The Effect of Dietary Crude Protein as Protected Soybean Meal on Mammary Metabolism in the Lactating Dairy Cow

J. A. METCALF,*,[1] D. WRAY-CAHEN,*,[2] E. E. CHETTLE,*,[3] J. D. SUTTON,*,[3] D. E. BEEVER,*,[3] L. A. CROMPTON,† J. C. MacRAE,‡ B. J. BEQUETTE,‡ and F.R.C. BACKWELL‡

*Agricultural and Food Research Council Institute of Grassland and Environmental Research, Hurley, Maidenhead, Berkshire, SL6 5LR, England
†Department of Biochemistry and Physiology, University of Reading, Whiteknights, PO Box 228, Reading RG6 2AJ, England
‡Rowett Research Institute, Greenburn Road, Bucksburn, Aberdeen, AB2 9SB, Scotland

ABSTRACT

Metabolism in the mammary gland was related to changes in milk output in response to changes in dietary protein intake. Three diets of grass silage and concentrate were fed to four lactating dairy cows equipped with intravascular catheters across the mammary gland. Concentrates differed in the inclusion of protected soybean meal and provided 11.3, 15.4, and 20.1% CP, respectively. Blood samples were taken to assess the effect of protein percentage on the nutrient fluxes across the gland and their relationship to milk production. Milk production, milk protein yield, and milk protein concentration were all increased as CP intake increased, although these responses were not linear. Concentrations of urea in milk reflected those in plasma and increased as dietary protein intake increased. Uptake of glucose and BHBA by the mammary gland tended to increase as milk production increased. Arterial supply of essential AA increased as the dietary protein increased. Supply and uptake of nonessential AA were unchanged by dietary treatment, and uptake was insufficient to account for output of nonessential AA residues in milk protein. The supply of essential AA was not limiting for milk protein synthesis, and some alternative mechanism must have existed for the control of milk protein yield.

(**Key words**: protein, mammary gland, metabolism, lactation)

Abbreviation key: **EAA** = essential AA, **HP** = high protein, **LP** = low protein, **MP** = medium protein, **NEAA** = nonessential AA, **PAH** = *p*-aminohippuric acid, $\mathbf{T_3}$= triiodothyronine, $\mathbf{T_4}$ = thyroxine.

INTRODUCTION

Previous studies (3, 9, 14, 16) have shown that milk protein output can be increased by dietary protein supplementation and by gastric and intravascular infusions of proteins or AA. This response suggests that the regulatory mechanism for milk protein synthesis may, at least in part, be substrate driven. Responses of cows to abomasal infusion of protein or AA have shown a variability that has not been satisfactorily explained (6) and may to some extent be related to limitations in available energy in the diets used. Casein infusions have yielded the largest response, and the AA contributing to this response have been largely identified by the elegant experimentation of Schwab et al. (19). The efficiency with which abomasal casein is used for milk protein also varies (12). Other protein supplements, when infused into the abomasum, have failed to show large increases in milk protein output (5).

Some of the problems in identifying a response to supplementary protein may be due to a reduction in response with advancing lactation (18). However, this factor is unlikely to be the only one involved, because sequential increases in casein supplementation via the abomasum are directed into milk protein with diminishing efficiency (23). The present experiment was one part of a coordinated trial undertaken in this laboratory to quantify the effect of sequential increases in dietary CP on duodenal flow, portal appearance, hepatic output, and mammary gland uptake of nutrients. Duodenal and hepatic measurements are reported separately, but some reference is made to those results in this paper. Our experiment

Received March 8, 1995.
Accepted December 1, 1995.

[1]Current address: ADAS Bridgets, Martyr Worthy, Winchester, Hampshire, SO21 1AP, England.

[2]Current address: USDA, Agriculture Research Service Children's Nutrition Research Center at Baylor College of Medicine, 1100 Bates, Houston, TX 77030-2600.

[3]Current address: University of Reading, Centre for Dairy Research, Arborfield Hall Farm, Church Lane, Arborfield, Reading, Berkshire RG2 9HX, England.

attempted to change only one variable, protein supply to the cow, while minimizing changes in metabolizable energy intake and other factors that might affect milk protein output because of changes in the diet. The current experiment measures metabolite flux across the mammary gland of cows given a basal diet alone or supplemented with two percentages of formaldehyde-treated soybean meal. A preliminary report of this work has already been published (17).

MATERIALS AND METHODS

Four Holstein-Friesian dairy cows in their second (n = 3) or third (n = 1) lactation were prepared with catheters inserted into the external pudic artery 7 to 10 d after calving as previously described (16). The cows were allowed to recover for 4 wk and then were fed diets of grass silage (177 g of CP/kg of DM) and one of three concentrates [113, 154, or 201 g of CP/kg of DM; low protein (**LP**), medium protein (**MP**), and high protein (**HP**), respectively] in a 40:60 ratio of DM in a 3 × 3 Latin square design with one repeated sequence. Each period consisted of 4 wk. During wk 1 and 2, the cows were fed to meet calculated energy requirements for maintenance, milk production, and 0.5 kg of BW gain/d (1); the ration at the end of the 2nd wk was then fixed for the remaining 2 wk. Milk production and composition were measured daily over d 15 to 28, and blood samples were taken on d 26 of each period. Milking was performed at 12-h intervals, and blood samples were taken hourly over a 12-h period between milkings. Arterial samples were paired with samples from the subcutaneous abdominal vein taken from a temporary catheter that had been inserted at least 24 h earlier. Silage was fed twice daily at 0930 and 1630 h, and the concentrates were automatically fed every hour. Concentrate formulations are shown in Table 1. Concentrates were calculated to contain similar amounts of metabolizable energy (3.0 Mcal/kg of DM); protein concentration was increased by the inclusion of protected soybean meal [Sopralin; Trouw Nutrition (UK), Northwich, Cheshire, England].

Blood flow was measured by the dye dilution technique using *p*-aminohippuric acid (**PAH**) infused at 90 mg/min. The extent of crossover of venous blood was also estimated (15). Blood samples were taken hourly and were prepared as previously described (14), except that 2-mercaptoethanol was not included in the laking solution, because of the problems previously identified (14). Blood samples were deproteinized to give a final sulfosalicylic acid concentration of 6.3% and were then analyzed for individual AA concentrations (Beckman 6300 AA analyzer; Beckman Instruments, High Wycombe, Buckinghamshire, England). Plasma samples were pooled and also analyzed for AA, because some AA (Asp and Thr) were not well resolved on whole blood samples; Arg was degraded by the release of arginase from the erythrocytes, which was caused by the laking of the whole blood. Individual plasma samples were analyzed for glucose (Trinder, Sigma test 315; Sigma Chemical Co. Ltd., Poole, Dorset, England), BHBA (Sigma test 310; Sigma Chemical Co. Ltd.), L-lactate (Sigma test 826; Sigma Chemical Co. Ltd.), urea (Roche Diagnostic Products, Welwyn Garden City, Hertfordshire, England), and PAH using a Cobas Mira® chemistry analyzer (Roche Diagnostic Products); concentrations of acetic acid were also determined by GLC as previously described (14). Arterial supply (concentration × blood flow) and uptake (arteriovenous difference × blood flow) of AA determined on whole blood was calculated using whole blood flow estimated from PAH concentration at the time of sampling for each sample, and plasma flow was used for the calculation of uptake and supply for metabolites and AA determined on plasma. Because PAH concentrations were determined in plasma, whole blood flows were calculated by correction of

TABLE 1. Concentrate formulation and chemical analysis of experimental diets.[1]

Composition	LP	MP	HP	Silage
Ingredient fresh weight, kg/tonne				
Barley	545	478	411	
Wheat	91	80	68	
Corn	109	96	82	
Molassed sugar beet pulp	100	88	75	
Molassine meal	64	56	48	
Protected soybean[2]	0	124	247	
Cassava[3]	90	80	68	
Chemical composition				
CP, g/kg of DM	113	154	201	177
NDF, g/kg of DM	170	157	166	463
Fiber, g/kg of DM[4]	103	95	107	324
OM, g/kg of DM	943	938	934	908
Water-soluble carbohydrate, g/kg of DM	74	81	86	113
Starch, g/kg of DM	510	462	408	ND[5]
NH_3 N, g/kg of DM	ND	ND	ND	3.1
NAN, g/kg of DM	ND	ND	ND	28.4
pH[4]	ND	ND	ND	4.22

[1]LP = Low protein (113 g of CP/kg of DM), MP = medium protein (154 g of CP/kg of DM), and HP = high protein (201 g of CP/kg of DM).

[2]Sopralin® [BR Nutrition (UK), Northwich, Cheshire, England].

[3]Alkali-treated straw and cassava (1:1, wt/wt).

[4]Determined by near infrared reflectance spectroscopy.

[5]Not determined.

plasma flows using the packed cell volume. Plasma flow was calculated as follows: plasma flow(liters/minute) = infusion rate of PAH (milligrams per minute)/downstream concentration − background concentration of PAH (milligrams per liter). Mammary extraction was calculated as arteriovenous difference divided by arterial concentration; the balance of AA was calculated from mammary uptake over 24 h, divided by daily milk protein yield minus 5% allowance for NPN, using literature values for milk AA composition as previously described (14). Concentration of CP was determined on whole milk samples after storage at −20°C as Kjeldahl N × 6.38. Milk samples were homogenized for 30 s before they were divided to ensure resuspension of fat globules after freezing. Milk urea was also determined on these samples after defatting by centrifugation at 1500 × *g* for 5 min at 4°C before analysis using the same procedure as for plasma urea.

The concentrations of insulin, IGF-I, cortisol, thyroxine (T_4), and triiodothyronine (T_3) were all measured using radioimmunoassay. The method for insulin was based on that described by Tindall et al. (22) using bovine insulin for iodination and standards (Novo Bio Laboratories, Guildford, Surrey, England) and guinea pig anti-insulin serum (Immuno Diagnostic Systems Ltd., Boldon, Tyne and Wear, England). The total concentration of IGF-I was measured in plasma after dissociation from the IGF-binding proteins using the modified acid ethanol extraction procedure described by Breier et al. (4). The assay used recombinant human IGF-I for iodination and standards (rh- IGF-I CGP 35126; Ciba Geigy Limited, Basel, Switzerland) and rabbit anti-IGF-I serum (UB3-189) obtained through the hormone distribution program of the National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases (Baltimore, MD). Total cortisol concentration was measured using the method described by Symonds et al. (20), using rabbit anticortisol serum (Biogenesis Ltd., Bournemouth, Dorset, England) and tritiated cortisol (Amersham International plc, Aylesbury, Buckinghamshire, England). The assays for T_4 and total T_3 concentrations used 8-anilinonaphthalene 1-sulfonic acid as the dissociation agent with rabbit anti-T_4 or anti-T_3 serum (Sigma Chemical Company Ltd.), respectively, and iodinated T_4 or T_3 [Du Pont (UK) Ltd., NEN Products, Stevenage, Hertfordshire, England].

Statistical Analysis

Statistical analysis was performed on mean values for each parameter by ANOVA using Genstat (General Statistical Package; Lawes Agricultural Trust, Rothampstead, Hertfordshire, England) based on a 3 × 3 Latin square with one repeated sequence in which cows and periods were the blocking factors. Four degrees of freedom existed for the error term for significance testing of treatment effects using the *F* distribution. Differences between the individual means for significant treatment effects were tested using the *t* distribution. Significance was declared at $P < 0.10$ unless otherwise specified; a trend was recognized at $P < 0.15$.

RESULTS

Milk production (Table 2) was higher for cows fed the LP diet than for those fed the MP diet, but the HP diet failed to stimulate any further increase. Milk protein concentrations were similar for cows fed the LP and MP diets, but were higher for cows fed the HP diet; LP milk protein yields of cows fed the two supplemented diets were similar and higher than that with the LP diet. Both the concentration ($P < 0.01$) and yield ($P < 0.05$) of urea in milk increased markedly as dietary CP increased.

The arterial supply of glucose to the mammary gland (Table 3) showed no trend as protein percentages increased, but glucose uptake tended to increase. The supply (and concentration, not shown) of urea in plasma increased markedly as dietary CP increased, but uptake was negligible and unaffected by diet. Neither supply nor uptake of BHBA was significantly affected by diet although both tended to be greatest for cows fed the HP diet. Supply and

TABLE 2. Milk production and composition and dietary DM and CP intakes at three percentages of CP intake.[1]

	LP	MP	HP	SED[2]
Intake				
DM, kg/d	16.5	17.5	16.4	0.62
CP, kg/d	2.33[c]	2.86[b]	3.14[a]	0.117
Milk production, kg/d	20.7[a]	22.1[b]	21.4[ab]	0.43
Milk protein				
Concentration, g/kg	32.6[b]	32.8[ab]	34.0[a]	0.51
Yield, g/d	675[b]	723[a]	722[a]	19.0
Milk urea				
Concentration, mg/kg	185[c]	248[b]	350[a]	25.8
Yield, g/d	3.9[b]	5.4[ab]	7.1[a]	0.80

[a,b,c]Means in the same row without a common superscript differ ($P < 0.10$).

[1]LP = Low protein (113 g of CP/kg of DM), MP = medium protein (154 g of CP/kg of DM), and HP = high protein (201 g of CP/kg of DM).

[2]Standard error of the difference.

output of acetic acid and lactate were also unaffected by diet.

Arterial supply of essential AA (**EAA**) in blood was increased from 5.5 (LP diet) to 7.1 (MP diet) and 8.2 (HP diet) mmol/min ($P < 0.05$; Table 4). Mammary uptake of EAA was not significantly different among diets but tended to reflect milk protein output for groups of EAA. The EAA have been grouped as previously suggested (8), based on the ratio of their uptake from blood to output in milk. Arterial supply of Phe, Tyr, and His (group 1 AA, for which uptake was considered to be equal to or less than output) tended to increase as CP intake increased, but only His reached significance. Mammary uptake of Met, another group 1 AA, was higher for the HP diet than for the LP diet, although supply was not affected. Arterial supply of branched-chain AA (Val, Ile, and Leu; group 2, for which uptake was considered to be greater than output) increased as dietary CP increased, but only Val ($P < 0.05$) and Leu ($P < 0.10$) reached significance, and only Ile showed a significant increase in uptake between the LP and HP diets. Of the other group 2 EAA, Lys supply increased between the LP and MP diets but not between the MP and HP diets, and uptake of Lys reflected arterial supply.

There were no significant changes in the supply and uptake of nonessential AA (**NEAA**), either individually or as a group. The overall tendency for a decrease in uptake of NEAA for cows fed the HP diet was largely due to the change from net uptake to output of Ala. Proline substantially increased in arterial supply as CP intake increased, but uptake of Pro did not change; extraction by the mammary gland decreased from 7% of supply (LP diet) to 6 and 5% (MP and HP diets, respectively).

Arterial concentrations and uptake (arteriovenous difference × plasma flow) of free AA that were determined on plasma have been included for reference (Table 5); those values show trends similar to those of free AA in blood (Table 4). The concentrations of AA were determined on 13 times more samples for whole blood than for plasma samples, which gave a greater certainty that erroneous or contaminated samples had been identified. Hence, the balance of AA between mammary uptake and milk output (Table 6) has been determined using blood and not plasma AA.

Arterial concentrations of insulin showed no response to protein intake, averaging 1.21 ng/ml, and, similarly, no significant responses were seen for IGF-1 (67.8 ng/ml), T_3 (1.33 ng/ml), or T_4 (48.9 ng/ml), although concentration of T_3 did tend to decline as CP intake increased (Table 7). Concentrations of cortisol were significantly different among dietary treatments; the MP diet produced lower concentrations than did the LP or HP diets, and the LP diet was significantly lower than the HP diet. However, there was a significant effect of period on the concentrations of cortisol; lower concentrations of cortisol occurred in later lactation: 19.1, 20.9, and 7.8 ± 2.60 ng/ml ($\bar{X}$ ± SE of difference; $P < 0.05$) in successive periods.

DISCUSSION

This experiment was designed to complement a previous study (14) that used one concentration of fish meal supplementation to increase duodenal supply of AA. In that earlier experiment, the response in milk protein output to dietary protein supplementation was small, even though circulating EAA concentrations were substantially increased. As the CP content of the basal diet increased in the current experiment, milk output was stimulated by almost 1.5 kg/d, which in turn led to the increased DMI of

TABLE 3. Plasma flows and arterial supplies of metabolites to, and uptake by, the whole mammary gland at three percentages of CP intake.[1]

	Supply				Uptake			
	LP	MP	HP	SED[2]	LP	MP	HP	SED
Plasma flow, L/min	7.5	7.5	7.4	1.14	. . .	. . .	. . .	. . .
Packed cell volume, %	26.1	26.9	27.0	1.32	. . .	. . .	. . .	. . .
Glucose, mmol/min	26.7	26.7	27.3	4.71	5.0	5.3	5.7	0.60
Lactate, mmol/min	3.0	2.7	2.8	0.52	−0.3	−0.1	−0.6	0.42
Acetic acid, mmol/min	13.4	12.9	13.0	1.36	9.8	9.1	9.2	0.98
BHBA, mmol/min	5.9	5.9	6.7	1.45	2.3	2.4	2.7	0.37
Urea, mmol/min	29.9[a]	39.6[ab]	56.6[b]	7.73	−0.5	0.1	−0.1	0.29

[a,b,c]Means in the same row without a common superscript differ ($P < 0.10$).

[1]LP = Low protein (113 g of CP/kg of DM), MP = medium protein (154 g of CP/kg of DM), and HP = high protein (201 g of CP/kg of DM).

[2]Standard error of the difference.

TABLE 4. Arterial supply and mammary uptake of selected free AA in whole blood at three percentages of CP intake.[1]

AA	Supply				Uptake			
	LP	MP	HP	SED[2]	LP	MP	HP	SED
	(μmol/min)							
Group 1								
Met	266	272	268	37.7	69[b]	80[ab]	83[a]	5.0
Phe	364	412	460	41.3	100	116	117	11.0
Tyr	430	504	530	75.6	102	101	108	11.1
His	478[b]	726[ab]	764[a]	99.0	60	62	65	8.2
Group 2								
Val	1276[b]	1942[a]	2386[a]	211.2	267	300	267	38.0
Ile	852	1012	1176	145.6	209[d]	240[cd]	271[c]	17.8
Leu	948[b]	1230[ab]	1514[a]	176.2	298	337	371	30.9
Lys	888[b]	1120[a]	1124[a]	83.8	268[d]	315[cd]	321[c]	16.8
Nonessential								
Gly	3486	3422	3530	605.6	−72	−50	−62	77.8
Ala	1580	1736	1720	346.6	78	44	−84	84.6
Pro	730	922	1004	168.6	53	59	51	14.6
Ser	870	966	956	161.4	60	102	46	35.9
Gln	1564	1642	1468	379.2	198	266	252	55.2
Glu	1258	1064	1052	176.4	294	130	310	105.4
Essential	5494[b]	7068[ab]	8249[a]	592.4	1374	1550	1604	98.7
Nonessential	9487	9756	9733	1652.9	610	550	513	295.7
Branched-chain	3076[c]	4186[b]	5076[a]	424.8	774	876	910	67.0

[a,b,c]Means in the same row without a common superscript differ ($P < 0.10$).

[1]LP = Low protein (113 g of CP/kg of DM), MP = medium protein (154 g of CP/kg of DM), and HP = high protein (201 g of CP/kg of DM).

[2]Standard error of the difference.

TABLE 5. Arterial concentration and mammary uptake of selected free AA in plasma at three percentages of CP intake.[1]

AA	Artery				Uptake			
	LP	MP	HP	SED[2]	LP	MP	HP	SED
	(μmol/L				(μmol/min)			
Group 1								
Met	20.6	21.2	19.0	2.00	61	72	64	5.9
Phe	40.5	46.0	50.2	3.94	126	137	129	6.5
Tyr	40.5	49.7	48.6	5.67	117	128	116	5.8
His	25.8[b]	56.9[a]	59.8[a]	4.56	64	78	75	8.8
Group 2								
Thr	85.1	96.1	92.7	10.02	187	199	196	51.9
Val	155.0[b]	210.0[ab]	262.1[a]	31.30	190[d]	431[c]	405[cd]	89.8
Ile	98.4[b]	120.6[a]	134.4[a]	6.67	249	282	285	19.3
Leu	92.4[a]	128.2[b]	156.9[a]	11.37	346[e]	402[de]	413[d]	21.3
Lys	74.2[b]	104.0[a]	102.6[ab]	10.70	311[d]	396[c]	415[c]	24.7
Arg	72.0[b]	104.6[a]	109.9[a]	7.23	198	200	226	49.9
Nonessential								
Gly	270.4	258.9	234.0	16.16	59	−7	−48	64.9
Ala	198.7	207.8	199.4	12.82	162[a]	86[ab]	−71[b]	80.5
Pro	70.4[b]	86.6[ab]	91.5[a]	6.33	60	51	44	41.9
Ser	82.9	90.3	85.1	9.53	89	96	75	40.6
Orn	28.6	39.7	44.2	7.54	94[b]	106[ab]	126[a]	5.8
Asp	13.0	13.6	15.0	3.13	94	109	115	101.1
Gln	217.0	196.9	181.4	13.94	248	265	159	40.4
Glu	85.9	81.7	79.5	6.65	372	307	328	72.0
Essential	705[b]	937[ba]	1036[a]	80.3	1850[d]	2325[c]	2325[c]	137.6
Nonessential	967	976	930	45.2	1118	963	684	256.0
Total	1672[b]	1913[ab]	1967[a]	94.0	2968	3288	3010	330.3

[a,b,c,d,e]Means in the same row without a common superscript differ ($P < 0.10$).

[1]LP = Low protein (113 g of CP/kg of DM), MP = medium protein (154 g of CP/kg of DM), and HP = high protein (201 g of CP/kg of DM).

[2]Standard error of the difference.

cows fed the MP diet, because the cows were rationed according to milk production. Milk protein content was not affected by the MP diet, and, thus, for an overall increased CP intake of 530 g/d, only 48 g/d (9/100 g) were identified in increased milk protein output. The magnitude of this response was similar to that recorded in the earlier trial of Metcalf et al. (14) in which the reported increase in milk protein output was equivalent to only 10% of the dietary protein increment. The relatively low milk yields observed for these cows were average for cows of this age within the herd. Because cows had their first calf at 18 mo, the three cows in second lactation were partitioning a higher proportion of nutrients to growth than the cow in third lactation.

The previous experiment (14) was limited to examination of only one percentage of dietary protein supplementation. In the present study, two protein supplementations were used but, at the higher protein inclusion, there was no response of milk production or of milk protein output above that seen for the lower supplementation, which in part might be due to the decreased DMI of cows fed the HP diet. However, despite a reduction of 1 kg of DM/d, dietary CP intake was increased by 230 g/d; yet none of this extra protein was apparent as milk protein.

TABLE 6. Mammary uptake of free AA as percentage of their output in milk protein minus 5% for NPN at three percentages of CP intake.[1]

AA	LP	MP	HP	SED[2]
	(%)			
Group 1				
Met	80	86	89	4.6
Phe	75	79	81	8.5
Tyr	65	68	75	11.2
His	77	75	90	16.7
Mean	76	77	83	3.4
Group 2				
Val	103	109	99	14.2
Ile	101	108	124	8.8
Leu	88	92	104	9.3
Lys	105	113	104	6.9
Mean	99	106	112	7.3
Nonessential				
Gly	−65	18	−9	68.2
Ala	47	28	−51	51.4
Pro	14	14	11	3.2
Ser	32	54	26	20.5
Gln	68	86	75	13.5
Glu	84	77	72	8.8
Mean	25	31	11	21.0

[1]LP = Low protein (113 g of CP/kg of DM), MP = medium protein (154 g of CP/kg of DM), and HP = high protein (201 g of CP/kg of DM).

[2]Standard error of the difference.

TABLE 7. Effect of three percentages of dietary CP intake[1] on the arterial plasma concentration of selected hormones in four lactating dairy cows.

Hormone	LP	MP	HP	SED[2]
	(ng/ml)			
Insulin	1.17	1.21	1.25	0.271
IGF-I	64.1	64.6	74.7	9.55
Cortisol	16.7[b]	7.3[c]	23.7[a]	2.60
T_4[3]	52.5	52.4	41.9	6.33
T_3[4]	1.51	1.34	1.13	0.351

[a,b,c]Means in the same row without a common superscript differ ($P < 0.05$).

[1]LP = Low protein (113 g of CP/kg of DM), MP = medium protein (154 g of CP/kg of DM), and HP = high protein (201 g of CP/kg of DM).

[2]Standard error of the difference.

[3]Thyroxine.

[4]Triiodothyronine.

Preliminary observations from another part of this trial to establish the effect of these diets on the flow of NAN and total AA to the small intestine confirmed that duodenal AA supply of all AA detected was increased in line with the dietary protein increase (J. A. Metcalf, 1992, unpublished results). This result appears to agree with the observations reported in an earlier study (14) and provides further evidence that a simple increase in duodenal supply of AA does not necessarily guarantee any substantial change in milk protein content or output.

The use of three percentages of CP in the diet of lactating dairy cows was designed to examine nutrient metabolism in the mammary gland of the cows when they showed a differential response to the increments in dietary CP. This situation was achieved in this experiment; efficiencies of conversion of the supplementary dietary protein into milk protein were 9 and 0% for the two percentages of CP.

Examination of nutrient flux to the mammary gland indicated some broad similarities with the data provided from earlier work (14) as well as some important differences. In both experiments, arterial supply of glucose was unaffected by dietary protein supplementation, but the values recorded in the present study were only 0.65 of those reported previously, despite similar milk production. In both experiments, net glucose extraction by the mammary gland averaged between 17 and 20% of arterial supply, such that in the present experiment mean glucose uptake across all treatments was only 0.68 of that reported earlier. Translated into daily production terms, however, data from the present experiment indicated a net glucose uptake of approximately 65 g/kg of milk produced. This value compared well with results from

another laboratory (9), which averaged 61 g/kg. Both of these studies have produced lower values than the 70 g/L previously proposed (12) for milk of average composition that was based on biochemical principles.

From results of the current study, the mammary gland appeared to be a net producer of lactate, in agreement with results of earlier work (14), although this agreement appears to be of limited biological significance. The suggestion made previously, that an increased supply of AA to the gland might reduce lactate output through increased availability of tricarboxylic acid cycle intermediates, has not been established.

Arterial supply of urea increased as dietary CP intake increased in both experiments, primarily because of increased plasma urea concentrations. Although there was no apparent trend in uptake or output of urea across the mammary gland in relation to the diets, output of urea in the milk increased from 3.9 to 7.1 g/d with the increases in protein intake. Concentrations of milk urea correlated well with arterial plasma concentrations [milk urea (millimolar) = 0.899 × plasma urea (millimolar) + .552; $r^2 = 0.90$] when individual daily mean values were compared (n = 12), confirming a direct relationship between urea in the blood and milk (10).

Concentrations of insulin, IGF-I, T_4, and T_3 were not significantly altered by either the dietary treatments or the changes in milk production. The effects of period were most pronounced for insulin (0.93, 1.17, and 1.53 ± 0.271 ng/ml for periods 1, 2, and 3, respectively; $\overline{X}$ ± SE of difference), although concentrations did not reach statistical significance. Cortisol concentrations, however, changed significantly because of diet and period effects. Although the lower cortisol concentrations in the final period of the experiment were probably due to the cows becoming more used to handling, explanation of the relationship to dietary effects is more difficult. Cows might have failed to respond to the HP diet above the response to the MP diet because of these high concentrations of cortisol, but the cause of the elevated concentrations remains unknown. Other researchers (9) have shown no changes in concentrations of insulin, prolactin, or growth hormone in response to duodenal casein infusion, and together these experiments indicate that there is little interaction between these hormones and the stimulation of milk output.

The increase in CP intake from the LP to MP diets was 530 g/d, which stimulated arterial EAA supply by 2.26 mol/d. Comparable values between the MP and HP diets were 280 g/d and 1.70 mol/d, respectively, suggesting a direct relationship between dietary CP intake and arterial supply of EAA similar to that previously observed (21). Because there was no change in blood flow with treatment, these increases in arterial supply reflect changes in arterial concentration of EAA. This result compares well with those of previous work in which an increase of CP intake by 437 g/d increased EAA concentration by 1.78 mol/d (14), but fewer AA were determined in that earlier study. Abomasal infusion of casein (9) tended to show similar increases in arterial supply of EAA (1.02, 0.75, and 1.43 mol/d) in plasma for smaller increments in CP intake (154, 136, and 380 g/d). The changes in milk protein yield resembled the increase in uptake of EAA, suggesting some relationship between these two, but did not reflect the increases in arterial supply, suggesting that EAA supply was not the primary limitation to milk protein synthesis. These findings are in agreement with those of Guinard et al. (9), who also found that uptake of EAA by the mammary gland reflected milk protein output but not arterial supply. Individual EAA generally showed similar trends; uptake of Met increased from the LP to MP diets, but was unchanged from the MP to HP diets, even though arterial supply was unchanged throughout the experiment. Davis et al. (7) have suggested that Met is limiting for milk production, and the 80 to 89% of Met output in milk protein provided from the blood (Table 6) confirmed that free Met was apparently insufficient to account for output as milk protein.

Hanigan et al. (11) suggested that mammary uptake of AA from blood is greater than that from plasma; however, our data tended to disagree. In our data, uptake from whole blood varied between 81 and 94% of that from plasma. However, this variation, although of interest, is not a good comparison because the plasma values were determined on pooled samples for each sampling day, and whole blood was from 13 individual samples. Thus, a comparison of these data with those of Hanigan et al. (11), which compared 948 paired samples from 21 different cows, may not be appropriate.

Arterial supply of the other group 1 AA (Phe, Tyr, and His) tended to increase as dietary protein increased, but only His was significant. Mammary uptake of Phe showed the largest increase with diet, and uptake of Tyr and His showed no significant changes, even though arterial supplies were increased. The supply of branched-chain AA increased with diet, and Leu and Ile showed similar increases in uptake, although only Ile reached significance. Supply and uptake of Lys also increased with differences between the LP and MP diets for supply and between the LP

and HP diets for uptake; the MP diet failed to reach significance at $P = 0.1$. Uptake of Leu, Ile, and Lys by the mammary gland might possibly be substantially regulated by supply because these results were similar to those reported previously (14).

In this study, changes in supply or uptake of NEAA with diet were not significant, although other researchers (9) have shown linear increases in arteriovenous difference of NEAA across the mammary gland as milk protein output increased. Supply of Pro increased with diet, but uptake did not change, indicating a decrease in mammary extraction of Pro from 7 to 5%. When a balance was constructed between AA uptake from blood and output as milk protein (Table 6), Pro uptake from the blood accounted for only 13% of Pro in milk protein, indicating a major shortfall for milk protein synthesis. The deficit of Pro in milk might be synthesized from Arg within the mammary gland (13), and uptake of Arg, which was only determined in plasma in this study, was 2.8 times its output in milk protein, regardless of dietary treatment. One molecule of Arg can provide N for two molecules of Pro and one molecule of urea. Thus, the excess Arg could provide an additional 369 μmol/min of the Pro output, which, together with Pro uptake, accounts for 94% of Pro output in the milk. The increase in urea production by the mammary gland at this rate of transamination would be approximately 185 μmol/min, which might be released into the bloodstream or into the milk. Because the arterial supply of urea was 170 times this rate of release for the LP diet, such a small increase would not have been detectable, and the appearance of this urea in the milk would be equivalent to 15 g/d, a value that was twice that for the HP diet. Hence, these calculations suggest that any urea produced by mammary metabolism of AA was released into the blood stream and that the appearance of urea in milk was related to arterial supply rather than to metabolism.

Calculated balances between uptake and milk output for most AA generally agreed with the groupings suggested by Davis and Mepham (8); group 1 EAA were removed at concentrations equal to or below the estimated minimal requirement, and group 2 EAA were removed above the estimated requirement. NEAA were taken up at an amount that, in all cases, was below output. No specific trends were evident for changes in balance with dietary treatment; however, uptake of Ile approached significance ($P = 0.110$) relative to output as dietary protein increased, and the other branched-chain AA, together with Lys, showed a similar trend. The fate of the excess AA taken up by the mammary gland was unclear, but those AA probably provided the N for NEAA synthesis. Mammary uptake of most AA was apparently higher from plasma than from blood (Tables 4 and 5). Although the data were not strictly comparable because of the differing numbers of individual analyses making up the mean values, constructing a balance for uptake and milk output did reduce the deficit for the group 1 AA. However, there was still a shortfall in AA uptake for all treatments. When the total amount of AA N provided in free AA was calculated, there was a deficit of 11% in uptake compared with milk protein AA N output. If all of the Arg, which has four N per molecule, were used to synthesize Pro, then the deficit in free AA N would increase to 29%. Such a large deficit, which assumes the conservation of all AA N except Arg, suggests that there is a substantial contribution of AA N from a peptide or small protein source (2).

CONCLUSIONS

Increased dietary CP typically produces a curvilinear response in milk protein output. One theory for this response is the limitation of AA supply to the mammary gland. In this study, EAA supply to the mammary gland increased linearly as CP intake increased, but NEAA supply was unchanged. Similarly, EAA uptake by the mammary gland was close to the estimated requirement for milk protein output but was not linear as supply increased; NEAA uptake was always well below requirement. The linear increases in uptake with increased supply of specific AA suggest that the uptake of these AA might be manipulated by appropriate dietary supplementation. The overall discrepancy between AA N uptake and output in milk suggests that a proportion of milk AA must be supplied in a bound form for milk protein synthesis and also that a high degree of interconversion of AA must occur within the lactating mammary gland.

ACKNOWLEDGMENTS

The authors thank past members of the Ruminant Nutrition and Metabolism Department at Hurley and M. A. Lomax for technical assistance and M. S. Dhanoa for statistical advice. This study formed part of a collaborative project funded by a consortium of the Ministry of Agriculture Fisheries and Food, BP Nutrition (UK), the Milk Marketing Board of England and Wales, the Scottish Office Agriculture and Fisheries Department, and the Agricultural and Food Research Council.

REFERENCES

1 Agricultural Research Council. 1980. Nutrient Requirements for Livestock. No. 2. Ruminants. Commonw. Agric. Bur., Farnham Common, Berkshire, England.

2 Backwell, F.R.C., J. A. Metcalf, B. J. Bequette, G. E. Lobley, D. E. Beever, and J. C. MacRae. 1994. A possible role for peptides as precursors for milk protein synthesis. J. Dairy Sci. 77(Suppl.1):1176.(Abstr.)

3 Beever, D. E., S. B. Cammell, M. C. Spooner, D. J. Humphries, and M. J. Haines. 1991. Effect of defined manipulations of nutrient supply on energy and protein utilization by dairy cows. Page 325 *in* Energy Metabolism of Farm Animals. C. Wenk and M. Boessinger, ed. Nutztierwissenschaften Gruppe Ernahrung, ETH-Zentrum, Zurich, Switzerland.

4 Breier, B. H., B. W. Gallaher, and P. D. Gluckman. 1991 Radioimmunoassay for insulin-like growth factor-I: solutions to some potential problems and pitfalls. J. Endocrinol. 128:347.

5 Choung, J.-J., and D. G. Chamberlain. 1992. Protein nutrition of dairy cows receiving grass silage supplements of protein and amino acids. J. Sci. Food Agric. 60:25.

6 Clark, J. H. 1975. Lactational responses to postruminal administration of proteins and amino acids. J. Dairy Sci. 58:1178.

7 Davis, S. R., R. Bickerstaffe, and D. S. Hart. 1978. Amino acid uptake by the mammary gland of the lactating ewe. Aust. J. Biol. Sci. 31:123.

8 Davis, S. R., and T. B. Mepham. 1974. Amino acid and glucose uptake by the isolated perfused guinea pig mammary gland. Q. J. Exp. Physiol. 59:111.

9 Guinard, J., H. Rulquin, and R. Verite. 1994. Effect of graded levels of duodenal infusions of casein on mammary uptake in lactating dairy cows. 1. Major nutrients. J. Dairy Sci. 77:2221.

10 Gustafsson, A. H., and D. L. Palmquist. 1993. Diurnal variation of rumen ammonia, serum urea, and milk urea in dairy cows at high and low yields. J. Dairy Sci. 76:475.

11 Hanigan, M. D., C. C. Calvert, E. J. DePeters, B. L. Reis, and R. L. Baldwin. 1991. Whole blood and plasma amino acid uptakes by lactating bovine mammary glands. J. Dairy Sci. 74:2484.

12 MacRae, J. C., P. J. Buttery, and D. E. Beever. 1988. Nutrient interactions in the dairy cow. Page 155 *in* Nutrition and Lactation in the Dairy Cow. P. Garnsworthy, ed. Butterworths, London, England.

13 Mepham, T. B., and J. L. Linzell. 1967. Urea formation by the lactating goat mammary gland. Nature (Lond.) 214:501.

14 Metcalf, J. A., D. E. Beever, J. D. Sutton, D. Wray-Cahen, R. T. Evans, D. J. Humphries, F.R.C. Backwell, B. J. Bequette, and J. C. MacRae. 1994. The effect of supplementary protein on in vivo metabolism of the mammary gland in lactating dairy cows. J. Dairy Sci. 77:1816.

15 Metcalf, J. A., S. J. Roberts, and J. D. Sutton. 1992. Variations in blood flow to and from the bovine mammary gland measured using transit time ultrasound and dye dilution. Res. Vet. Sci. 53:59.

16 Metcalf, J. A., J. D. Sutton, J. E. Cockburn, D. J. Napper, and D. E. Beever. 1991. The influence of insulin and amino acid supply on amino acid uptake by the lactating mammary gland. J. Dairy Sci. 74:3412.

17 Metcalf, J. A., D. Wray-Cahen, B. J. Bequette, F.R.C. Backwell, D. J. Humphries, D. E. Beever, and J. D. Sutton. 1993. The efficiency of amino acid utilization by the mammary gland of Holstein-Friesian cows fed three levels of crude protein. Anim. Prod. 56:419.

18 Schwab, C. G., C. K. Bozak, N. L. Whitehouse, and M.M.A. Mesbah. 1992. Amino acid limitation and flow to the duodenum at four stages of lactation. 1. Sequence of lysine and methionine limitation. J. Dairy Sci. 75:3486.

19 Schwab, C. G., L. D. Satter, and A. B. Clay. 1976. Response of lactating cows to abomasal infusion of amino acids. J. Dairy Sci. 59:1254.

20 Symonds, M. E., M. J. Bryant, and M. A. Lomax. 1986. The effect of shearing on the energy metabolism of the pregnant ewe. Br. J. Nutr. 56:635.

21 Tagari, H., and E. N. Bergman. 1978. Intestinal disappearance and portal blood appearance of amino acids in sheep. J. Nutr. 108:790.

22 Tindall, J. S., G. S. Knaggs, I. C. Hart, and L. A. Blake. 1978. Release of growth hormone in lactating and non-lactating goats in relation to behaviorus, stages of sleep, electroencephalograms, environmental stimuli and levels of prolactin, insulin, glucose and free fatty acids in the circulation. J. Endocrinol. 76: 333.

23 Whitelaw, F. G., J. S. Milne, E. R. Ørskov, and J. S. Smith. 1986. The nitrogen and energy metabolism of lactating cows given abomasal infusions of casein. Br. J. Nutr. 55:537.