Variations in Mammary Protein Metabolism During the Natural Filling of the Udder with Milk over a 12-h Period Between Two Milkings: Leucine Kinetics¹

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ABSTRACT

To define the temporal variations of whole body and mammary leucine kinetics over a 12-h period between two milkings, we used two groups of four Holstein cows, one in their second and the other in their third or fourth lactation. Cows were infused with L-[1-¹³C]leucine during the 12-h interval between two milkings. Blood was sampled every 30 min during that period from arterial and mammary sources. Time after milking did not affect whole body irreversible loss rate of leucine but affected whole body leucine oxidation, which broadly followed variations in arterial plasma leucine concentration. Similarly, mammary leucine irreversible loss rate and leucine used for protein synthesis were not affected by time after milking. Leucine oxidation by the mammary gland was, however, affected by time after milking. It increased by 15% from the first 2-h period to the following 4-h period and then decreased by 13% over the following 2-h period. A 21% increase in leucine oxidation was observed from 8 to 10 h after milking, and then it decreased by 26% over the last 2-h period. Protein degradation expressed as percentage of mammary leucine flux followed a similar temporal pattern. Leucine used for protein synthesis by the mammary gland was unaltered over time after milking, suggesting that the increased availability of leucine resulting from mammary protein breakdown would increase intracellular concentrations of leucine, which would have favored its catabolism. Overall, these results confirm the high metabolic activity of the

mammary gland, as protein synthesis by the mammary gland averaged 43% of whole body protein synthesis.

(**Key words:** dairy cow, kinetics, protein, metabolism)

Abbreviation key: APE = atom percent excess, IE = isotopic enrichment, FIL = feedback inhibitor of lactation, ILR = irreversible loss rate, MOP = 4-methyl 2-oxopentanoate.

INTRODUCTION

The mammary gland has the ability to regulate its nutrient uptake in order to maintain milk synthesis. This regulation is exerted by adjusting mammary blood flow (Cant et al., 1993; Bequette et al., 2000) or by adjusting the removal of milk precursors from arterial supply, as it has been well demonstrated when an amino acid limits protein synthesis (Bequette et al., 2000). The udder possesses intracellular controls that regulate milk synthesis, including a protein influencing milk output, the feedback inhibitor of lactation (FIL; Wilde et al., 1987; Wilde et al., 1989; Wilde et al., 1995). It is synthesized within the alveolar cells and is found in the whey protein fraction of milk (Wilde et al., 1989; Wilde et al., 1995). The inhibitory effect of FIL that is exerted on the synthesis of nonlipid milk components is concentration dependent (Wilde et al., 1987; Wilde et al., 1995) and is removed by milking (Henderson and Peaker, 1984). More specifically, the regulatory effect of FIL on milk protein secretion is mediated through an increased degradation of newly synthesized casein and a down regulation of protein synthesis (Wilde et al., 1987; Wilde et al., 1989; Rennison et al., 1993).

It has been demonstrated that the mammary net uptake of amino acids varied significantly over a 12-h period following milking, even in animals that were fed every 2 h (Thivierge et al., 2002). These temporal variations were characterized by an increase in amino acid net uptake between 2 to 8 h after the morning milking,

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followed by a decrease in net uptake between 8 to 12 h after milking in both whole blood and plasma. The natural filling of the udder occurring during the 12-h interval between two milkings was paralleled by a linear decrease in the mammary respiratory quotients (Thivierge et al., 2002). The mammary oxygen uptake tended to increase during the same period, suggesting that the requirements for oxidation increased in the mammary gland over time after milking.

The determination of amino acid kinetics is often made over a short period of time to avoid recycling of tracers. Temporal variations in mammary metabolism over time after milking would imply that short time measurements of mammary metabolism may not be representative of daily mammary protein metabolism. Therefore, the objectives of the present experiment were to determine the variation in protein synthesis, degradation, and oxidation at the whole body level and across the mammary gland during the 12-h interval between two milkings, using [1-¹³C]leucine in high yielding cows fed every 2 h.

MATERIALS AND METHODS

Animals, Care, and Feeding

One group of four Holstein cows in their second lactation and one group in their third or fourth lactation, averaging 29.2 (SEM 3.7) and 36.1 (SEM 1.2) kg of milk daily, respectively, were used to study the variation in mammary protein metabolism over a 12-h period following milking. Cows in their second lactation averaged 217 DIM (SEM 10) and 663 kg of BW (SEM 17) and cows in their third or fourth lactation averaged 93 DIM (SEM 16) and 620 kg of BW (SEM 45). All cows were fitted with permanent catheters in the left external pudic artery and in the lateral branch of the cranial mammary vein. An ultrasonic flow probe (Transonic Systems inc., Ithaca, NY) was placed around the left external pudic artery of all cows. Details on surgical procedures were previously reported (Thivierge et al., 2000). Experimental procedures were approved by a local animal care committee and conducted according to the Canadian Council on Animal Care (1993) guidelines. Cows were allowed at least a 2-wk recovery period following surgery before measurements began. Unfortunately one second-lactation cow had a large amount of orts for a few days including the day of blood sampling, during which time milk yield dropped by half. This cow was removed from statistical analyses, thus only the data from the remaining seven cows were considered.

Animals were maintained in tie stalls equipped with rubber mats and bedded with straw. A TMR meeting their nutritional requirements (1.53 Mcal/kg of NE_L, 16.2% CP, 27.6% ADF, and 44.8% NDF; NRC, 1989) was fed in 12 equal daily meals at 95% of their previous

individual ad libitum intake. The TMR contained, on a DM basis, 37.7% corn silage (8.2% CP; 23.0% ADF, and 41.3% NDF), 37.7% grass silage (17.3% CP; 29.9% ADF, and 45.9% NDF), 12.2% ground corn, 9.9% soybean meal, and 2.5% vitamin and mineral supplement. Rumen-protected methionine (72 g/d; Mepron M85, Degussa-Hüls, Canada) was fed simultaneously with the TMR in 12 daily meals. Cows were milked twice daily at 0700 and 1900 h.

Infusions and Sampling Procedures

Leucine kinetics. Temporal variation of protein metabolism was studied over a 12-h period between two consecutive milkings at the whole body level and across the mammary gland using L-[1-¹³C]leucine as a tracer. This particular tracer was chosen because we wanted not only to quantify the variation in irreversible loss rate (ILR) of an amino acid over time at the whole body level and across the mammary gland but also the variation in oxidation to allow estimation of protein synthesis and degradation. Leucine is an essential amino acid taken up in excess and oxidized by the udder, which made it an appropriate tracer to achieve our objectives. Twenty-four to 48 h before measurements, an indwelling catheter was placed in a jugular vein for tracer infusion. Starting 3 h before the morning milking, three simultaneous arterial and venous blood samples were withdrawn at 15-min intervals to determine natural abundance of leucine. Two hours before the morning milking, a priming dose of L-[1-¹³C]leucine (2.5 mmol; 99 atom %; MSD isotopes, Montréal, QC, Canada) was injected and was followed by a continuous infusion of the tracer (2.5 mmol/h). The infusion of L-[1-¹³C]leucine was maintained until the completion of the evening milking, at least 12 h later. At the morning milking, evacuation of residual milk was achieved by using an intravenous oxytocin injection (10 IU, Oxytocin Injection U.S.P., Sandoz Santé Animale Inc, Victoriaville, QC, Canada). Arterial blood flow was continuously recorded every second, starting at least 15 min before measurements began and ending 15 to 30 min after the end of the evening milking. The last blood sampling was followed by the evening milking, which was also completed by an oxytocin (10 IU) injection.

Blood sampling began 30 min after the end of the morning milking and samples were withdrawn simultaneously from both arterial and venous sites every 30 min over the entire 12-h period. We ensured that the cows were standing for at least 5 min before each blood sampling. Immediately after sampling, two 1-ml aliquots from each blood sample were injected into evacuated Vacutainers containing 1 ml of frozen lactic acid for measurement of blood CO₂ enrichment (Read et al., 1984).

The remaining blood samples were kept on ice until further processing. Whole blood aliquots (approximately 1 g) were precisely weighed and mixed with a weighed amount (1 g) of a hemolyzing standard 0.1 N HCl solution containing 181 μM L 1- ^{13}C leucine (MSD isotopes, Montréal, QC, Canada) and 30 μM oxo-hexanoate (Sigma-Aldrich Canada Ltd, Oakville, ON, Canada) to quantify leucine and 4-methyl 2-oxopentanoate (**MOP**) concentrations. Plasma was obtained from centrifugation of the remaining whole blood samples at $3000 \times g$ for 15 min at 4°C. Plasma aliquots (1 g) were precisely weighed and 0.2 g of a standard 0.1 N HCl solution containing 906 μM L 1- ^{13}C leucine was added for the determination of leucine concentration. The samples were kept frozen at -20°C until analyses.

During the infusion of labeled leucine, samples of expired air were taken with a face mask (Chevalier et al., 1984) with the same schedule used for blood samples. Three samples for natural abundance of expired CO_2 were taken at 15-min intervals before the labeled leucine infusion started. Carbon dioxide in breath samples was purified on a vacuum line with differential freezing with solid CO_2 -acetone mixture and liquid N_2 (Chevalier et al., 1984).

CO_2 production. A continuous infusion of $\text{NaH}^{13}\text{CO}_3$ (99 atom %; MSD isotopes, Montréal, QC, Canada) into a jugular vein was performed the day before or after leucine kinetics measurements for the determination of CO_2 production. Similar to the sampling procedures followed during the labeled leucine infusion, three samples for natural abundance of expired CO_2 were taken at 15-min intervals before the beginning of the $\text{NaH}^{13}\text{CO}_3$ infusion. The continuous infusion of the tracer began 2 h before the morning milking (0.4 mmol/h) and was preceded by a priming dose (0.56 mmol). The infusion of labeled bicarbonate was continued until the end of the evening milking. Sampling and processing of expired air were identical to that performed on the day of the leucine infusion.

Laboratory Analyses

The packed cell volume in blood was determined by the micro-hematocrit method (Strumia et al., 1954). Whole blood samples for quantification of leucine concentrations and isotopic enrichment (**IE**) were analyzed individually, giving 24 samples (30-min sampling over 12 h) per sampling site per cow. Measurements from blood for every four consecutive 30-min periods were averaged giving six values, each representing a 2-h period. Plasma samples for leucine concentrations and IE were pooled hourly before the analysis; therefore, 12 samples were analyzed per sampling site per cow. Every two consecutive 1-h pooled samples were averaged giving six values,

each representing a 2-h period. Determination of both plasma and whole blood leucine concentrations were carried out according to Calder et al. (1999). The IE of whole blood and plasma free leucine and the IE of MOP in whole blood were determined after deproteinization with sulfosalicylic acid and derivatization with *N*-(tert-butyl-dimethylsilyl)-*N*-methyltrifluoroacetate-acetonitrile (1:1, vol/vol), for *m/z* ions 302, 303 for leucine and 259, 260 for MOP, by gas chromatography-mass spectrometry (Hewlett Packard model HP6890, S973 Mass selective detector; Hewlett Packard, CA), as described by Calder and Smith (1988). Whole blood MOP concentration was determined from the gas chromatographic-mass spectrometric peak area, corrected for the known addition of oxo-hexanoate as the internal standard. Measurements for whole blood concentration and IE of MOP were averaged as outlined for whole blood concentration and IE of leucine.

Enrichment in ^{13}C of CO_2 in purified breath was analyzed using a triple collector isotope ratio mass spectrometer (Sira 12, VG, Manchester, UK) at *m/z* ions 44, 45, and 46. Blood frozen on lactic acid was thawed for 30 min and mixed. The CO_2 released was transferred to the triple collector isotope ratio mass spectrometer for the measurement of the CO_2 IE.

Calculations

Blood flow measured every second with ultrasonic blood flow probes was averaged over six consecutive 2-h periods. These averages were used to determine the effect of time after milking, as described previously (Thivierge et al., 2002). Average mammary blood flow (MBF) was also estimated according to the Fick principle using a correction factor of 3.5% for blood-borne proteins secreted directly in milk: $\text{MBF} = [(\text{milk Phe} + \text{Tyr}) \times 0.965] / (\text{AV difference Phe} + \text{Tyr})$, (Cant et al., 1993).

Literature values were used for milk concentration of phenylalanine and tyrosine (Jensen, 1995). Plasma flow could have been calculated directly with the Fick principle. However, given an 11% lower arteriovenous (**AV**) concentration difference for tyrosine measured in plasma compared with whole blood (Thivierge et al., 2002), we assumed that plasma flow would be overestimated this way. Thus, plasma flow was calculated by multiplying whole blood flow by its plasma fraction (1-hematocrit).

The continuous measurement of mammary blood flow shows that blood flow did not change over time after milking and was stable across the 12-h period (Thivierge et al., 2002). However, it underestimated mammary blood flow by 30% on average compared with the values obtained using the Fick principle in the same cows (Thivierge et al., 2002). Therefore, the average mammary

blood flow for the 12-h period estimated with the Fick principle was used to calculate mammary fluxes. Whole blood and plasma net fluxes were calculated as the product of net arteriovenous concentration difference of the component in whole blood or plasma and its respective flow.

For the following equations, infusion rates are in mmol/h, and the IE of the infusate, leucine, and CO₂ are in atom percent excess (APE). Whole body leucine kinetics were calculated using the IE of arterial plasma or whole blood leucine and whole blood MOP as representative of the precursor pool.

Whole body ILR of leucine was calculated as follows:

$$\begin{aligned} &\text{Whole body ILR (mmol/h)} \\ &= (\text{leucine infusion rate (mmol/h)} \times \text{infusate IE/IE}_{\text{pp}}) \\ &\quad - \text{leucine infusion rate (mmol/h)}, \end{aligned}$$

where IE_{pp} represents the IE of leucine in the appropriate precursor pool.

The whole body fractional rate of leucine oxidation (FO) was calculated using the following equation:

$$\begin{aligned} \text{FO} &= (\text{IE of expired CO}_{2[\text{leu. inf.}]}) \\ &\quad \times \text{CO}_2 \text{ production (mmol/h)} / \\ &\quad (\text{leucine infusion rate (mmol/h)} \\ &\quad \times \text{IE of the infusate}_{[\text{leu. inf.}]}) \times c \end{aligned}$$

The CO₂ production was estimated by a labeled bicarbonate infusion, defined as [bic. inf.], and calculated as follows:

$$\begin{aligned} \text{CO}_2 \text{ production (mmol/h)} &= \\ &(((\text{rate of bicarbonate infusion (mmol/h)} \\ &\quad \times \text{IE of the infusate}_{[\text{bic. inf.}]}) / (\text{IE of expired CO}_{2[\text{bic. inf.}]}) \\ &\quad - \text{rate of bicarbonate infusion (mmol/h)}) \times c. \end{aligned}$$

The coefficient "c" is the recovery rate of the labeled bicarbonate infused (%) in the animal. This considers that a part of the infused CO₂ is sequestered and not expired (Ram et al., 1999). This coefficient was not estimated in the present study, as it is present in both the numerator and denominator of the fractional oxidation of leucine and, therefore, cancels out. Whole body and mammary leucine kinetics estimations using whole blood as the precursor pool were not used to test the temporal variation. A plateau in IE was not reached for whole blood leucine across the 12-h infusion period (presented in the results and discussion section) but was reached for the plasma samples (no significant time effect). We therefore concluded that the increment observed over time for the IE of whole blood leucine resulted from the intracellular pool equilibrating with the

red blood cells and was not a reflection of decreased protein metabolism.

Mammary variations of plasma leucine kinetics over time after milking were studied using a simple two-compartment model using leucine and CO₂ compartments as previously described by Bequette et al. (1996b). The concentration of MOP and its IE were not measured in plasma samples given that mammary whole blood MOP ILR represented only 1.9% of mammary whole blood ILR of leucine (data not shown). Therefore, it was considered that mammary plasma MOP movement would not represent a significant loss of the tracer. For the comparison of the temporal variation of mammary leucine kinetics, mammary venous IE of plasma leucine was used as the precursor pool of intracellular IE given that it includes the dilution of leucine IE occurring within cells with unlabeled leucine coming from protein breakdown (Wolfe, 1992).

Mammary irreversible loss rate (ILR_{Leu,mam}) of leucine was calculated as follows:

$$\begin{aligned} \text{ILR}_{\text{Leu,mam}} &= ((\text{Leu}_a \times \text{IE}_{\text{Leu,a}}) \\ &\quad - (\text{Leu}_v \times \text{IE}_{\text{Leu,v}}) \times \text{PF}) / \text{IE}_{\text{Leu,v}} \end{aligned}$$

$$\begin{aligned} \text{Leucine oxidized} &= ((\text{CO}_{2,v} \times \text{IE}_{\text{CO}_2,v}) \\ &\quad - (\text{CO}_{2,a} \times \text{IE}_{\text{CO}_2,a}) \times \text{PF}) / \text{IE}_{\text{Leu,v}} \end{aligned}$$

where Leu and CO₂ indicate the respective plasma concentration whereas subscripts "a" and "v" indicate arterial or venous source and PF = plasma flow.

Utilization of leucine for protein synthesis and protein degradation by the mammary gland were calculated as follows:

$$\begin{aligned} \text{Protein synthesis} &= \text{ILR}_{\text{Leu,mam}} \\ &\quad - \text{Leucine oxidized} \end{aligned}$$

$$\begin{aligned} \text{Leucine from protein breakdown} \\ &= \text{ILR}_{\text{Leu,mam}} - \text{mammary leucine net flux} \end{aligned}$$

Plasma [1-¹³C]leucine kinetics, including whole blood [1-¹³C]MOP, movements were calculated according to the compartmental model of Oddy et al. (1988).

Statistical Analyses

The temporal variations were statistically analyzed according to a repeated measure design using the following model of six periods of 2 h with two groups of cows (three cows in second lactation and four cows in third and fourth lactation) including degrees of freedom in parentheses:

$$Y_{ijkl} = \mu + \text{lact}_i + \text{cow}(\text{lact})_{j(i)} + \text{time}_k \\ + (\text{time}_k \times \text{lact}_i) + e_{(ijkl)}$$

where:

Y_{ijkl} = dependent variable,

μ = overall mean,

lact_i = main effect corresponding to the i -th lactation (1 df)

$\text{cow}(\text{lact})_{j(i)}$ = effect of individual cow j nested within lactation i (5 df)

time_k = main effect corresponding to the k -th time (5 df)

$\text{time}_k \times \text{lact}_i$ = interaction between the k -th time and the i -th lactation (5 df)

$e_{(ijkl)}$ = residual error associated with each $ijkl$ observation (25 df).

Six periods of 2 h were compared in the statistical model given that the analysis as repeated measures requires that the number of periods compared be smaller than the number of experimental units used in the study (Milliken and Johnson, 1984). The statistics were performed using the GLM procedure of SAS (1985). This statistical design allowed us to control the variation in the mammary arteriovenous measurements due to a nonmammary venous backflux into the mammary vein measured previously in the third and fourth lactation cows used in the present study (Thivierge et al., 2000). Cow nested within lactation was used as the error term for determination of lactation number effect. Type III sum of squares was interpreted. Least square means are given in tables with pooled standard error of the mean. Highest polynomial degree of significant time effect or its interaction with lactation number is presented according to principles of nonlinear regression (Steel and Torrie, 1960) and $P \geq 0.05$ was considered significant and $0.05 < P \leq 0.10$ was considered as a tendency.

RESULTS AND DISCUSSION

Isotopic Enrichments

The IE of expired CO_2 measured during the infusion of labeled bicarbonate was stable across the 12-h period and averaged 0.0024 APE (Table 1). The IE of expired CO_2 measured when labeled leucine was infused varied according to a quadratic pattern over time ($P = 0.01$), which tended to differ with lactation number ($P = 0.06$). Two hours after the morning milking, the CO_2 IE was lower than in the following five periods, by 25 and 12% on average in second lactation and older cows, respectively. This suggests that the CO_2 pool had not reached the isotopic equilibrium at the beginning of the measure-

ments. Therefore, this 2-h period was removed to determine the effect of time on leucine oxidation and on leucine used for protein synthesis at the whole body level, given that the latter was estimated by subtracting leucine oxidation from leucine ILR. By removing the value of this first 2-h period of leucine oxidation, the statistical analysis still revealed a tendency ($P = 0.08$) for a cubic relationship over time that changed with lactation number.

During the $[1\text{-}^{13}\text{C}]$ leucine infusion, leucine IE in arterial and mammary venous plasma remained stable until the end of the 12-h period, averaging 2.10 and 1.91 APE, respectively. The ratios of arterial to mammary venous plasma leucine IE remained similar across all periods (Table 1). Thus, leucine kinetic calculations using either arterial or mammary venous plasma leucine IE as a representative precursor pool of intracellular enrichment will provide similar trends on the variation of leucine kinetics over time.

Leucine IE in arterial and mammary venous whole blood behaved differently than in plasma. It increased linearly ($P < 0.01$) over time and did not reach a plateau by the end of the 12-h $[1\text{-}^{13}\text{C}]$ leucine infusion period (Table 1). The IE of arterial and venous sites averaged 1.62 and 1.29 APE, respectively, during the first 2-h period. They both increased and reached 1.84 and 1.55 APE by the end of the last 2-h period. As leucine plasma IE did not change over time, this indicates that the free leucine in red blood cells became progressively labeled over time (Table 1). Furthermore, the arterial IE did not increase in a similar proportion as the mammary venous IE, as shown by the ratio of the arterial over the venous IE decreasing linearly over time by 7% ($P = 0.04$), from 1.28 to 1.19, 12 h later. Similar to whole blood leucine IE, whole blood MOP IE did not reach a plateau over the 12-h period (Table 1).

In our study, the labeled leucine equilibrated at a slower rate in whole blood than in plasma, as reported previously in dairy cows and goats (Bequette et al., 1996b, 1997), in lambs (Oddy and Lindsay, 1986), and in sheep (Lobley et al., 1996). This suggests very little direct exchange, if any, between plasma and red blood cells (Elwyn et al., 1972; Houlier et al., 1991). Rather, the exchange of amino acids with red blood cells would be exerted through transfer of amino acids from tissue to red blood cells as they cross tissue capillaries (Elwyn et al., 1972).

Across the mammary gland, arterial leucine IE is diluted with leucine from intracellular pools having a lower IE. The dilution in IE of intracellular leucine would originate from the mixing of arterial labeled leucine with unlabeled leucine released from mammary protein breakdown. This was expected to occur given the high protein turnover of mammary tissue (reaching 41%

Table 1. Temporal variation of CO₂ production, leucine arterial concentration in whole blood and plasma and the isotopic enrichment (IE) of whole blood leucine, plasma leucine, whole blood MOP, and breath CO₂ during a L-[1-¹³C]leucine or NaH¹³CO₃ infusion during a 12-h period following milking.

	Average period after the morning milking						P-value			
	0–2 h	2–4 h	4–6 h	6–8 h	8–10 h	10–12 h	SEM	PD ¹	T ²	I ³
Whole body CO ₂ production, mol/h	17.3	17.5	17.1	17.7	16.4	18.4	0.9		NS ⁵	NS
Breath CO ₂ IE, 1 × 10 ^{−3} APE ⁴ while infusing										
NaH ¹³ CO ₃	2.34	2.38	2.46	2.41	2.51	2.35	0.11		NS	NS
[1- ¹³ C]leucine	2.49	2.76	3.17	3.05	3.02	2.80	0.15	2	< 0.01	0.06
2 lac	2.42	2.80	3.56	3.08	2.98	2.74	0.23			
> 2 lac	2.57	2.73	2.78	3.02	3.06	2.86	0.20			
Whole blood leucine IE, APE										
Arterial (A)	1.62	1.66	1.65	1.77	1.78	1.84	0.03	1	< 0.01	NS
Venous (V)	1.29	1.32	1.37	1.48	1.48	1.55	0.03	1	< 0.01	NS
A/V	1.28	1.28	1.23	1.22	1.21	1.19	0.02	1	0.04	NS
Whole blood MOP ⁶ IE, APE										
Arterial (A)	1.47	1.54	1.43	1.53	1.53	1.54	0.03	5	0.03	NS
Venous (V)	1.35	1.44	1.31	1.46	1.30	1.40	0.05	5	0.01	NS
A/V	1.10	1.09	1.10	1.05	1.18	1.10	0.04	5	0.01	NS
Plasma leucine IE, APE										
Arterial (A)	2.08	2.15	1.98	2.09	2.17	2.12	0.05		NS	NS
Venous (V)	1.89	1.94	1.79	1.94	1.94	1.96	0.05		NS	NS
A/V	1.10	1.11	1.11	1.08	1.13	1.09	0.02		NS	NS
Arterial leucine concentration; μM ⁷										
Whole blood	187.1	194.4	196.8	191.2	193.8	191.2	3.5	2	0.04	0.02
2 lac	191.9	199.8	209.4	199.5	206.6	195.8	5.3			
> 2 lac	182.3	188.9	184.3	182.8	181.0	186.5	4.6			
Plasma	175.6	167.4	183.5	177.5	162.9	173.5	6.9	4	0.07	0.05
2 lac	170.0	183.7	191.0	176.1	181.8	180.9	10.5			
> 2 lac	181.3	151.2	176.0	178.8	144.0	166.2	9.1			

¹Highest polynomial degree (PD) of significant time effect or its interaction with lactation number (see Materials and Methods section).

²Probability of time (T) effect for the given polynomial degree (see Materials and Methods section).

³Probability of the interaction (I) between time effect and lactation number for the given polynomial degree; in addition to the mean values, data are presented for the two groups when the interaction is significant.

⁴Atom percent excess.

⁵Nonsignificant = $P > 0.10$.

⁶4-Methyl 2-oxopentanoate.

⁷From Thivierge et al. (2002).

daily) in lactating goats (Baracos et al., 1991; Champredon et al., 1990). Also, breakdown of peptides in erythrocytes by peptidases (Adams et al., 1952) as they cross mammary capillaries would also generate unlabeled leucine that could dilute whole blood mammary venous IE if the peptides are not taken up entirely by the udder.

The involvement of red blood cells in the amino acid exchange with the mammary tissue has been studied recently. The plasma pool is the main precursor pool of free amino acids in lactating cows and goats (Bequette et al., 1997; Hanigan et al., 1991; Mackle et al., 2000; Thivierge et al., 2002). The present results support these observations. The slow increase in leucine IE in red blood cells indicates a slow rate of amino acid exchange between the red blood cells and the tissues and a probable minor role of red blood cells in amino acid delivery to

the mammary gland. The involvement of the red blood cells in carrying amino acids away from the udder and their role in the transport of amino acids needs to be more clearly defined.

Whole Body Leucine Kinetics Using Plasma as the Precursor Pool

The variations in arterial leucine concentration have been discussed previously (Thivierge et al., 2002). Given that the IE of whole blood leucine and MOP did not reach a plateau over the 12-h period, the evolution of leucine kinetics with time after milking was not calculated using whole blood values as representative of the precursor pool. Whole body ILR estimated using arterial plasma leucine as a precursor pool remained stable across the 12-h period and averaged 119 mmol/h (Table

Table 2. Temporal variation of whole body leucine kinetics (mmol/h) using arterial plasma leucine isotopic enrichment as the precursor pool during a 12-h period after milking.

Parameters ⁴	Average period after the morning milking						P-value			
	0–2 h	2–4 h	4–6 h	6–8 h	8–10 h	10–12 h	SEM	PD ¹	T ²	I ³
ILR	120.2	116.2	127.1	120.7	114.6	117.0	3.3		NS ⁵	NS
LO	...	24.5	27.8	28.8	23.6	24.8	2.5	3	0.04	NS
FO, %	...	20.5	21.8	22.6	20.6	21.2	1.7		NS	NS
LS	...	91.6	99.3	91.9	90.6	91.7	3.3		NS	NS

¹Highest polynomial degree (PD) of significant time effect or its interaction with lactation number (see Materials and Methods section).

²Probability of time (T) effect for the given polynomial degree (see Materials and Methods section).

³Probability of the interaction (I) between time effect and lactation number for the given polynomial degree.

⁴Leucine kinetics: ILR: irreversible loss rate; LO: oxidation; FO: fractional rate of oxidation (LO/ILR, expressed in percentage); LS: used for protein synthesis.

⁵Nonsignificant $P > 0.10$.

2). This value is similar to the 90 to 101 mmol/h reported in lactating cows between 24 to 30 wk of lactation (Bequette et al., 1996b). It is also in agreement with the values of 118 and 113 mmol/h in cows at 6 and 25 wk of lactation, respectively (Lapierre et al., 1999b). The fractional oxidation rate of leucine was stable over time following milking and averaged 21% of whole body leucine ILR when the first 2-h period was removed from the analysis. However, whole body leucine oxidation varied according to a cubic relationship over time after milking ($P = 0.04$) despite an apparent nutritional steady state and averaged 26 mmol/h. The resulting leucine used for protein synthesis did not change over the 12-h period and averaged 93 mmol/h (Table 2). Whole body leucine oxidation broadly followed the changes in arterial plasma leucine concentration over time for each group of cows (Figure 1). This type of association has been reported previously in humans for leucine (El-Khoury et al., 1994) and for lysine (El-Khoury et al., 1998). Availability of tissue free amino acids would be an important determinant of their oxidation rate. When tissue free amino acid concentrations increase, their oxidation would increase concomitantly (Young and Marchini, 1990). This response would be mediated in part by a low affinity of oxidative enzymes for amino acids, favoring oxidation of amino acids at high concentrations (Young and Marchini, 1990). Regressions of whole body leucine oxidation against leucine arterial concentration indicate that the arterial concentration of leucine explains 47 and 28% of its oxidation in second-lactation and third- and fourth-lactation cows, respectively. Similar relationships between leucine oxidation and arterial leucine concentration were reported in fed ($r^2 = 0.55$) and fasted ($r^2 = 0.23$) humans (El-Khoury et al., 1994). These authors suggested that when arterial leucine is less abundant, factors other than the arterial concentration would have greater influence on leucine oxidation.

Figure 1. Temporal variation over 12 h after the morning milking of arterial plasma leucine concentration —□— and leucine oxidation◇..... at the whole body level in cows in second lactation (A) or in third and fourth lactation (B).

Table 3. Temporal variation of mammary leucine kinetics using mammary venous leucine isotopic enrichment as the precursor pool during a 12-h period after milking.

	Average period after the morning milking						<i>P</i> -value			
	0–2 h	2–4 h	4–6 h	6–8 h	8–10 h	10–12 h	SEM	PD ¹	T ²	I ³
Blood flow ⁴ , l/h	452	461	468	467	456	448	10		NS ⁷	NS
Plasma fluxes, mmol/h										
ILR ⁵	48.9	47.4	52.9	49.6	49.4	48.7	3.3		NS	NS
LO ⁵	5.16	6.02	5.85	5.19	6.27	4.67	0.60	4,5	0.08	0.05
2 <i>lac</i>	3.43	3.92	4.61	3.03	5.46	3.19	0.90			
> 2 <i>lac</i>	6.89	8.12	7.09	7.36	7.08	6.15	0.78			
LS ⁵	43.7	41.4	47.1	44.4	43.1	44.0	3.2		NS	NS
Net flux ⁴	39.1	37.7	41.2	42.0	37.9	40.4	2.2		NS	NS
LD ⁵	9.8	9.8	11.7	7.5	11.5	8.3	2.0		NS	NS
FO ⁵ , %	10.3	12.1	11.4	10.0	13.4	9.6	1.3	–,5	NS	0.08
2 <i>lac</i>	8.7	9.1	10.9	7.7	13.3	8.7	2			
> 2 <i>lac</i>	11.8	15.2	12.0	12.3	13.6	10.6	1.7			
LD/ILR ⁵	19.3	20.0	20.8	14.7	21.6	15.1	2.9	5,–	0.07	NS
Whole blood net flux, mmol/h										
Leucine ⁴	37.4	42.5	43.1	39.5	36.4	35.2	2.1	2,–	0.04	NS
MOP ⁶	–0.98	–0.97	–0.83	–0.90	–0.50	–0.67	0.10	1,–	0.06	NS

¹Highest polynomial degree (PD) of significant time effect (first number) or its interaction with lactation number (second number, see Materials and Methods section).

²Probability of time (T) effect for the given polynomial degree (see Materials and Methods section).

³Probability of the interaction (I) between time effect and lactation number for the given polynomial degree; in addition to the mean values, data are presented for the two groups when the interaction is significant.

⁴From Thivierge et al. (2002).

⁵Leucine kinetics: ILR: irreversible loss rate; LO: oxidation; LS: used for protein synthesis; LD: released from protein breakdown; FO: fractional rate of oxidation rate (LO/ILR; expressed as percentage of ILR); LD: released from protein breakdown; LD/ILR: expressed in percentage of ILR).

⁶4-Methyl 2-oxopentanoate.

⁷Nonsignificant: *P* > 0.10.

Mammary Leucine Kinetics Using Plasma as the Precursor Pool

Mammary blood flow measured with the flow probe did not change over time after milking (Table 3; Thivierge et al., 2002). The average mammary blood flow of the 12-h period used for all calculations and estimated using the Fick principle was 646 L/h (SEM 72) in second-lactation cows and 840 L/h (SEM 72) in cows in third or fourth lactation. Average plasma flow calculated from whole blood flow corrected for hematocrit was 465 L/h (SEM 58) in second-lactation cows and 613 L/h (SEM 51) in cows in third or fourth lactation.

Plasma leucine net flux was not affected by time after milking (Table 3; Thivierge et al., 2002). Mammary leucine ILR did not change over time after milking and averaged 49 mmol/h (Table 3). Leucine oxidized within the udder tended to exhibit a quartic relationship with time (*P* = 0.08) having a similar temporal pattern as mammary CO₂ net release (Thivierge et al., 2002). Leucine oxidation within the mammary gland increased by 15% from the 0- to 2-h period to the average of the 2- to 6-h period after milking, and then decreased again by 13% over the following 2-h period. From the 6- to 8-h to the 8- to 10-h period after milking, leucine oxidation rate

increased by 21% and decreased thereafter by 26% over the next 2-h period. The two groups of cows did not exhibit exactly the same temporal pattern of mammary leucine oxidation as showed by the interaction of time with lactation number (*P* = 0.05). However, this pattern differed only for the 4- to 6-h period after milking, where leucine oxidation decreased for the older cows but increased for the second-lactation cows.

Leucine used for protein synthesis and released from protein breakdown was not affected by time after milking and averaged 44 and 10 mmol/h, respectively (Table 3). Given that leucine oxidation represented only 11% of leucine ILR on average, variations in leucine oxidation were not large enough to generate a significant difference in leucine used for protein synthesis across the period studied. However, when leucine released from protein breakdown was expressed as a proportion of mammary ILR, it followed a pattern similar to that of mammary leucine oxidation. Given the unaltered mammary use of leucine for protein synthesis over time after milking, the increased availability of leucine issued from protein breakdown would have favored its catabolism accordingly (Benevenga et al., 1993).

Leucine is taken up by the udder in excess of its requirements for protein secretion (Mephram, 1982). Al-

though leucine oxidation does not appear to be a mandatory event for milk biosynthetic process (Bequette et al., 1996a), the present data suggest that its oxidation varies according to the different metabolic pathways promoted over the 12-h period following milking. Previous results have shown that from the 0- to 2-h to the 4- to 6-h period after milking, elevated respiratory quotients suggested promoted anabolic processes, including de novo fatty acid synthesis (Thivierge et al., 2002). The current data show that this is simultaneously associated with increases in leucine oxidation and protein breakdown. The mammary metabolism of leucine can support anabolic reactions by generating amino groups (DeSantiago et al., 1998) for de novo synthesis of alanine, glutamine, glutamate, glutamine (Harper et al., 1984; Roets et al., 1983). Also, its catabolism provides acetyl-CoA for fatty acid synthesis or precursors for energy. The catabolism of leucine in support of anabolism is also suggested by the increase in net mammary production of MOP. The period was also characterized by an increase in mammary leucine availability resulting from protein breakdown (LD/ILR) that was paralleled by leucine oxidation. During the last 4 to 6 h of 12-h milking interval, mammary catabolic processes appeared to be promoted as mammary oxygen uptake increased by 33% and mammary respiratory quotients decreased from 2.01 to 1.40 (Thivierge et al., 2002). While the nutrient catabolism was enhanced relative to lipogenesis, whole blood leucine net flux was decreased, as was mammary MOP release. The alteration of mammary metabolism occurring during that period is consistent with the regulatory effect of milk accumulation in the udder, which is expected to be exerted by FIL (Wilde et al., 1995). The decreased leucine net uptake during this period could arise from a down-regulation of A and L amino acid transport systems due to milk accumulation, as observed in rat mammary tissue (Shennan and McNeillie, 1994). The nonmammary backflux from the external pudic vein into the mammary vein in cows in third lactation and higher (Thivierge et al., 2000) appears to have no detectable effect on mammary leucine kinetics when mammary venous plasma is used as the precursor pool.

Precursor Pools and Protein Metabolism in Relation to Milk Production

For reasons discussed previously, variations in leucine kinetics over time have been calculated using plasma leucine as representative of the precursor pool. In addition, whole body ILR was estimated using the means of the IE for the entire milking interval of whole blood leucine and MOP (Table 4), to allow comparisons between the different precursor pools. Using MOP IE as the precursor pool led to a higher estimation of whole

body leucine flux compared to that calculated using the arterial whole blood leucine IE. The IE of MOP has been proposed as a reliable precursor pool of the IE of leucine bound to tRNA given that it is formed intracellularly from deamination of leucine (Wolfe, 1992). This seems mainly true when whole body metabolism is dominated by muscle, which would not be the case in lactating dairy cows. The present results show that mammary ILR represents 35 to 65% of whole body leucine flux. Lapierre et al. (1999a) showed that the splanchnic leucine flux approximates 40% of whole body leucine flux in growing steers. Together, these results account for most of the whole body leucine kinetics and indicate that MOP produced by leucine deamination in muscle does not provide a representative precursor pool of body protein metabolism in lactating cows.

Leucine IE was therefore preferred to estimate the IE of the precursor pool. Whole body leucine flux was not affected by lactation number, and the resulting difference in milk production when arterial plasma was used as the precursor pool (Table 4). This could be due in part to a similar DMI averaging 21 and 20 kg in second-lactation cows and in older cows, respectively. Similarly, whole body leucine oxidation, fractional rate of leucine oxidation, and leucine used for protein synthesis estimated using arterial plasma leucine as representative of the precursor pool, were not affected by lactation. This contrasts with the observations of Lapierre et al. (1999b), in which cows at 6 and 25 wk of lactation yielded 40.8 and 25.2 kg of milk daily, respectively. In that study, the cows also had a similar DMI, but whole body leucine flux, leucine oxidized, and leucine used for protein synthesis increased as milk yield increased. The smaller difference in milk yield between the two groups of cows used in the present study might have had a smaller impact on leucine kinetics at the whole body level.

As for whole body ILR, average mammary leucine ILR was also calculated with mammary venous plasma leucine (Table 4), mammary venous whole blood leucine, or MOP IE as the precursor pool for kinetic calculations. The two latter precursor pools increased variability of measurements and led to a nonsignificant numerical increase in mammary ILR in cows in their third and fourth lactations. The use of mammary leucine plasma as the precursor pool decreased the variability and leucine ILR tended to increase ($P = 0.07$) as milk yield increased with the lactation number (Table 4). For the kinetics across the mammary tissue, the IE of amino acids in milk casein appears to be an appropriate precursor pool to estimate the IE of amino acids incorporated into milk protein (Bequette et al., 1999). It should be a more appropriate precursor than the IE of free amino acids in mammary tissue homogenate, given that the IE in this pool was 26% lower than the IE of casein, 30 h

Table 4. Effect of the lactation number on whole body and mammary leucine kinetics (mmol/h).

Parameters ¹	Lactation number (milk yield, kg/d)		SEM	Lactation number effect ²
	2nd Lact (29.2)	> 2nd Lact (36.1)		
Whole body				
ILR (whole blood leucine)	144.3	142.2	9.1	NS
ILR (whole blood MOP) ³	167.5	162.1	14.3	NS
ILR (plasma leucine)	117.9	120.7	7.7	NS
LO ⁴	24.8	25.8	7.3	NS
LS ⁴	93.0	94.9	4.0	NS
FO ⁴ , %	21	21	5	NS
Mammary				
ILR (whole blood leucine)	54.1	92.7	17.1	0.15
ILR (whole blood MOP) ²	58.6	85.7	11.0	0.12
ILR (plasma leucine)	42.2	56.7	4.8	0.07
LO ⁵	3.9	7.1	0.9	0.05
FO, %	9.7	12.3	1.6	NS
MOP ILR ⁶	1.2	1.6	0.3	NS
LS ⁵	39.4	51.1	4.3	0.10
Leucine net flux ⁷	34.1	45.3	2.8	0.03
LD ⁵	9.0	12.4	2.3	NS
Milk Leu ⁸	28.4	37.3	2	0.09

¹Leucine kinetics: ILR: irreversible loss rate (within brackets the precursor pool used for calculations); LO: oxidation; LS: used for protein synthesis; FO: fractional oxidation rate (LO/ILR expressed as percentage of ILR); LD: released from protein breakdown (LD).

²Probability of lactation group effect; NS = nonsignificant, $P > 0.20$.

³4-Methyl 2-oxopentanoate.

⁴Arterial plasma leucine isotopic enrichment was used as the precursor pool.

⁵Mammary venous plasma leucine isotopic enrichment was used as the precursor pool.

⁶Calculated using MOP whole blood concentration and whole blood mammary venous leucine isotopic enrichment as the precursor pool.

⁷From Thivierge et al. (2002).

⁸Leucine secreted in milk over the 12-h period.

after the start of the labeled phenylalanine infusion in dairy goats (Bequette et al., 1999). In the present study, leucine IE in casein could not be determined. For this approach to work, the mammary gland has to be milked every hour over 11 to 13 h to get an enrichment plateau in milk casein (Bequette et al., 1994). Obviously, this goes against the objectives of the present study, in which milk had to accumulate in the udder over 12 h. However, mammary plasma leucine IE appears to be a reliable estimate of this precursor pool IE (Bequette et al., 1996b). Leucine IE in casein reached 99% of mammary venous leucine IE 13 h after the onset of the tracer infusion (Bequette et al., 1996b).

Also, as milk production increased, leucine oxidation rate increased ($P = 0.05$) and leucine used for protein synthesis tended to increase ($P \leq 0.10$; Table 4). The fractional oxidation rate of leucine remained similar between both groups of cows. In goats producing 2.2 and 4.6 kg of milk daily at 233 and 80 DIM, respectively, lysine used for protein synthesis and lysine absolute oxidation increased as milk production increased (Mabjeesh et al., 2000). Similar to our observations, the fractional oxidation rate of lysine remained similar between

both stages of lactation. The present results corroborate these observations. A higher utilization of leucine (ILR) within the mammary gland combined with a constant leucine fractional oxidation rate would allow more leucine available for milk protein synthesis as milk yield increases.

The present data shows that the mammary leucine flux as a percentage of whole body leucine flux tended to increase ($P = 0.08$) as milk yield increased when arterial leucine IE was used as the precursor pool for ILR calculations (Table 5). The use of mammary venous leucine IE for the mammary leucine ILR calculation, compared with the whole body leucine ILR, showed only a numerical increase with the increase in milk yield. Mammary leucine oxidation and mammary leucine used for protein synthesis expressed as a percentage of their respective values at the whole body level were numerically increased when milk yield increased using both approaches to calculate these ratios (Table 5). It was previously reported that mammary leucine flux represented 41% of whole body leucine flux in midlactation cows (Bequette et al., 1996b), and that mammary protein synthesis represented 27 to 31% of whole body protein syn-

Table 5. Mammary leucine irreversible loss rate (ILR), leucine oxidation (LO), and leucine used for protein synthesis (LS) expressed as a percentage of their respective value for the whole body.¹

Parameters	Lactation number (milk yield, kg/d)		SEM	Lactation number effect ²
	2nd Lact (29.2)	> 2nd Lact (36.1)		
Arterial Leu IE used as precursor pool for mammary kinetics				
ILR ³	34	44	3	0.08
LO ³	16	30	6	0.12
LS ³	38	49	5	0.15
Mammary venous Leu IE as precursor pool for mammary kinetics				
ILR	40	48	5	NS
LO	17	33	7	0.14
LS	46	53	7	NS

¹For both comparisons, plasma arterial leucine isotopic enrichment (IE) was used as the precursor pool for whole body leucine kinetic calculations.

²Probability of lactation group effect; NS = nonsignificant, $P > 0.20$.

³Leucine kinetics; irreversible loss rate (ILR); oxidation (LO); and used for protein synthesis (LS).

thesis in early-lactation goats (Champredon et al., 1990; Baracos et al., 1991). Overall, the increased use of leucine by the mammary gland relative to whole body protein metabolism as milk yield increased, despite a similar DMI, suggests that leucine used by the udder was spared by a decreased use by other tissues.

CONCLUSION

The present research deals with the effect of the natural milk accumulation in the udder over time between two milkings on mammary protein metabolism. The results show that time after milking had no significant effect on whole body leucine metabolism, with the exception of leucine oxidation, which broadly followed variations in arterial plasma leucine concentration. Mammary ILR of leucine, leucine used for protein synthesis, and leucine released from protein breakdown were not affected by time after milking. However, leucine oxidation as well as protein degradation expressed as a percentage of mammary leucine ILR exhibited similar temporal variations. Given that the mammary leucine ILR and leucine used for protein synthesis were unaltered over time after milking, it appears that the increased availability of leucine resulting from mammary protein breakdown would increase intracellular concentrations of leucine, which would have favored its catabolism.

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REFERENCES

- Adams, E., M. McFadden, and E. L. Smith. 1952. Peptidase of erythrocytes 1. Distribution in man and in other species. *J. Biol. Chem.* 198:663–670.
- Baracos, V. E., J. Brun-Bellut, and M. Marie. 1991. Tissue protein synthesis in lactating and dry goats. *Brit. J. Nutr.* 66:451–465.
- Bequette, B. J., F. R. C. Backwell, M. S. Dhanoa, A. Walker, A. G. Calder, D. Wray-Cahen, J. A. Metcalf, J. D. Sutton, D. E. Beever, G. E. Lobley, and J. C. MacRae. 1994. Kinetics of blood free and milk casein-amino acid labelling in the dairy goat at two stages of lactation. *Br. J. Nutr.* 72:211–220.
- Bequette, B. J., F. R. C. Backwell, J. C. MacRae, G. E. Lobley, L. A. Crompton, J. A. Metcalf, and J. D. Sutton. 1996a. Effect of intravenous amino acid infusion on leucine oxidation across the mammary gland of the lactating goat. *J. Dairy Sci.* 79:2217–2224.
- Bequette, B. J., J. A. Metcalf, D. Wray-Cahen, F. R. C. Backwell, J. D. Sutton, M. A. Lomax, J. C. Macrae, and G. E. Lobley. 1996b. Leucine and protein metabolism in the lactating dairy cow mammary gland: Responses to supplemental dietary crude protein intake. *Br. J. Nutr.* 63:209–222.
- Bequette, B. J., F. R. C. Backwell, A. G. Calder, J. A. Metcalf, D. E. Beever, J. C. MacRae, and G. E. Lobley. 1997. Application of a U-13C-labeled amino acid tracer in lactating dairy goats for simultaneous measurements of the flux of amino acids in plasma and the partition of amino acids to the mammary gland. *J. Dairy Sci.* 80:2842–2853.
- Bequette, B. J., S. J. Mabjeesh, and W. C. McNabb. 1999. Kinetics of free amino acid and protein pools in the lactating goat udder. Page 77 in *Abstracts of the VIII International Symposium on Protein Metabolism and Nutrition*. G. E. Lobley, A. White, and J. C. MacRae, eds. Wageningen Pers, The Netherlands.
- Bequette, B. J., M. D. Hanigan, A. G. Calder, C. K. Reynolds, G. E. Lobley, and J. C. MacRae. 2000. Amino acid exchange by the mammary gland of lactating goats when histidine limits milk production. *J. Dairy Sci.* 83:765–775.
- Benevenga, N. J., M. J. Gahl, and K. P. Blemings. 1993. Role of protein synthesis in amino acid catabolism. *J. Nutr.* 123:332–336.
- Calder, A. G., and A. Smith. 1988. Stable isotope ratio analysis of leucine and ketoisocaproic acid in blood plasma by gas chromatography/mass spectrometry. Use of tertiary butyldimethylsilyl derivatives. *Mass Spectrom.* 2:14–16.

- Calder, A. G., K. E. Garden, S. E. Anderson, and G. E. Lobley. 1999. Quantification of blood and plasma amino acids using isotope dilution electron impact gas chromatography/mass spectrometry with U-13C amino acids as internal standard. *Rapid Commun. Mass Spectrom.* 13:2080–2083.
- Canadian Council on Animal Care. I. Guide to Care and Use of Experimental Animals. Version 2 Edition. 1993. Bradda Printing Services, Inc., Ottawa, ON.
- Cant, J. P., E. J. DePeters, and R. L. Baldwin. 1993. Mammary amino acid utilization in dairy cows fed fat and its relationship to milk protein synthesis. *J. Dairy Sci.* 76:762–774.
- Champredon, C., E. Debras, P. Mirand, and M. Arnal. 1990. Methionine flux and tissue protein synthesis in lactating and dry goats. *J. Nutr.* 120:1006–1015.
- Chevalier, R., G. Pelletier, and M. Gagnon. 1984. Sampling technique for collection of expired CO₂ in studies using naturally labelled ¹³C in calves. *Can. J. Anim. Sci.* 64:495–498.
- DeSantiago, S., N. Torres, A. Suryawan, A. R. Tovar, and S. M. Hutson. 1998. Regulation of branched-chain amino acid metabolism in the lactating rat. *J. Nutr.* 128:1165–1171.
- El-Khoury, A. E., N. K. Fukagawa, M. Sanchez, and V. R. Young. 1994. The 24-h pattern and rate of leucine oxidation, with particular reference to tracer estimates of leucine requirements in healthy adults. *Am. J. Clin. Nutr.* 59:1012–1020.
- El-Khoury, A. E., A. Basile, L. Beaumier, S. Y. Wang, H. A. Al-Amiri, A. Selvaraj, S. Wong, A. Atkinson, A. M. Ajami, and V. R. Young. 1998. Twenty-four-hour intravenous and oral tracer studies with L-[1-13C]-2-aminoadipic acid and L-[1-13C]lysine as tracers at generous nitrogen and lysine intakes in healthy adults. *Am. J. Clin. Nutr.* 68:827–839.
- Elwyn, D. H., W. J. Launder, H. C. Parikh, and E. M. Wise. 1972. Roles of plasma and erythrocytes in interorgan transport of amino acids in dogs. *Am. J. Physiol.* 222:1333–1342.
- Hanigan, M. D., C. C. Calvert, E. J. DePeters, B. L. Reis, and R. L. Baldwin. 1991. Whole blood and plasma amino acid uptake by lactating bovine mammary gland. *J. Dairy Sci.* 74:2484–2490.
- Harper, A. E., R. H. Miller, and K. P. Block. 1984. Branched-chain amino acid metabolism. *Annu. Rev. Nutr.* 4:409–454.
- Henderson, A. J., and M. Peaker. 1984. Feed-back control of milk secretion in the goat by a chemical in milk. *J. Physiol.* 351:39–45.
- Houlier, M. L., P. P. Mirand, D. Durand, D. Bauchart, J. Lefaivre, and G. Bayle. 1991. Transport des acides aminés dans l'aire splanchnique par le plasma sanguin et le sang chez le veau préruminant. *Reprod. Nutr. Dev.* 31:399–410.
- Jensen, R. G. 1995. *Handbook of Milk Composition*. R. G. Jensen. London ed., Academic Press, Toronto, ON.
- Lapierre, H., J. F. Bernier, P. Dubreuil, C. K. Reynolds, C. Farmer, D. Ouellet, and G. E. Lobley. 1999a. The effect of intake on protein metabolism across the splanchnic tissues in growing beef steers. *Br. J. Nutr.* 81:457–466.
- Lapierre, H., G. E. Lobley, J. J. Matte, and C. Girard. 1999b. The effect of folic acid and rumen-protected methionine on protein metabolism in dairy cows. Page 84 in Abstracts of the VIII International Symposium on Protein Metabolism and Nutrition. G. E. Lobley, A. White, and J. C. MacRae, eds. Wageningen Pers, The Netherlands.
- Lobley, G. E., A. Connell, D. K. Revell, B. J. Bequette, D. S. Brown, and A. G. Calder. 1996. Splanchnic-bed transfers of amino acids in sheep blood and plasma, as monitored through use of a multiple U-13C-labeled amino acid mixture. *Br. J. Nutr.* 75:217–235.
- Mabjeesh, S. J., C. E. Kyle, J. C. MacRae, and B. J. Bequette. 2000. Lysine metabolism by the mammary gland of lactating goats at two stages of lactation. *J. Dairy Sci.* 83:996–1003.
- Mackie, T. R., D. A. Dwyer, K. L. Ingvarsen, P. Y. Chouinard, D. A. Ross, and D. E. Bauman. 2000. Evaluation of whole blood and plasma in the interorgan supply of free amino acids for the mammary gland of lactating dairy cows. *J. Dairy Sci.* 83:1300–1309.
- Mepharm, T. B. 1982. Amino acid utilization by the mammary gland. *J. Dairy Sci.* 65:287–298.
- National Research Council. 1989. *Nutrient Requirements of Dairy Cattle*. Version 6 Edition. Natl. Acad. Sci., Washington, DC.
- Milliken, G. A., and D. E. Johnson. 1986. Analysis of repeated measures designs for which the usual assumptions hold. Pages 323–340 in *Analysis of Messy Data. Volume 1: Designed Experiments*. Van Nostrand Reinhold Company, New York.
- Oddy, V. H., and D. B. Lindsay. 1986. Determination of rates of protein synthesis, gain and degradation in intact hind-limb muscle of lambs. *Biochem. J.* 233:417–425.
- Oddy, V. H., D. B. Lindsay, and I. R. Fleet. 1988. Protein synthesis and degradation in the mammary gland of lactating goats. *J. Dairy Res.* 55:143–154.
- Ram, L., R. Nieto, and G. E. Lobley. 1999. Tissue sequestration of C-labeled bicarbonate [HCO₃⁻] in fed and fasted young sheep. *Comp. Biochem. Physiol.* 122:323–330.
- Read, W. W., M. J. Read, R. C. Griggs, and D. Hallyday. 1984. Preparation of CO₂ from blood and protein-bound amino carboxyl groups for quantification of 13C-isotope enrichments. *Biomed. Mass Spectrom.* 15:467–472.
- Rennison, M. E., M. Kerr, C. V. P. Addey, S. E. Handel, M. T. Turner, C. J. Wilde, and R. D. Burgoyne. 1993. Inhibition of constitutive protein secretion from lactating mouse mammary epithelial cells by FIL (feedback inhibitor of lactation), a secreted milk protein. *J. Cell Sci.* 106:641–648.
- Roets, E., A. M. Massart-Leën, G. Peeters, and R. Verbeke. 1983. Metabolism of leucine by isolated perfused goat udder. *J. Dairy Res.* 50:413–424.
- SAS User's Guide: Statistics. Version 5 Edition. 1985. SAS Inst., Inc., Cary, NC.
- Shennan, D. B., and S. A. McNeillie. 1994. Milk accumulation down-regulates amino acid uptake via systems A and L by lactating mammary tissue. *Horm. Metab. Res.* 26:611.
- Steel, R. G. D., and T. H. Torrie. 1960. Nonlinear regression. Pages 332–345 in *Principles and Procedures of Statistics*. McGraw-Hill Company, Inc., Toronto, ON.
- Strumia, M. M., A. B. Sample, and E. D. Hart. 1954. An improved micro hematocrit method. *Am. J. Clin. Pathol.* 24:1016–1024.
- Thivierge, M. C., D. Petitclerc, J. F. Bernier, Y. Couture, and H. Lapiere. 2000. External pudic venous reflux into the mammary vein in lactating dairy cows. *J. Dairy Sci.* 83:2230–2238.
- Thivierge, M. C., D. Petitclerc, J. F. Bernier, Y. Couture, and H. Lapiere. 2002. Temporal variations of mammary metabolism as the udder gradually fills with milk during a 12-h period following milking. *J. Dairy Sci.* 85:1839–1854.
- Wilde, C. J., D. T. Calvert, A. Daly, and M. Peaker. 1987. The effect of goat milk fractions on synthesis of milk constituents by rabbit mammary explants and on milk yield in vivo. *Biochem. J.* 242:285–288.
- Wilde, C. J., C. V. P. Addey, and C. H. Knight. 1989. Regulation of intracellular casein degradation by secreted milk proteins. *Biochem. Biophys. Acta* 992:315–319.
- Wilde, C. J., C. V. P. Addey, L. M. Boddy, and M. Peaker. 1995. Autocrine regulation of milk secretion by a protein in milk. *Biochem. J.* 305:51–58.
- Wolfe, R. R. 1992. Selection of tracer infusion and sampling sites. Pages 167–188 in *Radioactive and Stable Isotope Tracers in Biomedicine: Principles and Practice of Kinetic Analysis*. Wiley-Liss, Inc., Toronto, ON.
- Young, V. R., and J. S. Marchini. 1990. Mechanisms and nutritional significance of metabolic responses to altered intakes of protein and amino acids, with reference to nutritional adaptation in humans. *Am. J. Clin. Nutr.* 51:270–289.