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ORIGINAL PAPER

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Biosynthesis of methionine from homocysteine, cystathionine and homoserine plus cysteine by mixed rumen microorganisms in vitro

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Abstract This study quantitatively investigated the biosynthesis of methionine (Met) and the production of related compounds from homocysteine (Hcys), cystathionine (Cysta), and homoserine (Hser) plus cysteine (Cys) by rumen bacteria (B) or protozoa (P) alone and by a mixture of these bacteria and protozoa (BP). Rumen contents were collected from fistulated goats to prepare the microbial suspensions and were anaerobically incubated at 39 °C for 12 h. Hcys, Cysta, and Hser plus Cys were catabolized by all rumen microbial fractions to different extents. B, P, and BP converted Hcys to Met with 2-aminobutyric acid (2AB) and methionine sulfoxide. The Met-producing ability of B (83.2 μmol g^{-1} microbial nitrogen; MN) from Hcys was about 3.6 times higher than that of P in a 6-h incubation period. The ability of BP, during the same incubation period, was about 30.0% higher than that of B. Hcys, Met, and 2AB were formed when Cysta was incubated with B, P, or BP. Rumen microbial fermentation of Hser plus Cys led to the formation of Cysta, Met (through Hcys), and 2AB. Thus the results indicated that a trans-sulfurylation pathway for Met synthesis was operating in the rumen bacteria and protozoa. The results mentioned above have been demonstrated for the first time in B, P, and BP in the present study.

Introduction

The ruminant animal must depend on microbial protein plus dietary protein that escapes digestion in the rumen for its supply for essential amino acids, of which 50–90% is of microbial origin (Bondi 1987). Methionine (Met) is believed to be an essential amino acid, the absence of which limits a ruminant's growth and production (Jacobson et al. 1967). Met is not only an important precursor in protein synthesis, but also serves as a methyl donor for trans-methylation reactions in the biosynthesis of lipids and other compounds (Patterson and Kung 1988).

With regard to Met synthesis in microorganisms, it has been reported that homocysteine (Hcys), a direct precursor of methionine, is synthesized from *o*-succinylhomoserine and cysteine (Cys), via cystathionine (Cysta) by coupled reactions of cystathionine γ-synthase (EC 4.2.99.9) and cystathionine β-lyase (EC 4.4.1.8; Soda 1987). *O*-Succinylhomoserine is produced by the conversion of homoserine (Hser; a key intermediate of the aspartate pathway) and succinyl-CoA (Old et al. 1991). Finally, Hcys is methylated to Met, either by a vitamin B_{12}-dependent Hcys-methyl transferase enzyme (EC 2.1.1.13; Foster et al. 1964), or by a vitamin B_{12}-independent enzyme (EC 2.1.1.14; Taylor and Weissbach 1973). Most microorganisms follow this common pathway. However, it has been reported that *Neurospora crassa* can produce Hcys enzymatically from Hser and H_2S (Wiebers and Garner 1960), which implies that Cysta is not an obligate precursor of Hcys. In *Salmonella*, it has also been shown to catalyze the formation of Met directly from *o*-succinylhomoserine and methyl mercaptan, bypassing Cysta and Hcys (Flavin and Slaughter 1967).

In spite of their great importance, these types of investigation as mentioned previously have not yet been conducted in rumen microorganisms. Although Sauer et al. (1975) postulated about the incorporation of labeled formate and acetate into Met, there has been no direct evidence until now concerning Met synthesis from the precursors mentioned above, by rumen microorganisms.

The present paper quantitatively describes the in vitro synthesis of Met and the production of other related compounds from Hcys, Cysta, and Hser plus Cys by mixed rumen bacteria and mixed rumen protozoa. A quantitative study was also carried out to investigate and compare the production of these compounds, including Met, in the actual rumen microbial ecosystem (important

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for the nutrition and physiology of the host animal), consisting of a combination of bacteria and protozoa mixed together with those of mixed bacteria and mixed protozoa alone. The use of HPLC for the quantitative determination of amino acids has become popular owing to its sensitivity and speed of analysis (Krishnamurti et al. 1984). Recently, we established a convenient, selective, sensitive, and reproducible HPLC method, based on precolumn derivatization using 9-fluorenylmethyl chloroformate for the quantitative determination of Met and its related amino compounds, which encompasses all the compounds within one chromatogram (Or-Rashid et al. 2000).

Materials and methods

Rumen microbial preparations

Samples of mixed rumen microorganisms for experiments were obtained from three mature goats (Japanese native breed, body weight of 35±5 kg), each fitted with a permanent cannula in the rumen. The animals were fed 370–450 g of lucerne hay cubes and 115–140 g of concentrate feed at 0900 hours and 1700 hours in two equal portions. Fresh water was available at all times.

Rumen digesta were removed before feeding in the morning and were strained through several layers of surgical gauze. The suspensions of mixed bacteria (B), and mixed protozoa (P), and B plus P (BP) were obtained using the procedure of Onodera et al. (1992). The P suspensions always included 0.1 mg ml^{-1} each of chloramphenicol, streptomycin sulfate, and penicillin G potassium to stop the biochemical activities of contaminating bacteria.

In vitro incubation and sample preparation

Microbial suspensions (20 ml) were incubated in 30-ml Erlenmeyer flasks at 39 °C for 12 h with 2 mM each of Hcys, Hser plus Cys, and 1 mM Cysta as substrate, after being flushed briefly with a mixture of 95% N_2 and 5% CO_2. Rice starch was added (0.5 mg ml^{-1}) as an energy source in all incubation media. A 1-ml sample of the suspension was withdrawn from the reaction vessel at 0, 6, and 12 h during the fermentation and was immediately added to equal volumes of 4% (w/v) sulfosalicylic acid (including 10 mM EDTA-3 K) in a 2-ml Eppendorf tube. The deproteinized samples were centrifuged at 27,000 *g* for 30 min at 4 °C. The supernatant fluids were filtered through a membrane filter (0.45 µm) and stored at 4 °C. The resulting pellet of mixed rumen microorganisms was washed three times in ice-cold saline (0.9% NaCl) to remove supernatant residues and was hydrolyzed with 4 M methanesulfonic acid (2 ml) containing 0.2% tryptamine (w/v) at 160 °C for 45 min (Chiou and Wang 1988). The hydrolysates were then transferred into another graduated tube and 2 ml of 3.5 M KOH solution was added to the tube. After mixing, the pH was 0.70–0.71. Finally, the volume was adjusted to 6 ml with distilled water and was filtered, first through filter paper (Whatman, No. 2), then by membrane filter (0.45 µm porosity) before being stored at 4 °C.

Methods of analysis

All of the supernatant fluids (after incubation) and the hydrolysates from the pellets were subjected to analysis by HPLC according to Or-Rashid et al. (2000). Samples of P and BP were preserved by the addition of nine volumes of methylgreen-formalin salt solution (Onodera et al. 1977) kept at room temperature and were then counted by direct light microscopy, using a Fuchs-Rosenthal hemocytometer. The microbial nitrogen (MN) content of the B, P, and BP suspensions was determined by the Kjeldahl method (Helrich 1990), using triplicate 1-ml samples of each microbial suspension.

Experimental design

In all experiments, the incubation of microbial suspensions was carried out using triplicate samples collected from one goat. Samples from the other two goats were used separately on successive days. A control incubation was always run without test substrate, to determine the endogenous products.

The following formula was used to calculate the net production of products from substrates: $X_i=(S_i-S_j)-(C_i-C_j)$, where X is the net amount of product, S is the amount of product in the incubated medium with substrate added, C is the amount of product in the incubated medium without substrate, i is the incubation period (either 6 h or 12 h), and j is 0 h.

The net production values of all components found in the supernatants and hydrolysates were expressed as the mean of nine observations, with their standard deviations.

Results

Synthesis of Met and production of methionine sulfoxide and 2-aminobutyric acid from Hcys

When Hcys (2 mM) was used as a substrate, 55.4% and 72.0% disappeared in B suspension, 33.8% and 52.0% disappeared in P suspension, and 72.8% and 84.1% disappeared in BP suspension, after 6 h and 12 h of incubation, respectively (Table 1). Calculated in terms of MN, the rate at which Hcys disappeared was greatest in B (141.2 µmol g^{-1} MN h^{-1}), followed by P (117.1 µmol g^{-1} MN h^{-1}), and BP (98.7 µmol g^{-1} MN h^{-1}) after 12 h of incubation. Rumen bacteria accumulated a small portion of the synthesized Met from Hcys in the incubation medium (Table 1), while a greater portion accumulated in the hydrolysate (Table 1). It was observed that 6.1% and 4.9% of the Hcys which disappeared remained as Met after 6 h and 12 h of incubation, respectively. The amounts of Met remaining in the medium in P suspension after 6 h and 12 h of incubation, were 2.5% and 1.2% of the disappeared Hcys, respectively. It was observed in BP that the Met produced was equivalent to 10.2% and 8.6% of the disappeared Hcys after 6 h and 12 h of incubation, respectively. About 60.8–66.9% of the total synthesized Met was accumulated as a free form in the supernatant of BP. When the production abilities of the different microbial fractions were calculated in terms of MN, the production of Met from Hcys in BP was higher than that in B or P.

As shown in Table 1, after 6 h of incubation, about 0.9, 1.0, and 1.0% of the disappeared Hcys were converted to methionine sulfoxide (MSO), which could be produced through Met in B, P, and BP. In terms of MN, the amounts of MSO formed from Hcys in 6 h of incubation in P and BP were almost similar; but in B, it was about 1.3 times higher than that in P or BP.

Table 1 also shows the formation of 2-aminobutyric acid (2AB) from Hcys by different microbial fractions after 12 h of incubation. When we consider the MN, the production of 2AB in P was about 3.6–3.9 times that in B or BP.

Table 1 Biosynthesis of methionine (*Met*) and production of methionine sulfoxide (*MSO*) and 2-aminobutyric acid (*2AB*) from homocysteine (*Hcys*) by mixed rumen bacteria (*B*), mixed rumen protozoa (*P*), and B plus P (*BP*). Average microbial nitrogen measurements in B, P, and BP suspensions were 0.85, 0.74, and 1.42 mg N ml^{-1}, respectively. Values are shown as the micromolar concentration of each product (mean value of nine determinations ± standard deviation) remaining after incubation. *D* decrease in substrate (micromoles of substrate per gram of microbial nitrogen), *I* increase in product (micromoles of product per gram of microbial nitrogen), *H* hydrolysate, *S* supernatant, *T* H and S together

Compound		Incubation mixture and duration of incubation					
		B		P		BP	
		6 h	12 h	6 h	12 h	6 h	12 h
Hcys	S	892.1±57.6	560.2±45.4	1324.0±65.3	960.3±72.3	544.1±35.4	317.9±36.5
	D	1,303.4	1,693.9	913.5	1,405.0	1,025.3	1,184.6
Met	S	12.8±3.76	8.3±2.64	18.0±3.61	14.5±2.18	98.9±14.5	87.7±10.5
	H	55.3±6.79	62.4±5.61	–1.4±0.63	–2.1±0.37	49.0±5.5	56.4±7.8
	T	68.1±8.87	70.7±7.31	16.6±3.05	12.4±2.15	147.9±17.3	144.1±16.9
	I	80.1	83.2	22.4	16.8	104.1	101.5
MSO	S	10.3±2.91	8.6±2.23	6.7±1.64	5.4±1.65	14.3±3.27	11.4±2.10
	I	12.1	10.1	9.1	7.3	10.1	8.0
2AB	S	7.3±2.6	5.3±1.87	24.8±5.23	16.7±3.53	14.3±2.79	8.2±1.67
	I	8.6	6.2	33.5	22.6	10.1	5.8

Table 2 Biosynthesis of Met and production of Hcys and 2AB from cystathionine (*Cysta*) by B, P, and BP. Average microbial nitrogen measurements in B, P, and BP suspensions were 0.89, 0.79, and 1.44 mg N ml^{-1}, respectively

Compound		Incubation mixture and duration of incubation					
		B		P		BP	
		6 h	12 h	6 h	12 h	6 h	12 h
Cysta	S	897.8±52.3	854.3±35.1	936.7±42.5	905.1±30.2	859.3±45.3	819.1±33.4
	D	114.8	163.7	80.1	120.1	97.7	125.6
Met	S	2.3±0.56	1.7±0.68	9.2±2.78	7.6±2.53	43.3±4.78	45.7±6.71
	H	34.1±4.12	42.3±3.17	–1.3±0.34	–2.1±0.67	32.1±4.76	37.2±5.32
	T	36.4±4.32	44.0±3.72	7.9±2.51	5.5±1.13	75.4±7.89	82.9±9.28
	I	40.9	49.4	10.0	6.9	52.4	57.6
Hcys	S	10.1±3.12	13.4±2.56	3.1±1.24	5.2±1.13	12.6±2.79	16.2±3.07
	I	11.34	15.05	3.9	6.6	8.8	11.25
2AB	S	1.7±0.31	0.0±0.0	6.3±1.49	3.6±1.31	4.1±1.43	2.4±0.63
	I	1.9	0.0	8.0	4.6	2.84	1.7

Synthesis of Met and production of Hcys and 2AB from Cysta

Table 2 shows the results when Cysta was used as substrate. Inclusion of Cysta (1 mM) in the suspension of B resulted in 10.2% and 14.6% degradation after 6 h and 12 h of incubation, respectively. In P and BP suspensions, these values were 6.3% and 9.5%, and 14.1% and 18.1%, respectively. However, the Cysta disappearance rate in B was about 1.3 times that of P or BP when calculated in terms of MN.

We noted that 35.7% and 30.1% of the disappeared Cysta were converted to and remained as Met in B suspension after 6 and 12 h of incubation, respectively. A greater portion of the Met produced by rumen bacteria was incorporated into body protein, whereas a small amount of Met was accumulated as a free form in the incubation medium (Table 2), as in the case of Met production from Hcys. In P, conversion of the degraded Cysta to Met was accounted for and the values were 12.5% and 5.8% in 6 h and 12 h of incubation, respectively. In BP, Met remained as 53.5% and 45.8% of the molar amounts of the disappeared Cysta after 6 h and 12 h of incubation, respectively. In this experiment, the Met-synthesizing ability of BP was about 1.2–1.3 times higher than that of B and 5.2–8.3 times higher than that of P.

The results summarized in Table 2 indicate that the amount of Hcys produced from Cysta remained as 9.9% and 8.9% of the disappeared Cysta after 6 h incubation in B or BP, respectively. These values were 9.2% and 9.0% after 12 h of incubation. In P suspensions, 4.9% and 5.5% of the disappeared Cysta remained as Hcys.

2AB was found to be produced in different microbial fractions, as shown in Table 2.

Table 3 Biosynthesis of Met and production of Cysta and 2AB from homoserine (*Hser*) plus cysteine (*Cys*) by B, P, and BP. Average microbial nitrogen measurements in B, P, and BP suspensions were 0.92, 0.85, and 1.55 mg N ml^{-1}, respectively

Compound		Incubation mixture and duration of incubation					
		B		P		BP	
		6 h	12 h	6 h	12 h	6 h	12 h
Hser	S	568.0±32.3	374.1±26.7	1117.8±40.1	890.2±42.3	812.4±30.4	456.2±35.6
	D	1,556.5	1,767.3	1,037.9	1,305.6	766.2	996.0
Cys	S	114.0±13.4	22.4±4.3	896.5±25.6	644.0±35.4	192.4±16.3	20.1±3.7
	D	2,050.0	2,149.6	1,298.2	1,595.3	1,166.2	1,277.4
Met	S	4.3±1.25	2.7±0.67	7.4±1.72	6.3±1.45	36.3±3.43	34.2±2.68
	H	27.1±3.78	33.3±3.52	–1.2±0.32	–1.6±0.27	31.2±4.2	32.7±3.8
	T	31.4±4.78	36.0±4.12	6.2±1.56	4.7±1.37	67.5±7.12	66.9±6.32
	I	34.1	39.1	7.3	5.5	43.5	43.2
Cysta	S	6.3±1.72	5.1±1.86	7.7±2.21	4.6±1.54	4.4±1.36	2.8±0.52
	I	6.84	5.5	9.1	5.4	2.83	1.8
2AB	S	70.3±6.21	56.3±6.57	0.0±0.0	0.0±0.0	40.70±5.37	33.10±4.73
	I	76.1	61.2	0.0	0.0	26.3	21.4

Synthesis of Met and production of Cysta and 2AB from Hser plus Cys

Disappearence of Hser plus Cys added in the same incubation media resulted in the synthesis of Met as shown in Table 3. After 6 h and 12 h of incubation, the level of Hser was found to have decreased by 71.6% and 81.3%, 44.1% and 55.5%, and 59.4% and 77.2% in B, P, and BP suspensions, respectively (Table 3). Cys in the same incubation media was 94.3% and 98.9% degraded in the B fraction, 55.2% and 67.8% in the P fraction, and 90.4% and 99.0% in BP fraction after 6 h and 12 h, respectively.

A comparatively large amount of synthesized Met was incorporated into the microbial cells of B and BP. In contrast, P cells did not incorporate Met, but liberated Met into the incubation medium (Table 3). BP showed the highest Met-producing ability, followed by B and then P. A small amount of Cysta was produced from Hser plus Cys by B, P, and BP; but a comparatively large amount of 2AB was produced in B and BP but not in P (Table 3).

Discussion

Synthesis of Met and production of MSO and 2AB from Hcys

To our knowledge, the present report is the first to deal with the metabolism of Hcys by the three different fractions of rumen microorganisms, i.e., mixed rumen bacteria, mixed rumen protozoa and their combined mixture.

In the present study, a significant amount of Met was synthesized from Hcys by rumen bacteria and the bacterial hydrolysate contained a greater portion of the synthesized Met, which indicated that rumen bacteria efficiently utilized Met for their cell protein synthesis (Broderick et al. 1991; Armstead and Ling 1993; Ling and Armstead 1995). In P, the amount of Met in the supernatant increased considerably after 6 h of incubation, followed by a decrease after 12 h of incubation, whereas the Met concentration in protozoal hydrolysate decreased after incubation (Table 1). It is known that rumen protozoa cannot grow in MB9 buffer and liberate endogenous amino acids into the medium (Onodera and Kandatsu 1970; Morgavi et al. 1993). Therefore, the total amount of free endogenous Met released by protozoa in the absence of Hcys was subtracted from the amount of the free Met released by protozoal metabolism in the presence of Hcys; and the net increase in Met was calculated.

The rumen microbial ecosystem mainly consists of bacteria and protozoa. In this experiment, a BP suspension was also incubated to speculate on the real activities of the rumen microbial ecosystem and to investigate any interactions between bacteria and protozoa. It was interesting that when rumen protozoa were mixed with rumen bacterial suspensions, the Met-producing capabilities of BP suspensions increased by 30.0% and 22.1%, compared with that of B after 6 h and 12 h of incubation, respectively. Interactions between the bacterial and protozoal populations in the rumen are important in maintaining the stability of the microbial community and in determining the fermentation pattern (Williams and Coleman 1997). The rumen fermentation is more stable when an active ciliate population is present with bacteria, because the protozoa exert some control over the rate of acid formation. The reason for the higher value of Met production in BP in this observation is not known for certain, but it can be regarded as a positive interaction between the bacteria and the protozoa. Thus, it is strongly suggested that the co-existence of P in B suspension may have a beneficial effect on Met production in the rumen microbial ecosystem. In this regard, Onodera and Koga (1987) demonstrated that Met in the goat rumen contents increased (0.024 g g^{-1} of total amino acids), both with time after feeding and also in comparison with

rations (0.018 g g^{-1} of total amino acids); and the Met values in the rumen contents of faunated animals tended to be higher than those in defaunated animals. One of the possible reasons for their findings may be that actual synthesis of Met by microorganisms occurs in the rumen; and this view seems to be supported by the present in vitro experiments. Although *Escherichia coli* has been reported to synthesize Met from Hcys (Foster et al. 1964), the evidence for Met biosynthesis from Hcys by rumen bacteria, protozoa, and their mixture, as shown in the present study was completely new.

In order to speculate on the possible ability of rumen microorganisms to produce Met from Hcys in the actual rumen ecosystem, the amount of Met produced by BP was calculated, using the value of the BP production rate (416.4 μmol Met g^{-1} MN day^{-1}) shown in Table 1, considering the volume of the goat rumen to be 10 l and that in cattle to be 150 l, and further considering that there was enough substrate in the rumen. The results were 0.88 g Met $head^{-1}$ day^{-1} in goat and 13.2 g Met $head^{-1}$ day^{-1} in cattle.

Met has been shown to be oxidized enzymatically by mixed rumen microorganisms (Salsbury et al. 1971) and by rumen protozoa (Onodera and Migita 1985) to produce MSO. In this regard, Met is susceptible to oxidation, which in turn is connected with a loss of protein activity (Teh et al. 1987). Our present experiment revealed that the MSO-producing ability of BP was 20.8% lower than that of B (10.1 μmol g^{-1} MN). It is known that protozoa consume oxygen and may have a significant role in ruminal oxygen-scavenging (Williams and Coleman 1992). Thus, the co-existence of P in B suspensions saved the produced Met from Hcys, by slowing the oxidation of Met to MSO. And this positive interaction between rumen protozoa and bacteria could be important for the host animal.

The present study revealed that 2AB was formed from Hcys by all of the rumen microbial suspensions and P accumulated a higher concentration, compared with B and BP. Kallio (1951) observed that Hcys could directly undergo desulfhydration to yield hydrogen sulfide, ammonia and 2-oxobutyric acid (2OB) in *Proteus morganii*. The anaerobic incubation of Met in mixed protozoa (Onodera and Migita 1985) and in *E. coli* (Meister 1965) also leads to the formation of 2AB via 2OB. Our observation, taken together with the facts mentioned above, would suggest that there are two possible routes for 2AB formation from Hcys in the rumen microbial suspensions: desulfhydration of Hcys and dethiomethylation of Met, first being formed by methylation of Hcys.

In B suspension, Hcys as a whole decreased by 55.4%; and 6.1% of the disappeared Hcys was converted to Met, 0.9% to MSO, and 0.7% to 2AB in 6 h of incubation. About 51.1% of the added Hcys was not recovered and may have been converted to other, non-determined compounds, such as homocystine (Ueland et al. 1993); 2OB, hydrogen sulfide, ammonia (Kallio 1951); methanethiol, *S*-methyl-L-cysteine, *S*-adenosylmethionine (Zikakis and Salsbury 1969); and 2-oxo-γ-methiolbutyric acid (Ishimoto et al. 1971). In the case of P and BP, these values were 31.4 % and 63.3%, respectively, and Hcys may have been converted to other non-detectable compounds as mentioned above.

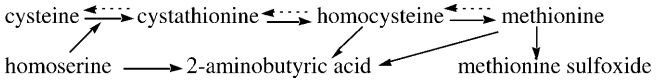
Synthesis of Met and production of Hcys and 2AB from Cysta

In the present study, incubation with Cysta in B, P, and BP resulted in the production of Met, Hcys, and 2AB. In the case of aerobic microorganisms, biosynthesis of Met by the methylation of Hcys derived from Cysta have been demonstrated with cell-free extracts of *E. coli* and *Aerobacter aerogenes* (Balish and Shapiro 1966). The present observation has revealed for the first time that not only aerobic microorganisms, but anaerobic rumen microorganisms, i.e., B, P, and BP could also convert Cysta to Met.

As described in the Introduction, Hcys is readily methylated to form Met in microorganisms (Foster et al. 1964) and there are some reports that Hcys can be synthesized from Hser (in presence of sulfur donor), either directly without the formation of Cysta (Wiebers and Garner 1960; Soda 1987), or indirectly through the formation of Cysta (Rowbury and Woods 1964). This may indicate that Cysta is not always the immediate precursor of Hcys. In the present experiment, when we added Cysta to the incubation media of B, P, and BP, Hcys was soon found in the incubation media (Table 2), which suggested that Cysta was the direct precursor of Hcys. As rumen microorganisms synthesize Hcys from Cysta, it seems probable that the trans-sulfurylation pathway may be operating for Met synthesis in rumen microorganisms. To confirm this point, Hser and Cys (as a sulfur donor) were also incubated in the present study, as will be discussed later, under another heading. It was also evident from the present study that: (1) Cysta was not produced from Hcys by rumen bacteria and protozoa when Hcys was incubated, and (2) Cys was not produced from Cysta. Therefore, it seems that the reverse trans-sulfurylation pathway was not present in rumen microorganisms, unlike the situation in *Neurospora* (Soda 1987) and *Pseudomonas aeruginosa* (Vermeij and Kertesz 1999).

A small amount of 2AB also accumulated in the incubation media of different microbial fractions (i.e., B, P, and BP) and the possible intermediates might be Hcys, with Met being synthesized from Cysta as described earlier.

In the experiment with Cysta as a substrate, MSO was not found in the incubation media of the different microbial suspensions. In this case, the amount of Met (a precursor of MSO) produced from Cysta was much lower than that produced from Hcys, because Cysta was not the immediate precursor of Met. The small amount of Met synthesized from Cysta might be converted into CH_3SH, 2-oxobutyric acid, 2-aminobutyric acid, and volatile fatty acids at rates higher than the oxidation rate. Therefore, MSO from Met might not be produced. Another possible



Scheme 1 A tentative reaction scheme for the synthesis of methionine and related compounds by rumen microorganisms. *Dashed arrows* indicate the pathways that do not occur in the rumen microorganisms.

cause might be that the oxidation step, using Met to produce MSO is much slower than the reduction of MSO to produce Met, if MSO from Met is produced to some extent by oxidation.

Synthesis of Met and production of Cysta and 2AB from Hser plus Cys

Rumen microbial incubation with Hser plus Cys increased Met production, as compared with the control. In microorganisms, e.g., *S. typhimurium* and *E. coli*, Cysta could be formed from Hser plus Cys, followed by cleavage of Cysta to Hcys, which then converted to Met (Old et al. 1991). The present results were consistent with those for *S. typhimurium* and *E. coli*, because Cysta was also found in the incubation media. In contrast, Hcys was not found in the media. Probably, the Hcys produced from Hser plus Cys via Cysta might be converted readily to Met and other compounds. Thus, the existence of the trans-sulfurylation pathway from Hser plus Cys to Met in the rumen bacteria and protozoa was indicated in the present study; and the formation of Hcys from Cysta, and Met from Hcys by rumen microorganisms has already been shown and discussed in previous sections.

It was observed that a (comparatively) large amount of 2AB was formed from Hser plus Cys in a different manner from the cases of Hcys and Cysta, especially in B and BP (Tables 1, 2, 3). It has been shown that 2OB, a precursor of 2AB, can be produced directly from Hser, as catalyzed by homoserine dehydratase [EC 4.2.1.15] in animals and general microorganisms (Ishimoto et al. 1971). In contrast, P did not produce 2AB. Therefore, it seems that homoserine dehydratase might not be present in rumen protozoa, although it is present in rumen bacteria.

Also, MSO was not found to be accumulated from Hser plus Cys via Met in the present study; and the reasons might be those already described.

On the basis of the present in vitro experiments, we can conclude that rumen bacteria, rumen protozoa and a mixture of both have the ability for de novo synthesis of Met by the main biosynthetic pathway (the trans-sulfurylation pathway) involving Hser plus Cys, Cysta, and Hcys. A tentative reaction scheme for the synthesis of Met and related compounds by rumen microorganisms may be presented as shown in Scheme 1. Dashed arrows indicate the pathways that do not occur in the rumen microorganisms.

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