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# Incorporation of endogenous urea nitrogen into amino acids of milk in goats fed diets with various protein levels

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**KEY WORDS:** endogenous urea, $^{15}N$, dietary protein, milk, amino acids, dairy goats

**ABSTRACT.** The aim of the study was to estimate how much endogenous urea nitrogen (EUN) was incorporated into various amino acids of milk protein when goats were fed low (LP), medium (MP), or high protein (HP) diets in a 3 × 3 Latin-square design. Three Alpine goats of about 35 kg body weight fitted with a catheter into the jugular vein were fed isoenergetic diets containing 11% (LP), 13% (MP), or 16% (HP) crude protein in dry matter. They were continuously infused with $^{15}N$ urea into the jugular vein for 6 days. Daily milk yield was 1.58, 1.49 and 1.77 g. Milk urea concentrations were 139, 342 and 451 mg $\cdot l^{-1}$, whereas plasma urea was 178, 356 and 667 mg $\cdot l^{-1}$ in groups LP, MP and HP, respectively. Samples of milk protein were hydrolysed with 6 M HCl, and then free amino acids were converted into butyl derivatives using HCl in butanol, followed by N-acylation using trifluoroacetic acid anhydride. The amino acid derivatives were analysed using a gas chromatograph equipped with a mass detector. $^{15}N$-excess after a six-day infusion of labelled urea was significantly higher ($P < 0.05$) in the vast majority of amino acids of milk protein from goats fed the LP diet in comparison with goats fed the HP diet. Therefore, the protein level of diets affects the incorporation of EUN into amino acids of milk protein. EUN was incorporated primarily into glutamic acid, methionine and arginine. At all levels of nitrogen in the diets, the incorporation of $^{15}N$ into phenylalanine was very low.

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## Introduction

In ruminants, endogenous urea nitrogen (EUN) is formed in the liver and can be transferred to the rumen (Kowalczyk et al., 1975a,b; Harmeyer and Martens, 1980; Kennedy and Milligan, 1980), where it is then hydrolysed (Gibbons and McKarthy, 1957) and the resulting ammonia enters the ruminal ammonia pool (Bunting et al., 1989). It is utilized by rumen bacteria as an important source of nitrogen for bacterial protein synthesis (Virtanen, 1964). This EUN is incorporated in various proportions into different amino acids of bacterial protein; the proportions depend on the composition of the diet (Havassy et al., 1982; Michalski et al., 2013). These amino acids can then be incorporated into milk protein.

The metabolism of the mammary gland is very complex. Although there are numerous studies on amino acid and protein metabolism in the mamma-

ry gland of the lactating ruminant (Bequette et al., 1998), little is known about the utilization of blood urea nitrogen during this period (Brun-Bellut, 1996; Al-Dehneh et al., 1997), especially on the influence of dietary protein level on incorporation of endogenous urea nitrogen into milk protein (Pfeffer et al., 2009; Michalski et al., 2012).

We were unable to find any studies on the incorporation of endogenous urea nitrogen into individual amino acids of ruminant milk. It is not known whether and to what extent the protein level in the diets affects the preference for and extent of incorporation of recycled nitrogen into individual amino acids of milk. The hypothesis of our study was that incorporation of endogenous urea nitrogen into various amino acids of milk depends on the dietary protein level. Therefore, the aim of the current study was to estimate the magnitude of incorporation of endogenous urea nitrogen into the individual amino acids of milk protein in goats fed diets containing low, medium, or high protein levels.

## Material and methods

### Animals and nutrition

The studies were conducted on three Alpine goats at 105 days of first lactation in a 3 × 3 Latin-square design. The goats were 12 months old with a 35 ± 2 kg body weight. The animals were housed and handled in accordance with protocols approved by the Local Animal Care and Use Committee. Animals fitted with a polyurethane catheter into the jugular vein (16G × 200 mm) were housed in individual cages. They were fed 1200 g of grass hay and 1000 g of one of three isoenergetic concentrates containing a low (LP), medium (MP), or high (HP) level of protein, respectively. The crude protein concentration in the whole diet was 11%, 13% and 16% in dry matter (DM), respectively, and soyabean made up most of the protein source. Almost 65% of the soyabean protein is degradable in the rumen (NRC, 2001), so diets differed mainly in rumen-degradable protein as well as rumen-undegradable protein. The diet containing the medium level of protein best met NRC norms (2007). Water was freely available. Concentrate composition was reported in detail in our previous paper (Michalski et al., 2013). The nutrient contents are given in Table 1.

**Table 1.** The nutrient content in feeds, % in DM

| Indices | Hay | Concentrate, protein level | | |
|---|---|---|---|---|
| | | low | medium | high |
| Dry matter | 91.53 | 89.21 | 89.68 | 89.68 |
| Crude protein | 11.27 | 11.07 | 15.05 | 21.60 |
| Ether extract | 2.15 | 0.95 | 1.65 | 2.21 |
| Crude fibre | 30.66 | 8.51 | 8.40 | 8.08 |
| NDF | 62.38 | 31.41 | 32.80 | 30.73 |
| Ash | 5.95 | 4.24 | 4.72 | 5.54 |
| ME, MJ · $kg^{-1}$ | 10.55 | 12.58 | 12.56 | 12.44 |

### Experimental design, sampling and $^{15}$N-urea infusion

After a 15-day adaptation period, samples of milk, blood and feeds were collected for respective analysis. Blood samples from the jugular vein were collected twice a day at 08.00 and 14.00 h in heparinized tubes, placed on crushed ice and centrifuged at 1450 *g* for 15 min at 4°C for preparation of plasma. Collected samples were stored at –20°C until analysis. Goats were milked twice a day at 06.00 and 18.00 h; milk was weighed and pooled for 24 h periods. A total of 144 blood samples and 72 milk samples were collected.

Blood urea nitrogen was labelled with the stable isotope of nitrogen ($^{15}$N) to estimate the efficiency of blood EUN utilization for synthesis of milk amino acids and estimation the losses of EUN as milk urea. From the second day of sample collection, for 6 days goats were administered $^{15}$N-labelled urea (98 atom % excess; VEB Berlin-Chemie, Germany); 1.6 g · $d^{-1}$ $^{15}$N-urea were continuously infused into the jugular vein as described in detail in a previous paper (Michalski et al., 2013). The amount of administered $^{15}$N-urea was chosen based on our previous studies (Michalski et al., 2012). Based on the detection capabilities, we found that the optimal amount was ~5 mg $^{15}$N · $kg^{-1}$ body weight per day.

### Analysis

The nutrient content of feeds and refusals were determined according to AOAC procedures (1990). The nutrient composition of consumed feed was calculated by subtracting the nutrient content of the refusals from the nutrient content of the diet. Milk composition was estimated by near-infrared spectroscopy (MilkoScan FT120, Foss). The concentration of urea in milk samples was quantified using high-performance liquid chromatography with pre-column derivation (Czauderna and Kowalczyk, 2009). Milk protein was prepared by precipitation with 20% trichloroacetic acid (2:1, v/v) and centrifugation (300 *g* for 5 min at 4ºC). The resulting precipitate was washed with methanol and dried. The supernatant obtained during centrifugation was used to obtain the free amino acids of milk. For this purpose the supernatant was filtered (30-SF-45N; Chromacol) and vacuum solid-phase extraction (SPE) was carried out on the respective stationary

phase (Strata-X-C 33u Polymeric Strong Cation 60 mg · 3 ml$^{-1}$ ; Phenomenex). The obtained eluent containing free amino acids was concentrated under a stream of argon.

The milk protein was hydrolysed in culture tubes, using 6 M HCl in water at 105ºC for 22 h, and the samples were dried with a rotary evaporator at 60ºC. Free amino acids were derivatized according to the procedure specified by Gehrke (2005) that was described in detail in a previous publication (Michalski et al., 2013). N-trifluoroacetyl n-butyl esters of amino acids were analysed using a gas chromatograph with a mass-selective detector using a capillary column (30 m × 0.25 mm × 0.25 µm). The excess of the $^{15}$N isotope in individual amino acids was calculated by reading the intensity of individual ions with a mass detector. Glutamine and glutamic acid as well as asparagine and aspartic acid were determined together as N-acylated butyl derivatives of glutamic and aspartic acids. $^{15}$N isotope enrichment analysis were performed on the samples collected pre-infusion and on day 6 of the infusion.

The data were statistically analysed using the non-parametric Mann-Whitney U test for comparing independent experimental groups. The Statistica v. 10 package (2010) was used (www.statsoft.pl).

## Results and discussion

No significant differences in dry matter intake or in the percentage of crude fibre were found among the LP, MP and HP diets of goats (Table 2). The crude protein contents in the dry matter of feed intake differed statistically. Milk yield was correlated with dry matter intake ($r$ = 0.982) and milk fat content increased with increasing levels of protein in the diets (Table 2). The level of protein in the diet did not affect the percentage of protein in milk. A positive correlation ($r$ = 0.707) was observed between the protein content of the diet and urea in milk (Table 2), which is in agreement with other studies (Zhai et al., 2007). The concentration of urea in milk was lower than in plasma, which is consistent with the results of Wang et al. (2007) and Zhai et al. (2007). Plasma urea concentrations differed significantly between groups ($P < 0.01$), and increased together with the level of protein in the diets (Table 2). At the medium level of protein, urea in blood plasma was increased by 100%, and at the high level, by 275%.

The amino acid profile of milk protein (Figure 1) was similar to results published by other authors (Davis et al., 1994; Rutherfurd et al., 2006). This profile was similar in all levels of protein in the diet. We found a numerical increase in the concentration of the analysed amino acids in milk protein.

$^{15}$N-labelled nitrogen was present in all analysed amino acids of milk protein. The protein level in the diets affects EUN incorporation in individual amino acids of milk protein. In the case of seven amino acids, the effect was statistically significant ($P < 0.05$). For low-protein diets, high enrichment was observed in methionine, isoleucine, and glutamic acid (Table 3). In high-protein diets, the greatest enrichment was also observed in glutamic acid, and in arginine and leucine. The level of protein in the diet had the least impact on the enrichment of phenylalanine.

It is noteworthy that the content of $^{15}$N was high in milk urea and quite high in arginine, but much lower in proline. The amount of arginine excreted in milk proteins (protein outputs) is much smaller than the quantity extracted from the blood by the mammary gland (Bequette et al., 1998). This means that only part of the arginine is used in milk protein synthesis, and most of it is broken down in the mammary gland (Mezl and Knox, 1977), resulting in milk urea. Proline extracted by the mammary gland does not meet the needs of milk synthesis and

**Table 2.** Diet intake, nutrient composition of feed intake, milk yield, milk urea and plasma urea concentration. Mean values ± SD[1,2]

| Indices | Protein level in diets | | |
|---|---|---|---|
| | low | medium | high |
| Diet intake, kg · DM · d$^{-1}$ | 1.66 ± 0.20 | 1.64 ± 0.23 | 1.78 ± 0.13 |
| Crude protein, % DM | 11.49$^{a}$ ± 0.41 | 13.27$^{b}$ ± 0.24 | 17.18$^{c}$ ± 0.42 |
| ME, MJ · kg$^{-1}$ DM | 11.92 ± 0.18 | 11.78 ± 0.02 | 11.73 ± 0.08 |
| Milk yield, kg · day$^{-1}$ | 1.58$^{a}$ ± 0.18 | 1.49$^{Aa}$ ± 0.30 | 1.77$^{Bb}$ ± 0.23 |
| Milk fat, % | 2.72$^{a}$ ± 0.28 | 2.78$^{ab}$ ± 0.50 | 2.94$^{b}$± 0.29 |
| Milk protein, % | 2.85 ± 0.27 | 2.82 ± 0.61 | 2.89 ± 0.17 |
| Milk urea, mg · l$^{-1}$ | 138.9$^{A}$± 41.9 | 341.9$^{Ba}$ ± 99.2 | 451.3$^{Bb}$± 156.1 |
| Plasma urea, mg · l$^{-1}$ | 178$^{A}$ ± 64 | 356$^{B}$ ± 161 | 667$^{C}$ ± 251 |

[1] some of the data were presented in our previous paper (Michalski et al., 2012); [2] mean values in rows having the different superscripts are significantly different at $^{a,b}P < 0.05$ and $^{A,B}P < 0.01$ levels, respectively

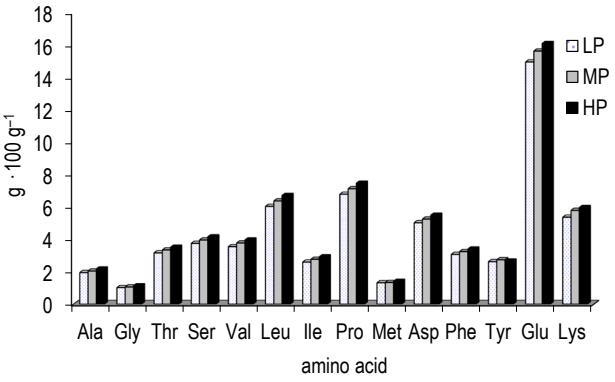


**Figure 1.** The mean concentrations of amino acids in milk protein, g · 100 · g$^{-1}$

**Table 3.** $^{15}N$ (at % excess) in milk urea and in individual amino acids of milk protein after infusion of $^{15}N$ labeled urea. Mean values ± SD (n=9)

| Indices | Protein level in diets[1] | | |
|---|---|---|---|
| | low | medium | high |
| Milk urea | 2.622 ± 1.265 | 3.077 ± 0.837 | 2.499 ± 0.636 |
| Alanine (Ala) | 0.450$^{a}$ ± 0.134 | 0.408$^{a}$ ± 0.079 | 0.196$^{b}$ ± 0.088 |
| Glycine (Gly) | 0.490 ± 0.383 | 0.508 ± 0.677 | 0.234 ± 0.229 |
| Threonine (Thr) | 0.400 ± 0.178 | 0.305 ± 0.257 | 0.185 ± 0.062 |
| Serine (Ser) | 0.361 ± 0.320 | 0.182 ± 0.173 | 0.170 ± 0.101 |
| Valine (Val) | 0.436$^{a}$ ± 0.114 | 0.305$^{ab}$ ± 0.128 | 0.102$^{b}$ ± 0.113 |
| Leucine (Leu) | 0.544$^{a}$ ± 0.173 | 0.397$^{a}$ ± 0.111 | 0.209$^{b}$ ± 0.016 |
| Isoleucine (Ile) | 0.702$^{a}$ ± 0.204 | 0.442$^{a}$ ± 0.212 | 0.000$^{b}$ ± 0.195 |
| Proline (Pro) | 0.397 ± 0.150 | 0.294 ± 0.142 | 0.154 ± 0.096 |
| Methionine (Met) | 0.895$^{a}$ ± 0.426 | 0.796$^{ab}$ ± 0.712 | 0.103$^{b}$ ± 0.245 |
| Arginine (Arg)) | 0.905 ± 0.321 | 0.513 ± 0.347 | 0.386 ± 0.498 |
| Aspartic acid (Asp) | 0.436 ± 0.358 | 0.329 ± 0.275 | 0.183 ± 0.292 |
| Phenylalanine (Phe) | 0.216 ± 0.099 | 0.268 ± 0.407 | 0.154 ± 0.218 |
| Glutamic acid (Glu) | 0.830$^{a}$ ± 0.252 | 0.878$^{a}$ ± 0.183 | 0.324$^{b}$ ± 0.240 |
| Lysine (Lys) | 0.660$^{a}$ ± 0.236 | 0.581$^{a}$ ± 0.150 | 0.126$^{b}$ ± 0.269 |

[1] values with different letters differ significantly, $^{a,b}$ $P < 0.05$

proline must be synthesized in the body (Mezl and Knox, 1977; Bequette et al., 2006).

The high content of the $^{15}N$ isotope in milk urea and its low content in proline suggests that the $^{15}N$ isotope from infused urea was incorporated mainly into the amidine group of bacterial arginine. Arginine was then utilized to produce proline in the mammary gland and the amidine group was used to form urea.

The relatively high $^{15}N$ enrichment of methionine and lysine at the low- and medium-protein content of the diet can be explained by the fact that they are essential amino acids, often considered to be the limiting amino acids, particularly when heated soyabean make up most of the protein source. So most of these amino acids can originate directly from the amino acids of bacterial protein, produced from EUN. Similarly, the high content of EUN in the branched-chain amino acids, such as leucine and isoleucine, can be due to these amino acids being produced by ruminal bacteria using urea nitrogen, as ruminants are unable to synthesize them. Incorporation of $^{15}N$ into these amino acids was low when the diet contained a high level of protein because the amount of dietary nitrogen was sufficient for microbial protein synthesis and only a small part of EUN was recycled to the rumen.

The high enrichment of glutamic acid can be explained by a large portion of arginine (which was highly enriched in the $^{15}N$ isotope) being converted

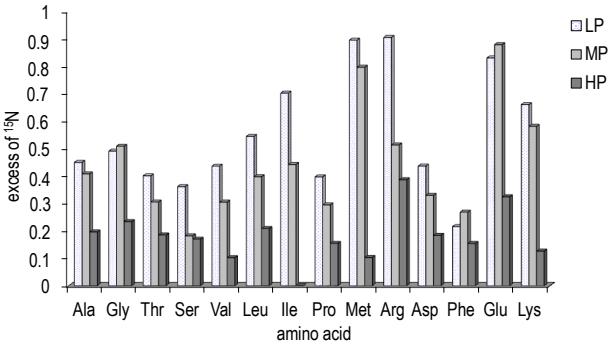


**Figure 2.** $^{15}N$ (at % excess) in individual amino acids of milk protein after infusion of $^{15}N$ labeled urea

in the mammary gland to glutamic acid and glutamine (Mezl and Knox, 1977).

The high enrichment with $^{15}N$ of glutamic acid, arginine, methionine, isoleucine, and lysine can be explained by the high incorporation of EUN in these amino acids of bacterial protein (Michalski et al., 2013).

The incorporation yield of endogenous urea nitrogen into amino acids of milk protein is reduced when the goat diet contains a high amount of protein.

The $^{15}N$ content in free amino acids of milk was highly variable and there were no statistically significant differences (Table 4). There were, however, numerical differences between the enrichment in $^{15}N$ of free amino acids of milk. The numerically higher content of $^{15}N$ from infused urea in free arginine of milk in goats receiving the low-protein diet compared with goats receiving the high-protein diet ($P = 0.0881$) is noteworthy.

The present study confirms that EUN is used for synthesis of bacterial amino acids and that these amino acids are utilized in the mammary gland for synthesis of milk protein. The results of this study correspond to our previous data (Michalski et al., 2012) in which increasing the protein content in the diet reduced the efficiency with which endogenous urea nitrogen was incorporated into the amino acids of milk protein.

**Table 4.** $^{15}N$ (at% excess) in free amino acids of milk after infusion of $^{15}N$ labeled urea. Mean values ± SD (n=9)

| Amino acids | Protein level in diets | | |
|---|---|---|---|
| | low | medium | high |
| Alanine (Ala) | 0.65 ± 0.25 | 0.77 ± 0.68 | 0.15 ± 0.20 |
| Glycine (Gly) | 1.20 ± 1.27 | 0.29 ± 0.11 | 0.50 ± 0.23 |
| Valine (Val) | 0.20 ± 0.44 | 0.24 ± 0.13 | 0.01 ± 0.12 |
| Leucine (Leu) | 0.61 ± 0.01 | 0.17 ± 0.26 | 0.00 ± 0.35 |
| Isoleucine (Ile) | 0.85 ± 0.89 | 0.41 ± 0.28 | 0.29 ± 0.08 |
| Proline (Pro) | 0.42 ± 0.23 | 0.34 ± 0.26 | 0.14 ± 0.06 |
| Arginine (Arg) | 2.16 ± 1.96 | 0.22 ± 1.56 | 0.27 ± 0.56 |
| Lysine (Lys) | 0.50 ± 0.31 | 0.37 ± 0.51 | 0.00 ± 0.36 |

## Conclusions

It can be concluded that endogenous urea nitrogen is primarily built into glutamic acid, methionine and arginine, and the yield of incorporation of $^{15}N$ into phenylalanine is very low, irrespective of the level of protein in the diets.

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