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Vascular Sources of Amino Acids for Milk Protein Synthesis in Goats at Two Stages of Lactation

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ABSTRACT

An arteriovenous technique, combined with a 30-h i.v. infusion of [5-$^{13}CH_3$]Met and [5,5,5-^{2}H]Leu, was used to monitor mammary uptake of free amino acid (AA) and to estimate the proportion of casein synthesized from circulating peptides in goats in early and late lactation. At both stages, kinetics was performed on the last day of consecutive 5.5-d periods. The first period was an i.v. infusion of saline and the second an i.v. infusion of lysine (8.9 g/h) plus methionine (2 g/h). Net uptake of essential AA and protein yields were higher in early than in late lactation. Uptake of free Met, His, and Pro was less than, uptake of Tyr and Lys was equal to, and uptake of Arg, Leu, Val, and Ile was greater than milk protein synthesis. Peptide uptake, estimated from the difference in casein and plasma free AA enrichment, accounted for a larger fraction of casein-Met (17 vs. 8%) and casein-Leu (27 vs. 12%) in late than in early lactation. Small decreases in mammary blood flow, AA transport activity, and AA concentrations accounted for the lower uptake of AA in late compared with early lactation. Based on our studies of several AA, the utilization of circulating peptides for casein synthesis appears to be a general phenomenon.
(**Key words:** mammary gland, amino acid metabolism, peptide, casein synthesis)

Abbreviation key: AV-difference = arteriovenous concentration differences, **BF** = blood flow, **GC-MS** = gas chromatography-mass spectrometry.

INTRODUCTION

Milk and mammary protein synthesis account for 20 to 40% of whole body protein synthesis in lactating cows and goats (Champredon et al., 1990; Bequette et al., 1996a). In consequence, large quantities of individual AA must be partitioned at variable rates to the mammary gland. At the level of the mammary gland, removal of AA for milk protein synthesis is a function of the AA supply (concentration and blood flow; **BF**) and the affinity of the udder for individual AA. Blood flow and AA transport activity appear to act in concert to allow the udder to cope with fluctuations in AA concentration and to assure that uptake of AA is adequate to meet the demands for milk protein synthesis (Fleet et al., 1985; Hanigan et al., 1998; Bequette et al., 2001; Hanigan et al., 2001). Growth hormone and insulin appear to modulate AA supply for milk synthesis by acting upon BF, AA transport activity, or both (Hanigan et al., 1998; Bequette et al., 2001). Such regulation has been examined at a specific stage of lactation; however, little is known regarding the role of regulation in determining BF and transport activity and, thereby, AA supply throughout the lactation cycle (Fleet et al., 1985).

Early observations by Mepham and Linzell (1966) suggested that circulating blood-free AA were the primary source of AA for casein synthesis, and this was later confirmed by Bickerstaffe et al. (1974), who found a close balance between free AA uptake and milk protein nitrogen and carbon outputs in cows. However, since these early observations it has become apparent that those AA (Arg, Leu, Ile, Val, and Lys) taken up in excess are also oxidized to CO_2 (Oddy et al., 1988; Bequette et al., 1998, 1999, 1996a, 1996b; Mabjeesh et al., 2000a). Thus, the carbon from these AA is unavailable for the synthesis of nonessential AA, which are not extracted in adequate quantities. Furthermore, certain essential AA (Phe, Met, Thr, and His) have been consistently observed to be extracted in amounts less than milk outputs (Guinard and Rulquin, 1994, 1995; Bequette et al., 1999, 1996b; Mabjeesh et al., 2000a,

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2000b). These two facts have led to speculation that to account for the deficit in free AA uptake, and to balance carbon and nitrogen inputs and outputs, the udder must also be extracting nonfree AA sources (i.e., peptides or proteins; Backwell et al., 1994; 1996; Bequette et al., 1994).

However, despite the simplicity of the net uptake to milk output measurement, there are potentially large sources of error associated with measurement of mammary BF, arteriovenous concentration differences (**AV-difference**) and of milk protein output (see Bequette et al., 1998), all of which add to the variability of the mammary balance data and thus the distinction of whether an AA is indeed taken up in excess or insufficient quantities. To acquire reliable estimates of free AA removal by the mammary gland we improved the AV-difference methodology (Bequette et al., 1999; Mabjeesh et al., 2000a) and developed stable isotope labeling techniques to indirectly demonstrate that the mammary gland can use synthetic peptides for casein synthesis (Backwell et al., 1994) and that the contribution of peptides to casein synthesis is probably significant (Bequette et al., 1994; Backwell et al., 1996; Backwell, 1997; Mabjeesh et al., 2000a). Employing the precursor-product labeling methodology in lactating goats (d 45 to 253), we provided evidence that peptides contributed to the supplies of Phe, Tyr, and Met for casein synthesis, which was compatible with the observations that these AA are often not taken up in adequate quantities by the udder (Bequette et al., 1994, 1999; Backwell et al., 1996). Surprisingly, it was also estimated that peptides contributed to the uptakes of Val and Lys, but not to Leu, and these are AA, which are almost always extracted in excess quantities by the udder (Bequette et al., 1994, 1999; Backwell et al., 1996; Mabjeesh et al., 2000a).

The present study extends our previous investigations to identify the key mechanisms controlling AA uptake by the lactating mammary gland (Hanigan et al., 1998, 2001; Bequette et al., 2001). Another objective was to further identify the sources of AA for casein synthesis and to examine whether free AA supply and stage of lactation affected the relative contributions of free AA and peptides to mammary metabolism (Backwell et al., 1994, 1996; Bequette et al., 1994, 1997; Mabjeesh et al., 2000a). The arteriovenous net balance and the precursor-product labeling techniques were used to examine AA extraction kinetics and the sources (free AA and peptides) of Met and Leu used for casein synthesis. Lysine metabolism and its precursor kinetics across the mammary gland were reported previously in a companion paper (Mabjeesh et al., 2000a), and some aspects of the current study have been reported elsewhere (Bequette et al., 1998; Mabjeesh et al., 2000b).

MATERIALS AND METHODS

Goats, Surgery, and Diets

The Institute's Ethical Review Committee and the veterinary inspectorate of the Home Office (United Kingdom) approved all surgical procedures and practices. Experimental details have been reported previously (Mabjeesh et al., 2000a). Four nonpregnant, multiparous British Saanen goats (BW = 65.1 ± 6 kg) were used in this study. Four goats were examined in late (233 ± 14 DIM) and in early (80 ± 17 DIM) lactation. Goats had been surgically prepared with a raised carotid artery, catheters placed into each of the external pudic arteries, and a flow probe fitted around an external pudic artery (6 mm; Transonic Systems Inc., Ithaca, NY) to allow arteriovenous measurements of mass and isotope fluxes across one-half of the mammary gland. At least 1 d prior to i.v. infusion of treatment solutions or kinetic measurements, temporary catheters (polyvinyl chloride, medical grade, i.d. 0.8 mm, o.d. 1.2 mm, Critchley Electrical Products) were inserted into each jugular vein and into the elevated carotid artery and the subcutaneous mammary vein ipsilateral to the probe. Catheters were kept patent by flushing once daily with a sterile heparin-saline (200 U/ml) solution.

Goats were placed in metabolism crates and allowed at least 10 d adaptation to the frequent feeding by automatic feeders (12 equal portions fed at 2-h intervals) and the daily routines of machine and hand-milking (0830 and 1630 h). Milk weights were recorded at each milking, and subsamples were preserved with 0.1 ml of formaldehyde (40% vol/vol) and stored at 4°C until analyzed for milk CP (milk N × 6.38). The diet was formulated (Mabjeesh et al., 2000a) to meet metabolizable energy and protein requirements for maintenance and milk production (AFRC, 1993; 1992). Daily feed refusals were removed and weighed, and feed intake was adjusted to allow 5% refusals.

Stable Isotopes

Specific mixtures of L-[5-$^{13}CH_3$]Met and L-[5,5,5-^{2}H]Leu (all 99 atoms %; Mass Trace, Somerville, MA) were dissolved in saline (9 g of NaCl/L, pH 7) and filter-sterilized (0.22-μm filter units) into sterile glass bottles.

Experimental Procedures

The same experimental protocol was employed at both stages of lactation. Goats were given a 5.5-d i.v. infusion of saline (15 g of saline/h) followed 3 d later

by a 5.5-d infusion of Lys plus Met (LM, 370 and 84 mg/h in 15 g of saline, respectively). Starting on d 5 of each treatment infusion period, goats received a 30-h continuous i.v. infusion (50 g of solution/h) of [$^{13}CH_3$]Met (10 mg/h) and [$^{2}H_3$]Leu (28 mg/h). From h 24 to 30 of isotope infusion, they received an i.v. infusion of sodium-heparinate (6.6 kU/h) to prevent clotting during continuous blood withdrawal. For goats in late lactation, *p*-aminohippurate (*p*-AH; 30 mg/g of solution) was infused into an external pudic artery from h 24 to 30 for measurement of blood flow by down stream dilution of dye. For all goats at both stages of lactation, BF was also monitored by flow probe to provide a comparison of dye dilution and flow probe techniques (Mabjeesh et al., 2000a). Starting at 23 h, each mammary gland was milked-out (no oxytocin given) by hand at 1-h intervals, the milk weighed and subsamples taken for AA analyses and for casein isolation to determine AA enrichment. Beginning at h 25, blood was continuously (10 ml/h) withdrawn from an arterial source and from the subcutaneous mammary vein for five consecutive 1-h periods. Samples were collected into sealed syringes submerged in an ice bath. Flow probe voltages were logged (every 10 s) and data integrated over the 1-h blood sampling periods. Flow probes were calibrated in vitro, employing gravimetric procedures outlined by the manufacturer. All goats were kept standing during the blood sampling period.

Samples of whole blood, plasma, whole milk, and isolated casein were prepared and analyzed for AA enrichment by gas chromatography-mass spectrometry (**GC-MS**, and for AA concentration as previously described (Heinrikson et al., 1984; Bequette et al., 1994; 1999; Backwell et al., 1996; Calder et al., 1998; Mabjeesh et al., 2000a). Gravimetric procedures were used to quantify blood *p*-aminohippurate concentrations employing the Bratton-Marshall procedure as described by Smith et al. (1945).

Kinetic Calculations

The flux of Met and Leu in plasma (F_p) was calculated from isotope dilution of [5-$^{13}CH_3$]Met and [5,5,5-^{2}H]Leu in plasma:

$$F_p \text{ (mmol/h)} = ((E_i/E_a) - 1) \times IR,$$

where E_i = the enrichment (molar percent excess) of the infused isotope, E_a = the enrichment of [5-$^{13}CH_3$]Met or [5,5,5-^{2}H]Leu in arterial plasma at plateau and IR = the rate of isotope infusion.

The comparison in late lactation of blood flow values measured by flow probe and dye-dilution indicated that blood flow is underestimated on average by 28% when using the flow probes. To account for this underestimation in the early lactation phase of the study (where *p*-AH was not given), probe values were adjusted using the equation reported in the companion paper (Mabjeesh et al., 2000a).

Kinetic calculations were only performed for the udder half that was monitored. Net uptake (NU) of AA was based on blood and plasma exchanges, calculated as:

$$NU \text{ } (\mu\text{mol/h}) = (C_a - C_v) \times BF$$

where C = the concentration (μM) of free AA in arterial (a) and mammary venous (v) blood (b) or plasma (p) and BF (l/h) = blood or plasma flow (blood flow × (1 – (packed cell volume/100)) as appropriate. Partition of plasma flux to the mammary gland (K_{mg}) was calculated by comparison of mammary gland gross removal to whole body flux as:

$$K_{mg} \text{ (per d)} = (((C_a \times E_a - C_v \times E_v)/E_a) \times BF_p)/F_p$$

where E_a and E_v = the enrichment of [5-$^{13}CH_3$]Met or [5,5,5-^{2}H]Leu in arterial and mammary venous plasma.

A rate parameter (*K*, ml/min) for removal of AA by an udder half was calculated from the model of Hanigan et al. (1998):

$$K_i = (C_{ai} \times BF/C_{vi}) - BF$$

where C_{ai} and C_{vi} represent arterial and venous plasma concentrations of the ith AA and BF represents mammary plasma flow (ml/min). The term *K* represents the ability of the tissue bed to clear plasma metabolite per unit time. In large part, K describe the activity of the transporter system, that is, the combination of the number and affinity of the transporters.

Statistic Analysis

Results were analyzed using a linear model involving a 2 × 2 factorial arrangement with a repeated measures procedures over the sampling time. The model included main effects of goat, infusion (saline vs. LM), stage of lactation (early vs. late), time of sampling, interactions (infusion × stage of lactation), and the residual error term. Because time effect was not significant, the model was reduced accordingly. The error term of the interaction effect (infusion × stage of lactation) was used to compare main effect means. Amino acids concentration in blood and plasma were compared by ANOVA from each treatment using a linear model that included the main effect of treatment and the residual error term. Means are presented as least square means. Means were considered significantly different at $P < 0.05$. Cal-

Table 1. Plasma concentrations of essential AA in lactating goats.[1]

	Early		Late			Main effect	
	Saline	LM	Saline	LM	SE	Lactation	Infusion
			μM			P	
His	30.5	30.7	33.5	30.2	4.4	NS	NS[2]
Thr	46.7	48.1	37.7	31.1	7.3	NS	NS
Arg	106.9	132.2	97.2	109.8	4.5	0.04	0.005
Pro	98.8	109.4	90.9	88.9	8.7	NS	NS
Tyr	46.7	46.0	39.2	38.6	5.5	NS	NS
Val	142.3	142.8	103.5	93.2	11.5	0.10	NS
Ile	89.4	90.1	67.7	58.2	6.2	0.09	NS
Leu	120.0	108.6	81.0	74.8	5.8	0.05	0.07
Phe	30.6	33.2	26.2	26.8	1.5	0.07	NS
Met	20.0	33.8	17.3	29.6	1.5	0.14	0.0001
Lys	145.5	233.4	111.4	221.4	22.8	NS	0.001

[1]In early and late lactation, goats (n = 4) were given i.v. infusions (5.5 d each period) of saline followed 3 d later by an infusion of lysine plus methionine (LM). Measurements were made on the last day of each infusion period.

[2]NS = Not significantly different ($P > 0.15$).

culations were done using the GLM procedure of SAS (1985).

RESULTS

Milk Production, Mammary Blood Flow, and AA Concentrations

Daily milk protein yield and secretion of AA in milk (measured during the arteriovenous kinetic period) were higher (by ~60%, $P < 0.001$) in early compared with late lactation (Mabjeesh et al., 2000a), and these were not affected by LM infusion. In contrast, mammary BF, while numerically lower in late lactation (36.55 vs. 34.60 L/h per udder half), did not differ significantly with stage of lactation. Infusion of LM also did not affect blood flow.

Plasma concentrations (Table 1, blood data not shown) of Arg, Val, Ile, Leu, Phe, and Met were higher ($P < 0.05$) or tended ($P < 0.15$) to be higher in early compared with late lactation. Infusion of LM increased plasma concentrations of Met and Lys, but also increased those of Arg and tended to decrease those of Leu.

Mammary Uptake Kinetics for Met and Leu

Kinetics of methionine are given in Table 2, and those of Leu are given in Table 3. Plasma fluxes of Met and Leu were higher in early than in late lactation, with Met flux increased (25 to 34%) by LM infusion. The proportion of plasma flux partitioned to the mammary gland (K_{mg}) was greater ($P < 0.01$) for Leu (0.18) than for Met (0.13) and stage of lactation did not affect K_{mg}. However, infusion of LM reduced K_{mg} for Met and Leu by 23 and 31%, respectively.

Net uptake of Met and Leu from blood and plasma did not differ, except for Met in late lactation during the LM infusion. Infusion of LM decreased plasma net uptake of Leu. Mammary net uptake of blood or plasma-free Met was less than milk output (average U/O = 0.84), and infusion of LM did not affect this relationship. Thus, for Met, mammary uptake of sources other than blood or plasma free AA appear to be required to make up this deficit. For Leu, the uptake to output ratio was often greater than unity (average U/O = 1.13), and as for Met, this relationship was not affected by LM infusion. Stage of lactation did not affect this ratio for either AA. The ratio of the plateau isotopic enrichment of the AA in casein to that of the free AA in plasma was used to estimate the contribution of plasma free AA to casein synthesis, and thus, by difference [i.e., 1 – (casein AA enrichment/plasma AA enrichment)], the proportion of casein-AA derived from unlabeled sources of AA, which at isotopic plateau should reflect the uptake and use of circulating peptides for casein synthesis (Backwell et al., 1996). For Met, the contribution of free AA to casein synthesis was on average 0.86, which is similar to the ratio of net uptake of blood free Met to milk output of Met (average U/O = 0.81). Consequently, when the estimate of peptide-bound Met (7 to 18%) was added to the U/O calculation, total Met (free plus peptide) uptake by the udder nearly balanced with milk output (average U/O = 0.95). The estimated proportion of casein-Met derived from peptides was greater in late than in early lactation, and infusion of LM reduced the proportion of Met derived from peptides (i.e., the C/P ratio was reduced, Table 2).

Table 2. Whole body flux and net metabolism of methionine by an udder half of lactating goats.[1]

	Early		Late			Main effect	
	Saline	LM	Saline	LM	SE	Lactation	Infusion
						P	
Plasma flux, mmol/h	3.23	4.05	2.57	3.44	0.16	0.0001	0.0001
K_{mg}[2]	0.13	0.12	0.14	0.10	0.02	0.10	0.001
Free AA net uptake (U), μmol/h							
Plasma	371	418	320	239	49	0.0001	NS[3]
Blood	387	375	304	196	59	0.0001	0.033
Blood vs. plasma, *P*<	NS	NS	NS	0.001			
Free AA + peptide uptake[4] (U), μmol/h							
Plasma	445	460	380	291	49	0.0001	NS
Blood	462	416	364	245	61	0.0001	0.006
Blood vs. plasma, *P*<	NS	NS	NS	NS			
Milk output[5] (O), μmol/h	486	500	323	295	68	0.0001	NS
Balance ratio (U/O)							
Free AA							
Plasma	0.79	0.88	1.04	0.78	0.13	NS	NS
Blood	0.81	0.80	0.97	0.64	0.14	NS	NS
Blood vs. plasma, *P*<	NS	NS	NS	NS			
Free AA + peptides							
Plasma	0.96	0.96	1.23	0.95	0.14	NS	NS
Blood	0.94	0.88	1.16	0.81	0.16	NS	NS
Blood vs. plasma, *P*<	NS	NS	NS	NS			
Casein:plasma enrichment (C/P)	0.85	0.93	0.82	0.84	0.04	0.0001	0.001

[1]In early and late lactation, goats (n = 4) were given i.v. infusions (5.5 d each period) of saline followed 3 d later by an infusion of lysine plus methionine (LM). Kinetic measurements were made on the last day of each infusion period.

[2]K_{mg} = Proportion of plasma flux partitioned to the udder.

[3]NS = Not significantly different ($P > 0.15$).

[4]Peptide uptake calculated as: milk AA output × [(1 – (C/P)], where C and P are the plateau enrichments in casein and plasma, respectively.

[5]Milk output was corrected for 4% of milk protein AA derived from nonmammary synthesized proteins appearing in the milk (Whitney et al., 1976).

The net uptake of free Leu from blood or plasma was either equal to or greater than the requirements for milk output (Table 3). However, based on the casein to plasma Leu enrichment ratio (average C/P = 0.81), we predicted that 19% of the supply of Leu for casein synthesis derived from circulating peptides. Similar to Met, the estimated proportion of casein-Leu derived from peptides was greater in late than in early lactation. Unlike Met though, the infusion of LM did not affect the contribution of peptides to casein-Leu synthesis. After adjusting the uptake to output ratio to include the estimation of Leu derived from peptides (7 to 28%), total Leu uptake (free plus peptides) was further in excess (by 17 to 62%) of milk protein output requirements.

Mammary Uptake Kinetics of Essential AA

Net uptake of blood AA by the udder did not differ from values based on plasma removals, except for His (LM infusion in late lactation), Lys (LM infusion in early lactation) and Thr (Table 4). Net uptake of essential AA was higher (range: 22 to 66%) in early compared with late lactation (most nonessential AA were not monitored). Infusion of LM decreased plasma net uptakes of Thr, Arg and Phe, and decreased blood net uptakes of all AA monitored, except for Leu. The ratios of net uptake to output in milk of AA are given in Table 5. In general, the net uptakes of His, Thr, Pro, and Phe (blood data) were less than milk outputs, the uptakes of Tyr, Phe (plasma data), and Lys were equal to milk outputs and the uptakes of Arg, Val, and Ile (blood and plasma data) were greater than milk outputs. Stage of lactation only affected this ratio for Lys, whereas in later lactation it was higher. Infusion of LM tended to reduce the uptake to output ratio for Arg (plasma data) and Lys (blood data).

For individual plasma and blood AA the rate constants (*K*) for mammary (udder half) apparent transport activity are given in Table 6. For plasma values, except for a tendency for *K* of His to be lower in late compared than in early lactation, there were no differences in transport activity at the two stages of lactation. At both stages, transport activity for Arg, Met, and Lys uptake was reduced by infusion of Lys plus Met.

Table 3. Whole body flux and net metabolism of leucine by an udder half of lactating goats.[1]

	Early		Late			Main effect	
	Saline	LM	Saline	LM	SE	Lactation	Infusion
						P	
Plasma flux, mmol/h	11.2	12.1	7.8	7.7	0.54	0.0001	0.109
K_{mg}[2]	0.21	0.16	0.20	0.15	0.03	NS[3]	0.002
Free AA net uptake (U), μmol/h							
Plasma	2194	1889	1462	1150	355	0.0001	0.063
Blood	1784	1906	1551	1065	236	0.0001	NS
Blood vs. plasma, *P*<	NS	NS	NS	NS			
Free AA + peptide uptake[4] (U), μmol/h							
Plasma	2460	2096	1743	1445	371	0.0001	0.019
Blood	2058	2115	1832	1361	212	0.0001	0.042
Blood vs. plasma, *P*<	NS	NS	NS	NS			
Milk output[5] (O), μmol/h	1757	1806	1169	1066	245	0.0001	NS
Balance ratio (U/O)							
Free AA							
Plasma	1.30	1.14	1.26	0.99	0.17	NS	NS
Blood	1.01	1.15	1.38	0.96	0.15	NS	NS
Blood vs. plasma, *P*<	NS	NS	NS	NS			
Free AA + peptides							
Plasma	1.45	1.22	1.56	1.29	0.14	NS	NS
Blood	1.17	1.23	1.62	1.24	0.15	NS	NS
Blood vs. Plasma, *P*<	NS	NS	NS	NS			
Casein:plasma enrichment (C/P)	0.84	0.93	0.76	0.72	0.04	0.0001	NS

[1]In early and late lactation, goats (n = 4) were given i.v. infusions (5.5 d each period) of saline followed 3 d later by an infusion of lysine plus methionine (LM). Kinetic measurements were made on the last day of each infusion period.

[2]K_{mg} = Proportion of plasma flux partitioned to the udder.

[3]NS = Not significantly different ($P > 0.15$).

[4]Peptide uptake calculated as: milk AA output × [(1 – (C/P)], where C and P are the plateau enrichments in casein and plasma, respectively.

[5]Milk output was corrected for 4% of milk protein AA derived from non-mammary synthesized proteins appearing in the milk (Whitney et al., 1976).

Table 4. Net uptake of AA by an udder half of lactating goats.[1]

	Early		Late			Main effect	
	Saline	LM	Saline	LM	SE	Lactation	Infusion
						P	
Plasma, μmole/h							
His	252	265	191	127[2]	46	0.0001	NS[3]
Thr	777[2]	787[2]	618	443[2]	84	0.0001	0.04
Arg	908	832	768	490	128	0.0002	0.005
Pro	854	563	863	484	153	0.0001	NS
Tyr	469	461	331	255	67	0.0001	NS
Val	1754	1794	1273	1007	195	0.0001	NS
Ile	1353	1411	1002	759	140	0.0001	NS
Phe	545	556	438	328	72	0.0001	0.07
Lys	1317[2]	1343	1167	1022	105	0.01	NS
Blood, μmole/h							
His	271	171	155	115	63	0.005	0.022
Thr	596	462	536	279	106	0.014	0.0002
Arg	1066	819	730	434	211	0.006	0.009
Pro	1036	825	582	418	187	0.0001	0.038
Tyr	529	440	342	243	85	0.0001	0.023
Val	1983	1755	1346	960	318	0.0001	0.045
Ile	1487	1431	1044	737	200	0.0001	0.059
Phe	617	530	495	304	88	0.0001	0.002
Lys	1781	1375	1272	943	225	0.001	0.001

[1]In early and late lactation, goats (n = 4) were given i.v. infusions (5.5 d each period) of saline followed 3 d later by an infusion of lysine plus methionine (LM). Net uptake measurements were made on the last day of each infusion period.

[2]Blood and plasma values were significantly different ($P < 0.05$) within a treatment.

[3]NS = Not significantly different ($P > 0.15$).

Table 5. Ratios of net uptake to milk secretion of free AA by an udder half of lactating goats.[1]

	Early		Late			Main effect	
	Saline	LM	Saline	LM	SE	Lactation	Infusion
						P	
Plasma, μmole/h							
His	0.63†	0.67†	0.78	0.55†	0.15	NS‡	NS
Thr	0.78†	0.80§	1.04	0.80	0.13	NS	NS
Arg	2.11†	1.93†	2.95†	1.94†	0.32	NS	0.120
Pro	0.42†	0.39†	0.44†	0.40†	0.07	NS	NS
Tyr	0.99	0.96	1.19†	0.91	0.16	NS	NS
Val	1.42†	1.67†	1.60†	1.32†	0.19	NS	NS
Ile	1.73†	2.05†	2.11†	1.72†	0.27	NS	NS
Phe	0.80	0.80	0.89	0.66	0.10	NS	NS
Lys	0.88	0.96	1.21	1.11	0.18	0.038	NS
Blood, μmole/h							
His	0.57†	0.40†	0.63†	0.57†	0.12	NS	NS
Thr	0.54†	0.50†	0.90	0.53†	0.13	NS	NS
Arg	2.25†	1.95†	2.76†	1.76†	0.41	NS	NS
Pro	0.49†	0.38†	0.45†	0.36†	0.08	NS	NS
Tyr	1.07	0.92	1.19†	0.89	0.16	NS	NS
Val	1.52†	1.65†	1.68†	1.28†	0.24	NS	NS
Ile	1.85†	2.10†	2.18†	1.69†	0.29	NS	NS
Phe	0.88	0.76†	0.97	0.61†	0.14	NS	NS
Lys	1.17	0.94	1.15	1.06	0.15	NS	0.10

[1]In early and late lactation, goats (n = 4) were given i.v. infusions (5.5 d each period) of saline followed 3 d later by an infusion of lysine plus methionine (LM). Net balance (uptake ÷ milk output) measurements were made on the last day of each infusion period. Milk output was corrected for 4% of milk protein AA derived from nonmammary synthesized proteins appearing in the milk (Whitney et al., 1976).

†$P < 0.05$ when $\mu \neq 1$ within a treatment.

‡NS = Not significantly different ($P > 0.15$).

§Blood and plasma values were significantly different ($P < 0.05$) within a treatment.

DISCUSSION

Milk protein yield was ~37% lower in late compared with early lactation, and net uptake of free AA (range: 23 to 39% lower) by the udder was also lower in late lactation. Fleet and Mepham (1994) also found that free AA uptake by the udder paralleled the changes in milk protein yield by ewes during the first 141 d of lactation. Uptake and use of AA by the udder is a multifaceted process involving changes in BF kinetics, plasma AA concentrations, AA transport activity by the udder, and endocrine control. Fleet and Mepham (1994) observed very little change in mammary BF during the first 141 d of lactation, leading them to conclude that AA uptake was probably regulated at the level of transport. We also did not detect significant differences in mammary BF (36.55 vs. 34.60 L/h per udder half) at the two stages of lactation examined (80 ± 17 vs. 233 ± 14 DIM), and, except for a tendency for His transport activity to be lower in late compared with early lactation, the ability of the udder to extract plasma and blood AA was also not different.

Transport activity was computed to account for effects on AA removal that are independent of changes in BF and arterial AA concentrations. As transport activity and BF were not significantly altered, then changes in plasma AA may be the driving variable. Only for Val, Ile, Leu, Phe, and Met were blood (plasma) concentrations lower (tendencies) in late lactation. Therefore, more than likely, the lower uptake of AA with advancing lactation was due to a combination of factors acting in a chronic fashion to control milk synthesis. Indeed, it appears that it was the subtle changes in plasma concentration (average: –20%), mammary BF (average: –6%), and mammary AA transport activity (average: –11%) that combined to produce the significant declines in AA uptake (average: –33%) as lactation progressed.

Inexplicably, the net removals by the mammary gland of blood-free Lys, Arg, Ile, Leu, and Val are often in excess of requirements for milk protein synthesis, whereas the uptakes of free Met, Phe, His, and Thr are often less than requirements (Mepham and Linzell, 1966; Bickerstaffe et al., 1974; Guinard and Rulquin, 1994; 1995; Bequette et al., 1999). Observations such as these for lysine and Met have been difficult to reconcile given that these AA are often considered to be limiting or colimiting for milk production by dairy cows (Schwab et al., 1992). Consistent with previous observations (Fleet and Mepham, 1985), we observed that the net uptakes of free Met, His, Thr, Pro, and Phe across the

Table 6. Rate constants (*K*, ml/min per udder half) for plasma and blood essential AA uptake by the mammary gland.[1]

	Early		Late			Main effect	
	Saline	LM	Saline	LM	SE	Lactation	Infusion
						P	
Plasma, ml/ min							
His	179	233	70	85	45	0.07	NS[2]
Thr	652	857	558	464	180	NS	NS
Arg	199	140	166	58	49	NS	0.05
Pro	173	192	141	105	52	NS	NS
Tyr	340	362	384	286	56	NS	NS
Val	339	399	438	319	77	NS	NS
Ile	550	607	691	474	119	NS	NS
Leu	877	812	1035	687	212	NS	NS
Phe	833	794	741	419	154	NS	0.15
Met	875	407	1341	204	197	NS	0.001
Lys	231	127	293	71	58	NS	0.005
Blood, ml/ min							
His	172	105	101	64	50	NS	NS
Thr	424	364	682	304	261	NS	NS
Arg	192	122	167	68	56	NS	0.11
Pro	218	158	138	96	44	NS	NS
Tyr	301	230	237	158	78	NS	NS
Val	384	383	410	325	86	NS	NS
Ile	562	552	574	480	107	NS	NS
Leu	589	572	778	500	169	NS	NS
Phe	672	586	824	328	233	NS	0.15
Met	720	324	919	149	321	NS	0.061
Lys	264	124	294	105	60	NS	0.013

[1]In early and late lactation, goats (n = 4) were given i.v. infusions (5.5 d each period) of saline followed 3 d later by an infusion of lysine plus methionine (LM). Values for *K* were based on measurements made on the last day of each infusion period.

[2]NS = Not significantly different ($P > 0.15$). There was an interaction for plasma methionine ($P < 0.05$).

udder were equal to or less than requirements for milk protein synthesis, whereas the net uptakes of free Leu, Arg, Val, Ile, and Lys were greater than milk output (Table 5). These relationships (excess and insufficient uptakes) were not significantly affected by stage of lactation, and therefore, level of milk protein yield (80 ± 17 DIM, 138 g of protein/d and 233 ± 14 DIM, 86 g of protein/d), suggesting that the individual milk protein precursor sources appear to remain constant with respect to the total precursor supply, regardless of the metabolic requirements for AA for protein synthesis and catabolism by the mammary gland. The consistency of these relationships should help to simplify future metabolic models of dairy cow metabolism (Hanigan et al., 2001).

Two questions arise. First, why is there a tendency for some AA to be extracted in excess by the udder? And, second, for those AA not extracted in sufficient quantities, where does the remainder of the AA come from and is the supply and uptake of this source regulated? So far, we have shown that for at least Lys and Leu, excess mammary uptake results in oxidation and this oxidative process does not appear to be linked to the regulation of milk protein synthesis (Bequette et al., 1996; Mabjeesh et al., 2000a). The present study examined the sources of the additional AA and how this may be regulated by AA supply and by stage of lactation.

At both stages of lactation, Met, His, Thr, Pro, and Phe were not extracted as free AA in adequate quantities to account for milk protein output and the magnitude of this deficit remained the same for each AA at both stages. Circulating peptides or proteins have been suggested as possible contributors to mammary uptake of AA and these could account for the deficit in free AA uptake reported herein (Backwell et al., 1994, 1996; Backwell, 1997; Bequette et al., 1998, 1999). Despite the widespread acceptance that many tissues possess peptide transporters and peptidase enzymes (see Webb, 2000), the quantitative significance of peptides in tissue metabolism has been debated. In large part, this stems from the fact that the methodology for quantifying free AA uptake by the mammary gland has potentially large errors, particularly in the measurement of arteriovenous difference, BF and milk AA output (see review Bequette et al., 1998). Further, direct measurement of peptide fluxes has also been problematic as isolation methods have been unable to conclusively demonstrate peptide or protein uptake by the udder, and indeed by other tissues (see review Backwell, 1997).

To overcome the methodological limitations, we developed alternative in vivo isotope kinetic techniques to show that the udder has the ability to utilize synthetic peptides for casein synthesis (Backwell et al., 1994) and to estimate the quantitative contribution of peptides to casein synthesis (Backwell et al., 1996; Bequette et al., 1999; Mabjeesh et al., 2000a). The precursor-product labeling technique involves the long-term (>20 h) i.v. infusion of a labeled AA until an isotopic steady state is reached (~12 to 15 h) in the plasma-free AA pool and in the secreted milk casein. At plateau, if the plasma-free pool is the only source of AA contributing to casein synthesis, then the isotopic enrichment of the plasma free AA and that in casein should be the same. Conversely, if the enrichment in casein is lower than that of the plasma free pool, then unlabeled sources of AA (circulating peptides or very slow turning over constitutive proteins) must have been used for casein synthesis. We have provided evidence to dismiss the possibility that the source of isotope dilution arises from intracellular protein turnover because 1) mammary tissue has a very high fractional rate of protein synthesis (goats: 40 to 130%/d; Oddy et al., 1988; Champredon et al., 1990) and 2) removals of isotopically labeled AA balanced with isotope secretion in milk (i.e., no sequestration of isotope, and thus nearly complete turnover of proteins) at the end of a 30-h infusion of isotope (Bequette et al., 1999). Thus, we have assumed that the main contributor to isotope dilution (casein vs. plasma) is the uptake and use of circulating unlabeled peptides. In a series of studies in goats employing this technique, we (Backwell et al., 1994, 1996; Bequette et al., 1999; Mabjeesh et al., 2000a) estimated that 2 to 16% of Phe, 12 to 19% of Lys, ~17% of Met, but none of the Leu and Val, for casein synthesis derived from circulating peptides. These observations are further supported by studies in vitro using mouse mammary tissue and cell lines, where peptide-bound Met and Lys were found to be equivalent, and often preferred, sources of AA for casein synthesis compared with the free AA (Wang et al., 1994, 1996). Herein, we estimated that 7 to 18% of Met in casein may be derived from peptides and, when this estimate was summed with free Met uptake, total (free plus peptide) uptake of Met by the udder came into close balance with milk Met output (Table 2).

In a previous study in midlactation goats employing the same precursor-product methodology as herein, we did not find that peptides contributed to casein-Leu (Backwell et al., 1996). By contrast, herein we estimated that 7 to 28% of Leu derived from peptides, and consequently, when free Leu and peptide-Leu uptakes were summed, total Leu uptake exceeded milk protein output by 17 to 62%. Though the present results conflict with that earlier report, they are consistent with our observation that ~10% of casein-Leu is derived from the synthetic peptide Gly-Leu when the dipeptide is infused close-arterial to the udder (Backwell et al., 1994).

Another possibility that could account for the dilution of the AA tracers from plasma to casein synthesis is the choice of the labeled AA and the position of the labeled atoms. Transmethylation of Met has been shown to occur in lactating goats (Emmanuel and Kelly, 1984), and thus the dilution of [$^{13}CH_3$]Met from plasma to casein could arise from the loss and exchange of the $^{13}CH_3$ group within the udder, in addition to dilution from unlabeled peptides. Dilution of the [5,5,5-^{2}H]Leu tracer could also have resulted from the plasma uptake and reamination of the keto-acid of leucine, 4-methyl-2-oxopentanoate. However, the net uptake of the keto-acid by the udder is small (<2% of Leu uptake; Bequette et al., 1994, 1996b), its plasma enrichment is only ~15% lower than that of arterial plasma Leu (Bequette et al., 1994, 1996b) and the rate of reamination of intracellular keto-acid is low (25% of Leu flux; Oddy et al., 1988). In consequence, intracellular dilution of the Leu tracer from uptake of the plasma keto-acid will be insignificant (<0.1%).

For both Met and Leu, the proportion of these AA in casein that derived from peptides was larger in late than in early lactation. By contrast, we reported in the companion paper that the proportion of Lys derived from peptides did not change with stage of lactation (~16%, Mabjeesh et al., 2000a). Interestingly, when one estimates the quantity of these AA that is contributed by peptides, for Met there is no difference between early and late lactation (58 vs. 53 μmoles/h), while for Lys the quantity is greater in early than in late (282 vs. 167 μmoles/h, $P < 0.01$), and for Leu the quantity from peptides is greater in late than in early (204 vs. 289 μmoles/h, $P < 0.05$). These differences between AA could suggest that the composition of the peptides extracted by the udder or the intracellular channeling of the AA (casein vs. tissue protein synthesis) varies with stage of lactation or the lactational demand. What can be surmised, however, is that the use of peptides by the mammary gland is probably a general feature of mammary metabolism, as it apparently is for other tissues (see Backwell, 1997; Webb, 2000), and this view is supported by findings that peptide transporters are relatively nonselective for the AA composition of peptides and, furthermore, that the presence of other peptides is costimulatory (McCollum et al., 1998).

Intravenous infusion of Lys plus Met did not significantly affect our estimation of the contribution of peptide-bound Lys to casein synthesis (Mabjeesh et al., 2000a). Herein, Lys plus Met infusion decreased the estimate of peptides for casein-Met, but not Leu. Simi-

larly, but to a greater extent, infusion of Phe reduced the proportion of Phe (and Tyr) in casein that was estimated to be derived from peptides (Bequette et al., 1996). The results for Leu were expected since the supply of Leu was not altered by the Lys plus Met infusion. Previously, Guinard and Rulquin (1994, 1995) observed that the uptake to milk output ratio for Phe and Met increased from less than to greater than unity when increasing amounts of casein were infused into the duodenum of lactating cows. Based on those data, we hypothesized that when the supply or concentration of free AA to the mammary gland increases, the requirement for peptide uptake decreases (Bequette et al., 1999). Our data for Phe, Tyr, and Met tend to support that hypothesis. Thus, in early lactation where the proportion of Met and Leu supplied as peptide was low, the concentration and supply of free Met and Leu were also high, and vice versa in late lactation. The data for Lys does not support this hypothesis, which may be an indication that the transporter mechanisms may operate differently for this AA.

CONCLUSIONS

The mammary gland, like many organs and tissues, has the capability to modulate local mechanisms to accommodate fluctuations in dietary and systemic AA availability to assure that adequate quantities of AA are extracted in support of protein synthesis. Results of the present study indicate that one mechanism that appears to operate in the udder to balance AA supply involves regulation of free AA and peptide-bound AA uptake. Along with evidence from our previous studies, the current study provides further quantitative data in support of a role for circulating peptides or proteins in mammary metabolism and milk protein synthesis. The list of AA that now appear to be supplied as peptides for casein synthesis includes Phe, Tyr, Leu, Val, Met, and Lys, not to mention Glu, Gly, and Cys, which may also be supplied via uptake of glutathione. Consequently, peptide utilization for casein synthesis may be a general feature of mammary metabolism, as it is for tissue like the gut and kidneys (Backwell, 1997; Webb, 2000). Further investigations are required to identify the endocrine and nutritional factors controlling mammary uptakes of free AA and peptides, the supplies of which could potentially become rate limiting.

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