Vascular Sources of Phenylalanine, Tyrosine, Lysine, and Methionine for Casein Synthesis in Lactating Goats

B. J. BEQUETTE,* F.R.C. BACKWELL,* C. E. KYLE,* A. G. CALDER,* V. BUCHAN,* L. A. CROMPTON,† J. FRANCE,† and J. C. MACRAE*

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

†The University of Reading, Department of Agriculture, Reading RG6 2AT, England

ABSTRACT

The contribution to casein biosynthesis of peptides derived from blood was examined in late lactation goats (254 to 295 d in milk). Ratios of mammary uptake of free amino acids (AA) in blood to output of AA in milk protein and ratios of the enrichments of Phe, Tyr, Met, and Lys at isotopic plateau in secreted milk casein to the free AA in arterial and mammary vein blood were monitored during the last 5 h of a 30-h continuous i.v. infusion of [1-^{13}C]Phe, [$^{2}H_4$]Tyr, [5-$^{13}CH_3$]Met, and [2-^{15}N]Lys on two occasions: before (control) and on d 6 of an i.v. infusion of Phe (6 g/d). During the control, uptakes of free Phe and Met were less than their output in milk. This result was comparable with the labeling kinetic results, suggesting that vascular peptides contributed 5 to 11% of Phe and 8 to 18% of Met. Free Tyr and Lys uptakes during the control were sufficient for milk output; however, the labeling kinetics indicated that 13 to 25% of the Tyr and 4 to 13% of the Lys were derived from peptides. Infusion of Phe increased the uptake of free AA but reduced the contribution of peptides toward Phe (0 to 3%) and Tyr (8 to 14%) supply for casein synthesis. Whole body hydroxylation of Phe to Tyr increased from 10 to 18% with the infusion of Phe; within the mammary gland, this conversion was lower (3 to 5%). Results suggest that the mammary utilization of peptides containing Phe and Tyr appears to depend on the supply of free AA in blood. (**Key words**: mammary gland, peptides, casein synthesis, goats)

Abbreviation key: **BF** = blood flow, **EPA** = external pudic artery, **GC-MS** = gas chromatography-mass spectrometry, ***p*-AH** = para-aminohippurate, **U:O** = ratio of net uptake to milk output.

Received June 12, 1998.
Accepted September 21, 1998.

INTRODUCTION

The mammary gland of a dairy cow that produces 35 kg/d of milk that contains 32 g/kg of protein requires at least 1.12 kg of AA to synthesize this protein. These AA must be obtained from circulating blood, or, in the case of some of the nonessential AA, they may be synthesized de novo in the gland. However, often times, certain AA that are not synthesized by the mammary gland (e.g., Phe, Met, His, and Thr) do not appear to be extracted as free AA in sufficient quantities to account for their output in milk proteins (18, 24, 25, 26, 35, 37, 40). Furthermore, the actual deficit of these AA may be even larger when they are used to synthesize some of the nonessential AA or when they contribute to the synthesis of constitutive proteins and other metabolites or compounds (8, 20, 31, 33, 48). Such observations suggest that the deficiency of free AA uptake must be accounted for by the utilization of peptides or proteins derived from blood for casein synthesis.

However, it is difficult to be unequivocal about such a phenomenon because of the technical limitations associated with the measurement of arteriovenous AA differences across the gland. Thus, extraction of Phe, Met, and His by the lactating gland results in only small (2 to 10 μM) AA differences in arteriovenous blood across the gland, which challenges the accuracy of most analytical methodologies and leads to a lack of confidence in the absolute values derived for metabolite uptake data (4, 6, 11). Consequently, it has been difficult to ascertain whether errors associated with the measurement of arteriovenous differences are compounded by inherent variabilities that are associated with the measurement of mammary blood flow (**BF**) (36).

In an effort to overcome the equivocal nature of the methodology that uses the ratio of net uptake to milk output (**U:O**), we developed an indirect isotope labeling technique (isotope enrichment of free AA from plasma compared with those in secreted casein over the plateau period) to provide additional evidence

that peptides supply Phe, but apparently not Leu, for milk casein synthesis in late lactation goats (6). In addition, this precursor to product tracer labeling technique has been employed in the current study to evaluate whether the lactating mammary gland of the goat utilizes circulating peptides containing Lys, Met, and Tyr for casein synthesis.

Many studies (24, 35, 37) have shown that, when supplemental protein or AA are administered to dairy cows, the mammary uptake of those AA previously observed to be in deficit is either sufficient or in excess of milk outputs. However, although it is tempting to suggest that this result may indicate a mechanism that allows the mammary gland to extract either more peptides or free AA to meet the net requirements for milk protein synthesis, such observations may be merely artifacts of the arteriovenous net uptake methodology and the inherent analytical difficulties mentioned previously. Therefore, we decided to incorporate a supplemental AA (Phe) infusion treatment into the present experiment and use the isotopic enrichment differentials between free AA in plasma and AA bound to casein to examine whether less Phe that is bound to peptides is used by the gland during supplemental Phe infusion. At the same time, modifications were made in the blood sampling techniques to improve the accuracy and precision of the U:O measurements. This approach was compared with the indirect tracer approach. Aspects of the experiment have been reported elsewhere (11, 13, 15).

MATERIALS AND METHODS

Stable Isotopes

L-[1-^{13}C]Phenylalanine, L-[5-$^{13}CH_3$]Met, and L-[2-^{15}N]Lys·HCl (all 99 atom % ^{13}C or ^{15}N) were purchased from Mass Trace (Somerville, MA). L-[2,3,5,6-$^{2}H_4$]Tyrosine (98 atom % ^{2}H) was purchased from CK Gas Products Ltd. (Berkshire, England). Specific mixtures of isotopes were dissolved in saline (9 g of NaCl/L); Tyr was dissolved at pH 10, the remaining AA were added, and the pH was adjusted to 7.4. Solutions were filter-sterilized (0.22-μm filter units) into sterile glass bottles. Intravenous infusion rates (micromoles per hour) were [^{13}C]Phe, 282; [$^{2}H_4$]Tyr, 84; [^{13}C]Met, 78; and [^{15}N]Lys·HCl, 132.

Goats and Diets

Four British Saanen goats (one first parity and three second parity; mean BW = 68.9 ± 4.6 kg) in late lactation (mean = 270 DIM; range = 254 to 295 DIM) were used. In two separate surgeries, the left carotid artery was raised to a subcutaneous position, and deep and percutaneous vessels (arteries and veins) transecting the udder halves were ligated. A transonic flow probe (6 mm; Transonic Systems Inc., Ithaca, NY) was fitted around each external pudic artery (**EPA**). The flow probe cables were secured in position to the lateral suspensory ligament by suturing collars that were attached to the probe cable onto polyester mesh (Ethicon Ltd., Edinburgh, Scotland) that had been sutured to the suspensory tissues. Cables were exteriorized directly beneath (5 to 6 cm) the vulva and protected in a pouch that was glued across the hindquarters. For two goats, catheters (medical grade polyvinyl chloride; i.d., 0.6 mm; o.d., 0.7 mm; Critchley Electrical Products, Auburn, New South Wales, Australia) were also positioned in each EPA via a small downstream arterial branch. The distal tip of the catheter was positioned downstream (2 cm) of the probe as previously described (12).

Goats were placed in metabolism crates and allowed at least 10 d to acclimate to the frequent feeding by automatic feeders (12 equal-sized meals/d at 2-h intervals) and the daily routines of machine-milking and hand milking (0830 and 1630 h). The goats were fed a diet of chopped grass hay containing 10% molasses (fresh weight basis) and a pelleted concentrate (45:55, wt/wt; DM basis) in amounts that matched or exceeded recommended (2, 3) intakes of energy and protein for maintenance plus milk production. 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.

Temporary catheters (medical grade polyvinyl chloride; i.d., 0.8 mm; o.d., 1.2 mm; Critchley Electrical Products) were inserted into each jugular vein for infusions and into the elevated carotid artery and a subcutaneous mammary vein for blood withdrawal. Catheters were kept patent by flushing once daily with a heparin-saline (200 U/ml) solution.

Experimental Protocol

Arteriovenous mass and isotope kinetics were performed before (control) and on the last day of a 6-d i.v. infusion of Phe [6 g/d in 480 g of saline (0.9% NaCl) solution (pH 7.4)]. Isotope infusions consisted of 30 h (nonprimed) of constant i.v. infusion (50 g of solution/h) of [^{13}C]Phe and [$^{2}H_4$]Tyr. During the control period, [^{13}C]Met and [^{15}N]Lys were also infused. Carry-over of isotope was minimized by allowing 9 to 12 d to pass between isotope infusions in the same

goat. After 23 h (0830 h; a.m. milking), sodium heparinate (47 kU in 2 ml of saline) was added to the isotope infusate to facilitate continuous withdrawal of blood. At this time, each mammary gland was milked out completely by machine and hand stripping after administration of an i.v. dose of oxytocin (2 IU). Thereafter, each gland was milked by hand every hour (without oxytocin). Milk production was recorded for both glands, and the milk from the gland that was monitored for arteriovenous kinetics was retained for the isolation of casein and measurement of AA concentration. Beginning at 25 h, samples of blood from the carotid artery and mammary vein were withdrawn continually (10 ml/h) by a peristaltic pump over consecutive 1-h periods for the next 5 h and collected into sealed syringes submerged in an ice bath. Mammary BF was logged every 10 s, and data were integrated for the 1-h blood sampling periods. Flow probes were calibrated in vitro, employing the gravimetric procedures outlined by the manufacturer. For the two goats with EPA catheters, mammary BF was also measured by infusion of para-aminohippurate (***p*-AH**; 600 mg/h in 20 ml of sterile saline) into the EPA catheter and measurement of the downstream dilution of the *p*-AH in the ipsilateral mammary vein. All goats remained standing during the 5-h sampling period.

Blood samples were mixed by gentle hand rolling, and duplicate 1-ml samples were injected into evacuated 10-ml vacutainers containing 0.5 ml of frozen lactic acid. The solutions reacted rapidly after vortexing and were kept at room temperature (20°C). Fresh blood samples were also analyzed for plasma HCO_3^- content and pH (Radiometer ABL3 blood gas analyzer; Radiometer Ltd., Crawley, England); packed cell volume (percentage) was determined by hematocrit. Blood samples (0.5 g) were accurately weighed, and a known similar weight of a hemolyzing solution containing L-norleucine (100 μM) was added to determine AA and *p*-AH concentrations. Whole blood was also prepared for gas chromatography-mass spectrometric (**GC-MS**) analyses by mixing 1 g with an equal weight of ice-cold distilled water. The remaining heparinized blood was centrifuged at 2000 × *g* for 15 min at 4°C. Plasma (0.5 g) was accurately weighed, and a known similar weight of the L-norleucine standard was added for AA analysis. Plasma (0.7 g) was also dispensed for GC-MS analyses. All samples were stored frozen at -20°C. Immediately prior to isotope infusion, blood and milk samples were taken to assess the natural (background) abundance of the various isotopes.

Whole milk samples (0.5 g) were accurately weighed, and a known similar weight of an L-norleucine standard (13.5 mM) was added for AA analysis. Samples were then hydrolyzed in 4 M HCl (3 ml) at 110°C for 18 h.

Analytical Procedures

Milk preparation. Milk N concentration was determined on subsamples by macro N (combustion) methods. Milk CP was calculated as milk N × 6.38. Casein was isolated from milk by a modification of the method of McKenzie (34). Hydrolyzed whole milk and casein were prepared for AA analyses or GC-MS analysis as previously described (14).

Concentration and enrichment of AA. Stored blood (plasma) was thawed (4°C) and deproteinized by centrifugation (13,000 × *g* for 15 min) through filter units (10,000 nominal molecular weight limit; Millipore Corp., Bedford, MA); free AA concentrations in the protein-free supernatant were determined as previously described (29). Gravimetric procedures were used to quantify blood *p*-AH concentrations assayed by the Bratton-Marshall procedure as described by Smith et al. (42).

Stored blood, plasma, and casein samples were prepared for GC-MS analysis as previously described (14). After isolation from the blood, plasma, or casein, the AA were treated with methyl-t-butyldimethylsilyl trifluoroacetamide to form their tertiary-butyldimethylsilyl derivatives based on the methods of Calder and Smith (19). The GC-MS analyses were performed using a quadruple mass spectrometer (HP5989A; Hewlett Packard, Avondale, PA). The temperatures of the gas chromatography injector and the interface line were 280 and 250°C, respectively. Separation of the tertiary-butyldimethylsilyl derivatives of Phe, Tyr, Met, and Lys was effected on a 30-m × 0.25-mm × 0.25-μm SE-30 CB capillary column (Alltech, Carnforth, England). The temperature was programmed at 220°C for 5 min and then increased 10°C/min to 260°C for 6 min. Injections (1 μl) were made in the split mode (40:1). Helium at a head pressure of 11 psi was used as the carrier gas, and the capillary column was connected directly to the ion source of the mass spectrometer. The source and analyzer temperatures were 200 and 100°C, respectively; the electron energy was 70 electron V, and the emission current was 300 μÅ. Selective ion monitoring was carried out with a 30-ms dwell time on each ion on the fragment ions corresponding to the m/z of 336 and 337 for natural and [^{13}C]Phe, respectively; a m/z of 466, 467, 468, and 470 for natural, [^{13}C], [$^{2}H_2$], and [$^{2}H_4$]Tyr, respectively; a m/z of 218 and 219 for natural and [^{13}C]Met,

respectively; and a m/z of 431 and 432 for natural and [^{15}N]Lys, respectively.

Plasma [$^2H_4$]Tyr enrichments were determined against a set of standard mixtures containing 0 to 6 molar percentage excess [$^2H_4$]Tyr. Upon hydrolysis of casein, however, loss of two deuterium from [$^2H_4$]Tyr occurred. Thus, for casein, fragment ions at a m/z of 466, 467, and 468, which correspond to natural, [^{13}C], and [$^2H_2$]Tyr, were monitored. The m/z of 468 to 466 was corrected for the naturally occurring m + 1 from Tyr enriched with ^{13}C, and the [$^2H_2$] enrichments were measured against a calibration curve generated from hydrolyzed [$^2H_4$]Tyr standards (0 to 6 molar percentage excess). Plasma and casein [^{13}C]Phe, [^{13}C]Met, and [^{15}N]Lys enrichments were determined from standard mixtures (0 to 20 molar percentage excess) of their respective tracers.

Blood $H^{13}CO_2$ enrichments were determination on an isotope ratio-mass spectrometer Sira-12 (VG Isogas, Middlewich, Cheshire, England) according to Read et al. (41). All enrichments were expressed as molar percentage excess against the appropriate natural abundance sample.

Calculations

Plasma kinetics. Plasma flux rates of AA (F; millimoles per hour) were calculated from isotope dilution in plasma (14):

$$F = [(E_i/E_a) - 1] \times IR_{aa}$$

where E_i = enrichment (molar percentage excess) of the infused isotope, E_a = enrichment of the free AA in arterial plasma at isotopic plateau, and IR_{aa} = rate of isotope infusion.

The rate of conversion (hydroxylation) of Phe to Tyr in the whole body was derived from the following expression:

$$F_{phe\rightarrow tyr} = [F_{tyr} \times (E_{a,tyr\wedge}/E_{a,phe})] \times [F_{phe}/(IR_{phe} + F_{phe})] \times C_f$$

where F_{tyr} and F_{phe} = values derived from the continuous infusion of [$^2H_4$]Tyr and [^{13}C]Phe, respectively; $E_{a,tyr\wedge}$ and $E_{a,phe}$ = plasma enrichments of [^{13}C]Tyr and [^{13}C]Phe, respectively; and IR_{phe} = infusion rate of [^{13}C]Phe. The term $F_{phe}/(IR_{phe} + F_{phe})$ corrects for the contribution of tracer Phe to F_{tyr}. The intracellular pool enrichment within the tissues is lower than that in plasma; thus, values of plasma flux rates of AA are underestimated to the extent of this error, depending on AA (9). The error in the estimation of $F_{phe\rightarrow tyr}$ is further compounded because of the underestimation of F_{tyr}, and the selection of arterial plasma overestimates the hepatic intracellular precursor enrichment at the site of Phe hydroxylation to Tyr. Because of its intracellular origin, the plasma enrichment of 4-methyl-2-oxopentanoate, the keto-acid of Leu, has often been used as a surrogate for the intracellular pool enrichment of Leu at the site of protein synthesis (i.e., AA tRNA) in the tissue. In lactating goats, the ratio of keto-acid to Leu enrichment in plasma is approximately 0.85 (16). This value was used to correct for the intracellular dilution of [$^2H_4$]Tyr. The hepatic intracellular dilution of [^{13}C]Phe was based on the enrichment of [$^2H_5$]Phe in the ratio of apolipoprotein B-100 to plasma [$^2H_5$]Phe enrichment, which was approximately 0.78 and was determined in growing sheep (21). Thus, the correction factor C_f was calculated as $1/(0.85 \times 0.78)$.

Mammary kinetics. Kinetic calculations were only performed for the udder half that was monitored. The rate of secretion of AA in milk was corrected for 4% of the AA derived from nonmammary synthesized proteins that appeared in milk (47). Net uptake (NU) of AA by the udder was calculated from both blood and plasma exchanges as

$$NU \text{ (micromoles per hour)} = (C_a - C_v) \times BF_{b \text{ or } p}$$

where C = concentration (micromolar) of the free AA in arterial (a) and mammary venous (v) blood or plasma, and BF (kilograms per hour) = mammary gland blood (b) or plasma (p) [i.e., BF × (1 – (packed cell volume/100))] flow rate as appropriate. The irreversible loss (sequestration) of AA across the mammary gland (Ra_a) was based on transfers from arterial plasma, calculated as

$$Ra_a \text{ (micromoles per hour)} = [(C_a \times E_a - C_v \times E_v) \times BF_p]/E_a$$

where E = enrichment of the AA in arterial and mammary venous plasma. Thus, partition of plasma flux to the mammary gland (K_{mg}) was calculated as $K_{mg} = Ra_a/F$.

Conversion of Phe to Tyr ($R_{phe\rightarrow tyr}$) by the mammary tissues was calculated from the increase in the ^{13}C enrichment of casein Tyr relative to the extracellular plasma Tyr pool and assuming that the ^{13}C enrichment of casein Phe ($E_{c,phe}$) was representative of the intracellular precursor pool at the site of Tyr synthesis. Thus,

$$R_{phe\rightarrow tyr} \text{ (micromoles per hour)} = [Ra_{a,tyr} \times E_{a,tyr\wedge} \times ((r_c/r_a) - 1)]/E_{c,phe}$$

where $Ra_{a,tyr}$ = irreversible loss of Tyr from plasma based on [^{2}H]Tyr kinetics. The term $((r_c/r_a) - 1)$, with symbols r_c and r_a representing the ratio of [^{13}C]Tyr to [^{2}H]Tyr enrichments in casein and arterial plasma, was used to correct for the contribution of [^{13}C]Tyr derived from plasma to casein [^{13}C]Tyr. Because the appearance of $^{13}CO_2$ across the mammary gland was undetectable, loss of Tyr via oxidation was assumed to be negligible.

An estimate of the minimum proportion of casein Tyr derived via Phe hydroxylation was calculated as casein Tyr from Phe = $R_{phe\rightarrow tyr}$/Tyr output in milk casein.

Statistics

The effect of Phe infusion on AA flux measurements was assessed by a one-sample paired *t* test. Differences in the enrichments of AA in blood, plasma, and casein were assessed by ANOVA. The ANOVA included a term for sampling time point (25 to 30 h of isotope infusion) to assess whether AA enrichments in blood, plasma, and casein had attained a plateau during the 5-h sampling period. The effect of sample time point was not significant, indicating that a plateau had been reached in these pools by 25 h.

RESULTS

Goats

The goats used in this experiment were in late lactation. To account for the normal decline in milk production, the effect of Phe infusion on milk production was assessed by regressing milk production and milk protein yield during the 5-d control and Phe infusion periods and comparing (paired *t* test) the slopes. The slopes for total milk production (–26 vs. –17; SEM = 12) and protein yield (–0.6 vs. –0.8; SEM = 0.8) were not significantly different between the control and Phe infusion periods. During the control and Phe infusion periods, daily milk production averaged 1846 ± 82 and 1711 ± 63 g, and milk protein yield averaged 69 ± 3 and 66 ± 2 g, respectively.

Phe and Tyr Kinetics

Plasma kinetics. Plasma Phe and Tyr kinetics are summarized in Table 1. Blood concentrations increased by 83% for Phe and by 51% for Tyr because of Phe infusion ($P < 0.05$). Flux rates and fractional and absolute rates of hydroxylation of Phe to Tyr and Phe available for protein synthesis (corrected for hydroxylation) in plasma were significantly increased because of the infusion of Phe. The increase in the flux of Phe in plasma when Phe was infused (47.8 mmol/d) was considerably greater than the additional amount of Phe infused (36.9 mmol/d), possibly because of the expansion of the Phe pools in plasma and tissue. Tyrosine flux and the proportion of Tyr derived from Phe hydroxylation were also higher during Phe infusion. Of the additional Phe infused, 43% (15.7 mmol/d) was hydroxylated to form Tyr. The infusion of additional Phe did not significantly alter the proportions of Phe flux (0.21 vs. 0.24) and Tyr (0.22 vs. 0.26) in the plasma that was partitioned to the mammary gland.

TABLE 1. Fluxes of Phe and Tyr in plasma of lactating goats in response to a 6-d i.v. infusion of Phe (36.9 mmol/d).

	Control	Phe Infusion	SED[1]	*P*[2]
Phe in Blood, μM	53.9	98.7	10.4	0.02
Phe Flux, mmol/d	90.2	138.0	1.3	<0.001
Fractional hydroxylation of Phe	0.097	0.178	0.014	0.013
Phe Hydroxylation, mmol/d	8.9	24.6	1.8	0.004
Phe for Protein synthesis, mmol/d	81.4	113.5	0.7	<0.001
K_{mg}[3] of Phe	0.21	0.24	0.02	NS[4]
Tyr in Blood, μM	71.9	108.4	11.2	0.05
Tyr Flux, mmol/d	70.0	91.9	3.4	0.008
Proportion of Tyr flux from Phe	0.091	0.185	0.010	0.002
K_{mg}[3] of Tyr	0.22	0.26	0.02	NS

[1]Standard error of the difference.

[2]Probability that control and Phe infusion values differ (paired *t* test).

[3]Proportion of plasma flux partitioned to the whole mammary gland.

[4]$P > 0.10$.

Enrichments of free AA in blood and milk casein enrichments. Figure 1 illustrates the mean (n = 4) isotopic enrichments of [^{13}C]Phe (A and D) and [^{2}H]Tyr (B and E) during the last 5 h of the 30-h isotope infusions in the control (A and B) and Phe infusion (D and E) periods. Both of the AA had attained a plateau in blood and plasma well before this period. Isotopic enrichments of casein AA reached the plateau usually by 25 or 26 h of infusion. Kinetic calculations were based on the values at the plateau level.

Table 2 gives the mean enrichments (molar percentage excess) of [^{13}C]Phe, [^{13}C]Tyr, and [^{2}H]Tyr in arterial and mammary vein blood and plasma and in secreted milk casein over the period of isotopic plateau. Thus, all values over the period of plateau were used to test for differences in AA enrichment between the blood pools and casein. [^{13}C]Phenylalanine, [^{13}C]Tyr, and [^{2}H]Tyr enrichments were sig-

nificantly lower in blood than in plasma. In the control period, the enrichment of [^{13}C]Phe in casein was significantly lower (by 2 to 11%) than that in arterial blood and plasma and mammary vein plasma enrichments and higher (by 9%) than the enrichment in mammary vein blood. In contrast, in the Phe infusion

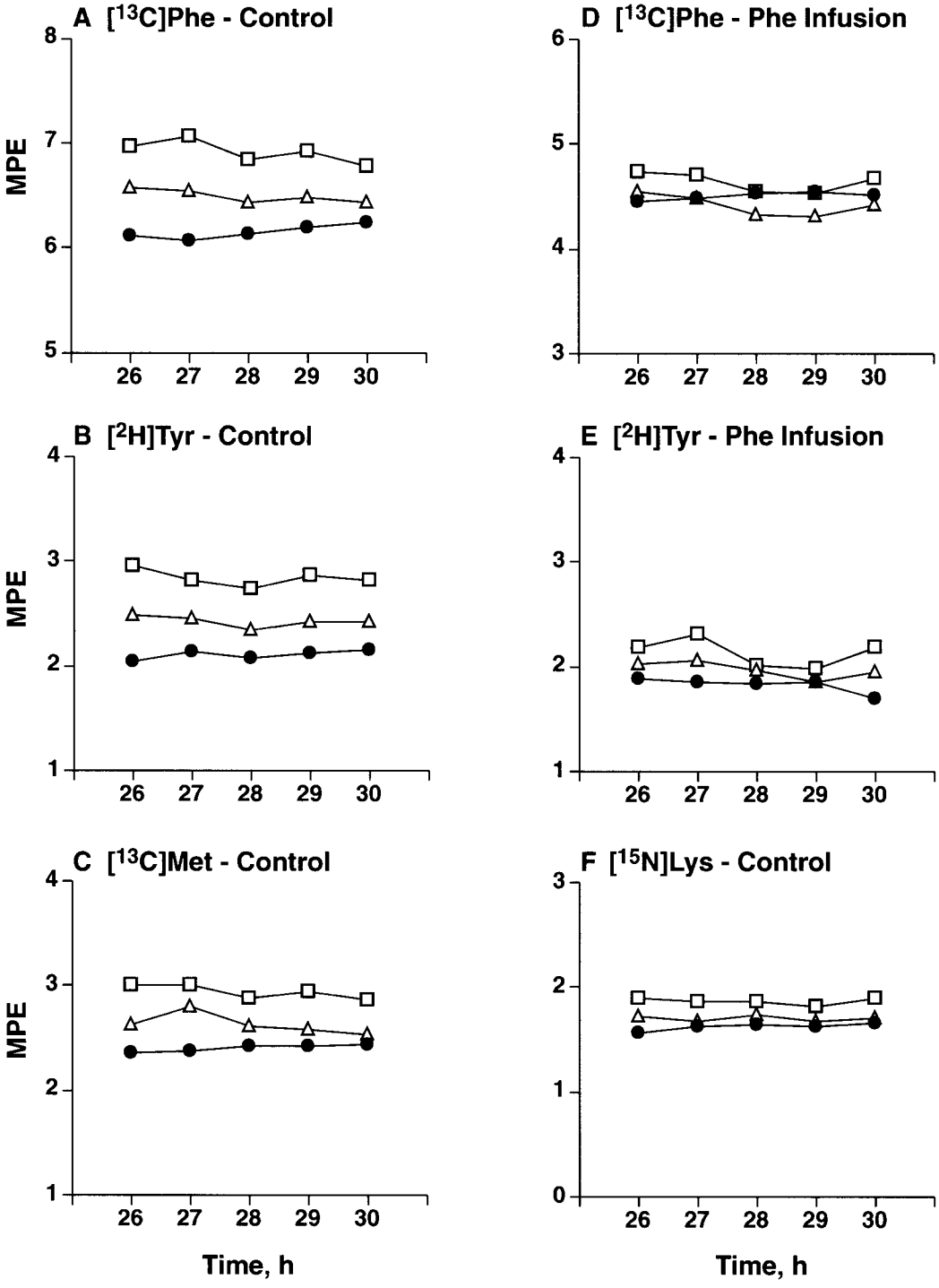

Figure 1. Temporal labeling (molar percentage excess; MPE) patterns of free Phe (A and D), Tyr (B and E), Met (C), and Lys (F) in arterial blood (△) and plasma (□) and in secreted milk casein (●) over the last 5 h of a 30-h continuous i.v. infusion of [1-^{13}C]Phe, [$^{2}H_4$]Tyr, [5-$^{13}CH_3$]Met, and [2-^{15}N]Lys in late lactation goats (n = 4). Phenylalanine and Tyr kinetics were examined before (control) and on the last day of a 6-d i.v. infusion of Phe (6 g/d of Phe); Met and Lys kinetics were examined only during the control period.

TABLE 2. Isotopic enrichment (molar percentage excess) of free AA in arterial and mammary vein blood and plasma and in secreted milk casein AA in goats before (control) and on the last day of a 6-d i.v. infusion of Phe.[1]

	Plasma		Blood			P[2]			
	Arterial	Mammary vein	Arterial	Mammary vein	Casein	A	B	C	D
[1-^{13}C]Phe									
Control	6.92	6.25	6.49	5.64	6.15	<0.001	0.07	<0.001	<0.001
Phe Infusion	4.65	4.44	4.42	4.23	4.51	0.02	NS[3]	0.09	<0.001
[1-^{13}C]Tyr									
Control	0.61	0.56	0.49	0.41	0.65	NS	0.02	<0.001	<0.001
Phe Infusion	0.86	0.94	0.82	0.79	0.94	0.015	NS	0.002	<0.001
[$^{2}H_4$] or [$^{2}H_2$]Tyr[4]									
Control	2.84	2.63	2.43	2.22	2.12	<0.001	<0.001	<0.001	0.025
Phe Infusion	2.14	2.06	1.98	1.89	1.83	<0.001	<0.001	<0.001	<0.001
[5-$^{13}CH_3$]Met[5]	2.94	2.52	2.63	2.11	2.41	<0.001	0.005	<0.001	<0.001
[2-^{15}N]Lys[5]	1.87	1.74	1.70	1.56	1.63	<0.001	<0.001	0.01	0.01

[1]Each value represents the mean of four goats calculated from five consecutive 1-h samples taken once a plateau was reached (i.e., the last 5 h of the 30-h isotope infusion).

[2]Probability that enrichments in arterial plasma (A) and blood (C) and mammary vein plasma (B) and blood (D) were different from casein values according to ANOVA. Standard errors of the differences between means were [1-^{13}C]Phe, 0.068 (151 df); [1-^{13}C]Tyr, 0.036 (151 df); [^{2}H]Tyr, 0.052 (151 df); [5-$^{13}CH_3$]Met, 0.043 (75 df); and, [2-^{15}N]Lys, 0.031 (75 df).

[3]$P > 0.10$.

[4]Plasma and blood enrichments were determined from a fragment ion containing all deuterium atoms; a fragment ion containing two deuterium was monitored for casein enrichments because of the loss of two deuterium upon hydrolysis.

[5]Infused during the control period only.

period, the differences in enrichment were much smaller; the enrichment of [^{13}C]Phe in casein was significantly lower than the arterial plasma value (by only 3%), and casein values were significantly higher (by 2 to 7%) than those in arterial and mammary vein blood. In the control period, [^{13}C]Tyr enrichment in casein was not different from the arterial plasma value, but casein values were significantly higher (by 16 to 59%) than those in arterial blood and in mammary vein blood and plasma. When Phe was infused, the casein [^{13}C]Tyr enrichment was higher (by 9 to 19%) than the arterial blood and plasma and mammary vein blood values but did not differ from that in mammary vein plasma. In the control and Phe infusion periods, the casein [^{2}H]Tyr enrichment was significantly lower than all of the vascular blood pools; however, these differences were much smaller during the Phe infusion period. Values in the control period were 5 to 25% lower compared with the 3 to 14% lower values during the Phe infusion period. Thus, the overall effect of the infusion of additional Phe was to enhance the transfer or equilibrium of the free Phe and Tyr pools in blood with the intracellular free pool at the site of casein synthesis.

Mammary metabolism. Phenylalanine and Tyr kinetics across one-half of the udder are presented in Table 3. Mammary BF rates (both blood and plasma) were higher (by 15 to 16%) in the Phe infusion period. When the flow probe and *p*-AH methods were compared in two goats in the present experiment, both methods detected similar increases in BF (kilograms per hour) because of Phe infusion [goat A: 20.11 vs. 24.09 (control) and 32.19 vs. 29.76 (Phe infusion); goat B: 18.47 vs. 19.90 (control) and 18.93 vs. 23.95 (Phe infusion)]. The BF values determined by the *p*-AH method were, on average, 16% higher than the flow probe values. A similar discrepancy between these methods has previously been reported in lactating dairy cows (36). For these two goats in the current experiment, the BF rates measured by the *p*-AH method were considered to be more appropriate for kinetic calculations (see Discussion).

In both periods, net uptake of Phe from blood and plasma did not differ, indicating that the packed cells (primarily erythrocytes) did not participate in the net exchange of AA. Net uptake of Phe tended (plasma: $P = 0.12$; blood: $P = 0.12$) to be higher because of the infusion of Phe; however, this additional uptake of Phe did not lead to an increase in the output of Phe in milk. The U:O for Phe (calculated based on blood removals) tended ($P = 0.068$) to be <1 (U:O = 0.81) in the control period, and, when additional Phe was infused, the U:O was not different from 1 (U:O = 0.94). The irreversible loss of Phe from plasma tended ($P = 0.085$) to be greater during the Phe infusion period. The fractional and absolute rates

of Phe hydroxylation by the mammary gland were low and not significantly affected by Phe infusion.

In contrast to Phe, the net uptake of Tyr from plasma was greater ($P < 0.05$; df 6) than that from blood, suggesting that the packed cells, via counter current exchange, transported Tyr away from the mammary gland. The infusion of additional Phe increased ($P = 0.039$) the net uptake of Tyr from blood, which was likely in response to the increase in blood Tyr concentrations. Tyrosine output in milk casein was also unaffected by Phe infusion. The U:O of Tyr did not differ from 1 and was not affected by Phe infusion. The irreversible loss of Tyr from plasma and the proportion of casein Tyr derived from Phe were not affected by Phe infusion.

Tracer balance. The rates of removal of [^{13}C]Phe and [^{2}H]Tyr from blood and plasma by the mammary gland were compared with rates of secretion in milk protein (Table 4). Tracer balance across the mammary gland was calculated to assess the extent that the mammary gland had reached isotopic steady state (i.e., no sequestration of tracer). Under these conditions, for those AA that are not significantly catabolized by the mammary gland (e.g., Phe, Tyr, and Met), net removal of isotope should equate to the rate of secretion of the isotope in milk. Blood and plasma transfers of [^{13}C]Phe were similar except during the Phe infusion period when transfer rates from plasma tended ($P = 0.09$) to be greater than those from blood. Again, this difference may be due to transport by the erythrocyte of unlabeled and labeled AA away from the gland. Similarly, plasma transfers of [^{2}H]Tyr were significantly greater than blood transfers in both periods. Thus, based on blood transfers of isotope, the rates of removal of [^{13}C]Phe and [^{2}H]Tyr by the mammary gland were not significantly different

TABLE 3. Blood flow and kinetic fluxes of Phe and Tyr across an udder half of lactating goats before (control) and on the last day of a 6-d i.v. infusion of Phe.[1]

Item[2]	Control		Phe Infusion		P[3]
	$\bar{X}$	SEM	$\bar{X}$	SEM	
BF_b, kg/h	21.38	1.41	24.86	1.57	0.046
BF_p, kg/h	16.99	1.10	19.56	1.01	0.07
Phe Metabolism, μmol/h					
Net uptake					
Blood	391	35	450	53	0.08
Plasma	382	34	489	41	0.12
Ra_a	424	35	559	43	0.085
$R_{phe\rightarrow tyr}$	16	3	26	4	NS[4]
Fractional hydroxylation	0.032	0.003	0.046	0.006	NS
Milk output	483	29	476	33	NS
U:O	0.81*	0.06	0.94	0.08	NS
Casein:plasma enrichment[5]	0.89**	0.04	0.98**	0.03	0.04
Phe Balance[6]	429	48	436	59	NS
Tyr Metabolism, μmol/h					
Net uptake					
Blood	267	25	353	24	0.039
Plasma[7]	336	42	437	48	NS
Ra_a	387	36	491	63	NS
Proportion of casein Tyr from Phe	0.054	0.014	0.085	0.012	NS
Milk output	314	29	310	33	NS
U:O	0.87	0.11	1.17	0.10	0.12
Casein:plasma enrichment	0.75**	0.04	0.86**	0.08	0.02

[1]Values are means (n = 4; ±SEM); one-sample paired t test, $\mu \neq 1$.

[2]BF_b = Blood flow, BF_p = plasma flow, Ra_a = irreversible loss from plasma, $R_{phe\rightarrow tyr}$ = rate of conversion of Phe to Tyr, and U:O = ratio of net uptake to milk output.

[3]Probability that values differ between control and Phe infusion (paired t test).

[4]$P > 0.10$.

[5]Calculated once values reached a plateau (25 to 30 h after isotope infusion began).

[6]Calculated as net uptake from blood + [(1 – ratio of casein to plasma Phe enrichment) × milk Phe output] – $R_{phe\rightarrow tyr}$.

[7]Net uptake of Tyr from plasma was greater ($P = 0.032$; df 6) than net uptake from blood.

*$P < 0.10$.

**$P < 0.05$.

TABLE 4. Comparison of isotope extraction across one udder half with isotope secretion in milk casein.[1]

	Blood extraction (A)		Plasma extraction (B)		Milk output (C)		P[2]		
							A vs. B	A vs. C	B vs. C
	$\bar{X}$	SEM	$\bar{X}$	SEM	$\bar{X}$	SEM			
[1-^{13}C]Phe									
Control	31.5	3.4	29.6	3.4	31.3	2.2	NS[3]	NS	NS
Phe Infusion	23.2	2.8	26.0	2.4	22.3	2.5	0.09	NS	0.05
[^{2}H]Tyr									
Control	8.0	1.4	11.1	1.2	7.0	0.8	0.05	NS	0.01
Phe Infusion	5.8	0.8	8.0	1.8	5.8	0.7	0.09	NS	0.09
[5-$^{13}CH_3$]Met	8.2	0.6	9.2	0.8	8.3	0.5	0.05	NS	0.07
[2-^{15}N]Lys	22.2	3.6	22.9	4.1	16.9	1.2	NS	0.05	0.05

[1]Values are means (n = 4; ±SEM).

[2]Standard errors of the differences between means were [1-^{13}C]Phe, 2.01 (12 df); [^{2}H]Tyr, 1.54 (12 df); [5-$^{13}CH_3$]Met, 0.51 (6 df); and [2-^{15}N]Lys, 2.62 (6 df).

[3]$P > 0.10$.

from their secretions in milk protein and were not affected by Phe infusion. These data suggest, first, that the free AA in the plasma pool, as opposed to that in the erythrocyte, represent the sole source of free AA uptake by the gland and, second, that the mammary gland had reached an isotopic equilibrium because sequestration (accretion) of isotope was negligible.

Lys and Met Kinetics

The kinetics of Lys and Met were monitored only in the control period. Figure 1 illustrates the mean (n = 4) isotopic enrichments of [^{13}C]Met (C) and [^{15}N]Lys (F) in arterial blood and plasma and in secreted milk casein over the last 5 h of the 30-h infusion of isotopes. Both tracers reached a plateau in the vascular pools well before the sampling period; for casein, a plateau was reached by 25 to 26 h. Comparison of AA enrichments in the vascular pools and in casein and kinetic calculations were based on enrichment values over the period of isotopic plateau. Listed in Table 2 are the enrichments of [^{15}N]Lys and [^{13}C]Met in arterial blood and plasma and in secreted milk casein over the period of plateau. The enrichment of [^{15}N]Lys in milk casein was significantly lower (by 4 to 13%) than that in arterial blood and plasma and mammary vein plasma and higher (by 4%) than values in mammary vein blood. Similarly, the enrichment of [^{13}C]Met in milk casein was significantly lower (by 4 to 18%) than that in arterial blood and plasma and mammary vein plasma and higher (by 14%) than values in mammary vein blood.

Plasma flux, partition to the mammary gland, and fluxes across the mammary gland of Lys and Met are presented in Table 5. For Lys, plasma flux and partition to the mammary gland were 166.2 mmol/d and 0.359, and the same values for Met were 61.4 mmol/d and 0.245, respectively. In this period, the net uptake of Lys from blood did not differ from that from plasma. By contrast, net uptake of Met from plasma was greater than that from blood, suggesting that the packed cells transported Met away from the gland. The U:O of Lys in the control period was numerically, but not statistically, >1 (U:O = 1.094). The U:O of Met was <1 (U:O = 0.76; $P < 0.05$). The irreversible loss of Lys across the mammary gland was 1199 μmol/h, and the irreversible loss of Met across the mammary gland was 314 μmol/h. Thus, similar to the Phe and Tyr data, removal of [^{13}C]Met from plasma was greater than that from blood, and net tracer removal by the gland did not differ from the rate of secretion in milk (Table 4). These data for Met also confirm that plasma exchange dominates and that no sequestration of isotope had occurred. In contrast, although blood and plasma transfers of [^{15}N]Lys were not different, they both exceeded (by 31 to 36%) the rate of isotope secretion in milk. This difference likely represents the loss of Lys through catabolism.

Mammary Uptake of Other AA

Presented in Table 6 is a comparison of the U:O of the remaining (mostly essential) AA based on blood or plasma exchanges. Infusion of Phe did not affect the U:O for the other AA. The U:O for His, Thr, Pro, Met, and Phe were significantly <1, and those for Arg, Val, Ile, Leu, and Lys were significantly >1. Across periods, net uptakes of Tyr, Leu, and Lys from plasma were greater than blood exchanges.

TABLE 5. Plasma flux and kinetics of Lys and Met across an udder half of lactating goats.[1]

Item[2]	Lys		Met	
	$\bar{X}$	SEM	$\bar{X}$	SEM
Plasma flux, mmol/d	166.2	9.4	61.4	0.8
K_{mg}	0.359	0.067	0.245	0.022
Mammary metabolism, μmol/h				
Net uptake				
Blood	1062	135	249	21
Plasma[3]	1098	134	284	28
Ra_a	1199	164	314	30
Milk output	994	73	328	23
U:O	1.094	0.179	0.76*	0.057
Casein:plasma enrichment[4]	0.88**	0.02	0.83**	0.02

[1]Measurements [means (n = 4); ±SEM] were made only during the control period; one-sample *t* test, $\mu \neq 1$.

[2]K_{mg} = Proportion of plasma flux partitioned to the whole mammary gland, Ra_a = irreversible loss from plasma, and U:O = ratio of net uptake to milk output.

[3]For Met, net uptake from plasma was greater ($P = 0.061$; paired *t* test) than that from blood.

[4]Calculated once values reached a plateau (25 to 30 h after isotope infusion began).

*$P < 0.05$.

**$P < 0.005$.

DISCUSSION

The often observed deficit in free AA uptake by the mammary gland has given rise to speculation that vascular peptides or proteins contribute to milk protein synthesis. However, direct unequivocal evidence has not been obtained to prove that peptides or proteins of dietary or endogenous origin make a significant contribution to casein biosynthesis or to absorptive and other anabolic (muscle gain or wool and hair growth) processes despite attempts to refine the analytical methodologies for measurement of transorgan peptide fluxes (4, 6, 7, 17). Thus, indirect methods have had to be developed to overcome such limitations. In the present experiment, one such indirect method, the ratio of precursor to product labeling technique, was used to quantitate the contribution of peptides to milk protein synthesis, which was then compared with the conventional approach of measuring free AA uptake but employing improved methodologies for acquiring arteriovenous blood samples and measuring net balance across the gland.

Methodological Considerations

Mammary arteriovenous balance. This technique, which involves the comparison of the net uptake of a nutrient (AA) by the mammary gland from blood with output in milk, depends mainly on the accuracy with which arteriovenous blood concentration differences of the nutrient and mammary BF can be measured and the ability to determine accurately the output of the milk proteins over this same period. In most studies, arterial and mammary venous blood samples have been withdrawn as spot samples, which themselves provide information on only a fraction of the total uptake of nutrients used for the biosynthetic

TABLE 6. Comparison of ratio of net uptake in blood and plasma to milk AA output (U:O) of selected AA in goats before (control) and on the last day of a 6-d i.v. infusion of Phe.[1]

	Control		Phe Infusion			*P*[2]		
	Blood	Plasma	Blood	Plasma	SED[3]	U:O ≠ 1[4]	Blood vs. plasma	Control vs. Phe infusion
His	0.50	0.65	0.81	0.73	0.224	<0.001	NS	NS
Thr	0.60	0.58	0.63	0.72	0.103	<0.001	NS	NS
Arg	1.83	2.07	2.52	2.79	0.685	<0.001	NS	NS
Pro	0.32	0.36	1.41	0.48	0.084	<0.001	NS	NS
Tyr	0.87	1.13	1.14	1.45	0.254	NS[5]	0.032	NS
Val	1.38	1.42	1.67	1.74	0.181	<0.001	NS	NS
Met	0.76	0.87	0.99	1.06	0.146	0.11	NS	NS
Ile	1.75	1.75	1.99	2.04	0.226	<0.001	NS	NS
Leu	1.08	1.14	1.27	1.38	0.146	0.005	0.015	NS
Phe	0.81	0.80	0.94	1.03	0.135	0.028	NS	NS
Lys	1.10	1.14	1.32	1.45	0.153	0.027	0.069	NS

[1]Output of AA in milk was measured directly and corrected for 4% of milk protein AA derived from nonmammary synthesized milk proteins (47).

[2]According to ANOVA.

[3]Standard error of the difference between means (source × treatment).

[4]Paired *t* test.

[5]$P > 0.15$.

process. To improve the precision of the measurement of arteriovenous difference, a procedure for the continuous withdrawal of arterial and mammary venous blood samples over consecutive 1-h periods (n = 5) and continuous monitoring of BF was adopted in the present experiment. At the same time, milk AA output was determined every hour by complete hand stripping of the udder halves. Thus, temporal fluctuations in the differences in arteriovenous concentrations of AA and BF (i.e., extraction rate) were integrated to coincide with the amount of milk protein synthesized and secreted over the same period of time.

To reduce the possibility that subcutaneous abdominal venous blood would become contaminated with blood draining from nonmammary tissues or the contralateral udder, the anastomosing vessels between the udder halves were ligated as were the subcutaneous abdominal veins and the perineal arteries and veins. Goats used in this experiment were in first or second lactation, minimizing the problems that can arise in later lactation when the valves of the external pudic vein become incompetent, allowing nonmammary blood to drain into and contaminate the subcutaneous abdominal venous blood drainage (32). Moreover, the goats were in late lactation when the flow in the external pudic vein was negligible in either direction anyway (38). The goats remained standing throughout the 5-h sampling period to minimize acute (5 to 10 min) hyperemic fluctuations (increased or decreased) in mammary BF that usually occur in response to postural changes (i.e., lying or standing) (36) and that could mask treatment effects.

Attempts were made to monitor mammary BF using an ultrasonic flow probe implanted around an EPA. To ensure that the EPA supplied most, if not all, of the arterial blood to one-half of the udder, the perineal and contralateral anastomosing arteries between the udder halves were ligated. However, the probes were calibrated in vitro before implantation and post-mortem using the water bath method outlined by the manufacturer (Transonics Systems Inc.). There were no differences in the calibration equations. Unfortunately, when these measurements were compared with those determined by downstream (subcutaneous abdominal vein) dilution of *p*-AH infused into the EPA, the probe values were, on average, 16% lower. A number of possible reasons for the lower probe data could be suggested. The lower data could have been due to an inappropriate (i.e., non in vivo) acoustical environment when the probes were calibrated in vitro; improper implantation of the probe around the EPA; or the fact that, in addition to the EPA, small anastomosing arteries were contributing to the mammary BF that the *p*-AH method had measured but that the probe could not. After consideration of the fact that most other comparable studies have used the dye dilution methods, the higher values determined by the *p*-AH method were used for these two goats. However, the choice of method did not influence the overall trend for AA balances across the gland (i.e., U:O were either significantly ≤1 for both methods) and the relative responses to Phe infusion.

Both the erythrocyte (packed cells) and plasma pools have been shown to exchange AA with the bovine and caprine mammary gland (12, 28). However, analytical problems (i.e., poor peak resolution) can be associated with analyses of AA in whole blood (4); as a result, most of the data on arteriovenous differences have been based on plasma measurements. Hanigan et al. (28) cautioned that net uptake values in the literature could be biased underestimates for some AA (e.g., up to 9% for Phe, 15% for Thr, 22% for Met, 28% for Lys, and 22% for Tyr) and overestimates for others (e.g., up to 40% for Leu), depending on the extent and direction of the erythrocyte and plasma exchanges (28). However, using the integrated sampling procedure, we detected significant differences between whole blood and plasma exchanges (net uptake from plasma was greater than that from whole blood) for Val, Leu, and Lys.

Net uptakes of free His, Thr, Pro, Met, and Phe were less than their rates of secretion in milk, but, for Arg, Val, Ile, Leu, and Lys, net uptake was greater than milk output. These results were consistent with those of a previous study using lactating goats that consumed a protein source based on corn (12). Furthermore, these results are comparable with those reported for dairy cows, including those for which only plasma fluxes were monitored (18, 24, 25, 26, 27, 35, 37, 40, 48). In all cases, uptake of Arg was in excess (two- to threefold) of its output in milk. The presence of several key urea cycle enzymes in the mammary tissues (10) probably facilitated the use of Arg as a major contributor to the de novo synthesis of Pro, which was also not extracted in adequate quantities (Table 6). The excessive extraction of the branched-chain AA (Leu, Ile, and Val) was probably accounted for as intramammary oxidation (16); the resulting carbon skeletons and amino groups contributed to the de novo synthesis of nonessential AA (48). The apparently excessive net uptake of Lys in the current experiment would appear to contradict its

status as the first-limiting or a colimiting AA for diets based on corn (25); however, despite this fact, recent data indicate that in addition to its role as a net contributor to milk protein synthesis, a considerable fraction of the Lys extracted by the gland is oxidized (13). Furthermore, whether the oxidative mechanism is maintained or diminished for Lys under more limiting conditions and indeed a milk production response situation, as is the case for Leu (16), will need to be established.

The apparent deficit in the uptake of certain AA needs further consideration. The deficiency of Phe would appear to be exacerbated by the small, but significant, proportion (0.03 to 0.05) of Phe that is converted to Tyr in the mammary gland. Similarly, as much as 10% of Met uptake appears to contribute to the synthesis of Cys (31); additional Met may possibly be required to supply methyl groups in support of the proliferative activity of the mammary gland and in the regulation of gene expression. Unknown quantities of His may be required to synthesize histamine. The intracellular concentrations of histamine appear to parallel those of His (33) (i.e., the function of histamine may be to regulate vasoactivity and milk secretion). Thus, little doubt exists that peptides or proteins must be used by the mammary gland to offset these deficits, and, in recent years, researchers have been able to demonstrate that the caprine mammary gland in vivo and bovine, caprine, and murine mammary tissues in vitro can and do utilize peptides containing Phe, Met, Lys, and Leu for mammary tissue and milk protein synthesis (5, 39, 44, 45).

However, the methodologies available for separating molecular weight fractions of peptides and proteins for the measurement of arteriovenous differences in AA concentration in the peptide fraction have proved inconsistent. Furthermore, even when refinements to methodology have been employed, the differences in arteriovenous concentrations of 2 to 3 μM that would be expected are probably beyond the precision of even the most technically advanced AA analyzer systems (4, 6). Consequently, it has been necessary to develop alternative methodologies to test the peptide theory.

Ratio of precursor to product tracer labeling techniques. Backwell et al. (6) developed an alternative, indirect approach to detect non-free AA use by the mammary gland based on comparison of the isotopic enrichment of plasma (primary precursor pool) free AA and the secreted milk casein AA. Given that the time interval between the synthesis and secretion of milk proteins is short [30 to 60 min; (14)] and the turnover rate of mammary tissue proteins is high [40 to 130%/d; (8, 20)], the isotopic enrichment of the AA in secreted milk casein can be expected to attain a pseudo-plateau (steady-state) enrichment after approximately 13 h of a constant i.v. infusion of isotope (14). If free AA in plasma contribute all of the supply of AA for casein synthesis, then, at isotopic equilibrium, the enrichment of the free AA in the vascular pools and in the secreted casein will be the same. A lower casein enrichment indicates that vascular non-free AA sources have contributed to casein synthesis.

The finding that the enrichment of Phe bound to casein was lower than that of free Phe in plasma (by 4 to 14%), although, for Leu, the enrichment was similar, appeared to substantiate many reports in which the net uptake of free Phe was 15 to 20% less than milk protein output, and, for Leu, the net uptake was in excess of milk protein output (24, 35, 37). In the study reported by Backwell et al. (6), it was necessary to subject the casein enrichment data to exponential analysis to predict the asymptote values because it was not readily discernible by visual inspection whether a plateau had been attained at the conclusion of the 24-h constant infusion of isotope. In the present experiment, the isotope infusion was extended to 30 h, and all tracer AA had attained a visually recognizable plateau in the vascular pools and in casein by 25 to 26 h (Figure 1, A to F). In addition to the longer infusion protocol, the earlier attainment of a plateau might have also been due to the frequent milking period (22 to 25 h) we incorporated into the sampling protocol, which aided in the removal of unlabeled casein material from the glands prior to making the balance measurements during the last 5-h of isotope infusion. To confirm this further, isotope balances across the gland were computed (Table 4). Except for [^{15}N]Lys, all tracer AA were in isotopic balance, suggesting that all intracellular protein pools that participate in the channeling of the extracted plasma AA were highly labeled (i.e., degradation products arising from their turnover would not dilute the enrichment of the AA in casein). The fact that 31 to 36% of the extracted [^{15}N]Lys could not be accounted for in milk protein is consistent with the fractional rate of Lys oxidation by the gland (13), although, if there are intracellular proteins with much slower rates of turnover that are rich in Lys, then sequestration of isotope could still have occurred.

One other possible source of unlabeled AA could arise when the AA is synthesized within the mammary gland from unlabeled carbon or nitrogen sources. De novo synthesis is an accepted fact for

many of the nonessential AA (48), but, of the AA studied in the present experiment, the only possibility of such synthesis was for Tyr. Tyrosine can be synthesized from Phe, but de novo synthesis appears to occur only to a very small and limited extent (5 to 9% in the present experiment) (30, 43). This degree of de novo synthesis falls considerably short of the 8 to 25% of Tyr (estimated by isotope dilution) that appears to be derived from the uptake of peptides.

Amino acid enrichments in whole blood were always lower than those in plasma (Table 2). There could be several reasons why this differential exists. Isotope is infused into the plasma (primary) pool; thus, a lower enrichment in whole blood would suggest an incomplete equilibration of the isotope across the erythrocyte membrane. However, upon transit through the tissue capillaries, there may be direct uptake by the erythrocytes of unlabeled AA derived from the intracellular tissue pool [present results; (22)] or there may be hydrolysis in the erythrocytes of peptides and proteins (1, 5). If the mammary gland can extract AA from both the erythrocyte and plasma pools and, for some AA, at different rates, then a greater or disproportionate rate of extraction from the lower isotopically enriched AA in the erythrocyte could lead to a lower enrichment of casein relative to plasma. However, based on the net uptake data (Table 6), there was no indication that AA had been extracted from the erythrocyte in this experiment. Nevertheless, the net measurements may misrepresent true transport phenomenon because it does appear that the erythrocyte can transport certain AA away from the gland (i.e., Tyr, Leu, and Lys; Table 6). Furthermore, there is some indication that erythrocytes hydrolyze dipeptides to free AA during passage of the packed cells through the mammary tissues (5), which could provide an additional and undetected supply of AA for mammary uptake. All of these processes could disguise the fact that there is unidirectional transfer of AA from the erythrocyte to the mammary gland. To determine the extent of this exchange would require isolations of the erythrocytes and a direct measurement of the mass and isotope transfers and exchanges by the erythrocytes, something that was not possible in the current experiment. However, at least during the control period, the fact that over the period of isotopic plateau the enrichments in casein failed to attain the same level as those in whole blood (Table 2) suggests that the free AA in blood (erythrocyte plus plasma) could not be the exclusive precursor of all of the AA incorporated into casein. Thus, other vascular non-free AA sources are required.

Nutrient Supply and Peptide Contribution to Casein Synthesis

Phe and Tyr. Data from the control periods (no infusion of Phe) indicated that the isotopic enrichments of the AA in casein were lower than those in the arterial free pools by 5 to 11% for Phe and by 13 to 25% for Tyr (based on [^{2}H]Tyr kinetics); these results for Phe were consistent with our previous findings also using late lactation goats (6). The labeling data are also consistent with the net uptake results for Phe, indicating that because Phe is not extracted as free AA in adequate quantities, uptake of peptides is required. In contrast, when the goats were given the constant i.v. infusion of the supplemental free Phe, the ratio of precursor to product enrichment for both Phe and Tyr were significantly smaller; casein enrichments were lower than arterial free pool values by 0 to 3% for Phe and 8 to 14% for Tyr, indicating that the contribution of peptides had been reduced. Furthermore, this lower contribution from peptides appeared to be counterbalanced by an increase in the net uptake of free Phe such that the net balance of total Phe remained unchanged (Table 3). These findings support data from dairy cows (24, 26, 35, 37). In those studies, under basal feeding conditions, the net uptake of some AA, often Phe and Met, failed to account for their output in milk protein, but, when additional protein or AA were infused (duodenal and intravenous), net uptake of these AA increased to equal or exceed milk protein output.

Met. The U:O and the ratio of precursor to product labeling methods both indicated that a large proportion (0.24 and 0.08 to 0.18, respectively) of Met used for casein biosynthesis had been derived from the uptake of peptides. These observations in vivo are supported by recent findings (39) that cultured mammary cells can utilize peptides containing Met as a substitute for free Met for mammary tissue and secretory protein synthesis. However, dilution of the [$^{13}CH_3$]Met tracer can also be reflective of transmethylation reactions (remethylation of homocysteine to Met) within the mammary gland, which are known to occur in lactating goats (23). Consequently, whether all of the 8 to 18% dilution of the Met tracer represents the proportional contribution of peptides containing Met for casein synthesis cannot be determined. In hindsight, it would have been possible to eliminate any dilution from transmethylation by using [1-^{13}C]Met as the tracer because the C-1 label position remains intact with the molecule beyond this reversible remethylation step.

Lys. The tracer data would suggest that 12% of Lys for casein synthesis is also derived from the uptake of

vascular peptides. Peptides containing Lys are utilized for secretory protein synthesis by mammary explants prepared from lactating mice (44). Yet, the uptake of Lys in a form bound to peptides would appear to contradict the fact that this AA is already extracted by the mammary gland as a free AA in adequate or often excess amounts [Table 6; (25)] and that this excess is probably then disposed of via oxidative mechanisms (13). The present results now suggest that one should consider not only the metabolic fate of the excess free AA, but also that which the uptake of peptides may contribute.

Metabolism of Phe and Tyr

Measurements of the flux in whole body plasma are often reported, but their interpretation requires some caution. First, the AA enrichment in plasma is unlikely to represent the enrichment at the site of protein synthesis or metabolite conversion (e.g., oxidation, hydroxylation, transmethylation, transsulfuration) within tissues. Another complication is the varying extents to which the absorbed AA are removed on first pass through the splanchnic tissues before first mixing (diluting) with the systemically infused tracer, which results in an underestimation of plasma flux. This latter feature is an important consideration in the estimation of plasma flux and hydroxylation of Phe because the major site of hydroxylation is the liver where the correct precursor pool measurement is the enrichment of Phe and Tyr in the hepatic intracellular free pool. In the current study, it was not possible to correct for splanchnic removal of AA on the first pass; however, a correction factor was computed to correct the extracellular arterial plasma enrichment to reflect the lower enrichment of the hepatic intracellular pool, correcting for the underestimation of hydroxylation.

Fractional and absolute rates of plasma Phe hydroxylation were increased (0.097 vs. 0.178 and 8.9 vs. 24.6 mmol/d) because of the i.v. infusion of Phe; the increase in the absolute rate represented 42% (15.7 mmol/d) of the additional Phe infused (36.9 mmol/d). The increase in Phe hydroxylation (+15.7 mmol/d), however, was less than the increase in Tyr flux (+21.9 mmol/d). This underestimation may be attributable to the previously mentioned considerations regarding first pass splanchnic removal or to our inability to measure directly the activity of the hepatic intracellular precursor pool and that in other peripheral tissues (e.g., mammary gland) that were also capable of converting Phe to Tyr. Another feature of the plasma flux measurement is the assumption that the size of the free AA pool (blood and tissue) does not change as a result of the treatment imposed. If the free pool is enlarged, this further dilution of the infused isotope cannot be distinguished from the effect of the treatment. In the current experiment, plasma Phe and Tyr concentrations were both increased, but to different extents, because of Phe infusion. This disproportionate increase in the free pools of Phe (+83%) and Tyr (+50%), and thus their plasma isotopic dilutions, might have been an additional contributing error to the estimation of plasma Phe flux and hydroxylation.

In the present experiment, the enrichment of the AA in secreted milk casein was selected as a closer approximation of the intracellular pool activity at the site of protein synthesis and metabolism in the mammary tissues compared with the other vascular choices (i.e., arterial or mammary venous plasma). The rate of hydroxylation of Phe to Tyr in the mammary gland was found to be low, representing only 3 to 5% of the Phe extracted and contributing to 5 to 9% of the Tyr molecules in casein. These data are the first quantitative estimates of Phe hydroxylation in vivo and are similar to the rates previously reported in vitro for the isolated perfused udder of the sheep (43).

CONCLUSIONS

Although the sources of peptides for ruminant metabolism remain equivocal (7, 17, 46), it is becoming clear that mammary tissues in vitro and in vivo appear to be able to utilize AA bound to peptides for tissue or milk protein synthesis (5, 6, 39, 44, 45). Thus, the extent to which the mammary gland utilizes vascular peptides for milk protein synthesis is an important issue to resolve, particularly if we are to delineate the mechanisms governing (limiting) the utilization of AA for casein biosynthesis and realize the potential of the gland to synthesize milk. In the current experiment, the contributions of free AA and peptides in blood to casein biosynthesis were assessed using the conventional arteriovenous net balance method and a novel ratio of precursor to product labeling technique. The results suggested that the deficit in the uptakes of free Phe and Met could be met by the uptake of these AA in forms bound to peptides. In contrast, although the uptake of free Tyr and Lys in blood was already adequate for casein synthesis, an additional supply of these AA appeared to be derived from vascular peptides. The most intriguing finding was that the use of Phe and Tyr that were bound to peptides for casein synthesis was

reduced when supply (rate or concentration) and uptake of the free AA in blood was increased. Although such a mechanism could allow the mammary gland to adapt to the prevailing blood supplies of free and bound AA, alternatively, this mechanism may impose a limitation on the total uptake of an AA. Nonetheless, the present results suggest that we may now have to consider that vascular peptides or proteins probably represent a general source of precursors for casein synthesis, even for those AA that apparently are already extracted as free AA in excess of milk protein output requirements. What remains to be resolved are the mechanisms of peptide metabolism by the lactating gland; whether peptide use is facilitated by a transporter or involves hydrolysis at the cell surface followed by free AA transport; and, ultimately, what is the source of their supply and how is it regulated.

ACKNOWLEDGMENTS

This work is part of a joint project at the Rowett Research Institute (Aberdeen, Scotland) and Reading University (Reading, England) cosponsored by a consortium of the Ministry of Agriculture, Fisheries and Food (England); the Biotechnology and Biological Sciences Research Council (England); the Scottish Office Agriculture Environment and Fisheries Department (Scotland); Purina Mills, Inc. (St. Louis, MO); Hendrix Voeders B.V. (The Netherlands); and the Milk Development Council (England). The advice of G. Horgan on the most appropriate statistical tests is gratefully acknowledged.

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