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Fermentation of Methanol in the Sheep Rumen

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Sheep fed a hay-concentrate diet were adapted to pectin administration and ruminal infusion of methanol. Both treatments resulted in a strong increase in the rate of methanogenesis from methanol. Quantitative data show that methanol was exclusively converted into methane. Treatments did not influence ruminal volatile fatty acid percentages.

Methanol is produced in the rumen as a result of the hydrolysis of methyl esters from pectins, which are abundant polysaccharides in plants. In vitro incubations pointed to subsequent ruminal fermentation (3, 13), probably into methane (3). Moreover, *Methanosarcina* spp. which may use methanol as a methanogenic substrate have been isolated from ruminal contents in several studies (1, 6, 9, 10).

However, Genthner et al. (6) isolated *Eubacterium limosum* from a sheep rumen and showed that this bacterium converted methanol mainly into acetate and butyrate. Conclusive quantitative data which ascertain the importance of *Methanosarcina* spp. or *E. limosum* in the rumen are lacking, however.

Therefore, we performed experiments to determine the ultimate fate of methanol in the rumen of hay-concentrate-fed sheep before and after adaptation to methanol infusion and to pectin administration.

Sheep were fed concentrates (100 g) and hay (400 g) twice daily. Sheep 15 received 100 g of pectin via its fistula just after the morning feed. Sheep 16 was continuously infused with a solution of methanol (1 M) at a rate of 19 ml/h. A 500-ml volume of ruminal contents was obtained before the morning feed, mixed, and filtered through gauze (1-mm pore diameter), and 40-ml portions were anaerobically (flushing with CO_2) dispensed in incubation flasks containing 10 ml of Burroughs saline (10% [vol/vol]) (2). Methanol was added by syringe through a septum after a preincubation period of about 10 min. Incubations were performed in a shaking water bath at 39°C. For zero-rate determinations, single gas samples were analyzed hourly during the first 5 h. For endpoint determinations, the analysis was done in duplicate.

Methanosarcina sp. was enriched on a medium with acetate as the sole organic substrate under an atmosphere of 80% N_2–20% CO_2 (11) and was subsequently isolated in agar roll tubes with the same medium; yeast extract, tryptone soy broth, and fatty acids were omitted from the medium.

Methane, hydrogen, and volatile fatty acids were determined as described previously (5). Methanol was analyzed as described by Van der Meijden et al. (12). Ruminal fluid volumes of sheep 15 and 16 were estimated to be 8 to 11 liters after a single injection of polyethylene glycol, analyzed as described by Decuypere et al. (4). The degree of esterification of pectin was 48%, which was determined as described by Katan and Van de Bovenkamp (7).

Five sheep (no. 2, 3, 14, 15, and 16) were used to determine the basal ruminal rate of methanogenesis from methanol. This was estimated by the difference between the zero rates of methane production with and without addition of methanol (1,230 μmol) to in vitro incubations. Virtually linear curves were obtained during several hours when ruminal fluid was used from animals which had been fasted for 24 h. All sheep showed rather low rates of methanogenesis from methanol. For sheep 2, 15, and 16 the maximal amounts of methane produced as a result of methanol addition were not obtained until after 2 days, and the corresponding molar ratios of methane produced to methanol added (CH_4/CH_3OH) were calculated (Table 1).

Infusion of methanol into the rumen of sheep 16 during a period of over a month resulted in a strong increase in the rate of methanogenesis from methanol, which was measured from day 5 on (Table 1). The maximal in vitro rates obtained, about 2 mmol/liter per h, could account for fermentation of the infused amount of methanol (19 mmol/h) in the rumen.

Pectin administration (about 540 mmol/day) to sheep 15 during the same period had the same effect, although apparently to a lesser extent. In this respect it should be noted that the theoretical maximum amount of methanol formed in the rumen from added pectin is about 260 mmol, which is about half the amount infused in sheep 16 and will therefore support less growth of methanol-fermenting organisms. The low value at day 37 may be explained by some residual ruminal pectin, resulting in the high background methanogenesis observed. The CH_4/CH_3OH ratios for sheep 15 and 16 after 5 days of adaptation were determined after 24-h incubations (Table 1).

After 9 days of adaptation, methanol utilization in sheep 16 was followed in vitro for 5 h (Fig. 1). Exhaustion of the limiting amount of methanol (370 μmol) coincided with slowing down of methanogenesis. The ratio of methane produced to methanol consumed was 0.77. This ratio and the ratios obtained as described above indicate a quantitative conversion of methanol into methane according to the stoichiometry $4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O$, as has been established for *Methanosarcina* cultures on methanol. The low partial pressure of H_2 in the present incubations (25 Pa) is comparable with that in the rumen and obviously does not lead to a direct reduction of methanol, as observed for *Methanosarcina* cultures under high H_2 pressure (8) and which would result in a ratio of about 1. The fact that 2-bromoethanesulfonic acid (150 μM), a specific inhibitor of methanogenesis, led to a complete inhibition of methanol consumption within 1 h after its addition (results not shown) further sustains the conclusion from the present results that methanol is exclusively converted into methane in the rumens of the sheep examined.

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TABLE 1. Ruminal in vitro methane formation

Sheep no.	No. of days[a]	CH_4 (μmol/ml per h)			Maximal amt of CH_4 formed[b]	Ratio, CH_4/CH_3OH[c]
		$-CH_3OH$	$+CH_3OH$	Difference		
2	0	1.24	1.50	0.26	970	0.79
3	0	2.25	2.34	0.09		
14	0	2.00	2.25	0.25		
15[d]	0	1.02	1.23	0.21	970	0.85
	5	0.81	2.12	1.31	963	0.78
	9	0.79	2.28	1.31		
	37	1.63	2.11	0.48		
16[e]	0	1.23	1.38	0.15	1,044	0.79
	5	0.76	2.50	1.74	840	0.68
	9	0.97	3.22	2.25	285	0.77
	15	1.44	1.82	0.38		
	37	1.16	2.87	1.71		

[a] Days of adaptation.
[b] Maximal amount of methane formed in the incubation.
[c] Ratio of methane formed to methanol added in the incubation (see the text), except for sheep 16 at day 9, where the methanol used is expressed.
[d] Sheep 15 received pectin.
[e] Sheep 16 was infused with methanol.

The organism most probably involved in this conversion is a *Methanosarcina* species which could be enriched and isolated on a medium with acetate as the sole growth substrate. Identification was based on its morphology and its fluorescence under UV light. The methanogen formed clumps of cells typical for *Methanosarcina* sp. which settled down very quickly. This made accurate counting and representative sampling from ruminal contents impossible and may be at least partly responsible for wide variations in the rates of methanogenesis observed. Unfortunately, the organism was lost before being studied further.

Any influence of methanol conversion on ruminal fermentation could not be detected from molar percentages of volatile fatty acids, which ranged between normal values: 72.1 to 78.4, 13.9 to 17.6, and 6.7 to 11.9 for acetate, propionate, and butyrate, respectively. An effect might have been expected if substantial secondary fermentation of acetate into methane by *Methanosarcina* sp. took place as observed by Rowe et al. (10), or if acetogenic methanol-utilizing organisms like *E. limosum* were involved as suggested by Genthner et al. (6). This should have resulted in an increased butyrate percentage.

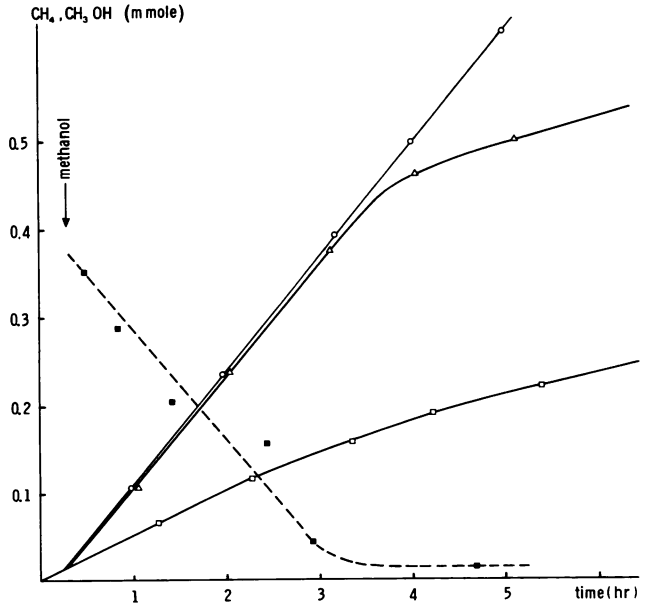

FIG. 1. Methane formation (——) and methanol utilization (----) in incubations of ruminal fluid of sheep 16 after 9 days of adaptation. Symbols for additions: □, none; ○, 1,230 μmol of methanol; △, 370 μmol of methanol. In the later incubation methanol was measured (■).

The results of this study and studies in which *Methanosarcina* spp. were enumerated (6, 9) suggest that these species are the predominant methanol-utilizing bacterium under normal feeding conditions. The aberrant nature of molasses diets may explain the great variability in the ruminal methanol-utilizing microbial population on these diets. While Rowe et al. (10) found *Methanosarcina* sp. as a large population (10^9/ml), Genthner et al. (6) and, very recently, Vicini et al. (14) found *E. limosum* in much higher numbers (10^8 to 10^9/ml and 10^5 to 10^6/ml, respectively) than *Methanosarcina* sp. (0 and 10^3 to 10^4/ml, respectively).

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