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Research Article

Preliminary Studies Demonstrating Acetoclastic Methanogenesis in a Rat Colonic Ring Model

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Washed rat colonic rings were incubated in closed flasks under N2 at physiologic pH and temperature levels. In the absence of an exogenous substrate, negligible H2 but some CH4 concentrations were detected in vitro after one hour of incubation, but high concentrations (H2 > 100 ppm, CH4 > 10 ppm) of both gases were found after 24 hours of culture. Production of H2 and CH4 by the washed colonic rings was stimulated by lactose addition. Maximum H2 production occurred at about pH 7.0, while maximum CH4 production occurred between pH 4.0 and 6.0. The increased production of both gases at 24 hours was associated with dramatic increases (104-fold) in anaerobic bacteria colony counts on the colonic rings and in the incubation media, as well as dramatic increases (100-fold) in acetate concentrations in the media, while lactate concentrations first rose and then fell significantly. These results suggest that gas production in colonic ring preparations is subject to quantitative changes in microbiota, pH, and metabolite formation analogous to in vivo conditions. In addition, microbiota firmly attached to colonic tissue appears to utilize colonic tissue to support its growth in the absence of an exogenous substrate.

1. Introduction

Humans and animal breath contains gaseous materials not found in normal air; these materials are thought to be products of bacterial and nonbacterial metabolism [1–3]. Two of these gases, hydrogen (H2) and methane (CH4), have attracted considerable attention in recent years, [4, 5] including a study suggesting that obese patients who have methane in their breath have significantly higher body mass indexes which may play a role in obesity [6–8]. Breath H2 has been shown (a) to be reduced with fasting, (b) to be responsive to exogenous substrate ingestion, (c) to vary with the various populations, (d) to be elevated in disease states (especially cancer), and (e) to be produced by the distal colonic bacteria.

Considerable attention has been devoted toward clinical applications for the measurement of breath H2 and CH4 levels. These studies have focused on H2 and CH4 generation in vivo by human or animal models or have used in vitro assays to monitor H2 and CH4 production using stool (feces) homogenates. Although the ultimate applicability of these breath tests have been improved, intrinsic limits exist to a complete understanding of the molecular mechanisms involved in the actual production of the gases at the site of origin, that is, the colon. In vivo fecal homogenate assays have clearly advanced our understanding of which substrates function in the production of which gases. However, these systems represent studies on bacterial populations that are “distal to” and “outside of” the colon. The present communication details an initial attempt to develop a model system to monitor the production of gaseous products in vitro using the bacteria adherent to the colon itself.
2. Materials and Methods

2.1. Animals. Female rats weighing 125–175 g (CD, Charles River Breeding Farms, Wilmington, MA, USA) or germ-free female rats of similar weight (Taconic Farms, NY, USA) were used throughout these studies and maintained in accordance with the guidelines of the committee on Animals of the Harvard Medical School and those prepared by the Committee on Care and Use of Laboratory Animals, the Institute of Laboratory Animal Resources, National Research Council (DHG, Publication No. NIH 78-23, Revised 1978). All animals were fasted overnight prior to use. Typically, six animals were used in each experiment.

2.2. Colonic Ring Preparation. Rats were sacrificed by cervical dislocation. The colon was removed, washed with 50 mL sterile saline, and then cut into 2-3 cm rings. All subsequent work was done inside an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI, USA). One gram of tissue was placed in 50 mL sterile saline, and then cut into 2-3 cm rings. All subsequent work was done inside an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI, USA).

2.3. Fecal Preparations. Fecal material (formed pellets) was taken from the colon of rats immediately after sacrifice and handled in the anaerobic chamber as described previously. The fecal material was homogenized (1 gram/10 mL) in a Waring blender for 1 min in the media described previously. Ten milliliters of homogenate was placed in a sterile 50 mL Erlenmeyer flask and gassed for 30 sec with N2 (10 liter/min). The flask was then capped and incubated at 37°C in a shaking water bath.

2.4. H2 and CH4 Analysis. The gaseous samples were analyzed for H2 and CH4 by gas chromatographic procedures described previously [1].

2.5. Colony Counts. Bacterial colony units were determined in both the bathing media and on the tissue using horse blood agar plates. Aliquots of media were serially diluted with sterile PBS. Aliquots (100 μL) were then transferred to the horse agar plates and anaerobically incubated at 37°C for 18 hours. Tissue was decanted from the flasks and lightly patted on sponges to remove excess media. Subsequently, the tissue samples were homogenized in 10 mL sterile PBS and serially diluted with additional sterile PBS. Again, 100 μL aliquots were transferred to the horse agar plates and anaerobically incubated at 37°C for 18 hours.

2.6. Acetate and Lactate Measurements. Acetate and lactate concentrations in the media were determined in the bathing media using Boehringer-Mannheim kits (West Germany). For these experiments the colonic rings and media were transferred to 15 mL plastic tubes, sealed and spun at 100 × g for 15 min at room temperature. The supernatant was deproteinized using perchloric acid for lactate and charcoal and boiling water for acetate.

2.7. Statistical Analysis. Analyses were performed by standard Student's unpaired T test.

3. Results

We first examined the ability of fecal homogenates made from the fecal colonic contents to produce H2 and CH4 with and without lactose addition after 1 or 24 hr of incubation; these two time points were chosen because human fecal homogenates have been shown to produce H2 or CH4 after 1 or 24 hr of incubation with lactate or glycoprotein, respectively [9]. As can be seen in Table 1, homogenates made from fecal material taken from the colon produced significant amounts of H2. Significantly more (P < 0.01) H2 was produced by the fecal colonic homogenates with lactose addition after either 1 or 24 hr incubation. In addition, the colonic fecal homogenates generated CH4, a production that was increased with time (24 hr of incubation), but not with lactose addition (Table 1). No H2 or CH4 was detected in flasks incubated with the sterilized media alone.

We next examined the ability of washed colonic rings to produce H2 and CH4 in the presence or absence of lactose at 1 and 24 hr incubation. As can be seen in Table 2, negligible amounts of H2 were detected with or without lactose after 1 hour of incubation, although small amounts of CH4 were detected. However, copious amounts of H2 and CH4 were detected after 24 hr of incubation. The addition of lactose increased H2 and CH4 production significantly at 24 hr (P < 0.01). Colonic preparations from germ-free rats incubated in the sterilized media did not produce H2 or CH4 in the absence or presence of lactose. This confirms a previous report that germ-free rats produce no methane in vivo [10]. The pH for maximum H2 production at 24 hr appeared to be 7.0, while the pH for maximum CH4 production was closer to 5 (Figure 1).

We next attempted to correlate the increases in H2 and CH4 production in the absence of lactose with actual bacterial colony counts as well as lactate and acetate concentrations, known metabolites of ruminant bacterial digestion [11]. As can be seen in Table 3, the colony counts observed in the media or on the colonic tissue increased some 104-fold after 24 hr of incubation. Lactate concentrations appeared to drop dramatically after 24 hr of incubation but acetate
concentrations increased to levels 100 times those seen at one hour.

Concentrations began to increase after 6 hr of incubation and incubation, but fell off rapidly after 6 hr. By contrast, acetate concentrations gradually increased during the first 4 hr of incubation, as described in Methods. Lactose (1.25 g% final concentration) was added where indicated. H₂ and CH₄ were determined as described previously [1]. The concentration of the gases is expressed as part per million. The results are the average of three experiments, mean ± SD, with six rats in each experiment.

Table 3: Bacterial counts, lactate, and acetate concentrations in rat colonic ring preparations.

<table>
<thead>
<tr>
<th>Assayed system</th>
<th>Concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 hour (N = 3, mean ± SD)</td>
</tr>
<tr>
<td>Bacterial counts</td>
<td>H₂</td>
</tr>
<tr>
<td>Tissue</td>
<td>4.0 ± 0.4 x 10⁸</td>
</tr>
<tr>
<td>Media</td>
<td>5.0 ± 0.5 x 10⁸</td>
</tr>
<tr>
<td>Lactate</td>
<td>20.0 ± 1.0</td>
</tr>
<tr>
<td>Acetate</td>
<td>5.0 ± 1.0</td>
</tr>
</tbody>
</table>

Table 1: H₂ and CH₄ production by rat fecal homogenates.

<table>
<thead>
<tr>
<th>Gas production (ppm)</th>
<th>H₂</th>
<th>CH₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 hour</td>
<td>24 hours</td>
<td>1 hour</td>
</tr>
<tr>
<td>Without added lactose</td>
<td>486 ± 220</td>
<td>380 ± 415</td>
</tr>
<tr>
<td>With added lactose</td>
<td>2,001 ± 706</td>
<td>14,235 ± 17,004</td>
</tr>
</tbody>
</table>

One gram of freshly collected feces in the colon was incubated as described in Methods. Lactose (1.25 g% final concentration) was added where indicated. H₂ and CH₄ were determined as described previously [1]. The concentration of the gases is expressed as part per million. The results are the average of three experiments, mean ± SD, with six rats in each experiment.

Table 2: H₂ and CH₄ production by rat colonic rings.

<table>
<thead>
<tr>
<th>Gas production (ppm)</th>
<th>H₂</th>
<th>CH₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 hour</td>
<td>24 hours</td>
<td>1 hour</td>
</tr>
<tr>
<td>Without added lactose</td>
<td>0</td>
<td>2.280 ± 1,695</td>
</tr>
<tr>
<td>With added lactose</td>
<td>0</td>
<td>34,280 ± 8,927</td>
</tr>
</tbody>
</table>

One gram of freshly collected feces in the colon was incubated as described in Methods. Lactose (1.25 g% final concentration) was added where indicated. H₂ and CH₄ were determined as described previously [1]. The concentration of the gases is expressed as part per million. The results are the average of three experiments, mean ± SD, with six rats in each experiment.

**4. Discussion**

Methanogens are microorganisms that produce methane as a metabolic byproduct in anoxic conditions. They are common in the guts of animals such as ruminants and humans, where they are responsible for the methane content of belching in ruminants and flatulence in humans [12]. Methanogens have been found to be present in the colons of rats [10]. Rodkey et al. [8] reported CH₄ production rates of as high as 29 mL/day with Sprague-Dawley rats. In a similar system of in vivo gas collection, we have observed both H₂ and CH₄ production in vivo by the rats in our facility (Carter, unpublished observations). The washed colonic tissue from the rats in our study produced both CH₄ and acetate. This may be related to acetoclastic methanogenesis (reaction 6, Table 2 [13]) based on the increase in the methane production by 400% under the same conditions.

It is generally felt that obesity disorders are the result, in part, of the gut microbiota which contributes to the energy imbalance because of its involvement in energy intake, conversion, and storage. Culture-independent methods have showed that high proportions of methanogens can comprise up to 10% of all anaerobes in the colons of healthy adults [14]. The development of *Methanobrevibacter smithii* in anorexic nervosa patients may be associated with an adaptive attempt towards optimal exploitation of the low caloric diet of anorexic patients [14]. Hence, an increase in *M. smithii* leads to the optimization of food transformation in low caloric diets. *M. smithii* could also be related to constipation, a common condition for anorexic patients [14].
It has been proposed that the role of *Methanobrevibacter smithii* in weight gain in animals is related to the ability of the *M. smithii* to scavenge hydrogen produced by syntrophic organisms for its hydrogen-requiring anaerobic metabolism, producing methane as a byproduct [7]. These authors proposed that the scavenging of hydrogen allows the syntrophic organisms to be more productive, increasing short chain fatty acids (SCFA) production and availability of calories for the host [7]. In their study [7], the presence of hydrogen and methane on breath test, but not either methane or hydrogen alone, was associated with higher BMI and percent body fat. The authors postulated that, in the subjects that had an abundance of hydrogen to fuel methane production, the intestinal methanogens could also contribute to enhanced energy harvest. These authors previously noted an association between breath methane and constipation in human subjects and, using an in vivo animal model, demonstrated that methane gas directly slows transit in the gut by 59% [7]. The authors hypothesized that the slowing of transit could result in greater time to harvest nutrients and absorption of calories, representing another potential mechanism for weight gain [7].

In summary, the present report details our initial studies to develop an *in vitro* model for monitoring H₂ and CH₄ production that will allow closer examination of the association of the evolution of these gases with changes in the tissue microbiota involved, particularly the methanogens, adherent to colonic tissue. Such a model may prove useful in the elucidation of the molecular mechanism(s) involved in the elaboration of these gases and the role of these microorganisms in the development of obesity.

**Acknowledgments**

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**References**


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**Figure 2:** Lactate and acetate production by washed rat colonic rings. Washed rat colonic rings were incubated, and lactate or acetate concentrations were determined in the incubation mixture, as described in the text. The results are the mean of two experiments at each time point.