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Inhibition of Rumen Methanogenesis by Methane Analogues

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Extremely low concentrations of chloroform and carbon tetrachloride and somewhat larger concentrations of methylene chloride inhibited the formation of methane by the rumen microbiota in the presence or absence of added substrate. The accumulation of hydrogen at these low concentrations indicates a selective inhibition of methanogenesis. Presumably, these inhibitors affect one or more of the reactions by which methane is formed from hydrogen and carbon dioxide.

The chief pathway of methane production in the bovine rumen is the reduction of carbon dioxide with molecular hydrogen (1, 3). In both rumen liquid and pure culture preparations (4), methane production is extremely sensitive to inhibition by oxygen. Wolin et al. (6) found that methane production by cell-free preparations of the culture known as *Methanobacillus omelianskii* was similarly affected by oxygen. Later, Wolin et al. (5) found that these same preparations were inhibited also by low concentrations of the viologen dyes, benzyl and methyl viologen.

The present work shows an inhibition by the chlorinated methanes.

MATERIALS AND METHODS

Rumen. Rumen contents were incubated in an 800-ml cylindrical gas washing bottle (Corning Glass Works, Corning, N.Y.) with a sintered-glass base through which gas was continuously circulated by means of a small pump. Controlled minute quantities of gas or liquid reagent could be injected via syringe through injection ports closed with a butyl rubber recessed stopper.

Rumen contents were freshly obtained from a cow fed on alfalfa hay. Rumen liquid was obtained by collecting rumen contents and incubating at 39 C until the particulate material either settled or was carried to the top by fermentation gases. The middle liquid containing bacteria with relatively few protozoa or food particles was drawn off and used, with or without addition of solids. When both liquid and solids were used, the material was designated rumen contents.

Small-scale experiments. To increase the number of experiments beyond those possible with the single gas recirculation apparatus, samples of rumen contents were also incubated in 150 by 16 mm roll culture tubes (Bellco, Glass, Inc., Vineland, N. J.) sealed anaerobically with recessed rubber stoppers. Substrates or inhibitors (0.1 ml) were pipetted into the tubes first, following the anaerobic culture-tube techniques of Hungate (3). A 10-ml amount of rumen liquid or rumen contents was then added to each tube which was stoppered anaerobically. The mixed microbiota protected the methanogenic bacteria during these operations, and the samples produced methane without appreciable lag. Except where indicated in the text, the gas phase in the tubes and in the recirculation apparatus was carbon dioxide. In some experiments, 1 g of total contents (solids) was substituted for 1 ml of the rumen liquid. Tubes were incubated at 39 C. In the tube experiments, 0.25 ml of gas was removed by syringe for analysis. Larger samples could be withdrawn from the recirculation experiments. Gases were analyzed at room temperature on a Perkin-Elmer Vapor Fractometer with a silica gel column and N_2 as carrier gas.

When formate was used, 0.1 ml of 1 M sodium formate was added to each tube.

Solutions of inhibitors. Carbon tetrachloride and methylene chloride were dissolved in absolute alcohol. Because 0.1 ml of ethyl alcohol occasionally stimulated methane production, a control tube with only ethyl alcohol added was run. Usually, the rate of methane production was identical with and without ethyl alcohol.

RESULTS AND DISCUSSION

The studies evolved from an accidental finding during an investigation of methane production by bovine rumen contents in vitro. Excessive foaming of the rumen liquid occasionally occurred in the gas circulation apparatus. On one such occasion, a small quantity of Antifoam A spray (Dow Corning, Corp. Midland, Mich.) was added. This reduced the foaming, but subsequent measurements disclosed that methane was no longer formed. The gas phase in this experiment con-

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sisted of 80% hydrogen and 20% carbon dioxide. In an attempt to investigate the antifoam effect further, the gas mixture was flushed out and replaced by carbon dioxide. Sodium formate was then fed into the incubation mixture at a constant rate of 10.2 μmoles per ml per hr. Normally, the addition of formate at this rate caused a constant rate of methane production while the concentration of hydrogen in the gas phase remained relatively constant (Fig. 1). With Antifoam A, however, no methane formed and hydrogen gas accumulated (Fig. 2).

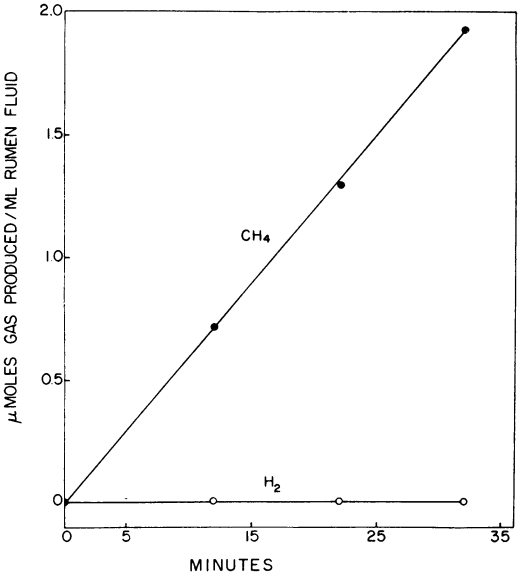

FIG. 1. *CH_4 and H_2 production from formate by rumen liquid. Gas circulation apparatus. Gas phase = CO_2. Formate feed rate = 10.2 μmoles per ml per hr.*

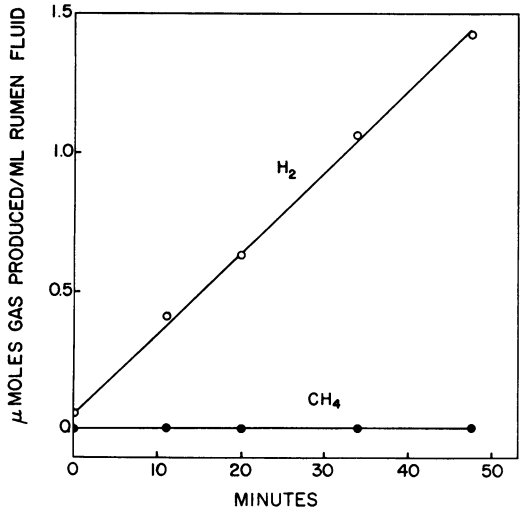

FIG. 2. *Effect of Antifoam A on CH_4 and H_2 production from formate. Experimental details as for Fig. 1.*

In rumen liquid, hydrogen is normally converted to methane. The Antifoam A preparation selectively inhibited methane formation and caused accumulation of the precursor hydrogen. This suggested inhibition at a site in the metabolic sequence between hydrogen production and its conversion into methane.

When a sample of Antifoam A was transferred to a beaker to quantitate its inhibitory ability, an odor resembling chloroform was detected. (This was in fact an incorrect conclusion. The Antifoam A spray, it was later learned, consisted of a silicone ingredient and a volatile Freon (CCl_2F_2) propellant. Although the active principle was not chloroform, it is probable that Freon acts in a similar manner.) The structural resemblance to methane supported the idea that chloroform might be the inhibitory agent in the antifoam.

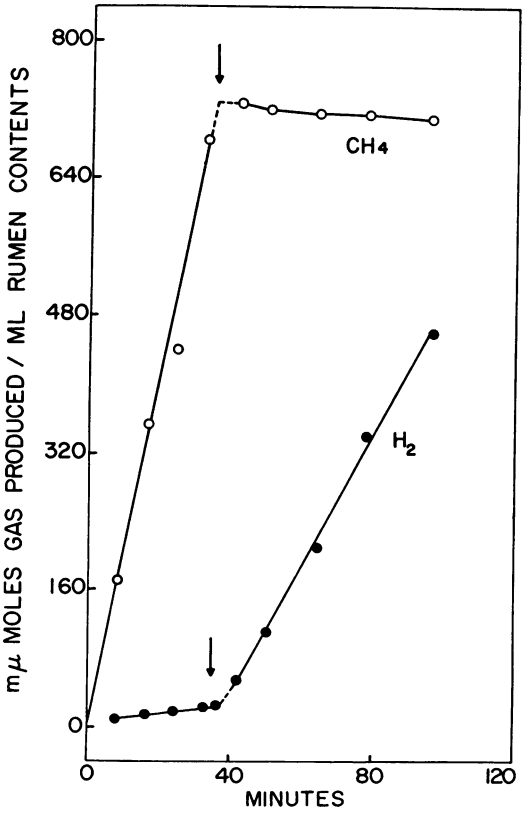

FIG. 3. *Effect of chloroform on CH_4 and H_2 production from rumen liquid plus solids. Chloroform = 20 mM. No formate added. Other experimental details as for Fig. 1. Chloroform added where indicated by the arrow.*

TABLE 1. *Effect of chloroform on the quantities of CH_4 and H_2 produced from solids in roll tubes*

Incubation time (hr)	Addition	Inhibitor concn (μM)	Gas produced (μmoles/ml)	
			H_2	CH_4
4	—	0	0.043	8.88
	0.1 ml of H_2O	0	0.026	9.92
	0.1 ml of $CHCl_3$ solution	490	4.31	2.25
11	—	0	0.064	15.66
	0.1 ml of $CHCl_3$ solution	49	6.15	1.46
	0.1 ml of $CHCl_3$ solution	490	7.07	1.36

This was tested by adding 0.5 ml of chloroform to 300 ml of rumen contents, liquid plus solids. Methanogenesis was completely inhibited and hydrogen accumulated (Fig. 3).

Similar results were obtained in experiments with rumen contents in tubes. Table 1 shows the H_2 and CH_4 produced during the incubation of rumen contents in tubes with and without chloroform. Also, in these experiments the chloroform inhibited methane production with hydrogen accumulation.

The accumulation of hydrogen was not stoichiometric with the equation $CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$. In experiment 1, the total hydrogen needed to account for the methane formed in the control was 35.6 μmoles/ml. With the inhibitor, 13.3 μmoles/ml was obtained. This suggests that chloroform, at these concentrations, also partially inhibits hydrogen production. An alternative explanation that the accumulated hydrogen

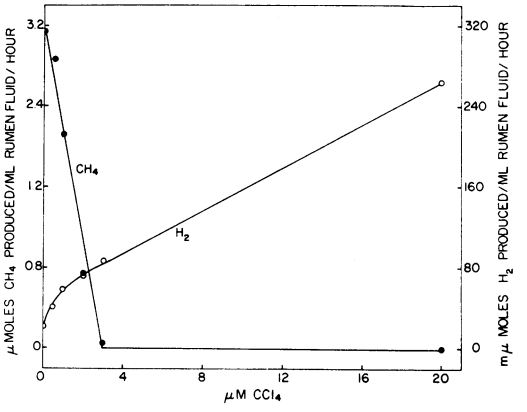

FIG. 4. *Effect of low concentrations of carbon tetrachloride on CH_4 and H_2 production from formate. Incubation was in tubes for a period of 1 hr during which time the rates of gas production were linear.*

TABLE 2. *Effect of chlorinated methanes on CH_4 and H_2 production from different substrates*[a]

Substrate added to rumen fluid	Inhibitor	Concn (mM)	Duration of expt (min)	Gas produced (μmoles per ml per hr)		Inhibition (%) of CH_4 production
				H_2	CH_4	
None	—	—	114	0.006	0.848	—
	CH_2Cl_2	15.8	114	0.012	0.080	91
	$CHCl_3$	0.5	114	0.007	0	100
	CCl_4	2.0	114	0.018	0.120	86
Rumen solids	—	—	180	0	1.72	—
	CH_2Cl_2	15.8	180	0.127	0	100
	$CHCl_3$	0.5	180	0.160	0	100
	CCl_4	2.0	180	0.212	0	100
Sodium formate	—	—	155	0	2.16	
	CH_2Cl_2	15.8	155	0.120	0.06	97
	$CHCl_3$	0.5	155	0.144	0	100
	CCl_4	2.0	155	0.200	0.04	98
Hydrogen	—	—	145	—	1.30	—
	CH_2Cl_2	15.8	145	—	0	100
	$CHCl_3$	0.5	145	—	0	100
	CCl_4	2.0	145	—	0	100

[a] Incubation was in roll tubes under an atmosphere of CO_2, except in the hydrogen experiment for which 80% H_2 and 20% CO_2 was used. Gas production was linear during at least the 1st hr of the incubation periods used. Sodium formate concentration was 10 mM.

was utilized in some other metabolic sequence was not explored. Diversion of hydrogen to propionate could be of considerable economic importance.

The related compounds methylene chloride and carbon tetrachloride produced effects similar to those obtained with chloroform (Table 2).

The rate of methane production by rumen fluid can be increased by the addition of formate, hydrogen, or simply the rumen solids which are the normal substrate. The inhibitors were effective also when methanogenesis was increased by adding the above substrates. Rumen fluid was incubated with added solids, with sodium formate, or under an atmosphere of 80% hydrogen and 20% carbon dioxide (Table 2). In each case, methane production was inhibited and hydrogen gas accumulated. With "solids" as substrate, formate and hydrogen were probably still the immediate precursors of methane. The accumulation of hydrogen from "solids" thus further illustrated the selectivity of this group of inhibitors for the methane-forming reactions.

The effect of adding low concentrations of carbon tetrachloride to rumen fluid is shown in Fig. 4. With increasing concentrations, the rate of methane production decreased and was accompanied by an increase in the rate of hydrogen production. However, again the amount of hydrogen evolved was less than that expected if it had been simply diverted from methane production.

Table 3 shows the inhibition of methanogenesis from formate by methylene chloride, chloroform, and carbon tetrachloride. Very low concentrations of chloroform and carbon tetrachloride were inhibitory. Methylene chloride was less effective. The concentrations of these compounds required to produce 50% inhibition of methane production were: 1.4 μM CCl_4; 7.8 μM $CHCl_3$; and 2.4 $\times$ 10^3 μM CH_2Cl_2.

TABLE 3. *Effect of low concentrations of chlorinated methanes on CH_4 and H_2 production from formate*

Addition	Concn (mM)	Duration of expt (min)	CH_4 produced (μmoles per ml per hr)	Inhibition (%)
CH_2Cl_2	0	90	2.81	—
	1.6	90	2.02	28
	2.0	90	1.40	50
	4.0	90	0.33	88
$CHCl_3$	0	79	2.60	—
	0.0049	79	1.90	27
	0.006	79	1.12	46
	0.012	79	0.54	79
CCl_4	0	60	3.13	—
	0.0005	60	2.86	9
	0.001	60	2.12	32
	0.002	60	0.74	76
	0.003	60	0.06	98

[a] Details as for Fig. 4 except where indicated in the table.

Inhibition by viologen dyes. Wolin et al. (5), working with *M. omelianskii*, demonstrated that low concentrations of methyl or benzyl viologen inhibited the formation of methane from ethyl alcohol and from a gas mixture of hydrogen and carbon dioxide. Hydrogen normally formed from ethyl alcohol accumulated in greater quantities when methane formation was inhibited by the viologen dyes.

Addition of benzyl viologen to rumen fluid produced similar effects. In one experiment (Fig. 5) with the gas circulation apparatus, benzyl viologen was added to the reaction mixture 36 min after the start of the experiment. The rate of methane production was immediately depressed, and at the same time the rate of hydrogen production increased. A concentration of 10 μM benzyl viologen had no effect on the methane production rate, whereas, in the experiments of Wolin et al. (5), 5 μM benzyl viologen completely in-

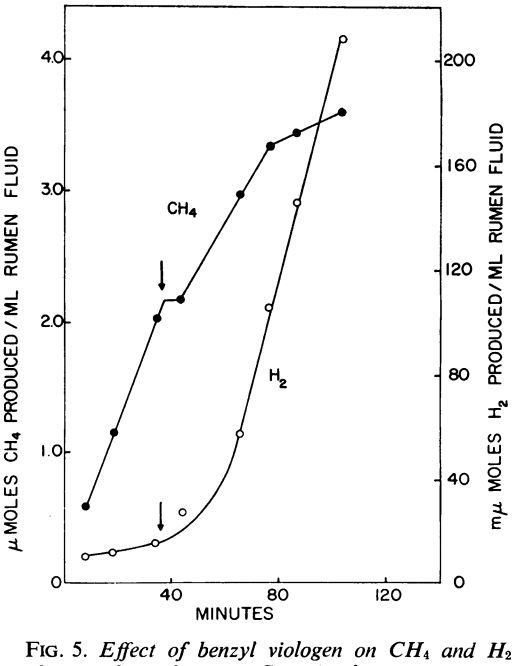

FIG. 5. *Effect of benzyl viologen on CH_4 and H_2 production from formate. Gas circulation apparatus. Gas phase = CO_2. Formate feed rate = 10.2 μmoles per ml per hr. A 1-ml amount of 40 mM benzyl viologen was added at the time shown by the arrow to give a final concentration of 133 μM.*

hibited methane production by washed cells of *M. omelianskii*. The difference may be a function of the mass of nonmethanogenic bacteria in the preparation, or of the lower sensitivity of the rumen methanogenic bacteria themselves.

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