

Effect of Chloral Hydrate on Methane and Propionic Acid in the Rumen

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Methane production from pyruvate by mixed rumen bacteria *in vitro* was nearly totally inhibited by chloral hydrate (0.1 μ mole/ml of incubation fluid). This effect was accompanied by an accumulation of gaseous hydrogen and an increase in propionic acid production. Infusion of chloral hydrate (4 g/day) into the rumen of a sheep produced the same effects. Evidence is presented for a direct toxic effect of chloral hydrate upon methane bacteria. Results are discussed in terms of fermentation balances.

In normal rumen fermentation, metabolic hydrogen produced during carbohydrate oxidation is used mainly in the production of methane, propionic acid, and butyric acid. Selective inhibition of methane production might be expected to be associated with an increase in the formation of at least one other reduced compound (10, 11, 18). In line with this theory, the production of methane and propionic acid varied in opposite directions when cell suspensions of rumen bacteria fermenting pyruvate were treated with unsaturated fatty acids (4). The same effect could be obtained *in vivo* by infusion of unsaturated fatty acids into the rumen of sheep (5a). These results encourage us to investigate the effect of chloral hydrate on production of methane and propionic acid by mixed rumen bacteria. Indeed, the stimulated production of propionic acid *in vitro* by this compound, reported by Prins (13), could be interpreted as a disposal of excess of hydrogen produced by inhibition of methane production. Furthermore, the same author reported that chloral hydrate can be converted to chloroform by rumen contents, a compound known to inhibit methane production *in vitro*. During this inhibition, hydrogen gas is formed (1).

The experiments described in this paper, carried out *in vitro* and *in vivo*, indeed established an opposite effect of chloral hydrate on methane production and production of propionic acid. In addition, hydrogen gas, a compound not normally accumulated in the rumen, accumulated in the presence of chloral hydrate.

MATERIALS AND METHODS

***In vitro* experiments.** Rumen contents were obtained from three fistulated wethers kept indoors and fed on hay (*ad libitum*) and concentrates (40 to 45% of carbohydrates other than crude fiber, 500 g/day). The animals were fed at 8:30 AM and 5 PM. Samples were withdrawn with the apparatus described by Hungate (9). The time period between feeding and sampling was at least 6 hr.

Cells suspensions. Cell suspensions were prepared as described elsewhere (4, 5). Total nitrogen content was determined on the suspension by the micro-Kjeldahl method and was usually between 0.9 and 1.5 mg of nitrogen per ml.

Incubations. A 5-ml (15 ml) amount of cell suspension was incubated with 5 ml (15 ml) of phosphate buffer (0.066 M, pH 6.9) containing formate or pyruvate as substrate. The incubation time varied from 3 to 15 hr as shown in the figures and tables. The incubation gases were N₂ or CO₂. Analysis of gases, volatile fatty acids, pyruvate, and formate was also carried out as described earlier (4, 5).

Assay of protein synthesis from NH₄HCO₃ in the presence of carbohydrates and of proteolytic activity with casein was carried out by using whole rumen liquid as described by Henderickx (7).

***In vivo* experiment.** The experimental animal was a 3-year-old fistulated wether which was kept in a metabolic cage and received 250 g of concentrates and 300 g of hay twice daily. Water was always available. Rumen gas expelled through the fistula by ruminal contraction and elevation of intraruminal pressure was collected over 0.1 N sulfuric acid as described elsewhere (5a). Gas production was measured 8 hr/day. Samples of rumen fluid (approximately 15 ml) were obtained through the fistula without disturbing gas collection and were used for the determination of volatile fatty acids after acidification and centrifugation (15 min; 20,000 \times g) as described by Van Eenaeme (17) and Cottyn (2). Samples were taken

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daily at 8:30 AM, 9:30 AM, 11:30 AM, 1:30 PM and 3:30 PM. Mean values were calculated for each day. Immediately after withdrawal of the samples, pH was measured by using a Radiometer 22 apparatus. The experiment involved two periods. In the first period of 18 days, no infusion was made; in the second period of 5 days, 4 g of chloral hydrate in 10 ml of 0.066 M phosphate buffer (pH 6.9) was infused daily through the fistula before the morning feeding.

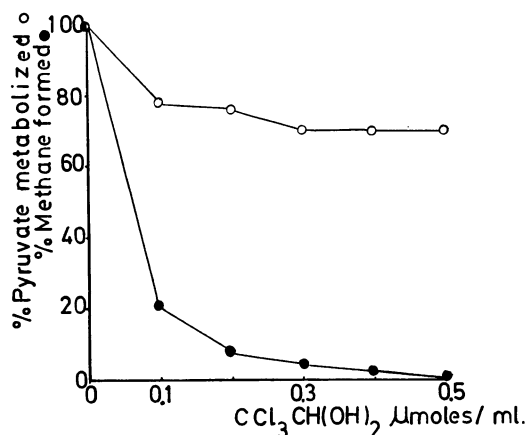


FIG. 1. Inhibition of methane production from pyruvate by chloral hydrate. A 5-ml amount of washed cell suspension was incubated with 5 ml of 0.066 M phosphate buffer (pH 6.9) containing 250 μmoles of pyruvate and increasing amounts of chloral hydrate. Time, 3 hr; gas, N₂. (Pyruvate metabolized in chloral hydrate-free control; 180 μmoles = 100%.)

RESULTS

In vitro. Addition of chloral hydrate to cell suspensions of rumen bacteria metabolizing pyruvate strongly inhibited methane production, whereas overall pyruvate breakdown was only slightly affected (Fig. 1).

Analysis of end products in large-scale incubations showed that inhibition of methane production was accompanied by an accumulation of gaseous hydrogen and an increase in propionic acid production. The latter effect was more pronounced when incubation time was increased to 15 hr or when CO₂ was used as incubation gas (Table 1). Addition of chloral hydrate to rumen bacteria metabolizing formate or gaseous H₂ + CO₂ also resulted in an inhibition of methane production (Table 2, Fig. 2).

Comparison with the effect of unsaturated fatty acids. The effect of chloral hydrate described here was very similar to that obtained with unsaturated fatty acids (4). However, important differences exist. On a molar basis, chloral hydrate was much more effective in inhibition of methane production than unsaturated fatty acids (Table 3).

It is also clear that addition of unsaturated fatty acids did not result in hydrogen accumulation for the concentrations used. Furthermore, both compounds showed striking differences in their effect on protein synthesis and proteolytic activity of rumen contents. Figure 3 shows that unsaturated fatty acids inhibit protein synthesis

TABLE 1. Effect of chloral hydrate on incubation of rumen bacteria metabolizing pyruvate as substrate

Incubation gas	Incubation time	CH added ^a	Pyruvate metabolized	Products formed ^b					
				CH ₄	CO ₂	H ₂	Acetic acid	Propionic acid	Butyric acid
N ₂	4	mM	μmoles						
CO ₂	4	mM	μmoles						

^a CH = chloral hydrate.

^b Expressed as micromoles formed per 100 μmoles of pyruvate fermented. A 15-ml amount of washed cell suspension was incubated with 15 ml of 0.066 M phosphate buffer (pH 6.9) containing 1 millimole of pyruvate. Values were corrected for blank incubations.

^c Mean value of at least three experiments ± SE.

^d Not determined.

and increase proteolytic activity, whereas chloral hydrate has exactly the opposite effect.

In vivo. The experiment in vivo confirmed the results obtained in vitro. Infusion of 4 g of chloral hydrate completely inhibited methane production (Fig. 4).

TABLE 2. Effect of chloral hydrate on incubations of rumen bacteria metabolizing formate as substrate

Formate (μ moles)		Products formed ^a					
Added	Fermented		Methane		Hydrogen		
	-CH	+CH	-CH	+CH	-CH	+CH	
553	530	199	17.6	0.3	0.1	20.7	
535	462	138	15.8	0.3	0.3	32.8	
520	490	178	19.8	0.1	0.5	30.5	

^a Expressed as micromoles formed per 100 μ moles of formate fermented. A 5-ml amount of washed cell suspension was incubated with 5 ml of 0.066 M phosphate buffer, pH 6.9. When added, 0.3 μ mole of chloral hydrate (CH) per ml of incubation fluid was present. Time, 3 hr; gas, N₂. All values were corrected for blank incubations.

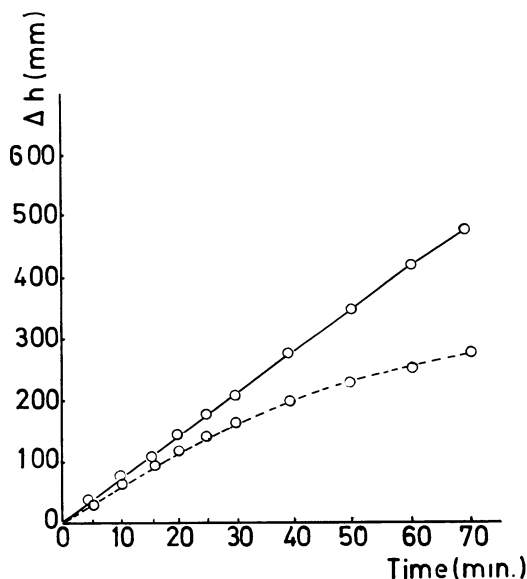


FIG. 2. Influence of chloral hydrate on methane production from CO₂ and H₂ (measured by pressure decrease). A 1-ml amount of washed cell suspension was incubated in a one side-arm Warburg vessel. After 15 min of equilibration, 0.5 ml of 0.066 M phosphate buffer, with or without 0.125 μ mole of chloral hydrate, was tipped in from the side arm. Readings were taken every 5 min; gas, 80% CO₂, 20% H₂.

TABLE 3. Comparison between the effect of chloral hydrate and linolenic acid on methane and hydrogen production in incubations of rumen bacteria with pyruvate

Addition	Products formed ^a	
	Methane	Hydrogen
None	14.3 ± 1.6 ^b	30.0 ± 5.0
CH (0.1 mM)	4.9 ± 1.4	2.0 ± 1.0
18:3 (2.0 mM)	9.1 ± 1.3	2.0 ± 1.0

^a Expressed as micromoles formed per 100 μ moles of pyruvate fermented. A 15-ml amount of washed cell suspension was incubated with 15 ml of 0.066 M phosphate buffer (pH 6.9) containing 1,000 μ moles of pyruvate, with and without chloral hydrate (CH 0.1 mM) or linolenic acid (18:3, 2.0 mM). Time, 3 hr; gas, N₂.

^b Mean value of three experiments ± SE.

This effect was observed as soon as 20 min after infusion. The total volume of gas collected remained unchanged, however, as methane was replaced by hydrogen. This is in contrast with results obtained earlier during infusion of unsaturated fatty acids (5a; Table 4). Changes in the proportions of rumen volatile fatty acids also followed a pattern similar to that obtained in vitro. Table 5 shows that the proportion of propionic acid increased whereas acetic acid decreased. A slight increase in butyric acid was observed. The pH and total volatile fatty acid concentration were not affected, indicating a net increase in propionic acid concentration.

DISCUSSION

As with unsaturated fatty acids, the results suggest a direct toxic effect of chloral hydrate on rumen methanogenic bacteria. Indeed, inhibition could be obtained in vitro with substrates specific for these bacteria, such as formate and gaseous carbon dioxide and hydrogen, whereas, in vivo, inhibition is almost instantaneous. It is striking that compounds differing so widely as chloral hydrate and long-chain unsaturated fatty acids have a similar effect on methane production. This observation does not support the hypothesis that depression of methane production is a function of molecules which have both polar and nonpolar characteristics (3).

The total effect on rumen metabolism is, however, different from that reported for unsaturated fatty acids (4, 5a). A linolenic acid concentration of 1.0 μ mole/ml of incubation fluid partially inhibits methane production in vitro, whereas with chloral hydrate, 0.2 μ mole/ml caused complete

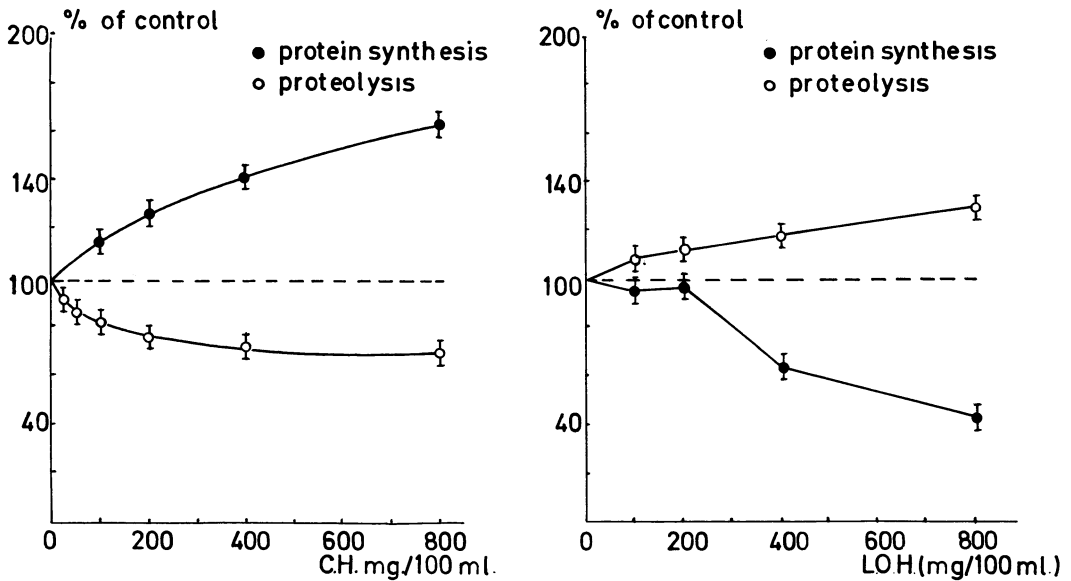


FIG. 3. Effect of chloral hydrate (C.H.) and linseed oil hydrolysate (L.O.H.) on protein synthesis from NH_4HCO_3 and proteolysis of casein in the artificial rumen. A 100-ml amount of strained rumen liquid was incubated with 10 ml of artificial saline and 90 ml of water. Incubation time, 4 hr; CO_2 bubbling. Substrate: 200 mg of casein or 140 mg of NH_4HCO_3 with 500 mg of glucose and 500 mg of soluble starch. Measurements of the increase or the decrease of the nitrogen soluble in $\text{Zn}(\text{OH})_2$. Mean values of at least three experiments \pm S.E.

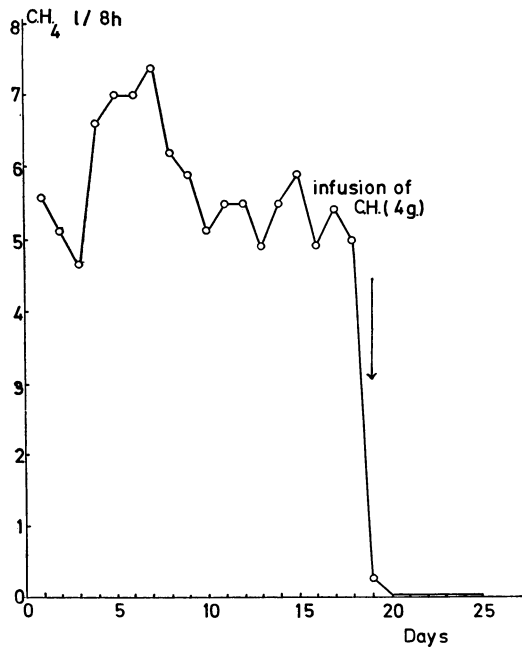


FIG. 4. Effect of infusion of chloral hydrate (C.H.) on daily methane production in vivo (measured 8 hr per day).

inhibition. For the unsaturated fatty acid concentration used, accumulation of hydrogen gas was never observed in vivo or in vitro. This accumulation was the major effect accompanying inhibition of methane production by chloral hydrate. This can explain the drop in rH value of rumen liquor observed by Prins (14) after administration of chloral hydrate to cows. Furthermore, with unsaturated fatty acids, the inhibition of methane and stimulation of propionic acid production in vivo and in vitro were always accompanied by a considerable decrease of butyric acid production (4, 5a). With chloral hydrate, the production of this acid was not always inhibited.

As in the case of unsaturated fatty acids, the increase in propionic acid production can be due to altered proportions of microorganisms in the rumen symbiosis. These proportions, however, are probably altered in a manner different from that brought about by unsaturated fatty acids. Indeed, in terms of fermentation balances, the excess of hydrogen resulting from inhibited methane production by chloral hydrate is not only disposed of as propionic acid but also as gaseous hydrogen. Furthermore, calculation of fermentation balances for pyruvate as described elsewhere (4) yielded too low hydrogen recoveries in the presence of chloral hydrate (Table 6).

TABLE 4. Effect of chloral hydrate (CH) on total daily gas production and per cent composition of gas in vivo

Period	Total gas production (liters/day)	% Composition				
		CH ₄	CO ₂	N ₂ ^a	O ₂ ^a	H ₂
Control (18 days)	22.5 ± 0.7	25.5 ± 0.2 ^b	57.6 ± 0.6	13.7 ± 0.4	3.2 ± 0.1	0
Infusion of CH (5 days)	22.1 ± 3.2	0	59.9 ± 0.7	11.9 ± 0.4	2.6 ± 0.1	25.6 ± 0.6

^a The reason for the rather high percentage of N₂ and O₂ is explained elsewhere (5a).

^b Mean value ± SE (control period, 18 determinations; infusion of CH, five determinations).

TABLE 5. Effect of chloral hydrate (CH) on pH, total concentration, and per cent composition of volatile fatty acids in vivo

Period	pH	Total volatile fatty acids (meq/100 ml)	% Composition				
			Acetic acid	Propionic acid	Butyric acid	Isovaleric acid	Valeric acid
Control	6.78 ± 0.02 ^a	9.5 ± 0.1	63.2 ± 0.2	23.6 ± 0.2	11.0 ± 0.1	1.2 ± 0.1	1.0 ± 0.02
Infusion	6.59 ± 0.03	9.0 ± 0.2	44.5 ± 0.6	38.9 ± 0.8	14.5 ± 0.4	0.5 ± 0.2	1.7 ± 0.3

^a Mean value ± SE (control period, 90 determinations; infusion of CH, 25 determinations).

This suggests that hydrogen is disposed of in the production of a compound(s) other than gaseous hydrogen or propionic acid. Indeed, small amounts of hydrogen probably used in reduction of chloral hydrate to trichloroethanol, methanol, and formaldehyde (13) are negligible. Again, this is in contrast with results for unsaturated fatty acids in which corresponding hydrogen and carbon recoveries were obtained (4). Although experimental errors can certainly be involved, it is tempting to speculate that this discrepancy is perhaps related to the difference in effect of chloral hydrate and fatty acids on protein synthesis and, hence, bacterial growth, as measured in vitro with whole rumen contents (Fig. 3).

Perhaps the most significant result of this work is the negative correlation obtained both in vitro and in vivo between propionic acid and methane production. Bauchop (1) obtained an inhibition of methane production by addition of methane analogues to incubations with formate as substrate. This inhibition was accompanied by a considerable accumulation of hydrogen gas. In view of the foregoing, it can be expected that these methane analogues will have a stimulating effect on propionic acid production. [While this paper was being reviewed, such a stimulation was reported for CCl₄ (16).]

On the other hand, it is possible that compounds such as potassium chlorate and propylene glycol (Baay, Ph.D. Thesis, State University,

TABLE 6. Hydrogen, carbon, and C₁ recovery in end products of pyruvate incubations^a

Incubation gas	Incubation time	CH added ^b	Hydrogen recovery (% of μmoles fermented)	Carbon recovery (% of μmoles fermented)	C ₁ recovery (100 × C ₁ observed/C ₁ calculated)
N ₂	hr	mm	96.1	104.2	89.0
		1.0	74.8	94.4	95.2
	15	1.0	99.3	103.3	96.8
		1.0	73.9	98.8	97.2
CO ₂	4	1.0	92.0	102.9	
		1.0	72.2	99.9	
	15	1.0	94.7	97.8	
		1.0	84.2	100.2	

^a Mean values reported in Table 1 were used to calculate fermentation balances as described elsewhere (4); 1 mole of hydrogen gas was assumed to originate from 1 mole of pyruvate.

^b CH = chloral hydrate.

Utrecht, 1959), which increased propionic acid production in the rumen, have an inhibitory effect on the methane formation.

Prins (13) reported an inhibited in vitro fermentation of glucose and cellulose by chloral hydrate, whereas unsaturated fatty acids inhibit glucose fermentation in vitro (4). In some cases, a decrease in rumen fermentation rate of crude fiber in vivo and in vitro was obtained by pelleting alfalfa (8, 15). This slower fermentation rate is

accompanied by an increase in the molar proportion of propionic acid (8). Furthermore, Ørskov et al. (12) calculated a decrease in the extent of digestible carbohydrate fermentation for rations yielding an increase in the molar proportion of rumen propionic acid. This tends to confirm that a decrease in the rate and (or) extent of rumen fermentation is associated with a microbial population producing more propionic acid, perhaps because of a larger relative availability of soluble carbohydrates.

In any event, the experiments reported here as well as in earlier work suggest that a negative correlation exists between the molar proportion of propionic acid in the rumen and methane production. Such a negative correlation was also obtained by Ørskov et al. (12) using data presented by Flatt et al. (6). However, before it can be stated that this correlation does occur in all the cases cited, much more experimental work is needed. Also, present knowledge of rumen metabolism does not suggest that such a correlation could be one of cause and effect.

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