Milk Production, Nutrient Utilization, and Endocrine Responses to Increased Postruminal Lysine and Methionine Supply in Dairy Cows

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ABSTRACT

The effect of increased postruminal supply of lysine and methionine was investigated in a production trial involving 64 dairy cows in early lactation. Within each of two basal rations, based on either corn silage or grass silage, rations were either naturally deficient in lysine or fortified with 24 g of lysine in a rumen-protected form and naturally deficient in methionine or fortified with 12 g of methionine in a rumen-protected form. The data were analyzed separately for the four lysine and the four methionine treatment groups. Milk production, body weight gain, and plasma concentrations of insulin-like growth factor-I, bovine somatotropin, insulin, glucose, nonesterified fatty acids, and urea were monitored over a 12-wk period. Supplementation with protected methionine led to increases in milk fat and protein contents of 2.4 and 1.8 g/kg of milk, respectively. Supplementation with protected lysine or methionine numerically increased protein yield comparable to values reported in the literature, but the treatment effects were not statistically significant. Efficiency of utilization of absorbed amino acids for milk protein synthesis and efficiency of utilization of metabolizable energy for milk production were not significantly altered in response to increased postruminal lysine and methionine flow, but a numerically increased efficiency of utilization of total amino acids was observed. No significant effect of lysine or methionine supplementation was observed on endocrine parameters nor on plasma metabolite concentrations. However, across treatment groups, high milk yield was correlated with low plasma insulin-like growth factor-I concentrations ($r = -0.44$) and partially with low plasma nonesterified fatty acids concentration and insulin levels ($r = -0.26$), while body weight gain was negatively correlated ($r = -0.33$) with elevated plasma bovine somatotropin concentrations.

(**Key words:** protein utilization, insulin-like growth factor-I, bovine somatotropin, insulin)

Abbreviation key: AAT = AA absorbed in the intestine, AAT-Lys = lysine absorbed in the intestine, AAT-Lys% = percent lysine in intestinally absorbed AA, AAT-Met = methionine absorbed in the intestine, AAT-Met% = percent methionine in intestinally absorbed AA, DCHO = digestible carbohydrates, DCF = digestible crude fat, ECM = energy corrected milk, EPD = effective protein degradability, HLG = high lysine, grass silage based, HLM = high lysine, corn silage based, HMG = high methionine, grass silage based, HMM = high methionine, corn silage based, k_{AAT} = efficiency of utilization of AAT for milk protein synthesis, k_I = efficiency of utilization of ME for milk synthesis, LLG = low lysine, grass silage based, LLM = low lysine, corn silage based, LMG = low methionine, grass silage-based, LMM = low methionine, corn silage-based, ME = metabolizable energy, PBV = protein balance in the rumen, RHCHO = rapidly hydrolyzable carbohydrates.

INTRODUCTION

Ruminants, on the tissue level, need the same essential AA as nonruminants, but due to the complicated nature of the ruminant gastrointestinal tract, progress in the field of individual AA nutrition of dairy cows has only been moderate. Infusion trials have often identified Lys and Met as the first limiting AA, under a variety of different conditions (Rulquin et al., 1993). The role of His as a potentially limiting AA has also been demonstrated (Kim et al., 1999; Vanhatalo et al., 1999), as has the beneficial effect of postruminal infusion of Pro (Bruckental et al., 1991). Improved N-balance has been observed in response to postruminal infusion of Leu in lactating cows (Iburg and Lebzién, 2000), while Robinson et al. (1999) observed a tendency towards increased milk yield when infusing Ile postruminally. A range of possibly limiting AA has been identified under different feeding conditions involving postruminal infusion of AA. The present experiment was undertaken to investigate whether supplementation with commer-

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cially available preparations of rumen-protected Lys or Met could elicit a production response when added to each of two rations composed from commonly occurring feeds but deliberately formulated to be low in absorbable Lys and Met, respectively. Furthermore, the purpose of the experiment was to investigate whether endocrine changes occur in response to AA manipulations. Parts of this study have been published in a preliminary form (Misciattelli et al., 1999b, 2001).

MATERIALS AND METHODS

The present experiment complied with the Danish Ministry of Justice, Law no. 382 (June 10, 1987) and Act no. 726 (September 9, 1993), regarding animal experimentation and care of animals.

Animals and Management

Sixty-four Danish Holstein-Friesian cows weighing on average 554 kg (SEM 7.2) at the onset of the experiment, were blocked in groups of eight on the basis of parity and expected calving date. Within blocks, the cows were assigned at random to one of eight different treatment groups for a 12-wk trial period following a 3-wk standardized preperiod initiated at parturition. All cows calved within a period of 73 d and had an average parity of 2.1 (SD = 0.9). The cows were tethered in tie stalls mounted with rubber comfort mats and bedded with straw. Cows were fed at approximately 0730 and 1530 h, had free access to water, and were milked in their tie stalls at approximately 0400 and 1600 h.

Diet Formulation

The treatments consisted of four basal rations, two deliberately formulated to be low in absorbable Lys, one based on corn silage (**LLM**), and the other on grass silage (**LLG**). These two rations included rumen-protected Met, 13 g Smartamine M (Rhône Poulenc Animal Nutrition, Antony, France), to ensure an adequate basal Met level. The two other basal rations were formulated to be low in absorbable Met, one based on corn silage (**LMM**) and the other on grass silage (**LMG**). All four basal rations had a corresponding equivalent ration, differing only by inclusion of a protected source of either Lys or Met, resulting in four AA-fortified treatments: Ration LLM plus rumen-protected Lys and Met (**HLM**), 62 g of Smartamine ML. Ration LLG plus rumen-protected Lys and Met (**HLG**), 61 g of Smartamine ML. Ration LMF plus rumen-protected methionine (**HMM**), 20 g of Smartamine M. Ration LMG plus rumen-protected methionine (**HMG**) from 20 g of Smarta-

mine M. The treatments were, thus, pair-wise comparable, differing only in postruminal Lys or Met supply as altered by inclusion of Smartamine M or ML in the concentrate mixtures. Corn and grass silage were fed *ad libitum*, and the cows fed the corn silage-based rations were additionally fed 1.5 kg of DM/d of clover grass hay to ensure an adequate physical structure of the diet. Vitamins and minerals were administered on a weekly basis with a dose of 150 g/cow of a commercial vitamin mixture, "Rød Solitren" (Leo Pharmaceutical Products, Vejle, Denmark). The vitamin mixture was declared to contain: vitamin A, 3500 IU/g; vitamin D₃, 350 IU/g; vitamin E (α -tocopherol), 11 g/kg; and sodium selenite, 20 mg/kg. Concentrate mixtures are given in Table 1, and chemical composition of grass silage, corn silage, hay, and the concentrate mixtures are given in Table 2.

Experimental Procedure and Sample Collection

Animals were fed individually, and feed allocation was determined twice daily, and daily refusals were quantified at the following morning feeding. Animals were weighed on d 2 and 3 after calving, and on two consecutive days in the first and last weeks of the experiment. Additionally, BW were recorded in wk 3, 5, 7, 9, and 11 after the onset of the experimental period, but complete datasets on BW were not obtained. Milk yield at morning and evening milking and composition of milk was recorded once weekly. Roughage and concentrates were sampled weekly and stored at -20°C until chemical analysis. Dry matter was determined weekly, while chemical analyses were performed on samples composed to represent each 14-d period. Blood was sampled on three occasions: in the pretrial period (d 18 to 20 after calving), first half of the experimental period (d 35 to 42 after calving), and second half of the experimental period (d 88 to 98 after calving). Blood was sampled from v. jugularis by venepuncture, 2 h after the morning feeding (0930 to 1000 h), using 10-ml Na-heparinized Vacutainers. Blood samples were stored on ice (less than 30 min) until plasma was obtained by centrifugation at 3000 rpm for 20 min at 4°C.

Sample Analysis

Feed analysis. Dry matter content of concentrate mixtures, roughages, and refusals were determined at 80°C for 20 h in a forced-air oven. The DM in all other samples was determined by oven drying at 100°C. Samples for nylon bag analysis were ground through a 1.5-mm screen before incubation; all other samples were ground through a 1-mm screen on a laboratory mill prior to analysis. Ash was determined after combustion

Table 1. Composition of concentrate mixtures (kg / 1000 kg feed). Low Lys concentrate for grass silage-based ration (LLG), low Lys concentrate for corn silage-based rations (LLM), HLG and HLM correspond to LLG and LLM, supplemented with rumen-protected Lys. Low Met concentrate for grass silage-based ration (LMG), low Met concentrate for corn silage-based rations (LMM), HMG and HMM correspond to LMG and LMM, supplemented with rumen-protected Met.

	LLG	HLG	LMG	HMG	LLM	HLM	LMM	HMM
Corn gluten meal	75	75			54	54		
Rape seed cake					286	286	166	164
Sunflower meal, decorticated	164	164			115	115		
Rape seed	63	63	99	98			79	78
Barley	618	614	472	475	457	453	88	95
Soybean meal							236	234
Soypass ¹			204	202			62	61
Peas (<i>Pisum sativum</i>)			137	136			285	283
Beet molasses	69	69	70	69	69	69	70	69
Limestone, ground	10	10	9	9	12	12	14	14
Urea					6	6		
Mineral premix ²			9	9				
Smartamine M ³	0.96			1.82	0.96			1.82
Smartamine ML ³		4.80				4.80		

¹Xylose-treated soybean meal, Borregård Lignotech, Sarpsborg, Norway.

²One kg contained: 60g of Ca, 100 g of P, 120 g of Mg, 50 g of Na, 80 g of Cl, 0.5 g of S, 4 g of Fe, 4.5 g of Zn, 0.9 g of Cu, 25 mg of Co and 20 mg of Se.

³Rumen-protected Met or Met and Lys, Rhône Poulenc Animal Nutrition, Antony, France.

at 525°C for 6 h according to AOAC (1990). Nitrogen was determined by the Kjeldahl method on an automated Kjel-Foss apparatus (Foss Electric, Hillerød, Denmark) according to AOAC (1990). Crude fat was determined gravimetrically as the mass extractable in petroleum ether in a Soxhlet-apparatus, after an initial hydrolysis in 3 M HCl, essentially as described by Stoldt (1952). Crude fiber was determined as outlined by Tecator (1978). Rapidly hydrolyzable carbohydrates (RHCHO) were analyzed by the method proposed by Jacobsen (1981), which involves a short gelatinization step, an incubation with amyloglucosidase and saccharase (1330 and 7684, Merck KGaA, Darmstadt, Germany) in a buffer and subsequent centrifugation, protein precipitation, and spectrophotometrical quantification of the reducing sugars after oxidation with ferricyanide. Free sugars were determined with the same method but omitting the initial gelatinization and enzyme incubation.

Digestible OM of forages was determined by incubation with rumen fluid and subsequent HCl and pepsin incubation as described by Tilley and Terry (1963). Enzymatically digestible OM of the concentrates were determined with a two-step method involving an initial HCl and pepsin incubation and a subsequent incubation with an array of cellulases as described by Weisbjerg and Hvelplund (1993).

Two distinct procedures were used for the analysis of AA in feeds; AA in blood and milk were not analyzed. All AA with the exception of Trp were assayed on a Biochrom B20 (LKB Biochrom, Ltd., Cambridge, England) automated AA analyzer using ninhydrin as re-

agent and running in conjunction with a S200 autosampler (Perkin Elmer, Foster City, CA). Hydrolysates were prepared as described by Mason et al. (1980) with an initial 16-h oxidation of the sample in performic acid and subsequent hydrolysis in 6M HCl at 90°C for 23 h under constant reflux. Due to the oxidation, Met was detected as methionine sulfone and Cys as cystic acid. Tyr was quantified on hydrolysates, where the oxidation step was omitted. The determined concentrations of Ser, Ile, and Val were multiplied by a factor of 1.06 to correct for losses during hydrolysis. Trp was quantified after alkaline hydrolysis of the sample in sealed tubes with 4.2N NaOH at 110°C for 16 h in an autoclave. The separation was performed on a S410LC HPLC-apparatus (Perkin Elmer) fitted with a hypersil ODS 3- μ m C-18 column (Waters, Milford, MA) and a LS-1 LC fluorescence detector, using 5-methyl-tryptophan as internal standard, as described by Bech-Andersen (1991). All integration was performed using the Turbochrom software packet (Hewlett Packard). All reference to concentrations of AA is given as grams of AA, including water, unless otherwise stated. Total AA-N was calculated as the sum of N in the determined proteinogenic AA, thus excluding Orn. Because Gln and Asn are converted to Glu and Asp during acid hydrolysis, only Glu and Asp were used in the calculations.

Milk analysis. Milk fat, lactose, and protein were determined by IR on a Milkoscan 104 (Foss Electric, Hillerød, Denmark) on samples of individual cows morning and evening milk pooled on a proportional volume basis. Milk composition was determined on

Table 2. Chemical composition of grass silage (GSI), corn silage (MSI), grass hay (GHA), and concentrate mixtures: Low Lys concentrate for grass silage-based ration (LLG), low Lys concentrate for corn silage-based rations (LLM), HLG and HLM correspond to LLG and LLM, supplemented with rumen-protected Lys. Low Met concentrate for grass silage-based ration (LMG), low Met concentrate for corn silage-based rations (LMM), HMG and HMM correspond to LMG and LMM, supplemented with rumen-protected Met. Not determined (n.d.).

Feed component	GSI	MSI	GHA	LLG	HLG	LMG	HMG	LLM	HLM	LMM	HMM
	Concentrates for grass diets							Concentrates for corn diets			
				−Lys	+Lys	−Met	+Met	−Lys	+Lys	−Met	+Met
DM, %	25	28	85	88	89	88	89	88	89	88	89
CP, g/kg DM	171	98	131	215	224	225	225	261	273	308	314
Crude fat, g/kg DM	34	35	14	57	62	69	72	64	69	78	80
Crude fiber, g/kgDM	268	206	331	66	69	50	53	81	81	73	70
Ash, g/kg DM	92	20	73	44	45	57	54	57	58	65	68
RHCHO ¹ , g/kgDM	n.d.	340	n.d.	462	440	426	425	376	358	302	297
Starch, g/kg DM ²	n.d	324	n.d	398	373	343	339	299	279	203	199
Sugar, g/kgDM	1.54	16	8.46	68	71	89	85	83	85	105	104
Ca, g/kgDM	5.0	2.7	4.1	5.2	5.4	7.4	6.4	7.7	8.2	8.2	8.9
P, g/kgDM	3.0	2.0	2.7	5.6	5.6	5.9	5.9	6.8	6.9	5.9	6.0
Mg, g/kgDM	1.3	1.3	1.3	2.1	2.2	3.3	2.8	2.6	2.7	2.3	2.4
Digestible OM, g/kg DM	792	734	620	844	842	876	870	832	831	881	878
NE _L , MJ/kg DM	7.1	6.6	4.5	9.3	9.4	9.8	9.8	9.3	9.4	10.2	10.2
ME ⁵ , MJ/kg DM	11.5	10.7	8.9	12.7	12.8	13.3	13.3	12.6	12.7	13.5	13.5
CP degradability (EPD), %	81	66	64	58	59	51	49	64	63	64	63
RUP intestinal digestibility, %	58	65	67	91	90	92	92	83	84	89	89
AAT ⁶ , g/kg DM	65	74	66	124	125	123	124	119	120	119	119
PBV ⁷ , g/kg DM	52	−35	4	41	41	45	45	70	71	137	135
AAT-Lys ⁸ , g/kg DM	4.69	5.20	4.64	6.16	7.67	7.63	7.61	6.35	7.85	7.58	7.56
AAT-Met ⁹ , g/kg DM	1.45	1.65	1.40	3.27	3.30	2.17	3.19	3.11	3.14	2.03	3.06
EAA ¹⁰ , g/ kg DM											
Arg	2.93	2.10	5.10	12.37	12.16	14.60	14.49	13.84	14.35	21.72	22.19
His	1.89	1.57	1.68	5.46	5.23	5.74	5.71	6.15	6.45	8.01	8.15
Ile	7.77	3.81	4.56	9.80	9.48	10.17	10.10	10.60	10.90	14.23	14.50
Leu	11.86	8.63	7.77	21.17	20.10	16.56	16.30	20.29	21.04	22.40	22.89
Lys	5.12	2.03	4.39	7.11	9.11	11.61	11.50	9.84	12.20	18.35	18.79
Met	2.79	1.67	1.69	9.53	5.03	2.95	4.93	5.49	5.96	4.07	5.74
Phe	6.02	3.75	4.86	11.82	11.48	10.91	10.94	11.48	11.79	14.12	14.42
Thr	6.25	2.92	4.65	8.23	8.01	8.47	8.54	9.72	10.01	12.15	12.37
Trp	1.11	0.55	1.60	2.35	n.d.	2.58	n.d.	2.89	n.d.	3.64	n.d.
Val	10.00	4.93	6.00	11.70	11.16	11.41	11.33	12.86	13.20	15.24	15.72

¹RHCHO = Rapidly hydrolyzable carbohydrates.

²Calculated as difference between RHCHO and sugar.

⁵ME = Metabolizable energy.

⁶AAT = Amino acids absorbed in the intestine.

⁷PBV = Protein balance in the rumen.

⁸AA-Lys = Lys absorbed in the intestine.

⁹AAT-Met= Met absorbed in the intestine.

¹⁰EAA = Essential AA.

three samples per cow in the pretrial period and 12 samples per cow from the trial period.

Plasma metabolite analysis. Plasma metabolite concentrations were determined colorimetrically on an autoanalyzer: opeRA, clinical chemistry system (Bayer Diagnostics, Tarrytown, NY) with a commercial kit for glucose (Bayer, 1996a), a commercial kit for urea (Bayer, 1996b), and a modified commercial kit for NEFA (Wako Chemicals GmbH, Germany).

Plasma hormone analysis. Both bST and insulin were assayed using TR-IFMA (time-resolved immunofluorometric assays) and an AutoDELFLIA fluorometer (Wallac, Turku, Finland). Determination of bST was in

a sandwich setup by two monoclonal antibodies, developed against synthetic bST (Løvendahl et al., 2000), the intraassay CV for low (4 ng/ml), medium (14 ng/ml), and high (49 ng/ml) bST controls was 3 to 10%, the interassay CV (four assays) was 1 to 9%. Insulin was determined with a modified DELFLIA kit (Wallac) developed against human insulin (Løvendahl and Purup, 2002), the intraassay CV for low (22 pmol/L), medium (88 pmol/L), and high (575 pmol/L) insulin controls was 3 to 8%, and the interassay CV (four assays) was 2 to 3%. Details on the modifications introduced in the insulin assay can be found in Ingvarsen et al. (1999). Samples for IGF-I analysis were assayed with

Table 3. Effect of Lys level on DMI, BW, weight gain, milk yield, and composition. Treatments were: low Lys concentrate and grass silage (LLG), high Lys concentrate and grass silage (HLG), low Lys concentrate and corn silage (LLM), and high Lys concentrate and corn silage (HLM).

	LLG	HLG	LLM	HLM	RMSE ¹	Type II SS $P > F$		
						Feed	Level	Feed × Level
DMI, kg/d	18.7	17.8	16.6	16.3	1.6	<0.001	NS	NS
Roughage intake, kg/d	7.4	6.5	5.1	4.8	1.6	0.002	NS	NS
Concentrate intake, kg/d	11.3	11.3	11.5	11.5	0.08	<0.001	NS	NS
NE _L ² intake, MJ/d	158	152	137	136	11	<0.001	NS	NS
ME ³ intake, MJ/d	229	220	196	193	18	<0.001	NS	NS
AAT ⁴ , g/d	1884	1842	1731	1720	104	<0.001	NS	NS
PBV ⁵ , g/d	848	802	679	706	95	<0.001	NS	NS
AAT-Lys ⁶ , g/d	104	118	99	114	7.4	0.1	<0.001	NS
AAT-Met ⁷ , g/d	48	47	44	44	2.3	<0.001	NS	NS
AAT-Lys, % of AAT	5.52	6.38	5.69	6.64	0.09	<0.001	<0.001	NS
AAT-Met, % of AAT	2.54	2.55	2.53	2.53	0.02	0.07	NS	NS
BW, kg	572	550	525	532	39	0.03	NS	NS
Weight gain, kg/d	0.11	0.30	0.07	0.06	0.43	NS	NS	NS
Milk yield, milk composition, and yield of constituents								
Milk yield, kg/d	32.8	33.7	31.4	32.4	2.7	NS	NS	NS
ECM ⁸ yield, kg/d	30.5	29.6	27.0	26.3	3.2	0.008	NS	NS
Protein content, %	3.10	3.15	3.10	3.05	0.12	NS	NS	NS
Protein yield, g/d	1015	1059	975	989	87	0.09	NS	NS
Fat content, %	3.42	3.06	2.89	2.70	0.65	0.07	NS	NS
Fat yield, g/d	1144	1035	933	853	209	0.02	NS	NS
Lactose content, %	4.91	4.87	4.75	4.71	0.08	<0.001	NS	NS
Lactose yield, g/d	1618	1631	1450	1516	149	0.04	NS	NS

¹RMSE = Residual mean squares error.²NE_L = Net energy for lactation.³ME = Metabolizable energy.⁴AAT = Amino acids absorbed in the intestine.⁵PBV = Protein balance in the rumen.⁶AAT-Lys = Lys absorbed in the intestine.⁷AAT-Met = Met absorbed in the intestine.⁸ECM = Energy corrected milk (3.14 MJ/kg).

a noncompetitive TR-IFMA (Frystyk et al., 1995), by use of a DELFIA 1234, research fluorometer (Wallac). The intraassay CV for low (84 ng/ml) and high (164 ng/ml) IGF-I controls was 5 to 6%, and the interassay CV (nine assays) was 6 to 7%.

Calculations

Body weight gain was estimated from linear regression analysis of weight data for each individual cow, and mean BW was calculated as the BW in mid trial period by the obtained equation.

The AA absorbed in the small intestine (**AAT**) and the protein balance in the rumen (**PBV**) were calculated according to the system described by Madsen et al. (1995), utilizing data on effective protein degradability (**EPD**) and intestinal digestibility of RUP of individual feed components obtained from in situ incubations and corrected for loss of small particles according to Hvelplund and Weisbjerg (2000). Microbial N synthesized was calculated as 29 g/kg carbohydrate digested in the

total tract (**DCHO**, Hvelplund and Madsen, 1985). The DCHO was calculated by the following formula:

$$\text{DCHO} = \text{digestible OM} - (\text{digestible CP} + \text{DCF}),$$

where digestible OM values were the corrected figures from the in vitro digestibility analysis as described by Weisbjerg and Hvelplund (1993) and Møller et al. (1989), and digestible CP was calculated from the percentage of CP in DM by the equation proposed by Thomsen (1979). Digestible crude fat (**DCF**) was calculated from the percentage of crude fat, by the equation proposed by Weisbjerg et al. (1991). Intestinally absorbed Lys (**AAT-Lys**) and Met (**AAT-Met**) were also calculated based on in sacco estimates of rumen degradability and intestinal digestibility of CP in conjunction with AA analysis on the individual feed components (Miscattelli et al., 2002), assuming that CP values also could be applied on individual AA. The Lys and Met proportions in RUP was assumed equivalent to the content of Lys and Met in the original protein expressed as: grams

Table 4. Effect of Met level on DMI, BW, weight gain, milk yield, and composition. Treatments were: low Met concentrate and grass silage (LMG), high Met concentrate and grass silage (HMG), low Met concentrate and corn silage (LMM), and high Met concentrate and corn silage (HMM).

	LMG	HMG	LMM	HMM	RMSE1	Type II SS $P < F$		
						Feed	Level	Feed × Level
DMI, kg/d	19.4	18.7	17.3	17.1	1.6	0.004	NS	NS
Roughage intake, kg/d	9.4	8.7	7.5	7.1	1.6	0.005	NS	NS
Concentrate intake, kg/d	9.8	10.0	10.0	9.9	0.08	NS	0.03	0.02
NE _L ² intake, MJ/d	164	159	148	147	11	0.002	NS	NS
ME ³ intake, MJ/d	241	233	209	207	18	<0.001	NS	NS
AAT ⁴ , g/d	1839	1800	1712	1703	113	0.01	NS	NS
PBV ⁵ , g/d	938	900	1147	1159	84	<0.001	NS	NS
AAT-Lys ⁶ , g/d	120	117	113	112	8.0	0.04	NS	NS
AAT-Met ⁷ , g/d	35	44	32	42	2.5	0.004	<0.001	NS
AAT-Lys, % of AAT	6.54	6.47	6.57	6.56	0.04	<0.001	0.005	0.08
AAT-Met, % of AAT	1.92	2.47	1.86	2.46	0.02	0.001	<0.001	0.005
BW, kg	558	555	563	554	43	NS	NS	NS
Weight gain, kg/d	0.20	0.13	-0.02	-0.10	0.40	NS	NS	NS
Milk yield, milk composition, and yield of constituents								
Milk yield, kg/d	36.3	34.2	30.6	30.9	3.1	<0.001	NS	NS
ECM ⁸ yield, kg/d	33.8	34.0	29.4	30.3	2.7	<0.001	NS	NS
Protein content, %	2.97	3.18	2.94	3.08	0.14	NS	0.002	NS
Protein yield, g/d	1070	1090	905	945	83	<0.001	NS	NS
Fat content, %	3.58	3.93	3.89	4.01	0.31	0.10	0.04	NS
Fat yield, g/d	1292	1354	1192	1229	114	0.01	NS	NS
Lactose content, %	4.99	4.90	4.82	4.81	0.12	0.006	NS	NS
Lactose yield, g/d	1802	1683	1472	1491	177	<0.001	NS	NS

¹RMSE = Residual mean squares error.

²NE_L = Net energy for lactation.

³ME = Metabolizable energy.

⁴AAT = Amino acids absorbed in the intestine.

⁵PBV = Protein balance in the rumen.

⁶AAT-Lys = Lys absorbed in the intestine.

⁷AAT-Met = Met absorbed in the intestine.

⁸ECM = Energy corrected milk (3.14 MJ/kg).

AA (excl. H₂O)/16 g of N, while contribution of Lys and Met from microbial protein was calculated as: 332 mg of Lys (excl. H₂O) per gram of microbial N and 95 mg of Met (excl. H₂O) per gram of microbial N, respectively. The supply of intestinally absorbable Lys and Met from the preparations of rumen protected AA were calculated based on rumen degradability and intestinal digestibility of 0.9.

Content of digestible energy and NE_L in concentrate mixtures and roughages were calculated based on the corrected in vitro digestibilities of OM and the calculated amounts of digested crude fat, CP, and carbohydrates as described by Weisbjerg and Hvelplund (1993). Content of metabolizable energy (ME) in individual feed components was estimated from in vitro digestibilities and chemical composition according to AFRC (1993).

Energy corrected milk (ECM) containing 3.14 MJ/kg was calculated from the determined concentrations of fat and protein in milk:

$$\text{ECM} = \text{milk yield (kg)} \times \{[0.383 \times (\text{fat \%}) + 0.242 \times (\text{protein \%}) + 0.7832] / 3.14\}$$

The efficiency of utilization of AAT for milk protein synthesis (k_{AAT}) was calculated, estimating maintenance requirement as 3.25 g AAT × BW^{0.75} (Madsen et al., 1995), net protein content in 1 kg of BW gain equivalent to 233 g of AAT, and BW loss equivalent to 138 g of AAT based on figures from AFRC (1993). The efficiency of utilization of calculated ME for lactation (k_l) was based on maintenance requirement of 0.41 MJ × BW^{0.75} (AFRC, 1993), assigning an energetic value of 17.3 MJ/kg of BW gain and 20.9 MJ/kg of BW loss (Gibb et al., 1992).

Statistical Analysis

Data were statistically analyzed independently for Lys and Met groups, as two continuous, completely randomized block designs, incorporating the effects of post-ruminal AA level, feed, and interaction between feed

Table 5. Effect of level of Lys on plasma hormone and metabolite concentrations. Treatments were: low Lys concentrate and grass silage (LLG), high Lys concentrate and grass silage (HLG), low Lys concentrate and corn silage (LLM), and high Lys concentrate and corn silage (HLM).

	LLG	HLG	LLM	HLM	RMSE ¹	Type II SS $P > F$		
						Feed	Level	Feed × Level
First half of trial								
Insulin, (pmol/L)	53	42	62	48	22.3	NS	NS	NS
IGF-I, (ng/ml)	91	94	108	94	20.5	NS	NS	NS
bST, (ng/ml)	2.19	3.17	1.93	2.62	1.97	NS	NS	NS
Second half of trial								
Insulin, (pmol/l)	62	53	119	95	54.3	0.02	NS	NS
IGF-I, (ng/ml)	97	98	133	139	18.9	<0.001	NS	NS
bST, (ng/ml)	1.65	2.16	1.71	2.42	1.75	NS	NS	NS
Glucose, mmol/L	3.7	3.7	3.6	3.7	0.39	NS	NS	NS
NEFA, meq/L	0.16	0.17	0.19	0.17	0.04	NS	NS	NS
Urea, mmol/L	7.3	6.9	9.0	9.8	0.99	<0.001	NS	0.10

¹RMSE: Residual mean square error.

and AA level. This approach was chosen because interactions between Lys and Met were considered nonrelevant, as concentrate mixtures used for Lys and Met rations, respectively, were not comparable. The corresponding pretrial variable was used as a covariate in the analysis of milk production, milk composition, metabolite concentrations in plasma, and hormone concentrations in plasma. All milk and BW parameters were analyzed as means obtained over the entire experimental period using PROC GLM (SAS, 1990). The statistical model used was:

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + \gamma_k + \delta_{jk} + \zeta_{ijkl} + \varepsilon_{ijkl},$$

where: Y_{ijk} is the dependent variable, μ was the overall mean, α was effect of block ($i = 1, 2, 3, 4, 5, 6, 7$, and 8), β was effect of ration ($j =$ grass silage and corn silage), γ was the effect of AA level ($k =$ low or high), δ is the interaction between ration type and AA level, ζ was the corresponding pretrial covariate, and $\varepsilon = N(0, \sigma^2)$ was the random variation which was assumed normally distributed with a variance σ^2 and a mean of zero. Results are reported as least squares means for each treatment combination together with the residual mean squares errors or standard error of the least squares means. Type II test was used to test main effects of ration type and AA level, thereby excluding the interaction term of the model when F -tests were made. Differences between least squares means were determined by conducting repeated t -tests, when significant main effects and interactions were observed. Partial correlation coefficients were calculated using the MANOVA procedure (SAS, 1990) either from a model including block and a covariate for the pre-treatment period (Table 3) or a complete model for the entire dataset ($n = 64$) containing all two- and three-way interactions (Table

4). Statistical significance was declared at $P < 0.05$ and tendencies were considered at $P < 0.10$.

RESULTS

Intakes, Weight Changes, Milk Production, and Composition

The intake of roughage differed significantly between corn and grass silage rations as did yield of ECM. The DMI of corn silage and hay varied from 4.8 to 7.5 kg/d, and grass silage DMI varied from 6.5 to 9.5 kg/d. Concentrate DMI was stable at 11.3 to 11.5 kg/d for treatments involving Lys supplementation and 9.8 to 10.0 kg/d for treatments involving Met supplementation. The actual DMI resulted in an increased amount of intestinally absorbable Lys of 14 and 15 g/d corresponding to 13 and 15% increases in Lys supply on supplemented grass- and corn silage-based rations, respectively. Intestinally absorbable Met supply was similarly increased by 9 and 10 g/d, corresponding to 25 and 31% increases on supplemented grass- and corn silage-based rations, respectively. Alterations in postruminal Lys level did not result in significant changes in milk output or daily gain. There was, however, a significant effect of postruminal Met supply on milk fat content and milk protein content. No significant interactions were observed between feeds and postruminal Lys or Met supply (Tables 3 and 4), except for intestinal Met and Lys supply expressed as a percentage of total AA absorbed in the intestine (Table 4).

Utilization of Energy and Protein

When increasing the postruminal Lys supply, efficiencies of utilization of AAT for milk protein synthesis rose from 0.71 and 0.73 in the unsupplemented rations

Table 6. Effect of level of Met on plasma hormone and metabolite concentrations. Treatments were: low Met concentrate and grass silage (LMG), high Met concentrate and grass silage (HMG), low Met concentrate and corn silage (LMM), and high Met concentrate and corn silage (HMM).

	LMG	HMG	LMM	HMM	RMSE ¹	Type II SS $P > F$		
						Feed	Level	Feed × level
First half of trial								
Insulin, (pmol/L)	69	55	53	36	35.4	NS	NS	NS
IGF-I, (ng/ml)	80	79	89	88	14.4	0.10	NS	NS
bST, (ng/ml)	2.47	2.99	3.67	3.63	1.10	0.07	NS	NS
Second half of trial								
Insulin, (pmol/L)	81	45	54	74	30.3	NS	NS	0.02
IGF-I, (ng/ml)	93	90	123	111	16.3	<0.001	NS	NS
bST, (ng/ml)	2.03	3.02	0.61	1.77	1.44	NS	NS	NS
Glucose, mmol/L	3.8	3.6	3.4	3.5	0.24	0.02	NS	0.06
NEFA, meq/L	0.15	0.16	0.15	0.16	0.02	NS	NS	NS
Urea, mmol/L	8.0	7.3	9.6	10.0	1.77	0.006	NS	NS

¹RMSE: Residual mean square error.

to 0.74 and 0.78 (SEM 0.03; $n = 8$) in the supplemented rations, for grass silage- and corn silage-based rations, respectively. When manipulating postruminal Met supply, efficiencies of utilization of AAT for milk protein synthesis were 0.76 and 0.70 in the unsupplemented rations and 0.79 and 0.75 (SEM 0.02; $n = 8$) in the supplemented rations based on grass silage and corn silage, respectively. The calculated efficiency of utilization of ME for lactation k_1 was 0.55 and 0.57 for the low Lys treatments and 0.55 and 0.61 (SEM 0.02; $n = 8$) for the supplemented rations for grass silage- and corn silage-based rations, respectively. For the low Met treatments, k_1 was 0.57 and 0.59 and was 0.59 and 0.63 (SEM 0.02; $n = 8$) with the supplemented diets for grass silage- and corn silage-based rations, respectively. Neither the effects observed on the efficiency of utilization of absorbed AA for milk protein synthesis in response to altered Lys ($P = 0.16$) or Met ($P = 0.08$), nor the effects on the efficiency of use of ME for lactation observed for increased postruminal Lys ($P = 0.36$) or Met ($P = 0.12$) were statistically significant.

Plasma Hormones and Metabolites

Within the treatments that involved manipulation of Lys supply (Table 5), a significant effect of basal ration was observed on plasma concentration of IGF-I, insulin and urea, resulting in higher concentrations on the rations based on corn silage. Within the treatments involving Met manipulation (Table 6), the corn silage-based rations were also characterized by higher IGF-I and urea levels, together with decreased glucose levels and a tendency towards higher plasma bST concentration in the first half of the experiment. Analysis of residual correlations obtained from a model containing the effects of block and the appropriate pretrial covariate

are shown in Table 7. The residual correlation coefficient identifies relations between variables that are mainly caused by the experimental treatments. Significant negative residual correlation coefficients were observed between ECM yield and plasma concentrations of insulin, IGF-I and NEFA. No significant residual correlations were observed between BW gain and plasma hormones or metabolite concentrations. Residual correlation coefficients obtained with a model containing all treatment effects, including two- and three-way interactions, thus, adjusting data with the complete model, are given in Table 8. These correlations demonstrate relations of a more general character, i.e., not attributable to the experimental treatments per se. Even after correction for effects attributable to the treatments, a negative correlation between ECM yield and plasma IGF-I concentration was still evident, while correlations between ECM yield and plasma levels of insulin or NEFA only tended to be significant. Plasma concentration of bST was negatively correlated to BW change, while no other significant correlations were observed between BW change and plasma concentrations of hormones and metabolites. The plasma concentrations of IGF-I were correlated ($r = 0.11$; $P = 0.01$) to the plasma insulin concentrations but not to bST.

DISCUSSION

Intakes, Weight Changes, Milk Production, and Composition

All diets had an AAT to energy ratio, where further increases in milk yield would not be expected from increasing the AAT supply (Aaes et al., 1991; Misciattelli et al., 1999a). Supplementation with protected Lys or Met only resulted in modest increases in milk protein yields varying from 14 to 44 g per d ($P = 0.36$ and $P =$

Table 7. Residual correlation coefficients (*r*) between yield of energy corrected milk (ECM) or BW change and plasma concentrations of hormones or metabolites after adjusting data for the effects of block and pretrial ECM yield or BW.

	Milk yield (kg ECM/d)		df ¹	BW change (kg/d)		df ¹
	<i>r</i>	<i>P</i>		<i>r</i>	<i>P</i>	
Insulin, pmol/l	-0.35	0.009	55	-0.05	NS	46
IGF-I, ng/ml	-0.62	<0.001	55	-0.02	NS	46
bST, ng/ml	0.09	NS	45	-0.17	NS	37 ^a
Glucose, mmol/l	-0.13	NS	55	-0.07	NS	46
NEFA, meq/l	-0.33	0.01	55	-0.15	NS	46
Urea, mmol/l	-0.23	0.08	55	-0.18	NS	46

¹Degrees of freedom from the MANOVA analysis.^aSamples where bST concentrations were below the level of reliable detection are left out.

0.33 for Lys and Met supplementation, respectively), but the numerical increases were of the same magnitude as predicted from published response curves (Rulquin et al., 1993) and has been depicted together in Figure 1. The treatments designed to be low in intestinally absorbable Lys were based mainly on rape seed cake and secondly on undegradable corn gluten meal protein. In experiments where corn protein has been the major source of RUP, responses to Lys supplementation have often been observed (King et al., 1991; Schwab et al., 1992). In the present experiment, the Lys-deficient rations were, to a large extent, based on RUP from rape seed cake (56 g Lys/16 g N), which compared to corn gluten meal (17 g Lys/16 g N), contains a much larger proportion of Lys. The extent of Lys limitation could, thus, have been marginal in the present experiment. Furthermore, the actual increases in Lys supply from the basal to the supplemented rations amounted to only 13 and 15%, which could have been too marginal an increase to detect a production response. The Met deficient rations were mainly based on RUP from soybean meal or protected soybean meal in the form of soypass (Borregård Lignotech, Sarpsborg, Norway) and to a minor extent from peas. Amino acid analysis revealed that

the protected soybean meal had a lower Met content than the normal soybean meal, but the unsupplemented grass silage-based rations resulted only in marginally lower levels of calculated intestinally absorbed Met than the corn silage-based ration, where Soypass was included only in small proportions. Responses to Met supplementation have been observed, mainly when the principal protein source was soybean (Robert et al., 1996; Armentano et al., 1997), and responses observed in the present experiment were within the levels observed earlier.

Utilization of Energy and Protein

The observed efficiencies of ME utilization for lactation were lower in all instances than those calculated according to AFRC (1993). Supplementation with rumen-protected AA resulted in a decrease in intake of ME, resulting in differences of ME intake from 2 to 9 MJ/d compared with the basal rations. Increased protein yields occurred despite a decreased energy intake. Differences in metabolizability ($q_m = [ME]/[GE]$) between supplemented and basal diets, due to different roughage intakes, could not explain the differences in k_l . Imposing corrections for decreasing ME availability at increasing feeding levels according to AFRC (1993) still confirmed the observed differences in k_l . The k_{AAT} varied from 0.70 to 0.79 and was consistently but non-significantly higher for treatments with increased post-ruminal Lys or Met flow. The efficiency of utilization of absorbed AA for lactation varies between 0.64 and 0.80 in the modern protein evaluations systems (Hvelplund and Madsen, 1995), and the observed k_{AAT} are, thus, within that range. The British metabolizable protein (MP) system (AFRC, 1993) operates with an efficiency of utilization of an "ideal" AA mixture (k_{aai}) of 0.85 and a relative value (RV) of 0.8 to scale the k_{aai} to arrive at efficiencies for MP utilization for lactation of 0.68. The RV varies according to the tissues specific AA requirements, but only one working value is used

Table 8. Residual correlation coefficients (*r*) between yield of energy corrected milk (ECM), BW change, and plasma concentrations of hormones and metabolites, after adjusting data for all effects, two-way and three-way interactions.

	Milk yield (kg ECM/d)		BW change (kg/d)		df ¹
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	
Insulin, pmol/l	-0.26	0.07	0.002	NS	48
IGF-I, ng/ml	-0.44	0.002	0.03	NS	48
bST, ng/ml	-0.02	NS	-0.33	0.07	31 ^a
Glucose, mmol/l	-0.17	NS	-0.19	NS	48
NEFA, meq/l	-0.26	0.07	-0.10	NS	48
Urea, mmol/l	0.06	NS	-0.05	NS	48

¹Degrees of freedom from the MANOVA analysis.^aSamples of bST concentrations that were below the level of reliable detection are left out.

Figure 1a and 1b. Observed changes in milk protein yield in response to alterations in a) proportion of lysine in intestinally absorbable protein (LysDI%) and b) proportion of methionine in intestinally absorbable protein (MetDI%). Grass silage-based rations (●), Corn silage based rations (□), Response curves to alterations in intestinally absorbable lysine or methionine according to Rulquin et al. (1993) (—). Axis of abscissa and ordinate axis does not intercept at zero.

in practice. The fact that the utilization of intestinally absorbed protein varies according to the absorbed AA profile has been demonstrated in trials with postruminal and isonitrogenous infusion of different protein sources (Rogers et al., 1984; Choung and Chamberlain, 1993). Improved k_{AAT} should, thus, be observed when supplementing a limiting AA, and the responses observed support this, although without statistical significance.

Plasma Hormones and Metabolites

The plasma hormone concentrations demonstrated a large variability, probably due to the large differences in DMI between animals, and the calculated LS-means for the treatments had considerable error terms associated. Lys and Met are not potent stimulators of bST, and insulin secretion in ruminants compared to other AA (Kuhara et al., 1991). While positive effects on plasma insulin levels have been observed in response to rumen-protected Met in the study of Blum et al. (1999), no significant effect of Lys or Met supplementation were observed on plasma insulin and bST levels in the present study. Plasma concentrations of IGF-I are dependent on nutritional status in growing (Breier, 1999) and lactating ruminants (Spicer et al., 1990; McGuire et al., 1992), but manipulating the nutritional status of lactating cows by dietary means or postruminal protein infusion has often failed to demonstrate alterations in levels of circulating IGF-I- and IGF-binding proteins, -2, -3, and -4 (Spicer et al., 1991; McGuire, 1992; Mackle et al., 1999). It has been speculated

that this lack of effect could be due to an effect of protein per se and due to the interactions between energy and protein in the rumen. In lactating cows, positive energy balance is associated with elevated levels of IGF-I in plasma (Spicer et al., 1990), while feed deprivation has been demonstrated to decrease plasma IGF-I levels and proportional distribution of IGF-I-binding proteins (McGuire et al., 1998). In the present study, no significant correlations were observed between energy intake or protein to energy level and plasma hormone and metabolite concentrations. But ANOVA resulted in significant effects of feed on plasma IGF-I levels, with higher IGF-I levels observed at the low energy intakes, which were also characterized by an increased proportion of AAT per unit of NE_L . Additionally a relatively strong negative correlation was observed between yield of energy corrected milk and plasma IGF-I concentration and, thus, emphasizes that the effect of IGF-I on lactation cannot be estimated only by the level of circulating IGF-I but also need to include the distribution of IGF-I on its different binding proteins. However, this was not measured in the present experiment. High-yielding dairy cows in early lactation would normally have higher plasma levels of bST than lower yielding animals (Cowie et al., 1980), but no correlations between milk yield and bST was observed in the present study. The episodic release of bST requires frequent sampling over time, which was not done in the present study; however, the large number of cows per treatment is believed to counterbalance this. Indeed a positive relationship between weight loss and plasma bST level was detected, which is in accordance with observa-

tions of increased plasma bST levels in feed restricted ruminants (McGuire et al., 1998; Breier, 1999). Plasma urea concentrations were not suppressed when the theoretical AA deficiency was alleviated by administration of rumen protected Lys or Met. The large differences in roughage intake within treatment groups had resulted in a substantial difference in the surplus of rumen degradable protein (PBV), especially on the corn silage-based rations. A small improvement in utilization of absorbed AA for productive purposes would, thus, be masked by the increased absorption of $\text{NH}_3\text{-N}$ over the rumen epithelium and the subsequently increased hepatic urea synthesis on the same treatments, thereby explaining the lack of reduction in plasma urea levels. Blum et al. (1999) observed a decrease in plasma NEFA concentration in response to rumen-protected Met, but in the present study no effects were observed on plasma NEFA, which exhibited a substantially larger variation than in the study of Blum et al. (1999).

CONCLUSIONS

A 25 to 31% increase in postruminal Met flows resulted in significantly increased protein and fat percentages in the milk, while 13 to 15% increases in postruminal Lys flow did not result in significant changes. The tendencies towards increases in milk protein yield occurred despite decreases in ME intake and a high protein to energy ratio, indicating an improved efficiency of utilization of absorbed AA and ME for milk production when increasing postruminal supply of Lys or Met. Even when rations were deliberately formulated to be theoretically deficient in intestinally absorbable Lys or Met, responses to supplementation with protected Lys or Met were marginal, thus indicating a need to also focus on AA other than Lys and Met as potential first limiting AA in future research. Effects of different postruminal levels of Lys and Met should also be studied at lower levels of total AA provision, especially to estimate the potential of reducing urinary nitrogen excretion from dairy cows. Tissue mobilization was correlated with high plasma bST concentrations. High milk yield was correlated with low plasma IGF-I concentrations and partially with low plasma NEFA and insulin levels. These parameters were mainly influenced by type of basal ration, while alterations in Lys or Met levels seemed to have no significant effect.

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