The Role of the γ-Glutamyl Cycle in Milk Protein Synthesis in the Ruminant

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The research presented in this thesis indicates milk protein production in the ruminant can be increased by supplying N-acetylcysteine to the mammary gland and this information would considerably benefit the dairy industry. AgResearch Limited has funded this research and it is requested that this thesis be embargoed to allow time to achieve a competitive advantage.

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Abstract

Dairy products are New Zealand's primary export commodity. The manufacturing efficiency for dairy products would be maximised if the Dairy Industry had the ability to control milk protein production to suit the manufacture of specific products. Understanding the role of amino acid transport in regulating milk protein synthesis may allow manipulation of proteins in milk. γ-Glutamyl transpeptidase (γ-GT), an enzyme thought to play a key role in mediating amino acid transport, has been demonstrated in mammary tissue, but the role of this enzyme and its associated biochemical pathway, the γ-glutamyl cycle, has not been fully elucidated. The γ-glutamyl cycle consists of synthetic and degradative enzymes for the cysteine-containing tripeptide glutathione. γ-GT transfers the γ-glutamyl moiety from glutathione to amino acids, and has a high affinity for cyst(e)ine. The vascular supply of cysteine is thought to be insufficient to maintain milk protein synthesis. In this study, the role of the γ-glutamyl cycle in amino acid transport for milk protein synthesis was investigated using two systems, firstly, in acini isolated from the udder of lactating sheep, and secondly in lactating goats. Milk protein secretion from isolated acini significantly decreased (70%) as a result of γ-GT inhibition with acivicin, and significantly increased (250%) when supplied with cysteine as N-acetylcysteine (NAC). In lactating goats, acivicin did not affect milk yield or total protein concentration or yield, but significantly increased α_{s2}- and κ-casein concentration in milk. This may have resulted from increased uptake of some amino acids by the mammary gland and suggests that γ-GT negatively regulates uptake of some amino acids for milk protein synthesis. NAC significantly increased milk yield, protein concentration and protein yield as a result of increased uptake of some amino acids, which may have been due to increased mammary blood flow. This increase was prevented by acivicin, however, suggesting that γ-GT plays an important role in amino acid supply. Inhibition of γ-GT may up-regulate sub-saturated transport systems leading to increased uptake of amino acids required for milk protein synthesis. Further testing of NAC and a greater understanding of the function and regulation of γ-GT may allow increased, and targeted, milk protein production as required by the Dairy Industry.

To my parents Graham and Jacqueline Cridland

'I have nothing but a book.
Nothing but that to prove your blood and mine.'

W B Yeats

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Mi amore.

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List of Abbreviations

^{3}H-AIB	α-amino-isobutyric acid [Methyl-^{3}H]
A	arterial
AA	amino acid
ATP	adenosine triphosphate
A-V	arterial venous difference
BBB	blood brain barrier
BBM	brush border membrane
Bq	becquerel (s^{-1})
BSO	buthionine sulphoximine
CA	cellulose acetate
Ci	Curie (37×10^9 Bq)
Da	Daltons
D-MEM	Dulbecco's modified eagle medium
DTT	dithiothreitol
EAA	essential amino acids
EDTA	ethylenediaminetetra-acetic acid di-sodium salt
FCS	foetal calf serum
FIA	flow injection analysis
g	grams
h	hours
HPLC	high performance liquid chromatography
kg	kilograms
LHS	left hand side
MBW	metabolic body weight ($kg^{0.75}$)
min	minutes
MQ H_2O	Millipore deionised water
NAC	N-acetylcysteine
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NEAA	nonessential amino acids
NIT	near infrared transmittance spectrophotometry
OTCA	(-)-2-oxo-4 thiazolidine-carboxylic acid
PBS	phosphate buffered saline

PCA	perchloric acid
PITC	phenylisothiocyanate
RHS	right hand side
rpm	revolutions per minute
SBD-F	4-fluro-7-sulfobenzo-furazan ammonium salt
SDS	sodium dodecyl sulphate
SRA	specific radioactivity
TCA	trichloroacetic acid
TEA	triethylamine
U	units
V	venous

Chapter 1. Introduction

Restriction of supply and uptake of certain amino acids to the mammary gland has been suggested as a factor limiting milk protein production (Schingoethe, 1996). In ruminants, the sulphur amino acid cysteine has been postulated to be a major factor affecting the capacity of the animal to increase milk protein production (Clark *et al.*, 1978). Restriction of cysteine supply for milk protein synthesis may occur at a number of levels but the transfer of cysteine across the plasma membrane of the mammary acinar epithelial cells may be a significant limiting factor for milk protein synthesis.

The γ-glutamyl cycle in acinar epithelial cells has been suggested to play a significant role in the transfer of amino acids from the blood to the cytosol where protein synthesis takes place (Viña *et al.*, 1989), and has been shown to mediate transport of cystine, the disulphide form of cysteine, into cultured human endothelial cells and pancreatic cells (Cotgreave and Schuppe-Koistinen, 1994; Sweiry *et al.*, 1995).

The γ-glutamyl cycle is a biochemical pathway that involves the cyclic synthesis and degradation of the tripeptide glutathione (GSH). A key enzyme in this cycle, γ-glutamyl transpeptidase (γ-GT) is bound to the membrane of secretory cells and has an extracellular active site. γ-GT transfers the γ-glutamyl moiety of GSH to amino acids that are free in blood plasma. These γ-glutamyl amino acids and the remaining portion of GSH (cysteinyl-glycine) are imported into the cell where they are converted to free amino acids. The role of γ-GT in the uptake of amino acids has been the subject of debate. Studies have confirmed the use of free amino acids and production of γ-glutamyl amino acids by γ-GT and while some support exists, many believe the key role of γ-GT is not as a supplier of amino acids.

Activity of γ-GT is high in mammary cells of lactating animals (Baumrucker and Pocius, 1978), and in lactating rats, γ-GT inhibition decreased amino acid uptake by the mammary gland (Viña *et al.*, 1989). Replication of this work in ruminants may clarify whether γ-GT could be manipulated to provide increased amino acid supply and milk protein production in the dairy cow.

γ-GT has a high affinity for cysteine and its disulphide, cystine (Thompson and Meister, 1977), and may control milk protein synthesis through supply of these amino acids. GSH contains cysteine (Beutler, 1989) so one possible control could be GSH degradation by γ-GT. Also, GSH synthesis inside the cell may be drawing cysteine away from milk protein synthesis pathways. An intermediate of the γ-glutamyl cycle, 5-oxoproline, has been shown to up-regulate amino acid transport systems and this may also control milk protein synthesis (Viña *et al.*, 1989). All of these findings suggest that understanding the regulation of the γ-glutamyl cycle may lead to a way of increasing the supply of amino acids to the mammary gland for milk protein synthesis.

The Dairy Industry in New Zealand, and around the world, is increasing its emphasis on the composition of protein in milk (Schingoethe, 1996; Valeur, 1997). The ability to control milk protein profiles would facilitate manufacture of specific products. As a reflection of the commercial value of milk protein, in 1993 the New Zealand Dairy Industry switched from paying farmers for milk fat to payment based on milk solids (www.dexcel.co.nz, September 2001).

New Zealand produces 1.3 million tonnes of a variety of dairy products per year (Figure 1.1) of which 90-95% is exported. In the year ending June 1999, dairy produce was New Zealand's leading export commodity. New Zealand is the world's largest exporter of casein and caseinate products, and is one of the four major world producers of whey protein concentrate (Statistics New Zealand, 2000). Continued research aimed at manipulating milk protein production and the production of novel milk products will allow New Zealand to remain on top of the world market.

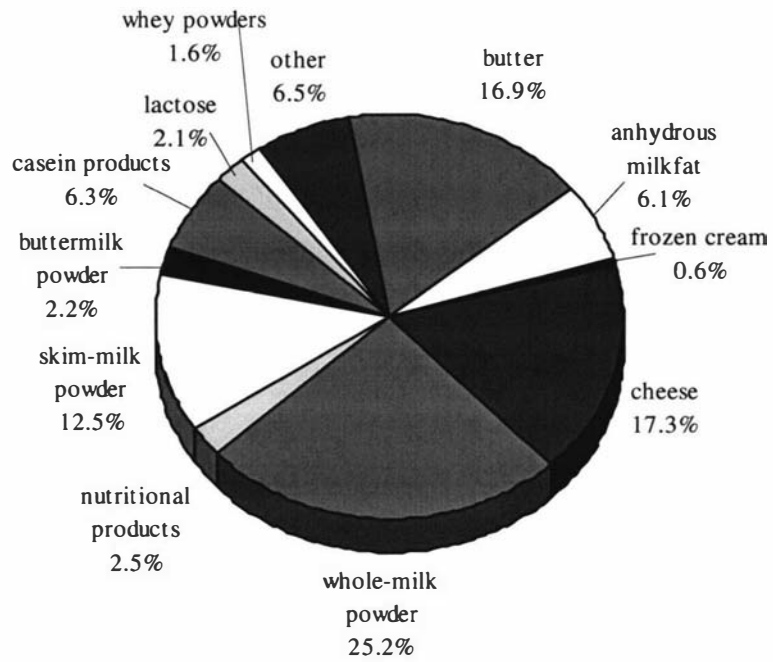


Figure 1.1 Products manufactured by New Zealand dairy factories in 1998-99.

From Statistics New Zealand (2000).

The manipulation of factors controlling milk protein synthesis in the dairy cow may allow an increase in the protein content of milk and one of these factors may be the supply of amino acids by the γ-glutamyl cycle. The role of the γ-glutamyl cycle in the supply of amino acids to the mammary gland of the ruminant needs to be clarified. In the present study the role of γ-GT, GSH synthesis, 5-oxoproline and the supply of cysteine from alternative sources were investigated for their effect on milk protein secretion of acini isolated from the udder of sheep. An attempt was made to replicate some of these results in lactating goats.

To help with the understanding of the factors involved in this study, a review of the literature has been prepared. This review covers milk composition and its effects on milk product manufacture, how milk protein composition may be controlled, the supply of amino acids to the mammary gland, and the role of the γ-glutamyl cycle in amino acid transport.

1.1. What is milk?

Milk is an excellent source of nutrients, which suits the purpose for which it was intended, the feeding of young mammals. Milk consists of water, protein, fat (mainly triglycerides) and a distinctive carbohydrate, lactose, which is a disaccaride of glucose and galactose. Milk also contains vitamins, minerals, organic acids such as citrate, and trace amounts of other miscellaneous components (Walstra *et al.*, 1999).

The composition of the major milk components depends on the species of animal (Table 1.1). Milk protein has been shown to range from 10 g/l in some primates to 200 g/l in some types of rabbit. Milk fat has been shown to range from trace to 500 g/l and carbohydrate from 0 to 100 g/l (Jenness, 1971). The milk protein content of mature bovine herd milks varies from 31 to 36 g/l (Swaisgood, 1995).

Table 1.1 Milk composition of several species.

The percentage composition of milks from some species (Allen, 1984; Jenness, 1986; Kumar *et al.*, 1994).

Animal	Genus species	Dry matter	Fat	Protein		Carbohydrate
				Casein	Whey	
Human	*Homo sapiens*	12.9	4.5	0.4	0.5	7.1
Cow	*Bos Taurus*	12.7	3.9	2.6	0.6	4.6
Goat	*Capra hircus*	13.3	4.5	2.6	0.6	4.3
Sheep	*Ovis aries*	18.0	7.2	3.9	0.7	4.8
Rat	*Rattus norvegicus*	27.6	9.3	7.2	1.0	3.7
Mouse	*Mus musculus*			7.9	1.9	4.1

Over centuries, man has domesticated animals to provide milk for food, specifically hoofed animals (including horse and camel) the most important of which are ruminants (cow, goat, sheep) (Walstra *et al.*, 1999). The selection by man of cows that perform according to use for milk or meat in a specific environment has produced large variability in the composition of milk, although variation is less between typical dairy breeds (Walstra *et al.*, 1999). Genetic variation between animals can, however, cause differences within breeds (see section 1.4.3).

Milk contains proteins that provide amino acids to meet the requirements of the growing young animal, proteins that confer immunity on the newborn and milk also contains a number of proteins that have as yet undefined or unknown functions such as lactoferrin, an iron binding protein (Jenness, 1971; Walstra *et al.*, 1999). Nomenclature of milk proteins is derived from studies of milk from the genus *Bos* (Eigel *et al.*, 1984). Acidification of milk to pH 4.6 at 20°C, or treatment with the enzyme rennin, causes the proteins called caseins to precipitate. The proteins that remain suspended in the solution (termed milk serum) after casein precipitation are collectively called whey proteins (McMeekin, 1971). The six major milk proteins are α_{s1}-, α_{s2}-, β-, and κ-casein, and β-lactoglobulin and α-lactalbumin (Table 1.2). These are described in the following sections, but refer to Eigel *et al.* (1984) for a detailed review of how genetic variants differ.

Table 1.2 Properties of major proteins in bovine milk.

From Eigel *et al.*, 1984; Swaisgood, 1995.

Protein	Genetic variant	Molecular weight (Da)	Amino acids	Content (g/100 ml milk)	Percentage of total protein
Casein				2.95	81.9
α_{s1}-casein	A,B,C,D,E	23300	199	1.19	33.1
α_{s2}-casein	A,B,C,D,E	25230	207	0.31	8.61
β-casein	A1,A2,A3,B,C,D,E	24000	209	0.98	27.2
κ-casein	A,B	19000	169	0.35	9.72
Whey protein				0.63	17.5
β-lactoglobulin	A,B,C,D,E,F,G,(Dr)	18300	162	0.12	3.33
α-lactalbumin	A,B,C	14200	123	0.32	8.89
serum albumin	A	66300	582	0.04	1.11
Immunoglobulins				0.08	2.22
IgG		146000-163000			
IgA		385000-417000			
IgM		1000000			

1.1.1. α_s-casein (α_s-CN)

α-casein proteins were originally resolved from β- and γ-casein by moving-boundary electrophoresis, but were then shown to be made up of proteins that either precipitate when sufficient calcium is bound (sensitive), α_s-casein, or those which were insensitive to calcium, κ-casein (Jenness, 1971). α_{s1}-casein, which predominates in bovine milk (Table 1.2), is highly charged and has the highest phosphate content of the caseins

(Walstra *et al.*, 1999). Five genetic variants have been described for α_{s1}-CN each differing by amino acid substitution or deletion. The major genetic variant in *Bos taurus* is α_{s1}-CN^{B}, which has 199 amino acid residues (Eigel *et al.*, 1984) and does not contain cysteine (Table 1.3).

α_{s2}-casein contains 207 amino acid residues (Eigel *et al.*, 1984) including two cysteine residues (Table 1.3) that can form intermolecular disulphide bridges creating dimers of this protein (Eigel *et al.*, 1984). There are four genetic variants of α_{s2}-casein (Table 1.2) and variant α_{s2}-CN^{A} has been shown to have four degrees of phosphorylation (Eigel *et al.*, 1984).

1.1.2. β-casein (β-CN)

β-CN is calcium-sensitive, has 209 amino acid residues (Table 1.2) and does not contain cysteine (Table 1.3). Half the peptide chain is hydrophilic and half is hydrophobic (Walstra *et al.*, 1999). The enzyme plasmin, found in milk, is responsible for proteolysis of milk proteins and gives rise to γ-casein, which is derived from the hydrophobic C-terminus of β-casein, and proteose peptone, the complementary N-terminal fragment (Eigel *et al.*, 1984). Proteose peptone is also derived from fat globule membrane glycoprotein proteolysis (Swaisgood, 1995). Seven genetic variants of β-CN have been described, differing by amino acid substitution and phosphorylation. The major species is β-CN^{A} (Eigel *et al.*, 1984).

1.1.3. κ-casein (κ-CN)

There are two genetic variants of the major component of κ-CN that contain 169 amino acids, differ by amino acid substitution, and are free of carbohydrate; the A variant predominates (Eigel *et al.*, 1984). There are about six minor components thought to be glycosylated forms of the major components (Eigel *et al.*, 1984; Walstra *et al.*, 1999). The presence of cysteine residues in κ-casein (Table 1.3) allows the formation of oligomers of this protein in milk (Eigel *et al.*, 1984).

Table 1.3. Bovine milk protein amino acid composition.

Amino acid composition (residues/protein) of the six major milk proteins in bovine milk determined from primary structures (Swaisgood, 1995).

	Casein				Whey protein	
	α_{s1}	α_{s2}	β	κ	β-lactoglobulin	α-lactalbumin
Essential amino acids						
Histidine	5	3	5	3	2	3
Lysine	14	24	11	9	15	12
Methionine	5	4	6	2	4	1
Phenylalanine	8	6	9	4	4	4
Threonine	5	15	9	14	8	7
Tryptophan	2	2	1	1	2	4
Isoleucine	11	11	10	13	10	8
Leucine	17	13	22	8	22	13
Valine	11	14	19	11	10	6
Conditionally essential amino acids						
Tyrosine	10	12	4	9	4	4
Cysteine	0	2	0	2	5	8
Nonessential amino acids						
Alanine	9	8	5	15	14	3
Arginine	6	6	4	5	3	1
Aspartate	7	4	4	3	11	9
Asparagine	8	14	5	8	5	12
Glutamate	25	24	19	12	16	8
Glutamine	14	16	20	14	9	5
Glycine	9	2	5	2	3	6
Proline	17	10	35	20	8	2
Pyroglutamate	0	0	0	1	0	0
Serine	8	6	11	12	7	7
Serine phosphate	8	11	5	1	0	0
Total	199	207	209	169	162	123

1.1.4. Micelles

Caseins have no tertiary structure (Euston *et al.*, 1997), but interact with each other and with salts (mainly calcium and magnesium) and phosphate to form spherical micelles or colloidal aggregates that make up approximately 80% of bovine milk protein (Table 1.2) (Jensen, 1995; Walstra *et al.*, 1999). Micelles keep the viscosity of milk low despite high protein concentration, and determine many physical characteristics of milk that are important for storage stability and processing properties. Models of micelle structure suggest the calcium sensitive caseins (α_{s1}-, α_{s2}-, and β-casein) are in the interior while κ-casein coats the micelle. Calcium interacts with the highly phosphorylated groups of calcium-sensitive proteins allowing poorly soluble calcium to be carried in milk (Kumar *et al.*, 1994). Individual micelles comprise 20-30 β-casein molecules, and over 30

molecules of κ-casein. The carbohydrate groups of the latter protrude into the surrounding milk serum (Walstra *et al.*, 1999). Hydration of κ-casein stabilises the micelles and prevents aggregation (Dalgleish, 1992).

1.1.5. Whey proteins

Milk with the fat globules separated off is sometimes called milk plasma and plasma without micelles is milk serum (Walstra *et al.*, 1999). Serum proteins are whey proteins excluding peptides derived from caseins. Milk serum proteins include components of blood as well as products of the mammary gland (Jenness, 1971), and can be separated into albumin and globulin fractions. α-lactalbumin and β-lactoglobulin are part of the albumin fraction that can be separated from the globulin fraction by acidification. The globulin fraction of whey, like that of blood, functions in the conferring of immunity (McMeekin, 1971). Whey proteins prepared commercially at acidity lower than pH 4.6 contain some caseins (Eigel *et al.*, 1984). The serum albumin and the immunoglobulins of milk are identical to the corresponding blood plasma proteins.

1.1.5.1. β-lactoglobulin (β-Lg)

The major whey protein in bovine milk is β-Lg (Table 1.2). β-Lg has seven genetic variants each with 162 amino acids including five cysteine residues (Eigel *et al.*, 1984) (Table 1.3) forming two disulphide bridges and leaving one free sulphydryl group reactive allowing formation of dimers in milk (Walstra *et al.*, 1999). Genetic polymorphism has been shown to be linked to differences in milk production (Winkelman, 1997).

1.1.5.2. α-lactalbumin (α-La)

Lactose synthase, the enzyme responsible for lactose production in milk, consists of a catalytic subunit (galactosyl transferase), and a modifier subunit (α-La) (Stryer, 1988). Galactosyl transferase alone catalyses a different reaction, while α-La is structurally similar to lysozyme (Walstra *et al.*, 1999) but does not have enzymatic activity. There are three genetic variants of α-La but only the B variant has been observed in Western cattle. α-La is calcium binding and contains 123 amino acids. It is rich in cysteine residues that form intramolecular disulphide bonds resulting in a compact globular structure (Walstra *et al.*, 1999).

1.1.6. Use of milk proteins in human nutrition

While cows' milk is not suitable as a complete food for human infants, milk is a highly nutritious food. The proteins in milk provide essential amino acids, calcium and inorganic phosphate, and the digestibility of milk proteins is superior to proteins from plant sources, as all amino acids supplied are available for absorption. With the correct intake, milk protein supplies sufficient amino acids (except histidine) for adult human requirements (Dibley, 1997).

Milk proteins confer on products their excellent nutritional quality and also provide desirable emulsification, solubility, heat stability, gelation, foaming and textural characteristics; are free from anti-nutritional factors and do not provide unpleasant flavours. The use of milk protein also allows a product to contain natural, rather than synthetic ingredients (Dibley, 1997; Valeur, 1997).

Food uses for milk protein are as diverse as sausages and drinks. Milk protein can replace soy and egg proteins, and hydrolysed whey can partly replace egg protein and sugar in baking (Tow, 1984). Milk proteins are becoming increasingly important in the production of modified foods for athletic performance, and in meal replacements for modern consumers, who have less time to prepare meals but have increased health awareness. Milk proteins are important ingredients in infant formulas, and may have health benefits for the aging population who lack the energy or motivation to prepare balanced meals (Tow, 1984; Valeur, 1997).

Whey proteins in particular appear to be beneficial in immuno-modulation and in animal studies have increased resistance to cancer and shown inhibition of HIV activity (Rattray and Jelen, 1996). However, human milk does not contain β-lactoglobulin (Great Britain working party on the composition of foods for infants and young children, 1980) and the presence of this protein in cows' milk can cause an allergic reaction in some individuals (Rattray and Jelen, 1996).

Peptides derived from milk proteins by protease action have been shown to have health benefits. These bioactive peptides include calcium phosphopeptides, which are derived from α_s-caseins and are claimed to enhance calcium and iron absorption from the small

intestine, and are used in soft drinks, confectionery and dietary supplement tablets. Small peptides with opioid (morphine-like) properties called casomorphins, bind receptors of the central and autonomous nervous systems, and endocrine and immune systems, and elicit numerous responses such as, anti-diarrhoeal action, nutrient absorption regulation, hormonal regulation, antithrombotic action, and reduction of blood pressure (Dibley, 1997). They may also have sedative properties.

Nutraceuticals are products purified from foods that have been shown to have physiological benefits and are sold in medicinal forms (Keeley, 2001). Nutraceuticals derived from milk could be used to enhance dairy products or be used to boost nutritional and health promoting profiles of other products (Dibley, 1997).

1.2. The effect of milk proteins on milk product manufacture

Compositional changes in milk are relatively unimportant for bottled milk, but have a major effect on the manufacture of milk products. The amount of cheese that can be produced from milk is dependent on the proportion of fat to casein, and the proportion of individual caseins affects cheese quality. Cheese milk is standardised by the addition of cream or skim milk to obtain the required protein to fat ratio and ensure optimal cheese quality throughout the season, but it is not economically sound to use cream or skim milk in this way (Banks and Muir, 1984). Differences in the physicochemical properties of milk protein variants can affect some processing properties making it desirable to manipulate their presence in milk for manufacture of particular products (Rattray and Jelen, 1996).

κ-casein, the micelle stabilising component, is cleaved by chymosin, the active component of rennet, to give para-κ-casein and glycomacropeptide. Para-κ-casein remains with the casein micelle but loss of glycomacropeptide causes micelles to aggregate and form a curd. Increased κ-casein content of milk results in the formation of a firmer curd (Dalgleish, 1992). The κ-casein phenotype BB has been associated with improved coagulation in less time resulting in increased cheese making efficiency, increased curd firmness, and increased yield (Horne *et al.*, 1997; Mayer *et al.*, 1997; Ostersen *et al.*, 1997). This is thought to occur through higher efficiency of fat retention by curds (Horne *et al.*, 1997). κ-casein BB milks have more κ-casein and therefore

smaller micelles (Horne *et al.*, 1997) and contain more calcium ions (Dalgleish, 1992). Increased calcium has been shown to increase the rate of coagulation of renneted casein micelles, which could be the reason why the B variant of κ-casein is superior for cheese manufacture (Dalgleish, 1992). κ-casein is one protein targeted for manipulation by the dairy industry because its gelling characteristics are desirable for cheese manufacture (Rattray and Jelen, 1996).

At high temperatures, β-Lg denatures and binds κ-casein micelles causing gel formation. This improves yoghurt, but is undesirable for sterilised (120°C, 20 min) and UHT (135°C, several seconds) milks (Dalgleish, 1992). Reduction of κ-casein from milk would allow increased heat stability for the production of heat-treated milks (Rattray and Jelen, 1996). β-Lg that had been modified with an extra cysteine residue increased the number of disulphide bridges in the protein to three and created a more heat-stable product (Dalgleish, 1992). Milk from cows of phenotype BB of β-Lg in combination with κ-casein BB has been described as having low heat stability (Hill *et al.*, 1997), and β-Lg and κ-casein have been shown to react more rapidly upon heating when the β-Lg variant is B (Andrén *et al.*, 1997).

κ-casein and β-Lg contain cysteine (Table 1.3) (McKenzie, 1971), an amino acid considered to be limiting for milk protein synthesis (Clark *et al.*, 1978). An increase in cysteine supply to the mammary gland may increase the κ-casein and β-Lg content of milk and enhance the manufacture of products that require gel formation such as cheese and yoghurt. The ability to control cysteine uptake by the gland may also lead to increased κ-casein and β-Lg and in addition may allow down-regulation of these proteins for manufacture of heat-stable products.

Several factors affect milk protein composition and thus milk product manufacture. These include season and stage of lactation, dairy herd management, cow age and parity, health, nutrition, breed, and genetics. The Dairy Industry would benefit from the ability to manipulate synthesis of individual milk proteins for the manufacture of specific dairy products.

1.3. Protein synthesis and secretion by the mammary gland

The synthesis of proteins in the mammary gland occurs by the same biochemical processes that occur in other eukaryotic cells (see Stryer, (1988) and refer to Mepham *et al.* (1992) for more detail). Secretory proteins are accumulated in the Golgi apparatus, transported in Golgi/secretory vesicles to the apical membrane of the cell and secreted by exocytosis (Figure 1.2). α-lactalbumin is part of the enzyme lactose synthase, which synthesises lactose in the Golgi apparatus and vesicles and is secreted from the cell with milk proteins. Lactose and milk ions cause water to enter Golgi vesicles by osmosis (Thomas, 1983).

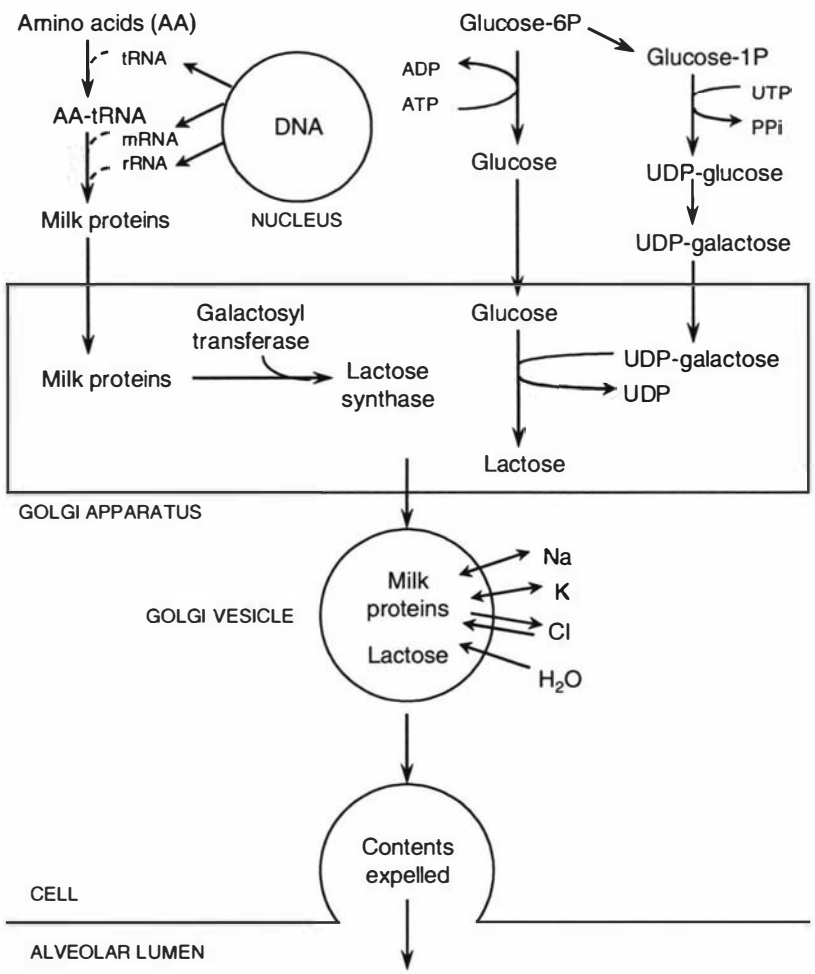


Figure 1.2 Schematic outline of milk protein and lactose synthesis and secretion by mammary secretory cells.

From Thomas (1983).

Protein synthesis requires precursor amino acids and the process is energy driven. The gland derives most of its ATP from oxidation of acetate, certain amino acids and

glucose, but blood fatty acids are major oxidative substrates during mid and late lactation. These play a crucial role in maintaining the secretory activity of the mammary gland (Thomas, 1984).

The amount of milk produced by an animal is determined by factors such as weight of tissue in the mammary glands, number of secretory cells per weight of mammary tissue, and synthetic activity of secretory cells. Maximum rates of reactions inside secretory cells are determined by amounts and activities of rate limiting enzymes, and concentration of precursor substrates, reaction products and coenzymes. Milk protein synthesis is sensitive to changes in the endocrine status of an animal, which changes with composition and amount of diet, the animal's physiological state, environment, and stage of lactation (Thomas, 1984).

1.4. Factors influencing milk protein production

1.4.1. The effect of dairy herd management on milk protein production

Milk protein composition may be controlled to some extent by effective management of dairy herds. The term management in this thesis is used to encompass variables that may be controlled by the farmer, such as milking schedules, calving dates, culling schemes and udder health.

Increased time between milkings reduces casein secretion, and incomplete milking rapidly reduces milk yield; the amount secreted adjusts to the amount of milk removed (Dodd, 1984) Increasing milking frequency increases milk yield but does not affect composition (Dodd, 1984). Increased body condition has been shown to positively affect renneting time and curd firmness (Ostersen *et al.*, 1997) so animals should be well fed. Non-fat milk solid content drops 4 g/kg milk with each lactation until the fifth lactation, and then drops 0.7 g/kg milk every lactation (Crabtree, 1984). In addition, the sum of α-, β-, and κ-casein concentrations in milk decreases with age/parity (Ng-Kwai-Hang *et al.*, 1987). Therefore, culling and replacement are necessary to maintain optimal milk production.

Total protein concentration of milk has been shown to drop during the second and third months of lactation, then increase to early lactation concentrations towards the end of

lactation (Ng-Kwai-Hang *et al.*, 1987). Milk from early and late lactation is superior for cheesemaking as it has a shorter renneting time and firmer curd, which is consistent with changes in protein concentration that occur with stage of lactation (Ostersen *et al.*, 1997).

Because the New Zealand dairy system uses fresh forage, the effects of season on pasture quality must be taken into account as well as the direct seasonal influence on the cow. An experiment with cows calved at different times of the year showed that cows in early lactation in spring produced more total protein and casein than cows at the same stage of lactation in winter. However, season did not affect total protein or casein from cows in mid or late lactation indicating that season and stage of lactation interact (Auldist *et al.*, 1997). Feed availability has been considered as the factor controlling changes in seasonal milk composition (Walstra *et al.*, 1999), but Auldist *et al.* (1997) did not find any association between dry matter, energy or protein intake, and milk composition.

Calving of animals in different seasons would smooth out differences in milk reaching the manufacturing plant (Walstra *et al.*, 1999), but this alone probably would not alter casein to whey ratios or manufacturing properties of milk and milk for cheese manufacture would still have to be standardised to obtain the required casein to fat ratio (Banks and Muir, 1984).

Infection of the udder (mastitis) decreases milk yield, alters composition, increases somatic cell count in milk (e.g. polymorphonuclear leukocytes), changes vitamin and mineral content, and changes enzyme activity in milk (Dodd, 1984; Walstra *et al.*, 1999). Increased tendency for lipolysis makes mastitic milk less suitable for some forms of processing (Dodd, 1984). Infection increases involution, affecting production from the entire quarter and causing milk to resemble blood serum (Walstra *et al.*, 1999). High somatic cell count in milk has been associated with decreased β-casein concentration and increased κ-casein (Ng-Kwai-Hang *et al.*, 1987). Treatment with antibiotics enables recovery of milk quality but yield will not recover until the next lactation and in severe cases the gland never recovers (Dodd, 1984).

It is in the best interests of the farmer and the manufacturer that efficient management practices are maintained so that differences in yield and composition are minimised. Even with excellent herd management, however, the farmer cannot increase the protein content or composition of milk beyond the cow's genetic capabilities.

1.4.2. The effect of dairy cow nutrition and amino acid supply on milk protein production

In the cow, proteins presented to the small intestine of the gut are proteins from the diet that have escaped digestion by rumen microbes, microbial proteins synthesised during rumen fermentation, and secreted endogenous proteins (Schingoethe, 1996). The supply of crude protein to dairy cows at levels above current recommendations has resulted in small increases in milk yield and milk protein yield but has usually not changed protein content. Restricted feed protein supply reduces milk yield and protein yield, but protein content does not decrease unless feed protein is severely restricted. Some studies have used proteins protected against rumen fermentation made from natural feeds treated with heat and/or formaldehyde, but increases in milk protein yield and/or content have not always resulted. Increased rumen-protected protein in the diet has not been effective in changing casein production (Backwell *et al.*, 1996).

Experiments carried out with cows on basal diets sufficient to maintain energy requirements, with additional supply of amino acids by intra-abomasal infusion of casein or supplementation of the diet with ground nut or fish meal, have increased milk protein yield (Thomas, 1984b; Metcalf *et al.*, 1994). A mixture of 10 amino acids mimicked the effect of casein infusion but single amino acids or combinations of fewer than 10 have not been as successful (Schingoethe, 1996).

Small increases in milk protein yield have been shown to be related to increases in mammary EAA uptake. Supply of histidine, phenylalanine, tyrosine, lysine, and branched chain amino acids (BCAA), isoleucine, leucine and valine, can be increased with rumen protected protein in the diet, but increased supply has only increased the uptake of some of these amino acids (phenylalanine, lysine, and isoleucine) (Metcalf *et al.*, 1996). That amino acid uptake is not reflective of increased supply indicates that

substrate supply is not limiting for milk protein synthesis and that there are regulatory factors inside the gland controlling uptake (Metcalf *et al.*, 1994).

Amino acids have been organised into groups on the basis of the ratios of uptake from blood to milk output (Mepham, 1982). Group one amino acids (methionine, phenylalanine, tyrosine, tryptophan and histidine) are taken up from blood in amounts that are only just sufficient for output in milk protein and are therefore considered potentially limiting for protein synthesis. Group two amino acids, which are taken up by the gland in excess of requirements for milk protein secretion, include the branched chain amino acids (BCAA) isoleucine, leucine and valine, together with lysine, threonine and arginine. The third group are nonessential amino acids (NEAA) that, while not taken up by the gland in sufficient amounts to meet output can be synthesized from EAA and other metabolites by the mammary gland.

Cysteine concentrations are difficult to determine and are often not included in studies targeting amino acids limiting milk protein synthesis. However, cysteine has been shown to be limiting for the synthesis of glutathione (GSH), a tripeptide with a number of physiological functions (see section 1.7.1), and there is also evidence to suggest that cysteine is limiting for milk protein synthesis. Cysteine has been shown to be limiting for wool protein production in sheep (Lee *et al.*, 1993), and cysteine concentration has been shown to correlate with coat colour in mice (Granholm *et al.*, 1996). Rats fed a sulphur amino acid deficient diet supplemented with the cysteine analogue, oxothiazolidine carboxylic acid, had superior coat condition to control animals (Jain *et al.*, 1995). Studies of isolated bovine mammary cells have shown that Eagle's minimal essential medium is limiting in the disulphide form of cysteine (cystine) for milk protein synthesis and that β-casein synthesis can be enhanced by supplementing the medium with cystine (Clark *et al.*, 1977). In the lactating goat, the cysteine concentration of plasma was not different from whole blood, and was lower than mammary tissue concentration indicative of active transport into the gland (Knutson *et al.*, 1994). However, the uptake of cysteine from blood in lactating goats (Lee *et al.*, 1999) and from plasma in lactating cows (Clark *et al.*, 1978) has been shown to be insufficient to account for output in milk protein. Further research is required to define the importance of cysteine supply in the control of milk protein production in dairy animals.

Uptake of EAA is not enough to account for output and conversion to NEAA, so amino acid N must be supplied from other sources such as peptides, small proteins and other intermediary metabolites (Metcalf *et al.*, 1996). The mammary gland has been shown to utilise peptides, and 10-30% of plasma amino acid was found to be present as peptides. Histidine, phenylalanine, proline, alanine and leucine have been found in plasma peptides with molecular weights less than 1500 Da (Backwell *et al.*, 1996).

An infusion of total amino acids or EAA both increased milk protein content and yield indicating NEAA are not limiting for milk protein synthesis. An infusion of a mixture of methionine, phenylalanine, tyrosine and histidine, however, did not increase milk protein yield to the same extent as the full complement of EAA, indicating that these amino acids alone cannot satisfy limits of supply (Beever, 1996) or that there is some other controlling factor. It appears that the mammary gland regulates uptake of amino acids to overcome limitation (Bequette *et al.*, 2000). The factors controlling amino acid uptake need to be understood if we are to manipulate milk protein synthesis in a predictable fashion either through dietary or genetic engineering approaches.

Milk from cows with β-Lg phenotype AA contains more cysteine, leucine, tryptophan, and lysine but less tyrosine, histidine and phenylalanine than milk from cows of the BB phenotype (Mackenzie, 1997). The ability to manipulate amino acid uptake by the mammary gland to promote synthesis of some protein variants may control their composition in milk and allow manipulation of milk composition for the manufacture of specific milk products.

Fat supplementation is an effective method of increasing energy and production efficiency but it reduces milk protein content by increasing total milk yield. Responses in milk protein production vary with amount and type of fat used as well as the type of basal diet being supplemented. A reduction in mammary blood flow has been observed with diets high in fat, which would reduce the uptake of amino acids (Schingoethe, 1996).

Injection of bovine somatotropin (bST) increases milk protein synthesis by 15-25% in association with an increase in substrate supply to the mammary gland via an increase in mammary blood flow. While the mechanism of action of bST is unknown, the rapidity

of its effects suggests that it stimulates productivity of the mammary secretory epithelium. The use of bST is controversial due to higher rates of mastitis occurring in treated animals (Schingoethe, 1996) but it does illustrate the potential to increase milk protein output.

Possibilities for increasing milk protein yield through the manipulation of the diet include, finding an efficient mechanism of supplying sufficient protein to the gland, by ruminally protected or unprotected protein and a method for increasing milk protein yield when diets are supplemented with fat must be obtained. While a cow's optimum milk production may be reached through excellent nutrition and herd management, these factors will not, in themselves, overcome a cow's genetic capabilities. In order to maximise milk protein production further, the breed of cow and genetic variation must be considered.

1.4.3. The effect of breed and genetic variation on milk protein production

The dominant breeds of dairy cow in New Zealand are the Friesian (58%), and the Jersey (16%) (www.nzholstein.org.nz, consulted September 2001). Composition of milk from these breeds is fairly constant (Table 1.4), relative to other animals (Table 1.1). However, the Friesian has often been selected because it is a larger cow with greater milk yield (Wilson and Lawrence, 1984), while Jersey milk has higher a fat content (Table 1.4). For any economic trait, a herd will have a few extremely poor animals and a few extremely good ones, with the majority being normally distributed around the mean (Mulholland, 1984). Selection of cows that have desirable milk characteristics should allow herd improvement over time.

Table 1.4 Milk composition of New Zealand cows.

Approximate average composition (% w/w) of milk from New Zealand's main breeds of cow (Walstra *et al.*, 1999).

Breed	Dry matter	Fat	Crude protein	Carbohydrate
Friesian[a]	13.2	4.4	3.4	4.6
Jersey	15.1	5.3	4.0	4.9

[a] derived from Dutch Friesians.

The ability to manipulate the concentration of individual proteins in milk has practical applications in the dairy industry. Genetic variants of α_{s1}-, β-, and κ-casein, and β-lactoglobulin have been associated with changes in milk yield and composition. The ability to use milk protein alleles as markers for the selection of cattle for increased milk yield and desirable milk composition will allow improvement of the dairy herd. Reports are conflicting and often contradictory, however, because of differences in breeds, season, stage of lactation, health status, age, calving month and year, population size, frequency distribution of genetic variants considered, measurement methods, and statistical model (Ng-Kwai-Hang, 1997). Conflicting results may also arise from the effects of linked genes rather than individual milk protein loci themselves (Bovenhuis *et al.*, 1992).

Ng-Kwai-Hang (1997) reviewed reports of the relationship between milk protein polymorphisms and composition and found that milk with α_{s1}-casein BC phenotype had a higher percentage of casein than BB due to increased concentration of α_{s1}-, β-, and κ-caseins, but had a lower percentage of whey proteins and was associated with lower milk yield than the BB phenotype. The A^2 variant of β-casein was associated with higher milk protein yield than A^1 or B. The κ-casein phenotype BB is associated with increased α_s- and κ-casein, serum albumin, and immunoglobulins but lower β-casein, β-Lg and α-La. Variant A of β-Lg is associated with increased protein content through increased β-Lg but lower concentrations of the other major milk proteins, while variant B confers lower protein content but higher concentration of caseins and α-La (Ng-Kwai-Hang, 1997).

The relationships of some milk proteins to milk yield and composition are still debated, but it is largely accepted that the B variant of κ-casein and the B variant of β-Lg are associated with increased casein content in milk (Ng-Kwai-Hang, 1997).

It is possible to breed dairy cattle for specific milk protein variants because these variants are inherited according to the Mendelian mode of inheritance (Ng-Kwai-Hang, 1997). There is greater chance of getting superior offspring from superior parents, but it is likely the same parents may produce suboptimal offspring and also that suboptimal

parents produce excellent offspring. It is not yet possible to estimate the breeding value of a bull for milk yield. This must be done by comparing milk yields of the bull's first daughters, and it would take nine years to breed a bull and test its progeny to determine whether it can be used to increase the appearance of milk protein genetic variants in dairy herds (Rendel and Harris, 1997). Selection and breeding for κ-casein B is being pursued in Europe (Ng-Kwai-Hang, 1997) and the economics of including β-Lg genotypes in New Zealand breeding programmes have been studied (Harris, 1997; Rendel and Harris, 1997).

There appear to be no unfavourable associations between β-Lg variant B and the other milk proteins supporting selection for this trait in breeding programmes (Lundén *et al.*, 1997). However, both κ-casein B and β-Lg B alleles are associated with decreased milk yield (Bovenhuis *et al.*, 1992). Selection of cattle based on β-Lg genotypes alone discards animals of high quantitative genetic merit (Harris, 1997), and valuable characteristics such as milk yield, fat or protein yield, or liveweight and survival may be compromised. These traits may not be able to be regained if the market changes its focus from β-Lg towards a different protein or milk characteristic. Genetic engineering provides a more specific method of altering β-Lg or κ-casein genotype and would prevent loss of valuable genes.

Breeding of cattle for improved milk protein production involves selection of desirable phenotypes over a long time scale, and although genetic modification may speed this up in the future it is currently unpopular. A greater understanding of the mechanisms restricting protein synthesis may allow milk protein production to be optimised to meet an animal's full output potential using chemical treatments and/or nutritional regimes. In the future, when deemed acceptable by the general population, genetic modification of these mechanisms may allow an animal's potential to be increased.

1.4.4. Summary of factors influencing milk protein production

Results from experiments involving manipulation of energy, protein and fat in the diet have been inconsistent. Desirable increases in milk protein content have been achieved in some experiments but this has been shown to be dependent on other factors such as stage of lactation, and the basal diet being supplemented which is most likely to be a

function of the country where the research is located. Use of fresh forage by the New Zealand farmer and lack of concentrate supplementation makes it difficult to extrapolate from international studies.

While increased milk protein content is achievable with the management of diet, stage of lactation, control of environment, and maintenance of optimum health in animals, we are faced with the requirement for a global increase in total protein content and have no control over the production of individual proteins as required. The ability to select for genetically superior animals for greater milk yield and milk protein content of desirable proteins is realistic but slow. The problem also arises that a herd of animals, once selected, is fixed for several generations. Greater control over the production of individual proteins, in the lifetime of the cow, is desirable.

The ability to manipulate milk protein production as the requirement arises without affecting milk yield or animal health would allow protein profiles to follow market trends for particular dairy products, year by year. Improved herd management and nutrition will not increase the protein content of milk beyond the genetic capabilities of the cow, and breeding is slow. We need to understand and be able to manipulate the factors controlling milk protein synthesis to improve a cow's maximum production. One of these factors is the supply of amino acids to the mammary gland, so we must understand the mechanisms of amino acid uptake by the gland.

1.5. Regulation of milk protein synthesis

Milk protein synthesis is a tightly regulated process responsive to nutritional status, milking frequency, and hormonal treatments such as bovine somatotropin. Milk protein synthesis is largely initiated at lactogenesis following stimulation of mammary epithelium by prolactin (Forsyth, 1984). In the lactating gland, there are several potential levels of regulation of milk protein synthesis and secretion. These include: amino acid supply; amino acid transport/intracellular amino acid supply; transcription; peptide chain initiation and mRNA translation; and protein degradation.

Amino acid supply in relation to milk protein synthesis has been widely discussed by others (section 1.4.2). While there are several key regulating points at the level of

mRNA translation, the rate of amino acid transport into the secretory cell has been proposed as a potential point of regulation. Several classical amino acid transport systems have been identified in mammary tissue (see section 1.6), which could be rate limiting for protein synthesis. In addition, it has been suggested that several peptides may contribute amino acids to protein synthesis.

Of great significance was the suggestion by (Viña *et al.*, 1989) that amino acid transport into mammary cells may be a function of the enzyme γ-glutamyl transpeptidase (γ-GT). This enzyme is active in tissues where amino acid transport rates are high, for example in the kidney and intestinal epithelium. Further, this enzyme increases in activity in mammary tissue at the time of lactogenesis, is controlled by prolactin and declines at involution (see section 1.8). Inhibitors of γ-GT provoke severe decreases in mammary uptake of amino acids at peak lactation.

It has been suggested that the γ-glutamyl cycle should not be considered a mechanism for amino acid transport but rather a generator of extracellular signals, γ-glutamyl amino acids that are converted intracellularly to 5-oxoproline, which activates uptake and/or metabolism of amino acids (Viña *et al.*, 1989). The significance of these observations has been controversial and the role of γ-GT in mammary amino acid transport requires clarification, including the potential role of the cycle in the supply of intracellular cysteine.

1.6. Amino acid transport in the mammary gland

Several systems have been described for the transport of amino acids into mammalian cells (Table 1.5) and for a detailed review refer to McGivan and Pastor-Anglada, (1994). Transporters are specific for L-isomers over D-isomers, have low specificity allowing a number of different amino acids to bind, and can be divided into two types: those that require Na^+ synport and use the electrochemical potential gradient for amino acid transport and those that act as amino acid uniport (McGivan and Pastor-Anglada, 1994).

Membranes of different cell types contain different complements of transporters that provide amino acids for specific tissue functions. The A, ASC and L systems have been shown to be expressed by most cells including mammary epithelial cells. The A and

ASC systems transport short straight chain (aliphatic) neutral amino acids, but the affinity of the A system for methylated amino-isobutyric acid (MeAIB) distinguishes the two. MeAIB is non-metabolisable and is often used in uptake studies as a competitive inhibitor. System A activity can be induced by amino acid starvation, hormones, growth factors, and hyperosmotic stress. Alanine, serine and glutamine are particularly good substrates of the A system, while alanine, serine and cysteine are preferred by the ASC system, which is not inducible. The L system has a broad specificity but aliphatic amino acids are poor substrates (McGivan and Pastor-Anglada, 1994).

Table 1.5 Mammalian amino acid transport systems.

The major amino acid transport systems of mammalian cells, their presence in mammary tissue and responsiveness to the major lactogenic hormone, prolactin (Baumrucker, 1985; McGivan and Pastor-Anglada, 1994; Shennan *et al.*, 1997).

Name	Specificity	Presence in mammary gland	Prolactin responsive
Na^+-dependent			
A	small aliphatic amino acids; methyl-AIB	mouse, rat, cow mammary tissue	yes
ASC	small aliphatic amino acids; not methyl-AIB	mammary tissue of cow, guinea pig but absent from rat, mouse	
N	glutamine, histidine, asparagines	thought to be present in cow mammary tissue	
Gly	glycine, sacrosine		
B^0	broad specificity; most neutral amino acids		
B	broad specificity; most neutral amino acids		
$B^{0,+}$	broad specificity; most neutral and basic amino acids		
X_{AG}^-	glutamate, aspartate	rat mammary tissue explants, perfused rat mammary gland	no
β	β-alanine, taurine, hypotaurine	rat mammary tissue explants	
Na^+-independent			
L	mainly branched-chain and aromatic amino acids	rat, mouse, guinea pig, cow mammary tissue	yes
y^+	lysine, histidine, arginine	related transporter found in rat and cow mammary tissue explants	yes
$b^{0,+}$	neutral and basic amino acids	}	
x_c^-	glutamate, cystine		

Other transport systems identified in mammary tissue are the anionic X_{AG}^-, and the β-system. A system for transporting lysine and arginine has been reported in mammary tissue and was thought to be the y^+ transporter system (Baumrucker, 1985) but has also been shown to interact with neutral amino acids (Shennan *et al.*, 1997). This indicates an amino acid transport system is present in mammary tissue that resembles both y^+ and $b^{0,+}$ (Shennan *et al.*, 1997). In addition to these transport systems, a mechanism for histidine, glutamine, and asparagine transport is thought to be present in the mammary gland, which may be the N system, although this has not been supported by experimental evidence (Baumrucker, 1985).

Cysteine supply may be limiting for milk protein synthesis (Clark *et al.*, 1978; Lee *et al.*, 1999). Transport of cysteine and cystine has been studied in rat lung alveolar epithelial type II cells and these amino acids have been shown to be transported by systems ASC, L and x_c^-. In addition to these, cysteine and cystine were also shown to be transported into rat alveolar epithelial type II cells by the X_{AG}^- system which was previously thought to be specific for glutamate and aspartate transport (Knickelbein *et al.*, 1997). These systems may also be responsible for the transport of cyst(e)ine in the mammary gland.

Glutamate is the most abundant amino acid in milk protein (Table 1.3). This amino acid may be partially supplied by glutathione (GSH) a tripeptide that also contains cysteine (Meister and Anderson, 1983). The membrane bound enzyme γ-glutamyl transpeptidase (γ-GT), an enzyme of the γ-glutamyl cycle, catalyses GSH breakdown and uses free amino acids to form γ-glutamyl amino acids that are taken up by the cell (Griffith *et al.*, 1979a). γ-GT has been shown to be present in mammary tissue and along with other components of the γ-glutamyl cycle, is thought to be involved in amino acid transport. The role of the γ-glutamyl cycle in supply of amino acids for milk protein synthesis is reviewed in the following sections.

1.7. The γ-glutamyl cycle: a novel amino acid transport system?

The γ-glutamyl cycle (Figure 1.3) was first proposed in 1970 by Orlowski and Meister (1970) through linkage of amino acid transport with degradation of glutathione (GSH) observed in kidney preparations (Schroeder and Woodward, 1937). Lytic action of an enzyme in this preparation produced cysteinyl-glycine (Binkley and Nakamura, 1948), and new peptides through interaction of free amino acids with GSH, and the reaction was termed transpeptidation (Hanes *et al.*, 1950). The newly formed peptides contained a γ-glutamyl moiety showing they were formed from transfer of the γ-glutamyl group from GSH or other γ-glutamyl donor, to an acceptor amino acid. Therefore, the enzyme was termed γ-glutamyl transpeptidase (γ-GT) (Hanes *et al.*, 1952).

Another enzyme, γ-glutamyl cyclotransferase from kidney preparations was shown to remove the γ-glutamyl moiety and restore the free amino acids (Connell and Hanes, 1956). γ-glutamyl cyclotransferase was purified from soluble fractions, while γ-GT was membrane bound, so the concept was proposed that γ-GT was transporting amino acids across the cell membrane (Orlowski and Meister, 1965).

The presence of these GSH degrading enzymes in kidney along with high glutathione concentrations led Orlowski and Meister to postulate that the machinery for glutathione synthesis must also be present. In 1970 Orlowski and Meister (Orlowski and Meister, 1970) found the enzymes γ-glutamylcysteine synthetase, and glutathione synthetase, which are required for glutathione formation, at high concentrations in rat kidney. Therefore, the catalytic events involving the synthesis and degradation of GSH were able to be linked, and the 'γ-glutamyl cycle,' was proposed for amino acid transport by γ-glutamyl linkage.

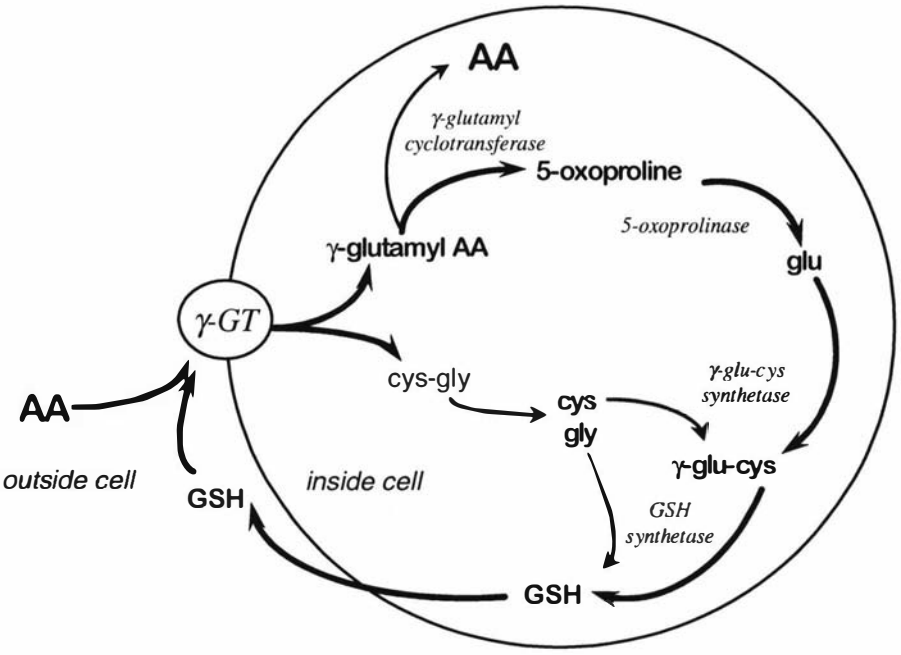


Figure 1.3 The γ-glutamyl cycle.

Glutathione is represented by GSH. Cysteinyl-glycine is degraded by dipeptidase to its amino acid components.

1.7.1. Glutathione (GSH)

The γ-glutamyl cycle consists of the enzymatic synthesis and degradation of glutathione (GSH). GSH is a tripeptide with the sequence γ-glutamyl-cysteinyl-glycine (Figure 1.4), and is the most abundant cellular peptide and major non-protein thiol in most cells (Kosower, 1976; McIntyre and Curthoys, 1980). Glutathione is widely distributed in most animals, plants, and microorganisms. It exists in a reduced form denoted GSH, and an oxidised form, GSSG, where the thiol groups of the cysteine residues of the two GSH molecules have formed a disulphide bond (Kosower, 1976).

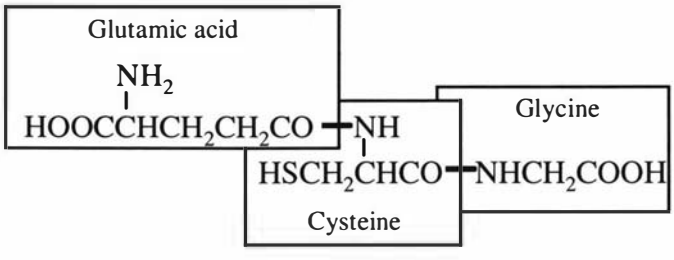


Figure 1.4 The structure of reduced glutathione.

The cysteine residue is bound to the side chain (γ) carboxyl group of glutamic acid (Beutler, 1989).

Synthesis of GSH involves γ-glutamylcysteine synthetase which catalyses formation of the peptide bond between glutamate and cysteine, and glutathione synthetase, which binds glycine to the cysteine residue of the dipeptide (Figure 1.3). The peptide bond between glutamate and cysteine uses the γ-carboxyl rather than the α-carboxyl group of glutamate (Figure 1.4) and confers peptidase resistance on the peptide (Kosower, 1976; Beutler, 1989). The only enzyme known to break this bond is γ-glutamyl transpeptidase (Beutler, 1989), a membrane bound enzyme that is part of the γ-glutamyl cycle, which will be discussed in detail in following sections.

Synthesis of GSH occurs in a variety of cells and largely depends on the presence of its constituent amino acids in the diet. It has been shown that a protein deficient diet causes a decrease in the concentration of GSH in various tissues (Tateishi and Sakamoto, 1983; Beutler, 1989). Cysteine and glutamate have been identified as limiting for GSH synthesis (Beutler, 1989; Meier and Issels, 1995). GSH deficiency can often be rectified by administration of cysteine, or methionine, which is converted to cysteine by transulphuration in animals (Tateishi and Sakamoto, 1983; Beutler, 1989). Low GSH concentrations in erythrocytes of a population of Finnish sheep is caused by a defect in cysteine uptake, and burn victims with demand for sulphur amino acids have decreased GSH concentrations. Increasing glutamate concentrations in erythrocytes of rabbits has increased GSH concentrations indicating the limiting role of glutamate in GSH synthesis (Beutler, 1989).

Intracellular GSH concentration ranges from 0.1 to 10 mM (Kosower, 1976). Many different types of cells release GSH into blood plasma. In ruminants' blood plasma GSH exists at micromolar concentrations (Pocius *et al.*, 1981, Knutson *et al.*, 1994). The kidney is the organ primarily responsible for the removal of GSH from plasma and its subsequent degradation, removing approximately 90% in a single pass. γ-GT is responsible for GSH extraction, which is four times faster than removal of GSH by filtration. γ-GT is found in its highest concentration in kidney, where it is concentrated in the brush border membrane of the proximal tubule (McIntyre and Curthoys, 1980).

Over 99% of intracellular glutathione is in the reduced form (GSH) but in plasma glutathione is mainly in its oxidised form (GSSG) (Meister and Anderson, 1983). Most

of the functions of GSH require the reduced form and this is oxidised to GSSG during physiological function. Glutathione reductase uses hydrogen from NADPH or NADH to reduce GSSG (Beutler, 1989).

GSH participates in maintenance of thiol groups of many proteins by preventing oxidation of -SH groups or reducing disulphides (-S-S-) once they have been formed. Through the action of glutathione peroxidase, GSH removes peroxides that can oxidise thiols. Thioltransferase catalyses exchange of disulphides from proteins to form GSSG. Glutaredoxin uses GSH as a hydrogen donor to reduce ribonucleotide reductase. In addition, GSH alone can reduce oxidised thiol groups (Beutler, 1989).

GSH is a cofactor for many enzymes involved in detoxification pathways. The glyoxylase reaction protects the cell against the mutagenic effect of 2-oxoaldehyde, which arises during intracellular oxidation, and uses GSH to conjugate the toxin methylglyoxal before its conversion to D, L-lactate. Methanol oxidation forms the toxin formaldehyde, which is removed in a GSH-dependent dehydrogenase reaction. Glutathione-S-transferases use GSH to conjugate toxins and produce mercapturic acids, which are considered less harmful (Beutler, 1989).

GSH has a role in the production of leukotrienes, which mediate inflammatory responses. Glutathione-S-transferase, γ-glutamyl transpeptidase, and a peptidase are involved in modifying leukotriene A_4 by addition and then cleavage of a GSH molecule to form leukotrienes C_4, D_4 and E_4 (Beutler, 1989).

GSH is released into blood plasma by many tissues such as the liver, and in addition to the kidney is taken up by many other tissues including the mammary gland. Therefore, GSH can be considered a form of transport for its constituent amino acids. Intracellular concentrations of cysteine are generally low (approximately 10^{-4} M) in comparison with glutamate and glycine (both approximately 10^{-3} M), and cysteine from GSH has been shown to be used in protein synthesis in the liver, implicating GSH as a storage form of cysteine (Beutler, 1989).

In addition to providing constituent amino acids, GSH is involved in the transport of other amino acids to cells through the γ-glutamyl cycle. This pathway, its use of GSH and its importance in providing amino acids for protein synthesis are the focus of the remainder of this review.

1.7.2. γ-Glutamyl transpeptidase (γ-GT)

γ-Glutamyl transpeptidase (γ-GT) catalyses three reactions: transpeptidation in which the γ-glutamyl moiety of the donor molecule, e.g. GSH, is transferred to an acceptor amino acid or dipeptide molecule; autopeptidation involving transfer of the γ-glutamyl moiety to GSH forming γ-glutamyl-GSH; and hydrolysis where water is the acceptor of the γ-glutamyl moiety. In the presence of high donor concentrations, donor molecules compete with acceptor molecules for the acceptor site of the enzyme, and autopeptidation becomes the major reaction (Allison, 1985). In addition to the transfer of γ-glutamyl moieties, γ-GT also plays a role in processing mercapturic acids, and in metabolic pathways involving prostaglandins, estrogens and leukotrienes (Meister and Anderson, 1983).

Products of transpeptidation, γ-glutamyl amino acids and cysteinyl-glycine, enter the cell (Figure 1.3). Cysteinyl-glycine is broken down to cysteine and glycine by a dipeptidase, and these then enter pathways for GSH synthesis or protein synthesis. γ-Glutamyl amino acids are converted to free amino acids plus 5-oxoproline by γ-glutamyl cyclotransferase. 5-Oxoproline is converted to glutamate and can be used for GSH synthesis, completing the cycle (Meister and Anderson, 1983).

GSH is the naturally occurring γ-glutamyl compound with greatest affinity for γ-GT (Table 1.6). γ-Glutamyl-*p*-nitroanilide is a good synthetic substrate that is used to determine enzyme activity spectrophotometrically (Thompson and Meister, 1977). L-isomers of amino acids, as well as many dipeptides, are good acceptors of the γ-glutamyl moiety (Meister and Anderson, 1983). Of the common amino acids L-cystine is the best acceptor with glutamine and methionine also showing high affinity for the enzyme (Table 1.7) (Thompson and Meister, 1977; Baumrucker and Pocius, 1978). Branched chain amino acids and those with aromatic side chains bind to the enzyme's acceptor

site with less affinity (Meister and Anderson, 1983). The enzyme functions by a ping-pong mechanism with the formation of a γ-glutamyl enzyme intermediate (Thompson and Meister, 1977).

Table 1.6. γ-GT donor molecules.

Kinetic constants for the hydrolysis of γ-glutamyl compounds. The acceptor substrate was glycyl-glycine. Kinetic experiments were carried out at pH 8.0 and 37°C (McIntyre and Curthoys, 1979).

Substrate	K_M (mM)	V_{max} (mmol $min^{-1}.mg^{-1}$)
Glutathione	5.7	9.1
S-substituted glutathiones		
R=(S)glutathione	8.1	5.4
R=-CH_3	5.4	7.1
R=-Benzyl	5.7	6.2
R=-Sulfobromophthalein	11.8	6.5
γ-Glutamate-glutamine	18	6.9
γ-Glutamate-alanine	10.8	5.7
Glutamine	430	1.3

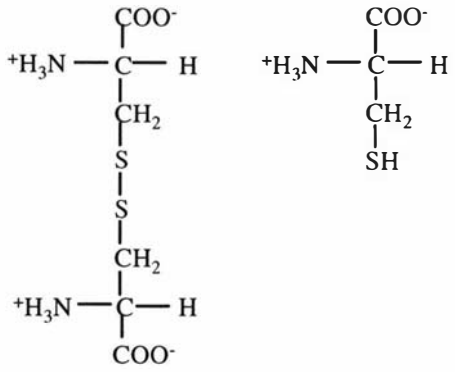
Table 1.7 γ-GT acceptor molecules.

Amino acid specificities of γ-glutamyl transpeptidase. The donor substrate was D-γ-glutamyl-*p*-nitroanilide. Kinetic experiments were carried out at pH 8.0 and 37°C. L-Proline and L-tyrosine were not studied (Thompson and Meister, 1977).

Amino acid	$V_t.K_t$[a] (min^{-1})
L-Cystine	250 ± 30[b]
L-Glutamine	23 ± 5
L-Methionine	12 ± 2
L-Alanine	6.8 ± 1.8
L-Glutamate	4.6 ± 0.8
L-Cysteine[c]	4.0 ± 0.8
L-Serine	3.3 ± 0.6
Glycine	2.6 ± 1.4
L-Asparagine	2.5 ± 0.3
L-Arginine	2.5 ± 0.3
L-Lysine	2.3 ± 0.3
L-Histidine	1.6 ± 0.5
L-Phenylalanine	1.1 ± 0.1
L-Tryptophan	1.0 ± 0.4
L-Leucine	0.6 ± 0.2
L-Threonine	< 0.6
L-Isoleucine	< 0.6
L-Valine	< 0.6
L-Aspartate	< 0.6

[a] $V_t.K_t$ ratios are pseudo-first order rate constants for the transfer reaction.
[b] SE
[c] Studied in the presence of equimolar concentrations of dithiothreitol.

Thompson and Meister (1977) found that the apparent K_M value for cystine was not far from the concentrations of L-cystine in rat blood plasma (0.3 mM) (although in ruminants cystine concentration in blood plasma is much lower (Knutson *et al.*, 1994)), and that γ-glutamyl-L-cystine is a good substrate for the next enzyme in the γ-glutamyl cycle, γ-glutamyl cyclotransferase. Blood plasma concentrations have been shown to be higher for cystine than for cysteine, although cells contain more cysteine (Figure 1.5). A number of cell types are able to reduce cystine to cysteine in a reaction requiring two GSH molecules yielding substrates for glutathione and protein synthesis (Figure 1.6) (Thompson and Meister, 1975).



Cystine
MW 240.30

Cysteine
MW 121.16

Figure 1.5 Structural forms of cystine and cysteine.

MW = molecular weight in Daltons

2 GSH + γ-glutamyl-cystine ⟶ GSSG + γ-glutamyl-cysteine + cysteine

Figure 1.6 Conversion of γ-glutamyl-cystine to cysteine.

Cystine is converted to γ-glutamyl-cystine by γ-glutamyl transpeptidase, then to cysteine inside the cell by a transhydrogenase reaction involving glutathione (GSH). GSSG is disulphide glutathione (Thompson and Meister, 1975).

GSH is highly concentrated inside the cell and since γ-GT is membrane bound and concentrated at sites of amino acid transport, with an extracellular active site, Meister was led to the conclusion that γ-GT uses extracellular amino acids and GSH derived from the inside of the cell to form γ-glutamyl amino acids, which are transported into the cell (Figure 1.3) (Meister, 1983).

Large amounts of disulphide forms of glutathione, γ-glutamylcysteine and cysteine are excreted in the urine of patients with γ-GT deficiency and animals treated with γ-GT inhibitors. This suggests that these products would normally be transported into tissues by the enzyme (Griffith and Meister, 1980). Increased γ-GT activity has been associated with cystinosis, a lethal autosomal recessive disorder where excessive intralysosomal storage of cystine occurs which also suggests that γ-GT is involved in amino acid transport. However, in one case where a patient exhibited severe glutathionuria and glutathionemia (Schulman *et al.*, 1983), known to be caused by γ-GT deficiency (Meister and Anderson, 1983), there was no amino aciduria and the uptake rates of four amino acids thought to be acceptor substrates were normal, questioning the role of γ-GT in amino acid transport (Schulman *et al.*, 1983). γ-GT is believed to transport 64% of cystine (Sweiry *et al.*, 1995), therefore, cystine transport would be occurring through the x_c^- system and this may have increased in response to absence of γ-GT activity.

γ-GT activity is concentrated in membranes of secretory and absorptive cells, such as epithelial cells of proximal renal tubules, jejunum, biliary tract, epididymis, seminal vesicles, choroid plexus, ciliary body, retinal pigment membrane, bronchioles, thyroid follicles, canalicular region of hepatocytes, pancreatic acinar and ductule cells. The kidney exhibits the greatest levels of γ-GT activity, along with pancreas, epididymis, seminal vesicles, jejunum epithelial cells, liver and spleen (Meister and Anderson, 1983). High levels of γ-GT activity also exist in the mammary gland epithelium (Baumrucker and Pocius, 1978). As well as the presence of γ-GT on cell membranes, activity has been found in endoplasmic reticulum and Golgi apparatus, and soluble activity is found in urine, seminal fluid, and pancreatic juice (Meister and Anderson, 1983) and also milk (Kitchen, 1974).

γ-GT is a heterodimer formed from cleavage of a proenzyme. It is anchored to the cell membrane by the N-terminus of its heavy chain. The active site is extracellular and is contained in the light chain of the enzyme. It is a highly glycosylated protein (Meister and Anderson, 1983; Viña and Viña, 1983; Smith *et al.*, 1995). The primary sequence of γ-GT has been determined from tissues of several species including rat kidney (Laperche *et al.*, 1989), and human liver (Sakamuro *et al.*, 1988), and from *Escherichia coli* K12 (Suzuki *et al.*, 1989). Human and rat amino acid sequences have approximately 79% identity (Sakamuro *et al.*, 1988), while identity scores for *E. coli* and mammalian γ-GTs are low (Suzuki *et al.*, 1989). From cDNA of human γ-GT the amino acid sequences for the heavy and light chains were estimated to be 351 amino acids (MW 38336) and 189 amino acids (MW 20,000) in length respectively (Sakamuro *et al.*, 1988).

The mouse γ-GT gene encodes six mRNAs that differ at the 5'-untranslated region but code for the same protein. These six mRNAs are expressed differentially in mouse tissue, all six being produced in kidney, but only types I and III in the eye and in the foetal liver respectively. Each transcript results from a different promoter, the activity of each being cell specific. This method of regulation of γ-GT expression is advantageous to an organism for the following reasons: *i*) it allows transcription in various cell types, through the use of tissue specific factors rather than ubiquitous ones; *ii*) it allows fine adjustment of the level of γ-GT expression depending of physiological needs; *iii*) it allows the tissue to respond to toxins. For example γ-GT is expressed at higher levels in animals exposed to xenobiotics (Sepulveda *et al.*, 1994). There are multiple γ-GT genes in humans (Siegrist *et al.*, 1990).

In addition to hydrolysis of GSH, there is a phosphate-independent glutaminase activity in the active site of γ-GT, indicating the presence of a glutamine binding site. Similarities have been found between human and rat light chain amino acid sequences, and glutamine binding sites of glutamine amido-N-transferases. Sakamuro *et al.* (1988) suggested that the active site of both human and rat enzymes is located at or near a single cysteine residue found in the light subunit which binds both glutamine and γ-

glutamyl moieties. The active residue in *E. coli* has been found to be serine (Suzuki *et al.*, 1989).

The γ-GT reaction is proposed to proceed through a γ-glutamyl enzyme intermediate. Investigation of active site residues that would form a covalent linkage with the γ-glutamyl moiety indicated ester linkage formation, probably to a serine hydroxy group. Ser-451 and Ser-452 residues were found to be conserved in human, rat, pig and two bacterial enzymes, and when substituted for alanine an enzyme with 1% of the wild-type activity resulted indicating that Ser-451 and Ser-452 are essential for catalysis (Ikeda *et al.*, 1995).

1.7.2.1. Acivicin inhibition of γ-GT

Acivicin (L-[αS,5S]α-amino-3-chloro-4,5-dihyhro-5-isoxazole acetic acid) (CAS RN No 42228-92-2) also known as AT-125, is a fermentation product of *Streptomyces sviceus*. Early studies investigated the effect of acivicin on glutamine amidotransferases and it was found to be a glutamine antagonist (Tso *et al.*, 1980). Acivicin has often been referred to as a glutamine analogue (Sastrasinh and Sastrasinh, 1986; Griffiths and Keast, 1991) but it shares the basic structure of any amino acid (Figure 1.7).

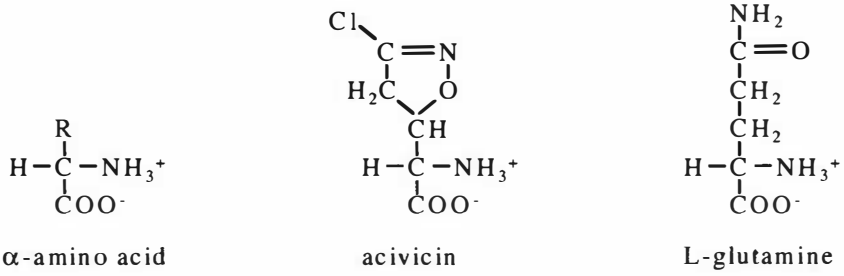


Figure 1.7 Structure of acivicin.

Comparison of the structures of an ionised (zwitterion) form of an amino acid, acivicin (Tso *et al.*, 1980), and glutamine (Stryer, 1988).

γ-GT has been shown to be inhibited by compounds that form analogues of the transition state such as L-serine + L-borate (Meister, 1983). Transition state analogues are compounds with structures resembling the hypothetical transition state or intermediate structure. They are unreactive but because of the structural similarity to the

transition state are held very tightly by the enzyme. Acivicin is a more effective inhibitor of γ-GT than L-serine + L-borate.

Acivicin has been described as a reversible (Stole *et al.*, 1994) and an irreversible inhibitor (Meister, 1983) of γ-GT. However, the slow release of the product of acivicin degradation indicates that acivicin is essentially an irreversible inhibitor or enzyme inactivator. Acivicin has been described as having an affinity labelling mechanism of action (Meister and Anderson, 1983). Affinity labelling agents are inhibitors that resemble the enzyme's substrate and generally alkylate or acylate active site residues (Silverman, 1995). However studies by Stole *et al.* (1994) have shown that acivicin itself does not inhibit γ-GT but rather a compound formed by γ-GT action on acivicin acts as the affinity label. This form of inhibition is consistent with that of a mechanism-based enzyme inactivator, which is an unreactive compound whose structure resembles that of either the substrate or product of the enzyme. This mechanism-based inactivator undergoes catalytic transformation to a species that inactivates the enzyme prior to release from the active site. The inhibitory species of a mechanism-based enzyme inactivator can be an affinity labelling agent, transition state analogue or tight-binding inhibitor (Silverman, 1995).

Incubation of purified enzyme with acivicin results in 99% inhibition of γ-GT activity and binding of γ-GT by acivicin is approximately 1:1. Acivicin is converted by γ-GT to *threo*-β-hydroxy-L-glutamate and hydroxylamine at a very slow rate; approximately 10^9 times slower than GSH. The inhibitory intermediate, a β-hydroxyl-γ-glutamyl hydroxamic ester, may lose hydroxylamine and then be hydrolysed like the normal γ-glutamyl enzyme intermediate to form β-hydroxyglutamate (Stole *et al.*, 1994). The intermediate is stabilised by the environment provided by the enzyme but would be unstable in an aqueous solution (Stole *et al.*, 1994) and can therefore not be used in γ-GT inhibition studies. Treatment of inactivated γ-GT with hydroxylamine results in increased release of the inhibitor (Stole *et al.*, 1994).

Acivicin was reported to bind outside of the γ-glutamyl active site and inhibit by causing a conformational change to hinder γ-GT activity (Smith *et al.*, 1995). However, in a

later report the same group use acivicin to investigate residues of the catalytic site essential for γ-GT activity, and describe the covalent linking of acivicin to active site residues. γ-GT is thought to act on acivicin in the same way as it does on γ-glutamyl substrates since they are similar in structure and both require a nucleophile for formation of intermediates (Ikeda *et al.*, 1995).

Acivicin has been reported to enter cells of a mouse leukemia line (P388) by the L transport system, but the uptake was also thought to be sodium-dependent, which is inconsistent with transport by this system (Table 1.5). However, uptake of acivicin by mouse leukemia cells and embryonic fibroblasts was inhibited by leucine supporting a role of the L system in acivicin transport (Rosenfeld and Roberts, 1981).

High levels of glutamine amidotransferases (Tso *et al.*, 1980) and γ-GT (Koss and Greengard, 1982; Meredith and Williams, 1986) in tumour cells have led to the study of acivicin for treatment of cancer. Acivicin has been studied as a chemotherapeutic agent in cultured cells (Griffiths and Keast, 1991; Weinberg *et al.*, 1992), whole animals (Poster *et al.*, 1981; McGovren *et al.*, 1988), and humans in phase I (McGovren *et al.*, 1985) and II (Eisenhauer *et al.*, 1987) clinical trials. These studies often investigate actions of acivicin that are secondary to targeted enzyme inhibition.

In one study, renal brush border membrane vesicles were treated with radiolabelled acivicin, followed by solubilisation of membrane proteins with papain. Subsequent gel filtration showed 95% of radioactivity was associated with γ-GT, indicating that γ-GT was the only protein modified by acivicin (Kozak and Tate, 1980). Another study with brush border membrane vesicles showed that acivicin did not immediately affect γ-GT activity, but rapidly decreased glutamine uptake by a high-affinity transport system. In that study the transport of glucose, alanine, glutamate, and proline was also reduced by acivicin (Sastrasinh and Sastrasinh, 1986). Hsu *et al.* (1984) showed acivicin inhibited 98% of γ-GT activity and reduced uptake of some amino acids but not uptake of cystine, glutamine, proline, glycine, methionine, leucine, lysine or glucose. These contrasting results obtained from similar experiments using brush border membrane vesicles brings

into question the validity of the technique. Acivicin inhibition of renal γ-GT in rats has been shown to occur without secondary effects (Capraro and Hughey, 1985).

Secondary effects of acivicin have been shown in other studies, with the enzymes involved in the *de novo* synthesis of nucleic acids inhibited by acivicin (Rosenfeld and Roberts, 1981). Acivicin has been shown to inhibit the growth of *Serratia marcescens* in culture (Tso *et al.*, 1980). Studies in cultured rat embryos indicated inhibition of γ-GT by anti-γ-GT antibodies produced malformations that were in contrast to those caused by acivicin, even though γ-GT was equally inhibited in each case (Stark *et al.*, 1987). The decrease in GSH export from cells when GSH synthesis is blocked may be a secondary effect of acivicin treatment (Dethmers and Meister, 1981).

Although acivicin has been shown to have effects secondary to γ-GT inhibition its effectiveness in inhibiting γ-GT makes acivicin a desirable chemical to study the role of this enzyme. Acivicin has been used successfully to study γ-GT in cultured cells (Cotgreave and Schuppe-Koistinen; 1994, Sweiry *et al.*, 1995) and in the whole animal (Araya *et al.*, 1989; Viña *et al.*, 1990).

1.7.2.2. Role of γ-GT in GSH transport

The γ-glutamyl cycle is proposed to utilise GSH as the donor of the γ-glutamyl moiety for amino acid transport (Meister and Anderson, 1983). Kinetic experiments comparing donor substrates support this, as γ-GT has a high affinity for GSH (McIntyre and Curthoys, 1979). Plasma concentrations of GSH in rodents (10-20 μM; Kannan *et al.* (1998)) and ruminants (goat 1.6 μM (Knutson *et al.*, 1994); cow 2.7 μM (Pocius *et al.*, 1981)) have been shown to be much lower than intracellular concentrations (0.1-10 mM; (Kosower, 1976)). Experiments have shown that γ-GT rapidly depletes intracellular GSH (Griffith *et al.*, 1978). This creates a problem because the active site of γ-GT is extracellular (Meister and Tate, 1976), unless there is a transport system for exporting GSH from the cell.

Initial observations resulted in the proposal that γ-GT itself was responsible for GSH transport. Inhibition of glutathione synthetase results in decreased intracellular GSH as

utilisation continues. Inhibition of γ-GT in mice increased GSH concentration in the kidney indicating that removal of GSH was blocked. With both enzymes inactive, the rate of intracellular GSH disappearance decreased, indicating that γ-GT is involved in export of GSH. This idea is supported by the observation that substrates that are good acceptors of the γ-glutamyl moiety increase γ-GT activity and decrease GSH concentrations inside kidney and liver cells (Griffith *et al.*, 1978).

A subsequent paper by Griffith and Meister (1979b) showed that inhibition of γ-GT in mice increased the GSH concentration in plasma, which corresponds with the role of γ-GT in GSH transport into the cell. This increase may arise from GSH released from cells that are not affected by γ-GT inhibition i.e. that have no or low γ-GT activity such as the liver (Meister and Anderson, 1983), but may also indicate that GSH export can occur when γ-GT is inhibited suggesting that γ-GT does not transport GSH out of cells. This is contrary to the observation that γ-GT itself was involved with GSH transport out of the cell. The latter suggests the presence of a system for transporting GSH out of the cell that is separate from γ-GT but in some way linked to the γ-GT cycle.

The decrease in intracellular GSH depletion in absence of both γ-GT and glutathione synthetase activities could be from a detrimental effect by γ-GT inhibitors on GSH transporters (Dethmers and Meister, 1981). Alternately the transporter of GSH could be sensitive to a GSH gradient across the membrane. In this case, when extracellular GSH concentrations increase as a result of γ-GT inhibition, the GSH transporter slows the rate of export causing an increase in intracellular GSH. When both GSH synthesis and γ-GT are inhibited, intracellular GSH concentrations slowly decrease as GSH inside the cell is consumed.

Cells with high levels of γ-GT activity show no translocation of GSH, as all exported GSH is rapidly imported by γ-GT. In cells with low γ-GT activity or when γ-GT is inhibited, GSH translocation can be measured (Griffith *et al.*, 1979b). Excess GSH export, like that occurring by the liver, gives rise to plasma GSH concentrations (Sze *et al.*, 1993). For this to be in agreement with a transporter sensitive to a transmembrane gradient of GSH, the transporter in the liver must have a higher activity in the presence

of extracellular GSH than the transporter in other tissues, or translocation is slow and many transporter molecules are present.

Specific transporters for intact GSH have been suggested for the brain, kidney, liver, intestine and eye lens but to our knowledge not in mammary tissue. GSH transporters of the liver canalicular (RcGshT) and sinusoidal (RsGshT) membranes are sodium independent and in physiological conditions are thought to export GSH out of cells (Kannan *et al.*, 1996; Favilli *et al.*, 1997). A report that RcGshT was expressed in the brain was later found to be incorrect. Although sodium-dependent and independent mechanisms for GSH transport have been demonstrated in the brain tissue and cells, the mechanisms remain to be elucidated (Kannan *et al.*, 1996; Kannan *et al.*, 1998).

GSH translocation is considered a discrete step in the γ-glutamyl cycle, as γ-glutamyl amino acid formation requires GSH interaction with free amino acids and γ-GT outside the cell. Increased intracellular 5-oxoproline production occurs after γ-glutamyl amino acid uptake, suggesting that γ-glutamyl amino acids are not hydrolysed at the membrane and taken up by classical transport systems as free amino acids. Rather, this indicates the presence of a distinct transport system for γ-glutamyl amino acids (Meister and Anderson, 1983). γ-GT appears to mediate the translocation of GSH from the cell for γ-glutamyl amino acid production, which controls amino acid appearance inside the cell. Alternately, γ-GT may mediate the import of γ-glutamyl amino acids. It is also possible that a single carrier system is responsible for the antiport of GSH and γ-glutamyl amino acids (Griffith *et al.*, 1979a).

1.7.3. Other γ-glutamyl cycle enzymes

γ-glutamylcysteine synthetase, the first enzyme involved in the synthesis of GSH is inhibited by GSH indicating a feedback mechanism. It is intracellular with a molecular weight of 100 kDa and has broad specificity. It can be separated into a heavy and a light subunit of molecular weights 74 kDa and 24 kDa respectively. The heavy subunit alone shows full enzyme activity and inhibition with GSH, whereas function of the light subunit is yet to be determined. The mechanism of action requires MgATP for formation of enzyme bound γ-glutamyl phosphate, which then interacts with the amino group of cysteine. The inhibitor L-buthionine sulphoximine is converted to buthionine

sulphoximine phosphate, which binds strongly and irreversibly to the enzyme (Meister and Anderson, 1983). Use of buthionine sulphoximine is effective at decreasing GSH and is relatively non-toxic (Dethmers and Meister, 1981).

Glutathione synthetase is thought to act in the same way as the previous enzyme. It is highly specific for glycine and the cysteine moiety of γ-glutamylcysteine. This enzyme is made up of two identical subunits that give a combined molecular weight of 118 kDa (Meister and Anderson, 1983).

γ-Glutamyl cyclotransferase deals with the products of γ-GT and produces 5-oxoproline. It is soluble and widely distributed in mammalian tissues. γ-Glutamyl cyclotransferase is highly specific to γ-glutamyl derivatives of cystine, cysteine, glutamine, methionine and several other protein amino acids. This is in parallel with the acceptor substrate specificities of γ-GT, which supports sequential action of these two enzymes. The cyclotransferase enzyme also acts on γ-glutamyl-γ-glutamyl amino acids. It is an unstable enzyme undergoing physical and catalytic changes during preparation and storage. Many forms of this enzyme have been separated by electrophoresis although activity is associated with a single protein. Multiple forms may result from intramolecular disulphide bond formation between accessible thiol groups, which may be associated with conformational changes involved in regulation (Meister and Anderson, 1983). β-aminoglutaryl-L-α-aminobutyrate is a specific inhibitor of γ-glutamyl cyclotransferase and administration to mice causes a decrease in renal 5-oxoproline indicating a role for this enzyme in 5-oxoproline formation (Bridges *et al.*, 1980).

5-oxoproline is removed by 5-oxoprolinase, which forms L-glutamate in an ATP and Mg^{2+} dependent reaction. 5-oxoprolinase was the last enzyme of the γ-glutamyl cycle to be characterised. In addition to its formation by γ-glutamyl cyclotransferase activity, 5-oxoproline arises from enzymatic cleavage of amino terminal 5-oxo prolyl residues of peptides and proteins, from the diet, and from nonenzymatic cyclisation of γ-glutamyl compounds. 5-oxoprolinase has a molecular weight of 325 kDa and is made up of two apparently identical subunits. It contains 27 thiol groups, two of which are required for

activity. Formation of L-glutamate from 5-oxoproline is essentially irreversible, although, in the presence of high L-glutamate concentrations very slow reversal is shown. 5-oxoprolinase is also responsible for formation of cysteine from 5-oxoproline analogues. For example, L-2-oxothiazolidine-4carboxylate (OTCA) is a substrate with high affinity for the enzyme and is rapidly metabolised. Administration of OTCA to mice has resulted in an increase in GSH concentrations indicating that it is a good method of cysteine delivery to the cell since administration of this amino acid itself is toxic (Meister and Anderson, 1983). However OTCA will competitively inhibit removal of 5-oxoproline leading to accumulation of the latter (Meister, 1983) and perhaps oxoprolinuria.

Cysteinyl-glycine, a product of γ-GT, is removed by dipeptidase activity, but the enzyme responsible has not been characterised. Dipeptides of this type are split by aminopeptidase M that is concentrated in membranes. However, there is little activity of this type in rat epididymis where γ-GT activity is high. Another membrane bound dipeptidase was isolated from rat kidney, but both this enzyme and aminopeptidase M only exhibit low activity towards L-cysteinyl-glycine. Extracellular oxidation of this cysteinyl-glycine is also thought to occur (Meister and Anderson, 1983).

Decreased circulating GSH concentrations have been found in patients with deficiencies of γ-glutamylcysteine synthetase and glutathione synthetase. Those with the latter exhibit oxoprolinuria, as γ-glutamylcysteine is acted on by γ-glutamyl cyclotransferase, forming oxoproline in excess of that which can be removed by 5-oxoprolinase. GSH that would normally be formed by glutathione synthetase is absent, and therefore so is the feedback inhibition on γ-glutamylcysteine synthetase, resulting in increased substrate for 5-oxoproline formation (Meister and Anderson, 1983). 5-oxoproline is excreted in large amounts in the urine of patients with glutathione synthetase deficiency, with main symptoms being metabolic acidosis and jaundice, and slow damage to the central nervous system (Larsson and Hagenfeldt, 1983).

1.8. Role of the γ-glutamyl cycle in milk production

Investigation of the role of the γ-glutamyl cycle in amino acid transport in the mammary gland began after research showed milk contained high levels of γ-GT (Kitchen, 1974). Lactating mammary tissue is a highly active secretory tissue and γ-GT was known to be present in high concentrations in membranes of cells involved in secretion (Meister and Anderson, 1983). Baumrucker and Pocius (1978) decided to investigate the presence of γ-GT in mammary tissue, as no data existed on possible transport systems responsible for amino acid extraction from blood plasma by the gland at that time. High γ-GT activity was found in bovine and rat lactating mammary glands and the bovine activity was found to be comparable with uptake of amino acids by the bovine mammary gland (Baumrucker and Pocius, 1978).

Early evidence for the γ-glutamyl cycle transporting amino acids in the mammary gland included work that showed increased γ-GT activity correlated with lactation (Figure 1.8). Rat γ-GT activity increases during pregnancy and dramatically at parturition to peak 10 to 15 days after birth, then declines rapidly to low concentrations 2 days after weaning (Puente *et al.*, 1979; Pocius *et al.*, 1980). The activity of γ-GT in the mouse mammary gland has been shown to follow a similar pattern (Siegrist *et al.*, 1990). In addition, malnutrition in lactating rats decreases γ-GT activity in the mammary gland with associated decreases in both milk output and milk protein content (Araya *et al.*, 1989).

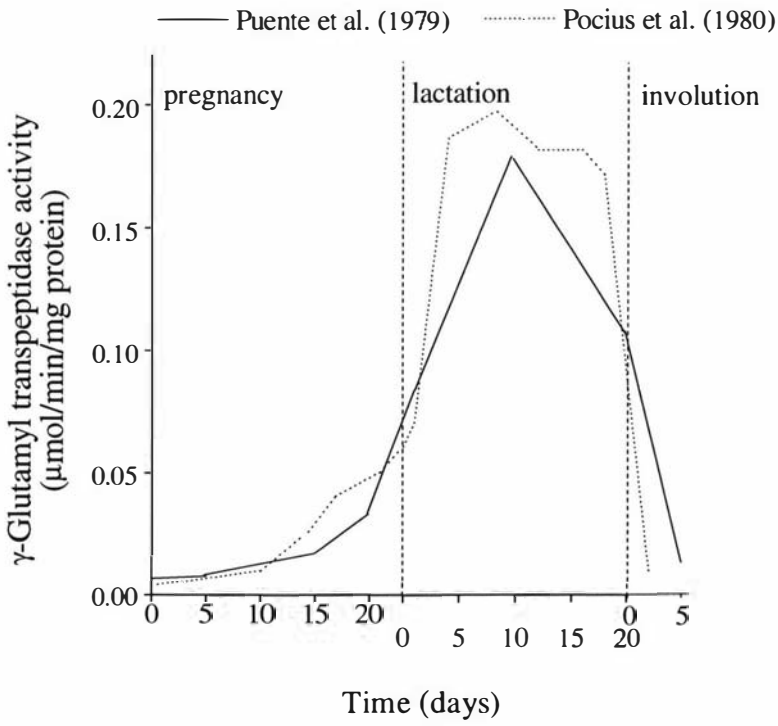


Figure 1.8 Activity of γ-glutamyl transpeptidase during the lactation of the rat.

γ-GT activity from rat mammary gland has been shown to be hormonally regulated. Puente *et al.* (1979) compared γ-GT activities of rat liver and mammary gland during pregnancy, lactation, and involution, and also hormonally induced pseudopregnancy. They concluded that γ-GT in the mammary gland was responsive to combined action of β-oestradiol and progesterone. Pocius *et al.* (1980) saw the same trend in γ-GT activity during lactogenesis of rats as Puente *et al.* (1979), and showed that prolactin was involved in increasing γ-GT activity at onset of lactation. This is consistent with observations of Puente *et al.* (1979) as prolactin release is stimulated by oestrogen (prolactin is a hormone necessary for the onset of lactation and milk biosynthesis). Bromocryptine, a specific inhibitor of the release of prolactin from the anterior pituitary, was used in lactating rats and caused a decrease in milk yield and also a decrease in γ-GT activity consistent with the requirement of prolactin for γ-GT induction (Pocius *et al.*, 1980).

In contrast to the mammary gland, liver γ-GT activity decreases during pregnancy with a similar decrease in cyclic AMP (cAMP) concentrations, suggesting that cAMP may be an inducer of γ-GT. However there was no relationship between changes in γ-GT activity and cAMP concentrations in the mammary gland, and the level of γ-GT in liver could not be altered with hormone injections, so these responses may be tissue specific (Puente *et al.*, 1979) or the parallel changes in cAMP and liver γ-GT activity may be fortuitous.

It was suggested that γ-GT levels in mammary gland were reflective of the amount of secretory epithelium, which increases during the lactogenic cycle through hormonal regulation, rather than being due to a direct effect of hormones on enzyme activity (Puente *et al.*, 1979). Consistent with this, the mRNA encoding γ-GT in mouse mammary tissue was shown to increase during pregnancy in a similar pattern to γ-GT activity (Siegrist *et al.*, 1990) suggesting that γ-GT gene expression is hormonally regulated.

The observation that γ-GT activity increases at the onset of lactation is consistent with its proposed role as a transporter of amino acids. The increase in γ-GT activity observed during pregnancy is unusual as several other enzymes of the mammary gland that are associated with milk production do not increase until the onset of lactation. However, the increase in γ-GT during pregnancy may be an anticipatory adaptation in preparation for greater demand of amino acid transport in milk protein synthesis (Puente *et al.*, 1979). Increased demand for amino acids would also arise from tissue synthesis as the mammary gland develops in readiness for lactation.

Continued research of the γ-glutamyl cycle in mammary tissue by Baumrucker and Pocius (Pocius *et al.*, 1981) looked at the role of glutathione in amino acid transport as suggested by Meister and co-workers (Griffith *et al.*, 1978). GSH was able to be taken up by lactating bovine mammary tissue slices, and treatment of isolated bovine mammary acini with GSH containing a radiolabelled cysteinyl moiety resulted in the appearance of radiolabelled protein (Baumrucker *et al.*, 1981). This indicates that GSH

is a source of amino acids for secretory protein synthesis. γ-GT activity would be required for GSH to be utilised in this way.

A breed of Finnish sheep inherits either high or low erythrocyte GSH concentrations. Those with higher GSH concentrations in their erythrocytes also produce more milk indicating a role for erythrocyte GSH in milk synthesis. Arterio-venous differences of erythrocyte GSH across the gland were greater after milking, when milk synthesis is thought to be higher (Atroshi and Sandholm, 1982). This is consistent with utilisation of GSH constituents for milk protein synthesis, or up-regulation of γ-GT activity and increased uptake of γ-glutamyl amino acids.

Investigation of GSH uptake by the bovine mammary gland showed that GSH was not taken up from plasma, as arterio-venous differences were not significantly different from zero (0.034 μg/ml, $P<0.05$). In lactating goats, the trend for net flux of GSH from whole blood by the mammary gland was negative indicating GSH release but this was also not significantly different from zero (-10 ± SEM 9 g/d) (Knutson *et al.*, 1994). However, the whole blood GSH concentration was 200-fold higher than plasma in the cow (Pocius *et al.*, 1981) and 500-fold higher in the goat (Knutson *et al.*, 1994). This was due to the high concentration of GSH found in erythrocytes. GSH cannot be transported out of erythrocytes in its reduced form, but can be in its oxidised/disulphide form when the cell is under oxidative stress (Srivastava, 1977). Xanthine oxidase is produced by lactating mammary cells (Walstra *et al.*, 1999) and generates superoxide (O_2^-) and peroxide (H_2O_2), which cause oxidative stress in cells. Therefore, erythrocytes passing the mammary gland are probably exposed to oxidative stress that may result in release of GSSG, which can be transported into mammary cells by the γ-glutamyl cycle.

In addition to the effect of oxidative stress on GSH supply to the mammary gland, uptake of cystine in human umbilical vein endothelial cells and human fibroblasts is enhanced when these cells are exposed to oxidative stress (Miura *et al.*, 1992). This probably occurs to replace cysteine for GSH synthesis but may also supply cysteine for milk protein synthesis.

Cysteine is thought to be limiting for milk protein synthesis and has been shown to be taken up from blood plasma in amounts insufficient to account for output in milk protein (Clark *et al.*, 1978; Lee *et al.*, 1999). γ-GT may regulate uptake of this amino acid by the mammary gland as it has high affinity for cysteine and cystine (Thompson and Meister, 1977). Cells that express γ-GT have been shown to use extracellular GSH as a source of cysteine, which can be used to increase intracellular GSH concentrations (Hanigan and Ricketts, 1993) or may be available for use in milk protein synthesis by the mammary gland. Thus, some of the components of GSH may not be recycled back into the tripeptide after its degradation by γ-GT. Erythrocyte GSH may provide a way of 'topping up' mammary cell GSH, may provide a source of cysteine, and/or may provide the γ-glutamyl moiety for transporting amino acids especially cystine.

1.9. Support and criticism of the role of γ-GT in amino acid transport

γ-GT activity has been shown to increase intracellular 5-oxoproline concentrations in the kidneys of mice (Griffith *et al.*, 1978). This indicates that γ-glutamyl amino acids formed by γ-GT are converted intracellularly to 5-oxoproline allowing transport by γ-GT to be distinguished from conversion of γ-glutamyl amino acids to free amino acids extracellularly, and their subsequent uptake by conventional transport systems. However, the role of the γ-glutamyl cycle in amino acid transport has received much criticism as well as support from many researchers.

Viña and co-workers showed that amino acid uptake by the gland could be decreased by inhibiting γ-GT using serine + borate in isolated mammary acini from the lactating rat gland (Viña *et al.*, 1981), and anthglutin and acivicin *in vivo* using lactating rats (Viña *et al.*, 1983). In addition, these authors were able to show that increasing the concentration of acceptor substrate (amino acids) could increase γ-GT activity and decrease donor substrate (GSH) concentrations inside cells (Viña *et al.*, 1981). Experiments using a cerebral endothelial cell line (ME-2) also support a role for γ-GT in amino acid transport as these experiments showed that increased γ-GT activity caused increased amino acid uptake by these cells (Cancilla and DeBault, 1983).

Continued research on the role of γ-GT in amino acid transport using rat mammary acini showed several factors that affect activity of γ-GT also induce changes in amino acid uptake by the gland (Viña and Viña, 1983). Viña and Viña (1983) concluded that the regulation of amino acid transport occurs through γ-GT activity. Viña *et al.* (1989) then proposed that γ-GT itself was not responsible for the transport of amino acids, but instead that it provides precursors for the production of signal molecules that in turn activate conventional amino acid transport systems. In rats, amino acid arterio-venous (A-V) difference decreases at day 19 of lactation, however, Viña *et al.* (1989) could restore the A-V difference to maximum using various intermediates of the γ-glutamyl cycle. The injection of γ-glutamyl amino acids or 5-oxoproline into lactating rats was effective, as was the inhibition of 5-oxoprolinase, the enzyme that removes 5-oxoproline. The use of anthglutin in rats to inhibit γ-GT decreased amino acid uptake at the peak of lactation but injection of 5-oxoproline prevented this decrease. In addition, infusion of 5-oxoproline into rats pretreated with acivicin restored transport of radiolabelled alanine into the gland (Viña *et al.*, 1989). Therefore, the results from this paper indicated that γ-GT was indirectly involved in amino acid transport as a producer of 5-oxoproline.

Viña *et al.* (1990) repeated some experiments in cultured human keratinocytes to show that inhibition of γ-GT by acivicin decreased amino acid uptake by these cells. However, only leucine and alanine were studied. In comparison with cystine these amino acids are poor acceptor substrates for γ-GT (Thompson and Meister, 1977). This paper supported the proposed involvement of γ-GT in the transport of amino acids but again the authors indicated that this role was indirect as a producer of signal precursors responsible for activation of conventional transport systems.

A direct involvement of γ-GT in the transport of amino acids was shown in human umbilical vein endothelial (HUVE) cells (Cotgreave and Schuppe-Koistinen, 1994). Cystine is transported by the x_c^- anionic amino acid transport system (McGivan and Pastor-Anglada, 1994). Other anionic amino acids, glutamate, aminoadipate and homocysteate, were used to competitively inhibit the use of this transport system by cystine, but administration of acivicin or anthglutin still caused a decrease in cystine

accumulation in these cells. Glutamate is a poor acceptor substrate for γ-GT (Thompson and Meister, 1977) so would not be expected to interfere with cystine transport by γ-GT. Inhibition of glutathione synthetase prevented cystine uptake indicating that cystine is transported into these cells by γ-GT using GSH (Cotgreave and Schuppe-Koistinen, 1994). This work was supported by Viña and co-workers (Sweiry *et al.*, 1995) who carried out similar experiments using human pancreatic cells (PaTu 8902). Cystine uptake was inhibited by acivicin after the x_c^- system was competitively inhibited for cystine use by glutamate. The γ-GT system was shown to account for 64% of cystine transport in this report (Sweiry *et al.*, 1995).

Strong support for a role of γ-GT in amino acid transport has come from research involving γ-GT deficient mice. These were produced by deleting promoter regulatory sites, splice sites, and coding exons from DNA coding for γ-GT, by homologous recombination. Embryonic stem cells containing this DNA construct were injected into blastocytes to produce chimeric male mice. These were bred to give mice homologous for the deleted γ-GT allele. γ-GT deficient mice were indistinguishable from wild-type at birth but gained weight more slowly, had a coat colour with a grey hue, and developed cataracts. Fifteen percent of γ-GT deficient mice survived to 9 months but had neurologic lesions. These effects were reversed by administering N-acetylcysteine (NAC) to the mice, which is a non-toxic form of cysteine, indicating that the disorders were the result of cysteine deficiency and that γ-GT functions in supply of cysteine (Lieberman *et al.*, 1996).

NAC was also able to reverse reproductive disorders in male and female γ-GT deficient mice, which became fertile and able to produce normal numbers of offspring when mated with wild-type mice (Kumar *et al.*, 2000). This indicates that γ-GT is itself not required for reproduction but is important for supply of cysteine to mouse reproductive organs.

Criticism of a role for γ-GT in amino acid transport has come from research using rabbit erythrocytes. In these cells, the authors expected to observe GSH degradation as a result of γ-GT mediated uptake of alanine, phenylalanine, and lysine. As intracellular GSH

concentrations remained constant, the researchers concluded that γ-GT was not using intracellular GSH to transport these amino acids. However, as GSH synthesis was not inhibited in this experiment, GSH would be regenerated as it was being used for amino acid transport by γ-GT (Young *et al.*, 1975).

The putative function of the γ-glutamyl cycle in amino acid transport was not supported by Sepúlveda and co-workers (1982) who investigated the involvement of γ-GT in the transport of alanine and leucine. A pig kidney cell line (LLC-PK_1) was shown to have increasing γ-GT activity after cells were seeded at low density. However, the uptakes of leucine and alanine decreased during this time, suggesting that γ-GT is not important in the transport of these amino acids (Sepúlveda *et al.*, 1982).

A paper by Hsu *et al.* (1984) also questions the role of the γ-glutamyl cycle in amino acid transport. Rat brush border membrane vesicles, which were used in the experiments described in this paper, contain γ-GT, and isolated rat renal cortical tubule fragments that contain all γ-glutamyl cycle components. Acivicin was used to inhibit γ-GT and emphasis was placed on cystine and glutamine uptake since they are both excellent acceptor substrates. Using membrane vesicles, no difference was observed between the uptake of glutamine or cystine and the controls with γ-GT inhibited 90% by acivicin. Uptake of proline, glycine, methionine, leucine, lysine and glucose by the vesicles was also unaffected by γ-GT inhibition (Hsu *et al.*, 1984).

The vesicles used by Hsu and co-workers (1984) were prepared according to the method used by Booth and Kenny (1974) who referred to them as microvilli, which are finger-like protrusions from the mucous membrane of the small intestine. Hsu *et al.* (1984) state that these vesicles showed significant capacity for binding substrate and that uptake decreases with increasing osmolarity. However, the results imply that the vesicles are not taking up the substrate. The membrane fragments in this preparation may not be sealed so it would not be possible to measure the uptake of amino acids across the membrane. The γ-glutamyl amino acids that would form from action of γ-GT on amino acid acceptor would become donors of the γ-glutamyl moiety and these would

continue recycling. In the presence of acivicin the amino acids would be stationary on either side of the membrane so uptake would not appear different from controls.

The isolated tubule fragments containing intact renal cells that were prepared by Hsu *et al.* (1984) also showed that uptake of cystine was not inhibited by acivicin. Although the authors tested γ-GT activity in the brush border membrane preparations they failed to do the same for the tubule fragments. Although these fragments would normally contain components of the γ-glutamyl cycle, it would appear from the results in this paper that they contain little γ-GT activity, so that no conclusion should be drawn from the paper for or against the involvement of the γ-glutamyl cycle in amino acid transport.

A lack of involvement by γ-GT in the transport of tyrosine was found in cultured bovine adrenal chromaffin cells. In these cells, γ-GT was inhibited using acivicin but tyrosine transport was shown to dramatically decrease with a concentration of acivicin insufficient to inhibit γ-GT (Morita *et al.*, 1994). This may indicate that acivicin inhibits transport of tyrosine by a method other than γ-GT inhibition. It is possible that acivicin damages chromaffin cells, altering cellular function and reducing amino acid transport. However, in human pancreatic duct cells it was found using ATP assays that acivicin does not affect cellular integrity (Sweiry *et al.*, 1995). If γ-GT was involved in signal production then tyrosine uptake should have decreased with γ-GT inhibition. Viña and co-workers (Viña *et al.*, 1989) showed that transport of amino acids was responsive to 5-oxoproline administration and it is unlikely that the signal is amplified for transport of tyrosine alone. The affinity of tyrosine as an acceptor substrate for γ-GT has not been described although it has been shown that γ-GT has low affinity for amino acids with aromatic side chains (Allison, 1985).

A paper by Kansal and Kansal (1996) also refuted the idea that the γ-glutamyl cycle has a role in amino acid transport. These researchers claimed that transport of methionine and alanine into mouse mammary gland tissue was not affected by γ-GT inhibition. However, there was a lack of statistical evidence to support this and the results could be interpreted differently from what the authors report. The results show that methionine uptake is increased when both γ-GT is inhibited and Na^+ supply is restricted. This

suggests that down-regulation of γ-GT increases the supply of methionine for uptake by a sub-saturated Na^+-dependent transporter, which was proposed by Kansal and Kansal (1996) to be the L transport system. The uptake of alanine decreased with γ-GT inhibition but was only evident when the A or L transport systems were blocked or when Na^+ was restricted. This suggests that γ-GT transports alanine but this activity is masked when other transport systems are operating. Thus, the results of Kansal and Kansal (1996) could be interpreted in a way that suggests γ-GT does play a role in methionine and alanine transport.

Hanigan (1998) interprets the work using γ-GT deficient mice differently and does not support the role of the γ-glutamyl cycle in amino acid transport. Hanigan (1998) implies that the disorders in γ-GT deficient mice are caused by GSH deficiency and that cysteine supplied by NAC allows GSH synthesis to continue. However, γ-GT deficient mice were found not be deficient in GSH (Lieberman *et al.*, 1996). NAC administration is an accepted method of increasing intracellular GSH (Lederman, 1995) but achieves this by increasing intracellular cysteine.

Research using microvessels prepared from porcine brain has led to criticism of the role of γ-GT in amino acid transport since uptake of ^{35}S-cystine was not affected by γ-GT inhibition (Wolff *et al.*, 1998). In this paper, alanine and isoleucine were treated as amino acids not bound by γ-GT, although data have been produced to the contrary (Thompson and Meister, 1977; Baumrucker and Pocius, 1978) and γ-GT inhibition was observed to decrease uptake of these amino acids (Wolff *et al.*, 1998). Efflux of ^{35}S into medium was observed and probably resulted from use of ^{35}S-cystine for GSH synthesis. ^{35}S-GSH export would have been observable since utilisation by γ-GT and re-entry into the cell would have been inhibited (Wolff *et al.*, 1998). This report supports the role of γ-GT in GSH metabolism and transport of alanine and isoleucine but not cystine in across the blood-brain barrier.

The role of the γ-glutamyl cycle in amino acid transport has been extensively researched. Criticism of the cycle has failed to rule out a role for γ-GT in amino acid transport, and investigations aimed at supporting the γ-glutamyl cycle have failed to

pinpoint the mechanism of action. An indirect role has been shown through the supply of 5-oxoproline which up-regulates amino acid transport by conventional systems. However, the production of 5-oxoproline requires γ-glutamyl amino acids to be taken up by the cell and the by-product of 5-oxoproline production would be free amino acids inside the cell. It is accepted that γ-GT binds GSH and free amino acids and produces γ-glutamyl amino acids, and since γ-glutamyl amino acids and the remaining portion of GSH are imported into the cell, it must be accepted that γ-GT is involved in amino acid transport. The mechanism of action will have to be defined to add credence to the role of the γ-glutamyl cycle in amino acid transport.

1.10. Summary and revision of the γ-glutamyl cycle

GSH is degraded to yield cysteine by γ-glutamyl transpeptidase (γ-GT), an enzyme of the γ-glutamyl cycle (Meister and Anderson, 1983). The products of GSH degradation are thought to be co-transported with amino acids (in the form of γ-glutamyl amino acids) into mammary cells. The amino acid that γ-GT has the highest affinity for is cystine (Meister and Anderson, 1983). Therefore, γ-GT may regulate the uptake of cyst(e)ine, which is thought to be potentially limiting for milk protein synthesis in the ruminant.

γ-GT activity increases at lactation (Puente *et al.*, 1979; Pocius *et al.*, 1980) supporting a role for this enzyme in milk protein production. Increased milk protein synthesis may result from generation by the γ-glutamyl cycle of intracellular signals that stimulate transport of other amino acids into the mammary gland (Viña *et al.*, 1989). Thus the γ-glutamyl cycle may be involved in control of amino acid supply for milk protein synthesis in three ways: the degradation of GSH to yield cysteine; the transport of cystine; and the up-regulation of conventional amino acid transport systems.

The γ-glutamyl cycle was first proposed in 1970 by Orlowski and Meister (1970) and although it has received much criticism (Young *et al.*, 1975; Sepúlveda *et al.*, 1982; Hsu *et al.*, 1984; Morita *et al.*, 1994; Kansal and Kansal, 1996; Hanigan, 1998; Wolff *et al.*, 1998), it has not been disproved and remains unchanged. However, there are some parts

of the cycle that require clarification, in particular the activities responsible for GSH export and γ-glutamyl import require elucidation.

The export of GSH from the cell where it is formed has been indicated not to be a function of γ-GT, although the actual transporter has not yet been identified and the two activities appear to be linked (Griffith *et al.*, 1979a; Dethmers and Meister, 1981). The γ-glutamyl amino acids that are formed through γ-GT activity have not been shown to be transported into cells by γ-GT although the two functions have not been disassociated. From a study of the literature, a revised γ-glutamyl cycle has been proposed (Figure 1.9) that includes a transporter for GSH export and a transporter for γ-glutamyl amino acid import into cells. This scheme suggests separate enzymes for GSH export, transpeptidation, and import of γ-glutamyl amino acids, with linked activities. These three enzymes may be bound in the membrane as a complex with γ-GT and the transport system for γ-glutamyl amino acids.

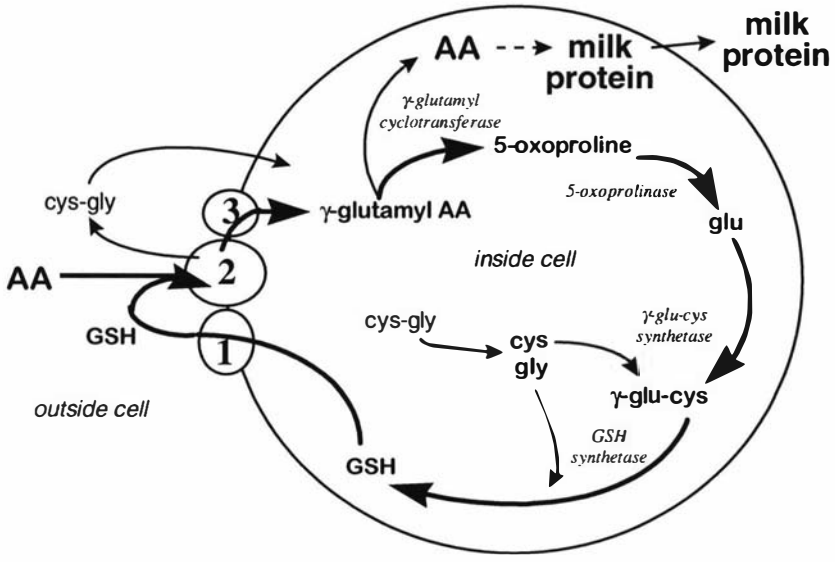


Figure 1.9 Revised γ-glutamyl cycle.

A revised version of the γ-glutamyl cycle and its role in amino acid supply for milk protein synthesis. Activities of enzymes 1, a glutathione (GSH) transporter, 2, γ-glutamyl transpeptidase, and 3, a transporter for γ-glutamyl amino acids appear to be linked.

1.11. Experimental objectives

The review of the literature shows that while the role of the γ-glutamyl cycle has been well researched, the mechanism of action of one enzyme of the cycle, γ-GT, has not been completely elucidated and it may mediate amino acid transport for milk protein synthesis. Activity of γ-GT in mammary tissue may be important to the Dairy Industry because this enzyme degrades GSH to produce cysteine, which is thought to be limiting for milk protein synthesis. γ-GT also has a high affinity for cystine, which is easily converted to cysteine. Further research is required to define the role of cysteine in regulating milk protein synthesis.

Studies in cultured cells have indicated an important role for γ-GT in cyst(e)ine supply. The role of γ-GT in amino acid supply for milk protein synthesis has been determined in rodents, and while γ-GT activity has been shown in the bovine mammary gland, further research is required to show how γ-GT is required for amino acid transport for milk protein synthesis in the ruminant mammary gland. The role of GSH synthesis, the extent of cyst(e)ine uptake by γ-GT, and the role of 5-oxoproline all need to be determined in the lactating ruminant mammary gland.

The aim of this research project was to determine the role of the γ-glutamyl cycle in milk protein secretion of the ruminant. Experiments were carried out using isolated acini from the udders of lactating sheep as a model for milk protein secretion *in vivo* and are discussed in Chapter 2. The objectives of the work described in Chapter 2 were to determine:

- the role of γ-GT in milk protein production by inhibiting this enzyme with acivicin
- whether inhibition of GSH synthesis with buthionine sulphoximine affects milk protein production
- whether administration of 5-oxoproline affects milk protein synthesis
- whether cysteine supply by alternative sources (OTCA and NAC) can override acivicin inhibition of γ-GT

Results using acivicin and NAC in isolated sheep mammary acini were successful, so an additional objective was formulated to validate *in vitro* results in lactating goats (Chapter 3). The objectives of the work described in Chapter 3 were to determine effects of acivicin and NAC on:

- milk yield and composition
- amino acid uptake by the gland by quantifying:
  - extraction %, which is the difference between arterial (A) and venous (V) concentrations as a percentage of arterial concentration
  - net uptake (mass per unit time), which takes into account mammary blood flow
  - ^{3}H-leucine to specifically measure mammary uptake and whole body leucine flux.

Chapter 2. The role of the γ-glutamyl cycle in milk protein synthesis *in vitro*

2.1. Introduction

The γ-glutamyl cycle may have a role in amino acid uptake although the mechanism of transport and in particular the function of the γ-glutamyl enzyme, γ-GT, in this role has not been fully established. Activity of γ-GT has been shown to increase in the rat mammary gland at lactation (Figure 1.8) suggesting that the γ-glutamyl cycle acts in milk production. However, it has not been proven that the γ-glutamyl cycle provides amino acids for milk protein synthesis. γ-GT has a high affinity for cyst(e)ine (Thompson and Meister, 1977) which is possibly limiting for milk protein synthesis (Clark *et al.*, 1978). Further investigation may reveal that the activity of enzymes in the γ-glutamyl cycle can be manipulated, allowing regulation of the appearance of individual proteins in milk so that the properties of milk can be targeted to produce specific milk products as required by the Dairy Industry.

Studies in the whole animal of the role of γ-GT in milk protein synthesis have been confined to lactating rodents given chemical treatments (Pocius *et al.*, 1980; Viña *et al.*, 1983). These have been informative but because of the differences that exist between rodents and ruminants in milk composition (Table 1.1) and hormonal regulation (Forsyth, 1984), the results from such studies cannot be extrapolated directly to the dairy cow. The ideal study would use dairy cows but these are expensive to use in a situation that requires the animal be killed to obtain mammary tissue for experiments.

Experiments in the whole animal often return positive results, but because of the complexity of the animal system, new questions often arise as to how the result came about. Experiments on cells from organs of interest allow the study of cellular events in the absence of whole body signals. *In vitro* methods using immortalised lines of cells derived from the mammary gland, or acini isolated from the mammary gland of slaughtered animals, are alternatives to the use of rodents and valuable dairy cattle.

A number of mammary cell lines exist including MAC-T cells derived from the bovine gland, and COMMA-D, isolated from the mouse gland. MAC-T are mammary epithelial cells that have been shown to produce α_s- and β-caseins (Huynh *et al.*, 1991). However in comparison to milk protein synthesis *in vivo*, the performance of this cell line is low (Wheeler *et al.*, 1995) and differs with each preparation (personal communication, S.R. Davis). COMMA-D cells derived from mice are a mixture of cell types with 10-20% synthesising caseins in response to hormonal stimuli (Beaton *et al.*, 1997). Since COMMA-D cells are not ruminant cells they may not accurately represent protein synthesis in the dairy cow.

The isolation of mammary cells from lactating ruminants in secretory units called acini provides a system for the study of milk production in the mammary gland. Tissue sampling from mammary glands of live animals can be performed (Farr *et al.*, 1996) but would be a distressing procedure that would not provide enough tissue or allow selection of particular parts of the tissue. Slaughter of the animal and removal of the udder allows dissection of adequate amounts of tissue. Dairy cows are far too valuable to be destroyed for removal of tissue for cell isolation, but sheep offer a good model and are less expensive.

Isolated ovine mammary acini have been shown to be a good model for *in vivo* mammary function. Davis and co-workers (1993) characterised lactose production and glucose uptake of acini isolated from sheep. Glucose uptake remained constant over 48 h indicating that acinar cells remained viable. Lactose production was lower for isolated acini than *in vivo*, and decreased to very low levels after 48 h of incubation. There was no evidence to suggest that this decrease was due to substrate depletion indicating that it may have resulted from the removal of *in vivo* signals that maintain milk production. Lactose production was not affected by the presence of foetal calf serum or the application of agents required for maintenance of milk secretion: insulin; hydrocortisone; prolactin; prostaglandin E. The work discussed above (Davis *et al.*, 1993) supports the use of isolated ovine mammary acini in the study of milk production, although the declining lactose production suggests acini should not be incubated for longer than 12 h.

Wheeler *et al.* (1993) investigated protein production of isolated ovine mammary acini and found that milk protein production was lost after 24 h of incubation. This was found to result from a decrease in RNA, as total RNA was only 23% of initial levels after 22 h of incubation. Transcription was thought to be shut down during isolation of the acini and was re-established during culture. Continuing milk protein production must therefore result from translation of the pre-isolation pool of RNA that is eventually exhausted, thus halting milk protein production. The secretion of casein was found to be greater in the presence of agents required for maintenance of milk secretion and foetal calf serum (Wheeler *et al.*, 1993). In a following report, isolated ovine mammary acini were shown to synthesise and secrete α- and β-caseins and β-lactoglobulin in amounts similar to those seen *in vivo* for up to 8 h (Wheeler *et al.*, 1995).

The use of ovine instead of bovine cells is supported by the similarity of the major proteins of ovine and bovine milk. Like bovine milk, ovine milk contains the four main caseins α_{s1}-, α_{s2}-, β-, and κ-caseins (Martin and Addéo, 1996). The ovine α_{s1}- and α_{s2}-caseins do not contain cysteine (Table 2.1) whereas in the bovine proteins α_{s2}-casein contains two cysteine residues (Swaisgood, 1995). Ovine β_1- and β_2-caseins have similar amino acid composition to bovine β-casein with 21 differences (Alichanidis and Polychroniadou, 1996). The ovine κ-caseins differ from bovine by 27 amino acid residues (Alichanidis and Polychroniadou, 1996) and are more like bovine β-casein in electrophoretic mobility (Alais and Jollès, 1967). Ovine milk has greater concentrations of β-lactoglobulin than bovine milk (Anifantakis, 1986) although the proteins have a high degree of similarity (Swaisgood, 1995).

Table 2.1 Ovine milk protein amino acid composition.

Amino acid composition (residues/protein) of five major proteins of ovine milk.

	Casein						Whey	
	α_{s2}[a]	α_{s3}[a]	β_1[a]	β_2[a]	κ_A[b]	κ_B[b]	β-lactoglobulin A[c]	β-lactoglobulin B[c]
Essential amino acids								
Histidine	4	4	5	5	3-4	3-4	2	3
Lysine	14	13	12	12	8	9	14	14
Methionine	5	4	5	5	2	2-3	4	4
Phenylalanine	7	6	9	9	4	4	4	4
Threonine	4	4	11	11	12	10	8	8
Tryptophan	2	2	1	1	-	1-2	2	2
Isoleucine	10	10	9	9	9	10	9	9
Leucine	17	17	21	21	7-8	8-9	20	20
Valine	9	10	22	22	9-10	10-11	10	10
Conditionally essential amino acids								
Tyrosine	10	9	3	3	8	8-9	4	3
Cysteine	-	-	-	-	1-2	1-2	5	5
Nonessential amino acids								
Alanine	12	12	4	5	17 ± 1	17	15	15
Arginine	6	5	3	3	5	5-6	3	3
Asparagine								
Aspartate	17	17	8	8	17	17	15	15
Glutamate	38	41	40	39	26	26 ± 1	24	24
Glutamine								
Glycine	9	10	5	5	2	2	5	5
Proline	22	22	37	37	23	21	8	8
Serine	16	18	13	14	12-13	11 ± 1	6	6

[a] (Richardson and Creamer, 1976).
[b] (Alais and Jollès, 1967).
[c] (McKenzie, 1971).

Ovine milk has been shown to have low concentrations of α-lactalbumin in comparison to bovine milk (Anifantakis, 1986). The ovine protein is similar to bovine α-lactalbumin but there is a greater percentage of cysteine in ovine milk compared to bovine, and since ovine κ-casein and β-lactoglobulin contain the same amount of cysteine as the bovine equivalents this suggests that ovine α-lactalbumin may contain a greater number of cysteine residues than bovine α-lactalbumin, which has eight cysteine residues (Alichanidis and Polychroniadou, 1996).

Isolated mammary acini from the sheep have been used in this study as a model for lactation in the dairy cow. This system allows acini from the mammary gland to be stimulated directly by adding chemicals to the medium, and the protein secreted into the culture medium can be harvested and analysed. In this study, attempts have been made to manipulate milk protein secretion of isolated ovine mammary acini using chemicals to target components of the γ-glutamyl cycle and provide an alternative supply of cysteine. The objectives of this chapter were to determine:

- the role of γ-GT in milk protein production by inhibiting this enzyme with acivicin
- whether inhibition of GSH synthesis with buthionine sulphoximine affects milk protein production
- whether administration of 5-oxoproline affects milk protein synthesis
- whether cysteine supply by alternative sources (OTCA and NAC) can override acivicin inhibition of γ-GT

2.2. Materials, equipment and animals

EDTA was obtained from Ajax Chemicals, Auburn, NSW, Australia.

3,4,5-^{3}H-L-leucine (4.44 GBq/μmol) in 2% ethanol, and α-amino-isobutyric acid [Methyl-^{3}H] (0.74 GBq/μmol) were obtained from American Radiolabelled Chemicals Ltd., St. Louis, MO, USA.

Sodium acetate.3H_2O and acetonitrile for HPLC were Hypersolv grade from BDH laboratory supplies, Poole, England. Crystal violet and trypan blue dyes,

orthophosphoric acid (H_3PO_4), sodium acetate, and universal pH indicator solution, were also obtained from BDH.

Falcon 12- and 24- well flat-bottom multi plates with lids, and Matrigel® basement membrane matrix were purchased from Becton Dickinson Labware, Bedford, MA, USA. Plates for use in the plate-reader were flat-bottomed 96-well Nuclon™ Nunc™ brand plates obtained from NalgeNunc International, Rochester, NY, USA.

SBD-F (4-fluro-7-sulfobenzo-furazan ammonium salt) was obtained from Dojindo Laboratories Kumamoto Techno Research Park, Kumamoto Japan.

Dulbecco's modified eagle medium (D-MEM), antibiotic/antimycotic (100 x lyophilized, 10,000 U/ml penicillin G sodium, 10,000 µg/ml streptomycin sulphate, 25 µg/ml amphotericin B as fungizone® (# 15245-012)) and foetal calf serum (FCS) were obtained from Life Technologies Inc., MD, USA.

Samples for scintillation counting were treated with Starscint™ scintillation amplifier and counted along side a ^3H quenched standard and a background standard all supplied by Packard BioScience BV, Groningen, The Netherlands.

Triethylamine (TEA) and Phenylisothiocynate (PITC) (#26922) were obtained from Pierce, Rockford, IL, USA. PITC derivatised samples were compared to Pierce A/N (# 20086) and Pierce B (# 20087) standard solutions. The BCA Protein Assay Reagent Kit (# 23225) was also from Pierce.

Hydroxylammonium chloride (hydroxylamine) and urea were obtained from Riedel-de Haën, Seelze, Germany.

The following chemicals were purchased from Sigma Chemical Company, St. Louis, MO, USA: cysteine/methionine depleted D-MEM; hepes; sodium bicarbonate; glutamine; phosphate buffered saline (PBS) tablets (10 mM phosphate buffer pH 7.4, 1.7 mM KCl, and 0.137 M NaCl); cycloheximide, hexokinase solid (18 units/mg); hexokinase liquid (150 units/mg); glucose-6-phosphate dehydrogenase (600 units/mg);

ATP and $NADP^+$; (-)-2-oxo-4 thiazolidine-carboxylic acid (OTCA); 5-oxo-L-proline; and tri-N-butyl phosphine (TBP). A diagnostic kit for the assay of γ-glutamyl transpeptidase activity was also obtained from Sigma (catalogue # 545-1).

Oxytocin was obtained from Vetpharm (NZ) Ltd., Glenfield, Auckland New Zealand.

All other chemicals and reagents were analytical grade or similar quality.

A Kinematica Polytron (Lucerne, Switzerland) was used to homogenise tissue samples.

Analysis of samples by HPLC used a Shimadzu LC4A (single module system) or LC10A system made up of a CBM-10A communications bus module, FCV-11AL solvent switching module, DGU-2A helium degassing system, two LC10A pumps, SIL-10AD auto injector including peltier cooled sample chamber, CTO-10A column oven, and SPD-10A UV-Vis detector or RF-10A spectrofluorometric detector obtained from Shimadzu Scientific Instruments Ltd., Columbia, MD, USA. The online scintillation counter (β-RAM) was obtained from IN/US, NJ, USA and had a counting efficiency for ^3H of 30%. Total counts were obtained using a scintillation counter from Packard BioScience BV, Groningen, The Netherlands.

Separation of PITC derived samples used an application-specified, Picotag C_{18} reverse phase column, quality controlled for rapid, high efficiency, bonded-phase separations (Waters Picotag® column 3.9 X 300 mm part # WAT 010950) obtained from Waters Corporation Milford MA USA. A Phenomenex Primesphere 250 x 4.6 mm 5 μm C18 HC column (part # 006-3034-EO) was used to separate caseins and whey proteins suspended in urea. A Phenomenex Jupiter 250 x 4.6 mm 5 μm C18 300 Å column was used to separate caseins and whey proteins in guanidinium.HCl suspended samples. The column for separation of SBD-F derivatised samples was an Applied Biosystems Brownlee™ column Spheri-5 ODS 5 μm 220 x 4.6 mm (part # 0711-0023 (ODS 224)).

New Zealand Romney ewes at a maximum of three weeks lactation were obtained from Massey University and AgResearch Grasslands, Ballantrae. Ethics approval was

obtained for this study from the Crown Research Institutes' Animal Ethics Committee under proposals numbered 26/97, 19/98, and 4/99.

2.3. Methods

2.3.1. Preparation of acini from ovine mammary tissue

The method used for extracting acini was modified from Davis *et al.* (1993). Standard incubation medium was prepared by supplementing D-MEM with 20 mM hepes, 2 mM $NaHCO_3$, 8.3 mM sodium acetate and 4 mM glutamine. Antibiotic/antimycotic (1 ml/l) was added to inhibit growth of microorganisms. The night before each experiment was carried out a 100 ml sterile Schott bottle was filled with standard medium using aseptic technique in a biohazard unit. Standard incubation medium, supplemented with 1% heat inactivated FCS in a 100 ml sterile Schott bottle was labelled 'wash', and a 25 ml sterile Schott bottle containing standard medium and 10% FCS was labelled 'digest'. These were stored at 4°C until the following morning. Collagenase (586 U/ml) was added to the 'digest' medium immediately before preparing the ewe for slaughter. All medium preparations were then incubated at 37°C.

Romney ewes at a maximum of three weeks lactation and with no obvious signs of mastitis were selected for acini incubation experiments. Ewes with single lambs were selected over ewes with twins. Lambs were left with ewes until immediately before slaughter of the ewe to maintain milk output at optimum levels. Ewes were hand milked 24 h before and immediately prior to slaughter following injection of 1 U oxytocin into the jugular vein to assist milk letdown. The ewe was slaughtered using a captive bolt gun followed by exsanguination. The udder was immediately excised and kept warm in a tinfoil-lined polystyrene box containing a plastic bag of water at approximately 37°C.

Approximately 6 g of mammary tissue was dissected, using sterile instruments, from the dorsal side of the glands in 1 cm^3 pieces avoiding blood vessels and milk ducts. Tissue was incubated in approximately 20 ml of 'wash' medium in a biohazard unit, before each piece was injected continuously for approximately 10 min with 1 ml of 'digest' medium, using a 25-G needle. Injected tissue was sliced and incubated with shaking for

80 min at 37 °C. Digested tissue was passed through a crude sterile filter made from net curtain (22 by 7 holes per cm^2). The filtrate underwent a series of washes to remove collagenase using 'wash' medium, with centrifugation for 5 min (850 x g) and then twice for 2 min (480 x g). Acini were pelleted (480 x g) for 2 min and resuspended in standard incubation medium.

2.3.2. Acini plating and incubation

A 100 µl aliquot of acini suspension was incubated with 900 µl of crystal violet solution (1 mM crystal violet in 0.1 M citric acid) for 5 min at 37 °C and then violently mixed using a vortex to break up acini so that individual cells could be counted using a haemocytometer at 400 x magnification. The proportion of viable acinar cells in the preparation was qualitatively assessed at 100 x magnification by staining 50 µl of acini suspension with 50 µl of trypan blue stain (1.7 mM trypan blue in PBS). Falcon tissue culture plates with 12- and 24-wells were coated with 60 µl and 32 µl respectively, of Matrigel® basement membrane matrix using the wide end of a sterile 200 µl pipette tip to evenly coat the surface of each well, and incubated at 37°C prior to use. Aliquots of 400 µl of an acini suspension of 1 x 10^6 cells / ml and 200 µl of an acini suspension of 2 x 10^6 cells / ml in 'control' medium were plated into prepared wells using 12- and 24-well plates respectively.

Experiments were set up with treatments in triplicate i.e. for one experiment n=3. A control set of wells where an equivalent volume of sterile MQ H_2O was added instead of chemical was present for each experiment and was regarded as a treatment. Treatments were carried out individually and in combination. Cycloheximide (25 µM), an antibiotic inhibitor of protein synthesis (Stryer, 1988), was added to a triplicate of background wells which accompanied each set of treatment wells so that treatments could be corrected for background levels of radioactivity. Mixing within the wells was achieved by gentle swirling of the plate.

A separate plate was set up for each harvest time point. Figure 2.1 shows the typical arrangement of a 24-well plate for two treatments. Plated acini were incubated at 37°C in the presence of 5% CO_2. In experiments where ^3H isotope was used, acini were incubated for 15 min prior to addition of 3,4,5-^3H-leucine (120 Ci/mmol) in 2% ethanol,

to wells. Initially 0.7 GBq of 3,4,5-^{3}H-L-leucine was added to wells but in later experiments this was reduced to 0.2 GBq. For isotope containing wells, 0 h was at the time of isotope addition. In experiments where wells did not contain isotope, 0 h was when acini were added to plates.

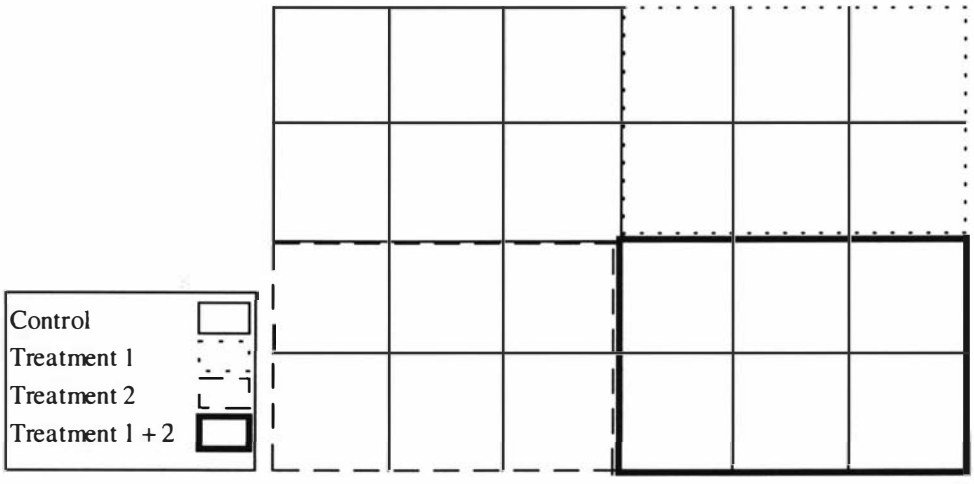


Figure 2.1 Incubation plate set up.

The typical set up is illustrated for a 24-well plate for an acini experiment involving two treatments. Each treatment included three wells containing cycloheximide (25 μM) the results of which were subtracted from treatments excluding cycloheximide to correct for background radioactivity. For each experiment n=3.

2.3.3. Harvesting of acinar cells and culture medium

Harvesting time points were predetermined for each experiment. At the end of incubation periods, plates were immediately placed on ice. The medium was removed from the wells and aliquoted into preweighed 1.5 ml microfuge tubes before undergoing centrifugation at 10000 x g for 5 min. This supernatant was retained for analysis of secreted milk protein and lactose. The well was washed twice using 200 μl ice cold PBS and scraped using a pipette tip. The cell pellet was resuspended with washings to remove Matrigel® and spun twice at 10000 x g for 5 min. This supernatant was discarded and the cell pellet was frozen at -85°C for analysis of intracellular protein, amino acids, ^{3}H-AIB uptake or ATP content at a later date.

2.3.4. Analysis of secreted milk protein

Skim milk, to be used as carrier protein for medium protein precipitation, was prepared by removing and discarding the fat layer from 50 ml of ovine milk following centrifugation at 3500 x g for 10 min in a swinging bucket type rotor.

Upon thawing of the ~250 µl cell culture medium sample, 25 µl of skim ovine milk and 100 µl of unlabelled leucine (76 mM), to dilute unincorporated isotope, was added. Protein was precipitated by adding 350 µl of 15% TCA. Samples were mixed and incubated on ice for 15 min before centrifugation for 15 min at 1600 g. The supernatant was discarded and the pellet was resuspended in 400 µl of 6 M urea overnight. The sample was washed two further times to remove unincorporated isotope by successive precipitation of protein using 500 µl 15% TCA in the presence of 100 µl 76 mM leucine and centrifugation at 1600 g for 15 min. The final protein pellet underwent a 20 sec pulse centrifugation to remove remaining TCA solution.

Two different methods were used for separating milk proteins, one that separated caseins from whey proteins before HPLC, and one that retained these together for HPLC separation. If caseins were to be separated from the whey proteins prior to separation of individual proteins by HPLC, the washed pellet was resuspended using 300 µl of 6 M urea. A 280 µl aliquot from one replicate was mixed with 400 µl of 100 mM sodium acetate pH 4.6 and incubated for 15 min at room temperature to precipitate caseins, which were then pelleted by centrifugation for 15 min at 1600 g. The supernatant was aliquoted into 1.5 ml microfuge tubes and treated with 1 volume of 15% TCA to pellet the remaining whey protein fraction, then mixed and incubated on ice for 15 min before centrifugation at 1600 g for 15 min. The supernatant was discarded. Whey and casein pellets were pulse centrifuged for 20 sec to pellet residual precipitant before overnight resuspension of the pellets in 300 µl of 6 M urea and separation by HPLC. The remaining 20 µl of replicate used for casein separation and 300 µl of the other two replicates were analysed for total radioactivity.

For HPLC separation, 200 µl of either casein or whey protein fraction suspended in urea was injected onto a Phenomenex Primesphere 250 x 4.6 mm 5 µm C18 HC column

plumbed into the Shimadzu LC10A system and heated in an oven set to 30 °C. A UV detector set at 280 nm was used to detect eluted proteins. The elution system consisted of 2 mobile phases, buffer A (10% acetonitrile, 89.9% MQ H_2O, and 0.1% TFA in MQ H_2O) and buffer B (90% acetonitrile, 9.9% MQ H_2O and 0.1% TFA). The separation gradient started with 20% buffer B increasing in a curve to 45% at 50 min then by linear increase to 100% at 60 min. Buffer B remained at 100% until 62 min then decreased to 20% at 62.1 min with run stop occurring at 70 min. The flow rate was set to 1.0 ml/min. All eluents in this and all HPLC experiments were kept under a blanket of $He_{(g)}$ to remove N_2 and O_2 from solution, and minimize on-column degassing

Alternatively, if the caseins and whey proteins were to be retained together for HPLC separation, the washed pellet was resuspended overnight in 400 µl casein dissolution buffer (6 M guanidinium.HCl, 0.1 M bis-tris, 5.37 mM sodium citrate, and 1.95 mM DTT) and then casein and whey proteins were separated by HPLC. A 100 µl aliquot of each replicate was taken for analysis of total radioactivity in the medium extract.

50 µl of the guanidinium.HCl suspended protein was injected onto a Phenomenex Jupiter 250 x 4.6 mm 5 µm C18 300 Å column plumbed into the Shimadzu LC10A system and heated in an oven set to 33 °C. A UV detector set at 254 nm was used. The elution system consisted of two mobile phases, buffer A (0.1% TFA in MQ H_2O) and buffer B (90% acetonitrile, 10% buffer A). The separation gradient started with 27% buffer B increasing to 33% at 2 min, increasing to 48.4% at 36.9 min, then to 50.2% at 40.9 min, and ended with a dump step of an increase to 90% at 43 min dropping to 27% at 46 min where it remained until the run stop at 68 min. The flow rate was set to 1.0 ml/min.

Following HPLC separation of individual proteins, the radioactivity of each peak was measured using an on-line β-radioactivity counter (β-RAM). Each protein peak, as a percentage of the sum of peaks obtained from the β-RAM, was used to calculate the individual protein radioactivity as a percentage of the total counts. Specific radioactivity could not be determined, as the sample preparation required the addition of skim milk to the sample as carrier protein.

2.3.5. Analysis of medium and cell total radioactivity by scintillation counting

Samples for total radioactivity counting were weighed into tared glass scintillation vials and mixed with 2 ml of Starscint™ scintillation amplifier. Samples were incubated in the dark for 48 h prior to the counting of ^3H radioactive decay using a Packard scintillation counter along side a ^3H quenched standard and a background standard supplied by Packard. Radioactivity of a blank sample containing H_2O and Starscint™ and also an empty scintillation vial were counted for background correction.

2.3.6. Analysis of medium and intracellular thiols by SBD-F derivatisation

The method for measuring thiols by SBD-F derivatisation was adapted from Toyo'oka et al (1988). Standards in 15% TCA were prepared by weighing out 5 mM stock solutions to give 50 μM cysteine, 10 μM homocystine, 20 μM GSH, and 50 μM N-acetylcysteine. Medium or standard (20 μl) was weighed into tubes. Cells were harvested into preweighed tubes and were reweighed to obtain the weight of the cell pellet. Samples and standards were diluted 2-fold using 0.375% SDS/ 4.5 M EDTA and incubated at room temperature for 15 min after vortex mixing. Samples were treated with 1 volume of 15% TCA. At this stage, cell samples were ground using a modified eppendorf tissue grinder fixed to an electric drill. Samples and standards were reduced using 10 μl of tri-N-butyl phosphine (TBP) kept under $N_{2(g)}$, prior to addition of the SBD-F reagent (0.4 mg/ml 4-fluro-7-sulfobenzo-furazan ammonium salt in borate buffer pH 9.30). Samples and standards were mixed and NaOH was added to final pH 8.5 - 9.0 before incubation at 60°C for 60 min in the dark. The pH was corrected to a pH 2.5 - 4.0 using 2 M HCl. Derivatised cells samples were centrifuged for 5 min at ~13500 g to remove cell debris.

Samples were analysed by fluorescence following separation of 50 μl of injected volume on an Applied Biosystems Brownlee™ column Spheri-5 ODS 5 μm 220 x 4.6 mm (part # 0711-0023 (ODS 224)) plumbed into either the LC4A or LC10A HPLC system. Mobile phase A was sodium phosphate (150 mM) made using 85% orthophosphoric acid (41.98 ml acid in 4000 ml MQ H_2O), corrected to pH 2.2 using NaOH pellets, and filtered through a CA 0.45 μm filter before use. Mobile phase B consisted of 85% phase

A buffer and 15% acetonitrile. The flow rate was set to 1.0 ml/min. The elution gradient began with 0% phase B. At 10 min, phase B increased until it reached 28% at 30 min. A dump step concluded the gradient with an increase to 100% phase B at 30.1 min dropping to 0% at 35.10 min to the run stop at 55 min.

2.3.7. Analysis of medium and intracellular amino acids by Picotag® derivatisation

A stock standard solution containing 0.5 mM amino acids was prepared using 200 µl Pierce A/N and 200µl Pierce B standard solutions together with 0.5 mM norleucine made up to a final volume of 1 ml using 0.1 M HCl. Stock standard was diluted 10-fold using 0.1 M HCl before use. A methionine sulphone (3 mM) standard was prepared in 0.1% phenol and 3.5 µl was added by weight to samples as an internal standard.

Analysis of amino acids in samples and standards involved pre-column phenylisothiocyanate (PITC) derivatisation and chromatography based on that described by Bidlingmeyer *et al.* (1984). A 50 µl aliquot containing free amino acids was dried under vacuum before addition of 20 µl redry solution (2:2:1, methanol: 1M sodium acetate: triethylamine (TEA) under $N_{2(g)}$), then mixed by vortex and again dried down. Derivatisation solution contained methanol, MQ H_2O, TEA (under $N_{2(g)}$), and PITC (under $N_{2(g)}$) (7:1:1:1) and was made fresh for each set of samples. The derivatisation reagent was stable for 2 h at room temperature. Derivatisation reagent (20 µl) was added to each dried sample. The samples were then vortex mixed and incubated at room temperature for 10 min after addition of reagent to final sample, and dried under vacuum. Dried derivatised samples were resuspended in 200 µl diluent containing 5% acetonitrile and 95% phosphate buffer (5 mM Na_2HPO_4 adjusted to pH 7.40 using 10% (v/v) H_3PO_4). Samples were vortex-mixed twice and transferred to 1.5 ml microfuge tubes and centrifuged at ~14000 g for 5 min. The supernatant was transferred to autosampler vials and placed in the peltier cooled autosampler of the Shimadzu LC10A HPLC system.

Derivatised samples (50 µl) were injected onto a Picotag C_{18} reverse phase column in an oven set to 46°C, with 90 min run time between each injection. The elution system consisted of 2 mobile phases, buffer A (70 mM sodium acetate.3 H_2O adjusted to pH

6.50 using 10% glacial acetic acid, containing 1.8% acetonitrile, and 2.5 µl EDTA) and buffer B (15% aqueous methanol, containing 45% acetonitrile). Buffer B was run in a gradient (Figure 2.2) starting with an increase from 0% at 13.5 min to 0.5% at 13.51 min, followed by an increase with a number 3 curve to 2% at 24 min, then by a linear increase to 6% at 30 min, then a number -2 curve to 28.5% at 50 min, and finally a linear increase to 36% at 62 min. Buffer B remained at 36% until 70 min when the gradient ended in a wash step of 100% B to remove residual sample from the column. The flow rate was set to 1.0 ml/min. Separated amino acids were detected by a UV detector set at 254 nm.

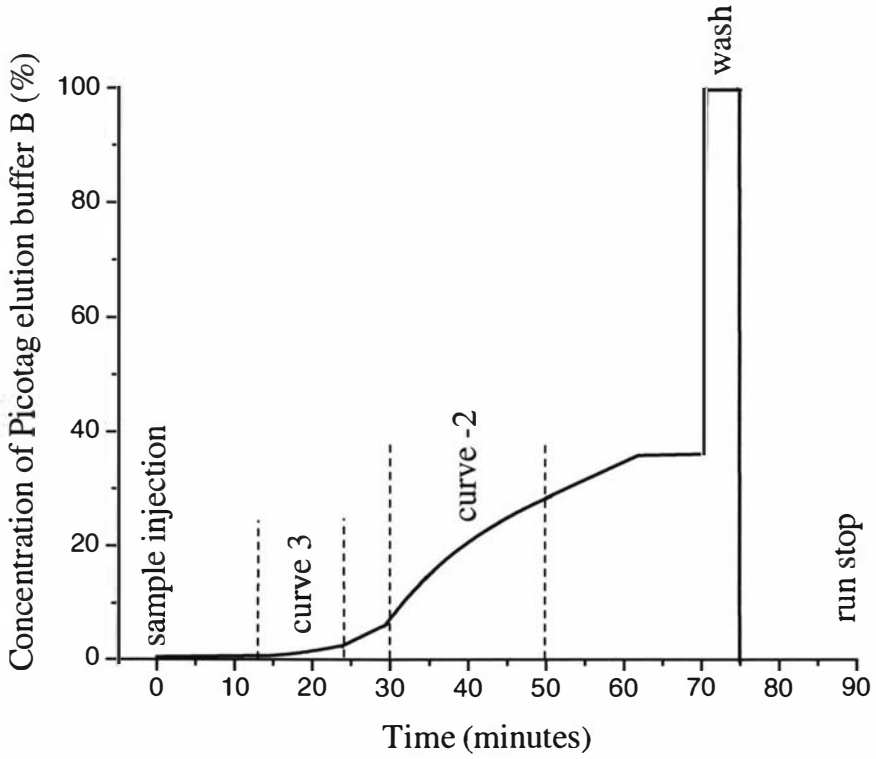


Figure 2.2 Buffer B gradient.

The gradient profile of buffer B for the elution of Picotag derivatised amino acids.

2.3.8. Validation of acinar cell viability using cell ATP content

Acini from three experiments that had been incubated for 0, 1.5, 3, and 5 h in the absence of isotope were stored at -85°C for later analysis. ATP content in mammary

cells from fresh tissue snap frozen in $N_{2(l)}$ 15 min after slaughter was also measured for comparison with *in vitro* levels. Samples of tissue were taken at various stages of the acini preparation procedure to characterise the effect of the procedure on cell viability. These samples were collected prior to digestion using collagenase, then immediately after injection with collagenase (t=0), and during the digestion at 30, 60, and 80 min (Table 2.1). Samples were also collected from acini suspensions that had been washed free of collagenase.

Table 2.2 Mammary tissue sampling times for ATP content.

ATP content was determined in mammary tissue at various stages of collagen digestion.

Stage of preparation	Time after slaughter (h)
Slaughter	0.00
Tissue excised as soon as possible after slaughter	0.25
Dissection of tissue pieces from gland	0.50
First piece of tissue after treatment with collagenase	0.75
Last piece prior to treatment with collagenase	1.75
Tissue digest 30 min after the start of incubation	2.25
Tissue digest 60 min after the start of incubation	2.75
Acini suspension in the absence of collagenase	3.25

Frozen mammary tissue was powdered using a modified French cell press and a hammer and stored at -85°C in preweighed microfuge tubes. Tubes were reweighed for tissue weight, and 1 volume of 10% PCA was immediately added and the sample was vortexed. Freshly prepared 1 mM ATP (100 µl) was added to some samples to monitor ATP loss through sample preparation. The samples underwent centrifugation at 10000 g for 10 min. The supernatant was collected in preweighed microfuge tubes, reweighed and then mixed with one drop of universal pH indicator solution. The pH was adjusted to pH 7.5 using KOH. The final weight of the neutralised sample was established for calculation of a dilution factor. An aliquot of 300 µl of neutralised tissue extract was assayed for ATP.

Tubes of known weight containing harvested cells were reweighed to obtain the weight of the cell pellet. The cell pellet was resuspended in 100 µl 10% PCA with vortex mixing. Cell samples did not undergo centrifugation. One drop of universal pH

indicator solution was added and the pH was adjusted to pH 7.5 using 0.1 M KOH. The final weight of the neutralised sample was established for calculation of a dilution factor. An aliquot of 200, 250 or 300 µl of neutralised cell extract was assayed for ATP.

ATP was measured using a reaction coupled to the production of NADPH, which can be monitored at 340 nm (Figure 2.3). Each sample was assayed in triplicate along with at least two standards, 17 µM and 33 µM of freshly prepared ATP. A reagent cocktail was made fresh each day containing 10 mM Tris base pH 7.6, 1.3 mM freshly prepared $NADP^+$, 10 mM $MgCl_2$, and 1 mM glucose. Samples were mixed with 2 ml reagent cocktail and made up to 3 ml using MQ H_2O in plastic cuvettes and vortex mixed. Absorbances were measured at 340 nm. Glucose-6-phosphate dehydrogenase (5 µl 7.5 U/mg) was added to each cuvette to remove all endogenous glucose-6-phosphate before its generation by hexokinase, so that the final change in glucose-6-phosphate is proportional to the ATP present in the sample. Samples were vortexed and incubated at room temperature for 5 min before the second absorbance reading. Hexokinase (5 µl 18 U/mg) was added finally to convert glucose to glucose-6-phosphate. Samples were vortexed and incubated at room temperature for 5 min then before the final absorbance reading was taken.

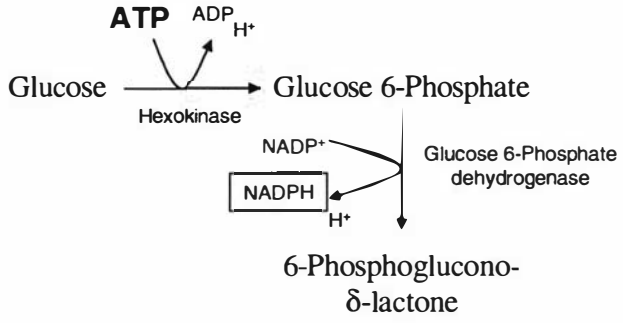


Figure 2.3 ATP assay reaction.

ATP was assayed spectrometrically by measuring NADPH formation at 340 nm.

The normal ATP content for sheep liver is well documented so liver samples from a Romney ram were collected to test the sensitivity of the assay. Liver tissue was prepared as described above for mammary tissue and stored at -85°C before analysis.

2.3.9. Validation of acinar cell viability by trypan blue dye exclusion

Viability of cells in treated acini was determined qualitatively as part of some experiments by trypan blue dye exclusion at harvest. A fourth well for each treatment was plated with acini and treated as per test wells and the contents of these wells were used only for trypan blue exclusion. When the test wells were placed on ice at harvest, the contents of the fourth well was resuspended by pipette and 50 µl was mixed with 50 µl trypan blue dye on a microscope slide using a 200 µl pipette tip, then viewed at 100 x magnification. Viable unstained cells were estimated as a percentage of the total cell count.

2.3.10. Validation of milk protein secretion by isolated ovine mammary acini

Milk protein synthesis was monitored by adding 3,4,5-^{3}H-L-leucine to wells to give a final specific radioactivity of 0.7 GBq/mol. An experiment was conducted using 12 and 24-well plates with cells harvested at 0, 1.5, 3, and 5 h after the addition of isotope. Acini were treated with cycloheximide or an equivalent volume of sterile MQ H_2O so that wells contained the same final volume. This experiment was repeated with 24-well plates and cells were harvested at times 0, 1.5, 3, 4, 5, 6, and 8 h after addition of isotope. Medium was collected at harvest and prepared for analysis of secreted protein and for total counts as described in sections 2.3.4 and 2.3.5 respectively.

2.3.11. Characterising γ-GT activity in mammary tissue

The preparation of mammary tissue for assay of γ-GT activity was based on the method by Baumrucker and Pocius (1978) and the enzyme extraction buffer (10 mM Tris HCl pH 8.0, 80 mM $MgCl_2$) was prepared as described by these authors. Mammary tissue was frozen in liquid nitrogen and crushed using a modified French cell press and a hammer. Powdered mammary tissue was stored at -85°C. Frozen sample was added to preweighed 50 ml centrifuge tubes and reweighed. Five volumes of extraction buffer was added and the mixture was homogenised using a Polytron with the sample tube in a beaker of ice. Samples were sonicated for 15 min then 0.5 ml was aliquoted into a 1.5 ml preweighed microfuge tube and weighed. The sample was weighed again after 20% (w/v) Triton was added to a final concentration of 2% (w/v). The sample was mixed using a vortex mixer at a very slow speed for 1 h and then centrifuged for 5 min at 9500

g. The supernatant was aliquoted into a preweighed 1.5 ml microfuge tube and weighed. The supernatant was diluted 5-fold using extraction buffer prior to assay for γ-GT activity, and 50-fold for protein assay.

Initially a diagnostic kit obtained from Sigma, which included γ-glutamyl-p-nitroanilide as the γ-GT substrate (4.6 mM), was used to determine γ-GT activity. Substrate (20 µl) was added to a test tube containing sample and incubated at 37°C for 20 min along with a reagent blank which contained substrate only. Acetic acid (2 ml 10% (v/v)) was added to each tube to terminate the reaction. Sample (20 µl) was added to reagent blanks after termination of the reaction to correct for background absorbance. Five standards (0 - 0.126 mM) were prepared using p-nitroaniline supplied in the kit. In timed sequence, 1 ml of 0.1% (w/v) sodium nitrite was added to each sample, blank and standard, which were mixed and incubated at room temperature for 3 min. Ammonium sulfamate solution (1 ml of 1% (w/v)) was then added in the same timed sequence and each tube was mixed and incubated for 3 min at room temperature. Naphthylethylenediamine solution (1 ml of 2.8 mM) was added and tubes were shaken vigorously to release air bubbles. Absorbance was determined at 545 nm against a water blank and γ-GT activity of samples was determined from the standard curve and corrected for background by subtracting the blank values.

The assay using the diagnostic kit gave inconsistent results and was modified according to assays documented in the literature (Baumrucker and Davis, 1980; Pocius *et al.*, 1980) to eliminate inconsistencies, and adapted for use in a plate-reader. This modified assay made use of the Sigma kit substrate to produce the yellow product p-nitroanilide. Validation of the assay is reported in Appendix A. The appearance of 1 µmol of p-nitroananiline per min was taken as 1 unit of enzyme activity. The plate-reader was prewarmed to 37°C and set to incubate and take absorbance readings from the plate every 2 min for 20 min at 405 nm. H_2O (286 µl) was added to one well as a blank. Substrate (275 µl 4.6 mM) was added to three reagent blank wells and sample wells. Immediately after addition of extraction buffer (11 µl) to reagent blank wells and 11 µl of sample to sample wells, the plate was mixed three times by the plate-reader and readings commenced.

The change in absorbance of each reagent blank and sample was plotted against time (minutes) and the slope was calculated. Total activity was calculated for reagent blanks and samples using the following formula:

$$\text{total activity}\,(\text{mmol/l/min}) = \frac{\Delta A \cdot \text{min}^{-1}}{\varepsilon \cdot l}$$

Eq. 2.1

where $\Delta A.min^{-1}$ is the slope, ε is the molar absorptivity for p-nitroaniline, and l is the pathlength calculated by dividing the total reaction volume by the volume of the well containing 1 ml (0.4 cm^3). For p-nitroaniline ε is 9.75 $mM^{-1}.cm^{-1}$, and for a reaction volume of 286 µl the pathlength (l) is 0.773 cm. The activity of reagent blanks was subtracted from sample activity to correct for background changes in absorbance.

Analysis of protein in samples was carried out using a BCA protein assay reagent kit. Standards and samples were prepared as per the manufacturer's instructions and were incubated at 37°C for 30 min prior to reading using a plate-reader. Specific activity (nmol/min/mg protein) of γ-GT was calculated by dividing the total activity by the concentration of protein in the sample. Total activity was also calculated per wet weight of tissue (µmol/min/g).

2.3.12. Characterising the role of γ-GT in milk protein synthesis by inhibition using acivicin

In an initial experiment using 24-well plates, medium was harvested at times 0, 2, 3, 5, 8, 10 and 18 h after the addition of 3,4,5-^{3}H-L-leucine at a final specific radioactivity of 0.7 GBq/mol. The γ-GT inhibitor acivicin (α-amino-3-chloro-4,5-dihydro-5-isoxazole-acetic acid) (CAS # 42228-92-2) was added to final concentration of 0.55 mM in wells. This experiment was repeated with harvests at 0, 2, 5, 8, and 18 h.

In three experiments using 24-well plates, acini were incubated in the presence of different concentrations of acivicin and 3,4,5-^{3}H-L-leucine (0.7 GBq/mol). In the initial experiment acini were incubated with acivicin at final concentrations of 0.7, 1.4, and 10.4 µM. The next two experiments were carried out using 1.4, 2.7, and 6.1 µM

acivicin. In each case acini incubation medium was harvested 0, 4, and 8 h after the addition of isotope.

In several of the following experiments acini were incubated with acivicin alone and in combination with other treatments. The concentration of acivicin used in each of these experiments will be discussed in the following sections.

2.3.12.1. Characterisation of γ-GT activity in acivicin treated acinar cells.

Acini that had been prepared for plating (14.25 ml 2 x 10^6 cells/ml) were treated with acivicin (0.75 ml, 2.5 mg/ml) to a final concentration of 0.7 mM, mixed and incubated at room temperature washed for 5 min. The acini underwent a series of washes to remove acivicin using 'wash' medium (as described in section 2.3.1), with centrifugation for 5 min (850 x g) and then twice for 2 min (480 x g). Acini were pelleted (480 x g) for 2 min and resuspended in 'control' medium. Samples were stored in 2 ml aliquots in preweighed tubes at -85°C along with acini remaining from the initial preparation that had not been washed with acivicin.

Tubes containing thawed samples were reweighed to determine the sample weight. The preparation of cells for assay of γ-GT activity was modified from Baumrucker and Pocius (1978) and the enzyme extraction buffer (10 mM Tris HCl pH 8.0, 80 mM $MgCl_2$) was prepared as described by these authors. Five volumes of enzyme extraction buffer were added before the sample was ground using an eppendorf tissue grinder mounted in an electric drill, and then sonicated for 15 min. Triton (20% (w/v)) was added to a final concentration of 2% (w/v) then samples were gently vortex mixed for 1 h at 1400 rpm. Cell debris was pelleted by centrifugation for 5 min at 10000 g. The supernatant was diluted 5-fold to assay for γ-GT activity and 10-fold to assay for protein. Samples were assayed for γ-GT activity and protein concentration as described in section 2.3.11.

2.3.12.2. *The use of labelled amino-isobutyric acid in characterising the action* of acivicin

The effect of acivicin on the A amino acid transport system was determined by incubating acini with acivicin and α-amino-isobutyric acid [methyl-^{3}H] (^{3}H-AIB), which

is know to be transported specifically by the A system (Lee *et al.*, 1996b). Plated acini were incubated with (76 MBq/mol) ^{3}H-AIB in the presence and absence of acivicin (6 μM). Acini were incubated with acivicin for 5 min before ^{3}H-AIB treatment and were placed on ice 0, 10, and 20 min after the addition of ^{3}H-AIB then harvested and analysed for total radioactivity.

2.3.12.3. Attempt to reverse acivicin inhibition of γ-GT using hydroxylamine

Hydroxylamine (7 mM) was added to acini during plating or at 4 h to acini plated with acivicin (6 μM). Medium was harvested at times 0, 4, 8, and 18 h after the addition of 3,4,5-^{3}H-L-leucine with a final specific radioactivity of 0.7 GBq/mol. Extra wells containing acini with each treatment were plated so that viability could be checked at each harvest point. This experiment was repeated using hydroxylamine at a final concentration of 13 μM, ~2-fold higher than the concentration of acivicin used.

2.3.12.4. Attempt to reverse acivicin inhibition of γ-GT by repeated washing and centrifugation.

Acini were washed with acivicin as described in section 2.3.12.1. After acini were resuspended in 'control' medium a volume of 200 μl per well was plated on Matrigel® coated 24-well plates and incubated for 0, 4, 8, and 18 h with 3,4,5-^{3}H-L-leucine with a final specific radioactivity of 0.2 GBq/mol. Harvested medium was analysed for total counts. This experiment was carried out twice.

2.3.13. Characterising the role of GSH synthesis in milk protein synthesis by buthionine sulphoximine inhibition of γ-glutamyl cysteinyl synthetase.

One experiment was carried out with acini incubated in 24-well plates for 0, 4 and 8 h before harvest of medium, in the presence of 0.5 mM buthionine sulphoximine (BSO), and 3,4,5-^{3}H-L-leucine (0.7 GBq/mol). This experiment was replicated twice using 1 mM BSO. For each of these experiments, extra wells containing acini with each treatment were prepared to monitor cell viability at each harvest time point by trypan blue dye exclusion.

Experiments where acini were treated with BSO were also carried out in combination with acivicin and with cysteine analogues. In one experiment a final concentration of 0.5 mM BSO was used in combination with acivicin (0.56 mM) for acini incubated 0, 4 and 8 h before the harvest of medium, in the presence of 3,4,5-^{3}H-L-leucine (0.2 GBq/mol). This experiment was repeated with medium harvested at 0, 2, 4, and 8 h after addition of 3,4,5-^{3}H-L-leucine. The effect of BSO on milk protein secretion was also studied when cysteine analogues were provided to acini in the medium as described in section 2.4.2.6.

2.3.14. Characterising the role of the γ-glutamyl cycle intermediate 5-oxoproline in milk protein synthesis

In two experiments, acini plated on 24-well plates and treated with 5-oxoproline (0.5 mM) in the absence and presence of acivicin (7 mM) were incubated for 0, 4 and 8 h in the presence of 3,4,5-^{3}H-L-leucine with a final specific radioactivity of 0.2 GBq/mol. Extra wells containing treated acini were plated so that viability could be monitored at each harvest time point by trypan blue dye exclusion.

2.3.15. Supplying intracellular cysteine for milk protein synthesis by alternative transport pathways

In experiments involving the use of cysteine analogues, cysteine depleted medium was used instead of standard medium to make the 'control,' 'wash' and 'digest' medium as described in section 2.3.1. Cysteine depleted medium was prepared using cysteine/methionine depleted D-MEM supplemented with 0.2 mM L-methionine as well as 20 mM hepes, 2 mM $NaHCO_3$, 8.3 mM sodium acetate, 4 mM glutamine, and antibiotic/antimycotic (1 ml/l).

2.3.15.1. Supplementing acini incubation medium with oxothiazolidine carboxylate

In an initial experiment final concentrations of 0.2 and 0.4 mM oxothiazolidine-carboxylic acid (OTCA) were used as treatments for acini incubated for 0, 4 and 8 h before harvest of medium, on 24-well plates in the presence of 3,4,5-^{3}H-L-leucine (0.7 GBq/mol). A similar experiment used 0.4, 0.8 and 1.6 mM OTCA, and two other experiments used 0.8 mM OTCA all with 0.2 GBq/mol 3,4,5-^{3}H-L-leucine.

In another experiment, acivicin (0.5 mM) was added to acini in 24-well plates that had been incubated with OTCA (0.4 mM) and 3,4,5-^{3}H-L-leucine (0.7 GBq/mol) for 4 h. Medium was harvested at 0, 4, 8 and 18 h. Two following experiments used OTCA (0.8 mM) with acivicin (0.58 mM) and medium was harvested at 0, 4 and 8 h. In these experiments, OTCA was added 2 h after the addition of 3,4,5-^{3}H-L-leucine (0.2 GBq/mol) and acivicin.

OTCA (0.8 mM) was also used in combination with BSO (0.5 mM) in the presence and absence of acivicin (0.5 mM). Medium of acini from 24-well plates was harvested at times 0, 4, and 8 h after the addition of 3, 4, 5-^{3}H-L-leucine (0.2 GBq/mol). Acini were treated with OTCA (0.8 mM), BSO (0.5 mM) and acivicin (0.5 mM) in one other experiment where acini were incubated for 0, 2, 4, 8 and 18 h after the addition of 3,4,5-^{3}H-L-leucine (0.2 GBq/mol) before medium was harvested.

2.3.15.2. Supplementing acini incubation medium with N-acetylcysteine

In an initial experiment, acini in 24-well plates were incubated for 0, 2, 4, 8, and 18 h with 0.2 GBq 3,4,5-^{3}H-L-leucine and in the presence of different concentrations of N-acetylcysteine (NAC) (1, 10 and 50 mM) and in the absence or presence of acivicin (0.5 mM). Three following experiments used the same conditions but with 1 mM NAC only, in the presence and absence of 0.5 mM acivicin.

NAC (1 mM) was used twice to treat acini in combination with BSO 0.5 mM for 0, 2, 4 and 8 h prior to medium harvest, on 24-well plates in the presence of 0.2 GBq 3,4,5-^{3}H-L-leucine.

2.3.16. Treatment of data

Results were corrected for background radioactivity by subtracting radioactivity of cycloheximide containing extracts. Each experiment had three background corrected samples and unless one sample was used for a different analysis results have n=3. Results combined from more than one experiment have greater values for n.

Each isolated acini preparation arose from one animal. While intra acini preparation variation was small there was a large amount of variation arising from either individual animals or individual acini preparations. In order to compare results between different

acini preparations secreted protein results from most acini experiments were standardised and have been labelled normalised. A representative control experiment was selected and each time point of the controls and treatments of all other experiments was multiplied by a correction factor to normalise the controls to the same level of protein secretion. This allowed direct comparison of chemical treatments on protein secretion relative to control treatments from several different acini preparations.

Each time point arose from a different plate not a single suspension so were not totally linear and in most cases there were only two time points, therefore, linear regression was not used. Results for experiments with increasing concentrations of acivicin, OTCA or NAC were fitted with trendlines to allow easier comparison between concentrations and further analysis.

The significance of treatment effects was determined using analysis of variance and least significant differences using Genstat 5.

2.4. Results and Discussion

2.4.1. Validation of acinar cell viability and protein secretion

The method used for extracting acini from the mammary gland of the ewe had been used successfully in previous studies (Davis *et al.*, 1993; Wheeler *et al.*, 1993). However, in the present study acinar cell viability and milk protein secretion by acini prepared in this manner was tested to justify use in experiments aimed at investigating the role of the γ-glutamyl cycle in milk protein synthesis. Initial acini incubation experiments were designed to establish whether isolated ovine mammary acini were viable in D-MEM cell culture medium after extraction and to determine the length of time that acini could be held in culture before milk protein secretion began to decline. Acinar cell viability was assessed by measuring cell ATP content and the ability of live cells to exclude trypan blue dye. Also, the function of acini was tested by incubating acini in the presence of ^{3}H-leucine, which was taken up and used in protein synthesis allowing milk protein secretion to be monitored.

2.4.1.1. Validation of acinar cell viability using cell ATP content

Oxygen deprivation leading to loss of viability rapidly reduces the ATP content of cells thus ATP measurement is a very sensitive indicator of cell viability. The reaction to determine ATP content was checked for completion by monitoring the absorbance of a 0.3 mM ATP standard at one min intervals for 15 min (Figure 2.4). The reaction was found to be near completion after 5 min but in subsequent sample analyses, assays were incubated for 15 min after the addition of each enzyme to ensure the reaction had gone to completion before the final absorbance reading was taken.

Initially mammary tissue samples gave lower concentrations of ATP than expected, so liver samples were collected from a sheep (slaughtered for an unrelated trial) to practice the extraction and assay procedures and to check that no ATP was being lost during the reaction. In the present study ATP content of sheep liver (0.66 μmol/g wet weight tissue) was found to be lower than that reported for rat in literature (2.5 μmol/g wet weight tissue; (Bergmeyer, 1974)). This could have resulted from the unavoidable delay in tissue collection rather than loss of ATP during sample preparation as ATP content in tissue begins to decline within seconds of loss of blood flow and oxygenation (personal communication K.E. Kitson).

Mammary tissue was collected from ewes at the same stage of lactation, and stored at -85°C. The ATP content of tissue obtained varied markedly with some results as low as 0.02 μmol/g wet weight tissue. These lower values may result from delays in obtaining samples post mortem. However, mammary samples were not easily homogenised using the modified French cell press, in comparison with liver tissue, and required more vigorous action with the hammer, which extended the time that tissue was exposed to ambient temperatures and may have resulted in ATP loss.

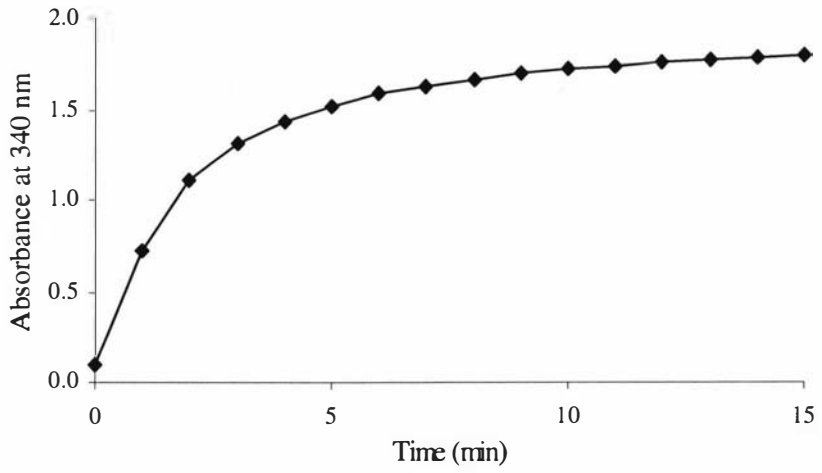


Figure 2.4 ATP assay.

The assay for ATP was followed to completion by monitoring the change in absorbance after hexokinase addition to an assay mixture containing glucose-6-phosphate dehydrogenase, 0.9 mM $NADP^+$ and 0.3 mM ATP.

The highest reading obtained after all samples were assayed was 0.70 ± 2.1 x 10^{-2} µmol/g wet weight tissue. Mammary tissue from an animal that was 100 days pregnant was also assayed and had an ATP content of 0.60 ± 4.1 x 10^{-3} µmol/g tissue. ATP content of mammary tissue in rats has been shown to be 0.23 µmol/g wet weight tissue (Bergmeyer, 1974) so the results for ewe tissue in the present study are realistic.

For one animal, samples were taken throughout the acini preparation procedure (Table 2.2) and compared to ATP concentrations of mammary tissue from the animal soon after slaughter. Unfortunately, samples were only assayed once for ATP while they should have been assayed in triplicate at least. Therefore the results shown in Figure 2.5 can be taken as an indication of ATP content only as there may be considerable variation between samples at each time point. The concentration of ATP for the tissue was found to be lower than expected which may have been because of the delay in obtaining the tissue post mortem. Figure 2.5 suggests a decrease in ATP content of mammary tissue between the time when the first sample was collected after slaughter (0.25 h), and the removal of the first piece of tissue for collagenase treatment 0.5 h after slaughter. This 0.25 h delay was the time taken to transport the excised mammary gland from the post mortem room to the room housing the biohazard hood, to prepare for work in the biohazard hood, and to prepare instruments prior to further work with the tissue.

Having an assistant excise and deliver the udder for collagenase treatment may speed up this step, and snap freezing tissue in $N_{2(l)}$ in the post mortem room may decrease ATP loss.

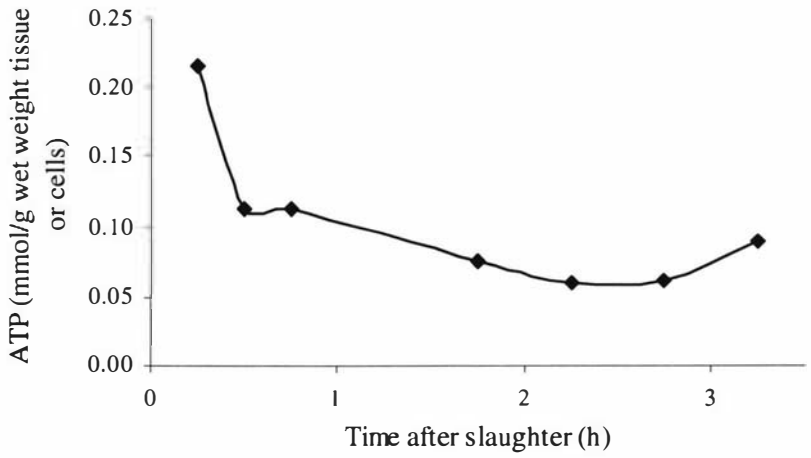


Figure 2.5 Sheep mammary ATP content

ATP content of mammary cells after slaughter (t=0), and during collagen digestion for isolation of mammary acini.

Each piece of mammary tissue took approximately 10 minutes to treat with collagenase, so there was a considerable delay between treatment of the first piece at 0.5 h and the final (sixth) piece at 1.75 h after slaughter of the animal. During this time, the pieces of tissue were kept in an unincubated beaker of control medium and therefore were allowed to cool. This delay is associated with a decrease in the concentration of ATP in the tissue as seen in Figure 2.5.

The digest, over the period 1.75 to 2.75 h, also appeared to cause a decrease in ATP content, but at a slower rate, possibly because tissue was incubated at 37°C. This decrease may have been a function of time rather than of collagenase treatment as collagenase treated tissue at 0.75 h had similar ATP content to tissue before treatment at 0.5 h.

The ATP content of mammary cells appeared to stabilise at 2.75 h and an increase is indicated 3 h after slaughter of the animal when acini had been washed free of collagenase. This suggested that a considerable proportion of acinar cells remain viable

after preparation and was supported by results from trypan blue dye exclusion tests that were carried out before plating (section 2.3.2). This confirmation of cell viability indicated the suitability of acini prepared using this method in subsequent experiments.

It was important to check cell viability at harvest, after experimental treatment and incubation of acini, in order to determine the effects of treatment on cell viability. For this purpose, cell pellets that were obtained at harvest were also assayed for ATP. The spectrophotometer used was known to be sensitive to changes in absorbance of 0.004 units, which enabled a change in NADPH of 0.64 μM to be measured. It was estimated that the ATP in the cell pellet would yield less NADPH than this with the assay method used, but an attempt was made to use this assay to measure ATP in cell pellets harvested from treatment wells of experiments.

There was no measurable change in absorbance in the 3 ml assay for ATP, although the standards revealed that the reactions had gone to completion. The concentration of cell extract in the assay was increased by scaling down the assay to 750 μl in 1 ml cuvettes. Reactions again went to completion but there were still no measurable changes in absorbance for assay mixtures containing cell extracts.

An attempt was made to scale the 3 ml ATP assay down to 300 μl to allow measurement of ATP in cell pellets using a plate-reader. An assay using an ATP standard (1 mM) was first conducted using the 3 ml assay volume and results were compared to those obtained from the 300 μl assay volume. Results showed that the assay was successfully modified for use in a plate-reader as expected; with 1.11 mM found in the 3 ml reaction mixture, compared to 1.14 mM in the 300 μl assay. Following this, cell extracts from two experiments were assayed. However, the results for these showed that the ATP concentrations in cell pellets from experiments were below the threshold for measurement by this method.

Although the ATP assay did not allow viability of plated acinar cells to be accurately measured, assays did show that acinar cells prepared using the method described were viable and could be used in further experiments. As a limited number of suitable

lactating sheep were available for obtaining mammary tissue samples, acini experiments had to continue without further attempts to measure ATP in cells.

2.4.1.2. Validation of acinar cell viability by trypan blue dye exclusion

The trypan blue dye exclusion method was used to give an indication of cell viability in the collagenase prepared acini suspensions (Figure 2.6). Experiments proceeded only if results from these tests showed that cells were at least 75% viable and often they were considered 80-85% viable. Production of milk protein and lactose provided additional confirmation of the viability of functional acini.

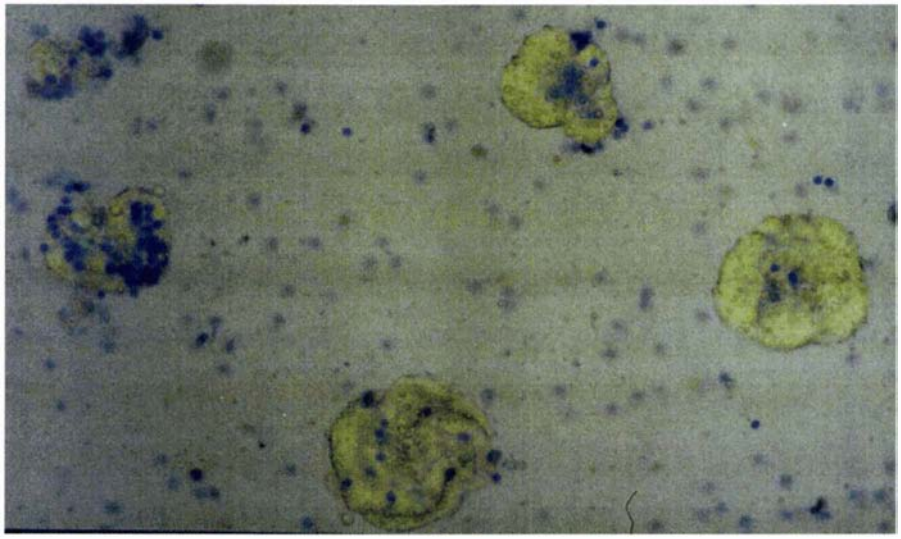


Figure 2.6 Isolated ovine mammary acini stained with trypan blue dye.

Mammary acini (secretory units), shown at approximately 100 x magnification, result from collagenase digestion of lactating sheep udder tissue. Acini were treated with trypan blue, which is excluded by live cells allowing cell viability to be assessed. Viability of acinar cells in this figure was considered to be approximately 80%.

Trypan blue was also used to stain incubated acini in wells and this showed that acini associated either with the lower surface of the plate or floated in the medium. Single cells floated in the medium and were not viable. Trypan blue staining of cells incubated for 8 h indicated that 80% of cells were viable, supporting the use of isolated ovine mammary acini in 8 h experiments. Viability of cells just before harvest was also assessed using trypan blue to determine the effect of treatment on viability. The results of these tests will be discussed along with results for treatments in following sections.

2.4.1.3. Validation of milk protein secretion by isolated ovine mammary acini

Preliminary experiments were conducted to justify the use of isolated ovine mammary acini in the study of milk protein secretion. ^{3}H-leucine was added to medium for uptake by acinar cells and incorporation into newly synthesised milk protein. Protein in the medium of plated acini was pelleted by strong acid (7.5% (w/v) TCA) and washed several times to remove unincorporated label. The remaining radioactivity was considered to represent ^{3}H-leucine in secreted protein.

Acini in the initial experiment were incubated for 5 h and showed an increase in secreted protein. In the second acini experiment (Figure 2.7), there was an initial lag period of 2 hr, then a constant increase in secreted protein. A significant amount of newly synthesised milk protein has been shown not to be secreted by isolated rat mammary acini within the first 3 h of incubation (Geursen *et al.*, 1987) and this may account for the lag in secretion of milk protein observed in the present study. Acini were incubated for 8 h without a decrease in the rate of protein secretion.

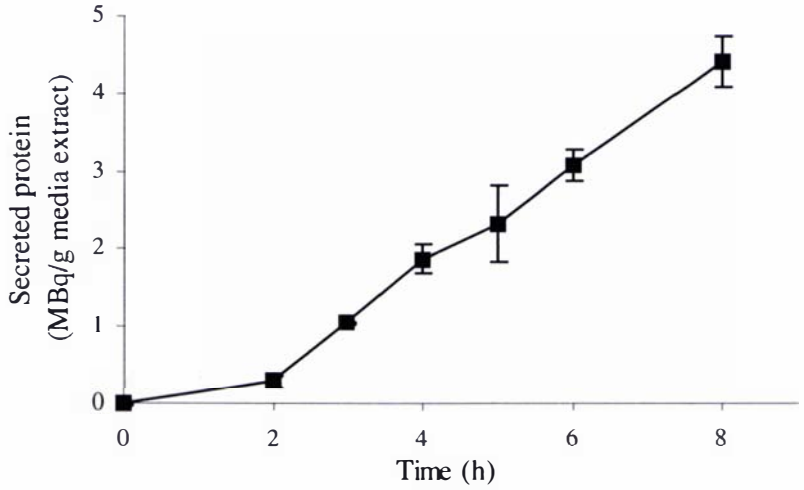


Figure 2.7 Protein secretion by isolated ovine mammary acini.

Incorporation of ^{3}H-leucine into protein allowed secretion by lactating acini to be monitored. Acini were incubated on 24-well plates. Error bars represent standard error of the mean (n=3).

Acini were incubated at different densities and in the absence and presence of Matrigel®. Milk protein secretion increased as plating density decreased but the improvement was not sufficient to outweigh the financial expense in using larger

amounts of Matrigel® to coat plates (Appendix B). Milk protein secretion was slightly higher in the absence than in the presence of Matrigel® after 18 h but Matrigel® use was continued to create an environment closer to that *in vivo* (Appendix C).

In a following experiment, acini were incubated for 8 h and the medium extract was separated by HPLC to determine whether the protein was secreted milk protein or microbial protein (Figure 2.8). The caseins and whey proteins of the medium extract were separated prior to further fractionation of individual proteins by separation by HPLC.

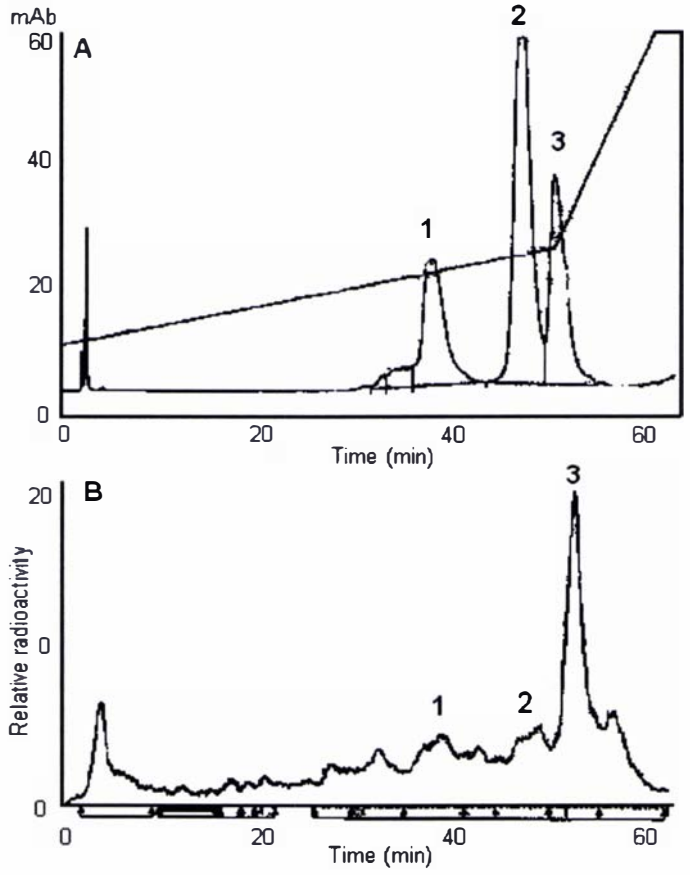


Figure 2.8 Scanned HPLC chromatographs of medium extract from acini incubated for 8 h.

Relative absorbance at 280 nm in (A) shows separated caseins from unlabelled ovine skim milk added to the sample during preparation. (B) shows separated radiolabelled (^{3}H-leucine) caseins from medium of isolated ovine mammary acini, detected by an online scintillation counter. Bovine protein standards indicated that peak 1 was κ-casein, peak 2 was α-casein, and peak 3 was β-casein.

The UV chromatographs of bovine casein standards were used to identify peaks in the casein fraction of medium extracts as κ- α- and β-caseins, and radioactively labelled peaks from the online scintillation counter (β-RAM) output were found to correspond to these peaks. The major protein present in the casein fraction of the acini medium was β-casein, with lower concentrations of α-casein and κ-casein present. In contrast to this result, α-caseins have been shown to be the major caseins of ovine milk (Anifantakis, 1986).

Separation of the medium whey protein fraction showed a large peak with the same retention time as the bovine β-lactoglobulin standard. β-lactoglobulin and β-casein have similar retention times by this method of separation, and although ovine milk has been reported to be richer in β-lactoglobulin than bovine milk (Anifantakis, 1986), the size of the peak suggested incomplete separation of caseins from whey proteins. The large peak in the whey protein fraction was probably β-casein. The pH of a mock acidified medium sample was found to be 4.6, which is correct for precipitation of caseins at room temperature (McKenzie, 1971). In the following experiments, caseins and whey proteins were not separated before undergoing HPLC separation. Instead the HPLC analysis resulted in complete separation of individual caseins and wheys.

The identification of individual caseins in medium extract from isolated ovine mammary acini in this study was strong evidence that the radioactivity from total count analysis of medium was the result of ^{3}H-leucine incorporation into milk protein. The increase in secreted milk protein in the medium indicated that acinar cells obtained from the mammary gland of ewes could be kept viable in incubated medium for at least 8 h, and could be used in experiments investigating milk protein synthesis.

2.4.2. Characterising the role of the γ-glutamyl cycle in milk protein synthesis

Isolated ovine mammary acini were found to remain viable in culture and secrete increasing α-, β-, and κ-caseins for at least 8 h. These acini were therefore considered to be a useful tool for studying milk protein synthesis *in vitro*, and a model for *in vivo* synthesis in the dairy cow. The following sections describe experiments where isolated ovine mammary acini were used to investigate the role of the γ-glutamyl cycle (Figure

2.9) in milk protein synthesis. Acini were treated with chemicals that have previously been shown to affect specific parts of the γ-glutamyl cycle and evidence to support this is discussed.

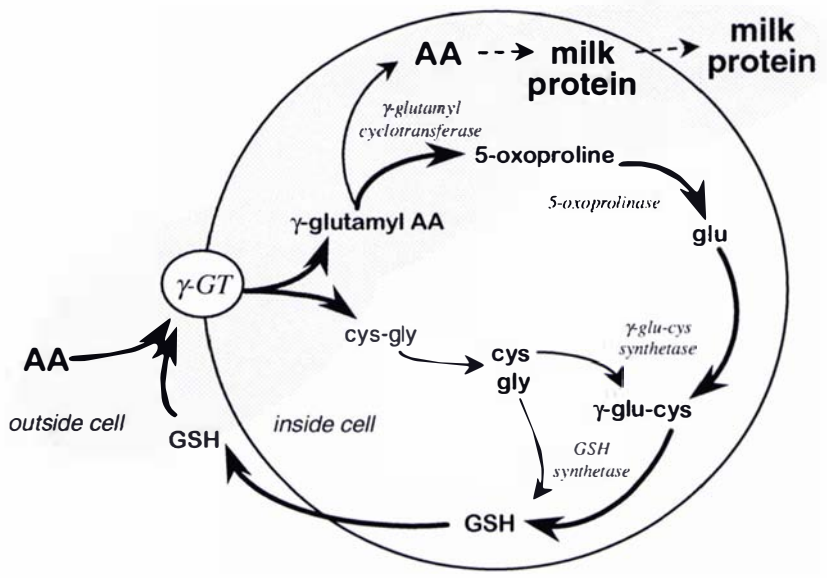


Figure 2.9 The proposed role of the γ-glutamyl cycle in milk protein synthesis.

Abbreviations: AA, amino acid; γ-GT, γ-glutamyl transpeptidase; GSH, glutathione.

The enzyme γ-GT has been proposed to act in amino acid transport (Cotgreave and Schuppe-Koistinen, 1994; Sweiry *et al.*, 1995) and was targeted for inhibition to investigate this role in milk protein synthesis. The enzyme γ-glutamylcysteinyl synthetase was targeted for inhibition to determine the effect of inhibition of GSH synthesis (Griffith and Meister, 1979a) on milk protein synthesis. The γ-glutamyl cycle intermediate 5-oxoproline (Griffith *et al.*, 1976) has been shown to up regulate amino acid transport (Viña *et al.*, 1989) and this was investigated in isolated ovine mammary acini to determine whether the γ-glutamyl cycle affects milk protein synthesis indirectly through 5-oxoproline. The amino acid that γ-GT has the highest affinity for is cystine (Thompson and Meister, 1977), which is easily converted inside the cell to cysteine, the limiting amino acid for GSH synthesis (Anderson and Meister, 1987). Investigation of the role of the γ-glutamyl cycle in supplying cyst(e)ine for milk protein synthesis and GSH synthesis involved the use of alternative pathways of cysteine supply. Chemical

treatments were often combined to test the roles of several reactions of the cycle in milk protein synthesis at once.

2.4.2.1. γ-GT activity in sheep mammary tissue

Mammary tissue was assayed for γ-GT activity for comparison with cell γ-GT levels (section 2.4.2.2).

A portion of γ-GT activity in mammary tissue is activity associated with milk membranes but correction for milk content only increases γ-GT activity slightly (Baumrucker and Pocius, 1978). Values from this experiment have not been corrected for milk content to allow comparison with several other reports where activity has also not been corrected (Puente *et al.*, 1979; Pocius *et al.*, 1980; Baumrucker *et al.*, 1981).

The results for total and specific activity from this experiment were approximately 3-fold higher than for mammary tissue from both lactating cows and rats (Table 2.3). This may be due to the treatment of samples in the present study with triton to release γ-GT from the cell membrane, which was not used in the methods described by Baumrucker and Pocius (1978), Puente *et al.* (1979) or Pocius *et al.* (1980).

Tissue was also collected from sheep that were slaughtered for other unconnected studies, which were at several stages of pregnancy and lactation. This enabled the characterisation of γ-GT activity over part of the lactogenic cycle for comparison with that previously described for rats (Figure 1.8). The present analysis shows that γ-GT activity in sheep mammary tissue increases slowly during pregnancy, then rapidly at parturition. As seen in rats, γ-GT activity reaches a plateau during lactation. γ-GT activity in mammary tissue has been shown to decrease during involution of the gland (Baumrucker and Pocius, 1978; Puente *et al.*, 1979; Pocius *et al.*, 1980). Tissue was not obtained from sheep with weaning lambs in the present study, so the decrease in γ-GT back to pre-pregnancy levels cannot be shown here.

Table 2.3 γ-GT activities in total homogenates of lactating sheep, cow and rat mammary tissues.

Animal	Stage of lactation	Total activity[a] (μmol/min/g wet wt)	Specific activity (μmol/min/mg protein)	n	Reference
Sheep	2-3 weeks	26 ± 2.4	0.49 ± 0.05	7	**Present study**
Sheep	6 weeks	26 ± 1.2	0.54 ± 0.02	11	**Present study**
Cow	11.4 kg milk/day	8.4 ± 1.4	0.16 ± 0.23	5	(Baumrucker and Pocius, 1978)
Cow	12 kg milk/day		0.17 ± 0.04	3	(Baumrucker *et al.*, 1981)
Rat	10-20 days	7.9 ± 1.1	0.09 ± 0.01	5-7	(Baumrucker and Pocius, 1978)
Rat	10-20 days		0.18	5	(Puente *et al.*, 1979)
Rat	10-20 days		0.18		(Pocius *et al.*, 1980)
Rat	10-20 days	13.1		3	(Baumrucker, 1985)

[a] total activity per g wet weight of fresh tissue.

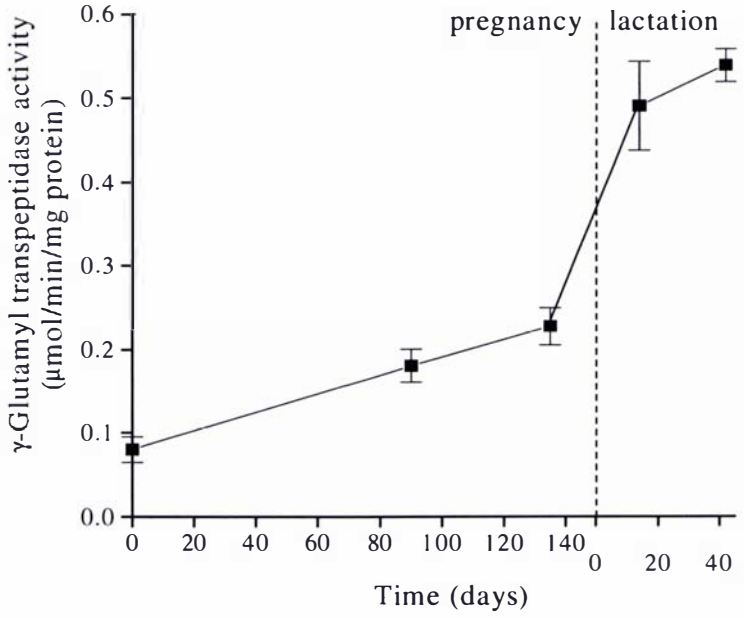


Figure 2.10 γ-Glutamyl transpeptidase activity in pregnant and lactating sheep mammary tissue.

Error bars indicate standard error of the means (day 0 n=4; pregnant day 90 n=2, day 135 n=6; lactating day 14 n=7, day 42 n=11).

The increase in γ-GT activity during pregnancy has been suggested as an anticipatory adaptation in preparation for the huge demand for amino acids with the onset of lactation (Puente *et al.*, 1979), and is considered similar to the changes in other enzymes involved in milk protein synthesis (Pocius *et al.*, 1980). Studies have shown that γ-GT activity is regulated directly and/or indirectly by the lactogenic hormones oestrogen, progesterone and prolactin. β-oestradiol and progesterone administration to ovariectomized rats increased γ-GT activities (Puente *et al.*, 1979) while administration of bromocryptine which blocks prolactin release from the anterior pituitary, prevented increased γ-GT activity in pregnant rats. Prolactin administered in combination with bromocryptine, allowed γ-GT to increase (Pocius *et al.*, 1980). Puente *et al.* (1979) suggest oestrogen and progesterone increase γ-GT activity by proliferation of secretory epithelium, although oestrogen stimulation of pituitary causing prolactin release may directly affect γ-GT activity (Pocius *et al.*, 1980).

The increase in γ-GT activity in the mammary gland that occurs with pregnancy and the onset of lactation coincides with a decrease in γ-GT activity in the liver (Puente *et al.*, 1979). Although liver γ-GT activity is low, this may suggest that the uptake of amino acids by the liver is down regulated to increase availability to the mammary gland.

2.4.2.2. Inactivation of γ-GT by acivicin

It is widely accepted that the enzyme γ-GT forms γ-glutamyl amino acids by transferring the γ-glutamyl moiety from GSH to an acceptor amino acid (Morita *et al.*, 1994; Hanigan, 1998; Wolff *et al.*, 1998). The chemical acivicin was used to treat isolated lactating acini in order to define the role of γ-GT in milk protein production (Figure 2.11). Acivicin has been well documented as an inhibitor of γ-GT in isolated enzyme studies (Stole *et al.*, 1994; Smith *et al.*, 1995), in cell culture studies (Rosenfeld and Roberts, 1981; Meredith and Williams, 1986; Cotgreave and Schuppe-Koistinen, 1994; Sweiry *et al.*, 1995), and in whole animal (Poster *et al.*, 1981; McGovren *et al.*, 1988; Araya *et al.*, 1989; Viña *et al.*, 1990) and human studies (McGovren *et al.*, 1985; Eisenhauer *et al.*, 1987).

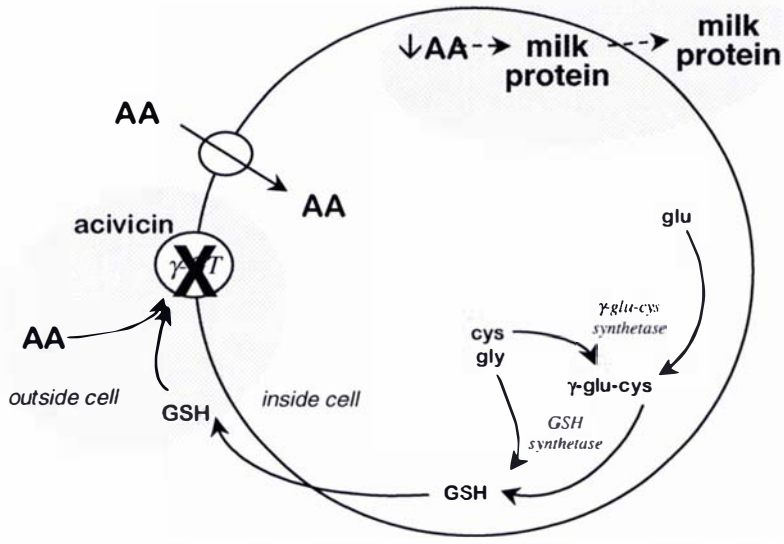


Figure 2.11 The effect of γ-GT inhibition on milk protein production.

Inactivation of γ-GT by acivicin may decrease amino acid (AA) supply for milk protein synthesis and glutathione (GSH) synthesis but has no effect on AA uptake by conventional transport systems.

In the present study, two initial experiments using isolated ovine mammary acini (Figure 2.12) involved adding acivicin (0.5 mM) to culture medium. This caused a decrease ($P<0.05$) in radiolabelled secreted protein in the medium of approximately 70% after 8 h and 80% after 18 h of incubation. In one experiment, acivicin decreased intracellular protein counts by 30% after 2 h incubation and 7% after 8 h incubation suggesting that acivicin inhibits protein synthesis and not just secretion by isolated mammary acini. The results of seven subsequent experiments showed that on average acivicin (0.5 mM) decreased milk protein secretion ($P<0.05$) by 74% at 8 h and 64% at 18 h (Figure 2.13).

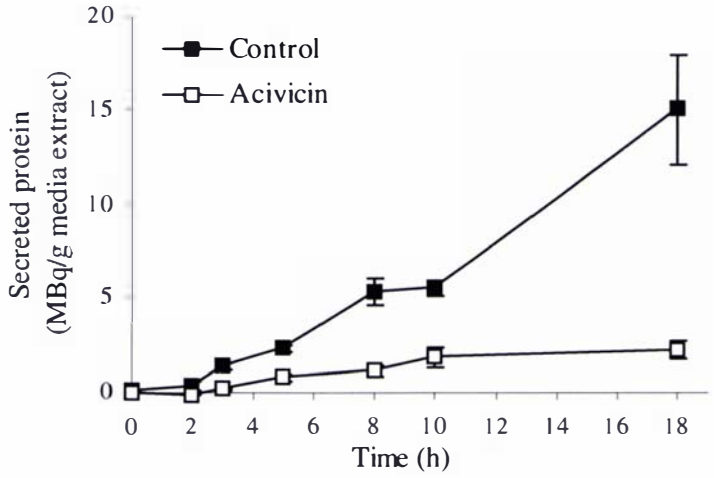


Figure 2.12 Acivicin inhibition of protein synthesis.

Radiolabelled (^{3}H-leucine) protein in the medium from two experiments where isolated ovine mammary acini were incubated in the absence or presence of acivicin (0.5 mM). Error bars represent standard error of the mean (n=3 for t_3 and t_{10}; n=4 for other points)

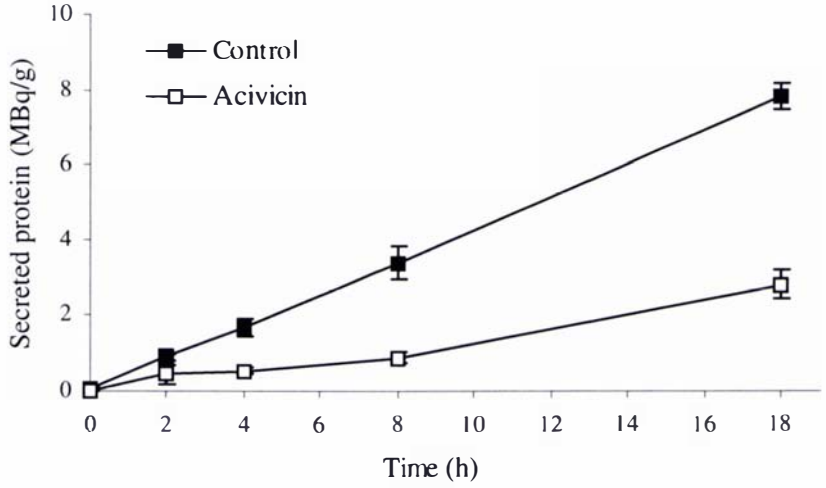


Figure 2.13 Acivicin inhibition of protein synthesis in seven experiments.

Combined results from seven experiments showing radiolabelled (^{3}H-leucine) protein secreted from isolated ovine mammary acini incubated for up to 18 h in the absence and presence of acivicin (0.5 mM). Results have been normalised as described in the text (section 2.3.16). Error bars represent standard error of the means (t_0 control n=19, acivicin n=13; t_2 control n=16, acivicin n=13; t_4 n=15; t_8 n=19; t_{18} n=16).

In a human pancreatic cell line (PaTu 8902), 0.5 mM acivicin was shown to decrease γ-GT activity by 60% (Sweiry *et al.*, 1995). A similar method to that used by Sweiry *et al.* (1995) was not sensitive enough to obtain γ-GT activities from incubated acinar cells in these experiments. However, acini remaining from two later experiments, where acini had been treated with 0.7 mM acivicin and washed, were analysed for γ-GT activity. Assays for γ-GT activity in these suspensions showed acinar cells retained on average 28% of γ-GT activity after treatment with acivicin (0.7 mM) (Table 2.4). This is consistent with the decrease in protein secretion of acini incubated with acivicin (0.5 mM) giving strong support for the suggestion that γ-GT inhibition is responsible for the decrease in secretion of radiolabelled milk protein.

Specific γ-GT activity reported in isolated bovine acini was much lower than that found in the present study (cf. 0.182 ± 0.031 μmol/min/mg protein (Baumrucker *et al.*, 1981)). The preparation of cells for assay of γ-GT activity was modified from that described by Baumrucker and Pocius (1978) for tissue. The addition of triton to sample extracts in

this experiment probably increased the effectiveness of γ-GT extraction, as assay results were low and variable without triton.

Table 2.4 γ-Glutamyl transpeptidase activity of isolated ovine mammary acini.

Acini were untreated or washed with acivicin (0.7 mM) (total activity experiment 1 n=3; experiment 2 control n=1, acivicin n=3; specific activity n=2).

	Experiment 1		Experiment 2	
	Control	Acivicin	Control	Acivicin
Total activity[a] (μmol/min/g wet weight)	927	188	1532	334
% of control		20.2		21.8
Specific activity (μmol/min/mg protein)	38.9	7.6	38.3	13.8
% of control		19.6		36.1

[a] total activity per g wet weight of fresh tissue.

Total and specific activities in tissue (2.4.2.1) were much lower than those for acini suspensions but this can be explained by the removal of milk, fat and collagen that occurs during the preparation of isolated acini. For acini suspensions, the total activity becomes rate per g of cells instead of per g of tissue and specific activity becomes rate per g of slightly purified protein.

Acini from three preparations were incubated in the presence of different concentrations of acivicin to determine whether acivicin action on milk protein secretion could be related to published data on the inhibition of γ-GT by acivicin. Medium extract radioactivity continued to increase over time even with the highest doses of acivicin indicating that acivicin is not toxic to acini and that milk protein synthesis can continue with γ-GT inhibition (Figure 2.14). Increases in secreted protein were fitted with trendlines to give rates that decreased as the concentration of acivicin added to the plates increased.

The dependence of protein secretion on acivicin dose can be seen clearly in Figure 2.15. Assuming that decreased milk protein secretion is a direct response to γ-GT inhibition by acivicin, the constant for 50% inhibition (IC_{50}) under these conditions has been estimated (from Figure 2.15) to be 3.9 μM for 4 h and 2.8 μM for 8 h incubation. The difference in IC_{50} for the different incubation times may indicate that acivicin does not come into immediate contact with all γ-GT molecules and that during the incubation period acini are penetrated by acivicin thus increasing inhibition and decreasing the dose required for 50% inhibition. This change in inhibition was not sufficient to cause an observed change in the rate of milk protein secretion when the effect of only one dose was studied (Figure 2.13), and was therefore averaged to give an estimated IC_{50} of 3.4 μM.

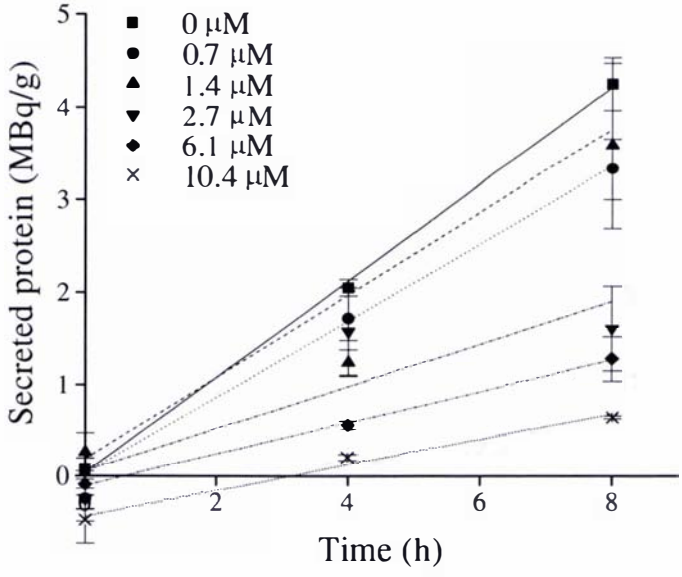


Figure 2.14 Protein synthesis with different concentrations of acivicin.

Radiolabelled (^{3}H-leucine) protein from the medium of three experiments where isolated ovine mammary acini were incubated in the presence of different concentrations of acivicin. Results have been normalised as described in the text (section 2.3.16). Error bars indicate standard error of the means (0 μM n=7; 0.7 μM n=3; 1.4 μM n=7; 2.7 μM n=4; 6.1 μM n=4; 10.4 μM n=3). Data have been fitted using linear regression.

The IC_{50} estimated for isolated ovine mammary acini under the conditions used in this study is much lower than the IC_{50} estimated for PaTu 8902 cells (0.3 mM) from γ-GT activity assays in the presence of acivicin (Sweiry *et al.*, 1995). As acivicin binds γ-GT in equimolar amounts (Smith *et al.*, 1995), this suggests that PaTu 8902 cells may contain more γ-GT molecules than mammary cells. However, tissue from lactating bovine mammary tissue has been shown to have greater γ-GT activity than pancreas (Baumrucker and Pocius, 1978). It is likely that acivicin is affecting factors other than γ-GT activity causing a decrease in milk protein secretion. Cystine transport in PaTu 8902 cells was found to decrease by 30% in the presence of approximately 440 μM acivicin, but the concentration of acivicin required to inhibit by 30% was much greater (approximately 1.3 mM) (Sweiry *et al.*, 1995). This difference in response to acivicin was not discussed by the authors of that report, but does suggest that decreased γ-GT activity is not the sole cause of reduced cystine uptake in PaTu 8902 cells, and that acivicin may affect some other mechanism of cystine uptake or may negatively affect cell function. Therefore, decreased milk protein secretion in isolated ovine mammary acini as a result of acivicin administration may be due to other factors as well as γ-GT inhibition and the IC_{50} estimated here must be accepted as a constant for milk protein secretion inhibition rather than specifically for inhibition of γ-GT activity.

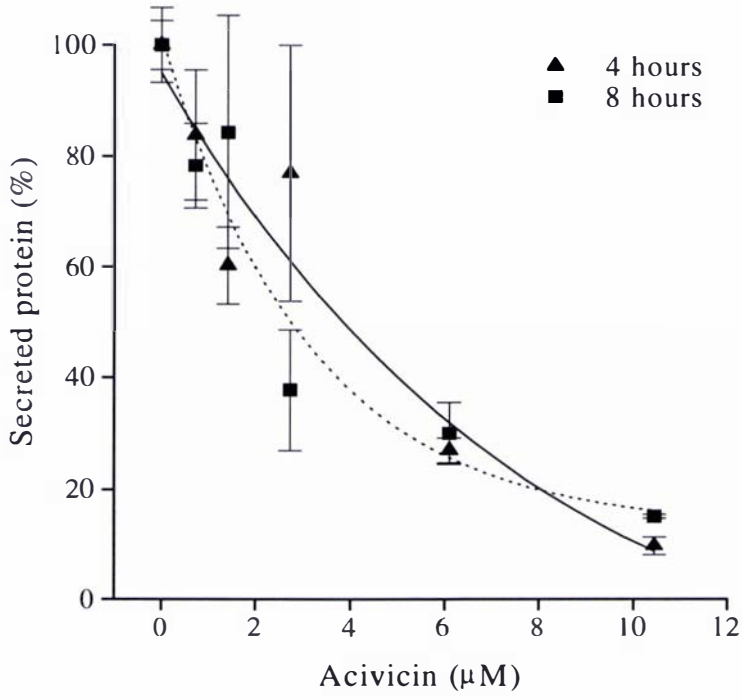


Figure 2.15 Inhibition of protein synthesis by different acivicin concentrations.

Concentration dependent acivicin inhibition of radiolabelled (^{3}H-leucine) protein in medium extract of isolated ovine mammary acini as a percentage of control. Error bars indicate standard error of the means (0 μM n=7; 0.7 μM n=3; 1.4 μM n=7; 2.7 μM n=4; 6.1 μM n=4; 10.4 μM n=3). Data have been fitted to first order exponential decay curves giving estimated IC_{50} (constant at 50% inhibition) of t_4 (—) 3.9 μM and t_8 (...) 2.8 μM.

It was hypothesised from the study of the literature (Thompson and Meister, 1977; Cotgreave and Schuppe-Koistinen, 1994; Sweiry *et al.*, 1995) that γ-GT was supplying cyst(e)ine for milk protein synthesis. Proteins in medium extract of acini of the present study were separated to determine the effect of γ-GT inactivation on milk proteins that do and do not contain cysteine. Like the equivalent bovine proteins (Table 1.3), ovine κ_A- and κ_B-casein have been shown to contain one to two residues of cysteine, β-lactoglobulin A and B contains five cysteine residues, and α-lactalbumin contains at least eight (Table 2.1). Unlike bovine α_{s2}-casein, ovine α_{s2}-casein does not contain cysteine.

In two experiments (Figure 2.16) where acini were incubated in the absence and presence of acivicin, medium extracts were separated by HPLC without prior separation of caseins from whey proteins (cf. section 2.4.1.3). Although this method allowed casein and whey proteins to be separated it did not give good resolution of the whey protein peaks. Therefore, only casein production can be reported, but there is greater certainty that all casein proteins have been accounted for unlike previous analyses (Appendix D).

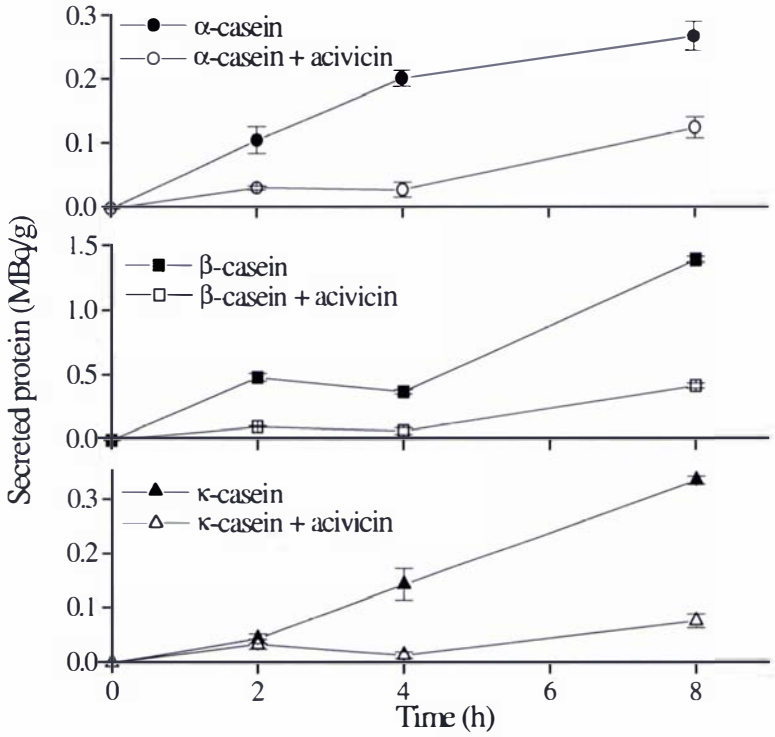


Figure 2.16 Acivicin inhibition of casein synthesis

Radiolabelled (^{3}H-leucine) casein secreted into the medium from two experiments where isolated ovine mammary acini were incubated for 8 h in the absence and presence of acivicin (0.5 mM). Standard error bars indicate standard error of the means ($t_{0,4,8}$ n=6; t_2 n=3).

β-casein was found to be the predominant protein produced by isolated ovine mammary acini in the present study (Figure 2.16). In the presence of acivicin, α-casein was reduced ($P<0.05$) to 30% of control values at 2 h, 13 % at 4 h and 40% of control values at 8 h. β-casein decreased ($P<0.05$) to 20 % of control values at 2 h, to 16 % at 4 h and to 31% at 8 h when acivicin was present. At 2 h κ-casein in acivicin treated wells was

not different from control wells but decreased ($P<0.05$) to 10% and 21% of control values at 4 h and 8 h respectively. The inactivation of γ-GT impaired secretion of all caseins without specificity for κ-casein, which contains cysteine.

These results indicate that while γ-GT may be supplying cyst(e)ine for milk protein synthesis, it may also supply other amino acids. This may be through direct uptake by γ-GT or indirectly through the γ-glutamyl cycle by the up-regulation of other amino acids by conventional amino acid transport systems. The γ-glutamyl cycle intermediate, 5-oxoproline has been shown to up regulate amino acid transport systems (Lee *et al.*, 1996b).

2.4.2.3. Characterisation of the effect of acivicin on isolated ovine mammary acini

Although acivicin is well accepted as an inhibitor of γ-GT, it also has actions on other enzymes and may affect cells in other ways. Acivicin has been shown to have high affinity for bacterial and mammalian glutamine amidotransferases and has inhibited growth in *Serratia marcescens* (Tso *et al.*, 1980). Although γ-GT homozygous mutations are non-lethal in mice, the treatment of rat embryos in culture with acivicin showed embryotoxicity. Attempted inactivation of γ-GT by acivicin and anti-γ-GT antibody gave different results indicating that the effects of acivicin are through mechanisms other than γ-GT inactivation alone (Stark *et al.*, 1987). Inactivation of γ-GT using acivicin has been shown to reduce GSH export from the cell, but this may indicate acivicin action on a GSH transport system in addition to γ-GT (Dethmers and Meister, 1981).

The following sections report experiments designed to define the action of acivicin on isolated ovine mammary acini in an attempt to further justify its use in γ-GT inactivation in the study of milk protein synthesis.

2.4.2.3.1. Lactose production of isolated ovine mammary acini in the presence of acivicin

Isolated ovine mammary acini, prepared in the same way as in the present study, have been shown to secrete lactose into medium. In a study by Davis and co-workers (1993) lactose concentration in the medium was shown to decrease from 3 fmol/cell/h in the first 6 h after plating, to 2 fmol/cell/h at 6-12 h, then 1 fmol/cell/h at 12-24 h (Davis *et al.*, 1993). Lactose production in isolated ovine mammary acini from two experiments in the present study was 10-fold higher than that reported by Davis *et al.* (1993), and remained constant at 27 fmol/cell/h, 8 h after plating (Figure 2.17).

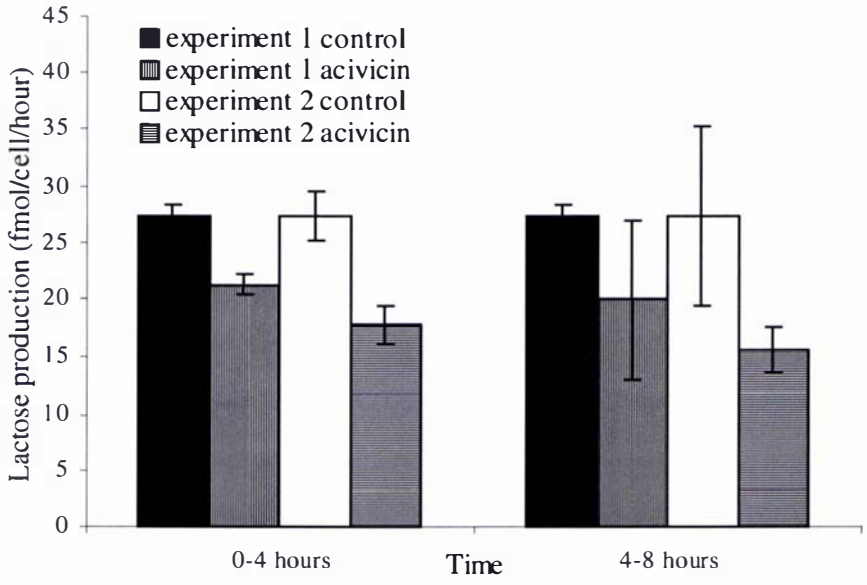


Figure 2.17 Lactose production in the presence of acivicin

Isolated ovine mammary acini from two experiments were incubated in the absence and presence of acivicin (0.5 mM). Results have been normalised so the controls are the same for each acini preparation. Error bars indicate standard error of the mean (n=6).

In both experiments, lactose production was lower ($P<0.05$) in the 0-4 h period for acini treated with acivicin (0.5 mM) (78% and 65% of control values respectively) (Figure 2.17). Lactose production for acivicin treated acini was not different ($P<0.05$) from controls in either experiment during 4-8 h of incubation.

The action of acivicin on lactose production may not be through adverse effects on cell viability but may be indirect via a decrease in α-lactalbumin synthesis. Together with galactosyl transferase, the whey protein α-lactalbumin forms lactose synthase, the enzyme which catalyses lactose production (Stryer, 1988). The appearance of α-lactalbumin in medium of acini treated with acivicin was decreased to 13% of control values at 2 h and 26% of controls at 8 h (Figure 5.4). This low synthesis of α-lactalbumin may cause lactose concentrations to be low in medium of acivicin treated acini. Thus, negative effects of acivicin on normal cellular processes cannot be confirmed or disproved by the study of lactose production in this study.

The fact that isolated ovine mammary acini successfully produce lactose at a constant rate over a time span of at least 8 h is further confirmation of the quality of the acini preparations achieved in this study.

2.4.2.3.2. The use of labelled amino-isobutyric acid in characterising the action of acivicin

α-amino-isobutyric acid (AIB) has been shown previously to be transported by the A system in the blood brain barrier (Lee *et al.*, 1996b). An experiment was designed to test whether acivicin affects transporters other than γ-GT by incubating acini for short periods with radiolabelled AIB in the absence and presence of acivicin. Intracellular radioactivity was high at 0 h indicating that uptake of ^{3}H-AIB is occurring very early in the experiment and suggesting that this experiment should have been run over a shorter time scale. Results from two experiments showed that acivicin does not decrease uptake of AIB. Acivicin actually increased the uptake of AIB after 10 min in comparison with 0 min ($P<0.05$) but was not significantly different from the control treatment at 10 min (Figure 2.18). This suggests that γ-GT inhibition may up-regulate sub-saturated amino acid transport systems. This indicates that decreased protein secretion by isolated acini results from the specific action of acivicin on γ-GT activity rather than a generalised negative effect on other cell functions.

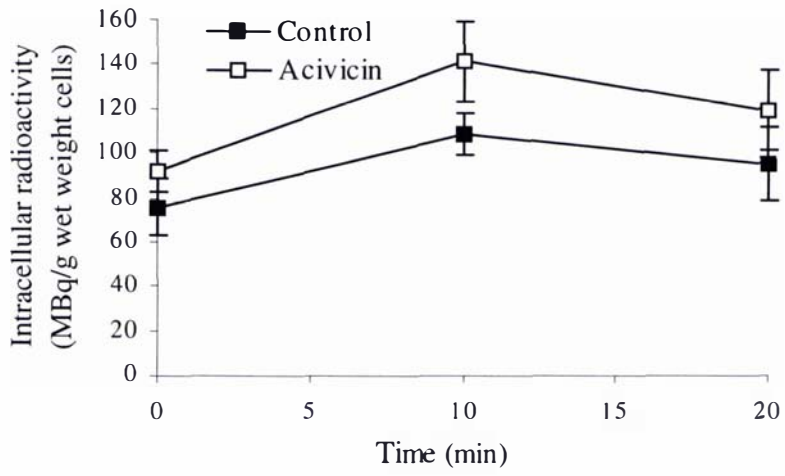


Figure 2.18 Accumulation of cellular ^{3}H-α-amino-isobutyric acid.

Combined results from two experiments where intracellular accumulation of ^{3}H-α-amino-isobutyric acid was determined in acini incubated in the absence and presence of acivicin (6 μM). Error bars represent standard error of the means (n=6).

2.4.2.3.3. Attempt to reverse acivicin inhibition of γ-GT using hydroxylamine

Studies using isolated enzyme have shown that γ-GT acts on acivicin but much more slowly than its normal substrate glutathione. Treatment of inhibited enzyme with the acivicin product, hydroxylamine, has been shown to release acivicin and restore γ-GT activity (Stole *et al.*, 1994).

In an experiment designed to help clarify whether γ-GT inhibition was responsible for reduced milk protein secretion, isolated acini were treated with acivicin (6 μM) and hydroxylamine (7 mM). The concentration of hydroxylamine in the present study was double that used by Stole *et al.* (1994) where the ratio of acivicin to hydroxylamine was 1 : 540. Trypan blue staining of acini gave a subjective indication that cells were 90% viable at 0 h and 4 h. At 8 h control and acivicin treated cells were 80-90% viable while hydroxylamine treated cells were 60% viable and cells incubated in the presence of acivicin and hydroxylamine were 80% viable indicating that hydroxylamine alone has some effect on cell viability. At 18 h cells incubated with hydroxylamine alone or in addition to acivicin were all dead while control and acivicin treated cells were 80% viable.

Secreted protein results for these experiments have not been normalised (as described in section 2.3.16). Control levels of radioactivity in medium extract increased as in other experiments but in the presence of hydroxylamine, radioactivity did not increase indicating that protein synthesis had been shut down (Figure 2.19). Radioactivity of medium extract from acini incubated in the presence of acivicin did not increase further after hydroxylamine was added. The results suggest that hydroxylamine was toxic to cells at the concentration used.

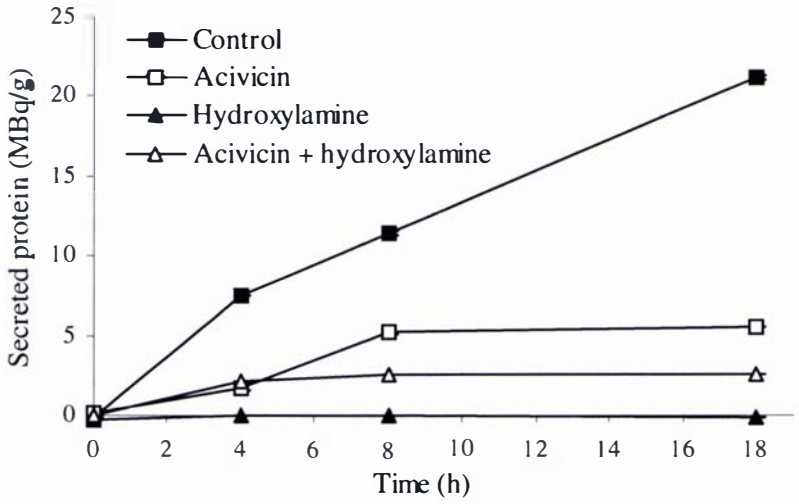


Figure 2.19 Acivicin inhibition of protein synthesis in the presence of 70 mM hydroxylamine.

Radioactivity of protein secreted from acini incubated in the absence and presence of acivicin (6 μM) and/or hydroxylamine (70 mM). Error bars represent standard error of the means (n=3).

In a second experiment hydroxylamine was diluted and added to wells to obtain a final concentration 2-fold higher than acivicin. Trypan blue staining of all incubated acini showed that cell viability remained at 80% from 0 h to 8 h while viability of hydroxylamine treated cells decreased to 70% at 18 h while control and acivicin treated cells remained at 80% indicating that hydroxylamine was not as toxic at this concentration compared to the concentration used in the previous experiment. Radioactivity in the medium extract of acini incubated with hydroxylamine was not as high as control levels and in combination with acivicin was the same level as acivicin alone (Figure 2.20).

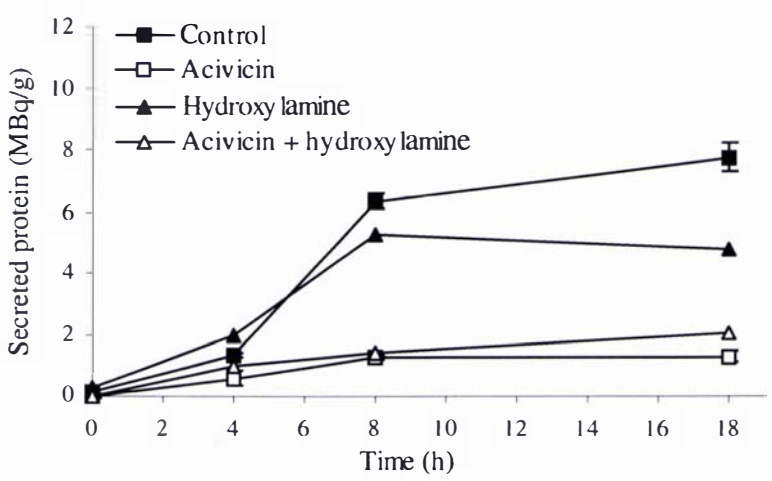


Figure 2.20 Acivicin inhibition of protein synthesis in the presence of 13 μM hydroxylamine.

Radioactivity of protein secreted from acini incubated in the absence and presence of acivicin (6 μM) and/or hydroxylamine (13 μM). Error bars represent standard error of the means (n=3).

The results of the second experiment indicate that the hydroxylamine may have been too dilute to cause a response. However, using higher concentrations of hydroxylamine resulted in cell death.

2.4.2.3.4. Attempt to reverse γ-GT inhibition by centrifugation and washing

An alternative experiment to determine whether protein secretion could be restored after acivicin treatment was designed and carried out twice. An aliquot of acini prepared for plating was treated with acivicin (0.7 mM) then washed to remove unbound acivicin by successive centrifugation and resuspension in fresh medium before being plated as normal. Acivicin treated and washed acini were incubated along side untreated acini from the same preparation in the absence and presence of acivicin (0.5 mM) for comparison. Medium radioactivity for acini plated after acivicin was removed, was lower than control values (29% of control levels at 6 h and 24% of control levels at 8 h) and lower ($P<0.05$) than that for acini incubated with acivicin (approximately 35% of control throughout incubation) (Figure 2.21). The difference in radioactivity between acivicin treated and washed acini and acivicin incubated acini probably arises from additional washing of the acini preparation. In hindsight it would have been useful to subject a control group of acini to the same extra washing procedure to compare with

acivicin treated and washed acini. Viability of cells in the acini preparation, as assessed by trypan blue dye exclusion, had reduced to 70% after washing with acivicin. Viability of cells was not assessed at each harvest in this experiment but this may have indicated decreased cell viability which would explain why radioactivity of acivicin treated and washed acini does not follow the same pattern of increase as that seen in acivicin incubated acini over 18 h. However, the results are consistent with the proposal that acivicin inhibits γ-GT by binding as a poor substrate that is only very slowly released from the enzyme, so that even at early time intervals where the cell viability should have been reasonably good, there was no restoration of protein synthesis when acini were washed.

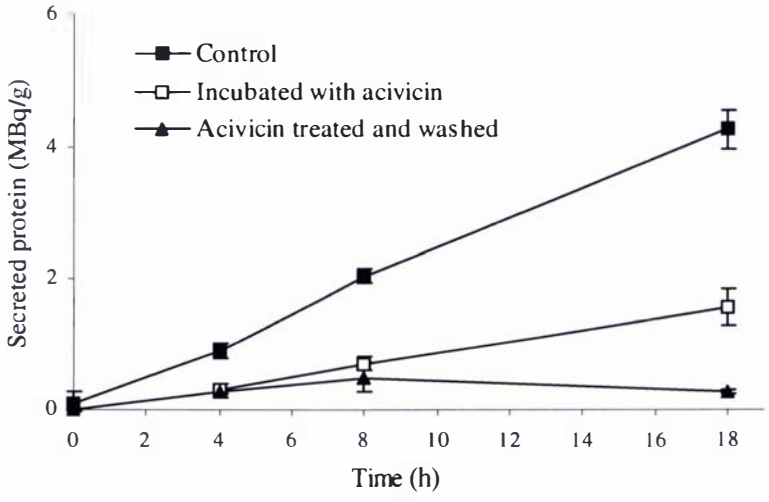


Figure 2.21 Acivicin inhibition of protein synthesis when acivicin is removed or left with acini.

Secreted protein radioactivity (normalised as described in section 2.3.16) from two experiments where acini were treated with acivicin, washed to remove unbound acivicin, then plated. Results from acini incubated in the absence and presence of acivicin (0.5 mM) have been plotted for comparison. Error bars represent standard error of the means (n=6).

2.4.2.4. The effect of inhibition of γ-glutamylcysteine synthetase and glutathione synthesis on milk protein secretion

GSH is synthesised within the cell (Meister and Anderson, 1983) where its production depletes intracellular glutamate, cysteine, and glycine pools, and this may potentially limit the supply of these amino acids for milk protein synthesis. Conversely, GSH production and export allows the formation of γ-glutamyl amino acids by γ-GT, which can be transported into the cell (Meister and Anderson, 1983) to supply milk protein synthesis (Figure 2.9).

In the present study, the effect of GSH synthesis on milk protein synthesis was investigated using an inhibitor of γ-glutamylcysteine synthetase, buthionine sulphoximine (BSO) (Figure 2.22). BSO has been shown to deplete tissue GSH when administered to rats by subcutaneous injection or when supplied in the drinking water (Griffith and Meister, 1979a). The viability of cultured lymphoid cells decreased during 96 h of culture when BSO (1 mM) was present in the medium, and this was found to result from GSH depletion (Dethmers and Meister, 1981). In another study, intracellular GSH in cultured lymphoma or mastocytoma cells was reduced by 50% after 4.5-5 h incubation in the presence of 0.2 mM BSO (Arrick *et al.*, 1982).

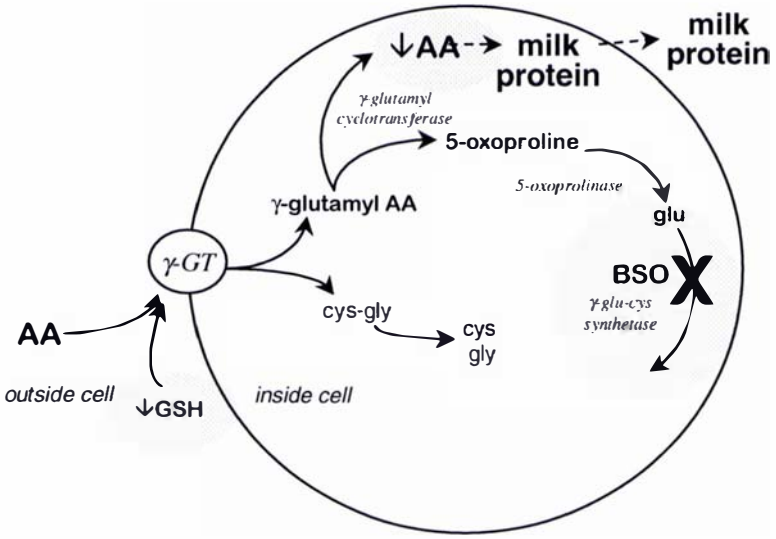


Figure 2.22 Effects of buthionine sulphoximine on milk protein synthesis.

Buthionine sulphoximine (BSO) inhibits γ-glutamyl synthetase activity and prevents glutathione (GSH) synthesis, leading to decreased GSH for γ-glutamyl transpeptidase (γ-GT) uptake of amino acids (AA).

In four experiments in the present study, isolated ovine mammary acini were incubated in the presence of 0.5 mM BSO, which was the same as the concentration of acivicin used regularly to cause a decrease in milk protein secretion. In one of these experiments, BSO was associated with a non-significant trend towards a decrease in milk protein secretion (Figure 2.23), and in another experiment, milk protein secretion was lower ($P<0.05$) in the presence of BSO than the control at 8 h but not different from the control at 2 and 4 h (Figure 2.24). The concentration of BSO was increased to 1 mM in two other experiments, but this higher concentration failed to alter milk protein secretion and results are shown in Figure 2.25 along with results from two experiments involving 0.5 mM BSO that also did not bring about a response.

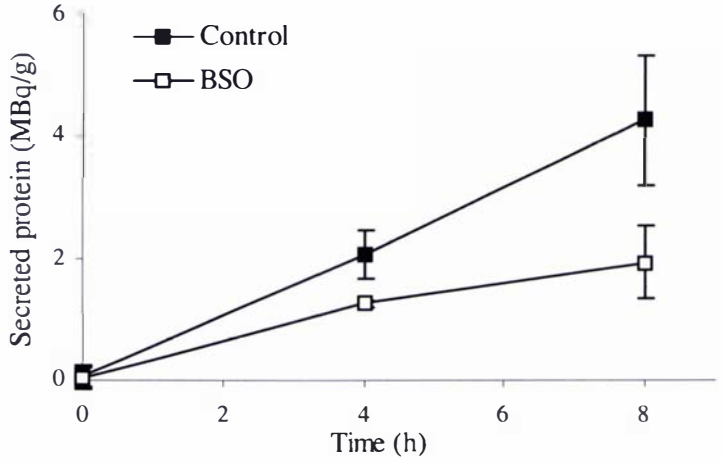


Figure 2.23 One experiment where buthionine sulphoximine did not significantly affect milk protein synthesis ($P<0.05$).

Radiolabelled (^{3}H-leucine) protein secreted from isolated ovine mammary acini incubated in the absence and presence of 0.5 mM buthionine sulphoximine (BSO). Results have been normalised as described in the text (section 2.3.16). Error bars indicate standard error of the means (n=3).

The effect of BSO on milk protein secretion was dependent on each acini preparation although there were no obvious differences between acini preparations and viability as estimated by trypan blue staining was consistently high (90 - 95%). The variation between experimental results may have arisen from differences in oxidative stress levels experienced by the acini in each preparation. Depletion of intracellular GSH by BSO in human lymphoid cells was found to increase cytolysis by reactive oxygen intermediates

(Meister and Anderson, 1983). A similar phenomenon may have caused differing results in the present study.

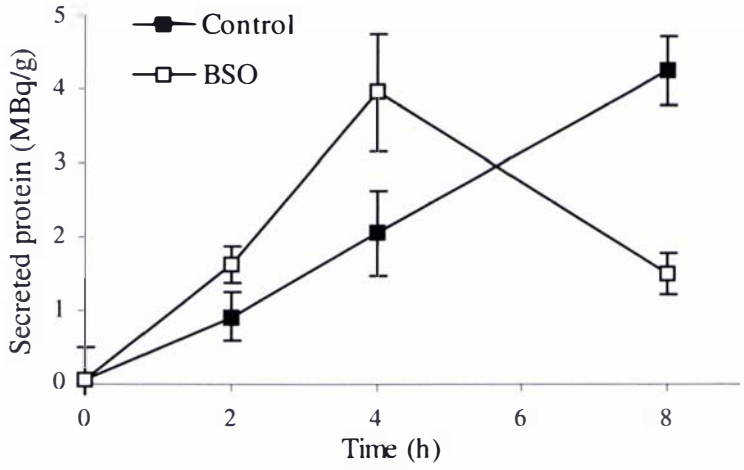


Figure 2.24 Buthionine sulphoximine significantly affected milk protein synthesis after 8 h incubation in one experiment (P<0.05).

Radiolabelled (^{3}H-leucine) protein secreted from isolated ovine mammary acini incubated in the absence and presence of 0.5 mM buthionine sulphoximine (BSO). Results have been normalised as described in the text (section 2.3.16). Error bars indicate standard error of the means (n=3). BSO did not significantly affect protein synthesis after 2 or 4 h incubation (P<0.05).

The decrease in protein synthesis in Figure 2.24 after 8 h incubation indicates that inhibition of GSH synthesis using BSO leads to reduced GSH export and reduced supply of GSH to γ-GT resulting in decreased γ-glutamyl amino acid formation and decreased uptake of amino acids by the cell. This would lead to a decrease in amino acid supply, which in turn would decrease milk protein synthesis. If extracellular concentrations of GSH were sufficient for γ-GT activity to continue without GSH synthesis and export, BSO would not decrease milk protein synthesis producing results shown in Figure 2.25.

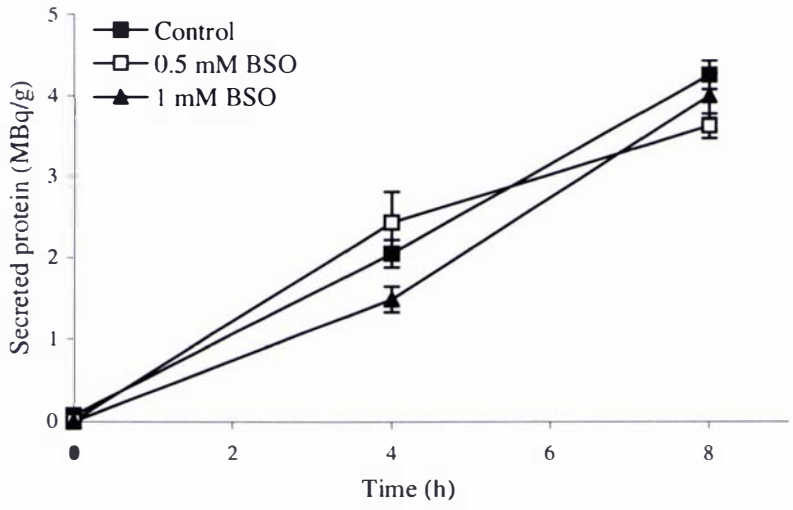


Figure 2.25 Buthionine sulphoximine did not affect milk protein synthesis in four experiments.

Combined results from four experiments showing radioactivity of protein secreted by isolated ovine mammary acini incubated in the absence and presence of two concentrations of buthionine sulphoximine (BSO). Results have been normalised as described in the text (section 2.3.16). Error bars represent standard error of the means (control $t_{0,4,8}$ n=12; 0.5 mM BSO t_0 n=1; 1 mM BSO t_0 n=6; BSO $t_{4,8}$ n=6; t_2 n=3).

A greater number of experiments of this type where acini were incubated in the presence of BSO are required to determine whether inhibition of GSH synthesis affects milk protein synthesis or not. The response to GSH depletion may depend on the condition of the isolated acini.

2.4.2.5. The effect of 5-oxoproline supply on milk protein secretion

5-Oxoproline is an intermediate of the γ-glutamyl cycle formed by γ-glutamyl cyclotransferase action on γ-glutamyl amino acids that are formed by γ-GT action on amino acids and GSH (Griffith *et al.*, 1976) (Figure 2.26). The appearance of 5-oxoproline inside the cell has been used to verify the link between GSH metabolism by γ-GT and the transport of γ-glutamyl amino acids (Griffith *et al.*, 1978), but has also been used to refute any role of the γ-glutamyl cycle in amino acid uptake. In rats, the decrease in amino acid transport into cells of the mammary gland and placenta with γ-

GT inhibition was reversed when 5-oxoproline was infused, and 5-oxoproline administration late in lactation restored amino acid transport to that of peak lactation (Viña *et al.*, 1989). This suggests that 5-oxoproline signals increased uptake of amino acids by conventional transport systems in the mammary gland and that the γ-glutamyl cycle is indirectly involved in amino acid transport by generating 5-oxoproline (Viña *et al.*, 1989).

Evidence of this role for 5-oxoproline has also been established in isolated plasma membrane vesicles derived from the blood-brain barrier (Lee *et al.*, 1996b). In these vesicles 5-oxoproline stimulated transport systems A and $B^{o,+}$ by 70% and 20% respectively, where system A transports small neutral amino acids and $B^{o,+}$ transports neutral and basic amino acids.

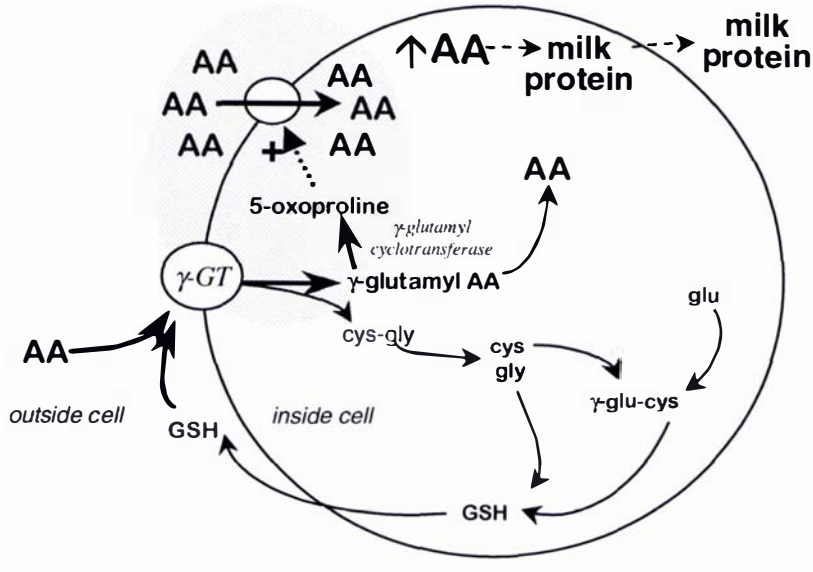


Figure 2.26 The proposed role of 5-oxoproline in up-regulating amino acid transport by conventional systems for milk protein synthesis.

Abbreviations: AA, amino acid; GSH, glutathione

In the present study, this alternative hypothesis for the role of the γ-glutamyl cycle was tested in isolated ovine mammary acini from the lactating sheep. In two experiments, acini were incubated with 5-oxoproline and with 5-oxoproline in combination with acivicin. Secreted protein results from these experiments, shown in Figure 2.27, indicate that under these experimental conditions 5-oxoproline does not enhance milk protein synthesis and does not reverse the effect of acivicin.

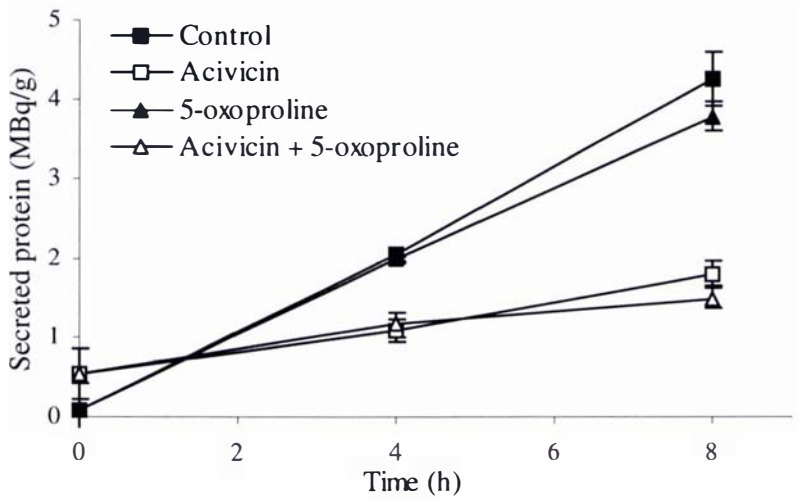


Figure 2.27 Protein synthesis in the presence of 5-oxoproline and acivicin.

Radiolabelled (^{3}H-leucine) protein secreted by isolated ovine mammary acini incubated in the absence and presence of 0.5 mM 5-oxoproline and/or 7 mM acivicin in two experiments. Results were normalised as described in the text (section 2.3.16). Error bars indicate standard error of the means (n=6).

Intracellular amino acid concentrations of harvested cells were analysed to test for changes in the presence of 5-oxoproline. In particular, the concentration of glutamate was expected to increase as 5-oxoproline is converted to glutamate by 5-oxoprolinase (Meister and Anderson, 1983). However, no pattern of change in intracellular concentrations of amino acids could be related to the use of 5-oxoproline (see Table 5.2 and Table 5.3 in Appendix E for detailed results).

The results from these experiments indicate that under the conditions used here, the γ-glutamyl cycle intermediate 5-oxoproline does not enhance milk protein secretion. A role for 5-oxoproline in enhancing amino acid uptake by conventional transport systems cannot be ruled out. However, three ATP are consumed during the γ-glutamyl cycle (Baumrucker, 1985), which must be considered an expensive way to produce 5-oxoproline. In addition, the formation of 5-oxoproline requires that γ-glutamyl amino acids be transported (Figure 2.26); that is, the transport of amino acids is already occurring. The action of 5-oxoproline may occur in concert with the uptake of γ-glutamyl amino acids by γ-GT and is probably a mechanism for up-regulating conventional amino acid transport systems when there is increased amino acid supply.

2.4.2.6. The effect of increasing intracellular cysteine by alternative supply pathways on milk protein secretion

The γ-glutamyl cycle has been shown to mediate cystine uptake in human endothelial cells (Cotgreave and Schuppe-Koistinen, 1994) and human pancreatic cells (Sweiry *et al.*, 1995) and γ-GT has been shown to have a high affinity for cystine (Thompson and Meister, 1977). Cystine is easily converted inside cells to cysteine, which is considered limiting for milk protein synthesis (Clark *et al.*, 1978). In this study, inhibition of γ-GT decreased milk protein secretion in isolated ovine mammary acini. This may be in response to decreased cyst(e)ine supply. To determine whether γ-GT plays a role in the supply of cyst(e)ine for milk protein synthesis, experiments were designed to study whether alternative supplies of intracellular cysteine would enable milk protein synthesis to continue in the presence of γ-GT inhibition. High concentrations of cysteine are toxic (Olney and Ho, 1970; Schonbeck *et al.*, 1975; Copper *et al.*, 1982) so non-toxic precursor forms, oxothiazolidine carboxylic acid and N-acetylcysteine, were used to supply intracellular cysteine by pathways alternative to γ-GT.

2.4.2.6.1. Increasing cysteine supply through oxothiazolidine carboxylic acid (OTCA)

OTCA is often referred to as a cysteine precursor (Chung *et al.*, 1990), cysteine prodrug (Anderson and Meister, 1987), or Procysteine® (Webb *et al.*, 1994). OTCA is converted to cysteine by the action of 5-oxoprolinase (Figure 2.28), an intracellular enzyme of the γ-glutamyl cycle that catalyses the conversion of 5-oxoproline to glutamate (Williamson and Meister, 1981) (Figure 2.9). It has been proposed that cysteine may limit milk

protein synthesis (Clark *et al.*, 1978) therefore the supply of OTCA to mammary acini may increase intracellular cysteine for milk protein synthesis (Figure 2.29).

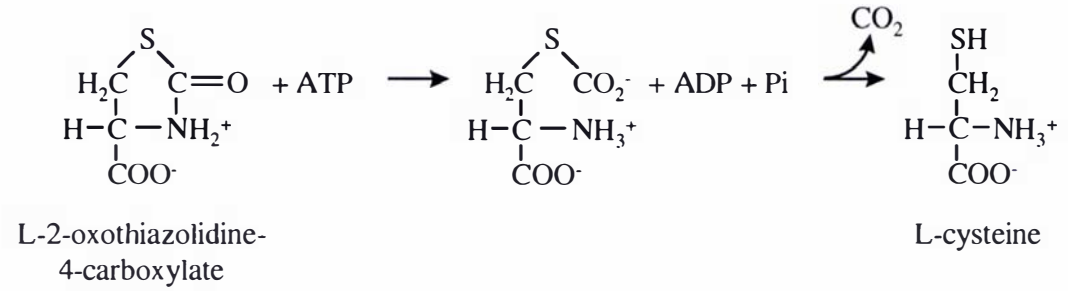


Figure 2.28 Reaction catalysed by 5-oxo-L-prolinase on oxothiazolidine carboxylic acid.

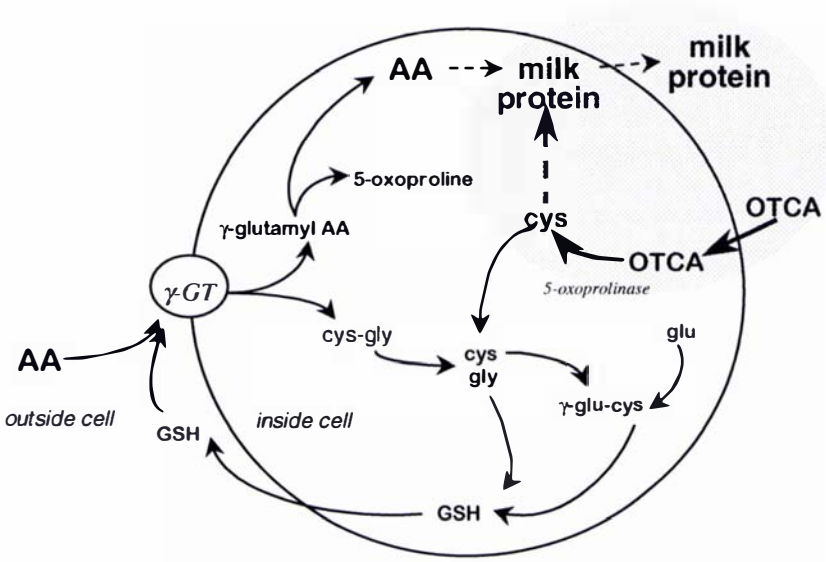


Figure 2.29 Oxothiazolidine carboxylic acid (OTCA) is converted to cysteine inside the cell.

Previous studies have shown that cysteine supplied in form of OTCA can increase intracellular GSH synthesis and restore depleted hepatic GSH in mice (Williamson and Meister, 1981; Williamson *et al.*, 1982). The rapid increase in intracellular GSH has been taken to mean that OTCA is readily transported into cells (Anderson and Meister, 1987). OTCA was suggested as a system for the delivery of intracellular cysteine for use in treatment of diseases, poisoning and toxicities associated with chemotherapy (Williamson and Meister, 1981; Williamson *et al.*, 1982). OTCA has since been shown

to decrease conjugation of the plastic manufacture by-product and water pollutant, 1,1-dichloroethylene, to tissues of rats (Moslen *et al.*, 1989); increase plasma cysteine and lymphocyte cysteine and GSH in human patients (Porta *et al.*, 1991); and replete tissue GSH following radiation without causing significant change in tumours (Rose *et al.*, 1996). Oral administration of OTCA was found to be more effective than dietary protein replenishment at reducing hyperoxia-induced oxidative tissue damage in the lung of malnourished rats (Levy *et al.*, 1998). OTCA may synergise with endothelium-derived nitric oxide in promoting dilation of the brachial artery. Dilation is impaired in patients with coronary artery disease (Vita *et al.*, 1998).

Supplementation of a sulphur amino acid deficient diet with either cysteine or OTCA resulted in similar weight gains (Chung *et al.*, 1990). In rat hepatocytes, enterocytes and renal cortical tubules, OTCA uptake was shown to be slower than uptake of cysteine and OTCA was not utilised as readily as methionine for GSH synthesis but was used preferentially over cysteine (Coloso *et al.*, 1991). In another study, OTCA increased tissue GSH and prevented weight loss in rats fed a sulphur amino acid deficient diet (Jain *et al.*, 1995).

OTCA has been shown to be a source of cysteine for protein synthesis as well as GSH synthesis. Chicks fed a low sulphur amino acid diet did not show an increase in hepatic GSH as dietary cysteine was increased from 0 to 0.1% of the diet (Chung *et al.*, 1990). With adequate to excessive cysteine supply, GSH increased linearly. It was suggested from this result that when cysteine was deficient, it was used primarily for protein synthesis and that GSH only began to increase when demands for protein synthesis were being met. Increasing supplementation of cysteine or OTCA in the diet of chicks and rats increased GSH concentrations above normal maxima. However, cysteine at high concentrations was toxic (Chung *et al.*, 1990). The role of OTCA in protein synthesis is supported by an observation that rats fed OTCA and a sulphur amino acid deficient diet have superior coat condition to control animals (Jain *et al.*, 1995).

As an analogue of 5-oxoproline, OTCA acts as a competitive inhibitor and blocks the γ-glutamyl cycle (Williamson and Meister, 1981). The build up of 5-oxoproline would be expected to increase amino acid transport as 5-oxoproline has been proposed to be an

activator of amino acid transport systems (Viña *et al.*, 1989). Therefore, OTCA may also indirectly increase milk protein synthesis by increased supply of amino acids other than cysteine.

In the present study, isolated ovine mammary acini were incubated in the presence of increasing concentrations of OTCA in order to determine whether OTCA would enhance milk protein secretion and to determine the concentration of OTCA to be used in experiments where acivicin was used to inhibit γ-GT activity. The culture medium for OTCA experiments did not contain cysteine and the concentration of OTCA added was determined from the concentration of cysteine in complete D-MEM medium that was used in previous experiments in this study (0.4 mM in wells). Two experiments were carried out using a total of four OTCA concentrations together with a control.

Since the culture medium was cysteine depleted and cysteine was thought to be limiting for milk protein synthesis, the control experiment was expected to have lower levels of protein secretion, whereas the concentrations of OTCA used were expected to significantly enhance milk protein secretion. However, milk protein secretion in the control (Figure 2.30) was similar to that in cysteine replete medium (Figure 2.7). This may have been because acini had enough residual intracellular cysteine or cysteine stored as GSH for milk protein synthesis to continue for the relatively short incubation times, or because the medium was replete with cysteine as a result of dead cells or the preparation procedure. In addition, cysteine can be made from transulphuration of methionine (Griffith, 1987; Stamler and Slivka, 1996) although this pathway has been shown to occur at low levels in the mammary gland of the lactating goat (Lee *et al.*, 1996a) and in sheep mammary cells (personal communication S.R Davis).

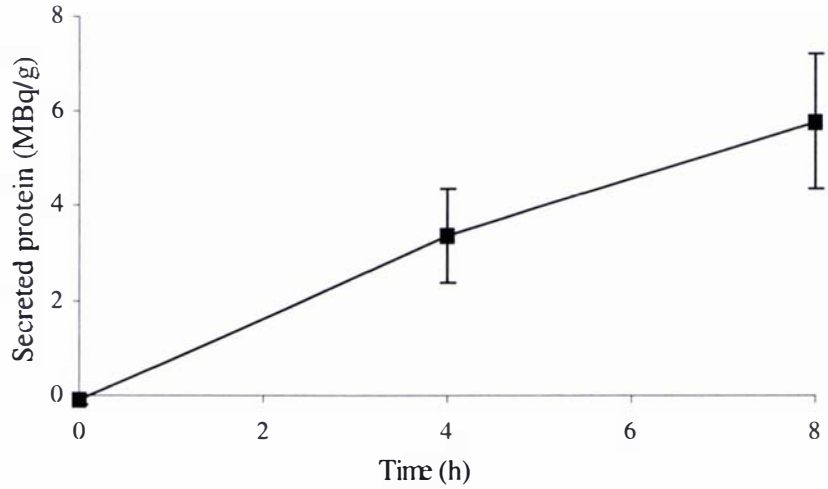


Figure 2.30 Protein synthesis by isolated ovine mammary acini incubated in cysteine depleted medium.

Radiolabelled (^{3}H-leucine) protein secreted from isolated ovine mammary acini incubated in cysteine depleted medium on 24-well plates. Error bars represent standard error of the mean (n=3).

To allow direct comparison between experiments, results were normalised. Protein secretion at each time point was multiplied by a correction factor determined from a data set considered to have representative rates of milk protein secretion in cysteine replete medium so that all controls were the same and the protein secretion with chemical treatments relative to controls could be directly compared. Only the highest concentration of OTCA used (1.6 mM) significantly increased milk protein secretion ($P<0.05$) (Figure 2.31).

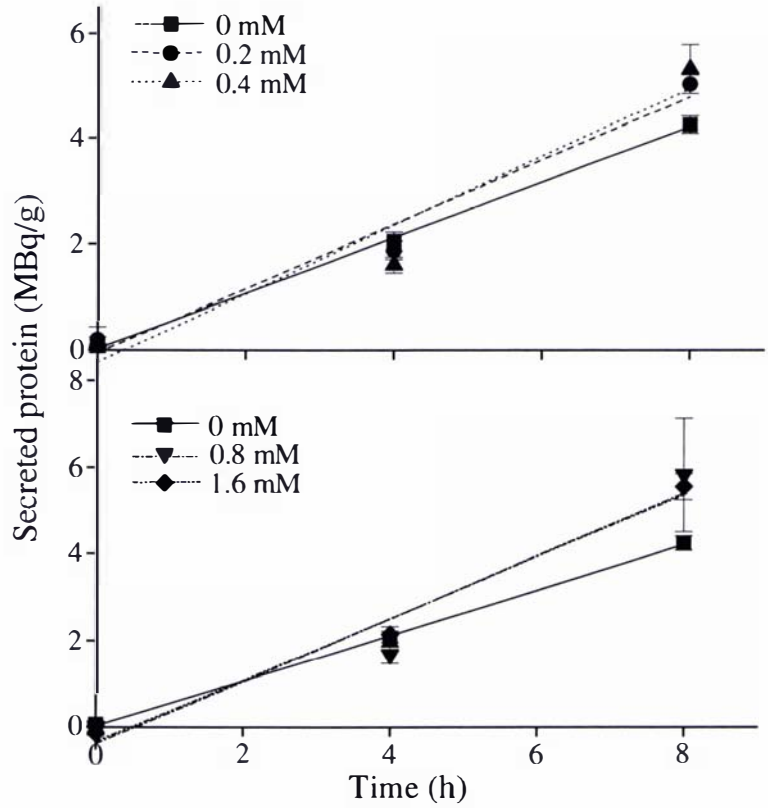


Figure 2.31 Protein synthesis with different concentrations of oxothiazolidine carboxylic acid.

Radiolabelled (^{3}H-leucine) protein secreted from isolated ovine mammary acini in two experiments where acini were incubated in cysteine depleted medium. Results have been normalised as described in the text (section 2.3.16). Error bars indicate standard error of the means (0 mM n=5; 0.2 mM n=2; 0.4 mM n=5; 0.8 mM n=3; 1.6 mM n=3). Data have been fitted using linear regression. Trendlines for 0.8 mM and 1.6 mM data are overlapping.

The main aim of the use of OTCA was to determine whether milk protein secretion could continue when γ-GT was inhibited by acivicin and cysteine was supplied by an alternative source. Therefore, isolated ovine mammary acini were incubated in the absence and presence of acivicin and/or OTCA. In the first experiment of this kind, acini for dual treatment with acivicin and OTCA were incubated for 4 h with OTCA alone and then acivicin was added to the culture medium (Figure 2.32). This experiment was carried out prior to results being known for experiments using increasing OTCA and only 0.4 mM OTCA was used. OTCA did not enhance milk protein secretion, which is consistent with the effect of this concentration in the previously described experiments (Figure 2.31). There was a decline in protein

secretion at 8 h associated with the addition of acivicin at 4 h. This was not significantly different from the control but was lower than when OTCA was supplied alone ($P<0.05$) indicating that protein synthesis had decreased with γ-GT inhibition. Protein synthesis, when OTCA was applied at 0.4 mM, may rely on γ-GT activity for cysteine supply (Figure 2.32).

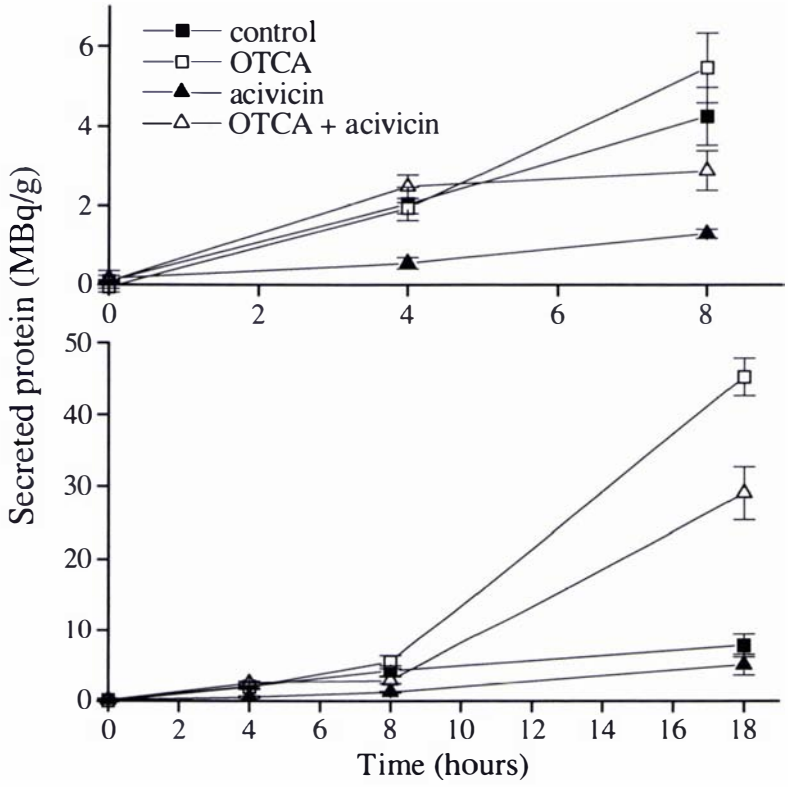


Figure 2.32 Protein synthesis in the presence of oxothiazolidine carboxylic acid and acivicin.

Radiolabelled (^{3}H-leucine) protein secreted from isolated ovine mammary acini from one experiment incubated in the absence and presence of oxothiazolidine carboxylic acid (OTCA) (0.4 mM) and acivicin (0.5 mM) in cysteine depleted medium. Acivicin was added at 4 h to OTCA + acivicin acini. The lower panel shows the same experiment as the top extended out to 18 h. Results have been normalised as described in text (section 2.3.16) Error bars indicate standard error of the means (n=3).

At 18 h, there was an increase in milk protein secretion of acini incubated in the presence of OTCA both with and without acivicin ($P<0.05$) (Figure 2.32). This relatively delayed increase was unexpected, as OTCA has been shown to increase hepatic GSH within 4 h of administration by injection, to mice (Williamson *et al.*,

1982). This indicates that after 18 h, intracellular cysteine becomes depleted and milk protein production becomes responsive to supply of cysteine by OTCA.

In a second experiment, acini were incubated with acivicin until 2 h when OTCA was added, and in a third experiment OTCA and acivicin were added together at 0 h. These experiments gave similar results and although they were carried out slightly differently, the results have been normalised (Figure 2.33). OTCA did not increase the rate of milk protein secretion although the concentration used was higher than that of the previous experiment. In addition, OTCA failed to alter the rate of milk protein secretion in the presence of acivicin. Acini from these experiments were only incubated for a maximum of 8 h.

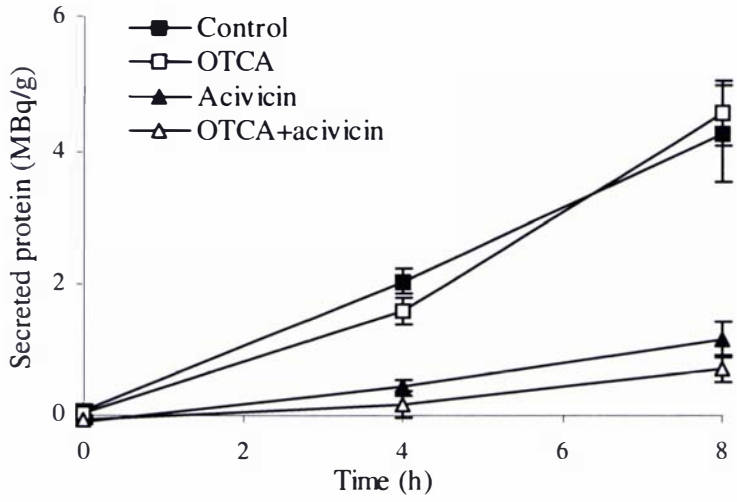


Figure 2.33 The effect of oxothiazolidine carboxylic acid and acivicin on milk protein synthesis.

Radiolabelled (^{3}H-leucine) protein secreted from isolated ovine mammary acini from two experiments where acini were incubated in the absence and presence of oxothiazolidine carboxylic acid (OTCA) (0.8 mM) and/or acivicin (0.6 mM) in cysteine depleted medium. In one experiment OTCA was added to OTCA + acivicin wells at 0 h, and in the other at 2 h. Results have been normalised as described in the text (section 2.3.16). Error bars represent standard error of the means (n=3).

At this stage of the study, questions were being raised as to the validity of running experiments for 18 h as other studies had reported that the production of mRNA

transcripts for milk proteins significantly decreased after 1 h of acini being plated (Wheeler *et al.*, 1995). In hindsight, it would have been better to incubate acini for 18 h in all experiments so that unexpected results such as these could have been compared to others.

Results from this study indicate that OTCA does not immediately affect milk protein synthesis but over longer periods, OTCA may overcome the effect of acivicin leading to increased milk protein synthesis in the presence of γ-GT inhibition. This suggests that γ-GT plays a role in cyst(e)ine supply for milk protein synthesis and also that cysteine is limiting for milk protein synthesis in isolated ovine mammary acini.

The supply of OTCA was not as effective at increasing milk protein output as expected, possibly due to channelling of cysteine from OTCA to GSH synthesis instead of protein synthesis. In order to prevent the cysteine derived from OTCA from entering the GSH synthesis pathway, acini were incubated in the presence of γ-glutamylcysteine synthetase inhibitor buthionine sulphoximine (BSO) which blocks GSH synthesis (see section 2.4.2.4), as well as OTCA. Acivicin was also added to determine whether milk protein synthesis could continue in the absence of γ-GT activity.

BSO decreased protein secretion at 8 h but this was not observed when OTCA was also present ($P<0.05$) (Figure 2.34). This suggests that the decrease in milk protein secretion observed in the atypical BSO experiment shown in Figure 2.24, may be caused through reduced cysteine supply because of decreased GSH for import by γ-GT. In experiments where BSO had no effect on milk protein secretion, there may have been a sufficient supply of GSH for γ-GT activity to continue. This indicates that cysteine is limiting for milk protein synthesis, that GSH is a requirement for cysteine uptake by the cells, and that the γ-glutamyl cycle plays a role in cysteine supply for milk protein synthesis.

In combination with acivicin and BSO, OTCA did not increase milk protein secretion suggesting that the supply of cysteine by this system cannot reverse the effect of acivicin even when cysteine provided by this procysteine molecule is prevented from entering the GSH synthetic pathway. However, this does not mean that cysteine derived from

OTCA does not enter a pool for GSH synthesis that may be unavailable for milk protein synthesis.

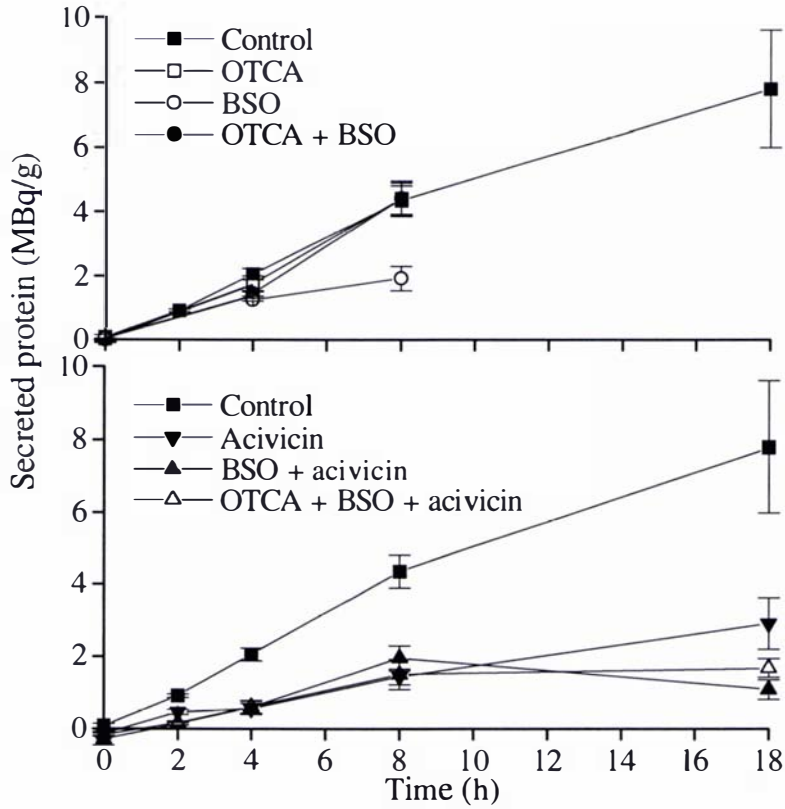


Figure 2.34 Protein synthesis in the presence of oxothiazolidine carboxylic acid, buthionine sulphoximine and acivicin.

Radiolabelled (^{3}H-leucine) protein secreted from isolated ovine mammary acini from two experiments. Acini were incubated in the absence or presence of oxothiazolidine carboxylic acid (OTCA) (0.8 mM), and/or buthionine sulphoximine (BSO) (0.5 mM), and/or acivicin (0.5 mM) in cysteine depleted medium. Results were normalised as described in the text (section 2.3.16). Error bars represent standard error of the means (all treatments t_2 n=3; t_{18} n=3; control $t_{0,4,8}$ n=6; OTCA, BSO, OTCA + BSO n=3; acivicin $t_{0.4.8}$ n=6; OTCA + acivicin n=3; BSO + acivicin, OTCA + BSO + acivicin t_0 n=3, $t_{4,8}$ n=6).

The concentration of cysteine in tissues has been shown to range between 10 and 100 μM (Gaitonde, 1967; Anderson and Meister, 1987). Intracellular GSH concentration varies between 0.5 and 10 mM (Meister and Anderson, 1983). In mammary tissue obtained from lactating goats, the cysteine concentration was 106 μM, and GSH was 0.9

mM (Knutson *et al.*, 1994). Lower concentrations of GSH are expected in mammary tissue since most GSH is produced in the liver (Meister and Anderson, 1983).

Cysteine and GSH concentrations were analysed to determine whether OTCA was affecting the concentration of these thiols inside cells. Unfortunately, cell samples were only obtained from the final experiment where OTCA did not affect milk protein secretion in the presence or absence of acivicin. As part of this experiment, acini were incubated in the absence and presence of BSO and/or acivicin. As usual, acini were plated in the presence of the individual treatments as well as in combination; so intracellular thiol data were obtained for OTCA, acivicin, and BSO alone and these treatments in combination.

Intracellular concentrations of cysteine in isolated ovine mammary acini in the present study (Table 2.5) were low but were in the range given by Gaitonde (1967) and Anderson and Meister (1987). However, the intracellular GSH concentrations obtained (Table 2.6) were much lower than tissue concentrations, perhaps because of loss of GSH during the acini preparation procedure. Depleted GSH would increase the responsiveness of acini to BSO and give rise to the results seen in the atypical BSO experiment (Figure 2.24) and the recovery to BSO treatment in the presence OTCA seen in Figure 2.34.

In untreated acinar cells, a trend towards decreased intracellular cysteine was observed between plating and 4 h but cysteine did not appear to change between 4 and 8 h (Table 2.5). Intracellular cysteine increased ($P<0.05$) with OTCA after incubation of acini for 4 h but not 8 h, indicating a short term effect for this level of administration. Acivicin was expected to decrease intracellular cysteine, but cysteine was not significantly affected by acivicin alone or when administered with OTCA. The latter is consistent with the lack of response in milk protein secretion of acivicin treated acini to OTCA administration (Figure 2.33).

An increase in intracellular cysteine might have been expected with administration of BSO because of decreased use for GSH synthesis, but intracellular cysteine was not affected by BSO alone, in combination with OTCA or in combination with acivicin

(Table 2.5). Intracellular cysteine concentrations at 8 h were not affected by treatment with OTCA, BSO or acivicin when administered separately or in combination.

Table 2.5 Intracellular cysteine concentrations (mean nmol/g cells ± SEM) with oxothiazolidine carboxylic acid, buthionine sulphoximine and acivicin.

Results are from one experiment (n=3) where isolated ovine mammary acini were incubated for 8 h in the presence and absence of oxothiazolidine carboxylic acid (OTCA) (0.8 mM) and/or buthionine sulphoximine (BSO) (0.5 mM) and/or acivicin (0.6 mM) in cysteine depleted medium.

Treatment	Time (h)		
	0	4	8
Control	32 ± 3.2	18^{a} ± 2.9	21^{a} ± 3.3
OTCA		37^{b} ± 3.5	19^{a} ± 8.8
Acivicin		15^{a} ± 2.8	18^{a} ± 7.3
OTCA + acivicin		25^{ab} ± 9.5	17^{a} ± 6.6
BSO		19^{a} ± 0.6	19^{a} ± 7.6
OTCA + BSO		21^{a} ± 1.0	13^{a} ± 2.2
BSO + acivicin		17^{a} ± 3.7	14^{a} ± 2.7
OTCA + BSO + acivicin		19^{a} ± 2.2	14^{a} ± 2.9
LSD		12.3	14.0

^{a,b} Means in the same column without a common superscript differ (P<0.05).

In untreated cells, a trend towards a decrease in intracellular GSH occurred between plating and 4 h incubation and a trend towards an increase was observed between 4 and 8 h incubation (Table 2.6). Acivicin alone did not affect intracellular GSH and OTCA alone did not appear to increase intracellular GSH. However, GSH export from the cell has been shown to be linked to γ-GT activity (Griffith and Meister, 1979b; Griffith *et al.*, 1979b) and by reducing GSH export through acivicin inhibition of γ-GT, increased intracellular GSH as a result of OTCA administration (Figure 2.35) was observed after 4 h incubation with both treatments together (Table 2.6). Both the intracellular concentrations of GSH and cysteine were affected by OTCA after 4 h but not 8 h incubation indicating that the concentration administered has only a short-term influence on intracellular thiols.

Table 2.6 Intracellular glutathione concentrations (mean nmol/g cells ± SEM) with oxothiazolidine carboxylic acid, buthionine sulphoximine and acivicin.

Results are from one experiment (n=3) where isolated ovine mammary acini were incubated for 8 h in the presence and absence of oxothiazolidine carboxylic acid (OTCA) (0.8 mM) and/or buthionine sulphoximine (BSO) (0.5 mM) and/or acivicin (0.6 mM) in cysteine depleted medium.

Treatment	Time (h)		
	0	4	8
Control	28 ± 0.8	11^{a} ± 1.7	17^{ab} ± 0.5
OTCA		12^{a} ± 1.2	13^{ab} ± 4.5
Acivicin		12^{a} ± 1.9	16^{ab} ±4.6
OTCA + acivicin		19^{b} ± 2.1	20^{b} ± 4.0
BSO		9.5^{a} ± 1.4	13^{ab} ± 4.1
OTCA + BSO		11^{a} ± 1.0	8.3^{a} ± 1.4
BSO + acivicin		12^{a} ± 3.2	16^{ab} ± 1.7
OTCA + BSO + acivicin		13^{a} ± 1.4	15^{ab} ± 2.8
LSD		5.5	8.5

[a, b] Means in the same column without a common superscript differ ($P<0.05$).

In previous studies when GSH synthesis was inhibited by BSO, GSH has been found to decrease inside cells and acivicin was shown to reduce this depletion (Griffith and Meister, 1979b). The decrease in milk protein secretion with BSO in an earlier experiment of the current study (Figure 2.34) suggested that intracellular GSH decreased when GSH synthesis was inhibited. However, this cannot be confirmed in this experiment, as intracellular GSH concentrations in the presence of BSO and acivicin were not different from BSO or acivicin alone, or controls (Table 2.6).

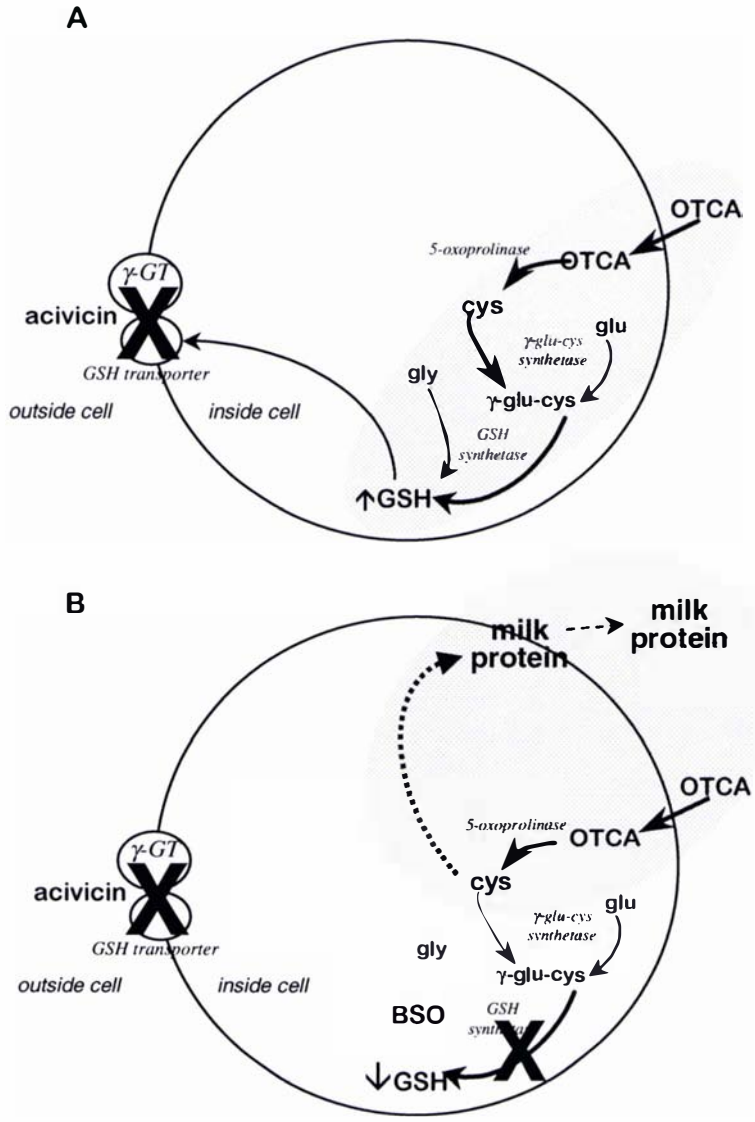


Figure 2.35 Mechanism of action of oxothiazolidine carboxylic acid and acivicin on intracellular glutathione.

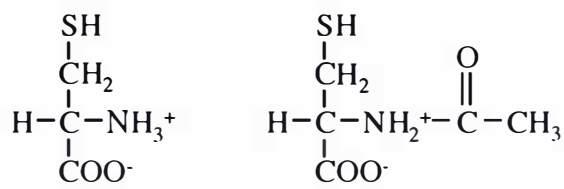
(A) Oxothiazolidine carboxylic acid (OTCA) increases glutathione (GSH) synthesis. GSH transport from the cell is prevented by acivicin inhibition of γ-glutamyl transpeptidase (γ-GT). (B) Inhibition of γ-glutamylcysteine synthetase using buthionine sulphoximine would decrease GSH production to increase availability of cysteine for milk protein production.

Intracellular GSH concentration was lower ($P<0.05$) when OTCA was supplied together with BSO and acivicin at 4 h in comparison to OTCA with acivicin (Table 2.6). This may be because BSO prevents OTCA-derived cysteine from being used in GSH synthesis (Figure 2.35).

These results indicate that OTCA may affect intracellular cysteine and GSH concentrations but treatment effects were not as marked as expected. Low concentrations of GSH in these isolated acini may have prevented greater responses. The low intracellular cysteine and GSH responses are consistent with the effects of OTCA on milk protein secretion for this experiment. Intracellular thiol analysis of cells incubated for 18 h with OTCA/acivicin in one experiment in this study (Figure 2.32) was not carried out but may have shown greater changes in cysteine and GSH concentrations because OTCA was associated with increased protein secretion at 18 h.

2.4.2.6.2. Increasing cysteine supply through N-acetylcysteine (NAC)

Since the administration of OTCA to isolated ovine mammary acini had not increased intracellular cysteine or GSH, or affected milk protein secretion in the expected manner, another alternative pathway for increasing intracellular cysteine was used. N-acetylcysteine (NAC) is an N-substituted cysteine derivative (Figure 2.36) that is transported into cells. NAC has been used to treat toxicity associated with treatment using acetaminophen, which decreases intracellular GSH, and it is therefore thought that NAC is transported into cells where it is deacylated (Anderson and Meister, 1987). The actual site(s) for NAC deacylation have not been determined but N-acylase activity has been found in kidney, liver and other tissues (Birnbaum *et al.*, 1952).



L-cysteine N-acetyl-L-cysteine

Figure 2.36 N-acetylcysteine is an N-substituted cysteine derivative

NAC has been shown to increase GSH concentrations in liver of mice, but with lower efficacy than OTCA (Williamson *et al.*, 1982). In a study of the vasorelaxant, nitroglycerine, in rats, NAC was administered to determine the effect of increasing extracellular cysteine and GSH while OTCA was administered to increase intracellular cysteine and GSH (Boesgaard *et al.*, 1993). NAC was found to increase intracellular cysteine but not GSH, and was more effective than OTCA at potentiating the nitroglycerine effect. NAC has also been used like OTCA to replete GSH in HIV patients where GSH is reduced, and can block HIV replication (Lederman, 1995).

Like OTCA, NAC can be administered orally. Dietary NAC has been used successfully in γ-GT deficient mice (Lieberman *et al.*, 1996). γ-GT deficient mice were indistinguishable from wild-type at birth but gain weight more slowly, have a coat colour with a grey hue, and developed cataracts. The 15% of γ-GT deficient mice that managed to survive to 9 months had neurologic lesions. NAC was administered to γ-GT deficient mice in drinking water and allowed mutant mice to grow at the same rate as wild-type mice, have normal coat colour and reversed early cataract formation. Wild-type mice fed NAC had enhanced weight gain. The ability of NAC to reverse γ-GT deficiency indicates that phenotypic changes result from cysteine deficiency (Lieberman *et al.*, 1996).

γ-GT is widely expressed in the reproductive organs and autopsies of γ-GT deficient mice revealed hypoplastic testes, seminal vesicles, epididymis in males, and hypoplastic ovaries, fallopian tubes, and uteri in females (Lieberman *et al.*, 1996). Recent studies involving γ-GT deficient mice have shown that when NAC is supplied in drinking water, homozygous mutant mice had reproductive tracts indistinguishable from wild-type mice, were fertile, and produced normal numbers of offspring when mated with wild-type mice (Kumar *et al.*, 2000). NAC withdrawal in male mice did not affect testes but caused atrophy of the seminal vesicles. Interestingly GSH was unchanged in γ-GT deficient mice. This indicates that γ-GT is itself not required for reproduction but is important for supply of cysteine to mouse reproductive organs (Kumar *et al.*, 2000).

In this study, NAC was tested as an alternative supply of cysteine for isolated ovine mammary acini to investigate if γ-GT inactivation by acivicin reduced supply of cysteine for protein synthesis. Acini were incubated in cysteine depleted medium for 18 h in all 4 experiments. In the first experiment, NAC was added to wells to give final concentrations of 1, 10, and 50 mM (Figure 2.37). Administration of 1 mM NAC to acini enhanced milk protein. NAC doses of 1 mM resulted in no change at 4 h, and significant increases ($P<0.05$) of approximately 250% and 165% at 8 h and 18 h respectively. Administration of 10 mM NAC did not significantly affect milk protein synthesis. Administration of 50 mM NAC resulted in decreased medium radioactivity indicating that at this level NAC was toxic to cells.

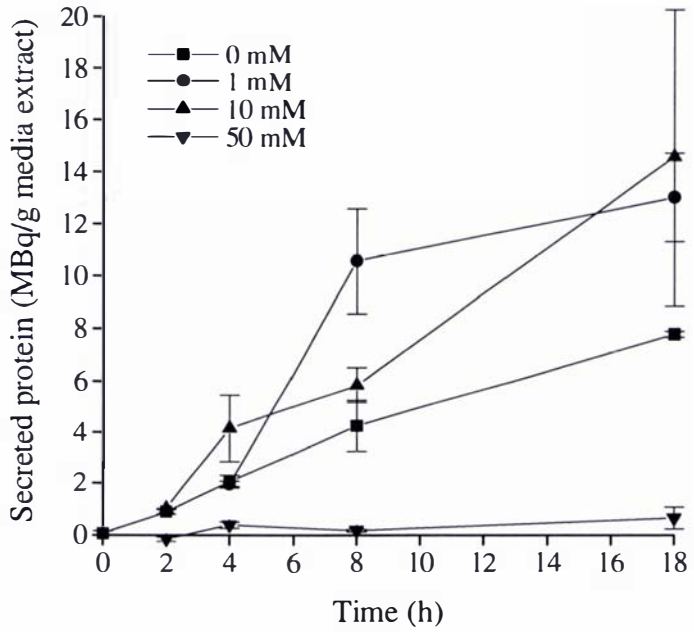


Figure 2.37 Protein synthesis with different concentrations of N-acetylcysteine.

Radiolabelled (^{3}H-leucine) protein secreted by isolated ovine mammary acini incubated in the presence of increasing concentrations of N-acetylcysteine in cysteine depleted medium. Results have been normalised as described in the text (section 2.3.16). Error bars indicate standard error of the mean (n=3).

In the first NAC experiment, acini were also incubated with acivicin in combination with each of the three concentrations of NAC (Figure 2.38). Acivicin with 50 mM NAC administration did not alter protein secretion by acini incubated with 50 mM NAC

alone, probably because of the apparent toxicity of 50 mM NAC, so data from this experiment have not been included in this report. When 1 mM NAC was combined with acivicin, protein secretion was not different from that of acivicin treatment alone up until 8 h, but after 8 h, there was a trend towards increased protein secretion so that levels were not different from the control and appeared to be greater than acivicin alone although the latter was not significant. At 18 h, the combined 1 mM NAC and acivicin treatment was not different from the control or from acivicin alone. The 10 mM NAC treatment combined with acivicin did not alter protein secretion until 8 h when the combined treatment was associated with a trend towards protein secretion that was greater than acivicin alone although not significantly different and still much less than the control. At 18 h, the combined treatment results were not different from acivicin alone and were much lower than the control.

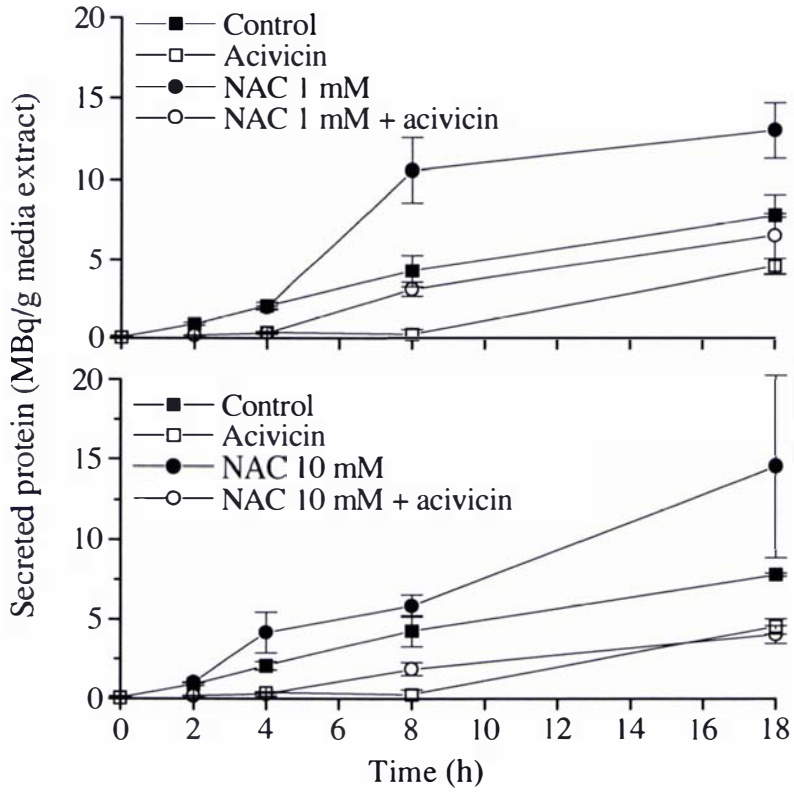


Figure 2.38 Protein synthesis in the presence of N-acetylcysteine and acivicin.

Radiolabelled (^{3}H-leucine) protein secreted by isolated ovine mammary acini from one experiment where acini were incubated in the absence and presence of two concentrations of N-acetylcysteine (NAC) and acivicin (0.5 mM) in cysteine depleted medium. Results have been normalised as described in the text (section 2.3.16). Error bars indicate standard error of the means (n=3).

The results suggest that it may be possible to maintain a higher level of protein synthesis when γ-GT is inhibited by acivicin by supplying cysteine to the mammary cell in the form of NAC. This suggests that γ-GT may play a role in cyst(e)ine supply for milk protein synthesis by the ruminant and that γ-GT inactivation may be bypassed by supplying cysteine through alternative pathways. It also supports studies that show cysteine is limiting for milk protein synthesis (Clark *et al.*, 1978).

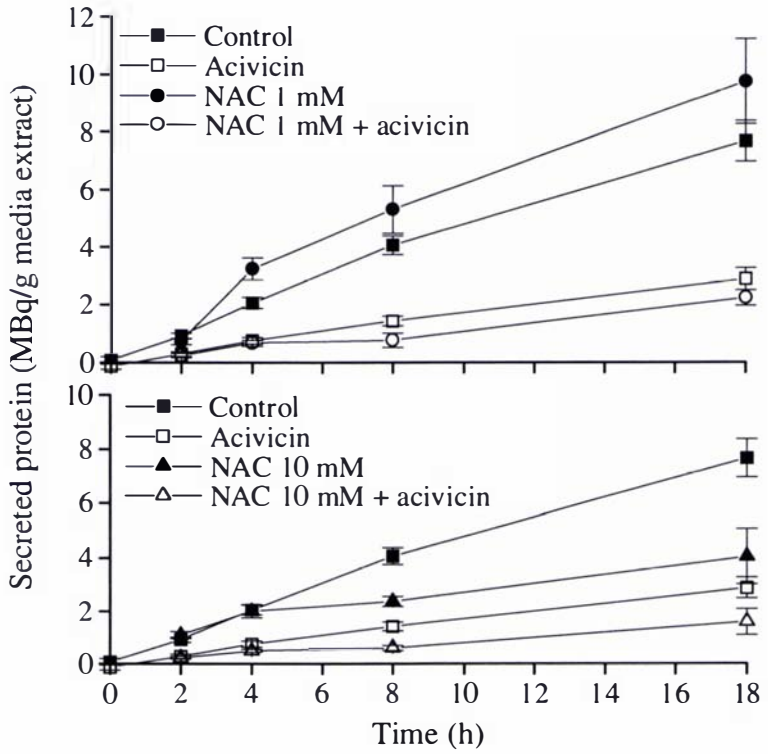


Figure 2.39 The effect of N-acetylcysteine and acivicin on milk protein synthesis.

Radiolabelled (^{3}H-leucine) protein secreted by isolated ovine mammary acini from three experiments, incubated in the absence and presence of two concentrations of N-acetylcysteine (NAC) and acivicin (0.5 mM) in cysteine depleted medium. Results have been normalised as described in text (section 2.3.16). Error bars represent standard error of the means (n=9 except NAC 10 mM n=3).

In three further experiments NAC administration produced results that were in contrast to the first experiment (Figure 2.39). In these experiments, administration of 1 mM NAC increased milk protein secretion at 4 h and a trend towards increased secretion was observed at 8 h and 18 h which is consistent with the first experiment and suggests that

cysteine is limiting for milk protein synthesis. However, in combination with acivicin, 1 mM NAC gave lower radioactivity than acivicin alone at 8 h and was not different from acivicin alone at other time points. Administration of 10 mM NAC resulted in lower radioactivity in comparison with the control and in combination with acivicin resulted in consistently lower radioactivity than acivicin alone.

Proteins in medium from experiments 3 and 4 where separated by HPLC and the effects of NAC and NAC + acivicin, as well as acivicin alone as a control, were determined on separated caseins. The whey proteins were not adequately resolved to allow quantitation. NAC increased secretion of all caseins, not just κ-casein, which is the only ovine casein that contains cysteine (Figure 2.40). This indicates that an increase in cysteine supply, via NAC, may increase GSH synthesis leading to increased γ-GT activity and increased amino acid uptake by this enzyme for milk protein synthesis (Figure 2.41). Alternatively, increased 5-oxoproline as a result of increased γ-GT activity may up regulate conventional amino acid transport systems (Viña *et al.*, 1989) (Figure 2.26). NAC did not restore secretion of caseins after acini were treated with acivicin in this experiment. This suggests that γ-GT activity is not supplying cysteine for casein synthesis. Cysteine has been shown to be limiting for GSH synthesis (Anderson and Meister, 1987). In the presence of NAC and acivicin intracellular GSH may increase but would not be used by γ-GT for amino acid transport. The analysis of individual proteins in experiment 1 was not carried out, but might have returned results to the contrary.

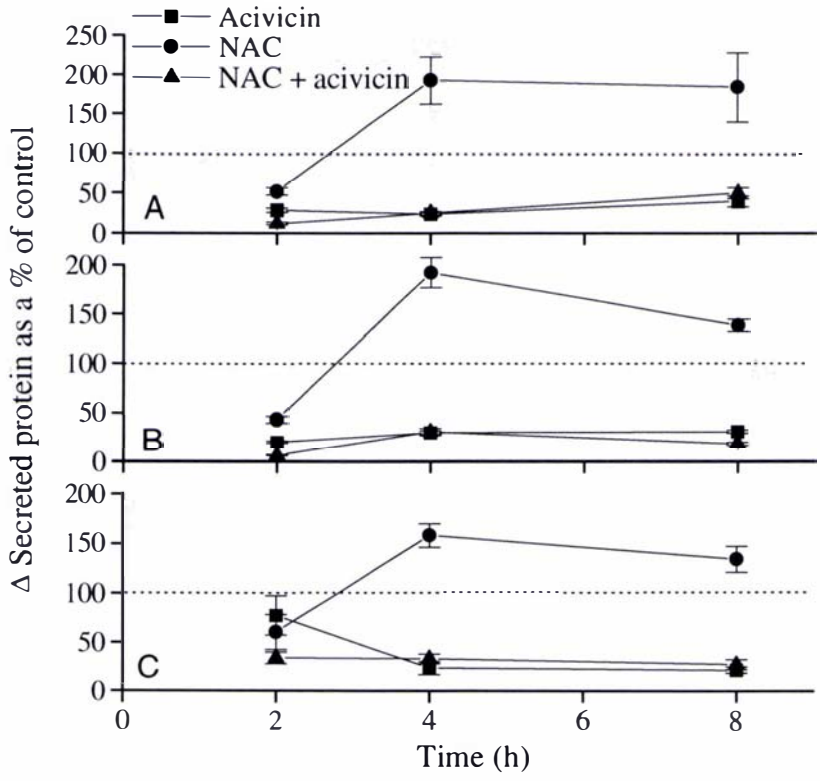


Figure 2.40 The effect of N-acetylcysteine and acivicin on casein synthesis.

Change in radiolabelled (^{3}H-leucine) casein secretion by isolated ovine mammary acini incubated in the presence of acivicin (0.5 mM) and/or N-acetylcysteine (NAC) (1 mM) in cysteine depleted medium as a percentage of control (untreated) protein secretion (...). A, α-casein; B, β-casein; C, κ-casein. Error bars indicated standard error of the means (t_2 n=3; $t_{4,8}$ n=6).

Differences in the response to NAC of acini between the first and the other three experiments, and the failure of NAC to reverse the effect of acivicin in the latter, may be because of differences in intracellular cysteine and/or glutathione concentrations of the individual acini preparations. Intracellular thiol concentrations were measured in all four experiments. It is customary to use an internal standard in the measurement of thiols so that loss during sample preparation may be corrected for. The best internal standard for this purpose is NAC since it is stable, elutes clear of other compounds, and is not present in physiological samples. But in the present study NAC was used to treat acini so it could not be included as an internal standard. Therefore, these samples are uncorrected and intracellular thiol concentrations should be regarded as approximate values only.

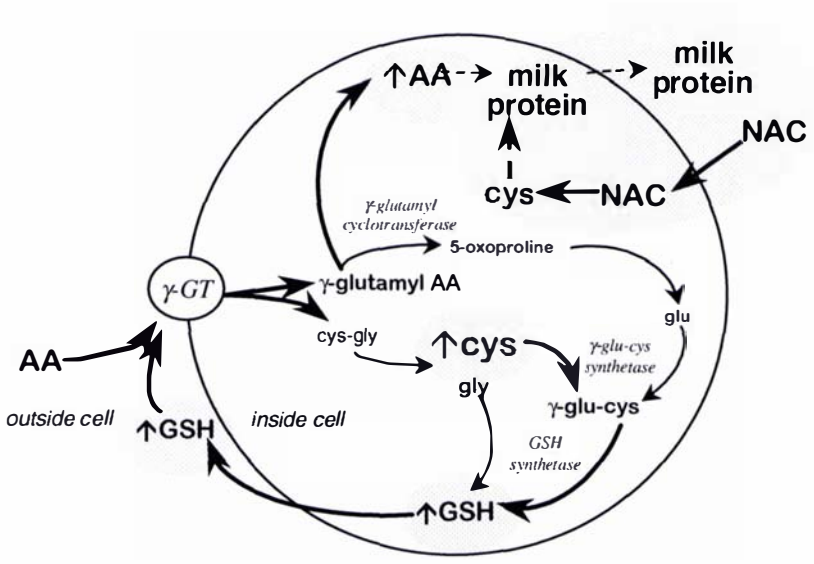


Figure 2.41 The effect of N-acetylcysteine on milk protein production.

Supply of cysteine by N-acetylcysteine (NAC) increases milk protein synthesis directly and through increased glutathione (GSH) synthesis leading to increased γ-glutamyl transpeptidase (γ-GT) activity and supply of amino acids (AA).

With the exception of experiment 2, intracellular cysteine concentrations in untreated isolated ovine mammary acini in the present study tended to decrease after plating (Table 2.7). The intracellular cysteine concentrations from the OTCA experiment (Table 2.5) are in agreement with this. The trends in intracellular cysteine do not appear to be related to differences in milk protein secretion between the first experiment (Figure 2.38) and the other three (Figure 2.39), but the cysteine content was different in cells from each acini preparation and this may have affected protein synthesis.

NAC successfully increased ($P<0.05$) intracellular cysteine in isolated ovine mammary acini in experiments 1 and 2 at each time point and in experiment 3 after 8 h incubation (Table 2.7). A trend towards increased intracellular cysteine was observed in experiment 4 with NAC administration. Intracellular cysteine increased by approximately 2-fold and 3-fold after 4 and 8 h incubation respectively in experiment 1 and in experiment 2 cysteine increased by approximately 3-fold after 4 h incubation and 4-fold after 8 h incubation with NAC.

Table 2.7 Intracellular cysteine concentrations (mean nmol/g cells) in the presence of N-acetylcysteine and acivicin.

Results from four experiments (n=3) where isolated ovine mammary cells were incubated in the absence and presence of N-acetylcysteine (NAC) (1 mM) and/or acivicin (0.5 mM) in cysteine depleted medium.

Experiment	Time (h)	Control	NAC	Acivicin	NAC + acivicin	LSD
1	0	29 ± 2.0				
	4	20^{ab} ± 2.6	34^{c} ± 5.1	15^{a} ± 1.5	28^{bc} ± 1.6	11
	8	16^{a} ± 1.9	50^{b} ± 8.9	18^{a} ± 3.5	53^{b} ± 5.1	20
2	0	31 ± 2.1				
	4	51^{ab} ± 16	148^{c} ± 24	34^{a} ± 2.2	89^{b} ± 10	51
	8	44^{a} ± 2.1	187^{c} ± 11	30^{a} ± 4.1	106^{b} ± 1.8	21
3	0	5.8 ± 1.3				
	2	6.2^{a} ± 0.6	10^{a} ± 0.2	6.3^{a}	12^{a} ± 1.7	7.9
	4	1.5^{ab} ± 0.3	3.8^{b} ± 0.8	1.4^{a} ± 0.3	1.3^{a} ± 0.1	2.2
	8	2.2^{ab} ± 0.5	3.2^{c} ± 0.2	2.9^{bc} ± 0.1	1.7^{a} ± 0.2	1.1
4	0	71 ± 4.6				
	2	15^{bc} ± 1.8	21^{c} ± 2.1	11^{ab} ± 1.4	4.4^{a} ± 0.8	7.6
	4	3.6^{b} ± 0.1	4.2^{b} ± 0.4	0.8^{a} ± 0.1	1.1^{a} ± 0.0	1.2
	8	4.2^{ab} ± 1.2	7.3^{b} ± 0.6	2.1^{a} ± 1.1	2.4^{a} ± 0.5	3.9

$^{a, b, c}$ Means in the same row without a common superscript differ (P<0.05).

Apart from experiment 2, acivicin administration tended to be associated with a trend towards decreased intracellular cysteine, but acivicin significantly decreased cysteine (P<0.05) in experiment 4 after 4 h incubation with acivicin. A decrease in intracellular cysteine is consistent with decreased transport of cysteine into the cell as a result of γ-GT inactivation (Table 2.7).

Acivicin and NAC administered together significantly increased intracellular cysteine (P<0.05) in experiments 1 and 2 after 8 h incubation in comparison to the controls. Also, intracellular cysteine with NAC in combination with acivicin in experiments 1 and 2 was greater than with acivicin alone after 4 and 8 h incubation which was expected as NAC should supply cysteine by an alternative pathway and overcome restricted cysteine uptake by cells as a result of acivicin inhibition of γ-GT. However, intracellular concentrations in experiment 3 were unaffected by acivicin in combination with NAC while intracellular cysteine in experiment 4 after 4 h incubation significantly decreased (P<0.05) indicating that NAC cannot always overcome γ-GT inhibition and this may

differ with each acini preparation. Intracellular cysteine concentrations were significantly greater in experiments 2, 3 and 4 for NAC treated acini than when NAC was administered in combination with acivicin suggesting that γ-GT inhibition restricts cysteine uptake.

The change in intracellular GSH that occurred over time with incubation of acini was dependent on each acini preparation (Table 2.8). The differences between experiments in intracellular GSH concentration and trends over time may have given rise to the difference in milk protein secretion in experiment 1 (Figure 2.38) compared to the other three experiments involving acivicin and NAC (Figure 2.39).

Table 2.8 Intracellular glutathione concentrations (mean nmol/g cells ± SEM) in the presence of N-acetylcysteine and acivicin.

Results from four experiments (n=3) where isolated ovine mammary cells were incubated in the absence and presence of N-acetylcysteine (NAC) (1 mM) and/or acivicin (0.5 mM) in cysteine depleted medium.

Experiment	Time (h)	Control	NAC	Acivicin	NAC + acivicin	LSD
1	0	10 ± 1.4				
	4	21^{b} ± 0.14	4.9^{a} ± 1.2	28^{b} ± 3.0	52^{c} ± 3.6	8.1
	8	8.4^{a} ± 1.2	2.1^{a} ± 0.8	21^{b} ± 0.4	99^{c} ± 17	34
2	0	9.8 ± 2.4				
	4	8.1^{a} ± 0.6	3.2^{a} ± 0.4	30^{b} ± 1.0	48^{c} ± 4.7	5.8
	8	11^{a} ± 0.4	2.7^{a} ± 0.3	30^{c} ± 2.4	46^{d} ± 1.1	4.7
3	0					
	2	1.4^{a} ± 0.2	9.1^{ab}	9.2^{ab} ± 0.9	13^{b} ± 2.9	8.5
	4	2.7^{a} ± 0.9	2.3^{a}± 0.1	13^{b} ± 1.1	14^{b} ± 0.0	3.5
	8	2.4^{a} ± 0.6	3.7^{a} ± 0.2	37^{b} ± 1.6	50^{c} ± 3.9	5.6
4	0	8.3 ± 0.9				
	2	6.1^{a} ± 0.8	2.0^{a} ± 0.4	19^{b} ± 3.1	42^{c} ± 0.3	4.6
	4	4.2^{a} ± 0.7	3.7^{a} ± 0.3	31^{b} ± 1.5	23^{b} ± 5.0	12
	8	3.9^{a}	9.4^{a} ± 0.3	25^{b} ± 2.4	48^{c} ± 2.9	7.8

$^{a, b, c, d}$ Means without a common superscript differ (P<0.05).

NAC has previously been shown to increase intracellular GSH concentrations (Williamson *et al.*, 1982). However, in experiment 1 NAC significantly decreased intracellular GSH after 4 h incubation (Table 2.8). There was no difference between control and NAC concentrations at 8 h in experiment 1 and no change in GSH

concentrations with NAC in the other experiments. This indicates that either GSH is rapidly exported from the cell or that cysteine derived from NAC may be entering the milk protein synthesis pathway and not the pathway for GSH synthesis consistent with the increase in milk protein secretion in acini incubated with NAC (Figure 2.37).

Acivicin significantly increased ($P<0.05$) intracellular GSH concentrations in all experiments at each time point except in experiments 1 and 2 after 4 h where trends towards increased GSH was observed. This is consistent with other studies where γ-GT inhibition has lead to accumulation of GSH inside the cells and suggests that γ-GT is closely linked to GSH export (Griffith *et al.*, 1978; Griffith and Meister, 1979b).

The combined administration of NAC and acivicin to acini resulted in either a trend towards increased intracellular GSH or a significant increase ($P<0.05$) in comparison to controls or NAC or acivicin alone. That NAC increased GSH in the presence of acivicin indicates that NAC alone also increased intracellular GSH but with active γ-GT GSH was rapidly exported from the cell. Reduced GSH export by γ-GT inactivation allowed this effect of NAC on GSH synthesis to be observed.

It is surprising that the changes in milk protein secretion of NAC experiments 1 and 2 are not more similar considering that the concentrations, and changes in concentration with treatment, of intracellular cysteine are similar but this suggests many factors besides cysteine regulate protein secretion. Experiment 3 had lower initial GSH concentrations inside cells and together with experiment 4, generally had lower intracellular cysteine, but these yielded similar milk protein secretion to experiment 2 and could be combined (Figure 2.39). The increase in intracellular cysteine in all experiments confirms NAC as an alternative cysteine supply. The absence of a corresponding increase in milk protein production when acivicin is also administered suggests that in some acini preparations a signal for synthesis may be absent. Wheeler *et al.* (1995) have shown that in isolated ovine mammary acini, mRNA encoding for α_{s1}-casein, β-lactoglobulin, and α-lactalbumin declines significantly 1 h after plating. This may be the case in experiments 2, 3 and 4, but a signal may be present in experiment 1 that maintains mRNA transcription and allows the increased cysteine supply to be used in translation.

This series of experiments suggest that NAC may enhance milk protein synthesis by increasing intracellular cysteine. Also the absence of γ-GT activity, through acivicin administration, decreases cyst(e)ine supply. NAC provides cysteine by an alternative route and can allow protein synthesis to continue in the absence of γ-GT activity.

2.5. Conclusions

- ATP content of lactating ewe mammary tissue was shown to be 0.7 μmol/g wet weight tissue. ATP content of ovine mammary cells confirmed viability after isolation from the gland. Trypan blue dye exclusion indicated that acini could be incubated for 8 h without a drop in viability.
- Incubation of acini in medium containing radiolabelled leucine resulted in increased protein secretion over time and this was confirmed to be milk protein rather than microbial protein. The major protein identified was β-casein with α-casein and κ-caseins also present
- Although the profile of caseins produced by isolated acini was different from that in ovine milk, these acini were considered a useful tool for monitoring milk protein secretion *in vitro* and were used in experiments to investigate the role of the γ-glutamyl cycle in milk protein synthesis.
- γ-GT activity in mammary tissue increased during pregnancy and peaked during early lactation consistent with bovine and rodent studies and this supports a role for γ-GT in milk production. γ-GT activity in the mammary gland of the Romney ewe at 2-3 weeks of lactation was established as 26 ± 2.4 μmol/min/g wet weight of tissue.
- Acivicin decreased γ-GT activity by 70% without affecting cell viability, as indicated by trypan blue dye exclusion, supporting a role for γ-GT in milk protein synthesis.
- Acivicin decreased secretion of α-casein by 60%, β-casein by 70%, and κ-casein by 80%. γ-GT has been shown to supply cyst(e)ine to cells but inactivation did not specifically decrease cysteine-containing κ-casein. Acivicin decreased intracellular cysteine indicating γ-GT has a role in cysteine supply.
- Acivicin decreased lactose production, which is consistent with decreased α-lactalbumin synthesis. Acivicin did not negatively affect uptake of amino-isobutyric acid (AIB) uptake, but actually increased uptake indicating that γ-GT inhibition may up-regulate conventional amino acid transport systems. γ-GT activity could not be

restored after acivicin treatment by using hydroxylamine, or by washing acini to remove acivicin.

- Increased acivicin concentrations decreased milk protein secretion without completely shutting down secretion, indicating milk protein synthesis can occur in the absence of γ-GT activity and suggesting that acivicin does not have adverse effects on acini. The inhibition constant calculated at 50% inhibition (IC_{50}) for acivicin on milk protein secretion was 3.4 μM.
- Inhibition of GSH synthesis using BSO decreased milk protein secretion in two of four experiments. The difference in response to BSO was probably due to oxidative stress, the degree of which could have varied in each acini preparation.
- The effect of the γ-glutamyl cycle intermediate, 5-oxoproline, on intracellular amino acid concentrations differed with each acini preparation but did support a role for 5-oxoproline in up-regulation of conventional amino acid transport systems.
- 5-oxoproline offers a mechanism for up-regulating amino acid transport when amino acid supply is adequate, and acts in concert with amino acid uptake by γ-GT. Changes in amino acid uptake by 5-oxoproline were not great enough to enhance milk protein secretion or reverse the effect of acivicin.
- Alternative cysteine sources, OTCA and NAC, increased intracellular cysteine and GSH. With OTCA intracellular cysteine increased further when GSH synthesis was inhibited. γ-GT inhibition increased intracellular GSH as a result of reduced export, and OTCA and NAC further increased GSH indicating these precursors provide cysteine for GSH synthesis.
- Inhibition of GSH synthesis decreased milk protein secretion but not when OTCA was present indicating that decreased GSH may lead to decreased cysteine uptake by γ-GT. After 18 h incubation, OTCA may overcome the effect of acivicin and increase milk protein secretion.
- In two experiments, NAC increased intracellular cysteine leading to increased milk protein secretion of up to 250% and increased α-, β-, and κ-casein secretion. NAC reversed the effect of acivicin on milk protein secretion in one experiment.
- The results of experiments using isolated ovine mammary acini varied with each individual preparation. A method for sustaining acini from a single preparation for use in several experiments may reduce this.

These experiments suggest that the γ-glutamyl cycle plays a role in the supply of cysteine and other amino acids for milk protein synthesis. In particular, γ-GT may play a role in cyst(e)ine supply for milk protein synthesis and inactivation of this enzyme can be overcome by supplying cysteine via NAC. Thus, cysteine appears to be limiting for milk protein synthesis in isolated ovine mammary acini and GSH appears to be required for cysteine uptake.

Chapter 3. The role of γ-GT and the effect of NAC on milk protein synthesis in the lactating goat

3.1. Introduction

Experiments using isolated ovine mammary acini reported in Chapter 2 suggest the possible involvement of the γ-glutamyl cycle in the transport of amino acids into mammary cells. This role may be in the supply of cysteine, which is thought to be limiting for milk protein synthesis (Clark *et al.*, 1978), given that the supply of cysteine through an alternative pathway (N-acetylcysteine (NAC)) was able to promote milk protein synthesis despite acivicin inhibition of γ-GT. However, acivicin decreased the secretion of all caseins not just cysteine-containing ones suggesting that γ-GT may have a more general regulatory role in the transport of amino acids required for milk protein synthesis.

The negative effect of acivicin on milk protein secretion by isolated acini may be an indirect one, through reduction of the γ-glutamyl cycle intermediate 5-oxoproline, which has been shown to stimulate amino acid transport (Lee *et al.*, 1996b). Extension of these experiments to the whole animal was required to verify whether the γ-glutamyl cycle regulates the net uptake of amino acid(s) and protein synthesis in the mammary gland *in vivo*.

In a whole animal study using goats as a model for the dairy cow, an experiment was designed in an attempt to replicate the results found in the *in vitro* isolated ovine acini experiments of Chapter 2. Lactating goats were chosen as a model for milk production because they are cheaper and easier to maintain than cows, and milk composition and hormonal regulation is similar to that of dairy cows (Forsyth, 1984). Amino acid uptake and milk protein synthesis were studied when NAC was administered by close-arterial infusion and acivicin was administered via the teat to lactating goats.

The composition of milk from goats is similar to that of cows' milk (Table 1.1) and each of the major proteins is present. However, production of α_{s1}-casein is high, medium, low, or zero depending on the genetic make-up of the goat (Alichanidis and Polychroniadou, 1996). Milk of high producers contains less α_{s1}-casein than bovine milk (11.9 mg/ml (Swaisgood, 1995)). Full length caprine α_{s1}-casein (α_{s1}-$CN^{A,B,C,E}$ 199 amino acid residues) has approximately 88% identity to bovine α_{s1}-casein, and approximately 97% identity to the ovine protein (Brignon *et al.*, 1989). Caprine α_{s2}-casein has 88% identity with the bovine protein (Trujilo *et al.*, 2000) and 99% identity with ovine α_{s2}-casein (Bouniol *et al.*, 1993). β-casein of goats' milk has 90% identity to the bovine variant A^2 and caprine κ-casein has 84% identity to bovine A variant (Trujilo *et al.*, 2000).

Some essential amino acids, including the sulphur amino acid cysteine, are thought to limit milk protein synthesis, as they are taken up from the blood in insufficient quantities relative to their output in milk proteins (Clark *et al.*, 1978). Cysteine is present in α_{s2}- and κ-casein, and the major whey proteins but not in α_{s1}- or β-casein (Table 3.1), which is the same as bovine milk proteins (Table 1.3) and similar to ovine proteins with the exception of ovine α_{s2}-casein which does not contain cysteine (Table 2.1).

Table 3.1 Amino acid composition of goat milk proteins (residues/protein).

	Casein				Whey protein[c]	
	α_{s1}[B]	α_{s2}[c]	β[c]	κ[c]	β-lactoglobulin	α-lactalbumin
Essential amino acids						
Histidine	4	5	5	4	2	3
Lysine	13	22	12	8	16	13
Methionine	5	4	6	1	4	0
Phenylalanine	7	8	9	4	4	4
Threonine	5	14	12	15	8	6
Tryptophan	2	2	1	1	2	4
Isoleucine	9	11	9	11	10	8
Leucine	17	12	20	8	21	13
Valine	9	12	21	11	10	6
Conditionally essential amino acids						
Tyrosine	11	11	4	9	4	4
Cysteine	0	2	0	3	5	8
Nonessential amino acids						
Alanine	12	10	5	16	16	5
Arginine	6	6	3	5	3	1
Asparagine	11					
Aspartate[a]	7	17	9	16	14	22
Glutamate[b]	20	45	43	26	24	13
Glutamine	15					
Glycine	9	4	6	1	5	5
Proline	19	18	33	19	8	2
Serine	18	14	15	13	6	6

[a] for α_{s2}- β-,and κ-casein, and whey proteins includes asparagines.
[b] for α_{s2}- β-,and κ-casein, and whey proteins includes glutamine.
[c] (Jenness, 1980).
[B] α_{s1}-casein B variant determined from the primary sequence given by (Brignon *et al.*, 1990).

Before administering acivicin to goats, a preliminary experiment was conducted in lactating sheep to verify the administration technique, the dose of acivicin required to produce an effect on milk protein production, and to clarify any possible secondary effects (Appendix F). The change in milk production and the absence of toxicity found in the preliminary experiment supported the use of acivicin, and the novel administration technique of injection up the teat.

In the present study, the role of γ-GT in the supply of amino acids for milk protein production was investigated by treating the lactating goat with acivicin, a potent inhibitor of γ-GT activity (Stole *et al.*, 1994). NAC was infused via the pudic artery into the mammary gland as a way of investigating the supply of cysteine in the lactating ruminant.

The objectives of the work described in this chapter were to determine the effects of acivicin and NAC on:

- milk yield and composition
- amino acid uptake by the gland by quantifying:
  - extraction %, which is the difference between arterial (A) and venous (V) concentrations as a percentage of arterial concentration
  - net uptake (mass per unit time), which takes into account mammary blood flow
  - ^{3}H-leucine to specifically measure mammary uptake and whole body leucine flux.

Part of this study has been published (Johnston *et al.*, 2001) (Appendix P).

3.2. Materials

Acivicin and dithiothreitol (DTT) were obtained from Sigma Chemical Co. St. Louis MO USA. Bomacillin (Procaine penicillin 300 mg/ml) was from Bomac Laboratories Ltd., Manukau City, Auckland, New Zealand. Ethylenediaminetetra acetic acid disodium salt (EDTA) was obtained from Ajax Chemicals, Auburn, NSW, Australia. Heparin sodium (ovine mucosa) (batch 5096113) was obtained from New Zealand Pharmaceuticals, Palmerston North, New Zealand. Pyrogen-free N-acetylcysteine (NAC) was obtained from BUFA B.V. Pharmaceutical Products, Holland. Oxytocin was from Vetpharm (NZ) Ltd., Glenfield, New Zealand. Saline (0.9% NaCl) was from Baxter Healthcare, Toongabbie, NSW, Australia. Seraprep™ was from Pickering Laboratories Inc. Mountain View, CA, USA. Sodium dodecyl sulfate (SDS) was obtained from BDH laboratory supplies, Poole, England.

A Watson Marlow 302-F pump (Watson Marlow Ltd., Falmouth, Cornwall, England) was used for infusion into animals, and a Watson Marlow 503-U variable speed pump was used for withdrawing blood for sampling over time. Pump tubing was from Elkay Products Inc., Worcester, MA, USA and Dural brand polyethylene tubing for acivicin administration (ID 0.58 mm OD 0.96 mm) was obtained from Critchley Electrical Products Pty. Ltd., Silverwater, NSW, Australia.

Transit-time ultrasonic 6 mm blood flow probes (6RS) and T106 research meter were from Transonic Systems Inc., Ithaca, NY, USA. Blood flow data was acquired using the Windaq/200 programme and data was analysed using the Windaq/EX programme both obtained from Dataq Instruments Inc., Akron, OH, USA.

A near infrared spectrometer from NIRSystems Inc., Silver Spring, MD, USA was used for analysis of milk samples. The nitrogen analyser for feed and milk protein total nitrogen analysis was a Carlo Erba NA1500 series 2, from Milan, Italy.

The HPLC used for ion exchange was a Shimadzu LC10Ai system with CBM-10A communications bus module, FCA-10AL solvent switching module, DGU-IUB helium degassing system, LC-10AD pump, SIL-10AD auto injector including peltier cooled

sample chamber, CTO-10A column oven, with a SPD-10A UV-Vis detector and a RF-10A spectroflurometric detector. A Pickering PCX3100 post-column reaction module (ninhydrin) was also used. The column was a 3 mm x 150 mm 5 μm lithium cation exchange column (part # 0353150) from Pickering Laboratories Inc., Mountain View, CA, USA. The Shimadzu LC4A (single module system) with a Waters refractive index detector, and a BioRad Aminex® anion cation exchange column HPX-87P, was used for the determination of lactose.

The flow injection apparatus for determination of cysteine in plasma samples comprised a Watson Marlow 505-U variable speed pump, Technicon voltage stabiliser, Technicon AutoAnalyser II manifold and temperature controller, AutoAnalyser 40 place sampler, AutoAnalyser colorimeter, and a chart recorder.

The online scintillation counter was an IN/US β-RAM, from NJ, USA and had a counting efficiency for ^{3}H of 30%.

One Saanen and three Toggenburg goats were obtained from Lynmwyra Dairy Goats, Woodville. Another Saanen goat was obtained from Eastwood Dairy, Takapau. All goats were non-pregnant and in mid-lactation (Appendix G). Ethics approval was obtained for this study from the Crown Research Institutes' Animal Ethics Committee Palmerston North, under proposal number 28/99.

3.3. Methods

3.3.1. Treatment of animals

Five goats were surgically modified by implantation of a transit-time ultrasonic blood flow probe around the right external pudic artery that feeds that side of the udder. A catheter was implanted into the same artery for infusion of NAC or physiological saline in controls. A catheter was also implanted into the mesenteric artery for arterial blood sampling. Surgical work was carried out by a team under the control of Dr Gordon Reynolds (Massey University) and Dr Nicole Roy (AgResearch). Animals received 2 ml antibiotic (Bomacillin) by intramuscular injection every day for 4 days after surgery. However, because goats had infections of the exit wound for the pudic artery catheter

and flow probe, each received 2 ml antibiotic everyday for the duration of the trial. In future this exit wound should be made above the tail rather than below to help avoid infection. Catheters were flushed to maintain competency every 2 weeks before the start of the trial with sterile 100 U/ml then 1000 U/ml heparin in saline and tied off. Forty days elapsed between surgery and the trial start date. One goat did not regain sufficient body weight after surgery (Appendix G) and was excluded from the trial.

Temporary catheters were inserted the day before the first experimental day, into both caudal superficial epigastric (milk) veins for venous blood sampling, and both jugular veins, one for infusion of ^{3}H-leucine, the other for oxytocin administration to aid milking. Goat 12 lost the mesenteric catheter shortly before the trial began. As a replacement, a catheter was implanted deep into the jugular vein until the blood could be seen pulsing in the tube, to collect mixed venous blood from the right ventricle of the heart. Arterial blood samples for Goat 12 were collected from this 'deep jugular' catheter in all experiments. Oxytocin for this animal was administered by subcutaneous injection on experimental days.

Animals were housed in an air-conditioned room, in metabolism crates with modified sides to narrow the crate and prevent nibbling of catheters (Figure 3.1 and Figure 3.5). Animals were fed chaffed lucerne hay and grain-based concentrate (Moozlee), *ad libitum* (10% refusal) by overhead continuous feeders with meadow hay and water freely available. Feed intake was monitored daily. Animals were acclimatised to conditions for 2 weeks before the start of the experimental trial.

On non-experimental days, goats were milked twice daily by machine. On the day before experimentation, in addition to morning and afternoon milking, goats were milked four times at 1.5-h intervals by hand using oxytocin (1 U via a jugular catheter). Animals were also acclimatised to slings on the day before experiments. Slings stopped the animals from sitting down and disrupting blood flow measurements on experimental days (Figure 3.2).

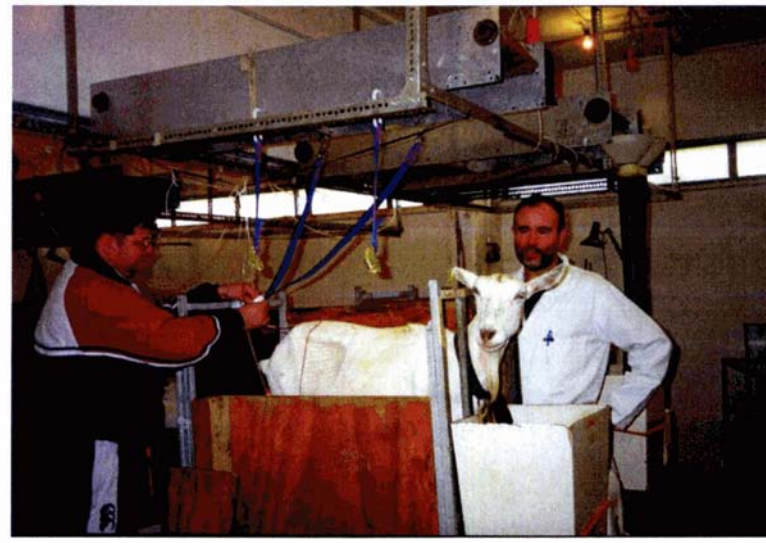


Figure 3.1 Metabolism crate set up.

Flossie (G11) in a metabolism crate with overhead continuous feeder. At left is Warren McNabb and at right is Julian Lee.

Figure 3.2 Goat prepared for experimental day.

Olga (G14) during an experimental day. Catheters were protected from the goats using surgical netting fitted around the body. Blue slings prevented goats from sitting and interrupting blood flow measurements. Goats had free access to food and water at all times. The overhead feeder for this metabolic crate had chutes that delivered feed concentrate (Moozlee) and chaffed lucerne directly to the trough.

A crossover experiment was designed with the help of AgResearch statistician David Baird so that each animal received four treatments (saline control, acivicin, NAC, NAC and acivicin) in random order. Animals were randomly allocated a number (11, 12, 13, 14) to fit with this design (Table 3.2). Two goats underwent treatment per experimental day with a 1-week rest between experiments.

Table 3.2 Experimental design.

Crossover design of experiment for four goats receiving a control saline treatment or acivicin and/or N-acetylcysteine (NAC).

Goat	Week			
	1	2	3	4
11	Acivicin	NAC	NAC + Acivicin	Control
12	Control	NAC + Acivicin	NAC	Acivicin
13	NAC + Acivicin	Acivicin	Control	NAC
14	NAC	Control	Acivicin	NAC + Acivicin

Based on an experiment with sheep that showed whole body infusion of 2 g of cysteine per day increased cysteine in peripheral blood plasma by approximately 50% (Roy & Lee, unpublished data), it was calculated from mammary blood flow that 0.5 g of NAC per day infused close-arterially would result in the same increase in cysteine supply (Appendix H). NAC prepared in saline (0.4 mg/ml) or saline alone was infused (1 ml/min) for 7.5 h via the pudic artery catheter.

Acivicin was administered 10 minutes after the udder was thoroughly stripped of milk by hand. Acivicin (5 ml of 14 mg/ml in saline) or saline was injected into the right hand side (RHS) lumen of the udder via the teat using 150 mm of polyethylene tubing, (ID 0.58 mm OD 0.96 mm) connected to a syringe by a 23G blunt needle. Saline (5 ml) was injected via the teat into the left hand side (LHS) of the udder. The glands was gently massaged for 5 min to distribute the treatment within the cistern.

^{3}H-leucine was infused to calculate total uptake of leucine and to monitor incorporation of label into individual milk proteins. A priming dose of infusate (2.96 MBq) and 25000 U heparin was injected into the animal via a jugular catheter using two 20 ml syringes immediately before the start of infusion (Appendix I). The infusate contained

^{3}H-leucine (37 MBq), 13 mg of unlabelled leucine, and 25000 U of heparin and was infused whole body (1 ml/min) into each animal via a jugular catheter for 7.5 h. Infusion of heparin prevented clotting of blood in catheters and collection tubes.

On experimental days, goats were milked by hand using oxytocin (1 U to Goats 11, 13, 14 via a jugular catheter and 10 U to Goat 12 by subcutaneous injection) to aid milk let down. Residual milk was removed by milking 4 times every 0.5 h before the start of infusion. After the start of infusion experimental animals were milked hourly. The udder was stripped 10 min before acivicin or control treatment and then animals were milked every 1.5 h to the end of infusion. Samples were collected from each side of the udder at the start of infusion, then 3, 5.5, 7, 8.5, and 24 h after the start of infusion as indicated in Figure 3.3.

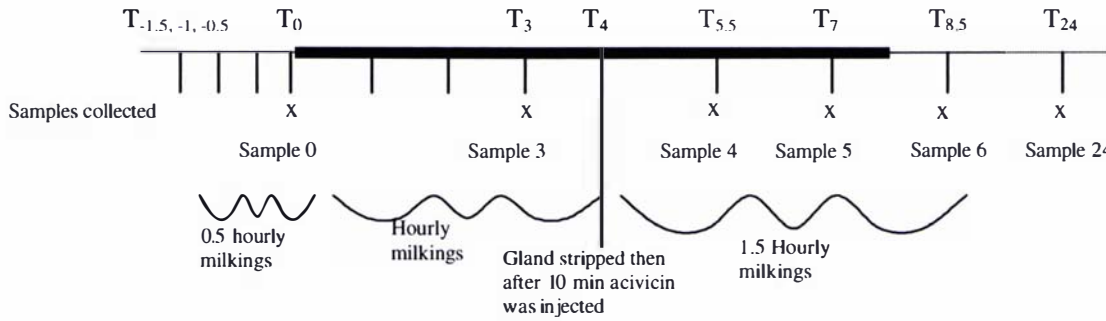


Figure 3.3 Milking schedule

On experimental days goats were milked by hand using oxytocin to aid milk let down.

A 10 ml blood sample was collected by syringe from the arterial catheter the day before and the day after the experiment. Arterial and venous blood samples were collected by 10 ml syringe 3-3.5 and 4 h after the start of infusion. Three 15 ml blood samples were collected immediately after acivicin or saline administration, by continual withdrawal using a peristaltic pump from arterial and venous catheters over 30, 60, then 120 minutes as described in Figure 3.4. Figure 3.5 shows the experimental set up for the collection of blood samples by pump.

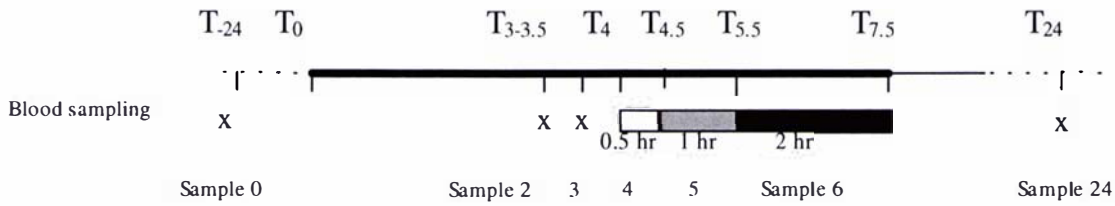


Figure 3.4 Blood sampling scheme.

Arterial and venous blood was sampled by syringe for samples 0, 2, 3 and 24, and by peristaltic pump for samples 4, 5 and 6.

Figure 3.5 Experimental set up.

Blood in collection tubing can be seen leaving the animal for collection in sample tubes in white plastic ice buckets.

Blood flow was measured by transonic probe for the duration of each experimental day except for the day Goat 11 received acivicin where blood flow was measured only for a few hours.

3.3.2. Preparation of milk samples

Milk fat was estimated immediately after collection by microcentrifugation (creamatocrit or fatocrit) (Fleet and Linzell, 1964). Skim milk was prepared by removing fat from centrifuged whole milk (900 g, 15 min, 4°C). Total protein in whole milk was analysed by near infrared transmittance spectrophotometry. As another measure of total protein, total nitrogen in skim milk was analysed using a nitrogen

analyser and nitrogen was converted to protein by multiplying by 6.38 (Great Britain working party on the composition of foods for infants and young children, 1980).

3.3.3. Determination of protein and SRA of ^{3}H-leucine in protein of milk

Milk protein was analysed by HPLC as described by Elgar *et al.* (2000) and radioactivity of proteins was determined after HPLC separation by an online scintillation counter (β-RAM) as described by Lee *et al.* (1993).

3.3.4. Determination of lactose in milk samples

Freeze dried milk (100 - 200 mg) was weighed into a preweighed centrifuge tube, diluted with 5 ml of MQ H_2O then mixed. Samples were mixed every 5 min during incubation for 15 min at 37 °C, and then centrifuged (850 g, 10 min). The supernatant was poured into preweighed tubes. The pellet was washed twice by resuspension in 2 ml MQ H_2O followed by centrifugation. The supernatants were combined and weighed. Lactose in the supernatant was determined by comparison with two lactose standards (1 and 5 mg/ml) after separation by HPLC using the LC4A system. A 50 µl of sample or standard was injected onto an Aminex® anion cation exchange column in a 60°C oven. The mobile phase was MQ H_2O with a flow rate of 0.6 ml/min.

3.3.5. Preparation of blood and plasma samples

Haematocrit (%) of blood was determined by microcentrifugation of blood immediately after collection.

Three types of sample were prepared from blood and labelled using in house nomenclature C, K or CP (Table 3.3). 'C' samples were prepared using blood (1 ml) that was added to preweighed centrifuge tubes and weighed, then mixed with 0.5 ml of a solution of 0.75% (w/v) SDS and 9 mM EDTA. Samples were incubated at room temperature for 15 min then mixed with 0.5 ml TCA (30% (w/v)), reweighed and centrifuged (3270 g, 15 min, 4°C). Supernatants were filtered through 0.45 µm CA disposable filters and labelled as 'C' samples stored at -85°C until analysis.

Table 3.3 In house nomenclature and uses of blood samples.

Sample	Measurements	Label
de-proteinised blood	GSH, cysteine, NAC by SBD-F derivatisation	C
DTT treated plasma	acivicin by ion exchange chromatography	K
de-proteinised plasma	amino acids by Picotag® derivatisation	CP
de-proteinised plasma	Cysteine using acid ninhydrin and photospectrometry	CP
de-proteinised plasma	^{3}H-leucine by HPLC and online scintillation counting	CP

Plasma was collected after red blood cells were pelleted by centrifugation (3270 g, 15 min, 4°C), and stored at -85°C until analysis. 'K' samples were prepared by adding 2 ml plasma to preweighed tubes and weighed, then mixed with DTT (3.7 mM) and weighed. Norleucine (50 µl of 3 mM in 0.1% (w/v) phenol) was added as an internal standard and the sample was reweighed. Samples were stored at -85°C until analysis.

'CP' samples were prepared from plasma (1 ml) that was added to preweighed centrifuge tubes and weighed, then mixed with 0.5 ml of a solution of 0.75% (w/v) SDS and 9 mM EDTA, and DTT (3.7 mM) and reweighed. Norleucine (50 µl of 3 mM in 0.1% w/v phenol) was added as an internal standard and the sample was mixed and reweighed. Samples were incubated at room temperature for 15 min then mixed with 0.5 ml TCA (30% (w/v)), reweighed and centrifuged (3270 g, 15 min, 4°C). The supernatant was filtered through 0.45 µm CA disposable filters and stored at -85°C until analysis.

3.3.6. Determination of thiols in goat blood

Glutathione, cysteine, homocysteine and NAC were determined in SBD-F derivatised C samples. Samples (20 µl) were treated and analysed in the same way as acini incubation medium samples as described in section 2.3.6.

3.3.7. Determination of cysteine in goat plasma

Cysteine concentration in plasma was determined from 'CP' samples reacted with acid ninhydrin after reduction using DTT, and determined photospectrometrically, at 570 nm, using a continuous flow analyser (Technicon AutoAnalyzer II) as described by Gaitonde (1967). Instrument set up and variables are shown in Figure 3.6. The variable speed

pump was set to 40% power giving 27 rpm. Sample cysteine concentrations were obtained from a standard curve.

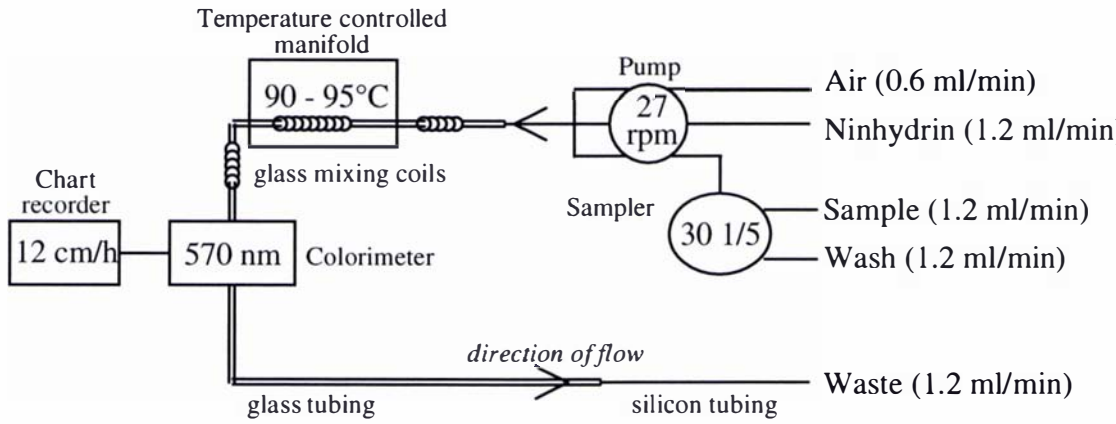


Figure 3.6 Flow injection apparatus.

Schematic diagram of the instrument set up for continuous flow injection analysis of cysteine in goat plasma samples. Solutions and air were pumped through silicon tubing at speeds in brackets, before reaching the glass tubing of the apparatus. The sampler was set to sample 30 samples per h with a wash ratio of 1/5 (20 sec sampling: 100 sec wash).

3.3.8. Analysis of plasma amino acids by Picotag® derivatisation

Amino acids in CP samples (50 µl) were analysed by Picotag derivatisation and separation by HPLC as described in section 2.3.7 with the exception of the standards. Standards for comparison with CP samples were matrix matched to samples by mixing 0.5 ml stock standard solution (section 2.3.7) with 2.5 ml matrix match solution (containing 13% w/w TCA, 0.33% SDS, 4 mM EDTA and 7 mM DTT) made up to 5 ml using MQ H_2O.

3.3.9. Detection of acivicin in goat plasma

'K' samples were prepared for separation by ion exchange by being diluted 1:1 with Seraprep and incubated on ice for 15 min, then centrifuged (13680 g, 5 min). Two additional samples were 'spiked' with 50 µl 0.5 µmol/l acivicin to allow positive identification of the acivicin peak.

The injection volume was set to 10 µl onto a lithium cation exchange column plumbed into the LC10Ai system in an oven set to 37°C. The elution system consisted of 4 mobile phases, buffer A Li 280 pH 2.750 (33 mM lithium citrate, 0.14 M lithium chloride, 0.16 mM sulpholane, 10.6 mM phenol, 0.03 mM HCl), buffer B Li 750 pH 7.50 (33 mM citric acid, 0.2 M lithium hydroxide, 0.44 M lithium chloride, 10.6 mM phenol), buffer C Li 750 high salt pH 7.50 (71.4 mM citric acid, 0.3 M lithium hydroxide, 1.5 M lithium chloride, 10.6 mM phenol) and buffer D (2 M LiOH) all filtered through 0.45 µm CA. Buffer B increased from 0% at 4 min to 31.3% at 40 min, then to 100% at 80 min, remaining at 100% until 95 min before dropping to 0% at 97 min. Buffer C increased from 0% at 97 min to 100% at 97.05 min then dropping to 0% at 122.10 min. Phase D increased from 0% at 122.2 min to 100% at 124 min, then dropped to 0% at 124.1 min and stopping at 152 min. The flow rate was set to 0.3 ml/min. Eluents were kept under a blanket of $He_{(g)}$ to remove N_2 and O_2 from solution. The UV detector was set to 570 nm and 440 nm.

3.3.10. Determination of SRA of ^{3}H-leucine in plasma

A 50 µl aliquot of CP sample was mixed with 2 ml of Starscint™ scintillation amplifier and analysed as described in section 2.3.5. CP samples (300 µl) were aliquoted into vials for online scintillation counting by a β-radioactivity counter (β-RAM) following separation by HPLC.

3.3.11. Treatment of data

The arterio-venous difference as a percentage of arterial supply (extraction %) of amino acids from plasma was calculated using the following equation:

$$\text{Extraction } \% = \frac{(\text{Conc}_A - \text{Conc}_V)}{\text{Conc}_A} \times 100$$

Eq. 3.1

where Conc is the concentration of amino acid in arterial (A) and venous (V) plasma. Positive values indicate uptake and negative values indicate release of amino acids from the gland.

Mammary blood flow (MBF) was calculated by applying the Fick principle as described by Davis and Bickerstaffe (1978) using the following equation:

$$MBF(l \cdot h^{-1}) = \frac{C \times P \times M}{(A - V)} \times \frac{10}{(100 - \text{haematocrit}\%)}$$

Eq. 3.2

where C is the content of amino acid in milk protein (g/100g), P is the protein content in milk (g/100g), M is the milk yield (g/h), and A-V is the difference between arterial and venous amino acid (AA) concentrations (mg/l). The AA content of protein used data from the literature for caprine milk (Appendix J). The protein content in milk was corrected to that synthesised in the mammary gland by multiplying by 97% (calculated from (Anifantakis, 1986; Swaisgood, 1995; Walstra *et al.*, 1999). MBF was an average of blood flows calculated from methionine, phenylalanine and tyrosine, because these amino acids are thought to be transferred directly from supply to milk (Davis and Bickerstaffe, 1978; Davis *et al.*, 1988).

Mammary blood flow (MBF) was used with arterio-venous difference of amino acids (AA) to calculate the net uptake or release (also know as net flux) of AA using the following equation:

$$\text{Net Uptake}(\mu mol \cdot min^{-1}) = \left(MBF(l \cdot min^{-1}) \times A - V(\mu mol \cdot l^{-1})\right) \times (1 - \text{haematocrit}\%)$$

Eq. 3.3

Positive values indicate net uptake of amino acids from the gland and negative values indicate net release.

The proportion of ^{3}H-leucine in plasma samples was determined from the β-RAM output and used to calculate Bq/ml leucine from results of total radioactivity counting of plasma. The specific radioactivity of ^{3}H-leucine in plasma was calculated as follows:

$$\text{Plasma SRA}\ (Bq \cdot \mu mol^{-1}) = \frac{^{3}H - \text{leucine in plasma}\ Bq \cdot ml^{-1}}{\text{leucine concentration in plasma}\ \mu mol \cdot ml^{-1}}$$

Eq. 3.4

The specific radioactivity of total milk protein used to describe the appearance of infused ^{3}H-leucine in milk protein was calculated using the following equation:

$$\text{Total protein SRA } (\text{Bq} \cdot \mu\text{mol}^{-1}) = \text{Bq} \cdot \text{ml sample}^{-1} \times \left(\frac{\text{mg protein} \cdot \mu\text{mol leucine}^{-1}}{\text{mg protein} \cdot \text{ml sample}^{-1}} \right)$$

Eq. 3.5

The specific radioactivity of individual milk proteins, was calculated as follows:

$$\text{Protein SRA } (\text{Bq} \cdot \mu\text{mol}^{-1}) = \text{Bq} \cdot \text{ml sample}^{-1} \text{ for individual protein} \times \left(\frac{\text{mg individual protein} \cdot \mu\text{mol leucine}^{-1}}{\text{mg protein} \cdot \text{ml sample}^{-1}} \right)$$

Eq. 3.6

Total uptake of leucine by the mammary gland was calculated as follows:

$$\text{Total uptake } (\mu\text{mol} \cdot \text{min}^{-1}) = \left(\frac{^{3}\text{H} - \text{leucine in plasma } (\text{Bq} \cdot \text{ml}^{-1})}{\text{SRA leucine in plasma } (\text{Bq} \cdot \mu\text{mol}^{-1})} \right) \times \text{MBF } (\text{ml} \cdot \text{min}^{-1}) \times 1 - \left(\frac{\text{haematocrit \%}}{100} \right)$$

Eq. 3.7

As an estimate of the whole body utilisation (flux) of leucine the irreversible loss rate (ILR) was calculated as shown by Lee *et al.* (1999) using the following equation:

$$\text{ILR } (\text{mmol} \cdot \text{h}^{-1}) = \frac{\text{Rate of } ^{3}\text{H - leucine infusion } (\text{Bq} \cdot \text{h}^{-1})}{\text{Plasma SRA } (\text{Bq} \cdot \text{mmol}^{-1})}$$

Eq. 3.8

where the plasma SRA was at pseudo plateau as estimated using the Michaelis-Menten equation (Appendix K).

Analysis of variance and 5% least significant differences (10% for blood flow as calculated using the Fick principle as this was not significant at the 5% level) (Genstat 5) were used to determine the significance of treatment and gland effects, and the occurrence of interactions between these factors.

3.4. Results and Discussion

3.4.1. The effect of acivicin and NAC on feed intake

Dry matter intake (Figure 3.7) was not affected by treatment and was 1860 ± 44 g/day (average ± SEM) for the four goats over the trial period. The decline in feed intake of Goat 12 on December 2 may have resulted from the loss of competency of the mesenteric catheter. For comparison with daily milk yield, see Appendix L.

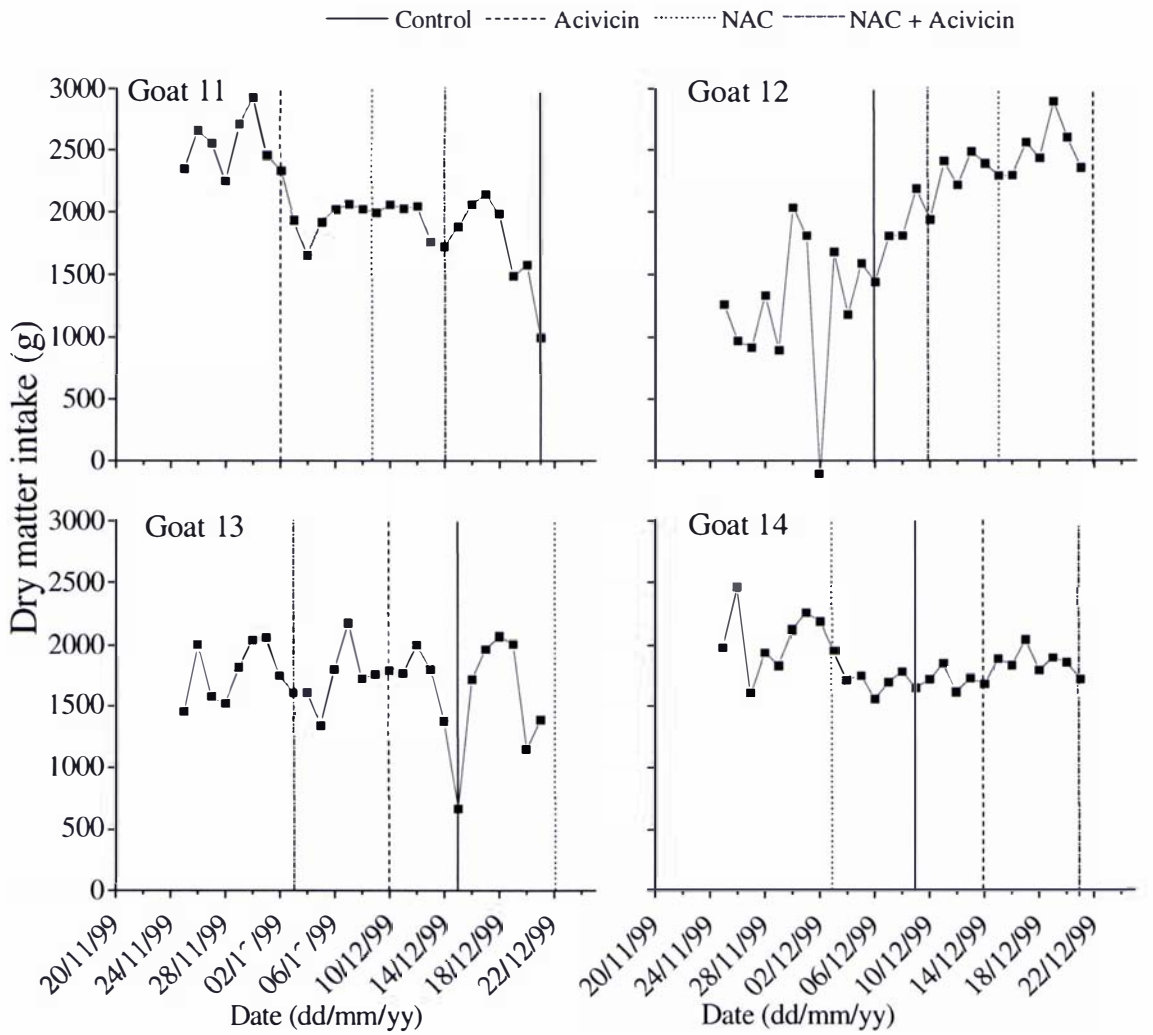


Figure 3.7 Daily dry matter intake.

Dry matter intake of four goats for the duration of a study in which they received a control saline treatment or acivicin and/or N-acetylcysteine (NAC).

3.4.2. The effect of acivicin and NAC on milk yield and composition

Acivicin administration alone did not affect total milk yield, protein concentration or yield, or fat concentration or yield (Table 3.4). There was no unilateral effect associated with the interductal injection of acivicin to the RHS of the udder. The infusion of NAC into the RHS of the udder via the pudic artery also did not result in any unilateral effect. Total milk yield from the RHS of the udder significantly increased ($P<0.05$) by approximately 20% of the control yield when NAC alone was administered but was not different from the LHS. NAC also increased ($P<0.05$) the protein concentration of milk from the LHS by 9.2%, and protein yield by 17% LHS and 18% RHS. Fat concentration significantly decreased ($P<0.05$) by approximately 20% in milk from the LHS. This corresponds with the increase in milk yield, as total fat yield did not change. Lactose concentration was not affected by NAC but a trend towards increased lactose yield was observed in milk from the LHS and lactose yield significantly increased (by 26%) on the RHS. These results indicate that cysteine supplied to the mammary gland in the form of NAC significantly increases milk production. This supports the results seen in experiments in isolated ovine mammary acini (Figure 2.38).

The increase in total milk yield, and protein concentration and yield with NAC treatment, was reversed with acivicin administration (Table 3.4). NAC may increase intracellular cysteine but this effect is reversed when acivicin is administered. Therefore, this study indicates that γ-GT has an important role in the supply of cyst(e)ine for milk protein synthesis.

Acivicin increased lactose concentration in milk by 9% from the LHS and 13.5% from the RHS ($P<0.05$). NAC in combination with acivicin also increased lactose with increases of 9% and 12% ($P<0.05$) from the LHS and RHS respectively (Table 3.4). Both sides of the udder were affected by treatments indicting a systemic affect. The mechanism for increased lactose with administration of both treatments together, appears to be different from NAC alone, which did not increase concentration but did increase yield, and probably results from acivicin action. The mechanism(s) for this increase cannot be determined from this experiment.

Table 3.4. Goats' milk yield and composition.

Mean milk yield and composition from each side of the mammary udder of four lactating goats that received acivicin and/or N-acetylcysteine (NAC) to the right udder half. Protein was determined from total nitrogen in skim milk.

	Control		Acivicin		NAC		NAC + Acivicin		LSD
	Left	Right	Left	Right	Left	Right	Left	Right	
Total yield (g/h)	50^{ab}	47^{a}	49^{a}	49^{a}	56^{b}	56^{b}	50^{ab}	48^{a}	6.5
Protein (g/100g)	2.8^{a}	2.9^{abc}	2.8^{a}	2.8^{a}	3.1^{c}	3.1^{bc}	2.9^{ab}	3.0^{abc}	0.20
Protein yield (g/h)	1.5^{ab}	1.4^{a}	1.4^{ab}	1.4^{ab}	1.7^{c}	1.7^{bc}	1.5^{ab}	1.4^{a}	0.24
Fat (g/100g)	4.8^{c}	4.5^{bc}	4.4^{bc}	4.5^{bc}	3.8^{a}	3.9^{ab}	4.9^{c}	4.9^{c}	0.64
Fat yield (g/h)	2.5^{a}	2.2^{a}	2.2^{a}	2.3^{a}	2.2^{a}	2.3^{a}	2.5^{a}	2.4^{a}	0.45
Lactose (g/100g)	4.0^{a}	3.9^{a}	4.4^{bc}	4.5^{c}	4.1^{a}	4.1^{ab}	4.4^{bc}	4.4^{c}	0.27
Lactose yield (g/h)	2.1^{ab}	1.9^{a}	2.2^{ab}	2.2^{ab}	2.4^{b}	2.4^{b}	2.2^{ab}	2.2^{ab}	0.37

$^{a, b, c}$ Means in the same row without a common superscript differ ($P<0.05$).
Means for total yield and fat are from 4 samples at different times after acivicin or saline treatment per goat.
Means for protein and lactose are from 3 samples at different times after acivicin or saline treatment per goat.

With the exception of α_{s1}-casein, the concentration of individual caseins in goats' milk was found to be similar to that of cows' milk (Table 3.5). The goats in the present study were found to produce different levels of α_{s1}-casein (Table 3.6) and can be classified as high, medium, and low producers as described by Mahé and Grosclaude (1993) and Alichanidis and Polychroniadou (1996). The effect of treatments on α_{s1}-casein concentrations has been statistically analysed separately for each goat.

Table 3.5 Individual casein concentration in goats' milk.

Concentration of individual caseins in milk from four goats receiving a control saline treatment compared with bovine milk.

Protein	Goats' milk (mg/ml)[a]	Cows' milk (mg/ml)[b]
α_{s1}-casein	2.4 ± 0.4	11.9
α_{s2}-casein	3.3 ± 0.1	3.1
β-casein	11.0 ± 0.3	9.8
κ-casein	3.0 ± 0.1	3.5

[a] Present study. Means are from 3 samples at different times after saline treatment per goat.
[b] (Swaisgood, 1995).

Table 3.6 Goats' milk α_{s1}-casein concentration.

Concentration of α_{s1}-casein in milk (mg/ml) from four goats receiving a control saline treatment. Production category was assigned according to reports by Mahé and Grosclaude (1993) and Alichanidis and Polychroniadou (1995).

Goat	Concentration	Production category
11	1.5[b]	medium
12	1.9[c]	medium
13	0.68[a]	low
14	5.4[d]	high

[a, b, c, d] Means without a common superscript differ ($P<0.05$; LSD 0.167).
Means are from 3 left and right samples at different times after saline treatment per goat.

There were no unilateral treatment effects on individual caseins. The results clearly indicate that the effect of treatment was minimal relative to the effect of genetic variation in terms of α_{s1}-casein concentration, and treatment had a different effect on α_{s1}-casein of each goat (Table 3.7). The α_{s1}-casein concentration in milk from Goat 11 was not significantly affected by any treatment, but decreased in Goat 12 and Goat 14 with acivicin and/or NAC (decrease in Goat 14 with NAC only on the LHS); and increased with acivicin in milk from the LHS of Goat 13.

It has not been determined why variants of α_{s1}-casein are expressed at different concentrations but from the present study it appears that γ-GT activity affects α_{s1}-casein production. Variants of α_{s1}-casein differ in amino acid content due to amino acid substitutions and differences in polypeptide chain length (Brignon *et al.*, 1989, Brignon *et al.*, 1990, Mahé and Grosclaude, 1993). Each variant may be affected differently by γ-GT inhibition depending on restricted supply of particular amino acids. Goats 11 and 12 were medium α_{s1}-casein producers but the effect of acivicin and NAC on α_{s1}-casein production by these two animals was different (Table 3.7). This may have been a statistical aberration. The significance of changes in α_{s1}-casein in relation to treatment may have improved if all four goats were the same type of α_{s1}-casein producer.

Cysteine transport into mammary cells has been hypothesised to occur through γ-GT activity, so inhibition of γ-GT by acivicin was expected to decrease concentrations of cysteine-containing proteins in milk such as α_{s2}- and κ-casein (Table 3.1). Similarly, supply of cysteine to the mammary gland by NAC infusion was expected to increase the concentration of cysteine-containing proteins in milk. Goat β-casein does not contain cysteine and as expected was not affected by acivicin or NAC treatment. However, acivicin treatment of goats increased α_{s2}-casein concentration in milk from both sides of the udder by 9% relative to the control, and increased κ-casein in milk from the RHS by 5.8%, and neither were affected by NAC (Table 3.7). This indicates that acivicin or inhibition of γ-GT is removing negative regulation from α_{s2}- and κ-casein synthesis and that cysteine supplied from NAC cannot be used for synthesis of these two proteins.

Table 3.7 The effects of acivicin and N-acetylcysteine on individual casein concentration in goats' milk.

Concentration (mg/ml) of α_{s1}-, α_{s2}-, β- and κ-caseins in milk of goats receiving acivicin and/or N-acetylcysteine (NAC) to the right udder half. α_{s1}-casein concentration has been shown for each goat separately because of genetic variation.

Protein	Goat	Control		Acivicin		NAC		NAC + Acivicin		LSD
		Left	Right	Left	Right	Left	Right	Left	Right	
α_{s1}-casein	11	1.4^{de}	1.5^{de}	1.7^{efg}	1.7^{efg}	1.4^{d}	1.4^{de}	1.6^{def}	1.7^{efg}	0.311
	12	2.0^{g}	1.9^{fg}	1.4^{de}	1.3^{cd}	1.6^{def}	1.4^{de}	1.5^{de}	1.4^{de}	
	13	0.68^{a}	0.69^{a}	1.0^{bc}	0.76^{ab}	0.79^{ab}	0.81^{ab}	0.73^{ab}	0.70^{a}	
	14	5.6^{k}	5.8^{jk}	4.6^{h}	4.6^{h}	5.0^{ij}	5.0^{j}	4.7^{hi}	4.7^{hi}	
α_{s2}-casein		3.3^{ab}	3.2^{a}	3.6^{c}	3.5^{bc}	3.4^{abc}	3.3^{ab}	3.3^{ab}	3.3^{a}	0.25
β-casein		11.8^{a}	11.5^{a}	11.6^{a}	11.9^{a}	11.3^{a}	11.1^{a}	11.7^{a}	11.9^{a}	0.92
κ-casein		3.0^{bc}	2.9^{ab}	3.1^{c}	3.1^{c}	2.9^{ab}	2.8^{a}	2.9^{ab}	2.9^{ab}	0.14

[a, b, c] Means in the same row without a common superscript differ ($P<0.05$). Also applies to columns for α_{s1}-casein.
Means are from 3 samples at different times after acivicin or saline treatment per goat.

This study indicates that the increase in milk protein concentration and yield when NAC was infused into the gland (Table 3.4) does not occur through increased casein production and indicates that increased supply of cysteine probably increases production of whey proteins. Whey proteins were not properly separated, and therefore, the effect of NAC on whey proteins cannot be confirmed in this study.

3.4.3. The effect of acivicin and NAC on mammary amino acid supply and extraction %

Previous reports of amino acid concentration in arterial plasma (Mepham and Linzell, 1966; Ranawana and Kellaway, 1977; Henderson and Peaker, 1983; Bequette *et al.*, 1997; Bequette *et al.*, 2000), and arterio-venous (A-V) differences (Ranawana and Kellaway, 1977; Backwell *et al.*, 1996) are similar to those for goats receiving a control saline treatment in this experiment (Table 3.8 and Appendix M respectively). This indicates that the administration technique using saline via the teat does not affect amino acid uptake or release. There were no reported values for tryptophan concentration in plasma from goats. The degree of tryptophan extraction shown here (LHS 9.4%, RHS 9.2%) was higher than that shown for cows (4.3%; Pacheco-Rios *et al.* (1998)).

Acivicin administration increased leucine concentration in A by 44% of the control concentration and in V by 51% ($P<0.05$) (Table 3.8). Decreases in glutamate (A 36%, V 25%; $P<0.05$) concentrations were also observed with acivicin treatment. These changes may result from normal variation in amino acid concentration, which may have been smoothed out with a greater number of animals involved in the trial. Only glutamate (18% less than control extraction % on the RHS) and serine (release almost doubled from both sides) extraction % were affected by acivicin ($P<0.05$) (Table 3.9). γ-GT has affinity for both these amino acids suggesting that acivicin decreases glutamate and serine extraction % by γ-GT inhibition. However, Barry (1964) observed that with natural variation A-V differences can vary day to day by ± 10%. Therefore, changes in extraction % must be greater than 20% to be considered biologically significant (Barry, 1964). These results indicate that γ-GT plays a role in serine uptake by the gland. A role for γ-GT in the regulation of uptake of other amino acids into the mammary gland cannot be ruled out since the changes caused by acivicin action on γ-GT could be masked by compensatory changes in the activity of other transporters.

Table 3.8. The effect of acivicin and N-acetylcysteine on amino acid concentrations in goat arterial and venous plasma.

Amino acid concentrations (mean μM ± SEM) in plasma from each side of the mammary udder of four lactating goats receiving acivicin or N-acetylcysteine (NAC) to the right udder half, compared with control. Means are from 3 samples at different times after acivicin or saline treatment per goat.

	Control			Acivicin			NAC			NAC + Acivicin		
	Arterial	Venous		Arterial	Venous		Arterial	Venous		Arterial	Venous	
		Left	Right		Left	Right		Left	Right		Left	Right
Essential amino acids												
Histidine	32 ± 2.7	26 ± 1.9	26 ± 2.0	36 ± 2.3	30 ± 1.9	30 ± 2.0	31 ± 3.9	27 ± 4.1	27 ± 4.0	31 ± 4.7	25 ± 4.0	25 ± 4.1
Lysine	73 ± 6.0	42 ± 5.1	42 ± 5.1	67 ± 4.7	38 ± 4.6	36 ± 4.0	91 ± 11	62 ± 11	60 ± 11	67 ± 6.2	36 ± 5.4	35 ± 5.6
Methionine	20 ± 2.2	9.5 ± 1.3	9.1 ± 1.4	21 ± 2.7	11 ± 1.9	11 ± 2.0	24 ± 2.1	15 ± 2.0	14 ± 2.0	21 ± 1.3	11 ± 0.6	9.8 ± 0.7
Phenylalanine	38 ± 1.3	25 ± 1.4	25 ± 1.3	37 ± 2.0	26 ± 1.3	25 ± 1.6	46 ± 2.9	33 ± 2.2	33 ± 1.9	37 ± 1.5	25 ± 1.2	25 ± 1.0
Threonine	53 ± 5.3	29 ± 3.7	30 ± 3.8	53 ± 4.5	32 ± 2.7	31 ± 3.0	68 ± 6.0	47 ± 6.0	45 ± 6.1	54 ± 3.4	31 ± 2.8	30 ± 2.8
Tryptophan	35 ± 2.1	32 ± 2.1	32 ± 2.0	40 ± 2.0	38 ± 2.0	38 ± 1.8	35 ± 1.7	32 ± 1.3	31 ± 1.1	38 ± 2.0	35 ± 1.8	35 ± 1.8
Isoleucine	61 ± 4.1	36 ± 3.2	35 ± 3.1	67 ± 4.5	41 ± 4.1	39 ± 3.6	78 ± 3.8	52 ± 2.3	51 ± 1.7	66 ± 3.5	40 ± 4.1	38 ± 3.8
Leucine	88 ± 5.3	49 ± 3.6	47 ± 3.0	127 ± 21	74 ± 12	71 ± 13	116 ± 7.5	75 ± 5.6	72 ± 3.8	113 ± 16	59 ± 5.1	56 ± 5.4
Valine	181 ± 21	125 ± 13	121 ± 9.8	182 ± 11	137 ± 8.1	140 ± 10	215 ± 16	164 ± 13	164 ± 12	156 ± 2.4	121 ± 4.0	120 ± 3.2
Conditionally essential amino acids												
Tyrosine	56 ± 3.7	43 ± 3.1	43 ± 3.1	61 ± 4.5	50 ± 5.0	49 ± 4.8	78 ± 8.6	67 ± 8.9	67 ± 8.6	59 ± 1.9	48 ± 2.1	47 ± 2.1
Cyst(e)ine	142 ± 6.4	134 ± 7.6	136 ± 9.9	124 ± 2.9	119 ± 3.3	120 ± 2.4	108 ± 6.4	104 ± 5.0	100 ± 5.5	115 ± 6.4	109 ± 4.5	106 ± 6.5
Nonessential amino acids												
Alanine	82 ± 5.4	63 ± 3.0	62 ± 3.6	77 ± 4.9	62 ± 3.1	59 ± 2.8	104 ± 13	89 ± 12	85 ± 13	88 ± 8.0	68 ± 6.2	67 ± 7.0
Arginine	90 ± 8.2	56 ± 8.0	57 ± 7.6	86 ± 8.1	57 ± 7.1	55 ± 6.5	98 ± 3.3	67 ± 3.0	67 ± 2.7	88 ± 7.8	55 ± 8.8	54 ± 8.9
Asparagine	30 ± 7.9	20 ± 6.0	20 ± 6.1	42 ± 4.0	31 ± 3.0	30 ± 2.8	45 ± 12	33 ± 12	33 ± 12	52 ± 4.3	41 ± 3.4	40 ± 3.7
Glutamate	95 ± 8.0	48 ± 7.6	40 ± 4.4	61 ± 3.9	33 ± 1.5	32 ± 1.3	96 ± 12	45 ± 5.6	51 ± 9.0	72 ± 5.1	37 ± 2.7	36 ± 2.9
Glutamine	253 ± 11	238 ± 12	240 ± 13	226 ± 11	209 ± 10	206 ± 8.8	260 ± 28	247 ± 29	250 ± 30	298 ± 19	280 ± 16	279 ± 17
Glycine	601 ± 56	603 ± 53	606 ± 52	507 ± 44	502 ± 41	506 ± 42	566 ± 53	576 ± 55	574 ± 54	666 ± 69	657 ± 67	658 ± 68
Proline	99 ± 6.0	82 ± 4.4	82 ± 4.7	92 ± 7.6	79 ± 5.9	77 ± 6.3	119 ± 11	104 ± 11	106 ± 11	108 ± 5.5	94 ± 4.0	93 ± 4.5
Serine	74 ± 6.2	83 ± 11	84 ± 9.6	66 ± 6.3	79 ± 7.7	81 ± 8.3	95 ± 11	111 ± 14	114 ± 14	86 ± 8.5	94 ± 8.0	96 ± 7.8
Taurine	77 ± 3.3	76 ± 2.4	77 ± 2.5	78 ± 4.4	77 ± 5.2	77 ± 4.4	65 ± 2.6	66 ± 2.8	65 ± 2.5	76 ± 5.9	75 ± 6.0	75 ± 6.0

Table 3.9 The effect of acivicin and N-acetylcysteine on amino acid extraction percentage from plasma in the goat.

Mean extraction % (A-V difference as a % of A) of amino acids from plasma by each side of the mammary udder of four lactating goats receiving acivicin and/or N-acetylcysteine (NAC) to the right udder half, compared with control. Means are from 3 samples at different times per goat.

	Control		Acivicin		NAC		NAC + Acivicin		LSD
	Left	Right	Left	Right	Left	Right	Left	Right	
Essential amino acids									
Histidine	19.2^{bcd}	18.8^{abc}	16.4^{abc}	17.7^{abc}	13.5^{a}	14.8^{a}	22.2^{cd}	24.6^{d}	5.70
Lysine	42.8^{b}	43.6^{bc}	43.9^{bc}	48.0^{cd}	34.8^{a}	37.0^{a}	48.1^{cd}	50.1^{d}	5.08
Methionine	54.6^{bc}	56.3^{c}	47.2^{ab}	48.8^{abc}	42.5^{a}	43.1^{a}	46.5^{ab}	52.2^{bc}	8.11
Phenylalanine	33.0^{ab}	33.4^{b}	27.6^{a}	30.9^{ab}	27.6^{a}	28.0^{ab}	31.0^{ab}	33.3^{b}	5.64
Threonine	45.5^{c}	45.5^{c}	39.0^{abc}	43.2^{bc}	34.0^{a}	36.3^{ab}	42.6^{bc}	45.1^{c}	7.67
Tryptophan	7.8^{a}	7.6^{a}	5.1^{a}	6.0^{a}	8.1^{a}	8.5^{a}	7.2^{a}	8.0^{a}	4.74
Isoleucine	41.8^{b}	42.8^{b}	40.0^{b}	43.4^{b}	33.0^{a}	33.1^{a}	40.9^{b}	43.6^{b}	4.55
Leucine	44.3^{cd}	45.5^{d}	40.3^{bc}	44.7^{d}	35.7^{a}	37.2^{ab}	44.6^{d}	48.0^{d}	4.19
Valine	27.5^{a}	29.9^{a}	24.2^{a}	22.5^{a}	23.6^{a}	23.3^{a}	22.8^{a}	23.3^{a}	8.98
Conditionally essential amino acids									
Tyrosine	22.9^{b}	22.8^{b}	18.4^{ab}	20.8^{ab}	17.3^{a}	16.6^{a}	19.0^{ab}	20.5^{ab}	5.10
Cysteine	6.1^{a}	5.0^{a}	4.4^{a}	2.9^{a}	3.2^{a}	7.1^{a}	3.5^{a}	7.2^{a}	8.41
Nonessential amino acids									
Alanine	21.8^{bcd}	23.3^{cd}	18.4^{ab}	22.2^{bcd}	16.7^{a}	20.2^{abc}	22.4^{cd}	24.3^{d}	3.96
Arginine	39.4^{bc}	38.2^{bc}	35.0^{ab}	37.6^{bc}	30.8^{a}	31.0^{a}	40.0^{bc}	41.6^{c}	5.64
Asparagine	25.6^{cde}	25.7^{cde}	26.9^{de}	28.6^{e}	19.06^{ab}	17.9^{a}	22.1^{bc}	23.7^{cd}	4.11
Glutamate	48.9^{ab}	55.6^{b}	43.3^{a}	45.7^{a}	48.9^{ab}	46.0^{a}	48.4^{ab}	50.5^{ab}	8.54
Glutamine	6.2^{a}	5.4^{a}	7.6^{a}	8.2^{a}	6.3^{a}	5.2^{a}	5.3^{a}	6.2^{a}	3.80
Glycine	-1.1^{a}	-1.8^{a}	0.48^{a}	-0.26^{a}	-1.7^{a}	-1.5^{a}	0.94^{a}	0.97^{a}	3.48
Proline	17.1^{c}	17.5^{c}	13.7^{abc}	16.0^{bc}	13.8^{abc}	11.4^{a}	12.6^{ab}	14.0^{abc}	3.90
Serine	-11.5^{c}	-13.8^{bc}	-22.1^{ab}	-26.4^{a}	-13.7^{bc}	-17.7^{abc}	-12.7^{bc}	-15.5^{bc}	9.61
Taurine	0.78^{a}	0.36^{a}	1.7^{a}	1.3^{a}	-1.6^{a}	-0.68^{a}	1.6^{a}	1.7^{a}	4.20

[a, b, c, d] Means in the same row without a common superscript differ ($P<0.05$).

Administration of acivicin into the teat of the RHS of the udder was expected to decrease extraction of AA by that side, but there was no unilateral effect. Acivicin was not detected in plasma but must have been entering the circulation to affect extraction % by the LHS of the udder. Leucine extraction % was different between LHS and RHS of the udder when acivicin was administered but these were not different from extraction % of the respective controls and therefore does not signify an acivicin effect. An improved method for acivicin administration may increase the response of the gland to this treatment.

A trend towards increased concentration of several essential amino acids (EAA) in A supply was observed with NAC treatment but only lysine (A 25% of control concentration, V 46%), and threonine (A 28%, V 56%) were significantly affected ($P<0.05$). Increases, in V concentrations only, were observed for methionine (56%), phenylalanine (22%), isoleucine 45%, leucine V 53%, and valine (34%) ($P<0.05$). Histidine and tryptophan were not affected (Table 3.8). Extraction % of EAA (Table 3.9), except histidine, decreased ($P<0.05$) on both sides of the udder by 17 to 22% of control values, but these reductions may not be biologically significant (Barry, 1964). Histidine decreased by 30% ($P<0.05$) but only on the LHS. Phenylalanine, tryptophan, and valine were not affected.

γ-GT has been shown to have no affinity for tyrosine (Thompson and Meister, 1977; Morita *et al.*, 1994) and as expected, inhibition of γ-GT using acivicin did not affect A and V concentrations (Table 3.8) or extraction % (Table 3.9) of tyrosine. Tyrosine A and V concentrations increased ($P<0.05$) by 39% and 56% of control concentrations respectively with NAC treatment but extraction % decreased by 26% on both sides of the udder.

Concentrations of alanine, proline and serine increased ($P<0.05$) with NAC treatment in A (27, 20, 28% respectively) and V (39, 28, 35% respectively) plasma, while taurine decreased significantly in A supply by 16% ($P<0.05$). NAC decreased ($P<0.05$) the extraction % of arginine (20%) and asparagine (28%) from both sides of the udder, alanine from the LHS (23%), and glutamate (17%) and proline from the RHS (35%). These changes were not unilateral.

NAC in combination with acivicin, increased histidine extraction % by the RHS (30%) of the udder and lysine on both sides of the udder (14%) ($P<0.05$).

Infusion of NAC into the RHS of the udder decreased extraction % of several EAA and NEAA by both sides of the udder. This indicates a systemic effect for NAC. That histidine and alanine were affected by NAC only on the LHS is difficult to explain but with a greater number of animals tested the trend towards a decrease observed on the RHS, might have been significant.

Changes in extraction % relate to either changes in AA uptake or release by the gland, or changes in A supply. The decrease in extraction % of AA that resulted following NAC treatment in this study could be explained by increased A supply, which may result from increased blood flow, leading to increased V concentrations, and does not suggest decreased uptake by the gland.

In the present experiment, cyst(e)ine concentrations in A plasma of control animals, were almost 2-fold higher than those previously reported by Lee *et al.* (1999). A trend towards decreased cyst(e)ine concentration was observed in both A and V plasma by 13% (Table 3.8) when acivicin was administered. γ-GT has a high affinity for both cystine and cysteine (Thompson and Meister, 1977) so a decrease in plasma extraction of cyst(e)ine was expected with acivicin. Cyst(e)ine extraction % by the RHS of the udder appeared to decrease with acivicin but because of large variation this observation was not statistically significant (Table 3.9). Thus, it was not possible to confirm the decrease in cyst(e)ine uptake caused by acivicin that has been shown previously in cultured human endothelial and pancreatic cells (Cotgreave and Schuppe-Koistinen, 1994; Sweiry *et al.*, 1995).

NAC increased total milk yield, and protein concentration and yield indicating that increased cysteine supply to the mammary gland can increase milk output. NAC did not alter milk production in the presence of acivicin (Table 3.4). This suggests the possibility that NAC was converted outside the mammary gland to cyst(e)ine and uptake into mammary cells was prevented by acivicin inhibition of γ-GT. Changes in milk production may result from NAC effect on the gland or increased blood flow.

While concentrations of glutamate, glycine and cysteine were not significantly affected by NAC an interesting trend was observed. Most of the amino acids measured showed trends towards increased A concentrations when NAC was administered, however, glycine and cysteine showed trends towards decreased supply and glutamate was unchanged. This suggests that these amino acids may be removed from circulation for GSH synthesis, probably by the liver.

The concentration of glutathione (GSH) in blood of goats in this study (Table 3.10) was lower than that reported by Knutson *et al.* (1994) (mean μM ± SEM) (823 ± 30) and Lee *et al.* (1999) (915 ± 69). GSH uptake from blood has been indicated in studies in cows (Pocius *et al.*, 1981) but the control extraction % values in the present study were negative indicating GSH was released and this is consistent with reports in human lymphoid cells (Dethmers and Meister, 1981). GSH was not significantly affected by NAC but a trend towards increased GSH concentration was observed in A blood, indicating either increased GSH production outside the gland or increased GSH supply to the gland by increased blood flow. Decreased GSH export with γ-GT inhibition has been shown in renal cells (Griffith and Meister, 1979b) but acivicin did not significantly affect GSH release in the present study.

Table 3.10 The effect of acivicin and N-acetylcysteine on glutathione concentrations and extraction percentage from goats' blood.

Arterial and venous concentrations (mean μM ± SEM) and mean extraction % (A-V difference as a % of A) (± SEM) of glutathione from blood by each side of the mammary udder of four lactating goats for control, acivicin and/or N-acetylcysteine (NAC). Treatment effects on extraction % were not significantly different (P<0.05; LSD 9.4).

Treatment	Vessel	Side of udder	Concentration (μM)	Extraction %
Control	**Arterial**		649 ± 20	
	Venous	**Left**	661 ± 37	-1.6 ± 3.90
		Right	681 ± 40	-4.6 ± 4.46
Acivicin	**Arterial**		672 ± 22	
	Venous	**Left**	700 ± 23	-4.6 ± 3.30
		Right	714 ± 35	-6.5 ± 4.43
NAC	**Arterial**		690 ± 28	
	Venous	**Left**	670 ± 33	3.0 ± 2.52
		Right	685 ± 30	0.81 ± 0.97
NAC + Acivicin	**Arterial**		653 ± 27	
	Venous	**Left**	692 ± 36	-6.3 ± 4.10
		Right	677 ± 27	-4.3 ± 3.22

Extraction % can give an indication of the relationship between supply and uptake, and what AA are required by the mammary gland. However, if arterial concentration of AA remains constant but blood flow increases, AA supply will increase and extraction % will decrease. Therefore, extraction % alone can be misleading. A better measure of AA uptake considering blood flow to provide the rate of mammary uptake for each amino acid is required.

3.4.4. The effect of acivicin and NAC on mammary blood flow and net uptake or release of amino acids

Each goat was fitted with a blood flow probe around the right pudic artery. However, when the experiment commenced the blood flow readings for Goats 13 and 14 were very low (10 - 20 ml/min) indicating that the probes in these animals were no longer measuring blood flow correctly. Data was collected from Goat 11 and 12 throughout the experiment, but was not measured for Goat 11 on the day of acivicin treatment. Blood-flow for Goat 11 on other days was consistent throughout the experimental day in the presence or absence of treatment (Figure 3.8). In Goat 12, blood flow was found to increase after treatment with acivicin and NAC alone, indicating that treatment may be affecting blood flow. However, in both animals blood flow did not change when treated with acivicin in combination with NAC.

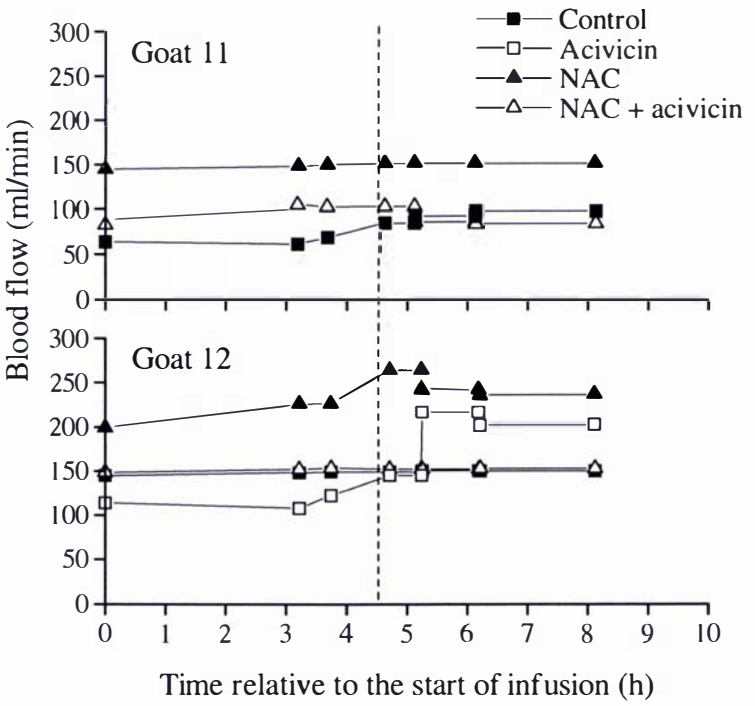


Figure 3.8 The effect of acivicin and N-acetylcysteine on blood flow measured by transonic probe.

Blood-flow was measured by transonic flow probe over experimental days in two goats treated with acivicin and or N-acetylcysteine (NAC). Dashed line indicates time of acivicin or saline administration. NAC infusion started at 0 h.

Blood-flow can also be calculated from the A-V difference of EAA that are considered to be transferred directly from supply to milk i.e. uptake to output ratio is close to 1, for instance methionine (Davis and Bickerstaffe, 1978), phenylalanine and tyrosine (Davis *et al.*, 1988). Blood-flow was calculated using Eq. 3.2. Treatment of goats with acivicin did not significantly affect blood flow as calculated by the Fick principle but NAC significantly increased blood flow on the RHS ($P<0.1$) and a trend towards increased blood flow was observed on the LHS (Table 3.11). Increased blood flow with NAC would account for the decrease in extraction % of amino acids that was observed with this treatment.

Table 3.11 The effect of acivicin and N-acetylcysteine on blood flow calculated using the Fick principle.

Blood-flow calculated using the Fick principle from methionine, phenylalanine and tyrosine arterio-venous differences, into the left and right sides of the mammary udder of four goats treated with acivicin and/or N-acetylcysteine (NAC) (ml/min). Means are from 3 samples at different times after acivicin or saline treatment per goat.

Treatment	Left	Right
Control	559^{ab}	507^{a}
Acivicin	639^{abc}	556^{ab}
NAC	656^{bc}	708^{c}
NAC + Acivicin	603^{abc}	510^{a}

[a, b] Means without a common superscript differ ($P<0.1$; LSD 145).

Milk yield has been shown to have a strong relationship with mammary blood flow. It is generally accepted that the ratio of blood flow to milk yield for goats is 500:1 (Linzell, 1974). For Goat 11 and Goat 12 the average milk yield was 0.97 ml/min giving a blood flow of 485 ml/min. The Fick principle calculated and an averaged blood flow for these two animals is 688 ml/min, while the probe measured blood flow was much lower (113 ml/min). However, the probes may not have been calibrated correctly and may have been measuring blood flow a factor too low. To check for this, probe measured blood flow was plotted along side blood flow calculated using the Fick principle, and a trendline was plotted showing a poor linear relationship between the two (Figure 3.9). This indicates that measurements from the ultrasonic probes must be corrected by a factor for use in establishing blood flow to the mammary gland. While probe blood flow was inaccurate, it was consistent throughout the experimental day with one exception (Goat 12 acivicin) supporting the use of probes for monitoring short-term changes in mammary blood flow.

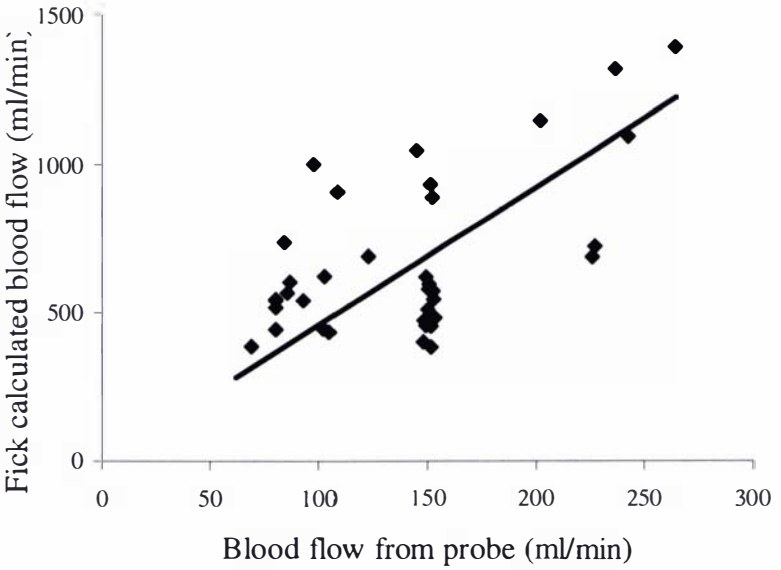


Figure 3.9 The comparison of blood flow measured using transonic probes and calculated using the Fick principle.

Blood-flow into one side of the mammary udder was measured by ultrasonic flow probe fitted around the right pudic artery in two animals treated with acivicin and or N-acetylcysteine. This has been plotted against blood flow calculated using the Fick principle. The fitted trend line ($R^2 = 0.23$) was forced through zero.

In the present study, blood flow from probes was not used and instead net uptake or release of amino acids was calculated using Fick principle blood flow data (Table 3.11). The net uptake or release of amino acids was calculated from Eq. 3.3. Net uptake of histidine, methionine, and tryptophan was not affected by treatment with acivicin or NAC (Table 3.12). Acivicin alone decreased ($P<0.05$) the net uptake of phenylalanine by 13% of the control by the LHS while NAC increased uptake ($P<0.05$) by 13% by both sides of the udder. γ-GT has affinity for phenylalanine so the effect of acivicin can be directly related to inhibition of uptake by γ-GT, although it is difficult to account for why the LHS was affected more than the RHS. There was a trend towards a decrease on the RHS and a greater number of animals may have improved the significance of this observation. Acivicin also affected leucine but caused an increase (44%; $P<0.05$) in uptake on both sides of the udder that is hard to explain. γ-GT has affinity for leucine so a decrease in uptake might have been expected. Phenylalanine and leucine are transported by the L transport system which is thought to transport acivicin also (Rosenfeld and Roberts, 1981), therefore competitive inhibition by acivicin might have

been expected to lead to decreased uptake of amino acids by this system. An increase in leucine indicates that acivicin or inhibition of γ-GT directly or indirectly, removed a factor that has negative control on amino acid uptake. For example, decreased γ-GT mediated uptake may increase the supply of amino acids and lead to up-regulation of sub-saturated transport systems. This is consistent with the increase in ^{3}H-AIB uptake in isolated ovine mammary acini in section 2.4.2.3.2 when acivicin was supplied.

In addition to phenylalanine, NAC also had a positive influence on the uptake of lysine (increased by 34% of control), threonine (21%), and isoleucine (26%) by the RHS and valine by both sides of the udder (142%) ($P<0.05$) (Table 3.12). Increased supply of these metabolisable amino acids, through increased blood flow, may affect mammary uptake by increasing cell metabolism. Alternatively, intracellular supply of cysteine delivered through NAC may signal mammary cells to increase uptake of other amino acids to match cysteine supply and lead to increased milk protein production.

Increased cysteine supplied from NAC may increase amino acid uptake via the γ-glutamyl cycle in three ways. i) Increased cysteine supply may increase intracellular GSH (Tateishi and Sakamoto, 1983) which is translocated to the plasma for use by γ-GT as a γ-glutamyl donor and this may lead to increased import of γ-glutamyl amino acids into the mammary gland (Griffith and Meister, 1979b; Bridges and Meister, 1985). ii) NAC may be converted to cyst(e)ine in the liver (Birnbaum *et al.*, 1952), which is an excellent γ-glutamyl acceptor (Thompson and Meister, 1977). Increased supply of acceptor may up regulate γ-GT activity (Griffith *et al.*, 1978) and increase AA uptake. NAC has been shown to induce expression of γ-GT in human mononuclear cells (Täger *et al.*, 1995). iii) Increased γ-GT activity through either (i) or (ii) may lead to increased production of 5-oxoproline which has been shown to up regulate AA transport by the A and $B^{o,+}$ transport systems (Lee *et al.*, 1996b).

Lysine was the only EAA affected by NAC treatment in combination with acivicin (Table 3.12). While uptake of lysine increased ($P<0.05$) on the RHS by 34% of the control with NAC alone, uptake increased on the LHS by 26% with NAC in combination with acivicin ($P<0.05$), but these changes may have been significant on both sides of the udder had a greater number of goats been included in this study.

Table 3.12 The effect of acivicin and N-acetylcysteine on net uptake or release of amino acids by the mammary gland.

Net uptake or release (μmol/min) of amino acids by the left and right sides of the mammary udder of four lactating goats receiving acivicin and/or N-acetylcysteine (NAC) to the right udder half. Means are from 3 samples at different times after acivicin or saline treatment per goat.

	Control		Acivicin		NAC		NAC + Acivicin		LSD
	Left	Right	Left	Right	Left	Right	Left	Right	
Essential amino acids									
Histidine	1.7^{a}	1.6^{a}	2.4^{a}	2.3^{a}	1.5^{a}	1.7^{a}	2.2^{a}	2.0^{a}	0.94
Lysine	10.0^{ab}	9.53^{a}	11.5^{abc}	11.9^{abc}	12.3^{bc}	12.8^{c}	12.6^{c}	11.3^{abc}	2.47
Methionine	3.7^{ab}	3.6^{ab}	3.6^{ab}	3.3^{a}	4.1^{b}	4.1^{b}	3.7^{ab}	3.7^{ab}	0.61
Phenylalanine	5.1^{bc}	4.9^{ab}	4.4^{a}	4.5^{ab}	5.8^{d}	5.5^{cd}	5.0^{abc}	4.8^{ab}	0.61
Threonine	8.3^{ab}	7.8^{a}	8.3^{ab}	8.4^{ab}	9.0^{ab}	9.4^{b}	9.5^{b}	8.7^{ab}	1.60
Tryptophan	1.0^{a}	1.0^{a}	0.7^{a}	1.0^{a}	1.4^{a}	1.2^{a}	1.2^{a}	1.2^{a}	0.70
Isoleucine	10.4^{ab}	9.8^{a}	12.0^{ab}	12.0^{ab}	12.4^{b}	12.3^{b}	11.6^{ab}	10.9^{ab}	2.17
Leucine	15.9^{a}	15.2^{a}	22.2^{b}	22.5^{b}	19.8^{ab}	20.4^{ab}	19.5^{ab}	21.1^{ab}	6.00
Valine	8.9^{a}	11.7^{a}	18.5^{abc}	15.7^{abc}	23.0^{bc}	26.4^{c}	13.2^{abc}	12.0^{ab}	10.5
Conditionally essential amino acids									
Tyrosine	4.2^{ab}	4.0^{a}	4.0^{a}	4.3^{ab}	4.8^{b}	4.2^{ab}	4.5^{ab}	4.3^{ab}	0.69
Cysteine	4.3^{a}	3.5^{a}	3.9^{a}	1.2^{a}	1.7^{a}	4.6^{a}	3.3^{a}	4.2^{a}	5.04
Nonessential amino acids									
Alanine	6.0^{ab}	6.0^{ab}	5.8^{a}	6.3^{ab}	6.5^{ab}	7.9^{b}	7.4^{ab}	6.9^{ab}	1.93
Arginine	12.4^{a}	11.1^{a}	11.3^{a}	11.4^{a}	13.3^{a}	13.1^{a}	12.9^{a}	11.4^{a}	2.30
Asparagine	8.7^{b}	6.6^{ab}	4.2^{a}	4.2^{a}	9.6^{b}	9.1^{b}	4.4^{a}	4.2^{a}	4.02
Glutamate	13.1^{ab}	17.3^{ab}	10.3^{a}	10.0^{a}	22.9^{b}	17.9^{ab}	13.1^{ab}	17.3^{ab}	10.8
Glutamine	1.8^{a}	1.9^{a}	7.3^{b}	7.4^{b}	4.8^{ab}	1.6^{a}	6.6^{ab}	6.9^{ab}	5.33
Glycine	-8.5^{a}	-7.4^{ab}	1.1^{abc}	-0.23^{abc}	-7.2^{ab}	-8.9^{a}	5.9^{c}	4.7^{c}	10.5
Proline	5.2^{ab}	5.4^{ab}	5.1^{ab}	5.3^{ab}	5.7^{b}	4.0^{a}	5.9^{b}	5.9^{b}	1.62
Serine	-3.8^{bc}	-3.7^{bc}	-6.0^{abc}	-6.4^{abc}	-7.7^{ab}	-10^{a}	-1.9^{c}	-2.0^{c}	4.85
Taurine	-0.14^{a}	-0.27^{a}	-0.38^{a}	0.00^{a}	-0.71^{a}	-0.70^{a}	0.50^{a}	0.64^{a}	1.51

$^{a, b, c}$ Means in the same row without a common superscript differ ($P<0.05$).

Changes in cysteine net uptake with acivicin or NAC were not significant (Table 3.12). Acivicin was expected to inhibit γ-GT and directly decrease cyst(e)ine uptake on the RHS. However, there was great variation in cyst(e)ine measurements so although there was a trend towards decreased uptake observed on the RHS this was not significantly different from uptake in controls. This high variation also affected uptake results for cyst(e)ine when NAC was infused as there was a trend towards increased uptake that was also not significant. This trend may have been the result of increased cyst(e)ine supply as a result of deacylation of NAC by the liver (Birnbaum *et al.*, 1952), but NAC did not appear to increase arterial concentrations of cysteine (Table 3.8). NAC has been shown to increase expression of γ-GT in human mononuclear cells (Täger *et al.*, 1995) and this may be the reason for the trend towards increased uptake of cysteine. The high variation associated with cyst(e)ine analysis will inevitably reduce as methods for measuring this thiol improve. In the present study, the chance of getting significant effects could have been improved if greater animal numbers were involved.

γ-GT has affinity for several NEAA but only asparagine and glutamine were affected by γ-GT inhibition with acivicin (Table 3.12). However, while asparagine uptake decreased (significant only on the LHS by 52% of control; $P<0.05$), the uptake of glutamine increased by almost 300%, which was unexpected since γ-GT has almost 10-fold greater affinity for glutamine than for asparagine. This increase may be related to the increase in leucine uptake but it is difficult to understand why this is occurring. It suggests acivicin may be removing some negative influence on the transport of AA by conventional systems. Serine was the only NEAA to be affected by NAC with an increase (176% of control; $P<0.05$) in net release on the RHS of the udder. Asparagine uptake by the LHS decreased 49% and glycine began to be released when NAC was supplied in combination with acivicin ($P<0.05$). While acivicin alone decreased asparagine, acivicin alone did not significantly affect glycine. The effect of NAC in combination with acivicin appears to be similar to the result of acivicin treatment alone and may be attributed to γ-GT inhibition.

Calculation of the net flux of GSH indicated GSH was released from the mammary gland (Figure 3.10). Treatment with acivicin and/or NAC did not significantly affect GSH net release although with NAC there was a trend towards decreased release. This

observation may be due to increased γ-GT activity because cells with high γ-GT activity such as renal cells have no measurable release of GSH (Griffith and Meister, 1979b). In renal cells inhibition of γ-GT has allowed GSH export from the cell to be measured. There was a trend towards increased release of GSH with acivicin indicating that γ-GT was inhibited but this observation was not significant due to high variability. GSH and cysteine were initially measured using the same HPLC method (section 3.3.6) but the cysteine recovery was low compared with other studies. Improved results were obtained from a method specific for cysteine (section 3.3.7) indicating that the GSH results are probably underestimated.

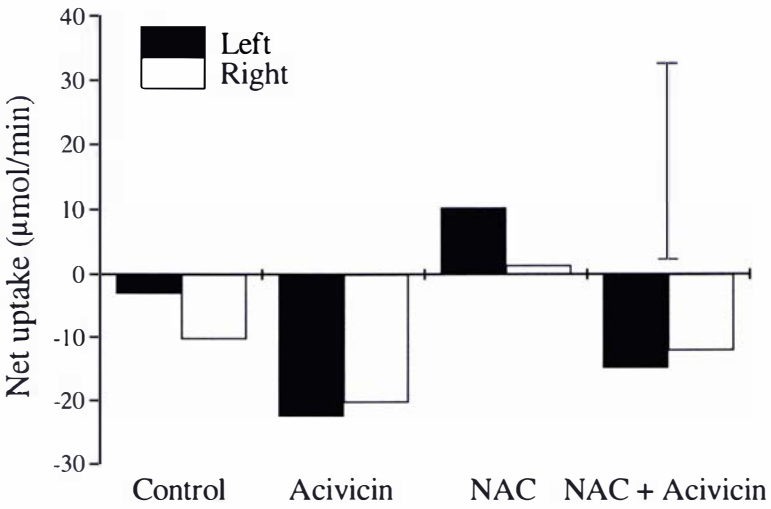


Figure 3.10 Net uptake or release of glutathione from whole blood by the mammary gland.

Mammary net flux of glutathione from the whole blood of four lactating goats treated with a saline control or acivicin and or N-acetylcysteine (NAC). Error bar indicates least significant difference.

The effect of acivicin on AA uptake was also investigated by comparing uptake (in the absence and presence of NAC) before and after treatment with acivicin or saline control. However, the uptake post treatment was not significantly different from control values.

While the supply of cyst(e)ine by NAC increased uptake of AA (phenylalanine, lysine, threonine, isoleucine and valine) by the mammary gland the effect of γ-GT inhibition by acivicin treatment on AA uptake was unpredictable. The decrease in uptake of phenylalanine and glutamine with acivicin treatment can be directly related to γ-GT inhibition. However, acivicin also led to an increase in uptake of leucine and asparagine. It is possible that acivicin blocks some factor that negatively controls conventional AA transport, but it is also likely that γ-GT inactivity causes increased rate of uptake by sub-saturated amino acid transport systems.

It is unlikely that NAC and acivicin are acting via the same mechanism since NAC has been shown to have many positive effects while acivicin has negative effects. NAC has been shown to protect embryos against acetaldehyde (Menegola *et al.*, 1995), and reduce dopamine toxicity and prevent apoptosis in rat pheochromocytoma (PC12) cells (Offen *et al.*, 1996). In contrast to NAC, acivicin has been shown to cause embryo toxicity (Stark *et al.*, 1987), inhibit the growth of *Serratia marcescens*, and has shown significant antineoplastic activity *in vitro* and *in vivo* (Rosenfeld and Roberts, 1981).

The effect of acivicin *in vivo* appears to be different from that *in vitro* (Chapter 2). In isolated ovine mammary acini, acivicin inhibited γ-GT and decreased milk protein secretion. Secretion in the goat was not affected by acivicin. Acivicin may not be inhibiting γ-GT *in vivo* because of less than effective binding of acivicin by γ-GT in the mammary gland, but also the complexity of the whole animal system may make it difficult to determine acivicin effects. An improved administration technique for acivicin is required.

3.4.5. Comparison of amino acid uptake and output in milk protein

The pattern of AA uptake from plasma to output in milk protein provides an indication as to which AA may be limiting for milk protein production. Net uptake and release of AA from plasma by the mammary gland in the present study was compared with AA output in protein of goats' milk calculated from data reported in Mepham and Linzell (1966), Jenness (1980) and Brignon *et al.* (1990) and is shown in Appendix J (Table 5.7). The net uptake of AA from plasma accounts for 60% of the AA that appears in milk protein (Appendix N). Although total nitrogen uptake is enough to account for

output in milk (Mepham, 1982), uptake of AA has previously been shown to be insufficient for output in milk (Backwell *et al.*, 1996; Bequette *et al.*, 1997). The uptake of arginine and branched chain amino acids (isoleucine, leucine and valine) has previously been shown to exceed output in milk protein (Mepham, 1982; Backwell *et al.*, 1996) supporting ratios found in the present study (Figure 3.11). Backwell *et al.* (1996) have reported that some histidine, phenylalanine, and proline is supplied to the mammary gland as small peptides present in arterial plasma and AA for milk protein production may also be supplied from erythrocytes (Hanigan *et al.*, 1991).

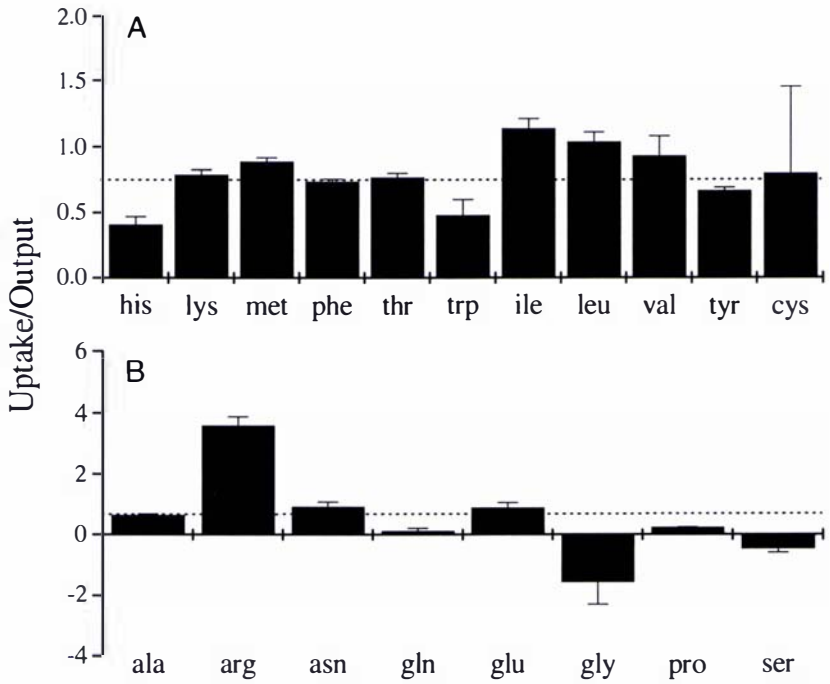


Figure 3.11 Mammary gland amino acid uptake to output ratios.

The ratio of amino acid uptake from plasma, to calculated output in milk protein from both sides of the udder of four lactating goats given control (saline) treatments. (A) Essential amino acids; (B) Nonessential amino acids. The dotted line is the average ratio of methionine, phenylalanine and tyrosine, which are considered to be transferred stoichiometrically from blood to milk. Error bars indicate standard error of the means.

The ratio of AA uptake to output has been used to indicate AA that are potentially first limiting for milk protein synthesis. Cysteine has been thought to be limiting for milk protein synthesis since it has been shown to be taken up from the blood in amounts insufficient to account for output in milk protein (Clark *et al.*, 1977; Lee *et al.*, 1999).

However, the uptake of cysteine in this study appears to be equal to output in milk although there is large variability. The present study suggests that histidine, in agreement with Bequette *et al.* (2000) and Pacheco-Rios (2000), and tryptophan may be first limiting for protein synthesis in the mammary gland.

3.4.6. The effect of acivicin and NAC on leucine kinetics

The uptake of amino acid by the gland can be investigated more accurately using radiolabelled AA (Figure 3.12). The infusion of a radiolabelled amino acid also allows the appearance of that amino acid in milk protein to be studied. ^{3}H-leucine was chosen for analysis because it was used in isolated ovine mammary acini experiments that led to the present study in goats, and is present in similar amounts in individual milk proteins (Table 3.1). Results that involve ^{3}H-leucine all involve specific radioactivity (SRA) of leucine in plasma or protein and are shown in Table 3.13.

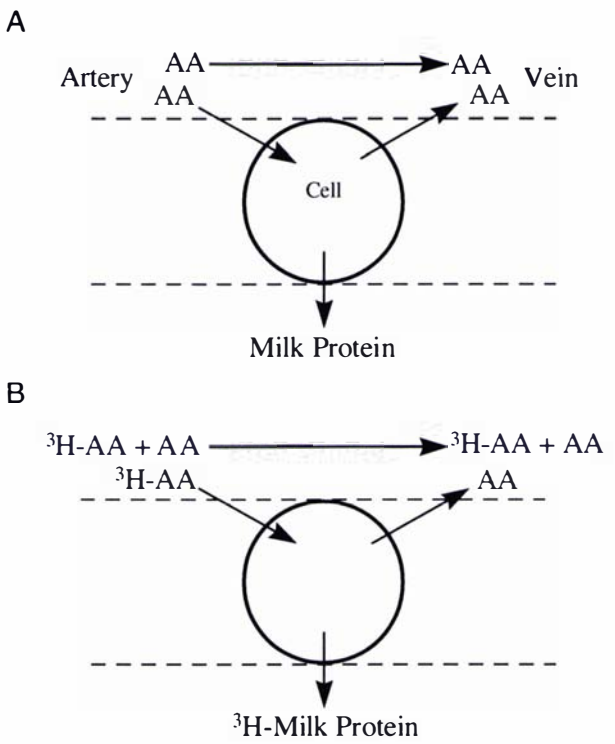


Figure 3.12 The use of radiolabelled AA in kinetic studies.

(A) Net flux of amino acids (AA) cannot distinguish amino acids that are not imported into the cell from those exported by the cell. (B) Total uptake requires supply of labelled AA (such as ^{3}H-AA) and can show the actual import of AA and incorporation into individual milk proteins.

Table 3.13 The effect of acivicin and N-acetylcysteine on leucine uptake kinetics.

^{3}H-leucine enrichment of plasma, mammary uptake and incorporation into milk protein of four goats receiving acivicin and/or N-acetylcysteine (NAC) treatments to the right udder half. Irreversible loss rate (ILR) has been corrected for metabolic body weight. Means are from 3 samples at different times after acivicin or saline treatment per goat, except for ILR. ILR was calculated from a single pseudo-plateau specific radioactivity (SRA) for each goat with each treatment. Pseudo-plateau of SRA in plasma was estimated using the Michaelis-Menten equation.

	Control		Acivicin		NAC		NAC + Acivicin		LSD
	Left	Right	Left	Right	Left	Right	Left	Right	
SRA Plasma (Bq/µmol)									
Arterial	488^{a}		521^{a}		536^{a}		512^{a}		124
Venous	529^{abc}	460^{abc}	432^{ab}	414^{a}	577^{bc}	590^{c}	486^{abc}	473^{abc}	155
SRA total milk protein (Bq/µmol)	27^{c}	26^{bc}	24^{bc}	24^{b}	19^{a}	19^{a}	26^{bc}	26^{bc}	3.3
SRA individual milk protein (Bq/µmol)									
α_{s1}-casein	42^{a}	45^{a}	46^{a}	42^{a}	36^{a}	35^{a}	41^{a}	46^{a}	13.8
α_{s2}-casein	21^{ab}	17^{ab}	21^{ab}	22^{b}	16^{ab}	16^{ab}	14^{a}	16^{ab}	6.9
β-casein	21^{bcd}	23^{d}	17^{a}	18^{ab}	18^{ab}	19^{abc}	23^{cd}	23^{cd}	3.7
κ-casein	25^{a}	29^{ab}	40^{b}	33^{ab}	22^{a}	25^{a}	35^{b}	37^{b}	11.5
Leucine ILR (µmol/min/kg$^{0.75}$)	7.2^{a}		8.0^{a}		6.6^{a}		6.6^{a}		3.75
Total uptake (µmol/min)	12^{a}	16^{a}	28^{b}	27^{b}	18^{ab}	29^{ab}	22^{ab}	21^{ab}	10.4
Total uptake as a % of ILR	9.3^{a}	12^{a}	19^{a}	18^{a}	15^{a}	14^{a}	17^{a}	16^{a}	9.4
Uptake:Output	0.71^{a}	1.0^{ab}	1.7^{bc}	1.9^{c}	1.0^{ab}	1.0^{ab}	1.3^{abc}	1.3^{abc}	0.75

$^{a, b, c}$ Means in the same row without a common superscript differ ($P<0.05$).

As expected, arterial SRA (Eq. 3.4) was not affected by NAC or acivicin treatment. The SRA in venous plasma with treatments was also not significantly different from the control, although on the RHS acivicin alone reduced the SRA of venous blood significantly below that of NAC alone.

After 5.5 h of isotope infusion the SRA of total protein (Eq. 3.6) was expected to be approximately 70% of the arterial plasma SRA cf. Bequette *et al.* (1994). The SRA of total protein in this study was approximately 5% of arterial plasma SRA. This low result may have arisen from the quenching of scintillations in skim milk samples (see Appendix O for a sample of data used to calculate protein SRA). The SRA of leucine in total milk protein decreased significantly with infusion of NAC indicating that NAC disrupts incorporation of leucine into milk protein.

The incorporation of infused ^{3}H-leucine into individual milk proteins was measured by calculating the SRA using Eq. 3.6. The incorporation of ^{3}H-leucine into the different milk proteins was not constant indicating that precursor leucine is being drawn from different intracellular pools. Incorporation of ^{3}H-leucine into α_{s1}- and α_{s2}-casein was not affected by either treatment. However, the SRA of β-casein decreased ($P<0.05$) with acivicin and NAC (RHS) alone, while κ-casein SRA increased ($P<0.05$) with acivicin alone (LHS) and acivicin in combination with NAC (both sides). This indicates that acivicin and NAC are affecting the use of leucine precursor pools for production of specific proteins.

Amino acids taken up from the blood can be used for the synthesis of protein which can be secreted in milk or degraded back into free amino acids, or can be oxidised inside the gland. Irreversible loss rate (ILR) of an amino acid (also known as whole body flux) based on SRA in blood, is a measure of utilisation of the labelled amino acid by the whole body and provides an estimate for net protein synthesis even though it is the sum of protein synthesis and oxidation (Lee *et al.*, 1995). ILR was corrected for metabolic body weight (MBW) ($kg^{0.75}$) (Lobley, 1994; Bequette *et al.*, 1997) to smooth out differences in weight and age of goats that may affect whole body calculations.

In this study, SRA of leucine was not determined in whole blood but in plasma, and this has been corrected using haematocrit to give ILR using equation Eq. 3.8. The mean ILR in this study, uncorrected for MBW (8.91 mmol/h = 28 g/d) compares well with the ILR for leucine in lactating goats (9.14 ± (SED) 0.54 mmol/h) reported by Bequette *et al.* (1996). Treatment of goats with acivicin and/or NAC did not significantly affect ILR (Table 3.13).

The estimation of total leucine uptake from
Eq. **3.7** shows that acivicin significantly ($P<0.05$) increases the uptake of infused leucine by both sides of the udder and that NAC alone and in combination with acivicin has no significant effect on leucine uptake (Table 3.13). This is in agreement with the affect of acivicin on net uptake (Table 3.12). Acivicin may increase uptake through up-regulation of transport systems that results from γ-GT inactivation because availability of amino acids for import may be increased. Total uptake as a percentage of ILR indicates that 9 to 19% of ^{3}H-leucine is partitioned to the gland (Table 3.13). Loss of ^{3}H-leucine to the gland was not affected by acivicin or NAC treatments.

The ratio of total uptake to output in milk protein was less than one on the LHS of the udder and one on the RHS showing that the uptake of labelled leucine is sufficient to meet demands of protein synthesis in agreement with the ratio of net uptake to output (Figure 3.11). However, previous reports indicate that leucine is normally taken up in excess with considerable oxidation occurring inside the gland (Bequette *et al.*, 1996). Uptake of leucine did exceed output when acivicin was supplied alone or in combination with NAC.

γ-GT has affinity for leucine, although this is not as great as for several other amino acids (Table 1.7). Acivicin transport has been reported in mouse P388 leukemia cells by the L transport system and is competitively inhibited by leucine (Rosenfeld and Roberts, 1981). Therefore, administration of acivicin was expected to cause a decrease in uptake of leucine by either γ-GT inhibition or competitive inhibition of other transporters. The fact that a decrease in total leucine uptake was not found in this experiment, but rather an increase, indicates that acivicin inhibition of γ-GT activity negatively regulates leucine uptake by other transport systems. The supply of cysteine through NAC did not

affect total uptake and increased net uptake of some amino acids but not leucine. The role of cysteine in uptake by the mammary gland would be easier to interpret with a more sensitive method for measuring this amino acid.

3.5. Conclusions

- Supply of cysteine to the mammary gland through NAC increased milk yield, protein concentration and protein yield suggesting that cysteine potentially limits milk production.
- Acivicin inhibition of γ-GT affected lactose yield, but also prevented NAC effects indicating γ-GT is important for NAC action on milk production and suggesting the possibility that NAC is converted to cysteine outside the mammary gland, then transported into cells by γ-GT.
- NAC and acivicin affected α_{s1}-casein production differently for each goat, which is probably due to genetic variation that has been well documented in goats (Grosclaude *et al.*, 1994), although the two medium α_{s1}-casein producers were also affected differently.
- The concentration of cysteine-containing proteins, α_{s2}- and κ-casein, in milk increased with acivicin. Inhibition of γ-GT may increase uptake of amino acids for protein synthesis by sub-saturated transport systems. NAC supply showed cysteine did not limit α_{s2}- or κ-casein production.
- Incorporation of leucine into β-casein decreased with acivicin suggesting that γ-GT inhibition restricts leucine uptake for incorporation into protein. However, κ-casein SRA increased with acivicin indicating γ-GT activity inhibits uptake by other transporters and suggests that amino acids for individual milk proteins come from different precursor pools.
- Casein production could not account for increased and decreased total milk protein with NAC and acivicin respectively, so these must result from changes in whey proteins, which unfortunately were not measured.
- Treatment with NAC and/or acivicin did not affect leucine ILR, an estimate for protein synthesis in the whole body, and did not affect the partitioning of leucine to the mammary gland.

- Measurement of blood flow by transonic probes was not accurate but was related to blood flow calculated using the Fick principle, and could be used to monitor relative changes in blood flow.
- NAC was associated with a trend in increased blood flow, which increased amino acid supply and decreased extraction %.
- Net uptake of phenylalanine, lysine, threonine, isoleucine and valine increased with NAC.
- Acivicin decreased phenylalanine and asparagine net uptake indicating γ-GT mediates uptake of some amino acids.
- Acivicin increased glutamine net uptake and leucine net and total uptake suggesting γ-GT suppresses a factor that negatively controls amino acid transport. γ-GT inhibition may increase leucine availability to transport systems that are sub-saturated when γ-GT is active, leading to increased rate of uptake (Figure 3.13).
- Comparison of net uptake to output in milk protein suggested histidine and tryptophan might be first limiting for milk protein synthesis. Cysteine uptake appeared to meet output suggesting that it would not limit milk protein production.
- Acivicin administration to the RHS did not produce unilateral effects indicating an improved method of administration is required.

This study in lactating goats suggests γ-GT has a regulatory role in amino acid uptake by the mammary gland. Further research targeting the manipulation of γ-GT activity may allow controlled production of individual proteins. Also, this research has for the first time shown the potential to manipulate protein yield in milk by cysteine supplementation by NAC supply. This has exciting prospects for the milk industry, in terms of increased protein yield in milk. However, the mechanism by which NAC affected protein yield is unclear, and needs to be fully understood before cysteine supplementation can realise commercial potential.

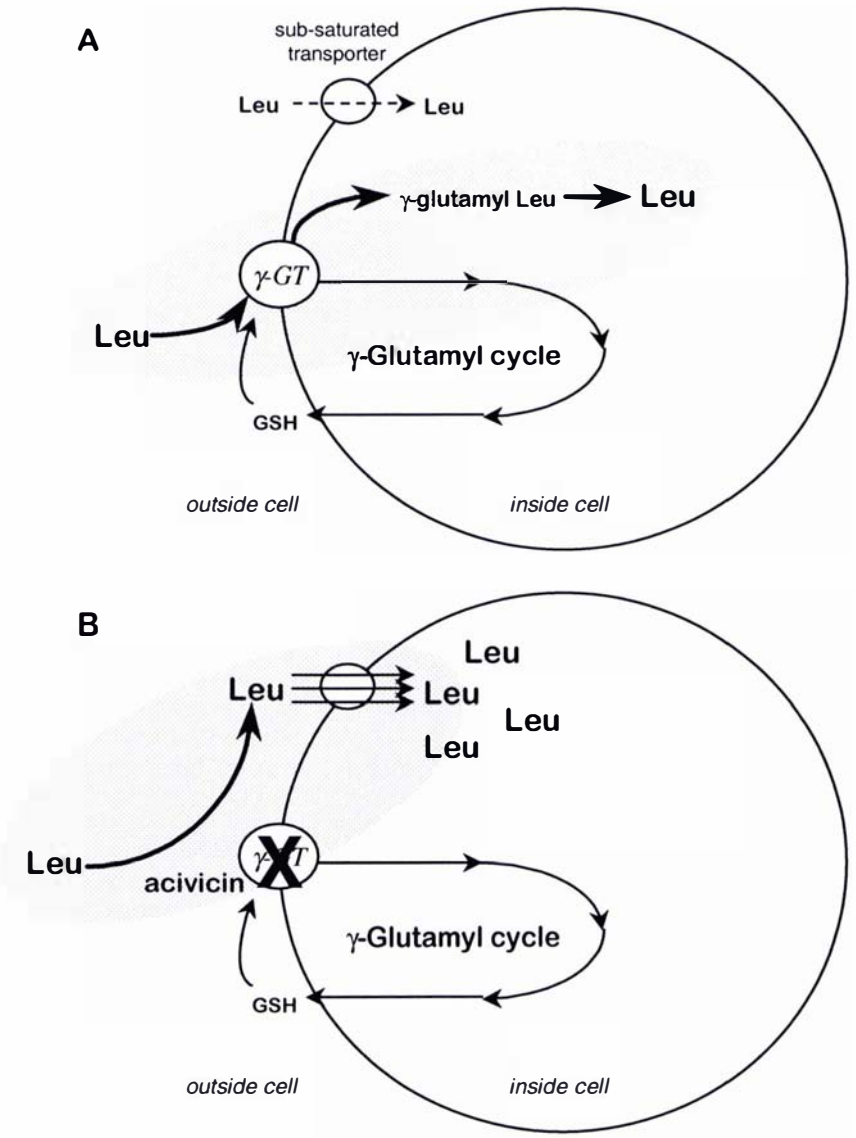


Figure 3.13 Proposed scheme for increased uptake of leucine by the mammary cell with acivicin.

(A) γ-GT mediates transport of leucine while other transport systems are sub-saturated. (B) γ-GT is inhibited by acivicin increasing the availability of leucine for transport by other systems, which are up-regulated and uptake is increased.

Chapter 4. General Discussion and Conclusions

Milk protein is increasingly important as an export commodity for New Zealand and the Dairy Industry would benefit from methods for producing an increase in the production of protein in milk. The mechanisms controlling milk protein production have proven difficult to characterise. Several amino acids have been suggested as limiting for milk protein production as they appear to be provided in amounts inadequate to meet output in milk. However, supply of these amino acids has not resulted in increased uptake by the mammary gland or increased milk production. This indicates the presence of a mechanism controlling amino acid uptake by mammary cells. The enzyme γ-glutamyl transpeptidase (γ-GT) was identified as a possible controlling factor for milk protein synthesis as it has been proposed to transport amino acids, has a high affinity for cyst(e)ine, which is considered limiting for milk protein synthesis, is the only enzyme know to degrade glutathione, providing cysteine and glutamate, and provides γ-glutamyl amino acids for production of 5-oxoproline by the γ-glutamyl cycle, which has been shown to up regulate amino acid transport by conventional amino acid transporters. Also, the activity of γ-GT has been shown to increase during pregnancy and peak during early lactation, then fall away at weaning, suggesting that this enzyme is important for milk production. The present study set out to clarify the role of components of the γ-glutamyl cycle, including γ-GT, in the supply of cysteine for milk protein synthesis in the ruminant.

4.1. Summary of in vitro and in vivo studies of the role of the γ-glutamyl cycle in milk protein synthesis

In the present study, the role of the γ-glutamyl cycle in milk protein production was studied in two systems. The first was a study *in vitro* using isolated ovine mammary acini. The second was a study *in vivo* in lactating goats.

In isolated acini the roles of several aspects of the γ-glutamyl cycle in milk protein synthesis were studied. These were: the role of γ-glutamyl transpeptidase (γ-GT) in amino acid transport; the role of 5-oxoproline in up-regulation of amino acid transport by conventional transporters; the effect of glutathione (GSH) synthesis on milk

secretion; and the role of cysteine as a limiting amino acid for milk protein synthesis studied using alternative sources of cysteine, oxothiazolidine carboxylic acid and N-acetylcysteine (NAC).

Key results from the study *in vitro* were decreased milk protein secretion with acivicin inhibition of γ-GT, which suggested this enzyme has an important role in amino acid uptake by mammary cells for protein synthesis, and increased milk protein secretion with NAC suggesting that cysteine is limiting for milk protein synthesis. It was not clear from studies using isolated ovine mammary acini whether or not the key role of γ-GT was to supply cysteine because results were inconsistent. One experiment with isolated acini clearly showed that cysteine supplied as NAC could overcome γ-GT inhibition indicating that under certain conditions γ-GT activity is essential for the supply of cysteine for milk protein synthesis.

An experiment was conducted in lactating goats to confirm whether the acivicin and NAC results in acini could be replicated in the whole ruminant. A summary of results found *in vitro* and *in vivo* is shown in Table 4.1. The supply of cysteine by NAC to the mammary gland of lactating goats caused an increase in milk yield and milk protein yield and concentration, supporting the results found *in vitro* and the use of isolated ovine mammary acini as a model for milk protein production in the whole ruminant. This result also adds strength to the hypothesis that cysteine is limiting for milk protein synthesis.

Changes in amino acid uptake in the goat experiment were difficult to interpret but suggest that the γ-glutamyl cycle through γ-GT has an important regulatory role in amino acid uptake by the mammary gland. Unfortunately changes in amino acid uptake with acivicin were not reflected by milk protein production. This is in contrast to results found *in vitro* and could be due to difficulty in delivering acivicin to the target site and does not necessarily refute the hypothesis that γ-GT is important for AA transport.

Table 4.1 Summary of effects of acivicin and N-acetylcysteine *in vitro* and *in vivo*.

Approximate changes with acivicin and N-acetylcysteine (NAC) treatment in studies *in vitro* studies of isolated ovine mammary acini, and *in vivo* studies using lactating goats.

ND, not determined.

	In vitro		*In vivo*	
	Acivicin	**NAC**	**Acivicin**	**NAC**
Total protein secretion	↓ 70%	↑ 250%	no change	↑ 17%
Individual protein secretion				
α_{s1}-casein	↓ 60%	↑ 200%	dependent on variant	
α_{s2}-casein			↑ 9%	no change
β-casein	↓ 70%	↑ 200%	no change	no change
κ-casein	↓ 80%	↑ 150%	↑ 6%	no change
Lactose	↓ 40%	ND	↑ 11%	↑ 10%
Intracellular cysteine	↓ 25-77%	↑16-190%	ND	ND
Intracellular GSH	↑ 33-638%	no change	ND	ND
γ-GT activity	↓ 70%	ND	ND	ND

4.2. *Schematic model of amino acid transport for milk protein synthesis*

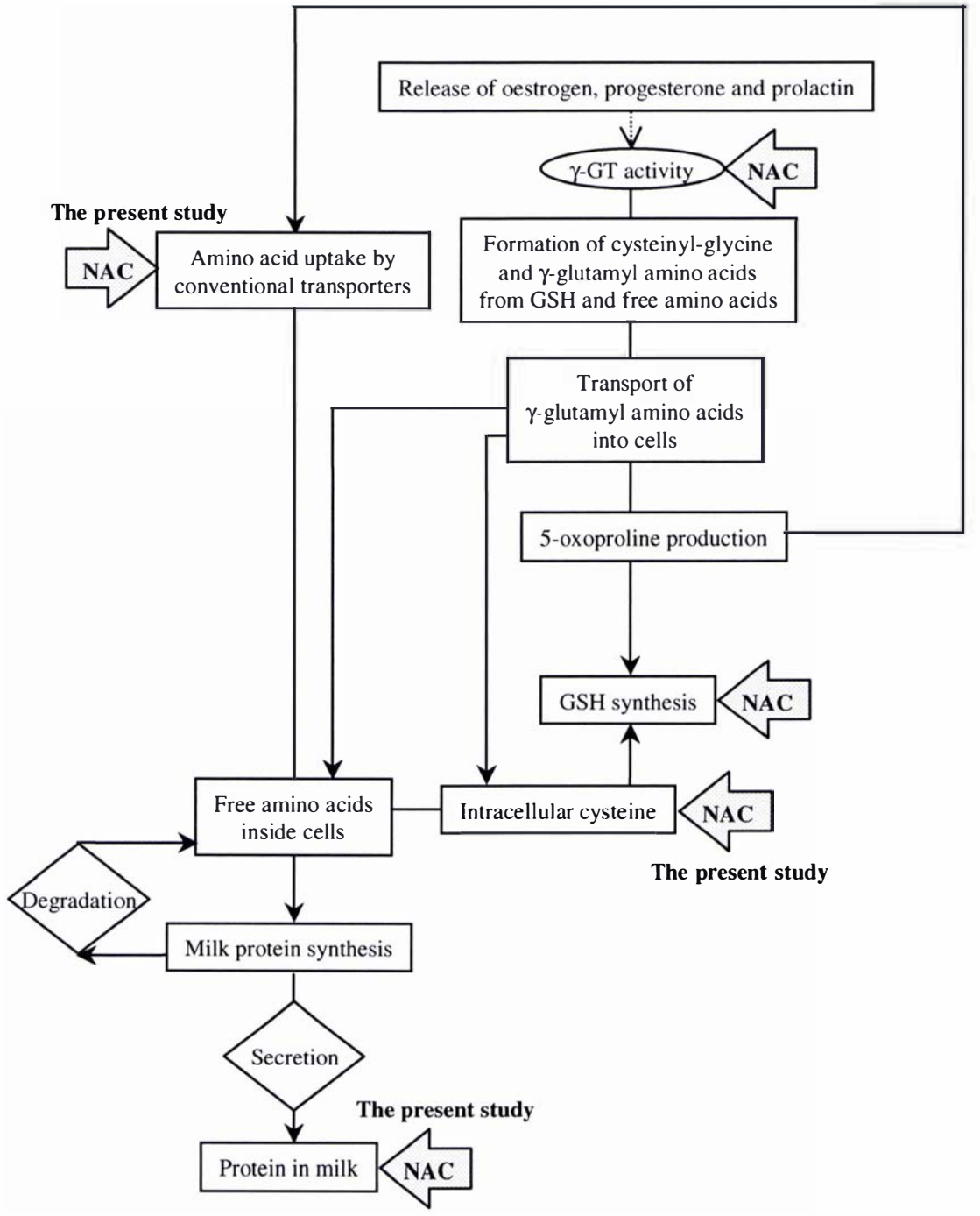


Figure 4.1 Flow diagram showing how γ-GT and N-acetylcysteine are involved in milk protein synthesis.

The levels where N-acetylcysteine (NAC) may positively affect milk protein synthesis, as shown in the literature and in the present study, are indicated by arrows.

4.3. Future Research

This study re-establishes the importance of the γ-glutamyl cycle in amino acid uptake but gaps in our knowledge of the cycle need to be clarified. A full understanding of the cycle and the factors regulating it is required for successful manipulation of increased milk protein synthesis.

Elucidation of mechanisms for the transport of γ-glutamyl amino acids into mammary cells, and GSH out of cells is required. The activity of both transporters appears to be linked to γ-GT activity and this observation does not disprove the existence of the γ-glutamyl cycle but rather increases the number of components present in the cycle. While γ-GT has an important regulatory role in the transport of amino acids, the transporter of γ-glutamyl-AA as the supplier of amino acids for milk protein synthesis is another the enzyme that needs to be characterised. This may lead to further understanding as to how to manipulate γ-glutamyl-AA transport and thus increase availability of amino acids in mammary cells for milk protein synthesis.

The study of milk protein production *in vitro* is restricted by the lack of a suitable model for *in vivo* synthesis. Short of the fashioning of a 'Friesian' mouse, isolated acini from mammary gland digestions are probably the best laboratory model for studying milk production in the ruminant. The greatest improvement that could be made to studies of milk production *in vitro* would be the implementation of a cell line that acted exactly like mammary secretory cells, or the repeated use of isolated acini from the same tissue digestion. The latter would involve the digestion of a large amount of mammary tissue, the acini of which would be cryogenically preserved for use in future experiments. This would rely on the preparation of acini that have optimal performance *in vitro*, and then validation that thawed acini were performing in the same way as the initial preparation. Each experiment uses a very small amount of isolated acini so a large digestion should provide enough acini for many replicated experiments. Removal of inconsistencies between acini in separate experiments would allow better reproducibility comparison between experiments and prevent the degree of variation that was observed occurred in the present study.

In the present study, inhibition of γ-GT has decreased milk protein secretion in isolated ovine mammary acini, which identifies the importance of this enzyme in the supply of amino acids for milk protein synthesis. Studies have shown that γ-GT activity is regulated directly and/or indirectly by the lactogenic hormones oestrogen, progesterone and prolactin (Puente *et al.*, 1979; Pocius *et al.*, 1980). Further investigation of the mechanisms involved in increasing γ-GT activity may allow targeted increase of γ-GT expression and activity perhaps through genetic manipulation.

The use of N-acetylcysteine (NAC) to increase the supply of cysteine to the mammary gland has resulted in increased milk protein secretion by isolated ovine acini, and increased milk yield and protein yield and concentration in milk of lactating goats. This was expected to result from increases in uptake and intracellular concentration of cysteine and glutathione, but these are difficult to analyse and concentrations in cells and goat plasma showed considerable variability, which reduced the significance of the results. A more sensitive, highly repeatable, method for measuring thiol-containing compounds needs to be established so that the effect of NAC treatment on cysteine and GSH can be reliably studied.

Supplementation of NAC in the diet of dairy animals may lead to increased milk protein production that is desired by the Dairy Industry. The current study using lactating goats should be repeated to fully establish the effect of NAC on milk protein production, using a greater number of animals to reduce variation and improve significance of the results. The goats used in the present study were found to produce different levels of α_{s1}-casein, which has been shown to affect milk protein production (Alichanidis and Polychroniadou, 1996). In the ideal experiment, goats should be of the same α_{s1}-casein production type to prevent differences resulting from this variation. The use of cloned animals would eliminate problems with between-animal variation. If the goat trial was to be repeated and a pudic artery catheter was required, the exit should be positioned above the tail rather than below it to help prevent infection and remove the need for prolonged antibiotic treatment. The present study showed that blood flow measurements by ultrasonic flow probes could not be relied upon for accurate measurements but could be used to monitor changes over time. The length of time that elapsed between surgery and the start of experimentation was too long and may have

resulted in the loss of competency of some catheters and the ultrasonic flow probes. While a recovery period after surgery is essential, it should be kept as short as possible.

The effect of acivicin and NAC on intracellular concentration of amino acids, the presence of acivicin and NAC in tissues, and the γ-GT activity of cells from the mammary gland of these goats, were not determined. Intracellular amino acid concentrations would have allowed the use of a mathematical model that has been designed to measure amino acid uptake and partitioning in the mammary gland (Lee *et al.*, 1999). Assays of γ-GT activity in isolated cells from lactating goats would have provided a comparison for activities in sheep, cows and rats and may have indicated that acivicin was not reaching cells, offering a reason for the lack of effect on amino acid uptake and milk protein secretion in the whole animal.

After a further trial in goats to test the hypothesis that NAC increases milk protein production, NAC should be tested on non-surgically modified goats in their normal working environment. Once the effect of NAC has been confirmed in the dairy goat a trial can be conducted using dairy cows so that results are not confounded by species differences.

NAC added to drinking water has been shown as an effective way of supplying cysteine to mice (Kumar *et al.*, 2000), but action of rumen microorganisms on NAC makes this an unsuitable method for dairy animals. A slow release capsule may be a more acceptable method for supplementation of NAC to lactating dairy animals. The use of NAC for increased milk protein production provides an alternative to modifying production by genetic manipulation.

4.4. Conclusions

The γ-glutamyl cycle was first proposed in 1970 but gaps in our understanding of the cycle have caused criticism of its proposed role in amino acid transport. The current study has re-established the importance of the γ-glutamyl cycle in amino acid supply for milk protein synthesis but further work is required to fully understand this role and allow its manipulation so that protein production can be modified as required by the Dairy Industry.

Inhibition of one enzyme of the γ-glutamyl cycle, γ-glutamyl transpeptidase (γ-GT), decreased secretion of individual milk proteins by isolated ovine mammary acini, indicating that γ-GT has an important role in milk protein synthesis. Inhibition of γ-GT in lactating goats altered amino acid uptake but this was not reflected in milk protein yield or concentration so further work is required to understand the mechanisms influencing the role of γ-GT in milk protein synthesis in the whole animal. Up-regulation of γ-GT activity in mammary cells may enhance milk protein synthesis.

The supply of cysteine to isolated ovine mammary acini, in the form of N-acetylcysteine (NAC), increased secretion of individual milk proteins indicating that cysteine is not provided in adequate amounts to allow optimal milk protein synthesis. Extension of this study to the whole animal showed that NAC increased milk yield and milk protein yield and concentration in lactating goats supporting the study in isolated acini. This research suggests that the supply of cysteine through NAC supplementation to dairy animals has the potential to meet the aims of the Dairy industry for increased milk protein production.

Chapter 5. Appendices

Appendix A. γ-Glutamyl transpeptidase activity assay validation

Validation of the enzyme assay to measure γ-glutamyl transpeptidase activity was carried out by measuring rates of reactions using the method described in section 2.3.11 with γ-glutamyl-p-nitroanilide (4.64 mM) as the substrate, but using ovine plasma as the source for γ-GT. There was no increase in absorbance at 405 nm when substrate was excluded from the assay resulting in zero rate of reaction indicating the reaction is substrate specific. To check the reaction was linear increasing amounts of plasma were added to the reaction mixture. Total γ-GT activity increased with increased plasma in the reaction mixture but the rate of reaction did not change (the rate was linear) indicating the enzyme was saturated with substrate (Figure 5.1).

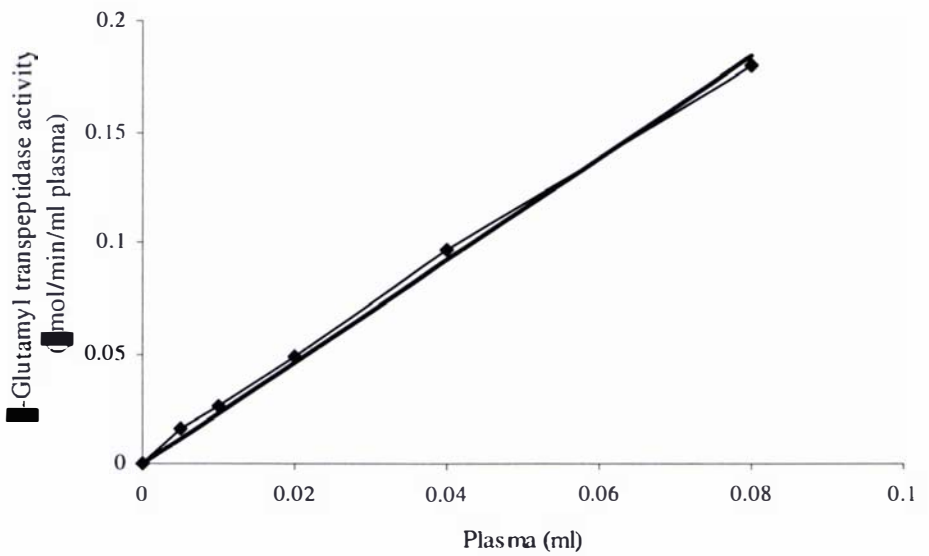


Figure 5.1 γ-Glutamyl transpeptidase activity assay linearity check.
(Trendline R^2=0.9961)

Appendix B. The effect of plating acini density on protein secretion.

Acini were plated on two sizes of plates, 24- and 12-well, but the number of cells per well was kept the same to test the effect of plating density and Matrigel® contact on milk protein secretion. Higher plating densities have previously been reported to have a negative effect on lactose output by isolated ovine mammary acini (Davis *et al.*, 1993). In the present study, there was no difference between secreted proteins of acini incubated on 12- or 24-well plates (Figure 5.2).

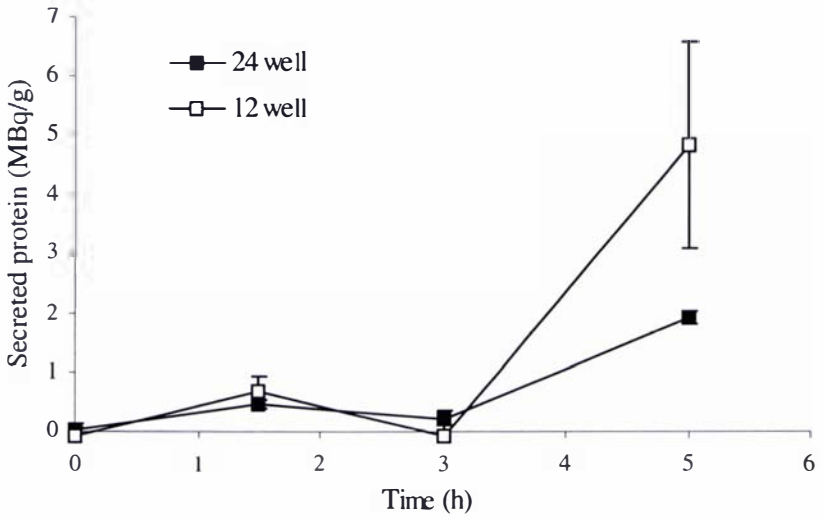


Figure 5.2 The effect of plating density on milk protein secretion by isolated ovine mammary acini.

Radioactivity of protein secreted by acini incubated on 24- or 12-well plates. Error bars represent standard error of the mean (n=3). Secreted protein of 24-well plates was not significantly different from 12-well plates at 5 h ($P<0.05$). The method was described in section 2.3.

Appendix C. The effect of coating plates with Matrigel® on protein secretion of acini.

The morphology and function of cultured rodent mammary cells, has been shown to be dependent on the type of extracellular matrix, with the expression of milk protein genes being affected (Talhouk *et al.*, 1990). To create an environment in cell culture plates that was more similar to that *in vivo*, in order to help maintain protein secretion, the lower surface of each well was coated with Matrigel®, a basement membrane extract. However Matrigel® is expensive and an experiment was designed to determine whether acinar cells could remain viable and secrete protein when incubated in the absence of Matrigel®.

Acini were prepared as described in section 2.3.1 and plated onto 24-well plates that had, and had not, been coated with Matrigel®. These were incubated in the absence and presence of cycloheximide in one experiment for 8 h, and in two other experiments for 0, 4, 8, and 18 h before the medium was harvested and prepared for total counts as described in section 2.3.4.

In an initial experiment, acini were incubated for 8 h in the absence and presence of Matrigel®. Trypan blue staining of incubated acini harvested at 0, 4 and 8 h, indicated that viability of isolated ovine mammary acini was not affected by the absence or presence of Matrigel® (Table 5.1.). There was no difference in secreted protein between acini plated on Matrigel® coated or uncoated plates. This is consistent with studies by Wheeler *et al.* (1993) who found that there was no difference in total RNA or levels of RNA coding for α-lactalbumin of acini incubated over 22 h in the presence or absence of Matrigel®.

Two further experiments (Figure 5.3) supported the initial experiment. Medium for acini incubated for 8 h on Matrigel® free plates had the same level of radioactivity as those incubated on Matrigel® coated plates. Acini were also incubated for 18 h in the presence and absence of Matrigel®, and those in the absence of Matrigel® had higher levels of radioactivity in the medium. This was an unexpected result and may be

because secreted protein adheres to Matrigel® which was removed during medium extract preparation.

Table 5.1. The effect of Matrigel® on isolated ovine acini in medium.

Observations were made after staining acini using trypan blue dye. Acini were incubated in the absence and presence of Matrigel®.

Time (h)	Matrigel®	Observations
0	+	Very dilute, expected clumps. Very nice - few dead single cells
	-	Clumps, with 25% dead cells. 1 dead clump. Not many clumps with no dead cells
4	+	Some dead clumps. Dead single cells around clumps. Some viable clumps. Live clumps may have settled. Therefore broke up Matrigel® and stained another 50 µl - lots of live clumps with a few dead single cells
	-	Some dead clumps but overall viability good
8	+	Viability 75-80%. Dead single cells around clumps. All clumps round. Still some clumps without dead cells
	-	Generally the same as with Matrigel® although < 75% viability. More dead singles. Some clumps completely surrounded by dead cells. Some clumps no dead cells.

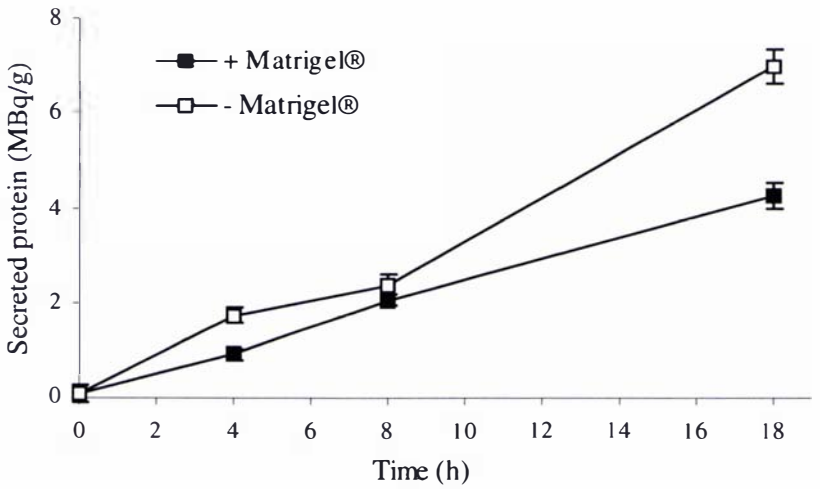


Figure 5.3 The effect of Matrigel® on milk protein secretion by isolated ovine mammary acini.

Combined results from two experiments showing radioactivity in medium extract of acini incubated for 18 h on Matrigel® free and coated plates in the absence of treatment. Error bars represent standard error of the mean (n=6). Protein secretion by acini + Matrigel® was significantly lower than - Matrigel® at 18 h.

Although these experiments showed that acini could be incubated on Matrigel®-free plates without adverse effects on the increase in medium radioactivity it was decided that all further acini experiments would be carried out on Matrigel® coated plates for consistency. Incubating acini on Matrigel® free or diluted Matrigel® coated plates has been found to cause a decrease in lactose production (personal communication; B.R. Sinclair) indicating that there may be some deleterious effects connected with not using Matrigel®. Lactose production was not compared for acini plated on Matrigel® free or coated plates in this study.

The use of Matrigel® may also help reduce differences between acini preparations by supplying interactions necessary to keep preparations viable when they were perhaps less than optimal. Keeping the acini environment as close to that *in vivo* as possible was considered important.

Appendix D. The effect of acivicin on individual caseins and whey proteins

The effect of acivicin on individual proteins was tested in an initial experiment where the casein fraction was acid precipitated (sodium acetate pH 4.6) from the whey proteins prior to further separation of individual proteins by HPLC. Methods are described in section 2.3.

Acini incubated in the presence of acivicin had reduced secretion of all six major milk proteins (Figure 5.4). The decrease observed was irrespective of whether the protein contained cysteine. At 8 h, α-casein had decreased to 13% of control levels, β-casein to 30%, and κ-casein to 10%. β-lactoglobulin and α-lactalbumin decreased to 19 and 26% of control levels respectively.

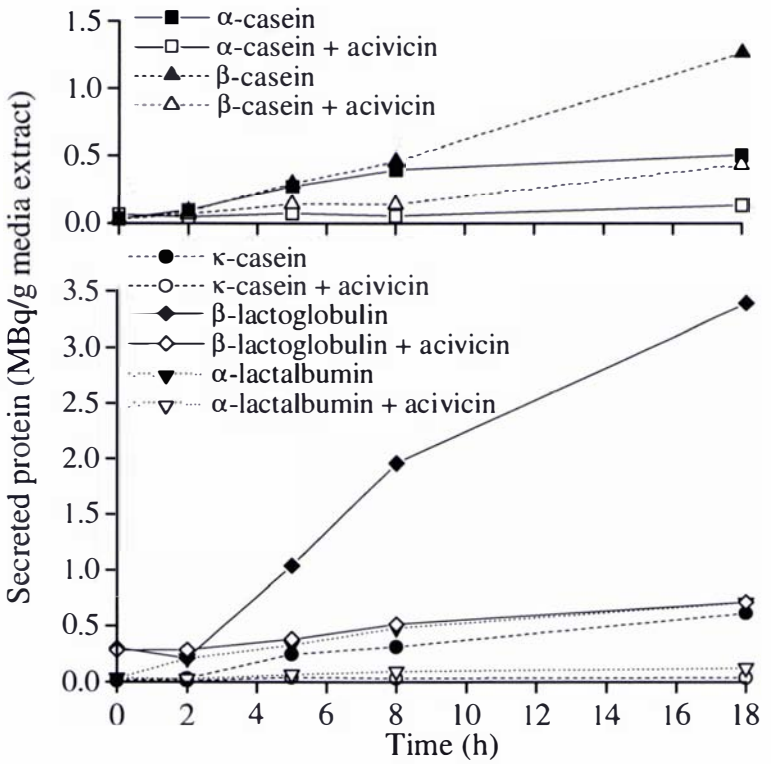


Figure 5.4 The effect of acivicin on individual milk proteins.

Radioactivity attributed to individual milk proteins from the medium of isolated ovine mammary acini incubated in the absence and presence of acivicin (0.5 mM). Casein and whey protein fractions were separated prior to HPLC.

From analysis of individual proteins by HPLC, β-lactoglobulin appeared to be the predominant fraction. This is in contrast to studies that have shown α-casein is the major ovine milk protein (Anifantakis, 1986). Although run separately through the HPLC system, β-lactoglobulin shares the same retention time as β-casein. It is likely that the β-lactoglobulin fraction contains β-casein as a result of incomplete acid precipitation. The pH of an acid precipitated sample was shown to be pH 4.6, which is correct for casein precipitation (McKenzie, 1971). This method of medium milk protein separation was not used for further sample analysis.

Appendix E. The effect of 5-oxoproline on intracellular amino acid concentrations in isolated ovine mammary acini.

Intracellular amino acids were measured in acini treated with 5-oxoproline in two experiments. Concentrations of alanine, arginine and leucine inside control cells from 5-oxoproline experiments 1 and 2 were below detectable levels (Table 5.2 and Table 5.3). This was because of reduced sensitivity in analysis of one batch, which contained control samples, as alanine, arginine and leucine were determined in other batches. Reduced sensitivity may have resulted from a slight change in elution buffer B composition. An error in the analysis of 5-oxoproline experiment 2 control samples prevented detection of lysine, methionine, phenylalanine, threonine, tryptophan, isoleucine, and leucine inside cells (Table 5.3). Sample volumes were small and prevented reanalysis so concentrations of these amino acids in controls for this experiment were lost. The error occurred because of incorrect pH of Picotag elution buffer B, which caused compression of some elution peaks, affecting threonine, and prevented separation of amino acids that eluted after 44 minutes (methionine, isoleucine, leucine, phenylalanine, tryptophan and lysine). A new pH indicator probe was obtained and further problems of this type were avoided.

Cells from 5-oxoproline experiment 1, after 4 h incubation in absence of treatment had increases in all measured intracellular amino acids with the exception of taurine and proline which were not different from concentrations at 0 h (Table 5.2.). Incubation of control acini for 8 h resulted in intracellular amino acid concentrations that were not different from 0 h except isoleucine which increased. Between 4 h and 8 h there were decreases in intracellular concentration of aspartate, serine, glycine, taurine, histidine, threonine, proline, tyrosine and tryptophan. The increase in intracellular amino acids at 4 h may result from acini recovery after depletion caused by acini isolation. The decrease at 8 h may have been because of declining cell metabolism that may occur prior to increased cell membrane permeability as measured by trypan blue staining, which showed the majority of cells were still viable at this time (section 2.4.2.3.1).

In 5-oxoproline experiment 2, acini incubated in the absence of treatment for 4 h had increased intracellular aspartate, glutamate, serine, histidine, tyrosine and valine, and

decreased taurine compared to concentrations at 0 h (Table 5.3.). Aspartate, serine, glycine, histidine, tyrosine and valine were higher at 8 h than at 0 h, while taurine was lower. Between 4 h and 8 h taurine decreased while tyrosine and valine increased.

In 5-oxoproline experiment 1 (Table 5.2) at 4 h, acivicin treatment of acini resulted in decreased serine, histidine, isoleucine and lysine inside cells compared to control concentrations at 4 h. At 8 h aspartate, serine, histidine, proline, isoleucine, phenylalanine and lysine were lower when acini were incubated with acivicin than controls. Acivicin treatment of acini in 5-oxoproline experiment 2 (Table 5.3) resulted in decreased aspartate, serine, histidine, proline, and tyrosine, and increased valine at 4 h and decreased tyrosine and increased valine at 8 h. All of these amino acids except proline and tyrosine have been shown to be acted on by γ-GT (Thompson and Meister, 1977), so a drop in intracellular concentration of these amino acids is consistent with inhibited uptake as a result of γ-GT inhibition by acivicin. γ-GT has been shown to have no affinity for tyrosine (Morita *et al.*, 1994) so the decrease in tyrosine with acivicin treatment must result from the effect of acivicin on other transport systems or on cell viability. The increase in valine in experiment 2 is unexpected but may result from increased uptake by other transport systems after the decrease or removal of a factor, perhaps another amino acid, which would otherwise suppress uptake. The steady state of other amino acids that γ-GT has affinity for (Table 1.7), indicates a feedback between intracellular concentration and uptake, otherwise ongoing protein secretion would result in decreased intracellular amino acid concentrations.

Table 5.2 The effect of 5-oxoproline on intracellular amino acid concentrations in experiment 1.

Intracellular amino acid concentrations (mean μM± SEM (n=3)) from 5-oxoproline experiment 1 where isolated ovine mammary acini were incubated for 0, 4 and 8 h in the absence and presence of 0.5 mM 5-oxoproline and/or 7 mM acivicin.

	0 h	4 h				8 h			
	Control	Control	5-Oxoproline	Acivicin	5-Oxoproline + Acivicin	Control	5-Oxoproline	Acivicin	5-Oxoproline + Acivicin
Essential amino acids									
Histidine	6.7 ± 1.1	21 ± 2.3	5.8 ± 0.76	6.0 ± 3.3	3.6 ± 2.2	11 ± 3.7	1.4 ± 0.72	4.1 ± 1.3	2.9 ± 1.5
Lysine	14 ± 4.6	21 ± 2.4	20 ± 8.3	37 ± 11	40 ± 1.8	19 ± 1.2	0.38[a]	10 ± 6.3	28 ± 0.96
Methionine	6.3 ± 2.2	11 ± 1.8	9.6 ± 1.7	11 ± 4.6	6.2 ± 2.1	8.4 ± 0.75	3.1 ± 0.44	6.5 ± 2.1	3.8 ± 1.9
Phenylalanine	11 ± 3.6	19 ± 2.3	16 ± 1.7	14 ± 4.9	9.4 ± 2.9	16 ± 1.2	5.9 ± 0.47	11 ± 3.2	9.9 ± 2.8
Threonine	26 ± 9.2	53 ± 7.7	51 ± 6.6	55 ± 21	31 ± 10	39 ± 4.9	21 ± 2.9	35 ± 8.9	28 ± 3.8
Tryptophan	1.5 ± 0.79	4.9 ± 0.70	3.6 ± 1.8	3.8 ± 1.9	1.6	1.1	ND[b]	2.4 ± 1.5	0.96 ± 0.96
Isoleucine	19 ± 5.8	56 ± 12	26 ± 1.8	23 ± 7.4	18 ± 1.9	46 ± 4.4	12 ± 1.0	20 ± 5.3	31
Leucine	ND	ND	33 ± 3.2	32 ± 12	19 ± 3.4	ND	13 ± 0.97	25 ± 6.7	28 ± 4.6
Valine	20 ± 6.6	32 ± 3.1	31 ± 2.8	29 ± 11	16 ± 5.6	27 ± 2.3	12 ± 1.1	23 ± 6.7	15 ± 1.3
Non essential amino acids									
Alanine	ND	ND	13 ± 2.9	17 ± 8.9	8.6 ± 3.9	ND	6.3 ± 0.48	8.6 ± 1.5	4.1 ± 0.44
Arginine	ND	ND	16 ± 2.8	17 ± 8.3	12 ± 5.7	ND	5.1 ± 0.53	11 ± 3.2	11 ± 1.7
Aspartate	9.1 ± 4.1	20 ± 2.8	10 ± 3.6	13 ± 5.0	ND	13 ± 3.6	3.7 ± 0.22	6.0 ± 1.4	ND
Glutamate	82 ± 38	181 ± 44	167 ± 57	224 ± 108	118 ± 43	132 ± 26	57 ± 1.8	86 ± 25	56 ± 4.1
Glycine	51 ± 22	134 ± 27	90 ± 31	146 ± 81	83 ± 37	66 ± 10	24 ± 1.8	48 ± 16	39 ± 4.2
Proline	9.2 ± 3.9.	15 ± 2.6	8.9 ± 2.1	12 ± 5.4	9.8 ± 3.8	11 ± 2.8	2.8 ± 0.11	4.8 ± 1.1	8.7 ± 3.3
Serine	27 ± 9.7	65 ± 6.3	29 ± 5.1	36 ± 13	24 ± 6.2	38 ± 11	13 ± 1.1	21 ± 5.1	24 ± 3.4
Taurine	31 ± 15	61 ± 18	45 ± 17	77 ± 46	44 ± 24	20 ± 2.0	7.1 ± 0.62	16 ± 5.9	9.7 ± 1.2
Tyrosine	12 ± 4.3	21 ± 2.7	20 ± 2.5	20 ± 7.8	13 ± 4.5	16 ± 1.7	7.1 ± 0.65	14 ± 3.8	12 ± 0.78

[a] Values without SEM occur when two of the three samples have concentrations too low to be detected.
[b] ND (not detected) indicates that concentrations were too low to be detected - alanine, arginine, leucine not determined due to experimental error.

Table 5.3. The effect of 5-oxoproline on intracellular amino acid concentrations in experiment 2.

Intracellular amino acid concentrations (mean μM± SEM (n=3)) from 5-oxoproline experiment 2 where acini were incubated for 0, 4 and 8 h in the absence and presence of 0.5 mM 5-oxoproline and/or 7 mM acivicin.

	0 h	4 h				8 h			
	Control	Control	5-Oxoproline	Acivicin	5-Oxoproline + Acivicin	Control	5-Oxoproline	Acivicin	5-Oxoproline + Acivicin
Essential amino acids									
Histidine	4.8 ± 0.63	9.7 ± 0.58	14 ± 2.8	7.0 ± 0.58	16 ± 0.82	13 ± 2.7	6.1 ± 1.2	9.4 ± 1.2	7.2 ± 1.2
Lysine	-[a]	-	54 ± 16	28 ± 3.2	56 ± 7.1	-	70 ± 9.4	75 ± 9.9	70 ± 9.5
Methionine	-	-	20 ± 4.2	9.9 ± 0.74	17 ± 5.1	-	8.8 ± 1.5	13 ± 1.6	8.7 ± 1.5
Phenylalanine	-	-	37 ± 7.4	18 ± 1.4	43 ± 2.9	-	18 ± 3.3	25 ± 2.3	20 ± 2.5
Threonine	-	-	84 ± 16	42 ± 3.0	98 ± 5.9	-	41 ± 6.7	59 ± 7.4	48 ± 6.5
Tryptophan	-	-	8.7 ± 1.6	4.5 ± 0.36	9.1 ± 0.78	-	4.3 ± 0.92	6.1 ± 0.61	3.8 ± 1.9
Isoleucine	-	-	64 ± 12	31 ± 2.0	74 ± 5.7	-	33 ± 5.1	39 ± 3.4	29 ± 8.9
Leucine	-	-	74 ± 15	36 ± 2.7	86 ± 6.4	-	30 ± 5.1	35 ± 2.8	36 ± 4.1
Valine	4.5 ± 0.43	12 ± 1.7	75 ± 15	36 ± 2.8	86 ± 7.1	18 ± 3.6	26 ± 5.9	34 ± 3.1	25 ± 5.7
Non essential amino acids									
Alanine	ND[b]	ND	14 ± 2.0	10 ± 0.74	20 ± 1.0	ND	3.5 ± 0.78	6.2 ± 0.50	2.6 ± 1.3
Arginine	ND	ND	32 ± 6.2	15 ± 1.2	35 ± 3.5	ND	18 ± 3.5	28 ± 5.8	23 ± 4.1
Aspartate	3.3 ± 0.56	6.5 ± 0.62	7.0 ± 0.87	4.0 ± 0.94	11 ± 0.76	6.3 ± 1.6	4.6 ± 0.74	6.7 ± 1.1	9.2 ± 3.7
Glutamate	47 ± 6.8	70 ± 3.6	81 ± 9.1	69 ± 8.5	103 ± 9.8	53 ± 13	34 ± 4.6	61 ± 10	46 ± 5.5
Glycine	31 ± 3.5	37 ± 2.0	53 ± 10	33 ± 3.3	65 ± 4.1	42 ± 7.3	26 ± 3.1	40 ± 2.6	31 ± 2.6
Proline	9.5 ± 1.5	8.4 ± 0.34	5.1 ± 0.8	6.6 ± 0.50	6.3 ± 0.78	8.3 ± 2.3	3.3 ± 0.55	9.0 ± 1.7	4.4 ± 0.31
Serine	21 ± 3.4	33 ± 2.2	49 ± 9.2	25 ± 1.7	59 ± 3.8	40 ± 6.7	25 ± 3.6	36 ± 2.5	29 ± 3.0
Taurine	15 ± 2.1	7.6 ± 0.41	7.0 ± 0.89	6.6 ± 0.87	8.7 ± 0.75	4.9 ± 1.3	3.7 ± 0.49	6.7 ± 1.3	5.0 ± 0.43
Tyrosine	11 ± 1.5	43 ± 4.0	38 ± 7.5	19 ± 1.5	45 ± 3.4	60 ± 12	22 ± 5.3	31 ± 3.4	25 ± 3.4

[a] indicates that concentrations were not obtained for these amino acids.
[b] ND (not detected) indicates that concentrations were too low to be detected.

In experiment 1, 5-oxoproline treatment caused a decrease in aspartate, serine, histidine, proline and isoleucine in cells of acini incubated for 4 h and a decrease in all measured amino acids in cells incubated for 8 h. In experiment 2 there was an increase in serine, glycine, histidine, and valine and a decrease in proline at 4 h, and a decrease in glutamate, serine, glycine, proline and tyrosine at 8 h. The increase in uptake seen in experiment 2 supports the work by Viña *et al.* (1989) who showed that 5-oxoproline infusion into rats increased amino acid transport into the lactating mammary gland and placenta. However, Viña *et al.* (1989) reported an increase in many amino acids that did not change in this study. That the intracellular concentration of several amino acids decreased with 5-oxoproline treatment is in contrast to the hypothesis suggested by Viña *et al.* (1989) and instead suggests that 5-oxoproline down regulates amino acid transport.

Intracellular amino acid concentrations from acini incubated in the presence of both 5-oxoproline and acivicin were compared with acivicin treated acini. In experiment 1, acini incubated with 5-oxoproline and acivicin for 4 h had reduced intracellular aspartate and glutamate compared to acivicin treated acini. After 8 h glutamate and alanine were reduced while serine, isoleucine and lysine increased. In experiment 2 after 4 h incubation, cells treated with 5-oxoproline and acivicin showed higher intracellular concentrations of all amino acids except proline indicating that the action of γ-GT on amino acid transport may be through the production of 5-oxoproline as suggested by Viña *et al.* (1989). However, serine, glycine, alanine, proline, methionine and phenylalanine in these cells had decreased after 8 h. This may have been due to the metabolism of 5-oxoproline.

Interestingly 5-oxoproline was associated with decreased intracellular glutamate except in experiment 2 in combination with acivicin where glutamate increased after 4 h incubation. Glutamate may be rapidly removed as it has been shown to be limiting for GSH synthesis (Beutler, 1989), which is why increases in glutamate were not always seen.

The increase in amino acid uptake in experiment 2, in the presence of 5-oxoproline alone and in combination with acivicin, when acivicin alone decreases uptake, suggests

that 5-oxoproline enhances amino acid transport and does reverse the action of γ-GT inhibition on cells in agreement with Viña *et al.* (1989). However, this reaction of cells to 5-oxoproline has been shown in this study to be dependent on the individual cell preparation.

Appendix F. Preliminary experiment with acivicin administration in sheep.

Lactating Romney cross ewes were used in a set of preliminary experiments to determine the amount of acivicin to administer, to establish a technique for acivicin administration that would produce a response, and monitor toxicity before attempting the study in goats. Tests in sheep were set up for clinical observations only. They did not take into account feed intake or external stimuli such as ambient temperature, and were not designed to provide enough data for statistical analysis.

The toxic effect of acivicin was of primary concern in this study. Acivicin has been evaluated as a treatment for cancer (McGovren *et al.*, 1985; Eisenhauer *et al.*, 1987) and as a lead up to phase I and II clinical trials has been studied in rodents, dogs and monkeys with different doses and schedules (Poster *et al.*, 1981; McGovren *et al.*, 1988) which provided adequate information on doses and toxicity for the present study.

In mice the elimination half-life of acivicin in blood was found to be 45 to 50 min with 53 to 82% of doses being recovered in urine 24 h after the doses were administered (Poster *et al.*, 1981) and toxicity was sex and age related. In cynomolgous monkey, rhesus monkey, beagle dog and humans acivicin was recovered from the urine at 25, 39, 10, and 21% of the dose respectively and total body clearance was 8.3, 15.8, 14.7 and 49 mg/min respectively. No sex dependent toxicity was noted in these species (McGovren *et al.*, 1988). Doses of 1.6 mg/kg and 3.1 mg/kg were delivered intravenously to the beagle dogs to study urinary excretion and sex dependence of acivicin pharmacokinetics and did not result in any toxic effects (McGovren *et al.*, 1988).

Of the toxicities that have been observed for acivicin, gastrointestinal toxicity predominated. Toxic concentrations of acivicin has been shown to cause diarrhoea, hematochezia, anorexia, adipsia, hyperthermia, oligodypsia, emaciation, convulsions, alopecia, rashes, emesis, gait disorders, pulmonary distress, somnolence and a variety of toxicities. Myelosuppression has been noted. Bone morrow hypoplasia is dose

dependent and histologically reversible however thrombocytopenia and reticulocytopenia were not reversible over the period that was observed. Renal abnormalities were also noted (Poster *et al.*, 1981).

In this preliminary experiment with sheep, catheters were surgically implanted into one pudic artery (supplies blood to one side of the udder) of each animal for the intravascular infusion of acivicin directly into one side of the mammary udder. The higher dose administered by McGovren *et al.* (1988) to elicit an effect in the whole body of dogs without toxicity (3.1 mg/kg body weight), was not exceeded in this study. In initial preliminary tests acivicin were injected as a bolus or continuously infused over 6 h. Milk yield was measured at 2 h intervals over an 8 h period and samples were analysed by near infrared transmittance for changes in protein, lipid, lactose and total solid concentration. The maximum dose (2.5 mg/kg) had no effect on milk yield or constituent concentrations when delivered to the gland via the pudic artery.

In the final preliminary test acivicin (1.5 mg/kg) was administered to one side of the udder by injection up the teat. This technique removed the possibility of blood flow sweeping acivicin into the whole body before enzyme binding sites of the mammary gland could be saturated. Administration of acivicin by this method caused a decrease in milk yield 4 h after treatment with acivicin and the next day. The concentration of milk constituents did not appear to change but were difficult to interpret with inconsistent feed intake perhaps interfering with production. The day following the test, milk protein concentrations were higher while lactose was much lower than pre test concentrations. Reduced lactose may have lead to decreased milk yield by reducing water in milk.

Secondary effects of acivicin on animal behaviour, heart rate, blood gas, temperature, intake, and faeces were monitored during treatment periods and blood samples for platelet counts were collected before, during and after treatment. There were no changes in any of the parameters measured supporting our use of acivicin at these doses, which have previously been shown not to be toxic (McGovren *et al.*, 1988).

Appendix G. Goat details

Table 5.4 Goat weight post-surgery pre-experiment.

Goat	Label	Breed	Age	Weight (kg)	Date of parturition
Flossie	G11	Saanen	8	58	12-08-99
Holly	G12	Saanen	6	65	19-09-99
Libby	G13	Toggenburg	2	53	14-08-99
Olga	G14	Toggenburg	7	56	13-08-99
Anna		Toggenburg	2	38.5	20-08-99

Appendix H. Estimation of the amount of N-acetylcysteine to infuse close arterially into the right hand side of the mammary udder of a goat.

Based on an experiment in lactating sheep (Roy & Lee unpublished data).

The whole body infusion of 2 g of cysteine per day in sheep resulted in an increase in peripheral cysteine of 55%. Mammary blood flow as a percentage of cardiac output was estimated to be between 10 - 35% depending on feed intake. 10 - 35% of 2 g cysteine per day equates to 0.2 - 0.7 g cysteine per day based on the partitioning of blood flow to both sides of the mammary udder, or 0.1 - 0.4 g cysteine per day based on the partitioning of blood flow to one half of the udder. From these estimations, it was decided that approximately 0.5 g of NAC per day should be infused close arterially via the pudic artery in to the RHS of the udder of each goat in this study.

Appendix I. ^{3}H-leucine infusion calculations.

The leucine in the infusate needed to be approximately the same concentration as leucine in plasma (200 μM) so as not to enhance supply of leucine to the gland. The amount of leucine added to the infusate bag containing sterile physiological saline (500 ml) was calculated as follows:

200 μmol/l	x	MW leu (131.2 g/mol)	=	0.026 mg/ml required
500 ml saline	x	0.026 mg/ml	=	13 mg leucine required

A 13 mg/ml solution of unlabelled leucine was prepared in physiological saline.

Animals needed to be infused with at least 2000 U/hr of sterile heparin to prevent blood from clotting in catheters and sample tubes. The stock sterile ovine heparin was 25000 U/ml.

The infusate was prepared by mixing approximately 30 ml of physiological saline with 1 ml 1 mCi/ml ^{3}H-leucine (120-190 Ci/mmol) and 1 ml (13 mg/ml) unlabelled leucine, which was filter sterilised into a 500 ml bag of sterile physiological saline. 1 ml of stock heparin was also sterile filtered into the infusate bag. A 4 ml sample of infusate for later analysis was withdrawn from the bag immediately prior to use. This sample was to be identical to the infusate so it was stored along side the infusate bag until the infusion had finished, and then the infusate sample was stored at -85°C. The infusate bag was weighed immediately before infusion, once during the infusion, and at the end of the infusion to determine the infusion rate and the exact amount infused.

To ensure specific radioactivity of ^{3}H-leucine was at plateau in goat plasma 4 h after the start of infusion (when acivicin or saline control was to be administered) a 40 ml bolus of the infusate (0.08 mCi) was administered to the goat before infusion commenced. One 20 ml syringe was filled with approximately 20 ml of infusate and 1 ml of stock heparin. The other syringe contained 20 ml infusate only. Each syringe was weighed before use, when filled with infusate, when filled with infusate and heparin, and after bolus administration so that the exact amount of infusate bolused could be determined.

The bolus was delivery slowly via the jugular catheter. A single larger syringe would have delivered the dose too quickly. The infusion commenced immediately after administration of the bolus. The total amount of radiolabelled infused was 1 mCi = 37 x 10^6 Bq.

Appendix J. Amino acid content of caprine milk.

Table 5.5 Amino acid (AA) content of protein in goats' milk.

AA content was calculated from content in α_{s1}-casein calculated from the primary sequence from Brignon *et al.* (1990) with the content in remaining proteins from Jenness (1980) except asparagine, aspartate, glutamine and glutamate, which were obtained from output in milk (Mepham and Linzell, 1966).

	AA content in milk protein (g/100g)
Essential amino acids	
Histidine	2.0
Lysine	6.2
Methionine	2.0
Phenylalanine	3.6
Threonine	4.1
Tryptophan	1.0
Isoleucine	4.0
Leucine	6.9
Valine	5.4
Conditionally essential amino acids	
Tyrosine	3.5
Cysteine	0.76
Nonessential amino acids	
Alanine	2.6
Arginine	2.0
Asparagine	3.5
Aspartate	2.3
Glutamine	8.5
Glutamate	8.5
Glycine	1.2
Proline	8.0
Serine	4.2

Appendix K. Specific radioactivity of ^{3}H-leucine in plasma of four goats treated with N-acetylcysteine and/or acivicin.

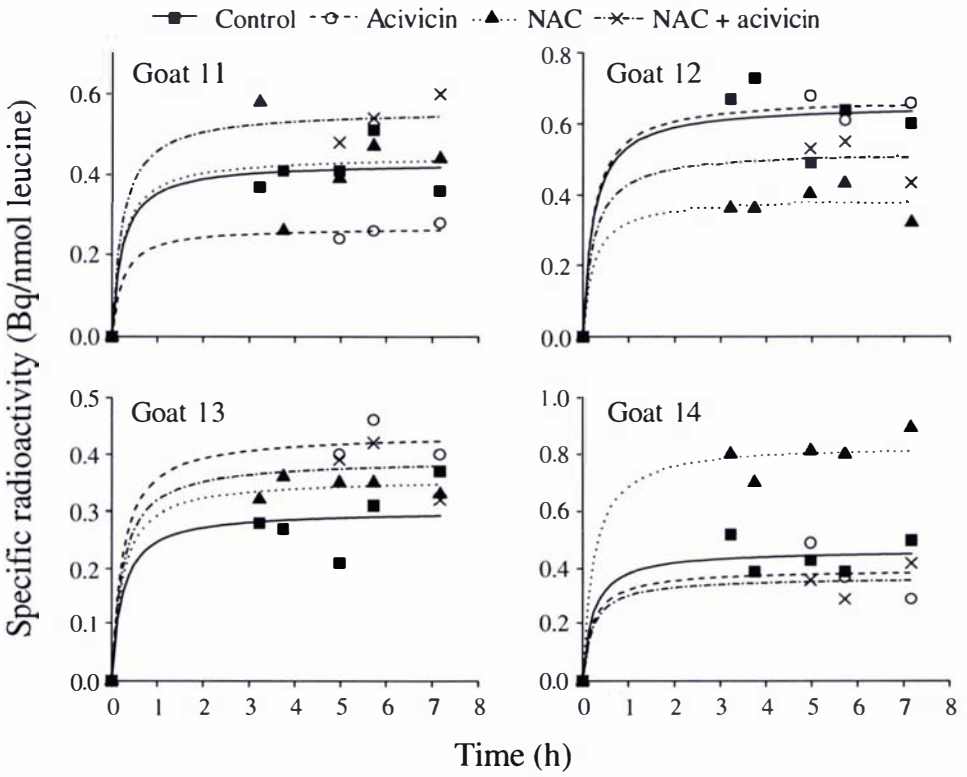


Figure 5.5 Specific radioactivity of ^{3}H-leucine in plasma of goats.

A bolus had been administered immediately prior to infusion to ensure that ^{3}H-leucine was at steady state at 4 h when acivicin or control saline was delivered. Michaelis-Menten curves were fitted to 5 data points for control and N-acetylcysteine (NAC), and 3 data points (post acivicin or saline administration at 4 h) for acivicin and NAC + acivicin treatments.

Appendix L. The effect of acivicin and N-acetylcysteine on milk yield.

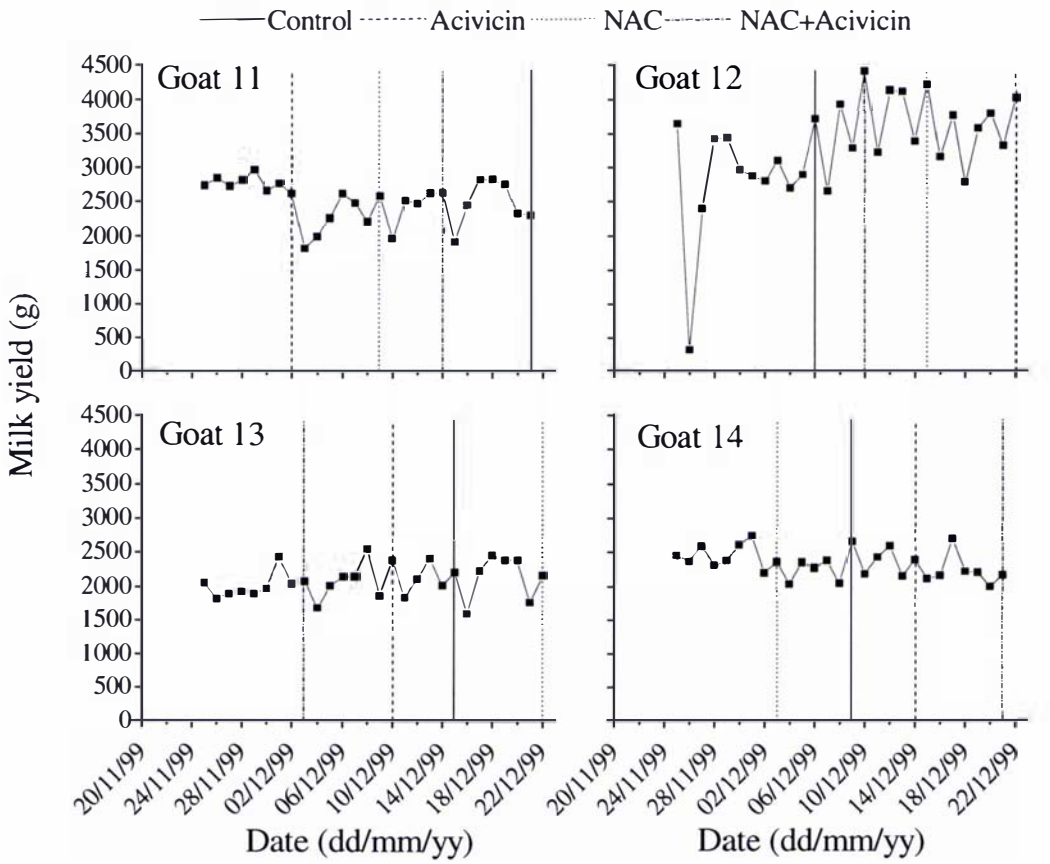


Figure 5.6 Daily milk yield.

Total daily milk yield of four goats for the duration of a study in which they received a control saline treatment or acivicin and/or N-acetylcysteine (NAC). The decrease in yield from Goat 12 on November 26 may have resulted from the flushing of catheters that occurred the day before.

Appendix M. Arterio-venous differences of goat plasma amino acids.

Table 5.6 Goat plasma amino acid A-V differences.

Arterio-venous differences (mean μM ± SEM) for amino acids in plasma of four lactating goats receiving a control saline treatment.

	Arterio-venous difference
Essential amino acids	
Histidine	6.4 ± 0.66
Lysine	31 ± 2.2
Methionine	11 ± 0.79
Phenylalanine	13 ± 1.5
Threonine	23 ± 1.5
Tryptophan	2.7 ± 0.43
Isoleucine	25 ± 1.1
Leucine	40 ± 2.2
Valine	57 ± 13
Conditionally essential amino acids	
Tyrosine	13 ± 0.96
Cysteine	7.2 ± 3.4
Nonessential amino acids	
Alanine	19 ± 2.0
Arginine	33 ± 2.0
Asparagine	16 ± 1.3
Glutamate	51 ± 5.9
Glutamine	14 ± 4.0
Glycine	-3.1 ± 6.1
Proline	18 ± 1.4
Serine	-10 ± 3.8
Taurine	0.86 ± 0.975

Means are from 3 samples from left and right sides of the mammary udder at different times per goat.

Appendix N. Comparison of amino acid uptake from plasma with output in milk protein of the goat.

Table 5.7 Comparison of amino acid net flux with output in milk.

Net uptake and release from the present study and output in milk calculated from Mepham and Linzell (1966), Jenness (1980) and Brignon *et al.* (1990) (Appendix J) of amino acids by both sides of the mammary udder of four goats receiving a control (saline) treatment.

	Uptake (μmol/min)	SEM	Output (μmol/min)	SEM
Essential amino acids				
Histidine	1.6	0.19	4.0	0.18
Lysine	9.7	0.37	13	0.6
Methionine	3.5	0.11	4.0	0.18
Phenylalanine	4.8	0.21	6.6	0.29
Threonine	7.8	0.34	10.	0.5
Tryptophan	0.84	0.16	1.5	0.07
Isoleucine	9.8	0.42	9.1	0.41
Leucine	16	0.7	16	0.7
Valine	12	1.8	14	0.6
Conditionally essential amino acids				
Tyrosine	3.9	0.18	5.8	0.26
Cysteine	2.1	1.35	1.9	0.09
Nonessential amino acids				
Alanine	5.4	0.33	8.9	0.40
Arginine	12	0.73	3.6	0.16
Asparagine	6.8	1.15	8.3	0.37
Glutamine	2.7	1.57	18	0.8
Glutamate	15	2.9	18	0.8
Glycine	-5.7	2.92	4.9	0.22
Proline	5.1	0.38	21	0.9
Serine	-4.3	1.24	12	0.5
Total	108		181	

Appendix O. Sample of data for protein SRA calculations

Total counts from scintillation counter

side of gland	time	Bq/ml
L	3	265
R	3	261
L	4	442
R	4	445
L	5	514
R	5	536
L	6	530
R	6	557

Concentration of protein in milk

side of gland	time	Protein mg/ml
L	3	32.5
R	3	26.2
L	4	28.1
R	4	27.4
L	5	28.1
R	5	28.1
L	6	24.9
R	6	26.2

Leucine in milk protein from Swaisgood (1995)

μmol leucine/mg protein
0.747

SRA of ^{3}H-leucine in total milk protein

side of gland	time	Bq/μmol leucine
L	3	10.91
R	3	13.35
L	4	21.09
R	4	21.72
L	5	24.52
R	5	25.57
L	6	28.49
R	6	28.50

Appendix P. γ-Glutamyl transpeptidase and amino acid transport for milk protein production *in vivo*.

A paper contributed to the New Zealand society of Animal production proceedings vol 61 by Johnston *et al.* (2001).

The results for protein composition of goats' milk in the paper by Johnston *et al.* (2001), obtained by analysing milk using near infrared transmittance spectrophotometry (NIT), are different from results for milk protein composition reported in the body of this thesis (Table 3.6 and Table 3.7), which were determined from total nitrogen analysis.

Initially, milk protein composition was analysed by NIT, but this was later considered to be less than ideal because the NIT was calibrated against bovine milk data. Also, chemical treatments in milk may have interfered with analysis by NIT. Samples of goats' milk were reanalysed by total nitrogen determination, which was considered to be a more robust measure of crude protein in milk than NIT. However, total nitrogen includes sources of nitrogen other than crude protein and therefore individual milk proteins were analysed to give a more specific indication of the effect of treatments on protein production and are discussed in section 3.4.2.

Appendix Q. Lab photo

Figure 5.7 Lab photo

Members of F3 1999 were from back left: Bruce Sinclair, John Rounce, Fiona Clark, Emma Bermingham, Penny Back, Malcolm Alley; and front: Sarah Johnston.

Appendix R. Supervisor mug shots

Chief supervisor: Associate Professor Kathy Kitson

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