Alternative pathways for hydrogen disposal during fermentation in the human colon

G R Gibson, J H Cummings, G T Macfarlane, C Allison, I Segal, H H Vorster, A R P Walker

Abstract

Hydrogen gas, which is produced during fermentation in the human colon, is either excreted in breath or metabolised by gut bacteria through a variety of pathways. These may include methanogenesis, dissipatory sulphate reduction, and acetogenesis. To determine which of these routes predominates in the large intestine, stools were taken from 30 healthy subjects and incubated as 5% (w/v) slurries with Lintner’s starch. In 23 of 30 subjects, methane production was the main method of hydrogen disposal. In the remaining seven, high rates of sulphate reduction were recorded together with raised production of H2S. All samples showed relatively low rates of hydrogen evolution and of acetate formation from CO2 and H2. Sulphate reduction and methanogenesis seem to be mutually exclusive in the colon and this is probably linked to sulphate availability. Sulphate reduction, methanogenesis, and acetogenesis were strongly influenced by pH. Sulphate reduction was optimal at alkaline pH values whereas methane production was maximal at a neutral pH and acetogenesis favoured acidic conditions. Faecal H2S values were related to carriage of sulphate reducing bacteria. These data show that a number of competing pathways for hydrogen disposal are possible in the large gut and that a variety of factors such as colonic pH and sulphate availability can determine which of these mechanisms predominates.

The aerobic metabolism of carbohydrate in mammalian cells requires oxygen as the terminal electron acceptor and produces carbon dioxide, water, and energy as the principal end products. In anaerobic systems such as the lumen of the human colon, however, starch, non-starch polysaccharides (dietary fibre), and other substrates are fermented by the resident microflora to yield short chain fatty acids, carbon dioxide, hydrogen, and energy.

Molecular hydrogen is used by intestinal methanogenic bacteria in many animal species to reduce carbon dioxide to methane. Methano-
BREATHE METHANE
Duplicate end-expiratory breath samples were collected into 20 ml plastic syringes and methane was determined by gas chromatography using a Pye Unicam PU-4500 with flame ionisation detector. Samples of room air were also taken and the value subtracted from the breath sample. A methane producer was defined as a subject with more than 1 ppm methane in breath above values in ambient air.

The study was approved by the Ethical Committee of the Medical School of the University of the Witwatersrand, Johannesburg.

FAECAL SLURRIES
Faecal slurries (5% w/v) were prepared by homogenising samples in anaerobic sodium phosphate buffer (0-1 mol/l, pH 7-0). For measurements of hydrogen production and methanogenic, sulphate reduction, and acetogenic rates, Lintner's starch (0-2% w/v) was incorporated into the slurries as the fermentable substrate.

METHANE FORMATION RATES
Methane production from faecal slurries was measured as described by Allison and Macfarlane. The linear part of the methane production plot during a 48 hour incubation period was used to calculate the rate of methanogenesis.

SULPHATE REDUCTION RATES
Triplicate sub-samples (5 ml) were removed from each faecal slurry and 5 µl of carrier free sodium (35S) sulphate added. Samples were then incubated anaerobically for 18 hours at 37°C before freezing and subsequent distillation using the method of Jørgensen. Rates of sulphate reduction were calculated from the amount of acid volatile (35S) sulphide formed.

ACETATE FORMATION RATES
Rates of acetogenesis in 5 ml sub-samples from faecal slurries were determined in triplicate using the method of Jones and Simon.

ENUMERATION OF SULPHATE REDUCING BACTERIA
Viable populations of sulphate reducing bacteria were counted using the agar shake dilution method with acetate, lactate, propionate, butyrate, and H2/CO2 as electron donors since various studies have shown that these are the major substrates that support the growth of sulphate reducing bacteria. Dilution tubes were incubated anaerobically at 37°C for 14 days. After this time, growth of sulphate reducing bacteria was indicated by a precipitation of ferrous sulphide and the number of black colonies formed was counted.

ISOLATION AND CHARACTERISATION OF SULPHATE REDUCING BACTERIA
Single colonies were removed from the highest agar shake dilution tubes and subcultured into liquid media. Pure cultures were obtained by successive passage through agar shakes and sulphate reducing bacteria were characterised using the criteria of Keith et al.

HYDROGEN SULPHIDE CONCENTRATIONS
Values of H2S in faeces were measured (after precipitation of sulphides in 10% w/v zinc acetate) using the spectrophotometric method of Cline.

EFFECT OF pH UPON METHANENOSIS, DISSIMILATORY SULPHATE REDUCTION, AND ACETOGENESIS IN FAECAL SLURRIES
To test whether colonic pH could significantly influence rates of methanogenesis, sulphate reduction, and acetogenesis, faecal slurries were prepared as described and adjusted to a range of pH values (5.5–8.5 in 0.5 increments). Methanogenic and acetogenic rates were calculated as before and sulphate reducing activity was determined by production of H2S.

Results
TRANSPORT OF FAECAL SAMPLES
Two approaches were tested to assess the most favourable method of transporting faecal samples to the UK for processing. Faeces incubated at ambient temperature for 24 hours under an atmosphere of oxygen-free nitrogen as well as samples frozen in a slurry (5% w/v) containing 10% w/v glycerol were prepared. Rates of hydrogen production and sulphate reducing activity were subsequently determined and compared with those found in fresh faeces. Data presented in Table 1 show that activities were always reduced in the incubated and frozen samples. The percentage inhibition of hydrogen release, sulphate reduction, and numbers of viable sulphate reducing bacteria, however, were appreciably greater in the frozen samples. Faeces were therefore transported from South Africa at ambient temperature in sealed plastic bags.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Hydrogen metabolism in faecal samples from 30 healthy subjects. Results are mean (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>Breath methane (ppm)</td>
</tr>
<tr>
<td>Group A</td>
<td>23 (3-7)</td>
</tr>
<tr>
<td>Group B</td>
<td>7 (0)</td>
</tr>
</tbody>
</table>

Group A = number of sulphate reducing bacteria <107/g dry wt faeces.
Group B = number of sulphate reducing bacteria >107/g dry wt faeces.
*9 hour incubation; 48 hour incubation.
TABLE III Percentage distribution of sulphate reducing bacteria (SRB) in human faecal samples. Results are mean (SEM)

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Percentage SRB utilising each substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate</td>
<td>14 (1-70)</td>
</tr>
<tr>
<td>Lactate</td>
<td>63 (1-24)</td>
</tr>
<tr>
<td>Propionate</td>
<td>9 (4-2)</td>
</tr>
<tr>
<td>Butyrate</td>
<td>9 (2-8)</td>
</tr>
<tr>
<td>H₂CO₂</td>
<td>4 (4-3)</td>
</tr>
</tbody>
</table>

HYDROGEN ACCUMULATION, METHAN GENESIS, SULPHATE REDUCTION/AND ACETOGENESIS IN FAecal SLURRIES

When faecal slurries were incubated with Lintner's starch a small amount of hydrogen accumulated during the first nine hours of incubation. The rate of hydrogen release during this time did not differ greatly in any of the samples tested (Table II). After nine hours, hydrogen concentrations gradually declined. To determine the route of hydrogen uptake during this period, rates of methanogenesis, sulphate reduction, and acetogenesis were measured and compared in each of the faecal slurries.

On the basis of methanogenesis rates and numbers of sulphate reducing bacteria (SRB) in faeces, the subjects divided readily into two groups (Table II). Most subjects (group A; n=23) shared high rates of faecal methanogenesis and had less than 10⁷ SRB/g dry weight faeces. In group A, 21 of the 23 subjects had methane in the breath. None of the subjects in group B (n=7) had methane in the breath, produced methane in vitro, or had more than 10⁷ SRB/g of faeces. Group B subjects had high rates of sulphate reduction in faeces and higher concentrations of sulphide. Low rates of sulphate reduction and H₂S formation were detected in some samples from group A but these were much less than those measured in the group B subjects (Table II).

Viable populations of sulphate reducing bacteria were enumerated with acetate, lactate, propionate, butyrate, and H₂CO₂ as electron donors to give total counts of faecal sulphate reducing bacteria. Sulphate reducer counts showed a strongly positive association with H₂S concentrations in faeces (Fig I). Hydrogen sulphide values in the four group A faecal samples that contained less than 10⁷ SRB/g were similar to those in which sulphate reducing bacteria were completely absent.

Rates of acetogenesis were relatively low in all samples tested (Table II). Those in group B, however, were approximately double those found in group A suggesting that acetate produc-

ISOLATION AND CHARACTERISATION OF SULPHATE REDUCING BACTERIA

The highest numbers of sulphate reducing bacteria were found using lactate as a source of carbon and energy (Table III). The dominant bacteria consisted of curved rods of various sizes, identified as belonging to the genus Desulfovibrio. Desulfovibrio species do not have a complete TCA cycle and acetate is therefore a normal end product of their metabolism. As a consequence, cocobacillar rods, identified as the acetate oxidizing Desulfobacter species were occasionally isolated from lactate-containing tubes. In dilution tubes containing acetate as the sole carbon source, Desulfobacter species were always numerically predominant.

Influence of pH upon Methanogenesis, Sulphate Reducing, and Acetogenic Activity

Data presented in Table IV show that the optimal pH values for methanogenesis and dissimilatory sulphate reduction were 7-0 and 7-5 respectively. At acidic pH values both processes were substantially inhibited whereas homoacetogenesis was highest at relatively low pH values.

Discussion

Dietary carbohydrate that escapes digestion and absorption in the small bowel passes into the caecum where fermentation by anaerobic bacteria occurs producing hydrogen. A number of fates for this hydrogen then exist. A small proportion may pass through the gut wall into the blood and be transported to the lungs where it is then excreted in breath. Alternatively, hydrogen may be metabolised by the large intestinal microflora. The removal of hydrogen allows a depletion of electron sink products such as lactate, succinate, and ethanol resulting in a higher energy yield from fermentation. Thus adequate removal of H₂ allows a more complete recovery of energy by bacteria from the degradation of organic substrates. An appreciable proportion of hydrogen may be removed by the action of methanogenic, sulphate reducing, and homoacetogenic bacteria. All three processes remove four moles of hydrogen per mole CO₂ or SO₄ reduced.

Hydrogen is essential for the growth of colonic methanogens and if this substrate is removed methanogenesis cannot occur. In the present study, considerable methane production occurred only when sulphate reducing bacteria were not active - that is group A subjects (Table II).
The metabolic end product of dissimilatory sulphate reduction (H₂S) is thought to be toxic to methanogenic bacteria, but at the low concentrations measured in faeces (Fig 1), it will not exert any direct inhibitory effect. When sulphate is available, sulphate reducing bacteria are known to have a higher substrate affinity for hydrogen than methanogenic bacteria, and this is a more likely explanation for the apparent mutual exclusion of methanogenesis and sulphate reduction in the large gut. We have shown that sulphate reducing bacteria outcompete methanogenic bacteria for hydrogen when faecal slurries from methane and non-methane producing subjects are mixed together.

In the group A samples, low rates of sulphate reduction and H₂S production occurred even in the presence of active methanogenesis. It is likely that the small amount of sulphate reduced is by assimilation into sulphur-containing amino acids and subsequently proteins. The H₂S produced in faeces from group A subjects was probably released from these amino acids during protein fermentation. Four of the group A subjects had low numbers of sulphate reducing bacteria in faeces but H₂S values were similar to those found in group B samples, so these sulphate reducers were active and values that did not affect hydrogen uptake by methanogenic bacteria. Some sulphate reducing bacteria can grow fermentatively in the absence of sulphate and in this case methanogenic bacteria may act as hydrogen scavengers to keep concentrations below thermodynamically unfavourable values. Sulphate reducing bacteria would then function as hydrogen producing acetogens.

An alternative route for hydrogen disposal is by reduction of CO₂ to acetate. A recent study has indicated that this may occur in man. Homooacetogenesis involves the utilisation of hydrogen and carbon dioxide to form acetate via acetyl CoA. Homooacetogenic bacteria are, however, competitively displaced by methanogenic bacteria for available hydrogen in other anaerobic ecosystems. Thus, these bacteria will only become active when there is little hydrogen uptake by sulphate reducing or methanogenic bacteria, explaining the low rates of acetogenesis recorded in this study. The fact that rates were generally higher in the non-methanogenic (group B) slurries may be linked to the concentration of available sulphate. If sulphate is limited and hydrogen is in relative excess, some of the hydrogen remaining after sulphate reduction could then be available for metabolism by homoacetogenesis (some other unknown factor would have to limit methanogenesis, however).

The concentration of sulphate present in the large gut is therefore critical for determining which of these processes occurs. If sufficient sulphate exists, sulphate reducing bacteria will predominate. If the colonic sulphate pool is low, however, these bacteria will not utilise appreciable amounts of hydrogen. During these conditions, methanogenic bacteria or perhaps acetogenic bacteria will become important. Recent studies have shown that a large variability in the amount of sulphate reaching the colon exists. The contribution of endogenous sulphate is approximately 1 mmol/d whereas dietary levels can range from 2–16 mmol/d, with maximal absorption occurring below 7 mmol/d.

Colonic pH may also be an important factor controlling the rate of hydrogen uptake in the large intestine. The right colon, where most carbohydrate fermentation occurs, is a region of low pH whereas conditions in the left colon and sigmoid rectum areas frequently approach neutrality. Homoacetogenesis may become important at low pH in the colon, because in vitro studies showed that faecal sulphate reducing and methanogenic bacteria were relatively pH-sensitive, preferring an environment that is neutral or slightly alkaline, whereas highest rates of acetogenesis occurred at acidic pH values (Table IV). Furthermore, we have shown previously using a three chambered fermentation system that at a pH of 6.0, hydrogen uptake can occur without any appreciable contribution from sulphate reducing or methanogenic bacteria.

A number of possible pathways for disposal of H₂ exist therefore in man and are summarised in Figure 2. What are the clinical consequences of this? Firstly, it makes it highly unlikely that simple relations can be drawn between fermentation of specified substrates, such as lactulose, and H₂ evolution in breath. In practice, widely differing responses to standard oral doses of fermentable carbohydrate are seen among subjects. Bjørnekleit and Jensen have shown that subjects who produce methane during fermentation produce appreciably less H₂ in breath in response to a standard dose of lactulose. Secondly, if H₂ is not further metabolised, fermentation may be incomplete and intermediates such as lactate, succinate, and ethanol are likely to accumulate. D-lactate, produced by colonic bacteria, is only partly metabolised in man and can cause severe metabolic disturbance on occasions. Thirdly, if H₂ gas is not metabolised the volume of gas accumulating in the gut will be substantially greater than if CH₄ is produced because the reaction:

\[
\text{CO}_2 + 4\text{H}_2 \rightarrow \text{CH}_4 + 2\text{H}_2\text{O}
\]
reduces five volumes of gas to one. Fourthly, the end products of these various terminal oxidative reactions differ in their toxicity. Methane is a harmless gas, readily expelled. Acetate is absorbed and metabolised by peripheral tissue such as muscle but H₂S is highly toxic and may poison colonic epithelial cells if not oxidised rapidly after absorption. The capacity for high rates of H₂S production exists in some people (Table II) and it may be that sulphate reducing bacteria play a part in the aetiology of some large gut disorders. We have previously shown, using in vitro faecal slurries, that up to 3 mM H₂S may be produced during a 48 hour incubation. In this situation acetic acid sulphate reducing bacteria did not rise above a concentration of 0.4 mM (Fig 1). This suggests that some detoxification mechanism for H₂S is operative in the large gut. Such a mechanism may include incorporation into sulhide containing amino acids or the production of mercaptans – for example, mercaptoproacetate or mercaptobutyrate.

26 Levin MD, Ingelsting FJ. Hydrogen and methane production in caecum of some NY Acad Sci 1968; 150: 75–81.