An in vitro method for assessment of amino acid bidirectional transport and intracellular metabolic fluxes in mammary epithelial cells

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ABSTRACT

Understanding uptake of AA by mammary tissue as supply varies is critical for predicting milk component production. Our objective was to develop an in vitro method to quantify cellular uptake, efflux, and intracellular metabolism of individual AA that could be implemented for evaluating these factors when AA supply and profile are varied. Bovine primary mammary epithelial cells were grown to confluency and exposed to medium with an AA profile and concentration similar to lactating dairy cow plasma for 24 h. Cells were then preloaded in medium enriched with ¹⁵N-labeled AA for 24 h followed by removal of the ¹⁵N-labeled medium and incubation with medium enriched with ¹³C-labeled AA for 0, 15, 60, 300, 900, 1,800, and 3,600 s. Extracellular free AA and intracellular free and protein-bound AA were analyzed for concentrations and isotopic enrichment by gas chromatography-mass spectrometry. A dynamic, 12-pool model was constructed representing extracellular and intracellular free and protein-bound pools of an AA, and their respective ¹⁵N and ¹³C isotopes. Markov chain Monte Carlo simulation ($n = 5,000$) was conducted to evaluate prediction errors by deriving standard errors and posterior distributions for rate constants, fluxes, and pools. Cellular Ala influx and efflux were higher than Leu, reflecting Ala role in driving system L transport and the high capacity of sodium-dependent transport. The Ala and Leu turnover rates were 181 and 95, 580 and 857, and 74 and 157% per hour for extracellular, intracellular, and fast protein-bound pools, respectively. The intracellular and extracellular Ala to Leu ratios were quite different, meaning the blood AA profile is not the AA profile provided for protein translation. The high level of exchange

and rapid turnover of pools provide a mechanism for matching the AA supplies to the precision necessary for translation. This also understates the importance of using experimental medium similar to what is observed in vivo given that some AA depend on other AA for influx (exchange driven). The average root mean squared prediction error across the isotope enrichments, pools, and concentrations was 9.7 and 14.1% for Ala and Leu, respectively, and collinearity among parameters was low, indicating adequate fit and identifiability. The described model provides insight on individual AA transport kinetics and a method for future evaluation of AA transport and intracellular metabolism when subjected to varying AA supplies.

Key words: amino acids, transport, isotope, Ala, Leu

INTRODUCTION

Udder AA uptake from arterial supplies and synthesis into milk proteins is relatively low. Only 22% of arterial AA daily influx is transformed into milk protein, although it varies considerably across AA (Arriola Apelo et al., 2014). If more AA could be taken up by the udder cells, then exiting venous blood would have lower AA concentrations. Lower AA venous concentrations will reduce splanchnic catabolism because these tissue-beds extract AA by mass action (Hanigan et al., 2004). The splanchnic catabolism of AA is the primary culprit of the low N efficiency with milk production from dairy cows (Hristov et al., 2004; Arriola Apelo et al., 2014).

Intracellular AA concentrations partially regulate milk protein synthesis rate (Cant et al., 2018). If linear (mass action) net AA uptake is assumed by the udder, decreasing or increasing dietary MP supplies would change intracellular AA concentrations and thus milk protein synthesis rate. To make a sizable increase in N efficiency (30% gross efficiency), maintaining or increasing udder cell AA concentrations would be required when MP supplies are reduced. This could be achieved by increased cellular AA transport efficiency.

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Amino acids can enter mammary epithelial cells via 3 mechanisms: passive diffusion, sodium-driven gradient, or AA exchange (Taylor, 2014). Active transporters lack high specificity. Some transporters work with up to a dozen AA and AA can use multiple transporter systems (Christensen, 1990). The active transporters can build up steep intracellular concentration gradients relative to extracellular concentrations (“loaders”), whereas others act as exchangers (“harmonizer”) to catalyze transport of other AA (Bröer and Bröer, 2017). In the mammary glands of cows, these “loader” transporters consist of system A, system Gly, system N, system X_{ag}, and system y+ (Shennan and Boyd, 2014). The “harmonizer” transporters consist of system ASC, system L, and system y+L. Another class of transporters reacts to short-term AA deprivation by increasing expression and activity. These are referred to as “rescue” transporters [i.e., SNAT2 within system A and CAT-1 within system y+ (Bröer and Bröer, 2017)]. Amino acid transporters likely have evolved to manage the variation in daily influx of AA so that intracellular concentrations are better matched to protein synthesis needs (Christensen, 1990). Net flux of an AA often can be quite different than influx and efflux, and the bidirectional flows confer more flexibility than that which could be achieved with a single, unidirectional flux.

Previously, net AA fluxes were observed to exhibit mass action responses to arterial AA supplies in lactating dairy cows and pig mammary tissue explants (Hanigan et al., 1992; Hurley et al., 2000; Jackson et al., 2000). Many experimental protocols have evaluated a single AA with various competitor AA or inhibiting molecules on kinetics and transporter activity (Tovar et al., 2000). However, the AA concentrations evaluated are usually quite different from in vivo, and the interaction with the transport of all the other 19 AA has not been assessed. Studies of AA transport should consider that individual AA uptake is not univariate, but likely a 20 dimension problem (Christensen, 1990). Additionally, measuring the enrichment of an isotope intracellularly will provide some information on net uptake, but it does not allow derivation of unidirectional uptake or intracellular metabolism. Knowledge of the AA unidirectional uptake kinetics are necessary for better predicting how intracellular AA concentrations change when MP supplies are varied.

The objectives of this study were to develop an in vitro method for simultaneous measurements of bidirectional transport and intracellular metabolism of multiple AA. We hypothesized that use of 3 unique isotopic labels per AA and the construction and fitting of a dynamic 12-pool model to the data will address our objectives.

MATERIALS AND METHODS

Experimental Design, Materials, and Protocol

Bovine primary mammary epithelial cells were obtained from the State Key Laboratory of Animal Nutrition, Institute of Animal Science, Chinese Academy of Agricultural Sciences, Beijing, China, and evaluated for being bovine and epithelial in origin (Yoder et al., 2019). Cells were seeded on two 89.4-mm × 19.2-mm tissue culture-treated polystyrene cell culture dishes (#353003, Corning Inc., Corning, NY) at a density of 1.9×10^6 cells per dish and incubated at 37°C under 5% CO₂ (HERAcell 150i, Thermo Fisher Scientific, Waltham, MA). The culture growth medium was DMEM/F-12 (#D8900, Sigma-Aldrich, St. Louis, MO) with the following additions: 10% fetal bovine serum, 5 mg/L bovine insulin (#700–112P, Gemini Bio-Products, Sacramento, CA), 5 mg/L prolactin (#L6520, Sigma-Aldrich), 5 mg/L progesterone (#P7556, Sigma-Aldrich), 5 mg/L transferrin (#T8158, Sigma-Aldrich), 0.01 mg/L epidermal growth factor (#E4127, Sigma-Aldrich), 1% of a 100 × antibiotic-antimitotic mix (#15240062, Gibco, Waltham, MA), and 1 mg/L hydrocortisone (#H0888, Sigma-Aldrich). All media were adjusted to a pH of 7.3 and sterile filtered through a 0.20-μm filter (Nalgene Rapid-Flow, #595–4520, Thermo Fisher Scientific). Cells were cultured for 5 d and then subsequently transferred to 27 culture dishes (89.4 mm × 19.2 mm, #353003, Corning Inc.) and incubated in growth medium. Culture dishes were weighed before addition of cells and medium. Cells were cultured to 90% confluency and then the growth medium was replaced with a steady-state medium. The steady-state medium consisted of 10% Earle’s balanced salts solution (#E7510, Sigma-Aldrich), sodium bicarbonate (2.2 g/L), D-glucose (2.151 g/L), 1% of a 100 × minimal essential medium vitamin solution (#M6895, Sigma-Aldrich), and the previously described hormones and antibiotics contained in the growth medium. Individual AA were added to the steady-state medium at the average plasma concentration of lactating dairy cows (DIM = 75 d, milk yield = 45.1 kg/d; Swanepoel et al., 2016; Supplemental Table S1, <https://doi.org/10.3168/jds.2020-18155>). Cells remained in this steady-state medium for 24 h before the experiment (medium changed every 12 h). A graphical outline of the experiment is provided in Figure 1.

Universally ¹³C-labeled AA derived from algae (U-[¹³C], 97–99% enriched, lot #30287, product #CLM-1548), universally ¹⁵N-labeled AA derived from algae (U-[¹⁵N], 98% enriched, lot #24163, product #NLM-2161), and ¹³C¹⁵N universally labeled AA derived from

Figure 1. Experimental outline, timeline, media treatments, and measurements of the cell culture experiment for assessment of AA transport. DMEM = Dulbecco's modified Eagle medium.

algae (U- $^{13}\text{C}^{15}\text{N}$, 98% enriched, lot #16824, product #CNLM-452) were purchased from Cambridge Isotope Laboratories Inc. (Tewksbury, MA). Amino acid concentrations and profiles of the 3 labeled algae were determined (Supplemental Table S2; <https://doi.org/10.3168/jds.2020-18155>) by measurement of the isotope dilution associated with the addition of a known amount of 18 unlabeled AA (#AAS18, Sigma-Aldrich) using GC-MS. The enriched algae previously underwent acid hydrolysis; hence, Gln, Asn, Cys, and Trp were not present in the algae. The $^{13}\text{C}^{15}\text{N}$ -enriched algae was also analyzed by HPLC at the University of Missouri for comparison purposes (Supplemental Table S2; <https://doi.org/10.3168/jds.2020-18155>). Media mimicking the steady-state medium components and concentrations, except that the AA were enriched with ^{13}C -labeled AA or ^{15}N -labeled AA, were formulated. These media will be referred to as ^{13}C -enriched medium or ^{15}N -enriched medium (Table 1).

Upon removal of the steady-state medium, cells were washed once with warm PBS (37°C) and 10 mL of the ^{15}N -enriched medium was added per plate. Cells were incubated 24 h in ^{15}N -enriched medium that was changed every 8 h to minimize the effects of AA depletion. During the ^{15}N incubation, 0.4 mL of medium was sampled at 8, 16, 22, and 23 h from 3 plates and stored at -20°C to assess extracellular AA concentrations and enrichment. Following 24 h of ^{15}N incubation, cells were washed twice with warm PBS (37°C), and 6 mL of warm ^{13}C -enriched medium (37°C) was added to the cells. Cells were incubated with the ^{13}C -enriched

medium for 0 s, 15 s, 1 min, 5 min, 15 min, 30 min, and 60 min. The zero time point was utilized to correct for extracellular medium contamination of intracellular AA during cell lysis (Darmaun et al., 1988). Other selected time points were based on preliminary experiments (data not shown). At each of the time points, 1 mL of medium was removed from the plate, placed into ice-cold 1.5-mL tubes, and stored at -20°C . Following medium removal, cells were rinsed 3 times with ice-cold PBS and the culture dish was weighed. Pre-weighed ice-cold 50% sulfosalicylic acid was then added, and the plate was vigorously agitated to lyse the cells. Cell debris were mechanically detached using a cell scraper, and the homogenate was pipetted into an ice-cold 2-mL tube and stored at -20°C . Upon removal of cells, the culture dish was weighed again.

Viable live cells from 3 culture dishes were counted following 60 min of incubation with the ^{13}C -enriched medium. Culture dishes were washed twice with warm PBS (37°C) and each plate was weighed. Next, 1 mL of 0.05% Trypsin EDTA (Sigma-Aldrich, St. Louis, MO) was added to the plate and incubated at 37°C for 5 min. Upon visible detachment of cells, cells were removed in warm PBS and centrifuged for 5 min at $500 \times g$ at room temperature. The supernatant was decanted, and the pellet resuspended in PBS and centrifuged for another 5 min at $500 \times g$ at room temperature. The supernatant was then discarded. The pellet was resuspended in PBS, the weight was recorded, and 100 μL of the suspensions was removed for cell counting using a hemacytometer and Trypan Blue solution, 0.4%

(#K940, Amresco, Solon, OH). The remaining sample was centrifuged at $2,500 \times g$ at room temperature for 5 min, the supernatant decanted, and the cells were lysed by the addition of 1 mL of 2% SDS lysis buffer containing phosphatase (#78426, Thermo Fisher Scientific) and protease (#G6521, Promega, Madison, WI) inhibitors. The samples were constantly agitated for 30 min at 4°C and centrifuged for 1 h at $21,130 \times g$ at 4°C to remove insoluble material. The supernatant was stored at -80°C. Total protein in the lysate was determined using a bicinchoninic acid assay (Smith et al., 1985).

AA Analyses

The extracellular medium contents were gravimetrically weighed, deproteinized using 7% sulfosalicylic acid, and centrifuged at $16,000 \times g$ for 15 min at 4°C. The intracellular lysates plus sulfosalicylic acid (added when cells were harvested) were gravimetrically weighed and centrifuged at $16,000 \times g$ for 15 min at 4°C. Supernatants of the extracellular medium and intracellular contents were gravimetrically weighed and combined with $^{13}\text{C}^{15}\text{N}$ universally labeled AA algae (97–99% enriched, Cambridge Isotope Laboratories Inc., Tewksbury, MA). The intracellular content pel-

let remaining following removal of the intracellular supernatant was gravimetrically weighed and combined with 250 μL of a mixture containing $^{13}\text{C}^{15}\text{N}$ universally labeled AA algae (97–99% enriched, Cambridge Isotope Laboratories Inc.) and 33.3 μL of 6 M HCl containing 0.1% phenol per mg of pellet and hydrolyzed for 20 h at 97.5°C. The acid hydrolysates from the extracellular and intracellular contents and the intracellular pellet were then desalted by ion chromatography (AG 50W-X8 resin, Bio-Rad, Hercules, CA), eluted with 2 N ammonia hydroxide into silanized glassware, and freeze-dried. Dried samples were resolubilized in 0.1 N HCl and derivatized in acetonitrile (J. T. Baker Inc., Phillipsburg, NJ) to form *N*-(*tert*-butyldimethyl) AA derivatives by incubation for 1 h at 70°C in *N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide (SELECTRA-SIL, UCT Inc., Bristol, PA). Amino acid derivatives were separated by GC (Trace GC Ultra, Thermo Fisher Scientific) and quantified by mass spectrometry (DSQII; Thermo Fisher Scientific) for ^{15}N , ^{13}C , and $^{15}\text{N}^{13}\text{C}$ enrichments of ions derived from each AA as previously described (Calder et al., 1999). Calibration curves for derivation of AA concentrations from the isotopic ratios were developed using the same labeled AA mixture spiked into the samples gravimetrically combined in varying ratios with an AA standard

Table 1. Amino acid concentrations and atom percent enrichment of the ^{13}C and ^{15}N media before exposure to the cells

AA	AA concentration and enrichment					
	^{15}N -enriched medium ¹			^{13}C -enriched medium ²		
	Mean, μM	SD, μM	AP ³	Mean, μM	SD, μM	AP ⁴
Ala	324	0.6	55	307	5.9	54
Arg	66	2.8	27	81	2.7	36
Asp	50	1.1	84	—	—	99
Glu	80	4.2	87	42	31.7	89
Gly	375	5.3	29	322	2.1	10
Ile	124	1.4	62	128	2.1	67
Leu	187	2.2	68	202	3.4	68
Lys	96	7.8	74	59	8.8	52
Met	19	1.5	68	19	0.7	65
Phe	70	3.5	73	59	6.2	99
Pro	115	1.7	54	117	1.1	34
Ser	190	17.5	37	139	8.8	32
Thr	195	0.6	38	168	0.4	58
Tyr	57	1.3	56	37	0.7	38
Val	285	1.2	42	274	5.9	41

¹Represents 3 samples.

²Represents 3 samples.

$$^3\text{AP: atom percent} = \left(\frac{^{15}\text{N}}{^{12}\text{C} + ^{15}\text{N}} \right) \times 100.$$

$$^4\text{AP: atom percent} = \left(\frac{^{12}\text{C}}{^{12}\text{C} + ^{13}\text{C}} \right) \times 100.$$

mix (#AAS18, Sigma-Aldrich) plus L-Asn, L-Gln, L-Trp, and L-Cys (Sigma-Aldrich).

Model Development

A dynamic system of differential equations and state variables was constructed to represent the transport and metabolism of a single AA within mammary epithelial cells. The described model was coded in R (version 3.3.2 and RStudio version 1.0.13; R Core Team, 2016). Primary-state variables were extracellular free AA ($Q_{xAA(i)}$), intracellular free AA ($Q_{nAA(i)}$), intracellular fast turnover protein-bound AA ($Q_{tfAA(i)}$), and intracellular slow turnover protein-bound AA ($Q_{tsAA(i)}$) to represent the mass of ^{12}C AA, ^{13}C AA, and ^{15}N AA resulting in 12 pools (Table 2). Rate constants and fluxes are described in Table 3. Mass (Q), flux (F), concentration (C), and volume (V) had units of nmol, nmol/min, μM , and mL, respectively. Fast and slow turnover protein pools were used to represent the variable return of protein-bound label to the intracellular free pool with respect to time. Such a representation is an oversimplification, as each protein has its own respective protein turnover pool, and likely ranges from nanoseconds to years in time. Previously, it has been demonstrated that specification of fast and slow protein turnover pools is needed to adequately represent isotopic movement within the pool over 30-h time periods (Hanigan et al., 2009; Estes et al., 2018).

The volume of the extracellular free AA pool (V_x) was assumed to be 6 mL of medium, whereas the intracellular free AA pool (V_n) and the intracellular protein-bound pool (V_t) were calculated from measurements of their respective weights. Three separate measurements of intracellular volume were conducted: volume observed before removal, volume of cells removed, and volume of cells mechanically harvested. For our purposes, we designated the latter as the volume of the intracellular free AA pool because of the high variation in the other 2 measurements (Supplemental Table S3, <https://doi.org/10.3168/jds.2020-18155>).

Following removal of the free AA during deproteinization, the pellet was weighed, and it was weighed again before acid hydrolysis. The weight of the pellet was assumed to be the average of these 2 measurements and designated as the intracellular protein-bound pool. The mass of the extracellular free AA, intracellular free AA, and total-bound protein pools for the i th AA (i = Ala and Leu) were determined as

$$Q_{xAA(i)} = V_{x(i)} \times C_{xAA(i)}, \quad [1]$$

$$Q_{nAA(i)} = V_{n(i)} \times C_{nAA(i)}, \quad [2]$$

$$Q_{tAA(i)} = V_{t(i)} \times C_{tAA(i)}, \quad [3]$$

where $Q_{xAA(i)}$, $Q_{nAA(i)}$, and $Q_{tAA(i)}$ represent the mass of extracellular AA, intracellular AA, and protein-bound AA of only ^{12}C AA. The volume and concentration of the intracellular and protein-bound pools were measured at each time point ($n = 24$). The mass of each isotope was calculated from the pool size and isotopic enrichment:

$$Q_{xAA(i)}^{13\text{C}} = Q_{xAA(i)} \times \left(\frac{C_{xAA(i)}^{13}}{C_{xAA(i)}^{12}} \right), \quad [4]$$

$$Q_{xAA(i)}^{15\text{N}} = Q_{xAA(i)} \times \left(\frac{N_{xAA(i)}^{15}}{C_{xAA(i)}^{12}} \right), \quad [5]$$

$$Q_{nAA(i)}^{13\text{C}} = Q_{nAA(i)} \times \left(\frac{C_{nAA(i)}^{13}}{C_{nAA(i)}^{12}} \right), \quad [6]$$

$$Q_{nAA(i)}^{15\text{N}} = Q_{nAA(i)} \times \left(\frac{N_{nAA(i)}^{15}}{C_{nAA(i)}^{12}} \right), \quad [7]$$

$$Q_{tAA(i)}^{13\text{C}} = Q_{tAA(i)} \times \left(\frac{C_{tAA(i)}^{13}}{C_{tAA(i)}^{12}} \right), \quad [8]$$

$$Q_{tAA(i)}^{15\text{N}} = Q_{tAA(i)} \times \left(\frac{N_{tAA(i)}^{15}}{C_{tAA(i)}^{12}} \right). \quad [9]$$

The initial proportion of the protein-bound pool resident in the fast and slow protein pools for each AA was estimated as that yielding the best model fit when varying the fast protein pool size by a fraction of 0.5 to 10% of the total protein-bound pool. The fast protein pool fraction was denoted f_{tf} :

$$Q_{tfAA(i)} = Q_{tAA(i)} \times f_{tf(i)}, \quad [10]$$

$$Q_{tsAA(i)} = Q_{tAA(i)} - Q_{tfAA(i)}, \quad [11]$$

where $Q_{tAA(i)}$, $Q_{tfAA(i)}$, and $Q_{tsAA(i)}$ represent the total protein turnover, fast protein turnover, and slow protein turnover pools, respectively. The mass of each isotope for the fast protein pool was then calculated using the fast protein pool enrichment and the fast protein pool mass. The slow protein pool isotope mass was then calculated by subtraction:

$$Q_{tfAA(i)}^{13C} = Q_{tfAA(i)} \times \left(\frac{C_{tfAA(i)}^{13C}}{C_{tfAA(i)}^{12C}} \right), \quad [12]$$

$$Q_{tsAA(i)}^{13C} = Q_{tAA(i)}^{13C} - Q_{tfAA(i)}^{13C}, \quad [13]$$

Table 2. Description of initial estimates for volume, concentrations, pool sizes, and isotopic ratios for Ala and Leu

Item ¹	Description	Unit	Ala ²	Leu ²
V_x	Extracellular	mL	6.0	6.0
V_n	Intracellular	mL	0.32	0.32
V_p	Protein-bound volume	mL	0.079	0.079
C_{xAA}	Extracellular, ¹² C free AA	μM	114	54
C_{nAA}	Intracellular, ¹² C free AA	μM	1,376	237
C_{tAA}	Protein-bound, ¹² C AA	μM	18,456	54,476
Q_{xAA}	Extracellular, ¹² C free AA	nmol	684	322
Q_{xAA}^{13C}	Extracellular, ¹³ C free AA	nmol	976	818
Q_{xAA}^{15N}	Extracellular, ¹⁵ N free AA	nmol	161	58
Q_{nAA}	Intracellular, ¹² C free AA	nmol	440	76
Q_{nAA}^{13C}	Intracellular, ¹³ C free AA	nmol	97	33
Q_{nAA}^{15N}	Intracellular, ¹⁵ N free AA	nmol	378	64
Q_{tAA}	Protein-bound, ¹² C AA	nmol	1,458	4,304
Q_{tAA}^{13C}	Protein-bound, ¹³ C AA	nmol	153	8.7
Q_{tAA}^{15N}	Protein-bound, ¹⁵ N AA	nmol	506	1928
Q_{tfAA}	Fast protein-bound, ¹² C AA	nmol	36.5	43.0
Q_{tfAA}^{13C}	Fast protein-bound, ¹³ C AA	nmol	3.82	0.09
Q_{tfAA}^{15N}	Fast protein-bound, ¹⁵ N AA	nmol	31.3	36.5
Q_{tsAA}	Slow protein-bound, ¹² C AA	nmol	1,422	4,261
Q_{tsAA}^{13C}	Slow protein-bound, ¹³ C AA	nmol	149.1	8.7
Q_{tsAA}^{15N}	Slow protein-bound, ¹⁵ N AA	nmol	475	1,891
E_{xAA}^{13C}	Extracellular isotope ratio of ¹³ C to ¹² C		1.43	2.54
E_{xAA}^{15N}	Extracellular isotope ratio of ¹⁵ N to ¹² C		0.24	0.18
E_{nAA}^{13C}	Intracellular isotope ratio of ¹³ C to ¹² C		0.22	0.43
E_{nAA}^{15N}	Intracellular isotope ratio of ¹⁵ N to ¹² C		0.86	0.85
E_{tAA}^{13C}	Protein-bound isotope ratio of ¹³ C to ¹² C		0.10	0.002
E_{tAA}^{15N}	Protein-bound isotope ratio of ¹⁵ N to ¹² C		0.35	0.45

¹Model abbreviations for volume (V), concentration (C), pools (Q), and isotope ratios (E).

²Alanine and leucine initial values represent the average of 6 plates of cells for volume and concentration, whereas mass of ¹²C, mass ¹³C, and ¹⁵N and isotope ratios represent the average of 3 plates of cells.

Table 3. Descriptions, abbreviations, and units of rate constants and fluxes

Variable ¹	Description	Unit
$k_{xAA nAA}$	Rate constant for uptake	min
$k_{nAA xAA}$	Rate constant for efflux	min
$k_{nAA tfAA}$	Rate constant for fast turnover protein synthesis	min
$k_{nAA tsAA}$	Rate constant for slow turnover protein synthesis	min
$k_{nAA nKA}$	Rate constant for transamination	min
$k_{nAA Ox}$	Rate constant for oxidation	min
$f_{synthesis}$	Fraction constant for NEAA synthesis	min
f_{tf}	Fast turnover fraction of protein-bound pool	%
f_{carbon}	Fraction of ¹³ C carbon used for reamination or synthesis	%
$f_{nitrogen}$	Proportion of ¹⁵ N nitrogen used for reamination or synthesis	%
$F_{xAA nAA(i,j,k)}$	Flux AA from extracellular to intracellular	nmol/min
$F_{nAA xAA(i,j,k)}$	Flux of AA from intracellular to extracellular	nmol/min
$F_{nAA Ox(i,j,k)}$	Flux of AA from intracellular to oxidation	nmol/min
$F_{nAA tfAA(i,j,k)}$	Flux of AA from intracellular to fast protein synthesis	nmol/min
$F_{tfAA nAA(i,j,k)}$	Flux of AA from fast protein degradation to intracellular	nmol/min
$F_{nAA nKA(i,j,k)}$	Flux of AA from intracellular to transamination	nmol/min
$F_{nKA nAA(i,j,k)}$	Flux of AA from transamination to intracellular	nmol/min
$F_{nSynAA(i,j,k)}$	Synthesis flux of AA to intracellular	nmol/min
$F_{nKA nAA u(i,j,k)}$	Flux of ¹² C AA from transamination of ¹³ C AA to intracellular	nmol/min

¹Subscripts i , j , and k represent the flux of ¹²C-, ¹³C-, or ¹⁵N-enriched AA, respectively.

$$Q_{tfAA(i)}^{15N} = Q_{tfAA(i)} \times \left(\frac{N_{tfAA(i)}^{15}}{C_{tfAA(i)}^{12}} \right), \quad [14]$$

$$Q_{tsAA(i)}^{15N} = Q_{tAA(i)}^{15N} - Q_{tfAA(i)}^{15N}. \quad [15]$$

Mass action kinetics were assumed for the fluxes among pools which included the following: AA influx and efflux between the extracellular medium and intracellular space; transamination, catabolism, or use for intracellular protein synthesis; and influx and efflux from the fast protein pool. Additionally, for Ala specifically, as an NEAA, intracellular Ala synthesis was considered. Flux descriptions are provided in Table 3. The differential equation describing the extracellular medium pool for the i th AA (i = Ala and Leu) was

$$\frac{dQ_{xAA(i)}}{dt} = F_{nAA xAA(i)} - F_{xAA nAA(i)}, \quad [16]$$

where $F_{nAA xAA(i)}$ represents efflux of AA from intracellular free pool to extracellular pool and $F_{xAA nAA(i)}$ the uptake of AA by the cell. The isotopic fluxes were calculated using the identical form as described above and for the remaining fluxes as follows:

$$E_a = \frac{Q_a^*}{Q_a}, \quad [17]$$

$$F_{a,b}^* = F_{a,b} \times E_a, \quad [18]$$

where E_a , Q_a^* , and Q_a represents the isotope ratio, mass of the isotope, and mass of ¹²C respective of a particular AA pool. A particular flux of a ¹²C AA from pool A to B is represented as $F_{a,b}$ and the flux of the isotope AA from pool A to B is represented as $F_{a,b}^*$. The AA influx was represented by the following for AA and the respective isotopes:

$$F_{xAA nAA(i)} = k_{xAA nAA(i)} \times Q_{xAA(i)}, \quad [19]$$

where $k_{xAA nAA(i)}$ represents the mass action rate constant for uptake of AA by the cells. The AA efflux was

$$F_{nAA xAA(i)} = k_{nAA xAA(i)} \times Q_{nAA(i)}, \quad [20]$$

where $k_{nAA xAA(i)}$ represents the mass action rate constant for AA efflux by the cells.

The differential for the intracellular free AA was also considered for the i th AA and the respective isotopes:

$$\begin{aligned} \frac{dQ_{nAA(i)}}{dt} = & F_{xAA nAA(i)} + F_{tfAA nAA(i)} + F_{nKA nAA(i)} + F_{nSynAA(i)} \\ & + F_{nKA nAA u(i)} - F_{nAA xAA(i)} - F_{nAA Ox(i)} - F_{nAA tfAA(i)} - F_{nAA nKA(i)}, \end{aligned} \quad [21]$$

where $F_{tfAA nAA(i)}$ represents the flux from degradation of the fast protein pool, $F_{nKA nAA(i)}$ represents reamination

of keto-acids to form AA in the intracellular free AA pool, $F_{nSynAA(i)}$ represents synthesis of AA, $F_{nAAOx(i)}$ represents oxidative loss, $F_{nAAtfAA(i)}$ represents AA incorporation into the fast turnover protein pool synthesis, $F_{nAAAnKA(i)}$ represents deamination of loss, and $F_{nAAAnKA(i)}$ represents deamination flux from the pool of carbon that was previously enriched with ^{13}C but underwent decarboxylation. Synthesis of fast and slow protein is described as

$$F_{nAAtfAA(i)} = k_{nAAtfAA(i)} \times Q_{nAA(i)}, \quad [22]$$

$$F_{tfAAAnAA(i)} = F_{nAAtfAA(i)}, \quad [23]$$

$$F_{nAAtsAA(i)} = k_{nAAtsAA(i)} \times Q_{nAA(i)}, \quad [24]$$

$$F_{tsAAAnAA(i)} = F_{nAAtsAA(i)}, \quad [25]$$

$$F_{nAAAnKA(i)} = k_{nAAAnKA(i)} \times Q_{nAA(i)}, \quad [26]$$

$$F_{nKAnAA(i)} = F_{nAAAnKA(i)}, \quad [27]$$

where $k_{nAAtfAA(i)}$ and $k_{nAAtsAA(i)}$ represent the mass action rate constants for protein synthesis. The mass of both protein pools was considered fixed; therefore, entry into each pool was set equal to the exit from each. The differential for the total protein-bound AA pool was the sum of the fast and slow pools:

$$\frac{dQ_{tAA(i)}}{dt} = Q_{tfAA(i)} + Q_{tsAA(i)}. \quad [28]$$

The differential for the fast protein-bound pool was calculated and the pool size was assumed to be fixed:

$$\frac{dQ_{tfAA(i)}}{dt} = F_{nAAtfAA(i)} - F_{tfAAAnAA(i)}. \quad [29]$$

The FME package of R (Soetaert and Petzoldt, 2010) was utilized to conduct local and global sensitivity, parameter identifiability, Markov chain Monte Carlo (MCMC) analysis, and model fitting as previously described (Wu et al., 2014). The time step was 15 s and Runge-Kutta 4th order was utilized for numerical integration. Initial intracellular pool sizes were set to the mean observed pool mass ($n = 6$) from time

0 and 15 s (Table 2). Both time points were utilized to minimize measurement error associated with this model input. The respective enrichments and calculated isotope pool masses were calculated from the mean enrichment at time 0 for extracellular and intracellular free AA pools ($n = 3$). The observed enrichment at time 0 for the intracellular free AA pool represented natural background abundance of ^{13}C AA and any ^{13}C AA medium contamination of the out-facing cellular membrane surface (Darmaun et al., 1988). The $Q_{xAA(i)}$, $Q_{nAA(i)}$, and $Q_{tAA(i)}$ were fitted to the measured mass of the respective ^{12}C AA, which represented 24, 21, and 21 observations, respectively. Additionally, these pools $Q_{xAA(i)}$, $Q_{nAA(i)}$, and $Q_{tAA(i)}$ were fitted to the measured mass of the respective ^{13}C and ^{15}N AA, which represented an additional 24, 21, and 21 observations each, respectively.

The initial total protein pool mass was set equal to the mean across all samples ($n = 21$), whereas the isotope enrichment was calculated from the average observed isotope ratio at time 0 and 15 s ($n = 6$). The fast protein turnover pool was initially set to 5% of the total protein pool and then varied between 0.5 and 10%. The fast turnover pool size proportion resulting in the best log-likelihood function value was then selected. The initial enrichment of the fast protein turnover pool was set to equal the intracellular free AA pool, which is based on the assumption that the pool would have achieved isotopic equilibrium with the free AA pool over the 24 h period of ^{15}N -enriched medium incubation. The initial enrichment of the slow protein turnover pool was calculated by the difference from the mass of isotopes in the total protein and fast turnover protein pools.

Parameter bounds were set to a minimum of 0 and maximum of 1 for all parameters except AA synthesis, which was set to a minimum of 0 and maximum of 100. Before parameter estimation, the linear dependence of the parameters was evaluated to determine collinearity using the *collin* function within the FME R package. A score of 1 indicates orthogonality between parameters (Brun et al., 2001). The maximum allowable score for a set of parameters was set to 15 for collinearity as higher scores indicate high collinearity and poor identifiability (Brun et al., 2001).

Model parameters were derived using the entire time series of measurements for pool size, volume, and enrichment using *modCost* and *modFit* functions within the FME package (Soetaert and Petzoldt, 2010). The model residuals were standardized based on the overall standard deviation of each respective variable when calculating residuals for model minimization:

$$Residual_a = \frac{Predicted_a - Observed_a}{SD_a}, \quad [30]$$

where $Predicted_a$, $Observed_a$, and SD_a are the modeled predicted, observed, and standard deviation of the observed data, respectively, of an individual variable within the model. Weighting of residuals is important in the situation in which some variables (i.e., isotope ratio) were measured with good precision versus others (i.e., AA mass) were measured with less precision (Soetaert and Petzoldt, 2010). Initial parameter estimates were derived using the Nelder-Mead algorithm, and final estimates using the initial estimates as starting values and the Marq algorithm, which in almost all cases resulted in identical parameter values. Parameter retention in the model was based on P -values ($P < 0.10$), collinearity, and biological relevance.

Standard errors for fluxes and rate constants were derived by simulation to provide a more robust assessment of uncertainty versus standard errors derived during model parameter estimation (Efron and Tibshirani, 1986). The hierarchical structure of the data (i.e., time series, small sample size, high dimensionality, and desire for assessment of predictive power more than parameter sampling distribution) suggested a Bayesian approach over traditional bootstrapping from residuals for derivation of standard errors (Alfaro et al., 2003; Cronin et al., 2010; Efron, 2011). Uncertainty estimates were derived using MCMC with the delayed rejection and adaptive Metropolis algorithm (**DRAM**; Haario et al., 2006; Laine, 2008). For prior specification, the distribution of parameters θ were assumed to be Gaussian, parameters must be between 0 and 1 except for synthesis, which was 100, and within this range, a uniform noninformative probability was specified. A prior error variance must be specified for each parameter (Soetaert and Petzoldt, 2010). This prior variance was derived from the mean of the unweighted squared residuals from the model previously solved by maximum likelihood and the distribution was assumed to be normal. Additionally, the prior error variance is weighted based on the number of observations and a user specified value, which we set to 0.1, which is quite low. For efficient burn-in, the initial parameter values were set to the maximum likelihood solution. The initial random walk-search-jumping distribution was specified using the variance matrix of the parameters when solved by maxima likelihood and scaled according to (Gelman et al., 2014) as follows:

$$J(\theta^*|\theta^{t-1}) = N(\theta^*|\theta^{t-1}, c^2\Sigma), \quad [31]$$

$$c = \frac{2.4}{\sqrt{d}} \times v, \quad [32]$$

where J , t , N , c , d , and v represent the target distribution kernel for the Metropolis algorithm, the function, normal distribution designation, the scaled covariance matrix, the number of parameters, and the unscaled covariance matrix. The proposal jumping distribution was set to update every 100 iterations. The burn-in length was set to one-half of the iterations ($n = 5,000$; Gelman et al., 2014) and the total number of iterations for Markov chain sampling of the probability distribution was set to 10,000. The DRAM (Haario et al., 2006) was implemented if efficiency was low for the MCMC algorithm search and rejection process (Malve et al., 2007). The targeted acceptance rate by the Metropolis algorithm was 23% given 6 parameters that were being sampled at once (Gelman et al., 2014). Fine-tuning to achieve this acceptance rate was conducted by varying the jumping rule scale (i.e., number of iterations per jump) and implementation of DRAM. Upon completion of the MCMC analysis, the observed standard deviation of the parameter posterior was considered the standard error of the estimates (Erixon et al., 2003) with the assumption that the posterior distribution was normal and asymptotic. The parameter posterior from the MCMC analyses ($n = 5,000$ rows) were then collected into a matrix and randomly sampled 1,000 times (R seed = 123). Using the randomly drawn parameters, the model was executed for each sample draw, and the distribution of the model predictions evaluated for the mean, standard deviation, and 95% confidence intervals, thereby providing estimates of prediction power that accounts for parameter uncertainty, random error, and measurement error. Root mean squared errors as a percentage of the mean (**RMSE**) and mean squared errors partitioned into mean bias, slope bias, and dispersion were calculated using the residuals for the maximum likelihood and MCMC solutions (Bibby and Toutenberg, 1977). The concordance correlation coefficient (**CCC**) was also calculated to provide a dimensionless evaluation of precision and accuracy (Lin, 1989).

RESULTS AND DISCUSSION

The objective of this experiment was to develop a method to evaluate AA transport by assessment of AA influx, efflux, and intracellular metabolism. For demonstration purposes, one NEAA (Ala) and one EAA (Leu) were arbitrarily selected. This method is applicable to all AA that are enriched separately with ^{13}C and ^{15}N .

The solved Ala and Leu models with fluxes, mass, and concentrations are provided in Figure 2.

Model Development

Development of a dynamic model to represent isotopic movement was necessary as isotopic equilibrium and steady-state across pools could not be assumed as has been done in previous transport kinetic experiments

(Biolo et al., 1995; Bequette et al., 2000; Miller et al., 2004). An advantage to our approach is the ability to delineate fluxes such as oxidation, transamination, synthesis, and degradation, all of which are difficult to define using steady-state modeling approaches (Biolo et al., 1995; Bequette et al., 2000). Fluxes mimicked biological expectations (Waterlow, 2006) and a previously described AA transport and metabolism model (Hanigan et al., 2009).

Figure 2. Alanine (A) and leucine (B) pool fitted pool masses (nmol), concentration (μ M), and fluxes (nmol/min). The extracellular AA concentrations were similar to in vivo blood plasma concentrations in lactating dairy cows.

Table 4. Rate constant estimates derived by maximum likelihood and Markov chain Monte Carlo simulation

Parameter	ML ¹				MCMC ²		
	Estimate	SE	CV % ³	P-value	Estimate	SE	CV % ³
Alanine							
$k_{xAA\alpha AA}$	0.032	0.002	7.6	<0.001	0.034	0.001	4.0
$k_{nAA\alpha AA}$	0.053	0.004	8.1	<0.001	0.065	0.004	6.1
k_{nAAOx}	0.029	0.004	12.8	<0.001	0.034	0.005	14.3
$k_{nAA\alpha fAA}$	0.001	0.000	31.8	<0.01	0.001	0.001	42.1
$k_{nAA\alpha nKA}$	0.044	0.003	6.6	<0.001	0.043	0.002	5.4
$f_{synthesis}$	8.914	1.662	18.6	<0.001	8.699	1.845	21.2
f_{if}	2.50	—	—	—	2.50	—	—
f_{carbon}	100	—	—	—	100	—	—
$f_{nitrogen}$	100	—	—	—	100	—	—
Leucine							
$k_{xAA\alpha AA}$	0.018	0.001	6.7	<0.001	0.021	0.001	6.6
$k_{nAA\alpha AA}$	0.087	0.007	8.3	<0.001	0.099	0.006	6.4
k_{nAAOx}	0.033	0.002	5.4	<0.001	0.030	0.002	6.4
$k_{nAA\alpha fAA}$	0.019	0.002	12.0	<0.001	0.016	0.004	22.0
$k_{nAA\alpha nKA}$	0.008	0.001	13.7	<0.001	0.006	0.001	8.7
f_{if}	1.00	—	—	—	1.00	—	—
f_{carbon}	—	—	—	—	—	—	—
$f_{nitrogen}$	100	—	—	—	100	—	—

¹ML = model solution obtained by maximum likelihood using nonlinear optimization.²MCMC = model solution represents the parameter posterior mean derived by Markov chain Monte Carlo simulation (n = 5,000 runs).³CV % = SE divided by parameter estimate.

Model parameters for uptake and efflux were well defined with standard errors that were less than 8% of the estimated values; whereas transamination, oxidation/protein export, turnover, and synthesis were derived with less confidence, standard errors were up to 32% of estimated values (Table 4). Although the correlations between uptake and efflux were high, 0.79 and 0.84 for Ala and Leu, respectively, the collinearity score indicated the parameters were identifiable (Brun et al., 2001; Supplemental Figures S1 and S2, <https://doi.org/10.3168/jds.2020-18155>). All other parameter correlations were less than 0.63 for Leu, indicating the ability to uniquely derive the parameters (Supplemental Figure S2). More problematic, Ala rate constants for oxidation or protein export, incorporation into the fast turnover protein pool, and de novo synthesis were highly correlated, ranging from 0.88 to 0.90 (Supplemental Figure S1). However, the collinearity score was 10, lower than the maximum of 15 recommended by Soetaert and Petzoldt (2010).

The maximum likelihood solution derived by FME reflected a deterministic model solution, whereas MCMC addresses the probability distribution of parameters (Wu et al., 2014). The uncertainty of predictions which is derived from posterior distributions is considered by some to be more important than derivation of a single set of parameters, as predictive power should account for parameter error, prediction random error, and

nonrandom system error (Laine, 2008). Simulating the prior parameter values using MCMC resulted in different rate constant estimates in most cases (Table 4). The Bayesian fitting process searches through the posterior space, which we specified as uniform within specified lower and upper bounds and seeks solutions that represent improved fit values compared with the prior by the Metropolis algorithm. Given the high dimensionality of the model, high probability fit solutions usually represent a low proportion of specified prior search space. The observed mean of the posterior might reflect the high dimensionality of this problem, poor starting prior specification, and highly contoured nature of the solution space. Parameters with low correlations tended to have lower standard error compared with maximum likelihood, whereas highly correlated parameters had higher standard error with MCMC, which represents the uncertainty around these parameters. The posterior distribution and correlation parameter matrices are provided in Supplemental Figures S1 and S2. The Ala rate constants for synthesis, fast protein turnover, and oxidation are highly correlated (Supplemental Figure S1), which was reflected in high uncertainty in these rate constants and respective fluxes.

The oxidation rate constant was derived considering net loss of the AA as well as the labeled AA (i.e., ¹³C and ¹⁵N). In contrast, transamination rate constant considers no loss of the AA but only loss of ¹³C, ¹⁵N,

Figure 3. Alanine observed and predicted isotope mass ratios of ^{13}C to ^{12}C and ^{15}N to ^{12}C within the medium, intracellular, and protein-bound pools. The y-axis labels refer to the following mass ratios: E_{xAAi} = extracellular isotope ratio of ^{13}C to ^{12}C ; E_{xAAii} = extracellular isotope ratio of ^{15}N to ^{12}C ; E_{nAAi} = intracellular isotope ratio of ^{13}C to ^{12}C ; E_{nAAii} = intracellular isotope ratio of ^{15}N to ^{12}C ; E_{lAAi} = protein-bound isotope ratio of ^{13}C to ^{12}C ; E_{lAAii} = protein-bound isotope ratio of ^{15}N to ^{12}C .

or both. For Ala, return of labeled C or N likely can occur and was specified using biological inference. The enrichment of these carbon and nitrogen precursor pools for reamination was set to the fast turnover pool enrichment as it improved model fit, reflecting a slight lag in equilibration with the intracellular free AA enrichment, and this lag was previously observed with casein enrichment (Hanigan et al., 2009).

The fraction of total protein that is fast protein constant (f_{if}) was iteratively varied between 0.5 and 10% and selection was based on best model fit. The greatest likelihood ratio was observed at 2.5 and 1% for Ala and Leu, respectively. At that setting, the fast protein pools for Ala and Leu reflected proteins that turned over within 128 and 34 min, respectively. Hanigan et al. (2009) observed the fast protein proportion to be 6% for Leu when fitting to in vivo data from a 30-h infusion, which is slightly higher than our derived estimates.

Protein turnover has high heterogeneity across the several thousand proteins present in a cell; hence, simplification to a 2-pool model is a major assumption. A minimum of 2 protein pools (i.e., fast and slow) ap-

pears to be necessary in these models to account for the changes in isotopic enrichment over time during the experiment. Intracellular free ^{13}C -Ala enrichment started decreasing after 15 min despite extracellular Ala entry to the intracellular pool other than from extracellular pool is occurring (Figure 3). In contrast, intracellular ^{15}N -Ala enrichment appears to reach equilibrium with extracellular ^{15}N enrichment within 30 min (Figure 3). This implies that ^{15}N enrichment of the fast turnover protein pool has apparently reached enrichment equilibrium. It is more difficult to determine if the slow turnover protein pool has reached equilibrium as the contribution to isotope dilution in the intracellular space is small, but this is highly unlikely based on the size of that pool, length of the experiment (24 h), and the turnover rate.

For Leu, de novo synthesis was not identifiable which was expected given its classification as an essential AA. Transamination to α -keto-isocaproate was observed as indicated by positive estimates for $k_{nAA nKA}$. The return of labeled N during reamination improved model fit for

Leu and hence was included, but a more encompassing model should account for labeled N across all AA, not just Leu. The decarboxylation of Leu is irreversible; hence, no carbon tracer should return during reamination and thus the return flux was set to zero.

For Ala, de novo synthesis was significant and different from zero, which reflects its importance for transporting N. The primary transamination product of Ala is pyruvate and this reaction is reversible. The return of labeled N and C improved model fit for Ala, which

reflects use of carbon and nitrogen precursor pools that are enriched during reamination of Ala.

Model fit was evaluated by several methods to assess the accuracy, precision, and robustness of the proposed model (Tables 5 and 6). For Ala and Leu, the RMSE was 12% or less for isotopic enrichment of the extracellular and intracellular free AA pools, indicating good fit relative to the observed values. The CCC values generally indicated model appropriateness in terms of accuracy and precision; however, the model poorly rep-

Table 5. Model fit statistics for Ala predictions

Item	ML ¹							MCMC ²	
	Mean		% of mean	% of mean square prediction error			CCC ⁵	% of mean	CCC
	Obs ³	Pred ⁴	RMSE ⁶	Mean	Slope	Dispersion		RMSE	
Isotope ratio									
E_{nAA}^{13C}	0.31	0.31	11.6	1.7	8.6	89.7	0.91	15.4	0.86
E_{xAA}^{13C}	1.01	1.01	4.9	1.1	34.5	64.4	0.99	3.8	0.99
E_{nAA}^{15N}	0.62	0.60	6.3	8.4	6.5	85.1	0.98	6.2	0.98
E_{xAA}^{15N}	0.34	0.33	5.9	19.1	0.2	80.7	0.96	4.4	0.98
E_{lAA}^{13C}	0.11	0.11	3.0	1.5	0.3	98.2	0.13	3.1	0.18
E_{lAA}^{15N}	0.35	0.35	3.8	2.3	0.1	97.6	0.03	3.8	0.04
Pool mass, nmol									
Q_{nAA}	445	451	15.5	0.4	22.2	77.4	0.17	19.8	−0.17
Q_{xAA}	712	716	6.6	0.8	2.2	97.0	0.44	6.5	0.46
Q_{lAA}	1,458	1,458	4.8	0.0	—	—	—	4.8	—
Q_{nAA}^{13C}	136	138	13.4	3.5	21.8	74.7	0.85	12.5	0.86
Q_{nAA}^{15N}	268	268	17.0	7.2	11.5	81.3	0.84	17.7	0.85
Q_{xAA}^{13C}	709	708	5.2	0.1	33.9	66.0	0.99	4.7	0.99
Q_{xAA}^{15N}	246	241	11.1	3.5	0.7	95.8	0.90	9.6	0.93
Q_{lAA}^{13C}	154	154	3.1	0.7	1.7	97.6	0.08	3.2	0.11
Q_{lAA}^{15N}	507	504	3.3	1.8	0.1	98.1	0.04	3.3	0.06
Concentration, μM									
C_{xAA}	278	278	5.5	0.0	0.5	99.5	0.85	5.4	0.86
C_{nAA}	3,036	2,677	43.0	5.9	7.6	86.5	−0.04	46.6	−0.10
C_{lAA}	26,929	26,624	10.4	1.2	0.0	98.8	0.00	10.4	0.00

¹ML = model solution obtained by maxima likelihood using nonlinear optimization.

²MCMC = model solution represents the parameter posterior mean derived by Markov chain Monte Carlo simulation (n = 5,000 runs).

³Obs = measured mean isotope ratio, pool size, and concentration.

⁴Pred = model predicted mean isotope ratio, pool size, and concentration.

⁵CCC = concordance correlation coefficient.

⁶RMSE = root mean square prediction error as a percentage of the observed mean.

resented isotopic enrichment in the protein pool. This was likely because data variation was minimal and only one pool was characterized with inputs and outputs, the fast turnover pool. Evaluation of MCMC derived parameters indicated similar fit quality in terms of RMSE and CCC despite different parameter estimates. The fitting of isotopic enrichment of Phe in an in vivo assessment of AA transport resulted in an average RMSE of 28.1% (Hanigan et al., 2009), which is higher than the average RMSE of 5.9 and 11.6% for Ala

and Leu isotopic enrichments in our study. Other AA transport models have not reported how well proposed parameters represent observed movement of AA (Biolo et al., 1995; Bequette et al., 2000; Miller et al., 2004).

AA Concentration, Transport, and Metabolism

The observed extracellular Ala and Leu concentrations were similar to in vivo, being within 3.7 and 6.1% of what is observed in the plasma of high-producing

Table 6. Model fit statistics for Leu predictions

Item	ML ¹							MCMC ²	
	Obs ³	Pred ⁴	% of mean	% of mean square prediction error			CCC ⁵	% of mean	CCC
			RMSE ⁶	Mean	Slope	Dispersion		RMSE	
Isotope ratio									
E_{nAA}^{13C}	0.97	1.10	9.7	49.7	13.6	36.7	0.98	16.7	0.95
E_{xAA}^{13C}	2.22	2.24	2.9	15.4	0.4	84.2	0.97	2.6	0.98
E_{nAA}^{15N}	0.56	0.53	8.3	0.1	41.5	58.4	0.98	7.3	0.98
E_{xAA}^{15N}	0.24	0.24	4.0	40.4	26.2	33.4	0.98	2.6	0.99
E_{tAA}^{13C}	0.01	0.01	29.1	0.0	3.2	96.8	0.94	29.2	0.93
E_{tAA}^{15N}	0.47	0.45	15.9	11.7	0.3	88.0	0.00	15.9	0.00
Pool mass, nmol									
Q_{nAA}	64	62	12.5	0.1	6.7	93.2	0.73	12.5	0.72
Q_{xAA}	303	307	4.2	12.1	0.0	87.8	0.80	4.2	0.81
Q_{tAA}	4,304	4,304	22.7	0.0	NA ⁷	NA	NA	22.7	NA
Q_{nAA}^{13C}	58	63	17.0	15.9	3.5	80.6	0.90	22.5	0.85
Q_{nAA}^{15N}	38	35	16.6	1.4	12.5	86.1	0.95	15.8	0.96
Q_{xAA}^{13C}	676	694	4.6	35.2	1.2	63.6	0.97	4.3	0.97
Q_{xAA}^{15N}	73	73	4.8	3.9	7.3	88.8	0.95	4.6	0.96
Q_{tAA}^{13C}	23	29	19.8	33.9	28.9	37.2	0.97	17.0	0.98
Q_{tAA}^{15N}	1,985	1,921	16.6	3.6	28.2	68.2	0.02	16.6	0.02
Concentration, μM									
C_{xAA}	175	179	4.1	26.0	1.9	72.1	0.94	4.0	0.95
C_{nAA}	571	503	41.7	6.1	3.7	90.3	−0.01	40.3	0.03
C_{tAA}	79,817	78,670	19.6	0.6	24.5	74.9	0.01	19.6	−0.01

¹ML = model solution obtained by maxima likelihood using nonlinear optimization.

²MCMC = model solution represents the parameter posterior mean derived by Markov chain Monte Carlo simulation (n = 5,000 runs).

³Obs = measured mean isotope ratio, pool size, and concentration.

⁴Pred = model predicted mean isotope ratio, pool size, and concentration.

⁵CCC = concordance correlation coefficient.

⁶RMSE = root mean square prediction error as a percentage of the observed mean.

⁷NA = not applicable.

Table 7. Cellular flux predictions for Ala and Leu (nmol/min)

Item	Ala			Leu		
	Mean ¹	SE ²	CV % ³	Mean ¹	SE ²	CV % ³
Entry	51.2	2.1	4.1	18.8	1.2	6.2
Exit	44.7	2.1	4.6	13.6	1.1	8.1
Net uptake	6.4	0.3	5.1	5.2	0.1	2.8
Fast turnover protein synthesis	0.6	0.3	61.8	3.0	0.3	9.6
Fast turnover protein degradation	0.6	0.4	60.0	2.4	0.3	11.1
Loss to transamination	37.2	1.9	5.1	1.3	0.1	9.3
Synthesis	17.4	3.2	18.7	—	—	—
Loss to oxidation or protein export	24.5	3.1	12.6	5.1	0.2	3.2
Extracellular turnover of free AA, % h	164.0			79.2		
Intracellular turnover of free AA, % h	548.7			776.3		
Fast turnover protein-bound AA, % h	47.0			179.1		

¹Average flux over 60 min using rate constants derived by maxima likelihood.

²SE represents the SD of 1,000 simulations randomly drawing parameters from the respective parameter posterior.

³CV % = SE divided by mean flux value.

dairy cows (Swanepoel et al., 2016; Table 1; Supplemental Table S1, <https://doi.org/10.3168/jds.2020-18155>). Intracellular concentrations were 10.9 and 3.3-fold higher than extracellular for Ala and Leu, respectively, indicating a steep concentration gradient (Tables 5 and 6). The vast majority of AA transport is mediated by active transport; hence, the observation of a significant gradient supports this theory. In human muscle, Ala and Leu concentrations were found to be 5.5- and 1.5-fold greater in the intracellular space than in the extracellular space (Miller et al., 2004). In lactating dairy goats, intracellular Leu concentrations were 3.3-fold higher than extracellular, exactly what we observed (Hanigan et al., 2009). The much higher ratio of Ala relative to Leu likely reflects de novo synthesis, importance for providing trans-stimulation to drive system L transport, and its role as a N carrier and mediator (Bender, 2012). Also, the ratio of Ala to Leu changed dramatically between extracellular and intracellular, 1.6 to 5.3; hence, assumptions that blood AA profiles match the intracellular AA profile are incorrect. The intracellular profile represents intracellular metabolism and the dynamics of AA transport, which greatly modifies the blood AA profile.

Within the protein-bound pool, our results indicate Leu was present 3-fold higher than Ala, which is somewhat unexpected given previous estimates of cattle (Wu, 2013) and mammary tissue AA profiles (Shenhan et al., 1997; Tables 5 and 6). However, neither of these studies specifically examined bovine epithelial cell protein.

The prediction of AA fluxes (i.e., influx, efflux, and metabolism) represents the primary objective of this method (Table 7). Standard errors based on the MCMC simulation indicated that entry, exit, and net uptake of Ala and Leu from the cell was predicted with

8.1% error or less (Table 7). Metabolic fluxes were predicted with slightly less confidence with the exception of the fast turnover protein synthesis, which had a CV of 61.8%. The high turnover rates of the Ala and Leu extracellular (i.e., 37 and 76 min) and intracellular free pools (i.e., 11 and 8 min) illustrate just how rapid exchange is and the inherent flexibility present to meet needs for protein synthesis. Extracellular Ala turnover was approximately twice as high as Leu, which likely reflects its role in driving system L transport, and the high capacity of system ASC, a sodium-dependent transporter, for taking up Ala (Bender, 2012; Bröer and Bröer, 2017). In lactating goats, approximately 55% of Leu that entered mammary tissue was released, which is lower than our observation of 72%. In human muscle, 104% of Ala and Leu entering muscle tissue was effluxed (Miller et al., 2004). An early study in human muscle observed that 136% of Ala that entered muscle cells was released (Biolo et al., 1995). The observed fluxes illustrate the very rapid exchange of Ala and Leu across the cell membrane that previous studies with arterial-venous difference measurements (Lapierre et al., 2012) are missing.

Whether this high level of exchange occurs in lactating dairy cows is unknown, but if confirmed, might provide a strong mechanism for matching intracellular specific AA needs in the face of daily variations in AA supply. High AA exchange rates do occur in the udder of lactating goats, with an estimated 69, 55, 53, and 69% of uptake of Phe, Leu, Met, and Val being effluxed (Hanigan et al., 2009). Competitive inhibition of transport systems is well documented (Baumrucker, 1984; Hurley et al., 2000; Jackson et al., 2000); hence, whether the AA profile or concentration affects exchange of intracellular AA by competing for transporters warrants investigation. Is there a specific profile and

concentration of AA that best optimizes transport (e.g., minimizing competitive inhibition, driving exchange by increasing NEAA) to meet the needs of intracellular protein synthesis? These questions will require further research in this area.

The turnover rates of the fast protein pool were quite rapid and likely reflect proteins with very short half-lives (i.e., 30 min). The finding that Leu turnover was nearly 4 times faster than Ala turnover confirms a previous study of high turnover rates of branched-chain AA (Hanigan et al., 2009), which reflects that proteins with high turnover rates are more highly enriched in branched-chain AA. In that previous study, the Leu fast turnover rate was 151% h, which is close to what we observed (i.e., 179% h; Hanigan et al., 2009).

Medium AA Concentrations and Enrichment

Extracellular AA profile and concentrations were set to mimic plasma concentrations of lactating dairy cows (Swanepoel et al., 2016). Evaluation of AA transport should consider all AA (Christensen, 1990). Transporter specificity is broad as AA can use multiple transporters. Competition for transporter sites and the synchrony of exchange driven transport ensures considerable interactions among AA with respect to transport. Transport kinetic experiments therefore should not be conducted using standard cell culture medium (i.e., Dulbecco's modified Eagle medium); they should be conducted using medium that more closely replicates *in vivo* AA concentrations and profile.

The measured AA concentration and profile of the ^{13}C - and ^{15}N -enriched experimental media were similar for most AA to the targeted concentrations (Table 1; Supplemental Table S1, <https://doi.org/10.3168/jds.2020-18155>). The discrepancies in some observed AA concentrations versus target concentrations stems from the inability to simultaneously achieve desired concentrations and enrichments and error. For Asp, Glu, and Phe, the concentrations in the algae (^{13}C -enriched and ^{15}N -enriched) and the amount of algae needed to achieve adequate enrichment of the other AA resulted in slightly higher concentrations in the experimental medium, particularly for Asp. For these AA, no ^{12}C was added to the medium, which resulted in very low assayed abundance of ^{12}C for concentration determination, particularly for Phe. Three forms of enriched algae from Cambridge Isotope Laboratories were used for isotope enrichment, but the AA concentration and profile of these algae were unknown before analysis. The observed differences in assayed AA concentrations between HPLC and isotope dilution measurements likely reflect methodology differences and sample size,

with the latter method having 9 replicates (Supplemental Table S2, <https://doi.org/10.3168/jds.2020-18155>). Our results indicate in some cases >25% differences in CP concentration and AA profile between the enriched algae, which might reflect different growing, harvesting, or acid hydrolysis recovery conditions of the enriched algae for each specific production lot. Future research should determine the isotope-enriched algae AA content as it may differ from the reported values herein.

Four AA were present in very low to immeasurable concentrations in the respective algae used, which is related to algae composition and loss during acid hydrolysis (i.e., Trp, Cys, Asn, and Gln). In our experiment, these 4 AA as well as His were added entirely in ^{12}C form to meet their targeted concentration in the ^{15}N medium. In the ^{13}C medium, individual ^{15}N -enriched Asn, Cys, Gln, and His were added to achieve isotope enrichment; however, only enrichment of Gln and His was observed, indicating the Asn and Cys amount were either too low or lost during the derivatization process. The AA concentration for AA not enriched in the $^{13}\text{C}^{15}\text{N}$ algae that were added as an external tracer were not determined as the addition of ^{15}N or ^{13}C tracers were not possible because cell and medium contents already contained these isotopes. Hence, this method is not applicable for 5 of the 20 AA.

Atom percent excess (APE) values were not used because reliance on only 3 background samples and integration of low abundance ions in a background sample on quadrupole MS increase variance of these measurements. Given that we measured enrichment in all pools at time zero, the enrichment change over time as an input for the model yields the same answer as using APE. The resulting ^{15}N experimental medium achieved APE values of 42.1 and 52.9 for Ala and Leu, indicating significant enrichment over background (Supplemental Table S4, <https://doi.org/10.3168/jds.2020-18155>). For the ^{13}C medium, APE values were 46.3 and 68.3 for Ala and Leu, respectively (Supplemental Table S4). Interestingly, ^{13}C enrichment of cellular intracellular free AA but not protein-bound AA at time zero was different than the background samples, which indicates extracellular attachment of AA (Supplemental Table S4). Extracellular AA attachment was expected, underscoring the importance of time zero measurements (Darmaun et al., 1988).

Cells were incubated for 24 h with ^{15}N -enriched medium in an attempt to achieve near stable enrichment of intracellular free AA and the fast turnover protein-bound pool of AA (Supplemental Table S5, <https://doi.org/10.3168/jds.2020-18155>). Enrichment assessment of Ala and Leu in the medium over time indicates that within 16 h, near steady state was achieved within

Figure 4. Leucine observed and predicted isotope mass ratios of ^{13}C to ^{12}C and ^{15}N to ^{12}C within the medium, intracellular, and protein-bound pools. The y-axis labels refer to the following mass ratios: E_{xAAi} = extracellular isotope ratio of ^{13}C to ^{12}C ; E_{xAAii} = extracellular isotope ratio of ^{15}N to ^{12}C ; E_{nAAi} = intracellular isotope ratio of ^{13}C to ^{12}C ; E_{nAAii} = intracellular isotope ratio of ^{15}N to ^{12}C ; E_{lAAi} = protein-bound isotope ratio of ^{13}C to ^{12}C ; E_{lAAii} = protein-bound isotope ratio of ^{15}N to ^{12}C .

the medium. Additionally, the observed turnover rate for the fast turnover protein pool indicates complete turnover within 128 and 34 min for Ala and Leu, respectively (Table 7). Incubation for 24 h thus achieved a stable plateau within the fast turnover protein and intracellular free AA pools.

A shortcoming of this method was the inability to solve for a slow turnover protein rate constant, which was reflected by high collinearity and high standard errors when attempting to derive this rate constant (data not shown). A longer-term incubation of particularly the ^{13}C tracer would greatly enhance our ability to derive a slow turnover rate constant. Isotopic equilibrium of medium, intracellular, and fast protein pools appeared to be reached within 60 min for Ala and Leu ^{15}N enrichment (Figures 3 and 4). In contrast, ^{13}C stable plateau enrichment was not achieved and likely reflected intracellular dynamics of protein turnover, particularly the Ala fast pool, still increasing in enrichment (Figures 3). However, this was apparently inadequate to define the turnover rate of the slow protein pool. This problem should be resolvable by the addition of one or more

sampling time points with much longer exposure to the ^{13}C -labeled medium to allow more time for accumulation of label in the slow turnover pool.

CONCLUSIONS

We have demonstrated a method to evaluate AA transport and metabolism in mammary epithelial cells. This method provides estimates of transport for multiple AA ($n = 15$), which can be modified to measure all 20 AA. The dynamic 12-pool model demonstrated adequate fit and prediction power for all flux assessments except for the slow protein turnover rate given the data collected. In general, observed concentration gradients and fluxes matched biological expectations, suggesting validity of the method and model. The observed fluxes illustrate the rapid exchange of Ala and Leu across the cell membrane. This exchange likely provides harmonization and buffering capacity of extracellular supplies of AA to ensure that cellular AA supply and use for protein translation are aligned. Application of this method to varying levels or profiles of AA supply

should provide more insight into AA transport activity and regulation and to the role it plays in regulating milk protein synthesis.

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