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Competition for hydrogen by human faecal bacteria: evidence for the predominance of methane producing bacteria

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Abstract

Studies of sludge have shown that some species of sulphate reducing bacteria outcompete methane producing bacteria for the common substrate H_2. A similar competition may exist in human faeces where the methane (CH_4) producing status of an individual depends on the faecal concentration of sulphate reducing bacteria. To determine if non-methanogenic faeces outcompete CH_4 producing faeces for H_2, aliquots of each type of faeces were incubated alone or mixed together, with or without addition of 10% H_2 and/or 20 mmol/l sulphate. Methane producing faeces consumed H_2 significantly more rapidly and reduced faecal H_2 tension to a lower value compared with non-methanogenic faeces. The mixture of the two types of faeces yielded significantly more CH_4 than CH_4 producing faeces alone (mean (SD) 8·5 (1·3) *v* 2·9 (0·45) mmol/l of homogenate per 24 hours, $p<0·01$). Faecal sulphide concentrations were similar in CH_4 producing and non-producing homogenates both before and after 24 hours of incubation. The addition of sulphate to the homogenates did not significantly influence CH_4 production or sulphide formation. Our results suggest that in human faeces methane producing bacteria outcompete other H_2 consuming bacteria for H_2.

Methane (CH_4), a metabolic product of a group of anaerobic bacteria, is excreted consistently in appreciable quantity by some subjects but not others. In various population groups the prevalence of CH_4 excretors has been found to range from 24%[1] to 95%.[2] Since CH_4 is not metabolised in man, the ability of subjects to excrete this gas reflects the number or activity, or both, of the methanogenic flora present in the colon.[3] Why only select subjects harbour a CH_4 producing flora has piqued the interest of numerous investigators.

The sole source of energy of most species of methane producing bacteria is via the oxidation of H_2 produced by other organisms and the activity of methanogens is limited by low H_2 availability.[4-6] Methanogenesis consumes 4 moles of H_2 to reduce 1 mole of CO_2 to CH_4, a process that greatly reduces the volume of gas that would otherwise be present in the colon. Thus, understanding the factors that regulate the activity of the CH_4 producing flora could provide both clinically relevant information with regard to flatulence, as well as basic knowledge concerning the factors that regulate the proliferation and/or activity of colonic bacteria.

Sulphate reducing bacteria (SRB) also use H_2 to reduce sulphate to sulphide, and studies of sludge and sediments have shown the SRB outcompete methane producing bacteria for H_2 when adequate sulphate is available.[4-6] As a result, the presence of a high concentration of SRB limits methanogenesis. The mystery of why some subjects consistently excrete CH_4, while others do not, was apparently solved by Gibson and co-workers who carried out a number of studies suggesting that a similar competition between methane producing bacteria and SRB exists in the human colonic lumen.[7-9] Thus the inability of a subject to excrete CH_4 apparently reflects the presence of a non-methanogenic colonic flora that outcompete methane producing bacteria for H_2.

If this hypothesis were correct, it follows that CH_4 formation by CH_4 producing faeces should be reduced appreciably by admixture with non-methanogenic faeces, and this reduction should be reversed in the presence of a large excess of H_2. This concept was tested in the present study by incubating CH_4 producing and non-methanogenic faeces, individually or mixed together, with and without the addition of H_2 and/or sulphate. Surprisingly, we found that methanogenesis actually was enhanced by the presence of non-methanogenic faeces, even when large quantities of sulphate were provided for SRB metabolism.

Methods

FAECAL HOMOGENATES

We studied faeces from eight healthy adult volunteers who were all on a conventional diet and who had not taken antibiotics during the two months before the study. On the basis of previous results, faeces of four of them were known to produce large quantities of CH_4, while faeces of the other four produced little or no CH_4.

Faecal homogenates were prepared by homogenising faeces (1:5 w/v) in 0·1 M phosphate buffer (pH 7·0). Strict anaerobiosis was maintained during the procedure and all vessels, syringes, and solutions were exhaustively purged with argon before use. The four CH_4 producing faecal samples were paired with the four non-producing samples and a series of four experiments were then carried out in which faeces from the producer and the non-producer were incubated singly or mixed together. Incubations were carried out in 12 50 ml gas-tight syringes sealed with stopcocks. Four syringes contained 5 ml aliquots of the CH_4 producing homogenate plus 5 ml of phosphate buffer, four

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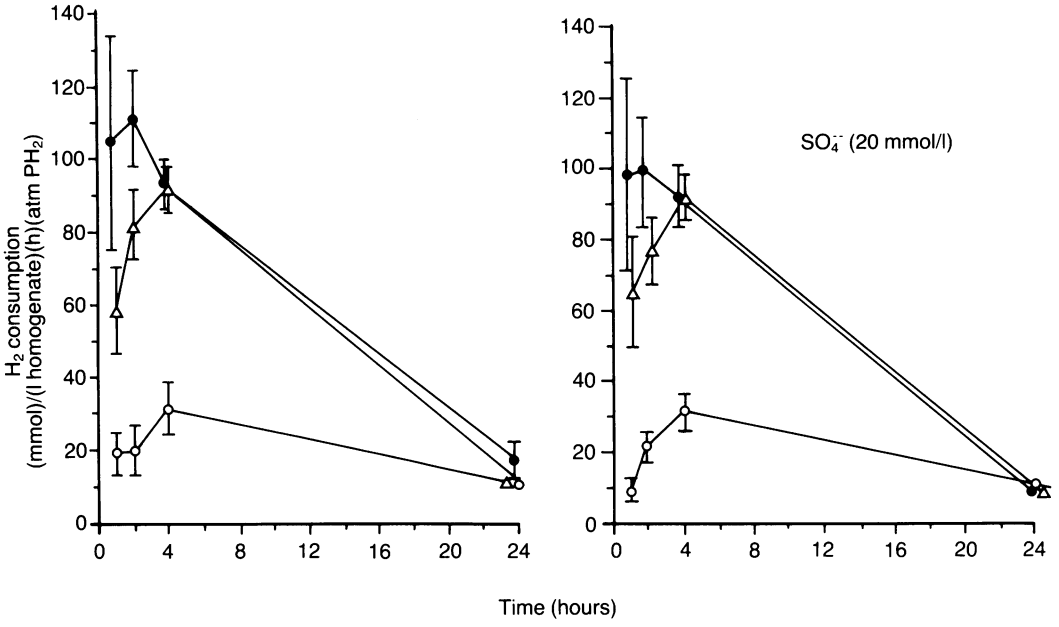

Figure 1: Hydrogen consumption (normalised for PH_2) by CH_4 producing faeces (●), non-methanogenic faeces (○), and by the mixture of the two types of faeces (△), during 24 hours of incubation with a gas space containing an initial H_2 concentration of 10%. The graph on the right shows the results obtained when the homogenates were supplemented with 20 mM Na_2SO_4. Data are represented as mean (SEM).

contained 5 ml aliquots of the non-methanogenic homogenate plus 5 ml of phosphate buffer, and four contained 10 ml of a mixture (1:1) of the two types of faeces. One of the following was then added to one of the four syringes that comprised the above sets: (a) argon (30 ml); (b) H_2 (3 ml) and argon (27 ml); (c) 20 mM Na_2SO_4 and argon (30 ml); or (d) 20 mM Na_2SO_4, H_2 (3 ml) and argon (27 ml). A rubber sleeve attached to a septum was slipped over the male end of the stopcock. At the time of removal of a gas sample from the syringe, the stopcock was turned from the position where the syringe was sealed, to a position where the syringe was open to the septum. A 21 gauge needle (attached to a 1 ml syringe) was then inserted through the septum and the stopcock into the gas space of the syringe, and a 1 ml gas sample was obtained.

Incubation was carried out at 37°C on a rotating wheel. Gas samples were obtained for analysis at 0, 1, 2, 4, and 24 hours of incubation. Aliquots of each homogenate were collected before and after 24 hours of incubation for sulphide analysis; 12% zinc acetate was anaerobically added to each aliquot in a ratio of 1:4 to prevent oxidation of sulphide.

ANALYSES

Gas samples were analysed for H_2 and CH_4 within six hours of collection using a gas chromatograph equipped with a molecular sieve column, a reduction detector for H_2, and a flame ionisation detector for CH_4.

The method of Cline for the measurement of sulphide in water was modified for faecal sulphide measurements.[10] Briefly, the homogenate was diluted 1:20 with distilled water and three aliquots of 0·909 ml were used. The first aliquot, that was treated with 0·72 µl of 50% HCl and vigorously stirred for 30 minutes to drive off all sulphide, served as a blank. The second was spiked with 18·2 µl of zinc acetate–sodium sulphide standard (2·6 mM) to evaluate sulphide recovery. The third aliquot was used for the determination of sulphide content of the specimen. The colorimetric reaction was carried out in 1·5 ml Eppendorf tubes that were immediately sealed following the addition of 0·80 µl of diamine–ferric chloride reagent made up in 50% HCl. At the time of reagent addition, 50% HCl (0·72 µl) was added to aliquots two and three and zinc acetate solution (18·2 µl) was added to aliquots one and three. After 30 minutes of incubation at room temperature, samples were centrifuged at 12 000$_g$ for three minutes and the absorbance of the supernatant was spectrophotometrically determined at 670 mµ. Percentage recovery of sulphide from spiked aliquots averaged 87% (range 73–99%). Sulphide concentration of a given sample of homogenate was calculated from the optical density of the sample minus that of the HCl treated sample, corrected for the percentage recovery determined from the spiked sample.

CALCULATIONS

The volume of H_2 or CH_4 present at any time point was calculated from the concentration of the respective gas and the volume of gas present in the syringe, plus the volume of H_2 or CH_4 calculated to have been previously removed for analysis. The consumption rate of H_2, determined from samples incubated with 10% H_2, was normalised for H_2 tension (PH_2) and expressed as µmol/hour per litre of homogenate per atmosphere of PH_2. The PH_2 of a given time period was considered equal to the arithmetic mean of the H_2 tensions at the beginning and end of the time period. Data were expressed as mean (SEM). Statistical analyses for significance were performed using the Student *t* tests for paired and for unpaired data.

Results

Figure 1 shows mean H_2 consumption (normalised for PH_2) by CH_4 producing faeces, non-methanogenic faeces, and the mixture of the two, in the absence and presence of additional Na_2SO_4. Methane producing faeces consumed

Methane formation by CH_4 producing faeces, non-methanogenic faeces, and by the mixture of the two during incubation with and without addition of H_2 and/or Na_2SO_4*

Faeces	Incubation (hours)	Substrate added: None	H_2 (10%)	Na_2SO_4 (20 mM)	H_2+Na_2SO_4 (10%) (20 mM)
CH_4 producing	1	0·30 (0·09)	1·35 (0·38)	0·23 (0·054)	1·26 (0·0045)
	2	0·54 (0·14)	1·98 (0·50)	0·43 (0·099)	1·89 (0·50)
	4	0·99 (0·22)	2·66 (0·41)	0·77 (0·14)	2·57 (0·45)
	24	2·88 (0·45)	4·37 (0·38)	2·25 (0·32)	4·14 (0·45)
Non-methanogenic	1	0·0 (0·0)	0·0 (0·0)	0·0 (0·0)	ND
	2	0·0 (0·0)	ND	ND	0·0059 (0·0045)
	4	ND	ND	ND	0·0086 (0·0068)
	24	0·013 (0·012)	ND	0·0027 (0·0026)	0·020 (0·011)
Mixture	1	0·68 (0·15)	1·22 (0·36)	0·72 (0·18)	1·35 (0·54)
	2	1·26 (0·18)	2·16 (0·54)	1·22 (0·25)	2·25 (0·54)
	4	2·30 (0·13)	3·87 (0·54)	2·12 (0·36)	3·51 (0·72)
	24	8·55 (1·26)	10·4 (1·3)	7·20 (1·49)	8·55 (1·62)

*Data are expressed as average (SEM) in mmol/l homogenate.
ND = <0·0045 mmol CH_4/l of homogenate.

H_2 significantly more rapidly than non-methanogenic faeces during the time periods 0–1 hour ($p<0·05$), 1–2 hours ($p<0·001$), and 2–4 hours ($p<0·001$). The mixture of the homogenates had a H_2 consumption rate comparable to that of CH_4 producing faeces, and significantly ($p<0·01$) higher than that of non-methanogenic faeces at 1, 2, and 4 hours of incubation. The addition of Na_2SO_4 had no statistically significant effect on H_2 consumption by any of the homogenates at any sampling time. After 24 hours of incubation the P_{H_2} of the CH_4 producing homogenates (1950 (325) ppm) was much lower ($p<0·0001$) than that of the non-methanogenic homogenates (39 200 (4600) ppm). The P_{H_2} reached in the mixture of homogenates (2900 (450) ppm) was comparable to that of the CH_4 producing homogenates and significantly lower ($p<0·0001$) than that of non-methanogenic homogenates. Similar results were obtained in the presence of added Na_2SO_4.

The mean CH_4 production by the different incubates is summarised in the Table. Trivial CH_4 production occasionally was observed in faeces considered to be non-methanogenic, but the highest value did not exceed 1% of the values observed with CH_4 producing faeces or with the mixture. This very low production was not significantly enhanced by the addition of 10% H_2 to the gas space, in contrast to the increase found with CH_4 producing homogenates.

In the absence of added H_2, CH_4 formation by CH_4 producing faeces was not inhibited by admixture with non-methanogenic faeces, but rather was enhanced in each of the four pairs of homogenates. This enhancement was statistically significant after 2, 4 and 24 hours of incubation (Fig 2). When H_2 was added, the increase in CH_4 production was statistically significant only after 24 hours. The addition of Na_2SO_4 had no significant effect on CH_4 production by any of the homogenates (Fig 2 and Table).

Before incubation, sulphide concentration averaged 0·18 (0·043) mM for non-methanogenic faeces and 0·15 (0·047) mM for CH_4 producing faeces (NS). Compared to the non-supplemented homogenates, neither the addition of 10% H_2, Na_2SO_4, nor both significantly influenced sulphide concentrations (Fig 3) after 24 hours of incubation. The tendency for faecal sulphide concentration to increase with incubation did not reach statistical significance in either the CH_4 producing or non-methanogenic homogenates. The greatest increase (0·17 mmol/l homogenate) was found in CH_4 producing faeces supplemented with sulphate. This sulphide production would have consumed only about one twentieth of the H_2 consumed via CH_4 formation.

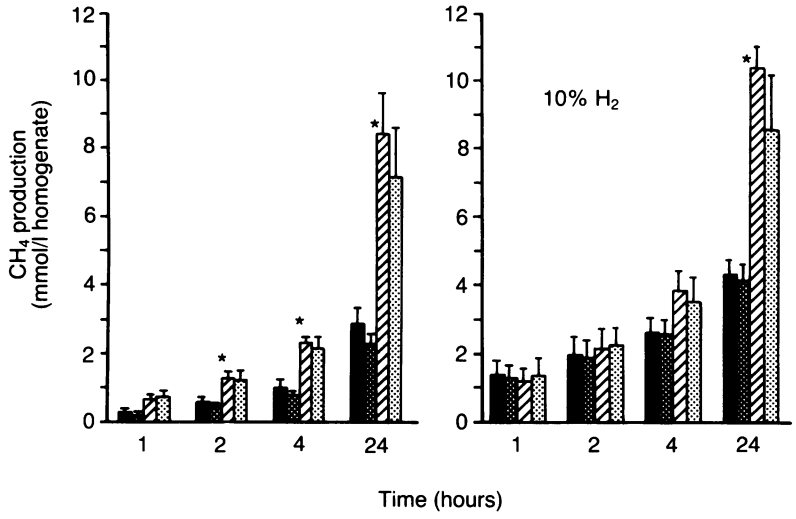

Figure 2: Methane production (mean (SEM)) by faecal homogenates during 24 hours of incubation without (left) and with 10% H_2 (right). From left to right, the four bars at each time point respectively represent: (a) CH_4 producing faeces without supplement; (b) CH_4 producing faeces supplemented with 20 mmol/l NA_2SO_4; (c) mixture of CH_4 producing faeces with non-methanogenic faeces without supplement; (d) mixture of CH_4 producing faeces with non-methanogenic faeces supplemented with 20 mmol/l Na_2SO_4. Asterisks indicate a significant difference ($p<0·02$) between the CH_4 producing homogenates and the mixture.

Discussion

The findings of our study sharply contrast with previous reports[7-9 11] suggesting that the absence of CH_4 production in the colon of certain individuals reflects the presence of high concentrations of organisms, such as SRB, that outcompete methanogens for H_2. These reports have shown that CH_4 producing faeces usually contained less than 10^7 SRB/g dry weight while non-methanogenic faeces always contained more than 10^7 SRB/g dry weight,[9 11] and that the sulphide concentration of CH_4 producing faeces was much lower than that of non-methanogenic faeces.[9] In addition, incubation of CH_4 producing with non-methanogenic faeces was reported to inhibit CH_4 formation.[7]

Our study provided two independent lines of evidence that led us to conclude that competition for H_2 does not explain why some subjects fail to excrete CH_4. First, if a lack of CH_4 production reflects very rapid H_2 consumption by non-methanogenic bacteria, one might expect that faeces that did not produce CH_4 would consume H_2 more efficiently than CH_4 producing faeces. To the contrary, we found that added H_2 was consumed about five times more rapidly by CH_4 producing faeces (see Fig 1). More important, after 24 hours of incubation, CH_4 producing faeces reduced the P_{H_2} of the homogenate to one twentieth of that observed in non-methanogenic faeces. Since the two type of faeces have been shown to have similar absolute H_2 production rates,[12] methanogens apparently are able to consume H_2 at a lower P_{H_2} than other H_2 consuming bacteria. These results agree with the in vivo observation that CH_4 producing subjects excrete

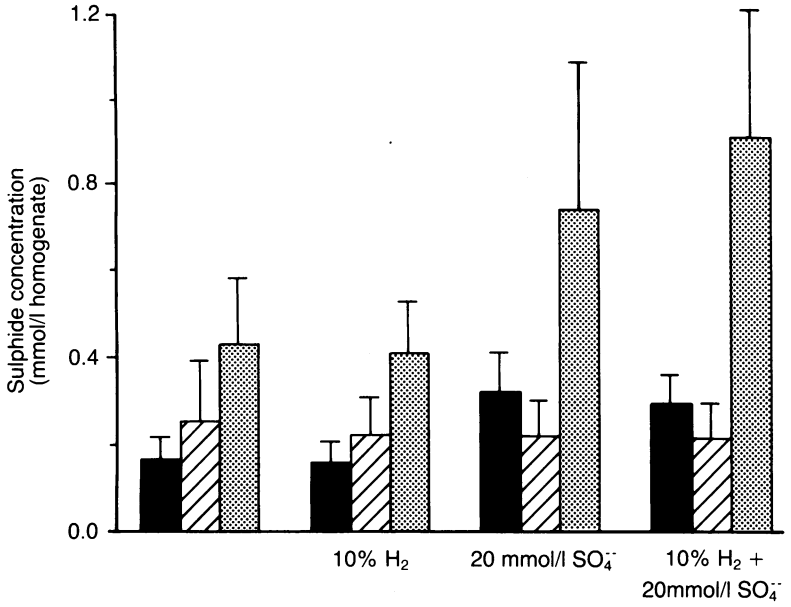

Figure 3: Sulphide concentration (mean (SEM)) after 24 hours of incubation without addition of either H_2 or sulphate, or with addition of 10% H_2, 20 mmol/l Na_2SO_4, or 10% H_2 plus 20 mmol/l Na_2SO_4. From left to right the series of three bars represent: CH_4 producing faeces, non-methanogenic faeces, and the mixture of the two types of faeces.

less H_2 than non-producers, both in the fasting state and after ingestion of non-absorbed carbohydrate.[13 14]

Second, the addition of non-methanogenic homogenates to CH_4 producing homogenates did not inhibit CH_4 formation, but indeed, roughly doubled it (see Fig 2). The most likely explanation for this result is provided by the finding that the addition of H_2 to the incubates significantly increased CH_4 production, indicating that H_2 availability was the rate limiting step in methanogenesis. Therefore the enhanced CH_4 production observed in the faecal mixture presumably resulted from the ability of the methanogens to pirate the additional H_2 liberated from the non-methanogenic homogenate.

While we did not enumerate SRB in our faecal samples, Gibson *et al*[11] demonstrated very high concentrations of these bacteria in the faeces of 17 consecutive subjects who did not produce CH_4. Since the rate of H_2 consumption by SRB is dependent on the availability of sulphate, we excluded the possibility that a lack of sulphate was limiting H_2 consumption by incubating each pair of homogenates in the presence of 20 mM sulphate. Sulphate addition did not affect the rate of H_2 consumption (Fig 1) and did not significantly reduce the rate of CH_4 production (Fig 2), although there was a trend in that direction. Therefore, the reported ability of sulphate feeding to stop CH_4 production in some subjects[15] presumably must be attributed to some inhibitory effect on methanogenesis rather than to the provision of substrate for H_2 consumption.

Our measurements of sulphide concentrations in freshly passed faeces differed appreciably from results reported by Gibson and co-workers in that our values were roughly 10 times higher and we did not find a significant difference between CH_4 producing and non-producing samples. These discrepancies presumably are attributable to our modifications of the standard technique for sulphide measurement in water[10] that made this technique more suitable for faecal analysis. We also found that the addition of sulphate (20 mM) and/or H_2 (10%) did not result in a significant increase in faecal sulphide concentration after 24 hours of incubation. Since sulphide may be converted to other compounds in faeces, sulphide concentrations are not a stoichiometric measure of sulphate reduction. However, to the extent that faecal sulphide is a semiquantitative indicator of sulphate reduction, it appears that this reaction may not have been a major route of H_2 consumption in our non-methanogenic (or CH_4 producing) homogenates.

We conclude that the methane producing bacteria present in human faeces outcompete other H_2 consuming organisms for H_2. This concept is compatible with the reported inverse relation between the faecal concentration of methane producing bacteria and SRB. However, in contrast to the prevailing hypothesis, the presence or absence of faecal methanogens would regulate SRB concentrations rather than vice versa. The burning question of what factor produces a colonic ecosystem favourable to methanogens still remains a 'mystery inside an enigma' (W S Churchill, unpublished observation, 1939).

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