The Metabolism of Branched-Chain Amino Acids by Starved Rumen Protozoa

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Abstract Regarding essential amino acids (for rat), little have been known about the metabolism of branched-chain amino acids (BCAA), phenylalanine and histidine by rumen ciliate protozoa. Thus isotopic tracer experiments were conducted in this study to examine the metabolism of BCAA such as L-leucine, L-isoleucine and L-valine using a starved mixed rumen protozoal suspension. The starved rumen protozoal suspension prepared from goat rumen contents was incubated in a buffer solution (MB9) containing ^{14}C -labelled BCAA with three kinds of antibiotics using a Warburg flask with a center well to trap CO_2 at 39°C for 12 hr. Before and after incubation, the concentrations of amino acids and volatile fatty acids (VFA) in the centrifuged supernatant fluid of the incubation medium and the concentration of amino acids in protozoal hydrolysates were assayed. Then the radioactivities of these fractions and CO_2 fraction were assayed. The protozoal suspension produced carbon dioxide and four- and five-carbon volatile fatty acids from those substrates. Although VFA, especially, branched-chain VFA could not be identified, the results obtained in the present experiments and some results previously reported by other investigators led us to presume that four-carbon VFA was isobutyrate produced from valine and five-carbon VFA were isovalerate and 2-methylbutyrate produced from leucine and isoleucine, respectively. The degradation rates of leucine and isoleucine in the protozoal suspension seemed to be intermediate, while that of valine was slow, compared to those of arginine, threonine and so on reported in previous papers.

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The fundamental study of the amino acid metabolism of rumen ciliate protozoa is important in understanding their role in the rumen microbial ecosystem and their contribution to the nutrition of their host animals. We have previously reported our studies of the metabolism of lysine⁷⁻¹⁰⁾, arginine and the related compounds¹⁴⁾, methionine and threonine^{13,15)} by mixed rumen ciliate protozoa.

There have been some studies on the metabolism of free branched-chain amino acids

(BCAA) by rumen microorganisms. For example, *Bacteroides rumenicola* and mixed rumen microbes have been shown to convert leucine, isoleucine and valine to isovalerate, 2-methylbutyrate and isobutyrate, respectively^{3,6)}. However there have been no studies on the free BCAA metabolism by rumen protozoa, except those on aminotransferase^{2,17,18)}, though COLEMAN⁵⁾ has reported that *Entodinium caudatum* produced isovalerate, 2-methylbutyrate and isobutyrate in the medium when *Escheri-*

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chia coli grown with ^{14}C -valine, ^{14}C -leucine and ^{14}C -isoleucine, respectively, was given to the protozoon.

This paper describes the metabolism of free BCAA by starved mixed rumen protozoa.

Materials and Methods

1. Preparation of starved rumen protozoa

Mixed rumen ciliate protozoa were obtained from a ruminally fistulated goat (Japanese-Saanen, castrated, 45 kg) fed a ration consisting of haycube (300 g) and concentrate (100 g, Chubu-Shiryo, Japan) twice daily (in the morning and the evening). Starved protozoal suspensions were prepared in order to make the mixed protozoa almost bacteria-free by treating them as reported earlier¹⁵. The starved protozoal suspensions when used, usually contain 0.1 mg/ml each of dihydrostreptomycin sulfate, penicillin G potassium and chloramphenicol sodium succinate.

2. Incubation procedure and sample treatment

At first, 4-ml portions of the starved rumen protozoal suspension prepared above were incubated at 39°C for 12 h with given amounts (see Table 1) of ^{14}C - and ^{12}C -BCAA in Warburg flasks with center wells to which 0.7 ml of 20% (w/v) KOH was added to trap carbon dioxide. As the controls, 4-ml portions of MB 9 buffer solution¹² (without protozoa) containing the same three antibiotics mentioned above were incubated under the same conditions. Before and after incubation, samples of the incubation medium were taken and radioactivities of

carbon dioxide and ether extracts of supernatant and radioactivities and concentrations of amino acids in the supernatant after being extracted with ether and in the protozoal hydrolysates were assayed in the same manner as in the previous paper¹⁵.

Simultaneously, 10-ml portions of the starved protozoal suspension were incubated similarly with given amounts (see Table 1) of ^{14}C - and ^{12}C -BCAA in 100-ml conical flasks with center wells to analyze the concentration and radioactivity of volatile fatty acids (VFA). After incubation, the medium was mixed with 2.5 ml of 5 N H_2SO_4 , left overnight and then treated using the procedure reported previously¹¹.

3. Analytical methods

Amino acids were determined by an automatic amino acid analyzer (AA-100, Sibata Chemical App. Mfg. Co., Ltd., Japan). The drained eluate of a peak of some amino acids, when necessary, was collected, and the radioactivity was determined after the removal of carbon dioxide by flushing it with air as described in the previous report¹⁶.

The VFA were analyzed by the method of BEUDING and YALE¹⁷ using a Celite column as modified by ONODERA and KANDATSU¹¹.

Radioactivity was determined with a liquid scintillation spectrometer (LSC-903, Aloka Co., Ltd., Japan) as reported previously¹⁵.

Results

According to what has been described previ-

Table 1. Composition of incubation medium

Additions	Protozoa	Incubation medium for		
		Leucine	Isoleucine	Valine
^{14}C -amino acid ^a ($\mu\text{Ci/ml}$)	With	1.1 ^b	0.8	0.7
	Without	0.8	0.8	0.8
^{12}C -amino acid (nmol/ml)	With	150	200	200
	Without	150	200	200

^a: Specific activity of each branched-chain amino acid is as follows: L-[U- ^{14}C] leucine, 346.0 Ci/mol; L-[U- ^{14}C] isoleucine, 331.0 Ci/mol; L-[U- ^{14}C] valine, 294.0 Ci/mol.

^b: For analysis of volatile fatty acids, 1.0 $\mu\text{Ci/ml}$ of leucine was added.

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ously¹⁵⁾, a starved rumen protozoal suspension, which has been shown to be almost bacteria-free, was also used in the present experiments. The metabolic activity and the protozoal composition of the suspension usually varied to some extent in every experiment, though the tendencies of the results were consistent. The data presented in this paper, therefore, are the most active out of three trials.

The species and numbers found in the cell suspensions of the starved rumen protozoa used in these experiments are shown in Table 2 with subfamily names or genus names. More than about 94% was *Entodiniinae*.

When the protozoal suspension was incubated with each radioactive BCAA for 12 h, radioactivities in the carbon dioxide and ether extracts in the supernatant of the medium increased, while those of the supernatant after being extracted with ether decreased (Table 3). Radioactivity in the protozoal hydrolysate also

slightly increased after incubation (Table 3).

The total amount of leucine, isoleucine and valine in the supernatant and protozoal hydrolysate in each medium decreased by 8.7, 15.3 and 7.3%, respectively, while the concentration of each amino acid in the supernatant increased about 2.6, 2.4 and 1.6 times, respectively (Table 4). The specific activity (SA) of each BCAA in the supernatant declined sharply, whereas the SA of the total (including ciliate hydrolysate) of each BCAA declined moderately (Table 5).

Analysis of VFA in the ether extracts of the medium after 12 h-incubation revealed that radioactive five-carbon VFA was produced from leucine, isoleucine and valine and radioactive four-carbon VFA was also produced from valine (Table 6). Among the VFAs, four-carbon acid (perhaps mainly butyrate) and acetate showed relatively higher concentrations in these protozoal suspensions.

Table 2. Protozoal composition in the media used in the present experiments

Incubation medium for	Composition of protozoa ($\times 10^3/\text{ml}$)			
	Entodiniinae	Diplodiniinae	<i>Isotricha</i>	<i>Dasytricha</i>
Leucine	512	14.2	1.4	0
Isoleucine	958	57.3	4.0	1.3
Valine	394	15.7	0.3	0.3

Table 3. Distribution of radioactivities in general fractions before and after incubation of radioactive branched-chain amino acids with or without starved mixed rumen protozoa (P)

(Unit: % of the initial radioactivity)

Fractions	Incubation time, (h)	Substrates					
		Leucine		Isoleucine		Valine	
		With P	Without P	With P	Without P	With P	Without P
Supernatant*	0	97.8	—	97.1	—	96.5	—
	12	71.0	100.3	64.3	94.5	82.7	99.7
Carbon dioxide	0	0	—	0	—	0	—
	12	5.2	0.2	0.7	0	0.5	0.1
Ether extracts	0	4.7	—	4.9	—	1.5	—
	12	18.3	5.5	23.5	5.0	7.1	2.5
Hydrolysate of protozoa	0	0	—	0.1	—	0.1	—
	12	0.3	—	1.6	—	2.5	—

*: Supernatant fluid after being extracted with ether.

Table 4. Change of the concentration (nmol/ml) of each branched-chain amino acid in each incubation medium

Fractions	Incubation medium for					
	Leucine		Isoleucine		Valine	
	0	(Incubation 12	0	time, 12	h) 0	12
Supernatant	133	344	229	557	193	309
Protozoa	2025	1627	2123	1435	746	561
Total	2158	1971	2352	1992	939	870

Table 5. Changes of specific activities of branched-chain amino acids during incubation

Amino acid added	Incubation time, (h)	Specific activity (dpm/nmol)		Decrement (%)	
		Supernatant ($\times 10^4$)	Total ^a ($\times 10^3$)	Supernatant	Total ^a
Leucine	0	2.136	1.330		
	12	0.455	0.830	78.7	37.6
Isoleucine	0	0.732	0.720		
	12	0.177	0.570	75.8	20.8
Valine	0	0.700	1.410		
	12	0.240	0.830	65.7	41.1

^a: Total value includes each corresponding amino acid in protozoal hydrolysate together with that in supernatant.

Table 6. Distribution of radioactivity in volatile fatty acid fractions after 12 h-incubation with radioactive branched-chain amino acids

Volatile fatty acid fractions	Amino acid added					
	Leucine		Isoleucine		Valine	
	$\mu\text{mol/ml}$	dpm/ml ($\times 10^4$)	$\mu\text{mol/ml}$	dpm/ml ($\times 10^4$)	$\mu\text{mol/ml}$	dpm/ml ($\times 10^4$)
C ₅ -acid	1.61	29.6	2.69	9.86	0.45	1.27
C ₄ -acid	3.42	0	7.04	0	0.61	3.15
Propionate	1.47	0	0.72	0	0	0
Acetate	6.16	0	4.20	0	1.46	0

Discussion

Radioactive BCAA were shown to be metabolized in the starved rumen protozoal suspension to produce common radioactive carbon dioxide and ether-soluble substances (Table 3). Degradation rates (nmol/ml/h) of leucine, isoleucine and valine calculated from the total values shown in Table 4 are 15.6, 30.0 and 5.8, respectively (Table 7). As shown in Table 7, arginine¹⁴, threonine¹⁵ and citrulline¹⁴ were indicated to be rapidly degraded in the protozoal

suspension and the former two were also shown to be rapidly-degradable even under *in vitro* and *in vivo* mixed rumen microbial conditions (CHALUPA)⁴¹. Therefore degradation rates of isoleucine and leucine in the present experiment were thought to be at an intermediate rank together with ornithine and lysine⁷, whereas valine was least rapidly degraded. These tendencies almost agreed with the results obtained by the mixed rumen microbial population⁴¹. Results of ammonia production by rumen protozoa also showed to include citrulline and

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Table 7. Ammonia production rates from and degradation rates of some amino acids so far tested in *in vitro* rumen protozoal suspension

Amino acid	Ammonia production (nmol/ml/h)	Amino acid	Decrease (nmol/ml/h)
Glutamine ^a	817	Arginine ^a	75.0
Citrulline ^a	333	Threonine ^b	70.7
Arginine ^a	267	Citrulline ^a	60.8
Asparagine ^a	267	Isoleucine ^c	30.0
Ornithine ^a	100	Ornithine ^a	20.0
Glutamate ^a	0	Leucine ^c	15.6
Aspartate ^a	0	Lysine ^d	15.5
Histidine ^a	0	Valine ^c	5.8

^a: From ONODERA *et al.*¹⁴⁾, where ammonia was assayed by CONWAY's microdiffusion method.

^b: From ONODERA and MIGITA¹⁵⁾.

^c: Calculated from the values shown in Table 4 in the present experiment.

^d: From ONODERA and KANDATSU⁷⁾.

arginine in the highest group together with glutamine and asparagine (Table 7).

Decrements (%) in the total concentrations of leucine, isoleucine and valine after incubation calculated from the values in Table 4 were 8.7, 15.3 and 7.3, respectively, and were not strictly consistent with the decrements in their radioactivities in the supernatant (29.0, 35.7 and 17.3%, respectively) calculated from the values in Table 3. This is because each free BCAA (¹²C) can be released from the protozoal protein to the medium as shown in Table 4. Thus the SA of each BCAA in the medium varied with incubation time as shown in Table 5. Namely the SA of each BCAA in the supernatant of the protozoal suspension decreased by about 66 to 79% after incubation, while the SA of each of the total BCAA including protozoal hydrolysate decreased by about 20 to 40 %.

Production of carbon dioxide (Table 3) suggests that these BCAA are firstly transaminated (or deaminated) and then decarboxylated like in the case of rumen bacteria^{3,6)}. Decarboxylated products seemed to appear mainly in ether extracts (Table 3). Chromatographic and radiologic analysis of the ether extracts after incubation revealed that the main radioactive metabolic products were four- and five-carbon VFA (Table 6). Of five-carbon acids, those produced from leucine and isoleucine were pre-

sumed to be isovalerate and 2-methylbutyrate, respectively, from the results with washed suspension of mixed rumen microbes⁶⁾ and with *Bacteroides ruminicola*³⁾, though we could not identify them in the present experiment. A five-carbon acid produced from valine may probably be 2-oxoisovalerate, as DEHORITY *et al.*⁶⁾ pointed out, though we are not sure whether the oxo-acid appear in this five-carbon VFA fraction in the Celite column fractionation method^{1,11)}. A four-carbon VFA produced from valine may be isobutyrate like in the case in mixed rumen microbes⁶⁾.

Protozoal cells incorporated radioactive substances from BCAA to some extent. This reflects the turnover of protein in the starved protozoal cells.

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絶食ルーメン繊毛虫類による分枝アミノ酸の代謝

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必須アミノ酸（ラット）のうち遊離の分枝アミノ酸（BCAA）、フェニルアラニンおよびヒスチジンについては、ルーメン繊毛虫類による代謝様式がまだ知られていない。そこで、本研究では分枝アミノ酸を取り上げ、ルーメン繊毛虫類によるそれらの代謝を検討した。

ヘイキューブおよび濃厚試料を給与している山羊（日本在来種）のルーメン内容物から常法により繊毛虫類を採取し、洗浄後これを絶食条件下の抗生物質を含む緩衝液（MB 9）中で、39°C、6時間インキュベーションを行なって細菌の影響を除去した後、再洗浄を行ない、¹⁴C 標識 BCAA 並びに抗生物質を加えた MB 9 中で、さらに12時間インキュベーションを行なった。インキュベーション用容器としては、中心井付きのワールブルグフラスコを用いた。インキュベーション前後に分析用試料を採取し、遠心上清液および虫体加水分解物中のアミノ酸および遠心上清液中の揮発性脂肪酸（VFA）を定量した。さらに、それらの放射能並びに CO₂ 画分の放射能を液体シンチレーションカウンターで測定した。

その結果、絶食繊毛虫類は、放射性の BCAA から CO₂ およびエーテル可溶物質、特に、C₄ および C₅ の VFA を生成していることが分かった。本研究ではそれらの VFA を同定できなかったが、過去のルーメン細菌などの結果から、C₄-VFA はイソ酪酸でバリンから生成されと考えられた。また、C₅-VFA には、少なくともイソ吉草酸および2-メチル酪酸が含まれ、それぞれ、ロイシンおよびイソロイシンから生成されと考えられた。さらに、BCAA の分解速度についても考察した。

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