Role of dietary sulphate in the regulation of methanogenesis in the human large intestine

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Abstract

Hydrogen produced during colonic fermentation may be excreted, or removed by H_2 consuming bacteria such as methanogenic and sulphate reducing bacteria. In vitro, sulphate reducing bacteria compete with methanogenic bacteria for hydrogen when sulphate is present. In this study the hypothesis that sulphate in the diet could alter CH_4 production in vivo has been tested. Six methane excreting volunteers were fed a low sulphate diet (1.6 mmol/d) for 34 days with the addition of 15 mmol sodium sulphate from days 11-20. Breath methane was measured and viable counts and metabolic activities of methanogenic bacteria and sulphate reducing bacteria determined in faeces. Whole gut transit time and daily stool weight were also measured. When sulphate was added to the diet, breath methane excretion decreased in three of the subjects while faecal sulphate reduction rates rose from 7.5 (0.5) to 20.3 (4.3) nmol SO_4 reduced/h/g faeces. Sulphate reducing bacteria, which were not detected during the control diet, were found and viable counts of methanogenic bacteria fell from 10^7 - 10^8 /g faeces to 10^6 /g. Methanogenic counts and breath CH_4 recovered after sulphate addition was stopped. No change was found in the other three subjects. Faecal weights and transit times were not different between study periods. It is concluded that methanogenesis is regulated by dietary sulphate if sulphate reducing bacteria are present. Dietary sulphate may allow growth of sulphate reducing bacteria which inhibit the growth of methanogenic bacteria. This may explain the absence of CH_4 in the breath of many people in western populations.

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The large intestine salvages energy from dietary residues and endogenous organic matter not absorbed in the small intestine, through fermentation by colonic bacteria.¹ This process generates short chain fatty acids which are absorbed, and hydrogen and carbon dioxide. Most people excrete hydrogen in breath and flatus but in 30-50% of western populations and in 80-90% of rural black Africans, methane (CH_4) is also excreted.²⁻⁵ CH_4 is produced by reduction of CO_2 with H_2 by methanogenic bacteria and epidemiological studies show that the carriage of these bacteria is determined in early childhood. No dietary factors have been shown to affect colonisation of the large intestine with methanogens.^{6,7}

Molecular hydrogen is produced by oxidation of $NADH_2$ and $FADH_2$ generated during

bacterial glycolysis. High partial pressures of H_2 , however, slow bacterial hexose breakdown by impairing regeneration of NAD.^{8,9} Energy efficient anaerobic ecosystems therefore usually have H_2 consuming organisms. In the rumen, H_2 is consumed by methanogenic bacteria⁸ and similarly, in methanogenic human subjects, 75% of the hydrogen excreted is as methane.¹⁰ In non-methanogenic subjects, sulphate reducing bacteria consume some of the hydrogen produced in fermentation.¹¹ In vitro data show that sulphate reducing bacteria outcompete methanogens for H_2 but only if sufficient sulphate is available.¹²

Sulphate availability may therefore be important in determining methanogenesis in man. Sulphate concentration in the colon is largely dependent on dietary intake.¹³ Changes in dietary sulphate could alter the activity of sulphate reducing bacteria and thus affect methanogenesis in the colon. To test this hypothesis, six methanogenic subjects have been fed a low sulphate diet for 34 days supplemented with sodium sulphate from day 11 to 20. Breath methane was monitored and metabolic activities of methanogenic bacteria and sulphate reducing bacteria studied in faecal samples.

Methods

SUBJECTS

Six healthy subjects (two men, four women, age 26-61 years) who had no history of gastrointestinal disease or use of antibiotics for at least three months took part in the study. All excreted methane in their breath at least 5 ppm above background.

STUDY DESIGN

The study was conducted in the metabolic suite of the MRC Dunn Clinical Nutrition Centre, Cambridge. The subjects were fed a controlled low sulphate diet for 34 consecutive days. From day 11 to day 20, 15 mmol sodium sulphate was

TABLE 1 Mean breath CH_4 (A) and H_2 (B) concentrations, ppm (SEM) during the last three days with basal diet and with 15 mmol sodium sulphate added in responders (n=3) and non-responders (n=3)

	Basal diet	High sulphate	Basal diet
A:			
Responders	22.2 (7.2)*	1.5 (0.2)*	17.0 (4.1)
Non-responders	21.6 (4.1)	22.7 (7.5)	21.0 (7.2)
B:			
Responders	14.7 (5.8)	19.7 (9.8)	16.0 (4.5)
Non-responders	25.5 (6.5)	19.9 (6.2)	21.4 (7.9)

*Significant difference ($p < 0.025$) between basal diet and high sulphate.

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added to the diet. End expiratory breath samples were collected three times daily throughout the study and analysed for hydrogen and methane concentration. On days 10, 20, and 34, a faecal sample was collected for measurement of bacterial counts and metabolic activity. Transit times were determined using a multiple dose marker technique. Informed consent was obtained and the study protocol was approved by the ethical committee of the MRC Dunn Nutrition Unit.

DIET

The diets were prepared in the metabolic kitchen of the unit. They consisted of three different, nutritionally balanced menus which were served in a three day rotation. Average nutrient composition (% energy) was protein, 15%; carbohydrate, 55%; and fat, 29%. The sulphate content of this diet has previously been measured at 1.7 mmol/day.¹³ The subjects were allowed to drink tea and coffee, prepared with deionised water ad libitum. From day 11 to 20 a dose of 5 mmol sodium sulphate in gelatin capsules, was given with each main meal (15 mmol/day).

GAS MEASUREMENTS

End expiratory breath samples were collected in duplicate every day at 800 am, 300 pm, and 1000 pm using a collapsible tube with an open end as previously described.¹⁴ Hydrogen concentrations were measured on a GMI Exhaled Hydrogen Monitor (GMI Medical Ltd, Ren-

frew, Scotland), and methane by gaschromatography (PYE 104, PYE-Unicam, Cambridge, England) using flame ionisation and a 2 m × 4 mm glass column packed with Poropak Q. Oven temperature was 50°C and the detector 100°C, with nitrogen as carrier gas. Methane concentrations were calculated as the difference between breath and a corresponding room air sample.

BACTERIAL COUNTS

Viable counts of methanogenic bacteria were enumerated using the Hungate roll tube technique.¹⁵ For enumeration of viable sulphate reducing bacteria the agar shake dilution method of Widdel *et al.*¹⁶ was used with media and conditions of cultivation as described by Gibson *et al.*¹¹ The dilutions used give a detection limit of 10² viable counts per gram faeces for both methanogenic bacteria and sulphate reducing bacteria.

MICROBIAL ACTIVITY

Faecal slurries were prepared by diluting the stool samples in anaerobic sodium phosphate buffer (0.1 mol/l, pH 7.0) to a final concentration of 5% (w/v). Lintners starch was added to a slurry portion of 60 ml in a serum bottle (70 ml capacity) to give a final concentration of 0.2% (w/v). The bottles were gassed out with argon and then incubated for 48 hours at 37°C on an orbital shaker. Hydrogen, methane and carbon dioxide production in the head space gas were measured by gas chromatography as described by Allison *et al.*¹⁷ and hydrogen sulphide was determined using a colorimetric method after precipitation of S²⁻ in 10% zinc acetate.¹⁸ For determination of sulphate reduction rates the ³⁵S-SO₄ core injection and distillation method of Jorgensen¹⁹ was used (incubation time 18 hours).

GASTROINTESTINAL TRANSIT TIME

Transit times were measured once on the basal diet and once during sulphate feeding. A modified version of the method described by Cummings *et al.*²⁰ was used. On four consecutive days at 800 am 20 radiopaque plastic shapes, packed in gelatine capsules, were given orally. The particles were distinctly shaped, and a different set was used for each day. All faeces were collected, the times recorded, and the markers determined by radiography. Transit time was calculated as the average passage time of all markers recovered.

STATISTICAL ANALYSIS

Variations are expressed as standard error of the mean. Analysis for significance were done using Student's *t* test for paired samples and the two group *t* test for unpaired samples.

Results

BREATH METHANE

Mean daily CH₄ concentrations in breath for

Figure 1: Breath methane concentrations (mean of three duplicate measurements daily) in responders (A, *n*=3) and non-responders (B, *n*=3) with basal diet and with the addition of 15 mmol sodium sulphate per day.

each subject throughout the study are shown in Figure 1. In three of the six subjects (responders) methane excretion showed a pronounced fall a few days after SO_4 was added to the diet, so that by 10 days with added sulphate methane concentration was less than 2 ppm in all samples measured in these three subjects. By seven days after returning to the low sulphate diet, breath CH_4 concentrations had increased again and returned almost to control levels by the end of the study. In the other three subjects (non-responders), methane excretion was not significantly affected by the sulphate supplement (Fig 1). The average breath CH_4 concentrations during the last three days of each test period were calculated for responders and non-responders separately (Table I).

BREATH HYDROGEN

Table I shows mean breath hydrogen concentrations for the last three days of each test period for

Figure 2: Pattern of hydrogen production and consumption in a faecal slurry incubation of a responder. (A) with basal diet. (B) with 15 mmol sodium sulphate per day added to the diet. $\triangle$ H_2 , $\square$ S^{2-} , $\circ$ CH_4 .

responders and non-responders. There was no effect of the low sulphate and high sulphate diets.

BOWEL HABIT

When all subjects were taken together there were no differences in mean transit time, faecal weight, and faecal pH between the control diet and the sulphate supplemented diet (Table II). In the three responders, initial faecal weight was higher, transit time was shorter, and pH was lower than in non-responders. Both the basal diet and the sulphate supplement were well tolerated by all the volunteers and no gastrointestinal symptoms were reported.

BACTERIAL COUNTS AND MICROBIAL ACTIVITY

Typical gas production data from a faecal slurry of one responder is shown in Figure 2. During the low sulphate period, there was active methanogenesis and little production of sulphide. The latter increased markedly, however, when sulphate was added to the diet, while methanogenesis became suppressed by approximately 60%. The complete data are shown in Table III. In responders, viable counts of methanogenic bacteria decreased in the presence of extra sulphate in the diet while sulphate reducing bacteria were detected in low numbers. Sulphate reducing activity and sulphide production rates in faecal slurries were significantly higher during the high sulphate period compared with the control diet (Table III). Counts and metabolic parameters reverted to approximately pre- SO_4 levels when sulphate was stopped. Non-responders had lower SO_4 reduction rates on the control diet than responders. In these subjects, there was no difference in methanogenic counts and activities remained stable in the presence or absence of 15 mmol/d sulphate. Viable counts of sulphate reducing bacteria were never found above the detection limit of 10^2 /g faeces in non-responders (Table III).

Discussion

In this study, the addition to the diet of 15 mmol sodium sulphate/day over a 10 day period inhibited methane production in three of six normally methanogenic subjects. As methanogenesis was reduced signs of sulphate reduction, previously absent during the control diet, became apparent in faeces of these subjects. When the supply of sulphate was stopped, methane production returned. This experiment shows a previously unknown dietary effect upon bacterial activity in the human colon.

Methane gas is regarded as a harmless byproduct of fermentation in the large intestine of man. CH_4 production is thought to be fairly constant in individuals and to be independent of diet.⁷ The reasons why western populations separate into methane producers and non-producers has never been explained. In recent studies, however, we have shown that methane production is more important than previously believed. Quantitative measurements show that in methanogenic subjects the majority of gaseous

hydrogen produced from fermentation is consumed by methanogenic bacteria.¹⁰

During fermentation, hydrogen gas production is used by bacteria to allow oxidation of reduced cofactors ($\text{NADH}_2 \rightarrow \text{NAD} + \text{H}_2$). H_2 must be rapidly removed from the fermentation system, however, because high partial pressures of H_2 inhibit the reactivation of these coenzymes which are essential in glycolysis.⁹ Reduction of CO_2 by H_2 to CH_4 by methanogenic bacteria is an effective way of lowering H_2 levels and allows bacteria to generate more oxidised products which result in higher energy yields.⁸ In the absence of methanogenesis, large intestinal fermentation is inefficient and produces different organic end products unless an alternative pathway exists to dispose of reducing equivalents.^{21, 22}

In previous studies we have shown that in the colon of non-methanogenic human subjects, significant populations of sulphate reducing bacteria exist.¹¹ These bacteria use molecular hydrogen as well as other electron donors, for the dissimilatory reduction of sulphate to sulphide. In marine sediments and faeces, sulphate reducing bacteria outcompete methanogenic bacteria because of their higher affinity for such substrates.^{12, 23, 24} Methanogenic bacteria tend to predominate in a sulphate depleted environment, however.²⁵ The present study provides further evidence for these interactions in the colon.

Under stable dietary conditions, western populations may be separated into predominantly methanogenic or sulphate reducing subjects.¹¹ A proportion of CH_4 producers (three of six in this study), however, may also harbour small numbers of sulphate reducing bacteria (not detectable with the methods used in this study). The presence of these bacteria was shown by higher sulphate reduction rates recorded in faeces of these subjects (responders) even on the basal diet. When sulphate availability is increased sulphate reducing bacteria proliferate and eventually may outcompete methanogens when the sulphate supply is sufficient. This is supported by our observation that sulphate reduction rates and sulphide production in faeces of the three responders increased when sulphate was supplemented to the diet, while methanogenesis became substantially reduced. Correspondingly, sulphate reducing bacteria which were not found during the basal diet, became detectable while counts of methanogenic bacteria decreased 100-fold during this period.

Metabolic activity of sulphate reducing bacteria was, however, well below values usually found in non-methanogenic subjects,¹¹ and

TABLE III Bacterial counts and microbial activity (SEM) on basal diet and with 15 mmol sodium sulphate added, for responders (A, n=3) and non-responders (B, n=3)

		Basal diet	High sulphate	Basal diet
Sulphate reduction rate (nmol/g/h)†	A	7.5 (0.5)*	20.3 (4.3)*	7.6 (1.3)
	B	2.8 (1.7)	4.4 (1.9)	3.3 (1.1)
Sulphide production rate (μmol/g/h)‡	A	0.35 (0.04)*	0.99 (0.11)	0.53 (0.14)
	B	0.22 (0.03)	0.26 (0.04)	0.25 (0.02)
Methane production rate (μmol/g/h)‡	A	1.43 (0.20)*	0.29 (0.23)*	1.73 (0.13)
	B	1.89 (0.09)	1.73 (0.07)	1.63 (0.10)
Sulphate reducing bacteria (log ₁₀ /g)	A	0	3.3 (0.3)	0
	B	0	0	0
Methanogenic bacteria (log ₁₀ /g)	A	9.2 (0.5)	6.3 (0.2)	8.5 (0.3)
	B	8.9 (0.6)	9.1 (0.5)	8.6 (0.5)

Weights are wet weights of faeces.

*Significant differences ($p < 0.05$) between basal diet and high sulphate; †Core injection method; ‡Slurry experiment.

viable counts of sulphate reducing bacteria were too low to explain the marked drop in methanogenesis. A reason for this may be the relatively short study period which was probably not sufficient for the bacterial population in more proximal areas of the colon to equilibrate with that measured in faeces. This may be important as a recent study²⁶ has revealed some differences between caecal and faecal flora, an observation which may limit the conclusions made from measurements with faecal inocula. Changes in both faecal sulphate reducing activity and sulphate reducing bacteria counts during the sulphate feeding period, however, strongly suggest a relation between the metabolism of these bacteria and the changes in methanogenesis observed in this study.

Breath hydrogen excretion was not significantly different between the low sulphate and the high sulphate diets, indicating that the volume of hydrogen consumed either by methanogenic bacteria or by sulphate reducing bacteria might be similar.

In three of the six subjects, sulphate reduction rates were low and did not show any change throughout the study. Sulphate reducing bacteria were never detected, and methane excretion was not affected by sulphate feeding. Small and undetectable numbers of sulphate reducing bacteria may have been present, unable to increase rapidly enough to be detected during the short period of this study. Alternatively, sulphate may have been absorbed more completely in the small intestine of these subjects with insufficient amounts being available for colonic sulphate reduction.

It was noted that breath methane concentrations were generally higher in non-responders than in responders. This may be explained by the fact that more hydrogen was used in a metabolism other than methanogenesis in these subjects even on the basal diet. Moreover, during the basal diet breath CH_4 concentrations increased in responders suggesting that their usual diet contained enough sulphate for sulphate reducing bacteria to reduce the amount of hydrogen available to methanogens, whilst on the sulphate depleted basal diet more hydrogen could be used for methanogenesis.

Intestinal transit times were measured to see whether the effect of sulphate upon methanogenesis might be the result of faster bowel transit, because the laxative properties of sodium

TABLE II Faecal wet weights, gastrointestinal transit times, and faecal pH (SEM) with basal diet and with 15 mmol sodium sulphate per day added. Differences not significant

		Basal diet	High sulphate
Faecal weight (g wet wt/d)	Responders	150 (12)	164 (16)
	Non-responders	99 (13)	112 (15)
	All subjects	125 (18)	138 (13)
Transit time (hours)	Responders	41.6 (4.6)	39.1 (3.0)
	Non-responders	62.8 (19.5)	59.7 (18.4)
	All subjects	52.2 (21.3)	49.4 (20.0)
Faecal pH	Responders	6.9 (0.19)	6.7 (0.14)
	Non-responders	7.1 (0.10)	6.7 (0.20)
	All subjects	7.0 (0.12)	6.7 (0.16)

sulphate are recognised. The amount used in this study given in three doses, however, is small compared with the usual laxative dose of about 60 mmol. Transit times were not different between the control period and the period with SO_4 supplementation.

The sulphate content of an average United Kingdom diet is about 5 mmol/d.^{13, 27} It has been shown that during intakes of up to 5 mmol/d most of the sulphate is absorbed in the small intestine and very little is found in ileal effluent¹³ although endogenous sources such as mucopolysaccharides provide an unknown additional amount of sulphate.²⁸ This is probably not enough to allow significant dissimilatory sulphate reduction. At intakes greater than 5 mmol/d, however, a greater proportion is delivered to the colon. Recent studies of sulphate absorption in man indicate that on a diet of 16.6 mmol/d about 12 mmol would be expected to reach the colon.¹³

During the bacterial reduction of one mole of sulphate, four moles of hydrogen are consumed. Thus the reduction of 12 mmol SO_4 would require a volume of 1100 ml H_2 . The average breath methane concentration in responders on the low sulphate diet was 25 ppm. If ventilation rate is estimated to be 4 l/min this is equivalent to an excretion of about 140 ml/24 h in breath. We have recently shown that at such an excretion rate, breath exhalation is about 50–60% of the total excretion.¹⁰ Total methane excretion can therefore be estimated at 300 ml/d. Thus, about 1200 ml of hydrogen were consumed in methanogenesis (4 mol H_2 /mol CH_4) when sulphate supply was limited. This is equal to the volume assumed to be consumed by the reduction of 12 mmol SO_4 in the three responders. Although this balance is not entirely based on direct measurements, it shows how the observed interactions between sulphate reducing bacteria and methanogenic bacteria in vivo can be understood as competition for the mutual substrate hydrogen. When both types of bacteria are present in the large intestine, hydrogen metabolism may be regulated by the availability of sulphate in the colon. We cannot exclude the possibility, however, that other unknown mechanisms may be involved in the effects observed in this study.

Some dietary components such as bread, food preserved with sulphur dioxide, and alcoholic beverages such as beer, wine and cider are rich in sulphate.^{29–31} Their consumption may increase sulphate ingestion naturally to quite high amounts. Thus, the amount of sulphate given in this study can be reached by dietary means in a proportion of people.

In conclusion, we have shown evidence for regulation of methanogenesis by dietary sulphate if sulphate reducing bacteria are present in the colon. Sulphate feeding allows growth of sulphate reducing bacteria which then may inhibit methanogenic bacteria by substrate competition. This may explain the absence of methane in the breath of 40–50% of western populations.

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