PROTEIN RECYCLING IN RABBITS: INCORPORATION OF MICROBIAL LYSINE IN GROWING RABBITS AS A METHOD OF MEASUREMENT

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ABSTRACT

The incorporation in body tissues of lysine synthesised by the microflora of the rabbit caecum was estimated by ^{15}N labelling of microbial protein in six animals receiving a conventional diet supplemented with $^{15}NH_4Cl$ (T_1), using a control group of four rabbits fed the same unlabelled diet (T_3). An additional group of six rabbits received the ^{15}N-labelled diet, but just during the last ten days and they were provided with a neck collar (T_2). The latter group was used to estimate caecotrophes production and find out if there is any microbial lysine incorporation in non-caecotrophagic animals. The experimental diets were administered for 35 days. Then animals were slaughtered, and caecal population and tissues were sampled. Lysine was isolated from tissues, microbes and food by ion exchange chromatography and its enrichment determined by Isotope Ratio Mass Spectrometry (IRMS). T_1-rabbits incorporated ^{15}N-lysine into the body tissues in significant amounts (0.11 (SE 0.005) atom % excess (APE)), although ^{15}N-lysine enrichment was significantly lower than in bacteria (0.21 (SE 0.010) APE), confirming the double origin of this essential amino acid (dietary and microbial). The contribution of microbes to tissular lysine was calculated from the ratio of ^{15}N-lysine enrichment between tissues and bacteria. So, it was estimated that the contribution of microbial labelled lysine to total lysine absorbed was 0.46. T_2-rabbits ingested the labelled diet only for ten days, but showed the same level of enrichment in caecal bacteria (0.22 (SE 0.034) APE) than T_1-rabbits. However, tissular ^{15}N-lysine enrichment was much lower (0.02 (SE 0.003) APE), although still significantly higher than in control group. This fact shows a certain absorption of microbial lysine by non-caecotrophagic routes in rabbits, but it represented only 14 % of total microbial lysine absorption, much lower than that obtained by the reingestion of soft faeces.

Key words: lysine, caecotrophy, rabbit, ^{15}N.

INTRODUCTION

Herbivores have not evolved adequate enzymes to degrade fibrous carbohydrates and then these ingredients must be digested symbiotically by gut microorganisms in specific

gut compartments. Fermentation compartment can be located before (rumen, pre-gastric fermenters) or after enzyme digestion area (caecum/colon, post-gastric fermenters), being the utilisation of microbial biomass the main difference between both fermentation systems. Although some absorption of microbial amino acid has been demonstrated in postgastric fermenters (TORRALLARDONA *et al.*, 2003), the re-ingestion of special faeces through caecotrophy process allows the lagomorphs to recycle most of microbial protein produced into the caecum. Quantitative estimation of this process is the largest element of uncertainty in lagomorph protein nutrition.

Lysine is an essential amino acid and thus unable to be synthesised by transamination (COX and DANCIS, 1995). Also it has been shown that inorganic ^{15}N ($^{15}NH_4Cl$) was never incorporated into tissue lysine in non-coprophagic rats, whereas incorporation was significant when rats were able to re-ingest microbial protein from its own faeces (TORRALLARDONA *et al.*, 1996). If ^{15}N is incorporated into the lysine pool only through microbial protein absorbtion, enrichment of body lysine must reflect the input of both components, microbial or dietary lysine sources. The aim of this study was to establish how much of the lysine comes from both sources, microbes and food, and to find out if microbial protein incorporated into tissues comes entirely by caecotrophy.

MATERIAL AND METHODS

Animals and diets

Sixteen New Zealand White male rabbits with a mean initial body weight (W) of 1.127 (SE 0.0395) kg and aged 45 days were used. Animals were penned individually in metabolism cages always under a 12:12 light cycle and with free access to drinking water.

The basal diet was formulated based on grass hay (35 %), barley grain (25 %), sugar beet pulp (20 %), soya bean meal (15.5 %), sunflower oil (3.5-4 %), and a vitamin-mineral mix (0.1-0.2 %), either supplemented or not with ^{15}N-labelled NH_4Cl (1 %; $^{15}NH_4Cl$, 10^+ atom % ^{15}N ISOTEC, Inc USA) and its chemical composition is presented in Table 1. Animals were fed once daily at a restricted level (55 g fresh matter/kg $W^{0.75}$) and the feed was sampled weekly.

Experimental design and sampling

Three experimental treatments were designed lasting for 35 days. In treatment 1 (T_1), six rabbits were fed the isotope supplemented diet during all the experimental period; in treatment 2 (T_2), six rabbits received the un-labelled diet for twenty-five days, then animals were fitted a neck collar (50 mm i.d. and 300 mm o.d., weighing approximately 65 g) and fed the labelled diet until slaughter (d 35); in the control group (T_3) four rabbits were fed only the un-labelled diet. Additionally, neck collars were fitted temporary (12 h) every six days to T_1 group rabbits in order to check ^{15}N-enrichment of the caecal population. Finally, animals were slaughtered between 08.00 and 12.00 by cervical dislocation, dissected and the caecum excised and weighed, caecal content was

sampled (20-50 g) for bacterial extraction following the procedure described by MINATO and SUTO (1981). Microbial extract was freeze-dried for subsequent analysis. The same technique was used to obtain the microbial extract from caecotrophes collected from T_1 animals. After removal of the gastrointestinal tract, liver and muscle (psemitendinose) were sampled.

Table 1. Chemical composition of the experimental diets.

Chemical composition (g/kg)	Unlabelled diet	Labelled diet
Dry matter	942.5	931.3
Organic matter	922.4	922.2
Crude protein	174.4	192.2
Neutral detergent fibre	297.0	320.5
Acid detergent fibre	158.4	165.4
Acid detergent lignin	23.3	20.4

Analytical procedures

Dry matter (DM) in food was determined by drying at 60ºC to constant weight. Organic matter (OM) was estimated by ashing samples at 550 ºC for 8 h. Nitrogen (N) was measured by the Kjeldhal method. Neutral and acid detergent fibre (NDF and ADF) and acid detergent lignin (ADL) were determined according to VAN SOEST *et al.* (1991), after amylase pre-treatment. Freeze-dried (extracted bacteria, liver and muscle) samples were ground with a coffee grinder for further analysis. Lysine from bacteria and tissues was isolated by ion-exchange chromatography and its enrichment, determined by IRMS.

Calculations and statistical analysis

The contribution of microbial lysine to tissular lysine (M_{lys}) was estimated as:
$M_{lys} = [E_{Tis}]-[E_{TisC}] / [E_{bac}]$ being E_{Tis}, ^{15}N-lysine enrichment (atom % excess, APE) in tissues, E_{bac} in bacteria in T_1-animals and E_{TisC} in the tissues of T_2 animals, respectively. Substrate enrichment *(E)* was calculated as the difference between the induced abundance and the background ^{15}N abundance of each substrate (T_3 animals) [$E_{Tis} = Ab_{Tis} - Ab_{Tis0}$].

Data were analysed as one-way classification with unequal replication per treatment. Animals were considered as a random variable. Significance was declared at $P<0.05$.

RESULTS AND DISCUSSION

The evolution of ^{15}N enrichment in total nitrogen of caecal bacteria extracted from caecotrophes of the isotope group is shown in Figure 1. The enrichment increased significantly after rabbits started to ingest the labelled diet and for practical purposes the ^{15}N enrichment plateau is reached after 10 days.

Table 4 shows the ^{15}N-lysine enrichments in tissue and caecal bacteria in those rabbits corresponding to T_1 and T_2 treatments, given that the ^{15}N-lysine abundance of those consuming the non-isotope diet (T_3) were taken as background for tissues (0.380 (SE 0.0022) %) and bacteria (0.382 (SE 0.0030) %). Rabbits in T_1 showed a significant enrichment of lysine in caecal bacteria (0.211 (SE 0.0097) APE) and in tissues (0.114 (SE 0.0054) APE). In the latter ^{15}N-lysine enrichment was about half than in bacteria. Finally, in T_2 rabbits, which were also fed the isotope-labelled diet simultaneously with neck collar fitting to avoid caecotrophy, lysine from caecal bacteria showed a similar enrichment (0.221 (SE 0.0335) APE) to that determined in T_1-rabbits, but only a low enrichment was detected in tissular ^{15}N-lysine (0.017 (SE 0.0026) APE). Considering lysine, as an essential amino acid, which comes from dietary or microbial sources, the contribution of microbial lysine to total synthesis was estimated to be 0.463 (SE 0.0237), and it represented nearly half of total absorbed lysine.

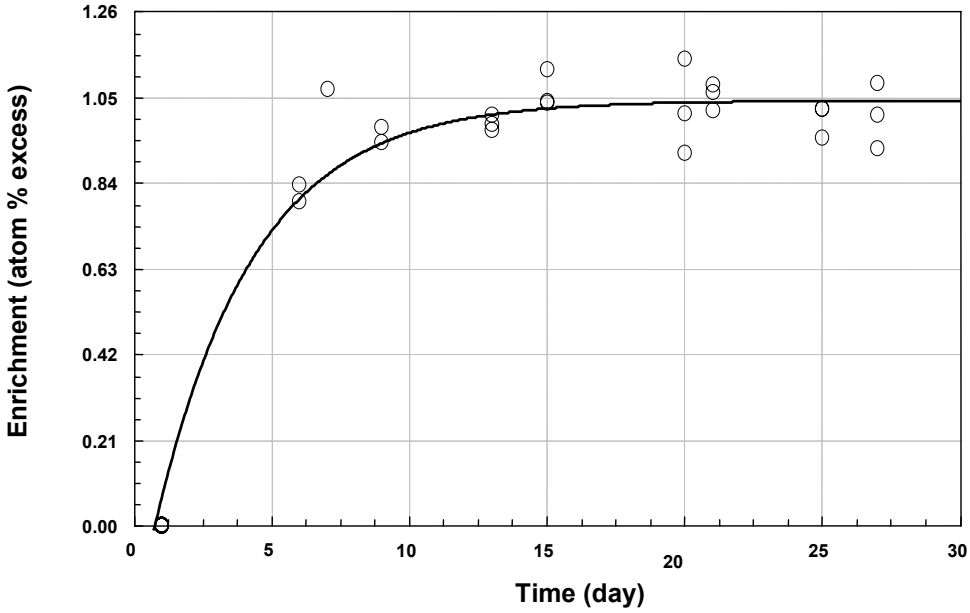

Figure 1. Evolution of the ^{15}N enrichment (atom % excess) in caecal bacteria of animals receiving isotope enriched diet (T_1).

Table 2. ^{15}N enrichment (atom % excess) in lysine of tissues and caecal bacteria of rabbits fed on $^{15}NH_4Cl$ supplemented diet without *(Isotope group*) or with a neck collar (*Collar group*).

	Tissue	SE	Caecal bacteria	SE
Isotope group	0.114	0.0054	0.211	0.0097
Collar group	0.017	0.0026	0.221	0.0335
Statistical significance (P)	<0.0001		0.750	

SE: Standard error of the means.

Initially when the experiment was planned we considered that N requirements of the microbial population of the caecum would be met mostly by endogenous nitrogen and recycled blood urea (FORSYTHE and PARKER, 1985). This moved us to attempt a significant label of the blood urea pool by supplying sufficient ^{15}N under the form of ammonium chloride in the diet. Figure 1 shows that the objective was clearly achieved.

Caecal bacteria was significantly enriched with ^{15}N and just after 6 days of isotope administration microbial-N enrichment almost reached a plateau level (Figure 1). It confirms that N requirements of the microbial flora would be met in an important proportion by blood urea (FORSYTHE and PARKER, 1985), while non-digestible N has a minor contribution. Isotopic enrichment of microbial-N was much higher than the enrichment recorded in microbial lysine-N (1 *vs* 0.211 and 0.221 APE, for T_1 and T_2 respectively). Results agree with those reported by MILLWARD *et al.* (2000) who found a much lower isotopic enrichment of lysine compared with glycine and alanine. A possible explanation would lie in labelled ammonia utilisation, in that sense the preferential incorporation of inorganic ^{15}N into non-essential (by transamination) than essential amino acids (DEGUCHI *et al.*, 1980) would confirm that fact. Moreover, lysine showed amongst the essentials the lowest enrichment .

Protein turnover is higher in liver than in muscle and it implies differential rate of tissue enrichment, which tend to equilibrate with long isotope administration period (DEGUCHI and NAMIOKA, 1989). The absence of differences (P=0.3032) in lysine ^{15}N-enrichment between liver (0.12 (SE 0.011) APE) and muscle (0.10 (SE 0.010) APE) seems to suggest that a steady estate in the isotope distribution was reached.

As mentioned, lysine cannot be synthesized “de novo” and microbial ^{15}N-lysine must be incorporated, either from the caecotrophy process or by direct intestinal absorption of microbial protein. In an attempt to measure both incorporation routes T_2 was designed. T_2-animals received the labelled diet but soft faeces re-ingestion was avoided by collar fitting, in that case a small, but significant, enrichment in tissues (either muscle or liver) was detected, whereas microbial lysine enrichment was not dissimilar than those values determined in T_1-animals (0.221 *vs* 0.211 APE for T_2 and T_1, respectively). The ^{15}N-enrichment in lysine of T_2 tissues (0.017 (SE 0.0026) APE) might reflect some amino acid absorption either through the hindgut (HOOVER and HEITMANN, 1975) or from the small intestine, where previously an active microbial fermentation has been described (GIDENNE *et al.*, 1998). Both possibilities would be taken into account in the present discussion, although some in-efficiency in the collar fitting with some intake of soft faeces could be not disregarded. In any case, in caecotrophagic animals direct intestinal absorption would have a minor relevance given that, from our results, that route would represent only 14 % of the total microbial lysine absorption. It is necessary to remark that in such calculation a similar ^{15}N-lysine enrichment in both microbial populations (small and large intestine flora) is assumed intrinsically, and that point requires a further development.

CONCLUSIONS

Our findings show that only caecotrophagic animals were able to incorporate ^{15}N to a significant extent into body lysine and thus microbial lysine contributes by nearly half of the total absorbed lysine what would confirm the significance of caecotrophy in rabbit protein metabolism.

ACKNOWLEDGMENTS

This work was financed by Cargill, S.L. Paseig Sant Joan 189, 08037 Barcelona, Spain and the Spanish Ministry of Science (Projects PTR 95/0405-OP and PB98-01601). Thanks are given to R. Redondo (Faculty of Sciences, Universidad Autónoma de Madrid) for isotope analyses in the Laboratory of stable isotopes and to the Servicio de Apoyo a la Investigación (Universidad de Zaragoza) for care of animals.

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