Microbial amino acid synthesis and utilization in rats: incorporation of ^{15}N from $^{15}NH_4Cl$ into lysine in the tissues of germ-free and conventional rats

BY DAVID TORRALLARDONA[1]*, C. IAN HARRIS[1], MARIE E. COATES[2] AND MALCOLM F. FULLER[1]

[1] *Rowett Research Institute, Greenburn Road, Bucksburn, Aberdeen AB2 9SB*
[2] *School of Biological Sciences, University of Surrey, Guildford, Surrey GU2 5XH*

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The absorption of lysine synthesised by the gastrointestinal microflora was estimated by comparing the ^{15}N incorporated into body lysine in four germ-free (^{15}N-GF) and four conventional (^{15}N-CV) rats. They were fed for 10 d on a protein-free diet containing fermentable carbohydrates and $^{15}NH_4Cl$; another four conventional rats (control), fed on the same diet but with unlabelled NH_4Cl, were used to estimate the natural abundance of ^{15}N. The eviscerated carcass of each rat was homogenized and a sample hydrolysed. Lysine was isolated by ion-exchange chromatography and its ^{15}N enrichment was measured by isotope-ratio mass spectrometry. The ^{15}N-CV rats significantly incorporated ^{15}N into their body lysine. The ^{15}N-GF rats had a statistically significant, although small, incorporation of ^{15}N into their body lysine, probably arising from a measurement artifact. It was concluded, therefore, that all [^{15}N]lysine was of microbial origin. The total lysine content in the body and the ^{15}N enrichment of lysine in the microbial fraction of the faeces of the ^{15}N-CV rats were also determined. The amount of microbial lysine absorbed by the ^{15}N-CV rats was estimated by dividing the total amount of [^{15}N]lysine in the body by the enrichment of microbial lysine. It was estimated that the daily absorption of microbial lysine by the conventional rats was 21·3 (SE 2·04) mg/kg body weight$^{0.75}$.

Microbial amino acid absorption: Gastrointestinal microflora: Amino acid requirement: Germ-free rats

It is generally accepted that in non-ruminants the main beneficial effect of their microflora is the so-called 'barrier effect' which prevents colonization of the gastrointestinal tract by pathogenic micro-organisms (Hentges, 1992). For some non-ruminant animals which eat relatively-indigestible low-protein diets (i.e. lagomorphs and horses), the intestinal microflora may also contribute to the amino acid supply to the host (Lang, 1981; Frape, 1986). In other non-ruminant animals (i.e. omnivores and carnivores), however, such nutritional benefits are not clear. The fact that, under domesticated conditions, such animals eat diets of relatively high digestibility and adequate protein contents may reduce the importance of any contribution of the intestinal microflora to their amino acid requirements. In malnutrition, however, this could be an important mechanism of nutritional adaptation.

In a pilot experiment (Torrallardona *et al.* 1993*a*), substantial absorption of microbial lysine was estimated in a rat and a pig. These estimates were based on the assumption that higher animals were not able to incorporate ^{15}N from $^{15}NH_4Cl$ into lysine and that all [^{15}N]lysine found in the body of animals fed on $^{15}NH_4Cl$ was, therefore, of microbial origin. Lysine and threonine are the only amino acids which are not considered to incorporate inorganic ^{15}N by transamination (Bender, 1985). This inability of lysine to transaminate is

* Present address: IRTA, Centre de Mas Bové, Apartat 415, E-43280 Reus, Spain.

supported by growth studies in which addition of α-oxo-ε-aminocaproate to lysine-deficient diets failed to improve growth (McGinty *et al.* 1924), and by isotopic studies in which ^{15}N labelling in lysine was not found after administration of inorganic ^{15}N (Fürst, 1972; Sheng *et al.* 1977). When larger doses of inorganic ^{15}N have been used, however, incorporation of ^{15}N into lysine has been measured in rats (Foster *et al.* 1939; Åqvist, 1951*a*, *b*; Tanaka, 1982), pigs (Deguchi *et al.* 1978, 1980) and human subjects (Giordano *et al.* 1968; Tanaka *et al.* 1980; Patterson *et al.* 1993). In general, all authors observed that more inorganic ^{15}N was incorporated into the dispensable amino acids (DAA) than into the indispensable amino acids (IAA). Amongst the IAA the lowest enrichments were generally found in lysine and threonine. From this work, it is not clear, therefore, whether the small ^{15}N enrichments found in body lysine and threonine are a result of microbial lysine being absorbed or are due to a certain degree of transamination. To check whether ^{15}N can be incorporated into lysine by transamination, inorganic ^{15}N should be fed to germ-free animals and its incorporation into body lysine measured. Deguchi & Namioka (1989) reported such an experiment with pigs, but unfortunately their results were not conclusive. They fed [^{15}N]diammonium citrate (DAC) to two germ-free (GF) piglets. One of them (piglet 5) received three doses of ^{15}N at days 13, 15 and 17 after birth, the other piglet (piglet 6) received one dose on day 21. Both piglets were killed and sampled at day 22. Piglet 5 showed substantial incorporation of ^{15}N into lysine and threonine, similar to that found in two specified-pathogen-free (SPF) piglets given the same three doses of [^{15}N]DAC. Piglet 6, however, did not incorporate ^{15}N into lysine or threonine. This cannot be entirely explained by the lower dose given to piglet 6, because the ^{15}N enrichments of DAA and IAA in the TCA precipitate of its liver were about one-third of those in the liver of piglet 5; therefore, if the lysine ^{15}N enrichment of piglet 5 was correct, detectable (although lower) enrichment would have been expected in piglet 6.

The first aim of the present experiment was to examine the ability of GF rats to incorporate ^{15}N from $^{15}NH_4Cl$ into body lysine (i.e. to establish whether the ^{15}N enrichment found in the body lysine of conventional (CV) animals is due to transamination or to absorption of microbial lysine). If it was found that inorganic ^{15}N can only be incorporated into body lysine via microbial lysine synthesis and absorption, the second aim was to estimate the amount of microbial lysine absorbed by CV rats.

Preliminary results from the present study have previously been published in abstract form (Torrallardona *et al.* 1993*b*).

MATERIALS AND METHODS

Animals and housing

Twelve Hooded Lister (six male and six female) rats from two GF litters were used. The GF procedures are described by Coates & Gustaffson (1984). The animals were divided at weaning into three groups of four rats (two male and two female), housed in standard cages. One of the groups (^{15}N-GF) remained under GF conditions in a polyvinyl chloride (PVC) isolator (Trexler & Reynolds, 1957); the other two groups (^{15}N-CV and control) were removed from the isolators to cages in an area where CV rats were already housed. They were left there for 2–3 weeks, to allow them to acquire the indigenous gut microflora of their CV companions. All animals were then moved into individual metabolism cages for a 4 d period of adaptation to the experimental diet followed by a 10 d experimental period.

Regardless of the previous mode of sterilization, the surfaces of all items entering the isolator were sprayed with peracetic acid (20 ml/l; PA) and left in the entry port for at least 20 min before being introduced into the isolator. The PA solution was prepared freshly every day with deionized water containing a few drops of detergent (Triton X-100).

Table 1. *Composition of experimental diets (g/kg)*

	Labelled	Unlabelled
Maize starch	336	336
Sucrose	100	100
Raw potato starch	350	350
Cellulose	100	100
Vegetable oil	60	60
Vitamins	9	9
Minerals	35	35
NH_4Cl	—	10
$^{15}NH_4Cl$ (10·3 atom %)	10	—

Monitoring of germ-free condition

At the beginning and end of the experiment, sterility tests were performed on fresh faecal samples from the rats. Inside the isolator the faeces were inoculated into four 30 ml universal containers containing thioglycollate broth, brain and heart broth ($\times 2$) and malt extract broth. The containers were securely closed before removal from the isolators, and incubated for 3 weeks either at 37° (thioglycollate and brain and heart broths) or at 25° (brain and heart and malt extract broths).

Diets

All rats had been fed on the same commercial diet GR3 EK.R20 (Special Diet Services, Witham, Essex) which was sterilized by γ-radiation at 50 kGy. During the adaptation period (4 d) the animals were gradually introduced to a protein-free diet containing fermentable carbohydrates and unlabelled NH_4Cl (unlabelled diet; Table 1). During the experimental period (10 d) the ^{15}N-GF and ^{15}N-CV groups were offered the ^{15}N-labelled diet and the control group continued on the unlabelled diet. The experimental diets were also sterilized by γ-radiation at 50 kGy. Since some vitamins are vulnerable to destruction during irradiation (Coates, 1984), the amount of vitamins was increased in order to compensate for any losses which might occur. All animals were offered deionized water which was sterilized by autoclaving at 1·05 kg/cm^2 for 1 h.

Measurements and sampling

During the experimental period the animals were weighed, the feed consumption was recorded and the faeces collected and frozen individually for each animal. At the end of the experiment the animals were slaughtered under anaesthesia. Blood samples were collected by heart puncture into sodium citrate (30 g/l). The cellular and plasma fractions from blood were separated by centrifugation and frozen until required. The gastrointestinal tracts were removed (in order to prevent contamination of the carcasses with microbial amino acids), emptied and weighed. The livers and carcasses were frozen individually.

Analytical procedures

The whole frozen carcasses of the rats were freeze-dried and ground with a coffee grinder. A subsample of about 2 g was then taken and finely ground with a freezer mill (Spex Industries Inc., Metuchen, NJ, USA) using liquid N_2. The liver samples were ground with the freezer mill directly after freeze-drying. The plasma samples were not processed before hydrolysis.

The microbial fraction of faeces was separated by differential centrifugation, essentially as described by Laplace *et al.* (1985). Faeces were defrosted overnight at 4° and homogenized in phosphate-buffered saline (PBS; 8·1 mM-Na_2HPO_4, 1·9 mM-$NaH_2PO_4.2H_2O$, 154 mM-NaCl, pH 7·4) using a blender. The homogenate was centrifuged (250 *g*) at 4° for 15 min using a Europa-24M centrifuge (6 × 300 head; Fisher, Loughborough, Leics.). The supernatant fraction obtained was transferred, using a pasteur pipette, to a suction flask connected to a vacuum pump, filtered through a sintered-glass funnel of porosity 4, and centrifuged (14500 *g*) at 4° for 30 min. After decanting the supernatant fraction, the pellet was resuspended in PBS and centrifuged (14500 *g*) at 4° for 30 min. The final pellet was frozen until required.

Microbial samples and freeze-dried tissues were hydrolysed under reflux with distilled constant-boiling 6 M-HCl at 137° for 18 h. Hydrolysates were filtered through a sintered-glass funnel of porosity 4, and dried under reduced pressure at 39° using a Büchi rotovapor (Büchi Laboratorium-Technik, Flowil, Switzerland). Samples were washed with deionized water, redried twice, and taken up in a small volume of deionized water.

The samples were taken to pH 11 with 10 M-NaOH and left in a desiccator under reduced pressure in the presence of concentrated H_2SO_4 for at least 1 h. This was to reduce the quantity of highly-^{15}N-enriched NH_3 present which could interfere with the measurement of the ^{15}N enrichment of lysine. The samples were acidified to pH < 2 with concentrated HCl before loading onto the preparative column. In some cases a precipitate formed when the sample was mixed with the eluting buffer, probably due to a saponification reaction between the fatty acids and NaOH. As this precipitate would cause an obstruction of the column, it was prevented by adding citrate buffer to the sample (1:1, v/v) 1 h before loading and removing the precipitate by centrifugation.

Portions of protein hydrolysate containing about 10 μmol lysine were loaded onto an ion-exchange chromatography preparative system (The Locarte Co., London) fitted with a 9 × 400 mm column. The column was eluted with a 0·13 M-trisodium citrate buffer, pH 4·18, at 29°, with a flow rate of 30 ml/h. The column was operated on a 12 h-cycle basis (amino acid elution for 11 h, regeneration with 0·2 M-NaOH for 20 min and equilibration with the eluting buffer for 40 min). Fractions of 150 drops (i.e. 6 min or 3 ml) were collected using a fraction collector. This system was preferred to others commonly used because it gave a good separation of resolved lysine and ornithine, which could be highly labelled.

The amino acid content of each fraction was measured using post-column derivatization with *o*-phthalaldehyde (OPA; Fig. 1) based on the method described by Church *et al.* (1985). The OPA solution (16 mg OPA in 400 μl methanol, 20 ml 0·3 M-borax (saturated), 40 μl mercaptoethanol) was prepared freshly every day. A 50 μl portion from each fraction was transferred into a multi-well plate and 80 μl OPA solution were added. The plate was left at room temperature for 2 min and the absorbance at 340 nm measured using a Titertek Multiskan MCC/340 (ICN Biochemicals, Thame, Oxon).

Fractions containing lysine were pooled. When pooling the fractions a few extra fractions before and after the peak were included in order to allow for the possible displacement of labelled relative to unlabelled amino acids (Spedding *et al.* 1955).

To desalt the amino acid fractions an adaptation of the chromatographic method (using HCl) of Stein & Moore (1950) was used. The pooled fraction containing lysine was loaded onto a 18 × 150 mm column of Dowex-50 (H^+ form, 100–200 mesh, 8% cross-linked; Sigma Chemical Co., St Louis, MO, USA). After loading the sample (approximately 10 μmol lysine, pH < 2), the citrate was removed with deionized water, and the column was washed with 100 ml 2·5 M-HCl, followed by 150 ml 6 M-HCl. Na^+ and any possible contaminating NH_4^+ was eluted with the 2·5 M-HCl wash, whereas lysine appeared in the 6 M-HCl wash (Fig. 2). The wide separation between lysine and NH_4^+ in the HCl system was

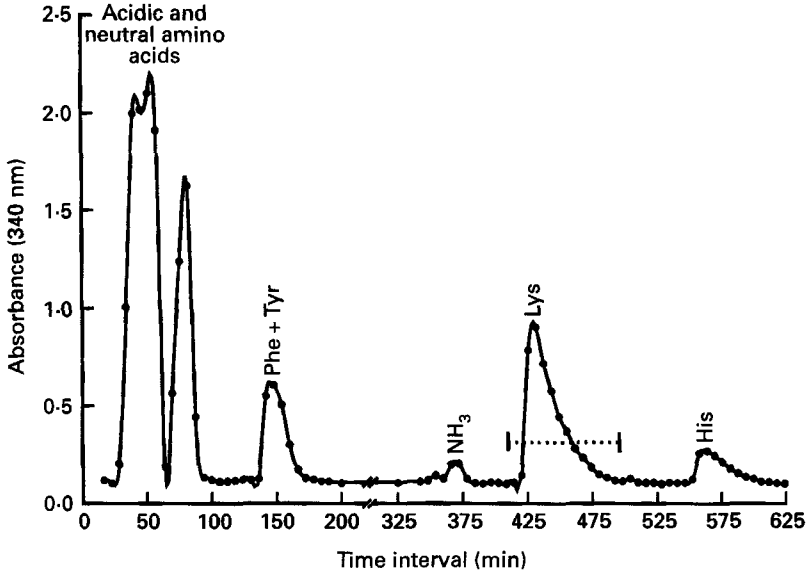

Fig. 1. Preparative separation of lysine by ion-exchange chromatography (9 × 400 mm column, 0·13 M-trisodium citrate, pH 4·18). Carcass sample containing approximately 10 μmol lysine. Amino acid peaks identified by post-column derivatization with *o*-phthalaldehyde. For details of experimental procedures, see pp. 691–692.

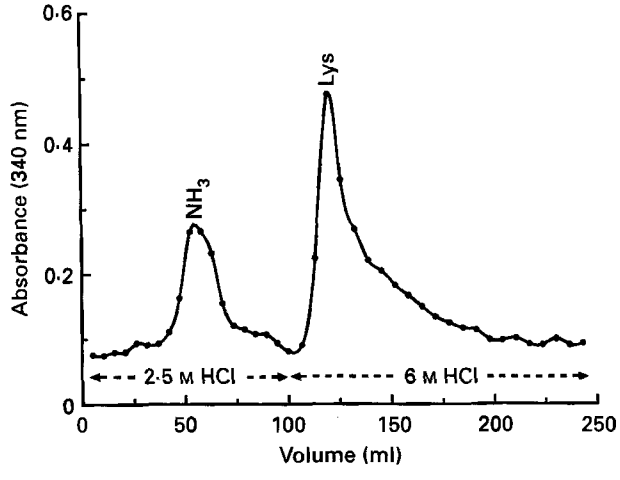

Fig. 2. Separation of lysine and NH_3 during desalting on a Dowex-50 column (18 × 150 mm). Standard containing 15 μmol NH_3 and 15 μmol lysine. Peaks identified with *o*-phthalaldehyde after neutralization with NaOH. For details of experimental procedures, see pp. 692–694.

very convenient, since NH_3 could interfere with the [^{15}N]lysine measurements and other systems do not separate them to the same extent.

After each sample the desalting column was regenerated. Excess HCl was removed with deionized water and the column washed with 100 ml 2 M-NaOH. The excess NaOH was removed with deionized water and the column was reconverted to the H^+ form with 100 ml 6 M-HCl. The column was given two additional washes with deionized water before loading the next sample.

The desalted lysine was dried under reduced pressure at 39°. A portion containing 0·5 mg N was digested to $(NH_4)_2SO_4$ by a micro-Kjeldahl method (1·5 ml concentrated H_2SO_4, 50 mg HgO in 1 ml 1·85 M-H_2SO_4, 1·5 g K_2SO_4). The sample was digested for 30 min after the solution clarified.

NH_3 was liberated in a Markham (1942) still with the addition of 10 ml 10 M-NaOH. Steam was generated from deionized water (acidified with a few drops of concentrated H_2SO_4). The distillate was collected into 2 ml 0·0025 M-H_2SO_4 for 10 min or up to 40 ml. Between samples the still was rinsed and left running with the cooling circuit off for at least 10 min to prevent contamination between samples. The distillate was taken to dryness by rotary evaporation, resuspended in 1·5 ml deionized water and dried in 4 ml glass vials in a vacuum oven (Fisher, Loughborough, Leics.) at 80° before mass spectrometry.

The dried samples containing 0·5 mg N were treated with alkaline hypobromite by the Sprinson and Rittenberg procedure as detailed by Hauck (1982). Enrichment of the liberated N was measured in a gas-isotope-ratio mass spectrometer (SIRA 12; VG Isogas, Middlewich, Ches.). The ^{15}N enrichment of lysine in the carcasses of the control group was taken to be the natural abundance.

In order to measure the lysine content in the carcass and tissues of the rats, approximately 150 mg dried tissue were carefully weighed, a measured volume of a standard solution (2·5 mM-ε-amino-caproic acid in 6 M-HCl) was added, and the mixture was hydrolysed in 200 ml 6 M-HCl (constant-boiling), under reflux at 137° for 18 h. The hydrolysate was filtered and washed through a sintered-glass funnel of porosity 4, and made up to 250 ml with deionized water. Portions of 10 ml were taken to dryness under reduced pressure at 39°, washed twice with deionized water and resuspended in 10 ml 0·1 M-HCl. A portion of the sample was loaded onto an analytical ion-exchange system (The Locarte Co.), fitted with a 6 × 215 mm column (Aminex A5 resin; Bio-Rad Laboratories Ltd, Hemel Hempstead, Herts.). The amino acids were analysed with an accelerated version (6 h cycle) of the two sodium citrate buffer system of Spackman *et al.* (1958) at 29° with a flow rate of 30 ml/h. The amino acids were quantified with ninhydrin, and corrected for the recovery of the internal standard (ε-amino-caproic acid).

Estimation of microbial lysine absorption

If all [^{15}N]lysine found in the body of the rats is of microbial origin (i.e. lysine does not transaminate), the total amount (mg) of microbial lysine absorbed (*AMLys*) can be estimated as:

$$AMLys = \frac{EBLys \times CBLys}{EMLys},$$

where *EBLys* is the enrichment in body lysine (atom % excess), *CBLys* is the total content of lysine in the body (mg) and *EMLys* is the enrichment in microbial lysine (atom % excess).

The estimation of microbial lysine absorption with the previously stated formula assumes that all microbial lysine entering the body is retained, but this is not strictly true. The loss (in endogenous secretions, skin desquamation, etc.) or catabolism of amino acids would result in an underestimation of the amount of microbial amino acids absorbed. By using protein-free diets in the present study, loss and degradation are likely to be minimal and will be neglected.

Statistical analysis

The statistical significance of the differences in lysine ^{15}N enrichments, body weights and feed intakes between the groups were assessed by ANOVA. Differences in weight loss (as a percentage of initial weight) were examined by analysis of covariance using total feed

Table 2. *Average initial and final body weights, feed intakes and weight losses of rats fed on ^{15}N-labelled or unlabelled diets and housed in a germ-free (^{15}N-GF) or a conventional (^{15}N-CV and control) environment**

	Initial wt (g)	Final wt (g)	Feed intake (g/10 d)	Wt loss† (g)
^{15}N-GF	96·5	83·3	48·1	12·1[a]
^{15}N-CV	88·5	73·5	49·1	14·9[b]
Control	78·5	65·7	49·3	14·0[b]
SED	19·02	17·16	6·01	0·69

[a, b] Mean values with unlike superscript letters were significantly different ($P < 0·05$).
* For details of diets and experimental procedures, see Table 1 and pp. 690–691.
† Adjusted to constant initial weight and feed intake.

intake (g/10 d) as the covariate. The statistical tests were applied using the statistical analysis package MINITAB (Minitab Inc., 1989).

RESULTS

No microbial growth was observed in any of the broths inoculated with faeces from the GF rats, so it was considered that the animals had remained GF during the experiment.

Feed intake and body weight changes

Relatively low feed intakes (Table 2) were observed in all groups; this was probably due to the characteristics of the diet (synthetic and protein-free). The total doses of ^{15}N consumed by the ^{15}N-GF and ^{15}N-CV rats were 13·0 (SE 1·69) and 13·3 (SE 0·52) mg respectively (i.e. 84·6 (SE 2·75) and 97·0 (SE 6·53) mg/kg body weight$^{0·75}$).

There were no differences in feed intake ($P = 0·983$) or in initial ($P = 0·652$) or final ($P = 0·610$) body weights between the different groups (Table 2). However, within each group there was a large range in initial body weight and in feed intake. In order to allow for this, and to compare GF and CV and control animals at constant intake, weight losses were analysed with initial weight and feed intake as covariates. Both had a significant effect on weight loss ($P < 0·001$). The adjusted values are given in Table 2 and the effect of feed intake is shown in Fig. 3.

The ^{15}N-GF rats showed a significantly smaller loss of body weight than the ^{15}N-CV and control rats ($P = 0·009$). The difference between ^{15}N-CV and control groups was not significant ($P = 0·239$).

^{15}N enrichments in lysine of tissues

The average [^{15}N]lysine enrichment (atom %) in the carcasses of the control group (0·3704 (SE 0·00009)) was used as the natural abundance. In the ^{15}N-CV group there was a significantly higher ^{15}N enrichment of lysine in the eviscerated carcass than in the ^{15}N-GF ($P = 0·0010$) or control ($P = 0·0008$) groups (Table 3). A small but statistically significant ($P = 0·042$) increase in the ^{15}N enrichment of body lysine was measured in the ^{15}N-GF rats.

The ^{15}N enrichments of lysine in liver and plasma protein of the ^{15}N-CV rats were also

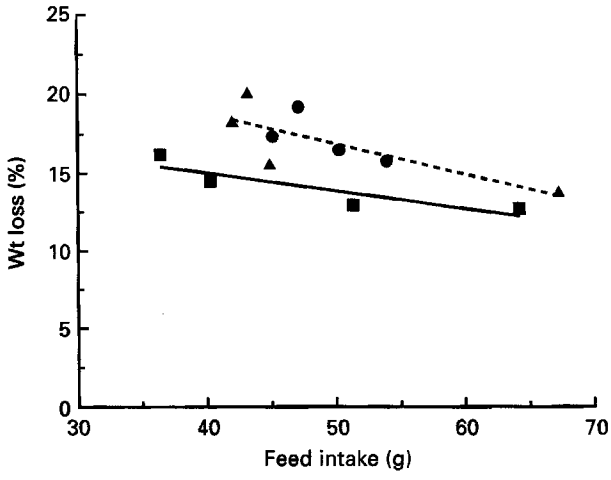

Fig. 3. Relationship between weight loss (%) and feed intake of germ-free (^{15}N-GF; ■) or conventional (^{15}N-CV (●) and control (▲)) rats fed on labelled (^{15}N-GF and ^{15}N-CV) and unlabelled diets. For the ^{15}N-GF animals: r^2 0·79, $P = 0·112$; for the conventional rats: r^2 0·59, $P = 0·026$. For details of diets and experimental procedures, see Table 1 and pp. 690–691.

Table 3. *^{15}N enrichments (atom% excess) in lysine of carcasses of germ-free (^{15}N-GF) and conventional (^{15}N-CV) rats fed on diet containing $^{15}NH_4Cl$, and of conventional rats (control) fed on diet containing unlabelled NH_4Cl**

Rat no.	Sex	Control	^{15}N-GF	^{15}N-CV
1	M	0·0002	0·0003	0·0054
2	M	0·0001	0·0002	0·0077
3	F	−0·0002	0·0003	0·0067
4	F	−0·0001	0·0007	0·0067
Mean	—	0·0000[a]	0·0004[b]	0·0066[c]
SE	—	0·00009	0·00010	0·00047

[a, b, c] Mean values with unlike superscript letters were significantly different: [a, b] $P < 0·05$, [b, c] $P < 0·001$, [a, c] $P < 0·001$.

* For details of diets and experimental procedures, see Table 1 and pp. 690–694.

measured. Lysine of liver protein was significantly more enriched than lysine of plasma protein ($P = 0·0034$) or carcass ($P < 0·0001$). Lysine of plasma protein was also significantly more enriched than lysine of carcass ($P = 0·0060$). The ^{15}N enrichment of lysine in the whole bodies of the ^{15}N-CV rats was estimated from the ^{15}N enrichments and relative contributions to total body lysine of carcass, liver and plasma (Table 4).

Estimates of microbial lysine absorption

The absorption of microbial lysine by the ^{15}N-CV rats (Table 5) was estimated from whole body and microbial lysine enrichments, and from the total lysine content in the body, as described previously. It was estimated that CV rats absorbed 21·3 (SE 2·04) mg microbial lysine/d per kg body weight$^{0·75}$.

Table 4. *^{15}N enrichments (atom % excess) in lysine of plasma, liver and carcass of conventional rats fed on diet containing $^{15}NH_4Cl$**

(The ^{15}N enrichment in lysine of the whole body was estimated from plasma, liver and carcass lysine enrichments and contents)

Rat no.	Plasma	Liver	Carcass	Whole body
1	0·0223	0·0309	0·0054	0·0064
2	0·0235	0·0338	0·0077	0·0088
3	0·0198	0·0320	0·0067	0·0077
4	—	0·0287	0·0067	0·0076†
Mean	0·0219[a]	0·0314[b]	0·0066[c]	0·0076[c]
SE	0·00109	0·00107	0·00047	0·00049

[a, b, c] Mean values with unlike superscript letters were significantly different: [a, b] $P < 0·01$, [b, c] $P < 0·001$, [a, c] $P < 0·01$.

* For details of diets and experimental procedures, see Table 1 and pp. 690–694.

† Calculated without using plasma values.

Table 5. *^{15}N enrichments (atom % excess) in lysine of the whole body and of the microbial fraction of faeces, total content of lysine in the body and absorption of microbial lysine in conventional rats fed on diet containing $^{15}NH_4Cl$**

Rat no.	Lysine ^{15}N enrichment		Body lysine content (g)	Lysine absorbed	
	Body	Microbial		mg/d	mg/d per kg body weight$^{0·75}$
1	0·0064	0·2914	1·45	3·19	17·1
2	0·0088	0·3135	1·00	2·81	21·6
3	0·0077	0·2705	0·83	2·37	19·8
4	0·0076	0·2102	0·92	3·32	26·7
Mean	0·0076	0·2714	1·05	2·92	21·3
SE	0·00049	0·02221	0·138	0·214	2·04

* For details of diets and experimental procedures, see Table 1 and pp. 690–694.

DISCUSSION AND CONCLUSIONS

^{15}N-CV rats showed a clear ^{15}N enrichment in lysine when compared with control and ^{15}N-GF rats. The small (although statistically significant) increase in ^{15}N enrichment of lysine found for the ^{15}N-GF group was of similar magnitude to that found when an unlabelled carcass sample (containing approximately 50 μmol lysine) was hydrolysed in the presence of $^{15}NH_4Cl$ (25 μmol; 10 atom ^{15}N %; Fig. 4). The ^{15}N enrichment found in the unlabelled carcass hydrolysed in presence of $^{15}NH_3$-N would indicate that 0·006% of the total N in the sample could have been derived from the labelled NH_3. A possible explanation for this is the contamination of the isolated lysine with NH_3 since the NH_3 peak, which might be highly labelled, appears just before the lysine peak in the preparative system used (Fig. 1). Another possibility is that during hydrolysis there is chemical exchange of lysine-amino groups with NH_3. Consequently, the small ^{15}N enrichment measured in the ^{15}N-GF rats was considered to be within methodological error.

The different lysine ^{15}N enrichments found in liver, plasma and carcass were expected because of differences in protein turnover in the different tissues. The higher enrichments in tissues with higher protein turnover have been reported by several authors (Vitti & Gaebler 1963; Vitti *et al.* 1964; Deguchi & Namioka, 1989).

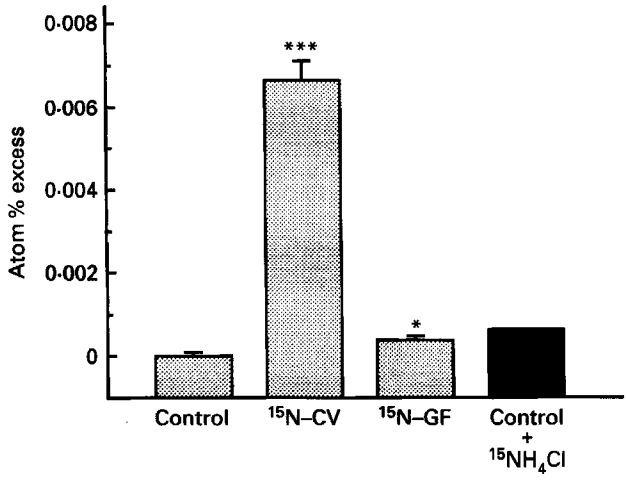

Fig. 4. ^{15}N enrichments in lysine of carcasses of conventional (^{15}N-CV) and germ-free (^{15}N-GF) rats fed on diet containing $^{15}NH_4Cl$, and of unlabelled carcasses hydrolysed with addition of $^{15}NH_4Cl$, compared with control carcasses of rats given diet containing unlabelled NH_4Cl. Values are means with their standard errors represented by vertical bars for four rats. Mean values were significantly different from those of the control group: * $P = 0{\cdot}042$, *** $P = 0{\cdot}0008$. For details of diets and experimental procedures, see Table 1 and pp. 690–694.

It was estimated that CV rats absorbed 21·3 (SE 2·04) mg microbial lysine/d per kg body weight$^{0{\cdot}75}$. This would represent two-thirds of the lysine maintenance requirements (32·2 mg/d per kg body weight$^{0{\cdot}75}$) estimated by the National Research Council (1978). This value is less than that estimated in a previous experiment (36·2 mg/d kg per body weight$^{0{\cdot}75}$; Torrallardona *et al.* 1993*a*), perhaps due to differences in diet composition. The diet in the previous experiment contained 671 g raw potato starch/kg, whereas in the present experiment there was only 350 g/kg. The higher content of raw potato starch might have resulted in an increase in the amount of microbial amino acids synthesized in the gastrointestinal tract and, therefore, an increase in the amount of microbial amino acids absorbed. This hypothesis is supported by Bergner *et al.* (1984) who observed that inclusion of partly-hydrolysed straw in the diet of pigs fed on $^{15}NH_4^+$ salts resulted in a higher incorporation of ^{15}N in both the TCA-soluble and -insoluble fractions of faeces.

It is concluded that GF rats are not able to incorporate substantial amounts (if any) of inorganic N into lysine, so all ^{15}N-labelled lysine in CV animals was of microbial origin and the model described is valid to estimate microbial lysine absorption.

Despite the absorption of amino acids of microbial origin by CV animals it did not appear that the presence of gastrointestinal microflora had a beneficial effect on growth. As shown in Table 2, the weight losses of the animals with gastrointestinal flora (CV and control) were significantly greater than those of the GF animals. However, these weight changes should be viewed cautiously. There were few animals and a large range in weight. Furthermore, body-weight comparisons between GF and CV rats are confounded by the gross caecal distension that develops in GF rodents (Coates & Gustafsson, 1984). It may be that the caeca of the CV and control rats were continuing to regress during the experiment, which would tend to increase their weight losses.

It seems that the gastrointestinal microflora confer both benefits and costs. As well as having this additional amino acid supply, CV animals have consistently smaller endogenous N losses than their GF counterparts (Salter, 1984). On the other hand, enterocyte turnover is more rapid in CV animals (Heneghan, 1984), which may result in greater energy

expenditure and the animals may mount an immune response against some of the gastrointestinal microflora, which may result in higher nutrient requirements (Klasing *et al.* 1991). Other physiological differences between GF and CV animals could also be important. However, these differences in no way affect the main conclusion of the present work, that, in rats, microbial amino acids contribute to an important extent to meeting the animals' metabolic requirements. In the following paper (Torrallardona *et al.* 1996) we consider the extent to which this process depends on coprophagy.

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