The effect of inhibitors of methane production on fermentation pattern and stoichiometry in vitro using rumen contents from sheep given molasses

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1. The fermentation in the presence of four known methane inhibitors was investigated in rumen contents from sheep given molasses *ad lib.* and showing two different fermentation patterns.

2. The average hydrogen recoveries obtained with the high butyrate and high propionate patterns were 93 ± 2 and 95 ± 2% respectively.

3. Sodium sulphite, chloral hydrate and a hemiacetal of chloral and starch (HCS) all inhibited methane production, and were associated with an accumulation of hydrogen and lactate in rumen contents showing the high butyrate fermentation pattern. Propionate production was slightly stimulated.

4. In rumen contents showing the high propionate fermentation pattern, linseed-oil hydrolysate depressed methane production at high levels only, increased hydrogen and lactate production but depressed all other products, and HCS depressed methane production and slightly increased propionate production.

5. Low hydrogen recoveries in the presence of the inhibitors were probably associated with the utilization of metabolic hydrogen in reactions not accounted for in the scheme under investigation.

Improvement in analytical techniques for the estimation of the end-products of rumen fermentation, together with increased understanding of metabolic pathways, has led to the development of theoretical schemes to describe the conversion of substrates into products such as volatile fatty acids (VFA), methane, hydrogen and incorporation into microbial cells (Wolin, 1960; Hungate, 1966; Baldwin, Lucas & Cabrera, 1970; Hungate, Reichl & Prins, 1971).

Demeyer, Henderickx & Van Nevel (1972) have recently proposed a comprehensive scheme incorporating cell synthesis, which is based on a theoretical fermentation balance described by Wolin (1960). This has been tested using in vitro incubations with rumen contents taken from a sheep given hay and concentrates (Van Nevel, Demeyer & Henderickx, 1972). To enable conclusions on its general applicability, further experiments with rumen contents showing different fermentation patterns are necessary. Our investigation into the application of the scheme was done with rumen contents of sheep given molasses but presenting two different fermentation patterns, one high in butyrate, the other high in propionate. The effect of some known inhibitors of methane production (Czerkawski, 1969; Marty, 1972) on the stoichiometry underlying the scheme was also investigated. However, as the amounts of end-products obtained with animals given molasses were different from those found by Van Nevel *et al.* (1972), the general equations were expanded to account for lactic acid and valeric acid as additional end-products of fermentation.

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EXPERIMENTAL

Animals. Two wethers (B and C), individually penned and fitted with rumen fistulas, were given diets containing hay (200 g/d) and molasses (composition, g/kg): molasses 900; fish meal 20; urea 20; NaCl 5; vitamin and mineral mixture (Kowalczyk, Ramirez & Geerken, 1970) 5; water 50; *ad lib.* (an average of 2 kg/d). This molasses mixture had a dry-matter content of 665 g/kg. Two weeks before the experiments began, the fermentation pattern of sheep C was changed by infusing 1 kg molasses in 15 min on 3 consecutive d. The expected change in microbial population due to the lowering of the pH would allow the testing of the scheme with two fermentation patterns (one high in butyrate, the other high in propionate) on the same type of diet. Both animals maintained their respective fermentation patterns and similar dry-matter intakes over the experimental period of 10 weeks.

Incubations. Samples of rumen contents were withdrawn before the animals were given the hay, using the apparatus described by Hungate (1950). The samples were filtered through stainless-steel wire gauze (16 mesh) and 25 ml filtrate were transferred anaerobically (continuous flushing with CO_2) to an incubation flask containing 25 ml buffer solution (pH 6·9) (Burroughs, Frank, Gerlaugh & Bethke, 1950) and 5 mg nitrogen in the form of NH_4HCO_3. All incubations were carried out anaerobically (under CO_2) for 3 h at 39 °C in a water-bath fitted with a shaker. The evacuation technique of Umbreit, Burris & Stauffer (1959) was used to fill the vessels with CO_2. The substrate (0·7 mmol sucrose (AR grade, UCB, Brussels) + 0·2 mmol D(+)-glucose (bacteriological grade, Merck, Darmstadt) + 0·1 mmol D(−)-fructose (AR grade, Merck, Darmstadt)) was dissolved in 25 ml Burroughs' buffer solution, the inhibitors were added, and the mixture was emulsified with a Bühler homogenizer (Virtis 45, Virtis Co., Gardiner, NY) (50000 rev./min; 30 sec). The four known inhibitors of methane production were sodium sulphite, chloral hydrate, linseed-oil hydrolysate (LOH), and a hemiacetal of chloral and starch (HCS) (LOH was a generous gift from Oléochim N.V. Brussels, and HCS from Smith, Kline & French Laboratories, Philadelphia, Pennsylvania). Incubation flasks were fitted with a silicone rubber septum to permit gas sampling with a Hamilton gas-tight syringe (Micromesure, N.V., The Hague). Incubations were stopped by the injection of 1 ml H_3PO_4 (AR grade, Merck, Darmstadt).

Analytical techniques. Methane and hydrogen contents were determined by gas-solid adsorption chromatography (Demeyer & Henderickx, 1967), and total and individual VFA by gas–liquid chromatography (Cottyn & Boucqué, 1968). Lactic acid production was determined by the microdiffusion method described by Conway (1957).

Calculation of fermentation balance. The amounts of acetic (A), propionic (P), butyric (B), valeric (V) and lactic (L) acids produced were obtained by subtracting the amounts present initially in the rumen contents from those determined at the end of the incubation period. From these values, the theoretical amount of hexose (C_6) metabolized was calculated as

$$C_6 = \tfrac{1}{2}A + \tfrac{1}{2}P + B + V + \tfrac{1}{2}L$$

(Wolin, 1960). As comparison of fermentation patterns requires correction for differences in rate of fermentation, production of acids, CH_4 and H_2 were expressed as μmol/mmol C_6 metabolized. From these values metabolic hydrogen (2H) produced and recovered was calculated as follows:

$$\text{total 2H produced} = 2A+P+4B+3V, \quad (1)$$

2H recovered:

$$\text{in end-products} = 2P+2B+4V+4CH_4+H_2, \quad (2)$$

$$\text{in cells} = (A+P+B+V+\tfrac{1}{2}L)\times 30\times 0{\cdot}0061. \quad (3)$$

The total amount of hydrogen recovered was expressed as a percentage of hydrogen produced. The equations on which these calculations are based were reported in earlier work (Wolin, 1960; Demeyer & Henderickx, 1967; Demeyer, Van Nevel, Henderickx & Martin, 1970; Demeyer, Henderickx & Van Nevel, 1972; Van Nevel *et al.* 1972). As significant amounts of lactic acid and valeric acid were regularly detected, the formation of these end-products was incorporated into the scheme, based on the equations:

$$C_6H_{12}O_6 \text{ —— } 2CH_3CHOHCOOH, \quad (4)$$

$$CH_3CH_2COOH+CH_3COOH+4H \text{ —— } CH_3(CH_2)_3COOH+2H_2O. \quad (5)$$

It was assumed that 30 g cellular dry matter were formed/mol VFA (Baldwin, 1970; Van Nevel *et al.* 1972), requiring $30\times 0{\cdot}0061$ mol 2H (Demeyer, Henderickx & Van Nevel, 1972). The cellular dry-matter yield/mol lactic acid was assumed to be half the yield/mol VFA, because according to the Embden–Meyerhof pathway, only 1 mol ATP/mol lactic acid is produced, whereas 2 mol ATP are gained for each mol VFA produced (Walker, 1968).

RESULTS

Differences in the fermentation pattern of the rumen contents from the two sheep were reflected in the VFA composition (Table 1). Sheep B maintained a higher production of butyrate than propionate which was typical for animals given molasses (Marty & Preston, 1970); this situation was reversed in sheep C.

This reversal was even more apparent in the end-products of fermentation obtained in incubations without inhibitors; total VFA, acetate and propionate production/mmol C_6 fermented were significantly higher, and production of methane, hydrogen and butyrate were significantly lower in rumen contents of sheep C (Table 2).

Methane production for the two sheep was inversely related to propionate production and acetate production was inversely related to butyrate production (Fig. 1). Average hydrogen recovery was approximately 95% in both experiments.

The effect of inhibitors on the fermentation pattern is shown in Table 3 for Expt 3 (rumen contents from sheep B) and in Table 4 for Expt 4 (rumen contents from sheep C).

All inhibitors, at the concentrations used, considerably reduced or completely inhibited methane production in vitro in rumen contents from sheep B and this was

Table 1. *Amounts of lactic acid and volatile fatty acids (VFA), and molar proportions of VFA in sheep B and C with high butyrate and high propionate fermentation patterns respectively*

(Mean values with their standard errors for ten determinations)

	Lactic acid (mmol/l)		Total VFA (mmol/l)		Molar proportions of VFA (%)							
					Acetic		Propionic		Butyric		Valeric	
Animal	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Sheep B	0·456	0·028	37·44	6·12	69·9	2·1	12·6	0·7	14·8	2·5	2·7	0·6
Sheep C	0·568	0·032	44·57	4·57	66·6	2·1	20·3	2·9	10·7	0·9	2·1	0·4

Table 2. *Effect of high butyrate and high propionate fermentation pattern of sheep B and C, respectively, on the fermentation balance and end-products of in vitro incubations of rumen contents with sugars* (1700 *μmol hexose*)

(Mean values with their standard errors for ten incubations)

	Expt 1 (sheep B)		Expt 2 (sheep C)	
	Mean	SE	Mean	SE
Hexose theoretically metabolized (μmol)	1157·0	16·42	1113·1	30·42
End-products formed*				
Lactic acid	7·6	1·3	8·1	0·5
Methane	359·3	13·2	224·5	30·3
Hydrogen	17·3	3·1	5·3	1·4
Total VFA	1200·9	23·2	1674·7	45·4
Acetic acid	290·1	43·1	722·8	35·6
Propionic acid	119·4	9·8	633·6	81·7
Butyric acid	759·9	20·9	297·0	44·2
Valeric acid	31·3	5·7	21·2	4·2
Hydrogen recovery (%)	92·63	1·54	94·70	2·04

VFA, volatile fatty acids.

* All end-products are expressed in μmol/mmol hexose theoretically metabolized, calculated from the end-products, after subtracting the initial contents of VFA and lactic acid in 25 ml strained rumen contents.

accompanied by a considerable increase in hydrogen and lactate production. Propionate production was slightly stimulated and the amount of substrate fermented, as calculated from VFA and lactate production was slightly depressed. Hydrogen recovery dropped to an average of 85% in the presence of inhibitors; the lowest recovery was obtained with the highest concentration of sodium sulphite.

With rumen contents from sheep C only very high levels of LOH depressed methane production, resulting in an increase in lactate and hydrogen production, and a depression in the amounts of other products. Hydrogen recovery was the lowest measured. HCS again completely depressed methane production, but there was neither a depression in the production of VFA nor an accumulation of hydrogen nor lactic acid; propionate production was, however, increased.

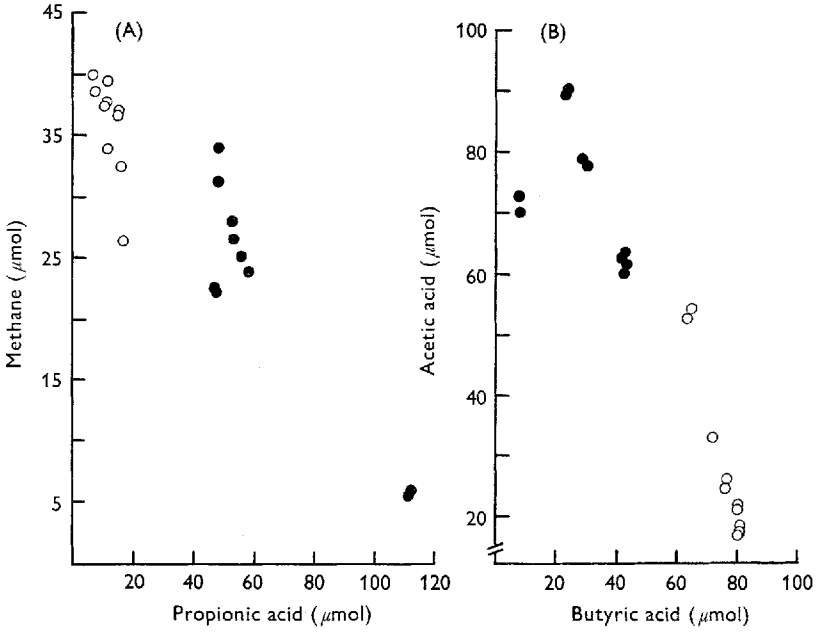

Fig. 1. Inverse relationship between methane and propionate production (A) and acetate and butyrate production (B), in vitro, in rumen contents from sheep B and C with high butyrate and high propionate fermentation patterns respectively. ○, Expt 1; ●, Expt 2.

Table 3. *Expt* 3. *Effect of inhibitors on the fermentation balance and end-products of in vitro incubations of sugars with rumen contents from sheep B*

Compounds tested	End-products formed (μmol/mmol hexose metabolized)*								Hexose fermented (μmol)	2H† recovery (%)
	Lactic acid	Methane	Hydrogen	Total VFA	Acetic	Propionic	Butyric	Valeric		
Sodium sulphite:										
Control	10	384	29	1154	214	104	808	28	1122	96·8
4 mmol/l	58	69	737	1099	142	114	816	26	1107	84·9
Control	5	368	14	1334	533	139	645	16	1201	87·5
8 mmol/l	223	1	799	1099	280	141	670	8	1059	78·4
Chloral hydrate:										
Control	8	361	9	1185	256	121	762	46	1122	94·6
2 mmol/l	283	0	741	929	7	135	763	25	976	86·3
Control	4	391	28	1163	257	73	777	56	1123	96·4
10 mmol/l	495	0	796	840	101	73	627	38	987	87·7
Hemiacetal of chloral and starch:										
Control	11	292	7	1169	190	159	808	12	1217	88·3
0·6 mmol/l	35	49	516	1092	26	194	857	16	999	82·6
1·2 mmol/l	48	14	628	1068	−26	210	869	15	914	84·1

VFA, volatile fatty acids.

* Calculated from the end-products, after subtraction of values for initial content of VFA and lactic acid in 25 ml strained rumen contents. Values are the average of four incubations.

† Metabolic hydrogen.

Table 4. *Expt 4. Effect of inhibitors on the fermentation balance and end-products of in vitro incubations of sugars with rumen contents from sheep C*

Compounds tested	End-products formed (μmol/mmol hexose metabolized)* Lactic acid	Methane	Hydrogen	Total VFA	Acetic	Propionic	Butyric	Valeric	Hexose fermented (μmol)	2H† recovery (%)
Linseed-oil hydrolysate:										
Control	7	326	14	1549	622	476	425	23	1000	99·9
2 mmol/l	21	290	24	1478	552	426	468	34	1179	96·2
Control	7	224	5	1534	608	467	440	18	1190	87·1
10 mmol/l	1049	29	183	732	344	168	235	−14	1033	72·5
Hemiacetal of chloral and starch:										
Control	11	57	1	1911	714	1118	79	−1	1025	103·5
3 mmol/l	8	0	2	1929	701	1156	70	−2	971	98·5
6 mmol/l	8	0	1	1920	707	1141	74	−2	990	97·2

VFA, volatile fatty acids.

* Calculated from the end-products, after subtraction of values for initial content of VFA and lactic acid in 25 ml strained rumen contents. Values are the average of four incubations.

† Metabolic hydrogen.

Table 5. *Effect of fermentation pattern on calculated hydrogen recovery for different in vitro incubations of hexoses with rumen contents from sheep*

(Mean values with their standard errors)

Substrate	Expt	(μmol/mmol hexose metabolized) Acetate Mean	SE	Propionate Mean	SE	Butyrate Mean	SE	Methane Mean	SE	2H* recovery (%) Mean	SE
Glucose	Van Nevel *et al.* (1972)	1250	30	430	50	160	20	530	40	102	4
Glucose + fructose + sucrose	Expt 1	290	40	120	10	760	20	360	10	93	2
	Expt 2	730	40	630	80	300	40	220	30	95	2

* Metabolic hydrogen.

DISCUSSION

The high values for hydrogen recovery in incubations without inhibitors were additional evidence for the validity of the fermentation balance scheme proposed by Demeyer, Henderickx & Van Nevel (1972), as they accounted for most of the end-products of carbohydrate metabolism. Hydrogen recoveries close to 100% were reported, with the same scheme, for in vitro fermentation of various sugars (Van Nevel *et al.* 1972), and in vitro fermentation of glucose in the presence of oxygen (Demeyer, Van Nevel & Henderson, 1972). In these earlier experiments the rumen contents of the animal, given hay and concentrates, had a high acetate fermentation pattern. In our investigation the scheme was tested with rumen contents showing a high butyrate or a high propionate fermentation pattern (Table 5). Furthermore, a high butyrate or high propionate fermentation pattern was established using the same substrate, but different sources of rumen contents. In this respect, our results

are similar to those described by O'Connor, Myers, Maplesden & Vander Noot (1971).

The production of total VFA in Expt 2 (1670 μmol/mmol C_6) was similar to the value reported by Van Nevel *et al.* (1972) (1870 μmol/mmol C_6) and that calculated from results presented by Czerkawski & Breckenridge (1969) (1780 μmol/mmol C_6). In Expt 1 the value was lower (1200 μmol/mmol C_6). The production of 1 mol butyrate requires 1 mol hexose, whereas the production of acetate or propionate requires 0·5 mol hexose. Since in these experiments the butyrate production was high one would expect low total VFA production per mol hexose. For methane production, our values of 220 and 360 μmol CH_4/mmol C_6 were lower than those of Czerkawski & Breckenridge (1969) (550 μmol) and of Van Nevel *et al.* (1972) (430 μmol), and were related to higher proportions of propionate and butyrate respectively. Furthermore, an inverse relationship between propionate and methane production was again apparent when results for the two experiments were pooled, although this relationship was not apparent from individual results from each experiment, where it was affected by variations in butyrate production and in the amount of metabolic hydrogen recovered (Van Nevel *et al.* 1972).

The inhibitory activity of the compounds tested merits little comment, as the results are similar to those reported by other workers, under different experimental conditions (Czerkawski, Blaxter & Wainman, 1966; Krabill, Alhassan & Satter, 1969; Van Nevel, Henderickx, Demeyer & Martin, 1969; Van Nevel, Demeyer & Henderickx, 1971; Trei, Scott & Parish, 1972). With most compounds, however, a less pronounced effect on propionate production was observed than could be expected from results obtained with animals given hay and concentrates (Demeyer, Van Nevel, Henderickx & Martin, 1969). Only HCS gave a consistent increase in propionate production, together with an inhibition of methane production.

It is interesting that there were lower calculated hydrogen recoveries in the presence of the inhibitors, as it indicates that metabolic hydrogen was used in reactions not accounted for by the scheme. Sodium sulphite (8 mmol/l) decreased the recovery to 78·4% and 10 mmol/l LOH decreased it to 72·5%. Both compounds can be reduced in the rumen (Lewis, 1954; Wilde & Dawson, 1966), which could account for the lower recoveries. Indeed, complete reduction of both compounds would require about 1·2 and 1·0 mmol 2H respectively and the actual amounts missing in the balance are approximately 0·9 and 0·6 mmol respectively. Furthermore, the accumulation of hydrogen, when methane production is inhibited, may induce its utilization in reactions not accounted for in the calculations. Low recoveries and accumulation of hydrogen were observed using chloral hydrate and HCS with rumen contents of sheep B (Table 3). These are not reduced in the rumen like sulphite or LOH and thus could not account for the missing hydrogen. On the other hand, using HCS with rumen contents from sheep C resulted in high recoveries and no accumulation of hydrogen was observed (Table 4).

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