NECESSITY OF READY ELECTRON DISPOSAL AND INTERSPECIES HYDROGEN TRANSFER FOR THE UTILIZATION OF ETHANOL BY RUMEN BACTERIA

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Summary

Ethanol was utilized by mixed rumen microbes, but addition of pentachlorophenol (25 mg/l), a methanogen inhibitor, suppressed the utilization of ethanol. Carbon monoxide (50% of the gas phase), a hydrogenase inhibitor, more strongly suppressed the utilization of ethanol, propanol, and butanol. These results suggest that the major ethanol utilizers are H_2 producers. Ethanol utilization was depressed at low pH (below 6.0). Since methanogens were shown to be relatively resistant to low pH, it appears that ethanol utilizers are particularly sensitive to low pH. *Ruminococcus albus* and *R. flavefaciens* in mono-culture produced ethanol from carbohydrate (glucose and cellobiose), even when a high level (170 mM) of ethanol was present. Ethanol was not utilized even in the absence of carbohydrate, but the co-culture of these bacteria with methanogens resulted in the utilization of ethanol, i.e., when H_2 was rapidly converted to CH_4, *R. albus* and *R. flavefaciens* utilized ethanol. These results suggest that ethanol is utilized when the electrons liberated by the oxidation of ethanol are rapidly removed, and ready electron disposal in ethanol-utilizing, H_2-producing bacteria is accomplished by the interspecies transfer of H_2.

(**Key Words**: Rumen Microbes, Ethanol, Interspecies Hydrogen Transfer)

Introduction

Alcoholic drinks have long been used in some districts of Japan for fattening beef cattle, expecting that ethanol may stimulate appetite and improve meat quality, i.e., increase the degree of marbling and the tenderness of meat. Recent examinations suggested that ethanol improves the meat quality of Japanese black cattle (Tsuyoshi, et al., 1990).

Ethanol ingested by ruminants is utilized to some extent in the rumen (Moomaw and Hungate, 1963; Hungate, 1966a; Orskov et al., 1967). We have confirmed that ethanol is metabolized by mixed rumen microbes *in vitro*, and mainly acetate is formed with small amounts of butyrate, valerate and caproate (Miyazaki et al., 1991). However, it is not known at present which microbes are responsible for the utilization of ethanol. Protozoa were unable to utilize ethanol (Miyazaki et al., 1989), and no single species of bacteria in pure culture utilized ethanol to any significant extent (Hino et al., 1991). We tried to isolate bacteria that possess high capacity to utilize ethanol, with the results in vain (Hino et al., 1991). These facts led us to the postulation that ethanol is utilized only when mixed microbes are present.

Some rumen bacteria form ethanol from carbohydrate as an electron-sink product (Hungate, 1966b). Iannotti et al. (1973) reported that *Ruminococcus albus* produces ethanol from glucose in mono-culture, but in the co-culture with *Wollinella (Vibrio) succinogenes*, a H_2 utilizing bacterium, *R. albus* does not produce ethanol. Electrons liberated by the oxidation of glucose are readily used to form H_2, since H_2 formation proceeds when the partial pressure of H_2 is extremely low (Wolin, 1975). This suggests that ethanol is formed when the "electron pressure" in the cells is high.

Since ethanol should be utilized by the reverse reaction, it may be possible that ethanol is metabolized when the "electron pressure" is low. Such a situation may be given when readily fermentable carbohydrate is not available, and electrons liberated by the oxidation of ethanol

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to acetate are disposed of by forming H_2 (figure 1). Most of the rumen bacteria known to produce ethanol produce H_2 (Hungate, 1966b).

Thus, if ethanol-producing bacteria are incubated in the presence of H_2-utilizing bacteria, ethanol may be utilized. To the contrary, if H_2 production is inhibited, ethanol utilization may be suppressed. The purpose of this study was to examine these problems. Effect of low pH on ethanol utilization was also examined, since we supposed that the main rumen bacteria that may produce ethanol and H_2 are relatively sensitive to low pH.

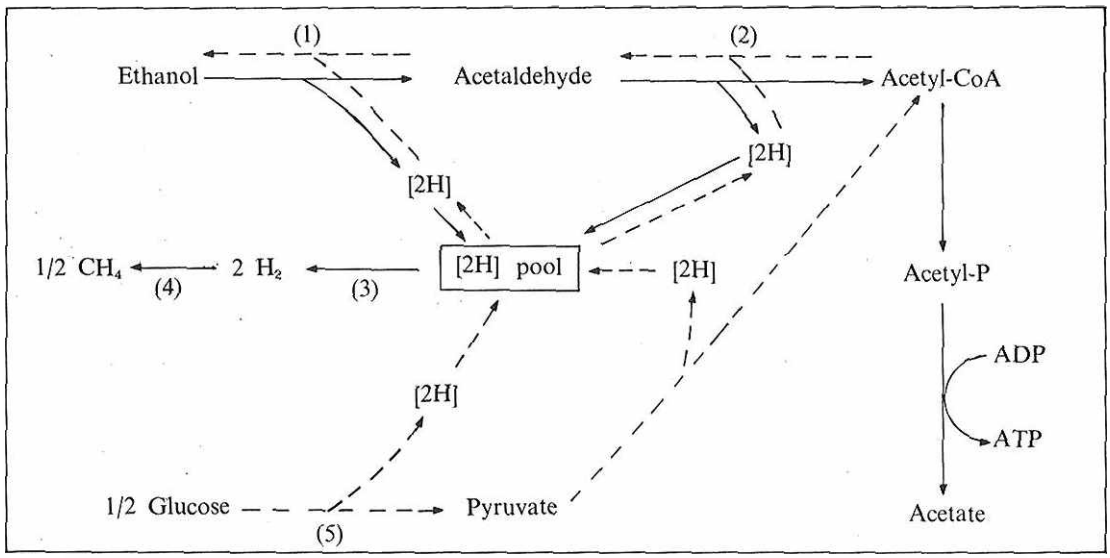

Figure 1. Main metabolic pathway and electron flow in the dissimilation of ethanol by *rumen microbes*. (Fermentation of glucose to ethanol is also shown: ----→)
(1) Alcohol dehydrogenase, (2) Aldehyde dehydrogenase, (3) Hydrogenase,
(4) Methanogenesis, (5) Embden Meyerhof pathway
$[2H] = 2e^- + 2H^+$

Materials and Methods

Source of mixed rumen microbes

A goat (body wt., 65 kg) fitted with a rumen fistula was given twice daily a diet of 350 g/d alfalfa haycubes, 350 g/d timothy hay, and 300 g/d commercial concentrate (formulated for dairy cattle). Rumen contents were taken before evening feeding, and squeezed through two layers of surgical gauze. This sqeezed rumen fluid was used as an inoculum for "mixed rumen microbes".

Incubation of mixed rumen microbes

Squeezed rumen fluid (mixed rumen microbes) was mixed with a basal medium (1:4), and the mixture was poured into 120-ml serum vials (60 ml/vial) under a stream of O_2-free CO_2, i.e., the headspace gas was 60 ml CO_2. Alcohols were anaerobically added using syringes. Pentachlorophenol (PCP), an effective methanogen inhibitor (Patel et al., 1991), was added before introducing the incubation mixture into vials: Diethyl-ether solution of PCP was added into vials, followed by evaporation of the solvent. In order to inhibit hydrogenase, carbon monoxide (CO, 60 ml/vial) was injected with a syringe, i.e., the gas phase was CO_2 and CO (1:1, 2 atm). In this experiment, 60 ml CO_2 was injected into control vials (2 atm).

The basal medium contained the following constituents (g/l): K_2HPO_4, 0.45; KH_2PO_4, 0.45; $(NH_4)_2SO_4$, 0.9; NaCl, 0.9; $CaCl_2 \cdot 2H_2O$, 0.12; $MgSO_4 \cdot 7H_2O$, 0.19; glucose, 1.25; Cellobiose, 1.25; Trypticase (BBL), 2.5; cysteine · HCl, 0.625. In this medium, the level of carbohydrate limits microbial growth; higher levels permit more growth. Unless otherwise stated, pH was adjusted to 6.9 with 8% Na_2CO_3.

Incubation was performed in triplicate for 24

h. Final pH after 24 h incubation was always 6.0-6.2. Details of the incubation procedures and conditions were described previously (Miyazaki et al., 1989; Hino et al., 1991). In the experiment dealing with the effect of pH, pH was readjusted every 6 h with anaerobic 8% Na_2CO_3 (prepared and stored under N_2).

Cultivation and incubation of known species of ethanol-producing bacteria and methanogenic bacteria

Ruminococcus albus NIAH-1188 was donated by Dr. H. Minato, and *Ruminococcus flavefaciens* FD-1 and *Streptococcus bovis* JB-1 were by Dr. K. Ogimoto. These three bacteria were grown in a medium composed of the basal medium described above and clarified rumen fluid (2 : 1). Clarified rumen fluid was prepared by centrifuging (12,000 × g, 15 min) squeezed rumen fluid.

Methanogenic bacteria were prepared as follows: Culture tubes (30 ml) specially manufactured for the cultivation of methanogens (Balch et al., 1979; Sanshin Kogyo, Inc., Japan) were used for selecting methanogens. Mixed rumen microbes were inoculated in the medium developed for the isolation and cultivation of methanogens (Balch and Wolfe, 1976; Lovley et al., 1984). The medium contained the following ingredients (g/l): KH_2PO_4, 0.45; K_2HPO_4, 0.45; $(NH_4)_2SO_4$, 0.45; NaCl, 0.9; $MgSO_4 \cdot 7H_2O$, 0.18; $CaCl_2 \cdot 2H_2O$, 0.012; $NaHCO_3$, 2.5; sodium formate, 2.5; sodium acetate, 2.5; Trypticase, 2.0; sodium selenite, 2×10^{-4}; coenzyme M (sodium 2-mercaptoethanesulfonate), 4.9×10^{-5}; vitamin solution and trace mineral solution (Balch et al., 1979); VFA (volatile fatty acid) mixture (Lovley et al., 1984); cysteine · HCl, 0.5; $Na_2S \cdot 9H_2O$, 0.5; clindamycin · HCl, 2.3×10^{-8}; vancomycin, 9.8×10^{-8}.

The medium (10 ml/tube) was introduced into culture tubes under CO_2 and subsequently twice the headspace-gas volume of H_2 (40 ml) was injected using a syringe, i.e., the gas phase was CO_2 and H_2 (1:2, 3 atm). Every week, one drop of the culture was withdrown with a syringe and transferred to fresh medium. This procedure had been continued for more than several months before the selected methanogens were subjected to the experiment. Microscopic examination showed that at least most of the bacteria were methanogens (Doddema and Vogels, 1978). These crude methanogens were grown in a larger amount (30 ml) in 120-ml serum vials, and used for the experiment described below.

R. albus, *R. flavefaciens*, and *S. bovis* were grown to full growth, and each of the cultures (30 ml) was combined with an equal volume (30 ml) of the methanogen culture at full growth. After the addition of 0.2% ethanol and adjustment of pH to 6.9, the mixtures were incubated for 24 h. The gas phase of this co-culture was CO_2. In the mono-culture of *R. albus*, *R. flavefaciens* and *S. bovis*, an equal volume of the uninoculated medium for methanogens was added. In the case of methanogens, an equal volume of the uninoculated medium for the other three bacteria was added.

Determinations of VFA, H_2, CH_4, and alcohols

VFA, H_2 and CH_4 were determined by GLC as described previously (Hino, 1981). Analytical procedure of alcohols was similar to that of VFA except that the column was packed with Flusin P coated with PEG 6000 (Gasukuro Kogyo Inc., Japan). Details were reported previously (Hino et al., 1991). Glucose and cellobiose in the cultures were estimated by TLC separation as described previously (Hino et al., 1991).

Results and Discussion

Effects of PCP and CO on the utilization of ethanol by mixed rumen microbes

As shown in table 1, methane production was significantly decreased by 25 mg/l PCP with concurrent accumulation of H_2. This concentration of PCP did not significantly affect H_2 production from carbohydrate fermentation by *R. albus* and *R. flavefaciens* (table 2), indicating that 25 mg/l PCP does not greatly inhibit H_2 production. Ethanol utilization and acetate production were also reduced by 25 mg/l PCP (table 1). These results suggest that the inhibition of methanogenesis lowers H_2 formation due to the accumulation of H_2, which in turn suppresses the fermentation of ethanol, i.e., the removal of H_2 by methanogens is important for the utilization of ethanol.

At 50 mg/l PCP, ethanol utilization, methane production, and acetate production were more severely suppressed. Less H_2 was accumulated at this level than at 25 mg/l PCP, indicating that H_2 production was also inhibited at high levels of PCP. It is obvious that the direct inhibition

TABLE 1. EFFECT OF PCP ON ETHANOL UTILIZATION, GAS PRODUCTION AND VFA PRODUCTION BY MIXED RUMEN MICROBES[a]

Ethanol addition (m mol/l)	PCP addition (mg/l)	Ethanol utilization (m mol/l)	Gas production (m mol/l)		VFA production (m mol/l)				
			H_2	CH_4	Acetate	Propionate	Butyrate	Others[b]	Total
34	0	5.3	0.1	9.6	27.6	7.6	6.3	2.6	44.0
	25	3.7*	2.2**	7.0*	23.2*	7.1	6.8	2.1	39.2
	50	1.2**	1.2*	5.5**	21.6**	5.1*	7.8*	1.3*	35.8*
0	0		0.0	7.8	22.0	8.4	6.9	2.3	39.7
	25		0.1	7.4	20.4	8.3	7.3	1.9	37.9
	50		1.3*	6.5*	19.3	5.2*	9.3*	1.0*	34.8

[a] Incubated with or without 0.2% (34 mM) ethanol in a carbohydrate-limited growth medium (5.5 mM glucose and 2.75 mM cellobiose) for 24 h.

[b] valerate + caproate + isobutyrate + isovalerate + 2-methylbutyrate

*, **: Inhibition by PCP is significant, evaluated by the t test (n = 3, * $p < 0.05$, ** $p < 0.01$).

TABLE 2. EFFECT OF PCP ON H_2 PRODUCTION FROM CARBOHYDRATE BY *R. albus*, AND *R. flavefaciens*[a]

PCP (mg/l)	H_2 Production (m mol/l)	
	R. albus	*R. flavefaciens*
0	2.15	1.83
25	1.97[b]	1.80[b]

[a] Incubated for 24 h in the rumen fluid medium described in the text.

[b] The difference from control is not significant statistically.

of H_2 production severely depresses ethanol utilization. Irrespective of the presence or absence of ethanol, propionate and other VFA were decreased by 50 mg/l PCP, while butyrate was increased. This is inexplicable at present.

Acetate production in cultures with ethanol was suppressed more greatly than that without ethanol (table 1). As described in the introductory paragraph, it may be likely that ethanol was fermented after sugars had been consumed. The observed results that PCP suppressed ethanol metabolism may be attributable to the effect in the later stage of incubation. When sugars are fermented, electrons can be used by forming electron-sink products other than H_2. On the other hand, electrons from ethanol oxidation should be used exclusively for H_2 formation, because little intermeditates are available to accept electrons. Therefore, it seems to be reasonable to infer that the effect of PCP is greater on the fermentation of ethanol than sugars.

Ethanol utilization was markedly suppressed by CO, with simultaneous suppression of methane production (table 3). In this case, H_2 was not accumulated, since CO is a hydrogenase inhibitor. The utilization of propanol and butanol was similarly inhibited by CO, indicating that these lower alcohols are metabolized in a similar manner by mixed rumen microbes (Hino et al., 1991). The inhibition by CO was greater in the conversion of alcohols to the corresponding VFA, compared to the case of carbohydrate fermentation (table 3, None). As mentioned above, in the metabolism of alcohols, there may be no feasible means for the rapid disposal of electorons other than H_2 formation.

These results suggest that H_2-producing bacteria play the major role in the utilization of alcohols, and electron disposal by means of H_2 production is necessary for fermenting alcohols.

Effect of pH on the utilization of ethanol by mixed rumen microbes

Figure 2-A shows that ethanol utilization was slightly decreased at pH 6.2, and markedly depressed below pH 6.0. Methane production was exactly paralled to ethanol utilization. However, methane production from H_2 by methanogens was not significantly suppressed at pH 6.2, and suppression below pH 6.0 was much less than

TABLE 3. EFFECT OF CO ON THE UTILIZATION OF ETHANOL, PROPANOL, AND BUTANOL BY MIXED RUMEN MICROBES[a]

Alcohol (m mol/l)	Gas phase	Alcohol utilization (m mol/l)	Methane production (m mol/l)	VFA production (m mol/l)				
				Acetate	Propionate	Butyrate	Others	Total
None	CO_2		10.6	26.5	16.1	11.4	1.7	55.7
	CO_2 + CO		6.5**	23.2	16.8	10.8	1.2	52.0
Ethanol (34)	CO_2	12.2	15.9	41.3	15.0	11.5	2.4	70.2
	CO_2 + CO	1.7**	5.0**	29.1**	14.5	9.4	3.0	56.0**
Propanol (27)	CO_2	7.7	13.6	28.4	24.2	11.5	1.4	65.5
	CO_2 + CO	1.1**	4.3**	25.0	17.2**	9.0	1.3	52.5**
Butanol (22)	CO_2	6.1	12.9	26.9	14.3	17.7	1.2	60.1
	CO_2 + CO	0.7**	4.5**	24.5	14.8	11.1**	1.1	51.5**

[a] Incubated with 0.2% alcohols in the medium noted in table 1 for 24 h.
**: Inhibition by CO is significant ($p < 0.01$).

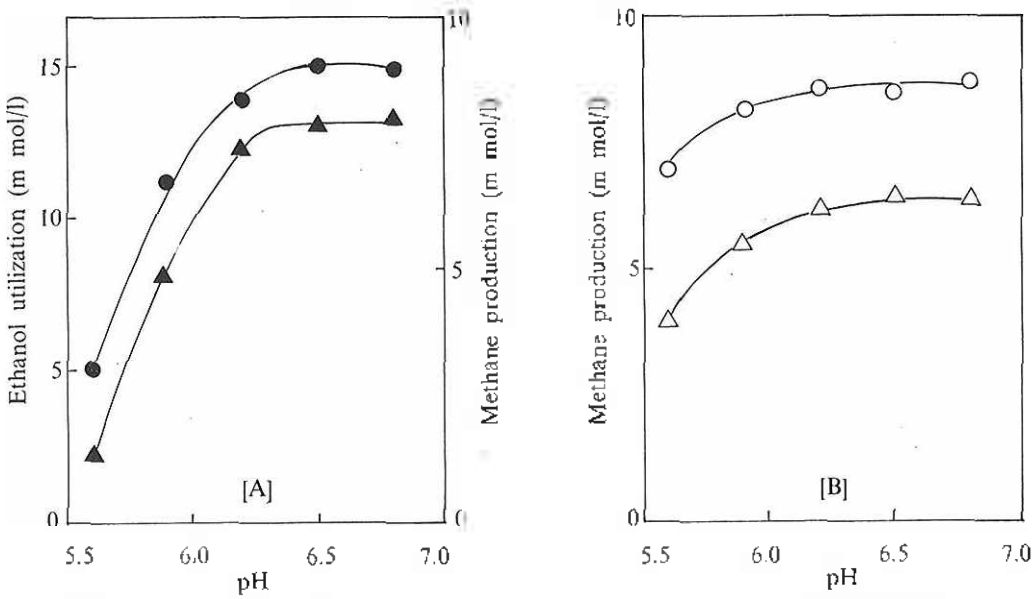

Figure 2. Effect of pH on the ethanol utilization and methane production by rumen microbes.
A. Ethanol utilization (●) and methane production (▲) by mixed rumen microbes incubated with 0.2% (34 mM) ethanol for 24 h.
B. Methane production by mixed rumen microbes incubated with 5.5 mM glucose and 2.75 mM cellobiose (○), and by methanogens incubated with H_2 + CO_2 (2:1, 3 atm) (△).

that observed for mixed rumen microbes (figure 2-B). This suggests that methanogens are relatively resistant to low pH. When mixed rumen microbes were incubated with carbohydrate, the effect of low pH on methane production was smaller than the case with ethanol (figure 2-B). These results indicate that ethanol-utilizing, H_2-producing bacteria are particularly sensitive to low pH.

Among the known species of ethanol-producing bacteria, cellulolytic bacteria are known to be sensitive to low pH (Russell and Dombrowski, 1980).

Effect of interspecies H_2 transfer on the utilization of ethanol

As shown in table 4, *R. albus*, *R. flavefaciens* and *S. bovis* produced ethanol from carbohydrate (glucose and cellobiose), even when as high as 1% (*ca.* 170 mM) ethanol was persent. After 24 h, substantially no sugars were detected, and the changes in ethanol concentration between 24 and 48 h were negligible, i.e., even after all the carbohydrate had been consumed, ethanol was not utilized.

TABLE 4. ETHANOL PRODUCTION BY *R. albus*, *R. flavefaciens* AND *S. bovis* CULTURED IN THE PRESENCE OF A HIGH CONCENTRATION OF ETHANOL[a]

Organism	Ethanol production[b] (m mol/l/d)	
	0-24 h	24-48 h
R. albus	0.65 + 0.06	0.07 ± 0.06
R. flavefaciens	0.53 ± 0.03	−0.02 ± 0.04
S. bovis	1.12 ± 0.12	0.09 ± 0.08

[a] Incubated with 1% (170 mM) ethanol in the medium noted in table 2 for 48 h. pH was adjusted to 6.7 after 24 h.

[b] Increase in ethanol in 24 h (mean ± SE, n = 3).

As shown in table 5, *R. albus*, and *R. flavefaciens* in mono-culture produced ethanol and evolved H_2. When these organisms were incubated in the presence of methanogens, ethanol was utilized and methane was produced without any accumulation of H_2. Since the methanogens used in this study did not utilize ethanol, these results suggest that rapid conversion of H_2 to CH_4 is necessary for the utilization of ethanol by *R. albus*, and *R. flavefaciens*.

On the other hand, *S. bovis* never utilized ethanol, even when methanogens were present. This can be explained by the fact that *S. bovis* does not produce H_2. Methane should have been produced from formate that had been formed by *S. bovis* through the pyruvate-formate lyase reaction. Since formate is not an electron-sink product, it is reasonable that the co-existence of methanogens did not have any significant effect on the utilization of ethanol by *S. bovis*.

TABLE 5. UTILIZATION OF ETHANOL BY *R. albus*, *R. flavefaciens* AND *S. bovis* IN THE PRESENCE OR ABSENCE OF METHANOGENS[a]

Organisms	Ethanol utilization[b] (m mol/l)	Gas production (m mol/l)	
		H_2	CH_4
Methanogens	0.00	0.00	0.67
R. albus	−0.44	1.54	0.00
R. albus + Methanogens	2.92**	0.00**	1.84**
R. flavefaciens	−0.37	1.78	0.00
R. flavefaciens + Methanogens	1.29**	0.00**	1.28**
S. bovis	−0.81	0.00	0.00
S. bovis + Methanogens	−0.62	0.00	0.89**

[a] Incubated with 0.2% (34 mM) ethanol in the medium noted in table 1 for 48 h. pH was adjusted to 6.7 after 24 h.

[b] Minus means production.

**: Effect of the co-existence of methanogens is significant ($p < 0.01$).

In conclusion, the main ethanol-utilizing bacteria in the mixed rumen microbes or in the rumen are H_2 producers, and it seems certain that as a whole interspecies H_2 transfer is necessary for the efficient utilization of ethanol.

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