
Formation of Lysine from α,ε-Diaminopimelic Acid Contained in Rumen Bacterial Cell Walls by Rumen Ciliate Protozoa

Ryoji ONODERA, Toshiharu SHINJO* and Makoto KANDATSU**

Laboratory of Nutritional Chemistry, Department of Agricultural Chemistry, Miyazaki University, Miyazaki
*Laboratory of Veterinary Bacteriology, Department of Veterinary Science, Miyazaki University, Miyazaki
**Azabu Veterinary College, Fuchinobe, Sagamihara-shi, Kanagawa

Received October 29, 1973

Cell walls containing α,ε-diaminopimelate-1,7-14C (DAP) was prepared from Escherichia coli isolated from the rumen. After incubation of ciliates with the cell walls, 22.0% of DAP contained in cell walls of E. coli was converted to lysine and pipecolate. Heat-treated mixed rumen bacteria and heat-treated cell walls of mixed rumen bacteria added to the culture medium of rumen ciliates increased 0.572 and 0.934 μmole/ml of sum of lysine and pipecolate, respectively.

From these results, it is clear that rumen ciliate protozoa can form lysine from DAP contained in the mucopeptide of bacterial cell walls. One of the nutritional significance of inhabitation of ciliates in the rumen was revealed.

In the nutrition of ruminants, it has been one of the problems whether mucopeptide of rumen bacterial cell walls have an nutritional value to the host animal or not. α,ε-Diaminopimelic acid (DAP) is known to be a constituent amino acid of mucopeptide contained in various bacterial cell walls.1) Weller et al.2) have shown that DAP nitrogen accounted for 0.57～0.64% of the total nitrogen of the mixed rumen bacteria drawn from sheep fed on a ration of wheaten hay. But the cell-wall mucopeptide of bacteria is resistant to digestion by trypsin,3) pepsin and chymotrypsin4) in vitro. Mason and White5) have revealed by in vivo experiment that the bacterial DAP-containing mucopeptide synthesized in the rumen of normal sheep were not digested in the small intestine and further that free DAP introduced into the peritoneum or jugular vein was not metabolized and excreted unchanged in the urine. Allison6) has stated that non-amino acid nitrogen and amino acid nitrogen in bacterial cell walls, which are poorly digested, is probably of little value to the host.

Rumen bacteria have, however, been known to be used to fall the prey to rumen ciliate protozoa.7)

In our previous reports, it has been proved that rumen ciliate protozoa could form a great deal of lysine from DAP8) and there was no difference of the ability to form lysine from DAP between Entodinium and Diplodinium, but they could hardly synthesize DAP from its precursors.9)

In this paper, formation of lysine from DAP contained in cell-wall mucopeptide of rumen bacteria by mixed rumen ciliate protozoa is reported.

MATERIALS AND METHODS

1. Isolation and identification of Escherichia coli from the rumen. One milliliter of rumen contents collected through the rumen fistula of a goat (female, body wt.: 42 kg), which was maintained on daily rations consisting of 3～5 kg of fresh grass and 200 g of mixed concentrate feed, was serially diluted with the diluent (A).10) Dilutions 10⁻¹ to 10⁻⁴ inclusive were plated out on the selected medium, McConkey (Nissan). After incubation at 37°C for 24 hr, lactose-fermenting colonies (red) were found in the media inoculated with dilution 10⁻¹ and 10⁻². Concentration of E. coli in the rumen of the goat was estimated to be 4.0 × 10⁹/ml of rumen contents.

The isolates were clearly identified as E. coli by

† Lysine and Pipecolic Acid Metabolism in the Rumen. Part III. See Refs. 9) and 14).
the methods of Cowan and Steel.11)

2. Cultural methods to obtain cell walls of E. coli labelled with DAP-1,7-14C. The colony of E. coli isolated on nutrient agar was inoculated into Tryptosoy broth (Eiken) and incubated at 37°C for 20 hr. A 6 ml of the cultures was inoculated into 200 ml of Tryptosoy broth containing 10 μCi of DAP-1,7-14C and 5 μmole/ml of L-lysine to inhibit to produce radioactive lysine from DAP-1,7-14C added and incubated at 40°C for 3 days after covering the cotton plug with 14CO2-recovering system.

3. Collection of cell walls
   a) In the case of E. coli. After incubation for 3 days and centrifugation of the medium at 9000~g for 20 min, precipitate was washed 3 times with 30 ml of 0.03 M Tris-HCl (pH 7.5) and broken down in 40 ml of the same Tris-HCl below 7°C for 90 min by Sonicator Oscillation Apparatus (Model: T-A-4201, Kaijo Denki Co., Ltd.). After centrifugation at 3000~g for 20 min, supernatant fluid was removed and centrifuged again at 27,000~g for 30 min. The precipitate was washed 5 times with 0.03 M Tris-HCl (pH 7.5) in order to obtain “Standard Membrane” described by Muñoz et al.12)

4. Isolation of rumen ciliate protozoa.
   Rumen ciliates were obtained by a method based on those described in previous paper14) from a goat (female, body wt.: 45 kg) maintained of daily rations consisting of 3~5 kg of fresh grass, 300 g of starch pulp and 200 g of mixed concentrate feed.

5. Cultural methods of ciliates and treatment of samples
   a) In the case of the addition of cell walls of E. coli labelled with DAP-1,7-14C. A 0.4 ml sample of wet living ciliates was suspended in heat-treated cell-wall suspension prepared with 12 ml of B-9 buffer solution described in 3 a). A 4 ml sample of the ciliate suspension thus obtained was removed as a sample before incubation. A 10 μl sample of the suspension was transferred into the vial to determine the total radioactivity before incubation. One milliliter of the ciliate suspension was supplied for determination of cell-wall nitrogen and protozoan nitrogen. Finally another 5 ml of the ciliate suspension was transferred into the Erlenmeyer flask to which 400 μg of dihydrostreptomycin sulfate was previously added. After incubation at 40°C for 6 hr, 4 ml of the medium was removed as a sample after incubation. After 4 ml samples before and after incubation were centrifuged at 120~g for 30 sec, 3 ml of the supernatant fluids containing cell walls were hydrolysed in 6 N HCl at 110°C for 20 hr in sealed tubes, desalted by ‘Amberlite’ CG-120 resin column (H form) and supplied as samples for paper chromatography. Precipitates (ciliates) were washed 5 times with B-9 buffer solution, hydrolysed in 6 N HCl at 110°C for 20 hr in sealed tubes and supplied as samples for paper chromatography.

   b) In the case of rumen mixed bacteria. Rumen contents (about 500 g) obtained from the same goat as mentioned above was filtrated with four layers of gauze and the filtrate was incubated in a separating funnel at 40°C for 60 min. Feed debris floated upwards during incubation. Thereafter, liquid layer was collected and centrifuged at 120×g for 5 min. After the supernatant fluid (200 ml) was centrifuged again at 27,000×g for 30 min, precipitate which almost consisted of bacteria was treated in the same way as in the case of E. coli.

---

**TABLE I. INCORPORATION OF DAP-1,7-14C INTO THE CELLS OF E. coli AFTER INCUBATION AT 40°C FOR 3 DAYS**

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Radioactivitya) (cpm/ml)</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before incubation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>462,000</td>
<td>100</td>
</tr>
<tr>
<td>After incubation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cells</td>
<td>120,800</td>
<td>26.1</td>
</tr>
<tr>
<td>Cell walls</td>
<td>54,600</td>
<td>11.8</td>
</tr>
<tr>
<td>DAP in cell-wall hydrolysate</td>
<td>51,870</td>
<td>11.2</td>
</tr>
</tbody>
</table>

a) Radioactivity was determined by a GM counter.
6. Analytical methods. Paper chromatography was carried out by using (1) n-BuOH: AcOH: H₂O (4:1:2) and (2) Phenol: H₂O (4:1) as solvents for two dimensions.

Autoradiogram was made by exposing X-ray film (25.4 cm × 30.5 cm, Fuji Photo Film Co., Ltd., Japan) to the paper chromatogram for 10 days in case of supernatant suspension hydrolysate and for 30 days in the case of protozoan hydrolysate.

Radioactivities of amino acids were determined after cutting off the spots of amino acids on the paper chromatograms (detected through their autoradiograms) and extracting them with water.

Radioactivity was determined by a GM counter (Aloka Handy Scaler, Model: TDC-101, Japan Radiation & Medical Electronics, Inc.) in the case of cultivation of E. coli, and by a Liquid Scintillation Spectrometer (Model: 3320, Packard) using 2,5-diphenyloxazole (4 g/liter) and 1,4-bis-(5-phenyloxazolyl)-benzene (0.1 g/liter) as fluorescent substances dissolved in mixed solvents consisting of toluene, ethyl cellosolve and dioxane (1:1:1) and of 10% (w/v) of naphthalene in the case of the incubation of ciliates.

Determination of lysine and pipecolic acid were carried out by Amino Acid Autoanalyzer (Model: AA-100, Sibata Chemical APP. MFG. Co., Ltd., Japan) and the authors' method, respectively.

7. Chemicals. α,β-Diaminopimelic acid-1,7-14C was supplied by International Chemical & Nuclear Corp., California, U.S.A.

RESULTS AND DISCUSSION

The tetrapeptide sequence, L-Ala-D-Glu-L-Lys (or meso-DAP)-D-Ala, in the mucopeptide has now been shown to occur in a wide variety of Gram-positive and Gram-negative bacteria. Although the dibasic amino acid in the tetrapeptide sequence is commonly L-lysine or meso-DAP, these may be replaced by other substances such as ornithine, 2,6-diamino-3-hydroxy-pimelic acid or 2,4-diamino butyric acid in some kinds of bacteria. E. coli is one of the DAP-containing bacteria and is known to be inhabiting the rumen though not to be dominant. And we tried to isolate E. coli from the rumen of a goat and to cultivate it with L-aspartic acid-U-14C in order to obtain cell walls containing radioactive DAP. Although we could obtain E. coli from the rumen, we failed to obtain radio-

active DAP-containing cell walls enough to prove the formation of lysine from them by rumen ciliates after incubation of E. coli with 20 μCi of L-aspartic acid-U-14C. After being tried to cultivate E. coli with 10 μCi of DAP-1,7-14C in the medium containing 5 μmoles/ml of lysine, radioactive DAP-containing cell walls enough to test the formation of lysine were obtained (Table I).

Mixed rumen ciliate protozoa were given the cell walls thus obtained and incubated at 40°C for 6 hr. Before incubation, radioactivities of DAP and lysine contained in the hydrolysate of the supernatant suspension of cell walls of the medium after centrifugation at 120 × g for 30 sec to remove ciliates were 95.0 and 2.1% of the total radioactivity contained in the cell walls, respectively (Table II). Autoradiogram of amino acids contained in the hydrolysate of the supernatant suspension of cell walls revealed also a strong spot of DAP and a trace of lysine (Fig. 1). After incubation, the spots of DAP, lysine and pipecolic acid were found in the autoradiogram of amino acids contained in the hydrolysate of the supernatant suspension of cell walls (Fig. 2). This picture shows that formation of lysine from DAP contained in the cell walls of E. coli was brought about not by bacteria but by ciliates, because pipecolic acid has been known to be the principal metabolite of lysine in rumen ciliates, while cadaverine and α-aminovaleric acid have been known to be the principal metabolites of lysine in rumen bacteria. Ciliate hydrolysate contained only lysine as a radioactive spot. Table II shows that 78.5% of DAP remained unchanged in the hydrolysate of the supernatant suspension and 22.0% of DAP contained in cell walls of E. coli was converted to lysine and pipecolic acid. Percentage of pipecolic acid to the amount of total lysine containing pipecolic acid produced in this experiment (27.8%) was a little higher than that produced from free DAP-1,7-14C in the previous experiment (9%). This may probably be due to the existence of radioactive lysine in cell walls before incubation. After incubation of cell walls
TABLE II. FORMATION OF LYSINE FROM DAP-1,7-14C CONTAINED IN CELL WALLS OF E. coli BY RUMEN CILIATES

Ciliates were incubated at 40°C for 6 hr.

Composition of ciliates in this expt.

*Entodiniinae, Diplodiniinae*

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Radioactivity detd. (cpm/ml)</th>
<th>Radioactivity calc'd. a) (cpm/ml)</th>
<th>Percentage b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before incubation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>70,900</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>DAP c)</td>
<td>67,382</td>
<td>67,382</td>
<td>100</td>
</tr>
<tr>
<td>Lysine c)</td>
<td>1,512</td>
<td>3,024</td>
<td>—</td>
</tr>
<tr>
<td>After incubation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DAP c)</td>
<td>52,900</td>
<td>52,900</td>
<td>78.5</td>
</tr>
<tr>
<td>Lysine c)</td>
<td>5,350</td>
<td>10,700</td>
<td>—</td>
</tr>
<tr>
<td>Pipolic acid c)</td>
<td>2,061</td>
<td>4,122</td>
<td>—</td>
</tr>
<tr>
<td>Lysine in ciliates</td>
<td>1,518</td>
<td>3,036</td>
<td>—</td>
</tr>
<tr>
<td>Total lysine formed d)</td>
<td>—</td>
<td>14,834</td>
<td>22.0</td>
</tr>
</tbody>
</table>

a) Values calculated so as to be equivalent to DAP-1,7-14C.
b) Percentage of the radioactivity a) of DAP c) and total lysine formed d) after incubation to that of DAP c) before incubation.
c) These fractions are those found in hydrolysates of supernatant suspensions contg. cell walls after centrifugation of the medium at 120 × g for 30 sec.
d) Difference between sum of the radioactivity a) of lysine c), pipolic acid c) and lysine in ciliates after incubation and that of lysine c) before incubation.

---

**Fig. 1.** The Autoradiogram of Amino Acids Found in the Hydrolysate of the Supernatant Suspension Containing Cell Walls of E. coli Labelled with DAP-1,7-14C after Centrifugation of the Culture Medium at 120 × g for 30 sec before Incubation.

**Fig. 2.** The Autoradiogram of Amino Acids Found in the Hydrolysate of the Supernatant Suspension Containing Cell Walls of E. coli Labelled with DAP-1,7-14C after Centrifugation of the Culture Medium at 120 × g for 30 sec after Incubation.
formation of lysine from DAP in bacterial cell walls by rumen ciliates

Without ciliates, DAP and a trace of lysine in the cell walls remained unchanged.

From these results, it appears clear that rumen ciliate protozoa can digest the mucopeptide of the cell walls of *E. coli* which is thought to be resistant to the digestive enzymes of the host animal and form lysine from DAP contained in the cell walls.

Next, increase of total lysine and pipecolic acid in the medium was determined after incubation of ciliates with heat-treated rumen bacteria or their cell walls at 40°C for 6 hr. Addition of heat-treated rumen bacteria increased 0.351 and 0.221 μmole/ml of lysine and pipecolic acid in the medium, respectively (Table III). Lysine must be produced from DAP contained in the rumen bacteria, and pipecolic acid must be formed from lysine and/or DAP in the rumen bacteria and from lysine in the ciliates. Therefore, heat-treated rumen bacteria added to the medium must have had at least more than 0.351 μmole/ml of DAP (66.8 μgDAP/ml). The culture medium contained 1.075 mg of bacterial nitrogen/ml. So it will be estimated that heat-treated rumen bacteria contained more than 62.1 mgDAP/g of bacterial nitrogen. This value seems to be higher than that proposed by Weller et al. (38.7–43.5 mgDAP/g of bacterial nitrogen). Such difference of DAP-content may arise from the difference of purity of rumen bacteria depending on how to obtain them, sorts of bacteria or further treatment (such as boiling). The amount of lysine in the control medium without heat-treated rumen bacteria decreased and pipecolic acid increased after incubation.

In the case of the addition of heat-treated cell walls of rumen bacteria, the amount of lysine produced was higher than that in the case of the addition of heat-treated rumen bacteria. These data will support a hypothesis that rumen ciliate protozoa can form lysine from DAP contained in cell walls of rumen bacteria.

From all of these results, it is thought that if ciliates inhabit the rumen, cell walls of rumen bacteria are rather available to the host animal, because they will become the source of lysine. It is also assumed that if lysine is contained in the tetrapeptide sequence of mucopeptide instead of DAP, it may be released as well as the case of DAP by digestion of mucopeptide by rumen ciliates and used for their nutrition.

One of the nutritional significance of inhabitation of ciliate protozoa in the rumen might be revealed.

In these experiment, isolation and identification of *E. coli* from the rumen was done by Dr. Toshiharu Shinjo.

Acknowledgement. The authors are grateful to Dr. Hiromitsu Ootsuka and Dr. Katsumi Hamana, Miyazaki University, for inserting permanent rumen fistula into goats, and to Dr. Michio Miura, Miyazaki University, for giving us facilities to use an Amino Acid Autoanalyzer.

This work was supported by Radioisotope Laboratory of Miyazaki University.

REFERENCES

6) M. J. Allison, "Physiology of Digestion and


