# STUDIES ON METHIONINE METABOLISM IN THE RUMEN BACTERIA OF GOATS

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## Summary

The metabolic fate of methionine in rumen bacteria was studied by intraruminal administration of ^{15}N and 1 ^{13}C labeled methionine in goats. Time course changes in isotopic abundance of amino acids in the rumen bacteria were determined with a computer-controlled gas-chromatograph mass spectrometer. The results from the transition of peak isotopic abundance in amino acids indicated that in rumen bacteria the ^{15}N or ^{13}C isotope in the methionine molecule was transferred rapidly to threonine, glycine, and subsequently to serine. It was concluded, therefore, that after incorporated into bacteria, methionine administered intraruminally may not be retained as it is, but would be converted quickly to other metabolites in the bacteria.

(**Key Words**: Goats, Rumen Bacteria, Methionine Metabolism, Isotope Abundance)

## Introduction

In ruminants, dietary supplemented methionine may not be utilized efficiently by the host animal when added to a diet due to substantial degradation by the rumen bacteria. Cottle and Velle (1989) found that in sheep the degradation rate of orally administered methionine fell somewhere between that of threonine and lysine, ranging from 70 to 88%/day depending on the dose employed. However, these estimates of methionine degradation rates might have been lower because no allowance was made for the fraction of dietary supplemented methionine that was incorporated into bacterial proteins, and subsequently reached the lower gut.

Dietary methionine is metabolized within rumen bacteria, and therefore the amount of methionine available to host ruminants may be lower than that incorporated in bacteria. Indeed, rapid conversion of methionine into metabolites within the rumen has been implicated by Doyle and Moir (1979). However, the metabolic fate of dietary methionine supplement in the rumen bacteria is not fully understood, nor is known the rate at which methionine is converted to other metabolites. The present study was conducted, therefore, to clarify the metabolic fate of methionine in rumen bacteria when given intraruminally to goats.

## Materials and Methods

### Animals

Three (experiment 1) and four (experiment 2) female Japanese Saanen goats at 15 to 17 months of age, weighing from 38 to 58 kg, were used, and were cared for under Guidelines for Animal Experimentation, established by the Committee of Experimental Animal Care, Nagoya University, Japan. They were fitted with a rumen fistula for rumen content collection. During an adaptation period of 8 days, the goats were kept individually in metabolism cages placed in a clean, air-conditioned room in which ambient temperature was kept at $21 \pm 2°C$, and fed on a diet based on ground alfalfa meal ($35 \ g/kg^{0.75}$ per day) and wheat bran ($35 \ g/kg^{0.75}$ per day). Digestible energy and crude protein intake were set as $0.184 \ Mcal/kg^{0.75}$ per day and $9.24 \ g/kg^{0.75}$ per day, respectively, to meet requirements for DE and DCP intake for goats gaining weight at 50 to 100 g/day, according to NRC (1981). Extra minerals were provided with a mineral block

(Koen, Nihon Zenyaku Co., Japan) which contained (/kg): NaCl, 955.5 g; ZnSO₄, 1,235 mg; MnCl₂, 1,146 mg; Fe₂O₃, 715 mg; CuSO₄, 377 mg; KI, 65.5 mg; CoSO₄, 6.6 mg. The daily meal was divided into 8 equal portions and given at 1:00, 4:00, 7:00, 10:00, 13:00, 16:00, 19:00, and 22:00 together with 250 ml water at each feeding time.

On day 9, one-twenty fourth of the daily feed was given every hour from 10:00 at which time either 5 mg/kg of DL-[$^{15}$N] methionine (94.6 atom % excess) (experiment 1) or 5 mg/kg of L-[1-$^{13}$C] methionine (98.9 atom % excess) (experiment 2) was administered through the rumen fistula. The reason for using different stable isotopes of methionine was to examine whether the N and 1-C molecules was metabolized to other intermediates at different rates, representing independent flux rates. If this happens, metabolic conversion of methionine should be estimated with a suitable tracer depending on objectives. Rumen contents were then sampled just before, and at 1, 2, 3, 4, 6, 8, 12, 16, 20 and 24 hours after administration of the tracer through the rumen fistula.

## Chemical analysis

The volume of the rumen content samples was measured, and a few drops of Hg₂Cl₃ were added to prevent microbial fermentation. The rumen contents were then filtered with four-ply gause to separate rumen fluid, to which 2.5 ml of 7.0 N H₂SO₄/100 ml of fluid was added to prevent the loss of ammonia. The rumen fluid was then stored at −20℃ until it was analyzed.

From the rumen fluid bacterial fraction was separated as a pellet by differential centrifugation at 1,500 × g for 10 min and at 20,000 × g for 60 min for three times. The bacterial fraction obtained from about 80 ml of the rumen fluid was hydrolyzed with 4 ml of 6 N HCl at 110℃ for 24 hours. A portion of hydrolyzed bacterial amino acids was used for measuring amino acid concentration by the method of Ishida et al. (1981) with an HPLC (LC-6A, Shimadzu Co. Ltd., Japan). The remaining hydrolyzed amino acids were derivatized to form n-trifluoroacetyl butyl (TFA) esters as described previously (Muramatsu et al., 1987a,b), and used for measurement of isotopic abundance of either [$^{15}$N] or [1-$^{13}$C] of the derivatized amino acids.

Approximately 80 ml of the bacteria-free supernatant of the rumen fluid was concentrated to 1 ml, to which 5 ml of 1% (w/v) picric acid was added to deproteinize, and centrifuged at 1,500 × g for 10 min. The supernatant was transferred onto Dowex 2 × 8 column of Cl form to remove picric acid. The eluate was evaporated to dryness, and dissolved in 2 ml of de-ionized water, and free amino acids thus obtained were converted to their TFA esters for measurement of isotopic abundance as done in hydrolyzed bacterial amino acids.

The isotopic abundance of TFA esters was measured with a seletected-ion-monitoring gas-chromatograph mass-spectrometer (QP 1000, Shimadzu Co. Ltd., Japan). After the analysis, detectable isotopic abundance was found only in glycine, methionine, serine and threonine, and therefore presentation of the results was limited to these amino acids. The $^{15}$N isotopic abundance was calculated by using relative intensity of the following mass fragment ions (m/z):glycine, 127 and 128; methionine 227 and 228; serine, 139 and 140; threonine, 153 and 154. The same combination of mass fragment ions was also used for determination of 1-$^{13}$C enrichment except for serine. The isotopic abundance of [1-$^{13}$C] serine was not analyzed because of lack of available samples for analysis.

## Statistical analysis

According to a randomized block design by taking goat as block, analysis of variance was conducted to assess the significance of sampling time effects. Significance of a difference between means at different time points was tested by Duncan's multiple range test by the GLM procedures of SAS (1988).

## Results

Changes in the time course of concentrations of methionine, threonine, glycine and serine in the rumen bacteria from experiments 1 and 2 are presented in table 1. The data were presented as pooled values of experiments 1 and 2 as inter-experimental variation was small and not significnat (p > 0.10). Between sampling times there were no significant changes (p > 0.10) in methionine, threonine, glycine or serine, indicating that bacterial amino acid composition was kept

constant over the measurement period under the present experimental conditions.

Table 2 shows the values for time course changes in $^{15}N$ isotopic abundance of free amino acids in the rumen fluid. It was clearly indicated that over the 24-hour period after the isotope administration, no detectable enrichment was observed ($p > 0.10$) except for methionine. The $^{15}N$ enrichment of methionine declined rapidly for the first 3 hours after the isotope administration ($p < 0.05$), followed by almost no detectable values.

TABLE 1. CHANGES IN THE TIME COURSE OF CONTENTS OF METHIONINE, THREONINE, GLYCINE AND SERINE IN THE RUMEN BACTERIA OF GOATS AFTER [$^{15}N$] METHIONINE ADMINISTRATION

| Time (hours) | Methionine | Threonine | Glycine | Serine |
|---|---|---|---|---|
| | | $\mu$mole/g bacteria | | |
| 1 | 63.0 | 153.3 | 259.8 | 154.5 |
| 2 | 59.3 | 155.6 | 266.7 | 155.1 |
| 3 | 51.0 | 166.3 | 267.0 | 157.3 |
| 4 | 50.5 | 158.3 | 264.3 | 160.8 |
| 6 | 52.3 | 162.3 | 270.7 | 156.1 |
| 8 | 52.4 | 162.3 | 263.5 | 162.0 |
| 12 | 63.0 | 156.0 | 271.1 | 166.9 |
| 16 | 57.5 | 158.7 | 260.9 | 155.5 |
| 20 | 53.3 | 163.8 | 279.6 | 157.6 |
| 24 | 50.3 | 156.7 | 271.3 | 165.3 |
| Pooled SEM | 5.7 | 8.3 | 11.3 | 5.2 |

DL-[$^{15}N$] methionine (94.6 atom % excess) was given through a rumen fistula at a dose of 200 mg.
No significant difference was detected between means (n=6) within a column ($p > 0.10$).

TABLE 2. CHANGES IN THE TIME COURSE OF SOTOPIC ABUNDANCE OF METHIONINE, THREONINE, GLYCINE AND SERINE IN THE RUMEN FLUID OF GOATS AFTER [$^{15}N$] METHIONINE ADMINISTRATION

| Time (hours) | Methionine | Threonine | Glycine | Serine |
|---|---|---|---|---|
| | | $^{15}N$ atom % excess | | |
| 1 | 0.37[a] | −0.00 | −0.00 | 0.02 |
| 2 | 0.33[a] | 0.01 | 0.01 | −0.02 |
| 3 | 0.23[b] | −0.00 | −0.00 | 0.00 |
| 4 | 0.03[c] | 0.01 | −0.01 | 0.02 |
| 6 | −0.01[c] | −0.00 | 0.01 | 0.00 |
| 8 | 0.00[c] | 0.01 | 0.01 | 0.01 |
| 12 | −0.00[c] | 0.01 | −0.01 | 0.01 |
| 16 | 0.01[c] | −0.00 | 0.01 | 0.00 |
| 20 | −0.00[c] | −0.00 | 0.00 | 0.00 |
| 24 | 0.00[c] | 0.01 | 0.00 | 0.02 |
| Pooled SEM | 0.02 | 0.01 | 0.01 | 0.01 |

DL-[$^{15}N$] methionine (94.6 atom % excess) was given through a rumen fistula at a dose of 200 mg.
[a,b,c] Means (n=3) not sharing a common superscript letter within a column are significantly different at $p < 0.05$.

Changes in the time course of $^{15}N$ enrichment of bacterial amino acids are given in table 3. The $^{15}N$ isotopic abundance of methionine decreased sharply for the first 3 hours ($p < 0.05$) with no

detectable enrichment afterwards. In threonine, the $^{15}N$ enrichment increased gradually ($p < 0.05$), having a peak at approximately 4 hours after the tracer administration, followed by a gradual decrease until 24 hours. In glycine, the peak isotopic abundance shifted later around 8 hours after the tracer administration, and in serine the enrichment seemed to reach a plateau value from 12 to 24 hours.

In tables 4 and 5, changes in the time course

TABLE 3. CHANGES IN THE TIME COURSE OF ISOTOPIC ABUNDANCE OF METHIONINE, THREONINE, GLYCINE AND SERINE IN THE RUMEN BACTERIA OF GOATS AFTER [$^{15}N$] METHIONINE ADMINISTRATION

| Time (hours) | Methionine | Threonine | Glycine | Serine |
|---|---|---|---|---|
| | $^{15}N$ atom % excess | | | |
| 1 | 0.89[a] | 0.05[a] | 0.07[a] | 0.00[a] |
| 2 | 0.42[a] | 0.07[ab] | 0.11[a] | −0.06[a] |
| 3 | 0.03[c] | 0.32[c] | 0.20[b] | 0.01[a] |
| 4 | −0.10[c] | 0.55[d] | 0.26[bc] | 0.10[b] |
| 6 | 0.07[c] | 0.38[c] | 0.41[ef] | 0.18[c] |
| 8 | 0.02[c] | 0.29[c] | 0.48[f] | 0.28[d] |
| 12 | −0.04[c] | 0.17[b] | 0.36[de] | 0.38[e] |
| 16 | 0.08[c] | 0.12[ab] | 0.32[cd] | 0.43[c] |
| 20 | 0.02[c] | 0.12[ab] | 0.19[b] | 0.40[e] |
| 24 | 0.07[c] | 0.06[a] | 0.09[a] | 0.40[e] |
| Pooled SEM | 0.09 | 0.03 | 0.02 | 0.02 |

DL-[$^{15}N$] methionine (94.6 atom % excess) was given through a rumen fistula at a dose of 200 mg.
[a,b,c,d,e,f] Means (n=3) not sharing a common superscript letter within a column are significantly different at $p < 0.05$.

TABLE 4. CHANGES IN THE TIME COURSE OF ISOTOPIC ABUNDANCE OF METHIONINE, THREONINE, AND GLYCINE IN THE RUMEN FLUID OF GOATS AFTER [1-$^{13}C$] METHIONINE ADMINISTRATION

| Time (hours) | Methionine | Threonine | Glycine |
|---|---|---|---|
| | [1-$^{13}C$] atom % excess | | |
| 1 | 0.40[a] | 0.02 | 0.02 |
| 2 | 0.31[a] | 0.02 | 0.02 |
| 3 | 0.21[b] | 0.02 | 0.01 |
| 4 | 0.04[c] | 0.02 | 0.02 |
| 6 | 0.00[c] | 0.02 | 0.02 |
| 8 | 0.01[c] | 0.02 | 0.02 |
| 12 | 0.02[c] | 0.02 | 0.02 |
| 16 | 0.01[c] | 0.01 | 0.01 |
| 20 | 0.01[c] | 0.01 | 0.01 |
| 24 | 0.01[c] | 0.02 | 0.02 |
| Pooled SEM | 0.01 | 0.002 | 0.002 |

L-[1-$^{13}C$] methionine (98.9 atom % excess) was given through a rumen fistula at a dose of 200 mg.
[a,b,c] Means (n=4) not sharing a common superscript letter within a column are significantly different at $p < 0.05$.

TABLE 5. CHANGES IN THE TIME COURSE OF ISOTOPIC ABUNDANCE OF METHIONINE, THREONINE, AND GLYCINE IN THE RUMEN BACTERIA OF GOATS AFTER [1-$^{13}C$] METHIONINE ADMINISTRATION

| Time (hours) | Methionine | Threonine | Glycine |
|---|---|---|---|
| | [1-$^{13}C$] atom % excess | | |
| 1 | 0.82[a] | 0.06[a] | 0.04[a] |
| 2 | 0.39[b] | 0.06[a] | 0.10[a] |
| 3 | 0.06[c] | 0.23[cd] | 0.19[bc] |
| 4 | −0.03[d] | 0.47[f] | 0.24[cd] |
| 6 | 0.03[cd] | 0.38[e] | 0.37[e] |
| 8 | 0.01[cd] | 0.29[d] | 0.44[f] |
| 12 | −0.01[cd] | 0.17[bc] | 0.37[e] |
| 16 | 0.00[cd] | 0.12[ab] | 0.28[d] |
| 20 | −0.02[d] | 0.11[ab] | 0.16[b] |
| 24 | −0.02[d] | 0.05[a] | 0.08[a] |
| Pooled SEM | 0.02 | 0.03 | 0.02 |

L-[1-$^{13}C$] methionine (98.9 atom % excess) was given through a rumen fistula at a dose of 200 mg.
[a,b,c,d,e,f] Means (n=4) not sharing a common superscript letter within a column are significantly different at $p < 0.05$.

of $^{13}C$ enrichment of free- and bacterial-methionine, threonine and glycine. Essentially the same results were observed as those found in the $^{15}N$ isotopic abundance of these amino acids, i.e. a rapid decrease in [1-$^{13}C$] methionine both in the free and bacterial fractions ($p < 0.05$), no detectable enrichment in free threonine and glycine, and transition of the peak [1-$^{13}C$] enrichment in threonine around 4 hours and in glycine around 8 hours after the isotope administration.

## Discussion

The results from tables 2 and 4 in which rapid disappearance of the labeled moiety of free methionine from the rumen fluid was shown suggested that the tracer was either incorporated into bacteria or flew out to the abomasum or both within the first few hours after the intraruminal administration of the isotope. The flow rate of free methionine from the rumen into the abomasum was not measured in the present study, and therefore the exact contribution of this to the disappearance of labeled free methionine is not known. However, the results for isotopic abundance of amino acids in the bacteria (tables 3 and 5) clearly demonstrated that once the labeled methionine was incorporated into the rumen bacteria, the $^{15}N$ or $^{13}C$ isotope in the methionine molecule was transferred rapidly to threonine, followed by glycine, and finally to serine according to the transition of peak isotopic abundance in the corresponding amino acids. From the present results and possible pathways published in the literature (Harper, 1975; Kikuchi, 1979; Yamaguchi and Ueda, 1979), expected metabolic fate of $^{15}N$ or 1-$^{13}C$ in the methionine molecule within the rumen bacteria is depicted (figure 1). In figure 1, a continuous flow of the tracers is assumed to be maintained, and metabolic conversion in boxes has been speculated from the above stated metabolic map in the literature.

The fact that after administering roughly similar doses of the $^{15}N$ and 1-$^{13}C$ tracers, approximately the same isotopic abundance was detected for both isotopes in bacterial methionine suggested that the side pathways by which $^{15}N$ but not 1-$^{13}C$ is to be released was not important in the metabolic map shown in figure 1. In this sense, metabolic conversion rates of methionine

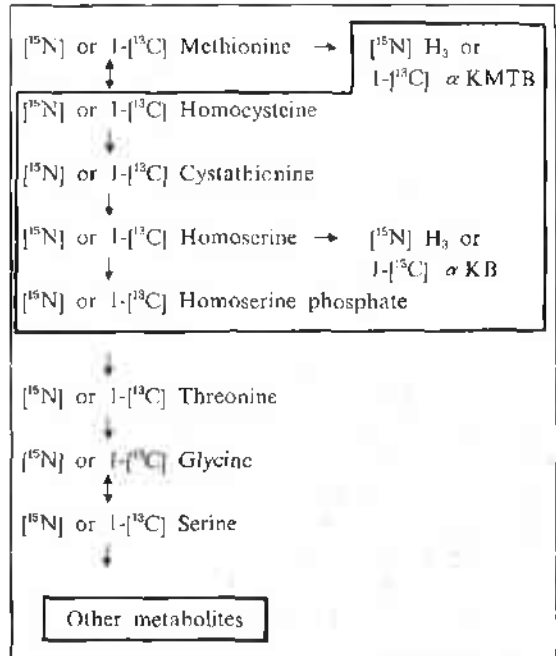in the rumen bacteria may well be represented by either the $^{15}N$ or 1-$^{13}C$ labeled tracer.



Figure 1. Metabolic fate of [$^{15}N$] of 1-[$^{13}C$] isotope in methionine molecule in the rumen bacteria when the isotope is administered intraruminally to goats. Conversion of metabolites in boxes was speculated from metabolic map (Harper, 1975; Kikuchi, 1979; Yamaguchi and Ueda, 1979). Abbreviations: α KMTB, α-ketomethylthiobutyric acid; α KB, α-ketobutyric acid.

Taken together with the rate at which methionine deamination occurs (Reis et al., 1978; Doyle and Moir, 1979; Doyle and Adams, 1980; Cottle and Velle, 1989), the present results emphasize the importance of protecting limiting amino acids from the rumen degradation. As has been repeatedly reported, protection of dietary supplemented methionine is in most cases useful for improving ruminant performance (Papas et al., 1984; Illg et al., 1987; Rogers et al., 1989; Muramatsu et al., 1989, 1991). A part of the reason for this would be that bacterial amino acid profiles in which methionine tends to be deficient are relatively constant, and even if nonprotected methionine is added to a diet, methionine would be converted rapidly to other

metabolites within the rumen bacteria once methionine is incorporated into the bacteria.

In the present study, no prescise estimation of the rate at which $^{15}N$ or 1 $^{13}C$ in methionine molecule is transferred to other amino acids has been attempted. By taking account of the following assumptions, however, rough estimation of methionine degradation rates in the present study could be given. The assumptions are as follows: (a) bacteria content in the rumen fluid is constant; (b) volume of the rumen fluid is constant; (c) amino acid concentration in the rumen bacteria is constant, (d) outflow of the rumen bacteria from the rumen into the duodenum is constant; and (e) substance transfer follows the first-order reaction kinetics. It would be reasonable to assume that the first two assumptions are held under the present experimental conditions because the daily meals with drinking water were divided into 24 equal portions, and evenly distributed within the 24-hour measurement period. Indeed, under the similar experimental conditions, no detectable changes in bacteria content in the rumen fluid was found (unpublished results). The third assumption was validated from the results shown in table 1. In a preliminary study with goats fitted with a duodenal cathether, it was found that there were no significant changes in the flow rate of the rumen fluid from the rumen into the duodenum throughout the feeding period under the present conditions, implying the validity of the fourth assumption. The last assumption cannot be easily substantiated, and in reality it may not be true, although this assumption has been frequently used to describe amino acid metabolism. The reason for the use of the first-order reaction kinetics is mostly its simplicity in many cases to explain biochemical reactions. With these assumptions, approximate estimation for the methionine flux in the rumen bacteria was attempted by computer-assisted simulation in which simultaneous differential equations were solved by numerical integration. The results suggested that almost 90% of intraruminally administered methionine in the present study may have been either deaminated or its nitrogen and carboxyl carbon transferred to other amino acids within 1 hour. This is not surprising because bacteria can grow fast and undergo mitosis to proliferate within a matter of minutes (Japanese Biochemical Society, 1979), and therefore amino acid metabolism should take place at a substantially fast rate in the rumen bacteria.

Cottle and Velle (1989) reported that in sheep the degradation rate of orally administered methionine ranged from 70 to 88%/day depending on the dose used: the higher the dose, the lower the degradation rate. However, their estimates may not be directly relevant to our observation not only because their conclusion with sheep was based on a dose of orally administered methionine as 10 times high as that employed in the present study with goats, but also because our results were exclusively related to methionine metabolism in the bacteria. A more detailed study is necessary to determine the rate of methionine degradation in the rumen to clarify an optimum dose of this amino acid when given orally to ruminants under a variety of feeding conditions.

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