AMP-PK PHOSPHORYLATES SERINE-79 ON ACC

Munday et al (1988 a,b) has shown that AMP-PK from rat liver exclusively phosphorylates serine-79 and that cAMP-PK

Figure 4.8 PHOSPHORYLATION OF ACC BY AMP-PK - effect on Vmax and correlation with phosphorylation of peptide TC1



Time	2 mins	5 mins	20 mins	60 mins
Vmax	1.24 (58%)	0.94 (44%)	0.27 (13%)	0.20 (9%)
moles Pi/ mol/ACC	0.26	0.54	1.10	1.77
$\frac{\text{cpm TC1}}{\text{cpm TC2}}$	1.94	1.40	0.85	0.79

The profiles show radioactive peptides from ACC prepared and separated by reversed phase HPLC as described in Materials and Methods. The 100% Vmax of ACC was 2.14μmol/min/mg ACC in the experiment illustrated.

exclusively phosphorylates serine-77 of TC1 and that the phosphorylation of the two sites is mutually exclusive. Both kinases phosphorylate serine 1200 of TC2. Phosphorylation of serine 1200 is considered to be silent as it does not correlate with the changes in the kinetic parameters of ACC (Munday et al 1988; Davies et al 1990).

A synthetic peptide substrate specific for liver AMP-PK has been described by Davies et al (1989). It has been termed 'SAMS' and corresponds to residues 73 to 85 of ACC but with serine-77 (the cAMP-PK site) conservatively replaced with alanine.

^{73}His-Met-Arg-Ser-Ala^{77}-Met-Ser^{79}-Gly-Leu-His-Leu-Val-Lys^{85}. This 'SAMS' peptide is phosphorylated by AMP-PK (Fig. 4.9) from mammary gland with a Km of 20-25 μM, similar to that reported for liver AMP-PK. Furthermore the mammary AMP-PK does not phosphorylate a synthetic peptide 'SSMA' (results not shown) based upon residues 73-85 of ACC which has the putative AMP-PK site serine-79 replaced with alanine, but the cAMP-PK site serine-77 intact. (^{73}His-Met-Arg-Ser-Ser^{77}-Met-Ala^{79}-Gly-Leu-His-Leu-Val-Lys^{85}). Thus mammary AMP-PK is specific for serine-79 on ACC and does not phosphorylate the cAMP-PK site-serine 77.

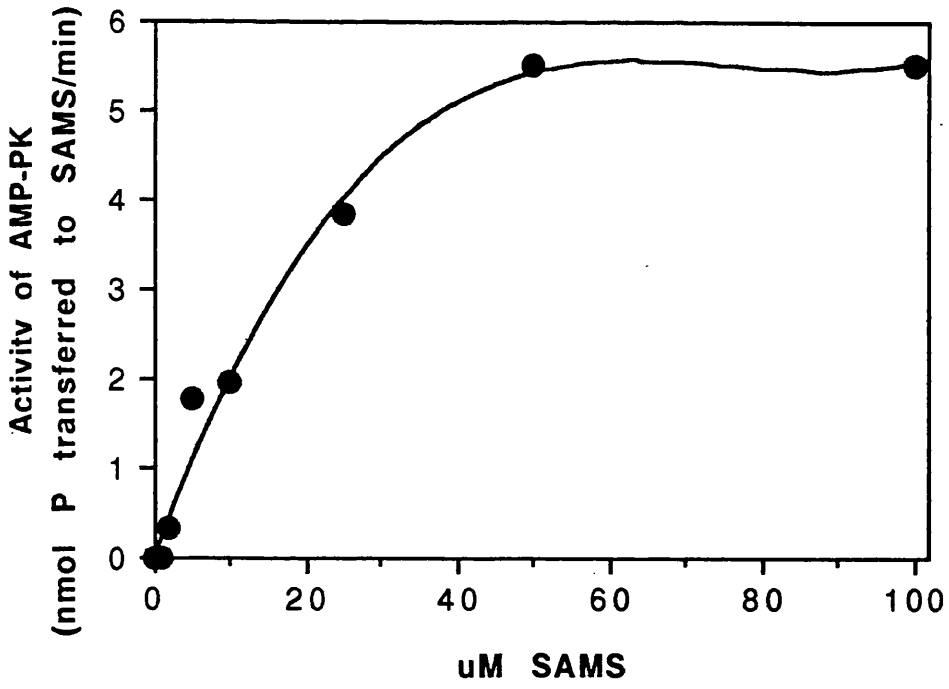
Table 3. SUMMARY OF THE EVIDENCE THAT AMP-PK PHOSPHORYLATES SERINE-79 ON ACC.

Synthetic Peptide	Substrate for:	
	AMP-PK	cAMP-PK
^{73}HMRSSMSGLHLVK^{85}	yes	yes
^{73}HMRSAMSGLHLVK^{85}	yes	no
^{73}HMRSSMAGLHLVK^{85}	no	yes

In Table 3 the first peptide corresponds to the native sequence of ACC between residues 73 and 85 inclusive. No phosphate has ever been found *in vivo* in serine-76 (Munday et al 1988a) and so the different specificity of the

Figure 4.9 DEPENDENCE OF AMP-PK ON SAMS CONCENTRATION

The initial activity of AMP-PK was determined as described in Materials and Methods but with varying concentrations of the synthetic peptide 'SAMS' as the phosphate acceptor. 'SAMS' is the term used to denote the peptide His-Met-Arg-Ser-Ala-Met-Ser*-Gly-Leu-His-Leu-Val-Lys-Arg-Arg which corresponds to residues 73 to 85 of ACC but with serine-77 replaced by alanine thus making it a specific substrate for AMP-PK. AMP-PK phosphorylates the peptide on serine-79 (marked with an asterisk).



peptides is a consequence of their modification indicated by the underlined Alanine. The lack of effect of cAMP-PK on the Vmax of ACC can be rationalised on the basis that because it phosphorylated serine-77 and this site does not greatly affect Vmax. AMP-PK however produced its profound inhibition of ACC by the specific phosphorylation of serine-79, it is in this respect identical to the AMP-PK isolated from rat liver (Davies et al 1989).

4.3 CHARACTERISATION OF MAMMARY AMP-ACTIVATED PROTEIN KINASE

4.3.1 ACTIVITY VERSUS pH

Figure 4.10 shows that the peak of kinase activity (with the specific AMP-PK peptide as substrate) occurred at pH 7.0 with more than 70% of activity retained at 0.5 pH units either side of pH 7.0. The rapid increase in activity observed between pH 5.0 and pH 7.0 suggests that the ionisation of amino acids with pKa's below 7.0 (aspartate pKa 3.90; glutamate pKa 4.07; and histidine pKa 6.04) are important for catalysis. It is known that an aspartate residue is concerned in the nucleotide binding site of all protein kinases sequenced to date (Taylor, 1987). This residue is necessary for the binding of Mg^{++}.

Activity decreased more gradually above pH 7.0. The kinase retains 50% of its activity up to pH 8.5; even at pH 11.0 23% of maximum activity is seen.

4.3.2 DEPENDENCE OF AMP-PK ACTIVITY ON ATP

The maximal activity of AMP-PK was achieved at approximately 200 μM ATP with a Km of 36 μM (Fig. 4.11). As with all kinases reported to date the Km is far below the intracellular ATP concentration (approx. 2 mM) and so the modulation of ATP concentration offers no mechanism for physiological regulation of kinase activity. The Km for ATP is lower than the 70 μM reported for liver AMP-PK.

Figure 4.10 EFFECT OF pH ON ACTIVITY OF AMP-PK

Determination of activity was performed as detailed in Materials and Methods using 'SAMS' as the substrate. Buffering components were used at 40 mM with 0.5 mM-EDTA, 0.5 mM-EGTA, 2 mM-DTT and 10% (w/v) glycerol. The buffers used were: pH 4.0 - 5.5 Na Acetate/AcCOOH; pH 5.5 - 7.0 PIPES/NaOH; pH 7.0 - 8.0 MOPS/NaOH; pH 8.0 - 9.0 Tris/HCl and pH 9.0 - 11.0 CAPS/NaOH. All buffers were prepared at 25°C.

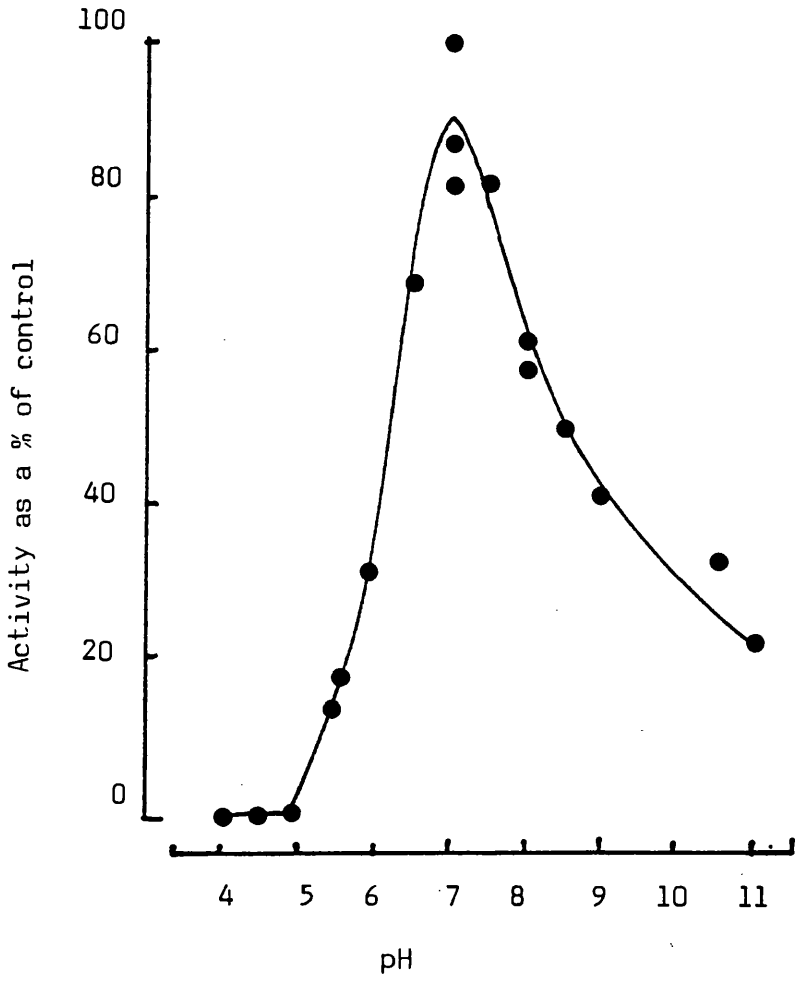
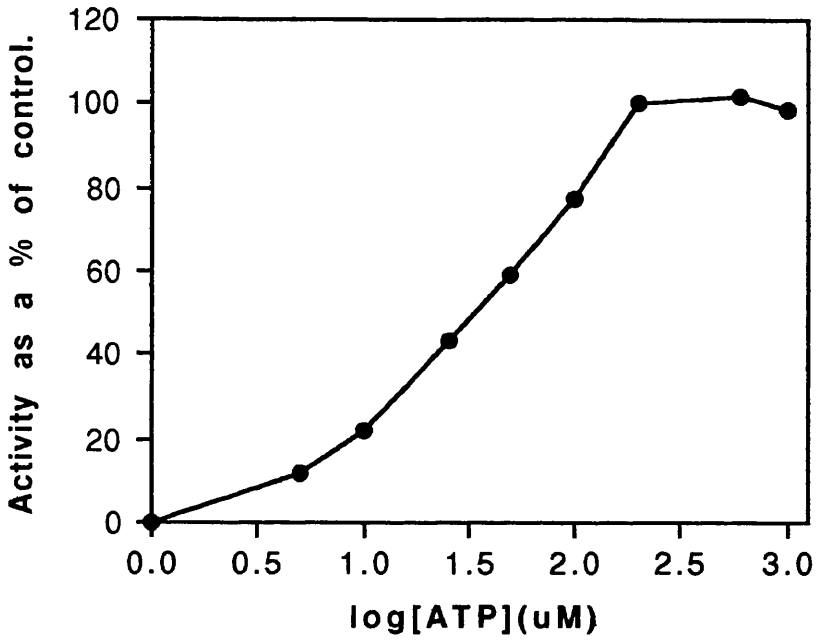


Figure 4.11 DEPENDENCE OF AMP-PK ACTIVITY ON ATP

Initial activity was determined as described in Materials and Methods with 'SAMS' as the substrate except that Mg^{++} was kept constant at 4 mM and ATP was varied from zero to 1.0 mM.



(Carling et al 1989), but for the reasons stated this is probably of no physiological significance.

4.3.3 DEPENDENCE OF AMP-PK ACTIVITY ON Mg^{++}

AMP-PK activity was maximal at 2 mM-Mg^{++} (Fig. 4.12). Thereafter activity was inhibited by higher concentrations of Mg^{++}. Thus the standard assay which contains 200 μM ATP and 4 mM-Mg^{++} is measuring a submaximal activity. High concentrations of Mg^{++} lead to precipitation of ATP and so a fall in the free ATP:Mg concentration is the probable cause of the diminished activity.

4.3.4 SUBSTRATE SPECIFICITIES OF ACC KINASES

Table 4 compares the substrate specificity and other properties of AMP-PK with cAMP-PK and Acetyl-CoA Carboxylase Kinase-2 (ACK2). The latter is a partially characterised kinase activity identified in lactating mammary gland (Munday and Hardie 1984). It phosphorylates ACC but produces only modest effects on Vmax (Table 2). AMP-PK showed a greater specificity for ACC than either ACK2 or cAMP-PK and it should be noted that the cAMP-PK used for Table 4 was purified from bovine heart and so its activity versus ACC is, although low, greater than is seen with cAMP-PK from mammary gland (Chapter 3, Table 7). As with ACK2, cAMP-PK has only small effects on the Vmax of ACC (Table 3).

The relative molecular weight of AMP-PK as determined by gel filtration was estimated to be 160,000 which is higher than the 100,000 $\pm$ 30,000 reported by Carling et al (1989) for the liver AMP-PK but the same as that reported by Shiao et al (1981) for an ACC kinase from rat liver which inactivated ACC and is itself phosphorylated, thus making it similar to AMP-PK. The molecular weight of mammary gland AMP-PK is clearly distinct from both cAMP-PK catalytic subunit and ACK2 which have MWr of 40,000 and

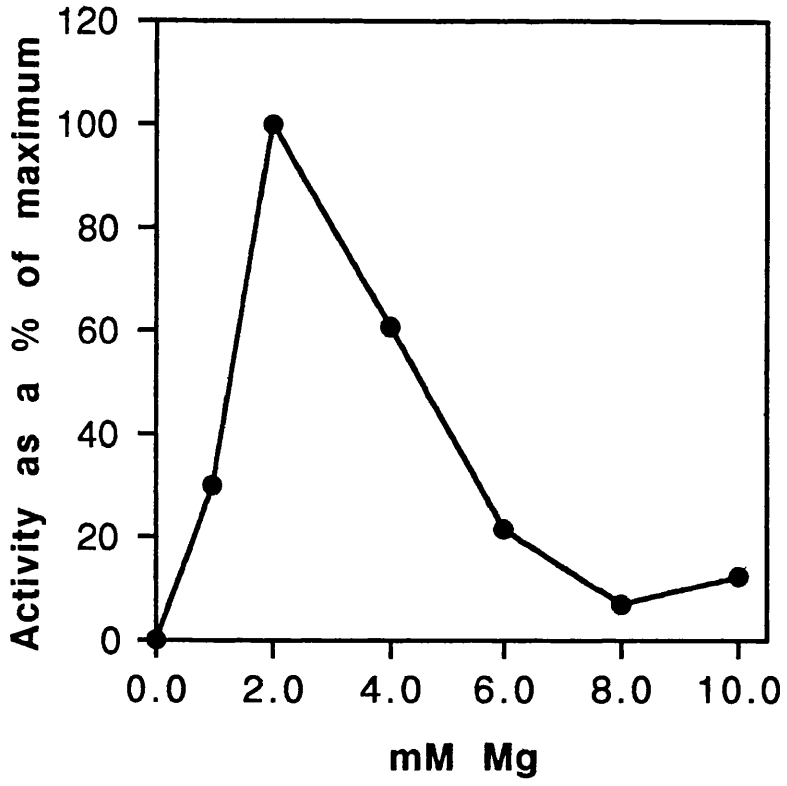
Table 4 PROPERTIES OF ACC KINASES

Protein Kinase	cAMP Kinase	ACK2	AMP-PK
Apparent MW_r by gel filtration	40,000	76,000	160,000
Substrate specificity initial Rate of Phosphorylation as % of that with ACC			
Acetyl-CoA Carboxylase	100	100	100
ATP-citrate lyase	10	23	15
Glycogen Synthase	500	54	29
Phosphorylase Kinase	1,000	10	-
Casein	78	80	15
Sensitivity to the protein inhibitor of cAMP kinase	Yes	No	No
Activation by AMP	No	No	Yes

(cAMP Kinase was purified from bovine heart, ACK2 and AMP-PK were purified from rat mammary gland).
Kinase activities were assayed in duplicate as described in Materials and Methods.

Figure 4.12 DEPENDENCE OF AMP-PK ACTIVITY ON Mg^{++}

Initial activity was determined as in Materials and Methods except that ATP was kept constant at 200 μM and Mg^{++} was varied from zero to 10 mM.



76,000 respectively. AMP-PK is insensitive to the specific inhibitor of cAMP dependent protein kinase thus showing that AMP-PK is not the catalytic subunit of cAMP-PK. The activity of AMP-PK was increased in a concentration dependent manner by 5'AMP; this property is discussed further in sections 4.4.2 and 4.5. The other kinases are not activated by this nucleotide.

4.4 REGULATION OF AMP-PK

4.4.1 REGULATION OF AMP-PK BY REVERSIBLE PHOSPHORYLATION

AMP-PK is itself regulated by phosphorylation. This was first noted during development of the purification procedure. Phosphatase inhibitor (NaPPi) was required in all buffers used for purification otherwise activity was lost. When the DEAE fraction (Section 4.1.2) was dialysed overnight into pyrophosphate free buffer, endogenous phosphatases dephosphorylated the kinase and produced up to an 80% fall in activity in comparison to an identical fraction dialysed in buffer containing NaPPi. The activity returned to 90% of the activity of the control (which had not been dephosphorylated) after 40 minutes incubation of the fraction with ATP/Mg^{++}; 0.2 mM/4 mM (Fig. 4.13).

Incubation of the DEAE fraction purified in the presence of NaPPi with ATP/Mg (0.33/6.6 mM) plus phosphatase inhibitors (NaPPi) produced at least a 2 to 3 fold increase of the initial activity of the kinase compared to that incubated with water as a control (Fig. 4.14). This shows that the kinase as isolated from lactating mammary tissue was not maximally active.

Endogenous reactivation could not be reproduced after the DEAE step indicating that the phenomenon is due to an 'AMP-PK-Kinase' which has co-purified to this stage and not autophosphorylation. The identity of AMP-PK-Kinase is unknown. It is not cAMP-PK as the addition of cAMP-PK to AMP-PK purified beyond the DEAE step neither phosphorylates nor increases the activity of the latter. Addition of

Figure 4.13 REACTIVATION OF DEPHOSPHORYLATED AMP-PK BY INCUBATION WITH ATP/Mg^{++}

DEAE eluate was dialysed overnight into AMP-PK buffer lacking phosphatase inhibitor (NaPPi). A fraction of DEAE eluate was dialysed simultaneously against normal AMP-PK buffer containing 5 mM-NaPPi and the activity of this preparation used as the control. The kinase was reactivated by incubation at 30°C with 0.2 mM-ATP and 4 mM-$MgCl_2$ in AMP-PK buffer. At the times indicated aliquots from the preincubation were stopped with EDTA to a final concentration of 20 mM before desalting into AMP-PK buffer lacking NaPPi but containing 50 mM-NaF to remove $MgCl_2$, EDTA and unreacted ATP as described in Materials and Methods. The preincubations were desalted into buffer containing NaF rather than $NaPP_i$ as the former does not interfere with the subsequent phosphorylation assay. The amount of reactivation was followed by the increase in the initial rate of phosphorylation of histone IIA.

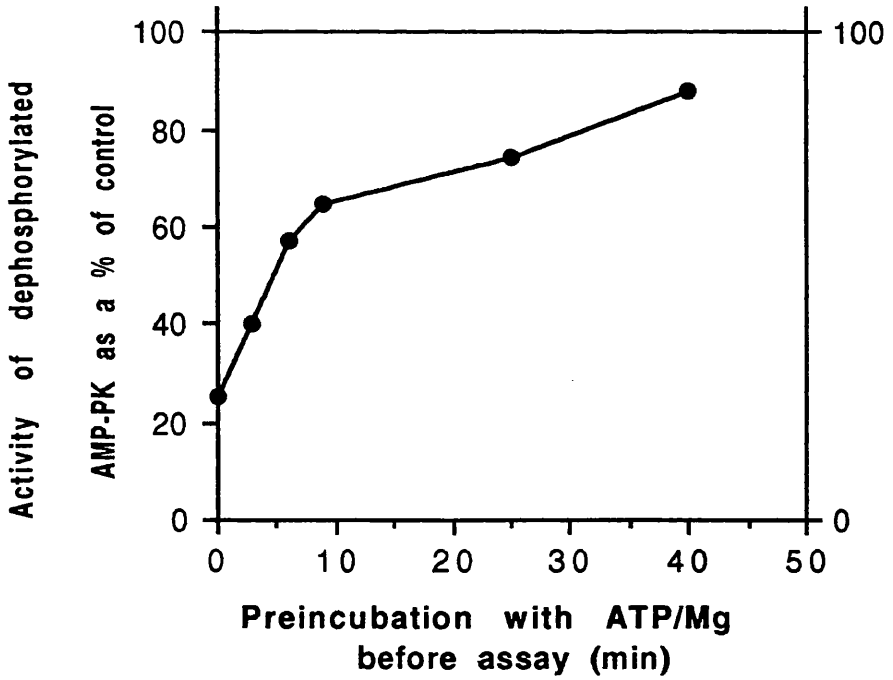
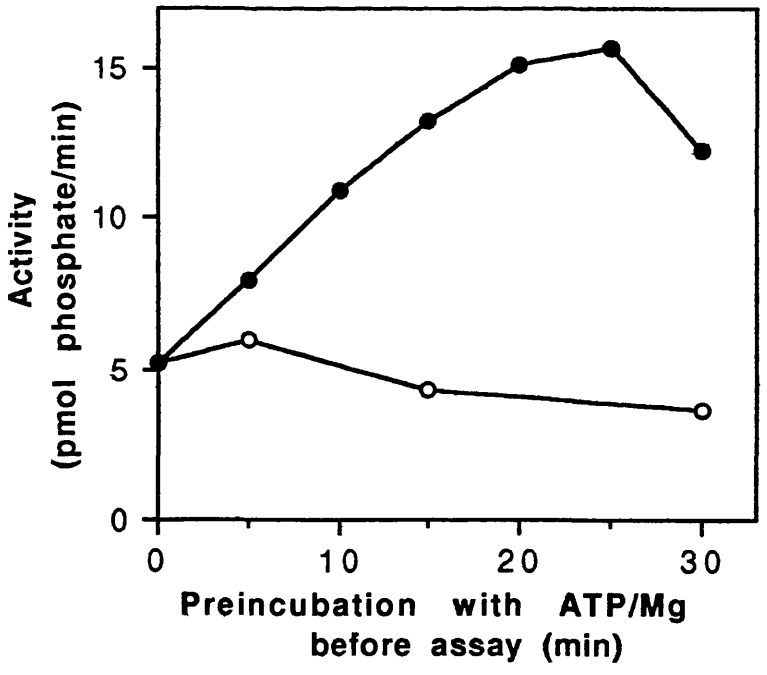


Figure 4.14 EVIDENCE TO SHOW THAT THE ACTIVITY OF AMP-PK AS PURIFIED IS SUBMAXIMAL

DEAE eluate prepared in the presence of phosphatase inhibitor was pre-incubated with 0.33 mM-ATP and 6.6 mM-$MgCl_2$ at 30°C (●). Simultaneously a fraction of DEAE eluate was incubated with no additives as a control (O). At the times indicated aliquots of the preincubation were stopped by addition of EDTA to a final concentration of 20 mM then desalted into AMP-PK buffer lacking NaPPi but containing 50 mM-NaF, to remove $MgCl_2$, EDTA and unreacted ATP, as described in Materials and Methods. Changes in the activity of the AMP-PK were followed by measuring the changes in the initial rate of phosphorylation of Histone IIA.



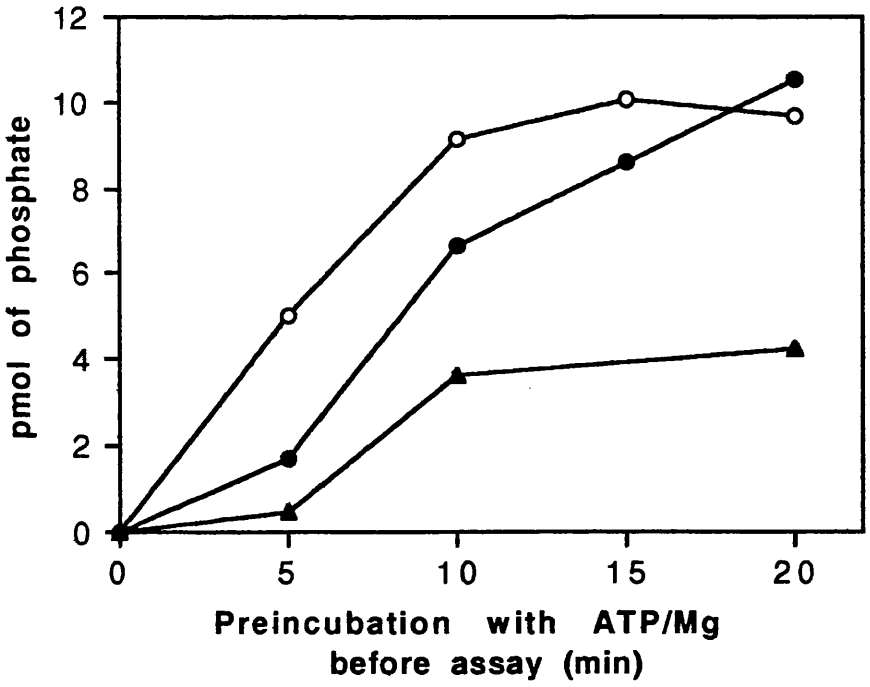
cAMP to the DEAE fraction (to activate endogenous cAMP-PK holoenzyme) increased the rate of activation of AMP-PK and the specific inhibitor of cAMP-PK decreased reactivation (Fig. 4.15). This suggests that cAMP-PK may be involved via a cascade in the activation of AMP-PK and hence phosphorylation and inhibition of ACC. The physiological significance in the mammary gland is unclear considering that cAMP-PK activity in the mammary gland does not increase in 24 hour starvation (Table 2, Chapter 3) and there is no correlation *in vivo* between the activity of cAMP-PK and ACC; but as discussed in Chapter 3 the *in vitro* and *in vivo* effects of cAMP-PK are not always the same. Moreover it is unlikely that the very high activity of cAMP-PK, typically a four fold increase over basal levels in the presence of 5 μM cAMP, would occur *in vivo*. However such a mechanism could explain the inhibition of ACC observed in isolated hepatocytes challenged with glucagon (which activates adenylate cyclase) especially as it has been shown that phosphorylation of ACC purified from such cells is exclusively due to AMP-PK (Sim and Hardie 1989). The same laboratory has also reported that AMP-PK from rat liver is not directly activated by cAMP-PK *in vitro* (Davies et al 1989) and they find no evidence for an involvement of cAMP-PK in the reactivation of AMP-PK. This is in contrast to Lent and Kim (1983b) who find that cAMP-PK caused activation of an ACC-kinase from rat liver which, as their purification procedure (Lent and Kim 1982) is similar to that from the Hardie laboratory (Carling et al 1989) is probably AMP-PK. It is also possible that the effects of glucagon in liver are mediated by the rapid breakdown of the cAMP to generate 5'AMP by the action of phosphodiesterases. This would activate AMP-PK and cause the phosphorylation and inactivation of ACC observed (See sections 4.4.2 and 4.5).

4.4.2 ACTIVATION OF AMP-PK BY 5'AMP

Mammary gland AMP-PK like the liver enzyme is activated in a concentration dependent manner by 5'AMP

Fig. 4.15 ACTIVATION OF AMP-PK IN THE DEAE FRACTION IN THE PRESENCE OF cAMP OR THE SPECIFIC INHIBITOR OF cAMP-PK

Identical DEAE fractions were preincubated at 30°C with 0.2 mM-ATP and 4 mM-Mg^{++} in AMP-PK buffer in the presence of 1 μM cAMP (○) or 2.1 U/μl cAMP-PK specific inhibitor (▲) or with no additions (●). At the times indicated 45 μl aliquots were removed and stopped by the addition of 15 μl of EDTA to a final concentration of 12.5 mM before desalting into AMP-PK buffer lacking NaPPi but with 50 mM-NaF to remove Mg EDTA and unreacted ATP as described in Materials and Methods. The amount of reactivation was followed by the increase in the initial rate of phosphorylation of histone IIA in the presence of cAMP-PK specific inhibitor.

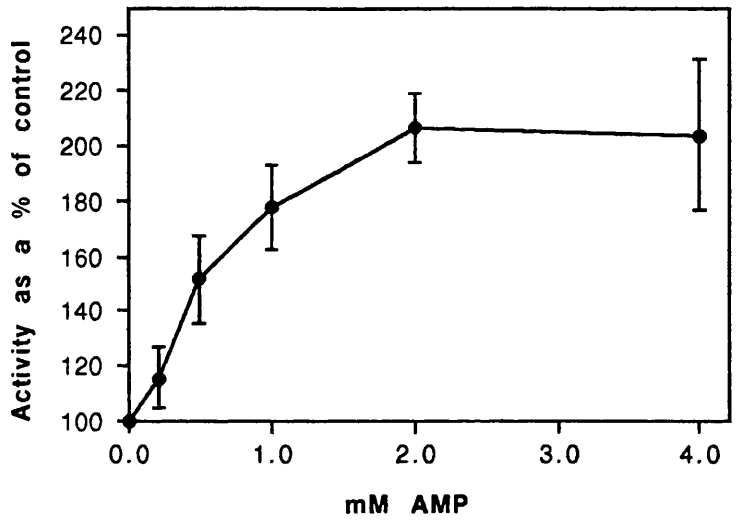


(Fig. 4.16 a and b). In the DEAE fraction the Km for activation is 0.53 mM and maximal activation occurs at 2 mM of the nucleotide. In purer fractions however the maximum activation is achieved at 50 μM with a Ka of approx. 1.5 μM; values similar to those reported for AMP-PK from liver (Carling et al, 1989). Typically a 2 fold activation is seen in both the DEAE and the purer fractions. This is comparable to the 3 to 5 fold increase in the Vmax of liver AMP-PK reported by Carling et al (1989). The difference in Ka between the relatively crude DEAE fraction and purer fractions is probably due to the presence of nucleotide binding proteins in the crude DEAE fraction 'mopping up' the exogenous 5' AMP.

Figure 4.16a and b ACTIVATION OF AMP-PK BY 5'AMP

AMP-PK activity was assayed using ACC or 'SAMS' peptide as substrate as described in Materials and Methods with 5'AMP present at the concentrations indicated. Figure 4.14a shows the mean ± SEM of at least four separate experiments conducted using AMP-PK purified up to and including the DEAE anion exchange step. Figure 4.14b shows the results of one typical experiment using AMP-PK purified up to and including the blue agarose affinity separation.

a



b

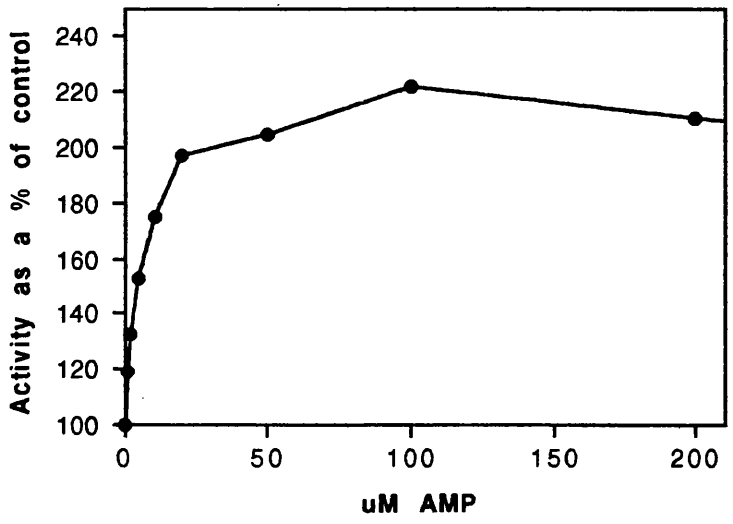
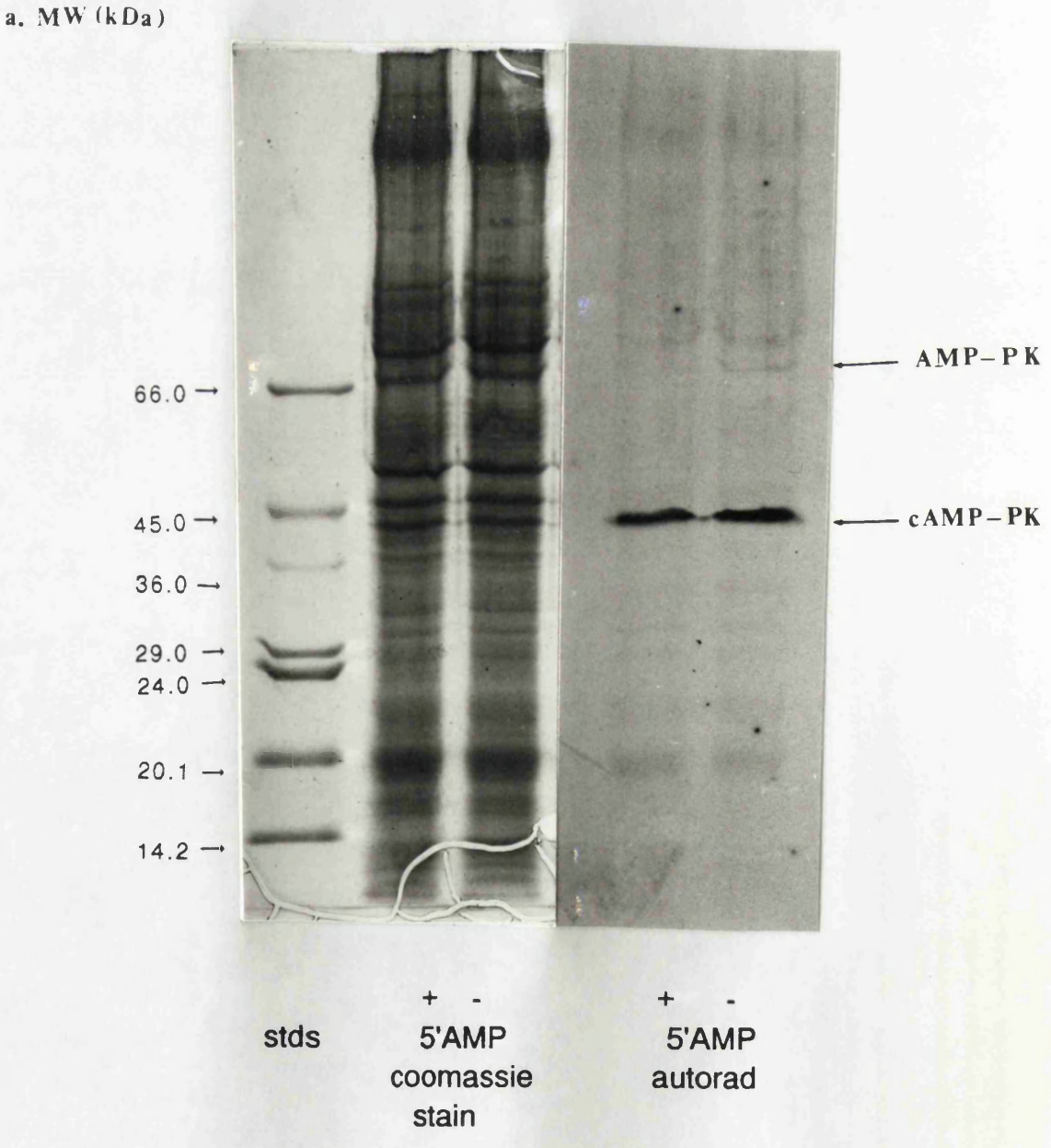
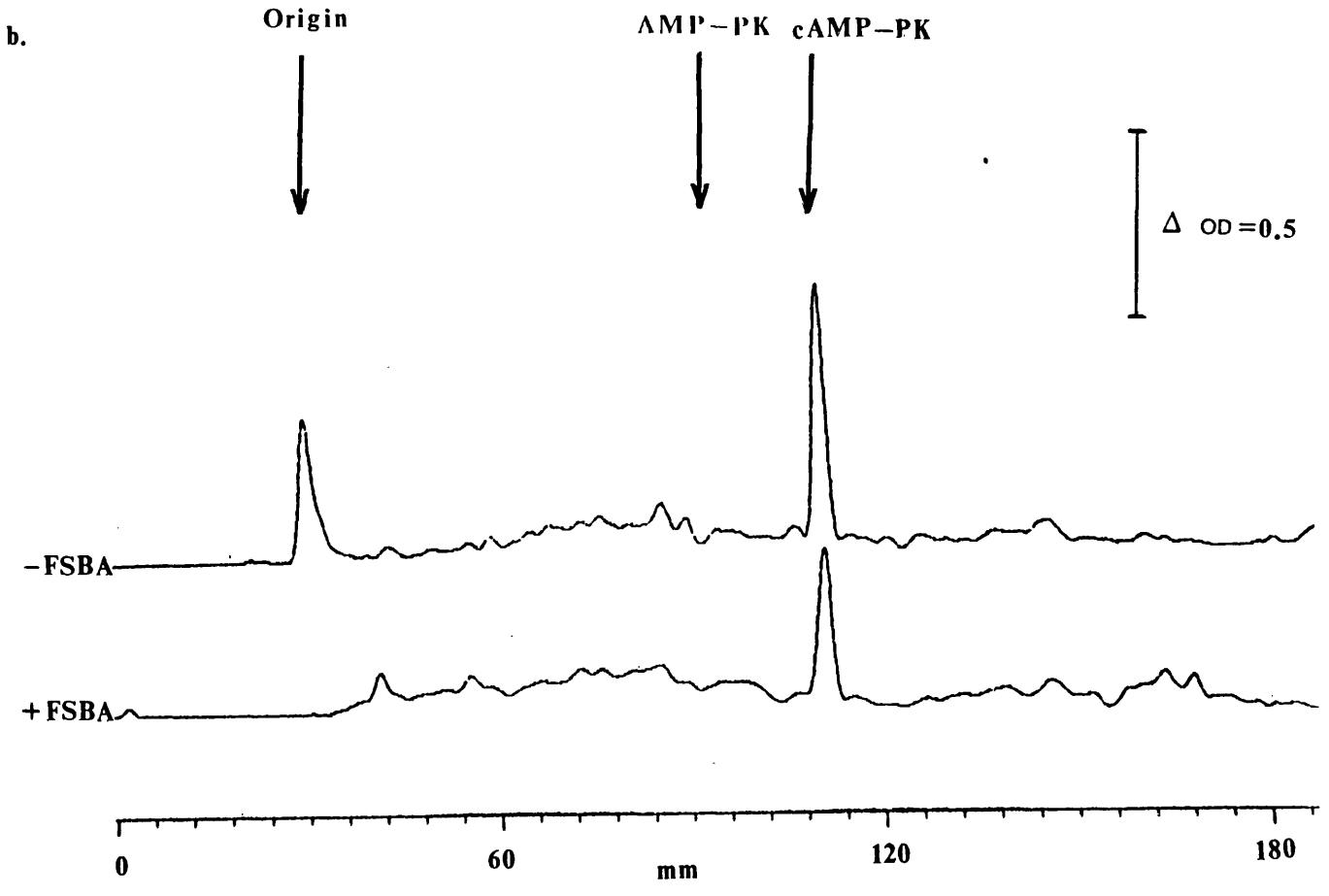


Figure 4.17a and b [^{14}C]-FSBA LABELLING OF AMP-PK

AMP-PK was incubated for one hour with [^{14}C]-FSBA as described in Materials and Methods in the presence and absence of 5'AMP. The photograph and scan of the autoradiograph shows that 5'AMP completely prevented modification of a 75 kDa protein (4.17b). The heavily labelled band corresponds to the catalytic subunit of cAMP-PK; incorporation of radioactivity was reduced by 5'AMP but this was due to non specific effects of 5'AMP as the nucleotide does not affect the activity of cAMP-PK.




b.
Origin
AMP-PK
cAMP-PK
Δ OD=0.5
-FSBA
+FSBA
0
60
120
180
mm

4.4.3 5'AMP BLOCKS THE COVALENT MODIFICATION OF AMP-PK BY [^{14}C]-FLUOROSULPHONYLBENZOYL ADENOSINE ([^{14}C]-FSBA)

The ATP analogue FSBA has been shown to be an effective covalent modifier of nucleotide binding sites on several proteins (Zoller et al 1981; Carling et al 1989). The latter authors have shown that in the presence of 5'AMP covalent modification of AMP-PK by [^{14}C]-FSBA is reduced; these studies also identified the kinase activity as a 64 kDa protein, after SDS-PAGE. This suggests that the native liver AMP-PK is a dimer as on gel filtration the kinase activity eluted at 100 $\pm$ 30 kDa (Carling et al 1989).

In contrast the enzyme from mammary tissue consistently showed an apparent molecular weight of approx. 160 kDa. In order to confirm the molecular weight of mammary AMP-PK a fraction of the kinase preparation taken to the blue Sepharose stage was incubated with [^{14}C]-FSBA in the presence and absence of 5'AMP as described in Materials and Methods. After separation by SDS-PAGE and autoradiography it was seen that the 5'AMP sensitive covalent modification corresponded to a protein of 75 kDa (Fig. 4.17a,b). Assuming the kinase is a homodimer this corresponds to a molecular weight of 150 kDa which is close to the 160 kDa as estimated by gel filtration (Fig. 4.5). Thus the mammary AMP-PK does appear to be larger than the liver enzyme and probably represents an isozymic form of the enzyme. The heavily labelled band corresponds to the catalytic subunit of cAMP-PK (MWr 42.7 kDa). 5'AMP was also able to reduce the amount of radioactivity incorporated into the catalytic subunit. This was probably due to non-specific interactions between the ATP binding site and 5'AMP as the latter enzyme has no physiological effect on cAMP-PK activity.

4.5 5' AMP AND THE REGULATION OF AMP-PK AND ACC ACTIVITY

The intracellular concentration of 5'AMP in the mammary gland can be elevated by arresting the blood flow thus making the tissue anoxic. This was achieved by ligating the blood vessels serving the mammary gland before freeze clamping as described in Materials and Methods. In mammary tissue thus treated the concentration of AMP increased sixfold and the ATP concentration fell by nearly 80% (Table 5): ADP concentration was essentially unchanged. Iles et al (1985) from ^{31}P-NMR studies on living tissue *in situ* (liver) suggest the following mechanism which though derived from ischaemia induced in liver may also be true for mammary gland since like liver, it has no phosphocreatine to serve as an ATP substitute:

Initially there is depletion of ATP:

(1) $2ATP \rightarrow 2ADP + 2Pi$

Adenylate kinase then 'recycles' the ADP:

(2) $2ADP \rightarrow ATP + AMP$

So the final effect of anoxia on adenine nucleotides is:

(3) $ATP \rightarrow AMP + 2Pi$

Thus there is no significant change in ADP concentration.

The increase in AMP concentration in anoxic mammary gland should activate AMP-PK and hence decrease the activity of ACC. There is a 50% fall in the Vmax of ACC purified from ligated mammary gland compared to non-ligated tissue from the same animal. (Table 6). This fall is comparable to that achieved after 24 hour starvation and is too large to be due to any known ACC kinase other than AMP-PK. Table 5 also shows that the AMP concentration changes from 0.19 mM to 1.19 mM during anoxia. This increase is across the same range which produces a doubling of AMP-PK activity in the DEAE extract (Fig. 4.16a) and further supports the conclusion that AMP-PK is responsible for the decreased activity of ACC observed. The Ka for citrate increased by 43% in response to ligation and the Hill coefficient did not change significantly. Both these effects are also consistent with AMP-PK being the kinase

Table 5 EFFECTS OF ANOXIA ON THE ADENINE NUCLEOTIDE CONCENTRATION IN MAMMARY TISSUE.

	CONTROL non-ligated	ANOXIC ligated
DNA		
(mg/g tissue)	4.26±0.28	3.62±0.11
ATP		
(μmol.mg DNA)	0.38±0.04	0.10±0.01**
(mM)	1.60	0.38
ADP		
(μmol/mg DNA)	0.14±0.02	0.14±0.04
(mM)	0.58	0.52
AMP		
(μmol/mg DNA)	0.04±0.02	0.33±0.66**
(mM)	0.19	1.19

$HCLO_4$ extracts of freeze clamped mammary tissue were prepared and assayed for adenine nucleotides as described in Materials and Methods. Concentrations of nucleotide in mM units were derived from the mean values of μmol/mg DNA by multiplying the DNA values and nucleotide values and making the assumption that 1g tissue = 1 ml water.
Results are the mean ± SEM of six separate experiments.
The control and ligated mammary glands were taken from the same animals as described in Materials and Methods therefore differences between control and anoxic tissue were assessed by Students t-test for paired samples.
**P<0.0005.

Table 6 EFFECTS OF ANOXIA ON THE KINETIC PARAMETERS OF PURE ACETYL-CoA CARBOXYLASE (ACC) IN MAMMARY TISSUE

	CONTROL non-ligated	ANOXIC ligated
Vmax (μmol/min/mg ACC)	1.96±0.48	1.03±0.21*
Ka citrate (mM)	1.43±0.27	2.04±0.31*
Hill coefficient	0.95±0.09	0.92±0.07

ACC was purified from freeze clamped tissue and its activity assayed as described in Materials and Methods. Results are the mean ± SEM of 15 separate experiments. Differences between control and anoxic tissue were assessed by Student's t-test for paired samples. *$P<0.02$.

producing the inhibition of ACC. (c.f. Table 2). The anoxia did not induce proteolysis of ACC, as in crude homogenates total ACC activity was unchanged between control and ligated mammary gland but the expressed activity was decreased by 50% (Clegg and Calvert 1988).

Jones and Williamson (1984) have shown that blood flow in the mammary gland has decreased by 45% after 6 hours starvation. Assuming this would persist (if not actually become lower), after 24 hours starvation one might expect the tissue to show signs of anoxia. However the concentration of 5'AMP is unchanged after 24 hours starvation when ACC activity has fallen by 50% (Table 7).

These results might suggest that 5'AMP may not be responsible for the inactivation of AMP-PK in the mammary gland during starvation. However it must be borne in mind that the method of measuring 5'AMP employed measures the total cellular 5'AMP content. Thus any changes in the free cytosolic concentration of 5'AMP which may be occurring at micromolar levels will be masked. The only way to test this is in living tissue using scanning ^{31}P-NMR. (Iles et al 1985,a,b; Karczmar et al 1989).

The possibility that AMP-PK is activated during starvation by phosphorylation was investigated. No significant change in AMP-PK activity was apparent after 24 hours of starvation or 3 hours refeeding (Table 8). This is in contrast to the situation in the liver of virgin female rats where AMP-PK activity doubles after only 6 hours of starvation. (S. Takhar unpublished results).

Thus at present the involvement of AMP-PK in the regulation of the phosphorylation state and hence activity of ACC in the lactating mammary gland <u>in vivo</u> must be inferred by indirect evidence:

i) The phosphorylation of ACC <u>in vitro</u> by AMP-PK produced a decrease in Vmax similar to that which occurs <u>in vivo</u> after 24 hours starvation.

Table 7 EFFECT OF 24 HOUR STARVATION ON ADENINE NUCLEOTIDE CONCENTRATION AND ACETYL-CoA CARBOXYLASE ACTIVITY

	CONTROL	24HR-STARVED
DNA (mg/g tissue)	3.37±0.24 (5)	0.16±0.37 (5)
ATP (μmol/mg DNA)	0.23±0.04 (4)	0.16±0.03 (5)
ADP (μmol/mg DNA)	0.33±0.02 (4)	0.25±0.03 (5)
AMP (μmol/mg DNA)	0.12±0.02 (4)	0.13±0.02 (5)
TOTAL (μmol/mg DNA)	0.67±0.05 (4)	0.53±0.06 (5)
ACC, Vmax (μmol/min/mg ACC)	3.48±0.23 (7)	1.67±0.13 (10)

$HC10_4$ extracts of freeze clamped mammary tissue were prepared and assayed for adenine nucleotides as described in Materials and Methods. Results are expressed as the mean ± SEM for the number of samples shown in parenthesis.

Table 8 EFFECT OF DIETARY MANIPULATION ON THE ACTIVITY OF AMP-PK IN MAMMARY GLAND

		ACTIVITY AMP-PK nmol ^{32}P transferred/min/mg DNA
CONTROL FED	(n=5)	0.73±0.12
24HR STARVED	(n=4)	0.83±0.23
3HR REFED	(n=4)	0.58±0.18

n = number of experiments

5% PEG pellets were prepared from freeze clamped mammary tissue which had been homogenised in buffer containing phosphatase and kinase inhibitors. The pellets were resuspended in AMP-PK assay buffer, and the resuspension assayed against SAMS the specific peptide for AMP-PK (Davies et al, 1989). Assays were conducted in conditions where phosphorylation was linear with respect to both time and quantity of extract added.

ii) The amount of phosphate incorporated into the TC1 peptide Ser-Ser-Met-Ser-Gly-Leu (the sequence of ACC containing the specific AMP-PK site) correlates with the fall in the Vmax of ACC.

iii) AMP-PK *in vitro* is activated up to four-fold by 5'AMP; a similar effect apparently occurs *in vivo*, as inferred from the observation that the Vmax of ACC is reduced by 50% when intracellular 5'AMP is elevated.

CHAPTER 5

SHORT TERM REGULATION OF LIPOGENESIS IN THE LACTATING MAMMARY GLAND

In the lactating mammary gland the rate of lipogenesis is very sensitive to dietary manipulation and, of the major milk constituents, fat is the most variable both in quantity and composition. Fatty acid synthesis falls by 98% after 24 hour starvation and is rapidly restored by refeeding for 2 hours (Robinson et al 1978).

The response of mammary gland to starvation is rapid, after 6 hours lipogenesis had fallen by 88% (Williamson, 1983). This is accompanied by many extra-mammary changes of relevance to lipogenesis in the mammary gland. For example plasma insulin falls, circulating ketone bodies increase (Jones et al 1984), serum free fatty acid concentration rises (Seitz et al 1977; Page and Kuhn 1986) and mammary blood flow decreases by 45% (Jones and Williamson 1984). Within the gland glucose transport is depressed by starvation (Threadgold and Kuhn 1984) as are the activities of hexokinase, phosphofructokinase (Jones et al 1984), pyruvate dehydrogenase (Baxter and Coore 1978) and acetyl-CoA carboxylase (McNeillie and Zammit 1982, Munday and Williamson 1982). The enzymes listed all catalyse essentially irreversible reactions and because of their low relative activities in the cell (Gumąa et al 1973) are, together with glucose transport, potential control points for the supply or utilisation of lipogenic substrates within the mammary gland. The interplay between the enzymes and intermediates of both glucose metabolism and lipogenesis as well as the precise intracellular actions of circulating effectors such as insulin, fatty acids and ketone bodies is unclear. The aim of the experiments described in this chapter was to assess the relative contributions of the various metabolic changes which occur within the lactating rat during starvation to

the observed depression of lipogenesis in the mammary gland, and also to assess the possible role of ketone bodies or fatty acids as 'signals' which bring about the inhibition of lipogenesis by covalent modification of ACC and PDH.

5.1 STUDY OF METABOLIC CHANGES IN THE MAMMARY GLAND OF THE LACTATING RAT DURING STARVATION

Rats at peak lactation were starved for various times up to 24 hours. The pups were not separated from the dams so accumulation of milk within the gland did not occur. Considerations of inhibitory components in milk leading to reduced metabolism within the gland (Wilde et al 1987) are therefore not important. Food was withdrawn at 10.00h. The concentration of key glycolytic intermediates, ketone bodies and also the activity of both ACC and PDH was measured at several time points.

5.1.1 THE EFFECT OF FASTING ON THE RATE OF LIPOGENESIS IN THE LACTATING MAMMARY GLAND

The decrease in the rate of lipogenesis that occurs when food is withdrawn is shown in Table 1. The fall in lipogenesis seen in the first 6 hours of starvation is significantly greater than that due to the normal diurnal decline that is already happening at this time (Munday and Williamson 1983). From these authors' results it can be calculated that lipogenesis in the mammary gland of fed lactating rats falls by approximately 30% between 10.00h and 16.00h. However, starvation over a similar time (10.00h to 16.00h) caused an 87% inhibition of lipogenesis. It is probable that the rapid fall in lipogenesis was facilitated by the metabolism of the mammary gland already being a "downward slope".

The fall in lipogenesis may be due to decreased substrate supply caused by either a fall in glucose availability and/or uptake, or inhibition of glycolysis.

Table 1 THE CHANGES IN MAMMARY GLAND FATTY ACID SYNTHESIS DURING STARVATION

Hours starvation	Fatty acid synthesis	% inhibition
Fed	191.8 ± 16.9	0
1.5	151.9 ± 11.6	20.8
3.0	109.9 ± 9.6*	43.0
6.0	25.1 ± 2.7**	87.0
9.0	16.86 ± 1.01**	91.2
24	5.6 ± 0.57**	97.1

Fatty acid synthesis was measured as ^{3}H incorporated/hour/g fresh weight.

Results are expressed as mean ± SEM and were derived from at least 4 separate experiments. Those which were significantly different from those for fed animals as calculated by Student's t-test are denoted by *P<0.005; **P<0.001.

Lipogenesis may also be decreased by direct inhibition of lipogenic enzymes. These factors are considered in the following sections.

5.1.2 CORRELATIONS BETWEEN LIPOGENESIS AND GLUCOSE SUPPLY AND UPTAKE.

In agreement with several authors (eg Hawkins and Williamson 1972, Robinson and Williamson 1977a, Threadgold and Kuhn 1984) glucose uptake is decreased during starvation. Table 2 shows that over 9 hours the glucose uptake by the gland fell by 83%. Arterial glucose fell by only 22% over the same time. This represents a five fold decrease in the percentage extraction of glucose from 58% down to 12%. Percentage extraction = AV difference divided by the arterial concentration then multiplied by 100. This value is a measure of the efficiency of glucose uptake. The changes in glucose uptake correlate well with the decrease in lipogenesis and Prosser (1988) has shown that in mouse mammary gland fasting for 16 hours decreases the number of functional glucose transporters on the plasma membrane by 70%. It is likely that a similar mechanism occurs in the rat but the rate of decline of glucose transporters is unknown. The relative importance of glucose metabolism and glucose transport in determining glucose uptake during starvation cannot therefore be formally assessed. However, the sharp decline in glycolytic flux as indicated by the 90% drop over 9 hours in the concentration of fructose-1,6-bisphosphate (F-1,6-P_2) was probably sufficient to explain the reduced glucose uptake.

Crossover analysis of the results in Table 2 show that during short term starvation phosphofructokinase-1 (PFK-1) is the rate limiting step in glycolysis. Mammary gland PFK-1 has an absolute requirement for fructose-2,6-bisphosphate (F-2,6-P_2) (Ward and Kuhn 1985) and is inhibited by citrate at physiological concentrations (Zammit 1979). Inhibition by citrate can be discounted as

Table 2 The Changes in Glucose Uptake and the Concentration of Key Glycolytic Intermediates in the Lactating Mammary Gland During Starvation.

Hours Starvation	A-V difference for glucose	Glucose-6-phosphate	Fructose-1,6-bisphosphate	G-6-P / F1-6-P_2
Fed	3.14±0.17	0.018±0.002	0.157±0.002	0.115
1.5	2.43±0.12*	0.020±0.002	0.116±0.005	0.172
3.0	1.08±0.08++	0.026±0.002**	0.091±0.010**	0.286
6.0	0.058±0.05**	0.038±0.004**	0.068±0.002**	0.559
9.0	0.052±0.05**	0.053±0.005**	0.016±0.002**	3.313
24	0.034±0.03**	0.306±0.02**	0.011±0.001**	27.818

Values are expressed as μmol/g fresh weight for metabolites and μmol/ml blood for A-V difference. Results are mean ± SEM for at least 5 separate experiments. Those which were significantly different from fed (control) values are denoted by *P <0.005; **P<0.001.

the citrate concentration does not increase significantly until 9 hours starvation (Table 3). The concentration of mammary F-2,6-P_2 falls during overnight starvation (Ward and Kuhn 1985) but this change alone (40%) is thought to be insufficient to account of the fall in PFK-1 activity and glycolytic flux (Ward and Kuhn 1985). Both PFK-1 and PFK-2 are stimulated by AMP and there is marked synergy between F-2,6-P_2 and AMP (Van Schaftingen et al 1981). In prolonged starvation the intracellular free fatty acid concentration could be expected to rise due to elevated plasma levels (Page and Kuhn 1986) and the formation of acyl-CoA esters would therefore cause an increase in AMP levels that would, in turn, activate PFK-1 . Thus an alternative mechanism for the inhibition of PFK-1 in starvation seems more likely. PFK-1 purified from rabbit muscle (Foe and Kemp 1982) or rat liver (Sakakibara and Uyeda 1983) can be phosphorylated by cAMP-PK <u>in vitro</u>. Phosphorylated PFK-1 has an unchanged Vmax but altered allosteric properties so that it is more sensitive to inhibition by citrate and ATP and less sensitive to activation by AMP and F-2,6-P_2. (Sakakibara and Uyeda 1983). It is not known whether mammary PFK-1 can be regulated by phosphorylation and although cAMP-PK activity does not increase (Section 3.5) phosphorylation by other protein kinases would provide an attractive mechanism for rapid decrease in PFK-1 activity observed during starvation.

Glucose-6-phosphate levels rose 3 fold over 9 hours starvation and 17 fold after 24 hours. After 9 hours the concentration of glucose-6-phosphate would not yet have been sufficient to inhibit hexokinase. Hexokinase type II is the predominant form in lactating mammary gland and has a Ki of 0.16 mM for glucose-6-phosphate (Grossbard and Schimke 1966).

5.1.3 <u>THE EFFECTS OF FASTING ON THE ACTIVITY OF ACC AND PDH IN LACTATING MAMMARY GLAND.</u>

Table 3 shows that changes in the activity of PDH

Table 3 The effects of Fasting on the Activities of ACC and PDH in Lactating Mammary Gland.

Hours Starvation	Purified ACC (Units/mg protein)	% inhibition	Tissue Citrate (μmol/g fres tissue)	PDH (munits/unit citrate synthase)	% inhibition	A-V for Lactate (μmol/ml blood
Fed (control)	3.48±0.23 (7)	-	0.21±0.01(4)	127.8±4.8(6)	-	1.10±0.11(6)
1.5	2.70±0.12 (3)	22	0.21±0.01(5)	104.6±4.3(6)***	20	0.63±0.05(6)
3.0	3.21±0.12 (3)	6	0.21±0.01(6)	64.0±3.8(6)***	50	0.54±0.03(6)
6.0	3.06±0.22 (8)	12	0.22±0.03(4)	26.3±1.8(6)***	80	0.36±0.03(6)
9.0	2.40±0.16 (5)**	40	0.35±0.01(4)***	22.8±1.7(6)***	83	0.26±0.02(6)
12.0	2.07±0.24 (6)**	58	0.37±0.02(3)***	ND	-	0.13±0.01(3)
18.00	1.39±0.09 (5)***	60	0.43±0.02(5)***	ND	-	0.25±0.03(5)
24.00	1.67±0.13 (10)***	52	0.53±0.02(4)***	10.7±0.7(6)***	92	-0.44±0.03(6)

ACC activity is expressed as Vmax where 1 unit of activity = 1 μmol/min. Results are expressed as mean ± SEM.
The numbers in parentheses indicate the number of observations made.
Values which are significantly different from the relevant control are denoted by:

***P<0.001; **P<0.005; *P<0.025; ND = not determined.

unlike ACC, correlate exactly with the decrease in lipogenesis (Table 1). The arterio-venous differences for lactate also fell which is consistent with decreasing PDH activity (but see section 5.2.3). The activity of purified ACC did not fall significantly until between 6 and 9 hours starvation. Covalent modification of ACC is therefore clearly not involved in the short term (< 6 hours) metabolic adaptations to starvation. This is in agreement with the conclusions of Munday and Hardie 1986a, and Williamson et al 1983.

These changes in PDH and ACC activities are associated with changes in some metabolite levels. The effect of starvation on the pyruvate-lactate couple in the mammary gland is shown in Table 4. The ratio of pyruvate/lactate had decreased by 50% after 1.5 hour starvation, and then fell a further 10% after 6 hours. The change in the ratio was primarily due to a fall in pyruvate concentration. Pyruvate and lactate are in equilibrium according to the following equation:

$$\text{pyruvate} + \text{NADH} \xrightarrow{\text{LDH}} \text{lactate} + \text{NAD}^+$$

LDH = lactate dehydrogenase

and as lactate dehydrogenase is an exclusively cytoplasmic enzyme the observed decrease in the ratio of pyruvate/lactate indicates an increased cytoplasmic $NADH/NAD^+$ ratio. The increased $NADH/NAD^+$ ratio will reinforce the inhibition of glycolysis produced by decreased PFK-1 activity by inhibiting the production of glyceraldehyde-1,3-bisphosphate.

In starvation, changes in the cytoplasmic $NADH/NAD^+$ ratio correlate positively with changes in the mitochondrial ratio (Newsholme and Start 1981). The link between the mitochondrial and cytoplasmic $NADH/NAD^+$ couples is the malate-aspartate shuttle which is the mechanism whereby cytosolic NADH is oxidised to NAD^+ (Fig. 5.1) and the reducing equivalents transferred to the mitochondrial

Figure 5.1 THE MALATE-ASPARTATE SHUTTLE

The figure (from Nicholls, 1987) shows the mechanism for the oxidation of cytosolic NADH. TCA cycle intermediates 2-oxoglutarate and a low mitochondrial NADH/NAD^+ ratio are required for the malate-aspartate to function. Thus depletion of the intermediates or an unfavourable mitochondrial NADH/NAD^+ ratio as occurs in starvation will cause an elevated cytosolic NADH/NAD^+ ratio.

I - cytosolic malate dehydrogenase oxidises NADH
II - exchange of malate for 2-oxoglutarate
III - mitochondrial malate dehydrogenase oxidises malate; regenerates NADH
IV - aspartate and 2-oxoglutarate formed by transamination of oxaloacetate with glutamate
V - transamination of 2-oxoglutarate and aspartate in cytosol regenerates oxaloacetate.
VI - the glutamate formed re-enters the mitochondrial matrix by proton symport in exchange for aspartate.

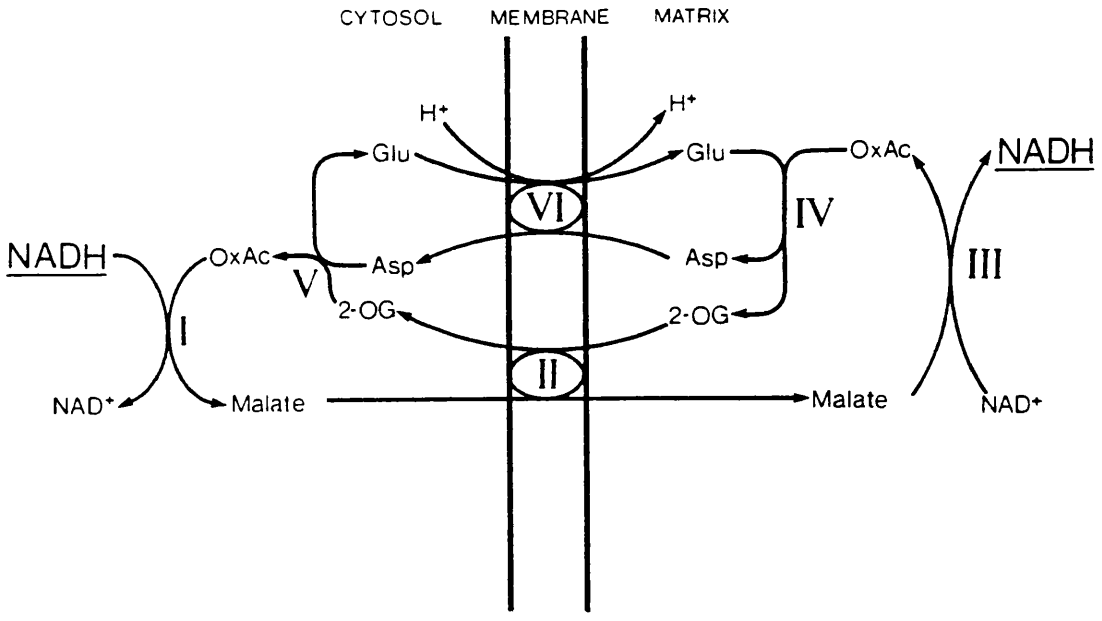


Table 4 The Changes in Mammary Gland Lactate and Pyruvate Concentration During Starvation.

Hours Starvation	Pyruvate (μmol/g)	Lactate (μmol/g)	Pyruvate/Lactate
Fed	0.150±0.01	2.54±0.46	0.059
1.5	0.070±0.001*	2.34±0.41	0.030
3.0	0.053±0.002*	2.27±0.03	0.023
6.0	0.045±0.005*	2.18±0.03	0.021
9.0	0.041±0.004*	1.77±0.29	0.023
24.0	0.040±0.004*	1.54±0.16*	0.026

Experimental details are described in text.
Values are the result of at least 5 experiments and expressed as the mean ± SEM.
Results which are significantly different from the fed (control) values as assessed by Student's t-test are denoted:
*$P<0.05$

matrix. When ß-oxidation of fatty acids is high, as in starvation, the mitochondrial NADH/NAD^+ ratio will rise and the malate-aspartate shuttle decrease due to the inhibition of the TCA cycle (Williamson and Cooper 1980). This is due primarily to the increased NADH/NAD^+ ratio reducing the concentrations of α-ketoglutarate and malate, both essential components of the carrier systems. Although fatty acid levels were not measured in these studies it is well documented that the concentration of plasma and tissue fatty acids rises when animals are starved (Weiland et al 1972; Seitz et al 1977; Page and Kuhn 1986). In lactating rats, increases in serum fatty acids are discernible after 3 hours and are significant after 6 hours (Page and Kuhn 1986). These changes will be reflected intracellularly as there is rapid equilibration of fatty acids across the plasma membrane (Noy et al 1986). Thus the decreased PDH activity (Table 3) is partly due to the activation of PDH kinase by the elevated NADH/NAD^+ and acetyl-CoA/CoA ratios associated with increased intracellular fatty acid concentration and ß-oxidation (Seiss and Weiland 1976). The proposed role of fatty acids in the regulation of mammary metabolism was studied further using the mammary gland perfused in situ and also isolated acini (Sections 5.2 and 5.3).

The circulating concentration of insulin in lactating rats falls by 48% after 6 hours starvation and 64% after 24h starvation (Jones et al., 1984a). Short term insulin deficiency decreases lipogenesis in the mammary gland (Robinson et al 1978; Bussmann et al 1984). Initial PDH activity was also reduced though its 'total' activity was unchanged indicating that the reduced activity was due to phosphorylation (Field and Coore 1976). The latter authors also show that the plasma free fatty acid levels were doubled by streptozotocin treatment for 3 hours. Thus it is possible that the effect of insulin deficiency on PDH activity was due to the elevated plasma free fatty acid levels leading to an increased intracellular ratio of NADH/NAD^+ and acetyl-CoA/CoA and inhibition of PDH as

Table 5 The Changes in Mammary Gland Ketone Body and Citrate Concentration During Starvation.

Hours starvation	Total Ketone bodies (μmol/g)	Citrate (μmol/g)
Fed	0.12±0.02	0.21±0.01
1.5	0.15±0.02	0.21±0.01
3.0	0.16±0.02	0.22±0.01
6.0	0.18±0.02	0.22±0.03
9.0	0.25±0.03*	0.35±0.01***
24.0	0.52±0.54***	0.53±0.02***

Total ketone bodies are acetoacetate plus ß-hydroxybutyrate.
Experimental details are as described in text and values are the mean ± SEM from at least four experiments.
Results which were significantly different from fed (control) values as assessed by Student's t-test are denoted:
*P<0.01; ***P<0.001.

described earlier. The same authors (Baxter and Coore 1979a) have also reported that 24 hour starvation and 3 hour insulin deprivation causes a decreases in PDH phosphatase activity which is rapidly reversed by refeeding or the administration of insulin. Thus changes in insulin may play a direct role in the regulation of PDH activity through PDH phosphatase.

The significant decrease in mammary gland ACC activity between 6 and 9 hour starvation was coincident with an increase in the tissue concentration of both ketone bodies (acetoacetate plus ß-hydroxybutyrate) and citrate (Table 5). Ketone body concentration has been proposed as a signal for reducing the utilisation of glucose in lactating mammary gland (Hawkins and Williamson 1972). Acetoacetate reduces glucose uptake in mammary gland in vivo (Robinson and Williamson 1977b) and has been shown to inhibit glucose incorporation into lipid in isolated acini (Robinson and Williamson 1977c). However, after 6h starvation the uptake of glucose had already declined to 1.8% of fed control values (Table 2) in the absence of any significant increase in tissue ketone body concentration (Table 5). The possibility that ketone bodies inhibit ACC is suggested by the increase in citrate concentration in acini in response to acetoacetate (Robinson and Williamson 1977c). Furthermore, the inhibition of ACC and the increase in mammary gland ketone body concentration occur over similar time courses of starvation (i.e. from 6h onwards, Table 3 and Table 5). Therefore the effects of acetoacetate on the activity of ACC were studied further in the mammary gland perfused in situ (Section 5.4).

The 50% increase in citrate concentration which occurs between 6 hours and 9 hours starvation (Table 5) was almost certainly a consequence of the inhibition of ACC. Reports describing experiments using 5-(tetradecycloxy)-2-furoic acid (TOFA) in hepatocytes (McCune and Harris 1979) show that the compound inhibits ACC directly by being converted to TOFA-CoA intracellularly. This inhibition of ACC caused

a doubling of tissue citrate concentration. Similarly Robinson and Williamson (1977c) measured a doubling in citrate levels when isolated mammary acini were incubated with TOFA.

The results support the proposal that the declining rate of lipogenesis in the first 6 hours of starvation was a result of reduced substrate supply rather than changes in the activity of ACC. As described earlier (5.1.1) the activity of PFK-1 (as judged by the increased G-6-P/F-1,6-P_2 ratio) declined rapidly upon starvation (Table 2). The rate of decline increased after 6 hours when citrate levels started to rise (Table 5). So citrate appears to play a small part in the regulation of glycolysis (and thus lipogenesis) in the lactating mammary gland during starvation in that it inhibits PFK-1 but as with the inhibition of ACC, this effect occurs when glycolysis and lipogenesis are already profoundly inhibited.

5.2 PERFUSION OF MAMMARY GLAND WITH PALMITATE

Observations made in this study and by others on the inhibition of mammary gland lipogenesis by starvation suggests that fatty acids and ketone bodies may be important signals. This hypothesis was tested in the perfused mammary system. The left inguinal mammary gland of lactating rats was perfused using a non recirculating system as described in Materials and Methods. The perfusate was three parts medium 199 containing 8 mg/ml fatty acid free BSA mixed with one part (by volume) of goat or human erythrocytes. The major respiratory substrates in medium 199 were 5.5 mM glucose and 0.6 mM sodium acetate. 0.1 mUnits/ml of insulin was added to the perfusate to elevate glucose uptake and the flux through the glycolytic and lipogenic pathways and thus make the effects of fatty acids easier to see.

5.2.1 UPTAKE OF PALMITATE BY PERFUSED MAMMARY TISSUE

Table 6 shows that there is significant uptake of palmitate by the perfused mammary gland. The A-V difference for palmitate across the perfused gland of 0.16 meq/l (Table 6) is similar to the values A-V difference for the fatty acids across the mammary gland *in vivo* measured by Hawkins and Williamson (1972). The perfusate contained 0.96 ± 0.16 mM (mean ± SEM, 8 perfusions) of palmitate complexed with albumin.

Significant efflux of fatty acids was seen in the control perfusions which contained no added palmitate. This was probably due to the diffusion from the mammary epithelial cells of *de novo* synthesised fatty acids although it is possible that some of the fatty acid in the post mammary perfusate was released from damaged cells. This net efflux of fatty acids by perfused mammary tissue was probably not physiological as *in vivo* the arterial concentration of unesterified fatty acids in the fed lactating rat is 0.61 ± 0.11 meq/l (Hawkins and Williamson, 1972) rather than the 0.034 ± 0.006 meq/l of the perfusate. Thus *in vivo* there would be net diffusion of fatty acids into the mammary gland (Cooper et al 1987). Efflux of fatty acid from dispersed mammary cells has been observed by Hansen and Knudsen (1987a).

5.2.2 EFFECT OF PALMITATE ON THE ACTIVITY OF ACC AND PDH

Perfusion of the mammary gland with palmitate had no effect on the activity of purified ACC (Table 7) but produced a 60% fall in the activity of PDH (Table 8). The inhibition of PDH in mammary gland by long chain fatty acids has been inferred because its activity is decreased by starvation and diabetes (Kankel and Rienauer 1976; Baxter and Coore 1978; Field and Coore 1976), two states associated with elevated serum fatty acid levels (Seitz et al 1977). However inhibition of PDH by increasing fatty acid levels has not been previously demonstrated in mammary

Table 6. Uptake of Palmitate by Perfused Mammary Gland

Additions to perfusate	Arterio-venous difference, meq/l	Flow ml/min	Uptake μeq/min
none, n=8	-0.08±0.01	1.73±0.14	-0.12±0.01
1 mM palmitate n=8	+0.16±0.01	2.01±0.21	+0.33±0.05

0.1mUnits/ml of insulin was present in all perfusions. Uptake was calculated by division of the arterio-venous difference by the perfusion flow rates '-' denotes efflux from the gland; '+' denotes uptake. meq = millequivalents of free fatty acid.
Results are expressed as the mean ± SEM; n=number of observations.

Table 7. The Effect of Palmitate on the Activity of Acetyl-CoA Carboxylase purified from Perfused Mammary Gland.

Additions to perfusate	Vmax (μmol/min/mg/ACC)	Ka Citrate (mM)	Hill Coefficient
None n=8	1.17±0.17	2.55±0.33	1.32±0.05
1mM palmitate n=8	1.57±0.34	3.07±0.32	1.42±0.08

0.1 m Units/ml insulin was included in the perfusate.
Experimental details are described in text.
Results are the mean ± SEM. n = number of observations.

Table 8 The Effect of Palmitate on the Activity of Pyruvate Dehydrogenase (PDH) and Citrate Synthase (CS) in Perfused Mammary Gland.

Additions to Perfusate	PDH	CS	Ratio PDH:CS
None n=6	9.00±0.79	80.97±4.13	0.111
1mM palmitate n=7	3.76±0.12***	100.35±18.40	0.037

0.1 m Units/ml insulin was included in the perfusate. Other experimental details are described in text. Activity is expressed as μmol/min/g wet weight of tissue. Results are expressed as mean ± SEM; n = number of observations.
Differences between palmitate perfusions and the relevant controls were assessed by Student's t-test: ***P<0.001.

tissue. This result contrasts with that of Robinson and Williamson (1978c) who found no change in lactate and pyruvate production in isolated rat mammary acini incubated with oleate. From this they inferred that the PDH activity was not inhibited by high fatty acid levels. (See section 5.2.3)

The observed decrease in the activity of PDH in palmitate perfusions was clearly due to phosphorylation of the complex as it was stable to dilution to 1:150 in the PDH assay. Degradation is unlikely to have occurred as even after 24 hour starvation total PDH in mammary gland is unchanged (Baxter and Coore 1978). End product inhibition of the complex by acetyl-CoA or NADH which may occur <u>in vivo</u> would be negligible at the dilutions used in the assays. There is no evidence in liver for the direct activation of PDH kinase by fatty acids. PDH kinase is activated by increased ratios of acetyl-CoA/CoA and $NADH/NAD^+$ (Pettit et al 1975, Kerbey et al 1976) and PDH phosphatase is inhibited by an elevated $NADH/NAD^+$ ratio. These changes in metabolite levels would be produced by increased ß-oxidation. The rate of mitochondrial ß-oxidation is limited by the transfer of the long chain fatty acids into the mitochondria from the cytosol and is associated with changes in the activity of ACC. Inhibition of the latter reduces intracellular malonyl-CoA levels which in turn relieves the inhibition of long chain acyl carnitine acyltransferase I (CAT I), (McGarry and Foster 1979). Miller et al (1970) have also shown that ACC from rat mammary gland is inhibited by fatty acyl-CoA esters. Carling et al (1989) have proposed that in liver long chain fatty acids induce the inhibition of ACC by stimulating a cascade system which results in the phosphorylation and activation of AMP-PK which then in turn phosphorylates and inhibits ACC. 5'AMP generated by the formation of fatty acyl-CoA esters might also have been expected to activate AMP-PK and thus increase the phosphorylation of ACC. However this is clearly not occurring in the palmitate perfused mammary gland as ACC purified from both control

and palmitate perfused animals had the same kinetic parameters (Table 7). Thus increased ß-oxidation of fatty acids and the subsequent inhibition of PDH probably occurs in the palmitate perfused mammary gland at least partly as a result of the allosteric inhibition of ACC by fatty acids. Profound inhibition of ACC would not be necessary under these experimental conditions for the relief of CAT-I inhibition because the high intracellular concentrations of fatty acyl-CoA esters (as evidenced by the high A-V difference in the palmitate perfused animals, [Table 6]) could reverse the inhibition of CAT-I by successfully competing with malonyl-CoA for the transferase (McGarry et al 1978 a,b) and thus directly increasing their own ß-oxidation. In lactating rats the Vmax of ACC is reduced by 64% (Munday and Hardie 1986b) by a high fat diet so the long term effects of high intracellular fatty acid concentrations are different from the short term.

5.2.3 EFFECT OF PALMITATE ON GLUCOSE AND LACTATE UPTAKE

Perfusion of the mammary gland with palmitate produced only a 10% fall in glucose uptake (Table 9). A minimal effect of fatty acid on glucose transport has also been observed in palmitate perfused rat liver (Kohout et al 1971) and in isolated rat mammary acini with oleate (Robinson and Williamson 1978c). The maintenance of glucose uptake is necessary to provide glycerol-3-phosphate for increased esterification. It is well documented that palmitate stimulates triglyceride synthesis in adipocytes (Evans and Denton 1977), hepatocytes (Mayorek and Bar Tana 1983) and bovine or goat mammary cells (Hansen and Knudsen 1987a). This is because the acyltransferases which catalyse the synthesis of phosphatidate use the CoA esters of palmitate and (to a lesser extent) oleate as their preferred substrates (Abou-Issa and Cleland, 1969; Yamashita et al 1973; Kinsella and Gross 1973; Marshall and Knudsen 1977). In addition it has been shown in liver that long chain fatty acids promote the translocation of

Table 9 The Effects of Palmitate on the Uptake of Glucose and Lactate

Additions to perfusate	Arterio-Venous difference (mM)		Uptake (μmol/min)	
	glucose	lactate	glucose	lactate
none, n = 8	0.90 ± 0.15	1.85 ± 0.34	1.60 ± 0.33	3.32 ± 0.65
1 mM palmitate n=8	0.70 ± 0.06	1.47 ± 0.13	1.44 ± 0.21	3.07 ± 0.56

Experimental details are described in Materials and Methods section. Uptake was calculated by dividing the arterio-venous difference by the flow rate of the perfusion. Results are expressed as mean ± SEM. n = number of experiments.

phosphatidate phosphohydrolase from the cytosol to the endoplasmic reticulum membranes where it is believed to be physiologically active (Brindley 1984).

Lactate uptake was also unchanged by palmitate perfusion (Table 9). This result was surprising because a change in PDH activity is usually associated with, and has been inferred from, inverse changes in lactate utilisation (Robinson and Williamson 1978, Munday and Williamson 1981). This observation shows that in the perfused mammary gland at least, A-V differences for lactate do not necessarily reflect changes in the activity of PDH. The constancy of lactate uptake clearly indicates that despite a 60% fall in PDH activity in the palmitate perfusions the flux of pyruvate through the enzyme was unchanged. The activity of PDH has clearly not been reduced to limiting levels. The addition of insulin to the perfusate doubled the activity of PDH and increased ACC activity by 50% (Table 13). Therefore the 50% fall in PDH activity observed would only reduce the PDH activity to levels 20% below levels observed in tissue perfused in the absence of insulin. The effect of palmitate perfusion on lipogenesis could not be tested in the perfused tissue due to problems of containment of the amounts of ^3H_2O required. Therefore the effects of palmitate on isolated mammary acini were investigated. (See section 5.3)

The observation that a drop in PDH activity is not necessarily reflected by decreased flux through the glycolytic pathway or decreased lactate uptake indicates that PDH is not always a limiting enzyme for fatty acid synthesis. This has implications for the interpretation of data where correlations between PDH and observed effects are being made. (See section 5.1)

5.3 METABOLIC CHANGES IN ACINI INCUBATED WITH LONG CHAIN FATTY ACIDS

Mammary acini were incubated with 1 mM palmitate or 1

mM oleate complexed with albumin as these are the major serum fatty acids available to the mammary gland *in vivo*.

5.3.1 THE EFFECT OF FATTY ACIDS ON THE ACTIVITY OF ACC AND PDH AND THE RATE OF LIPOGENESIS.

Table 10 shows that in acini, as in perfused tissue, palmitate had no effect on the activity of ACC measured in crude extracts. In contrast, however, oleate produced a 20% ($p<0.005$) fall in the activity of ACC. These changes in ACC activity correlate with changes in the rate of lipogenesis (Table 11): oleate produces a 27% ($P<0.025$) fall in lipogenesis. This result supports the generally held view that the rate of lipogenesis is limited by the activity of ACC.

These results also show that palmitate and oleate cannot be regarded as 'equivalent' fatty acids with respect to mammary metabolism. Differential effects between fatty acids have also been observed in bovine and goat mammary cells (Hansen and Knudsen 1987 a,b) and can be attributed to the substrate specificity of diglyceride acyl transferase. Although diglyceride acyl transferase can esterify palmitate to the sn-3 position, palmitate is a poorer substrate than oleate or medium and short chain fatty acids (Marshall and Knudsen 1977b, 1980; Mayorek and Bar-Tana 1983; Hansen et al 1984; Rao and Abraham 1980). The results with acini are consistent with this and indicate that with 1 mM-palmitate, the intracellular levels of its CoA ester were not raised enough, probably because of the fast rate of phosphatidate synthesis, to compete effectively with the fatty acids produced *de novo* for diacylglycerol acyltransferase, but the same concentration of oleate was able to do so. Exogenous oleate was therefore esterified to the sn-3 position of diacylglycerol causing accumulation of *de novo* synthesized fatty acids and the observed inhibition of ACC. Clarke et al (1977) have also reported that palmitate did not inhibit lipogenesis in rat liver or adipose tissue whereas oleate, linoleic acid

Table 10 The Effect of Fatty Acids on the Activity of ACC in Crude Extracts of Isolated Rat Mammary Acini

Additions to incubations	Initial Activity 0mM citrate	Initial Activity 20mM citrate	Total Activity 20 mM citrate
None, n = 14	0.132±0.016	0.202±0.016	0.407±0.023
Palmitate, n=6	0.129±0.036	0.204±0.032	0.443±0.040
Oleate, n=8	0.105±0.016**	0.169±0.021*	0.371±0.033

Experimental details are described in text. Activity is expressed as μmol/min/ug homogenate DNA
Results are the mean ± SEM; n=number of experiments.
Measurements for activity of ACC were analysed by Student's t-test for paired data. Acini incubated with fatty acid were compared with those incubated simultaneously in fatty acid free medium (*P<0.01, **P<0.005)

Table 11 Fatty Acid Synthesis in Isolated Mammary Acini Incubated with Palmitate and Oleate.

Acini incubation MEDIUM	Fatty Acid Synthesis μmol ^3H_2O/min/100mg defatted dry weight
CONTROL n=14	0.592 ± 0.091
Palmitate 1mM n=6	0.583 ± 0.184
Oleate n=7	0.433 ± 0.066**

Results are expressed as mean ± SEM. n = number of observations.
Differences between the acini incubated with fatty acid and control were analysed using Student's t-test for paired data:
**P<0.025

(18:2) and linolenic acid (18:3) all did. Furthermore they found that 18:2 and 18:3 were better inhibitors of lipogenesis than oleate (18:1). The superiority of unsaturated over saturated fatty acids as lipogenic inhibitors has also been observed in isolated hepatocytes (Wilson et al 1990).

5.3.2 THE EFFECT OF FATTY ACID ON PDH AND ACC ACTIVITY IN ISOLATED ACINI

In contrast to what was observed in perfused mammary gland, PDH activity in isolated acini is unaffected by incubation with fatty acids (Table 12). However, direct comparison with the perfused mammary gland is difficult because insulin did not activate PDH in isolated acini as it did in perfused tissue (compare Table 8 and Table 13). The insensitivity of PDH to insulin in isolated mammary acini has been observed by others (Munday and Williamson 1981). Storer et al (1980) have reported that in livers perfused *in situ* with perfusate of 50% reduced haematocrit, the effects of insulin on glucose uptake and ketogenesis (stimulation and inhibition respectively) were abolished. So the insensitivity of some metabolic parameters to insulin seen in acini may be caused by decreased oxygen supply. The reasons why, in isolated acini, some pathways retain their insulin sensitivity (e.g. ACC Table 13) while others do not will require the study of insulin post receptor events especially the generation of chemical mediators which have been shown in adipose tissue (Kiechle et al 1980, 1981) and liver (Saltiel et al 1982, Saltiel 1987) to stimulate PDH activity. The full characterisation of the receptor interactions on mammary cells will also be needed. The mammary gland synthesises autocrine effectors, for example 17ß-oestradiol (Walker and Peaker 1978) and catecholamines (Marchetti and Labrie 1990), and it is possible that these (or other) locally produced effectors interact with insulin in perfused tissue and *in vivo* in a manner not possible in isolated acini. When tissue or acini were exposed to insulin the activity of ACC

Table 12 The Effect of Fatty Acids on the Activity of Pyruvate Dehydrogenase (PDH) and Citrate Synthase (CS) in Isolated Mammary Acini

Addition to incubations	PDH	CS	PDH:CS
None, n=14	4.82±0.27	84.01±4.73	0.057
Palmitate, n=6	5.43±0.29	90.57±4.64	0.060
Oleate, n=8	5.15±0.44	74.88±5.35	0.069

Activity is expressed as μmol/min/g acini. Results are the mean ± SEM; n=number of experiments.

in crude extracts was increased (Table 13). The increase in the initial activity of ACC was expected but the 'total' activity of ACC also rose. Changes in the total activity of an enzyme are usually interpreted to indicate a change in the absolute amount of the enzyme. It is not possible that the 90 minute perfusion or 15 minute incubation would have brought about new synthesis of ACC sufficient to double or increase by 50% the total amount of ACC present in the tissues. The best explanation for this observation is provided by Allred and co-workers (Allred et al 1985, 1989; Allred and Roman-Lopez 1988), who report the existence of an inactive mitochondrial form of ACC which is shifted to an active cytoplasmic form in lipogenic conditions.

5.4 PERFUSION OF MAMMARY GLAND WITH ACETOACETATE

The left inguinal mammary gland was perfused with 1 mM acetoacetate as described in section 5.2, but insulin was not added to the perfusate because insulin abolishes some of the effects of ketone bodies in mammary tissue (Williamson et al 1975, Robinson and Williamson 1977c).

5.4.1 EFFECTS OF ACETOACETATE ON MAMMARY METABOLISM

Acetoacetate had no effect on PDH or ACC activity; lactate uptake was also unchanged (results not shown). Glucose uptake however increased by 40% ($P<0.025\%$) from 0.61 ± 0.06 μmol/min (mean $\pm$ SEM, n=8) to 1.02 ± 0.14 μmol/min (mean $\pm$ SEM n=8) when acetoacetate was perfused. These results are contrary to those of the Williamson group (Williamson et al 1975, Robinson and Williamson 1977 b,c) who reported decreased lactate and glucose uptake. Ketone bodies are both lipogenic and oxidative substrates in lactating mammary gland (Williamson et al, 1975, Robinson and Williamson 1978a) and these results from perfused tissue are consistent with acetoacetate increasing lipogenesis. Stimulation of glucose uptake by acetate has been observed in mammary gland slices (Williamson et al

Table 13 Activation of ACC and PDH by Insulin:- occurs when mammary gland is perfused with media containing 0.1mU/ml insulin but the same concentration of insulin in isolated acini elevates the activity of ACC but has no effect on PDH.

Enzyme activity	Isolated Acini insulin (mU/ml) 0	0.1	Perfused Tissue insulin (mU/ml) 0	0.1
ACC				
'Initial'activity 0mM citrate	0.07±.01	0.15.0.01***	0.12±0.02	0.19±0.002*
'Initial'activity 20mM citrate	0.11±0.01	0.22±.01***	0.20±0.04	0.30±0.02*
'Total'activity 20mM citrate	0.24±0.02	0.41±.03***	0.44±0.07	0.61±0.04*
Pyruvate Dehydrogenase	4.07±0.03	4.82±0.27	4.58±1.01	9.00±0.79**

Data for ACC activity in perfused tissue from Clegg and Calvert.
Units of Activity:ACC μmol/min/mg DNA;
PDH μmol/min/g wet wt
Differences between treatments +/- insulin were analysed using Student's t-test for unpaired data: *P,0.05 **P<.005 ***P<.001

1975) and also isolated acini (Katz et al 1974).

Thus in the perfused mammary gland acetoacetate may be providing a substantial pool of cytosolic acetyl-CoA, through the concerted activity of acetoacetyl-CoA synthase and acetoacetyl-CoA thiolase, (Buckley and Williamson 1975) or butyryl-CoA (Robinson and Williamson 1977c), a better primer than acetyl-CoA for fatty acid synthesis in rat mammary gland, which was not accompanied by increased cytosolic citrate. The observed increase in glucose uptake would be consistent with a stimulation of the pentose phosphate pathway in order to ensure an adequate supply of reduction equivalents for fatty acid synthesis and glycolysis to supply glycerol-3-phosphate for esterification.

5.5 SUMMARY AND CONCLUSIONS

1) The activities of PFK-1 and PDH are the most probable control points during starvation for the limitation of substrate for fatty acid synthesis as their activities decline coincident with lipogenesis.

2) Free fatty acids are the probable mediators of PDH inhibition during starvation as its activity can be reduced by 60% by perfusing mammary gland with palmitate for 90 minutes.

3) Inhibition of PFK-1 may be through the reduction of F-2,6-P_2 as the concentration of the physiological inhibitors citrate and ATP did not change during short term (< 6 hours) starvation. Direct inhibition of PFK-1 through phosphorylation is also a possibility.

4) The cytoplasmic NADH/NAD^+ couple becomes more reduced during starvation this will reinforce the inhibition of glycolysis and is indicative of an inhibited TCA cycle as would be expected because anaplerotic substrates such as pyruvate, and anabolic pathways are

severely reduced during starvation. (Bussmann et al, 1984).

5) The inhibition of ACC through phosphorylation plays no part in the large inhibition of lipogenesis that occurs in the first 6h starvation. The inhibition which occurs at the later stages of starvation (>6 hours) may, be promoted by free fatty acids reaching a critical level and thus activating AMP-PK via the AMP-PK Kinase.

However, it must be noted that while a stable increase in mammary AMP-PK activity after 24h starvation (Chapter 4, Table 8) was observed this increase was not statistically significant.

6) Palmitate and oleate show different properties with respect to their effects on ACC activity and lipogenesis. Namely oleate causes inhibition of ACC and lipogenesis but palmitate under the same conditions does not. It is proposed that a higher rate of palmityl-CoA esterification prevents the free fatty acid CoA ester reaching inhibitory levels at the concentrations used but that oleoyl-CoA is not esterified so rapidly and so can accumulate and thus inhibit ACC presumably through activation of AMP-PK as postulated for the liver enzyme by palmitoyl-CoA, Hardie (1989).

7) Ketone bodies are not signals for the inhibition of ACC or PDH. There is no correlation between ketone body concentration and the activity of PDH during starvation and when mammary tissue was perfused with acetoacetate the activities of ACC and PDH were unchanged. Unexpectedly, acetoacetate stimulated glucose uptake in the perfused mammary gland which suggests that it was functioning as a lipogenic substrate and also promoting esterification. One can speculate that the phosphorylation of ACC <u>in vivo</u> after 6 hours starvation occurs to prevent the ketone bodies (whose tissue concentrations rise steeply at the same time) being channelled into fatty acid synthesis.

These studies were concerned with the elucidation of the factors within the mammary which caused the decline in lipogenesis associated with starvation. The precise initiator of the changes described was not investigated but it is almost certainly insulin perhaps acting in conjunction with other effectors. Insulin levels are halved in 24 hour starved lactating rats (Robinson and Williamson 1978b). It has also been shown that the presence of food in the gut stimulates the secretion of peptides which can in turn stimulate insulin secretion (Unger and Eisentraut 1969; Douglas et al 1990; Page 1989). Thus during starvation the decrease in insulin levels are larger than the decrease in serum glucose would suggest (22% after 9 hours starvation). Klein et al (1990) have shown in starved humans that even when serum glucose levels were maintained by glucose infusion during the fast plasma insulin still declined and the serum free fatty acids concentration still increased. Thus decreased glucose concentrations per se are not responsible for the physiological manifestations of starvation. These observations suggest that it is the absence of food in the gut rather than the decrease in energy supply that precipitates the fall in serum insulin (and perhaps other factors) which then causes increased lipolysis from adipose tissue and decreased triglyceride esterification in the mammary gland. The intracellular levels of fatty acids would then rise and lead to the decreased mammary gland metabolism observed. Such a mechanism acting in reverse would also explain the rapid reactivation of lipogenesis which occurs upon refeeding (Robinson and Williamson 1978b)

Further work

Several avenues of further research have been suggested by the work described in this thesis. The elucidation of the regulation of the AMP-PK especially with regard to phosphorylation by AMP-PK kinase would be particularly interesting especially as inhibition of ACC is 'slow onset' when the animal is starved but activity is rapidly restored when it is refed. The elucidation of the molecular structure of cAMP-PK would confirm the biochemical evidence presented which suggests that the mammary gland expressed a novel isoform of the kinase.

The differential effects of oleate and palmitate on fatty acid synthesis are worthy of further investigation as a possible mechanism for the dietary regulation of lipogenesis; and may have implications for the study of obesity and its control.

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