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High Rates of Mammary Tissue Protein Turnover in Lactating Goats Are Energetically Costly[1–3]

Mark D. Hanigan,[4]* James France,[5,9] Sameer J. Mabjeesh,[6] Warren C. McNabb,[7] and Brian J. Bequette[8]

[4]Department of Dairy Science, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061; [5]Centre for Dairy Research, School of Agriculture, Policy and Development, University of Reading, Reading, Berkshire RG6 6AR, UK; [6]Department of Animal Science, Faculty of Agriculture, Hebrew University of Jerusalem, Rehovot, Israel; [7]Food, Metabolism and Microbiology, Food and Textiles Group, AgResearch Grasslands, Palmerston North 4442, New Zealand; and [8]Department of Animal and Avian Sciences, University of Maryland, College Park, MD 20742

Abstract

The high energetic demands and metabolism of amino acids (AA) within the lactating mammary gland have been ascribed to the requirements for milk component synthesis and tissue maintenance. Our objective in this work was to assess rates of protein synthesis from several AA so that the energetic costs of tissue maintenance could be better reflected. Lactating goats ($n = 4$) were given staggered infusions of 5 labeled forms of phenylalanine (Phe) initiated at 30, 12, 9, 6, and 3 h before goats were killed. [5-$^{13}CH_3$] Methionine (Met), [1-^{13}C] leucine, and [1-^{13}C] valine were also infused for 30 h, during which time, the glands were milked hourly and arteriovenous flux measurements were performed the last 6 h. A dynamic, compartmental model capable of simulating fluxes of AA through extracellular and intracellular free, slow and fast turnover tissue-bound, and milk protein pools was developed and fitted to the observed data. The udder removed 81% of the Phe present in plasma using 31% for milk protein synthesis and releasing 66% back into plasma. Transamination accounted for 40% of Phe flux in the mammary and transmethylation accounted for a portion of mammary Met flux. Mammary tissue protein synthesis was >300% the value of milk protein synthesis with fractional protein synthesis rates >130%/d. Assuming 4 mol of ATP/mol of peptide bond formed, we estimate that ~50% of ATP generated by the lactating mammary glands is used for synthesis of tissue (nonmilk) protein. J. Nutr. 139: 1118–1127, 2009.

Introduction

Lactation is a demanding process that affects dietary and maternal nutrient supplies and use and overall energy balance. Although current feeding systems provide reasonable estimates of the protein and energy requirements of lactating animals to sustain a given level of milk production, the empirical nature of these estimates does not allow the prediction of milk and milk component production responses to changes in dietary nutrient intake. This is largely due to the limited quantitative data and mechanistic description of the postabsorptive metabolic fates of absorbed nutrients and metabolites.

During lactation, the mammary gland becomes a metabolically active and energetically demanding tissue (1,2). Balance studies indicate an excess net uptake by the mammary gland of glucose, acetate, and amino acids (AA)[10] relative to milk lactose and casein output, respectively. These excess nutrients are oxidized to support energy demands of the tissue. Hanigan and Baldwin (3) constructed a detailed model of energy metabolism in the lactating cow udder and found that ATP that was generated exceeded use for milk component synthesis by 2-fold (312 mol ATP generated vs. 102 mol used for milk synthesis). Approximately 60 mol could be expected to be used to maintain membrane potential and synthesize nucleic acids (4), leaving

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[3] An appendix describing in detail the model used for isotope interpretation, Supplemental Figures 1 and 2, and Supplemental Table 1 are available with the online posting of this paper at jn.nutrition.org.
[9] Present address: Centre for Nutrition Modeling, Department of Animal and Poultry Science, University of Guelph, Guelph, ON N1G 2W1, Canada.
* To whom correspondence should be addressed. E-mail: MHanigan@vt.edu.

[10] Abbreviations used: AA, amino acid; BF, blood flow; $F_{xAa,nAa}$, flux of extracellular to intracellular; $F_{nAa,xAa}$, flux of intracellular to extracellular; $K_{xAa,nAa}$, rate constant for AA uptake; $K_{nAa,mAa}$, rate constant for incorporation into milk protein; $K_{nAa,tsAa}$, rate constant for synthesis of tissue protein (nonmilk) in the slow turnover protein pool; $K_{nAa,nKa}$, rate constant for deamination; $K_{nAa,tfAa}$, rate constant for synthesis of tissue protein (nonmilk) in the fast turnover protein pool; $K_{nAa,xAa}$, rate constant for efflux from the cell; *Lag*, lag in time from the start of a tracer infusion until appearance in milk protein; m/z, mass:charge ratio; RMSPE, root mean square prediction error.

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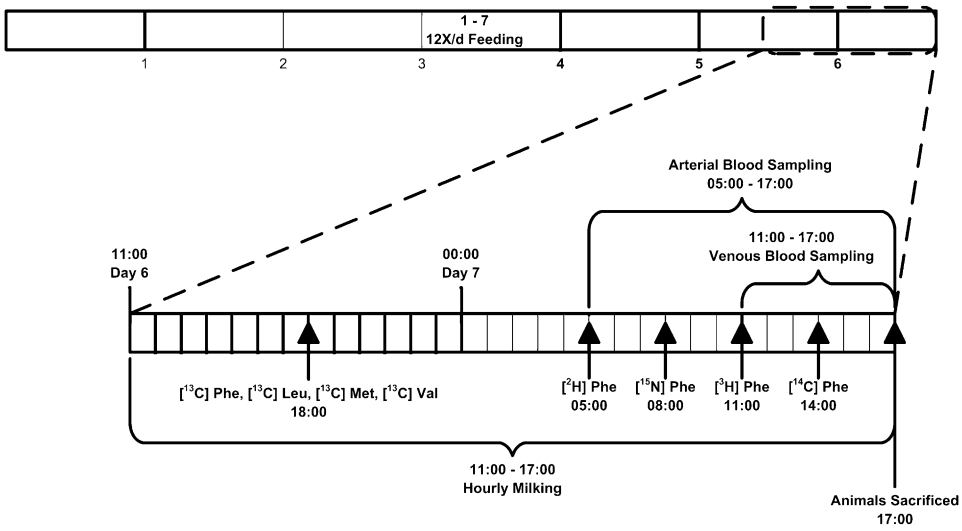

FIGURE 1 Experimental timeline. The upper timeline has units of days. The lower timeline lists events by hour during the last 30 h of the experiment.

almost one-half of the generated ATP for use in protein synthesis and other undefined uses. Such a high rate of ATP use would infer very high rates of protein turnover and high maintenance energy costs. Given that mammary tissue mass and protein turnover increases from gestation into lactation and subsequently declines as lactation progresses and ceases (5,6), maintenance costs of the tissue likely change significantly, which could be expected to affect body energy balance and weight change. Protein synthesis in other highly active tissues such as liver, gut, and skin may also be much higher than current estimates, which would similarly affect estimates of energy requirements.

Using constant infusion methods, it was observed that mammary protein fractional synthesis rates were 42%/d when plasma samples were used to represent the precursor pool and 130%/d when calculated from tissue homogenate samples in lactating animals (7,8). The former underestimate true rates and the latter are generally thought to overestimate true rates. Estimates derived from long-term, constant infusions are also thought to underestimate the true rate due to label recycling (9).

The objective of this work was to more accurately estimate mammary synthesis of nonmilk tissue protein rates based on tracer kinetics for phenylalanine (Phe[10]), leucine (Leu), valine (Val), and methionine (Met). As part of this effort, a compartmental model representing the various pools contributing to mammary protein turnover and AA exchange was constructed.

Materials and Methods

Stable and radio-isotopes

[5-$^{13}CH_3$]Met, [1-^{13}C]Val, [1-^{13}C]Leu, and [1-^{13}C], [^{15}N] and [ring-$^{2}H_5$]Phe (all 99 atoms %) were purchased from Mass Trace and [U-^{14}C] (453 mCi/mmol; 1 Ci = 3.7 × 10^{10}Bq) and [ring-$^{3}H_5$]Phe (115 Ci/mmol) were purchased from Amersham. Prior to i.v. infusion, specific mixtures of isotopes were dissolved in saline (9 g of NaCl/L, pH 7) and filter sterilized (0.22-μm filter units) into sterile glass bottles.

Animals, diet, and isotope infusions

This study was conducted in 1998 at the Rowett Research Institute (Aberdeen, Scotland). The Institute's Ethical Review Committee and the veterinary inspectorate of the Home Office (UK) approved all surgical procedures and practices.

A timeline for the experiment is provided (**Fig. 1**). Goats used in this experiment were used in a previous study (10). Briefly, 4 British Saanen goats were surgically prepared with indwelling arterial catheters, transonic flow probes placed around each external pudic artery, and a carotid artery elevated to a subcutaneous position. The current experiment was conducted on these goats during their second lactation when they weighed 73 ± 12 kg and at d 128 ± 43 of lactation. The diet offered in this experiment was formulated to meet metabolizable energy and protein requirements for maintenance and milk production (11) as described by Mabjeesh et al. (10) and goats were acclimated to the diet prior to the trial. Daily feed refusals were collected and weighed, and feed intake was adjusted to allow 5% refusals. Six days prior to the isotope infusions (adaptation period), the goats were placed in metabolism crates and fed at 2-h intervals and milked twice daily at 0830 and 1630 h.

One day before tracer kinetic measurements, temporary catheters were inserted into each jugular vein, the elevated carotid artery, and a subcutaneous mammary vein, respectively. Prior to initiating isotope infusions (1100 h), goats were given an i.v. dose of oxytocin (1 IU) and hand milked. Immediately, a constant (15 mL/h) i.v. infusion of a mixture of stable isotope-labeled AA ([5-$^{13}CH_3$]Met, 29 mg/h; [1-^{13}C]Val, 24 mg/h; [1-^{13}C]Leu, 50 mg/h; [1-^{13}C]Phe, 34 mg/h) and unlabeled Phe (68 mg/h) was initiated. Constant i.v. infusions of [ring-$^{2}H_5$]Phe (34 mg/h), [^{15}N]Phe (34 mg/h), [U-^{14}C]Phe (200 μCi/h), and [ring-$^{3}H_5$]Phe (25 μCi/h) were initiated at 18, 21, 24, and 27 h, respectively. These additional tracers were infused in place of equimolar amounts of unlabeled Phe to maintain a constant rate of total Phe infusion. Throughout the infusion period, goats were milked at 1-h intervals. Milk from the udder halves was combined for analysis during the first 24 h but collected and processed separately from each udder half over the last 6 h. Milk weights were recorded at each milking and milk was subsampled for determination of composition, AA concentration, and isotopic enrichment of AA in casein.

Arterial blood samples (4 mL) were taken at 1-h intervals during the first 24 h of isotope infusion. Over the last 6 h of isotope infusion, sodium-heparinate (6.6 kU/h) was constantly infused i.v. and blood was continuously (8 mL/h) withdrawn from the carotid artery and from the mammary vein over 6 consecutive 1-h periods. Samples were collected into sealed syringes submerged in an ice bath and subsequently stored at −20°C. Throughout the 30-h isotope infusion, blood flow (BF) was electronically recorded at 10-s intervals.

Mammary tissue sampling. Immediately after the last blood sample and milking, goats were killed by lethal injection. Mammary glands were excised and weighed, and within 1–2 min, samples (10–20 g) of tissue (excluding skin) were dissected from the core of 2 separate regions (rear and fore aspects) of the mammary gland that was monitored for arteriovenous fluxes. Tissues were minced (0.5 g), washed twice in ice-cold saline containing oxytocin to remove blood and residual milk, and then plunged into liquid N_2. Tissues were stored at −80°C until further processing.

Analytical methods

Plasma free AA concentrations were determined after deproteinization by spin filtration (12) at 13,000 × *g*; 45 min (10,000 nominal molecular weight limit; Millipore). For plasma Phe specific radioactivities (^{3}H and

^{14}C) and enrichments, samples were processed and analyzed according to previously described methods (9,13). Whole-milk AA content was determined after hydrolysis in 4 mol/L HCl for 18 h as described previously (12). Casein was isolated from whole milk (14), acid hydrolyzed, and prepared for GC-MS analysis (15).

Frozen mammary tissue was pulverized in liquid N_2 and deproteinized using sulfosalicylic acid (48%, wt:v). Tissue free AA concentrations, enrichment, and specific activities were determined using the acid-supernatant (15). A portion of the precipitated pellet was hydrolyzed in 3 mol/L HCl (containing 0.5 g dithiothreitol/L) at 110°C for 18 h, desalted, and subjected to GC-MS analysis to determine AA enrichments. We analyzed another portion of the precipitated pellet for tissue-bound, Phe-specific radioactivity. The remainder of the precipitated pellet was hydrolyzed and analyzed for AA composition.

For GC-MS analyses, free AA were converted to their *t*-butyldimethylsilyl derivative and isotopic enrichments were determined in the electron impact mode by selective ion monitoring (Trio-1, VG Masslab) (16). The following ions of mass:charge (m/z) were monitored: Leu, 302 and 303; Met, 292 and 293; and Val, 288 and 289. For the various label positions in Phe, fragment ions at m/z 234 (unlabeled) and 235 (^{15}N) and ions at m/z 336 (unlabeled), 337 (^{15}N+^{13}C), and 341 ($^{2}H_5$) were monitored. [1-^{13}C]Phe enrichment was calculated by difference (^{15}N+^{13}C minus ^{15}N). Crude ion ratios were converted to molar percent excess based on calibration curves generated from gravimetric mixtures of labeled and unlabeled AA.

Model derivation

The model represented the metabolism of a single AA within the udder. It was based on a set of dynamic, differential equations coded in Advanced Continuous Simulation Language (Aegis Technologies Group). The tissue was assumed to be constant in size with intracellular (V_n) and extracellular (V_x) compartments of fixed fluid volume (L). The model contained 5 primary-state variables representing extracellular AA, intracellular AA, a pool of AA in tissue protein with a rapid rate of turnover, a pool of AA in tissue protein with a slow rate of turnover, and AA in milk protein. An intermediate turnover protein pool was attempted, but attempts at parameter estimation resulted in nonunique solutions, indicating the model was overparameterized. These protein pools were aggregates of the various proteins present in an average mammary cell. It is likely the fast turnover pool included enzymes and other proteins that were degraded rapidly, whereas the slow turnover pool represented more structural proteins that were degraded more slowly; however, the pools were purely empirical descriptions defined by the data. Because the udder was assumed to be fixed in size, these protein pools were also assumed to be fixed in size with respect to time, i.e. rates of degradation equaled rates of synthesis. Each primary-state variable was replicated 5 times to represent each of the 5 isotopes of Phe, resulting in 25 secondary-state variables. A schematic of the model is provided in **Figure 2**.

Fluxes between pools were assumed to occur by mass action. AA uptake and efflux from the extracellular space, catabolism and use for protein synthesis in the intracellular space, and secretion of AA in milk protein were explicitly represented. Inputs to the model were arterial AA concentrations (labeled and total), BF, initial pool sizes (labeled and total), and the protein content and proportion of the given AA in milk protein. From these inputs, the model predicted unidirectional AA uptake and efflux from the cell, venous AA output, intracellular AA concentrations, milk protein output, tissue protein synthesis rates, and rates of AA transamination. A full description of the model is provided in the Appendix and stoichiometric constants used in the model are summarized in **Supplemental Table 1**.

The model was applied and parameterized using Leu, Met, Phe, and Val measurements. However, it is applicable to additional AA and to whole udders or other tissues provided metabolism of the AA are consistent with that described in the model.

Parameter estimation

Model rate constants were fitted to the data using the Nelder-Mead and generalized reduced gradient optimization algorithms available within ACSL Optimize (Ver. 11.8, Aegis Technologies Group) to maximize the log likelihood function. Model inputs required to conduct the simulations

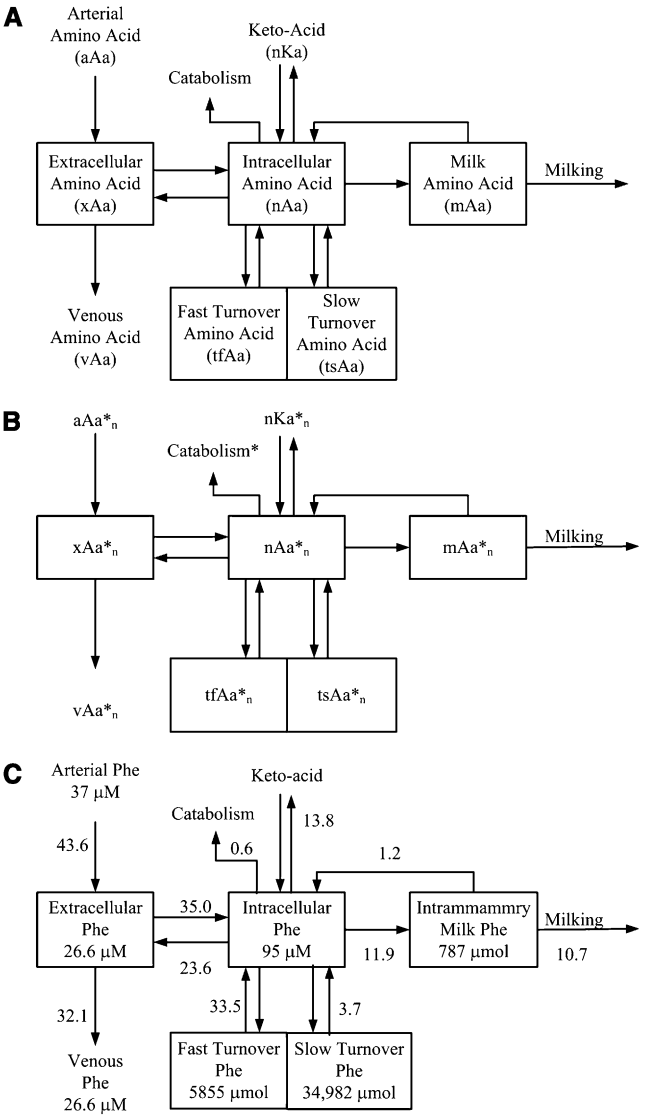

FIGURE 2 Flow diagram depicting a model of total (*A*) and labeled (*B*) AA flux in mammary tissue. Boxes represent pools and arrows represent fluxes. The entire structure in *B* is repeated for each isotope used as reflected in the subscript *n*, where $n = 1$–5. See the text for a complete description of abbreviations. *C* represents the model after fitting to Phe observations. Values associated with the arrows indicate fluxes (μmol/min). Pool sizes or concentrations are denoted by numbers below the description.

are summarized in **Table 1**. Not all model parameters could be uniquely deduced from the observed data and thus required 4 assumptions.

First, during development efforts, we deduced that the measured rate of Phe uptake by the gland was insufficient to account for the quantity of total and labeled Phe secreted as milk protein. Because Phe cannot be synthesized on a net basis in the udder, the latter observations suggest an underestimate in 1 or more of the measurements. This observation is consistent with previous observations of flow measured by dye-dilution that were greater than flow measured by trans-sonic flow probe in these goats (10), indicating alternative arterial sources of blood. To account for this problem and to balance Phe uptake with milk output, a factor (λ) was introduced to adjust mammary BF:

$$BF_{Mammary} = BF_{Observed} + (BF_{Observed} \times \lambda),$$

where λ was set to 0 initially and derived by fitting to the observed Phe data.

TABLE 1 Mean inputs used for simulations of mammary metabolism for each goat

Input variable	Equation	Goat				Mean	SD
		9G	1G	15F	10F		
$Q_{Tissue,Wet}$, g	(32)	0.95	1.80	0.86	1.19	1.20	0.42
f_{DM}, g/g	(32)	0.18	0.22	0.22	0.22	0.21	0.02
$f_{CP,DM}$, g/g	(32)	0.70	0.79	0.71	0.66	0.71	0.05
$BF_{Mammary}$, L/min	(1)	0.82	1.01	0.64	0.36	0.71	0.28
C_{aAa},[1] μmol/L	(7)	34	40	43	33	37	4
iC_{nAa},[1] μmol/L	(31)	92	121	105	63	95	25
iQ_{ttAa},[1] μmol	(31)	26,060	69,841	29,229	38,220	40,838	20,010

[1] Observed values for Phe.

Second, newly synthesized milk protein undergoes cleavage of the export signal peptide in the Golgi prior to secretion into the alveoli (17). Further, as the udder fills with milk, milk secretion is inhibited by the soluble feedback inhibitor of lactation (18), resulting in disruption of the secretory process that leads to degradation of milk protein and recycling of AA to the intracellular pool. Degradation was negligible when the inhibitor was absent and ranged from 10 to 25% when the inhibitor was present. Based on these observations, we assumed that 10% of synthesized milk protein was degraded and returned to the intracellular AA pool.

Third, a portion of the secreted milk remains in the udder after complete milking. This residual milk provides a pool of unlabeled AA (time = 0) or less enriched AA (time > 0) that will cause an apparent dilution of the Phe in the newly synthesized milk protein, i.e. protein present in the pool is made at a prior time point and is less enriched. Bruckmaier (19) observed that ~10% of milk present in the udder remained after milking without oxytocin administration and this was reduced to ~5% when oxytocin was used. Oxytocin was used herein and so we assumed that 5% of milk produced in a 12-h period remained in the udder after milking. Mean milk production was 1.63 kg/12 h, resulting in an estimated residual milk volume of 82 g.

Finally, we (13) have previously observed that Phe is converted to tyrosine within the udder and that this rate of conversion scales with milk protein output, equating to 4.6% of Phe flux in the mammary gland. This loss could be derived by mass Phe difference across the tissue; however, due to the first assumption, the catabolic rate for Phe was assumed to be 4.6% of milk Phe output, as both the catabolic rate and λ could not be derived from the data.

Rate constants describing AA uptake ($K_{xAa,nAa}$) and incorporation into milk protein ($K_{nAa,mAa}$) were derived using additive factors (α and δ, respectively) to adjust for animal effects. It is likely that catabolism also varies by animal, but in the absence of unique measures of catabolism, derivation of individual animal estimates was not possible. Parameters were derived in 2 phases. An initial estimate of $K_{xAa,nAa}$ and estimates of $K_{nAa,mAa}$, λ, α, and δ were derived from the concentrations of venous and intracellular AA and milk protein output, which yielded predicted AA mass balances that were in accordance with the observed values. $K_{nAa,mAa}$, λ, α, and δ were then fixed and $K_{xAa,nAa}$ plus parameters describing efflux from the cell ($K_{nAa,xAa}$), synthesis of tissue protein (non-milk) in fast turnover ($K_{nAa,tfAa}$) and slow turnover protein ($K_{nAa,tsAa}$) pools, deamination ($K_{nAa,nKa}$), the fraction of tissue protein AA present in the fast turnover pool, and the lag in time from the start of a tracer infusion until appearance in milk protein (*Lag*) were estimated from concentrations of venous and intracellular AA, milk protein output, and enrichments of AA in the mammary vein, intracellular free pool, milk protein, and tissue protein. These latter parameter estimates were all associated with the rate of cycling among pools. The 2-step parameter estimation prevented the much greater frequency of milk protein enrichment measurements from overwhelming those with fewer observations (milk yield and venous concentrations), a problem that was encountered when attempting to fit all the parameters at once. Parameter estimates were reported with their respective SE with the latter calculated as the square root of the variance estimate.

Parameterization followed the same pattern for Leu, Met, and Val; however, estimates for λ, *Lag*, and $K_{nAa,xAa}$ were retained from the Phe derivations; the former 2 are common to all AA and the latter could not be uniquely defined from the data due to the lack of blood sampling early in the infusion period. Because λ was fixed by Phe observations, $K_{nAa,Ox}$ could be derived for these AA.

Statistical analysis

Enrichments or specific activities of the intracellular free AA, tissue protein-bound AA, and milk protein-bound AA pools at the time of killing were expressed as percentages of the plateau values achieved in the corresponding plasma free AA pool. These percentages were then tested for significant differences within an AA by ANOVA with animal and pool treated as fixed effects. No interactions between animal and pool were considered. Because each isotope represented a single time point, time was also not considered in the model. Differences among sites were tested by construction of contrast statements. Differences were considered significant at $P < 0.05$. Observed values were reported as means ± SD, with SD being calculated between animals.

Residual errors associated with the final predictions from the metabolic model were calculated and used to generate mean square prediction errors. The latter was partitioned into mean bias, slope bias, and random dispersion elements and used to calculate root mean square prediction errors (RMSPE) according to Bibby and Toutenberg (20). RMSPE were expressed as a percentage of the mean predicted value so that comparisons across variables were standardized.

Results

The temporal labeling patterns of Leu, Met, Phe, and Val in milk casein were similar during the first 9 h of infusion (**Fig. 3**), which is consistent with our previous observations in goats (13). Staggering the Phe tracer infusions and terminal sampling allowed for an evaluation of the relative (percent of arterial plateau) labeling of Phe in casein, tissue-free, and tissue protein pools (**Fig. 4**). From this evaluation, it was apparent that the [^{15}N]Phe labeling (9-h time point) deviated from the other Phe tracers. Compared with the other Phe tracers, [^{15}N]Phe enrichment in casein and mammary tissue was less than expected, indicating that a proportion of the ^{15}N label was lost or diluted via a transamination reaction. The enrichments of Phe, Val, and Leu in casein and arterial plasma reached similar values by the end of the 30-h isotope infusions (**Fig. 5**), whereas [5-^{13}CH$_3$]Met enrichment was lower (13%; $P < 0.05$) in casein than in arterial plasma. For all 4 AA, their enrichments in casein were higher ($P < 0.001$), but to varying extents, than in the tissue homogenate free and tissue-bound pools at the end of the 30-h infusion.

Most parameter estimates were well defined by the Phe data (**Table 2**), with SE that were <15% of estimated values. This included α and δ when expressed as a percent of the parent parameter estimate. Correlations among parameters were generally low except for between *Lag* and $K_{nAa,tfAa}$ (−0.71), $K_{xAa,nAa}$ and $K_{nAa,xAa}$ (0.49), $K_{nAa,tfAa}$ and f_{tfAa} (−0.39), and

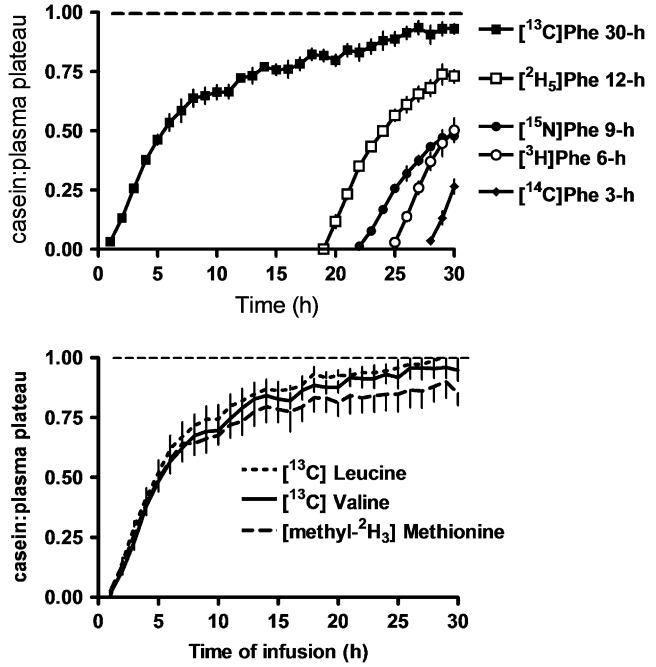

FIGURE 3 Observed casein enrichments or specific activities for different isotopes of Phe (top panel) and Leu, Val, and Met (bottom panel) over a 30-h period. Continuous infusions of the Phe isotopes were initiated at time 0, 18, 21, 24, and 27 h of the 30-h infusion period. Leu, Val, and Met were infused for the entire 30 h. All isotopes were infused at a constant rate into the jugular vein. Values are the mean ± SD, $n = 4$ proportion of the plasma enrichments or activities, and the dashed line at 1.0 represents the normalized plasma mean. Error bars denote ± 1 SD.

$K_{nAa,tfAa}$ and $K_{nAa,tsAa}$ (0.39). These did not appear to be problematic as evidenced by the lack of variance inflation for any of the parameter estimates. Although animal effects (α and δ) were numerically important with respect to Phe uptake and deposition in milk protein, they did not all differ from 0, as evidenced by SE for parameter estimates that were greater than one-half of the estimate (21).

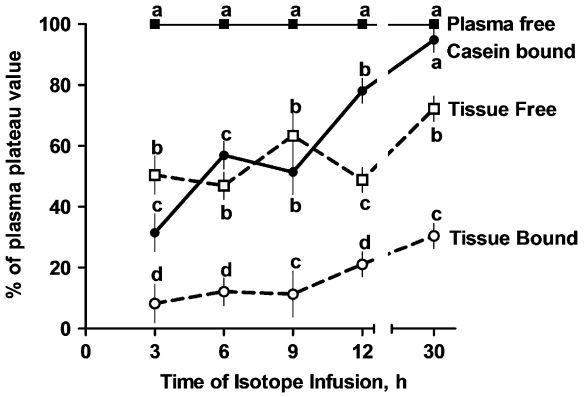

FIGURE 4 Temporal relationship of the labeling of various Phe pools of the mammary gland over a 30-h time course with 5 different Phe tracers infused into the jugular for 3 [^{14}C], 6 [^{3}H], 9 [^{15}N], 12 [$^{2}H_5$], or 30 [^{13}C] h. Values are the mean ± SD, $n = 4$ percentages of the plasma enrichment or specific activity at the end of all the tracer infusions. For each tracer, means at a time without a common letter differ, $P < 0.05$. Error bars denote ± 1 SD.

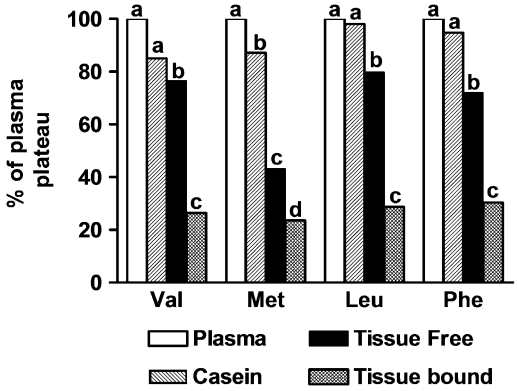

FIGURE 5 Relationship of enrichments or specific activities in plasma, tissue-free, tissue-bound, and casein pools for Val, Met, Leu, and Phe at the end of a 30-h jugular infusion of isotope. Values are expressed as a percentage of the plasma enrichment value. SE of the treatment differences were 0.05 for leucine and methionine and 0.04 for phenylalanine and valine. For each AA, means between pools without a common letter differ, $P < 0.05$.

Flux values and pool sizes for Phe are presented in **Table 3** and Figure 2C. The data were adequate to define unidirectional fluxes for Phe uptake from blood and efflux from the cell to blood (Table 3). Mean flux values for Phe uptake by ($F_{xAa,nAa}$) and efflux ($F_{nAa,xAa}$) from the organ were 35 and 24 μmol/min, respectively, resulting in a net uptake of 11 μmol Phe/min.

Predictions of venous and intracellular concentrations and milk Phe output had errors of <15%, with no apparent systematic bias (**Table 4**; **Supplemental Fig. 1**). RMSPE for venous and milk ^{13}C- and ^{2}H-Phe enrichments and milk ^{3}H-Phe–specific activities were <15%. However, RMSPE were greater for all other predictions. In some cases, such as ^{15}N-Phe in milk, there was no apparent systematic bias and the error was due solely to dispersion. However, there were systematic overpredictions of intracellular Phe and underpredictions of total tissue Phe enrichments and specific activities.

The lag in appearance of label in milk casein relative to the start of the isotope infusion was estimated at 81 ± 5 min, which is consistent with initial estimates of protein processing and export in pancreatic cells (22) and subsequent work in ruminants (13,23).

The mean total Phe pool size in tissue protein was 40,838 μmol, of which 14.3 ± 1.5% or 5855 μmol was estimated to reside in a rapid turnover pool. The rate constants for incorporation of Phe into rapid and slow turnover protein-bound Phe pools were 0.49 ± 0.06 and 0.05 ± 0.01 L/min, respectively, yielding mean flux values of 33.5 and 3.7 μmol/min, respectively (Table 3). Summing those rates yielded a rate of Phe incorporation in total tissue protein (nonmilk) that was 350% of the rate of milk protein synthesis (milk output). Rates of Leu and Val incorporation into total tissue protein were 133 and 225 μmol/min, respectively, values 5.8 and 7.1 times the rate of incorporation into milk protein (**Table 5**). However, the rate of Met incorporation into tissue was only 1.25 that of the rate of milk protein incorporation.

Phe deamination was estimated at 13.8 μmol/min compared with the assumed oxidation rate of 0.6 μmol/min. Thus, nearly all of deaminated Phe is apparently reaminated and this is further supported by the close balance of ^{13}C-Phe uptake to output in milk.

Sensitivity analyses indicated that parameter estimates for $K_{nAa,tfaa}$ were sensitive to the assumption regarding residual

TABLE 2 Parameter estimates derived from fitting the model to the observed Phe data

Parameter	Description	Equation	Estimate	SE
$K_{xAa,nAa}$, min^{-1}	Rate constant for uptake	(13)	5.10	0.45
α_2	Differential for goat 15F	(13)	1.12	0.55
α_3	Differential for goat 1G	(13)	−0.21	0.52
α_4	Differential for goat 9G	(13)	0.82	0.57
$K_{nAa,xAa}$, min^{-1}	Rate constant for efflux	(12)	0.342	0.001
$K_{nAa,mAa}$, min^{-1}	Rate constant for milk protein synthesis	(14)	0.192	0.025
δ_2	Differential for goat 15F	(14)	0.082	0.033
δ_3	Differential for goat 1G	(14)	−0.085	0.026
δ_4	Differential for goat 9G	(14)	0.020	0.032
Lag, *min*	Lag between introduction of label and appearance in milk	(30)	80.8	5.1
f_{tfAa}, *mol/mol*	Fraction of tissue protein in the fast turnover pool	(33)	0.143	0.015
$K_{nAa,tfAa}$, min^{-1}	Rate constant for fast turnover protein synthesis	(19)	0.487	0.062
$K_{nAa,tsAa}$, min^{-1}	Rate constant for slow turnover protein synthesis	(20)	0.054	0.010
$K_{nAa,xKa}$, min^{-1}	Rate constant for transamination	(16)	0.200	0.031
λ, *L/min*	BF adjustment	(1)	0.626	0.094

milk volume where greater residual milk resulted in lesser estimates for $K_{nAa,tfAa}$. This is not surprising given that residual milk protein represents a pool of unlabeled AA that will mix with newly synthesized milk protein resulting in an apparent dilution of the Phe label in exported milk protein. At a mean observed milk yield of 66.8 ± 15.6 g/h, the assumed 82 mL of residual milk represents 74 min of production. Combining residual milk with the derived estimate for *Lag* results in a residual milk protein pool of ~5 g compared with the observed total mammary tissue protein pool of 179 g. The residual milk protein pool would have needed to be >30 g to account for all of the label dilution in the early stages of the infusion if no fast turnover tissue pool were present.

Discussion

Parameter estimation and methods. Although no direct comparison was made with a flooding-dose technique, the approach herein provides precise, unbiased estimates of protein turnover and AA metabolism while avoiding the impacts of infusing large doses of a tracer AA (24). When used to assess a tissue where repeated measures of export protein enrichment can be collected, a single tracer would be adequate to assess rates of tissue protein synthesis. It would also be adequate to derive bidirectional fluxes of AA transport across the cell membrane provided the blood supply is sampled throughout the infusion period. If repeated measures of export protein enrichment are

TABLE 3 Flux predictions generated from the model when parameterized with the observed Phe data

Flux		Equation	Goat					
			9G	1G	15F	10F	Mean	SD
			μmol/min					
$F_{Aa,In}$	Arterial AA flux	(7)	46	65	44	19	44	19
$F_{xAa,vAa}$	Venous AA flux	(8)	35	52	31	11	32	17
	Net AA uptake	(7,8)	11	13	14	8	11	3
$F_{xAa,nAa}$	AA uptake	(11)	29	56	32	23	35	14
$F_{nAa,xAa}$	AA efflux	(12)	18	43	18	15	24	13
$F_{nAa,mAa}$	AA to milk protein	(18)	11	13	14	9	12	3
$F_{mAa,nAa}$	Milk protein to AA flux	(23)	1.1	1.3	1.4	0.9	1.2	0.3
dQ_{mAa}/dt	Net AA to milk AA flux	(4)	10	12	13	8	11	2
$F_{nAa,tfAa}$	AA to tissue protein, fast	(19)	26	61	26	22	34	18
$F_{nAa,tsAa}$	AA to tissue protein, slow	(20)	2.9	6.7	2.9	2.4	3.7	2.0
$F_{nAa,Ox}$	AA oxidation	(15)	0.5	1.1	0.5	0.4	0.6	0.3
$F_{nAa,nKa}$	AA transamination	(17)	11	25	11	9	14	7
	Turnover,[1] *%/d*		161	139	141	90	131	30
	Tissue protein synthesis, *g/d*		189	434	185	155	241	130

[1] AA turnover in the total tissue protein pool.

TABLE 4 RMSPE and a partition of those errors into mean bias, slope bias, and dispersion after fitting the model to the observed Phe data[1]

Variable	*n*	Mean observed	Mean predicted	RMSPE	Mean bias	Slope bias	Dispersion
				% of Observed mean	*% of Mean square prediction error*		
C_{vAa}, μmol/L	24	26.8	26.6	9.6	0.3	11.2	88.4
C_{nAa}, μmol/L	8	95.5	95.1	11.1	0.2	1.0	98.8
dQ_{mAa}/dt, μmol/min	24	10.8	10.7	13.3	0.1	1.1	98.8
E_{vAa1}	24	4.11	4.23	6.5	20.7	5.8	73.4
E_{vAa2}	24	5.04	4.83	8.0	27.5	2.6	69.9
E_{vAa3}	24	2.95	2.24	26.2	84.3	1.6	14.1
E_{vAa4}	14	33.7	36.5	23.2	12.6	24.9	62.6
E_{vAa5}	6	19.4	18.6	20.3	4.1	30.6	65.4
E_{nAa1}	8	3.14	3.87	26.7	76.1	0.3	23.6
E_{nAa2}	8	2.42	4.15	74.6	92.9	0.8	6.3
E_{nAa3}	8	1.81	1.43	31.5	43.7	22.6	33.8
E_{nAa4}	8	20.8	28.9	48.9	64.6	23.8	11.7
E_{nAa5}	8	11.3	11.9	29.9	3.5	51.2	45.2
E_{mAa1}	119	3.11	3.05	11.4	2.6	2.0	95.5
E_{mAa2}	47	2.54	2.61	12.6	4.9	1.2	93.9
E_{mAa3}	35	0.83	0.87	24.1	4.5	4.6	90.9
E_{mAa4}	24	14.2	12.9	13.3	46.4	1.6	52.1
E_{mAa5}	12	3.44	2.87	32.4	26.5	17.2	56.3
E_{ttAa1}	8	1.34	1.07	27.9	53.0	2.7	44.3
E_{ttAa2}	8	1.03	0.76	27.4	96.4	1.9	1.7
E_{ttAa3}	8	0.29	0.24	44.2	14.9	11.4	73.7
E_{ttAa4}	8	5.14	3.86	31.0	65.0	2.1	33.0
E_{ttAa5}	8	1.65	1.12	41.8	59.1	10.1	30.7

[1] Subscripts 1–5 for enrichments (atom percent excess) represent [^{13}C]-, [^{2}H]-, [^{15}N]-, [^{3}H]-, and [^{14}C]-Phe, respectively. Predictions were of venous (C_{vAa}) and intracellular (C_{nAa}) concentrations, Phe output in milk (dQ_{mAa}/dt), venous enrichment (E_{vAa}), intracellular enrichment (E_{nAa}), milk protein enrichment (E_{mAa}), and total tissue protein enrichment (E_{ttAa}).

not possible, such as for muscle, skin, and gut, use of multiple isotopes provides the needed temporal observations (25). Although liver is not considered an export tissue, it does secrete various proteins into blood that have been monitored in the past and could be used for this technique.

Under the assumption that the mammary gland extracts only free AA, we observed that to achieve total and labeled Phe balance required 62 ± 9% greater BF than was measured by flow probe. It is likely that collateral blood supplies not measured by the arterial flow probe had developed after surgical implantation, which is consistent with our previous findings for these goats (10). Although this adjustment affects parameter estimates for uptake and efflux from the udder, it does not alter estimates of flux rates for milk and tissue protein synthesis and AA metabolism within the organ, because these values are determined from isotopic enrichments and specific activities.

AA transport and metabolism. Unidirectional uptake of Phe from the extracellular space represented 81% of the mammary arterial supply of free Phe but 66% of that taken up was returned to the blood circulation. Such high rates of uptake and efflux confers significant flexibility in matching uptake to needed intracellular supplies. These rates are greater than the previously observed rates of 11.8 and 3.6 μmol/min for uptake and efflux, respectively, in goats fed a similar diet (26). However, the Biolo model (27) used in the prior work to derive estimates did not consider an extracellular pool; rather, it calculated uptake and efflux rates from arterial and venous concentrations and enrichments. Given the high rate of exchange between vascular and interstitial spaces, it is more appropriate to utilize venous concentrations and enrichments to calculate uptake, because the capillary and interstitial pools will be very close to equilibrium (28). Given the lack of plasma observations in the early stages of the infusion, estimates of cycling of the other AA could not be determined uniquely.

Deamination of Phe within the mammary glands was estimated to be 13.8 μmol/min, which represents almost 40% of Phe uptake. This was a surprising observation, because the primary route of Phe catabolism was thought to be hydroxylation to form tyrosine (29,30). However, hydroxylation would result in the loss of both ^{15}N and ^{13}C labels. The loss of the ^{15}N label with retention of the ^{13}C label in the current work indicates that deamination and subsequent reamination must have occurred, which is consistent with previous observations (13).

While consideration of Phe deamination in the model reduced systematic bias in milk protein ^{15}N enrichments, it resulted in greater mean bias in intracellular and extracellular enrichments of free ^{15}N-Phe compared with observed enrichments for other labeled forms of Phe (Table 4). Based on the latter, one must conclude that deamination of Phe occurs in a compartment separate from the compartments that exchange with the extracellular space and supply Phe for milk protein synthesis. Previous work has demonstrated that glutamine can act as a transamination donor for conversion of phenylpyruvate to Phe and the activity is present in both the cytosol and mitochondria of kidney and liver cells (31). If mitochondrial transamination occurs in mammary tissue, the compartmentalization of this activity could lead to the observed prediction errors as a common intracellular compartment was assumed (Table 4).

Prediction errors for concentrations and enrichments or specific activities of intracellular Leu and Met and intracellular

TABLE 5 Parameter estimates and mean model predictions derived from fitting the model to the observed Leu, Met, and Val data[1]

Parameter[2]	Leu	Met	Val
		min^{-1}	
$K_{xAa,nAa}$	7.2 ± 1.2	10.8 ± 2.0	4.8 ± 0.4
α_2	6.4 ± 2.9	3.8 ± 2.5	3.4 ± 0.8
α_3	−3.2 ± 1.3	−7.7 ± 2.0	3.1 ± 0.7
α_4	1.5 ± 1.1	−2.3 ± 2.1	0.3 ± 0.47
$K_{nAa,mAa}$	0.28 ± 0.1	0.54 ± 0.22	0.24 ± 0.03
δ_2	0.51 ± 0.31	−0.32 ± 0.19	0.19 ± 0.06
δ_3	0.47 ± 0.39	−0.01 ± 0.25	−0.12 ± 0.03
δ_4	−0.14 ± 0.07	−0.18 ± 0.18	−0.05 ± 0.03
$K_{nAa,Cd}$	0.12 ± 0.02	0.2 ± 0.06	0.03 ± 0.01
f_{tfAa}, *mol/mol*	0.06 ± 0.01	0.24 ± 0.06	0.2 ± 0.02
$K_{nAa,tfAa}$	1.55 ± 0.48	0.37 ± 0.06	1.2 ± 0.4
$K_{nAa,tsAa}$	0.07 ± 0.01	0.03 ± 0.02	0.04 ± 0.01
Mean predictions		$\mu mol/min$	
$F_{Aa,In}$	82	17	118
$F_{xAa,vAa}$	45	9	81
Net AA uptake	37	8	38
$F_{nAa,0x}$	14	3.1	5.4
dQ_{mAa}/dt	23	5	32
$F_{nAa,tfAa}$	127	5.6	218
$F_{nAa,tsAa}$	5.8	0.4	7.6
C_{xAa}, $\mu mol/L$	38	7	70
C_{nAa}, $\mu mol/L$	124	24	249
Turnover,[3] %/*d*	255	59	481
Tissue protein synthesis, g/d	379	89	866

[1] Values are parameter estimates ± SE. *Lag* and $K_{nAa,xAa}$ were assumed equal to values derived for Phe.
[2] Parameter abbreviations are defined in Table 2.
[3] AA turnover in the total tissue protein pool.

enrichments of Val and minimal prediction errors for venous enrichments and concentrations and milk enrichments of each also suggested some compartmentalization was occurring for these AA.

Protein synthesis. Mean Phe incorporation into tissue protein was 37 μmol/min (Table 3), whereas incorporation into milk protein was estimated to be 12 μmol/min. The mean fractional tissue protein synthesis rate based on Phe kinetics was 131%/d. On a protein equivalent basis, tissue protein synthesis would be 240 g/d using the observed Phe content of total tissue protein. This estimate is greater than a previous estimate of mammary tissue protein synthesis employing tracer Phe (107%/d) in lactating goats (8) when rates were calculated using the tissue homogenate-free pool activity as precursor. Given that the tissue homogenate-specific activity is lower than the true milk protein precursor pool, the previous estimates were thought to represent an overestimate of the true rate of protein synthesis. However, it appears that the underestimates of protein synthesis associated with the inevitable return of incorporated label to the precursor pool when using labeling periods longer than a few minutes partially offset errors associated with using the cell homogenate as a precursor pool. Use of a dynamic, compartmental representation that considered 2 pools of tissue protein herein accommodates the return of label from synthesized protein to the precursor pool and thus provides more accurate estimates of protein synthesis rates.

The majority (90%) of the Phe turnover in tissue protein was associated with a pool of rapidly degraded protein estimated at 5855 μmol of Phe. At a synthesis rate of 33.5 μmol/min of Phe, this pool would turnover in 174 min. Thus, even if a 15-min flooding dose technique were used, the high rate of turnover of this protein pool would result in significant return of tracer from the bound pool within the experimental time frame, which violates the key assumption that label return to the free pool is insignificant. If a classical steady-state infusion approach were taken, with measurements after 12 h, the pool would be labeled almost to the same enrichment as the arterial pool (**Supplemental Fig. 2**), resulting in almost equal rates of label incorporation and return to the free pool. Conversely, if one is attempting to estimate the contribution to milk protein synthesis of blood-derived nonfree Phe sources (e.g. peptides, proteins) (13) by comparison of milk protein and arterial Phe enrichments (dilution of label is an indicator of an alternative source of Phe), a number of days of isotope infusion would be required to completely label the slowly exchanging Phe pool of tissue protein. Assessment of the ratio of milk protein:blood Phe enrichments would only be valid after ~40 d of continuous isotope infusion. Prior to that, observed differences in enrichment may be partially or entirely due to unlabeled Phe released within the cell from protein degradation.

The differences in milk protein labeling patterns for the several AA during the 30-h isotope infusion (Fig. 3) at least partially represent the relative proportions and fractional incorporation rates for each AA in the fast and slow turnover protein pools. The overall fractional synthesis rates for Leu, Met, and Val were 255, 59, and 481%/d, respectively (Table 5). The Met rate was significantly less than the rate of 131%/d previously observed for lactating goats using tissue homogenate as a precursor pool and constant infusion techniques (7). A significant difference between the studies was the use of methyl-labeled Met herein compared with [^{35}S]Met in the previous work (7). It seems likely that the difference between studies may be due to trans-methylation of Met within the organ, which would dilute the label, resulting in lower enrichments in milk and tissue protein and reduced rates of apparent protein synthesis.

The fast turnover pools of Leu, Met, and Val had estimated fractional synthesis rates of 151, 8, and 97%/h (3614, 192, and 2328%/d), respectively, with pool sizes that were 6, 24, and 20% of their respective total tissue AA pools. The fractional synthesis rates for the slow turnover pools of Leu, Met, and Val were 10, 5, and 20%/d, representing <8% of tissue protein synthesis. The observed differences in synthesis rates at least partially reflect the heterogeneity of the aggregated protein pools. Obviously some proteins that are highly enriched in Leu and Val have high turnover rates, whereas other proteins that are enriched in Phe have relatively slower turnover rates. As noted above, the rates of synthesis calculated from Met may reflect transmethylation activity more than the composition of Met in tissue protein. This is supported by the observation that casein enrichments at 30 h for Leu, Phe, and Val all approximated their corresponding values in plasma (Fig. 5), whereas those for Met enrichment in casein were less ($P < 0.05$) than in plasma at 30 h.

The tissue free AA pool activity has often been monitored as a surrogate to the direct measurement of the immediate precursor pool for protein synthesis (i.e. amino-acyl-tRNA). As has been previously observed (32,33), the homogenate free AA pool activity was less than that of the synthesized protein, indicating that amino-acyl-tRNA are being charged from a pool more highly enriched than the average intracellular pool. Evidence indicates that liver export proteins are synthesized from AA

transported into the hepatocyte before they completely mix with the mixed intracellular pool, whereas AA for tissue protein synthesis derive from the mixed intracellular pool (34,35). To test this hypothesis for mammary, we modified equations describing isotope entry into the intracellular pool and protein synthesis pathways to reflect the potential for a diversion of newly transported AA directly into milk protein synthesis. This was accomplished using a variable to proportion transported Phe between intracellular entry and direct use for milk protein synthesis. The results indicated that approximately one-half of the Phe used for milk protein synthesis derived from Phe newly transported into the cell; however, a unique solution could not be determined. The challenge was that solving the intracellular AA bias resulted in the introduction of bias in other predictions, which is likely related to additional compartmentalization of Phe. The overall results clearly indicate that some channeling of Phe destined for export protein synthesis and for deamination must occur. Use of isotopomer analyses for casein enrichment would provide the true precursor enrichment, which could be used to solve for the proportion of AA channeled directly into protein. This approach could also be applied to tissue protein turnover in a tissue such as muscle where export proteins are absent provided multiple tracers were used. Analyzing 1 or more proteins in the tissue extract would provide an estimate of the precursor enrichment at a few time points, which should be adequate to determine the fraction of AA channeled directly into the monitored protein and thus allow a model solution. Channeling into other proteins may not be identical, but this would at least be a step in the correct direction toward more accurate estimates of tissue protein synthesis.

Energetic implications of tissue protein synthesis. When the current estimates for Phe are scaled to a lactating cow possessing a 20-kg udder comprised of 3 kg of tissue protein, the daily rate of tissue protein synthesis approximates to 3.93 kg compared with perhaps 1.2 kg of milk protein synthesis. Assuming a requirement of 4 mol of ATP to form 1 mol of peptide bond and a mean AA molecular weight in tissue protein of 110 g (4), the daily synthesis of 3.93 kg of tissue protein would consume 143 mol of ATP. Milk protein synthesis would consume an additional 48 mol of ATP. The estimate for tissue protein synthesis is considerably greater than the 9 mol of ATP required to support an 8% turnover rate used in the model of Baldwin (36) and possibly explains challenges that model has with predicting energy balance over a complete lactation cycle (37). It also does not support the use of basal energy requirements determined in a nonlactating animal to calculate maintenance requirements for a lactating one. However, this estimate is highly influenced by the rate of turnover of the fast protein pool and thus the relative proportions of Phe and other AA in that pool must be known to derive a more accurate estimate of ATP use. For example, if Phe is highly represented in the pool, the number of total peptide bonds formed per mole of Phe incorporated would be less that the above estimate. These differences likely explain the different apparent rates of total protein synthesis calculated from individual AA (Table 5).

Hanigan and Baldwin (3) used a model of mammary metabolism to estimate ATP synthesis and use associated with mammary metabolism and milk synthesis. It was predicted that an average cow generates 312 mol of ATP daily in the udder, but only 102 mol of explicit ATP use could be accounted for in general metabolism and milk component synthesis. The remaining 210 mol of ATP were assigned to cell maintenance functions that were not explicitly represented, such as tissue protein turnover, maintenance of membrane potential, and nucleic acid synthesis. If the above estimate of 143 mol of ATP to support tissue protein synthesis is used, the undefined ATP use would be reduced to 67 mol/d (210 – 143 = 67), which represents ~21% of the estimated total ATP supply. Rolfe and Brown (4) estimated that maintenance of membrane potential consumes 10–15% and RNA plus DNA synthesis consumes ~5% of total tissue oxygen consumption by hepatic tissues of rats in a resting, nonproductive state. Although the udder of a cow producing milk is clearly not in a resting state, application of these fractional energy use values to total ATP production yields an estimated ATP use of 47 mol/d for membrane potential and 16 mol/d for DNA and RNA turnover, which almost perfectly matches the remaining undefined use of 67 mol of ATP. Thus, the energy needed to support such high rates of protein turnover in the udder is consistent with predicted energetic balance and has significant implications for calculating daily maintenance requirements of mammary tissue at differing physiological states.

In conclusion, rates of tissue protein synthesis were successfully estimated from temporal labeling patterns in blood AA and milk protein. Fractional rates of AA incorporation into tissue protein ranged from >250%/d for Leu and Val to 131%/d for Phe These rates are equal to or greater than the upper range of previous measurements and represent between 55 and 88% of the total protein synthesis (tissue plus milk) in the udder. Such high rates of synthesis could be expected to consume almost one-half of the available ATP supply generated in the lactating udder on a daily basis. Compartmentalization of intracellular free AA was apparent and 2 pools of Leu, Met, Phe, and Val in tissue protein could be discerned, one with a rapid rate of turnover and the other with a slow rate of turnover. Unidirectional transport of Phe from the extracellular compartment removed ~80% of the arterial supply to the udder, but one-half of that taken up was returned back to the extracellular space. Phe deamination was very active in mammary tissue, but the majority of deaminated Phe is reaminated. Substantial trans-methylation of Met occurs in mammary tissue.

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