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# Casein synthesis is independently and additively related to individual essential amino acid supply

**S. I. Arriola Apelo,*[1] L. M. Singer,* W. K. Ray,† R. F. Helm,† X. Y. Lin,‡ M. L. McGilliard,* N. R. St-Pierre,§ and M. D. Hanigan***
*Department of Dairy Science, and
†Department of Biochemistry, Virginia Tech, Blacksburg 24061
‡Animal Science and Technology College, Shandong Agriculture University, 271018, China
§Department of Animal Sciences, The Ohio State University, Columbus 43210

## ABSTRACT

Specific AA affect rates of milk protein synthesis in the mammary glands of lactating cows. The objective of this study was to quantify the rate of $\alpha_{S1}$-casein synthesis in response to Ile, Leu, Met, and Thr supplementation, and to test the single-limiting AA theory for milk protein synthesis by exploring interactions among these AA. Effects of Ile, Leu, Met, and Thr were studied in vitro with a composite design containing a central point repeated 4 times, with 2 axial points per AA and a complete $2^4$ factorial. Other AA were at the concentration in Dulbecco's modified Eagle medium/F12 medium (DMEM). The experiment was replicated with mammary tissue from 5 lactating cows. Mammary tissue slices (0.12 ± 0.02 g) were incubated for 4 h at 37°C in 5 mL of treatment medium containing $^2H_5$-Phe. Caseins were precipitated from cell homogenate supernatants. Enrichment with $^2H_5$-Phe of the N[34]LLRFFVAPFPE $\alpha_{S1}$ peptide was determined by matrix-assisted laser desorption/ionization-tandem time-of-flight (MALDI-TOF-TOF), which was used to determine enrichment of Phe in the transfer (t)RNA pool and $\alpha_{S1}$-casein fractional synthesis rates (CFSR). Data were analyzed with a polynomial mixed model containing linear, quadratic, and 2-factor interactions for Ile, Leu, Met, and Thr, and cow and residual as random factors. Interactions were not significant at $P < 0.1$ and were removed from the model. Increasing concentrations of Ile, Leu, Met, and Thr simultaneously increased CFSR curvilinearly with a predicted maximum response of 4.32 ± 0.84%/h at 63% of DMEM concentrations. The maximum response to each of the 4 AA was at 71, 49, 60, and 32% of the concentration in DMEM, for Ile, Leu, Met, and Thr, respectively. These values correspond to 270, 120, 440, and 140% the plasma concentrations of Ile, Leu, Met, and Thr observed in lactating cows fed to meet National Research Council requirements, respectively. The CFSR estimated at those maxima were similar among AA (3.6 ± 0.6%/h). Individual AA effects on CFSR did not correlate with mammalian target of rapamycin (mTOR) signaling. Independent responses of CFSR to individual essential AA observed in this study contradict the single-limiting AA theory assumed in current requirement systems. The saturable responses in CFSR to these 4 AA also highlight the inadequacy of using a fixed postabsorptive AA efficiency approach for determining AA requirements for milk protein synthesis.
**Key words:** essential amino acid, casein, protein synthesis, mammary gland

## INTRODUCTION

Ruminants are especially efficient in converting low-quality feed into high-quality product (i.e., milk, meat, and wool). This is explained by their synergism with microbes that allow them to degrade cell-wall components, and to incorporate nonprotein N into carbon skeletons to synthesize AA that mammalian cells cannot. However, when fed typical North American diets, dairy cattle only capture 25 ± 4% of dietary N in milk protein, with the remaining being excreted in feces and urine (Hristov et al., 2004). Furthermore, after duodenal absorption, utilization of microbial and dietary AA is significantly less efficient (43%; Lapierre et al., 2010) in dairy cattle than in swine and poultry. Nitrogen export to the environment affects soil, water, and air quality, with potential effects on aquatic ecosystems, global warming, and human health (Wolfe and Patz, 2002).

Baker (1996) demonstrated that postabsorptive use of N in pigs can be up to 85% efficient when the supply of AA matches tissue needs. Similarly, in lactating cows, the efficiency of conversion of extracted EAA by the mammary glands into casein can be as high as 81%, with even higher values for individual EAA (Guinard and Rulquin, 1994). However, EAA extraction by the mammary glands averages 43% of that supplied (Hani-

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[1]Corresponding author: sebuy@vt.edu

gan et al., 1992), and EAA not extracted by the glands returns to central circulation and can be catabolized by the liver and other tissues. Metabolizable protein efficiency for milk protein, therefore, is determined by the rate of milk protein synthesis relative to the rates of AA utilization for other processes (Hanigan et al., 1998). Given that extraction rates of EAA by splanchnic tissues appear to be a fixed proportion of total supply (Hanigan et al., 2004a,b), stimulating milk protein synthesis would increase extraction by the mammary glands and reduce recycling to splanchnic tissues, thereby reducing splanchnic removal and increasing postabsorptive efficiency of EAA for milk protein.

Among factors reported to affect casein synthesis rate are insulin (Bellacosa et al., 1998), which reflects systemic energy status, and intracellular AMP:ATP ratios (Hardie, 2004), which is a local indicator of energy status. These 2 factors have been shown to regulate protein synthesis through the mammalian target of rapamycin (**mTOR**) pathway, which regulates mRNA translation initiation and elongation rates (Mahoney et al., 2009). Essential AA have also been shown to control mTOR phosphorylation and regulate protein synthesis in mammary tissue of lactating cows (Appuhamy et al., 2011a). Among EAA, Leu has been shown to increase β-lactoglobulin synthesis in mammary epithelial cells (Moshel et al., 2006). In vivo, Leu alone increased milk protein yield curvilinearly, with a maximum estimated at 140 g/d of digestible Leu (Rulquin and Pisulewski, 2006). Toerien et al. (2010) did not observe the same effect after 9 h of jugular infusion of Leu (1.87 g/h). However, Leu plasma concentrations in the saline treatment were significantly higher than those observed by Rulquin and Pisulewski (2006). Methionine and Lys increased milk protein yield, but addition of Leu and Ile had no effect (Appuhamy, 2010). In vitro, Ile, Leu, Met, and Thr were the only EAA that had an effect on casein synthesis when removed from the culture medium (Appuhamy et al., 2012). Other than identifying the EAA that affect milk protein synthesis, little is known about quantitative responses to those EAA. Therefore, the objectives of this study were to quantify the effect of Ile, Leu, Met, and Thr on casein synthesis rate in vitro, to determine the relation between casein synthesis and protein signaling responses reported previously (Arriola Apelo et al., 2014), and to explore interactions among these AA on casein fractional synthesis rate (**CFSR**).

## MATERIALS AND METHODS

Tissue collection, treatment medium preparation, tissue slice incubation, and postincubation processing were performed as previously described (Arriola Apelo et al., 2014). Briefly, mammary tissue slices from 5 lactating dairy cows (0.121 ± 0.016 g) were incubated in high glucose (17.5 m*M*), high insulin (0.01 mg/L) Dulbecco's modified Eagle medium/F12 medium (**DMEM**; 0.0215 m*M* phenol red), in which Phe was substituted with $^{2}H_{5}$ Phe (98% purity; Cambridge Isotope Laboratories Inc., Andover, MA). The effects of Ile, Leu, Met, and Thr on $^{2}H_{5}$-Phe transfer (**t**)RNA$^{Phe}$ enrichment and CFSR were studied with a composite design consisting of a central point repeated 4 times, 2 axial points per AA, and a complete $2^{4}$ factorial (28 treatments). Preliminary data indicated saturable responses around 50% of the concentrations in DMEM. Thus, the central points were set at 35% of the AA concentration in DMEM to center the design in the curvilinear region of the response. Axial points for each AA were set at 0 and 100% of the concentrations in DMEM, holding the other AA at the central points. Factorial points were set equidistant from central points at 20 and 50% of DMEM concentrations. Threonine concentrations in treatment media were set to 0, 10, 17, 25, and 49% of those in DMEM to better describe response curves based on preliminary data. Two additional treatments with all 4 AA at 0 or 100% DMEM were added to the experiment to analyze the response to the bulk of AA. For that analysis, the 2 added treatments were used in conjunction with the central points and the 2 factorial treatments with the 4 AA at 20 and 50% of the concentrations in DMEM (10 and 25% for Thr; 8 treatments total). The effects of individual EAA and interactions were determined using the 28 treatments of the central composite design, without the 2 treatments with the 4 AA at 0 and 100% of DMEM concentrations.

Following a 4-h incubation in treatment medium, slices were homogenized in lysis buffer (7:1, vol/wt; 50 mmol of Tris HCl, pH 7.4; 150 mmol of NaCl; 1 mmol of EDTA; 1 mmol of phenylmethylsulfonyl fluoride, 1 mmol of $Na_3VO_4$, 1 mmol of NaF, 0.001 μg of aprotinin, 0.001 μg of leupeptin, 0.001 μg of pepstatin, 10 mL of Nonidet P-40, and 2.5 g of Na-deoxycholate per liter) and centrifuged for 5 min at 16,000 × *g* at 37°C. The pellet was discarded.

### *Intracellular Casein Enrichment*

Liquid chromatography (**LC**)-MS solvents were from Spectrum Chemical (New Brunswick, NJ). All other reagents were from Sigma-Aldrich (St. Louis, MO) unless indicated otherwise. Caseins were precipitated from media and cell homogenate supernatants, as described by Cuollo et al. (2010) with modifications. Briefly, 100 μL of cell lysate was acidified by addition of 100 μL of 1 *M* sodium acetate (pH 4.6), incubated for 30 min at 37°C, and centrifuged for 10 min at 4,000 × *g*. Pellets were washed twice with ice-cold 0.15 *M* sodium acetate (pH