Ureolytic Activity of the Washed Cell Suspension of Rumen Ciliate Protozoa

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Ureolytic activity of rumen ciliate protozoa was examined in comparison with that of rumen bacteria. Freshly obtained rumen ciliates which were washed five times with B-9 buffer solution previously, decomposed 1.3 μ mole/ml of urea to give ammonia after incubation for 6 hr in the buffer solution containing streptomycin, while rumen bacterial suspension decomposed 84.6~97.8 μ mole/ml of urea after incubation for 2 hr. The amount of ammonia-N/bacteria-N produced from urea by the suspension of rumen bacteria was usually higher than that by the coexistent suspension consisting of rumen bacteria and ciliates. When the freshly obtained rumen ciliates were previously incubated in the buffer solution containing 100 μ g/ml each of streptomycin, penicillin and chloramphenicol (starved ciliates), ureolytic activity of the ciliates decreased with the incubation time and reached zero after 24 hr of incubation. 14 C-Urea remained unchanged in the medium of the starved ciliates even after incubation for 6 hr. All these results led us to the conclusion that rumen ciliate protozoa did not have ureolytic activity and that ureolytic activity of the freshly obtained ciliates might be due to contaminating bacteria.

Whether rumen ciliate protozoa decompose urea, which is known to be used as a nitrogen source for ruminants, may be one of the problems to be dissolved.

Formerly, Kikuchi *et al.*¹⁾ have shown the urease activity of washed cell suspension of rumen ciliate protozoa. They did not use any antibiotics, however, to inhibit the bacterial action contaminating the ciliate suspension. Abou Akkada and Howard²⁾ have reported later that *Entodinium caudatum*, previously starved in buffer-chloramphenicol solution for 36 hr, did not produce ammonia from urea during 6 hr of incubation in the presence of chloramphenicol. Kandatsu and Takahashi³⁾ have reached the conclusion similar to that of Abou Akkada and Howard²⁾ by incubating the mixed rumen ciliates, which were not starved in advance, in the absence of antibiotic for 2 hr. Naga and El-Shazly⁴⁾ have similarly denied the ureolytic activity of *Eudiplodinium medium* which was not starved previously but incubated with chloramphenicol. Lately, Onodera *et al.*⁵⁾ have shown that L-arginine- U - 14 C was degraded to give radioactive ornithine together with a large amount of 14 CO₂ by washed suspension of freshly obtained mixed rumen ciliates after incubation with streptomycin for 6 hr and have suggested that 14 CO₂ might be produced from radioactive urea liberated from arginine added.

These results could not lead us to the clear conclusion that rumen ciliates did not have ureolytic activity, because the experimental conditions in which they^{2~4)} denied the ureolytic activity in the ciliates were different. Furthermore, there was a disagreement between the results of Naga and El-Shazly⁴⁾ and those of Onodera *et al.*⁵⁾ in spite of their almost similar experimental conditions using not starved but only washed protozoa in the presence of antibiotics.

Considering these problems, the aim of the present study is to reinvestigate and clarify whether or not rumen ciliates have an ureolytic activity.

MATERIALS AND METHODS

1. Isolation of rumen ciliate protozoa

Mixed rumen ciliates were obtained by the method

based on those described in our previous paper⁹⁾ from a goat (female, body wt.: 42 kg) maintained on daily rations consisting of 2~4 kg of fresh grass and 200 g of mixed concentrates. Ophryoscolecoid ciliates (expressed as *Ophryoscolecidae* in Table III) were obtained after incubation of the mixed ciliates in 1.2% D-mannose solution⁷⁾ (partially modified B-9 buffer solution⁸⁾) at 39°C for 5 hr to kill isotrichid ciliates. Mixed ciliates (II) shown in Table III were obtained after incubation at 39°C for 5 hr in B-9 buffer solution alone and washing five times with the buffer solution.

2. Preparation of mixed rumen bacterial suspension

Mixed rumen bacterial suspension was prepared by the centrifugal method described in the previous paper⁹⁾ from the rumen contents of the same goat as mentioned above.

3. Preparation of "coexistent system" consisting of mixed rumen bacteria and mixed rumen ciliates

Rumen contents obtained through the rumen fistula of the goat mentioned above were strained by four layers of surgical gauze and the filtrate was incubated in the separating funnel at 39°C for about 60 min after replacing the air phase of the funnel with nitrogen gas. Fine food debris floated to the upper phase of the filtrate during incubation. The lower liquid phase which was chiefly consisting of bacteria and ciliate protozoa with probably a small number of other microorganisms such as fungi^{10,11)} was used as "coexistent system."

4. Preparation of rumen fluid

The lower liquid phase just mentioned in 3. was centrifuged at $27,000 \times g$ for 30 min and the supernatant fluid was supplied for the test as rumen fluid.

5. Cultural methods and treatment of samples

1) In the case of non-radioactive urea added to the medium

a) *Ciliate suspension and their homogenate.* Ten ml of ciliate suspension (ciliate concentration: 4% (v/v) in B-9 buffer solution⁸⁾) or 10 ml of homogenate of ciliates, which were obtained after breaking down one ml of the living ciliates in 24 ml of B-9 buffer solution below 7°C for 20 min by an Ultrasonic Generator (Model: T-A-4201, Kaijo Denki Co., Ltd., Japan), were incubated with or without 10 μ mole/ml of urea and with 100 μ g/ml of streptomycin sulfate alone or with 100 μ g/ml each of the mixture of streptomycin sulfate, penicillin G potassium and chloramphenicol sodium succinate at 39°C for 3 or 6 hr. Before and after incubation, 0.5 ml of the medium was immediately taken out with stirring and supplied for determination of ammonia produced.

b) *Bacterial suspension and coexistent system.* Ten ml of each suspension were incubated with or without 100 μ mole/ml of urea except for the case of Table I, in which case 10 μ mole/ml of urea were added, at 39°C for indicated hours in each Table. Before and after incubation, 0.1 ml of the medium was sampled with stirring and submitted to the determination of ammonia.

2) In the case of ¹⁴C-urea added to the medium

In this case, were used starved ciliates which were obtained after incubation of 2 ml of freshly isolated mixed rumen ciliates in 50 ml of B-9 buffer solution containing 100 μ g/ml each of streptomycin sulfate, penicillin G potassium and chloramphenicol sodium succinate at 39°C for 24 hr. The same amount of each of the three kinds of antibiotics was added again to the medium after 12 hr of incubation. Eight ml of the suspension of the starved rumen ciliates in B-9 buffer solution were transferred to 30 ml of conical flask, to which 80 μ mole/ml of ¹²C-urea, 10 μ Ci of ¹⁴C-urea and the mixture of the three kinds of antibiotics mentioned above (800 μ g each) were previously added, and shaken for a few minutes to dissolve them. Four ml of the suspension containing urea and antibiotics were immediately transferred into a Warburg flask with a center well to which about 0.7 ml of 20% KOH was added previously to trap carbon dioxide. As soon as the air phase of the Warburg flask was replaced with nitrogen gas, the flask was sealed and incubated at 39°C for 6 hr. After incubation, microbial activity was stopped by adding one ml of 50% trichloroacetic acid (TCA) into the medium and the flask was left overnight at room temperature. The remaining starved ciliate suspension (4 ml) was similarly transferred into another Warburg flask as completely as possible and immediately subjected to addition of one ml of 50% TCA to make the zero-time control. Five μ l of sample were taken out for the determination of total radioactivity.

Radioactivities in carbon dioxide, ciliates and some components (shown in Table IV) of the supernatant medium were determined by the same manners as those described in 2. a) of the MATERIALS AND METHODS of the previous paper⁹⁾ with some modifications that centrifugation to get ciliate fraction was carried out at $120 \times g$ for 30 sec in this case instead of $27,000 \times g$ for 30 min in the previous case⁹⁾ and that the desalting of the water-soluble fraction of the supernatant fluid was made as follows: The water-soluble fraction after extraction with ether under alkaline condition was concentrated to about 5 ml and desalted at first by percolation through a bed (2 cm $\times$ 6 cm) of 'Amberlite' CG-120 (H form) resin. After washing the bed with about 1000 ml of water, the washings were concentrated *in vacuo* to about 50 ml, poured again into an another column (2.5 cm $\times$ 15 cm) of

'Amberlite' CG-400 (OH form) resin and washed with about 1500 ml of water. The washings thus obtained were evaporated and used for the determination of radioactivity and for paper chromatography. The fraction containing amino acids adsorbed in the bed of 'Amberlite' CG-120 resin was eluted with 300 ml of 2 N NH_4OH . The eluate (Eluate-120) was taken to dryness and used for the determination of radioactivity. The substances adsorbed on 'Amberlite' CG-400 resin were eluted with 500 ml of 2 N HCl. The eluate (Eluate-400) was concentrated to 10 ml and radioactivity was determined.

6. Analytical method

Urease activity was determined by the amount of ammonia-N liberated from urea by rumen microorganisms and expressed as a molar amount of decomposed urea.

Ammonia-N was determined by Conway's micro-diffusion method.¹²⁾

Bacterial nitrogen was determined by micro Kjeldahl method after treating the samples as follows; 1). Bacterial nitrogen of the mixed rumen bacterial suspension: Firstly 1 ml of the mixed rumen bacterial suspension mentioned in 2. was supplied for wet ashing and the nitrogen content (A) was determined. Secondly about 5 ml of the same suspension were centrifuged at $27,000 \times g$ for 30 min and one ml of the supernatant fluid was taken out to determine the nitrogen content (B). The difference between A and B was conveniently thought as the bacterial nitrogen. 2). Bacterial nitrogen of the "coexistent system": About 10 ml of the microbial suspension of the "coexistent system" shown in 3. were centrifuged at $120 \times g$ for 30 sec to remove ciliate protozoa from the suspension and 1 ml of the supernatant fluid was supplied for determination of nitrogen content (A). The remaining supernatant fluid which still contained microorganisms such as bacteria was centrifuged again at $27,000 \times g$ for 30 min and 1 ml of the supernatant fluid was taken out to determine the nitrogen content (B). The difference between A and B was conveniently thought as the bacterial nitrogen of the "coexistent system."

Paper chromatography, autoradiography and the determination of radioactivity were carried out by quite the same methods as those of our previous paper.⁹⁾

7. Chemicals

Urea was supplied by Wako Pure Chemical Industries, Ltd. Streptomycin sulfate, penicillin G potassium and chloramphenicol sodium succinate were supplied by Meiji Seika Kaisha, Ltd., Banyu Pharmaceutical Co., Ltd., and Sankyo Co., Ltd., respectively. ^{14}C -Urea (Sp. Act.: 50.0 mCi/mm) was supplied by

Daiichi Pure Chemicals Co., Ltd.

RESULTS AND DISCUSSION

At the beginning, ureolytic activity of the washed cell suspension of freshly obtained rumen ciliate protozoa was examined in the medium containing 10 $\mu\text{mole/ml}$ of urea and 100 $\mu\text{g/ml}$ of streptomycin sulfate. After incubation for 6 hr, 13% of added urea were decomposed to give ammonia by the ciliates (Table I). Results of trials over five times showed almost similar activity. Thereafter, ureolytic activity of ciliates was compared with those of bacteria, coexistent system of bacteria and ciliates and rumen fluid alone. As shown in Table I, rumen fluid was shown to be slightly ureolytic, while rumen bacterial suspension and coexistent system decomposed almost all urea added.

TABLE I. UREOLYTIC ACTIVITIES OF RUMEN MICROORGANISMS AND RUMEN FLUID

These values were obtained after incubation with 10 $\mu\text{mole/ml}$ of urea for 6 hr.

Incubation system	Activity Decompd. urea $\mu\text{mole/ml}$
Ciliates ^{a)}	1.3
Bacteria ^{b)}	10.5
Coexistence ^{c)}	9.1
Rumen fluid ^{d)}	0.06

Ciliate composition in these expt.

Entodiniinae, *Diplodiniinae*, *Isotricha*, *Dasytricha*

a)	162	13	5	$25 \times 10^8/\text{ml}$
c)	1104	15	3	34 "

b) The word 'Bacteria' used here means rumen microorganisms except for ciliate protozoa.

c) Coexistence: Coexistent system of ciliates and bacteria.

d) Supernatant fluid after centrifugation of rumen juice at $27,000 \times g$ for 30 min.

As it was impossible to compare the difference of ureolytic activities between ciliates and bacteria from these results, ureolytic activity of the bacterial suspension was further examined by increasing the amount of urea to be added to 100 $\mu\text{mole/ml}$. From the results shown in Table II, most of all urea added

TABLE II. UREOLYTIC ACTIVITIES OF RUMEN MICROORGANISMS

These values were obtained after incubation with 100 μ mole/ml of urea.

Incubation time, hr		0.5		1		2		3	
Decompd. urea		μ mole/ ml	μ mole/ mgN ^{a)}	μ mole/ ml	μ mole/ mgN ^{a)}	μ mole/ ml	μ mole/ mgN ^{a)}	μ mole/ ml	μ mole/ mgN ^{a)}
Incubation system									
Bacteria	1	34.6	54.9	54.8	87.0	84.6	134.3	94.4	149.8
	2	—	—	—	—	97.8	128.9	—	—
Coexistence ^{b)}	1	—	—	—	—	77.8	89.6	—	—
	2	—	—	—	—	96.9	100.9	—	—

^{a)} Bacterial nitrogen.^{b)} Coexistent system of ciliates and bacteria.

Ciliate composition in these expt.

	<i>Entodiniinae</i> ,	<i>Diplodiniinae</i> ,	<i>Isotricha</i> ,	<i>Dasytricha</i>	
^{b)} 1	1000	6	3	13	$\times 10^3$ /ml
^{b)} 2	920	3	1	2	$\times 10^3$ /ml

was known to be decomposed during incubation for 3 hr by the rumen bacteria. These values, therefore, made us presume at first that ureolytic activity of rumen bacteria might be much larger than that of rumen ciliates, even if the ciliates really had urease. By the way, it is interesting that the amount of urea decomposed by bacteria usually showed higher value than that by coexistent system of bacteria and ciliates. In this regard, it was assumed that rumen ciliates might produce some valuable compounds with which rumen bacteria could synthesize amino acids and further their body protein by using ammonia liberated from urea and/or that rumen ciliates might be able to utilize ammonia for the synthesis of some amino acids such as citrulline, arginine, glutamine and so on.

Then ureolytic activity of rumen ciliates was further examined. It was firstly tested whether or not there were differences of ureolytic activities between mixed ciliates and ophryoscolecoid ciliates. In this test, mixed ciliates, which had previously been incubated in B-9 buffer solution for 5 hr and washed five times with the buffer solution (starved ciliates), were used simultaneously as well as the freshly obtained ciliates. From the results of Table III, though there apparently seemed to be difference between freshly obtained ciliates and ophryoscolecoid ciliates, no true difference was presumed to exist between the

two, because the value for starved ciliates was almost equal to that for ophryoscolecoid ciliates. Ureolytic activity of homogenate of ciliates was 3.5~4.6 fold higher than that of intact cells, but there was no difference between the activity of homogenate of starved ciliates and that of ophryoscolecoid ciliates, in agreement with the result of intact cells. In this experiment, it seemed to be rather strange that the ureolytic activity of the starved ciliates (both mixed and ophryoscolecoid ciliates) fell off to the level less than a half of that of freshly obtained ciliates in the cases of not only intact cells but also homogenates. These results made us infer that ureolytic activity of rumen ciliates which was apparently seemed to be of ciliates themselves might be of mingled rumen bacteria which might probably survive outside and/or inside ciliate bodies in spite of five-times-washing of the ciliates, because the ureolytic activity of rumen bacteria was much higher beyond comparison (Table II), in contrast to their activity of metabolism of amino acids such as lysine.⁹⁾

In the next experiment, the relation of ureolytic activity of rumen ciliates to starved time was examined and the result was shown in Fig. 1. Ureolytic activity of the ciliates declined with increasing of starved time and reached zero after 24 hr of incubation though the ciliates were microscopically confirmed to be still surviving and retaining their motilities.

TABLE III. UREOLYTIC ACTIVITIES OF RUMEN CILIATES AND THEIR HOMOGENATES

These values were obtained after incubation with 10 μ mole/ml of urea for 6 hr.

Kinds of ciliates	Decompd. urea	Intact cells μ mole/ml	Homogenates μ mole/ml
Mixed ciliates (I) ^{a)}		2.0	9.1
Mixed ciliates(II) ^{b)}		0.9	3.5
<i>Ophryoscolecidae</i> ^{c)}		1.0	3.4

^{a)} Washed rumen ciliates freshly obtained were immediately used.

^{b)} Mixed ciliates after incubation for 5 hr in B-9 buffer solution with streptomycin sulfate and washing (starved ciliates) were used.

^{c)} *Ophryoscolecidae* were obtained after incubation for 5 hr in 1.2% mannose solution to kill *Isotrichidae*.

Ciliate composition in these expt.

Entodiniinae, *Diplodiniinae*, *Dasytricha*

^{a)} 486 23 8 $\times 10^3$ /ml

^{b)} 468 25 9 "

^{c)} 471 28 0 "

FIG. 1. Relation between the Ureolytic Activity of Rumen Ciliates and the Starved Time.

of incubation. This assumption has, in fact, been demonstrated and the results will be published later.¹³⁾

To certify the fact that rumen ciliates have

TABLE IV. FATE OF ¹⁴C-UREA IN THE MEDIUM OF STARVED RUMEN CILIATES

Incubation time, hr	0		6	
	Radioactivity (cpm/ml)	Percentage (%)	Radioactivity (cpm/ml)	Percentage (%)
Before incubation				
Total	2,268,590	100	2,268,590	100
After incubation				
Carbon dioxide	9,510	0.4	10,103	0.5
EF1 ^{a)}	19,398	0.9	24,476	1.1
EF2 ^{b)}	17,362	0.8	20,493	0.9
Washings ^{c)}	2,188,590	96.5	2,182,187	96.2
Urea in washings ^{c)}	2,157,870	95.1	2,158,500	95.1
X ₁ in washings ^{c)}	24,500	1.1	23,450	1.0
Eluate-120 ^{d)}	22,656	1.0	23,906	1.1
Ciliates	1,148	0.05	1,113	0.05

^{a)} EF1: Ether-soluble fraction under an acidic condition.

^{b)} EF2: Ether-soluble fraction under an alkaline condition.

^{c)} Washings: Water effluent at desalting by anion-exchange resin of the first water effluent at desalting of the supernatant fluid of the medium by cation-exchange resin.

^{d)} Supernatant fluid of the medium desalted by cation-exchange resin.

Composition of ciliates in this expt.

Entodiniinae, *Diplodiniinae*, *Dasytricha*
1050 1 2 $\times 10^3$ /ml

From this fact, rumen ciliates seemed to have really no ureolytic activity and the mingled bacteria were assumed to be completely killed and/or digested by the ciliates during 24 hr

no ureolytic activity, mixed ciliates starved for 24 hr were incubated with ¹⁴C-urea for 6 hr. As shown in Table IV, there was no difference of radioactivity of each fraction between

before and after incubation. Most of the radioactivity was found in washings *i.e.* final water effluent at desalting by anion-exchange resin of the first water effluent at desalting of the supernatant fluid of the medium by cation-exchange resin (Table IV) and almost all radioactivity of this fraction was revealed to occur in urea by paper chromatography and autoradiography (Fig. 2). In this regard,

FIG. 2. Autoradiogram of the Paper Chromatogram of Washings (Shown in Table IV) at Desalting of the Supernatant Fluid of the Medium after Incubation of Starved Ciliates with ^{14}C -Urea for 6 hr.

urea had been known not to adsorb on both cation- and anion-exchange resins by our preliminary experiment. The spot of urea on the paper chromatogram was detected as cleaner white one compared with surroundings after spraying of ninhydrin solution and heating at $80\sim 100^\circ\text{C}$ for 15 min. The autoradiogram of the paper chromatogram of the washings after incubation showed an unidentified spot (X_1) besides the spot of urea. But the spot (X_1) was also found on that before incubation, so the spot (X_1) was not thought to be formed from urea by rumen ciliates. Radioactivities of Eluate-120 and of ciliates were too weak to make a spot in autoradiogram. Thus most of all urea was revealed to remain unchanged in the medium of starved rumen ciliates even after incubation.

All these results led us to the conclusion that rumen ciliate protozoa did not have

ureolytic activity. In connexion with the result of Onodera *et al.*⁵⁾ that a large amount of radioactive carbon dioxide had been produced from L-arginine- $\text{U-}^{14}\text{C}$ with ornithine by freshly obtained mixed rumen ciliates, it was presumed that radioactive carbon dioxide might be produced by the rumen bacteria mingled in the ciliate suspension from radioactive urea which might be formed from L-arginine- $\text{U-}^{14}\text{C}$ by probably rumen ciliates because our unpublished data that will be published in the near future revealed an intensive arginase activity of starved (24 hr) rumen ciliates.

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