Current Concepts of Amino Acid and Protein Metabolism in the Mammary Gland of the Lactating Ruminant

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ABSTRACT

Milk protein responses to protein nutrition are typically poor and, in part, may be due to the low efficiency (~25 to 30%) of converting dietary N into milk. Posthepatic availability of amino acids (AA) is not limited, yet only ~30% is converted into milk. The poor capture of AA by the mammary gland may relate to the imbalanced and uncoordinated timing of nutrient delivery to the gland. The infusion of essential AA improves the efficiency of utilization (0.31); however, further catabolism of AA within the mammary gland suggests that AA transport is not a major limitation. These losses may serve ancillary or functional roles, but mammary oxidation of some AA occurs only when AA extraction exceeds the stoichiometric requirements for milk protein synthesis. Intracellular substrate supply may be more limiting than is the appartus for protein synthesis. Studies utilizing isotope labeling and conducted in vitro and in vivo now suggest that circulating peptides and proteins can serve as sources of perhaps all AA for casein synthesis, but the source of these remains elusive. Constitutive protein and casein turnover contribute significantly (42 to 72%) to mammary protein synthesis. All AA are extensively channeled through an intermediary protein pool or pools that have rapid turnover rates. The AA are then incorporated into casein, which appears to be fixed in association with protein turnover. The mammary gland is a major controller of its metabolism, and the mechanisms of AA extraction and conversion into milk protein are linked to secretion events. Blood flow may be a key point of regulation whereby mechanisms sense and respond to nutrient supply and balance to the gland via alterations in hemodynamics.

(**Key words**: amino acid, mammary gland, casein, lactation)

Abbreviation key: **BCAA** = branched-chain AA, **EAA** = essential AA, **EPV** = external pudic vein, **GIT** = gastrointestinal tract, **GSH** = glutathione, **MG** = mammary gland, **NEAA** = nonessential AA.

INTRODUCTION

Dairy producers and researchers have the goals of increasing the conversion efficiency of dietary nutrients (energy and protein) into milk and improving its healthful properties (reduced fat and higher protein) and processing characteristics (e.g., >3% milk protein, higher κ-CN and lower β-LG contents, and reduced lactose). An ability to achieve these goals, preferably jointly and through approaches that are acceptable to consumers, should translate into greater profit margins at the marketplace.

The efficiency of converting dietary N into milk protein output is poor at 25 to 30%. Milk protein content and yield can be increased by dietary protein supplementation or by gastrointestinal infusion of protein or AA; however, the responses attained are often unpredictable and are considerably less than would be predicted by current feeding schemes for dairy cows (1, 53, 82). This result is largely due to the incomplete understanding and the inadequate mathematical representation of the metabolic transformations of AA during absorptive and postabsorptive processes (2). Currently, a fixed factor (0.64 to 0.80 on an incremental basis) is used to convert absorbed AA into milk protein despite evidence that the observed response to supplemental protein AA is curvilinear [i.e., diminishing partial efficiencies (47, 80, 110)]. For example, Guinard et al. (47) observed that the conversion of absorbed essential AA (**EAA**) into milk protein decreases from 0.44 to 0.34 as levels of duodenal infusion of casein increase up to 762 g/d. A large proportion of this loss occurred across the mammary gland (**MG**) such that the udder conversion of EAA decreased from 0.88 to 0.49 with infusion. These responses probably would not have been predicted by the current protein feeding schemes. Thus, in the future, the dynamics of postabsorptive metabolic events, especially those governing the availability of AA to the MG and the utilization of AA by the MG for milk protein synthesis, will need to be

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represented so that responses in milk protein output from changes in dietary nutrient availability can be predicted more accurately. As a consequence, dairy producers might be better able to formulate diets that are economical and biologically efficient to attain desired production goals.

Our two research groups have been working jointly on a project investigating the organ tissue-specific metabolism of nutrients, primarily AA, in the lactating dairy cow and goat to elucidate the mechanisms and quantify the important nutrient interactions occurring in the gastrointestinal tract (**GIT**), liver, and MG. The overall aims of this project are to develop the framework of a mechanistic model of dairy cow metabolism for on-farm use in managing expensive feed resources and to identify ways of altering the protein content of milk (71). Aspects of studies conducted on the MG in vivo form part of this review, which briefly considers mammary AA metabolism in the context of the whole body and attempts to identify limits to AA conversion into milk protein. Many of our studies have coupled stable isotope kinetics to the conventional arteriovenous difference technique across the MG to investigate the dynamics of AA and protein metabolism and the possible role of blood-derived peptides and proteins as sources of AA for milk protein synthesis.

TISSUE PROTEIN METABOLISM BY THE WHOLE BODY

Milk protein secretion immediately postpartum occurs at such high rates that, except for the equally dramatic increases (by ~300%) in feed intake and the modulations (both up- and down-regulation) that occur in nonmammary tissue AA and protein metabolism, the cow could quickly reach a metabolic crisis by depleting critical protein reserves. The establishment and coordination of this higher plane of metabolism forms the basis of the homeorhesis concept proposed by Bauman and Currie (11) whereby acute and chronic interactions among nutrients, hormones, and tissues orchestrate the metabolism and physiology of the whole body in a way that allows for high rates of milk production while also allowing the animal to partition nutrients for vital functions that are unrelated to the MG. The net result is that nutrients are delivered at adequate rates to the MG from exogenous (i.e., diet) or endogenous (e.g., fat depots and muscle tissue) sources to support synthesis of milk constituents while sufficient quantities of nutrients are held in reserve to protect the health and reproductive status of the cow during the current and subsequent lactations.

For example, the tissues of the dairy goat and cow undergo dramatic changes in metabolism within the first few weeks of lactation, including substantial increases in the protein mass of the MG (+1000%) and of the splanchnic tissues (rumen, small and large intestines, and liver; +11 to 28% vs. dry animals); conversely, decreases (18 to 21% vs. dry animals) occur in carcass, head, and feet protein mass (8, 25, 43). These changes have been linked to alterations in the fractional and absolute rates of tissue protein synthesis, but rates of protein degradation have not yet been monitored at this early stage. During later lactation, however, there is some evidence that muscle tissue depletion and repletion are modulated mainly by the regulation of proteolytic activity. Insulin is thought to inhibit protein hydrolysis in the muscle of lactating goats by selectively acting on skeletal muscle tissues, resulting in a decrease in the mRNA levels of ubiquitin, a component of the ATP-ubiquitin-dependent proteolytic pathway (62). The inhibitory actions of insulin appear to increase as stage of lactation advances, but this activity appears to be augmented by AA supply (102). Thus, through the interdependent influences of tissue sensitivity and substrate supply, the depletion or repletion of muscle protein stores could be tightly regulated to maintain a balanced and continuous supply of precursors to the MG. Perhaps a clearer understanding of how this process is regulated through metabolite receptor interactions and postreceptor events could lead to the development of nutritional regimens to up- or down-regulate the proteolytic pathway and make better economic use of these potentially rate-limiting reserves of AA.

In early lactation, energy and N intake are often less than adequate to support the high rates of milk protein output. In these circumstances, the depletion of carcass tissue protein is important to supplement dietary and microbial protein AA and to maintain an adequate supply of AA to the MG and carbon for gluconeogensis by the liver. In the dairy cow, mobilization of protein from tissue is capable of supplying 90 to 430 g of AA/d (43, 60); in the dairy goat, this contribution could range from 2 to 66 g/d (8, 9, 25, 102). Thus, for a cow producing 1 kg of milk protein/d, the contribution of these reserves can be considerable in terms of the quantity supplied; equally important however, is the timeliness of this contribution with respect to the requirements of the MG in early lactation to synthesize parenchymal (secretory) tissue (57) as well as milk proteins.

The contribution of tissue mobilization has been assessed in cows by employing an isotope dilution technique, which exploits the natural differences in

the ^{13}C concentration of C_3 and C_4 in plants (115). The proportion of endogenous reserves contributing to the pool of carbon for casein synthesis was greater (0.34 vs. 0.25) for cows of high genetic merit than for cows of low genetic merit. With this data set, it is not possible to quantify the exact contributions to casein synthesis, but relative comparisons suggest that one possible reason for the greater persistency of milk production that is associated with dairy cows of high genetic merit is the size of their body protein reserves and the inherent ability of these cows to mobilize such reserves readily during early lactation for the synthesis of casein and lactose (from glucogenic AA).

The net availability of absorbed AA for milk protein synthesis, however, may also be inherently limited. For example, Black et al. (23) monitored whole body oxidation of 20 AA in lactating cows and observed that the AA were oxidized to varying (3 to 42%) degrees. The AA that are likely to be more limiting for milk production (i.e., His, Lys, Met, Phe, and Thr) constituted a group that was oxidized the least (4 to 7%). These losses may seem relatively trivial, but, if the efficiency of utilization of individual AA for milk protein synthesis is as varied as it is in growing sheep for muscle gain [Thr, 0.32 to Arg, 0.88; (69)], then the consequences of reducing or eliminating oxidative losses could be sufficient to alleviate the limitation on AA supply. Thus, a large production response would be expected when the partial efficiency of utilization of the limiting AA is also large, or, conversely, the response may be small if the efficiency is lower. Other specialized functions may exist for an AA that may be as rate-limiting to milk production as is the supply of the AA as a substrate for protein synthesis. By i.v. infusion of a mixture of U-^{13}C-labeled AA, we (14) monitored the plasma flux in the whole body and the partition of a range of AA to the MG of goats. The proportion of flux that was diverted to the MG varied with His, Ser, Phe, and Ala directed to less than 0.20; Arg, Thr, Tyr, and Leu from 0.20 to 0.30; and Pro, Ile, Lys, and Val from 0.30 to 0.40. These differences suggest a divergence of priorities for AA utilization for MG and non-MG tissue. The relative pattern of this partitioning remained consistent for all animals, indicating that the tissue processes contributing to the plasma flux measurement for each AA (i.e., protein synthesis, oxidation, metabolite interconversion, and formation) may share common regulators, perhaps to balance the metabolic demands of whole body tissue.

Where do these metabolic losses occur? Are they actively or passively regulated? Conceptually, these questions are important to consider because their answers will determine where research efforts may need to be focused, that is, whether attempts should be directed at reducing non-MG losses or on improving the ability of the MG to retain AA and to convert them into milk proteins. The MG receives a pattern of supply that reflects the cumulative metabolism of the GIT and hepatic tissues (2, 68, 70, 94). The GIT derives approximately 20 and 80% of its AA requirements from the luminal and arterial supplies, respectively, to the gut (70). These tissues, therefore, have the potential opportunity to influence both the amount and pattern of AA that are available to the MG during first pass digestive absorption and then as a competitor of posthepatic AA supplies.

The protein mass of the goat liver increases by 25% within a week of parturition (8, 25). Data for rats indicate that, despite the greater protein mass of the liver, net removal of AA is lower than that from nonlactating counterparts, presumably because AA is directed toward the MG (40). Few studies such as this have been conducted using the lactating dairy cow. From a survey of several studies of cows (2), the utilization of AA N by the liver has been estimated to represent, on a net basis, approximately half of the nonmammary tissue utilization of AA entering the duodenum.

Most of what is known of hepatic metabolism derives from growing sheep, for which net removal represents considerable catabolic losses (67) and use for the synthesis of export proteins (26). Quantification of these processes for dairy cows has not been extensively examined; however, Reynolds et al. (93) recently observed that the splanchnic tissues (GIT plus liver) removed 15% of the 600 g/d of a total AA mixture that they had infused into the mesenteric vein of lactating cows. Of the AA escaping splanchnic metabolism (510 g/d), only 36% was recovered as milk protein. The remainder may represent alternative uses associated with metabolism in non-MG tissues or catabolic losses within the MG that are unrelated to milk production, or both. There are two ways to interpret these findings. The first is that the metabolism of the splanchnic tissue plus other non-MG tissues dictates the amount of AA available for milk protein synthesis, supporting the concept that these tissues actively regulate net tissue use, or, alternatively, that these tissues are passively regulated (i.e., in response mode), in which case, AA catabolism occurs in response to the surpluses that the MG does not utilize. Perhaps by redirecting efforts to improve the competitiveness and the efficiency of the MG to

remove and convert AA into milk protein, these losses (70% of AA available post-absorptively) might be avoided or at least reduced.

The remainder of this review focuses on the mechanisms that control the delivery to the MG and uptakes of AA by the MG, whether or not these mechanisms pose limitations on AA availability for extraction, and the metabolic fates of AA.

THE ARTERIOVENOUS DIFFERENCE TECHNIQUE

Criteria for Application

The ability to measure directly the net anabolism of the MG as secreted milk proteins, fat, and lactose provides an opportunity that is not available with studies of other tissues and organs. The net removal of nutrients from the blood supply can be compared—or, rather, balanced—with milk outputs. Balance studies can provide meaningful information if the arteriovenous difference of blood (plasma) metabolites, MG blood flow rate, and milk outputs are accurately measured. Mepham (75) highlighted many of the important considerations and problems that he and the late J. L. Linzell had identified when applying the arteriovenous technique to the bovine and caprine MG. Probably the most important criterion is that blood leaving the gland via the subcutaneous abdominal veins (the milk veins) is not contaminated by blood originating outside the MG. The sources of this contamination include contributions from the external pudic veins (**EPV**) as a result of valvular incompetence in these vessels (64) and minor contributions arising from the perineal veins. It is generally assumed that blood in the subcutaneous abdominal veins of the lactating goat (84) and cow (22) is solely of MG origin if the animals are in the first or second lactation and if the animals are kept standing during blood flow and sampling procedures. This assumption does not exclude the possibility that blood is leaving the gland via the EPV. Nielsen et al. (84) examined whether flow in the EPV of lactating goats changed with stage of lactation (wk 2, 10, and 12 postpartum) and observed that manual occlusion of the EPV caused external pudic artery flow rate to decrease (by 36, 19, and 3%) and blood velocity in the subcutaneous abdominal veins to increase (by 24, 8, and –2%). The nearly reciprocating responses of the subcutaneous abdominal veins versus external pudic artery suggests that 1) EPV flow in the standing goat is probably always away from the udder, 2) blood flow in the EPV is less with advancing lactation, and 3) manual occlusion of the EPV can disrupt the normal arterial blood flow rate either by direct occlusion of the external pudic artery or from changes in vascular resistance caused by venous back pressure. Presumably, the acute effects of these manipulations, especially on blood flow, would not be compatible with the chronic responses that they are being compared with, for example, milk output over hours or days.

Under the assumption that EPV blood flow is always away from the udder, then consideration must be given to whether the composition of blood nutrients is the same in the subcutaneous abdominal veins and EPV (i.e., is the metabolic activity of the regions drained by the respective veins pro rata with blood flow rate?). To our knowledge, there is only one report (108) addressing this issue, and those researchers observed that the EPV of one multiparous cow in early lactation had higher concentrations of acetate (+13%), propionate (+9%), butyrate (+5%), and β-hydroxybutyrate (+8%) than did the subcutaneous abdominal veins. Unfortunately, the direction of flow in the EPV was not reported. However, it cannot be assumed that the blood composition is the same in these vessels. What is required is some measure of the direction of flow in the EPV and measurement of AA differences. One possible way to ensure, under all circumstances, that the blood flow and metabolite concentrations of the subcutaneous abdominal veins are exclusively of MG origin would be to ligate all inflowing and outflowing vessels except the external pudic artery and subcutaneous abdominal veins; ligation of the EPV and the perineal arteries and veins would be required. Researchers in Australia (J. M. Gooden, 1996, personal communication) have been successful in performing ligations of the EPV without causing adverse effects on health and milk production, and we have now routinely included ligation of the perineal arteries and veins as part of our surgical preparation in goats, also without influence on normal milk production.

Arteriovenous Blood Sampling

Measurements of blood substrate uptake in most studies involved the withdrawal of blood samples from arterial and subcutaneous abdominal veins at 0.5- to 1-h intervals for 4 to 8 h during the day (44, 47, 76, 80). The difference in arteriovenous blood metabolites of the sample as well as MG blood flow, usually measured by a dye-dilution technique at the time of sampling, was assumed to represent the average of MG metabolism. In fact, the measurement

corresponds to MG metabolism during the 2 to 5 min that the sample was being withdrawn and blood flow was being determined. The assumption when employing this approach must be that blood flow and the arteriovenous difference remain constant during the sampling period; otherwise, net uptake would not be comparable with the hourly or daily milk output measurement.

Posture and the regularity of feed consumption appear to have a major influence on MG blood flow and possibly nutrient uptake. The MG blood flow (monitored by flow probe) and arteriovenous blood differences of volatile fatty acids, glucose, and oxygen have been monitored in cows, both standing and lying (90). Those authors observed that, although blood flow increased by 18% when cows were lying down, net substrate uptake remained constant. A preliminary study (B. J. Bequette, 1995, unpublished data) examined whether meal consumption or milking affect MG blood flow. Daily (24-h) patterns of MG blood flow were compared in goats under three milking and feeding regimens. Goats were milked twice daily (0800 and 1600 h) and were fed equal portions (90 g) of molasses-treated hay every 2 h (12 times daily). In addition, the goats received the concentrate part of their diet in one of three regimens: as two equal portions (800 g) fed during milking (Figure 1A), as two equal portions (800 g) fed 4 h after milking (Figure 1B), or as equal portions (133 g) fed with the hay (Figure 1C). The most striking difference was the 4- to 5-h shift of the two blood flow peaks when twice daily concentrate feeding was moved forward by 4 h (compare Figure 1A vs. 1B), suggesting a strong involvement of signals related to nutrient intake in regulating MG blood flow. No discernible patterns or relationships were apparent when the ration was fed 12 times daily (Figure 1C). These temporal blood flow responses to feeding compare closely with the hormone and metabolite profiles characterized for dairy cows fed at different frequencies (2 vs. 6 times daily) (101). Identification of the mechanisms that signal nutrient intake and blood flow could be important to enhance the partition of nutrients to the MG.

Although it is unclear whether postural and feeding effects on blood flow also alter the net utilization of substrates by the MG, these effects must be considered to avoid misinterpretation of the exact mechanisms (blood flow vs. nutrient extraction) involved. In our studies with goats, we have often attempted to minimize blood flow variability by ensuring that goats remain standing for the sampling periods and are fed frequently (12 times daily) (14, 16, 18). In addition, arteriovenous blood samples are continuously withdrawn over 1-h periods to provide a direct

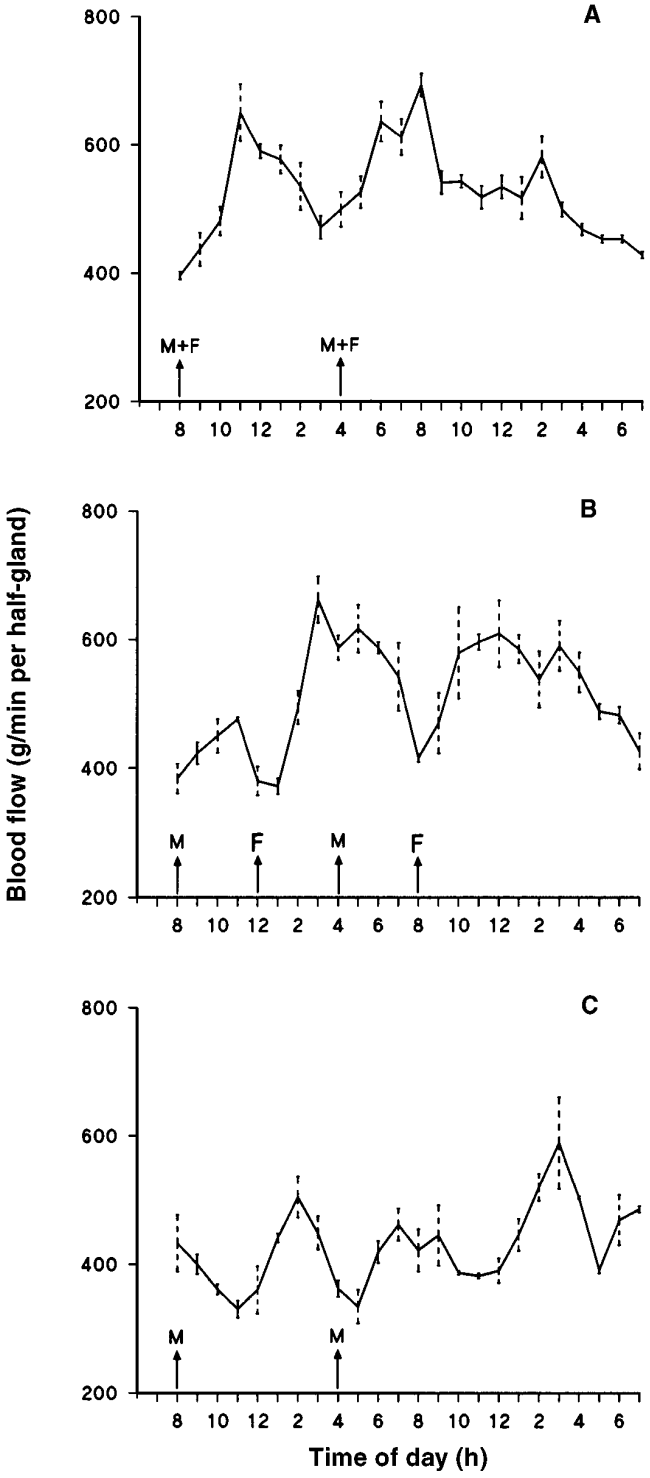

Figure 1. Comparison of the 24-h blood flow pattern of one-half the mammary gland of a goat milked (M) twice daily (0800 and 1600 h), fed molasses-treated hay every 2 h in equal portions (90 g), and fed the concentrate portion either in equal parts (A; 800 g) at the milking times (M + F), in equal parts (800 g) 4 h after (M, F) the milking times (B), or in equal parts (133 g) every 2 h along with the hay (C). Blood flow was averaged over 1-h periods during 24 h, and each 1-h time point represents the mean (±SE) of 3 consecutive d on the respective feeding regimens.

comparison with measurements of blood flow and milk output integrated during the sampling period.

AA SUPPLY AND MG UPTAKE: ARE THESE LIMITED?

There are numerous reports (18, 47, 76, 80) in the literature of small or often no increases in milk yield and protein output (content) when supplemental dietary protein or AA are given and when poor recovery (13 to 17%) of the supplemental AA in milk protein is the norm. Such observations serve to highlight that the knowledge is incomplete concerning how dietary nutrients influence or limit milk protein synthesis. We have conducted a series of intragastric and i.v. AA infusion studies to begin to delineate whether tissue partition (competition), pattern, amount supplied, stage of lactation, or MG uptake of AA limit milk protein synthesis (see Table 1).

GIT and i.v. Supplementation of AA

A logical first step to identify AA limitations to milk protein synthesis is one that considers the relative importance of EAA versus nonessential AA (**NEAA**) supplies. To test whether the NEAA supply is limiting to milk protein synthesis, midlactation cows were fed a low protein diet (140 g of CP/kg of DM) and given jugular infusions (5-d) of either a total AA (400 g/d) or an EAA (208 g/d) mixture, both being equivalent in composition to bovine milk protein (78). Milk yield was not affected, but milk protein content increased by 2.6 and 4.4 g/kg with total AA and EAA infusion translating into +87 and +143 g of milk protein/d, respectively (Table 1). Recovery of the infused AA in milk protein was greater for EAA (36%) than for total AA (22%) infusion, and both were higher than has been observed in studies of dietary supplementation (76, 80) and GIT infusion (44, 45, 46, 47). Thus, there appears to be no additional advantage to increasing the posthepatic supplies of the NEAA. For midlactation cows that were fed the same low protein diet, mesenteric vein infusion (3 d) of total AA (600 g/d) increased (+2.8 kg/d) milk yield, but infusion of the NEAA (288 g/d) reduced milk yield slightly (93). Both total AA and EAA (312 g/d) infusions increased milk protein content (3.8 and 4.5 g/kg); however, the recovery of the infused AA was greater for total AA (31%) than for EAA (21%) infusion. This result may suggest that some relationship exists between NEAA and EAA metabolism by the liver, which may be required to ensure that posthepatic availability of EAA is adequate for milk protein synthesis. Indeed, NEAA may be more important when EAA supplies are augmented prehepatically. When midlactation cows were fed a medium CP diet (170 g/kg of DM) and were given duodenal infusions of EAA at two levels (312 and 468 g/d), maximal responses in milk protein output were achieved at the lowest rate of infusion (+86 vs. +74 g/d), and the recovery of the infused EAA in milk protein was also highest at the lower rate (14% vs. 8%) (29).

The responses to AA supply may also be dependent upon stage of lactation, perhaps as a consequence of changes in tissue depot depletion and repletion and hormone sensitivity, which may alter the pattern and availability of AA to the MG. For cows that were fed a high protein diet (200 g/kg of DM) [(28); L. A. Crompton, 1996, unpublished data], EAA infusion (312 g/d) during early lactation (wk 8) resulted in a greater increase (+2.0 g/kg) in milk protein content than when EAA were given to the same cows in midlactation (wk 16; +1.2 g/kg). The efficiency of converting the additional EAA into milk was higher in early lactation (14%) than in midlactation (8%). Responses to AA infusion are also dependent upon the CP content of the basal diet. In midlactation cows that were fed a medium protein diet (170 g of CP/kg of DM), larger increases in milk protein content (2.4 vs. 1.2 g/kg) and output (80 vs. 49 g/d) and greater efficiencies of recovery (13 vs. 8%) were achieved in response to a jugular infusion of 312 g of EAA/d than when cows were fed a higher protein diet (200 g of CP/kg of DM) (L. A. Crompton, 1996, unpublished data). Comparison across studies also seems to suggest that milk protein content is increased more when AA are infused via i.v. rather than the intragastric route (2.9 vs. 1.1 kg of milk/d, 0.7 vs. 0.2 g of protein/kg of milk) but that milk protein output and recoveries are identical or similar. These conclusions will require further confirmation by direct testing in the same animals by site infusions.

AA Supply and MG Uptake

Metcalf et al. (76) observed that only the arterial supply of Lys and the net uptakes of Lys, Leu, and Tyr were increased significantly in response to supplemental fish meal. In a subsequent study (80) in which three levels of CP were examined, Lys (and Val) uptake by the MG was also increased by supplementation, despite the fact that the concentrations of most EAA, except for Met, Phe and Thr, were increased. In both of these studies, however, Lys was

TABLE 1. Comparison of studies examining responses of dairy cows fed grass silage diets to infusion of AA mixtures.[1]

Study	Lactation	CP in diet	Infusion[2] site	Infusion rate[3]	Milk yield		Milk protein content		Milk protein yield		Recovery[4]
	(wk)	(g/kg of DM)		(g/d)	(kg/d)	(Δ)	(g/kg)	(Δ)	(g/d)	(Δ)	(g/g)
Crompton et al. (28)	7–9	200	JV	EAA 312	31.4	0.5	31.0	2.0	986	83	0.14
Metcalf et al. (78)	11–28	140	JV	EAA 208	22.4	1.1	32.5	4.4	726	143	0.36
				TAA 400	23.8	0.6	32.4	2.6	765	87	0.22
Reynolds et al. (93)	13–18	140	MES	EAA 312	22.0	0.7	32.4	4.5	709	126	0.21
				TAA 600	21.8	2.8	32.4	3.5	706	183	0.31
L. A. Crompton et al. (1996, unpublished)	13–18	170	JV	EAA 312	22.9	0.7	36.7	2.4	841	80	0.13
		200	JV	EAA 312	24.1	0.7	36.8	1.2	887	49	0.08
Crompton et al. (29)	13–18	170	DUO	EAA 312	23.7	0.9	36.0	1.1	833	86	0.14
				EAA 468	24.4	0.6	35.2	1.9	857	74	0.08

[1]Milk production values represent the control (saline infusion) mean and the response (Δ) to the infused AA.

[2]JV = Jugular vein, MES = mesenteric vein, and DUO = duodenum.

[3]EAA = Essential AA; TAA = total AA (both equivalent to AA composition in milk protein).

[4]Calculated as grams AA infused divided by equivalent casein AA output.

always extracted in excess, which increased still further with supplementation. Conversely, Phe, Tyr, His, and Met uptakes were consistently less than milk protein outputs.

The intragastric infusion studies of Guinard and Rulquin (44) and Guinard et al. (47) are among the few available studies that have allowed a comparison of the partition and utilization by the MG of controlled known rates of AA supply. Removal of EAA by the MG was increased in response to incremental duodenal infusions of casein. The net removal of EAA represented 0.81 of the casein EAA infused at the lowest rate but only 0.50 at the highest level of casein infusion. The efficiency of converting the extracted EAA into milk protein was also reduced considerably (from 0.88 to 0.49) with nearly all the EAA being extracted far in excess of net requirements for milk protein synthesis. Excess uptake may have been utilized for the de novo synthesis of many of the NEAA for which uptake was found to be less than or equal to milk outputs at all levels of infusion. In a related study (45), Lys was infused into the duodenum of cows on the same diet as just described. The net uptake of Lys exceeded milk protein outputs at the level of Lys infusion producing the maximum increase in milk protein output. At this level, presumably, other AA or factors had become limiting. This limitation was probably not due to a limitation of Met supply because, in a companion study (46), there was no milk response to infusion of Met on this same diet despite the substantial increase in arterial concentrations and supplies of Met to the MG. In contrast to the response to Lys infusion, however, the uptake of Met by the MG was not enhanced by Met infusion, which may be one reason for the lack of a milk protein response.

PRECURSOR AA FOR MILK PROTEIN SYNTHESIS

Arteriovenous balance has been widely used to quantify the AA requirements of the MG for metabolism and milk protein synthesis; based upon this comparison, AA have been proposed that limit milk protein synthesis. The needs of the MG would appear to be greater for EAA such as Val, Leu, and Ile (branched-chain AA; **BCAA**) and Arg, Lys, and Thr because their extraction generally exceeded milk protein outputs. Quantification of Met, His, Phe, and Trp, for which uptake is usually considerably less (10 to 70%) than milk protein outputs, has proved to be more troublesome. In part, this situation may be due to the relatively low concentrations of these AA in blood and their greater instability during derivatization procedures and AA analysis. Apparent deficits in the net uptake of these AA therefore has resulted in speculation that non-free AA sources, such as peptides and proteins, may contribute to the supply of AA for milk protein synthesis. Whether non-free AA contribute to mammary metabolism is of particular importance to our attempts to develop mechanistic models of organ and tissue metabolism for which the fluxes of all AA must be represented (35, 36, 71).

Arteriovenous Measurement of Peptides

The direct quantification of peptides in blood and plasma has proved difficult to assess because of the

TABLE 2. Comparison of mean plateau enrichments of [13C]Leu and [13C]Phe in casein (CN) and in arterial blood (B) and plasma (P) of goats in early (2 to 3 wk) and late (15 to 28 wk) lactation and of cows in early (7 wk) lactation during long-term (16 to 24 h) infusion of isotopes.[1]

Species	Lactation stage	B		P		CN		B/P		CN/B		CN/P	
		$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE
		[13C]Leu (mol/100 mol excess)											
Goats	Early[2]	5.22	0.79	5.85	0.86	5.84	0.91	0.89	0.02	1.12	0.01	0.99	0.01
Goats	Late[3]	2.68	0.11	3.06	0.14	3.00	0.13	0.87	0.01	1.12	0.02	0.98	0.02
								(P = 0.0002)		(P = 0.043)		(P = 0.029)	
Cows	Early[2]	2.53	0.10	3.34	0.18	3.11	0.17	0.76	0.01	1.23	0.02	0.94	0.01
								(P = 0.001)		(P = 0.002)		(P = 0.008)	
		[13C]Phe (mol/100 mol excess)											
Goats	Early[2]	5.2	0.88	5.77	0.94	5.53	0.95	0.90	0.03	1.06	0.02	0.96	0.01
								(P = 0.07)		(P = 0.1)		(P = 0.08)	
Goats	Late[3]	2.71	0.16	3.02	0.17	2.59	0.12	0.90	0.01	0.96	0.01	0.86	0.02
								(P = 0.0012)		(P = 0.046)		(P = 0.0018)	
Cows	Early[2]	3.76	0.13	4.32	0.13	3.93	0.17	0.87	0.01	1.05	0.01	0.92	0.02
								(P = 0.002)		(P = 0.032)		(P = 0.019)	

[1]Means (± SE) for goats in early lactation (n = 3) or cows in early lactation and goats in late lactation (n = 4). Probability values were determined by one sample t test, $P < 0.05$ indicates that $\mu \neq 1$.

[2]From F.R.C. Backwell (1995, unpublished data).

[3]From Backwell et al. (4).

limited availability of techniques to separate accurately and reproducibly free AA from small peptides. Hence, quantification has generally involved measurement of AA concentrations in deproteinized blood or plasma before and after complete acid hydrolysis; the difference in these values represent peptide-bound AA (59, 100). The relative inefficiency of deproteinization techniques is a major flaw of this approach; thus, residual proteins in the samples give rise to artificially high peptide concentrations. We have overcome this problem by incorporating a gel filtration step into the purification procedure prior to acid hydrolysis that removes the residual proteins to yield a fraction containing peptides of molecular mass <1500 Da. Application of this methodology to arterial plasma samples of lactating goats revealed that 10 to 30% of the total AA (free AA plus peptides) in the <1500-Da fraction was bound to peptides (4). The arteriovenous difference of AA in the plasma peptide fraction (<1500 Da) was found to be positive and small for some AA (His, Ala, Leu, Pro, and Phe). Uptakes of the individual AA as peptides could not be demonstrated statistically, but, when these were summed, they represented 15% ($P < 0.05$) of the total AA extracted by the MG. It is not surprising that the quantification of the peptide contributions of individual AA is limited when one considers that a 15% contribution of peptides to casein output would require an arteriovenous difference in the peptide fraction of 2 to 3 μM for an AA such as Phe. Detection of this small amount of AA, in addition to the existing free AA, would stretch the limits of sensitivity of most AA analyzers. Moreover, these analyses exclude the contributions arising from larger peptides and proteins, such as liver export proteins, which may be transport vehicles of milk substrates and serve hemodynamic functions in the MG. The possibility that such proteins may also act as sources of AA for mammary metabolism has yet to be examined.

Stable Isotope Labeling Studies

In the absence of sensitive techniques for the direct quantification of the uptake of non-free AA by the MG, we have adopted an alternative strategy of using a number of techniques for stable isotope labeling to assess indirectly the involvement of these sources of AA in milk protein biosynthesis. Using these techniques, we have been able to examine whether the MG has the ability to utilize peptides for milk protein synthesis and the extent to which this occurs in vivo at different stages of lactation and when nutrition or arterial substrate supplies are altered. A dual-label tracer technique was used to demonstrate that the MG can utilize AA from ^{13}C-labeled synthetic dipeptides (glycyl-Phe and glycyl-Leu) for casein synthesis (3). Further support that this process occurs in vivo has been obtained from analysis of the relationships of precursor to product ^{13}C-labeling of free AA in plasma (primary precursor pool) and secreted caseins (product). This assessment is based upon comparison of the steady-state (plateau) isotopic enrichment of

the AA in secreted milk casein with that of the free AA in blood or plasma over the period (>20 h) when the enrichment of infused stable isotopes have reached a plateau. For goats in late lactation (15 to 28 wk), 10 to 20% of Phe in casein was found to be derived from vascular non-free AA sources, but this contribution was negligible and often zero for Leu (4). These observations (Table 2) would be compatible with other reports showing net uptakes of Phe that were less than milk outputs and those of Leu that were in excess of milk outputs. Moreover, because casein and plasma Leu enrichments were similar but those for Phe were not, the possibility that isotope dilution occurred as the result of intracellular protein turnover can be eliminated because this dilution should have contributed similarly to the labeling kinetics of every AA, not just to those of Phe. In contrast to late lactation, the contribution of non-free AA to Phe supplies (<4%) in early lactation (2 to 3 wk) of goats appeared to be considerably less, suggesting that peptide contributions may differ with stage of lactation (Tables 1 and 5). In dairy cows in early lactation (7 wk), the contributions of peptides also appear to be low, supplying 8% of Phe and 6% of Leu (5).

We (16) have recently examined whether Lys, which was assumed to be extracted in excess, and Met, which was assumed to be transferred stoichiometrically into casein, might also be supplied as peptides. For goats in late lactation (38 to 40 wk), the examination of the precursor:product kinetics during a 30-h infusion of [^{15}N]Lys and [^{13}C]Met suggested that 12% of Lys and 17% of Met in casein arises from vascular sources of non-free AA. Recent demonstrations (106, 107) in vitro that the provision of these AA as peptides in culture medium of mouse mammary tissue explants can serve as a replacement of the free AA for milk protein synthesis support these findings. Moreover, these latest observations require some groups to evaluate whether the use of Met uptake as a predictor of MG blood flow, based upon the Fick principle, is still valid. More importantly, it should be considered whether peptide-bound AA contributions to milk protein synthesis occur generally for a larger range of AA than had previously been considered.

In a number of studies (44, 76, 80), it appears that, as additional AA are supplied by dietary or infusion routes, the net uptake of many AA, including those AA (Phe, Tyr, Met, Thr) extracted at levels lower than requirements, begins to exceed milk outputs to a greater extent with each increment that is fed or infused. These observations suggest a switch from a requirement for uptake of peptide-bound AA under basal feeding conditions to one in which uptake of free AA predominates during supplemental conditions. To test this hypothesis, we (16) examined whether, during supplementation of Phe by i.v. infusion (6 g/d for 5 d), the precursor:product kinetics of Phe would also reflect a diminished use of Phe-containing peptides as the supply of free Phe was increased. The enrichment ratio of casein to plasma Phe increased significantly from 0.89 to 0.98 (i.e., a reduction of peptide-bound Phe from 11 to 2%) upon Phe supplementation; this decrease in peptide utilization was counterbalanced by an increase in the net uptake of free Phe.

Sources of Circulating Peptides and Proteins

Given that the MG does appear to use peptide-bound AA as precursors for milk protein synthesis and that, for a number of AA, circulating concentrations of smaller molecular mass peptides represent a substantial (0.20 to 0.30) proportion of the total AA, it is perhaps important to consider the source of these peptides. Peptide transport systems in the GIT are now recognized as important mechanisms for the absorption of the end products of dietary protein digestion (39). Some of these absorbed peptides likely escape peptide hydrolase activity in the enterocyte and are intact when they enter the portal circulation (42), where they may serve as vehicles for interorgan exchange of AA. Indeed, Webb et al. (109) have reported work showing a substantial appearance of peptide AA N in the portal circulation of growing steers (59); furthermore, this group has also demonstrated that carnosine (β-alanyl-His) and methionyl-Gly peptides can be transported intact across ovine epithelial (rumen and omasum) tissue in vitro (73). Peptide transporter activity has also been identified in the ovine intestine (6, 74), but, as yet, the extent to which these absorbed peptides contribute to net anabolism in the ruminant is still a subject of controversy.

Employing the simple techniques mentioned earlier for blood and plasma deproteinization, it has been reported (59) that there is a large net flux of peptide AA across the GIT of growing steers. With the exception of Ile, Tyr, and Lys, the net fluxes (positive or negative) of AA were not different from zero. The net fluxes of free AA and peptide-bound AA N were, surprisingly, approximately three times the intake of dietary N. In another study (100) employing slightly different methodologies, similarly large concentra-

tions of circulating peptides that represented up to 80% of the total AA measured (peptide-bound plus free AA) were found, but there was no significant net portal appearance of peptides. Employing more rigorous deproteinization techniques, much lower peptide concentrations have been reported in studies with rats (38), lactating goats (4), and growing sheep (5). In the latter study, peptide fluxes were also assessed in plasma that had been prepared by a previous method (59) to establish whether differences in techniques may account for the varying results reported. In this analysis, as expected, peptide concentrations were much higher, but there was still no evidence that peptide uptake occurred across the GIT of the sheep. Recently, we have confirmed this observation in two related studies where experimental methodologies (21) and the influence of dietary components (20) on peptide and AA fluxes in the GIT were examined in sheep.

It appears, therefore, that it may be necessary to look elsewhere for the origin of much of the circulating peptide AA. One obvious other source is the liver export proteins. Albumin is the major contributor to the export protein pool. Based upon the mean fractional synthesis rate (0.05/d) reported for growing sheep [52 kg of BW (26)], plasma volume (0.05 of BW), and concentrations of albumin (29 to 38 g/L) in the normal lactating goat (65 kg of BW), the potential supply of albumin for peripheral tissue use could be 5 to 6.7 g/d. This amount could provide 0.280 to 0.367 g of Phe daily, which, if transferred to the MG, could make up 50 to 100% of the deficit in Phe supply that is required by goats (16). It is probably unlikely that all of the albumin synthesized is transported to the MG, and so other sources of the bound AA supply need to be considered, which may include contributions of peptides derived from the turnover of endogenous sources such as collagen and skeletal muscle proteins [(41), review; (85)].

PROTEIN METABOLISM OF THE MG

Milk Protein Synthesis

The MG tissues comprise secretory and nonsecretory cells. In the lactating goat, secretory cell mass is substantially increased between parturition and peak lactation. Most of this increase and the accompanying up-regulation of mammary metabolic activity is the result of a threefold increase in cellular differentiation (112). The reduction in milk yield during later phases of lactation appears to be due to the loss of secretory tissue through apoptosis, rather than by dedifferentiation of the epithelial cells (41).

The primary function of the MG is to synthesize and assemble the constituents of milk and, relative to the neonate, to allow bulk transfer of water after birth. Milk proteins are synthesized at a phenomenal rate compared with the constitutive protein mass of the MG [ratio of milk protein secretion (grams per day) to mammary tissue protein mass (grams): cows, 0.45 (43); and goats, 0.50 (8, 25)]. The major milk proteins that are synthesized by ruminant species are the caseins (α-CN, β-CN, κ-CN, and γ-CN) and whey proteins (β-LG, α-LA, and proteose-peptones), representing approximately 92% of bovine milk proteins; the remainder is represented by BSA and the Ig, which are transported into milk via transcellular mechanisms. All epithelial cells of the MG are thought to contribute to this biosynthetic process, and each cell expressing all of the genes is thought to synthesize and secrete the casein and whey proteins. What remains unclear, however, is whether all of the cells of the gland synthesize milk proteins at a constant rate or whether synthesis is phasic. Researchers in New Zealand (33, 81) have examined the distribution of milk protein gene expression in the MG of lactating sheep, beef cattle, and dairy cows. Gene expression of α_{s1}-CN, α-LA, and lactoferrin and the immunohistochemical staining of α-LA were found to be distributed heterogeneously in core biopsies taken throughout the MG of these lactating animals. This group also observed two classes (fatty and nonfatty) of alveoli having contrasting levels of α-LA gene expression, suggesting that there may be specialized cell types that are capable of synthesizing milk proteins versus fat or that some cells within an alveolus are actively lactating (nonfatty) while others are dormant (fatty). Both scenarios probably occur, and, thus, the challenge is to delineate the exquisite local mechanisms controlling these events so that fat-producing cells can be converted into cells producing milk protein, or the proportion of these cells that are actively lactating can be increased.

Intracellular Channeling of AA

The intracellular compartmentalization of AA pools and the protein synthetic and catabolic apparatus have been demonstrated in many tissue types (heart, skeletal muscle, and liver) from different species [humans, sheep, mice, swine, and rabbits (26, 34, 99, 117)] under a variety of nutritional conditions (starved vs. fed; protein intake level). Moreover, compartmentalization of AA pools for the synthesis of different cellular proteins [acid-soluble vs. muscle protein (117); albumin vs. ferritin (34)] has also

been observed. In studies of the MG and other tissues, the selection of the appropriate precursor pool for protein synthesis and catabolism is paramount to assess the qualitative and quantitative significance of these pathways.

In order to determine the precursor pool(s) for synthesis of the caseins, we compared the rates of labeling of the free AA in blood and plasma with that of secreted milk casein. In studies with lactating cows (19, 35) and goats (early and late lactation) (15) that were given a constant jugular infusion of ^{13}C-labeled AA (Leu, Phe, Val, and Met) for 12 to 16 h, the free AA pool in blood and plasma rapidly (1 to 3 h) reached an isotopic plateau. The rise to plateau (rate of incorporation) of the [^{13}C]AA in milk casein and whey proteins lagged substantially behind, reaching an asymptote with the blood and plasma only after 10 to 13 h of infusion. At 1-h intervals, the glands were milked out completely (using oxytocin injections in the cow studies). Thus, the rapid (<1 h) appearance of isotope in secreted milk proteins is indicative that the intervals between synthesis and secretion are short and that storage or residual pools are small. Similarly, slow rates of incorporation into milk casein have now been observed to occur for 13 AA (14). In fact, this may be the case for all AA because the number of AA evaluated in that study was limited by the capabilities of the mass spectrometric analyses. The overall slow rate of appearance of [^{13}C]AA in the secreted milk proteins suggests that there is channeling of intracellular degradation products arising from the turnover of milk proteins, constitutive proteins, or both, into the immediate precursor pool for milk protein synthesis.

In a study of goats (17), the casein proteins were separated by FPLC® (Pharmacia Biotech, Uppsala, Sweden) into three crude fractions representing predominantly κ-CN, α-CN, and β-CN. The incorporation rates were slightly different between casein fractions, and κ-CN had the slowest rate (i.e., lower enriched). Similarly, others (63) have observed in two lactating goats that the [^{35}S]Met specific activity of whole casein was 19 to 33% lower than in β-LG after 7 to 9 h (nonplateau conditions) of isotope infusion. These observations suggested that the casein and whey proteins may be synthesized by specialized cells receiving a source of AA that is of lower specific activity (diluted), that these proteins are synthesized by the same cells but with different sources of AA (intracellular, extracellular free vs. peptide-bound AA) contributing to their precursor pools, or that these proteins are synthesized or processed more slowly than the other proteins. One

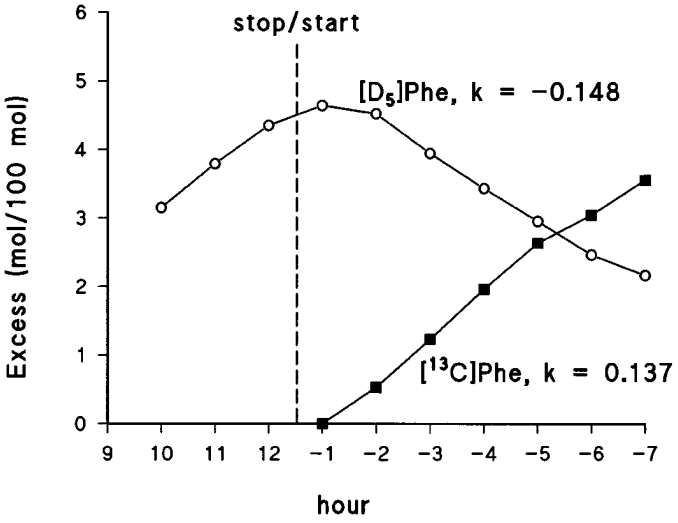

Figure 2. Comparison of the temporal labeling patterns of [D_5]Phe and [^{13}C]Phe in secreted milk casein. The 12-h constant intravenous infusion of [D_5]Phe was terminated at the same time as the 7-h infusion of [^{13}C]Phe was initiated. Beginning at h 10 of [D_5]Phe infusion and continuing until the end of the [^{13}C]Phe infusion, the goat was milked out completely by hand, and, at the first 3 milking times (h 10, 11, and 12), an intravenous dose of oxytocin (1 IU) accompanied milking. The rate constants (k) for the decay and incorporation curves were derived by fitting the data to the equation form: $y = A(m)$, where m denotes $(1 - e^{-kt})$ and (e^{-kt}) for the two curves, respectively.

approach to differentiate further between these mechanisms would be to examine the labeling kinetic relationships under plateau conditions. Nonetheless, substantiation of the observations of Lee et al. (63) would raise the possibility that AA could be specifically targeted to certain milk proteins.

The channeling hypothesis has been challenged (113) on the basis that residual milk had not been completely removed in the latter studies because animals were not given repeated injections of oxytocin. Rather, those authors suggest that the more likely explanation is that unlabeled residual milk at the start of isotope infusion had become progressively diluted with highly labeled, newly synthesized caseins. Some milk resides in the secretory cells and in the alveolar lumen and smaller ducts even after apparent complete milk removal; however, several pieces of evidence continue to support the process of precursor channeling.

First, even when oxytocin is administered, the time lag and rate of labeling of secreted milk casein are the same as in other studies. Illustrated in the modeling paper of France et al. (35) is the temporal labeling pattern of casein from a dairy cow that had been given oxytocin (5 to 10 IU) every time that she had

been milked (machine-milking and hand-stripping at 1-h intervals) during a 20-h infusion of [^{13}C]Leu. The rate constant for casein labeling for this cow (0.31/h) is similar to those results reported in studies (15; mean: 0.25/h) in which the goats were not given oxytocin at each hourly milking time.

Second, in a preliminary experiment, we (B. J. Bequette and F.R.C. Backwell, 1994, unpublished observations) found that the rate constants for label disappearance from (–0.148/h) casein and incorporation into (0.137/h) casein were similar in a goat that had been milked out three times with oxytocin during the final 2 h prior to ending a 12-h constant infusion of [D_5]Phe and the start of a 7-h constant infusion of [^{13}C]Phe (Figure 2). The similar kinetic behaviors of the isotopes suggested that they labeled the same newly synthesized casein pool, which would suggest that the residual pool was small or absent.

Third, the size of the residual casein pool would have to be extraordinarily large to account totally for the kinetics observed. For instance, if isotope dilution of secreted casein is assumed to have occurred only as the result of a residual pool in the gland (cellular, luminal, and ductal) and that the average rate constant for casein labeling was 0.31/h for a cow producing 1 kg of milk protein, then the turnover of milk protein on a daily basis would be nearly equivalent to the daily milk protein output (i. e., a protein pool in the gland that would be three times the size of the hourly rate of milk protein output (130-g pool vs. 42 g/h). This turnover would have to represent the mixing of old caseins with newly synthesized caseins. A more probable explanation, but one that would also be insufficient to account for the labeling kinetics observed, would involve the constant replacement of casein by degradative mechanisms rather than by the mixing of casein molecules. Nascent and mature caseins have been shown to be degraded intracellularly. Proteolytic cleavage of the signal (docking) sequence [7 to 10% of the molecule (27)] from nascent proteins occurs as part of the recognition process for targeting proteins destined for export, and studies in vitro have shown that mature caseins are also degraded (92). Based on a model of Leu kinetics of the lactating goat MG in vivo (87), it has been estimated that one-third of the milk proteins synthesized are degraded. Indeed, a group from the Hannah Research Institute (111, 114) has identified and isolated a protein from the whey fraction of residual milk, termed the feedback inhibitor of lactation, which appears to be involved in the regulation of casein degradation through its inhibitory actions on casein secretion in vitro.

Finally, the high turnover rate (42 to 130%/d) that has been reported for mixed mammary tissue proteins of the early lactating goat (8, 25) would appear to be substantial enough to result in the dynamic mixing events of the intracellular pool. These measurements probably also include a population of tissue proteins that have much higher rates than the average values reported. However, even when based upon the rates reported for mixed proteins, significant recycling of labeled AA into the intracellular pool will occur, leading to the curvilinear pattern of casein labeling observed (15). In consequence, although it is likely that some cells may retain or fail to secrete milk proteins after initiation of milk letdown and that a significant portion (maximum of 40%) of the caseins may be degraded intracellularly, it is highly unlikely that the gland stores a pool of milk proteins three times the rate of output. Thus, channeling through intermediary constitutive or casein pools must occur.

The existence of an intracellular residual protein pool or pools has been implicated in previous studies with mammary tissue explants (111) and with the perfused MG of the goat (95, 96). Intracellular channeling phenomena are not restricted to observations in ruminants because similar rate constants of casein labeling (employing [^{13}C]Leu) of secreted milk have been observed in the lactating sow [goats 0.21 to 0.27/h (15) vs. sows 0.24/h (52)]. Channeling has also been observed in other tissues. For example, 60% of AA for protein synthesis in muscle is derived from products of protein degradation (99). The reasons for the extensive channeling in the MG remains to be elucidated. However, based upon the fact that all AA are involved (14, 15) and that these kinetics are maintained under a range of physiological conditions, this obligatory process may be necessary to guarantee an adequate rate of delivery and concentration of AA reaching the site of casein synthesis, even through periods of AA deficiency (13). In other words, this process may represent a natural buffering system. Constitutive protein channeling would serve two purposes. First, it would prioritize the maintenance of cell integrity and the synthetic machinery; otherwise, unregulated casein synthesis would deplete the cell of vital structural and regulatory proteins (e. g., enzymes, transporters, receptors, and growth factors) that are required to guarantee that milk is produced every hour and every day for the suckling young. Second, channeling of metabolically related pathways is more efficient than are free ones. This process would allow local concentrations of substrates to be increased without destruction of unstable intermediates or losses from competitive side reactions, thus

allowing combined regulation of multiple synthesis activities (i. e., synthesis of all caseins for micelle formation).

Mammary Tissue Protein Synthesis

In addition to milk proteins, the MG also synthesizes a variety of constitutive proteins (structural proteins and enzymes). The fractional synthesis rate of mixed mammary tissue proteins from goats in early lactation ranges from 42 to 130%/d, depending upon the precursor pool (free plasma or tissue homogenate) selected for calculation of protein synthesis rate. The turnover of these mixed proteins, therefore, contributes 42 to 72% of the total (milk plus constitutive) protein synthesized in the MG. This additional protein synthesis and degradation (assuming zero or negligible net gain by the gland) represents an energetic cost. For example, a cow producing 0.95 kg of milk protein/d requires oxidation of substrates to yield the 40 mol of ATP [5.0 mol of ATP/mol of peptide bond formed (48)] that are required to synthesize the milk protein. This calculation does not include the energetic cost associated with protein turnover. Using the relationship of 1.55 to 2.60:1 for total protein synthesis to milk protein output (assuming 1 mol of ATP/mol of hydrolyzed peptide bond, 65), inclusion of protein turnover raises the energy costs by 27 to 79 mol of ATP to a new value of 67 to 119 mol needed to synthesize 0.95 kg of milk protein. Comparison of this result with the 290 mol of ATP expended (calculated stoichiometry) by an MG producing 30 kg of milk containing 0.95 kg of milk protein (48), one begins to realize the impact the protein turnover process can have on the energetic efficiency of the gland.

If protein turnover in the MG acts as a buffering system to ensure the constant availability of AA, then would this relationship be maintained when supplies are in excess? Total MG protein synthesis in the cow, as monitored by an arteriovenous kinetic ([1-^{13}C]Leu) technique, increased only slightly (nonsignificantly) in response to dietary protein supplementation [+490 g of crude protein/d, (19)]. However, because milk protein output did not change with supplementation, one might not have expected that total gland protein synthesis would change. In a subsequent experiment with dairy cows, J. A. Metcalf (1994, unpublished data) also employed the [1-^{13}C]Leu kinetic model across the MG and found that jugular infusion (208 g/d) of a mixture of 10 EAA increased total protein synthesis in the MG according to a fixed (2.5:1) relationship with milk protein output, which was increased by 92 g/d. This relationship (2.5:1) is similar to that observed for early lactating goats in which protein synthesis by mammary tissue was determined by the incorporation of radiolabeled AA into mixed tissue proteins (8, 25). This fixed relationship should help simplify model predictions of the intracellular metabolic fate of AA and the energy required to synthesize milk proteins.

MAMMARY AA CATABOLISM AND ANABOLISM

The MG has long been recognized as a site of extensive synthesis and degradation of AA. Tracer studies with the perfused MG (30, 95, 96, 103, 104, 105) and tissue explant and cell culture systems (56, 116) have been instrumental in identifying many of the metabolic transformations of AA that can occur within the MG. These pathways are the same or similar to pathways that occur in other tissues. Although this information has been invaluable, it is of limited application for quantifying the fluxes through these pathways in vivo, mainly because the mammary organ and tissue and cell preparations often do not synthesize or secrete milk proteins at a constant rate for sufficient periods of time to perform studies involving nutrient and hormone perturbations. Also, the metabolic activity (protein secretion) of cell lines and tissue explants is considerably reduced compared with that of the MG in vivo. In recent years, a wider range of radiolabeled and stable isotopically labeled AA have become available at an affordable cost, which has led to a number of metabolic studies using the lactating cow and goat MG in vivo. Results of these studies have begun to provide quantitative information of the influence of different regulators on primary and intermediary routes of AA metabolism in a normal MG with high rates of milk protein synthesis.

Traditionally, AA and their metabolism have been categorized according to the balance between net arteriovenous uptake and milk casein and AA output. Excess uptake is assumed to represent catabolism, and, for this reason, those AA taken up in excess are usually not considered to be limiting for milk protein synthesis. This point has also been argued on the basis that the Michaelis constant for activation of acyl-tRNA synthetases are 100 times lower than those for catabolic enzymes [1×10^{-6} vs. 1×10^{-4}; (98)], and, therefore, catabolism should proceed only after the acyl-tRNA have become fully charged (31). This argument assumes that none of the metabolites

or products of AA catabolism serve as rate-limiting substrates for protein synthesis or serve as regulators of protein synthesis. In addition to milk protein synthesis and catabolism, however, other routes may exist for AA utilization by the MG. To assess this possibility, we simultaneously monitored the isotope kinetic transfers (unidirectional flux) of a range of AA across the goat MG and then compared this pattern, standardized to Lys, with that required (casein composition) for milk protein output (14). Thus, any deviations from the Lys relationship would indicate fewer or additional fluxes of an AA compared with Lys and its metabolic relationship with milk protein synthesis. Some AA (Arg plus Pro, Phe plus Tyr, His, and Thr) had fewer metabolic requirements and others (Val, Ile, and Leu) had greater metabolic requirements than did Lys. It is critical to assess whether these additional metabolic fluxes are obligatory for milk synthesis; we have begun to test this hypothesis (18).

BCAA

Leucine, Val, and Ile are catabolized by mammary cells along pathways found in other tissues (95, 96, 116) to yield organic acids (oxo- and iso acids, propionate, acetate, and citrate), carbon skeletons for NEAA (Glu, and Asp) synthesis, and CO_2. The rate-limiting step of the BCAA catabolic pathway is decarboxylation of the respective branched-chain keto acid catalyzed by the branched-chain keto acid dehydrogenase (EC 1.2.4.4) shared by all the BCAA and Met (50). Substantial transamination of BCAA is known to occur in the MG of the goat with reamination of the oxo acid representing 30 to 35% of Leu (87) and 10% of Val (96) flux. Net catabolism of the BCAA results in the contribution of $+NH_3$ to NEAA synthesis (i. e., Glu, and Asp). The regulation of branched-chain keto acid dehydrogenase is dependent upon phosphorylation status, and, thus, when insulin levels or tissue sensitivity is high or BCAA concentrations are low (91), the enzyme is inactive (phosphorylated), and catabolism is inhibited. Both insulin and Leu have been shown to be modulators of Leu oxidation by the hind limb tissues of sheep (51, 86). Nonetheless, the role of insulin and Leu in regulating Leu oxidation by the MG is presently speculative and requires more examination by direct manipulation of these at the level of the MG by close arterial infusion (79).

The oxidation of Leu by the MG is lower (0.08 vs. 0.34 of Leu uptake) for early lactation goats yielding 4.3 kg of milk/d than for late lactation goats yielding 1.5 kg of milk/d (87). We (19) monitored Leu metabolism by the dairy cow MG and observed an increase in both the fractional (0.047 vs. 0.136) and the absolute (5 vs. 18 g of Leu/d) rates of oxidation in response to dietary protein supplementation (+490 g/d). Milk protein output was not altered by protein supplementation in these midlactation dairy cows. Results of this study and a study of goats (87), suggest an inverse relationship between changes in milk protein output and Leu catabolism whereby the oxidative process may be competitive with milk protein synthesis by limiting substrate availability. This hypothesis was subsequently tested using goats given a jugular infusion of either saline or a complete mixture of AA not including Leu in order to alter the mammary availability of Leu relative to other AA while maintaining the net whole body supply of this AA (18). In that study, the proportion of Leu that was oxidized by the gland was substantially reduced (0.19 vs. 0.07) by the infusion of the AA mixture; however, milk protein output did not change, and, therefore, it appears that oxidation, at least of Leu, is not competitive with the process of milk protein synthesis. That is, oxidation is a function of the differential of supply and demand. Moreover, this study demonstrated that Leu oxidation is probably not an obligatory event as part of the milk synthesis process, although perhaps a basal level may still be necessary because Leu oxidation was not eliminated.

Phe and Tyr

Earlier observations that cultured bovine mammary tissues did not require Tyr in order to synthesize casein suggested that sufficient quantities of Tyr could be generated via the Phe hydroxylase pathway and that, by addition of Tyr to the medium, this conversion could be reduced (56). Studies with the perfused sheep MG began to give qualitative information of the significance of this pathway whereby 10% of casein-Tyr was found to be derived via Phe hydroxylation but could be reduced 30-fold when more Tyr, but less Phe, is supplied (105). Similarly, low rates of conversion have now been demonstrated in vivo. Employing an arteriovenous kinetic approach ([^{13}C]Phe and [$^{2}H_4$]Tyr), the contribution of Phe to casein-Tyr was examined in response to a jugular infusion of Phe (6 g/d for 5 d) in late lactation goats (16). The fraction of casein-Tyr residues derived via the Phe hydroxylation pathway in the gland was similar to that in the perfused MG (105) but did not change much with Phe infusion, approximating 0.05 to 0.09.

On a whole body basis, however, the contribution was much greater and increased from 0.10 to 0.18. The greater conversion rates observed peripheral to the MG may suggest a greater requirement for Tyr outside the MG tissues or may reflect the role of the liver in eliminating excess AA supplies in the body.

Lys

Despite many reports demonstrating the rate-limiting status of Lys on most corn-based dairy rations, there is little information regarding its metabolism by the gland. This AA presents an anomaly in that, despite its often limiting situation, net extraction of Lys is significantly greater than milk protein output (45). Net accretion of Lys may occur during the latter stages of lactation when the gland undergoes apoptosis and remodeling whereby Lys could be accreted in ϵ-(γ-glutamyl)-Lys crosslinkages (83). Alternatively, Lys may be oxidized in a regulatory or passive manner to provide ketogenic intermediates. To determine whether the excess uptake represented oxidation, we (S. J. Mabjeesh and B. J. Bequette, 1997, unpublished data) monitored Lys metabolism by the MG of the goat in late lactation by employing the arteriovenous kinetic technique and found that, in response to a 5-d i.v. infusion of Lys plus Met (9 and 3 g/d, respectively), Lys oxidation increased from 16 to 30%, representing a nearly twofold increase in the absolute rate of oxidation. Milk protein output was not affected by infusion of these two AA; thus, Lys oxidation also appears to respond to AA supply. However, a test that is similar to that conducted in the Leu study with goats (18) needs to be undertaken to determine the obligatory nature of this process.

Sulfur AA

Along with Lys, Met is often considered to be one of the limiting AA of corn-based rations, particularly when heated soybeans make up most of the protein source. In addition to incorporation into protein, Met is involved in a multitude of pathways leading to synthesis of specialized compounds, such as phospholipids, carnitine, creatine, and the polyamines. At the same time, Met provides methyl groups for a number of transmethylation reactions involved in the regulation of DNA activity and oncogene status and sulfur groups for the synthesis of Cys. In goats, 28% of the methyl group of Met contributes to the choline pool, and 10% of Met molecules are irreversibly lost through oxidation. One consequence of the latter process is the synthesis of Cys (32). The difference in these contributions reflects the remethylation of homocysteine to produce Met. The net demands of the transmethylation routes versus transsulfuration routes may place limits on each of these pathways while the sum of the pathways alter the availability of Met for protein synthesis; thus, identification of the regulators balancing these opposing processes is important. It has been examined whether provision of choline plus creatinine to sheep fed to maintenance requirements could alleviate the recycling of Met through the transmethylation pathway (66). Although less recycling was observed to occur with supplementation of the methyl donors, this decrease did not lead to a greater incorporation of [^{35}S]Cys into wool.

Recent in vitro (88, 107) and in vivo (16) evidence supports a role for peptides and proteins in the extracellular supply of Met for milk protein synthesis. Thus, the total uptake of Met probably balances with its output in milk protein. Conversely, the net uptake of Cys is often inadequate to meet the requirements for the synthesis of milk proteins and possibly for glutathione (**GSH**) synthesis. Thus, additional inputs of Cys may be required either from blood-derived peptides containing Cys or via MG synthesis from Met. These latter possibilities have been investigated by a group in New Zealand. During jugular infusion of [^{35}S]Cys in goats, intracellular specific activity of GSH in MG was 30-fold greater than the blood GSH activity, implying that the MG synthesizes considerable GSH (58). Conversely, GSH uptake can be a source of mammary Cys. Glutathione is concentrated in the erythrocyte and appears to be transferred to tissues such as the MG via this pool (89). Glutathione may also be synthesized within the MG and transported extracellularly, and, thus, net uptake measurements may underestimate GSH influx. One approach to assess the uptake of GSH from the erythrocyte and the contribution toward Cys supply would be to infuse [^{35}S]GSH-labeled red blood cells.

The contribution of sulfur-Met to the MG synthesis of Cys via Cys synthetase has also been examined (63). In that study in goats, α-LA, which does not contain Met residues, had greater incorporation of [^{35}S] when [^{35}S]Met was infused close arterially (i. e., into the external pudic artery) than when the isotope was infused peripherally via the jugular vein. Also, the specific activity of the intracellular pool of free [^{35}S]Cys was higher than that in plasma. It was estimated (63) that roughly 10% of the Met-sulfur is transferred to Cys in the whey proteins.

Arg

Arginine is extracted in the greatest quantities relative to milk protein outputs. Arginine has other metabolic functions in addition to protein synthesis. Recently, the role of Arg as a precursor of nitric oxide has received attention because of the potential effect of nitric oxide in regulating the nutrient perfusion of mammary tissue through dilation of the microvasculature (61). The endothelial cells of the mammary vasculature and the epithelium lining alveoli and ducts exhibit nitric oxide synthase III activity, and secretory cells may therefore be capable of regulating their own local nutrient environment through alteration of the capillary blood supply.

The MG possesses a partial urea cycle, which suggests an intermediary role for Arg and other intermediates of the cycle in MG function. In rat mammary tissue, the activity of arginase, which hydrolyzes Arg to form Orn and urea, increases 3-fold during lactation (55). There are two forms of the enzyme in the MG; the AII form is found in greatest quantities at midlactation. Arginase activity appears to be under the influence of other AA, and in studies (37) of rat MG (37) Pro, Orn, Lys, and certain BCAA inhibited the enzyme. The activity of this pathway may be important for the synthesis of Pro. In studies utilizing labeling of perfused sheep and goat udders, citrulline, Arg, and Orn contributed approximately 20% of casein-Pro (97, 104). This pathway may provide an alternative and perhaps critical supply of Pro that is typically not extracted in adequate quantities for casein synthesis (80). The synthesis of Pro via Arg may be inherently limited, however, because, in bovine mammary tissue, the key enzyme in this pathway, ornithine-δ-transferase (EC 2.6.1.13), has a high Michaelis constant (8.4 m*M*), which would require that high pools of Orn be maintained to achieve maximal rates of conversion through this pathway (10). Alternatively, the requirement for de novo synthesis of Pro may restrict the availability of Arg for other functions (e.g., polyamine synthesis via Orn). Indeed, in vitro studies indicate that the contribution of Arg to Pro synthesis can be reduced when additional Pro is supplied (49). This possibility has been considered (24), and milk protein output was increased by 16% when two midlactation cows were given duodenal infusion of Pro (80 g/d). Although MG uptake of Arg was decreased with Pro infusion, this decrease could not be linked to the effects of Pro on mammary metabolism because changes in Pro uptake were not reported. Nevertheless, the responses to Pro infusion are intriguing and warrant further investigation.

His and MG Blood Flow

The relationship between His uptake and milk output is quite variable, and, in part, this variation may be related to whether concentrations in whole blood or plasma are monitored for uptake comparisons (14). In the perfused guinea pig MG (30), His is not oxidized, and so utilization for protein synthesis or nonoxidative pathways (e.g., carnosine synthesis and deamination via histidase) predominate. However, the guinea pig MG appears to express the His catabolic pathways and enzymes differently from expression in the cow (72). The two species vary primarily because of differences in the His degradative enzymes for the synthesis and inactivation of histamine, the latter having been shown to cause constriction of mammary pudendal arteries of goats (54). In contrast to the laboratory species, bovine mammary tissue contains concentrations of histamine that parallel those of His, and levels of the enzyme responsible for this conversion, His decarboxylase (EC 4.1.1.22), also appear to parallel these changes (72). Those authors also found histamine to be localized in mast and nonmast cell tissues of the MG, but whether histamine functions here to initiate milk secretion and letdown, or in regulation of MG blood flow, or both, has not been determined. We have evidence that may link the His supply to the regulation of MG blood flow. Employing a goat model in which milk production response was limited by His (B. J. Bequette, 1995, unpublished data), we observed upon removal of His from the AA mixture that was abomasally infused (77 g/d for 7-d periods) that milk protein output decreased by 15 to 35%. In contrast, MG blood flow (monitored 24-h/d by flow probes) increased by 26 to 34% upon removal of His. Upon replenishment of His, milk protein output increased, and MG blood flow decreased, both returning to normal levels. Arterial plasma His was also reduced from 50 to 15 μM during the limitation period. One might speculate that blood flow decreased because of lower mammary tissue concentrations of histamine, which may have occurred in response to the lower concentrations of plasma His. Conversely, a reduced rate of histamine synthesis and, thus, milk protein secretion may have been the initial response to the limiting His supply. The changes in blood flow did influence MG function because, despite the lower plasma His, MG fractional extraction of His was considerably enhanced (15 to 20% vs. 85 to 95% extraction), resulting in extremely low (1 to 2 μM) plasma venous concentrations of His for some of the goats. Moreover, these findings may suggest that the MG has the ability to sense a substrate limitation and respond through mechanisms controlling blood flow.

IMPLICATIONS AND FUTURE PERSPECTIVES

Are scientists getting any closer to identifying consumer acceptable ways to increase milk protein percentage (>3%) and output consistently and to improve the efficiency of milk production above the current upper limit of 30%? The answer to this question is a resounding yes, but progress has been and will continue to be cautiously slow, at least to those in search of the "magic bullet".

The MG is the major controller of its own metabolic fate, but, as yet, we have not been able to identify the right combination of factors that the MG requires to take full advantage of its potential to synthesize and secrete milk. Although many have continued, sometimes exhaustively, to search for limiting AA, others have taken a different approach and have begun to ask important questions such as what these limitations are and why and where these limitations occur. Finding the answers may require looking in places not previously contemplated and looking for metabolic roles not yet identified. The recent evidence supporting a role for peptides or proteins in interorgan AA exchange and as transport vehicles of possibly every AA to the MG for milk protein synthesis may be one of these unforeseen places. The ability to extract AA from the blood supply, a function of transporter activity, may not be as much a limitation as the surface area that is exposed to AA in the capillary networks. Optimizing this situation may be a function of the relative distribution of blood flow to the MG on a whole body basis and that passing through the capillary microvasculature [i.e., the nutritive blood flow concept advanced by the New Zealand group (61)], which appears to be regulated locally within the mammary tissues, possibly via mechanisms that are capable of sensing substrate deficiencies. The cellular metabolism of the MG is quite active, with many synthetic and catabolic mechanisms to be considered, and yet we have probably only now begun to identify how these mechanisms interrelate and how important, necessary, or rate-limiting they can be. What is the potential of molecular biology to eliminate or enhance the throughput of some of these enzymatic pathways? The role of energy substrates was not considered in this review, but may be equally as important as rate-limiting sources for ATP and NADPH production or for intermediary metabolic carbon supplies.

The metabolism and functions of the MG are much more complex than we have considered. As more information accumulates, greater emphasis will be placed on mathematical modeling to collate and simplify all of the interrelated mechanisms of metabolism and regulation (7). The ability to represent MG biochemistry and physiology in mathematical terms is close to becoming a reality, but there is still the challenge of integrating these terms within the whole-animal system. Obviously knowledge and representation of the endocrine systems and their components will be required as well as ways in which these integrate or are integrated by incoming dietary nutrients (11, 12). The challenge will then be to test and represent these processes at the different organ, tissue, cellular, and subcellular levels so that ultimately it will be possible to evaluate quantitatively the influence of dietary nutrient intake on the patterns of substrate assimilation into milk components.

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