Amino acid supply and metabolism by the ruminant mammary gland

BY BRIAN J. BEQUETTE AND F. R. COLETTE BACKWELL

Rowett Research Institute, Bucksburn, Aberdeen AB21 9SB

Metabolism in the gastrointestinal tract (MacRae *et al.* 1997) and liver (Lobley & Milano, 1997) has been shown to be important in determining the amount and pattern of amino acids (AA) available post-hepatically for mammary gland utilization as well as for muscle, skin and, indeed, the gastrointestinal tract. High rates of metabolism by the non-mammary tissues have been suggested as one reason for the low efficiency (approximately 20 %) of conversion of dietary N into milk protein (MacRae *et al.* 1995). Thus, ways to reduce these losses could lead to a larger proportion of AA being partitioned to the mammary gland for milk protein synthesis. Recent studies (Guinard & Rulquin, 1994; Bequette *et al.* 1996*b*), however, now suggest that once AA are extracted by the mammary gland considerable losses in efficiency of conversion of these AA into milk protein also occur (i.e. net mammary AA uptake greater than milk AA outputs), but little is known of the factors controlling intracellular metabolism. Thus, a better understanding of how catabolic and alternative routes of metabolism within the gland are regulated, and how these might be avoided or reduced, could lead to an increase in the efficiency of milk production and a reduction in non-mammary tissue N losses. The present paper will discuss what is currently known about the processes controlling arterial AA delivery (supply) to the mammary gland, and the mechanisms and metabolic pathways the mammary gland employs to balance the supply of AA at the site of milk protein synthesis during times of inadequacy and excess.

PARTITION OF AMINO ACIDS TO THE MAMMARY GLAND

At the onset of lactation in the dairy goat, whole-body protein synthesis is considerably enhanced, with a substantial proportion attributable to the increased metabolism of the mammary gland. This appears to occur at the expense of other tissues as protein synthesis is relocated from the carcass tissues to the mammary gland, changing from 0·28 and 0·01–0·03 of whole-body protein synthesis respectively in the non-lactating, non-pregnant animal to 0·08 and 0·34–0·43 respectively during early (week 2) lactation (Champredon *et al.* 1990; Baracos *et al.* 1991). Hence, it has often been said that during full lactation the body of the dairy cow is an appendage to the mammary gland. The various factors responsible for initiating and maintaining this dramatic shift are poorly understood, but probably involve ontological regulatory mechanisms and changes in the recognition and sensitivity of nutrient–hormonal signals at the different organ, tissue and cellular levels. These types of regulatory mechanisms will not be discussed in the present paper, which will instead concentrate on sources and delivery of AA to the gland, and subsequently their extraction and metabolism within the mammary gland in support of casein biosynthesis.

In support of protein synthesis in the mammary gland, an increase in the metabolic partition of AA to the gland is required, and when the partition of individual AA to the mammary gland are considered, a different picture emerges compared with the shifts in protein synthesis referred to previously. Utilizing a mixture of [U-^{13}C]-labelled AA as a tracer, plasma flux (irreversible loss rate) and partition to the mammary gland of twelve AA were simultaneously monitored in mid-lactation (week 16) goats (Bequette *et al.*

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1997). On average, 25 % of flux was partitioned to the mammary gland, with values for histidine, serine, phenylalanine and alanine being less than 20 %, arginine, threonine, tyrosine and leucine from 20 to 30 % and proline, isoleucine, lysine and valine from 30 to 40 %. Although the average AA partition differed between individual animals in this study, the relative pattern of AA partition remained consistent between animals (Fig. 1). Thus, the metabolic processes that comprise the plasma flux measurement for each AA (i.e. protein synthesis, oxidation, inter-conversions and metabolite formations) appear to share common regulators, perhaps to maintain the balance of AA available in the whole animal and at the mammary gland level.

BLOOD OR PLASMA SOURCES OF AMINO ACIDS?

The supply of AA to the gland is the product of arterial AA concentration in blood (plasma) and mammary blood flow rate. Quantification of the partitioning process requires *in vivo* methodology, such as the arterio–venous technique, to monitor the exact AA inputs for metabolism by the mammary gland. There has always been uncertainty as to the relative extractions of AA from erythrocyte and plasma pools. Few studies have attempted to address this issue, primarily due to the analytical challenge to detect small concentration differences between erythrocytes and plasma, which when compared on an arterio–venous basis begins to test the sensitivity of most AA analyser systems. Perhaps the most comprehensive analysis to date is that conducted by Hanigan *et al.* (1991), in which erythrocyte and plasma arterio–venous differences across the mammary gland were compared in seventy-nine studies conducted on twenty-one lactating dairy cows involved in a bovine growth-hormone trial. The major finding from this study was the variability found between AA, with only valine extracted just from plasma, while for all other AA both erythrocyte and plasma exchanges occurred, and in some cases (leucine, aspartate,

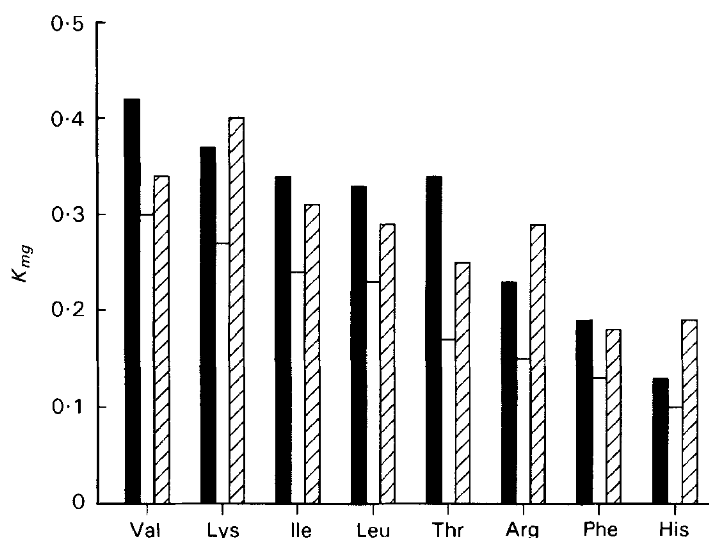

Fig. 1. Coefficients (K_{mg}) for the partition of plasma essential amino acid fluxes to the mammary gland in three lactating goats (■, □, ▨, individual goats). Measurements were made during a 7 h constant intrajugular infusion of a mixture of [U-^{13}C]-labelled amino acids. (Data from Bequette *et al.* 1997.)

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glutamate) counter-current (carriage from the mammary tissues) transport via the erythrocyte was also observed.

IMPORTANCE OF PEPTIDES IN AMINO ACID SUPPLY

One further question regarding the supply of AA to the gland relates to the potential utilization of circulating peptides as a source of AA for milk protein synthesis. The suggestion that peptides may be involved in tissue protein metabolism has provoked much controversy; but, at the same time, there are several pieces of information relating to mammary AA and peptide utilization which cannot be ignored. Thus, on a simple arterio–venous difference basis, certain essential AA (often phenylalanine (and tyrosine), methionine and histidine) are extracted in insufficient amounts in the free form to account for their outputs in milk protein (Bickerstaffe *et al.* 1974; Guinard & Rulquin, 1994; Metcalf *et al.* 1994, 1996; Backwell *et al.* 1996*b*), leading to the suggestion that this apparent deficit may be met by the uptake of circulating peptides. Unfortunately, direct measurement of peptide fluxes across tissue beds is fraught with difficulties because of the lack of reliable and reproducible methodology available for their quantification (for review, see Backwell, 1997).

In an attempt to overcome these difficulties, we used a combination of chemical measurement and tracer techniques in studies in lactating goats and cows to provide further support that the mammary gland can and does utilize peptides and(or) proteins for milk protein synthesis. Employing a dual-labelled tracer technique, direct utilization of arterially-supplied dipeptides (glycyl-phenylalanine and glycyl-leucine) as a source of AA for casein synthesis in goats was demonstrated (Backwell *et al.* 1994). Subsequently, evidence for the utilization of naturally-occurring peptides was obtained in a companion study in goats (Backwell *et al.* 1996*b*). During a long-term (24 h) intravascular infusion of [1-^{13}C] phenylalanine, the free AA enrichments in blood and plasma were compared with plateaux values in secreted milk casein. The casein-bound phenylalanine enrichment attained only 80–90 % of the blood or plasma value at plateaux, implying that 10–20 % of casein-phenylalanine was derived from the uptake of peptide or protein-bound (i.e. unlabelled sources) phenylalanine. By contrast, in these same animals, casein : plasma relationships for [1-^{13}C]leucine indicated that this AA was not extracted in peptide or protein-bound form, as might be expected since this AA is consistently extracted in excess of milk protein output and, thus, additional sources are not required. Similar observations (casein : plasma enrichment of 1) have since been obtained for leucine in the dairy cow (Boirie *et al.* 1995; France *et al.* 1995; Bequette *et al.* 1996*b*). The contrasting observations between leucine and phenylalanine eliminate the possibility that the plateaux differentials were related to intracellular protein turnover, since this process should have contributed, by supplying unlabelled material, equally to both AA pools. In addition, the apparent selection of phenylalanine rather than leucine-containing peptides implies a degree of peptide specificity.

In the studies of Guinard & Rulquin (1994) and Metcalf *et al.* (1994, 1996), where the extractions of certain AA were shown to be less than their requirements for milk protein outputs, it was subsequently shown that when additional supplies of AA are provided (dietary protein supplementation, intragastric casein or intravascular free AA infusion), many of the AA are extracted in far greater excesses than previously; thus, for those AA whose uptake was less than requirements (phenylalanine, tyrosine), uptake now exceeded milk output. The latter suggests that the gland is capable of changing the proportion of AA derived from the blood free AA or peptide pools, depending on supply in order to balance its net requirements.

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Recently, we (B. J. Bequette, F. R. C. Backwell and C. E. Kyle, unpublished results) conducted a study to further examine this phenomenon. In late-lactating (from weeks 24 to 40) goats, casein : plasma plateaux enrichments of phenylalanine and tyrosine were compared during the last 5 h of a 30 h intravenous infusion of [1-^{13}C]phenylalanine and [^{2}H$_4$]tyrosine. Plateaux comparisons were made during a control period (no infusion) and during a 5 d period of intravenous infusion of free phenylalanine (6 g/d) with treatment periods separated by 1 week. The hypothesis under examination was that if, during the control period, a differential existed between casein and plasma plateaux values, then during free phenylalanine infusions this differential would be reduced if the gland utilized free phenylalanine in replacement of the peptide-derived AA. Preliminary data (Table 1) from three goats indicates that for those animals demonstrating phenylalanine uptake as peptide (i.e. casein : plasma enrichment < 1) during the control period, the requirement for peptide uptake was diminished or disappeared (i.e. casein : plasma enrichment approximately 1) when additional phenylalanine was provided in the free form. The results for phenylalanine in this study are not as consistent as those in our previous study (Backwell *et al.* 1996*b*) in which, for all goats on the two diets compared, phenylalanine uptake as peptide was observed. Interestingly, a casein : plasma differential was also observed for tyrosine, which would be compatible with its net uptake : output value being < 1 (Guinard & Rulquin, 1994; Metcalf *et al.* 1994, 1996), and this differential was also reduced on infusion of the free phenylalanine, but on the condition that the differential had already existed for phenylalanine during the control period (goats nos. 1 and 2, Table 1). This may suggest animal variability for general peptide uptake capabilities, which may explain the differences observed between this experiment and our previous experiment (Backwell *et al.* 1996*b*). For tyrosine, isotope dilution (casein : plasma) can occur due to peptide (unlabelled AA) uptake and/or from *de novo* mammary gland conversion of phenylalanine to tyrosine. Thus, either or both routes of tyrosine supply were reduced in favour of free tyrosine uptake when arterial supply of tyrosine was enhanced.

If the latter observations on peptide phenylalanine uptake can be confirmed and extended to other AA in the dairy cow, such as those often implicated as being limiting on most dairy rations (i.e. methionine and lysine), then it will be important to be able to quantify these contributions and characterize the mechanisms involved in their regulation. Indeed, studies *in vitro* of the utilization of methionine-containing peptides by MAC-T cells and tissue explants from lactating mice indicate that most of the methionine-containing peptides examined could replace free methionine and, for some of the peptides, promoted greater rates of milk protein synthesis compared with free methionine (Pan *et al.*

Table 1. *The effect of a 5 d intravenous infusion (6 g/d) of phenylalanine on the plateaux ratios (casein : plasma) of [1-^{13}C]phenylalanine and [^{2}H$_4$]tyrosine in casein and in arterial plasma during the last 5 h (1 h sampling intervals) of a 30 h constant intravenous infusion of these isotopes in three late-lactation goats* (Data from B. J. Bequette, F. R. C. Backwell and C. E. Kyle, unpublished results)

(Each infusion period was separated by at least 1 week)

	Control (no infusion)		Phe infusion	
Goat no.	[1-^{13}C]Phe	[^{2}H$_4$]Tyr	[1-^{13}C]Phe	[^{2}H$_4$]Tyr
1	0·94	0·77	1·00	0·92
2	0·84	0·80	0·99	0·92
3	0·98	0·88	0·97	0·88

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1996; Wang *et al.* 1996). Demonstration of the stimulatory effects or advantages of such peptides *in vivo* could open up a whole new area in the targeting of specific (limiting) AA for milk protein synthesis through the development of feeding regimens or supplements which promote the production of peptides or proteins in the rumen or in the body, or by dietary supplementation of specific peptides. In fact, intra-jugular infusion of glycyl-histidine into goats with an induced histidine deficiency has been shown to increase milk protein output more consistently than free histidine infusion alone (Backwell *et al.* 1996*a*).

BLOOD FLOW AND AMINO ACID SUPPLY: PUSH OR PULL?

A key question regarding the supply of AA to the mammary gland relates to the importance of mammary blood flow: whether increases in blood flow drive mammary protein synthesis by increasing AA availability (i.e. the 'push' hypothesis), or if increases in mammary metabolism result in subsequent changes in blood flow in order to meet the gland's additional substrate or other requirements (i.e. the 'pull' hypothesis). With respect to the partition of absorbed AA, perhaps mammary gland blood flow and its relationship with cardiac output and blood flow through the hepatic, gastrointestinal, and other peripheral tissues (muscle, skin) will prove to be just as crucial when it comes to predicting the quantities of AA directed to the mammary gland. To date, however, other than as a measurement to achieve the equation for nutrient uptake by the mammary gland (and other tissues), little is known of the role of blood flow in regulating nutrient supply. Recently, there has been some interest in identifying the hormones and other factors responsible for regulation of mammary blood flow (Prosser *et al.* 1996).

We (B. J. Bequette and C. E. Kyle, unpublished results) have now started to obtain evidence to support the concept that blood flow is driven, at least in part, by mammary metabolism and its substrate requirements, i.e. the pull mechanism. In contrast to an earlier study (Maltz *et al.* 1984), it was observed that during unilateral frequent (eight times daily for 5 d) milking of goats, subsequent increases (6–36 %) in milk and protein yields were mirrored by increases (3–35 %) in mammary gland blood flow (monitored continuously by ultrasonic flow probes). The blood flow to the contralateral gland (milked once daily and blood flow logged continuously) was reduced in relation to its milk yield; thus, it is likely that blood flow was regulated locally through changes in capillary perfusion efficiency in response to the increased or decreased metabolic activity of the respective glands, as others have suggested (Davis *et al.* 1988). Work from the latter group (Prosser *et al.* 1996) suggests that, in addition to arterial blood flow, regulation also occurs at the capillary level, with changes in perfusion efficiency occurring to alter nutrient extraction under limiting nutritional conditions (fed *v.* 24 h starved). What is not known, however, is whether the increased blood flow and the changes in capillary vaso-activity are regulated directly through substrate supply.

EFFECT OF AMINO ACID LIMITATIONS ON MAMMARY BLOOD FLOW

The effects of a single AA limitation, histidine, on mammary AA metabolism and blood flow were examined in lactating goats (B. J. Bequette, J. C. MacRae and G. E. Lobley, unpublished results). An induced histidine deficiency was created by intra-abomasal infusion of a complete AA mixture (77 g/d) either with or without histidine. Removal of histidine resulted in a decrease in arterial plasma histidine (50 nM–15 μM) and milk protein output (26–34 %). By contrast, mammary blood flow increased (46–58 %) gradually and inversely in relation to the daily changes in milk protein output. The effects on milk protein output, arterial histidine and blood flow were all reversed on addition of histidine.

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Interestingly, despite the reduced plasma histidine levels during its withdrawal, mammary gland fractional extraction of histidine was enhanced considerably (15–20 *v.* 85–95 % extraction, i.e. 1–2 μM-histidine in venous plasma) in order to maintain a net rate of uptake of histidine commensurate with the uptakes of other AA for milk protein synthesis. This is the first evidence suggesting that the mammary gland may be capable of sensing and responding to a single nutrient limitation through alterations in its rate of blood flow. The observations with histidine are the reverse of that predicted by the theoretical model of mammary gland blood flow developed by Cant & McBride (1996); given a higher concentration of substrate, blood flow would decrease to maintain the arterio–venous difference, i.e. the net requirement of substrate. The data of Guinard & Rulquin (1995) would also appear to support the latter prediction, as they observed a quadratic decrease in mammary blood flow in response to incremental (0, 8, 16 and 32 g/d) amounts of methionine infused into the duodenum of dairy cows. Perhaps the most intriguing aspect of our study was the efficient extraction of histidine during the deficiency period, and this questions why under normal circumstances extraction is less efficient (10–60 %) not only for histidine but also for all other AA. One possibility is that mammary metabolism and utilization of AA becomes more limiting than extraction (uptake).

THE PARTITION OF AMINO ACIDS FOR PROTEIN METABOLISM IN THE MAMMARY GLAND

The lactating mammary gland is a site of considerable metabolic turnover, with high rates of protein synthesis and degradation and continuous AA catabolism and anabolism. The extent to which these processes are an advantage or disadvantage to the mammary gland in its drive to synthesize and secrete milk protein will need to be considered, if attempts to predict the AA output into milk protein and improve the efficiency of conversion of absorbed AA into milk protein are to be successful.

Studies in dairy goats and cows indicate that total protein synthesis in the lactating mammary gland is 1·3–2·5-fold the rate of milk protein secretion (Oddy *et al.* 1988; Champredon *et al.* 1990; Baracos *et al.* 1991; Bequette *et al.* 1996*b*). In the absence of protein accretion (gland growth), this would suggest high rates of protein turnover, representing an 'additional' energetic cost, which may also compete with milk protein synthesis by either competing for precursors or altering the pattern of AA precursors for casein synthesis. Degradation of both nascent and mature caseins are known to occur, the former involving proteolytic cleavage to remove signal (docking) sequences (7–10 % of the molecule; Craig *et al.* 1979), while the latter is proposed to occur in response to incomplete milk removal reported to be under regulation by an inhibitor of milk secretion (feedback inhibitor of lactation; FIL) found in the whey fraction of residual milk (see Wilde *et al.* 1995). To date, however, the potential loss of casein through the proposed FIL-regulated pathway, particularly where this occurs in practice (i.e. on-farm), has not been quantified. Another contributor to protein turnover in the gland is the continuous synthesis and degradation of constitutive proteins (apoptosing cells, cell-wall debris lost during secretion, enzyme turnover, aberrant protein repair and/or replacement). Fractional rates of protein synthesis in the lactating goat mammary gland, measured directly by incorporation of isotope into mixed tissue proteins, ranges from 40 to 130 %/d (Champredon *et al.* 1990; Baracos *et al.* 1991). What will be important to ascertain is whether the turnover of these proteins serves an obligatory role in the milk protein biosynthetic process, perhaps as part of the repair and replacement processes following every secretory event (Larson, 1979).

Current information suggests that the turnover process may be linked to milk protein synthesis. In a study in goats (Bequette *et al.* 1994) examining the transfer kinetics of ^{13}C-

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labelled AA (leucine, valine, phenylalanine, methionine) from blood into secreted milk protein, the blood (plasma) free AA pool reached an isotopic plateau rapidly (1–3 h), whilst the rate of incorporation into casein was much slower and did not reach an asymptote with the blood (plasma) until after 12–13 h. A similar pattern (rate) of casein labelling was observed for all four AA monitored, and this rate did not differ between animals, or between mid- and late-lactation when milk protein output had been reduced by 50 % (130 *v*. 66 g/d). We have now extended these observations on leucine to the dairy cow (France *et al.* 1995; Bequette *et al.* 1996*b*), and in the goat have observed a similar temporal casein-labelling pattern for thirteen AA (some AA could not be monitored by mass spectrometry) during a 7 h infusion of a mixture of [U-^{13}C]-labelled AA (Bequette *et al.* 1997). The appearance of isotope in casein within 1 h suggests that the time interval between synthesis and secretion is short, with small storage or residual pools of casein. Consequently, the continued (diminishing) dilution of isotope with non-blood sources suggests that before incorporation into casein a substantial proportion of the total AA pool is 'channelled' through an intermediary pool(s), presumably either rapidly turning over constitutive proteins and/or casein. More importantly, it appears that this channelling process may be an obligatory event in the milk synthetic process, perhaps as a 'buffer' system to maintain sufficient rates of delivery and concentrations of AA at the site of casein synthesis (i.e. the local pool of acyl-tRNA) during periods of deficiencies.

In a study conducted by our group, protein metabolism in the dairy cow mammary gland, monitored by an arterio–venous kinetic technique with infusion of [1-^{13}C]leucine, was observed to increase only slightly in response to supplemental dietary protein intake (Table 2; Bequette *et al.* 1996*b*). This was perhaps not surprising since milk protein output was also not altered by supplementation. Subsequently, J. C. Metcalf, L. A. Crompton, J. D. Sutton, M. A. Lomax, E. E. Chettle, D. E. Beever, J. C. MacRae, F. R. C. Backwell and B. J. Bequette (unpublished results) monitored mammary protein metabolism (employing the arterio–venous [1-^{13}C]leucine kinetic model) in the dairy cow in response to intravascular infusion of a mixture of ten essential AA. It was found that total gland protein synthesis increased according to a fixed relationship with the increase in milk protein output (Table 2). Thus, on an incremental basis, the increased total gland protein synthesis was 2·5-fold the increase in milk protein output and this would compare directly with the earlier relationships observed in lactating goats (Champredon *et al.* 1990; Baracos *et al.* 1991). These observations suggest that this additional protein synthesis is a required event in the synthesis and secretion of milk protein which, when it comes to modelling the dynamics of metabolism in the mammary gland, can be assumed to be fixed, especially in relationship to the energetics of the milk protein synthetic process.

CATABOLISM AND SYNTHESIS OF AMINO ACIDS WITHIN THE MAMMARY GLAND

Numerous studies in dairy animals, under basal feeding conditions and when given supplemental sources of AA, have now demonstrated that for several AA (leucine, valine, isoleucine, arginine, lysine, threonine, histidine) net extractions by the mammary gland are in excess of milk protein outputs (Bickerstaffe *et al.* 1974; Guinard & Rulquin, 1994; Metcalf *et al.* 1994, 1996). For many years, this phenomenon formed the basis for the argument that these AA were the 'limiting ones', since they may have a role other than as precursors of milk proteins. Through the years, attempts were made to reconcile these observations, and in studies conducted with perfused glands (Verbeke *et al.* 1968; Roets *et al.* 1974) and mammary explants (Wohlt *et al.* 1977) these AA were found to be catabolized along pathways similar to other tissues, utilized in the synthesis of non-

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Table 2. *Comparison of studies examining the effects of protein supplementation or intravenous amino acid (AA) infusions in dairy cows and goats on leucine metabolism by the mammary gland*

Study	Oxidation (g/d)	Total gland protein synthesis (g/d)	Milk leucine output (g/d)
Dairy cow			
Bequette *et al.* (1996*b*)			
Basal diet (140 g CP/kg)	5	112	89
Protein supplemented diet (180 g CP/kg)	18*	119	86
Metcalf and co-workers† (unpublished results)			
Basal diet (140 g CP/kg)	8·1	70·5	63
Basal diet + essential AA infusion (208 g/d)‡	14·4*	96	73
Dairy goat			
Bequette *et al.* (1996*a*)			
Basal diet (110 g CP/kg)	1·2	4·9	–§
Basal diet + total AA infusion (65 g/d) without leucine	0·6*	7·6	–

CP, crude protein (N × 6·25).

* Values were significantly different from those for the basal diet ($P < 0{\cdot}05$).

† J. C. Metcalf, L. A. Crompton, J. D. Sutton, M. A. Lomax, E. E. Chettle, D. E. Beever, J. C. MacRae, F. R. C. Backwell and B. J. Bequette.

‡ AA were administered via jugular infusion.

§ Milk protein output was not significantly different between treatments; leucine output was not measured directly.

essential AA and specialized compounds, as well as being used for casein synthesis. Despite this information, we know very little of the contribution and relevance of these processes to the metabolism of the mammary gland *in vivo*. The following discussion will highlight recent studies in this and other laboratories, conducted on the mammary gland of the lactating goat and cow *in vivo*, in which these issues were considered.

In one study in which protein supplements were given to dairy cows (Bequette *et al.* 1996*b*), leucine oxidation by the gland increased with supplementation (Table 2). Nearly all the excess leucine taken up was oxidized and there was no response in milk protein leucine output, suggesting that leucine oxidation occurs as a passive response to its excess supply. The uptakes of several other AA were also increased and this raised the possibility that the oxidative process was competitive with milk protein synthesis. Oddy *et al.* (1988) had also observed an inverse relationship between leucine oxidation and milk protein output in comparisons of mammary leucine oxidation in early- and late-lactating goats. To test this hypothesis, we (Bequette *et al.* 1996*a*) monitored mammary leucine oxidation in early lactation goats given a jugular infusion of either saline (9 g NaCl/l) or a complete mixture of AA but not including leucine. Surprisingly, milk protein output was not increased by infusion of the AA mixture; however, leucine oxidation was nearly halved, suggesting that oxidation, at least of leucine, was not competitive with milk protein synthesis (Table 2). The study also demonstrated that leucine oxidation is probably not an obligatory event in the milk synthetic process, although there may be a basal level of oxidation required since leucine oxidation was not eliminated (19 *v.* 7 %). The fact that milk protein output was not increased with AA infusion suggests that either leucine had become limiting, or that there was a limitation on the utilization of these AA within the gland.

In general, under basal feeding conditions, non-essential AA are not extracted in sufficient quantities to account for their milk protein outputs; thus, the deficit must be made-up, either by uptake of these AA in peptide and/or protein-bound forms or they must

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be synthesized by the mammary gland from C, S (for cysteine synthesis) and N sources. At present, there is no direct evidence demonstrating uptake of non-essential AA in peptide form. The tripeptide glutathione (γ-glutamyl-cysteinyl-glycine) has been implicated as a possible source of cysteine (Pocius *et al.* 1981), and perhaps for glutamate and glycine. This theory might appear to be dismissable because there is limited incorporation of ^{35}S into casein during a close-arterial (external pudic artery) infusion of [^{35}S]glutathione into the goat mammary gland (Knutson *et al.* 1994). In that study, however, tracer glutathione did not equilibrate with the erythrocytes, the major intravascular pool of the glutathione for uptake, thus the possibility for glutathione contribution cannot be eliminated.

Methionine could also supply S for the *de novo* mammary synthesis of cysteine. However, in recent experiments conducted in New Zealand, this contribution in the goat was found to be small (Lee *et al.* 1997). The net uptake of methionine rarely exceeds milk outputs, even under supplemental conditions (Guinard & Rulquin, 1995); thus, transport of methionine into the mammary cell and the contribution of its S group via trans-sulfuration could be the limiting step in the synthesis of cysteine. The C skeleton for cysteine synthesis is donated by serine, but the supply of this AA may also be limiting since its net uptake is always inadequate to account for milk protein outputs. Alternatively, the real limitation for cysteine synthesis may be the low activity of the trans-sulfuration pathway. This places a limitation on the intracellular availability of cysteine; at least under basal feeding conditions (see below) when the protein supply to the animal will be dominated by microbial protein, which is limiting in the S AA. A better understanding of the regulation of the trans-sulfuration pathway, or how the enzyme is expressed, could lead to nutritional or genetic approaches to manipulate milk protein production.

Tyrosine, also, is not extracted in adequate quantities. It is assumed, but not proven *in vivo*, that this deficit can be made-up through the mammary conversion of phenylalanine to tyrosine by the enzyme phenylalanine hydroxylase (*EC* 1.14.16.1; Jorgensen & Larson, 1968). However, one questions the extent to which this occurs in practice, since phenylalanine is itself often not extracted in adequate amounts in the free-form, and tyrosine may also be extracted in peptide-bound form (B. J. Bequette, F. R. C. Backwell and C. E. Kyle, unpublished results).

In contrast, the uptakes of both tyrosine and cysteine from blood can be increased to match or exceed their outputs in milk protein when dairy cows are given additional dietary protein or post-ruminal infusion of casein (Guinard & Rulquin, 1994; Metcalf *et al.* 1994, 1996). Despite the increased arterial supply of all AA in these studies, the extractions of serine, proline, glutamine, glutamate, glycine and aspartate, although increased, were still insufficient to balance with their outputs in milk protein. Many of these AA can apparently be synthesized from the C skeletons of some of the essential AA (Wohlt *et al.* 1977). However, where the source of N comes from for this synthesis is uncertain and open to speculation. The branched-chain AA are taken up in excess by the mammary gland and would appear to be candidates to contribute some of this N because of their involvements in transamination reactions. Arginine, also extracted in quantities three times greater than its output in milk, and ornithine and citrulline, which are not incorporated into milk protein but are extracted in large quantities, have been shown to contribute 20 % of the proline residues in casein via a partial urea cycle (Verbeke *et al.* 1968; Roets *et al.* 1974). The key enzyme in this metabolic conversion, ornithine-δ-aminotransferase (*EC* 2.6.1.13), has been characterized in bovine mammary tissue and found to have a high K_m (8·4 mM), necessitating high pools of ornithine to achieve maximal rates of conversion through this pathway (Basch *et al.* 1995). Consequently, this route of proline supply in the gland may be inadequate to balance the demands of proline for casein synthesis. In this respect, it is

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interesting to revisit the study of Bruckental *et al.* (1991) in which proline infused (80 g/d) into the duodenum of two mid-lactation cows increased milk protein output by 16 % and reduced arginine uptake by the mammary gland, although the authors did not report on proline uptake. Despite the limitations of these data, they do raise some intriguing questions as to the limitations some of the so-called intermediary routes of metabolism may have on milk protein synthesis.

FUTURE PERSPECTIVES

The mammary gland has a unique metabolism that allows it to synthesize proteins, AA and other energy substrates and secrete these in milk. The features of this metabolism may give the gland a competitive edge over other tissues of the body to assure its continued priority during lactation. On the other hand, many of these metabolic features may contribute to the inefficiency of milk production. Thus, the mammary gland has the ability to extract large quantities of all AA. However, proportions of some of these AA may be needed to synthesize 'non-essential' AA and specialized compounds (polyamines), and some AA may be lost through catabolic pathways, all of which may divert AA away from casein synthesis or create an imbalance in the local intracellular AA pools. Whilst apparent deficiencies of certain AA for net anabolism may be overcome by the uptake of peptides and/or proteins, direct demonstration of their utilization is limited to phenylalanine (and possibly tyrosine). There is a need for further investigation, especially in terms of demonstrating the mechanisms of peptide uptake and utilization *in vivo*. In this respect, it is not surprising that even the most current metabolic model of the dairy cow (for example, see Hanigan, 1997) fails to provide acceptable predictions of the utilization of AA for milk protein synthesis.

Thus, more *in vivo* examination is needed to ascertain, in the context of the milieu of events in the animal and in relationship to milk protein outputs, how AA metabolism at the levels of the whole body (non-mammary tissues) and mammary gland is regulated. The partition of individual AA or groups of AA to, and within, the mammary gland between anabolic (milk protein synthesis) and catabolic pathways will need to be quantified and understood. In addition, information is needed on the limits to AA and/or peptide or protein transport across the mammary capillary–epithelial cell-wall barriers, especially the interactions that may be occurring at the transporter levels which ultimately could determine the pattern and size of the intracellular AA pool for milk protein synthesis. In this connection, perhaps an alternative strategy to identify limiting AA for milk protein synthesis will need to consider AA × transporter interactions in relation to the pattern and local pool levels of AA necessary to initiate and maintain synthesis of the caseins, beginning at the gene level. Consideration of the contributors to the supplies of C and N for *de novo* mammary synthesis of non-essential AA and the regulation of the key enzymes in these pathways is needed, with one potential spin-off involving genetic approaches (e.g. selection, transgenesis, gene insertion) to alter the limiting or catabolic steps. There is still the question of AA supply; how much and what (free AA *v.* peptides) does the mammary gland receive relative to other tissues. Consequently, an understanding of the regulation of blood flow at the whole-animal and mammary gland levels will be important in determining the amount and rate of AA supply.

Integrating these mechanisms of AA delivery and metabolism by the mammary gland with those occurring throughout the whole animal will be the next step. The mammary gland obviously plays a major role in determining (pull hypothesis) the delivery and metabolism of nutrients, but this is certainly only part of the regulation. Homeorhetic and

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homeostatic hormonal regulations will be important in maintaining the 'checks and balances' throughout the body (Bauman & Curie, 1980). What is not fully understood is how, on the one hand, up-regulation of the partition, extraction and metabolism of nutrients by the mammary gland occurs, while, on the other hand, the non-mammary tissues (e.g. muscle, skin) are down-regulated, but only to the extent that they are still allowed to meet metabolic requirements and prevent complete tissue depletion. We are just now beginning to get a glimpse of how these mechanisms unite through nutrient–hormone (e.g. substrate-hormonal potentiation; McGuire *et al.* 1995; Crompton *et al.* 1997) and hormone–receptor–tissue interactions (Houseknecht *et al.* 1995).

These are the challenges facing scientists now and in the future, which, hopefully, will drive efforts in lactation research towards achieving the goals of increased efficiency of conversion of dietary N into milk protein and improving the quality of milk products to satisfy today's consumers and the milk manufacturing industry's processing requirements.

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