Application of a U-^{13}C-Labeled Amino Acid Tracer in Lactating Dairy Goats for Simultaneous Measurements of the Flux of Amino Acids in Plasma and the Partition of Amino Acids to the Mammary Gland

B. J. BEQUETTE,* F.R.C. BACKWELL,* A. G. CALDER,* J. A. METCALF,†,1 D. E. BEEVER,† J. C. MACRAE,* and G. E. LOBLEY*

*Rowett Research Institute, Greenburn Road, Bucksburn, Aberdeen, AB21 9SB Scotland

†University of Reading, Centre for Dairy Research, Hall Farm, Arborfield, Reading, RG2 9HX England

ABSTRACT

A preliminary study was conducted using lactating British Saanen goats (n = 5) at 109 to 213 d in milk that yielded 1.67 to 3.68 kg of milk/d to examine the application of a U-^{13}C-labeled amino acid (AA) mixture obtained from hydrolyzed algal proteins as a tracer for measuring plasma flux (n = 5) and partition to the mammary gland (n = 3; arteriovenous difference) of 13 AA simultaneously. Except for Ile and Ser, there was incomplete (6 to 54%) equilibration of the tracer with AA from packed blood cells (>90% erythrocytes) during the 6-h infusions. This result agreed with the large ratio of packed cells to gradients for plasma AA concentration that was also observed. However, net mass and isotope removals by the mammary gland were predominately from plasma, indicating that the erythrocytes did not participate in kinetic exchanges. Plasma AA fluxes (millimoles per kilogram of metabolizable protein intake per kilogram of body weight$^{0.75}$) differed among goats that consumed different protein sources; however, overall rates were lowest for Met (5 to 14) and His (8 to 17) and highest for Leu (48 to 70) and Ala (53 to 88). On average, 25% of plasma flux was partitioned to the mammary gland. Less than 20% of His, Ser, Phe, and Ala were directed to the mammary gland; 20 to 30% of Arg, Thr, Tyr, and Leu were directed to the mammary gland; and 30 to 40% of Pro, Ile, Lys, and Val were directed to the mammary gland. The unidirectional AA flux in the mammary gland (AA apparently available for protein syntheses, oxidation, and metabolite formation) did not match the pattern that is required for casein synthesis, suggesting differences in the metabolic requirements of AA for nonmilk protein synthesis.

(**Key words**: lactating goats, stable isotope, amino acid, mammary gland)

Abbreviation key: **BF** = blood flow, **GC-MS** = gas chromatography-mass spectrometry, **ME** = metabolizable energy, **MP** = metabolizable protein, ***p*-AH** = *p*-aminohippurate, **PCV** = packed cell volume.

INTRODUCTION

During the past 20 yr, considerable progress has been made in the ability to predict the supply of protein to the small intestine of ruminants (2, 3, 26, 33). Despite this progress, the ability to predict responses in milk protein output by dairy animals is still limited because the understanding of the metabolic requirements and events at the absorptive (J. C. MacRae, 1997, unpublished data) and postabsorptive (27) stages remains limited.

Kinetic tracer techniques that were originally developed for laboratory animals and humans have been applied to the studies of AA and protein metabolism of lactating ruminants (ewes, goats, and cows); however, few studies (5, 6, 8, 9, 10, 34) have examined the metabolic events that occur in vivo across the lactating mammary gland. In many of those studies, only a single tracer AA was infused, and the tracer AA usually was selected based on cost. Although those studies have provided valuable information on the dynamics of specific AA during lactation, those data are somewhat limited regarding an integrated view of AA metabolism. To improve this situation, researchers (6, 8) examined simultaneously whole body kinetics and mammary gland transfer kinetics of up to three labeled AA (Phe, Leu, Met,

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[1]Current address: Agricultural Development Advisory Board Bridgetts, Martyr Worthy, Winchester, Hampshire, SO21 1AP England.

or Val) in lactating goats to compare AA that differed in metabolism and relative supplies (limitation) for milk protein synthesis.

Recently, in our laboratory (27; J. C. MacRae, 1997, unpublised data), a tracer technique was developed based on a mixture of U-^{13}C-labeled AA obtained from hydrolyzed algal proteins. The AA mixture could be infused into growing sheep to allow simultaneous examination of whole body kinetics and tissue kinetics of up to 16 AA. The objective of this study was to adapt this methodology for use in lactating goats and to derive data from the flux of AA in plasma and the partition of a number of AA to the mammary gland. The transport kinetics of AA by erythrocytes and plasma were also examined. The compartmentalization of the mass of AA under examination was compared with the compartmentalization of the tracer in the plasma and packed cells (erythrocytes, leukocytes, and reticulocytes) and their exchanges across the mammary gland. Preliminary results have been reported elsewhere (7, 31).

MATERIALS AND METHODS

Preparation of the U-^{13}C-Labeled AA Mixture

Enriched algal biomass (dilipidated and destarched; 99 atom % ^{13}C; Martek Corp., Columbia, MD) was hydrolyzed in 6*N* HCl (1.25 g of biomass/250 ml of acid) containing dithiothreitol (100 mg) and phenol crystals (5 to 10 mg) at 110°C for 18 h. The mean recovery of AA from the algal biomass after hydrolysis was 450 g/kg of dried powder. Samples were filtered (Whatman no. 4 filter paper; Whatman, Clifton, NJ) and evaporated to dryness under reduced pressure following three washes with 50 ml of water. Samples were reconstituted in 0.2 *M* phosphate buffer and adjusted with 2*N* NaOH to pH 7.4. Aliquots (50 ml containing 2 g of biomass) were filtered (0.2-μ nitrocellulose filters) under aseptic conditions into sterilized glass bottles and stored frozen (–20°C) until use (<1 mo). One day prior to infusion, aliquots were thawed, reconstituted up to 140 ml with sterilized distilled water, and then filtered as described previously under aseptic conditions into sterilized glass bottles for intrajugular infusion.

Goats and Diets

British Saanen goats in mid to late lactation (nonpregnant) were used for these experiments. Two multiparous goats (mean BW, 56 kg) were used in Experiment 1, and three primiparous (mean BW, 75 kg) goats were used in Experiment 2. During the current or previous lactation, goats were surgically prepared with an exteriorized carotid artery and a mammary venous (superficial epigastric vein), skin-covered loop, including ligation of anastomotic vessels that transected the udder halves (8, 9). The goats used in Experiment 2 underwent a second surgery (<2 mo prior to experimentation) to position permanent catheters (polyvinyl chloride; i.d., 0.8 mm; o.d., 1.2 mm; Critchley Electrical Products, New South Wales, Australia). One catheter was inserted into each of the external pudic arteries. The 6 cm of catheter inside the artery had been reduced to an i.d. of 0.4 mm and an o.d. of 0.7 mm prior to insertion by steam-heating and stretching. One day before kinetic measurements, temporary catheters (as described previously) were inserted into a jugular vein and into the carotid artery and mammary vein loops. In Experiment 2, a temporary catheter was also placed into the contralateral jugular vein (6 d prior to kinetic measurements) for the infusion of Met.

All goats were allowed free access to rations comprising fixed proportions (60:40, wt/wt) of an air-dried pelleted concentrate and molassed hay (10% air dry) [79 g of metabolizable protein (**MP**), calculated from relationships described by the Agricultural and Food Research Council (3, 4), and 11.9 MJ of metabolizable energy (**ME**)/kg of DM (89%)]. Daily orts of concentrate and hay were recorded separately. In Experiment 1, the concentrate provided 129 g of MP and 12.9 MJ of ME/kg of DM (89%) and consisted of 395 g/kg of barley, 150 g/kg of whole maize, 150 g/kg of wheat feed, 150 g/kg of soybean meal, 50 g/kg of white fish meal, 50 g/kg of linseed flakes, and a vitamin and mineral premix (Norvite Feed Supplies, Insch, United Kingdom). In Experiment 2, the concentrate provided 124 g of MP and 12.1 MJ of ME/kg of DM (85%) and consisted of 450 g/kg of corn gluten meal (19% CP), 150 g/kg of barley, 150 g/kg of wheat feed, 90 g/kg of prairie meal (corn gluten feed; 60% CP), 70 g/kg of beet pulp, and a vitamin and mineral premix (Norvite Feed Supplies). The proportions of concentrate and molassed hay consumed varied by goat; however, the daily intakes of MP (Table 1) and ME (data not shown) were sufficient to meet the daily recommended intakes (2, 4) needed to support maintenance and the lactational demands of each goat.

Goats were housed in metabolic crates and were allowed a 10-d period to adapt to the daily routines of machine-milking and hand milking (0830 and 1630 h) and frequent feeding (every 2 h by autofeeders). Milk weights were recorded, and subsamples were taken at each milking during the 2 to 5 d prior to isotope infusion. Subsamples were preserved with 0.1 ml of formaldehyde (40%, vol/vol) and stored at 4°C until analyzed for total N (macro-Kjeldahl method).

The CP content in milk was calculated as milk N × 6.38. Data for the milk yield of each goat are presented in Table 1.

Experimental Protocols

Experiment 1. This experiment with two goats established the appropriate intravenous (jugular) infusion rate of the U-^{13}C-labeled AA mixture to enrich AA in arterial blood to concentrations that permitted analyses with suitable accuracy and precision by gas chromatography-mass spectrometry (**GC-MS**). Goat 19b received a nonprimed, continuous infusion and goat 24b received a primed, continuous infusion. The isotopic enrichments of each AA in arterial blood were monitored.

The goat that received the nonprimed infusion also received the U-^{13}C-labeled AA mixture for 6 h at 65% of the rates reported for Experiment 2. During the last 4 h, at 30-min intervals, blood samples were withdrawn from the arterial catheter. Goat 24b received a priming dose (equivalent to the 1-h rate reported in Experiment 2) at the beginning of the experiment and then the same continuous infusion rate as that for goat 19b for 4 h. Blood samples were taken at 20-min intervals throughout this infusion. On all occasions, the goats were standing for at least 5 min prior to and during blood withdrawal.

Samples were dispensed into ice-cold tubes containing heparin (40 IU). Aliquots (1 g) were hemolyzed with an equal volume of ice-cold distilled water and stored at –20°C until GC-MS analyses. Immediately prior to commencing isotope infusion, the mammary glands were milked-out by machine and by hand following an initial intrajugular dose of oxytocin (1 IU) and then again by hand at 1-h intervals (without oxytocin) throughout the infusions. Milk casein was isolated to determine the extent of ^{13}C incorporation. To determine the natural abundance of AA, blood and milk casein samples were taken at least 24 h prior to isotope infusion.

Experiment 2. This experiment examined the kinetics of the U-^{13}C-labeled AA mixture across one-half of the udder of three lactating goats. Each goat was fed a ration containing a concentrate based on corn gluten meal and was supplemented with an intrajugular infusion of Met (1.1 g/d for 5 d). Because rations based on corn protein are typically colimiting in Lys and Met, the Met was infused to eliminate its limiting status and to enhance the limiting status of Lys. On d 5 of Met infusion, each goat received a 6-h nonprimed, continuous infusion of the U-^{13}C-labeled AA mixture. The rate of isotope infusion was increased by 50% to ensure that the enrichments of AA in blood reached a satisfactory concentration for GC-MS analyses. The infusate also contained heparin (200 IU/g of infusate) as an anticoagulant to facilitate continuous blood withdrawal. Over the last 5 h of the tracer infusion, a solution of *p*-aminohippurate (***p*-AH**; 35 mg/g of solution) was infused (20 g of solution/h) into an external pudic artery ipsilateral to the mammary vein catheter to determine the mammary blood flow (**BF**) by downstream dilution. Over the last 4 h of infusion, arterial and mammary venous blood samples (9 ml/30 min) were withdrawn continuously by a proportioning pump. Samples were

TABLE 1. Daily feed intakes, milk yield, and blood flow (BF) in the mammary gland of individual goats in Experiments 1 and 2.

	Experiment 1		Experiment 2		
	Goat 19b	Goat 24b	Goat 3c	Goat 4c	Goat 6c
BW, kg	56	56	76.5	82	54.5
DIM	213	189	109	114	120
DMI, kg	2.55	1.91	3.10	1.57	1.20
MP Intake,[1] g	288	206	356	186	133
MP Requirement,[1] g	141	133	216	125	136
Milk yield,[2] kg/d	2.22	1.94	3.68	1.67	2.10
CP Yield,[3] g/d	75	61	110	52	75
BF,[4] kg/min	. . .	. . .	1.258	0.684	0.650
SE			0.034	0.034	0.052
BF:Milk yield	. . .	. . .	493:1	591:1	446:1

[1]Metabolizable protein (MP) intake and requirements were calculated from relationships described by the Agricultural and Food Research Council (3, 4).

[2]Values represent the means during the 5 d preceding isotope infusion.

[3]Calculated as milk N × 6.38.

[4]Blood flow in one-half of the mammary gland was converted to BF in the total mammary gland based on the ratio of BF to milk yield of the udder halves.

collected into sealed syringes and were submerged in an ice bath. Goats remained standing throughout the blood sampling period.

Blood samples were thoroughly mixed by gentle hand rolling, and the packed cell volume (**PCV**) was then determined by hematocrit. For *p*-AH determination, two samples (~0.5 g, accurately weighed) were diluted by the addition of an equal weight of ice-cold distilled water. Four further samples (~1 g, accurately weighed) were mixed with an equal weight of hemolyzing solution containing L-norleucine (200 μM). The remaining heparinized blood was centrifuged at 2000 × *g* at 4°C for 15 min to harvest plasma. Four plasma samples (~0.7 g, accurately weighed) were mixed with equal weights of the hemolyzing solution. Blood and plasma samples that were used to determine natural abundance were taken at least 24 h prior to isotope infusion. All samples were kept frozen at –20°C until analyzed.

Analytical Methodology

Gravimetric procedures were used to quantify blood *p*-AH concentrations assayed by the Bratton-Marshall procedure as described previously (36). Free AA concentrations in blood and plasma samples were determined by reverse-phase HPLC as described previously (24). Casein was isolated from milk samples and hydrolyzed; AA were separated by ion-exchange chromatography as described previously (8).

Carbon-13 enrichments of AA in blood, plasma, and casein were determined by GC-MS under electron impact or chemical ionization conditions as reported previously (15, 27, 28, 29). During the derivatization process, Gln and Arg are hydrolyzed to Glu and Asp, respectively, which then appeared with lower enrichments. Glutamic acid and Asp were excluded from the present results. During GC-MS determinations, a contaminant coeluted with Met (venous samples only); therefore, arteriovenous isotope exchanges of Met were not computed. Chiral column analysis of the AA mixture indicated the presence of considerable ($\overline{X}$ = 22%) D-Lys, occurring either as a natural product of the algal biomass or as a consequence of acid hydrolysis conditions. The D-isomer of the other AA represented less than 4%. L-Lysine enrichments were determined by chiral column separation by GC-MS.

The GC-MS determinations monitored only fragment ions that contained all of the carbon atoms of the AA (27). Only the m + 0 and m + n ions, where n is the number of carbon atoms in the molecule, were monitored. Therefore, the molar percentage excess could not be calculated. Rather, values represented the relative enrichments of (m + n)/[(m + 0) + (m + n)]. The calculation was based on the following equation:

$$(R_s - R_0)/[1 + (R_s - R_0)]$$

where R_s and R_0 are the ratios of (m + n) to (m + 0) ions in an enriched and natural abundance sample, respectively (16). Because greater than 99% of the molecules in the AA mixture that was obtained from hydrolysis of algal proteins were in the m + n form (all carbon atoms were fully labeled), the relative enrichment of essential AA would approximate the molar percentage excess. For the nonessential AA, this result might not be apparent because the m + n species can be formed in the body from other AA or metabolites in the algae, and molecules with fewer than n labeled ^{13}C atoms may also arise (11).

Calculations

The AA fluxes in blood (F_b) and plasma (F_p) were calculated from the relative enrichment of free AA in arterial blood or plasma at a plateau (assessed by visual inspection); corrections were made to accommodate the infusion of nontracer quantities of AA, including an allowance for the presence of the D-isomer (Lys only). Values were then normalized to the daily MP intake and metabolic BW (kilogram$^{0.75}$): $F_{b\ or\ p}$ (millimoles per kilogram of MP intake per kilogram of BW$^{0.75}$) = [((0.99/$E_{b(p)}$) – 1) × I × 24]/(kilograms of MP × kilograms of BW$^{0.75}$), where $E_{b\ or\ p}$ is the relative enrichment of the free AA in arterial plasma (p) or blood (b), and I is the rate of infusion (millimoles per hour) of the L-U-^{13}C-labeled AA mixture. The daily intakes of MP and the BW of each goat are presented in Table 1. For Experiment 2, infusion rates for individual L-U-^{13}C-labeled AA were 0.015 mmol/h for His, 0.061 mmol/h for Thr, 0.048 mmol/h for Arg, 0.066 mmol/h for Val, 0.015 mmol/h for Met, 0.042 mmol/h for Ile, 0.101 mmol/h for Leu, 0.045 mmol/h for Phe, 0.102 mmol/h for Lys, 0.057 mmol/h for Ser, 0.130 mmol/h for Ala, 0.050 mmol/h for Pro, and 0.024 mmol/h for Tyr. Infusion rates for Experiment 1 were 65% of these values.

The concentration of AA in the packed blood cells (C_{pcv}; erythrocytes, leukocytes, and reticulocytes), per unit volume, was calculated as

$$[C_b - C_p \times (1 - PCV)]/PCV$$

where $C_{b\ or\ p}$ is the concentration (micromoles per kilogram) of the AA in arterial blood (b) or plasma (p).

The relative enrichment of AA in the arterial packed cells was calculated similarly:

$$[(C_b \times E_b - C_p \times E_p \times (1 - PCV))/PCV]/C_{pcv}.$$

The BF (kilograms per minute) for one-half of the udder was measured by close arterial (external pudic artery) infusion of *p*-AH and measurement of the downstream dilution of *p*-AH in the mammary vein loop. Milk yield was not different between the gland halves; therefore, BF was doubled to yield total mammary BF.

Mass transfers (MT) across the mammary gland of the unlabeled AA under examination and of the isotope (^{13}C AA) from blood (b) or plasma (p) were calculated (micromoles per minute) as

$$MT_b = (C_{a(b)} - C_{v(b)}) \times BF$$

and

$$MT_p = (C_{a(p)} - C_{v(p)}) \times BF(1 - PCV).$$

Isotope transfer (IT) from blood (b) and plasma (p) were calculated as

$$IT_b = (C_{a(b)} \times E_{a(b)} - C_{v(b)} \times E_{v(b)}) \times BF \times 1000$$

and

$$IT_p = (C_{a(p)} \times E_{a(p)} - C_{v(p)} \times E_{v(p)}) \times BF(1 - PCV) \times 1000$$

where the subscripts a and v represent arterial and mammary venous blood. Mass transfers of unlabeled AA were balanced against their calculated outputs into milk protein, assuming 92% of milk AA N is synthesized in the mammary gland (20) and based on the AA composition of goat casein (D. Brown and C. E. Kyle, 1996, personal communication) [1.23 mmol/g of His, 2.78 mmol/g of Thr, 1.12 mmol/g of Arg, 4.15 mmol/g of Val, 1.29 mmol/g of Met, 2.55 mmol/g of Ile, 4.73 mmol/g of Leu, 2.04 mmol/g of Phe, 3.49 mmol/g of Lys, 3.18 mmol/g of Ser, 1.96 mmol/g of Ala, 6.79 mmol/g of Pro, and 1.60 mmol/g of Tyr].

Unidirectional AA fluxes in the mammary gland (F_{mg}) were calculated from plasma isotope transfers [transfer of the mass of AA under examination and the isotope (^{13}C AA) transfers in blood and plasma did not differ; $P > 0.05$] and were converted to AA flows assuming the corresponding relative enrichment of the arterial plasma was representative of the exchanging intravascular precursor pool and that the unlabeled AA were utilized by the tissue in a manner similar to the U-^{13}C-labeled AA mixture:

$$F_{mg} \text{ (micromoles per minute)} = [(C_{a(p)} \times E_{a(p)} - C_{v(p)} \times E_{v(p)})/E_{a(p)}] \times BF(1 - PCV).$$

Partition of plasma flux to the mammary gland (K_{mg}) (i.e., the proportion of plasma flux irreversibly removed from the plasma pool by the mammary gland) was calculated as

$$K_{mg} = F_{mg}/F_p.$$

Statistics

The current data file was limited by the number of goats and the large differences among goats in some parameters, especially ^{13}C enrichments of blood and plasma that were caused by differences in the rate of isotope infusion. To overcome these problems, ratios of parameters (e.g., ratios of blood to plasma) were computed, and tests of statistical difference from unity were assessed using Student's *t* distribution (one- or two-sided, whichever was most appropriate).

RESULTS

Goats

All goats consumed sufficient quantities of the ration to meet or exceed the calculated MP and ME requirements for maintenance (data not shown) plus requirements for milk yield (Table 1). In Experiment 1, the goat administered the nonprimed, continuous infusion showed no signs of reaction to infusate, but the goat administered the primed, continuous infusion coughed periodically and refused portions of each meal provided at 2-h intervals. In Experiment 2, all goats consumed meals immediately and showed no reaction to the nonprimed infusion of the isotope mixture. The ratios of mammary BF to milk yield for goats in Experiment 2 were within the expected range of values reported for goats at a similar stage of lactation and similar level of milk yield (34).

Experiment 1

Isotopic plateaus for individual AA in blood were achieved within 1 to 4 h for the goat that received the nonprimed infusion (Figure 1) and within 20 to 40

min for the goat that received the primed infusion. Individual AA enrichments in arterial blood varied over a fivefold range (0.15 to 0.7 relative enrichment), indicating that the AA composition of the mixture derived from infused algal proteins differed considerably from the proportional fluxes of AA in goats. Illustrated in Figure 1 are the temporal labeling patterns of His, Ile, Pro, and Tyr in arterial blood and in casein from goat 19B. Labeling patterns were similar for the other goat and the other AA monitored. By 1 to 2 h of infusion, ^{13}C could be detected in all AA secreted in milk casein, and, throughout the 4- or 6-h infusion period, incorporation continued to increase in a nearly linear fashion. Relative enrichment of the U-^{13}C-labeled AA in casein at 4 or 6 h of infusion attained only 30 to 100% of the plateau blood value.

The AA flux in blood and estimated AA flux in plasma for each goat are listed in Table 4. Standardization of the flux values according to MP intake and metabolic body size (kilograms of $BW^{0.75}$) greatly reduced variability among goats. Thus, blood and plasma fluxes of His and Met were lowest, and blood and plasma fluxes of Ala, Leu, and Ile were highest.

Experiment 2

Concentrations and relative enrichments of AA. A comparison of the AA concentrations in arterial plasma and packed cells (calculated) and the relative enrichments during infusion of the U-^{13}C-labeled AA mixture is shown in Table 2. Concentrations of His, Thr, Arg, Met, Phe, and Lys were significantly higher in packed cells than in plasma. And, except for Ile and Ser, relative enrichments in blood were always lower than those in plasma. Very little of the U-^{13}C-labeled AA was detected in the packed cells; thus, for all AA, relative enrichments in packed cells were significantly lower than those in plasma. The negative value reported for His enrichment in the packed cells was likely due to a contaminant that coeluted during either the AA analysis or during GC-MS determinations on blood samples.

Flux of AA in plasma. All flux calculations were based on enrichments in plasma that were caused by the incomplete equilibration of the ^{13}C AA mixture and, therefore, the uncertainty of the exchanges by erythrocytes in blood. Values for each goat are reported in Table 4. Values were slightly different

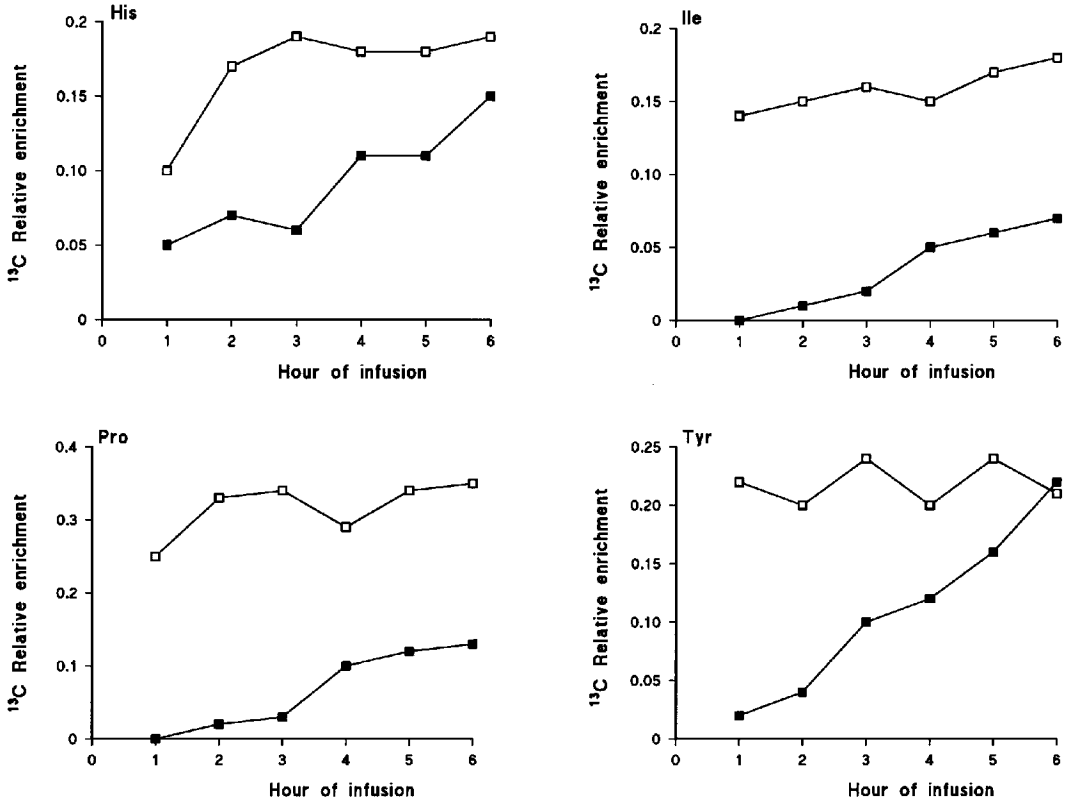

Figure 1. Comparison of the temporal labeling patterns of His, Ile, Pro, and Tyr in arterial blood (□) and in secreted milk casein (■) during a 6-h nonprimed, continuous intravenous infusion of a U-^{13}C-labeled AA mixture in one lactating goat (goat 19b; Experiment 1).

from those found in Experiment 1 (plasma fluxes of Met and Tyr were the lowest, and those of Leu and Ala were the highest). However, the most notable differences were the much higher plasma fluxes of Val, Met, Ile, Leu, and Tyr and the lower flux of His in plasma in Experiment 1.

Flux of AA in the mammary gland. Transfer of the net mass of AA under examination and that of the isotope (U-^{13}C-labeled AA) in blood and plasma across the mammary gland are presented in Table 3. Except for isotope transfers of Arg (blood greater than plasma; $P < 0.05$), there were no differences in the net mass and isotope transfers from blood and plasma. This result suggested that neither mass nor the U-^{13}C-labeled AA were exchanged via packed cells. Mammary gland partition coefficients, based on plasma AA exchanges and calculated from mammary gland unidirectional and plasma fluxes, are presented in Table 4. Although the mean coefficient for mammary gland partitioning of AA differed by goat (goat 3c, 0.29; goat 4c, 0.18; and goat 6c, 0.26), the order that AA were partitioned to the mammary gland was highly conserved. Histidine and Phe always had the lowest partition coefficient, and Ile, Val, and Lys always had the highest values. Values of unidirectional flux of AA in the mammary gland and output of AA into milk protein were standardized to the Lys fluxes for comparisons of unidirectional fluxes and milk AA output. Fluxes of Arg and Pro and those of Phe and Tyr were summed to reflect their common metabolic pathways. These relationships are illustrated in Figure 2. Thus, in Figure 2, AA to the left of Lys (Arg and Pro, Phe and Tyr, His, and Thr) reflect those AA that have a lower nonmilk protein metabolic flux, and those to the right (Val, Ile, and Leu) reflect a greater or additional nonmilk protein metabolic flux than that of Lys. For all goats, net mass transfers of AA from plasma were always less than the AA output in milk for His, Met, Phe, Ser, Pro, and Tyr and greater than the AA output in milk for Ala and Arg (Table 3).

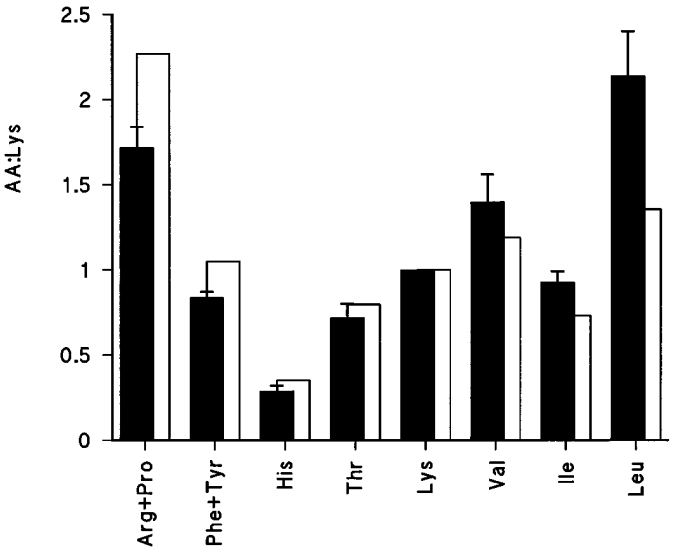

Figure 2. Comparison of the relationships between unidirectional fluxes of AA in the mammary gland (■) and AA output in milk (□) in Experiment 2. Values were standardized to fluxes and milk outputs of Lys. Bars for unidirectional fluxes represent the standardized mean (±SE) of the mean.

DISCUSSION

Present schemes for feeding protein to dairy ruminants (2, 3, 4, 26, 33) attempt to predict the absorbable protein or MP supply to the small intestines and the protein requirements for maintenance, growth, and milk protein output based on a combination of empirical and factorial data. With one exception (26), these schemes assume a series of fixed efficiencies for directing AA toward the various functions in the whole animal [1.0 for maintenance, 0.59 (variable) for growth, and 0.64 to 0.70 for lactation], despite a volume of literature that demonstrates that marginal milk protein responses to AA supply are much lower than these values [~0.2; (22, 32)], which is probably dependent on the physiological stage of lactation (early vs. late, i.e., negative N balance vs. positive N balance), interactions between dietary protein and energy sources and supplies, and the pattern of the AA absorbed and supplied to tissues. However, protein and its metabolism are referred to generically because the AA pattern of the absorbed protein and functional requirements for the individual AA in tissues are largely unknown. In the present study, we attempted to provide a metabolic description of the ration consumed by lactating goats by monitoring the metabolic partition of 13 AA simultaneously and equating these kinetic fluxes to the intake of MP as predicted from the current protein feeding system described by the Agricultural and Food Research Council (3, 4).

Methodological Considerations

The U-^{13}C-labeled AA mixture was originally used as an oral tracer to determine the essentiality of dietary AA in chickens (11) and humans (12), and, recently, the algal biomass has been processed to yield a free AA mixture that is suitable for intravenous infusion into growing sheep to monitor the kinetics of AA exchange across the gastrointestinal tract (J. C. MacRae, 1997, unpublished data) and liver (27). The major advantages of the U-^{13}C-labeled

AA mixture include the ability to monitor a large number (n = 16) of AA simultaneously (27), the substantial gains in mass spectrometric sensitivity associated with monitoring > m + 1 ions, and the consequent lower (10-fold) required rates of mass isotope infusion, which reduce isotope costs (27) and reduce the potential for substrate (metabolic) effect from the infused isotope (i.e., when nontracer quantities are infused).

The U-^{13}C-labeled AA are produced by growing the phototrophic microalgae in an atmosphere of $^{13}CO_2$. Thus, because >99% of all carbon atoms of every AA are enriched with ^{13}C, it is not possible to determine the oxidative fate of individual AA. For the nonessential AA that are derived from ^{13}C-enriched AA (e.g., Phe → Tyr, Gly ↔ Ser), their flux rates in plasma and mammary gland will be underestimated. In the present analyses, measurements of the kinetic fluxes of Met in the mammary gland were not possible because of an unidentified coeluting substance found only in the venous plasma during GC-MS determinations; however, this problem can be overcome by supplementing the U-^{13}C-labeled AA mixture with a lower mass isotope (<m + 5) of Met (e.g., [$^{13}CH_3$]-Met, [1-$^{13}CO_2$]-Met, or [CD_3]-Met).

Except for Ile and Ser, free AA enrichments in blood were always lower than those in plasma, for several reasons. Isotope is continuously infused into the plasma compartment, reaching a pseudoplateau when the rate of isotope exit (into blood cells and extravascular tissue pools) from the plasma equals the rate of infusion; thus, a permanent differential exists between packed cells (>90% erythrocytes) and AA pools in plasma. We observed (calculated) limited or no entry of isotope into the packed cells (Table 2), which could have occurred because of the higher AA concentrations (calculated) found in the packed cells than in plasma, thus limiting AA entry. The AA gradient could be the result of in situ synthesis [nonessential AA only; (19)], peptide and protein hydrolysis (1, 5, 35), or uptake of AA by the erythrocytes upon passage through tissues. In the present study, the ratio of packed cells to differences in plasma concentration and enrichment were particularly large for His, Thr, Lys, and Arg. Lobley et al. (27) also reported these differences in blood from growing sheep, except for Arg, which, because of ex-

TABLE 2. Concentrations of AA and relative enrichments of AA in arterial plasma and packed cells of three lactating goats fed a ration based on corn gluten meal and administered a continuous intravenous infusion of a U-^{13}C-labeled AA mixture (Experiment 2).

	Concentration of AA[1]				Relative enrichment of AA					
AA	Plasma	Packed cells	Plasma:packed cells[2]		Plasma	Blood:plasma[3]		Packed cells	Packed cells: plasma[3]	
	(μM)				(×10^2)					
			$\bar{X}$	SE		$\bar{X}$	SE		$\bar{X}$	SE
Essential										
His	44.3	112.3	0.47*	0.12	0.49	0.46**	0.04	–0.30[4]	. . .	. . .
Thr	37.6	80.1	0.48*	0.06	1.39	0.68**	0.02	0.16	0.09**	0.06
Arg	90.5	334.2	0.27**	0.01	0.91	0.52**	0.04	0.01	0.06**	0.01
Val	142.1	112.0	1.65	0.80	1.16	0.94**	0.01	0.79	0.63*	0.10
Met	27.5	51.4	0.54*	0.08	1.12	0.80*	0.06	0.49	0.45*	0.15
Ile	61.7	56.1	1.15	0.10	0.91	0.96	0.02	0.56	0.59*	0.08
Leu	159.9	155.9	1.03	0.20	0.95	0.86*	0.05	0.30	0.35*	0.05
Phe	33.0	72.1	0.46**	0.03	1.20	0.91*	0.02	0.85	0.72*	0.04
Lys	65.2	165.3	0.40**	0.03	2.31	0.61**	0.04	0.02	0.04**	0.03
Nonessential										
Ser	62.4	78.6	0.96	0.22	0.62	0.83	0.08	0.18	0.32*	0.17
Ala	110.0	124.0	1.03	0.21	0.87	0.81*	0.03	0.002	0.08**	0.04
Pro	144.2	240.5	0.62	0.13	1.21	0.69*	0.05	0.01	0.05**	0.04
Tyr	52.9	65.7	0.85	0.23	1.16	0.77*	0.04	0.03	0.07**	0.06

[1]Concentrations of AA in plasma and packed cells were calculated on a volume equivalent basis.

[2]Significant differences between concentrations of AA in plasma and packed cells were assessed by testing the ratio of plasma to packed cells = 1 using a two-tailed *t* test.

[3]Significant differences between relative enrichments of AA in blood and plasma or packed cells and plasma were assessed by testing the latter ratios <1 using a one-tailed *t* test.

[4]The negative value for His might have been due to an unidentified contaminating substance that appeared during the AA or mass spectrometric analyses of blood.

*$P < 0.05$.

**$P < 0.005$.

TABLE 3. Comparison of net mass and isotope (U-^{13}C-labeled AA) transfers of AA in plasma and blood across the mammary gland of three lactating goats fed a ration based on corn gluten meal (Experiment 2).

AA	Net mass transfer[1]						Isotope transfer[2]			
	Plasma	Blood	Blood:plasma		U/O[3]		Plasma	Blood	Plasma:blood	
	(μmol/min)						(nmol/min)			
			$\overline{X}$	SE	$\overline{X}$	SE			$\overline{X}$	SE
Essential										
His	7.0	7.5	1.00	0.19	0.81	0.05	33	27	1.27	0.28
Thr	18.2	17.5	0.93	0.03	0.88	0.12	229	223	1.03	0.03
Arg	22.2	22.4	0.98	0.08	2.89	0.02	163	168*	0.97	0.01
Val	39.9	37.5	0.95	0.08	1.35	0.22	354	375	0.96	0.09
Met	5.7	6.8	1.17	0.20	0.73	0.11	ND[4]	ND	. . .	. . .
Ile	25.3	24.6	0.95	0.04	1.39	0.17	186	185	1.02	0.04
Leu	56.8	57.2	0.98	0.04	1.68	0.28	427	422	1.02	0.02
Phe	9.5	11.0	1.11	0.17	0.67	0.06	114	132	0.90	0.11
Lys	24.5	24.4	0.97	0.04	1.00	0.08	521	551	0.98	0.09
Nonessential										
Ser	–11.5	–10.9	–0.20	1.09	–0.56	0.30	99	84	1.15	0.11
Ala	17.5	21.6	1.14	0.18	1.32	0.03	290	289	1.02	0.06
Pro	26.4	23.4	0.89	0.08	0.51	0.09	241	238	1.01	0.03
Tyr	9.5	9.4	0.95	0.07	0.83	0.07	109	111	1.02	0.10

[1]Significant differences between net mass transfers of AA in plasma and blood were assessed by testing the ratio of blood to plasma = 1 using a two-tailed *t* test. Net mass transfers of AA in plasma and blood did not differ ($P > 0.05$).

[2]Significant differences between isotope transfers in plasma and blood were assessed by testing the ratio of plasma to blood <1 using a one-tailed *t* test.

[3]Net mass transfer of plasma AA divided by the calculated output of AA in milk (N × 6.38), assuming that 92% of milk AA N is synthesized in the mammary gland (20).

[4]Not determined for Met because of a contaminating peak found in venous samples during the gas chromatography-mass spectrometric analyses.

*$P < 0.05$.

TABLE 4. Comparison of AA fluxes in blood (F_b) and plasma (F_p) (millimoles per kilogram of metabolizable protein intake per kilogram of BW$^{0.75}$) for goats in Experiments 1 and 2 and the proportion of F_p partitioned to the mammary gland (K_{mg}).

AA	Experiment 1[1]				Experiment 2					
	Goat 19b		Goat 24b		Goat 3c		Goat 4c		Goat 6c	
	F_b	Estimated F_p	F_b	Estimated F_p	F_p	K_{mg}	F_p	K_{mg}	F_p	K_{mg}
Essential										
His	18.7	8.6	18.0	8.3	12.8	0.13	14.2	0.10	17.4	0.19
Thr	42.9	29.2	50.7	34.4	17.1	0.34	20.1	0.17	23.9	0.25
Arg	ND[2]	ND	ND	ND	22.6	0.23	26.7	0.15	26.6	0.29
Val	53.3	50.1	52.3	49.1	23.6	0.42	30.5	0.30	27.3	0.34
Met[3]	17.8	14.2	15.7	12.6	6.0	ND	5.0	ND	7.1	ND
Ile	92.5	88.8	90.6	87.0	19.5	0.34	22.2	0.24	23.3	0.31
Leu	77.7	66.8	81.8	70.4	48.6	0.33	57.6	0.23	48.3	0.29
Phe	20.4	18.6	23.5	21.3	14.8	0.19	18.7	0.13	19.0	0.18
Lys	32.7	20.0	48.0	29.3	17.0	0.37	20.6	0.27	23.1	0.40
Nonessential										
Ser	59.6	49.4	56.1	46.5	28.3	0.19	38.0	0.05	62.0	0.11
Ala	76.4	61.9	77.3	62.6	52.5	0.18	67.2	0.10	88.0	0.16
Pro	60.8	42.0	46.5	32.1	19.4	0.38	20.6	0.24	22.4	0.29
Tyr	24.1	18.6	22.1	17.1	8.4	0.34	10.7	0.20	12.5	0.29

[1]The F_b values were converted to F_p from the relative enrichment of AA determined in Experiment 2 and presented in Table 2 as the ratio of blood AA to plasma AA.

[2]Not determined.

[3]Because of an unidentified contaminating peak in venous plasma during mass spectrometric determinations for Met, K_{mg} was not computed.

tensive arginase activity in the erythrocytes that were released during laking of blood, was barely detectable in whole blood samples. By employing laking procedures that were similar to those of Lobley et al. (27) and employing those in the present study, Metcalf et al. (32) were also unable to detect Arg in whole blood samples from dairy cows. It is unknown whether this inability represents a species difference in arginase activity, which could either be less active or even nonexistent in the goat.

An alternative explanation for the differences in the ratio of packed cells to plasma enrichment and concentration might be related to the uptake of AA by the erythrocytes, which are the product of intracellular protein turnover in the tissues as the blood perfuses through the tissue capillary beds. However, based on data in other species (dogs, sheep, and cattle) across the gastrointestinal tract, the consensus is for the exchange and transport of AA mainly via plasma; however, across the hepatic tissues, despite earlier conflicting reports (21, 25), recent data in sheep (27) indicate that only Ile and Pro are exported by erythrocytes. Comparison of mass and isotopic transfers in blood versus plasma across the mammary gland (Table 3) yields no indication that erythrocytes participate in AA exchange with the mammary gland of a lactating goat. This result is in conflict with observations of the dairy cow for which several net arteriovenous differences in AA concentration in blood versus plasma suggested that erythrocytes carry AA to and from the mammary gland (23). In the present study using goats, however, based on net mass and isotope exchanges and the incomplete equilibration of isotope with the packed cells, only exchange kinetics in plasma were considered relevant for examining the kinetics of intravascular AA.

An important consideration in the application of tracer techniques is that the tracer AA mixes with, and is indistinguishable from, the unlabeled AA pool under consideration. In the current investigation, although net mass and isotope exchanges with the mammary gland via the packed cells did not occur, it is apparent from previous studies (6, 13, 20, 22, 32) of arteriovenous balance across the mammary gland, which the current data supports (Table 3), that plasma-free AA uptake does not adequately account for the output of several essential AA (His, Phe, Met, and Thr) into milk protein. Indeed, direct and indirect evidence now supports a role for peptides or proteins in the supply of AA for milk protein synthesis by the mammary gland of the lactating goat (5, 6). The infused tracer AA mixes completely only with the plasma-free AA pool and, therefore, represents only exchanges and the kinetics of plasma-free AA, which, because of the potential utilization of peptides and proteins by the goat mammary gland (5, 6), underestimates the true AA exchange rate.

In Experiment 1, the ^{13}C labeling kinetics of casein AA secreted in milk were monitored during the 4- or 6-h infusions. For many casein AA, incorporation of ^{13}C could be detected by GC-MS at the 1-h sample, and, during the 4 or 6 h of infusion, ^{13}C incorporation continued to increase (Figure 1). These findings are in direct agreement with those in a previous study in this laboratory (8) that compared the labeling kinetics of Leu, Val, Phe, and Met in blood and casein of goats. Thus, the time from casein synthesis to secretion is short (<1 h) with small intermediary storage pools of casein. In addition, the temporal labeling pattern of the 13 AA in casein suggests that the channeling phenomenon originally proposed in our earlier study occurs generally for all AA.

Plasma Fluxes and Mammary Gland Kinetics of AA

Recent studies have highlighted the limitations of using a single AA as a tracer for the whole body metabolism of other AA and protein (17, 18, 27). To circumvent these problems, we simultaneously compared the labeling kinetics of three AA (Leu, Phe, Val, or Met) in blood, plasma, and casein in goats during early and late lactation (6, 8), and, in the current study, we have substantially improved the measurements of the rates of plasma flux for 13 AA.

Rates of AA flux in plasma (Table 4) were standardized to MP intake and metabolic body size to provide a metabolic description in relation to protein intake and to create a reference for comparisons across rations and between goats that differed in body size and, thus, overall metabolic capacity. Although the number of observations ($n = 2$ and $n = 3$) in the two experiments was limited, the data for AA flux in plasma were fairly consistent within the experiment, which provided some basis for comparisons. In Experiment 1, the two goats that consumed the ration containing soybean meal and fish meal had higher (estimated) flux rates for Leu, Ile, Val, Met, and Tyr in plasma, and a lower flux rate for His than did the three goats in Experiment 2 that consumed the ration containing corn gluten meal. If these observations can be confirmed in further comparisons, then the implications are that, despite the similar MP content of the two rations, metabolic utilization differed. Moreover, current protein feeding systems (2, 3, 4, 26, 33) may now need to consider that all MP or absorbable protein intake, regardless of dietary protein contributions (e.g., soybean meal and fish meal vs. corn gluten meal proteins) or microbial protein contributions, is not metabolized or utilized in the same way (i.e., according to a single, fixed efficiency). For in-

stance, if these differences in plasma AA flux between rations (irreversible loss into protein synthesis and catabolism) relate to a change in the availability of AA for milk protein synthesis, partition coefficients could be altered, particularly if a limiting AA is involved.

The partition of plasma AA flux to the mammary gland (Table 4) varied among AA. The lowest proportinal contributions were for His (0.10 to 0.19) and Phe (0.13 to 0.19), and the highest contributions were for Lys (0.27 to 0.40) and the branched-chain AA (Leu, Ile, and Val) (0.23 to 0.42). Although the overall AA partitioning varied among goats, the relationships among AA were similar. One component of flux is oxidative disposal, and, interestingly, whole body fractional oxidation rates of AA in the dairy cow appeared to maintain fixed relationships among AA (14). If these observations can be extended and are found to be unaltered by stage of lactation, milk yield, or quantity and quality of feed intake, then perhaps the use of a single reference AA may be justified, provided that information is available on the anabolic and catabolic fates of the various AA at the different tissue levels. In the dairy cow, partition of plasma Leu to the mammary gland does not appear to change with protein intake [0.40 vs. 0.42; (9)]. However, in that study, the partitioning of Leu within the mammary gland between anabolic and catabolic fates did not remain constant. Leucine fractional oxidation increased as protein intake increased [(0.047 vs. 0.136; (10)], although studies of goats showed an increase in Leu oxidation as stage of lactation advanced [0.083 vs. 0.336; (34)]. Furthermore, a study from our laboratory using lactating goats (9) suggested that the arterial supply of Leu relative to other AA and, therefore, the extent of Leu availability (limitation or excess) could also influence Leu oxidation. If this relationship exists for other AA, then the ability to predict milk protein output by the gland may require, in addition to an estimate of the mammary gland partition coefficient, a measure of the relative availability of AA for milk protein synthesis versus other potentially competitive processes (constitutive protein and nonessential AA synthesis, oxidation, and metabolite formation) in the mammary gland.

In the present study, we compared the unidirectional fluxes of AA across the mammary gland to their secretion in milk protein as an indication of the relative metabolic availability of AA within the mammary gland (Figure 2). To provide a reference for comparisons among AA, values were standardized against Lys, which was assumed to be first-limiting under the feeding and infusion conditions established in the present study, and, thus, any deviations from the Lys relationship would indicate fewer or additional fluxes of an AA compared with Lys flux and its obligatory metabolic relationship with milk protein synthesis. Fluxes of Arg and Pro and those of Phe and Tyr were summed in this comparison to reflect the relationships in their metabolism by the gland, but the other nonessential AA were excluded because of transfer of ^{13}C among AA, which would lead to an underestimation of their fluxes. According to this comparison, Arg and Pro, Phe and Tyr, and probably His had fewer metabolic requirements for nonmilk protein synthesis (turnover), oxidation, and metabolite formation than did Lys, although metabolic fluxes of the branched-chain AA were greater. Essentially, however, all of the AA might have additional metabolic fluxes, which, if not crucial to the milk protein synthetic process, and there is evidence that Leu oxidation by the gland is not obligatory (9, 10), could become competitive with milk synthesis, particularly if limiting conditions prevailed (e.g., negative nutrient balances in early lactation, reduced feed intake, and limiting AA situations).

CONCLUSIONS

This study employed a U-^{13}C-labeled AA mixture that was used as a tracer to acquire simultaneous estimates of AA flux in plasma and partition of AA to the lactating mammary gland. Preliminary results indicated that plasma flux rates differ considerably among AA and that these AA are partitioned to the mammary gland in varying proportions (0.05 to 0.42). Within the mammary gland, the partition of AA between milk protein synthesis and other metabolic functions differs by AA. This information and the results of others (27; J. C. MacRae, 1997, unpublished data) could be incorporated into the current protein feeding systems for dairy ruminants to provide a better description of the metabolic availability and partition of AA for milk protein synthesis in response to MP or absorbable protein intake. Perhaps then we will be able to predict responses in milk protein output from changes in nutrient availability.

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