Effect of Casein and Propionate Supply on Mammary Protein Metabolism in Lactating Dairy Cows

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ABSTRACT

The effects of casein (CN) and propionate (C3) on mammary AA metabolism were determined in 3 multiparous Holstein cows fitted with both duodenal and ruminal cannulas and used in a replicated Youden square with six 14-d periods. Casein (743 g/d in the duodenum) and C3 (1,041 g/d in the rumen) infusions were tested in a factorial arrangement. For each period, L-[1-¹³C]Leu (d 11) and NaH[¹³C]O₃ (d 13) were infused into a jugular vein, and blood samples were taken from the carotid artery and the mammary vein to determine Leu kinetics and net uptake of AA. Both CN and C3 treatments separately increased milk protein concentration and yield. With CN there was a general response in mammary protein metabolism, involving increases in Leu net uptake (30%), the uptake:output ratio (8%), protein synthesis (11%), secretion in milk protein (21%), and oxidation (259%). In contrast, C3 treatments tended to increase only Leu in milk protein (7%) and, when in combination with CN, to reduce Leu used for protein synthesis (5%). Across all treatments, most Leu uptake by the mammary gland was accounted for as Leu in milk or oxidized, and the Leu balance was therefore achieved without involvement of either net peptide use or production. Mammary uptake of group 1 AA increased to match milk output with all infusions. In contrast, mammary uptake of group 2 AA exceeded output to a greater extent with CN than with C3 infusions, whereas the increment in uptake of group 3 AA increased with C3 treatments. Overall, these data suggest that different mechanisms operate to improve milk protein production when either protein or energy is supplied.

Key words: casein, propionate, mammary gland, protein metabolism

INTRODUCTION

In dairy cows during midlactation, milk protein yield can be manipulated by varying either energy or protein intake (MacRae et al., 2000). In mechanistic terms, however, changes in either energy or protein supply have been shown to exert different effects on whole-body protein metabolism. For example, CN infusion increased both whole-body protein synthesis and Leu exported in milk to a greater extent than did propionate (C3; Raggio et al., 2006). Furthermore, although whole-body Leu oxidation increased with CN treatment, the addition of C3 to the CN treatment reduced Leu oxidation compared with CN alone. Whether these different responses at the whole-body level are also reflected in mammary gland (MG) metabolism is unknown, however.

More studies have examined responses in MG protein metabolism to altered AA supply than impacts of changed energy provision. Additional protein, via CN infusion, has been shown to increase milk protein output in a wide range of studies (Hanigan et al., 1998). Furthermore, it is well established that the MG can adjust both protein synthesis and Leu oxidation in response to either a high protein intake (Bequette et al., 1996a) or a deficiency in Leu provision (Bequette et al., 1996b). To date, however, no studies have related the impact of energy supply alone to mammary protein metabolism in dairy cows. Partly this may relate to the relatively moderate responses in milk protein yield observed with either glucose or C3 supplementation (Huh-tanen et al., 2002; Rigout et al., 2003). Nonetheless, in view of the known interaction between energy and protein supply in determining milk output, it is clearly necessary to understand the separate and combined effects of these major macronutrients.

In studies in which protein supply has been increased, the uptake:output ratio for the branched-chain AA and Lys is usually elevated (Metcalf et al., 1991; Guinard and Rulquin, 1994; Bequette et al., 1996a; Vanhatalo et al., 2003b; Raggio et al., 2004). This raises the important question of what the fates of this excess AA uptake are. Some reports suggest that these AA

Received January 27, 2006.

Accepted April 5, 2006.

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may be predominantly oxidized (Bequette et al., 1996a) or used to provide N for synthesis of other AA (Lapierre et al., 2005), or both. It has also been proposed that under certain conditions, the MG may release the excess AA taken up as peptides into the mammary vein (Guinard and Rulquin, 1994; Rulquin et al., 2004). The importance of these alternative fates needs to be resolved to increase our understanding of how the MG responds to changes in nutrient supply.

Therefore, the objectives of this study were 1) to differentiate how increased milk protein secretion in response to a change in energy and protein supply relates to changes in MG protein metabolism and net uptake of AA, and 2) to quantify, for Leu, the metabolic fate of that taken up in excess relative to milk output. These objectives were achieved by coupling the arterio-venous technique with infusion of labeled (stable isotope) Leu and bicarbonate. Whole-body protein kinetics from this study have been reported previously (Raggio et al., 2006).

MATERIALS AND METHODS

Animals and Treatments

Three multiparous Holstein cows, averaging 615 ± 24 kg of BW and 65 ± 4 DIM at the beginning of the study, were fitted with both a proximal T-shaped duodenal cannula, placed 10 to 15 cm from the pylorus, and a ruminal cannula (Hurtaud et al., 1998) before calving. Approximately 20 d after calving, cows were surgically prepared with an ultrasonic flow probe (Transonic Systems Inc., Ithaca, NY) implanted around the right external pudic artery as previously described (Rigout et al., 2002), and 2 permanent catheters were placed in the right carotid artery and in the right subcutaneous abdominal vein (for a description of catheters see Guinard et al., 1994, and Raggio et al., 2006). During the experiment, problems were encountered with 2 catheters. One arterial catheter failed and a replacement catheter was inserted in the left carotid artery. In another cow the subcutaneous abdominal vein catheter was nonfunctional and a temporary catheter was inserted (for 3 d) during the second week of each period. In addition, one ultrasonic flow probe failed after the second period. For the tracer infusions, a catheter was inserted in the left jugular vein for the whole study. Catheter maintenance was performed as described previously (Guinard et al., 1994). The surgical preparations were reviewed and approved by the Animal Care Committee of the French Ministry of Agriculture.

The same basal diet (Raggio et al., 2006) was fed during the whole study, balanced to provide limited intestinal glucose (Rigout et al., 2003). The basal diet was estimated to supply 1,593 g/d of protein truly di-

gested in the small intestine (**PDI**) and 124 MJ/d of NE_L (INRA, 1989), or 1,624 g/d of MP and 119 MJ/d of NE_L (NRC, 2001). The quantity of wet feed offered was adjusted every day to ensure the same delivery of DM on each experimental day, based on an estimation of DM content of the silage. The concentrate was supplied every 3 h in equal portions from automatic feeders, starting at 0715 h. Grass silage was fed 3 times per day: 25% at 0715 h, 25% at 1315 h, and 50% between 1715 and 1915 h. However, to maintain a better steady state, the silage was fed 5 times per day during the kinetic days: 12.5% at 0715, 1015, 1315, and 1615 h and then 50% at 1915 h. Throughout the study, access to the diet was limited to 1 h after the concentrate distribution times. Cows had free access to water and were housed in individual tie stalls. Every day before 0715 h, feed refusals were collected, weighed, and sampled for later analyses.

The infusion of 2 nutrients was tested, separately or in combination, according to a 2×2 factorial arrangement: duodenal infusion of calcium caseinate (743 ± 7 g/d, estimated to provide 687 g/d of PDI, and 7.9 MJ/d of NE_L ; Guinard et al., 1994) or ruminal infusions of propionic acid (**C3**; $1,042 \pm 8$ g/d, estimated to provide 15.6 MJ/d of NE_L ; INRA, 1989), or both. Therefore, the 4 treatments were 1) control, 2) CN, 3) C3, and 4) CN and C3 combined (**CN + C3**). Infusion of CN increased the PDI balance from 97% for the control cows to an average of 120% for the cows receiving CN infusions. To optimize the effect of C3, it was infused at the dose that yielded the maximal effect on milk protein observed by Rigout et al. (2003). Each treatment period lasted 14 d, and the first 2 d of each period served as a transition for the infusions (the cows received, on d 1, 33% of the total subsequent infusion and, on d 2, 66% of the total subsequent infusion). Preparation of the infusions is detailed in a separate paper (Raggio et al., 2006).

Sampling and Laboratory Analyses

Cows were milked twice daily (0630 and 1830 h), with yield recorded at each milking and assayed for fat and true protein composition by infrared analysis (ISO 9622: ISO, 1999, 2000; MilkoScan, Foss Electric, Hillerød, Denmark). During the second week of each treatment period, the udder halves of each cow were milked separately, and the right udder samples were analyzed as described above for composition. Feed samples were taken daily and stored at -20°C until analyzed. Stored samples were pooled by period prior to analysis.

On d 11 of each experimental period, mammary Leu kinetics were determined with a primed (4.3 mmol) continuous infusion of L-[1- ^{13}C]Leu (4.3 mmol/h; Cam-

bridge Isotope Laboratories, Andover, MA; 99 atom%) for 7.5 h (starting at 1000 h, 3.5 h after the morning milking). Cows were made to stand for at least 15 min before blood sampling. Hourly samples were taken from the carotid artery and mammary vein starting 3 h after initiation of the infusion (1300, 1400, 1500, 1600, and 1700 h) and were analyzed to determine the concentration and the isotopic enrichment (IE) of Leu, 4-methyl-2-oxopentanoate (MOP), and CO₂. In addition, samples for AA and urea concentrations were taken at 0700, 0900, 1100, 1300, 1500, and 1700 h.

On d 13, a mixture of [¹³C]bicarbonate (Cambridge Isotope Laboratories; 99 atom %; 4.7 mg/mL) and [6,6-²H₂]glucose (Cambridge Isotope Laboratories; 99 atom %; 60 mg/mL) was infused. The rate of [¹³C]bicarbonate infusion was 4.2 mmol/h for 4 h between 1200 and 1600 h, preceded by a priming dose (3.2 mmol). Glucose kinetics will be described in a separate paper. Samples were taken from the carotid artery and mammary vein starting 1.75 h after initiation of the infusion (1345, 1430, 1515, and 1600 h) and were analyzed for concentration and IE of CO₂. On d 11, samples were also collected from the artery prior to the infusions to determine the natural abundance of Leu and MOP (2 samples). On both days of infusion, 3 samples were taken from the artery and the mammary vein to determine the natural abundance of CO₂ because it differs between blood vessels (Read et al., 1984).

Blood samples for AA concentrations were collected in heparinized syringes and kept on ice. Plasma was prepared by centrifugation (2,000 × *g* at 4°C for 15 min). To 0.7 g of plasma was added 0.4 g of an internal standard solution, and the mixture was stored at -80°C. The internal standard solution was a U-¹³C algal hydrolysate plus the following AA: [5-¹⁵N]Gln (501 nmol/g), [indole-¹⁵N]Trp (252 nmol/g), and [1-¹³C]Cys (260 nmol/g). The AA concentrations were determined by isotope dilution as previously described (Calder et al., 1999).

For MOP and [1-¹³C]Leu IE (expressed as molar percent excess, **mpe**) and MOP concentration, 0.7 g of plasma was weighed and 0.3 g of an oxohexanoic acid solution (33.3 nmol/g) was added, and the mixture was stored at -80°C until analysis. Determinations of MOP and Leu IE were as described by Calder and Smith (1988). For CO₂ IE, triplicate 1-mL blood samples were injected into evacuated Vacutainers (Becton Dickinson, Plymouth, UK) containing 1 mL of lactic acid, immediately mixed, and kept at room temperature until analysis. Determination of IE was as previously described (Lobley et al., 2003) based on Read et al. (1984).

Calculations

In addition to the blood flow probe, mammary plasma flow (**PF**) was estimated according to the Fick principle,

using Phe and Tyr as internal markers with an allowance for a 3.5% contribution from blood-derived proteins (Linzell, 1974; Cant et al., 1993). For this paper, the Fick principle was adopted to estimate PF and calculate mammary AA fluxes because the probe was not functional for one cow. For the 2 functional cows, the probe measurements were 24% lower than mammary PF estimated with the Fick principle. Similar underestimates with ultrasound probes have previously been observed when compared either with the Fick principle (Thivierge et al., 2002) or with *p*-aminohippurate (Bequette et al., 1999). Nevertheless, there was no effect of treatment on the uptake:output ratio of Phe and Tyr when the uptake was estimated with the PF from the probe for the 2 functional cows. Secretions of AA into milk were calculated using published AA concentrations in milk (Swaigood, 1993) except for Leu, Phe, and Tyr, for which milk samples were acid-hydrolyzed with 6 *N* phenol-HCl for 18 h at 110°C (AOAC, 2000) and AA concentrations of the hydrolysates were determined by the isotope dilution method (Calder et al., 1999). Concentrations of Leu, Phe, and Tyr averaged 98, 49, and 52 g/kg of CP, respectively.

For the following equations, infusion rates and fluxes of Leu, MOP, or CO₂ are in millimoles per hour. The concentrations of Leu, MOP, and CO₂ are expressed in millimoles per liter. The IE of ¹³C Leu, ¹³C MOP, and ¹³CO₂, are given in mpe/100. In all cases, *m* refers to the mammary vein, and *a* to the artery. Plasma flow is given in L/h.

Net Leu uptake was calculated as

$$\text{net uptake} = ([\text{Leu-}a] - [\text{Leu-}m]) \times \text{PF}$$

using the 6 samples collected every other hour, because milk protein yield over the 12 h was used to calculate the PF with the Fick principle. Net uptakes measured during infusion of the tracer were corrected for the extra oxidation in the MG caused by infusion of the tracer as described below (**CorOx**).

Mammary gland Leu kinetics were studied using a 3-compartment model with Leu, CO₂, and MOP as previously described by Bequette et al. (2002), but with IE of MOP from the mammary vein taken as representative of IE of the precursor pool.

The MG irreversible loss rate (**ILR**) of Leu was calculated as follows:

$$\text{MG Leu ILR} = \{([[\text{Leu-}a] \times \text{IE}_{\text{Leu-}a} - [\text{Leu-}m] \text{IE}_{\text{Leu-}m}) \times \text{PF}\} / \text{IE}_{\text{MOP-}m} - \text{CorOx}.$$

Dynamic transfers of MOP to Leu or CO₂ (positive values) or Leu to MOP (negative values) were calculated from

$$\text{MG MOP to Leu or CO}_2 = \{([MOP-a] \times IE_{MOP-a} - [MOP-m] \times IE_{MOP-m}) \times PF\} / IE_{MOP-m}$$

where [Leu] and [MOP] refer to concentrations of Leu and MOP, respectively, IE refers to their isotopic enrichment, and PF refers to plasma flow.

Blood flow was used to calculate MG $^{13}\text{CO}_2$ fluxes and was estimated as follows:

$$\text{BF} = \text{PF} / (1 - \text{hematocrit} - \alpha)$$

where BF is blood flow. The MG $^{13}\text{CO}_2$ flow was calculated as follows:

$$\text{MG } ^{13}\text{CO}_2 = ([\text{CO}_2-m] \times IE^{13}\text{CO}_2-m - [\text{CO}_2-a] \times IE^{13}\text{CO}_2-a) \times \text{BF}$$

To correct for $^{13}\text{CO}_2$ sequestration (**Sq**) that occurred during the infusion of ^{13}C Leu, $^{13}\text{CO}_2$ Sq was estimated during the bicarbonate infusion as the arterial inflow of $^{13}\text{CO}_2$ not recovered in the mammary vein. During period 6, Sq was not determined because of technical problems with the measurements of IE of $^{13}\text{CO}_2$ during the bicarbonate infusion. We used an average of Sq for each cow (1.4, 1.2, and 4.8%) because no significant differences were observed between treatments.

Therefore, the corrected MG $^{13}\text{CO}_2$ flux was calculated as follows:

$$\text{MG } ^{13}\text{CO}_2 \text{ corrected} = (\text{arterial inflow of } ^{13}\text{CO}_2 \times \text{Sq}) + \text{MG } ^{13}\text{CO}_2$$

Oxidation of Leu (Leu Ox) across the MG was calculated as

$$\text{MG Leu Ox} = \text{MG } ^{13}\text{CO}_2 \text{ corrected} / IE_{MOP-m} - \text{CorOx}$$

Tracer kinetics assumes the animal does not distinguish between ^{13}C -AA and ^{12}C -AA. In addition, at the whole-body level, it was assumed that all Leu tracer infused was in excess, and that a corresponding amount of Leu was thus oxidized by the animal (Raggio et al., 2006). Accordingly, it was assumed that mammary Leu oxidation measured during the tracer infusion included a proportion of this extra oxidation, calculated to be in proportion to the measured MG oxidation as a fraction of the measured whole-body oxidation (before correction was made to account for the tracer infusion) multiplied by the infusion rate. This factor (CorOx) was subtracted from the measured Leu oxidation across the MG as well as the ILR and net flux, which should have all been increased by this amount during the tracer infusion.

Fractional oxidation was calculated as this corrected oxidation divided by the ILR.

Leucine used for MG protein synthesis was calculated as the difference between the MG Leu ILR, including conversion to or from MOP, and MG oxidation. Once Leu used for milk protein secretion was subtracted from Leu used for protein synthesis, the remainder was converted into kilograms of constitutive protein, applying the factor 63 g of Leu/kg of constitutive protein (Lobley et al., 1980).

“Residual” Leu was obtained by the difference of the amount of Leu taken up by the MG plus the dynamic transfer of MOP to Leu or CO_2 minus oxidation and secretion as milk protein. This represents the net mammary Leu inflow not accounted for by the measured outflows:

$$\begin{aligned} \text{residual} &= \text{net Leu uptake} + \text{MOP to Leu or CO}_2 \\ &\quad - \text{MG Ox} - \text{Leu in milk} \end{aligned}$$

If positive and significantly different from zero, this “residual” would suggest that an additional metabolic fate occurred (e.g., release in peptide form into the mammary vein).

Net mammary uptake of other AA was calculated using the 6 samples collected every other hour as follows:

$$\text{net uptake} = ([\text{AA}-a] - [\text{AA}-m]) \times \text{PF}$$

where [AA] refers to concentrations of individual AA and PF refers to plasma flow.

Experimental Design and Statistical Analyses

The original design was a 4×4 Latin square, but the loss of one cow before the start of the experiment led to a redesign of the experiment as 2 incomplete 4×3 Youden squares, with 3 periods for each square (i.e., 6 periods per cow), for a total of 18 observations distributed as follows: control, $n = 5$; CN, $n = 4$; C3, $n = 5$; CN + C3, $n = 4$. During the third period, one cow had a problem with her catheter during the CN treatment, and as a consequence, $n = 3$ for CN.

Analyses of variance were made using the MIXED procedure of SAS (SAS Institute, 1999) according to the following statistical model:

$$\begin{aligned} Y_{ijkl} &= \mu + \text{SQUARE}_i + \text{PERIOD}_j(\text{SQUARE}_i) \\ &\quad + \text{COW}_k + \text{TREAT}_l + e_{ijkl} \end{aligned}$$

Differences among treatments were compared using orthogonal contrasts, estimating the main effect of CN,

Table 1. Effect of CN and propionate (C3) supply on milk yield and composition of the right-half udder in dairy cows¹

Item	Treatment				SEM	P		
	Control	CN	C3	CN + C3		CN	C3	CN × C3
Milk, kg/12 h	6.38	7.39	6.55	7.38	0.19	<0.001	0.68	0.60
True protein, g/kg	29.3	30.2	30.6	32.8	0.8	0.04	0.03	0.35
True protein, g/12 h	186	222	197	240	7	<0.01	0.06	0.59
CN, % of true protein	82.4	83.5	82.0	82.3	0.4	0.07	0.05	0.31
NPN, % of CP	2.13	2.39	2.05	2.24	0.09	0.02	<0.01	0.17
Fat, g/kg	44.8	42.3	46.1	39.0	2.7	0.07	0.71	0.36
Fat, g/12 h	282	304	299	286	24	0.82	0.97	0.40

¹Least squares means presented with pooled SEM, given for n = 3; from the half udder in the evening (12 h) of the Leu kinetic day (17 observations: control = 5, CN = 3, C3 = 5, CN + C3 = 4). P = probability corresponding to the null hypothesis with CN, C3, and CN × C3 contrasts.

C3, and the interaction between the 2 nutrients. Results are expressed as least square means with the highest standard error of the mean. Significance was set at $P \leq 0.05$ and the tendency at $0.05 \leq P \leq 0.10$.

RESULTS

Milk production and composition for the last week of the experimental periods were presented in a previous paper (Raggio et al., 2006). In the present paper, milk production and composition were based on the right-half udder at the evening milking (12 h) of the Leu kinetic day (Table 1). Milk yield increased with CN treatments ($P < 0.001$). True protein concentration and yield both increased with CN ($P = 0.04$; $P < 0.01$) and C3 ($P = 0.03$; $P = 0.06$) infusions.

Mammary Leu Kinetics

The mammary PF tended to decrease by an average of 16% ($P = 0.07$) when CN was infused and increased by 32% ($P = 0.02$) when C3 was supplied (Table 2). The MG Leu kinetics are presented in Table 2. The net uptake of Leu and MOP increased with CN infusions ($P < 0.01$) but did not show an effect when C3 was infused. The MG Leu ILR was increased by CN infusions ($P < 0.001$) and tended to increase with C3 treatments ($P = 0.09$). In addition, there was a CN × C3 interaction ($P < 0.01$), because the response with combined treatments was smaller than the sum of the response to each nutrient supplied separately. Both dynamic transfer of MOP to Leu or CO₂ (MOP to Leu or CO₂) and mammary oxidation of Leu (Leu Ox) increased when CN was supplied ($P = 0.04$; $P < 0.001$, respectively). The mammary fractional oxidation of Leu increased ($P < 0.001$) with CN supply. Leucine used for protein synthesis (Leu PS) increased with CN ($P = 0.02$) but, as with the ILR, there was a tendency for a CN × C3 interaction ($P = 0.09$). Leucine in milk protein (Leu

Milk) increased with CN treatments ($P < 0.001$) and tended to increase with C3 infusions ($P = 0.06$). In addition, differences were observed for Leu derived from protein degradation (estimated as Leu PS minus Leu Milk), with an increase with CN alone compared with the control, but a decrease with CN and C3 combined compared with C3 (interaction CN × C3, $P < 0.001$).

AA Arterio-Venous Concentration Differences

The AA arterio-venous concentration differences are presented in Table 3. When CN was supplied, the arterio-venous difference increased for all AA ($P \leq 0.05$), except for Asp and Glu, for which there was no effect, and for Ala, for which there was a decrease ($P = 0.03$). In contrast, during C3 infusions the arterio-venous differences decreased for all AA ($P \leq 0.06$) except Asp, Cys, Glu, Ser, and Thr, which were not affected, and Ala, for which there was an increase ($P < 0.01$). There was also a trend ($P \leq 0.10$) for an interaction with the branched-chain AA, with a larger decrease with C3 and CN compared with CN than when comparing C3 with the control.

AA Uptake and Uptake:Output Ratio

The MG uptake data are presented in Table 4. During CN infusions, the uptake increased ($P \leq 0.01$) for all the essential AA (EAA) as well as for Gln, Gly, and Pro, whereas uptakes were reduced for Ala ($P < 0.001$) and Glu ($P = 0.02$). With C3 treatments, uptake of the nonessential AA (NEAA) Ala, Glu, and Pro, as well as the EAA Met, Phe, and Thr, increased ($P < 0.05$). There was also a CN × C3 interaction ($P = 0.02$) for Ala uptake, with a larger decrease when CN was added to the control diet than when CN was added to C3.

The AA uptake:output ratios are presented in Table 5. If the ratio exceeded unity, then the mammary uptake was more than sufficient to account for milk pro-

Table 2. Effect of CN and propionate (C3) supply on Leu kinetics and plasma flow of the right-half udder in dairy cows¹

Item, mmol/h	Treatment				SEM	P		
	Control	CN	C3	CN + C3		CN	C3	CN × C3
Net Leu ²	13.48	17.63	14.11	18.37	0.83	<0.001	0.40	0.94
Net MOP ³	0.22	0.37	0.19	0.29	0.03	<0.01	0.19	0.39
Leu ILR ⁴	18.65	24.48	21.07	23.97	0.51	<0.001	0.09	0.01
MOP to Leu or CO ₂	0.13	0.29	0.16	0.25	0.06	0.04	0.84	0.45
Leu Ox ⁵	1.52	4.28	1.69	4.04	0.38	<0.001	0.90	0.54
Fractional Ox	0.08	0.18	0.08	0.16	0.01	<0.001	0.55	0.54
Leu PS ⁶	17.25	20.49	19.54	20.18	0.76	0.02	0.21	0.09
Leu Residual ⁷	0.44	-0.28	0.20	-0.42	0.76	0.30	0.81	0.95
Leu Milk ⁸	11.64	13.93	12.34	15.01	0.43	<0.001	0.06	0.61
Leu Milk/Leu PS	0.75	0.77	0.71	0.83	0.01	<0.001	0.62	0.02
Leu PS - Leu Milk	4.54	4.99	6.02	3.62	0.33	<0.01	0.85	<0.001
MG Leu Ox/WB Leu Ox ⁹	0.49	0.43	0.4	0.47	0.11	0.98	0.85	0.49
MG ILR/WB ILR ¹⁰	0.42	0.43	0.43	0.38	0.02	0.22	0.40	0.10
Plasma flow, L/h	307	241	386	338	32	0.07	0.02	0.75

¹Least squares means presented with pooled SEM, given for n = 3 (17 observations: control = 5, CN = 3, C3 = 5, CN + C3 = 4). P = probability corresponding to the null hypothesis with CN, C3, and CN × C3 contrasts.

²Based on the 6 samples collected every other hour, corrected for increased oxidation due to tracer infusion.

³Net 4-methyl-2-oxopentanoate (MOP) uptake during Leu kinetic measurement.

⁴Leucine irreversible loss rate (ILR), corrected for increased oxidation due to tracer infusion.

⁵Leucine oxidation (Ox), corrected for increased oxidation due to tracer infusion.

⁶Leucine used for protein synthesis (PS).

⁷Calculated as net Leu + MOP to Leu or CO₂ - Leu Ox - Leu Milk.

⁸Leu in milk protein, from milk collected from the right-half udder at the evening milking on the day of kinetic measurements.

⁹Mammary gland (MG) oxidation (half udder × 2)/whole-body (WB) oxidation, adjusted to use the same precursor pool.

¹⁰Mammary gland ILR (half udder × 2)/WB ILR, adjusted to use the same precursor pool.

tein secretion; if smaller than unity, then there was a need for either de novo synthesis in the gland or uptake in peptide form. Uptake of EAA was sufficient to account for milk output, except for Met when CN was infused. The uptake:output ratio of NEAA varied greatly between AA, from a large excess for Arg to ratios as low as 0.14 for Asp. With CN treatments, the uptake:output ratios decreased ($P < 0.001$) for Ala and Glu, whereas they increased for Pro ($P < 0.001$), with a tendency to increase ($P \leq 0.08$) for Ile and Leu. However, when C3 was supplied, the ratio for Ala increased ($P < 0.001$) and tended to either increase ($P = 0.07$) for Glu or decrease ($P = 0.08$) for Gln.

Nitrogen Balance Across the MG

Nitrogen balances are presented in Table 6. This calculation is based on mammary uptake and milk output of AA used for protein synthesis, except for Asn, which was not measured. Total N uptake increased ($P < 0.01$) when CN was supplied ($P < 0.01$), with a tendency to increase when C3 was infused ($P = 0.07$). Nitrogen uptake as EAA (EAA-N) increased ($P < 0.001$) with CN but C3 had no effect. Nitrogen uptake as NEAA (NEAA-

N) was increased by both CN ($P = 0.05$) and C3 ($P = 0.03$). Total-N, EAA-N, and NEAA-N in milk were all increased for CN ($P < 0.001$) and tended to increase for C3 ($P = 0.07$). In terms of N balances across the MG, the NEAA-N deficit increased ($P = 0.05$) when CN was infused. Overall, however, uptake of total AA-N from the plasma matched the N output as milk proteins (Table 6), with the small positive values not being significantly different from zero except for a tendency ($P = 0.08$) for the C3 infusion alone.

DISCUSSION

This study had 2 objectives. The first was to examine whether supplemental protein and energy, the latter as C3, had similar mechanistic effects on protein and AA metabolism within the MG to increase milk protein yield. The second was to ascertain the fate of excess AA taken up by the MG and help resolve the issue of whether the MG can produce a net secretion of peptides or whether oxidative pathways are the major routes of removal. In both cases, clear answers to these questions were obtained.

Table 3. Effect of CN and propionate (C3) supply on plasma arterio-venous differences of AA on the right-half udder in dairy cows¹

AA, μM	Treatment				SEM	P		
	Control	CN	C3	CN + C3		CN	C3	CN × C3
Ala	28.5	16.5	33.4	33.1	2.64	0.03	<0.01	0.39
Arg	24.5	32.4	22.3	27.8	1.34	<0.01	0.03	0.32
Asp	1.8	2.5	2.3	2.5	0.40	0.27	0.54	0.63
Cys	2.4	3.4	2.2	2.9	0.30	0.01	0.25	0.73
Gln	36.2	54.2	26.1	40.2	4.27	<0.01	0.02	0.60
Glu	39.5	36.5	40.6	36.9	2.61	0.17	0.74	0.88
Gly	-3.9	4.2	-5.4	0.1	1.62	<0.01	0.10	0.37
Pro	9.3	20.4	8.3	18.0	0.79	<0.01	0.05	0.33
Ser	6.9	15.2	7.4	11.7	3.14	0.05	0.61	0.49
Tyr	14.5	21.6	11.7	16.7	1.78	<0.001	0.06	0.50
His	9.5	13.5	8.2	11.3	0.75	<0.01	0.04	0.49
Ile	29.9	47.6	25.2	37.6	1.60	<0.001	<0.01	0.10
Leu	45.6	73.1	38.4	58.1	2.60	<0.001	<0.01	0.12
Lys	37.7	56.9	32.2	48.2	3.00	<0.001	0.04	0.55
Met	9.7	13.4	8.1	11.2	0.78	<0.001	0.04	0.65
Phe	15.6	22.4	13.8	19.3	1.07	<0.01	0.04	0.47
Thr	19.6	27.1	17.3	23.6	0.00	<0.01	0.15	0.71
Trp	3.4	5.8	3.2	4.4	0.32	<0.01	0.01	0.18
Val	37.1	57.4	30.5	44.5	1.73	<0.001	<0.001	0.07

¹Least squares means presented with pooled SEM, given for n = 3 (17 observations: control = 5, CN = 3, C3 = 5, CN + C3 = 4). P = probability corresponding to the null hypothesis with CN, C3, and CN × C3 contrasts.

Protein Metabolism

Leu Net Uptake. There were obvious differences in how the net uptake of Leu and secretion as milk were affected by the supply of CN and C3. With CN treatments, the increment in net uptake of Leu was greater than the increase in milk protein yield, with only 59% of the increment in net uptake transferred to milk. This result supports earlier observations that the difference between mammary net uptake of Leu and secretion in milk protein becomes greater with an increased protein supply. [For example, in terms of the uptake:output ratio, this increased from 1.04 to 1.87 with CN infusion (Guinard and Rulquin, 1994); from 1.05 to 1.66 with Leu infusion (Rulquin and Pisulewski, 2000); and from 1.10 to 1.40 with a high-RUP supplement (Raggio et al., 2004).] This indicates that metabolic pathways other than milk protein synthesis are stimulated by the protein supply and that these can be determined using tracer infusion, as discussed below.

In contrast, when C3 was infused, the nonsignificant increase in Leu net uptake (4.4%) was less than the extra Leu secreted in milk protein. In consequence, the apparent efficiency of use of the extra uptake was 130%. Similarly, Vanhatalo et al. (2003a) did not observe an increase in the uptake of Leu, although milk protein yield was improved by the infusion of glucose. Together, these data suggest that when an increased energy supply elevates the milk protein yield, without any concomitant increase in the protein supply, the MG uses its Leu uptake more efficiently toward milk protein secretion.

However, energy does not always exert a positive effect, because when a very high dose of glucose was infused as an energy substitute, the uptake:output ratio of Leu increased because of a combined decrease in the milk protein yield coupled with an increased Leu net uptake (Rulquin et al., 2004).

Leu Kinetics. The current kinetic data were quantified assuming that the enrichment of MOP in the mammary venous plasma was similar to that of the precursor intracellular pool. Because MOP is formed intracellularly from Leu deamination, MOP release into the venous drainage ideally should reflect intracellular enrichment of Leu. In practice, only part of the venous metabolite is derived from MG intracellular outflow while the rest arises from the bypass arterial supply (Biolo et al., 1995), so the IE of venous MOP will be lower than MOP or Leu in the cells of the MG. Indeed, the intracellular Leu enrichment will lie between those of MOP and Leu in the venous plasma. Consequently, use of venous MOP enrichment as a precursor will slightly overestimate the true rates of protein synthesis but will still allow comparison between treatments.

Compared with the control, CN alone increased the MG ILR by 22%, confirming the general response observed previously by Bequette et al. (1996a) in dairy cows with increased dietary protein supply. For the current cows, the dynamic increase (5.8 mmol/h) was partitioned approximately equally between oxidation and protein synthesis, of which 80% went to exported proteins (milk) and the rest to constitutive sources.

Table 4. Effect of CN and propionate (C3) supply on AA uptake by the right-half udder in dairy cows¹

AA, mmol/h	Treatment				SEM	P		
	Control	CN	C3	CN + C3		CN	C3	CN × C3
Ala	8.8	3.6	12.8	10.9	0.64	<0.001	<0.001	0.02
Arg	7.4	8.1	8.5	9.3	0.83	0.33	0.18	0.94
Asp	0.5	0.6	0.9	0.8	0.18	0.99	0.13	0.71
Cys	0.7	0.8	0.8	0.9	0.09	0.11	0.24	0.66
Gln	11.1	13.7	9.8	13.2	0.66	<0.001	0.21	0.52
Glu	12.1	8.9	15.6	12.2	1.29	0.02	0.03	0.95
Gly	-1.3	1.0	-2.4	0.0	0.51	<0.01	0.07	0.99
Pro	2.7	5.1	3.2	5.8	0.23	<0.001	0.02	0.45
Ser	2.0	3.6	2.6	3.8	0.89	0.11	0.67	0.76
Tyr	4.4	5.4	4.4	5.4	0.25	<0.01	0.89	0.98
His	2.9	3.3	3.1	3.7	0.17	0.01	0.12	0.69
Ile	9.1	11.9	9.5	12.4	0.57	<0.001	0.34	0.93
Leu ²	13.5	17.6	14.1	18.4	0.83	<0.001	0.40	0.94
Lys	11.4	14.1	12.3	15.9	0.84	<0.001	0.14	0.51
Met	2.9	3.3	3.1	3.7	0.10	<0.001	0.05	0.26
Phe	4.7	5.5	5.2	6.3	0.20	<0.01	0.01	0.43
Thr	5.9	6.7	6.5	7.8	0.37	0.01	0.05	0.52
Trp	1.2	1.5	1.2	1.4	0.09	0.01	0.92	0.64
Val	11.2	14.3	11.5	14.7	0.69	<0.01	0.59	0.94

¹Least squares means presented with pooled SEM, given for n = 3 (17 observations: control = 5, CN = 3, C3 = 5, CN + C3 = 4). P = probability corresponding to the null hypothesis with CN, C3, and CN × C3 contrasts.

²Corrected for increased oxidation due to tracer infusion.

In contrast, with C3 infusions all of the ILR increase (0.96 mmol/h) was diverted to protein synthesis with a minor effect on oxidation (0.04 mmol/h). This indicates that the MG adjusts differently to increased protein vs. energy supply. Although energy supply is known to influence plasma insulin concentrations, these were unaltered in the current study (unpublished data); thus,

other mechanisms must operate. Because energy appears to induce different responses than protein, the possibility thus arises of additive, or even synergistic, effects when both nutrients are supplied together. In practice, however, the interaction between CN and C3 on protein synthesis indicates that the cosupply of CN and C3 was not completely additive.

Table 5. Effect of CN and propionate (C3) supply on AA uptake:milk output ratio in dairy cows¹

AA ²	Treatment				SEM	P		
	Control	CN	C3	CN + C3		CN	C3	CN × C3
Ala	1.05	0.50	2.08	1.46	0.09	<0.001	<0.001	0.06
Arg	2.44	2.23	2.64	2.33	0.24	0.23	0.53	0.83
Asp	0.14	0.12	0.21	0.16	0.04	0.39	0.23	0.61
Cys	0.80	0.78	0.83	0.83	0.07	0.86	0.63	0.84
Gln	1.09	1.14	0.92	1.02	0.07	0.30	0.08	0.67
Glu	0.93	0.58	1.14	0.74	0.09	<0.001	0.07	0.73
Pro	0.20	0.31	0.22	0.33	0.01	<0.001	0.16	0.93
Ser	0.21	0.32	0.26	0.30	0.08	0.30	0.89	0.70
His	1.06	1.02	1.08	1.06	0.05	0.50	0.64	0.91
Ile	1.29	1.41	1.28	1.37	0.05	0.07	0.61	0.76
Leu	1.16	1.27	1.14	1.22	0.05	0.08	0.50	0.76
Lys	1.29	1.33	1.30	1.40	0.07	0.29	0.51	0.59
Met	1.00	0.95	0.99	0.97	0.02	0.09	0.94	0.42
Thr	1.05	0.99	1.09	1.06	0.04	0.27	0.26	0.72
Trp	1.02	1.07	1.00	0.97	0.07	0.88	0.43	0.52
Val	1.28	1.36	1.24	1.29	0.06	0.23	0.36	0.78

¹Least squares means presented with pooled SEM, given for n = 3; milk output was estimated from milk collected from the half udder on the evening of the Leu kinetic day (17 observations: control = 5, CN = 3, C3 = 5, CN + C3 = 4). P = probability corresponding to the null hypothesis with CN, C3, and CN × C3 contrasts.

²Phe and Tyr were not included because they were used to calculate plasma flow.

Table 6. Effect of CN and propionate (C3) supply on the nitrogen balance in the right-half udder in dairy cows¹

Nitrogen, ² mmol/h	Treatment				SEM	<i>P</i>		
	Control	CN	C3	CN + C3		CN	C3	CN × C3
Uptake								
Total-N	163.0	189.5	178.0	212.8	9.43	0.01	0.07	0.62
EAA-N	81.3	100.7	86.4	109.2	4.89	<0.001	0.18	0.69
NEAA-N	81.7	88.8	91.7	103.6	4.81	0.05	0.03	0.57
Group 1	23.0	27.0	24.5	29.5	0.88	0.00	0.05	0.54
Group 2	56.7	72.1	59.7	77.4	3.70	0.00	0.26	0.73
Group 3	79.6	86.4	89.7	101.1	4.98	0.09	0.03	0.59
Milk								
Total-N	156.1	186.8	165.2	200.9	5.74	<0.001	0.07	0.61
EAA-N	68.9	82.6	73.0	88.8	2.54	<0.001	0.07	0.61
NEAA-N	87.1	104.3	92.2	112.2	3.20	<0.001	0.07	0.61
Group 1	22.5	26.9	23.9	29.0	0.83	<0.001	0.07	0.61
Group 2	45.3	54.2	47.9	58.3	1.67	<0.001	0.07	0.61
Group 3	84.9	101.7	89.9	109.3	3.11	<0.001	0.07	0.61
Balance								
Total-N	6.9	2.7	12.8	11.8	8.33	0.72	0.36	0.83
EAA-N	12.3	18.2	13.3	20.3	4.17	0.11	0.69	0.88
NEAA-N	-5.4	-15.5	-0.5	-8.6	4.51	0.05	0.20	0.79
Group 1	0.5	0.1	0.6	0.5	0.54	0.54	0.60	0.81
Group 2	11.4	17.9	11.8	19.1	3.20	0.04	0.79	0.89
Group 3	-5.3	-15.3	-0.2	-8.3	4.80	0.01	0.21	0.82

¹Least squares means presented with pooled SEM, given for n = 3; milk output was estimated from milk collected from the half udder on the evening of the Leu kinetic day (17 observations: control = 5, CN = 3, C3 = 5, CN + C3 = 4). P = probability corresponding to the null hypothesis with CN, C3, and CN × C3 contrasts.

²Total nitrogen (total-N) = N-His + N-Ileu + N-Leu + N-Lys + N-Met + N-Phe + N-Thr + N-Trp + N-Val + N-Ala + N-Arg + N-Asp + N-Cys + N-Glu + N-Gln + N-Gly + N-Pro + N-Ser + N-Tyr; essential AA nitrogen (EAA-N) = N-His + N-Ileu + N-Leu + N-Lys + N-Met + N-Phe + N-Thr + N-Trp + N-Val; nonessential AA nitrogen (NEAA-N) = N-Ala + N-Arg + N-Asp + N-Cys + N-Glu + N-Gln + N-Gly + N-Pro + N-Ser + N-Tyr; group 1 = N-His + N-Phe + N-Met + N-Tyr + N-Trp; group 2 = N-Ileu + N-Leu + N-Val + N-Lys; group 3 = N-Ala + N-Arg + N-Asp + N-Cys + N-Glu + N-Gln + N-Gly + N-Pro + N-Ser.

In this study, MG ILR accounted for 38 to 43% of whole-body ILR, as previously reported in dairy cows (37 to 44%, Bequette et al., 1996a; 41 to 43%, Thivierge et al., 2002), confirming the major contribution of the MG to whole-body metabolism. The MG also played a major role in whole-body Leu oxidation, accounting for 40 to 50% across the range of treatments studied. Certainly the gland can remove close to half of any excess supply, and in the lactating animal may therefore be more important than the other ruminant tissues known to be involved in Leu catabolism (Goodwin et al., 1987). Estimated synthesis of constitutive protein by the MG averaged 975 g/d, indicating the high turnover rate of constitutive protein in the MG.

Net Uptake of AA. The use of precise analytical techniques to measure AA concentrations in the current study also allows examination of how CN and C3 modified the net mammary uptake of individual AA to support the increased milk protein yield generated.

Effect of CN. Treatment effects on arterial AA concentrations have already been discussed in a previous paper (Raggio et al., 2006). Across the MG, the arterio-venous differences increased for almost all EAA with

CN infusions, as previously reported with infusions of CN ranging from 177 to 762 g/d (Clark et al., 1977; Guinard and Rulquin, 1994; Vanhatalo et al., 2003b). In these studies, net uptake, calculated from reported arterio-venous differences and PF, increased with CN infusions. Despite the CN-induced decrease in PF in the current experiment, CN treatments also increased the net uptake of all EAA.

Mepharm (1982) divided AA into 3 groups, each having a different metabolism within the MG. Group 1 includes His, Met, Phe + Tyr, Thr, and Trp, and for these AA their stoichiometric transfer yields uptake:output ratios close to unity (e.g., 1.14, Guinard and Rulquin, 1994; 1.02, Miettinen and Huhtanen, 1997; 0.95, Raggio et al., 2004). The differences between studies might be due to the different energy or protein status of the animals as well as to different methodologies, including blood flow measurement (probe, Fick principle, *p*-aminohippurate), AA analyses (number of samples taken, use of individual or pooled samples, analysis technique), and frequency of blood sampling relative to feeding protocols. Because of uncertainties of whether Thr belongs to this group (Lapierre et al.,

2005), Thr was not included in group 1 calculations. In line with the concept of Mephram, MG uptake of group 1 AA increased to maintain at unity (0.99) the uptake:output ratio as milk protein production changed. This supports previous reports in which, with additional protein supply, the udder extracted these AA in ratios equal to their secretion in milk protein (Guinard and Rulquin, 1994; Vanhatalo et al., 2003a; Raggio et al., 2004).

The second group discussed by Mephram (1982) included EAA usually taken up in excess by the MG relative to milk output, namely, Ile, Leu, and Val (the branched-chain AA) and Lys (Clark et al., 1977; Guinard and Rulquin, 1994; Raggio et al., 2004). Arginine was not considered an EAA and was therefore not included in group 2 calculations. Casein infusions resulted in an increased uptake:output ratio for the group 2 AA, from 1.28 to 1.34, comparable to other findings (e.g., from 1.41 to 1.53, Clark et al., 1977; from 1.21 to 1.64, Guinard and Rulquin, 1994; from 1.57 to 1.61, Vanhatalo et al., 2003a; from 1.33 to 1.50, Vanhatalo et al., 2003b).

For many of the AA of the third group, which constitute the NEAA (except Tyr), uptake was far less than output under basal conditions (Table 5), except for Gln and Ala (close to balance) and Gly (net release). For Arg (here considered as a NEAA), uptake was consistently in excess of output. Casein infusions increased net uptakes of Gln, Gly, and Pro, whereas Ala and Glu net uptakes decreased. Vanhatalo et al. (2003a) also observed a decrease of Glu uptake, but no change in Ala uptake, with 350 g of CN. Overall, for group 3 AA, net uptake increased less than the output in milk in response to CN infusions, leading to a greater deficit in uptake relative to output (Table 6).

Therefore, the MG responded to the increased demand for NEAA for milk protein synthesis with CN supply by increasing the uptake of the EAA of group 2 to a greater extent than the net uptake of the NEAA per se. Based on transfers of AA present in milk protein, these increases were enough to achieve a close overall balance for N transactions in the MG. Why the gland increases uptake of group 2 AA to restore balance instead of extracting more NEAA from the blood during CN supplementation remains a mystery. Obviously, this is not due to a lack of NEAA, as both NEAA and EAA were supplied through infusion of CN. In addition, it has been shown, using tracer kinetics, that for certain NEAA in which the net uptake:output ratios were less than unity across the caprine MG, there was isotopic dilution between the artery and vein (Bequette et al., 1997). This means that although the MG extracted only a proportion of the NEAA needed to support milk protein output, there was sufficient *de novo* synthesis of

these AA within the MG to create an excess that was then released into the mammary vein. This may suggest that *de novo* synthesis is an important process within the MG when there is a general excess of AA. Whatever the reason, the metabolism of the MG adjusts to produce more milk protein from an extra protein supply by using additional N from those EAA extracted in excess to synthesize NEAA rather than increasing, on a net basis, the extraction of NEAA.

Effect of C3. When C3 was infused, there were differences in how the MG achieved N balance. Here, PF increased, regardless of whether determined by the Fick principle (Clark et al., 1977; Huhtanen et al., 2002; Vanhatalo et al., 2003b) or through the use of flow probes (Rulquin et al., 2004). Although net uptake of group 1 AA increased to parallel the extra milk protein output, the largest changes were observed with group 3 AA. The uptake:output ratio of both Ala and Glu increased to considerably exceed unity. These changes, coupled with an increased net uptake of some of the other NEAA, as also observed for Ala (Clark et al., 1977) and Pro (Vanhatalo et al., 2003b; Rulquin et al., 2004), were sufficient to reduce the deficit for group 3 AA (Table 6). Therefore, the mechanisms that operate to stimulate milk protein output in the absence of an extra protein supply (i.e., solely with energy provision) involve increased MG uptake of some AA of group 3. This contrasts with the response to an increased protein supply, in which uptake of group 2 AA is augmented. Again, this suggests that the MG may be an important site within the body for removal of excess group 2 AA, and the transfer of their N and C structures to form group 3 AA may be part of the homeostatic process. Confirmation of this concept requires more detailed studies on transport kinetics and N transfer within the MG. Such approaches would also help unravel how different pathways respond to changes in the protein and energy supply.

Peptide Release

Our second objective was to examine the fate of Leu uptake under conditions similar to those reported in which uptake of Leu was greatly in excess of milk output and in which there was an unaccounted for AA balance, hypothesized as a release of peptides by the MG (Guinard and Rulquin, 1994; Rulquin et al., 2004). Within the current study, the various metabolic fates of Leu were quantified, permitting a complete balance for this AA. In the control cows, all the Leu extracted by the MG was secreted as milk or oxidized. Furthermore, the extra uptake observed with an increased protein supply was also directed toward these 2 metabolic pathways. In all treatments, the Leu unaccounted for

across the MG (the difference between uptake plus movement from MOP minus oxidation minus milk) was small and not significantly different from zero. Similarly, the N balance across the MG for AA used for protein synthesis (without Asn) was not different from 0. Thus, there was no need to invoke an additional metabolic route via peptide release into the mammary vein. This is similar to the finding of Lapierre et al. (2005) in which increased Lys-N uptake was quantitatively used for mammary synthesis of NEAA and in which it was also unnecessary to hypothesize the export of peptides.

CONCLUSIONS

The responses in milk protein output when CN or C3 was supplied to a basal ration involve different mechanisms within the MG. Both treatments increased the mammary Leu ILR, but the response was greater when CN was infused, and was accompanied by increased oxidation. During CN infusions, N balance across the MG was mainly achieved by increased uptake of group 1 and 2 AA, but during C3 supplementation this was accomplished by increased uptake of AA from groups 1 and 3. No evidence was found for the release of peptides by the MG.

ACKNOWLEDGMENTS

The authors gratefully thank the UMR PL, INRA, France; Y. Lebreton for surgeries; P. Lamberton and his team for technical support and animal care during the experiment; C. Nahuet, L. Finot, N. Huchet, I. Jicquel, and S. Rigault for their help in laboratory analyses; and, from the Rowett Research Institute, A. G. Calder and S. E. Anderson for mass spectrometric analyses. The authors also wish to acknowledge the financial support of the INRA, the European Union for the award of Marie Curie Training Site funding to the Rowett Research Institute, the Scottish Executive Environment and Rural Affairs Department (Edinburgh, Scotland, UK), the National Science and Engineering Research Council of Canada (Ottawa, Ontario, Canada), and Agriculture and Agri-Food Canada (Dairy and Swine R&D Centre Contribution no. 895).

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